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ABSTRACT

Title of Dissertation: Characterization and Function of the Inflammatory Response to Infection by a Gastrointestinal Nematode Parasite: New Insights into Protective Th2 Responses.

Name: Robert McCullough Anthony, Molecular and Cell Biology Graduate Program, USUHS, Bethesda, MD 20814

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Effective immune responses to infectious diseases involve recognition of invading pathogens and result in appropriate primary and secondary reactions mediating host protection. Although these responses against many bacteria and viruses have been characterized extensively, Th2 effector mechanisms leading to host-protection remain elusive. Using an infectious model employing a natural murine gastrointestinal nematode parasite, *Heligmosomoides polygyrus*, we characterized the immune cell infiltrate surrounding invasive larval parasites in the small intestinal muscosa and submucosa (host:parasite interface) during early stages of a secondary infection. A primary is chronic, with established adult parasites detectable up to four months post infection, where as the parasites are naturally cleared from the intestinal lumen by 14 days follow challenge. This distinction between primary and secondary *H. polygyrus* infections allows a clear

readout of protective immunity, making this infectious model useful for examining protective secondary Th2 responses.

A distinct and highly reproducible leukocyte architecture developed by the fourth day post challenge, which included $Gr1^{+}$ neutrophils amassing adjacent to the parasite, and $CD4^{+}$ T cells, $CD11c^{+}$ dendritic cells, and MBP-1^{+} eosinophils surrounding the parasite in the lamina propria. Additionally, laser capture microdissected (LCM) samples from the host:parasite interface featured upregulated Th2 cytokine mRNAs relative to untreated intestinal tissue. This localized inflammatory response differed during primary infection, as $CD4^{+}$ T cells did not infiltrate the host:parasite interface, and there were no increases in cytokine expression. These findings were extended to show that the peripheral inflammation during the memory Th2 response at the host:parasite interface is essential for host-protection leading to worm expulsion. Memory $CD4^{+}$ T cells that express Th2 cytokines rapidly accumulate around the invading parasite in the intestinal submucosa, and induce the alternative activation of macrophages (IL-$4R^{hi}$, $CD206^{+}$, arginase-1^{+}, $Fizz1^{+}$, $Ym1^{+}$, $iNOS^{-}$).

Alternatively activated macrophages metabolize the amino acid, arginine, by the enzyme arginase-1, which is essential for their differentiation and effector functions. Through intervention experiments, our findings demonstrate that macrophages and arginase contribute to the natural clearance of a secondary *H. polygyrus* infection. These observations provide new insights into mechanisms of host-protection mediated by Th2 responses, and establish a novel, protective role for alternatively activated macrophages.

Characterization and Function of the Inflammatory Response to Infection by a Gastrointestinal Nematode Parasite: New Insights into Protective Th2 Responses.

by

Robert McCullough Anthony

Dissertation submitted to the faculty of the Molecular and Cell Biology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, May 2006.

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-/-	deficient.
1'Hp	primary *H. polygyrus* infection; this infection is chronic with established adult worms in the intestinal lumen 3 months post infection.
2'Hp	secondary *H. polygyrus* infection; this infection is naturally cleared by day 14 post infection.
AAMΦ	alternatively activated macrophage.
Ab	antibody; immune product produced by B cells, featuring antigen binding portion (Fab) and isotype portion (Fc).
AMCase	acidic mammalian chitinase; expressed by AAMΦ.
APC	antigen presenting cell; traditionally including DCs, MΦ, and B cells, these cells present antigen to T cells on MHC II molecules.
Arg-1	arginase-1; gene expressed by AAMΦ.
Ascaris sp.	nematode parasites; *A. lumbricoides, A. suis.*
BCR	B cell receptor; gives B cells ability to distinguish between antigens.
BEC	S-(2-boronoethyl)-L-cystein; an arginase inhibitor
CD	common domain; surface markers used to identify leukocyte populations.
CD4	a common marker for helper T cells.
CD8	a common marker for cytotoxic T cells.
CD25	part of the IL-2 receptor, and sometimes used as a marker for regulatory T cells.
CD206	the macrophage mannose receptor; a non-signaling PAMP receptor recognizing mannose; a marker for alternatively activated macrophages.
Cl_2MDP	clodronate; a chemical loaded into liposomes, which are used to deplete macrophages.
CpG	metholated DNA motif characteristic of bacterial DNA; a PAMP recognized by TLR-9.
CTLA-4	cytotoxic lymphocyte antigen-4; an inhibitor co-stimulatory molecule that interacts with CD80 and CD86.
D4	day 4 post infection.
D14	day 14 post infection.
DAPI	4',6-Diamidino-2-phenylindole; a fluorescent nuclear stain which excites at 350nm and emits at 450nm.
DC	dendritic cell; a highly effective APC.
DFMO	α-difluoromethylornithine; an ODC inhibitor.
dsRNA	double stranded RNA, characteristic of some viral genomes; PAMP recognized by TLR-3.

F4/80	a common surface marker for macrophages.
FACS	flow activate cell sorting; used to phenotype cells.
Fab	fragment antigen binding; antibody portion which recognizes antigen epitopes.
Fc	fragment crystallizable; antibody portion which determines isotype of antigen.
FcR	Fc receptor; allows innate immune cells to bind Abs with antigen binding portion facing out, away from the cell.
FITC	fluorescein isothiocyanate; a fluorochrome which excites at 488nm and emits at 520nm.
FOX	forkhead box protein; transcription factor family sharing a common DNA binding domain.
FoxP3	forkhead box protein 3; a common marker expressed by some regulatory T cells.
GABA	γ-amino butyric acid; a neurotransmitter with activation and inhibitory properties, depending on the receptor type expressed by target cells.
GFP	green fluorescent protein; shares fluorescent properties with FITC.
H&E	hematoxylin and eosin staining.
HIV	Human Immunodeficiency Virus.
host:parasite interface	region during invasive stages of infection where host and parasite tissue adjoin.
H. polygyrus	natural murine gastrointestinal nematode parasite; *Heligmosmoidies polygyrus.*
Hp	*H. polygyrus*.
IBD	inflammatory bowel disease; an autoimmune disease targeting the colon.
IF-LCM	immunofluorescent laser capture microdissection.
IFN	interferon; a cytokine family originally identifies with resistance to viral infection.
Ig	immunoglobulin; Abs.
IL	interleukin; a generic term for cytokines.
IL-4R	IL-4 receptor.
iNOS	inducible nitric oxide synthase; produced by phagocytes in type 1 inflammatory response, which is responsible for bacterialcidal NO burst.
i.v.	intravenous injection.
L1	first stage larvae.
L2	second stage larvae.
L3	third stage larvae.
L4	fourth stage larvae
L5	fifth stage larvae.
LCM	laser capture microdissection.

L. monocytogenes	intracellular pathogenic bacteria capable of escaping MΦ phagocytic pathway; *Listeria monocytogenes.*
Leishmannia sp.	single celled, intracellular parasites; *Leishmania major, Leishmania donovani.*
LOHA	L-hydroxy-arginine; intermediate product during conversion of L-arginine to NO by iNOS.
Lp	lamina propria.
LPS	lipopolysaccharide; a product of gram negative bacterial cell wall; a PAMP recognized by TLR-4
M	muscularis.
MΦ	macrophage; a phagocytic and APC cell.
MBP-1	major basic protein-1; a marker for eosinophils.
MHC	major histocompatibility complex; complex on which antigens are presented.
MLN	mesenteric lymph node; the major draining lymph node of the intestines.
NK	natural killer cell; a leukocyte important in viral and cancer immunity.
NO	nitric oxide; antimicrobial product produced by iNOS.
OAT	ornithine aminotransferase; enzyme downstream of Arg-1, which converts L-ornithine to proline.
ODC	ornithine decarboxylase; enzyme downstream of Arg-1, which converts L-ornithine to polyamines.
PAMP	pathogen associated molecular pattern (mannose, LPS, CpG, dsRNA).
PBS	phosphate buffered saline.
PE	phycoerythrin fluorochrome, which is excited at 488nm and emits at 580nm.
RT-PCR	reverse transcriptase polymerase chain reaction
Rx	drug treatment.
Salmonella sp.	intracellular pathogenic bacteria capable of proliferating inside of MΦs; *S. typhimurium, S. paratyphimurium.*
SCID	severe combined immunodeficiency.
sp.	species.
STAT	signal transducer and activator of transcription; family of signaling molecules associated with cytokine receptor signaling.
TCR	T cell receptor; conveys antigen recognition ability to T cells.
Th1	T helper type 1 cell; producers of IFN-γ, and are important in anti-bacterial, viral, and cancer responses.
Th2	T helper type 2 cell; producers of IL-4, IL-13, and IL-5, and are involved in anti-helmith and allergic responses.

TLR	toll like receptor; a family of innate PAMP receptors
TNF	tumor necrosis factor; produced by T cells and MΦ during Th1 responses.
Trichuris sp.	gastrointestinal nematode parasites;*T. trichuris, T. muris, T suis.*
WT	wild type.

Dissertation Introduction

Background and Significance

Infectious diseases are caused by diverse types of pathogens, including bacteria, viruses, fungi, and parasites[1]. Of these pathogens, parasites are the most highly evolved, as these organisms are eukaryotic and often developed complicated life cycles frequently involving multiple environmental niches and hosts[2]. Infectious parasites are responsible for a wide range of human diseases, including Leishmanisis, Malaria, Schistosomaisis, Giardia, and Ascariasis, although most morbidity and mortality caused by these infection types occurs in the third world[1,2].

Parasitic helminths are large, extracellular, bilateral worms, ranging from 1mm to 1 meter in length, and possess rudimentary nervous and excretory systems, but no circulatory systems[3]. These parasites are further characterized by their body shape: nematodes (including *Ascaris* sp., *Trichuris* sp.,) are round worms, trematodes (*Schistosoma* sp.) have leaf-shaped bodies, and cestodes (*Taenia* sp., *Diphyllobothrium* sp.) are flat and ribbon shaped tapeworms[3].

With more than two billion of the world's population infected with parasitic helminths[4], understanding the mechanisms of host defense against these organisms is compelling. While infection by these pathogens is generally not fatal, they are associated with high rates of morbidity, with chronic infections often leading to anemia and malnourishment[4]. Children in the developing world are most affected, exhibiting the most detrimental responses to these infections,

which has prompted the World Health Organization in 1993 to rank intestinal helminths as the main cause of disease burden in school-aged children[4].

Westernized countries have been able to control these infections through primary health care programs and effective public sanitation, but third world nations lack resources for such programs resulting in endemic and intractable gastrointestinal parasitism[5-7]. Therefore, immunotherapeutic approaches may provide the most feasible method to controlling these types of infection, which mandates a greater understanding of the immune response elicited against these parasitic worms.

Additional interest in the mechanisms and cellular populations involved in immune responses elicited by these parasites has stemmed from the "hygiene hypothesis." Over the last 20 years in Westernized nations, the incidences of allergic, asthmatic, and autoimmune diseases have steadily increased[8,9]. In contrast, diseases of this sort are rarely seen in the third world. To explain this trend, the hygiene hypothesis[10,11] was put forth, which postulates that growing up in an environment that is too clean leads to the development of an under-stimulated immune system, which consequently responds inappropriately to innocuous antigens including ragweed, animal dander, pollen, self, etc.

This hypothesis has been extended beyond merely being too clean, to living in areas devoid of parasitic helminthes and consequentially not being exposed to these pathogens [12-14]. The epidemiology of helminth infections follows the inverse relationship outlined by the hygiene hypotheses: they are

endemic in the third world, and effectively nonexistent in Western countries where allergy, asthma, and autoimmune disease are prevalent.

Additionally, a rapidly growing body of literature supports the ability of helminth infections to down-modulate immune responses[15-20]. School children in Gabon that were infected with *schistosome* parasites were found to be less likely to develop contact dermatitis to dust mite allergens than uninfected children, and anti-helminthic treatment resulted in an increased likelihood of developing contact dust mite allergen induced dermatitis[21]. Taking this observation to the clinic, researchers at the University of Iowa effectively treated sufferers of inflammatory bowel disease (IBD) by orally administering pig whipworms (*Trichuris suis*)[22]. This parasite is capable of only a transitory infection in humans, and its administration lead to a temporary reduction in the symptoms of IBD. While there is tremendous potential in these observations and in the immunoregulatory potential of helminthes, a potential concern that arises pertains to the ability of helminth-infected individuals to develop appropriate immune responses to vaccines against other infectious pathogens. An underlying helminth infection might effect the development of immunity to malarial, HIV, or Tuberculosis vaccines, as these target populations reside in helminth-endemic regions[23].

Much of our understanding of the immune responses elicited by helminth parasite infections stems from studies of allergic and asthmatic diseases; all are polarized Th2 responses, but atopic diseases target inappropriate or innocuous antigens[24-29]. Sensitization with allergens leads to the production of IgE by B

cells; this antibody isotype can bind receptors on the surface of tissue-residing mast cells[30,31]. Upon re-exposure, allergen-specific IgEs on the surface of mast cells are cross-linked, resulting in mast cell activation and degranulation leading to the release of soluble mediators, including histamines, leukotrienes, and prostaglandins, which are responsible for acute allergy, and in extreme cases, anaphylactic shock[32-34]. Recent studies have begun to uncover the individual roles of type 2 cytokines in asthma and have assigned an essential role to IL-4 in the generation of primary Th2 responses to allergens, whereas both IL-4 and IL-13 play a role in mediating airway hyperresponsiveness, mucus hypersecretion, and subepithelial fibrosis[35]. Insights into the mechanisms leading to asthmatic attacks and allergies have resulted in many treatments for these diseases, including anti-histamines, anti-leukotrienes, anti-IL-5 (to partially block eosinophilia), and immunotherapies to downmodulate the allergen-specific Th2 response [32,36,37].

Thus, understanding the mechanisms of protective Th2 responses will be beneficial for many reasons; it may lead to the development of vaccines for gastrointestinal parasites, as well as shed light on novel immunoregulatory mechanisms to treat diseases of inappropriate immune responses which include allergy, asthma, and autoimmune diseases, as well as some harmful inflammatory reactions to viral and bacterial infections.

The Immune System

The immune system is essential for controlling infectious diseases and cancer in the mammalian host. All leukocytes, or white blood cells, are derived

from a common bone marrow stem cell precursor[33,38]. Typically characterized as two subsystems, the immune system contains both innate and adaptive systems[33,39]. The adaptive immune system is further categorized as humoral and cell-mediated, which consists of B cells which produce antibodies, and cytotoxic and helper T cells, respectively[1,33].

The innate immune system is defined by the inability to recognize specific antigens, and comprises macrophages (MΦs), dendritic cells (DCs) neutrophils, eosinophils, mast cells, basophils, and cytotoxic natural killer cells[33,40]. While these cell types have distinct effector functions and roles in immunity, they are all found throughout the body or circulation and rapidly accumulate at sites of inflammation[33].

The cells of the adaptive immune system, or lymphocytes, express antigen-specific receptors which allow the recognition of specific peptides and provides the mammalian host with the ability to differentiate between distinct antigens[33,40]. The receptors expressed by T and B cells are generated by tightly controlled mutations in the genes encoding the receptors, allowing an essentially infinite number of different receptors[41-43]. T cells which undergo thymic selection express αβ TCRs and have been extensively characterized[41-43]. Less is understood about T cells that express TCR composed of γσ chains, which have effector functions bridging innate and adaptive systems by recognizing non-peptide antigens, including lipids, self-antigens, and potentially presenting antigen on MHC II molecules[44]. αβ T cells are subdivided into two classes: $CD8^+$

or $CD4^+$, which recognize antigen complexed with MHC I or MHC II molecules, respectively[45].

All nucleated cells in the body express MHC I molecules, and therefore all can present antigens to $CD8^+$ T cells. Cytotoxic $CD8^+$ T cells are important for killing of cells infected with intracellular pathogens. MHC II molecule expression is generally restricted to professional antigen presenting cells (APCs), which are DCs, MΦs, and B cells. These APCs have phagocytic and opsonizing abilities that allow them to present peptide antigens to $CD4^+$ T cells, who in turn direct or orchestrating an immune response. More recently, populations of regulatory T cells have been described, which down-modulate immune responses to a variety of antigens[46-49]. They are thought to prevent harmful or inappropriate responses, including autoimmune disease, asthma, and allergy. Some regulatory subsets are identified by the expression of the transcription factor forkhead box protein 3 (*FoxP3*)[50,51], which belongs to the FOX family of protein transcription factors sharing a DNA-binding motif of 80 to 100 amino acids, known as *forkhead box*.

B cells also express antigen specific receptors (BCRs) on their surface, in the form of IgD molecules. The BCR allows B cells to recognize and opsonize antigens, which are then presented on MHC II molecules. Activated effector CD4+ T cells that recognize the antigen presented by B cells can induce isotype class-switching in B cells, a mechanism by which B cells produce distinct antibody types. Antibodies are secreted into the circulation and extracellular space, and can bind and neutralize infectious agents[33]. Also, a number of innate cells express specific Fc receptors, which bind the isotype-determining portion of

the antibodies, allowing innate cells the ability to recognize specific antigens via the antibodies on their surface.

Natural killer cells (NK cells) also express receptors for recognition of virally infected cells and play a role in killing infected and cancerous cells[52,53]. Naïve T and B cells, which have not encountered the specific antigen their receptors recognize, reside in secondary lymphoid organs, including the spleen, lymph nodes, and Peyer's patches[33]. Typical lymphoid morphology features distinct zones or areas where resting T or B cells reside (**fig. 1**).

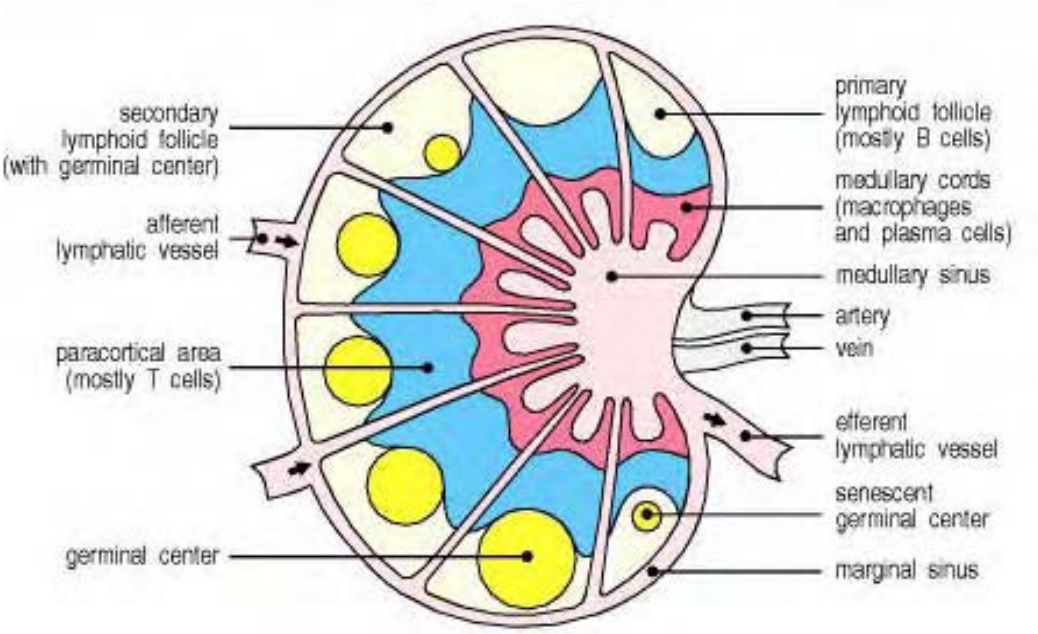

Figure 1. The anatomy of a lymph node[33]. T and B cells reside in distinct zones within a node. Antigens are brought into a lymph node by DCs or the lymphatics themselves in the case of soluble antigens via the afferent lymphatic vessel and presented to T cells in the T cell zone.

DCs reside throughout the body, continually phagocyzing antigens. In the presence of bacteria or viruses, DCs are activated through receptors recognizing pathogen associated molecular patterns (PAMPs) including Toll Like Receptors (TLR), which recognize bacterial and viral motifs. This activation of DCs leads to

reduced phagocytic ability, and enhanced expression of antigen-loaded MHC II molecules, costimulatory molecules, and lymph node homing receptors. The combined upregulation of these molecules allows the activated DCs to traffic to the closest "draining" lymph node and interact with naïve T cells[33,54]. Soluble antigens can also be carried to lymph nodes by the lymphatics, where they are phagocytosed, and presented to T cells by lymph node resident DCs[54,55].

T cells become activated following appropriate stimulation from DCs, which, in general, involves recognition of appropriate antigen presented on the MHC molecule and a secondary costimulatory signal (via CD80 and CD86) through the co-receptor CD28[56,57] (**fig. 2**). These molecules are upregulated by DCs upon activation, and provide a primary, antigen-specific signal through MHC II-TCR interactions, and a secondary positive signal through CD80/86 and CD28, leading to T cell activation. In the absence of a secondary signal, T cells can become anergic, non-responsive, or regulatory. Therefore, this "two signal hypothesis" is considered to be a protective mechanism against the development of inappropriate immune responses (as in principle, only pathogen-activated DCs should upregulate CD80 and CD86 expression)[50,58]. Activated $CD4^+$ T cells go on to direct other cell types to elicit specific effector functions, including inducing class-switching in B cells to produce appropriate antibody isotypes, and activate macrophages.

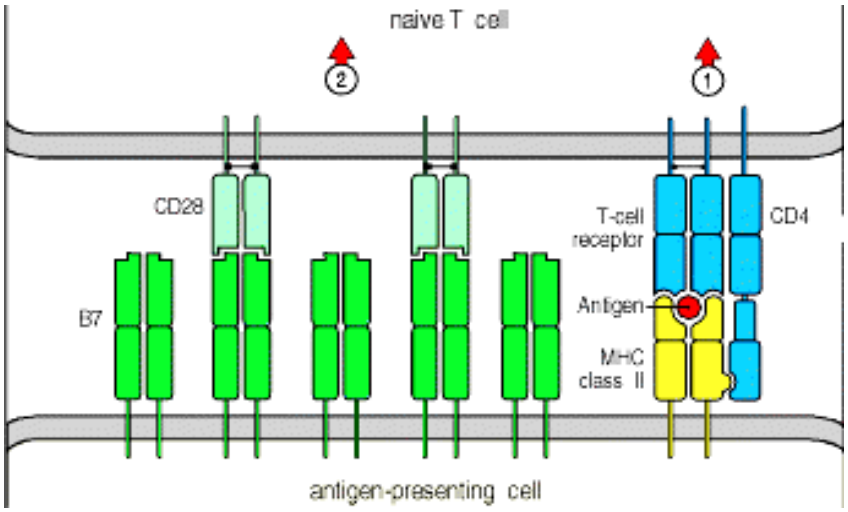

Figure 2. The two-signal hypothesis of T cell activation. Naïve T cells are activated upon TCR recognition of MHC loaded with appropriate antigens (signal 1) and signaling through CD28 following recognition of B7 (CD80 and CD86) molecules on APCs {Janeway, 2001 #32}.

Effector CD4$^+$ T Cells

Upon appropriate signaling involving antigen recognition and costimulation, naïve CD4$^+$ T cells become activated and differentiate into at least two distinct effector phenotypes, defined by their cytokine expression profiles[59]. Th1 responses are initiated by DCs that are stimulated through TLRs, which recognize patterns associated with bacterial and viral pathogens (PAMPs) and express the cytokine IL-12, which drives differentiation of naïve CD4$^+$ T cells into Th1 effector cells[60,61]. The prototypic cytokine produced by Th1 cells is IFN-γ. Additionally, Th1 cells induce class switching in B cells to make the antibody isotype IgG2a, and upregulate iNOS expression by macrophages essential for killing intracellular pathogens (**fig. 3**). Polarization towards a type 1 response is very effective at clearing bacterial and viral infections, and intracellular parasites including *Leishmania* sp. and malarial parasites [62-64].

