

ANTIGEN SPECIFIC CD4+ T CELL RESPONSES AGAINST A GASTROINTESTINAL
NEMATODE

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A DISSERTATION

in

Immunology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2021

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For my grandfather, Buck Shamer. I hope I've made you proud.

ACKNOWLEDGMENTS

For starters, De'Broski – I came to graduate school to learn how to do science, and you have given me a training environment to learn so much more. Thank you for challenging me to think big, be creative, and lighten up. Your enthusiasm, support, and devotion to my development as a scientist are unwavering and I am forever grateful.

To the Herbert lab – thank you for being the absolute best. Special thanks to Li-Yin, for teaching me so much and having patience with me; Kelly, for supporting me through prelims and early thesis days; and Chris, for keeping the lab running and for the constant reminders that you are younger and cooler than me. To all of the newer members, and especially fellow grad students Annabel, Evonne and Jay – I'm so excited to be leaving the lab in such good hands. Keep killing it.

To my committee, as well as faculty and trainees in Hill 3rd floor joint lab meeting, Worm Enthusiast's meeting, and across Penn Vet – thank you for listening to me talk about this project and giving such phenomenal advice and encouragement. Your excitement and feedback were invaluable and I'm so appreciative of the time and energy you put into my training. Thanks also to those of you who shared expertise, techniques, and reagents when I needed protocols, antibodies, or to be talked down from going Office Space on a clogged Fortessa.

To IGG – first, to Mary and Thalia – thank you for running the show, but also for your constant positivity and caring in spite of what I can only imagine is a very demanding and difficult job. Dave, Igor, Taku, Jorge, and especially my first-year advisor Paula – thank you for the countless hours you devote to this program and its students. To the IGG community as a whole – thanks for the practice prelims, the student RIPs, the happy hours, the post-happy hours, and the companionship.

To my dear friends in and outside of IGG – thank you for keeping me sane, and for encouraging me to grow from adversity and not be broken by it. Especially to Andrew, Austin, Joey, and Sophia – thanks for listening, providing snacks, drinks, and memes, being shoulders to cry on, and not letting me quit. Let's do Research in Beer again soon.

To my family – thank you for all of the love and encouragement you have given me over the years. I know I'm the odd one out in a family of musicians, but I so value your ceaseless enthusiasm and support for my interests anyway.

To Steve – you're the reason I'm still here. Thank you for getting me through this, love ya.

Finally, to the animals. To my cat, Lupin – you are endlessly frustrating but I love you anyway, thank you for keeping me entertained. To my dog Hank – thank you for giving me something to care for when I was at my most burnt out. And to all the research animals who made this work possible – thank you for allowing us to learn.

ABSTRACT

ANTIGEN SPECIFIC CD4+ T CELL RESPONSES AGAINST A GASTROINTESTINAL NEMATODE

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Helminths are distinct from microbial pathogens in terms of size and complexity, and are likely the evolutionary driving force for type 2 immunity. CD4+ helper T cells can both coordinate worm clearance and prevent immunopathology, but issues of T cell antigen specificity in the context of helminth-induced Th2 and T regulatory cell (Treg) responses have not been addressed. Herein, a novel transgenic line of the gastrointestinal nematode *Strongyloides ratti* was generated that expresses the immunodominant CD4+ T cell epitope 2W1S as a fusion protein with green fluorescent protein (GFP) and FLAG peptide in order to track and study helminth-specific CD4+ T cells. C57BL/6 mice infected with this stable transgenic line (termed *Hulk*) underwent a dose-dependent expansion of activated CD44+CD11a+ 2W1S-specific CD4+ T cells, preferentially in the lung parenchyma. Transcriptional profiling of 2W1S-specific CD4+ T cells isolated from mice infected with either *Hulk* or the enteric bacterial pathogen *Salmonella* expressing 2W1S revealed that pathogen context exerted a dominant influence over CD4+ T cell phenotype. Interestingly, *Hulk*-elicited 2W1S-specific CD4+ T cells exhibited both Th2 and Treg phenotypes and expressed high levels of the EGFR ligand amphiregulin, which differed greatly from the phenotype of 2W1S-specific CD4+ T cells elicited by 2W1S-expressing *Salmonella*. While immunization with 2W1S peptide did not enhance clearance of *Hulk* infection, immunization did increase total amphiregulin production as well as the number of amphiregulin-expressing CD3+ cells in the lung following *Hulk*

infection. Altogether, this new model system reveals that helminth-specific CD4+ T cells can adopt effector as well as immunosuppressive and wound reparative phenotypes.

This report establishes a new resource for studying the nature and function of helminth-specific T cells.

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CHAPTER 1: INTRODUCTION

1.1 *Parasitic helminths in global public health*

Humans and helminths have coexisted for millennia, with evidence of helminth infections in human remains dating back to 10,000 years before present day (Cox, 2002; Sianto et al., 2009). This coexistence has driven the evolution of both host and parasite: the host immune system has developed tools for damaging, containing and expelling worms while mitigating infection-induced tissue damage, and the parasite has evolved means by which to manipulate and evade host immune responses. In the present era, human helminthiases are widespread within the developing world, with over one billion individuals estimated to harbor one or more parasitic helminth infections (P. J. Hotez et al., 2008). In industrialized countries, improved sanitation and access to healthcare has largely eliminated helminth infections, but in the absence of these parasites the prevalence of allergies and autoimmune diseases has increased (Smits & Yazdanbakhsh, 2007; Weinstock, 2006). Thus, there is a need to develop new treatments and prophylactics to alleviate the burden of helminth infections and to harness the host-modulatory capabilities of helminths as therapeutics for aberrant inflammatory conditions. Underpinning both of these goals is the need to improve our basic understanding of the interplay between the host immune system and helminth parasites.

While parasitic helminths are commonly grouped together as metazoan parasites, there is tremendous heterogeneity within this class of pathogen. There are nearly 300 species of helminths that infect humans alone, and these vary greatly in terms of their genetics, body plans, and infection cycles (Cox, 2002). Taxonomically, parasitic helminths can be divided into the nematodes (roundworms), which include gastrointestinal (GI) and filarial nematodes; trematodes (flukes), which include

trematodes such as schistosomes; and cestodes, which include tapeworms (Brindley, Mitreva, Ghedin, & Lustigman, 2009). For the purposes of epidemiologic studies, GI nematodes that spend any portion of their life cycle in the soil are referred to as soil-transmitted helminths (STH). Sections 1.1 and 1.2 of this introduction will focus on STH and GI nematodes, respectively, as the parasitic infection model used in this study falls under both of these categories.

STH are the most common causes of human helminthiases worldwide, and include the roundworm *Ascaris lumbricoides*, hookworms *Necator americanus* and *Ancylostoma duodenale*, whipworm *Trichuris trichiura* and threadworm *Strongyloides stercoralis* (Weatherhead, Hotez, & Mejia, 2017). STH heavily parasitize children and cause delayed or stunted growth, cognitive impairment, reduced school attendance and performance, and ultimately lower future wage-earning potential. As individuals in endemic areas age, they typically acquire some level of protective immunity that results in an inverse correlation between age and infection severity. However, even low-level chronic infections still result in morbidity, including malnutrition, anemia, and fatigue. Moreover, not all STH infections wane with age: in the case of hookworm infections, infection intensity often peaks in adulthood, suggesting that the immune response against these pathogens is insufficient to fully clear existing infections or prevent new infections from occurring (P. J. Hotez et al., 2008).

Fortunately, there are a number of anthelmintic drugs available to treat STH infections. These include albendazole and mebendazole, which are commonly used to treat infection with *A. lumbricoides*, *N. americanus*, *A. duodenale*, and *T. trichiura*, and ivermectin, which is potent against *S. stercoralis* (Buonfrate et al., 2019; P. J. Hotez et al., 2008). Delivery of these pharmaceutical agents via mass drug administration (MDA) has proven effective at curbing infection intensity and associated morbidities, albeit to

varying degrees based on the methods used (Farrell et al., 2018). However, there is growing evidence of emerging drug resistance that could render MDA efforts futile (Moser, Schindler, & Keiser, 2017). Further, even after drug treatment, there are high rates of reinfection by the same parasite(s) in endemic areas, highlighting the need not only for curative treatment options but also preventative measures such as vaccines (Dunn et al., 2019; Jia, Melville, Utzinger, King, & Zhou, 2012).

1.2 Biology of gastrointestinal nematodes

In order to better understand how to treat or prevent infections by soil-dwelling GI nematodes, it is helpful to first understand the biology of these organisms. Morphologically, GI nematodes bear many similarities to their free-living counterparts, but have evolved distinct life histories that require parasitism for reproduction. Contrary to microbial pathogens, GI nematodes are multicellular and have multiple organ systems. Their life cycles are complex, with different life stages occupying various environmental and host tissue niches. These features make it challenging to design effective therapeutics and vaccines, and pose a unique set of challenges to the host immune system.

1.2.1 Life cycles

As their name suggests, soil-dwelling GI nematodes share two common life cycle features: they exist in the soil as eggs or larvae, and ultimately reach sexual maturity as parasites in the GI tract of their hosts. How they infect their hosts, what (if any) intermediate tissues they traverse on their way to the GI tract, and how they reproduce varies by species. Since the studies presented in subsequent chapters will utilize the *S. ratti* infection system, this section will focus on *Strongyloides spp.* life cycles specifically. However, differences between these life cycles and those of other GI nematodes will also be highlighted. A diagram of the *S. ratti* life cycle is provided in Figure 1.1.

Strongyloides spp. begin their life cycles in the soil, when eggs or first-stage larvae (L1) from infected hosts are deposited into the environment through the feces. Once in the soil, two developmental pathways are possible. One pathway is referred to as *direct* or *asexual* development, whereby L1 larvae mature through a second (L2) and third (L3) larval stage and then arrest until encountering a host. Importantly, this pathway is only accessible to female larvae. Conversely, both males and females can develop into free-living, sexually-reproducing adults via *indirect* or *sexual* development. These adults can then mate to produce a new generation of all-female L1s, which themselves develop through the L2 stage and arrest as L3s until they encounter a host. Whether they originated from direct or indirect development, only female L3 are capable of infecting new hosts (M. E. Viney & Lok, 2015).

At the infective L3 stage, *Strongyloides spp.* infect new hosts by penetrating the skin. In the wild, this typically occurs when exposed skin comes into contact with the ground. However, in the laboratory, rodent-adapted species such as *S. ratti* or *S. venezuelensis* can be used to infect mice or rats either by applying larvae directly to the skin or by injecting larvae subcutaneously. Larvae quickly migrate through the skin and into blood vessels, where they utilize the vasculature to travel predominantly to the lungs or the nasofrontal region of the head within 24 hours (Dawkins & Grove, 1981; Nutman, 2017; Tindall & Wilson, 1988). Here, they undergo a molt from an L3 to an L4 stage between 1-3 days post-infection, before moving to the GI tract by crawling up the trachea or migrating through the nasal cavity to the esophagus (Dawkins & Grove, 1981; Tindall & Wilson, 1988). Fifth-stage larvae (L5) and adult parasitic females take up residence in the proximal small intestine, where they burrow into the intestinal epithelium, reproduce asexually through parthenogenesis, and release eggs into the host intestinal lumen to propagate the life cycle (Dawkins, Robertson, Papadimitriou, &

Grove, 1983; M. E. Viney & Lok, 2015). *S. stercoralis* infections in immunocompetent humans result in chronic, often subclinical infections that may last for the life of the host, in part due to the unique ability of *S. stercoralis* to develop autoinfective L3 that can infect the same individual without re-entering the environment (Nutman, 2017). Notably, neither *S. ratti* or *S. venezuelensis* have been observed to autoinfect hosts. In experimental settings, *S. ratti* and *S. venezuelensis* infections usually clear within 2 weeks post-infection (M. Viney & Kikuchi, 2017).

The *Strongyloides* life cycle is typical of most GI nematodes, with a few important exceptions. Firstly, not all GI nematodes are skin-penetrating: *Trichuris spp.*, for example, infect hosts orally and are strictly enteric pathogens. Secondly, as mentioned above, *S. stercoralis* is unique even among *Strongyloides spp.* in its ability to autoinfect hosts. Finally, the *Strongyloides* genus is one of only two parasitic clades in Phylum Nematoda known to reproduce sexually in the environment, and they are the only known GI nematode which can undergo asexual reproduction in host (Stasiuk, Scott, & Grant, 2012). These last two features make *Strongyloides spp.* particularly amenable to genetic manipulation, which will be discussed in more detail in Section 1.3.

1.2.2 Anatomy and physiology

The complexity of GI nematode life cycles poses many challenges to the immune system, including cellular migration to and from multiple tissues and detection of extracellular parasites that are orders of magnitude larger than individual immune cells. Moreover, GI nematode transcriptomes and proteomes change as parasites develop through distinct life stages, implying that host immune cells are exposed to temporally and spatially restricted antigens (Hunt, Hino, Yoshida, & Kikuchi, 2018; Soblik et al., 2011). Host-parasite interactions are further complicated by the fact that helminths are comprised of multiple cell types and organ systems, which are presumed to be variably

accessible to host immune cells in different host tissues. How the immune system interacts with different nematode anatomic compartments, and which nematode antigens confer protection against GI nematode infection, remain largely unknown. Yet understanding the anatomy and physiology of GI nematodes is important for hypothesizing how the immune system might interact with these metazoan parasites.

Nematode body plans are relatively simple: their digestive tract or alimentary canal runs the length of their body, and is surrounded by a pressurized fluid cavity called the pseudocoelom that helps maintain the organism's shape and motility. The pseudocoelom is then contained by a body wall, which is comprised of a muscle layer and an outer cuticle (Basyoni & Rizk, 2016). The nervous system is centralized at the nerve ring, which encircles the pharynx near the mouth of the nematode, and extends out to the lengths of the worm body via ventral and dorsal nerve cords that control motor function (Basyoni & Rizk, 2016). Sensory neurons also protrude from the nerve ring toward the mouth region, and help to sense and integrate environmental cues (Schafer, 2016). The reproductive tract, which is present in immature forms in larvae, becomes fully developed in adult stages of nematodes and is made up of tube structures that run parallel to the digestive tract (Basyoni & Rizk, 2016). In addition to these somatic tissues, nematodes also release into their surroundings a wide variety of excretory/secretory (ES) products and extracellular vesicles (EVs) through the digestive system, reproductive tract, and excretory/secretory system (Coakley, Maizels, & Buck, 2015; Hewitson, Grainger, & Maizels, 2009). A diagram of the nematode body plan is provided in Figure 1.2.

Among these compartments, the nematode secretome (including ES products and EVs) has garnered perhaps the most research interest due to its high potential to interface with host cells and factors. Whereas nematodes themselves are spatially

restricted in host tissues, ES products and EVs can diffuse into host tissues, interact with host soluble factors or membrane-bound receptors, and even deliver cargoes of proteins, carbohydrates, and nucleic acids directly to host cells (Coakley et al., 2015; Hewitson et al., 2009). One example of how ES products influence host-parasite interactions is through proteases. Metallo-, cysteine and serine proteases are prevalent in nematode ES products (Hewitson et al., 2009). While these proteases are necessary to breakdown and reform the nematode cuticle during molting, evidence suggests that they also have the ability to bind to and degrade host structural proteins (e.g. fibronectin, plasminogen, laminin) (P. Hotez et al., 1990; Jiang et al., 2019; Page, Stepek, Winter, & Pertab, 2014). While it is unclear whether this enzymatic activity is necessary for tissue invasion in mammalian hosts, work in the plant-parasitizing nematode *Globodera rostochiensis* supports that venom allergen-like (VAL) proteins, which are highly conserved between animal and plant-parasitic nematodes, are required for establishment of infection (Lozano-Torres et al., 2014). In addition to their purported roles in tissue invasion and migration, ES products and EVs also interface with components of the host immune system and greatly impact host immune responses.

This topic will be discussed in greater detail in Section 1.4.

Aside from the nematode secretome, the cuticle is likely the most accessible somatic tissue to the immune system on account of its location on the outside of the worm and its large surface area. The cuticle is an essential part of the body wall that helps maintain nematode shape and motility. It is made up predominantly of collagen and cuticulin proteins, and is covered by a thin epicuticle that is composed of lipids and glycoproteins (Page et al., 2014). As nematodes develop through successive larval stages, the cuticle must be shed and reformed (Page et al., 2014). Shedding of the cuticle in the L3 or L4 stages likely provides a source of antigen for the host immune

system. Work in insect (entomopathogenic) nematodes also suggests that lipid components of the epicuticle may suppress host immunity by binding directly to soluble host immune factors (Brivio, Mastore, & Nappi, 2010; Brivio, Pagani, & Restelli, 2002; M, Moro, & Mastore, 2006). While this phenomenon has not been reported in vertebrate or mammalian nematode models, these findings support that the cuticle is likely a dynamic site of host-parasite interactions during nematode infections.

Though it may not be as directly accessible to host immune cells during infection as the cuticle, the digestive tract is also likely to influence host-parasite interactions. As mentioned previously, the digestive system releases soluble factors and EVs into the host-parasite interface, either as waste or as an active means of virulence or host immune suppression (Hewitson et al., 2009). However, this system has also been hypothesized as an ideal target for vaccines or anthelmintic drugs. While an array of digestive antigens are likely released and accessible to the host immune system, there are presumably a number of antigens that are not released in high concentrations, or that are released into spaces where they cannot readily be recognized by cells of the immune system (e.g. host intestinal lumen). The latter are referred to as “hidden” antigens, and these are considered advantageous therapeutic and vaccine targets for 2 reasons. First, because these antigens are not naturally accessible to the host immune system, it is unlikely they have undergone selective pressure to evade host immune recognition. Second, if these antigens serve essential roles in nutrient absorption or digestion, blocking their function via antibody binding or small molecule inhibition could quickly kill infective nematodes (Munn, 1997). Vaccines targeting hidden antigens have been modestly successful in protecting sheep from infection by the nematode *Haemonchus contortus*, and identifying and characterizing intestinal antigens has

become a priority for vaccine and novel anthelmintic development (Bassetto, Silva, Newlands, Smith, & Amarante, 2011; Wang, Rosa, Jasmer, & Mitreva, 2015).

In addition to the digestive tract, the neuromusculature of nematodes is a known target of several anthelmintic drugs important in both human and veterinary medicine. Ivermectin, for example, acts as an agonist on inhibitory glutamate-gated chloride channels that are expressed predominantly in neurons and muscle cells, as well as in cells of the excretory pore and reproductive tract. By depolarizing these cells, ivermectin disrupts worm motility and pharyngeal pumping, which is necessary for digestion, as well as secretion through the excretory pore and release of eggs from the reproductive tract – ultimately reducing reproductive fitness and worm survival (Martin, Robertson, & Choudhary, 2021). Similarly, anthelmintic drugs like levamisole and pyrantel paralyze worms by agonizing nematode nicotinic acetylcholine receptors (AChR), causing muscle contractions, loss of motility, and detachment from host tissue (Harrow, 1985).

While impairing neuromuscular function is a well-established strategy for clearing GI nematodes from hosts, it is less clear how disrupting the sensory nervous system affects GI nematode physiology, survival and infection persistence. However, there is increasing evidence that nematode chemical and thermal sensing facilitates host seeking behavior in skin-penetrating GI nematodes. Infective-stage larvae of skin-penetrating nematodes *S. stercoralis*, *S. ratti*, *N. americanus* and *Ancylostoma spp.* exhibit migratory behavior toward heat (up to 42° C), near-physiological concentrations of sodium chloride (NaCl), and host sweat and serum (Bhopale, Kupprion, Ashton, Boston, & Schad, 2001; Forbes, Ashton, Boston, Zhu, & Schad, 2004; Koga et al., 2005; Koga & Tada, 2000; Lopez, Boston, Ashton, & Schad, 2000). Further, laser microbeam ablation studies have demonstrated that sensory neurons in the amphidial channel are responsible for thermo- and chemotaxis, as well as activation of arrested third-stage *S.*

stercoralis larvae into their infective forms (Ashton, Zhu, Boston, Lok, & Schad, 2007; Bhopale et al., 2001; Forbes et al., 2004; Lopez et al., 2000). Accordingly, these neurons may also play roles in sensing other chemoattractants or repellants in host secretions and tissues.

With recent advances in parasitic nematode genetics, it has been possible to begin isolating not only neurons but specific genes that facilitate chemotaxis. An early candidate for study was the *S. stercoralis* gene *Ss-tax-4*, a homolog of the *Caenorhabditis elegans* *tax-4* gene which encodes a cyclic-nucleotide-gated channel and is important for chemosensing in that model organism (Bryant et al., 2018; Gang et al., 2020). Knocking out *Ss-tax-4* using the CRISPR/Cas-9 system revealed that this gene is necessary for both thermotaxis and migration toward the host sweat odorant, 3-methyl-1-butanol (Bryant et al., 2018; Gang et al., 2020). Still, much work remains to identify which host molecules serve as chemoattractants or repellants at distinct sites of infection, and to elucidate the signaling cascades responsible for host sensing in GI nematodes. Further, whether ablation of amphidial neurons or their signaling pathways is sufficient to prevent GI nematodes from initiating or maintaining infection in hosts remains unknown. However, studying the GI sensory nervous system will likely identify new drug and vaccine targets as well as improve our understanding of the dynamic interplay between parasite and host during infection.

1.3 Genetic manipulation of parasitic helminths

Genetic gain and loss of function studies have been invaluable in advancing our understanding of model organisms, including the free-living nematode *C. elegans* and rodent hosts of well-studied GI nematode species. Unfortunately, genetic manipulation in parasitic nematodes, and helminths in general, has historically proven challenging - in large part due to their requirement for host passage, developmental and anatomical

complexity, and endogenous gene silencing machinery (J. Lok, 2013; J. B. Lok, Shao, Massey, & Li, 2017). However, recent studies utilizing new tools for genetic modification of parasitic helminths have demonstrated how powerful these approaches can be in terms of understanding both helminths and their hosts. As these techniques become more tractable and accessible, they have the potential to revolutionize both basic research and clinical treatment of parasitic helminth infections.

Among the many obstacles to establishing genetic techniques in parasitic helminths is delivering sufficient genetic material to cells of relevant tissues without significant worm mortality. A number of different methods have been used in various helminth species to transiently silence or overexpress genes, with varying efficiencies and survival rates: these include electroporation, biolistic gene transfer, transfection, and culturing worms in media containing nucleic acids (Correnti, Jung, Freitas, & Pearce, 2007; Correnti & Pearce, 2004; Davis et al., 1999; Higazi & Unnasch, 2013; Issa, Grant, Stasiuk, & Shoemaker, 2005; Kines et al., 2010; S. Liang, Knight, & Jolly, 2013; Shu, Katholi, Higazi, & Unnasch, 2003; Xu et al., 2011; Yang et al., 2010). These techniques have been used extensively to facilitate RNA interference (RNAi) in the blood fluke *Schistosoma mansoni*, and have identified genes involved in *S. mansoni* development, reproduction, infection persistence, and drug resistance (Beckmann, Buro, Dissous, Hirzmann, & Grevelding, 2010; Collins & Collins, 2016; Correnti, Brindley, & Pearce, 2005; Valentim et al., 2013; Wendt et al., 2018). Further, combining electroporation or transduction with retro- or lentiviral or CRISPR/Cas9 gene editing has allowed for transgenesis in *S. mansoni* and a similar liver fluke, *Opisthorchis viverrine*. These tools have thus far been used to knockout or knock-in mutations to genes whose products influence host immunity and pathogenesis, and have helped establish methods for antibiotic selection in order to generate stable, heritable transgenesis in *S. mansoni*.

specifically (Arunsan et al., 2019; Ittiprasert et al., 2019; Rinaldi et al., 2012; Suttiprapa et al., 2016).

While genetic techniques are probably most well developed in the *S. mansoni* model, transgenesis in GI nematode species has been accomplished. Recently, Hagen et.al. developed a method for RNAi using the Vesicular Stomatitis Virus glycoprotein G-pseudotyped lentivirus to transduce third-stage larvae (L3) of the rodent hookworm *Nippostrongylus brasiliensis*. Transduction of L3 with lentivirus encoding an mCherry reporter and microRNA-adapted short hairpin RNAs (shRNAmirs) targeting β -tubulin isotype 1 or acetyl-cholinesterase B led to detectable mCherry transcription and a 40-50% reduction in transcription of each gene. Importantly, though mCherry fluorescence was predominantly detected in the intestinal epithelium of transduced L3s, mCherry transcription could be observed in F1 progeny of transduced L3 after passage through rats, indicating germline integration and transgene heritability (Hagen, Sarkies, & Selkirk, 2021). Though it remains to be seen whether this technique will be tractable for delivery of larger transgenes, or in other GI nematode species, this method could significantly enhance genetic study of genes involved in *N. brasiliensis* pathogenesis.

A more established method for transgenesis in GI nematodes involves delivering genes directly to germline cells via microinjection, a technique developed in the *C. elegans* model nearly 40 years ago (Stinchcomb, Shaw, Carr, & Hirsh, 1985). First adapted for use in the entomopathogenic nematode *Heterorhabditis bacteriophora*, microinjection is now also used in *Strongyloides* and *Parastonyloides* spp. (Grant et al., 2006; Hashmi, Hashmi, & Gaugler, 1995; J. B. Lok & Massey, 2002). *Strongyloides* spp. are particularly well-suited for this type of delivery because of the accessibility and morphology of the free-living adult stage, which bears anatomical similarity to *C. elegans*. Further, because the adult parasitic stage reproduces through parthenogenesis,

their progeny are all clonal – allowing for higher frequencies of transformed parasites to be recovered during host passage. Using this method, transgenes encoded with a 5' *S. stercoralis* or *C. elegans* promoter and a 3' *C. elegans* untranslated region (UTR) can be directly injected into the gonads of the free-living female and expressed in egg stages of F1 progeny (J. B. Lok & Massey, 2002). Stable, heritable transgenic lines of *S. stercoralis* have since been established by replacing the *C. elegans* 3' UTR with the 3' UTR region of the *S. stercoralis* intestinal *era-1* gene, which allows for continued transgene retention and expression through multiple generations (X. Li et al., 2006).

Such transgenic constructs delivered alone via microinjection, in the form of PCR products or in the context of plasmid vectors, are assumed to integrate into multi-copy extrachromosomal arrays, or episomes (J. Lok, 2013). While genes encoded in episomal arrays *can* be robustly expressed and vertically transmitted, expression is often unstable over time and subject to endogenous gene silencing mechanisms, making it difficult to generate germline-encoded, stable mutant lines (Boulin & Hobert, 2012). However, heritable transgenesis in *Strongyloides spp.* has become more attainable through the use of either transposon-mediated chromosomal integration or CRISPR/Cas-9 gene editing. The former was first achieved in *S. ratti* by microinjecting two plasmids simultaneously into free-living females: one encoding the promoter-transgene-UTR construct, and one encoding the *piggyBac* transposase (Shao et al., 2012). The *piggyBac* transposase is known for its ability to integrate relatively large portions of DNA into actively-transcribed regions of the genome at high copy numbers, allowing for continuous and heritable gene expression (J. Lok, 2013). Using this approach, stable, GFP-expressing transgenic lines of *S. ratti* were generated, each bearing between 40-50 genome-integrated copies of the transgene and reaching 95-100% penetrance after 4 generations and successive selection and passage of GFP+ progeny (Shao et al.,

2012). More recently, heritable transgenesis has been performed in both *S. stercoralis* and *S. ratti* by microinjecting either plasmid-encoded Cas9 and short guide RNAs (sgRNA) or a ribonucleoprotein (RNP) complex containing Cas9 and sgRNA into the gonads of free-living females (Gang et al., 2017). This approach has helped to identify and characterize genes involved in *S. stercoralis* muscle function and chemosensing (Bryant et al., 2018; Gang et al., 2017; Gang et al., 2020).

Transgenesis in parasitic nematodes, whether transient or permanent, opens avenues for more sophisticated study of both parasite and host. Most immediately, these tools will allow for thorough interrogation of parasitic nematode biology and hopefully begin to address the genetic and molecular basis for parasitism. For example, in both *Strongyloides spp.* and *N. brasiliensis*, genetic manipulation has identified important roles for *daf* genes, which are involved in insulin-like signaling via the nematode steroid dafachronic acid, in larval development in the environment (Ayoade et al., 2020; Castelletto, Massey, & Lok, 2009; Dulovic & Streit, 2019; Massey, Nishi, Chaudhary, Pakpour, & Lok, 2003; Z. Wang et al., 2015). Moreover, dafachronic acid signaling via DAF receptors has been implicated in suppressing L3 development within hosts during *S. stercoralis* autoinfection (Patton et al., 2018). While *daf* genes are conserved between parasitic nematodes and the free-living nematode *C. elegans*, such studies could also elucidate new genes or pathways in parasitic nematodes that may function to facilitate host-dependent life cycles.

Additionally, studies focused on host-parasite interactions and anti-helminth immune responses could greatly benefit from transgenesis. As new tissue-specific promoters are discovered in different helminth species, it will become possible to engineer stable transgenic lines that express fluorescent reporters or immunogenic molecules in different anatomical compartments to test how accessible different tissues

are to host immune cells. Of interest in *Strongyloides spp.* particularly are the intestine-specific promoter *Ss-era-1* and the amphidial neuron-specific promoter *Ss-gpa-3p*, which induce expression in compartments that I hypothesize to be highly accessible to the host as discussed in Chapter 1.2.2 (Junio et al., 2008). Moreover, even genes that are necessary for nematode development may now be targeted to study their effects on infection using inducible Cas9 expression systems. After infecting hosts with worms that transgenically express Cas9 under promoters inducible by small molecules (e.g. tetracycline), genes can be discreetly deleted in different life stages upon administration of that small molecule to the host (J. B. Lok, 2019). As will be discussed in the next section, this tool could be hugely beneficial for studying TGF- β mimics and other nematode-derived host immunomodulatory molecules that are also believed to be necessary for worm development (Johnston et al., 2017). Parasitic helminth transgenesis is still in its early days, but has the potential to completely change how the field approaches the study of parasitic helminth infections.

1.4 Immune responses to parasitic helminth infection

Whereas transgenesis in parasitic helminths has long been intractable, genetic manipulation in model host organisms – particularly mice – has offered tremendous insight into the immune mechanisms at play during helminth infection in mammals. Helminths are distinct from microbial pathogens (e.g. bacteria, viruses, protozoan parasites), most notably in terms of size, morphological and ontogenic complexity, and the infectious niches they occupy within hosts. These features pose unique challenges to the host immune system with regards to both pathogen clearance and host homeostasis, as worms cause tremendous damage to host tissues as they develop, migrate and feed. Furthermore, because parasitic helminths require years, even decades-long survival in their hosts to sustain reproduction, they have evolved ways of

suppressing and evading host immune responses to promote establishment and chronicity of infection. Altogether, the co-evolution of host and helminth is believed to be the driving force behind type 2 immune responses. This section will give an overview of both inflammatory and wound reparative aspects of type 2 immunity against GI nematodes, and will highlight elements of this immune response that are particularly important in the context of the *S. ratti* model system used in this study.

1.4.1 Innate recognition and control of helminth infection

As is the case in other infections, the first lines of defense against GI nematode infections are often physical and chemical barriers: epithelial barriers at the skin, lung, and gut, stomach acid, mucus, etc. Although their necessity has not been proven empirically, nematode proteases are thought to be essential for breaking down cell-cell junctions, extracellular matrices, and other structural components at physical barriers to allow for invasion and establishment of infection (P. Hotez et al., 1990; Jiang et al., 2019; Page et al., 2014). Invasion is often highly injurious, and leads to death of structural cells like epithelial and endothelial cells at sites of infection (Andronicos, McNally, Kotze, Hunt, & Ingham, 2012; Craig & Scott, 2014). As structural cells die or sense damage, they release damage-associated or alarmin cytokines like Interleukin (IL)-25, IL-33, and thymic stromal lymphopoitin (TSLP) to activate a variety of immune cells (Fallon et al., 2006; Fort et al., 2001; Schmitz et al., 2005; Soumelis et al., 2002). Given the relative dearth of information regarding molecular patterns associated with GI nematodes and their corresponding pattern recognition receptors, the prevailing theory in the field is that these damage-associated signals are key initiators of type 2 immune responses to parasitic nematodes.

