A zebrafish model to study the schistosome egg granuloma

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Preface

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and List of Contributions and specified in the text.

This thesis is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

This thesis does not exceed the prescribed word limit for the Clinical Medicine and Veterinary Medicine Degree Committee.

Summary

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Schistosomiasis is a disease caused by parasitic flatworms which reside within the venules of their human host. The disease pathology is caused by the eggs which they produce, and is primarily characterized by the granulomas which form around them. While the granulomas have pathological consequences to the host, they are thought to be essential to facilitate egg expulsion and completion of the parasite life cycle. Here, I have developed a larval zebrafish model to study the formation of the schistosome egg granuloma in detail within an optically transparent animal. I have developed the tools and techniques for implantation of individual *Schistosoma mansoni* eggs into zebrafish, followed by intravital microscopy to observe the formation of the schistosome egg granuloma. Within the zebrafish, eggs induce the formation of epithelioid granulomas, as in mammalian models. I find that while mature schistosome eggs induced granuloma formation, immature eggs do not, and this is due to their eggshell functioning as an immunologically inert barrier between the parasite and host. Complemented by the finding that only mature eggs are shed in both mice and humans, these findings indicate that immature parasite eggs avoid foreign body granuloma formation to prevent premature expulsion during their host-dependent development. Then, after completing development, the mature egg secretes antigens through its eggshell to promote granuloma formation and expulsion to complete its life cycle. I investigate the host and parasite factors involved in granuloma formation, and demonstrate that TNF receptor 1 signaling is not required for either initial macrophage recruitment or granuloma formation, but does contribute to granuloma enlargement. In contrast, the major egg antigen, omega-1, utilizes its RNase activity to induce initial macrophage recruitment.

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Contents

Chapter 1. Introduction

1.1. Introduction to Schistosomiasis

1.1.1. Schistosomiasis: The disease, its species, and prevalence

Schistosomiasis is a parasitic disease caused by flatworms of the genus *Schistosoma* (McManus et al., 2018). The disease has ancient origins (Barakat, 2013), having been diagnosed in 5,000 year old Egyptian mummies based on the presence of parasite eggs, DNA, and antigens which were preserved within their tissues (Matheson et al., 2014; Miller et al., 1992; Ruffer, 1910). During the same time period, symptoms of the disease, the hematuria (blood in urine) caused by the *S. haematobium* species endemic to Egypt, were described in The Kahun Gynaecological Papyrus; an ancient Egyptian medical text written in hieroglyphics and dating back nearly 4,000 years (Shokeir and Hussein, 1999). In modern times, schistosomiasis is still endemic to Egypt, as well as various tropical and subtropical regions of the world, where it affects more than 200 million people (Hotez et al., 2014; McManus et al., 2018), making it the world's second most devastating parasitic disease, after malaria (King, 2015).

There are six known species which cause schistosomiasis in humans, with the majority of infections caused by three species; *S. haematobium*, *S. japonicum*, and *S. mansoni* (McManus et al., 2018).

S. haematobium is thought to be the most prevalent cause of the disease (McManus et al., 2018), and is found throughout Africa and the Middle East where it causes the urogenital form of schistosomiasis (causing the hematuria described by the ancient Egyptians)(McManus et al., 2018). It causes bladder cancer and is classified as a definitive biological carcinogen by the World Health Organization (Colley and Secor, 2014; Humans, 2012; Ishida and Hsieh, 2018; van der Werf et al., 2003; WHO, 2002). It is a cofactor which increases the risk of HIV acquisition three-fold in women, due to the genital lesions it causes which are prone to contact-induced bleeding (Wall et al., 2018). Accumulation of eggs along their transit route can lead to obstructive fibrosis and calcification of the urinary tract, leading to hydronephrosis and chronic interstitial nephritis. This in turn can lead to the renal failure which is the major cause of death with *S. haematobium* (Barsoum, 2003, 2013; WHO, 2002). Despite its prevalence and significant morbidity, *S. haematobium* is insufficiently studied due to the lack of adequate animal models (Fu et al., 2012).

S. japonicum is found in East- and Southeast Asia where it causes intestinal schistosomiasis (McManus et al., 2018). Unlike the other major species, *S. japonicum* is considered a true zoonotic parasite with considerable animal reservoirs of infection, largely associated with agriculture due to the close working conditions of humans and the water buffalo used in the cultivation of rice (Kajihara and Hirayama, 2011; Tanaka and Tsuji, 1997). *S. japonicum* causes intestinal schistosomiasis, as with *S. mansoni* (Burke et al., 2009; McManus et al., 2018).

And lastly, *S. mansoni* is the most researched and the most widespread geographically, being found in Africa and the Middle East, and following its spread during the slave trade is now also found in Brazil, the Caribbean islands, Puerto Rico, Suriname and Venezuela (Crellen et al., 2016; McManus et al., 2018). *S. mansoni* causes intestinal schistosomiasis (McManus et al., 2018) and will be the focus of this research project.

It should be noted that while there are avian-infecting species of schistosomes which are much wider-spread geographically, being found also in North America and England, they do not cause stable infection in humans but instead die shortly after penetration of the skin, resulting in raised papules known as cercarial dermatitis and more commonly referred to as “swimmer’s itch” (Horak et al., 2015).

1.1.2. Treatment and prevention of schistosomiasis

The only recommended treatment for schistosomiasis is the anti-helminth drug, praziquantel, which has been in use since its development in the 1970s (Andrews, 1985; Gonnert and Andrews, 1977; WHO, 2002). Although Praziquantel is efficacious against all species of *Schistosoma*, and can be administered in a single dose, it does have its disadvantages. Praziquantel is ineffective against the juvenile infecting stage, affording the parasite an untreatable window of time; treatment only affords a partial cure rate of 60-90%, necessitating re-evaluation and re-treatment; and re-infection rates in endemic regions are high, often times necessitating re-treatment (Andrews, 1985; Cioli et al., 2014). Before the advent of praziquantel, elimination of schistosomiasis in Japan was achieved following approximately 70 years of multifaceted eradication efforts initiated in the early 1900s (Kajihara and Hirayama, 2011; Stothard et al., 2017; Tanaka and Tsuji, 1997). While complete elimination of schistosomiasis in other areas of the world have yet to be achieved, efforts have reduced the prevalence and burden of the disease (McManus et al., 2018). Some resistance against reinfection develops during

chronic long-term infection (>10 years), thought to occur as an IgE-mediated cross recognition of antigens on adult worms which had died naturally, which are also present on early infecting schistosomula (McManus et al., 2018; Pearce and MacDonald, 2002). Despite the promise this holds for immunity-based protection, there is still no vaccine to prevent schistosomiasis (Cioli et al., 2014; Tebeje et al., 2016).

1.1.3. Lifecycle of the *Schistosoma* parasite

The *Schistosoma* parasite has a complex lifecycle requiring the parasitism of two hosts; an aquatic snail which it utilizes as its intermediate host for asexual replication, and a human which it utilizes as its definitive host for sexually reproduction (Figure 1.1). Beginning at the egg stage, the egg hatches within freshwater where it releases its miracidium; a ciliated larva which infects its specific species of aquatic snail (*Biomphalaria spp*, *Bulinus spp*, and *Oncomelania spp* in the case of *S. mansoni*, *haematobium*, and *japonicum*, respectively)(2018 Primer). Within its intermediate snail host the parasite will reproduce asexually, forming sporocysts, and after 3-12 weeks depending on the species, shedding of the cercarial stage of the parasite will begin (Lewis, 2001).

Cercariae are free-swimming using their forked tail, and have evolved to detect lipids released from human skin, which they utilize to locate their human host (Shiff et al., 1993). Following contact with their human host, cercariae digest and penetrate through the skin using a combination of proteases released from specialized (acetabular) penetration glands (Ligasova et al., 2011) and mechanical effort (Inobaya et al., 2014; McKerrow and Salter, 2002; McManus et al., 2018). During entry they shed their forked tail and their glycocalyx coat; a carbohydrate rich envelope which protects cercariae from osmotic stress during the free-swimming stage of their lifecycle, but is highly antigenic and so requires shedding to avoid immune attack following entry into their human host (Da'dara and Krautz-Peterson, 2014; Dunne, 1990).

The parasites, now referred to as a schistosomula, will migrate deeper into the skin where they locate and enter into a blood vessel, and from within the circulation they passively travel to the lungs (McManus et al., 2018) where they will undergo further development, including the masking of surface epitopes and the acquisition of host antigens, which render them insusceptible to both cell- and humoral-mediated immunity (Gobert et al., 2007; McLaren and Terry, 1982). From the lungs, schistosomula will then migrate to the heart and then liver, where

they mature into adults. Adult male and female worms, now referred to as schistosomes, will form mating pairs and migrate to the mesenteric venules (or bladder venules in the case of *S. haematobium*) where they will reside in copula. The adult parasites are uniquely adapted to evade host immune responses (Keating et al., 2006; Sepulveda et al., 2010; Skelly and Alan Wilson, 2006) affording them long-term survival typically lasting for 5-10 years, and with case reports of infections persisting for decades (Colley and Secor, 2014; Pearce and MacDonald, 2002; Warren et al., 1974). During this time, adult worms sexually reproduce, releasing up to 300 eggs per day (3,000 for *S. japonicum*) into circulation (Cheever et al., 1994; IARC, 1994; Moore and Sandground, 1956). These eggs, and not the worms themselves, are responsible for disease pathology and morbidity (Burke et al., 2009; Colley and Secor, 2014). Each egg carries a living miracidium and secretes highly inflammatory antigens which co-opt the host immune system to facilitate its passage from circulation into the intestine (or bladder in the case of *S. haematobium*), where they are released back into the environment in feces (or urine) (Amiri et al., 1992; Costain et al., 2018; Doenhoff et al., 1978; Dunne et al., 1983; Karanja et al., 1997; McManus et al., 2018; Pearce and MacDonald, 2002; Schwartz and Fallon, 2018).

Approximately half of the eggs do not reach their intended location, but instead become lodged in other tissues, the liver in particular, where they induce the formation of the core pathological structure of schistosomiasis, the granuloma (Colley and Secor, 2014; Hutchison, 1928; Moore and Sandground, 1956; Warren, 1978).

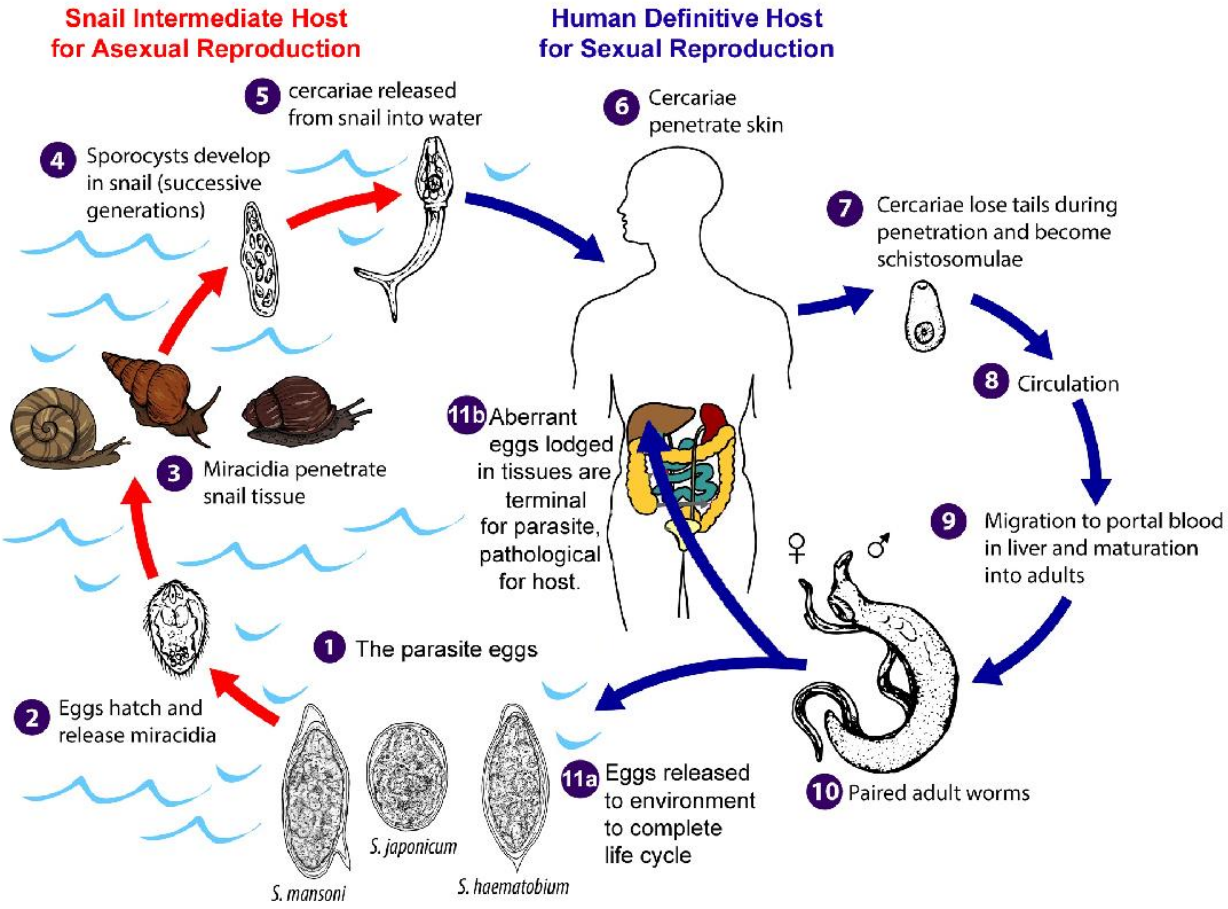


Figure 1. 1. Parasitic life cycle of the three major human-infecting species of *Schistosoma*
(**1**) The three species of parasite eggs are distinguished morphologically by their lateral and terminal spines in *S. mansoni* and *S. haematobium*, which is absent in the more ovoid *S. japonicum*. Eggs in water hatch to release (**2**) miracidia which infect their (**3**) intermediate snail host. (**4**) Within their intermediate snail host, the parasite replicates asexually to form self-regenerative sporocysts, and (**5**) cercaria which emerge from the snail to penetrate their (**6**) definitive human host. (**7**) Cercaria shed their tail and become schistosomulae which (**8**) migrate via circulation and (**9**) mature into adult worms in the liver. (**10**) Adult worms reside in copula within the venules of the mesentery (for *S. mansoni* and *S. japonicum*) or bladder (for *S. haematobium*). There they produce eggs which (**11a**) transit to the intestines (*S. mansoni* and *S. japonicum*) or bladder (*S. haematobium*) to be released into the environment in feces or urine, respectively. (**11b**) Aberrant eggs become lodged in other tissues, where they are terminal for the parasite, and pathological for the host. Illustration modified from the life cycle of the *Schistosoma* species, CDC Division of Parasitic Diseases.

1.2. Granulomas and their roles

1.2.1. Introduction to granulomas

Granulomas are organized aggregates of immune cells which have evolved to recognize and encase a diverse range of foreign materials, or chronically infecting inflammatory agents which the immune system is unable to eliminate (Pagan and Ramakrishnan, 2018). These include infectious agents such as bacteria, fungi, protozoa, helminthes, insects, and viruses; non-living foreign bodies such as beryllium, silica, and tattoo pigments; and those of unknown etiology thought to arise from chronic inflammatory or autoimmune responses as in the case of Crohn's and Sarcoidosis (Pagan and Ramakrishnan, 2014, 2018).

Granulomas are always composed of macrophages, which play a prominent role in shaping the immune response. They express chemokines which define the cellular composition through recruitment of specific cell types, they express cytokines which shape the immune response, and they can perform a structural function through specialized forms of differentiation to more effectively wall-off the inciting agent (Pagan and Ramakrishnan, 2018).

One such form of specialized differentiation is macrophage epithelioid transformation, in which they interdigitate their cell membranes together resulting in a tight multicellular macrophage aggregate with enhanced phagocytic, bactericidal, and degradative abilities (Adams, 1976; Cronan et al., 2016; Pagan and Ramakrishnan, 2018). This process is observed in various types of granulomas (including the tuberculous and schistosome egg granulomas), and although the signaling pathways leading to its formation are not fully understood, they are thought to involve cytokines IL-4 and IL-13, transcription factor STAT6, and possibly chronic activation of the metabolic sensor, mTORC1 (Pagan and Ramakrishnan, 2018). In some cases, cell-to-cell fusion of macrophages leads to cytoplasmic fusion to produce multinucleated giant cells (Helming and Gordon, 2007; Pagan and Ramakrishnan, 2018).

In addition to macrophages, and depending on the inciting agent and subsequently activated signaling pathways, the granuloma will have a specific cellular composition containing other cells of the immune system of both innate and adaptive origin; cells such as neutrophils, eosinophils, T-cells, and B-cells, whose roles will vary within the context of the inciting agent and who will together that shape the immune response and outcome of infection (Adams, 1976; Boros, 2003; Pagan and Ramakrishnan, 2018).

1.2.2. Cellular structure and cytokine milieu of the schistosome egg granuloma

The granulomas which form around the *Schistosoma mansoni* eggs are composed predominantly of macrophages (and their epithelioid and multinucleated counterparts), eosinophils, fibroblasts, and T cells and B cells, aggregated together within a Th2-dominant cytokine milieu characterized by high levels of IL-4, IL-13, and IL-5, but with significant contribution from the Th1 cytokine, TNF (Figure 1.2) (Pagan and Ramakrishnan, 2018; Reiman et al., 2006; Weinstock and Boros, 1983b).

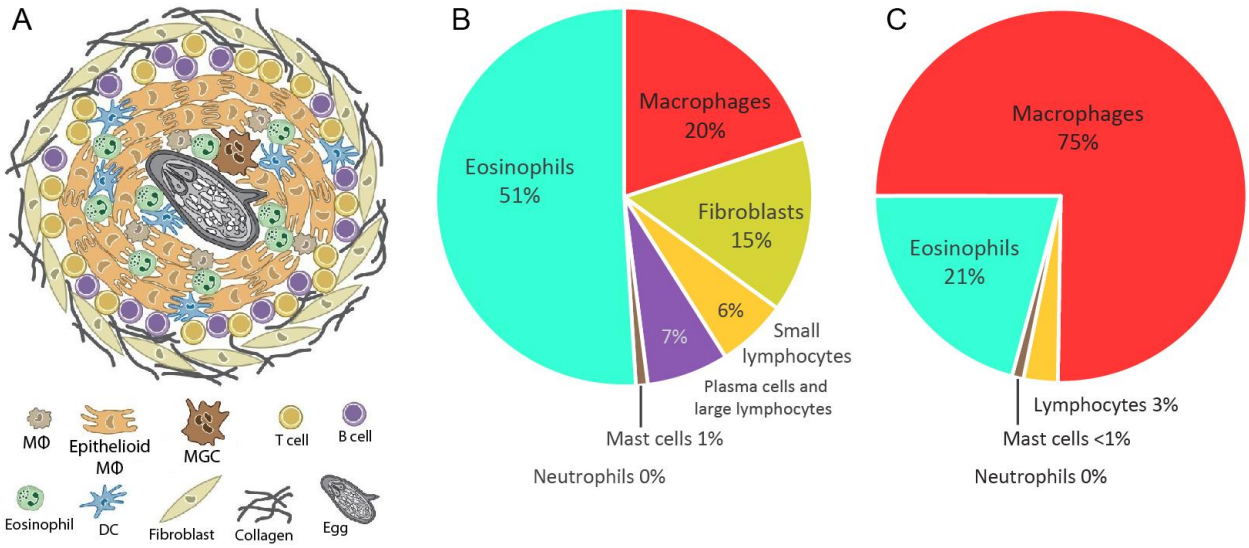


Figure 1. 2. Cellular structure and composition of the *Schistosoma mansoni* granuloma
(**A**) Illustration of the composition of the *S. mansoni* granuloma. (**B**,**C**) Quantification of the cellular composition of (**B**) hepatic and (**C**) intestinal *S. mansoni* granulomas. (**A**) Modified from 2018 Pagán with permission of author and publisher. (**B**,**C**) Graphs produced using data from Reiman et al., 2006 and Weinstock and Boros, 1983, respectively. Fibroblasts were not included in the quantification in (C).

As with other granulomatous diseases, macrophages are a key player in schistosomiasis. During the course of infection, liver-entrapped eggs induce the accumulation of macrophages through both the local recruitment of resident macrophages (also known as Kupffer cells), and from the CCR2-dependent recruitment of circulating inflammatory monocytes (also known as Ly6C^{hi} monocytes) (Girgis et al., 2014; Nascimento et al., 2014). Antigens released from eggs induce the expression of IL-4 and IL-13 (Grzych et al., 1991; Pearce et al., 1991; Pearce and

MacDonald, 2002), which bind IL-4 receptor complexes (containing the IL-4rα) on macrophages resulting in their alternative activation to adopt a distinct macrophage phenotype associated with parasitic infections, wound-healing responses, and allergies (Gordon, 2003), and which can also induce their differentiation into epithelioid macrophages and multinucleated giant cells (Pagan and Ramakrishnan, 2018)(Figure 1.2A). Within the context of liver-entrapped eggs, these alternatively activated macrophages protect the hepatocytes from egg-induced damage by organizing the cellular encasement of eggs (Herbert et al., 2004). While macrophages are essential for host survival during schistosomiasis (Nascimento et al., 2014), it is specifically these alternatively activated macrophages which are essential, as loss of the signaling pathway for their development; IL-4, IL-4rα, and the macrophage-specific IL-4rα, all result in the same acute lethal disease (Fallon et al., 2000; Herbert et al., 2004; Nascimento et al., 2014). In addition to their role in physical encapsulation of the schistosome egg, macrophages can also contribute to cytokine production through antigen-induced expression of TNF and IL-33 (Hams et al., 2016; Nascimento et al., 2014).

As with other helminth infections, eosinophils are a prominent component of the immune response to schistosome eggs; egg-induced expression of IL-5/13 is required for eosinophilia and recruitment to the granuloma, where they are the major cellular component making up approximately 50% of cells of the hepatic granuloma (Figure 1.2B) (Brunet et al., 1999; Hams et al., 2013; Moore et al., 1977; Sher et al., 1990; Swartz et al., 2006), and 21% of the cells of the intestinal granuloma (Weinstock and Boros, 1983b)(Figure 1.2B). However, despite their prominence in the granuloma, ablation of eosinophils has no effect on granuloma development, size, fibrosis, or its ability to protect hepatocytes from damage, indicating that either they are bystander cells and their function is dispensable, or that their function is rescued by compensatory increases of other cell types, chiefly macrophages (Brunet et al., 1999; Sher et al., 1990; Swartz et al., 2006). Although eosinophils may not alter granuloma formation, they may function to directly induce damage to the schistosome egg, a function which possibly explains their higher proportion in hepatic granulomas (de Brito et al., 1984; Hsu et al., 1980; Kazura et al., 1985; Schwartz and Fallon, 2018).

