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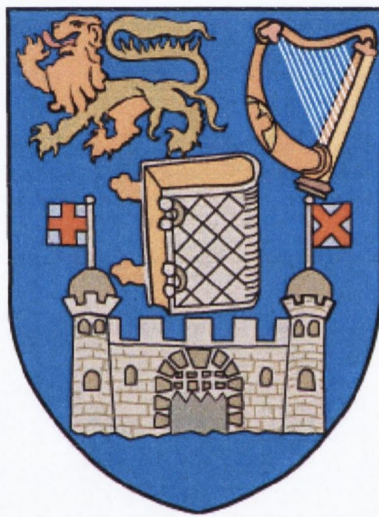
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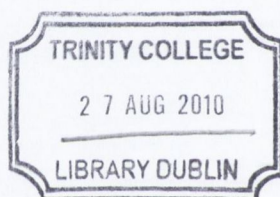
Exploiting a mouse model to identify the mechanistic basis of
resistance/susceptibility to a major gastrointestinal (GI) nematode
infection of man and livestock

Christina Dold



A thesis submitted in the fulfilment for the Degree of Doctor in
Philosophy to Trinity College, University of Dublin

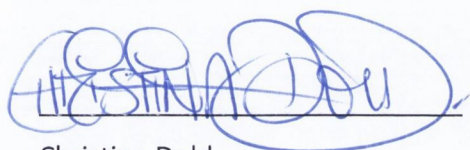
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Christina Dold

Summary

Ascaris lumbricoides and *Ascaris suum* are widespread parasitic nematodes of humans and pigs respectively. *Ascaris* worms exhibit an overdispersed frequency distribution in their hosts. In addition, observation of re-infection patterns post-chemotherapy has indicated that individuals display a degree of predisposition to their worm burden status. The underlying reasons for the observed variation in worm burden within host populations have yet to be fully defined and are difficult to elucidate in humans and pigs for ethical and logistical reasons. Comparative studies on larval migration demonstrated that the mouse is a suitable model for the early phase of *A. suum* infection. A murine model was recently developed and optimised and two inbred strains were identified as putatively susceptible (C57BL/6j) and resistant (CBA/Ca) to infection. The present study was focused upon the mechanism underlying the generation of resistance and susceptibility to *A. suum* infection in the mouse model. All experiments conducted involved the comparison of different host parameters in the two different host strains during single-pulse infections.

The first set of experiments investigated the dispersal of larvae in intestinal-hepatic-pulmonary migratory stages, with particular focus on the composite lobes of the liver and lungs. Live larvae were isolated from these tissues and a similar number of larvae were recovered in the livers of both strains, while significant differences were observed in the lungs. Heterogenous lobar distribution of larvae was evident in the two mouse strains. In C57BL/6j mice, the majority of larvae were recovered from the middle and caudal lobes of the right lung, accounting for the higher pulmonary larval numbers in the susceptible strain. Despite this, strain-specific imaging using optical projection tomography (OPT) indicated that there are no obvious differences in pulmonary arterial structure that would facilitate a higher level of larval dispersal in the lungs of the susceptible strain.

A backcross experiment was undertaken with the two mouse host strains to test the association between a gene known as Intelectin-2, which is absent from the C57BL/6j mouse genome, and susceptibility to *A. suum*. Co-habitation of male and female mice

prior to and during infection in this experiment resulted in further increases in pulmonary larval burdens for male C57BL/6j mice, indicating a possible immunosuppressive effect of sex hormones. The emergence of this so-called “sex effect” further pointed towards a factor with an immunological rather than anatomical basis accounting for variation in pulmonary larval burdens.

Histopathological examination of lung sections from timepoints representing pulmonary larval migration did not reveal any evidence of a pulmonary mechanism, as the inflammatory response mirrored the observed larval burden. However, an earlier, more severe hepatic inflammation was detected on day 4 p.i. in the resistant CBA/Ca mice, which coincided with reduced numbers of larvae successfully migrating from the liver to the lungs. In contrast, a more delayed severe inflammatory reaction was evident on day 6 p.i. in the susceptible C57BL/6j mice, when the majority of the larvae had already migrated to the lungs. In addition, the resolution of the necrosis and inflammation in CBA/Ca mice was more rapid following the reduction in larval numbers. The more effective and timely inflammatory response in the resistant strain was mirrored by higher levels of the inflammatory cytokine IL-6 measured in liver tissue homogenate. Coupled with this, the rapid tissue repair appears to be induced by increased levels of IL-10 and IL-13 over time in CBA/Ca mice.

The work presented in this thesis has elucidated a novel inflammatory mechanism of resistance, focused in the liver. Collectively, these results greatly advance our knowledge of both the impact and response to *Ascaris* larval migration at the hepatic stages of infection in lightly- and heavily-infected hosts.

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1.1. Introduction

The human roundworm *Ascaris lumbricoides* is one of the most common parasites in the world, infecting 1.2 billion people globally (De Silva *et al.*, 2003). The spectrum of disease associated with *A. lumbricoides* infection is known as ascariasis, and morbidity assessed as disability-adjusted life years (DALYs) is approximately 10.5 million (Chan, 1997). Furthermore, morbidity with serious health consequences is observed in 122 million cases per year (Chan, 1997). However, ascariasis is still considered a neglected tropical disease (NTD) (Hotez *et al.*, 2008).

Ascaris suum is a widespread parasitic nematode that causes infection in pigs with high prevalence rates in host populations (Roepstorff *et al.*, 1998; Nansen and Roepstorff, 1999). Porcine ascariasis interferes with health and performance of pigs while resulting in reduced feed to gain ratios and liver condemnation incurring economic losses (Stewart and Hale, 1988).

Similarly to most macroparasites, *Ascaris* of both human and pig origin exhibit an overdispersed frequency distribution, which results in the worms aggregating in few heavily infected hosts (Croll and Ghadirian, 1981; Croll *et al.*, 1982; Thein-Hlaing, 1984; Elkins *et al.*, 1986; Bundy *et al.*, 1987; Guyatt *et al.*, 1990; Boes *et al.*, 1998; Holland and Boes, 2002). Furthermore, observations of re-infection patterns post-chemotherapy have indicated that individuals display a degree of predisposition to their worm burden status (Haswell-Elkins *et al.*, 1987; Holland *et al.*, 1989; Boes *et al.*, 1998). The generative mechanism(s) of these observed phenomena are currently unknown and are difficult to elucidate in humans and pigs for ethical and logistical reasons.

Helminthiasis are frequently coendemic with malaria and HIV/AIDS (Druilhe *et al.*, 2005; Hotez *et al.*, 2006b). There has been renewed interest in the public health significance of *A. lumbricoides* in recent years, as observations suggest geohelminth

infection modifies the host's immune response to HIV and TB, which increases the susceptibility and spread of these diseases in the developing world (Bundy *et al.*, 2000).

Comparative studies on larval migration have demonstrated that the mouse is a suitable model for investigating the early phase of *A. suum* infection (Murrell *et al.*, 1997; Slotved *et al.*, 1998). Mitchell *et al.* (1976) reported that mouse strains vary in their susceptibility to *Ascaris* infection and therefore provide a convenient model for investigating the genetic basis of variation in migratory kinetics and immune responses to infection. A murine model was recently developed and optimised, and two inbred strains were identified as putatively susceptible (C57BL/6j) and resistant (CBA/Ca) to infection (Lewis *et al.*, 2006). This was reflected in the distinct, repeatable difference in *A. suum* larval burden in the lungs on day 7 post-infection (Lewis *et al.*, 2006). The contrast of resistance and susceptibility between CBA/Ca and C57BL/6j mouse strains respectively represents the extremes of host phenotype displayed in an aggregated distribution. The underlying mechanism responsible for the loss of *A. suum* larvae during primary infections and its precise location within the body are currently unknown.

The established mouse model facilitates research on the impact of larval infection, which is not possible in human hosts due to obvious ethical considerations, and investigation of the underlying mechanism of resistance/susceptibility. Work conducted on the mouse model to date has indicated that a hepatic/post-hepatic factor varies between susceptible and resistant mice, which results in a large discrepancy in pulmonary larval numbers between the two mouse strains (Lewis *et al.*, 2006; 2007). Therefore, the overall objective of the present study was to exploit the optimised and established *A. suum* mouse model in order to identify the underlying mechanism(s) of resistance and susceptibility. Various parameters of early *A. suum* infection were compared in C57BL/6j and CBA/Ca mice, and explored as possible explanatory mechanisms of resistance and susceptibility.

The aims of the present study, therefore, were to identify whether there existed a quantifiable difference in both the rate and extent of larval attrition and accumulation

in the intestines, liver and lungs of each strain, with particular focus on the hepatic and pulmonary lobes. Additionally, we sought to elucidate whether differences observed in these parameters between strains were attributable to immunological factors, such as the hepatic and pulmonary cellular inflammatory response and the hepatic cytokine immune response, or alternatively occur as a consequence of a differing anatomical structure between strains, with respect to branching of the pulmonary arteries of the left and right lungs. In conjunction with this, we sought to investigate the association between susceptibility to infection and a candidate gene, *Intelectin-2* which is absent in C57BL/6j mice.

1.2. Literature Review

1.2.1. Taxonomy, morphology and life cycle of *Ascaris*

Ascaris lumbricoides, Linnaeus, 1758 and *Ascaris suum*, Goeze, 1782 are parasitic nematode (Family Ascarididae) infections of humans and pigs respectively.

Morphologically indistinguishable, human and pig *Ascaris* have been shown to differ by only six (1.3%) nucleotides in the first internal transcribed spacer (ITS-1) (Zhu *et al.*, 1999; Abebe *et al.*, 2002) and differ in sequence by 3-4% in the mitochondrial genome (mtDNA) sequence (Anderson *et al.*, 1993), indicating that the species are closely related at a phylogenetic level.

Even though both parasitic nematodes display strong affinity for their conventional hosts (Crompton, 2001), experimental cross-transmission studies have demonstrated that *A. lumbricoides* can infect pigs and vice versa (Takata, 1951; Galvin, 1968). In *A. lumbricoides* non-endemic areas in North America and Denmark, infected human hosts were found to harbour worms of pig origin (Anderson, 1995; Nejsun *et al.*, 2005), indicating that pigs are a potential reservoir of infection for the human host population. However, molecular epidemiological studies in *Ascaris* endemic regions of Guatemala and China indicate that the level of cross-infection between host species is low or absent (Anderson *et al.*, 1993; Anderson and Jaenike, 1997; Peng *et al.*, 1998; 2003) and that gene flow is limited between/among different genotypes (Peng *et al.*, 1998; Anderson, 2001; Peng *et al.*, 2003; 2006).

Other work was focused upon clustering of worm genotypes in host populations. Anderson *et al.* (1995) demonstrated that in Guatemalan host populations, worms bearing identical mtDNA genotypes are found within the same host. Furthermore, the proportion of *A. suum* larvae with four differing mtDNA haplotypes varied in their porcine hosts in experimental infections (Nejsun *et al.*, 2008), supporting the

hypothesis of Anderson *et al.* (1995) that the clustering of particular genotypes within individual hosts may be a result of natural selection against certain genotypes.

1.2.2. Life cycle of *Ascaris* species

Hosts contract *Ascaris* infection via the faecal-oral route. It is known that when infective eggs are ingested and hatch, *Ascaris* larvae develop in host parenteral tissues. Knowledge of the pattern of *A. suum* larval migration stems from a range of studies systematically examining the intestinal, hepatic and pulmonary larval counts in both pigs (Roberts, 1934; Douvres and Tromba, 1971; Keittivuti, 1974; Murrell *et al.*, 1997; Roepstorff *et al.*, 1997) and abnormal hosts (see section 1.2.6).

The pattern of extra-intestinal migration in the pig is illustrated in Fig. 1.1. Following ingestion of infective ova, L₃ larvae covered by the L₂ cuticle (Maung, 1978; Geenen *et al.*, 1999), hatch in the small intestine and migrate to the caecum and proximal colon where they penetrate the mucosa (Murrell *et al.*, 1997). The larvae then migrate via the portal blood to reach the liver, where the L₂ cuticle is shed. After migration in the liver, the larvae advance to the lungs on days 6-8 p.i. (Roepstorff *et al.*, 1997). The larvae penetrate the alveolar space and move to the pharynx where they are swallowed, resulting in the helminths returning to the small intestine on days 8-10 p.i. (Douvres *et al.*, 1969; Roepstorff *et al.*, 1997). *A. suum* moult again to L₄ stage larvae in the small intestine on day 10 p.i. (Pilitt *et al.*, 1981). Larvae mature and reach sexual maturity in the small intestine, moulting again (L₅ stage larvae) on day 24 p.i. (Pilitt *et al.*, 1981).

In the most detailed study examining the migratory pattern of *A. suum* in pigs, Roepstorff *et al.* (1997) divided the intestines into sections. When returning to the small intestine, the worms demonstrated an affinity for the first three quarters of the organ but particularly the latter portion of the first half. On day 14 p.i., few larvae were recorded in the first quarter and the majority was found in the second half of the intestine. This trend appeared to continue as Roepstorff *et al.* (1997) recovered the majority of the worms in the third and fourth section with only few larvae residing in

the first two sections. The pattern of posteriorly located worms on days 14-21 p.i. coincided with expulsion of *A. suum* from the intestines and thus the host. By day 28 p.i., at which point worm populations were established and loss of worms had ceased, the majority of parasites were recovered in the second quarter of the intestine distal to the stomach (Roepstorff *et al.*, 1997).

Adult worms may reside in the intestines for approximately one year, but the majority of worms are expelled by the 23rd week of infection in pig infections (Olsen *et al.*, 1958). Male and female adult worms measure 15-25cm and 20-35cm respectively. Estimates of daily *Ascaris* female egg production generally are in the range of 200,000 eggs (Brown and Cort, 1927; Sinniah, 1982) but the number of eggs a female produces decreases with worm load (Sinniah and Subramaniam, 2009). Unembryonated ova enter the environment via the faeces and can remain viable in the soil for up to 15 years (WHO, 1967; Krasnonos, 1978; Storey and Phillips, 1985), and their viability is influenced by variables such as moisture, oxygen and shade (Crompton, 1994). During embryonation, larvae undergo two moults in the egg (Geenen *et al.*, 1999; Fagerholm *et al.*, 2000)

The hepato-tracheal migration (Sprent, 1954) takes place over a 10 to 14- day period after the uptake of eggs in pigs and humans respectively. It is not clearly understood why a parasite undertakes a migratory route within its definitive host which results in the eventual return to the site of larval emergence. Smyth (1994) speculated that the observed extraintestinal migration represented “evolutionary baggage”, a vestigial behaviour that may have once been a part of the life cycle in another host. Later, authors argued that migratory behaviour must confer a specific survival strategy (Read and Skorpington, 1995; Mulcahy *et al.*, 2005), as during migration in parenteral tissues larvae risk becoming lost or killed by host immune responses. Migratory behaviour may serve as an immuno-evasive strategy to avoid an immunological response in the intestine, where ova hatch (Mulcahy *et al.*, 2005). As stated by Parker *et al.* (2009), “if growth rate/mortality rate is higher in the tissues, it may pay to migrate to the tissues for growth and return to the gut for propagule release”. In a review by Read and Skorpington (1995), the larger growth seen in migratory parasites is argued as the associated fitness benefit despite the inherent cost to survival. When pigs are

administered an intravenous dose of larvae hatched *in vitro*, *A. suum* larvae do not undergo hepatic migration and develop slower (Jungersen *et al.*, 1999a), thus indicating the importance of migration for helminth growth.

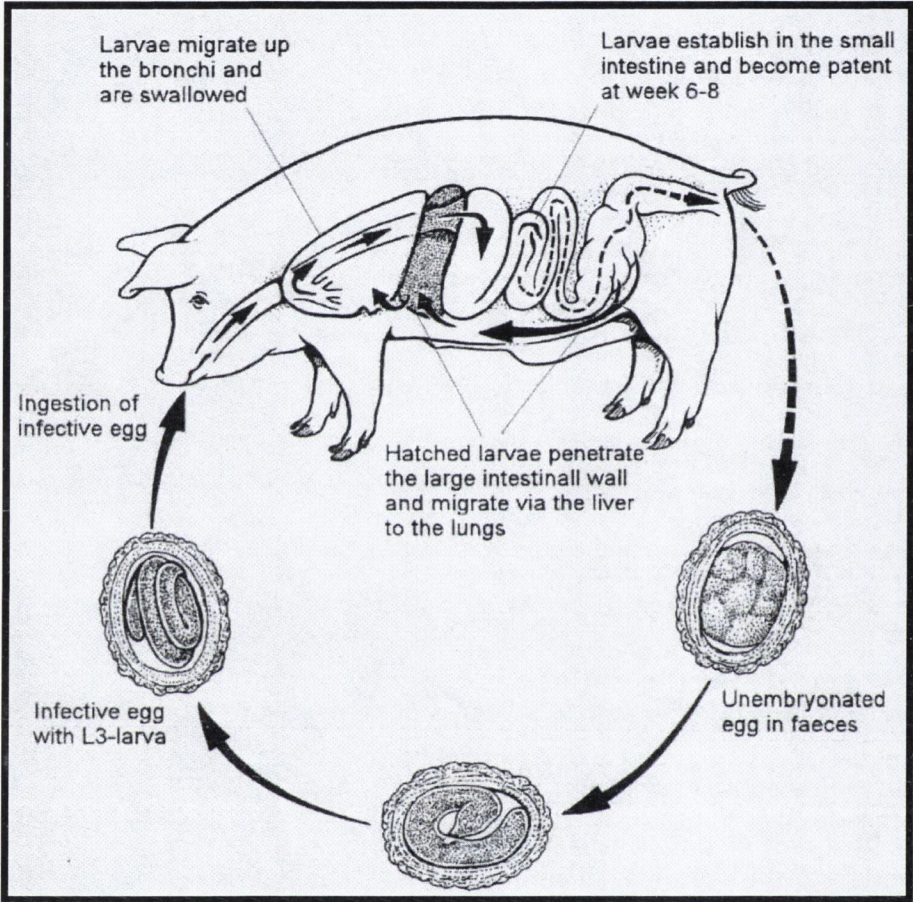


Figure 1.1. A schematic representation of the life cycle of *A. suum* in the pig (illustration by Wm P Hamilton CMI) (Roepstorff and Nansen, 1998)

1.2.3. Epidemiology of *Ascaris*

Recent figures estimate that 1.2 billion people are infected with *A. lumbricoides* (De Silva *et al.*, 2003). The parasitic nematode is distributed in over 150 countries (Crompton, 1989). A joint Nordic survey on swine endoparasites revealed that *A. suum* is one of the most common parasites in swine hosts in the countries studied (Roepstorff *et al.*, 1998). The prevalence of *A. suum* infection varies with geographical region and

farm management practices but, as pointed out by Nansen and Roepstorff (1999) and reviewed by Roepstorff (2003), few swine herds are totally free of infection.

Helminthic infections are characteristically overdispersed in their host populations, with a small proportion of people harbouring the majority of parasitic worms present (Anderson and May, 1985). *Ascaris* of both human and pig origin exhibit an overdispersed frequency distribution, which results in the worms aggregating in few heavily infected hosts (Croll and Ghadirian, 1981; Croll *et al.*, 1982; Thein-Hlaing, 1984; Elkins *et al.*, 1986; Bundy *et al.*, 1987; Guyatt *et al.*, 1990; Roepstorff *et al.*, 1997). This epidemiological pattern in which 'wormy persons' (Croll and Ghadirian, 1981) harbour disproportionately large worm burdens is also high within groups stratified by age and sex (Croll *et al.*, 1982; Thein-Hlaing, 1984; Elkins *et al.*, 1986; Bundy *et al.*, 1987).

Furthermore, studies involving the provision of anthelmintic treatment and subsequent observation of intensity of re-infection have shown that individuals tend to re-acquire similar worm burdens to those harboured before treatment. This phenomenon, which is termed predisposition, has been demonstrated in longitudinal studies for ascariasis in both humans (Elkins *et al.*, 1986; Haswell-Elkins *et al.*, 1987; Thein-Hlaing *et al.*, 1987; Holland *et al.*, 1989; Forrester *et al.*, 1990; Chan *et al.*, 1992; Hall *et al.*, 1992) and pigs (Boes *et al.*, 1998), and can be detected over multiple rounds of chemotherapy (Holland *et al.*, 1989; Chan *et al.*, 1992). Predisposition to *Ascaris* infection has important implications for transmission, morbidity and control (Anderson and May, 1985). The consistent variability in infection intensity observed between hosts generates the over-dispersed frequency distribution displayed in host populations discussed above.

While predisposition to *A. lumbricoides* is observed within age cohorts, changes in the average intensity of *A. lumbricoides* infection with age are convex in form with intensity peaking in the 5-10 year old age groups (Thein-Hlaing, 1984; Elkins *et al.*, 1988; Holland *et al.*, 1989; Peng *et al.*, 1996). Furthermore, the strength of predisposition decreases with age (Elkins *et al.*, 1986).

In a review of the phenomenon, Keymer and Pagel (1990) collated the data obtained in 12 studies of helminth infection demonstrating predisposition and noted that the relationship between initial and final infection levels is seldom strong. However, the authors also identified factors such as the influence of age on intensity, the means of measuring intensity and the duration of the reinfection period as important contributors to the detection of predisposition, which varies among the studies evaluated (Keymer and Pagel, 1990).

The bases for heterogeneity of infection or predisposition are not yet fully understood. Much focus has been placed on investigating whether variation in infection intensity is a result of differences in environmental exposure to infection or susceptibility. Determinants of infection intensity may be divided into two categories – long-term effects that operate on the time-scale of the host life expectancy (e.g. host genetics, host socio-economic status), and short-term effects that operate on the time-scale of the parasite life expectancy (e.g. host acquired immune response). In an attempt to estimate the relative importance of long- and short-term effects on infection intensity, McCallum (1990) used probability theory and concluded that both categories have an approximately equal contribution to the observed heterogeneity.

Differential exposure to infection in humans is difficult to quantify and requires a detailed behavioural study (Bundy and Blumenthal, 1990). Kightlinger *et al.* (1998) assessed exposure to infection in a population of children in S.E. Madagascar by environmental, demographic, behavioural, and socio-economic indicators. Results suggested that intensity of *A. lumbricoides* infection is influenced by gender-related behavioural and environmental factors that contribute to exposure. Furthermore, longitudinal studies in human host populations indicate that a range of socio-economic conditions such as housing conditions (Holland *et al.*, 1988) and cultural practices such as unhygienic defecation practices (Haswell-Elkins *et al.*, 1989a) influence infection intensity.

Behavioural-mediated reduction in exposure with age is a likely determinant of the observed age-intensity profiles (Keymer, 1982). Adults are also known to harbour *A.*

lumbricoides worms, but generally at a lower intensity than children (Thein-Hlaing, 1984; Elkins *et al.*, 1988; Holland *et al.*, 1989; Peng *et al.*, 1996). This has led to the suggestion that less marked aggregation in older age cohorts reflects a slow build-up of specific immunity or variation in susceptibility to infection over time. However, overdispersed worm frequency distributions are also recorded within age classes as age is not the only source of variation. Coupled with this, as Bundy (1988) points out the epidemiological pattern of predisposition shows that hosts with the greatest prior experience of infection are subsequently re-infected. This indicates that acquired immunity cannot be the only primary determinant of variability in infection intensity.

McCallum (1990) reported that genetic factors also play a significant role in predisposition to *Ascaris* infection. However Chan *et al.* (1994b) noted that environmental or behavioural features of the family household were found to be a major determinant of infection status. Nevertheless, there are many lines of evidence indicating that the underlying mechanism of resistance/susceptibility to *Ascaris* infection is also influenced by host genetics, the host's immune repertoire (which is ultimately under genetic control) and concurrent infections. The role of each of these determinants in the observed heterogeneity of infection intensities is discussed below (see section 1.2.11).

1.2.4. Strategies for control of *Ascaris*

For human hosts, there are three major strategies for the control of soil transmitted helminths; reducing parasite intensity (and consequent morbidity) by means of improvements in sanitation, health education and anthelmintic treatment (chemotherapy) (Hotez *et al.*, 2006a).

The long-term control and eradication of *A. lumbricoides* infection lies in the provision of sanitation for the safe disposal of human faeces (Hotez *et al.*, 2006a). Sanitation aims to interrupt transmission, prevent re-infection and gradually reduce worm loads. The construction of sanitary facilities may be encouraged through health education

(Albonico *et al.*, 1999), which aims to stimulate changes in behaviour related to environment and family hygiene. Furthermore, education programmes have been successful in parasite control. For example, the Japanese Association of Parasite Control (JAPC) implemented education programmes in schools and has contributed to the recent successful control of soil transmitted helminths (Dickson *et al.*, 2000) and schistosomiasis in Japan (Ohta and Waikagul, 2007).

Since STH infections are coendemic, deworming programmes are targeted at all three helminth infections. WHO (2002) implemented the combined approach of integrated control of both schistosomiasis and STHs. Therefore, most commonly administered drugs to treat STH infections are albendazole, levamisole and mebendazole, which are combined with praziquantel in areas with schistosomiasis (Urbani and Albonico, 2003). Several papers highlight the potential interaction of STH infection on promoting the “big three” diseases (HIV/AIDS, TB and malaria) (e.g. Nacher *et al.*, 2000; Wolday *et al.*, 2002). Therefore, the importance of integrated parasite control of STH with other debilitating infections has also been highlighted (Molyneux *et al.*, 2005).

Anthelmintic drug treatment programmes are aimed to reduce morbidity as opposed to eradicate helminths, which is not a feasible goal (WHO, 2002). Regular treatment is necessary due to high reinfection rates in endemic regions. For example, *A. lumbricoides* reached 55% of pre-treatment rates within 11 months (Elkins *et al.*, 1988). Several approaches to treatment programmes exist; mass (universal), selective and targeted treatment (Albonico *et al.*, 2006). Mass universal treatment is offered to all individuals in an affected population, regardless of age, gender, worm burden or other social characteristics. In contrast, targeted treatment is selectively offered to a particular group within the affected population, defined by the age, gender, worm burden or other social characteristics shared among the targeted group. In the selective treatment approach, hosts with the highest worm burdens are preferentially offered chemotherapy. As described earlier, children tend to acquire heavier worm burdens and so are considered a suitable group for targeted treatment (Haswell-Elkins *et al.*, 1989a).

Studies on the efficacy of the various approaches to treatment programmes support the concept of mass treatment (Stephenson *et al.*, 1980b; Asaolu *et al.*, 1991), while targeted treatment for children aged 5-15 years also results in a significant reduction in post-treatment egg counts (Asaolu *et al.*, 1991). Furthermore, since children act as significant contributors of infective stages into the environment targeted treatment for children aged 5-15 years led to a significant difference in pre- and post-treatment egg counts in untreated adults (Asaolu *et al.*, 1991). Current efforts to control STH are targeted at the school age population (Brooker *et al.*, 2006) as 25-35% of school-age children are infected with one or more parasitic worm species (Bundy, 1997; De Silva *et al.*, 2003) so are considered as a high risk group. Therefore, in an effort to reduce morbidity by 80% by 2010, WHO (2002) recommended regular administration of anthelmintics to at least 75% of all school children. Coupled with this, the need to incorporate younger age cohorts (less than 24 months) in large scale deworming programmes has recently been highlighted (WHO, 2003; Kirwan *et al.*, 2009).

There are concerns about periodic deworming with anthelmintics and the emergence of anthelmintic resistance, which is now widespread in nematode parasites of livestock (Bethony *et al.*, 2006). The development of drug resistance in these nematode populations results from frequent treatment of animals kept in close proximity with little gene flow (Geerts *et al.*, 1997). Humans are more heterogeneous and are subjected to less frequent treatment, which is targeted at certain populations and so reduces selection pressure (Albonico *et al.*, 2004).

Transmission of *A. suum* among pig populations is dependent on factors such as housing systems, hygiene, management practices and anthelmintic treatment (Nansen and Roepstorff, 1999). Therefore, as Roepstorff (2003) discussed, there is a need for multivariate analyses encompassing a range of variables in order to assess the most important risk factors for controlling *A. suum* infection in pigs. Such analyses have been undertaken on three occasions (Roepstorff and Jorsal, 1990; Dangolla *et al.*, 1996; Roepstorff *et al.*, 1999) yet only one study encompassed a wide range of age categories (Roepstorff *et al.*, 1999). The variables tested that were significantly associated with *A. suum* infections were 'age-group', with large fatteners and gilts (young female swine)

recorded to have the highest infection intensity (Roepstorff *et al.*, 1998), 'country', 'weaning age' as late weaning was associated with higher prevalence of infection, and 'water supply', which indicated that drinking facilities located in the lying area was a risk factor.

A. suum is the most prevalent intestinal parasite in Danish herds (Roepstorff *et al.*, 1998) and therefore concurrent infections are common. The importance of control strategies for *A. suum* infection was highlighted recently as the parasite was shown to reduce the efficacy of the *Mycoplasma hyopneumoniae* vaccine, which is commonly used to prevent pneumonia in pig herds (Steenhard *et al.*, 2009).

1.2.5. *Ascaris* associated pathology and morbidity

Ascariasis is the term used to describe the spectrum of disease symptoms observed in infected humans and pigs. Morbidity and mortality increases with worm burden (Pawlowski and Davis, 1989). Aggregation leads to relatively few individuals harbouring sufficient worms to precipitate life-threatening or severe morbidity (Anderson and May, 1991). As observed by Crompton (1999), most *A. lumbricoides*-induced morbidity is borne by school-age children due to the age-related intensity patterns, discussed above. Furthermore, as *Ascaris* larvae develop, different stage-specific antigens are observed (Kennedy and Qureshi, 1986) and various tissues are invaded, so therefore the effects of infection differ over the course of larval migration and development.

While the majority of infections are asymptomatic, an estimated 8-15% (120-220 million cases) of those infected with *A. lumbricoides* demonstrate associated morbidity (Chan, 1997; Albonico *et al.*, 1999). The manifestations of ascariasis can be broadly characterised into acute and chronic symptoms. Human hosts tend to experience acute lung inflammation, difficulty in breathing and fever as a result of larval migration through the pulmonary tissue. Abdominal distension and pain, nausea and diarrhoea are also characteristic symptoms of adult worm infection and chronic ascariasis (Crompton, 2001). Entangled adult worms have also been documented as leading to

mechanical intestinal obstruction (Pawlowski and Davis, 1989; Crompton, 1994; Villamizar *et al.*, 1996; de Silva *et al.*, 1997) in 0.005-2 per 1000 infections per year (Pawlowski and Davis, 1989). In an analysis of published hospital reports, de Silva *et al.* (1997) revealed that intestinal obstruction was the most common complication of chronic human ascariasis with an associated mean case fatality rate of 5.7 %. Intestinal obstruction occurs most frequently in children under five years old despite peak infection intensity occurring in 5-10 year olds (de Silva *et al.*, 1997), which may be due to the narrower intestinal lumen in younger hosts (Villamizar *et al.*, 1996). Biliary ascariasis is the second most documented complication of chronic infection at 5-39% of hospital cases (de Silva *et al.*, 1997).

