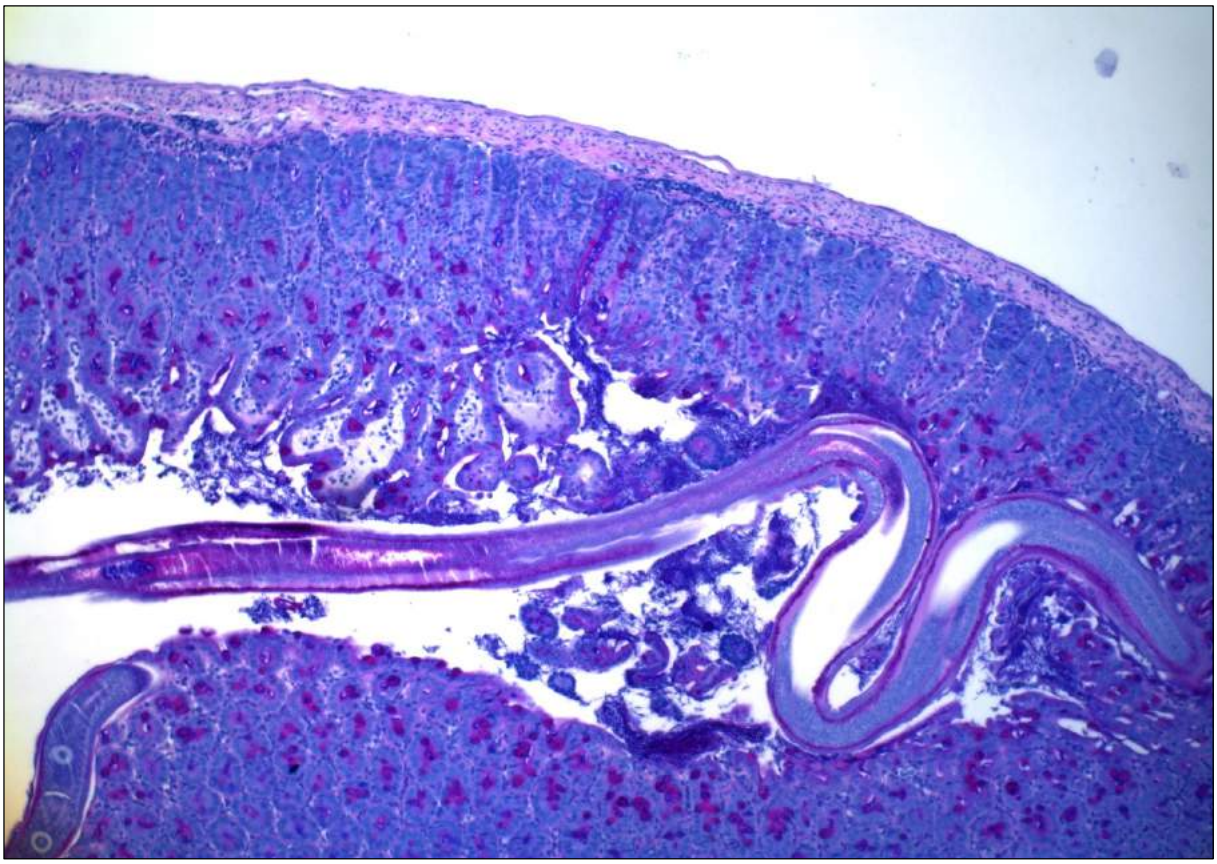
Regulation of immunity and inflammation during parasitic helminth infection by inflammasomes



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Abstract

More than 1.5 billion people, or 24% of the world's population, are infected with helminths worldwide. Infections are highly epidemic in tropical and subtropical areas, with the greatest numbers occurring in sub-Saharan Africa, the Americas, China and East Asia. Helminths are strongly immunomodulatory, which has facilitated their ability to resist attack by their host's immune system, which has made attempts to vaccinate against parasitic helminths an enormous challenge. In order to develop a vaccine, we need to understand more about the nature of the protective immune response against helminths, and how helminths have evolved to control the host immune system to enable their survival.

Inflammasomes are important for immunity against invading microbial pathogens such as bacteria, viruses and protozoa by promoting the maturation and secretion of inflammatory cytokines such as IL-18 and IL-1β and resultant Type 1-mediated immunity. However, the roles for inflammasomes in Type 2 immunity to parasitic helminths are less well studied. Therefore, this dissertation has focused on studying the roles of distinct inflammasome activation pathways (NLR Family Pyrin Domain Containing (NLRP3 and NLRP1) in immunity and inflammation following infections with multiple species of gastrointestinal helminth. Firstly, we show that intestinal whipworm infection, *Trichuris muris*, and its excretory/secretory proteins can promote NLRP3-dependent IL-18 and IL-1β secretion *in vivo* and *in vitro*, in concert with signals from bacteria. Critically, we have identified a novel mechanism by which NLRP3-dependent IL-18 suppresses innate and adaptive immune responses to *T. muris*, resulting in immunopathology and more persistent infections. This suggest that worm-mediated NLRP3 inflammasome activation is one potential mechanism of how parasitic worms may enhance their survival in their host. We next assessed whether NLRP3 plays a similar or different role in regulation of immunity to a different species of parasite helminth,

Nippostrongylus brasiliensis, a model of hookworm that infects both the lung and intestine. Similar to results we observed with whipworm infections, *N. brasiliensis* infections promoted NLRP3-dependent increases in IL-18 and IL-1β levels in the lungs and gut and ablation of NLRP3 resulted in increased anti-parasitic immunity in the lung. NLRP3-deficient mice displayed marked increases in neutrophil recruitment to the lung, a cell type that is potently toxic to parasite larvae. Thus we demonstrate that NLRP3 may restrain anti-parasitic neutrophil responses within the lung. Lastly, we investigated for the first time the role for a distinct inflammasome regulator, NLRP1, in immunity to a parasitic helminth. We found that NLRP1 does suppress immunity to both *T. muris* and *N. brasiliensis* infection in a similar way to NLRP3, however NLRP3 appears to play a greater role in regulating innate immunity to *N. brasiliensis*, in particular.

Together these data provide novel insights into the activating and regulatory pathways of both the innate and adaptive immune systems following gastro-intestinal helminth infections. By understanding the precise mechanism of how inflammasomes regulate immunity and inflammation following helminth infection, it may help inform the development of novel vac vaccines against human infections.

Publications relevant to this thesis

1. **Al-Hallaf, Rafid**, Agha, Zainab, Miller, Catherine M, Robertson, Avril A B, Sotillo, Javier, Croese, John, Cooper, Matthew A, Masters, Seth L, Kupz, Andreas, Smith, Nicholas C, Loukas, Alex and Giacomin, Paul R (2018) *The NLRP3 Inflammasome Suppresses Protective Immunity to Gastrointestinal Helminth Infection*. *Cell reports, 23* 4: 1085-1098.

Publications not directly relating to, but relevant to this thesis

2. Giacomin, Paul, Zakrzewski, Martha, Jenkins, Timothy P., Su, Xiaopei, **Al-Hallaf, Rafid**, Croese, John, De Vries, Stefan, Grant, Andrew, Mitreva, Makedonka, Krause, Lutz, Loukas, Alex, and Cantacessi, Cinzia (2016) *Changes in duodenal tissue-associated microbiota following hookworm infection and consecutive gluten challenges in humans with coeliac disease.* Scientific Reports, 6. pp. 1-10.

3. Sundaraneedi, Madhu, Eichenberger, Ramon M., **Al-Hallaf, Rafid**, Yang, Dai, Sotillo, Javier, Rajan, Siji, Wangchuk, Phurpa, Giacomin, Paul R., Keene, F. Richard, Loukas, Alex, Collins, J. Grant, and Pearson, Mark S. (2018) *Polypyridylruthenium(II) complexes exert in vitro and in vivo nematocidal activity and show significant inhibition of parasite acetylcholinesterases.* International Journal for Parasitology: Drugs and Drugs Resistance, 8 (1). pp. 1-7.

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CHAPTER 1

INTRODUCTION

1. CHAPTER 1: INTRODUCTION

1.1 Parasitic helminth infections: Global health burden

Parasitic helminth infections are among the most common and debilitating causes of chronic disease in humans. Helminths can be classified into two major groups; N**ematodes** (roundworms) that include the gastrointestinal worms (also known as soil-transmitted helminths) and filarial worms that cause lymphatic filariasis (LF) and onchocerciasis; and **Platyhelminths** (flatworms) that include the flukes such as schistosomes, and tapeworms such as the pork tapeworm that causes cysticercosis (Hotez et al., 2008). Among these parasites, soil-transmitted helminths (STHs), such as *Ancylostoma duodenale, Necator americanus, Ascaris* spp. and *Trichuris* spp. are the most common parasitic helminth that infects humans, where approximately one-sixth of all humans are affected (Chanco and Vidad, 1978; Hotez et al., 2009). Helminth infections are most common in developing countries, including those in sub-Saharan Africa, South America and South-East Asia (Figure 1.1), where infections are especially problematic in children (Crompton, 1999). Furthermore, chronic intestinal helminth infections can also impact the pathology associated with other viral and bacterial diseases such as human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* (Bentwich et al., 1995; Chatterjee and Nutman, 2015). Prevalence, mortality and morbidity of intestinal helminth infections are influenced by genetics, public health measures and the environment. Children suffering from helminth infections exhibit signs of stunted growth, intellectual retardation, educational deficits and malnutrition (Bethony et al., 2006). Moreover, helminth infections negatively affect infant birth weight, survival as well as increased occurrence of maternal anemia during pregnancy (Bethony et al., 2006). Helminth infections are also an enormous problem in the livestock industry, leading to billions of dollars of production losses annually (Balic et al., 2000; Claerebout and Vercruysse, 2000).

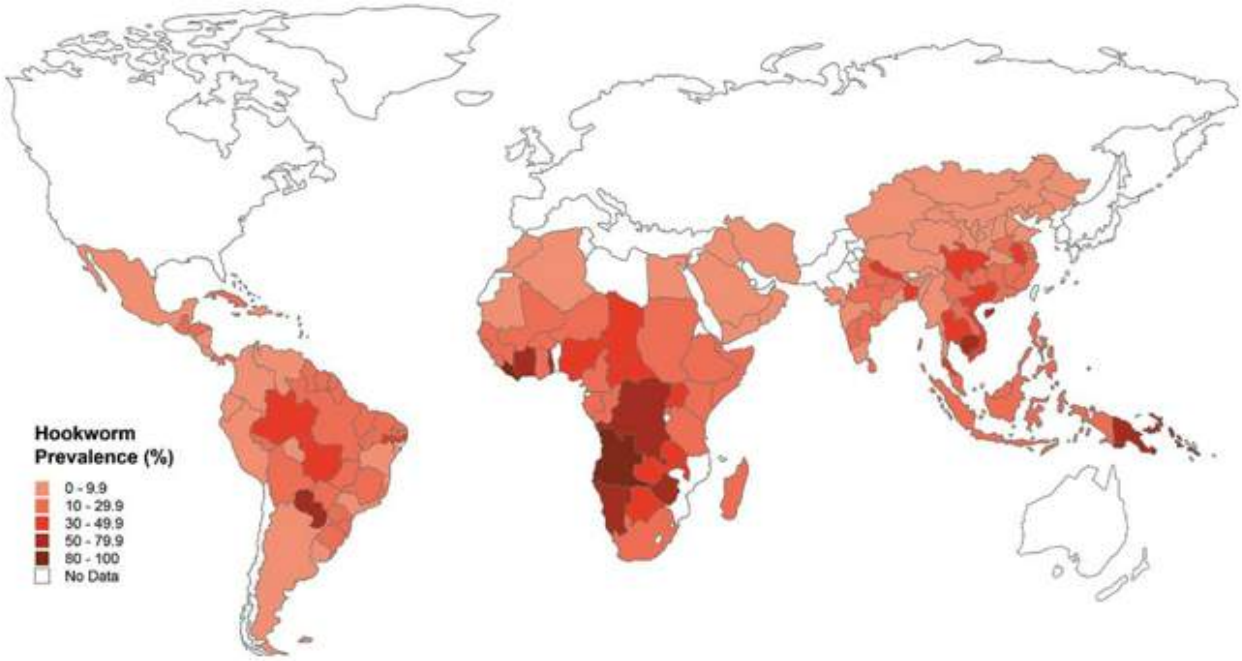

Figure 1.1 Global Distribution of Human Hookworm Infection (Hotez et al., 2005)

1.2 Current control methods for worm infections

The most effective control methods of helminth infections in humans are hygiene improvement throughout health education and appropriate sanitation procedures. In addition, chemotherapy via the use of anthelminthic drugs can also reduce morbidity and prevalence of infection (Gazzinelli et al., 2012). Chemotherapy such as ivermectin, albendazole and praziquantel are widely used for helminth control and eradication when taken individually or as combinations (ivermectin and albendazole or albendazole and praziquantel), and are a safe tool against schistosomiasis and STH in endemic regions (Olds et al., 1999). However, the number of available anthelmintic drugs is limited and the costs of drug production and the necessity of repeated administrations are problematic. This is especially true for the livestock industry, where repeated drug treatments cost around one thousand millions pound sterling (£1000 million) per annum in Britain alone (Newton and Munn, 1999). Currently, effective control depends on continuous use of anthelmintic drugs to protect humans and animals from infections. However, some important issues such as parasitic resistance against anthelmintic drugs have arisen in livestock, highlighting the importance for developing alternative control strategies (Balic et al., 2000; Hagel et al., 1999; Newton and Munn, 1999; Ruso et al., 2015). Therefore, more effective preventative treatments are required, such as- vaccine or vaccine/drug dual therapies.

1.3 Current state of vaccine research

There have been many examples of successful development of vaccines against viral and bacterial pathogens (Cowling et al., 2016; Mcaleer et al., 1984; Ruso et al., 2015), to date there are no vaccines that can limit the immense burden of human helminth infections. This may in part be due to the ability of worms to manipulate the immune system by suppressing immune responses, which is essential for the survival of the parasites. In many cases, the immune

responses that are mounted against parasitic helminths are ineffective, resulting in an inability to clear the infection. This could be due to a number of factors, including a failure to mount a protective memory T cell or antibody-mediated immune response that could protect against reinfection. The development of a vaccine would be critically important for assisting the existing chemotherapy methods for controlling infections in helminth-endemic areas.

1.3.1 Vaccines in livestock and animals

Vaccinations against helminths are an important element of disease prevention in an animal health program. Helminth-excreted proteases can protect sheep against *Haemonchus contortus* (Knox et al., 2003; Smith et al., 2003) and *Ostertagia ostertagi* (Geldhof et al., 2002). Similarly, substantial protection against livestock nematodes has been demonstrated with a purified recombinant protease vaccine (Knox et al., 1999). There are also successful metazoan parasite vaccines against the gastrointestinal nematode *Haemonchus contortus* (Knox, 2000) as well as cestodes, *Taenia ovis* (Johnson et al., 1989) and *Echinococcus granulosus* (Lightowlers et al., 1996). It has also been shown that canines can be vaccinated against hookworm *Ancylostoma caninum* via immunization with third-stage infective larvae (L3) that were attenuated by ionization radiation (Miller, 1965a, b). Irradiated schistosomes are also effective vaccines for animals (Eberl et al., 2001). Furthermore, antigens released by hookworm larvae are able to protect hamsters (Goud et al., 2004; Mendez et al., 2005) and dogs (Hotez et al., 2003) from hookworm infections, where larval excretory/secretory (ES) proteins can promote a strong protective Th2 response (Allen and MacDonald, 1998). Further studies have identified purified recombinant proteins that are released by parasites that are effective vaccines for hookworm infections, including then cysteine hemoglobinase, *Ac*-CP-2 (Loukas et al., 2004), *Ac*-APR-1 and Ac-ASP-2 (Loukas et al., 2005).

1.3.2 Human vaccines against helminths

Development of a vaccine against human helminth infections remains elusive despite the tremendous progress made in vaccinology for other infectious diseases. It has been shown in some trials that *Necator americanus* ASP-2 adjuvanted with Alhydrogel are safe and immunogenic against hookworm infection (Goud et al., 2005), However, during phase 1 testing in a hookworm endemic area of Brazil, ASP-2 resulted in urticarial responses after a single vaccination (Diemert et al., 2012). Human *Schistosoma mansoni* TSP-2 is being developed as a recombinant protein to prevent heavy *Schistosoma mansoni* infection (Curti et al., 2013). Further, a human hookworm vaccine is being developed as a bivalent substance containing two recombinant proteins (GST-1 and APR-1 from *N. americanus*), to reduce moderate and heavy hookworm infections (Pearson et al., 2010). These vaccines will target a pediatric population, as this age group is more susceptible to chronic infections (Bethony et al., 2006; Brooker et al., 2004; Hotez et al., 2005; Hotez et al., 2008; Hotez and Brooker, 2004). The previous failures in vaccine development have been due to many reasons, including the ability of parasites to evade immune responses and our lack of understanding of how these parasites interact with their hosts to induce immunity. In order to develop vaccine, it is important to understand the immune response to infection in both human and animal models.

1.4 Protective immune responses to helminth infections

1.4.1 Lessons from humans in endemic areas

Humans living in endemic areas are constantly exposed to parasites. However, there have been observations that some people are highly resistant to infection, while others develop chronic and recurring infections (Elliott et al., 2007). Some of this variation may be attributed to differences in nutrition, genetics and immune status. Susceptibility can be correlated to

variation of the genetic regulation of B cell activation as well as immunoglobulin secretion (Hagel et al., 1999). Serum antibody levels against hookworms are predominantly the Type-2-immune associated immunoglobulins such as IgG1, IgG4 and IgE (Jarrett and Bazin, 1974). Furthermore, eosinophil and mast cell numbers are increased during hookworm human infection, in addition to increase of Type 2 cytokines (MacDonald et al., 2002). However, the study of immune responses against helminths in endemic areas is complicated due to coinfections with other infections such as bacteria and viruses, as well as infestations with multiple helminth species. Further, it is difficult to monitor patients over a long period of time without using chemotherapeutical intervention and it is impossible to know the frequency or timing of these infections. Only a few publications have described controlled infection of volunteers (Gaze et al., 2012; Maxwell et al., 1987; White et al., 1986), demonstrating that worm infections do indeed induce a biased Type 2 immune response.

1.4.2 Lessons from experimental animal models

Animal models have been critical for defining the immunological mechanisms of protection against helminths. For example, studies on the parasitic helminth infections such as *Trichuris muris* (a model of human whipworm *Trichuris trichiura*) and *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* (mouse models of human hookworm) have greatly contributed to our knowledge of the components of immune responses that are responsible for resistance and susceptibility to infection (Camberis et al., 2003; Farah et al., 2001; Klementowicz et al., 2012). Helminths infections are often accompanied by polarized T helper Type 2 (Th2) immune response (Anthony et al., 2007; Maizels and Yazdanbakhsh, 2003; Mulcahy et al., 2004), characterized by increased production of interleukins (IL-4), IL-5, IL-9 and IL-13 by $CD4^+$ T-helper Type 2-cells. Experimental models revealed that the protection against nematode infection can be a $CD4^+$ T cell-dependent process, with T cell-derived IL-4 being essential (Finkelman et al., 1997). Th2 cell-derived cytokines elicit activation of cells such as

macrophages, mast cells, eosinophils, basophils and neutrophils that coordinate immunity to the parasite. Th2 cell-derived cytokines also induce B-cells to produce helminth specific IgE and IgGs that, together with the persistence of memory Th2 cells, can provide long-lasting immunity to reinfection (Anthony et al., 2007; Erb, 2007; Finkelman et al., 1997; Maizels and Yazdanbakhsh, 2003; Mulcahy et al., 2004), However, the helminth- host immune system interaction is the result of co-evolution, thus helminths had to develop mechanisms to deal with the host's immune system (Bentwich et al., 1995). There are different strategies for helminth immunomodulation leading to down-regulation of T cell immune responses, including stimulation of regulatory T-cell (Treg) immune responses, inhibition of effector cells as well as inhibition of immune cell proliferation (Allen and Maizels, 2011; Erb, 2007; Finkelman et al., 1997; Mulcahy et al., 2004). Helminth infections in humans and animal models are associated with expansion of regulatory T-cell populations and it is believed that these cells may directly or indirectly suppress the anti-parasitic immune responses directed against the parasite (Ludwig-Portugall and Layland, 2012; Maizels and Smith, 2011; Metenou and Nutman, 2013). Regulatory T cells possess an array of mechanisms by which they may regulate immune responses, including the release of anti-inflammatory cytokines such as IL-10 and TGF-β that can inhibit the development and function of pro-inflammatory Th1 and Th17 responses, but also restrain anti-parasitic Th2 responses (Flynn and Mulcahy, 2008; Maizels et al., 2004). This can enhance the ability of the worm to survive (Anthony et al., 2007), which is beneficial both for the parasite and the host, by the suppression of potentially pathogenic, tissue-destructive immune responses such as Type 1 immune responses or unrestrained Type 2 immune responses (Anthony et al., 2007). However, Type 2 immune responses are not associated with immunity to all helminths. In schistosomiasis, the host can develop protective Th1 immune responses to parasites during early stages of infection, where Th2 immune responses occur later in response to eggs laid by the parasite (Stadecker et al., 2004). Together, the complete nature of the

immune response against helminth infection still remains unknown. The following section provides a more detailed about the mechanisms of worm expulsion as well as the role of immune cells and cytokines towards infection.

1.4.3 Physiological mechanisms of worm expulsion

While T cells play a crucial role in promoting the inflammatory response against infection, T cells do not kill the parasite directly. Rather, they orchestrate a downstream effector biological response that expels the worms. Epithelial turnover and mucin production are considered two of the key mechanisms of worm expulsion, which has been primarily demonstrated using the *T. muris* model of whipworm infection (Cliffe et al., 2005). Goblet cells within the crypts of colon and caecum produce mucins that form the major component of the mucus layer of the intestine and prevent close association of *T. muris* with the epithelial barrier (Klementowicz et al., 2012). Goblet cell hyperplasia occurs following exposure to *T. muris* infections in genetically-resistance mice such as C57BL/6 or BALB/c mice (Artis et al., 2004), and does not occur in susceptible mice such as AKR mice (Hasnain et al., 2011a; Hasnain et al., 2011b; Hasnain et al., 2010). Mucin production by goblet cells is regulated by Th2 cytokines such as IL-9 and IL-13 (Kondo et al., 2002; Taube et al., 2002; Whittaker et al., 2002). Furthermore, epithelial cells of intestine have a close relationship with gastrointestinal parasites such as *T. muris* (Tilney et al., 2005). The formation of an “Epithelial escalator” and increased epithelial turnover, where cells migrate from the bottom of the crypt “proliferation zone” to the shedding zone at the top of crypt, is an important mechanism that accelerates *T. muris* expulsion in resistant animals, and is delayed in susceptible mice (Cliffe et al., 2005). Moreover, Th2 cytokines such as IL-4 and IL-13 promote increased intestinal smooth muscle contractility and also increased intestinal permeability and responsiveness to mediator including prostaglandin

that stimulates fluid secretion following *H. polygyrus* and *N. brasiliensis* infections (Zhao et al., 2003).

1.5 Adaptive immune responses to helminths

1.5.1 T-helper cells and cytokines

The immune system consists of 2 arms: The innate immune system and the adaptive immune system. Activation of adaptive immunity requires that foreign antigens must be recognized by innate immune cells, which process them into peptides that are presented to lymphocytes such as T cells and B cells through MHC molecules. For small foreign pathogens, such as bacteria, viruses, fungi and protozoan parasites, killing of the pathogen can be achieved following engulfment of the invading pathogen. However, helminths are large and impossible to be engulfed by immune cells. Thus, helminth proteins excreted during infection are processed and presented instead to adaptive immune cells (Gause et al., 1999). However, the precise cellular mechanisms of how helminth antigens are recognized by the immune system to promote adaptive immunity are incompletely understood.

Within the adaptive immune system there are many distinct lymphocyte subsets that are important for immunity to different pathogens. CD8+ T cells have important roles in immunity to some infectious diseases via MHC-1 expression and induction of cell-mediated killing (Jordan and Hunter, 2010). However, the importance of CD8+ T cells during helminth infections is less clear, since helminths may be resistant to CD8-mediated attack due to their large size (Metwali et al., 2006). In contrast, CD4+ T helper cells are known to play a central and essential role in orchestrating immunity to large pathogens such as parasitic helminths. There are many different subsets of CD4+ T cells that play different roles depending on the nature of the infection, and the cytokines they release.

The development of a T-helper Type 1 (Th1) cell response is important for immunity to small, easily phagocytosed pathogens such as bacteria, viruses, fungi and protozoan parasites (Kidd, 2003). However aberrant Th1 cell responses are associated with various autoimmune and inflammatory diseases (Liblau et al., 1995). Th1 cell development begins with the secretion of IL-12 and IL-18 by cells such as macrophages and DCs upon recognition of intracellular or extracellular pathogens (Farrar et al., 2002). IL-12 can induce the differentiation of naïve $CD4^+$ T lymphocytes to become interferon-gamma (IFN-γ)-producing Th1 cells (Trinchieri, 1994), which can activate macrophages to become "classically activated macrophages" that can phagocytose and kill pathogens (Kidd, 2003). IL-12 also acts to up-regulate IL-18 receptor expression.

Th17 cells represent a subset of CD4 effector T cells that are important for immunity to extracellular bacteria and fungi, but also associated with autoimmune diseases (Bettelli et al., 2007; Weaver et al., 2006). Th17 cell development is dependent on RORγt transcription factor (Dong, 2006; Ivanov et al., 2006). IL-23 production by dendritic cells and macrophages is an important factor in production of a robust Th17 response; however, studies *in vitro* and *in vivo*, have shown that it is not responsible for the initial induction or commitment to differentiation of the Th17 phenotype (Mangan et al., 2006; Veldhoen et al., 2006). Other factors such as IL-6 and TGFβ also play important roles in Th17 cell development (Kimura and Kishimoto, 2010).

Th2 cells are important for immunity to large pathogens such as parasitic helminths, but are also associated with detrimental inflammation during allergies and asthma (Cooper, 2009). The differentiation of Th2 cells primarily involves the transcription factor GATA-3 and the action of IL-4 produced by a variety of cells such as natural killer T cells, eosinophils, mast cells, basophils and innate lymphoid cells (Kidd, 2003; Lafaille, 1998; Pelly et al., 2016; Wang et al.,

2006). After Th2 cells become mature they increase IL-4 production, which generates a paracrine loop and induces naive T cells to become Th2 cells (Kidd, 2003). Further, IL-6 also secreted during the early stages of a Th2 immune response, that induces the Th2 activation via the up-regulation of IL-4 and inhibition of STAT1 phosphorylation, through inhibition IFN-γ gene expression (Detournay et al., 2005; Dodge et al., 2003).

1.5.2 B cells and antibody

Production of IL-4 following helminth infection can promote generation of antigen-specific IgG1 and IgE secreting plasma cells (Gause et al., 1999). The relative importance of antibody in immunity to worms is still unclear, however antibody binding could directly neutralize the ability of the parasite to feed or migrate, or could activate downstream cellular immune responses (Liu et al., 2010; McCoy et al., 2008). Consistent with this, purified IgG and serum transfer from immune animals can confer protection against some species of helminth (Liu et al., 2010; McCoy et al., 2008). IgE is an important effector molecule that can activate immune effector cells during helminth infections, where mast cells and basophils are activated by IgE binding and subsequently release their granules and cytokines. However, it has been proposed that IgE is not essential for the protective immune response against parasites like *H. polygyrus bakeri* or *N. brasiliensis* (Liu et al., 2010; McCoy et al., 2008; Watanabe et al., 1988). Interestingly, depletion of B cells increases the severity of infections with helminths such as *H. polygyrus bakeri* (McCoy et al., 2008; Watanabe et al., 1988), suggesting that B cells have additional functions that may promote immune resistance, B cells also produce cytokines that have effect on the ongoing Th2 response and induce immune regulation (Wojciechowski et al., 2009).

1.6 Innate immune responses to helminths

The innate immune system plays important role as the first line of defense against parasitic infection. Cells and cytokines of this system can initiate and direct the adaptive immune response, in addition to controlling the early stages of infection. Dendritic cells, neutrophils, eosinophils, basophils, mast cells and macrophages are the main innate immune cells that immediately respond to invading pathogens. In addition to their role as the first effector cells, they can sense danger signals and pathogen-associated molecular patterns, producing cytokines that direct the immune response.

1.6.1 Dendritic cells

Dendritic cells (DCs) are important in stimulation and modulation of the host immune system following helminth infections, by acting as the primary cell that processes and presents antigens to the adaptive immune system (Pulendran et al., 2010). Helminth products can modulate DC activation and prevent their ability to promote Th1 and Th17 responses, but also induce Th2 or regulatory T cell responses (Aranzamendi et al., 2012; Donnelly et al., 2010; Goodridge et al., 2003; Haçariz et al., 2009; Layland et al., 2007; Van der Kleij et al., 2002; Zaccone et al., 2011; Zaccone et al., 2003). DCs are crucial antigen presenting cells and are involved in T-cell activation and regulation (Mulcahy et al., 2004). Their activation results in increased surface expression of MHC class II and co-stimulation molecules including CD40, CD80 and CD86 that modulate the developing adaptive immune response (Kumamoto et al., 2013). The DC-signals responsible for inducing Th2 cell responses *in-vivo* are still unclear (Gao et al., 2013). Other studies have demonstrated that DC-mediated Th2 cell differentiation required the Notch ligand jagged (Krawczyk et al., 2008) and up-regulation of the molecules CD40 and OX40L (Ganley-Leal et al., 2006). But whether these pathways are required for DCs to induce CD4+ Th2 cell differentiation *in vivo* remains undefined. Further, the *in vivo* role of DCs in helminth infections is controversial. In some experimental systems, DCs alone are not sufficient to induce

a Th2 response (Paul and Zhu, 2010), suggesting that other cells also contribute. This may be due in part to active regulation of DC function by helminths, where the release of parasite enzymes can polarise DC responses and establish infections (Hewitson et al., 2009).

1.6.2 Eosinophils

Increases in eosinophil numbers in blood and tissues is a hallmark of parasitic helminth infections (Ganley-Leal et al., 2006; Huang and Appleton, 2016) Interlukin-5 (IL-5) is important for growth and differentiation of eosinophils (Dent et al., 1990; Lopez et al., 1986). Eosinophils provide immune protection against some helminth species by releasing their toxic granules that damage the parasite when they recruited to the site of infection (Makepeace et al., 2012; Shamri et al., 2011; Voehringer et al., 2006). For example, it has been highlighted that eosinophil-derived major basic protein (MBP) has toxicity against some species of helminth (Stone et al., 2010). A recent report demonstrated that during a secondary immune response, eosinophils are activated by memory Th2 cells to express even higher levels of MBP, which makes them more toxic to *N. brasiliensis* larvae in the lung (Obata-Ninomiya et al., 2018). In addition to toxic granule proteins, eosinophils can also express other proteins, such as cytokines, chemokines, growth factors and lipid mediators (Rothenberg and Hogan, 2006). For instance, eosinophils have been suggested to support Th2 immune response through releasing IL-4 (Voehringer et al., 2004). However it is clear that due to the chronic nature of some helminth infections, in the face of ongoing eosinophils, it suggests that worms may be able to evade eosinophil attack to prolong their survival (Maizels et al., 2012). For example, it has been proposed that eosinophils can be effective at killing infective larval stages but not the adult worms (Meeusen and Balic, 2000). Furthermore, studies using mice that over-express IL-5, and hence display constitutive eosinophilia, have revealed that eosinophils may not be toxic to all species of helminth. For example, IL-5 transgenic mice display increased immunity to *N.*

brasiliensis but not other parasites such as *Toxocara canis* (Dent et al., 1999), which suggests that eosinophils can promote increased resistance to some helminth species but not others.

1.6.3 Mast cells

Mast cells are also considered to be a major hallmark of protective immunity to parasitic helminths (Weller et al., 2011). Mast cells can respond to circulating parasite-specific IgE via the expression of FcεRI receptors, which stimulates the release different mediators including histamine, cytokines or chemokines that induce Th2 immune responses (Melendez et al., 2007; Pearce, 2007). However the relative roles of mast cells in resistance to helminth infections varies greatly depending on the model (Anthony et al., 2007). It has been suggested that mast cells have a key role in decreasing the fecundity of worm species (Anthony et al., 2007), whilst they are not essential for clearance of challenge infection by *H. polygyrus bakeri* (Hashimoto et al., 2010). Mast cells are mostly implicated in the clearance of gut helminths, however they do not appear to have important role in the clearance of other tissue dwelling helminths like *S. mansoni* (Gerken et al., 1990). However, in mice infected with *N. brasiliensis*, IL-3 and IL-4 depletion lead to mucosal mast cell depletion but had no impact on worm expulsion (Madden et al., 1991). Furthermore, mast cells can orchestrate Type 2 immune reponses to helminths by regulation of tissue-derived cytokines (Hepworth et al., 2012) and inducing Type 2 cytokine producing-innate lymphoid cell responses (Shimokawa et al., 2017).

1.6.4 Basophils

Basophils have substantial role in context of Th2 immune responses, especially in allergic diseases and helminth infections (Ohnmacht and Voehringer, 2009; Voehringer, 2011). They can be activated by IgE, cytokines, TLR ligands and proteases, which leads to the production of mediators including histamine, chemokines, heparin or antimicrobial peptides and lipid

mediators like leukotriene C4 or prostaglandin D2 (Schroeder et al., 2001). Basophils also synthesize and produce cytokines, including IL-4, IL-5, IL-13 and TSLP and are thus considered as an early, innate regulator of Type 2 immunity (Min et al., 2004; Schroeder et al., 2001). Several studies have also suggested that basophils can act as antigen-presenting cells via MHC-II expression (Perrigoue et al., 2009; Sokol et al., 2008; Yoshimoto et al., 2009) and recent studies showed that this may happen due to acquisition of MHC-II molecules from dendritic cells via cell contact-dependent trogocytosis (Miyake et al., 2017). Depletion of basophils *in vivo* results in impaired protective immunity to some helminth species during secondary infections (Ohnmacht et al., 2010; Ohnmacht and Voehringer, 2009) and adoptive transfer of basophils increases CD4+ T cell proliferation against *Schistosoma mansoni* egg injection. However, other studies have shown that basophils are not necessary for the primary Th2 response to *N. brasiliensis* infection (Ohnmacht et al., 2010), suggesting that the relative role for basophils in immunity to helminths depends on the species of parasite and the frequency of exposure.

1.6.5 Macrophages

Macrophages play multiple roles in immunity to parasitic helminths. Macrophages are efficient at detecting danger signals in early infections with pathogens (Dunn et al., 1985). They express pattern recognition receptors (PRRs) that enable recognition of pathogen- associated molecular patterns (PAMPs) (Plüddemann et al., 2007). Macrophages have an important role as phagocytic cells and as antigen presenting cells (APC) and migrate to sites of infection and secrete mediators including cytokines and chemokines. Macrophages are important regulatory and suppressor cells during parasitic infections as well as in tumour-bearing hosts (Noel et al., 2004; Sica and Bronte, 2007). Macrophages have two distinct types, M1 or classically activated macrophages and M2 or alternatively activated macrophages (AAMacs) that both have

important roles during Type 1 or Type 2 immune responses, respectively (Mills et al., 2000). IFN-γ plays crucial role in promoting M1 cell development in association with microbial products or tumor necrosis factor α (TNF-α), thus leads to secret pro-inflammatory cytokines such as (IL-12, IL-1β, TNF-α, IL-6 and IL-23) and chemokines (CXCL-9, 10, 11 and 16). M1 are essential in polarized Th1 responses and induced resistance against intracellular microorganisms (Mantovani et al., 2005). M2 are less effective at killing microorganisms, but are important for inducing tissue remodelling and repair. For example migration of helminths through tissues can cause remarkable tissue damage, and macrophages might participate in wound healing by cleaning cell debris, releasing cytokines, growth factors which promote fibroplasia and angiogenesis (Martin and Leibovich, 2005). Further, it has been highlighted that the M2 phenotype of macrophages are important for immune killing of *N. brasiliensis* (Chen et al., 2014) and filarial parasites (Turner et al., 2018), and that macrophage-derived arginase is important for trapping *N. brasiliensis* larvae in the skin (Obata-Ninomiya et al., 2013) and killing in the lung (Anthony et al., 2006). Macrophages also play a critical role as providers of many of the molecules necessary for tissue repair, including arginase-1, transforming growth factor- (TGF-) β, and Resistin-like molecule (RELM)α (Forbes and Rosenthal, 2014). RELMα is a highly secreted protein in type-2 cytokine-induced inflammation including helminth infection and allergy (Batugedara et al., 2018). It has been suggested that RELMα acts as an immune brake that provides mutually beneficial effects for the host and parasite by limiting tissue damage and delaying parasite expulsion (Batugedara et al., 2018).

1.6.6 Neutrophils

Neutrophils have a central role in the type 1 immune responses, as they are often the first line of defense against pathogens such as bacteria, viruses and parasites. Neutrophils are phagocytic cells that release lytic enzymes, as well as produce reactive oxygen species with anti-microbial potential (Cassatella et al., 1997; Mollinedo et al., 1999). These substances can act in association with cells in the affected tissue, including macrophages and mast cells, thus exaggerate the initial inflammatory responses (Fialkow et al., 2007). However, some studies have been examined their role in case of metazoan parasites like helminths (Bonne-Anneé et al., 2011; Bonne-Année et al., 2013). Neutrophils are activated during sub-mucosal invasion of parasite larvae, and immediately recruited to the site of infection (Anthony et al., 2007). In *Strongyloides stercoralis* infections, neutrophils play important roles in destroying parasites (Galioto et al., 2006). Following *N. brasiliensis* infections neutrophils can infiltrate the lung at day 2 in primary inoculation, and depletion of these cells resulted in remarkably impaired parasite expulsion (Chen et al., 2014). Interestingly, it has recently been demonstrated that helminth infections can promote the development of a functionally-distinct "alternatively activated neutrophils" ('N2') population that distinct from classical ('N1') neutrophils, suggesting that neutrophils can have different functions depending on the nature of the infectious stimulus (Chen et al., 2014). Further, it has demonstrated that neutrophils and macrophages cooperate to drive *N. brasiliensis* killing in the lung (Sutherland et al., 2014). However, another study has not been able to demonstrate any role for neutrophils in mechanism of *N. brasiliensis* killing in the lung (Bouchery et al., 2015).

1.6.7 Innate lymphoid cells

Innate lymphoid cells (ILCs) are cells of the innate immune system that represent a small portion of the immune cell population in lymphoid organs, at epithelial barrier surfaces, as well as in other tissues (Spits and Cupedo, 2012; Spits and Di Santo, 2011). ILCs express similar transcription factors and effector molecules expressed by T cell populations, but do not express specific antigen receptors, so they can be rapidly activated following infection before the development of the adaptive immune response (Spits and Cupedo, 2012; Spits and Di Santo, 2011). There are at least 3 types of ILCs, including ILC 1 populations that express transcription factor T-bet and release IFN-γ (Spits and Cupedo, 2012; Spits and Di Santo, 2011). ILC2s express RORα and GATA3, release interleukin 5 (IL-5) and IL-13 (Spits and Cupedo, 2012a; Spits and Di Santo, 2011). ILC3 populations induce lymphoid organogenesis and analogous to Th17 cells which rely on the transcription factor RORγt, in addition to releasing IL-17A, IL-17F as well as IL-22 (Spits and Cupedo, 2012; Spits and Di Santo, 2011). There has been considerable recent interest in ILC2s in their ability to promote immunity to parasitic helminths, since they may be critical early sources of IL-4, IL-5 and IL-13 (Moro et al., 2010; Neill et al., 2010; Price et al., 2010). These cells are activated by epithelial cell-derived cytokines such as TSLP, IL-25 and IL-33. However, the precise roles of ILCs in helminth infections, including the factors that regulate their function, remain to be defined. It has been highlighted that Arginase-1 is a key regulator of ILC2 proliferative capacity and proinflammatory functions (Monticelli et al., 2016). Further, it has demonstrated the potential of both ILC2 and $CD4^+$ T-cell-mediated regulation of M2 macrophages-mediated *N. brasiliensis* killing through the expression of IL-4 and/or IL-13 (Bouchery et al., 2015). More recent work reveals that sensory neurons are a critical regulator of ILC2 responses during helminth infections (Cardoso et al., 2017), suggesting that in addition to tissue resident epithelial cells and their

secreted cytokines, cells of the nervous system are important for sensing infection with helminths and directing downstream ILC2 responses and anti-parasitic immunity.