After macrophages and eosinophils, fibroblasts are the third most prominent cell type, making up 15% of the cellular composition of the *mansoni* hepatic granuloma (Reiman et al., 2006). There, they contribute to egg-induced, IL-13 (and IL-5)-mediated pathology through excessive

formation of connective tissue, resulting in fibrosis (Chiaramonte et al., 2001; Fallon et al., 2000; Hams et al., 2013; Herbert et al., 2004; Pagan and Ramakrishnan, 2018).

Whereas neutrophils are a major component of *Schistosoma japonicum* granulomas, they are rarely found in *S. mansoni* granulomas (Moore et al., 1977; Swartz et al., 2006; Von Lichtenberg et al., 1973)(Figure 1.2). This is possibly due to the absence of neutrophil-recruiting antigens in *S. mansoni* eggs which are present in *S. japonicum* eggs (Wu et al., 2014), the presence of IL-8 neutralizing antigens secreted from the *mansoni* egg (Smith et al., 2005), or the relative absence of necrosis which is prominent in both the *S. japonicum* and tuberculous granulomas, and which is known to recruit neutrophils (Yang et al., 2012). The lack of neutrophils in the *Schistosoma mansoni* granuloma might be an evolutionary mechanism of the parasite to enhance its survival, as in vitro experiments have shown that neutrophils are deleterious to *S. mansoni* egg survival, reducing respiration rate, viability, and granuloma formation, and directly inducing eggshell fragmentation leading to destruction of the miracidium (de Brito et al., 1984; Kazura et al., 1985).

Although few basophils are found in the *Schistosoma* granuloma, they are stimulated by egg antigens to release IL-4 and IL-13, contributing to the characteristic cytokine profile of the granuloma (Knuhr et al., 2018).

Together, the cells of the innate immune system are sufficient to form granulomas, as evidenced by granulomas forming in the sole context of innate immunity in infected SCID mice in response to tissue-entrapped schistosome eggs (Amiri et al., 1992; Cheever et al., 1999), and in non-sensitized WT mice in response to injected eggs (Chensue et al., 1995a) or beads coated with schistosome soluble egg antigens (SEA) within 3 days post-injection, before adaptive immunity develops (Chiu et al., 2004).

The adaptive immune response as mediated by T cells plays an important role, complementary to this process. T cells (CD4 helper T cells) are stimulated by egg antigens to express both Th1 and Th2 cytokines; TNF, and IL-4, IL-13, and IL-5 (Amiri et al., 1992; Cheever et al., 1999; Chensue et al., 1994; Everts et al., 2012; Grzych et al., 1991; Pagan and Ramakrishnan, 2018; Pearce et al., 1991; Sher et al., 1990). These T cells and the cytokines they produce have been found to enhance the formation of the granuloma, with their loss in both nude and SCID mice resulting in smaller granulomas, increased hepatic disease and mortality, and decreased egg expulsion (Amiri et al., 1992; Cheever et al., 1993; Cheever et al., 1999; Fallon

and Dunne, 1999). In humans, some studies have found that diminished CD4 T cell levels in HIV co-infected schistosomiasis patients correlated with reduced rates egg expulsion, supporting the role of T cells in this process (Colombe et al., 2018; Karanja et al., 1997). While the T cell-mediated enhancement of granuloma formation is clear, it is not always clear through which cytokines and pathways they function (Amiri et al., 1992; Cheever et al., 1999). In one study, the deficient granuloma formation and egg excretion in SCID mice were both rescued with supplemental TNF as well as activated T cell medium, but not with T cell medium pre-absorbed with anti-TNF antibody, indicating that their T cells were mediating granuloma formation and egg excretion through expression of TNF (Amiri et al., 1992). However, in a following publication in which they directly assessed the role of TNF in knockout mice, they found no defect in granuloma formation (Davies et al., 2004). Yet mice lacking the two receptors for TNF signaling, TNFR1 and TNFR2, had a reduction in granuloma formation, which was proposed to be due to diminished signaling through the TNF receptors via another ligand, such as lymphotoxin alpha (2004 Davies). Consistent with the TNF knockout mice, another group found that the defective granuloma formation in their SCID mice were not rescued with supplemental TNF (Cheever et al., 1999). Subsequently, they found that the diminished granuloma formation in their SCID mice was not due to TNF deficiency as this cytokine was at near normal levels, but was instead attributed to deficiency in IL-4, IL-5, IL-13, and gamma interferon (Cheever et al., 1999). The role of IL-4 and IL-13 was confirmed in other studies in which combined IL-4 and IL-13 deficiency in mice resulted in smaller granulomas with an associated increase in hepatocyte damage and mortality, and an accumulation of eggs in the intestinal wall as they failed to translocate into the lumen of the intestines (Fallon et al., 2000).

In contrast to the role of T cells in the adaptive immune response to egg, B cells function to produce antibodies which directly neutralize cytotoxic egg antigens, limiting their tissue-damaging effects, and limiting granuloma formation (Dunne et al., 1991; Dunne et al., 1981; Hams et al., 2013; Jankovic et al., 1998; Pearce and MacDonald, 2002).

1.2.3. Dual roles of granulomas in schistosomiasis

While the granuloma has evolved as a protective mechanism, some pathogens usurp it for their own benefit, in which case the granuloma itself may contribute to pathology, taking on a complex, dually protective and pathogenic role. The best example of this is with perhaps the

oldest and longest-studied of the granulomatous diseases, tuberculosis (Cambier et al., 2014a; Davis and Ramakrishnan, 2009; Pagan et al., 2015; Ramakrishnan, 2012). This disease as caused by the bacterium, *Mycobacterium tuberculosis*, primarily affects the lungs where it establishes chronic infection leading to the formation of its hallmark structure, the TB granuloma. Its dually protective and pathogenic role is such that while the tuberculous granuloma can successfully isolate and eradicate their entrapped bacteria, it may also become a site for dissemination of infection to newly recruited, uninfected macrophages, thus increasing the intracellular niche for bacterial growth while also spreading the infection to other parts of the body following emigration of infected macrophages (Davis and Ramakrishnan, 2009; Pagan and Ramakrishnan, 2018; Ramakrishnan, 2012). Furthermore, whereas the immune response relies on granulomas to clear bacteria, the bacteria rely on the granuloma for dissemination to a new host. Granulomas within the lung may undergo cell death and necrosis, rupturing and releasing bacteria into the alveolar space of the lungs which are then transmitted to others through coughing (Doenhoff, 1998; Pagan and Ramakrishnan, 2018). While TB was used as an example, the complex nature in which granulomas may be protective and pathogenic is a feature in many granulomatous diseases, and so too has parallels in schistosomiasis.

During the course of schistosomiasis also, the granulomas play roles in both protection and pathogenesis (Hams et al., 2013). Granulomas which form in response to liver-entrapped eggs protect the host by sequestering harmful egg secretions which would otherwise damage the hepatocytes (Hams et al., 2013). As a result, despite an abundance of accumulated eggs, the liver parenchyma is healthy (Hams et al., 2013). In contrast, the disruption of granuloma formation in immunocompromised animals leads to the development of liver pathology (Abdulla et al., 2011; Amiri et al., 1992; Dunne et al., 1981; Fallon et al., 2000; Hams et al., 2013; Herbert et al., 2004; Meleney et al., 1953; Symmers, 1904). In the intestines, granulomas are thought to protect the host by preventing translocation of intestinal bacteria back into the host during egg expulsion (Schwartz and Fallon, 2018).

However, as aberrant eggs accumulate along the portal tract of the liver, the granulomas form through a period of expansion and then resolution (Chensue et al., 1995a; Chiu et al., 2004), eventually leaving behind a remnant eggshell within a fibrotic plaque (Symmers, 1904). As these fibrotic plaques accumulate over 5-15 years during chronic schistosomiasis, they produce periportal fibrosis (Burke et al., 2009; Colley and Secor, 2014; Symmers, 1904). This periportal

fibrosis acts as an obstructive bottleneck, causing portal hypertension and the development of esophageal varices which can rupture and lead to the major cause of death with schistosomiasis, internal hemorrhage (Cheever and Andrade, 1967; Weinstock, 1992; WHO, 2002 ; Wilson et al., 2011). Additionally, obstructive fibrosis can also result in portosystemic shunting of eggs through alternative vascular pathways, bypassing the liver and lodging the eggs in other tissues such as the lungs, brain, and spinal cord, often with much more severe and immediate consequences (Carod-Artal, 2008; Papamatheakis et al., 2014; Schwartz, 2002; Weinstock, 1992). For example, eggs accumulated in the arteries of the lungs induce granulomas, leading to pulmonary fibrosis and pulmonary hypertension, which in turn places excessive pressure on the right ventricle of the heart leading to heart failure and death, in a condition known as cor pulmonale (Schwartz, 2002). Other complications such as paralysis from aberrant egg deposition and granuloma formation around the spinal cord, although statistically rare, are well-recognized in endemic regions due to high prevalence of the disease (Nascimento-Carvalho and Moreno-Carvalho, 2004).

Lastly, whereas the schistosome egg granuloma can be both protective and pathogenic for the host, from the perspective of the parasite, granulomas are thought to be essential for the completion of its lifecycle cycle by facilitating the translocation of its eggs from the vasculature to the intestines where they are released back into the environment (Amiri et al., 1992; Cheever et al., 1993; Costain et al., 2018; Dunne et al., 1983; Hams et al., 2013; Karanja et al., 1997; McManus et al., 2018; Pearce and MacDonald, 2002; Schwartz and Fallon, 2018). The evidence for this granuloma-mediated translocation of eggs comes from tissue histopathology, in which eggs were directly observed to be in various stages of transit through the intestinal wall, and extravasating into the lumen of the intestines, within granulomas (Costain et al., 2018; Domingo and Warren, 1969; Fallon and Dunne, 1999; Fallon et al., 2000; Schwartz and Fallon, 2018). That the granuloma is an active participant in this translocation was best illustrated in a comparison of WT and immunocompromised (athymic, Cytoxan-treated, egg-tolerized) mice. Whereas the eggs in immunocompromised mice in were found primarily in the submucosa with little or no granulomatous response, the eggs in WT mice were found encased within granulomas, and at various stages of translocation throughout the submucosa, lamina propria, and along the villi, and extravasating into the lumen of the intestines (1999 Dunne).

In support of this finding is that various immunocompromised animal models, including hydrocortisone-treated (Dunne et al., 1983), athymic (Doenhoff, 1998; Dunne et al., 1983), egg-tolerized (Fallon and Dunne, 1999), IL-4/13-deficient (Fallon et al., 2000), and TNF-deficient SCID mice (Amiri et al., 1992) were all defective not only in granuloma formation, but also in egg excretion, further linking the granulomatous immune response with the translocation of eggs (Costain et al., 2018; Schwartz and Fallon, 2018).

There is also evidence for immune-mediated translocation of eggs in humans. Although this observation was not uniform in all studies (Kallestrup et al., 2005), some studies found that *Schistosoma* co-infection in HIV-positive patients with acquired immunodeficiency had reduced egg expulsion which correlated with reduced CD4 T cell levels (Colombe et al., 2018; Karanja et al., 1997; Muok et al., 2013). Taken together, these results suggest that the mature egg secretes antigens so as to induce the formation of granulomas which facilitate its translocation through the tissues in order to complete its lifecycle.

1.3. The parasite egg and its antigens

1.3.1. Development of the parasite egg into a mature, antigen-secreting egg

As the male and female adult schistosomes mate within the venules, the ovum is fertilized within the oviduct of the schistosome female, followed by the formation of the eggshell which encases the zygote along with 30-40 vitaline cells (Dewalick et al., 2011). Tyrosinases within the ootype catalyze its formation through the cross-linking of localized proteins which form its rigid, yet porous, protease-resistance matrix of proteins (deWalick et al., 2012; Fitzpatrick et al., 2007). The most abundant protein, which comprises 70% of the eggshell, is the eggshell-specific protein, p14 (Dewalick et al., 2011). This protein is highly expressed in the female worm, and only during ovulation (Ojopi et al., 2007). The remaining 30% of the eggshell is composed of an assortment of non-eggshell-specific, common cellular proteins (Dewalick et al., 2011). The eggshell once formed is unaltered chemically during, or following egg maturation (Dewalick et al., 2011; deWalick et al., 2012). After encapsulation within the eggshell is complete, the egg is then laid into the vasculature in its zygotic form (Jurberg et al., 2009).

The newly laid immature egg absorbs nutrients from its host through 30 nm pores in its eggshell, which it utilizes for development (Ashton et al., 2001), until reaching maturity within approximately 7 days (Ashton et al., 2001; Jurberg et al., 2009; Mann et al., 2011; Michaels and

Prata, 1968). During the developmental process, a subshell syncytium forms, referred to as the inner envelope or Von Lichtenberg's envelope, and it is this structure, present in the mature egg and absent in the immature egg which synthesizes antigens (Ashton et al., 2001; Jurberg et al., 2009; Neill et al., 1988; Schramm et al., 2006). This fully formed mature egg is granuloma-inducing (Jurberg et al., 2009), a property that is attributed to the appearance of these immunogenic antigens which are secreted through the eggshell of the mature egg into the surrounding environment (Ashton et al., 2001; Fitzsimmons et al., 2005; Mathieson and Wilson, 2010; Schramm et al., 2006).

1.3.2. Antigen-mediated granuloma formation

Several decades of experiments suggest that antigens secreted by the mature parasite through its eggshell induce granuloma formation. Early observations of eggs in tissues found that minimal immune reactions were most commonly associated with immature eggs which had not reached developmental maturity, as well as those which were heat-killed and injected into mice and rabbits (Lichtenberg, 1964; Sorour, 1929). This lack of granuloma formation around immature and dead eggs was associated with the relative absence of immunostainable antigenic secretions, which in contrast were found in maximal secretory capacity in viable mature eggs, and corresponded with maximal granuloma development (Jurberg et al., 2009; Lichtenberg, 1964; Sorour, 1929; Von Lichtenberg et al., 1973). Based on these observations, a link was suggested between the presence of these egg secretions and the degree of granulomatous immune response.

That these antigens were responsible for granuloma formation was directly validated in a body of work by Stephen Chensue in which the transfer of egg antigens to agarose beads made the beads highly granulomatous when injected into mice, demonstrating that the antigens were inducing the granulomas which formed around them (Chensue, 2013; Chensue et al., 1994; Chensue et al., 1995a; Chensue et al., 1995b; Chiu and Chensue, 2002; Chiu et al., 2004). Further, the antigen-mediated formation of granulomas around the beads was attributed to their ability to induce a variety of inflammatory cytokines and chemokines, consistent with those induced by eggs (Chensue, 2013; Chensue et al., 1994; Chensue et al., 1995a; Chensue et al., 1995b; Chiu and Chensue, 2002; Chiu et al., 2004).

More recently, the role of egg antigens in granuloma formation was further validated using genetic knockouts targeting the major egg antigens, omega-1, alpha-1, and kappa-5, which

diminished granuloma formation, thus providing additional evidence that the antigens produced by the eggs are mediating the formation of granulomas around them (Hagen et al., 2014; Ittiprasert et al., 2019).

1.3.3. Antigens secreted by the mature egg

Early immunohistochemistry experiments utilizing sera from infected mice identified the presence of immune-reactive secretions around the tissue-entrapped eggs, the abundance of which corresponded with granuloma intensity (Lichtenberg, 1964). Identification of these secreted egg antigens followed the fractionation of egg homogenates which revealed cationic fractions containing proteins which reacted strongly with serum from chronically infected mice (Dunne et al., 1981). These proteins were deemed omega, alpha, and kappa (Dunne et al., 1981). Later, the schistosome egg secretome was characterized as producing a simple 6-band pattern by SDS-PAGE (Ashton et al., 2001), followed by a more sensitive proteomics approach which identified a larger array of 188 secreted proteins (Cass et al., 2007). In addition to the protein secretome, more recently, the schistosome egg lipodome has been characterized as containing various secreted lipids, including prostaglandins, which are enriched in the egg stage of the parasite life cycle (Giera et al., 2018). Despite the array of various proteins and lipids found in the schistosome egg secretome, the vast majority of immunogenic activity and granuloma induction is still attributed to the proteins initially identified within the cationic fractions, omega-1, alpha-1, and kappa-5 (Abdulla et al., 2011; Dunne et al., 1981; Everts et al., 2012; Everts et al., 2009; Hagen et al., 2014; Ittiprasert et al., 2019; Steinfelder et al., 2009).

1.3.4. Omega-1, its properties and role in granuloma formation

During its initial discovery, omega-1 was found to be the major target of the humoral immune response against eggs, with anti-omega-1 antibody being the most abundant antibody detected, and detectable in all chronically infected mice (Dunne et al., 1981). Furthermore, it was found that injection of serum containing this antibody protected against egg-induced hepatotoxicity in immunocompromised animals (Dunne et al., 1981), indicating that omega-1 is hepatotoxic, and indicating that the granuloma might form as a protective response to prevent hepatotoxicity (Dunne et al., 1981). Further testing in hepatocyte tissue cultures verified that omega-1 is responsible for the majority of egg-induced hepatotoxicity (Abdulla et al., 2011; Dunne et al.,

1991; Dunne et al., 1981). Experiments testing the immunological properties of omega-1 found it to induce IL-4/13 expression in vitro in DC and T cell co-cultures, and in vivo following injection into the footpads of mice (Everts et al., 2012; Everts et al., 2009; Steinfelder et al., 2009). Additionally, injection of omega-1 into the intraperitoneal cavity of mice induced cell death and IL-33 expression in macrophages and DCs (Hams et al., 2016). IL-33 is known to function as a DAMP/alarmin to alert the immune system to tissue damage or stress, and is a strong inducer of Th2 cytokines from both innate immune cells and Th2 cells (Gajardo Carrasco et al., 2015; Oboki et al., 2011).

Recent advancements in gene knockdown approaches in eggs have found omega-1 to be the major component responsible for granuloma formation around them (Hagen et al., 2014; Ittiprasert et al., 2019). In 2014, a lentivirus-based transduction system was developed to deliver microRNA adapted short hairpin RNAs (shRNAmirs) into the parasite eggs to silence the expression of omega-1. Injection of these omega-1-silenced eggs into the tail vein of mice resulted in reduction in macrophage, DC, and T cell recruitment to the lungs, and a reduction in the size of the pulmonary granulomas which formed around the eggs (Hagen et al., 2014). In 2019, a new approach was developed in which lentiviral transduction of a CRISPR/Cas9 construct into the parasite eggs was performed to knockout the expression of omega-1 (Ittiprasert et al., 2019). This methodology was approximately 80% efficient, with both the omega-1 transcript and the omega-1 RNase activity reduced to about 20% of normal levels (Ittiprasert et al., 2019). Antigens harvested from these eggs were likewise reduced in their ability to induce Th2 polarization in macrophage/T cell co-cultures, with a reduction in IL-4, IL-5, and TNF induction, and a trend of reduced IL-13 induction (Ittiprasert et al., 2019). When these eggs were injected into the tail vein of mice, their ability to induce pulmonary granulomas formation was greatly diminished (Ittiprasert et al., 2019).

At the molecular level, omega-1 is a glycosylated (Dunne et al., 1991; Meevissen et al., 2010) ribonuclease (Everts et al., 2012; Fitzsimmons et al., 2005; Steinfelder et al., 2009) which gains entry into dendritic cells through recognition of its glycosylation by cell surface mannose receptors, and once internalized, co-localizes with and degrades host mRNA and rRNA resulting in inhibition of protein synthesis (Everts et al., 2012). Additionally, testing of omega-1 in cell cultures found that it alters DC cytoskeletal organization resulting in a rounder, less spread-out morphology with reduced adhesion to glass (Steinfelder et al., 2009). Similarly, omega-1

treatment also reduced their conjugation affinity with T cells, and this was proposed as a possible mechanism for Th2 polarization (Steinfelder et al., 2009). However, it is unclear if reduced conjugation affinity is causative of Th2 polarization, or an effect stemming from inhibition of protein synthesis. Despite the various known functions of omega-1, exactly how it induces granuloma formation is currently not known.

1.3.5. Alpha-1 and Kappa-5

The second major egg antigen is alpha-1, which contributes to the formation of granulomas around tissue-entrapped eggs (Hagen et al., 2014). Alpha-1 is the most abundant egg secretion, making up 83% of secreted egg antigens (Mathieson and Wilson, 2010) and found secreted in abundance around tissue-entrapped eggs (Schramm et al., 2003). Like omega-1, alpha-1 is a major hepatotoxic egg antigen (Abdulla et al., 2011), and likewise, might contribute to granuloma formation through the induction of a protective immune response to sequester this antigen (Abdulla et al., 2011). Alpha-1, also known as the interleukin-4 inducing principle of *Schistosoma* mansoni eggs (ISPE), may also contribute to the induction of granuloma formation based on its ability to induce IL-4 and IL-13 release from basophils (Knuhr et al., 2018; Schramm et al., 2003; Schramm et al., 2007). ISPE binds to all antibody isotypes, with the highest affinity for IgE (Meyer et al., 2015), and its induction of IL-4/13 release requires the presence of IgE (bound to the FcεRI) on basophils (Haisch et al., 2001). Alpha-1/ISPE is glycosylated (Wuhrer et al., 2006), and also contains a C-terminal nuclear localization sequence (NLS); however, neither internalization nor nuclear localization are required for induction of IL-4/13 release from basophils (Kaur et al., 2011; Schramm et al., 2003). A mutant of alpha-1, named the *Schistosoma* mansoni chemokine-binding protein (smCKBP), binds to CXCL8 and inhibits neutrophil recruitment (Smith et al., 2005), which may contribute to shaping the cellular composition of the immune response to eggs. This smCKBP variant does not induce IL-4 release from basophils (Smith et al., 2005).

The potential role of the third major egg antigen, kappa-5, is not clear. It is unique among the major egg antigens in that it is present both in the egg and in the miracidium (Everts et al., 2009). It contains structurally distinct LDN- glycan motifs, rather than the LeX glycan motifs of omega-1 and alpha-1 (Meevissen et al., 2011; Wilbers et al., 2017), which were shown to be sufficient to induce granuloma formation when bound to beads (Van de Vijver et al., 2006). Knockdown of

kappa-5 expression in eggs results in a minor reduction in granuloma size, indicating that any contribution it has is minor relative to that of omega-1 and alpha-1 (Hagen et al., 2014). However, its potential role in granuloma formation is unclear, as while kappa-5 accumulates within the subshell envelop, it does not diffuse through the eggshell, and subsequently is not found among the secreted egg antigens, nor is it found secreted around tissue-entrapped eggs (Mathieson and Wilson, 2010; Schramm et al., 2009).

1.4. Human and animal models for schistosomiasis

1.4.1. Initial schistosomiasis etiology and life-cycle discoveries gleaned from humans and animals

While the disease symptoms of schistosomiasis and its waterborne transmission were documented over 2,000 years ago, separately in both ancient Egypt and China (Mao and Shao, 1982; Shokeir and Hussein, 1999), its parasitic origin and pathology was not discovered until the application of science in the 1800s. The history of schistosomiasis research began with Theodor Bilharz, a German physician and pioneer in the field of parasitology who discovered and characterized the schistosome worms and eggs of *S. haematobium* in a human cadaver while performing an autopsy in Egypt in 1851, and hypothesized a link between the parasite and the disease (Di Bella et al., 2018; Tan and Ahana, 2007). Subsequent pathology of the disease within the definitive human host, was likewise obtained primarily in the clinical setting and from autopsies; Symmers in 1904 characterized the periovular liver pathology as “pipe-stem fibrosis” based on the similar appearance to the clay pipes used for smoking at the time (Symmers, 1904).

