Hepatic ascariasis in a mouse model of relative susceptibility

By

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Summary

The parasitic nematode *Ascaris lumbricoides* infects 800 million people worldwide. The age group most affected by this parasite are children between the ages of 5 and 15 years. The symptoms of ascariasis can be broadly divided into acute and chronic. The acute symptoms are more severe, but relatively rare. It is in fact the chronic symptoms that are more common, but less severe. The impact of these chronic symptoms, which include malnutrition and diminished cognitive development, can however, not be underestimated, as these contribute to the significant morbidity associated with ascariasis. Severity of disease is linked to the number of worms a host harbours, with some people harbouring heavy infections whereas other only experience light infections with few worms. People that are found to have heavy infections, tend to regain similar high worm burdens upon reinfection, even after several rounds of chemotherapy. The reasons why some people harbour many worms, and others only few, are unknown. This body of work aims to elucidate some of the mechanisms behind this difference.

The porcine ascarid, *Ascaris suum*, has been used previously to develop a mouse model where one mouse strain is a model for heavy infection and the other mouse strain is a model for light infection. Using this model, previous work was able to identify the liver stage of the parasitic life cycle as the likely stage at which this difference in infection rate occurs. Chapter 2 is a review of the available literature of the importance of the liver as an organ during parasite migration. It investigates the incorporation of the liver in the life cycle of three parasites of global significance: *Ascaris*, *Schistosoma*, and *Plasmodium*.

In chapter 3, the mouse model is used to investigate the differences between the livers of the two mouse strains during *A. suum* infection, from a proteomics perspective. The results show that there are some important intrinsic differences between the two mouse strains, with the relatively resistant mouse strain having a higher abundance in proteins involved in oxidative phosphorylation and complement activation.

Building on the mouse model, chapter 4 explores the potential for this model to be used to examine the human ascarid *A. lumbricoides*. Due to relative ease of obtaining *A. suum* eggs, this has so far been the preferred parasite, but little research has been performed to investigate if there are any differences in a mouse model between these two

parasites. This chapter thus concludes that resistance and susceptibility in the mouse model, as observed in *A. suum* infection, are retained for *A. lumbricoides* infection and therefore that *A. lumbricoides* is suitable for use in this model.

The final data chapter, chapter 5, focusses on the hepatic immune response against *A. suum* and *A. lumbricoides* in this mouse model. These results indicate that at the investigated time point, of day 7 post infection, the innate immune system plays an important role, more specifically the eosinophils, dendritic cells and monocytes. This was found to be true for both ascarid species and for both mouse strains.

In all, this body of work has contributed novel data on the understudied aspect of the hepatic resistance to *Ascaris* infection in a mouse model.

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Finished, it's finished, nearly finished, it must be nearly finished. Grain upon grain, one by one, and one day, suddenly, there's a heap, a little heap, the impossible heap.

Samuel Beckett, Endgame

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List of abbreviations

ABF	Adult body fluid
AIC	Akaike Information Criterion
APC	Antigen-presenting cells
ASC	Antibody secreting cells
ATP	Adenosine triphosphate
atRA	All-trans-retinoate or retinoic acid
BAL	Bronchoalveolar lavage fluid
Ben-Ho	Benjamini-Hochberg
C8g	complement component C8 gamma chain
Cfh	Complement factor h
Cfi	Complement factor i
CSP	Circumsporozoite protein
DAMP	Danger-associated molecular pattern
DC	Dendritic cells
Epg	Eggs per gram faeces
EPO	Eosinophil peroxidase
ES	Excretory/secretory products
ETC	Electron transport chain
EV	Extracellular vesicles
FDR	False discovery rate
Fgg	Fibrinogen gamma chain
GLM	General linear models
GO	Gene ontology
GST	Glutathione S-transferase
HDM	House dust mite
HPSG	Heparin sulphate proteoglycans
HSC	Hepatic stellate cells
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant NKT
Irf	Interferon-regulatory factors
ITS	Internal transcribed spacer
KC	Kupffer cells

KEGG	Kyoto Encyclopaedia of Genes and Genomes
L2	Second larval stage
L3	Third larval stage
LB	Lysis buffer
LFQ	Normalised protein intensities
LogLik	Log likelihood
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cells
MAC	Membrane attack complex
MAVS	Mitochondrial antiviral signalling
Mbl1	Mannose-binding protein A
Mbl2	Mannose-binding protein C
MDA	Mass drug administration
MDA5	Melanoma differentiation-associated gene 5 protein
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MNC	Mononuclear cell
mROS	Mitochondrial ROS
mtDNA	Mitochondrial DNA
Mup	Major urinary proteins
NK	Natural killer cells
NKT	Natural killer T cells
NRROS	Negative regulator of reactive oxygen species
NTD	Neglected tropical diseases
OXPHOS	Oxidative phosphorylation
p.i.	Post infection
PBS	Phosphate buffered saline
PC	Principal components
PCA	Principal components analysis
PCF	Pseudocoelomic fluid
pDC	Plasmacytoid dendritic cell
pfam	Interpro and protein family
PMNC	Polymorphnuclear cell
PV	Parasitophorous vacuole
RAR	Retinoic acid receptors

Retsat	All-trans-retinol 13,14-reductase
ROS	Reactive oxygen species
S.E.M.	Standard error of the mean
SD	Standard deviation
SPECT2	Sporozoite microneme protein essential for cell traversal 2
SSDA	Statistically significant differentially abundant
STHs	Soil-transmitted helminths
STRING	Search Tool for Retrieval of Interacting Genes/Proteins
Tapbp	Tapasin
TRAP	Thrombospondin-related anonymous protein
Treg	Regulatory T cells

Chapter 1 - Introduction

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Chapter 1

1.1 General remarks

Ascaris lumbricoides, a member of the soil-transmitted helminths, infects a staggering 800 million people yearly (Hotez *et al.*, 2008, Hotez, 2013, Pullan *et al.*, 2014, Global Burden of Disease Study 2013 Collaborators, 2015). Ascariasis, however, is not a new disease. Recent developments in the field of archeoparasitology, sometimes referred to as paleoparasitology, have shown the presence of *Ascaris* eggs in the Roman empire (Mitchell, 2017), pre-Colombian South America (Leles *et al.*, 2008), Korean mummies (Hong *et al.*, 2017) and even king Richard the 3rd (Mitchell *et al.*, 2013).

It is hard to fully comprehend the worldwide impact of this nematode, but Dr. Peter Hotez, an acknowledged expert on Neglected Tropical Diseases (NTDs) described it best as "the most important disease you have never heard of" (Hotez, 2013). Despite its high prevalence, *A. lumbricoides* is still classified as a neglected tropical disease (Hotez *et al.*, 2020). Neglected tropical diseases affect poor communities disproportionately, but are often forgotten when it comes to resources and research funding (Kvalsvig and Albonico, 2013). Many questions therefore remain unanswered, such as, why are children disproportionately infected? What is the role of host genetics in predisposition, where individuals reacquire similar worm burdens upon reinfection? What is the true degree of cognitive impairment suffered by children because of *A. lumbricoides*? How does the immune response against *Ascaris* larvae unfold?

Ascariasis can induce severe, acute symptomatology such as intestinal obstruction and morbidity. However, the chronic aspect of the disease, including growth retardation and cognitive impairment, has a more significant impact at the population level (Watkins and Pollitt, 1997, Olsen *et al.*, 1998, Stephenson *et al.*, 2000). As the severity of symptoms correlates to worm burden, it is essential to investigate why some people are more heavily infected than others (Croll and Ghadirian, 1981, Holland, 2009). Children in particular, are most adversely affected by *Ascaris* infection and consequently suffer more severely from chronic symptoms (Watkins and Pollitt, 1997, Olsen *et al.*, 1998). Eradication of the parasite itself might prove to be difficult without a vaccine, control through mass drug administration (MDA) is therefore the best way to reduce the effects of acute and chronic symptoms on the population (WHO, 2012).

1.2 Life cycle

The life cycle of *A. lumbricoides* in humans is very similar to the life cycle of *A. suum* in pigs (Cooper and Figueiredo, 2013). The infective eggs (Figure 1.2), which contain L3 larvae covered with the L2 cuticle (Geenen *et al.*, 1999, Fagerholm *et al.*, 2000), are ingested and hatch in the small intestine (Douvres *et al.*, 1969). The larvae migrate to the caecum where they penetrate the mucosal barrier (Murrell *et al.*, 1997) and migrate to the liver via the portal blood vessels. In the liver, the L2 cuticle is shed after which the larvae migrate to the lungs on day 6-8 post infection (p.i.) (Fagerholm *et al.*, 2000). These L3 larvae continue to develop and penetrate the alveolar spaces and migrate to the pharynx. Subsequently the larvae are swallowed and return to the small intestine at day 8-10 p.i. (Douvres *et al.*, 1969). On day 10 p.i., most of the L3 stage larvae have reached the small intestines and moult into the L4 stage. Here larvae moult again, on day 24 p.i., resulting in the L5 stage (Pilitt *et al.*, 1987), which will eventually mature into adult worms. After roughly six weeks, the worms reach sexual maturity. Adult female worms measure between 20-35 cm, in contrast to smaller males that range from 15-20 cm (Dold and Holland, 2011a). Adult worms can survive for 1-2 years in the gut (Anderson and May, 1992).

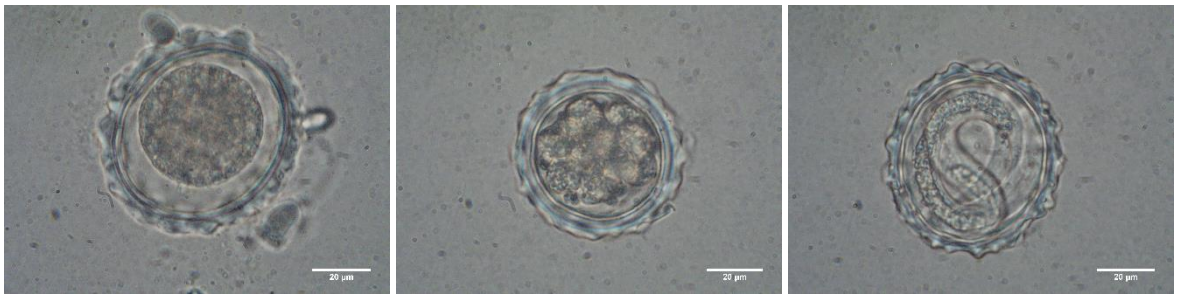

Figure 1.1: Development of *A. lumbricoides* egg to larva in culture , photographed by author. Left: A fertilized *A. lumbricoides* egg as extracted from the uteri of an adult worm. Middle: Embryonation has started in *A. lumbricoides* egg in an in vitro culture in 0.05M H2SO4. Right: Fully developed, infectious *A. lumbricoides* larva in its egg shell.

Female worms can produce in the order of 200,000 eggs per day, however, egg production is variable (Sinniah, 1982). Wet and dark environments were found to be optimal conditions for survival of infective eggs, whereas a dry, sunny environment kills the eggs after a few weeks (Gaasenbeek and Borgsteede, 1998). The eggs are sticky and can therefore get stuck to various objects such as utensils, furniture, money, fingers, door handles, and food such as fruit and vegetables (Kagei, 1983). In the soil, the larvae within the eggs undergo two moults (Geenen *et al.*, 1999), developing into

L3 larvae, which takes around 10 days at optimal conditions (28-32°C) (Crompton, 1989). This delay means that it is not fresh faeces, but rather old faeces, that contains infectious material (Hall *et al.*, 2008).

Eggs are ingested frequently in endemic areas. Wong *et al.* (1991) monitored children of two households that received treatment at the start of the trial. Using the Kato-Katz thick smear, they were able to subsequently confirm that the treatment had worked and the children were in fact parasite-free. The authors then determined the amount of soil the children consumed by measuring the quantity of non-dietary silica present in the stool. After determining the density and distribution of the eggs in the play area of the children, the authors were able to estimate that the children on average ingest 9-20 *Ascaris* eggs per year. Three months later the children received another round of mebendazole, allowing for the measurement of their new worm burden. The authors then compared the estimated infection rate with the observed infection rate and found that between 12 and 90% of ingested eggs developed into adult worms. Although ingestion is the most common way of infection, inhalation and swallowing of eggs from the air are also possible routes of entry in hyperendemic regions (WHO, 1967, Bidinger *et al.*, 1981, Kroeger *et al.*, 1992).

The exact reason why the parasite goes through such complex cycle i.e. larval migration is not fully known (Holland *et al.*, 2013a). One theory (Smyth, 1994) suggests it is an evolutionary remnant from when the parasite used to penetrate the skin. Other theories suggest that the complex life cycle provides fitness benefits to the parasite due to a reduced risk of immune-mediated damage and death occurring in the different tissues (Read and Skorping, 1995, Mulcahy *et al.*, 2005). Evidence for this theory was found through introducing infective larvae in the blood stream in the pigs, which resulted in a lack of larval migration and slower larval development (Jungersen *et al.*, 1999).

1.3 Two species

There are two species of *Ascaris*: *Ascaris lumbricoides* Linnaeus, 1758 and *A. suum* Goeze, 1782 with the former infecting humans and the latter infecting pigs (Maung, 1973). However, there has been considerable debate in the literature about whether these ascarids are truly separate species (Leles *et al.*, 2012, Peng and Criscione, 2012). The adults of both species are morphologically very similar, with only small differences

in denticle and lip morphology observed (Sprent, 1952a, Ansel and Thibaut, 1973, Maung, 1973). The use of molecular techniques was therefore an essential development, with mitochondrial DNA (mtDNA) and the first internal transcribed spacer (ITS-1), located on the ribosomal DNA (Zhu *et al.*, 1999), being the most frequently used molecular markers. mtDNA is often used for identifying cryptic species, with a 2% sequence variation between individuals of the same species and 10-20% between closely related species being observed (Blouin *et al.*, 1998, Blouin, 2002, Cavallero *et al.*, 2013). ITS-1, conversely, has ≤1% sequence variation between individuals of the same species and between closely related nematode species (Stevenson *et al.*, 1995, Nadler and Hudspeth, 2000). mtDNA has a higher mutation rate than ITS-1, making it preferable when analysing small sample sizes (Blouin, 2002).

As for *Ascaris*, a difference was observed between the two species in both ITS-1 and the mtDNA (Anderson *et al.*, 1993, Zhu *et al.*, 1999) - with a 1.3% difference in the ITS-1 (Zhu *et al.*, 1999) and 3-4% for the mtDNA (Anderson *et al.*, 1993), which the authors interpret as evidence for two separate species. Liu *et al.* (2012), however, compared whole mtDNA extracted from *A. lumbricoides* and *A. suum* from both human and porcine sources respectively and found a 1.9% sequence difference, indicating a single species. A more recent study performed in Brazil sequenced the cox1 and nad1 genes from *Ascaris* from pigs and humans, in settings where humans and pigs live closely together (Monteiro *et al.*, 2019). The authors found that, using these markers, it was not possible to distinguish the parasites obtained from pigs from those obtained from humans. Whole genome sequencing of *A. lumbricoides* and *A. suum* revealed two distinct clusters, indicating two different species (Zhou *et al.*, 2020). Although the evidence isn't conclusive yet, there are strong indications that *A. suum* and *A. lumbricoides* are two species.

Another area of debate has been the possibility of cross-transmission. In non-endemic areas, pig-to-human infections have been demonstrated, however, in endemic regions such cross-infections are harder to verify (Anderson, 1995, Nejsum *et al.*, 2005, Arizono *et al.*, 2010, Betson *et al.*, 2014). Evidence for cross-transmission was identified in both endemic and non-endemic settings (Nejsum *et al.*, 2005, Criscione *et al.*, 2007, Zhou *et al.*, 2012, Betson *et al.*, 2014, Palma *et al.*, 2019). The question as to whether *Ascaris* can cross-infect is an important one for the development of appropriate control measures as it determines if *Ascaris* infection should be considered as a zoonosis (Criscione *et al.*,

2007). Although rare, cross-infections could have important implications regarding drug-resistant gene transfer, formation of hybrid genes, potentially increasing parasite virulence or host immune evasion (Anderson, 2001, Arnold, 2004). The presence of hybrids does not immediately imply a single species. It is possible that after mating the eggs die off straight away, or that the offspring of the hybrids could be sterile or have lower fitness, all of which would be supporting the two species theory (Betson *et al.*, 2013). In the case of *Ascaris*, however, it is thought that the hybrids are able to produce fertile offspring (Criscione *et al.*, 2007, Zhou *et al.*, 2012, Betson *et al.*, 2013). A more recent study found distinct mitochondrial lineages, indicating species differentiation (Easton *et al.*, 2020). However, the nuclear genome indicated an admixture of the different *Ascaris* lineages. The authors conclude that *A. suum* and *A. lumbricoides* form a genetic complex.

The next question is then about the last common ancestor. Anderson and Jaenike (1997) first found that 17% of genetic variation between *Ascaris* obtained from pig samples was explained by differences in geographical location. Criscione *et al.* (2007) confirmed these results through the investigation of *Ascaris,* isolated from both human and porcine origin from different parts of the world. The authors found that *Ascaris* tends to cluster together depending on the region rather than based on their host organism, i.e. *A. suum* and *A. lumbricoides* cluster together per region. The authors thus concluded that *Ascaris* diverged into infecting humans and pigs separately in different locations and that this evolutionary event therefore must have happened several times. The high similarity observed by Easton *et al.* (2020) in *Ascaris* samples geographical locations and in different hosts (pigs and humans), indicates a rapid spread of the parasite around the world. Betson *et al.* (2014) found evidence for a single host switch and subsequent geographical differentiation, however, these authors further suggest that an alternative model of multiple host switches is also a possibility (Criscione *et al.*, 2007, Betson *et al.*, 2014). The above, taken together with evidence for restricted gene flow between the species, suggests the possibility of the presence of multiple species of *Ascaris* in humans and pigs (Søe *et al.*, 2016). Solving these species problems will prove to be essential for transmission and control purposes (Søe *et al.*, 2016).

1.4 Clinical features

Most individuals infected with *A. lumbricoides* will only harbour a few worms (Croll *et al.*, 1982, Thein-Hlaing *et al.*, 1984) and will not experience significant symptoms (Thein-Hlaing *et al.*, 1991, de Silva *et al.*, 1997c). Moderate infections can cause acute illness with symptoms such as nausea, diarrhoea, and abdominal pain, which usually disappear shortly after onset (Brooker, 2010). Heavily infected individuals, however, can have a wide range of symptoms which are divided into chronic and acute symptoms (de Silva *et al.*, 1997b). Acute symptoms are rare but more serious (de Silva *et al.*, 1997b) with an increased risk of mortality. de Silva *et al.* (1997a) estimated a case fatality rate of 5%.

Both intestinal obstruction and biliary complications are the most common acute symptom of *Ascaris* infection (O'Lorcain and Holland, 2000). Intestinal obstruction accounts for 57% of all complications, it is most often found in young children (5-10 years) because of their smaller intestinal lumen (de Silva *et al.*, 1997b).

Chronic symptoms of *Ascaris* infection have a major public health impact (Stephenson *et al.*, 2000). The most common symptoms of chronic disease are growth impairment, and malnutrition (Stephenson *et al.*, 2000, Hall *et al.*, 2008). Malabsorption of nutrients, caused by the parasite damaging the intestinal villi, is in combination with anorexia the main causes of malnutrition (Stephenson, 2002). Malnutrition in children can cause impairment of growth and physical ability, together with reduced work capacity and cognition (Stephenson *et al.*, 2000, O'Lorcain and Holland, 2000). This has also been suggested by Blouin *et al.* (2018), using a 'critical period framework approach' the authors found that children that had been infected with *Ascaris* between one and two years of age, went on to have a decrease in cognitive and verbal scores between two and five years of age, when compared to children that had had no *Ascaris* infection.

The effects of larval migration through internal organs in the early stages of infection is difficult to study in humans. However, a range of symptoms, caused by this larval migration, have been identified these include asthma, coughing, skin rashes, fever, eosinophilia and substernal pain, with dying larvae causing more harm than the living ones (Pawłowski, 1978, Pawloski and Arfaa, 1984, Coles, 1985). While the larvae are migrating through the lung, as part of their life cycle, they can cause symptoms which include blood eosinophilia, respiratory symptoms, and pulmonary infiltrates termed

"Loeffler's syndrome" (Loeffler, 1932, Loeffler, 1956). Respiratory symptoms, such as dyspnoea and bronchospasms, can potentially become severe (Ribeiro and Fischer, 2002). Even after death, the worms can still release their eggs which can cause a granolumatous reaction and ultimately even induce liver abscesses (Brooker and Pullan, 2013).

The impact of larval migration on the liver is much harder to study. In pigs, however, white spots, pathological lesions formed by leukocytes, can often be observed as a result of the immune response against these larvae (Schwartz and Alicata, 1932, Eriksen, 1981, Pérez *et al.*, 2001, Frontera *et al.*, 2003). In humans, inflammatory responses have been observed (Cooper *et al.*, 1992). Javid *et al.* (1999) investigated liver abscesses over a 10 year period in India. From a total of 510 patients, 74 were found to be caused by biliary *Ascaris*. Furthermore, 11 patients were found with intact *Ascaris* in the liver. An in-depth analysis of larval migration and the liver can be found in chapter 2.

1.5 Epidemiology

1.5.1 Patterns of infection

In endemic areas *Ascaris* infections follow a similar age pattern. Although children of all ages can be infected, including children as young as 6 months (Kirwan *et al.*, 2009), it is mainly children between 5-15 years that have the highest prevalence and intensity of infection, even after several rounds of anthelmintic treatment and reinfection (Bethony *et al.*, 2006, Wright *et al.*, 2018). In India children were found to have a high prevalence of *A. lumbricoides* compared to adults and a high rate of reacquisition of worms after anthelmintic treatment (Elkins *et al.*, 1986, Haswell-Elkins *et al.*, 1989). The authors suggest that this could be due to age-dependent transmission, build-up of immunological resistance, or a combination of both. This was also found in a study performed in Burma, where children reached their initial worm burden much more quickly after a round of anthelmintic treatment compared to adults (Hlaing *et al.*, 1987). Using previously obtained data from Bangladesh, Walker *et al.* (2011) found that children 're-acquire their pre-treatment worm burdens more rapidly than adults'. They found that children between the ages of 1 and 2 reacquire double their initial worm burden. This was in contrast to adults and teenagers, who upon reinfection only had 50% of their original worm burden, even after 2 rounds of chemotherapy and

reinfection (Walker *et al.*, 2011). The observed decrease in worm burden in older children is thought to be driven by a behavioural change by young children exploring their environment more and therefore potentially being more exposed to infectious eggs (Walker *et al.*, 2011). This effect seems to be particularly strong in the first three life years. Older teenagers and adults therefore tend to have lower intensity levels (Scott, 2008). Age dependence of intensity of *A. lumbricoides* infection was found in populations worldwide in Mexico (Forrester *et al.*, 1990), the Caribbean (Bundy *et al.*, 1987), Thailand (Suntaravitun and Dokmaikaw, 2018), Nigeria (Okeke and Ubachukwu, 2015) and even very recently in Ethiopia (Dana *et al.*, 2020).

People over the age of 60 are often placed in the 'adult' group, however, they are a distinct group with different immunocompetence than younger adults (Scott, 2008). A study performed in Morocco found that those over 60 had prevalence more similar to that of children aged between 3 and 14 (El Kettani and Azzouzi, 2006). It would therefore be interesting for future studies to make this distinction between over 60s and adults under 60.

In addition to age related patterns of infection, gender, was found to be a risk factor for reinfection (Halpenny *et al.*, 2013) and predisposition (Wright *et al.*, 2018). In general, females tend to have higher infections compared to males in each age category (Elkins *et al.*, 1986). Women were found to have a 'higher average intensity of infection,' and ' greater frequency of moderate infection' (Haswell-Elkins *et al.*, 1989). Kightlinger *et al.* (1998) found evidence for higher infection rate of *A. lumbricoides* in girls compared to boys in a population in Madagascar. The authors identified the behaviour of girls staying closer to home and boys away from home in the fields and forests, as contributor to this difference. They conclude that differences in worm intensity is the result of differences in behavioural and environmental exposure. However, Traub *et al.* (2004) did not find that gender was risk factor for *Ascaris* infection, however, females between the ages of 16 and 20 did show an 'over-proportional' parasite intensity. This observed sex variation was again attributed to a behavioural difference in work environments, with men mostly working in factories, or other indoor environments and the women mainly being outdoors tea-pickers (Traub *et al.*, 2004). Women in the Thai-Burmese border region, were also found to have a higher prevalence of *A. lumbricoides* than men (Nacher *et al.*, 2003). In a Bangladeshi study, adult women were found to have higher worm burdens than adult men (Walker *et al.*, 2011). This sex

difference was not observed in children, and is likely to be ascribed to the sociological aspects of the society, where women tend to stay at home (exactly the area that has been identified as the place where most infections occur by the authors) and teenage boys and men tend to go to work during the day.

1.5.2 Risk factors

Reinfection and intensity of infection have been linked to a range of social and environmental risk factors. A study in St. Lucia, identified crowding and income as significant risk factors for *Ascaris* infection (Henry, 1988). Holland *et al.* (1988) found that children in Panama, living in 'houses of bamboo or wood with dirt floors and lacking inside plumbing and toilets' had a significantly higher risk of *A. lumbricoides* infection. The presence of adequate flooring as a risk factor has also been found in low-income households in Bangladesh, where finished flooring was associated with a 44% decrease in *Ascaris* infection (Benjamin-Chung *et al.*, 2015). The authors concluded that a combination of deworming, increased hygienic sanitation and flooring may have resulted in a stronger reduction of *Ascaris* prevalence than deworming on its own. Walker *et al.* (2011) confirmed the association between that heavy worm burden and earth flooring in the house, together with absence of latrine and a common tap for washing water. The authors therefore conclude that *Ascaris* infection is associated with 'acute poverty'.

Poor sanitary conditions have also been linked to *Ascaris* infection in children between 3-12 years in government-owned tea plantations in Sri Lanka, although only in some parts of the country (Sorensen *et al.*, 1994). The positive effect of latrine availability in *Ascaris* infection has further been identified in studies in Brazil (Gonçalves *et al.*, 2016), China (Xiao *et al.*, 2015) and Tanzania (Schüle *et al.*, 2014). This latter study also identified socio-economic status as a risk factor.

The influence of household conditions and socio-economic status on *Ascaris* infection was found to have a positive correlation between average worm burden and increasing number of family members in an Indian fishing village near Madras (Haswell-Elkins *et al.*, 1989). Traub *et al.* (2004) further identified a range of risk factors linked to *Ascaris* infection in an Indian population: Hindu religion, lower education level (or maternal education in the case of children), higher density of people in one household, owning pigs, and lack of anthelminthic treatment. Gunawardena *et al.* (2011) found similar risk

factors for *Ascaris* infection in Sri Lankan children: lower altitude of below 500 meters, maternal education, household sanitation, and gender.

Several studies in Africa confirmed that the influence of socio-economic factors on *Ascaris* infection are not geographically bound. A study performed in Ebonyi State, Nigeria which investigated the *Ascaris* prevalence in children between 5 and 15 years old, found that risk factors included parents being farmers and the household having more than 6 people sleeping in one room (Okeke and Ubachukwu, 2015). Similar findings were observed in Osun state in Nigeria, where children whose father's occupation was identified as farmer had increased *A. lumbricoides* prevalence compared to children whose fathers were businessmen (Kirwan *et al.*, 2009).

In a population in Panama, Halpenny *et al.* (2013) investigated whether the height-for-age Z-score (as a measure for malnutrition) had an influence on reinfection rate. The authors point out that is usually the opposite that is investigated, namely how *Ascaris* infection influences malnutrition, but here the authors identified that having a low height-for-age Z-score was associated with *Ascaris* reinfection. Additionally, low maternal education and low average asset-based household wealth index were also associated with higher *Ascaris* reinfection. However, the authors observed that these differences in height-for-age Z-score and wealth index were only true for one of the two studied time periods.

Interestingly, Kightlinger *et al.* (1998) found that rainforest communities in Madagascar did not show a household centred infection pattern, but instead, exposure to *A. lumbricoides* eggs was distributed among the community, with changes in worm burden visible between communities rather than between households. Control measures applied to single households would therefore not be sufficient, but would need to be applied to entire communities in order to be effective.

In short, factors such as gender-related behaviour, environment, and the socio-economic situation including housing conditions, cultural practices, defecation habits and available infrastructure all have an influence on *Ascaris* infection (Holland *et al.*, 1988, Haswell-Elkins *et al.*, 1989, Sorensen *et al.*, 1994, Kightlinger *et al.*, 1998, Traub *et al.*, 2004, Gunawardena *et al.*, 2011, Benjamin-Chung *et al.*, 2015).

1.5.3 Aggregation & predisposition

Regardless of these age- and sex-related differences, it is clear that certain people are more heavily infected than others (Croll and Ghadirian, 1981, Thein-Hlaing *et al.*, 1984, Elkins *et al.*, 1986, Holland *et al.*, 1989). This means that most of the worms in an endemic area aggregate in a small set of people (Bethony *et al.*, 2006). These people, carrying the majority of the worm burden, are often described as 'wormy people' (Croll and Ghadirian, 1981), with heavily-infected individuals often aggregating in one family (Chai *et al.*, 1983, Forrester *et al.*, 1988). The over dispersed frequency distribution was first described for parasites in general by Crofton (1971) and for *Ascaris* specifically by Croll and Ghadirian (1981).

Subsequent to anthelminthic treatment, patients re-acquire a similar worm burden to that which they possessed before treatment, a phenomenon known as predisposition (Croll *et al.*, 1982). Predisposition has subsequently been demonstrated in humans, for a variety of geographical locations, ranging from India to Nigeria (Seo *et al.*, 1979, Croll *et al.*, 1982, Anderson and May, 1982, Elkins *et al.*, 1986, Holland *et al.*, 1989), and in naturally and experimentally-infected pigs (Boes *et al.*, 1998b). The mechanisms that determine predisposition are not yet fully elucidated and are likely to be multifactorial (Holland, 2009). Using probability theory, McCallum (1990) found that both long- (i.e. host genetics and socio-economic status) and short-term (i.e. host acquired immune system) variables were involved. However, Chan *et al.* (1994) suggested that genetic predisposition is likely to be 'overwhelmed by environmental or behavioural' factors.

Once household clustering was taken into account, the importance of individual predisposition was reduced (Walker *et al.*, 2011). Chan *et al.* (1994) also concluded that environmental factors probably outweigh familial predisposition.

It is this population, of heavily infected individuals, that is most at risk of having severe symptoms, morbidity and mortality due to heavy worm burdens (Holland, 2009). Additionally, because of their high parasite burden, such individuals can potentially excrete more eggs and thus enhance the parasite spread (Holland, 2009). Understanding the underlying mechanisms for this diversity in parasite load is therefore important with respect to enhancing parasite control and diminishing morbidity and mortality.

One of the unanswered questions regarding heterogeneity is at which point in the life cycle of *Ascaris* does predisposition occur? As much of the parasite's early life cycle is impossible to study in humans, the use of an animal model becomes essential. Early work by Mitchell *et al.* (1976) showed differences in lung larval burden between inbred mouse strains. Lewis *et al.* (2006) expanded on these finding and assessed a range of inbred mouse strains in order to find the most dissimilar cases of *Ascaris* susceptibility and resistance to *Ascaris* infection. The authors identified two mouse strains as model organisms for heterogeneity in *Ascaris* infection with one strain susceptible (C57BL/6J) and one relatively resistant (CBA/Ca) to *A. suum* (Lewis *et al.*, 2006). Furthermore, by experimenting with different infective doses, the authors found that dose did not affect relative susceptibility. The authors observed that with increased dosage, there was an increased larval recovery.

Two main factors have been identified as contributors to the observed predisposition at the individual level of infection (Walker *et al.*, 2013): host genetics and host immunity. As for the role of the immune system, a study showed that putatively immune children demonstrated higher levels of *Ascaris*-specific anti-ABA IgE antibodies and pro-inflammatory proteins (Holland *et al.*, 1989, McSharry *et al.*, 1999). In work performed before the commencement of this thesis, we also used C57BL/6J and CBA/Ca mice and identified an intrinsic difference in susceptibility between the two strains (Deslyper *et al.*, 2016). We investigated the protein abundances in the liver of the mice at day 4 post-infection and found that the relatively resistant mouse strain, CBA/Ca, had an intrinsically higher abundance of oxidative phosphorylation cycle (OXPHOS) proteins. These proteins are part of the cellular respiration process and produce reactive oxygen species (ROS) as a by-product (Adam-Vizi, 2005, Kowaltowski *et al.*, 2009). Under infection, both mouse strains had an increased relative abundance of OXPHOS proteins, but with the resistant strain still having a higher relative abundance than the susceptible strain (Deslyper *et al.*, 2016). These results indicate a potential role of ROS in early elimination of *Ascaris*. Additionally, CBA/Ca mice had an intrinsically higher relative abundance of ribosomal proteins than C57BL/6J mice. This difference became more pronounced under infection (Deslyper *et al.*, 2016).

At the genetic level, the major histocompatibility complex (MHC) genes were among the first to be implicated to play a role in predisposition. Holland *et al.* (1992) found that the presence of the A30-31 antigen was more prevalent in consistently infected

children. Subsequently, Williams-Blangero *et al.* (1999) identified, in the Jirel population of East Nepal, that 30-50% of difference in worm burden between individuals could be attributed to host genetics. Using variance component linkage analysis, the authors measured the covariance between infection and genotype, which resulted in the identification of three loci on chromosomes 8, 11 and 13 as having an influence on susceptibility (Williams-Blangero *et al.*, 2002, Williams-Blangero *et al.*, 2008). An additional three suggestive loci were recognised in a later study, using a bigger sample size and thus enhancing the statistical power. This subsequent analysis indicated a total of four chromosomes of interest: 13, 11, 8, and 1 (Williams-Blangero *et al.*, 2002, Williams-Blangero *et al.*, 2008). The authors propose the TNFSF13B gene as a candidate for further investigation. This gene is known to be involved in immunoglobulin (Ig) secretion and B cell activation (Moore *et al.*, 1999, Schneider *et al.*, 1999, Yan *et al.*, 2000) Other studies confirmed these findings, suggesting TNFSF13B, together with LIG4, as important factors that might explain heterogeneity in *Ascaris* infection (Acevedo *et al.*, 2009), with a possible role of these two genes in the modulation of an IgE and IgG response against the parasite in favour of a protection against *Ascaris* infection. DNA ligase IV, the protein product from the LIG4 gene, is essential in the so called V(D)J recombination (Grawunder *et al.*, 1998). This V(D)J recombination mechanism is the driver behind the wide variety of antibody and T-cell receptor diversity, by combining a random combination of several available variable (V), diversity (D) and joining (J) exons (Roth, 2014). LIG4 additionally plays a role in the class-switch recombination, or isotype switch, in B cells (Pan-Hammarström *et al.*, 2005). Additionally Peisong *et al.* (2004) identified a STAT6, a Th2 signalling transducer, gene variant in a Chinese population that was linked with lower *Ascaris* burden. Furthermore, one specific haplotype in the STAT6 gene, a shorter allele, has been found to be associated with higher levels of allergy in British children, such as rhinitis, dermatitis and asthma (Moller *et al.*, 2007). However, this same haplotype was associated with lower levels of *Ascaris* burden in a Chinese population. The authors suggest that a variation in Th2 signalling could be involved in differences in worm burden.

In order to understand the role of the household, the influence of exposure on predisposition was examined (Walker *et al.*, 2011), using data obtained from Hall *et al.* (1992) who investigated a population in Bangladesh, using new Bayesian statistical

model methods. Walker *et al.* (2011) found that individual differences play a minimal role in predisposition, whereas the household was found to be the main risk factor.

1.6 Animal models

Although pigs are a natural host for *A. suum* infection, which in itself is closely related to the human *A. lumbricoides*, their use as model system is challenging because of cost, husbandry, size of the pigs and the lack of inbred strains (Holland *et al.*, 2013a). A large range of animal models has been explored for *Ascaris* infection (see Table 1.1): mice, guinea-pigs, rabbits, lambs, goats, gerbils, rats and cows (Holland *et al.*, 2013a). Because only pigs are natural hosts, all other model organisms have incomplete life-cycles, with only the early stages of infection occurring (Holland *et al.*, 2013a), making them abnormal hosts. Additionally, Holland *et al.* (2013a) found "that in the vast majority of these models, susceptibility and resistance to *Ascaris* infection in either the liver or the lungs has not been clearly established."

Experiments on rats were conducted by Davaine as early as 1863 (Davaine, 1863). Stewart continued Davaine's experiments, using rats and mice (Stewart, 1917b), where he identified the presence of *Ascaris* larvae in the gut, the liver and lungs. Slotved *et al.* (1998) subsequently recognised that the migratory path of *A. suum* in both pig and mouse were the same, making mice an ideal model organism. Furthermore, the migratory path of the *A. suum* larvae in mice is similar to *A. lumbricoides* in humans, although truncated, with larval migration arrested at the lung stage (Figure 1.2) (Murrell *et al.*, 1997, Slotved *et al.*, 1998). Mice also have a higher larval recovery rate than other animals such as rats, guinea-pigs and rabbits (Douvres and Tromba, 1971) and even pigs (Roepstorff *et al.*, 1997). This is due to their relative host size and parasite size (Lewis *et al.*, 2006).

Table 1.1: Parameter(s) investigated in experimentally infected model organisms excluding pigs. Reproduced and modified from (Holland *et al.*, 2013b) with permission from the authors.

Model organism	**Parameter(s) investigated**	**References**
Guinea-pig	Larval migration Immunological response	(Stewart, 1916a, Ransom and Foster, 1919, Yoshida, 1919a, Yoshida, 1919b, Kerr, 1938, Fallis, 1948, Soulsby, 1957, Beraldo *et al.*, 1961, Taffs, 1968, Douvres and Tromba, 1971, Khoury *et al.*, 1977)
Rabbit	Larval migration Immunological response Hepatic pathology Pulmonary pathology	(Arean and Crandall, 1962, Taffs, 1968, Galvin, 1968, Douvres and Tromba, 1971, Berger, 1971, Yoshida *et al.*, 2012)
Gerbil	Larval migration	(Cho *et al.*, 2007)
Rat	Larval migration	(Stewart, 1916b, Yoshida, 1919a, Yoshida, 1919b, Beraldo *et al.*, 1961, Galvin, 1968, Berger, 1971, Cho *et al.*, 2007, Yoshida *et al.*, 2012)
Cow	Larval migration Immunological response Pathology	(Greenway and McCraw, 1970a, Greenway and McCraw, 1970b, McCraw and Greenway, 1970, McCraw, 1975)
Lamb	Larval migration	(Ransom and Foster, 1919)
Goat	Larval migration	(Ransom and Foster, 1919)
Mice	Larval migration Immunological response Pathology Host parasite genetics	(Stewart, 1917a, Stewart, 1917b, Ransom and Foster, 1919, Ransom and Foster, 1920, Ransom and Cram, 1921, Sprent, 1949, Sprent and Chen, 1949, Sprent, 1952b, Sinha, 1967, Jenkins, 1968, Bindseil, 1969b, Bindseil, 1969a, Guerrero and Silverman, 1969, Bindseil, 1970a, Bindseil, 1970b, Douvres and Tromba, 1971, Crandall and Crandall, 1971, Keittivuti, 1974, Mitchell *et al.*, 1976, Brown *et al.*, 1977, Eriksen, 1981, Bindseil, 1981, Song *et al.*, 1985, Kennedy *et al.*, 1987a, Jeska and Stankiewicz, 1989, Slotved *et al.*, 1998, Lewis *et al.*, 2006, Lewis *et al.*, 2007, Dold *et al.*, 2010, Dold *et al.*, 2011, Pineda and Ramos, 2012, Peng *et al.*, 2012)

Lewis *et al*. (2006), building on the early mouse work of Mitchell *et al*. (1976), developed a standardised method for preparing the inoculum. A 10 µl volume was counted 10 times in order to closely estimate the number of eggs per µl. They

subsequently tested a range of different mouse strains, with this standardised inoculum, in order to mimic the susceptibility and resistance to *Ascaris* found in humans. The authors first showed a difference in susceptibility between different inbred mouse strains, making mice, despite their truncated life cycle of *Ascaris*, suitable model organisms to study predisposition. Nine different mouse strains(A/J, SJL, DBA/2, SWR, C3H/HeN, C57BL/6J, CBA/Ca, NIH, BALB/c) were tested to mimic the susceptibility and resistance found in humans (Lewis *et al.*, 2006). The authors identified three levels among the strains with the C57BL/6J strain being the most susceptible. They also identified an intermediate category and a resistant category. The latter included the CBA/Ca strain, which was identified as the most resistant. The mouse strains C57BL/6J and CBA/Ca mice, were therefore identified as susceptible and resistant respectively against *Ascaris* infection (Lewis *et al.*, 2006), with C57BL/6J mice demonstrating a peak in larval numbers at 7 days p.i., and CBA/Ca mice remaining relatively resistant (Holland *et al.*, 2013a). A change in dose of infective eggs did not modify the relative susceptibility, indicating an intrinsic difference in host factors between the two strains (Lewis *et al.*, 2006). The authors concluded that a dose between 500 and 1000 eggs was optimum, as this did not generate 'an unnecessarily heavy larval burden in the mice and place undue stress upon the mice'. Additionally, administering the 1000 egg split over 2 or 4 doses did not change the relative susceptibility of the two mouse strains.

