ANALYSIS OF IMMUNE INTERACTIONS, HISTOPATHOLOGY, AND VIRAL LOAD DURING *SCHISTOSOMA MANSONI* AND INFLUENZA A COINFECTION

by

HILLARY ROSE DANZ

(Under the Direction of Donald A. Harn)

ABSTRACT

Approximately three billion people in the world are infected with helminth parasites. *Schistosoma mansoni* is a blood fluke that accounts for over 85 million infections across sub-Saharan Africa, Middle East, and parts of Brazil. *S. mansoni* is often found in areas with high rates of viral infections such as dengue, rotavirus, HCV, and HIV, and this co-endimicity greatly increases the risk of coinfection. Chronic *S. mansoni* infection biases the immune system to a CD4+ Th2 and anti-inflammatory phenotype. In contrast, a robust pro-inflammatory response is needed to effectively resolve most viral infections. Therefore, it is important to understand how a Th2/anti-inflammatory biased immune system influences a secondary viral infection requiring a pro-inflammatory response. Several studies have evaluated helminth, influenza coinfections, with varied results dependent on the helminth species. We hypothesize that this variation is related to the ability of the helminth to induce localized immune biasing, in particular in the lung, during subsequent infection with influenza. During chronic *S.*

mansoni infection, schistosome eggs travel to the pulmonary vasculature, become embedded in pulmonary tissue, and secrete products that induce localized immune biasing. Here we show that mice chronically infected with *S. mansoni* had significantly reduced morbidity and mortality to influenza infection. Coinfected mice also had significantly reduced pro-inflammatory responses from influenza-specific CD8+ T cells in the lung milieu. Additionally, histopathology showed lungs of *S. mansoni* infected mice had smaller lesions of alveolitis and pneumonitis as a result of influenza infection. Together, these data indicate chronic *S. mansoni* infection is beneficial for influenza infection due to decreased pro-inflammatory responses that limit cellular infiltration to the lungs resulting in decreased pneumonitis. Additionally, this study adds to the literature suggesting a relationship between the location(s) of localized helminth biasing and location of viral infections.

INDEX WORDS: *Schistosoma mansoni*; Influenza A; coinfection; lung granuloma; alternative activation; macrophage; Relmα; immunohistochemistry

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DEDICATION

This work is dedicated to my mother, Marilyn. You have been an ear to listen, a shoulder to cry on, and a voice to cheer me on. You have given me strength when I was weak and have always been my biggest source of support and encouragement. Without you I would not have been able to achieve the successes (and failures) that have made me who I am.

Thank you for everything.

This is also dedicated to my brother, Brian. You have been my biggest fan since the day I was born. I have always looked up to you and I always will. I couldn't have wished for or imagined up a better brother.

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CHAPTER 1

INTRODUCTION

Prevalence of parasitic helminth infection is high in many developing nations. Schistosomes are trematode helminth parasites that are long-lived, infecting approximately 207 million people in 2010, according to the WHO [1]. *Schistosoma mansoni* is one of three species of schistosomes responsible for serious disease burden in humans [2]. Adult *S. mansoni* worms reside in the mesenteric vasculature of humans, where female worms produce from 200-300 eggs per day [3]. Eggs laid by female worms have two potential fates; one is to pass through the gut wall and enter the environment in feces, the second is to get trapped in tissues, primarily the liver, where the host immune response forms granulomas around the eggs, leading to fibrosis and hepatomegaly [4, 5]. Schistosome eggs also damage the intestines and lead to enlargement of the spleen, all of which contributes to distention of the abdomen, which may be accompanied by ascites [6, 7]. Finally, eggs can travel to the lungs where tissue damage and fibrosis result in pulmonary hypertension [8, 9].

During the initial stages of the schistosome life cycle in mammalian hosts, a strong CD4+ Th1-type pro-inflammatory response is dominant [6]. However, subsequent to egg-laying and deposition in tissues, the response begins to shift towards a CD4+ Th2 immune bias [6, 10-12]. In addition to CD4+ Th2 cells, schistosome infection is often associated with an increase in eosinophils and anti-inflammatory, alternatively activated (M2) macrophages. Together these different cell types, along with additional cells, form

dense granulomas around schistosome eggs deposited in tissues [13-16]. As the infection progresses to chronic, elevated levels of FoxP3+ Tregs are observed [6]. Chronically infected individuals generally have high levels of IgE and IgG1 antibodies as well as IL-4, IL-5, IL-10 and IL-13 [10, 11, 17, 18]. Induction of this Th2/anti-inflammatory immune state is largely in response to schistosome egg proteins and glycoproteins including the Lewis-X moiety of LNFPIII and Omega-1 [11, 14, 19-21].

The immune biasing just described is not specific to schistosomes among helminth parasites. Similar Th2 and anti-inflammatory biasing of the host immune response has been observed in helminth infected individuals in general. This then poses multiple questions regarding how helminth infected individuals respond to various coinfections. This is an important concept, as high prevalence rates of helminth infection are widespread in Sub-Saharan Africa, Southeast Asia, and South America [1]. These same geographic regions also have a high prevalence of HIV, malaria, measles, tuberculosis, and many other infectious diseases [22-25]. Comorbidity in these areas is a common issue and while the immunomodulatory effects of helminth infections are well known, how this altered immune state affects the outcome of infection with other pathogens is only just now being investigated in detail, at both the patient and experimental animal levels.

A common comorbidity during helminth infection is influenza infection. Annually, influenza infects the upper respiratory tract of 5-15% of the world's population, resulting in 3-5 million severe cases and up to 500,000 deaths [26]. Influenza induces a strong pro-inflammatory response from CD4+ Th1 and CD8+ T cells, which produce high levels of IFNγ [27-32]. *S.mansoni* is an ideal helminth parasite to

investigate the influence of helminth infection on infection with influenza not only because *S. mansoni* infection induces a systemic biasing of the host immune response, but in addition, the parasite enters the lungs at two different times. Initially, three to five day old schistosomula enter the lungs, where they remain and in essence "molt" into the lung worm stage over a four to six day period. Secondly, schistosome eggs enter the lungs of hosts during chronic infection. Therefore, it is likely that the immunobiology of host lung tissues are significantly influenced by exposure to schistosome infection/antigens. This is why we suggest that schistosome infection represents a good model to investigate how helminth infection may influence influenza infection, and this is the focus of my dissertation. Here I present my evaluation on the effects a schistosome-induced Th2/anti-inflammatory biasing has on pro-inflammatory immune responses to influenza infection.

CHAPTER 2

LITERATURE REVIEW

***Schistosoma mansoni*- Background**

Schistosome Biology

Helminth (worm) parasites can be found in nearly one third of the human population [33]. Taxonomic classification divides helminths into four phyla: Nematoda (nematodes), Platyhelminths (cestodes and trematodes), Annelida (ectoparasites), and Acanthocephala (spiny-headed worms) [34, 35]. The majority of worldwide human helminth infections are caused by soil-transmitted nematodes such as *Ascaris lumbricoides*, *Trichuris trichiura*, and *Necator americanus* [36, 37]. Approximately 10% of human helminth infections are caused by blood flukes of the genus *Schistosoma* [1, 38]. *Schistosomes* belong to the class Trematoda from the diverse phylum of platyhelminths (Fig. 2.1).

Schistosomes are unique among the platyhelminthes as the only dioecious species in a sea of hermaphrodites [39, 40]. Schistosomes have unsegmented bodies that are bilaterally symmetrical and comparatively small in size, with adult parasites being ~1-2 cm in length [41]. Male and female worms pair in the liver, then migrate to and permanently reside in the veins of the mesentery or bladder, depending on species, where they latch onto the vessel wall using a ventral sucker [42]. They position themselves to face the flow of circulation in order to reduce energy consumption while feeding on

- Kingdom Animalia
- Phylum Platyhelminthes
- Class Trematoda
- Sub-class Digenia
- Order Strigeidida
- Family Schistosomatidae
- Genus *Schistosoma*
- Species *mansoni*

FIG 2.1 Taxonomic classification of *Schistosoma mansoni*

blood using their oral suckers [43]. Females require substantially more blood to sustain egg production, consuming over 300,000 red blood cells (RBCs) per hour, equivalent to 880nl of whole blood per day, while males consume approximately 40,000 RBCs/hour [44].

RBCs are lysed in the esophageal glands with lysed RBC components being further digested by numerous proteases and peptidases within the elongated gut of adult worm parasites [45-47]. One byproduct of RBCs is hemozoin, an inert crystal formed of hydrogen-bonded heme dimers from digested hemoglobin [48, 49]. One theory on restriction of schistosome species to certain hosts suggests that their ability to digest hemes of different mammalian origin as the restriction factor that limits their ability to maintain an infection [50]. Schistosomes possess a closed gut, thus they lack an anal canal and therefore must regurgitate digested waste products [46]. Though this process is not well understood, it appears to be a result of anterior gut activity and relaxation of the

esophageal wall rather than reverse peristalsis [47]. In addition to hemozoin, the vomitus also contains immunoregulatory components, for example, circulating anodic antigen (CAA) and circulating cathodic antigen (CCA), both of which have linear repeats of the Lewis X trisaccharide [51]. These antigens are only found circulating in the host during an active schistosome infection as they originate from the gut mucosa of adult worms [52]. This makes these antigens ideal for immunodiagnostic assays targeting the presence of adult worms [52, 53].

In addition to nutrient uptake via the gut, schistosomes acquire additional nutrients by transportation or diffusion across the tegument. The tegument of schistosomes is an atypical membrane structure, consisting of a double lipid bilayer which surrounds/covers the parasites as a syncytium, without individual cell bodies; there is also a single lipid bilayer basal membrane [54, 55]. Male worms transport more glucose through the tegument than female worms. One study suggests that male worms obtain 3872-fold more glucose via tegumental transport than by oral ingestion [56]. Glucose transport across the tegument is facilitated by two proteins, schistosome glucose transport protein 1 and 4 (SGTP1 and SGTP4) that are located in different areas of the tegument [57]. SGTP4 is localized in the apical membrane where it is exposed to serum glucose that it passively transports into the tegument [58]. SGTP1 is localized in the basal membrane where it can transport the glucose brought in my SGTP4 from the tegument into the interstitial fluids of the worm, where it can be metabolized [55].

The tegument is the primary layer of direct host-parasite interaction and also functions as the first line of defense to protect schistosomes against the host immune system. This is important as schistosomes reside in the host blood vascular system and

are constantly exposed to host immune cells and immune mediators. Interestingly, host antigens, including MHC, have been detected on the tegument [59-61]. The presence of host antigens on the schistosome tegument is considered a means to "mask" parasite antigen detection by the host. The classic study demonstrating that adult worms have host antigens on them was the "mouse worms" into monkeys experiment performed by Smithers *et al*. [62]. Smithers and collaborators demonstrated that transplant of worms from mice into monkeys resulted in a drop in worm fecundity, and these transplanted worms appeared unhealthy. They followed this initial experiment by next immunizing monkeys against mouse red blood cells, then repeating the transplant of mouse worms into mouse RBC immunized monkeys, compared to monkeys receiving a transplant of monkey worms. They found that monkeys immunized with mouse RBCs killed the transplanted mouse worms, whereas transplanted monkey were not attacked and were normal [62]. Lastly, they further demonstrated acquisition of host antigens by maintaining transplanted mouse worms monkeys for a period of time sufficient for them to lose mouse antigens and acquire monkey antigens. When these worms were collected from monkeys, then transplanted again into naïve monkeys, the worms did not exhibit reduced fecundity and appeared normal, suggesting that they had acquired monkey antigens and were not attacked by the immune system. Taken together, Smithers and team demonstrated that adult worms acquire antigens from the host, and that these host antigens remain in the tegument for a finite period of time. One method that adult schistosomes use to acquire host antigens is by lysis of host cells on the tegument via a tegument expressed detergent-like membrane lipid [63]. The lysis of host cells on the worm surface allows for rapid incorporation of host cellular components into the

tegument bilayer. In addition, schistosomes can replace their tegument rapidly, shedding not only their membrane, but any immune activating antigens attached to it [64, 65]. These mechanisms allow schistosomes to adapt to hostile host immune environments.

Parasite Life Cycle

S. mansoni has a complex life cycle requiring a snail intermediate host and a definitive mammalian host (Fig. 2.2). The end result of the asexual development in the snail intermediate host is production of cercariae. Cercariae are the aquatic stage of the parasite that are able to infect mammalian hosts by direct penetration through the skin. Immediately upon penetration, the cercarial glycocalyx is shed along with the cercarial tail. The parasite undergoes membrane transformation to adapt to a saline environment, forming the double lipid syncytial bilayer. The parasite is now called a schistosomula. The schistosomula migrates to find a venule to penetrate. The venous blood flow carries schistosomula to the lungs, where they elongate and develop a primitive gut over a four to six day period. This stage is called the lung worm, and they exit the lungs between five and nine days post-infection. From the lungs, larval worms migrate to the liver where they grow and mature to male or female worms, which begin pairing from four to five weeks post-infection coincident with migrating to the mesenteric plexus, where they remain *in copula*, with the female worm producing eggs [66]. One pair of adult *S. mansoni* worms produces approximately 350 eggs per day. The eggs are considered the major inducers of CD4+ Th2-type immune bias observed in schistosome infection [12, 67, 68]. Approximately one third of schistosome eggs successfully traverse the gut wall and end up excreted in feces. The remaining eggs either get trapped in the intestinal walls

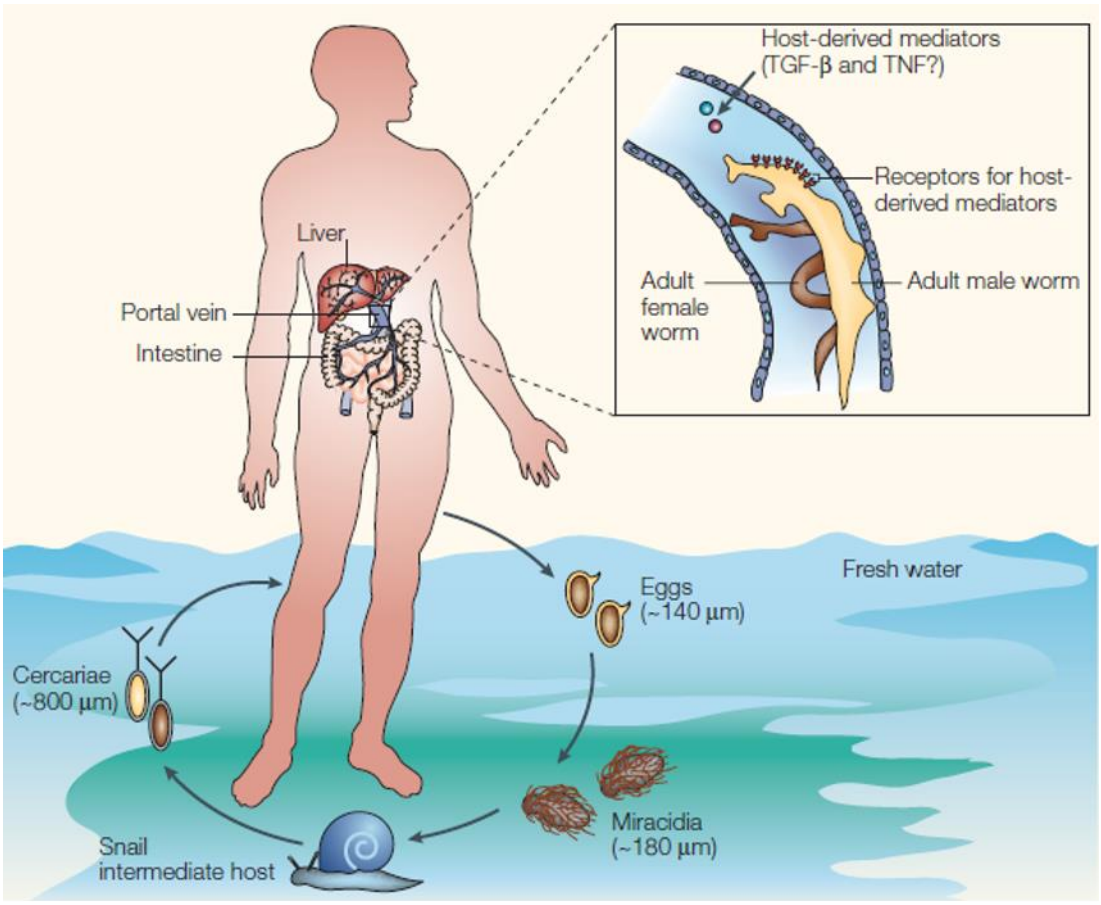

FIG 2.2 *Schistosoma mansoni* life cycle. Reprinted by permission from Nature Publishing Group: *Nature Reviews Immunology* (**5**, 420-426), Copyright (2005)

or enter venous blood flow and travel to the liver and lungs [69]. The exact mechanism behind egg transport from veins to intestinal lumen has yet to be deciphered. Older studies demonstrated that host immune cells, such as eosinophils and macrophages, form small granulomas that chaperone eggs across the gut wall and a more recent study has shown Peyer's patches are involved in this process [70-72]. Further, host CD4+ T cells are required for egg passage through the gut wall and into the lumen of the gut [71, 73].

Schistosome eggs passing into the environment in feces, contain the ciliated miracidium that will hatch upon entry into fresh water [74, 75]. Alterations in

environmental osmolarity trigger the ciliated miracidium to spin until the egg shell splits creating a vent that allows the miracidium to escape into the water, in search of an intermediate snail host [76]. *S. mansoni* miracidia can only infect aquatic snails of the *Biomphalaria* genus [77].

Miracidia penetrate through the snail body where they transform into a mother sporocyst, initiating asexual reproduction in the snail [78]. The mother sporocyst gives rise to daughter sporocysts and the daughter sporocysts yield the infectious cercariae stage, all of this via asexual reproduction, wherein a single miracidium can result in thousands of daughter sporocysts and tens of thousands of cercariae [5, 39]. From the time a miracidia infects a snail till the first cercariae emerge from the snails takes four to six weeks. Cercarial emergence from infected snails is photoperiodic, with cercariae emerging from snails in response to sunlight [5, 76]. Cercariae do not feed or obtain nutrients and therefore have a lifespan of less than 24 hours [79]. Cercariae move through water by way of their bifurcated tail in an ATP driven process. They swim to the water surface and then fall down until they come into contact with a vertebrate animal. If they do not come into contact with a vertebrate, they eventually run out of energy and die [76]. When a cercaria makes contact with human skin, it will first glide across to find an easy site to gain entry, such as a hair follicle, then utilize lipids on the skin to induce penetration [80, 81].

Skin penetration by cercariae is assisted by pre- and post-acetabular gland secretions including elastase and cathepsins B and L [80, 82]. Penetration induces loss of the cercarial tail and shedding of the glycocalyx, accompanied by manufacture of the double-lipid bilayer syncytial membrane that covers the entire parasite, which is now

called a schistosomula [76]. Schistosomula reside in the dermal skin layer for up to 3 days adjusting, maturing, and finding a blood vessel [2, 43, 83]. After gaining access to a blood vessel, schistosomula pass through the right side of the heart and into small lung capillaries where they often get stuck, with one study showing that up to 70% never make it back into circulation [84].

After several days schistosomula exit the lungs, travel through the left ventricle, and get pumped out into general circulation, eventually arriving in the hepatic portal system [85, 86]. Schistosomula reside in the portal system/liver for four to five weeks maturing into adult male and female schistosomes, which then pair [85, 87]. Interestingly, male schistosomes can fully mature and survive without pairing with a female. In contrast, female schistosomes do not fully mature, nor can they survive unless paired with a male worm [40, 88]. Paired worms migrate to the mesenteric vasculature and begin producing eggs by five to six weeks post-infection [68].

Human Disease

There are nineteen species of schistosomes, with five able to infect humans [42]. Three of the five species of schistosomes that infect humans, *S. haemotobium, S. japonicum,* and *S. mansoni,* are responsible for the bulk of disease [66]. Among these three species, *S. mansoni* accounts for 85 million cases and is endemic in sub-Saharan Africa, the Middle East, and South America [66, 89]. Schistosome infections can be long-lived, some reports state 30 yrs or more. Regardless of long-lived or repeated re-infections, patients often are chronically infected with schistosomes and chronic, long-lived infections can result in debilitating disease with hepatosplenomegaly [76, 90, 91].

Complications of schistosome infection range from failure to thrive in young children to severe portal hypertension and death in adults [92].

Schistosome infection affects multiple organ systems due to parasite eggs causing damage to blood vessels, the gut wall, and the lumen of the gut resulting in scarring and polyp formation as the eggs traverse the gut wall [43]. These events coincide with an increase in translocation of intestinal bacteria across the gut wall and into the bloodstream [93-96].

For eggs caught up in the mesenteric venous flow towards the portal vein, the liver is the first major organ, other than the gut wall, where schistosome eggs will become trapped in host tissues [43]. A patient harboring only six adult worm pairs will be exposed to 1,000 to 2,000 newly produced eggs each day. Due to the host response to parasite eggs trapped in tissues, the portal vein and smaller hepatic veins can become partially obstructed or blocked entirely [4, 97]. Blockages of smaller vessels results in high hepatic blood pressure, known as portal hypertension. The end result may be peritoneal ascites caused by blood plasma inappropriately passing through hepatic blood vessels into the peritoneal cavity resulting in abdominal distension [4, 84, 98, 99].

Deoxygenated blood originating from vessels located below the diaphragm, travels through the hepatic portal system before being pumped to the lungs. Unfortunately, portal hypertension due to schistosome infection can lead to formation of anastomoses, where mesenteric vessels develop connections directly to the vena cava and completely bypass the portal system [84, 100, 101]. This shunting of blood allows eggs to travel to, and be deposited in, the lungs. As in the liver, eggs will obstruct pulmonary

blood vessels and lead to pulmonary hypertension, one of the symptoms associated with chronic schistosomiasis [8, 9, 100].

When eggs become lodged in tissues, the body responds by forming dense granulomas and stimulating collagen production, which in chronic stages of infection leads to liver fibrosis and adhesions of the lobes [12, 102, 103]. In severe infections, other organs within the peritoneal cavity may also be affected by adhesions, such as the spleen, bile ducts, stomach, and kidneys [104-106]. Splenomegaly becomes an increasingly high-risk problem as the spleen attempts to cope with constant immune stimulation and increased circulating blood products [105, 107, 108]. The enlarged spleen can rupture resulting in internal bleeding with a low chance of survival [109, 110]. As noted earlier, chronic infection with both liver and spleen enlargement is referred to as heptosplenic schistosomiasis [13, 97]. In addition to pathology induced by tissue trapped eggs, blood loss/anemia, due to consumption of blood by adult worms can occur [44, 111]. Anemia is problematic in young children, affecting growth and brain/cognitive development [112, 113].

***Schistosoma mansoni*- Immune Responses**

As depicted in Figure 2.3, host immune responses to schistosome infection are complex. This is in part due to the multiple developmental stages of the parasite the host encounters, including infectious cercariae, skin schistosomules, lung worms, immature adult worms, male and female worms, schistosome eggs, adult worm vomit and multiple other excretory and secretory molecules. Post-infection, and after transforming into schistosomula, parasites initiate evasion of the host immune system by producing an

anti-inflammatory protein called Sm16, which inhibits IL-1α and IL-1β transcription, inhibits IL-2 production from cells in lymphoid tissues, and stimulates IL-1RA production in keratinocytes [114-116]. Further, Sm16 and a cysteine protease, SmCB1, inhibit TLR activation [117, 118]. Despite these evasion mechanisms, within two to four weeks of the infection the host immune system mounts a Th1-type immune response to the parasite, characterized by the production of IL-2, IFNγ, and TNFα.

IL-2 production by Th1 cells is vital for proliferation of all T helper subsets [119, 120]. IFNγ is primarily produced by Th1 cells, however during schistosome infection, IFNγ can also be produced by iNKT cells [121]. TNFα is produced by classically activated macrophages, and is a major pro-inflammatory cytokine of innate responses [120]. Interestingly, TNFα production was shown to have a positive influence on worm fecundity [122]. Unfortunately, this finding could not be reproduced by other investigators.

In addition to TNFα, several other host cytokines have been linked to worm development and fecundity. Similar to TNFα, IL-7 has been linked to schistosome development, as male and female worm development is impaired in IL-7 KO mice, including reduced fecundity. Impaired worm development is not a direct result of IL-7 acting on the worms, rather the defect in IL-7 deficient mice that leads to altered worm development is thought to be due to an impairment in CD4+ T cell maturation [123]. This is interesting as chronically infected IL-7 KO mice have an enhanced Th1 phenotype compared to wild-type infected mice [124]. These studies demonstrate that host immune responses, while intended to eliminate infection, can be beneficial and even required for schistosomes to mature properly and effectively produce eggs.

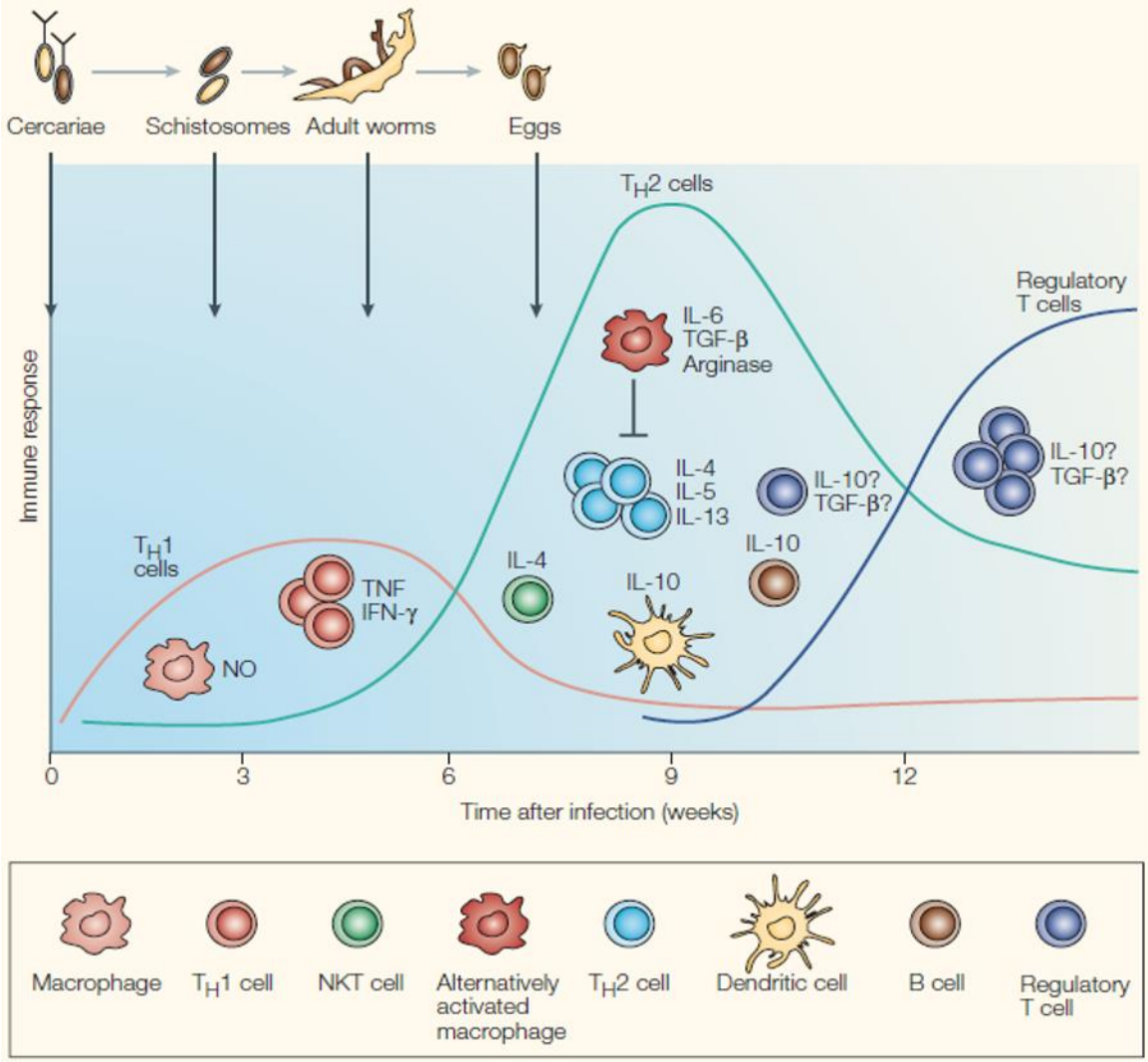

FIG 2.3 Induction of T-helper-1 and T-helper-2 cell responses and development of regulatory T cells after infection with *Schistosoma mansoni*. Reprinted by permission from Nature Publishing Group: *Nature Reviews Immunology* (**5**, 420-426), Copyright (2005)

The Th1-type immune responses seen early in infection can be dangerous to the host when not controlled, resulting in extensive inflammation and damage to host tissues. Fortunately, one to two weeks post initiation of egg production, the host immune profile

begins to shift from a pro-inflammatory Th1-type to a Th2 and anti-inflammatory phenotype [125, 126]. Th2 cells are induced by IL-4 from cells like ILC2s, discussed below, and produce the characteristic cytokines IL-4, IL-5, and IL-13 [120]. Eggs, or more specifically egg products, induce Th2-type and anti-inflammatory immune responses in chronically infected hosts [12, 67, 69, 70, 102, 127-131]. Omega-1 is a glycoprotein found in soluble egg antigens (SEA) and in excretory/secretory products (ESPs) of eggs [129, 130]. Purified Omega-1 stimulates Th2 cell polarization similar to T cells stimulated with SEA [129, 130]. Recently, using a lentivirus delivered short-hairpin micro-RNA, it was established that knocking down Omega-1results in reduced granuloma size and pathology, likely as a result of reduced stimulation of T cells and macrophages [132]. Another product of schistosome eggs is the immunomodulatory Lewis X (LeX) trisaccharide, initially reported as the pentasaccharide Lacto-N-fucopentaose III [133]. Intranasal, intraperitoneal, or subcutaneous administration of LNFPIII conjugates leads to increased production of IL-4, IL-5, and IL-10 as well as decreased production of IFNγ by lymphocytes [20]. Th2 cells induced by these and other egg antigens have broad effects on other cells of the immune system during infection [119]. Th2 cells reduce severe pathology and death associated with continuous pro-inflammatory Th1 and innate cell responses and are also required for granuloma formation [125, 134, 135]. Similarly, blocking the IL-2 receptor (CD25) during schistosome infection results in diminished granuloma formation, indicating that T cell proliferation is required [136]. Additionally, Th2 production of IL-13 and IL-4 stimulates and maintains alternatively activated macrophages [127, 137-139].