Patrick Manson was the first to propose that there were separate species of parasite based on the distinct location of worms in cadavers, their route of egg transmission (bladder or intestines), and the distinct morphology (terminal or lateral spines) of the eggs found within each anatomical site. Thus, distinguishing a separate species from *S. haematobium* which was later to be named *S. mansoni* in his honor. Based on the collection of eggs from patients, their hatching and the swimming behavior of miracidia, he also predicted the requirement of an intermediate host for the parasite, and proposed it might be a mollusk (Di Bella et al., 2018; Manson, 1902; Manson, 1905).

Meanwhile, in isolation from western research, in 1887 a Japanese physician found eggs in the feces of patients who were ill with their regional variety of schistosomiasis; a perplexing waterborne disease of unknown etiology which had been documented in Japan at least as far back as the 1500s (Kajihara and Hirayama, 2011; Tanaka and Tsuji, 1997). In 1898, parasite eggs were found in the liver of an autopsy patient who had died from the disease, and then in 1904, the schistosome worm was found during autopsy of a farmer from the Katayama district, an endemic region for the disease (Kajihara and Hirayama, 2011; Tanaka and Tsuji, 1997), thus linking together the disease with the parasite and its eggs. Following these human autopsies, a series of experiments were performed using animal models as a surrogate for the human host (Kajihara and Hirayama, 2011; Tanaka and Tsuji, 1997). By submerging bovines, dogs, cats, rabbits, and mice into infected water, followed by autopsies and the study of thousands of histological sections, they uncovered the lifecycle stages within the definitive host, from percutaneous infection, development and maturation to adult worms, and then sexual reproduction and the transmission of eggs (Kajihara and Hirayama, 2011; Tanaka and Tsuji, 1997). Then, by 1913 they had identified the intermediate host, an aquatic snail abundant within infected waters, within which they detailed the parasite stages; from percutaneous infection of the snail by miracidia, formation of sporocysts, asexual reproduction, and the emergence of cercaria and their transmission back into animals, thus completing their understanding of the lifecycle of *Schistosoma* (Kajihara and Hirayama, 2011; Stothard et al., 2017; Tanaka and Tsuji, 1997).

1.4.2. Animal models of schistosomiasis

While much of the basic pathology had been determined in the clinical setting from patients and cadavers, and from the basic use of animals (bovines, dogs, cats, rabbits, and mice) in research during the early 1900s, more advanced animal models were required to gain a deeper understanding of the disease at the molecular and immunobiological level. There are many potential animal models for schistosomiasis, as human-infecting schistosomes are also found naturally in variety of other mammals, including; non-human primates such as chimpanzees, gorillas, monkeys, and baboons; domesticated animals such as water buffalo, horses, pigs, dogs, and cats; wild rodents and procyonids (Standley et al., 2012). While non-human primates such as chimpanzees and monkeys have been used as experimental animal models in the study of

schistosomiasis due to their genetic and physiological similarity with humans (Cheever et al., 2002; Farah et al., 2001; Von Lichtenberg and Sadun, 1968), their use has long been unviable due to high costs and ethical concerns. To circumvent this, a variety of smaller and more affordable rodent models have been used, including guinea pigs, hamsters, rats, and mice (Cheever et al., 2002; Farah et al., 2001; Hsu et al., 1973; Von Lichtenberg et al., 1973). Among these, most of what we have learned about the immunology of schistosomiasis came from the mouse model of infection due to the availability of genetic tools and mutants, and their susceptibility to natural infection (Farah et al., 2001). Mice can be infected naturally by percutaneous exposure to cercaria, after which the parasites will mature to adulthood, establishing natural infection and egg-laying (Farah et al., 2001). The eggs will translocate through the intestinal wall into the lumen of the intestines to be shed in feces, or in the liver the eggs will induce the formation of hepatic granulomas and fibrosis (Dunne et al., 1983; Fallon and Dunne, 1999; Fallon et al., 2000). Whereas natural infection produces asynchronous granulomas as the eggs are laid continuously and at different time points (Moore and Sandground, 1956; Schwartz and Fallon, 2018), synchronous granulomas can be produced following the injection of eggs or beads into the tail vein to establish pulmonary granulomas, or into the portal vein to establish hepatic granulomas (Cheever et al., 2002; Chensue et al., 1995b; Eltoum et al., 1995). Despite their advantages, experiments performed with rodents are typically limited to fixed timepoint analyses of euthanized animals following removal of tissue samples, organs, and cells (Chensue et al., 1995b; Pearce et al., 1996), making the earliest response difficult to determine, with analysis only reliable after days following infection (Lichtenberg, 1962). In essence, there is no perfect animal model, with the best solution being the usage of various animal models to complement each other to build a bigger picture.

1.4.3. Zebrafish as an animal model

Zebrafish (Danio rerio) are small freshwater fish native to the rivers and paddy fields in India (Arunachalam et al., 2013). Initially brought to the west as an aquarium pet, zebrafish were introduced as a vertebrate animal model for laboratory research in the 1960s, and then later popularized by the work of George Streisinger in the 1980s for their use in genetics and developmental biology (Varga, 2018). As a vertebrate organism, they share the same major organs and tissues as humans, including epithelial, blood, bone, muscle, heart, kidney, intestines,

and eyes (Santoriello and Zon, 2012; Takaki et al., 2018). At the genetic level, 70% of human genes, and 84% of disease-associated genes have an orthologue in zebrafish (Cambier et al., 2014b; Clay et al., 2008; Howe et al., 2013; Oehlers et al., 2017; Tobin et al., 2010; Yang et al., 2012). Zebrafish are teleosts, and as such, have both innate and adaptive immune systems similar to those in humans (Renshaw and Trede, 2012). While experiments in the context of adaptive immunity can be performed on juveniles and adults following the development of their adaptive immune system after 4-6 weeks post-fertilization, experiments in larvae allow for the analysis of immune responses in the sole context of innate immunity (Lam et al., 2004; Novoa and Figueras, 2012; Traver et al., 2003; Trede et al., 2004).

In addition to their genetic, physiological, and immunological similarities with humans, zebrafish possess many additional traits that make them highly useful as a vertebrate animal model in the study of human disease. They are highly fecund and develop rapidly, with a single mating between an adult pair producing hundreds of fertilized eggs, enough for several experiments (Lieschke and Currie, 2007). Additionally, each of these embryos develop rapidly, allowing experiments to be performed as early as 1 day post-fertilization at which time they have already developed into larvae with their heart and most organ systems formed, including a functioning innate immune system composed of monocytes, resident macrophages, and neutrophils (Meyers, 2018; Takaki et al., 2013; Traver et al., 2003).

Most importantly, zebrafish are small and optically transparent during their first few weeks of life, allowing for high resolution, multi-timepoint intravital microscopy to track and visualize immunological processes in individual living animals, as they occur. To facilitate this, several fluorescently labeled transgenic fish lines were developed in which cell-specific expression of fluorescent proteins allows for the color-based visualization of distinct cell types such as macrophages and neutrophils (Hall et al., 2007; Pagan et al., 2015). Complementing this are a variety of injectable and immersible dyes commercially available for identifying cellular subsets (Berg et al., 2016; Davis and Ramakrishnan, 2009).

Advantageous in the study of the genetic component of disease, zebrafish are genetically tractable with a variety of tools to easily manipulate gene expression de novo, both transiently and permanently. Embryos can be injected at the 1-cell stage with the newly adapted CRISPR/Cas9 system to mutagenize a target gene (Wu et al., 2018). The resulting crispant larvae can be used the next day for experiments (McManus et al., 2018; Wu et al., 2018), or they can be

raised and bred to propagate the mutation in a stable fish line (Wu et al., 2018). The full genome has been sequenced and annotated relative to the human genome (Howe et al., 2013), and is complemented by additional genetic tools which include the Zebrafish Mutation Project (ZMP); a large library of zebrafish mutants generated by ENU-mutagenesis during a forward genetics approach at the Wellcome Sanger Institute (WSI) (Kettleborough et al., 2013). It is due to these various traits that the use of zebrafish as an animal model has expanded to encompass a variety of infectious diseases.

1.4.4. Zebrafish as a model to understand the formation and function of the tuberculosis granuloma

The use of zebrafish as a model organism expanded further to encompass infectious diseases with its development as an animal model for tuberculosis pathogenesis by L. Ramakrishnan since 2000 (Ramakrishnan, 2020). This led to new understanding of tuberculosis pathogenesis as well as a deeper insight into granuloma biology (Davis and Ramakrishnan, 2009; Pagan and Ramakrishnan, 2018; Ramakrishnan, 2020; Takaki et al., 2018; Volkman et al., 2010).

Within the zebrafish it was found that at the onset of infection, mycobacteria were controlling the cellular response that formed to them. Whereas the first responders to the initial infection are resident macrophages, it was found that through the coordinated usage of two virulence-associated lipids, PDIM and PGL, that the bacteria selectively control cellular recruitment (Cambier et al., 2017; Cambier et al., 2014b). In the former case, PDIM functions to mask bacterial pattern-associated molecular patterns (PAMPs) from TLR/MyD88-dependent recruitment of microbicidal macrophages, while the other lipid, PGL, activates the STING pathway, leading to the expression of CCL2 to selectively recruit the more infection-permissive monocytes (Cambier et al., 2017; Cambier et al., 2014b). While initially discovered in the zebrafish, these finding were complemented using the mouse aerosol model of tuberculosis infection, and cultured human alveolar macrophages (Cambier et al., 2017; Cambier et al., 2014b), and taken together, were used to explain the observation in human tuberculosis as to why infections initiate deep in the lower respiratory tract of the lungs, away from the inhaled microflora and the microbicidal macrophages which respond to them. Additional detailing of the early recruitment steps found that whereas macrophages and then monocytes are recruited during the early stages of infection, neutrophils are not (Yang et al., 2012). That only later when the

infection progresses to cellular necrosis that neutrophils are recruited, not by the bacterium, but by signals produced by dead and dying macrophages (Yang et al., 2012).

Within the zebrafish it was also found that the tuberculous granuloma, long thought to function solely as a host-protective immune response, could also be a structure exploited by the bacteria to serve as a niche for bacterial growth and dissemination to newly recruited, uninfected macrophages (Davis and Ramakrishnan, 2009). Moreover, it was found that mycobacteria actively induce the formation of this immune structure through expression of a virulence locus, ESX-1 (Volkman et al., 2004; Volkman et al., 2010). The ESX-1 locus encodes a type VII secretion system, and confers the mycobacteria with the ability to induce contact-dependent lysis of host cells (Conrad et al., 2017), and through induction of MMP9 expression in epithelial cells and macrophages, induces the formation of the granuloma (Volkman et al., 2010).

Using the zebrafish model, other host factors influencing the infection outcome were discovered, such as the finding that infection induces localized hypoxia and the subsequent expression of the angiogenic factor, Vegfa, which causes vascularization of the tuberculous granuloma (Oehlers et al., 2015). This pathogen-induced host response was found to benefit the bacteria, with pharmacological inhibition of angiogenesis limiting bacterial burden and dissemination of infection (Oehlers et al., 2015).

During analysis of granuloma formation, it was found that the specialized differentiation of macrophages into epithelioid macrophages observed in human tuberculosis granulomas, also occurred in the fish (Cronan et al., 2016). Further, that this was yet another mechanism exploited by the bacteria, in which the epithelialization of the granuloma limited macrophage access resulting in poor control of bacterial growth and worse disease outcome (Cronan et al., 2016).

During a forward genetics screen to identify host determinants for resistance and susceptibility to infection, it was discovered that balanced expression of leukotriene A4 hydrolase (LTA4H), an enzyme which catalyzes the synthesis of the pro-inflammatory lipoxin, LTB4, was essential for host defense against infection – a finding which translated to human cohorts in which homozygotes with a single functional allele for LTA4H had the greatest level of resistance to infection (Tobin et al., 2010). It was later found that this heterozygous advantage functioned through optimal expression of TNF levels, with both TNF deficiency and TNF excess resulting in worse disease outcome (Roca and Ramakrishnan, 2013; Tobin et al., 2012). TNF deficiency led to poor microbicidal control of infection and uncontrolled extracellular bacterial

growth, and TNF excess resulted in induction of programmed cell death resulting in the same uncontrolled extracellular bacterial growth as TNF deficiency (Clay et al., 2008; Roca and Ramakrishnan, 2013; Tobin et al., 2012).

Following the development of the zebrafish as an animal model for tuberculosis pathogenesis, their use has expanded to encompass a variety of other infectious diseases, including various bacterial, viral, fungal, and parasitic infections (Brannon et al., 2009; Gratacap and Wheeler, 2014; Rosowski et al., 2018; Sullivan et al., 2017; Takaki et al., 2018; Varela et al., 2017).

In this thesis, I report the development of the zebrafish as an animal model for studying the schistosome granuloma, which I use to gain new insight into host and parasite factors required for the modulation of the timing of granuloma formation by the *Schistosoma mansoni* egg so as to promote its transmission.

Chapter 2. Materials and Methods

2.1. Experimental animal model details

2.1.1. Ethics statement

All animal experiments were conducted in compliance with guidelines from the UK Home Office and approved by the Wellcome Sanger Institute (WSI) Animal Welfare and Ethical Review Body (AWERB).

2.1.2. Husbandry of zebrafish

All zebrafish lines were maintained on a recirculating aquaculture system with a 14 hour light - 10 hour dark cycle. Fish were fed dry food and brine shrimp twice a day. Zebrafish embryos were housed in fish water (reverse osmosis water containing 0.18 g/l Instant Ocean) at 28.5°C. Embryos were maintained in 0.25 μg/ml methylene blue from collection to 1 day post-fertilization (dpf). At 24 hours post-fertilization 0.003% PTU (1-phenyl-2-thiourea, Sigma) was added to prevent pigmentation.

2.1.3. Zebrafish lines

Experiments requiring larvae with red-fluorescent macrophages were performed using Tg(mpeg1:Brainbow)^{w201} (Pagan et al., 2015). For experiments requiring analysis of neutrophils, Tg(lyz:EGFP)^{nz117} (Hall et al., 2007) were crossed with Tg(mpeg1:Brainbow)^{w201} (Pagan et al., 2015) to produce larvae with green neutrophils and red macrophages. Experiments assessing early macrophage recruitment in response to beads or ruptured immature eggs utilized Tg(mfap4:nlsVenus-2A-tdTomato-CAAX)(A. Pagán, unpublished). All zebrafish lines were produced in an AB background, with the exception of Tg(mfap4:nlsVenus-2A-tdTomato-CAAX) which utilized a mixed AB/TLF background.

2.1.4. Generation of the TNFR1 mutant and its usage

The zebrafish TNFR1 mutant (*tnfrsf1a*^{rr19}) was generated using CRISPR Cas9 technology, targeting the sequence TGGTGGAAACAAGACTATGAA of the third exon of the gene (ENSG00000067182) using a T7 promoter-generated guide RNA. Sequencing verified the mutation as a 25 bp deletion (ATGAAGGGAAATTGTCTTGAAAATG) and 6 bp insertion

(TGGTGG), resulting in a frame shift and introduction of a premature stop codon soon after the start codon. HRM genotyping was performed using the TNFR1-HRM1- forward and reverse primer set (5'-GTTCCCCACAGGTTCTAACCAG-3' and 5'-CTTGATGGCATTTATCACAGCAGA-3', respectively). TNFR1 heterozygotes in the macrophage reporter background, *Tg(mpeg1:YFP)*^{w200} (Roca and Ramakrishnan, 2013), were incrossed, genotyped, and sorted as fluorescence-positive, homozygous TNFR1 mutants or WT siblings. Homozygous TNFR1 mutants or WT siblings were then incrossed to generate larvae for experiments.

2.2. Preparation of eggs, egg antigens, and recombinant omega-1

2.2.1. Isolation and manipulation of schistosome eggs

The complete life cycle of *Schistosoma mansoni* NMRI (Puerto Rican) strain is maintained at the WSI by breeding and infecting susceptible *Biomphalaria glabrata* snails, and mice. Schistosome eggs were harvested as previously described (Mann et al., 2010). Briefly, anesthetized Balb/c female mice were infected by tail submersion in water containing 250 *S. mansoni* cercariae collected from experimentally-infected snails, and 6 weeks later euthanized by an overdose of Euthasol (sodium pentobarbital and sodium phenytoin, 40 mg per mouse) delivered by intraperitoneal injection. Mixed-sex adult worms were collected by portal perfusion, washed and maintained in culture for *in vitro* laid eggs (IVLE) collection (below). The mouse livers were removed after the portal perfusion, minced with a sterile razor blade in 1X PBS containing 200 U/ml penicillin, 200 μg/ml streptomycin and 500 ng/ml amphotericin B (i.e. 2% antibiotic-antimycotic - ThermoFisher Scientific), and incubated with 5% clostridial collagenase (Sigma) in 1X PBS with 2% antibiotic-antimycotic at 37°C with shaking for 16 hours. The digested liver tissue mixed with the *Schistosoma* eggs was washed three times with 1X PBS with 2% antibiotic-antimycotic by centrifugation at 400 g for 5 min at room temperature and serially filtered through a sterile 250 μm and 150 μm sieve. The eggs were then separated from the liver tissue by a sucrose-based Percoll gradient and washed three times as above. The eggs were kept at 37°C, 5% CO_2 in DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic. All the procedures were performed in sterile conditions inside a biological safety cabinet. For experiments comparing eggs from liver and intestinal tissue, and small and large intestinal luminal content (feces), liver and intestinal tissue eggs were isolated as above, but with serial

passage through 300 μm and 200 μm filters (pluriSelect, 43-50300-01 and 43-50200-01). For isolation of eggs from the lumen of the small and large intestines, the luminal content was gently extracted by squeezing the intestines, which contained both intestinal feces and translocated eggs. *S. mansoni* IVLE were harvested as previously described (Mann et al., 2010; Rinaldi et al., 2012). Briefly, schistosome mixed-sex worms collected by portal perfusion were washed with sterile 1X PBS and 2% antibiotic-antimycotic, placed in 6-well plates and cultured in modified Basch's medium (Mann et al., 2010) at 37°C, 5% CO_2. Two days later, the eggs laid *in vitro* by the cultured worm pairs were collected from the bottom of the well. For experiments using immature IVLE, eggs were implanted into zebrafish larvae soon after collection, and for experiments using mature IVLE, eggs were cultured in modified Basch's medium at 37°C, 5% CO_2 for 6 days before being implanted into zebrafish larvae. For experiments using heat-killed eggs, the eggs were killed at 90°C for 15 minutes and incubated in 1 mL of modified Basch's medium for 3 days to wash away residual egg antigens. Old dead eggs were created by stored at 4°C for >12 months and were verified as unviable based on lack of miracidial movement and hatching. For experiments using ruptured immature eggs, the CAIN was used to apply downwards pressure in combination with a sideways motion over the glass slide.

2.2.2. Soluble Egg Antigens (SEA)

SEA was prepared by Gabriele Schramm as previously described (Schramm et al., 2018). Briefly, isolated eggs were homogenized in PBS, pH 7.5, using a sterile glass homogenizer. The homogenate was then centrifuged at 21 krcf for 20 minutes. Supernatants were pooled and then dialyzed overnight in PBS using a 3.5 kDa molecular weight cutoff dialyzer. Sample was then centrifuged at 21 krcf for 20 minutes, and supernatant (SEA) was aliquoted and stored at -80°C. SEA was quantified for protein concentration using the Micro-BCA assay (Pierce, 23225), and quality controlled by SDS-PAGE and western blotting against the *S. mansoni* antigens, omega-1, alpha-1, and kappa-5. Quality control for low LPS content was performed using the Chromo-LAL assay (Associate of Cape Cod, Inc., C0031-5). SEA from WT and omega-1 knockout eggs were prepared by Wannaporn Ittiprasert as previously described (Ittiprasert et al., 2019).

2.2.3. Recombinant omega-1

Recombinant plant-expressed omega-1 engineered to contain native-like LeX glycans were prepared by Ruud Wilbers as previously described (Wilbers et al., 2017). WT and RNase mutant omega-1 were prepared by Gabriel Schramm as previously described ((Everts et al., 2012). SEA from WT and corresponding omega-1 knockout eggs were injected at 1 ng per hindbrain ventricle. For comparison of SEA and plant-expressed omega-1, SEA was injected at 2 ng per hindbrain ventricle (1.5 nL injection of 1.4 mg/mL SEA), and plant-expressed omega-1 with LeX glycans (Wilbers et al., 2017) was injected at 0.02 ng per hindbrain ventricle, the relative concentration of omega-1 present in SEA (G. Schramm, personal communication). For DEPC inactivation of plant-expressed omega-1, 1 μL of 0.07 M DEPC (1/100 dilution of Sigma, D5758) was added to 5 μL of 1.5 mg/mL omega-1 (12 mM final concentration of DEPC), and then incubated for 1 hour at 37°C. Because the small volume of protein did not allow for ultrafiltration and requantification of protein, the sample was simply diluted 1/100 in PBS and then 0.02 ng of protein injected into the hindbrain ventricle. For comparison, control sample was incubated at 37°C (without DEPC-treatment) and then diluted 1/100 in PBS. Because the HEK-expressed WT and RNase mutant omega-1 (H58F) lack the native-like LeX glycans in plant-expressed and natural omega-1 (Everts et al., 2012; Everts et al., 2009), they were injected at a 5-fold higher concentration of 0.1 ng per hindbrain ventricle. All hindbrain injections of antigens were assayed at 6 hours post-injection.

2.3. Preparation of bacteria

2.3.1. Preparation of *Mycobacterium marinum*

The culture and preparation of *Mycobacterium marinum* was previously described in detail (Takaki et al., 2013). Briefly, *Mycobacterium marinum* M strain (ATCC #BAA-535) constitutively expressing EBFP2 (strain KT30)(Takaki et al., 2013) was cultured in 7H9OADC, syringed to disrupt clumps, and then passaged through a 5 μm filter to generate a single-cell suspension of bacteria. The filtrate was concentrated to 100 CFU/nL by centrifugation and then prepared as single-use 5 μL aliquots and stored at -80°C. Before use, each aliquot was thawed, and zebrafish larvae were infected with 20 CFU (Figure 3.1 and 3.2) or 75 CFU (Figure 4.1) of *Mycobacterium marinum* via injection into the hindbrain ventricle.

2.3.2. Preparation of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (strain MPAO1, courtesy of Professor Gordon Dougan) was grown overnight in LB medium at 37°C with shaking, and then prepared as 5 µL aliquots containing 400 CFU/nL and stored at -80°C. Before use, each aliquot was thawed, and zebrafish larvae were infected with 200 CFU of *Pseudomonas aeruginosa* via injection into the hindbrain ventricle.