At present, it is unknown what signals dendritic cells to drive the development of naïve $CD4^+$ T cells into Th2 cells[62,65]. The prototypic cytokines produced by Th2 cells are IL-4 and IL-13 (fig. 2)[33,59,66]. These effector cells induce class switching in B cells to make the antibody isotypes IgE and IgG1, trigger eosinophila through IL-5 production, and upregulate arginase-1 expression by macrophages leading to proline and polyamine production[67-69]. This response type is very effective at clearing large, extracellular worm parasites, and is also responsible for the symptoms associated with allergy and asthma[70].

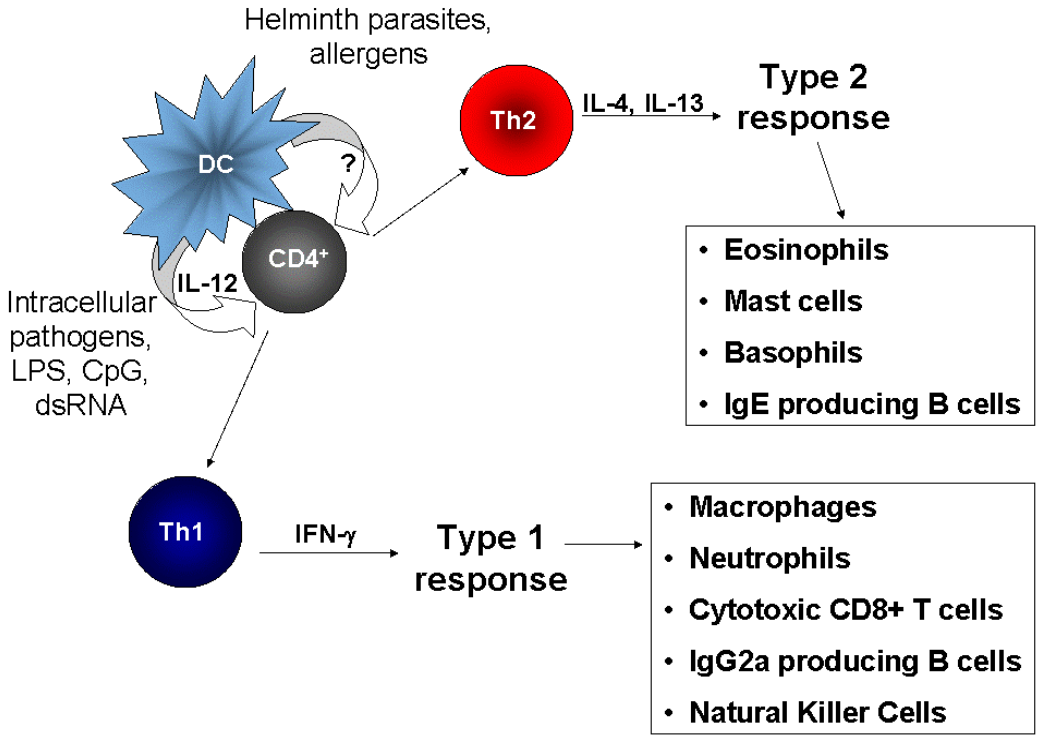

Figure 3. T helper adaptive immune responses. The presence of Toll Like Receptor ligands will activate dendritic cells, resulting in increased MHC II expression, B7-1 and B7-2 upregulation and IL-12 production. This will drive CD4+ T cell differentiation to a Th1 effector cell type. These cells make IFN-gamma, and invoke an immune response featuring inflammatory populations of macrophages, neutrophils, cytotoxic CD8+ T cells, natural killer cells, and induce class switching in B cells to produce IgG2a antibodies. Helminth parasites and

allergens result in the generation of Th2 effector cells, which make IL-4 and IL-13, triggering a response including activation of eosinophils, mast cells, basophils, and IgE production by B cells.

Memory T cell Development and Populations

Upon clearance of the antigen, a large proportion (~90%) of effector T cells undergo apoptosis; however, a small number of these cells persist and differentiate into memory cells, which reside throughout the body for long periods, and are able to respond quickly to subsequent challenges with the same antigen (**fig. 4**)[71-73].

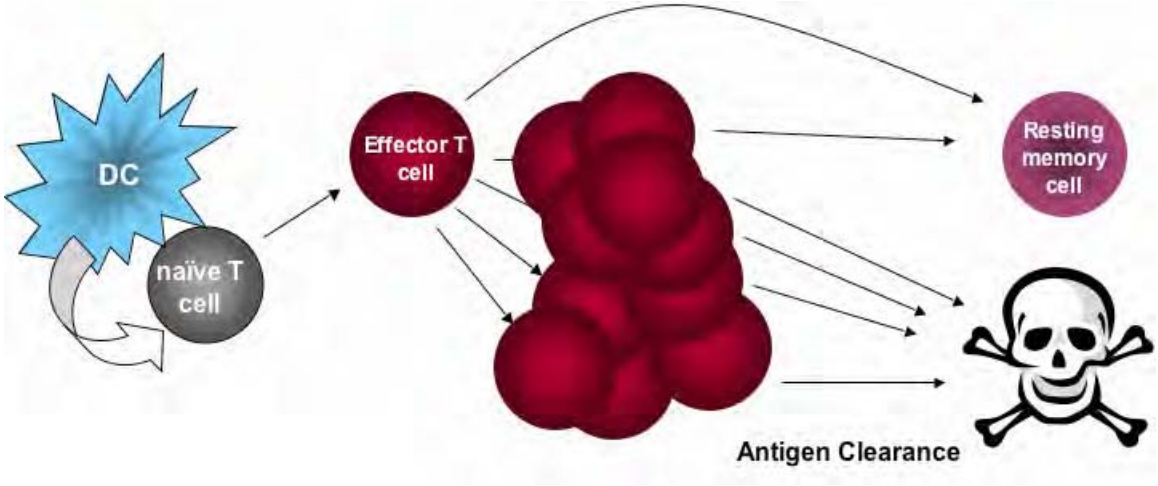

Figure 4. The generation of immunological memory. During primary immune responses, there is a massive clonaltypic expansion of effector T cells. Upon clearance of antigen, ~90% of these effector T cells enter apoptosis and die. The remaining cells make up the precursors of the memory T cell pool.

Because several models exist for their study *in vivo* and their effector cytotoxic function is easily quantified, the development and function of memory $CD8^+$ T cells is much better understood than memory $CD4^+$ T cells. Two distinct $CD8^+$ memory cell populations have been described in humans and mice. Central memory cells reside in secondary lymphoid organs, are identified based

on their expression of the lymph node homing receptors CCR7 and CD62L ($CCR7^+$ $CD62L^{high}$), and are thought to be a less polarized, longer-term memory population[74,75]. In contrast, effector memory cells do not express lymph node homing receptors ($CCR7^-$ $CD62L^{low}$) are found in non-lymphoid tissue (liver, lungs, and skin), and are described as a more easily activated and polarized population[74,76,77].

While memory $CD4^+$ T cells have been detected throughout the body, their differentiation during the primary response into central and effector memory cell populations remains unclear[72]. It has been postulated that there are distinctions between memory cells developing from Th1 and Th2 lineages, including events leading to the development of effector cells into memory cells and the maintenance of these different memory populations[78,79]. Most likely, resting memory Th2 cells develop during a polarized type 2 primary response, and exist in that polarized state, as the addition of exogenous IL-12 during nematode parasite challenge has no effect on the ensuing memory Th2 response[80].

Regulatory T cells

While the immune system is a potent mediator against invading pathogens, it has developed numerous mechanisms to down-modulate responses, thereby preventing harmful inflammation. A failure in these inhibitory mechanisms is often harmful to the host, and can lead to the development of inappropriate immune responses, some of which include autoimmune disease, allergies, and asthma. Of particular interest in recent years are regulatory T cells, which have been broadly categorized into three classes: thymus-derived

naturally occurring $CD25^+$ $CD4^+$ T cells, and two induced populations, anergic and tolerized regulatory cells[50]. Anergic cells develop in the absence of a secondary co-stimulatory signal, and tolerized cells result from oral tolerance, low-dose antigen, or are primed by inhibitory dendritic cells[50]. While these populations are difficult to distinguish *in vivo*, the transcription factor *FoxP3* is considered the best indicator of certain regulatory T cell subtypes. Other regulatory cell markers, including CD25 and CTLA-4 are also expressed by activated effector T cells, making these markers useful only in untreated mice.

A number of studies in both humans and mice have demonstrated the immunoregulatory potential of helminth infections. Clinical studies have shown infection or administration of helminthes leads to the reduced inflammation in patients suffering from IBD and contact dermitis. These studies suggest a potential mechanism behind the hygiene hypothesis, where regulatory T cells induced by helminth infections can inhibit unrelated immune responses. It is possible that this induction of regulatory T cells also explains why infections of these types are often chronic, as the presence of regulatory T cells may inhibit an effective response.

The Th2 Response

In the presence of helminth parasites and allergens, naïve $CD4^+$ T cells differentiate into Th2 cells. These effector cells make the cytokines IL-4, IL-13, IL-9, and IL-5 among others[81,82]. A growing body of literature has documented a number of other leukocytes that also adopt a type 2 activation state following exposure to Th2 cytokines produced by Th2 cells; B cells undergo class

switching to make the antibody isotypes IgE and IgG1; dendritic cells thought to initiate type 2 responses are termed DC2; and additional macrophage effector types have been described including alternatively activated macrophages and type II macrophages, which express high levels are arginase-1 or the immumosuppressive cytokine IL-10, respectively[83-87].

Some effector mechanisms associated with type 2 responses have been described thoroughly. Following the production of IgE by B cells (**fig. 5a**), the Fc (fragment crystalizable) region of the IgE molecule binds high affinity FcεR on mast cells and basophils (**fig. 5b**), allowing the antigen binding portion of the molecule to point outward, away from the mast cell or basophil[33,88]. Thus, the stage is set for the prototypic acute type 1 immediate hypersensitivity response, where re-exposure to antigen induces cross-linking of the IgE molecules on the surface of the mast cell (**fig. 5c**), leading to mast cell activation, degranulation, and the release of soluble mediators in the surrounding environment (**fig. 5d**). These factors include leukotrienes, prostaglandins, and histamine, which cause vasodilation, smooth muscle contractions, and the recruitment of eosinophils and memory Th2 cells to the site (**fig. 5e**). This response is generally associated with a deleterious acute atopic allergic response. Recent studies have suggested the Th2 cytokines IL-4 and IL-13 are essential in allergic airway responses directly binding receptors on airway tissues itself, and can directly induce mucus secretion in the absence of IL-4, IL-5 and mast cells[35].

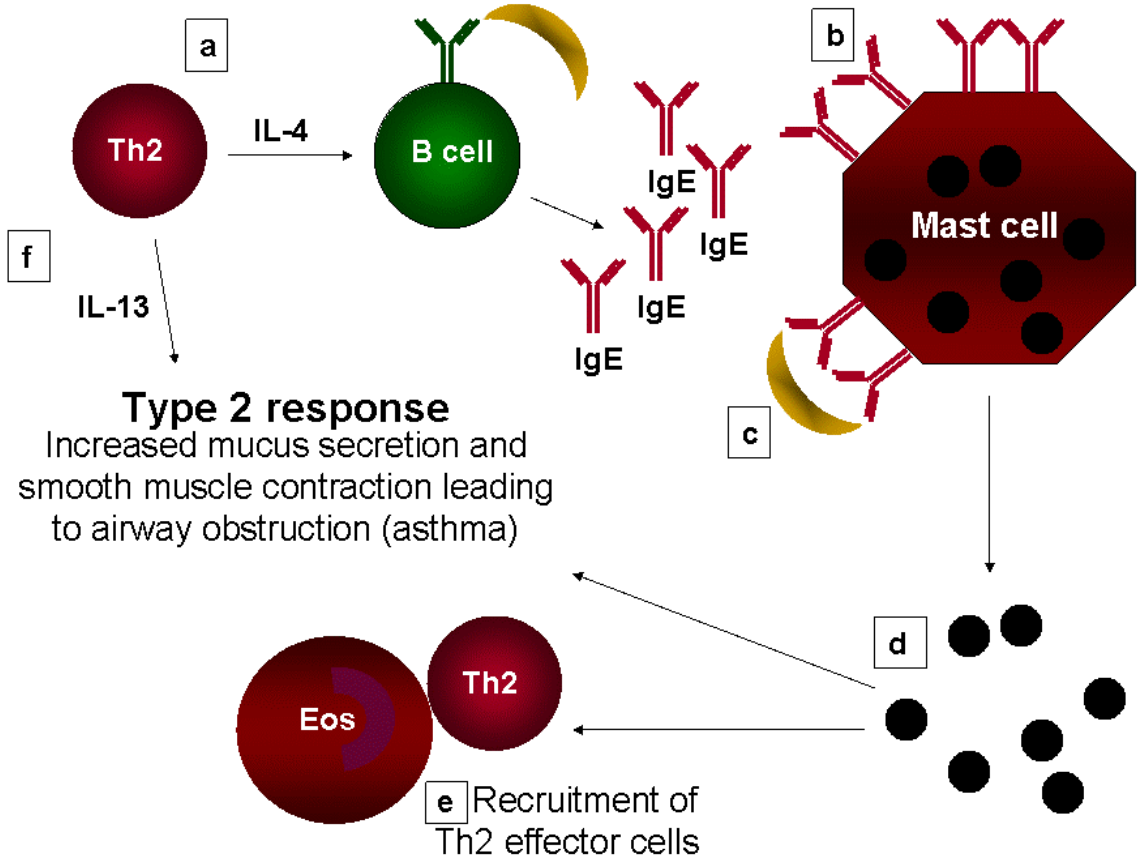

Figure 5. The mechanisms of acute and chronic allergic responses. a. Th2 responses lead to the production of IgE antibody isotypes by B cells. b. The Fc portion of IgE binds surface FcεRI on the surface of tissue residing mast cells. c. Upon re-exposure to the allergen, IgE molecules on the surface of mast cells are cross-linked, activating the mast cells leading to their degranulation (d), and release of prostaglandins, leukotrienes, and histamine. This causes immediate vasodilation, and the smooth muscle contraction associated with acute allergic reactions. e. Additionally, Th2 cells and eosinophils are recruited to the site of degranulation leading to swelling, and itching, typically of chronic allergies. f. Upon challenge, IL- 4 and IL-13 production by Th2 effector cells can also lead to airway hyperresponsiveness, and increased mucous secretion.

Th2 responses are induced following infection by gastrointestinal helminthes, and this response type is effective at expelling large, extracellular worm parasites[89-91]. However, the mechanisms leading to expulsion have largely been elusive[92]. Perhaps the best studied effector mechanisms are the changes induced by IL-4 and IL-13 on the smooth muscle of the gut, which increase

contractility and luminal fluid secretion, both proposed to make the intestinal lumen an inhospitable environment for gastrointestinal parasites[93-96]. Because $CD4^+$ T cell depletion during nematode infection blocks IL-4 and IL-13 production, these cells are thought to be the primary producers of these cytokines[97,98].

An Infectious Th2 Memory Model

The gastrointestinal trichostrongylid nematode *Heligmosomodes polygyrus*, is a natural parasite of mice, and triggers a highly polarized Th2 response in the draining mesenteric lymph node[82,90] (**fig. 7**). The use of a natural murine helminth is advantageous because this nematode evolved along with its mammalian host, and thus both host and parasite affected each other's development, resulting in a symbiotic relationship that closely mimics natural parasites of humans[99].

Mice are infected through oral ingestion of third stage larvae (**fig. 6a**), and the parasites travel to the small intestine, and invade the duodenum to take residence in the muscularis by day 3 post infection(**fig. 6b**)[100]. The larvae undergo two molts over the next five days, and by day 8 migrate to the gut lumen as sexually mature adults[101]. The adult worms mate in the lumen, and excrete eggs which are passed along with feces[101]. In the soil, the eggs hatch, and develop into the infectious L3 stage over two weeks[101].

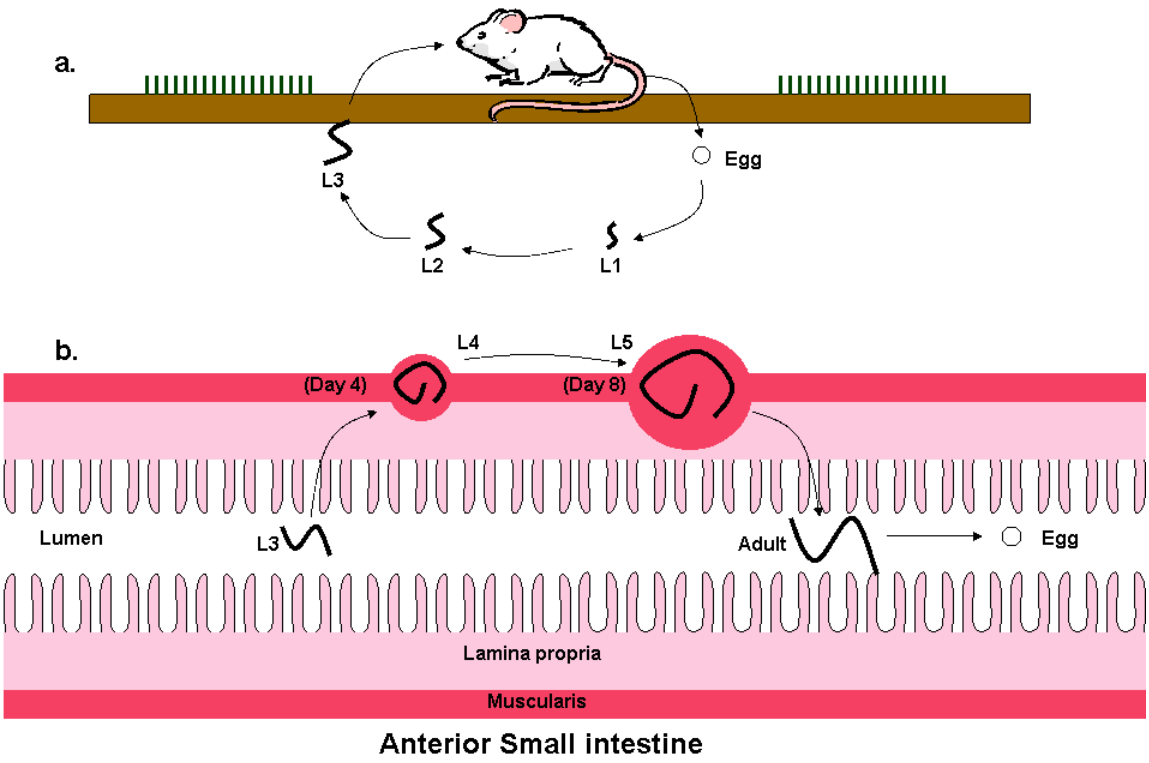

Figure 6. The life cycle of *H. polygyrus*. Following oral infection (a), *H. polygyrus* L3 larvae migrate to the small intestine, where they invade the epithelium and submucosa and take up residence in the muscularis (1b). The parasites undergo two molts there, and enter into the gut lumen as adults eight days post infection. The luminal female adult parasites excrete eggs, which are passed along with feces. The eggs hatch in the soil, and develop into the infectious L3 larvae over the next 14 days. Infection in naïve mice of all backgrounds results in a chronic luminal infection, with adult parasites teeming in the intestinal luminal by 14 days post infection, which can be cleared by the administration of an antihelminthic drug. A subsequent infection of primed and drug treated mice results in the clearance of parasites from the intestinal lumen by 14 days post infection.

There is a striking difference in the ensuing immune response to *H. polygyrus*, depending on whether the infection results from a primary or secondary inoculation. The former generates a chronic infection, cleared only by the administration of an anti-helminthic drug[98,101,102]. However, secondary infections after experimentally induced worm expulsion are naturally cleared from the

mouse after two weeks. The clearance is dependant upon IL-4 production: IL-4 deficient mice fail to expulse *H. polygyrus* upon challenge, while administration of the cytokine to infected SCID mice results in parasite clearance[97,103]. IL-4 production during a secondary response is abrogated upon depletion of $CD4^+$ T cells, which indicates a role of memory $CD4^+$ Th2 cells in worm expulsion. This distinction between primary and memory responses makes this infectious model one of the few functional $CD4^+$ T cell peripheral memory responses readily studied.

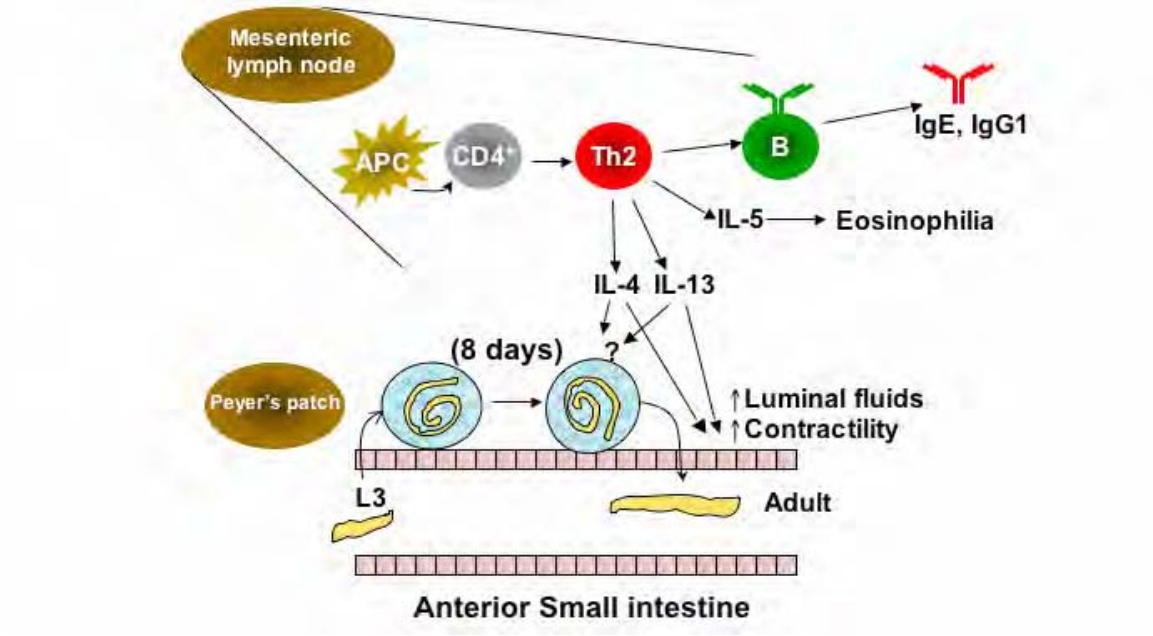

Figure 7. Infection with *H. polygyrus* triggers a highly polarized Th2 response, in all backgrounds strains of mice examined. Following oral inoculation, $CD4^+$ T cells differentiate into Th2 effector cells, producing IL-4 and IL-13. IL-5 production leads to eosinophilia, and IL-4 and IL-13 induce physiological changes on the small intestine, including increased luminal fluid secretion and gut contractility, which are thought to make the intestinal lumen inhospitable to the invading adult parasites[62].