1.6.8 Epithelial cells, TSLP, IL-25 and IL-33

Intestinal epithelial cells (IECs) support a primary physical barrier against commensal and pathogenic microorganisms in the gastrointestinal tract, but also produce an array of cytokines and chemokines that can regulate inflammation (Nagler-Anderson, 2001). It has also been shown that epithelial microRNAs induce the mucosa–immune system crosstalk important for mounting protective T helper type 2 (TH2) responses (Biton et al., 2011). Furthermore, specialized epithelial cells can release anti-parasitic effector molecules at the mucosal barrier such as resistin-like molecule β (RELM β) that participate in protection against helminths (Herbert et al., 2009). For example, NF-κB signaling within intestinal epithelial cells (IECs) promotes $CD4^+$ T-cell-dependent immunity to *T. muris* via the secretion of cytokines such as thymic stromal lymphopoietin **(**TSLP) (Zaph et al., 2007). TSLP in particular is a potent regulator of Type 2 immunity (Friend et al., 1994; Schramm et al., 2007; Sokol et al., 2008), by activating cells of the innate and adaptive immune system, including T cells, dendritic cells, basophils and mast cells (Ziegler et al., 2013). TSLP is critical for the detrimental airway inflammation associated with asthma, where TSLPR-deficient mice showed impaired type 2 responses and reduced asthma symptoms (Zhou et al., 2005). *In vivo* studies have shown the importance of TSLP signaling in the clearance of *T. muris* (Massacand et al., 2009; Taylor et al., 2009). In contrast, in other helminth infections the role of TSLP in initiating type 2 immune responses is unclear, as TSLPR deficient mice mount normal type 2 immune responses and expel *N. brasiliensis* and *H. polygyrus* infections (Massacand et al., 2009).

Interleukin 25 or IL-17E is another epithelial-derived cytokine that plays a crucial role in the initiation of type 2 immune responses. Intraperitoneal injection of recombinant IL-25 leads to features associated with type 2 immune responses (Fort et al., 2001). Moreover, in allergic lung diseases, IL-25 is overexpressed in airway epithelial cells, leading to exaggerated type 2 responses (Angkasekwinai et al., 2007). It has been shown that IL-25 is beneficial for host resistance in helminth infections. For instance, in *N. brasiliensis* or *T. muris* infections, animal deficient in IL-25 signalling exhibit delayed or absent expulsion of the these helminths (Fallon et al., 2006; Schmitz et al., 2005). Similarly, administration of IL-25 can induce worm expulsion in *N. brasiliensis* infections (Neill et al., 2010) via regulation of an intestinal ILC2-epithelial response against the parasite (von Moltke et al., 2016). Recent research has highlighted how specialised epithelial cells, called tuft cells, may be the key IL-25 producing cells, where tuft cells constitutively express IL-25 to sustain ILC2 homeostasis in the resting lamina propria in mice (von Moltke et al., 2016).

Interleukin-33 is an alarmin that is released by epithelial cells following cell damage or stress. IL-33 has an important role in initiation of type 2 immune response, where injection of recombinant IL-33 results in eosinophilia, IgE class switching, releases of type 2 cytokines in addition to changes in mucus of the lung and gastrointestinal system (Schmitz et al., 2005). Furthermore, during *T. muris* infections, IL-33 administration promotes type 2 responses (Humphreys et al., 2008), and other studies have demonstrated that IL-33 promotes the production of type 2 cytokines such as IL-5 and IL-13 by ILC2s (Moro et al., 2010; Neill et al., 2010). IL-33 may also activate human basophils to increase IL-4, IL-13 and histamine expression (Pecaric-Petkovic et al., 2009; Suzukawa et al., 2008). IL-33 also helps initiate type 2 immune responses via activation of mast cells in humans and promotes differentiation of alternatively macrophages (Kurowska-Stolarska et al., 2009). Together, epithelial cells and the

cytokines that they produce are central regulators of the innate and adaptive immune responses to helminth infections.

1.7 Type 1 immune responses and helminth infections

Th1 immune responses are induced by invading microorganisms such as viruses and bacteria, promoting IFN-γ expression by CD4+ T cells and cytotoxic CD8+ T cells, and activation of neutrophils and M1 macrophages. However, Type 1 immune responses also play substantial roles during helminth infections. The development of robust Type 1 immune responses following helminth infections can be harmful for host as well as to helminth, where subsequent activation of pro-inflammatory cells such as macrophages and neutrophils may be sufficient for killing the parasite, but could come at the expense of damage to host tissues where the parasite resides (Allen and Maizels, 2011). As such, infections with most species of helminth are associated with down-regulation of potentially damaging Type 1 immune responses (Anthony et al., 2007).

Type 1 responses can also directly mediate parasite killing. For example, in schistosomiasis, a vaccine can promote macrophage and IFN-γ-dependent killing of schistosomula and limiting infection (Goud et al., 2004). Conversely, IFN-γ expression can promote parasite chronicity, where depletion of IFN-γ in *T. muris*-infected mice changed the immune response from a Th1 response to a Th2 response, resulting in the reduced levels of the Th1 associated antibody IgG2a/c and higher levels of Th2 associated antibody IgG1 and enhanced parasite expulsion (Else et al., 1994). Hence, understanding the nature of how Th1 immune responses are regulated is important for understanding how infections can become chronic in some individuals, while other individuals can efficiently expel parasites. However, to date, most researchers have concentrated on either the early events that promote Type 2 immunity, or the effector

mechanisms that promote parasite expulsion.

1.8 Interleukin -1 cytokines and regulation of immunity

Interleukin-1 (IL-1) is a key mediator of innate immunity and inflammation, particularly promoting Type 1 cytokine responses. Within this family, there are IL-1-β, IL-1α, IL-18 and IL-33 that are similar by mechanism of origin, receptor structure, and signal transduction pathways utilized (Arend et al., 2008). Some studies have demonstrated the requirement for IL-1 in the development of Th2 cells in vitro (Greenbaum et al., 1988; Lichtman et al., 1988). However, other studies have highlighted an involvement of IL-1 in inducing IFN-γ producing Th1 cells (Schmitz et al., 1993) and inhibiting effect of IL-1 on IL-4 producing by human T cells. Hence, individual members of the IL-1 cytokine family have varying functions. Excessive production of IL-1β can causes widespread tissue damage and is associated with acute and chronic inflammatory human diseases, such as auto-inflammatory or autoimmune pathologies (Dinarello, 2011; Masters et al., 2009).

The IL-1 family of cytokines also plays a pivotal role in immunity to parasites, however their relative roles depends on the nature of the infection. It has been shown that IL-18 and IL-33 can activate basophils resulting in the production of Type 2 cytokines such as IL-4 via a MyD88 dependent pathway (Kroeger et al., 2009). Further, IL-18 can also act on CD4 T cells, for example, daily administration of IL-18 in particular with IL-2 induced a marked increase in serum IgE levels in a $CD4^+$ T cell- and IL-4/IL-4R/STAT6-dependent manner (Yoshimoto et al., 2000). *In vivo* studies have shown that IL-1R deficiency resulted in enhanced Th2 immune responses following *Leishmania major* infection (Satoskar et al., 1998), suggesting that IL-1β may suppress Type 2 responses. Consistent with this, in follow up studies IL-1β was shown to induce Th1 biased immune resistance to *Leishmania major* (Von Stebut et al., 2003). Consistent

with this, one study had reported that IL-1β suppresses Type 2 immunity to *H. polygyrus* by inhibiting IL-25 responses in the gut (Zaiss et al., 2013). In direct contrast, studies in other models of parasitic helminth infection have revealed that IL-1β $^{-/-}$ mice showed defective Th2 mediated induced resistance to *T. muris* (Helmby and Grencis, 2004; Humphreys and Grencis, 2009), suggesting that IL-1β may in this context promote Type 2 responses. This is supported by a recent study, that reported that IL-1β secretion is important positive regulator of group 2 innate lymphoid cell function and plasticity (Ohne et al., 2016). A recent report has demonstrated a novel immune modulatory strategy used by *Fasciola hepatica* involves secretion of the FhHDM-1, a cathelicidin-like protein, which impairs the activation of NLRP3 by lysosomal cathepsin B protease, preventing the downstream production of IL-1β and Type-1 immune responses (Alvarado et al., 2017). Together, it is evident that the role for IL-1β in immunity to parasites, including parasitic helminths remains unclear and depends on the nature of the infection and the tissues affected.

IL-18 is another member of the IL-1 family of cytokines that has varying functions depending on the inflammatory context. IL-18 has direct antimicrobial and immunomodulatory effects that are IFN-γ and/or IL-12 independent and has a significant impact on the development of intestinal immunity against *T. spiralis* infection (Helmby et al., 2001; Neighbors et al., 2001). Interestingly, other studies have documented a role for IL-18 in inducing Th2 responses *in vivo* or *in vitro* by increasing levels of IgE, IL-4, and IL-13 (Hoshino et al., 2000; Yoshimoto et al., 2000). Further, IL-18 can induce the production of T_H2-type cytokines by both naive and T_H1-polarized cells (Moller et al., 2001). IL-18 is a pleiotropic cytokine that can regulate both Type-1 and Type-2 responses, depending on the nature of the cytokine milieu, infectious stimuli and genetic background of the host (Nakanishi et al., 2001; Wei et al., 2004; Xu et al., 2000). IL-18 can promote Th1 immune responses and resistance to one species of the *Leishmania*

parasite (Li et al., 2004), but induce non-protective Th2 responses to another species (Bryson et al., 2008). Further, one study highlights how IL-18 induces Type-2 responses when IFN-γ is absent (Liu et al., 2006), another study implicates IL-18 in suppressing Type-2 cytokine responses to *T. muris* (Helmby and Grencis, 2004). In conclusion, IL-1β and IL-18 can induce either a Th1 or Th2 response, depending on the cytokine milieu and genetic background of the host (Wei et al., 2004; Xu et al., 2000). Because of this, the IL-1 family of cytokines appear to have key and diverse roles in helminth infections. Understanding the mechanism of how these cytokine responses are initiated and regulated might help us understand why some infections are acutely resolved, and how some infections become chronic.

1.9 Regulation of IL-1 cytokine production and secretion

IL-1β and IL-18 production is a result of cell activation and promotes a multitude of metabolic, physiologic, inflammatory, hematologic and immunologic effects. The synthesis of IL-1 cytokines is dependent on factors such as nuclear factor kappa B NF-kappa B activation pathway, following stimulation by pattern recognition receptors on the surface of immune cells, for example TLR (Croston et al., 1995). However, these cytokines are not functional and cannot be released from the cell until a second signal is activated. This second signal, and the subsequent maturation and release of IL-1β and IL-18 are dependent on the activation of inflammasome complexes (Cruz et al., 2007).

1.10 Inflammasomes

The inflammasome is a part of the innate immune system, and consist of an intracellular sensor such as nod-like receptor (NLR) proteins that is coupled with caspase-1 and the adaptor apoptosis-associated speck-like protein containing a carboxy-terminal (ASC) and Caspase activation and recruitment domain (CARD) (Meylan et al., 2006; Yu and Finlay, 2008) (Figure1.2). Inflammasome expression is well characterised in immune cells of myeloid origin, but their components are also expressed by some non-professional immune cells (Schroder and Tschopp, 2010).Furthermore, different inflammasomes can have distinct tissue- or cell type-specific functions such as epithelial cells (Broz and Dixit, 2016; Kummer et al., 2007). Generally, expression of inflammasomes is constitutive, however cells can increase their expression of the various inflammasome protein components under some conditions (Latz et al., 2013).

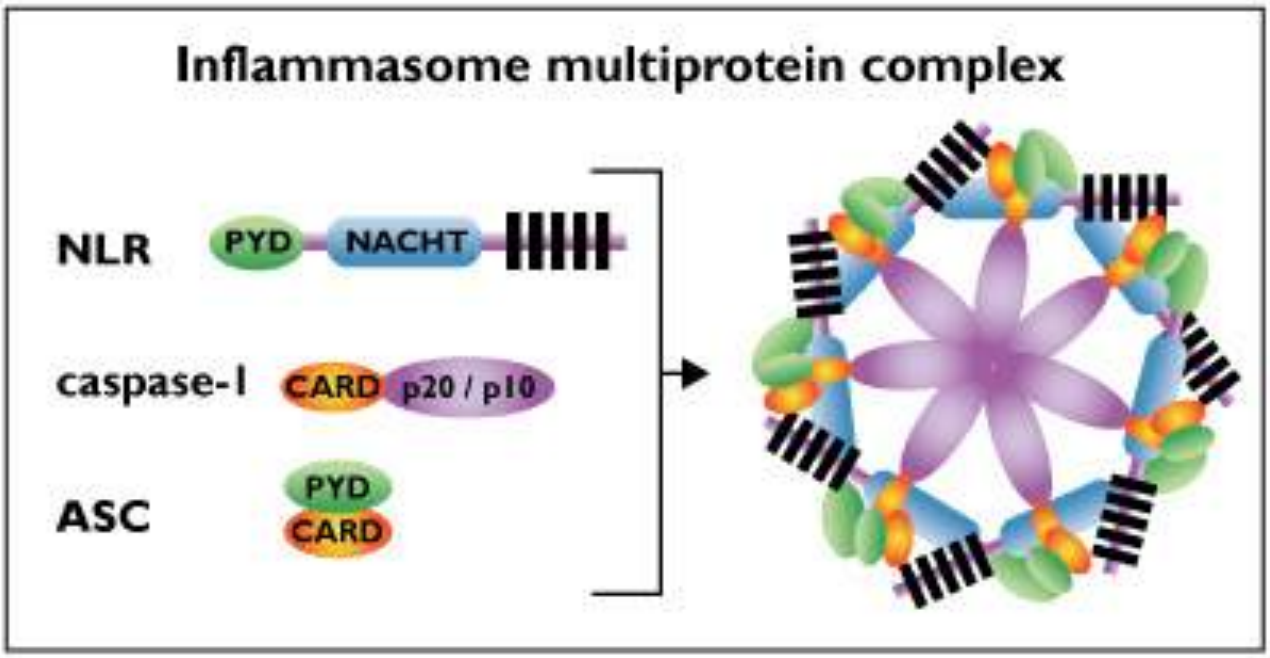

Figure 1.2 Generic structure of inflammasome complexes, including Toll like receptor (NLR) that activates following interaction with pathogen molecule leading to activation of caspase1 via interaction with ASC (apoptosis-associated speck-like protein containing a carboxy-terminal CARD). From: www.invivogen.com

Inflammasomes have two families, NLR and PYHIN (pyrin and hematopoietic interferon-inducible nuclear antigens domain-containing protein) (Broz and Monack, 2011; Fernandes-Alnemri et al., 2009). The NLR family is composed of a central nucleotide-binding and oligomerization (NACHT) domain, which is flanked by C-terminal leucine-rich repeats (LRRs) and CARD or pyrin (PYD) domains (Schroder and Tschopp, 2010). It has been shown that LRRs are involved in ligand sensing and autoregulation, however CARD and PYD domains induce homotypic protein-protein association for downstream signalling (Schroder and Tschopp, 2010). The NACHT domain is the common domain to all NLR families, and promotes activation of the signalling complex in an ATP-dependent manner. There are 3 distinct subfamilies within the NLR family which are the NODs (NOD1-2, NOD3/NLRC3, NOD4/NLRC5, NOD5/NLRX1, CIITA), the NLRPs and the IPAF subfamily, consisting of IPAF (NLRC4) and NAIP (Schroder and Tschopp, 2010).

ASC is an adaptor molecule that recognises the insoluble cytosolic fraction, known as speck, which is formed in cells that are undergoing apoptosis (Taniguchi and Sagara, 2007). It is characterized of an N-terminal PYD domain and a CARD, that acts directly with multiple PRRs, for instance NLRPs, NLR caspase recruitment domain-containing protein (NLRC), and AIM2, leads to caspase 1-activating platforms known as inflammasomes (Martinon et al., 2002; Masumoto et al., 1999).

Activation of inflammasomes promotes a programmed cell death process (Fink and Cookson, 2005) whereas the dying cell induces potent cytokine production eventually leads to inflammation. Importantly, this activation needs to a “second signal” that triggers maturation and secretion of IL-1β and IL-18 that dependent on the assembly of the inflammasome, activation of Caspase-1 enzyme which cleaves pro-inflammatory IL-1 cytokines into their maturation and release them from the cell (McKee et al., 2009).

The hallmarks of inflammasome activation are the processing of caspase-1, the maturation and release of IL-18 and IL-1β and the intiation of pyroptosis, a lytic inflammatory cell death (Broz and Monack, 2013). These represent the primary ways in which researchers can detect and quantify the activation of inflammasomes. Moreover, it is possible to visualize endogenous ASC specks in infected cells and study the release of processed caspase-1, caspase-11 and mature cytokines into the cell supernatant by Western blotting (Broz and Monack, 2013), further intracellular staining of IL-18 expression by flow cytometry (Gerdes et al., 2002).

1.10.1 Types of inflammasomes

It has been shown that the NLR family has more than 20 members identified in humans that such as nucleotide-binding domain (NBD) and leucine-rich repeat (LRR) proteins include NLRP1, NLRP3, NLRC4, and AIM2 (Allen et al., 2013; Chen, 2014; Correa et al., 2012) (Figure 1.3)

Name	Activator	Adapter/ binder	Function
NLRC3	?	TRAF6	Negative TLR regulator?
NLRC4	Flagellin, T3SS components	ASC, Caspase-1	Inflammasome formation
NLRC5	virus	?	Transcription MHC class I related genes
NLRX1	RNA?	UQCRC2, MAVS, TUFM	ROS production, autophagy, Negative TLR regulator and MAVS-dep. signalling
NLRP1	LF, MDP	ASC, Caspase-1	Inflammasome formation
NLRP2	?	ASC?	Inflammasome formation?
NLRP3	Pore-forming toxins, nucleic acids, ATP, uric acid, hyaluronan..	ASC, Caspase-1	Inflammasome formation
NLRP4	?	Beclin-1	Negative regulator of NK-Kß activation and autophagy
NLRP6	?	ASC	Inflammasome formation and Negative regulator of TLR
NLRP7	Microbial acylated lipopeptide	ASC	Inflammasome formation
NLRP10	?	?	Negative regulator of NK-Kß activation and DC migration
NLRP12	Lipopeptide (*Yersinia pestis*)	ASC	Negative regulator of TLR
CIITA	?	?	MHCII regulator

Figure 1.3: Summary of the main characteristics of the NLRs.(Chaput et al., 2013)

NLRP1, the first inflammasome to be characterized, has only been described to be activated in a physiologically relevant manner by a single signal, after exposure to the anthrax lethal toxin (LeTx). LeTx is consist of protective antigen and lethal factor. Lethal factor is a putative metalloprotease and a zinc metalloprotease-like consensus sequence that is essential for NLRP1 activation (Fink et al., 2008). LeTx is important for the assembly of the NLRP1 inflammasome, which is crucial for immunity of mice to *Bacillus anthracis* spore infections. NLRP1 also contains a Function-to-Find domain (FIIND) and autoprocessing of NLRP1 within this FIIND is a prerequisite for the activation (Frew et al., 2012).

NLRP3 is the most-studied inflammasome owing to its involvement in immunity to small pathogens such as bacteria, viruses and fungi and its important roles in sterile inflammation and metabolic diseases such as Type 2 Diabetes. The precise mechanism of how these stimuli activate NLRP3 is still unclear, however studies have demonstrated that NLRP3 can be activated via different pathways, for example canonical and non-canonical (Kayagaki et al., 2013). The canonical NLRP3 pathway is activated by Gram-positive bacteria including *Staphylococcus aureus* and viruses such as Influenza, pore-forming toxins including hemolysin and pneumolysin as well as endogenous ligands and crystalline substances such as ATP, silica and alum (Fitzgerald, 2010; Lamkanfi and Dixit, 2014; Rathinam et al., 2012). (Figure 1.4).

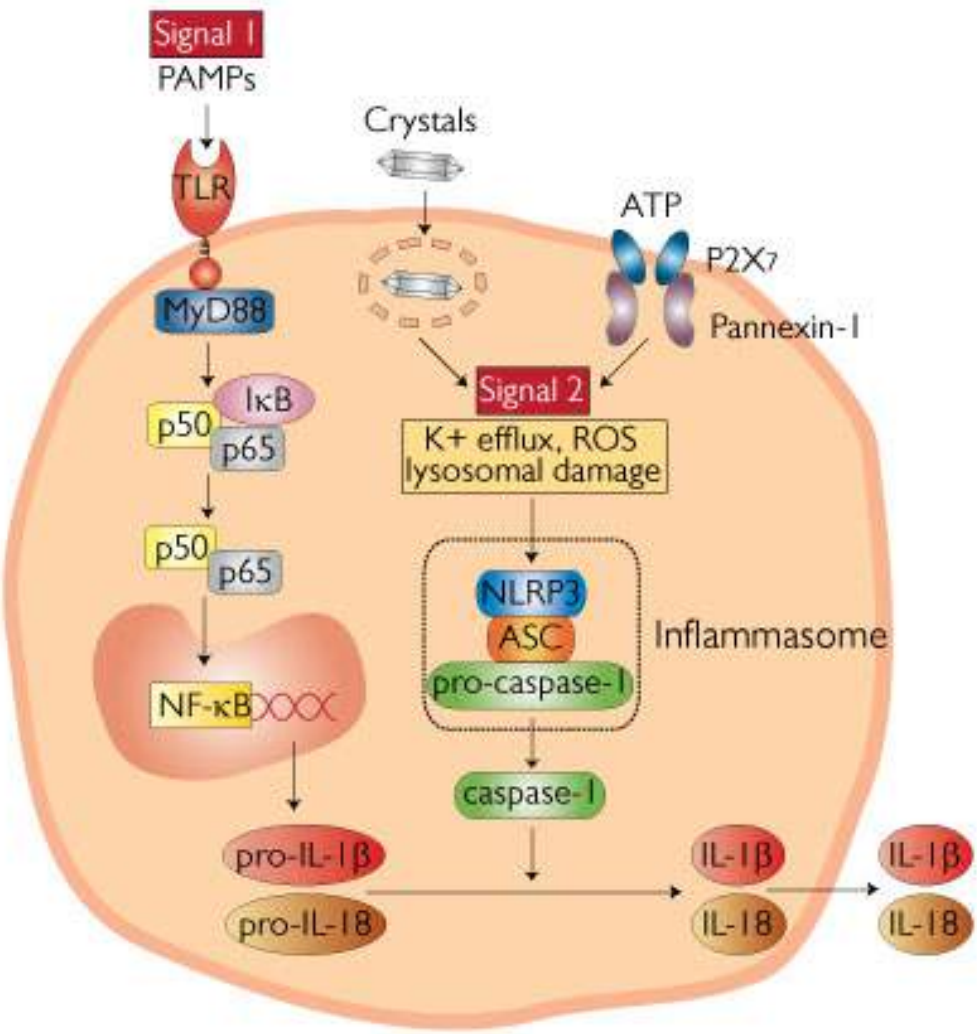

Figure 1.4: Describing that NLRP3 responds to a wide range of DAMPs and PAMPs, including infectious pathogens and exogenous materials. Activation of the NLRP3 inflammasome requires two signals and is controlled at transcriptional and post-translational levels. The first signal, also referred to as the priming signal, is the induction of the toll-like receptor (TLR)/nuclear factor (NF)-κB pathway to upregulate the expression of NLRP3 and pro-IL-1β. Signal 2 is transduced by various PAMPs and DAMPs to activate the functional NLRP3 inflammasome by initiating assembly of a multi-protein complex consisting of NLRP3, the adaptor protein ASC, and pro-caspase-1, association of NLRP3 with ASC is required for recruitment of pro-caspase-1. From: www.invivogen.com

NLRC4 has a variance requirement for the adaptor protein, ASC; ASC is important for NLRC4-induced caspase-1 and IL-1β maturation, however it is dispensable for NLRC4-promoted pyroptosis (Broz et al., 2010c; Van Opdenbosch et al., 2014). It is mostly activated by a different set of ligands including bacterial flagellin and components of the bacterial type III secretion system (Zhao et al., 2011). Further, NLRC4 activation needs another NLR protein, NAIP, which is important as a receptor for the NLRC4 stimulators (Freeman et al., 2017). NAIP has four different proteins in C57BL/6 mice and among them, NAIP1 binds to needle proteins of the type III secretion system, NAIP2 binds to the *Salmonella* SPI-1 basal rod component PrgJ, and NAIP5 and NAIP6 sense flagellin (Kofoed and Vance, 2011; Rayamajhi et al., 2013; Yang et al., 2013; Zhao et al., 2011).

ALR inflammasomes are another type of inflammasome, which is responsible for inducing caspase-1 activation and IL-1β cytokine secretion. But, unlike NLR inflammasomes, ALR inflammasomes directly bind their ligand, dsDNA, via HIN-200 domains (Schattgen and Fitzgerald, 2011).

AIM2 and IFI16 do not have CARD domains and hence need ASC recruitment via their PYD for inflammasome activation (Schattgen and Fitzgerald, 2011). AIM2 mostly senses double stranded DNA in the cytosol from DNA viruses for instance, mouse cytomegalovirus and vaccinia virus as well as cytosolic bacteria including *Francisella tularensis* and *Listeria monocytogenes* (Fernandes-Alnemri et al., 2010; Hornung et al., 2009; Rathinam et al., 2010). However, IFI16 typically senses DNA from Kapsosi's sarcoma-associated herpes virus (KSHV) (Kerur et al., 2011). AIM2 has also been demonstrated to play a role in the adjuvanticity of DNA vaccines (Suschak et al., 2015) and is essential to autoimmune disorders

such as systemic lupus erythematosus through recognition of host DNA (Panchanathan et al., 2011).

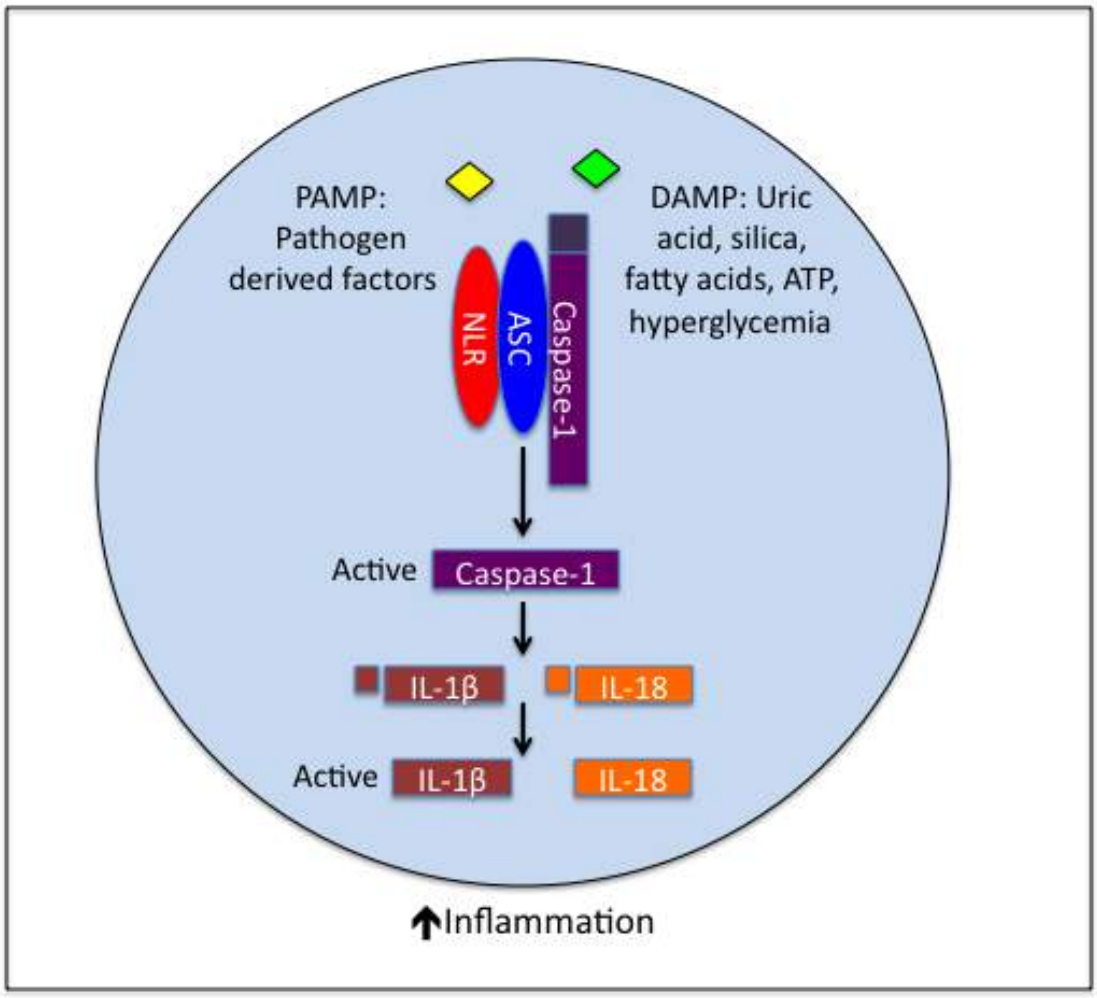

Figure 1.5 Inflammasome activation. Pathogen-associated molecular patterns (PAMPs) interact with NLRs in the cytosol. Caspase1 is then activated which cleaves the immature pro-forms of IL-1β and IL-18, and allowing maturation and release from the cell. *(Discov Med. July 26, 2011).*

Recent studies using animal models of disease where specific inflammasome components have been targeted for deletion (i.e. gene deficient mice, chemical inhibitors, CRISPR/Cas9) have been instrumental in defining the roles for the various inflammasomes in biology (Coll et al., 2015; Fenini et al., 2018). While much has been learned recently about how important various inflammasome complexes are for promoting maturation of these pro-inflammatory cytokines, the mechanisms of how inflammasomes are activated, and their relative roles in various inflammatory and infectious diseases remain largely unexplored.

1.11 Role of inflammasome in inflammatory diseases

Inflammasomes have a key role in the pathobiology of a number of inflammatory disorders, where the most widely studied inflammasome thus far has been the NLRP3 inflammasome. For instance in diabetes, exogenous substances like the saturated fatty acids activate the NLRP3 inflammasome and promote release of IL-1β (Vilaysane et al., 2010). NLRP3 inflammasome activation has also been shown in chronic kidney disease and diabetic nephropathy (Vilaysane et al., 2010). Atherosclerotic lesions have abundant cholesterol crystals have also been demonstrated to be a strong activator of NLRP3 (Duewell et al., 2010). Some studies have suggested that inflammasome activation and expression of IL-1β and IL-18 may be linked to susceptibility to inflammatory bowel diseases (IBDs) (Villani et al., 2009). It has been indicated that caspase-1, ASC, or NLRP3 deficiencies were associated with an increased severity of colitis in mice (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Hirota et al., 2011). However, other studies have examined the potential role of NLRP3 inflammasome in murine models of colitis, and have found different results (Bauer et al., 2010; Zaki et al., 2010). The NLRP3 inflammasome also regulates graft-versus-host disease GvHD resulting in shaping of Th17 responses in the intestines of the recipient (Fulton et al., 2012). The NLRC4 inflammasome is a direct negative regulator of colonic epithelial cell tumorigenesis that is not driven by the

microbiota (Hu et al., 2010). Hence targeting inflammasomes, particularly the NLRP3 inflammasome, may be a rational approach for new therapies against autoimmune diseases. Consistent with this, a recent study used a potent, selective, small-molecule inhibitor of NLRP3 (MCC950) that minimized IL-1β production and reduced the severity of experimental autoimmune encephalomyelitis (EAE), a disease model of multiple sclerosis, cryopyrin-associated periodic syndrome (CAPS) and Muckle–Wells syndrome (Coll et al., 2015).

1.12 Role of inflammasome in infectious diseases

Inflammasomes, including the NLRP3 inflammasome, have a significant role in immune responses against small, easily phagocytosed infectious pathogens such as bacteria, viruses, fungi and protozoan parasitic infections. For example, activation of inflammasomes and secretion of IL-18 and IL-1β are important to eradicate bacterial infections (Broz et al., 2010; Maher et al., 2013; Muruve et al., 2008; Song-Zhao et al., 2014), viral infections (Allen et al., 2009; Ermler et al., 2014; Kamada et al., 2014; Thomas et al., 2009) and fungal infections (Hise et al., 2009; Kistowska et al., 2014).In Candida infection, it has been demonstrated increase in mucosal expression of NLRP3 and NLRC4 inflammasomes, and up-regulation of these molecules is impaired in NLRP3 and NLRC4 knockout mice. These results documented that the NLRC4 plays a crucial role in limiting mucosal candidiasis (Tomalka et al., 2011). However, another study has showed, that dectin-1 can induced both IL-1β production and maturation via caspase-8-dependent inflammasome for protective immunity against fungi and mycobacteria (Gringhuis et al., 2012).

There has been considerable research into the role for inflammasomes in immunity to parasites. Inflammasome activation promoted bias of adaptive immunity toward Th2-type responses against some parasitic infection such as is leishmaniasis (Gurung et al., 2015).

Two studies were documented the protective role of the NLRP3 inflammasome activation against *Trypanosoma cruzi* infection in the mouse model of Chagas' diseases. They highlighted that the IL-1β production following *T. cruzi* infection was rely upon NLRP3, ASC, and caspase-1 utilizing knockout mice and macrophages, which showed sever immunopathology and greater parasite burdens in ASC and caspase-1 deficient mice compared to wild type (Silva et al., 2013). In malaria infections, the NLRP3 inflammasome is activated by the crystalline malarial byproduct hemozoin (Hz), which results in cerebral malarial symptoms in mouse models (Griffith et al., 2009). Macrophages from wild type mice produced IL-1β and promoted caspase1 when incubated in hemozoin, however the impact was absent in macrophages from NLRP3 or ASC deficient mice (Griffith et al., 2009). *In vivo*, NLRP3 deficient mice had reduced *Plasmodium berghei* ANKA-promoted cerebral malaria without any change in parasite burden, highlighting the role of NLRP3 in immunopathology but not necessarily infection (Dostert et al., 2009). It has been reported that dual engagement of the NLRP3 and AIM2 inflammasomes by plasmodium-derived hemozoin and DNA occurs during malaria (Kalantari et al., 2014). A recent paper demonstrated that targeting the IL33-NLRP3 axis improves therapy for experimental cerebral malaria (Strangward et al., 2018).

In Toxoplasma rodent models it has been shown that the NLRP1b inflammasome was activated during *T. gondii* infection of mice and rats, resulting in protective immunity against oral challenge infection (Ewald et al., 2014). NLRP1 has recently been shown to be important for immunity to protozoan parasite infections (Clipman et al., 2018; Ewald et al., 2014) .

It has revealed that the NLRP3 inflammasome as well as the NLRP1 are important in murine resistance to *T. gondii* infection. *In vivo*, *T. gondii* activated the NLRP3 inflammasome in macrophages. NLRP3, ASC, and NLRP1 deficient mice all showed increased parasite burden and mortality. These studied highlighted that IL-18 may be a key cytokine in inflammasome-mediated resistance to *T. gondii* infection (Gorfu et al., 2014) . Moreover, P2X7 receptor mediates *T. gondii* control in macrophages through NLRP3 inflammasome activation and reactive oxygen species secretion (Moreira-Souza et al., 2017). Further, NLRP3 and potassium efflux drive rapid IL-1β secretion from primary human monocytes against *T. gondii* Infection (Gov et al., 2017).

1.13 Role of inflammasome in helminth infections and Type 2 immunity

While the role for inflammasomes in Type 1-dependent immunity to small, easily phagocytosed infectious pathogens is becoming well established, the role of inflammasomes in Type 2 immunity and inflammation remains unclear. Inflammasomes are suggested to play a role in the Type 2 inflammation in patients with asthma (Oliphant et al., 2011). Inhaled substances are linked to asthmatic inflammation but their role with the inflammasome is still unclear (Nadeem et al., 2008). It was demonstrated that extra- cellular ATP could be responsible for inflammasome activation in asthma. For instance, dust mites, a common asthmatic allergen, were shown to activate and release of ATP from macrophages, epithelial and dendritic cells (Suzuki et al., 2009). Furthermore, increases in ATP were observed in patients with asthma as a result of an allergen (Idzko et al., 2007). IL-1β activity is also elevated in asthma models and can be related to the increased caspase-1 activity (Thomas and Chhabra, 2003). Moreover, patients with asthma have increased levels of IL-1β in their sputum compared with patients without asthma (Thomas and Chhabra, 2003). Interestingly, it has been highlighted that NLRP3 expression in CD4+ T cells can act as a transcription factor, independent of its ability to activate

inflammasomes, and can stimulate a T helper type 2 response (Bruchard et al., 2015). Hence, there is some evidence that inflammasomes may promote Type-2 immune responses in asthma.

While inflammasomes are generally associated with promotion of Type 2 immune responses in asthma, the role for inflammasomes in Type 2 inflammation during helminth infections is more controversial. For example, the NLRP3 inflammasome can mediate IL-1β production in schistosomasis, and genetic ablation of ASC and NLRP3 in mice lead to reduced liver pathology and down-regulated Th1, Th2, and Th17 adaptive immune responses (Ritter et al., 2010). Hence in the context of Schistosoma infection, NLRP3 appears to promote inflammation. Moreover, NLRP3 inflammasomes can be activated in mouse hepatic stellate cells against *S. japonicum* infection (Meng et al., 2016). *S. mansoni* T2 ribonuclease omega-1 can modulate inflammasome-dependent IL-1β secretion in macrophages (Ferguson et al., 2015). However, a recent paper highlighted that **t**he immune modulatory peptide FhHDM-1 secreted by *Fasciola hepatica* inhibits NLRP3 inflammasome activation by suppressing endolysosomal acidification in macrophages (Alvarado et al., 2017). Another study using a different helminth model has demonstrated the opposite phenomenon, i.e. that infection by *Heligmosomoides polygyrus bakeri* and IL-1β production attenuates type 2 immunity and promotes parasite chronicity, however a role for specific inflammasome proteins was not defined (Zaiss et al., 2013). Further, it has been reported that cattle with deletion mutations in the NLRP3 gene display increased anti-parasitic resistance, highlighting the potentially critical role for NLRP3 in influencing immunity to helminths (Xu et al., 2014).

Together, there has been very little research into roles of inflammasomes in immunity to helminths, and most of that has concerned the roles in *schistosome* infections, with very little research into the roles for inflammasomes in immunity to gastrointestinal helminths. Hence, there are still a lot of questions to address regarding whether inflammasomes play an important

role in regulating immunity and inflammation associated with parasitic helminth infection. This includes: (1) whether worm infections activate or inhibit inflammasomes *in vitro* and *in vivo*, (2) whether inflammasomes are important for *in vivo* immunity to helminths and (3) whether distinct inflammasome plays similar or different roles in immunity to helminths. Hence, future work should investigate the roles for specific inflammasome proteins, such as NLRP3, NLRP1 in immunity to a variety of species of parasitic helminths and inflammation in multiple tissue sites. These two inflammasomes have been shown to play a dual role in immunity against protozoan infections (Gorfu et al., 2014), hence more research needs to be conducted to understand whether antigens derived from worms similarly influence inflammasome activation and immunity.

1.14 General Conclusion:

Helminth parasites are incredibly successful pathogens, infecting a quarter of the world's population, causing high morbidity. Protective immunity and expulsion of helminths is activated by Th2 cytokines and cells, in addition to macrophages, ILC2, basophils and eosinophils. Failure to mount these type 2 immune responses can lead to immunopathology mediated by Th1 cytokines such as IL-1β, IL-18 and IFN-γ. The mechanisms by which host immunity is initiated are still unknown. Clearly, understanding the early events that initiate and regulate immunity to helminths is critical, including the worm-derived antigens that are recognized by the immune system and how these signals are processed to evoke immune resistance, which would be informative for the rational development of vaccines against worm infections. In addition, it will help us understand how worms and worm-derived proteins may modulate host immunity, which may have implications for future developments of novel worm-based therapies for inflammatory diseases in the first world.