Alarms released at the site of infection signal first to cells seeded in the tissue microenvironment: while there are many immune cell types present in tissues that can

respond to these alarmins, this section and the next will highlight mast cells, group 2 innate lymphoid cells (ILC2s), and dendritic cells. Mast cells mature and reside in tissue, and are present at virtually all mucosal sites – albeit at low frequencies – at homeostasis. They express a wide array of cytokine, chemokine, and growth factor receptors, as well as the receptor for IgE (Fc ϵ R1), and possess granules with pre-formed compounds that can be released rapidly. These features make them well-poised to respond quickly to diverse stimuli in distinct ways.

During helminth infections, mast cells can become activated in response to IL-33 and TSLP signaling, which can promote their survival and proliferation in tandem with cytokines like IL-4 and IL-3 (da Silva, Jamur, & Oliver, 2014; Madden et al., 1991; Saluja, Zoltowska, Ketelaar, & Nilsson, 2016). While there is conflicting evidence regarding whether mast cells are universally required for GI nematode expulsion, some reports have demonstrated this myeloid lineage to be necessary for the timely clearance of *Trichinella spiralis*, *Trichuris muris*, *Heligmosomoides polygyrus*, and *S. ratti* (Anthony, Rutitzky, Urban, Stadecker, & Gause, 2007; Donaldson, Schmitt, Huntley, Newlands, & Grencis, 1996; Grencis, Else, Huntley, & Nishikawa, 1993; Ha, Reed, & Crowle, 1983; Hepworth et al., 2012; Nawa, Kiyota, Korenaga, & Kotani, 1985; Ohnmacht & Voehringer, 2010; Reitz et al., 2017). In these contexts, mast cells regulate early alarmin cytokine levels, support early Th2 cell polarization and tissue remodeling, and promote expulsion of worms through cell-cell contacts and, predominantly, release of cytokines, proteases, histamine, and lipid mediators (da Silva et al., 2014; Hepworth et al., 2012). Multiple signals induce mast cell cytokine release and degranulation, and though IgE-mediated crosslinking of Fc ϵ R1 is probably most well-known, there is also evidence in the *S. ratti* model that both IL-33 and IL-9 are crucial for driving mast cell-dependent worm expulsion (Meiners et al., 2020; Reitz et al., 2017). However, work remains to be

done to discern exactly what signals drive specific anti-helminth effector functions of mast cells in relevant tissues during GI nematode infection.

Although developmentally and morphologically distinct from mast cells, ILC2s are similarly seeded in tissues and are sensitive to a wide variety of stimuli (Ricardo-Gonzalez et al., 2018). Initially described as non-B, non-T lymphoid cells that expand in response IL-25, it is now clear in the context of GI nematode infection that IL-25 from the intestinal epithelium, and specifically from the Tuft cell lineage, is crucial for early ILC2 expansion (Fallon et al., 2006; Gerbe et al., 2016; Howitt et al., 2016; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010; von Moltke, Ji, Liang, & Locksley, 2016). In addition to IL-25, IL-33 is critical for ILC2 expansion in the lung and gut during infection with both *N. brasiliensis* and *S. ratti* (Hung et al., 2013; Meiners et al., 2020; Yasuda et al., 2012). Though it is unclear whether IL-33, like IL-25, is required in a specific epithelial cell lineage, clearance of *N. brasiliensis* is dependent upon expression of IL-33 in the intestinal epithelial cell compartment (Hung et al., 2020).

Once activated, ILC2s are crucial players in the anti-helminth immune response, largely for their ability to quickly release large amounts of IL-5, IL-9 and IL-13 (and, to a lesser degree, IL-4) that recruit eosinophils, promote mast cell function, and feedback on the epithelium and smooth muscle to promote Tuft cell and goblet cell hyperplasia, mucus production, and enhanced gut motility – all of which facilitate worm expulsion (Fallon et al., 2006; Gerbe et al., 2016; Howitt et al., 2016; Hung et al., 2013; Moro et al., 2010; Price et al., 2010; Saenz et al., 2010; von Moltke et al., 2016). Additionally, ILC2s provide help to CD4+ T cells via class II major histocompatibility complex (MHCII) and OX40L-dependent cell-cell interactions to drive protective Th2 responses (Fallon et al., 2006; T. Y. F. Halim et al., 2018; Hung et al., 2013; Moro et al., 2010; Oliphant et al., 2014; Price et al., 2010; Saenz et al., 2010).

1.4.2 Initiation of type 2 adaptive immune responses

While the innate effector functions carried out by mast cells, ILC2s, and other innate cells early at the site of infection are important for limiting parasite burden, the adaptive immune system – and particularly CD4+ T cells – are crucial for worm clearance (Katona, Urban, & Finkelman, 1988; Reitz et al., 2017; Urban, Katona, & Finkelman, 1991). Given that CD4+ T cells must see antigen in the context of MHCII, there has been much interest in identifying the requisite MHCII-expressing antigen presenting cell (APC) for priming Th2 responses during helminth infections. Macrophages, dendritic cells (DCs), B cells, ILC2s, basophils and even epithelial cells can all express MHCII. While macrophages and MHCII-expressing ILC2s play necessary roles in supporting Th2 responses at sites of infection, it seems most likely that DCs are responsible for initially priming Th2 responses (Anthony et al., 2006; Walker & McKenzie, 2018). This is supported by data showing that loss of CD11c+ cells, and specifically loss of IRF4 or Klf4 expression in the CD11c+ compartment, leads to impaired Th2 responses and susceptibility to *T. muris* and *S. mansoni* infections (Mayer et al., 2017; Phythian-Adams et al., 2010; Tussiwand et al., 2015). Further evidence showing that loss of Batf3, a key transcription factor in type 1 conventional dendritic cells (cDC1s), enhances Th2 responses points to a requirement for cDC2s for Th2 priming (Everts et al., 2016). However, these reports are complicated by data demonstrating that Th2 responses are still impaired when MHCII expression is restricted to the CD11c+ compartment, suggesting that DCs alone are not sufficient to prime Th2 cells (Perrigoue et al., 2009).

One reason DCs may not be sufficient for Th2 priming is that, while they can be induced to express Th2-promoting costimulatory molecules like OX40L and Notch ligand Jagged 1 by TSLP and IL-33, they are poor producers of the Th2-skewing cytokine IL-4

(Walker & McKenzie, 2018). A likely source of IL-4 in this context are basophils, which themselves produce IL-4, are responsive to cytokines like TSLP, and expand in infection site-draining lymph nodes during GI nematode infections (Perrigoue et al., 2009; Reitz, Brunn, Voehringer, & Breloer, 2018). Though basophils are not required for secondary immunity against *S. ratti*, basophil-depleted or deficient mice do exhibit slightly higher worm burdens in the intestine following primary *S. ratti* infection and significantly higher worm burdens following secondary *H. polygyrus* and *N. brasiliensis* challenge (Ohnmacht et al., 2010; Reitz et al., 2018; Schwartz et al., 2014). Further, loss of basophils is associated with reduced Th2 responses in *H. polygyrus* secondary infection, and IL-4+MHCII+ basophils are capable of enhancing Th2 cytokine production *in vitro* and proliferation *in vivo* following injection of *S. mansoni* eggs (Perrigoue et al., 2009; Schwartz et al., 2014).

The role of basophils in Th2 priming is controversial, however. Other groups have shown no defects in Th2 expansion in basophil-deficient or depleted mice during papain or OVA-induced allergic inflammation or helminth infection, and many strategies for depleting basophils (e.g. Fc ϵ R1 blockade) also deplete mast cells, making it difficult to discern the individual contributions of these two cell types (Hammad et al., 2010; Ohnmacht et al., 2010). Moreover, while IL-4 is necessary for *in vitro* Th2 polarization, it is dispensable for Th2 priming *in vivo*, suggesting that DCs require other signals to prime Th2 responses (T. Y. Halim et al., 2014; van Panhuys et al., 2008). Although this process is still not fully characterized, more recent work suggests ILC2s, in addition to directly engaging CD4+ T cells via MHCII, may also be necessary to support initial DC migration to lymph nodes via IL-13 production in the context of allergic inflammation in the lung (T. Y. Halim et al., 2014).

1.4.3 Adaptive immune functions in parasitic helminth clearance

Despite gaps in knowledge regarding their initial priming, conventional CD4+ T cells are known to play an integral role in coordinating helminth clearance (Katona et al., 1988; Urban, Katona, & Finkelman, 1991; Urban, Maliszewski, Madden, Katona, & Finkelman, 1995). Th2 cells expressing the transcription factor GATA3 are required for immunity to *N. brasiliensis* and presumably other GI nematodes, although it remains unclear what the requisite function(s) of these cells are (Zheng & Flavell, 1997; Zhu et al., 2004). Historically, it was assumed that Th2 cells were necessary producers of cytokines like IL-4 and IL-13. This assumption was based on evidence showing that IL-4 and/or IL-13 signaling through IL-4 receptor alpha (IL-4R α) and downstream transcription factor STAT6 is necessary for worm expulsion, and that Th1 polarization and subsequently enhanced interferon gamma (IFN- γ) production is associated with susceptibility to chronic GI nematode infections (Else, Finkelman, Maliszewski, & Grencis, 1994; Urban, Katona, Paul, & Finkelman, 1991; Urban et al., 1998). However, mice in which CD4+ T cells lack IL-4 and IL-13 retain immunity to *N. brasiliensis* infection (Oeser, Schwartz, & Voehringer, 2015; Voehringer, Reese, Huang, Shinkai, & Locksley, 2006). Altogether, these data suggest that, while IL-4/13 are required for expulsion of GI nematodes, CD4+ T cell-derived IL-4/13 are not necessary – at least for clearance of *N. brasiliensis* infection.

Aside from IL-4 and IL-13, Th2 cells can also produce other signaling molecules, namely IL-2, IL-5 and the epidermal growth factor (EGFR) ligand amphiregulin (Cherwinski, Schumacher, Brown, & Mosmann, 1987; Zaiss et al., 2006). CD4+ T cell-derived IL-2 has been shown to be critical for supporting ILC2 function during infection in an MHCII-dependent manner, but since other T helper subsets (i.e. Th1s) produce IL-2 it is not clear whether Th2 cells exclusively engage in this crosstalk (Dong & Flavell, 2000;

Oliphant et al., 2014). IL-5 is crucial for eosinophilia, and both IL-5 and eosinophil numbers increase during helminth infection (Maizels & Balic, 2004). However, the role of IL-5 and eosinophils in helminth infection is controversial, varying between helminth species, host tissue context, and primary and secondary infections, and it is completely unclear whether IL-5 from CD4+ T cells is necessary for helminth immunity (Urban, Katona, Paul, et al., 1991)(Betts & Else, 1999; Brunet, Sabin, Cheever, Kopf, & Pearce, 1999; Dent et al., 1999; Filbey et al., 2019; Herbert et al., 2000; Herndon & Kayes, 1992; Shin et al., 1997). Amphiregulin has been shown to be necessary for clearance of *T. muris*, and its receptor (EGFR) is necessary on CD4+ T cells for timely expulsion of *N. brasiliensis* (Zaiss et al., 2006) (Minutti et al., 2017). Yet it is not known whether CD4+ T cell-derived amphiregulin is necessary for GI nematode expulsion.

Perhaps the most well-defined role of CD4+ T cells in helminth immunity is their ability to support production of antibodies such as IgG1 and IgE via IL-4 production (Finkelman et al., 1988; Katona et al., 1988; Voehringer et al., 2006). In this context, T follicular helper cells (Tfh) – which are developmentally related to, but functionally and phenotypically distinct from, Th2s – are a key source of this cytokine to promote class-switching in lymph nodes (King & Mohrs, 2009; Meli, Fontes, Leung Soo, & King, 2017). However, whether antibodies or B cells are necessary for immunity against helminths is also controversial. Passive transfer of immune serum can accelerate clearance of *T. muris*, but this is highly dependent upon the time of transfer and can be enhanced by co-transfer of cells from the mesenteric lymph nodes (Wakelin, 1975). In the *H. polygyrus* model, studies in *JH*-/- and μ MT mice reveal that B cells are necessary for secondary immunity, but reports are conflicting as to whether or not this is due to their ability to produce antibody or their ability to present antigen to and support CD4+ T cell responses (Liu et al., 2010; McCoy et al., 2008; Wojciechowski et al., 2009). Indeed,

while immunization with *H. polygyrus* ES products can elicit protective immunity, this cannot be conferred to naïve mice through passive serum transfer (Hewitson et al., 2011). While B cells are dispensable for clearance of primary *N. brasiliensis* infection, accelerated expulsion upon secondary infection is B cell-dependent and specifically requires IL-4Ra and MHCII expression on B cells (Horsnell et al., 2013). This suggests that B cells may play a more important role as antigen-presenting cells than as antibody-producers in secondary *N. brasiliensis* infection.

Altogether, while effector CD4+ T cells are necessary for clearance of a variety of GI nematodes, their requisite functions likely change in different infectious contexts. Though it's easy to consider all GI nematodes as equals, in reality there are differences in how they infect hosts (*i.e.* skin penetration vs. ingestion), where they molt (*i.e.* lungs vs. intestinal tract), and what cell types and cytokines are required for their clearance. The issue is further complicated by the considerable functional overlap between CD4+ T cells and other cells like ILC2s, for example. However, unlike these other cell types, CD4+ T cells can assert their functions in an antigen-specific manner, which likely allows them to act in unique locations and in concert with MHCII-expressing cells specifically. Understanding the impact of antigen specificity on CD4+ T cell phenotype and function in the context of GI nematode infection may help to elucidate CD4+ T cell-dependent mechanisms of clearance.

1.4.4 Immune suppression and tissue repair during parasitic helminth infection

Type 2 inflammation in the context of GI nematode infection must be tightly regulated to prevent immunopathology. Moreover, regulation and suppression of inflammation allows for host tissue repair, which is necessary following nematode-induced tissue damage. While hosts have evolved endogenous immunosuppressive and wound reparative mechanisms, GI nematodes can also produce immunomodulatory

factors that interact and sometimes interfere with productive host responses. This section will discuss immunosuppression and tissue repair, with an emphasis on the role of Tregs in these processes.

Tregs are an important subset of CD4+ helper T cells that were first identified as CD25+ CD4+ T cells and are now more specifically characterized by their expression of Foxp3 (Fontenot, Gavin, & Rudensky, 2003; Hori, Nomura, & Sakaguchi, 2003; Khattri, Cox, Yasayko, & Ramsdell, 2003). They are crucial for enforcing tolerance at baseline: both mice and humans with mutations at the *Foxp3* locus develop severe lymphoproliferative disorders and multiorgan autoimmune diseases (Blair et al., 1994; Clark et al., 1999; Wildin, Smyk-Pearson, & Filipovich, 2002). Moreover, upon infection, Tregs regulate inflammation to prevent host immunopathology and terminate immune responses after successful pathogen clearance. Tregs enforce tolerance and immunosuppression through an ever-increasing number of mechanisms, including but not limited to: release of IL-10, IL-35 and TGF- β , competition for co-stimulatory signals from B7 molecules via CTLA-4, inhibitory receptor signaling, and disruption of effector T cell activation, proliferation and metabolism through depletion of IL-2 and release of cyclic AMP (Campbell & Rudensky, 2020) (Zhao, Liao, & Kang, 2017). During helminth infection, these functions help direct initial immune responses while preserving tissue function. Depletion of Tregs prior to infection with *H. polygyrus* leads to increased interferon- γ (IFN- γ) production and subsequently increased weight loss early in infection (Smith et al., 2016). In *N. brasiliensis* and *S. mansoni* infection, loss of IL-4R α on Tregs impairs effector Treg function and recruitment and ultimately exacerbates tissue damage in the lung and liver, respectively (Abdel Aziz, Nono, Mpotje, & Brombacher, 2018).

Despite their importance in limiting immunopathology, Tregs have also been implicated in prolonging GI nematode infection. Infection with *H. polygyrus*, *N.*

brasiliensis or *S. ratti* leads to increased Treg numbers at sites of infection and site-draining lymph nodes (Finney, Taylor, Wilson, & Maizels, 2007)(Smith et al., 2016)(Blankenhaus et al., 2014)(Halim et al., 2018). Notably, when Tregs are expanded by administration of IL-2 complex, *H. polygyrus* and *N. brasiliensis* parasite loads are significantly increased (Smith et al., 2016)(Hung et al., 2020). Conversely, when Tregs are specifically deleted during infection with *S. ratti* or the filarial nematode *Litosomoides sigmodontis*, worm burden and fecundity are reduced (Blankenhaus et al., 2014; Taylor et al., 2009).

An open question remains as to whether Tregs that prolong or promote chronic infection are induced in the periphery (pTregs) or derive from the thymus (tTregs). Using Helios as a marker for tTregs, studies in *H. polygyrus* have shown expansion of both Helios- pTregs and Helios+ tTregs post-infection, but only a positive correlation between Helios+ tTregs and worm burden, suggesting tTregs may predominantly promote helminth chronicity (Smith et al., 2016)(Reynolds & Maizels, 2012). However, this notion can be difficult to square with evidence that GI nematodes produce immunosuppressive molecules and EVs, some of which can directly induce *de novo* Treg polarization. While ES products from multiple nematode species can induce Tregs *in vitro*, the most well-characterized molecule is a TGF- β mimic called *Hp-TGM* from *H. polygyrus*. Like host TGF- β , which potently induces Treg polarization, *Hp-TGM* signals through TGF- β receptors, induces SMAD2/3 phosphorylation, and strongly polarizes naïve mouse CD4+ T cells to become Foxp3+ Tregs *in vitro* (Grainger et al., 2010; Johnston et al., 2017; Marie, Letterio, Gavin, & Rudensky, 2005). Moreover, *in vivo* administration of *Hp-TGM* prolonged survival of allogeneic skin grafts (Johnston et al., 2017). Accordingly, *Hp-TGM* is thought to exploit endogenous host signaling machinery to promote pTreg expansion, allowing *H. polygyrus* to establish or maintain chronic infection. Of course, this does not

necessarily contradict a role for tTregs in facilitating chronic infection – these two Treg subsets could both support helminth persistence. Alternatively, it may benefit the parasite to induce Tregs that can perform other roles recently ascribed to this T helper cell subset, such as host tissue repair.

Host injury occurs early in GI nematode infection during larval migration through tissues. In the case of enteric nematodes like *H. polygyrus*, this stage occurs as larvae burrow into the submucosa of the small intestine and later emerge into the lumen. In skin-penetrating models like *N. brasiliensis* and *S. ratti*, damage is most evident during L4 migration in the lung. As early as 2 days post-infection, and 24-36 hours after larvae arrive in the lung, gross lung hemorrhage is evident (Chen et al., 2014; Douglas et al., 2021). This correlates with development of airway hyperresponsiveness (Marsland, Kurrer, Reissmann, Harris, & Kopf, 2008). In the *N. brasiliensis* model, this damage is resolved – at least at a histological level – by day 7 post-infection, and I no longer observe lung hemorrhage 14 days post-*S. ratti* infection (Chen et al., 2014; Douglas et al., 2021).

In addition to other cell types that have been more thoroughly characterized in the wound healing process (e.g. macrophages), Tregs have recently been appreciated as important players in tissue repair. Recent evidence suggests that amphiregulin is involved in both promoting Treg suppression, via autocrine signaling through EGFR, and in reducing tissue damage or promoting tissue regeneration in the lungs, muscle and skin (Arpaia et al., 2015; Burzyn et al., 2013; Nosbaum et al., 2016; Zaiss et al., 2013). However, it is unclear whether this amphiregulin must come from Tregs; though this appears to be the case for lung repair following influenza infection, others report mast cells as a crucial source of amphiregulin for promoting Treg immunosuppression in the B16 melanoma model (Arpaia et al., 2015; Zaiss et al., 2013). Moreover, evidence to

address whether Tregs directly participate in wound healing through amphiregulin production or other mechanisms is lacking. Studies showing that Tregs are necessary for wound healing in the skin, for example, propose that this function is still dependent upon their ability to suppress interferon gamma (IFN- γ) production and inflammatory macrophage populations, and not necessarily through direct signaling to damaged structural cells (Nosbaum et al., 2016). Though subtle, this distinction is important in GI nematode infections, where Tregs can antagonize clearance but may be crucial for restoring host tissue function and survival. If immunosuppression and tissue repair can be uncoupled – perhaps performed by distinct Treg subsets, or carried out in response to different stimuli – new therapies could be designed to specifically target immunosuppression to promote infection clearance while preserving wound healing.

1.5 Antigen specificity in immunity to infection

Antigen specificity is a hallmark of the adaptive immune system. This feature allows B and T lymphocytes to distinguish pathogens from one another and form memory of specific pathogens to mount more effective immune responses upon subsequent encounters. The consequences of antigen recognition in the context of microbial infections – specifically intracellular microbial infections – have been well-studied. During a type 1 immune response to an intracellular pathogen, CD8+ T cells produce cytokines like IFN- γ and TNF and lyse infected cells presenting peptide on MHC I through release of perforin and granzyme. CD4+ T cells interact with MHC II-expressing cells, also producing IFN- γ and TNF to support microbicidal functions in infected APCs and helping B cells in germinal centers via cytokine and surface receptors and ligands (e.g. CD40 ligand). B cells produce antibodies that can opsonize microbes for phagocytosis, inhibit growth, and prevent entry into new cells (Annunziato, Romagnani, & Romagnani, 2015).

How antigen recognition promotes clearance of or prevents infection by GI nematodes is less clear. GI nematodes occupy extracellular niches and are orders of magnitude larger than host cells, so CD8+ T cell-mediated cytolysis is not thought to play an appreciable role in this context. CD4+ T cells produce type 2 cytokines (e.g. IL-4, IL-5, IL-13) necessary to induce the “weep-and-sweep” response that ultimately expels worms, but their functions are largely redundant with those of ILC2s. They are unique, of course, in their ability to interact with and support B cell affinity maturation and antibody class-switching, but as discussed previously antibodies are variably protective in GI nematode infection and it is also unclear how they might prevent infection, block migration, or inhibit worm survival. This section will discuss studies of antigen specificity in microbial models and consider how antigen specificity may be necessary – or not – in the context of helminth infections, with emphasis on antigen-specific CD4+ T cells.

A wide variety of tools exist to identify and track antigen-specific CD4+ T cells in microbial infections. One set of these tools is comprised of MHCII tetramers that can identify CD4+ T cells specific for endogenous epitopes on pathogens, for example viruses like influenza and lymphocytic choriomeningitis virus (LCMV) and intracellular protozoan parasites like *Toxoplasma gondii* and *Leishmania spp.* (Brincks et al., 2013; Grover et al., 2012; Homann et al., 2007; Mou et al., 2015). Another common approach is to engineer transgenic pathogens that express model antigens such as ovalbumin or the E α peptide 2W1S, for which TCR transgenic mice or tetramer reagents exist that can be used to study antigen-specific CD4+ T cell responses during infection (Benoun et al., 2018; Ertelt et al., 2009; Pepper, Dzierszinski, Crawford, Hunter, & Roos, 2004; Prickett et al., 2006). Admittedly, both of these approaches have caveats: MHCII tetramers are often unable to detect low-affinity antigen-specific CD4+ T cells, CD4+ T cells specific for model antigens may behave differently than endogenous epitope-specific CD4+ T cells

due to intrinsic differences in TCR signaling strength, and adoptive transfer of high numbers of TCR transgenic CD4+ T cells does not accurately represent physiological numbers of naïve antigen-specific CD4+ T cells that exist normally prior to infection (Crawford, Kozono, White, Marrack, & Kappler, 1998; Martinez, Andargachew, Martinez, & Evavold, 2016).

Despite these limitations, these reagents have provided tremendous insights into the accessibility of antigens for CD4+ T cell recognition as well as the function of CD4+ T cells that recognize these antigens. In the context of protozoan parasite infection, expression of ovalbumin in different subcellular compartments of *L. major* or *T. gondii* revealed that surface-bound or secreted antigens are more accessible than antigens contained within the parasite's cytosol (Pepper et al., 2004; Prickett et al., 2006). Further, these tools have enhanced our understanding of antigen-specific CD4+ T cell expansion and cytokine production, revealing a protective role for IFN- γ -producing antigen-specific cells in *T. gondii* as well as a dual role for *L. major*-specific CD4+ T cells in producing IFN- γ and IL-10 (Grover et al., 2012; Pagan et al., 2013; Pepper et al., 2004). Generation of an E α peptide-expressing *T. gondii* strain allowed for the identification of plasmacytoid dendritic cells as an important APC early during infection (Pepper et al., 2008). Work with transgenic 2W1S-expressing bacteria such as *Listeria monocytogenes* has revealed that the route of infection can impact on T helper cell differentiation and memory, with mucosal infection promoting more short-lived Th17 responses and systemic infection promoting more long-lived Th1 responses (Pepper et al., 2010). However, given that these infections predominantly drive type 1 and/or type 17 immune responses, they do not allow for interrogation of antigen-specific Th2 cells or Tregs that expand in type 2 settings. Moreover, since a majority of these systems utilize intracellular pathogens, there is a dearth of information on how antigen-specific CD4+ T

cells play a role in killing or expelling extracellular pathogens, especially multicellular pathogens like helminths.

To my knowledge, no such transgenic systems or tetramer reagents exist to study CD4+ T cells specific for model or endogenous antigens expressed by GI nematodes. One TCR transgenic mouse line has been generated in which all CD4+ T cells are specific for the liver fluke *S. mansoni* egg antigen Sm-p40. However, these cells predominantly drive liver pathology through IL-17 and IFN- γ production during *S. mansoni* infection (Shainheit et al., 2011). Thus, while this model provides insight into pathological antigen-specific CD4+ T cells functions during helminth infection, it does not seem suitable to study Th2 cells or Tregs that participate in protection or immunosuppression. There is, however, evidence for antigen-specific Th2 and Treg responses during GI nematode infection. Multiple groups have shown that stimulation of cells from lymph nodes of mice infected with *H. polygyrus*, *N. brasiliensis*, or *S. ratti* with ES products or crude antigen preparations from the correspondent nematode species leads to production of one or multiple type 2 cytokines (e.g. IL-4, IL-5, IL-13) as well as IL-10 (Eschbach et al., 2010; Finney et al., 2007; Mearns et al., 2008). While these experiments do not rule out cytokine production by other cell types in response to innately-recognized nematode molecular patterns, more sophisticated work has demonstrated similar findings by co-culturing sorted CD4+ T cells with *H. polygyrus* antigen-pulsed dendritic cells, again suggesting that antigen-specific CD4+ T cells can produce both Th2 and Treg-associated cytokines (Rausch et al., 2008).

While these studies are useful for understanding polyclonal CD4+ T cells responses to a heterogenous mix of GI nematode antigens, they do not shed light on which antigens specifically are recognized by CD4+ T cells. They also cannot provide insight into antigen-specific requirements for clearance: that is, what is the minimal

number of T cell clones needed to confer protection? Can a single GI nematode-specific CD4+ T cell clone expand to promote clearance, or is a polyclonal repertoire required?

To begin to address this question, one group compared immune responses to and expulsion of *N. brasiliensis* in wild-type polyclonal mice, DO11.10 or SMARTA TCR transgenic mice with oligoclonal TCR repertoires (due to leakiness in TCR α chain allelic exclusion), and mice with a monoclonal TCR repertoire (DO11.10/*Rag*^{-/-} mice).

Interestingly, though DO11.10 mice with an oligoclonal repertoire supported normal IgE, basophil and eosinophil levels, oligoclonal DO11.10 and SMARTA mice and monoclonal DO11.10/*Rag*^{-/-} mice all failed to clear worms. This failure corresponded with significant reductions in IL-4-producing CD4+ T cells in lymph nodes and lungs of infected mice (Seidl, Panzer, & Voehringer, 2011).

Importantly, this study does not rule out that a monoclonal CD4+ T cell repertoire *specific for an N. brasiliensis antigen* could promote normal clearance. Indeed, DO11.10 T cells recognize ovalbumin and SMARTA T cells recognize the LCMV GP₆₁₋₈₀ epitope – neither of which are known to share any similarity with *N. brasiliensis* epitopes. Moreover, these two TCRs could have intrinsic defects in Th2 polarization relative to other clones which prevent them from sufficiently orchestrating clearance. Caveats aside, however, this study provides evidence that a polyclonal CD4+ T cell repertoire is necessary for clearance. This leaves an important question: does this polyclonal CD4+ T cell repertoire even need to recognize GI nematode antigens to carry out protective effector functions?

Perhaps the most obvious way in which CD4+ T cell-mediated antigen-recognition could be necessary is through CD4+ T cell-dependent antibody production. Although their protective capacity is controversial, one could imagine antibodies raised against a secreted larval protease could potentially block enzymatic activity and prevent

nematode tissue invasion in the skin. Antibodies raised against receptors or enzymes involved in digestion could inhibit nematode feeding and be lethal. Since CD4+ T cells can support antibody affinity maturation and class-switching in germinal centers for B cells presenting their cognate antigen, nematode-specific CD4+ T cells could select for B cells that produce potentially neutralizing antibodies (Figure 1.3). Yet evidence from both mice and humans suggests that a predominance of antibody responses against GI nematodes are either directed toward decoy antigens, such as venom allergen *Ancylostoma*-secreted protein-like (VAL) proteins, or class-switched to non-inflammatory isotypes such as human IgG4, and are therefore non-protective (Hewitson et al., 2011; Maizels & McSorley, 2016). Importantly, these data do not necessarily mean that antigen-specific antibodies are not involved in protection, and some studies have demonstrated protective roles of GI nematode-specific antibodies during infection of rodents with human-adapted parasites (Abraham et al., 2011; Herbert, Nolan, Schad, Lustigman, & Abraham, 2002). However, in their natural hosts, it may be that GI nematodes have evolved effective mechanisms to evade specific antibody responses that could otherwise afford protection.

In addition to supporting B cell affinity maturation and class switching in germinal centers, CD4+ T cells are also important cytokine producers. However, many cytokines produced by Th2 cells (e.g. IL-4, IL-5, IL-9, IL-13, amphiregulin) can also be produced by ILC2s (Fallon et al., 2006; Moro et al., 2010; Neill et al., 2010; Price et al., 2010; J. E. Turner et al., 2013). Given their ability to survive long-term and remember specific antigens, CD4+ T cells are unique from ILC2s in their ability to form memory to protect against secondary infections. ILC2s, on the other hand, are seemingly better suited to respond quickly to primary GI nematode infection: they are seeded in tissues such as skin, lung and gut during development, express a wide array of receptors to respond to

environmental changes, and can rapidly become activated *in situ* (Ricardo-Gonzalez et al., 2018)(Kotas & Locksley, 2018). Yet ILC2s are not sufficient to clear primary GI nematode infections, as evidenced by the susceptibility of RAG- and T cell-depleted mice (Katona et al., 1988; Reitz et al., 2017; Urban, Katona, & Finkelman, 1991; Urban et al., 1995). What, then, are CD4+ T cells doing to promote GI nematode clearance?

When considering the function of CD4+ T cells in contexts like intracellular bacterial infections, TCR-MHCII mediated cell-cell contact is important for delivering CD4+ T cell help to effector cells. For example, delivery of IFN- γ to infected macrophages at the T cell-macrophage synapse induces nitric oxide production, which is directly microbicidal and helps macrophages kill bacteria, as well as GTPases that can disrupt intracellular bacterial niches and make them more susceptible to host killing mechanisms (Annunziato et al., 2015; Kim et al., 2011). IFN- γ can also induce cell death in structural cells, such as epithelial cells infected with *Salmonella* (Ingram et al., 2018). In the context of GI nematode infection, CD4+ T cells have been similarly shown to support ILC2 proliferation and IL-13 secretion in an MHCII, contact-dependent manner via IL-2 production (Figure 1.3)(Oliphant et al., 2014). However, other cells may benefit from MHCII-mediated contact with CD4+ T cells.