Alternatively Activated Macrophages

Macrophages (MΦ) are phagocytic cells that reside throughout the body, and along with neutrophils are thought to be among the first responders to insulting microbes[33]. While these cells are conventionally defined as professional antigen presenting cells, their primary function is now thought to be as phagocytic effector cells capable of delivering a toxic intracellular respiratory burst of nitric oxide (NO) against phagocytosed pathogens[104]. Upon activation via Toll Like Receptor (TLR) stimulation (including TLR-4 by LPS) or exposure to IFN-γ from T cells, MΦ's upregulate the enzyme Inducible Nitric Oxide Synthase (iNOS) which converts L-arginine ultimately to NO and citrulline (**fig. 8**)[105]. This reaction is essential for a protective response against a number of intracellular pathogens, including *Listeria monocytogenes*, *Salmonella* sp., and *Leishmania* sp[106-109].

However, an additional activated phenotype has recently been identified in macrophages, which downregulates type 1 inflammatory responses and promotes type 2 inflammatory responses[83,104,110-113]. Macrophages exposed to Th2 cytokines express the enzyme arginase-1, which out-competes iNOS for their common substate L-arginine[114]. L-Arginine is converted to L-ornithine, which is further catabolized to proline by ornithine amino transferase (OAT) and polyamines by ornithine decarboxylase (ODC).

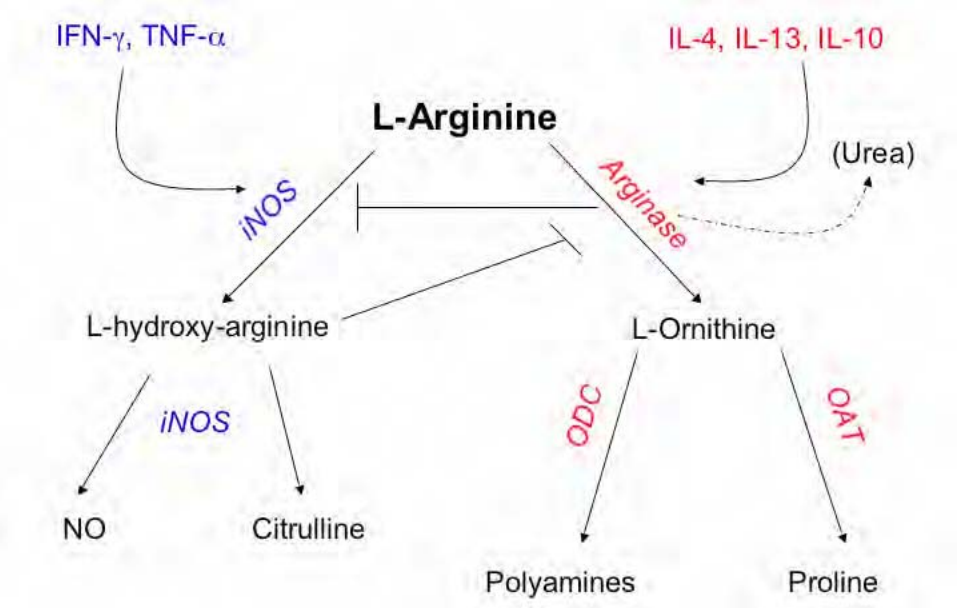

Figure 8. L-Arginine catabolism by macrophages. Upon exposure to type 1 inflammatory cytokines, macrophages upregulate the enzyme iNOS, which converts L-arginine to L-hydroxy-arginine (LOHA); LOHA is then converted by iNOS into NO and citrulline. LOHA inhibits arginase-1. In the presence of type 2 inflammatory cytokines, the enzyme arginase-1 is expressed, which has a higher affinity for L-arginine, and therefore out competes iNOS for the common substrate. Arginase-1 converts L-arginine into L-ornithine, which is further converted into proline by ornithine aminotransferase (OAT) and polyamines by ornithine decarboxylase (ODC).

Alternatively activated macrophages have been identified in a number of type 2 responses, including the hepatic fibrosis associated with Schistosomiasis, pulmonary inflammation of asthma, and mouse filariasis[115-117]. These cells recruit fibroblasts and produce the collagen precursor, proline, resulting in fibrosis necessary for tissue repair. This fibrosis becomes life-threatening in the case of *Schistosome* hepatic granulomas[116,118-121]. The production of polyamines is essential for cellular proliferation, as these amines aid in DNA-protein interactions required for DNA replication[122]. A number of characteristic genes have been associated with these cells, many of which are chitinases and

chitinase-like, including Ym1 and 2, Fizz1 and 2, and Acidic Mammalian Chitinase (AMCase)[117]. The high expression of genes of this type suggested these MΦs may play a role in anti-helminth responses; however, no protective role has been assigned.

These MΦs adopt the alternative phenotype following exposure to type 2 cytokines, specifically IL-4 and IL-13[104,113,123]. Thus, they express the IL-4 receptor (IL-4R). Following exposure to type 2 cytokines, AAMΦ upregulate surface expression of the macrophage mannose receptor (CD206), which is therefore considered a useful marker for this activation state[112,124]. Two IL-4R's exist in humans and mice; the IL-4Rα, which couples with either the common gamma chain or the IL-13Rα1 to form a type 1 or type 2 receptor, capable of binding IL-4 or both IL-4 and IL-13, respectively (**fig. 9**)[125-127]. Both receptors signal through the common adaptor protein, STAT6. A number of bone marrow derived populations (including T cells, B cells, eosinophils, mast cells, basophils, DCs, and MΦs), and non-bone marrow derived cells (including smooth muscle tissue and goblet cells) express IL-4 receptors and are therefore responsive to type 2 cytokines[93,96,128]. Exposure to IL-4 or IL-13 promotes the development of Th2 cells and class-switching to IgE in B cells, activates mast cells and eosinophils, and upregulates arginase-1 expression in DCs and MΦs; goblet cells secrete increased amounts of mucous, and smooth muscle contractility intensifies upon stimulation through the IL-4 receptor[91,93,96].

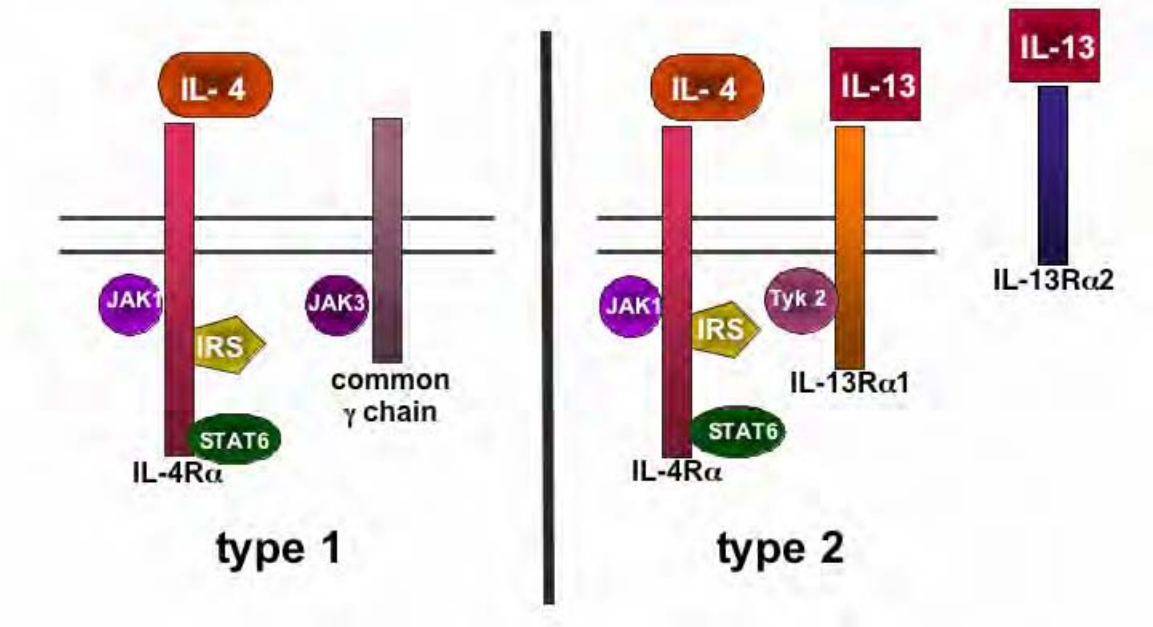

Figure 9. The proposed structures of the IL-4 receptors. The IL-4Rα couples with either the common γ chain or the IL-13Rα1 to form a type 1 or type 2 IL-4R. IL-13Rα2 is thought to be a soluble decoy receptor, binding excess IL-13 to dampen the cytokines potential detrimental effects.

Specific goals of this study

Little is know about protective Th2 responses and the mechanisms by which gastrointestinal helminths are expelled from the mammalian intestinal tract have remained elusive. Using an infectious model which elicits a natural protective immunity to a nematode parasite, we set out to define the role of memory Th2 cells in this response. The studies outlined herein identified early events in this response as essential for maximal host-protection. Initially, $CD4^+$ T cells were depleted at distinct intervals during challenge, to examine when $CD4^+$ T cell dependent immune events occurred during infection. These depletion experiments showed $CD4^+$ T cells were required during the first seven days of challenge, as mice depleted of these cells at later stages (days 7, 9, or 11 post infection) of the response retained their ability to expel the parasites, suggesting

events leading to eventual worm expulsion were set in place during the first 7 days of the memory response. These findings were extended using a Baermann apparatus to recover larvae in the intestinal tissue at days 4 and 7 post infection, as the developing parasites had difficulty migrating out of the tissue during a secondary response compared to those recovered during a primary infection[129].

Our initial studies showed the immune response during the first 7 days of infection were essential for host-protection, when larval parasites develop in the small intestinal submucosa, and therefore the host:parasite interface was examined. Four days after challenge, a characteristic and highly reproducible immune cell architecture was identified at the host:parasite interface, with $Gr1^+$ neutrophils accumulating immediately adjacent to the parasite, memory $CD4^+$ T cells accumulating in a region surrounding the parasite and neutrophils, and Th2 cytokine mRNA detected in a $CD4^+$ T cell dependent fashion[78,130,131]. LCM preformed in combination with fluorescent immunohistochemistry allowed the dissection $Gr1^+$, $CD4^+$, and $Gr1^-$ $CD4^-$ cells from this microenvironment, and demonstrated that both $Gr1^+$ and $CD4^+$ populations expressed high levels of IL-4 and IL-13 mRNA relative to untreated Peyer's patches. These findings were extended further by the development of a novel approach to detect IL-4 protein *in situ*, which showed that the accumulating CD4+ T cells were associated with IL-4 protein at the host:parasite interface, strongly indicating this population was the main producer of the prototypical type 2 cytokine. $F4/80^+$ macrophages accumulating around the parasite during challenge expressed high levels of IL-4Rα and the macrophage mannose receptor (CD206), consistent with an

alternatively activated phenotype (IL-4R^{hi} CD206^{+}). Microdissection of the F4/80+ cells from the host:parasite interface confirmed their alternatively activated phenotype, as these cells featured high arginase-1, Fizz1, Ym1 and AMCase-1 mRNA, and undetectable iNOS. Adoptive transfer of memory CD4^{+} T cells into STAT6 deficient ($^{-/-}$) recipient mice failed to induce alternative macrophage activation, as did depletion of CD4^{+} T cells at the time of infection; taken together, these results indicated that the macrophages accumulating at the host:parasite inteface were becoming alternatively activated by type 2 cytokine producing CD4^{+} T cells.

These AAMΦs were then shown to be essential effector cells of the protective immune response against *H. polygyrus* challenge in corroborative intervention experiments. Selective depletion of these cells using clodronate liposomes and inhibition of their effector function by an arginase inhibitor abrogated the protective response, indicating that these cells are essential effectors in this anti-helminth response. These studies provide a new model for protective Th2 responses, where memory Th2 cells rapidly accumulate and express IL-4 and drive the alternative activation of macrophages. This work provides new insights into mechanisms of expulsion of gastrointestinal parasites, and identifies macrophages as important effector cells in protective Th2 responses, providing a possible explanation for the evolution of their alternatively activated state.

Peripheral CD4 T Cells Rapidly Accumulate at the Host:Parasite Interface during an Inflammatory Th2 Memory Response[1]

Motoko Morimoto,* Masahiro Morimoto,† Jeannette Whitmire,* Shiyun Xiao,* Robert M. Anthony,* Hiroshi Mirakami,‡ Robert A. Star,‡ Joseph F. Urban, Jr.,† and William C. Gause[2]*

Memory peripheral Th2 immune responses to infectious pathogens are not well studied due to the lack of suitable models and the difficulty of assessing Th2 cytokine expression at sites of inflammation. We have examined the localized immune response to a nematode parasite that encysts in the small intestine. An unexpected architecture was observed on day 4 of the memory response, with granulocytes and macrophages infiltrating the cyst and $CD4^+$, TCR-$\alpha\beta^+$ T cells surrounding the cyst. Laser capture microdissection analysis showed a pronounced CD4-dependent Th2 cytokine pattern at the cyst region only during the memory response, demonstrating that the Th2 memory response is readily distinguished from the primary response by the rapid accumulation of Th2 effector cells at the host:parasite interface. ***The Journal of Immunology,*** **2004, 172: 2424–2430.**

During an effective adaptive immune response it is thought that naive CD4 T cells differentiate to effector memory T cells that migrate to peripheral nonlymphoid tissues where the memory cells can rapidly respond to a subsequent challenge. Accumulation of CD8 T cells or IFN-γ-producing Th1 cells at peripheral sites of infection is well established (1). However, accumulation of polarized, IL-4-producing Th2 cells in peripheral nonlymphoid tissues during infectious disease is not well examined, partly because of the paucity of suitable models. Indeed, several reports have suggested that the Th2 response is more associated with B cell help in lymphoid tissue and that effector T cell migration to peripheral nonlymphoid sites of infection is more associated with inflammatory Th1 responses (2).

The primary response to the murine gastrointestinal nematode parasite, *Heligmosomoides polygyrus* (HP),[3] is associated with chronic infection. However, if the parasite is cleared from the mouse with an antihelminthic drug, subsequent reinfection of the mouse with HP results in a memory response and worm expulsion by ~2 wk after inoculation (3, 4). This clear distinction between primary and memory responses makes this infection a useful model for study of a functional memory Th2 response. Infection of mice with HP involves oral inoculation with third-stage larvae (L3) that migrate into the muscularis at the submucosal border of the jejunum of the small intestine and develop into adults over an 8-day period, after which the parasites migrate back to the gut lumen. An inflammatory cell infiltrate develops around the invading parasitic larvae, resulting in a border of cells that delineate a loosely defined, cyst-like structure (5, 6). Although the mechanism of worm expulsion remains uncertain, direct effects of IL-4 and IL-13 on gut tissue are probably important (7–9), and some studies have suggested that an effective memory response is also associated with delayed larval development before emergence into the lumen (5, 10). The CD4 T cell response has not been examined at peripheral sites of infection, but pronounced increases in CD4 T cell IL-4 and IL-13, but not IFN-γ, are detected in the mesenteric lymph node (MLN) as early as day 8 after primary inoculation and secondary challenge (11, 12).

Investigation of cytokine production at the localized site of parasite infection is difficult due to the short half-life and low expression level of IL-4 and IL-13 mRNA and protein. The relatively small number and size of cysts in the intestinal jejunum and the presence of other lymphoid tissue, including lamina propria and Peyer's patch, make it difficult to extrapolate cytokine expression patterns in whole gut to changes that may be occurring at the host:parasite interface. The recent development of laser capture microdissection (LCM), which permits microscopic visualization and selection of specific tissue regions for mRNA analysis (13), provides a technology that makes it possible to assess Th2 cytokine gene expression in localized tissue microenvironments.

In the studies described in this report, we characterized a peripheral Th2 memory immune response. Our findings unexpectedly indicate that the Th2 response is initially associated with pronounced infiltration of neutrophils and macrophages to regions immediately adjacent to the parasite. In the memory, but not the primary, response, $CD4^+$ T cells also accumulated as early as 4 days after HP inoculation, and LCM analysis showed pronounced CD4-dependent elevations in Th2 cytokines. These studies demonstrate that a highly polarized Th2 memory response can rapidly develop in peripheral nonlymphoid tissues during infectious disease.

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[3] Abbreviations used in this paper: HP, *Heligmosomoides polygyrus*; DAPI, 4′,6-diamido-2-phenylindole hydrochloride; L3, third-stage larvae; LCM, laser capture microdissection; MLN, mesenteric lymph node; Rx, inoculation.

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Materials and Methods

Animals and treatments

Female BALB/c mice (Small Animal Division, National Cancer Institute, Fredrick, MD) were used for each experiment and were inoculated orally with 200 L3 as previously described (14). Separate groups of HP1°-infected mice were treated with the antihelminthic drug, pyrantel pamoate, at 14 days after inoculation (Rx), and were reinfected orally with 200 L3 at 50 days after inoculation (HP2°) or were not infected (HP1°+Rx). The gut tissue was collected on day 4 after secondary inoculation. Some groups of HP-challenged mice were treated with anti-CD4 Ab (GK1.5) on day 3 and examined on day 4 after secondary inoculation.

Preparation of frozen tissue blocks and sectioning

The gut tissues were taken from the jejunum of HP-infected mice, slit longitudinally, prepared using the Swiss-roll technique (15, 16), embedded in Tissue-Tek OCT compound (Sakura Finetek U.S.A., Torrance, CA), frozen on dry ice-acetone, and stored at −80°C. For LCM, 4-μm tissue sections were cut from frozen blocks using an HM505E cryostat (Richard-Allan Scientific, Kalamazoo, MI).

Immunohistological analysis

Four-micron frozen tissue sections of Swiss-rolled jejunum of the small intestine were fixed in cold acetone and then stained with anti-CD11c-PE, anti-Gr-1PE, anti-Gr-1-FITC, anti-B220(6B2)-FITC, anti-TCR-$\gamma\delta$-PE, anti-CD8-PE, anti-TCR-β-PE, and anti-CD4 PE (BD PharMingen, San Diego, CA) or F4/80-PE (5 μg/ml; Caltag, Burlingame, CA), followed by 10 μg/ml of 4′,6-diamido-2-phenylindole hydrochloride (DAPI; Roche, Indianapolis, IN) for fluorescence analysis as previously described (14). For composite photos, a 4-μm section of intestinal jejunum prepared as a Swiss roll was mapped using scanning software designed by J. Czege (Biomedical Instrumentation Center, Unformed Services University of the Health Sciences). As previously described for lymph node tissue (17), mapped regions of the intestinal tissue section were then individually photographed at ×200 magnification using a SPOT2-cooled CCD camera (Diagnostic Instruments, Sterling Heights, MI) mounted on a Leica DMRXA (Leica Microsystems, Bannockburn, IL) computerized fluorescence microscope and using SPOTAdvance software (Diagnostic Instruments). Each fluorescent channel was photographed separately, and the three sets of ×200 images were merged using TIFFany3 software (Caffeine Software, Santa Clara, CA) to create the final composite photo of intestinal tissue.

LCM and real-time RT-PCR

Cryosectioned tissue sections of a Swiss roll preparation of the jejunum of the small intestine were dehydrated and stained for H&E (Sigma-Aldrich, St. Louis, MO). LCM of stained sections was performed on a PixCell II (Arcturus Engineering, Mountain View, CA), and captured cells were transferred to CapSureTM LCM Caps (Arcturus Engineering, Mountain View, CA). The LCM cap was inserted into a 0.5-ml microcentrifuge tube containing RNA isolation solution, and total RNA was extracted using an RNA isolation kit (Stratagene Cloning Systems, La Jolla, CA). In other experiments whole MLN tissue was homogenized in RNAzol B (Cinn/Biotecs, Friendswood, TX) and purified according to the manufacturer's instructions. Total RNA was then reverse transcribed as previously described (18). Real-time PCR kits (PE Applied Biosystems, Foster City, CA), specific for individual cytokines or rRNA, were used to quantitate differences in gene expression; all data were normalized to constitutive rRNA values and a PE Applied Biosystems 7700 sequence detector was used for target mRNA amplification.

Results

A specific immune cell infiltrate architecture develops at the host parasite interface of HP-challenged mice

The HP L3 encyst within the muscularis that borders the submucosal region of the jejunum of the small intestine. Previous studies have reported that an inflammatory infiltrate develops around the worm, which includes both macrophages and polymorphs, and that resistance after secondary challenge may be associated with delayed maturation of parasitic larvae within the intestinal mucosa (4, 5, 10). To further characterize the cells infiltrating the cyst, we used immunofluorescent staining with specific Abs detecting cell surface markers on cells associated with the immune response. Fig. 1*A* shows a section of jejunum from a Swiss roll preparation on

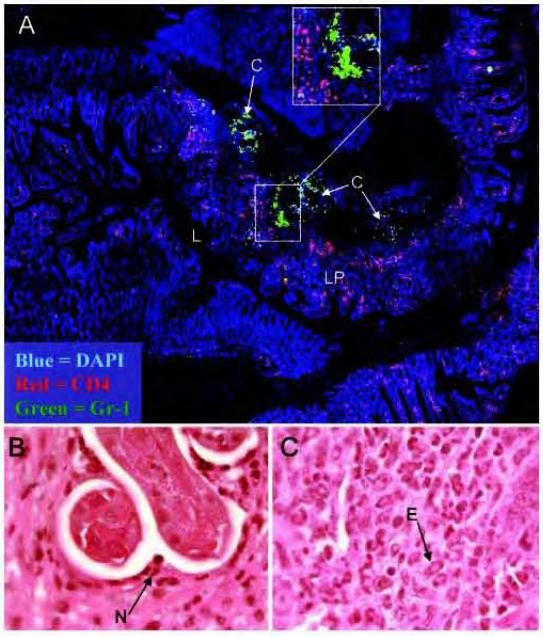

FIGURE 1. CD4 T cells migrate to the cyst region on day 4 after HP secondary inoculation, surrounding the neutrophil-infiltrated cyst region. BALB/c mice were orally infected with 200 HP L3 larvae and then treated with the anthelminthic, pyrantel pamoate, at 2 wk postinfection. Fifty days after the initial inoculation, the mice were administered a challenge dose of HP. Four days after challenge or priming (depending on the group), intestines were collected (using the Swiss roll technique), prepared for frozen sectioning, and subsequently sectioned and stained for anti-CD4-PE (red), anti-Gr-1-FITC (green), and DAPI (blue). *A*, The surface of the entire tissue section was digitally mapped and photographed at ×200 magnification with a Leica DMRXA fluorescence microscope, and individual fluorescent channels and images were merged using TIFFany3 software to create the final picture, as described in *Materials and Methods*. *B*, High power H&E stain of cyst region showing infiltrating neutrophils. *C*, High power H&E stain of lamina propria showing abundant eosinophils. *C*, Cyst; LP, lamina propria; N, neutrophil; E, eosinophil.

day 4 after HP challenge inoculation that was stained for CD4 (red), Gr-1 (green), and DAPI (blue) as a background stain. The high resolution microphotograph shown is actually a composite of multiple photos (×200) taken individually and tiled together as described in *Materials and Methods*. Areas of cysts containing individual larva are readily observed as Gr-1$^+$ cells specifically infiltrate them. Dark regions inside the cyst are sections of the actual parasite. CD4$^+$ T cells are greatly increased in regions of the lamina propria near the cyst, indicating specific localization of CD4$^+$ T cells to the area surrounding the invading parasite and associated granulocytes during challenge inoculation. High power analysis (×1000) of H&E-stained, paraffin-embedded sections showed predominantly neutrophils and macrophages, with occasional, but scarce (<1%), eosinophils (Fig. 1*B*). Surprisingly, although eosinophils were rare in the region around the cyst, they were abundant in the lamina propria (Fig. 1*C*). As expected, eosinophils in the lamina propria did not stain for Gr-1.