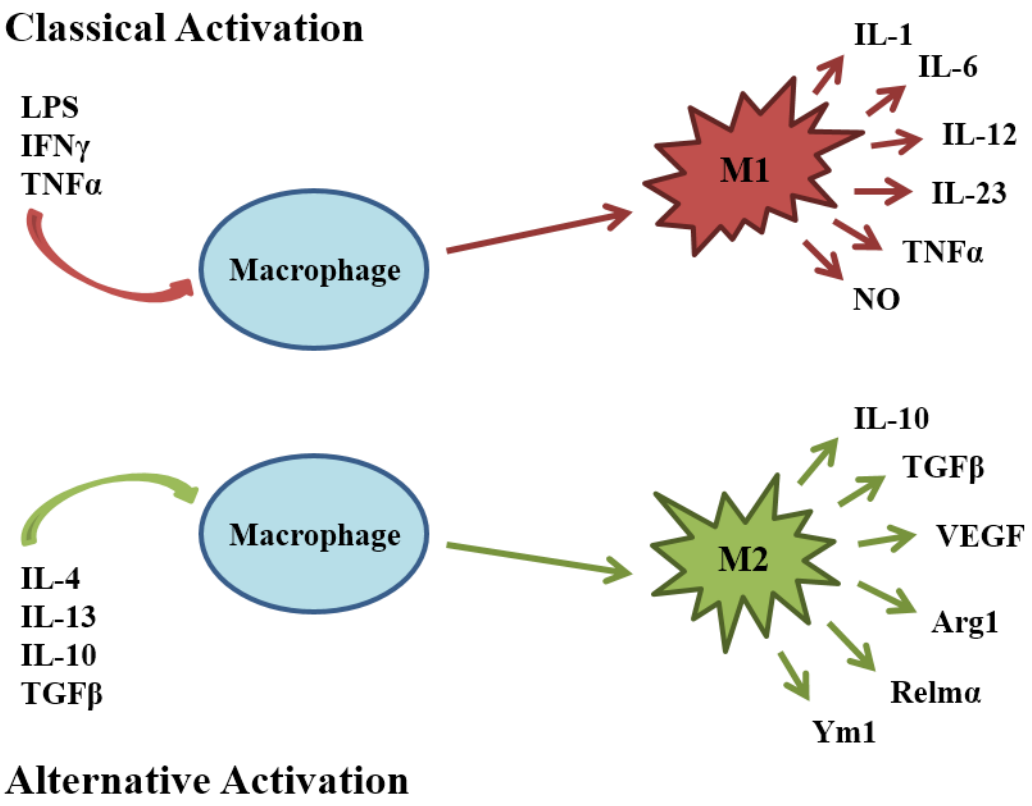

FIG 2.4 Differential macrophage activation state induction and responses

Macrophages can be divided in two broad categories, classically activated (M1) and alternatively activated (M2) (Fig. 2.4). Classically activated macrophages are pro-inflammatory and produce nitric oxide (NO) when stimulated with IFNγ [140]. M1 macrophages are seen early during schistosome infection and are characterized by the enzyme inducible nitric oxide synthase (iNOS), which oxidizes L-arginine into NO [21, 138, 140, 141].

Alternatively activated macrophages are further categorized as M2a, M2b, M2c, and M2d [142-144]. M2 macrophage subtypes are based on the factors that activate them; M2a by IL-4 and IL-13, M2b by lipopolysaccaride (LPS) and immune complexes

(ICs), M2c by IL-10, TGFβ and glucocorticoids, and M2d by IL-6 and adenosines. Induction of M2a macrophages by binding of IL-4 and IL-13 to IL-4Rα, has been shown to be required for survival of schistosome infected mice [127, 145]. Characteristic M2 activation markers are Arginase-1 (Arg-1), Chitinase-3-like molecule 3 (Chi3l3 or Ym1) and Resistin-like molecule alpha (Relmα) [17, 146]. Arginase-1 competes for substrate with iNOS, and hydrolyzes L-arginine into ornithine, which is further processed into polyamines that can be utilized by the worms [147]. M2 produced Arg1 has been shown to downregulate Th2 responses during schistosome infection in mice, resulting in reduced granuloma pathology and fibrosis [148]. Ym1 is a protein considered responsive to chitin, which is produced by schistosomes, and induces recruitment of eosinophils [149-151].

In the lungs, Relmα induces vascular remodeling, reduces Th2 cell associated inflammation, and promotes tissue repair by stimulating fibroblasts to produce collagen [152-156]. Relmα is produced by alternatively activated macrophages, alveolar and bronchiolar epithelial cells, adipocytes, and smooth muscle cells [154, 157-159]. In addition to helminth infection, Relmα can be induced by allergic pulmonary inflammation, where it is referred to as FIZZ1 (found in inflammatory zone 1), and can be produced in response to hypoxia where it is referred to as HIMF (hypoxia-induced mitogenic factor).

Whether IL-4 and IL-13 are required to stimulate polarization of macrophages to an M2 state, these cytokines are required to maintain this activation state. Consistent stimulation is necessary as macrophages are phenotypically plastic and able to alter functional phenotypes based on the cytokine environment [160]. Cytokines produced by

Th2 cells confer maintenance of M2 macrophages [161]. Within lung tissue, M2 macrophages are most often found in granulomas that surround schistosome eggs, however they can also be seen independently of eggs in scattered clusters. Egg granulomas also consist of T cells, eosinophils, and fibroblasts [162, 163]. M2 macrophages induce collagen deposition by fibroblasts, and depending on infection stage, egg granulomas can be highly fibrotic [4, 15, 164].

In addition to Arg1, Relmα, and Ym1, M2 macrophages produce various cytokines including IL-10 and TGFβ as well as VEGF (vascular endothelial growth factor) [165]. IL-10 is an important cytokine during schistosome infection as it regulates inflammation, whether Th1, Th2, or innate cell derived. Hoffmann *et al.* demonstrated that, during schistosome infection, Th2 deficient mice had severe pathology resulting in death and Th1 deficient mice, while surviving infection, had significantly more granuloma related fibrosis. Importantly, IL-10 KO mice experienced symptoms related to both extremes indicating the importance of IL-10 in regulating both Th1-type and Th2-type inflammation [166]. A study of Gabonese children showed that those infected with schistosomes had a reduced prevalence of allergic atopy and that this was correlated with serum IL-10 levels [167].

In addition to M2 macrophages, T regulatory cells (Tregs) also produce IL-10 during schistosome infection [168]. Tregs can be divided into two main subtypes, natural Tregs (nTregs) and inducible Tregs (iTregs). Thymus derived nTregs circulate in the periphery monitoring self-reactive T cells, while iTregs are a product of conventional CD4+ T cells that receive a differentiation signal by stimulated APCs producing IL-10 and TGFβ [169]. Both Treg subtypes are characterized by expression of CD4, CD25,

CTLA4 and FoxP3, however nTregs can be distinguished by their expression of neuropilin-1 (Nrp1) and, to a lesser degree, Helios [170-172].

Innate lymphoid cells (ILCs) are a recently described type of cell that, like T and B cells, originate from common lymphoid progenitors (CLPs). However, unlike B and T cells, ILCs do not express an antigen receptor. Additionally, ILCs do not express typical lineage (Lin) markers of lymphocytes (Lin^- in mice: CD3ε, CD4, CD8α, CD19, B220, Ter119, Gr1, CD11b, CD11c, and FcεRIα) [173]. ILCs are further categorized by cytokine expression profiles and surface receptors. In 2010, three groups independently described Group 2 innate lymphoid cells (ILC2s), which are Lin^- cells producing the Th2-type cytokines IL-4, IL-5, IL-13 [18, 174, 175]. ILC2s, also called nuocytes, innate helper cells, or natural helper cells develop from CLPs in a GATA3 dependent manner [176, 177].

ST2 is a receptor found on ILC2s and Th2 cells and is a member of the IL-1 receptor family (IL-1R). Cytokine production by ILC2s is initiated by IL-33 binding to the ST2 receptor. IL-33 is a member of the IL-1 family that potently drives production of Th2-type cytokines [178-180]. Low level expression of ST2 has also been demonstrated for eosinophils, basophils, mast cells, and dendritic cells, each of which are associated with helminth-induced immune responses [181-183]. In addition to ST2, ILC2s express surface MHC-II molecules and low levels of co-stimulatory molecules CD80 and CD86 [175, 184].

ILC2s are considered to "bridge the gap" between innate and adaptive responses by producing IL-13, which signals CD4+ T cells to differentiate into Th2 cells and stimulates M2 macrophage differentiation and maintenance [185-188]. The role of ILC2s

during helminth infection has been investigated for the nematode parasite *Nippostongylus brasiliensis.* Infection of IL-33 KO mice with *Nippostongylus brasiliensis* revealed no defects in IL-4 mediated responses, however IL-13 levels were significantly diminished as a result of an impaired induction of IL-13+ ILC2s and IL-13+ Th2 cells in lungs [189]. IL-33 KO mice compensated for the loss of IL-13+ ILC2s by upregulating IL4+ Th2 cells, though this was not sufficient to kill the worms indicating that IL-33 induced IL-13+ ILC2s are required for worm expulsion in this model. Administration of rIL-33 to Rag1 KO mice resulted in ILC2s that were able to induce M2 macrophages independently of Th2 cells and these M2 macrophages were sufficient to kill lung stage *N. brasiliensis* [187]. Importantly, surface expressed MHC-II on ILC2s was shown to bind to CD4+ T cells and, in the presence of IL-2 and antigen, induce polarization and proliferation of IL-4 and IL-13 producing Th2 cells as well as increase numbers of IL-4 and IL-13 producing ILC2s [184]. Together these studies show that, in response to *N. brasiliensis* infection, ILC2s can act independently to stimulate M2 macrophage differentiation via production of IL-13, which is necessary to successfully expel the worms. Additionally, they showed that ILC2s can act in conjunction with CD4+ T cells to induce activated Th2 cells independently of DCs.

Relevance to schistosome infection was shown in a study examining the schistosome egg granuloma model (described below). In this study, IL-13 producing ILC2s were implicated in inducing pulmonary fibrosis [190]. However, in light of the recent studies on ILC2s and M2 macrophages mentioned above, it is more likely that fibrosis was induced by M2 macrophages stimulated by IL-13+ILC2s. In support of this concept is a study examining pulmonary fibrosis in *N. brasiliensis* infection in mice

[191]. ILC2s constitutively express Arg1, a signature enzyme of M2 macrophages. Using the Cre-Flox system, these investigators eliminated Arg1 in ILC2s, which did not affect the degree of pulmonary fibrosis induced by *N. brasiliensis* infection, indicating that Arg1+ILC2s are not a factor in fibrosis.

As noted earlier, schistosome infection can result in pulmonary fibrosis. Therefore it is important to understand the underlying mechanisms behind induction of collagen deposition leading to fibrosis. Schistosome eggs trapped in lungs are thought to be the primary cause of lung pathology during schistosome infection. The schistosome egg granuloma model was developed as an alternative experimental model to natural infection. The schistosome egg granuloma model induces similar Th2-type induction and lung granuloma responses as are seen in schistosome infected mice [192-194]. This model was initially developed in 1962 by Franz von Lichtenberg using *Ascaris suis* eggs [195, 196] and then for schistosome eggs in 1967 [197]. The egg granuloma is a two-stage process wherein the immune system is sensitized to schistosome egg antigens by intraperitoneal injection of purified schistosome eggs. Two weeks later, mice are challenged with schistosome eggs via intravenous injection, resulting in egg deposition in the lungs and a significant granulomatous response at 8 days post IV injection [193].

Influenza A- Background

Influenza

Influenza is a negative sense single stranded RNA ((-)ssRNA) virus from the family orthomyxoviridae [198]. In humans and other mammals it results in a respiratory infection, however in birds it infects the gastrointestinal tract [199]. Infections in humans

can cause mild to severe symptoms depending on age, overall health, and various other factors [200]. Symptoms will last approximately two weeks and generally include cough, nasal discharge or congestion, fever, malaise, body aches, and fatigue [200, 201]. However, certain symptoms like cough and nasal involvement can last for a month or more.

Influenza has a 2-4 day incubation period after infection with infected people shedding virus for an average of 4-6 days [202-205]. Transmission routes of infectious virus include direct/person-to-person contact, fomites, and droplets [206, 207]. Fomites are anything capable of transferring infectious virus from one person to another including tissues, door knobs, and children's toys [208]. Influenza virus can survive on non-porous surfaces for up to 48 hours and porous surfaces up to 12 hours, however successful transmission of infection only occurs during the first 8 hours and approximately 15 minutes, respectively [209]. A temporal evaluation of when infection was most likely in daycare facilities and households was conducted over a two year period and found that more than 50% of fomites tested were positive for influenza virus, with the highest proportion positive (59%) during March [210].

Droplet transmission occurs when an infected person coughs or sneezes infectious virions suspended in liquid particles of $\geq$5μm in diameter, which will either come in contact with another person or settle on surfaces resulting in fomites [211]. Aerosols (also known as droplet nuclei) are liquid particles $\leq$5μm and are light enough that they can remain in the air indefinitely [211, 212]. Traditionally, aerosol transmission of influenza was only thought to occur under experimental and clinical settings, such as by centrifugation or ventilation, however there is strong evidence that this may not be

accurate [213]. A case study of an outbreak on a grounded airplane with a broken ventilation system found that within three hours a single influenza infected person infected 72% of the passengers with influenza [214]. Under experimental conditions, influenza has been detected in particles ≤5μm during coughing and tidal breathing and can be detected in particles <1μm, often with greater copy numbers than found in 4μm particles [215-217]. However, this topic is hotly debated and while aerosol transmission of influenza is not completely dismissed, it is viewed as "unlikely to be of significance in most clinical settings" [206].

There are currently four known genera of influenza viruses; Influenza A, B, C, and D are distinguished by their matrix and nucleoprotein antigenic variations [218]. Influenza A causes the vast majority of disease in humans and has been the cause of all known human influenza pandemics [206, 219-221]. Influenza A infects humans, pigs, and water fowl, among various other species [199]. Influenza A will be the primary focus of this review and, unless otherwise noted, all information provided will be in regards to this genus.

Influenza B circulates along with seasonal Influenza A, although it generally does not result in severe infection. Influenza B has a slower mutation rate compared to Influenza A and an antigenic shift has never been detected [222, 223]. Additionally, the only other animal species Influenza B has been detected in is the harbor seal [224]. Together this indicates limited potential for a pandemic caused by Influenza B. One to two strains of Influenza B are included in the influenza vaccine each year as it is still a common infection and preventative measures should be taken to reduce spread whenever possible [213, 225-227].

Influenza C infects humans and pigs, however outbreaks are rare and result in mild symptoms [200]. When outbreaks do occur, children under the age of 5 are in the highest risk group for infection [228]. Unfortunately, research on Influenza C is limited as a result of difficulties in isolating it as well as issues in finding a suitable cell type for *in vitro* culture [200].

Influenza D is a recently discovered genus that was isolated from swine in 2011 [229]. Influenza D was initially thought to be a variation of Influenza C, however genetic testing comparing multiple isolates of this virus to various Influenza C isolates, determined that the virus was unique and deemed Influenza D [230]. Since its discovery, Influenza D has also been recovered from cattle, sheep, and goats [231, 232].

Influenza A is unique from other Influenza generas as it has multiple subtypes based on surface protein expression. Hemagglutinin (HA) and neuraminidase (NA) proteins are responsible for entry and exit, respectively, from a host cell and cover the virion exterior [233-235]. There are currently 18 known HA and 11 known NA subtypes of Influenza A that are expressed in various combinations and it is this combination that determines how Influenza A viruses are identified [236]. Currently, there are two Influenza A virus subtypes circulating in the human population; H1N1 (hemagglutinin type 1 with neuraminidase type 1) and H3N2 [237]. These two viruses are referred to as seasonal strains since they reoccur in populations every winter. Additionally, an H2N2 virus circulated from the 1950s-1960s and an H1N2 virus has sporadically circulated over the past century [238]. Many other Influenza A subtypes are of concern to humans including H5N1 (“bird flu”/high-path influenza), H7N7, H7N9, and H9N2 [239-242]. These four viruses preferentially bind to avian cell surface glycoproteins rather than

human, however influenza is highly mutagenic and very few mutations in the HA binding site are required to result in a change in binding specificity [234, 243-245]. Additionally, pigs express glycoproteins of humans and avian species making it possible for two different species-specific viruses to infect the same cell in a pig host allowing for recombination of viral genes to occur resulting in unique, and possibly dangerous, influenza virus [246]. Details regarding aspects of cell surface binding, mutagenesis, and recombination will be discussed later in this chapter.

Human Impact

Seasonal Influenza A infections account for ~5 million severe cases and up to 500,000 deaths per year, however influenza can also result in pandemics [26]. Five influenza pandemics have occurred over the past 100 years [247]. The1918 H1N1 pandemic, also known as the "Spanish flu", is considered to be the worst influenza pandemic in recorded history with a broad estimate of 50-100 million associated deaths worldwide and 675,000 within the United States [220, 248, 249]. However, as a result of WWI, under-reporting, and various other confounders, it is difficult to get an accurate estimate on worldwide infections and mortalities during the 1918 pandemic [249, 250].

Additional pandemics occurred in 1957 (H2N2 "Asian flu"), 1968 (H3N2 "Hong Kong"), and 1977 (H1N1 "Russian flu"), however none had the same impact on the human population as the 1918 pandemic [251-256]. The most recent pandemic was the H1N1 "Swine flu" of 2009, which spread rapidly from Mexico City to North America [257]. Although this virus was highly infectious, it was not very deadly due to a gene

mutation that resulted in non-transcription of a protein linked to the high virulence of the 1918 strain [258-263].

Influenza infection can negatively impact anyone in the population, however infection related deaths most often occur in the young, old, and immunocompromised [264]. Influenza induced pneumonia, particularly in these populations, often results in death [264-266]. A study comparing influenza related hospitalizations during the 1968 pandemic against a non-pandemic year showed that during the pandemic, people 65 years or older with an underlying disease were admitted at a rate of 476-636/100,000 people in excess over that seen in the non-pandemic year [265]. Further, incidence of acute respiratory disease increased 30-50% and influenza related pneumonia increased by 140-150%, with 11-13 excess deaths per 100,000 people during the pandemic. Surveillance of hospitals over two separate influenza seasons found persons over 85 years of age had the highest risk of hospitalization due to infection and children were found to have a similar risk to those aged between 50 and 64 [267]. The elderly also have poor vaccine responses making them high risk for susceptibility to infection. During a nursing home outbreak, despite a 90% influenza vaccination rate among residents, 30% become infected with influenza and 3 died as a result of influenza induced pneumonia [264].

Influenza induced pneumonia is directly brought on by viral infection. Histopathlogy of viral pneumonia shows dense cellular infiltration, hemorrhagic blood vessels, and sloughing off of epithelial cells deep within the bronchial tree and down to the alveoli [201, 247, 265, 266]. Patients with viral pneumonia can experience acute respiratory distress, which requires additional oxygen or ventilation as a result of reduced lung capacity [268, 269]. Importantly, viral pneumonia leaves patients particularly

susceptible to acquiring secondary bacterial infections [270, 271]. This combination is highly lethal as the patient's immune system is already compromised from influenza induced responses [258].

Influenza Structure

Influenza A is a small, pleomorphic virus with a lipid envelope measuring 50-120nm in diameter [272]. It has a segmented genome comprised of eight (-)ssRNA segments that code for 10-12 proteins as a result of two segments having multiple open reading frames [273].

Out of the nine proteins that are expressed in an influenza virion, there are three proteins found on the surface (Fig. 2.5). The most abundant protein is hemagglutinin (HA), which binds to host epithelial cells and initiates the entry process [274]. Hundreds of HA proteins, in a trimeric conformation, protrude through the virion envelope and are dispersed evenly across the surface [275]. Neuraminidase (NA), which facilitates virion egress from an infected cell, is expressed on the surface as a tetrameric protrusion and is outnumbered by HA at a 5:1 ratio [276]. Also on the surface is the tetrameric M2 ion channel, which functions to maintain a low pH during virion uncoating [277]. The M1 matrix protein is located underneath the viral envelope where it interacts with both the envelope and viral core, which contains ribonucleoprotein segments [275, 278].

Within the viral core, each of the eight RNA gene segments is encapsidated by nucleoprotein (NP) and associated with the viral RNA-dependent RNA polymerase (RdRp) complex comprised of the PA, PB1, PB2 proteins, which together form the ribonucleoprotein (RNP) segments [279, 280]. The 8th protein expressed in a virion is

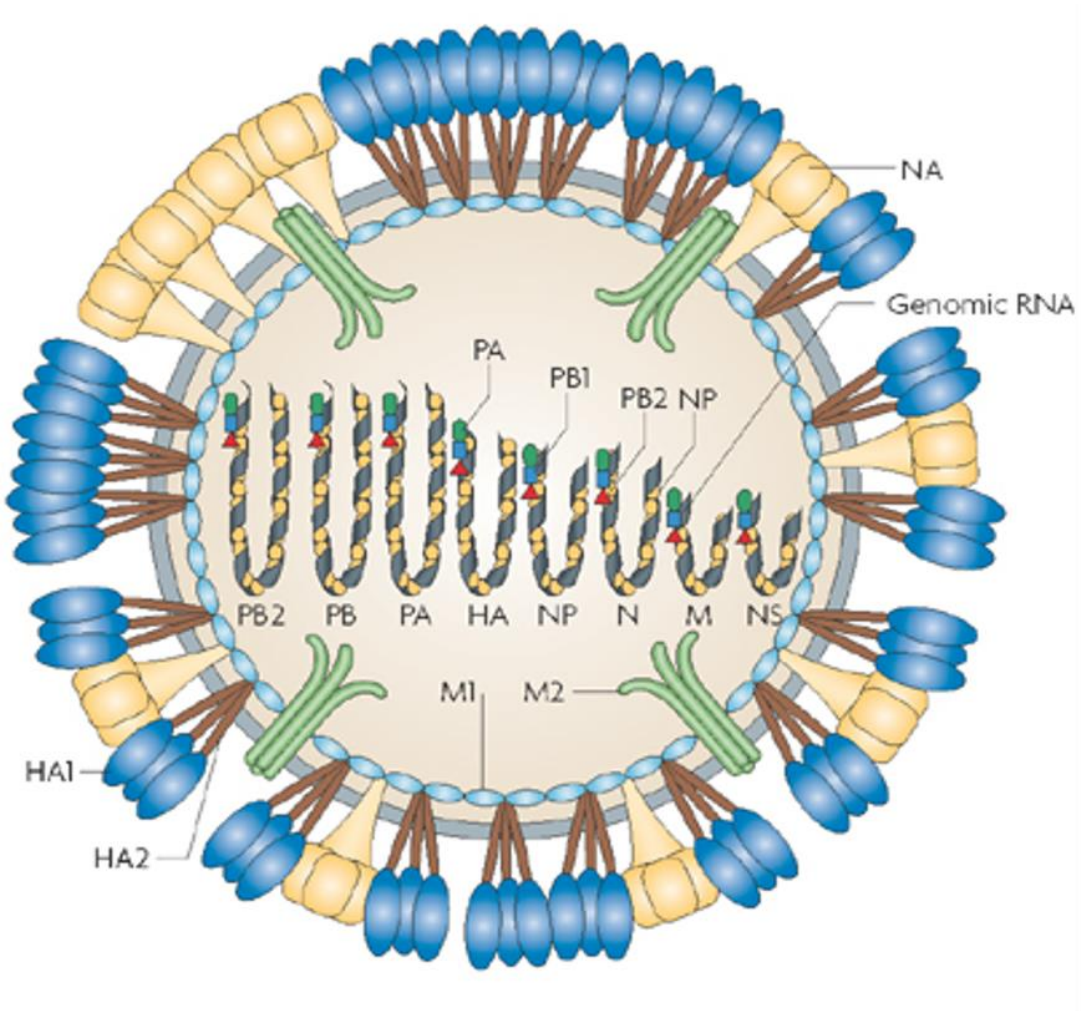

FIG 2.5 Schematic diagram of HIV-1 and Influenza A virus. Modified and reprinted by permission from Nature Publishing Group: *Nature Reviews Microbiology* (**6**, 143-155), Copyright (2008)

the nuclear export protein (NEP, formally NS2), which is present in low concentrations within the viral core [281]. NEP functions to export new RNPs out of the nucleus after they are synthesized [282, 283]. In addition to these eight proteins, there are one to four other proteins that are only expressed in an infected cell during viral replication. Non-structural 1(NS1) protein is encoded by the same gene segment as NEP and is always expressed during viral replication [284, 285]. NS1 serves two primary purposes; it negatively affects host interferon responses and inhibits export of host RNA from the

nucleus [283, 286]. In 2012 the PA gene was found to possess a second ORF, deemed X-ORF [287]. As a result of ribosomal frameshifting, X-ORF codes for the PA-X protein, which functions to suppress various host immune responses [288]. PA-X is found in 75% of known isolates. In the remaining 25% of isolates, a variant of PA-X is present where the last 20 amino acids are deleted, resulting in the name PAXΔ20 [289-291]. Expression of the PAXΔ20 variant has been shown to reduce pathogenicity and viral replication [291]. The last 20aa of PA-X are involved in restricting host protein synthesis. Full length PA-X may also be a virulence factor as it can increase viral polymerase activity and inflammatory responses in mice [292].

Like PA, the PB1 gene segment codes for additional proteins due to a ribosomal frameshift. PB1-F2 is a pro-apoptotic virulence factor protein that was found while looking for peptides recognized by CD8+ T cells [293]. N40 (or PB1-N40) is expressed when the first 40aa of PB1 are truncated [273]. Although there is not yet a definitive function, it has been shown that the N40 protein may have a role in viral RNA synthesis [294].