2.4. Implantation of schistosome eggs and beads

2.4.1. Implantation of schistosome eggs

Capillary-Assisted Implantation Needles (CAIN) were created by pulling borosilicate thin wall with filament capillaries (GC100TF-10, Harvard Instruments) using a micropipette puller (Sutter Instruments, P-2000) with the following settings: Heat = 350, FIL = 4, VEL = 50, DEL = 225, PUL = 150. The tips of pulled needles were opened with jeweler's forceps and then double-beveled using a MicroForge-Grinding Center (MFG-5, Harvard Instruments). Micromanipulation was achieved using a 3-axis micromanipulator (Narishige, M-152) with pressure control using a FemtoJet Express microinjection unit (Eppendorf). The VAMP (Vacuum-Assisted MicroProbe) was previously described (Takaki et al., 2013). Larval zebrafish were anesthetized and implanted at 30 hpf in 0.252 g/L tricaine (Sigma, A5040) in a modified Schistosomula Wash medium (500 ml DMEM, 5 ml 1M HEPES and 2% antibiotic-antimycotic) to prevent egg hatching during implantation. Anesthetized larvae were grasped using the VAMP and an incision was made in the forebrain region using the CAIN. After making an incision, a single schistosome egg was picked up using the capillary action of the CAIN, and passed through the incision and deposited into the hindbrain ventricle (Movie 2).

2.4.2. Implantation of beads

Zebrafish larvae were implanted with Sepharose (Sigma, C9142), polyethylene (Cospheric, CPMS-0.96 63-75um and CPMS-0.96 27-32um), and polystyrene (Generon, 07314-5) microspheres in fish water containing 0.252 g/L tricaine (Sigma, A5040) using the same technique as with schistosome egg implantations.

2.5. Hindbrain ventricle microinjection

Hindbrain ventricle injection of bacteria and soluble reagents were performed under anesthesia with 0.252 g/L tricaine (Sigma, A5040) using a microinjection needle supplied to a FemtoJet Express microinjection unit (Eppendorf), with larval manipulation performed using the VAMP (Takaki et al., 2013) (Movie 1).

2.6. Confocal microscopy

Zebrafish were anesthetized in fish water containing tricaine and then and mounted onto optical bottom plates (MatTek Corporation, P06G-1.5-20-F) in 1% low melting point agarose (Invitrogen, 16520-100) as previously described (Takaki et al., 2013). Microscopy was performed using a Nikon A1 confocal laser scanning confocal microscopy with a 20x Plan Apo 0.75 NA objective and a Galvano scanner, acquiring 30-80 µm z-stacks with 2-3 µm z-step intervals. Timelapse microscopy was performed at physiological temperature using a heat chamber set to 28°C (Okolab) with an acquisition interval of 2.5-3 minutes. For multi-day timelapse imaging, zebrafish larvae were carefully removed using jeweler's forceps and returned to their standard housing (see husbandry) for imaging at later timepoints.

2.7. Immunofluorescence staining

Immunofluorescence was performed as previously described (Cronan et al., 2016). Briefly, zebrafish larvae were fixed in Dent's fixative overnight at 4°C, rehydrated in PBS containing 0.5% tween 20, and then blocked for 1 hour in PBDTxGs (PBS containing 1% BSA, 1% DMSO, 0.1% Triton X-100, 2% goat serum). Mouse anti-E-cadherin antibody, clone 36 (BD, 610181) was added at a 1/500 dilution followed by incubation overnight at 4°C. Larvae were washed in PBDTxGs and then Alexa Fluor 647 Goat Anti-Mouse IgG (H+L) antibody (ThermoFisher, A-21236) added at a 1/500 dilution followed by incubation overnight. Larvae were washed 5 times in PBDTxGs before analysis.

2.8. Analysis of eggs from liver, intestinal tissue, and feces

All eggs were analyzed by microscopy and scored as immature or mature based morphological differences in size and shape as characterized by Jurberg (2009 Jurberg). Eggs from the liver and intestinal tissue were analyzed in 1x PBS. Eggs from the luminal content of the small intestine were imaged in a petri plate using a glass coverslip to create a thin section of sample to image through. Eggs from the large intestines were diluted in 2% methyl cellulose and spread thinly across a petri plate to dilute the fecal matter and create a thin section of sample for analysis. Mature and immature eggs were imaged by brightfield microscopy, and then their lengths and widths were determined using the measurement function within the ImageJ analysis software. Volumes were calculated from dimensions using the formula for the volume of a prolate spheroid ($v=4/3\pi ab^2$).

2.9. Quantification and statistical analysis

2.9.1. Phagocyte recruitment

For quantification of phagocyte recruitment, fluorescence confocal microscopy was performed, capturing z-stack images at the designated timepoint following implantation of eggs or beads, or the injection of soluble antigens or bacteria. Experimental groups were then blinded, and 3D rendering of confocal images were used to count the number of phagocytes in contact with the schistosome egg or bead, or the number of phagocytes within the hindbrain ventricle following injection of soluble antigens or bacteria.

2.9.2. Determination of egg and bead volumes

Schistosome eggs were stained with Coomassie InstantBlue dye (Sigma, ISB1L) and imaged by confocal microscopy with the 641 nm laser and CY5 HYQ filter, 590-650 nm excitation and 663-738 nm emission. Using Imaris X64 (Bitplane) 3D surface rendering of the eggs were then generated and used to calculate the egg volumes. Bead volumes calculated using the median radius (1/2 diameter) and formula for the volume of a sphere ($v=4/3\pi r^3$).

2.9.3. Quantification of infection burden

Bacterial infections and quantification of infection burden was performed as previously described (Takaki et al., 2013). Briefly, 75 CFU *Mycobacterium marinum* was microinjected into

the caudal vein of zebrafish larvae at 36 hours post-fertilization. At 4 days post-infection larvae were imaged by inverted fluorescence microscopy and bacterial fluorescence quantified from images.

2.9.4. Granuloma measurement

Confocal images were used for quantifying the number of macrophages in contact with the egg, and subsequent classification of the immune response. Granuloma size was quantified by fluorescence analysis of confocal z-stacks which were flattened, and then fluorescent macrophages comprising the granuloma area was measured by fluorescent pixel counts (FPC) (Takaki et al., 2013).

2.9.5. Statistical analysis

Statistical analyses were performed using Prism 5.01 (GraphPad Software), with each statistical test used specified in the corresponding figure legend. Post-test p-values are as follows: ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Where the n value is given and not represented graphically in the figure, n represents the number of zebrafish used for each experimental group.

Chapter 3. *Schistosoma mansoni* eggs modulate the timing of granuloma formation to promote transmission

3.1. Background and significance

Human schistosomiasis, caused by parasitic flatworms of the genus *Schistosoma*, affects more than 200 million people worldwide (WHO, 2019). Adult schistosomes live in the mesenteric venules of their definitive hosts, humans, where they produce eggs that are shed into the environment through feces or urine, depending on the schistosome species (Colley and Secor, 2014). Upon reaching fresh water, the eggs hatch releasing free swimming larvae, miracidia, that can then infect their intermediate snail hosts (Colley and Secor, 2014). In the snails, they reproduce asexually and mature to produce cercarial larvae, which are released into the water, and infect humans by penetrating the skin (Colley and Secor, 2014). In the case of *Schistosoma mansoni*, the most studied and geographically widespread species, the egg-laying adult pair resides in the mesenteric venous plexus where they produce eggs (Nation et al., 2020). The eggs are shed by translocation through the venule and then the intestinal wall into the feces; however, many become lodged in the intestinal wall or the liver (Hams et al., 2013; McManus et al., 2018; Nation et al., 2020; Schwartz and Fallon, 2018).

As the egg matures, it secretes antigens that provoke the formation of the granuloma which encases it (Ashton et al., 2001; Boros and Warren, 1970; Chiu and Chensue, 2002; Jurberg et al., 2009). For the host, the granuloma may play a dual function - both protective and pathogenic (Hams et al., 2013). On the one hand, it may protect the host by sequestering toxic egg antigens, and by preventing translocation of bacteria from the intestinal lumen into the tissues as the egg breaches the intestinal wall to exit the host (Costain et al., 2018; Hams et al., 2013; Pagan and Ramakrishnan, 2018; Schwartz and Fallon, 2018). On the other hand, the chronic granulomas around tissue-trapped eggs, particularly those in the liver, are the principal drivers of disease pathogenesis and morbidity (Hams et al., 2013; Pagan and Ramakrishnan, 2018). The chronic *Schistosoma* granuloma has a complex cellular composition with an abundance of myeloid cells, lymphocytes, eosinophils, and fibroblasts that act in concert to cause tissue pathology (Hams et al., 2013; Pagan and Ramakrishnan, 2018). The fibrogenic granulomatous response to the liver-entrapped eggs causes periportal fibrosis leading to portal hypertension and the development of

esophageal varices which can rupture leading to internal bleeding and death (Colley and Secor, 2014; Pagan and Ramakrishnan, 2018).

While the granuloma has mainly been studied from a host centric view, it has also been hypothesized that the early granuloma is critical for the parasite by facilitating the translocation of its eggs from the vasculature to the intestines where they are released into the environment in feces for continuation of its life cycle (Dunne et al., 1983; Hams et al., 2013; Schwartz and Fallon, 2018). Because insights into the *Schistosoma* granuloma have been derived from single time point histologic studies of human clinical samples and animal models - hamsters, mice and monkeys (Cheever et al., 2002; Hutchison, 1928), its role in translocation is understudied. The optical transparency of the zebrafish larva has enabled detailing of the early events of tuberculous granuloma formation in real-time using non-invasive, high resolution, serial intravital microscopy (Pagan and Ramakrishnan, 2018; Ramakrishnan, 2020; Takaki et al., 2013). Here, I have used the zebrafish larva to detail the events of early granuloma formation to *S. mansoni* eggs. I find that macrophage-dense epithelioid granulomas form rapidly around mature eggs. In striking contrast, I find that immature eggs are immunologically silent, failing to provoke even minimal macrophage recruitment. Given that inert beads induce epithelioid granulomas, this finding provides insight into how the egg might actively manipulate the timing of granuloma formation so as to prevent immune destruction or premature extrusion from the host. This idea is supported by my findings that *S. mansoni*-infected mice have both mature and immature eggs in their liver and intestinal wall but shed only mature eggs into the intestinal lumen.

3.2. Results

3.2.1. *S. mansoni* eggs induce epithelioid granuloma formation in the context of innate immunity

To study *Schistosoma* granulomas I used the zebrafish hindbrain ventricle (HBV), an epithelium-lined cavity to which phagocytes are recruited in response to chemokines and bacteria (Cambier et al., 2017; Cambier et al., 2014b; Takaki et al., 2013; Yang et al., 2012)(Figure 3.1A). It has previously been shown that beads coated with *S. mansoni* soluble egg antigens (SEA) injected intravenously into mice get deposited in the lung where they induce macrophage recruitment and aggregation around them (Boros and Warren, 1971; Chiu et al., 2004). Using

transgenic zebrafish with red fluorescent macrophages, I found that injection of SEA into the HBV induced macrophage recruitment within six hours (Figure 3.1B)(Movie 1). Next, I implanted *S. mansoni* eggs into the HBV. Because the mature egg is relatively large (>50 μm diameter), I used a large bore borosilicate needle which allowed me to make an incision, grasp the egg and implant it into the HBV cavity in rapid succession (Appendix 1, Movie 2 and Methods, Figure 3.1C). Implantation of the eggs had no deleterious effect on larval survival; larvae implanted with either one or two eggs had a survival rate of 98%-100% at 5 days post-implantation (dpi), identical to the mock-implanted control group (n=50 per group). Implantation also did not change larval swimming behaviors or responses to tactile stimuli.

I examined macrophage responses to the egg at 5 dpi. Eight independent experiments showed a consistent pattern of varying levels of macrophage recruitment: some eggs (32%, range 17 to 44%) had minimal macrophage recruitment with 0-6 macrophages found in contact with the egg (Figure 3.1D and 3.1E and Appendix 2). The majority (69%, range 56 to 83%) elicited robust macrophage recruitment with 41% (range 11 to 67%) having several isolated macrophages or small clusters of macrophages in contact with them and 28% (0 to 45%) eliciting organized granulomas that had either partially or fully enveloped them (Figure 3.1D and 3.1E and Appendix 2).

To determine the macrophage recruitment events leading to granuloma formation, I imaged nine implanted eggs sequentially over seven days, and then analyzed retrospectively the progression of recruitment in the three that had formed granulomas (Figure 3.2A and Appendix 3). For the egg shown in Figure 3.2A, by 1 dpi, macrophages had arrived in response to the egg and were in contact with it (Figure 3.2A; Movie 3). By 3 dpi, macrophages had formed loose aggregates on one part of the egg (Figure 3.2A), an intermediate stage that is likely to represent a transition to granuloma formation as it was not seen in the 5 dpi single timepoint analyses. By 5 dpi, an organized granuloma partially covering the egg was apparent, which had expanded to encapsulate the entire egg by 7 dpi (Figure 3.2A; Movie 3). In the remaining two eggs that elicited granulomas, one had a similar sequence of events except that the granuloma which formed by 5 dpi had still not enveloped the egg completely at 7 dpi (Appendix 3A). The other egg had already formed a small partial granuloma by 3 dpi but could not be monitored further owing to failure to recover the animal following imaging on this day (Appendix 3B). Thus, the sequence of events leading to granuloma formation seemed consistent in all cases.

Likewise, in all three cases, even the partial granulomas had macrophages which appeared confluent with indistinct intercellular boundaries, suggesting they had already undergone the characteristic epithelioid transformation associated with mature *Schistosoma* granulomas (Moore et al., 1977; Von Lichtenberg et al., 1973)(Figure 3.2A and Appendix 3). To confirm this, I identified 8 eggs that had elicited partial or complete granulomas and assessed these for epithelioid transformation using immunofluorescence staining for E-cadherin, the expression of which is its cardinal feature (Cronan et al., 2016). All 8 eggs had E-cadherin staining, confirming that they had undergone epithelioid transformation as exemplified by Figure 3.2B and Movie 3.

In mammals, *S. mansoni* eggs invoke macrophage-rich granulomas with very few neutrophils in contrast to *S. japonicum* eggs, which recruit both macrophages and neutrophils (Chensue et al., 1995b; Moore et al., 1977; Swartz et al., 2006; Von Lichtenberg et al., 1973). Likewise, I found that in the zebrafish, granulomas forming to *S. mansoni* eggs contained very few neutrophils (Figure 3.2C and D). In contrast, similarly-sized *Mycobacterium marinum* granulomas all contained neutrophils as expected (Figure 3.2C and D) (Yang et al., 2012). This pattern was established at the onset of egg implantation with the recruitment of macrophages but not neutrophils at 6 hours post-implantation (hpi), whereas the Gram-negative bacterium *Pseudomonas aeruginosa* recruited both types of cells, as expected (Figure 3.2.E)(Yang et al., 2012). The lack of neutrophil recruitment has been attributed to the egg-secreted, interleukin-8 neutralizing *S. mansoni* chemokine binding protein (smCKBP), more commonly known as alpha-1 (Smith et al., 2005). Accordingly, the injection of SEA recruited macrophages but not neutrophils, in contrast to *P. aeruginosa* which recruited both (Figure 3.2F and 3.2G).

Next, I asked if the miracidium could survive within an epithelioid granuloma. I imaged individual eggs containing mature miracidia within organized granulomas at 5 dpi, and found they were still alive; the miracidium could be seen moving within the eggshell (Appendix 4A; Movie 4). E-cadherin staining immediately after imaging confirmed that the granuloma macrophages had indeed undergone epithelioid transformation (Appendix 4B). I also saw that in those cases where the eggshell had ruptured either during or after implantation, macrophages had entered into the eggshell and destroyed the miracidium (Appendix 4C; Movie 4). These findings were consistent with those in mammals showing that the intact eggshell protects the miracidium against destruction by host macrophages (Bunnag et al., 1986; Hutchison, 1928; Von

Lichtenberg et al., 1973). Further confirming this, miracidia implanted after removal from the egg rapidly recruited macrophages that destroyed them (Appendix 4D).

In sum, I found that the key features of early mammalian responses to *S. mansoni* eggs are replicated in the zebrafish: selective macrophage recruitment to form bona fide epithelioid granulomas within days, which formed in the sole context of innate immunity. These findings highlight that the miracidium tolerates granuloma formation as long as the eggshell is intact, a critical aspect of the *Schistosoma* life cycle that depends on granulomas to enhance egg extrusion from the host. These granulomas most closely resemble intestinal granulomas in mice, which comprise mostly macrophages with fewer lymphocytes and eosinophils (Weinstock and Boros, 1983a).

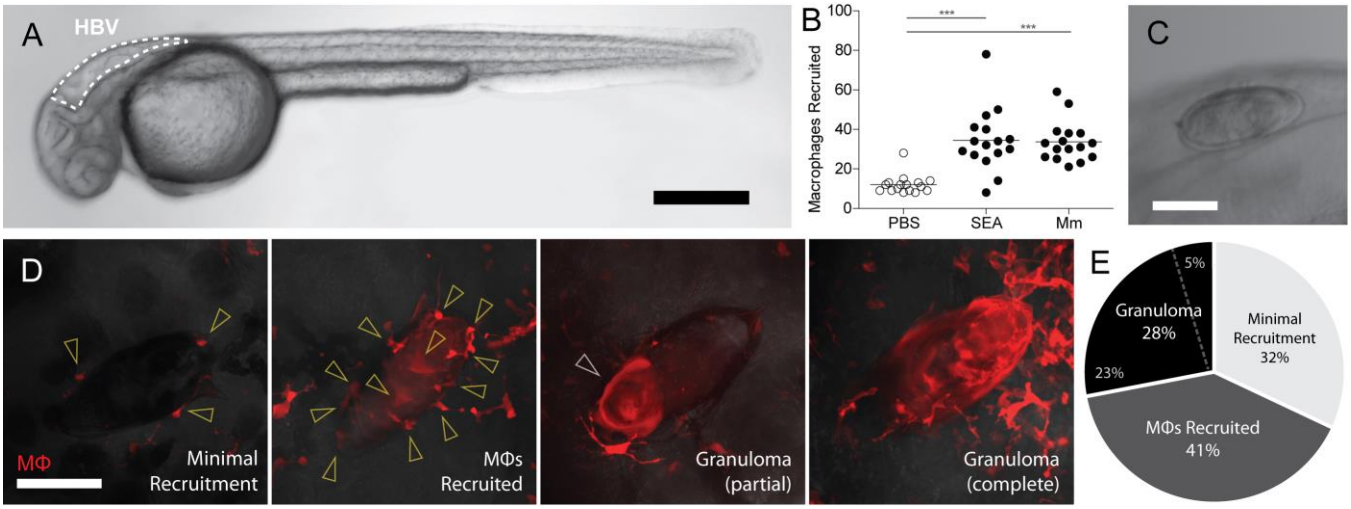


Figure 3. 1. Macrophage responses to SEA and *S. mansoni* eggs

(A) Zebrafish larvae at 30 hpf with hindbrain ventricle (HBV) outlined. Scale bar, 300 μm. (**B**) Mean macrophage recruitment to HBV 3 hours post-injection with PBS, SEA or Mm; ANOVA with Dunnet's post-test. (**C**) *S. mansoni* egg in HBV immediately after implantation. Scale bar, 75 μm. (**D**) Representative images of macrophage responses to *S. mansoni* eggs observed 5 dpi; Minimal recruitment, few if any macrophages recruited with ≤6 in contact with the egg (arrowheads); Macrophages recruited, several macrophages recruited with >6 in contact with the egg (arrowheads) but without aggregation; Granuloma; macrophage aggregation in which individual macrophages cannot be distinguished as separate, either partially (arrowhead) or completely encasing the egg. Scale bar, 75 μm. (**E**) Prevalence of macrophage responses to implanted eggs as defined in **D**, representing 8 experiments, each constituting a separate batch of eggs and a separate clutch of zebrafish larvae, as detailed in Appendix 2. Dotted line divides the proportion of partial (23%) and complete (5%) granulomas. Also see Appendix 1, Appendix 2 and Movie 2.

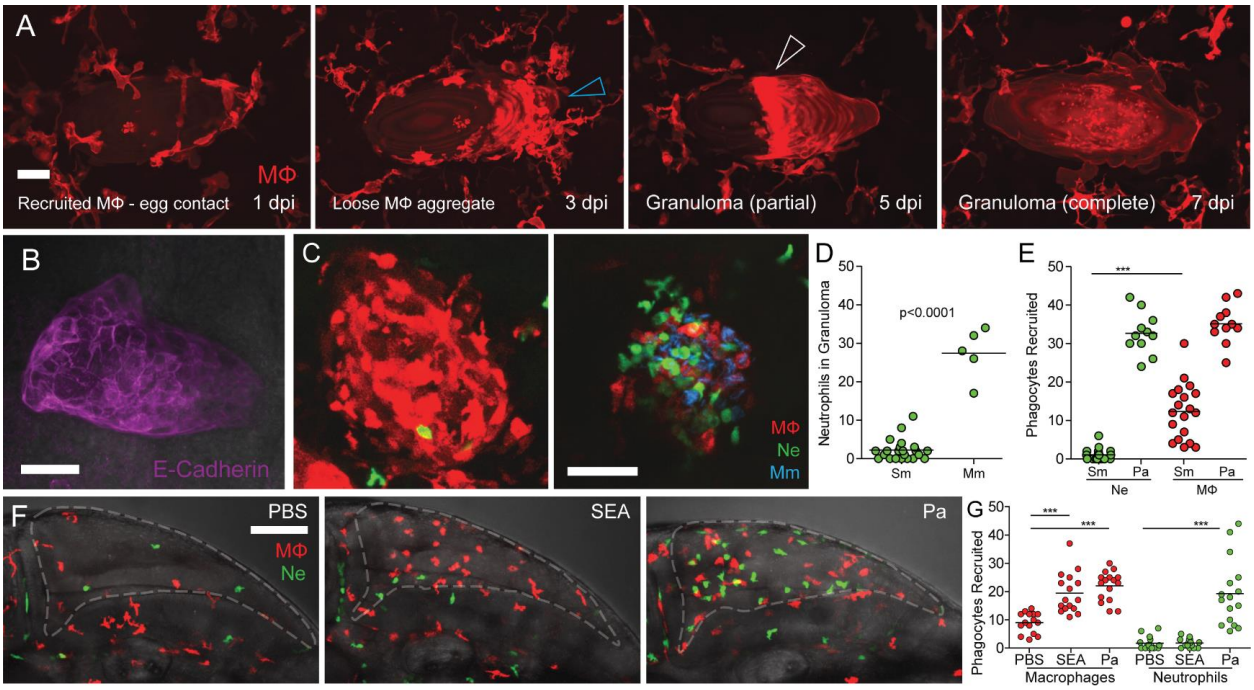


Figure 3. 2. *S. mansoni* eggs induce epithelioid granulomas in larval zebrafish

(**A**) Timelapse microscopy of egg monitored at 2 day intervals from 1-7 dpi showing in the four panels, respectively, sequential macrophage recruitment, aggregation (blue arrowhead), formation of the partial granuloma (white arrowhead), and its expansion to encase the egg. Scale bar, 25 μm. (**B**) Epithelioid granuloma immunostained using E-cadherin antibody. Scale bar, 50 μm. (**C**) Confocal images of granulomas in representative transgenic zebrafish larvae with red-fluorescent macrophages (MΦ) and green-fluorescent neutrophils (Ne) at 5 dpi with *S. mansoni* eggs (Sm) (left), or 5 days post-infection with *M. marinum* (Mm) (right); Scale bar, 50 μm. (**D**) Quantification of neutrophils recruited to Sm and Mm granulomas. (**E**) Quantification of phagocytes recruited to Sm and *P. aeruginosa* (Pa) 6 hours post-injection. (**F**) Confocal images of HBV of representative larvae showing phagocyte recruitment at 6 hours post-injection with phosphate buffered saline (PBS) (left), *S. mansoni* soluble egg antigens (SEA) or *P. aeruginosa*. Scale bar, 100 μm. (**G**) Quantification of phagocytes recruited to Sm and *P. aeruginosa* (Pa) at 6 hours post-injection. Horizontal lines in (**D**,**E**,**G**) depict mean values. Student's t-test (**D**) or one-way ANOVA with Bonferroni's post-test (**E**,**G**). Experiments in (**A-B**,**E**) were done once each, those in (**C-D**,**F-G**) are representative of two experiments. See also Appendix 3 and Movie 3.