Additional phenotyping was performed and is shown in Fig. 2. Specific staining for macrophages (F4/80) showed that cells of this phenotype were localized to regions of the lamina propria immediately surrounding the cyst and that they also infiltrated the cyst

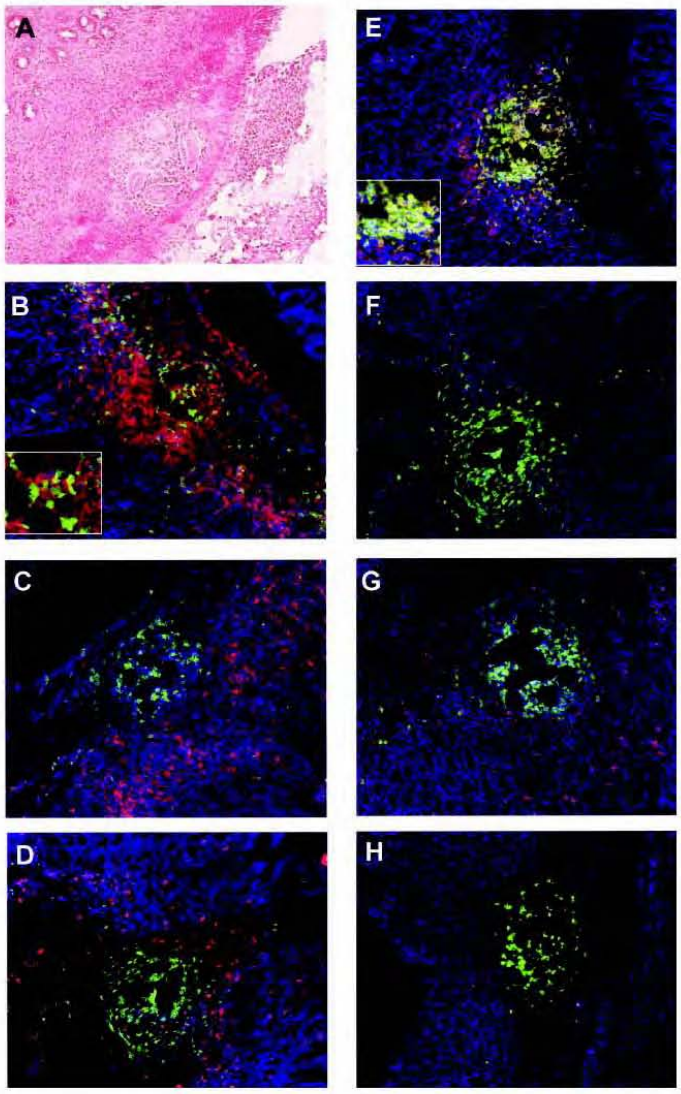

FIGURE 2. A characteristic inflammatory Th2 response rapidly develops around the invading larval cyst by day 4 after HP inoculation. HP-inoculated BALB/c mice were primed and challenged as described in Fig. 1. On day 4 after challenge (*A–F*), primary immunization (*G*), or challenge plus anti-CD4 treatment (*H*), the small intestine was collected as described in Fig. 1, and frozen sections were stained for: *A*, H&E; *B*, GR-1 FITC and macrophages (F4/80 PE); *C*, GR-1 FITC and dendritic cells (CD11c PE); *D*, GR-1 FITC and T cells (TCRβ PE); *E*, GR-1 FITC and CD11b (MAC-1 PE); *F*, GR-1 FITC and TCR-$\gamma\delta$ (GL3 PE); *G*, GR-1 FITC and CD4 (GK1.5 PE); and *H*, GR-1 FITC and CD4 (GK1.5 PE). Enlarged regions of cellular infiltration in the cyst region are shown as *insets* in *B* and *E*. All images were taken of 4-μm sections at $\times$100, and images are representative of cysts obtained from more than four mice and stained with all Abs shown.

region (Fig. 2*B*). Gr-1$^+$ cells also infiltrated the cyst, but did not dual stain with F4/80, indicating that these two Ags were expressed on distinct populations (Fig. 2*B*, *inset*). Gr-1$^+$ cells also stained CD11b$^+$ (Fig. 2*E*). The *inset* of Fig. 2*E* shows considerable yellow fluorescence resulting from both cell surface Ags being expressed on the same cell. The observation that Gr-1$^+$ cells were CD11b$^+$ and F4/80$^-$ is consistent with a neutrophil phenotype. Both the fluorescent and the H&E staining indicated that there was pronounced infiltration by macrophages and neutrophils in approximately equal numbers. CD11c$^+$ dendritic cells were restricted to the lamina propria area outside the cyst region, showing a similar distribution to CD4$^+$ cells (Fig. 2*C*). TCR-$\gamma\delta$ cells (Fig. 2*F*), B cells (data not shown), CD8 T cells (data not shown), and NK cells (data not shown) were not detected. TCR-$\alpha\beta^+$ T cells (Fig. 2*D*) showed the same distribution as CD4$^+$ T cells, and two-color staining confirmed that the CD4$^+$ cells were predominantly TCR-$\alpha\beta^+$ (data not shown). Jejunum samples were also examined on day 4 after primary HP inoculation. Similar, but markedly reduced, populations of infiltrating cells were observed (data not shown), and CD4 T cells had not accumulated to a significant degree around the cyst (Fig. 2*G*). Thus, an acute inflammatory response associated with high numbers of CD4$^+$ T cells and a highly specific immune cell architecture develops as early as day 4 after HP challenge inoculation.

IL-4 and IL-13 gene expression in different microenvironments of the small intestine after HP priming and challenge

Resistance to HP, which occurs after secondary challenge, but not after primary inoculation, is immune mediated and CD4$^+$ T cell dependent (19). The early activation and maintenance of this robust type 2 immune response, which includes increased expression of IL-4, but not IFN-γ, in the draining lymph nodes on day 10 after

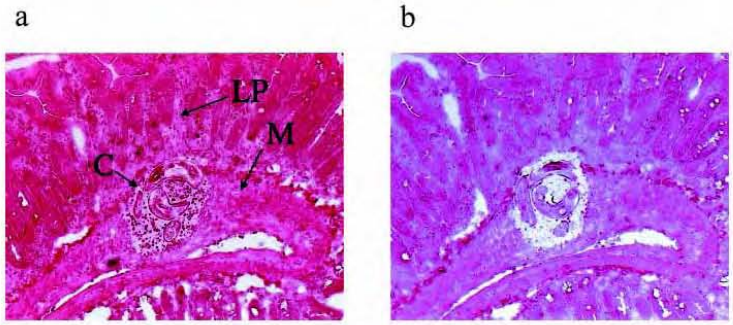

FIGURE 3. Use of LCM to sample microenvironments in the small intestine after a secondary challenge infection with HP. BALB/c mice were primed and challenged with HP as described in Fig. 1. On day 4 after challenge, the small intestine was collected as described in Fig. 1 and prepared for LCM as described in *Materials and Methods*. In this example, multiple 7.5-μm samples were collected inside the cyst region, including samples immediately adjacent to the invading larvae. An H&E-stained cyst is shown before (*a*) and after (*b*) LCM sampling. LCM was performed on a PixCell II (Arcturus Engineering), and target cells were transferred to CapSureTM LCM Caps (Arcturus Engineering). Cells were also captured from the lamina propria, Peyer's patch, and immediately surrounding the cyst. LP, Lamina propria; C, cyst; M, muscularis.

challenge inoculation are required for controlling the level of infection (4, 7, 12, 20).

To identify mucosal microenvironments outside the secondary lymphoid tissues, where Th2 cytokines were expressed, we used LCM. This technique allows microscopic collection of individual tissue samples ~7.5 μm in diameter, which usually includes one to three cells (see Fig. 3). To examine the gene expression of individual microenvironments in the small intestine of HP-infected mice, cells were captured from 1) lamina propria; 2) Peyer's patch; 3) the area immediately surrounding the cyst, including $CD4^+$, TCR-$\alpha\beta^+$ cells in HP-challenged mice; and 4) the area inside the cyst, containing primarily macrophages and neutrophils, but no T cells. Multiple samples (~1000) were taken in the selected regions for each mouse and pooled for RNA isolation.

RNA was purified from pooled LCM samples and reverse transcribed, and IL-4, IL-13, IFN-γ, and rRNA were assessed by real-time RT-PCR. All data were normalized to constitutive rRNA values. In each experiment, all samples were measured in the same assay for each cytokine, allowing direct comparison between different groups or tissues. In all groups and tissues examined, no elevations were detected for IFN-γ (data not shown). On day 4 after primary HP-inoculation, IL-4 and IL-13 were detected in the Peyer's patch, but not in the lamina propria, inside the cyst, or in the area immediately surrounding the cyst (Fig. 4). Similarly,

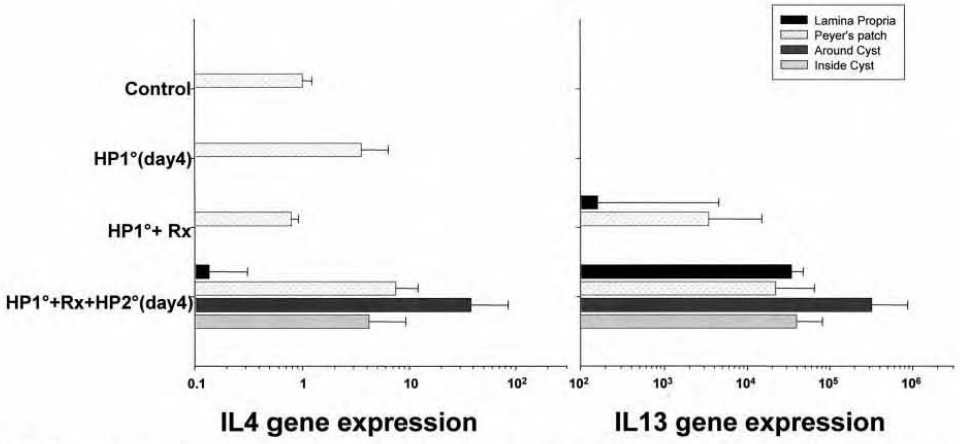

FIGURE 4. IL-4 and IL-13 mRNAs are markedly elevated in and around the larvae-induced cyst on day 4 after HP secondary, but not primary, inoculation. BALB/c mice (five per treatment group) were primed and challenged as described in Fig. 1. Two groups received a primary infection, followed 14 days later with an anthelminthic treatment (Rx); tissues were taken from one group at 54 days after primary inoculation (HP1° + Rx), and the other was given a challenge infection at 50 days after the primary infection, and tissues were collected 4 days later (HP1° + Rx + HP2° (day 4)). A third group received only a primary HP infection, and tissues were taken 4 days later (HP1° (day 4)), at the same time as in the other groups of mice. LCM samples were collected from individual mice (five per treatment group) as described in Fig. 3, and RNA was purified, reverse transcribed, and analyzed for the expression of rRNA, IL-4, IL-13, and IFN-γ mRNA using real-time PCR. All data were normalized to constitutive rRNA values, and no elevations in IFN-γ were detected. All samples were expressed relative to untreated Peyer's patch. mRNA levels were determined for all treatment groups shown; in groups in which levels were undetectable or below graph values (IL-13 control PP), no bar is shown. The results are expressed as the mean and SE for each treatment group, and similar results were obtained in two experiments.

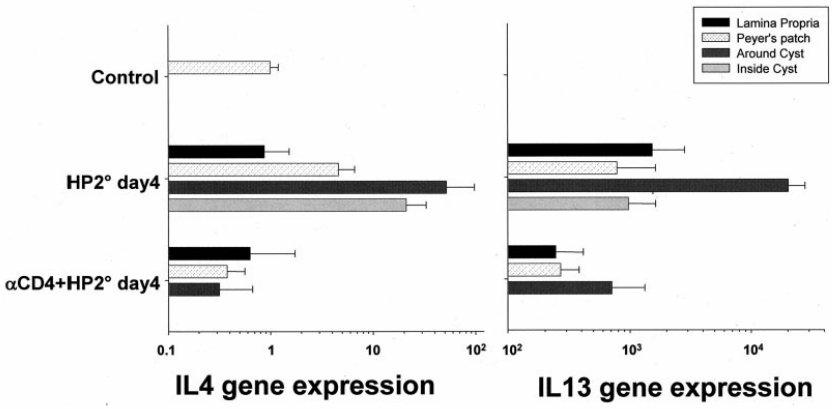

FIGURE 5. Elevations in IL-4 and IL-13 at the host:parasite interface are CD4 dependent. BALB/c mice (five per treatment group) were primed and challenged with HP as described in Fig. 1, except that anti-CD4 mAb was given on day 3 after challenge to deplete $CD4^+$ T cells. All data were expressed relative to untreated Peyer's patch, and the values are presented as described in Fig. 4. The results are expressed as the mean and SE for each treatment group, and similar results were obtained in two experiments.

although residual cysts were present in the intestinal muscularis of the control group on day 54 after primary inoculation (did not receive a challenge inoculation), no elevations in IL-4 and IL-13 were detected in Peyer's patch, lamina propria, or inside or surrounding the residual cysts (data not shown). However, on day 4 after HP secondary challenge, pronounced IL-4 and IL-13 elevations occurred in Peyer's patch and lamina propria and inside the cyst. Even higher elevations in these Th2 cytokines were detected in the nonlymphoid tissue immediately surrounding the cyst. Thus, at this early time point of the memory response, but not the primary response, Th2 cytokine gene expression was pronounced at the localized site of the host:parasite interface. Using the LCM technique, we have also examined cytokine gene expression at a later time point after secondary challenge (day 12). Our results showed that IL-4 and IL-13 mRNA elevations were substantially reduced in the area surrounding and inside the cyst, with no elevations in IFN-γ detected (data not shown). This is most likely a consequence of the immune stimulus, namely the worm, leaving the cyst region to occupy the intestinal lumen on day 8 after challenge inoculation.

Anti-CD4 treatment inhibits Th2 cytokine elevations

To examine the contribution of $CD4^+$ T cells to the increased Th2 cytokine gene expression in tertiary tissue sites after secondary challenge with HP, HP-challenged mice were administered anti-CD4 Abs at doses (1 mg) that have previously been shown to effectively inhibit the host protective memory response and block elevations in MLN Th2 cytokine gene expression (19). As shown in Fig. 5, mice that received anti-CD4 Abs on day 3 after secondary HP inoculation and were examined on day 4 showed markedly reduced IL-4 and IL-13 elevations (>50-fold) in the region surrounding the cyst, where the $CD4^+$, TCR-$\alpha\beta^+$ T cells were localized. It should be noted that although gene expression was reduced after CD4 T cell depletion, residual elevations were still detectable, suggesting that blockade was not complete or that non-CD4 cells may contribute slightly to elevations in cytokine gene expression. After anti-CD4 Ab treatment, marked decreases were also observed inside the cyst, suggesting that the presence of $CD4^+$ T cells surrounding the cysts was required to induce increased cytokine gene expression by cell populations inside the cyst. It should also be noted that granulocyte infiltration was not reduced or changed after anti-CD4 Ab administration during the HP challenge response (Fig. 2*H*). This is similar to our findings for the HP primary response, where neutrophils infiltrated the cyst on day 4 after HP primary inoculation, although $CD4^+$ T cell expansion was not observed (Fig. 2*G*).

Th2 cytokines are up-regulated as early as day 2 in the MLN during the HP memory response

To examine whether a Th2 memory response in the draining lymph node precedes the Th2 memory response observed in the cyst on day 4 after HP inoculation, MLNs were collected from HP-primed and -challenged mice at several time points after inoculation. As shown in Fig. 6, IL-4 and IL-13 gene expression were elevated as early as day 2 after HP secondary challenge, whereas greatly reduced increases were observed after primary immunization. These early Th2 cytokine elevations in the MLN,

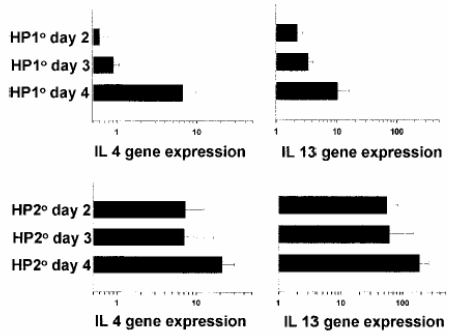

FIGURE 6. Pronounced elevations in IL-4 and IL-13 mRNA are observed at early time points of the HP memory response. BALB/c mice (five per treatment group) were primed and challenged with HP as described in Fig. 1. On days 2, 3, and 4 after primary or secondary inoculation, MLNs were collected and individually analyzed for cytokine gene expression using quantitative real-time RT-PCR. Data were individually normalized to rRNA values, and treatment group means are expressed relative to the mean of uninfected control MLNs. The results are expressed as the mean and SE for each treatment group.

specifically associated with the memory response, suggests that memory T cells initially stimulated in the draining lymph nodes may provide a source for Th2 memory cells at the host:parasite interface.

Discussion

The peripheral memory CD4 T cell response is one of the most important outcomes of adaptive immunity during infectious disease, providing the basis for rapid natural immune protection and vaccine development. We have characterized the cell populations at the host:parasite interface during a peripheral memory Th2 immune response to an intestinal nematode parasite and used LCM to characterize localized cytokine gene expression at specific sites in peripheral mucosal tissues from infected mice. Our findings demonstrate that 1) during the Th2 memory response a specific microenvironment rapidly develops with neutrophils and macrophages infiltrating and $CD4^+$, TCR-$\alpha\beta^+$ T cells surrounding the cyst-like structure associated with the invading parasite; 2) after challenge, the CD4 T cells at the host:parasite interface are highly polarized, expressing pronounced elevations in Th2 cytokines; and 3) the rapid CD4 T cell response and associated cytokine elevations are not observed during the primary response, although neutrophils and macrophages still accumulate at the host:parasite interface.

Previous studies have suggested that effector memory T cells develop in lymph nodes during the primary response and migrate to peripheral tissues, where they can rapidly respond to secondary stimulation. This model is based on responses that stimulate CD8 memory T cells (1, 21) or, more recently, CD4 Th1 memory cells (22, 23); studies of the latter population frequently rely on potentially nonphysiological approaches involving responses of Ag-specific transgenic T cells that have been transferred to Ag-immunized recipients. In the results reported in this paper we have defined an experimental system for examining peripheral memory Th2 effector cell function at the host:parasite interface. The response to this natural murine parasite has previously been shown to be highly polarized, with elevations in IL-4, but not IFN-γ or IL-10, in the draining lymph nodes after either primary or challenge HP inoculation (11, 12). We have used this model system to describe for the first time the development of a highly polarized peripheral Th2 memory response during infectious disease. The rapid development of this response after challenge, but not primary, inoculation suggests that Th2 memory cells have the capability to rapidly migrate to peripheral nonlymphoid sites and differentiate into potent sources of Th2 cytokines. This scenario is analogous to models for peripheral memory Th1 responses, suggesting that Th2, as well as Th1, memory cells are capable of rapid migration and effector cell function at sites of inflammation. As elevations in Th2 cytokines were detected in the MLN as early as day 2 after challenge inoculation, it remains uncertain whether the CD4 cells at the site of infection migrated from the lymph nodes or from peripheral nonlymphoid tissues. The observation that IL-4 and IL-13 are elevated, whereas IFN-γ is not, in both lymphoid and peripheral nonlymphoid tissues after challenge inoculation suggests that in the Th2 response to HP, central memory cells as well as peripheral memory cells exhibit a highly polarized Th2 response. This is in contrast to studies in some other model systems, where central memory cells in the lymph nodes may initially produce a mixed Th1/Th2 cytokine pattern after challenge immunization (24, 25).

In the memory response, pronounced increases in IL-4 and IL-13, but not IFN-γ, were observed in lamina propria, Peyer's patch, and the microenvironment of the invading parasite, indicating that this particular pathogen triggers a highly polarized Th2 cytokine pattern that rapidly develops in different mucosal secondary lymphoid and tertiary tissue sites. Interestingly, although the developing larval parasite resides in nonlymphoid tissue, infiltrating cells at the host:parasite interface raised Th2 cytokine mRNA to levels at or above those obtained in mucosal lymphoid tissues. The degree of Th1/Th2 skewing appears to vary greatly depending on the particular invading pathogen. For example, the peripheral response to influenza is polarized toward a Th1 pattern (26), whereas the response to *Listeria* is associated with elevations in both Th1 and Th2 cytokines (27). However, a caveat is that cytokine expression in these reports was examined using in vitro restimulation assays, which may result in the production of cytokines not expressed in vivo.

The use of LCM permitted direct assessment of cytokine mRNA levels using highly sensitive fluorogenic RT-PCR analysis at the specific site where the parasite encysted in the small intestine. Less sensitive techniques, such as in situ hybridization or protein staining, are usually not practical with Th2 cytokines due to their short mRNA and protein half-life. Conventional analysis of gene expression using more sensitive techniques are limited to much larger regions of tissue (28), making it difficult to distinguish specific microenvironments, in this case regions where host:parasite interactions actually occur in the intestinal tissue. Other approaches for the detection of IL-4 expression have used transgenic reporter mice or knockin mice expressing green fluorescent protein linked to IL-4 (29). However, in these studies the reporter protein may not be secreted similarly to the cytokine, or its half-life may be different. LCM has the distinct advantage, in that one can actually examine individual regions of interest from undisturbed specific tissue microenvironments in normal mice and directly assess in situ cytokine gene expression using highly sensitive, PCR-based techniques.

A characteristic and consistent pattern of specific immune cell infiltrates was observed at the host:parasite interface during the memory Th2 response. Although macrophages both infiltrated and surrounded the cyst region, neutrophils were predominantly observed only in the region immediately around the parasite. Of note, eosinophils were restricted to the lamina propria and were rare to absent at the host:parasite interface on day 4 after inoculation. Macrophages and neutrophils are generally associated with Th1 inflammatory responses and down-regulated production of Th2 cell cytokines (30–33). However, recent studies have suggested that IL-4 and IL-13 can induce alternatively activated macrophages that show distinct phenotypes from macrophages activated by IFN-γ (34–36). Recent findings have also indicated that activated neutrophils can produce IL-4 (37) and that they accumulate during chronic allergic responses (38). Our findings indicate that neutrophils and macrophages are major infiltrating populations at early stages of the peripheral Th2 as well as the peripheral Th1 inflammatory response, demonstrating the plasticity of these responding populations during infection. Interestingly, a previous study has suggested that neutrophils may be important in protective immunity against HP (39). Macrophage infiltration is also observed in the granulomatous response to schistosome eggs; however, at early stages of this primary response a mixed Th1/Th2 cytokine pattern is observed (40, 41), whereas the memory response to HP is not associated with elevations in Th1 cytokines even at early stages of the response. It should be noted that the HP infectious model system is particularly useful for CD4 Th2 cell memory studies, as the parasite can be completely cleared from the gut with anthelminthic drugs before subsequent challenge inoculation. It is of interest that a number of cell populations, often associated with Th1 mucosal immune responses, including NK T cells and TCR-$\gamma\delta$ cells (42–44), were not detected at the host:parasite interface, suggesting that in this highly polarized Th2

memory response these cells do not play a major role at the site of infection. In terms of adaptive immunity, it appears that the major infiltrating population is $CD4^+$, TCR-$\alpha\beta^+$ T cells. This distinct response should provide a useful model for examining immune cell interactions at the host:parasite interface during the acute memory Th2 inflammatory immune response.

The studies described in this report demonstrate that a polarized Th2 peripheral memory response associated with a distinct immune cell architecture rapidly develops in the localized microenvironment of an invading parasite, and they establish the use of LCM as a technique for examining peripheral memory Th2 cell distribution and function in situ.

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Memory Th2 cells induce alternatively activated macrophages to mediate protection against nematode parasites.