While immune cells are crucial for orchestrating clearance, the key effector cells in GI nematode immunity are thought to be intestinal epithelial cells (IECs) and smooth muscle cells. Epithelial cell mucus production, release of anti-helminth factors such as Relm- β , and proliferation are thought to physically dislodge worms from the epithelial surface, which, when coupled with increased transit time induced by smooth muscle cell hypercontractility, leads to expulsion of worms from hosts (Akiho, Blennerhassett, Deng, & Collins, 2002; Cliffe et al., 2005; Herbert et al., 2009). Importantly, these functions can all be driven by IL-4R α signaling (Akiho et al., 2002; Herbert et al., 2009). Furthermore,

both IECs and intestinal smooth muscle cells have been demonstrated to express MHCII constitutively or inducibly, and IECs significantly upregulate expression of MHCII during *H. polygyrus* infection (Biton et al., 2018; Hogaboam, Snider, & Collins, 1996). Perhaps CD4+ T cells are uniquely poised to deliver localized help – in the form of IL-4, IL-13, other type 2 cytokines, or even surface receptor ligation – to these effectors in an antigen-dependent manner (Figure 1.3). Since epithelial cells are often in closest physical proximity to nematodes at sites like the intestine, they may have the greatest access to helminth ES products, EVs, and other antigens. Though epithelial cells in the skin and lungs have not yet been implicated in larval killing, both of these cell types have been shown to express MHCII, and so could also cooperate with CD4+ T cells to promote immunity at earlier points of infection (Tamoutounour et al., 2019; Toulmin et al., 2021).

Antigen-dependent interactions between epithelial or muscle cells and Tregs could also potentially help direct wound reparative cytokines (e.g. amphiregulin, TGF- β) where they are most needed (Figure 1.3). Since antigen-specific Tregs are thought to have enhanced suppressive capabilities compared to bystander, polyclonal Tregs, such interactions could also enhance immunosuppression at sites near worms where inflammation might be greatest (Tang et al., 2004; Tarbell, Yamazaki, Olson, Toy, & Steinman, 2004).

Admittedly, the idea that CD4+ T cells direct worm expulsion via MHCII-mediated interactions with epithelial or smooth muscle cells has not been directly tested. Investigating worm clearance in mice that lack MHCII specifically in epithelial or smooth muscle cells would help address this question. It is also possible that non-nematode-derived antigens could be presented by these cells, such as commensal microbial antigens. However, it would stand to reason that epithelial and/or muscle cells nearest to

worms would have the greatest access to antigens, since these cells stay fixed and do not migrate over long distances. CD4+ T cell recognition of nematode antigens could therefore help concentrate these cells in regions where worms are present and where effector responses are most needed. Expansion of nematode-specific CD4+ T cells as opposed to unrelated specificities would also bode well for effective memory responses, allowing for rapid memory reactivation in response to nematode antigens and not in response to environmental or commensal antigens that may be present without nematode infection.

Importantly, this hypothesis still does not address why CD4+ T cell-derived IL-4/13 are dispensable – at least in *N. brasiliensis* infection (Oeser et al., 2015; Voehringer et al., 2006). One would imagine that these cytokines would still be crucial to help IECs and smooth muscle cells given that their effector functions are largely IL-4R α -dependent. Indeed, other groups have shown that treatment of *H. polygyrus* or *N. brasiliensis*-infected, T cell-deficient mice with IL-4 complex is sufficient to cure infection, suggesting that T cells are needed to produce this cytokine at a high enough level (and, potentially, in close enough proximity to IECs or smooth muscle cells) to promote expulsion (Urban et al., 1995). Nevertheless, this model could partially explain why CD4+ T cell antigen recognition is still necessary despite their overlap in function with ILC2s. Moreover, uncoupled from the prospect of antibody responses and antibody-dependent memory, tissue-resident memory CD4+ T cell recognition of GI nematode antigens on epithelial cells directly at sites of infection could help facilitate the faster recall responses upon re-exposure to helminths observed in laboratory models of infection.

Summary

GI nematodes are metazoan organisms that are orders of magnitude larger than the host cells tasked with coordinating their elimination. Their complex anatomy and life cycles makes genetic manipulation difficult, although emerging technologies have made transgenesis more tractable. Such advances stand to improve our understanding of basic parasitic nematode biology, but also of host-parasite interactions, which to date have most benefited from genetic modification of host model organisms. Though we have developed a deep knowledge of the individual players in GI nematode clearance over the last several decades, large gaps remain in our understanding of how these parasites are actually cleared and how antigen-specific responses to them form, contribute to clearance, and perpetuate memory – when protective memory develops at all. In the present study, I have sought to combine transgenic methods in the nematode *S. ratti* with immunological analyses to interrogate, for the first time, how antigen-specific CD4+ T cells function during infection. These studies reveal novel insights into the nature of antigen-specific CD4+ T cells, and serve as an important proof-of-concept for future investigations using transgenic GI nematodes to study both parasite and host.

Figures

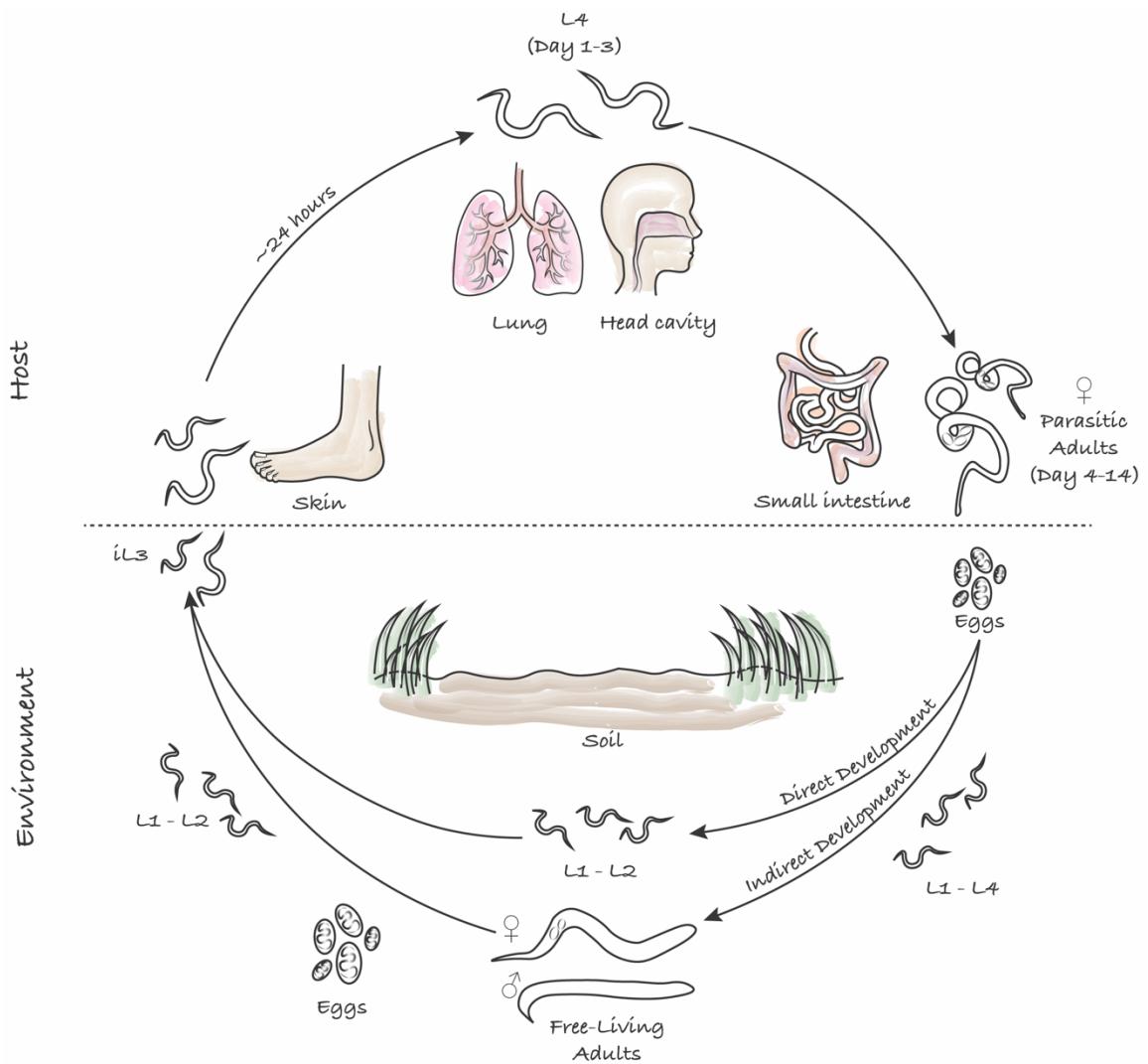


Figure 1. 1. *S. ratti* life cycle.

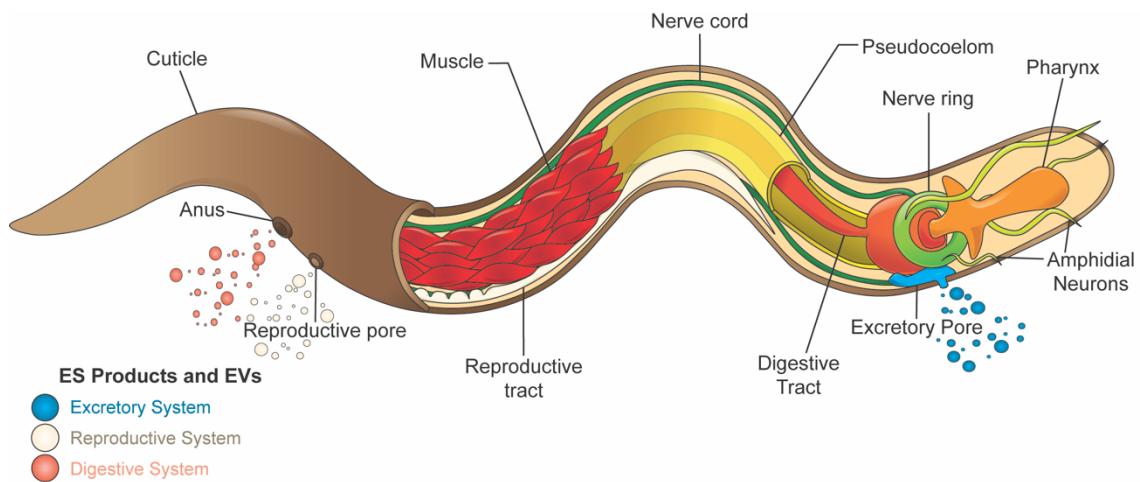


Figure 1. 2. Gastrointestinal nematode body plan.

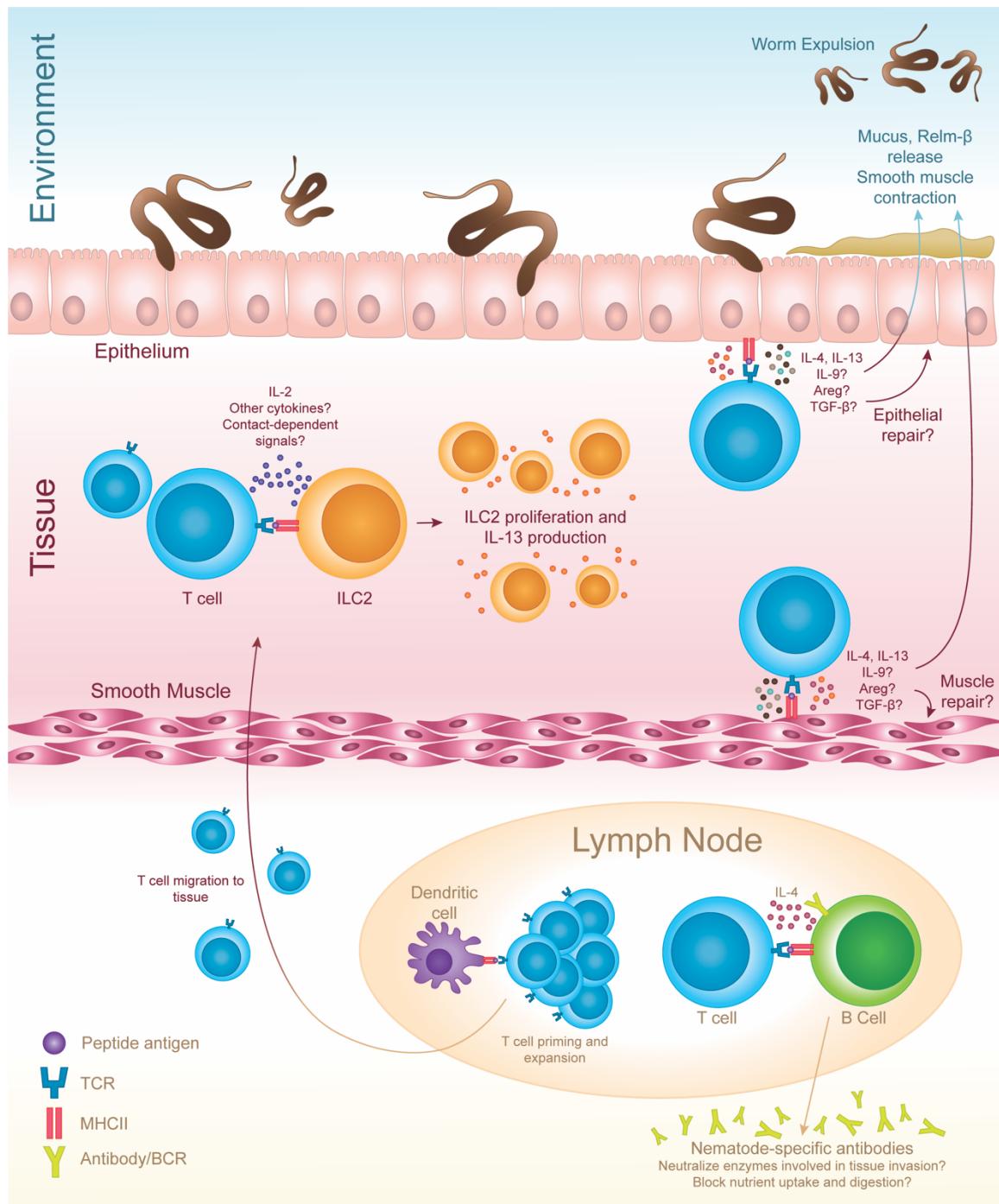


Figure 1. 3. Potential protective roles of antigen-specific CD4+ T cells in GI nematode infection.

CHAPTER 2: GENERATION AND VALIDATION OF TRANSGENIC MODEL

ANTIGEN-EXPRESSING *S. RATTI* (*HULK*)

Parts of this chapter were previously published in *PLoS Pathogens*:

Douglas, B., Wei, Y., Li, X., Ferguson, A., Hung, L., Pastore, C., Kurtz, J.R, McLachlan, J.B., Nolan, T.J., Lok, J., & Herbert, D.R. (2021). Transgenic expression of a T cell epitope in *Strongyloides ratti* reveals that helminth-specific CD4+ T cells constitute both Th2 and Treg populations. *PLoS Pathog*, 17(7), e10097909.

Background

Unlike microbial pathogens, which are relatively small in terms of both size and genomic complexity, parasitic nematodes are metazoan organisms that are orders of magnitude larger than both microbes and host cells and express a more diverse antigenic repertoire across multiple life stages within a single host (Hewitson et al., 2013; Soblik et al., 2011; M. Viney & Kikuchi, 2017). Moreover, GI nematode infection induces considerable host tissue damage at sites of infection, including the respiratory and GI tracts. CD4+ T cells are required for clearance, and also play roles in immunosuppression and tissue repair, but it remains unknown how CD4+ T cells that specifically recognize helminth-derived antigens behave during infection with these parasites (Bouchery, Kyle, Ronchese, & Le Gros, 2014; Urban et al., 1995; Katona, Urban, & Finkelman, 1988; Urban, Katona, & Finkelman, 1991; Abdel Aziz, Nono, Mpote, & Brombacher, 2018; Arpaia et al., 2015; Blankenhaus et al., 2014; Burzyn et

al., 2013; D'Elia, Behnke, Bradley, & Else, 2009; Finney et al., 2007; Smith et al., 2016).

This gap in knowledge persists due to a lack of tools available to identify and track helminth-specific CD4+ T cells.

As discussed in Chapter 1.5, the field has taken 2 main approaches to address these questions in microbial infection models: a) to identify endogenous peptide epitopes that can be used to generate novel MHCII tetramers or b) to genetically engineer pathogens to express model antigens for which MHCII tetramers already exist. These strategies have led to significant advances in our understanding of antigen-specific CD4+ T cell function, localization, and memory formation in various bacterial, viral, and protozoan parasite infections (Benoun et al., 2018; Ertelt et al., 2009; Grover et al., 2012; Homann, Teyton, & Oldstone, 2001; Mooney et al., 2015; Pepper et al., 2004). While it is possible, in theory, to develop MHCII tetramers against endogenously expressed helminth antigens, much work remains to be done to identify immunodominant epitopes from the wide array of those expressed by these metazoan organisms. Accordingly, to my knowledge, no such reagents exist to label CD4+ T cells specific for helminth-derived antigens.

Likewise, while transgenically expressing model epitopes in parasitic helminths would allow the field to take advantage of existing tetramer reagents, helminths – and particularly GI nematodes – are difficult to genetically modify for reasons discussed in Chapter 1.3. However, because of the accessibility of their free-living, dioecious adult stage and their ability to reproduce asexually in hosts to produce high frequencies of genetically transformed progeny, *Strongyloides spp.* are particularly amenable to transgenesis (J. B. Lok et al., 2017; Shao et al., 2012). Microinjecting free-living *Strongyloides* females with both a plasmid encoding a model antigen and a plasmid encoding the *piggyBac* retrotransposase allows for high-copy, random genome

integration into the germline, yielding transformed F1 progeny. Serial passage of selected transgenic progeny through appropriate hosts can then lead to the generation of stable, germline-transmitting transgenic lines.

In this study, I worked with collaborators to take advantage of this innovative system of transgenesis and generate a novel strain of transgenic *S. ratti* that expresses a known CD4+ T cell epitope. We utilized the 2W1S peptide, a variant of the E α ₅₂₋₆₈ peptide with two tryptophans and one serine substitution at amino acid residues 3, 9, and 13, respectively, because there is a relatively high frequency of circulating naïve CD4+ T cells in C57BL/6 mice that recognize this epitope and a well-established MHCII tetramer allowing for detection and purification of 2W1S-specific CD4+ T cells (Moon et al., 2007; Rees et al., 1999). By expressing 2W1S peptide as a fusion protein with green fluorescent protein (GFP) and FLAG peptide in the body wall of *S. ratti*, we developed a stable line (*Hulk*) that elicits expansion of 2W1S-specific CD4+ T cells predominantly in the lungs of infected C57BL/6 mice.

Results

Hulk is a novel strain of *S. ratti* that expresses a GFP-2W1S-FLAG fusion protein

To create a system for *in vivo* tracking of helminth-specific CD4+ T cells, a plasmid vector was designed to encode 2W1S peptide (EAWGALANWAVDSA) as a fusion protein with GFP followed by the FLAG peptide epitope (schematic in Fig 2.1A). The *S. sterocoralis* actin-2 (Ss-actin-2) promoter was used to drive high-level expression in the body wall muscle, which underlies the cuticle. This construct was co-injected with a vector encoding the *piggyBac* retrotransposase into the gonads of free-living females to yield transformed, GFP-expressing progeny. From this F1 generation, 44 GFP+ third-stage larvae (iL3) were selected and used to infect a Wistar rat. Feces were collected beginning 6-7 days after infection and were used to generate coprocultures, from which

subsequent generations of GFP+ iL3 were again selected and passaged through a second, naïve Wistar rat. This process was repeated 6 times until the frequency of GFP+ iL3 was consistently 90-95% (Fig 2.1C). The resulting transgenic line was named “*Hulk*”.

Once *Hulk* was determined to stably express the transgene in the iL3 stage over multiple generations, I next sought to confirm expression of the transgene in multiple life stages of the parasite. The N-terminal GFP reporter could be detected by fluorescence microscopy in first- and third-stage larvae (L1 and iL3), as well as the free-living female stage and in eggs (Fig 2.1B). Consistent with prior data on expression of *Ss-actin-2* promoter-controlled genes (Shao et al., 2012), I observed GFP expression predominantly in the body wall muscle (Fig 2.1B). Interestingly, while up to 95% of iL3 were GFP expressing, observation of parasitic females under fluorescence microscopy revealed that only 85% were GFP+, suggesting either loss of the transgene in a portion of adult parasites or outcompetition of transgene-expressing parasites by their GFP- counterparts (Fig 2.1C).

To ensure that the length of the fusion protein was transcribed and translated in parasitic life stages, I next performed Western blots to detect FLAG and/or GFP in skin-penetrating iL3, lung-residing fourth-stage larvae (L4), and gut-dwelling parasitic adult female stages of *Hulk*. Consistent with microscopic analysis, *Hulk* iL3, lung-dwelling L4, and gut-dwelling parasitic adults all exhibited GFP expression at the protein level (Fig 2.2A-C). However, I observed a reduction in C-terminal FLAG expression from the iL3 stage to the parasitic adult stage relative to loading controls. Whereas a robust FLAG band was detected in protein lysates from iL3, this band was barely detectable in the L4 stage and could not be detected in the parasitic adult stage (Fig 2.2A-C). While I did not observe complete loss of GFP expression in parasitic adults by Western blotting, this finding is consistent with the reduction in frequency of GFP+ parasitic adults relative to

GFP+ iL3 (Fig 2.1C). Importantly, neither FLAG nor GFP were detected in the parental strain in any of the life stages examined (Fig 2.2A-C).

Finally, I sought to ascertain whether transgene insertion impaired the virulence of *Hulk* relative to the parental strain. I did not observe differences in worm burdens in the lung and gut on days 2 and 7 post-infection, respectively, between *Hulk* and parental-infected mice (Fig 2.3A-B). Moreover, there were no significant differences in egg deposition in the feces over time when comparing *Hulk* with the parental strain (Fig 2.3C). Lung hemorrhage, which is apparent 2 days post-infection with parental *S. ratti* and largely resolved by day 14, was comparable following infection with *Hulk* or the parental strain (Fig 2.S1). These data demonstrate that there is no appreciable difference in the infectivity of *Hulk* in comparison to parental *S. ratti*.

Hulk infection expands 2W1S-specific CD4+ T cells in the lung and lung-draining lymph nodes

After confirming that *Hulk* could establish infection in mice while maintaining some degree of transgene expression, I next asked whether *Hulk* infection could elicit adaptive immune responses against 2W1S peptide as well as the GFP reporter, for which a small number of naïve CD4+ T cells in C57BL/6 mice are specific (Nelson et al., 2015). Activated (CD44+) 2W1S-specific CD4+ T cells were found to expand in the lung parenchyma of *Hulk*-infected mice 14 days post-infection, whereas this did not occur in mice infected with the parental strain or in naïve controls (Fig 2.4B, gating strategy shown in Fig 2.4A). Though the N-terminal GFP reporter was more robustly detected by Western blot than the C-terminal FLAG region in the lung L4 stage, I was not able to detect expansion of GFP-specific CD4+ T cells in the lung using a GFP tetramer loaded with the peptide sequence HDFFKSAMPEGYVQE (Nelson et al., 2015) (Fig 2.S2A-B). However, anti-GFP IgG and IgM antibodies could be detected at higher titers in *Hulk*-

infected mice than in parental-infected or naïve control mice (Fig 2.4C).

To determine whether the clonal burst size of *Hulk*-elicited 2W1S-specific CD4+ T cells could be increased by increasing antigen load via multiple infections, I also quantified 2W1S+CD4+ T cell expansion following three successive inoculations with 1,000 iL3 (schematic in Fig 2.4D). Notably, egg production kinetics over 3 infections was similar to that observed following 1 infection, with a peak between days 6-7 post-initial infection and no subsequent peaks correspondent to subsequent infections (Fig 2.S3), and no adult worms found 14 days post-infection. However, multiple live infections resulted in a 30-fold increase in the frequency and a 6.5-fold increase in the number of activated (CD11a+) 2W1S+CD4+ T cells in the lung over mice given a single dose of 1,000 *Hulk* iL3 (Fig 2.4E). Expansion of CD11a+2W1S+CD4+ T cells also occurred to a lesser extent in the lung-draining mediastinal lymph nodes (mdLN) following one infection, and was similarly proportional to antigen load following 3 *Hulk* inoculations (Fig 2.4F).

In order to corroborate this finding, I also quantified the frequency and number of activated and proliferating CD4+ T cells by CD11a and Ki67 expression following *in vivo* 2W1S peptide restimulation of *Hulk*-infected, parental-infected or naive mice (schematic shown in Fig 2.5A). Since a predominance of 2W1S-specific CD4+ T cells were found in the lung, I used irradiated larvae which arrest in the lung and delivered peptide to the lung by intratracheal injection. As expected, I observed an increase in the frequency of CD11a+Ki67+ CD4+ T cells following 2W1S peptide restimulation in *Hulk*-infected mice relative to control mice (Fig 2.5B-D). While the frequency of CD11a+Ki67+ CD4+ T cells was non-zero following intratracheal 2W1S injection in naïve mice, note that frequencies of activated, CD11a+CD44+CD4+ T cells comprise between 30-50% of all CD4+ T cells in completely naïve mouse lungs as well (Fig 3.1B, D). Since these mice are all housed

in specific-pathogen free facilities and are not completely germ-free, it is perhaps unsurprising that I see some level of T cell activation in mice exposed to commensal microbes, food antigens, dust from bedding, etc.

Following a molt in the lung, *S. ratti* migrates to the small intestine where it burrows into the epithelium to form sexually mature adults. Accordingly, I sought to determine whether 2W1S-specific CD4+ T cells expanded in the gut in addition to lung tissue. Interestingly, I found that irrespective of whether mice received 1 or 3 infections, a negligible number of 2W1S-specific CD4+ T cells could be detected in gut-draining mLN or the Peyer's patches compared to parental-infected or naïve mice (Fig 2.6A-B). These data revealed that among the tissues evaluated, the lung parenchyma holds the predominance of the 2W1S-specific CD4+T cell response. This response pattern is also consistent with my observation that the C-terminal region of the GFP-2W1S-FLAG fusion protein is expressed in the lung-dwelling L4 but not the gut-dwelling adult stage of *Hulk*.

Protein analysis by Western blotting would suggest that the full length of the transgene is expressed most robustly in the iL3 stage of *Hulk*, which first penetrates the skin. Using a percutaneous infection method, by which larvae are applied to the footpad of mice and allowed to penetrate the skin, I assessed 2W1S-specific CD4+ T cell expansion in the paw-draining popliteal lymph node (pLN) after 2 live infections (infection scheme shown in Fig 2.S4A). Preliminary data reveal very few CD4+ T cells with dim 2W1S:I-A^b tetramer staining in the pLN following 2 *Hulk* infections (Fig 2.S4B-C). Nevertheless, the number of 2W1S-specific CD4+ T cells in the pLN is greater than the number observed in *Hulk* mLNs, where I have not previously observed expansion of this population following subcutaneous *Hulk* infection (Fig 2.S4C). The number of 2W1S-specific CD4+ T cells is also comparable in *Hulk*-infected and 2W1S-immunized pLNs (Fig 2.S4B-C).

Discussion

To my knowledge, *Hulk* is the first transgenic GI nematode that stably expresses a tractable model antigen. Infecting mice with *Hulk* allowed us to identify and characterize helminth-specific CD4+ T cells, predominantly in lung tissue and lung-draining lymph nodes. This expansion profile was consistent with transgene expression data showing waning C-terminal FLAG peptide expression at the protein level over subsequent parasitic life stages. Having the ability to identify and track helminth-specific CD4+ T cells *in vivo* during infection opens a number of avenues for future investigation, and I think this tool will help advance the field's understanding of both helminth immunity and CD4+ T cell biology.

While 2W1S-specific CD4+ T cells could be observed to expand in the lung tissue, few if any of these cells could be observed in gut-draining mesenteric lymph nodes and Peyer's patches. This distribution pattern of 2W1S-specific CD4+ T cells was consistent with the expression pattern of the full-length transgene: while the C-terminal FLAG region could be detected at low levels in the lung L4 stage, it was undetectable in adult parasites in the intestine. I interpret this to mean that expression of the 2W1S antigen is likely lost in the gut-dwelling adult stage, although interestingly GFP can still be detected in the parasitic adult stage by fluorescence microscopy and Western blotting.

Though it is not clear why the C terminal region of the transgene is lost as *Hulk* develops within its host, there are two, non-mutually exclusive explanations: (a) the transgene, though constitutively transcribed, becomes truncated or silenced at the transcript or protein level in the L4 and parasitic adult stages, and/or (b) the *Ss-act-2* promoter does not drive constitutive expression throughout the *S. ratti* life cycle. The first explanation is consistent with recent data showing that lentiviral-transduced *N.*

brasiliensis worms can retain an mCherry transgene in their genomic DNA throughout ontogeny, but that transcription of this transgene is lost in later, parasitic developmental stages of the worm (Hagen et al., 2021). Given the immunogenic nature of the 2W1S peptide, there may be selective pressure in hosts for adult parasites to silence or edit the transgene at the transcript or protein level. Conversely, expression of this transgene in the free-living adult stage of *Hulk* does not appear to alter survival or reproduction, and so transcription and translation of the full-length fusion protein is permissible.

In support of the second possibility, the free-living nematode *Caenorhabditis elegans* has 5 actin genes, and multiple actin genes have been annotated in the *S. ratti* genome (MacQueen et al., 2005; Nemetschke, Eberhardt, Viney, & Streit, 2010). Whether expression of different actins fluctuates throughout *S. ratti* ontogeny is not known, but it may be that each actin gene is differentially regulated at different stages of nematode development. Indeed, my data in Fig 2.2 show a reduction in levels of protein detected by an anti-mouse β-actin antibody in the parasitic adult stage relative to the iL3 and L4 stages, which may indicate that whichever *S. ratti* actin is recognized by this antibody is downregulated and perhaps compensated for by another isoform in this life stage. Moreover, while the GFP portion of the fusion protein likely persists in the adult parasitic stage due to its long half-life (~26 hours), the *Ss-act-2* promoter may not drive high-level *de novo* expression in the parasitic adult stage of *S. ratti*.

Despite this drawback to the *Hulk* model, studies of helminth-specific CD4+ T cells in the lung will still be incredibly helpful for both parasitological and immunological investigations. The lung has previously been demonstrated as an important location for memory responses against *N. brasiliensis*, a GI nematode that mimics the infection route of *S. ratti* (Harvie et al., 2010; Thawer et al., 2014). There is attrition of larvae migrating between the lung and the intestine in primary responses, and few, if any parasites

escape the lung parenchyma during secondary infection (Dawkins & Grove, 1981). It is likely that antigen-presenting cells in the lung have greater access to nematode body wall components, and in this case 2W1S, from either dead or developmentally arrested larvae during primary infection, allowing for activation and infiltration of 2W1S CD4+ T cells to this site. Further, the added attrition of larvae in the lungs upon secondary *N. brasiliensis* challenge is mediated by lung-resident CD4+ T cells (Thawer et al., 2014). Whether these cells are specific for helminth-derived antigens, or whether CD4+ T cells that recognize antigens from different anatomical compartments of the helminth (e.g. body wall/muscle, ES products) are more or less effective at stopping larval migration out of the lung, is not known. However, these questions could potentially be addressed either with *Hulk* or by generating new transgenic lines using the same transgenic method.

My lab became interested in the antigen-specific CD4+ T cell response in the skin because of data showing that *S. ratti* iL3 penetration is reduced in secondary infections compared to primary infections. Ultimately, we would like to understand whether CD4+ T cells – and particularly those that recognize helminth antigens – play a role in this process. Preliminary data show a very low number of dim 2W1S-specific CD4+ T cells in the pLNs of mice infected percutaneously on the footpad with *Hulk*. Further experiments will be necessary to determine whether this staining is reproducible, and how this frequency and number of 2W1S-specific CD4+ T cells in *Hulk*-infected mice compares to naïve mice. However, it may be that at the time point examined, a predominance of 2W1S-specific CD4+ T cells are present in the skin tissue rather than in the skin-draining lymph nodes. Indeed, when mice are analyzed 14 days after 1 or 3 *Hulk* infections, most 2W1S-specific CD4+ T cells are seen in the lung parenchyma and not in the lung-draining mediastinal lymph nodes. Since iL3 are not

known to migrate through lymph nodes, CD4+ T cells likely migrate to inflamed tissues like the skin shortly after priming. Moreover, since many iL3 are prevented from migrating into the skin during a secondary infection, the amount of body wall antigen present in the skin upon secondary infection may be quite low. Accordingly, it would be helpful to assess the kinetics of 2W1S-specific CD4+ T cell expansion at the tissue site of infection and in the lymph node. Nevertheless, if 2W1S can be recognized by CD4+ T cells at this infection site, future experiments could assess their role in preventing larval penetration by first immunizing mice with 2W1S and then performing percutaneous injection to assess whether prior expansion of CD4+ T cells specific for a nematode body wall-antigen increases the frequency of non-penetrating iL3. Since intraperitoneal immunization did not robustly expand 2W1S-specific CD4+ T cells in the pLN as it does in other lymph nodes and the lung, I would also consider footpad immunizations in this model for future studies.