For my PhD studies outlined in this thesis, my hypothesis was that inflammasome activation following helminth infection would be a critical influence on the nature of the innate or adaptive immune response to gastrointestinal helminths.

To address this hypothesis, my aims were to:

1. Determine whether gastrointestinal helminths activate inflammasomes *in vivo* and *in vitro* and the mechanism by which this occurs.
2. Investigate the role of NLRP3 inflammasomes in controlling immunity to the murine model of whipworm, *T. muris.*
3. Define the molecular and cellular mechanism of NLRP3 inflammasome mediated immunity to *T. muris.*
4. Assess the role for inflammasomes in regulation of innate immunity to a different species of helminth, the murine hookworm *N. brasiliensis.*
5. Assess the role of the NLRP1 inflammasome in regulating immunity to distinct models of gastrointestinal helminth infection.

By addressing these aims my studies will have for the first time addressed the roles for distinct inflammasome in immunity to multiple species, *T. muris* and *N. brasiliensis,* and the molecular and cellular immunological mechanisms by which inflammasome regulate the immunity against these infections.

1.15 Murine models of helminth infection used in these studies:

1.15.1 T. muris

T. muris utilizes a fecal/oral route of transmission and has a direct lifecycle whereby eggs are ingested by the host, hatch in the cecum and undergo three sequential moults before reaching sexual maturity, after which adult worms release eggs into the lumen of the gut to continue the cycle (Cliffe and Grencis, 2004). Mice are infected by oral gavage with 100-200 embryonated *T. muris* eggs, which typically results in an acute infection dependent on Type 2 immune responses that resolves within 3-5 weeks. If mice are administered lower doses of embryonated *T. muris* eggs (between 5 and 30), it causes a Type 1-dominated immune response and results in chronic infections that can last up to a year. Experimentally, analysis of worm burdens after sacrificing mice at day 21 or 35 post-infection allows for analysis of immune responses and how they may promote or suppress immunity to *T. muris*.

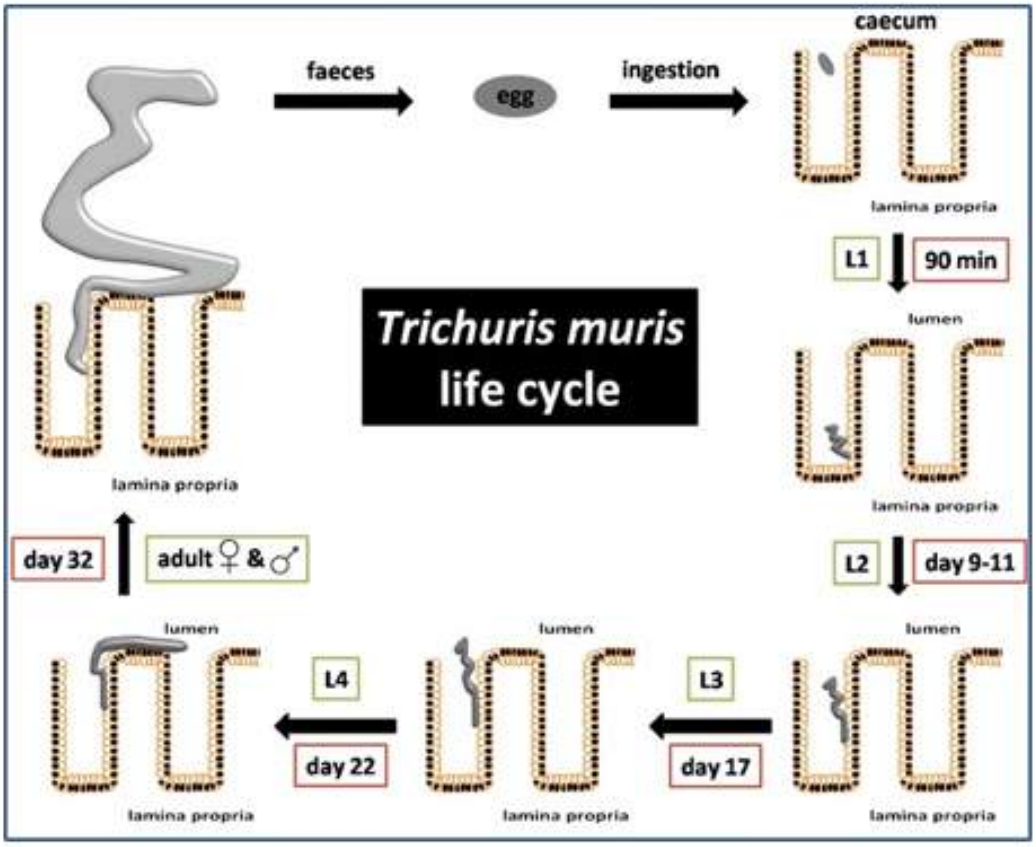

Figure 1.6 *T. muris* life cycle. Infection occurs by the ingestion of infective eggs which hatch in the caecum 90 min post infection (p.i.) releasing the first larvae (L1). L1penetrate the caecum and proximal colon wall, dwell in the epithelial layer and undergo three more moults to L2 (9–11 days p.i.), L3 (17 days p.i.) and L4 stage (22 days p.i.). By day 32 p.i. female and male adult forms of T. muris can be observed in the caecum and proximal colon of infected mice. Eggs, which leave the host organism with faeces, need 2 months to embryonate and become infective (Klementowicz et al., 2012)

1.15.2 N. brasiliensis.

The *N. brasiliensis* life cycle begins when the elliptical eggs of the parasite are passed in the feces that hatch to first, second and third-stage larvae (L3). Infection can be by oral ingestion, but skin penetration or sub-cutaneous injection is most efficient, with penetration within 5 min after placement on the skin. Parasitic larvae move deep into the epidermis, then migrate to the loose subcutaneous connective tissue where they find blood vessels that carry them to the lungs as early as 11 hr after invasion. Experimental inoculations are typically given by subcutaneous injection of between 250 and 600 infective L3. The third moult occurs in the lung between 19 and 32 hr, and the emerging L4 remain in the lung up to 50 hr post-infection before migrating via the trachea to the intestine. The final molt in the intestine begins at 90 to 108 hr post-inoculation and results in the L5, or mature adult. Adult worms are found loosely attached to the proximal half of the small intestine (Camberis et al., 2003). Mice are infected subcutaneously with 500 L3 which is result in an acute infection associated with a strong Type 2 immune responses and the parasites can be recovered from the lungs within day 1-2 and gut within day 4-7 to determine levels of immunity to infections.

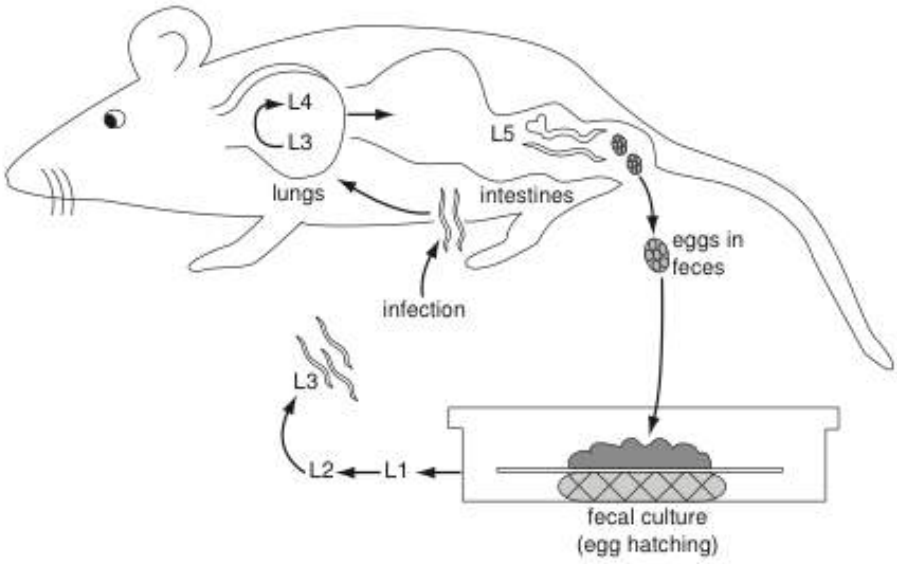

Figure 1.7 *N. brasiliensis.* Infection occurs by the skin penetration of L3 which migrates to lung at day 1 post infection (p.i.) to become L4, L4 that migrate to small intestine to become adults males and females at day 6 post infections (Camberis et al., 2003)

CHAPTER 2

2. The NLRP3 inflammasome suppresses protective immunity to gastrointestinal helminth infection

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2.1 Abstract

Inflammasomes promote immunity to microbial pathogens by regulating the function of IL-1-family cytokines such as IL-18 and IL-1β. However, the roles for inflammasomes during parasitic helminth infections remain unclear. We demonstrate that mice and humans infected with gastrointestinal nematodes display increased IL-18 secretion, which in *Trichuris*-infected or worm antigen-treated mice and in macrophages co-cultured with *Trichuris* antigens or exosome-like vesicles, was dependent on the NLRP3 inflammasome. NLRP3-deficient mice displayed reduced pro-inflammatory Type 1 cytokine responses and augmented protective Type 2 immunity, which was reversed by IL-18 administration. NLRP3-dependent suppression of immunity partially required $CD4^+$ cells but was apparent even in $Rag1^{-/-}$ mice that lack adaptive immune cells, suggesting that NLRP3 influences both innate and adaptive immunity. These data highlight a role for NLRP3 in limiting protective immunity to helminths, suggesting that targeting the NLRP3 inflammasome may be an approach for limiting the disease burden associated with helminth infections.

Keywords

Inflammasome, NLRP3, IL-18, helminth, exosomes, immunopathology, goblet cells

2.2 Introduction

The IL-1 family of cytokines, in particular IL-1α, IL-1β and IL-18, are important for the initiation and amplification of innate and adaptive immune responses and resistance against microbial infections. These cytokines are produced by innate immune cells such as macrophages, dendritic cells and monocytes, as well as non-hematopoietic cells such as epithelial or endothelial cells (Sims and Smith, 2010). Because of their potent and diverse functions, the biological activity of IL-1 family cytokines must be tightly controlled, since dysregulation of these responses is associated with the detrimental inflammation associated with autoimmune diseases (Dinarello, 2011). Regulation of the activity of many IL-1 cytokines is achieved by inflammasomes; multi-protein immune complexes that control the cleavage and subsequent activation of immature versions of these cytokines by the intracellular cysteine protease Caspase-1 (Netea et al., 2015).

Inflammasomes play a critical role in immune defense against small, easily phagocytosed infectious pathogens by promoting Type 1 immune responses via secretion of IL-1β and IL-18 (Arbore et al., 2016; McIntire et al., 2009; Strowig et al., 2012). The NLR family, Pyrin Domain Containing 3 (NLRP3) inflammasome is the most well-characterized inflammasome and is important for immunity to diverse pathogens such as bacteria (Broz et al., 2010; Muruve et al., 2008), viruses (Allen et al., 2009; Kamada et al., 2014; Thomas et al., 2009), protozoan parasites (Gorfu et al., 2014; Silva et al., 2013) and fungal pathogens (Hise et al., 2009). Other inflammasomes, such as NLRP1, AIM2 and NLRC4, are similarly important for anti-microbial defense (Ewald et al., 2014; Rathinam et al., 2010; Tomalka et al., 2011). Inflammasomes are also implicated in the immunopathology of a number of inflammatory disorders, including diabetes (Lee et al., 2013), chronic kidney disease and diabetic nephropathy (Vilaysane et al., 2010) and inflammatory bowel diseases (IBDs) (Villani et al., 2009).

There is emerging evidence that, in addition to promoting pro-inflammatory Type 1 or Type 17 immune responses, inflammasomes can directly or indirectly influence Type 2 immune responses. For example, activation of the NLRP3 inflammasome promotes allergic Type 2 inflammation in some murine models (Besnard et al., 2011; Ritter et al., 2014), and NLRP3 promotes non-protective Type 2 immune responses to infections with the protozoan parasite *Leishmania major* (Gurung et al., 2015). These observations are consistent with a report that NLRP3 can directly promote T-helper Type 2 (Th2) cell differentiation by acting as a transcription factor (Bruchard et al., 2015). Other studies suggest that NLRP3 may suppress Type 2 immune responses, for example by inactivation of the alarmin IL-33 (Cayrol and Girard, 2009; Moulin et al., 2007), or indirectly via promotion of Type 1 biased immune responses (Ritter et al., 2010). Hence, the role of inflammasomes in regulating Type 2 immune responses remains incompletely defined.

Type 2 immune responses are associated with protective immunity to helminths, while Type 1 responses promote chronic infections and immunopathology, although very little is known about whether inflammasomes control immunity to parasitic helminth infection. The blood fluke *Schistosoma mansoni* secretes proteins that directly activate the NLRP3 inflammasome and thereby promote IL-1β secretion (Ferguson et al., 2015; Ritter et al., 2010), and genetic deficiency of NLRP3 results in suppressed Th1, Th2, and Th17 immune responses and reduced liver pathology (Ritter et al., 2010). Similarly, secreted products from the intestinal nematode *Heligmosomoides polygyrus* activate the NLRP3 inflammasome *in vitro* and increase IL-1β secretion *in vitro* and *in vivo* (Zaiss et al., 2013); however, the *in vivo* role for inflammasomes in regulating anti-parasitic immunity was not determined. Hence, while the NLRP3 inflammasome is a critical determinant of immunity and immunopathology associated to

diverse pathogens, a role for any inflammasome complex in controlling Type 2 immunity during infections with large metazoan parasites like gastrointestinal helminths is unclear.

In the present study, we describe a role for the NLRP3 inflammasome in suppressing protective innate and adaptive immune responses to infection with the gastrointestinal nematode *Trichuris muris*. *Trichuris* infection resulted in elevated IL-18 secretion in mice, and similar results were seen in human subjects experimentally infected with gastrointestinal nematodes. *In vivo* and *in vitro* murine studies revealed that proteins and exosome-like extracellular vesicles secreted by *Trichuris* enhance NLRP3-dependent IL-18 and IL-1β secretion, in combination with microbial signals such as LPS. Targeted ablation of NLRP3 caused reduced Type 1 cytokine responses, increased Type 2 responses, accelerated worm expulsion and lessened intestinal immunopathology, which was reversed by rIL-18 administration. While the presence of $CD4^+$ cells was important for the ability of NLRP3 to influence anti-parasitic immunity, NLRP3 could suppress protective immunity to *Trichuris* even in the absence of a fully-functional adaptive immune system, highlighting a complex mechanism by which the NLRP3 inflammasome regulates inflammation and immunity to parasitic helminth infections.

2.3 Results

2.3.1 Helminth infections promote Caspase1-dependent IL-18 expression that limits anti-parasitic immunity.

To investigate whether helminth infection was associated with inflammasome activation, wild type (WT) C57BL/6 mice were infected with *T. muris* and IL-18 and IL-1β levels were quantified in the serum at different time points post infection (p.i.). Caspase 1/Caspase 11 double-deficient mice were included as a control due to their impaired ability to be activated by most inflammasome complexes (Schroder and Tschopp, 2010). *Trichuris* infection in WT mice caused significant elevations in serum IL-18 levels at day 21 p.i. (**Figure 2.3.1 A**), while IL-1β levels were not significantly increased at any time point (**Figure 2.3.1 B**). Caspase-1/11-deficient mice had significantly reduced serum IL-18 and IL-1β levels compared with WT mice, suggesting that *Trichuris* elicits Caspase-1/11 dependent IL-18 expression (**Figure 2.3.1 A-B**). Following infection, Caspase-1/11-deficient mice had increased *Trichuris* antigen (Ag)-specific serum IgG1 titers (**Figure 2.3.1C**) and significantly reduced worm burdens at day 21 post-infection (p.i) compared with WT mice (**Figure 2.3.1D**), suggesting improved anti-parasitic immunity. Consistent with an increased anti-parasitic response, Caspase-1/11-deficient mice displayed significantly increased numbers of goblet cells in the colon compared with infected WT mice (**Figure 2.3.1 E-F**). Together, these data suggest that Caspase1/11-dependent inflammasome activation suppresses immunity to *Trichuris* infection.

We next aimed to determine whether gastrointestinal nematode infection of humans was also associated with inflammasome activation. Nineteen otherwise healthy subjects with celiac disease (on a gluten-free diet) from a non-helminth endemic area (Brisbane, Australia) were either infected with 15 *Necator americanus* (human hookworm) infective third stage larvae (L3, n=9) or were treated with a placebo (chili pepper solution, n=10) and serum was collected

at week 0 (day of treatment) and week 20 post-inoculation. Interestingly, helminth-infected humans, but not placebo-treated humans, displayed significant increases in serum IL-18 levels at week 20 p.i. (**Figure 2.3.1 G**). IL-1β levels were typically below the level of detection of the assay and not impacted by helminth or placebo treatment (**Figure 2.3.1 H**). These data suggest that gastrointestinal helminth infections in humans are also associated with inflammasome activation, specifically resulting in elevated IL-18 production.

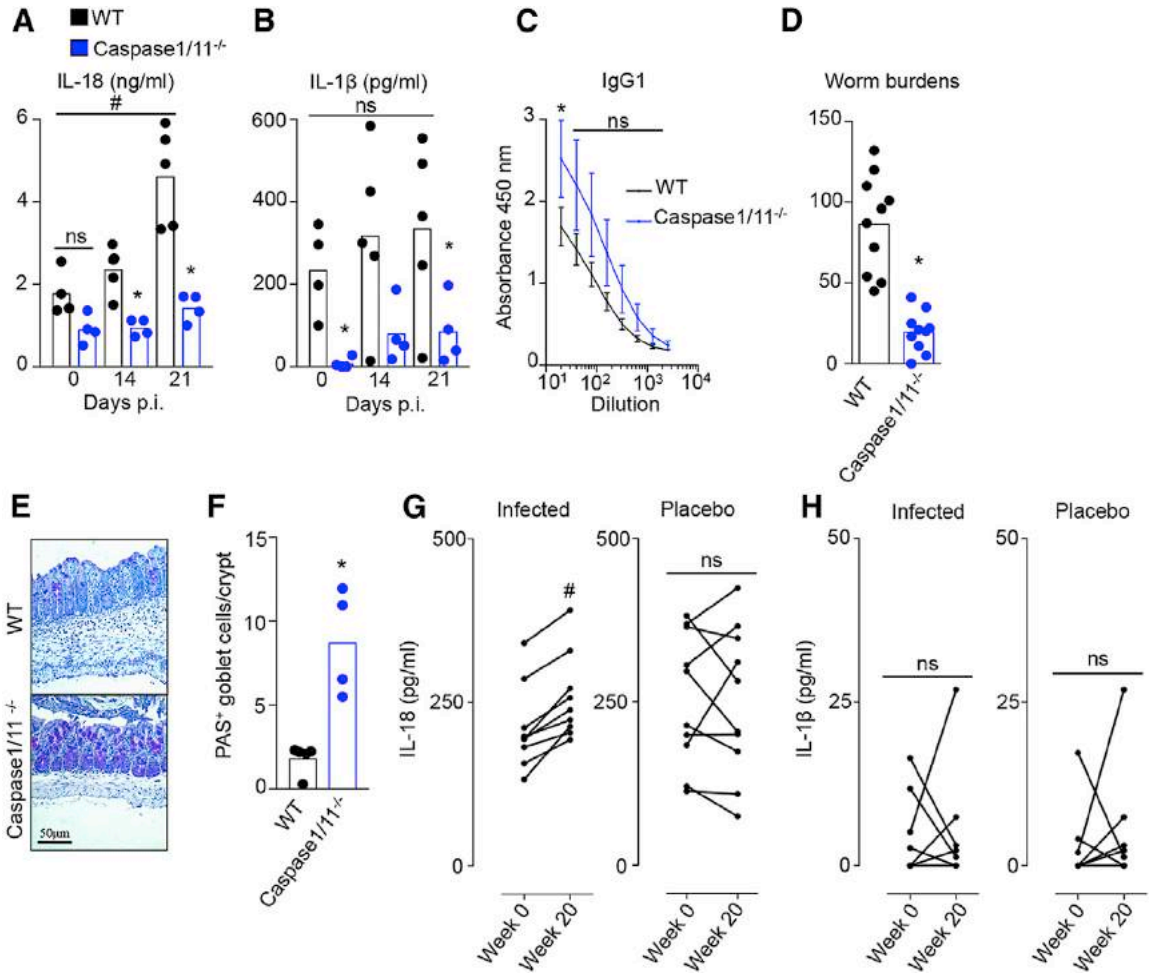


Figure 2.3.1 Helminth infections promote Caspase 1/11-dependent IL-18 expression that limits anti-parasitic immunity. C57BL/6 wild-type (WT) and Caspase1/11$^{-/-}$ mice were infected with *Trichuris* and sacrificed at d 14 or d 21 post-infection (p.i.). **(A)** Serum IL-18 and **(B)** IL-1β levels. (**C**) *Trichuris* antigen-specific IgG1 titers at d 21 p.i. (**D**) Cecal worm burdens d 21 p.i, pooled from 2 experiments. (**E**) Representative images of PAS/Alcian Blue stained cecum at d 21 p.i. Scale bar = 50μm. (**F**) Quantification of goblet cell numbers per cecal crypt unit. Data are expressed as mean with individual data points or mean±SEM and are representative of 4 experiments (n=4-5/group). Human clinical trial volunteers with celiac disease were infected with 15 *Necator americanus* larvae (n=9) or a mock infection (placebo, n=10). (**G**) Serum IL-18 and (**H**) IL-1β levels in individuals pre-infection (wk 0) or 20 weeks p.i. **p* <0.05 compared to WT. #*p*<0.05 compared to day 0 or week 0.

2.3.2 Trichuris and its secreted factors promote NLRP3-dependent in vivo and in vitro secretion of IL-18 and IL-1β.

Inflammasomes are multi-protein complexes that contain members of the NLR family of proteins (i.e. NLRP3, NLRP1, NLRC4) or non-NLR proteins such as AIM2, which give the various inflammasomes their distinct properties and effector functions (Davis et al., 2011). Since the ligands for NLRP1, NLRC4 and AIM2 have been defined and are not likely to be contained within *Trichuris* (other than DNA), the well-characterized NLRP3 inflammasome was a likely mediator. Hence, we examined the potential role for the NLRP3 inflammasome in *Trichuris*-mediated stimulation of IL-18 and IL-1β secretion. WT and NLRP3$^{-/-}$ mice were infected with *T. muris* and serum IL-18 and IL-1β levels were measured at day 21 p.i. While WT mice exhibited significant increases in serum IL-18 and a trend towards increased IL-1β levels compared to naïve mice, NLRP3$^{-/-}$ mice displayed significantly reduced levels of these cytokines (**Figure 2.3.2 A**), suggesting that NLRP3 is important for *in vivo* IL-18 and IL-1β secretion. Previous studies have demonstrated that factors excreted and secreted by worms can activate inflammasomes *in vitro* (Ferguson et al., 2015; Ritter et al., 2010; Zaiss et al., 2013). To examine whether products released by *Trichuris* similarly activate inflammasomes, we purified excretory/secretory (ES) products from *Trichuris* adult worms and injected either WT mice or NLRP3$^{-/-}$ mice intra-peritoneally (i.p.) daily for 6 days and measured circulating IL-18 and IL-1β levels at days 2 and 6. Injection of WT mice with ES products resulted in significantly increased levels of serum IL-18 and IL-1β at 2 days post-injection, with even higher levels at day 6 post-injection (**Figure 2.3.2 B)**. In contrast, NLRP3$^{-/-}$ mice exhibited no increases in these cytokines following ES injection, suggesting that factors excreted or secreted by *Trichuris* may promote *in vivo* NLRP3 inflammasome-mediated IL-18 and IL-1β secretion.

We next adopted *in vitro* experiments to determine which cell type may be responsive to *Trichuris* ES, and whether ES products are sufficient for NLRP3-dependent IL-18 and IL-1β secretion. Macrophages are key cells responsible for inflammasome activation in response to pathogens (Sims and Smith, 2010), including helminths (Ferguson et al., 2015). Therefore, we generated bone marrow derived macrophages from WT mice and cultured them in the presence and absence of *Trichuris* ES products, known inflammasome activators (LPS and nigericin toxin) or culture media alone. Culturing macrophages from WT mice with ES, LPS or nigericin alone did not cause significant increases in IL-18 or IL-1β secretion compared to media control (**Figure 2.3.2 C**). As expected, LPS and nigericin stimulation did significantly increase IL-18 or IL-1β secretion compared to media, however these levels were increased substantially if *Trichuris* ES was also included in the cultures (**Figure 2.3.2 C**). We next aimed to determine whether ES-mediated IL-18 or IL-1β secretion was dependent on NLRP3 and whether a specific fraction of *Trichuris* ES may exert these effects. Parasitic worm ES products are highly complex mixture of proteins, lipids, carbohydrates, microRNAs and small molecules, and recent studies have highlighted that these can be contained within extracellular vesicles such as exosomes, which facilitate host-pathogen interactions and immunomodulation (Eichenberger et al., 2018). Similar to results from Figure 2C, ES products or purified exosomes alone or in combination with LPS did not significantly increase secretion of IL-18 or IL-1β compared to treatment with media or LPS only (**Figure 2.3.2 D**). However, if WT macrophages were stimulated with ES or exosomes in the presence of known inflammasome activators (LPS and nigericin), the levels of both IL-18 and IL-1β rose significantly compared to stimulation with LPS + nigericin alone (**Figure 2.3.2 D**). Critically, this synergistic stimulation of IL-18 or IL-1β secretion by *Trichuris* ES products and exosomes was not apparent when cells from NLRP3$^{-/-}$ mice were used, indicating that molecules and extracellular vesicles secreted by *Trichuris* augment NLRP3-dependent secretion of IL-18 or IL-1β, in concert with microbial signals and toxins.

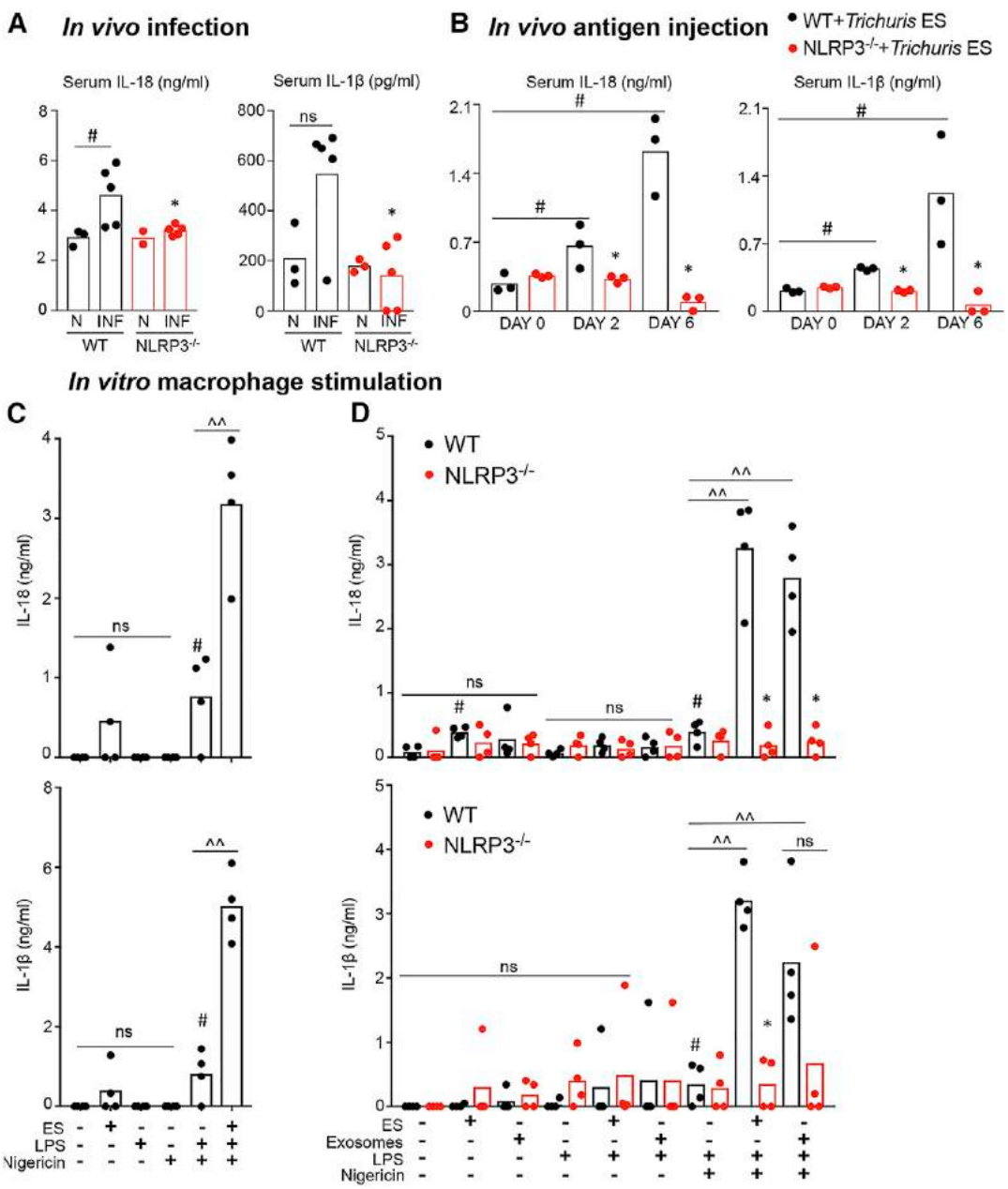


Figure 2.3.2 *Trichuris* and its secreted factors promote NLRP3-dependent *in vivo* and *in vitro* secretion of IL-18 and IL-1β. (A) Wild-type (WT) and NLRP3$^{-/-}$ mice were infected (INF) with *T. muris* and serum IL-18 and IL-1β levels were determined at d 21 post-infection (n=3-5 mice/group). N=naive mice. (**B**) WT and NLRP3$^{-/-}$ mice were injected intraperitoneally with 50 μg of *Trichuris* excretory/secretory (ES) products and were bled immediately (day 0) or 2 or 6 days post-injection and serum levels of IL-18 and IL-1β levels were determined (n=3/group). **(C)** Bone marrow-derived macrophages from WT mice were cultured with combinations of either 25 μg/ml of Trichuris ES, 1 μg/ml LPS or 20 μM nigericin. Plus (+) symbols denote inclusion of reagent, minus (-) symbol denotes media control. (**D**) Bone marrow-derived macrophages from WT or NLRP3$^{-/-}$ mice were cultured with combinations of either 25 μg/ml of *Trichuris* ES, 25 μg/ml purified *Trichuris* adult worm exosomes, 1 μg/ml LPS or 20 μM nigericin (n=4 wells/treatment). IL-18 and IL-1β were measured in supernatants or sera by ELISA. All results are representative of 3 experiments. *p <0.05 compared to WT. # p<0.05 compared to N, DAY 0 or media only control, ^^ p<0.05 compared to LPS+nigericin, ns denotes not significantly different.

2.3.3 NLRP3 limits protective Type 2 immunity and promotes IFN-γ responses following Trichuris infection.

Given the critical role for NLRP3 in *Trichuris*-mediated inflammasome activation and IL-18 secretion, we assessed the *in vivo* role for NLRP3 in protective immunity to *Trichuris* infection. Similar to results seen in Caspase $1/11^{-/-}$ mice (**Figure 2.3.1**), $NLRP3^{-/-}$ mice had significantly increased *Trichuris* Ag-specific serum IgG1 titers (**Figure 2.3.3 A**) and reduced worm burdens at day 14, 21 and 35 p.i compared to WT mice (**Figure 2.3.3 B**). $NLRP3^{-/-}$ mice also displayed significantly increased frequencies and total numbers of eosinophils in the mesenteric lymph node (mLN) compared to WT mice (**Figure 2.3.3C-D**), consistent with an elevated cellular Type 2 immune response. Further analysis of cellular immune responses at the site of infection (cecum) revealed that *Trichuris*-infected $NLRP3^{-/-}$ mice displayed elevated eosinophil frequencies in the cecum compared to WT mice (**Supplementary figure 2.3.1 A**). Frequencies of other cell types typically associated with Type 2 immunity such as basophils (**Supplementary figure 2.3.1 B**), mast cells (**Supplementary figure 2.3.1 C**) and macrophages (**Supplementary figure 2.3.1 D**) were not elevated in the ceca of infected $NLRP3^{-/-}$ mice. The degree of intestinal immunopathology was significantly decreased in $NLRP3^{-/-}$ mice compared to WT mice (**Figure 2.3.3 E-F**), which corresponded with significantly increased goblet cell responses (**Figure 2.3.3 G-H**). One of the most well studied mechanisms by which the NLRP3 inflammasome is activated is by engagement of the $P2X_7R$ by extracellular ATP (Riteau et al., 2010). However, mice deficient in $P2X_7R$ do not display the same enhanced immunity phenotype as $NLRP3^{-/-}$ mice during *Trichuris* infection, suggesting that a different pathway to NLRP3 inflammasome activation may be more important (**Supplementary figure 2.3.2**). Together, these data suggest that the NLRP3 inflammasome inhibits protective Type 2 associated immune responses and promotes immunopathology following *Trichuris* infections, likely via a $P2X_7R$-independent mechanism. To investigate the immunological mechanisms by

which NLRP3 controls immunity to *Trichuris* infections, we assessed antigen-specific cytokine responses in the mLN of WT and NLRP3$^{-/-}$ mice and cytokine responses in the colon. NLRP3$^{-/-}$ mice displayed increased IL-4 and IL-13 expression by Ag-stimulated mLN cells compared to WT mice (**Figure 2.3.3 I**), consistent with an augmented Type 2 immune response in the intestinal draining lymph nodes. This was associated with increased levels of IL-13 and TSLP in the colon tissue (**Supplementary figure 2.3.3 A**). Levels of the Type 2-associated cytokines IL-25 and IL-33 in the colon were not significantly elevated in infected NLRP3$^{-/-}$ mice, nor were there increases in innate lymphoid cell Type 2 (ILC2) responses in either the mLN or colonic lamina propria, since frequencies of CD3^{-} CD19^{-} CD90^{+} GATA3^{+} cells in these tissues were not significantly different to those in infected WT mice (**Supplementary figure 2.3.3 B**). Together, these data suggest that NLRP3 limits Type 2 cytokine responses to *Trichuris* infection. The augmented Type 2-associated immune responses observed in NLRP3$^{-/-}$ mice was accompanied by a significant reduction in Type 1 cytokine responses, measured by IFN-γ expression (**Figure 2.3.3 I**). Given that IFN-γ is associated with the development of chronic infections and suppression of protective Type 2 immune responses to helminths, including *Trichuris* (Coomes et al., 2015; Else et al., 1994), we investigated the cellular sources of IFN-γ. While WT mice displayed significant increases in frequencies (**Figure 2.3.3 J**) and total numbers (**Figure 2.3.3 K**) of CD4^{+} T cells that express IFN-γ, these responses were significantly diminished in NLRP3$^{-/-}$ mice. Similarly, IFN-γ responses by non-T cells (CD3^{-} cells) were also significantly reduced in the absence of NLRP3 (**2.3.3 L-M**), together suggesting that NLRP3 promotes innate and adaptive IFN-γ responses following helminth infection, potentially representing a mechanism by which inflammasomes limit protective Type 2 immunity to helminths.

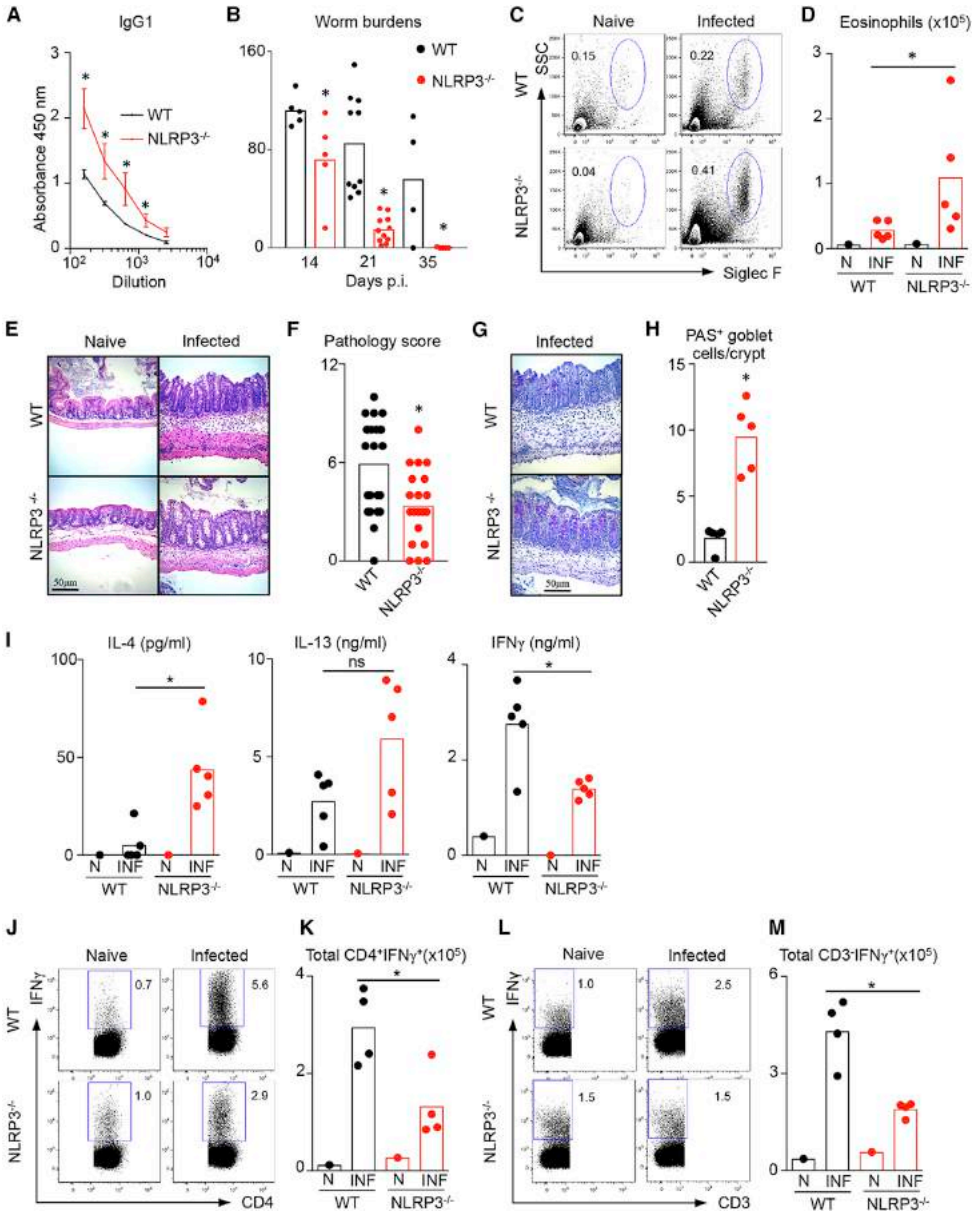


Figure 2.3.3 NLRP3 limits protective Type 2 immunity and promotes IFN-γ responses following *Trichuris* infection. Wild-type (WT) and NLRP3$^{-/-}$ mice were infected with *T. muris* and sacrificed at d 14, d 21 or d 35 post-infection (p.i.). N= naive mice. (**A**) *Trichuris* antigen-specific IgG1 titers at d 21 p.i. (**B**) Cecal worm burdens at d 14, 21 and d 35 p.i. pooled from 2 experiments. (**C**) Representative plots displaying Siglec F^{+} eosinophils frequencies in the mesenteric lymph nodes (mLN) at d 21 p.i. (**D**) Total mLN eosinophils. (**E**) Representative images of H&E stained cecum at d 21 p.i. Scale bar=50μm. (**F**) Blinded scores of cecal pathology, pooled from 4 experiments. (**G**) Representative images of PAS/Alcian Blue stained cecum tissue at d 21 p.i. (**H**) Quantification of goblet cell numbers per cecal crypt unit. (**I**) *Trichuris* antigen-specific IL-4, IL-13 and IFN-γ production by restimulated mLN cells. (**J**) Frequencies of CD3^{+} CD4^{+} IFN-γ^{+} cells in the mLN following ex vivo stimulation. (**K**) Total mLN CD4^{+} IFN-γ^{+} cells. (**L**) Frequencies of CD3^{-} IFN-γ^{+} cells in the mLN. (**M**) Total mLN CD3 IFN-γ^{+} cells. Data are expressed as mean with individual data points, or mean±SEM, and are representative of 7 experiments where n=4-5/group. *p <0.05 compared to WT, ns denotes not significantly different.