Chan (1997) described a model in which the worm burdens were separated into two threshold levels based on the disabilities experienced by the human hosts. The model facilitated calculation of the disability-adjusted life years (DALYs), which translates disabilities experienced into years of healthy life lost. As calculated by Chan (1997), the DALYs lost due to ascariasis are 10.5 million, while the combined DALYs for the soil transmitted helminths, *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms are 39.0 million, higher than the DALYs estimated for malaria (35.7 million).

While *Ascaris* is most prevalent in areas of low socioeconomic status and thus poverty and malnutrition, studies indicate that *Ascaris* infection exerts a chronic influence on host nutrition (Crompton and Nesheim, 2002). Various intervention and clinical studies, the majority of which are focused on school children, demonstrated that infection is associated with appetite loss (Hadju *et al.*, 1996), lactose maldigestion (Carrera *et al.*, 1984; Taren *et al.*, 1987) and impaired weight gain (Stephenson *et al.*, 1980c; Hadju *et al.*, 1996). Nutritional consequences of infection are generally most pronounced in those who harbour the heaviest burdens. In contrast, results in a study of Mexican school children conducted by Quihui-Cota *et al.* (2004) indicated that intestinal parasitic infections were not the causal factor but are rather associated with malnutrition in the host population. Intestinal malabsorption is considered a potential cause of nutritional impairment in *Ascaris*-infected children, particularly by Tripathy *et al.* (1972) who studied worm-induced gastrointestinal tract physiopathology in children. Abnormalities

in jejunal biopsies such as broadening and shortening of villi, elongation of crypts and decrease in villus-crypt ratio were observed in infected patients but the morphology of the intestines reverted to normal post-chemotherapy simultaneously to increased protein and fat absorption. Later studies showed that *A. lumbricoides* infection can induce vitamin A and C deficiency (Thein-Hlaing, 1993), which may be a result of inhibited intestinal absorption.

While *A. lumbricoides* is of considerable public health importance, porcine ascariasis is also known to interfere with the health and performance of pigs and is responsible for reduced feed to gain ratios resulting in considerable economic losses (Stewart and Hale, 1988). Similarly to human infection, porcine hosts display stunted growth and consume less food than uninfected controls (Stephenson *et al.*, 1980c; Forsum *et al.*, 1981).

As stated by Boes and Helwich (2000) *A. suum* infections in young malnourished pigs provide an useful model for *A. lumbricoides* infection in children. *Ascaris*-infected pigs also display reduced food intake and growth rate (Stephenson *et al.*, 1980c; Stephenson *et al.*, 1980a; Forsum *et al.*, 1981), and impaired lactase activity in the intestinal mucosa (Forsum *et al.*, 1981), all of which are significantly correlated with the intensity of infection (Stephenson *et al.*, 1980a; Forsum *et al.*, 1981). Stephenson *et al.* (1980c) examined the effects of adult *Ascaris* infection in pigs on controlled diets and demonstrated that the effects on the nutritional status of the hosts was most evident in pigs on marginal protein diets. Furthermore, the efficiency of feed utilisation in *Ascaris*-infected pigs was decreased, which was a product of the observed reduction of the villar height to crypt depth ratio in the intestines.

There is a proposed association between helminth infection and a reduction in host cognitive abilities (reviewed by Kvalsvig, 2002). However, confounding variables such as age, gender, socio-economic status, baseline nutritional status, food intake and polyparasitism pose difficulties when designing a well-controlled study. In two recent intervention studies, whereby children were assessed within a 6 month time frame post-treatment, conflicting results on the influence of *A. lumbricoides* on cognitive function were observed. Watkins *et al.* (1996) demonstrated that anthelmintic

treatment in Guatemalan children did not improve reading, vocabulary or school attendance. However, a study conducted by Hadidjaja *et al.* (1998) in Indonesia indicated that mebendazole-treated children showed significant improvements in learning ability, concentration and eye-hand co-ordination.

The impact of larval migration within human hosts remains an elusive topic for obvious ethical reasons (Cooper *et al.*, 1992). An inflammatory reaction in the liver has been observed in *A. lumbricoides*- (Javid, 1999) and *A. suum*- (Sakakibara *et al.*, 2002; Kakiyama *et al.*, 2004) infected humans, pigs (Schwartz and Alicata, 1932; Ronéus, 1966; Copeman and Gaafar, 1972; Pérez *et al.*, 2001; Frontera *et al.*, 2003) and model organisms such as calves (McCraw and Greenway, 1970), guinea pigs (Fallis, 1948; Leventhal *et al.*, 1978), rabbits (Arean and Crandall, 1962) and mice (Bindseil, 1981; Eriksen, 1981). In *A. suum* infections, white spots (WS) are white pathological lesions that are formed by the mechanical injury and inflammatory response induced by migrating larvae in the liver (Ronéus, 1966). WS formation over the superficial hepatic surface and within the liver tissue is characteristic of porcine infections in response to larval migration through the liver (Schwartz and Alicata, 1932; Copeman and Gaafar, 1972). WS are also induced by other ascarids such as *Toxocara canis*, *Toxocara cati* and *Parascaris equorum* (Ronéus, 1966; Helwig *et al.*, 1999), as well as immature *Stephanurus dentatus* (Batte *et al.*, 1975), metacestodes of *Echinococcus multilocularis* (Sydler *et al.*, 1998) and *Schistosoma japonicum* eggs (Willingham *et al.*, 1994). Studies of the location, distribution and cellular infiltrate composition of WS in pigs have been described in detail previously (Ronéus, 1966; Pérez *et al.*, 2001).

There are three types of WS observed in *A. suum*-infected pigs. The granulation tissue type WS (GT-WS) have been suggested to form along the larval migration routes (small GT-WS) or encapsulate trapped larvae (large GT-WS) (Ronéus, 1966; Copeman and Gaafar, 1972; Pérez *et al.*, 2001) and therefore have been proposed to play a role in immunity to *A. suum* infection in pigs (Copeman and Gaafar, 1972; Eriksen *et al.*, 1980). *A. suum* larval debris has been previously detected within GT-WS (Schwartz and Alicata, 1932; Sprent and Chen, 1949; Copeman and Gaafar, 1972; Pérez *et al.*, 2001), centrally located within the granular mass (Schwartz and Alicata, 1932). The larvae-induced GT-

WS have been proposed as a precursor to the lymphonodular types WS (LN-WS) (Ronéus, 1966; Copeman and Gaafar, 1972), as the appearance of the latter on day 10 p.i. in pigs coincides with the healing of GT-WS (Roepstorff *et al.*, 1997).

Larval migration in the host lung tissue induces pulmonary distress in both porcine (Matsuyama *et al.*, 1998) and human (Beaver and Danaraj, 1958; Spillmann, 1975) hosts. The respiratory distress experienced during pulmonary ascariasis is referred to Löfflers syndrome (Löffler, 1956), which is a recognised eosinophilic disease (Allen and Davis, 1994; Ribeiro and Fischer, 2002). In *A. lumbricoides* infection, dyspnoea (difficult or laboured breathing) and bronchospasm may be severe (Ribeiro and Fischer, 2002). Severe dyspnoea has also been documented in porcine infections (Taffs, 1968; Eriksen, 1981; Yoshihara *et al.*, 1983). Short dry coughs are also a typical feature of *A. suum*-induced respiratory distress in pigs (Yoshihara *et al.*, 1983) and have been reported in experimental infections of cows (Greenway and McCraw, 1970a).

1.2.6. Animal models of *Ascaris* infection

Studies of *A. lumbricoides* worms in the human host are limited due to ethical considerations. Therefore, research involving human hosts is confined to the adult stage of *Ascaris* infection. Furthermore, difficulties exist in determining the complex immunoepidemiological patterns or importance of host genetics in disease development under field conditions, due to the multiplicity of environmental factors which must be considered (Woolhouse, 1993; Dessein *et al.*, 2001). For these reasons, many researchers have turned to exploring *Ascaris* infection in a wide variety of model organisms (Table 1.1), which has contributed to the current knowledge of the larval migratory pattern in early infection as well as the immune response to *Ascaris* spp.

As discussed by Boes and Helwigh (2000), an appropriate model of helminth infection should mimic the human host, the parasite and the human host parasite system. *A. suum* infection in the pig is a naturally occurring host parasite system and as discussed earlier *A. suum* is closely related to the human parasite species, *A. lumbricoides* (e.g.

Anderson, 1995). Therefore, the pig serves as a suitable model for human *Ascaris* infection. The pig model has contributed to demonstrating that epidemiological patterns of infection are comparable in humans and pigs (Boes *et al.*, 1998).

Table 1.1. Parameter(s) investigated in experimentally infected model organisms excluding mice (shown in Table 1.2)

Model organism	Parameter(s) investigated	Reference
guinea pig	larval migration	(Stewart, 1916a; Ransom and Foster, 1919; Yoshida, 1919; 1920; Kerr, 1938; Fallis, 1948; Soulsby, 1957; Taffs, 1965; Douvres and Tromba, 1971)
	immunological response	
rabbit	immunological response	(Arean and Crandall, 1962; Taffs, 1965; Galvin, 1968; Berger, 1971; Douvres and Tromba, 1971)
	larval migration	
	hepatic pathology	
	pulmonary pathology	
gerbil	larval migration	(Cho <i>et al.</i> , 2007)
rat	larval migration	(Stewart, 1916b; Yoshida, 1919)
cow	larval migration	(Greenway and McCraw, 1970a; 1970b; McCraw and Greenway, 1970)
	immunological response	
	pathology	
lamb	larval migration	(Ransom and Foster, 1919)

Model organism	Parameter(s) investigated	Reference
goat	larval migration	(Ransom and Foster, 1919)
pig	larval migration immunological response epidemiological patterns hepatic pathology pulmonary pathology concurrent infections <i>Ascaris suum</i> genetics host genetics	(Stewart, 1917a; Stewart, 1919; Schwartz and Alicata, 1932; Kelley <i>et al.</i> , 1957; Ronéus, 1966; Galvin, 1968; Taffs, 1968; Douvres <i>et al.</i> , 1969; Douvres and Tromba, 1971; Copeman and Gaafar, 1972; Keittivuti, 1974; Eriksen <i>et al.</i> , 1980; Eriksen, 1981; Yoshihara <i>et al.</i> , 1983; Urban and Romanowski, 1985; Eriksen <i>et al.</i> , 1992b; Eriksen <i>et al.</i> , 1996; Jungersen <i>et al.</i> , 1996; Murrell <i>et al.</i> , 1997; Roepstorff <i>et al.</i> , 1997; Boes <i>et al.</i> , 1998; Jungersen <i>et al.</i> , 1999b; Coates, 2000; Pérez <i>et al.</i> , 2001; Boes <i>et al.</i> , 2002; Frontera <i>et al.</i> , 2003; Eriksen <i>et al.</i> , 2004; Frontera <i>et al.</i> , 2004; Frontera <i>et al.</i> , 2005; Nejsum <i>et al.</i> , 2005; Nejsum, 2008; Nejsum <i>et al.</i> , 2008; Nejsum <i>et al.</i> , 2009a; Nejsum <i>et al.</i> , 2009b)

Furthermore, model organisms facilitate a greater degree of control over the different parameters under study, since the ecological parameters that impact helminth infection can be controlled or eliminated (Boes and Helwig, 2000).

1.2.7. The mouse host as a model of early *Ascaris* infection

The mouse has been extensively studied as a model of early *Ascaris* infection. Infection in mice only represents larval stages of the life cycle as worms do not return to the small intestine to mature, therefore this constitutes an abnormal host-parasite relationship. However, Slotved *et al.* (1998) confirmed by means of comparative work with the pig that the migratory pattern of *A. suum* is similar in murine and porcine hosts. Therefore, the mouse is deemed an appropriate host of early *Ascaris* infection (Slotved *et al.*, 1998). Prior to this, Kennedy *et al.* (1986) concluded that *Ascaris* in the mouse was a suitable model for genetic control of host immune responses to defined antigens. Therefore, the mouse has been confirmed as an appropriate organism for modelling the early *Ascaris* migratory pattern, and also host genetics and immunology as determinants of infection intensity.

The migratory pattern of *Ascaris* larvae has been studied extensively in mice and previous studies (Jenkins, 1968; Keittivuti, 1974) have documented the preferential penetration of the caecal and colon wall during early migration. This migratory stage was recognised in mice before it was recorded in pigs (Murrell *et al.*, 1997), highlighting the important role abnormal host model organisms have played in understanding the *Ascaris* life cycle. Furthermore Ransom and Foster (1920) detected larvae in the portal vein on day 1 p.i. so were the first researchers to document the role of a blood vessel in facilitating the onward migration from the intestines to the liver.

Timing of *Ascaris* migration is similar in mice and pigs (section 1.2.2) yet migration extends over a slightly longer time in pigs (Murrell *et al.*, 1997) When infective embryonated ova are administered to a mouse (Fig. 1.2 (1)) into the stomach via gastric intubation (Fig. 1.2 (2)), larvae hatch in the small intestine (Fig. 1.2 (3)). Larvae migrate to the large intestine (Fig. 1.2 (4-5)) and within two to three hours p.i. a large number of

larvae are recovered in the caecal and colonic tissue (Jenkins, 1968; Keittivuti, 1974; Slotved *et al.*, 1998). From here, larvae migrate to the liver (Fig. 1.2 (6)) as early as four hours p.i. (Slotved *et al.*, 1998). While the first appearance of larvae in the lungs (Fig. 1.2 (7)) has been documented as early as 6 hours (Slotved *et al.*, 1998) but more commonly on days 4-5 p.i in mice (Douvres and Tromba, 1971; Lewis *et al.*, 2006), the numbers do not tend to peak in this tissue until approximately days 5-7 p.i in mice (Douvres and Tromba, 1971; Keittivuti, 1974; Slotved *et al.*, 1998; Lewis *et al.*, 2006).

Working with the mouse model is advantageous in terms of economical and practical considerations. Furthermore, as stated by Slotved *et al.* (1998) there is a greater availability of reagents for mice which makes the mouse an attractive model organism especially for immunological studies. The migratory pattern of *Ascaris* in the mouse can be studied over a shorter time-frame than in the pig. Moreover, as stated by Behnke *et al.* (2009) in mouse models “the mechanism of resistance can be conveniently dissected at a variety of highly sophisticated levels.” Furthermore, sequencing of the mouse genome (Waterston *et al.*, 2002) has provided a valuable tool for investigating the role of particular gene(s) that influence resistance to infection.

Inbred strains of mice that vary in their susceptibility to *Ascaris* infection have been identified (Mitchell *et al.*, 1976; Lewis *et al.*, 2006) and two inbred strains were recently recognised to represent hosts susceptible or predisposed to infection (C57BL/6j) and those that are resistant (CBA/Ca). These mouse strains, in the absence of environmental variables, represent the extremes of outbred populations containing individuals that vary in their ability to resist *Ascaris* infection.

C57BL/6j and CBA/Ca mice have different MHC haplotypes (C57BL/6j: H-2^b, CBA/Ca: H-2^k). C57BL is thought to be the most widely used of all inbred strains and the sub-strain C57BL/6 alone accounts for over 14% of occasions on which an inbred strain is used for a study (Mouse Genome Informatics www.informatics.jax.org).

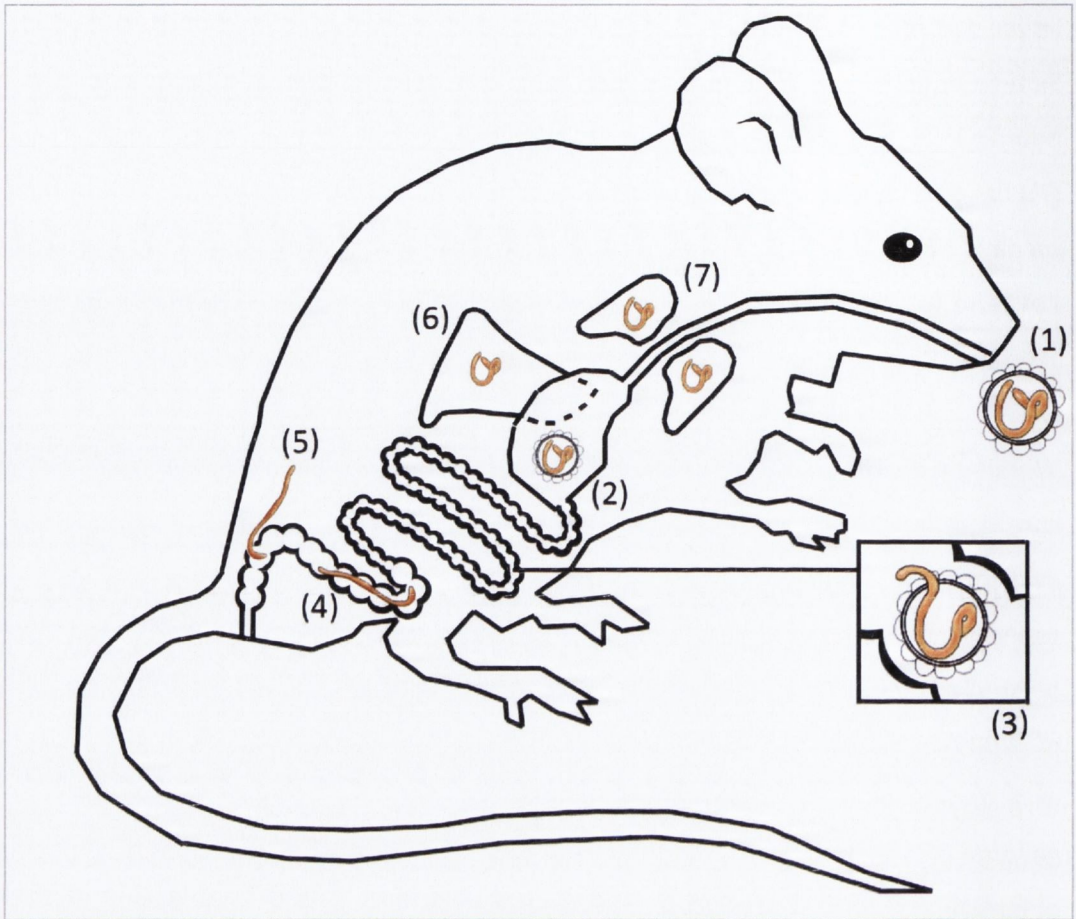


Figure 1.2. Migration of *Ascaris suum* in the mouse.

In experiments involving the mouse as a model organism, embryonated ova (1) are typically administered by means of gastric intubation into the stomach (2). Larvae hatch in the small intestines (3) and migrate to the large intestine (4), where they penetrate the caecal and colonic wall (5). From here, larvae migrate to the liver (6) and then the lungs (7). (Original illustration)

Table 1.2. Selection of studies which the mouse was used as a model for early *Ascaris* migration and the host immunological response to *Ascaris* infection

Study Type	Reference
intestinal-hepatic migratory pattern	(Ransom and Foster, 1919 (strain not specified); 1920 (strain not specified); Ransom and Cram, 1921 (strain not specified); Jenkins, 1968 (albino mice); Keittivuti, 1974 (IKR strain); Slotved <i>et al.</i> , 1998 (BALB/c strain))
hepatic-pulmonary migratory pattern	(Stewart, 1917b (strain not specified); Stewart, 1917a (strain not specified); Sprent, 1952 (white mice, strain not specified); Sinha, 1967 (white mice, strain not specified); Douvres and Tromba, 1971 (general purpose Swiss mice); Song <i>et al.</i> , 1985 (strain not specified); Lewis <i>et al.</i> , 2006 (A/J, BALB/c, CBA/Ca, C57BL/6j, C3H/HeN, DBA/2, NIH, SJL, and SWR strains))
hepatic pathology	(Bindseil, 1969b; Crandall and Crandall, 1971; Bindseil, 1981 (NMRI/Bom strain); Eriksen, 1981 (albino mice, nu/nu, nu/+, +/+))
pulmonary pathology	(Sprent, 1949 (strain not specified); Crandall and Crandall, 1971; Eriksen, 1981 (albino mice, nu/nu, nu/+, +/+); Lewis <i>et al.</i> , 2007 (C57BL/6j and CBA/Ca))
immunological response	(Sprent and Chen, 1949 (strain not specified); Bindseil, 1969a (NMRI strain); 1969b (NMRI strain); Guerrero and Silverman, 1969 (strain not specified); Bindseil, 1970b (NMRI strain); 1970a (NMRI strain); Mitchell <i>et al.</i> , 1976 (C57BL/6 and BALB/c strains); Brown <i>et al.</i> , 1977 (CBA/N, CBA/H and C57BL/6j strains); Crandall <i>et al.</i> , 1978 (C57BL/6j); Eriksen, 1981 (albino mice, nu/nu, nu/+, +/+); Kennedy <i>et al.</i> , 1986 (NIH, C3H/He, BALB/c, SJL, CBA/Ca, DBA/2 and C57BL/6 strains); Jeska and Stankiewicz, 1989 (NFR/N strain))

1.2.8. The immunological response to infection

Immunity can be broadly categorised into innate and adaptive immunity. Innate immunity represents the first line of defence, affording protection by recognising “non-self”. Combat with a pathogen by the innate immune system does not confer long-lasting memory, whereas a unique feature of the adaptive immune system is generation of immunological memory. Therefore, once a vertebrate is exposed to an infectious agent, the adaptive immune response is immediate and stronger against subsequent exposure to the particular infectious agent.

The innate immune system’s recognition of “non-self” is mediated by a detection system composed of pathogen recognition receptors (PRRs) (Akira *et al.*, 2006). PRRs are cell receptors that recognise specific common structures known as pathogen-associated molecular patterns (PAMPs) (Janeway Jr and Medzhitov, 2002) found on exogenous pathogens (e.g. flagellin, viral RNA and bacterial DNA) as well as endogenous ligands (e.g. heat shock proteins released by damaged necrotic host cells). The pathways that follow recognition of PAMPs initiate the activation of principal effector cells of innate immunity such as monocytes, macrophages, neutrophils and/or dendritic cells.

Effector cells of the innate immune response are activated once non-self is detected then communicated by means of cytokines, soluble peptides secreted by cells that modulate a wide range of potent immunoeffector mechanisms. Chemokines, a subset of cytokines, attract cells such as neutrophils and monocytes by promoting chemotaxis from the bloodstream into infected tissues (Borish and Steinke, 2003). Therefore, cytokines play a key role in the initiation and promotion of the inflammatory response. Inflammation is a general term used to describe the recruitment of cells and molecules of innate immunity in response to host tissue damage.

Macrophages and neutrophils are the chief cell types involved in the initial phase of an inflammatory response. The latter cell type are recruited early in response to pathologic

conditions to the site of injury in the host by means of chemokines (Cyster, 1999; Mackay, 2001). Monocytes are recruited shortly after neutrophils and rapidly differentiate into macrophages while eosinophils are recruited at a later stage. Cytokines also influence the permeability of blood vessels, thus further facilitating the migration of cells to the site of injury.

The effector cells of the innate immunity which are involved in the inflammatory response function in destroying both invading infectious agents and damaged host tissue. Both macrophages and neutrophils phagocytose invading pathogens while the former cell type acts as general scavenger cells, clearing dead cells and cell debris. Eosinophils are believed to be involved in the destruction of parasites too large to be phagocytosed by neutrophils and macrophages (Meeusen and Balic, 2000).

During the inflammatory response, cytokines also promote activation of the synthesis and release of further soluble mediators of innate immunity, such as acute phase proteins (APPs) and complement, which bind to pathogens and target them for destruction and phagocytosis. APPs are produced in the liver and contribute to the systemic immune response. A raised level of the APP C-reactive protein (CRP) is a widely used marker of inflammations. As reviewed by Gao *et al.* (2008), liver hepatocytes are also responsible for the biosynthesis of 80-90% of complement components.

Adaptive immunity is mediated by clonally distributed T and B lymphocytes. The first and crucial step of adaptive immunity is the activation of T lymphocytes. By becoming activated by pathogen recognition receptors, professional antigen presenting cells (APCs) (dendritic cells, macrophages and B cells), which form part of the innate system, stimulate naïve antigen-inexperienced T lymphocytes. The APCs present fragments of the pathogen, now called antigen, to T lymphocytes in peripheral lymphoid organs (Trombetta and Mellman, 2005). APCs also produce cell-surface proteins known as co-stimulatory molecules, which provide signals to stimulate T lymphocytes to proliferate and differentiate. Antigens are presented on APCs cell surfaces, complexed with MHC molecules.

Different classes of T cells recognise intracellular and extracellular antigens. MHC class I proteins present peptides derived from endogenous antigens, generated from within a cell such as viral proteins, while MHC class II proteins bind and display proteins from exogenous antigens living in macrophage vesicles or internalised by phagocytic B-cells. MHC class I proteins are recognised by cytotoxic T cells ($CD8^+$) and MHC class II proteins are recognised by helper T (Th) cells ($CD4^+$), which in turn activate other cells of the adaptive response. All elements of the MHC:antigen complex are required for T cell recognition – neither is effective alone (Murphy *et al.*, 2008).

The differentiation of naïve T cells into functional effector cells is controlled by signals from the innate immune system (Abbas *et al.*, 1996; Murphy and Reiner, 2002). $CD4^+$ T cells are classified as T-helper 1 (Th1) and Th2 cells on the basis of contrasting patterns of cytokines they secrete (Mosmann and Coffman, 1987). The selective differentiation of precursor $CD4^+$ T cells into effector Th1 and Th2 cells is based on the cytokine environment and the antigenic peptide presented to the cells by specialised APCs (Fig 1.3). Release of IL-12 and IL-18 by macrophages and dendritic cells and IFN- γ by natural killer (NK) cells stimulates the development of $CD8^+$ T cells and Th1 cells. Release of IL-4 and IL-6 promotes the development of Th2 cells. Furthermore, Th1 cells are generally induced by viruses and intracellular bacteria, while Th2 cells are induced by allergens and helminths.

Th1 and Th2 cells are important sources of cytokine production and the Th1 and Th2 arms of the immune response are modulated by a highly integrated network of molecular and cellular interactions driven by cytokines. Table 1.3. shows the signature cytokines of the Th1 and Th2 subsets, which are known to inhibit each other's secretions. Therefore, the highly integrated network of cytokines maintains a balance between the Th1 and Th2 response.

In general, Th1 cells are responsible for generating strong cellular immunity against intracellular pathogens. Th2 cells activate differentiation and class-switching of B-cells thus promoting the development of humoral responses that direct effector functions using specific antibodies against extracellular pathogens (Fig 1.3, Table 1.3) (Abbas *et*

al., 1996; Murphy and Reiner, 2002). Allen and Maizels (1997) noted that Th1 and Th2 responses represent extremes of a continuum of cytokine production profiles, and warned that the strong existing preconceptions that exist that regarding the roles of Th1 and Th2 cytokines in parasite clearance may threaten the process of discovery. Therefore, the authors recommend that researchers assess the immune response of each by the individual cytokines and the effector pathways that are subsequently induced.

Recently a third population of CD4⁺ T cells with a regulatory function known as Th3 or T regulatory 1 (Tr1) cells were described. These cells produce IL-10 and transforming growth factor- β (TGF- β) and suppress Th1 responses (Mills, 2004; Dunne *et al.*, 2008).

Table 1.3. Cytokines and their roles in immunity

Cytokine	Producer cells	Function
IL-1	macrophages epithelial cells APCs	Stimulates macrophages and T-cells Stimulates acute phase response
IL-2	Th1 cells	Stimulates T cell proliferation Co-stimulates B-cell differentiation
IL-4	Th2 cells NK cells	Simulates B cell activation Stimulates differentiation of Th2 cells Enhances IgG1 and IgE synthesis Inhibits Th1 differentiation
IL-5	Th2 cells	Stimulates eosinophil proliferation Stimulates B cell growth and IgA switch
IL-6	macrophages Th2 cells hepatocytes	Stimulates T and B cell growth and differentiation Stimulates acute phase response
IL-8	macrophages	Stimulates cell migration
IL-9	Th2 cells	Stimulates Th2 cells and mast cell activity
IL-10	macrophages B cells Th2 cells	Inhibits macrophages and NK cell functions Inhibits Th1 cytokine production
IL-13	Th2 cells NK cells	Stimulates B-cell growth and differentiation Stimulates IgE switch Inhibits macrophage inflammatory cytokine production Inhibits Th1 cell functions
TNF- α	macrophages NK cells	Stimulates macrophages Stimulates acute-phase response
TGF- β	macrophages Th3 cells	Inhibits B,T, and NK cells and macrophages Stimulates fibrogenesis and B cell IgA switch
IFN- α	macrophages	Inhibits viral replication Stimulates NK cells
IFN- γ	Th1 cells NK cells	Inhibits viral replication and Th2 differentiation Stimulates Th1 and NK cell activation
IFN- β	fibroblasts	Inhibits viral replication
IL-18	macrophages	Stimulates Th1 cell differentiation

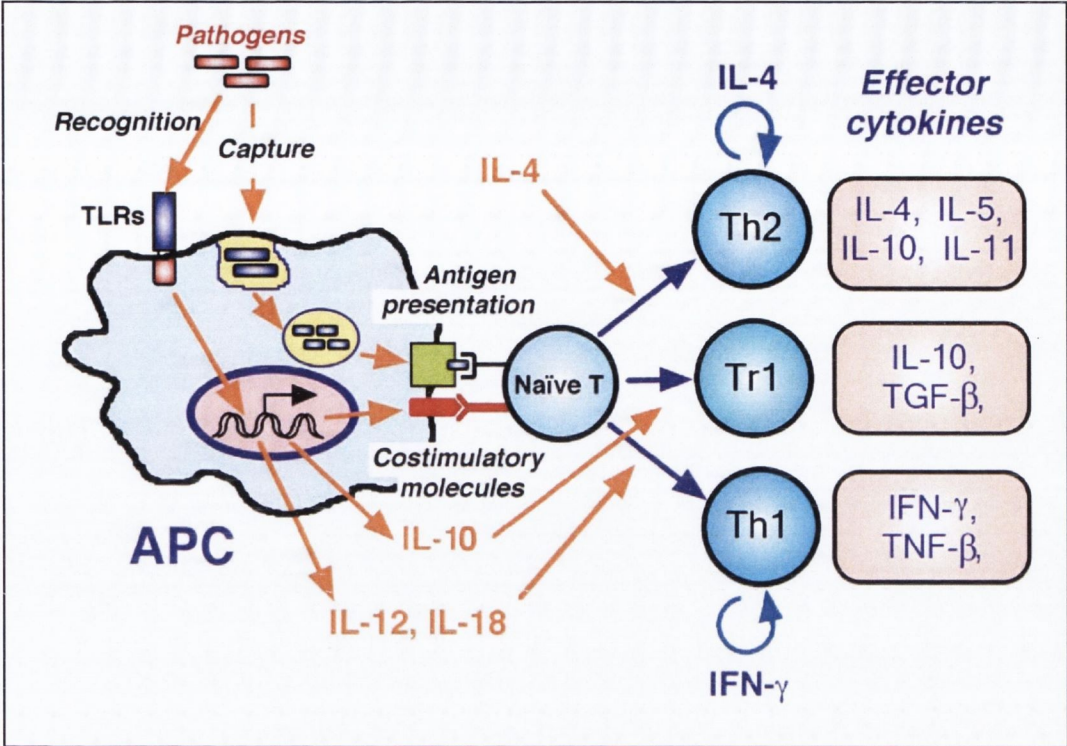


Figure 1.3. Early detection of pathogen by means of TLR and, activation and regulation of naïve CD4+ T cells
(adapted from Akira *et al.*, 2001)

1.2.9. The immunological response to helminth infection

Despite variations in the experimental outcome using different host systems, it is known that helminth parasites induce a stereotypical host response: Th2-type immunity (Grencis, 1997; Cooper *et al.*, 2000; Finkelman and Urban, 2001; Turner *et al.*, 2003), which is broadly characterised by the activation of eosinophils, basophils and mast cells and the production of IgE. As reviewed by Urban *et al.* (1992), Th2 cytokines play an important role in protective immunity to helminth infection as their secretion stimulates the major immunologic “hallmarks” of helminth infection. For example, IgE is stimulated by IL-4 (Finkelman *et al.*, 1988), eosinophilia by IL-5 (Coffman *et al.*, 1989) and mastocytosis by IL-3, IL-4 and IL-10 (Madden *et al.*, 1991; Thompson-Snipes *et al.*, 1991).