Influenza A Replication

Viruses require a host cell to replicate in, and to do this the virus must first bind to a cell surface receptor (Fig. 2.6). HA proteins covering influenza virions target sialic acid moieties expressed on epithelial cell surfaces [295]. Sialic acids are the terminal sugar on host cell glycoproteins and are most commonly attached to a galactose by either an α2,3-linkage or α2,6-linkage [295].

The human respiratory tract has epithelial cells that primarily express sialic acids with an α2,6-linkage whereas birds express an α2,3-linkage [296]. The HA of influenza viruses is highly specific for either one linkage type or the other and will either not bind, or weakly bind to the incorrect linkage [234]. Details regarding this will be discussed in the mutagenesis section of this chapter. Each globular head of a trimeric HA structure will bind to a sialic acid, initiating attachment [274]. The virus then utilizes host cell trypsin to cleave HA into two subunits; one that stays bound to the sialic acid and one that is flexibly tethered called the fusion peptide [297]. Binding signals the cell to take up the virus by clathrin-mediated endocytosis [298]. The host cell will attempt to destroy the endosomal contents by fusing it with a low pH lysosome. However, low pH is required for inducing a conformational change in the HA subunits. The acidic environment releases HA from the sialic acid, allowing binding of the fusion peptide to the endolysosomal membrane [298]. Protons pass through the M2 ion channel of the virion inducing acidification of the viral core, which dissociates M1 from the RNPs [278]. The bound fusion peptide initiates fusion of the viral envelope to host endolysosome membrane, permitting the release of RNPs into the host cell cytoplasm. The NP proteins covering each RNP, act as a nuclear localization signal that allows importation of RNPs to the cell nucleus [279, 299].

Within the nucleus there is a replication cycle and a transcription cycle to produce new genome segments and new proteins, respectively. Replication of genome segments does not require a primer, only the viral RNA-dependent RNA polymerase (RdRp) complex (PA, PB1, PB2) [300]. Negative sense vRNA is copied into complementary (+)cRNA by RdRp, which can then be copied back to (-)vRNA by RdRp [280]. For

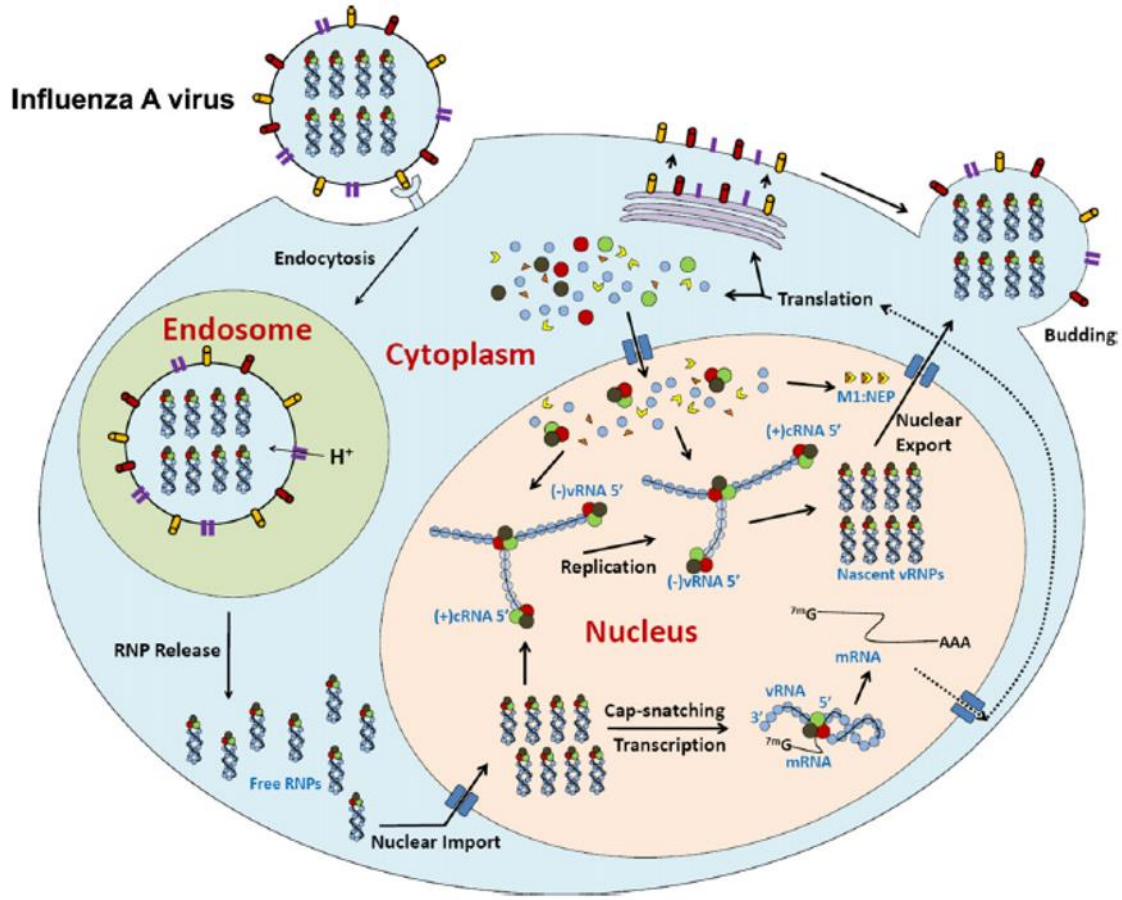

FIG 2.6 The Influenza A virus lifecycle. Reprinted by permission from Elsevier: *FEBS Letters* (**587**, 1206-1214), Copyright (2013)

transcription to occur, a host cell primer is required and obtained in a mechanism called cap-snatching [301]. Cap-snatching is achieved by bind of the PB2 subunit of RdRp to cellular pre-mRNA allowing PA, which has endonuclease activity, to cleave the 5'cap and use as a primer for viral RNA transcription [302, 303]. The RdRp will utilize the 5'cap as a primer to initiate transcription of viral (+)mRNA gene segments, which will have a 5'cap and a poly-A tail [302]. Gene segments can then be transported to the cytoplasm and translated into proteins by hijacking host cell ribosomes [283, 300, 304].

HA, NA and M2 are transported to the cell surface for virion assembly, Other proteins, such as RdRp and NP, are imported back into the nucleus to either be utilized for further replication or form new RNPs [279]. NEP forms a pore in the nucleus by which RNPs can be transported to the cytoplasm and packaged into virions. New virions leave the host cell by a process called budding. They remain membrane bound due to HA binding to sialic acid [305]. Complete budding of the virions requires the enzymatic activity of NA to cleave sialic acid from the cell surface glycoprotein [306].

Influenza Mutagenesis

Negative sense RNA viruses like influenza have high mutation rates as a result of requiring an RdRp during replication. RdRp lacks proofreading capabilities of DNA polymerases. Influenza mutates at a rate of ~$2x10^{-6}$ substitutions per site per replication cycle [223]. Certain genes, and even specific regions within these genes, mutate at a faster rate than others. Hemagglutinin is the most mutagenic gene in influenza and is more likely to mutate at areas corresponding to antigenic sites than anywhere else [307]. This is primarily due to immune pressure, specifically by neutralizing antibodies against the globular head regions of HA [233]. Targeted mutagenesis of viral genes as the result of immune pressure is called antigenic drift [276, 308-310]. Antigenic drift is the reason a new influenza vaccine must be made every year; to adjust for changes in the HA proteins in order to ensure effective neutralizing antibody responses against the circulating strains [311].

In addition to the antigenic sites having high mutation rates, HA binding domains are specific for linkages of sialic acid to galactose, also mutate at higher rates [234].

Glasser *et al*. demonstrated *in vitro* that only a single amino acid change is required to alter sialic acid receptor specificity of the pandemic 1918 H1N1 virus from α-2,6 to α-2,3 [243]. Tumpey *et al.* genetically altered the HA from the 1918 virus by two amino acids to change sialic acid binding specificity from α-2,6 to α-2,3 and found that while the mutant 1918 virus could maintain normal replication efficiencies, infected ferrets were not able to transmit the virus to naïve animals [245]. Importantly, Pappas *et al.* recovered a virus isolate from a ferret that naturally mutated from an α-2,3 to an α-2,6 linage specificity, which allowed for successful transmission to a naïve animal [244].

These examples demonstrate how small genetic changes can induce protein alterations that are significant for transmission. More concerning than antigenic drift is the potential for antigenic shift, where genetic material from two viruses is exchanged, resulting in an entirely new virus. Antigenic shift can be the result of one virus copying small stretches of nucleic acid template from another virus called recombination or exchanging of entire gene segments called reassortment, which is more commonly seen in influenza due to its segmented genome [312]. Every pandemic influenza virus has arisen from antigenic shift and there is a great deal of trepidation surrounding a scenario where a seasonal strain has a reassortment event with a highly pathogenic virus like the avian H5N1 or H7N9 viruses [313].

Although humans primarily express the α-2,6 linkage on respiratory epithelial cells, α-2,3 can be found deep within the lungs beginning at the junction of respiratory bronchioles and alveoli as well as the alveolar wall [314]. Additionally, *ex vivo*, α-2,3 expressing human respiratory cells are susceptible to infection with avian influenza viruses [296]. Even so, experimental human infections with avian viruses resulted in a

27.5% infection rate with minimal symptoms and undetectable antibody responses [315]. Natural human infection with avian influenza has not been any more successful, with only 645 confirmed cases of H5N1 over a ten year period in people with frequent exposure to poultry [316, 317]. Since humans have a low likelihood of infection with avian viruses as a result of the location of α-2,3 linked epithelial cells, why is there such fear surrounding these viruses obtaining the ability to circulate in the population?

In order for viral gene reassortment to occur, two different viruses must infect the same cell and therefore must infect an animal that equally expresses both sialic acid linkages. Livestock pigs are deemed "mixing vessels" for avian and human influenza as they express sialic acid receptors for both virus types [246]. Many human viruses, both circulating and pandemic, have swine influenza strain genes. The 2009 H1N1 pandemic virus had genes from an avian virus, human H3N2, classic swine H1N1, and a Eurasian "avian-like" swine virus. Thus, a quadruple reassortment combination that had not been seen before [318]. Although this virus was a result of multiple reassortments over time rather than four independent viruses infecting the same cell, it serves as an example of antigenic shift potentials of influenza.

Influenza A Immune Responses

Innate Response

In humans, influenza virus predominantly infects epithelial cells of the respiratory tract. After successfully penetrating through physical and chemical barriers, such as mucins and surfactants, a virion can infect an epithelial cell [319, 320]. During cellular infection virion contents are available for recognition by host immune receptors like Toll-

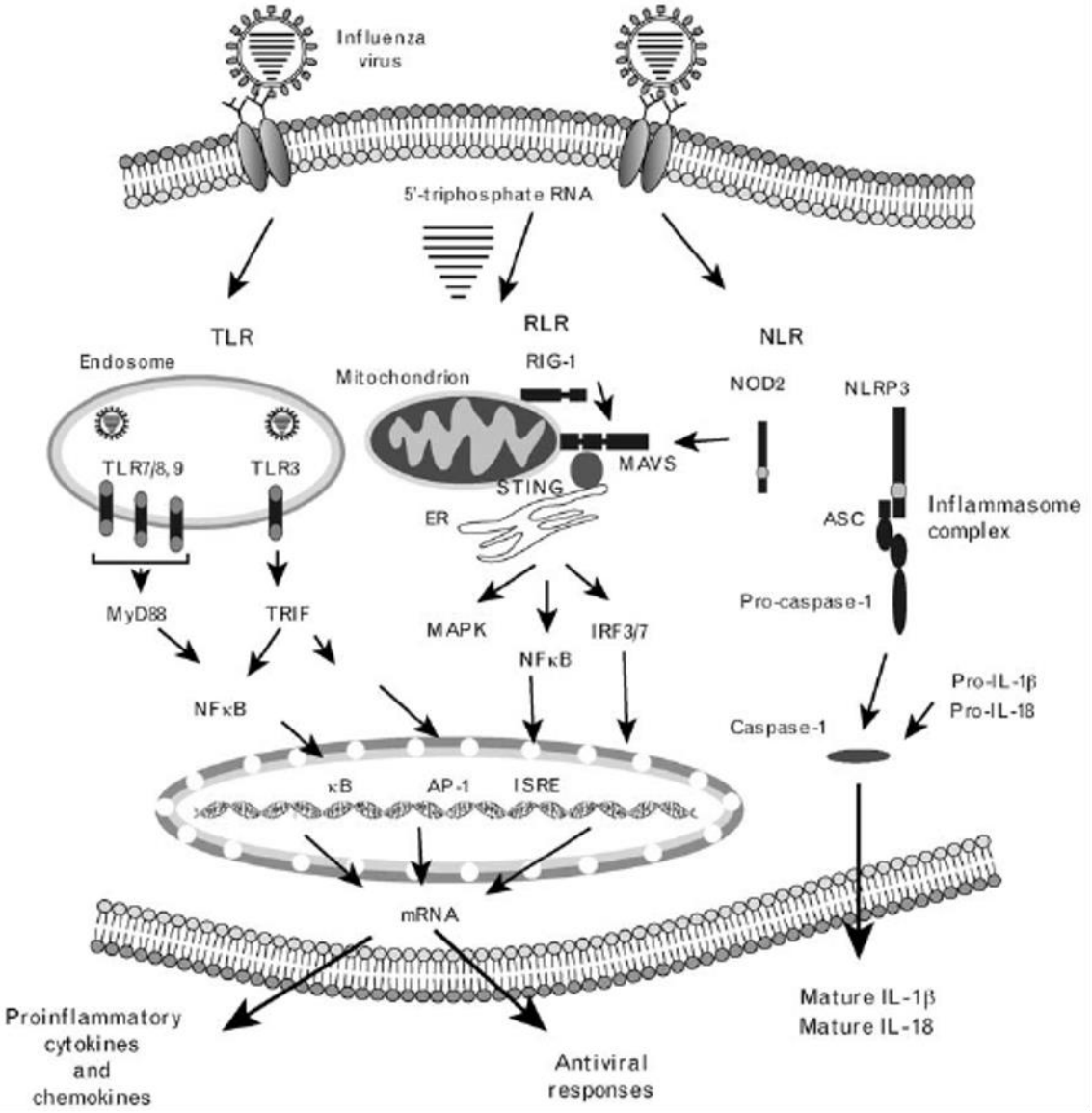

FIG 2.7 Current model of the innate immune response to influenza virus. Reprinted by permission from Wolters Kluwer Health, Inc.: *Current Opinion in Infectious Diseases* (**24**, 235-240), Copyright (2011)

like receptors (TLRs), RIG-like receptors (RLRs), and NOD-like receptors (NLRs) (Fig. 2.7) [321-325].

In the TLR family, influenza is primarily recognized by TLR3 and TLR7, both of which are expressed in endosomes in the cell cytosol [326]. TLR3 recognizes viral

dsRNA in both alveolar and bronchial epithelial cells and induces a proinflammatory response by producing IL-6, IL-8, and IFNβ [327]. Airway epithelial cells do not readily express TLR7 and therefore rely on TLR3 to recognize viral RNA and signal via the TRIF pathway to induce Type I Interferons IFNα and IFNβ, which inhibit viral replication [323, 324, 328]. However, other cell types express TLR7 including plasmacytoid dendritic cells (pDCs) and alveolar macrophages [322, 329, 330].

Additionally, RIG-I recognizes dsRNA in the cytosol and activates transcription factors IRF3/7 and NF-κB to induce production of Type I Interferons and pro-inflammatory cytokines, respectively [321]. Pro-inflammatory cytokines are responsible for the significant lung pathology that can be seen during an influenza infection, including IL-1β [331]. Signaling by RLRs and TLRs results in cellular transcription of the pro-form of IL-1β, which is enzymatically cleaved to produce its active form [321]. When activated by influenza infection, the NLRP3 inflammasome produces caspase-1, which cleaves pro-IL-1 as well as pro-IL-18 into their active forms [332]. These cytokines signal for cellular recruitment to the lungs, including neutrophils, monocytes, and natural killer cells (NKs) [331].

Natural killer cells can detect surface expressed hemagglutinin on infected cells and directly kill by releasing cytolytic granules that result in cell lysis [333, 334]. In addition, NK cells produce large amounts of IFNγ, which promotes CD4+ T cell differentiation to Th1 cells as well as stimulates an increase in the phagocytic abilities of macrophages [120, 333]. Dendritic cells are stimulated by Type I Interferons to become better antigen presenting cells (APCs) to CD4+ and CD8+ T cells in the lymph nodes [335].

Upon infection with influenza, APCs located at the site of infection directly phagocytose virions and/or viral particles and process the proteins down to peptides which can then be expressed on MHC-I or MHC-II on the cell surface [336]. In brief, peptide processing for CD4+ T cell activation is restricted to APCs and involves endosomal uptake of proteins which get proteolytically cleaved into small pieces of approximately 14-20 amino acids and loaded onto MHC-II molecules for APC presentation [32, 337].

CD8+ T cells are important for dealing with intracellular pathogens like influenza therefore, all nucleated cells must be able to process peptides for CD8+ T cell recognition [338]. Proteosomes within the cytosol process viral proteins into fragments of 8-10 amino acids that are loaded on to MHC-I molecules for presentation to CD8+ T cells [339]. During influenza replication host protein synthesis is suppressed; this can result in a loss of MHC-I expression on the cell surface and, consequently, a loss of viral peptide presentation for CD8+ T cell recognition [340]. This could result in poor viral clearance, however a function of NK cells is to inspect other cells for MHC-I expression, which is normally constitutively expressed, and destroy cells not expressing it [340, 341]. Unique for MHC-I peptide presentation, DCs can phagocytose an infected cell and process the peptides in a mechanism called “cross-presentation” [342].

Naïve T cells must encounter antigen to be activated become effector cells capable of resolving the infection; in influenza this primarily occurs in draining lymph nodes [343]. After exposure to virions or virion infected cells, epithelial residing DCs begin to express CCR7, directing their migration to T cell zones of draining lymph nodes

[344]. Here DCs present processed antigens to naïve T cells of both CD4+ and CD8+ lineages.

Adaptive Response

In addition to an innate response, a robust adaptive response is also required in order to clear a primary influenza infection. Activated CD4+ T cells have multiple potential terminal differentiation states, each with a range of functions. Importantly during influenza infection, is T cell help in the production of neutralizing antibodies. During influenza infection, production of serum IgG2a antibodies are produced by B cells that were "helped" by IFNγ producing Th1 cells within lymphoid tissue [345]. Mucosal IgA antibodies are produced in a T-independent manner, with B cell activation occurring at the site of infection by APCs and stimulated by TGFβ [346]. Antibodies play a key role in secondary influenza infection, however during a primary infection CD8+ T cells aide in efficiently clearing the infection and, in turn, preventing additional damage to the host [27, 343].

As previously discussed, DCs present antigens to CD8+ T cells via MHC-I inside the draining lymph node. CD8+ T cells clonally expand in the lymph nodes within 2-4 days post influenza infection [347]. Activated CD8+ T cells will migrate back to the lungs beginning at 5-7 dpi and reach peak numbers around 9-11 dpi before they begin the contraction phase around 14 dpi [348, 349]. When activated antigen specific effector CD8+ T cells, called cytotoxic T cells (CTLs) migrate from lymph nodes back to the lungs they will destroy any infected cell that expresses their cognate peptide presented by MHC-I.

The primary mechanisms of CTL killing is by Fas/FasL binding and exocytosis of cytotoxic granules [350, 351]. Fas/FasL mediated killing occurs when CTL expressed FasL binds to Fas on target cells and induces cellular apoptosis by activation of caspase 8 [352]. However, there are reports of caspase 8-independent Fas/FasL killing by CTLs, which is necrotic in nature [353]. Cytotoxic granules of CTLs contain numerous proteases and granzymes as well as perforin [354-356]. Perforin 1 is a Ca2+-dependent protein which functions to form holes in target cell membranes allowing granule contents to pass in and cytoplasmic contents to flow out [350, 357]. The serine-protease Granzyme B is one of the most abundant granzymes in cytotoxic granules and is responsible for activating caspase 3-mediated apoptosis [358-360]. Granzyme B is able to pass through the cytoplasmic membrane independently of perforin 1, however it is not able to translocate to the nucleus in the absence of perforin 1, indicating there is an as yet undiscovered additional interaction/activation taking place between these two proteins [361, 362]. The effect of perforin and Granzyme B together is what many call the "lethal hit" of CTLs [354].

Coinfections

Unlike the United States and Europe, people living in developing countries may be concurrently infected with multiple pathogens. People infected with schistosomes are often coinfected with other soil-transmitted helminths, gut protozoa and malaria. Depending on the geographic region, schistosome infected individuals can also be infected with *Mycobacterium tuberculosis*, and HIV-1 [37, 363-365]. Infection with helminth parasites induces a Th2/anti-inflammatory immune environment. Therefore it is

important to understand how this immune biasing may influence infections with other pathogens, in particular, those that are thought to require a Th1 or mixed Th1/Th2 environment. Coinfection has become a major topic over the past decade and there is a growing amount of literature on the subject, including studies showing the effects of helminth infections on viral infections.

A number of model systems have been studied to evaluate the effects of helminth infection on secondary viral infections, with varied results. Infection with the intestinal helminth *Taenia crassiceps* increased susceptibility to infection with *Streptococcus pneumoniae*, but not to Influenza A [366]. Infection with the intestinal parasite, *Heligmosomoides polygyrus*, was shown to reactivate latent Mouse Herpes Virus (MHV) as a result of decreased IFNγ responses [367]. *Trichinella spiralis* infections resulted in reduced anti-Influenza A CD8+ T cell populations, however, only during lung stages of parasite infection and not chronic stages [368, 369]. In addition to reduced CD8+ T cell responses, coinfection of *S. mansoni* and vaccinia virus resulted in viral replication within liver granulomas and increased viral clearance time [370]. *S. mansoni* and LCMV coinfection also resulted in increased viral replication in the liver despite a potent IFNγ producing CD8+ T cell response against the virus [371].

This is further complicated when we evaluate helminth-virus coinfections in human populations. Human field studies on Hepitits C Virus (HCV) showed schistosome coinfection decreased HCV-specific T cell responses and schistosome drug treatment resulted in increased transmission of HCV in Egypt [372, 373]. In Brazil, coinfection of schistosomes and HCV lead to increased liver disease and pathology [374].

These examples highlight the complexity of interactions between infections, immune responses, and disease outcomes. They also illustrate that not all helminth-virus models are equal or representative of what may be occurring in a real-world setting. For influenza and helminth coinfections, based on the lack of detectable differences when animals harbor an intestinal worm, it may be that in addition to systemic helminth biasing, there also needs to be local lung biasing.

CHAPTER 3

CHRONIC INFECTION WITH *SCHISTOSOMA MANSONI* REDUCES CELLULAR RESPONSES TO INFLUENZA A[1]

[1] Danz HR, Shollenberger LM, Tundup S, Nagy,T, Harn DA. To be submitted to *Journal of Virology*

Abstract

Helminths infect over 2.5 billion people worldwide, with *Schistosoma* species accounting for approximately 10% of those infections. Chronic infection with helminth parasites biases the host immune system to a CD4+ Th2 and anti-inflammatory phenotype to suppress helminth-induced pro-inflammatory responses. Many helminth-endemic populations are at risk for infection with malaria, tuberculosis, HIV, and multiple other pathogens. Many viral pathogens require host-pro-inflammatory responses to resolve infection. Therefore we evaluated the effect of chronic *Schistosoma mansoni* infection on acute pro-inflammatory Influenza A infection. We observed that schistosome infected mice subsequently infected with influenza had significantly reduced influenza mortality and were able to withstand an 8.5 fold higher dose of influenza compared to schistosome uninfected mice. Similarly, influenza associated morbidity, measured by weight loss and symptom severity scores, was significantly reduced in schistosome infected mice. Specifically, a sublethal influenza infection in schistosome infected mice yielded significantly reduced influenza-specific IFNγ production from mediastinal lymph node (MdLN) cells. Similarly, *ifng* transcript levels from lung tissue of helminth infected mice were significantly reduced during influenza infection compared to helminth uninfected counterparts. Further, helminth infected mice maintained a Th2/anti-inflammatory phenotype in the lungs throughout influenza infection. Together, these data suggest the Th2/anti-inflammatory environment induced during *S. mansoni* infection protects mice from deleterious pro-inflammatory responses induced by influenza, providing a better disease outcome.

Importance

How biased immune systems influence host responses to secondary infections is an area of intense investigation. Mice infected with the helminth parasites *T. spiralis* and *H. polygyrus* and subsequently infected with influenza has yielded conflicting findings. This was likely due to whether the parasites traverse through the lungs. For schistosome, both early larval stages and parasite eggs, traverse or are trapped in lungs, influencing local immune responses. Therefore, the goal of this study was to infect mice with *S. mansoni* to evaluate immune responses to influenza infection. Our findings show that chronic *S. mansoni* infection reduces influenza-specific pro-inflammatory responses systemically and locally in lung tissue. Further, simple injection of schistosome eggs, allows mice to better tolerate influenza infection compared to control mice. These results suggest that infection with helminth parasites having an obligate lung stage, are important in individuals infected with respiratory viruses.

Introduction

Infection with helminth parasites is a global public health problem, with an estimated 2.5 billion cases annually. Approximately 10% of these infections are with *Schistosom*a *spp*. parasites [34, 38]. Schistosome infection is found in approximately 78 tropical and subtropical countries where communities have inadequate water supply systems. Ninety percent of schistosome infections occur in sub-Saharan Africa. *Schistosoma mansoni* is one of the three schistosome species that account for the majority of human infections [34]. Chronic infection with *S. mansoni* is characterized by a systemic biasing of the host immune response towards CD4+ Th2-type and anti-

inflammatory phenotype dominated by an abundance of IL-4, IL-13, and IL-10 [67, 131, 375, 376]. Immune biasing begins at the time of oviposition [11, 130]. Over time, dense granulomas comprised of T cells, eosinophils, and anti-inflammatory alternatively activated macrophages (M2) form around schistosome eggs lodged in liver, lungs, and other organs [4, 9, 377]. M2 macrophages play a role in tissue repair and immune suppression and are distinguished by their expression of Arginase-1 (Arg-1), Relmα/Fizz-1, and Ym-1, as well as their production of anti-inflammatory mediators IL-10 and TGFβ [15, 21, 127].

In contrast to what is observed during schistosome infection, most viral infections induce pro-inflammatory responses. Notably, infection with Influenza A virus results in an acute upper respiratory illness characterized by high levels of pro-inflammatory cytokines including Type I (α/β), Type-II (γ) and Type III (λ) interferons, TNFα, and IL-6 [30, 31, 324, 378]. During influenza infection, the innate immune system and the cellular and humoral arms of the adaptive immune response work together to eliminate the virus [379]. The innate response can control the virus but not eliminate it, whereas the humoral response can take weeks to be fully effective. Therefore, a potent cellular response is necessary to clear the infection before significant damage to the host occurs [380-382]. The cellular immune response to influenza expands influenza-specific CD8+ T cells to conserved immunodominant epitopes from proteins such as NP [27, 383]. Flu-specific CD8+ T cells are cytotoxic and produce high levels of IFNγ.

Schistosome infected individuals may be coinfected with soil-transmitted helminths, *Plasmodium* species, *Mycobacterium tuberculosis*, HIV, and other pathogens, making schistosomiasis a relevant disease for evaluating the effects of helminth-induced

immune modulation on the host response to infection with other pathogens [37, 363-365]. Therefore, in this study, we asked if, and how, the dominant Th2 and anti-inflammatory immune environment found during *S. mansoni* infection in mice would influence pro-inflammatory cellular immune responses to secondary Influenza A infection.

Material and Methods

Infectious agents and animals. For the schistosome life cycle, we obtained *S. mansoni* infected (NMRI strain) *Biomphalaria glabrata* snails from the NIAID Schistosome Reagent Program. Influenza A virus strain A/PR/8/34 (PR8) was a gift from Dr. S. Mark Tompkins (UGA). Infectious virus was propagated in 10 day old specific pathogen-free embryonated chicken eggs for 48 hours and allantoic fluid was recovered. We enumerated virus titer by plaque assay and $TCID_{50}$ using MDCK cells. Six to eight week old female C57BL/6 mice were purchased from Harlan Laboratories. All animals were housed in pathogen-free conditions and allowed to acclimate for 1 week prior to manipulation. All animal work was performed in accordance with UGA and federal regulations and approved by the institutional animal care and use committee.