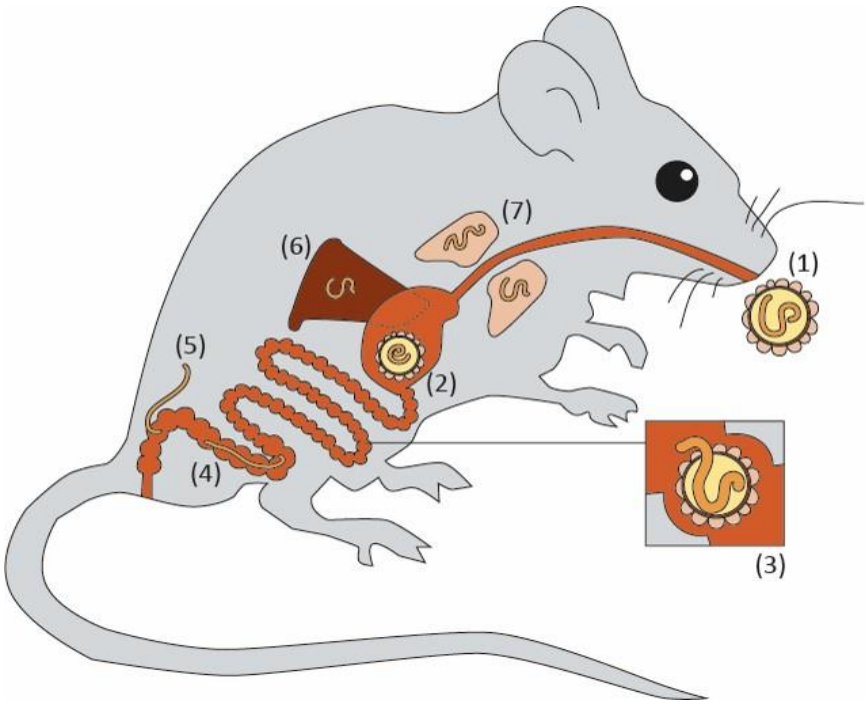

Figure 1.2: The life cycle of *Ascaris* in a mouse model. The life cycle in the mouse model is very similar to the human life cycle, albeit truncated. The ingested eggs migrate to the gut where the larvae hatch. The larvae subsequently migrate to the liver and onwards to the lungs. In the mouse, however, the life cycle is truncated and larval migration is arrested at the lung stage. Figure reproduced with permission from the authors (Holland *et al.*, 2013b).

1.7 Immune response

Upon oral infection, *Ascaris* larvae migrate through the digestive tract to the caecum, the site where the larvae penetrate the gut (Murrell *et al.*, 1997). In general, gut dwelling helminths trigger the innate immune system through damaged epithelial cells, which secrete cytokines called alarmins, they are interleukin (IL)-25 and IL-33 (Neill *et al.*, 2010, Maizels *et al.*, 2012). These alarmins activate nuocytes, which are non-B non-T cells (Neill *et al.*, 2010, Allen and Maizels, 2011, Maizels *et al.*, 2012). Nuocytes can secrete IL-4 and IL-13 and alternatively activate macrophages (Allen and Maizels, 2011). Neill *et al.* (2010) demonstrated the importance of these nuocytes and their cytokine production in the expulsion of the intestinal nematode *Nippostrongylus brasiliensis* from rats.

Little is known about the response to L3 stage larvae of *Ascaris* during the early migratory path in the gut. However, there is some evidence for the role of eosinophils during this early stage, from repeat infection experiments in pigs. Masure *et al.* (2013a) found that pigs continuously exposed to *A. suum* eggs for 14 weeks manage to develop immunity against subsequent infections, resulting in a 99.7% reduction in larvae. This

was confirmed by Vlaminck *et al.* (2016) , using a trickle infection in pigs, the authors found a 100% reduction in recovery of worms from the gut. This observed reduction in larval burden has been linked to immunological changes in the caecum including eosinophilia, mastocytosis and a hyperplasia of goblet cells (Masure *et al.*, 2013a). This was paired with an increase in IL-5, IL-13, CCL11 and EPO transcripts, see summary table 1.2. *In vitro* experiments showed that *A. suum* larvae induce degranulation of eosinophils. In all, their results indicate an important role for eosinophils and eosinophil degranulation, measured by ROS levels, in the development of immunity against *Ascaris* and subsequent larval expulsion (Masure *et al.*, 2013a).

1.7.1 Liver

After moving through the gut, the larvae migrate to the liver, however, very little is known regarding the immune response at this stage. The liver shows macroscopic signs of an innate immune response, with the presence of white spots in the livers of pigs infected with *A. suum* (Ronéus, 1966) and humans infected with *A. lumbricoides* (Javid *et al.*, 1999). There are two types of these white spots: granulation tissue and lymphonodular (Pérez *et al.*, 2001). The former mainly contains eosinophils, neutrophils and macrophages, the latter mainly contains lymphoid cells.

The liver is an immunomodulatory organ (Jenne and Kubes, 2013), which could explain why *Ascaris* incorporated this organ as part of its life cycle. This is a route which is also used by *Plasmodium*, Bertolino and Bowen (2015) suggested that the malaria parasite goes through a hepatic, pre-erythrocytic stage, potentially to evade the immune system. Additionally, *Schistosoma*, after oral ingestion, migrates to the lungs first and subsequently to the liver (McManus *et al.*, 2018)(see also Chapter 2).

1.7.2 Lungs

The surviving *Ascaris* larvae subsequently move from the liver to the lungs (Roepstorff *et al.*, 1997). The lung stage is characterized by an increase in neutrophils, eosinophils and monocytes (Enobe *et al.*, 2006, Nogueira *et al.*, 2016). Lewis *et al.* (2007) showed, using hydrocortisone to suppress inflammation, that this inflammatory response is not responsible for the observed difference in heterogeneity of infection.

Yoshida *et al.* (2012) identified a novel C-type lectin of *A. suum* called *A. suum* C-type lectin-1 (*As*-CTL-1) in the lung tissue of infected rabbits. As-CTL-1 was found to have

greater similarity to mammalian C-type lectins than those of *Caenorhabditis elegans*, indicating it could potentially interfere with host cell receptors and therefore potentially play a role in immune response interference.

Using BALB/c mice infected with *A. suum*, Gazzinelli-Guimarães *et al.* (2013) found an increase in IL-5 concentration at day 4 post-infection, coinciding with the migration of the larvae towards the lung. Interestingly, IL-5 has been shown to play a role in the eosinophilia observed in helminth infections (Gazzinelli-Guimarães *et al.*, 2013). IL-5 is involved in the development of eosinophils and their recruitment from the bone marrow into the blood (Rosenberg *et al.*, 2007).

Weatherhead *et al.* (2018) found similar results in the bronchoalveolar lavage fluid (BAL) of *A. suum*-infected BALB/c mice at day 12 p.i., with a significant increase for macrophages, eosinophils, lymphocytes and neutrophils. Additionally, an increase of IL-4, IL-5 and IL-13 in both the lung tissue and BAL and IL-17 in lung tissue were also observed.

In a recent study, Gazzinelli-Guimaraes *et al.* (2019) investigated the influence of presensitizing BALB/c mice with house dust mite (HDM) on *Ascaris* infection. The authors found that during *Ascaris* infection, without the influence of HDM, there was a significant increase of IL-4, leukocytes, eosinophils, Th2 cells and alternatively activated macrophages in the lungs. However, these increases were all observed at day 18 p.i., so rather late in the infection and after the lungs have been cleared of larvae. At day 8 p.i., there was an increase in IL-6, IL-33, IL-5 and eosinophil peroxidase (EPO) (a measure of eosinophil activity)- EPO is involved in ROS production (Malik and Batra, 2012). So despite a lack of expansion of the eosinophil population until day 18 p.i., the activity of these cells increased at day 8 p.i. The only difference observed at the earliest time point, day 5 p.i., was an increase in IL-5.

But when comparing *A. suum* infection with and without HDM presensitization, the authors found a reduction in larval numbers and size of the parasite, indicating an inhibition in larval migration as well as larval development. Interestingly, they did not find a difference in larval burden in the livers. At the earliest time point, day 5 p.i., the

Table 1.2: Summary table of the immune system during *Ascaris* infection. Immune cells noted were found to be increased under infection, unless otherwise specified. The three larval stages of *Ascaris* infection are described for the two most common model organisms pig (✓) and mouse (Δ), in addition to findings from infected humans, which used serum (Θ). A † next to a cell type indicates evidence for immunomodulation by *Ascaris*.

	Larvae in gut	Larvae in liver	Larvae in lungs	Human serum	References
Innate					
Eosinophils	✓	✓ (white spots)	Δ		✓Masure *et al.*, 2013a, ✓Vlaminck *et al.*, 2016, ✓Pérez *et al.*, 2001, Δ Enobe *et al.*, 2006, Δ Nogueira *et al.*, 2016, Δ Gazzinelli-Guimaraes *et al.*, 2019, Δ Nogueira *et al.*, 2016, Δ Weatherhead *et al.*, 2018
Epo (transcription factor) †	✓		Δ		✓Masure *et al.*, 2013a, Δ Gazzinelli-Guimaraes *et al.*, 2019 † Itami *et al.*, 2005
CCL11	✓				Masure *et al.*, 2013a
Neutrophils		✓ (white spots)	Δ		✓Pérez *et al.*, 2001, Δ Enobe *et al.*, 2006, Δ Nogueira *et al.*, 2016, Δ Weatherhead *et al.*, 2018
Macrophages		✓ (white spots)	Δ		✓Pérez *et al.*, 2001, Δ Enobe *et al.*, 2006, Δ Nogueira *et al.*, 2016, Δ Weatherhead *et al.*, 2018
Monocytes			Δ		
Alternatively activated macrophages			Δ		Δ Gazzinelli-Guimaraes *et al.*, 2019
Mast cells	✓				✓ Masure *et al.*, 2013a
Goblet cells (hyperplasia)	✓				✓ Masure *et al.*, 2013a
Dendritic cells †					† Favoretto *et al.*, 2014
Adaptive					
IL-6 †			Δ		Δ Gazzinelli-Guimaraes *et al.*, 2019, † Jakobsen *et al.*, 2019
Th1 †					† Arora *et al.*, 2020
Th2			Δ		Δ Gazzinelli-Guimaraes *et al.*, 2019, Δ Nogueira *et al.*, 2016
IL-5	✓		Δ		✓Masure *et al.*, 2013a, Δ Gazzinelli-Guimarães *et al.*, 2013, Δ Weatherhead *et al.*, 2018
IL-13	✓		Δ		✓ Masure *et al.*, 2013a, Δ Gazzinelli-Guimaraes *et al.*, 2019, Δ Weatherhead *et al.*, 2018
IL-4			Δ		Δ Gazzinelli-Guimaraes *et al.*, 2019, Δ Weatherhead *et al.*, 2018
IL-33			Δ		Δ Gazzinelli-Guimaraes *et al.*, 2019
Th17 †			Δ (with Th2)		Δ Nogueira *et al.*, 2016, † Arora *et al.*, 2020
Treg †				Θ	Θ Matera *et al.*, 2008, Θ Antunes *et al.*, 2015, Θ Titz *et al.*, 2017, † Antunes *et al.*, 2015, † Titz *et al.*, 2017
IL-10				Θ	Θ Turner *et al.*, 2003, Θ Jackson *et al.*, 2004, Θ Turner *et al.*, 2005, Θ Antunes *et al.*, 2015, Θ Titz *et al.*, 2017
Immunoglobulins					
IgE				Θ	Θ Hagel *et al.*, 1993, Θ McSharry *et al.* 1999
IgA	✓			Θ	✓ Miquel *et al.*, 2005, Θ Turner *et al.*, 2003, Θ Jackson *et al.*, 2004

presensitized group showed an increase in eosinophils, macrophages, Th1, Th17 and Th2 cells compared to the unsensitised group. This was paired with an increase in IL-4, IL-13, IL-33 and IL-5. The levels of cells and cytokines in the presensitized group were back to baseline level at day 18 p.i. Additionally, at the two early time points, i.e. day 5 and 8 p.i., there were significantly more innate lymphoid cells in the presensitized mice, however, this flips with more innate lymphoid cells in the unsensitised mice at day 18 p.i.

The authors did not observe this difference in larval burden and size in HDM sensitized eosinophil-deficient mice, indicating that eosinophils are essential for larval inhibition. Interestingly, a study by Nogueira *et al.* (2016) demonstrated that this rise in eosinophils, combined with a Th2/Th17 mixed response, was more pronounced in re-infected mice than in single-infections. These authors observed that this increase in eosinophils correlated with a decrease in parasite burden, they thus also found a potential role for eosinophils in the clearance of infection. The allergic response evoked by HDM (Gazzinelli-Guimaraes *et al.*, 2019) could therefore be quite similar to a single, primary infection with *A. suum*. It appears that lungs primed, either by *A. suum* infection or HDM allergic response, respond to an *A. suum* challenge infection with an increase in Th2 and eosinophils, allowing for a reduction in larval numbers and size.

1.7.3 A focus on different cell types

1.7.3.1 *Ascaris targets dendritic cells*

Dendritic cells (DC) are an important link between the innate and adaptive immune system and are essential for a Th2 response activation (MacDonald and Pearce, 2002, Balic *et al.*, 2004). High molecular weight antigens from adult *A. suum* extract have been found to be internalized by murine DCs and modulate DC activation by lowering the usual increase in costimulatory molecules under lipopolysaccharide (LPS) activation, which ultimately resulted in reduced proliferation of T cells (Favoretto *et al.*, 2014).

Adult body fluid (ABF) from both *A. suum* and *A. lumbricoides* has been shown to interfere with LPS-induced maturation and functionality of DCs from both humans and mice. Human monocyte-derived DCs exposed to both ABF from *A. suum* and LPS were found to suppress IL-6, IL-12p70, and TNF-α secretion (Summan *et al.*, 2018). However, expression of molecular surface markers CD86 and MHC-II were not altered.

Midttun *et al.* (2018) found similar results with reduced secretion of IL-23, IL-12p70, TNF-α, IL-6, IL-10, and CXCL1 in monocyte-derived human macrophages cultured with ABF and stimulated with LPS. The authors also identified an upregulation in negative regulator of ROS (NRROS), when culturing human monocyte-derived DCs with ABF from *A. suum*. The authors therefore hypothesize 'that *A. suum* inhibits inflammatory responses by modulating ROS production' (Midttun *et al.*, 2018). This is an interesting find, and correlates with our own findings in the proteome of the liver in a mouse model of hepatic resistance (Deslyper *et al.*, 2016). Here we identified that the relatively resistant strain had an intrinsically higher abundance in proteins involved in OXPHOS, than the relatively resistant strain. We hypothesized that because of these differences in OXPHOS proteins, the two mouse strains have an inherent difference in ROS, which could influence the observed difference in larval burden.

Midttun *et al.* (2018) also found that ABF suppresses lactate production in DCs. Maturation and activation of immune cells, including DCs, is driven by changes in the cell metabolism. Naïve DCs rely on OXPHOS and switch to anaerobic glycolysis during activation (Everts *et al.*, 2014). The authors conclude that *Ascaris* could therefore induce DC immune suppression in the gut.

Recently, pseudocoelomic fluid (PCF) from *A. suum* has been found to downregulate genes involved in Th1 and Th17 responses by monocyte derived DCs *in vitro* (Arora *et al.*, 2020). This is an interesting find, as we previously noted that in the lungs, *Ascaris* induces a Th2 response.

1.7.3.2 *Eosinophils in humans*

Interestingly, eosinophilic involvement in human *Ascaris* infection has been found by McSharry *et al.* (1999). They found that Nigerian children, putatively immune to *Ascaris* had a higher concentration of the inflammatory markers C-reactive protein, ferritin, and eosinophil cationic protein, when compared to a group predisposed to infection. Eosinophil cationic protein was also found to be upregulated in Ecuadorian children chronically infected with *Ascaris*, i.e. they had *A. lumbricoides* eggs in their stool at all investigated time points (Reina Ortiz *et al.*, 2011). Eosinophilic cationic protein has been shown to be able to paralyse *S. mansoni* (McLaren *et al.*, 1984) and kill both *Trypanosoma cruzi* and *Brugia malayi in vitro* (Hamann *et al.*, 1990).

1.7.4 Regulation of the immune system

Regulation of the immune response is an important part of the immune system. Several immune modulating cells were identified to play a role in helminth infections such as alternatively activated macrophages, regulatory T (Treg) cells, and regulatory B cells (Allen and Maizels, 2011). Matera *et al.* (2008) found a statistically significant increase of innate $CD4^+$ $CD25^+$ Treg cells in *Ascaris* infected individuals compared to controls. The authors suggest this regulation of the immune response could be beneficial for both the host and the parasite. The parasite would be able to sustain prolonged infection. The host on the other hand, would benefit from a dampened immune response, which would otherwise induce pathology (Matera *et al.*, 2008). However, Matera *et al.* (2008) found that in *Ascaris*-infected patients, despite an increase in Treg cells, no expected rise in IL-10 was observed. This is in contrast to other studies performed in human populations, which found that age dependent heterogeneity of *A. lumbricoides* infection was associated with an increase in IL-10 (Turner *et al.*, 2003, Jackson *et al.*, 2004, Turner *et al.*, 2005).

The above research in humans highlights the potential of immune modulation of *Ascaris* infections. Mouse models enable further exploration of specific components and proteins involved in *Ascaris*-induced immune modulation. PCF, in combination with LPS stimulation, was found to induce Treg responses, increase IL-10 levels, reduce LPS induced pro-inflammatory cytokines and increase anti-inflammatory cytokines (Antunes *et al.*, 2015, Titz *et al.*, 2017). However, the presence of an LPS stimulation is not essential as a Treg induction without an LPS stimulus has also been found (McConchie *et al.*, 2006, Rocha *et al.*, 2008).

Several proteins have been studied to identify their immunomodulatory properties. PAS-1, a component of the PCF of *A. suum*, was observed to induce an inhibition of LPS-induced inflammation and leukocyte migration in an IL-10 mediated manner (Oshiro *et al.*, 2005, Antunes *et al.*, 2015), decreased anti-ovalbumin antibody secretion (Oshiro *et al.*, 2004) and reduced EPO activity (Itami *et al.*, 2005). Interestingly, PAS-1 is homologous to ABA-1 from *A. lumbricoides* (Antunes *et al.*, 2015). It is secreted during both larval development and adult life stages, but secretion is highest during the early stages of larval development (L3).

As discussed in the dendritic cells section, ABF also has immune modulatory functions. Porcine peripheral blood mononuclear cells treated with ABF and stimulated with LPS, showed a reduction in IL-6 compared to LPS induced without ABF cells (Jakobsen *et al.*, 2019). However, no difference in IL-10 production was observed.

ABF from *A. suum* was also found to influence the phenotype of human macrophages *in vitro* (Almeida *et al.*, 2018). Undifferentiated macrophages were inhibited from M1 differentiation by ABF. LPS stimulation of M1 macrophages, in the presence of ABF, reduced both pro- and anti-inflammatory cytokines. However, this was not the case in M2 macrophages, where IL-10 secretion was not affected.

Other noteworthy molecules are phosphorylcholine secretion by *A. suum*, which have immunomodulatory properties by disturbing lymphocyte proliferation pathways (Bethony *et al.*, 2006), and glycosphingolipids, which have been shown to inhibit LPS-induced Th1 cytokine production (IFN-γ). Mucin12 has been identified in the mucous secretory organs and the epidermis of hypodermis and adult *A. lumbricoides* worms (Hayashi *et al.*, 2019). Human transmembrane mucin12, secreted in the small intestines, was found to have a common antigenicity with mucin12 from *A. lumbricoides*. This mucin12 could therefore play an important role in the immune evasion strategies of the parasite. A more recent study found the presence of plasminogen binding proteins present on the cuticle and in the excretory/secretory products of L3 stage *A. suum* larvae (Diosdado *et al.*, 2020). Plasminogen binding proteins have been found in different helminths, where they are found on the surface of the parasite (Ayón-Núñez *et al.*, 2018). These plasminogen binding proteins were found to be able to stimulate plasmin production. Interestingly, plasmin is an inhibitor of complement activation through inactivation (Barthel *et al.*, 2012). The presence of these plasminogen binding proteins in could therefore indicate that the immunomodulatory mechanism of the parasite are already present at this early stage.

To conclude, the immunomodulatory effect of *Ascaris* makes it hard for the host to develop natural immunity (Hewitson and Maizels, 2014).

1.7.5 Immunoglobulins in adult stages

An increase in IgE concentration is associated with *Ascaris* infection. High levels of specific IgE antibodies against a recombinant *Ascaris* protein in particular (anti-ABA-1) have been associated with natural immunity against *Ascaris* infection (Hagel *et al.*,

1993, McSharry *et al.*, 1999). Paterson *et al.* (2002), however, found that ABA1 is 'not intrinsically allergenic'. The authors argued that the IgE response observed in *Ascaris* infections is a combination of several factors: exposure route, antigen dose, and other Th2 promoting molecules, which together make ABA-1 a bystander antigen (Paterson *et al.*, 2002). IgE was found to bind the Fc epsilon RI (high-affinity IgE) receptor on eosinophils and induce eosinophilic degranulation (Gounni *et al.*, 1994).

IgE has been found to play an important role in eosinophil-mediated cytotoxicity in *S. mansoni* infection (Gounni *et al.*, 1994). The role of IgE, however, is a source of debate, with some evidence suggesting that high levels of *Ascaris* specific IgE antibodies have a protective effect (Hagel *et al.*, 1993), whereas a study performed in Bangladesh found that heavily infected children have higher anti-*Ascaris* IgE than lightly infected children (Palmer *et al.*, 1995).

Miquel *et al.* (2005) found an increase in anti-*Ascaris* IgA antibody secreting cells (ASC) at day 10 p.i. in the *lamina propria* of the proximal and distal jejunum of pigs, which the authors attribute to a delayed response to larval penetration after infection. These ASCs started decreasing at day 21 p.i. The authors additionally measured increased serum anti-*Ascaris* IgA, with IgA anti-ABF serum levels remaining high throughout infection and IgA anti-L3-ES serum levels dropping after day 21 p.i. Secreted anti-IgA antibodies play an important role in the gut as they form a protective barrier for pathogens and toxins to enter the gut epithelium, through trapping and eliminating potential pathogens (Mantis *et al.*, 2011).

The presence of a strong Th2 response in *Ascaris* infection has been found to be important for worm clearance, with an increased Th2 response playing a significant role in age-dependent resistance to *Ascaris* infection (Turner *et al.*, 2003, Jackson *et al.*, 2004). A Th2 immune response is associated with IL-4 and IL-5 production, two cytokines that induce IgE production and eosinophilia (Cooper *et al.*, 2000b), with IL-4 in particular mediating the class switch to IgE B cells (Anthony *et al.*, 2007).

To conclude, the immune response to *Ascaris* infection is still largely unknown. In general, parasitic infections are associated with a Th2 response. This has also been found for *Ascaris* infection in humans (Cooper *et al.*, 2000b, Turner *et al.*, 2003, Jackson *et al.*, 2004, Anthony *et al.*, 2007) and mice (Nogueira *et al.*, 2016, Gazzinelli-Guimaraes *et al.*, 2019). Additionally, in humans, high levels of IgE have been associated with

natural immunity against *Ascaris* (Hagel *et al.*, 1993, McSharry *et al.*, 1999). As for the early immune response, little is known about the liver stage. *A. suum* infection during the lung stage, on the other hand, has been found to induce a Th2 response with alternatively activated macrophages, leukocyte infiltration and eosinophilia (Gazzinelli-Guimarães *et al.*, 2013, Nogueira *et al.*, 2016, Weatherhead *et al.*, 2018, Gazzinelli-Guimaraes *et al.*, 2019). Two cell types in particular have received more attention, eosinophils and dendritic cells. Eosinophils were found to be important in reduction in number and seize of *A. suum* larvae in the lungs of a HDM sensitized mouse model (Gazzinelli-Guimaraes *et al.*, 2019) and in reduced larval numbers in trickle infections in the gut of pigs (Masure *et al.*, 2013a, Vlaminck *et al.*, 2016). Dendritic cells are important as they form a bridge between the innate and the adaptive immune system. Although the role of dendritic cells in *Ascaris* infection is not fully understood, it has been found that this cell population is a target for *Ascaris* excreted/secreted products (Favoretto *et al.*, 2014, Midttun *et al.*, 2018, Summan *et al.*, 2018, Arora *et al.*, 2020). But not only DCs experience the strong immune modulatory properties of *Ascaris*. Certain excretory/secretory products induce a decrease in pro-inflammatory cytokines and increase in anti-inflammatory cytokines in mouse models (Antunes *et al.*, 2015, Titz *et al.*, 2017) and an increase Treg populations in humans (Matera *et al.*, 2008).

1.8 Prevention & control

Ascaris prevention is performed through regular administration of chemotherapeutic drugs to vulnerable populations: pre-school aged children, school-aged children, women of reproductive age, and particular at risk groups, with the aim to control morbidity (WHO, 2012, Werkman *et al.*, 2020). The preventative treatment of school-aged children is important for all age groups as this demographic harbours the most heavy infections, and potentially excretes a disproportionate amount of infective eggs into the environment (Anderson *et al.*, 2013). The administration of chemotherapy to this group will therefore benefit the entire community. Holland *et al.* (1996) demonstrated the cost effectiveness of administering anthelmintics to a targeted group. The authors selected 4 different villages in Nigeria and gave each a different treatment regime (Asaolu *et al.*, 1991), a first village received selective treatment where 20% of the most heavily infected individuals received treatment. A second

village received targeted treatment, here all children between 2 and 15 years of age received treated. In the third village everyone was given treatment (MDA) and the fourth village acted as control with no treatment being given. The results of the study showed that targeted treatment and MDA gave statistically significant reductions in mean eggs per gram faeces (epg) per person. Both treatment regimens were also found to be cost efficient. However, selective treatment was found to be the worst intervention, it did not give any statistically significant reductions in mean epg, and it was not cost efficient. Furthermore, the selective option proved to be unpopular with the village community. Kirwan *et al.* (2009) investigated the often ignored group of preschool age children. The authors concluded that many of these young children (0-25 months) are infected with *Ascaris*. As this is a critical age of their development, it would be beneficial to include this age group in MDA treatment.

The use of MDA, however, has come under scrutiny (Taylor-Robinson *et al.*, 2012, Taylor-Robinson *et al.*, 2015, Taylor-Robinson *et al.*, 2019). Cochrane systematic reviews found 'little to no effect' of MDA on height, weight, haemoglobin, cognition, school performance and mortality (Taylor-Robinson *et al.*, 2019). They propose that screening and targeted treatment of infected individuals should be preferred over MDA. However, there are some problems with the review and its conclusions. Firstly, the analysis did not differentiate between the soil-transmitted helminths (STHs), by pooling the STHs together one does not take into account the varying effects of different MDA treatments on this diverse group of parasites (Bundy *et al.*, 2013, Majid *et al.*, 2019). Additionally, they did not take the intensity of infection into account, with heavy infection causing more severe pathologies. Taylor-Robinson *et al.* (2019) agree that a subgroup of heavily infected children could have a beneficial effect from treatment on their weight gain, but that this effect could be undetected due to dilution.

A recent study again confirmed the effectiveness of MDA, here the authors investigating the usefulness of serological tests as a diagnostic tool for *Ascaris* infection (Dana *et al.*, 2020). The authors found that after three years of bi-annual MDA of school aged children, both prevalence and infection intensity were decreased (Dana *et al.*, 2020). This study was performed over a three year period, which is relatively long. On the other hand, some of the studies used in the Cochrane review were as short as 6 months (Bundy *et al.*, 2013). As the effects of worm infections are often chronic rather than acute, more longitudinal studies would be necessary to determine the effects of

MDA (Bundy *et al.*, 2013). For one of these chronic symptoms, cognitive development, the Cochrane reviews found not 'enough evidence' for benefits of MDA. However, comparing cognition between studies is notoriously challenging due to a lack of a consistent test battery (Bundy *et al.*, 2013). Ultimately, Bundy *et al.* (2013) point out that there are practical benefits to MDA treatment of school aged children in particular. This is group often has the highest worm burden in a population and therefore excretes most eggs into the environment. This group is also easily accessible for MDA treatment. These two factors combined make it the ideal target population for MDA treatment while having a beneficial effect on the whole population (Bundy *et al.*, 2013). The proposed targeted treatment can result in significantly higher costs than MDA (Majid *et al.*, 2019). Introducing a baseline for level of infection and intensity could potentially be useful, where if a population is above this baseline, MDA is more cost effective (Majid *et al.*, 2019).

Furthermore, MDA treatment is said to have 'collateral benefits' (Hotez *et al.*, 2019). It is often given in a population against one particular prevalent STH. This MDA will also be useful against other STHs in that population, even if the efficacy is lower. But MDA is not only beneficial against STHs, MDA administered for one parasite will also help in reducing the prevalence of other NTDs (Hotez *et al.*, 2019), including yaws (Mitjà *et al.*, 2015), scabies (Engelman *et al.*, 2013) and potentially even has a positive effect on *Plasmodium vivax* transmission (Smit *et al.*, 2018). Furthermore, mass treatment of trachoma with azithromycin was associated with reduced child mortality (Keenan *et al.*, 2018). Hotez *et al.* (2019) explains: 'Expanding the public health impact of preventive chemotherapy would significantly increase years of healthy life for people in affected regions and would be highly cost-effective.'

Anthelmintic resistance in *A. lumbricoides* has not been observed so far (Bennett and Guyatt, 2000, Vercruysse *et al.*, 2011), although some studies found reduced efficacy during treatment, especially in areas with a history of distributing preventative anthelmintics (Krücken *et al.*, 2017, Vlaminck *et al.*, 2019). Although this is not widespread at the moment, it is a concerning development which needs to be monitored for future preventative programs. A meta-analysis investigated the efficacy of all four anthelmintic drugs, albendzole, mebendazole, levamisole, pyrantel pamoate, used against *A. lumbricoides*, based on their estimate average cure and egg reduction rates (Moser *et al.*, 2017). The authors concluded there were no statistically significant

differences between the drugs in their efficacy to treat *A. lumbricoides*. A more recent meta-analysis found similar results, with no differences in treatment efficacy between single albendazole, mebendazole, ivermectin and multiple albendazole treatment (Conterno *et al.*, 2020).

Interference with transmission is difficult, given that hardy, infective eggs of *Ascaris* are present in the environment (Cooper and Hollingsworth, 2018). In fact, eggs can survive for up to 15 years (Crompton, 1989, O'Lorcain and Holland, 2000). It is thought, however, that most of the eggs die during shedding (Stephenson and Holland, 1987). A vaccine would therefore be a great help to *Ascaris* elimination. Several vaccines have been trialled on pigs (Urban and Tromba, 1984). Other vaccines, trialled in mice, target specific egg antigens of *A. suum* (As14&As16, As26, As37, As-Enol-1) (Hewitson and Maizels, 2014, Zhan *et al.*, 2014, Wei *et al.*, 2017, Versteeg *et al.*, 2020), antigen from the adult worm (As-GST-1) (Zhan *et al.*, 2014), crude extracts from different *Ascaris* life stages (Gazzinelli-Guimarães *et al.*, 2018) and homologues from other parasites (Hewitson and Maizels, 2014). However, so far no vaccine has shown sufficient efficacy in mouse trials.

It is still more feasible to eliminate morbidity rather than the parasite itself (WHO, 2012). This is for two reasons, the first being that anthelmintic treatments are very effective against heavy infections and can thus reduce morbidity efficiently (WHO, 2012). Secondly, chemotherapy is easy to administer in countries with limited resources, and are sometimes donated and therefore available for free (WHO, 2012). Health education and improved sanitary facilities (Montresor *et al.*, 2013) should also be considered in order to reduce infection. MDA treatment could therefore be used in combination with water, sanitation, and hygiene (WASH) improvements (Strunz *et al.*, 2014). Economic development in general, however, remains the best possible route to eliminating STH (O'Lorcain and Holland, 2000).

Using mathematical models, it is possible to predict if *Ascaris* transmission can be halted using conventional chemotherapy (Hollingsworth *et al.*, 2013). Due to aggregation of parasites in few hosts, the breakpoint at which the *Ascaris* population would go extinct is rather low, with an average of close to zero worms per host (Hollingsworth *et al.*, 2013). Although some studies have shown that these targets could potentially be reached through preventative chemotherapy alone. Preventative chemotherapy has been shown to be able to reduce STH prevalence to a very low

number in addition to an 'almost complete' elimination of STH associated morbidity (Midzi *et al.*, 2020). However, due to the robust nature of the *Ascaris* eggs and their subsequent long-term survival in the environment, preventative chemotherapy will need to be coupled to a serious investment in WASH (Jourdan *et al.*, 2017). In all, achieving *Ascaris* – or STH in general – elimination through a combination of preventative chemotherapy and WASH may prove to be costly and could take a long time (Jourdan *et al.*, 2017), a vaccine might be the final push necessary to really break the transmission cycle.

1.9 Thesis structure

The aim of this thesis is to investigate the role of the liver in susceptibility to *Ascaris* infection.

Chapter 2: The role of the liver in the migration of parasites of global significance

A comprehensive review of the role of the liver during the migration of *Ascaris* and two other parasites of major public health significance – *Plasmodium* and *Schistosoma*. These three parasites migrate through the liver during their early life cycle and the status of the liver as an immunotolerant organ is highly likely to play a role in the migratory process. This opens the question whether these parasites use this immunotolerance to hide from the host's immune response and the time in the liver to mature and grow within the host. Chapter 2 investigates what is known about the molecular and immune mechanisms occurring in the liver during the passage of these three parasites.

Chapter 3: The liver proteome in a mouse model for *Ascaris suum* resistance and susceptibility: evidence for an altered innate immune response

This chapter examines the role of the liver in susceptibility to *Ascaris* from a proteomic perspective. A mouse model for relative susceptibility and resistance to *A. suum* using one strain, CBA/Ca, as a model for relative resistance and another strain, C57BL/6J, as a model for relative susceptibility. At day 7 p.i. the livers were extracted and their proteins examined using shotgun mass spectrometry.

This study identifies several differences between the mouse strains. Firstly, a difference was observed in oxidative phosphorylation proteins and proteins involved

in retinol metabolism. Secondly, a difference was also observed under infection, where the CBA/Ca strain was found to have more proteins involved in activating the complement system, whereas the C57BL/6J mouse strain had higher protein abundances for complement inhibiting proteins.

These results therefore indicate the necessity to investigate the immune response in the liver during *Ascaris* infection.

Chapter 4: *Ascaris lumbricoides* and *A. suum* vary in their larval burden in a mouse model

This next chapter explores whether the previously used mouse model for hepatic resistance is suitable for *A. lumbricoides*. So far the mouse model of hepatic resistance had only been used for *A. suum* infection, this chapter therefore investigates whether the model can be used for *A. lumbricoides* infection. To investigate this, both mouse strains (CBA/Ca and C57BL/6J) were infected with either *A. suum* or *A. lumbricoides*. Animals were sacrificed at different time points, starting at 6 hours p.i. up until day 8 p.i., and the number of larvae in the liver and lungs were counted. Furthermore, the length of the larvae in the lungs were measured for each species on day 6, 7 and 8 p.i.

The main finding was that the difference in susceptibility between the two mouse strains was maintained under *A. lumbricoides* infection compared to the previously established *A. suum* infection. Interestingly, during the liver stage there was a higher burden of *A. lumbricoides* in the liver than for *A. suum*, however, this changed in the lungs where the reverse was true. As for larval length in the lungs, which can be considered as a level of fitness of the parasite, larval length in C57BL/6J mice were consistently greater than in the CBA/Ca mice, for both ascarid species. Additionally, larval length of *A. suum* was greater than *A. lumbricoides* in both mouse strains. The study found that this mouse model of hepatic resistance is suitable for both ascarid species.

The higher number of larvae in the liver therefore indicates that *A. lumbricoides* potentially has greater infectivity than *A. suum*. However, the reversal in the lungs, with lower larval burdens for *A. lumbricoides*, indicates a greater antigenicity for this species with a greater immune response from the host, leading to a reduced number of smaller larvae in the lungs.

Chapter 5: Distinct hepatic myeloid and lymphoid cell repertoires associated with susceptibility and resistance to Ascaris infection

This chapter investigates the hepatic immune response in a mouse model of hepatic resistance to both *A. suum* and *A. lumbricoides* infection. So far the literature has mainly focused on the host immune responses to adult worms and less so, in response to lung larvae, however, the hepatic life stage has previously been identified as the most likely stage where the difference in larval burden occurs. This chapter therefore describes the immune cells activated during *Ascaris* infection in the liver.

Compared to uninfected C57BL/6J mice, uninfected CBA/Ca mice had higher $CD4^+$ and γδ T cell counts in their spleens and higher B cell and lower eosinophil and Kupffer cell counts in their livers. Infection with *A. suum* led to expansions of eosinophils, Kupffer cells, monocytes and dendritic cells in the livers of both mouse strains and depletions of hepatic natural killer cells in CBA/Ca mice. Infection with *A. lumbricoides* led to decreases in the numbers of $CD8^+$, αβ, natural killer and natural killer T cells in the livers of CBA but not C57BL/6J mice.

Chapter 6: General discussion

This final chapter draws together the main findings of this thesis and will outline unanswered questions for future research.

Chapter 2 - The role of the liver in the migration of parasites of global significance

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2.1 Abstract

Many parasites migrate through different tissues during their life-cycle, possibly with the aim to enhance their fitness. This is true for species of three parasite genera of global importance, *Ascaris*, *Schistosoma* and *Plasmodium*, which cause significant global morbidity and mortality. Interestingly, these parasites all incorporate the liver in their life-cycle. The liver has a special immune status being able to preferentially induce tolerance over immunity. This function may be exploited by parasites to evade host immunity, with *Plasmodium* spp. in particular using this organ for its multiplication. However, hepatic larval attrition occurs in both ascariasis and schistosomiasis. A better understanding of the molecular mechanisms involved in hepatic infection could be useful in developing novel vaccines and therapies for these parasites.

2.2 Background

The life-cycle of many parasites in their final host often involves migration from one tissue to another. The reasons for this are unclear; however, tissue migration has been linked to increased body size and maturation, which leads to improved parasite survival (Read and Skorping, 1995). One study (Mulcahy *et al.*, 2005) suggested that tissue migration enables the parasite to avoid eliciting an immune response, which is often raised at mucosal surfaces. If evading the host's immune response is in fact a key purposes of this migration, the potential role of the liver becomes clear. The liver is an immunotolerant organ and therefore an ideal place for parasites to hide from the immune system. We will use human parasites of three genera, *Ascaris*, *Schistosoma* and *Plasmodium*, to illustrate the essential role of the liver in the life-cycle of these parasites. We will demonstrate that the liver is a crucial step in their life-cycle, a point at which infection appears to go unnoticed, but a potential bottleneck where vaccination/treatment could be most effective.