While antibody production, particularly IgE is typically detected in individuals considered resistant to helminth infection (Anthony *et al.*, 2007), antibody-independent mechanisms have also been demonstrated to be important in mediating protection from chronic helminth infection. For example, the role of intestinal mucins in expulsion of helminths, notably for *Trichinella spiralis* (Knight *et al.*, 2008) and *Nippostrongylus brasiliensis* (Miller *et al.*, 1981; Ishikawa *et al.*, 1994; Nawa *et al.*, 1994), has been previously demonstrated. Eosinophils are characteristic of the inflammatory response to helminth infection yet, as reviewed by Meeusen and Balic (2000) there is conflicting evidence as to whether or not eosinophils mediate protective immunity or expulsion of helminth parasites. Eosinophilia appears to be more effective against tissue-migrating helminths (Finkelman *et al.*, 1997; Maizels and Holland, 1998; Maizels and Balic, 2004).

1.2.10. The immunological response to *Ascaris* infection

While resistance to helminth infection tends to be associated with Th2 responses, the precise underlying immunological mechanisms that lead to the expulsion of *Ascaris* worms are yet to be defined.

It is known that gastrointestinal nematodes induce immune responses in their human hosts, although the mechanisms involved in putative functional resistance are not known. Due to ethical reasons, direct experimental studies are not possible in humans. However, various surveys of endemic host antibody and cellular immune response patterns to antigens have been undertaken, typically prior to and after chemotherapy. Such studies generally correlate the immune signalling or effector molecules with infection intensity and/or predisposition.

Early field surveys examined the relationship between specific immunoglobulin responses in *A. lumbricoides*-infected humans and intensity of infection. While the magnitude of the antibody response is likely to be determined by age, infection intensity, history of infection and individual host genetics, studies have shown that antibodies of all isotypes (IgM, IgG, IgA and IgE) are induced during human infections

with *A. lumbricoides* (Cooper *et al.*, 2000). Antibody responses appear to be stimulated by *Ascaris* larvae as demonstrated by studies involving experimental infection whereby antibodies were detected shortly after infection, lasting 3-4 months p.i. (Lejkina, 1965). In studies where interruption of transmission during the winter has been documented, host antibody levels coincide with parasite transmission in the remaining months (Lejkina, 1965).

The age-related changes in intensity patterns of *A. lumbricoides* infections observed in human populations (e.g. Elkins *et al.*, 1988), discussed earlier, are mirrored by the antibody response levels recorded, indicating that the humoral immune response is dependent on burden as opposed to being protective (Haswell-Elkins *et al.*, 1989b; Haswell-Elkins *et al.*, 1992). Coupled with this, in studies conducted in Bangladesh and Ecuador, Palmer *et al.* (1995) and Cooper *et al.* (2000) both demonstrated that antibody levels are reflective of parasite burdens.

Contrasting IgG and IgE responses have been detected in lightly- and heavily-infected hosts. Geiger *et al.* (2002) recorded that both total IgG and the subclass IgG4 were significantly elevated in response to *A. lumbricoides* in infected patient groups. In contrast to this, a minimal IgG4 response was detected in infected hosts in a Nigerian population (McSharry *et al.*, 1999), while Cooper *et al.* (2000) also showed that levels of *Ascaris*-specific IgG, IgG1 and IgG2 antibodies are positively correlated with parasite burdens. High circulating levels of IgE, in excess of 10,000 IU/ml (Cooper, 2002), and *Ascaris*-specific IgE have been consistently associated with human ascariasis (Johansson *et al.*, 1968; Jarrett and Miller, 1982; Hagel *et al.*, 1993; Lynch *et al.*, 1993; Cooper, 2002). It has been speculated that the heightened IgE response in *A. lumbricoides*-infected hosts is stimulated by larval migration (Radermecker *et al.*, 1974) because non-invasive helminths are not associated with significant increases in IgE levels (Orren and Dowdle, 1975). Heightened levels of IgE in infected hosts has been considered to be a result of direct mitogenic effects of *Ascaris* allergens on B cells responsive to *Ascaris* allergens (Lee and Xia, 1995), as many allergens are secreted during larval migration (Kennedy and Qureshi, 1986; Fraser *et al.*, 1993) or are associated with the production of large amounts of IL-4 (Cooper *et al.*, 2000). A negative association between IgE and

susceptibility to *A. lumbricoides* reinfection was reported in a Venezuelan population (Hagel *et al.*, 1993), and McSharry *et al.* (1999) found that levels of specific and total IgE were related to protection rather than to exposure to infection, indicating that high IgE producers have natural immunity to *A. lumbricoides* infection.

Focus has also been placed on cytokine expression and *A. lumbricoides* infection patterns in human populations. Cooper and colleagues (2000) first described the cytokine phenotypes associated with *A. lumbricoides* infection in endemic Ecuadorian populations and thus demonstrated that the cellular immune response to *Ascaris* antigens is characterised by a highly polarised Th2 cytokine response. In this study, frequencies of peripheral blood mononuclear cells (PBMCs), expressing IL-4 and IL-5 were significantly greater in the infected group, whereas the frequencies of IL-10 and interferon- γ (IFN- γ) were similar in the both the infected and control groups. While levels of IFN- γ were not raised in infected subjects, there was no evidence for impaired IFN- γ expression, which may indicate a mixed Th1/Th2 response (Cooper *et al.*, 2000).

A study on a Cameroonian population further highlighted the importance of a Th2 response and resistance to *Ascaris* infection, as hosts considered persistently susceptible were characterised by a weak Th2 response (Jackson *et al.*, 2004). Resistance to reinfection at 8-9 months post-chemotherapy was positively related to pre-treatment levels of IL-5 (Jackson *et al.*, 2004), indicating a negative association of this Th2 cytokine with general susceptibility. This is consistent with the effectiveness of IL-5 driven Th2 cellular responses being greatest against larvae, rather than established adult infections (Maizels and Balic, 2004). Furthermore, IL-13 levels have been documented to be negatively associated with general susceptibility (Jackson *et al.*, 2004). In addition a separate study recorded increased IL-13 levels in the older age classes, in which lower infection intensities were observed (Turner *et al.*, 2003).

Contrasting levels of IL-10 have been documented in endemic populations, with some studies showing no difference in expression between the infected and uninfected groups (Cooper *et al.*, 2000), while other authors document an inverse correlation with intensity of *A. lumbricoides* infection (Turner *et al.*, 2003). It has been speculated by

Turner *et al.* (2003) that the presence of IL-10 may be “reflective of a regulatory network that is induced to hold potentially damaging inflammatory responses in check as has been proposed by the hygiene hypothesis”.

The studies detailed above which evaluated cytokine responses to *Ascaris* infection all revealed that Th2 cytokines are important in mediating resistance to *Ascaris* infection (Cooper, 2002; Turner *et al.*, 2003; Jackson *et al.*, 2004), although the way in which the Th2 response coordinates this protection is still unknown. Both Turner *et al.* (2003) and Jackson *et al.* (2004) conducted their studies in Cameroon albeit in different provinces. Despite this, when results from the two studies were compared, variations in the Th2 cytokines were observed with different age-dependent trends at different localities. This led the authors to conclude that age- and location-related differences may impact cytokine responses to infection, adding another layer of complexity to the immunological response to *A. lumbricoides* and an additional factor to consider when implementing control strategies.

An inflammatory reaction in the liver has also been observed in humans infected with *A. lumbricoides* (Javid, 1999) and *A. suum* (Sakakibara *et al.*, 2002; Kakiyama *et al.*, 2004). Furthermore, increased levels of the indices of inflammation, C-reactive protein (CRP), ferritin and eosinophil cationic protein, found in putatively immune children, indicate that an ongoing inflammatory response may represent the antiparasite effector mechanism (McSharry *et al.*, 1999). However, since direct experimental work is not possible on humans it is still unknown whether this ongoing inflammatory response is localised or occurring in multiple tissues invaded by *Ascaris*.

Studies on the immunology of *Ascaris* infection in the pig model have contributed to our understanding of the response in human hosts. Various immunological studies in the pig have focused on the possibility of stimulating a pre-hepatic barrier. Reduced recovery of hepatic larvae and associated inflammation indicating protective intestinal immunity has been observed in naturally-infected pigs (Urban *et al.*, 1988; Eriksen *et al.*, 1992a; Eriksen *et al.*, 1992b). Furthermore, on return to the small intestine post migration, pre-adult larvae shift distally and are expelled between days 14-21 p.i. This pattern of self-

cure expulsion was observed during experimental single infections and did not appear to be influenced by the size of the inoculation dose (Roepstorff *et al.*, 1997).

It has been speculated that expulsion of larvae on return to the intestine is a result of either a density-dependent self-reduction of the larval population or a specific immune-mediated reaction. Eriksen (1981) noted that, during primary infections, increased mucus secretion and desquamation of the intestinal epithelial cells occurs concurrently with the re-arrival of larvae to the intestinal lumen. Once larvae left the intestine, an increase in mast cells was observed and normal tissue architecture was rapidly re-established. During secondary infections, the nature of the reaction in the intestine and all migratory route tissues was similar yet heightened and was observed earlier. Miquel *et al.* (2005) examined the dynamics of the immune responses before, during and after expulsion. An increase in *A. suum*-specific IgM and IgA antibody-secreting cells was found over the course of the expulsion period, with a more pronounced increase in IgA levels. The antibody response to *Ascaris* antigens in the luminal small intestine was also more prominent in the proximal than in the distal jejunum, which may be related to the longer antigen exposure time at this site, as large numbers remain at the former site for 5-7 days prior to expulsion.

While an increase in localised IgA in the intestine may be involved in the process of intestinal expulsion, recent work conducted by Nesjum and colleagues (2008) has indicated that parasite genetics may also play a role, as an uneven distribution of four different genotypes was documented in the intestine under controlled experimental infections. Furthermore, circumvention of the hepatic stages of migration may decrease intestinal expulsion (Jungersen *et al.*, 1999a), indicating a possible immunological role of the liver in initiating larval expulsion on return to the small intestine.

While it is known that the development of strong immunoglobulin E (IgE) is characteristic of helminth infections, the lack of porcine anti-IgE prohibits studies on the dynamics of the serum IgE during helminth infection in porcine hosts. However, biological activities related to IgE production, such as degranulation of intestinal mucosal mast cells, have been observed in trickle-infected pigs (Ashraf *et al.*, 1988;

Urban *et al.*, 1988). However, this response was not observed in a later single infection experiment (Miquel *et al.*, 2005), indicating that repeated exposure is necessary. Increased serum levels of IgA and IgG have been detected in response to antigens in infected pigs (Lind *et al.*, 1993; Hill *et al.*, 1994; Frontera *et al.*, 2004; Nejsum *et al.*, 2009b), and Frontera *et al.* (2004) also demonstrated that IgG was positively correlated with the severity of the hepatic inflammatory reaction but negatively correlated with the number of larvae recovered in the lungs, indicating a protective function.

The characteristic hepatic inflammatory reaction in pigs, known as white spot formation (described above), is observed during the larval stages of porcine infections (Schwartz and Alicata, 1932; Eriksen, 1981; Pérez *et al.*, 2001; Frontera *et al.*, 2003). Induced by migrating larvae in the liver (Schwartz and Alicata, 1932; Copeman and Gaafar, 1972), early lesions are composed of necrotic haemorrhagic foci, surrounded by infiltrates of numerous eosinophils, a smaller number of neutrophils and small numbers of macrophages (Pérez *et al.*, 2001). WS have been proposed to play a role in immunity to *A. suum* infection in pigs (Copeman and Gaafar, 1972; Eriksen *et al.*, 1980). *A. suum* larval debris has been previously detected within GT-WS (Schwartz and Alicata, 1932), centrally located within the granular mass (Schwartz and Alicata, 1932). Numerous macrophages, some fibroblasts and lymphocytes have been observed in the granulomatous lesions. In some cases, Pérez *et al.* (2001) reported that larger granulomas were surrounded by fibrous connective tissue. The diffuse lymphocyte infiltrate of granulomatous lesions was composed mainly of CD3+ T cells, with only a few CD79a+ B cells. The older lymphoid follicles were mainly composed of CD79a+ B cells with IgG+ plasma cells at the periphery of the lesions, indicating that this immunoglobulin is important in the local response to *Ascaris* hepatic tissue invasion. IgA+ and IgM+ plasma cells were rare. Anti-MHC class II mAb reacted with the majority of the macrophages and lymphocytes in granulomatous lesions, but this cannot be interpreted as activation as this antigen is constitutively expressed by porcine T lymphocytes.

The larvae-induced GT-WS have been proposed as a precursor to the lymphonodular type WS (LN-WS) (Ronéus, 1966; Copeman and Gaafar, 1972), as the appearance of the

latter on day 10 p.i. in pigs coincides with the healing of GT-WS (Roepstorff *et al.*, 1997). Pérez *et al.* (2001) likened the LN-WS cellular distribution to that of the cortex of lymph nodes, with the majority of lymphoid cells being CD79a⁺ B cells with a few CD3⁺ T cells. A variable number of the B cells were shown to be IgG⁺ plasma cells, while only a few IgM⁺ and IgA⁺ cells were detected. Dendritic-like cells with cytoplasmic projections were also recorded, which may be responsible for enhancing antigen presentation.

Pérez *et al.* (2001) noted that the macrophages and neutrophils observed in the haemorrhagic foci often expressed alpha-1-antitrypsin (A1AT), which is known to protect host tissues from enzymatic damage, and Mac387. In contrast, the granulomatous lesions were composed of a marked infiltrate of eosinophils and macrophages yet the majority of the latter did not express A1AT or Mac387, which agrees with previous results in which mature macrophages in tissues did not react with these markers (Mombello *et al.*, 1989; Mozos *et al.*, 1999).

Increased liver pathology has been shown to be related to a reduction in lung larval numbers in repeated experimental inoculations (Eriksen, 1981; Eriksen *et al.*, 1992a; Jungersen *et al.*, 1999b) and in consistently naturally-exposed herds (Eriksen *et al.*, 1992a). Studies in which pre-hepatic protective immunity was not observed involved immunization with ultraviolet-irradiated *A. suum* eggs (Urban and Tromba, 1982) or with different antigen preparations (Urban and Romanowski, 1985; Hill *et al.*, 1994).

Eosinophil infiltrates are typically observed during the course of *A. suum* infection in pigs, and levels of peripheral blood eosinophilia are elevated in secondary infections (Eriksen, 1981). A localised response of eosinophils around larvae may be reflective of the extent of larval invasion rather than necessarily being involved in overall pathology. Eosinophils have been observed in histopathological sections of liver and lung tissue in pigs and also mice infected with *Ascaris* (Bindseil, 1981; Eriksen, 1981; Pérez *et al.*, 2001; Lewis *et al.*, 2007) yet it is not clear if these cells were present at the time of parasite killing or a consequence of larval death and tissue damage.

The inflammatory reaction in response to invading *Ascaris* larvae has been examined previously in model organisms such as calves (McCraw and Greenway, 1970), guinea pigs (Fallis, 1948; Leventhal *et al.*, 1978), rabbits (Arian and Crandall, 1962) and mice (Bindseil, 1981; Eriksen, 1981). Eriksen (1981) demonstrated that WS in mice were detected in the liver tissue and omentum, some of which contained immobilised larvae. This reaction, however, was less marked in athymic mice, indicating that WS were influenced by a thymus-dependent immune response as was also later demonstrated in pigs (described earlier) (Pérez *et al.*, 2001). Crandall *et al.* (1978) have examined the quantitative changes in the splenocyte populations in *A. suum*-infected C57BL/6j and reported an increase in lymphocytes, eosinophils, neutrophils and plasma cells at 11 days p.i. Coupled with this, an increase in IgG and IgM levels was observed in the third week of infection. Further work on the humoral response to *Ascaris* infection was undertaken in guinea pigs, whereby protection (significantly reduced larval numbers) was observed in recipient animals once administered IgG1+IgE or IgG2 (Khoury *et al.*, 1977).

Experiments on guinea pigs also involved donating cells drained from the hepatic, mediastinal and mesenteric nodes of animals demonstrating protection and transplanting them to recipient animals, which were then infected once. Cells from the hepatic nodes were shown to be significantly more effective in conferring protective immunity upon normal recipients. Therefore, Khoury *et al.* (1977) concluded that “the major protective immune mechanisms to *A. suum* take place at the hepatic level”.

Nematode parasites are known to have a tough outer cuticle that confers a degree of protection against host immune responses but is also antigenic. Antigenic deposits have been found in the livers of immunised and infected pigs (Frontera *et al.*, 2003), which were thought to be the remains of L₃ stage larvae which have been trapped by inflammatory cells since the body wall antigen was the strongest antigen detected in these livers. Throughout larval migration through host tissues, *A. suum* complete an important metabolic process (Guerrero and Silverman, 1969; Kennedy and Qureshi, 1986), in which excretory-secretory (ES) materials are produced. These metabolites

have since been shown to be stage-specific during development (Kennedy and Qureshi, 1986).

Furthermore, a proportion of these secreted products have been shown to differ in *A. suum* and *A. lumbricoides* (Kennedy *et al.*, 1987). *Ascaris* ES antigens have been also detected in the liver of pigs, which again may account for liver pathology (Frontera *et al.*, 2003). *Ascaris* body fluid is also antigenic, and the 14.6kDa body fluid protein ABA-1 being the major allergen of *Ascaris*. ABA-1 is the most abundant protein in the parasite's body fluid (Tomlinson *et al.*, 1989) and is present in all stages of the *Ascaris* life cycle (Kennedy and Qureshi, 1986; Kennedy *et al.*, 1987). The amino acid sequence is highly conserved between *A. suum* and *A. lumbricoides* (Christie *et al.*, 1990). Recognition of ABA-1 in naturally infected humans varies considerably (Haswell-Elkins *et al.*, 1989b; Kennedy *et al.*, 1990; McSharry *et al.*, 1999), while recognition is highly restricted in both naturally and experimentally infected pigs (Jungersen *et al.*, 1999b). Nevertheless, work with *Ascaris* ABA-1 has provided evidence for a significant relationship between predisposition status and parasite-specific IgE, as described above (McSharry *et al.*, 1999). McSharry *et al.* (1999) also tested a further two antigens; *Ascaris* body/pseudocoelomic fluid (ABF) and a commercially prepared crude allergen extract from the helminth known as *Ascaris* p1, were also tested in order to observe whether they induced a response in consistently lightly- and heavily- infected individuals. Interestingly only the refined ABA-1 antigen provided evidence for a significant relationship between predisposition status and parasite-specific IgE as the putatively immune individuals tended to have higher levels of recombinant ABA-1-specific IgE in contrast to the susceptible group who had low levels.

While it is now accepted that *Ascaris* spp. hatch as L3 stage larvae (Geenen *et al.*, 1999; Fagerholm *et al.*, 2000), previous studies have demonstrated a low response to L₂/L₃ ES antigen significantly correlated with high worm burdens in the intestine (Lind *et al.*, 1993). This, in combination with the differing results obtained by McSharry *et al.* (1999) dependent on the target antigen assessed, indicates that antigen response studies would benefit from the use of standard antigens. Many of these antigens have been characterised but yet few studies in humans and pigs involving parenteral application

have induced a protective response in the host comparable to that induced by egg inoculation.

1.2.11. The role of genetics in helminth infection

The host immunological response is an important determinant of host variation in susceptibility to helminth infection. Differences in host immunological responses are ultimately determined by host genetics. However, the genetic constitution of both host and parasite has fundamental implications for the immunity, epidemiology and evolution of helminth infection (Wakelin, 1985; Wakelin and Walliker, 1996). Differential host immune responses to infection, which regulate parasites and thus control resistance or susceptibility at the individual and population level, are genetically determined. Coupled with this, parasite genes determine characters such as virulence and vary in their capacity to induce host responses (immunogenicity). Furthermore, helminths vary in their ability to suppress the host's immune response (immunomodulation) or alternatively evade the host's immune response (Read and Viney, 1996).

Dissecting the genetic control of resistance to helminth infection can prove problematic for various reasons. The complexity of the host-parasite relationship and multiple possible host immune responses indicates that the genetic factors influencing the host's capacity to respond protectively to infection are polygenic. The field of immunogenetics, which incorporates the link between genetics and immune related diseases (reviewed by Geraghty, 2002) has indicated that much of the genetic component accounting for interindividual infection intensity is specified by many minor susceptibility genes rather than primarily by a few major loci (Hill, 2001).

As reviewed by Quinnell (2003), susceptibility to helminth infection in human populations has been shown to vary among ethnic groups, yet reliable studies investigating the effect of genetic relatedness on helminth infections requires pedigree studies. In a pedigree study one can trace a variant phenotype in related individuals

living in different households and unrelated individuals living in the same household, therefore eliminating environmental factors that may mask genetic effects on the phenotype.

Pedigree studies not only provide information on the extent of genetic determination of susceptibility to infection but also the pattern of inheritance of susceptibility genes. Within pedigree populations or laboratory model studies, there are two main approaches to investigating the nature and extent of the host genetic contribution to variation in susceptibility to helminth infection; genome-wide linkage studies and candidate gene association studies. Genome-wide scanning involves the use of genetic markers, regions of repetitive 'microsatellite' DNA which provide positional reference points from which one can determine the chromosomal regions linked with resistance. Further analysis is required in this technique to identify the specific alleles within the loci which are linked to the trait. Genetic linkage studies analyse the segregation of genetic markers and a disease in large multigenerational pedigrees.

Investigating the association of host candidate genes with resistance is generally based on previous genetic studies or is suggested on the basis of immunological grounds (Quinnell, 2003). Since the MHC gene products are involved in regulation of T-cell function, there has been much focus on investigating the involvement of MHC-linked genes coupled with genes for cytokine and chemokine receptors in controlling resistance to helminth infection (Hill, 1998).

As discussed by Charbonnel *et al.* (2006), heritability values "provide a measure of the cumulative effect of all genes involved in resistance, but cannot provide any information about the specific genes involved." Such measures have been recorded for various helminth parasites and range from 0.21 to 0.44 (reviewed by Behnke *et al.*, 2003).

1.2.12. The role of genetics in *Ascaris* infection

While the generative mechanisms of predisposition are currently unknown, it is believed that host genetics may play a major role (Holland *et al.*, 1989; 1992; Peng *et al.*, 1996; Williams-Blangero *et al.*, 1999; 2002; 2008). Ascariasis has been referred to as a familial disease by Williams *et al.* (1974) and infection has been shown to cluster in households and within families (Chai *et al.*, 1983; Forrester *et al.*, 1990; Chan *et al.*, 1994a). However, studies of familial aggregation in human host populations are confounded by environmental, sanitation and family behavioural factors, which are difficult to disentangle from the effects of host genetics. Therefore, further work has been aimed at determining the genetic loci involved in variation of susceptibility and resistance. Such work involves initially observing the phenotypic differences between the individuals in the study population and conducting a genome scan or observing differences in the expression of a candidate gene (reviewed by Hill, 1998; Quinnell, 2003).

The link between MHC genes and the immune response stimulated research on the association of MHC antigens and resistance to infection. Holland *et al.* (1992) undertook an associational study in which the distribution of human leukocyte antigen HLA-A, HLA-B and HLA-C was assessed in consistently uninfected, lightly infected and heavily infected Nigerian children. The frequency of occurrence of the A30/31 antigen was significantly higher in the hosts who were consistently infected, indicating a role of the MHC in determining resistance to *Ascaris* infection. Coupled with this mouse strains sharing MHC haplotypes had identical recognition profiles when exposed to *Ascaris* antigens, while the response observed in mice with differing MHC haplotypes was readily apparent (Tomlinson *et al.*, 1989). Further to this, relationships of the candidate gene, the non-MHC gene signal transducer and activator of transcription 6 (STAT 6) which promotes expression of Th2 associated genes has been shown to be linked with *Ascaris* resistance (Peisong *et al.*, 2004). Furthermore, polymorphisms in the β_2 -adrenoreceptor gene were found to correlate with varying *Ascaris* egg loads in Venezuelan children (Ramsay *et al.*, 1999). The latter candidate study is of particular interest as one of the polymorphisms observed accounted for 25% of the variation in

the *Ascaris* egg loads, and also the gene is located on the same chromosome 5q region as the SMI gene, which is known to influence variation in schistosome infection (Marquet *et al.*, 1996; 1999)

Detailed research has been conducted in East Nepal (Williams-Blangero *et al.*, 1999; 2002; 2008) in genome scanning individual human hosts from a Jirel population (Jiri Helminth Project). The worm burden of the individuals in the study group was first assessed to determine the intensity level of infection. The study design, which utilizes a single large pedigree, allows for discrimination between the effects of shared environment and genetics. The initial research with the pedigree focused on clarifying whether host genetics in fact played a role in predisposition. High within-individual correlations indicated that there is individual predisposition, which also appeared to be heritable. From this data it was calculated that a host genetic component accounted for 30-50% of variation in the worm burden harboured, while the effects of a shared environment accounted for 3-13% of variation in helminth load (Williams-Blangero *et al.*, 1999). A similar heritability measure (0.35 for total *A. suum* eggs excretion and 0.44 for actual worm counts) was later identified in pigs (Nejsum *et al.*, 2009a).

Conducting further research on the Jiri pedigree, Williams-Blangero *et al.* (2002) undertook a genome scan project in which they found strong evidence for two distinct quantitative trait loci (QTL) influencing the susceptibility to *Ascaris* infection. The genes are on chromosomes 1 and 13 and there was also suggestive evidence for a third locus influencing *Ascaris* burden on chromosome 8. Evidence for the latter quantitative trait locus was significantly less than that for the former two. Further genome scanning in a higher number of host individuals of the Jirel pedigree was subsequently undertaken, in which six and three chromosomal regions were identified respectively, with evidence for QTLs influencing the intensity of and susceptibility to *Ascaris* infection (Williams-Blangero *et al.*, 2008).

The significant QTL on chromosome 13, influencing susceptibility to *A. lumbricoides* infection contains a major candidate gene called *TNFSF13B* (also known as *BlyS*) (Williams-Blangero *et al.*, 2002). The pedigree studies conducted by Williams-Blangero

et al. (2002; 2008) indicated that *TNFSF13B* is involved in determining susceptibility and resistance to *A. lumbricoides* infection. *TNFSF13B* is a member of the tumor necrosis factor (TNF) superfamily of cytokines and is a major regulator of B cell activation and immunoglobulin secretion (Moore *et al.*, 1999; Schneider *et al.*, 1999; Yan *et al.*, 2000). This cytokine is also involved in promoting the survival of immunoglobulin-secreting cells and so may be active in enhancing antibody responses to *Ascaris* infection. Williams-Blangero *et al.* (2002; 2008) have also recognised two further quantitative trait loci (QTL), the first of which is involved is a membrane-bound inhibitor of the cytolytic membrane attack complex (MAC) of complement (Kimberley *et al.*, 2007). There are two potential genes in the region of the second QTL, one of which is a member of the collectin family of soluble PRRs (Gupta *et al.*, 2007), while the second gene is a T cell differentiation protein (Bello-Morales *et al.*, 2005). The high number of genes in the regions of the identified loci indicates that it would be beneficial to assess the contribution of such candidates to resistance in experimental infection studies.

As discussed by Shaw and Quinnell (2009), “the similarity between immune responses in asthma and helminth infection has led to the hypothesis that alleles predisposing to asthma provide resistance to helminth infection.” Recently, the relationship between three genes (LIG4, *TNFSF13B* and *IRS2*) and the IgE and IgG response to *Ascaris* infection and a resistance marker, the ABA-1 allergen was investigated in an endemic Columbian population (Acevedo *et al.*, 2009). Coupled with this, a case control study was conducted to investigate whether observed polymorphisms were associated with predisposition to asthma and atopy. In this study, two of the assessed genes’ genomic location (13q33.3) was implicated in resistance to *Ascaris* infection (Williams-Blangero *et al.*, 2002; 2008). The two genes *TNFSF13B* and *LIG4* were shown to modulate the IgE and IgG response to *A. lumbricoides* infection, thus appearing to be involved in conferring protective immunity to the helminth parasite (Acevedo *et al.*, 2009).

In contrast with previous work in which exposure to infection appeared to mask the influence of host genetic factors on predisposition (Chan *et al.*, 1994b), Williams-Blangero (1999; 2002; 2008) clearly demonstrated a role for host genetics in determining infection intensity. In their most recent paper, Williams-Blangero and

colleagues concluded that efforts will now focus on the characterisation of the specific genes underlying the three significant QTL regions that have been identified (Williams-Blangero *et al.*, 2008). The importance of this work was recently highlighted, as identifying the underlying genes could provide new approaches to the treatment or prevention of infection (Petri *et al.*, 2008).

1.3. Objectives of this thesis

The work detailed in the present thesis was undertaken in two mouse strains that represent opposite response phenotypes, mimicking the extremes of predisposition detected in humans and pigs. Overall, the objective of this thesis was to investigate the underlying mechanism of resistance/susceptibility to *Ascaris* infection. All experiments conducted involved the comparison of different host parameters in the two different host strains during single-pulse infections.