This novel transgenic line is both an important tool in and of itself and also a proof of concept for future model antigen-expressing GI nematode strains. As will be discussed in the next chapter, *Hulk* has allowed us to begin to characterize the heterogeneity of CD4+ T cells specific for an antigen expressed in the body wall of a GI nematode. However, much more can be learned by expressing model antigens in different anatomical compartments – for example, on the cuticle or as ES products – or by identifying endogenous helminth epitopes that can be used to generate novel tetramers. Such studies will not only inform our understanding of helminths and their co-evolution with hosts, but will also further our understanding of CD4+ T cells and antigen specificity in the context of helminth infections.

Figures

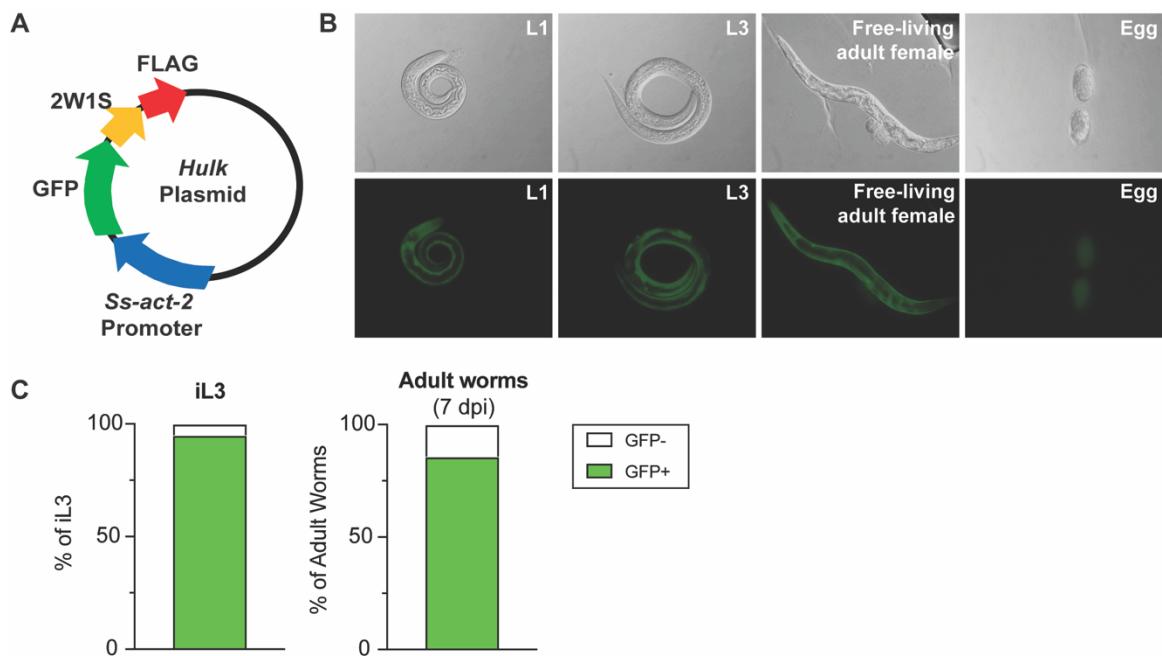


Figure 2. 1. *Hulk* is a transgenic line of *S. ratti* that expresses GFP as a fusion protein with 2W1S and FLAG. (A) Schematic depicting *Hulk* plasmid encoding green fluorescent protein (GFP), 2W1S peptide, and FLAG peptide under control of the *S. stercoralis* actin (Ss-act-2) promoter. (B) Brightfield DIC (top) and green fluorescent (bottom) images of the first and third larval stages, adult free-living female stage, and egg stage of *Hulk* (L1, L3, eggs: 40x magnification; Free-living adult: 20x magnification). (C) Frequency of GFP+ and GFP- parasites at the iL3 and parasitic adult female stages (7 days post-infection).

iL3

L4

Parasitic Adult

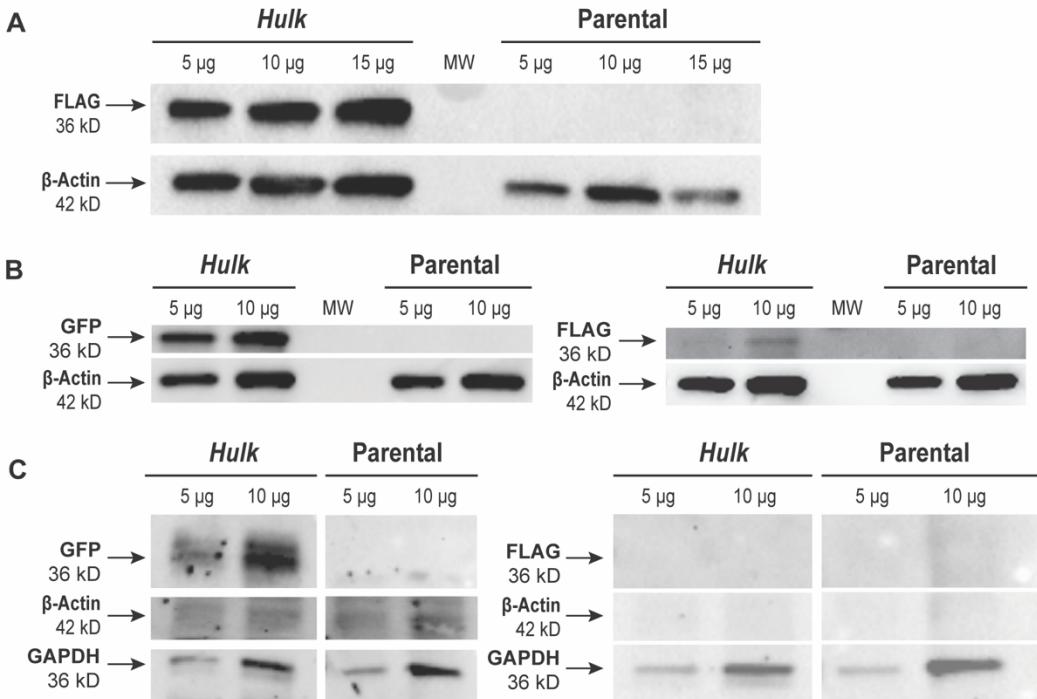


Figure 2. 2. *Hulk* expresses GFP protein in all parasitic life stages, but FLAG expression is diminished in lung L4 and lost in intestinal parasitic adults. (A-C)

Western blot analysis of FLAG and/or GFP protein expression in *Hulk* and parental *S. ratti* at the indicated protein amounts in iL3 (A), L4 (B) and parasitic adult females (C). Results are representative of at least 2 independent experiments for each life stage examined. β-actin and/or GAPDH were detected as loading controls.

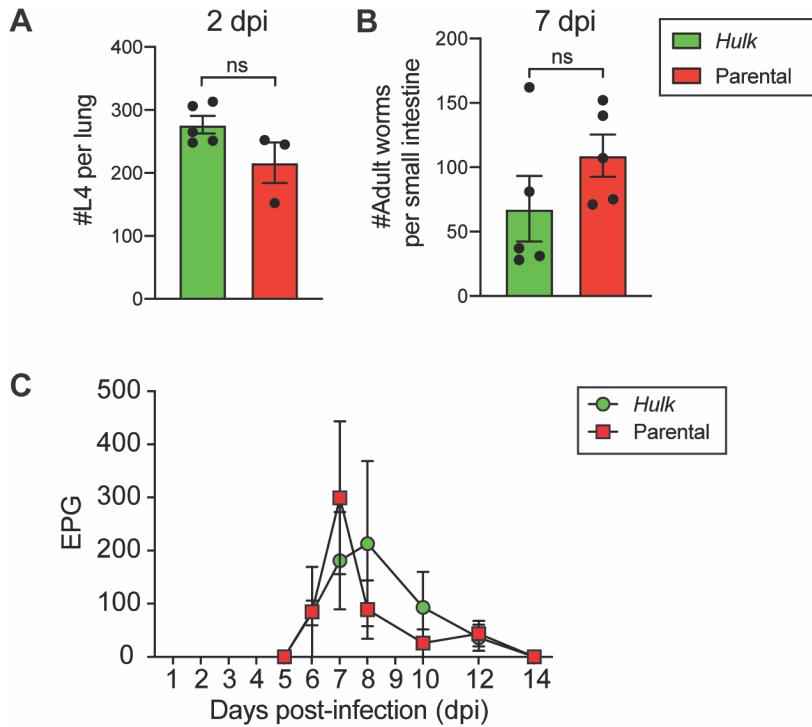


Figure 2. 3. Parental and *Hulk* *S. ratti* exhibit comparable infection kinetics in the lungs and intestines of infected mice. (A-B) Enumeration of L4 (A) or parasitic adult females (B) at the indicated days post-infection in the lungs (A) or small intestine (B). C57BL/6 mice were infected with 3,000 iL3 in (A) and with 1,000 iL3 in (B). Data representative of 2 independent experiments, n = 3-5 per experiment. (C) Fecal egg enumeration per gram of feces (EPG) for mice infected with 1,000 infective L3 (iL3) *Hulk* or the parental *S. ratti* strain. Data is representative of 3 independent experiments, n = 4-5 per experiment. Significance tested using Mann-Whitney test.

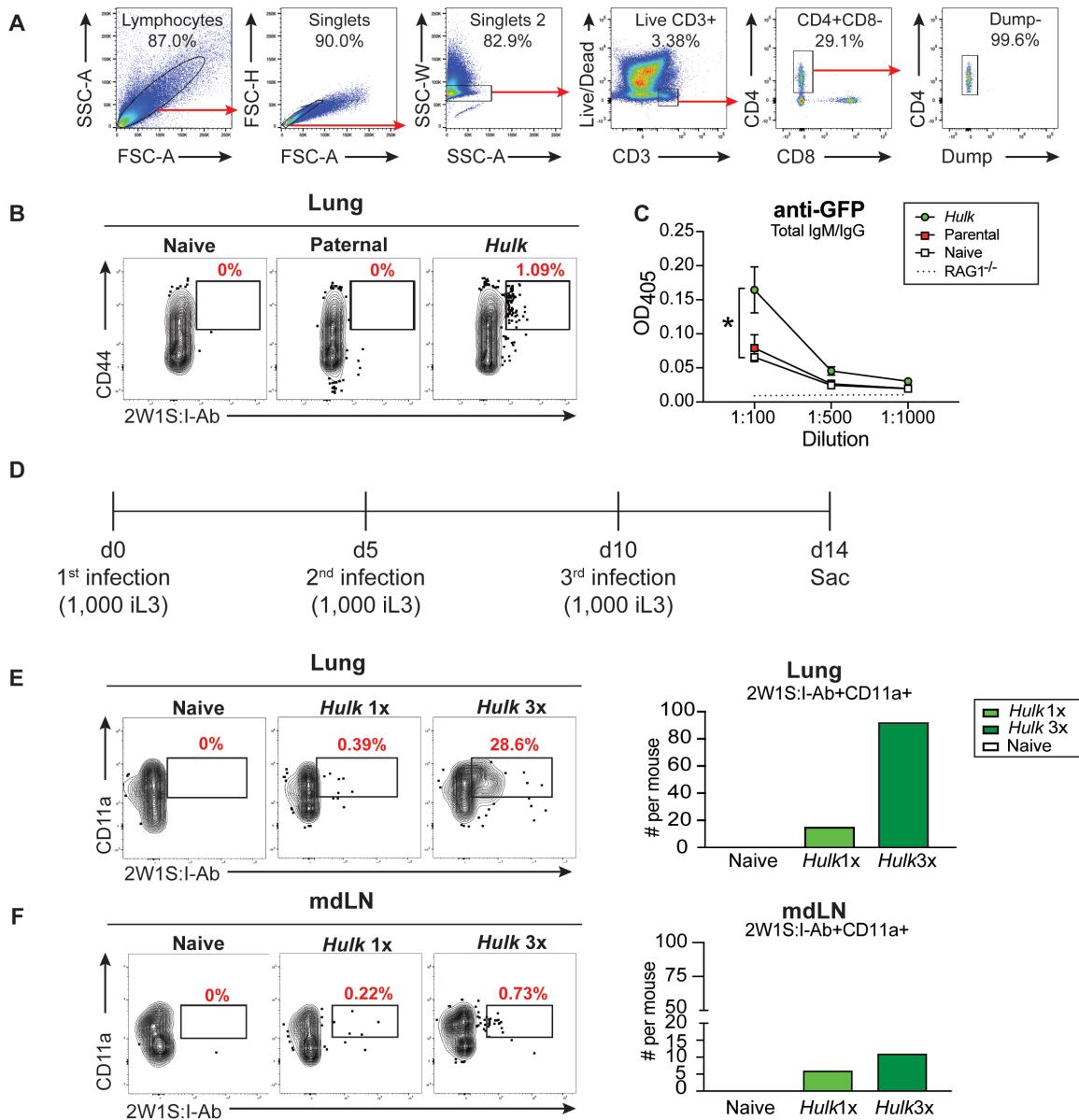


Figure 2. 4. Infection with *Hulk* elicits expansion of activated 2W1S-specific CD4+ T cells in the lung and lung-draining lymph nodes in a dose-dependent manner. All mice were analyzed 14 days post-initial infection, and 2W1S-specific cells were enriched using a fluorophore-specific MACS bead sorting pulldown technique prior to flow cytometry. (A) Representative gating strategy, shown in lung. Dump gate includes B220/CD45R, CD11b, and CD11c. (B) 2W1S:I-A^b+CD44+ CD4+ T cell frequency in the lungs of naive mice and mice infected once with *Hulk* or parental *S. ratti* (n=3-4 mice

pooled per group). Data are representative of 2 independent experiments. (C) Anti-GFP IgM+IgG serum titers from naïve, parental *S. ratti*-infected and *Hulk*-infected mice. Significance determined using an unpaired T-test; *p < 0.05. (D) Infection scheme used in Figs 2.4E-F. (E) 2W1S:I-A^b+CD11a+ CD4+ T cell frequency and number in the lungs of naive mice and mice infected once or three times (days 0, 5, 10) with *Hulk* (n = 1-3 mice pooled per group). (F) 2W1S:I-A^b+CD11a+ CD4+ T cell frequency and number in the lung-draining mediastinal lymph nodes (mdLN) of naive mice or mice infected once or three times with *Hulk* (n=1-3 mice pooled per group).

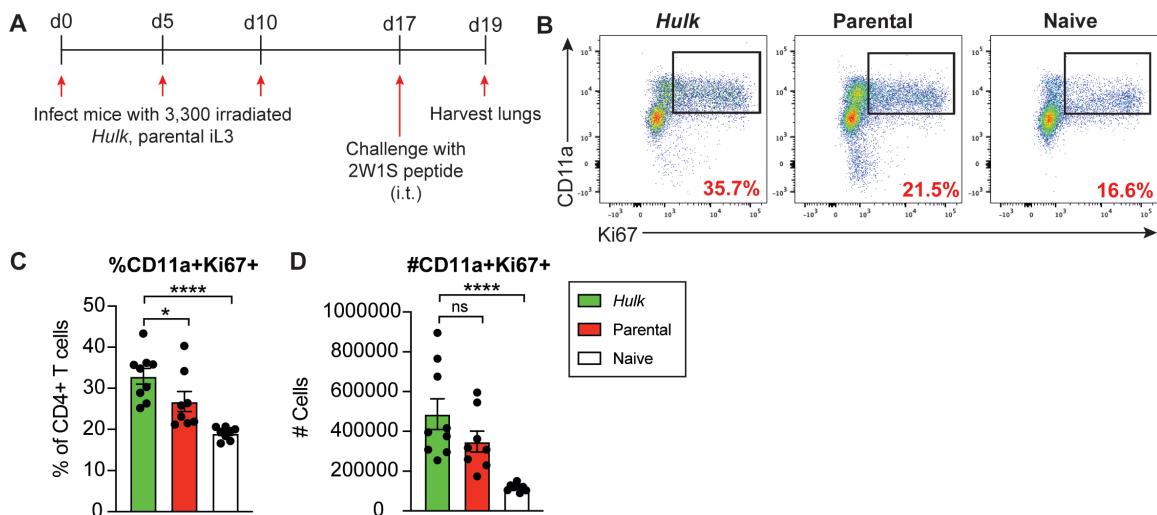


Figure 2. 5. *In vivo* restimulation with 2W1S peptide yields greater frequencies and numbers of activated, proliferating CD4+ T cells in the lungs of *Hulk*-infected mice than in parental-infected or naïve controls. (A) Schematic of experimental design used in (B-D). (B) Representative flow plots showing CD11a+Ki67+ CD4+ T cells in lungs of mice infected and peptide challenged as in (A). (C-D) Frequency and number of CD11a+Ki67+ CD4+ T cells in the lungs of mice infected and peptide challenged as in (A). Significance tested using Mann-Whitney test; *p < 0.05, ****p < 0.0001. Data combined from 2 independent experiments, n = 8-9.

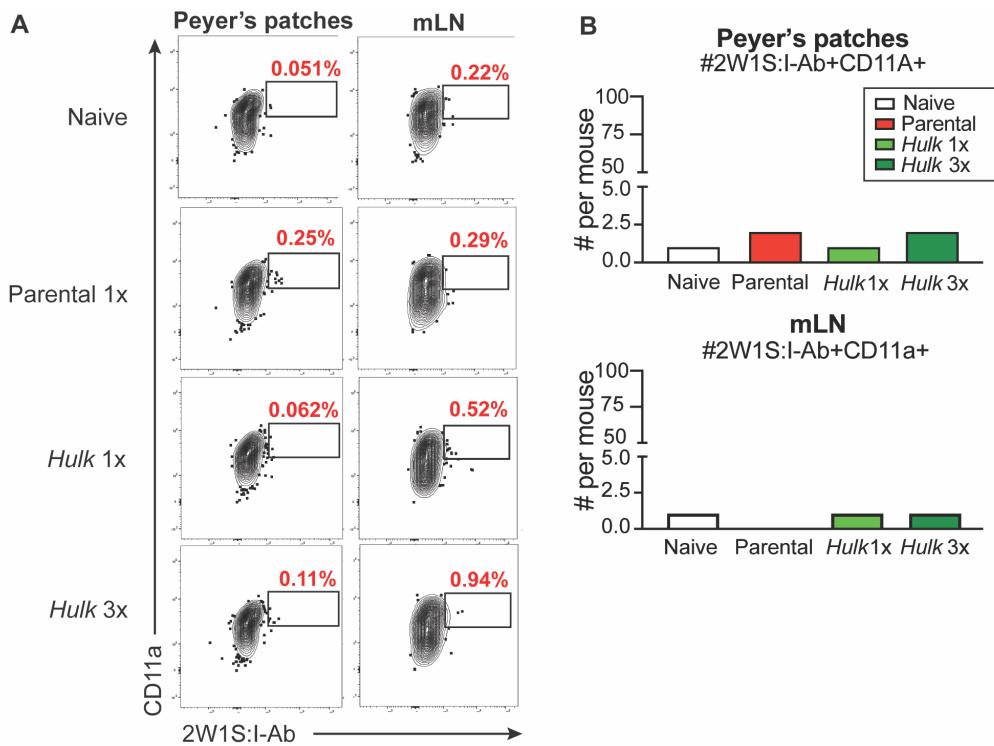


Figure 2.6. *Hulk* infection does not elicit substantial expansion of activated 2W1S-specific CD4+ T cells in gut-associated lymphoid tissue or draining lymph nodes.

(A) 2W1S:I-A^b+CD11a+ CD4+ T cell frequency in Peyer's patches and mesenteric lymph nodes (mLN) in naïve mice or mice infected once with *Hulk* or parental *S. ratti* or three times with *Hulk* (n=3-4, pooled). (B) Number of 2W1S:I-A^b+CD11a+CD4+ in Peyer's patches and mLN in naïve, 1x *S. ratti*-infected or *Hulk*-infected, and 3x *Hulk*-infected mice. Data are representative of 2 independent experiments in the Peyer's patches and 3 independent experiments in the mLN.

Supplemental Figures

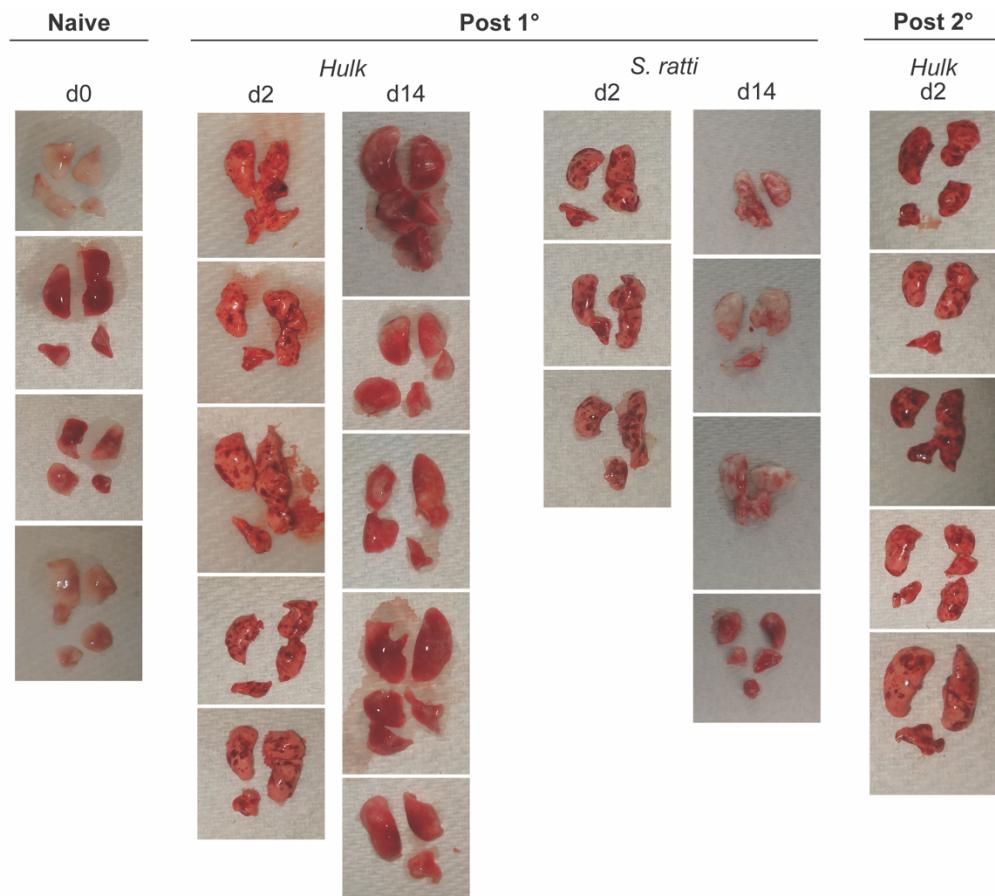


Figure2.S 1. Infection with parental or *Hulk* *S. ratti* causes gross lung pathology 2 days post-infection that is largely resolved 14 days post-infection. Images of lungs excised from naïve or infected mice at the indicated days post-infection. Infected mice were infected with 3,000 live iL3 of either *Hulk* or parental *S. ratti*. Images are representative of 2 independent experiments on day 2 post-infection and greater than 3 independent experiments at 0 and 14 days post-infection.

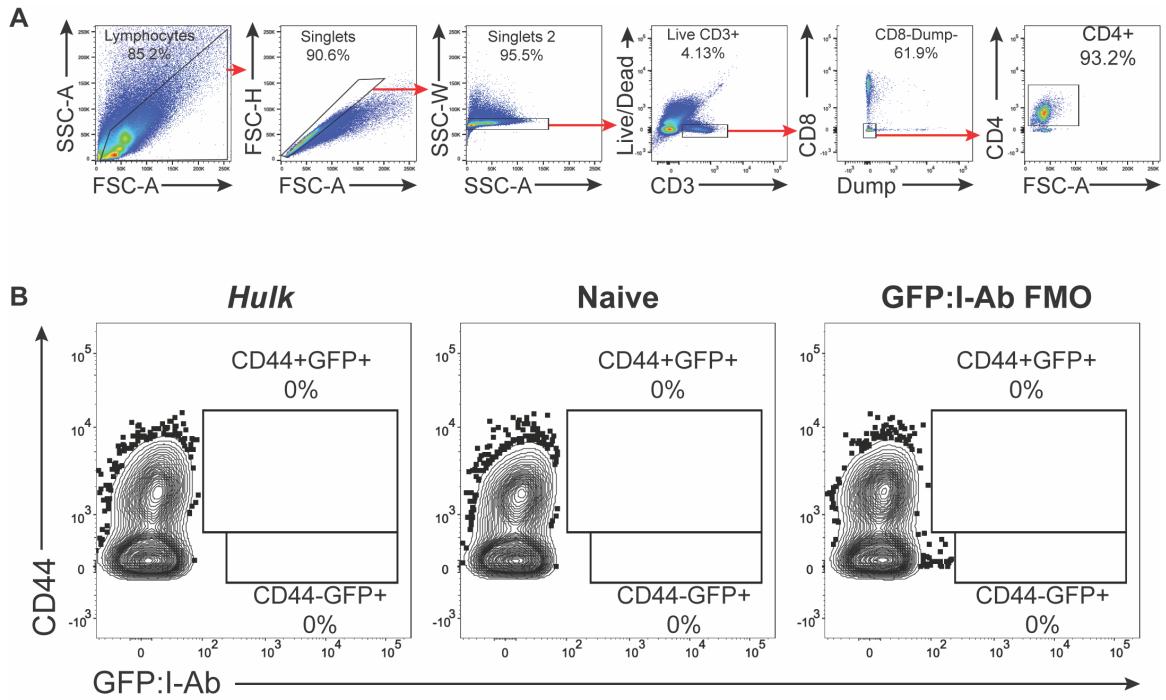


Figure 2.S 2. Hulk infection does not elicit notable expansion of GFP-specific CD4+ T cells in the lungs of infected mice 7 days post-infection. Mice were infected with 1,000 live iL3 Hulk or injected with 1 mg/mL alum adjuvant alone (Naïve) and analyzed 7 days after infection or treatment. Lungs were pooled ($n = 3-4$ mice per group) and GFP:I-Ab^b tetramer-bound cells were enriched using a fluorophore-specific MACS bead sorting strategy prior to flow cytometric analysis. (A) Gating strategy used to analyze activated GFP+CD4+ T cells from lungs. (B) Frequencies of CD44+ or CD44- GFP+ CD4+ T cells.

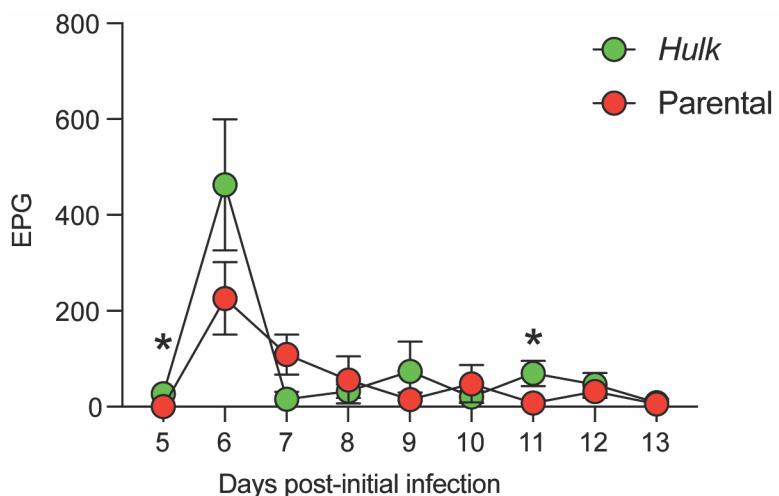


Figure2.S 3. Infecting mice 3 times with *Hulk* or parental *S. ratti* results in only a single peak in fecal egg deposition. Egg production in feces (denoted as eggs per gram (EPG)) over time following 3 infections with *Hulk* or parental *S. ratti*. Data are representative of 2 independent experiments, n = 4-5 per experiment. Significance determined using a Mann-Whitney test; *p < 0.05.

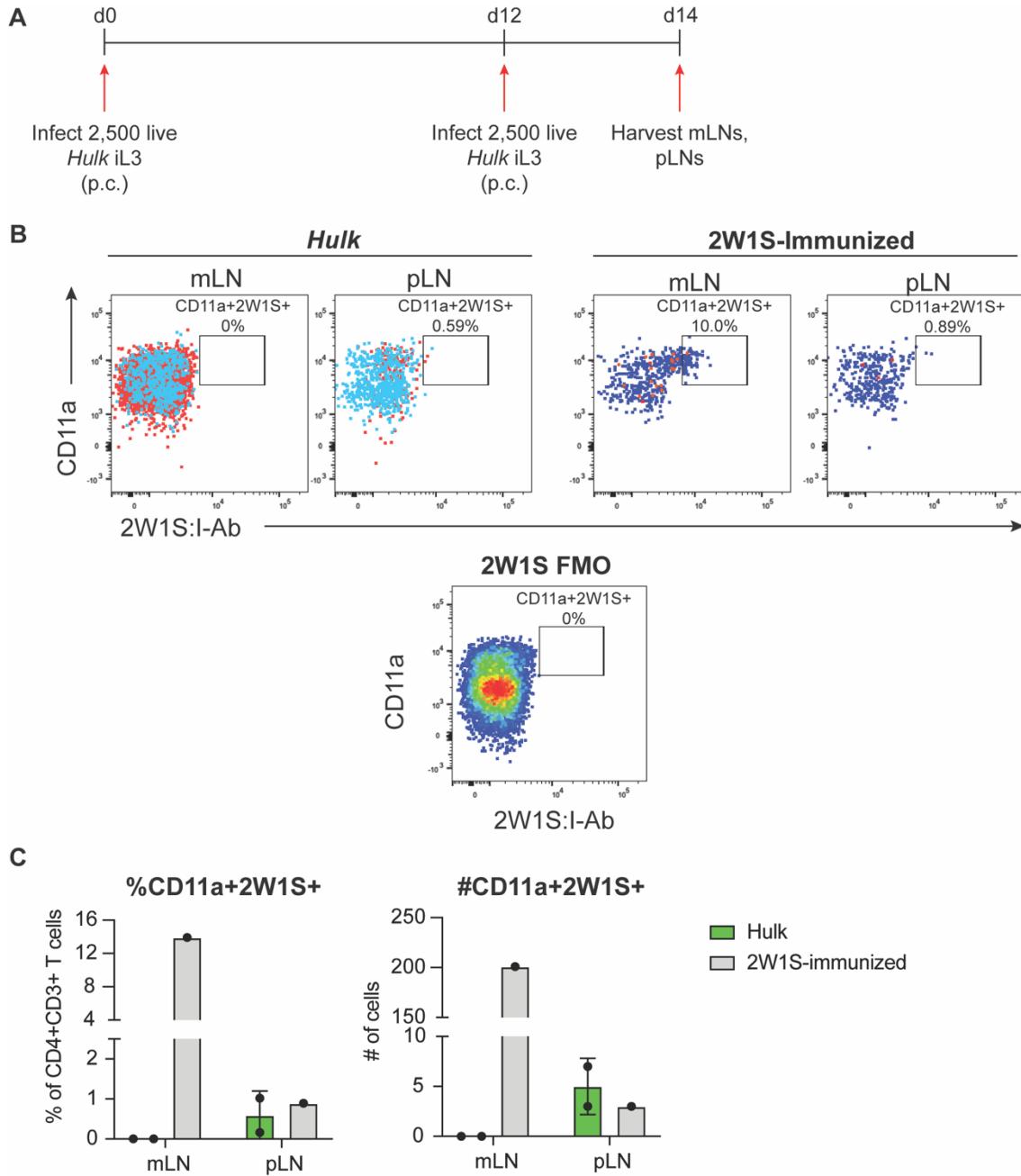


Figure 2.S 4. Percutaneous *Hulk* infection in the footpad results in a small number of activated 2W1S-specific CD4+ T cells in the skin-draining popliteal lymph node.