2.3 Immunotolerance in the liver

The special immune status of the liver was first identified in transplantation experiments in pigs, where allogeneic liver transplants were not rejected as was the case with other organs (Calne, 2000, Doherty, 2016). In humans some tolerance is observed whereby transplanted livers recover spontaneously after a rejection reaction (Calne, 2000) and some liver allograft recipients can even be completely withdrawn from immunosuppression (Devlin *et al.*, 1998).

Hepatic immunotolerance occurs through a combination of unique anatomical and histological features of the liver. Most of the blood that enters the liver comes directly from the portal system, making it the first organ to be exposed to gut-derived molecules including harmless bacterial products and nutrients (Brandl *et al.*, 2017). The smallest unit of the liver, the hexagonal lobule, consists of a small layer of hepatocytes around a central vein. The capillary bed of the liver, the sinusoids, does not form tight junctions, instead it forms fenestrations that are known as sieve plates (Doherty, 2016). The perisinusoidal space, also called the space of Disse, replaces the basement membrane to separate the liver sinusoidal endothelial cells (LSECs) from the hepatocytes (Doherty, 2016).

The liver sinusoids are home to multiple populations of resident immune cells. These include myeloid leukocytes and liver parenchymal cells that express receptors that sense pathogens, and myeloid and lymphoid cells capable of phagocytosis and cytotoxicity (Doherty and O'Farrelly, 2000). Central to the tolerogenic nature of the liver, the sinusoids contain multiple populations of antigen-presenting cells (APC) which present antigenic peptides bound to MHC molecules to T lymphocytes of the adaptive immune system (Doherty and O'Farrelly, 2000, Thomson and Knolle, 2010, Crispe, 2011). Hepatic APCs are capable of activating T cells *in vitro*, inducing cytotoxicity and inflammatory cytokine secretion (Doherty and O'Farrelly, 2000). However, in the environment of the liver, hepatic APCs are more likely to inactivate T cells or induce their maturation into regulatory T (Treg) cells that suppress immune responses (Doherty, 2016).

Antigen presentation to T cells is typically mediated by DCs (Guermonprez *et al.*, 2002). DCs express pathogen receptors that enable them to recognise components of microorganisms that are not found in mammalian systems. They also express costimulatory and/or coinhibitory receptors and release cytokines that determine the nature of T cell activation or T cell tolerance. Antigen presentation by hepatic DC generally results in T cell inactivation by anergy or exhaustion. These tolerogenic DC can also drive the differentiation of naïve T cells into Treg cells, which release immunosuppressive cytokines and suppress the activities of other immune cells in an antigen-specific manner (Steinman *et al.*, 2003, Tiegs and Lohse, 2010, Crispe, 2014).

Macrophages, known as Kupffer cells, are also abundant in the liver sinusoids. Similar to other macrophages, two subsets of Kupffer cells (KC), defined by their phagocytic and cytokine-producing properties, have been described (Kinoshita *et al.*, 2010). 'Inflammatory' or M1 macrophages secrete high levels of the proinflammatory cytokine IL-12 and low levels of the regulatory cytokine IL-10, whereas 'alternatively-activated' or 'repair' M2 macrophages, produce high levels of IL-10, TGF-β and low levels of IL-12 (Davies *et al.*, 2013). Upon pathogen receptor ligation, KCs most frequently act as M2 macrophages (Knolle *et al.*, 1995) and antigen-presentation by these cells is frequently associated with expression of inhibitory ligands and cytokines and the induction of Treg cells (Wiegard *et al.*, 2005, Breous *et al.*, 2009).

LSECs also express MHC and costimulatory molecules and are capable of presenting antigen to $CD8^+$ T cells leading to tolerance (Lohse *et al.*, 1996, Limmer *et al.*, 2000, von

Oppen *et al.*, 2009) and to $CD4^+$ T cells leading to their differentiation into Treg cells (Kruse *et al.*, 2009). Hepatic stellate cells (HSC), also called Ito cells, can also present antigen to T cells (Winau *et al.*, 2007), but again, antigen presentation by this APC preferentially promotes T cell tolerance (Jiang *et al.*, 2008, Yang *et al.*, 2009). HSC can also promote the differentiation of monocytes into myeloid-derived suppressor cells (MDSC), which have potent T cell inhibitory activities (Höchst *et al.*, 2013). Hepatocytes also express pathogen receptors, MHC and costimulatory molecules, although it is not clear these cells can present antigens to T cells leading to their activation (Bertolino *et al.*, 1998, Chen *et al.*, 2005).

2.4 Three parasite genera of global importance: *Ascaris*, *Schistosoma* and *Plasmodium*

Species of the three parasite genera discussed in the present review all use different ways to enter their final hosts, oral ingestion of eggs (*Ascaris* spp. (Murrell *et al.*, 1997)), skin penetration by free-swimming cercariae (*Schistosoma* spp. (McManus *et al.*, 2018)), and injection into the blood stream *via* mosquito bites (*Plasmodium* spp. (Ashley *et al.*, 2018)) (Figure 2.1). Despite entering different tissues, the parasites migrate to the liver rather quickly.

Additionally *Ascaris* is a nematode, whereas *Schistosoma* is a trematode and *Plasmodium* is a protozoa. Despite belonging to completely different classes, these parasites all migrate through the liver.

After egg-hatching in the gut, *Ascaris* spp. larvae are transported to the liver *via* the portal vein. Subsequently the larvae migrate to the lungs, where they are coughed up and swallowed thereby re-entering the gut (Roepstorff *et al.*, 1997). The complexity of this life-cycle, in addition to the fact that both the start and end organ of the life-cycle are one and the same, the gut, indicates the importance of tissue migration, potentially related to increased fitness of the parasite.

Schistosoma spp. are slightly different, as the parasites migrate to the a different organ, the lungs first, before entering the liver and ultimately reaching the mesenteric vessels (McManus *et al.*, 2018). This is therefore the opposite path to *Ascaris* spp. larvae take. Although *Schistosoma* spp. reach the lungs first and the liver second, we will show that the role of the liver in the migratory path is still very important in the larval

development. In particular, in non-immune animals, the liver appears to play an important role in parasite attrition.

Plasmodium sporozoites are injected into the skin by an infected mosquito and migrate to the liver *via* the bloodstream (Cowman *et al.*, 2016) where the parasites mature into merozoites and multiply. Subsequently the merozoites are released in great numbers into the bloodstream and infect erythrocytes where they mature into trophozoites, schizonts which release merozoites during asexual multiplication (Cowman *et al.*, 2016). Ultimately trophozoites will mature into male and female gametocytes that can be picked up by a mosquito bite. The liver is therefore used by the parasite as a 'safe haven' for the parasites to evade the hosts' immune system. It is also the place where hypnozoites from *P. vivax* can remain unnoticed for years before restarting their life-cycle and causing malaria pathology.

2.5 *Ascaris*: self-cure and differential burden

A. lumbricoides is a soil-transmitted helminth which infects 800 million people worldwide (Pullan *et al.*, 2014). The eggs have a thick shell, making them highly resilient to various environmental factors such as temperature and desiccation (O'Lorcain and Holland, 2000). Infections usually peak in children between 5–15 years-old, who can experience symptoms ranging from growth retardation to diminished cognitive development (O'Lorcain and Holland, 2000, Deslyper and Holland, 2017).

Most infected individuals carry light worm burdens but a relatively small proportion harbour heavy infections, a distribution described as aggregated (Croll and Ghadirian, 1981). Predisposition to infection has also been observed in ascariasis, a phenomenon whereby individuals are prone to a particular intensity of infection and regain similar worm burdens after treatment (Holland *et al.*, 1989). Although the exact mechanism of predisposition is unknown, it has been found to involve various factors, such as host genetics and adaptive immunity (Holland, 2009).

While the liver stage appears to be clinically silent, the presence of macroscopical white spots, areas of inflammation formed around the larvae due to injury during migration, in the liver of both humans infected with *A. lumbricoides* (Javid *et al.*, 1999) and pigs infected with the porcine ascarid *A. suum* (White, 1941) is a clear indication that an immune response is generated. When treating experimentally infected pigs with anthelmintics during the liver stage (day 2, 3 and 4 post-infection, p.i.) of *A. suum*

infection, an increase of 22% in their feed conversion rates, was found when compared to treating the pigs at the lung stage of infection (day 6, 7 or 8 p.i. (Stewart *et al.*, 1984)). As for humans, a prospective study of 510 Indian patients with liver abscesses during a 10-year period identified *A. lumbricoides* as the causative agent in 14.5% of the cases (Javid *et al.*, 1999). Similarly, a study in South African children demonstrated that *A. lumbricoides* was the causative agent of liver abscesses in 2% of the cases (Hendricks *et al.*, 1997).

2.5.1 Self-cure in pigs

Self-cure is a phenomenon observed in some pigs, where pigs receiving an oral trickle infection with *A. suum* eggs do not develop intestinal worms. The potential role of the liver in this phenomenon has received considerable attention.

In order to assess the role of the liver, an experiment was performed which bypassed the liver by infecting pigs with L3 stage (the lung stage) larvae through intravenous injection (Jungersen *et al.*, 1999). At day 21 p.i., a time at which self-cure usually has occurred, pigs did not show the typically observed decreased larval burden. This led the authors to believe that the liver played a crucial role in self-cure. However, the lack of a control group of orally infected pigs makes it hard to confirm this hypothesis. Another study approached the question of the role of the liver in self-cure by orally infecting pigs with lung-stage larvae(Masure *et al.*, 2013b). The authors found first that self-cure still occurred, and secondly, that it happened at the same rate as their controls, i.e. pigs orally infected with embryonated eggs. A weep and sweep response has been attributed to this observation, whereby increased mucus secretion and gut movement eliminates the larvae before they can penetrate the gut wall (Masure, 2013). Additionally, intestinal eosinophils and T cells were found to potentially play an important role. The mechanisms behind self-cure appear to be diverse and are not yet fully understood.

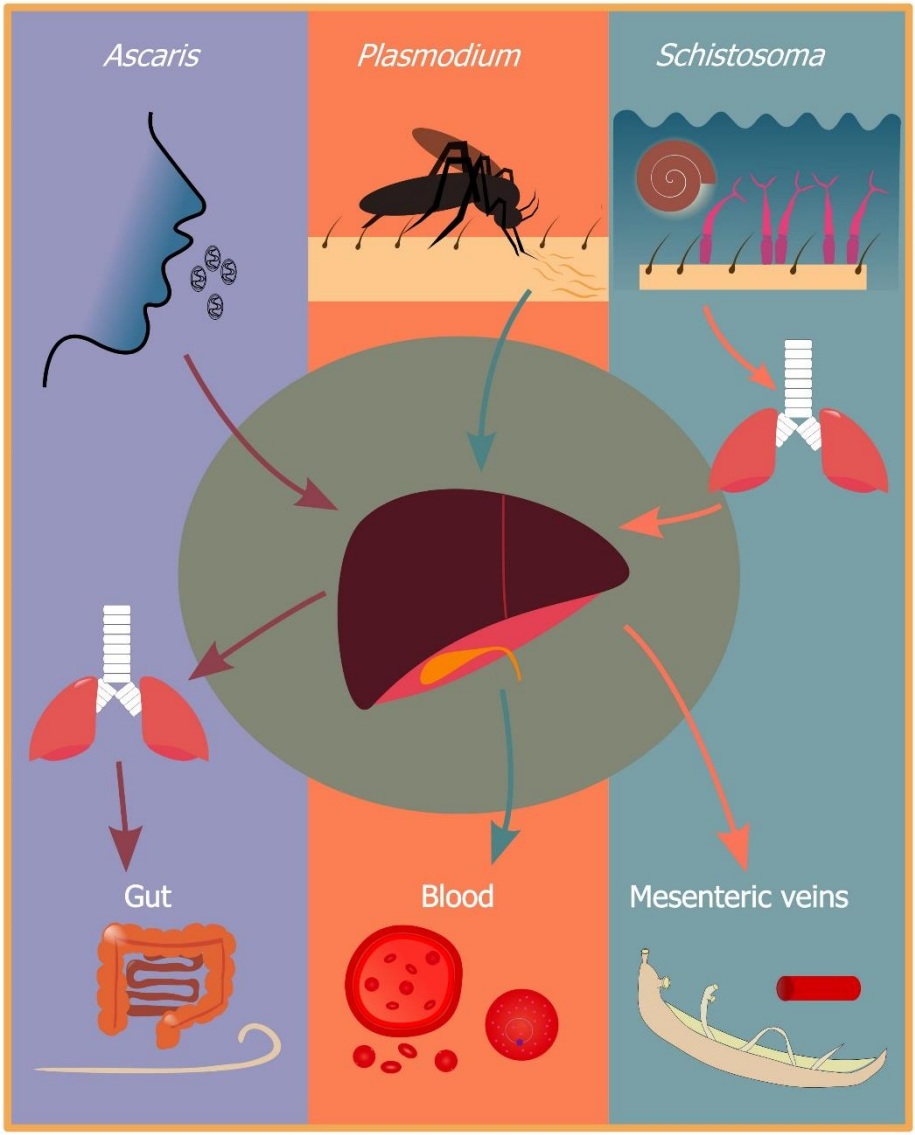

Figure 2.1: The migratory path of *Ascaris*, *Plasmodium* and *Schistosoma*. *Ascaris* eggs are ingested orally and after hatching, larvae penetrate the gut wall. The larvae subsequently move to the liver and the lungs from where they are coughed up and swallowed thereafter establishing as adult worms in the gut. *Plasmodium* sporozoites are injected into the skin; from there they migrate to the liver where they multiply into merozoites. Merozoites leave the liver and infect erythrocytes where they eventually mature into gametocytes to continue the life-cycle. Schistosome cercariae are released into water from infected snails. Cercariae penetrate the skin and then migrate to the lungs and then the liver. Ultimately, these parasites establish themselves as adult worms in the mesenteric veins

2.5.2 A mouse model for hepatic resistance

In order to study the liver stage of ascariasis, an appropriate animal model is necessary. Building on earlier work (Mitchell *et al.*, 1976), a mouse model has been developed specifically to study the predisposition phenomenon by using two inbred mouse strains which mimic the extremes of predisposition to light and heavy infection (Lewis *et al.*, 2006, Dold *et al.*, 2010, Deslyper and Holland, 2017). CBA/Ca and C57BL/6J were found to have a substantial and consistent difference in lung larval burden, with the former as the relatively resistant strain and the latter as the relatively susceptible strain.

This mouse model was subsequently used to investigate when, during migration, this difference in larval burden occurs (Lewis *et al.*, 2007). Mice of both strains were infected with the same number of *A. suum* eggs. At regular intervals from 6 hours p.i. until 8 days p.i., mice were sacrificed and their organs (gut, liver, lungs) removed. The larvae were retrieved from those organs, using the modified Baermann method. The larvae were subsequently enumerated and counted. This allowed for detailed tracking of larval migration throughout the various organs. Using this mouse model, similar larval burdens were found in the liver of both strains (Lewis *et al.*, 2007); however, once the larvae reached the lungs a significant difference in larval burden was observed, with the relatively resistant strain having a lower burden than the relatively susceptible strain. Another study found similar results, with no difference in total larval burden in the liver between the two strains (Dold *et al.*, 2010). The first larvae appear in the liver at 6 hours p.i. with a peak appearing between days 3–6, at this stage the larval burden is similar in both strains (Lewis *et al.*, 2007). The larvae subsequently migrate to the lungs, with the first larvae appearing as early as day 1 p.i.; however, the majority of the larvae arrive around day 6 p.i. At this point it becomes clear that one strain is relatively susceptible (C57BL/6J) and another relatively resistant (CBA/Ca), as there is a statistically significant difference in lung larval burdens between the two strains. Interestingly, the authors pointed out that there is a steady increase in mean larval burden in the liver between days 2 and 5 p.i., long after the larvae should have penetrated the gut wall and made their way to the liver. The authors speculated that "larvae were arriving from other locations and perhaps had become temporarily lost or trapped in other host tissues", indicating a strong instinct for the larvae to make their way to the liver and highlighting the importance of this organ during migration.

The question then remains, which organ contributes most to this difference in infection rate, is it the departing organ, the liver, or the organ where the larvae arrive, the lungs. In order to explore this question in more detail, an investigation of the presence of various leukocytes in the BAL in both strains of mice, infected with *A. suum* (Lewis *et al.*, 2007). This study showed that an increase in leukocytes occurred at days 8–9 p.i., so only after the difference in larval burden had already occurred. Additionally, it was found that the increase in leukocytes in BAL was higher in the susceptible strain than in the resistant strain, indicating that this increase did not contribute to the difference in larval burden between the two mouse strains but reflected the observed larval burden. This was confirmed in the same study by examining the lung tissue, in which was observed a greater inflammatory response in the susceptible strain. Taken together, these data indicate the importance of the liver stage and that it plays a key role in the difference in infection rate between these two mouse strains.

Shotgun mass spectrometry was used to explore the liver proteome in the above mentioned mouse model at day 4 p.i. (Deslyper *et al.*, 2016). We observed a difference in abundance of mitochondrial proteins involved in OXPHOS. The relatively resistant strain (CBA/Ca) had, both intrinsically and under infection, higher levels of this protein group than the relatively susceptible strain (C57BL/6J). This led us to believe that a potential intrinsic difference in ROS in the liver gives the relatively resistant strain an advantage in contending with the parasite.

A subsequent study investigated the difference in liver proteome at day 7 p.i. This study confirmed the earlier findings of day 4 p.i., demonstrating that the relatively resistant strain had a higher abundance of proteins involved in OXPHOS. However, the later day experiment revealed an important difference in the immune response to *A. suum* in the liver, with the relatively susceptible strain showing a higher abundance of proteins involved in complement inactivation compared to the relatively resistant strain which had a higher abundance of proteins involved in complement activation (Deslyper *et al.*, 2019b). These two studies demonstrate the importance of delineating host responses to helminth infection at different time points post-infection.

During a reinfection experiment (Nogueira *et al.*, 2016), BALB/c mice were infected with *A. suum*, the authors found no difference in the liver larval burden when comparing mice that were infected once or reinfected. However, a lower larval burden was observed in the lungs in the reinfected mice, and more importantly, lesions caused

by hepatocyte necrosis and infiltration of eosinophils and neutrophils were more pronounced in the reinfected group. These results indicate that the observed, more pronounced hepatic immune response in the reinfected group results in a decrease in lung larval burden.

To conclude, the presence of white spots indicates the presence of a strong immunological response in the liver. The liver stage becomes particularly important when investigating predisposition in pigs, mice and humans. Novel therapies could therefore focus on the liver in order to stop larval migration in its tracks, prevent increased damage due to larval migration and ultimately inhibit the development of adult worms which have significant impact on the nutritional and developmental status of the host (Holland *et al.*, 2013b, Deslyper *et al.*, 2016).

2.6 The liver as a site for attrition in *Schistosoma* infection

Species of *Schistosoma* infect over 250 million people worldwide (McManus *et al.*, 2018). Reinfections are common in endemic regions, with children often having suffered their first infection by the age of two, followed by a steady increase in worm burden with every new infection (Colley *et al.*, 2014). After these early infections, the worm burden decreases with increased exposure to the parasite that is thought to increase immunity, combined with the death of older worms (McManus *et al.*, 2018).

Schistosoma spp. interact with the liver during two distinct parts of their life-cycle. First, for all human *Schistosoma* species, the immature worms pass through the liver's vasculature after lung migration. Secondly, after the migratory path has been completed and the adult worms are located in the mesenteric vessels, some excreted eggs however do not end up in the faeces but instead travel to the liver *via* the hepatic vessel, this is true for all human *Schistosoma* species except *Schistosoma haematobium*. *S. haematobium* is the only species that does not cause liver pathology, as the adult worms are located in the *venus plexus* of the bladder (McManus and Loukas, 2008). In order to break down the eggs, granulomas are formed around them, causing hepatic fibrosis, which is the main source of mortality and morbidity in chronic schistosomiasis (Olveda *et al.*, 2017). Liver-associated disease in schistosomiasis is more common in *S. japonicum* and *S. mansoni* infections (Burke *et al.*, 2009). The liver is also profoundly impacted due to the longevity of the adult worms with an average life span of 3-10

years (Warren *et al.*, 1974). In essence, the most damaging symptoms associated with *Schistosoma* spp. infection, hepatic granuloma formation does not occur until after larval migration is completed and can be considered an unwanted side effect.

2.6.1 The liver as a site for maturation, pairing and sexual development

The liver stage is a crucial phase in the life-cycle of schistosomes, it is here that they can increase their biomass and develop into mature life stages (Barbosa *et al.*, 1978, Rocha and Coelho, 1980). These important life-cycle changes do not occur at any other stage, in fact, schistosomulae of *S. mansoni* which were trapped in the pulmonary vasculature were not able to reach maturity (Barbosa *et al.*, 1978, Jeremias *et al.*, 2017). Parasites that reached the liver, however, showed exponential growth, thus demonstrating that the liver's vasculature alone can provide the parasites with an adequate environment to reach maturity. Additionally, it was found that culturing *S. mansoni* schistosomulae in the presence of human portal serum showed a significant increase in cell proliferation when compared to schistosomula cultured in the presence of human peripheral serum (Shaker *et al.*, 1998, Draz *et al.*, 2008), again confirming that the liver vasculature provides the optimal environment for these parasites. However, so far it is not clear which components contribute, or might be essential for these processes to occur.

Additionally, it is at the liver stage that male and female parasites pair, which allows the females to reach sexual maturity; females cannot reach sexual maturity without the presence of males (Lu *et al.*, 2016). The paired schistosomes subsequently migrate against the blood flow to the mesenteric veins (Steinauer, 2009). The fact that these parasites need to manoeuvre against the blood flow, a process that female parasites cannot do by themselves (Steinauer, 2009) highlights again the importance of the liver stage for this parasite.

2.6.2 Attrition: when, where and how?

The site of attrition has been a topic of contention for decades, it was studied intensively in the 1980s thanks to the development of the autoradiographic tracking (Georgi *et al.*, 1982) which greatly improved the sensitivity of these experiments. Using this technique, it was found that between 86% and 90% of the skin-penetrant cercariae

in naïve mice (i.e. mice that received a single round of infection) had migrated to the lungs, indicating that the skin was not the site of attrition (Georgi *et al.*, 1983, Mangold and Dean, 1983). Further investigation of the migration of *S. mansoni*, using autoradiographic tracking in C57BL/6 mice showed a peak in the lungs at day 8 and in the systemic organs at day 12 (Wilson *et al.*, 1986). All this indicates attrition occurring between the pulmonary and hepatic life stages of the parasite (Georgi *et al.*, 1983). This raises the question as to why these parasites move to the liver next - perhaps the liver is a safe haven - a place where the parasites are no longer under attack and can reach sexual maturity safely hidden from the immune system.

As for immunized animals, which were immunized through either gamma-irradiated cercariae or establishing a chronic infection, the attrition is largely similar to that of naïve animals, except that everything appears to go more slowly, i.e. migration from the skin to the lungs takes longer and peak burden in the lungs was at a later time point. The big difference with naïve animals is the lower number of parasites reaching the systemic and splanchinic organs (Mastin *et al.*, 1983, Mangold and Dean, 1984, Von Lichtenberg *et al.*, 1985, Wilson *et al.*, 1986).

The location of attrition varies between *Schistosoma* species. For *S. mansoni* and *S. haematobium* attrition mainly occurs in the lungs (Georgi *et al.*, 1986, Wilson, 2009). However, five times more *S. mansoni* larvae reached the liver than is the case for *S. haematobium*. This observed greater larval death in *S. haematobium* infection can be partially attributed to the fact that mice are non-permissive hosts for this parasite (Imbert-Establet *et al.*, 1992). Attrition in *S. japonicum* was found to occur in both the lungs and the liver (Laxer and Tuazon, 1992).

Regardless of whether the lungs or the liver are the main site of larval removal, it is still crucial to investigate the role of the liver in the life-cycle, which could also be a potential target for vaccination. Early removal of the parasite, before it reaches sexual maturity and therefore egg-laying, would reduce granuloma formation.

2.7 Multiplication of *Plasmodium* in the liver

The protist parasites *Plasmodium* spp. are the causative agents of malaria in humans, resulting in an estimated 429,000 deaths in 2015 (WHO, 2016). There are five species that infect humans: *P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae* and *P. knowlesi*. Of these,

P. falciparum infection is the most common and pathogenic, causing 99% of malaria associated deaths (WHO, 2016).

Malaria-associated pathology is often divided into uncomplicated and severe *P. falciparum* malaria. Uncomplicated malaria results in general malaise (Ashley *et al.*, 2018). Severe falciparum malaria is caused by *P. falciparum* sequestering in the small and medium-sized blood vessels of different organs particularly the brain (Ashley *et al.*, 2018). Pathology in humans is therefore mainly associated with the blood stages of the parasite and complications thereof such as cerebral malaria (Ashley *et al.*, 2018). The lack of symptoms and the brief duration of the liver stage, make it a particularly difficult stage to study. However, a recent study using malaria-naïve human volunteers who were infected with *P. falciparum* through infective bites observed an increase in total leukocyte, lymphocyte and monocyte count during the liver stage followed by a decrease in the aforementioned counts when the blood stage is initiated (van Wolfswinkel *et al.*, 2017).

The liver is the only organ necessary for *Plasmodium* spp. maturation. At this stage a low number of sporozoites proliferate into a large numbers of merozoites, ready to invade erythrocytes and start the process of the well-known cyclic fever bouts. Due to this bottleneck, the liver stage forms an ideal target for vaccination purposes. However, before a vaccine can be developed, an in-depth understanding of this stage is necessary. The National Institute of Allergy and Infectious Diseases recently identified 'greater understanding of parasite liver-stage biology and development' as a key challenge in malaria vaccine development (Mo and McGugan, 2018).

2.7.1 The path to the liver

After being deposited in the skin from a mosquito bite, sporozoites migrate to the liver *via* the blood. To enter the liver, the sporozoites must cross the fenestrated endothelial layer; however, the fenestrations are too small for the sporozoites to pass through, thus passage must go through the sinusoidal cells (Ejigiri and Sinnis, 2009). Circumsporozoite protein (CSP) and thrombospondin-related anonymous protein (TRAP), expressed by sporozoites, binds human heparin sulphate proteoglycans (HSPGs), the signal for sporozoites to leave the blood stream (Frevert *et al.*, 1993, Sinnis *et al.*, 1996). Whether the sporozoites invade the liver through KC invasion or not is still under debate, with some studies suggesting a necessary step through KCs

(Pradel and Frevert, 2001, Frevert *et al.*, 2005), whereas others indicating that hepatocytes can be invaded directly (Tavares *et al.*, 2013). Sporozoites have been shown to directly infect hepatocytes *in vitro* and develop into merozoites; however, due to the cellular structure of the liver it has been suggested that *in vivo* the sporozoites must pass through KCs (Pradel and Frevert, 2001). Frevert *et al.* (2005) described the migration of the sporozoites with an abrupt speed change, at which point they glide along LSECs, followed by a long pause for the sporozoites to enter the KCs, which the authors attribute to formation of nonfusogenic vacuole formation and a relatively slow passage through the KCs. CSP from sporozoites has been shown to elevate cAMP levels in KCs, thereby inhibiting the cells from producing a respiratory burst and thus protecting the sporozoites (Usynin *et al.*, 2007). Additionally, sporozoite microneme protein essential for cell traversal 2 (SPECT2) deficient sporozoites were not able to infect the liver *in vivo* (Ishino *et al.*, 2005). These data suggest the need of KCs in hepatocyte infection.

However, a recent study provided evidence for the alternate hypothesis (Tavares *et al.*, 2013). The authors, building on earlier work (Mota *et al.*, 2001), identified that 17% of sporozoite cell traversals exclusively involve endothelial cells (Tavares *et al.*, 2013). They also identified that 15% of crossing events were independent of cell traversal and KCs, which could be the sporozoite moving between two endothelial cells or between an endothelial cell and a KCs. Furthermore, the authors found that sporozoites KCs traversal is associated with cell death of those KCs.

Regardless of this, sporozoites will eventually migrate through several hepatocytes, for yet unknown reasons, using a transient vacuole to ensure passage (Frevert *et al.*, 2005). Three theories exist as to why the *Plasmodium* parasites travel through several hepatocytes before forming the parasitophorous vacuole (PV) and ultimately differentiating into merozoites. The first theory argues several rounds of migration through hepatocytes might be a protective mechanism by the parasite to ensure that formation of the PV and subsequent merozoites formation only occurs in the liver and not in the other tissues (skin, etc.) (Mota *et al.*, 2001). A second theory builds on the observation that hepatocyte growth factor is released when the parasite migrates through hepatocytes this induces neighbouring hepatocytes to be more susceptible to infection (Carrolo *et al.*, 2003). Thirdly, it has been suggested that upon detection of high sulphated HSPGs, the sporozoites turn off their traversal machinery and activate

the invasion machinery; however, this has been shown to take between 30–60 min in *Plasmodium yoelii*, which would explain the invasion of multiple hepatocytes (Coppi *et al.*, 2007, Risco-Castillo *et al.*, 2015). When reaching their final hepatocyte, a PV is formed and the sporozoites can differentiate into merozoites (Frevert *et al.*, 2005).

The merozoites will then need to pass the space of Disse, at which stage they are vulnerable to KC phagocytosis (Sturm *et al.*, 2006). To avoid this, the infected hepatocytes release merozoite-filled vesicles, which are derived from the plasma membrane of hepatocytes, into the liver sinusoids (Sturm *et al.*, 2006, Graewe *et al.*, 2011). The phosphatidylserine switch is prevented in these merozoites-filled vesicles so as not to alert the KC and other immune cells, granting an escape route for the parasite (Sturm *et al.*, 2006).

2.7.2 Hypnozoites

Hypnozoites have only been observed in *P. vivax* (Richter *et al.*, 2010), although a handful of cases of relapsing *P. ovale* have been seen as well, but without confirmation of hypnozoites (Groger *et al.*, 2017). This life stage arguably exploits the liver's special immune status to the fullest. By going into a dormant state in the liver, for weeks or months, these parasites can hibernate and resume infection (Adams and Mueller, 2017). No study so far have been able to identify the triggers for hypnozoites reactivation, nor its specific relationship to the liver.

The importance of these hypnozoites, which can remain dormant for 6–9 months, cannot be overstated if the ultimate goal is malaria elimination (Battle *et al.*, 2014, White *et al.*, 2016). Up to 80% of all blood stage *P. vivax* infections are attributed to relapses; however, not all relapses can be attributed to hypnozoites and other mechanisms are thought to be at play (Betuela *et al.*, 2012, Robinson *et al.*, 2015, Markus, 2018). Investigating these hypnozoites more in depth could give a better insight in to why *Plasmodium* spp. incorporate the liver in the life-cycle and how they exploit hepatic immune tolerance.

2.7.3 Hepatic immunity

Naturally acquired immunity is probably not achieved at the liver stage, rather it is more likely an antibody-mediated response to the blood stage (Cowman *et al.*, 2016). However, the liver stage offers a great opportunity to activate the immune system and

eliminate the relatively few sporozoites before they multiply greatly in numbers and spread all over the body via the blood.

Innate recognition of *Plasmodium* RNA by melanoma differentiation-associated gene 5 protein (MDA5) and mitochondrial antiviral signalling (MAVS) pathway in infected hepatocytes induces activation of the transcription factors interferon-regulatory factors-3 (Irf3) and IRF7 (Gowda and Wu, 2018). Additionally, a recent *in vivo* study showed that protection against clinical malaria in children is associated with C1q-fixing antibodies against CSP in *P. falciparum* sporozoites (Kurtovic *et al.*, 2018). These antibodies inhibited hepatocyte cell traversal and ultimately induced sporozoite death.

$CD8^+$ T cells play an important role in immunity to malaria, with $CD8^+$ T-cell depleted mice being unable to develop immunity (Weiss *et al.*, 1988). The efficacy of parasite inhibition is therefore dependent on the availability of effector $CD8^+$ T cells (Silvie *et al.*, 2017). Some $CD8^+$ T cells get primed by dendritic cells in the skin draining lymph nodes before moving to the liver where they eliminate antigen presenting hepatocytes (Chakravarty *et al.*, 2007). No evidence exists that hepatocytes can successfully present antigen and activate naive $CD8^+$ T cells, however, hepatocytes can present a CSP epitope of *P. berghei* to primed $CD8^+$ T cells (Silvie *et al.*, 2017).

To ensure full inhibition of blood stage malaria development 100% immunity is required. To achieve this, the very low number of hepatocytes that are actually infected has to be fully eliminated in the relatively short hepatic period, meaning that there is a need for a very large threshold of memory T cells to ensure immunity after epitope-specific immunization (Silvie *et al.*, 2017).

In short, the immune response in the liver is not fully elucidated yet and knowledge appears to be lacking. *Plasmodium* spp. likely exploit the immunotolerance of the liver to increase the relatively small number of sporozoites to a much larger number of merozoites. The liver stage therefore forms an ideal target for vaccine development. If successful, a vaccine would be able to stop the infection before any symptoms occur and would eliminate the spread of malaria, thus also contributing to its elimination.

While the liver offers an immunotolerogenic environment for the maturation of species of *Ascaris*, *Schistosoma* and *Plasmodium,* it should be noted that the liver has many other attributes that make it an attractive residence for these parasites. The liver sinusoids comprise a network of capillaries containing nutrient-rich blood from the

intestine. The low blood pressure in the sinuses may offer an environment that supports growth, maturation and/or multiplication. Furthermore, the liver has a unique ability to regenerate and remodel itself, an attribute which could be exploited by the parasites to limit the deleterious effects of infection and inflammation and preserve their host tissue. The propensity of the liver to induce tolerance of foreign antigens, rather than immunity, is another attribute that may attract parasites, as well as other microorgansims, to this organ.

2.8 Conclusions

Immunologically the liver is a special organ where immune activation is reduced. This forms the ideal environment for species of *Ascaris*, *Schistosoma* and *Plasmodium* to mature to their next life stages, and multiply as is the case for *Plasmodium* spp. only. When migrating to the liver, the parasites are able evade the immune system. However, this does not always go according to plan. In the case of both *Schistosoma* spp. and *Ascaris* spp., the liver has been identified as a site of larval attrition. Understanding the molecular mechanisms behind this attrition could lead to the development of novel therapies. As for *Plasmodium* spp., the liver is a true bottleneck. It is at this life stage that the parasites multiply rapidly, before being released in the blood and spreading all over the host. The liver stage is therefore the ideal vaccine/drug target, as the parasite is still in relatively low numbers and concentrated in one organ. In short, the liver stage is understudied and more research is necessary to fully understand the molecular mechanisms and immune responses activated during parasitic invasion.

Chapter 3 - The liver proteome in a mouse model for *Ascaris suum* resistance and susceptibility: evidence for an altered innate immune response

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3.1 Abstract

Background: Ascariasis is a neglected tropical disease that affects 800 million people worldwide. Whereas most people only experience light worm burden, some people experience heavy worm burdens even after several rounds of chemotherapy, a phenomenon known as predisposition. Such heavy infections are associated with more severe symptoms and increased chronic morbidity.

Methods: In order to investigate potential mechanisms that may explain the observed predisposition, we infected mice with the porcine ascarid *Ascaris suum* using an established mouse model with two different mouse strains, where the C57BL/6J strain is more susceptible to infection and therefore a model for heavy infection and the CBA/Ca strain is more resistant and thus a model for light infection. At day 7 post-infection we investigated the liver proteome, using shotgun mass spectrometry, of both infected and control mice of each strain.

Results: We identified intrinsic differences, between the two mouse strains, in both oxidative phosphorylation proteins and proteins involved in retinol metabolism. Additionally, we found differences between the two mouse strains in activation of the complement system, where the CBA/Ca strain has higher protein abundances for lectin pathway proteins and the C57BL/6J strain has higher protein abundances for complement inhibiting proteins. The CBA/Ca strain had a higher abundance of proteins involved in the activation of the complement cascade *via* the lectin pathway. In contrast, the C57BL/6J strain demonstrated a higher abundance of proteins involved in arresting the complement pathway.

Conclusions: We observed clear differences between the two mouse strains both intrinsically and under infection.

3.2 Background

The neglected tropical disease ascariasis, caused by the parasitic nematode *A. lumbricoides*, infects an estimated 800 million people worldwide (Pullan *et al.*, 2014). The disease is widespread in Africa, Asia and South America (Jourdan *et al.*, 2018) and is particularly prevalent in children between the age of 5 and 12 who suffer the majority of the worm burden (Jourdan *et al.*, 2018, Wright *et al.*, 2018). Ascarids are also of agricultural significance as pigs are infected with *Ascaris suum*, which has a profound economic impact due to increased feed to gain ratio and liver condemnation (Boes *et al.*, 2010).

Symptoms of ascariasis can be divided into acute and chronic (Deslyper and Holland, 2017). Acute symptoms, though rare, can be severe and include intestinal obstruction and a characteristic allergic response, due to larvae migrating through the lungs, called Loeffler syndrome (Loeffler, 1932, Loeffler, 1956, Ribeiro and Fisher, 2002). Chronic symptoms are more common and although these are less severe, they have a significant impact at the population level. These include malnutrition, with associated stunting, and decreased cognitive development (O'Lorcain and Holland, 2000).

A small proportion of the population become heavily infected and are termed 'wormy people', whereas the majority of the people only experience a light infection (Croll and Ghadirian, 1981). This phenomenon is termed aggregation and is a key epidemiological pattern observed in macroparasites including *Ascaris* infection (Holland *et al.*, 1989, Crofton, 1971, Shaw and Dobson, 1995). Increased worm burden is associated with more severe symptomatology and can lead in extreme cases, especially in young children, to intestinal obstruction and even death (de Silva *et al.*, 1997b, O'Lorcain and Holland, 2000, Holland, 2009). Additionally, certain individuals were found to regain a similar worm burden upon reinfection, a phenomenon known as predisposition (Holland, 2009). Identifying the factors responsible for predisposition and aggregation may enable the development of new therapies that will halt the parasite during its larval migration and before it causes extensive tissue damage to the host.

In order to examine the factors responsible for predisposition, a better understanding of the host's response to the migrating larvae is necessary. Due to limitations with epidemiological studies, where it is impossible to study a large part of the parasite's life-cycle in its natural host, it is necessary to use alternative model organisms (Holland

et al., 2013b). Although pigs are natural hosts of *A. suum*, their husbandry, cost, size and lack of inbred strains makes them challenging to use under experimental conditions (Holland *et al.*, 2013b). Other model organisms, such as mice, rats, guinea pigs, gerbils, rabbits, goats and cows are abnormal hosts, meaning that the parasite has an incomplete life-cycle (Holland *et al.*, 2013b). In mice, the parasite follows a similar migratory pathway as in humans and pigs, making mice suitable as a model organism for early *Ascaris* infection (Slotved *et al.*, 1998). Lewis *et al.* (2007) developed a mouse model to explore *Ascaris* aggregation. They identified the C57BL/6J mouse strain as a model for relative susceptibility and the CBA/Ca strain for relative resistance. The difference in larval burden between the two strains was found to occur at the hepatic/post-hepatic stage of the migratory path (Lewis *et al.*, 2007).

Using this mouse model, we previously used label-free quantitative mass spectrometry to investigate the liver proteome between the two mouse strains with and without *A. suum* infection at day 4 post-infection (Deslyper *et al.*, 2016). This study revealed an inherent difference between the two mouse strains in mitochondrial proteins, more precisely for proteins involved in the OXPHOS, with the CBA/Ca mouse strain having a higher abundance of these proteins than the C57BL/6J strain both with and without infection. Furthermore, a decrease in abundance of ribosomal proteins was observed under infection for both strains, when compared to their respective controls.

In the present study, we build upon our previous data and examine the livers of CBA/Ca and C57BL/6J mice with and without *A. suum* infection at day 7 p.i., using high throughput quantitative mass spectrometry. At this time point, the majority of larvae have migrated from the liver to the lungs, with a peak larval burden observed in the lungs for both strains (Dold *et al.*, 2010). Our original study at day 4 p.i. illuminated the potential molecular determinants of resistance and susceptibility between the two mouse strains and a more restricted immune response than expected. It is anticipated that by examining the response in the livers at day 7 a stronger immune signature will be apparent and provide an enhanced view of the overall response to the sustained presence of *Ascaris*.