Experiments were undertaken in order to fulfil the following individual aims:

- To clarify precisely the locations and times of larval attrition during migration of *A. suum* in C57BL/6j and CBA/Ca mice. This was undertaken by means of enumerating larvae recovered from sections of the intestines, and composite lobes of the liver and lungs. Larval burdens, over an eight day period were then compared in order to identify the organ in the parasite's migratory route at which resistance is manifested (Chapter 3).
- To investigate whether differences existed between the broad pulmonary artery architecture in C57BL/6j and CBA/Ca mice. Optical Projection Tomography (OPT) was used to assess whether the pulmonary vasculature varies between the mouse strains, which could then account for a lower pulmonary larval burden in CBA/Ca mice (Chapter 3).
- To observe the timing and severity of granulocyte infiltration during hepatic and pulmonary larval migration in C57BL/6j and CBA/Ca mice. Histopathological assessment of hepatic and pulmonary tissue was undertaken in order to determine differences in the response to infection that may account for varying larval burdens between strains (Chapter 4).

- To investigate whether the Intelectin-2 gene, which is absent in C57BL/6j mice influences susceptibility to *Ascaris* infection. A backcross experiment was undertaken with the two mouse host strains to test the association between the gene and susceptibility to *A. suum* infection (Chapter 5).
- To observe cytokine production of hepatic tissue in response to *A. suum* infection. Production levels of cytokines in the liver of infected mice were assessed by means of ELISA and compared (Chapter 6).

2. General Materials and Methods

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2.1. Maintenance of mice

Mice were housed in animal maintenance rooms of the Bioresources Unit (Trinity College Dublin) for the duration of the experiments. The room was maintained at approximately 22°C, and operated on a 12 hour light/dark photoperiod (8am lights on – 8pm lights off). On arrival, mice were randomly assigned to groups of four mice or less per standard plastic cage (35 x 15 x 13 cm). Each cage contained isopod bedding and plastic Perspex tubing (approximately 10cm). Water and pelleted commercial food were supplied *ad libitum*, and cages were cleaned on a regular basis. In order to identify individual mice within a cage, each subject's ear was punched with a different marking (Fig. 2.1). If aggressive behaviour was observed during the course of experiments, mice were transferred to individual cages.



Figure 2.1. Ear-punch marking system used to identify individual mice in each cage.

(A) Full circle in right ear (B) Full circle in left ear (C) Half circle in right ear (D) Half circle in left ear (E) No markings. (adapted from Duke University and Medical Center Animal Care and Use Program homepage:

<http://vetmed.duhs.duke.edu/index.htm>)

2.2. Preparation of *Ascaris suum* inocula

Approximately 5,000,000 embryonated *Ascaris suum* ova (batch no AP 04.04) were supplied by the Danish Centre for Experimental Parasitology (CEP), Copenhagen. The suspension was gently agitated by hand and placed on a magnetic stirrer (Labinco L32) in order to ensure homogeneity. To calculate the number of eggs in suspension, 10µl was transferred to a glass slide and examined under a Nikon light microscope, using a x10 objective. Embryonated and unembryonated eggs were separately enumerated as the slide was scanned, so that the total number of eggs and the percentage of embryonated eggs per 10µl could be calculated. This was repeated for ten 10µl aliquots, the average number of embryonated eggs was calculated and the appropriate calculations were carried out to determine the number of infective eggs in suspension. A corresponding volume of suspension to provide a dose of 1000 infective eggs was then aliquoted into clean 1.5ml microcentrifuge tubes. Each aliquot was supplemented with 0.1M H₂SO₄ to 100µl. During each preparation of inocula, five extra doses were always prepared and the dose was checked prior to infection. Placebo doses for control mice consisted of 100µl of 0.9% saline or 0.1M H₂SO₄ only.

2.3. Infection of mice

In all experiments, mice were purchased at 7 weeks old and were allowed to acclimatise for one week prior to experimental procedures. On the day of infection, all mice were weighed prior to inoculation. Prepared doses were drawn up using a 1ml graduated syringe attached to a slightly curved needle with a bulb at the tip, used for intragastric intubation. Mice were restrained gently at the nape of the neck and the intubation needle was inserted into the mouth and guided down the oesophagus and into the stomach. If any resistance was felt, the needle was removed and repositioned. Once in the stomach the syringe was depressed slowly, which released the egg suspension into

the stomach and the intubation apparatus was removed. Control mice were handled in the same manner, but inoculated with placebo doses rather than infective ova.

2.4. The modified Baermann technique

All living larvae were isolated from mouse tissue using the modified Baermann Technique (Eriksen, 1981; Lewis *et al.*, 2006). The modified Baermann apparatus (Fig. 2.2) was constructed using a 20ml universal sample tube from which the base was removed. A mesh, made from a double layer of gauze, was fixed to the bottom of the sample tube with a rubber band. The sample tube was placed in a 50ml centrifuge tube so that the end with the gauze reached the 35ml mark. Prior to the addition of the sample tube, 35ml of 0.9% saline was added to the centrifuge tube and placed in a water bath at 37.5°C overnight. At post-mortem, mouse tissue was removed and placed in a 10 ml flat bottom glass beaker with approximately 1ml of warm 0.9% saline. Tissues were macerated using a fine scissors and poured into an individual modified Baermann apparatus. Each beaker was rinsed with warm 0.9% saline and poured into the apparatus to ensure the entire tissue sample was completely collected. Saline was subsequently added up to the 50ml mark on the centrifuge tube and sealed in order to prevent evaporation. Each apparatus was held in a water bath at the 35ml mark for 24 hours at a constant temperature of 37.5°C.

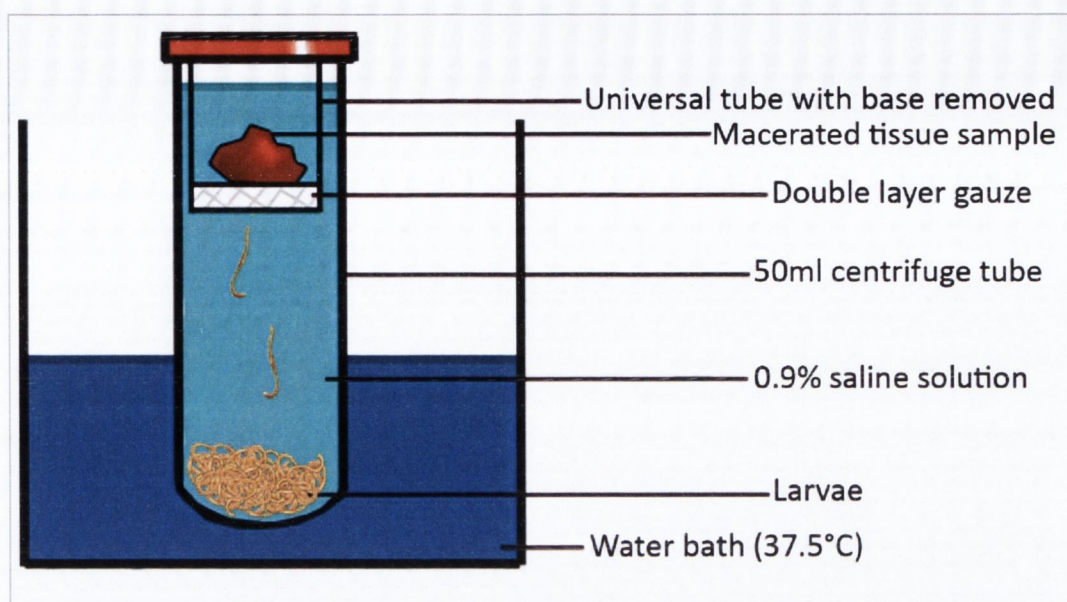


Figure 2.2. The modified Baermann apparatus
(Original illustration)

2.5. Larval recovery and enumeration

Each modified Baermann apparatus was removed from the water bath after 24 hours. The sample tube and gauze were removed and saline was added to the 50ml mark if required. Samples were centrifuged at 3000 rpm for five minutes. Saline was aspirated to the 10ml mark and 10ml of 10% formalin was added. On the day of larval enumeration, samples were centrifuged for five minutes at 805g. The solution was aspirated to the 5ml mark whilst taking care not to disturb the pellet of isolated viable larvae. Prior to larval counts, the 5ml solution was agitated to ensure a homogenous distribution of larvae within the sample. Larval counts were performed on 1ml of each sample. The amount of debris in samples differed between the tissue type examined so therefore the quantity of solution examined on a slide varied. In order to enumerate the live larvae in the liver, large and small intestinal samples, twenty 50 μ l, ten 100 μ l and five 200 μ l aliquots respectively were screened under x40 magnification (Table 2.1). The average number of larvae recorded in the aliquot volume was calculated and the total

number of larvae in the 5ml sample was determined using the appropriate multiplication factor (Table 2.1).

Table 2.1. Number of aliquots required to enumerate larvae in intestines and liver

Organ	Quantity of sample solution per aliquot (μl)	Number of aliquots	Multiplication factor
large intestine	50	20	100
small intestine	100	10	50
liver	200	5	25

When recording the larval burden in the lung samples, 2ml was pipetted into the chamber of a nematode counting slide (Chalex Corporation) (Fig. 2.3). The number of larvae present in the grid area, which represents 1ml, was counted under x40 magnification. The number of larvae in a 1ml solution was multiplied by total volume in order to approximate the number of larvae in the tissue sample.

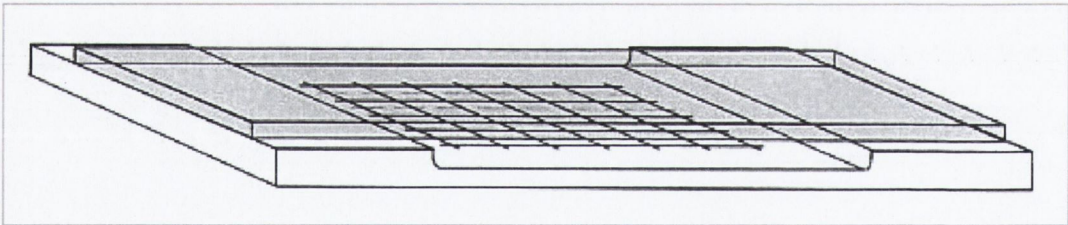


Figure 2.3. Nematode counting slide
(<http://www.vetslides.com>)

3. The kinetics of early *Ascaris suum* infection in C57BL/6j and CBA/Ca mice, with particular focus on the composite lobes of the liver and lungs

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3.1. Introduction

The course of *A. suum* larval migration has been extensively studied in the parasite's definitive host, the pig (Kelley *et al.*, 1957; Douvres and Tromba, 1971; Keittivuti, 1974; Eriksen *et al.*, 1992b; Murrell *et al.*, 1997; Roepstorff *et al.*, 1997; Slotved *et al.*, 1998; Boes *et al.*, 2002). Prior to establishment in the small intestine as adult worms, *Ascaris* larvae grow in host parenteral tissues. When pigs ingest infective ova, L₃ larvae hatch in the small intestine and migrate to the caecum and colon, where they penetrate the mucosa (Murrell *et al.*, 1997). The larvae then migrate via the portal blood to reach the liver, which they subsequently leave via the hepatic veins and migrate to the lungs. Larvae enter the lungs through the pulmonary artery and traverse the capillary beds into the neighbouring alveoli (Roepstorff *et al.*, 1997). From here, microcilliary movements allow the larvae to move to the pharynx where they are swallowed resulting in the helminths returning to the small intestine (Douvres *et al.*, 1969; Roepstorff *et al.*, 1997).

Experiments investigating the migratory route of *Ascaris* larvae in abnormal hosts have also been undertaken. *Ascaris* larval migration has been extensively studied in the mouse (Ransom and Foster, 1919; Jenkins, 1968; Douvres and Tromba, 1971; Keittivuti, 1974; Song *et al.*, 1985; Slotved *et al.*, 1998; Lewis *et al.*, 2006; 2007). As observed in porcine hosts, *Ascaris* larvae in mice also penetrate the caecum (Keittivuti, 1974; Slotved *et al.*, 1998). Furthermore in mice, larvae migrate to the liver and later accumulate in the lungs, however over a shorter time course (Slotved *et al.*, 1998). In the murine host, *Ascaris* worms do not return to the small intestine post migration and do not reach sexual maturity. Therefore, since the course of migration undertaken by *Ascaris* larvae is similar in both murine and porcine hosts, albeit over different timescales, the mouse is deemed a suitable model host of early *Ascaris* migration (Murrell *et al.*, 1997; Slotved *et al.*, 1998).

Past studies on the migratory route in the pig have played a key role in contributing to the understanding of *Ascaris* migration. However large scale experiments examining

larval burdens in the mouse can be conducted with greater ease due to the host's small body size and can be undertaken over shorter time scales due to disparities in the timing of migration between the pig and the mouse. Recent studies undertaken by Lewis *et al.* (2006, 2007) revealed that similar numbers of larvae were detected in the liver of both susceptible C57BL/6j and resistant CBA/Ca mouse strains. However, the majority of the larvae observed in the liver successfully migrated to the lungs in the C57BL/6j, while far fewer larvae accumulated in the lungs of CBA/Ca mice. The contrast of resistance and susceptibility between CBA/Ca and C57BL/6j mouse strains respectively was reflected in the distinct, repeatable difference in *A. suum* larval burden in the lungs on day 7 post-infection (p.i.) (Lewis *et al.*, 2006; 2007).

The underlying mechanism responsible for the loss of *A. suum* larvae during primary infections and its precise location within the body are currently unknown. The degree of pulmonary inflammatory immune responses, marked by the infiltration of innate immune cells such as granulocytes, macrophages and leukocytes in the pulmonary tissue bronchoalveolar lavage fluid, reflected the observed larval burdens in C57BL/6j and CBA/Ca mice (Lewis *et al.*, 2006; 2007). Combined with the reduction in the lung larval burden in resistant mice, the pulmonary inflammatory response led Lewis *et al.* (2007) to conclude that a hepatic/post-hepatic factor varying between the two mouse strains is likely to account for the divergence in pulmonary larval burdens.

In previous experiments, larvae have been recovered and enumerated from whole organs. Therefore, there is a lack of knowledge regarding the exact lobar or segmental locations of attrition and accumulation within the organs of the parasite's migratory path. However selected studies involving the porcine model have divided the intestinal tissues in order to gain a more definitive insight into the parasite's migratory path. Murrell *et al.* (1997) broadly divided the small intestine into the upper and lower half for examination while additional studies have separated the small intestine into three (Keittivuti, 1974; Eriksen, 1981) or four (Eriksen *et al.*, 1992b; Roepstorff *et al.*, 1997) segments. In mice, Keittivuti (1974) identified and separated the duodenum, jejunum and ileum. The division of the large intestine has been rarely undertaken whilst examining the *Ascaris* spp. migratory route. Jenkins (1968) enumerated larvae in the

caecum, colon and rectum and Keittivuti (1974) divided the large intestine of both infected mice and pigs into the caecum and colon. Later, Murrell *et al.* (1997) similarly divided the large intestine of infected pigs. Very little focus has been placed on the larval distribution within the various lobes of the liver and lungs. McCraw and Greenway (1970) recovered *A. suum* larvae from lung lobes in calves but stated that no significant difference between the pulmonary lobes was detected.

The main aim of the present study was to further pinpoint the location at which the mechanism of resistance to *Ascaris* infection manifests itself. In order to assess the precise times and locations of larval attrition and accumulation during the tissue-migratory phase of *Ascaris* infection in C57BL/6j and CBA/Ca mice, the distribution of larval numbers was quantified in the small and large intestines and in individual hepatic and pulmonary lobes. This detailed approach to isolating and enumerating larvae in individual hepatic and pulmonary lobes enabled the sites and times of worm accumulation and attrition to be determined accurately and worm attrition to be quantified.

A secondary aim of the present study was to assess whether an anatomical difference existed in the pulmonary artery between mouse strains, hindering larval migration into the lungs. Therefore the fundamental architecture of the pulmonary artery was examined in the left and right lungs of C57BL/6j and CBA/Ca mouse strains.

3.2. Materials and Methods

3.2.1. Experiment 1: Examination of the lobar distribution of larvae in the organs of the migratory path in C57BL/6j and CBA/Ca mice

3.2.1.1. Collection of tissues post-mortem

A total of 90 (45 C57BL/6j and 45 CBA/Ca) male inbred mice were purchased for experiment 1. A sample number of five mice of each strain were sacrificed at 6 hours and days 1-8 p.i. At post-mortem the lungs, liver and intestines were removed from each individual. The small intestine was separated into 7cm segments (1-7cm, 8-14cm, 15-21cm and the remainder, 22-x cm) from the pylorus of the stomach. The large intestine was also divided into the caecum and colon. The liver was separated into its multiple lobes, the median, caudate, left and right lobes (Fig. 3.1A). Similarly, the right lung was separated into its composite lobes, the cranial, middle, caudal and accessory lobes. The left lung cannot be further separated (Fig. 3.1B). All lobes and intestinal segments were evaluated as separate entities. Living larvae were recovered from each lobe and segment by means of the Modified Baermann Technique (Lewis *et al.*, 2006) (chapter 2, section 2.4). Larvae were then enumerated as detailed in chapter 2 (section 2.5).

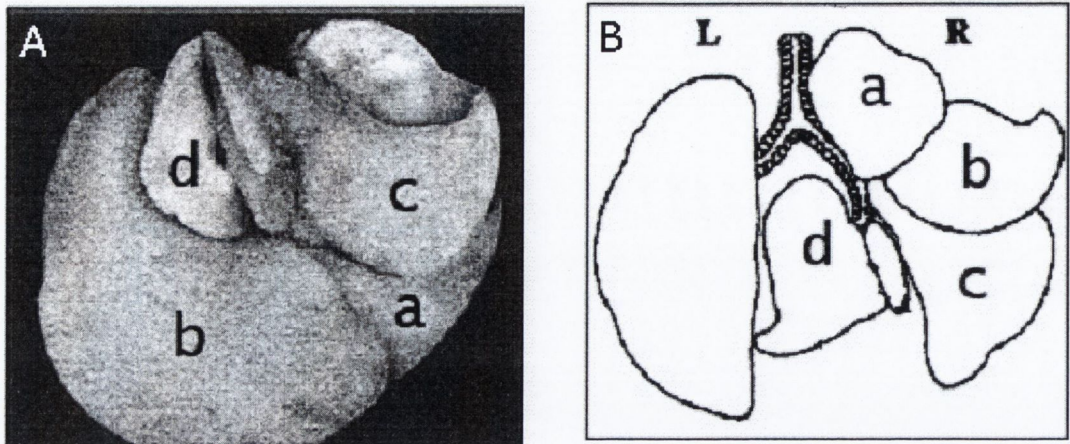


Figure 3.1. The composite lobes of the (A) murine liver (a = median lobe, b = left lobe, c = right lobe, d = caudate lobe) and (B) murine left (L) and right (R) lungs (a = cranial lobe, b = middle lobe, c = caudal lobe, d = accessory lobe)

(adapted from Maronpot *et al.*, 1999)

3.2.1.2. Statistical analysis

All statistical analysis was carried out at the 95% confidence limit. Larval recovery data were assessed for normality and were subsequently log + 1 transformed. Intestinal larval numbers were not subjected to statistical analysis as larvae were almost exclusively recovered at one post-mortem time-point. The influence of the factors, time and strain on the mean larval recoveries from the liver and lungs were investigated using a two-way General Linear Model (GLM) (SPSS 15.0). To investigate the influence of the factors, time, strain and the distribution of larvae within organs on the mean larval recoveries in the lobes of the liver and the lungs, a MANOVA (SPSS 15.0) was conducted. In order to gauge whether a larval predilection for a specific lobe in an organ exists, a MANOVA was undertaken on the proportion of total larvae in each mouse recovered from each hepatic and pulmonary lobe. Proportional data were arcsine, square root transformed prior to analysis.

3.2.2. Experiment 2: Examination of the location of the pulmonary artery in the lungs of C57BL/6j and CBA/Ca mice using Optical Projection Tomography (OPT)

3.2.2.1. Collection of tissues post-mortem

A total of four mice (two C57BL/6j and two CBA/Ca) were purchased for the second experiment. All mice were administered 1000 *A. suum* ova and were sacrificed on day 6 (p.i.), as at this post-mortem time-point in previous experiments interlobar septa were more distinct than normal and atelectasis (collapse of part or all of the lung) was also evident in CBA/Ca mice. An intraperitoneal injection of 0.5ml of Butatyl was administered to each mouse, which was returned to a separate cage until terminally anaesthetised. Reflexes were monitored and mice were not dissected until deemed unresponsive. The abdominal and thoracic cavities were exposed and the heart was held in place at the apex using a fine forceps. A 23G needle, connected to a perfusion pump (5ml/min), was inserted approximately 5mm into the left ventricle. The vena cava was immediately cut open using fine scissors to allow excess perfusate to flow out. Once the liver had changed colour from red to tan and the excess perfusate ran clear (approximately 5-8 minutes), the tube was inserted into a bottle of 0.2% glutaraldehyde and 4% paraformaldehyde in phosphate buffered saline (PBS) while the needle was held in place. The fixative was pumped through each mouse for ten minutes at which point the needle was removed. The lungs were removed and placed in 10ml of 0.01% sodium azide, 0.2% glutaraldehyde and 4% paraformaldehyde in PBS, and stored at 4°C.

3.2.2.2. Embedding tissues for Optical Projection Tomography (OPT)

The left and right lungs were individually weighed and embedded in 1% filtered low melting point agarose. Once the agarose had set, a blade was used to cut around the tissue sample with care to leave approximately 4mm of agarose on all sides. The embedded tissue was then attached to a metal mount using superglue, which was then placed in a glass bottle and dehydrated in methanol. On the day prior to scanning, the

tissue and mount were removed and placed in a clean glass bottle and covered in benzyl alcohol/benzyl benzoate (1:2) (BABB) in order to clear the tissue.

3.2.2.3. Scanning tissue

Each specimen, embedded in agarose, was individually placed in a cuvette containing BABB. The cuvette was held in a prototype OPT scanner constructed at the MRC Human Genetics Unit, Edinburgh (Sharpe *et al.*, 2002) and installed in the Zoology Department Trinity College Dublin. The specimen was simultaneously attached to the OPT scanner by means of a magnet and the attached metal mount. Whilst the specimen was rotated 360° around a single axis, a single projection image of the specimen was captured for each of 400 rotated positions (0-9° apart). Scanning was performed at different optical magnifications for the left (1.6x) and right (1.25x) lungs due to the larger size of the right lung. All specimens were scanned using the visible light channel. Autofluorescence was also tested but this light channel did not provide images of the pulmonary vasculature due to the lack of blood, which was removed during perfusion at post-mortem.

3.2.2.4. Examination of the position of the vessels entering the lungs

The pulmonary tissue was fixed as described above to ensure that the lungs would not collapse once removed from the mouse. Staining of the vasculature was not undertaken. At the time of experiments, it was thought that the fixative would prevent successful staining. Therefore, it was necessary to determine the position of the pulmonary artery so that it could be identified in the OPT images. However, recent work has shown that alcian blue staining of cartilage can be undertaken post paraformaldehyde fixation (Dr. Paula Murphy, personal communication)

In order to assess the position of the bronchi relative to the blood vessels on entry into the left and right lungs in a mouse, two male 3 month old CD1 mice were euthanised by cervical dislocation. The 'pluck' (trachea, heart and lungs) was removed and the organs

positioned and pinned to a dissection board as *in vivo*. Ventral aspect photos were taken and the position of the bronchus entering each lung relative to the blood vessels and cranial and caudal end of the tissue was examined.

The projection images, obtained using the OPT scanner were uploaded onto a Linux workstation and reconstructed using various algorithms provided by the Mouse Atlas Project, Edinburgh. Reconstructed lungs were viewed using the MAPaint programme in which the X-Y, Z-X and Y-Z plans of view were visualised simultaneously in separate windows. The point at which the pulmonary vasculature enters the lung, known as the hilar area was located in each specimen and from this point the pulmonary artery's path through each lung lobe was visualised. Furthermore the primary branches of the pulmonary artery in each lobe were also enumerated in order to assess whether differences existed between the two strains.

3.3. Results

3.3.1. Experiment 1: Examination of the lobar distribution of larvae in the migratory path in C57BL/6j and CBA/Ca mice

3.3.1.1. Larval burdens recovered in the intestines

Larvae were observed at 6 hours and on days 1 to 8 p.i. in the intestines, liver and lungs in both mouse strains, but larval burdens varied over time and between mouse strains.

The highest incidence of larvae in the large intestine of both mouse strains was recorded at 6 hours p.i. At this time-point, the majority of larvae were recovered from the large intestine and had a strong predilection for the caecum (Table 3.1). Mean larval burdens were higher in the resistant CBA/Ca mice in the caecum at 6 hours p.i. The mean larval numbers in the large intestine then decreased by day 1 p.i. in both strains and remained low over days 1 to 8 p.i. No consistent pattern distinguishing the two strains was evident (Table 3.1).

Larval numbers in the segments of the small intestines were both sporadic and very low (one to two larvae per segment per post-mortem time-point) (Table 3.2). On days 7-8 p.i., an increase in larval numbers in the lower small intestine of CBA/Ca mice was observed.

Table 3.1. Mean larval numbers ($\pm$ S.E.M) recovered at 6 hours and days 1 to 8 p.i. in the large intestine in C57BL/6j and CBA/Ca mice, following inoculation with 1,000 *Ascaris suum* ova

Day p.i.	Caecum		Colon		Total	
	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca
6h	102 $\pm$ 24.0	180 $\pm$ 45.3	7 $\pm$ 4.4	8 $\pm$ 4.9	109 $\pm$ 24.4	188 $\pm$ 45.3
1	1 $\pm$ 1.0	0	0	2 $\pm$ 1.2	1 $\pm$ 1.0	2 $\pm$ 1.2
2	1 $\pm$ 1.0	0	3 $\pm$ 1.2	1 $\pm$ 1.0	4 $\pm$ 1.9	1 $\pm$ 1.0
3	0	0	1 $\pm$ 1.0	1 $\pm$ 1.0	1 $\pm$ 1.0	1 $\pm$ 1.0
4	0	0	0	2 $\pm$ 1.2	0	2 $\pm$ 1.2
5	0	2 $\pm$ 1.2	1 $\pm$ 1.0	1 $\pm$ 1.0	1 $\pm$ 1.0	3 $\pm$ 1.2
6	2 $\pm$ 1.2	0	1 $\pm$ 1.0	3 $\pm$ 2.0	3 $\pm$ 1.2	3 $\pm$ 2.0
7	0	0	0	0	0	0
8	4 $\pm$ 2.9	1 $\pm$ 1.0	2 $\pm$ 1.2	0	6 $\pm$ 2.9	1 $\pm$ 1.0

Table 3.2. Mean larval numbers ($\pm$ S.E.M) recovered at 6 hours and days 1 to 8 p.i. in the small intestine in C57BL/6j and CBA/Ca mice, following inoculation with 1,000 *Ascaris suum* ova

Day p.i.	1-7 cm		8-14 cm		15-21 cm		22-x cm		Total	
	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca
6h	0	2 $\pm$ 2.0	0	0	0	1 $\pm$ 1.0	2 $\pm$ 1.2	0	3 $\pm$ 1.2	3 $\pm$ 2.0
1	0	0	0	1 $\pm$ 1.0	0	0	0	0	0	1 $\pm$ 1.0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	1 $\pm$ 1.0	0	0	0	0	0	1 $\pm$ 1.0	0
4	0	0	0	0	0	0	0	0	0	0
5	1 $\pm$ 1.0	0	0	0	0	0	2 $\pm$ 1.2	0	3 $\pm$ 2.0	0
6	2 $\pm$ 1.2	0	1 $\pm$ 1.0	0	0	0	1 $\pm$ 1.0	0	4 $\pm$ 1.9	0
7	0	1 $\pm$ 1.0	0	0	0	0	0	6 $\pm$ 4.9	0	7 $\pm$ 5.8
8	0	0	1 $\pm$ 1.0	0	1 $\pm$ 1.0	1 $\pm$ 1.0	1 $\pm$ 1.0	4 $\pm$ 2.9	3 $\pm$ 3.0	5 $\pm$ 2.7

3.3.1.2. Larval burdens recovered in liver

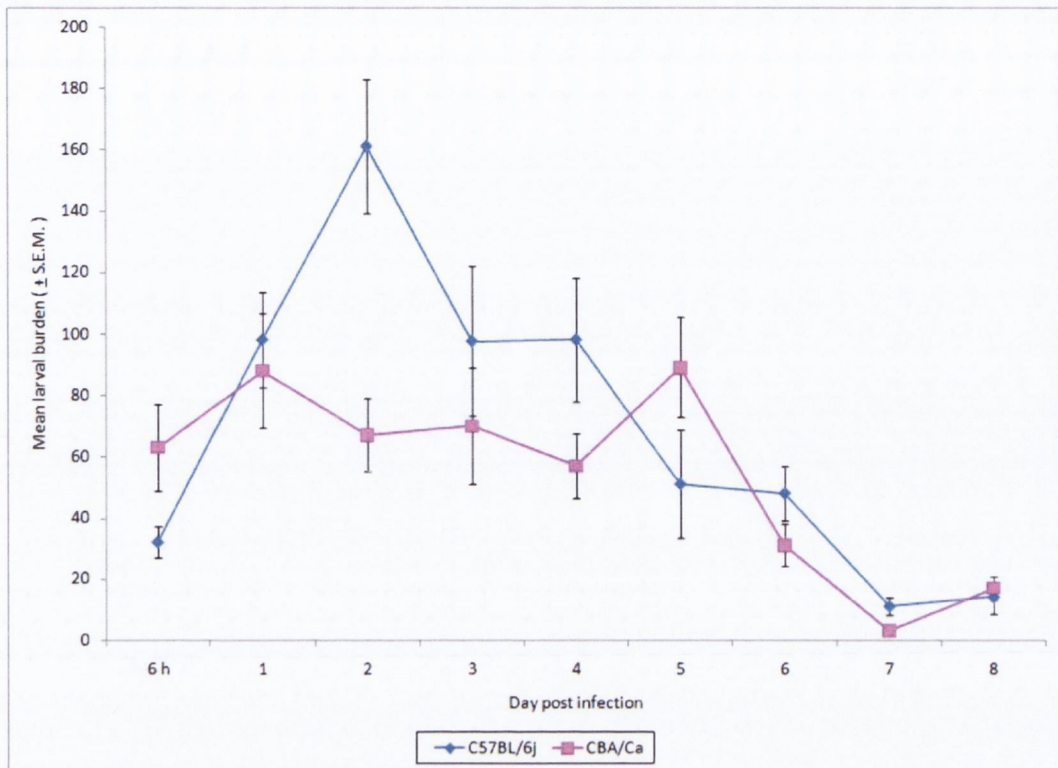


Figure 3.2. Changes in total mean larval burden ($\pm$ S.E.M.) in the liver of C57BL/6j and CBA/Ca mice, following inoculation of 1,000 *Ascaris suum* ova

A. suum larvae were recovered from the liver at every post-mortem time-point in each mouse strain. Appreciable numbers were recovered in this organ at 6 hours and marked decreases were observed on days 5 and 6 in C57BL/6j and CBA/Ca mice respectively. A two-way GLM (Table 3.3) demonstrated that the total numbers of larvae observed in the liver did not differ significantly between the two strains ($P=0.085$). However, recoveries of larvae differed significantly over post-mortem time-points ($P<0.001$). A significant strain*day interaction was found ($P=0.011$) as the larvae recovered from each strain displayed different migratory patterns through this organ (Fig. 3.2).