(A) Infection schematic for (B-C). Mice were anaesthetized with ketamine and xylazine, and were infected percutaneously (p.c.) with 2,500 live iL3. 2W1S-immunized mice were injected intraperitoneally with 100 µg 2W1S and 1 mg alum 7 days prior to harvest. (B)

Flow cytometry plots showing frequency of CD11a+2W1S+ CD4+ T cells. The gating strategy for the experiment was the same as shown in Fig 2.S2. Cells from mLN or pLN were pooled from 2 mice (*Hulk*, 2W1S mLN) or 4 mice (2W1S pLN) and enriched using a fluorophore-based MACS enrichment strategy. (C) Frequency and number of CD11a+2W1S+ CD4+ T cells in the mLN and pLN of *Hulk*-infected or immunized mice. Cell numbers are normalized to the number of mice pooled per sample.

CHAPTER 3: ANTIGEN-SPECIFIC CD4+ T CELL PHENOTYPE AND FUNCTION DURING *HULK* INFECTION

Parts of this chapter were previously published in *PLoS Pathogens*:

Douglas, B., Wei, Y., Li, X., Ferguson, A., Hung, L., Pastore, C., Kurtz, J.R, McLachlan, J.B., Nolan, T.J., Lok, J., & Herbert, D.R. (2021). Transgenic expression of a T cell epitope in *Strongyloides ratti* reveals that helminth-specific CD4+ T cells constitute both Th2 and Treg populations. *PLoS Pathog*, 17(7), e10097909.

Background

Decades of work using genetic loss-of-function models and antibody-mediated ablation have implicated CD4+ T cells as critical players in clearance of GI nematodes (Katona et al., 1988; Reitz et al., 2017; Urban, Katona, & Finkelman, 1991). Upon GI nematode invasion at mucosal sites like the lung or small intestine, tissue damage signals such as IL-25, IL-33 and TSLP activate dendritic cells to present antigen to and activate CD4+ T cells, typically toward a Th2 phenotype (Allen & Maizels, 2011). Activated Th2 cells then migrate to affected tissues, where they both secrete type 2 cytokines like IL-4, IL-5 and IL-13 and also support ILC2 production of these cytokines in a contact-dependent manner (T. Y. F. Halim et al., 2018; Harris & Loke, 2017; Oliphant et al., 2014). Signaling through IL-4R α and IL-5R can recruit and activate eosinophils, basophils, and mast cells to produce pharmacologically active compounds that damage worms, and IL-4 and IL-13 expand alternatively-activated macrophages (Aamann, Vestergaard, & Gronbaek) which can trap and sometimes kill larvae (Harris & Loke, 2017) (Anthony et al., 2006; Esser-von Bieren et al., 2015; Herbert et al., 2004). In

tandem with IL-4R α -dependent goblet cell hyperplasia and smooth muscle hypercontractility, these mechanisms drive the “weep and sweep” response that expels worms from the intestinal tract (Allen & Maizels, 2011; Herbert et al., 2009). However, whether the Th2 cells that mediate these clearance mechanisms are specific for GI nematode antigens is not known.

In addition to coordinating pathogen clearance, CD4+ T cells also play important roles in preventing immunopathology and in tissue repair (Bouchery et al., 2014; Urban et al., 1995; Katona, Urban, & Finkelman, 1988; Urban, Katona, & Finkelman, 1991; Abdel Aziz et al., 2018; Arpaia et al., 2015; Blankenhaus et al., 2014; Burzyn et al., 2013; D'Elia et al., 2009; Finney et al., 2007; Smith et al., 2016). IL-4R α -expressing Tregs are essential for repairing tissue and restoring lung function in mice during infection with the GI nematode *N. brasiliensis* (Abdel Aziz, Nono, Mpotje, & Brombacher, 2018). Further, when Foxp3-expressing cells are depleted prior to infection with *H. polygyrus*, host animals lose more weight and have worsened pathology associated with high levels of IFN- γ production (Smith et al., 2016). However, when Tregs are expanded by administration of IL-2 complex, *H. polygyrus* expulsion is significantly delayed, and when Tregs are specifically deleted during *S. ratti* or *Litosomoides sigmodontis* infection, worm burden and fecundity are reduced (Blankenhaus et al., 2014; Smith et al., 2016; Taylor et al., 2009). Altogether, this implicates Tregs as being essential not only for host tissue preservation but also for fine-tuning cytokine secretion and regulating worm burden. Whether the Treg compartment contains helminth-specific CD4+ T cells is largely unexplored due to a lack of tools to identify and track these cells during infection.

The *Hulk* model provided us with the means to address the question of GI nematode-specific CD4+ T cell phenotype and function in the lungs of infected mice. My data suggests that CD4+ T cells specific for a body wall antigen (in this case 2W1S

peptide) are phenotypically heterogenous, with a majority of cells expressing Th2 lineage-defining transcription factor GATA3 but a portion expressing Treg lineage-defining transcription factor Foxp3 alone or in combination with GATA3. Interestingly, the predominant cytokine produced by these cells upon peptide restimulation is amphiregulin, an EGFR ligand produced by Th2 cells and Tregs and implicated in worm clearance and in tissue repair (Arpaia et al., 2015; Burzyn et al., 2013; Zaiss et al., 2006). While prior expansion of the 2W1S-specific CD4+ T cell pool through peptide immunization was insufficient to accelerate *Hulk* clearance, immunization did increase amphiregulin levels and amphiregulin-expressing CD3+ cells in the lungs relative to unimmunized mice. Altogether, I hypothesize that CD4+ T cells specific for body wall antigens may be more involved in mitigating tissue damage or promoting tissue repair in the lung than in worm clearance mechanisms.

Results

S. ratti activates and expands polyclonal Th2s and Tregs, eliciting associated cytokine production

While others have demonstrated increased type 2 cytokine production and Treg expansion in gut-draining mesenteric lymph nodes (mLN) during *S. ratti* infection, I first sought to confirm that polyclonal CD4+ T cells in infected tissue exhibit the expected Th2 and Treg phenotypes following infection with the parental *S. ratti* strain (Eschbach et al., 2010). *S. ratti* is a skin-penetrating soil-transmitted helminth, and once it enters the skin it travels to the lungs and naso-frontal region of the head for 1-2 days before migrating to the proximal small intestine (Figure 1.1)(M. Viney & Kikuchi, 2017). Upon analysis of the lung at 14 days post-infection, activation markers CD11a and CD44 were both upregulated on polyclonal CD4+ T cells (Fig 3.1B, D with gating strategy shown in Fig 3.1A). As expected, the frequency of GATA3+ CD4+ T cells also increased with infection

(Fig 3.1C, E). Though Foxp3+ Treg expansion has been observed in the mLN of *S. ratti*-infected mice (Blankenhaus et al., 2014), I did not observe increased frequencies of polyclonal Foxp3+ and Foxp3+GATA3+ CD4+ T cells in the lungs of mice 14 days post *S. ratti*-infection relative to naïve mice (Fig 3.1C, E).

Type 2 and immunomodulatory cytokine production is enhanced in mLN and spleen during *S. ratti* infection, but the extent to which CD4+ T cells contribute to this cytokine production is presently unknown (Eschbach et al., 2010). To assess cytokine production by CD4+ T cells from site-draining lymph nodes, I sorted CD4+ T cells from pooled draining lymph nodes of infected mice and stimulated cells with anti-CD3/CD28 for 3 days and assessed cytokine release. As expected, CD4+ T cells robustly upregulated type 2 cytokines IL-4, IL-5 and IL-13 by day 7 post-infection (Fig 3.2A). This response was significantly diminished by day 14, at which point the mice cleared a majority of worms from the small intestine. In addition, I observed enhanced secretion of IL-10, as well as the EGFR ligand amphiregulin, a molecule expressed by both Tregs and Th2 cells (Fig 3.2A). Similar to the kinetics of type 2 cytokine production, both IL-10 and amphiregulin peaked at 7 days post-infection and had returned completely to baseline by day 14. Interestingly, there was also an increase in IFN- γ production by CD4+ T cells 7 days post-infection (Fig 3.S1A), but this increase in type 1 cytokine production was not associated with an increase in canonical Th1 or Th17 transcription factor expression. Indeed, transcript levels of both *Tbx21* (*Tbet*) and *Rorc* (*ROR γ t*) were diminished in infected mice on day 7 relative to naïve mice (Fig 3.S1B).

Since CD4+ T cells also provide help to B cells in lymph node germinal centers, I also assessed levels of polyclonal type 2-associated antibody isotypes in the serum over the course of infection. As expected, I observed an increase in total serum IgE by day 7 post-infection that was maintained at day 14 (Fig 3.2B). Surprisingly, I did not observe

an increase in serum levels of IgG1, which is an isotype that is largely dependent on T cell help and IL-4 signaling (Madsen et al., 1999). This finding correlated with data showing an unexpected increase in IFN- γ secretion, which antagonizes IL-4 signaling and inhibits Th2-mediated inflammation (Fig 3.S1A) (Cohn et al., 1999; Huang, Xin, Coleman, & Huang, 2005). However, IFN- γ -regulated isotypes IgG2b and IgG2c levels were not upregulated (Fig 3.S1C), suggesting that IgE is the dominant isotype induced by *S. ratti* infection.

Finally, *S. ratti* clearance was significantly impaired in RAG1-deficient mice that lack both T and B cells, as evidenced by continued fecal egg deposition and presence of intestinal worms up to 25 days post-infection (Fig 3.3A-B). These results confirm that the adaptive immune response is required for timely elimination of *S. ratti* infection, as has been shown previously (Reitz et al., 2018). Altogether, these data indicate that *S. ratti* elicits both inflammatory and immunomodulatory CD4+ T cell cytokine production and B cell production of IgE, and that these responses are necessary for immunity.

2W1S-specific CD4+ T cells exhibit distinct transcriptional profiles during Hulk vs. 2W1S-Salmonella infection

Having determined that *S. ratti* infection elicited canonical type 2 CD4+ T cell responses, I next wanted to employ the *Hulk* model to interrogate the phenotype and function of antigen-specific CD4+ T cells responding to 2W1S expressed by *Hulk*. More specifically, I asked whether altering the infectious context in which the 2W1S epitope was recognized would skew the phenotype and/or effector phenotype of CD4+ T cells specific for this epitope. Transcriptional profiling was performed on activated 2W1S-specific CD4+ T cells isolated from mice infected 3 times with *Hulk* or infected with the type 1/type 17-skewing 2W1S-expressing vaccine strain of *Salmonella typhimurium*, LVS strain BRD509-2W1S (Δ aroA) (2W-Salmonella) (Fig 3.4A). *Hulk* and 2W-

Salmonella infections drove expansion of 2W1S-specific CD4+ T cells that were transcriptionally distinct by principle component analysis, with 22.3% of variation driven by infectious context (Fig 3.4B). Predictably, 2W-*Salmonella* infection induced T cell-intrinsic expression of genes involved in type 1 and type 17 immune responses, including *Tbx21* (*Tbet*), *Rorc* (*ROR γ t*), *Il17d/f* and *Il22* (Fig 3.4C). However, *Hulk* infection was associated with upregulation of both canonical type 2 genes, including *Gata3*, *Il4*, *Il5*, and *Il13*, as well as immunosuppressive genes like *Il10* (Fig 3.4C). Though I expected these canonical type 2 and immunomodulatory genes to be highly expressed in *Hulk*-expanded 2W1S-specific CD4+ T cells, to my surprise the most significantly upregulated gene in this population was amphiregulin, an EGFR ligand known to be expressed by both Th2 cells as well as Tregs (Fig 3.4C)(Arpaia et al., 2015; Burzyn et al., 2013; Zaiss et al., 2006).

To further understand how two different CD4+ T cell populations with shared antigen receptor specificity were altered by infectious context, gene set enrichment analysis (GSEA) was performed using an in-house curated Th2 gene set (Table 3.S1) and gene ontology (GO) terms. Notably, and consistent with the differentially expressed gene list, *Hulk*-expanded 2W1S-specific CD4+ T cells were enriched for signature Th2 genes, including transcription factors *Gata3*, *Bhlhe4* (*Dec2*), *JunB*, *Gfi1* and *Maf* (c-Maf), cytokines IL-4 and IL-13, and receptors for IL-33 (*Il1rl1*) and IL-4 (*Il4ra*) (Fig 3.4D). Both *Hulk*- and *Salmonella*-expanded 2W1S-specific CD4+ T cells also showed multiple significantly enriched GO pathways (FDR < 0.01) (Fig 3.S2-S3). Interestingly, two pathways enriched in *Hulk*-expanded cells were 1) Negative Regulation of I κ B and NF κ B signaling and 2) SMAD binding, which include genes often associated with Tregs, including *Il10*, *Tgfbr2* (TG β RII), and *Il1rl1* (IL-1Rap, a component of the IL-33 receptor) (Fig 3.4E). These pathways also include genes such as *Tnfaip3* (A20), which is

implicated in T cell activation and activation induced cell death, and *Tob1*, a negative regulator of T cell activation, suggesting that *Hulk*-expanded 2W1S-specific CD4+ T cells may be intrinsically negatively regulated to a greater extent than those expanded during 2W-*Salmonella* infection (M. Li, Zhu, M., Linghu, E., Feng, F., Zhu, B., Wu, C., Guo, M., 2016; May et al., 2014).

Altogether, these results indicated that both *Hulk* and 2W-*Salmonella* infections drove expansion of 2W1S-specific CD4+ T cells that adopted prototypic type 2 or type 1/17 effector functions, respectively, but that *Hulk* infection specifically expanded 2W1S-specific cells with putative suppressor function. These data reveal that, whereas 2W-*Salmonella*-elicited 2W1S-specific CD4+ T cells resembled type 1 and type 17 effector cells, *Hulk*-elicited cells resembled type 2 effectors and immunosuppressive Tregs.

Hulk-elicited 2W1S-specific CD4+ T cells produce amphiregulin in response to peptide restimulation and express both GATA3 and Foxp3

I next wanted to determine the phenotype and function of 2W1S-specific CD4+ T cells during *Hulk* infection. Based on RNA sequencing, *Hulk*-elicited 2W1S-specific CD4+ T cells upregulate type 2 cytokines IL-4, IL-5, IL-13, and amphiregulin. In order to validate that this was also true at the protein level, I performed *in vitro* 2W1S peptide restimulation of cells from lung tissue of naïve mice or mice infected 3 times with live *Hulk* or parental *S. ratti*. After 72 hours in culture, amphiregulin was significantly upregulated in cells from *Hulk*-infected mice upon peptide restimulation relative to controls (Fig 3.5A). Notably, the level of amphiregulin production from 2W1S stimulated cells was roughly one-third of the concentration measured in supernatants from cells polyclonally stimulated with anti-CD3, suggesting that 2W1S-specific CD4+ T cells contribute robustly to overall CD4+ T cell-derived amphiregulin levels in the lung.

To further confirm this finding, I also performed *in vivo* peptide restimulation of

Hulk-infected, parental-infected or naïve mice. In this experiment, I infected mice 3 times with irradiated larvae, which arrest in the lung, to further enhance the amount of antigen present in this tissue (schematic shown in Fig 2.5A). One week after the last infection, mice were administered 2W1S peptide intratracheally, and 2 days later I isolated whole lung cells and assayed their cytokine production over 48 hours *in vitro*. As observed with *in vitro* peptide restimulation, *in vivo* peptide restimulation of *Hulk*-infected lungs resulted in slightly enhanced amphiregulin production by lung cells relative to those from parental-infected mice and significantly enhanced production relative to naïve mice (Fig 3.5B). Amphiregulin levels in the bronchoalveolar lavage fluid (BALF) were also significantly elevated in *Hulk*-infected mice relative to parental controls (Fig 3.5C). Flow cytometric characterization of cells from BAL and lungs of these mice further suggested that the cells responding to 2W1S peptide were Th2 cells, since proliferating (Ki67+) GATA3+ CD4+ T cells were significantly enriched by frequency in *Hulk*-infected mice relative to parental-infected and naïve controls (Fig 3.5D-I). Interestingly, however, type 2 cytokines IL-4, IL-5 and IL-13 were not significantly upregulated in *Hulk*-infected mice relative to controls whether stimulated *ex vivo* or *in vivo* with 2W1S peptide (Fig 3.S4). Though surprising given the increased frequency of GATA3+CD4+ T cells in *Hulk*-infected mice, this result is consistent with the fact that amphiregulin was more highly enriched in *Hulk* 2W1S-specific CD4+ T cells than any other type 2 cytokine by RNA sequencing.

RNA sequencing analysis revealed that *Hulk*-elicited 2W1S-specific CD4+ T cells upregulate GATA3 and type 2 cytokines, but also some genes associated with Tregs. Further, amphiregulin is known to be produced by both Th2 and Tregs. Accordingly, I asked whether *Hulk*-expanded 2W1S-specific CD4+ T cells heterogeneously expressed Th2 and Treg lineage-defining transcription factors. To this end, 2W1S-specific CD4+ T

cells from mice inoculated 3 times with live *Hulk* were evaluated for expression of the Th2 lineage-defining transcription factor GATA3 and the Treg lineage-defining transcription factor Foxp3. Total CD4+ T cells were isolated from the lungs and lymphoid tissues of infected and naïve mice and stimulated for 3 days on anti-CD3/CD28-coated plates to enrich the 2W1S-specific CD4+ T cell population. Using this *in vitro* expansion method, I observed that a majority (60%) of 2W1S-specific CD4+ T cells from *Hulk*-infected mice were GATA3 single positive, 1.0% were Foxp3 single positive, and 1.7% were double positive for GATA3 and Foxp3 (Fig 3.6A-B). Furthermore, when 2W1S-specific CD4+ T cells were analyzed for Foxp3 expression directly *ex vivo*, up to 30% of all 2W1S-specific CD4+ T cells expressed Foxp3 (Fig 3.6C-D). In contrast, immunization with 2W1S peptide and alum adjuvant did not expand Foxp3+ 2W1S-specific CD4+ T cells to the same extent (Fig 3.6C-D). Taken together with my transcriptional analysis, I interpret these data to indicate that *Hulk* selectively expands a heterogenous population of 2W1S-specific CD4+ T cells with the potential to function as Th2s or Tregs.

Immunization with 2W1S peptide enhances amphiregulin-expressing CD3+ cells in the lung parenchyma following Hulk infection

Given that a proportion of 2W1S-specific CD4+ T cells express GATA3 and amphiregulin, a molecule necessary for clearance of the GI nematode *T. muris* (Zaiss et al., 2006), I questioned whether these cells could promote *Hulk* clearance. However, mice immunized with 2W1S peptide and alum adjuvant had comparable worm burdens as unimmunized mice 3 and 6 days following primary *Hulk* infection (Fig 3.7A-B). This result was in spite of the increased number and frequency of activated 2W1S-specific CD4+ T cells in the lungs of immunized mice relative to unimmunized mice (Fig 3.S5). As expected, mice given a secondary infection with *Hulk* had significantly reduced worm burdens compared to mice given a primary infection (Fig 3.7A-B), but these mice still

had significantly fewer activated 2W1S-specific CD4+ T cells than 2W1S peptide-immunized mice (Fig 3.S5).

While amphiregulin has been implicated in GI nematode clearance, it has also been shown to be integral for wound repair at mucosal sites (Arpaia et al., 2015; Burzyn et al., 2013; Monticelli et al., 2015). Accordingly, I sought to determine whether 2W1S immunization led to increased amphiregulin in *Hulk*-infected lungs at a timepoint when larvae usually cause injury as they migrate out of the lungs and into the gut (Fig 2.S1) (Chen et al., 2014; Douglas et al., 2021). Interestingly, prior immunization with 2W1S peptide was associated with elevated spontaneous release of amphiregulin by lung cells isolated 3 days post-infection following a 72 hour culture period (Fig 3.7C). Additionally, I observed an increased number of both CD3+ and Areg+CD3+ cells in the lung parenchyma of 2W1S-immunized mice relative to non-immunized primary and secondary *Hulk*-infected lungs (Fig 3.7D-F). Though these amphiregulin-producing T cells do not correlate with enhanced *Hulk* clearance, these data may indicate that 2W1S immunization can improve lung tissue repair later following infection.

Given reports of amphiregulin's role in ameliorating lung damage, I asked whether increases in amphiregulin corresponded to decreased lung damage in 2W1S-immunized mice early post-infection (Arpaia et al., 2015). To do this, I quantified the number of red blood cells (RBCs) in the BAL of immunized or unimmunized mice 3 days post-infection with *Hulk*. Though there was a slight decrease in RBCs in immunized mice compared with unimmunized, primary-infected mice, this difference was not significant (Fig 3.S6). Moreover, using RBC counts as a proxy for lung hemorrhage, I did not detect any remarkable effect of immunization on lung damage at this time point post-infection.

Discussion

The *Hulk* model has herein provided direct insight for the first time into the phenotype and function of CD4+ T cells specific for a GI nematode-derived antigen. Lung CD4+ T cells specific for 2W1S are predominantly Th2 cells, but a portion exhibit characteristics of Tregs as well (namely Foxp3 expression). Relative to 2W1S-specific CD4+ T cells expanded in the context of 2W-*Salmonella* infection, *Hulk*-expanded 2W1S-specific cells strongly upregulated amphiregulin at the transcript level – a finding confirmed at the protein level following peptide restimulation *in vitro* and *in vivo*.

Interestingly, though prior immunization with 2W1S peptide and type 2-skewing adjuvant alum did not accelerate *Hulk* clearance, immunization led to increased amphiregulin levels in the lungs and may reduce tissue damage as *Hulk* passes through the lung tissue.

That a majority of 2W1S-specific CD4+ T cells in the lung express GATA3+ is consistent with the expected CD4+ T cell phenotype during most helminth infections (Harris & Loke, 2017). Th2 cells have long been thought to orchestrate worm clearance via type 2 cytokine production, but 2W1S-specific CD4+ T cells expanded by peptide immunization are not sufficient to accelerate *Hulk* expulsion. Though a protective monoclonal CD4+ T cell response against helminths would have tremendous implications for vaccination strategies, it is perhaps unsurprising that a single CD4+ T cell specificity fails to confer protection against a complex, metazoan parasite. Previous studies that compared monoclonal versus oligoclonal T cell repertoires in host immunity against *N. brasiliensis* showed neither could drive expulsion (Seidl et al., 2011). However, that work utilized TCR transgenic mice with TCRs specific for helminth-irrelevant LCMV GP₆₁₋₈₀ epitope or ovalbumin (OVA₃₂₃₋₃₃₉) epitopes, respectively. Thus, this study is the first to demonstrate that CD4+ T cells specific for a helminth-derived antigen do not drive worm killing or expulsion. Nevertheless, it remains a possibility that

a monoclonal CD4+ T cell repertoire specific for 2W1S expressed in a different compartment, or specific for an endogenous helminth epitope, could better promote worm clearance. Resolving the degree of CD4+ T cell repertoire complexity required for protection against nematodes is a critical topic for vaccine development and is the topic of future work.

Foxp3+ Tregs have been reported to expand in *S. ratti* and other GI nematode infections (Blankenhaus et al., 2014; Finney et al., 2007), but finding that a proportion of these Tregs are helminth antigen-specific is intriguing and novel. There is evidence that both thymic Tregs (tTregs), defined by expression of Helios, and peripherally induced Tregs (pTregs) expand or differentiate in the context of helminth infections (Grainger et al., 2010; Reynolds & Maizels, 2012). The latter are thought to be at least partially induced by helminth-derived secretory products that can drive Treg differentiation, some of which act through the TGF- β signaling pathway (Grainger et al., 2010; Johnston et al., 2017; Smyth et al., 2018). It would be particularly interesting to investigate whether 2W1S-specific CD4+ Tregs elicited by *Hulk* infection share the same TCR sequences as a) 2W1S-specific Tregs present in naïve mice or b) 2W1S-specific Th2 effectors that arise during *Hulk* infection (Ertelt et al., 2009). These investigations could shed light on the expansion of tTregs vs. pTregs in infection and the relative contributions of cell intrinsic (*i.e.* TCR) vs. cell extrinsic (*i.e.* cytokine/co-stimulatory) signals in driving Th2 vs. pTreg differentiation, respectively.

Furthermore, expansion of Tregs is somewhat unique to helminth infections, as infection with intracellular microbes such as *L. monocytogenes* or *T. gondii* results in reduction of both bulk and antigen-specific Tregs during acute inflammation (Ertelt et al., 2009; Oldenhove et al., 2009). Yet the functional significance of Tregs that recognize pathogen-derived antigen, particularly in the context of helminth infection, is largely

unexplored. My data show that 2W1S-specific Foxp3+ T cells express *Il10* transcripts, but I have not demonstrated that 2W1S-specific Foxp3+ T cells are functionally suppressive during *Hulk* infection. If helminth-specific Tregs are immunosuppressive, this could pose significant challenges to the development of lasting protective immunity following natural infection and/or vaccination. However, these cells may also be crucial for preventing tissue damage and preserving organ function, particularly in the lung (Abdel Aziz et al., 2018).

I found it particularly interesting that *Hulk*-elicited 2W1S-specific CD4+ T cells upregulated amphiregulin transcript and preferentially produced amphiregulin upon peptide restimulation. Amphiregulin is necessary for immunity against the whipworm *T. muris*, and expression of amphiregulin's receptor, EGFR, is required on CD4+ T cells for clearance of *N. brasiliensis* (Minutti et al., 2017). However, data from the 2W1S immunization model show that amphiregulin levels and numbers of amphiregulin-producing CD3+ T cells in the lung are not associated with reduced worm burden. Given findings showing that amphiregulin promotes epithelial cell repair and tissue remodeling in models of influenza or dextran sulfate sodium (DSS)-induced colitis, it may be that these cells instead promote enhanced tissue repair during *Hulk* infection (Arpaia et al., 2015; Monticelli et al., 2015). Contrary to this hypothesis, quantification of RBC counts in the BAL of immunized mice did not differ significantly from their unimmunized counterparts, suggesting that lung hemorrhage was similar between these two groups at the early timepoint analyzed. However, it would be valuable to assess the lungs of immunized and unimmunized mice histologically and at different timepoints post-infection to gain a better understanding of how immunization might impact other features of lung damage, such as bronchial epithelial loss, lymphocyte aggregation, or edema (Arpaia et al., 2015). Further, if amphiregulin can protect from lung damage in this

context, whether that amphiregulin must come from CD4+ T cells remains an open avenue for investigation.

Hulk represents a unique tool for the study of antigen-specific Th2s and Tregs in the context of GI nematode infection. Unlike antigen-specific cells that have been studied in intracellular microbial infections, 2W1S-specific CD4+ T cells elicited by *Hulk* are heterogenous and may functionally support tissue repair or reduce tissue damage, though their phenotypes also suggest roles in immunosuppression and type 2 inflammation as well. In addition to the applications described herein, I propose that *Hulk* has the potential to be used to study T-B cell interactions and helminth-specific antibody production, visualization of parasite-immune interactions using fixed or live imaging, and compartmentalization or localization of CD4+ T cell responses during helminth infection.

Figures

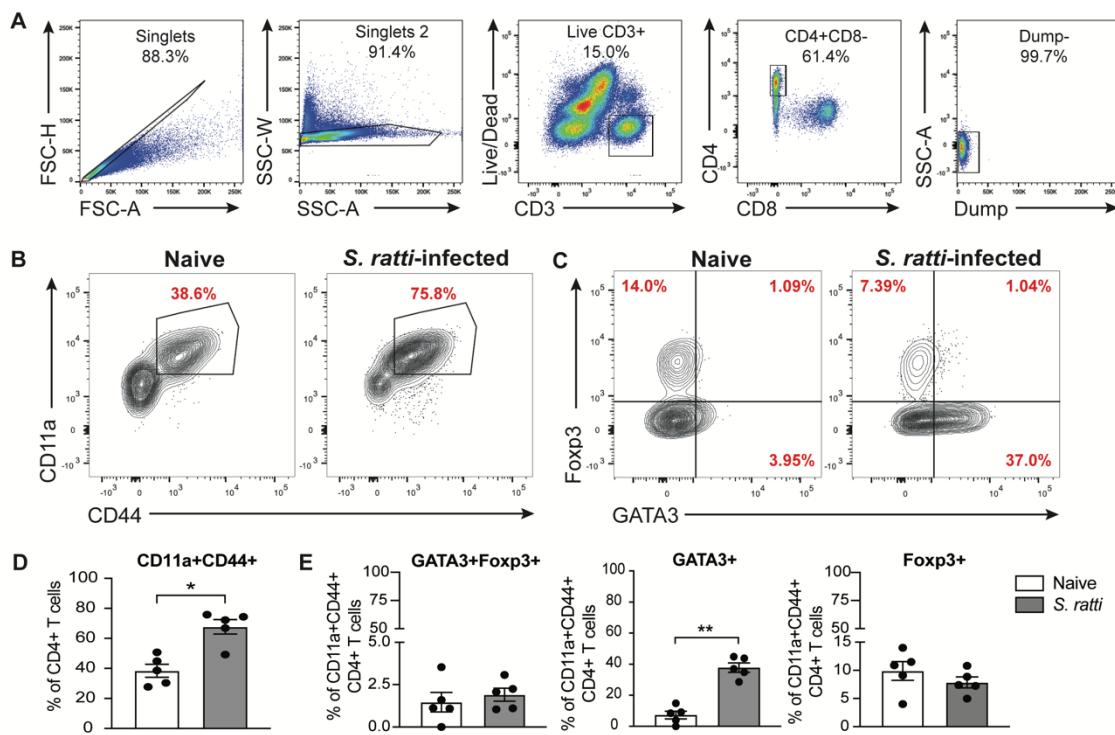


Figure 3. 1. Activated CD4+ T cells expand and express Treg and Th2 transcription factors during *S. ratti* infection. (A) Gating strategy for CD4+ T cells in the lungs. Dump gate includes B220/CD45R, CD11b, and CD11c. (B) Activation status and (C) transcription factor expression in CD4+ T cells from lungs of *S. ratti* infected mice 14 days post-initial infection (3 total infections, 1,000 iL3 per infection). (D) Frequency of activated or (E) GATA3+, Foxp3+, and Foxp3+GATA3+ cells in the lungs of *S. ratti*-infected and naïve mice. Significance determined by Mann-Whitney test; *p <0.05, **p < 0.01, n = 5.

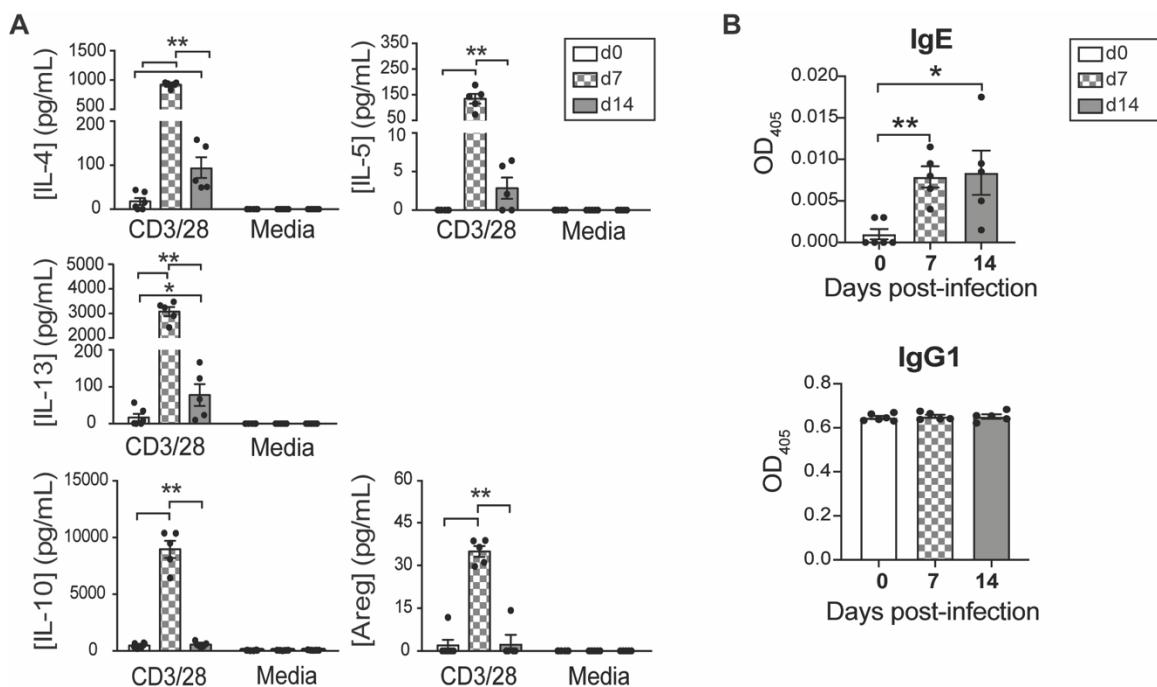


Figure 3.2. *S. ratti* elicits canonical type 2 and regulatory cytokine production from CD4+ T cells and induces polyclonal IgE. (A) Type 2 and regulatory cytokine production by MACS-sorted CD4+ T cells from pooled lymph nodes at 0, 7, and 14 days post-infection following 72 hours of culture in the presence or absence of anti-CD3/CD28 antibody. Data representative of 2 independent experiments, n = 3-5 per experiment. (B) Total serum IgE and IgG1 levels at 0, 7 and 14 days post-infection, n = 5-6. Significance determined by Mann-Whitney test; *p <0.05, **p < 0.01.