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Abstract

Although primary and memory responses against bacteria and viruses have been studied extensively, Th2 effector mechanisms leading to host-protection against helminthic parasites remain elusive. Examination of the host:parasite interface following primary and secondary infections of a natural murine gastrointestinal parasite revealed a distinct immune cell infiltrate following challenge, featuring IL-4 expressing memory $CD4^+$ T cells which induced IL-4 receptor^{hi} (IL-$4R^{hi}$) $CD206^+$ activated macrophages. In turn, these alternatively activated macrophages (AAMacs) functioned as important effector cells of the protective

memory response contributing to worm expulsion, demonstrating a novel mechanism for host protection against intestinal helminthes.

Introduction:

The immune system's ability to adapt to and remember antigens from previous exposures is essential for host protection against infectious disease[1]. Productive immune responses result in $CD4^+$ T cell polarization into effector phenotypes defined by differing cytokine milieus[2]; Th1 cells produce IFN-γ and TGF-β, triggering IgG2a production by B cells and the upregulation of inducible nitric oxide synthase (iNOS) in classically activated macrophages[3,4]. This response type is very effective at clearing intracellular pathogens, including bacteria, viruses, and *Leishmania* sp[5]. Conversely, helminth parasites and allergens induce Th2 responses, leading to IL-4, IL-13, and IL-5 production by $CD4^+$ T cells resulting in eosinophilia, isotype switching to IgE and IgG1 in B cells, and upregulation of arginase-1 in macrophages[6,7]. While these arginase-1 expressing macrophages accumulate during asthmatic inflammation[8,9] and helminth parasite infections[10-13], down-regulate type 1 inflammation[14], and promote recruitment and collagen deposition by fibroblasts[15], a protective role fo them remains undefined.

We examined the immune response to a natural murine gastrointestinal helminth parasite, *Heligmosomoides polygyrus*, which triggers a highly-polarized Th2 response[6,16,17]. *H. polygyrus* provides an excellent model of a protective Th memory response as all parasites are readily cleared from the intestinal lumen o the host through anti-helminthic drug treatment, terminating an otherwise chronic

primary infection. Subsequent challenge results in memory $CD4^+$ T cell and IL-4 dependent worm expulsion by day 14 post infection[18]. The best-studied mechanisms of protective Th2 responses have been to intestinal nematode parasites, focusing on the roles of IL-4 and IL-13 in inducing changes in gut physiology, including increased gut contractility and luminal fluid secretion, proposed to promote adult worm expulsion by creating an inhospitable luminal environment[16,19,20]. Although mast cells may play a role in the induced physiological changes, mast cells and eosinophils are thought to play a minor role in host protection as their inhibition during *H. polygyrus* challenge has little effect on parasite burdens[21-23] (and unpublished data).

Our studies examined early events in this memory response to *H. polygyrus* larvae developing in the intestinal submucosa, and suggest a novel mechanism for protective $CD4^+$ T cell-dependent type 2 memory responses: rapid cytokine production at the host:parasite interface by memory Th2 cells mediates the accumulation of IL-$4R^{hi}$ $CD206^+$ AAMacs[11,12] around the invading larvae, which in turn are important for eventual parasite expulsion. These findings indicate a novel role for AAMacs as important effector cells in this localized, protective memory Th2 response.

Results:

Immune events directed against invasive larval stages of *H. polygyrus* are important for host-protection.

H. polygyrus progresses through distinct environmental niches during the course of infection (fig. 1a, 1b), which suggests multiple host-protective

mechanisms may be required for maximal protection[16,24]. To examine which stages of the memory response required $CD4^+$ T cells, anti-CD4 antibody was administered at specific intervals following secondary infection, including: (*i*) days 0 and 7; (*ii*) day 7; (*iii*) day 9; (*iv*) day 11 (fig. 2a). Invading larvae develop into adults and migrate into the lumen at day 8 after inoculation (fig. 1b), therefore the anti-CD4 treatments at day 9 and particularly day 11 specifically examined effects of memory $CD4^+$ T cells on adult worms only. The anamnestic response to *H. polygyrus* (fig. 2b, 2'HpD14) effectively expelled the parasites compared to mice receiving primary infections (1'HpD14). Administration of anti-CD4 Ab at days 0 and 7 after inoculation resulted in pronounced increases in worm burden, while treatment at day 7 only partially blocked the host protective response shown by a 2 fold reduction in worm burden and egg production compared to mice depleted of $CD4^+$ cells on days 0 and 7. $CD4^+$ T cell depletion at later stages in the secondary response (groups treated on days 9 or day 11) had minimal effects on the host protection, as these mice effectively cleared the infection. These results indicate $CD4^+$ T cells are required at early stages of a secondary infection for effective expulsion of the parasite, and for the first time implicate the adaptive immune response during larval development in the intestinal tissue as crucial for host protection.

The requirement of CD4+ T cells during early stages of the memory response for efficient expulsion of parasites suggested a potential protective immune element during the invasive stages of the infection. Muscularis-residing larvae can be recovered from infected small intestines by a Baermann

apparatus[25], as removal of infected intestines from the host provokes premature evacuation of the tissue by the invading parasites. Comparing the recovery of larvae from primary and secondary infected intestines as an indicator of larval health and mobility, we found significantly fewer larvae were recovered from secondary infections, compared to primary infected tissues. Over 80 larvae were recovered from the small intestines of primary infected mice at both days 4 and 7 after infection (fig. 2c). In marked contrast, fewer than 20 larvae were recovered from the intestines of challenged mice at either day analyzed, further confirming that early events in this memory Th2 immune response during invasive stages play a role in host protection.

Both Gr-1$^+$ cells and CD4$^+$ T cells accumulating at the host:parasite interface upregulate Th2 cytokine gene expression after *H. polygyrus* challenge inoculation.

Previous studies described a distinct immune cell architecture at the host:parasite interface and localized elevations in Th2 cytokines 4 days following challenge[26]. Further analyses necessitated the use of recently developed histological methodologies; FACS analysis was not practical since the leukocytes at the host:parasite interface represented only a small fraction of the diverse immune cell phenotypes in the intestine. To identify the leukocyte populations expressing IL-4 and IL-13, immunofluorescence used in combination with Laser Capture Microdissection (IF-LCM) allowed individual microdissection of Gr-1$^+$ cells, CD4$^+$ cells, and Gr-1$^-$ CD4$^-$ cells from the regions immediately surrounding the parasite. Increased IL-4 and IL-13 mRNA (determined by quantitative real

time fluorogenic RT-PCR, expressed relative to untreated Peyer's Patches) was detected in $CD4^+$ and $Gr\text{-}1^+$ populations (fig. 3a). IFN-γ elevations were not detected (data not shown).

To determine if any infiltrating leukocyte populations were capable of responding to the Th2 cytokines expressed in the submucosal microenvironment, infected small intestine sections were assayed for IL-4Rα expression, a component of both type 1 and type 2 IL-4 receptors[27]. Four days following challenge, $Gr\text{-}1^+$ neutrophils (fig. 3b, green) accumulated adjacent to the parasite, and $CD4^+$ T cells (blue) surrounded the parasite along the muscularis border. A ring of IL-4Rα^{hi} cells (red) encircled the parasite and $Gr\text{-}1^+$ neutrophils within the band of $CD4^+$ T cells, suggesting another infiltrating cell was expressing sufficient IL-4Rα to be readily detected by immunofluorescence.

Immunohistological staining has proven useful for detecting *in situ* expression patterns of particular cytokine proteins, including IL-12 and IFN-γ; however, IL-4 protein has proven difficult to detect. To enhance the sensitivity of IL-4 staining, directly conjugated monoclonal antibody pairs recognizing distinct epitopes of the IL-4 molecule were used simultaneously to stain tissues of interest, to amplify the fluorescent IL-4 signal. Using this methodology, substantial $IL\text{-}4^+$ staining (green) was detected in the band of $CD4^+$ T cells (blue) 4 days following challenge (fig. 3c). A serial tissue section stained with an isotype control antibody showed minimal nonspecific staining (fig. 3d). IL-4 protein was not detected in the vicinity of the parasite 4 days following primary inoculation or in challenged IL-4 deficient ($^{-/-}$) mice (not shown). These findings

suggest that memory $CD4^+$ T cells are the primary source of IL-4 protein at the host:parasite interface, although both $CD4^+$ T cells and $Gr\text{-}1^+$ neutrophils express IL-4 and IL-13 mRNA.

Memory Th2 cells drive macrophages to adopt an alternatively activated phenotype ($IL\text{-}4R^{hi}$ $CD206^+$) at the host:parasite interface.

The IL-4Rα^{hi} cells surrounding developing *H. polygyrus* larvae 4 days after challenge did not express lymphocyte markers (CD4, CD8, B220, DX5), granulocyte markers (Gr-1, MBP-1, DX5), or dendritic cell markers (CD11c) (fig. 3c, 4a and data not shown), but were abundant (Fig. 4b, red) in the region immediately surrounding the parasite. When serial tissue sections were also stained for macrophages (F4/80, blue), a ring of double-positive (purple) cells encircling the parasite emerged (Fig. 4c), demonstrating that infiltrating macrophages express high levels of IL-4Rα. While macrophages at the host:parasite interface preferentially stained bright for the IL-4Rα, other leukocyte populations, including $CD4^+$ T cells (fig. 3c, 4a), stained dull for the IL-4R. Macrophages exposed to Th2 cytokines become alternatively activated, express arginase-1, IL-4Rα, and upregulate the macrophage mannose receptor (CD206)[11,12,14]. $F4/80^+$ macrophages (fig. 4d, red) dual stained with CD206 (green), as indicated by the yellow and orange staining cells. In contrast, $F4/80^+$ macrophages at the host:parasite interface 4 days after primary infection were much lower in number, as was expression of CD206 (fig. 4f) and IL-4Rα (fig. 4e). The host:parasite interface 4 days post challenge in $IL\text{-}4^{-/-}$ mice resembled the interface following primary infections of wild type mice, with reduced numbers of

CD4+ T cells (fig. 4g), macrophages, and CD206^{+} cells (fig. 4h), demonstrating that IL-4 was of particular importance in alternative macrophage activation during the memory response. It should be noted that the selective development of AAMacs during the memory response was also observed in BL/6 mice (data not shown), demonstrating that the accumulation of alternatively activated macrophages was not dependent genetic background.

To directly implicate memory Th2 cells in the development of alternatively activated macrophages, the effect of CD4^{+} T cell depletion at the time of challenge on infiltrating immune cell populations was examined. While accumulation of Gr-1^{+} neutrophils (green) was unaffected, very few CD4^{+} T cells (blue) or IL-4Rα^{hi} cells (red) were present (fig. 4i). The phenotypes and numbers of infiltrating macrophages in these mice (fig. 4j, F4/80 (red), CD206 (green)) resembled those seen during a primary response (fig. 4f), confirming that memory CD4^{+} T cells were required for the *in vivo* accumulation of AAMacs at the host:parasite interface.

AAMacs metabolize L-arginine using the enzyme arginase-1, instead of iNOS which is associated with type 1 inflammatory macrophages[4,28]. A number of chitinases and chitinase-like genes have also been associated with AAMacs[8]. Using IF-LCM, accumulating F4/80^{+} cells were dissected from the host:parasite interface 4 days following challenge. Expression profiles characteristic of AAMacs were identified, with high levels of arginase-1 (fig. 4k), Fizz1 (fig. 4l), Ym1, and acidic mammalian chitinase, and no detectable iNOS (fig. 4k), further confirming their alternatively activated state.

Upon ligation of the IL-4R, the signaling molecule stat6 is phosphorylated, allowing its dimerization and migration into the nucleus[29,30]. To determine if IL-4 receptor signaling was required for the alternative activation of macrophages by $CD4^+$ T cells, $CD4^+$ T cells were purified from Hp primed and cured mice and adoptively transferred to naïve WT or stat6 deficient ($stat6^{-/-}$) recipients, which were subsequently infected. Thus, wild type memory $CD4^+$ cells were transferred into animals with functional (wild type) or disabled ($stat6^{-/-}$) IL-4 receptors. Both wild type and $stat6^{-/-}$ recipients exhibited similar $Gr\text{-}1^+$ neutrophil (red) and $CD4^+$ T cell (blue) infiltrates at the host:parasite interface (Fig. 4m, o, respectively). Macrophages (F4/80, red) in wild type recipients upregulated CD206 (green) as indicated by the yellow population (Fig. 4n). In contrast, macrophages in the $stat6^{-/-}$ recipients (fig. 4p) did not express high levels of the mannose receptor.

Arginase-1 expression results in proline production, fibroblast recruitment, and subsequent collagen deposition, characteristics of Th2 inflammation[15]. Small intestine tissue sections collected 4 or 12 days after challenge were stained using the picrosirius red stain for fibrosis[31], which stains collagen bright red to examine collagen deposition following *H. polygyrus* primary and secondary infections. Small intestines normally feature a wall of collagen providing structural support, separating the muscularis and lamina propria[32]. Collagen was detected only along this border 4 days following challenge (supplemental fig. 1a). However, by 12 days after challenge collagen fibers extended into the muscularis region (supplemental fig. 1b), surrounding the invading parasite. Collagen deposition at the host:parasite interface at later stages of the *H. polygyrus*

secondary response is a characteristic of inflammation mediated by AAMacs in this microenvironment.

Macrophages are required for the development of the host protective Th2 memory response.

The combined observations of the importance of CD4 T cells during the first seven days after *H. polygyrus* secondary challenge, and the preferential accumulation of AAMacs during the memory response suggested that macrophages might play a role in mediating protective immunity at the host:parasite interface. To examine this possibility, macrophages were depleted *in vivo* using clodronate liposomes (Cl_2MDP), a technique widely and effectively used *in vivo* to selectively kill macrophages[33,34]. As shown in Fig. 5a, effective worm expulsion was observed in the control group challenged with *H. polygyrus* treated with mock-PBS-loaded liposomes. However, challenged mice treated with Cl_2MDP-liposomes were unable to effectively clear the parasites, with parasite burdens equivalent to primary infected animals; it should be noted that similar results were observed when macrophages were depleted in *H. polygyrus*-challenged BL/6 mice (data not shown). Interestingly, egg burdens remained decreased in clodronate-liposome treated animals, indicating that an additional host-protective mechanism remains intact and effects adult worm fecundity. This is not surprising as there may be multiple host defense mechanisms targeting different stages of the life cycle.

To further examine the protective role of macrophages during *H. polygyrus* challenge, premature larval evacuation from infected intestines was examined in

animals depleted of macrophages. Mice treated with PBS-loaded liposomes generated a productive memory response, and had low numbers of recovered larvae compared to primary infected controls at days 4 and 7 post infection (fig. 5b and c). In contrast, Cl_2MDP-liposome treated mice showed dramatic increases in larval recovery during the memory response, indicating that larval retention is inhibited when macrophages are depleted. Examination of the host:parasite interface 4 days after challenge demonstrated F4/80$^+$ macrophages were selectively depleted from clodronate treated animals (fig. 5g) compared to PBS treated controls (fig. 5f), and that other infiltrating leukocytes, including CD11c$^+$ dendritic cells, Gr1$^+$ neutrophils, and CD4$^+$ T cells were unaffected by either treatment (fig. 5 d and e).

No significant changes in cytokine gene expression were detected in the draining mesenteric lymph nodes of challenged mice receiving clodronate-liposome treatment compared to those receiving PBS-liposomes (fig. 5h), indicating that CD4$^+$ T cells were appropriately activated, and the Cl_2MDP liposome treatment did not systemically inhibit antigen presentation. These findings indicated that the Cl_2MDP-liposome treatment selectively and effectively depleted macrophages in the microenvironment immediately surrounding the invading parasite during the *H. polygyrus* memory Th2 immune response, leaving other phagocytic populations (Gr-1$^+$ neutrophils and CD11c$^+$ dendritic cells) unaffected, and that this treatment abrogated the protective response.

Arginase is required for a protective memory response to *H. polygyrus*.

To further confirm a role for alternatively activated macrophages in host protection, challenged mice were administered an arginase inhibitor S-(2-boronoethyl)-l-cysteine (BEC)[35] to block the effector functions of AAMacs. Mice treated with BEC (2'HpD14+BEC) showed significantly increased parasite burdens compared to challenged controls (2'HpD14) (figure 6a), but had reduced egg burdens. Larval recovery by Baermann apparatus at both 4 and 7 days post challenge was enhanced when arginase was inhibited by BEC (Fig. 6b and 6c).

To directly examine the effect of arginase on the parasites *in situ*, parasite cross-sections taken 7 days post-infection were stained using cytochrome oxidase. Staining for cytochrome oxidase has been used as an indicator of stress[36], and observed in several helminthes including *Caenorhabditis elegans* and *Arscaris suis*[37-40]. Cytochrome oxidase activity was markedly increased 7 days following challenge (fig. 6e) compared to samples obtained 7 days following primary infection (fig. 6d) or challenged tissue treated with BEC (fig. 6f). Taken together these findings indicate that arginase expression is required for expulsion of *H. polygyrus*, and is associated with strained parasites, further implicating alternatively activated macrophages as essential effector cells in this response.

Discussion

Most studies of recall Th2 responses have focused on inappropriate or harmful reactions including asthma and allergy[9,41-44]; consequentially, little is known about protective Th2 mechanisms[13]. We have examined a memory Th2 response developing at the host-parasite interface, and its contribution to host defense against a natural murine gastrointestinal nematode parasite. A

substantial and distinct type 2 inflammatory response develops, comprised of Gr-1$^+$ neutrophils in the immediate vicinity of the invading parasite, and CD4$^+$ T cells, macrophages, eosinophils and dendritic cells surrounding the neutrophils. Our studies show that alternatively activated macrophages, stimulated by IL-4 producing memory CD4$^+$ T cells in a stat6-dependant manner, are an essential effector population in the protective Th2 response against this important group of pathogens.

In infectious diseases, macrophages are usually associated with responses against intracellular pathogens, as their activation via Toll-Like Receptors and IFN-γ results in iNOS expression and NO production crucial for killing *Leishmania sp.* and *Listeria monocytogenes*[14,28]. Arginase-1 expression by macrophages exposed to type 2 cytokines leads ultimately to production of polyamines and proline, essential for cellular proliferation and collagen production, respectively[11,14,45]. Arginase-1 competes with iNOS for L-arginine, preventing the intracellular nitric oxide (NO) burst essential for killing intracellular pathogens[46]. However, its protective role has not been described. Previous studies have shown that arginase blockade *in vivo* promotes resistance to intracellular parasites by enhancing classical macrophage activation and increased iNOS activity. Our studies demonstrate that arginase, can also mediate host protective responses, in this case against intestinal nematode parasites. These results, taken together with the macrophage depletion studies, strongly indicate that, as in the Th1 response, in a protective Th2 memory

response, macrophages are an essential effector population; our findings thus provide an evolutionary role for their alternatively activated state.

Protective mechanisms against nematode parasites have remained elusive, although studies have suggested Th2 responses enhance gut contractility and luminal fluid secretion leading to eventual worm expulsion[16,19,20,47]. Also, increased gut epithelial cell turnover was shown to aid in expulsion of *Trichuris muris*[48]. These mechanisms may also be important for *H. polygyrus*, as the adult parasites that were not expelled in challenged mice depleted of macrophages or treated with BEC still produced reduced egg numbers, suggesting an additional mechanism of host-protection remains intact and effects adult worm fecundity. This is perhaps not surprising as *H. polygyrus* passes through multiple sites during the course of infection, potentially eliciting multiple mechanisms for maximal host-defense. For example, resistance during trickle infection is associated with pronounced mastocytosis[22,49].

Detection of collagen at the host:parasite interface is a characteristic of macrophage alternative activation and Th2 inflammation[15,50], and further confirms the activation phenotype of this macrophage population. It is unlikely that collagen plays a role in host-defense, as it was not detected until later stages in the response, after the larvae left the submucosa and entered into the intestinal lumen. Future studies should address the arginase-dependent mechanisms through which they function. Increased polyamine synthesis and iNOS reductase activity leading to reactive oxygen intermediates are both potential mechanisms;

however, *in vitro* analyses in other experimental systems may be required as many inhibitors of these pathways have broad effects *in vivo*.

Elevations in CD4-dependent Th2 cytokine mRNA were previously detected at the site of parasite invasion. We have now extended these findings through IF-LCM to show that *in situ* both $CD4^+$ T cells and neutrophils express IL-4mRNA. Although the use of LCM is a major advance for the detection of *in situ* mRNA levels, the relationship of IL-4 mRNA to protein expression remained uncertain. To address this, we developed a novel approach for detecting IL-4 protein directly, by staining relevant tissue sections with antibodies recognizing different epitopes of the IL-4 protein simultaneously, and showed that IL-4 protein was associated with $CD4^+$ T cells and not neutrophils. These studies indicate that, although both populations express IL-4 mRNA *in vivo*, only the CD4 T cells express IL-4 protein in situ, consistent with previous studies indicating that non-T cells cultured *in vitro* can express IL-4 mRNA without secreting high levels of IL-4 protein[51].

The studies described in this report suggest a novel model for the development of a protective Th2 memory response. Memory $CD4^+$ T cells, in contrast to $CD4^+$ T cells during the primary response, rapidly accumulate at the host:parasite interface where they secrete IL-4 and there induce the localized development of alternatively activated macrophages that have been recruited to this site. These macrophages impair larval parasite health and mobility and contribute to eventual adult worm expulsion.

Materials and Methods

Mice, Parasites, and Injections

Female BALB/c mice were purchased from the Small Animal Division, NCI, and kept under specific pathogen-free conditions in filter top cages in the animal facility at BHNRC, ARS, USDA. $Stat6^{-/-}$ mice were purchased from Jackson Laboratories, Bar Harbor, Maine. 200 infective third stage *H. polygyrus* larvae were orally inoculated (1'Hp), as previously described[18,23]. Infected mice were treated with the antihelminthic drug, pyrantel pamoate 14 days post inoculation to clear the parasites, and shelved for at least 5 weeks to allow for the development of resting memory cells. These mice were then either re-infected (2'Hp) or used as donors for memory $CD4^+$ T cells. Some mice were treated with 1mg of anti-CD4 Ab (GK1.5), or with 0.15ml of PBS- or Cl2MDP-liposomes, all of which were injected I.V. Liposomes were administered at days 0, 1, 3, 5, 7, and 9. For experiments involving BEC, mice were given 0.2% BEC via drinking water days 2-11 post challenge[35].

Immunohistochemistry

Small intestine tissue sections were collected, opened longitudinally, prepared using a Swiss roll method[26,52,53], embedded in TissueTek OCT (Sakura Finetek USA, Torrance, CA), frozen in a dry ice-acetone bath, and stored at -80°C. 4-µm tissue sections were cut from the frozen blocks using an HM505E cryostat (Richard-Allan Scientific, Kalmazoo, MI), fixed in cold acetone, stained with antibody cocktails prepared in 0.1% bovine serum albumin/10% rat sera/PBS for 45 minutes at room temperature, and washed in PBS and dH_2O. Fluoromount G (Southern Biotechnology Associates, Birmingham, AL) was added along with a

coverslip prior to imaging. Antibodies included anti-Gr-1-FITC, anti-IL-4Rα-PE (M1), anti-CD11c-FITC (BD PharMingen, San Diego, CA), anti-CD4-Alexa647, F4/80-Alexa647 (Caltag, Burlingame, CA), and anti-CD206-FITC (MR5D3; Serotek, Raleigh, NC). 200x images were taken on a Zeiss Axiovert 200M inverted microscope (Zeiss), using a 20x objective controlled by a TILLVISTRAC imaging system (TILLVISION 4.0 software (TILL Photonic, Planegg, Germany)). Fluorescent channels were photographed separately, merged together, and overlaid atop corresponding normarski images. Some images were taken using a Leica DM6000B microscope with an automated stage attached to a Leica DFC350FX camera, and tiled together using Image-Pro stitching software (Media Cybernetics). Exposure times and fluorescence intensities were normalized to appropriate control images.