Table 3.3. Results of two-way GLM examining the effects of strain and day on larval burdens recovered in hepatic tissue of C57BL/6j and CBA/Ca mice

Source	df	F-ratio	P value
strain	1	3.060	0.085
day	8	23.785	<0.001
strain*day	8	2.745	0.011
error	71		

Larvae were recovered in each liver lobe as early as 6 hours p.i. In C57BL/6j the mean number of larvae peaked on days 1, 2 and 3 p.i. in the left, median and caudate, and right lobes respectively and subsequently declined to comparatively low larval numbers on days 7 and 8 p.i. (Fig. 3.3). As in C57BL/6j mice, low larval numbers were observed on the later days of infection in the CBA/Ca liver lobes (Fig. 3.3).

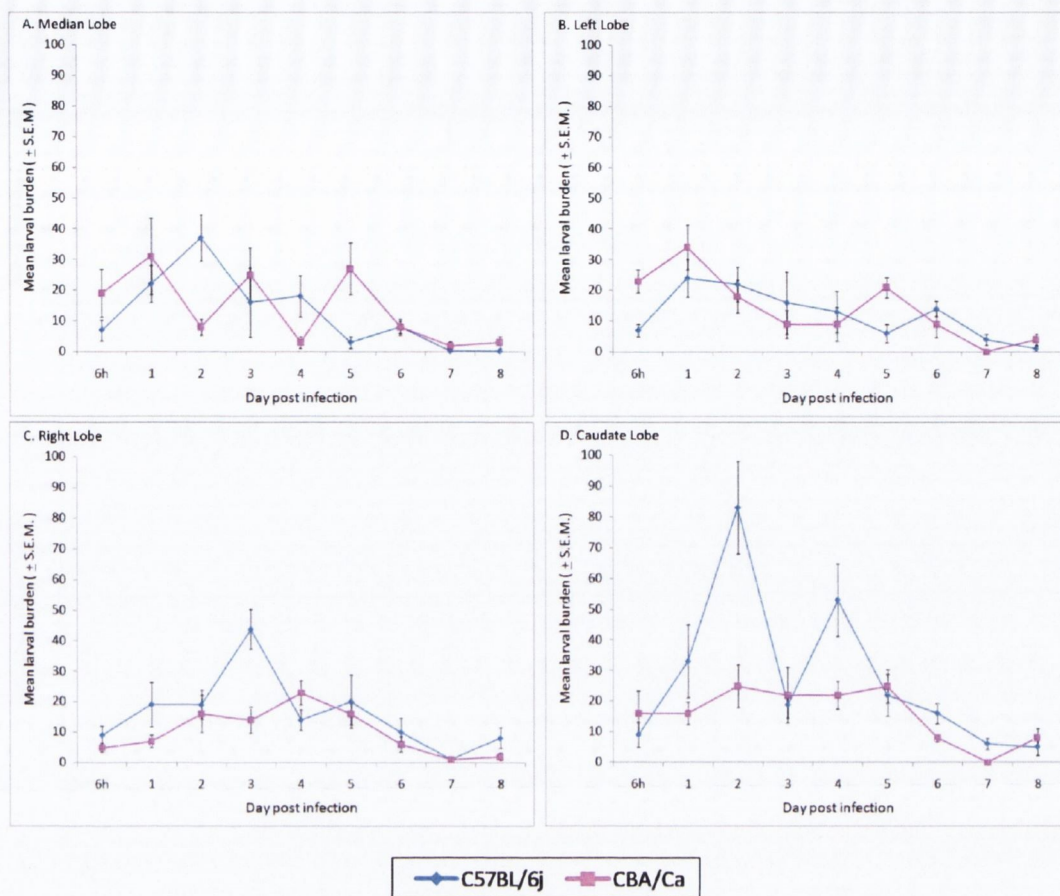


Figure 3.3. Changes in total mean larval burden ($\pm$ S.E.M.) in the hepatic (A) median, (B) left, (C) right and (D) caudate lobes of C57BL/6j and CBA/Ca, following inoculation with 1,000 *Ascaris suum* ova

Table 3.4. Results of two-way MANOVA examining the effects of strain, day and lobe on proportion of total larvae recovered in the four hepatic lobes of C57BL/6j and CBA/Ca mice

Source	Lobe	df	F-ratio	P value
strain	-	4	3.196	0.018
error	-	68		
day	-	32	3.465	<0.001
strain*day	-	32	2.089	0.001
error	-	252		
strain	median	1	5.519	0.022
	left	1	0.792	0.377
	right	1	4.238	0.043
	caudate	1	3.732	0.057
day	median	8	1.803	0.091
	left	8	2.046	0.053
	right	8	2.95	0.007
	caudate	8	2.437	0.022
day*strain	median	8	2.091	0.048
	left	8	1.951	0.065
	right	8	3.085	0.005
	caudate	8	1.894	0.074
error		71		

The results from a two-way MANOVA (Table 3.4) on the proportion of total larvae recovered in the four hepatic lobes as opposed to the absolute mean numbers revealed that a significant strain effect was detected in the median ($P=0.022$) and right ($P=0.043$) lobes. The proportion of total liver larvae recovered in the median lobe of C57BL/6j mice increased to a peak on day 2 p.i. (0.26 ± 0.07) and then fluctuated until days 7 and 8 p.i. at which point no larvae were recovered in this lobe (Fig. 3.4). In CBA/Ca mice the proportion recovered in the median lobe fluctuated consistently with the lowest proportion recovered on day 4 p.i. (0.04 ± 0.03) and the highest on day 3 p.i. (0.42 ± 0.10). With the exception of days 2 and 4 p.i. a higher proportion of larvae was recorded in the median lobe of CBA/Ca mice. In the right lobe, the proportion of total liver larvae observed in C57BL/6j mice fluctuated consistently, peaking on day 8 p.i. (0.61 ± 0.42). However in CBA/Ca mice the proportion of total larvae in the liver recovered in the right

lobe increased to a peak on day 4 p.i. (0.46 ± 0.10) and then decreased steadily (Fig. 3.4).

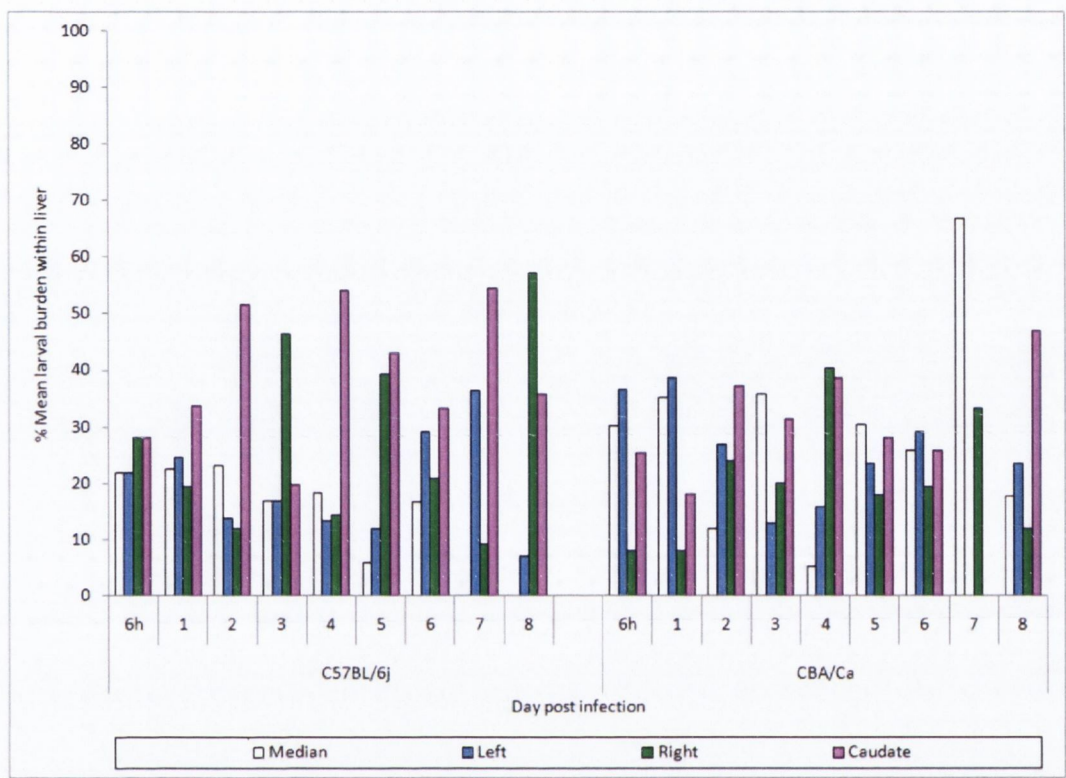


Figure 3.4. Changes in proportion of the mean larval burden in the hepatic lobes of C57BL/6j and CBA/Ca mice, following inoculation with 1,000 *Ascaris suum* ova

The strain effect was approaching significance in the caudate lobe ($P=0.057$), in which the proportion of total liver larvae fluctuated slightly in both mouse strains. However, a noteworthy decrease in the proportion of larvae recovered in the caudate lobe in CBA/Ca mice was observed on day 7 p.i. (0.00) followed by a considerable increase on day 8 p.i. (0.43 ± 0.17) (Fig. 3.4). The proportion of total liver larvae recovered in the right and caudate lobe significantly changed over time (right lobe: $P=0.007$, caudate lobe: $P=0.022$). A significant day*strain interaction was observed in the median ($P=0.048$) and right ($P=0.005$) lobes.

3.3.1.3. Larval burdens recovered in pulmonary tissues

The larval migratory kinetics observed in the liver and lungs of C57BL/6j and CBA/Ca indicated a reduction of larvae in the liver coinciding with the arrival of larvae in the lungs on days 4 and 5 p.i. (Fig. 3.5). In C57BL/6j mice, larvae accumulated in the lungs to a peak on day 7 p.i. (125 ± 21.74) and subsequently fell by day 8 p.i. (50 ± 21.51). Larvae consistently increased to a maximum on day 8 p.i. (31 ± 7.48) in CBA/Ca mice (Fig. 3.6). A two-way GLM (Table 3.5) conducted on larval numbers in the lungs on days 5 to 8 p.i. indicated a highly significant effect of strain ($P<0.001$) and time ($P<0.001$). No strain*day interaction was detected ($P=0.077$) as the pattern of larval migration in this organ was relatively similar in both strains.

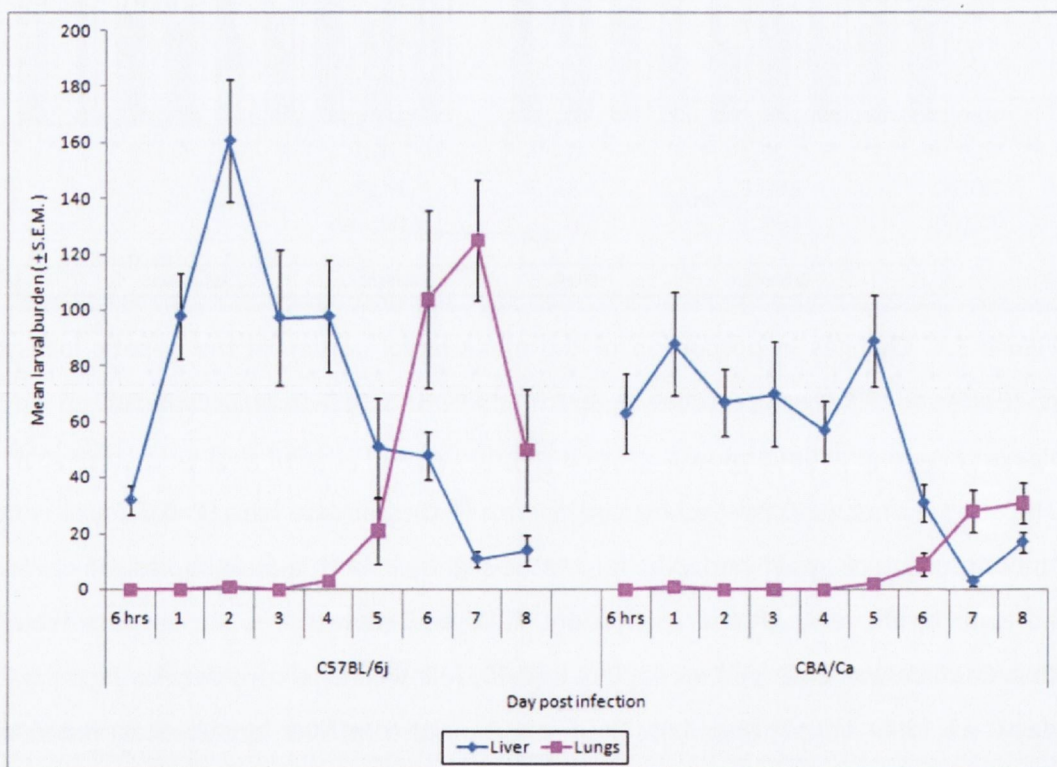


Figure 3.5. Changes in the mean larval burden ($\pm$ S.E.M.) in the liver and lungs in C57BL/6j and CBA/Ca mice, following inoculation with 1,000 *Ascaris suum* ova

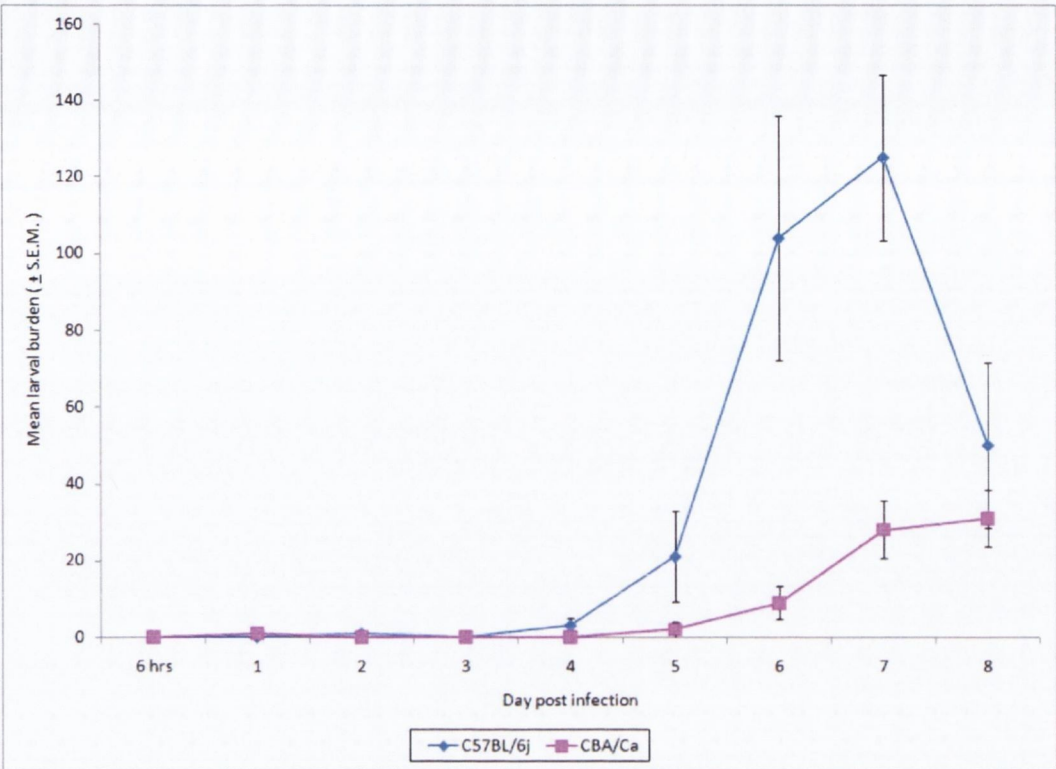


Figure 3.6. Changes in total mean larval burden ($\pm$ S.E.M.) in lungs of C57BL/6j and CBA/Ca mice, following inoculation with 1,000 *Ascaris suum* ova

Table 3.5. Results of two-way GLM examining the effects of strain and day on larval burdens recovered in pulmonary tissue of C57BL/6j and CBA/Ca mice

Source	df	F-ratio	P value
strain	1	16.164	<0.001
day	3	9.025	<0.001
strain*day	3	2.507	<0.001
error	32		

Appreciable numbers of larvae entered the left and right lungs on day 5 p.i. In C57BL/6j mice, larvae in each lung increased to a peak on day 7 p.i. and subsequently declined on day 8 p.i. Larvae recovered in the left lung of CBA/Ca mice increased to a maximum on day 8 p.i. A similar burden was recovered on days 7 and 8 p.i. in the right lung of CBA/CA mice (day 7 p.i.: 23 ± 4.90 , day 8 p.i.: 22 ± 6.04) (Fig. 3.7).

A two-way MANOVA (Table 3.6) conducted on proportional larval burdens on days 5 to 8 p.i. in the left and right lungs indicated that a strain effect was not detected in either the left ($P=0.659$) or right ($P=0.155$) lungs. The proportion of larvae in the lungs, which were recovered in the left and right lungs varied over time (left lung: $P=0.013$, right lung: $P=0.002$). Fluctuations over time in the proportion of larvae recovered in each lung was particularly evident in CBA/Ca mice as illustrated in Fig. 3.8. However, very low numbers were recorded on days 5 and 6 p.i. in the lungs of CBA/Ca mice (Fig. 3.7). At some post-mortem time-points, the proportion of larvae recovered in the right lung differed significantly between C57BL/6j and CBA/Ca mice ($P=0.006$).

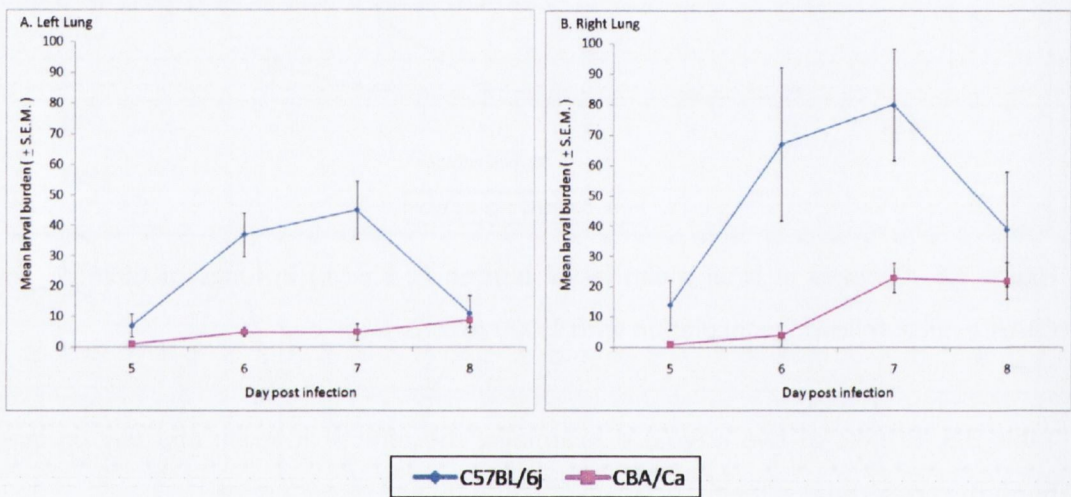


Figure 3.7. Changes in total mean larval burden ($\pm$ S.E.M.) in the left (A) and right (B) lungs of C57BL/6j and CBA/Ca, following inoculation with 1,000 *Ascaris suum* ova

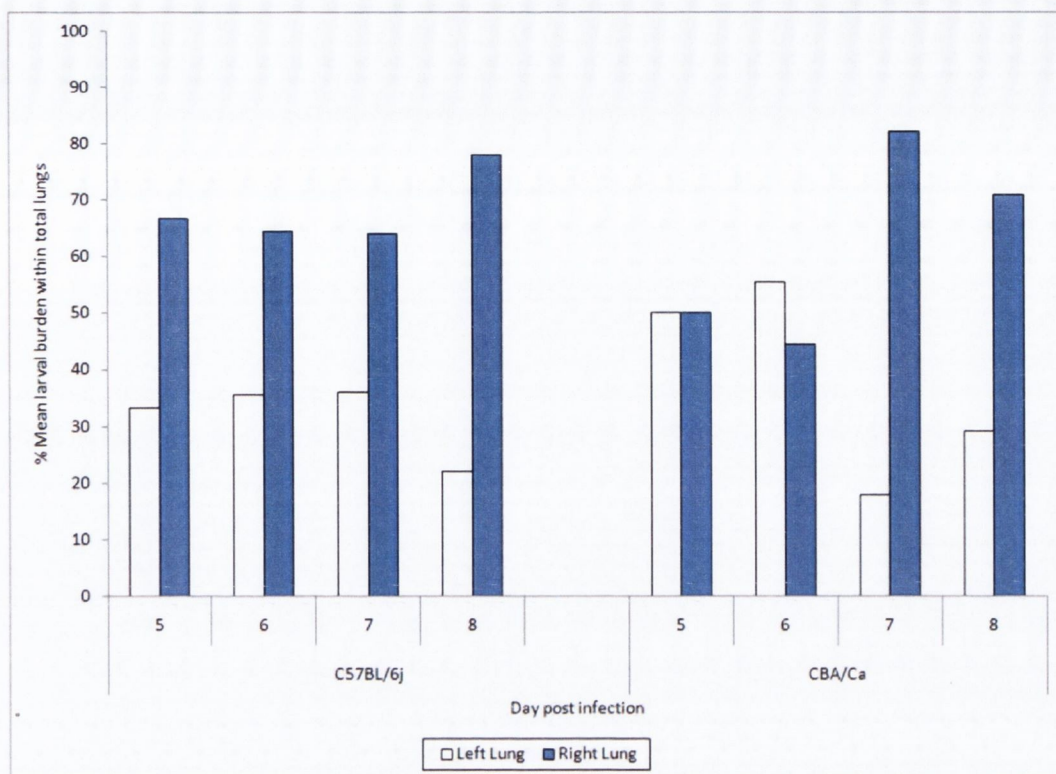


Figure 3.8. Changes in proportion of the mean larval burden in the left and right lungs of C57BL/6j and CBA/Ca mice, following inoculation with 1,000 *Ascaris suum* ova

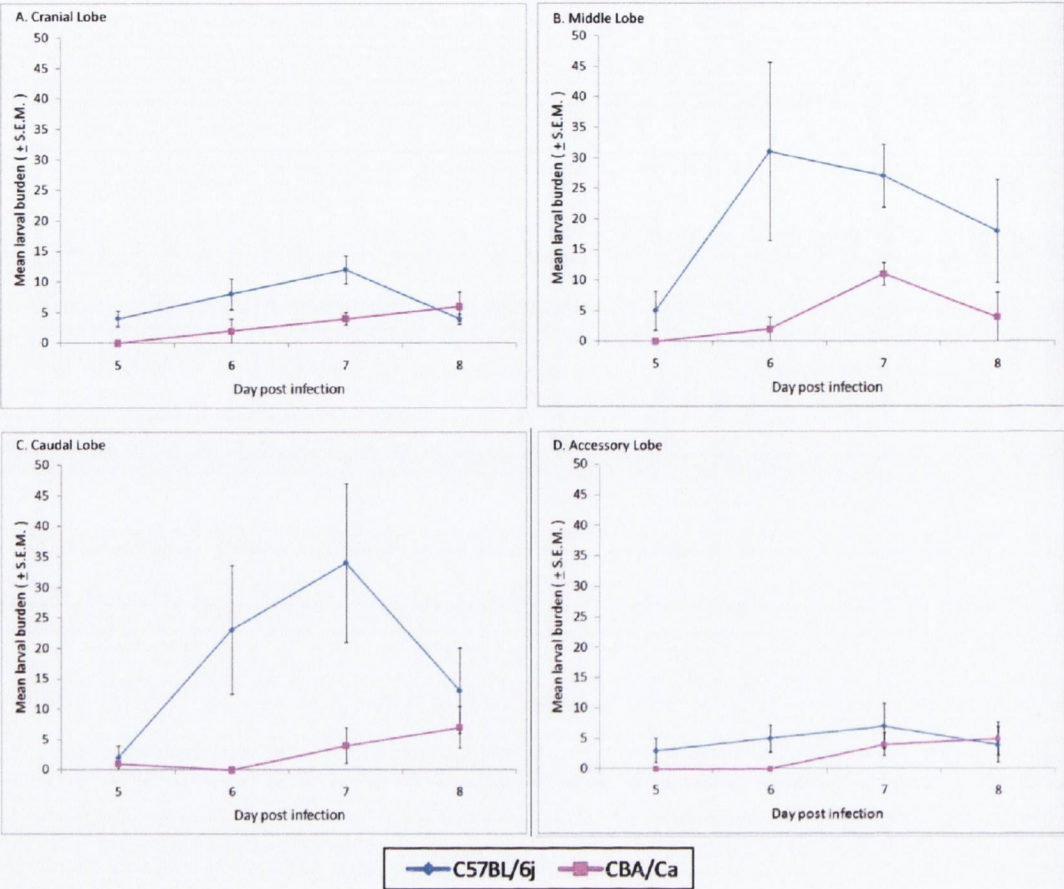


Figure 3.9. Changes in total mean larval burden ($\pm$ S.E.M.) in the pulmonary (A) cranial, (B) middle, (C) caudal and (D) accessory lobes of the right lung of C57BL/6j and CBA/Ca mice, following inoculation with 1,000 *Ascaris suum* ova

Table 3.6. Results of two-way MANOVA examining the effects of strain, day and lung on the proportion of total larvae recovered in the left and right lungs of C57BL/6j and CBA/Ca mice

Source	Lung	df	F-ratio	P value
strain	-	2	1.292	0.289
error	-	31		
day	-	6	4.879	0.000
strain*day	-	6	3.055	0.011
error	-	62		
strain	left	1	0.198	0.659
	right	1	2.124	0.155
day	left	3	4.193	0.013
	right	3	5.994	0.002
day*strain	left			
	right	3	5.060	0.006
error		32		

The mean larval burden recovered on days 5 to 8 p.i. in the right lung lobes is illustrated in Fig. 3.9. On days 5 and 6 p.i., the larval burdens in the right lung lobes of CBA/Ca mice are low. Therefore, similarly to Fig. 3.8, the proportions illustrated in Fig. 3.9 on these days are not as relevant as those on the later post-mortem time-points. In C57BL/6j, larvae increased to a peak on day 7 p.i. in each lobe, which was also observed in the middle lobe in CBA/Ca mice. However, in the cranial, caudal and accessory lobe of CBA/Ca mice, larvae increased to a maximum on day 8 p.i.

A two-way MANOVA (Table 3.7) conducted on the proportional data confirmed the effects of strain ($P=0.014$) and time ($P=0.007$) on lobar distribution in the right lung. A strain*day interaction was observed ($P=0.001$) as the proportion of larvae in the right lung recovered in the four lobes varied between strains at some post-mortem time-points. From the four right lung lobes, only the larval burden in the middle lobe changed over time ($P<0.001$). The migratory pattern of larval burdens differed significantly between the two mouse strains in the cranial ($P=0.043$) and middle ($P=0.029$) lobes. The proportional larval burden recovered in the caudal lobe was highly significantly different between C57BL/6j and CBA/Ca mice ($P<0.001$). The proportion of larvae in this lobe

was consistently higher in C57BL/6j and the main sites for recovery in this mouse strain were the middle and caudal lobe. The disparity was most pronounced on day 6 p.i., when larvae were absent from the caudal lobe of CBA/Ca mice, and day 7 p.i., at which point 42.5% and 17.4% of the larvae were recovered from the caudal lobes of C57BL/6j and CBA/Ca mice respectively (Fig 3.10).

Table 3.7. Results of two-way MANOVA examining the effects of strain, day and lobe on proportion of total larvae recovered in the four right lung lobes of C57BL/6j and CBA/Ca mice

Source	Lobe	df	F-ratio	P value
strain	-	4	3.576	0.014
error	-	29		
day	-	12	2.565	0.007
strain*day	-	12	3.285	0.001
error	-	77		
strain	cranial	1	0.715	0.404
	middle	1	0.022	0.882
	caudal	1	15.331	0
	accessory	1	0.13	0.721
day	cranial	3	1.156	0.342
	middle	3	8.546	<0.001
	caudal	3	1.312	0.297
	accessory	3	0.418	0.741
strain*day	cranial	3	3.046	0.043
	middle	3	3.41	0.029
	caudal	3	1.682	0.191
	accessory	3	2.477	0.079
error		32		

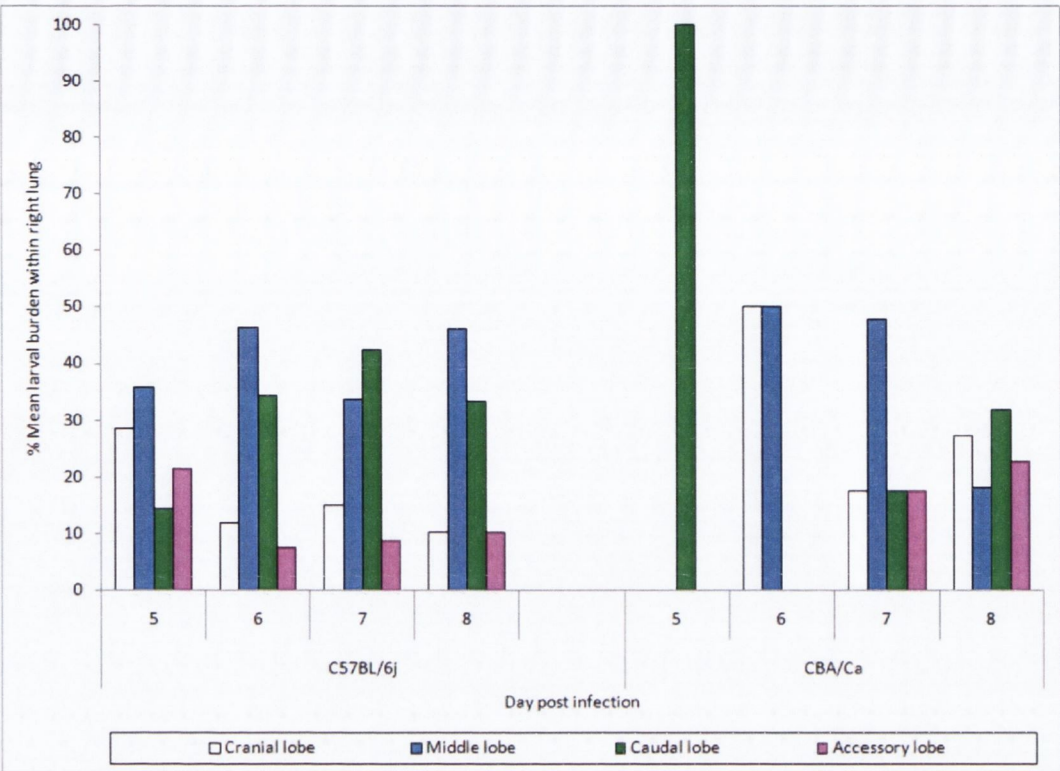


Figure 3.10. Changes in proportion of the mean larval burden in the right lung lobes of C57BL/6j and CBA/Ca mice, following inoculation with 1,000 *Ascaris suum* ova

3.3.2. Experiment 2: Examination of the pulmonary artery in the lungs of C57BL/6j and CBA/Ca mice using Optical Projection Tomography (OPT)

The position of the bronchus, pulmonary artery and vein entering the left and right lungs was assessed macroscopically. Plate 3.1 illustrates the pluck removed from a male CD1 mouse. The bronchus was identified as the most cranial of these structures entering each lung with the blood vessels located caudaloventrally to the airway.