Schistosome infection. The infectious cercariae were collected from water holding infected snails that had been exposed to direct light for 30-60 mins. Mice were infected by intraperitoneal (IP) injection of 60-75 cercariae. To validate infection, sera were collected from mice eight weeks post infection, and tested by ELISA for binding to schistosome soluble egg antigen (SEA) or adult worm antigen (SWAP), plated at 20ug/ml or 10ug/ml respectively on 96 well plates.

Influenza infection. Age-matched naïve mice and mice infected with schistosomes for nine weeks were infected with influenza. Using 2,2,2-tribromoethanol (TBE, 250mg/kg i.p.) as anesthesia, mice were infected with PR8 influenza virus in PBS by intranasal instillation of 25ul. For median lethal dose (LD_{50}) studies, mice were infected with doses ranging from 10^1 to 10^6pfu of PR8 or were mock infected with PBS alone. For sublethal infection studies, mice were infected with 45pfu of PR8. Post-influenza infection, mice were monitored daily for clinical symptoms; Mice were assigned symptom severity scores based on weight loss [20% (3), 25% (4), 30% (5)] and symptoms, including hunched posture (1), ruffed fur (1), lethargy (2), paralysis (5), seizure (5), and cyanosis (5). A cumulative score of 5 or greater resulted in humane euthanization, which was considered a "death" for mortality plots.

Lymphocyte analysis. Mediastinal lymph nodes (MdLN) were mechanically disrupted and passed through a 70um cell strainer using complete T cell media (RPMI 1640 with 10%FBS, L-glutamine, penicillin, streptomycin and amphotericin B). Cell counts were determined using a TC10 (Bio-Rad). Single cell suspensions were plated at $1.5x10^6$/ml in 200ul/well in 96 well plates. Cells were stimulated for 5hrs with 2ug/ml of irrelevant HPV oncoprotein peptide E7 (aa49-57, RAHYNIVTF), 2ug/ml of specific Influenza A nucleoprotein peptide NP (aa366-374, ASNENMETM), or a mix of 500ng/ml phorbol 12-myristate 13-acetate (PMA) and 50ng/ml ionomycin (PMA/Ionomycin) in the presence of GolgiStop (BD Pharmingen).

Flow cytometry. For surface staining, cells were incubated with Fc Block (BD Pharmingen) on ice followed by anti-CD8α (clone 53-6.7, BD), anti-CD44 (clone IM7, BD), and an H2-D^b restricted tetramer loaded with $NP_{366-374}$ (NIH Tetramer Core Facility,

Emory University). Cells were then stained using a Live/Dead Fixable Staining Kit (Life Technologies) and fixed (CytoFix, BD Pharmingen). Fixed cells were permeabilized (Perm Buffer, BD Pharmingen), followed by incubation with anti-IFNγ (clone XMG1.2, BD). One hundred thousand cells were acquired using an LSRII (BD Biosciences) running FACSDiva (version 6.0, BD) at the CVM Cytometry Core Facility. Data were analyzed using FlowJo software (Version 7.6.5, Tree Star). See Supp. 1 for gating scheme.

Cytokine production by ELISA. Cells from MdLN were recovered, processed, and plated as described above, except GolgiStop was not added. Briefly, cells were stimulated with 2ug/ml irrelevant or specific peptide, 25ug/ml schistosome soluble egg antigens (SEA), or PMA/Ionomycin for 72 hrs, then supernatants collected and stored at -80°C. Levels of IFNγ (BD), IL-10 (BD), and IL-13 (eBiosciences) were determined by ELISA, according to manufacturer's instructions.

Quantitative RT-PCR. The cardiac lobe of the lung was removed and homogenized using a 5mm stainless steel ball in RNA lysis buffer for 15 sec at 25Hz using a TissueLyser II (Qiagen). RNA was isolated using an EZNA Total RNA kit I (Omega) and the concentration measured with a NanoDrop1000 (Thermo Scientific). Samples were normalized to equal concentrations of RNA and converted to cDNA with a High Capacity cDNA kit (Applied Biosystems) using recommended cycling on the DNA Engine Thermal Cycler (Bio-Rad). Real-time qRT-PCR was performed using TaqMan primer/probes (Applied Biosystems) and TaqMan Sensifast HiRox Master Mix (Bioline). Transcript levels of *il10* (Mm00439614_m1), *il4* (Mm00445259_m1), *il13* (Mm00434204_m1), *ifng* (Mm01168134_m1), and *retn1a/Relma* (Mm00445109_m1)

were evaluated against the endogenous control □-Actin (*actb*, Mm00607939_s1). Samples were run on a StepOne Plus (Applied Biosystems) for 40 standard cycles on a 2 hour program and analyzed by $2^{-\Delta\Delta CT}$ [384].

Plaque assays. Whole lungs were homogenized with a 5mm stainless steel ball in 500ul of cold PBS with penicillin, streptomycin, and amphotericin B, using a TissueLyzer II (Qiagen) for 2 min at 25Hz. Homogenized tissue was centrifuged and supernatants aliquoted and stored at -80°C. Plaque assays were performed with MDCK cells by standard protocol [385]. Briefly, monolayers of MDCK cells in 12-well plates were infected with 10-fold dilutions of lung supernatants and incubated for 1hr at 37°C. Wells were overlayed with a mix of 2.4% Avicel (FMC BioPolymer) and MEM containing TPCK-treated trypsin and incubated for 72hr at 37°C. Wells were washed, fixed with methanol/acetone (40/60 v/v), and stained with crystal violet for plaque visualization and enumeration.

Histology and immunohistochemistry (IHC). The left lobe of the lung was removed and put in 10% neutral buffered formalin for 24 hours, then processed, embedded in paraffin blocks, cut into serial sections (5μm thickness), and processed for H&E or IHC staining by the Department of Pathology (UGA College of Veterinary Medicine). H&E sections were blindly scored by Dr. Tamas Nagy, Dept. of Pathology, College of Veterinary Medicine, on a scale of 0-4 (0 = not observed, 4 = severe) for bronchiolitis, peribronchiolitis, vasculitis, perivasculitis, and alveolitis/pneumonitis.

For IHC staining, sections were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was performed using Citra Solution (BioGenex) and proteins were blocked with Power Block (BioGenex). A primary antibody mixture of polyclonal

goat anti-Influenza (Meridian Life Sciences) and polyclonal rabbit anti-Relmα (PeproTech) was incubated for 1hr followed by Alkaline Phosphatase conjugated horse anti-goat IgG (Vector Labs) for 30 min and chromogen development with Vulcan Red (Biocare Medical). Biotinylated goat anti-rabbit IgG (Vector Labs) was applied for 15 min followed by 4+ Streptavidin HPR (Biocare Medical) and chromogen development with DAB/DAB+ (Dako). Slides were counterstained with Gill's Hematoxylin, left to dry and mounted with EcoMount (Biocare Medical). Similar to the histology, IHC sections were evaluated in a blinded manner by Dr. Tamas Nagy, for Relmα staining of macrophages and epithelial cells and were assigned a degree of frequency based on their prevalence in the tissue.

Statistical analysis. A two-way ANOVA with Bonferroni post test was used to assess statistical significance in all figures except Fig. 1A-B for LD_{50} [Trimmed Spearman-Karber method [386]] and survival experiments (Log-rank Mantel-Cox test) using GraphPad Prism version 5. A p value of less than 0.05 was considered significant. P values comparing schistosome infected and uninfected groups on the same day are shown as a horizontal bar with asterisks. Vertical asterisks represent p values comparing a single group against day 0 post influenza infection. P values are represented as *$p<0.05$, **$p<0.01$, ***$p<0.001$, unless otherwise stated.

Results

Coinfected animals exhibit reduced morbidity and mortality at the same viral dose. Previously, infection of mice with the helminth *T. spiralis* was shown to

reduce morbidity to an influenza infection. However, this was only observed during the stages of larval migration, where some larvae pass through the lungs, not during chronic stages [368]. To determine if mice chronically infected with a helminth parasite differed in their ability to survive a secondary viral infection, naïve and *S. mansoni* infected mice were infected with 10^1 to 10^6pfu PR8 strain of Influenza A, then monitored for 14 days to determine the LD_{50}.

Schistosome uninfected mice had an LD_{50} of $10^{2.65}$pfu (Fig. 1A) whereas schistosome infected mice had an increased LD_{50} of $10^{3.58}$pfu (Fig. 1B). We observed that schistosome infected mice fared better overall to influenza infection compared to schistosome uninfected mice, surviving 12 days post-flu infection (dpfi) at 10^3pfu,

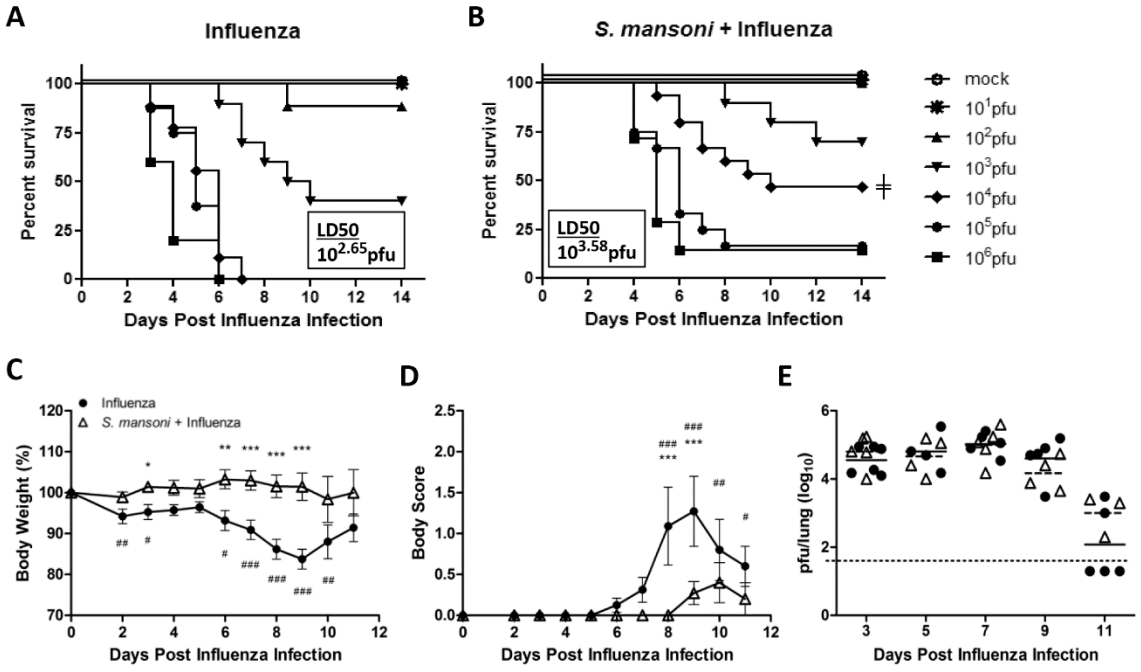

FIG 1 Morbidity and mortality during Influenza A infection. (A) Schistosome uninfected and (B) chronically infected mice were given either PBS or a dose of A/PR/8/34 Influenza virus ranging from 10^1 to 10^6pfu in 10 fold increments via IN route in 25ul and monitored for 14 days for signs of disease. The LD_{50} value was determined by Spearman-Karber calculation. Data was pooled from 2 (A) or 3 (B) independent experiments, n=8-15 per dose of influenza. ǂp= 0.0001 by Log-rank (Mantel-Cox) Test. Mice were infected with a sub-lethal dose (45pfu) of PR8 in 25ul of PBS via IN route. Mice were monitored daily for (C) body weights and for (D) disease severity. (E) Viral lung titers were determined by plaque assays. Representative data shown from duplicate experiments, n≥5. Asterisk (*) denotes significance between groups, pound sign (#) denotes significance against 0 dpfi of the same treatment group.

compared to 10 dpfi respectively, at the same viral dose. The most striking difference was seen at 10^4pfu where schistosome infected mice had significantly increased survival ($p=0.0001$), with 46.7% of the animals surviving compared to no animals surviving in helminth naïve mice.

We next examined morbidity and viral loads during a sublethal infection of PR8 in schistosome infected and uninfected mice. Based on the LD_{50} results, 45pfu ($0.1LD_{50}$) was selected as a dose that would induce productive infection without death. Schistosome uninfected mice began losing weight at 6 dpfi with peak weight loss at 9 dpfi and recovery beginning at 10 dpfi (Fig. 1C). In contrast, schistosome infected mice maintained their weight throughout influenza infection with significantly higher body weights from 6 dpfi through 9 dpfi. Additionally, schistosome infected mice had reduced symptom severity scores over the course of influenza infection (Fig. 1D). Schistosome uninfected mice presented with increased symptom severity scores beginning on 6 dpfi and persisting until 11 dpfi with significant differences at 7, 8, and 9 dpfi. No differences in lung viral loads were detected between the two groups over the course of infection (Fig. 1E). We must note,that at 11 dpfi, virus was detected in lungs of all schistosome infected mice, whereas 60% of schistosome uninfected mice had successfully cleared the virus. Thus, schistosome infection prolonged viral clearance.

Coinfected animals have reduced proportions of IFNγ+ NP-specific CD8+ T cells. Having observed that schistosome infected mice had an increased ability to tolerate subsequent influenza infection, we wanted to next evaluate if schistosome infection altered immune responses to influenza virus. We initially measured responses of CD8+ T cells, as they are important in the response to primary influenza infection. Activated

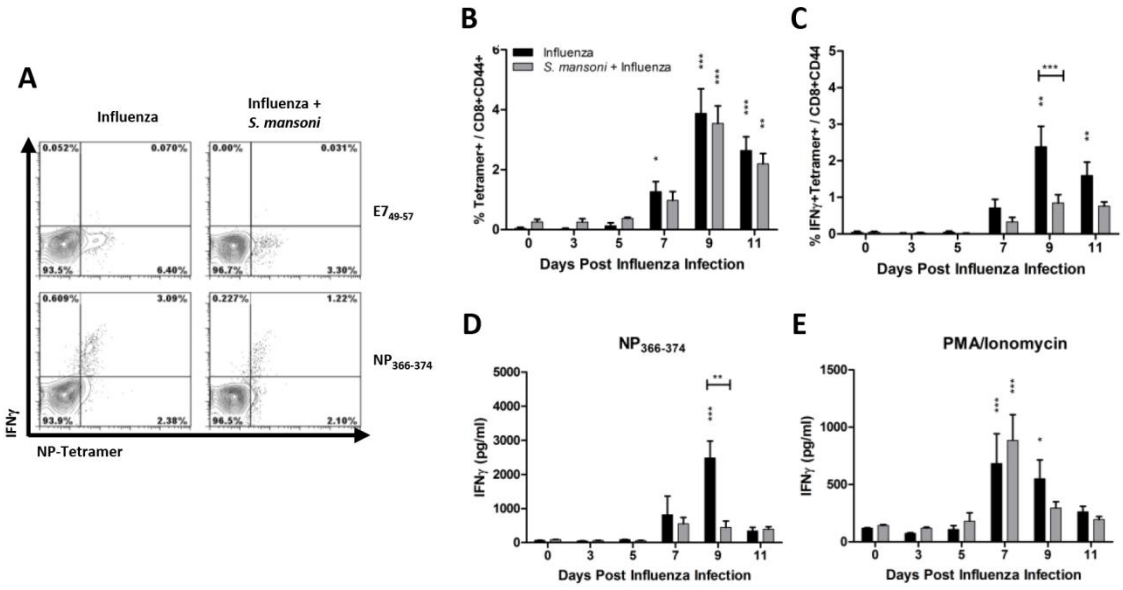

FIG 3.2 Virus specific CD8+ T cells responses in the MdLN. MdLNs were recovered from schistosome infected and uninfected mice over the course of a sublethal infection with PR8 virus. (A-C) Cells were stimulated for 5hrs with either irrelevant peptide or specific peptide in the presence of GolgiStop and stained for evaluation by flow cytometry. (A) Representative flow cytometry contour dot plots from 9 dpfi after a 5hr stimulation with an irrelevant peptide or $NP_{366-374}$ in schistosome infected and uninfected mice. (B) Cells were stained for flow cytometry and analyzed for proportions of NP-tetramer+ cells and (C) NP-tetramer+IFNγ+ after gating on the CD44+CD8+/Live cell population. (D-E) Cells were stimulated for 72hrs to determine cytokine production levels by ELISA. (D) Levels of IFNγ in the supernatant after recall with specific peptide and (E) after stimulation with PMA/Ionomycin (E). Representative data is shown from 2 independent experiments, n=3-5.

MdLN CD8+ T cells were assessed for IFNγ expression and NP specificity. Representative flow cytometric plots are shown for MdLN CD8+CD44+ T cells from schistosome infected and uninfected mice at 9 days post influenza infection after *ex vivo* stimulation with either irrelevant or specific peptide (Fig. 2A). Schistosome infected and uninfected mice had comparable proportions of tetramer-specific cells among activated CD8+ T cells throughout the course of influenza infection (Fig. 2B). For both groups,

tetramer positive cells began to increase at 7 dpfi, peaked at 9 dpfi, and began to contract at 11 dpfi. Further analysis of CD8+ T cells showed that schistosome infected mice had lower proportions of NP-specific IFNγ+ cells, among activated CD8+ T cells, throughout influenza infection (Fig. 2C). Importantly, schistosome infected mice had a significant reduction in proportions of NP-specific IFNγ+ cells, among activated CD8+ T cells, as compared to schistosome uninfected mice at 9 dpfi. Additionally, schistosome infected mice displayed a limited increase in NP-specific, IFNγ+CD8+ T cells over the course of influenza infection, whereas schistosome uninfected mice had significantly increased proportions of these cells at both 9 and 11 dpfi.

MdLN cells from coinfected animals have reduced pro-inflammatory responses to influenza. To further evaluate influenza-specific pro-inflammatory responses, MdLN cells were stimulated with NP peptide, irrelevant peptide or PMA/Ionomycin, and cytokines quantified by ELISA. From day 0 through day 5 post-influenza infection, NP stimulated MdLN cells from schistosome infected and uninfected mice produced limited but comparable amounts of IFNγ (Fig. 2D). Levels of IFNγ began to increase in both groups at 7 dpfi. In schistosome infected mice, concentrations of IFNγ were maintained at 7 dpfi levels for the remainder of the influenza infection. Conversely, schistosome uninfected mice had an increase in IFNγ at 9 dpfi, which was significant both in relation to starting levels and in comparison to schistosome infected mice. Interestingly, at 11 dpfi the level of IFNγ produced by MdLN cells from schistosome uninfected mice was reduced to the level of schistosome infected mice.

In order to determine if schistosome infected mice had a general defect in their ability to produce IFNγ, MdLN cells were non-specifically stimulated with

PMA/Ionomycin (Fig 2E). There was no defect in the ability of schistosome infected mice, as we observed no differences in IFNγ production upon PMA/Ionomycin stimulation of MdLN cells between schistosome infected mice and schistosome uninfected mice.

Coinfected animals have reduced IFNγ transcript levels in lungs and maintain a Th2/anti-inflammatory phenotype during peak influenza responses. Cells from MdLNs of schistosome infected mice infected with influenza had decreased influenza-specific IFNγ responses compared to schistosome uninfected mice infected with influenza. To evaluate responses of the local lung environment, RNA transcript levels in lung tissue were assessed during a sublethal infection with influenza by quantitative RT-PCR. As shown in Figure 3A, *ifng* transcript levels in schistosome infected mice were elevated (7.5 fold at 0 dpfi, 9 fold at 3 dpfi, and 3.5 fold at 5 dpfi) compared to schistosome uninfected mice. At 7 dpfi, transcript levels of *ifng* in both groups rose, though only schistosome uninfected mice had a significantly higher increase compared to starting levels. Transcript levels for *ifng* began to decline in both groups at 9 dpfi.

Transcript levels for the anti-inflammatory cytokine *il10* were low in schistosome infected mice, yet elevated over schistosome uninfected mice at 0 through 5 dpfi (Fig. 3B). Both groups began to display increased *il10* transcript levels at 7 dpfi, however at 9 dpfi, levels of *il10* from schistosome uninfected mice had significantly increased and exceeded that of schistosome infected mice by over 2 fold. At 11 dpfi, *il10* levels decreased to the 0 dpfi level in schistosome infected mice. Though *il10* fell in schistosome uninfected mice, it did not return to starting levels. In schistosome

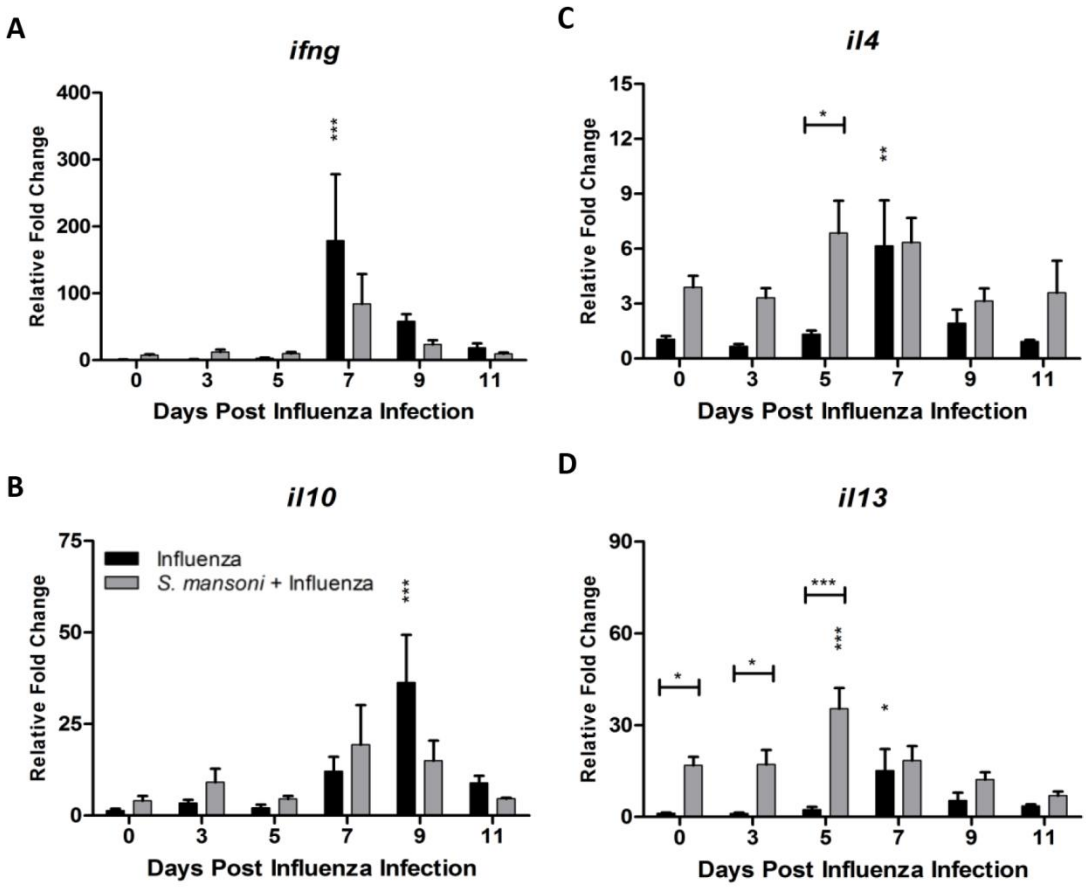

FIG 3.3 Cytokine transcript levels in lungs of influenza infected mice. RNA was recovered from the cardiac lung lobe of schistosome infected and uninfected mice over the course of a sublethal PR8 viral infection and evaluated via quantitative RT-PCR. (A) Transcript levels for *ifng*, (B) *il10*, (C) *il4*, and (D) *il13* were graphed as relative fold change over *actb* levels and normalized to samples from schistosome uninfected mice on day 0 . Representative data is shown from 2 independent experiments, n=3-5.

uninfected mice, transcript levels of *il4* were low early during influenza infection, then rose at 7 dpfi to comparable levels as schistosome infected mice before declining at 9 dpfi (Fig. 3C).

Levels of *il13* were significantly higher in schistosome infected mice secondarily infected with influenza, with 15 fold higher levels at day 0 pfi, 16 fold higher at 3 dpfi

and 15 fold higher at 5 dpfi, compared to schistosome uninfected mice (Fig. 3D). At 7 dpfi, transcript levels for *il13* in schistosome uninfected mice increased to levels similar to schistosome infected mice. The increase in *il13* transcript was short lived, declining by 9 dpfi when *il13* levels in schistosome infected mice were 2.5 fold higher. Even at 11 dpfi *il13* levels in schistosome and flu infected mice remained 2 fold higher than in schistosome uninfected mice.

Inflammation of alveolar tissue is reduced in coinfected animals. Lung homogenates from schistosome infected mice expressed reduced transcript levels of *ifng* at 7 through 11 dpfi. To determine if this reduced pro-inflammatory response affected cellular infiltration to the lungs, a histopathologic analysis of lung tissue was performed. Tissues were scored for bronchiolitis, peribronchiolitis, vasculitis, perivasculitis, and alveolitis/pneumonitis (Supp. 2) and a combined score was tabulated (Fig. 4A). Expectedly, some schistosome infected mice had a low observed score at 0 dpfi, primarily due to perivasculitis (Supp. 2) as a result of chronic helminth infection. Histological micrographs of 0 dpfi from schistosome uninfected and schistosome infected mice show there is ample alveolar airway space in both groups (Fig. 4B). Histology scores for schistosome infected and uninfected groups began to rise at 3 dpfi and by 5 dpfi both groups had significantly higher scores as compared to their 0 dpfi scores, an observation that was maintained through 14 dpfi. Though there are no statistically significant differences in combined scores between groups, representative micrographs from 11 dpfi show increased cellular infiltration and reduced alveolar airway space in schistosome uninfected mice as compared to schistosome infected mice (Fig. 4B).