3.2.2. Immature *S. mansoni* eggs do not induce macrophage recruitment or granuloma formation

The egg matures six days after it is fertilized at which point it begins to secrete antigens (Ashton et al., 2001; Jurberg et al., 2009; Mann et al., 2011; Michaels and Prata, 1968). Accordingly, only viable mature eggs are found to induce granulomas (Jurberg et al., 2009; Von Lichtenberg et al., 1973). I sorted immature and mature eggs based on their size and appearance (Appendix 5A)(Jurberg et al., 2009). None of the immature eggs had reached maturity by 5 dpi and importantly all invoked only minimal macrophage recruitment (Figure 3.3A and 3.3B). To corroborate this result, I implanted in vitro laid eggs (IVLE) at 2 and 6 days post-fertilization in which the developmental stages were synchronized so that the two day eggs were immature and the six day eggs mature (Appendix 5B). Again, the majority of the six day old mature eggs induced macrophage recruitment, including granuloma formation, whereas the two-day eggs elicited only minimal macrophage recruitment (Figure 3.3C). These results were consistent with antigens secreted from the mature egg being the trigger for granuloma formation (Ashton et al., 2001; Boros and Warren, 1970; Chiu and Chensue, 2002). To test this, I asked if dead eggs elicited a macrophage response. Freshly heat-killed eggs produced fewer granulomas than live eggs (Figure 3.3D). This finding is consistent with prior observations that some egg antigens are heat stable and that heat-killed eggs retain a thin layer of antigens which can induce granulomas, albeit less than living eggs (Freedman and Ottesen, 1988; Klaver et al., 2015; Lichtenberg, 1964). Accordingly, I found that eggs that had been killed by storage at 4°C for 12 months (old dead eggs) so as to potentially inactivate all their antigens did not induce granulomas, and only a minority (10%) recruited any macrophages at all (Figure 3.3E).

I next asked if immature and dead eggs, although failing to form granulomas, could still induce early transient macrophage recruitment. At 6 hours post-implantation, immature, heat-killed, and old dead eggs all recruited fewer macrophages than live mature eggs (Figure 3.3F-I). These findings suggested that mature egg antigens enhance macrophage recruitment from the earliest stages, and subsequently activate the recruited macrophages to form the granuloma.

Finally, I found that if I ruptured immature eggs prior to implantation, they rapidly recruited macrophages (Figure 3.3J and 3.3K). Similar to the case with ruptured mature eggs, these macrophages entered the ruptured immature egg and killed the embryo (Figure 3.3J and data not

shown). Together these results suggest that while the exposed embryo and fully-mature miracidium elicit macrophage recruitment similarly, the intact egg at the two stages is fundamentally different in its ability to recruit macrophages, the initial step that is required for granuloma formation.

To ask if egg antigen secretion was also required for the subsequent steps of macrophage aggregation into granulomas, I implanted an immature egg together with a mature egg in each animal. If mature egg antigens were required only to recruit macrophages to the egg, then the presence of the mature egg should recruit macrophages to the vicinity of the immature egg, allowing granulomas to form. In both instances, macrophages were recruited to and settled on the mature egg, with hardly any on the adjacent immature egg (Figure 3.3L). Thus, macrophage recruitment in response to the presence of egg antigens in the vicinity of the immature egg is not sufficient to induce macrophage adherence and granuloma formation. Rather, egg-intrinsic antigen is required for both macrophage recruitment and adherence to the egg with subsequent granuloma formation.

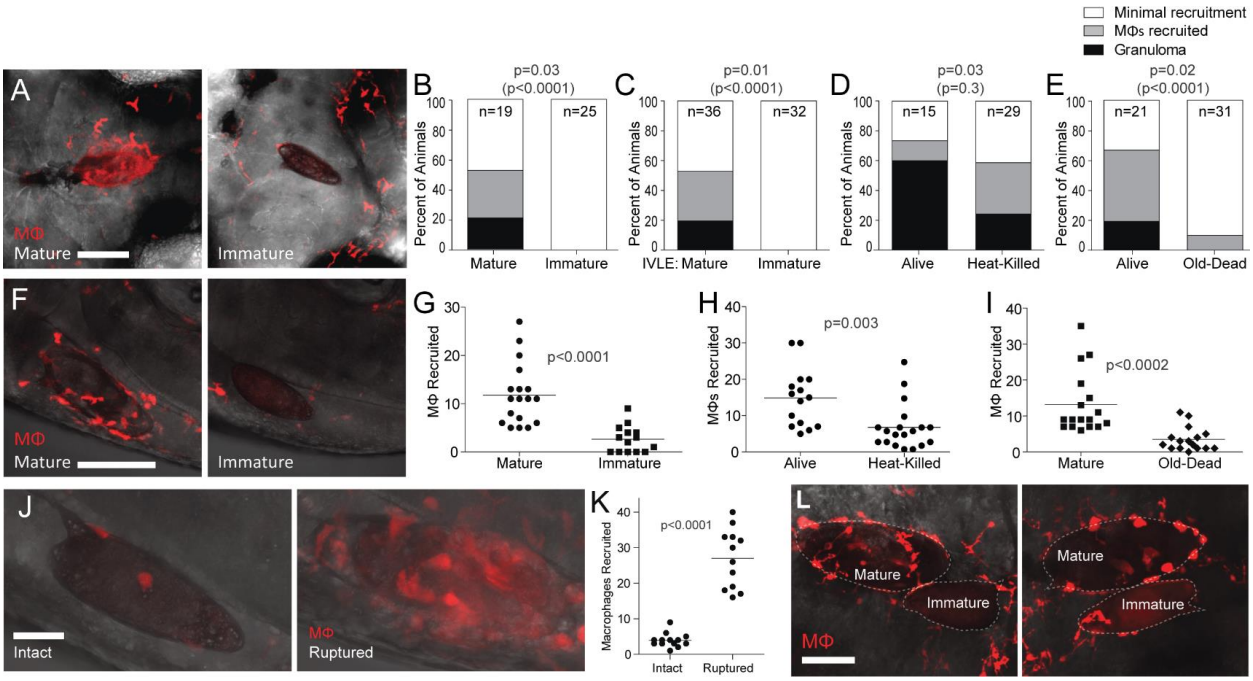


Figure 3. 3. Immature eggs do not induce macrophage recruitment or granuloma formation

(**A**-**E**) Granuloma formation and macrophage recruitment at 5 dpi comparing mature eggs with (**A**,**B**) immature eggs, (**C**) immature IVLE, (**D**) heat-killed eggs, or (**E**) old-dead eggs. Representative images in (**A**), scale bar 100 μm. (**B**-**E**) Percent of animals with different levels of macrophage recruitment to the egg. (**F**-**I**) Macrophages recruited to mature eggs at 3 hpi compared to (**F** and **G**) immature eggs, (**H**) heat-killed eggs, and (**I**) old-dead eggs.

Representative images in (**F**), scale bar, 100 μm. (**G-I**) Quantification of macrophages recruited. (**J**) Confocal images showing macrophage recruitment to intact and mechanically ruptured immature eggs 6 hpi. Scale bar, 25 μm. (**K**) Quantification of macrophage recruitment to intact and ruptured immature eggs 6 hpi. (**L**) Confocal images of macrophage recruitment 5 dpi to co-implanted mature and immature eggs into the same hindbrain ventricle of two different larvae. Enumeration of recruited macrophages showed 19 and 2 macrophages recruited respectively to the mature and immature egg (left panel), and 23 and 6 macrophages recruited respectively to the mature and immature egg (right panel). Scale bar, 50 μm. (**G-I**) Horizontal bars, mean values. Statistics, (**B-E**) Fisher's exact test comparing the proportion of eggs which induced granuloma formation (black bars), or granuloma formation with macrophage recruitment (black and gray bars combined, in parentheses); (**G-I**, **K**) Student's t-test. (**B-E**) n, number of animals. All experiments performed once, except for **F**,**G**,**J**, and **K** which are representative of two experiments. Also see Appendix 5.

3.2.3. The immature *Schistosoma* egg evades foreign body granuloma formation

These findings were consistent with macrophage recruitment occurring only in response to antigens secreted from the mature egg rather than to the eggshell itself. Granulomas form in response to inert foreign bodies (Pagan and Ramakrishnan, 2018), so why would the eggshell not induce a foreign body granuloma? I considered three possibilities. First, that it was too small to invoke a foreign body response; this seemed unlikely as very small inert particles, e.g. a tiny thorn, can provoke a robust foreign body response (Pagan and Ramakrishnan, 2018). Second, that the mechanisms to form foreign body granulomas were not yet operant in the developing zebrafish larvae; this too seemed unlikely given that the foreign body granuloma response is evolutionarily ancient, and epithelioid granulomas form in response to foreign bodies in invertebrates (Pagan and Ramakrishnan, 2018). Third, that the immature schistosome egg has specific mechanisms to evade foreign body granuloma formation. To distinguish between these possibilities, I implanted beads of three different chemically inert materials of the same size as the schistosome egg (Appendix 6). I chose sepharose, which is hydrophilic, and polystyrene and polyethylene, which are hydrophobic. All recruited macrophages within six hours (Figure 3.4A and 3.4B). By five days, epithelioid granulomas had formed on most of the sepharose and polystyrene beads (Figure 3.4C-E). The polyethylene beads were less granuloma inciting, with only 11% inducing bona fide granulomas, and most of the remaining beads failing to retain recruited macrophages (Figure 3.4C and 3.4D). However, even this weaker response was more robust than that of the immature eggs, which did not even transiently recruit macrophages. I confirmed these findings with a head-on comparison of macrophage recruitment and granuloma

formation in response to immature eggs or similarly sized polystyrene beads in the same experiment (Appendix 6). Again, the polystyrene beads recruited macrophages by six hours and formed granulomas by five days, whereas the immature eggs did neither (Figure 3.4F and G). This result suggested that the immature egg specifically avoids being recognized as a foreign body. This could be because the immature egg secretes a specific product to inhibit macrophage recruitment, or that the eggshell is immunologically inert. To distinguish between these possibilities, I implanted an immature egg and a polystyrene bead adjacent to each other in the same animal. In every case, at six hours, macrophages were recruited only to the bead and not to the egg (Figure 3.4H and 3.4I). By five days post-implantation, granulomas had formed around the beads but none of the immature eggs (Figure 3.4J). These results support the idea that the eggshell evolved to be immunologically inert so as to evade the ubiquitous foreign body granulomatous response.

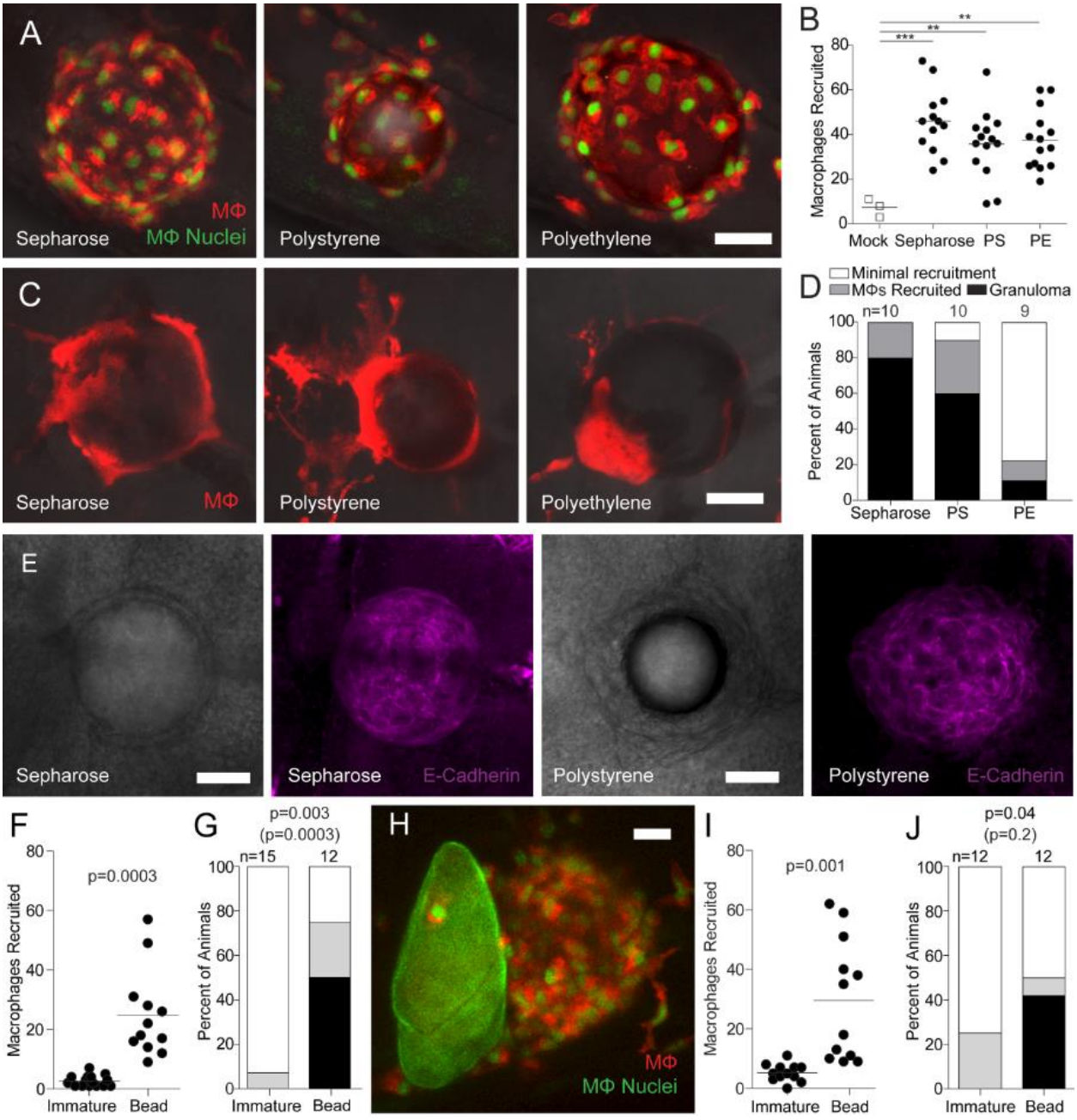


Figure 3. 4. Chemically inert beads induce epithelioid granulomas

(**A**) Representative confocal images of macrophages recruited 6 hpi of sepharose, polystyrene or polyethylene microspheres into the HBV of transgenic zebrafish larvae carrying red-fluorescent macrophages with green nuclei. (**B**) Enumeration of macrophages recruited to these microspheres in multiple animals. (**C**) Representative confocal images of granulomas formed around the three types of microspheres 5dpi into the HBV of transgenic larvae carrying the transgene for red-fluorescent macrophages (without green nuclei). (**D**) Stages of macrophage recruitment to microspheres 5 dpi into HBV of multiple larvae. (**E**) Brightfield (panels 1,3) and fluorescence confocal (panels 2,4) microscopy of sepharose and polystyrene bead granulomas following immunofluorescence staining with the E-cadherin antibody. (**F** and **G**) Macrophage recruitment to immature eggs or microspheres implanted into the HBV at 6 hpi (**F**) and 5 dpi

(**G**). (**H-J**) Macrophage recruitment following co-implantation of an immature egg and a polystyrene microsphere into the HBV of larvae transgenic for red-fluorescent macrophages with green nuclei. (**H**) Representative confocal image of an immature egg next to a microsphere. (**I**,**J**) Quantification of macrophage recruitment at 6 hpi (**I**), and at 5 dpi (**J**). Scale bars, 25 μm. (**B**,**F**,**I**) Horizontal bars, means. (**D**,**G**,**J**) n, number of animals. Statistics, one-way ANOVA (**B**), unpaired (**F**) and paired (**I**) Student's t-test and Fisher's exact test comparing granulomas (black bars) or granuloma formation with macrophage recruitment (black and gray bars, in parentheses) (**G-J**). Experiments in **E** and **F-J** were performed once. **A-D** are representative of three experiments. Also see Appendix 6.

3.2.4. Only mature eggs translocate into the intestinal lumen of *S. mansoni*-infected mice and humans

The observation that immature eggs, unlike mature eggs, are immunologically silent, led us to hypothesize, as P.D. Ashton et al. did before (Ashton et al., 2001), that timing granuloma formation to egg maturation prevents the expulsion of immature eggs while they are still dependent on the absorption of nutrients from the host for development. Moreover, only a mature miracidium can survive in the aquatic environment and invade its snail host. If this hypothesis were true, I would expect to find in *S. mansoni*-infected mice, an enrichment of mature eggs in the intestinal lumen as compared to intestinal wall and liver. To test this prediction, mice were naturally infected with *S. mansoni* by cutaneous exposure to cercaria, and at 6 weeks post-infection, eggs were analyzed from liver, intestinal tissue, and small and large intestinal luminal content (feces). I quantified and categorized eggs as mature or immature by size and morphology (Jurberg et al., 2009)(Appendix 5 and Appendix 7). As a test of my scoring accuracy, I then measured the size of the eggs and confirmed that my visual inspection had correctly separated the immature and mature eggs (Appendix 7). I next assessed the distribution of mature and immature eggs for each collection site in each mouse. I found that while the liver and the intestinal tissue contained roughly equal proportions of both immature and mature eggs (Figure 3.5A-D), hardly any immature eggs were found in the small intestinal lumen (6% average for all six animals; Figure 3.5E). Moreover, only 2 out of 11 immature eggs were at the very early stage of development, with the remaining ones nearing maturity (2009 Jurberg)(Figure 3.5E-G). All eggs scored from the lumen of the large intestines (feces) were morphologically mature and contained fully mature miracidia (Figure 3.5H and 3.5I). Statistical analysis of the pooled data from four mice confirmed an enrichment of mature eggs in the lumen of the small and large

intestines (Figure 3.5J). These results confirmed that virtually all eggs shed by infected mice are mature.

Do humans also shed only mature eggs? I was unable to find a direct answer to this question in the literature. However, a paper was published which had assessed the length and width of 30 eggs shed in the feces of *S. mansoni*-infected humans (Martinez., 1916). Because I found that immature and mature eggs differ in size with immature eggs being much smaller (Appendix 5 and Appendix 7)(Ashton et al., 2001), I was in a position to determine if the eggs shed by humans were mature or immature. I plotted the sizes of the eggs shed in human feces alongside the eggs from the mouse intestinal wall and lumen and found that all of the eggs from human feces were in the mature egg size range (Figure 3.5K). Thus, humans also shed only mature eggs.

These results support the hypothesis that the timing of granuloma formation and subsequent egg expulsion is modulated so as to prevent premature expulsion of immature eggs, which would be terminal for the parasite were it to occur.

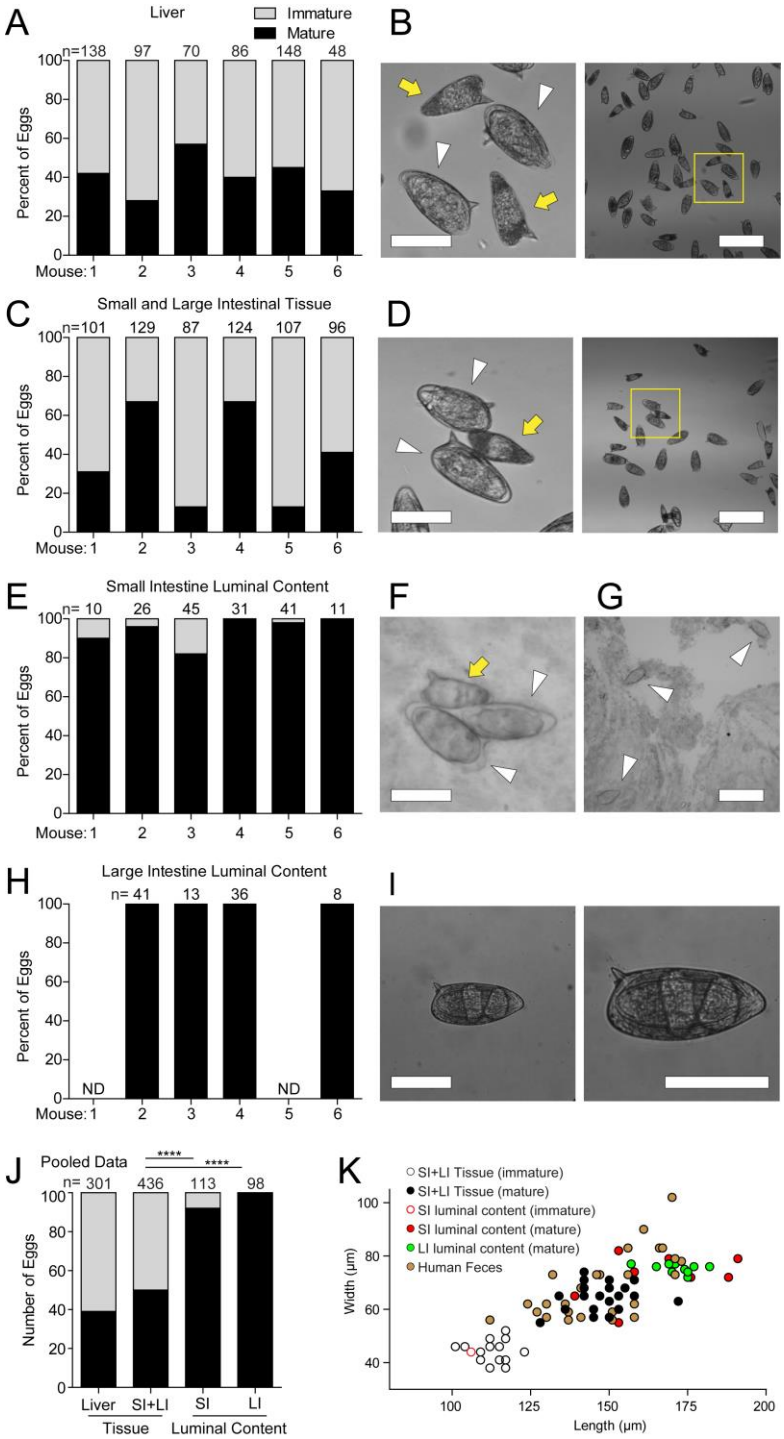


Figure 3. 5. Mature eggs translocate into the lumen of the intestines

(**A-I**) Quantification (**A**,**C**,**E**,**H**) and representative brightfield images (**B**,**D**,**F**,**G**,**I**) of mature and immature eggs found in the liver (**A**,**B**), small and large intestinal wall tissue and vasculature (**C**,**D**), small intestinal luminal content (**E-G**) and large intestinal luminal content (**H**,**I**) for six individual *S. mansoni*-infected mice. (**B**,**D**) Representative images with image (left panel) showing immature (yellow arrow) and mature (white arrowhead) magnified from yellow square in wide-field image (right panel). (**F**,**G**) Images of eggs from the lumen of the small intestine, showing two mature eggs in contact with one immature egg (**F**), and a wide-field image showing

three mature eggs (**G**). (**I**) Representative image of an egg recovered from feces at low resolution (left) and higher resolution with developed miracidia visible (right). (**J**) Pooled data for mice 2,3,4, and 6 from panels (**A**,**C**,**E**,**H)**. SI, small intestine; LI, large intestine. (**K**) Dimensions of eggs from this experiment which were classified as immature or mature (open or closed circles, respectively) plotted with eggs shed in the feces of *S. mansoni*-infected humans (Martinez., 1916). All scale bars 100 μm except for (**G**) and the right panels of (**B**,**D**) which are 300 μm. ND, not determined. Statistics, Fisher's exact test. Also see Appendix 7.