For IL-4 *in situ* staining, 20µg/ml of 11B11-Alexa488 (BD PharMingen, San Diego, CA), and 10µg/ml BDV6-24G2-Alexa488 (Caltag, Burlingame, CA) were used in tandem, and normalized to serial sections stained with 30µg/ml of Rat IgG1-Alexa488 isotype control (BD PharMingen, San Diego, CA). Two additional wash steps with 0.5% Tween-20 in PBS was added for this procedure.

Picroserius red and cytochrome oxidase staining was performed as previously described[36,54], and imaged using Leica DM6000B microscope; exposure times and light intensities were constant between samples.

IF-LCM

IF-LCM was performed with modifications to previously described methods[55]. Briefly, freshly cut 4µm tissue sections were stored on dry ice until use. Sections

were fixed in 75% ethanol for 30 seconds, air dried, washed in PBS, stained with antibody cocktail (5ug/ml anti-Gr-1-FITC (BD PharMingen, San Diego, CA), 20ug/ml anti-CD4-Alexa647 (Caltag, Burlingame, CA), 400 units/ml RNasin (Promega, San Luis Obispo, CA)) for 30 seconds, rinsed in PBS, incubated in 75% ethanol for 30 seconds, 100% ethanol for 30 seconds, and desiccated by two 1 minute xylene incubations. Stained slides dissected using PixCell II and CapSureTM LCM Caps (Arcturus Engineering, Mountain View, CA), and dissected samples analyzed by RT-real time PCR as previously described. Arginase-1 and iNOS primers and probes were kindly provided by Dr. Harry Dawson. Fizz1, Ym1, and AMCase primers were previously described[4].

Parasite and Egg Quantification

To quantify adult parasite burdens, the contents of the small intestines were collected 14 days post infection, and parasites were counted using a dissecting microscope. For egg burdens, fecal contents were collected in PBS, and counted under a light microscope. For larval parasite counts, small intestines were collected, opened longitudinally, and incubated in PBS at 37°C for 3 hours in a Baermann apparatus as previous described. Briefly, intestines collected 4 or 7 days post infection were placed in a sieve suspended atop a pilsner glass, and parasites that collected at the bottom of the glass were counted.

Liposome-Mediated Macrophage Depletion

0.2ml of clodronate- (Cl_2MDP) or control PBS- liposomes were administered to deplete macrophages. Liposomes were generated as previously described[34] using phosphatidylcholine (LIPOID E PC; Lipoid GmbH,

Ludwigshafen,Germany), and cholesterol (SIGMA, St. Louis, MO). Cl_2MDP was a kind gift of Roche Diagnostics GmbH (Mannheim, Germany).

Cell preparations and adoptive transfers

$CD4^+$ T cells were isolated from the lamina propria (LP) as previously described[56], with modifications. Small intestines from Hp primed mice were removed, opened longitudinally, and all mucus and debris manually removed. The tissue was cut into 5mm pieces, and washed twice in PBS, incubated in HBSS with 0.5mM EDTA for 20 minutes at 37°C with, washed and the incubation repeated. Next, the tissue was digested in 0.25mg/ml Liberase C (Roche, Indianapolis, IN) and 30µg/ml DNase (Sigma, St. Louis, MO). The digest was further disrupted by repeated forced passage through a needle, and lymphocytes purified by gradient centrifugation using Lympholyte M (CedarLane Laboratories, Ontario, Canada). For MLN preparations, single cell suspensions were generated in RPMI fortified with 2.5% fetal calf serum, and washed multiple times. CD4+ cells from both LP and MLN preparations were isolated using MACS CD4+ beads (Miltenyi Biotech, Auburn, CA); $5x10^5$ cells were injected I.V. into sex and age matched recipients, which were infected 2 days following transfer.

Statistical analysis

Statistical analyses were preformed using SigmaStat 3.0 (SyStat Software, Inc.), employing student's T tests to compare 2 groups, and ANOVA followed by Tukey's post hoc to compare multiple groups, when needed.

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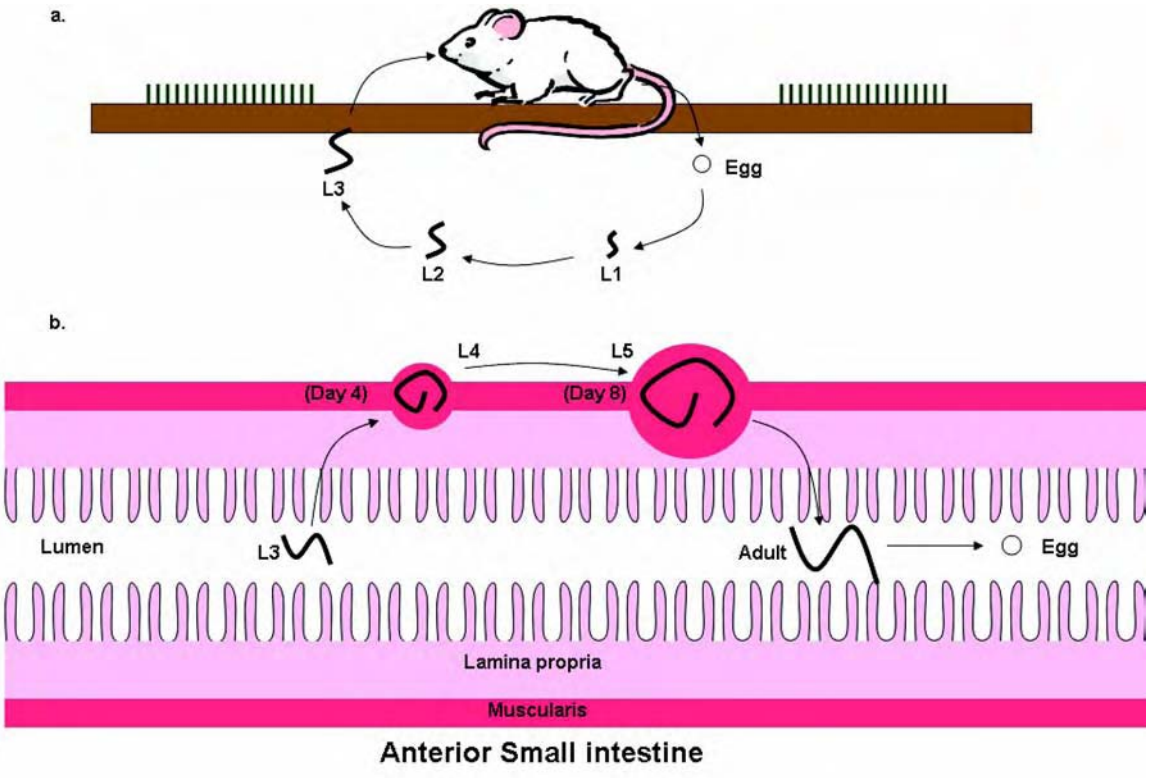


Figure 1. The life cycle of *H. polygyrus*. Following oral infection (a), *H. polygyrus* L3 larvae migrate to the small intestine, where they invade the epithelium and submucosa and take residence in the muscularis (1b). The parasites undergo two molts there, and enter into the gut lumen as adults 8 days post infection. In the lumen, female adult parasites excrete eggs which are passed along with feces. The eggs hatch in the soil and progress to the infectious L3 larvae over the next 14 days. Infection of naïve mice with L3 results in a chronic luminal infection, which can be cleared by the administration of an antihelminthic drug. A subsequent infection of primed and drug treated mice results in the clearance of parasites from the intestinal lumen by 14 days post infection.

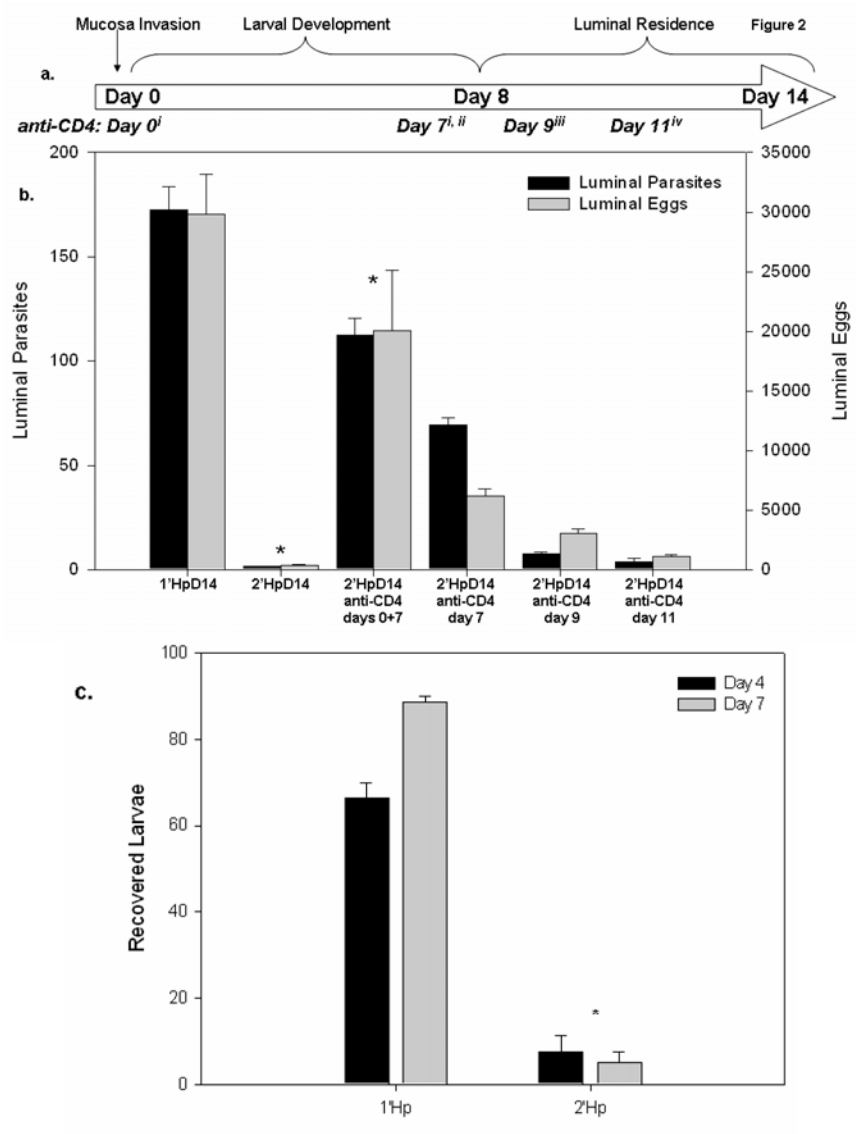
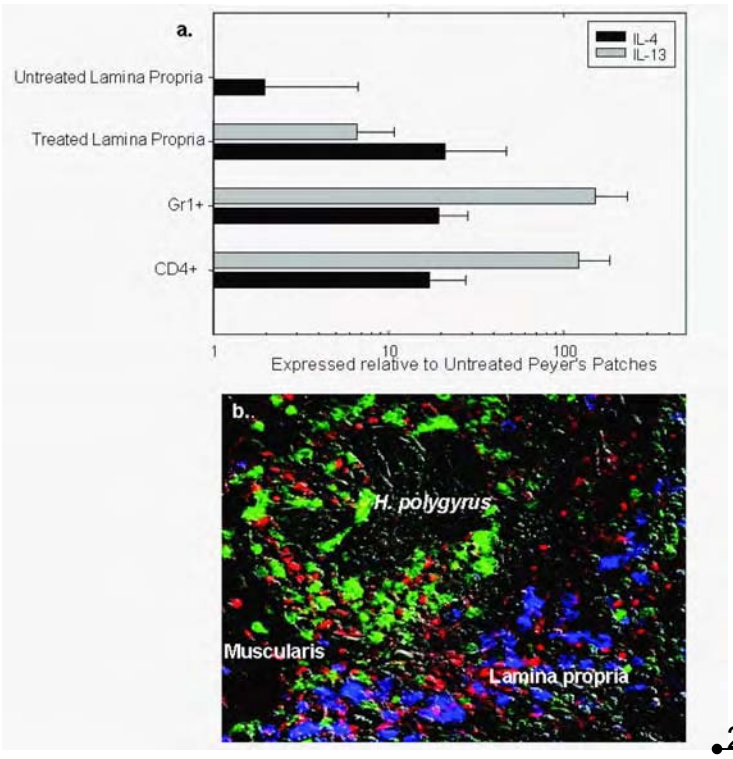

Mucosa Invasion
Larval Development
Luminal Residence
Figure 2
a.
Day 0
Day 8
Day 14
anti-CD4: Day 0[i]
Day 7[i, ii]
Day 9[iii]
Day 11[iv]
b.
Luminal Parasites
Luminal Eggs
Luminal Parasites
Luminal Eggs
1°HpD14
2°HpD14
2°HpD14 anti-CD4 days 0+7
2°HpD14 anti-CD4 day 7
2°HpD14 anti-CD4 day 9
2°HpD14 anti-CD4 day 11
c.
Day 4
Day 7
Recovered Larvae
1°Hp
2°Hp

Figure 2. CD4+ T cell-dependent host protective mechanisms occur at early stages after *H. polygyrus* challenge inoculation. a. H. *polygyrus* L3 larvae invade the small intestinal submucosa, molt twice, and emerge into the gut lumen as adults 8 days post infection. Anti-CD4 Ab was administered to challenged mice at days 0 and $7^{(i)}$, $7^{(ii)}$, $9^{(iii)}$, or $11^{(iv)}$ post inoculation. b. Worm and egg burdens were determined 14 days post challenge in mice receiving different anti-CD4 treatments described above. The mean and standard error of 5 mice per treatment group are shown; the results are representative of 3 separate experiments. *$p<0.05$ was determined using Kruskall-Wallace test followed by Dunn's post hoc. c. Larvae have difficulty evacuating tissue during the secondary but not primary infections. Parasite larvae were recovered from the small intestines of primed or challenged mice at the indicated intervals using a Baermann apparatus. The mean and standard error of 4 or 5 mice per treatment group is shown and is representative of three separate experiments. *$p<0.001$ using a student's T test.


a.
IL-4
IL-13
Untreated Lamina Propria
Treated Lamina Propria
Gr1+
CD4+
1
10
100
Expressed relative to Untreated Peyer's Patches
b..
H. polygyrus
Muscularis
Lamina propria
20µm

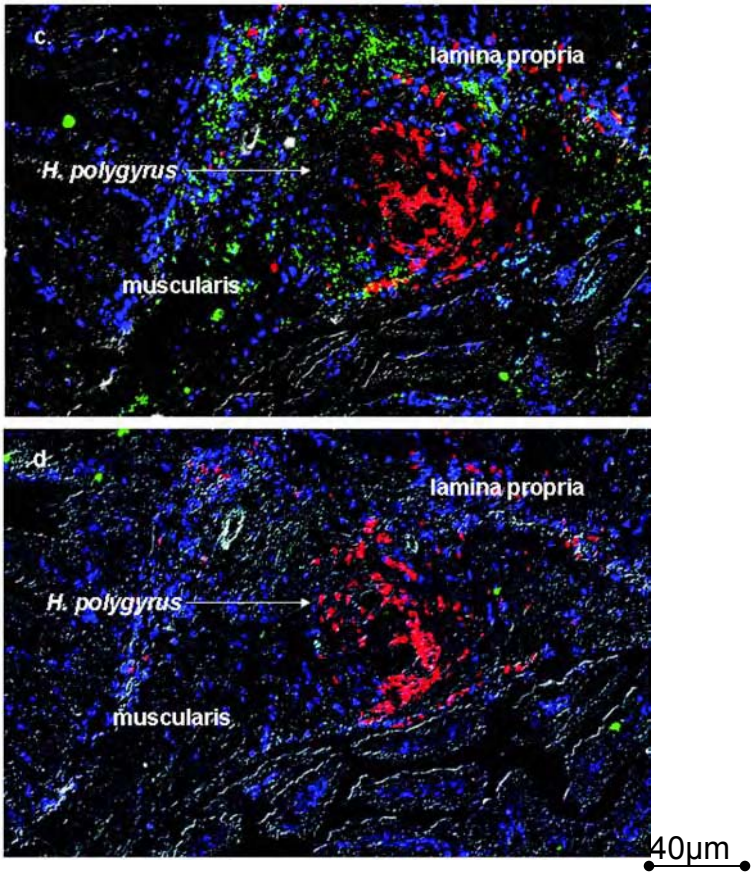
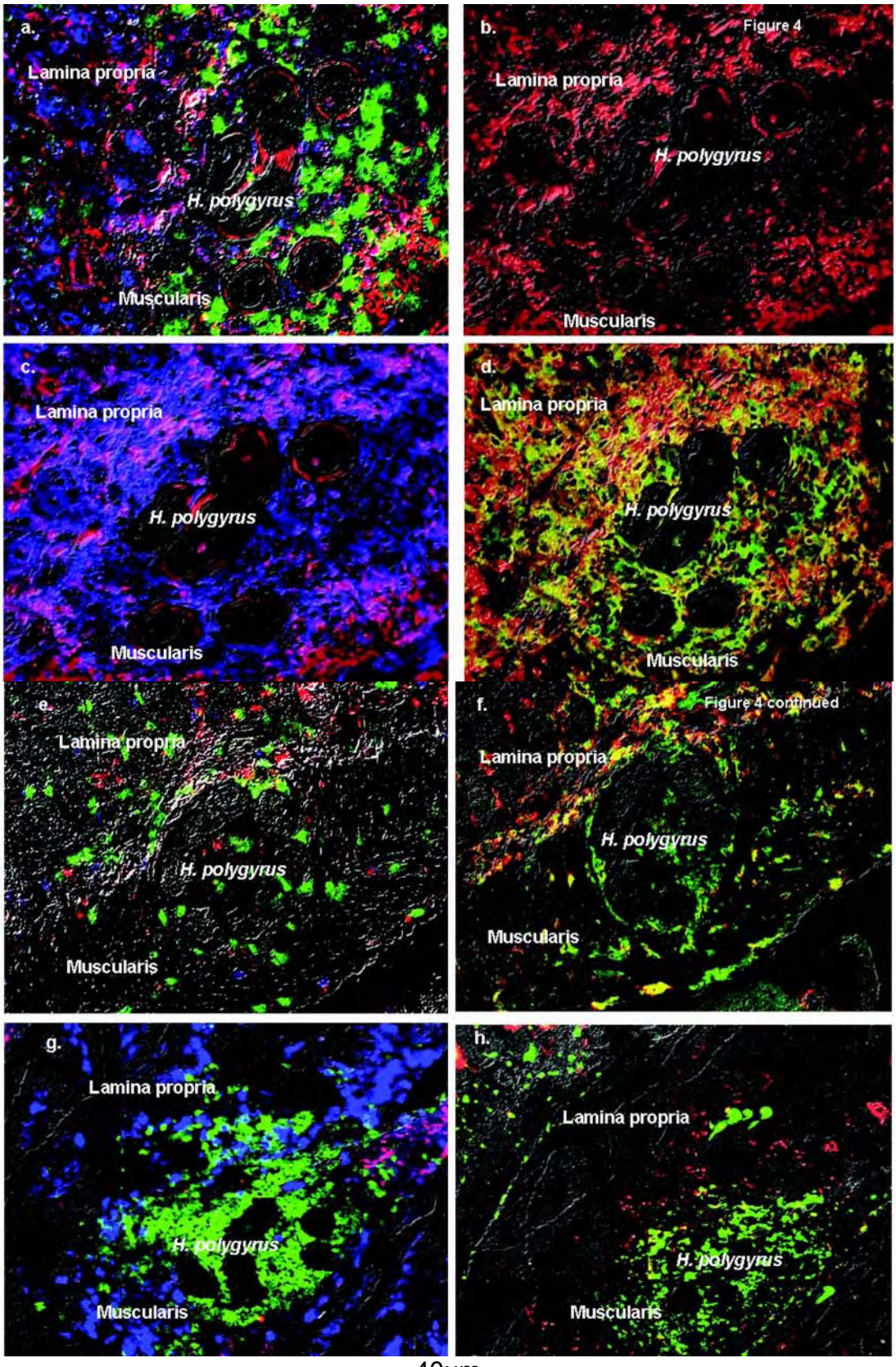
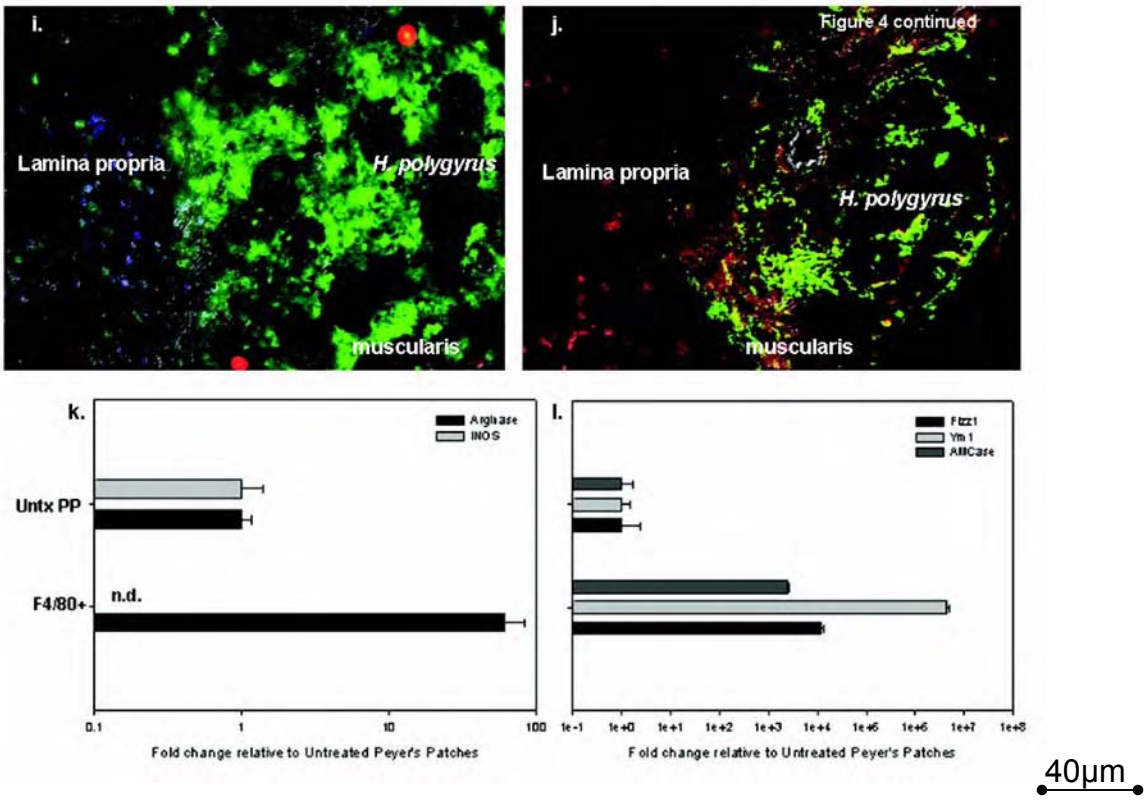

c.
lamina propria
H. polygyrus
muscularis
d.
lamina propria
H. polygyrus
muscularis
40µm