Within the alveolitic/pneumonitic lesion, no significant differences between schistosome infected and uninfected mice were found (Supp. 2), however, percent tissue involvement did differ between groups (Fig. 4C). The proportion of tissue the alveolitis/pneumonitis lesion encompassed grew steadily during influenza infection in schistosome uninfected mice from 5 dpfi through 14 dpfi. In contrast, schistosome

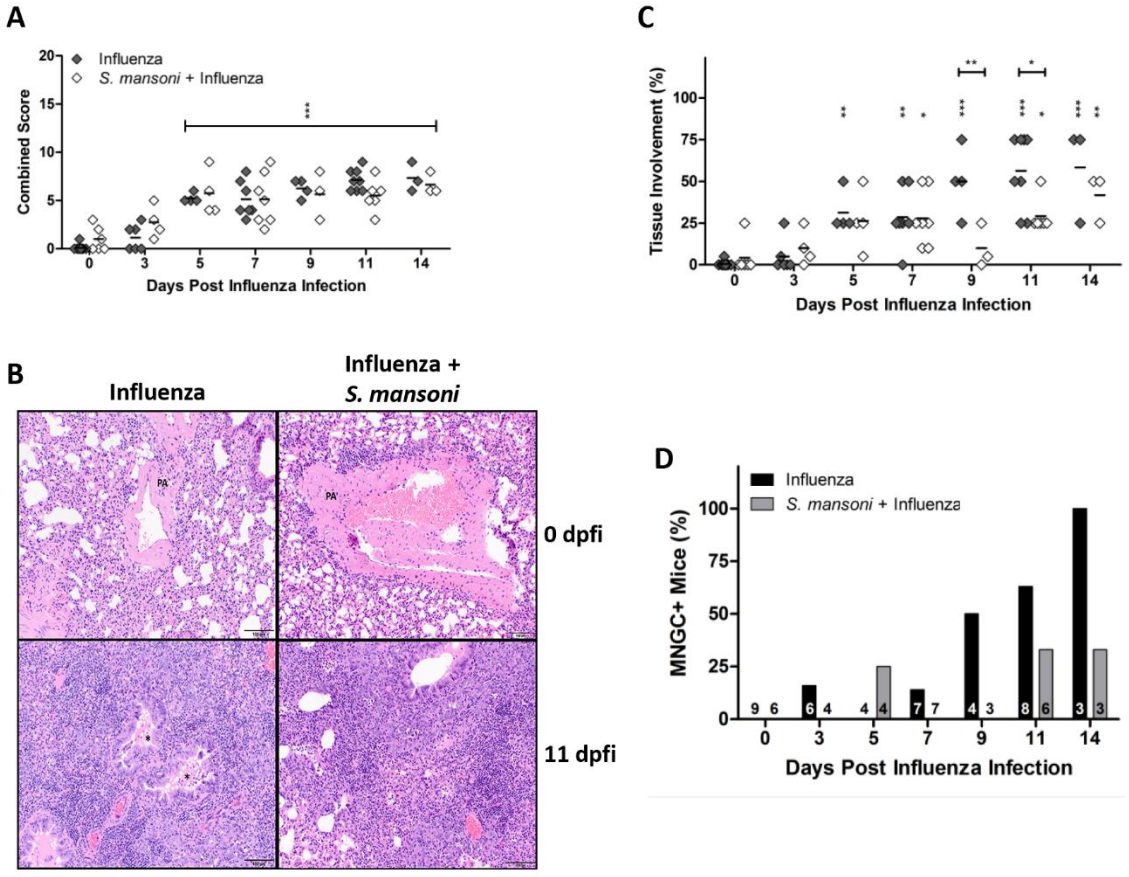

FIG 3.4 Histopathology of pulmonary tissue. H&E stained lung tissue was evaluated for inflammation during a sublethal PR8 infection in schistosome infected and uninfected mice. (A) Combined histopathologic score. Closed diamonds, influenza; open diamonds, *S. mansoni* + influenza. (B) Representative micrographs of tissue sections from schistosome infected and uninfected mice at 0 dpfi and 11 dpfi. PA, pulmonary artery; asterisk, edema; 10X magnification. (C) Proportion of tissue affected by primary inflammatory lesion. (D) Percentage of lung sections that were positive for MNGCs. Pooled data from 3 independent experiments , n=3-9.

infected mice had no significant increases in lesions until 7 dpfi. Additionally, schistosome infected mice had significantly reduced lesion proportions at 9 dpfi and 11 dpfi, remaining reduced until 14 dpfi, compared to schistosome uninfected mice.

We next evaluated multinucleated giant cells (MNGCs) in lung tissues (Fig. 4D). The proportion of schistosome uninfected mice that had MNGCs increased during influenza infection with 14% at 7 dpfi, 50% at 9 dpfi, 63% at 11 dpfi, and 100% by 14 dpfi. In contrast, only a limited number of schistosome infected mice had MNGCs with 33% at 11dpfi and 14 dpfi.

Expression of Relmα by macrophages and alveolar epithelial cells is increased in lungs of coinfected mice. M2 cells contribute to the anti-inflammatory state during chronic schistosome infection, therefore, we wanted to evaluate whether M2 cells influence a secondary influenza infection, or vice versa [152, 153]. M2 macrophages can be characterized by expression of Relmα.

We assessed *relma* RNA transcript levels in lungs by qRT-PCR and found that schistosome infected mice had higher expression than schistosome uninfected mice over the course of influenza infection (Fig. 5A). Schistosome infected mice had significantly higher levels of *relma* at 0 dpfi (14 fold), 3 dpfi (4.5 fold), and 5 dpfi (4 fold) and maintained increased expression over schistosome uninfected mice at 7 dpfi (3.5 fold), 9 dpfi (3.5 fold), and 11 dpfi (2.5 fold). Though not statistically significant, expression of *relma* in schistosome uninfected mice increased by 4 fold at 3 dpfi and this level was maintained throughout influenza infection.

In addition to M2 macrophages, pulmonary epithelial cells can produce Relmα; therefore, to determine which cells are responsible for the increased transcript levels, we

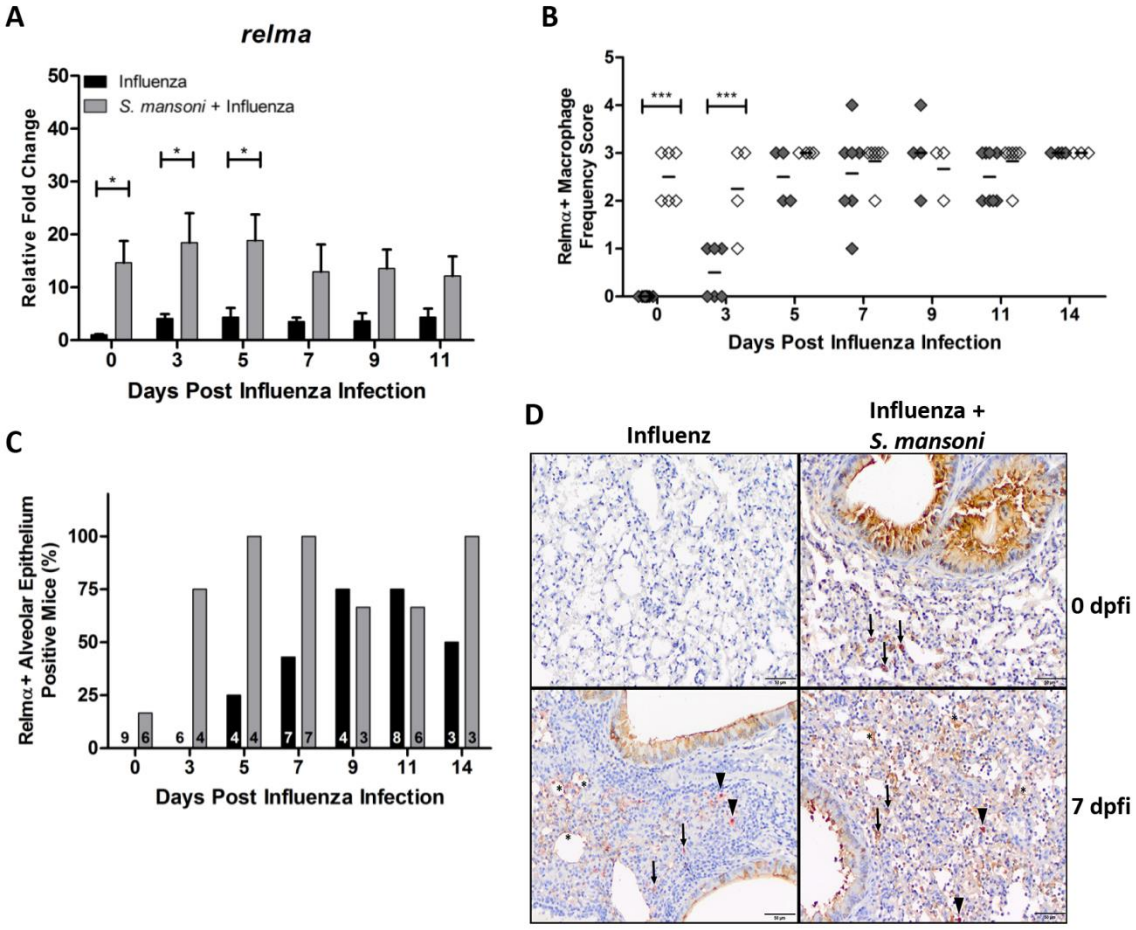

FIG 3.5 Immunohistochemical analysis of Relmα in pulmonary tissue. Lung tissue was evaluated for Relmα expression during a sublethal PR8 infection in schistosome infected and uninfected mice by (A) qRT-PCR and (B-D) immunohistochemistry. (A) Cardiac lung lobe was recovered and evaluated by qRT-PCR. Transcript levels of *relma* were graphed as relative fold change over *actb* and normalized to schistosome uninfected at day 0. Representative data is shown from 2 independent experiments, n=3-5. (B) Left lung lobe sections were evaluated for the frequency of Relmα positive macrophages in the tissue. Closed diamonds, influenza; open diamonds, *S. mansoni* + influenza. (C) Percentage of lung sections that had Relmα positive alveolar epithelial cells. (D) Representative micrographs of lung tissue sections from schistosome infected and uninfected mice at 0 dpfi and 7 dpfi. DAB stained, Relmα; Vulcan red stained, Influenza A virus; arrows, Relmα+ macrophages; arrow heads, Relmaα+influenza+ macrophages; asterisk, Relmα+ alveolar epithelium; 20X magnification. Pooled data from 3 independent experiments, n=3-9 .

performed IHC staining of lung sections for Relmα during influenza infection. Immunohistochemical analysis of lung tissue sections revealed differential Relmα expression in both macrophages and alveolar epithelial cells. Prior to influenza infection, schistosome uninfected mice did not have any detectable Relmα positive macrophages while schistosome infected mice had frequent positive staining of macrophages and this changed very little throughout influenza infection (Fig. 5B). Though the frequency was significantly lower than schistosome infected mice, Relmα positive macrophages were detected in half of schistosome uninfected mice at 3 dpfi, and by 5 dpfi, all schistosome uninfected mice had Relmα positive macrophages. The frequency of Relmα positive macrophages increased slightly at 7 dpfi and achieved peak levels at 9 dpfi.

We also observed Relmα staining of the alveolar epithelium during influenza infection in schistosome infected and uninfected mice (Fig. 5C). At 0 dpfi, alveolar epithelium staining was absent in schistosome uninfected mice, whereas we observed Relmα staining in alveolar epithelium of a single mouse in the schistosome infected group, (16.7%). Schistosome uninfected mice remained negative for alveolar epithelial expression of Relmα at 3 dpfi, while 75% of schistosome infected mice were positive. At 5 dpfi and 7 dpfi 100% of schistosome infected mice were positive for alveolar epithelial expression of Relmα, compared with only 25% and 43% positive at 5 and 7 dpfi respectively in schistosome uninfected mice., Peak levels for schistosome uninfected mice were 75% of mice at days 9 and 11 dpfi. Schistosome infected mice had a temporary decline in detection of Relmα positive alveolar epithelium staining to 67% of on 9 and 11 dpfi, which increased to 100%, by 14 dpfi. Conversely, Relmα staining of alveolar epithelium decreased to 50% at 14 dpfi in schistosome uninfected mice.

Representative micrographs of lung sections from 0 dpfi and 7 dpfi schistosome uninfected and infected mice are shown (Fig. 5D). As previously noted, we did not detect Relmα positive macrophages or alveolar epithelial cells at 0 dpfi in schistosome uninfected mice. Not surprising, schistosome infected mice had Relmα positive macrophages present in clusters and scattered throughout the lung tissue at 0 dpfi. In addition to Relmα, we also co-stained for Influenza A, which can be seen at the site of inflammation at 7 dpfi in lung tissue from both schistosome infected and schistosome uninfected mice. Relmα positive macrophages were primarily localized to areas with virally infected cells and the alveolar epithelium was diffusely positive for Relmα. In schistosome infected mice, intensely stained Relmα positive macrophages and alveolar epithelial cells accumulated. Both groups were positive for Relmα in alveolar epithelial cells though staining was limited to sites of inflammation.

Discussion

How coinfections influence disease morbidity, immune and vaccine responses, is an important problem. In particular, the mechanism by which chronic helminth infections may alter the ability of the immune system to respond to acute secondary infections is an important area of research that remains largely unexplored. *Trichinella spiralis* and *Heligmosomoides polygyrus* are helminth model infections used to examine coinfection immune alterations. However, both of these model helminth infections have some limitations, notably for *H. polygyrus*, is the lack of a lung stage during the parasite life cycle [366]. Organisms that pass through/or reside in the lungs will bias local immune responses. Thus, helminthes that do not traverse or reside in the lungs may not be the best

models to examine subsequent immune responses to respiratory infections. For example, Reese *et al.* found that mouse herpesvirus (MHV), a respiratory pathogen with a splenic latency phase, is reactivated following infection with *H. polygyrus* [367, 387]. In contrast, Apiwattanakul *et al.* determined that *H. polygyrus* infection had no effect on influenza infection morbidity or mortality [366]. Interestingly, when examining the influence of *T. spiralis* on influenza induced responses, Furze *et al.* found reduced virus-specific responses in mice coinfected with *T. spiralis* [368]. Importantly, Furze determined that the alterations were limited to the early stage of the helminth life cycle (7 days post helminth infection), when females produce larvae that transiently migrate through pulmonary blood vessels, eliciting a Th2-type response [388]. Sixty day patent *T. spiralis* infections where the lung phase has been absent for about seven weeks, do not show an altered immune response to secondary influenza infection. These studies establish that helminth infections can result in altered immune responses to secondary viral infections, and may be partly dependent on what organs the helminth parasites traverse through, or reside in. Thus, there appears to be significant outcome differences depending on whether the helminth parasite has no lung stage, has a lung stage, has a transient lung stage, or has a chronic lung stage as in *S. mansoni* infection, with eggs gradually being trapped in lungs over the course of chronic infection. This observation likely will hold true for other respiratory viruses such as RSV, measles, nipah, MERS, and parainfluenza, all of which are important in helminth endemic regions.

In this study, we observed that schistosome infected mice had reduced morbidity and mortality during an infection with the PR8 strain of Influenza A. In studies to establish the median lethal dose of PR8 influenza we found that *S. mansoni* infected mice

were able to withstand significantly higher doses of influenza virus, compared to schistosome uninfected mice, indicating that schistosome infection provides some level of protection. Importantly, this is the first time the median lethal dose of influenza has been determined in mice chronically infected with a helminth parasite compared to that of normal mice. The starkest contrast in morbidity/mortality was seen with the 10^4pfu dose of influenza, with all schistosome uninfected mice succumbing to infection, compared to nearly 50% survival in schistosome infected mice. Weight loss and symptom severity scores illustrated the "positive" effects a concurrent schistosome infection had on secondary influenza infection morbidity. Schistosome infected mice continued to eat, were active, and showed little outward signs of disease. Attempting to correlate these observations to human disease, it may mean that helminth coinfected individuals will not exhibit all symptoms of illness due to influenza, resulting in a reduction in negative impact of influenza infection on daily life. In a negative sense, coinfected people might attend work or school, where they would spread influenza infection.

Prior to the 2009 H1N1 Influenza A pandemic (caused by the pH1N1 strain), there was minimal influenza monitoring in Africa. A post-pandemic surveillance study of pH1N1-specific neutralizing antibodies determined the number of infections in Djibouti, Africa was drastically under-reported [389]. Only 9 cases were reported for the entire country over the combined 2009/2010 and 2010/2011 circulating influenza seasons, however, based on circulating antibody levels, an estimated 29% of the population was exposed,[389]. If helminth-infected individuals exhibit less symptoms of illness during

influenza infection, they are less likely to seek medical attention, which could result in the apparent under reporting of infection in Djibouti.

We observed no differences in the kinetics of viral clearance between helminth-infected and uninfected groups, which agrees with an earlier study in *T. spiralis* infected mice using H3N2-X31 Influenza A strain [368]. Taken together, the decreased morbidity and mortality observed in schistosome infected mice was not the result of reduced viral replication, as both schistosome infected and uninfected mice had similar viral titers until day 11 post influenza infection. At this time point, schistosome infected mice still had detectable virus in the lungs, whereas schistosome uninfected mice had cleared the virus.

The cellular arm of the adaptive immune system is integral in resolving influenza infection [390-392]. Because schistosome infected mice have CD4+ Th2-type and anti-inflammatory biased responses, we hypothesized there would be a reduction in pro-inflammatory responses to influenza infection, particularly in CD8+ T cells. Support for such a hypothesis comes from the study by Osborne *et al.*, showing a reduction in the total number of virus-specific CD8+ T cells in the lungs during early stage *T. spiralis* infection [369].

Here, we found no differences in proportions of NP-specific CD8+ T cells between schistosome infected and uninfected mice post-influenza infection. Schistosome infected mice did have significantly decreased proportions of NP-specific CD8+ cells producing IFNγ. The observed defect in CD8+ T cell responses was influenza specific and not a general defect, as non-specific stimulation of MdLN cells with PMA/ionomycin revealed no differences in IFNγ production between schistosome infected and uninfected mice. The observation that PMA/ionomycin stimulation induced equivalent levels of

IFNγ production from MdLN cells from schistosome infected and uninfected mice is interesting in light of higher levels of IL-10 and IL-13 from these same PMA/Ionomycin treated cells (Supp. 3). Further studies aimed at determining if there are differences in CD8+ T cell cytotoxic capability, via killing assay or detection of perforin and/or granzyme B, would help in evaluating the functionality of these cells in the context of schistosome infection.

The immune response to chronic helminth infection is complex. In schistosome infection while there is overall systemic biasing to a Th2/anti-inflammatory phenotype, there are pro-inflammatory responses to eggs freshly deposited into host tissues [393-396]. Because schistosome eggs can be found in the lungs of chronically infected mice and human patients [8, 377, 394], we were not surprised to find elevated transcript levels of *ifng* in the lung tissue of schistosome infected mice at day 0 post-influenza infection.

In contrast to pro-inflammatory responses, IL-4, IL-13 and IL-10 are prototypic Th2/anti-inflammatory cytokines, all of which are upregulated during helminth infection. Therefore, the observed elevated transcript levels of these cytokines in schistosome infected mice prior to influenza infection was expected. That the levels of these cytokines in schistosome infected mice were maintained over the course of the influenza infection was an unexpected finding, and likely contributes to the reduced IFNγ levels and symptom severity scores. Since IL-10 is a master regulatory cytokine, its later rise correlates with the reductions in *ifng*, *il4* and *il13* levels. Overall, these results indicate that the cellular milieu in the lungs of schistosome infected mice given a secondary influenza infection is predominantly CD4+ Th2-type and anti-inflammatory while the

cellular milieu in the lungs of schistosome uninfected mice is considerably more pro-inflammatory.

The observation of increased transcript levels for both *il4* and *il13* in the schistosome uninfected group at 7 dpfi may be partly explained by earlier studies showing that influenza infection can result in IL-4- and IL-13-producing type-2 innate lymphoid cells (ILC2s) [397, 398]. One other explanation for the increased levels of *il4* and *il13* could be the presence of M2 macrophages. Induction of M2 macrophages *in vivo* using the DcR3 decoy receptor was demonstrated to reduce inflammation mediated pathology due to influenza infection [399]. However, the influence of M2 macrophages on disease induced by viral lung pathogens varies based on the virus. For example, Osborne *et al.* demonstrated that M2 macrophages induced by the helminth parasite *T. spiralis* actually impaired mouse norovirus clearance [369]. A putative role for M2 macrophages as anti-inflammatory cells was shown during, RSV infection of IL-4Rα deficient mice (required for IL-4/IL-13-dependent M2 activation) [400]. The IL-4Rα -/- mice had more severe lung inflammation due to RSV than wild-type mice. It was additionally shown that adoptive transfer of wild type M2 macrophages into IL-4Rα -/- mice reduced RSV-induced lung pathology [400]. One of our observations was that influenza infection induced M2 macrophages in the lungs of schistosome uninfected mice, where they appeared quickly post-influenza infection and were maintained throughout the monitoring period. This profile of M2 upregulation is consistent with previous findings based on flow cytometry data during PR8 infection prior to a secondary bacterial infection (*Streptococcus*) [271]. By comparison, we found that the frequency of M2 macrophages in schistosome infected mice was not greatly altered by influenza

infection, likely due to the significantly elevated levels in schistosome infected mice prior to influenza infection.

Another lung cell type that can produce Relmα, a marker we used to identify alternative activation (M2), are alveolar epithelial cells. We observed a time dependent increase of alveolar epithelial cells positive for Relmα. Though typically associated with alternative activation of macrophages, expression of Relmα by lung epithelial cells has been known since its discovery [157, 158]. Most studies evaluating epithelial Relmα in lungs have focused on induction during hypoxic conditions and allergic responses [155, 401-404]. In an IFN-γR-/- model of γ-hepesvirus infection, an increase of epithelial Relmα positive cells in lungs was detected by IHC staining, however, these are Th2 biased mice, likely influencing upregulation of Relmα [405]. Our results demonstrate that Relmα expression is not limited to M2 macrophages or a Th2-type environment, rather it can be induced in alveolar epithelial cells isolated from areas of severe inflammatory lesions in response to influenza infection. In the aforementioned models of hypoxia and allergy, epithelial cell expressed Relmα and Relmβ, a sister protein, were implicated in stimulating airway remodeling and fibrogenesis [155, 406]. Therefore, we suggest that Relmα may have a similar effect in our model.

We expected the influence of a Th2 and anti-inflammatory biased lung environment prior to and during influenza infection would be a reduction in influenza associated inflammation in the lungs. To that end, histological staining to of lungs for inflammation showed little difference in terms of histological scores for schistosome infected or uninfected mice. What we did find was a difference in the size of the alveolitic/pneumonitic lesions in the two groups. Pneumonitis is one of the most severe

symptoms of influenza infection and is characterized by lesions of cellular infiltration and edema of the alveolar spaces [201, 247]. These severe lesions, which are generally contiguous in nature, spread through the tissue, reducing available alveolar space. This results in poor blood oxygenation and an increased likelihood of death [407, 408]. Thus, the smaller lesion proportions we observed in schistosome infected mice may be directly responsible for reduced symptom severity scores and weight loss detected during influenza infection. As IFNγ is a known inducer of severe lung pathology in response to influenza infection [28, 409, 410], it is likely the lower *ifng* transcript levels detected in lungs of schistosome infected mice resulted in reduced cellular recruitment to the inflammatory lesions, diminishing overall lesion size and improving or maintaining oxygenation levels. Further tests would be necessary to evaluate this.

Histopathologic and IHC analysis also revealed an unexpected observation. We noted very few MNGCs of monocyte origin in coinfected mice compared to schistosome uninfected mice. MNGCs are typically found in immune responses of a granulomatous nature against pathogens such as *M. tuberculosis*, however, they have also been found in response to SARS virus and HIV-1 [411, 412]. Additionally, MNGCs have been shown to be induced by IFNγ both *in vitro* and *in vivo* [411-414]. Coinfected mice exhibited reduced *ifng* transcript levels in lung homogenates and, based on the aforementioned SARS and HIV-1 research, this correlates with reduced numbers of coinfected mice which were positive for MNGCs. Though we present no data to show the function of MNGCs in this system, this is the first documentation of these cells being present in lung tissue during influenza infection.

Taken together, our results demonstrate that induction and maintenance of a Th2 and anti-inflammatory lung environment during chronic *S. mansoni* infection provides protection against Influenza A infection. An increased LD_{50} value and reduced morbidity suggest there is a defect in pro-inflammatory responses in schistosome coinfected mice. We demonstrated this defect is in IFNγ production by CD8+ T cells, resulting in reduced cellular activation and recruitment to the site of infection, thereby reducing severe lung pathology. This study demonstrates that chronic *S. mansoni* infection alters anti-viral immune responses during influenza infection. Worldwide, billions of people are infected with one or more immune modulatory helminth parasites. Thus, the implications of these results are vast considering the number of other pro-inflammatory viruses circulating in these populations. Additional studies examining the influence helminth parasites may have on host immune responses and pathology to secondary infections need to be performed so that we can gain a greater understanding regarding their potential benefit or harm to patients, depending on the secondary infecting pathogen.

Acknowledgements

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B.glabrata snails were provided by the NIAID Schistosomiasis Resource Center of the Biomedical Research Institute (Rockville, MD) through NIH-NIAID Contract HHSN272201000005I for distribution through BEI Resources. Flow cytometry data was acquired at the CVM Cytometry Core Facility at the University of Georgia, School of

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Supplemental Figures

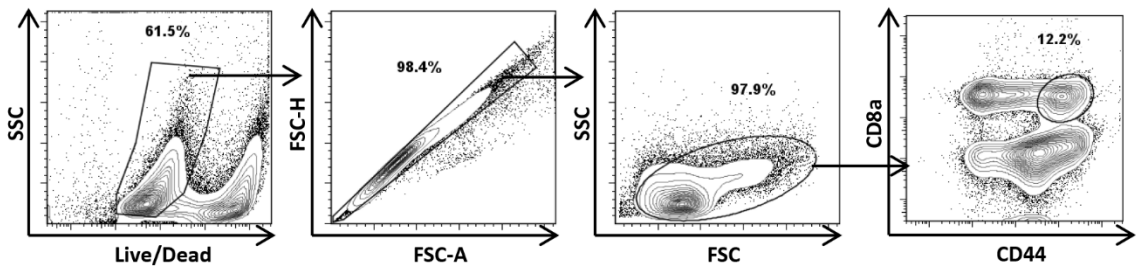


FIG S3.1 Flow cytometry gating. Gating scheme for evaluation of CD8+ T cells from the MdLN used in FIG 2.

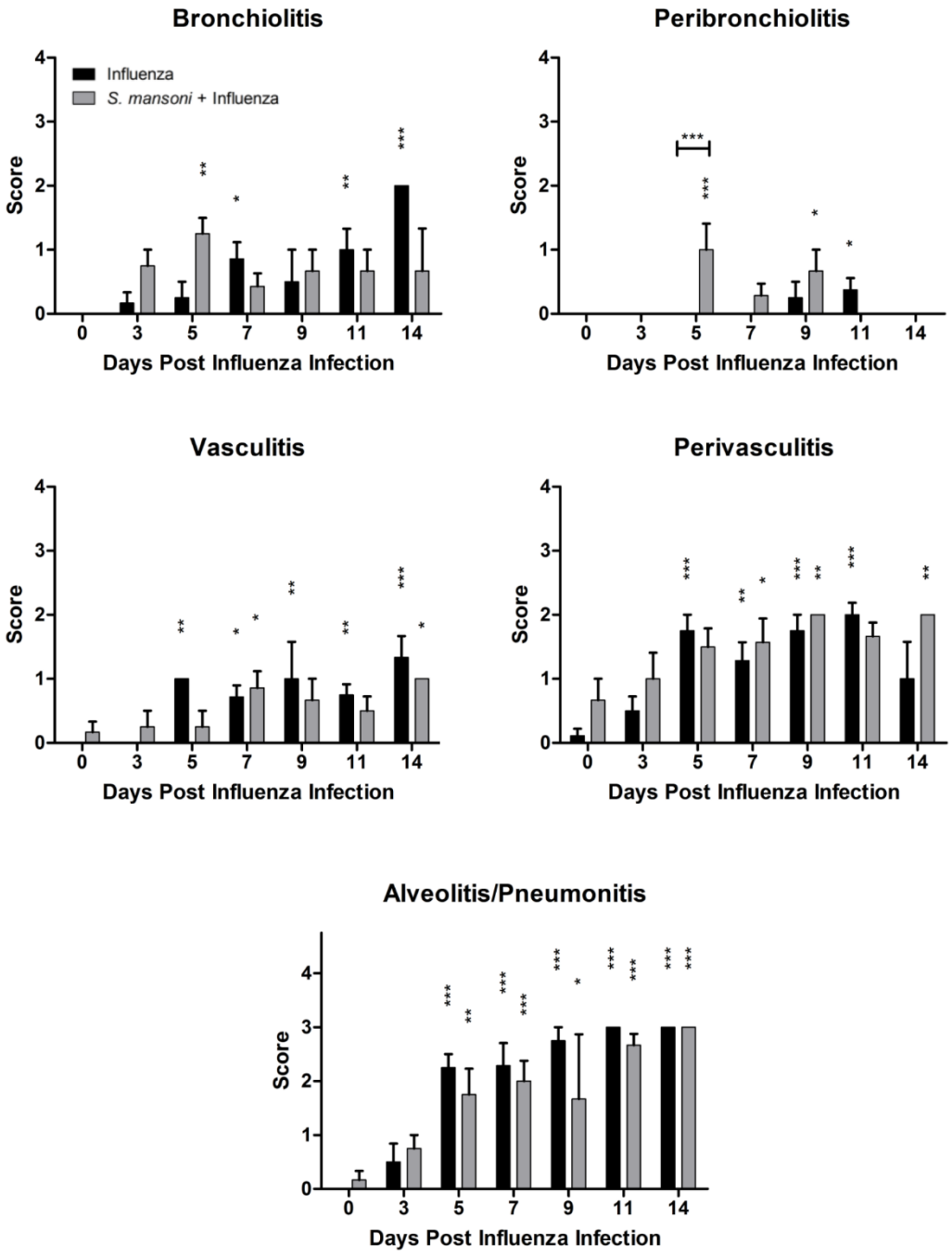


FIG S3.2 Histopathology evaluation. H&E stained lung tissue was evaluated for inflammation during a sublethal PR8 infection in schistosome infected and uninfected mice and scored on a scale of 0-4 for (A) bronchiolitis, (B) peribronchiolitis, (C) vaculitis, (D) perivasculitis, and (E) alveolitis/pneumonitis. (F-G) Representative tissue lesions of alveolitis/pneumonitis at 11 dpfi in (F) schistosome uninfected and (G) schistosome infected at 2X magnification. Pooled data from 3 independent experiments is shown, n=3-9. *p<0.05, **p<0.01, ***p<0.001 by two-way ANOVA with Bonferroni post-test.