3.3. Discussion

Research on *S. mansoni* granulomas has focused mainly on the organ-damaging fibrosis that ensues from granulomas forming around tissue-lodged eggs (Colley and Secor, 2014). Yet most *S. mansoni*-infected individuals are either asymptomatic or only mildly symptomatic (Hams et al., 2013), possibly because their granulomatous response is more tempered. These individuals shed parasite eggs, highlighting that disease per se does not benefit the parasite's evolutionary survival. Rather, as in the case with many infectious diseases, human disease represents collateral damage stemming from the host-pathogen interaction, harming the host with little benefit to the pathogen (Relman et al., 2020). On the other hand, early granuloma formation in appropriate anatomical locations is thought to benefit both host and parasite for the same reason, expelling the parasite egg from the human host so as to enable it to continue its life cycle in its intermediate snail host (Dunne et al., 1983; Hams et al., 2013). While this idea is appreciated, it has been difficult to study extensively because of experimental limitations. Early or asymptomatic human infection seldom presents itself for study, and existing animal models are less suitable for the study of early granuloma-associated pathology.

This work explores the earliest steps of *Schistosoma* granuloma formation that have not been captured in existing animal models. I show that as is the case with mycobacterial granulomas, bona fide epithelioid granulomas form in response to the Schistosoma egg in the sole context of innate immunity (Cronan et al., 2016; Davis et al., 2002). This should not be surprising given that epithelioid granulomas form in multiple invertebrate species in response to retained foreign bodies or even their own dead eggs (Pagan and Ramakrishnan, 2018). Yet, there has been at best a limited appreciation that adaptive immunity is not required for the formation of such an organized structure in the context of infectious granulomas, and indeed, the emphasis of schistosomiasis research on the late-stage granuloma has caused the focus to be on how the

granuloma is modulated by adaptive immunity to become pathogenic (Hams et al., 2013; Pagan and Ramakrishnan, 2018). Given that Schistosoma eggs begin to be shed into the feces within days following maturation (deWalick et al., 2012), egg shedding must occur even in the absence of adaptive immunity and is likely promoted by these innate epithelioid granulomas. The rapid epithelioid transformation of the granuloma also has relevance for granuloma-induced transmission later in infection when adaptive immunity is operant. Moreover, intestinal granulomas, the ones that extrude the eggs, are smaller than those in the liver, with a paucity of the lymphocytes and eosinophils that characterize liver granulomas (Weinstock and Boros, 1983a). The rapid epithelioid transformation of the *Schistosoma* granuloma may help it to more efficiently extrude the eggs and hence propagate the parasite.

These experiments have also led to an increased understanding of the mechanics of early granuloma formation. Broadly speaking, granuloma formation in response to the mature egg proceeds in two discrete steps. In the first step, macrophages are attracted to secreted parasite antigens, and upon contact with the egg, appear to gain a chemotactic activity that outstrips that of the egg. This results in the subsequent macrophages being recruited to the existing macrophages forming a tight, aggregate that then pulls itself together to encapsulate the egg. It is noteworthy that epithelioid transformation precedes the complete covering of the egg, highlighting that this specialized macrophage transformation (Pagan and Ramakrishnan, 2018) constitutes an early response.

While these new details on how granulomas form around mature eggs are thought-provoking, more striking is the lack of even minimal macrophage recruitment by the immature egg. Given that like-sized beads recruit macrophages robustly and induce epithelioid granulomas, this finding reveals further nuance to the exploitation of the granuloma by the parasite. Not only must the parasite induce granuloma formation through secretion of antigens, but it must also prevent the granuloma from forming too soon. Premature granuloma formation may be detrimental for two reasons. The egg is laid into the blood stream, and needs to reach the wall of the blood vessel from which it extravasates and then penetrates the gut wall to be shed (deWalick et al., 2012). Perhaps, premature granuloma formation might encumber its passage to the intestinal wall. Second, premature extrusion would remove the egg from the human tissue environment that is conducive to its maturation (Ashton et al., 2001).

Prior work has noted that the granuloma-inducing schistosome egg antigens are secreted from the egg, rather than being incorporated into the eggshell, and that secretion occurs only after egg maturation (Ashton et al., 2001; Schwartz and Fallon, 2018). This work adds the key insight that the immunologically inert nature of the eggshell is a requisite counterpart of the *Schistosoma* transmission strategy. The ability to directly compare granuloma formation around eggs and beads has been key to this insight. It will be interesting to determine how the eggshell remains immunologically inert in the context of adaptive immunity, particularly because eggshell proteins induce antibodies in humans (Dewalick et al., 2011; deWalick et al., 2012). Foreign body granuloma formation is a major complication of implanted devices (Pagan and Ramakrishnan, 2018). Identifying the chemical basis of the granuloma-silencing mechanism of the eggshell may have therapeutic implications in the design of inert materials for medical implants that alleviate this problem.

3.4. Summary

Schistosome eggs provoke the formation of granulomas, organized immune aggregates, around them. For the host, the granulomatous response can be both protective and pathological. Granulomas are also postulated to facilitate egg extrusion through the gut lumen, a necessary step for parasite transmission. I used zebrafish larvae to visualize the granulomatous response to *Schistosoma mansoni* eggs and inert egg-sized beads. Mature eggs rapidly recruit macrophages, which form granulomas within days. Beads also induce granulomas rapidly, through a foreign body response. Strikingly, immature eggs do not recruit macrophages, revealing that the eggshell is immunologically inert. These findings suggest that the eggshell inhibits foreign body granuloma formation long enough for the miracidium to mature. Then, parasite antigens secreted through the eggshell trigger granulomas which facilitate egg extrusion into the environment. In support of this model, I find that only mature *S. mansoni* eggs are shed into the feces of mice and humans.

Chapter 4. Tumor Necrosis Factor and *Schistosoma mansoni* egg antigen Omega-1 shape distinct aspects of the early egg-induced granulomatous response

4.1 Background and significance

Schistosomiasis is a major granulomatous disease, caused by parasitic flatworms of the genus *Schistosoma* with *Schistosoma mansoni* being the most widespread agent of the disease (McManus et al., 2018). The events of *Schistosoma* egg-induced granulomas have been deduced mainly from histological assessments of human clinical samples and the use of experimental mammalian models (Cheever et al., 2002; Hutchison, 1928). In the previous chapter I have shown the use of the optically transparent and genetically tractable zebrafish larva as a model to study early macrophage recruitment and granuloma formation in response to *S. mansoni* eggs. Because the zebrafish larva lacks adaptive immunity during their first few weeks of development, this model can be used to dissect mechanisms in the sole context of innate immunity (Davis et al., 2002; Takaki et al., 2013). I found that while epithelioid granulomas form rapidly around mature eggs, immature eggs fail to provoke granulomas, consistent with the mature stage-specific secretion of antigens and their function to induce granuloma formation in mammalian models (Ashton et al., 2001; Boros and Warren, 1970; Chiu and Chensue, 2002; Jurberg et al., 2009; Lichtenberg, 1964; Schramm et al., 2006).

In the zebrafish, I can additionally examine macrophage recruitment within hours of implantation, and I find that whereas injections of schistosome soluble egg antigen (SEA) obtained from mature eggs induce early macrophage recruitment, implantation of immature eggs do not. Together these findings both validate the zebrafish model to study *S. mansoni* egg-induced granuloma formation and reveal new insights into the underlying molecular mechanisms.

In mice, the cytokine Tumor Necrosis Factor (TNF) and the *S. mansoni* secreted antigen omega-1 have been identified as host and parasite factors, respectively, that promote granuloma formation around the egg (Amiri et al., 1992; Chensue et al., 1994; Chensue et al., 1995b; Hagen et al., 2014; Ittiprasert et al., 2019). However, the role of TNF remains controversial and the

mechanism by which omega-1 exerts its role is unresolved. In this work, I use the zebrafish model to explore their roles in macrophage recruitment and innate granuloma formation.

4.2. Results

4.2.1. TNF signaling through TNF Receptor 1 promotes macrophage recruitment to nascent *S. mansoni* egg-induced granulomas but is dispensable for initial macrophage recruitment to the eggs

The role of TNF in *S. mansoni* egg-induced granulomas remains unresolved after two decades of studies in the murine model of schistosomiasis. Early findings showed that *S. mansoni*-infected SCID mice were deficient in both granuloma formation and egg extrusion, phenotypes which was rescued by recombinant TNF and activated T cell medium, but not by TNF-depleted T cell medium (Amiri et al., 1992). These findings suggested a role for TNF in granuloma formation and egg excretion (Amiri et al., 1992). However, subsequent work from this group found that TNF knockout mice did not have a defect in granuloma formation (Davies et al., 2004). Mice lacking both receptors through which TNF signals did exhibit a mild granuloma deficit, leading the authors to propose that it might be due to a defect in signaling of lymphotoxin (Davies et al., 2004). However, this would not explain their earlier findings that exogenous TNF rescued granuloma formation in SCID mice (Amiri et al., 1992). Meanwhile, a different group reported that TNF did not rescue granuloma formation in SCID mice (Cheever et al., 1999). Additionally, *S. mansoni*-infected SCID mice displayed normal levels of TNF expression, suggesting that other cells may be the major source of TNF during the infection (Cheever et al., 1999). It has been suggested that Ly6C^{hi} monocytes, which are known to express TNF in response to the schistosome egg, might be the innate source of TNF (Nascimento et al., 2014).

To delineate the role of TNF in macrophage recruitment and granuloma formation around S. mansoni eggs, I used a TNFR1 zebrafish mutant created by CRISPR-Cas technology (see Methods). I first confirmed that the lack of TNFR1 signaling rendered zebrafish larvae susceptible to *Mycobacterium marinum* infection, consistent with previous findings using TNFR1 morpholino (Clay et al., 2008)(Figure 4.1).

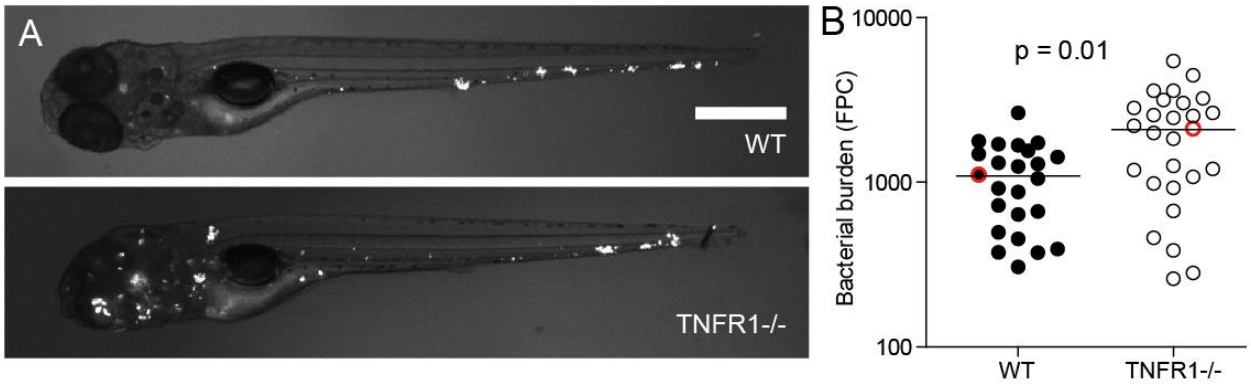


Figure 4. 1. TNFR1 mutant zebrafish larvae have increased infection burden
WT and TNFR1 mutant zebrafish larvae were systemically infected at 36 hours post-fertilization via caudal vein injection with 75 CFU *Mycobacterium marinum*, and then imaged at 4 days post-infection for bacterial burden. (**A**) The two animals closest to the mean. Scale bar, 300 μm. (**B**) Quantification of bacterial burden, with the two red data points corresponding to the animals in (A). Horizontal bar, means. Statistics, Student's t test. FPC: fluorescent pixel counts.

Next, I implanted the Hindbrain Ventricle (HBV) of wildtype and TNFR1 mutant larvae with *S. mansoni* eggs and evaluated granuloma formation after five days (Figure 4.2A-D). In the previous chapter I categorized early macrophage recruitment and granuloma formation based on the number and characteristics of macrophages in contact with the egg: Minimal recruitment, 0-6 macrophages; Macrophages recruited, >6 macrophages; Granulomas, confluent epithelioid macrophage aggregates. At 5 days post-implantation of the eggs, the TNFR1 mutants had similar macrophage responses to wildtype animals with ~50% of the animals forming epithelioid granulomas in each group (Figure 4.2B). However, I found that the TNFR1 deficient granulomas were significantly smaller than wildtype granulomas, with the mean granuloma size being 62% smaller than in wildtype (Figure 4.2C and D). Also notable is that the TNFR1 mutant granulomas, though smaller, showed a characteristic epithelioid morphology with confluent macrophages and loss of intercellular boundaries. This finding suggests that epithelioid transformation occurs independent of TNFR1 signaling (Chapter 3.2.1)(Figure 4.2D). Because the *S. mansoni* granuloma is comprised solely of macrophages at this early stage (Chapter 3.2.1), this finding implies that TNFR1 signaling promotes macrophage recruitment to the nascent granuloma around the egg. As shown in chapter 3.2.2, the initiation of macrophage recruitment to *S. mansoni* eggs in zebrafish can be observed within hours of implantation. However, I found that TNFR1 signaling is not required for initiation of macrophage recruitment (Figure 4.2E).

Together, these results show that TNF signaling through TNFR1 is required specifically for macrophage recruitment after the initial macrophages reach the egg through other signal(s). Thereby, TNF mediates granuloma enlargement rather than granuloma initiation. Furthermore, TNF is not required for epithelioid transformation. Finally, TNF plays a role in the granulomatous response in the sole context of innate immunity.

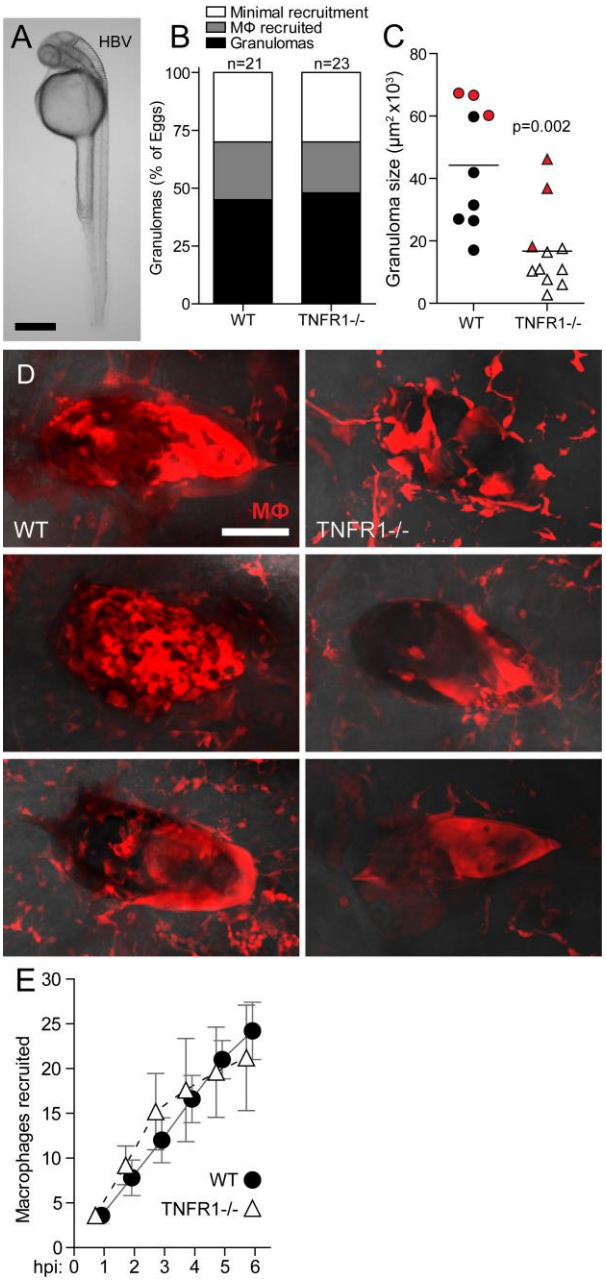


Figure 4. 2. TNF affects late-stage granuloma formation
Comparison of macrophage recruitment and granuloma formation in WT and TNFR1 mutant zebrafish larvae following implantation with a single schistosome egg into their hindbrain

ventricle. (**A**) Zebrafish larva at 36 hours post-fertilization with the hindbrain ventricle (HBV) site of injection and implantation outlined. Scale bar, 300 μm. (**B**-**D**) Granuloma formation at 5 days post-implantation. (**B**) Percent of animals with; granuloma formation (confluent epithelioid macrophage aggregates), macrophages recruited (>6 macrophages in contact with the egg), or minimal recruitment (0-6 macrophages in contact with egg) (Chapter 3.2.1). (**C**) Granuloma size and (**D**) images, with each image from top to bottom corresponding with each red data point, top to bottom, respectively. Scale bar, 50 μm. Horizontal bars in (**C**), means. Statistics, Student's *t*-test. (**E**) Mean macrophage recruitment kinetics during the first 6 hours post-implantation. Error bars, SEM. Sample size, n=5 animals per group.

4.2.2. *S. mansoni* omega-1 promotes initial macrophage recruitment to the egg through its RNase activity

Next, I sought to probe the parasite determinants that induce granuloma formation. In the previous chapter, it was shown that immature *S. mansoni* eggs invoked neither granuloma formation nor even initial macrophage recruitment, indicating that mature egg antigens were required for the first macrophages to be recruited to the egg. Mature eggs express a variety of antigens (Ashton et al., 2001; Cass et al., 2007; Dunne et al., 1981), of which omega-1 is known to be the major contributor to granuloma formation, as knockdown of its expression leads to greatly diminished granuloma formation around eggs (Hagen et al., 2014; Ittiprasert et al., 2019). Omega-1 is an RNase which effects DCs in several ways, by inhibiting protein synthesis, altering morphology, inducing IL-33 expression, and reducing their conjugation affinity with T cells (Everts et al., 2012; Everts et al., 2009; Fitzsimmons et al., 2005; Steinfelder et al., 2009). If and how this leads to granuloma formation is not known. However, it is well-established that its RNase activity is essential for inducing the Th2 polarization of granulomas (Everts et al., 2012; Everts et al., 2009; Fitzsimmons et al., 2005; Steinfelder et al., 2009). This in turn induces expression of IL-4 and IL-13, known egg-induced host factors that can mediate granuloma formation (Cheever et al., 1999; Fallon et al., 2000; Jankovic et al., 1999). Additionally, omega-1 is a major hepatotoxin (Abdulla et al., 2011; Dunne et al., 1991; Dunne et al., 1981), and it has been proposed that the granuloma forms as a protective immune response to prevent the cytotoxic effects of this egg antigen on the host liver.

My attempts to test the role of omega-1 by implanting omega-1 knockout (KO) eggs (Ittiprasert et al., 2019) into the larvae failed, as the genetically modified eggs did not survive shipment. As an alternative approach, I tested if the SEA obtained from omega-1 KO eggs could recruit macrophages. I examined macrophage recruitment at 6 hours post-injection with SEA

into the hindbrain ventricle (Figure 4.3A). Omega-1-deficient SEA recruited macrophages similar to wildtype SEA (Figure 4.3A). Omega-1-deficient SEA retains ~20% of the omega-1 RNase activity (not shown), suggesting that even though reduced compared to wildtype eggs, it is may still be sufficient for macrophage recruitment (Ittiprasert et al., 2019). Alternatively, the omega-1 activity may be redundant with other SEA components (Kaisar et al., 2018). To investigate these hypotheses, I used a recombinant omega-1 that contain the native-like LeX glycosylation, which is important for its uptake by dendritic cells and subsequent Th2-polarization (Everts et al., 2012; Wilbers et al., 2017). Injection of 0.02 ng of omega-1, the approximate amount of omega-1 in the corresponding SEA injections (Chapter 3.2.1, G. Schramm, personal communication), induced macrophage recruitment, although less than SEA, consistent with other components inducing macrophage recruitment (Figure 4.3B and C).

Next, I asked if omega-1-associated recruitment of macrophages is dependent on its RNase activity. The inhibition of RNase activity in the recombinant omega-1 with diethyl pyrocarbonate (DEPC) (Steinfelder et al., 2009), led to loss of macrophage-recruiting activity (Figure 4.3D). Because DEPC inhibits RNase function through covalent binding to the essential histidine in the catalytic domains of RNase, one caveat is that it can create off-target modifications to the protein structure and function through binding to other histidine residues, as well as to a lesser extent, tyrosine, lysine, and cysteine (Wolf et al., 1970). To validate these findings, I used recombinant omega-1 mutant lacking RNase activity due to a phenylalanine substitution of the essential histidine of the catalytic domain (Everts et al., 2012; Irie and Ohgi, 2001). As expected, the omega-1 mutant failed to recruit macrophages (Figure 4.3E). These findings confirmed that the omega-1 macrophage chemotactic activity is mediated through its RNase activity.