Figure 3. An inflammatory Th2 microenviroment develops around *H. polygyrus* larvae 4 days after challenge. a. $CD4^+$, $Gr\text{-}1^+$, and treated lamina propria ($CD4^-$ $Gr\text{-}1^-$) samples were removed from the host:parasite interface using IF-LCM, and cytokine mRNA expression levels determined by RT-real time-PCR. Samples were standardized to their own rRNA, and treated samples were expressed relative to untreated lamina propria; mean and standard errors are derived from 5 animals per treatment group, and represent 2 independent experiments. b. Fluorescent immunohistochemistry of the host:parasite interface 4 days post challenge depicts IL-4Rα^{hi} (red) cells encircling the parasite, $Gr\text{-}1^+$ (green) cells accumulating adjacent to the parasite, and $CD4^+$ T (blue) cells surrounding both the parasite and the $Gr\text{-}1^+$ population. c. IL-4 protein (green) is detected *in situ* in the region occupied by CD4+ T cells (blue), which surround $Gr\text{-}1^+$ neutrophils (red). d. Rat IgG1-Alexa488 (isotype control for anti-IL-4-Alexa488 antibodies used in figure 3C) antibody displays minimal non-specific binding in a serial section of the tissue shown in Fig. 2c.


a.
Lamina propria
H. polygyrus
Muscularis
b.
Figure 4
Lamina propria
H. polygyrus
Muscularis
c.
Lamina propria
H. polygyrus
Muscularis
d.
Lamina propria
H. polygyrus
Muscularis
e.
Lamina propria
H. polygyrus
Muscularis
f.
Figure 4 continued
Lamina propria
H. polygyrus
Muscularis
g.
Lamina propria
H. polygyrus
Muscularis
h.
Lamina propria
H. polygyrus
Muscularis
40µm


i.
Lamina propria
H. polygyrus
muscularis
j.
Figure 4 continued
Lamina propria
H. polygyrus
muscularis
k.
Arginase
iNOS
Untx PP
F4/80+
n.d.
0.1
1
10
100
Fold change relative to Untreated Peyer's Patches
l.
Fizz1
Ym1
AMCase
1e-1
1e+0
1e+1
1e+2
1e+3
1e+4
1e+5
1e+6
1e+7
1e+8
Fold change relative to Untreated Peyer's Patches
40µm

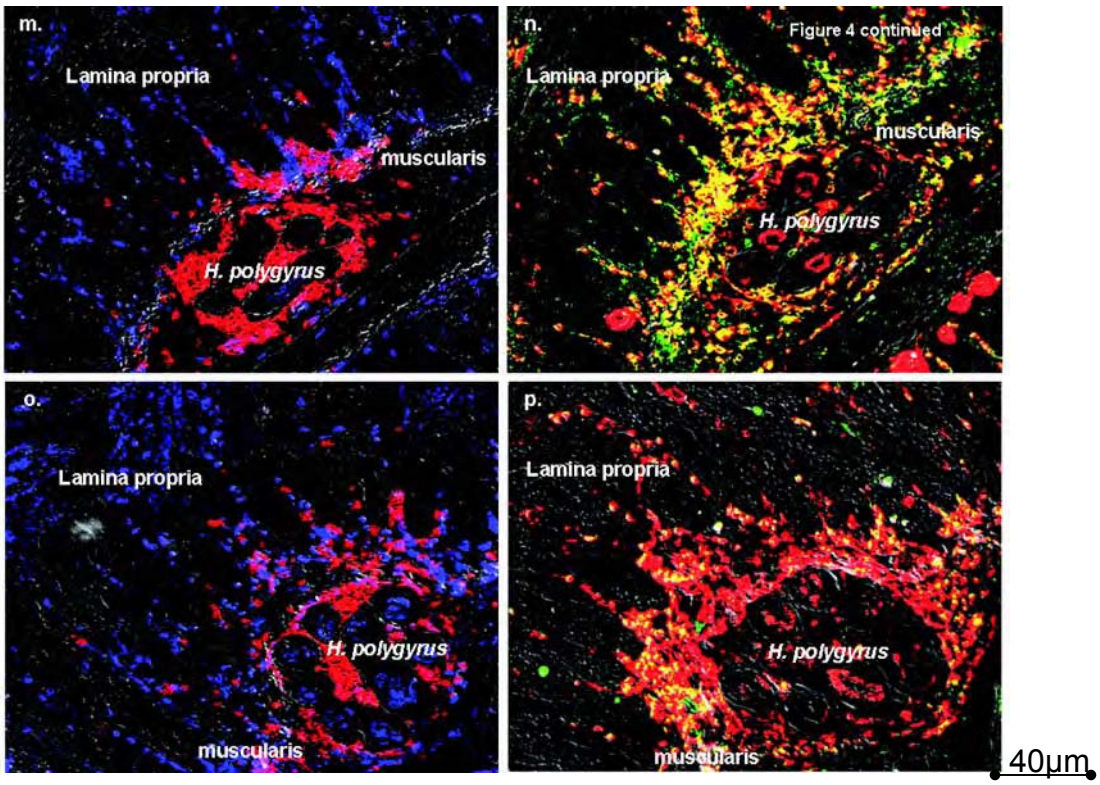
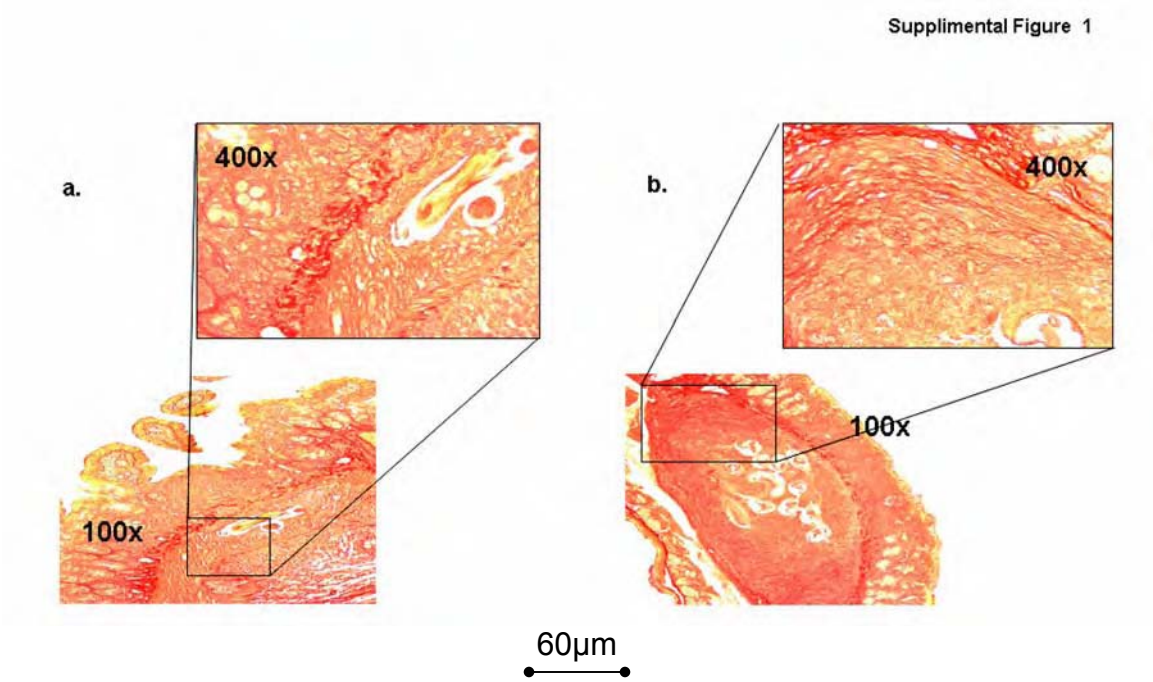


Figure 4. Macrophages accumulating at the host:parasite interface 4 days after challenge express high levels of the IL-4Rα, and adopt an alternatively activated phenotype dependant on stat6 signaling. a-d: Immunofluorescent images of the host:parasite interface 4 days after challenge. a. IL-4Rα^{hi} cells (red) do not stain positive for Gr-1 (green) or CD4 (blue). b. A serial tissue section depicts the ring

of IL-4Rα^{hi} cells (red) encircling the parasite. c. F4/80^{+} macrophages (blue) co-stain for the IL-4Rα (red) demonstrated by the ring of purple cells around the parasite. d. F4/80^{+} macrophages (red) accumulating at the host:parasite interface express CD206 (macrophage mannose receptor (green)), resulting in a yellow/orange stain indicative of their alternatively activated state. e. Four days following primary infection, Gr-1^{+} neutrophils (green) accumulate adjacent to the parasite, with substantially less CD4^{+} T cells (blue) or IL-4R^{hi} cells (red), compared to a secondary infection (fig. 4a). Also, fewer F4/80^{+} macrophages (f, red) and CD206^{+} (f, green) cells were detected. The host:parasite interface in challenged IL-4$^{-/-}$ mice (g, h) display Gr-1^{+} neutrophils (g, green) and CD4^{+} T cells (g, blue), but minimal IL-4Rα^{hi} (g, red) cells. Additionally, there is a great reduction in accumulating F4/80^{+} (h, red) or CD206^{+} (h, green) cells. Tissue sections taken 4 days after challenge in mice treated with anti-CD4 Ab (i, j) show the accumulation of Gr1+ neutrophils (i, green), but a marked reduction in CD4^{+} T cells (i, blue) and IL-4R^{hi} cells (i., red). Serial tissue sections stained for alternatively activated macrophages show reduced numbers of F4/80^{+} (j, red) and CD206^{+} (j, green) cells. K, l. F4/80+ cells dissected from the host:parasite interface by IF-LCM 4 days after challenge exhibit gene expression profiles characteristic of AAMacs, including undetectable iNOS (k), and high levels of arginase (k), Fizz1 (l), Ym1 (l), and AMCase (l). m-p: CD4+ T cells transferred from Hp primed mice induce alternatively activated macrophages in WT recipients (m, n) but not stat6$^{-/-}$ recipients (o, p). Gr-1+ neutrophils (red) and CD4+ T cells (blue) accumulate around the parasite 4 days post infection

following adoptive transfer of Th2 memory cells into wild type (m) and stat6$^{-/-}$ (o) recipients. Serial tissue sections show staining for macrophages (F4/80, red) and macrophage mannose receptor (CD206, green), demonstrating that wild type recipients (n) develop alternatively activated macrophages (yellow) while stat6$^{-/-}$ recipients (p) do not. Exposure times and fluorescence intensities of all fluorochromes were normalized to appropriate controls.



Supplemental figure 1. Collagen is deposited around the parasite at later stages of a challenge infection and is associated with the Th2 inflammatory response. Tissue sections of the host:parasite interface, stained with picrosirius red for fibrosis, display red strands, indicative of collagen, extending into the muscularis region and encircling the parasite 12 days (b), but not 4 days (a) after challenge.

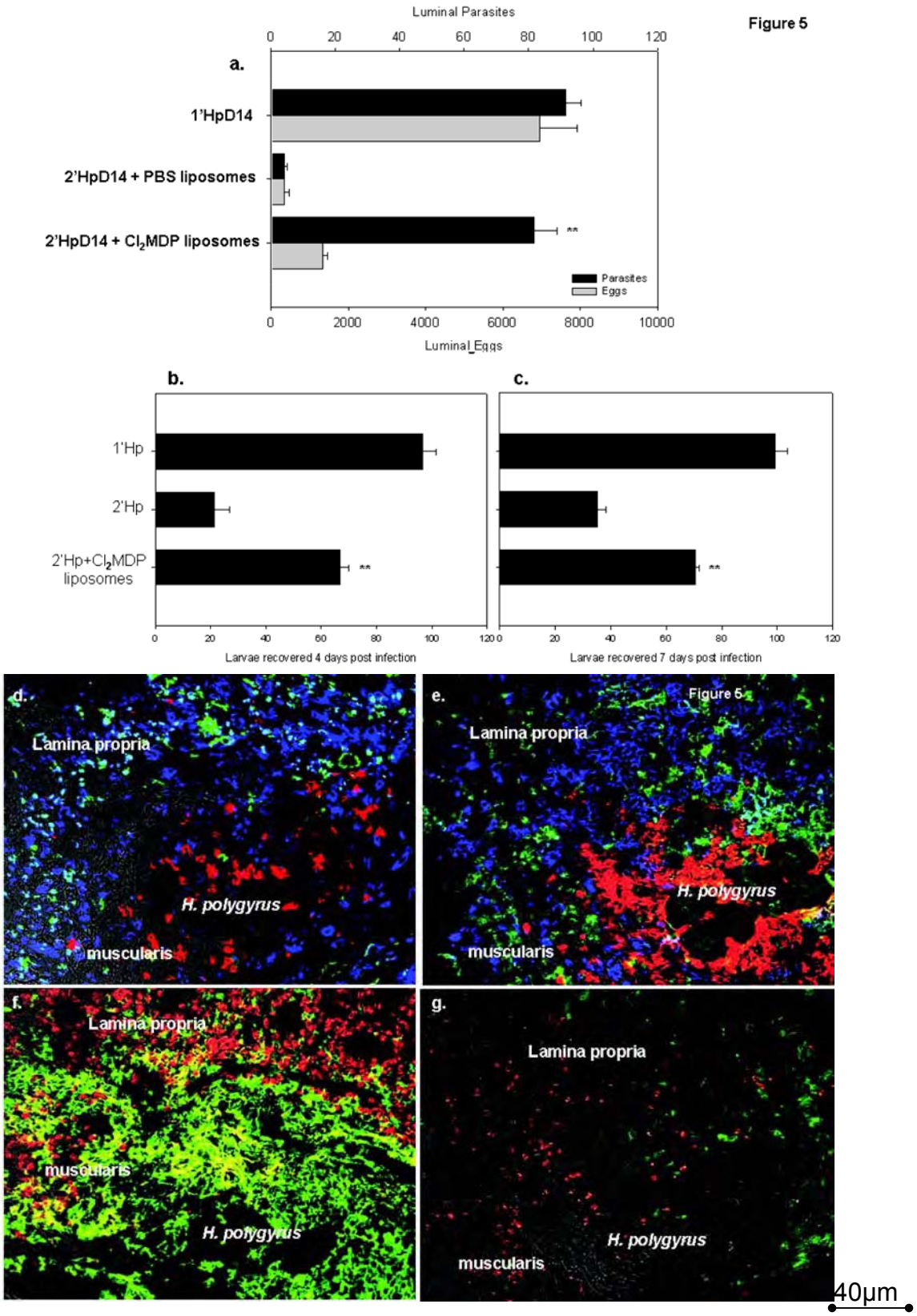

Luminal Parasites
0
20
40
60
80
100
120
a.
1'HpD14
2'HpD14 + PBS liposomes
2'HpD14 + Cl2MDP liposomes
**
Parasites
Eggs
0
2000
4000
6000
8000
10000
Luminal Eggs
b.
c.
1'Hp
2'Hp
2'Hp+Cl2MDP liposomes
**
**
0
20
40
60
80
100
120
Larvae recovered 4 days post infection
Larvae recovered 7 days post infection
d.
Lamina propria
H. polygyrus
muscularis
e.
Figure 5
Lamina propria
H. polygyrus
muscularis
f.
Lamina propria
muscularis
H. polygyrus
g.
Lamina propria
H. polygyrus
muscularis
40µm

Figure 5

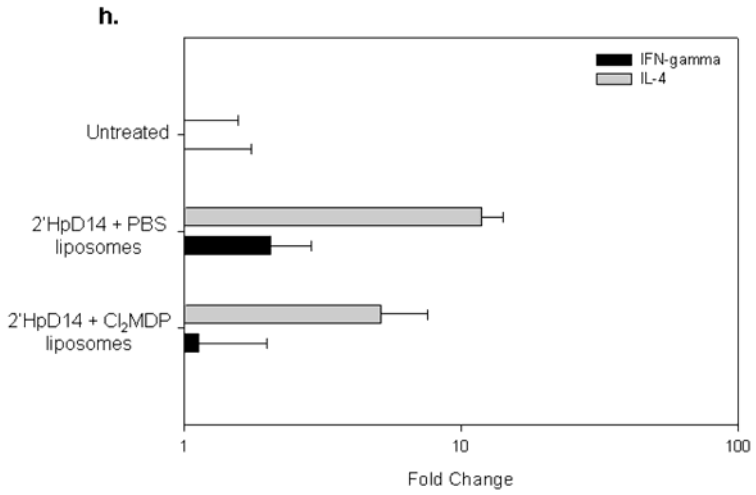
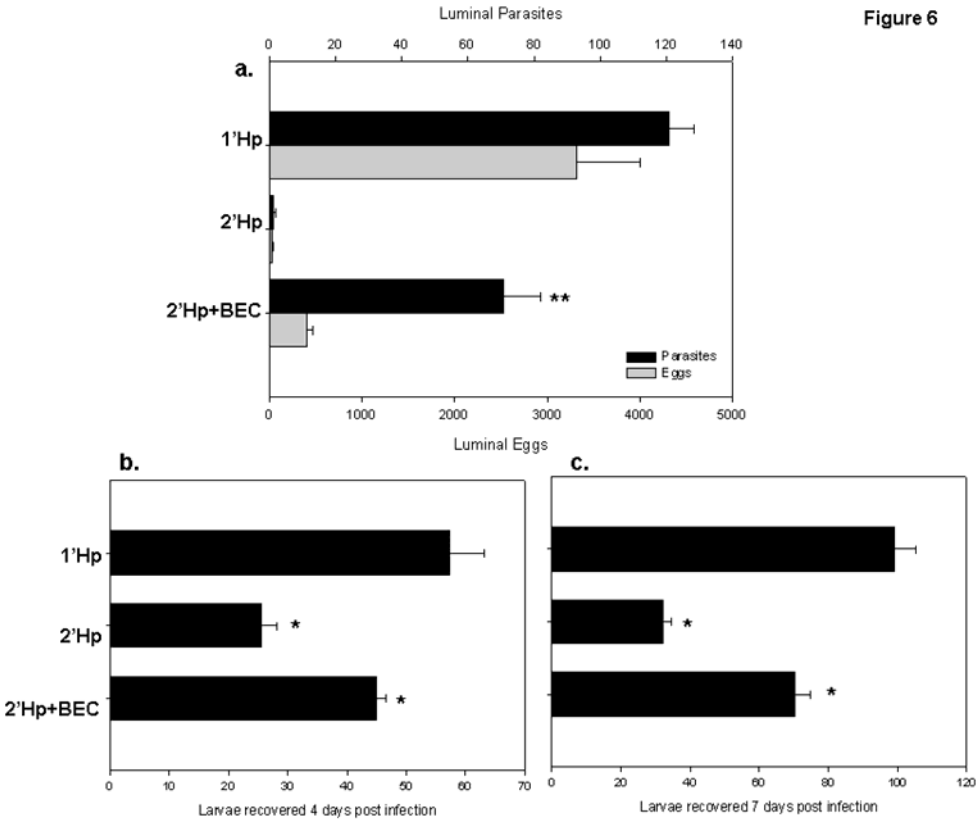


Figure 5. Macrophage depletion abrogates a protective Th2 memory response. a. Challenged mice were treated with clodronate- (2'HpD14+Cl2MDP) or PBS-liposomes (2'HpD14+PBS), and parasite and egg burdens were determined 14 days post infection. Larvae were collected from infected intestines using a Baermann apparatus at 4 (b) and 7 (c) days following infection from challenged mice receiving clodronate- or PBS liposome treatment. The mean and standard for each treatment group (5 animals per group) is shown, and the results are representative of 3 separate experiments. *$p<0.01$ determined using ANOVA followed by Tukey's post hoc test. d-g. Fluorescent immunohistochemistry of the host:parasite interface 4 days post challenge showed that neither clodronate- (d) nor PBS- liposome (e) treatment affected infiltration of Gr-1^+ (red), $CD4^+$

(blue), or $CD11c^+$ (green) cells at the host:parasite interface, while $F4/80^+$, $CD206^+$ macrophages were specifically depleted in the CL2MDP liposome (g) treated group compared to the PBS-liposome treated group (f). Image exposure times and fluorescent intensities were normalized to appropriate controls. h. Mesenteric lymph nodes (MLN) were collected 14 days post infection, cytokine gene expression was determined by real time PCR, and fold changes determined by comparison to untreated MLNs. Highly polarized Th2 cytokine profiles were detected in secondary infections, in both PBS and Cl2MDP treated mice, indicating that clodronate liposome treatment did not affect the polarization of the response.

Figure 6


Luminal Parasites
a.
1'Hp
2'Hp
2'Hp+BEC
**
Parasites
Eggs
Luminal Eggs
b.
1'Hp
2'Hp
2'Hp+BEC
*
*
Larvae recovered 4 days post infection
c.
*
*
Larvae recovered 7 days post infection

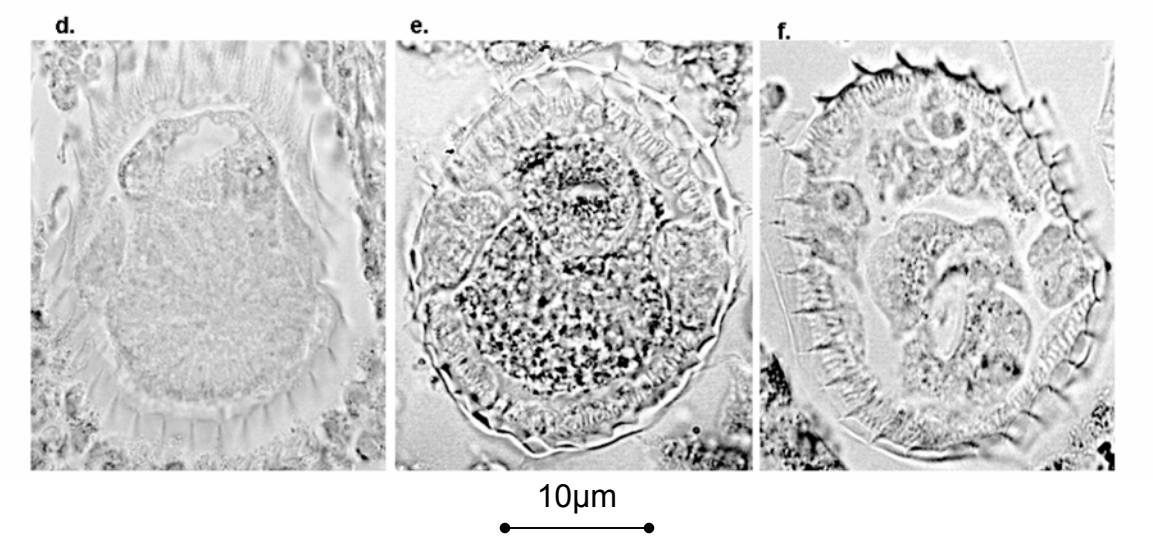


Figure 6. Arginase inhibition abrogates protective memory response to *H. polygyrus*. Challenged mice were given 0.2% BEC (2'HpD14 + BEC) in their drinking water, and parasite and egg burdens determined 14 days post injection (a), and larvae were recovered from infected small intestines 4 (b) or 7 (c) days post infection using a Baermann apparatus. Bars represent mean and standard errors of 5 mice per group, and black dots represent individual parasite burdens. **$p<0.001$ and *$p<0.01$ determined using ANOVA following by Tukey's post hoc test. Parasite cross-sections were stained for cytochrome oxidase (black granules) and obtained 7 days following primary infection (d), secondary infection (e), and secondary infection treated with BEC (f).

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Dissertation Discussion and Summary

A greater understanding of the immune response elicited by helminth parasites is important for a number of reasons: it may identify novel protective mechanisms or cell populations that can be targeted for vaccines against this important group of pathogens; it may illuminate regulatory mechanisms that could be exploited to control the inappropriate responses of allergic, asthmatic, or autoimmune disease; it may elucidate therapies to control aberrant, and potentially detrimental inflammatory responses triggered by bacterial or viral infections. Despite these wide-reaching implications, little is known about the host-defense mechanisms that lead to expulsion of these parasites.