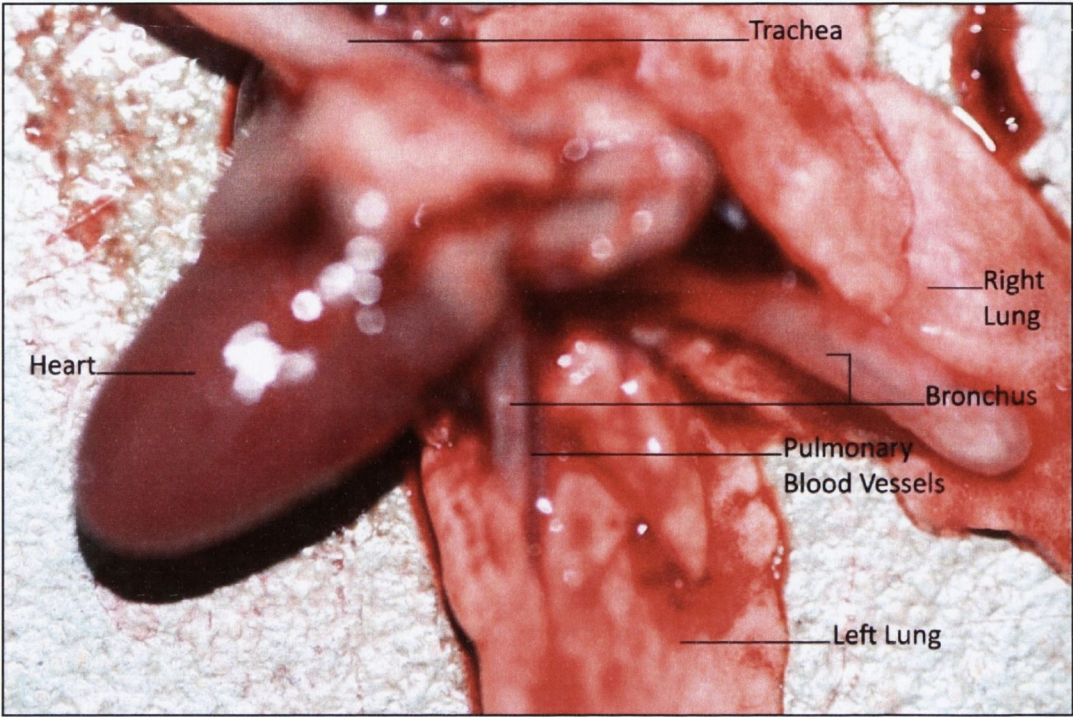


Plate 3.1. The “pluck” (lungs, trachea and heart) removed from a three month old CD1 mouse

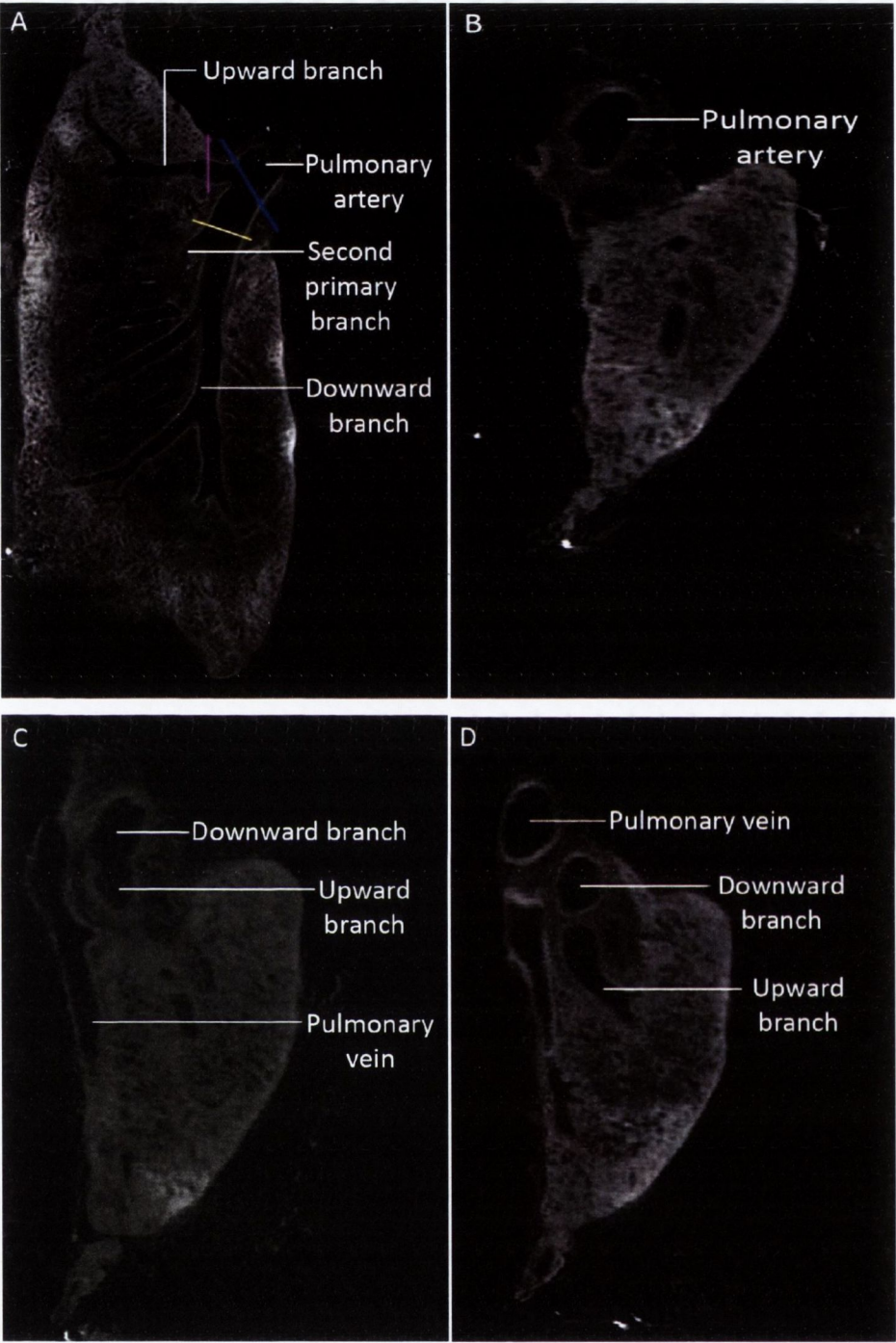


Plate 3.2. Cont.

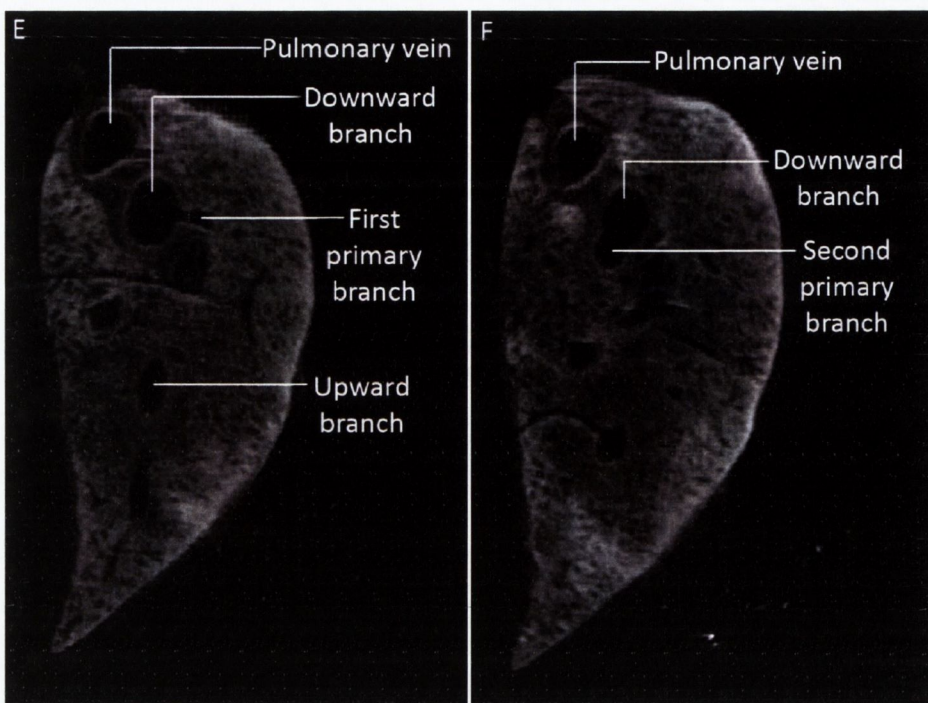


Plate 3.2. Sections of reconstructed left lung depicting the pulmonary vasculature.

(A) Pulmonary artery on the dorsoventral plane. Lines indicate angles at which the left lung was viewed to observe the entry of the pulmonary artery into the left lung (blue line) and its division into an upward (pink line) and downward branch (yellow line), the primary branches of the upward branch (pink line), and the branches of the downward branch (yellow line). (B) Pulmonary artery entering the left lung viewed on the anteroposterior plane (indicated by blue line in A). (C-D) Pulmonary artery dividing into upward and downward branches (indicated by pink and yellow lines in A respectively). (E) First primary branch off the downward branch of the pulmonary artery. (F) Second primary branch off the downward branch of the pulmonary artery.

On entry to the left lung the pulmonary artery immediately divided into an upward and downward branch (Plate 3.2 B-D). As one moved towards the caudal end of the lung along the downward branch on the dorsoventral plane (angle indicated by yellow line on Plate 3.2 A), primary branches were seen arising from the main vessel on the anteroposterior plane (Plate 3.2 E-F).

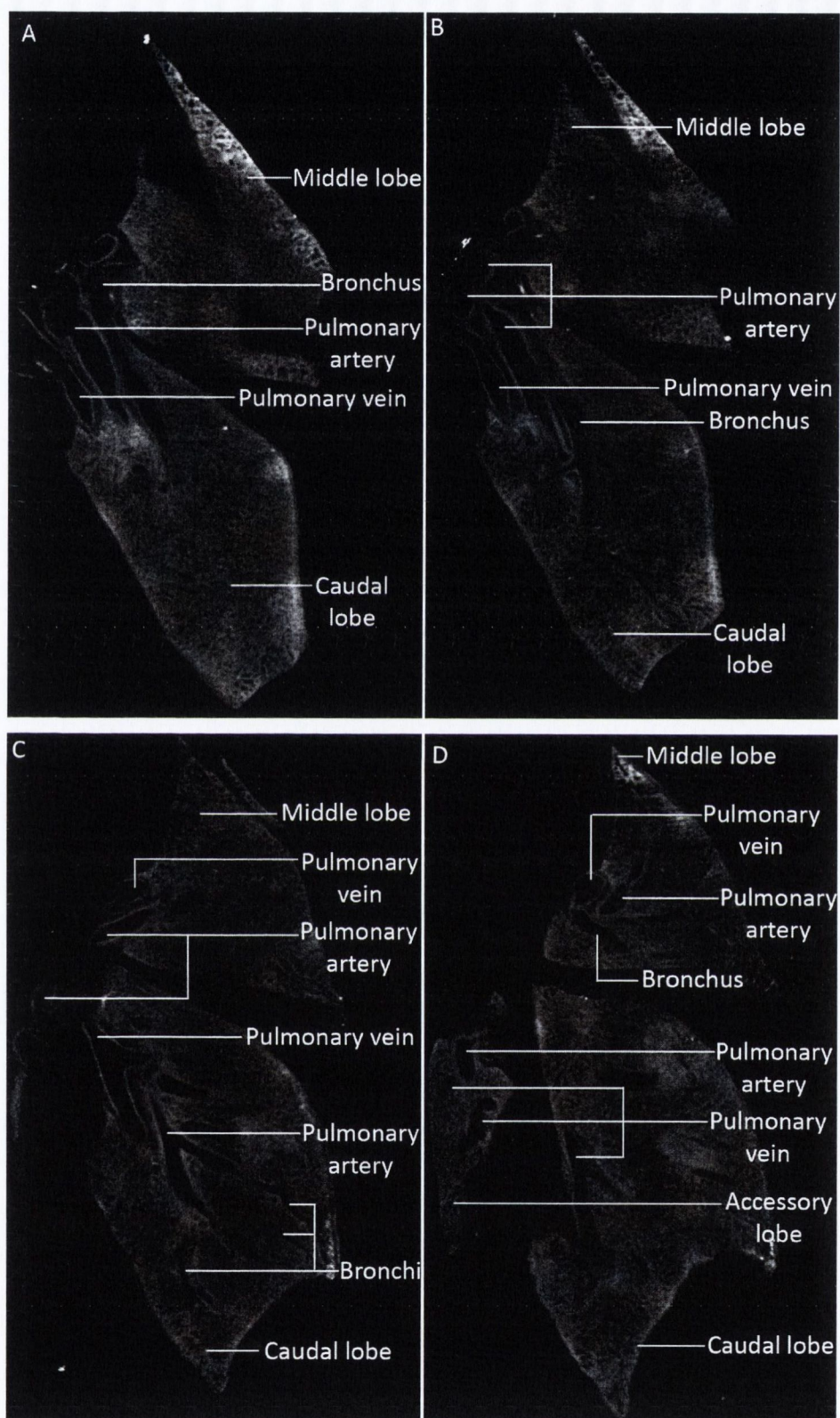


Plate 3.3. Cont.

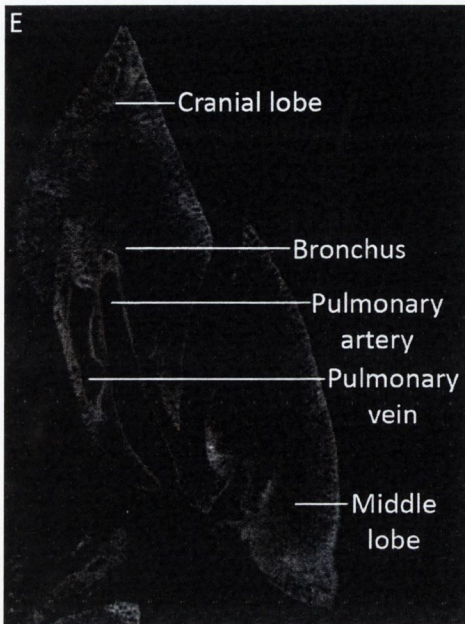


Plate 3.3. Sections of reconstructed right lung depicting the pulmonary vasculature on the dorsoventral plane.

The pulmonary artery branches down into (A) the caudal lobe, up into (D) the cranial lobe and (B) divides to enter (C) the middle lobe and (D) the accessory lobe.

The pulmonary artery divided at the hilus of the right lung, located proximal to the middle and caudal lobes (Plate 3.3 A-B), and entered each lobe separately (Fig. 3.11). As seen in Plate 3.3. (A-D) and Fig 3.11 the pulmonary artery branched upwards into the cranial lobe, relatively straight into the middle lobe, and downwards into the caudal and accessory lobes.

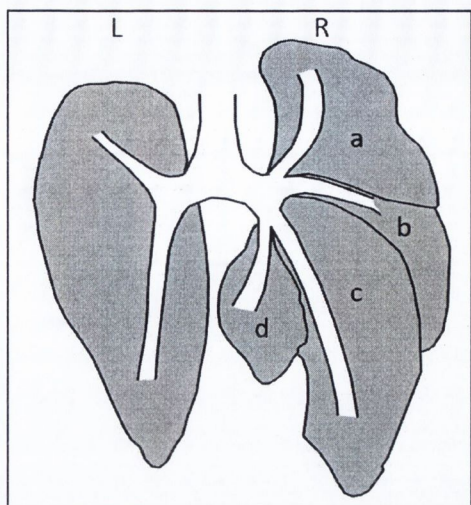


Figure 3.11. Schematic of main branches of pulmonary artery in left (L) and right (R) lungs observed in C57BL/6j and CBA/Ca mice by means of OPT.

The right lung can be separated into the cranial (a), middle (b), caudal (c) and accessory (d) lobes.

No striking differences in the position or number of primary branches of the pulmonary artery between C57BL/6j and CBA/Ca mice were observed in the left or right lungs (Table 3.8). The mass of the right lung was greater in each mouse strain (Table 3.9).

Table 3.8. The observed number of primary branches of pulmonary artery in each pulmonary lobe of C57BL/6j and CBA/Ca mice

Strain	Mouse	Tissue	No. of primary branches
C57BL/6j	1	left lung	upward branch: 2 downward branch: 10
		right lung	cranial 12
			middle 10
			caudal 11
			accessory 8
C57BL/6j	2	left lung	upward branch: 2 downward branch: 8
		right lung	cranial 12
			middle 9
			caudal 11
			accessory 6
CBA/Ca	1	left lung	upward branch: 2 downward branch: 10
		right lung	cranial 12
			middle 10
			caudal 12
			accessory 5
CBA/Ca	2	left lung	upward branch: 2 downward branch: 11
		right lung	cranial 12
			middle 9
			caudal 11
			accessory 5

Table 3.9. Mass of lung tissue used to observe the number of primary branches of the pulmonary artery in each pulmonary lobe of C57BL/6j and CBA/Ca mice, prior to embedding in agarose

Strain	Mouse	Tissue	Mass (g)
C57BL/6j	1	Left lung	0.1248
		Right lung	0.3450
C57BL/6j	2	Left lung	0.0921
		Right lung	0.2282
CBA/Ca	1	Left lung	0.0948
		Right lung	0.1786
CBA/Ca	2	Left lung	0.0971
		Right lung	0.2080

3.4. Discussion

During migration of *A. suum*, larvae were found to preferentially accumulate in particular lobes (caudal and middle) of the right lung in susceptible mice, thereby contributing to the difference in total larval burdens between strains of mice. Analysis of larval burdens in the organs, through which the parasite migrates, particularly the significant disparity in pulmonary larval numbers between strains, indicated a significant differential response, adding weight to the hypothesis that the liver of resistant CBA/Ca mice plays an important role in the impediment of onward larval migration.

The importance of the large intestine in the early stages of the tissue migratory phase in *A. suum* infection in mice has been highlighted previously (Keittivuti, 1974; Slotved *et al.*, 1998; Lewis *et al.*, 2007). In the current study, the number of larvae in the intestines was monitored over an 8-day period in susceptible and resistant mouse strains yet a high number of larvae was only recovered in the intestines at the 6-h p.i. time-point and was predominantly localised in the large intestine. The observed burden in C57BL/6j mice at this time in the large intestines was comparable to that of a previous study (Lewis *et al.*, 2007). However, the larval burden detected in the large intestine of CBA/Ca mice was comparatively higher than in the C57BL/6j mice and was also higher relative to that previously recorded (Lewis *et al.*, 2007). The observed high caecal numbers at the 6-h p.i. time-point was documented in mice by Keittivuti (1974) and Jenkins (1968), and in both of these studies a predilection for the caecal wall was noted. Despite higher numbers in the large intestine of CBA/Ca mice at 6 h p.i., equivalent numbers in the liver suggest a similar number of larvae successfully migrate from the large intestine to the liver in both mouse strains. This finding is consistent with earlier studies on our mouse model (Lewis *et al.*, 2007) and in immunised and control guinea pigs (Soulsby, 1957). Timing, therefore, may account for the strain difference in the early intestinal burden as suggested by Slotved *et al.* (1998). These authors noted that individual mice of the same strain show variation in the rate at which larvae passed through the large intestine. While large intestinal *A. suum* larval burdens have been shown to peak at 6 h p.i. in mice (Keittivuti, 1974), larvae have been recorded in this

tissue as early as 2 h p.i. (Keittivuti, 1974; Slotved *et al.*, 1998). Furthermore, in the current study, larvae were recovered at 6 h p.i. in liver, indicating earlier hatching in the intestines. A more comprehensive examination of larval numbers in the large intestine at hourly intervals p.i. would indicate whether there is a temporal difference in hatching and larval penetration of the large intestinal wall. Nevertheless, if a temporal difference in hatching exists between the two strains, it is confined to a short period of time. This is reflected in the inconsistency displayed in the intestinal larval burdens on days 1–8 p.i. observed in our mouse model and up to 10 days p.i. in a previous study in the ICR strain of mouse (Keittivuti, 1974).

A. suum larvae have been speculated to migrate within the liver of their host, for a period of days, in an attempt to find a suitable blood vessel for onward migration to the lungs. This was reflected in the erratic pattern of larval burden in each hepatic lobe, particularly the right and caudate lobes, in both strains. The pattern of migration within the liver was shown to differ between the two mouse strains in the median and right lobes. Furthermore, a difference in the proportion of total liver larvae was observed in the median and right lobes and was shown to be approaching significance in the caudate lobe. In an anatomical study of the mouse liver, the caudate lobe was found to be the smallest, accounting for $8.1 \pm 1.0\%$ of the total liver in BALB/c mice, while the left lobe was shown to be the largest ($34.4 \pm 2.0\%$) (Inderbitzin *et al.*, 2006). However, while no anatomical studies have been undertaken on the lobes of C57BL/6j and CBA/Ca mice, the large size of the left lobe does not explain the lack of disparity in the larval burdens in this lobe between the two mouse strains. The higher vascular density of the caudate lobe, observed in BALB/c mice (Inderbitzin *et al.*, 2006) may vary between the two mouse strains exploited in the current study, and may account for the increased larval accumulation in C57BL/6j mice. However, since BALB/c mice were found to be in the intermediate susceptibility category in previous *A. suum* experiments (Lewis *et al.*, 2006), this explanation seems unlikely. It is currently not possible to determine if the difference in larval recovery from the hepatic caudate lobe is due to anatomical differences between the two mouse strains. Furthermore, the increased onward migration of *A. suum* larvae in C57BL/6j mice from the liver to the lungs may be explained by a hepatic or pulmonary anatomical difference between the two strains.

The present study recovered similar larval burdens as previous work (Lewis *et al.*, 2006; 2007) in the key whole organs of interest involved in migration of *A. suum* in C57BL/6j and CBA/Ca mice. However, in depth analysis of larval numbers in sub-divisions of the liver and lungs revealed that particular lobes are major sites of recovery and account for disparities in parasitic burdens observed between susceptible and resistant mice, particularly in the lungs. Mitchell *et al.* (1982) commented that “expression of host resistance need not have an immunological basis”. Physical barriers operate predominantly externally, protecting the host from initial infection. However, there are examples of non-immunological factors, influencing “permissiveness” or relative natural resistance. For example, as briefly described earlier, studies on migration and attrition of *S. mansoni* in mice have indicated that physical problems associated with migration can result in larval trapping. A body of work, reviewed by Wilson (1990; 2009) revealed that granulomas which form around schistosome eggs in the liver effectively block portal blood flow, leading to portal hypertension. The integrity of the portal system is compromised due to a build-up of pressure resulting in alternative vascular channels opening through the sinusoids resulting in “leaky livers”, which the schistosome worms exploit to pass to the lungs.

Given the lack of any other research detailing the migration or establishment of helminthic parasites within particular hepatic or pulmonary lobes, it is difficult to build coherent explanations to why there are different lobar larval burdens within and between mouse strains in the present study. Strain-specific imaging by means of OPT was restricted to the lungs since changes in the gross size of the CBA/Ca lungs was observed during later post-mortem time-points (C. Dold, personal observations). Furthermore, the number of larvae recovered in the middle and caudal lobes of C57BL/6j mice appeared to largely account for the higher pulmonary larval burden in the susceptible strain. The lack of any obvious difference in the positioning of pulmonary artery on entry into each right lung lobe, or in the number of primary branches of the artery between the two strains, indicates that it is unlikely that larvae are restricted entry to the lungs in the resistant strain. Based upon two animals per mouse strain, it appears that the right lung is the bigger of the two lungs in the mouse

and is therefore likely to receive increased blood flow, which may account for the larger proportionate larval burden in both mouse strains. The positions and angles at which branches of the pulmonary artery enter each right lung lobe are similar to that described and illustrated in a model for the relative positions of the pulmonary arterial branches by Lim *et al.* (2002), who focused on the fusion of lung lobes and vessels in mouse embryos. The relatively direct entry to the middle lobe and the proximity of the caudal and middle lobes to the hilus may explain the greater burden in these two right lung lobes as the reduced turbulent blood flow to these lobes here would facilitate increased parasite accumulation.

OPT is commonly used for embryonic development and previous gene expression studies (eg. Sharpe *et al.*, 2002; Sharpe, 2003) in both the chick (eg. Roddy *et al.*, 2009) and the mouse (eg. Summerhurst *et al.*, 2008). Alanentalo *et al.* (2007) not only stained adult murine pancreatic tissue in order to enumerate islets, but also successfully stained and scanned liver, stomach, brain, gluteus muscle and heart. To the best of our knowledge, the present study is the first to scan adult murine lung tissue without fluorescence or antibody staining to visualise the feature of interest. OPT enabled visualisation of the pulmonary artery and surrounding pulmonary vasculature and tissue in three dimensions. Furthermore, this imaging technique facilitated enumeration of the primary branches of the pulmonary artery within the lung lobes. However, further analysis of secondary or tertiary branches and the finer detail of the pulmonary tissue, if required, would prove difficult as resolution at a smaller scale was not optimal. Therefore if further comparative studies of the vasculature within the pulmonary tissue are to be undertaken, different techniques should be sought and tested. For example, Coulson and Wilson (1989) examined the layout of hepatic portal vessels by means of latex casts in mice infected with *Schistosoma mansoni*. Similarly, Elsaghier and McLaren (1989) prepared casts of hepatic and pulmonary vasculature from uninfected mice. Micrographs of the hepatic casts of CBA/Ca mice indicated highly branched peripheral vessels, which contrasted with the truncated and much-reduced peripheral hepatic vessels in 129/Ola mice. The vascular abnormalities were proposed to account for the chronic schistosomiasis in CBA/Ca mice and possible “trapping” of schistosomes in resistant 129/Ola mice. Therefore, latex infusion appears to be a suitable technique for

imaging organs to examine differences in vasculature that might account for “permissiveness” to parasite migration.

The similarities observed in the structure of the pulmonary artery, the major blood vessel supplying the lungs in both strains indicates the larvae are not differentially impeded from entering the lungs of CBA/Ca mice. This further suggests that the reduction in larval burdens in the resistant CBA/Ca mice is due to a pre-pulmonary or hepatic/post-hepatic factor or event that varies between the two strains. Therefore, the results of the current study, taken together with earlier data from the *A. suum* mouse model (Lewis *et al.*, 2006; 2007), indicates that an immune-mediated hepatic/post-hepatic factor varies between C57BL/6j and CBA/Ca mice and accounts for the divergence in the lung larval burdens between the mouse strains. In consequence in the next chapter, the infiltration of innate inflammatory cells in the liver will be assessed to investigate if and at which time-point during infection, a difference in the fundamental hepatic and pulmonary inflammatory response is observed between strains.

4. The hepatic and pulmonary inflammatory response to *Ascaris suum* infection in C57BL/6j and CBA/Ca mice

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4.1. Introduction

Clinical symptoms of ascariasis depend on the stage of invasion due to the succession of developmental stages, which can carry stage-specific antigens (Kennedy and Qureshi, 1986) and the different locations of the host encountered, and the intensity of infection (Pawlowski and Davis, 1989). Since the frequency distribution of *Ascaris* worms is aggregated in the host population (Croll and Ghadirian, 1981; Boes *et al.*, 1998; Holland and Boes, 2002), many hosts tend to be asymptomatic (Chan *et al.*, 1994c) due to their low levels of infection intensity. Adult worms in the small intestine in chronic infections cause malnutrition in humans and pigs (Stephenson *et al.*, 1980c; Stephenson *et al.*, 1980a; Forsum *et al.*, 1981; Carrera *et al.*, 1984; Taren *et al.*, 1987; Hadju *et al.*, 1996). Abdominal distension and pain, nausea and diarrhoea are also characteristic symptoms of adult worm infection. As previously highlighted by Cooper *et al.* (1992) and Lewis *et al.* (2009), the impact of *Ascaris* larval migration is less clearly understood than that of mature worms in the gut. A recent study, conducted in the mouse indicated that larval migratory ascariasis has a significant negative impact on host growth (Lewis *et al.*, 2009).

There is indirect evidence that larval migratory ascariasis in porcine hepatic tissue impacts host fitness (Stewart *et al.*, 1984). Stewart and colleagues (1984) demonstrated that febendazole treatment during the liver and pulmonary phase of migration led to improvements in the feed conversion ratios of 22% and 80% respectively (Stewart *et al.*, 1984). Coupled with this, migrating larvae are known to induce an inflammatory reaction in the lungs, and the resultant respiratory distress (Loeffler's syndrome) (Löffler, 1956) is a characteristic symptom of early *Ascaris* infection (Matsuyama *et al.*, 1998). As described by Ribeiro & Fischer (2002), Loeffler's syndrome is classed as an eosinophilic lung disease as the eosinophil count in humans can reach up to 70% of all leucocytes. In *A. lumbricoides* infection, dyspnoea (difficult or laboured breathing) and bronchospasm may be severe (Ribeiro and Fischer, 2002). Severe dyspnoea has also been documented in porcine infections (Taffs, 1968; Eriksen, 1981; Yoshihara *et al.*, 1983). Short dry coughs are also a typical feature of *A. suum*-induced respiratory

distress in pigs (Yoshihara *et al.*, 1983) and have been reported in experimental infections of cows (Greenway and McCraw, 1970a).

The pathological consequences of *A. lumbricoides* and *A. suum* infection underlying clinical expression have been documented in natural infections through use of clinical data from hospital cases (Khuroo *et al.*, 1992; Gul *et al.*, 1999; Javid, 1999). However, controlled experimental infections in animal models facilitate investigation of the pathogenesis of infection and resultant pathology in particular host organs at particular stages of infection.