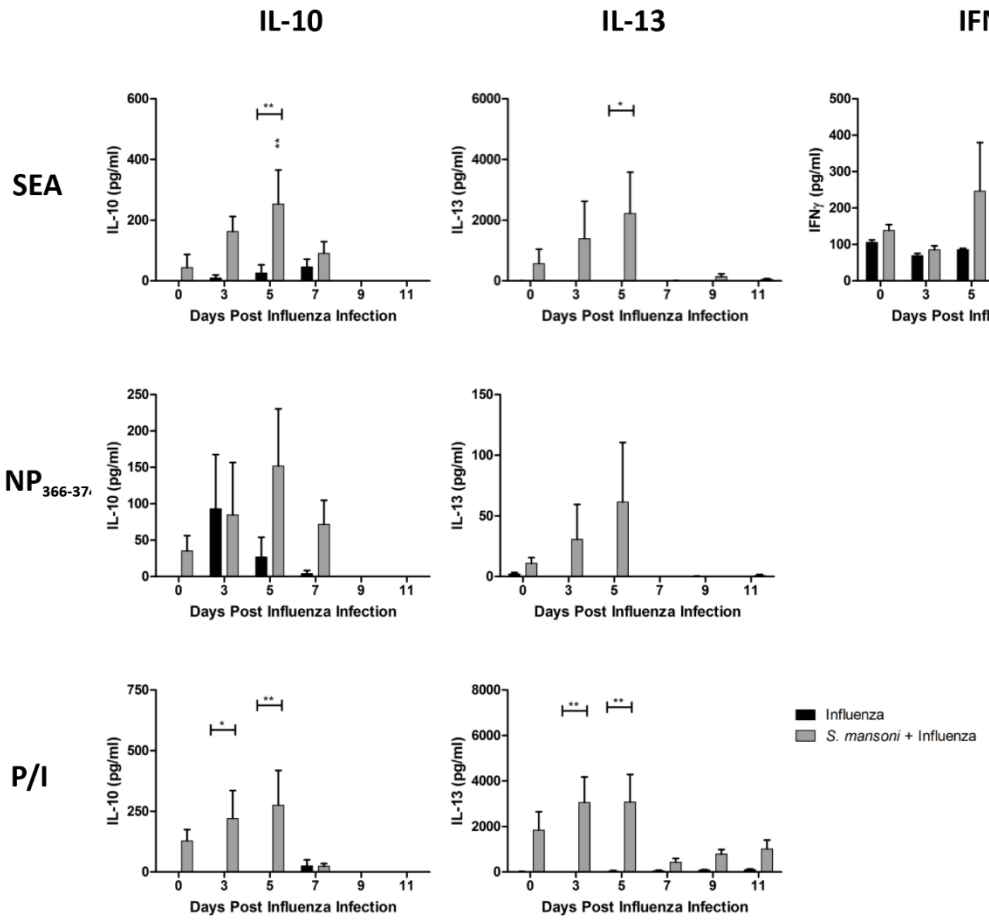


FIG S3.3 Cytokine production by stimulated MdLN cells. MdLNs from schistosome infected and uninfected mice were recovered over the course of a sublethal infection with PR8 virus and lymphocytes were stimulated with specific peptide, SEA, or PMA/Ionomycin (P/I) for 72hrs to determine levels of IL-10, IL-13, and IFNγ by ELISA. Representative data from 2 independent experiments is shown, n=3-5. *p<0.05, **p<0.01, ***p<0.001 by two-way ANOVA with Bonferroni post-test.

CHAPTER 4

INVESTIGATE THE MECHANISM(S) OF PROTECTION AGAINST INFLUENZA A IN SCHITOSOME EGG-BIASED MICE

Introduction

Helminth parasites are found worldwide resulting in billions of human infections [33]. *Schistosoma* species account for more than 250 million infections, with *S. mansoni* accounting for nearly one third of those infections [1, 34, 38]. Adult schistosomes reside in mesenteric plexus blood vessels, or those of the urinary bladder, producing hundreds of eggs per day. Many of these eggs get lodged in tissues of the intestines, liver, and lungs [4, 8, 13, 102, 134]. Dense granulomas form around implanted eggs and consist of macrophages, T cells, and eosinophils [163]. Schistosome eggs secrete glycoproteins and glycans, including Omega-1 and Lewis^X, which bias the immune system to a Th2 and anti-inflammatory state characterized by an abundance of IL-4, IL-5, IL-10, and IL-13 [8, 12, 67, 68, 377, 394].

During schistosome infection, IL-4 and IL-13, produced by CD4+ T cells, stimulates macrophage polarization to M2s that produce IL-10 and TGFβ, promoting an anti-inflammatory environment that influences Treg differentiation [138, 415]. M2 macrophages also function to promote tissue repair by inducing collagen production by fibroblasts [148]. Activation markers of M2 macrophages include Arginase-1, Ym1, and Resistin-like molecule-alpha (Relmα), all of which modulate the host environment [146,

148]. Relmα can also be produced by pulmonary epithelial cells and is upregulated in response to infection with both *S. mansoni* and Influenza A, as shown in Chapter 3 [154, 155].

Seasonal Influenza A virus infects approximately 15% of the world's population each year resulting in mild to severe respiratory symptoms [26]. Various cell types are involved in mediating a response against a primary influenza infection. This includes a strong CD8+ T cell response for eliminating the infection before significant damage to the host occurs [383, 390, 416, 417]. CD8+ T cells destroy infected cells by releasing cytotoxic granules, which contain proteins that destroy cytoplasmic and nuclear membranes [358, 359, 418]. Additionally, CD8+ T cells produce IFNγ to facilitate differentiation and activation of cell types such as CD4+ Th1 cells and classically activated macrophages (M1) [410].

In Chapter 3 we demonstrated that mice chronically infected with *S. mansoni* had reduced morbidity and mortality when coinfected with Influenza A. Coinfected mice exhibited suppressed IFNγ responses from influenza-specific CD8+ T cells and immunohistochemical analysis of lung tissue revealed increased proportions of Relmα positive M2 macrophages as well as decrease in size of pneumonitis lesions. These data indicate that schistosome infection dampens IFNγ-mediated responses against influenza and this suppressed response may be due to anti-inflammatory Relmα expressing M2 macrophages. Therefore, we performed this study to determine if M2 macrophages contribute to the reduction in pro-inflammatory responses and increased survival during influenza infection.

Schistosome infection results in strong biasing to a Th2 and anti-inflammatory immune phenotype, however it is well documented that pro-inflammatory responses also occur as a result of egg deposition in host tissues [393]. Schistosome egg deposition results in IL-6 and TGFβ induction and triggers differentiation of CD4+ T cells to IL-17 producing Th17 cells [394, 419]. That Th17 cells contribute to increased egg granuloma pathology was suggested by a study showing that egg granuloma pathology can be reduced by anti-IL-17 treatment [394, 395, 420]. Of note, IFNγ has been shown to be a negative regulator of IL-17 induced granuloma immunopathology [396].

Post-influenza infection, IL-17 is produced by lung resident γδT cells [421]. Upregulation of IL-17 producing Th17 cells results in reduced mortality to influenza [422]. Furthermore, human survival rates to influenza infection have been correlated to increased plasma levels of IL-17 [423]. Therefore, because IL-17 producing cells are common during schistosome and influenza infections, we asked if IL-17 producing cells were involved in mediating protection in coinfected mice.

During chronic *S. mansoni* infection, egg granuloma alterations in hepatic portal pressure results in angiogenesis, including hepatic portal shunts, allowing eggs to traffic directly to the pulmonary vasculature [84, 100, 101]. Injection of mice with schistosome eggs is a longstanding and valuable model for studying egg-specific effects on pulmonary immunopathology [193, 194, 424-427]. Therefore, in this Chapter we utilized the schistosome egg-induced lung granuloma model (lung granuloma mice) to evaluate the role of M2 macrophages and IL-17 producing cells. Here we report our findings on specific cell types induced as a result of schistosome egg injection, and whether or not these cells influence secondary influenza infection.

Materials and Methods

Infectious agents and animals. We obtained *S. mansoni* infected (NMRI strain) *Biomphalaria glabrata* snails from the NIAID Schistosome Reagent Program. These snails were maintained in a BSL2 facility and shed under light to induce production of the infectious cercariae stage. Influenza A virus strain A/PR/8/34 (PR8) was a gift from Dr. S. Mark Tompkins (UGA). Infectious virus was propagated in 10 day old specific pathogen-free embryonated chicken eggs for 48 hours afer which we recovered allantoic fluid and determined viral titers using plaque assay and $TCID_{50}$ with MDCK cells.

Six to eight week old female C57BL/6 and BALB/c mice were purchased from Harlan Laboratories. Eight week old male and female breeding pairs of IL-4Rα -/- mice were from Taconic Biosciences. IL-4Rα -/- breeding was synchronized to ensure age matched litters. Pups were separated from Dams at 21 days old, then used at six to eight weeks old. Mice were housed in pathogen-free conditions and allowed to acclimate for 1 week prior to manipulation. All animal work was performed in accordance with UGA and federal regulations and approved by the institutional animal care and use committee.

Acute schistosome infection. Infectious cercariae were obtained by exposing patent, infected snails to direct light for 30-60 mins. Water containing cercariae was collected and the numbers of cercariae/ml determined by direct count. Mice were infected by intraperitoneal (IP) injection of 150-200 cercariae. *S. mansoni* infected mice were maintained for six to seven weeks at which time they were euthanized, livers collected, and eggs isolated as previously described [193].

Schistosome egg injection. Six to eight week old mice were injected with *S. mansoni* eggs to generate egg-induced lung granuloma mice, as previously reported [193, 428]. Briefly, schistosome eggs were diluted to 25,000 eggs/ml in 1xPBS and then 5000 eggs were intraperitoneally injected in 200ul. Two weeks later, mice were intravenously injected with 5000 eggs. One week post intravenous egg injection, mice were used experimentally.

Adoptive transfer of macrophages. We obtained classically activated macrophages (M1) from mice by peritoneal lavage, three days post-IP injection with 1ml of 3% thioglycolate. We obtained alternatively activated macrophages (M2) by peritoneal lavage of mice seven days post-IP injection with 5000 schistosome eggs. M1 and M2 donor mice were sacrificed by CO_2 inhalation and peritoneal exudate cells (PECs) were recovered by washing the peritoneal cavity with cold PBS. Three milliliters PECs were plated at 1-2x10^6/ml in 6 well tissue culture plates and incubated for 1hr at 37^o. Next, non-adherent cells were discarded, plates gently washed and then incubated on ice for 10 mins. After 10 mins on ice, cells were scraped from the wells, collected, washed and then counted. Live cells were adjusted to 5x10^6/ml in PBS. Recipient mice were injected with 200ul of adherent cells by IV tail injection. Cells were kept on ice at all times, unless otherwise stated.

Influenza infection. One week post-IV injection of eggs, aged matched naïve and egg injected mice were infected with influenza. Mice were anesthetized using 2,2,2-tribromoethanol (TBE, 250mg/kg IP), then infected with PR8 influenza virus in PBS by intranasal instillation of 25ul. For median lethal dose (LD_{50}) studies, mice were infected with doses ranging from 10^1 to 10^6pfu of PR8 or mock infected with PBS alone. For

sublethal infections, mice were infected with 45pfu of PR8, unless otherwise stated**.** Mice were monitored daily for body weights and signs of disease to enumerate symptom severity. Mice were assigned scores based on weight loss [20% (3), 25% (4), 30% (5)] and symptoms, including humped back (1), ruffled fur (1), lethargy (2), paralysis (5), seizure (5), and cyanosis (5). A cumulative score of 5 or greater resulted in humane euthanization, which was considered a "death" for mortality plots.

Lymphocyte analysis. Mediastinal lymph nodes (MdLN) were mechanically disrupted and passed through a 70um cell strainer using complete T cell media (RPMI 1640 with 10%FBS, L-glutamine, penicillin, streptomycin and amphotericin B). Cell counts were determined using a TC10 (Bio-Rad), then single cell suspensions were plated with $1.5x10^6$/ml using 200ul/well in 96 well plates. Cells were stimulated for 5hrs with 2ug/ml of irrelevant HPV oncoprotein peptide E7 (aa49-57, RAHYNIVTF), 2ug/ml of specific Influenza A nucleoprotein peptide NP (aa366-374, ASNENMETM), or a mix of 500ng/ml phorbol 12-myristate 13-acetate (PMA) and 50ng/ml ionomycin (PMA/Ionomycin) in the presence of GolgiStop (BD Pharmingen).

Flow cytometry. For surface staining, cells were incubated with Fc Block (BD Pharmingen) on ice followed by anti-CD8α (clone 53-6.7, BD), anti-CD44 (clone IM7, BD), and an H2-D^b restricted tetramer loaded with $NP_{366-374}$ (NIH Tetramer Core Facility, Emory University). Cells were then stained using a Live/Dead Fixable Staining Kit (Life Technologies) then fixed with CytoFix (BD Pharmingen). Fixed cells were permeabilized in (Perm Buffer BD Pharmingen), followed by incubation with anti-IFNγ (clone XMG1.2, BD). One hundred thousand cells were acquired using an LSRII (BD

Biosciences) running FACSDiva (version 6.0, BD) at the CVM Cytometry Core Facility. Data were analyzed using FlowJo software (Version 7.6.5, Tree Star).

Quantitative RT-PCR. Cardiac lobes of lungs were removed and homogenized using 5mm stainless steel balls in RNA lysis buffer for 15 sec at 25Hz using a TissueLyser II (Qiagen). Aliquots of adherent cells used for adoptive transfers were lysed for RNA analysis. RNA was isolated using the EZNA Total RNA kit I (Omega) and the concentration measured using a NanoDrop1000 (Thermo Scientific). Samples were normalized to equal concentrations of RNA and converted to cDNA with a High Capacity cDNA kit (Applied Biosystems) using recommended cycling on the DNA Engine Thermal Cycler (Bio-Rad). Real-time qRT-PCR was performed using TaqMan primer/probes (Applied Biosystems) and TaqMan Sensifast HiRox Master Mix (Bioline). Transcript levels of *nos2* (Mm00440502 _m1), *arg1* (Mm00475988 _m1), *ym1* (Mm00657889_m1), and *il17* (Mm00439618_m1) were evaluated against the endogenous control β-Actin (*actb*, Mm00607939_s1). Samples were run on a StepOne Plus (Applied Biosystems) for 40 standard cycles on a 2 hour program and analyzed by $2^{-\Delta\Delta CT}$ [384].

Plaque assays. Whole lungs were homogenized with the TissueLyser II (Qiagen) for 2 min at 25Hz using 5mm stainless steel balls in 500ul of cold PBS with penicillin, streptomycin, and amphotericin B. Homogenized tissue was centrifuged and supernatants, collected, aliquoted and stored at -80°C. Plaque assays were performed with MDCK cells by standard protocol [385]. Briefly, monolayers of MDCK cells in 12-well plates were infected with 10-fold dilutions of lung supernatants and incubated for 1hr at 37°C. Wells were overlayed with a mix of 2.4% Avicel (FMC BioPolymer) and MEM

containing TPCK-treated trypsin and incubated for 72hr at 37°C. Wells were washed, fixed with methanol/acetone (40/60v/v), and stained with crystal violet for plaque visualization and enumeration.

Histology and immunohistochemistry (IHC). The left lobe of lungs were removed and placed in 10% neutral buffered formalin for 24 hours, then processed and embedded in paraffin blocks. Following paraffin embedding, blocks were cut into serial sections (5μm thickness), and processed for H&E or IHC staining by the Department of Pathology (UGA College of Veterinary Medicine).

For IHC staining, sections were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was performed using Citra Solution (BioGenex) and proteins were blocked with Power Block (BioGenex). Polyclonal rabbit anti-Relmα (PeproTech) or polyclonal rabbit anti-IL-17 (Abcam) antibodies were incubated with lung sections for 1hr then washed with 0.05% TBST. HRP conjugated goat anti-rabbit IgG (Cell Signaling) was applied for 30 mins and washed with 0.05% TBST before chromogen development with DAB/DAB+ (Dako). Slides were counterstained with Gill's Hematoxylin, left to dry and mounted with EcoMount (Biocare Medical).

Statistical analysis. A two-way ANOVA was used to assess statistical significance in all experiments except for LD_{50} [Trimmed Spearman-Karber method [386]] and survival experiments (Log-rank Mantel-Cox test) using GraphPad Prism version 5. A p value of less than 0.05 was considered significant. P values comparing schistosome infected and uninfected groups on the same day are shown as horizontal bars with asterisks. Vertical asterisks represent p values comparing a single group against day

0 post-influenza infection. P values are represented as *p<0.05, **p<0.01, ***p<0.001, unless otherwise stated.

Results and Discussion

Lung granuloma mice are highly resistant to influenza induced mortality.

The lung granuloma mouse model was used to evaluate the effects of schistosome egg-induced immune biasing on secondary influenza infection. Mice were injected intraperitoneally with purified eggs, followed two weeks later by an intravenous injection of eggs which traffic to and are trapped in lungs. This leads to a large number of egg granulomas in the lungs making this model especially useful for evaluating the effect local egg-induced responses have on a pulmonary infection with influenza.

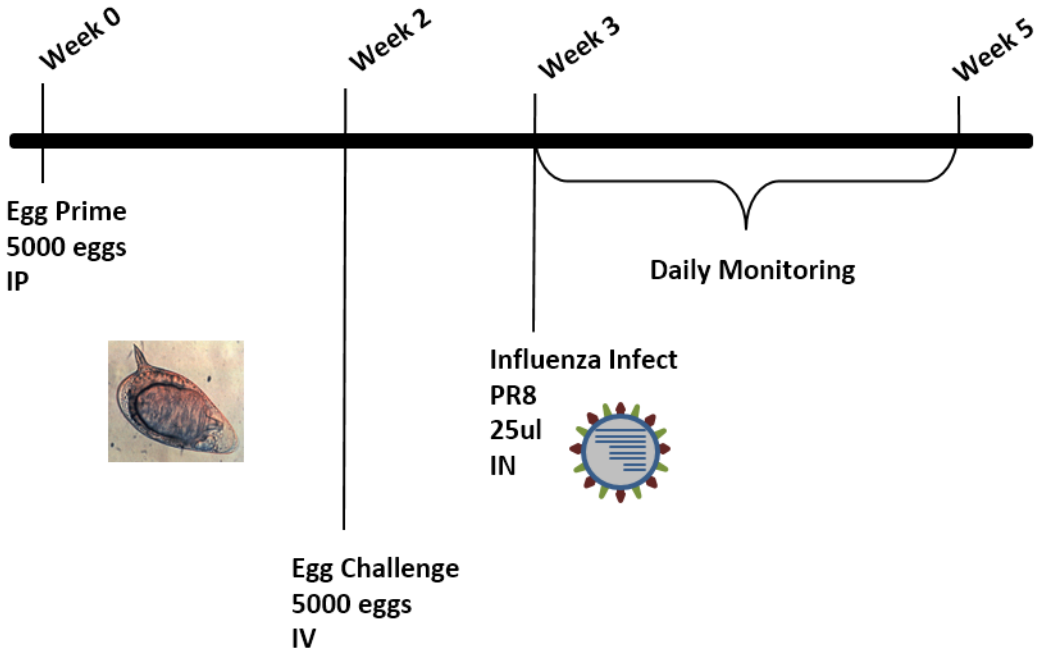


Fig 4.1 Injection timeline. Mice were IP injected with *S. mansoni* eggs followed two week later with an IV injection of S. mansoni eggs. Mice were left to rest for one week before infection with Influenza A

Chapter 3 demonstrated that mice infected with *S. mansoni* were able to withstand higher infecting doses of Influenza A virus coincident with reduced IFNγ responses as compared to naive mice infected with influenza. We hypothesize that this is the result of Th2 and anti-inflammatory biasing induced by schistosome eggs. To properly employ the egg granuloma model for these experiments, we initially determined the LD_{50} for influenza in lung granuloma mice. Mice sensitized and then IV injected with schistosome eggs were subsequently infected with 10^1 to 10^6pfu PR8 strain of Influenza A and monitored for 14 days for signs of disease (Fig. 4.1). Egg granuloma mice were found to have an LD_{50} of $10^{3.99}$pfu (Fig 4.2). When contrasted against the LD_{50} values reported in Chapter 3 for both naïve ($10^{2.65}$pfu) and schistosome infected ($10^{3.58}$pfu) mice, lung granuloma mice had a 22 fold and 2.5 fold higher LD_{50}, respectively. These data show that the schistosome egg granuloma model results in increased tolerance to influenza infection and confirms that schistosome eggs likely contribute to this effect.

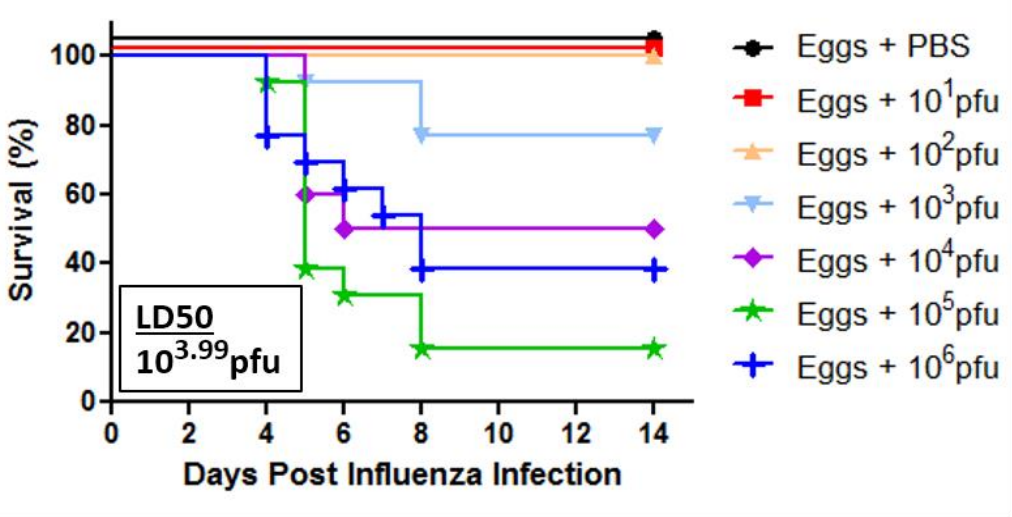


Fig 4.2 Lung granuloma mouse survival during Influenza A infection. Egg injected mice were given PBS or a dose of A/PR/8/34 Influenza virus in 10-fold increments ranging from 10^1 pfu to 10^6 pfu by IN route in 25ul. Mice were monitored for 14 days for signs of disease. Data was pooled from 3 independent experiments, n=3-13 per dose of influenza

Schistosome egg-induced CD8+ T cells are cross-reactive to influenza antigens. To further characterize the response of lung granuloma mice to secondary influenza infection, egg injected mice were infected with a sublethal dose of influenza and monitored for signs of disease. In addition, mediastinal lymph nodes (MdLNs) were evaluated for CD8+ T cell responses to determine what role this cell type has during influenza infection in egg injected mice.

During a sublethal influenza infection, lung granuloma mice had significantly reduced loss of body weight compared to normal mice (Fig. 4.3A). In fact, lung granuloma mice maintained their body weights through 9 dpi with the greatest weight loss seen at 8 dpi (–1.5%). Moreover, lung granuloma mice actually began to gain weight at 10 dpi, and by 12 dpi had gained a significant amount of weight (+6%) compared to day 0 weight. In contrast, naive mice infected with influenza began to lose weight at 5 dpi, lost a significant amount of weight starting at 6 dpi and had the greatest weight loss

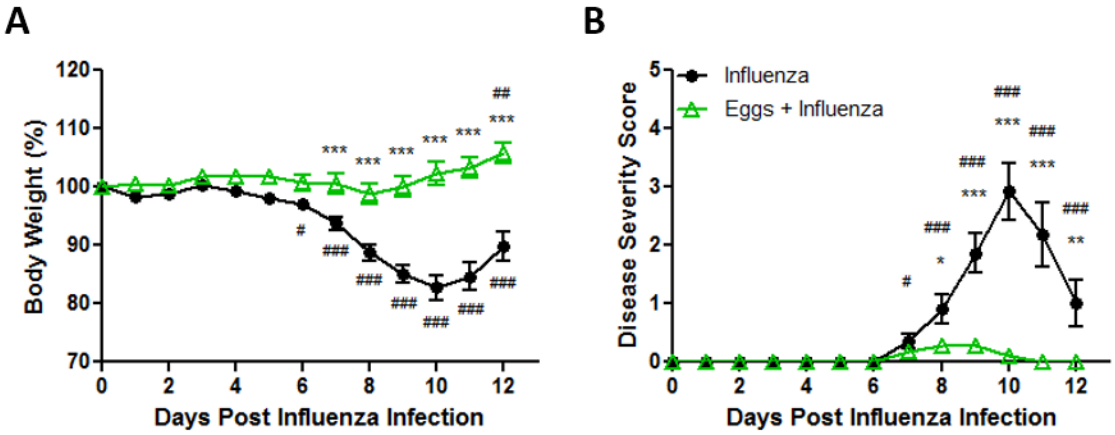


Fig 4.3 Morbidity of lung granuloma mice during Influenza A infection. Mice were infected with a sub-lethal dose (45 pfu) of PR8 influenza virus. (A) Mice were monitored for body weights and (B) disease severity daily for 12 days. Data from single experiment shown, n≥5. Asterisk (*) denotes significance between groups, pound sign (#) denotes significance against 0 dpi in the same treatment group.

at 10 dpi (–17%), compared to lung granuloma mice secondarily infected with influenza. Naive mice infected with influenza began to recover body weight at 11 dpi, however remained significantly lower at 12 dpi as compared to their starting weights (–10%).

Symptom severity scores reflected body weight loss for both groups (Fig. 4.3B). Lung granuloma mice exhibited only mild signs of disease from 7 dpi to 10 dpi. Naïve and influenza infected mice began to show signs of disease at 7 dpi, with symptom severity rising significantly by 8 dpi. Symptom severity continued to increase significantly and peaked at 10 dpi, corresponding with the timepoint of greatest body weight loss (Fig. 4.3A). For egg granuloma mice, symptom severity quickly declined, whereas influenza infected naive mice showed significantly greater signs of disease even at 12 dpi as compared to lung granuloma mice.

Both lung granuloma and naïve mice had significantly higher proportions of NP-specific CD8+ cells at 9 and 12 dpi as compared to day 0. However, lung granuloma mice had significantly higher proportions of these cells compared to influenza infected naive mice (Fig 4.4A). Interestingly, lung granuloma mice had elevated proportions of NP-specific cells from the activated CD8+ T cell population at 0 and 3 dpi.

Further evaluation of NP-specific activated CD8+ T cells that were IFNγ positive showed no significant differences between naive and lung granuloma mice during influenza infection (Fig. 4.4B). As compared to day 0, normal and lung granuloma mice began to produce increased proportions of NP-specific, IFNγ positive, activated CD8+ T cells at 9 dpi and both groups had significantly higher proportions at 12 dpi.