3.3 Methods

3.3.1 Ethical approval

The samples used in this study were approved by the TCD Animal Research Ethics Committee and the Health Products Regulatory Authority, the Irish regulator for scientific animal research in Ireland, under Directive 2010/63/EU and its Irish transposition, SI no. 543 of 2012 (Project Authorisation ID AE19136/P008 ID; Case Reference 7015826).

3.3.2 Power calculation

Animal numbers used in experiments were based on the following power analysis.

Formula for replication:

CV = coefficient of variation
D = % difference of importance
(Note: a 50% i.e. d=50 is the difference of importance)
R = replication per treatment

CV = 100 * (S.E./Mean)
$R = 16(CV/d)^2$

Table 3.1 Strain variation in susceptibility to *Ascaris suum* larvae (Lewis *et al*., 2006, Dold *et al*., 2010, Mitchell *et al*., 1976)

Mouse strain	Dose administered	Number of mice	Number of larvae per lungs, day 7
C57BL	1000	5	314 ± 105
C57BL/6	1000	5	164 ± 21.6
CBA/Ca	1000	5	38.4 ± 8.5
C57BL/6	1000	5	125 ± 21.74
CBA/Ca	1000	5	28 ± 7.52

Using the values in Table 3.1, the replication required per treatment can be calculated. It is possible to calculate r for varying doses. However, the experiments which will be undertaken in this study will only involve administering 1000 ova per mice as this has been calculated as the optimum dose of infection (Lewis *et al.*, 2006).

Table 3.2: Calculated replication per treatment based on variation in susceptibility to *Ascaris suum* larvae

Mouse strain	Dose administered	Number of mice	Number of larvae per lungs, day 7	CV	r
C57BL	1000	5	314 ± 105	33	7
C57BL/6	1000	5	164 ± 21.6	13	1
CBA/Ca	1000	5	38.4 ± 8.5	22	3
C57BL/6	1000	5	125 ± 21.74	17	5
CBA/Ca	1000	5	28 ± 7.52	27	9

When we examine variation between C57BL, C57BL/6 and CBA/Ca mice we find a mean r = 5. This replication size has previously been stated as the minimal acceptable sample size (Lewis, 2006).

3.3.3 Sample collection

Five mice of both C57BL/6JOlaHsd (Comparative Medicine Unit, Trinity College Dublin, Dublin, Ireland) and CBA/Ca (Harlan Laboratories, Blackthorn, United Kingdom) strains were infected with 1000 embryonated *A. suum* ova (kindly supplied by Peter Nejsum, Faculty of Health and Medical Sciences, The University of Copenhagen, Denmark). Control mice received oral intubation of 0.1M sulfuric acid solution, which was used to store the eggs. The animals were euthanized using cervical dislocation at day 7 p.i. and the livers of these mice and their matching controls were extracted and each lobe was snap frozen in liquid nitrogen separately and stored at -80 °C. Larval counts on lung tissue were performed on day 7 p.i. on five mice of both strains in order to confirm the difference in larval burden between the strains using the modified Baermann technique (Lewis *et al.*, 2006). The larval pellet was suspended in 5 ml of 0.9% v/v saline and 6% v/v formalin. The solutions were agitated prior to larval count in order to obtain a homogenous larval distribution in the samples and a 2 ml aliquot was applied to a nematode counting slide (Chalex Corporation, Park City, UT, USA) (Dold *et al.*, 2010) and the number of larvae was estimated.

3.3.3.1 *Sample preparation for mass spectrometry*

The left lobes of day 7 p.i. livers were homogenized in lysis buffer (LB) comprising 6M urea and 2M thiourea, supplemented with a protease inhibitor cocktail (Complete Mini, Roche, Citywest, Ireland). In order to remove cellular debris, the samples were centrifuged for 5 min at room temperature at 10,000× *g* to pellet any cellular debris. Five hundred microliters of this lysate was added to 500 µl of LB and quantified using the Qubit System (Invitrogen, Dun Laoghaire, Ireland) following the manufacturer's

instructions. Using the calculations from the Qubit System, seventy-five micrograms of total protein was removed and purified using a 2D Clean Up Kit (GE Healthcare, Belfast, United Kingdom) following the manufacturer's instructions. The resulting pellet was resuspended in 50 µl of resuspension buffer (6M urea, 2M thiourea, 0.1 MTris-HCl, pH 8.0), requantified, using the Qubit System, and 20 µg was removed for in solution trypsin digest. One hundred and five microliters of 50 mM ammonium bicarbonate and 1 µl of DL-dithiothreitol was added to each protein sample and the resulting mixture was incubated for 20 min at 56 °C to reduce cysteine disulphide bridges. Cysteines were alkylated by adding 2.7 µl of iodoacetamide (0.55M) and incubated at room temperature (20–25 °C) for 15 min in the dark. One microliter of both 1% (w/v) Proteasemax (Promega, Kilkenny, Ireland) and 0.5 µg/µl trypsin (Promega) were added and the samples were incubated overnight at 37 °C. The next day the samples were briefly spun to collect any condensate and acidified with 1 µl of trifluoroacetic acid. The samples were vortexed briefly and incubated for 5 min at room temperature (20–25 °C). Samples were centrifuged at 13,000× *rcf* for 10 min and the supernatant, containing the peptides, was removed to a fresh tube and purified using C18 Spin Columns (Pierce, Thermo Fisher Scientific, Dublin, Ireland) following the manufacturer's instructions. Peptides were lyophilised using a Speedyvac (Savant DNA120, Thermo Fisher Scientific), medium setting, for 2 h at 39 °C and stored at 4 °C. On the day of mass spectrometry, the peptides were brought to room temperature for 10 minutes, resuspended in 30 µl loading buffer for QExactive (2% acetonitrile, 0.05% trifluoroacetic acid), sonicated for 2 min and centrifuged for 5 min at room temperature at 15,460× *g*. The remaining supernatant was used for mass spectrometry analysis.

3.3.3.2 *Mass spectrometry*

One microgram of tryptic peptides was loaded onto a QExactive (Thermo Fisher Scientific) high-resolution accurate mass spectrometer, connected to a Dionex Ultimate 3000 (RSLCnano, Thermo Fisher Scientific) chromatography system. A 50 cm column was used to separate the proteins using a 4 to 40% acetonitrile gradient with a 130 min reverse-phase gradient at a 250 nl/min flow rate. Data was collected using the automatic data dependent switching mode. A full MS scan was set at a resolution of 70,000 with a scan range of 400–1600 m/z, selecting the 15 most intense ions.

Subsequently, an MS/MS scan was performed, with a resolution of 17,500 and a range of 200–2000 m/z.

MaxQuant v.1.5.6.5 (http://www.maxquant.org) was used to perform protein identification and LFQ normalisation on the MS/MS data following Hubner *et al.* (2010). The MS/MS data were searched against both the *Mus musculus* SWISS-PROT database (accessed July 2017, containing 16,716 sequences; 9,390,804 residues) (UniProt Consortium, 2015) and a contaminant sequence set supplied by MaxQuant, using the Andromeda algorithm (Cox *et al.*, 2011). The search was performed using the first search peptide tolerance of 20 ppm, second search peptide tolerance of 4.5 ppm with cysteine carbamidomethylation as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modifications. A maximum of two missed cleavage sites was allowed. The false discovery rate (FDR) was set at 1% for both peptides and proteins. The FDR was estimated using searches against a target-decoy database. Using the MaxLFQ algorithm (Cox *et al.*, 2014), LFQ intensities were calculated from razor and unique peptides with a minimum ratio count of two peptides across samples. A minimum length of seven amino acids was needed in order to be considered for identification. Additionally, proteins were only considered for identification if more than one unique peptide for each protein was found. The MS proteomics data and MaxQuant search output files have been deposited to the ProteomeXchange Consortium (Côté *et al.*, 2012) *via* the PRIDE partner repository with the dataset identifier PXD014508.

3.3.3.3 *Data analysis*

Normalised protein intensities (LFQ intensities) were used for the subsequent quantitative analysis in Perseus v.1.6.0.7. Proteins were filtered for contaminants: only identified by site, reverse and potential contaminants. The LFQ intensities were $\log_2$ transformed (Quackenbush, 2002) and individual replicates were allocated their respective sample groups (CBA infected, CBA control, C57 infected and C57 control) prior to protein annotation. One outlier in each group was eliminated, leaving four samples for further analysis. Only proteins found in all four replicates of at least one group were retained for analysis. Subsequently, a data-imputation was performed (Deeb *et al.*, 2012) to replace any missing values. The imputated values are chosen to simulate values of low abundant proteins and are chosen randomly from a distribution

using a downshift of 1.8 times and a width of 0.3 times the mean standard deviation (SD) of all measured values.

Using these imputated data, two sample t-tests and volcano plots were generated for the relevant comparisons using the standard settings of FDR cut-off at 0.05 and $S_0 = 0.1$. The statistically significant differentially abundant (SSDA) obtained values from these t-tests were used to generate volcano plots. Using the 'categories' function in Perseus, different pathways and processes could be visualised on these volcano plots which could indicate trends. A principal components analysis (PCA) was completed on normalised intensity values. Hierarchical clustering, with Euclidean distance and complete linkage, was performed using Z-score normalised intensity values.

All proteins were annotated using the UniProt gene ID. Terms for Kyoto Encyclopaedia of Genes and Genomes (KEGG) name, KEGG pathway, Interpro and protein family (pfam) were extracted for each protein. Enrichment for these terms was performed on hierarchical clustering using the Fisher's exact test with a Benjamini-Hochberg (Ben-Ho) corrected FDR of 2%. FASTA files of all FDR positive proteins were made using Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Hall, 1999). Protein network analysis was completed using the online tool: Search Tool for Retrieval of Interacting Genes/Proteins (STRING) (Szklarczyk *et al.*, 2017) v.10.5 (http://string-db.org), with a high confidence setting (0.5–0.7). KEGG pathway analysis was performed using BlastKOALA (Kanehisa and Goto, 2000, Kanehisa *et al.*, 2014) (http://www.kegg.jp/blastkoala/). FASTA sequences were uploaded with taxonomy identified as animals and family eukaryotes and the enriched pathways were identified.

3.3.4 UniProt accession numbers for proteins mentioned in text

25-hydroxycholesterol 7-alpha-hydroxylase: Q60991; 40S ribosomal protein S30: P62862; acetoacetyl-CoA synthetase: Q9D2R0; acyl-CoA desaturase 1: P13516; aldehyde oxidase 3: G3X982; all-trans-retinol 13,14-reductase: Q64FW2; C4b-binding protein: P08607; complement C3: P01027; complement C4-B: P01029; complement component C8 alpha chain: Q8K182; complement component C8 beta chain: Q8BH35; complement component C8 gamma chain: Q8VCG4; complement component C9: P06683; complement factor B: P04186; complement factor H: P06909; complement factor i: Q61129; cytochrome P450 3A13: Q64464; cytochrome P450 3A13: Q64464; cytochrome P450 4A10: O88833; cytochrome P450 4A14: Q62264; eosinophil granule

major basic protein: Q61878; fibrinogen alpha chain: E9PV24; fibrinogen beta chain: Q8K0E8; fibrinogen gamma chain: Q8VCM7; haptoglobin: Q61646; haemoglobin subunit beta-2: P02089; major urinary protein 1: P11588; major urinary protein 17: B5X0G2; major urinary protein 2: P11589; major urinary protein 20: Q5FW60; major urinary protein 3: P04939; major urinary protein 6: P02762; mannose-binding protein A: P39039; mannose-binding protein C: P41317; plasminogen: P20918; platelet glycoprotein 4: Q08857; protein S100-A1: P56565; protein S100-A10: P08207; protein S100-A8: P27005; protein S100-A9: P31725; putative hydroxypyruvate isomerase: Q8R1F5; S-methylmethionine-homocysteine S-methyltransferase BHMT2: Q91WS4; tapasin: Q9R233; thyroid hormone-inducible hepatic protein; tyrosine-protein phosphatase non-receptor type 6: P29351

3.4 Results

The mean larval burden in the lungs of the C57BL/6J mice on day 7 post-infection (n = 5) was 188 ± 78.7 (mean ± SD) and the mean larval burden for CBA/Ca mice (n = 5) was 12 ± 8.5 confirming the susceptible and resistant phenotype in the mice used in our experiment.

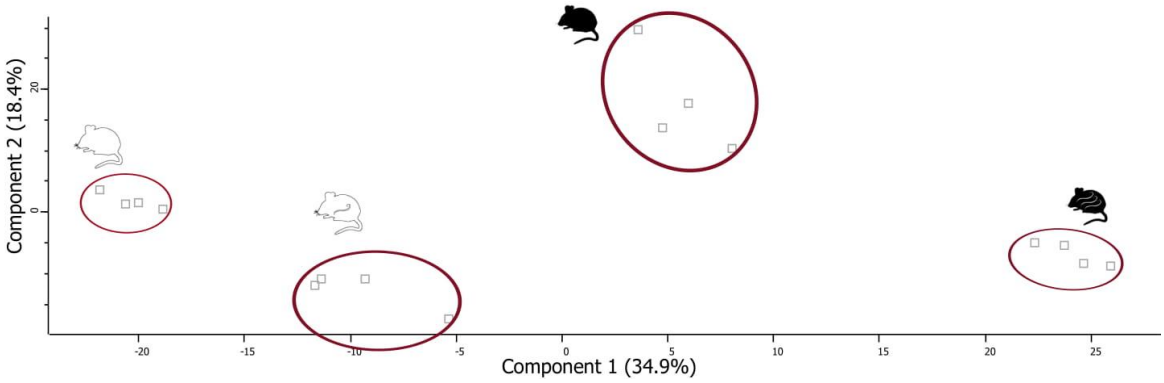

Figure 3.1: PCA of the proteome of the left liver lobes of CBA/Ca (the resistant strain - white mice on figure) and C57BL/6J (the susceptible strain - black mice on figure) infected with *A. suum* at 7 days post-infection . All four groups are clearly distinct from each other. The groups from left to right are: CBA/Ca control, CBA/Ca infected, C57BL/6J control, C57BL/6J infected. CBA/Ca mice are represented by the white mouse symbol and C57BL/6J mice are represented by the black mouse symbol. When infected, there are *Ascaris* larvae drawn in the mice.

A PCA was performed on all proteins (Figure 3.1), resolving a clear difference between the four groups and highlighting an intrinsic difference between the two strains. A PCA reduces the information of the initial variables into new variables, or principal components. These principal components are linear combinations of the initial

variables and take the total variance of the data into account. This allows visualisation of a multivariate table, which is reduced to a few principal components without losing too much information from the initial variables. The PCA shows a clear difference between all four groups with no overlap. The first two principal components (PC) explain 53.3% of the total variation. PC1 (34.9%) explains most of the variation, clearly dividing the groups into their respective clusters. PC2 (18.4%) mainly explains the variation between control and infected samples. This difference is clear when comparing control samples of both mouse strains as well as for infected samples. Most evident is the clustering per mouse strain, with each strain having the clusters of both infected and control samples close together. Ultimately it appears that CBA/Ca control and C57BL/6J infected are the two extremes in this PCA with C57BL/6J control resembling CBA/Ca infected more closely than it does CBA/Ca control.

Using hierarchical clustering on Z-scored normalised LFQ intensities, a heatmap was produced (Figure 3.2a) comprising eleven clusters (A-K) (Additional file 1: Tables S1–S3). Each cluster represents proteins that are expressed in the same pattern in our four groups (Figure 3.2b). Within each cluster, the number of proteins that are statistically significantly (ANOVA, Ben-Ho FDR <0.05) differentially abundant proteins between the groups are found in Figure 3.2b in the 'number in cluster' column. The proteins within each cluster were interrogated for gene ontology (GO) and KEGG term statistical enrichment (Fisher's exact test with Ben-Ho adjustment, $P < 0.05$) in Perseus and a number of clear trends became evident. CBA/Ca mice both with and without infection had higher abundances of proteins involved in oxidative phosphorylation (Ben-Ho FDR < 0.0001) and associated mitochondrial proteins (Ben-Ho FDR < 0.0001) (Cluster E). Conversely, C57BL/6J mice both with and without infection had higher abundances of proteins involved in drug metabolism (cytochrome P450) (Ben-Ho FDR < 0.01) and the metabolism of xenobiotics (Ben-Ho FDR < 0.05) (Cluster H). Immune system process ($P < 0.0001$), phagosome (Ben-Ho FDR < 0.0001), cytoskeleton ($P < 0.0001$), mRNA processing (Ben-Ho FDR < 0.01), cell chemotaxis (Ben-Ho FDR < 0.01) and positive regulation of cell death (Ben-Ho FDR < 0.01) are all enriched under infection for both strains.

Table 3.3: Pathways found to be enriched in two-sample t-tests. The numbers of proteins in each t-test are shown below

Pathway	**Control**		**Infected**		**C57**		**CBA**		**Total no. of proteins**
	CBA	C57	CBA	C57	Control	Infected	Control	Infected	
Mitochondrial part	34	8	63	24	25	25	2	5	326
Oxidative phosphorylation	11	2	23	5	4	4	0	1	74
Retinol metabolism	3	7	4	10	4	5	0	0	26
Immune system process	7	10	19	26	5	41	1	25	116
Innate immune response	4	1	9	7	2	17	1	12	39
Complement and coagulation cascade	2	1	9	4	1	11	0	6	27
Peroxisome (GOCC)	10	4	22	9	13	7	1	1	60
Ribosome	0	0	0	1	1	3	7	0	70
Cell death	3	5	8	10	3	14	0	7	71
Cytochrome P450	4	5	5	9	7	4	1	0	28

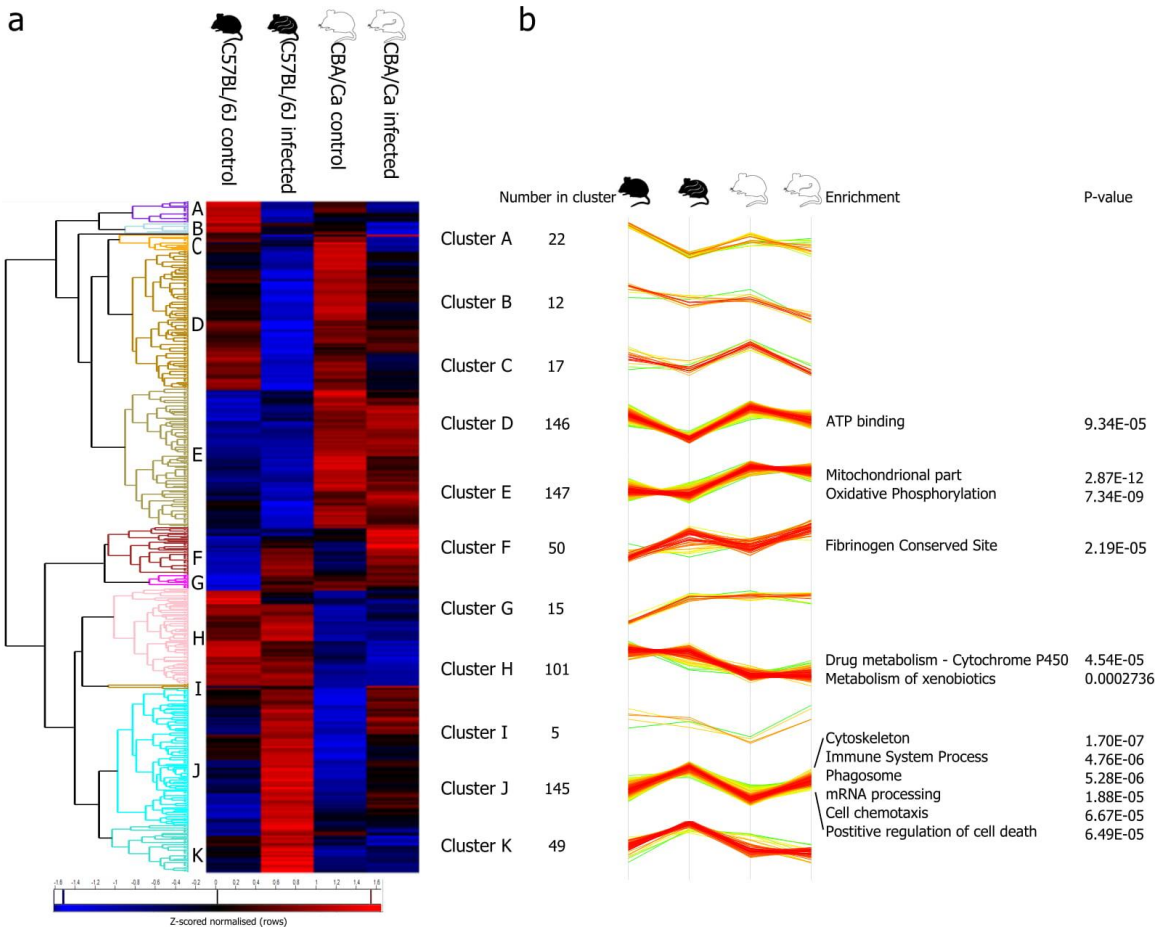

Figure 3.2: Two-way unsupervised hierarchical clustering of the median protein expression values of all statistically significant differentially abundant proteins. **a** Hierarchical clustering (columns) showed 11 (A-K) distinct clusters. **b** A more detailed view of the clusters with the number of proteins in each cluster, enrichments with associated P-values are shown where applicable. If no enrichments are outlined, this indicates no statistically significant enrichments were found. The line graph shows the profiles of the proteins within each cluster, where every line is a protein.

In total, 2065 unique proteins were identified in the samples (Additional file 2: Table S4). Two-sample t-tests were performed between strains and within strains using an FDR of 0.05 and S_0 = 0.1 in Perseus software (Table 3.3). From these between strain and within strain t-tests, the proteins that were found to be statistically significant differentially expressed between groups were analysed using STRING, Perseus and KEGG.

3.4.1 CBA/Ca control compared to C57BL/6J control

In total, 236 proteins were found to be statistically significant differentially expressed with a $\log_2$ fold change ranging from -7.3 to 11.5 (Additional file 2: Table S5). The top 20 most differentially expressed abundant proteins can be seen on the volcano plot (Figure 3.3a).

The three most differentially expressed abundant proteins for CBA/Ca were: haemoglobin subunit beta-2, S-methylmethionine-homocysteine S-methyltransferase BHMT2 and putative hydroxypyruvate isomerase. The protein haemoglobin subunit beta-2 had a $\log_2$ fold change of 11.5, the highest difference in the dataset. Functional enrichment in Perseus revealed that mitochondrial proteins, more precisely, proteins involved in OXPHOS, were more abundant in the CBA/Ca strain. This was confirmed using STRING analysis (KEGG pathway ID: 00190, FDR < 0.0001).

For C57BL/6J the three most differentially expressed abundant proteins were: aldehyde oxidase 3, cytochrome P450 3A13 and complement C4-B. Clusters involved in retinol metabolism (KEGG pathway ID 00830, FDR < 0.0001) and drug metabolism: cytochrome P450 (KEGG pathway ID 00982, FDR < 0.0001) were also identified (Figure 3.3c).

3.4.2 CBA/Ca infected compared to C57BL/6J infected

This pairwise comparison identified 580 statically significant differentially expressed proteins with a $\log_2$ fold change range from -7.4 to 11.3 (Additional file 2: Table S6). The 20 most statistically significant differentially expressed abundant (SSDA) proteins within this comparison, can be found in Figure 3.3b. The three most differentially expressed abundant proteins for CBA/Ca were: haemoglobin subunit beta-2, S-methylmethionine-homocysteine S-methyltransferase BHMT2, and 25-hydroxycholesterol 7-alpha-hydroxylase (Figure 3.3b). The CBA/Ca strain showed a higher abundance of mitochondrial proteins, in particular, proteins involved in the OXPHOS (KEGG pathway ID: 00190, FDR < 0.0001). CBA/Ca also have more differentially abundant proteins involved in the immune response, more precisely the membrane attack complex (MAC) (cellular component GO ID: 0005579, FDR < 0.0001).

As for the C57BL/6J strain, the three most differentially expressed abundant expressed proteins were: cytochrome P450 4A14, aldehyde oxidase 3 and complement C4-B. These C57BL/6J mice have more differentially expressed abundant proteins involved in cytochrome P450 complex (Figure 3.3d). C57BL/6J showed upregulation in cell-junction proteins (Cellular Component GO pathway ID: 0030054, FDR < 0.0001) and lysosome in (KEGG pathway ID: 04142, FDR < 0.0001). One cluster in particular includes the proton-transporting V-type ATPase complex (cellular component GO pathway ID: 003176, FDR < 0.0001). A cluster in glutathione metabolism (KEGG

pathway ID: 00480, FDR < 0.0001) and a small cluster of the small subunit of mitochondrial ribosomal proteins (cellular component GO pathway ID: 0005763, FDR: 0.00746) were also identified. Finally, retinol metabolism is also upregulated compared to CBA/Ca (KEGG pathway ID: 00830, FDR < 0.0001).

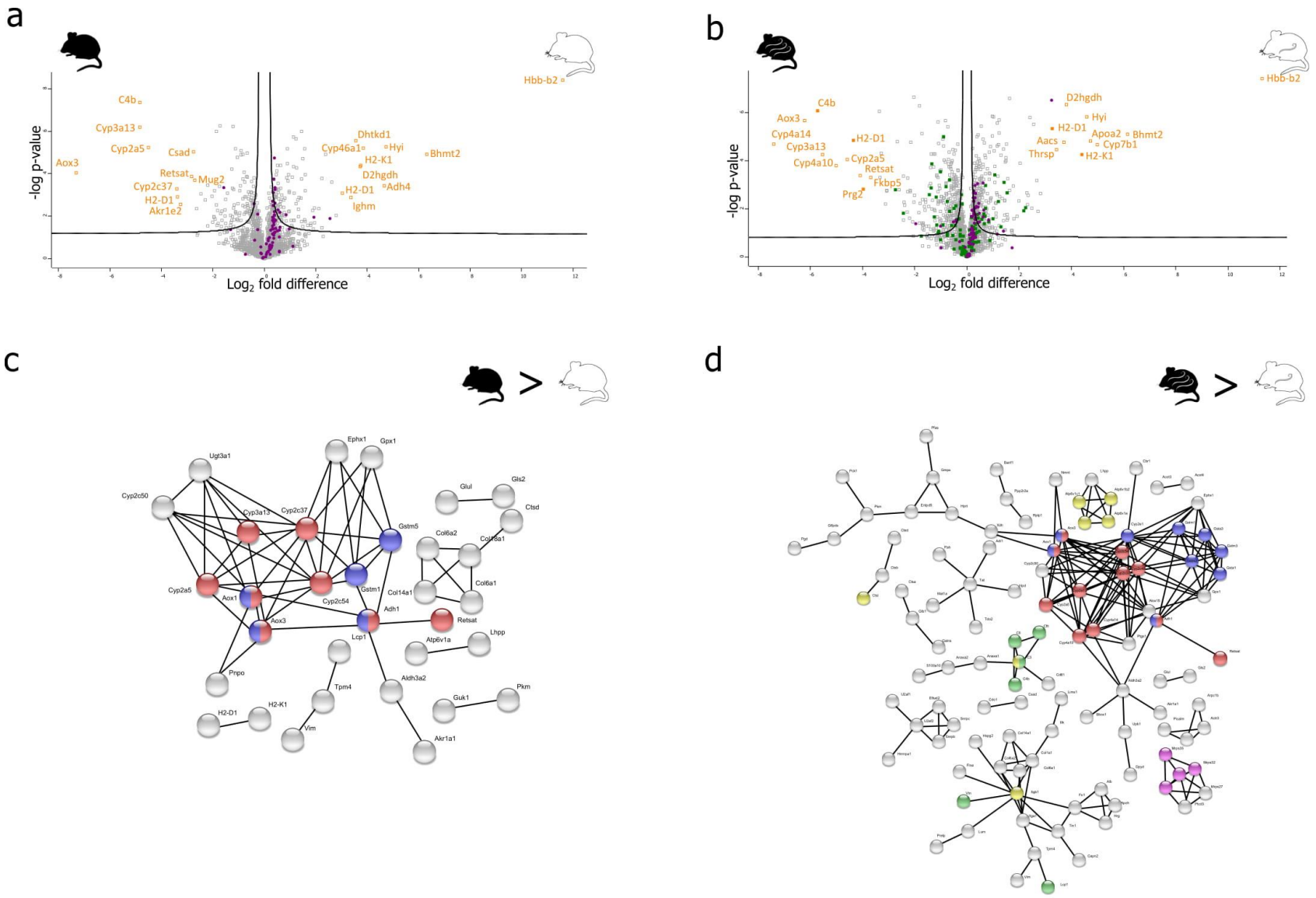
a
b
c
d
-log p-value
Log2 fold difference
Hbb-b2
C4b
Cyp3a13
Cyp2a5
Csad
Aox3
Retsat
Mug2
Cyp2c37
H2-D1
Akr1e2
Dhtkd1
Cyp46a1
Hyi
H2-K1
Bhmt2
D2hgdh
Adh4
Ighm
Cyp4a14
Cyp3a13
Cyp4a10
Cyp2a5
Retsat
Fkbp5
Prg2
D2hgdh
Hyi
H2-D1
Apoa2
Bhmt2
Aacs
Cyp7b1
Thrsp
H2-K1
Ephx1
Gpx1
Ugt3a1
Cyp2c50
Cyp3a13
Cyp2c37
Glul
Gls2
Ctsd
Gstm5
Col6a2
Col3a1
Col6a1
Cyp2a5
Aox1
Cyp2c54
Gstm1
Col14a1
Adh1
Aox3
Retsat
Lcp1
Atp6v1a
Lhpp
Pnpo
Tpm4
Aldh3a2
Guk1
Pkm
H2-D1
H2-K1
Vim
Akr1a1

Figure 3.3: Between strain analysis of abundant proteins. **a** Volcano plot of control mice. On the X-axis are the log2 fold differences and on the Y-axis is the -log(P) value. The black curve indicates the cutoff for statistically significant proteins with FDR of 0.05 and S0 = 0.1. The top 20 most abundant proteins are highlighted in orange. Proteins involved in the oxidative phosphorylation are highlighted in purple. **b** Volcano plot of infected mice. On the X-axis are the log2 fold differences and on the Y-axis is the -log(P) value. The black curve indicates the cutoff for statistically significant proteins with FDR of 0.05 and S0 = 0.1. The top 20 most abundant proteins are highlighted in orange. Proteins involved in the oxidative phosphorylation are highlighted in purple. Immune system proteins are highlighted in green. **c** String network analysis of differentially abundant proteins upregulated in C57BL/6J control mice when compared to CBA/Ca control mice, indicated as C57BL/6J>CBA/Ca. Proteins marked in red are part of retinol metabolism. Blue marked proteins are part of the cytochrome P450. If proteins are involved in both pathways, their representative dots are coloured half and half. **d** String network analysis of C57BL/6J infected mice when compared to CBA/Ca infected mice. Red proteins are part of retinol metabolism. Blue marked proteins are part of the cytochrome P450 proteins. Proteins in purple are part of the mitochondrial small ribosomal subunit. Immune system proteins are green and phagosome proteins are yellow.

3.4.3 C57BL/6J infected compared to C57BL/6J control

In total 451 proteins were statically significant differentially expressed for this two sample t-test, (Additional file 2: Table S7) with a $\log_2$ fold change range from -5.5 to 6.4 (Figure 3.4a). For the control samples, the three most significantly expressed abundant proteins were: 25-hydroxycholesterol 7-alpha-hydroxylase, acetoacetyl-CoA synthetase, and thyroid hormone-inducible hepatic protein. Functional enrichment revealed a trend towards translational upregulation; however, most proteins were under the FDR cutoff. A similar situation was seen for proteins involved in oxidative phosphorylation and peroxisomal proteins. STRING analysis confirmed the increased abundance of mitochondrial proteins (cellular component GO ID: 0005739, FDR < 0.0001) and proteins involved in the metabolism of xenobiotics by cytochrome P450 (KEGG pathway ID: 00980, FDR < 0.0001). Additionally, retinol metabolism proteins were found to be upregulated (KEGG pathway ID: 00830, FDR < 0.0001).

For the infected samples, the most differentially expressed abundantproteins were: cytochrome P450 4A14, cytochrome P450 4A10 and eosinophil granule major basic protein. An increase in RNA and DNA binding proteins and proteins involved in RNA processing under infection was identified (biological process GO: 0006396, FDR < 0.0001). A higher abundance of differentially expressed abundant proteins involved in the regulation of cell death and immune system process in the infected samples was also identified, including immune system process (biological process GO: 0002376, FDR < 0.0001) and more specifically the complement and coagulation cascade (KEGG pathway ID: 04610, FDR < 0.0001) (Figure 3.4c). Finally, an increase in phagosomal proteins under infection was also found (KEGG pathway ID: 04145, FDR < 0.0001).

3.4.4 CBA/Ca infected compared to CBA/Ca control

In this two sample t-test, 117 proteins were statically significant differentially expressed (Additional file 2: Table S8), with a $\log_2$ fold change range from -2.7 to 5.3. The most abundant differentially expressed proteins for the control samples were: acyl-CoA desaturase 1, major urinary protein 20, and 40S ribosomal protein S30 (Figure 3.4b). Functional enrichment of the control samples revealed an upregulation of ribosomal proteins and the translational machinery in control samples analysis (KEGG pathway ID: 03010, FDR: 0.0031).

The most abundant differentially expressed proteins for the infected samples were: haptoglobin, protein S100-A8 and protein S100-A9, proteins involved in transcription those of the innate immune system in particular. The proteins of the immune response (biological process GO: 0006955, FDR < 0.0001) were upregulated in infected samples compared to control samples (Figure 3.4d). Phagosome proteins were found to be upregulated (KEGG pathway ID: 04145, FDR < 0.0001). Additionally, the analysis revealed RNA binding proteins to be upregulated in the infected group (molecular function GO ID: 0003723, FDR < 0.0001). Regulation of cell death was found to be upregulated, and although no clear cluster existed in STRING analysis the regulation of cell death process (biological process GO: 0010941, FDR < 0.0001) was found to be enriched. Additionally, changes in cell movement were observed including upregulation of proteins involved in cytoskeleton organisation, cell migration and cell-cell adhesion.

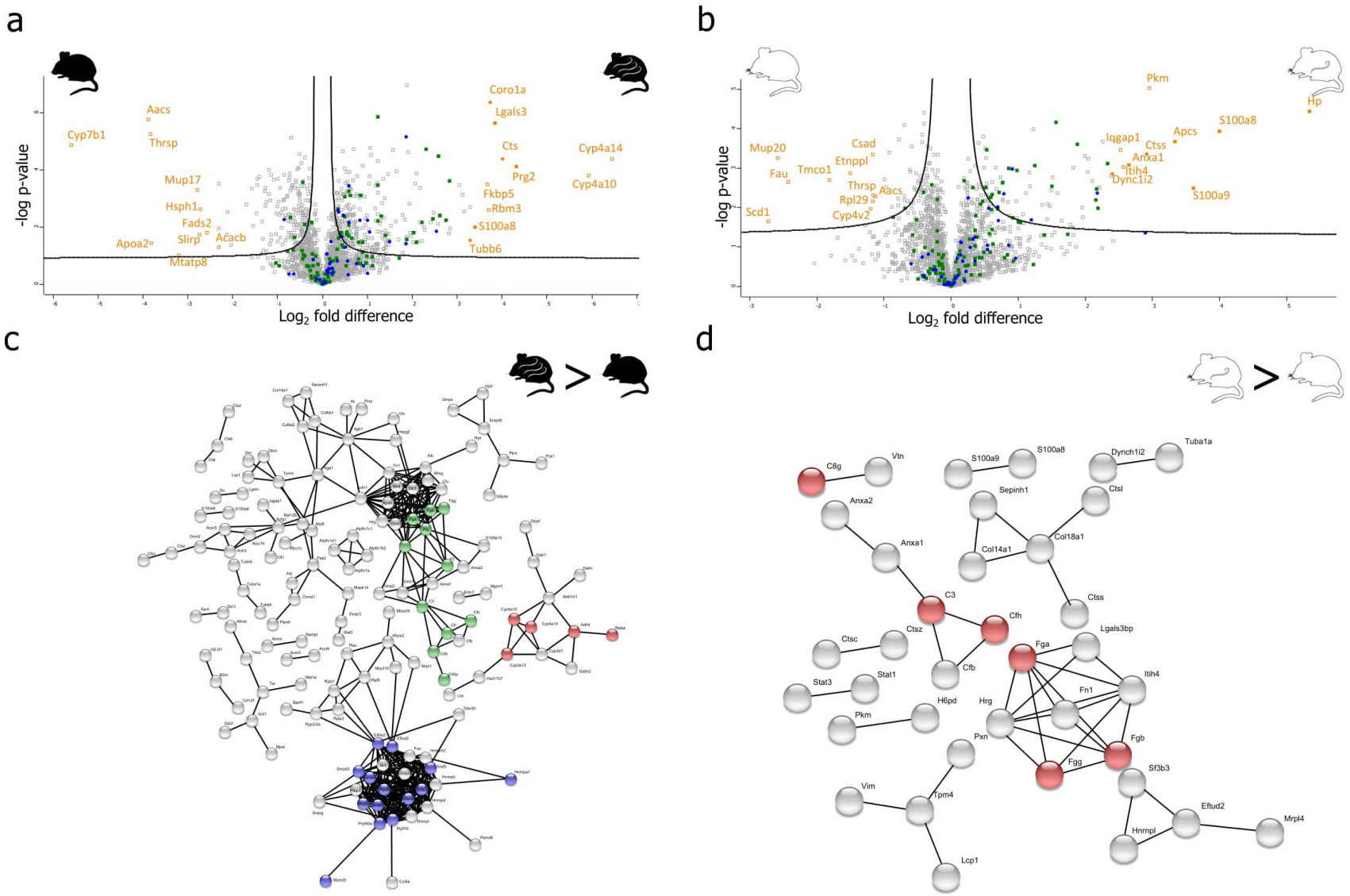
a
-log p-value
Log2 fold difference
Cyp7b1
Aacs
Thrsp
Mup17
Hsph1
Fads2
Slirp
Acacb
Apoa2
Mtatp8
Coro1a
Lgals3
Cts
Prg2
Fkbp5
Rbm3
S100a8
Tubb6
Cyp4a14
Cyp4a10
b
-log p-value
Log2 fold difference
Mup20
Scd1
Csad
Etnppl
Fau
Tmco1
Thrsp
Rpl29
Aacs
Cyp4v2
Pkm
Iqgap1
Ctss
Anxa1
Itih4
Dync1i2
Apcs
S100a8
S100a9
Hp
c
>
d
>
C8g
Vtn
S100a9
S100a8
Dynch1i2
Tuba1a
Anxa2
Sepinh1
Ctsl
Anxa1
Col18a1
Col14a1
C3
Cfh
Ctss
Ctsc
Ctsz
Lgals3bp
Fga
Cfb
Stat1
Stat3
Itih4
Fn1
H6pd
Hrg
Pkm
Pxn
Fgb
Fgg
Sf3b3
Vim
Tpm4
Eftud2
Mrpl4
Hnrnpl
Lcp1

Figure 3.4: Within strain analysis of abundant proteins **a** C57BL/6J infected compared to C57BL/6J control. Volcano plot showing the differentially abundant proteins of C57BL/6J mice. On the X-axis are the log2 fold differences and on the Y-axis is the -log(P) value. The black curve indicates the cutoff for statistically significant proteins with FDR of 0.05 and S0 = 0.1. Proteins abundant in control mice are on the left of the plot and abundant proteins present in infected mice are on the right of the plot. The top 20 most abundant proteins are highlighted in orange. Immune proteins are green squares and proteins involved in the phagosome are blue circles. **b** Volcano plot of CBA/Ca infected compared to CBA/Ca control. On the X-axis are the log2 fold differences and on the Y-axis is the -log(P) value. The black curve indicates the cutoff for statistically significant proteins with FDR of 0.05 and S0 = 0.1. Proteins abundant in control mice ae on the left of the plot and abundant proteins present in infected mice are on the right of the plot. The top 20 most abundant proteins are highlighted orange. Immune proteins are green squares and proteins involved in the phagosome are blue circles. **c** C57BL/6J infected compared to C57BL/6J control. String network analysis of the proteins found to be differentially abundant in C57BL/6J infected mice when compared to C57BL/6J controls. Red is retinol metabolism. Blue is spliceosome proteins and green is complement pathway. **d** CBA/Ca infected compared to CBA/Ca control. Visualisation of a STRING network analysis performed on the differentially abundant proteins of CBA/Ca in infected mice when compared to CBA/Ca control mice. Proteins highlighted in red are the complement pathway.