While the clinical signs of liver migration may not be evident, the pathological consequences of *Ascaris* invasion of the liver has been studied in pigs (Schwartz and Alicata, 1932; Oldham and White, 1944; Ronéus, 1966; Taffs, 1968; Copeman and Gaafar, 1972; Eriksen *et al.*, 1980; Eriksen, 1981; Nakagawa *et al.*, 1983; Yoshihara *et al.*, 1983; Pérez *et al.*, 2001; Frontera *et al.*, 2003), mice (Sprent, 1949; Bindseil, 1969b; 1981; Eriksen, 1981), hamsters (Mazumder *et al.*, 1992), cows (McCraw and Greenway, 1970), rabbits (Arean and Crandall, 1962; Taffs, 1965) and guinea pigs (Fallis, 1948; Taffs, 1965). An inflammatory reaction in the liver has also been observed in humans infected with *A. lumbricoides* (Javid, 1999) and *A. suum* (Sakakibara *et al.*, 2002; Kakiyama *et al.*, 2004).

In *A. suum* infection, 'white spots' (WS) are white pathological lesions, composed of leucocytic infiltrations that form in the liver consequent to the mechanical injury and inflammatory response induced by migrating larvae (Schwartz and Alicata, 1932; Copeman and Gaafar, 1972). Early lesions are composed of necrotic haemorrhagic foci, surrounded by infiltrates of numerous eosinophils, a smaller number of neutrophils and small numbers of macrophages (Pérez *et al.*, 2001). Granulation tissue type WS (GT-WS) have been suggested to form along larval migration routes (small GT-WS) or encapsulate trapped larvae (large GT-WS) (Schwartz and Alicata, 1932; Ronéus, 1966; Copeman and Gaafar, 1972; Pérez *et al.*, 2001). Numerous macrophages, some fibroblasts and lymphocytes have been observed in these granulomatous lesions. Cases

where larger granulomas are surrounded by fibrous connective tissue have been reported (Pérez *et al.*, 2001).

Greater numbers of lesions have been observed with more larvae surrounded by inflammatory cells in experimental mice and guinea pigs considered resistant due to previous exposure to *A. suum* (Sprent, 1949; Taffs, 1965). Therefore, since WS are associated with trapped larvae and are more abundant in resistant animals, the liver has previously been cited as a key site in the immobilisation of migrating *Ascaris* larvae (Kerr, 1938; Fallis, 1948; Sprent, 1949; Taffs, 1968; Copeman and Gaafar, 1972; Eriksen *et al.*, 1980).

The pathology underpinning the respiratory distress experienced in *Ascaris*-infected hosts has also been investigated in animals such as guinea pigs (Taffs, 1965), rabbits (Arean and Crandall, 1962; Taffs, 1965), pigs (Schwartz and Alicata, 1932; Taffs, 1968; Frontera *et al.*, 2004) and cows (McCraw and Greenway, 1970)

Inflammatory cells such as eosinophils and macrophages have been shown to increase in numbers in infected lungs. Granulomas are more abundant in animals deemed resistant due to repeated infections or antigen stimulation (Taffs, 1965; 1968; Frontera *et al.*, 2004). Furthermore, intra-alveolar haemorrhage, oedema and emphysema are also typical features of *Ascaris*-induced pulmonary pathology (Arean and Crandall, 1962; Taffs, 1965; 1968).

Lewis *et al.* (2007) examined and graded the pulmonary inflammatory response on days 8-10p.i. in C57BL/6j and CBA/Ca mice and found that the former strain displayed a more intense inflammatory reaction. It was concluded that the heightened response in the susceptible strain was not protective but a result of a heavier pulmonary burden. Lewis *et al.* (2007) selected post-mortem time-points on the later days of larval migration (days 8-10 p.i.) as in a previous experiment by Eriksen (1981), in which a pulmonary inflammatory response was first observed on day 8p.i., which peaked on day 10p.i.

Ascaris larvae migrate to the lungs via the pulmonary artery and penetrate the alveoli through the alveolar wall capillaries. As reviewed by Rogers (1994), goblet cells which line the epithelium of the bronchi discharge mucus in response to a range of stimuli. Furthermore, goblet cells increase in numbers (hyperplasia) during chronic airway insult (Rogers, 1994). The role of intestinal mucins in expulsion of microbial parasites (Deplancke and Gaskins, 2001) and helminths, notably for *T. spiralis* (Knight *et al.*, 2008) and *N. brasiliensis* (Miller *et al.*, 1981; Ishikawa *et al.*, 1994; Nawa *et al.*, 1994), has been previously demonstrated. Abundant secreted mucus surrounds and entraps the helminth in the gut lumen, preventing anchorage to the intestinal surface.

As Miller (1987) noted, one must take care when extrapolating between bronchial and enteric mucus secretion due to differences in mucus-secreting glands in the two tissues. Very few studies have investigated the role of mucus hypersecretion induced by helminth migration in the lung. Silveira *et al.* (2002) noted an increase in mucus production in response to *Strongyloides venezuelensis* in the lungs and commented how such a response requires further investigation. It is possible that larval invasion of the lungs induces production of mucus to protect mucosal surfaces but may ultimately be counter-productive if in the process of facilitating worm expulsion, the excess mucus obstructs airways leading to respiratory distress

Lewis *et al.* (2007) proposed that a hepatic/post-hepatic factor, which varies between C57BL/6j and CBA/Ca mice, plays a critical role in restricting further larval migration through host tissues. The liver has previously been cited as a key site in the immobilisation of migrating *Ascaris* larvae (Kerr, 1938; Fallis, 1948; Sprent, 1949; Taffs, 1968; Copeman and Gaafar, 1972; Eriksen *et al.*, 1980). Furthermore, the early inflammatory reaction prior to day 8 p.i. in pulmonary tissues has not yet been investigated in C57BL/6j and CBA/Ca mice and may play a role in impeding onward larval migration.

Therefore the primary aim of the present investigation was to compare the inflammatory response, as characterised by the infiltration of granulocytes and macrophages, in the hepatic and pulmonary migration phases of *A. suum* larvae in

susceptible C57BL/6j and resistant CBA/Ca mice. This approach will determine if hepatic inflammation and early pulmonary inflammation play a significant role in parasite attrition in resistant mice. Results obtained in chapter 3 indicate that lobar differences in larval burdens exist, which may be a consequence of contrasting inter-lobe inflammation. Therefore, a secondary aim was to assess whether a difference in distribution and severity of inflammation exists in the hepatic and pulmonary lobes during primary infection in the susceptible C57BL/6j and resistant CBA/Ca mice.

A further objective of the current study was to examine the level of mucus production in the left and right lungs of both mouse strains during the lung-migration phase in order to assess whether mucus production facilitates *A. suum* expulsion in early pulmonary invasion.

4.2. Materials and Methods

4.2.1. Collection of tissues at post-mortem

4.2.1.1. Tissues collected for assessment of inflammation

A total of 90 (45 C57BL/6j and 45 CBA/Ca) male inbred mice were purchased for this experiment. A sample number of five mice of each strain were sacrificed at 6 hours and days 1-8 post infection (p.i.). At post-mortem the lungs and liver were removed from each mouse. The liver was separated into its multiple lobes, the median, caudate, left and right lobes. Similarly, the right lung was separated into its composite lobes, the cranial, middle, caudal and accessory lobes. The left lung cannot be further separated (Fig. 4.1). All lobes were evaluated as separate entities.

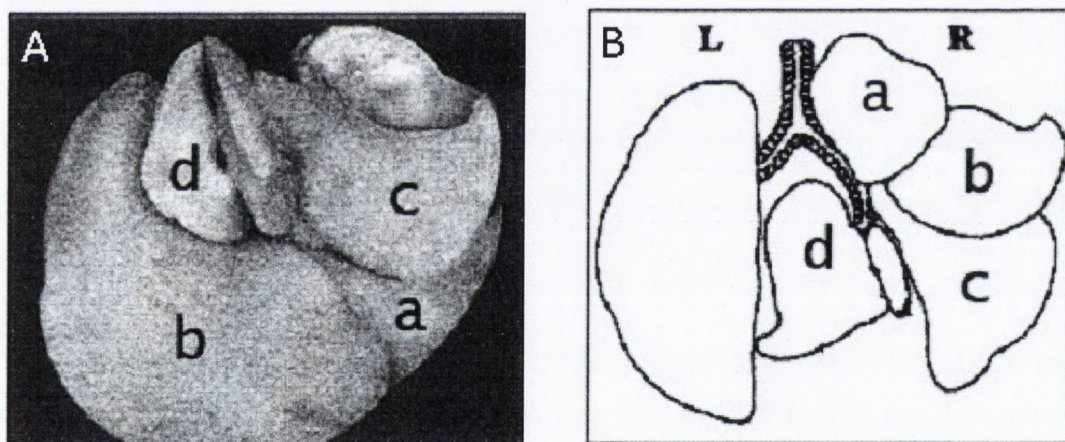


Figure 4.1. The composite lobe of the (A) murine liver (a=median lobe, b=left lobe, c=right lobe, d=caudate lobe) and (B) murine left (L) and right (R) lungs (a=cranial lobe, b=middle lobe, c=caudal lobe, d=accessory lobe)

(adapted from Maronpot *et al.*, 1999)

Each pulmonary lobe was secured in individual histocassettes and placed in 10% formalin for 24 hours. Prior to hepatic lobe separation, each mouse's liver was placed in a 10ml tub containing 10% formalin. After 24 hours fixation each liver was separated into its composite lobes, which were placed in individual histocassettes. This process was undertaken to ensure penetration of the fixative throughout the hepatic tissue. All

tissues within histocassettes were preserved in 70% ethanol after fixation. Tissue samples were placed in a series of alcohol dilutions, embedded in paraffin wax and 5µm sections of each lobe were cut using a microtome (Thermo Scientific HM340E rotary microtome). Hepatic and pulmonary sections were stained with haematoxylin and eosin (appendix 1, section 1.2).

4.2.1.2. Tissues collected for examination of mucus secretion

A total of 56 (28 C57BL/6j and 28 CBA/Ca) male inbred mice were purchased for this experiment. A sample number of seven mice (five experimental and two control) of each strain were sacrificed on 5-8 days p.i. At post-mortem the lungs were removed from each individual.

The left and right lungs were processed for histopathological examination as for the liver samples above. In addition, pulmonary sections were stained with the periodic acid Schiff stain (appendix 1, section 1.3).

4.2.2. Histopathological examination

The selection of time-points examined was based on the distribution of larvae recorded in the tissues of euthanised mice (chapter 3, section 3.3.1). Histopathological changes were determined in each hepatic lobe (median, left, right and caudate) sampled at 6 hours and on days 1-8 p.i. and in the left lung and right pulmonary lobes (cranial, middle, caudal and accessory) sampled on days 4-8 p.i.

4.2.2.1. Examination of haematoxylin and eosin stained sections

Hepatic and pulmonary injury and inflammation was scored semi-quantitatively on a scale ranging from +/- (no inflammation) and + (very mild inflammation) to ++++ (severe inflammation). Examining sections of each liver and lung lobe from each animal reduced

the margin of error in scoring that could have resulted from a localised response in one region of the organ. Histopathological examination was performed without prior knowledge of sample identity.

To assess changes in the hepatic inflammatory reaction, an initial histopathological section from each liver lobe sampled at 6 hours and on days 1-8 p.i. was examined and graded. A further three histopathological sections, each 100µm apart were examined from livers sampled on days 2-5 p.i. The scores allotted to the three further serial histopathological sections examined were subjected to statistical analysis. The pulmonary inflammatory response was assessed in a single section on days 5-8 p.i.

4.2.2.2. Examination of periodic acid Schiff stained sections

The degree of mucus production on days 5-8 p.i. in the left lung and entire right lung was scored semi-quantitatively ranging from +/- (no mucus present) and + (very little mucus present) to ++++ (abundant mucus well distributed). Sections of lung tissue were compared and calibrated with samples from cat, dog and other mice (provided by the Pathology laboratory, Veterinary Sciences Centre, University College Dublin) in which mucus production was previously confirmed.

4.2.3. Statistical analysis

The serial sections cut from the hepatic lobe samples on days 2-5 p.i. were subjected to statistical analysis. Analysis of the semi-quantitative pathological scores (converted into a scale ranging from zero (+/-) to 4 (++++)) in the liver was carried out using a mixed-GLM model, with time and strain as fixed factors, mouse as a random factor nested within the 2-way interaction of day*strain and liver lobe as a fixed effect. The model was therefore time + strain + day*strain + mouse (day*strain) + liver lobe, with mouse as a random factor. Models involving interactions between liver lobe and time and strain were also explored but none of these proved significant. Appropriate statistics are reported in the text.

4.3. Results

4.3.1. Examination of haemotoxylin and eosin stained sections

4.3.1.1. Examination of hepatic histopathological sections

4.3.1.2. General observations

None of the uninfected control animals exhibited histopathological changes in the liver (Plate 4.1. A). No marked difference in the character of infiltrate was observed between the two strains and thus, it was concluded that the inflammatory response observed was qualitatively similar in each. Focal accumulations of granulocytes, macrophages and multinucleated giant cells were observed in infected animals from both strains with a predominant peri-portal and centrilobular distribution in each lobe. However such accumulations were also detected within the intervening hepatic lobular parenchyma (Plate 4.1. I). Necrotic tracts were widely distributed throughout the tissue with a particular association with portal and perilobular blood vessels (Plate 4.1. F). Longitudinal and transverse sections of *A. suum* larvae, both surrounded by or devoid of cellular infiltrate, were frequently identified within blood vessels and sinusoids (Plate 4.1. G and H).

4.3.1.3. Changes in the hepatic inflammatory reaction on days 1-8 p.i.

Semi-quantitative grading of sections from liver lobes (Plate 4.1. B-E) revealed a contrast in timing of the inflammatory reaction in C57BL/6j and CBA/Ca mice (Fig. 4.2). A low inflammatory score was recorded in both mouse strains at the initial stages of infection but was relatively greater in the CBA/Ca mice (mixed model as explained in Materials and Methods, main effect of strain $P=0.04$) (Table 4.1). Although C57BL/6j mice had a higher percentage of lobes with mild inflammation (grade +) than did CBA/Ca mice, the C57BL/6j animals had extensive necrotic tracts radiating from blood vessels. The parasites and necrosis observed in the susceptible strain at the initial stages did not appear to elicit a local cellular inflammatory response. Comparatively severe inflammation was observed in CBA/Ca mice on day 2 p.i., which became more

pronounced on day 3 p.i. On day 4 p.i., a higher incidence (58.3%) of more severe inflammation (grade +++) was observed in more hepatic lobes of CBA/Ca animals. A similar response in the hepatic lobes in C57BL/6j mice was not detected until day 6 p.i. at which point severe inflammation (grade +++) was detected in 75% of the liver lobes examined. Comparatively less severe inflammation was detected on days 7 and 8 p.i. in CBA/Ca mice (Fig. 4.2).

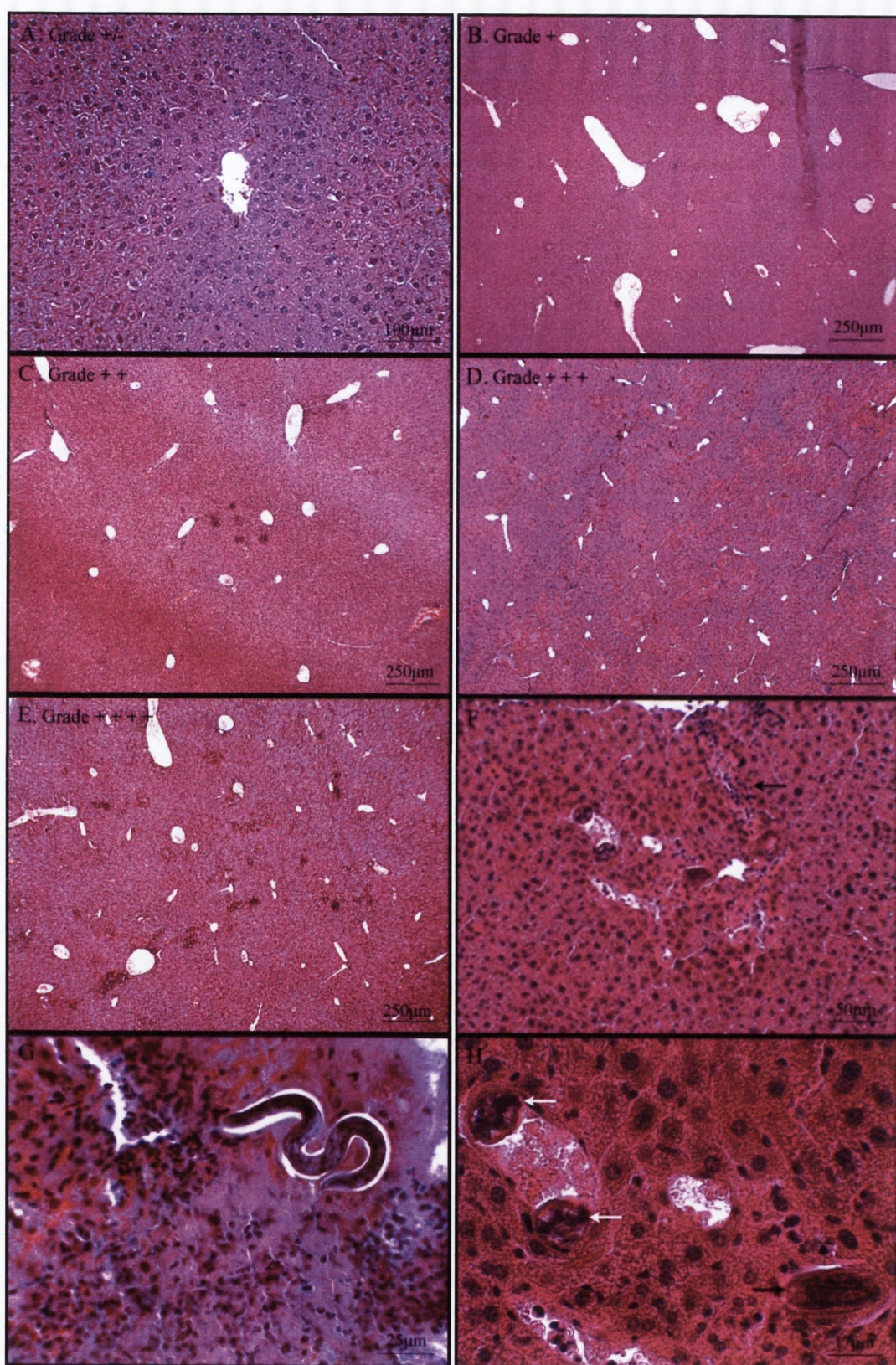


Plate 4.1. Cont.

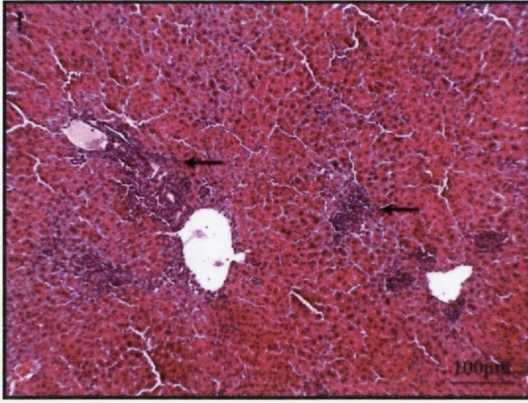


Plate 4.1. Photomicrographs of haematoxylin and eosin stained liver sections.

No accumulations of granulocytes were apparent in (A) control uninfected liver lobe (grade +/-). The number and distribution of accumulations of granulocytes increased in livers scored + to ++++ as shown in (B) mild inflammatory reaction (grade +), (C) moderate inflammatory reaction (grade ++), (D) strong inflammatory reaction (grade +++), (E) severe inflammatory reaction (grade ++++). (F) larvae with associated necrotic larval tracts (indicated by black arrow), Larvae were observed in the liver tissue as (G) longitudinal sections in hepatic parenchyma and adjacent granulocytes, (H) transverse (within blood vessel) (indicated by white arrows) and longitudinal (indicated by black arrow) sections of larvae, (I) focal accumulations of granulocytes (indicated by black arrows).

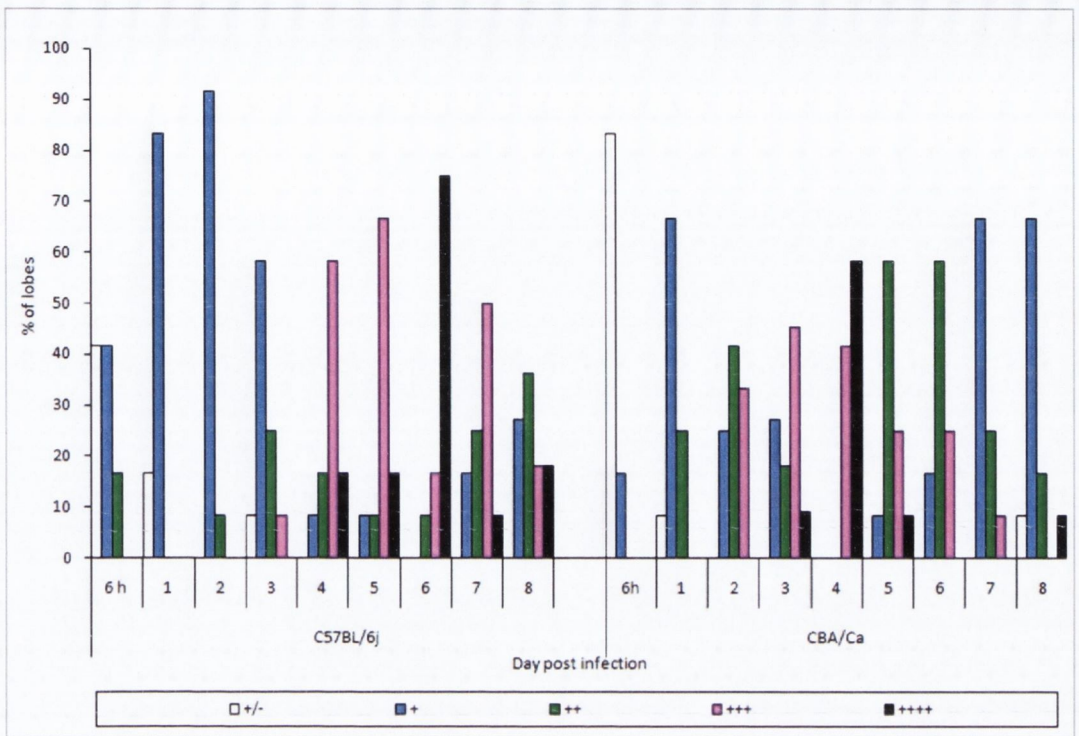


Figure 4.2. Changes in the percentage of hepatic lobes allotted each score based on examination of one tissue section

4.3.1.4. Changes in the inflammatory reaction in individual hepatic lobes

A weak initial reaction, which increased steadily over the early days of infection, was observed in each of the hepatic lobes of C57BL/6j mice. The response observed in the median lobe (Fig. 4.3 A) increased steadily on days 3-5 p.i. and severe inflammation was noted on days 6-8 p.i. Severe inflammation was observed earlier on days 4 and 5 p.i. in the left, right and caudate lobes (Fig. 4.3 B-D) and peaked on day 6 p.i. but the reaction became weaker on days 7 and 8 p.i. A striking reduction in the degree of inflammation was observed on day 7 p.i. in the left lobe as the severe inflammation (grade +++) present in 100% of C57BL/6j mice on day 6 p.i. was reduced to relatively mild inflammation (grade + and grade ++) in 67% of mice on day 7 p.i.

Similarly low-grade inflammation was recorded during the initial post-mortem time-points in each lobe of the CBA/Ca mice yet this increased more rapidly, particularly on

days 2 and 3 p.i., relative to C57BL/6j mice. Severe inflammation (grade +++ and +++) was detected in a proportion of mice in all hepatic lobes on day 3 p.i. especially in the right lobe in which the reaction was most intense (Fig. 4.4 C). The reaction peaked on day 4 p.i. in each lobe. At this post-mortem time-point the inflammatory reaction was identical in the median, left and right lobes (67% grade ++++ and 33% grade +++). A somewhat less marked response was observed on day 4 p.i. in the caudate lobe (67% grade +++ and 33% grade ++++). On day 5 p.i., the inflammatory reaction decreased in each CBA/Ca hepatic lobe particularly the left lobe (Fig. 4.4 B), in which a mild inflammatory reaction (grade + and grade ++) was only observed on days 5-7 p.i. and particularly on day 8 p.i. The resolution of hepatic injury was more gradual on days 5-6 p.i. in the remaining hepatic lobes yet the inflammation in the median, right and caudate lobes on days 7 and 8 p.i. was generally milder than in the left lobe. On day 8 p.i. the inflammation recorded in the median and right lobe of CBA/Ca mice was comparatively milder than that of C57BL/6j mice. In general, infiltration of inflammatory cells occurred earlier in CBA/Ca mice as did the resolution of the inflammation.

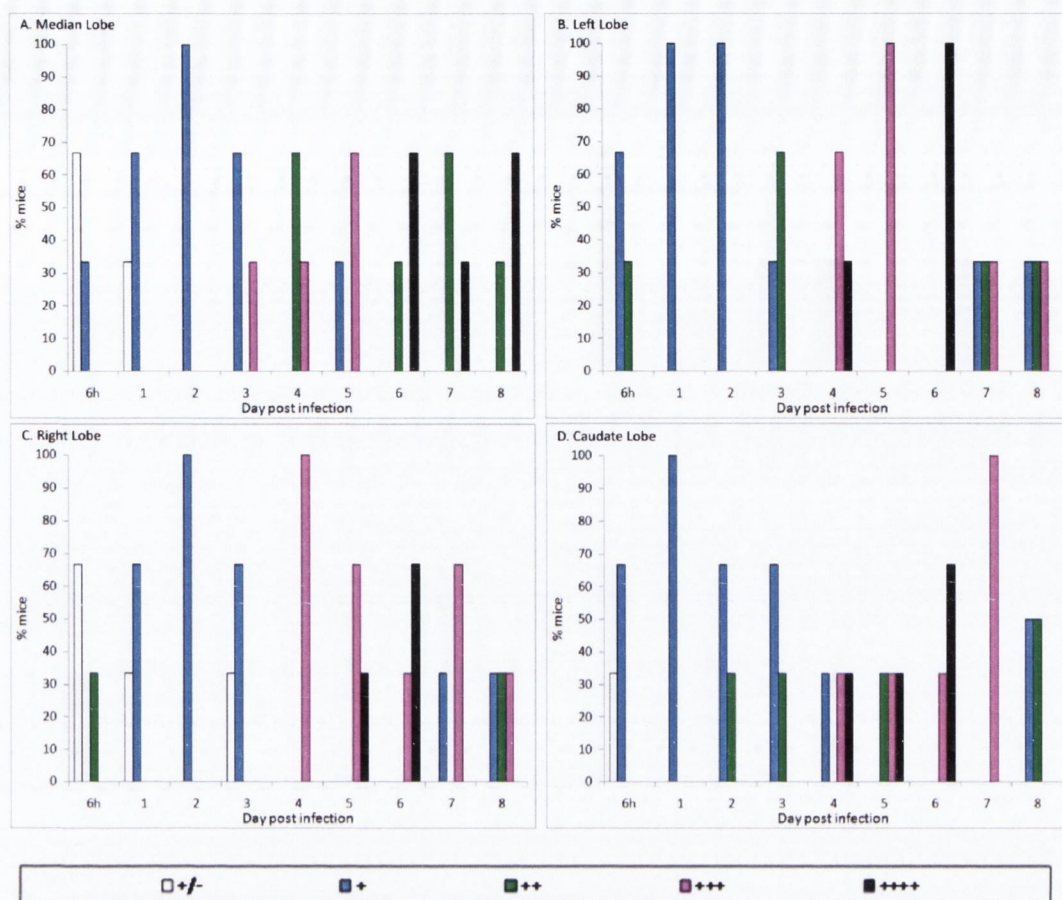


Figure 4.3. Changes in the percentage of individual hepatic lobes of C57BL/6j mice allotted each score based on examination of one tissue section.
(A) median lobe, (B) left lobe, (C) right lobe, (D) caudate lobe.

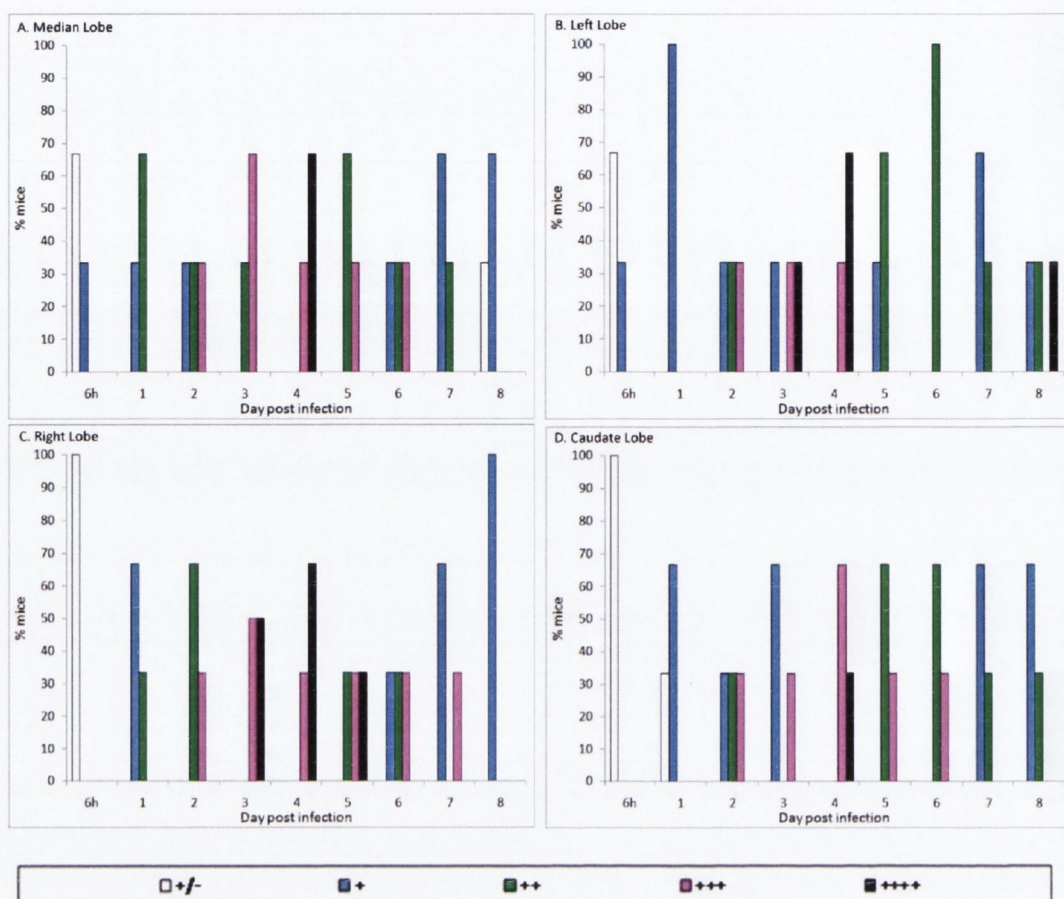


Figure 4.4. Changes in the percentage of individual hepatic lobes of CBA/Ca mice allotted each score based on examination of one tissue section.