2.3.4 Therapeutic NLRP3 inhibition suppresses Type 1 immune responses and promotes resistance to Trichuris infection.

To confirm the role for NLRP3 in regulating immunity to *Trichuris* infection, and to avoid potential impacts of developmental abnormalities in gene knockout mice, we next targeted the NLRP3 inflammasome therapeutically with a selective chemical inhibitor MCC950 (Coll et al., 2015). C57BL/6 WT mice were i.p. treated daily with either PBS vehicle control or 20 mg/kg/day of MCC950 starting on the initial day of *Trichuris* infection. Treatment with MCC950 lowered serum IL-18 and IL-1β levels in *Trichuris*-infected mice but these reductions were not statistically significant (**Figure 2.3.4 A-B**). MCC950 treatment significantly increased serum titers of *Trichuris* Ag-specific IgG1 (**Figure 2.3.4 C**), significantly lowered worm burdens at day 21 p.i. (**Figure 2.3.4 D**) and resulted in a non-significant trend towards increased mLN eosinophil numbers compared to the PBS control-treated mice (**Figure 2.3.4 E**), consistent with enhanced anti-parasitic responses. *Trichuris*-infected mice treated with MCC950 displayed evidence of decreased intestinal inflammation compared to PBS control mice, however blinded pathology scores were not significantly reduced (**Figure 2.3.4 F-G**). MCC950-treatment resulted in elevated *Trichuris* Ag-specific Type 2 cytokine responses in the mLN, and concurrent significant reductions in IFN-γ responses (**Figure 2.3.4 H**). Analysis of the cellular sources of IFN-γ revealed that IFN-γ responses by $CD4^+$ $CD3^+$ T cells and non-T cells ($CD3^-$ cells) were both significantly reduced by MCC950-treatment (**Figure 2.3.4 I-J**). Together, these data suggest that targeting the NLRP3 inflammasome therapeutically with a chemical inhibitor has a similar phenotypic effect to genetic NLRP3 deficiency.

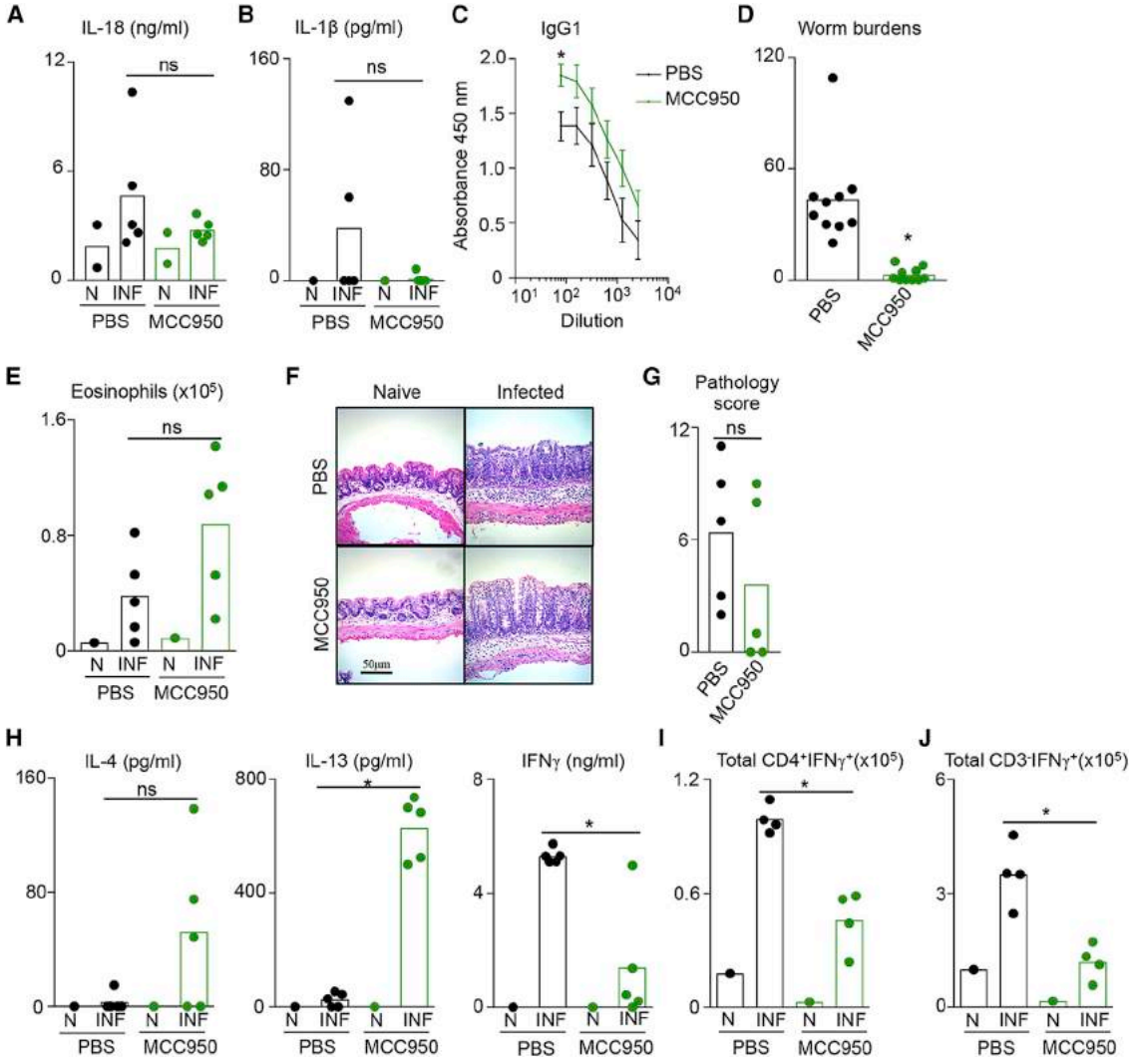


Figure 2.3.4 Therapeutic NLRP3 inhibition suppresses Type 1 immune responses and promotes resistance to *Trichuris* infection.C57BL/6 WT mice were treated intraperitoneally (i.p) daily with either PBS or 20 mg/kg/day of MCC950 following *T. muris* infection (INF) and sacrificed at d 21 post-infection (p.i.). N=naive mice. (**A**) Serum IL-18 and (**B**) IL-1β levels. (**C**) *Trichuris* antigen-specific serum IgG1 titers. (**D**) Worm burdens, pooled from 2 experiments. (**E**) Total mesenteric lymph node (mLN) eosinophils. (**F**) Representative images of H&E stained cecum tissue. Scale bar=50μm. (**G**) Blinded scores of cecal pathology. (**H**) *Trichuris* antigen-specific IL-4, IL-13 and IFN-γ secretion by restimulated mLN cells. (**I**) Total mLN $CD4^+$ $IFN\text{-}\gamma^+$ cells. (**J**) Total mLN $CD3^-$ $IFN\text{-}\gamma^+$ cells. Data are expressed as mean with individual data points or mean±SEM and are representative of 4 experiments, n=4-5 infected or 1-2 naive mice/group. *p <0.05 compared to PBS control, ns denotes not significantly different.

2.3.5 Exogenous delivery of rIL-18 reverses the enhanced anti-parasitic immunity of NLRP3-deficient mice.

Activation of the NLRP3 inflammasome can have multiple downstream effects, including the release of mature IL-18, IL-1β and regulation of IL-33 secretion (Strowig et al., 2012). Data thus far has indicated that *Trichuris* infection increases IL-18 expression, and ablation of NLRP3 diminishes this response, suggesting that NLRP3 may control immunity or susceptibility to this parasite by regulating IL-18. To more precisely define the molecular mechanism of how NLRP3 controls immunity to *Trichuris*, we examined whether delivery of exogenous IL-18 to NLRP3-deficient mice could reverse the robust immunity of NLRP3-deficient mice. $NLRP3^{-/-}$ mice were either treated every 3 days with PBS vehicle or recombinant murine IL-18 (200 ng/mouse i.p.) from days 7–19 post-*Trichuris* infection. C57BL/6 WT mice were included as a control and received PBS only. Results demonstrated that, as expected, the levels of IL-18 in the serum were increased in $NLRP3^{-/-}$ mice treated by rIL-18 (**Figure 2.3.5 A**). Furthermore, while $NLRP3^{-/-}$ mice treated by PBS exhibited a trend towards increases in *Trichuris* Ag-specific serum IgG1 titers (**Figure 2.3.5 B**) and significantly increased eosinophil numbers in the mLN (**Figure 2.3.5 C**), rIL-18 treatment of $NLRP3^{-/-}$ mice normalized these responses to WT+PBS levels (**Figure 2.3.5 B-C**). Similarly, rIL-18 treatment of $NLRP3^{-/-}$ mice resulted in significantly higher worm burdens (**Figure 2.3.5 D**) and increased $CD4^{+}$ IFN-γ responses (**Figure 2.3.5 E**) and a trend toward increased $CD3^{-}$ IFN-γ responses (**Figure 2.3.5 F**) compared to $NLRP3^{-/-}$ mice treated with PBS. Lastly, treatment of $NLRP3^{-/-}$ mice with rIL-18 led to increased inflammation in the cecum (**Figure 2.3.5 G**) and a significant increase in pathology score compared to PBS-treated mice (**Figure 2.3.5 H**). Together, these data suggest that NLRP3 may limit immunity to *Trichuris* by enhancing IL-18 production, which promotes Type 1 cytokine responses and resultant immunopathology associated with *Trichuris* infections.

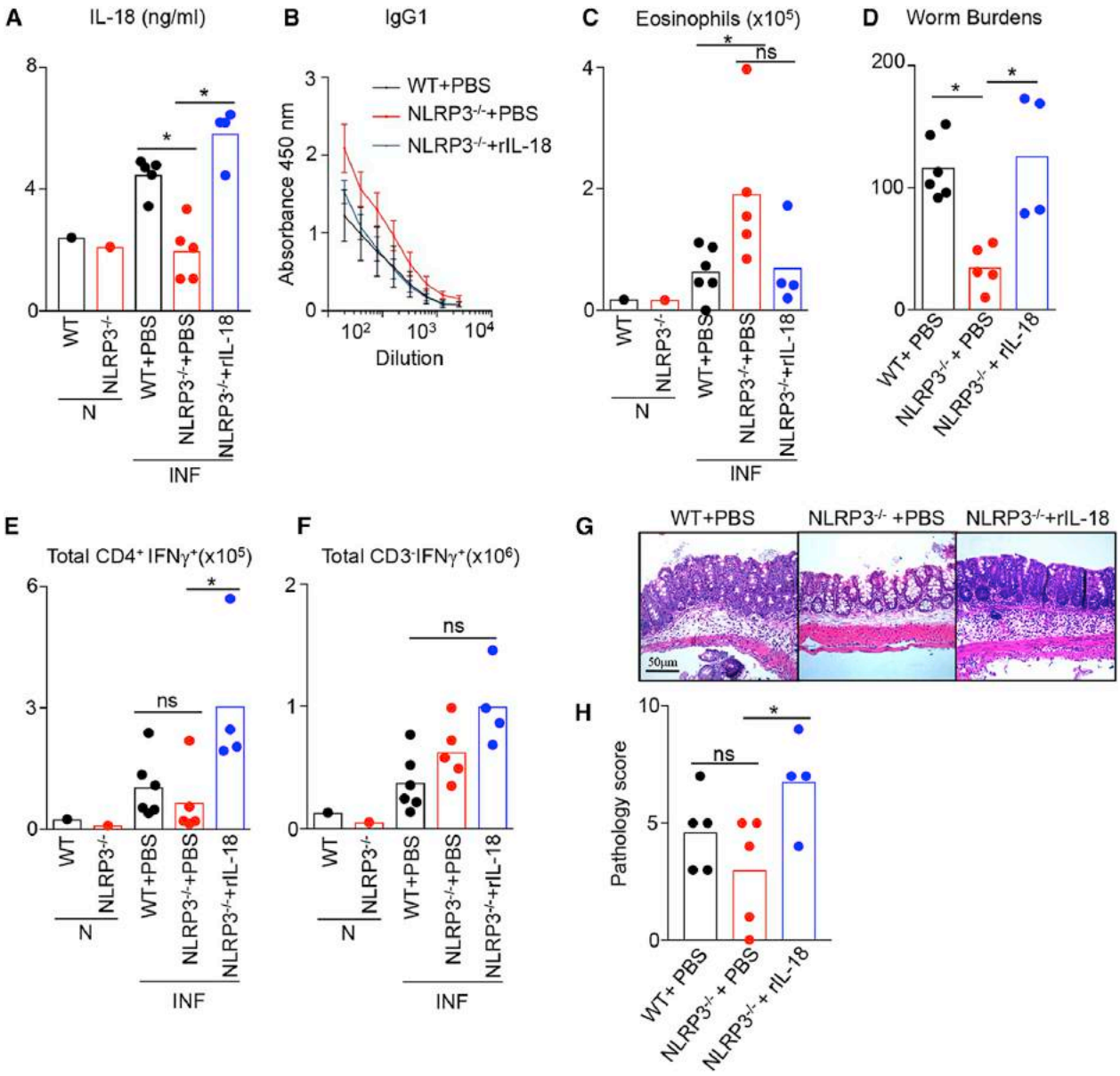


Figure 2.3.5 Exogenous delivery of rIL-18 reverses the enhanced anti-parasitic immunity of NLRP3-deficient mice. NLRP3$^{-/-}$ mice were either daily treated with PBS vehicle or recombinant murine IL-18 (200 ng/mouse i.p.) from days 7–19 post-*Trichuris* infection (INF) and were sacrificed at day 21 post-infection (p.i.). WT control mice received PBS only. (**A**) Serum IL-18 levels. (**B**) *Trichuris* antigen-specific serum IgG1 titers. (**C**) Total mLN eosinophils. N=naive mice. (**D**) Worm burdens. (**E**) Total CD3^{+} CD4^{+} IFN-γ^{+} cells in the mesenteric lymph node (mLN). (**F**) Total mLN CD3^{-} IFN-γ^{+} cells. (**G**) Representative images of H&E stained cecum tissue from infected mice. Scale bar=50µm. (**H**) Blinded scores of cecal pathology. Data are expressed as mean with individual data points or mean±SEM and are representative of 3 experiments, n=4-6 mice/group. *p <0.05, ns denotes not significantly different.

2.3.6 NLRP3 partially requires CD4$^+$ T cells to limit protective immunity to T. muris infection.

Inflammasomes are critical mediators of innate immunity to infections (Pang and Iwasaki, 2011), however they can also influence the development of adaptive immunity via regulation of T cell responses (Ciraci et al. 2012). Further, CD4+ T cells can also express NLRP3 inflammasomes, which can promote Th1 cell differentiation (Arbore et al., 2016). Immunity to *Trichuris* is dependent on the generation of adaptive CD4$^+$ T cell responses, hence to assess whether NLRP3-dependent regulation of immunity to *Trichuris* is dependent on CD4$^+$ T cells, WT or NLRP3$^{-/-}$ mice were treated every 3 days with either 0.5 mg of a neutralizing anti-CD4 mAb or a control rat IgG following *Trichuris* infection. As expected, treatment with anti-CD4 mAb was able to effectively deplete CD3$^+$ CD4$^+$ cells in infected mice (**Figure 2.3.6 A**). While NLRP3$^{-/-}$ mice treated with control Ig exhibited characteristic significant increases in total mLN eosinophils compared to WT mice (**Figure 2.3.6 B**), these responses were significantly diminished when CD4$^+$ cells were ablated. Anti-CD4 mAb treatment caused a significant increase in worm burdens in NLRP3$^{-/-}$ mice at d 21 p.i. compared to control IgG treated mice (**Figure 2.3.6 C**), which corresponded with significantly increased cecal pathology (**Figure 2.3.6 D-F**) and significantly reduced presence of goblet cells (**Figure 2.3.6 E-G**). Together, these data suggest that CD4$^+$ cells are partially required for the ability of NLRP3 to suppress immunity to *Trichuris* infection.

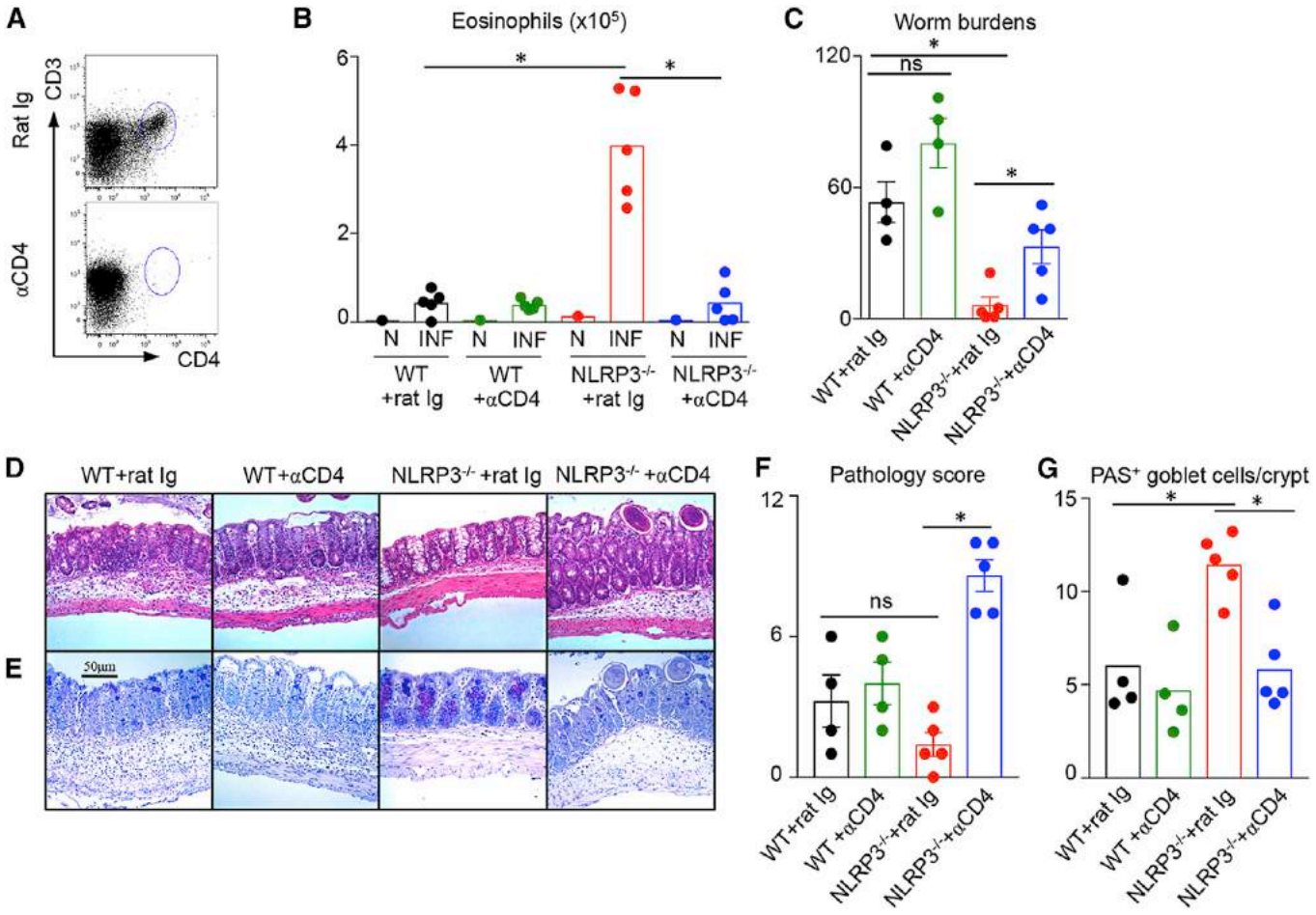


Figure 2.3.6 NLRP3 partially requires CD4+ T cells to limit protective immunity to *T. muris* infection. WT or NLRP3$^{-/-}$ mice were treated every 3 days with either 0.5mg of a neutralizing anti-CD4 mAb or a control rat IgG following *T. muris* infection and were sacrificed at day 21 post-infection (p.i.). (**A**) Representative plots demonstrating successful depletion of CD3^{+} CD4^{+} cells in mesenteric lymph nodes (mLN) of infected mice following anti-CD4 treatment. (**B**) Total mLN eosinophils. (**C**) Worm burdens. (**D**) Representative images of H&E stained cecum tissue. (**E**). Representative images of PAS/Alcian Blue stained cecum tissue. (**F**) Blinded scores of cecal pathology. (**G**) Blinded quantification of goblet cell numbers per cecal crypt unit. Scale bar=50μm. Data are expressed as mean with individual data points and are representative of 2 experiments, n=4-5 mice/group. *p <0.05, ns denotes not significantly different.

2.3.7 NLRP3 can regulate immunity to Trichuris independent of the adaptive immune system.

To further define the cellular mechanisms of NLRP3-dependent regulation of immunity to infection, we aimed to assess whether NLRP3 could influence innate immunity to *Trichuris*. C57BL/6 Rag1$^{-/-}$ mice (which do not possess a functional adaptive immune system) were infected with *Trichuris* treated daily with either PBS or 20 mg/kg/day i.p. of MCC950. C57BL/6 WT mice were included as a control for assessment of worm burdens. Critically, MCC950 treatment was able to significantly reduce worm burdens in Rag1$^{-/-}$ mice at day 21 p.i. compared to PBS treated mice (**Figure 2.3.7 A**), which corresponded with significantly increased eosinophil numbers in the mLN (**Figure 2.3.7 B**). Analysis of cytokine responses in the mLN revealed that while Rag1$^{-/-}$ mice treated with PBS displayed increased IFN-γ responses within cells that express CD90 following infection, mice that received the MCC950 had a reduced IFN-γ response in these cells (**Figure 2.3.7 C**). Further analysis of this cell population demonstrated that IFN-γ-expressing CD90^{+} cells following *Trichuris* infection co-expressed the innate lymphoid cell (ILC) markers Sca-1 and CD127 as well as T-bet (**Figure 2.3.7 D**), a phenotype consistent with ILC Type 1 cells (ILC1). Histological analyses demonstrated that while MCC950 treatment did not result in significant reductions in immunopathology in the cecum (**Figure 2.3.7 E-G**), there were significant increases in goblet cell responses (**Figure 2.3.7 F-H**). These data suggest that the NLRP3 inflammasome can influence innate immunity to *Trichuris* infection, potentially acting via regulation of the function of innate cells such as ILC1s and when taken into consideration with results that CD4^{+} T cells are also important for NLRP3-dependent immune regulation (**Figure 2.3.6**), implies that the NLRP3 inflammasome can influence elements of both the innate and adaptive immune system to limit protective immunity to helminths.

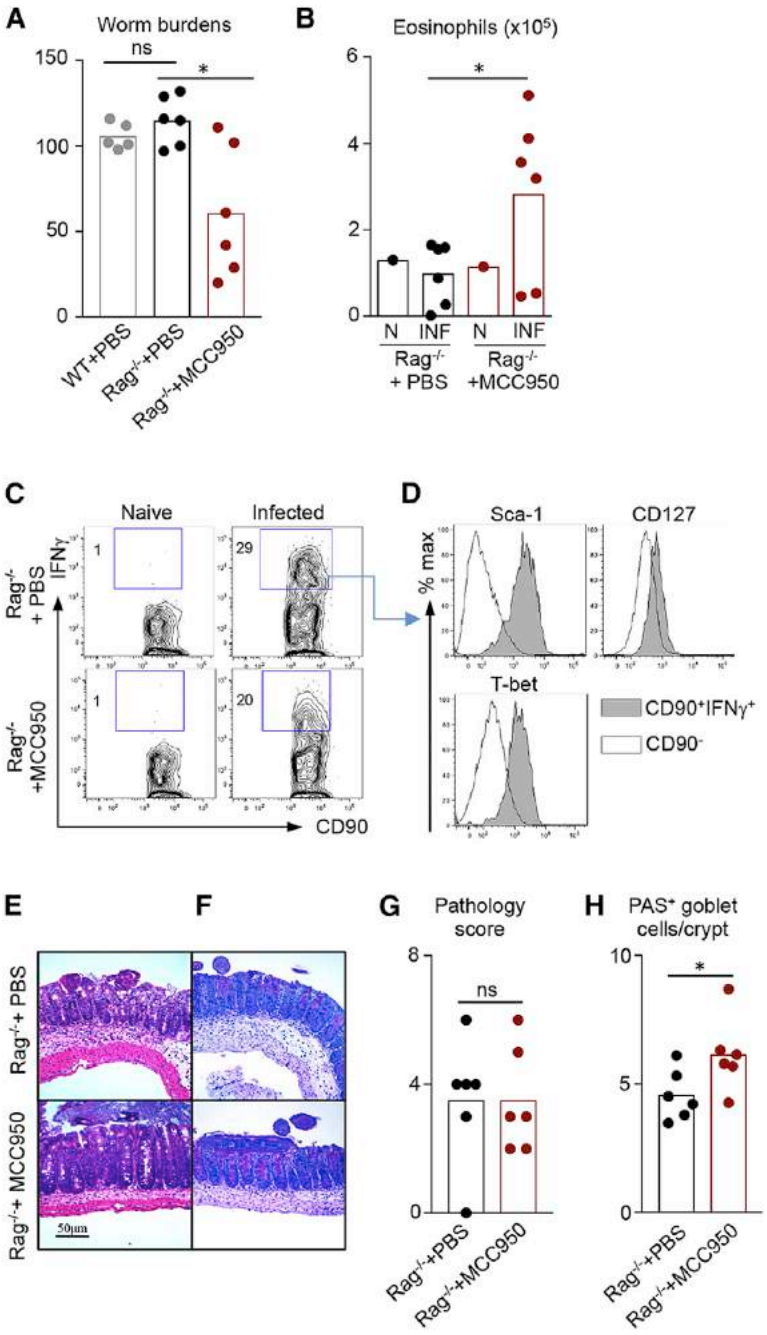
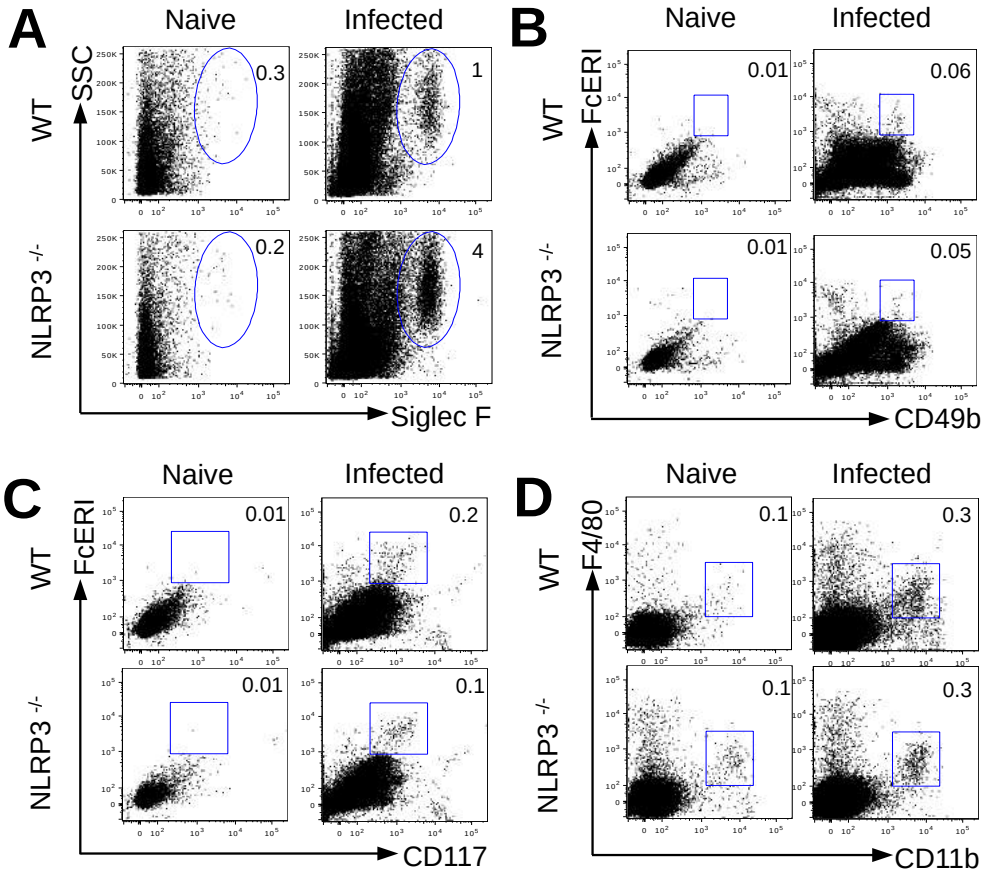
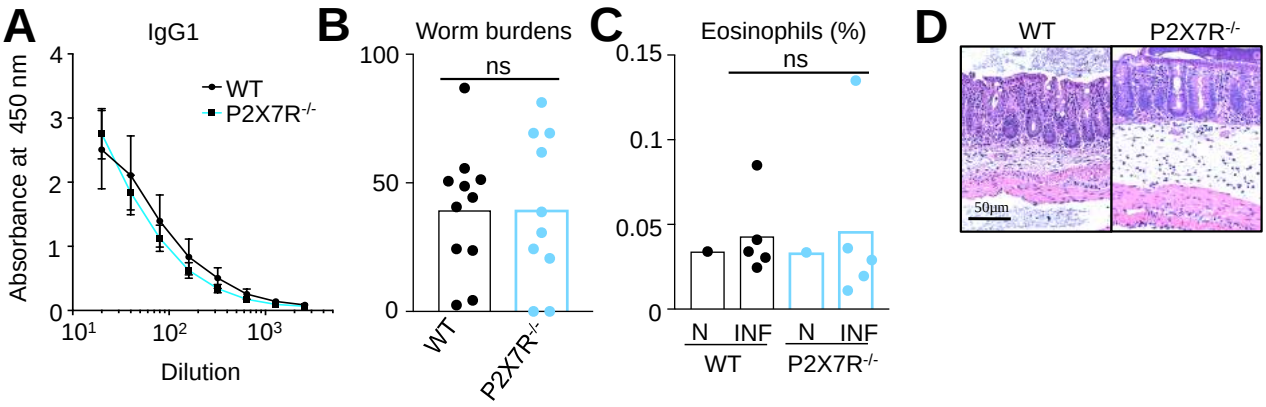


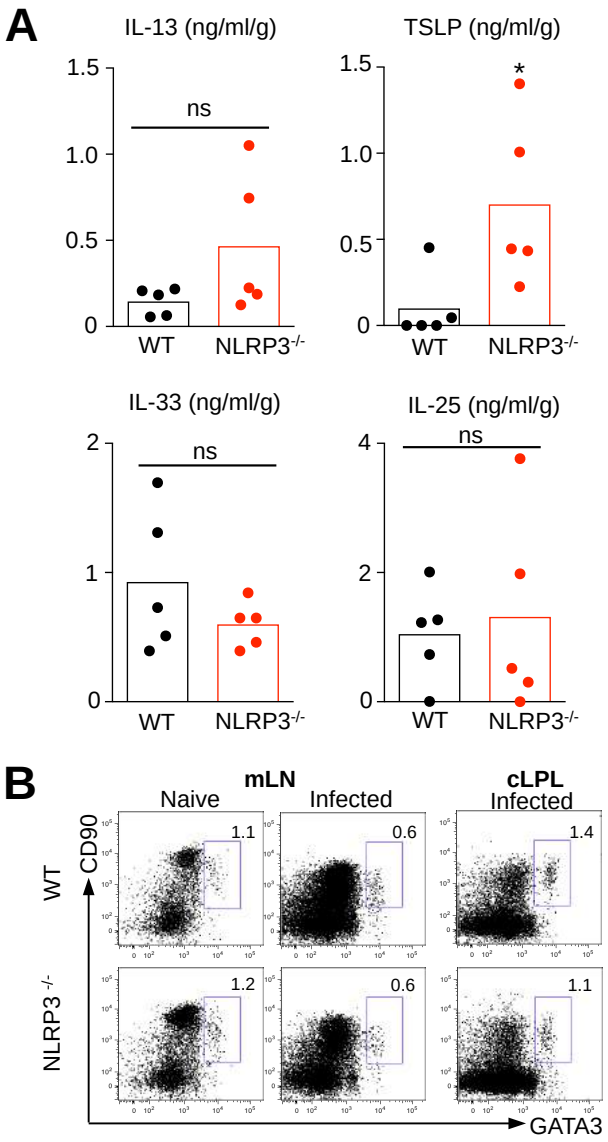
Figure 2.3.7. NLRP3 can regulate immunity to *T. muris* independent of the adaptive immune system. C57BL/6 Rag1$^{-/-}$ mice were treated daily with either PBS or 20 mg/kg/day i.p. of the NLRP3 inhibitor, MCC950 following *Trichuris* infection and were sacrificed at day 21 post-infection (p.i.).. C57BL/6 WT mice received PBS only and were only used as a control for worm burden. (**A**) Worm burdens. (**B**) Total mLN eosinophils. (**C**) Frequencies of CD90^{+} IFN-γ^{+} cells in the mLN. (**D**) IFN-γ-expressing CD90^{+} cells (shaded histograms) were gated and analysed for co-expression of innate lymphoid cell markers Sca-1, CD127 and T-bet. Clear histograms represent gated CD90^{-} cells from the same sample. (**E**) Representative images of H&E stained cecum tissue. (**F**) Representative images of PAS/Alcian Blue stained cecum tissue. (**G**) Blinded scores of cecal pathology. (**H**) Blinded quantification of goblet cell numbers per cecal crypt unit. Scale bar=50μm. Data are expressed as mean with individual data points and are representative of 3 experiments, n=5-6 mice/group. *p<0.05 compared to NLRP3$^{-/-}$+rat Ig, ns denotes not significantly different.



Supplementary figure 2.3.1 NLRP3$^{-/-}$ mice do not display elevated frequencies of basophils, mast cells or macrophages in the intestine following *T. muris* infection. Related to Figure 2-3.3.3. C57BL/6 WT and NLRP3$^{-/-}$ mice were infected with *T. muris* and sacrificed at d 21 p.i. Frequencies of (**A**) Siglec F^{+} eosinophils, (**B**) CD3^{-} CD19^{-} CD49b^{+} FcERI^{+} basophils, (**C**) CD3^{-} CD19^{-} CD117^{+} FcERI^{+} mast cells and (**D**) CD3^{-} CD19^{-} CD11b^{+} F4/80^{+} macrophages in cecal patch tissue. Data are expressed as mean cell frequency and are representative of 2 experiments, n=1 naive or pooled tissue samples from 5 infected mice/group



Supplementary figure 2.3.2 P2X$_7$R-deficient mice do not exhibit enhanced anti-parasitic immunity to *T. muris*. Related to Figure 2-3.3. C57BL/6 WT and P2X7R$^{-/-}$ mice were infected with *T. muris* and sacrificed at d 21 p.i. (**A**) *Trichuris* antigen-specific serum IgG1 titers. (**B**) Cecal worm burdens, pooled from 2 experiments. (**C**) Frequencies of mLN eosinophils. (**D**) Representative images of H&E stained cecum tissue, scale = 50µm. Data are expressed as mean and individual data points or mean±SEM and are representative of 4 experiments, n=4-5 mice/group.



Supplementary figure 2.3.3. NLRP3-/- exhibit elevated intestinal TSLP expression but no increases in IL-33, IL-25 or ILC2 responses following *Trichuris* infection. Related to Figure 2-3.3. C57BL/6 WT and NLRP3$^{-/-}$ mice were infected with *T. muris* and sacrificed at d 21 p.i. (**A**) Levels of IL-13, TSLP, IL-33 and IL-25 protein in colon tissue homogenates, measured by ELISA and normalized to weight of tissue in grams (g), expressed as mean and individual data points (n=5). (**B**) Representative plots demonstrating frequencies of CD3^{-} CD19^{-} CD90^{+} GATA3^{+} cells in the mesenteric lymph node (mLN) or pooled colonic lamina propria (cLPL) of naive or infected mice. Data are expressed as mean cell frequency and are representative of two experiments (n=1 naive or pooled tissue samples from 5 infected mice/group).

2.4 Discussion

Parasitic helminth infections are among the most common and debilitating causes of chronic disease in humans, infecting over a billion people worldwide. However, there are no vaccines that can protect humans against infection, owing in part to the complexity of helminth life cycles, the immune-suppressive capacity of helminth antigens and our relative lack of understanding of how protective Type 2 immune responses are initiated and regulated. Inflammasomes are critical regulators of immunity to pathogens, yet their roles in immunity to parasitic helminths have been largely unexplored. The present study has identified a role for the NLRP3 inflammasome in limiting both innate and adaptive immunity of mice to a gastrointestinal helminth, via regulation of IL-18 expression. We demonstrate that chronic helminth infections of humans are similarly associated with elevated IL-18 expression. Critically, targeting this pathway therapeutically with a chemical inhibitor was able to accelerate Type 2 immune-mediated clearance of the parasite, shedding light on how innate immune receptors control immunity to helminths and potentially identifying a strategy for limiting the disease burden in helminth-infected animal or human populations.

NLRP3 has been implicated in promoting Type 1 immune-mediated protection against pathogens and detrimental inflammation in some disease settings (Silva et al., 2013; Thomas et al., 2009). In addition, recent studies have highlighted that NLRP3 can have important roles in promoting Type 2 immune responses (Bruchard et al., 2015; Gurung et al., 2015). Our findings herein are consistent with a role for NLRP3 in promoting non-protective Type 1 immune responses to *Trichuris*, potentially by eliciting IL-18 secretion, a cytokine also known as "IFN-γ inducing factor" (Micallef et al., 1996). IL-18 is a pleiotropic cytokine that can promote either a Type 1 or Type 2 response, depending on the nature of the cytokine milieu, infectious stimuli and genetic background of the host (Xu et al., 2000). For instance, IL-18 can induce Type 1

immune responses and resistance to one species of the *Leishmania* parasite (Li et al., 2004), but induces non-protective Type 2 responses to another species (Bryson et al., 2008). Previous studies have demonstrated that IL-18 is a critical regulator of immunity to *Trichuris,* with differences in the relative role depending on the inflammatory context. While one report highlights how IL-18 promotes Type 2 responses when IFN-γ is absent (Liu et al., 2006), another report implicates IL-18 in suppressing Type 2 cytokine responses to *Trichuris* (Helmby et al., 2001), which is more consistent with our findings in NLRP3$^{-/-}$ mice. Hence it is likely that in the context of the present study, lowered IL-18 expression in the absence of NLRP3 may contribute to the elevated Type 2 responses and diminished Type 1 responses following *Trichuris* infection. However, it remains unclear whether elevations in Type 2 immunity were a consequence, or a cause, of reductions in Th1 cell or ILC1 responses observed in NLRP3$^{-/-}$ mice (i.e cross-regulation of Type 1 vs. Type 2 responses), or whether this was a direct effect of IL-18 or other factors.

Our data are supportive for a central role for NLRP3-dependent IL-18 in regulating immunity and inflammation following *Trichuris* infection but we have also demonstrated that IL-1β levels are similarly regulated by NLRP3, MCC950 and Caspase-1/11, raising the question as to the role for IL-1β in this system. While IL-1β can have a role in suppressing protective immunity to other helminth species (Zaiss et al., 2013), this contrasts with what is observed during *Trichuris* infections, where IL-1/IL-1R interactions promote Type 2 immunity (Helmby and Grencis, 2004; Humphreys and Grencis, 2009). IL-1β also has an emerging role in stimulating ILC2 responses (Ohne et al., 2016) suggesting that, at least in the context of *Trichuris* infection, defective IL-1β production in NLRP3$^{-/-}$ mice would be unlikely to be responsible for the increased Type 2 immune responses we observed in the present study.