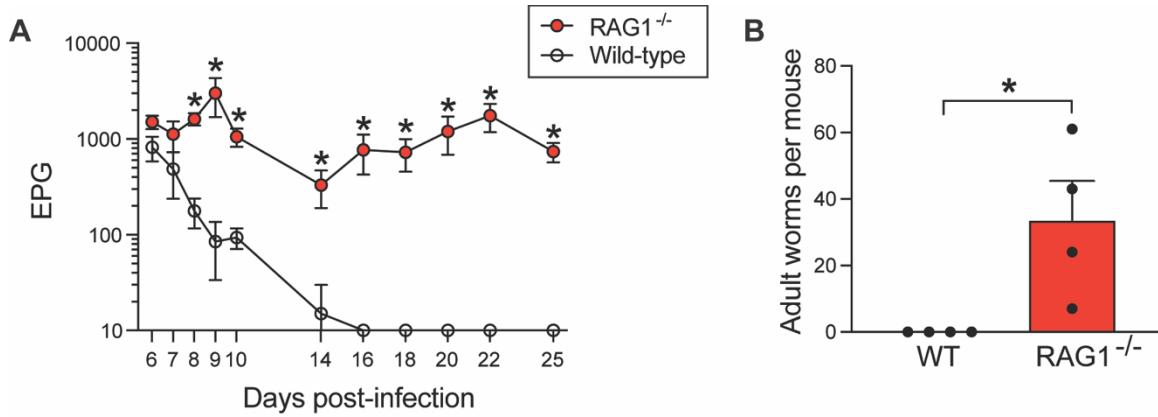


Figure 3.3. Adaptive immune responses are necessary for clearance of *S. ratti* infection. (A) Fecal egg deposition and (B) adult worm counts from wild-type and RAG1^{-/-} mice infected with 1,000 iL3 *S. ratti*. Adult worms were collected from the entire length of the small intestine on day 25 post-infection. Data representative of 2 independent experiments, n = 4 per experiment. Significance determined by Mann-Whitney test; *p <0.05.

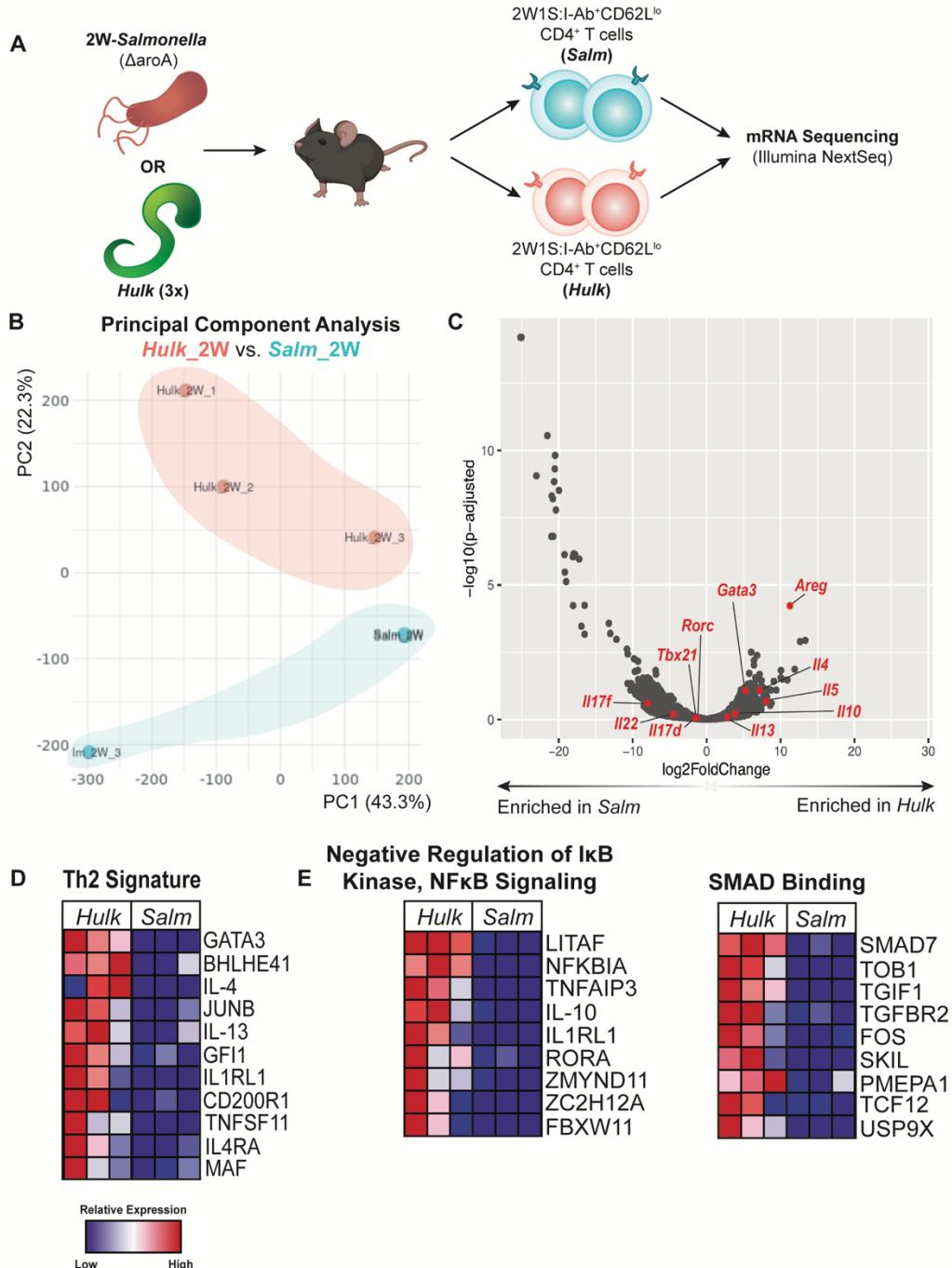


Figure 3. 4. *Hulk*, but not 2W-Salmonella, infection enriches type 2 and immunosuppressive programs in 2W1S:I-A^b+CD4+ T cells. (A) Schematic of experimental design for mRNA sequencing. 2W1S:I-A^b+CD4+ T cells were isolated from pooled lungs and mdLN of *Hulk*-infected mice and pooled mLN and Peyer's patches of

2W-*Salmonella*-infected mice; n = 3 biological replicates per group. Schematic created with mouse image from BioRender.com. (B) Principal component analysis comparing 2W1S-specific CD4+ T cells from *Hulk* (*Hulk_2W*) and 2W-*Salmonella* (*Salm_2W*)-infected mice. (C) Volcano plot depicting genes of interest enriched in *Hulk* or *Salm*-elicited 2W1S-specific CD4+ T cells. (D) Heatmap depicting enrichment of curated Th2 Signature gene set in *Hulk*-expanded 2W1S-specific CD4+ T cells. (E) Heatmaps depicting enrichment of Negative Regulation of I κ B and NF κ B Signaling and SMAD Binding gene sets (Gene Ontology) in *Hulk*-expanded 2W1S-specific CD4+ T cells.

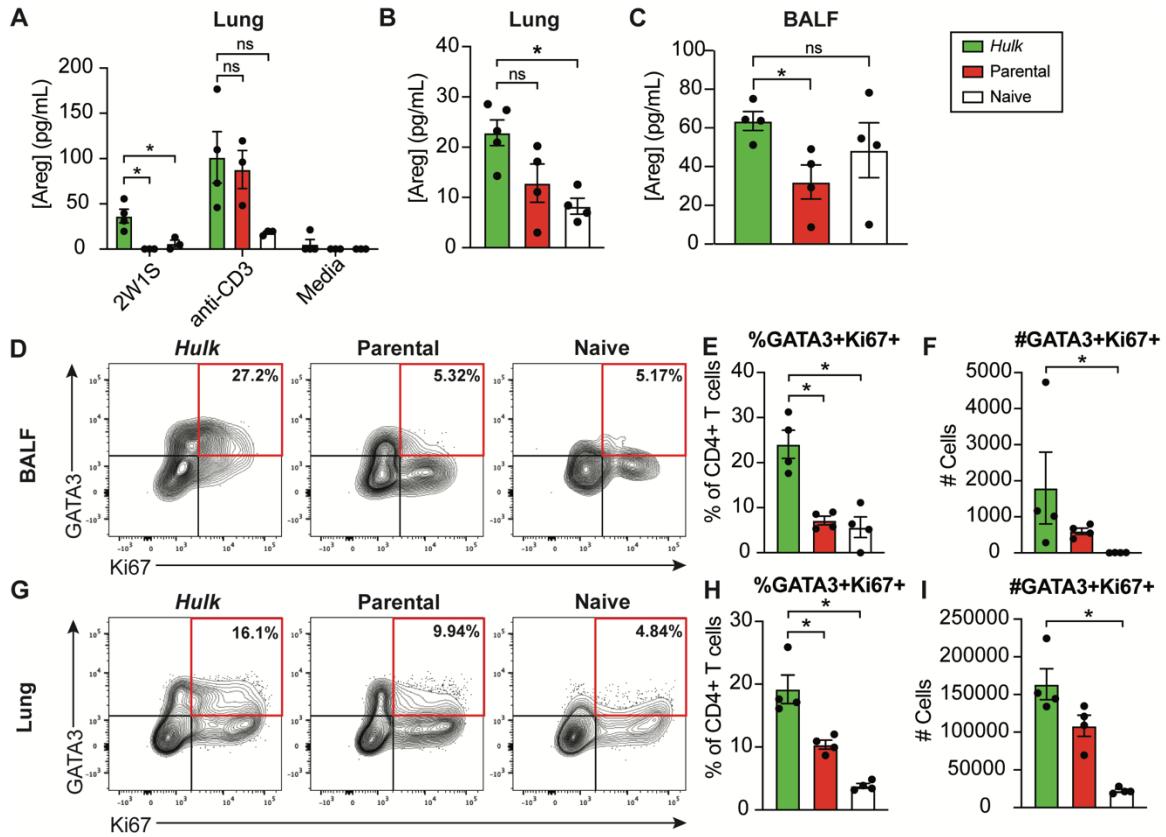


Figure 3. 5. 2W1S-specific CD4+ T cells that expand during *Hulk* infection in the lung proliferate, express GATA3, and produce amphiregulin in response to peptide stimulation. (A) Amphiregulin production by lung cells from naïve mice or mice infected 3 times with live *Hulk* or parental *S. ratti* following 72 hours restimulation with 2W1S or anti-CD3 (n = 3-5). (B) Spontaneous amphiregulin production by lung cells from naïve mice or mice infected 3 times with irradiated *Hulk* or parental *S. ratti* and challenged intratracheally with 2W1S peptide after 48 hours in culture. Data representative of 2 independent experiments, n = 4-5 per experiment. (C) Amphiregulin levels in bronchoalveolar lavage fluid (BALF) from mice infected and challenged as in (B) (n = 4). (D) Representative flow plots showing GATA3+Ki67+ CD4+ T cells in BALF of mice infected and peptide challenged as in (B). Parent gates were set according to the gating strategy shown in Fig 3A. (E-F) Frequency and number of GATA3+Ki67+

CD4+ T cells in BALF of mice infected and peptide challenged as in (B) (n = 4). (G)
Representative flow plots showing GATA3+Ki67+ CD4+ T cells in lungs of mice infected
and peptide challenged as in (B). Parent gates were set according to the gating strategy
shown in Fig 3A. (H-I) Frequency and number of GATA3+Ki67+ CD4+ T cells in lungs of
mice infected and peptide challenged as in (B) (n = 4). Significance determined using
Student's T-test with Welch's correction (A) or Mann-Whitney test (B-C, E-F, H-I); *p <
0.05.

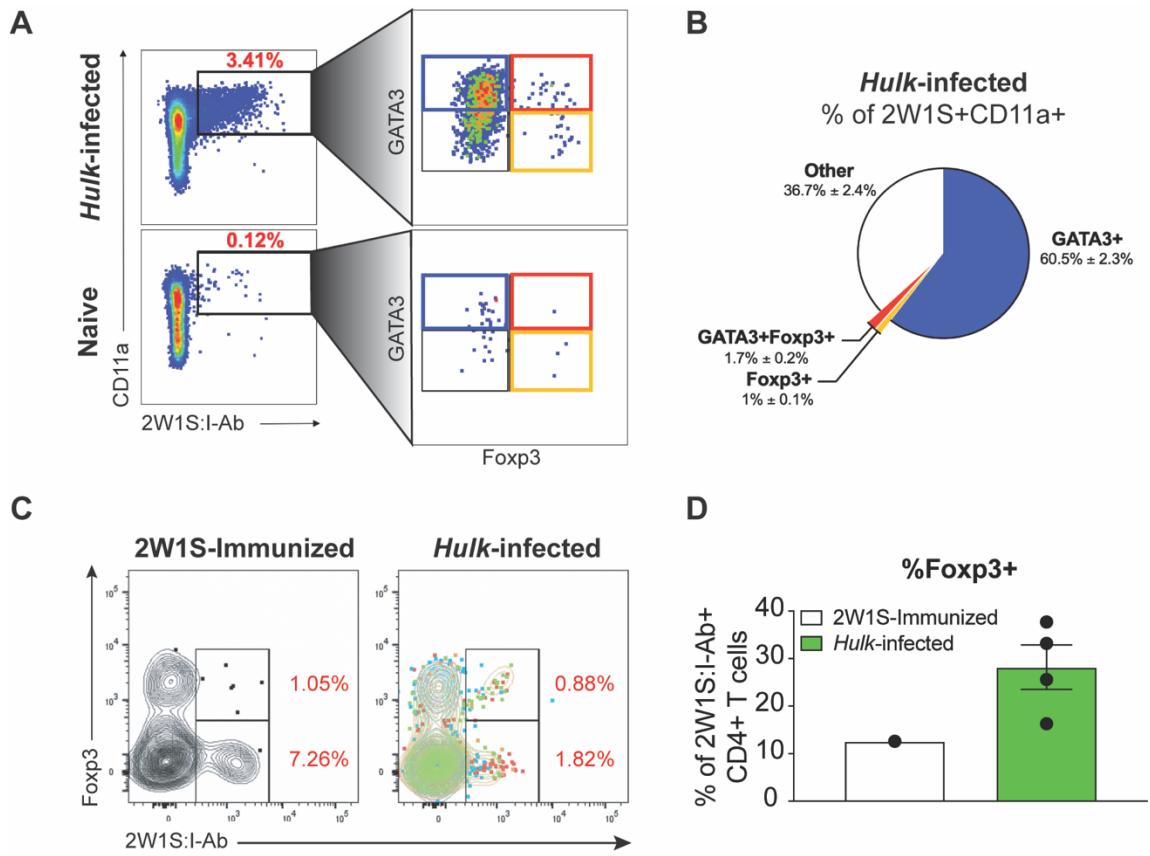


Figure 3.6. *Hulk*-expanded 2W1S-specific cells express both GATA3 and Foxp3.

(A) GATA3 and Foxp3 expression in CD11a⁺2W1S:I-A^b⁺ CD4+ T cells from lymph nodes and lungs of naïve mice or mice infected 3 times with live *Hulk* following 72 hours of anti-CD3/CD28 stimulation. (B) Relative frequencies of GATA3+, GATA3+Foxp3+, Foxp3+ or GATA3-Foxp3- cells within 2W1S:I-A^b⁺ CD4+ T cells shown in (A). (C) Foxp3-expressing 2W1S:I-A^b⁺ CD4+ T cells following 2W1S-immunization or 3 live *Hulk* infections. (D) Frequency of Foxp3+ cells within 2W1S:I-A^b⁺ CD4+ T cells. Observation of Foxp3⁺2W1S:I-Ab^b⁺CD4+ T cells expanded in *Hulk*-infected lungs analyzed directly ex vivo representative of 2 independent experiments, n = 4-5 per experiment.

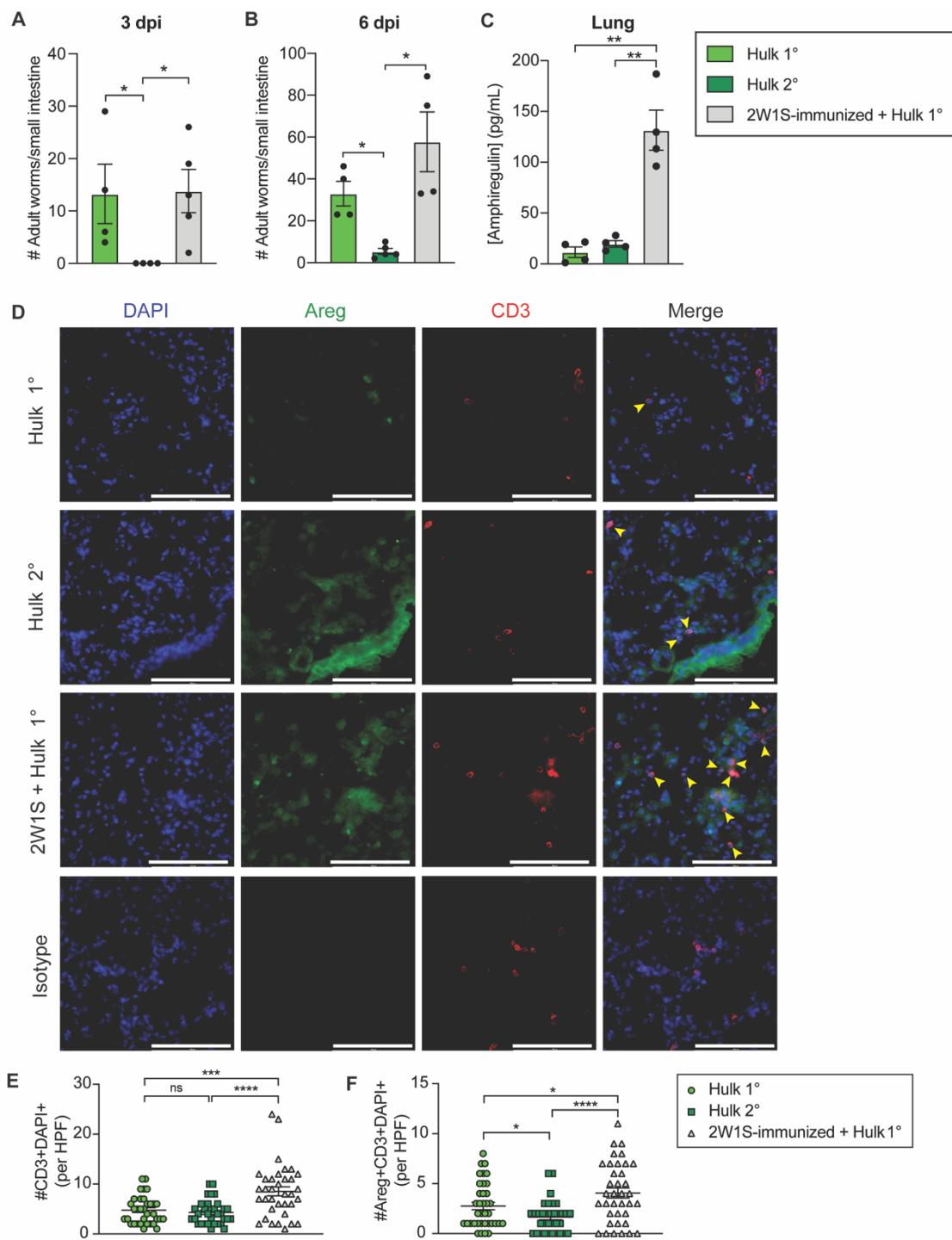


Figure 3. 7. 2W1S peptide immunization prior to *Hulk* infection does not confer protection, but enhances amphiregulin production in the lung. Mice immunized with

2W1S peptide and alum were subsequently infected with *Hulk* and compared to mice given a primary or secondary *Hulk* infection. (A-B) Adult worm burdens in the small intestine of mice on day 3 (A) or day 6 (B) post-primary (1°) or secondary (2°) *Hulk* infection. (C) Spontaneous amphiregulin production in supernatants from lung cells from indicated groups isolated 3 days post-infection and cultured for 72 hours. (D) Representative images of lung tissue from 1°, 2W1S-immunized + 1° or 2° infected mice 3 days post-infection (63x). DAPI is shown in blue, CD3 is shown in red and Areg is shown in green. Yellow arrows indicate cells that were counted as DAPI+CD3+Areg+. Scale bars represent 100 um. (D) Number of CD3+DAPI+ or (E) Areg+CD3+DAPI+ cells counted per high-powered field (HPF) at 63x magnification, with 37-38 HPFs counted from 3-4 biological replicates per group. Significance determined using (A-B) Mann-Whitney test or (C, E-F) Student's T-test with Welch's correction; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplemental Figures and Tables

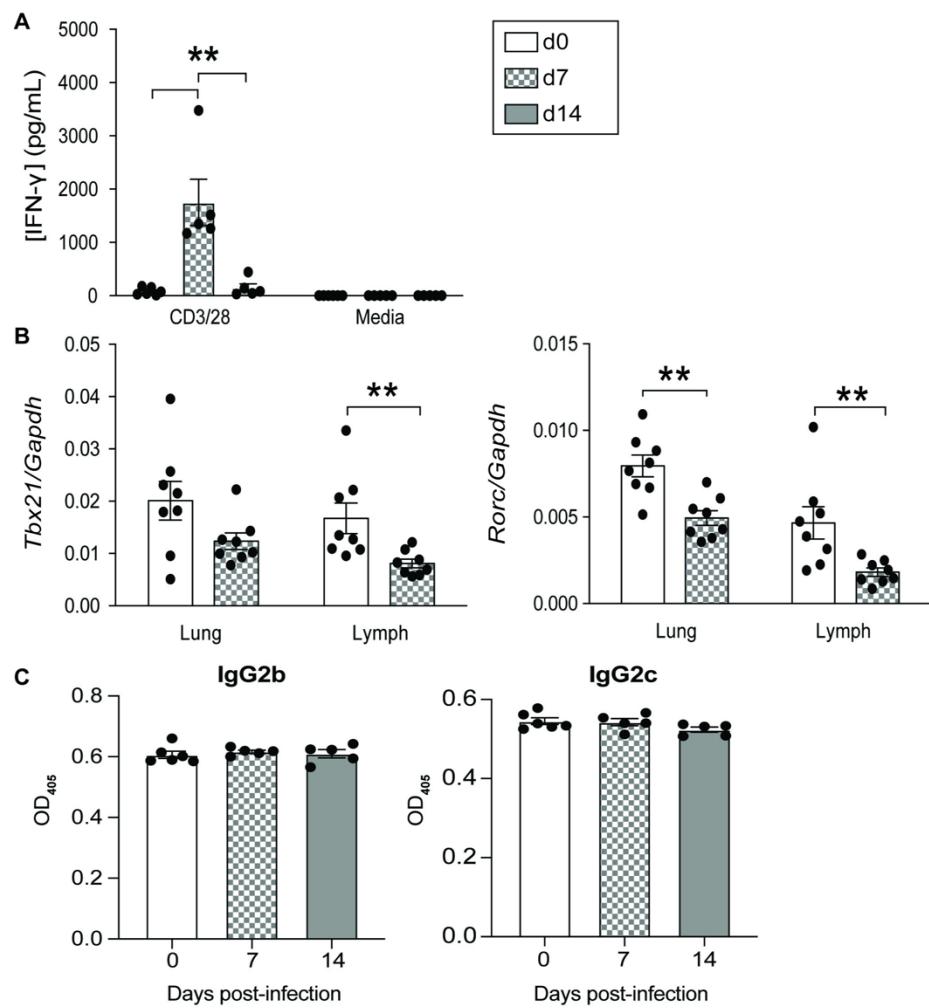


Figure 3.S 1. IFN- γ , but not *Tbx21*, *Rorc* or IFN γ -dependent antibodies, increase during *S. ratti* infection. (A) IFN- γ levels in anti-CD3/CD28-stimulated CD4+ T cells from secondary lymphoid organs of *S. ratti*-infected or naïve mice 72 hours post-stimulation. Data representative of 2 independent experiments, n = 3-5 per experiment. (B) *Tbx21* (Tbet) and *Rorc* (ROR γ t) expression in CD4+ T cells from lungs and secondary lymphoid organs of naïve and *S. ratti*-infected mice. Data are combined from 2 independent experiments, n = 8. (C) IgG2b and IgG2c antibody absorbance values in

sera from naïve and *S. ratti*-infected mice (n = 5-6). Significance determined using a Mann-Whitney test for significance; **p<0.01.

| Th2 Gene Signature |
|---------------------------|
| GATA3 |
| TNFRSF4 |
| IL1RAP |
| IL1RL1 |
| IL4RA |
| IL4 |
| IL5 |
| IL9R |
| IL13 |
| PLAC8 |
| IGFBP7 |
| CD200R1 |
| PDCD1 |
| TNFSF11 |
| STAT6 |
| STAT5 |
| BHLHE41 |
| GFI1 |
| IKZF1 |
| JUNB |
| MAF |
| PRDM1 |

Table 3.S 1. Th2 signature gene set used for gene set enrichment analysis.

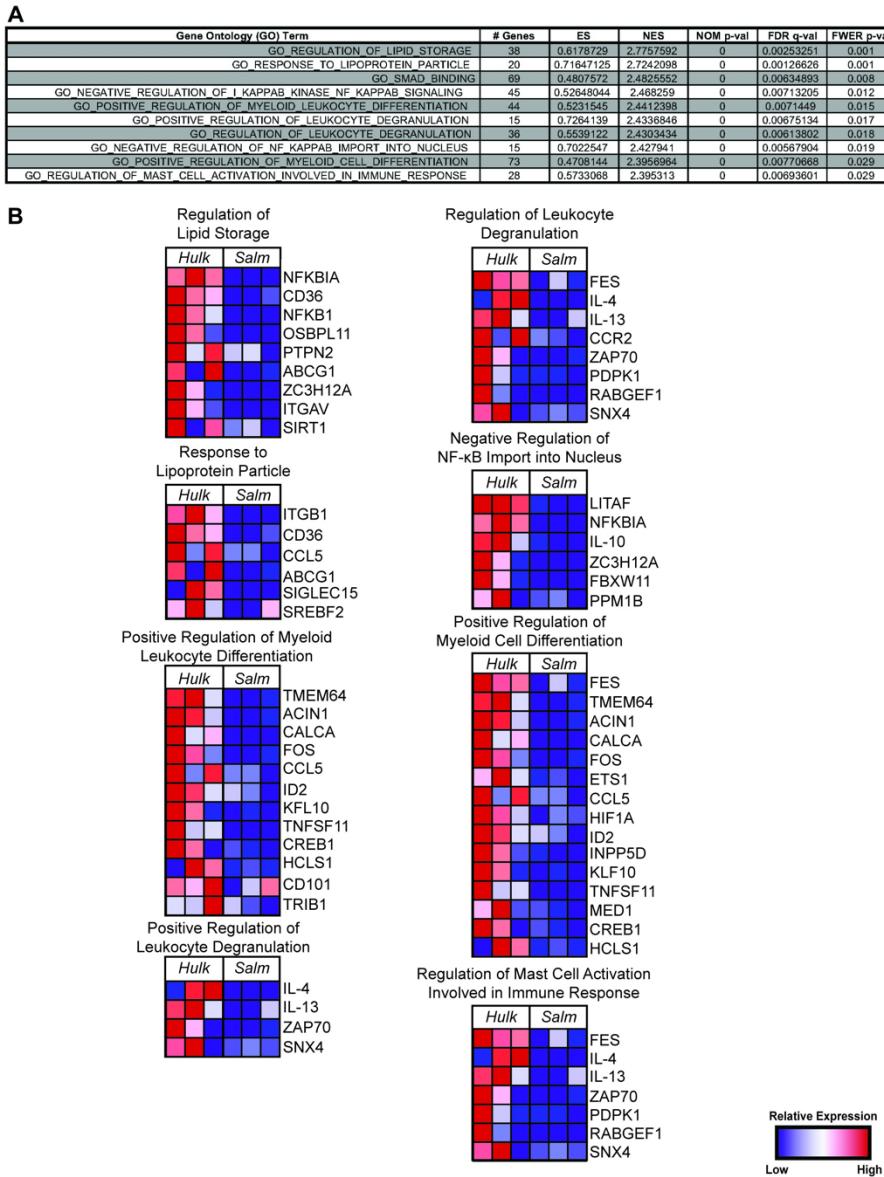


Figure 3.S 2. Gene set enrichment analysis using GO terms for *Hulk*-expanded 2W1S-specific CD4+ T cells. (A) Table depicting the top ten most significantly enriched gene ontology terms within 2W1S+CD4+ T cells from *Hulk*-infected mice relative to 2W1S+CD4+ T cells from 2W-*Salmonella*-infected mice. (B) Heat maps showing representative genes upregulated in 8/10 gene ontology pathways upregulated in 2W1S+CD4+ T cells from *Hulk*-infected mice in addition to those shown in Fig 4 (n = 3).

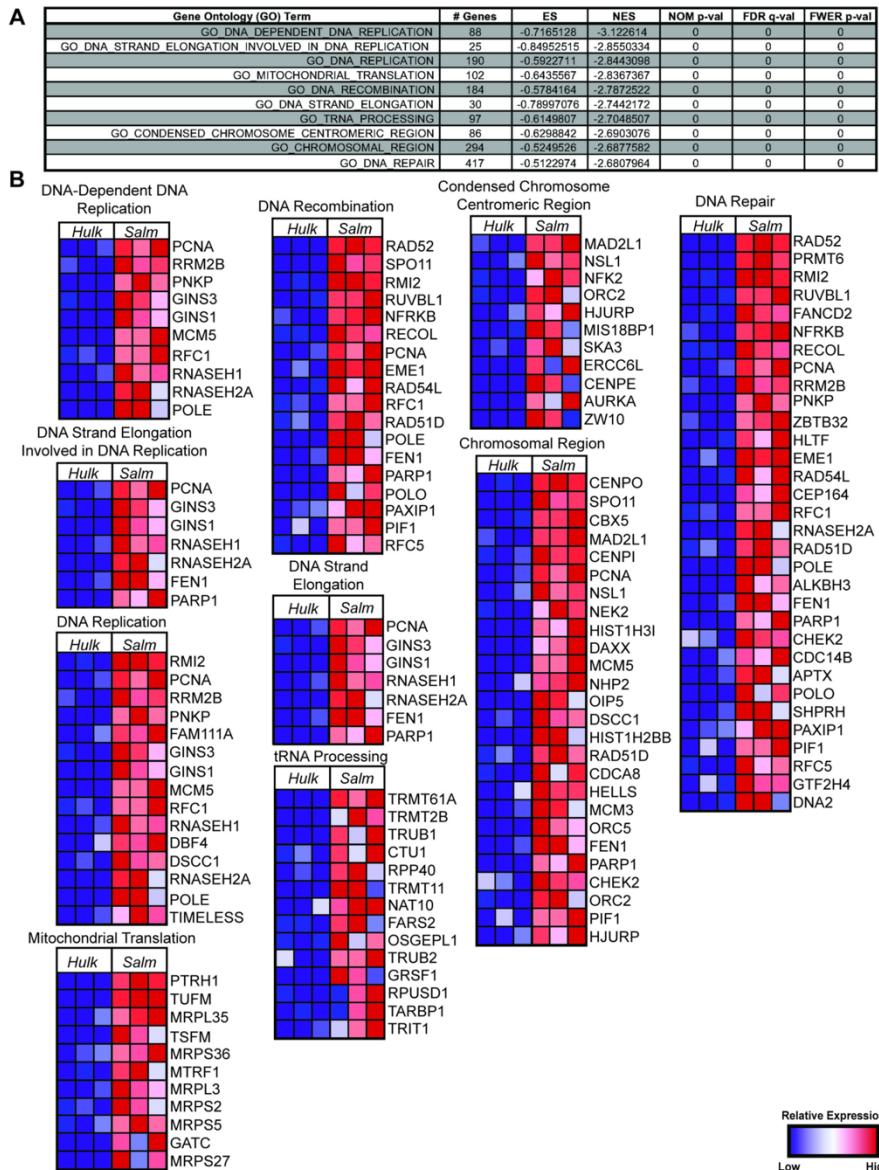


Figure 3.S 3. Gene set enrichment analysis using GO terms for 2W-Salmonella-expanded 2W1S-specific CD4+ T cells. (A) Table depicting the top ten most significantly enriched gene ontology terms within 2W1S+CD4+ T cells from 2W-Salmonella-infected mice relative to 2W1S+CD4+ T cells from *Hulk*-infected mice. (B) Heat maps showing representative genes upregulated in each gene ontology pathway upregulated in 2W1S+CD4+ T cells from 2W-Salmonella-infected mice relative to *Hulk*-infected mice in addition to those shown in Fig 4 (n = 3).