The work presented in this dissertation employed an infectious gastrointestinal nematode model to examine protective Th2 immune responses. *H. polygyrus*, a natural murine parasite, establishes a chronic infection in naïve mice, while a challenge infection is naturally and rapidly cleared by the host. This distinction between primary and secondary responses provides an easy readout of functional immunity based on parasite and egg burdens. Initial experiments showed that the first 7 days of the secondary response were essential for expulsion of the parasites and prompted further analysis of the early events occurring during challenge infection. A localized inflammatory response was identified surrounding the parasite during these early invasive stages, which was distinct from a primary response, and featured $CD4^+$ T cells surrounding the parasite as early as 4 days post infection, and localized expression of Th2 cytokine mRNA. Further characterization of this polarized type 2 inflammatory

response identified $CD4^+$ T cells as the predominant producers of IL-4 protein at the host:parasite interface, and infiltrating MΦs as the primary responders to these cytokines. Accumulating MΦs adopted an alternatively activated phenotype (IL-$4R^{hi}$ $CD206^+$ arginase-1^+ $Fizz1^+$ $Ym1^+$ $iNOS^-$) that was dependent on $CD4^+$ T cells and IL-4R signaling, which demonstrates that the MΦs were in fact responding to the T cell derived cytokines expressed at the host:parasite interface. A number of intervention experiments, where macrophages were either depleted or inhibited during challenge infections, resulted in the blockade of protective immunity as characterized by high parasite burdens at day 14 and enhanced larval recovery at days 4 and 7 post infection. These studies implicate alternatively activated macrophages as essential effector cells in this protective Th2 response, and mucosal inflammation as a novel protective mechanism against gastrointestinal parasites.

Discussion of results

Early immune events are important for protection against *H. polygyrus* challenge

Our efforts focused on immune mechanisms elicited against the invasive phase of *H. polygyrus* infection. Depletion of $CD4^+$ T cells at day 0 post challenge, and to a lesser extent on day 7-post challenge, inhibited the protective memory response, while $CD4^+$ cell depletion at later stages of challenge (on day 9 or 11 post infection) had little effect on day 14 adult worm and egg burdens. These results suggested the first 7 days of the immune response were essential for maximal worm expulsion. During this time, the parasite larvae reside in the

intestinal submucosa, which suggested the protective immune response may be targeting invasive larvae.

There has been little evidence suggesting that responses directed against invasive larval stages of *H. polygyrus* are important for host-protection[89,91,94,95,103]. Studies have demonstrated that termination of primary infections during larval stages has no effect on the development of protective immunity to challenge infections. By administering the anti-helminthic agent ivermectin as early as four days post primary infection, investigators showed mice were able to effectively clear a challenge infection[132,133]. It is possible that there are common antigens expressed by larval and adult stages, thereby providing a potential explanation for this observation: mice from these studies might have been exposed to the protective antigen despite the premature termination of the infection, ultimately resulting in development of protective memory T cells. Regardless, these findings clearly show host exposure to adult worms is not required for the development of protective immunity against *H. polygyrus*.

To confirm that early events during the invasive stage of infection were essential to the secondary response for host-protection, larval recovery at days 4 and 7 were assessed following primary and secondary infections using a Baermann apparatus. High numbers of larvae were recovered from primary infected intestines, while in comparison significantly fewer larvae were collected from challenged intestines at both intervals. These findings prompted further

evaluation of the early events in the secondary response, focusing on the inflammatory reaction surrounding invasive larvae.

Analysis of the host:parasite interface following *H. polygyrus* challenge

A distinct immune cell architecture surrounds the invading larvae 4 days following secondary infection, with $Gr1^+$ neutrophils accumulating adjacent to the parasites in the muscularis, and $CD4^+$ T cells surrounding the parasite, accumulating along the muscularis border in the lamina propria. The $CD4^+$ T cells express IL-4 and IL-13 mRNA, are associated with IL-4 protein, and their depletion ablates type 2 cytokine expression at the host:parasite interface; taken together, these results suggest $CD4^+$ T cells are the primary producers of Th2 cytokines in the microenvironment surrounding the parasite. A number of IL-4 producing non-lymphocytes have been described, including eosinophils, basophils, and mast cells[134-136]; however, of these cell types, only eosinophils were detected at the host:parasite interface, and our analysis strongly suggests that $CD4^+$ T cells are the primary producers of type 2 cytokines in this microenvironment. Neutrophils and MΦs are often considered to be the first leukocyte responders at a sight of type 1 inflammation[33]. Therefore, the populations accumulating around the parasite by day 4-post infection have been previously implicated in early responses to infection; however, their role in type 2 inflammation remains undefined.

The MΦs gathering at the host*:H. polygyrus* interface were determined to be the principal responders to these cytokines; these cells expressed high levels of the IL-4Rα, and adopted an alternatively activated phenotype ($IL\text{-}4R^{hi}$ $CD206^+$

arginase-1^{+} Fizz1^{+} Ym1^{+} iNOS^{-}) in a stat6-dependent manner, indicating signaling through the IL-4 receptor was required for the development of this phenotype. The accumulation of MΦs at the host:parasite interface has been extensively reported in a number of infectious diseases, including during *Myocobacterium tuberculosis* infection[137,138]. This respiratory pathogen induces a potent Th1 response, characterized by high levels of IL-12, IFN-γ, and TGF-β, leading to iNOS induction by MΦs.

Examination of diseases mediated by intracellular pathogens have elucidated an essential, protective role for MΦs, activated through exposure to IFN-γ from T cells leading to the production of bactericidal NO. Infections with *Salmonella typhimurium*, *Listeria monocytogenes*, and *Leishmania sp*. all require IFN-γ activation of MΦs for host protection[63,106-109,139-141]; these pathogens have acquired mechanisms to evade the lysosomal pathway, and require super oxide bursts to control infection. Closely mirroring this model of MΦ activation, our studies show that during the type 2 responses, cytokine-producing Th2 cells also activate MΦs that accumulate at the site of infection; however, although the cell populations are similar, the activated phenotype and effector functions are quite different from those seen in a type 1 response.

Macrophages are essential effector cells during protective secondary responses to *H. polygyrus.*

Roles for MΦs in Th2 responses are beginning to be understood, as AAMΦs have only been described recently. In *schistosomaisis*, MΦs play an important role in host survival; the initial infection by these parasites triggers a

Th1 response directed against the larvae and adult stages[142]. As the parasites mature and produce eggs, the immune response repolarizes, and a Th2 response develops that targets the parasite eggs[142]. AAMΦs are important in the down-modulation of the initial Th1 response: blockade of the development of AAMΦs results in intense type 1 inflammation, hepatic and intestinal pathology, and death[110]. However, *H. polygyrus* infection triggers a highly polarized Th2 response, with no detectable IL-12 or IFN-γ, and thus it is unlikely AAMΦs are playing a similar role in this response[90].

Our studies showed that AAMΦs were essential effector cells in our infectious model, as their selective depletion (by administration of clodronate liposomes) and inhibition (using an arginase inhibitor, BEC) blocked adult parasite expulsion 14 days post challenge; additionally, both of these interventions enhanced larval migration out of the small intestinal tissue at 4 and 7 days post infection compared to challenged controls. These are the first studies to show that arginase can be protective in infectious diseases; studies examining infection with the intracellular parasite *Leishmania major* convincingly show that arginase expression is harmful to the host, preventing killing of the parasite leading to propagation of disease[109]. A protective role of arginase has been described in non-infectious immune responses, by its ability to inhibit or downmodulate inappropriate type 1 inflammation in an IBD model[143].

To demonstrate the larval parasites themselves were under duress in the intestinal tissue, tissue sections taken 7 days following primary and secondary infections were stained for cytochrome oxidase activity, an indicator of aerobic

metabolism and oxidative stress[144-148]. Interestingly, parasites from the challenge infection stained darkly for cytochrome oxidase, compared to those in a primary infection. Treatment with the arginase inhibitor, BEC, markedly reduced cytochrome oxidase staining. Taken together, these findings indicate that AAMΦs were causing a cellular stress response in the invading larvae, and support a novel model of protective Th2 inflammatory responses (fig. 8), where accumulating memory $CD4^+$ T cell expressing Th2 cytokines induce the alternative activation of MΦs, which in turn trigger a stress response in invading parasites in an arginase-dependent manner, ultimately contributing to worm expulsion. This model of inflammation has many parallels to models of Th1 inflammation, where $CD4^+$ T cell are recruited to the sight of infection following neutrophils and macrophages, and T cell derived cytokines direct inflammatory populations to mediate appropriate immunity (**fig. 10**).

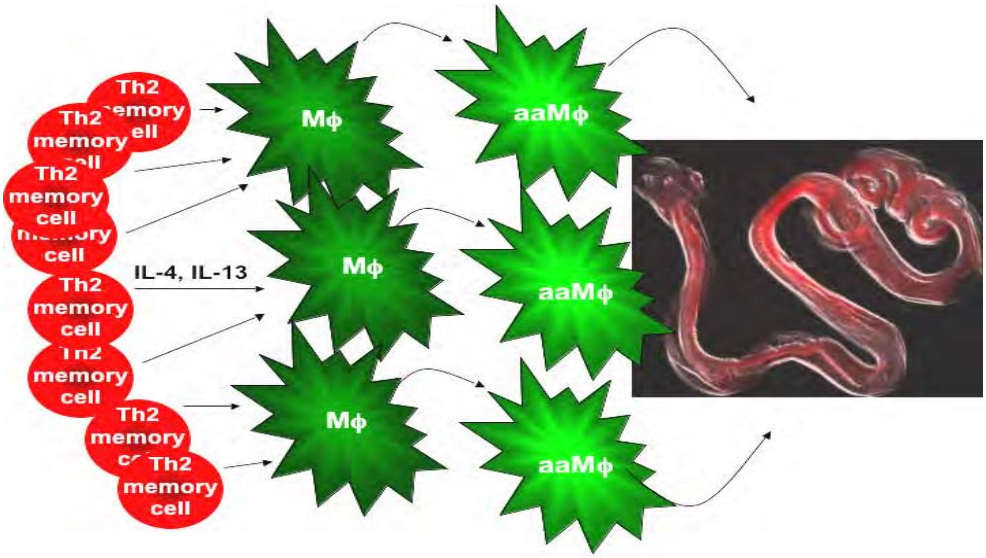

Figure 10. A model of Th2 protective inflammation against *H. polygyrus*. Accumulating memory Th2 cells expressing IL-4 and IL-13 alternatively activate macrophages, which in turn contribute to parasite expulsion.

A number of hallmark or characteristic genes have been described for AAMΦs that are chitinases or chitinase-like genes, including Ym1, Fizz1, and AMCase[112,117]. F4/80$^+$ cells dissected from the host:parasite interface 4 days after challenge by IF-LCM highly upregulated these genes, while expressing no detectable iNOS mRNA, further confirming that these accumulating macrophages were alternatively activated. This characteristic expression of chitinases and chitinase-like genes suggests a potential effector mechanism, as some helminth parasites feature a chitin cuticle that might be degraded by chitinases expressed by AAMΦs[115]. However, this is an unlikely protective mechanism against *H. polygyrus*, because chitin is expressed only in the non-infectious parasite eggs, and not by the invasive larvae. Our studies show an important aspect of the protective immune response is directed against the invasive stages of the infection[149].

AAMΦs have been described in a non-infectious Th2 response as key mediators of asthmatic inflammation in the lung, actively recruiting eosinophils and T cells to that mucosal site[115,150]. MΦs from human asthmatic lung biopsies upregulated AMCase mRNA; additionally, neutralizing antibodies to the murine AMCase protein inhibited asthmatic inflammation in a mouse model of asthma[115]. Other studies have described a recruitment role for AAMΦs, where Ym1 acted as a chemotactic agent for eosinophils in a Brugia malayi model[151]. Initial studies of protective Th2 responses to *H. polygyrus* challenge highlighted the importance of CD4$^+$ T cells and Th2 cytokines, as depletion of CD4$^+$ T cells or inhibition of the IL-4R by administration of a blocking antibody resulted in blockage of

protection[97,98,103]. The Th2 cytokines IL-4 and IL-13 have overlapping functions, are expressed in similar patterns, and signal through the type 2 IL-4 receptor (although only IL-4 signals through the type 1 receptor) (figure 7 of introduction)[126]. These similarities make deciphering the individual functions of IL-4 and IL-13 difficult. In the case of *H. polygyrus*, IL-4 was cited as the primary protective cytokine when IL-4$^{-/-}$ mice were found to be unable to clear secondary infections, and as the administration of exogenous IL-4 to immunocompromised mice resulted in the clearance of infection[97]. These studies, and others, highlight the principle that IL-4 and IL-13 have distinct functions *in vivo*[97,103].

To elucidate the roles of IL-4 and IL-13 in protection, studies have focused on their ability to alter gut physiology[90,91,93-96]. These cytokines increase luminal fluid secretion and gut contractility, which have been proposed to make the gut lumen an inhospitable environment to the lumen-dwelling adult parasites. Studies have convincingly shown these changes can be induced in a stat6-dependent manner, thereby implicating signaling through the IL-4R, and suggesting Th2 cytokines directly affect the smooth muscle of the intestines to induce increased contractility and intestinal goblet cells to increase luminal fluid production. On going studies (by Fred Finkelman, Terez Shea-Donohue and others) are teasing out the specific effects of IL-4R signaling on these distinct anatomic sites by engineering tissue-specific deletions of the IL-4R.

More recently, investigators have begun to look beyond the induced mechanical changes of the gut to define more clearly protective mechanisms against gastrointestinal nematodes. Using a mouse whipworm model of *Trichuris*

muris, Cliffe *et al.* [152] showed that resistant mice have accelerated rates of intestinal epithelial cell turnover two weeks post infection, and this turnover contributes to expulsion of the parasites. However, it is unlikely similar protective mechanisms are employed during resistance to *H. polygyrus* challenge. *T. muris* partially embeds in the epithelial lining of the colon, leaving its tail extending in the gut lumen, and thus occupies a distinct microenvironment and niche[90,153]. *H. polygyrus* invades the muscularis region of the small intestines for approximately a week, followed by a chronic infection in the lumen of the small intestine, thereby making epithelial cell turnover an unlikely mechanism leading to expulsion[100].

Alternatively activated macrophages and L-arginine catabolism

The catabolism of the amino acid L-arginine is essential for macrophage function; during a Th1 response, MΦs are activated via Toll Like Receptors or through exposure to IFN-γ, leading to upregulated expression of iNOS, which converts L-arginine to L-hydroxy-arginine and finally into citruline and nitric oxide (NO), essential for the killing of intracellular pathogens[154-156]. Conversely, MΦs exposed to hallmark Th2 cytokines (IL-4 and IL-13) upregulate arginase-1; this enzyme has a higher affinity for L-arginine than iNOS, and therefore out-competes iNOS for the common substrate[105,114,122,154-156]. Arginase-1 converts L-arginine to L-ornithine and urea; L-ornithine is further processed by two enzymes, ornithine decarboxylase (ODC) and ornithine aminotransferase (OAT). ODC converts L-ornithine into polyamines (putrescine, spermidine, and spermine), which enhance cellular proliferation by stabilizing DNA-protein interactions[157,158].

OAT catabolizes L-ornithine into proline and glutamate. Proline is used by fibroblasts to synthesize collagen, assigning a role of AAMΦs in tissue repair and fibrosis[114,116]. Glutamate is further processed into the inhibitory neurotransmitter γ-amino butyric acid (GABA)[159-161] .

Regulatory T cells during *H. polygyrus* infections

Infection with *H. polygyrus* has been shown to induce regulatory T cells; CD4+ T cells recovered from the lamina propria of infected mice expressed high levels of the inhibitory cytokine IL-10[17]. Adoptive transfer of these cells into recipient mice with an induced colitis reduced the inflammation in an IL-10 dependent fashion. Another study addressed the effect of *H. polygyrus* infection on asthmatic responses, and demonstrated that airway hyperresponsiveness was reduced in mice infected with *H. polygyrus*[19]. While it is possible that regulatory T cells play a role in the establishment of a chronic primary infection, interestingly, these cells do not seem to affect the response to challenge with *H. polygyrus*, which is acutely expelled (Anthony, Urban, and Gause, unpublished data).

Effector $CD4^+$ T cells circulate systemically following primary infection with *H. polygyrus*, although it is not clear if these cells have regulatory potential[162]. However, it does suggest that systemic regulatory T cells might develop following infection with gastrointestinal parasite infection. The identification of regulatory cells, while providing insights into potential treatments for allergy, asthma, and autoimmune disease, suggests additional difficulties for the development of

vaccines against tropical diseases. Presently, it is not clear if AAMΦ play a role in the induction of regulatory T cells during helminth infection.

Advancements in IL-4 detection

The prototypic Th2 cytokine IL-4 is essential for the development of Th2 responses, as it promotes the differentiation of T helper cells into type 2 effector cells and inhibits type 1 responses. The expression of this important cytokine is very tightly regulated, and IL-4 is also at very low quantities compared to other cytokines (it is expressed 1000-fold lower than its Th1 counterpart, IFN-γ), making its detection extremely difficult[163]. This technical hurdle has been overcome by the use of robust techniques, including real time RT-PCR for examination of mRNA levels, and ELISPOT assays and intracellular FACS analysis for identification of IL-4 producing cells. Recent studies have employed IL-4 reporter knockin mice, featuring an IL-4 promoter driving the expression of GFP to identify IL-4 expressing cells, and have described IL-4 expression by basophils and eosinophils, as well circulating T cells[134,135,162,164]. However, no known function of the IL-4 expression by these populations is known. Potential caveats with these mice are that expression of the GFP may not be similar to IL-4 protein, or even reflect the cytokine's mRNA levels, as the GFP is processed differently by the cell and has a much longer half-life. An additional shortcoming of these techniques is that they require the generation of single cell suspensions or whole tissue homogenates, resulting in the loss of immune cell architecture within the organ of interest.

Our studies employed two novel methods for the detection of IL-4 *in situ*, IF-LCM coupled with RT-PCR, and immunofluorescent antibody staining. IF-LCM allowed the microdissection of specific cell populations identified by immunofluorescence within the microenvironment of the host:parasite interface. The dissected populations were analyzed by RT-real time PCR, and allowed for detection of IL-4 mRNA express within the individual populations. To examine the IL-4 protein expression patterns, we developed a new methodology, where two antibodies recognizing distinct epitopes of the IL-4 proteins were used in tandem (**fig. 11**). These antibodies were conjugated to the same fluorochrome, resulting in amplification of the fluorescent IL-4 signal enough to be detected by conventional fluorescent microscopy.

Figure 11. IL-4 *in situ* staining. Antibody pairs (red and blue) conjugated to the same fluorochrome (green) amplify the IL-4 signal enough to be detected by convention fluorescent microscopy.

These methods identified CD4+ T cells as the primary producers of IL-4 at the *H. polygyrus* host:parasite interface, and have been extended to analysis of lymph nodes during Th2 responses, while preserving the structure of the tissue.

Future Directions

To further examine the role of L-arginine catabolism during this response, we have conducted preliminary experiments using inhibitors of the enzymes downstream of arginase-1: OAT and iNOS. OAT converts L-ornithine into proline, essential for collagen production, and glutamate that is further converted to glutamine and GABA. Th2 responses have been implicated in fibrotic disease during *Schistosomaisis*, with IL-13 being a key profibrotic cytokine in the development of liver granulomas [116,119,165]. Indeed, collagen was detected at the host:*H. polygyrus* interface , but only at stages after the larvae matured and migrated out of the submucosa and into the intestinal lumen. For this reason, collagen deposition itself is an unlikely protective mechanism, but is instead a marker for Th2 inflammation mediated by alternatively activated macrophages. It has been proposed that profibrotic actions of macrophages are important in wound healing[166,167].

Interestingly, inhibition of OAT by the chemical inhibitor gabaculine resulted in blockade of worm expulsion (data not shown). Thus, it is possible that one protective mechanism of AAMΦs is through the production of neurotransmitters which might act to induce changes in gut physiology; GABA has been shown increase intestinal smooth muscle contractility, although there are multiple receptors for the neurotransmitter in the gut with distinct

properties[168]. However, there is emerging evidence for crosstalk between the immune and nervous systems[169-172].

ODC converts the amino acid L-ornithine into polyamines, resulting in the production of spermidine, putrescine, and spermine[160]. The polyamines aid in cellular proliferation by stabilizing DNA-protein interactions[173-176]. It is possible AAMΦs throughout the intestines produce polyamines to aid in the proliferation of lymphocytes or intestinal epithelial cells[152]. To examine the role of ODC and polyamine production in the protective immune response, the selective inhibitor α-difluoromethylornithine (DFMO) can be administered in drinking water[143,177,178].

An additional consequence of arginase-1 expression involves iNOS, which is traditionally associated with classically activated MΦs. Resting MΦs express low basal levels of iNOS, and in the absence of intracellular L-arginine (depleted by arginase-1), iNOS adopts a reductase function, leading to the generation of oxygen and nitrogen radicals[114]. Increases in systemic oxygen radicals during Th2 responses have been detected during filarial infections, and therefore may be an addition mechanism of protection exerted by AAMΦs[124]. To directly examine the interplay of iNOS during the protective response to *H. polygyrus* challenge, we employed the iNOS inhibitor 1400W. We found little effect on resulting parasite burdens 14 days post infection, suggesting iNOS is not required for *H. polygyrus* clearance, which is not entirely surprising, as the enzyme is conventionally associated with responses to intracellular pathogens.

Conclusions

This dissertation has focused on the localized inflammatory response during early stages of *H. polygyrus* challenge, and infection that is acutely cleared by the host in a $CD4^+$ T cell and IL-4 dependent fashion. Memory Th2 cells accumulating at the host:parasite interface expressed IL-4 and IL-13 mRNA, and were associated with IL-4 protein, while infiltrating MΦs were found to be the primary responders to this localized cytokine production by adopting an alternatively activated phenotype (IL-$4R^{hi}$ $CD206^+$ arg-1^+ $Fizz1^+$ $Ym1^+$ $iNOS^-$). The early stages of this response were found to be important for host protection, based on both CD4+ T cell depletion and larval recovery studies. Similar results were found in both the MΦs depletion (via clodronate liposomes) and inhibition (by inhibiting arginase), studies both of which resulted in blockade of parasite expulsion 14 days post infection and increased larval recoveries at days 4 and 7 post infection. Interestingly, in the intervention studies targeting macrophages, while adult luminal parasite burdens were significantly increased compared to controls, the egg production by these parasites was markedly reduced suggesting that additional macrophage-independent protective mechanisms remained intact that affected parasite fecundity.

The protective inflammatory response to *H. polygyrus* superficially resembles inflammatory responses at the site of infection described in many Th1 models of infectious disease, where neutrophils and macrophages also rapidly accumulate followed by $CD4^+$ T cells. The T cells then direct the inflammatory populations' activation state and effector functions through the production of cytokines. In the Th2 response, however, the cell populations are quite similar,

although their activation state and function are different. These studies represent the first demonstration that AAMΦs are critical effector cells during protective Th2 responses and they assign an evolutionary role to the MΦs' alternatively activated state.

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Appendix

Statement of Author's Contribution:

The author was personally involved in the planning and execution of all experiments described in this dissertation, and played a crucial role in the writing of both papers presented here. The author also developed the IF-LCM technique, *in situ* IL-4 staining method, and was directly involved in additional experimental assays used for these studies. Furthermore, the author has presented different stages of this work in oral presentations at a number of scientific meetings, in addition to coauthoring papers not directly related to this dissertation. This dissertation serves as partial fulfillment of the dissertation requirements for the Molecular and Cell Biology program of USUHS, Bethesda, MD 20814.