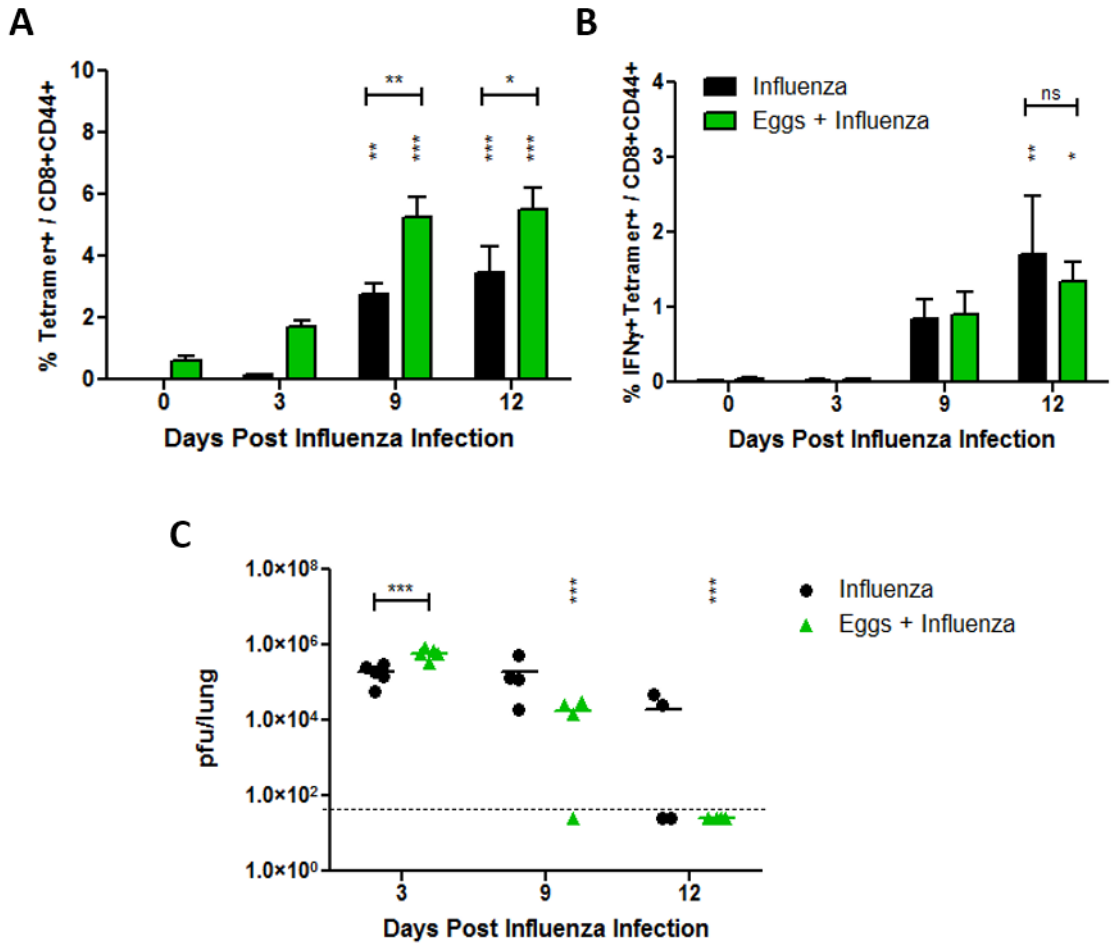


Fig 4.4 Influenza specific CD8+ T cell responses in lung granuloma mice. Normal and egg injected mice were infected with a sub-lethal dose of PR8 virus (A-B) MdLNs were recovered and cells were stimulated with specific or irrelevant peptide for 5 hrs in the presence of GolgiStop then stained for evaluation by flow cytometry. (A) Cells were analyzed for proportions of NP-tetramer+ cells and (B) NP-tetramer+IFNγ+ after gating on the CD44+CD8+/Live cell population. (C) Whole lungs were harvested and viral titers were determined by plaque assay. Data from a single experiment is shown, n=4-6.

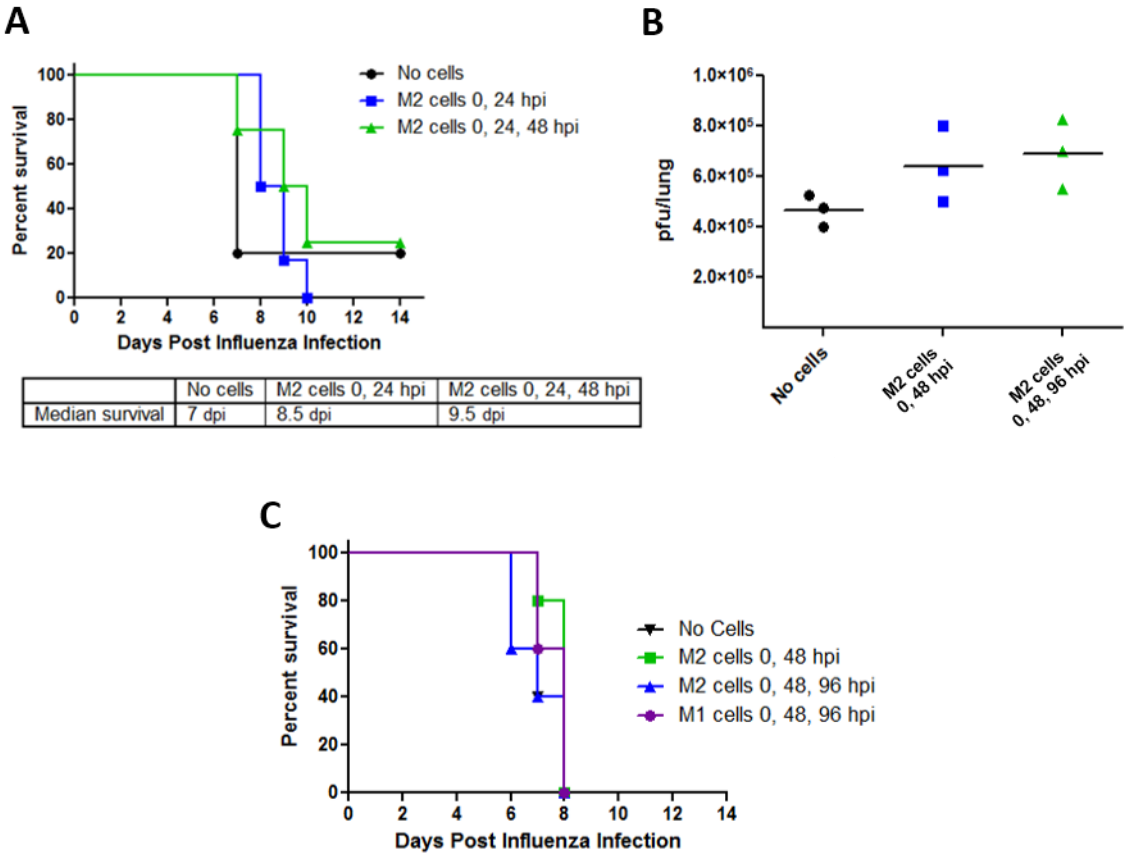
We also evaluated viral lung titers over the course of a sublethal influenza infection (Fig. 4.4C). Lung granuloma mice had significantly higher viral titers at 3 dpi as compared to normal mice. At 9 dpi, 25% of lung granuloma mice had cleared the virus and there was a significant reduction in viral load as compared to 3 dpi. In contrast, all

influenza infected naive mice remained infected at 9 dpi with similar titers as 3 dpi. One hundred percent of lung granuloma mice cleared the virus by 12 dpi, whereas 50% of influenza infected naive mice remained infected at this timepoint.

These data suggest that schistosome egg antigen stimulated CD8+ T cells cross-reactive to influenza-specific peptides may be capable of attacking virus infected cells early during infection. Correlating this observation to viral titer data, lung granuloma mice possessing a population of cross-reactive CD8+ effector cells in addition to infection induced influenza-specific CD8+ effectors, may explain the observed faster viral clearance in lung granuloma mice compared to influenza infected, naïve mice. Further investigation on whether schistosome eggs are able to induce influenza cross-reactive CD8+ T cells needs to be performed to confirm this.

Beneficial influence of Adoptive transfer of M2 macrophages into WT mice during influenza infection may depend on time of transfer post-influenza infection. Cross-reactive CD8+ T cells may assist in protection against secondary influenza infection in lung granuloma mice. Interestingly, we did not see this in secondary influenza infection of schistosome infected mice. Therefore, to continue with our analysis of potential cell types involved in response to schistosome eggs, we next evaluated the roles M2 macrophages may play during influenza infection.

Adherence purified egg-induced M2 macrophages were adoptively transferred into naïve recipients. M2 macrophage recipient mice were then infected with 10^3pfu of influenza and monitored for signs of disease. Using this dose of influenza we normally observe moderate death in untreated mice. Thus, if M2 macrophages are protective, this dose should result in reduced mortality to infection.



	No cells	M2 cells 0, 24 hpi	M2 cells 0, 24, 48 hpi
Median survival	7 dpi	8.5 dpi	9.5 dpi

Fig 4.5 Adoptive transfer of M2 macrophages to WT mice. (A-C) Naïve mice were administered PBS or 10^6 macrophages by IV injection and infected with 100 pfu of PR8 influenza virus. (A) M2 macrophages were injectedat 0 and 24 hpi or 0, 24, and 48 hpi and monitored for signs of disease, (B) Lungs were recovered at 5 dpi from the three groups and viral titers were determined by plaque assay. (C) M2 macrophages were injected at 0 and 48 hpi or 0, 48, and 96 hpi or M1 macrophages were injected at 0, 48, and 96 hpi and mice were monitored for signs of disease

Two studies were performed where 10^6 M2 macrophages were injected into naïve WT mice. In the first study, cells were injected twice at 0 and 24 hours post-infection (hpi) or three times at 0, 24, and 48 hpi and compared against PBS injected mice (Fig. 4.5A). Mice that received three cell transfers had a median survival of 9.5 days, which was 1 day longer than mice receiving two transfers and 2.5 days longer than PBS mice.

Enumeration of viral lung titers at 5 dpi revealed a modest but discernible increase detected in mice receiving M2 cell transfers (Fig. 4.5B). This experiment suggests that adoptive transfer of M2 macrophages is protective but does not reduce viral lung titers.

The second experiment not only added a third injection, but we also included an additional group injected with classically activated M1 macrophages (Fig 4.5C). Mice were given two M2 macrophage injections at 0 and 48 hpi, three M2 macrophage injections at 0, 48, and 96 hpi, three M1 macrophage injections at 0, 48, and 96 hpi, or injected with PBS. Unfortunately, in this experiment we observed no differences in survival against influenza infection between any treatment groups. The major difference in the two experiments is the lack of an adoptive transfer at 24 hpi in the second experiment where we did not see any differences. A third experiment comparing the adoptive transfers performed in experiment 1 with experiment 2 needs to be performed to confirm that this timepoint is the critical difference. If we see that this is the case, it would argue that M2 macrophages can provide protection if administered early in infection, but not when administered later.

Macrophages are among the cell types that influenza can infect, therefore an increase in viral lung burdens in macrophage recipient mice was anticipated as these mice were provided with additional cells that could be infected. Survival outcomes of influenza infected M2 macrophage injected mice appeared promising, yet even a small variation in injection schedules ablated any observed effects. Additionally, these studies were performed using fully immune competent wild-type mice making it difficult to determine if M2 macrophages were specifically responsible for any observed effects. In

order to isolate the effects of M2 macrophages during influenza infection we employed IL-4Rα -/- mice.

Using an altered adoptive transfer schedule we find that M2 macrophages are detrimental to survival of mice during influenza infection. M2 macrophages use IL-4Rα signaling to induce polarization to an alternative activation state. IL-4Rα -/- mice

A

MΦ donor mouse	MΦ activation state	MΦ recipient mouse
WT	M2	WT
WT	M2	KO
KO	null	KO
—	—	KO

B

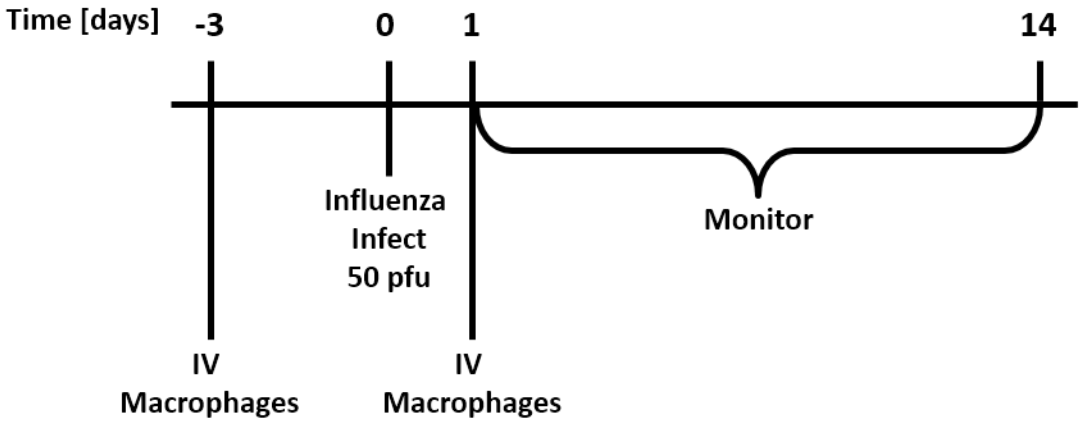


Fig 4.6 Adoptive transfer matrix and injection timeline. (A) Matrix of macrophage donor mouse strain, macrophage recipient mouse strain, and activation state of transferred macrophages. (B) Experimental timeline of macrophage injection and influenza infection. WT, wild type strain; KO, IL-4Rα -/- strain.

are unable to respond to IL-4 or IL-13 making this knockout mouse a useful model for evaluating the requirement of M2 macrophages during influenza infection. Following a transfer schedule adapted from previous work using the immunomodulatory helminth glycan LNFPIII [429], egg-induced M2 macrophages were adoptively transferred by IV injection from WT or IL-4Rα -/- donor mice into WT or IL-4Rα -/- recipient mice at –3 and +1 days post infection with influenza (Fig. 4.6A-B). In this manner, an M2 macrophage-driven lung environment is induced by the -3 dpi injection and then

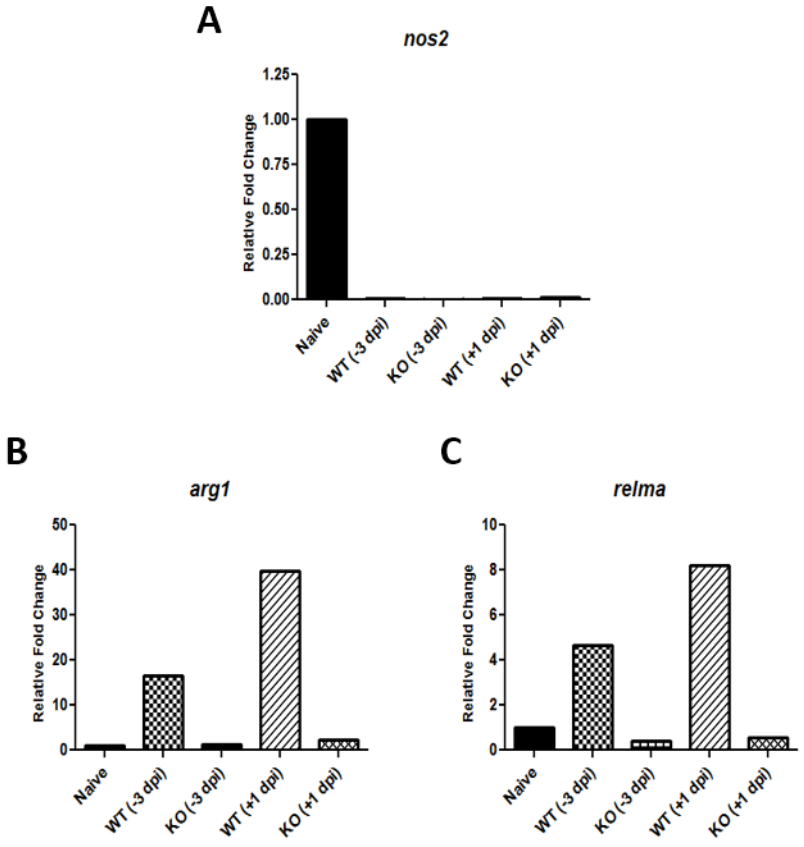


Fig 4.7 qRT-PCR of adoptively transferred macrophages. An aliquot of pooled cells from each adoptive transfer group and day were lysed for RNA analysis by qRT-PCR. Transcript levels for M1 the marker (A) *nos2* and M2 markers (B) *arg1* and (C) *relma* were graphed as relative fold change over *actb* and normalized to naïve cell. Data from a single experiment, n=1.

reinforced by the +1 dpi injection. Additionally, in this experiment we also used male and female recipient mice to determine if sex of the recipient influences the outcome.

Macrophages were obtained by peritoneal lavage of WT and IL-4Rα-/- mice that had been IP injected with purified *S. mansoni* eggs. One week post-injection, peritoneal cells were collected and macrophages isolated by plate adherence purification. Macrophages were analyzed by qRT-PCR for phenotypic M1 (*nos2*) and M2 (*relma* and *arg1*) gene markers (Fig. 4.7A-C). Relative expression of M2 macrophage transcripts were increased in WT donor macrophages with expression of M1 macrophage transcripts severely diminished. Macrophages isolated from IL-4Rα -/- mice did not have increased transcripts of M1 or M2 phenotypic genes, indicating these macrophages were not associated with either activation state and therefore referred to as null macrophages.

Recipient mice were given 10^6 macrophages by IV injection at -3 and +1 dpi, as depicted in the injection schedule (Fig. 4.6B). Mice were infected with a sublethal dose (50pfu) of influenza and monitored daily for signs of disease. We expected to find that IL-4Rα -/- mice receiving IL-4Rα -/- macrophages (KO→KO) would have a poor response to infection, whereas WT mice receiving WT macrophages (WT→WT) would be unaffected. Further that IL-4Rα -/- mice receiving WT macrophages (WT→KO) would fare better than KO→KO, as a result of receiving fully competent WT M2 polarized macrophages.

Surprisingly, we found that both WT and IL-4Rα -/- mice receiving WT M2 macrophages were more susceptible to influenza with significantly increased mortality in

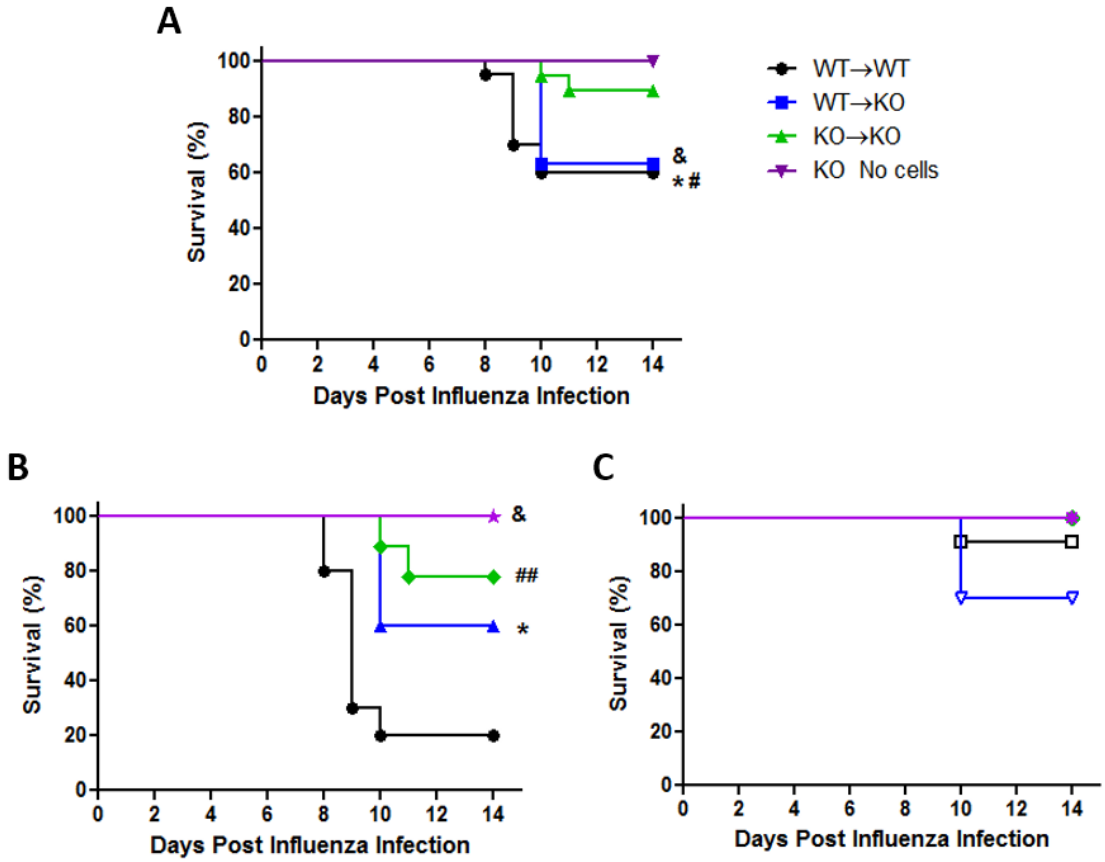


Fig 4.8 Adoptive transfer of WT and KO M2 macrophages. Macrophages from BALB/C WT and KO mice were recovered and transferred as depicted in Fig. 4.6. All mice were infected with 50 pfu of PR8 virus and monitored for signs of disease. Survival outcomes were graphed as (A) male and female mice, (B) female only, and (C) male only. Asterisk (*) denotes significance between WT→WT and WT→KO; pound sign (#), between WT→WT and KO→KO; ampersand (&) between WT→WT and KO no cells by Log-rank (Mantel-Cox) Test. Pooled data from two independent experiments, n=5-11 per sex, per group.

response to a sublethal dose of virus (Fig 4.8A). The KO group that did not receive any donor macrophages was the only group with 100% survival following influenza infection. Groups receiving WT M2 macrophages had a significant reduction in survival with mortality rates of 40% in WT→WT and 37% in WT→KO. KO mice receiving KO null macrophages had a mortality rate of 10%, the lowest of any group receiving

macrophages. These results did not fit with the results seen in WT mice in either experiment 1 or experiment 2 discussed in the previous section. Unfortunately, here, we used a different adoptive transfer schedule than was used in experiment 1 or experiment 2 of the preceeding section with the major difference being the –day 3 timepoint used here. Therefore, to draw conclusions from these studies, repeat experiments should be performed using the timeline for adoptive transfer employed in experiment 1 of the previous section, where we saw a protective influence of adoptively transferred M2 macrophages.

Furthermore, when data were analyzed by sex of the recipient mice, we saw striking differences in survival outcomes. Comparing female KO mice not given cells to female WT→WT, significant differences in survival were observed in 20% of female WT→WT mice surviving to 14 dpi (Fig. 4.8B). Female WT→KO and female KO→KO groups had significantly greater survival rates of 60% and 78%, respectively.

In contrast, male mice had limited responses to infection with influenza and no significant differences in survival were found between any groups (Fig. 4.8C). Male KO mice that received WT M2 macrophages had the lowest survival proportion of 70%. In the WT→WT group there was a single mortality resulting in 91% survival. There were no mortalities in the male KO→KO group or in the male KO mice that did not receive macrophages. Comparing mortality rates between sexes it is clear that females fare worse than males in all groups receiving macrophages (Fig. 4.9). This disparity is most apparent when comparing male and female WT→WT groups where there is a significant 70% difference in survival post-influenza infection. As noted earlier, these data suggest that

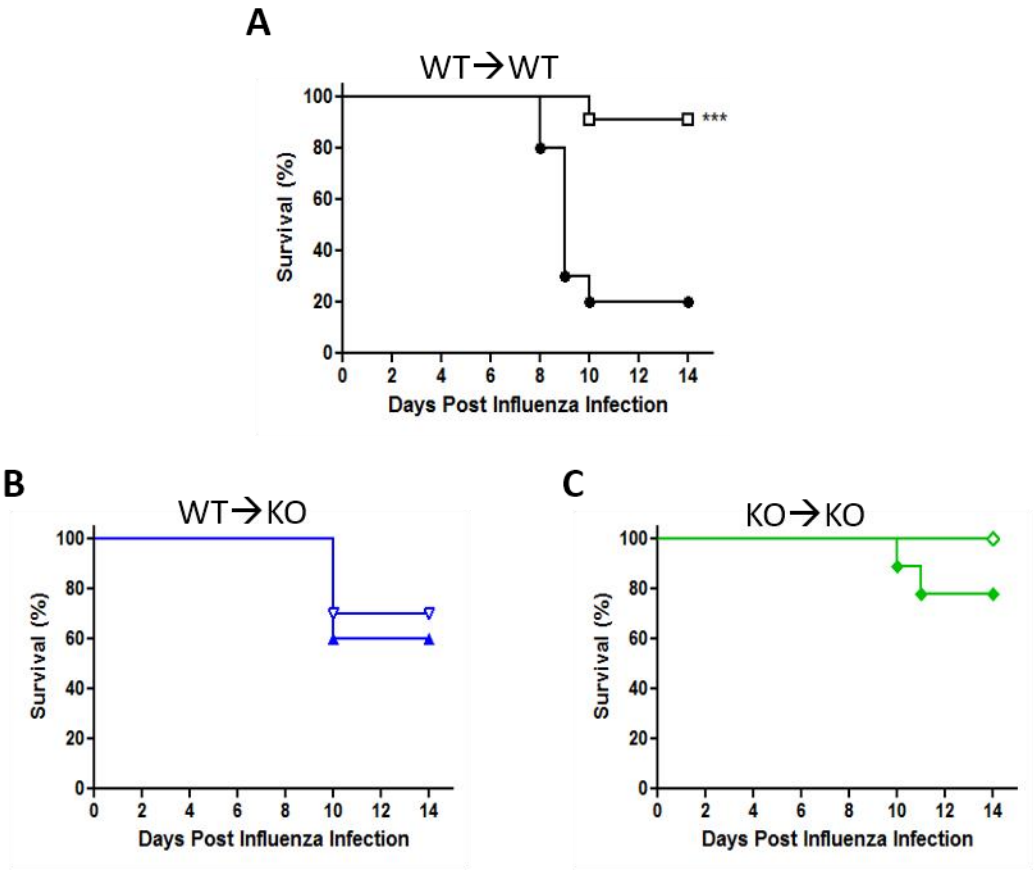


Fig 4.9 Comparison of male and female mice adoptively transferred with macrophages. Survival outcomes from Fig. 4.8 were graphed comparing male and female mice from (A) WT→WT, (B) WT→KO, and (C) KO→KO. Solid shapes, females; open shapes, males. Data was pooled from two independent experiments, n=9-11. ****p*=0.0006 by Log Rank (Mantel Cox) Test.

the –day 3 adoptive transfer point might be responsible for the altered outcomes post-influenza infection.

Some differences in results between sexes were expected as broad variations in male and female responses to immune activation are well documented [430-433].What was unexpected were the large differences in response to influenza infection between male and female mice. Similar results were responsible for the 2014 NIH implementation

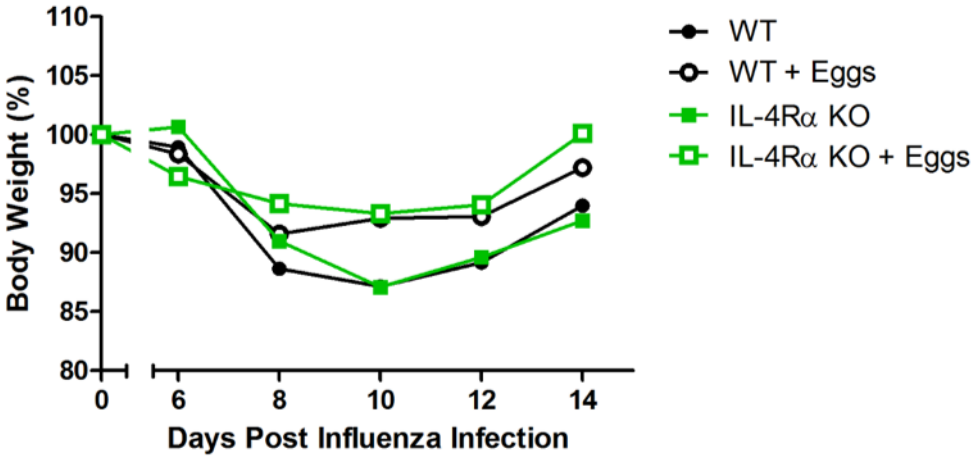


Fig 4.10 Body weights of WT and KO mice during influenza infection. BALB/c WT and KO mice +/– *S. mansoni* eggs were infected with 25 pfu of PR8 virus via IN with 25ul and monitored for signs of disease. Data from a single experiment, n≥4.

of new regulations on pre-clinical research funding to ensure findings are evaluated in both sexes [434].