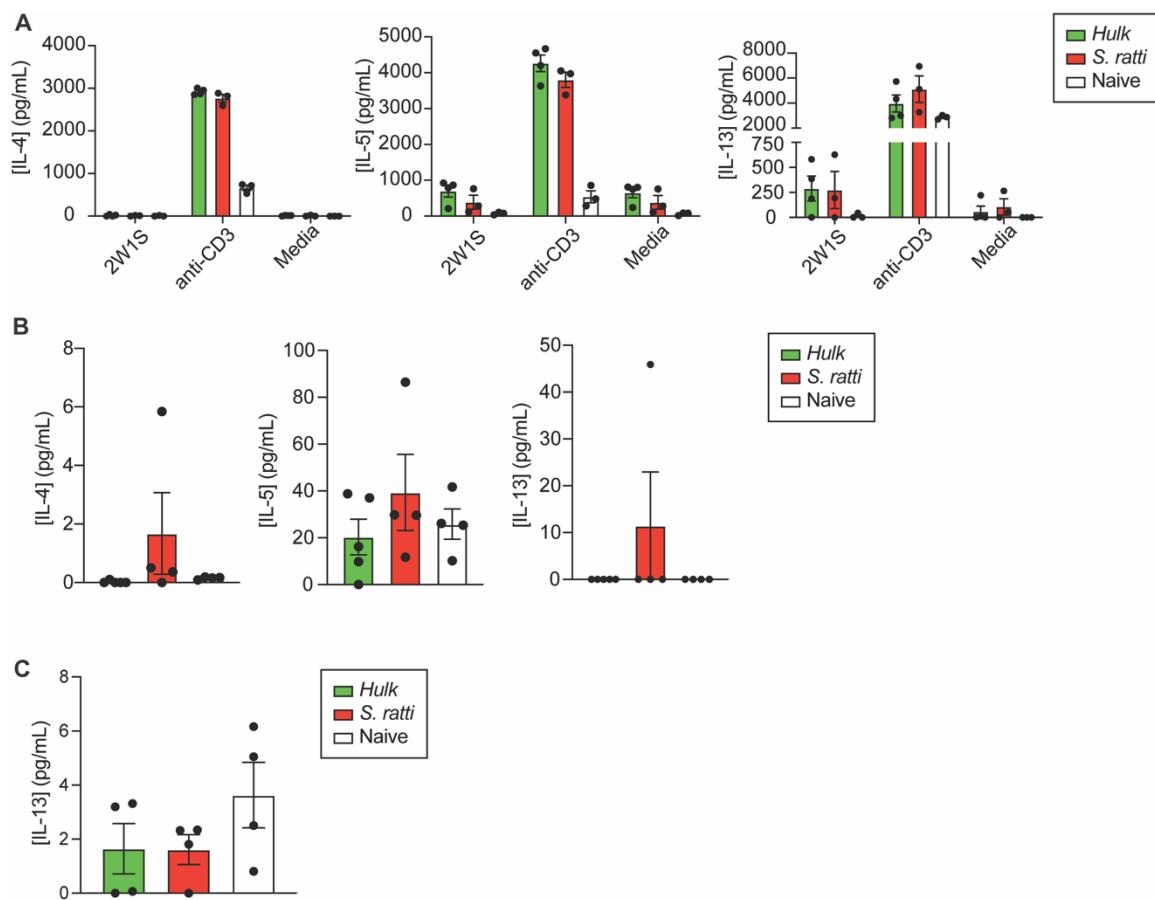


Figure 3.S 4. Canonical type 2 cytokine production is unchanged in *Hulk* lungs, bronchoalveolar lavage fluid (BALF) relative to controls following 2W1S restimulation. (A) IL-4, IL-5 and IL-13 production by lung cells from naïve mice infected 3 times with live *Hulk* or parental *S. ratti* after 72 hours stimulation with 2W1S peptide or anti-CD3. Data representative of 2 independent experiments, n = 3-5 per experiment. (B) Spontaneous IL-4, IL-5 and IL-13 production by lung cells from naïve mice or mice infected 3 times with irradiated *Hulk* or parental *S. ratti* and restimulated with 2W1S peptide intratracheally after 48 hours *in vitro* culture. Data representative of 2 independent experiments, n = 4-5 per experiment. (C) IL-13 levels in BALF of naïve mice or mice infected 3 times with irradiated *Hulk* or parental *S. ratti* and restimulated with

2W1S peptide intratracheally. Data representative of 2 independent experiments, n = 4-5 per experiment.

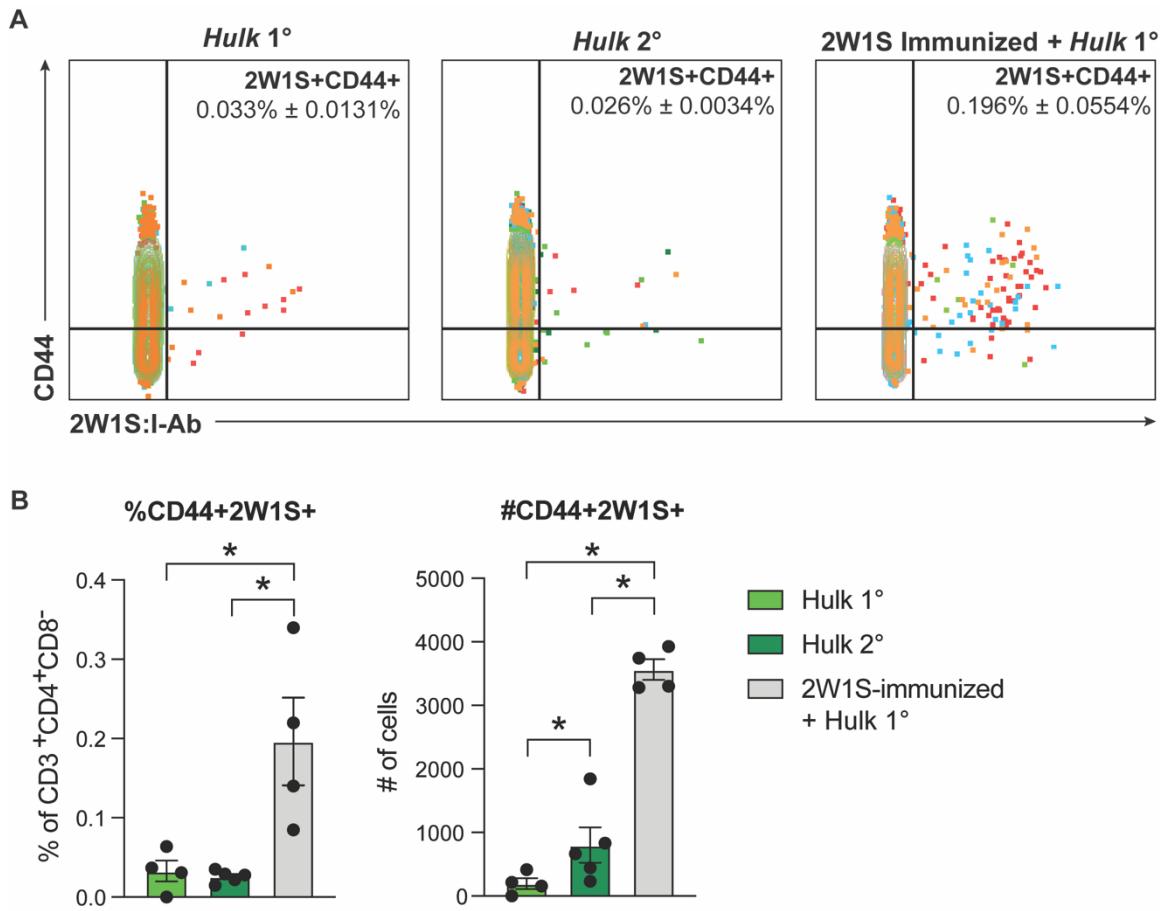


Figure 3.S 5. 2W1S-specific CD4+ T cell frequencies and numbers in the lungs of mice following secondary *Hulk* infection or primary *Hulk* infection with or without prior 2W1S immunization. Mice immunized with 2W1S peptide and alum were subsequently infected with *Hulk* 6 weeks post-immunization and compared to mice given primary or secondary *Hulk* infections alone ($n = 4-5$). (A) Concatenated flow plots showing the frequency of 2W1S+CD44+ CD4+ T cells in the lungs of each group 6 days post-challenge. Note: Lung cells were not enriched for 2W1S:I-A^b + cells prior to analysis as in main figures. (B) Frequency and number of 2W1S:I-A^b+CD44+ CD4+ T cells in each group 6 days post-challenge. Significance was determined using a Mann-Whitney test; * $p < 0.05$.

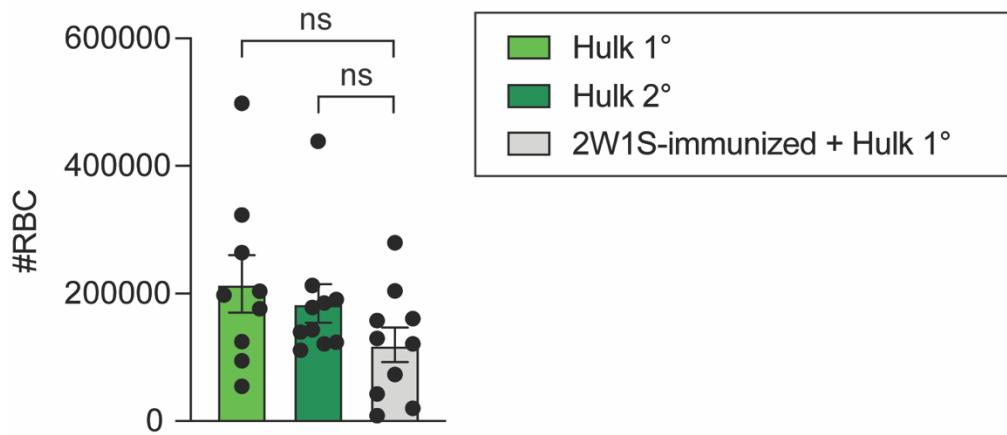


Figure 3.S 6. Immunization with 2W1S peptide does not significantly alter red blood cell numbers in the BAL of *Hulk*-infected mice. Mice were immunized with 2W1S and alum or infected with 1,000 iL3 *Hulk*. 3 weeks later, these mice and a naive cohort were all challenged with 1,000 iL3 *Hulk*. Red blood cells (RBCs) in the BAL were enumerated pre- and post-ACK lysis 3 days post-infection. Data combined from 2 independent experiments, n = 9-10.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

4.1 Roles of antigen-specific CD4+ T cells in helminth infection

To my knowledge, this is the first study to utilize a model antigen-expressing GI nematode to identify and characterize CD4+ T cells recognizing the same nematode-derived antigen. Using the *Hulk* model, I find 2W1S-specific CD4+ T cells expanding predominantly in the lung parenchyma with a small proportion in the mediastinal lymph nodes. My data suggests that these cells are heterogeneously Th2 cells and Tregs, and that they largely produce amphiregulin, and express other type 2 cytokines like IL-4, IL-5 or IL-13 at the transcriptional level. Interestingly, though I find an increased number of CD3+ amphiregulin-producing cells and greater total amphiregulin levels in the lungs of 2W1S-immunized, *Hulk*-infected mice relative to unimmunized primary or secondary *Hulk*-infected mice, prior expansion of 2W1S-specific CD4+ T cells by immunization does not accelerate *Hulk* clearance (Fig 4.1).

I have demonstrated that *Hulk* is a particularly useful model for studying antigen-specific CD4+ T cells in the lungs of infected mice. While few immunological studies in *S. ratti* have focused on the lung, work in *N. brasiliensis* – which similarly migrates through the skin and lungs before reaching the intestine – reveals that CD4+ T cells at this site are crucial for memory responses against secondary challenge (Harvie et al., 2010; Thawer et al., 2014). Accordingly, tools to study antigen-specific CD4+ T cells in this tissue are of great utility. That said, I do not find evidence that 2W1S-specific CD4+ T cells mediate protection against subsequent *Hulk* infection. Instead, I hypothesize that these cells help ameliorate tissue damage and promote repair, predominantly through production of amphiregulin.

While this study constitutes the first investigation into antigen-specific CD4+ T cell function in the context of GI nematode infection, it is important to emphasize that

these findings may not apply to all antigen specificities that can arise in response to *S. ratti* or other GI nematode infections. Since 2W1S is expressed in the muscular body wall of *Hulk*, I hypothesize that this antigen is only accessible from worms that have died or arrested in tissue. Body wall antigens like 2W1S can then be acquired by APCs that are clearing debris from tissue and presented to CD4+ T cells, presumably first in the draining lymph node. Whereas actively secreted, soluble worm antigens may be taken up almost immediately after worms enter tissues (referred to subsequently as “early” antigens), I think that body wall antigens (as well as persistent soluble antigens) may be recognized later, once worms have not only entered tissues but have also been trapped and degraded (referred to subsequently as “late” antigens). This difference in timing may be inconsequential for parasite clearance in the lung during primary infections, since it takes ~2-3 days for naïve CD4+ T cells to be fully activated and a majority of L4 larvae leave the lungs within 48 hours after their arrival (Dawkins & Grove, 1981; Kaech, Wherry, & Ahmed, 2002). However, this timing difference may impact the functions that CD4+ T cells adopt: whereas CD4+ T cells specific for “early” antigens become activated in a highly inflammatory environment during or shortly after larval migration out of the lung, the cytokine milieu present during activation of “late” antigen-specific CD4+ T cells may favor more immunosuppressive or tissue-reparative functions (Reece, Siracusa, & Scott, 2006). Moreover, upon restimulation during secondary infection, “early” antigen-specific CD4+ T cells may be primed to produce cytokines involved in worm clearance, whereas “late” antigen-specific CD4+ T cells like 2W1S-specific CD4+ T cells in the *Hulk* model may be more primed to participate in damage control, tissue repair, or regeneration (Fig 4.2). This concept of temporal antigen expression has been explored using transgenesis in the *Salmonella* model, where it influences Th1 vs. Th17

differentiation, but exploration in GI nematode models may provide novel insights into CD4+ T cell fate decisions and functions in type 2 immunity (Lee et al., 2012).

Just as CD4+ T cell function may depend on the timing of antigen exposure, the location in which CD4+ T cells see their antigen likely also influences their function. I and others have shown that larval migration through the lung causes severe hemorrhaging in *S. ratti* and *N. brasiliensis* infection (Chen et al., 2014; Douglas et al., 2021; Reece et al., 2006). While *S. ratti* does burrow into the epithelium in the intestine, it does not blood feed and does not encyst in the submucosa, meaning that worm clearance and/or immunosuppression may be prioritized in the intestine rather than wound repair (Dawkins et al., 1983). Moreover, clearance mechanisms in the gut are thought to detach worms from the intestine into the lumen where they can be passed from the host (termed expulsion). If nematode tissues are physically expelled from the intestine rather than becoming trapped in parenchymal tissues as happens in the lung, body wall and other somatic tissue antigens may be less likely to be recognized in the gut. Accordingly, while *Hulk* provides insight into the phenotype and function of CD4+ T cells specific for body wall antigens in the lung, many questions remain regarding CD4+ T cells that recognize antigens from other nematode anatomical compartments in different host tissues. Such questions could be addressed with transgenic models that express model antigens in different nematode compartments (e.g. ES products), or by designing tetramers that can identify CD4+ T cells specific for endogenous nematode antigens expressed in gut-resident stages of the parasite.

It is not clear from these data whether the increased amphiregulin observed in response to 2W1S peptide stimulation in the lung during *Hulk* infection is produced by 2W1S-specific Th2 cells, Tregs, or both. Treg-derived amphiregulin has already been shown to play roles in tissue repair following injury in the lung, skin and muscle, so it is

not terribly surprising to find potentially amphiregulin-producing Tregs in the lung following a highly injurious GI nematode infection (Arpaia et al., 2015; Burzyn et al., 2013; Nosbaum et al., 2016). Further, a portion of the Foxp3+ Tregs I observe also co-express GATA3, which, in addition to being a critical transcription factor for T cell commitment to the Th2 subset, regulates expression of a component of the IL-33 receptor, ST2 (Zheng & Flavell, 1997; Zhu et al., 2004) (Guo et al., 2009). Interestingly, I observed an increase in ST2 gene transcript (*Il1rl1*) in *Hulk*-expanded 2W1S-specific CD4+ T cells by RNA sequencing. Since lung Tregs were shown to produce amphiregulin largely in response to IL-33 as well as IL-18 stimulation, and since IL-33 is induced upon GI nematode infection, it would stand to reason that 2W1S-specific Tregs could be strong producers of amphiregulin in this system in response to IL-33 signaling (Arpaia et al., 2015). It is of great interest whether these cells are also functionally suppressive, as ST2+GATA3+ Tregs have been reported to exhibit a highly activated and strongly suppressive phenotype (Siede et al., 2016).

The consequences of Th2-derived amphiregulin in the lung are slightly more complicated. Since amphiregulin is crucial for timely elimination of *T. muris* infection, and since Th2 cells are known to produce amphiregulin, it is tempting to think that Th2-derived amphiregulin would promote worm clearance. However, my data demonstrates that despite increased 2W1S-specific CD4+ T cells and numbers of amphiregulin-producing CD3+ cells in the lung, mice immunized with 2W1S peptide are not able to clear *Hulk* infection any more quickly than unimmunized, primary-infected mice. Moreover, neither total lung amphiregulin levels nor numbers of CD3+ amphiregulin-producing cells in the lung correlated with protection in mice during secondary *Hulk* infection. While these data do not allow solid conclusions to be drawn regarding the necessity of CD4+ T cell-derived amphiregulin in *Hulk* clearance, they suggest that

CD4+ T cell-derived amphiregulin in the lung is not sufficient to enhance worm clearance. This may reflect a more general difference between the lung and the gut: indeed, the *T. muris* model in which amphiregulin was shown to be necessary for clearance is strictly enteric and does not infect the lung. This discrepancy opens the possibility for tissue-specific differences in the biological importance of amphiregulin during GI nematode infection.

Assuming 2W1S-specific Th2 cells do produce amphiregulin in the lung, this finding may implicate amphiregulin-producing Th2 cells in a broader range of functions than was previously appreciated – specifically in reducing tissue damage or coordinating tissue repair. Th2 cells have long been known to participate in normal wound healing and pathological fibrosis through production of cytokines like IL-4 and IL-13 that can alternatively activate macrophages or act directly on structural cells like myofibroblasts (Gieseck, Wilson, & Wynn, 2018). Data from the *N. brasiliensis* model also demonstrates that lung damage is exacerbated and repair delayed in SCID mice, suggesting a necessary role for lymphocytes in this process (Reece et al., 2006). However, amphiregulin-dependent Th2-mediated tissue repair mechanisms have not been thoroughly explored in the context of GI nematode infection. While ILC2s are also known to produce amphiregulin, reserving a pool of memory, antigen-specific CD4+ T cells primed to produce amphiregulin after a primary infection may further mitigate damage in the lung upon subsequent infections (Monticelli et al., 2011). Conversely, the prospect of having memory CD4+ T cells whose primary role is to protect or repair host tissue is interesting in the context of other type 2 pathologies like asthma, where these cells could either promote productive repair or drive pathological fibrosis (Morimoto et al., 2018).

As was discussed in Chapter 1.5, orchestrating wound repair in an antigen-specific manner in tissues could also help concentrate amphiregulin-producing Tregs

and/or Th2 cells at sites where the most damage has occurred. Whereas delivery of cytokines such as IFN- γ to structural cells infected with intracellular bacteria like *Salmonella* can lead to cell death, engagement of TCR by MHCII on structural cells in helminth infections could induce expression of wound reparative programs in these cells (Ingram et al., 2018). This process could occur either through reactivation of CD4+ T cells and TCR signaling-dependent cytokine production, or TCR-MHCII engagement could simply prolong CD4+ T cell dwell time in close proximity to cells producing signals like IL-33, which has been shown to induce both amphiregulin and IL-13 in a TCR signaling-independent manner (Arpaia et al., 2015; Minutti et al., 2017). Restricting CD4+ T cell amphiregulin production geographically could also prevent excessive EGFR signaling on and hyperplasia of alveolar epithelial cells, which is associated with emphysema or COPD-like pathologies (Fig 4.2) (Mercer, Lemaitre, Powell, & D'Armiento, 2006; Yokohori, Aoshiba, Nagai, & Respiratory Failure Research Group in, 2004).

However, the above scenario relies on the ability of structural cells like epithelial cells to effectively present nematode antigens for which amphiregulin-producing (or otherwise wound reparative) CD4+ T cells are specific. This question could be addressed in the *Hulk* model using an existing antibody for the 2W1S peptide:MHCII I:A^b complex, which would label any 2W1S-presenting cells in the lung during infection. Admittedly, given that 2W1S is a body wall antigen and not known to be soluble, I would not predict robust uptake of this antigen by non-migratory structural cells like epithelial cells that have low phagocytic capacity relative to other, professional APCs (e.g. macrophages, DCs) (Sharma, Feng, Britto, & Dela Cruz, 2020). However, as discussed in Chapter 1.3, new transgenic models in which antigens are expressed in different nematode compartments might allow for more in-depth interrogation of this question.

Finally, while I have emphasized the potential role of 2W1S-specific CD4+ T cells in wound healing and tissue repair, my studies have not explored the possibility that 2W1S-specific CD4+ T cells could play a role in antibody production as T follicular helper cells (Tfh). Indeed, I do observe an increase in serum GFP-specific antibody production in *Hulk*-infected mice relative to parental *S. ratti*-infected and naïve mice, suggesting that CD4+ T cells specific for the GFP-2W1S-FLAG fusion protein may be involved in GFP-specific B cell selection in germinal centers. Furthermore, given the small population of 2W1S-specific CD4+ T cells in the lung draining mediastinal lymph nodes, and given that IL-4-producing Tfh are known to predominate in the mediastinal lymph node as opposed to the lung during *N. brasiliensis* infection, it is logical to think those cells may be Tfh in the *Hulk* system as well (Fig 4.1)(H. E. Liang et al., 2011). Assessing expression of Tfh transcription factor Bcl-6 and surface markers CXCL5 and PD-1 on these cells could address this question (Crotty, 2014).

4.2 Helminth transgenesis as a means of exploring anti-helminth immunity

Hulk is the first transgenic GI nematode model to express a model CD4+ T cell epitope, and has proven useful for elucidating functions of antigen-specific CD4+ T cells during infection. Admittedly, however, this model could be improved upon to provide insights into CD4+ T cell antigen recognition at other infectious sites and at different time points during infection. As was discussed previously, new transgenic lines in which 2W1S is expressed under different tissue specific promoters would allow for a more comprehensive investigation into how antigen-specific CD4+ T cell phenotype and function changes with the localization and temporal restriction of the cognate antigen.

Alternatively, expressing different model epitopes under the *Ss-act-2* or other tissue-specific promoters presented in Chapter 1.3 would be beneficial in a few ways. First, while there is no TCR transgenic mouse that recognizes 2W1S, transgenic mice

have been made to recognize other epitopes like ovalbumin (e.g. OT-II, DO11.10 mice). Whereas the 2W1S system relies on endogenous T cell precursor frequencies and can result in relatively low numbers of antigen-specific cells for downstream analyses, congenically-labeled TCR transgenic T cells can be transferred at low or high numbers depending on the investigator's needs into wild-type recipients and recovered post-infection for study. Since the 2W1S-specific CD4+ T cell pool is diverse in its V β usage, obtaining CD4+ T cells from TCR Tg mice crossed onto a RAG-deficient background would also allow for more controlled studies of how a single TCR clone behaves following antigen recognition during helminth infection (Moon et al., 2007). Along the same lines, a second benefit to changing the model epitope would be to gain further insight into how T cell-intrinsic signals dictated by TCR specificity impact on antigen-specific CD4+ T cell function in this context. In other words, would CD4+ T cells activated in the same context behave differently if they recognize an antigen distinct from 2W1S? Third, while 2W1S is an MHCII I-A b -restricted epitope, using epitopes that are restricted to other MHCII molecules or haplotypes would allow for the study of antigen-specific CD4+ T cell function in different mouse strains (e.g. Balb/c mice) that are differentially resistant or susceptible to GI nematode infections (Else & Grencis, 1991; Else, Hultner, & Grencis, 1992; Prowse, Mitchell, Ey, & Jenkin, 1979).

While transgenesis in GI nematodes is an extremely powerful tool for interrogating questions of CD4+ T cell antigen specificity, this is hardly the extent of its utility. Early studies have already implemented CRISPR/Cas-9 mediated gene deletion to understand the role of specific genes in helminth motility, worm-induced host tissue damage and host immune activation during infection (Arunsan et al., 2019; Gang et al., 2017; Ittiprasert et al., 2019). As genetic manipulation of GI nematodes and other helminth taxa becomes more tractable, these studies could be expanded to identify

genes and pathways involved in host sensing and seeking, invasion into host tissues, migration through various organ systems, and host immune evasion or suppression. Particularly attractive candidates for mutagenesis studies would be genes with no clear homologs between parasitic nematodes and their free-living counterparts (or, in *Strongyloides spp.*, those not shared between free-living and parasitic life stages), as these may be involved in parasitism. Such studies could also help uncover ligands for host pattern recognition receptors, which have thus far remained elusive. These investigations would advance basic knowledge of nematode biology and host immunology as well as reveal new drug and vaccine targets for treating and preventing nematode infection.

4.3 Forming protective memory against parasitic helminths

Understanding how immunological memory of parasitic helminths forms is crucial for developing effective vaccines to prevent these infections. However, type 2 memory formation in the context of helminth infection remains poorly understood. In murine GI nematode models, partial immunity against secondary infections is commonly observed, as challenge infections are cleared much more rapidly than primary infections (Dawkins & Grove, 1981; Harvie et al., 2010; Urban, Katona, & Finkelman, 1991). On the other hand, human infections are chronic, and even individuals who receive curative drug treatment are susceptible to re-infection with the same parasite. Notably, with the exception of human hookworm infection, worm burdens tend to decline with age – suggesting that some level of immunity does develop over time (P. J. Hotez et al., 2008). Evidence from mouse models suggests that this immunity is dependent upon CD4+ T cells (Urban, Katona, & Finkelman, 1991). Yet evidence of complete, sterilizing immunity is difficult to find in humans, and our lack of understanding of how to facilitate even partial immunity in humans continues to thwart vaccine efforts.

Though I have not explored whether 2W1S-specific CD4+ T cells enter the memory pool post-*Hulk* infection, the *Hulk* system would potentially be a strong tool to study formation, longevity, and localization of GI nematode-specific CD4+ T cell memory responses. However, my work using 2W1S peptide immunization suggests that CD4+ T cell responses directed against this epitope alone are not sufficient to improve immune clearance upon subsequent *Hulk* infection. Of course, given data showing that these cells largely produce amphiregulin, and that a portion are Tregs, it could be that these cells simply do not participate in worm clearance and are instead more involved in wound repair and immunosuppression. Ongoing work seeks to understand whether immunization with 2W1S peptide can reduce tissue damage or promote repair by assessing lung histology in immunized and unimmunized mice post-infection. Another possibility is that immunization fails to protect from challenge infection because immunizing with 2W1S peptide and alum adjuvant does not exactly recapitulate the T cell priming environment of *Hulk* infection. This possibility could be addressed through adoptive transfer of 2W1S-specific CD4+ T cells from *Hulk*-infected mice into naïve recipients prior to *Hulk* infection.

It is also possible that the CD4+ T cell response against 2W1S contributes to clearance, but that CD4+ T cells specific for just one antigen are insufficient to independently drive clearance. As discussed earlier, this could be a result of the localization of 2W1S within Hulk, and does not rule out the possibility of protection by one or multiple endogenous helminth epitopes. Indeed, immunization of mice with *H. polygyrus* ES products is sufficient to confer immunity against challenge infection, and immunization of mice with one (Ss-IR molecule) or multiple *S. stercoralis* antigens leads to enhanced larval killing in the skin (Abraham et al., 2011; Herbert et al., 2002; Hewitson et al., 2011). While one of these studies showed protective IgG bound to

nervous and muscle tissue on fixed *S. stercoralis* larval cross-sections under electron microscopy, a later study suggested that protective antibodies bind the surface of live, intact *S. stercoralis* worms despite evidence of internal binding by electron microscopy studies (Abraham et al., 2011; Herbert et al., 2002). Moreover, antibodies from *H. polygyrus*-infected mice predominantly bind HES rather than antigens from whole somatic tissue extracts, again suggesting that secreted antigens like those in ES products or surface-bound antigens are more immunogenic than internal antigens like those in the muscle or body wall layer (Hewitson et al., 2011). Additionally, if sterilizing immunity is the goal, it is likely that these antigens would need to be expressed by the larval stages seen first by the host during infection. While immunity against molecules produced by more mature life stages in the lung and gut may suffice to kill or clear worms at those locations, it would likely not be able to prevent invasion of larvae into the skin or migration through the vasculature (Diemert, Bethony, & Hotez, 2008).

Sterilizing immunity is a common goal in clinical immunology and vaccinology, which makes sense in the context of bacterial or viral infections that can quickly replicate in hosts and cause overt pathology and mortality. This goal is also often discussed in terms of developing vaccines for GI nematodes and other helminths to prevent establishment of infections altogether. However, while GI nematode infections can cause morbidity over time, infections often remain asymptomatic for long periods and rarely cause death (P. J. Hotez et al., 2008; McSorley & Maizels, 2012). Though new worms can be acquired from the environment, GI nematodes (with the exception of *S. stercoralis*) cannot reproduce new infective stages to autoinfect the same host. On the other hand, strong type 2 responses – such as those associated with severe asthma, allergy, and anaphylaxis - can be life-threatening (P. J. Turner et al., 2017). From a teleological perspective, this may explain why humans rarely form natural sterilizing

immunity against natural GI nematode infection, and why vaccine development has been so challenging. Perhaps in addition to immunosuppressive mechanisms enforced by nematodes themselves, hosts mitigate their own type 2 responses to both existing and newly-acquired parasite infections to balance immunity with self-preservation. Limiting worm burden through type 2 effector mechanisms and repairing worm-induced tissue damage is necessary to maintain host tissue function, but maintaining high-affinity antigen-specific memory cells for one or more antigens – as is observed in allergic models with allergen-specific IgE – might actually cause more damage to hosts upon re-exposure to GI nematodes than infection itself (Gowthaman, Chen, & Eisenbarth, 2020). Indeed, vaccine trials in which endemic (and presumably helminth pre-exposed) populations have been vaccinated with immunogenic hookworm antigens have reported redness, rash and inflammation at the site of injection, consistent with an allergic response (Diemert, Bottazzi, Plieskatt, Hotez, & Bethony, 2018). Therefore, while some level of protective immunity to GI nematodes is likely advantageous to curb worm burdens and parasite-associated morbidity, strong sterilizing immunity may actually be deleterious. Developing systems to not only track but also manipulate the affinity of antigen-specific lymphocytes for target antigens could allow for exploration of how memory cells with different affinities for different helminth-derived antigens affect the balance of immunity and immunopathology.