The NLRP3 inflammasome can be activated by a wide variety of stimuli, ranging from pathogen-associated molecular patterns, danger signals (ATP, ADP, DNA), toxins and crystals (Schroder and Tschopp, 2010). We found that *Trichuris* adult worms release factors that can support NLRP3-dependent IL-18 and IL-1β secretion *in vivo* and in cultured macrophages. Interestingly, worm secreted factors do not appear to induce NLRP3-dependent IL-1β and IL-18 secretion alone, since signals from known inflammasome activators (e.g. LPS and nigericin) were also required. It is therefore unclear whether worm-derived molecules are operating as a "first signal" to promote pro-IL-1β/pro-IL-18 generation, or as a "second signal" to activate the NLRP3 inflammasome and promote cytokine maturation and release, or both. We also cannot definitively say that *Trichuris* and its secretions definitively elicit *in vivo* IL-18 secretion, since it remains possible that IL-18 secretion is diminished in the absence of NLRP3 due to an inability to respond to the microbiota. Regardless, our results are consistent with the premise that worm-derived factors may be able to augment NLRP3 activation, provided that signals from commensal microbes are also present.

While our study has not pinpointed the precise worm-derived molecule(s) responsible for enhancing NLRP3-dependent IL-1β and IL-18 secretion, as has been demonstrated for schistosomes (Riteau et al., 2010), we found that purified exosome-like extracellular vesicles released from the worm can mediate these effects. Helminth-derived exosomes have been recently implicated in immune modulation (Buck et al., 2014) and pathogenesis (Chaiyadet et al., 2015) by parasitic helminths. *T. muris* exosomes contain hundreds of proteins, micro-RNAs and mRNAs that are postulated to mediate host-pathogen interactions (Eichenberger et al., 2018), and the present study suggests that some of these factors may regulate activation of inflammasomes by host cells such as macrophages. Given that activation of this pathway is associated with greater parasite persistence in the intestine, it is possible that parasites such as

Trichuris may have evolved strategies to selectively target inflammasomes to regulate localized inflammation and enhance their survival within a host (Hewitson et al., 2009). In addition to worm-secreted factors and signals from commensal microbes, there are likely other mechanisms by which a worm infection could elicit inflammasome activation. For instance, helminth infections can induce substantial tissue damage during colonization and feeding that can release danger signals such as ATP, which is a well-described activator of the NLRP3 inflammasome (Riteau et al., 2010). However, our finding that the ATP-$P2X_7R$ pathway does not appear to control *Trichuris* infections in the same way as NLRP3 suggests that other activation pathways are likely to play more substantial roles. Other danger signals such as ADP can also activate inflammasomes (Baron et al., 2015) and can promote immunity to helminths by interacting with A2B receptors (Patel et al., 2014).

While inflammasomes are critical regulators of early innate responses to infection, by permitting rapid maturation and release of inflammatory cytokines, there is substantial evidence that inflammasomes are involved in shaping adaptive immunity (Ciraci et al., 2012). Our results are consistent with multiple roles for the NLRP3 inflammasome in controlling immunity to gastrointestinal helminths. $NLRP3^{-/-}$ mice display enhanced parasite antigen-specific humoral and Type 2 cytokine responses and the presence of $CD4^+$ T cells is important for the optimal ability of NLRP3 to suppress protective immunity to *Trichuris,* demonstrating that NLRP3 regulates adaptive immunity. Our results could not distinguish a potential role for CD4+ T cell-intrinsic NLRP3 activation in regulating immunity to *Trichuris*, as has been shown in other models of infection and inflammation (Arbore et al., 2016), hence it remains possible that CD4 cells could either be an upstream mediator (inflammasome activation) or downstream mediator (Th2-driven inflammation) of immunity. However, the fact that NLRP3 could suppress immunity to *Trichuris* in the absence of functional T cells and B cells (in $Rag1^{-/-}$ mice)

highlights the importance of NLRP3 in innate immunity. The precise mechanisms by which NLRP3 suppresses the innate response to *Trichuris* was not defined, but the selective elevation in eosinophil numbers in lymph nodes and intestinal tissues, increased goblet cell responses and the modulation of ILC1 function that arose following NLRP3 inhibition suggests that there may be multiple mechanisms involved. Previous reports have shown that NLRP3-dependent IL-1β negatively regulates immunity to different helminth species by suppressing IL-25 and IL-33 (Zaiss et al., 2013), factors that are known to promote ILC2 responses. However, none of these factors were elevated in NLRP3$^{-/-}$ mice in the present study, suggesting that other mechanisms may promote Type 2 immunity in the context of *Trichuris* infection. Interestingly, TSLP expression was elevated in infected NLRP3$^{-/-}$ mice, and TSLP is strongly associated with Type 2 immune responses and protective immunity to helminths, including *Trichuris* (Taylor et al., 2009), *v*ia associations with a range of accessory cells including dendritic cells, basophils, ILCs and mast cells. It is also possible that the absence of NLRP3-dependent cell death or pyroptosis in NLRP3-deficient mice may result in increased survival of innate immune cells (McIntire et al., 2009), which could provide increased immunity to parasitic helminth infection. Further, NLRP3 inhibition may influence the functions of non-hematopoietic cells in the intestine that promote worm expulsion, including epithelial cell turnover, goblet cell expansion and mucus production and smooth muscle hyper-contractility.

Our observations that humans infected with a gastrointestinal nematode exhibit elevated serum IL-18 levels are consistent with activation of inflammasomes. Hence, it is conceivable that targeting the NLRP3 pathway in helminth-infected humans using MCC950 may be a rational approach for boosting Type 2 immunity to helminths, lowering worm burdens and limiting the health problems these infections cause in the developing world. However, people living in helminth endemic regions are often exposed to co-infections with bacterial, viral and parasitic

pathogens such as tuberculosis, HIV and malaria and previous studies have demonstrated a protective role for NLRP3 in these infection models (Dorhoi et al., 2012b; Shio et al., 2009). Hence, caution would be warranted to prevent such therapeutic strategies from having unwanted effects of reduced ability to fight other infections. Nevertheless, administering therapies that enhance a patient's natural ability to mount protective Type 2 immune responses would have benefits over the use of anthelmintic drugs alone, for example NLRP3 inhibition could promote the development of more robust parasite-specific memory Th2 cells and IgG1 responses that could protect an individual from reinfection. In conclusion, we have provided information on how the NLRP3 inflammasome regulates immunity and inflammation in helminth infections. Further work is necessary to define the role of this pathway in immunity to other helminth species and the roles for other inflammasomes (e.g. NLRP1, AIM2, NLRP6, NLRC4) in regulation of Type 2 immune responses.

2.5 Materials and methods

2.5.1 Mice and treatments

Caspase-1/11 double-deficient, NLRP3-deficient, $P2X_7R$-deficient and Rag-1-deficient mice, all on C57BL/6 genetic background, and C57BL/6 wild-type mice were bred and maintained at James Cook University (JCU) Cairns Campus. Male and female mice between 6 and 10 weeks of age were used. Experimental protocols were approved by the JCU Animal Ethics Committee (approval A2213). Mice were injected intraperitoneally (i.p) with 50 µg of *Trichuris muris* adult worm excretory/secretory (ES) antigens in PBS. Mice were i.p treated with 20 mg/kg/day of MCC950 (Coll et al., 2015) or PBS vehicle, daily starting on day 0 of *Trichuris* infection. Mice were treated with neutralizing antibodies against CD4 (GK1.5, Bioxcell, 0.5 mg/mouse) starting on day 0 and continuing every 3 days. Recombinant murine IL-18 (R&D systems) or

PBS vehicle were injected i.p. at a dose of 200 ng/mouse daily from day 7-19 post-*Trichuris* infection.

2.5.2 Human ethics and experimental procedures

The design and clinical results of the placebo-controlled clinical trial using *N. americanus* to treat celiac disease have been described elsewhere (Daveson et al., 2011). The trial was registered at ClinicalTrials.gov as NCT00671138. Briefly, otherwise healthy people with HLA-$DQ2^{+}$ celiac disease on a gluten-free diet were recruited, randomised into two groups and either infected percutaneously with 15 infective larvae of *N. americanus* or given placebo treatment with topical chili pepper solution. Blood was collected by venepuncture and serum was collected and stored at -80°C.

2.5.3 Parasitological techniques

T. muris was maintained in genetically susceptible mouse strains and *T. muris* ES antigens were obtained as described previously (Artis et al., 1999). Mice were infected by oral gavage with approximately 200 embryonated *T. muris* eggs. *Trichuris*-specific serum IgG1 titers were assayed by ELISA on plates with coated with *T. muris* antigen (5 µg/ml). Maintenance of the lifecycle of *Necator americanus* and experimental infections of human subjects were performed as described previously (Daveson et al., 2011).

2.5.4 Bone marrow macrophage assays

Bone marrow was isolated from hind legs of C57BL/6 or $NLRP3^{-/-}$ mice by flushing bones with DMEM and cells were cultured at 1×10^{6} cells/ml in DMEM supplemented with 10% FBS, 5% horse serum (Invitrogen), 2 mM L-glutamine, 10 mM HEPES, 1mM sodium pyruvate and 10 ng/ml M-CSF. On day 6, macrophages were harvested and plated overnight at a concentration

of 2×10^5 cells/ml. The following day, cells were treated with media alone, or LPS (1 µg/ml) for 4 h, 20 µM nigericin for 2 h, 25 µg/ml of *Trichuris* adult worm ES products or 25 µg/ml worm exosomes for 6 h, and cell-free supernatants were stored at -80°C.

2.5.5 Mouse tissue collection, processing and pathology scoring

Blood was collected by cardiac puncture for terminal procedures, or submandibular bleeding for non-terminal procedures, and serum was stored at -80°C. Single cell suspensions of mLN or cecal patch tissue were prepared by passing through 70 µm nylon mesh filters. Colonic lamina propria lymphocytes (LPL) were isolated as described previously (Zaph et al., 2007). For colon tissue homogenates, 1 cm of tissue was homogenized mechanically in 0.5 mL of PBS using a Tissue Lyzer (QIAGEN) and supernatants stored at -80°C. Cecal tissue was fixed in 4% paraformaldehyde and embedded in paraffin, and 5 µM sections were stained with H&E or PAS/Alcian Blue stains. Scoring of pathology was performed from H&E-stained slides by an individual blinded to the experimental group. Tissue sections were scored on a scale of 0-5 for the following parameters; (1) epithelial pathology (crypt elongation, hyperplasia, erosion), (2) mural inflammation and (3) oedema for a maximal score of 15. Goblet cell responses were assessed by counting PAS-positive cell number per crypt unit, with a minimum of 20 crypt units assessed for each tissue sample. For determination of worm burdens, ceca were collected and worms were counted using a dissecting microscope.

2.5.6 Statistical analyses

Statistical analyses for murine *in vitro* and *in vivo* studies were performed using unpaired Mann-Whitney U tests. Human cytokine data were compared using paired Mann-Whitney U tests. *In vivo* mouse experiments included n=1-3 naïve control mice and a minimum of n=4-6 infected mice and were repeated at least twice to demonstrate reproducibility. Results are expressed as

mean ± SEM or as individual data points and the differences were considered significant at a *p* value of <0.05.

2.5.7 Supplementary Experimental Procedures

2.5.7.1 Exosome-like extracellular vesicle purification

Exosome-like vesicles were purified by differential centrifugation using a protocol based on ultracentrifugation coupled to membrane filtration to eliminate large contaminating extracellular vesicles (Mathivanan et al., 2010). Following culture of *Trichuris* adult worms, the culture media was collected and centrifuged at different speeds (500 *g*, 2,000 *g* and 4,000 *g* for 30 min each) to remove larger debris, and the resulting supernatant was centrifuged at 15,000 *g* for 45 min at 4°C to remove larger microvesicles. Supernatants were filtered through a 0.2 μm ultrafiltration membrane and centrifuged at 120,000 *g* for 3 h at 4°C in an ultracentrifuge. The pellet was washed in PBS, ultracentrifuged again and the final pellet containing the exosome-like vesicles was resuspended in 250 μl of PBS and kept at -80°C until use.

2.5.7.2 Cell culture and ELISA

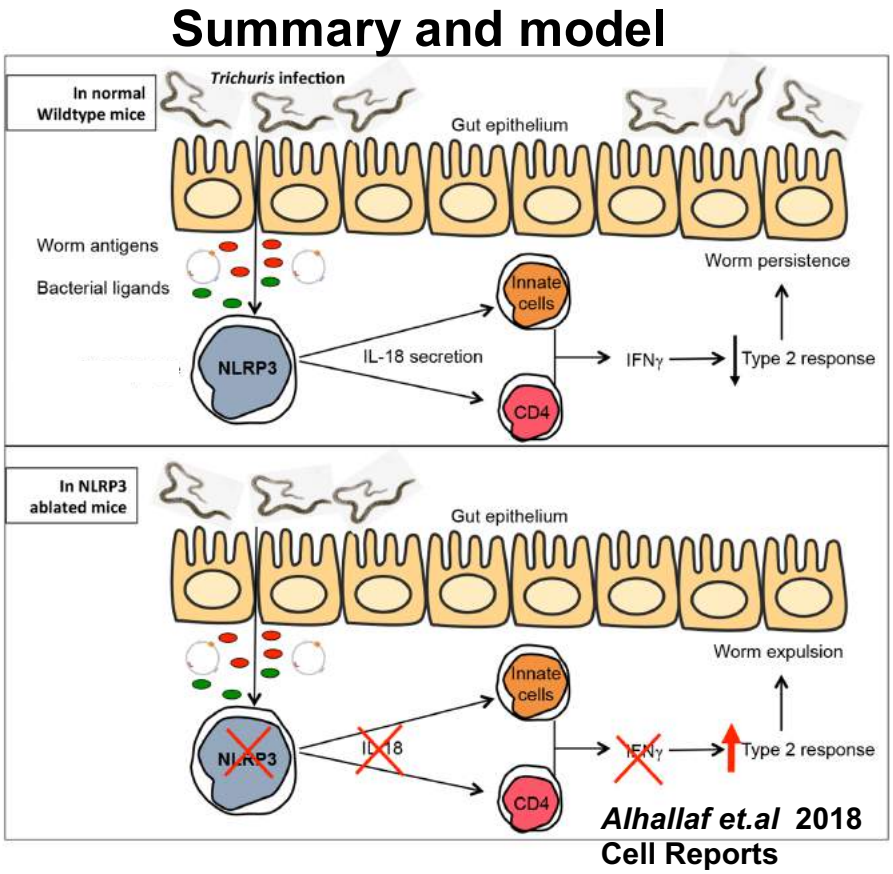
Single cell suspensions of mLN were plated at 5×10^6 cells/ml in complete medium (RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 50 μM 2-ME) and stimulated for 48 h with 50 μg/mL *Trichuris* Ag. Supernatants or sera were assayed for IL-4, IL-13, IL-18, IL-1β, IL-25, IL-33, TSLP and IFN-γ using standard sandwich ELISA protocols (eBioscience or ELISAkit.com.au). For colon tissue homogenate analyses, cytokine levels were normalised to weight of colon tissue.

2.5.7.3 Flow cytometry

Cell preparations were surface-stained with anti-mouse fluorochrome-conjugated mAbs against CD3(145-2C11), CD4 (RM4-5), CD11b (M1/70), CD90.2 (53-2.1), CD117 (2B8), CD127 (SB/199), FcεRI (MAR-1), F4/80 (T45-2342), Sca-1 (D7) and Siglec F (E50-2440). For staining with intracellular molecules, cells were stimulated for 4 h with 50 ng/mL PMA and 500 ng/mL ionomycin in the presence of 10 μg/mL brefeldin A (Sigma Aldrich). Cells were first stained with antibodies to cell surface antigens and then with fluorochrome-labeled anti-IFN-γ, anti-GATA3 and anti-T-bet Abs following fixation and permeabilization using a commercial kit (eBioscience). Cells were analysed by flow cytometry using a FACS Canto II or Aria III cytometer (BD Biosciences) and further analysis was performed using FlowJo software (Tree Star, Inc).

Linker to chapter 3

In the previous chapters 2 we aimed to determine how a gastrointestinal helminth (*T. muris*) activates the NLRP3 inflammasome and the role of NLRP3 in regulating immunity and inflammation to infection. We showed that *T. muris* and its excretory antigens promote NLRP3-dependent IL-18 secretion *in vitro* and *in vivo* and IL-18 is a critical factor in suppressing innate and adaptive immunity to the parasite (See summary model figure below).



However, there are still significant unknowns about how NLRP3 controls immunity to helminth parasites. First, it is unclear whether the same IL-18-dependent mechanisms operate during infections with other species of gastrointestinal helminth, or whether NLRP3 can control immunity via distinct mechanisms. A second key question is whether NLRP3 has a role in rapid, innate immunity to infections.

To address these key research questions, the aim of the next Chapter is to investigate the role of the NLRP3 inflammasome in regulating early immune responses to a different species of gastrointestinal helminth, the murine model of hookworm, *N. brasiliensis.* Immunity to this parasite is critically dependent on the innate immune response that occurs in the first few days of infection, in a variety of different tissues (skin, lung and small intestine). Hence it is a good opportunity to examine the role of NLRP3 in regulating innate immunity at a variety of tissue sites.

CHAPTER 3

3. NLRP3 suppresses neutrophil-dependent lung-stage immunity to hookworm infection

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3.1 Abstract

Inflammasomes have a key role in promoting Type 1-mediated immunity to infections with viruses, bacteria, fungi and protozoan parasites, by regulating the function of pro-inflammatory IL-1-family cytokines such as IL-18 and IL-1β. However, recent studies have also highlighted an important role for inflammasomes in promoting or inhibiting Type 2 immune responses, such as those seen in allergic disease or helminth infections. We recently identified an important role for the NLRP3 inflammasome in suppressing protective Type 2 immunity of mice to gastrointestinal whipworm infection via regulation of IL-18 secretion. However, the role of NLRP3 in other models of helminth infection and the mechanisms by which NLRP3 control anti-parasitic immunity remain incompletely defined. In the present study we demonstrate that infection with a rodent model of hookworm, *Nippostrongylus brasiliensis,* results in NLRP3-dependent increases in IL-18 and IL-1β secretion in the lung and intestine, consistent with inflammasome activation in these tissues. NLRP3 deficient mice displayed elevated protective Type 2 immune responses compared to wild type mice, including elevated IL-4, *Retnla* and *Arg1* expression in the lung that was associated with reduced lung larval burdens as early as 2 day post-infection. Examination of lung cellular infiltrates revealed a significant increase in neutrophil recruitment to the lung in NLRP3$^{-/-}$ mice and co-culture of sort-purified neutrophils with *N. brasiliensis* larvae resulted in killing of the parasite, potentially representing a mechanism of how neutrophils may provide enhanced innate protection against primary infection. Our findings suggest that NLRP3 may control the early innate immune response to helminth infection, suggesting that targeting NLRP3 to promote anti-parasitic neutrophil responses may provide enhanced protection against infections with these pathogens.

3.2 Introduction

The innate immune system defends against internal and external pathogens and often involves detection of the invader by pattern-recognition receptors on innate immune cells such as monocytes, macrophages, neutrophils, and dendritic cells (DCs) (Fullard and O'Reilly, 2015; Schroder and Tschopp, 2010). These cells can either kill the pathogen directly, or play a crucial role in directing an adaptive immune response that provides long-lasting protection (Iwasaki and Medzhitov, 2015). Many of these innate immune responses are stimulated by the secretion of members of the IL-1 family of cytokines such as interleukin-1β (IL-1β) and interleukin-18 (IL-18) (Netea et al., 2015). Secretion of these cytokines is tightly controlled by inflammasomes, which are multi-protein immune complexes that control the cleavage and subsequent activation of immature versions of these cytokines by the intracellular cysteine protease Caspase-1 (Netea et al., 2015). Numerous inflammasomes have been studied, such as nucleotide-binding and oligomerization domain-like receptor pyrin domain containing protein-1 (NLRP1), NLRP6, NLRP3 and double-stranded DNA (dsDNA) sensors absent in melanoma 2 (AIM2) and NLRC4 (Ozaki et al., 2015). The most well-characterised inflammasome is the NLRP3 inflammasome, which is expressed by multiple cell lineages (Eigenbrod and Dalpke, 2015; Guarda et al., 2011; Shao et al., 2015; Zhong et al., 2013), and is critical for defense against small, easily phagocytosed infectious pathogens such as bacteria, viruses, protozoa and fungi by enhancing Type-1 immune responses via secretion of IL-1β and IL-18 (Allen et al., 2009; Broz et al., 2010a; Dostert et al., 2009; Ermler et al., 2014; Gorfu et al., 2014; Kamada et al., 2014; Maher et al., 2013; McIntire et al., 2009; Muruve et al., 2008; Song-Zhao et al., 2014; Strowig et al., 2012; Thomas et al., 2009).

In addition to influencing Type-1-mediated immunity to pathogens, NLRP3 plays an important role in regulating Type-2 immune responses to infections with large metazoan pathogens such as parasitic helminths. For instance, it has been demonstrated that cattle with deletion mutations

in the NLRP3 gene display increased anti-parasitic resistance, highlighting the potentially critical role for NLRP3 in influencing immunity to helminths (Xu et al., 2014). Consistent with this, murine studies have shown that NLRP3 suppresses Type 2 immune responses to infection with *schistosomes* (Ritter et al., 2010) and may also suppress protective immunity to gastrointestinal helminths by promoting IL-1β responses (Zaiss et al., 2013). Despite this supporting evidence, the precise roles of NLRP3 inflammasomes in immunity to diverse species parasitic helminths, and the immunological mechanisms of how they influence innate and/or adaptive immunity remain incompletely defined. We recently showed that decreased IL-18 expression in the absence of NLRP3 may contribute to the amplified Type-2 responses and diminished Type-1 responses following *T.muris* infection (Alhallaf et al., 2018). While $CD4^+$ cells were partially required for the ability of NLRP3 to inhibit immunity to *T. muris* infection, NLRP3 could also inhibit immunity to *T. muris* in the absence of adaptive immune cells, highlighting the potential importance of NLRP3 in regulating anti-parasitic innate immunity (Alhallaf et al., 2018). However, the role of NLRP3 in regulating innate immune responses against helminths remains incompletely defined and may depend on the type of helminth, tissue site of infection and the time point of assessment.

In the present study, we used a murine model of hookworm infection (*Nippostrongylus. brasiliensis*) to assess the role for NLRP3 in regulating the early innate immune responses to infection. *N. brasiliensis* is a tissue-invasive helminth that enters the body through the skin and then migrates to the lung via the bloodstream over the course of 1-2 days. The parasite then traverses the trachea and oesophagus to reach the small intestine where it becomes fully mature over the course of 6-7 days. We found that *N. brasiliensis* infection resulted in NLRP3-dependent elevations in levels of IL-1β and IL-18 in the lung and small intestine, consistent with inflammasome activation in both tissue sites. NLRP3-deficient mice exhibited increased

Type 2 immune responses and reduced parasite burdens in the lung as early as day 2 post-infection, which correlated with increased neutrophil recruitment to the lung, highlighting a potential anti-parasitic role for neutrophils in the absence of NLRP3. Co-culture of sort-purified neutrophils with *N. brasiliensis* larvae resulted in potent killing of the parasite *in vitro*. Our findings suggest that NLRP3 may suppress early neutrophil responses in the lung following *N. brasiliensis* infection, thus targeting NLRP3 may be a novel approach for enhancing innate immunity to hookworm-like parasites.

3.3 Results

3.3.1 NLRP3 is critical for in vivo inflammasome activation following N. brasiliensis infection.

Multiple species of helminth and the molecules they excrete or secrete can activate the NLRP3 inflammasomes *in vivo* and *in vitro* (Alhallaf et al., 2018; Ritter et al., 2010; Zaiss et al., 2013). Hence we first aimed to assess whether *N. brasiliensis* infection can similarly mediate NLRP3-dependent inflammasome activation. C57BL/6 WT or NLRP3$^{-/-}$mice were infected with 500 *N. brasiliensis* L3 and IL-1β and IL-18 levels were measured in the small intestine at day 6. While *N. brasiliensis* infection in WT mice caused significant increases in IL-1β and IL-18 levels in the gut, consistent with inflammasome activation, NLRP3$^{-/-}$ mice displayed no significant infection-induced response (**Figure 3.3.1 A-B**). These data suggest that *N. brasiliensis* infection is associated with NLRP3 activation in the intestine, which consistent with other models of gastrointestinal helminth infection (Alhallaf et al., 2018).

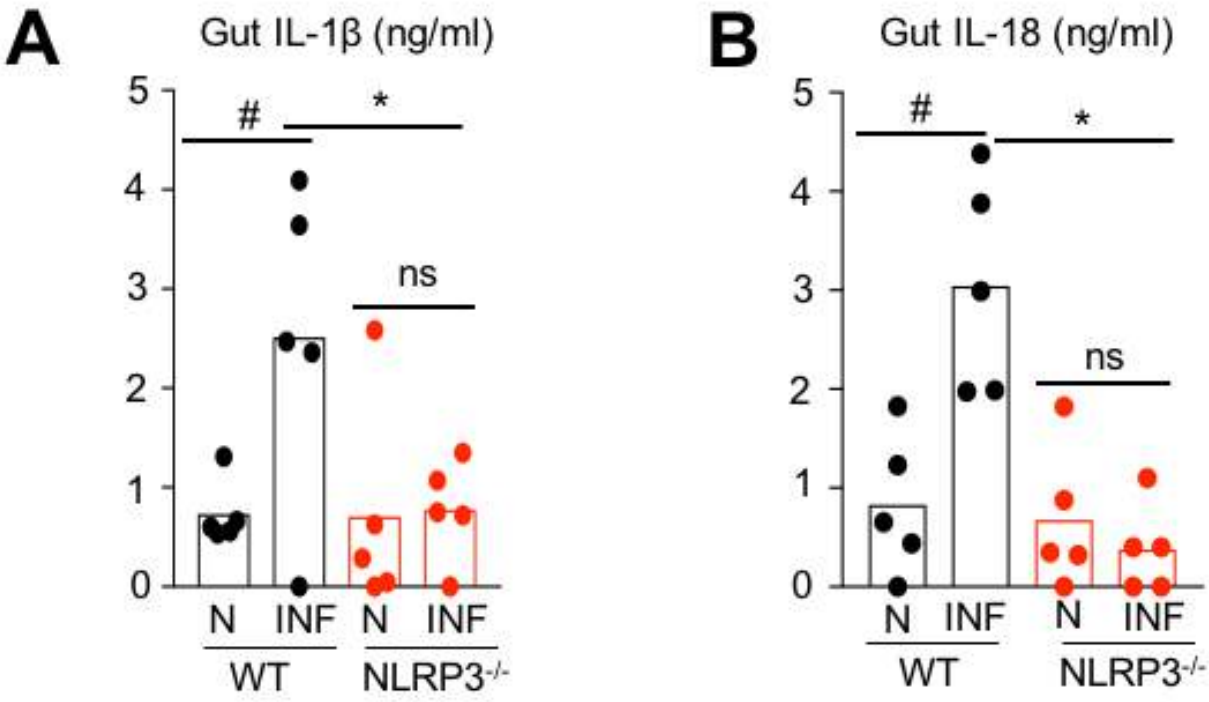


Figure 3.3.1 NLRP3 is critical for *in vivo* inflammasome activation following *N. brasiliensis* infection. C57BL/6 WT and NLRP3$^{-/-}$ mice were infected with *N. brasiliensis* and sacrificed at d 6 post-infection (p.i.). (**A**) IL-1β and (**B**) IL-18 were measured in small intestinal tissue homogenate supernatants by ELISA. Data are expressed as mean with individual data points and are representative of 3 experiments, n=5 mice/group. # $p<0.05$ compared to naïve (N). * $p<0.05$ significant compared to WT. ns denoted not significantly different

3.3.2 NLRP3 inflammasome inhibits type 2 gut-stage immunity to N. brasiliensis.

Given the critical role for NLRP3 in *N. brasiliensis*-mediated inflammasome activation in the gut, we next wanted to assess the *in vivo* role for NLRP3 in protective immunity to *N. brasiliensis* infection. WT or NLRP3$^{-/-}$ mice were infected with 500 *N. brasiliensis* L3 and the small intestine was removed at day 6 post infection. Analysis of worm burdens at day 6 post-infection revealed that NLRP3$^{-/-}$ mice had significantly reduced worm burdens **(Figure 3.3.2 A)** and presence of parasite eggs in faeces **(Figure 3.3.2 B)** at day 6 p.i compared to WT mice, consistent with increased anti-parasitic immunity. This was associated with significantly increased frequencies and total numbers of eosinophils in the mesenteric lymph node (mLN) **(Figure 3.3.2 C-D)** and significantly increased goblet cell responses compared to WT mice (**Figure 3.3.2 E-F**). Together, these data suggest that NLRP3 may suppress protective Type-2 immune responses during intestinal *N. brasiliensis* infection.

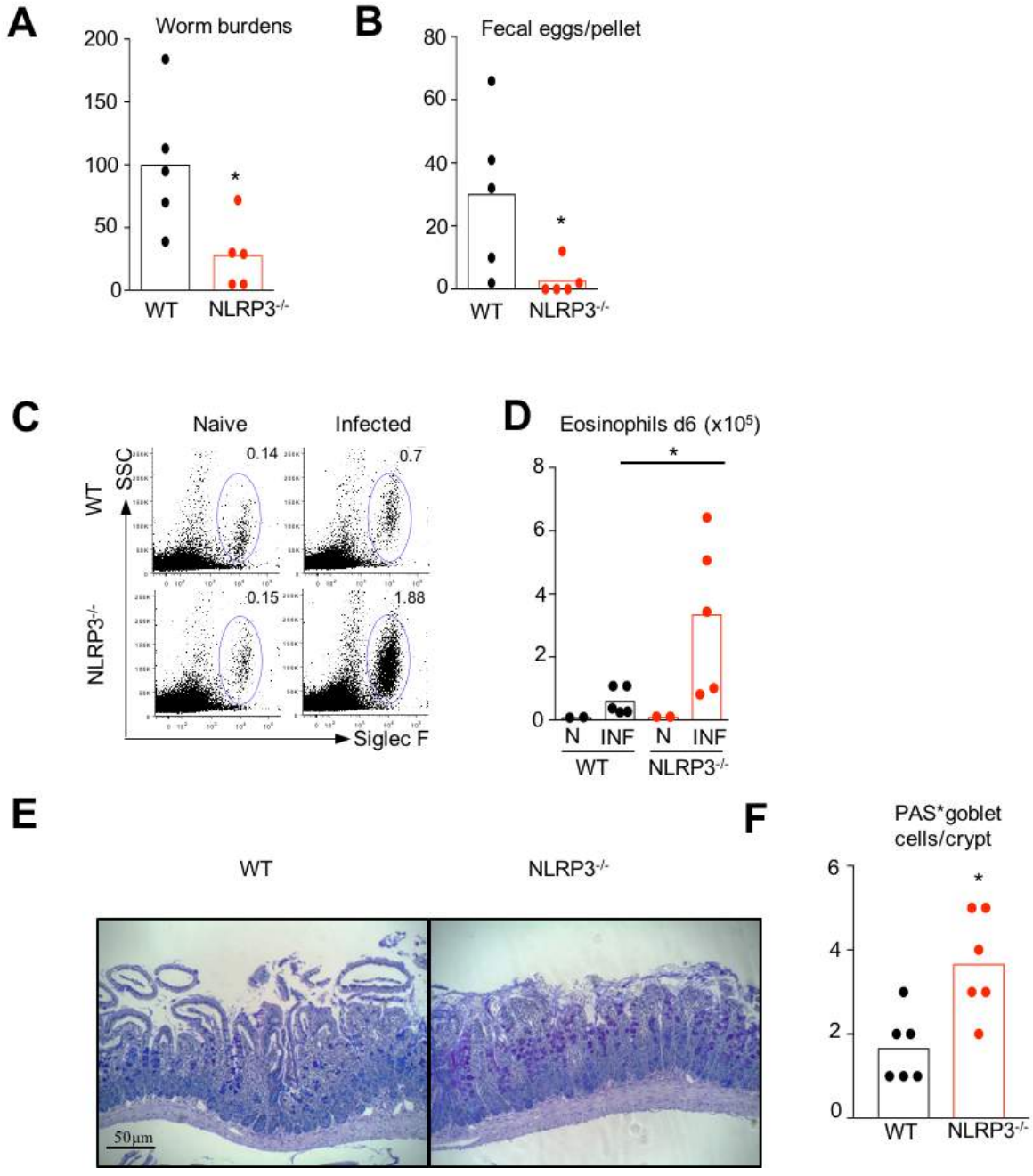


Figure 3.3.2 NLRP3 inflammasome inhibits type 2 gut-stage immunity to *N. brasiliensis.* WT or NLRP3$^{-/-}$mice were infected with 500 *N. brasiliensis* L3 and small intestine were removed at day 6 post infection. (**A**) small intestine worm burdens. (**B**) Fecal eggs numbers per pellet. (**C**) Representative plots displaying Siglec F^{+} eosinophils frequencies in the mesenteric lymph nodes (mLN) at d 6 p.i. (**D**) Total mLN eosinophils (**E**) Representative images of PAS/Alcian Blue stained small intestine (**F**) Blinded quantification of goblet cell numbers. Data are expressed as mean with individual data points and are representative of 5 experiments, n=5 mice/group. *p <0.05 compared to WT.

3.3.3 NLRP3 inflammasome limits lung-stage innate immune responses to N. brasiliensis infection

We next assessed whether NLRP3 inflammasome activation and increased Type-2 mediated immunity to *N. brasiliensis* was evident prior to the intestinal stage, i.e. in the lung. C57BL/6 WT and NLRP3$^{-/-}$ mice were infected with *N. brasiliensis* and lungs were removed at day 2 post infection. Analysis of IL-1β and IL-18 levels in lung homogenates revealed that while infected WT mice exhibited significant increases in levels of these cytokines compared to naïve WT mice (**Figure 3.3.3 A-B**), NLRP3$^{-/-}$ mice did not display any infection-induced elevations in IL-1β or IL-18 (**Figure 3.3.3 A-B**), hence lung-stage infection with *N. brasiliensis* induces NLRP3 inflammasome dependent IL-1β and IL-18 secretion. NLRP3$^{-/-}$ mice displayed a significant reduction in lung larval burdens (**Figure 3.3.3 C**) and increased mRNA levels of IL-4, *Retnla* and *Arg1* compared to WT mice (**Figure 3.3.3 D-F**), suggesting that NLRP3 may suppress innate Type 2 immune responses against *N. brasiliensis* in the lung. *Retnla* and *Arg1* are important M2-derived factors that resolve inflammation and repair damaged tissue (Mantovani et al., 2013; Murray et al., 2014).

Our previous study demonstrated that NLRP3-mediated suppression of immunity to *T .muris* was partially dependent on CD4+ cells and adaptive immunity (Alhallaf et al., 2018). To assess whether NLRP3-dependent regulation of immunity to *N. brasiliensis* is also dependent on CD4^{+} T cells, WT C57BL/6 mice or NLRP3$^{-/-}$ mice were treated one day before *N. brasiliensis* infection and every 2 days thereafter with either 0.5mg of a neutralizing anti-CD4 mAb or a control rat IgG following *N. brasiliensis* infection. Interestingly, depletion of CD4+ cells did not significantly affect the lung larval burdens in NLRP3$^{-/-}$ mice at day 2 post-infection **(Fig 3.3.3 G)**, suggesting that CD4+ cells were not required for the increased lung-stage anti-parasitic immunity of NLRP3$^{-/-}$ mice. In contrast, depletion of CD4+ cells in NLRP3$^{-/-}$ mice did significantly ablate the normally robust protective immunity to *N. brasiliensis* at the level of

the gut at 6 p.i. **(Figure 3.3.3 H).** Together, these data suggest that NLRP3 suppression of protective immunity to *N. brasiliensis* in the lung may be independent of adaptive CD4+ cells, however NLRP3 may influence downstream CD4+-dependent protective immunity at the level of the gut.

One of the mechanisms by which the NLRP3 inflammasome is activated is by engagement of the $P2X_7R$ (Purinergic Receptor P2X 7) by extracellular ATP (Riteau et al., 2010), which may be released from damaged cells. To determine if the $P2X_7R$ was similarly associated with suppressed immunity to *N. brasiliensis,* WT C57BL/6 mice or $P2X_7R^{-/-}$ mice were infected with 500 *N. brasiliensis* L3 and the small intestine was removed at day 6 post infection. Consistent with results seen in $NLRP3^{-/-}$ mice, $P2X_7R$-deficient mice display a significant decreased in worm burdens during *N. brasiliensis* infection, suggesting that P2X7R may be involved in NLRP3-mediated suppression of protective immunity to *N. brasiliensis* **(Supplementary Figure 3.3.1 A)** .

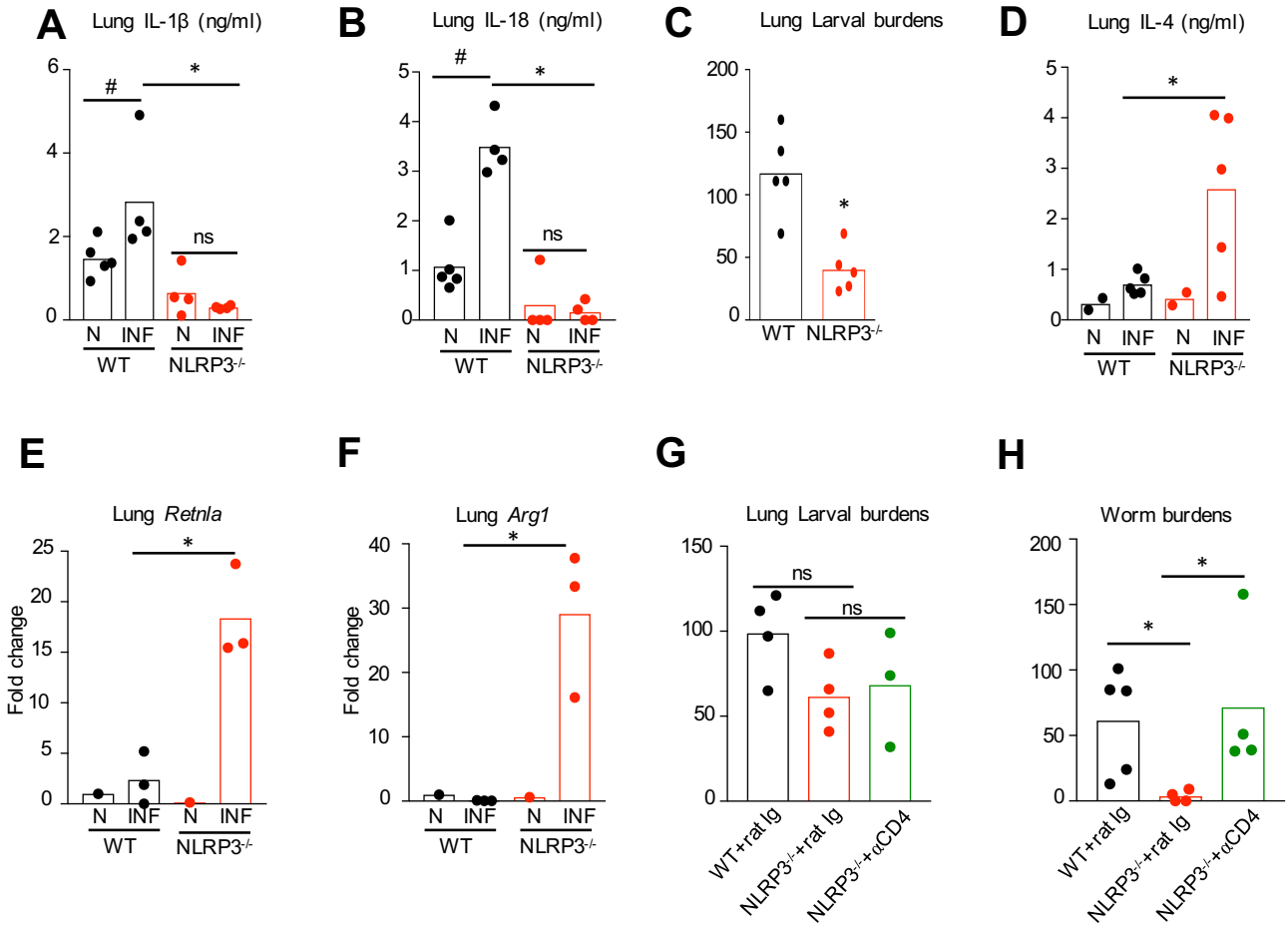


Figure 3.3.3 NLRP3 inflammasome limits lung-stage innate immune responses to *N. brasiliensis* infection. WT and NLRP3$^{-/-}$ mice were infected (INF) with *N. brasiliensis* and lungs were harvested at d 2 p.i. (**A**) Lung IL-1β levels and (**B**) lung IL-18 levels in tissue homogenates measured by ELISA (**C**) Lung larval burdens. (**D**) IL-4 protein, (**E**) *Retnla* and (**F**) *Arg1* mRNA levels in lung homogenates measured by ELISA or qPCR. Data are expressed as mean with individual data points and are representative of 4 experiments, n=5 mice/group. *p <0.05 compared to WT. WT C57BL/6 mice or NLRP3$^{-/-}$ mice were treated one day before infection and every 2 days thereafter with either 0.5 mg of a neutralizing anti-CD4 mAb or a control rat IgG following *N. brasiliensis* (**G**) lung larval burdens day 2 (**H**) gut worm burdens day 6. Data are expressed as mean with individual data points and are representative of 2 experiments, n=5 mice/group. # p<0.05 compared to naive (N). *p <0.05 compared to rat Ig control. ns denotes not significantly different.