3.4.5 Enriched pathways

3.4.5.1 *The susceptible strain (C57BL/6J) has more immune system process proteins*

A total of 116 proteins were identified from the immune system process, including 39 from the innate immune response and 27 from the complement and coagulation cascade (Table 3.3). Under infection, C57BL/6J mice produce more immune response-associated proteins (26 proteins) than their CBA/Ca counterparts (19 proteins). C57BL/6J infected mice also showed a higher abundance of immune system process proteins (41 proteins) when compared to their controls. CBA/Ca infected mice also have a higher abundance for proteins associated with immune system process (25 proteins) compared to their own controls.

Complement factor i (Cfi) and h (Cfh) were upregulated in C57BL/6J infected mice compared to CBA/Ca infected mice. These proteins are involved in inhibiting the complement response. In contrast, mannose-binding protein A (Mbl1) and mannose-binding protein C (Mbl2), both proteins essential for activation of the lectin complement pathway, are found in CBA/Ca infected mice when compared to C57BL/6J infected mice. The same is true for part of the membrane attack complex (MAC), complement component C8 gamma chain (C8g), C8b, C8a and C9 and tapasin (Tapbp).

When comparing C57BL/6J infected mice with their own controls, more immune system-associated proteins are found to be upregulated under infection: Cfb, involved in alternative complement cascade; and C4bp, involved in controlling the classical complement cascade. Additionally, factors involved in blood clotting are upregulated

[plasminogen, fibrinogen gamma chain (Fgg), Fga, Fgb, C3 and tyrosine-protein phosphatase non-receptor type 6] and platelet glycoprotein 4 (CD36).

As for CBA/Ca, Cfb, a protein of the alternative pathway, is upregulated under infection when compared to its counterpart control, together with C8g, C3 and Cfh. The latter is an inhibitor of the complement cascade.

3.4.5.2 *Mitochondrial proteins are upregulated in the resistant (CBA/Ca) strain*

Of the 2065 identified proteins, 326 were found to be associated with the mitochondria, with 204 belonging to the mitochondrial membrane and 74 involved in OXPHOS. Increased mitochondrial protein abundance was observed in the CBA/Ca strain in comparison to the C57BL/6J strain, both intrinsically and under infection (Table 3.3). Furthermore, no change in absolute protein numbers was observed when comparing the C57BL/6J group between its controls and infected. For the CBA/Ca strain, however, a small increase was observed.

3.4.5.3 *Differences in retinol metabolism between the two mouse strains*

In total, 26 of the 2065 proteins identified are involved in retinol metabolism (Table 3.3). The C57BL/6J strain showed a higher abundance of these proteins when compared to the CBA/Ca mice both with and without infection. When comparing infected livers of the CBA/Ca strain with corresponding controls, no difference in retinol metabolism was observed. The C57BL/6J mice, however, express different proteins between control and infection. The protein all-trans-retinol 13,14-reductase (Retsat) is among the 10 most abundant proteins in C57BL/6J control when compared to CBA/Ca control.

3.4.5.4 *Major urinary proteins*

Major urinary proteins (Mup) are downregulated under infection. Mup17 is present in the top 10 most differentially upregulated proteins in the control group of the C57BL/6J when compared to its infected counterparts. The same is true for Mup 20 in the CBA/Ca group. In total, 6 Mups are upregulated in C57BL/6J controls when compared to C57BL/6J infected. As for the CBA/Ca group, 2 Mups are found to be upregulated in controls when compared to the infected group. When comparing both control samples, Mup20 is found to be upregulated in C57BL/6J mice. This same protein remains more abundantly expressed in C57BL/6J infected samples, when

compared to CBA/Ca infected mice. However, CBA/Ca infected mice also showed an increase in Mup17, Mup1, Mup3 and Mup2 when compared to C57BL/6J infected mice. Our dataset shows a downregulation of Mup20 and Mup2 under infection for CBA/Ca when compared to their own controls. As for C57BL/6J mice, Mup6, Mup20, Mup2, Mup3, Mup1 and Mup17 are downregulated under infection when compared to their own controls. In essence, the C57BL/6J strain downregulates more Mups (6) under infections when compared to its own control than the CBA/Ca strain (2).

3.4.5.5 *S100*

S100a8 and S100a9, two components of the heterodimer calprotectin, were found to be highly abundant in this dataset. S100a8 and S100a9 are in the top 3 most abundant proteins in CBA/Ca infected when compared to its control counterparts. For C57BL/6J infected mice, S100a8, S100a9, S100a10 and S100a1 are differentially expressed compared to their controls.

To conclude, CBA/Ca mice show an increase in complement activating proteins under infection, whereas the C57BL/6J infected mice have a higher abundance of complement inhibiting proteins. Additionally, CBA/Ca mice have a higher abundance of OXPHOS proteins compared to the C57BL/6J, both intrinsically and under infection. Furthermore, a difference was observed in the retinol metabolism proteins, where the C57BL/6J strain shows a higher abundance in these proteins than the CBA/Ca strain.

3.5 Discussion

Ascariasis affects 800 million people worldwide (Pullan *et al.*, 2014) and, although anthelmintic drugs are available for its treatment, reinfections occur frequently, due principally to the persistence of infective eggs in the environment. Despite the extensive number of infected people, our knowledge of the molecular mechanisms involved in larval migration and the host immunological response remains incomplete. The role of the liver in ascariasis has been understudied, yet this appears to be the primary location where the difference in resistance and susceptibility manifests. This study extends our previous work that focused on the liver at day 4 p.i. (Deslyper *et al.*, 2016), by examining the liver at a later time point, day 7 p.i. At day 4 p.i., we identified molecular determinants that were possibly associated with resistance whereas in this study, with a focus on day 7 p.i., we attempt to obtain insight into the responses to, and effects of *Ascaris* infection at the molecular and immunological level at a time where

the biggest difference in larval burden between the CBA/Ca and C57BL/6J mouse strains is observed.

Four different groups of proteins were identified including those associated with mitochondrial processes, the immune response, retinol metabolism and haemoglobin, all of which showed both an intrinsic difference between the two strains as well as a difference between control and infected groups.

3.5.1 The immune response to the parasite differs between the two strains

In comparison to the responses previously reported for day 4 p.i., a more pronounced innate immune response is observed at day 7 p.i. with differences observed between the C57BL/6J and CBA/Ca strains. The C57BL/6J mouse strain had a higher abundance of immune system process proteins under infection. However, this does not necessarily translate into a higher activation of the immune system. A more thorough investigation of exactly which type of proteins are involved reveals a difference in innate immune response activity.

In particular, we detected a difference in the complement system, with a higher abundance of activating and inactivating proteins in the CBA/Ca and C57BL/6J strains, respectively. The CBA/Ca strain displayed an increased abundance in proteins involved in the lectin pathway of the complement system, more specifically, with both Mbl1 and Mbl2 being statistically significantly upregulated in CBA/Ca infected when compared to C57BL/6J infected. Conversely, C57BL/6J infected had more proteins upregulated involved with complement inhibition, Cfi and Cfh in particular, when compared to its CBA/Ca infected counterpart. Additionally, Cfb was upregulated in C57BL/6J infected mice compared to their uninfected controls. This suggests a downregulation of complement activation in the C57BL/6J strain and an upregulation of the lectin pathway in the CBA/Ca strain. So we observed a different activation of the immune system, with the C57BL/6J strain actually downregulating the immune response. It is unclear whether this is a response from the host or immune modulation by the parasite.

Complement is one of the first steps in the innate immune system and plays an important role not only in directly killing a pathogen (MAC) but also in the activation of various other members of the immune system (Murphy, 2012). It is especially a

powerful mechanism that provides a link between the innate and adaptive immune response (Dunkelberger and Song, 2010). Complement is thus an important first step in the activation of the immune response against invading pathogens. Unsurprisingly, some parasites are capable of inhibiting this mechanism, such as *B. malayi* which has a serine protease that inactivates C5a, a key component in the cascade (Rees-Roberts *et al.*, 2010). Early granulocyte-mediated larval attrition of *N. brasiliensis* appears to be in part complement-driven as well, with the alternative pathway being activated during early invasion and a switch to the lectin pathway during late infection (Giacomin *et al.*, 2005). Similarly, *Onchocerca volvulus* microfilariae have been shown to bind factor H, which can cleave C3b to iC3b in the presence of factor I, therefore inactivating the central C3b protein (Meri *et al.*, 2002). This resistance to complement activation throughout maturation, with a switch to a different mechanism, has also been seen in *O. volvulus* and *Dirofilaria immitis* as the parasites mature into the next life cycle stages (Abraham *et al.*, 1988, Brattig *et al.*, 1991). Recombinant *Trichinella spiralis* paramyosin can bind C8 and C9 and therefore inhibit MAC formation (Zhang *et al.*, 2011). More specifically, Mbl has been found to bind oligosaccharides on the surface of *T. spiralis* larvae (Gruden-Movsesijan *et al.*, 2003). Additionally, Mbl-A was found to bind to the surface of *B. malayi* and activate C3 (Carter *et al.*, 2007).

In short, a range of parasites have developed mechanisms to inhibit or modify complement activation. Little evidence is available for the role of complement in *Ascaris* infection. However, one *in vivo* study stimulated alveolar macrophages from male Wistar rats with somatic antigens from *A. suum* adult worms and L3 stage larvae, produced nitric oxide (NO) in a dose-dependent manner (Andrade *et al.*, 2005). As for mouse studies, C3 from both human and guinea pig serum has been shown to bind *A. suum* larvae (Leventhal and Soulsby, 1977). There are no uniform specialized pattern recognition receptors (PRRs) for helminths or *Ascaris* (Perrigoue *et al.*, 2008) which could be recognized by the complement system; this is in stark contrast with the highly specialized PRRs for viruses and bacteria. However, the presence of C-TL like protein on *A. suum* (Loukas and Maizels, 2000) could indicate a role in immune evasion or tissue recognition (Perrigoue *et al.*, 2008).

As described above, complement modulatory mechanisms have been observed for several parasites and it may be that *Ascaris* either directly or indirectly modulates host immune components during infection also. Components of adult *A. suum* body extract

and fluid have both been shown have a strong immune modulatory effect inhibiting DC maturation, cytokine production and results in the alteration of human macrophage surface markers (Loukas and Maizels, 2000, Favoretto *et al.*, 2014, Almeida *et al.*, 2018). It appears that the ability of *Ascaris* to modulate the host response is particularly mouse strain dependant. However, the mechanisms of how *Ascaris* evades or suppresses the immune system and the factors involved in immunomodulation have yet to be determined.

3.5.2 Intrinsic, between-strain differences in oxidative phosphorylation

Proteins of OXPHOS were found to be upregulated in the CBA/Ca strain, both with and without infection, when compared to the C57BL/6J strain. The OXPHOS proteins are part of the mitochondria, which function as the cells' powerhouse, by producing adenosine triphosphate (ATP) at the end product of the electron transport chain (ETC) (Weinberg *et al.*, 2015). As ROS are produced during OXPHOS we speculate that the CBA/Ca strain, therefore, could have higher ROS concentrations. This higher ROS concentration could be of great benefit in order to ward off the invading parasite. ROS could directly damage the parasite but it is also known to act as a signalling molecule, including in the activation of the immune system. Mitochondrial ROS (mROS) specifically has been shown to be able to act as a danger-associated molecular pattern (DAMP) (Weinberg *et al.*, 2015) and is known to direct the immune system by polarizing macrophages and differentiating $CD4^+$ T cells during infection (Kaminski *et al.*, 2010).

The mitochondrial role in parasite immunology is as yet poorly characterised with only a small number of studies performed. In *Toxoplasma gondii* the parasitic vacuole binds with the host mitochondrial proteins (Sinai *et al.*, 1997, Pernas *et al.*, 2014). This is mediated by the parasites' secreted mitochondrial association factor 1 (MAF1). Certain *T. gondii* strains are able to bind mitochondrial proteins, this could indicate that the different strains have altered susceptibility to ROS (Pernas *et al.*, 2014). In general, uncoupling proteins are involved in reducing the production of mROS by decreasing the mitochondrial membrane potential (Vozza *et al.*, 2014). In another example, livers of Wistar rats infected with the parasitic helminth *Cysticercus fasciolaris* showed an increase of ROS compared to their uninfected counterparts (Giri and Roy, 2016).

In order to maintain a normal redox status within the parasite and to contain ROS attacks from hosts, the parasite needs a good detoxification strategy. *A. suum* adult worms are known to have peroxiredoxin (Tsuji *et al.*, 2000), glutathione S-transferase (GST) (Liebau *et al.*, 1997) and catalase (Eckelt *et al.*, 1998). Additionally, peroxiredoxin was found to be present in an L3 stage larvae cDNA library of *A. suum*, indicating the importance of this protein as a ROS defence mechanism (Tsuji *et al.*, 2000). GST was found in the excretory/secretory products of *A. suum* L4 lung stage larvae (Wang *et al.*, 2013). Catalase activity was found to be highest in the unembryonated *A. suum* eggs and decreased steadily during embryonation (Lesoon *et al.*, 1990). The catalase activity during the L3 life stage was found to be similar to that of the adult life stages. To summarise, *A. suum* larvae have been shown to possess antioxidant activity which could be used as a defence mechanism against ROS attacks from the host.

3.5.3 Retinol pathways involving cytochrome P450 are more pronounced in the susceptible (C57BL/6J) strain

The appearance of proteins involved in retinol metabolism was of particular interest. The names retinol and vitamin A are sometimes used interchangeably, but in general vitamin A is a broader term which includes all 'compounds having the biological activity of retinol or its metabolic products' (Saeed *et al.*, 2017). The vitamin A status of people infected with STH, *Ascaris* in particular, has been studied extensively, with some studies indicating that vitamin A supplementation reduced reinfection rates (Long *et al.*, 2006, Payne *et al.*, 2007). However, one randomised controlled trial in a high prevalence STH endemic area dispute this claim (Al-Mekhlafi *et al.*, 2014).

In our study, we found that C57BL/6J have a higher abundance of proteins involved in retinol metabolism when compared to CBA/Ca, both with and without infection. Additionally, C57BL/6J infected mice have a higher abundance of these proteins when compared to their controls. As for CBA/Ca, no difference in the abundance of proteins involved in the retinol metabolism was found between infected and uninfected controls.

We found more proteins involved in retinol metabolism in C57BL/6J control mice compared with CBA/Ca mice. In general, more proteins are involved with the

formation and degradation of all-trans-retinoate or retinoic acid (atRA) in the C57BL/6J strain when compared to its CBA/Ca controls.

This difference remains true when comparing both strains under infection. In particular, the cytochrome P450 Cyp3A13 is the fourth-most abundant protein in this comparison. Other studies have identified a change in abundance in this protein, with hepatic Cyp3A being downregulated in CBA/Ca mice infected with *T. gondii* (Berg-Candolfi *et al.*, 1996). This is in contrast with our own findings where the CBA/Ca mice showed an upregulation of proteins involved in retinol-retinal conversion. In another study, male ICR mice infected with *Babesia microti* showed a similar reduction in hepatic CYP3A at 12 days p.i. (Shimamoto *et al.*, 2012).

atRA is transcriptionally active and has a wide range of functions through binding with the retinoic acid receptors (RAR) and retinoid X receptors (Hurst and Else, 2012). In the C57BL/6J mice under infection, when compared to CBA/Ca infected mice, we observed a higher abundance of Cyp3A and Cyp1A proteins. These proteins degrade the atRA and therefore could play a role in reducing its transcriptional function (Nebert and Russell, 2002). atRA has a wide range of functions including immunity, vision and reproduction (Vilhais-Neto and Pourquié, 2008). The influence of atRA on the immune system during parasitic infection is variable (Larange and Cheroutre, 2016). Vitamin A insufficient mice orally infected with *T. gondii* showed impaired Th1 and Th17 response on the mucosal level (Hall *et al.*, 2011). A similar outcome was seen in $Rara^{-/-}$ mice, indicating that the atRA/RAR is essential for T-cell priming (Hall *et al.*, 2011).

Some data regarding the importance of atRA in *Ascaris* infection is already available. Pigs fed atRA showed, an increased abundance of hepatic mRNA of Th2-associated cytokines; this was true for both pigs infected with *A. suum* and their uninfected controls (Dawson *et al.*, 2009). The presence of atRA might, therefore, indicate an early push for a Th2 shift. This could be what is absent in the C57BL/6J mice if atRA is being degraded by Cyp3A proteins. Interestingly, ABA-1, an allergen (Tomlinson *et al.*, 1989, Christie *et al.*, 1990) found in somatic tissues of *Ascaris* and secreted by the parasite (Kennedy and Qureshi, 1986, Kennedy *et al.*, 1987b), has been found to bind retinol among other substances such as fatty acids (Kennedy *et al.*, 1995). This could indicate a regulatory mechanism of the parasite on retinol levels in the host and therefore control the Th2 response mounted by the host (Hurst and Else, 2012).

In short, it is unclear what the exact role of the retinol metabolism is in this mouse model of *A. suum* infection. However, based on previous findings in human populations it appears that there might be an important role for vitamin A, its storage and metabolism. Additionally, there is a link between Retsat protein, which is one of the ten most abundant proteins in C57BL/6J control when compared to CBA/Ca control, and oxidative stress (Pang *et al.*, 2017). Retsat is an enzyme involved in retinol metabolism. Its role in ROS production is currently unclear, but the enzyme could be a potential link between retinol metabolism and the immune system.

3.5.4 Other proteins that are not part of the above processes

3.5.4.1 *Major urinary proteins*

At day 7 p.i. we found that in general Mups were downregulated under infection for both strains when compared to their respective controls. A total of six Mups were observed in the dataset. Mups are pheromone transporters and are used as chemical cues for individual recognition (Hurst *et al.*, 2001, Brennan and Keverne, 2004, Manivannan *et al.*, 2010). They are produced in the liver, under the regulation of testosterone and subsequently released in the plasma, from where they are excreted through the urine (Hurst and Beynon, 2004). Mups bind small volatiles (Hurst and Beynon, 2004), a process that is necessary to ensure their slow evaporation once released through urine (Hurst *et al.*, 1998). Both males and females produce Mups. However, male adult mice produce more Mups than females (Beynon and Hurst, 2003). A decrease in Mups in mice livers is also observed during *S. mansoni* infection (Isseroff *et al.*, 1986, Manivannan *et al.*, 2010) highlighting that a potentially conserved response to parasitic infection may be present and localised to the liver.

3.5.4.2 *S100*

In our dataset, the proteins S100a10, S100a9, S100a8 and S100a1 were all upregulated in C57BL/6J infected compared to its own control. Only s100a8 and s100a9 are upregulated in CBA/Ca infected compared to their own control. In short, S100a10 and S100a1 are only significantly upregulated in C57BL/6J. These proteins were absent in the dataset of the day 4 p.i. proteomes.

The S100 group is a large group of proteins involved in a number of cellular functions including cell motility and cell differentiation (Marenholz *et al.*, 2004). The S100a8 and S100a9 proteins can be found as homodimers, but can also form a heterodimer, called

calprotectin, when in the presence of calcium. Calprotectin can act as a DAMP (Vogl *et al.*, 2007, Ehrchen *et al.*, 2009) and it can also recruit neutrophils to the site of infection. Such is the case in *S. japonicum* infection, where S100a8 and neutrophils were found near fibrotic areas of granulomas (Burke *et al.*, 2010). During *Litomosoides sigmodontis* infection, BALB/c mice were found to have an increase in S100a9 transcription in lung tissue within hours after the infection (Karadjian *et al.*, 2017). The authors believe the role of s100a9 in filarial infection is either as a danger signal or as part of neutrophil extracellular traps (NETs) (Urban *et al.*, 2009) NETs have been found to play a role in the clearance of pathogens, such as trapping *Leishmania amazonensis in vivo* (Guimarães-Costa *et al.*, 2009) and activating TLR 2 and 4 during *T. cruzi* infection (Sousa-Rocha *et al.*, 2015).

Regardless of whether the S100a8 and S100a9 proteins are DAMPs or recruit neutrophils, they appear to play an important role in leucocyte (neutrophilic) defence against the invading larvae.

3.6 Conclusions

These results confirm our previous findings where we identified an innate difference between the two mouse strains with the CBA/Ca strain showing a higher abundance of OXPHOS proteins both with and without infection. Here, we focused on a later time point and were able to identify novel hepatic responses to the invading parasite. We found that the initiation of the complement system of the innate immune response differs between the two strains. We distinguished an increased abundance of proteins involved in the activation of the complement system *via* the lectin pathway in the CBA/Ca strain. This was in contrast to the C57BL/6J strain which showed a higher abundance of proteins involved in inhibition of this cascade. This difference in immune activation is an interesting new finding with potentially important consequences for the overall immune response towards the parasite. Furthermore, our finding regarding the retinol pathway is remarkable given the many human studies that have been performed on vitamin A supplementation and STH (re)infection. Our results confirm a potential role for retinol and its metabolites during *Ascaris* infection in the liver. However, it is unclear what this role is and how retinol metabolism influences the hosts' response. Further studies will be essential to unravel these questions. In short, this study confirms that both mouse strains show a different hepatic proteomic

response to *A. suum* infection which could give more insight into the predisposition observed in both pigs and humans.

3.7 Additional files

Due to large size, the additional files are available online on https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-019-3655-9#Sec27

Additional file 1: Table S 1: ANOVA statistics (*P*- and q-values) for all proteins Benjamini-Hochberg FDR < 0.05.

Table S 2: All proteins involved in the assembly of the heatmap.

Table S 3: Enriched GO, KEGG and protein family terms from heatmap clusters.

Additional file 2: Table S 4: Proteins identified from the liver left lobes of C57BL/6J and CBA/Ca mice with and without *Ascaris* infection.

Table S 5: t-test ($P < 0.05$) results and relative fold differences between CBA/Ca control and C57BL/6J control mice.

Table S 6: t-test ($P < 0.05$) results and relative fold differences between CBA/Ca infected and C57BL/6J infected mice.

Table S 7: t-test ($P < 0.05$) results and relative fold differences between C57BL/6J control and infected mice.

Table S 8: t-test ($P < 0.05$) results and relative fold differences between CBA/Ca control and infected mice.

3.7.1 Availability of data and materials

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD014508, available at http://www.proteomexchange.org/submission/index.html.

3.8 Acknowledgements

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Chapter 4 - *Ascaris lumbricoides* and *A. suum* vary in their larval burden in a mouse model

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Author contributions for the publication: Celia Holland and Gwendoline Deslyper designed the experiment. Oluyomi Sowemimo collected *A. lumbricoides* worms. Gwendoline Deslyper preformed the mouse experiments and collected the larvae. Gwendoline Deslyper performed the mouse work, larval retrieval and larval counts. Gwendoline Deslyper performed statistical analysis of the larval counts and produced associated figures and tables. Jessica Beresford measured the larval lengths, performed some larval counts and performed statistical analysis on the larval lengths with associated tables. Gwendoline Deslyper wrote the manuscript. Gwendoline Deslyper, Celia Holland and Oluyomi Sowemimo performed final revisions of the manuscript.

4.1 Abstract

Ascariasis is a neglected tropical disease, caused by *Ascaris lumbricoides*, affecting 800 million people worldwide. Studies focused on the early stage of parasite infection, occurring in the gut, liver and lungs, require the use of a mouse model. In these models, the porcine ascarid, *Ascaris suum*, is often used. The results obtained from these studies are then used to draw conclusions about *A. lumbricoides* infections in humans. In the present study, we sought to compare larval migration of *A. suum* and *A. lumbricoides* in mouse models. We used a previously developed mouse model of ascariasis, which consists of two mouse strains, where one mouse strain – C57BL/6J – is a model for relative susceptibility and the other – CBA/Ca –for relative resistance. Mice of both strains were infected with either *A. suum* or *A. lumbricoides*. The larval burden was assessed in two key organs, the liver and lungs, starting at 6h post infection (p.i.) and ending on day 8 p.i. Additionally, we measured larval size of each species at days 6, 7, and 8 p.i. in the lungs. We found that larval burden in the liver is significantly higher for *A. lumbricoides* than for *A. suum*. However, the inverse is true in the lungs. Additionally, our results showed a reduced larval size for *A. lumbricoides* compared to *A. suum*.

4.2 Introduction

Ascariasis is a neglected tropical disease widespread in Asia, Africa and South America (Jourdan *et al.*, 2018) with an estimated 800 million people infected with *A. lumbricoides* worldwide (Pullan *et al.*, 2014). Children, between the ages of 5 and 15 years, suffer the majority of the worm burden (Jourdan *et al.*, 2018, Wright *et al.*, 2018). Ascariasis causes both chronic and acute symptoms. The latter are often more severe, but less common, and include intestinal obstruction and the so-called Loeffler syndrome, a characteristic allergic response due to larval migration (Loeffler, 1932, Loeffler, 1956, Ribeiro and Fisher, 2002). The chronic symptoms, which occur more frequently, include malnutrition and associated stunting (O'Lorcain and Holland, 2000, Deslyper and Holland, 2017). In addition, *A. suum* is of considerable economic importance due to an increased feed to gain ratio and liver condemnation associated with porcine infection (Boes *et al.*, 2010).

Embryonated eggs, containing a third stage larva (L3) with a second stage larva (L2) cuticle, are orally ingested by the host animal (Murrell *et al.*, 1997, Geenen *et al.*, 1999). These eggs hatch in the intestines and migrate via the portal blood to the liver. Here, the larvae shed their L2 cuticle and increase in size (Roepstorff *et al.*, 1997). Subsequently they migrate to the alveolar space in the lungs, where, again, they increase in size before moving to the pharynx. The larvae will be coughed up, swallowed again and migrate to the intestines where they mature into adult worms (Dold and Holland, 2011a).

An important aspect of many macroparasites, is the aggregated distribution of worm burden whereby most hosts harbour few worms and a small proportion of hosts harbour heavy worm burdens (Crofton, 1971, Shaw and Dobson, 1995). Such aggregated distributions are observed in both humans and pigs infected with *Ascaris* (Crofton, 1971, Croll and Ghadirian, 1981, Holland *et al.*, 1989, Shaw and Dobson, 1995, Boes *et al.*, 1998b). The same people – and pigs – reacquire similar worm burdens upon reinfection, this is termed predisposition (Holland, 2009).

Both *A. suum* and *A. lumbricoides* are morphologically similar (Sprent, 1952a, Maung, 1973, Ansel and Thibaut, 1973). This has led to speculation whether *A. suum* and *A. lumbricoides* are in fact two separate species. Cross transmission has been observed in non-endemic areas, where pig-to-human transmission can be verified (Anderson,

1995, Nejsum *et al.*, 2005, Arizono *et al.*, 2010, Betson *et al.*, 2014). However, human-to-pig transmission is more difficult to prove (Zhou *et al.*, 2012, Betson *et al.*, 2013, Criscione *et al.*, 2007). Fertile hybrids have been identified (Criscione *et al.*, 2007, Zhou *et al.*, 2012, Betson *et al.*, 2013), giving some weight to the one species theory, however, the advancement of genetic technology has been able to shed new light on this question. The use of mtDNA and ITS-1 has shown that *A. suum* and *A. lumbricoides* are in fact two separate species (Anderson *et al.*, 1993, Zhu *et al.*, 1999). However, a comparison of whole mtDNA (Liu *et al.*, 2012) showed high similarities and again pointed to one species. In short, 'based on the phonetic, phylogenetic, and evolutionary species concepts', *A. lumbricoides* and *A. suum* would be considered one species (Betson *et al.*, 2013). Conversely, when approaching this question from the biological concept of species, *A. lumbricoides* and *A. suum* would be considered two species, and it is this theory that currently has more support (Betson *et al.*, 2013, Easton *et al.*, 2020).

Despite being abnormal hosts, and therefore having a truncated migratory path, mice have proven to be excellent model systems for *Ascaris* research (Holland *et al.*, 2013b). Mice have some advantages over the use of natural porcine host, the latter being larger in size, having a more complex husbandry and ethical constraints and a lack of inbred strains. Other potential model organisms, such as guinea pigs, rabbits and rats, all have lower larval recovery rates than mice (Douvres and Tromba, 1971, Roepstorff *et al.*, 1997). Our group has previously developed a mouse model of *A. suum* aggregation (Lewis *et al.*, 2006, Lewis *et al.*, 2007, Dold *et al.*, 2010). The migratory path is truncated in the mouse, allowing therefore, only the study of early migration (Holland *et al.*, 2013b). Two mouse strains were identified with contrasting phenotypes of resistance/susceptibility to *A. suum* infection, where one mouse strain – C57BL/6J – is a model for relative susceptibility and another mouse strain – CBA/Ca – for relative resistance (Lewis *et al.*, 2006). Using this model, our group was able to identify the liver stage during the parasite's life cycle as the period during which this difference in larval burden develops (Lewis *et al.*, 2007, Deslyper *et al.*, 2019a). This model has subsequently proven useful for the study of the liver proteome in order to help identify the underlying mechanisms of predisposition (Deslyper *et al.*, 2016, Deslyper *et al.*, 2019b).

Most mouse studies use *A. suum* as infective agent when studying ascariasis, undoubtedly because this species is easier to obtain through abattoirs worldwide.

However, the suitability of its use as a model for *A. lumbricoides* infection has, to our knowledge, never been thoroughly investigated.

In the present study, we used our previously developed mouse model of hepatic resistance (Lewis *et al.*, 2006, Lewis *et al.*, 2007) to compare firstly the migratory path, with a specific focus on the liver and lungs, and larval burden of *A. lumbricoides* and *A. suum* infection. Secondly we measured the larval sizes during the lung stage of infection.

4.3 Materials and methods

4.3.1 Ethical approval

The samples used in this study were approved by the TCD Animal Research Ethics Committee and the Health Products Regulatory Authority, the Irish regulator for scientific animal research in Ireland, under Directive 2010/63/EU and its Irish transposition, SI no. 543 of 2012 (Project Authorisation ID AE19136/P008 ID; Case Reference 7015826).

4.3.2 Power calculation

Animal numbers used in experiments were based on the following power analysis. Formula for replication:

CV = coefficient of variation
D = % difference of importance (Note: a 50% i.e. d=50 is the difference of importance)
R = replication per treatment

$CV = 100 * (S.E./Mean)$
$R = 16(CV/d)^2$

Table 4.1: Strain variation in susceptibility to *Ascaris suum* larvae (Lewis *et al*., 2006, Dold *et al*., 2010, Mitchell *et al*., 1976)

Mouse strain	Dose administered	Number of mice	Number of larvae per lungs, day 7
C57BL	1000	5	314 ± 105
C57BL/6	1000	5	164 ± 21.6
CBA/Ca	1000	5	38.4 ± 8.5
C57BL/6	1000	5	125 ± 21.74
CBA/Ca	1000	5	28 ± 7.52

Using the values in Table 4.1, the replication required per treatment can be calculated. It is possible to calculate r for varying doses. However, the experiments which will be undertaken in this study will only involve administering 1000 ova per mice as this has been calculated as the optimum dose of infection (Lewis *et al.*, 2006).

Table 4.2: Calculated replication per treatment based on variation in susceptibility to *Ascaris suum* larvae

Mouse strain	Dose administered	Number of mice	Number of larvae per lungs, day 7	CV	r
C57BL	1000	5	314 ± 105	33	7
C57BL/6	1000	5	164 ± 21.6	13	1
CBA/Ca	1000	5	38.4 ± 8.5	22	3
C57BL/6	1000	5	125 ± 21.74	17	5
CBA/Ca	1000	5	28 ± 7.52	27	9

When we examine variation between C57BL, C57BL/6 and CBA/Ca mice we find a mean r = 5. This replication size has previously been stated as the minimal acceptable sample size (Lewis, 2006).

4.3.3 *Ascaris* eggs

The eggs from *A. lumbricoides* were obtained from dewormed children in Ile-Ife, Nigeria. Deworming was performed using pyrantel pamoate (Albendazole damages egg development (Boes *et al.*, 1998a)). Worms were obtained from several hosts and pooled together. In total, 28 female worms were transported in 4% formalin. Female worms were transported on ice in 4% formalin. Upon arrival in Dublin, the worms were dissected by making an incision along the length of the worm. Subsequently, the uteri removed and mechanically broken up with scissors before being sieved (425 µm). The sieved eggs were placed in 0.05M H_2SO_4 (Aldrich, 32,050-1) in T175 culture flasks with ventilated cap at 26°C and they were oxygenated twice per week. The development of the eggs was microscopically checked on a weekly basis. The percentage of embryonated eggs was calculated. After 8 weeks, approximately 70% of the eggs were embryonated and were ready for use in experimental infection.

Embryonated *A. suum* eggs were kindly donated by Dr. Johnny Vlaminck (Ghent University). These were shipped in a water solution, stored at 26°C in 0.05M H_2SO_4 and oxygenated twice per week.

The eggs used for infection were therefore, for both species, from a mixture of worms, mimicking the situation as it would occur naturally.

4.3.4 Infection experiment

Ninety (90) male C57BL/6J mice (Comparative Medicine Unit, Trinity College Dublin, Dublin, Ireland) and 90 male CBA/Ca mice (Envigo, the Netherlands) were infected with 1,000 eggs each via oral gavage (FTP-20-38-50, Instech, Plymouth Meeting, PA USA). In total, 45 mice of each strain were infected with *A. suum* and 45 were infected with *A. lumbricoides*. The mice were 8 weeks old at the time of infection. Animals were provided *ad libitum* with sterilized water and feed.

4.3.5 Larval recovery and enumeration

Five mice from each group were sacrificed daily via cervical dislocation, starting at 6 hours p.i. until and including 8 days p.i. Subsequently the mice were dissected, livers and lungs removed, and larvae were recovered using the modified Baermann method (Lewis *et al.*, 2006). For ease of use, the lungs were split into the left and right lung. The resulting saline solution, containing the larvae, was spun at 1389 *g* for 5 min. The supernatant was decanted to a level of 10 ml and then 10 ml of 70% ethanol was added. Preceding the larval counts, the samples were spun at 805 *g* for 5 min and 15 ml of the supernatant was decanted.

The pellet was resuspended and larval counts were performed on the remaining 5 ml. For the lungs, larvae in 1 ml of each sample was counted on a nematode counting chamber (Chalex Corporation, Park City, UT, USA). The number obtained for the left lung and the right lung were added to provide the total number of larvae recovered from the lungs from each mouse. For the liver, 100 µl aliquots were screened for the presence of larvae, this was repeated five times per sample.

4.3.6 Measuring of larval lengths

Larval lengths from the lungs were measured at days 6, 7 and 8 p.i. The larvae were photographed (Olympus digital camera C-5050, Shinjuku, Tokyo, Japan) and their length from anterior to posterior calculated using ImageJ (v.1.52a) (Lewis *et al.*, 2006).

4.3.7 Statistical models

General linear models (GLM) were run in R (version 3.5.1) (R Core Team, 2018) for the numbers of liver and lung larvae , separately. All models used a combination of the three categorical independent variables, *Ascaris* species, mouse strain and day p.i. The

data for larvae recovered from the liver and lungs were over dispersed. The MASS package (Venables and Ripley, 2002) and pscl package (Zeileis *et al.*, 2008) were used to build the negative binomial (log link) and zero-inflated negative binomial (logit link) models respectively. Model selection was based on a combination of Akaike Information Criterion (AIC), log likelihood (LogLik) values and number of expected zeroes.

Table 4.3: Model comparison for liver data. All models in table are zero-inflated negative binomials and have a logit link. Additive models have a '+' sign between the variables whereas interaction models have a '*' sign between variables. A '|' sign denotes a logit link between variables.

No	Model	Df	LogLik	AIC
1	Count ~ *Ascaris* species + mouse strain + days p.i.	23	-672.21	1390.417
2	Count ~ *Ascaris* species + mouse strain | days p.i.	13	-707.40	1440.806
3	Count ~ *Ascaris* species * mouse strain + days p.i.	25	-671.51	1393.029
4	Count ~ *Ascaris* species * mouse strain | days p.i.	14	-706.29	1440.583

For the liver, the total number of zeroes in the data was 59, meaning the zero-inflated models were a better fit. Model number 1 & 3 were very similar (Table 4.3). The likelihood ratio test did not reveal statistical significant differences between the two (Pr(>Chisq) = 0.4996). The interaction was not significant in model number 3. Using the parsimony principle, the simpler of the two models, number 1, was chosen.

The data for the lungs were not zero inflated so a negative binomial model was preferred. Model 1 & 2 had the best AIC scores (Table 4.4) and the likelihood ratio test (Pr(>Chisq) = 0.9343) did not reveal statistical significant differences between the two. As the interaction between strain and day was not significant, the simpler model – number 1 – was chosen.

Table 4.4: Model comparison for lung data, all models in the table are negative binomials and have a log link. Additive models have a '+' sign between the variables whereas interaction models have a '*' sign between variables. A '|' sign denotes a logit link between variables.

No	Model	Df	LogLik	AIC
1	Count ~ *Ascaris* species + mouse strain + days p.i.	12	-193.02	410.0377
2	Count ~ *Ascaris* species * mouse strain + days p.i.	13	-193.01	412.0309
3	Count ~ *Ascaris* species * mouse strain * days p.i.	37	-188.96	451.9133

4.3.8 Statistical analysis larval length

Larval lengths were analysed at day 8 p.i. Other time points did not yield enough larvae/data points for statistical analysis. We used a two-way ANOVA with *Ascaris* species and mouse strain as factors.

4.4 Results

4.4.1 Higher *A. lumbricoides* counts in the liver

The number of larvae recovered from the CBA/Ca strain were consistently lower than those of C57BL/6J. The C57BL/6J mouse strain had, for both ascarid species, much higher mean larval numbers than the CBA/Ca mouse strain. Our results show that C57BL/6J had an increase in larval numbers at day 6 p.i. of 208 ± standard error of the mean (S.E.M.) 71.7 larvae for mice infected with *A. lumbricoides*. As for the same mouse strain, but with *A. suum* infection, day 3 p.i. was identified as the day with the highest number of larvae with 56 ± S.E.M. 26.8 larvae. For the relatively resistant strain, CBA/Ca, the highest number of larvae were observed for *A. lumbricoides* and *A. suum* respectively at day 1 p.i. with 82 ± S.E.M. 18.8 larvae and day 2 p.i. with 46 ± S.E.M. 26.2 larvae.

Overall, higher counts of *A. lumbricoides* than *A. suum* larvae (see Figure 4.1a-b) were observed in the liver. This was true for nearly all time points, except on day 2 and 3 in CBA/Ca mice. However, the difference in mean larval burden on those days was found to be quite low. CBA/Ca mice, on day 2 and 3, infected with *A. lumbricoides* had a mean of 34 ± S.E.M. 10.3 and 20 ± S.E.M. 6.3 respectively, whereas *A. suum* infected mice had a mean of 46 ± S.E.M. 26.2 and 24 ± S.E.M. 11.2 for those days respectively.

The model revealed that the difference in larval burden between the two mouse strains and the two species was statistically significant ($p < 0.01$). The theta value of the model was 1.3873 or an alpha value of 0.7208. The coefficients of the zero-inflated negative binomial model (Table 4.5) indicated that, for the counts portion of the model, the difference in the logs of expected larval counts decreased with 0.52 units if the mouse was infected with *A. suum* compared to *A. lumbricoides*. As for the CBA/Ca mouse strain, a decrease of 0.53 units was observed compared to C57BL/6J. Both these values were statistically significant, with observed lower larval counts for *A. suum* infection, compared to *A. lumbricoides* infection. The same was true for the CBA/Ca mouse strain,

indicating this mouse strain had statistically significantly lower larval numbers than the C57BL/6J mouse strain.

In the logistic portion of the model, or the zero-inflated portion, the difference in the logs of expected zeroes increased by 2.69 units for *A. suum* infected mice compared to *A. lumbricoides* infected mice. The model predicted an increase of 1.20 units for every difference in the logs of the larval counts if it was a CBA/Ca mouse rather than a C57BL/6J mouse. Again, these values were statistically significant, meaning that a higher number of zeroes was expected in mice infected with *A. suum* or of the CBA/Ca species.