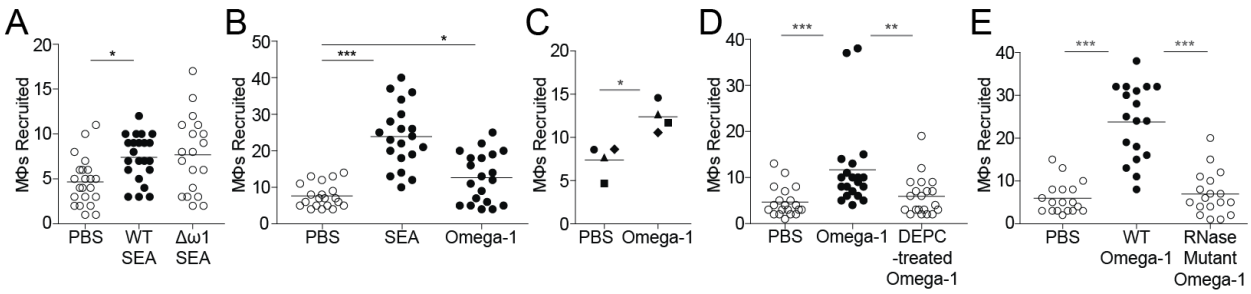


Figure 4. 3. Omega-1 recruits macrophages via its RNase activity
Macrophage recruitment at 6 hours post-injection (hpi) with egg antigens. (**A**) Macrophages recruited to SEA from WT or omega-1 knockout eggs (Δω1). (**B**) Macrophages recruited to SEA or omega-1. (**C**) Mean macrophage recruitment to omega-1 for each of four experiments.

Individual experiments represented with unique symbols; triangles and squares represent means of panels B and D. (**D**) Macrophages recruited to omega-1 or DEPC-treated omega-1. (**E**) Macrophages recruited to WT or omega-1 mutant. All omega-1 injections were performed using 0.02 ng of plant-expressed omega-1, with the exception of (**E**) which used HEK-expressed WT or mutant omega-1 injected at a 5-fold higher concentration of 0.1 ng to compensate for lack of LeX glycosylation in plant-expressed and natural omega-1. Statistics, ANOVA with Dunnett's post-test comparing all samples to PBS (**B**) or WT omega-1 (**A**,**E**); (**D**) non-parametric ANOVA with Dunn's post-test comparing all samples to omega-1; (**C**) paired *t*-test. All horizontal bars, means.

4.3. Discussion

This study reinforces the use of the zebrafish model to study molecular pathways involved in *S. mansoni*-egg-induced granuloma formation. Particularly, it provides new insights on host and parasite factors modulating this critical process that drives the pathology associated with schistosomiasis.

I have demonstrated that TNF signaling is required for granuloma enlargement but not initiation, in agreement with previous observations in the mouse (Amiri et al., 1992; Chensue et al., 1994; Chensue et al., 1995b; Ehlers and Schaible, 2012). Further, I show that TNF is dispensable for the first wave of macrophage recruitment to the egg. These findings are consistent with TNF not being a direct chemotactic agent, but mediating cell recruitment through interactions with other cells that, in turn, synthesize macrophage chemokines (Kalliolias and Ivashkiv, 2016; Mukaida et al., 2011). SEA is known to induce the expression of TNF (Chensue et al., 1994; Chiu et al., 2004; Nascimento et al., 2014), therefore, it might be only after granuloma initiation, at which point significant numbers of macrophages are in contact with the egg, that TNF is produced above the threshold to induce these chemokines. In addition, the close cell-to-cell contacts following the initiation of granuloma formation and epithelioid transformation may be vital; if TNF is acting in both an autocrine and paracrine manner, then the cell-to-cell interaction would allow for maximal signal exchange between cells, the optimal amplification of this signal and subsequent expression of chemokines (Blasi et al., 1994; Caldwell et al., 2014). Epithelioid transformation is primarily associated with Th2-polarized immune responses involving IL-4/IL-13, expression of which can occur in the context of innate immunity alone (Bottiglione et al., 2020; Chiu et al., 2004; Mitre et al., 2004; Pagan and Ramakrishnan, 2018). Therefore, it is not surprising to observe epithelioid transformation in the

absence of TNF. Chronic mTORC1 signaling, which does not require adaptive immunity, can also induce epithelioid transformation (Linke et al., 2017).

As shown in the previous chapter, *S. mansoni* eggs, upon reaching maturity, induce granuloma formation that benefits the parasite by extruding the egg into the environment. This would be achieved by mature egg stage-dependent secretion of antigens such as omega-1 (Ashton et al., 2001; Schramm et al., 2006). Here, I show that recombinant omega-1 recruited macrophages rapidly, similar to SEA. This finding supports the hypothesis that omega-1 is sufficient yet dispensable for early macrophage recruitment. This may have parallels in observations regarding its role in granuloma formation; omega-1 knockdown eggs form granulomas in the mouse, albeit smaller ones, suggesting other egg antigens such as IPSE could contribute to this process (Hagen et al., 2014; Ittiprasert et al., 2019).

In addition, I have demonstrated that the omega-1 RNase activity is required for macrophage recruitment. Prior work has shown that its RNase activity mediates Th2 polarization through inhibition of protein synthesis in dendritic cells (Everts et al., 2012; Everts et al., 2009; Steinfelder et al., 2009). In the context of the 6-hour recruitment assay performed herein, I speculate that the protein is taken up by epithelial cells that line the hindbrain ventricle cavity, perturbing cellular homeostasis by an RNase-induced inhibition of protein synthesis and in turn, inducing cell stress signals which trigger macrophage recruitment.

As with tuberculous granulomas (Pagan and Ramakrishnan, 2018; Ramakrishnan, 2012), I expect that the development of the zebrafish model for studying the schistosome egg granuloma will stimulate its use to dissect the mechanisms underlying the genesis of schistosome egg-induced granulomas, the main driver of schistosomiasis pathogenesis and transmission.

4.4. Summary

Infections by schistosomes result in granulomatous lesions around parasite eggs entrapped within the host tissues. The host and parasite determinants of the *Schistosoma mansoni* egg-induced granulomatous response are areas of active investigation. Some studies in mice implicate TNF produced in response to the infection whereas others fail to find a role for it. In addition, in the mouse model, the *S. mansoni* secreted egg antigen omega-1 is found to induce granulomas, but the underlying mechanism remains unknown. Following the development of the zebrafish larva as a model to study macrophage recruitment and granuloma formation in response to *Schistosoma mansoni* eggs, I have investigated the mechanisms by which TNF and omega-1 shape the early granulomatous response. I find that TNF, specifically signaling through TNF receptor 1, is not required for macrophage recruitment to the egg and granuloma initiation but does mediate granuloma enlargement. In contrast, omega-1 mediates initial macrophage recruitment, with this chemotactic activity being dependent on its RNase activity. These findings further the understanding of the role of these host- and parasite-derived factors and show that they impact distinct facets of the granulomatous response to the schistosome egg.

Chapter 5. Discussion and future directions

In this work, I have developed the zebrafish larva as a model to examine the early stages of granuloma formation in response to *S. mansoni* eggs. This work has provided insights into the dynamics of granuloma formation and the host and pathogen factors that mediate this. My finding that immature eggs avoid eliciting granulomas has led to insights about how *S. mansoni* manipulates the formation of these structures to promote the exit of the egg from the host to continue its life cycle. I have tested this hypothesis by showing that *S. mansoni*-infected mice shed only mature eggs in their feces and have analyzed old human data to show the same is the case for humans. Below, I discuss the broader implications of my findings and future experiments suggested by them.

5.1. Stages of granuloma formation

The formation of the granuloma was dependent on egg maturation, and was observed to occur in distinct stages, summarized in Figure 5.1. Immature eggs elicited neither macrophage recruitment nor granuloma formation, due to the absence of secreted antigens and the eggshell acting as an immunologically silent barrier (Figure 5.1, stage 0). After development, the mature egg induced the recruitment of macrophages, but not neutrophils (Figure 5.1, stage I); an observation consistent with the known cellularity of the schistosome egg granuloma in mammalian models, and consistent with its mature stage-specific antigens being the recruiting factor. While I found this macrophage-specific recruitment to be driven by both the mature egg and injection of egg antigens, it remains to be determined if some of the antigens were acting directly as chemoattractants, or only indirectly by triggering the action of host chemokines and chemoattractants. These possibilities can be distinguished using in vitro chemotaxis assays to assess macrophage recruitment in the absence of other cells or tissues in which the antigen might react.

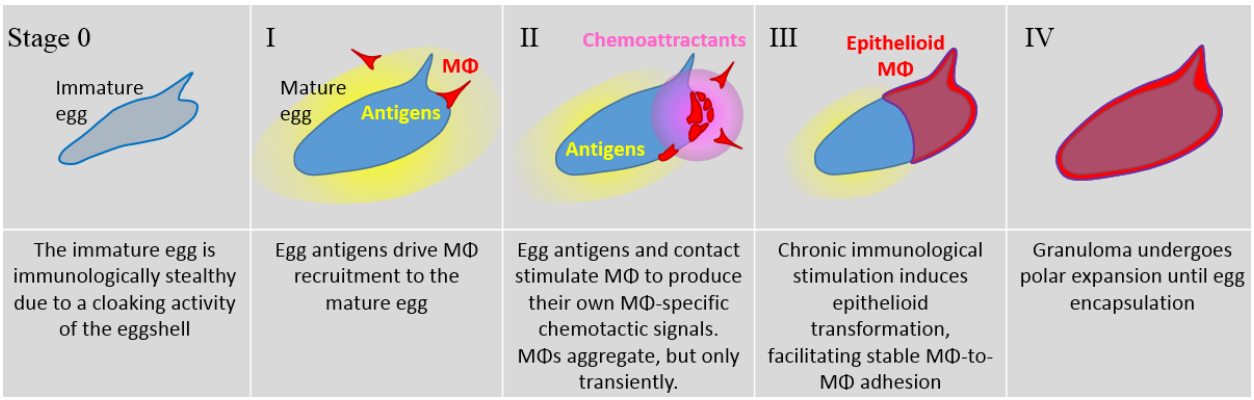


Figure 5. 1. Model of the stages of granuloma formation
Model for the progression of egg maturation and granuloma formation compiled from observations and inferences.

Whether or not the egg antigens act directly as chemoattractants, it is known that egg antigens recruit macrophages indirectly through the induction of host inflammatory responses and chemokine expression. In the zebrafish too, there was evidence for this in some eggs which I monitored closely, although not in all. Following the initial seeding of the first macrophages to arrive to the egg, I observed a preference of secondary macrophages to migrate directly towards these initial macrophages, rather than to the egg itself (Figure 5.1, stage II). This observation coincided with the first arriving macrophages adopting a flattened morphology against the egg, which resembled frustrated phagocytosis (Herant et al., 2006). Perhaps this flattened morphology enhanced the ability of these macrophages to produce their own chemoattractants, either by maximizing contact with the egg and its antigens therefore maximizing antigenic stimulation, or perhaps the adoption of the frustrated phagocytosis morphology might itself be a stimulating factor. Although these macrophages showed a preference for aggregation during this early stage of granuloma formation, the aggregates were transient in nature for the first day or so.

It was not until later, typically between 3-5 days post-implantation that the macrophages were no longer transiently aggregated, but instead had undergone epithelioid transformation to form the confluent aggregate comprising the nascent epithelioid granuloma (Stage III). The timing of which was consistent with epithelioid transformation in the tuberculous granuloma (Cronan et al., 2016). That epithelioid transformation occurred between 3-5 days, and never before then, may have biological relevance. Perhaps the delayed timing of epithelioid transformation has evolved to occur only when absolutely necessary, in response to chronic inflammatory agents

that the immune system cannot eradicate. For comparison, in the case of implanted miracidia, despite their large size and the robust macrophage response they elicited, epithelioid granulomas did not form. Instead, macrophages rapidly recruited and surrounded the miracidium, and within 3 days the miracidium was completely degraded followed by macrophage dispersal and resolution of the immune response. In this scenario, had these macrophages undergone epithelioid transformation, they would have lost their agility and been unable to quickly disperse to address other potential threats. So perhaps the delayed timing is an evolutionary mechanism to ensure that epithelioid transformation does not occur too quickly, and only when absolutely necessary during chronic infection with an inciting agent that is recalcitrant to degradation.

The delayed timing of epithelioid transformation also raises the question about the mechanism through which the timing of epithelioid transformation is controlled. Understanding the temporal control of epithelioid transformation will be an interesting aspect to uncover. The more immediate aspect is to dissect the signaling pathways through which epithelioid transformation occurs, which mechanistically, has been proposed to occur either through IL-4/STAT6, or through chronic mTORC1 signaling (Pagan and Ramakrishnan, 2018). In preliminary experiments I tested a zebrafish STAT6 mutant from the Sanger Zebrafish Mutation Project, and found that the epithelioid granuloma still formed. This mutant was verified as being homozygous for the mutation, however, there was not sufficient time perform additional quality controls to confirm the phenotype by immunofluorescence against E-cadherin, and to verify the STAT6 mutation with other known phenotypes. The independence of STAT-6 in epithelioid transformation will be confirmed in future experiments, after which the potential role of mTORC1 in epithelioid transformation can be explored using a currently available mTORC1 mutant. If epithelioid transformation in response to the Schistosoma egg does occur independent of STAT-6, it raises the question of whether epithelioid transformation in the context of innate immunity is generally STAT-6 independent and this has been missed by studying the phenomenon in later stages. I observed rapid epithelioid transformation to inert beads as well, raising the question of whether that is also STAT-6 independent. If mTOR is ruled out as the mediator of epithelioid transformation, a single cell RNA-seq approach may help identify the determinants involved. I propose collecting granuloma macrophages by FACS sorting just before they undergo epithelioid transformation and again after epithelioid transformation in the same cohort of animals. Comparison of either bulk RNA-seq or single cell RNA-seq analysis of the

isolated macrophages should reveal candidate genes that can then be tested by mutational analysis for their impact on epithelioid transformation. Any identified genes can then be tested for their impact on epithelioid transformation that occurs in response to inert beads.

Following epithelioid transformation and formation of the nascent epithelioid granuloma, I found that encapsulation of the egg occurred through a process of expansion of the initial focus or occasionally two foci that then coalesced (Figure 5.1, stage IV). In attempts to visualize this expansion process in greater detail, I used high temporal resolution timelapse microscopy to directly observe macrophage recruitment to the epithelioid granuloma, but I observed few if any macrophages recruited within the 3-6 hour observation windows. So it is unclear if the focal expansion occurred through macrophage recruitment and adhesion to the nascent granuloma, or if the macrophages of the nascent granuloma were undergoing proliferation. Both possibilities are logical as the schistosome egg granuloma is characterized as inducing a mixed Th1/Th2 cytokine response; whereas Th1 responses are associated with inflammation and recruitment, the hallmark of Th2 responses is not macrophage recruitment, but proliferation (Jenkins et al., 2011). Because the rate of epithelioid granuloma expansion may be slower than the initial recruitment, future experiments will require longer periods of observation to directly observe if macrophages are recruited to the epithelioid granuloma. Alternatively, staining with the synthetic nucleoside, BrdU, will be performed to determine if the macrophages of the epithelioid granuloma are proliferating.

5.2. The temporal control of granuloma formation to promote transmission

The granulomas which formed were specific to mature, viable eggs. In contrast, granuloma formation was diminished in non-viable mature eggs, consistent with previous findings in mammalian models in which killed eggs were less granulomatous, only retaining a thin layer of antigens which did not regenerate (Lichtenberg, 1964; Sorour, 1929). In contrast to the mature egg, immature eggs were immunologically silent, not only failing to form granulomas, but failing to recruit macrophages at all. While it has been long known that antigen secretion is specific to the mature egg, a finding which was proposed as a mechanism to prevent premature expulsion while the immature egg is still reliant on the host for nutrients for development (Ashton et al., 2001), what has been overlooked is that the immature egg, parasite-derived and itself a parasite,

is remarkably immunologically silent. This became evident in the comparison of immature eggs with all other implanted materials, which induced rapid and robust immune responses.

I found that this immunological silence of the immature egg was mediated by the eggshell acting as an immunologically inert barrier, masking the parasite antigens within. How the eggshell does this is not currently known. The eggshell is primarily composed of the eggshell-specific protein, p14, in combination with a smaller proportion of assorted, glycine-rich cellular proteins (deWalick et al., 2012). The structure of p14 has been proposed to consist of short anti-parallel beta strands forming a glycine rich backbone and with tyrosine and cysteine located at the bends, available for cross-linking during eggshell synthesis (Rodrigues et al., 1989). Given that glycine is the simplest amino acid, the eggshell may achieve its immunological silence through its simple structure and lack of activating motifs. I found only one research team that published on the purification of p14, which they found difficult to express and to purify, and they did not have any available material. As an alternative approach to using p14, I tried coating beads with purified polyglycine to mimic the glycine-rich composition of the eggshell proteins. However, the beads still recruited macrophages. This result is inconclusive as it cannot determined whether it is not the polyglycine on the eggshell that is responsible for its immunological silence or whether I was unsuccessful in fully masking the bead surface. Regretfully, I had to drop this line of experiments.

The finding that the immature egg was immunologically silent indicated that the parasite utilizes a two-step strategy to tightly regulate the timing of granuloma formation. Initially, the immature egg uses its eggshell to achieve a minimal baseline of detection to avoid eliciting an immune response while it is still reliant on absorption of nutrients from its host, and then after developing to maturity, secretion of antigens through the eggshell of the mature egg induces the formation of the granulomas which facilitate its translocation through the tissues into the lumen of the intestines, so that it can be expelled into the environment to complete its life cycle (Figure 5.2). To test this, I analyzed the eggs from the feces of mice, and found that whereas a mixture of mature and immature eggs was found in the intestinal wall, only mature eggs were found in the lumen of the intestines. Having demarcated the sizes of mature versus immature eggs, I was able to use the published sizes of *S. mansoni* eggs in human feces from a 1916 publication (Martinez., 1916), to show that they corresponded with those of mature eggs. My work has led to the insight that both in humans and in mice, it is specifically the mature eggs which are shed.

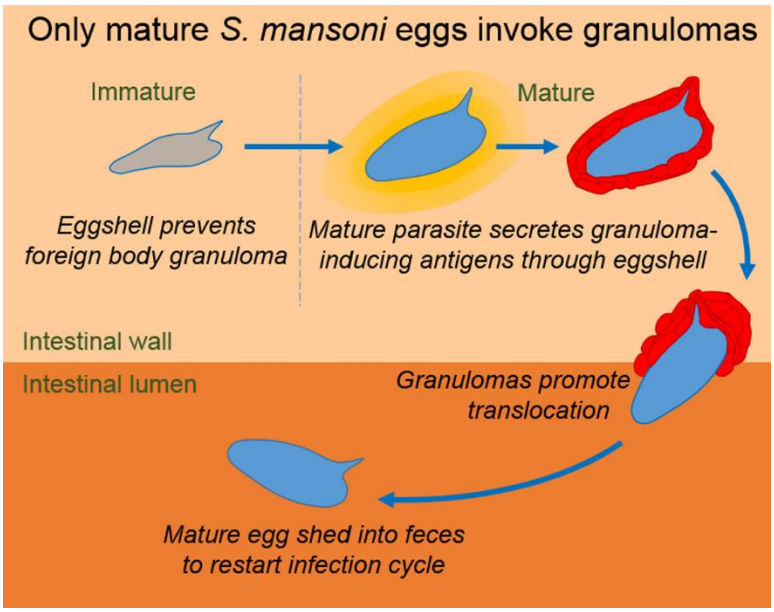


Figure 5. 2. Developmental stage-specific induction of granulomas

5.3. Role of omega-1 in granuloma formation

Granuloma formation around the eggs are driven by their secreted antigens which are strong inducers of Th2 responses, IL-4 and IL-13, with the two major egg antigens, omega-1 and alpha-1, contributing to this process. While it is known that omega-1 is internalized into DCs where it degrades host RNA leading to inhibition of protein synthesis, a clear connection to how this activity induces Th2 polarization and granuloma formation has not been established (Everts et al., 2012; Everts et al., 2009; Steinfelder et al., 2009).

In the zebrafish I found that injected omega-1 elicited RNase-dependent macrophage recruitment. This finding indicates that omega-1 does not act directly as a chemoattractant, and that recruitment must be mediated through downstream effects stemming from its RNase activity. Further, this indicates that at least some of its contribution to granuloma formation is through inducing macrophage recruitment.

Omega-1 alters DC morphology and reduces their conjugation affinity with T cells (Steinfelder et al., 2009). However, this may not be the cause of Th2 polarization, but a side-effect stemming from inhibition of protein synthesis. Omega-1 is hepatotoxic, and it has been suggested that the granuloma might form as a protective response to sequester it. There is an overlap in the involvement of Th2 responses to helminth infections and to wound-healing (Gause et al., 2013), raising the possibility that macrophage recruitment, Th2 polarization, and

granuloma formation are all induced by the cytotoxicity of egg antigens, and subsequent activation of a wound-healing response. Clues as to how omega-1 could induce such responses and subsequent granuloma formation come from a 2016 paper investigating the use of omega-1 to induce weight loss and improve glucose homeostasis in obese mice (Hams et al., 2016). These authors found that injection of omega-1 into the intraperitoneal cavity of mice induces RNase-dependent cell damage, and the expression of the IL-33 in DCs and macrophages within 3 hours post-injection (Hams et al., 2016), a timeframe consistent with my observations of omega-1-induced macrophage recruitment. IL-33 is known to function as a DAMP/alarmin to alert the immune system to tissue damage or stress, and is a strong inducer of Th2 cytokine production from both innate immune cells and Th2 cells (Miller, 2011; Oboki et al., 2011). Taken together, this may bridge the gap between omega-1 activity and induction of Th2 responses, and to provide a molecular explanation to complement the initial hypothesis that the granuloma forms as a response to minimize the cytotoxic activity of the eggs. In preliminary experiments to detect cell damage during granuloma formation, I found some evidence for this in epithelial cells, but not in macrophages. Using the Sytox stain for cell permeability, I did not find cell damage in the macrophages of the *S. mansoni* granuloma. This is in contrast to substantial cell death seen in mycobacterial granulomas at the same time point (Davis and Ramakrishnan, 2009). It might be that the cell damage or stress is below the threshold of permeabilization of their cell membranes, or that macrophages are resistant to damage by omega-1. In contrast, at earlier timepoints preceding granuloma formation, I observed Sytox staining in the epithelial cells adjacent to the egg. Possibly this indicates that at the initiation of macrophage recruitment omega-1 is initially internalized by epithelial cells resulting in expression of chemokines or DAMPs, which would be analogous to the initial interaction of egg antigens with hepatocytes, preceding granuloma formation. Future experiments will require assessment of IL-33, IL-4/5/13 expression in the zebrafish before and after granuloma formation in response to schistosome eggs, and in comparison to SEA beads and *Mycobacterium marinum*. Initially this will be done by qPCR, and then ideally using a fluorescent reporter line to determine in which cells these are expressed, and when.

5.4. Studying *S. japonicum* and inert granulomagenic materials in the zebrafish larva

Following the analysis of the *S. mansoni* granuloma, the obvious next use of this zebrafish model would be to assess granuloma formation in response to another schistosome species, *Schistosoma japonicum*. In the case of *Schistosoma mansoni*, I found that neither its egg nor its egg antigens recruited neutrophils, resulting in granulomas composed of macrophages but few neutrophils, consistent with mammalian models. Whereas neutrophils are rarely seen in these *S. mansoni* granulomas, they are a common cellular component of the *S. japonicum* granulomas, where they are thought to cause its characteristic tissue damage (Chuah et al., 2013; Hsu et al., 1973). Following their recruitment via the japonicum-specific secreted egg antigen, Sje16.7 (Wu et al., 2014), as well as egg-induced IL-8 expression (Chuah et al., 2014), the neutrophils are stimulated by the japonicum egg to release NETs in the granuloma (Chuah et al., 2013) and to induce the expression of MMP9 (Chuah et al., 2014), a host factor which was found to enhance the formation of the tuberculous granuloma in zebrafish (Volkman et al., 2010).