3.3.4 NLRP3$^{-/-}$ mice display elevated neutrophil recruitment to the lung following N. brasiliensis infection

We next aimed to profile the nature of the cellular infiltrate within the lung of WT and NLRP3 mice to determine what innate immune cells may be associated with the increased immunity to *N. brasiliensis*. Analysis of cellular immune responses in lung tissue enzymatic digests using flow cytometry revealed that while WT mice exhibited elevations in frequencies **(Figure 3.3.4 A)** and significant increases in total numbers **(Figure 3.3.4 B)** of Siglec F^{+} CD11b^{+} eosinophils and Siglec F^{-} CD11b^{+} neutrophils following infection, these responses were exaggerated in NLRP3$^{-/-}$ mice **(Figure 3.3.4 A-B)**. Frequencies and total numbers of Siglec F^{+} CD11b^{int} alveolar macrophages were not elevated in NLRP3$^{-/-}$ mice (**Figure 3.3.4 A-B**). Further confirmation of the cellular phenotypes using additional markers demonstrated that neutrophils co-expressed Ly6G, macrophages co-expressed CD11c and eosinophils were SSC-high **(Supplementary Figure 3.3.2 A).**

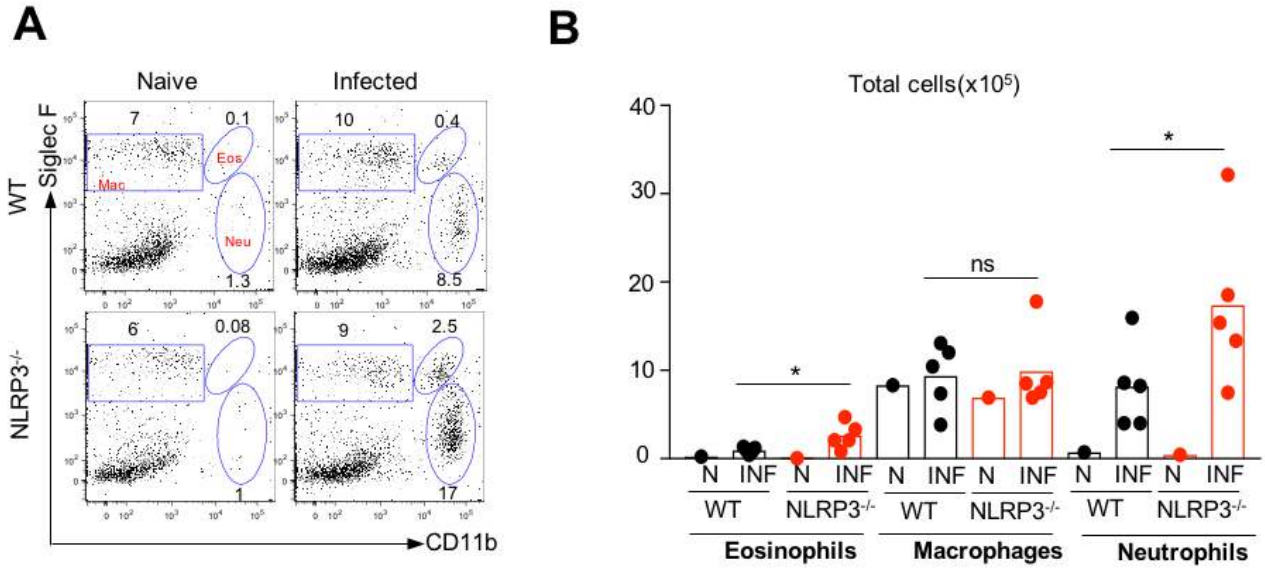


Figure 3.3.4 NLRP3$^{-/-}$ mice display elevated neutrophil recruitment to the lung following *N. brasiliensis* infection. C57BL/6 WT and NLRP3$^{-/-}$ mice were infected with 500 *N. brasiliensis* L3 and sacrificed at d 2 post-infection (**A**) Representative plots displaying of Siglec F^{+} CD11b^{+} eosinophils, Siglec F^{-} CD11b^{+} neutrophils and Siglec F^{+} CD11b^{int} macrophages frequencies in lung tissue at d 2 p.i. (**B**) Total lung neutrophils, eosinophils and macrophages. Data are expressed as mean with individual data points and are representative of 3 experiments, n=5 mice/group. *p <0.05 compared to WT. ns denotes not significant

3.3.5 NLRP3$^{-/-}$ mice display elevated neutrophil chemokines in the lung

Given that neutrophils were the most abundant infection-induced immune cell in the lung of NLRP3$^{-/-}$ mice, we next assessed the levels of key neutrophil chemoattractants in lung tissue homogenates. C57BL/6 WT and NLRP3$^{-/-}$ mice were infected with *N. brasiliensis* and *Ccl3*, *Cxcl1* and *Cxcl2* mRNA levels were measured at day 2 post infection using qPCR. While infected NLRP3$^{-/-}$ mice displayed approximately 2-fold increased levels in *Cxcl1* compared to WT but no apparent increase in *Ccl3* (**Figure 3.3.5 A-B**), expression of Cxcl2 was elevated nearly 20-fold compared to WT (**Figure 3.3.5 C**). These data suggest that elevated neutrophil recruitment to the lung of NLRP3$^{-/-}$ mice may be as a result of increased expression of chemokines, especially CXCL2.

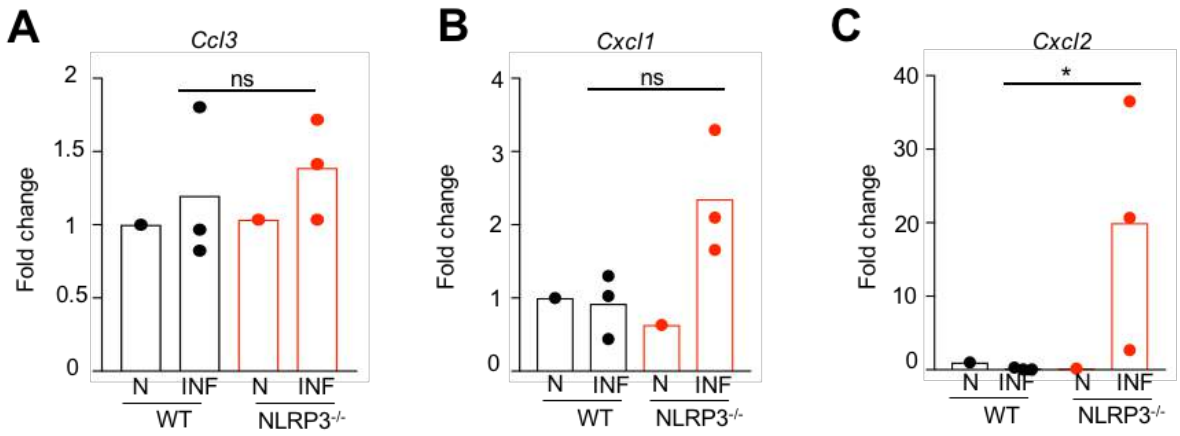


Figure 3.3.5. NLRP3$^{-/-}$ mice display elevated neutrophil chemokines in the lung. C57BL/6 WT mice were infected with 500 *N. brasiliensis* L3 and sacrificed at d 2 post-infection and *Ccl3*, *Cxcl1* and *Cxcl2* mRNA levels were measured at day 2 post infection using qPCR (**A**) Lung *Ccl3* (**B**) Lung *Cxcl1* (**C**) Lung *Cxcl2*. Data are expressed as mean with individual data points and are representative of 2 experiments, n=4 mice/group. *p <0.05 compared to WT. ns denotes not significant.

3.3.6 Neutrophils promote rapid immunity to Nippostrongylus brasiliensis lung-stage infection

Given the critical role for neutrophils in immunity to *N. brasiliensis* (Chen et al., 2014), and also the role of NLRP3 in regulating the lung neutrophil response to infection, we next wanted to assess whether elevated neutrophilia in NLRP3$^{-/-}$ mice promoted enhanced protection against primary *N. brasiliensis* infection. WT and NLRP3$^{-/-}$ mice were treated with either anti-Gr1 mAb (to deplete neutrophils), or a control Ab (rat Ig), infected with 500 L3 and sacrificed at day 1 p.i. As expected, treatment with anti-Gr1 was able to effectively deplete neutrophils in infected mice (**Figure 3.3.6 A**). Also as expected, administration of anti-Gr1 resulted in death of WT and NLRP3$^{-/-}$ mice between 18-48 hours post-infection **(Supplemental Figure 3.3.3 A),** consistent with previous reports that highlight a critical role for neutrophils in preventing *N. brasiliensis* larvae-associated bacterial dissemination (Pesce et al., 2008). Interestingly however, this Gr-1 cell depletion in both WT and NLRP3$^{-/-}$ mice was associated with increased lung larval burdens (**Figure 3.3.6 B**), suggesting impaired anti-parasitic immunity in neutrophil-depleted mice. This was associated with lowered eosinophil responses in the lung **(Figure 3.3.6 C)** and lowered IL-4 expression in anti-Gr-1 treated NLRP3$^{-/-}$ mice **(Figure 3.3.6 D**). Together these data suggest that elevated neutrophil responses in NLRP3$^{-/-}$ mice may be at least partially responsible for elevated Type 2 immune responses and anti-parasitic immunity following *N. brasiliensis* infection.

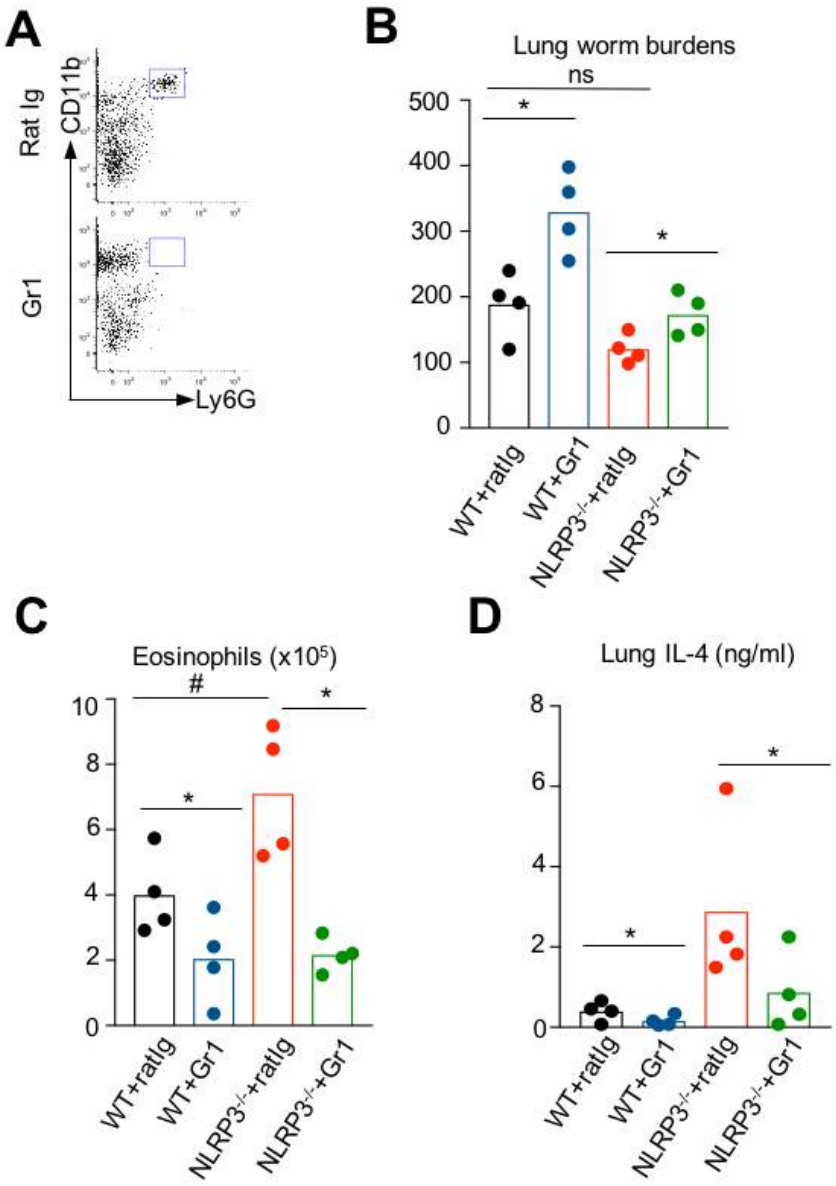


Figure 3.3.6. Neutrophils promote rapid immunity to *N. brasiliensis* lung-stage infection
C57BL/6 WT and NLRP3$^{-/-}$ mice were either daily treated with control rat Ig or anti-Gr1 antibody 1d before *N. brasiliensis* infection and were sacrificed at day 1. (**A**) Representative plots displaying successful depletion of CD11b^{+} Ly6G^{+} neutrophils frequencies in the WT mouse lung at d2 following anti-Gr1 antibody. (**B**) Lung larval burdens. (**C**) Total lung eosinophils (**D**) Lung IL-4. Data are expressed as mean with individual data points and are representative of 3 experiments, n=5 mice/group. # $p<0.05$ compared to WT. *p <0.05 compared to rat Ig control. ns denotes not significant.

3.3.7 Neutrophils are potently anti-parasitic to N. brasiliensis larvae in vitro

To assess whether neutrophils in this model may be directly killing *N. brasiliensis* larvae, as neutrophils and macrophages have been shown to collaborate in killing various helminth species (Bonne-Annee et al., 2013; Bonne-Annee et al., 2014; Jong et al., 1984), we conducted *in vitro* co-culture experiments with *N. brasiliensis* L3 and sort-purified cell populations. Neutrophils were sorted from bone marrow of WT mice and macrophages were enriched from bone marrow cultures. *N. brasiliensis* L3 were cultured for 24 h with either neutrophils, no cells (control) or bone-marrow-derived macrophages for 24h in the presence of normal mouse serum. As expected, L3 cultured with no cells remained highly motile and curved with near 100% viability **(Figure 3.3.7 A).** However, inclusion of macrophages into the cultures resulted in an average motility of 65%, suggesting macrophages were partially toxic to L3. Most strikingly, the presence of the neutrophils resulted in an almost complete reduction in parasite viability **(Figure. 3.3.7 A),** which was supported by the presence of straight motionless larvae with evidence of cells adhering to parasites as well as fibrous extracellular material, consistent with neutrophil extracellular traps (Behrendt et al., 2010; Gabriel et al., 2010). **(Figure 3.3.7 B-C).** These data support the notion that neutrophils may be directly cytotoxic to infective parasitic larvae, hence elevated neutrophil responses in NLRP3 deficient mice and direct parasite killing may be one mechanism by which NLRP3 mice are more resistant to infection.

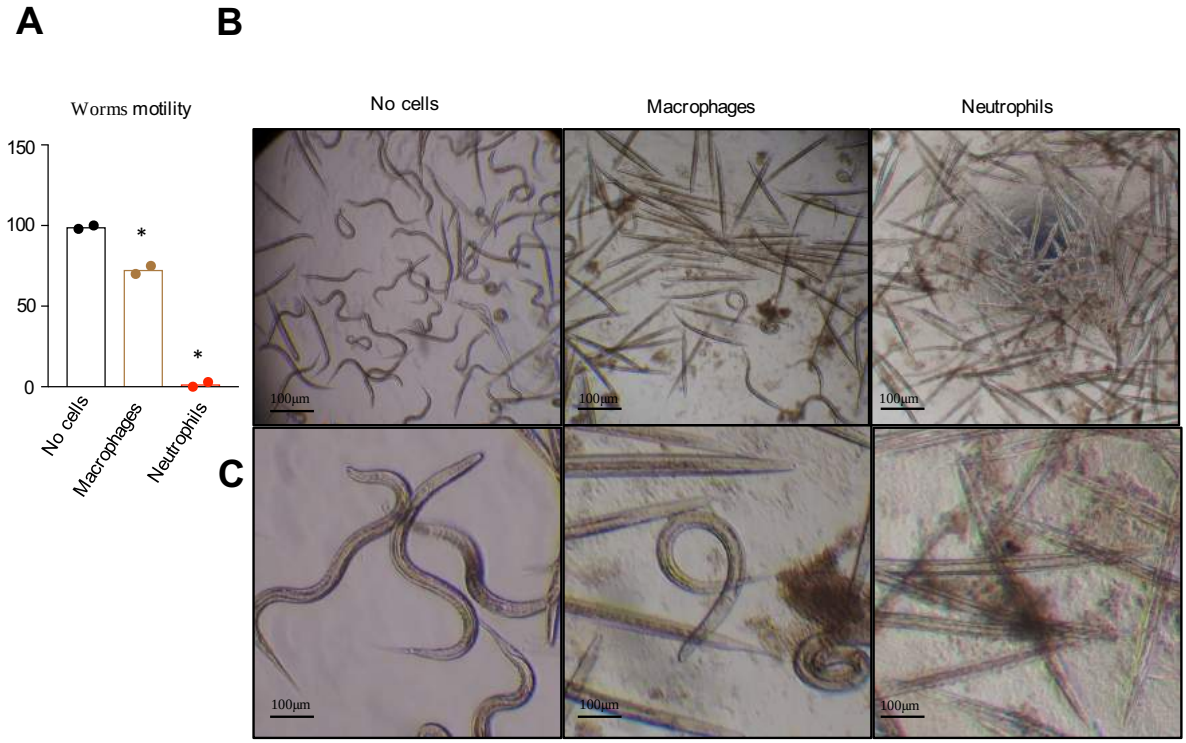
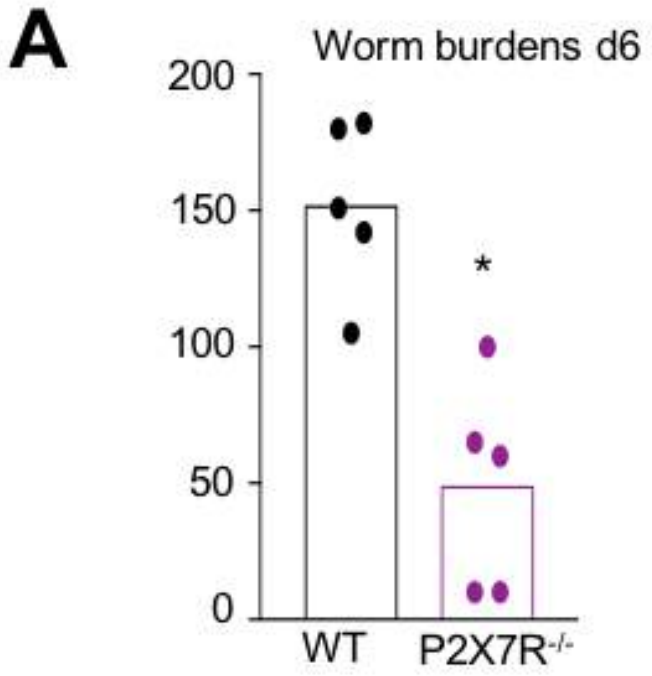


Figure 3.3.7. Neutrophils are potently anti-parasitic to *N. brasiliensis* larvae *in vitro*

Neutrophils were sorted from bone marrow of WT mice and macrophages were enriched from bone marrow cultures. *N. brasiliensis* L3 were cultured for 24 h with either neutrophils, no cells control or bone-marrow-derived macrophages for 24h in the presence of normal mouse serum. (**A**). Worm motility. (**B-C**) Representative pictures displaying no cells, macrophage and neutrophils. Data are expressed as mean with individual data points and are representative of 2 experiments, n=2 cell culture replicates/treatment. *p <0.05 compared to WT.



Supplementary figure 3.3.1 $P2X_7R$-deficient mice display enhanced immunity to *N. brasiliensis* infection. C57BL/6 WT and $P2X_7R^{-/-}$ mice were infected with *N. brasiliensis* and sacrificed at d 6 p.i. (**A**) Worm burdens. Data are expressed as mean with individual data points and are representative of 2 experiments, n=5 mice/group. *p <0.05 compared to WT.

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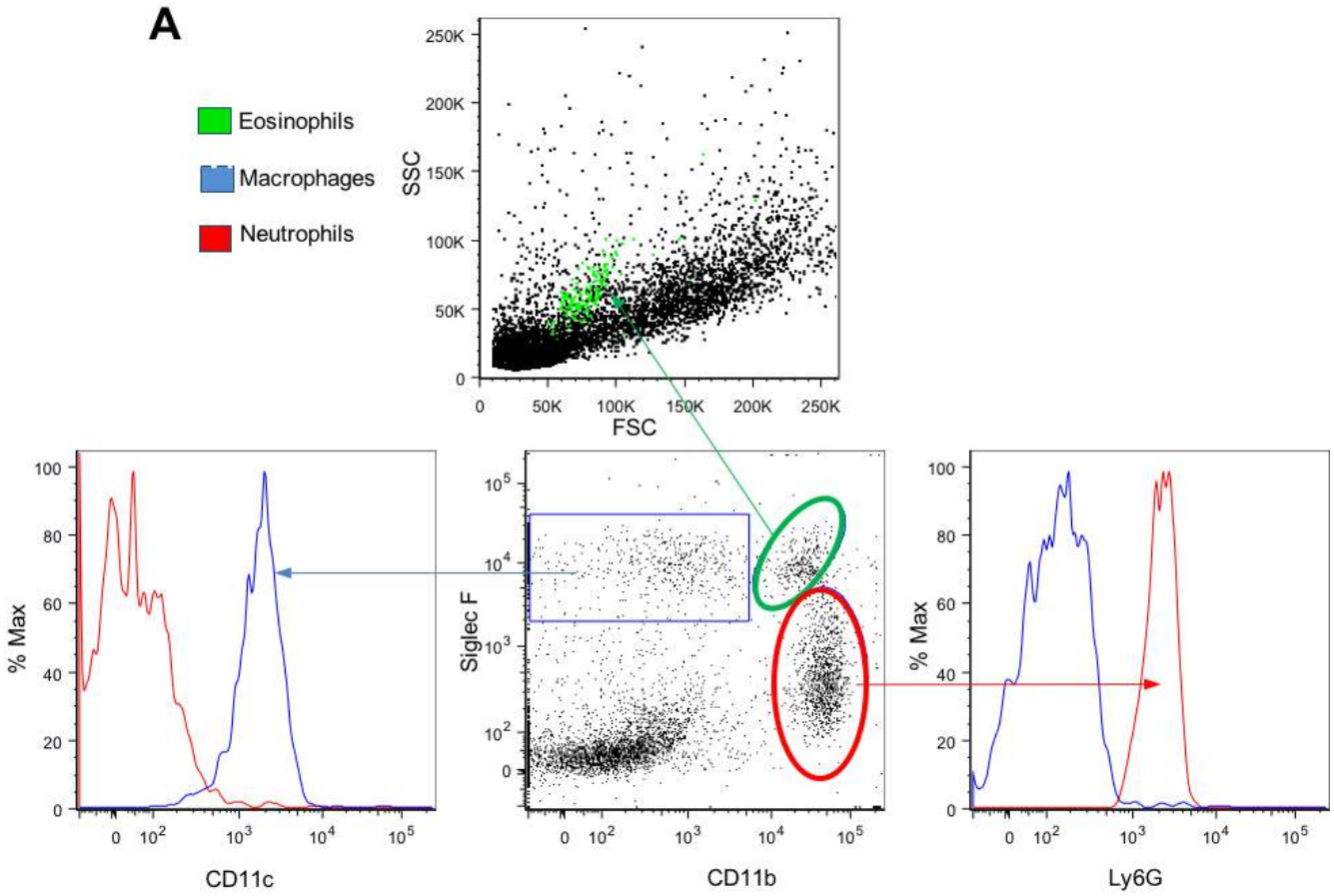
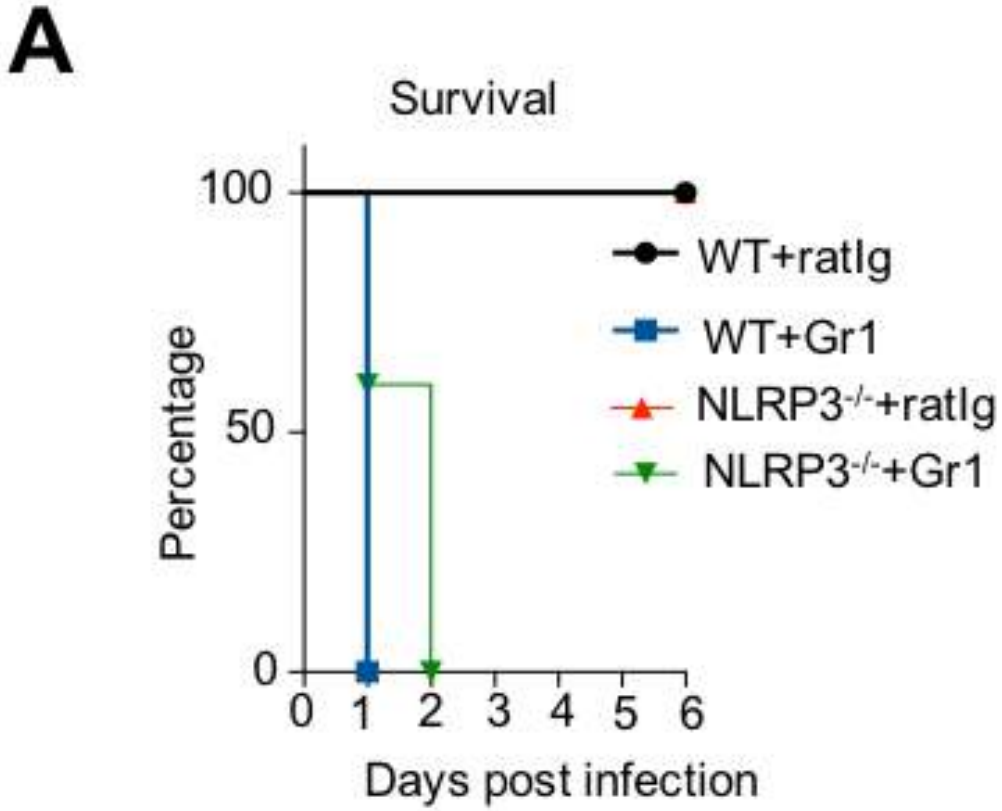


Supplementary figure 3.3.2. Phenotyping of lung innate immune cells. Further confirmation of the cellular phenotypes described in Fig. 4.3.4. Using additional markers demonstrated that gated neutrophils (red gate) co-expressed Ly6G, macrophages (blue gate) expressed CD11c and eosinophils (green gate) were SSC-high.



Supplementary figure 3.3.3. Neutrophils is important against *N. brasiliensis* infection. WT and NLRP3$^{-/-}$ mice were treated with either anti-Gr1 mAb (to deplete neutrophils), or a control Ab (rat Ig), infected with 500 L3. (**A**) Survival rate in infected mice. Data are expressed as mean with individual data points and are representative of 2 experiments, n=5 mice/group.

3.4 Discussion

Understanding the nature of the immune response to parasitic helminths is critical for the rational design of preventative therapies against infection, such as vaccines. Helminths have evolved to manipulate and limit elements of the host immune response to enhance their ability to survive, which may explain why effective vaccination against helminths has proven to be an enormous challenge. While inflammasomes are a crucial element of the protective immune response against a variety of pathogens, recent literature suggests that inflammasomes may in fact suppress the protective immune response against parasitic helminths. Consistent with this, in the present study we identified an additional mechanism of how the NLRP3 inflammasome may limit innate immunity of mice to a hookworm-like parasite, potentially via limiting the recruitment of anti-parasitic neutrophils into the lung.

NLRP3 is important in inducing Type 1 immune responses against microbial pathogens (Silva et al., 2013; Song-Zhao et al., 2014), however other studies have demonstrated that NLRP3 is promoting Type 2 immune responses (Bruchard et al., 2015; Gurung et al., 2015). In the present study, infection-induced NLRP3 activation is associated with reduced Type 2 immune responses including IL-4 and eosinophils in the lung and gut, intestinal goblet cell responses and markers of alternatively activated macrophages (AAMac) in the lung (*Retnla* and *Arg1*) that have been associated with direct killing of helminths and tissue repair (Gobert et al., 2000; Jang and Nair, 2013). Our previous results have demonstrated that NLRP3 may suppress Type-2 immune responses via regulation of IL-18 secretion and promotion of Th1 and ILC1 responses (Alhallaf et al., 2018). In the present study we have reported infection-induced elevations in lung and gut IL-18 but did not observe increased Th1 cytokines in either tissue (data not shown), suggesting that there may be different mechanisms by which NLRP3 can regulate the immune responses. Consistent with this, compared to results seen in *Trichuris*

infections (Alhallaf et al., 2018), CD4 cells do not appear to be an essential downstream modulator of NLRP3-dependent suppression of immunity to *N. brasiliensis*, particularly lung-stage immunity. This suggests that NLRP3 may be regulating an early innate component of the immune response to *N. brasiliensis* and not adaptive immune cells or antibodies that would take much longer than 1-2 days to develop. We did observe increased eosinophil responses in NLRP3$^{-/-}$ mice and these cells are important for immunity against *N. brasiliensis* infections (Al-Dahwi et al., 2006; Knott et al., 2007; Shin et al., 1997), however we did not demonstrate a causal role for these cells in direct killing of parasites, such studies could involve depletion of eosinophils using anti-CCR3 antibodies or by crossing NLRP3$^{-/-}$ mice with eosinophil deficient mouse strains (Knott et al., 2007). Similarly, macrophages are important for *N. brasiliensis* killing (Bouchery et al., 2015; Chen et al., 2013). While we did not observe increased numbers of macrophages in the lung (Fig 4), we did observe increased AAMac markers *Arg1* and *Retnla* that have been associated with protective immunity to *N. brasiliensis* (Jang and Nair, 2013), in concert with ILC2 cells. This suggests that NLRP3 may influence the phenotypes of macrophage responses within the lung, which may regulate anti-parasitic responses. However further studies are required to delineate the precise role for NLRP3-dependent regulation of macrophage function in the context of *N. brasiliensis* infection.

While we were not able to demonstrate a causal role for eosinophils or macrophages in NLRP3-dependent suppression of immunity to *N. brasiliensis*, we did observe correlations between rapid neutrophil recruitment to the lung and enhanced anti-parasitic immunity. Neutrophils are well studied during microbial infections in the context of type 1 immune responses (Mantovani et al., 2011), and they are also important against helminth infection (Chen et al., 2012; Chen et al., 2014; Morimoto et al., 2004). It has demonstrated that neutrophils are recruited to site of infection and arrive following the production of CXCL1, CXCL8 and CXCL2 (Miotla et al., 2001; Ribeiro et al., 1990). We found that CXCL2 levels were the most strongly upregulated

in NLRP3-deficient mice, implicating this chemokine as likely to mediate elevated neutrophil chemotaxis to the lung. How NLRP3 regulates expression of CXCL2 was not determined. CXCl2 expression in dependent on NF-kappa B, TNF, platelet-activating factor (PAF) and IKK kinase alpha (IKKα) (Burke et al., 2014; da Silva et al., 2012; De Plaen et al., 2006; Goktuna et al., 2014), however it is unclear how NLRP3 may influence expression of CXCL2 in response to these factors. CXCL2 is expressed primarily by epithelial cells, mast cells and macrophages (De Filippo et al., 2013; Ohtsuka et al., 2001). It is possible that following *N. brasiliensis* infection in the lung, CXCL2 is expressed by intestinal epithelial cells (IECs) and this mediates recruitment of neutrophils to the lung tissue, however further work examining cell-specific expression of CXCL2 is required to demonstrate which cell type is responsible for recruiting neutrophils.

Our *in vitro* culture data suggest that neutrophils are highly toxic to *N. brasiliensis* larvae, rendering the larvae straight and immotile. Neutrophils possess an array of mechanisms by which they could damage helminths, including complement-mediated cytotoxicity, production of oxygen free radicals or the release of extracellular traps (Bonne-Annee et al., 2013; Galioto et al., 2006). Neutrophils are similarly associated with killing other species of helminth, including larval stages of *Strongyloides stercoralis*, where neutrophils alone could kill the parasite, however eosinophils and macrophages were also required for optimal killing (Bonne-Annee et al., 2013; Chen et al., 2014; Galioto et al., 2006). In contrast, neutrophils seem to have a limited role during infection with *S. mansoni*, as their depletion did not influence the severity of disease (Herbert et al., 2004). While neutrophils have been shown to be important *in vitro*, there is relatively little evidence that they are important *in vivo* (Pesce et al., 2008). The present study showed that neutrophil depletion by anti-Gr-1 mAb injection had a partial reduction in anti-parasitic immunity. Hence while neutrophils appear to be at least partially responsible for

increased parasite killing in absence of NLRP3, other factors may also be involved. Additionally, anti-Gr-1 treatment is known to deplete other immune cells subsets such as monocytes, macrophages and some T cells subsets (Daley et al., 2008), hence we cannot be completely certain that the observed effects were due to neutrophil depletion alone. Further studies involving more specific depletion of neutrophils (for example using anti-Ly6G antibodies) as well as other cell subsets mentioned earlier (eosinophils, macrophages) but also ILC2s and basophils that are also associated with immunity to *N. brasiliensis* may reveal more information about the range of immune factors that promote innate resistance to the parasite.

The role for inflammasome activation during helminth infections is becoming increasingly appreciated, however it remains unclear how helminths activate inflammasomes. Further, there is evidence that some helminth-derived factors may also inhibit inflammasome activation (Ritter et al., 2010). Previous *in vitro* studies have highlighted that factors excreted and secreted by other parasites can directly activate inflammasomes (Ferguson et al., 2015; Ritter et al., 2010; Zaiss et al., 2013). Further, our recent work have highlighted that factors excreted or secreted by *Trichuris* may activate the NLRP3 inflammasome *in vivo* and *in vitro* and some of these factors may be contained within extracellular vesicles or exosomes (Alhallaf et al., 2018). In the current study we demonstrate that *N. brasiliensis* infection is similarly associated with NLRP3-dependent inflammasome in lung and gut, but we did not establish whether this was mediated by worm-secreted molecules, or other factors such as parasite-induced tissue damage. For example, damage to the lung tissue may result in ATP release, which is a well-known activator of NLRP3 via interaction with the $P2X_7R$. Furthermore, helminths such as *Trichinella spiralis* also can directly modify host-derived ATP (Gounaris et al., 2004), which may activate the $P2X_7R$. Interestingly, we found that $P2X_7R$ deficient mice had similar phenotypes to

NLRP3-deficient mice (e.g increased anti-parasitic immunity) suggesting that the $P2X_7R$ may be associated with NLRP3 activation in this model.

In conclusion, we have provided novel information on how the NLRP3 inflammasome regulates innate immune cells like neutrophils during helminth infections. We have identified that activation of NLRP3 inflammasome is important for controlling early immune responses potentially by suppressing the recruitment and function of innate immune cells such as neutrophils. By identifying the precise worm-secreted molecules that activate or inhibit inflammasome activations and which cellular and molecular pathways are important, we may be able to create a novel treatment for boosting immunity to helminth infections.

3.5 Materials and Methods

3.5.1 Parasitological techniques

N. brasiliensis was maintained in Sprague–Dawley rats (Animal Resources Centre, Perth, WA, Australia) and infective L3 were prepared from rat fecal cultures. Mice were infected by 500 L3 subcutaneously.

3.5.2 Mice and treatments

NLRP3 and $P2X_7R$ -deficient mice all on C57BL/6 genetic background, and parental C57BL/6 wild-type (WT) control mice were bred and maintained at James Cook University (JCU), Cairns Campus. Male and female mice between 6 and 8 weeks of age were used. All experimental protocols were approved by the JCU Animal Ethics Committee. Mice were treated with neutralizing antibodies against CD4 (GK1.5, Bioxcell, 0.5 mg/mouse) starting on day -1 and continuing every 2 days until necropsy. For neutrophil depletion, Anti-Gr1 antibody (BioXcell) or isotype control rat Ig was administered to mice both intraperitoneally (i.p.) (0.5 mg in 0.2 ml)

3.5.3 Mouse tissue collection and processing

At necropsy, lung tissue was minced and incubated with stirring at 37 °C for 30 min in HBSS with 1.3 mM EDTA (GIBCO), followed by treatment at 37 °C for 1 h with collagenase (1 mg/ml; Sigma) in RPMI 1640 (Mediatech) with 5% FCS (Biowest) and with 100 µg/ml of DNase (Sigma) for 10 min. Cells were lysed with ACK Lysing Buffer (Lonza) to remove erythrocytes, blocked with Fc Block (BD Biosciences). Single cell suspensions of mesenteric lymph node (mLN) were prepared by passing through 70um nylon mesh filters. For lung and colon tissue homogenates, 1 cm of colon tissue or one lobe of lung tissue was homogenized

mechanically in 0.5 mL of PBS using a Tissue Lyzer (QIAGEN) and supernatants stored at -80°C. Gut or lung homogenates were assayed for IL-4, IL-1β and IL-18 using standard sandwich ELISA protocols (eBioscience or ELISAkit.com.au).

Gut was fixed in 4% formaldehyde and embedded in paraffin, and 5 μM sections were stained with PAS/Alcian Blue stains using the standard protocol of an institutional histology service provider (QIMR Berghofer Medical Research Institute). For determination of worm burdens, small intestines were collected and worms were counted using a dissecting microscope. Lung larval burdens were assessed by mincing the mouse lungs with scissors in PBS and counting emigrated larvae under a dissecting microscope.

3.5.4 In vitro larval killing assay.

In vitro killing assays were performed by plating of 100 larvae into 48-well plates. Normal mouse serum collected from naive C57BL/6J mice via exsanguination or cardiac puncture was immediately filter sterilized prior to freezing. Serum was added to plates, as a source of complement, at a concentration of 25% of the total well volume. Macrophages Bone marrow was isolated from hind legs of C57BL/6 or NLRP3$^{-/-}$ mice by flushing bones with DMEM and cells were cultured at 1×10^6 cells/ml in DMEM supplemented with 10% FBS, 5% horse serum (Invitrogen), 2 mM L-glutamine, 10 mM HEPES, 1mM sodium pyruvate and 10 ng/ml M-CSF. On day 6, macrophages were harvested and plated overnight at a concentration of 2×10^5 cells/ml. The following day, cells were treated with media alone, or LPS (1 μg/ml) for 4 h, 20 μM nigericin for 2 h, 25 μg/ml of *N. brasiliensis* L3 ES products and cell-free supernatants were stored at -80°C.