4.4 Therapeutic implications

Vaccines against GI nematodes and other helminths would be greatly beneficial for global public health, but remain an elusive goal. While vaccines utilizing irradiated whole larvae, or even single recombinant larval epitopes, have shown some promise in veterinary and human clinical trials, concerns about the feasibility of safely irradiating sufficient larvae for mass production or about side effects such as allergic responses to

helminth antigens have halted their progression into mainstream use (Diemert et al., 2018). While the purpose of my study was not to develop a vaccination strategy against GI nematode infection, I believe it provides two important takeaways that may inform future vaccine development efforts. First, my finding that immunization against a body wall antigen does not confer protection supports the notion that antigens from this anatomical compartment are not suitable vaccine targets. While this immunization strategy may help dull tissue damage during larval migration, it does not appear to promote protective immunity to any appreciable degree – at least in combination with alum adjuvant, at the dose given and with the amount of time between immunization and infection. Further, though my studies do not rule out that immunization against a single epitope could be protective, the failure of a single epitope to provide protection from infection suggests that vaccines with multiple epitopes may be more effective than single epitope vaccines at preventing infections in other non-murine hosts.

Second, the finding that a proportion of 2W1S-specific CD4+ T cells express Foxp3 during infection raises the possibility that vaccine-elicited, helminth-specific CD4+ T cells could also become immunosuppressive in the context of infection. Indeed, there is already concern within the helminth vaccinology field about the potential for endogenous, pro-regulatory helminth epitopes that preferentially engage tTregs or intrinsically promote pTreg differentiation (Hewitson & Maizels, 2014). Further, helminth infections are known to reduce the efficacy of other, unrelated vaccines such as the BCG vaccine against tuberculosis (Chatterjee & Nutman, 2015). That CD4+ T cells specific for an antigen expressed by a GI nematode can themselves become Tregs during active infection further complicates vaccine design, as it suggests immunosuppressive mechanisms enforced by helminths or enacted by hosts could

thwart antigen-specific memory by directing it instead to regulatory or suppressor functions.

While the idea of helminth-specific Tregs may be detrimental for vaccine design, this concept may actually be beneficial in other contexts. Namely, in efforts to harness the immunomodulatory capacity of helminths to control autoimmune or autoinflammatory disorders. Helminths – and predominantly GI nematode species – have been shown to ameliorate diseases like multiple sclerosis (MS), rheumatoid arthritis (RA), asthma, allergy, and inflammatory bowel disease (IBD) in mouse models and in humans, either through active infection or via administration of ES products (Bohnacker, Troisi, de Los Reyes Jimenez, & Esser-von Bieren, 2020; Smallwood et al., 2017; White et al., 2020; Wolff, Broadhurst, & Loke, 2012). By treating patients with a combination of helminths/ES products and antigens targeted in these diseases, perhaps it would be possible to expand populations of Tregs that could specifically inhibit pathological immune responses against host tissues, commensals, or allergens, thereby promoting more long-term tolerance against these antigens.

Summary

To my knowledge, this study is the first of its kind to utilize a model antigen-expressing GI nematode to study antigen-specific CD4+ T cell responses. While these antigen-specific CD4+ T cells do not confer protection based on my work, they appear to be both Th2 cells and Tregs that produce amphiregulin. I hypothesize they may be involved in preventing tissue damage or promoting repair during infection in the lung, and it would be worth investigating in the future whether they play functional roles in repair processes or in supporting antibody production. This study constitutes an important proof of concept for how transgenic GI nematode systems can be used to further our understanding of antigen specific responses, CD4+ T cell biology and

memory, and also emphasizes how transgenic modification of these parasites could be used to explore other pertinent parasitological and immunological questions. Moreover, my findings have important implications for both helminth vaccine development as well as the development of novel, helminth-based treatments for inflammatory disorders and autoimmune diseases.

Figures

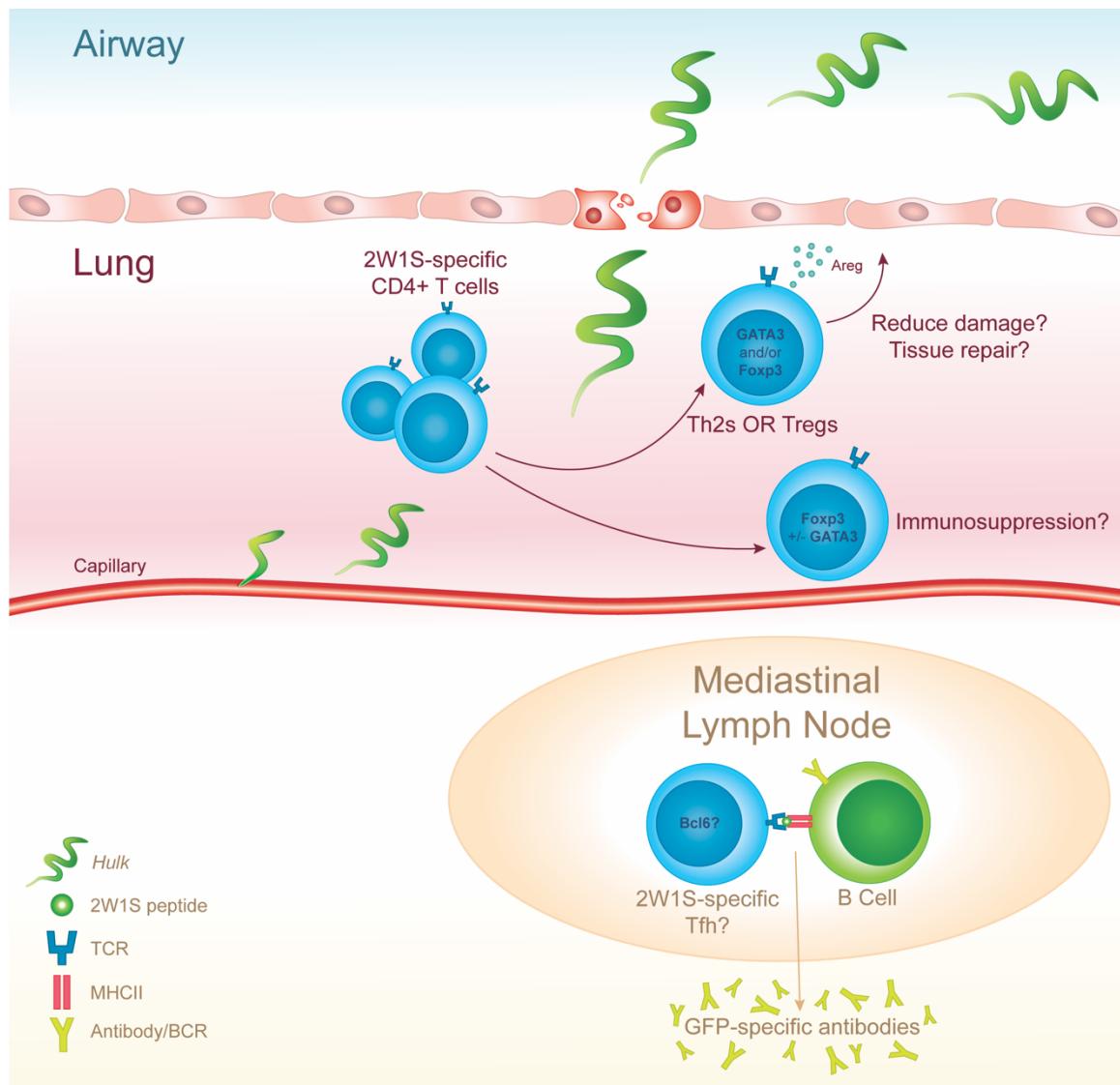


Figure 4.1. Model of 2W1S-specific CD4⁺ T cell localization and function during *Hulk* infection.

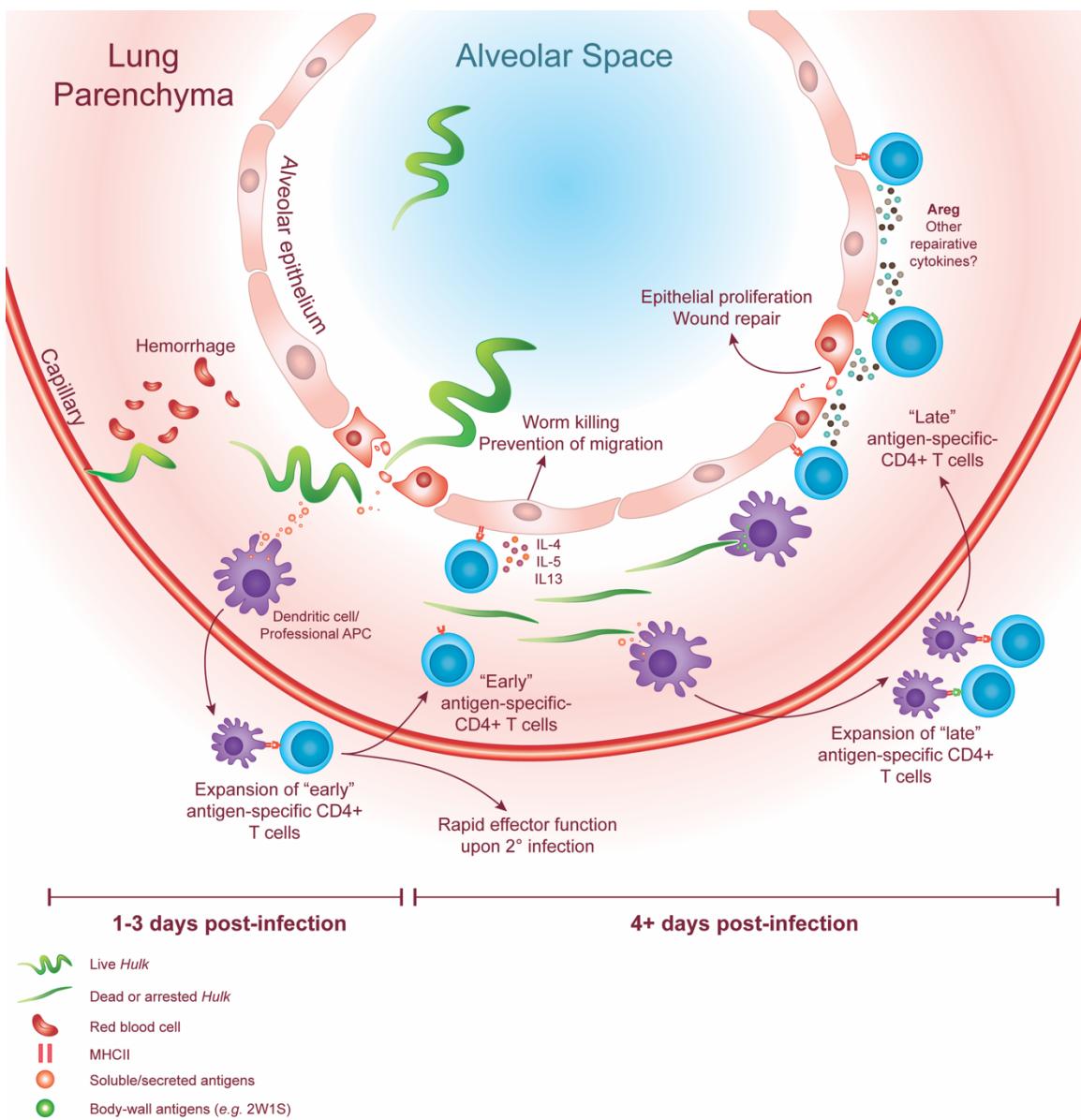


Figure 4. 2. Initiation and outcomes of diverse antigen-specific CD4+ T cell functions in the lung during *Hulk/S. ratti* infection.

CHAPTER 5: MATERIALS AND METHODS

This chapter has been modified slightly from methods published in *PLoS Pathogens*:

Douglas, B., Wei, Y., Li, X., Ferguson, A., Hung, L., Pastore, C., Kurtz, J.R, McLachlan, J.B., Nolan, T.J., Lok, J., & Herbert, D.R. (2021). Transgenic expression of a T cell epitope in *Strongyloides ratti* reveals that helminth-specific CD4+ T cells constitute both Th2 and Treg populations. *PLoS Pathog*, 17(7), e10097909.

Ethics statement

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania (Protocol No. 805911). Crl:WI (Wistar) rats were used for generation of the transgenic *Hulk* line. Crl:NIH-*Foxn1^{mu}* rats and Crl:MON(Tum) gerbils were used for maintenance of both parental and transgenic *S. ratti* strains, as these hosts maintain infections for up to 6 months. All rats and gerbils were purchased from Charles River. For experimental purposes, wild-type C57BL6 mice were bred in-house or purchased from Taconic. Prior to subcutaneous infections, mice were anesthetized using isoflurane in an induction chamber. Euthanasia was performed by exposing mice to carbon dioxide (CO₂) for 10 minutes, after which time euthanasia was confirmed by cervical dislocation. Animals were housed under specific-pathogen free barriers in an AAALAC-accredited vivarium at the University of Pennsylvania. All IACUC protocols and routine husbandry and medical care of animals at the University of Pennsylvania were conducted in strict accordance with the *Guide for the Care and Use of Laboratory Animals* of the US National Institutes of Health (National Research Council, 2011).

S. ratti strain generation, maintenance, and animal infection

The *Hulk* strain of *S. ratti* was derived using published methods (Shao et al., 2012). Briefly, the GFP-2W1S-FLAG transgene contained in plasmid vector pPV691 was integrated into the parental *S. ratti* genome using the *piggyBac* transposon system, where the transposase was provided in trans by non-integrated vector pPV402. pPV691 encodes the GFP-2W1S-FLAG fusion protein under the promoter for *Ss-act-2* and utilizes the *Ss-era-1* 3' UTR, a multi-purpose terminator for transgenes in *S. stercoralis* and *S. ratti* (Junio et al., 2008; X. Li et al., 2011). Transgenic progeny were selected based on GFP-expression and passaged serially through Wistar rats until the GFP+ frequency was roughly 90-95%, which was achieved after 6 passages. Parental (ED321) and *Hulk* strains of *S. ratti* were maintained in Crl:NIH-*Foxn1^{mu}* rats and gerbils and cultured as previously described (M. E. Viney, 1996). For infections, infective third-stage larvae (iL3) were isolated via the Baermann funnel technique from charcoal coprocultures grown at 22°C for 5 or more days. In cases where secondary infections were administered, mice were infected once with 1,000 live iL3 and allowed to rest for 3-6 weeks prior to challenge with 1,000 iL3 live iL3. Larvae were washed three times in phosphate-buffered saline (PBS) with 1% Penicillin-Streptomycin (P/S) to minimize carryover of fecal bacteria and enumerated under a light microscope. When indicated, iL3 were irradiated at a concentration of 16,500 iL3 per mL in PBS with 90 Gy using a Cs-137 irradiator (Reitz et al., 2017). Infective larvae were confirmed to be alive post-irradiation by motility under a microscope, and developmental arrest in-host was confirmed by absence of fecal eggs on days 6-9 post-infection. Mice were infected with indicated numbers of iL3 by subcutaneous injection of the flank skin. In the case of percutaneous infections, larvae were applied to the footpad by placing the hind paw in a 0.5 mL tube containing 100 µL of worms suspended in PBS for 40 minutes while mice were anesthetized with ketamine and xylazine.

2W-Salmonella culture and infection

Prior to infections, the 2W1S-expressing vaccine strain of *Salmonella* Typhimurium, LVS strain BRD509-2W1S (Δ aroA), was streaked onto LB agar plates supplemented with streptomycin and incubated at 37°C overnight. LB broth supplemented with streptomycin and kanamycin was then inoculated with a single colony and incubated at 37°C in a bacterial shaker for 16-18 hours. Mice were fasted overnight prior to infection, and dosed twice orally (5×10^9 CFU in 5% NaCHO₃) on days 0 and 2 and once intraperitoneally (1×10^5 CFU in PBS) on day 2 with 2W-*Salmonella*. All mice were euthanized 7 days post-initial oral infection. Dose estimates were confirmed by serially diluting the inoculum onto LB agar supplemented with streptomycin and quantifying 24 hours later. Infection was confirmed by plating spleen homogenates from infected mice on LB agar plates supplemented with streptomycin and counting colonies 24 hours later.

2W1S peptide administration

For *in vivo* peptide restimulations, 100 µg 2W1S peptide (EAWGALANWAVDSA, GenScript) was administered intratracheally using a nebulizing syringe 7 days following the third of 3 infections with irradiated *Hulk* or parental *S. ratti* iL3. Mice were euthanized 2 days post-peptide challenge. For 2W1S+ CD4+ T cell identification by flow cytometry and immunization, mice were immunized with 5 or 100 µg 2W1S peptide (respectively) and 1 mg alum (Invivogen) intraperitoneally. CD4+ T cell responses were measured 5 days post-immunization, and immunized mice were challenged with Hulk 3-6 weeks following immunization.

Tissue dissection and digestion

Bronchoalveolar lavage (BAL) was performed by flushing lungs with 0.5 mL PBS with 2 mM EDTA and 0.5% bovine serum albumin (BSA). Cells were isolated by centrifugation at 1,700 RPM for 5 minutes, and red blood cells were lysed in 1 mL ACK lysis buffer for 5

minutes at room temperature. BAL fluid (BALF) was removed after the first centrifugation for analysis by ELISA. Lungs were perfused with 10 mL cold PBS. For prefixation and cryosectioning, lungs were inflated with 0.75 mL 4% paraformaldehyde (PFA) and removed. For tissue digestion, lungs were removed and minced using dissection scissors prior to a 40 minute digest at 37°C in digestion buffer (0.4 g/L Dispase II, 0.15 g/L Liberase TM, and 0.014 g/L DNase I in serum-free DMEM). After 40 minutes, digests were extruded through a 16G needle 10 times and returned to incubate for an additional 15 minutes before being extruded again through an 18G needle 10 times. Cell suspensions were then strained over a 70 µm strainer, centrifuged at 1,700 RPM for 5 minutes at 4°C, and red blood cells were lysed in 2 mL ACK lysis buffer for 5 minutes at room temperature. Cells were centrifuged again at 1,700 RPM for 5 minutes at 4°C and resuspended in complete RPMI (cRPMI) (10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin (P/S)). Lymph nodes and Peyer's patches were dissected, homogenized over a 70 µm cell strainer (Fisher Scientific), centrifuged at 1,500 RPM for 5 minutes at 4°C and resuspended in cRPMI. Cells were enumerated using a Muse Cell Analyzer (EMD Millipore).

Flow Cytometry and Fluorescence-Associated Cell Sorting (FACS)

APC-conjugated MHCII:I-A^b EAWGALANWAVDSA (2W1S) tetramer was obtained from the NIH Tetramer Core. Tetramer-labeled 2W1S-specific CD4+ T cells were enriched and analyzed using the protocol previously described (Moon et al., 2009). Briefly, single cell suspensions were stained for 1 hour at room temperature at a final tetramer concentration of 16.7 nM in Fc block (0.2 mg/mL goat γ globulin, 5 µg/mL 2.4G2 in PBS) and enriched over a MACS LS column using magnetic-conjugated anti-APC beads (MACS Miltenyi). Following tetramer enrichment, cells were stained with LIVE/DEAD Aqua (Invitrogen) and the following surface markers: CD3 BV650, BV711 or Pe-Cy5 (17A2 or 145-2C11, BioLegend), CD4 APC-Cy7 (GK1.5, BioLegend), CD8 PE-

TexasRed (5H10, Thermo Fisher), CD44 AF700 (IM7, BioLegend), CD11a Pe-Cy7 (2D7, BD Biosciences) CD62L BV421 (MEL-14, BioLegend), B220 PerCP-Cy5.5, BV711, or FITC (RA3-6B2, BioLegend, ThermoFisher), CD11b PerCP-Cy5.5, BV711, or FITC (M1/70, BioLegend), and CD11c PerCP-Cy5.5, BV711 or FITC (N418, BioLegend, Thermo Fisher) diluted in FACS buffer (2% BSA, 0.1% NaNa₃) for 20-30 minutes. After surface stain, cells were washed with permeabilization buffer and fixed for 30 minutes using the Foxp3/Transcription Factor Staining Buffer set (ThermoFisher). After fixation, cells were washed with permeabilization buffer and stained overnight in permeabilization buffer with the following markers: Ki67 eF450 (SolA15), Foxp3 PE or Pe-Cy5.5 (FJK-16s, ThermoFisher), and GATA3 AF488 (TWAJ, ThermoFisher). All staining steps after tetramer enrichment were done at 4°C. All flow cytometry data were acquired on an LSR Fortessa (BD) and analyzed in FlowJo 10.6.1 (TreeStar).

For sorting, tetramer-labeled cells were enriched as for flow cytometry and stained as described above in azide-free sorting buffer (2% BSA in PBS). Roughly 1,000 live, singlet CD3+CD4+CD8-CD62L-2W1S+ events from each biological replicate were collected into lysis buffer (SMART-seq v4 Ultra Low-Input RNA Kit, Takara Bio) in 1.5 mL Eppendorf tubes. 2W1S+CD4+ T cells were sorted from pooled lungs and mLN of *Hulk*-infected mice, and from pooled mLN and Peyer's patches of 2W-*Salmonella*-infected mice. Cells were frozen until ready to proceed with cDNA synthesis. Sorting was performed on an Aria II (BD).

Tissue Pre-Fixation and Cryosectioning

Following perfusion and inflation with 4% PFA, lungs were removed with the trachea attached and incubated in 4% PFA for 1 hour at room temperature. Lungs were then washed once with PBS and incubated in PBS for up to 4 hours at room temperature prior to being incubated overnight at 4°C in 30% sucrose with 0.02% sodium azide. The

next day, lungs were placed in 50% optimal cutting temperature (OCT; Scigen) medium with 15% sucrose for 2 hours at room temperature and then embedded in 100% OCT on dry ice.

OCT cryoblocks were sectioned at a thickness of 7 μ m on a Leica cryostat. Sections were placed onto poly-L-lysine-coated, positively-charged slides (Fisher Scientific) and kept frozen until immunostaining.

Immunofluorescence

Slides were thawed at room temperature for 20 minutes and then washed 3 times in 1X tris-buffered saline (TBS) for 5 minutes each wash. Slides were then blocked and permeabilized for 1 hour at room temperature in block/perm buffer (3% BSA, 3% normal horse serum (Jackson Immunoresearch), 0.3% mouse-on-mouse blocking reagent (R&D Systems), and 0.3% TritonX-100 (Millipore-Sigma) in 1X TBS). Primary antibodies were incubated overnight at 4°C in block/perm buffer, and include: anti-CD3 (Clone 145-2C11, eBioscience, 1:250) and anti-Amphiregulin (polyclonal, Bioss Antibodies, 1:200). The next day, slides were washed 3 times in 1X TBS for 5 minutes per wash and then incubated with the appropriate secondary antibodies for 2 hours in block/perm buffer. These antibodies include Cy-3 anti-Armenian hamster, Cy-3 anti-rat, and AF488 anti-rabbit (Jackson Immunoresearch, 1:1000). Slides were then washed as before and stained for 30 minutes with DAPI (1 μ g/mL, ThermoFisher). After DAPI staining, slides were washed again as before and mounted with ProLong Gold antifade reagent (Invitrogen) and a covered with coverslips (Fisher Scientific). All slides were imaged on a DMi8 microscope (Leica), and analyzed using LAS X software (Leica) and ImageJ (NIH).

RNA Sequencing

cDNA was synthesized directly from cell lysates using the SMART-seq v4 Ultra Low-Input RNA Kit (Takara Bio). Libraries were prepared using NexteraXT library prep

(Illumina) kits. The samples were loaded onto a platform for single read 75 cycles at the depth of 33 million reads per sample. Sequencing reads were trimmed for quality (phred score < 33) and to remove adapter sequences using the Trimmomatic software (Bolger, Lohse, & Usadel, 2014). High quality reads were aligned to the GRCm38.p6/mm10 reference genome using the STAR aligner (Dobin et al., 2013), and quantified to genes with HTSeq-count (Anders, Pyl, & Huber, 2015). Differentially expressed genes were obtained using the DESeq2 tool in the R statistical software (Love, Huber, & Anders, 2014) and gene set enrichment analysis was assessed using the GSEA R tool (Mootha et al., 2003; Subramanian et al., 2005).

RNA Isolation, cDNA Synthesis and Quantitative Real-Time (qRT)-PCR

RNA was isolated from pelleted MACS-sorted CD4+ T cells using the NucleoSpin RNA Plus kit according to the manufacturer's instructions (Macherey-Nagel). cDNA was prepared by combining RNA, random primers, and dNTPs and heating for 5 minutes at 65°C for 5 minutes. Samples were then chilled briefly, mixed with 5x RT buffer (Invitrogen) and incubated at 25°C for 2 minutes. Maxima H Minus reverse transcriptase (Invitrogen) was then added at a 1:1 ratio with molecular-biology grade water, and cDNA was synthesized by incubating at 25°C for 10 minutes, 50°C for 50 minutes, and terminating at 85°C for 15 minutes. qRT-PCR was run and analyzed on a CFX96 platform (BioRad). Primers: *Gapdh* FWD 5' – TGTGTCCGTCGTGGATCTGA – 3', REV 5' – CCTGCTTCACCACCTTCTTGA – 3', *Rorc* (ROR γ t) FWD 5' – AGGAGCAATGGA AGTCGTCC – 3', REV 5' – CCGTGTAGAGGGCAATCTCA – 3', *Tbx21* (Tbet) FWD 5' – CCAAGTTCAACC AGCACCAG – 3', REV 5' - GCCTTCTGCCTTCCACACT – 3'.

CD4+ T cell culture

For polyclonal CD4+ T cell stimulation, single cell suspensions from pooled infection site-draining secondary lymphoid organs (mediastinal, cervical, inguinal, and

mesenteric) or lungs were sorted using the L3T4 Positive CD4+ T Cell Selection kit and LS Columns from MACS Miltenyi according to the manufacturer's instructions. After sorting, cells were washed once in MACS buffer, counted using a Muse Cell Analyzer (Millipore Sigma), and plated at a density of 1×10^6 cells per well of a 96-well round-bottom plate. Plates were either uncoated or coated with 1 $\mu\text{g}/\text{mL}$ each of anti-CD3 (145-2C11, BioLegend) and anti-CD28 (37.51, BioLegend). Cells were cultured in cRPMI for 72 hours at 37°C at 5% CO₂.

For whole lung cell stimulation with 2W1S peptide or anti-CD3, single cell suspensions from lungs were prepared and plated at a density of 2×10^5 cells per well in a 96 well round-bottom plate. For anti-CD3 stimulation, plates were pre-coated with 1 $\mu\text{g}/\text{mL}$ anti-CD3 (145-2C11, BioLegend) and cells were resuspended in CD4+ T cell media (cRPMI + 1 mM Sodium Pyruvate, 1 mM L-Glutamine, 50 μM MEM non-essential amino acids (NEAA), 5 mM HEPES, and 50 μM β -mercaptoethanol, 10 ng/mL mouse recombinant IL-2). For peptide stimulation, cells were resuspended in CD4+ T cell media containing 100 $\mu\text{g}/\text{mL}$ 2W1S peptide and 1 $\mu\text{g}/\text{mL}$ anti-CD28 (37.51, BioLegend).

Enzyme-Linked Immunosorbance Assay (ELISA)

Supernatants from cultured CD4+ T cells were harvested after 48-72 hours of culture, as described above. Analytes were probed for using the following kits: IL-4 (R&D, Invitrogen), IL-5 (Invitrogen), IL-10 (Invitrogen), IL-13 (Invitrogen), IFN- γ (Invitrogen), and amphiregulin (R&D).

For serum immunoglobulin ELISAs, serum was isolated from naive mice or infected mice at the indicated timepoints and diluted 1:1000 in PBS. Plates were coated with 1 $\mu\text{g}/\text{mL}$ of the appropriate anti-Ig antibody (Bethyl Laboratories, Inc.), and immunoglobulins were detected using 1 $\mu\text{g}/\text{mL}$ of horseradish peroxidase (HRP)-conjugated anti-Ig antibody (Bethyl Laboratories, Inc.). For anti-GFP ELISAs, serum was isolated from naive mice or

infected mice 14 days post-infection and diluted as indicated in PBS. Plates were coated with 10 ug/mL recombinant GFP (Abcam), and GFP-specific IgM and IgG were detected using 1 ug/mL of HRP-conjugated anti-IgM/IgG antibody (Jackson ImmunoResearch).

Western blotting

Hulk or parental *S. ratti* iL3, L4 or adult parasites were homogenized using a Dounce shaved glass homogenizer on ice in 2x RIPA buffer (Millipore-Sigma) with 1x cOmplete Mini Protease Inhibitor Cocktail (Roche). Debris was removed via centrifugation, and supernatants containing protein were quantified using a BCA assay (Invitrogen). Protein was diluted with PBS to obtain the amount indicated in Fig 2, and mixed with 4x Laemmli sample buffer (Bio-Rad) with 10% β-mercaptoethanol before being boiled for 5 minutes. Samples were then loaded onto NuPAGE 4-12% bis-tris gels (Thermo Fisher) and run in 1x NuPAGE MOPS SDS running buffer (Invitrogen) at 110V for 2 hours until the dye front reached the bottom of the gel. Protein was then transferred to a nitrocellulose membrane at 211 mAmps for 5 hours at 4°C using NuPAGE transfer buffer (Invitrogen). Membranes were incubated in: 5% BSA in PBS + 0.1% Tween20 (Fisher Scientific) (PBS-T) for 1 hour at room temperature (RT) to block; 5% BSA in PBS-T with primary antibody for 2 hours (RT) or overnight (4°C); and in 5% BSA in PBS-T with secondary antibody for 1-2 hours at RT. Membranes were washed 3x with PBS-T following primary and secondary antibody incubations. The following antibodies were used: mouse anti-FLAG (Clone M2, Millipore Sigma, 1:500), rabbit anti-GFP (Torrey Pines Biolabs, 1:1000), mouse anti-β-actin (Clone C4, Santa Cruz Biotechnology, 1:2000), rabbit anti-GAPDH (Bethyl Laboratories, Inc., 1:1000), HRP-linked horse anti-mouse IgG (Cell Signaling Technology, 1:2000), and HRP-linked goat anti-rabbit IgG (Cell Signaling Technology, 1:2000). Membranes were developed using the SuperSignal West Femto

Maximum Sensitivity Substrate (ThermoFisher) and exposed on a Bio-Rad ChemiDoc XRS System.

S. ratti fecal egg enumeration

Fecal pellets (3-7) were collected from infected mice on the days indicated over a 10-20 minute period. Feces were weighed and then incubated for 40-60 minutes in 750 µL warm PBS at 37°C before being homogenized using a wooden stick and added to 10 mL saturated NaCl solution. Once fecal slurries were added, salt solutions were capped, shaken, and incubated at room temperature for 20 minutes. Eggs were counted by pipetting 650 µL from the meniscus of the salt float onto a McMaster slide and counting using a light microscope. Eggs per gram of feces were calculated using the following equation:

$$\text{EPG} = \frac{1}{\text{Fecal Weight}} \times \left(\frac{750}{150} \right) \times \text{No. Eggs Counted}$$

Fourth-stage larval (L4) enumeration

Infected mice were euthanized 2 days post-infection with parental or *Hulk S. ratti*, and lungs were removed without perfusion. Lungs were minced in 7 mL PBS in 60 x 15 mm petri dishes, and incubated at 37°C for 2 hours. Following incubation, L4 were counted using a stereoscope.

Adult S. ratti enumeration

At the indicated days post-infection, the entire length of the small intestine was removed from infected mice, cut longitudinally, and placed over a wire-mesh Baerman apparatus in a 250 mL beaker with enough PBS to cover the tissue. After a brief (5-10 second) agitation, tissues were incubated at 37°C for several hours to allow adult worms to migrate out of the epithelium. Adult worms that had settled to the bottom following this incubation were then counted using a stereoscope.

Statistics

Statistical analyses were performed using Mann-Whitney tests or Student's T-test with Welch's correction in Prism7 (GraphPad). Statistical probabilities where $p < 0.05$ were considered significant: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

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