Future experiments to contrast the *mansoni* and *japonicum* granulomas in the zebrafish may provide insight into the granulomas of both species. Further, japonicum-specific pathology could be investigated by utilization of the neutrophil-defective WHIM mutant zebrafish (Yang et al., 2012), allowing us to determine what proportion of necrosis and pathology is mediated by neutrophils, and which proportion is induced directly by the egg. Such results would be complimented by serial intravital timelapse microscopy to visualize the formation of the japonicum granuloma over time, with clues potentially gleaned from the timing of events. For example, if neutrophil recruitment precedes necrosis, that would be consistent with egg-mediated direct neutrophil recruitment, and would also be expected if neutrophils were the cause of necrosis.

During the course of this project I developed the tools and techniques for implantation of schistosome eggs and beads into zebrafish larvae to visualize the formation of their granulomas in a living animal. In future experiments I would like to expand on the usage of these tools and techniques to access the granuloma which form in response to other foreign materials known to induce epithelioid foreign body granulomas, such as pine pollen and beryllium (Pagan and Ramakrishnan, 2018). In the former case it will be interesting to compare the formation of epithelioid granulomas around pine pollen with those of the schistosome egg. Whereas in the latter case, beryllium is thought to require T cells for granuloma formation, and so assessment in

the sole context of innate immunity will be revealing. Other possibilities include the injection of lipids and alum to visualize the formation of lipid and alum granulomas, respectively.

While this project has focused on the formation of granulomas in response to large, extracellular objects (eggs and beads), our laboratory focus is on the tuberculous granuloma as it forms in response to intracellular infection. In this case, the tuberculous bacteria reside within the endosome of the macrophage and induces the inflammatory response which induces granuloma formation. This is in contrast to the schistosome egg which is too large to be phagocytosed by the macrophages, which instead undergo frustrated phagocytosis, spreading out and flattening over the surface of the egg. By juxtaposition of these two inciting agents, the question which arises is that of size; does the size of the inciting agent (large vs small) and its cellular localization (intracellular vs extracellular) have specific consequences? Or is size irrelevant given that the inciting agent induces a persistent inflammatory response? One possible way to address this question is by implanting objects of the same material, but of differing sizes, and then assessing if granuloma formation is altered. This could be done using sepharose, polystyrene, and polyethylene beads, as the large versions were found to induce granulomas on their own. However, to assess the contribution of specific antigens to this process will require finding a material to which proteins or lipids can be bound, yet does not induce granuloma formation on its own. Alternately, I have previously generated and implanted bacteria-entrapped microspheres using *Pseudomonas aeruginosa*. In contrast to injection of the bacteria alone which results in their phagocytosis and clearance, their encagement within the microsphere protects them from phagocytosis, allowing them to persist chronically and to induce granuloma formation. A similar approach could be used to compare the granulomas of intracellular tuberculous bacteria or extracellular tuberculous bacteria encaged with microspheres.

List of Figures

List of Movies

Movie 1. Microinjection into the hindbrain ventricle
Hindbrain ventricle microinjections for the introduction of egg antigens or bacterial infection, using the standard microinjection needle and VAMP (2013 Takaki). Linked to Figure 3.1 and 3.2 and Methods 2.5.

Movie 2. Schistosome egg implantation
Implantation of the schistosome egg into the hindbrain ventricle using the CAIN and VAMP. Linked to Figure 3.1 and Appendix 1.

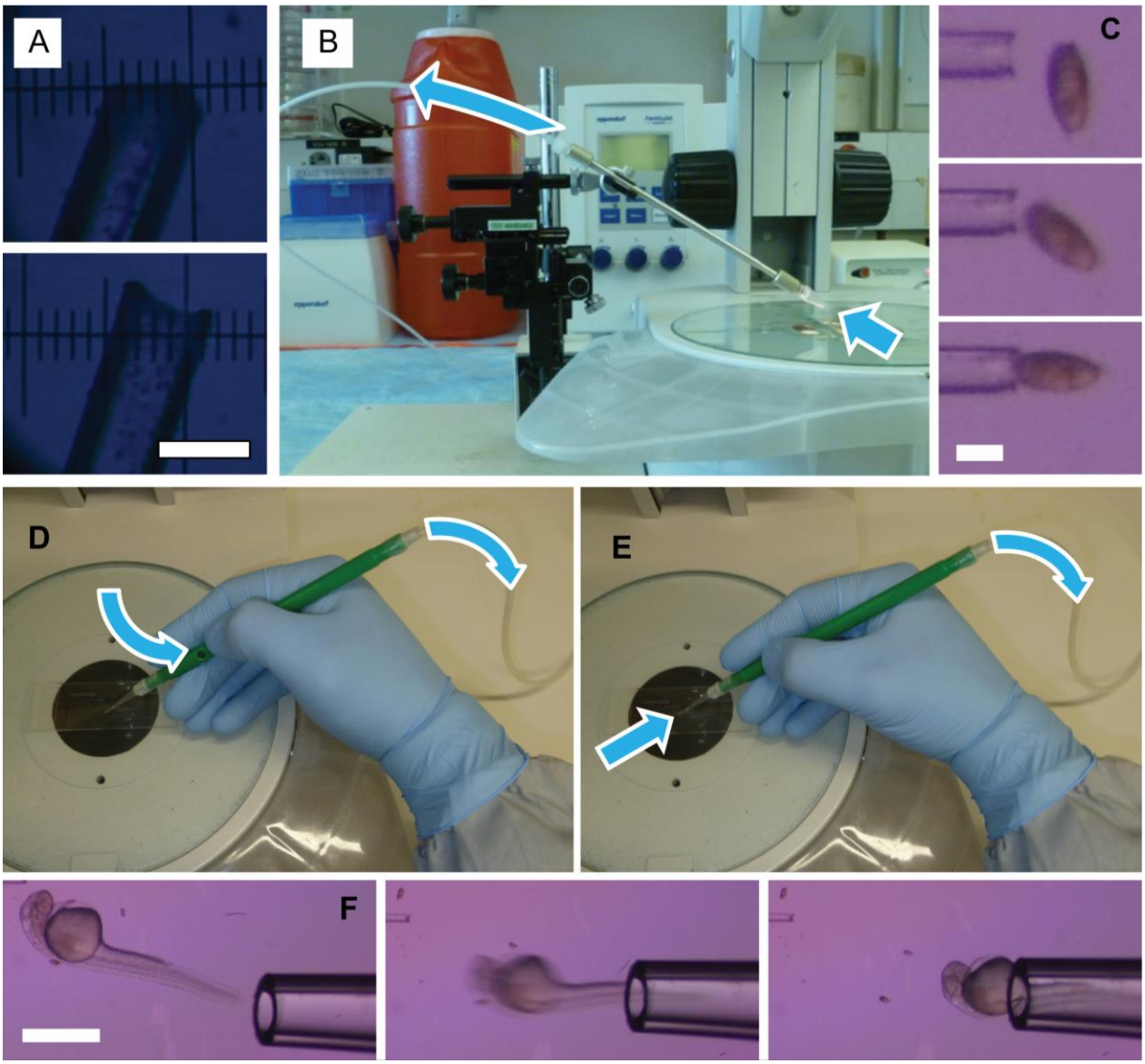
Movie 3. Formation of the schistosome egg granuloma
Timelapse 3D microscopy from 1-7 days post-implantation showing macrophage recruitment to the egg and granuloma formation around the egg. The last series of images shows E-cadherin staining around the same egg, done at the end of the time-lapse imaging. Linked to Figure 3.2.

Movie 4. The parasite can withstand granuloma formation if the eggshell is intact
The first series of images show a moving miracidium within an intact egg surrounded by an epithelioid granuloma five days post-implantation. The second series of images shows a ruptured egg which has been infiltrated by macrophages that are seen moving within the egg. Linked to Appendix 4.

Table of Contributions

Contributor	Contribution
Gabriel Rinaldi	Prepared liver-derived and IVLE schistosome eggs, and mouse intestine and luminal contents
Gabriele Schramm	Prepared SEA, and WT and RNase mutant HEK-expressed recombinant omega-1
Ruud Wilbers	Prepared plant-expressed omega-1
Wannaporn Ittiprasert	Prepared SEA from Omega-1 knockout eggs
Francisco Roca	Generated the TNFR1 mutant zebrafish

Appendices

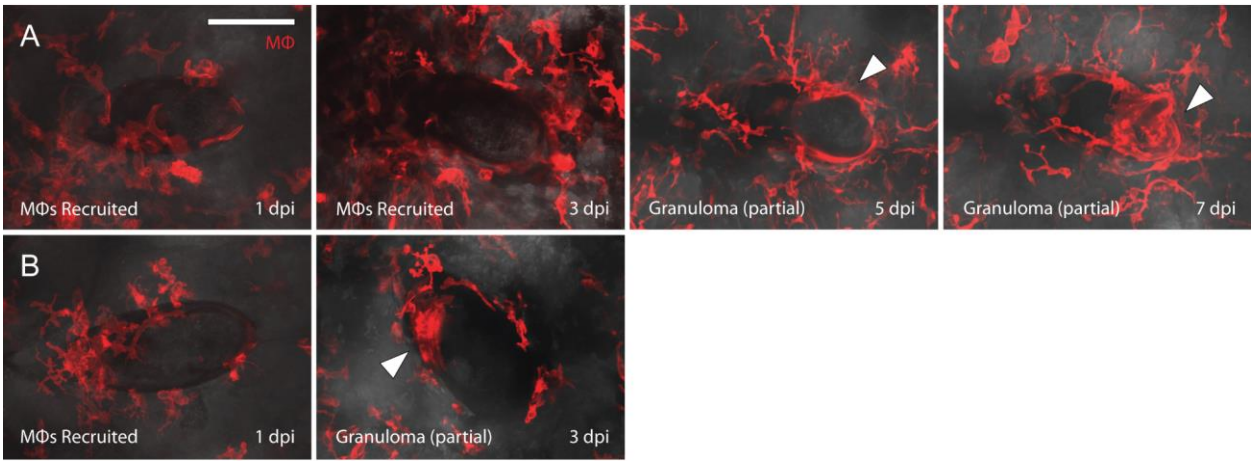


Appendix 1. Implantation of *Schistosoma mansoni* eggs into zebrafish larvae
(**A-C**) Capillary-Assisted Implantation Needle (CAIN). (**A**) Side and front profile of CAIN showing double-beveled point. Scale bar 50 µm. (**B**) CAIN attached to micromanipulator for X,Y, and Z control, as used by left hand of operator. Arrows indicated upward flow of fluid during grasping of egg. (**C**) Function of CAIN demonstrated by grasping *S. mansoni* egg. Scale bar, 50 µm. (**D-F**) Vacuum-Assisted MicroProbe (VAMP). (**D**) Occlusion of thumb hole re-routes aspiration pressure to tip (**E**), allowing for grasping of the larvae (**F**). Scale bar, 1000 µm. VAMP as previously described (Takaki et al., 2013). Linked to Figure 3.1.

Exp	Minimal Recruitment (%)	MΦs Recruited (%)	Granuloma (partial)(%)	Granuloma (complete)(%)	Granuloma (%)	n
1	7 (44%)	5 (31%)	3 (19%)	1 (6%)	4 (25%)	16
2	1 (20%)	2 (40%)	2 (40%)	0 (0%)	2 (40%)	5
3	8 (44%)	2 (11%)	7 (39%)	1 (6%)	8 (44%)	18
4	5 (25%)	6 (30%)	6 (30%)	3 (15%)	9 (45%)	20
5	15 (38%)	11 (28%)	10 (25%)	4 (10%)	14 (35%)	40
6	2 (25%)	5 (63%)	1 (13%)	0 (0%)	1 (13%)	8
7	2 (17%)	8 (67%)	2 (17%)	0 (0%)	2 (17%)	12
8	3 (43%)	4 (57%)	0 (0%)	0 (0%)	0 (0%)	7
Total	43	43	19%	6%	40	126
Mean %	32%	41%	23%	5%	28%	---

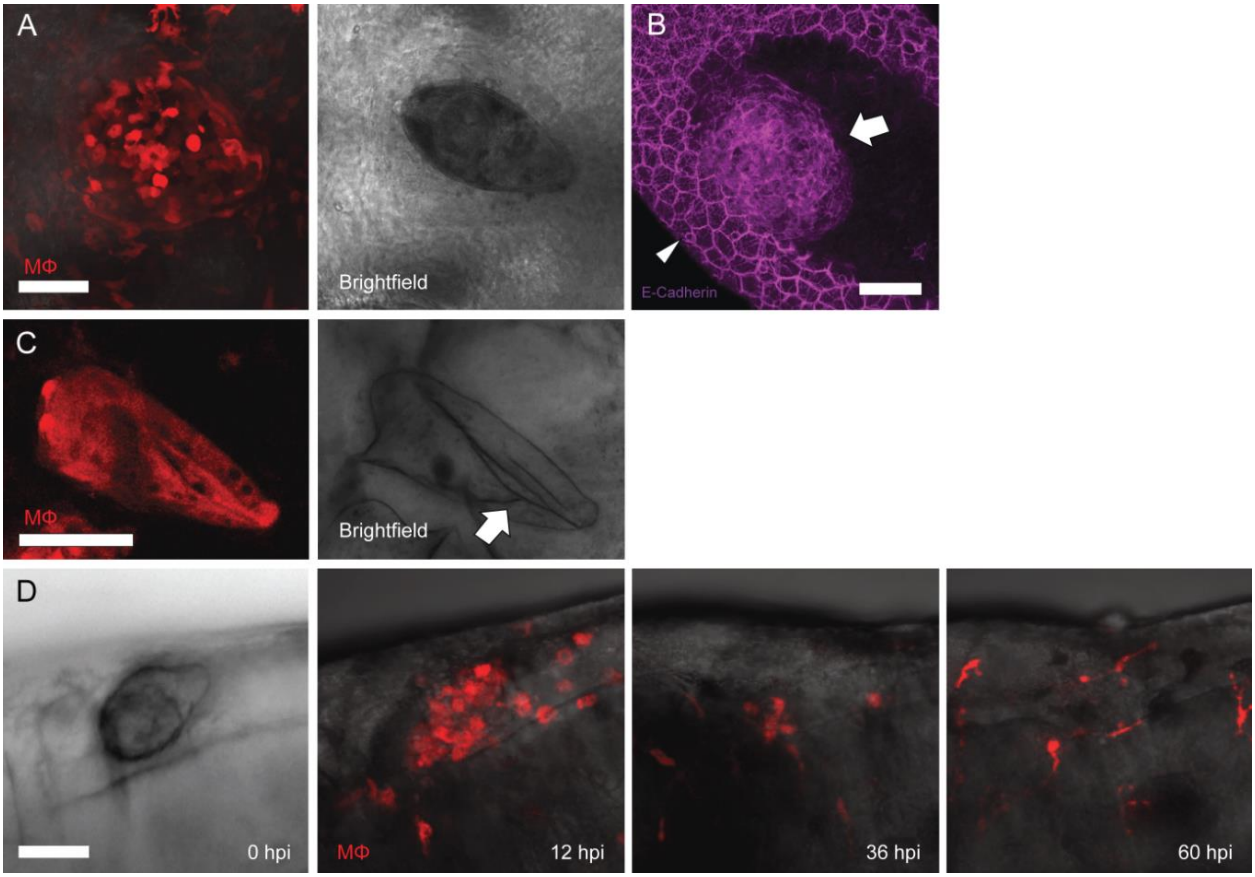
Appendix 2. Prevalence of granuloma formation

The number and rounded percentages of implanted eggs in each category of immune response as defined in **Figure 3.1D**, for each of 8 experiments. Each experiment constitutes a separate batch of eggs and a separate clutch of zebrafish larvae. Mean percent is the mean of the percent of eggs in each category for each individual experiment. Sample size, n, the total number of assessed eggs per experiment. Linked to Figure 3.1D and E.

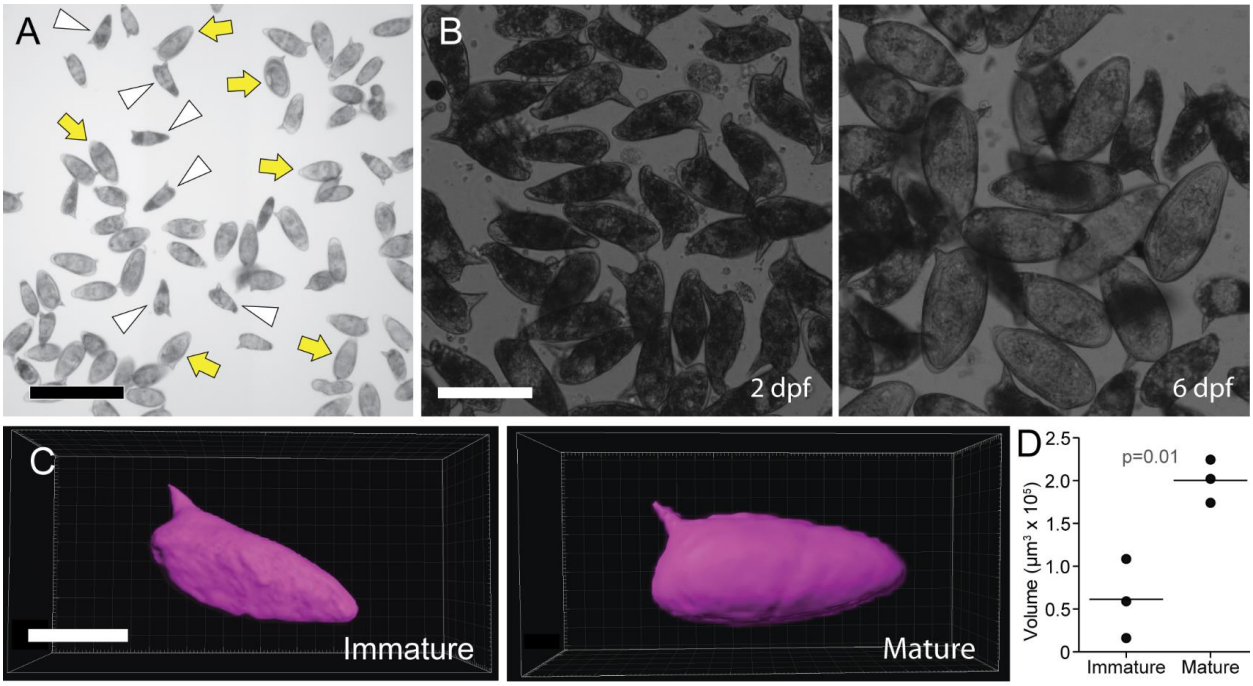


Appendix 3. Formation of the epithelioid granuloma

Timelapse microscopy following the formation of the epithelioid granuloma (white arrowhead) from 1-7 dpi, imaged at 2 day intervals. Two examples shown, (**A**) and (**B**). Animal in (**B**) was not recovered after the 3 day time point. Scale bar, 100 μm. Linked to Figure 3.2A.



Appendix 4. The eggshell protects the miracidium from being killed by host macrophages (**A** and **B**) The parasite is alive within an epithelioid granuloma at 5 dpi. (**A**) Fluorescence and brightfield intravital microscopy. (**B**) Immunofluorescence staining with E-cadherin antibody. The outer-most stained structure is the epithelial lining of the hindbrain ventricle (arrowhead), and is not in contact with the epithelioid granuloma (arrow). (**C**) Fluorescence and brightfield microscopy of ruptured egg showing macrophage infiltration and the absence of an intact parasite. Arrow, rupture point of eggshell. (**D**) Representative brightfield and fluorescence timelapse microscopy of a miracidium following implantation into the HBV. (**A-C**) Representative of routinely observed miracidia alive within intact eggs within granulomas, and of occasionally ruptured eggs. (**D**) Representative of two experiments, each with a sample size of 10 animals. Scale bars, 50 μm. See also Movie 4.

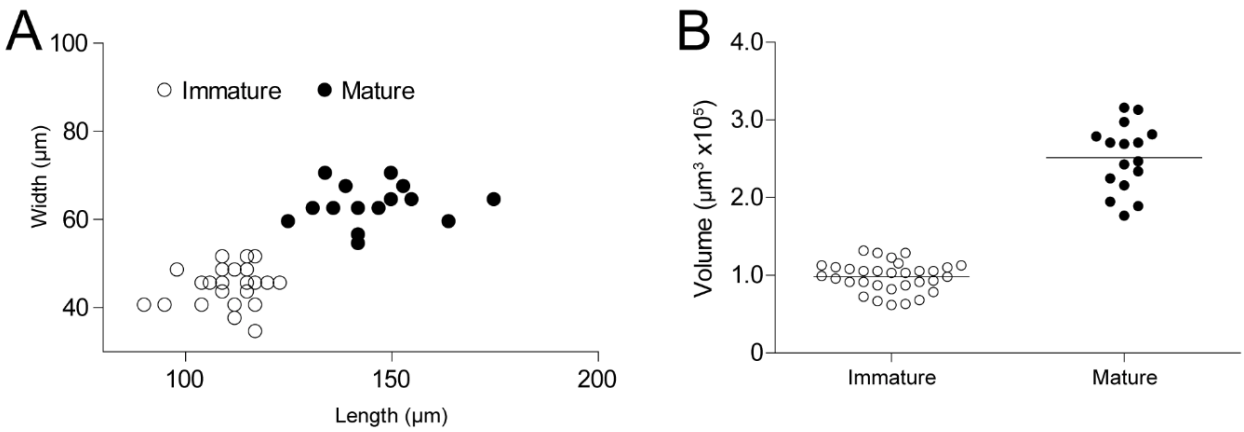


Appendix 5. Morphology and volume of mature and immature eggs

(**A**) *S. mansoni* eggs isolated from mouse livers. Immature and mature eggs, arrowheads and arrows, respectively. Scale bar, 300 µm. (**B**) Immature IVLE at 2 days post-fertilization (dpf), and mature IVLE at 6 days post-incubation in nutrient medium at 37°C. Scale bar, 100 µm. (**C**) 3D rendering of Coomassie-stained eggs following confocal microscopy, and (**D**) volumetric analysis of three immature and mature eggs using 3D renderings shown in (**C**). Scale bar, 50 µm. Statistics, Student's t-test. Linked to Figure 3.3.

Implanted Material	Diameter (median, µm)	Volume (median, µm³)
Mature Schistosome egg	---	200,000
Immature Schistosome egg	---	60,000
Sepharose Agarose beads	65	146,346
Polystyrene beads	45	47,713
Polyethylene beads (large)	70	175,909

Appendix 6. Sizes of implanted materials



Appendix 7. Dimensions and volume of mature and immature eggs

(A) Measurements of *S. mansoni* eggs isolated from mouse livers that were imaged and classified as immature (open circles) or mature (closed circles) based on visual estimate of size and morphology. (B) Egg volumes calculated from egg dimensions in (A). Linked to Figure 3.5.

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