CD11b$^+$ and Ly6G$^+$ neutrophils were sorted from bone marrow of WT mice and (1×10^6) cells were added to select wells in a final volume of 250 μl with RPMI 1640 (Mediatech)

supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin plus 100 µg/ml streptomycin and incubated for 24 h. The contents of the wells were then assessed for larval viability of every larva in the culture wells where larvae were considered viable if they were visibly motile or curved. If larvae were straight and not motile then they were classified as non-viable.

3.5.5 Flow cytometry

Cell preparations were surface-stained with anti-mouse fluorochrome-conjugated mAbs against CD11b (M1/70), CD11c (HL3), Gr-1 (RB6-8C5), Ly6G (1A8), and Siglec F (E50-2440) Cells were first stained with cell surface and analysed by flow cytometry using a FACS Canto II or Aria III cytometer (BD Biosciences) where dead and doublet cells were excluded by the use of a live gate and doublet discrimination. Further analysis was performed using FlowJo software (Tree Star, Inc).

3.5.6 Real-Time PCR and relative quantification analysis

Total RNA was extracted from excised lungs, at the sites where the worms were located. Total RNA from each tissue obtained mice was isolated using Trizol Reagent (Sigma-Aldrich), according to the manufacturer instructions, according to the manufacturer instructions. The cDNA was synthesised using SuperScript III First-Strand Synthesis System (Invitrogen).

For quantitative PCR, 40 ng total RNA reverse transcribed to cDNA was added to 10 μL of TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2x), 1 μL of the specified TaqMan® Gene Expression Assay, and water to final reaction volume of 20 μL. Reac- tions were performed on the Abi Prism 7000 (Applied Biosystems), with the following thermal cycler conditions: initial setup of 2 min UNG activation at 50 °C, followed by 10 min at 95 °C, and 40 cycles of 15 s denaturation at 95 °C and 1 min of anneal/extend at 60 °C each. Samples were

amplified in a 96-well plate. On each plate endogenous control, samples and negative control were analyzed in triplicate. Cycle threshold (Ct) value was calculated for each sample, house-keeping and uninfected control. To normalize for differences in efficiency of sample extraction or complementary DNA synthesis we used β-actin as housekeeping gene. To estimate influence of infection in the expression levels of cytokines we use a comparative ($2^{-\Delta\Delta CT}$ – method) (Livak and Schmittgen, 2001). The fold change in the target gene was normalized to β-actin and standardized to the expression of naïve mice to generate a relative quantification of the expression levels.

3.5.7 Statistical analyses

Statistical analyses for murine studies were performed using unpaired Mann-Whitney U tests. Human cytokine data were compared using paired Mann-Whitney U tests. Results represent the mean ± SEM and the differences were considered significant at a p value of <0.05.

Linker to chapter 4

In the previous chapter 3 we aimed to address how a rodent model of hookworm, *N. brasiliensis,* activates the NLRP3 inflammasome, and the role of NLRP3 in regulate early immune responses against hookworm infections. We showed that NLRP3 may suppress the early innate immune response to helminth infection, and NLRP3 can potentially inhibit anti-parasitic neutrophil responses, thereby preventing the development of optimal immune protection against *N. brasiliensis* infections. Together with the results from Chapter 2, thus far we have demonstrated a critically important role for NLRP3 in suppressing immunity to two distinct species of helminths, via two distinct immunological mechanisms. However, NLRP3 is just one member of an increasingly large family of inflammasome complexes, which also includes members such as NLRP1, NLRP6, AIM2 and more, which have shown to play different biological roles depending on the inflammatory context. Hence in my last Chapter, I aim to determine if the NLRP1 inflammasome plays an important role in immunity to gastrointestinal helminths, and to begin to establish whether distinct inflammasomes play similar or different roles in controlling and innate and adaptive immune response to parasitic helminths.

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CHAPTER 4

4. The role of the NLRP1 in immunity to diverse gastrointestinal helminths

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4.1 Abstract

Type 2 immune responses are critical for protective immunity to gastrointestinal helminths, however helminths are proficient at suppressing host immune responses to enhance their survival. One mechanism by which helminths may modulate the host immune system is via activation of inflammasomes in innate immune cells, which can initiate non-protective Type 1 immune responses. We recently demonstrated roles for worm-induced activation of the NLRP3 inflammasome in suppressing innate and adaptive immunity to models of hookworm and whipworm infection in mice, however the roles for other types of inflammasomes during helminth infections are completely unknown. In this study, we examined the role of the NLRP1 inflammasome in immunity to the gastrointestinal helminths *Nippostrongylus brasiliensis* and *Trichuris muris*. Following *N. brasiliensis* infection, NLRP1 deficient mice displayed elevated protective Type 2 immune responses compared to wild type (WT) mice and reduced gut worm burdens, similar to what has been previously observed in NLRP3-deficient mice. However, unlike what is observed in NLRP3$^{-/-}$ mice, absence of NLRP1 did not result in enhanced early immunity to lung stages of *N. brasiliensis* infection suggesting that NLRP1 may be more involved in regulating gut-stage immunity to *N. brasiliensis* infection. Consistent with these results, NLRP1$^{-/-}$ mice also display increased gut-stage Type 2 immunity to infection with the gastrointestinal helminth *T. muris*. Together, these findings reveal that NLRP1 may be an important negative regulator of immunity to diverse gastrointestinal helminths.

4.2 Introduction

Type-2 immune responses are associated with the development of $CD4^+$ T helper Type 2 (Th2) cells, expression of the cytokines interleukin-4 (IL-4), IL-5, IL-9 and IL-13, IgE production and expansion of innate immune cells like eosinophils, mast cells and basophils, which coordinate a protective immune response against helminth infection (Anthony et al., 2007). Conversely, interferon-γ (IFN-γ)-dominant Type-1 immune responses to helminth infections can result in chronic infections and immunopathology(Anthony et al., 2007; Stadecker et al., 2004). The molecular and cellular mechanisms by which helminth infection results in acute resolving infections (and Type 2 immune responses), or chronic infections (and Type 1 responses) are highly complex and incompletely understood. Further, it is clear that helminths have evolved sophisticated mechanisms by which they manipulate the host immune response to enhance their survival (Maizels and Yazdanbakhsh, 2003). The cellular basis of this immunoregulation involves the modulation of both the innate and adaptive immune responses and the promotion of regulatory cell responses (Maizels and Yazdanbakhsh, 2003).

Recent research has highlighted one of the mechanisms by which helminths influence the host immune system to promote chronic infections is to activate innate immune receptors known as inflammasomes (Zaiss et al., 2013). Inflammasomes are multiprotein complexes expressed by a variety of immune cells, where their activation results in release of the proinflammatory cytokines IL-1β and IL-18, which are critical for inducing protective immunity to microbial pathogens (Dinarello and Fantuzzi, 2003; Sahoo et al., 2011; Sims and Smith, 2010). In contrast, multiple recent reports suggest that activation of these pathways by helminths is associated with suppression of protective Type 2 immune responses (Alhallaf et al., 2018; Zaiss et al., 2013). For example, we have recently showed that NLRP3 can suppress both innate and adaptive immunity against *T. muris* infection (Alhallaf et al., 2018), which is consistent with

reports from other models of helminth infection (Ritter et al., 2010; Zaiss et al., 2013). We also have highlighted the importance of the NLRP3 in limiting the early neutrophil-mediated immune responses against *N. brasiliensis* (unpublished). However, the innate immune system is comprised of many different types of inflammasomes that all respond to varying stimuli and exert diverse effector functions (Martinon et al., 2002). For example, the AIM2, NLRP1 and NLRC4 inflammasomes are important for anti-microbial defense (Ewald et al., 2014; Rathinam et al., 2010; Tomalka et al., 2011), but to date the only inflammasome that has been studied in the context of helminth infections is NLRP3. Hence whether distinct inflammasomes play different roles during helminth infections has never been examined.

Roles for NLRP1 in immunity and cell biology are being increasingly appreciated, where NLRP1 is a central regulator of programmed cell death (pyroptosis) in response to microbial stimuli (Masters et al., 2012; Moayeri et al., 2003; Terra et al., 2010). In addition, NLRP1 has recently been shown to be important for immunity to protozoan parasite infections (Clipman et al., 2018; Ewald et al., 2014). Comparison of the relative roles for NLRP1 with NLRP3 revealed that both inflammasome proteins play important roles in immunity of mice to *Toxoplasma gondii* (Gorfu et al., 2014), suggesting that along with NLRP3, NLRP1 may be an important sensor or regulator to infections with protozoan parasites, where there may be overlapping functions of distinct inflammasomes in our immune system. Whether NLRP1 plays a role in immunity to parasitic helminths has yet to be determined.

In the present study we have examined the role for the NLRP1 inflammasome in immunity to a model of hookworm *N. brasiliensis* and whipworm *T. muris*. Infections with *N. brasiliensis* resulted in NLRP1-dependent elevations in IL-18 and IL-1β secretion in the intestine, however *in vitro* activation of inflammasomes by worm-secreted antigens was primarily NLRP3-

dependent. Similar to results seen in NLRP3$^{-/-}$ mice, NLRP1$^{-/-}$ mice exhibited enhanced intestinal Type 2 immune responses and accelerated worm expulsion compared to wildtype mice. Interestingly however, NLRP1 does not play a substantial role in controlling the early innate immune response to *N. brasiliensis* infection in the lung, suggesting that there may be distinct mechanisms by which NLRP1 and NLRP3 control immunity to helminths in different tissues. Together these results highlighting the importance of the NLRP1 inflammasome regulates intestinal inflammation and immunity to diverse parasitic helminth infections.

4.3 Results

4.3.1 N. brasiliensis infection elicits NLRP1-dependent elevations in IL-18 and IL-1β secretion in the intestine.

Activation of the NLRP1 inflammasome has been demonstrated by anthrax lethal toxin (Moayeri et al., 2003) and *T. gondii* infection (Gorfu et al., 2014), however it is unclear if parasitic helminths also activate NLRP1 to promote secretion of IL-1β and IL-18. Hence we first assessed whether NLRP1 was important for *N. brasiliensis*-mediated inflammasome activation. C57BL/6 WT and NLRP1$^{-/-}$ mice were infected with *N. brasiliensis* and small intestinal IL-18 and IL-1β levels were measured at day 6 p.i. While WT mice exhibited significant increases in both gut IL-18 and IL-1β levels compared to naive control mice, NLRP1$^{-/-}$ mice displayed significantly reduced levels of these cytokines (**Figure 4.3.1 A-B**), suggesting that there is still evidence of NLRP1-independent activation which is likely due to NLRP3.

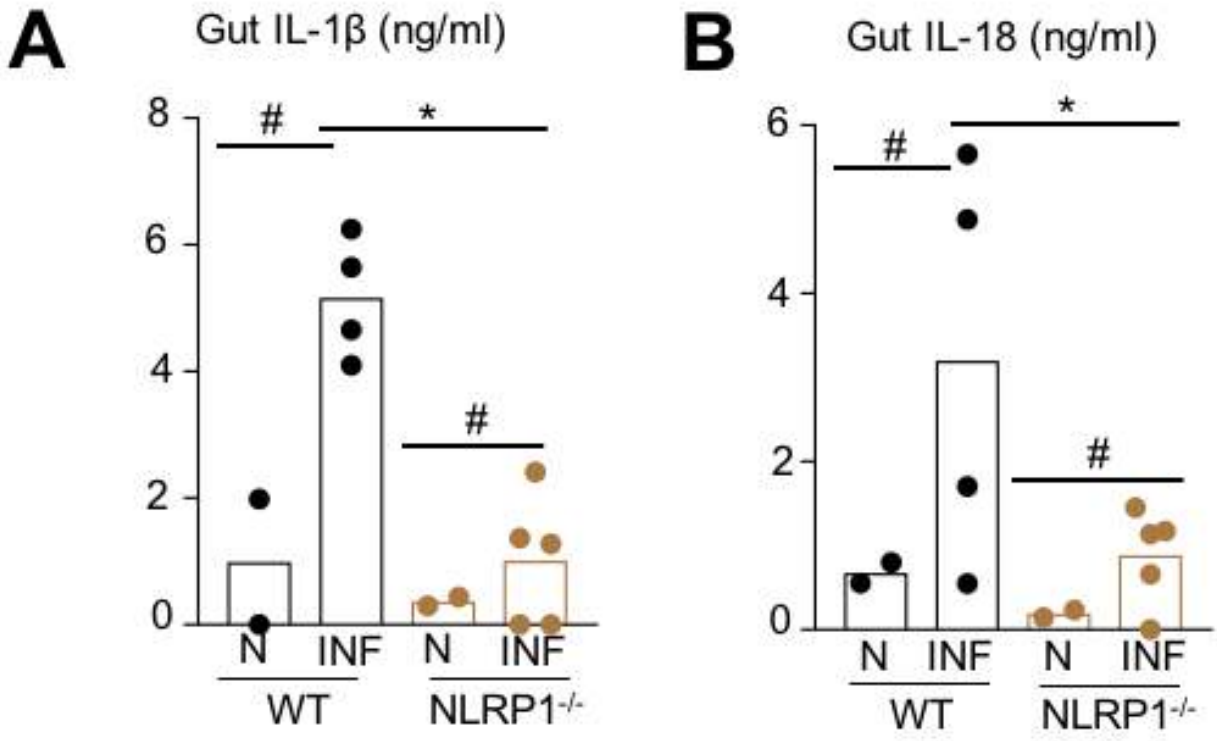


Figure 4.3.1. *N. brasiliensis* infection elicits NLRP1-dependent elevations in IL-18 and IL-1β secretion in the intestine. C57BL/6 WT and NLRP1$^{-/-}$ mice were infected with *N. brasiliensis* and sacrificed at d 6 post-infection (p.i.). (**A**) gut IL-1β and (**B**) IL-18 levels in small intestinal tissue homogenates were measured by ELISA. Data are expressed as mean with individual data points and are representative of 3 experiments, n=5 mice/group. # $p<0.05$ compared to naive (N). *$p<0.05$ compared to WT.

4.3.2 N. brasiliensis antigens do not significantly activate NLRP1 inflammasomes in vitro.

Our previous report demonstrated that one of the ways in which worms may activate inflammasomes is via the release of excretory/secretory (ES) proteins (Alhallaf et al., 2018). Hence we next addressed whether *N. brasiliensis* was able to activate inflammasomes *in vitro* in cultured macrophages, and whether this was dependent on NLRP1 or NLRP3. Bone marrow derived macrophages from WT, NLRP1$^{-/-}$ and NLRP3$^{-/-}$ mice were cultured for 6 hours in the presence and absence of native *N. brasiliensis* ES larvae, known inflammasome activators (LPS and nigericin), or culture media alone. Similar to what have been seen in *T. muris* (Alhallaf et al., 2018), culturing macrophages from WT mice with *N. brasiliensis* ES alone did not substantially increase secretion of IL-1β compared to treatment with culture media only. However, if known inflammasome activators (LPS and nigericin toxin) were included the levels of IL-1β rose significantly (**Figure 4.3.2 A**). Critically, this synergistic stimulation of IL-1β secretion by *N. brasiliensis* ES was significantly dependent on NLRP3. Absence of NLRP1 only caused a modest reduction in IL-1β secretion, suggesting that *in vitro* inflammasome activation by worm antigens appears to be more dependent on NLRP3 than NLRP1 (**Figure 4.3.2 A**).

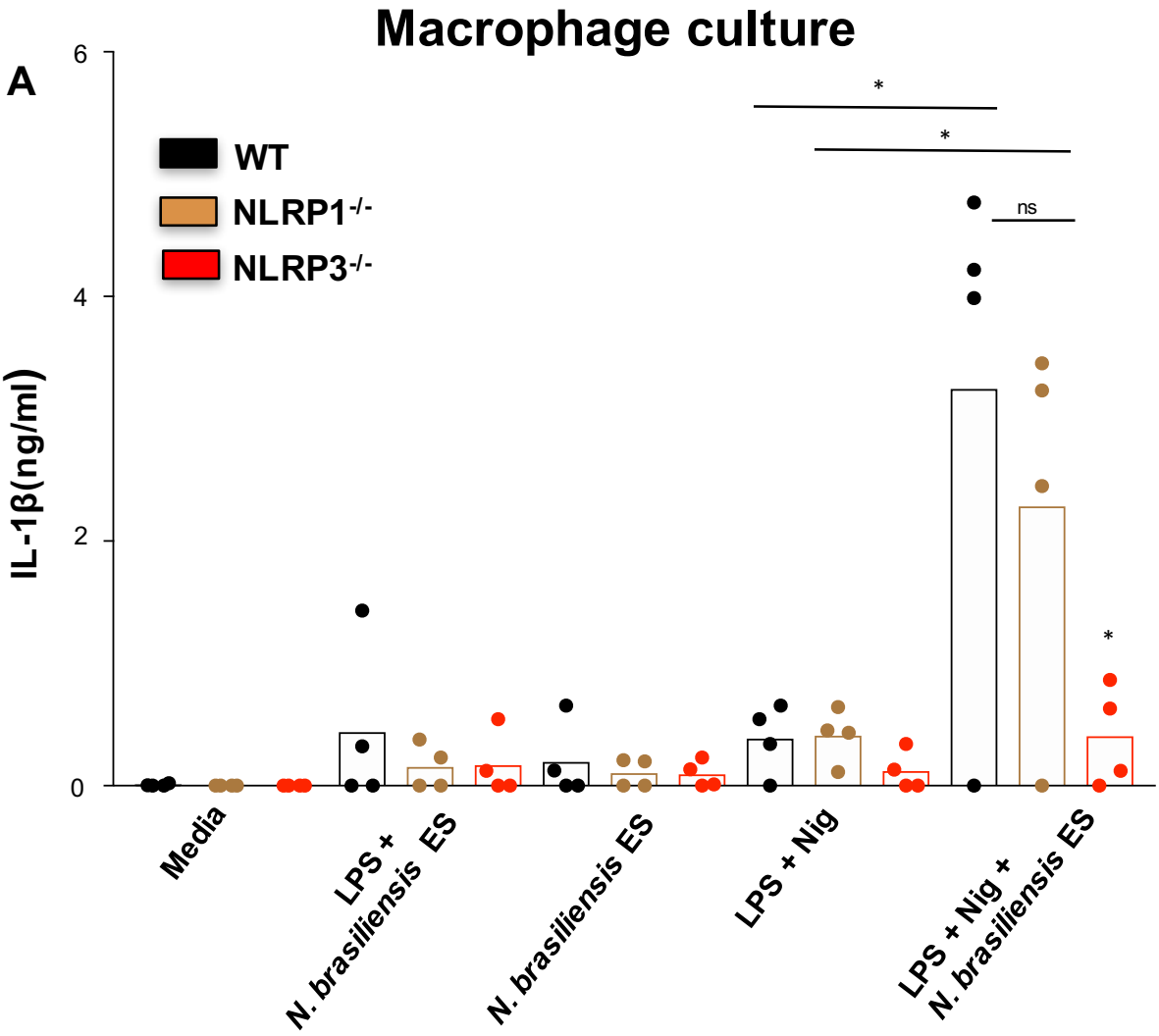


Figure 4.3.2. *N. brasiliensis*-mediated modest NLRP1 inflammasome activation *in vitro*. Bone marrow-derived macrophages were obtained from WT C57BL/6 mice, NLRP1$^{-/-}$ and NLRP3$^{-/-}$ mice and were cultured for 6 hours with combination of either 25 µg/ml of *N. brasiliensis* larvae, LPS (1 µg/ml)+nigericin (20 µM), or media only. IL-1β were measured in supernatants by ELISA. *p <0.05 compared to WT. ns denotes not significantly different.

4.3.3 NLRP1-deficient mice displayed reduced intestinal worm burdens following N. brasiliensis infections.

We next investigated whether NLRP1 plays a role *in vivo* in immunity to *N. brasiliensis.* C57BL/6 WT and NLRP1$^{-/-}$ mice were infected with *N. brasiliensis* and sacrificed at day 6 post infection, NLRP3$^{-/-}$ mice were included as a comparison. NLRP3$^{-/-}$ and NLRP1$^{-/-}$ mice had significantly reduced gut worm burdens (**Figure 4.3.3 A)**, and had significantly reduced faecal egg numbers, compare to WT mice (**Figure 4.3.3 B)**, and had visible increases in the presence of goblet cells compared to WT mice (**Figure 4.3.3 C)**. Together, these data suggest that both NLRP1 and NLRP3 play significant roles in suppressing immunity to *N. brasiliensis* infections at the level of the intestine. To determine whether NLRP1 can similarly suppress immunity to other gastrointestinal helminths, C57BL/6 WT and NLRP1$^{-/-}$ mice were infected with *T. muris* and we assessed the *in vivo* role for NLRP1 in protective immunity to *T. muris* infection. Similar to results seen in *N. brasiliensis* infections, NLRP1$^{-/-}$ mice had significantly reduced *T. muris* worm burdens (**Supplementary Figure 4.3.1 A)**, and displayed significantly increased frequencies and total numbers of eosinophils in the mesenteric lymph node (mLN) compared to WT mice (**Supplementary Figure 4.3.1 B-C**), suggestive of an increased Type 2 cellular immune response. This was consistent with increased IL-5 and IL-13 expression by Ag-restimulated mLN cells in NLRP1$^{-/-}$ mice compared to WT mice (**Supplementary Figure 4.3.1 D-E**)**,** had a trend toward reduced IFN-γ expression by Ag-restimulated mLN cells compare to WT (**Supplementary Figure 4.3.1 F**), and had displayed evidence of decreased intestinal inflammation compared to WT mice, and significantly reduced caecal pathology scores. Together these data suggest that NLRP1 limits Type 2 immune responses following *T. muris* infections (**Supplementary Figure 4.3.1 G-H**).

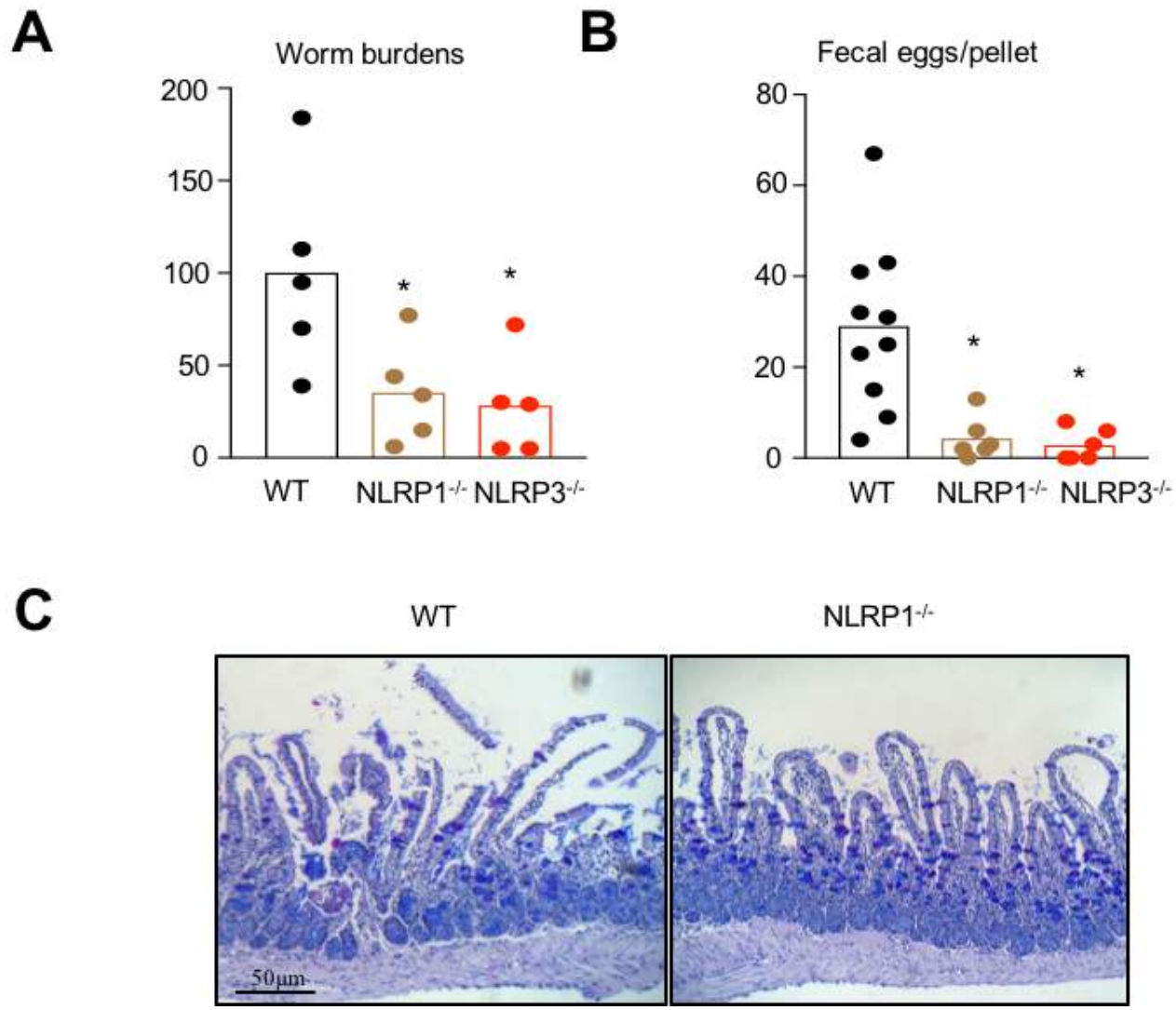


Figure 4.3.3. NLRP1 deficient mice displayed reduced intestinal worm burdens following *N. brasiliensis* infections. C57BL/6 WT and NLRP1$^{-/-}$ mice were infected with *N. brasiliensis* and sacrificed at d 6 post-infection (p.i.). (**A**) small intestine worm burdens and. (**B**) Fecal eggs numbers per pellet. (**C**) Representative images of PAS/Alcian Blue stained small intestine. Data are expressed as mean with individual data points and are representative of 4 experiments, n=5 mice/group. *p <0.05 compared to WT.

4.3.4 NLRP1 deficient mice display a trend toward to increase type 2 immune responses following N. brasiliensis infections.

Previous reports have shown that NLRP3 can suppress Type 2 immunity to both *T. muris* and *N. brasiliensis* infections, however it is unclear if NLRP1 plays a similar or different role in immune regulation. To investigate the immunological mechanisms by which NLRP1 controls immunity to *N. brasiliensis* infections. C57BL/6 WT and NLRP1$^{-/-}$ mice were infected with *N. brasiliensis* and we assessed the immune responses in WT and NLRP1$^{-/-}$ mice. NLRP1$^{-/-}$ mice displayed a trend toward increased frequencies and total numbers of eosinophils in the mesenteric lymph node (mLN) compared to WT mice (**Figure 4.3.4 A-B**), consistent with an elevated cellular immune response. NLRP1$^{-/-}$ mice displayed increased IL-5 and IL-13 expression in the small intestine compared to WT mice (**Figure 4.3.4 C-D)** and had a trend toward reduced IFN-γ levels in the small intestine (**Figure 4.3.4 E)**. Together, these data suggest that NLRP1 may play a limited role in inhibiting protective Type-2 immunity following *N. brasiliensis* infections.

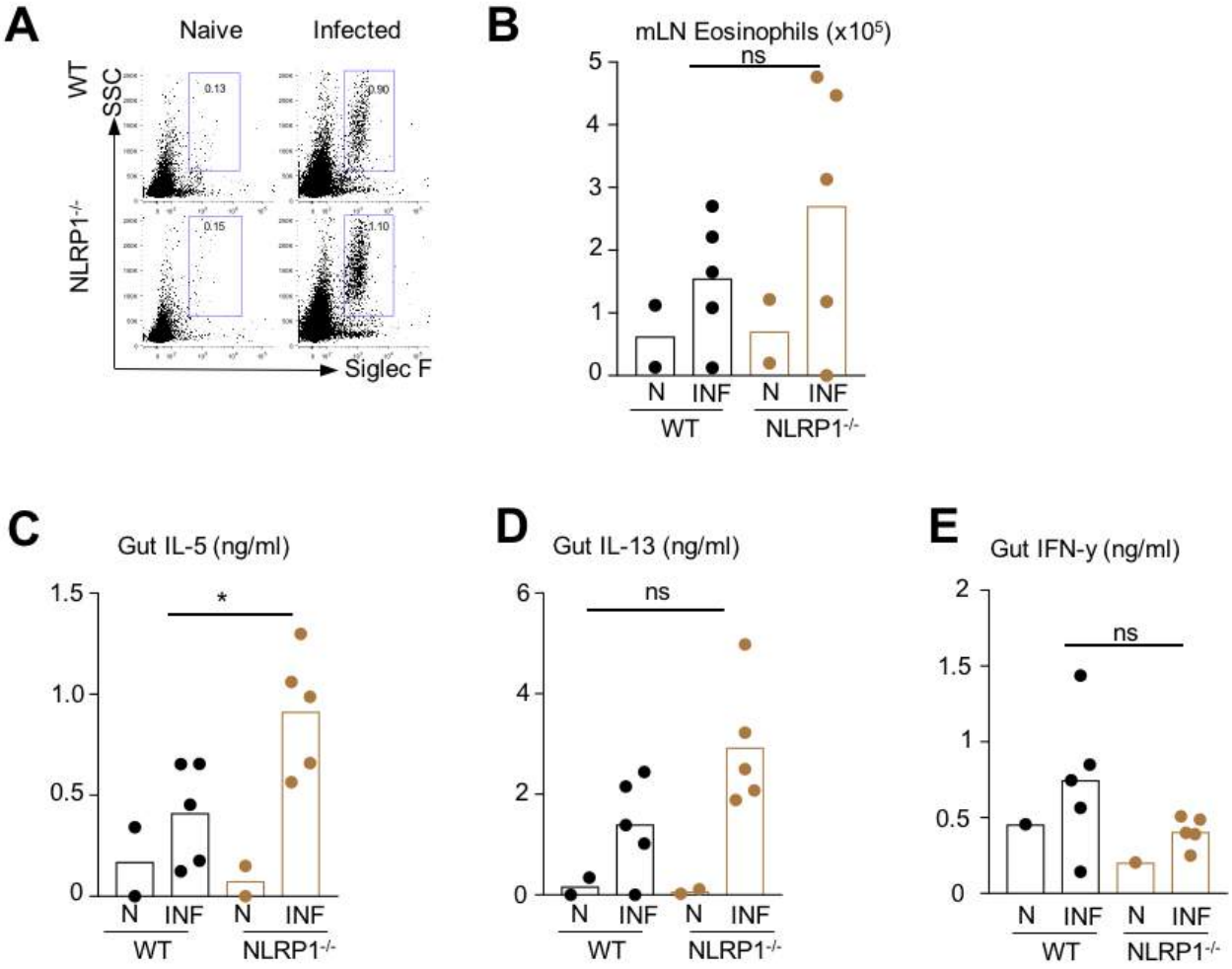


Figure 4.3.4. NLRP1 deficient mice display a trend toward to increase type 2 immune responses. C57BL/6 WT and NLRP1$^{-/-}$ mice were infected with *N. brasiliensis* and sacrificed at d 6 post-infection (p.i.). (**A**) Representative plots displaying Siglec F^{+} eosinophils frequencies in the mesenteric lymph nodes (mLN) at d 6 p.i. (**B**) Total mLN eosinophils (**C**) Small intestine homogenate IL-5 (**D**) Small intestine homogenate IL-13 and (**E**) Small intestine homogenate IFN-γ. Data are expressed as mean with individual data points and are representative of 3 experiments, n=5 mice/group. *p <0.05 compared to WT. ns denotes not significantly different.

4.3.5 NLRP3 is more important than NLRP1 for early immune responses in the lung following N. brasiliensis infection.

We have previously highlighted that NLRP3 suppresses very early innate immunity to *N. brasiliensis* in the lung, potentially by limiting neutrophil recruitment to the lung. Hence, we next aimed to address whether NLRP1 plays a similar role at the lung stage of *N. brasiliensis* infection. WT, NLRP1$^{-/-}$ and NLRP3$^{-/-}$ mice were infected with *N. brasiliensis* and lung larval burdens and cellular infiltrates were assessed at day 2 post-infection. As expected NLRP3$^{-/-}$ mice had significantly reduced worm burdens, however NLRP1$^{-/-}$ mice had modest, non-significant reductions in worm burdens compared to WT mice (**Figure 4.3.5 A**), suggesting a limited role for NLRP1 in suppressing lung-stage immunity to *N. brasiliensis*. Comparison of lung neutrophil, macrophage and eosinophil numbers showed that as expected NLRP3$^{-/-}$ mice had significantly increased frequencies and total number of neutrophils compared to WT mice (**Figure 4.3.5 B-C**). However, NLRP1$^{-/-}$ mice did not display elevated neutrophil responses in the lung (**Figure 4.3.5 B-C**), suggesting that there are distinct mechanisms by which the different inflammasome proteins NLRP1 and NLRP3 control tissue-specific immunity to worms.

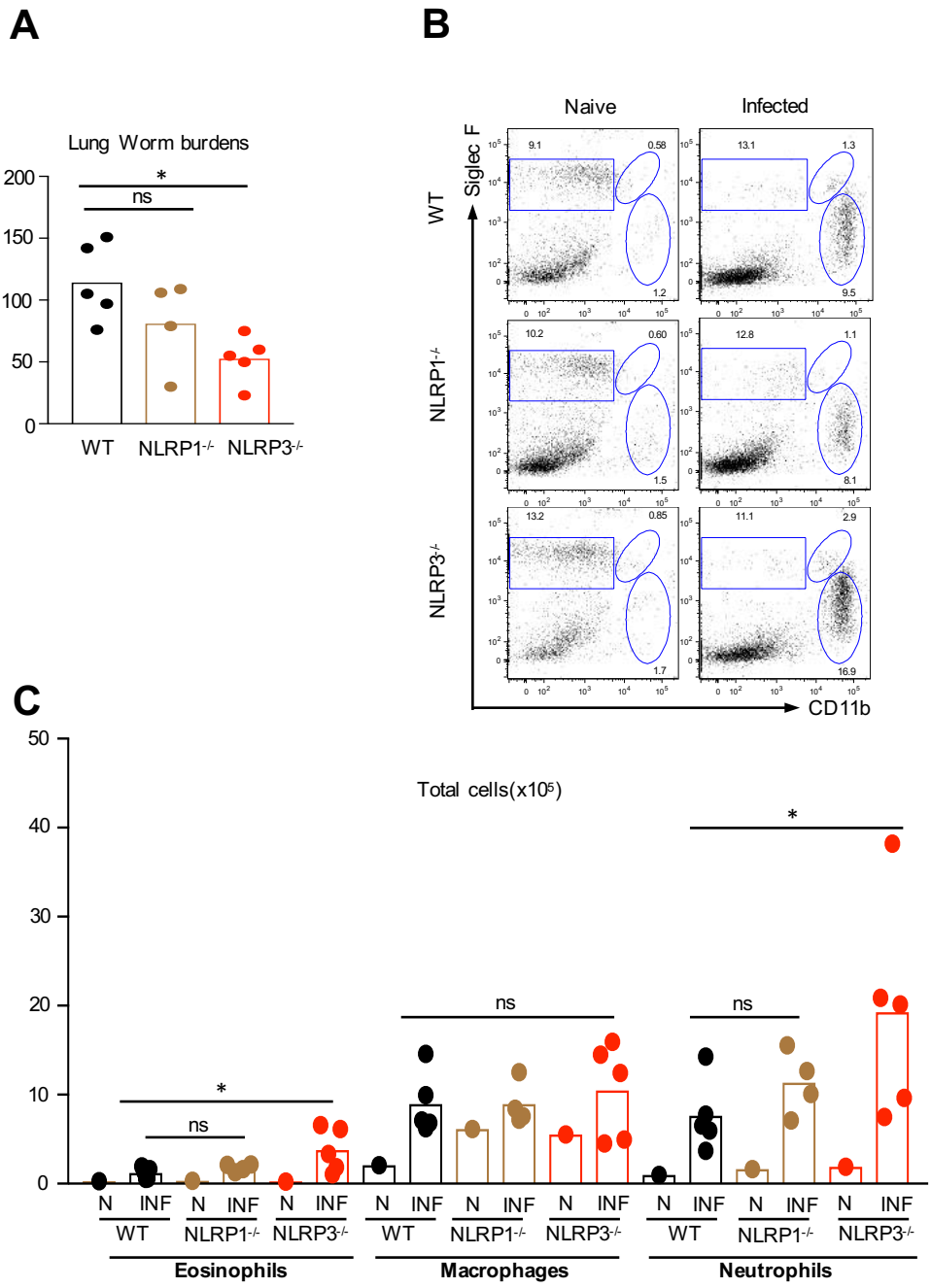
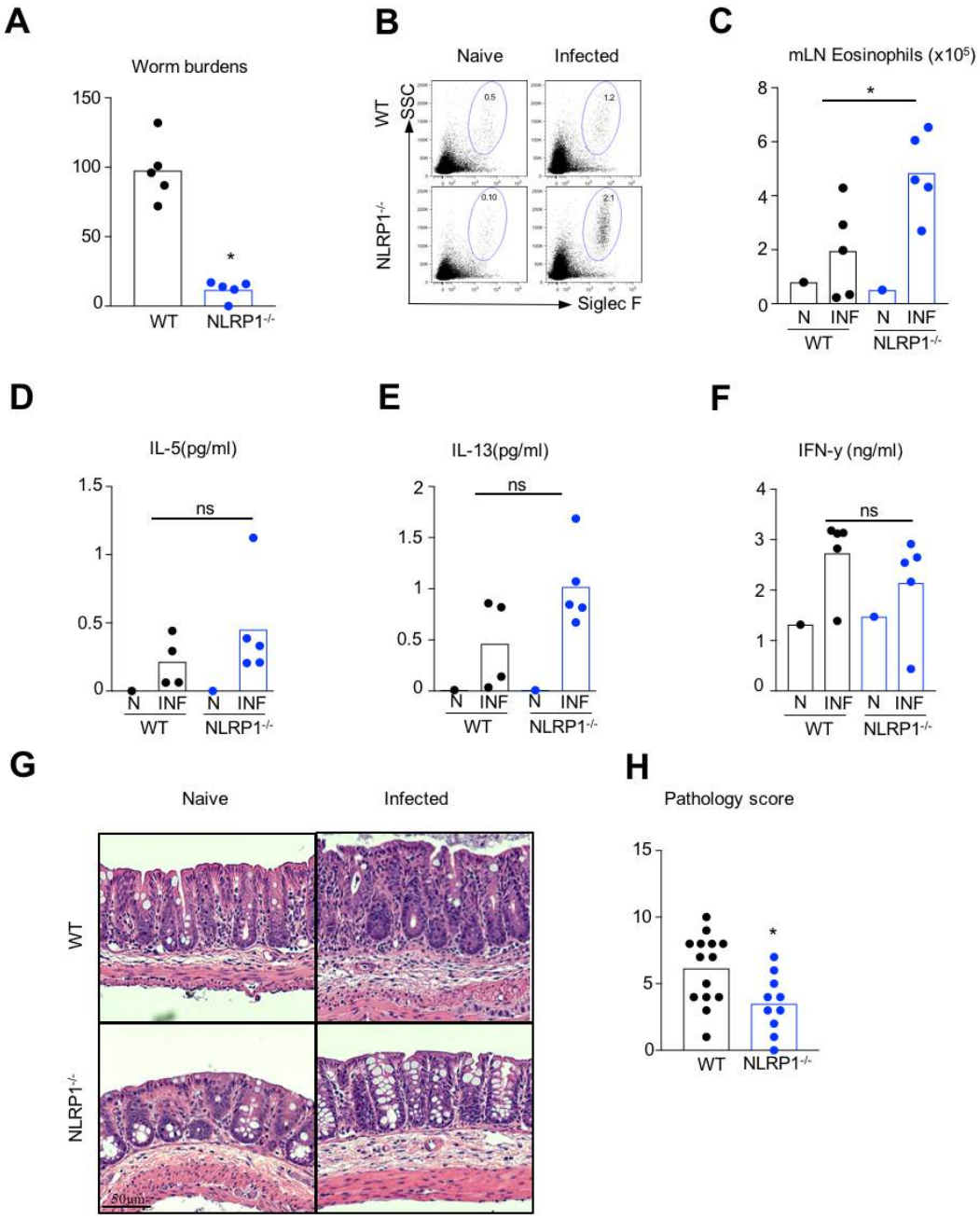


Figure 4.3.5. NLRP3 is more important than NLRP1 for early immune responses in the lung following *N. brasiliensis* infection. WT, NLRP1$^{-/-}$ and NLRP3$^{-/-}$ mice were infected (INF) with *N. brasiliensis*, worm burdens and lung cellular immune responses were determined at d 2 p.i. (n=5 mice/group). (**A**) Lung worm burdens. (**B**) Representative plots displaying of Siglec F^{+} CD11b^{+} eosinophils, Siglec F^{-} CD11b^{+} neutrophils and Siglec F^{+} CD11b^{int} macrophages frequencies in lung tissue at d 2 p.i. (**C**) Total lung eosinophils, macrophages and neutrophils. Data are expressed as mean with individual data points and are representative of 2 experiments, n=5 mice/group. *p <0.05 compared to WT. ns denotes not significantly different.