4.4.2 A drop of *A. lumbricoides* counts in the lung

In the lungs, there was a consistently higher larval count for *A. suum*, compared to *A. lumbricoides* (See Figure 4.1c-d). Larval counts were much lower overall for both mouse strains compared to the liver. Additionally, the CBA/Ca strain had consistently lower larval numbers compared to the C57BL/6J strain. Additionally, both *A. suum* and *A. lumbricoides* infected mice had higher larval burdens in C57BL/6J compared to CBA/Ca. With the highest number of larvae for C57BL/6J mice infected with *A. lumbricoides* of 47 ± S.E.M. 29.1 larvae at day 8 p.i. and 86 ± S.E.M. 37.2 larvae for *A. suum* infected mice at the same time point. The highest number of larvae observed for the CBA/Ca mouse strain was observed at day 8 p.i. and was found to be much lower, with 5 ± S.E.M. 4.1 and 3 ± S.E.M. 1.8 larvae for *A. lumbricoides* and *A. suum* respectively.

The difference between the two species was found to be statistically significant ($p < 0.01$). Additionally there was a statistically significant difference between the two mouse strains ($p < 0.01$). The binomial model for the lungs predicted (Table 4.5) that the difference in the logs of larval counts increased with 1.18 units if the mouse were infected with *A. suum*. If the mouse strain was CBA/Ca, the difference in the logs of expected larval counts decreased with 2.14 units compared to C57BL/6J. However, no statistical difference was found regarding the different time points.

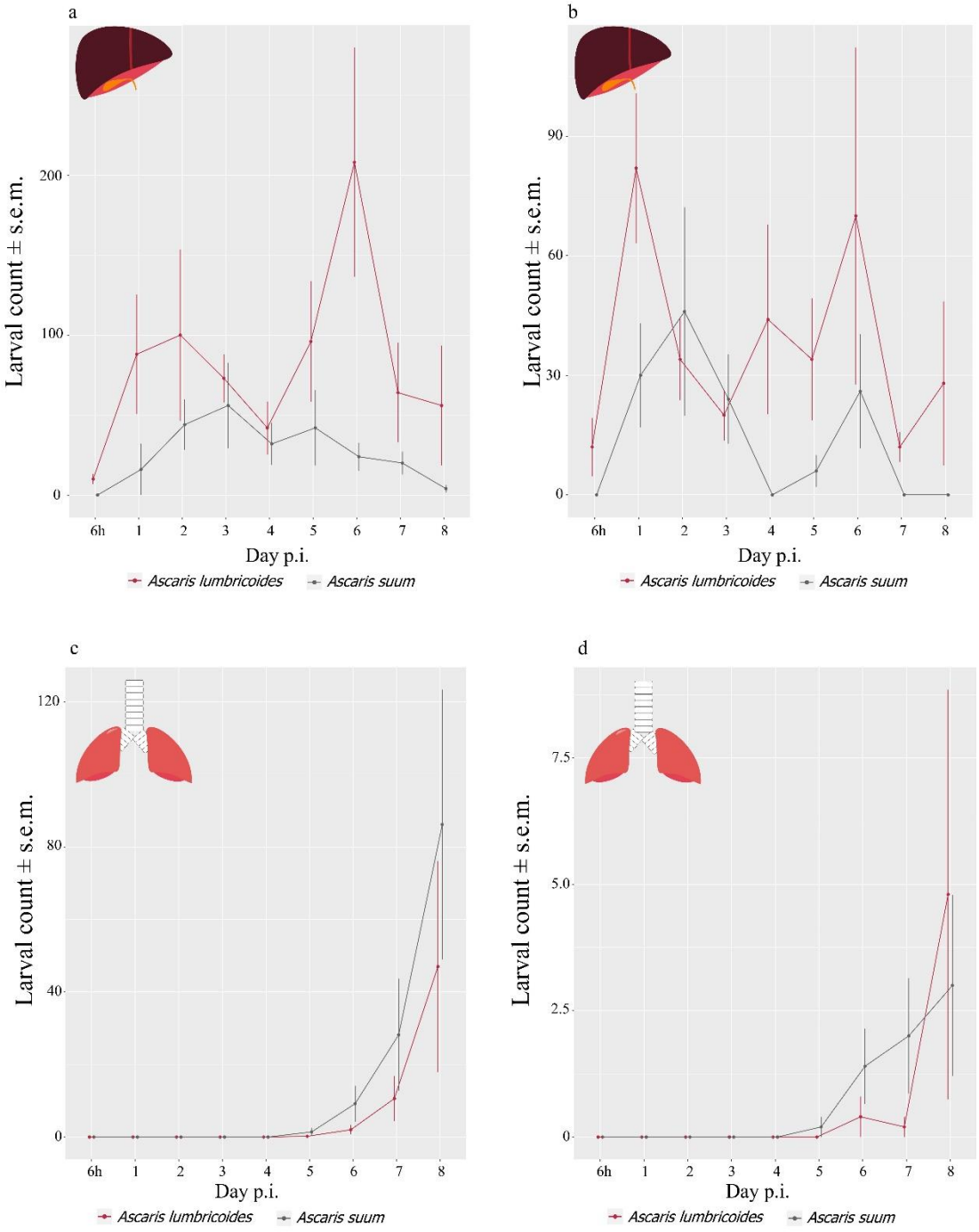

Figure 4.1: Mean (± S.E.M.) larval counts of *A. lumbricoides* and *A. suum* in C57BL/6J mice – a relatively susceptible strain – and CBA/Ca mice – a relatively resistant strain – for both the liver and the lungs. The red line are the counts for *A. lumbricoides* and the grey line are the counts for *A. suum* a: Liver counts of C57BL/6J b: Liver counts of CBA/Ca c: Lung counts of C57BL/6J d: Lung counts CBA/Ca

Table 4.5: The coefficients from the models for both liver and lungs. Ascarid species is A. suum compared to A. lumbricoides. Mouse strain is CBA/Ca compared to C57BL/6J and time points are compared to day 1 p.i. ** Pr(>|z|) < 0.01 * Pr(>|z|) < 0.05.

	Liver – Zero-inflated negative binomial				**Lungs – Negative binomial**	
	Counts portion of model		Logistic Portion of model			
	Coefficient	Standard error	Coefficient	Standard error	Coefficient	Standard error
Intercept	4.66**	0.25	-3.95**	0.85	-33.87	2.852e+06
Ascarid species (*A. suum*)	-0.52**	0.18	2.69**	0.51	1.18**	0.38
Mouse strain (CBA/Ca)	-0.53**	0.17	1.20**	0.43	-2.41**	0.38
6 hours p.i.	-1.46**	0.42	3.21**	0.93	4.28e-03	4.034e+06
2 days p.i.	0.05	0.30	-0.98	1.04	4.28e-03	4.034e+06
3 days p.i.	-0.31	0.31	-0.44	0.96	4.28e-03	4.034e+06
4 days p.i.	-0.32	0.34	1.67	0.87	4.28e-03	4.034e+06
5 days p.i.	-0.28	0.31	0.36	0.89	34.48	2.852e+06
6 days p.i.	0.32	0.31	0.37	0.89	35.06	2.852e+06
7 days p.i.	-0.86*	0.33	1.02	0.87	35.90	2.852e+06
8 days p.i.	-0.66	0.35	1.97*	0.87	37.40	2.852e+06

4.4.3 Lung larval length differs between *Ascaris* species and mouse strain

In total, 559 larvae from the lungs were measured for days 6, 7, and 8 p.i (Table 4.6). These data showed larval growth over the course of the measured days for all studied groups. Overall, the mean length of *A. suum* was greater than for *A. lumbricoides*. This was true for both mouse strains. Additionally, *A. lumbricoides* had a lower larval length in the relatively resistant strain at day 8 p.i., when compared to the relatively susceptible strain. As for *A. suum*, this difference was not found at day 8 p.i., however, it was observed in the earlier days, where the larvae had lower length in the relatively susceptible strain when compared to the relatively resistant strain.

Due to insufficient number of larvae available in the samples, analysis (two-way ANOVA) was only performed on the results from day 8 p.i. This analysis revealed that both mouse strain ($F_{1,401}$ = 8.37, p = 0.004) and *Ascaris* species ($F_{1,401}$ = 71.15, p < 0.0001) had a statistically significant effect on larval length for this day. The interaction between the two factors was found not to be statistically significant.

4.5 Discussion

In a mouse model of ascariasis, *A. suum* has tended to be the species of choice, with *A. lumbricoides* much less commonly used (Stewart, 1917b, Stewart, 1918, Sprent, 1952b, Bhowmick, 1964, Cho, 1967, Buske and Engelbrecht, 1968, Kumar and Singh, 1968, Maung, 1978, Massara *et al.*, 1990, Massara *et al.*, 1991, Peng *et al.*, 2012, Gazzinelli-Guimarães *et al.*, 2013). To the best of our knowledge, no previous study has provided a detailed comparison of the larval burden of the two ascarid species derived from the two most important organs in early migration, the liver and the lungs. Additionally, we measured the larval length in the lungs at the later time points of day 6, 7, and 8 p.i., considering this parameter as a measure of larval fitness. Using our mouse model of hepatic resistance, and thus examining two mouse strains, we were able to observe whether any differences were strain specific or not.

Table 4.6: Larval length of *A. suum* and *A. lumbricoides* in C57BL/6J and CBA/Ca mouse strains on day 6, 7 and 8 post-infection in µm of lung samples.

		Day 6	Day 7	Day 8
C57BL/6J with *A. suum*	Mean ± S.D.	810 ± 124	809 ± 207	1122 ± 253
	Median	815	764	1116
	Range	395	936	1476
	Sample Size	28	83	253
C57BL/6J with *A. lumbricoides*	Mean ± S.D.	527 ± 91	717 ± 146	932 ± 184
	Median	503	747	938
	Range	188	548	1018
	Sample Size	3	28	131
CBA/Ca with *A. suum*	Mean ± S.D.	719 ± 191	754 ± 201	1104 ± 141
	Median	676	703	1124
	Range	424	538	343
	Sample Size	4	6	8
CBA/Ca with *A. lumbricoides*	Mean ± S.D.	-	681	767 ± 162
	Median	-	-	769
	Range	-	-	563
	Sample Size	0	1	11

4.5.1 Larval counts

The larval counts differed significantly between the two *Ascaris* species in both the lungs and liver as well between the two mouse strains. In the liver, *A. lumbricoides* was found to have consistently higher larval numbers. In the lungs, however, the inverse was observed, with *A. suum* counts being consistently higher than *A. lumbricoides* counts. Taken together, these could indicate a delayed, but more pronounced immune defence against *A. lumbricoides*. The inversion of the larval recoveries is quite interesting. It confirms our previous findings, indicative of a role of the liver in larval attrition (Lewis *et al.*, 2007, Dold *et al.*, 2010, Deslyper *et al.*, 2016, Nogueira *et al.*, 2016, Deslyper *et al.*, 2019b). Our results indicate that *A. lumbricoides* larvae have a higher infectivity rate, potentially associated with higher antigenicity, and therefore reach the liver in higher numbers. However, upon reaching the liver, this presumed higher

infectivity and antigenicity becomes a burden as the immune system ramps up and targets *A. lumbricoides* larvae at a higher proportion than *A. suum* larvae. Based on those findings, we conclude that *A. suum* infection in a mouse model is not a perfect substitute for *A. lumbricoides* infection and results obtained with *A. suum* eggs should be interpreted carefully.

We also observed that the relatively susceptible strain, C57BL/6J, had consistently higher larval counts than the relatively resistant strain, CBA/Ca. This was true for both Ascarid species. We can therefore conclude that our mouse model of hepatic resistance can also be used for *A. lumbricoides* infection.

There is a paucity of comparative data on the differences in larval burden between *A. suum* and *A. lumbricoides* in a mouse model. However, a previous study reported that 5-6 week old male (non pathogen-free white) mice were infected with what was described as '*A. lumbricoides* from man' or '*A. lumbricoides* from pig' (Sprent, 1952b). The author found that both species had a similar migratory path. However, it was observed that the 'human strain appeared to have about twice the infectivity' (Sprent, 1952b). These data therefore showed a similar pattern to our own observations, specifically the higher number of larvae in the liver of the human ascarids. However, the author observed a higher number of larvae of this human ascarid in the lungs as well, the exact opposite of our own findings.

A more recent study (Peng *et al.*, 2012) infected C57BL/6 mice and pigs with *Ascaris* eggs. The authors did identified the *Ascaris* as a genotype which mainly infects humans or one that mainly infects pigs rather than as *A. suum* or *A. lumbricoides*. The findings were similar to results obtained in this study, with a higher larval count in the liver of the genotype, mainly associated with infecting humans compared to the genotype mainly infecting pigs. Conversely, Peng *et al.* (2012) also found this to be true in the lungs where, in contrast to our results, they identified a higher larval burden for *A. suum* in the lungs. However, their lung larval counts did not show the typical slow increase in larval counts that we observed, rather they observed several days with higher numbers, with larvae detected in this organ as early as 8 hours p.i.

Another study, investigated how the age of an *Ascaris* egg culture influences infectivity, briefly touches on the question of the use of *A. lumbricoide*s in a mouse model (Gazzinelli-Guimarães *et al.*, 2013). The authors found no statistically significant

differences between *A. suum* and *A. lumbricoides* larval counts in male BALB/c mice, however, larval counts were only compared from lung samples derived from day 8 p.i.

In short, our study showed similar results to previous studies regarding larval counts in the liver, where the human ascarid has a higher larval count than the porcine ascarid. However, our study differs from these studies regarding the lung. Where we found higher *A. suum* larval counts for this organ, other studies found higher *A. lumbricoides* burdens.

4.5.2 Larval length

When measuring the larval length in the lungs, we found, interestingly, that the mean larval length for *A. suum* was longer in both mouse strains. Taken together with the data from the larval counts, it appears that *A. lumbricoides* may provoke a stronger immune response, which therefore results in smaller larvae in the lungs.

One study immunized 8-week old male C57BL/6 mice with *A. suum* via oral infection, followed by a challenge infection (Johnstone *et al.*, 1978). The larvae recovered from these mice were compared to mice which had received one single dose of eggs. The authors found that for the liver the larval counts were quite similar, however, the difference in larval counts was quite substantial in the lungs with the non-immunized having a much higher larval count compared the immunized animals. The authors therefore confirm the idea that 'the mechanism of immunity against *A. suum* operates primarily in the liver rather than in the gastrointestinal tract'. Interestingly, between days 5 and 9 p.i. the difference in larval lengths in the liver was significantly lower for the immunized animals compared to non-immunized animals. So despite there being no significant difference in larval burden in the liver, the larvae are already smaller at this point in time. This trend continues in the lungs, where a statistically significant difference in larval lengths is observed.

A later study, using a reinfection experiment, developed a simpler version of the above experiment (Song *et al.*, 1985). The authors compared reinfected mice with primary infected mice and found that the larval length in both liver and lungs was lower for the reinfected mice compared to the primary infected mice. As for the liver specifically, the difference in larval length was relatively small during early infection, however, it increased over time. As for the lungs, the initial differences were quite big, but the difference actually decreased. The authors concluded that the 'development of larvae

in the liver of immune mice were probably repressed by the immune mechanisms being r(a)ised in the livers' (Song *et al.*, 1985).

Lewis *et al.* (2006) identified that infection dose influenced the length of the larvae, with a higher egg dose resulting in higher larval burden in the lungs, but their length was reduced. They explain that increased larval count could be due to increased tissue damage allowing for more larvae to migrate to the lungs and reduced size could occur due to a greater innate response hampering larval growth in the case of an increased infective dose. This could explain our own observations, where the decreased mean larval size of *A. lumbricoides* in both mouse strains could be related to the observed higher larval burden in the liver and the potentially associated pronounced immune response.

In addition to using a mouse model, some research groups have used the natural host, the pig, to study *A. suum* adult worm length. Pigs were immunized with *A. suum*, through repeat infection, with some pigs receiving fenbendazole treatment after each infection (Stewart *et al.*, 1985). After an *A. suum* challenge infection, the adult worms were measured and counted. The authors found that fewer and smaller adult worms were recovered from the groups that received the anthelmintic after each vaccination infection, compared to those that were immunized but did not receive any anthelmintic. The authors concluded that the fenbendazole treatment probably heightened the immune response against the parasite.

Another group infected pigs with different *A. suum* haplotypes and found that there was a consistent statistically significant difference in the worm length between the haplotypes, with one haplotype consistently being larger than the others (Nejsum *et al.*, 2009). Furthermore, the effect of the different hosts – i.e. different pigs – on worm length was statistically significant. This therefore implies that not only the genetics of the specific haplotype, but also the interaction with the host has an effect on worm size. The authors subsequently found that worm length was highly correlated to the length of the female worm from which the eggs were taken. The authors suggested, assuming all eggs from one female are fertilized by one male, that 18-46% of the worm length can be explained by heritability.

We see similar results in our CBA/Ca mouse strain, where mice infected with *A. lumbricoides* had an earlier increase in larval numbers in the lungs - at day 1 p.i. -

compared to *A. suum* infected mice - at day 2 p.i. Additionally, we found that larval length and burden is lower for *A. lumbricoides* in the lungs. This would indicate that there is a more pronounced immune response against this species, compared to *A. suum*, much like the case for the immunized or reinfected animals in the previous experiments. One could argue that the observed reduction in larval size of *A. lumbricoides* in the lungs, is a consequence of density-dependent growth retardation. However, we could argue that evidence from the reinfection experiments (Johnstone *et al.*, 1978, Song *et al.*, 1985) points towards a more pronounced immune response against *A. lumbricoides*. We postulate that the antigenicity and infectivity of *A. lumbricoides* is higher in this mouse model, with higher larval counts in the liver than *A. suum*, followed by lower larval counts in the lungs. However, *A. lumbricoides* may evoke a much stronger immune response than *A. suum*, leading to a reduced larval count and length in the lungs compared to *A. suum*. These results therefore indicate that more research is necessary to compare the early immune response to *A. suum* and *A. lumbricoides* in a mouse model.

However, it cannot be excluded that a different growth rate between the two species is (in part) responsible for the observed differences in larval length. The ascarid species are morphologically very similar, with only few morphological differences between the two species (Deslyper and Holland, 2017). However, distinguishing the larvae and eggs of the two species can only be performed using molecular techniques. Morphological similarities does not necessarily infer an identical development timeline, and this would need to be further investigated.

Additionally it needs to be noted that geographical differences can play a role in this experiment. The *A. lumbricoides* eggs were obtained from a Nigerian human population, whereas the *A. suum* eggs were obtained from a Belgian porcine population. Using microsatelite markers, genetic differences for *Ascaris* on a macro-scale – i.e. between countries – have been observed (Betson *et al.*, 2014, Betson *et al.*, 2011, Betson *et al.*, 2012, Criscione *et al.*, 2007) with some studies attributing up to 17% of genetic diversity to these geographical differences (Anderson and Jaenike, 1997). It can therefore not be excluded that (part of) the differences observed in size and larval numbers between *A. suum* and *A. lumbricoides* are in fact caused by the geographical origin of the worms/eggs used in this experiment.

In conclusion, we observed a difference in larval burden and length between the two ascarid species both in the liver and the lungs. Our results therefore indicate a potentially different host response towards *A. suum* compared to *A. lumbricoides*. This has previously been investigated by proteomics, using an *A. suum* infection in the mouse model (Deslyper *et al.*, 2016, Deslyper *et al.*, 2019b). However, in order to fully understand the mechanisms behind this difference we will need to understand the immune response in the liver against *Ascaris*. We are currently undertaking an experiment using flow cytometry to determine which immune cells are activated in the liver during *Ascaris* infection. We are investigating if there is a different immune response between the two mouse strains of the mouse model and if there is a different immune response against *A. suum* and *A. lumbricoides*.

4.6 Acknowledgements

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Chapter 5 - Distinct hepatic myeloid and lymphoid cell repertoires associated with susceptibility and resistance to *Ascaris* infection

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Author contributions for the publication: Gwendoline Deslyper, Celia Holland and Derek Doherty designed the experiment and wrote the manuscript. Oluyomi Showemimo collected the *A. lumbricoides* worms. Gwendoline Deslyper performed the mouse work, larval retrieval and larval counts. Gwendoline Deslyper, Derek Doherty and Dearbhla Murphy performed the flow cytometry. Gwendoline Deslyper and Dearbhla Murphy performed FlowJo analysis. Gwendoline Deslyper performed statistical analysis of the results and produced associated figures and tables. Derek Doherty produced the gating strategy figure. Gwendoline Deslyper, Derek Doherty, Celia Holland and Dearbhla Murphy performed final revisions of the manuscript.

5.1 Abstract

Background: The soil-transmitted helminth *Ascaris lumbricoides* infects ~800 million people worldwide. Some people are heavily infected, harbouring many worms, whereas others are only lightly infected. People regain similar worm burdens even after several rounds of chemotherapy. The mechanisms behind this difference in worm burden has yet to be elucidated. However, a mouse model of resistance and susceptibility to *Ascaris* has been developed. C57BL/6J mice experience heavy larval burdens whereas CBA/Ca mice experience low larval burdens and the liver stage has been identified as a key time for the development of this difference in larval burden. This study was undertaken to explore the cell types involved at the liver stage of *Ascaris* infection.

Methodology: CBA/Ca and C57BL/6J mice were infected with either the porcine ascarid, *A. suum* or the human ascarid, *A. lumbricoides*. At day 7 p.i., immune cells in the livers and spleens of the mice was enumerated using flow cytometry.

Principal findings: Larval counts confirmed that C57BL/6J mice are more susceptible than CBA/Ca mice to infection by both ascarid strains. Compared to uninfected C57BL/6J mice, uninfected CBA/Ca mice had higher $CD4^+$ and γδ T cell counts in their spleens and higher B cell and lower eosinophil and Kupffer cell counts in their livers. Infection with *A. suum* led to expansions of eosinophils, Kupffer cells, monocytes and dendritic cells in the livers of both mouse strains and depletions of hepatic natural killer (NK) cells in CBA/Ca mice. Infection with *A. lumbricoides* led to decreases in the numbers of $CD8^+$, αβ, natural killer and natural killer T cells in the livers of CBA but not C57BL/6J mice.

Conclusions: Differences in liver cell type between control and infected mice were mainly found to be of the myeloid lineage, with expansions of monocytes, Kupffer cells, dendritic cells and eosinophils. However, intrinsic differences between the two mouse strains were found to reside in the lymphocyte compartment.

5.2 Author summary

Ascaris is a parasite which infects 800 million people worldwide, in particular children between the ages of 5 and 15. Some people experience heavy infections with many adult worms in their gut. Such individuals are at a higher risk of experiencing severe

symptoms which could be acute or chronic. The acute symptoms are less common and include intestinal obstruction, which can lead to death. However, the chronic symptoms are more common and cause significant morbidity, such as malnutrition and impaired cognitive development, especially in children. To investigate this difference in infection intensity, we utilised a mouse model with one mouse strain as a model for heavy infection and another strain as a model for light infection. Previously the liver stage was identified as the likely stage for this difference in larval burden to occur. Here we investigated changes in the numbers of immune cells in the spleens and livers of the two mouse strains during liver stage infection with two strains of *Ascaris*. We found that eosinophils, monocytes and dendritic cells expand in the livers of both mouse strains during early *Ascaris* infection. However, subsets of cytotoxic lymphocytes in the livers appear to distinguish the relatively-resistant from the relatively-susceptible mice, suggesting that they play key roles in immunity against *Ascaris* larvae.

5.3 Introduction

It is estimated that approximately 800 million people are infected with the human roundworm *Ascaris lumbricoides* (Pullan *et al.*, 2014). Despite this high number of infected individuals, ascariasis remains a neglected tropical disease (Hotez *et al.*, 2020, World Health Organization, 2020). *Ascaris* infection is associated with a wide range of poverty related risk factors including a lack of adequate sanitary facilities and poor socio-economic conditions (Deslyper and Holland, 2017). Symptoms of ascariasis can be divided into two categories, acute and chronic. The acute symptoms, such as intestinal obstruction, are severe but rare. Chronic symptoms, including growth retardation and cognitive impairment, are more common and have a more severe impact on the population level (Watkins and Pollitt, 1997, Olsen *et al.*, 1998, Stephenson *et al.*, 2000).

Intensity of infection is not evenly distributed among the population, with children between the ages of 5 and 15 in particular carrying the majority of the worm burden (Bethony *et al.*, 2006, Wright *et al.*, 2018). Additionally, within each age group, some people are more heavily infected than others (Holland *et al.*, 1989, Croll and Ghadirian, 1981, Elkins *et al.*, 1986, Thein-Hlaing *et al.*, 1984). In essence, a small subset of the population carries the majority of the worm burden (Bethony *et al.*, 2006). These 'wormy people' (Croll and Ghadirian, 1981) are often found together in one family (Chai *et al.*, 1983, Forrester *et al.*, 1988). Furthermore, people regain similar worm burdens upon reinfection, even after several rounds of chemotherapy (Elkins *et al.*, 1986, Holland *et al.*, 1989, Croll *et al.*, 1982, Seo *et al.*, 1979, Anderson and May, 1982). This is known as predisposition and appears to be multifactorial in origin (Holland, 2009) with both long-term (host genetics and socio-economic status) and short-term (host acquired immune system) factors involved (McCallum, 1990).

Pigs can get infected with the porcine ascarid *Ascaris suum*. The human and porcine species are very similar with only small morphological differences in the denticle and lip (Ansel and Thibaut, 1973, Maung, 1973, Sprent, 1952a). Although there is still an ongoing debate about whether *A. suum* and *A. lumbricoides* are the same species, current genetic evidence suggests that they are, in fact, separate species reviewed in Deslyper and Holland (2017).

The migratory path of *A. suum* in pigs and *A. lumbricoides* in humans is very similar (Cooper and Figueiredo, 2013). Infection occurs via ingestion of infective eggs. The larvae hatch in the small intestines and penetrate the caecum (Douvres *et al.*, 1969, Murrell *et al.*, 1997). They migrate to the liver via the portal vein from where they migrate to the lungs, penetrate the alveolar space and move up the pharynx (Fagerholm *et al.*, 2000). The larvae are then swallowed and migrate back to the small intestines where they moult twice and mature into adult worms (Pilitt *et al.*, 1987). Although egg release is highly variable, it is estimated that female worms can release around 200,000 eggs per day (Sinniah, 1982). These highly resistant eggs can remain infectious in the soil for up to 15 years contributing to enhanced transmission and the challenge of parasite control (Crompton, 1989, O'Lorcain and Holland, 2000).

As heavy worm burden is associated with more severe symptoms (Croll and Ghadirian, 1981, Holland, 2009), it is important to understand the underlying molecular mechanisms associated with the observed predisposition. Because the early life cycle of the parasite includes internal organs, it is necessary to use animal models (Holland *et al.*, 2013b). Building on earlier work (Mitchell *et al.*, 1976), our group (Lewis *et al.*, 2006) developed a mouse model for resistance to *A. suum* infection. We identified two mouse strains, one (C57BL/6J) as a model for susceptibility to heavy infection and another (CBA/Ca) as a model for resistance to *Ascaris* infection. Using this mouse model, it was subsequently determined that the hepatic stage during larval migration is the likely time at which the observed differences in larval burden between the two mouse strains occur (Lewis *et al.*, 2007, Dold *et al.*, 2010). In fact, an earlier intense immune response was observed via histopathological examination of the liver in the CBA/Ca strain when compared to the C57BL/6J strain (Dold *et al.*, 2010).

The liver has special immunological properties. It receives blood directly from the gut via the hepatic portal vein. This blood carries with it antigens from both gut commensals and dietary products (Doherty, 2016). Because these antigens could cause unwanted chronic inflammatory responses, the hepatic immune system favours tolerance over immunity. This feature makes it the ideal organ for several parasites, including *Ascaris*, to incorporate the liver in their migratory path, as it could be used for immune evasion (Deslyper *et al.*, 2019a). We investigated the liver proteomes of the relatively resistant (CBA/Ca) and relatively susceptible (C57BL/6J) mouse strains, infected with *A. suum* (Deslyper *et al.*, 2016, Deslyper *et al.*, 2019b). We found an

intrinsic difference between the two mouse strains at the level of the OXPHOS, at both day 4 and day 7 p.i. Additionally, at day 7 p.i., a difference in immune response proteins was observed. The relatively resistant strain had a higher abundance of proteins associated with complement activation, whereas the relatively susceptible strain had a higher abundance in proteins associated with complement inhibition.

These mouse models were also found to be suitable for infection with the human ascarid, *A. lumbricoides* (Deslyper *et al.*, 2020). In the liver, higher numbers of *A. lumbricoides* were found than *A. suum*. The inverse was true for the lungs, where higher numbers of *A. suum* were found than *A. lumbricoides*. Furthermore, the mean length of *A. lumbricoides* larvae was shorter in the lungs at days 6-8 p.i. compared to the *A. suum* in both mouse strains. This difference in larval length was even more pronounced in the relatively resistant strain.

Much about the immune response during *Ascaris* infection is unknown. Studies on human serum have found an association between the presence of IgE antibodies specific for the parasite antigen ABA-1 and natural immunity against *Ascaris* infection (Hagel *et al.*, 1993, McSharry *et al.*, 1999). Anti-ABA-1 IgE is thought to protect by inducing eosinophilic degranulation (Gounni *et al.*, 1994). Eosinophils contribute to the elimination of *A. suum* from pigs, (Masure *et al.*, 2013a) and mice (Gazzinelli-Guimaraes *et al.*, 2019). IgE production, eosinophilic degranulation and immunity against parasitic infections require the actions of Th2 cells (Iwasaki and Medzhitov, 2015). In fact, the observed age dependent decrease in *A. lumbricoides* intensity in humans is associated with the production of Th2 associated cytokines (IL-9, IL-10 and IL-13) (Turner *et al.*, 2003, Turner *et al.*, 2005, Jackson *et al.*, 2004). This Th2 response has also been observed during *A. suum* infection in the lungs of mice, where it is characterized by the presence of eosinophils, Th2 cells, alternatively activated macrophages, and elevated levels of IL-4, IL-5, and IL-13 (Weatherhead *et al.*, 2018, Gazzinelli-Guimaraes *et al.*, 2019, Nogueira *et al.*, 2016). Interestingly, some mouse studies, using *A. suum* infection, found evidence of the involvement of Th17 responses at a later time point during the lung stage (i.e. day 12 p.i.) (Weatherhead *et al.*, 2018) or during repeat infections (Nogueira *et al.*, 2016).

To our knowledge, little research has been performed on the immune response in the liver to *Ascaris* infection. The only evidence of the immune response in the liver is the presence of white spots which have been observed in *A. suum* -infected pigs (Ronéus,

1966), *A. lumbricoides*-infected humans (Javid *et al.*, 1999) and *A. suum*-infected mice (Dold *et al.*, 2010).

In this study we performed flow cytometry on liver and spleen samples of the mouse model of hepatic resistance. We selected the time point, day 7 p.i., because we previously found evidence for an altered immune response between the two mouse strains during *A. suum* infection (Deslyper *et al.*, 2019b). Additionally, given our previous finding of a difference in larval burden in both the liver and lungs between *A. lumbricoides* compared to *A. suum* (Deslyper *et al.*, 2020), we infected the mice with either of the two parasites.

5.4 Materials and methods

5.4.1 Ethics statement

The mouse samples used in this study were approved by the TCD Animal Research Ethics Committee and the Health Products Regulatory Authority, the Irish regulator for scientific animal research in Ireland, under Directive 2010/63/EU and its Irish transposition, SI no. 543 of 2012 (project authorization ID: AE19136/P078 ID; case reference 7026410).

5.4.2 Power calculation

Animal numbers used in experiments were based on the following power analysis.

Formula for replication:

CV = coefficient of variation
D = % difference of importance
(Note: a 50% i.e. d=50 is the difference of importance)
R = replication per treatment

CV = 100 * (S.E./Mean)
$R = 16(CV/d)^2$

Table 5.1 Strain variation in susceptibility to *Ascaris suum* larvae (Lewis *et al*., 2006, Dold *et al*., 2010, Mitchell *et al*., 1976)

Mouse strain	Dose administered	Number of mice	Number of larvae per lungs, day 7
C57BL	1000	5	314 ± 105
C57BL/6	1000	5	164 ± 21.6
CBA/Ca	1000	5	38.4 ± 8.5
C57BL/6	1000	5	125 ± 21.74
CBA/Ca	1000	5	28 ± 7.52

Using the values in Table 5.1, the replication required per treatment can be calculated. It is possible to calculate r for varying doses. However, the experiments which will be undertaken in this study will only involve administering 1000 ova per mice as this has been calculated as the optimum dose of infection (Lewis *et al.*, 2006).

Table 5.2 Calculated replication per treatment based on variation in susceptibility to *Ascaris suum* larvae

Mouse strain	Dose administered	Number of mice	Number of larvae per lungs, day 7	CV	r
C57BL	1000	5	314 ± 105	33	7
C57BL/6	1000	5	164 ± 21.6	13	1
CBA/Ca	1000	5	38.4 ± 8.5	22	3
C57BL/6	1000	5	125 ± 21.74	17	5
CBA/Ca	1000	5	28 ± 7.52	27	9

When we examine variation between C57BL, C57BL/6 and CBA/Ca mice we find a mean r = 5. This replication size has previously been stated as the minimal acceptable sample size (Lewis, 2006).

5.4.3 Parasite eggs

The eggs from *A. lumbricoides* were obtained from dewormed children in Ile-Ife, Nigeria. Deworming was performed using pyrantel pamoate (Albendazole damages egg development (Boes *et al.*, 1998a)). Worms were obtained from several hosts and pooled together. In total, 28 female worms were transported in 4% formalin.Female worms were transported on ice in 4% formalin. Upon arrival in Dublin, the worms were dissected by making an incision along the length of the worm. Subsequently, the uteri removed and mechanically broken up with scissors before being sieved (425 µm). The sieved eggs were placed in 0.05M H_2SO_4 (Aldrich, 32,050-1) in T175 culture flasks with ventilated cap at 26°C and they were oxygenated twice per week. The development of

the eggs was microscopically checked on a weekly basis. The percentage of embryonated eggs was calculated. After 8 weeks, approximately 70% of the eggs were embryonated and were ready for use in experimental infection.

Embryonated *A. suum* eggs were kindly donated by Dr. Johnny Vlaminck (Ghent University). These were shipped in a water solution, stored at 26°C in 0.05M H_2SO_4 and oxygenated twice per week.

5.4.4 Infection of mice with *Ascaris* eggs.

Fifteen male mice of both CBA/Ca OlaHsd (Envigo, Blackthorn, United Kingdom) and C57BL/6J OlaHsd (Comparative Medicine Unit, Trinity College Dublin, Dublin, Ireland) were purchased, all mice were eight weeks old at the time of the experiment. Five mice of each strain received oral gavage (Instech, FTP-20-38-50, Plymouth Meeting, PA USA) with either 1000 eggs of *A. suum*, 1000 eggs of *A. lumbricoides* or 100 µl 0.05 M of H_2SO_4. The mice were culled at day 7 post infection and the livers and spleens removed for flow cytometric analysis of immune cells. Additionally, the lungs were removed for larval counts using the modified Baermann method (Lewis *et al.*, 2006).

5.4.5 Larval recovery and enumeration for the lungs

After 24 hours, the resulting samples from the Baermann method were centrifuged at 1389 *g* for 5 minutes. The supernatants were removed and 70% ethanol was added (50% v/v). Subsequently, the larvae were counted in 1 ml of sample solution, using the nematode counting chamber (Chalex Corporation, Park City, UT, USA). All mice (five per group) were analysed on both Baermann method and flow cytometry.

5.4.5.1 Retrieval of spleen and liver immune cells

After dissection, livers and spleens were kept in ice cold PBS. Both organs were mechanically minced using sterile scalpels and sieved through a 70 µm-gauge mesh in complete RPMI medium (cRPMI) (RPMI with GlutaMAX™ supplemented with 1.25 mM HEPES and 10% FCS, pH = 7.4). This resulted in single suspensions of spleen cells.

The liver cell suspensions were suspended in 50 ml cRPMI and centrifuged for 1 min at 60 *g* without brake to remove undissociated tissue. The top 45 ml was removed and centrifuged again at 530 *g* for 10 min at 4°C with brake on. The resulting pellet was resuspended in 10 ml of digestion buffer (0.2 g/l collagenase from *Clostridium histolyticum* (Sigma-Aldrich) and 0.02 g/l DNase I (Sigma-Aldrich)) and incubated at

37°C for 30 min while shaking. Next, 30 ml of phosphate buffered saline (PBS) was added and left to rest on ice for 5 min before centrifugation, with brake on, at 528 *g* for 10 min at 4°C. The pellet was resuspended in PBS and layered over Lymphoprep™ (STEMCELL Technologies) and centrifuged at 400 *g* for 25 min without brake. The buffy coat layer, containing the mononuclear cells (MNCs), was removed and kept aside. The pellet, containing the polymorphonuclear cells (PMNCs) was incubated for 5 min at room temperature in red cell lysis buffer (0.1 mM EDTA, 155 mM NH_4Cl, 10 mM $KHCO_3$). Both MNCs and PMNCs were centrifuged, with brake on, for 8 min at 480 *g* and the pellet resuspended in PBS and counted.

5.4.5.2 Antibodies and flow cytometry

Approximately $0.5x10^6$ of liver and spleen cells were pelleted by centrifugation and stained with a dead cell stain (Flexible viability dye; eBioscience; diluted 1/1000 in PBS) for 15 min at room temperature in the dark. Cells were then washed in PBA buffer (PBS containing 1% bovine serum albumin and 0.02% sodium azide) and blocked with FcR blocking reagent (Miltenyi Biotec) and washed again. Next, the antibody panels (see Table 5.3 and Table 5.4) were added and incubated for 15 min at room temperature in the dark. The panel for staining lymphocytes (Table 5.3) consisted of the following antibodies: APC/Cy7 conjugated anti-mouse NK-1.1 (PK136), PerCP/Cy5.5 conjugated anti-mouse CD19 (1D3/CD19), APC conjugated anti-mouse CD69 (H1.2F3), FITC conjugated anti-mouse CD4 (GK1.5), PE/Cy7 conjugated anti-mouse CD8 (53-5.8), PE conjugated anti-mouse TCR γ/δ (UC7-13D5) and Pacific Blue ™ conjugated anti-mouse CD3ε (145-2C11). The panel for the myeloid cells (Table 5.4) consisted of the following antibodies: PerCP/Cyanine5.5 conjugated anti-mouse F4/80 (BM8), APC/Cyanine7 conjugated anti-mouse CD11c (N418), APC conjugated anti-mouse CD170 (Siglec-F) (S17007L), PE conjugated anti-mouse CD200R3 (Ba13), Pacific Blue™ conjugated anti-mouse CD45 (30-F11), FITC conjugated anti-mouse CD317 (BST2, PDCA-1) (927), PE/Cy7 conjugated anti-mouse/human CD11b (M1/70). All antibodies were purchased from BioLegend® (San Diego, CA, USA). After staining, the samples were washed, fixed with 1% paraformaldehyde, washed again and analysed on a Becton Dickinson FACSCanto II flow cytometer. Compensation beads were used to set correct voltages and gating parameters. Background fluorescence was measured by taking one of the samples and adding the antibody cocktail, without the dead cell stain, without the antibody for which dye the background needs to be

measured. This was repeated for every antibody. A negative control, an unstained sample, was measured for each of the 6 groups (i.e. each combination of control/*A. suum*/*A. lumbricoides* with C57BL/6J and CBA/Ca). Data were analysed using Flow Jo software (Tree Star). Gating strategies for the detection and enumeration of lymphoid and myeloid cells are shown in Figure 5.1.