Thus overall, we cannot draw any conclusions on the role of M2 macrophages as protective against influenza infection as each of the three trials used different adoptive transfer timepoints. The two most similar experiments simply swapped the 24 hpi timepoint for a 48 hpi timepoint with the former being protective, and the latter not. However, during the time we were performing these adoptive transfer studies, Osborne *et al*. published a study on coinfection with *T. spiralis* and mouse norovirus (MNV) showing that effective antiviral immunity is dependent on suppression of Ym1 expressing macrophages. A conditional macrophage IL-4Rα -/- mouse [127] would be useful to further elucidate the effects of M2 macrophages during viral infection.

IL-4-independent M2 macrophages are not induced in lungs of IL-4Rα -/- mice. Using one particular adoptive transfer schedule, we demonstrated that adoptive

transfer of WT M2 macrophages into IL4Rα -/- mice resulted in increased mortality in response to influenza infection. Interestingly, egg injected IL4Rα -/- mice had reduced body weight loss during a sublethal influenza infection compared to IL4Rα -/- mice that were not injected with eggs (Fig. 4.10). Thus, injection of schistosome eggs provided some level of protection to subsequent influenza infection in IL4Rα -/- mice.

Previously, our lab demonstrated that egg products were able to induce M2 macrophages independently of IL-4Rα signaling [14]. Therefore, we asked if IL-4-independent M2 macrophages were induced in egg injected IL4Rα -/- mice and if so, what happens in subsequent influenza infection. To test this, we stained lung sections with Relmα, a marker associated with M2 macrophages.

Immunohistochemical analysis of Relmα was performed on lung sections from WT and KO mice injected with eggs, infected with influenza, or both. Appropriate mice were infected with 100pfu of influenza and lungs were harvested 3 dpi for all animals. Egg injected WT mice had widespread Relmα expression throughout lung tissue, including macrophages, bronchiolar epithelial cells, and alveolar epithelial cells (Fig. 4.11A). These mice had mild inflammation of the alveolar space and well formed granulomas. In contrast, egg injected IL-4Rα -/- mice had considerably reduced Relmα expression that was largely restricted to the bronchiolar epithelium (Fig. 4.11B). In contrast to egg injected WT mice, egg injected IL-4Rα -/- mice had minimal inflammation and granuloma sizes were greatly reduced. Influenza infected WT mice had localized areas of dense inflammation along with Relmα staining of bronchiolar epithelial cells and infrequent macrophages (Fig 4.11C). Dense sites of inflammatory cells were absent in influenza infected IL-4Rα -/- mice, however, increased cellular infiltration was

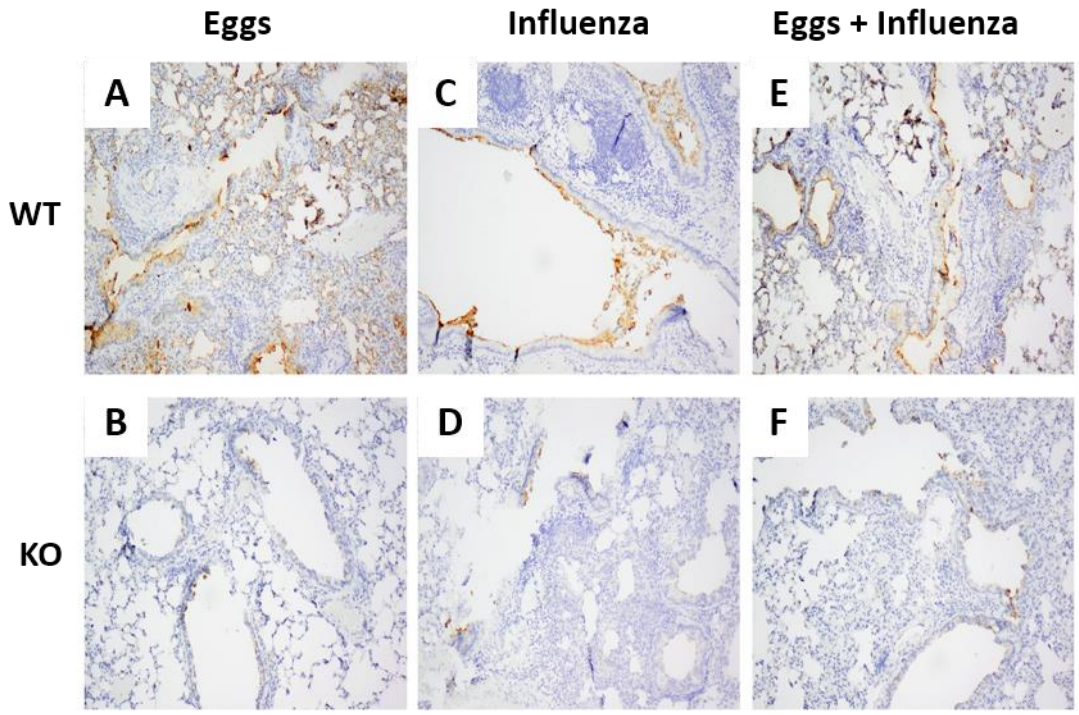


Fig 4.11 Immunohistochemistry of Relmα from pulmonary tissue of WT and KO mice. Lung tissue from the left lobe of BALB/c WT and KO mice was evaluated for Relmα expression 3 days post (A-B) induction of lung granuloma model by injection of *S. mansoni* eggs, (C-D) infection with 100 pfu of PR8 influenza virus, and (E-F) induction of lung granuloma model followed by infection with 100 pfu PR8. Representative micrographs of tissue sections; DAB stained, Relmα; 10x magnification. Data shown from a single experiment, n=4-5.

observed around bronchioles which impacted the alveoli (Fig. 4.11D). Though uncommon, Relmα staining was detected in bronchiolar epithelial cells of influenza infected IL-4Rα -/- mice. Egg injected and influenza infected WT mice exhibited features of both independent treatments (Fig. 4.11E). Diffuse inflammation was present across lung tissue with areas of dense inflammation forming around bronchioles, which infiltrated into the alveolar space, and large well formed granulomas were well distributed. Relmα was detected in macrophages, which were scattered throughout lung tissue. Relmα was also detected in alveolar and bronchiolar epithelial cells. In contrast,

egg injected and influenza infected IL-4Rα -/- mice had minor pathology, with small patches of dense inflammation localized around bronchioles, mildly affecting the alveolar space, and with egg granulomas considerably smaller than influenza infected and egg injected WT mice (Fig. 4.11F). Finally, the bronchiolar epithelium was sparsely positive for Relmα with no involvement of the alveolar epithelium.

Relmα positive macrophages were not detected in egg injected IL4Rα -/- mice indicating IL-4Rα-independent macrophages may not be responsible for the reduced weight loss that we observed in these animals. Previous studies from our group on IL-4Rα-independent M2 macrophages used either schistosome soluble egg antigens (SEA) or purified LNFPIII glycan, neither of which results in tissue damage after administration. This is different from the temporary inflammation seen in response to eggs deposited in the lungs. One consideration is that we needed to wait for a longer period post-egg injection in IL4Rα -/- mice to give rise to IL-4Rα-independent induction of M2 macrophages.

In chapter 3 we showed that Relmα expression is upregulated in pulmonary epithelial cells in mice infected with schistosomes, influenza, or mice that are coinfected. Therefore, the strong staining we observed in WT groups was expected. However, the near total loss of Relmα positive epithelial cells in all IL4Rα -/- groups was not anticipated. Since all cells in IL4Rα -/- mice are able to produce, but not respond to IL-4/IL-13, it is likely that an IL-4/IL-13 dependent cell type is required for induction of Relmα in epithelial cells.

IL-17 producing cells are not altered in lungs of IL-4Rα -/- mice.

Immunohistochemistry of egg injected IL4Rα -/- mice showed that M2 macrophages are

not correlated with weight loss reduction following subsequent influenza infection. Similar to M2 macrophages, Th17 cells are upregulated during schistosome infections and IL-17 is associated with increased egg granulomatous pathology [394, 395, 419, 435-437]. However, IFNγ is a negative regulator of IL-17 during schistosome infection [396]. In response to influenza infection, increased IL-17 and Th17 cells results in reduced mortality [422, 423]. Egg injected IL-4Rα -/- mice should have increased IFNγ and therefore, decreased IL-17 due to an inability to induce Th2 cells. As IL-17 is correlated with protection during influenza infection, we wanted to evaluate the effect on IL-17 producing cells in egg injected IL4Rα -/- mice during subsequent influenza infection.

Lungs of schistosome infected and uninfected mice were assessed by qRT-PCR for transcript levels of *il17* at days 0 and 7 post-influenza infection (dpfi) (Fig. 4.12). We

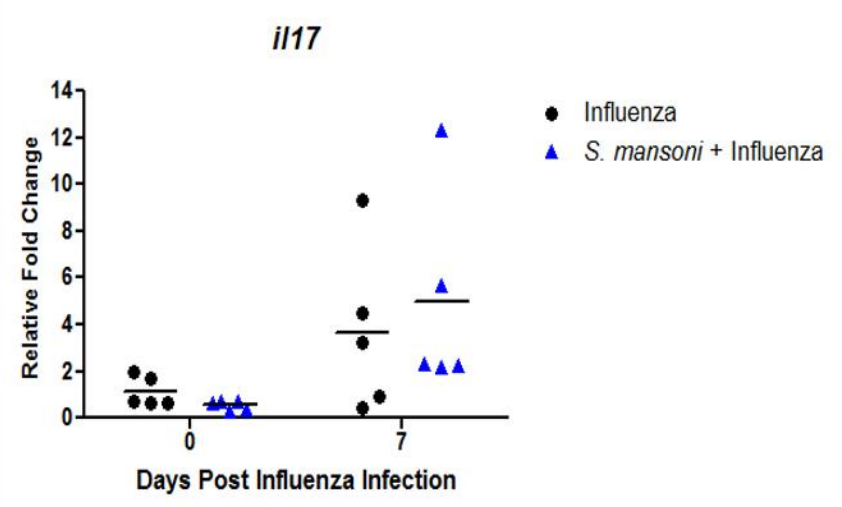


Fig 4.12 Transcript levels of *il17* in lungs of influenza infected mice. Cardiac lung lobe was recovered and evaluated by qRT-PCR from schistosome infected and uninfected mice at day 0 and day 7 post sublethal influenza infection (45 pfu of PR8 virus). Transcript levels of *il17* graphed as relative fold change over *actb* and normalized to schistosome uninfected at day 0. Data shown from a single experiment, n=5.

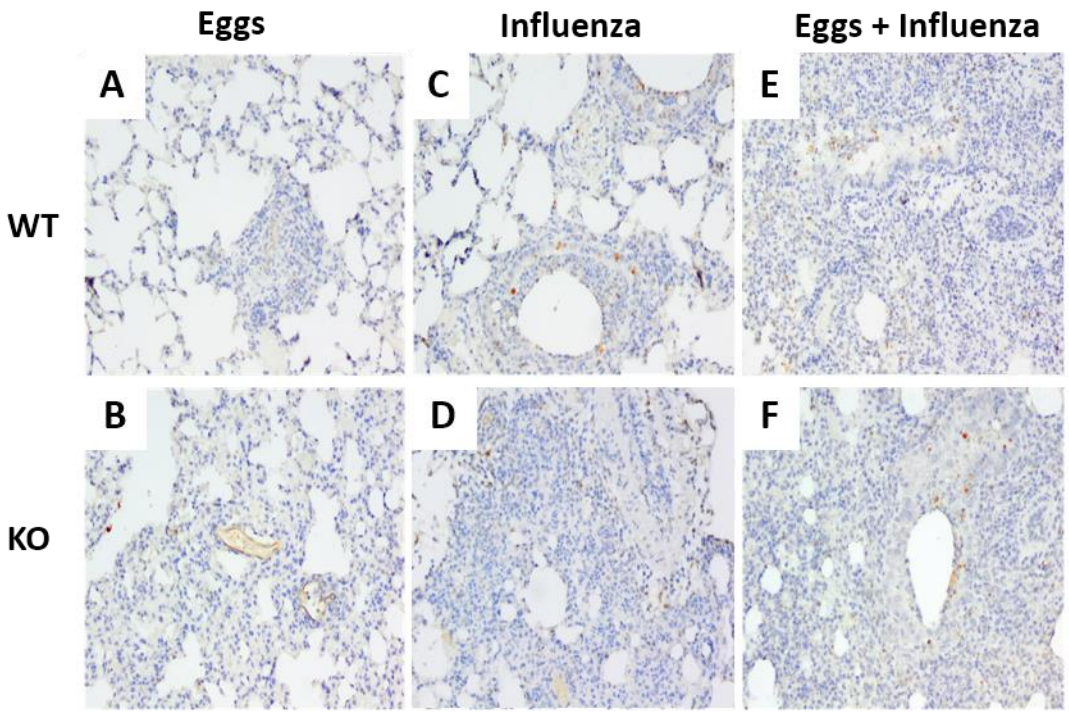


Fig 4.13 Immunohistochemistry of IL-17 from pulmonary tissue of WT and KO mice. Lung tissue from the left lobe of BALB/c WT and KO mice was evaluated for IL-17 expression 3 days post (A-B) induction of lung granuloma model by injection of *S. mansoni* eggs, (C-D) infection with 100 pfu of PR8 influenza virus, and (E-F) induction of lung granuloma model followed by infection with 100 pfu PR8. Representative micrographs of tissue sections; DAB stained, IL-17; 20x magnification. Data shown from a single experiment, n=4-5.

observed no significant differences in levels between schistosome infected and uninfected mice. However, at 7 dpfi, coinfected mice had mildly elevated levels of *il17* compared to naïve, influenza infected mice. Increased levels of *il17* transcripts in lungs of coinfected mice was a promising result and encouraged further investigation.

Additional sections of lung tissue were cut from WT and IL-4Rα -/- mice and immunohistochemical analysis for the presence of IL-17 was performed. Egg injected WT mice had limited staining for IL-17 expressing cells which were found sporadically in lung tissue (Fig. 4.13A). IL-17 expression in egg injected IL-4Rα -/- mice was similar

to what we observed in egg injected WT mice (Fig. 4.13B). Influenza infected WT mice had strongly positive IL-17 cells that were localized in clusters within areas of bronchiolar inflammation (Fig. 4.13C). Influenza infected IL-4Rα -/- mice had similar expression of IL-17 as influenza infected WT mice (Fig. 4.13D). Egg injected and influenza infected WT and IL-4Rα -/- mice, had intensely positive IL-17 expressing cells localized in areas of bronchiolar inflammation (Fig. 4.13E-F).

When comparing WT and IL-4Rα -/- mice from each treatment group, our data suggest that IL-17 producing cells are not upregulated in IL-4Rα -/- mice and therefore it is unlikely that this cell type is involved in protecting egg-biased mice against influenza. Interestingly, although egg administration was not found to induce IL-17 producing cells,

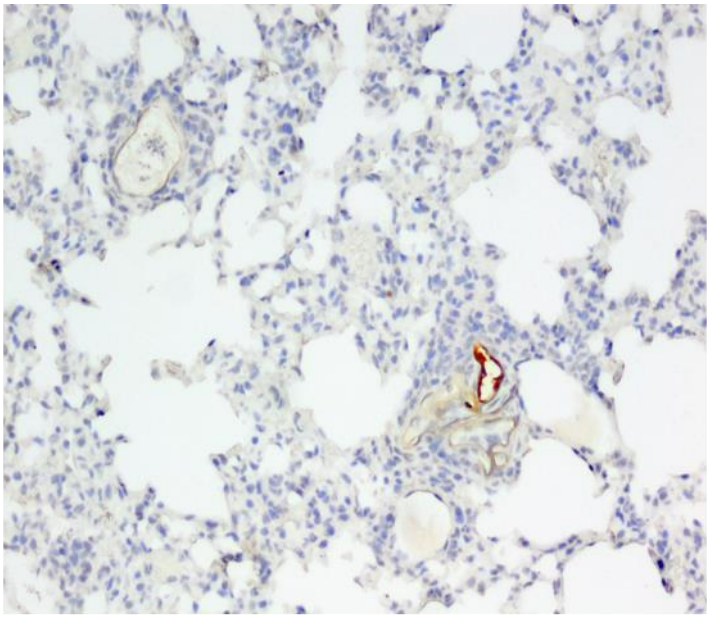


Fig 4.14 Positive immunohistochemical staining of IL-17 *on S. mansoni* egg shells. Lung tissue from the left lobe of BALB/c WT and KO mice was evaluated for IL-17 expression 3 days post induction of lung granuloma model by injection of *S. mansoni* eggs. Micrograph of lung tissue from KO mouse shown; representative of WT and KO lung granuloma mice. DAB, IL-17; 20x magnification. Data shown from a single experiment, n=4-5.

some schistosome egg shells stained positive for IL-17 while other eggshells on the same section did not (Fig. 4.13B and Fig. 4.14).

Unfortunately for our study, we were not able to find an anti-IL-17a antibody compatible for formalin fixed tissues. Also, due to the small foci of positively stained cells, an eosin counterstain overpowered the DAB chromogen and therefore neutrophils cannot be ruled out as IL-17 producing cells. Nonetheless, human studies have found a positive correlation between high serum IL-17 levels and survival from severe influenza infection [423, 438].

Conclusion

Immune responses to helminth infections are complex and interconnected. Dissecting out specific cellular responses during a schistosome infection is made exponentially more difficult upon secondary infection with a pathogen like influenza. Here, we selected CD8+ T cells, M2 macrophages, and Th17 cells as cell types that might be elicited via schistosome egg injection to further evaluate regarding a potential protective role during a subsequent influenza infection.

CD8+ T cells are an integral component of the immune response against influenza infection. In Chapter 3 we reported a reduction in NP-specific IFNγ production from activated CD8+ T cells, which could have contributed to the reduced cellular infiltration to the lungs of influenza coinfected mice. Here we show that egg injected mice have increased proportions of NP-specific cells from activated CD8+ T cell populations before the animals are infected with influenza (Fig. 4.4A). These cross-reactive CD8+ T cells may be responsible for the high LD_{50} to influenza challenge that we observed in egg

injected mice (Fig. 4.2), coincident with the almost non-existent symptoms during sub-lethal influenza infection (Fig. 4.3A-B) and faster viral clearance (Fig. 4.4C). However, these results are not consistent with the CD8+ T cell data reported in Chapter 3. Therefore, although cross-reactive CD8+ T cells may play a role in egg injected mice, we cannot confirm this is the cell type responsible for protection during coinfection.

M2 macrophages were the next logical cell type to evaluate as they are substantially upregulated during schistosome infections and are potently anti-inflammatory. High levels of IL-10 produced by M2 macrophages in lungs of schistosome infected mice could inhibit pro-inflammatory effects of influenza infection and reduce pulmonary cellular infiltration. However, we determined that addition of M2 macrophages via adoptive transfer was actually detrimental to survival of influenza infected mice (Fig. 4.6A). It was also apparent that female mice had significantly increased mortality compared to male mice (Fig. 4.6D).

Lastly, we considered IL-17 producing cells as the cell type mediating protection of egg-biased mice against influenza infection. IHC stained lung sections revealed that these cells are an unlikely candidate. The studies presented here illustrate the complicated nature of immune responses in helminth-virus coinfections. We were not able to pin down a particular cell type among the three types we investigated as being responsible for the observed reduced mortality seen in schistosome egg injected mice infected with influenza. For M2 macrophages, this may be due to altering the schedule for adoptive transfer of M2 macrophages in each of the three different experiments that were performed. It seemed the only timepoint related to protection by adoptively transferred M2 macrophages was the 24 hr timepoint. Although we had a 24 timepoint in experiment

3, we also had a day -3 timepoint that could have altered several parameters. Thus, to clarify the role of M2 macrophages we need to repeat the adoptive transfer experiments using the 24 hr schedule in both WT and IL-4Rα-/- mice. For IL-17 producing cells, the results are inconclusive.

CHAPTER 5

CONCLUSIONS

Infection with helminth parasites induces biasing of the host immune response towards CD4+ Th2, and anti-inflammatory responses, which can include immune anergy [138]. Coendemicity of helminth parasites with other pathogens such as dengue, malaria, and HIV, results in an increased likelihood of coinfection. With hundreds of millions of people at risk, it is important to understand the immunological factors involved in host responses during coinfection.

Similar to other helminth parasites, infection with schistosomes biases host immune responses to a CD4+ Th2/anti-inflammatory state characterized by high levels of IL-4, IL-10, IL-13, and TGFβ, produced by CD4+ Th2 cells, M2 macrophages, Tregs, and ILC2s. Although immune biasing to a CD4+ Th2/anti-inflammatory state is a shared trait of all helminth infections, the difference in levels of these cytokines, duration of the response, as well as locations in the host where they are produced, differ dramatically as a result of complex life cycles.

Influenza A infection induces a strong pro-inflammatory immune response dominated by IFNγ producing CD4+ Th1 and CD8+ cytotoxic T cells. A handful of studies have evaluated the effects of helminth infection on secondary influenza infection with conflicting results. In one study the investigators examine the influence of mice infected with the intestinal helminth *Heligmosomoides polygyrus* on subsequent influenza

infection and reported that they saw no significant differences in the response to influenza between *H. polygyrus* infected and uninfected mice [366]. Conversely, a separate study reported that *Trichinella spiralis* infected mice did have reduced influenza-specific immune responses, however these effects were limited to the *T. spiralis* lung stage [368]. Taken together, each of these studies suggest that the location of infection, for helminth and virus, is an important factor to consider when evaluating immune response interactions during coinfection.

Here we present data on immune responses to a secondary Influenza A infection in mice chronically infected with *S. mansoni*. *S. mansoni* was used as the helminth infection model for several reasons: 1) *S. mansoni* is a natural and common human helminth responsible for over 85 million infections; 2) An *S. mansoni* infection is chronic and induces strong and continuous immune stimulation as a result of immunomodulatory eggs produced by adult worms; 3) *S. mansoni* eggs have been found in pulmonary tissue of humans, which would result in a localized and robust immune biasing, in addition to systemic biasing; 4) *S. mansoni* infection is similar in mice.

Indeed, we show that chronic *S. mansoni* infection effects influenza induced immune responses. *S. mansoni* infection decreased morbidity and mortality to influenza infection, as demonstrated by an 8.5 fold higher influenza LD_{50} compared to schistosome uninfected mice. Additionally, schistosome infected mice had significantly less weight loss and symptom severity scores as a result of a secondary, sublethal influenza infection. These data suggest that *S. mansoni* infection in mice protects against influenza induced morbidity and mortality. Further, our results show that *S. mansoni* is an appropriate

model helminth to investigate immune how helminth coinfection influences secondary infection with respiratory pathogens.

In the study examining the influence of *T. spiralis* infection on subsequent influenza infection, they demonstrated that during the lung-phase of migratory, new larvae of *T. spiralis* there was a decrease in influenza-specific CD8+ T cell responses. Interestingly, this was not observed in the chronic, muscle cell encystment phase of *T. spriralis* infection [368]. To determine if chronic *S. mansoni* infection affected T cell responses to influenza infection, MdLNs were recovered at multiple timepoints during influenza infection and evaluated for influenza-specific CD8+ T cells. Proportions of activated CD8+ T cells specific for the immunodominant epitope of influenza ($NP_{366-374}$) were found to be the same among schistosome infected and uninfected mice, however, cells from coinfected mice produced significantly less IFNγ. This result shows that, unlike chronic *T. spiralis* infection, chronic *S. mansoni* infection does alter influenza specific CD8+ T cell responses. The data presented here do agree with the results seen when *T. spiralis* larvae were in the lungs, suggesting that when evaluating coinfection with a pulmonary pathogen, lung involvement by the helminth or helminth products is necessary.

As both *S. mansoni* and influenza affect pulmonary tissue, we next examined lung responses during coinfection. In coinfected mice we saw significantly reduced levels of *ifng* expression in lungs, as well as increased levels of *il4*, *il10*, and *il13* as measured by qRT-PCR. In addition, expression of the M2 macrophage marker *relma* was significantly higher in coinfected mice compared to influenza only mice. These data show that the

Th2/anti-inflammatory phenotype established during schistosome infection was maintained in the lungs of *S. mansoni* infected mice during influenza infection.

Histology was performed on lung tissue recovered from schistosome infected and uninfected mice during influenza infection to further evaluate effects of *S. mansoni* infection on secondary influenza infection. Histological analysis revealed that pneumonitis lesions were significantly smaller in coinfected mice than schistosome uninfected mice. The reduced pulmonary pathology likely results in better blood oxygenation and fewer symptoms associated with influenza infection. These results, taken together with reduced transcript levels of *ifng* in lung tissue and reduced IFNγ production by influenza-specific CD8+ T cells in MdLNs, show an overall pattern of reduced inflammation in coinfected mice, and the lowered levels of inflammation are likely related to the observed reduction of infiltration of cells to the lungs, resulting in smaller pneumonitis lesions and a better outcome for the mouse.

The observation that lungs of *S. mansoni* infected mice maintained a Th2/anti-inflammatory biasing during influenza infection as well as IHC analysis showing an abundance of Relmα positive macrophages, suggested to us that M2 macrophages might be a candidate cell contributing to reduced pro-inflammatory responses in lungs during secondary influenza infection. As *S. mansoni* egg-induced M2 macrophages are potent anti-inflammatory cells, we evaluated if M2 macrophages were mediators of reduced influenza induced pathology. Using WT and IL-4Rα -/- (KO) mice, we found, in contrast to our hypothesis, that adoptive transfer of egg-induced M2 macrophages actually increased influenza associated mortality instead of reducing it. Further evidence that the protective cells are not macrophages was shown in WT mice that received WT

macrophages having a dramatic and significant reduction in survival to influenza infection. The final evidence that macrophages are likely not the protective cell type was shown in IL-4Rα-/- mice that received IL-4Rα-/- macrophages, which had the best survival outcome. IL-4Rα-/- mice were unable to stimulate alternative activation of macrophages, however WT mice were fully capable. Therefore, decreased survival of WT→WT mice indicated that *S. mansoni* egg-induced M2 macrophages were detrimental during influenza infection. Recently, Osborne, *et al.* showed bone marrow-derived rIL-4-induced M2 macrophages, inhibited mouse norovirus-specific CD8+ T cell responses [369]. As we did not evaluate T cell responses in adoptively transferred mice, it is possible that adoptive transfer of WT or M2 macrophages severely impaired CD8+ T cells, resulting in increased morbidity and mortality.

Notably, a striking contrast was observed between male and female mice during adoptive transfer experiments. Male mice from all three adoptive transfer groups had increased survival compared to female mice in response to influenza infection. This dichotomy in survival between sexes is an important observation and one that requires additional investigation.

Although egg-induced M2 macrophages were determined to have a negative effect on survival during influenza infection, we confirmed that *S. mansoni* eggs were driving the protective effect during schistosome infection. Utilizing the lung granuloma model, we found that egg injected mice were highly tolerant to influenza, even gaining a significant amount of weight over the course of influenza infection. Additionally, lung granuloma mice had significantly higher influenza-specific CD8+ T cells and faster viral clearance than normal mice. However, the findings on increased CD8+ T cells and faster

viral clearance in lung granuloma mice than normal influenza infected mice does not agree with data obtained during *S. mansoni* infection and therefore, further experimentation is required.

Here we have presented data demonstrating that *S. mansoni* is a valid helminth to use for evaluation of pulmonary coinfection. Our results suggest that studies on the influence of helminth infection on subsequent respiratory infections need to be interpreted in the context of whether or not that particular helminth parasite has a lung stage, or is depositing parasite antigens into the lungs, as results of coinfection may vary significantly based on this parameter alone. Additionally, we confirmed that *S. mansoni* egg-induced M2 macrophages negatively impact influenza induced disease pathology and to this end, illustrated a clear difference between male and female responses to influenza infection.

Together this work shows that chronic *S. mansoni* infection is beneficial and protects against secondary influenza infection, in part, by decreasing pro-inflammatory responses that limit cellular infiltration to the lungs resulting in decreased pneumonitis and increased survival.

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