Supplementary figure 4.3.1. NLRP1 inflammasome inhibits Th2 immunity and induce worm burdens. WT and NLRP1$^{-/-}$ mice were infected with 200 *T muris* eggs and sacrificed at d 21 post-infection (p.i.). (**A**) Cecal worm burdens at d 21 p.i. (**B**) Representative plots displaying Siglec F^{+} eosinophils frequencies in the mesenteric lymph nodes (mLN) at d 21 p.i. (**C**) Total mLN eosinophils at d 21 p.i. (**D, E and F**) *T . muris* antigen-specific IL-5, IL-13 and IFN-γ production by restimulated mLN cells. (**G**) Representative images of H&E stained cecum at d 21 p.i. Scale bar=50µm. (**H**) Blinded scores of cecal pathology, pooled from 2 experiments. Data are expressed as mean with individual data points and are representative of 2 experiments, n=5 mice/group. *p <0.05 compared to WT. ns denotes not significantly different.

4.4 Discussion

Type 2 immune responses are important for immunity to parasitic helminths, however it is clear that helminths are able to evade this immune response to enable their survival within their host (Maizels and McSorley, 2016). Previous research shown that inflammasomes, particularly NLRP3 may be important for suppressing protective immune responses to helminth infection, resulting in immunopathology and chronic infections. In present paper we showed that a different inflammasome pathway, involving NLRP1, is also important for controlling intestinal immune responses following infection with a mouse model of hookworm, *N. brasiliensis* and a model of whipworm infection, *T. muris*. However, data from this report and previous studies support the notion that NLRP3 appears to play a more substantial role in immunity to helminths, in particular lung-stage immunity to *N. brasiliensis* and activation of inflammasomes *in vitro.* Together it is evident that there are multiple distinct inflammasomes that may play important roles in immunity to invading pathogens such as parasitic helminths, and these inflammasomes may play different functions depending on the nature of the pathogen and the tissue that is infected.

Our results suggest that *N. brasiliensis* infection induce NLRP1 and NLRP3 inflammasomes activation *in vitro* and *in vivo*, therefore this study represents the first report of activation of multiple inflammasomes by a helminth pathogen. These results are consistent with those seen during other infectious diseases, such as *Salmonella* infection (Qu et al., 2016), and in *Toxoplasma* infection. In these studies it was observed *Toxoplasma* infection of murine bone marrow-derived macrophages primarily activates the NLRP3 and NLRP1 inflammasomes, leads to rapid secretion of IL-1β (Gorfu et al., 2014). Similar activation of NLRP1 was observed in rat macrophages (Cirelli et al., 2014). Together, these studies using diverse pathogens have shown that exposure to infectious stimuli can activate multiple pathways to inflammasome activation, which may lead to distinct downstream mechanisms of immunity to the pathogen.

Data from the present study suggest that NLRP1 may be important for controlling the intestinal phases of both *N. brasiliensis* and *T. muris* infections, where NLRP1 deficient mice displayed increased cellular immune responses, measured by mLN eosinophils, IL-5, IL-13 and accelerated worm expulsion. However, taking into consideration data from previous studies and the present study, it appears as if NLRP3 may be more important for innate immunity within the lung. For example, NLRP3 is more essential for the rapid neutrophil and eosinophil response in the lung. We were not able to define the precise mechanism of how NLRP1 controls innate or adaptive immunity to gastrointestinal helminths within the intestine. NLRP1 may influence the balance of Type 1 and Type 2 cytokine responses, where in the absence of NLRP1 there is a more biased Type 2 immune response and enhanced protective immunity. There is also a possibility that NLRP1 and NLRP3 may be expressed on different cells that may explain their different roles in regulating immunity. For example, the reason why NLRP3 is more important for innate immunity to *N. brasiliensis* is that it may be expressed on innate immune cells, while NLRP1 is more restricted to adaptive immune cells. Further studies should examine the nature of the intestinal cellular immune responses and immune gene expression in NLRP1 deficient mice that would help define a specific molecular or cellular pathway that is up-regulated in NLRP1-deficient mice that may contribute to enhanced Type 2 immunity.

Our results from chapter 2 highlighted the importance of *T. muris* in activate the NLRP3 inflammasome *in vitro* and in *vivo*, however, in present chapter we found that in macrophages, activation of inflammasomes by *N. brasiliensis* antigens was more dependent on NLRP3 than NLRP1 (see Fig 4.3.2). However, our *in vivo* studies revealed that NLRP1 may control infection-induced IL-18 and IL-1β levels in the gut, similarly to NLRP3 (see chapter 3 Fig 3.3.1), hence there must be some NLRP1-dependent activation of inflammasomes that is occurring *in vivo*. There may potentially be other gut-resident cells that are activating inflammasomes *in vivo,* for example epithelial cells, dendritic cells and neutrophils.

Alternatively, intestinal macrophages may differ from bone marrow macrophages in their responsiveness to *N. brasiliensis* and its secreted antigens. Overall, there are very few known factors that stimulate NLRP1 inflammasome activation, hence further studies to investigate the potential worm-derived factors, or host-derived factors (such as damage-associated molecules or commensal bacteria) would be important for fully elucidating the mechanism of how NLRP1 controls anti-parasitic immune responses.

In summary, we establish that both NLRP1 and NLRP3 are important *in vivo* regulators of gut stage following *N. brasiliensis* infection. Our findings also indicate that *in vivo* and *in vitro* innate resistance to lung stage following *N. brasiliensis* infection is more NLRP3 dependent than NLRP1, indicate that there are distinct mechanisms by which different inflammasomes control immunity *N. brasiliensis* infection. Further investigations are needed to identify the mechanisms of the NLRP1 pathway in regulation of Type 2 immune responses and whether other inflammasomes (such as AIM2, NLRP6, NLRP12 and NLRC4) also play roles in immunity to parasitic helminths.

4.5 Materials and Methods

4.5.1 Parasitological techniques

4.5.1.1. Nippostrongylus brasiliensis

N. brasiliensis was maintained in Sprague–Dawley rats (Animal Resources Centre, Perth, WA, Australia) and infective L3 were prepared from rat fecal cultures. Mice were infected by 500 L3 subcutaneously.

4.5.1.2 Trichuris muris

T. muris was maintained in genetically susceptible or immunocompromised mouse strains and *T. muris* excretory/secretory (ES) antigens were obtained as described previously (Artis et al., 1999). Mice were infected by oral gavage with approximately 200 embryonated *T. muris* eggs. *Trichuris*-Ag specific cytokine responses in mLN cells were assayed by ELISA.

4.5.2 Mice and treatments

NLRP1-deficient, NLRP3-deficient mice on C57BL/6 genetic background, and parental C57BL/6 wild-type control mice were bred and maintained at James Cook University (JCU), Cairns Campus. Male and female mice between 6 and 8 weeks of age were used. All experimental protocols were approved by the JCU Animal Ethics Committee

4.5.3 Mouse tissue collection and processing

At necropsy, Lung tissue was minced and incubated with stirring at 37 °C for 30 min in HBSS with 1.3 mM EDTA (GIBCO), followed by treatment at 37 °C for 1 h with collagenase (1 mg/ml; Sigma) in RPMI 1640 (Mediatech) with 5% FCS (Biowest) and with 100 µg/ml of DNase (Sigma) for 10 min. Cells were lysed with ACK Lysing Buffer (Lonza) to remove erythrocytes, blocked with Fc Block (BD Biosciences).

Single cell suspensions of mLN tissue were prepared by passing through 70 μm nylon mesh filters. For small intestine or colon tissue homogenates, 1 cm of tissue was mechanically homogenized in 0.5 mL of PBS using a Tissue Lyzer (QIAGEN) and supernatants were assayed for IL-5, IL-13, IL-1β and IL-18 using standard sandwich ELISA protocols (eBioscience or ELISAkit.com.au). Small intestine or colon tissue was fixed in 4% formaldehyde and embedded in paraffin, and 5 μM sections were stained with H&E or PAS/Blue stains using the standard protocol of an institutional histology service provider (QIMR Berghofer Medical Research Institute). For determination of worm burdens, small intestines or colons were collected and worms were counted using a dissecting microscope. Lung larval burdens were assessed by mincing the mouse lungs with scissors in PBS and counting emigrated larvae under a dissecting microscope.

4.5.4 In vitro bone marrow macrophage assays

Bone marrow was isolated from hind legs of C57BL/6, NLRP1 and NLRP3$^{-/-}$ mice by flushing bones with DMEM and cells were cultured at 1×10^6 cells/ml in DMEM supplemented with 10% FBS, 5% horse serum (Invitrogen), 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate and 10 ng/ml M-CSF. On day 6, macrophages were harvested and plated at a concentration of 2×10^5 cells/ml. The following day, cells were treated with 25 μg/ml of *Nippostrongylus*, LPS (1μg/ml)+nigericin (20 μM) Sigma-Aldrich, or media only for 6 hours, and cell-free supernatants were stored at -80°C.

4.5.5 Flow cytometry

Cell preparations were surface-stained with anti-mouse fluorochrome-conjugated mAbs against

CD11b (M1/70), CD11c (HL3), Gr-1 (RB6-8C5), Ly6G (1A8), and Siglec F (E50-2440) Cells were stained with cell surface Ags and then with fixation and permeabilisation using a commercial kit (BD Biosciences). Cells were analysed by flow cytometry using a FACS Canto II or Aria III cytometer (BD Biosciences) where dead and doublet cells were excluded by the use of a live gate and doublet discrimination. Further analysis was performed using FlowJo software (Tree Star, Inc).

4.5.6 Statistical analyses

Statistical analyses for murine studies were performed using unpaired Mann-Whitney U tests. Mice cytokine data were compared using paired Mann-Whitney U tests. Results represent the mean ± SEM and the differences were considered significant at a p value of <0.05.

5. General Discussion

5.1 Overview

Helminth parasites infect almost one-third of the human population, rendering them among the most predominant infectious diseases in the world (Hotez et al., 2008). Additionally, helminths are a prevalent problem in the livestock industry where they are considered a major economic burden. These infections can also be responsible for promoting long-term chronic infections with other pathogens due to downregulation of immune responses (Elliott et al., 2007; Hoerauf et al., 2005; Maizels and Yazdanbakhsh, 2003). The eradication of helminthic infections remains difficult because of a lack of effective vaccines, inadequate anthelminthic drug treatment efficacy, emerging drug resistance, and rapid reinfection. Since helminth parasites are masters of evading the immune system, it is imperative that more research goes into the immunological basis of why some helminth infections become chronic and some are efficiently expelled, so that the power of the immune system can be unleashed to fight these pathogens.

Data presented in this dissertation provide evidence that inflammasomes, which had previously been known for being important for immunity to small microbial pathogens, play an important role in whether infections with helminths are acutely expelled, or become chronic with increased worm burdens. Specifically, I have shown that the NLRP3 and NLRP1 inflammasomes regulate cellular and molecular immune responses to multiple species of gastrointestinal helminths, the whipworm *T. muris* and hookworm *N. brasiliensis*, by a range of potential biological mechanisms including actions on the innate and adaptive immune system. I have identified that targeting the NLRP3 inflammasome therapeutically results in a boost in protective Type-2 immune responses, reduced worm burdens and decreased immunopathology associated with infection. Furthermore, we have identified that inflammasomes may also regulate the innate neutrophil-mediated immune responses to the

hookworm *N. brasiliensis.* Together, my research project has shed new light on a previously under-researched topic, that is how innate immune receptors such as inflammasomes are activated or inhibited and most importantly identified some molecular and cellular mechanisms by which these inflammasomes regulate immunity to infections.

5.2 Activation of inflammasomes by helminths.

Inflammasomes are important mediators of immunity to pathogens, however their roles in immunity to parasitic helminths has been under-studied. Inflammasome formation is triggered by a range of substances that emerge during infections, tissue damage or metabolic imbalances (Pelegrin and Surprenant, 2007; Vilaysane et al., 2010). ASC is composed of an N-terminal PYD domain and a CARD, which interacts directly with multiple PRRs, such as NLRPs, NLR caspase recruitment domain-containing protein (NLRC), and AIM2, to form caspase 1-activating platforms termed inflammasomes (Martinon et al., 2002; Masumoto et al., 1999). Once the protein complexes have formed, the inflammasomes activate caspase 1, which proteolytically activates the pro-inflammatory cytokines IL-18 and IL-1β (Martinon et al., 2002; Masumoto et al., 1999). However, the mechanisms underlying both the activation and regulation of this inflammatory pathway during helminth infections has been unclear. Here we have provided evidence that helminths such as *T. muris* and *N. brasiliensis*, and their excretory/secretory products and bacteria serve as an integral activating signal for the NLRP3 and to a lesser extent, NLRP1 inflammasomes.

The NLRP3 inflammasome can be activated in response to a range of stimuli including those of microbial, endogenous and exogenous origins. However, the precise pathway at which these stimuli converge to activate NLRP3 still unknown. For instance, exogenous pathogens can activate the NLRP3 pathway such as Sendai virus (Kanneganti et al., 2006), influenza virus (Ichinohe et al., 2009; Kanneganti et al., 2006), adenovirus (Muruve et al., 2008),

Staphylococcus aureus (Mariathasan et al., 2006), *Listeria monocytogenes* (Mariathasan et al., 2006; Warren et al., 2008) and *Shigella flexneri* (Willingham et al., 2007) . Further, bacterial pore forming toxins can also activate this pathway; nigericin (*Streptomyces hygroscopicus*), maitotoxin (*Gambierdiscus toxicus*), aerolysin (*Aeromonas hydrophila*) and listeriolysin O (*L. monocytogenes*) are also responsible for activating caspase-1 in a NLRP3 dependent manner (Gurcel et al., 2006; Mariathasan et al., 2006). In contrast, less is known about how the NLRP1 inflammasome can be activated, where the *B. anthracis* lethal toxin (LeTx) (Boyden and Dietrich, 2006), and peptidoglycan (PGN) or its degradation product muramyl dipeptide (MDP) (Faustin et al., 2009), have been shown to be responsible. In our studies, we reported that adult worms from *T. muris* and *N. brasiliensis* release factors that can promote NLRP3-dependent IL-18 and IL-1β secretion *in vivo* and *in vitro*. A role for NLRP1 in *in vitro* inflammasome activation by helminth antigens was not clearly defined. Interestingly, we found that worm secreted factors do not induce NLRP3-dependent IL-1β and IL-18 secretion alone, since signals from known inflammasome activators (e.g. LPS and nigericin) were also needed. These data suggest that worm molecules may have a role as a "second signal" in this system that boosts NLRP3 activation in the presence of signals from other microbes. This may be important in the context of an intestinal bacterial infection where the parasite is feeding and disrupting the epithelial barrier, potentially exposing the host to increased stimuli from resident commensal or pathogenic bacteria. It would be interesting to conduct *in vivo* studies to determine whether bacteria are also necessary for *in vivo* inflammasome activation by helminths (i.e. using germ-free mice or antibiotic treatments), however these studies would have some practical limitations in that helminths such as *T. muris* can't establish efficient infections in the absence of commensal flora (Hayes et al., 2010).

Our research has not addressed the precise worm-derived molecule(s) responsible for inflammasome activation, or the receptor for the worm-derived molecules which has been highlighted in schistosomes (Ritter et al., 2010). However we did make some progress in identifying that purified extracellular vesicles (exosomes) produced by the *T. muris* worm can promote NLRP3 activation as efficiently as native ES proteins. Helminth-derived exosomes are becoming increasingly appreciated for their roles in immune modulation by parasitic helminths (Buck et al., 2014), where they contain hundreds of proteins, micro-RNAs and mRNAs that can mediate host-pathogen interactions (Eichenberger et al., 2018). Our study was the first evidence that helminth-derived exosomes can activate inflammasomes and future studies could explore the potential factors within exosomes that mediated these effects. Understanding which molecules activate /inhibit the inflammasomes may lead to significant outcome for the development of new strategies cure gastro-intestinal helminth infections. For instance, a novel immune modulatory strategy used by *F. hepatica* involves secretion of the FhHDM-1, a cathelicidin-like protein, which impairs the activation of NLRP3 by lysosomal cathepsin B protease, preventing the downstream production of IL-1β and Type-1 immune responses (Alvarado et al., 2017). It is conceivable that other worms may secrete an array of factors that may inhibit inflammasome activation, which could be identified, purified and used in human autoimmune diseases such as IBD, rheumatoid arthritis or Type 1 diabetes to dampen damaging Type-1 cytokine responses. Alternatively, if we can identify a specific worm-derived molecule that activates inflammasomes, which may act to enhance its ability to survive in its host, there may be an opportunity to design a vaccine or chemical inhibitor that will block worm-induced inflammasome activation and maybe accelerate immunity to the worm. This would have advantages over current methods of inhibiting NLRP3 using chemical inhibitors such as MCC950 which would block all inflammasome activity and hence may have side effects such as increased susceptibility to infectious pathogens. Together, there is much work to do beyond

my research project to define how a range of species of worms, and their secreted products, influence activation of the various inflammasome pathways and progress in this area could lead to significant outcomes for both parasitic diseases, and inflammatory diseases.

In addition to worm-secreted factors and signals from commensal microbes, there are likely other mechanisms by which a worm infection could promote or inhibit inflammasome activation. It has revealed that pannexin hemichannel can activate the NLRP3 inflammasome mostly in response to ATP, through the P2X7 receptor, nigericin and maitotoxin (Pelegrin and Surprenant, 2006, 2007). We found that the ATP-$P2X_7R$ pathway does appear to control *N. brasiliensis* infections in the same way as NLRP3, suggesting that during *N. brasiliensis* infections, the presence of larvae within the lungs and the damage they cause when they traverse from the lung vasculature into the airways (potentially generating damage-associated factors like ATP) may be an important factor in inflammasome activation within the lung. This was in contrast to what we observed in *T. muris* infections in the gut, where $P2X_7R$-deficient mice did not have the same phenotype as NLRP3-deficient mice, suggesting that there are clear differences in the mechanisms by which worms activate inflammasomes, depending on the type of worm and tissue infected.

Our data demonstrated that *N. brasiliensis and T. muris* molecules activate NLRP3 and promote IL-18 and IL-1β secretion by macrophages *in vitro.* However, were we unable to determine if macrophages were responsible for *in vivo* infllammasome activation. Further studies such as looking at intestinal macrophages in naive and helminth infected mice to assess inflammasome activation by cleavage of Caspase-1, ASC speck formation and IL-18/IL-1β production would more convincingly show that macrophages respond to helminth infection to activate inflammasomes, or whether other cells may be also important for the inflammasome activation *in vivo*. For example, it has been reported that antigen-specific $CD8^+$ T cell feedback activates

NLRP3 inflammasome in antigen-presenting through perforin (Yao et al., 2017). Moreover, it has been revealed that dendritic cells activate NLRP3 inflammasome against *Chlamydia* infection (Omosun et al., 2015), and against *Histoplasma capsulatum* (Chang et al., 2017). Further, It has demonstrated that human neutrophils express NLRP3 and secreted IL-1β against *H. Pylori* infections (Perez-Figueroa et al., 2016). Further, Natural killer cells can also activate NLRP3 in response to colorectal cancer (Dagenais and Saleh, 2016). Hence, it would be interesting to conduct *in vitro* studies to determine whether other cells such as T cells, dendritic cells, NK cells and neutrophils are also important for the NLRP3 inflammasome activation in responsible to helminths.

5.3 Mechanism(s) by which NLRP3 and NLRP1 regulate immune responses following helminth infections

In the present studies we identified a novel role for the NLRP3 inflammasome in manipulating both innate and adaptive immunity of mice to a whipworm *T. muris* infection, where regulation of IL-18 expression may be the primary mechanism. Targeting this pathway therapeutically with a chemical inhibitor MCC950 was able to exaggerate Type-2 immune-mediated clearance of the parasite, demonstrated the importance of NLRP3 in suppressing immunity to *T. muris.* Studies outlined in Chapter 4 demonstrated that NLRP1 appears to play a similar role in suppressing immunity to *T. muris*, where NLRP1-deficient mice displayed elevated protective Type 2 immune responses associated with reduced gut worm burdens at day 21 post-infection. However, there is less evidence that this mechanism is mediated by IL-18 and IFN-γ-dominated Type-1 immune responses, which is what was observed for NLRP3 (see results from Chapter 2). In contrast, NLRP1 may act via some other mechanism, for example by directly inhibiting the Type 2 immune response. This may be a role for NLRP1-mediated IL-1β, which would be consistent with a previous study where IL1β suppresses Type 2 immunity by inhibiting IL-25

responses in the gut (Zaiss et al., 2013). Similarly, another study has reported that IL-1β secretion is a critical regulator of group 2 innate lymphoid cell function and plasticity (Ohne et al., 2016). Unfortunately, I was unable to confirm whether IL-1β secretion was directly associated with boosting Type-2 immune responses, or if there was a different NLRP1-mediated mechanism of how immunity to *T. muris* was suppressed. In any case, ours is the first study to report the roles for multiple distinct inflammasomes in immunity to helminths and they support findings from studies with protozoan parasite infections where both NLRP1 and NLRP3 both play significant roles(Gorfu et al., 2014) . These finding suggest that significant redundancy exists within the immune system where multiple proteins/factors play similar or overlapping roles. This has the benefit of enabling protection of the host from infectious challenge if a particular inflammasome is non-functional due to genetic polymorphism, i.e. another inflammasome may be sufficient to help fight the infection and ensure survival of the affected host.

My studies have shown that IL-18 may be a critical downstream regulator of NLRP3-dependent immune responses but questions still remain as to how IL-18 functions to regulate immunity to helminths. IL-18, which was originally termed IFN-γ-inducing factor, was fundamentally considered a T_H1-inducing cytokine, and IL-18R is expressed by NK cells, Th1 cells, macrophages and dendritic cells in response to IL-12 stimulation (Yoshimoto et al., 1998). Hence in our *T.muris* studies is remains possible that IL-18 may be engaging with one of these target cells to exert its biological effect. For example, NK cells or ILCs promptly produce effector cytokines such as IFN-γ in response to stimulation with IL-18 and data from Chapter 2 support a potential role for ILC1s in helminth-induced immune regulation via NLRP3 (Cella et al., 2014; Diefenbach et al., 2014; McKenzie et al., 2014). In addition, IL-18 is critically important in controlling intestinal immune function where intestinal epithelial cells

can produce, and respond to IL-18 via expression of the IL-18R (Nowarski et al., 2015; Siegmund, 2010). As part of our studies we were not able to ascertain if IL-18 is acting on immune cells (ILCs, Th1 cells) or non-haematopoietic cells such as epithelial cells but this could be investigated further by studies using bone-marrow chimeric mice, or mice with cell specific deletions in IL-18R.

The role for IL-18 in the immune system is very complex, since IL-18, is unessential for the initial polarization of T_H1 cells, but delivers an accelerating and amplifying signal for their proliferation and IFN-γ secretion. Further IL-18 can induce the production of T_H2-type cytokines by both naive and T_H1-polarized cells (Moller et al., 2001). IL-18 is a pleiotropic cytokine that can regulates both Type-1 and Type-2 responses, relying on the nature of the cytokine milieu, infectious stimuli and genetic background of the host (Nakanishi et al., 2001; Wei et al., 2004; Xu et al., 2000). Our results from chapter 2 have highlighted the importance of IL-18 in promoting Type-1 immune responses. However other studies revealed that IL-18 can promote Th1 immune responses and resistance to one species of the *Leishmania* parasite (Li et al., 2004), but induce non-protective Th2 responses to another species (Bryson et al., 2008). Further, one study highlights how IL-18 induces Type-2 responses when IFN-γ is absent (Liu et al., 2006), another study implicates IL-18 in suppressing Type-2 cytokine responses to *T. muris* (Helmby and Grencis, 2004). Hence it is possible that in the context of the present study, lowered IL-18 level in the absence of NLRP3 may participate in the elevated Type-2 responses and diminished Type-1 responses against *T. muris* infection. It is possible that NLRP3 suppress immune responses to *T. muris* infection via inhibiting IL-25, IL-33 and TSLP secretion.

5.4 Mechanism by which NLRP3 and NLRP1 regulate early innate immune responses following helminth infections.

While our studies have shown that NLRP3 and NLRP1 may play important roles in regulating adaptive immune responses to *T. muris* infections, which develop over weeks, we have also evidence that roles for inflammasomes are evident even in the first 1-2 days of infection, which would likely involve regulation of innate immune responses. For example, we have demonstrated that NLRP3 can also suppress the early innate immune responses against hookworm *N. brasiliensis* infections in the lung, and absence of NLRP3 lead to increased resistance to the parasite. Similar to results seen in *T. muris* infections, this increased anti-parasitic immunity was associated with increased eosinophilia and Type 2 cytokines such as IL-4, however it was the rapid increase in neutrophil recruitment to the lung that was most striking. This suggests that NLRP3 may have diverse functions in immunity to different helminth pathogens, depending on the type of parasite and the tissue affected. We found that NLRP1 is less involved in innate immunity to *N. brasiliensis*, potentially because it does not suppress neutrophil responses into the lung. We could not identify the precise mechanism of how NLRP1 is activated and how it suppressed gut-specific immunity to *T. muris* and *N. brasiliensis*. It would be important in future to conduct a detailed analysis of immune responses in the gut of WT and NLRP1-deficient mice via gene expression analysis and flow cytometry to determine if there is a specific biological pathway is up or down regulated in NLRP1-deficient mice, which would provide a clue about how NLRP1 influences anti-parasitic immunity.

While we have shown that neutrophils were present in increased numbers in the lung in the absence of NLRP3, and that neutrophils can potently kill *N. brasiliensis* larvae, there are questions that still remain regarding how neutrophils are regulated by NLRP3. For example,

neutrophils have a potential role in parasite killing, however they may also participate in initiating inflammation and perhaps even being responsible for inflammasome activation and function as a feedback loop for lung neutrophil responses (Amulic et al., 2012; Cassatella, 1995; Coeshott et al., 1999; Joosten et al., 2009). The mechanism of how neutrophils are recruited into the lung remain undefined. It has been revealed that neutrophils are recruited to site of infection and arrive following the production of CXCL1, CXCL8 and CXCL2 (Miotla et al., 2001; Ribeiro et al., 1990). We have demonstrated that one of the key neutrophil chemoattractant molecules, CXCL2, is significantly upregulated in the absence of NLRP3, implicating this chemokine as likely to mediate elevated neutrophil chemotaxis to the lung. How NLRP3 regulates the level of CXCL2 was not determined. CXCL2 expression is dependent on factors such as NF-kappa B, TNF, PAF and IKKα (Burke et al., 2014; da Silva et al., 2012; De Plaen et al., 2006; Goktuna et al., 2014), however it is unclear based on the current literature how NLRP3 may influence expression of CXCL2 in response to these factors. Further, the cell type that expressed CXCL2 and promotes neutrophil recruitment was not determined. CXCL2 is expressed by a variety of cell types including epithelial cells, mast cells and macrophages (De Filippo et al., 2013; Ohtsuka et al., 2001), hence it is possible that following *N. brasiliensis* infection in the lung, CXCL2 is expressed by epithelial cells and this mediates recruitment of neutrophils to the lung tissue, however further work examining cell-specific expression of CXCL2 is required to demonstrate which cell type is responsible for recruiting neutrophils. To further define a causal role for CXCL2 in enhanced neutrophil recruitment to the lung in absence of NLRP3, more studies involving such as blocking CXCL2 receptor could be conducted, to determine whether this prevents neutrophil recruitment. Lastly, other factors can influence neutrophil activation and recruitment, including IL-17A which promotes neutrophil-mediated immunity by activating microvascular pericytes (Liu et al., 2016). Similarly, neutrophils can also be recruited into tissues in response to segmented

filamentous bacteria, which is dependent on both IL-17A and CXCR2 (Flannigan et al., 2017). However, I was unable to detect the correlation between IL-17 expression and neutrophils recruitment but recent research has highlighted how IL-17A is important for neutrophil responses in the lung in *N. brasiliensis* infections (Sutherland et al., 2014). Further work should concentrate on the relative roles for IL-17A in the lung and how this may be regulated by parasite-mediated inflammasome activation.

We have reported that neutrophils are immediately recruited to the lung site of infection which were consistent with other study who demonstrated that neutrophils can recruited to the lung at day two after primary *N. brasiliensis* infection (Chen et al., 2012). Further Chen. et.al have demonstrate that macrophages primed during a Type-2 immune response and under the influence of neutrophils maintain a long-lived phenotype that facilitates accelerated helminth eradication in the absence of T or B cells. (Chen et al., 2014). These studies suggest that in addition to acting alone, neutrophils may also be able to cooperate with other lung-resident cells, such as macrophages, to promote anti-parasitic immunity. While we were not able to demonstrate a causal role for macrophages in NLRP3-dependent suppression of immunity to *N. brasiliensis*, we did show that depletion of neutrophils only partially suppressed the robust protective immunity to *N. brasiliensis*, hence other cells such as macrophages are likely to play a role. The potential synergistic innate immunity afforded by neutrophils and macrophages in the lung was supported by a recent study where Chen. et.al demonstrated that *N. brasiliensis* infection triggers the development of an alternatively activated neutrophil (‘N2’) population that shows a characteristic global transcriptional profile distinct from LPS-activated neutrophils (‘N1’) (Chen et al., 2014). These N2 cells produce Type 2 cytokines that are important for promoting alternative-activated macrophage-mediated parasite killing. Hence, it remains possible that in the absence of the NLRP3 inflammasomes, neutrophils may exhibit a distinct

N2 phenotype that orchestrates the innate Type 2 immune responses, and this could be followed up in further studies undertaking detailed gene expression analyses of lung neutrophils sorted from WT and NLRP3-deficient mice.

My *in vitro* culture results suggest that neutrophils alone are highly toxic to *N. brasiliensis* larvae, rendering the larvae straight and immotile. Neutrophils possess an vary of mechanisms by which they could kill helminths, including complement-mediated cytotoxicity, production of oxygen free radicals or the release of extracellular traps (Bonne-Annee et al., 2013; Galioto et al., 2006). Neutrophils are also associated with killing other species of helminth, such as larval stages of *Strongyloides stercoralis*, where neutrophils alone could eradicate the parasite, however eosinophils and macrophages were also required for optimal killing (Bonne-Annee et al., 2013; Chen et al., 2014; Galioto et al., 2006). However, neutrophils seem to only have a partial role during infection with *S. mansoni*, as their depletion did not influence the severity of disease (Herbert et al., 2004). Further, while neutrophils have been shown to be important *in vitro*, there is relatively little evidence that they are important *in vivo* (Pesce et al., 2008). Hence neutrophils seem to be at least partly responsible for increased parasite killing in absence of NLRP3, other reasons may also be involved. It would be interesting to conduct a detailed analysis such as depletion of other cell including eosinophils, macrophages, ILC2s and basophils that are also associated with immune responses to *N. brasiliensis* may demonstrate more information about the range of immune factors that induce innate resistance to the helminth.

5.5. Potential role for other inflammasomes in regulating immune responses following helminth infections.

My studies have focussed on the roles for the NLRP3 and NLRP1 inflammasomes in regulating immunity to helminth infections, however there are different inflammasomes each with different functions. Hence it is possible that these other inflammasomes may also be involved in regulation the immune responses against helminth infections. For example, NLRP6 is play an important role against infectious diseases (Anand et al., 2012; Elinav et al., 2011; Wlodarska et al., 2014). Moreover, NLRP12 has also been shown to play an important role against invading pathogens (Ataide, 2014; Vladimer et al., 2012). AIM2 is similarly critical for immune responses after infection with various viral and bacterial infectious agents, such as vaccinia virus, mouse cytomegalovirus (Rathinam et al., 2010), *Francisella tularensis* (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010) and *Listeria* (Kim et al., 2010; Rathinam et al., 2010). It would be interesting to conduct a detailed analysis of the relative roles for different inflammasomes following helminth infections. For example, investigate the activation of different inflammasomes *in vitro* by exposing macrophages or dendritic cells from the various inflammasome-deficient mice to different helminth-secreted antigens and seeing which inflammasome pathways are activated. Similar studies could be performed *in vivo*, by examining which of the various inflammasome-deficient mouse strains exhibit differences in worm-induced inflammasome activation (IL-18, IL-1β secretion) and downstream anti-parasitic immune responses, using different murine models of helminth infections.

5.6. Potential role of the NLRP3 inflammasome as a transcription factor following helminth infections.

Our data have supported the role of NLRP1 and NLRP3 in regulating the innate and adaptive immune responses following helminth infections, which we argue is dependent on the activation of inflammasome complexes and release of the products of this activation, i.e. IL-18 and IL-1β. However, it is becoming apparent that some inflammasome proteins have biological roles that are independent of their abilities to activate inflammasomes. For example, NLRP3 can act as a direct transcription factor in promoting Th2 cell differentiation (Bruchard et al., 2015). Other studies have suggested that NLRC5 is also a transcriptional regulator, orchestrating the concerted expression of critical components in the MHC-I pathway (Meissner et al., 2010). Further, Class II transactivator (CIITA) has been shown to regulate the transcription of genes encoding major MHC but does not act as a classical transcriptional factor because it does not bind to DNA (Devaiah and Singer, 2013). Hence, it remains possible that NLRP3 and NLRP1 may also play a role as transcription factors or in some other biological process, it was impossible for us to distinguish these potential roles in the studies described in this thesis. As a first step to address this, further experiments could be conducted such as using mice deficient in Caspase-1/11 and testing various inflammasome inhibitory molecules (e.g. MCC950). For example, if Caspase 1 deficient mice are treated with MCC950 and it still has an effect, then NLRP3 may play an inflammasome independent role, such as descried in other papers. This could also be done by making double mutant Caspase 1/11-deficient, NLRP1/3 deficient mice to see if the absence of specific inflammasome proteins has a biological effect that is independent of its activity to activate inflammasomes and Caspase 1.

Recent studies have highlighted that inflammasomes are associated with determining the nature of the microbiome (Elinav et al., 2018), and also that worm infection can alter the microbiome

(Zaiss and Harris, 2016). Further, recent studies have shown that the effects of NLR deficiency can vary depending on the research facility (Robertson et al., 2013). This raises the question as to whether the effects of NLRP1 and NLRP3 deficiency on immunity to *N. brasiliensis* and *T. muris* were direct (via changes in immune function), or indirect i.e. via changes in composition of the microbiota. I didn't directly assess whether there were differences in the microbiota of the deficient mice I used in my studies, hence I was not able to determine whether the microbiota played a significant role in my findings. However, I did perform studies involving targeting the NLRP3 inflammasome acutely with a selective chemical inhibitor MCC950, where all mice were on the same genetic background, age and sex matched and thus would have had very similar microbiomes. In these studies (described in Chapter 2), acute ablation of NLRP3 had a similar effect as genetic NLRP3 deficiency, hence it is unlikely that microbiome status alone determined the phenotype we observed. Unfortunately, we could not perform similar studies with *N. brasiliensis* experiments or NLRP1 studies and should be a focus of future research. For example, co-housing studies should be performed to determine if any of my observed effects could be due to differences in microbiome.

5.7. Potential role of inflammasomes in human helminth infections.

We have investigated the molecular mechanisms of how NLRP3 and NLRP1 inflammasome are activated by using different model of infections *in vivo* and *in vitro,* mostly using an animal model of helminth infections. Some of my data from Chapter 2 support the notion that some of these mechanisms may operate in human infections with hookworms, but these studies were very limited in scope. It would be important to follow up my human studies in more details to see whether helminths activate inflammasomes in humans. Firstly, it would be important to

conduct *in vitro* studies using human monocyte-derived macrophages or dendritic cells exposed to antigens from different species of helminth, to see if the results I observed using mouse cells translate to humans. This would represent a great opportunity to identify if there are molecules from human pathogens that activate or inhibit inflammasomes. Logical follow-up studies could involve an analysis of humans naturally infected with helminths, from endemic areas, to investigate whether there is evidence of inflammasome activation. This could involve collection of blood and serum from these people and an analysis of IL-18 and IL-1β levels as well as Caspase-1 cleavage or ASC speck formation. However, there is a significant limitation for these studies given that co-infection would be a major confounder, for instance people with tuberculosis or malaria co-infections, which are known to activate inflammasomes (Dorhoi et al., 2012; Shio et al., 2009). Equally, it would be difficult to know how many times they have been exposed to the worm and how many types of worm they have, how long they have been exposed to infections. Lastly, there would be no suitable controls for these studies of un-infected individuals within the same population. More appropriate for these human *in vivo* studies would be the human helminth challenge studies that involve exposure of previously helminth naive individuals to safe, low doses of helminth in a controlled clinical setting (Giacomin et al., 2016). Collection of blood, serum and even gut biopsy tissue as part of these trials would be a unique resource in which to study the impact of helminth infections on inflammasome activation. For example, samples could be collected pre- and post-infection and cells such as macrophages, neutrophils, dendritic cells and epithelial cells. could be sorted by using flow cytometry and analysed for evidence of worm-induced caspase-1 activation and cytokine secretion. Together, better understanding of the roles for inflammasomes in human helminth infections would identify whether targeting inflammasomes is a viable strategy to treat helminth infections of humans. However, people living in helminth endemic regions are often exposed to co-infections with infectious pathogens such as tuberculosis, HIV and malaria

and previous studies have demonstrated a protective role for NLRP3 in these infection models (Dorhoi et al., 2012b; Shio et al., 2009).

Hence, caution would be warranted to prevent such therapeutic strategies from having unwanted effects of reduced ability to fight other infections. Similarly, I have shown that blocking NLRP3 results in increased Th2 responses, highlighting a possible risk of therapeutic NLRP3 inhibition for people with allergic diseases such as asthma. While it is too early to determine whether this is a considerable risk, given that NLRP3 in particular is associated with promoting allergic lung inflammation in mice it is possibly unlikely that blocking NLRP3 would lead to increased allergic disease. However much more research into this possibility would be warranted if inflammasome inhibition therapy becomes more widespread in future.

5.8 Conclusion

The principle aim of my thesis was to provide evidence that different gastrointestinal helminth such as *T. muris* and *N. brasiliensis* activate NLRP3 and NLRP1 inflammasomes *in vitro* and *in vivo* and how these influence helminth-induced immunity and inflammation. We have identified a novel mechanism by which NLRP3 regulates both innate and adaptive immune responses following *T. muris* infections via regulation of IL-18 expression. In follow up studies, we have confirmed that NLRP3, but not NLRP1, can regulate the early anti-parasitic neutrophil responses following *N. brasiliensis* infection. Additionally, we provided evidence that there is a dual role for NLRP3 and NLRP1 in suppressing the generation of gut protective immune responses against *N. brasiliensis* and *T. muris* infections. Together, my studies herein have provided significant new information in the understanding of how immune responses against helminths are initiated and regulated and have lead to the identification of potential new avenues to progress research in this important area in future. Future work should be focus on

defining other signals or associated molecules that may facilitate or direct inflammasome activation. Also, the roles of other inflammasomes in regulating immunity and inflammation following helminth infection are required, which would be important to develop a potential treatment against such infections. Understanding these precise mechanisms may allow the development of treatments that can specifically target the function of individual inflammasome, by inhibiting their suppressive effects on anti-parasitic immunity and potentially reducing the burden of helminth infections in humans.

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