Table 5.3: Antibody panel used for detection of lymphocytes for both liver and spleen

	NK1.1	CD19	CD3	CD4	CD8	CD69	γδ TCR
NK cells	+		-				
B cells	+		-				
$CD4^+$ T cells			+	+	-		
$CD8^+$ T cells			+	-	+		
γδ T cells			+				+
αβ T cells			+				-
Activated cells						+	
NKT cells	+		+				

Table 5.4: Antibody panel used for detection of liver myeloid cells

	CD45	F4/80	CD11b	CD11c	CD317	CD170	CD200R3
Kupffer cells	+	+	+/-			-	
Eosinophils	+	+	+			+	
Monocytes	+		+				
Myeloid dendritic cells	+			+	-		
Plasmacytoid dendritic cells	+			+	+		
Mast cells and basophils	+						+

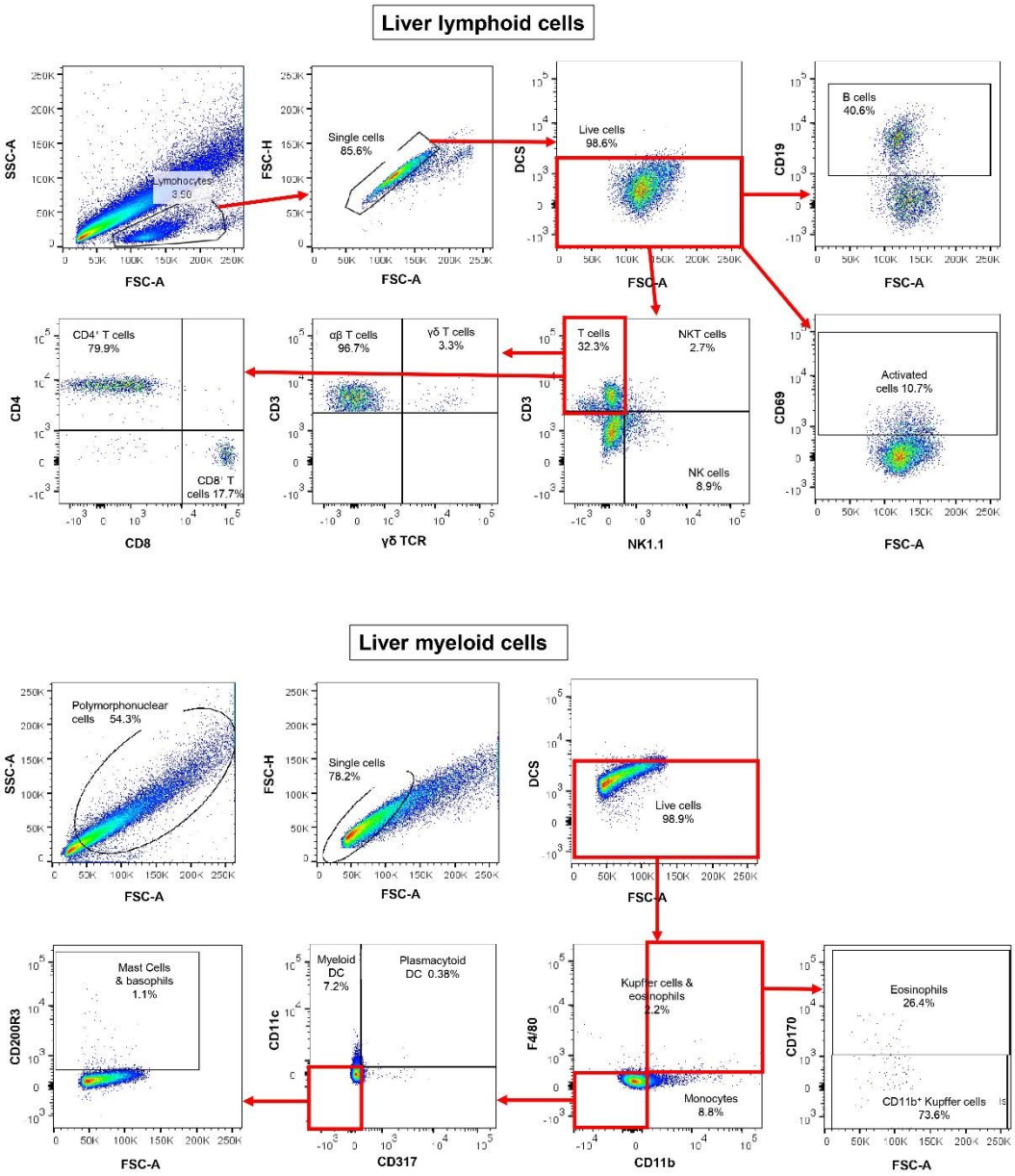

Figure 5.1: Gating strategies for the definition of lymphoid and myeloid cell populations. Following flow cytometric acquisition of MNCs, an electronic gate was placed on the lymphocytes based on forward and side scatter areas (SSC-A vs FSC-A) followed by gating of singlets (FSC-H vs FSC-A). Next, the live cells were gated upon (eFluor vs FSC-A). From these live cells, the activated cells were identified (CD69 vs FSC-A) and $CD3^+$ cells were gated (CD3 vs NK1-1) with $CD3^+$ and $NK1\text{-}1^+$ cells identified as natural killer T cells (NKT). B cells were gated from the $CD3^-$ and $NK1\text{-}1^-$ population (CD19 vs FSC-A). αβ T cells ($CD3^+$ and TCR γ/δ^-) and γδ T cells ($CD3^+$ and TCR γ/δ^+) were gated from $CD3^+$ and $NK1\text{-}1^-$ cells. Finally the αβ T cells were used to identify $CD4^+$ and $CD8^+$ T cells (CD4 vs CD8). For analysis of myeloid cells, debris was eliminated by gating based on (SSC-A vs FSC-A) followed by isolation of singlets (FSC-H vs FSC-A) and live cells (eFluor vs FSC-A). Next, the monocytes were identified (FM4/80 vs CD11b). Eosinophils and KCs (CD170 vs FSC-A) were gated from the $FM4/80^+$ and $CD11b^+$ population. DCs and plasmacytoid dendritic cell (pDCs) (CD11c vs CD137) were identified from $FM4/80^-$ and $CD11b^-$ cells. Finally, basophils and mast cells (CD200R3 vs FSC-A) were gated on $CD11c^-$ and $CD137^-$ cells. Gates for spleen and liver lymphoid and myeloid cells were manually adjusted for every sample.

5.4.6 Statistical analysis

5.4.6.1 Principal component analysis

PCA were run in R (version 3.5.1) (R Core Team, 2018). A PCA was performed to visualize the immune profile of the different samples. The PCA was performed on all variables from liver lymphocytes, liver myeloid cells and spleen lymphocytes. The data were scaled to unit variance. The analysis was performed using the FactoMineR (Lê *et al.*, 2008) package and visualization of PCA's and bar charts of variable contributions were done using the factoextra package (Kassambara and Mundt, 2019) with 95% confidence ellipses.

5.4.6.2 Statistical model

The flow cytometry data was found to be overdispersed, and therefore a negative binomial distribution was found to be more appropriate. The MASS package (Venables and Ripley, 2002) was used for the negative binomial (log link) on each cell type. The most parsimonious model, a negative binomial without interaction between mouse strain and infection status of the mouse, was considered the default model. This model was compared to an interaction model, with an interaction between the mouse strain and infection status of the mouse. If the difference in absolute values of the AIC of the models ($\Delta_i = AIC_i - AIC_{min}$) was greater than two, then the interaction model was used (Burnham and Anderson, 2004). Posthoc tests with a multivariate testing adjustment were performed using contrasts in the emmeans package (Lenth, 2019). Posthoc tests were performed between mouse strains for each species.

5.5 Results

5.5.1 Lung Larval counts

The mean number of larvae recovered from the C57BL/6J strain was higher for both *A. suum* and *A. lumbricoides* infection, compared to the respective CBA/Ca infected mice. The larval counts were as follows: C57BL/6J infected with *A. suum* 31±37.3 (mean±SD), C57BL/6J infected with *A. lumbricoides* 3±2.74, CBA/Ca infected with *A. suum* 7±8.37 and CBA/Ca infected with *A. lumbricoides* 1±2.24.

5.5.2 PCA

The PCA was generated using data from cell counts from liver myeloid and lymphoid cells and splenic lymphocytes. The PCA visualized the differences in cell populations between the mouse strains and between infected samples and uninfected control samples. The first PC and second PC together explained 53.1% of the variation (Figure 5.2). The 95% confidence ellipses were created around the group mean points per mouse strain. The 95% confidence ellipses did not overlap for mouse strain. The contribution of the variables for the first and second PC showed an above expected average contribution for liver and spleen lymphocytes (data not shown).

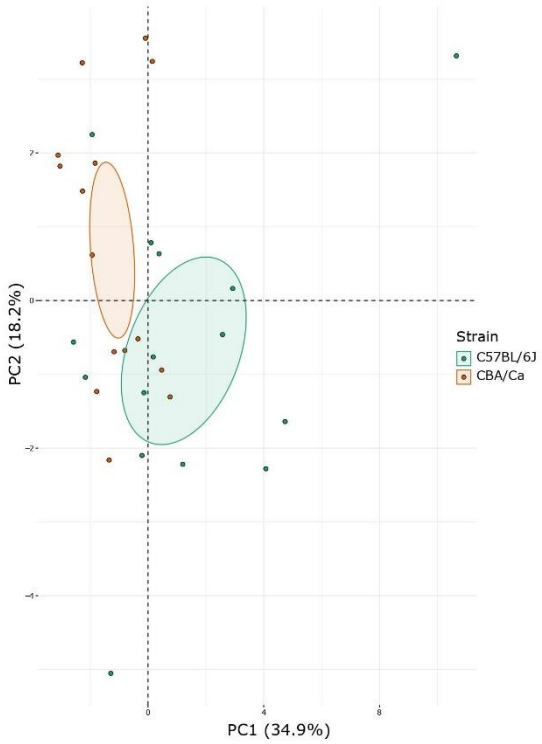

Figure 5.2: PCA of all samples. C57BL/6J mice are represented in green and CBA/Ca are represented in red. Each dot represents a single mouse. PC1 is on the x-axis and accounts for 34.9% of the variation. PC2 is on the y-axis and accounts for 18.2% of the variation. The 95% confidence ellipses are applied for both mouse strains.

In short, these PCAs indicate that the observed differences between the cell populations can be mainly attributed to differences between the two mouse strains. This may be attributed to differences in liver and spleen lymphoid cells. Differences in cell populations observed between the infection status of the mice were found to be mainly driven by liver myeloid cells.

In the following sections the differences between *A. suum* control samples and the differences between *A. lumbricoides* and control samples will be discussed. An initial comparison between *A. suum* and *A. lumbricoides* was examined. This involved a more complex model (not mentioned in the methods) and did not show any differences in the lymphoid cells between the two ascarid species. As for the myeloid cells, the comparison of the two ascarid species did not yield any statistically significant results. Only for the susceptible strain, C57BL/6J three cell types were found to be statistically significantly different: Kupffer cells, monocytes and dendritic cells. All were found to be higher for *A. suum* infected mice compared to *A. lumbricoides* infected mice. However, biological interpretation of these results was found to be confusing and in the interest of presenting the results in a coherent manner, a simpler model was chosen where this comparison between ascarid species was excluded. Table 5.5 summarises the main findings.

5.5.3 Effect of *A. suum* infection on spleen and liver cell numbers and phenotypes

5.5.3.1 Differences in spleen lymphoid cells

Infection with *A. suum* did not elicit a statistically significant change in the numbers of any of the investigated spleen cell populations, $CD8^+$ T cells, $CD4^+$ T cells, αβ T cells, B cells, NK cells, γδ T cells, NKT cells or activated T cells (Figure 5.3). However, an intrinsic difference between the two mouse strains was observed for $CD4^+$ T cells (z ratio: 2.505, $p<0.05$) (note: the ratios are not back transformed) and γδ T cells (z ratio: 5.644, $p<0.01$). For these cell populations, there were statistically significantly higher numbers in the CBA/Ca mouse strain, compared to the C57BL/6J mouse strain, both with and without infection.

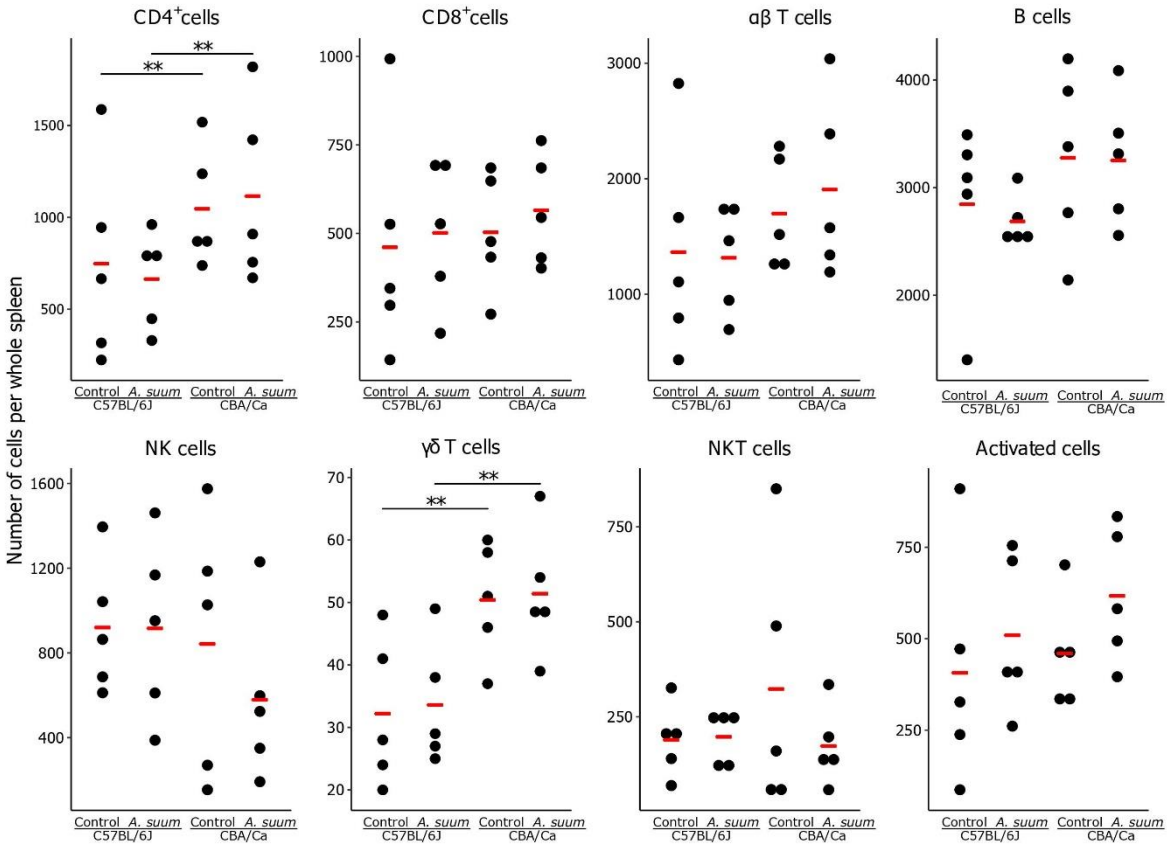

Figure 5.3: Lymphoid cell subtype number in the spleens of uninfected and *A. suum* infected C57BL/6J and CBA/Ca mice. The number of cells per whole spleen for the different cell types for each sample. The means are indicated with the red horizontal bar. *P<0.05; **P<0.01.

5.5.3.2 Differences in liver lymphoid cell numbers are mainly between mouse strains

For the liver lymphocytes, (Figure 5.4), the numbers of only one cell type, NK cells was found to be statistically significantly different (z ratio: -2.766, p<0.05) when comparing infected samples to their uninfected controls, and that occurred only for the CBA/Ca strain. Here the control samples were found to have more NK cells than the infected samples.

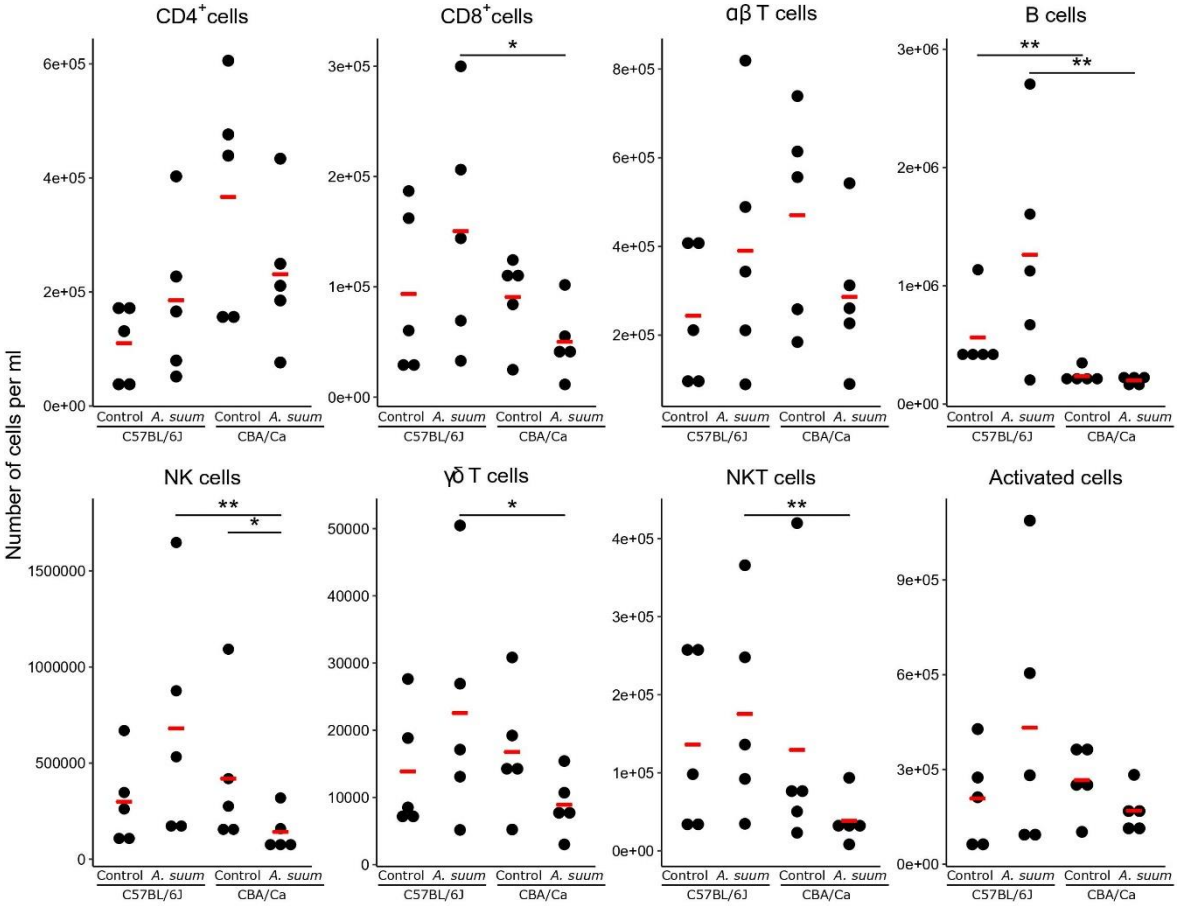

Figure 5.4: Lymphoid cell subtype numbers in the livers of uninfected and *A. suum* infected C57BL/6J and CBA/Ca mice. The numbers of cells per mL of liver extract for the different cell types for each sample are shown. The means are indicated with the red horizontal bar. *P<0.05; **P<0.01.

An intrinsic difference between the two mouse strains, was found for hepatic B cell numbers (z ratio: -7.054, p<0.01), with the C57BL/6J strain having a higher number of B cells compared to the CBA/Ca strain. Although the numbers of the other lymphoid cell populations tested were similar in both mouse strains, after infection with *A. suum*, the numbers of several cell types were found to be present in higher numbers in the livers of C57BL/6J mice compared with those of CBA/Ca mice. These are: $CD8^+$ T cells (z ratio: -2.783, p< 0.05), B cells (z ratio: -7.054, p<0.01), NK cells (z ratio: -4.003, p<0.01), γδ T cells (z ratio: -2.761, p<0.05) and NKT cells (z ratio: -3.293, p<0.01).

5.5.3.3 Differences between hepatic myeloid cells are mainly between control and infection

Analysis of liver myeloid cells, revealed higher numbers of eosinophils (z ratio: -5.070, p<0.01), and KCs (z ratio: -4.143, p<0.01) in uninfected C57BL/6J compared to uninfected CBA/Ca livers (Figure 5.5). For the eosinophils, this difference disappeared under *A. suum* infection. However, for the KCs this difference remained, with the

C57BL/6J mouse strain having a higher cell count (z ratio: -4.143, p<0.01) than the CBA/Ca strain.

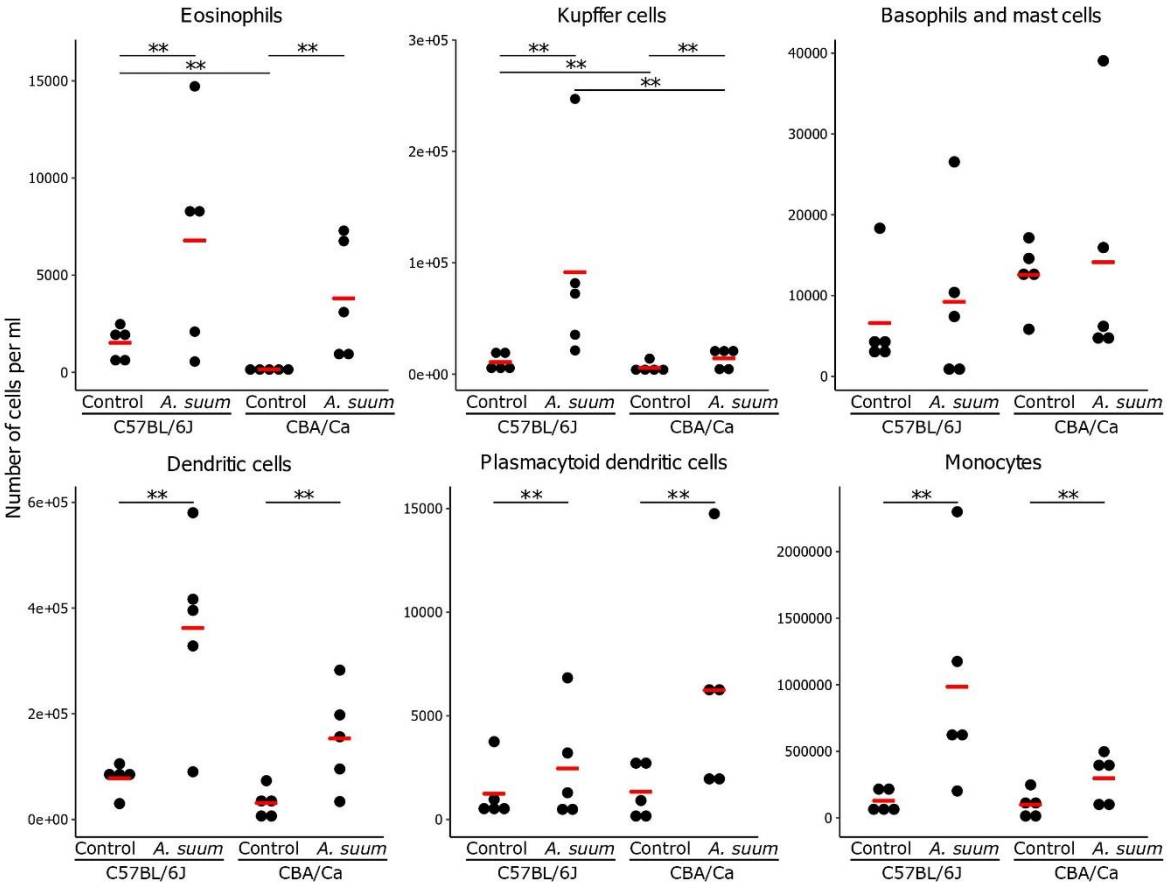

Figure 5.5: Myeloid cell subtypes in the livers of uninfected and *A. suum* infected C57BL/6J and CBA/Ca mice. The numbers of cells per mL of liver extract for the different cell types for each sample are shown. The means are indicated with the red horizontal bar. *P<0.05; **P<0.01.

In both mouse strains, the numbers of eosinophils (C57BL/6J: z ratio: 3.309, CBA/Ca: z ratio: 7.095, both: p<0.01), KC (C57BL/6J: z ratio: 4.648, CBA/Ca: z ratio: 4.648 , both: p<0.01), monocytes (C57BL/6J: z ratio: 4.962, CBA/Ca: z ratio: 4.962, both: p<0.01) and dendritic cells (C57BL/6J: z ratio: 4.401, CBA/Ca: z ratio: 4.553, both: p<0.01) were significantly higher in *A. suum*-infected livers compared to uninfected. In contrast, basophils and mast cells were found in similar numbers when comparing uninfected and infected mice.

5.5.4 Effect of *A. lumbricoides* infection on spleen and liver cell numbers and phenotypes

5.5.4.1 Differences between splenic lymphocytes

When the immune cell composition in spleens of C57BL/6J and CBA/Ca mice infected with *A. lumbricoides* were examined, the frequencies of $CD4^+$ T cells, $CD8^+$ T cells, αβ T cells, B cells, NK cells, γδ T cells, NKT cells and activated T cells were found to be similar to those in uninfected mice. The only statistically significant differences (Figure 5.6) found were higher numbers of $CD4^+$ cells (z ratio: 2.505 , $p<0.05$) and γδ T cells (z ratio: 5.644, $p<0.01$) in the CBA/Ca strain compared to the C57BL/6J strain. These higher numbers of $CD4^+$ and γδ T cells were found in both uninfected and infected C57BL/6J mice.

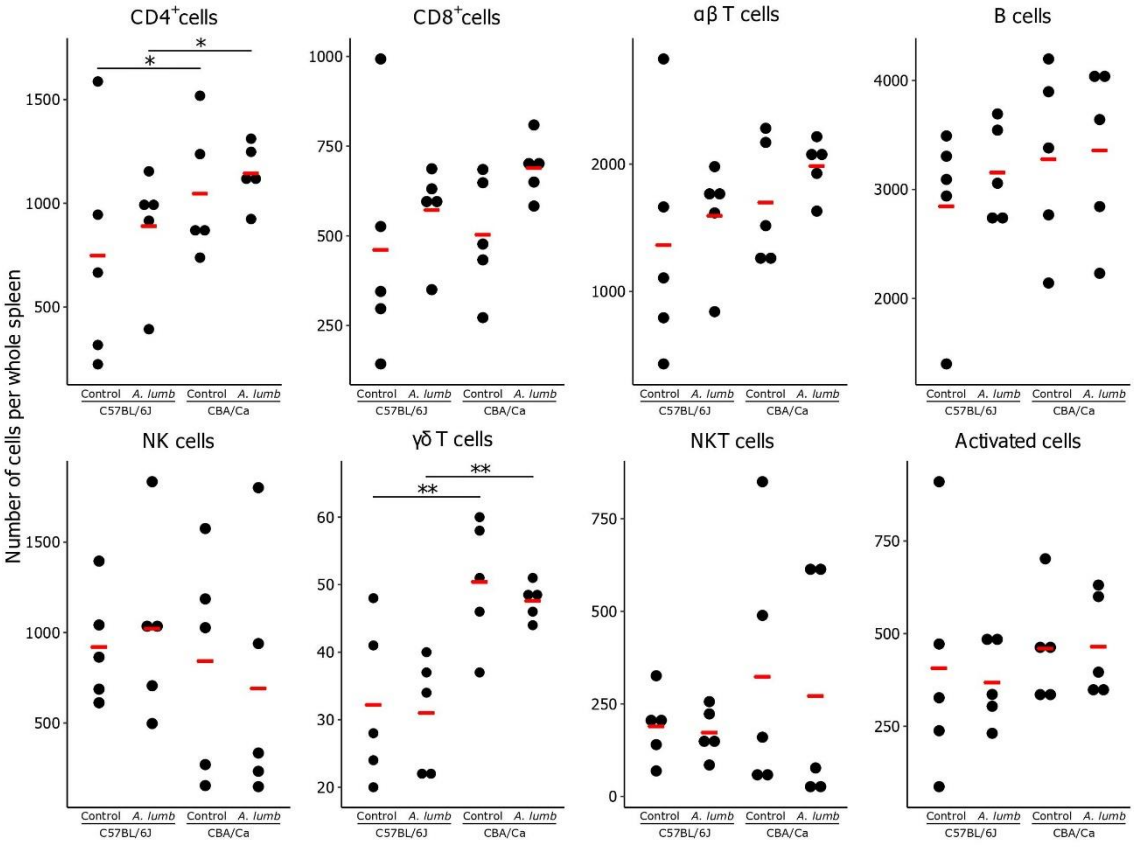

Figure 5.6: Lymphoid cell subtypes in the spleens of uninfected and *A. lumbricoides* infected C57BL/6J and CBA/Ca mice. The number of cells per whole spleen for the different cell types for each sample. The means are indicated with the red horizontal bar. *P<0.05; **P<0.01.

5.5.4.2 Differences between liver lymphocytes

Infection with *A. lumbricoides* did not lead to any statistically significant changes in numbers of hepatic lymphoid cells in C57BL/6J mice (Figure 5.7). For the CBA/Ca

strain, infection with *A. lumbricoides* led to significant decreases in the numbers of: $CD8^+$ T cells (z ratio: -2.939, $p<0.05$), αβ T cells (z ratio: -2.895, $p<0.05$), NK cells (z ratio: -3.049, $p<0.05$), and NKT cells (z ratio: -3.783, $p<0.01$). Compared to infected CBA/Ca mice, *A. lumbricoides*-infected C57BL/6J mice had higher numbers of $CD8^+$ T cells (z ratio: -3.893, $p<0.01$), B cells (z ratio: -7.054, $p<0.01$), NK cells (z ratio: -3.345, $p<0.01$), γδ T cells (z ratio: -2.918, $p<0.05$) and NKT cells (z ratio: -4.258, $p<0.01$).

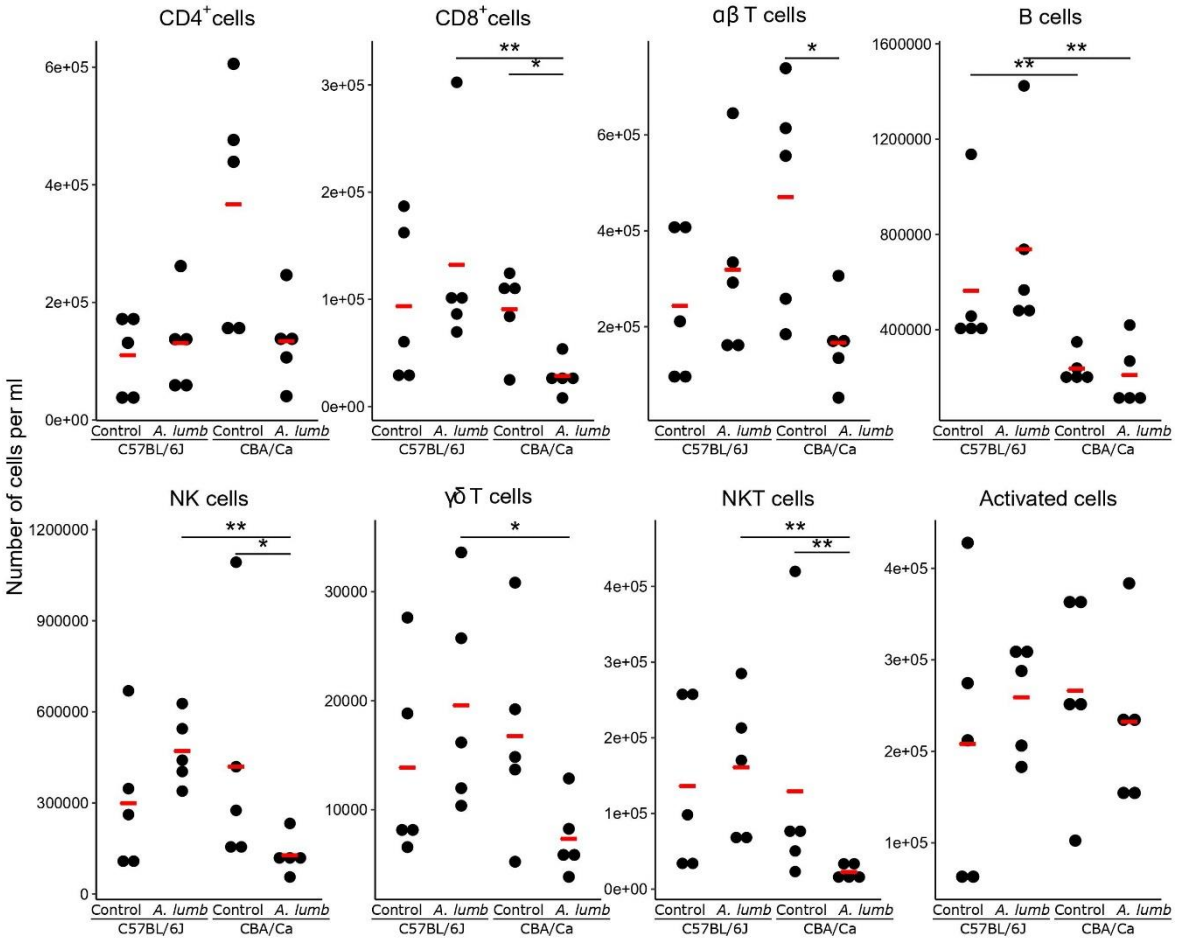

Figure 5.7: Lymphoid cell subtype numbers in the livers of uninfected and *A. lumbricoides* infected C57BL/6J and CBA/Ca mice. The numbers of cells per mL of liver extract for the different cell types for each sample are shown. The means are indicated with the red horizontal bar. *P<0.05; **P<0.01.

5.5.4.3 Differences between liver myeloid cells

Analysis of hepatic myeloid cell numbers in control and A. lumbricoides-infected mice revealed that eosinophils (z ratio: 5.978, $p<0.01$), DC (z ratio: 3.969, $p<0.01$) and monocytes (z ratio: 2.483, $p<0.05$) were expanded in infected CBA/Ca mice, whereas only monocytes (z ratio: 2.483, $p<0.05$) were expanded in infected C57BL/6J mice. KCs were the only cell type whose numbers differed significantly in the 2 mouse strains after infection with A. lumbricoides (z ratio: -4.143, $p<0.01$), with the C57BL/6J

infected samples having a higher number of cells compared to CBA/Ca infected samples (Figure 5.8).

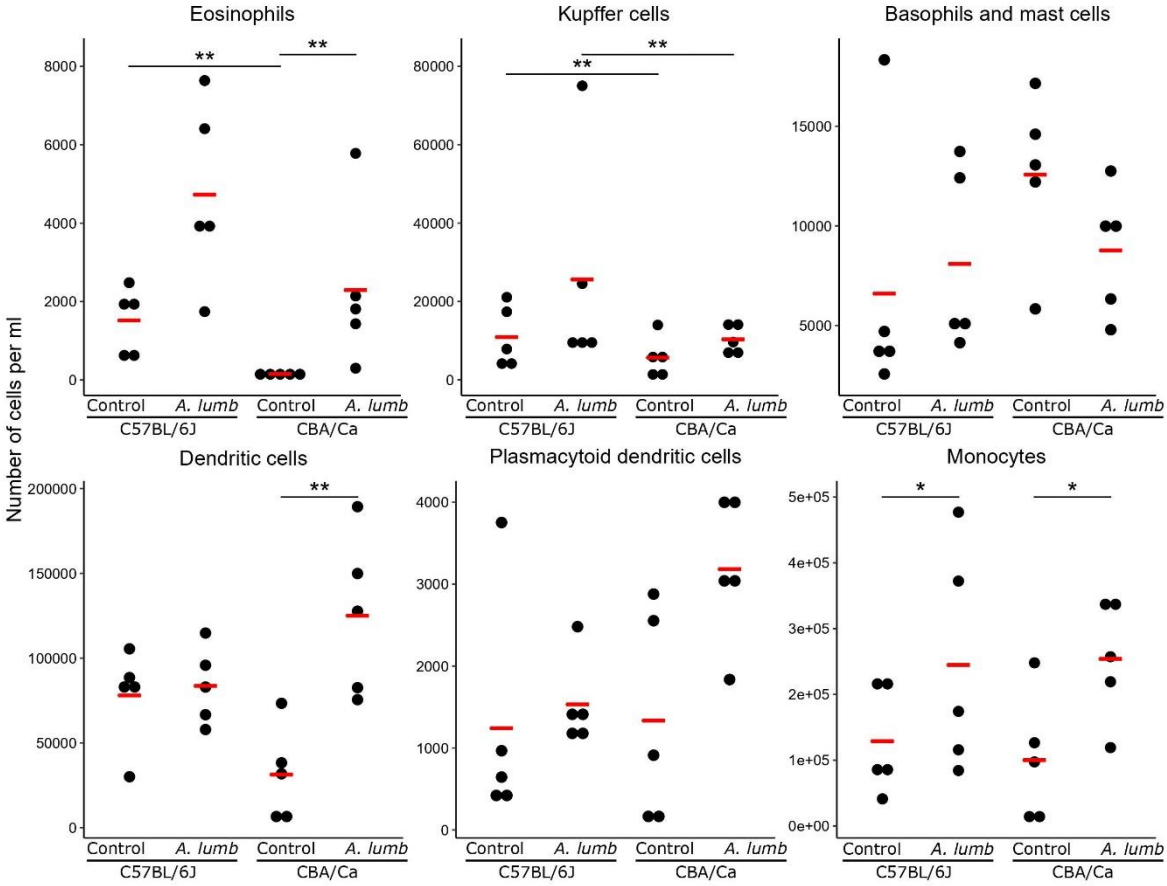

Figure 5.8: Myeloid cell subtype number in the livers of uninfected and *A. lumbricoides* infected C57BL/6J and CBA/Ca mice. The numbers of cells per mL of liver extract for the different cell types for each sample are shown. The means are indicated with the red horizontal bar. *P<0.05; **P<0.01.

Table 5.5: Summary table of key findings flow cytometry experiment. Each column is a comparison between two conditions. The text in the table indicates for which condition an increase for a specific cell type was seen. Cell types for which there were no statistically significant changes observed are not shown in the table. *P<0.05; **P<0.01.

		A. suum			*A. lumbricoides*		
	CBA/CA control compared to C57BL/6J control	CBA/CA infected compared to C57BL/6J infected	CBA/CA control compared to CBA/CA infected	C57BL/6J control compared to C57BL/6J infected	CBA/CA infected compared to C57BL/6J infected	CBA/CA control compared to CBA/CA infected	C57BL/6J control compared to C57BL/6J infected
Spleen lymphoid cells							
$CD4^+$ cells	CBA/CA**	CBA/CA**			CBA/CA*		
γδ T cells	CBA/CA**	CBA/CA **			CBA/CA**		
Liver lymphoid cells							
$CD4^+$ cells							
$CD8^+$ cells		C57BL/6J*			C57BL/6J**	Control*	
B cells	C57BL/6J**	C57BL/6J**			C57BL/6J**		
NK cells		C57BL/6J**	Control*		C57BL/6J**	Control*	
γδ T cells		C57BL/6J*					
NKT cells		C57BL/6J**			C57BL/6J*		
Liver myeloid cells							
Activated cells					C57BL/6J**	Control**	
Eosinophils	C57BL/6J**		Infected**	Infected **		Infected **	
Kupffer cells	C57BL/6J**	C57BL/6J**	Infected **	Infected **	C57BL/6J**		
Dendritic cells			Infected **	Infected **		Infected **	
Plasmacytoid dendritic cells			Infected **	Infected **			
Monocytes			Infected **	Infected **		Infected *	Infected *

5.6 Discussion

In this study we investigated the immune response in the liver during *Ascaris* infection. For this study, we used a model of hepatic resistance, where one mouse strain (C57BL/6J) is a model for relative susceptibility and another mouse strain (CBA/Ca) is a model for relative resistance to *Ascaris* infection (Lewis, 2006, Deslyper *et al.*, 2020).

The larval burdens in the lungs at day 7 p.i. indicate the difference in hepatic resistance between the two mouse strains. For *A. suum* in the relatively resistant mouse strain we observed 7 larvae, compared to 31 for the relatively susceptible strain. As for *A. lumbricoides* we observed 3 larvae for the relatively susceptible strain and 1 larva for the relatively resistant strain. This corresponds with our previous findings where we extensively investigated the suitability of *A. lumbricoides* in this mouse model (Deslyper *et al.*, 2020). In this previous study we found that the larval counts, for both ascarid species, were lower in the resistant strain.

The main differences observed between the immune cell populations can be attributed to the different mouse strains. These differences are both intrinsic, i.e. when comparing splenic or hepatic leukocyte subsets in uninfected mice, but they also occur under infection. A study which investigated the liver proteome between the two mouse strains under *A. suum* infection found similar results, with a clear difference between the two mouse strains, both with and without infection (Deslyper *et al.*, 2016, Deslyper *et al.*, 2019b).

In short, our data suggest that the drive behind the differences in susceptibility to *Ascaris* infection between the two mouse strains is mainly attributed to differences in the distributions of liver and spleen lymphoid cells.

Our data indicate that infection with *Ascaris* is mainly associated expansions or influxes of populations of myeloid cells in the livers. As for the eosinophils, there is an intrinsic difference between the two mouse strains, with the relatively susceptible strain having more eosinophils than the relatively resistant strain. However, under infection, it is the relatively resistant strain that showed a greater expansion of eosinophils than the relatively susceptible strain. This is true for both *A. suum* and *A. lumbricoides* infection. Given the importance of eosinophils in parasite immunity in the lung and the gut, this is an expected finding (Nogueira *et al.*, 2016, Enobe *et al.*, 2006, Weatherhead *et al.*,

2018, Gazzinelli-Guimaraes *et al.*, 2019, Masure *et al.*, 2013a, Vlaminck *et al.*, 2016). Eosinophils have also been found to be increased in murine lungs during *A. suum* infection (Nogueira *et al.*, 2016, Enobe *et al.*, 2006, Weatherhead *et al.*, 2018, Gazzinelli-Guimaraes *et al.*, 2019) and have been shown to be involved in a reduction of larval numbers and size in a HDM presensitized mouse model (Gazzinelli-Guimaraes *et al.*, 2019). Additionally, eosinophils have been shown to be activated during *A. suum* infection in the lungs at day 8 p.i., although the authors did not observe an increase in eosinophil numbers at that time point (Gazzinelli-Guimaraes *et al.*, 2019). In pigs the presence of eosinophils has been linked to elimination of *Ascaris* in the gut (Masure *et al.*, 2013a, Vlaminck *et al.*, 2016). Furthermore, eosinophils have also been found to play a role in human ascarid infections, where putatively immune Nigerian children were found to have an upregulation in this cell type, together with C-reactive protein and ferritin (McSharry *et al.*, 1999). Ecuadorian children that were found to be chronically infected with *A. lumbricoides*, i.e. had *A. lumbricoides* eggs in their stool at all investigated time points, were found to demonstrate an upregulation of eosinophil cationic protein (Reina Ortiz *et al.*, 2011). Eosinophils have been found to paralyze *Schistosoma mansoni* (McLaren *et al.*, 1984) and kill both *Trypanosoma cruzi* and *Brugia malayi in vitro* (Hamann *et al.*, 1990). A recent *in vivo* experiment found that the motility of L3 *A. suum* larvae was inhibited when cultured with human eosinophils in the presence of pig immune serum (Coakley *et al.*, 2020). The results of the present study further implicate hepatic eosinophils in the immune response to *A. suum* and *A. lumbricoides* in both mouse strains.

As for liver monocytes, both *A. suum* and *A. lumbricoides* infection have similar patterns, where there were significantly more of this cell type under infection for both *Ascaris* strains, compared to controls. However, a different pattern was observed for Kupffer cells. For *A. lumbricoides* infection, there was a significant difference only between the two strains, both with and without infection. The relatively susceptible strain had higher numbers of this cell type. Under *A. suum* infection, however, there was a significant increase in Kupffer cell numbers for both mouse strains. However, the relatively susceptible strain still had more of this cell type than the relatively resistant strain. Alternatively activated macrophages have been observed in lung samples for mice infected with *A. suum*, however, this was at day 18 p.i., so at a much later time point than our study (Gazzinelli-Guimaraes *et al.*, 2019). Additionally, ABF from *A.*

suum has been found to change the polarizations of human macrophages *in vitro* (Almeida *et al.*, 2018). Human macrophages which were cultured *in vivo* with *A. suum* larvae and porcine immune serum overnight, were found to recognize and adhere to the larvae, impairing larval motility (Coakley *et al.*, 2020).

DCs are another cell type of particular interest as these cells are required for the induction of Th2 responses. Studies have found that *A. suum* has immunomodulatory effects on human DCs *in vitro* (Favoretto *et al.*, 2014, Summan *et al.*, 2018, Midttun *et al.*, 2018, Arora *et al.*, 2020). No difference was observed between the two strains, however their numbers were significantly higher in *A. suum* infected livers for both the relatively susceptible and relatively resistant strain. For *A. lumbricoides* infection, the same was observed, an increase in cell numbers under infection, however, this is only statistically significant for the relatively resistant strain. Human monocyte-derived DCs stimulated *in vitro* with LPS and ABF from *A. suum* upregulated NRROS, a negative regulator of ROS (Midttun *et al.*, 2018). The authors hypothesized 'that *A. suum* inhibits inflammatory responses by modulating ROS production' (Midttun *et al.*, 2018). We previously identified a difference in abundance of proteins involved with OXPHOS between the two mouse strains, with the relatively resistant strain having a higher abundance of these proteins than the relatively susceptible strain (Deslyper *et al.*, 2016, Deslyper *et al.*, 2019b). This led us to speculate that the relatively resistant strain had more ROS, a product of OXPHOS, intrinsically. Our results together with the findings of the immunomodulatory effects of *A. suum* on human DCs *in vitro* could therefore indicate that the an intrinsic higher amount of ROS in the relatively resistant strain makes it harder for the parasite to reduce this ROS and therefore give this strain an advantage in eliminating the parasite.

Interestingly, the pDCs, immune cells usually activated during viral infections, see similar changes as the myeloid DCs, but only statistical significance is observed under *A. suum* infection.

For both comparisons (i.e. *A. suum* vs control and *A. lumbricoides* versus control) in the spleen we found that the relatively resistant mouse strain had more $CD4^+$ and γδ T cells than the relatively susceptible strain before infection and this difference did not change with infection. While both $CD4^+$ and γδ T cells can release Th2 cytokines, future studies are required to determine if the increased numbers of these cells in CBA/Ca mice compared to C57BL/6 mice reflect expansions of Th2 cells. A previous study, however,

observed a reduction in $CD4^+IL\text{-}4^+$ T cells in the spleen of *A. suum* infected BALB/c mice (Gazzinelli-Guimarães *et al.*, 2013), suggesting that the parasite may inhibit Th2 cell differentiation.

For the liver lymphocytes there is a general pattern whereby there is an increase for every investigated cell population under infection by either *Ascaris* species for the relatively susceptible strain. The opposite is true for the relatively resistant strain, where there is a decrease for these cell types under infection. This observation suggests, surprisingly, a more pronounced immune response to *A. lumbricoides* by these lymphoid cells in the susceptible C57BL/6 mice.

In the liver, the numbers of γδ T cells differed between the two mouse strains, but under infection only. For both *Ascaris* species, there was a non-significant increase in this cell population in infected livers from the relatively susceptible strain, but a decrease in infected livers from the relatively resistant strain. Little is known about the role of γδ T cells in *Ascaris* infection. However, they have been implicated in other parasitic infections. Whole blood samples from patients with cutaneous leishmaniasis were found to have more γδ T cells than treated and control patients (Darabi *et al.*, 2002). Peripheral blood mononuclear cells from pregnant women with a primary *Toxoplasma gondii* infection had increased levels of γδ T cells compared to uninfected control subjects (Prigione *et al.*, 2006). Increased numbers of γδ T cells have also been found in the circulation of patients with *Schistosoma* infections (Schwartz *et al.*, 2014) and in the mesenteric lymph nodes of *Schistosoma*-infected mice (Yu *et al.*, 2014). The typical granuloma formation in *Schistosoma* infection, however, was not found to be dependent on γδ T cells in one study (Iacomini *et al.*, 1995), however, another study found that these cells were recruited to granulomas (Sandor *et al.*, 1995). Another study found that the Vγ2 subset of γδ T cells prevented hepatic fibrosis, recruitment of neutrophils and IL-17 secretion (Zheng *et al.*, 2017). During *Plasmodium* infection, an increase in αβ cells was observed during the acute parasitemia, followed by an increase in γδ T cells after the acute phase abated (Mamedov *et al.*, 2018). Additionally, γδ T cells were found to play an important role during *Plasmodium* vaccination studies in mice, where the absence of these cells impaired protection (Zaidi *et al.*, 2017). However, no evidence has been found for the role of these cells during the liver stage of malaria infection (Deroost and Langhorne, 2018).

In the present study, we observed a similar pattern for the $CD8^+$ T cells and NKT cells in the liver as for the γδ T cells, where there was an increase for both *A. suum* and *A. lumbricoides* infection, for the relatively susceptible strain, but a decrease for the relatively resistant strain. Invariant NKT (iNKT) cells (Yang *et al.*, 2016) were found to be activated *in vivo* in a mouse model and were able to produce IFN-γ and IL-4 during egg deposition in the liver during *Schistosoma mansoni* infection (Mallevaey *et al.*, 2006). The authors suggest this cytokine production was DC dependent (Mallevaey *et al.*, 2006). Interestingly, iNKT cells promote Th1 differentiation during *S. mansoni* infection in mice, and non-invariant NKT promote Th2 differentiation (Mallevaey *et al.*, 2007). These effects were all at 2-7 weeks p.i., so at a much later time point than in our study. However, infection of C57BL/6 mice with *Brugia pahangi* resulted in an increase in NKT cells within 24 hours of infection, in the spleen and lymph nodes (Balmer and Devaney, 2002).

The NK cells again followed a similar pattern, with an additional significance for the relatively resistant strain infected with *A. suum* compared to its own control. Overall, our results show that infection with either *Ascaris* species led to increased numbers of $CD8^+$ T cells, γδ T cells, NKT cells and NK cells in the livers of susceptible C57BL/6 mice, but decreased numbers of these cells in the relatively resistant CBA/Ca mice. A common feature of these cell types is their ability to kill infected and transformed host cells, a function unlikely to contribute to immunity against extracellular parasites, such as *Ascaris*. If these cells contribute to immunity against *Ascaris*, they most likely do so through cytokine production. Not all changes in cytokine production, due to infection, are associated with cell proliferation. Quantifying cytokine production could therefore be necessary to determine the activity of these cells and their potential influence on *Ascaris* infection. These studies could also be used to determine if any of these cell types exhibit skewed Th2 phenotypes in response to *Ascaris* infection, which has previously been assumed to be the case (Cooper and Figuieredo, 2013).

Taken together, our data demonstrate that the main difference in cell populations are between the two mouse strains. Intrinsically these mouse strains have different numbers of lymphoid cells present in the liver and the spleen. However, under infection, the main changes observed are liver myeloid cells, with significant increases in the numbers of monocytes, dendritic cells and eosinophils in the livers in response to *Ascaris* challenge. These cells are all likely to contribute to parasite elimination,

whereby monocytes and dendritic cells promote Th2 cell activation through antigen presentation. The cells, in turn, promote the production of IgE, which targets *Ascaris* for destruction by eosinophils. Clearly, more research is needed to thoroughly investigate the influence of these intrinsic immunological difference on *Ascaris* infection.

5.7 Acknowledgments

We would also like to thank Dr Johnny Vlaminck (Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium) for kindly donating the *A. suum* eggs. We would also like to thank the following people for their support during the larval recovery stage, from Trinity College Dublin: Alison Boyce, Sinead Kelly, Dr. Peter Stuart, Ciarán Ó Cuív, and Sheila Gargan. We would like to thank the following people for their support during the flow cytometry experiment, from Trinity College Dublin: Eamon Breen, and Gráinne Jameson.

Chapter 6 - Discussion & Conclusion

6.1 Summary main findings

This thesis investigated the role of the liver during *Ascaris* infection using a mouse model. Some people infected with *A. lumbricoides* experience a high worm burden, whereas others experience a light worm burden. Heavy infection is associated with increased morbidity, and therefore understanding the molecular mechanisms associated with this differential worm burden is an important step in lowering *Ascaris*-associated morbidity.

An investigation of the molecular determinants behind the role of the liver during *A. suum* infection was first approached using shotgun proteomics in Chapter 3. This analysis confirmed earlier findings regarding OXPHOS. A previous study performed as part of my taught MSc thesis demonstrated that there was an intrinsic difference between the two mouse strains with regards to OXPHOS (Deslyper *et al.*, 2016). More specifically, that the relatively resistant strain both with and without infection, had a higher abundance of these proteins. These results were confirmed in Chapter 3, with the relatively resistant strain again having a higher abundance of these proteins, with and without infection, than the relatively susceptible strain. During OXPHOS ROS are produced, which could therefore indicate that the relatively resistant strain has higher ROS levels, and could be a potential benefit in eliminating invading larvae (Deslyper *et al.*, 2019b).

The analysis also revealed the presence of immune proteins at day 7 p.i. during *Ascaris* infection for two inbred mouse strains. However, for these proteins a difference between the two mouse strains was observed. The relatively resistant strain showed a higher abundance in proteins involved in the activation of the complement cascade, more specifically the lectin pathway. For the relatively susceptible strain, the immune-associated proteins were found to be involved in suppression of the complement cascade activation. The complement cascade is an important part of the innate immune system, it is activated by the sensing of danger signals and can lead to opsonisation, phagocytosis or the direct killing of invading pathogens via the MAC (Merle *et al.*, 2015a, Merle *et al.*, 2015b). Additionally, complement has been shown to play an important role linking the innate and adaptive immune system (Merle *et al.*, 2015b). Due to these important roles of the complement system, several intracellular parasites, such as *Plasmodium*, *Leishmania* and *Trypanosoma*, have been shown to have

developed complement evasion strategies (Puentes *et al.*, 1990, Norris *et al.*, 1991, Kennedy *et al.*, 2016, Sikorski *et al.*, 2019). Macroparasites, such as *Schistosoma*, have been found to be able to alter complement activation (Skelly, 2004). Therefore in the present work, the relatively resistant strain therefore had an increased abundance of proteins involved in complement activation, whereas the relatively susceptible strain had an increased abundance of proteins involved in immune suppression.

The presence of these different proteins involved in the immune response could indicate a different immune response to the invading larvae in the liver between the two mouse strains. The immune response in the lungs has been investigated in several studies (Gazzinelli-Guimarães *et al.*, 2013, Gazzinelli-Guimaraes *et al.*, 2019), however, little is known about the immune response in the liver. One first step would be to investigate which immune cells are increased under *Ascaris* infection in the liver.

Building on this proteomics work, the next chapter in this body of work investigated the immune response during *Ascaris* infection. However, another question had to be addressed first and that was whether *A. suum* is an appropriate as a model organism for human ascariasis caused by *A. lumbricoides*. Previous work (Lewis *et al.*, 2006, Lewis *et al.*, 2009, Dold and Holland, 2011b, Dold *et al.*, 2011, Deslyper *et al.*, 2016, Deslyper *et al.*, 2019b), including that outlined in Chapter 3, used the porcine ascarid *A. suum* in murine infections. The availability of *A. suum* from pigs in abattoirs makes it an ideal study organism, however, the literature was lacking an in-depth study comparing *A. lumbricoides* and *A. suum* in a mouse model. Therefore the larval migration of both ascarids was simultaneously explored in the susceptible and resistant mouse strains in order to validate the mouse model for the human ascarid.

This comparative study of larval migration (Chapter 4) revealed that the mouse model previously used for *A. suum* was also suitable for *A. lumbricoides*. The model of resistance and susceptibility held true for *A. lumbricoides* with the relatively resistant mouse strain demonstrating a lower larval burden of the human ascarid than the relatively susceptible mouse strain in both the liver and lungs. However, interesting differences between the two ascarid species were also observed. There was a higher number of *A. lumbricoides* larvae in the liver for both mouse strains, but the inverse was true for the lungs, where more *A. suum* larvae were observed. Additionally, *A. suum* larvae were found to be statistically significantly larger at day 8 p.i. in the lungs. These

results were confirmed for the liver, where on day 5 and day 6 p.i. the larval length of *A. lumbricoides* was found to be significantly smaller than that of *A. suum* (Gargan, 2020). Due to the higher larval burden of *A. lumbricoides* compared to *A. suum* in the liver, but the opposite occurring in the lungs in addition to the *A. lumbricoides* larvae being smaller in the lungs, it could be speculated that these results indicate that *A. lumbricoides* has a higher antigenicity, with the human species generating a more pronounced immune response, resulting in reduced larval numbers in the lungs. But these observations could also point towards a more pronounced immune response against *A. lumbricoides*, due to the fact that the larval lengths of *A. lumbricoides* are significantly smaller than those of *A. suum*. These conclusions are drawn tentatively, and more studies would need to be performed to elucidate the mechanism behind the observed differences. However, the main finding of this part of the study was that the mouse model is suitable for an exploration of resistance and susceptibility for *A. lumbricoides* as it was for *A. suum*.

In Chapter 5, the mouse model was used for infection with both ascarid species resulting in control mice of each strain and mice of each strain infected with either *A. suum* or *A. lumbricoides*. These groups were used to investigate the host's immune response during the liver stage of infection. This was approached using flow cytometry and investigated a range of different cell types from both the innate and adaptive immune response at day 7 p.i. Very little is known about the early stages of the immune response during *Ascaris* infection and this paucity of information is particularly pronounced with respect to the hepatic immune response. Flow cytometry is a useful technique to obtain a broad knowledge of what is happening in the liver during early *Ascaris* infection. A wide range of antibodies allows the differentiation of cells from both the adaptive and innate immune system. The main differences in the cell types observed were between the mouse strains, rather than between the two *Ascaris* species. Additionally, the main cell types that were activated under infection were of the innate immune response - the eosinophils, monocytes and dendritic cells. No substantial differences were found when comparing the immune cells between the two species. These results therefore did not indicate that there was a more pronounced immune response against *A. lumbricoides* as was originally hypothesised after the results of the comparative migratory path experiment. However, this does not entirely exclude the possibility that the immune response differs between the two ascarid

species. These findings, however, do not exclude the possibility of a different immune response mounted against the different species, however, a more in-depth investigation would be necessary. The measurement of cytokine levels could indicate if there is a difference in immune cell activation, rather than immune cell proliferation. Additionally, a difference in immune response may only occur during the adaptive immune response. Investigating a later time point would be able to answer that question.

Further research will need to elucidate whether the difference in larval length between the two ascarid species, observed in the lungs during the migratory path experiment in Chapter 4, continues after day 8 p.i. This will be important to look further into the host immune response during *Ascaris* infection with these two species. If the difference in larval length continues after day 8 p.i., then this would contribute to our theory that *A. lumbricoides* has higher antigenicity and evokes a more pronounced immune response. This will need to be combined with a more in-depth flow cytometry study. Our results indicated that the difference in immune response could be mainly attributed to mouse strain rather than ascarid species. This in itself is an interesting find and would need to be further explored, how much do these differences in immune response contribute to the observed difference in larval burden? But it will also be important to further investigate whether there are any differences in immune response at a later time point. What happens past 7 days p.i.? Another aspect would be the investigation of the eosinophils in more detail. This cell type has been implicated as important during *Ascaris* infection in pigs and mice both during the gut stages and the lung stage (Masure *et al.*, 2013a, Gazzinelli-Guimaraes *et al.*, 2019). A thorough investigation of the activation status of these cells would be of interest in the liver, in addition to their any potential cytokine secretion. In general, an investigation of cytokine secretion, not just for eosinophils, in the liver during *Ascaris* infection would be very interesting. This has been performed in the lungs of the mice (Gazzinelli-Guimarães *et al.*, 2013), however, nothing is known about cytokines in the liver.

Another aspect worthy of investigation is looking at the differences in *Ascaris* sources. Here, *Ascaris* eggs were obtained from two countries only, but previous studies have found evidence of genetic differences worldwide. Therefore repeating these experiments with *Ascaris* eggs from different countries would be of interest.

In summary, the proteomics work identified a key difference in OXPHOS associated proteins between the two mouse strains intrinsically and under infection. Additionally, this method elucidated a difference between the two strains in response to an *A. suum* infection, with the relatively resistant strain having a higher abundance in proteins associated with complement – lectin pathway – activation, whereas the relatively susceptible strain has a higher abundance of proteins associated with complement inhibition. The flow cytometry was then able to reveal some of the immune cells that are activated during *Ascaris* infection, with the increase of eosinophils, monocytes and dendritic cells during infection being the most important findings. Additionally, this flow cytometry work was performed on both *Ascaris* species thanks to the work of Chapter 4, where it was found that *A. lumbricoides* is also suitable for use in the mouse model.

This body of work can be considered exploratory in nature, with the use of shotgun mass spectrometry and a broad antibody panel for the flow cytometry. It did not target specific pathways or immune cells and therefore forms the basis for more in depth investigations. Such avenues could include the activation status of the immune cells. The presence of immune cells, and increased cell populations, does not say anything about whether or not these cells are active. Additional questions are: Are the eosinophils releasing their granules? What is the exact antigen in *Ascaris* that causes the lectin pathway activation in the relatively resistant strain? And why is that antigen not starting the complement cascade in the relatively susceptible strain? What are the cytokines that are released in the liver during early infection? Additionally, a further exploration of potential differences in the immune response between *A. suum* and *A. lumbricoides* would be interesting. In the work in Chapter 5 no differences in the immune response against the ascarid species were observed, however, this study looked at a limited number of immune cell populations, and a more in depth analysis is still required. The levels of cytokine production and a further look at a later time point into the adaptive immune response may still reveal differences between the two ascarid species. If so, these differences could be significant in developing treatments and ultimately a vaccine, where it will be crucial to understand which epitopes elicit the best responses. These epitopes could be different for the different *Ascaris* species. A more in depth analysis of these differences, therefore, will prove important during vaccine development.

6.2 Looking ahead

6.2.1 The utility of mouse models

The use of inbred mouse strains in biomedical research goes back to the early 20th century (Masopust *et al.*, 2017). Ever since their first use, inbred mouse models have proved to be useful in the study of human diseases. Mouse models of disease are often induced artificially by, for example, administering certain reagents (e.g. colitis in a mouse model is induced via dodecyl sodium sulfate (Sellers, 2017)), this method therefore results in similar phenotypes, but does not reflect the underlying conditions when humans acquire this disease. However in our mouse model, the ascarid eggs are ingested orally (via gastric intubation), as would happen in human infection.

In fact, mouse models are especially useful to study certain infectious diseases like *Ascaris*, where the infectious agent needs to migrate through different tissues (Holland *et al.*, 2013b). The early migration of *Ascaris* is impossible to study in humans, it would require an experimental infection combined with sampling of internal organs – liver and lungs – all of which are ethically unacceptable. Mouse models are, therefore, an excellent alternative. Mice, however, are abnormal hosts for *Ascaris* infection. The life cycle of the parasite is therefore arrested at the lung stage (Holland *et al.*, 2013b). The mouse model is therefore only useful to study the early stages of infection. At the same time, the mouse model is necessary to study these early stages of infection, which would be impossible to study in humans.

Recently a new system has been developed, organoids. These organoids are *in vitro* multicellular clusters which could potentially be used to study host-parasite interactions (Duque-Correa *et al.*, 2020). Exosome-like vesicles derived from *A. suum* have been shown to be phagocytosed by canine enteroids, intestinal organoids from the small intestine (Chandra *et al.*, 2019). So far, however, these models have not been used with live helminths (Duque-Correa *et al.*, 2020). These organoids have the advantage over tissue culture, in that they include different cell types, whereas tissue cultures consist of only one cell type. An important limitation of organoids is that the parasites could be restricted in their moulting process (Duque-Correa *et al.*, 2020). However, organoids could be developed from small tissue biopsies, therefore allowing the possibility of studying *Ascaris* in human (liver) organoids. This could open the doors to making organoid from extra tissue taken during human biopsies and

investigating the effect of *Ascaris* infection in the human liver directly. Even more, if possible to get biopsy tissue from people identified as being predisposed to *Ascaris* infection, this could potentially be compared to control tissue or ideally to tissue from people identified with low larval burden.

Organoids are currently not widely used due to their cost and the difficulty associated with their production and maintenance. This area of research is still in its infancy, however its use could become more widespread in the future and help in the first of the '3Rs' (replacement, reduction, and refinement) of animal research.

Additionally, the immunological findings in murine models have to be interpreted appropriately. Although similar, the mouse immune system is quite different from the human immune system. There are differences in, for example, circulating leukocyte populations, antibody production and receptor expression (Haley, 2003, Mestas and Hughes, 2004). Furthermore, in some cases for therapeutics developed in mice, the jump from mouse to human was unsuccessful or in some cases even fatal (Jameson and Masopust, 2018). It is therefore important to keep the limitations of mouse models in mind when performing this type of research and to not automatically translate the obtained findings to humans.

Furthermore, the usefulness of mouse models becomes even more apparent in vaccine research. Mouse models play an important role during the development of vaccines, including vaccine development against *Ascaris* (Hewitson and Maizels, 2014).

There are numerous inbred mouse strains available, each with its own unique genetic background, leading to morphological and immunological differences between the strains (Haley, 2003). For example, one such morphological difference is in the alveolar size between C3H/HeJ, C57BL/6J, and A/J strains (Soutiere *et al.*, 2004). Therefore, the selection of a mouse strain to investigate lung diseases should therefore take such intrinsic differences into consideration. Immunological differences have also been observed between strains, with the most clear example the differences in the MHC genotypes between mouse strain (Vaz and Levine, 1970). Other important differences have been found between BALB/c and C57BL/10J mouse strains in terms of the different immune cells being observed (Vogelsang *et al.*, 2009). Each strain is likely to respond differently to infections, a property which can be exploited (Haley, 2003), such as in the mouse model of relative resistance and susceptibility to *Ascaris* infection.

Here, both CBA and C57BL/6 are thought to be Th1 biased strains (De Vooght *et al.*, 2010). This is an interesting observation as helminth infections are associated with a Th2 response. However, these findings do tie in with the results from Chapter 5, where no significant differences between the two strains for the adaptive immune system was observed, and in fact, our results suggests that the adaptive immune system is not involved in the mechanism behind the difference in larval burden. However, only a select number of cells were investigated and no cytokine levels or cell activity were measured. Measuring these cytokines would give a better insight into if and how the immune cells are activated and how they respond to *Ascaris* infection. Cytokine levels have previously been measured in *Ascaris* studies in the lungs of mice and indicated a Th2-associated immune response (Gazzinelli-Guimarães *et al.*, 2013, Nogueira *et al.*, 2016, Gazzinelli-Guimaraes *et al.*, 2019).

In summary, mouse models are a useful tool for infectious disease modelling. They are especially useful for diseases such as *Ascaris*, where the infection is acquired in a similar manner to that in humans – via oral ingestion – and where the pathogen needs to migrate through several tissues to reach maturity. This migration cannot, for now, be mimicked in tissue culture. The development of novel technologies, such as organoids, may in the future allow for these type of studies to be performed *in vitro*, thereby reducing the use of mice. When using mouse models, it is important to bear in mind the differences between humans and mice when extrapolating the results from a mouse study to humans. Again, the above mentioned organoids could offer a solution to this problem, as these could be made from human biopsy tissue.

6.2.2 Immune response

Comparatively little is known about the immune response against *Ascaris* larvae in general, but particularly the liver stage of infection remains a particularly understudied part of the life cycle. However, this is an important stage during which the observed difference in worm burden potentially occurs (Lewis *et al.*, 2007, Dold *et al.*, 2010). The data presented in this thesis therefore enhances our knowledge of the immune response during larval migration in the liver. It also highlights the importance of the early immune response, with eosinophils standing out in particular. This cell population was found to be increased under infection for both the relatively resistant and relatively susceptible strain of mouse during the flow cytometry study in Chapter

5. Eosinophils have previously been identified as having an important role in self cure in pigs (Masure *et al.*, 2013a, Vlaminck *et al.*, 2016) and in the lungs of mice infected with *A. suum* (Nogueira *et al.*, 2016, Enobe *et al.*, 2006, Gazzinelli-Guimarães *et al.*, 2013, Weatherhead *et al.*, 2018, Gazzinelli-Guimaraes *et al.*, 2019). It is therefore interesting to fill this knowledge gap, where previous studies have identified the presence of these immune cells in the lungs and the gut, the present work has now been able to show the presence, and expansion under infection, of these cells in the liver.

Eosinophilic degranulation, ROS production and cytokine production to only name a few ways in which eosinophils can play a role during parasite infection (Shin *et al.*, 2009). Eosinophils produce a broad range of cytokines (Shin *et al.*, 2009), and therefore investigating which cytokines are released during *Ascaris* infection could be informative with respect to the next stages of the immune response of the host during infection. Additionally, immune modulation targeting eosinophils or their secretions have been identified in other parasites (Shin *et al.*, 2009), such as *Toxocara canis* (Giacomin *et al.*, 2008), *Paragonimus westermani* (Shin *et al.*, 2001), *Fasciola hepatica* (Carmona *et al.*, 1993), and *Necator americanus* (Culley *et al.*, 2000).

Furthermore, a full understanding the immune response associated with *Ascaris* infection will prove essential in developing a vaccine against this parasite.

6.2.3 Immune modulation

Immune modulation against eosinophils in other parasites was mentioned above, however, immunomodulatory properties of *Ascaris* have already been discovered (Rocha *et al.*, 2008, Titz *et al.*, 2017, Almeida *et al.*, 2018, Jakobsen *et al.*, 2019). These proteins can modulate the immune response by modifying several mechanisms, including cytokine secretions (Jakobsen *et al.*, 2019), immune cell differentiation (Almeida *et al.*, 2018) and proliferation (Bethony *et al.*, 2006). The immunomodulatory properties of *Ascaris* will be essential in the development of a vaccine.

A new field of study has developed in the last few years, with many groups turning their research to the excretory/secretory products (ES) of parasites. These molecules could be excreted waste products released by the parasite, or actively secreted (Harnett, 2014). Although the existence of such molecules has been known since the 1970s, it is only recently that such research has gained renewed interest, thanks to technological advances to study these products, and their potential immune modulatory functions

(Harnett, 2014). The ES products of several parasites have been shown to modulate the Th2 response, IL-10 production, macrophage function, lymphocyte activation, Treg response and inhibit the Th1/Th17 response (Harnett, 2014). Exactly how these immune modulations happen is relatively unknown, but it likely involves, on some level, the interactions of these ES products with DCs (Harnett, 2014). *Ascaris* has been shown to have a wide range of ES products for different life stages, with potentially immune modulatory properties (Wang *et al.*, 2013, Chehayeb *et al.*, 2014, Ebner *et al.*, 2020). However, these products can also have the potential to be used against the parasite in vaccines, currently these ES products, such as the OV17 and PABA1 antigens or the As16 protein, of *Ascaris* are being tested for usefulness in a vaccine (Tsuji *et al.*, 2003, Tsuji *et al.*, 2004, Wei *et al.*, 2017, Ebner *et al.*, 2020).

Extracellular vesicles (EVs), membrane-enclosed nanoparticles which carry molecules such as miRNAs, have been shown to be released by parasites and fuse with host cells, thus delivering molecules which could potentially contribute to immune modulation (Hansen *et al.*, 2019). These EVs have been identified for different life stages of *A. suum* and they were found to contain miRNAs which could potentially modulate the immune response (Hansen *et al.*, 2019).

It would be interesting to test these products, ES, EV or miRNAs, on tissue cultures of livers, or perhaps even the above mentioned organoids. This would allow us to explore their influence on the immune system and their potential role in predisposition.

In short, as with most parasites, *Ascaris* has been shown to have immune modulatory potential. Understanding these various mechanisms will greatly enhance our knowledge to develop a potential vaccine, perhaps even by utilizing some of these immune modulatory mechanisms, such as the ES. Furthermore, the immune modulatory potential of these ES products could make them suitable for therapeutics in inflammatory and autoimmune diseases (Harnett, 2014).

6.2.4 The gut microbiome

The role of the gut microbiome in health and disease is still a relatively new field with many unanswered questions. The gut microbiome has been found to play a role in a wide range of different diseases, ranging from type 1 diabetes, rheumatoid arthritis and irritable bowel syndrome (Round and Mazmanian, 2009), but also depression, Parkinson's disease, and autism (Dinan and Cryan, 2017).

The gut microbiome also influences extra-intestinal organs, of which the liver is one of the most important ones (Albillos *et al.*, 2020). Alterations in the gut are therefore linked to the liver, making this an important aspect to investigating the liver stage of *Ascaris* infection. Alterations in permeability of the gut, which can be caused by alcohol consumption or inflammation of the gut, enhance the possibility of transport of microbial metabolites to the liver, which in turn can cause liver inflammation. *Ascaris* larvae in the gut evoke an immune response and cause damage to the gut wall itself. Both events therefore increase the possibility of gut permeability and possible transfer of microbial metabolites to the liver, which in turn can induce hepatic inflammation.

Household clustering has been found to be an important factor in the predisposition to heavy *Ascaris* infection (Walker *et al.*, 2011), and interestingly people sharing a household have been found to have a similar microbiome (Dill-McFarland *et al.*, 2019). It would therefore be very interesting to investigate the differences in the microbiome of individuals predisposed for heavy infection, compared to individuals predisposed for light infection. It is therefore possible that the microbiome could also be influence *Ascaris* larval clearance in the liver (and beyond).

The fact that two different mouse strains were used in this work leads to an interesting question, what are the differences in the microbiome between these two strains? What if a microbiome transfer was performed and the mice basically 'swapped' microbiome, would this have an influence on the outcome of *Ascaris* infection?

Studies in humans have found that the microbial biodiversity – a measure for a 'healthy gut' – is in fact increased during STH infection (Lee *et al.*, 2014, Cantacessi *et al.*, 2014, Giacomin *et al.*, 2015, Giacomin *et al.*, 2016, Chabé *et al.*, 2017, Gordon *et al.*, 2020). However, an experimental study of pigs infected with *A. suum* found a decrease in microbial diversity in the colon (Wang *et al.*, 2019). The exact influence of these adult worms on the gut microbiome will still need to be untangled, but it is clear that the presence of *Ascaris* in the gut will have an influence, with one study indicating that *A. suum* released antimicrobial factors which could inhibit biofilm formation (Midha *et al.*, 2018). It would therefore be interesting if studies which collect faecal samples for *Ascaris* research, would also perform microbiome analysis on these faeces.

The study of the microbiome is still in its infancy. The complexity of the bacteria involved and the lack of knowledge about the influence of the different bacterial taxa

on human health make it hard to interpret these studies. However, it is clear that the microbiome plays an important role in human health. Given the fact that the microbiome influences the immune system (Spencer *et al.*, 2019) and a dysbiosis causes many diseases, it is important to investigate the exact influence of the microbiome on both the hepatic response to early *Ascaris* infection and the influence of chronic intestinal *Ascaris* infection on the microbiome and its potential consequences.

6.2.5 Co-infections

Infections with multiple parasites is 'the norm rather than the exception' (Pullan and Brooker, 2008). Infections with different parasites can have a positive or a negative influence on each other (Viney and Graham, 2013). In general, a positive association between STH species has been observed (Howard *et al.*, 2001, Pullan and Brooker, 2008, Donohue *et al.*, 2019), leading to an increased egg deposition of the parasites (Brooker *et al.*, 2000), which has been found to be the case for *Ascaris* and *N. americanus* (Lepper *et al.*, 2018). Polyparasitism of STHs has, therefore, been found to increase the risk of morbidity and worse health outcomes compared to single infections (Booth *et al.*, 1998, Donohue *et al.*, 2019).

STH infection often occurs with other infectious diseases, such as intestinal protozoa, malaria, tuberculosis, HIV, and malaria (Bundy *et al.*, 2000, Kirwan *et al.*, 2010, Naing *et al.*, 2013, Salgame *et al.*, 2013, Donohue *et al.*, 2019). The effects of co-infections between STHs and the above mentioned pathogens have not been clearly defined yet, with some studies indicating protective effects of helminths, whereas others indicate an exacerbating effect (Salgame *et al.*, 2013). However, what is clear is the importance of understanding the effects of these co-infections, as mass drug treatment of helminths could have a beneficial/detrimental effect on the above mentioned (and potentially other) diseases (Donohue *et al.*, 2019).

It would therefore be interesting to use the mouse model of hepatic resistance, described in the previous chapters, for a co-infection experiment and examine the effect these co-infections have on the larval burden in the mouse strains. A co-infection of *Ascaris* with *Schistosoma* or *Plasmodium* would be of particular interest, as both these parasites migrate through the liver as well. This could answer the question whether a co-infection with these parasites increases the larval burden of *Ascaris*

infection or decreases it. Furthermore, the immune activity in the livers could be investigated using flow cytometry.

Furthermore, helminths have been shown to have an influence on vaccinations, with several studies indicating a detrimental effect of *Ascaris* infection on vaccine efficacy (Cooper *et al.*, 2000a, Cooper *et al.*, 2001, Elias *et al.*, 2001). In fact, a growing body of evidence suggests that antenatal or early childhood parasitic infection has lifelong consequences on immune status, potentially affecting any future vaccine efficacies (Labeaud *et al.*, 2009).

In short, *Ascaris* infection in the 'real world' does not happen in a bubble. As said by Viney and Graham (2013) 'The challenge for us all is to become scholars of co-infections'.

6.2.6 Reinfection

People often get reinfected with *Ascaris* after anthelmintic treatment, with prevalence reaching 94% of pre-treatment levels at 12 months post treatment (Jia *et al.*, 2012). Additionally, intensity of infection was found to be positively correlated to pre-treatment levels (Jia *et al.*, 2012). Reinfection therefore occurs quite regularly during the lifetime of people living in endemic areas. It would, therefore, be interesting to investigate in more depth, the adaptive immune response to a repeat infection and the potential presence of memory T cells or B cells.

The observed age-related reduction during *Ascaris* infection could in part be explained by such immune mechanisms, although behavioural aspects have also been identified to play a role in this age-dependent reduction (Scott, 2008). Human studies showed evidence for Th2-associated cytokines age-dependent reduction in larval burden (Jackson *et al.*, 2004). Additionally, the potential increase in infections for people over the age of 60 should be viewed and investigated in the context of reinfection (Scott, 2008).

A mouse model of *A. suum* infection found that upon reinfection there was no difference in larval burden in the liver, but there was a reduction in larval numbers in the lung (Nogueira *et al.*, 2016). However, larger hepatic lesions were observed in multiple infected animals compared to single infected animals. Increased tissue damage was observed in the lungs, potentially due to a chronic inflammation.

These studies could be undertaken in the established mouse model of hepatic resistance, used in this work. A reinfection experiment in this model would allow us to investigate more in depth the adaptive immune response involved in *Ascaris* (re)infection. This reinfection could be done quite quickly, or left over a longer period of time to see how long any potential memory T or B cells remain present. Such studies could be quite important, given the fact that humans often become reinfected with *Ascaris* occur frequently in humans. These studies could therefore be of great biological importance for the elimination efforts of *Ascaris* in humans.

6.3 Conclusion

The body of work described in this thesis investigated the molecular and immunological determinants behind the difference in larval burden in a mouse model of relative resistance and susceptibility. Chapter 3 approached this *via* the use of shotgun proteomics at day 7 p.i. This technique identified the differences between the two mouse strains of the model of relative resistance and susceptibility. Some of these differences were intrinsic to the mouse strains, such as OXPHOS. However, some differences in protein abundances between the two mouse strains were in response to an *Ascaris* infection and hinted towards a differing innate immune response. Chapter 5 therefore built on this work, investigating the immune response at day 7 p.i. during *Ascaris* infection in the mouse model, using flow cytometry. However, before this work was undertaken, the work in Chapter 4 validated the mouse model of relative susceptibility and resistance against *A. suum* for the human ascarid *A. lumbricoides*. This allowed for a full flow cytometric investigation in Chapter 5 of both ascarid species in the mouse model. These results, obtained in liver samples, tie in with findings of other research groups investigating the murine lungs during *A. suum* infection, they also found that eosinophils, monocytes and dendritic cells played an important role during infection (Gazzinelli-Guimaraes *et al.*, 2019). Further investigation of the immune response in this mouse model are needed to fully elucidate the mechanisms behind the observed difference in larval burden.

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