(A) median lobe, (B) left lobe, (C) right lobe, (D) caudate lobe.

4.3.1.5. Comparison of semi-quantitative scores observed in serial sections

The mean semi-quantitative scores allotted to the three serial histopathological sections of each hepatic lobe are illustrated in Fig. 4.5, in C57BL/6j mice, the inflammatory reaction increased steadily over days 2-5 p.i. in the left, right and caudate lobes. A similar inflammatory reaction was observed in the median lobe of C57BL/6j mice on days 3-5 p.i.

In CBA/Ca mice, the severe inflammation on day 4 p.i. and its subsequent resolution by day 5 p.i. was observed in the left, right and caudate lobes (Fig. 4.5). A noteworthy

increase in the mean semi-quantitative score in the right and caudate lobes of CBA/Ca mice was observed from days 3 to 4 p.i. In the left lobe of the resistant strain, a more steady increase in the mean score was detected. While a similar mean score was observed in the median and left lobes of CBA/Ca mice on day 3 p.i., a lower mean score was observed in the median lobe on day 4 p.i. (Fig. 4.5).

Changes in the mean score over time (Fig. 4.5) were significant (main effect of day $P=0.001$) (Table 4.1), and both strains showed a similar pattern, if not intensity, of changes, over time (day*strain $P<0.153$). There was no significant difference between liver lobes (main effect of liver lobe $P=<0.128$) (Table 4.1) but with time, strain, the interaction between time and strain, and the main effect of liver lobe taken into account, there was a highly significant component of variation attributed to between-mouse variation in scores (mouse (day*strain), $P<0.001$) (Table 4.1).

Table 4.1. Results from a mixed model GLM, which examined the effects of time, strain and liver lobe on the mean semi-quantitative score of inflammation for liver serial sections during *Ascaris suum* larval migration

Source	df	F-ratio	P value
strain	1	5.008	0.040
day	3	8.785	0.001
day*strain	3	2.010	0.153
Error	16		
liver lobe	3	1.913	0.128
mouse (day*strain)	16	10.390	<0.001
Error	257		

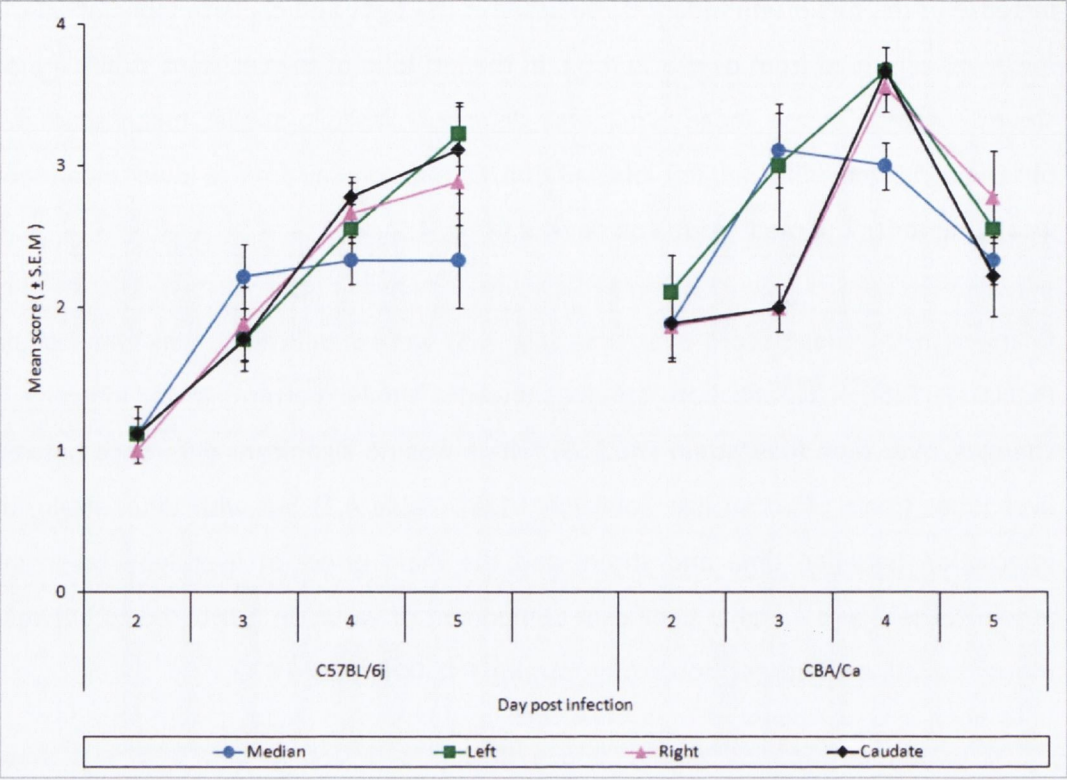


Figure 4.5. Changes in the mean score allotted to each hepatic lobe based on examination of three serial tissue sections

4.3.1.6. Examination of pulmonary lobe sections

4.3.1.7. General observations

No significant changes were observed in the pulmonary lobe sections of control animals (Plate 4.2 A). Following infection, focal accumulations of neutrophils, eosinophils, macrophages and lymphocytes were observed around blood vessels, bronchioles and alveoli in both mouse strains (Plate 4.2 F). Furthermore, emphysema, haemosiderophages and intra-alveolar haemorrhage was also detected (Plate 4.2 F and G). Longitudinal and transverse sections of larvae were detected in bronchioles and alveoli in both strains (Plate 4.2 D, F and I). An inflammatory response was often observed in the walls and surrounding area of the vessel containing the larva but not in all cases (Plate 4.2 D, F and I). Inflammatory cells were also seen to infiltrate the alveolar space (Plate 4.2 H) and at times closely abutted larvae (Plate 4.2 I).

4.3.1.8. Changes in the pulmonary inflammatory reaction on days 4-8 p.i.

Changes in the inflammatory reaction were detected in each pulmonary lobe of experimental animals examined and are illustrated in Fig. 4.6. At the initial stages (days 4-6 p.i.) of pulmonary larval invasion, mild inflammation (grade + and grade ++) was noted in the majority of lobes in C57BL/6j mice, while a low percentage of pulmonary lobes displayed a more intense inflammatory reaction (grade +++). A weaker initial reaction was observed in the lung lobes of CBA/Ca mice, as a strong inflammatory reaction was only observed in 6.67% of lobes on day 5 p.i. A varied reaction was observed in both strains on day 7 p.i., at which point a strong inflammatory reaction (grade +++) was detected in 27% and 47% of pulmonary lobes in C57BL/6j and CBA/Ca mice respectively. Severe inflammation was first noted on day 7 p.i. in 20% of C57BL/6j and 67% of CBA/Ca pulmonary lobes. An increase in inflammation was observed on day 8 p.i. as a more severe reaction was noted in both strains, particularly CBA/Ca mice. However, comparatively more C57BL/6j lobes were allotted a high semi-quantitative score (grade +++ and grade ++++) while mild inflammation (grade ++) was recorded in 20% of CBA/Ca pulmonary lobes on day 8 p.i. also (Fig. 4.6).

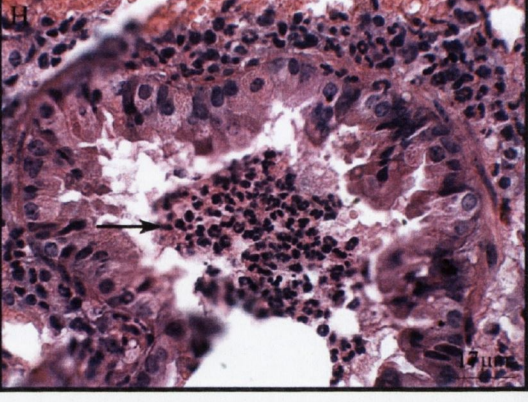
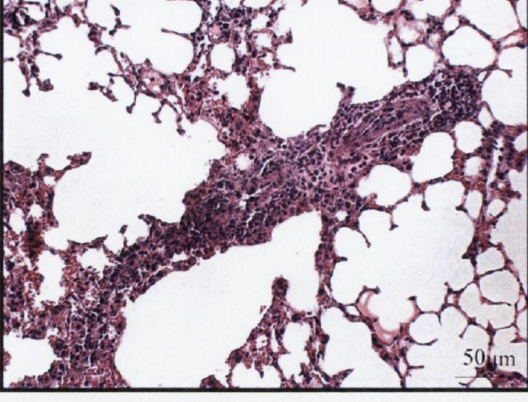
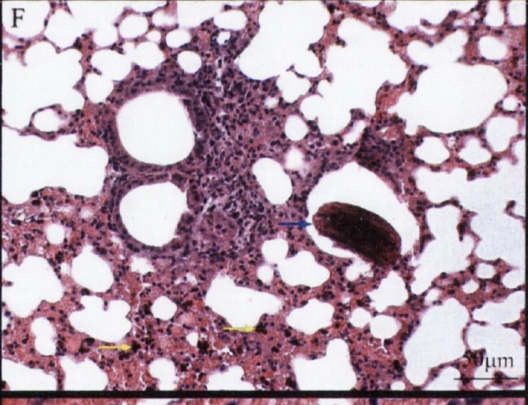
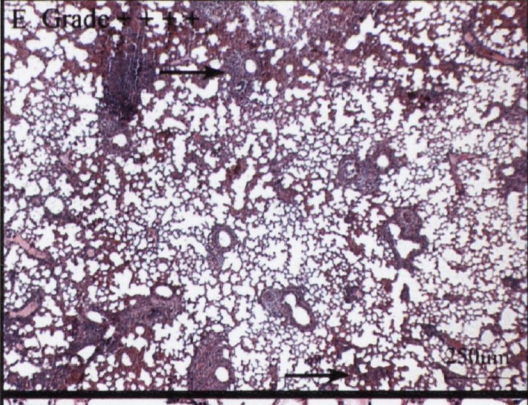
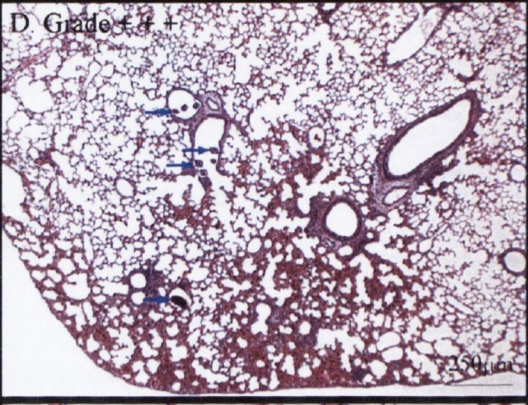
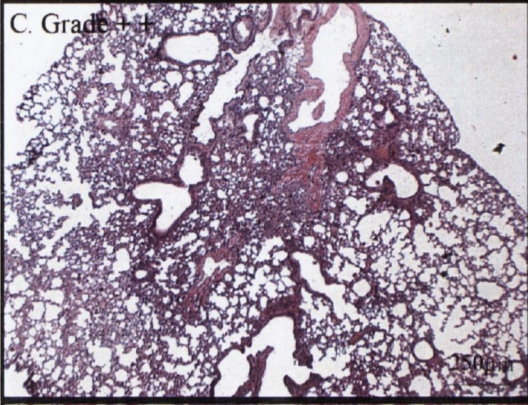
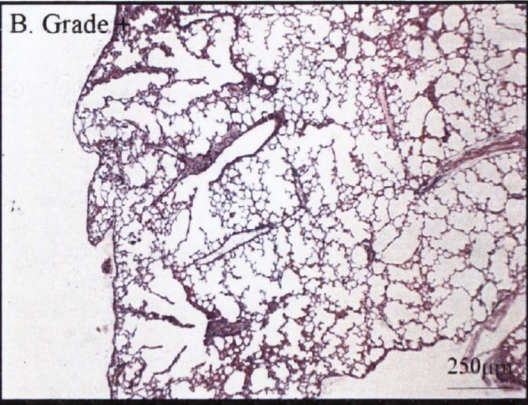


Plate 4.2. Cont.

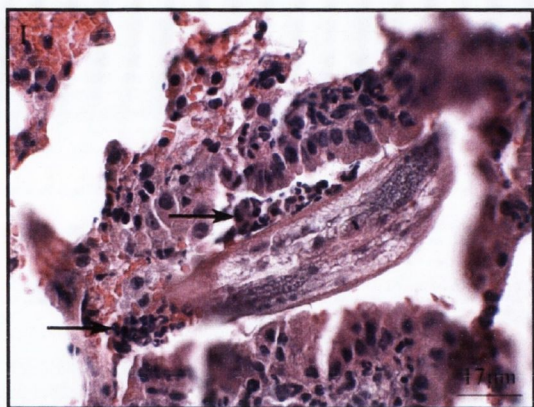


Plate 4.2. Photomicrographs of haematoxylin and eosin stained lung sections.

Grades of inflammation were based on an increased amount of granulocytes accumulating in the pulmonary tissue, particularly surrounding the vasculature. (A) Control, uninfected lung lobe (grade +/-), (B) mild inflammatory reaction (grade +), (C) moderate inflammatory reaction (grade ++), (D) moderately-severe inflammatory reaction (grade +++), (E) severe inflammatory reaction (++++), (F) Longitudinal section of larva in alveolus (indicated blue arrow) and haemosiderophages (indicated by yellow arrows), (G) emphysema observed in pulmonary parenchyma, (H) inflammation within a bronchiole (indicated by black arrow), (I) inflammation in an alveolar space adjacent to a larva.

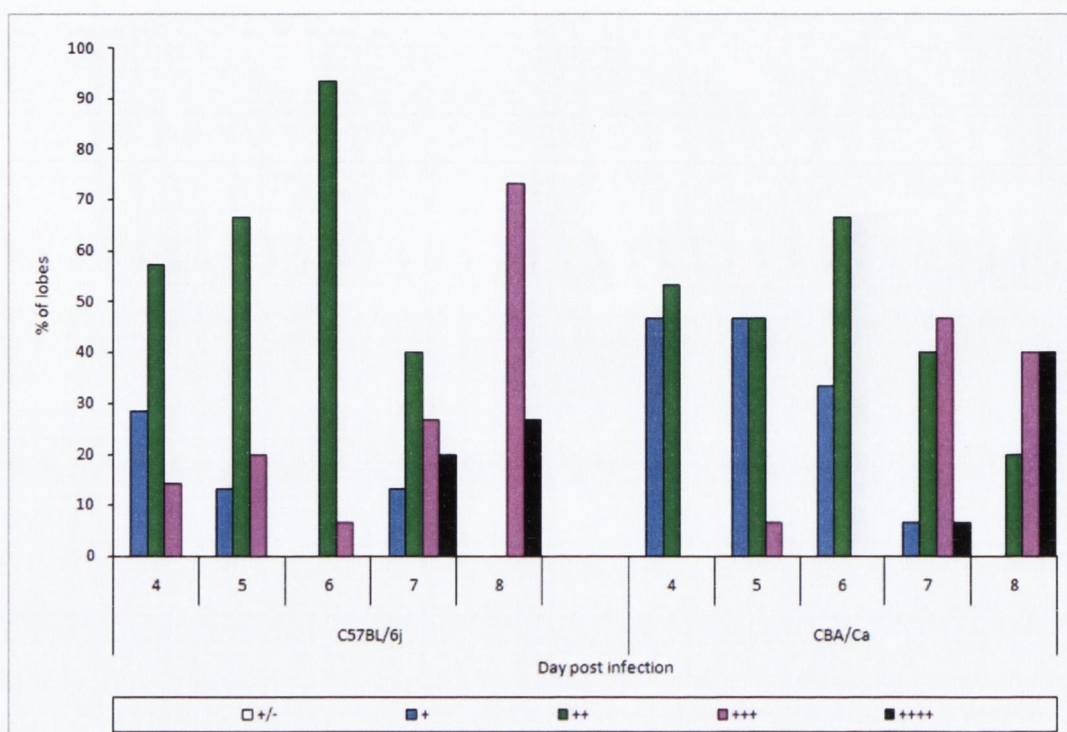


Figure 4.6. Changes in the percentage of pulmonary lobes allotted each score based on examination of one tissue section

4.3.1.9. Changes in the inflammatory reaction in individual pulmonary lobes

A relatively weak inflammatory reaction was observed on days 4-6 p.i. in the left and right lung lobes of C57BL/6j mice (Fig. 4.7 A-E). Severe inflammation was noted in a low proportion of sections over the early time-points in all lobes with the exception of the accessory lobe. Severe inflammation (grade +++ or grade +++) was observed on day 7 p.i. in the left and right lung cranial, caudal and accessory lobes but was most striking in the left lung and caudal lobe. On day 8 p.i., an identical inflammatory response (67% grade +++ and 33% grade +++) was recorded in every lobe excluding the accessory lobe (100% grade+++).

In CBA/Ca mice, a weak inflammatory reaction was also noted in the early stages of pulmonary larval invasion (days 4-6 p.i.) yet was generally milder than the inflammation observed in C57BL/6j mice. Severe inflammation (grade +++ and grade +++) was

evident on days 7 and 8 p.i. particularly in the accessory lobe (Fig 4.8 A-E). The reaction observed on these later time-points was similar in both strains in the middle and caudal lobes (Fig 4.8 C-D).

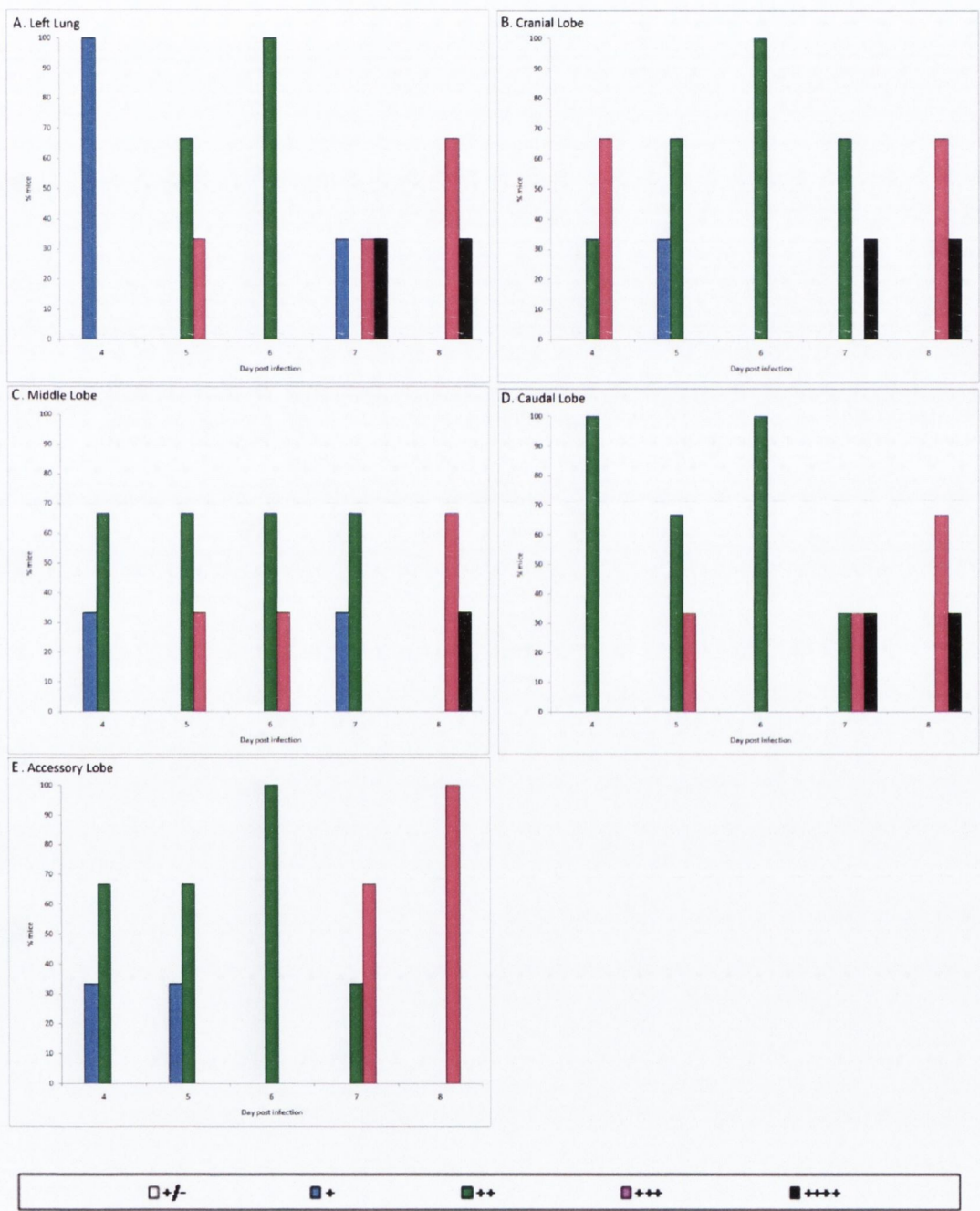


Figure 4.7. Changes in the percentage of individual pulmonary lobes ((A) left lung, and right lung (B) cranial, (C) middle, (D) caudal and (E) accessory lobes) of C57BL/6j mice allotted each score based on examination of one tissue section

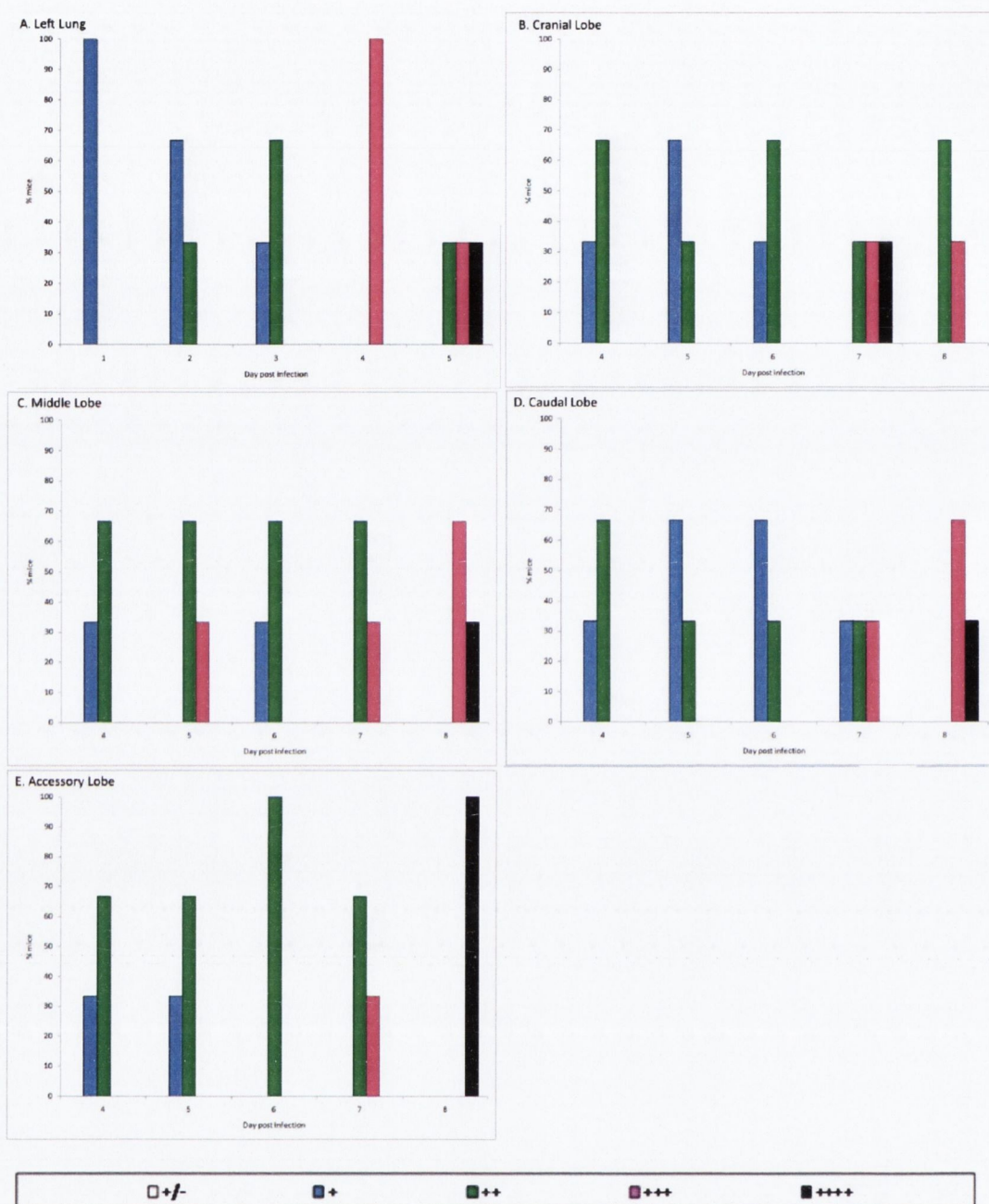


Figure 4.8. Changes in the percentage of individual pulmonary lobes ((A) left lung, and right lung (B) cranial, (C) middle, (D) caudal and (E) accessory lobes) of CBA/Ca mice which were allotted each score based on examination of one tissue section

4.3.2. Examination of periodic acid Schiff stained sections

4.3.2.1. General observations

No mucus secretion was observed in uninfected control animals (score +/-) (Plate 4.3 A). Varying degrees of mucus secretion were observed in lungs of both C57BL/6j and CBA/Ca mouse strains and the abundance observed was graded accordingly (Plate 4.3 B-E). Larvae were frequently detected in proximity to, but not adherent to, mucus (Plate 4.3 F).

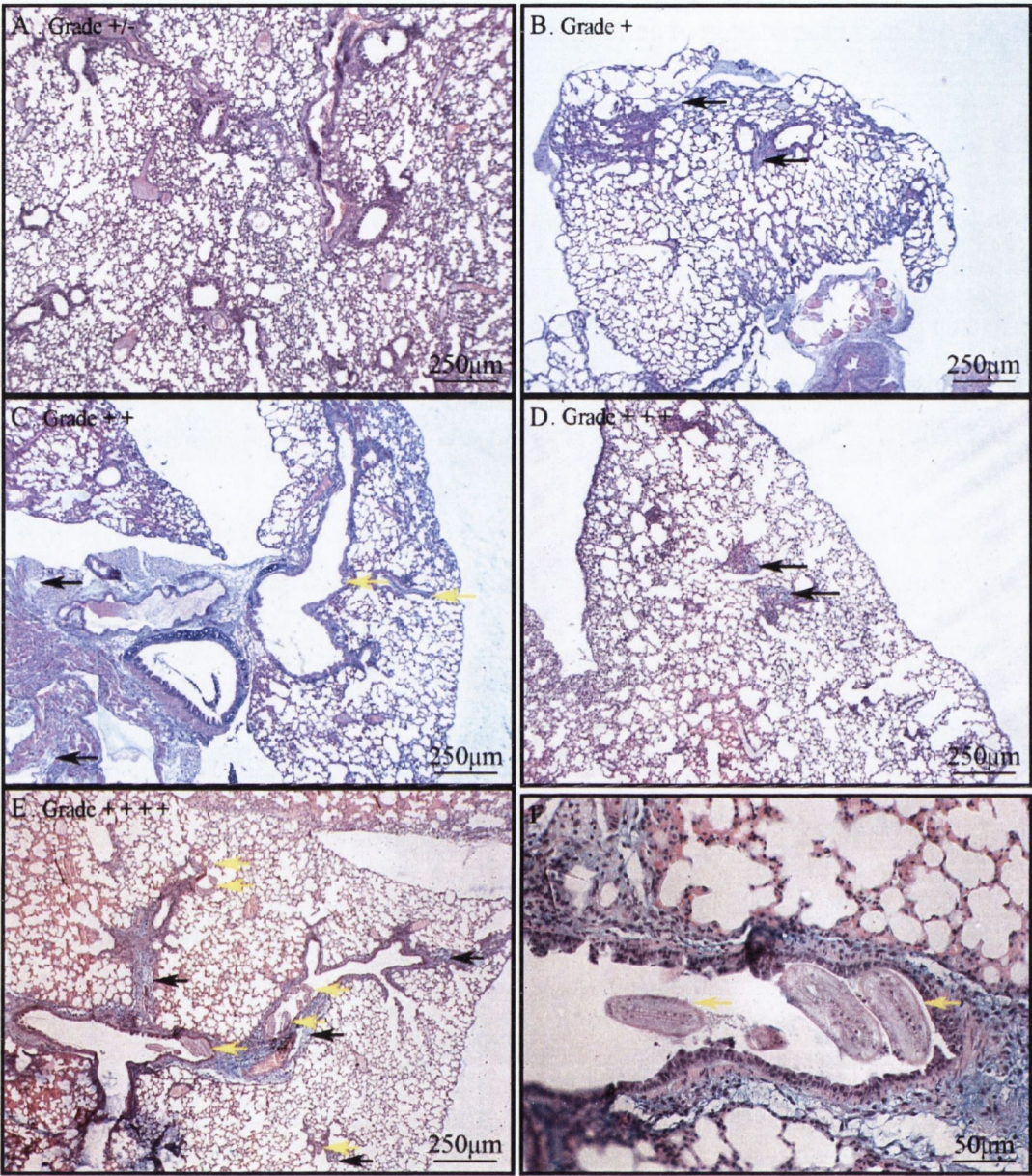


Plate 4.3. Photomicrographs of periodic acid Schiff stained lung sections.

Foci of mucus are indicated by black arrows in the various grades of mucus secretion observed. (A) Control uninfected lung lobe (grade +/-), (B) mild mucus secretion (grade +), (C) moderate mucus secretion (grade ++), (D) moderately-severe mucus secretion (grade +++), (E) severe mucus secretion (grade ++++), (F) Longitudinal section of larva in bronchiole (indicated yellow arrows) with adjacent, non-adherent mucus.

4.3.2.2. Changes in mucus production in individual pulmonary lobes

The percentage of total lung sections allotted scores based on mucus secretion are illustrated in Fig 4.9. In general, a higher level of mucus was evident in C57BL/6j mice as grade ++++ levels were detected as early as day 6 p.i. in this strain. The percentage of lung sections assigned a grade +++ gradually increased over time in C57BL/6j mice but peaked in CBA/Ca mice on day 6 p.i. (Fig 4.9). Very high levels of mucus were not observed in the resistant CBA/Ca strain until day 8 p.i. Furthermore, on days 5-7 p.i. consistently low levels of mucus were observed in CBA/Ca mice as a maximum of 10% of lung sections examined were allotted a grade +++ (Fig. 4.9).

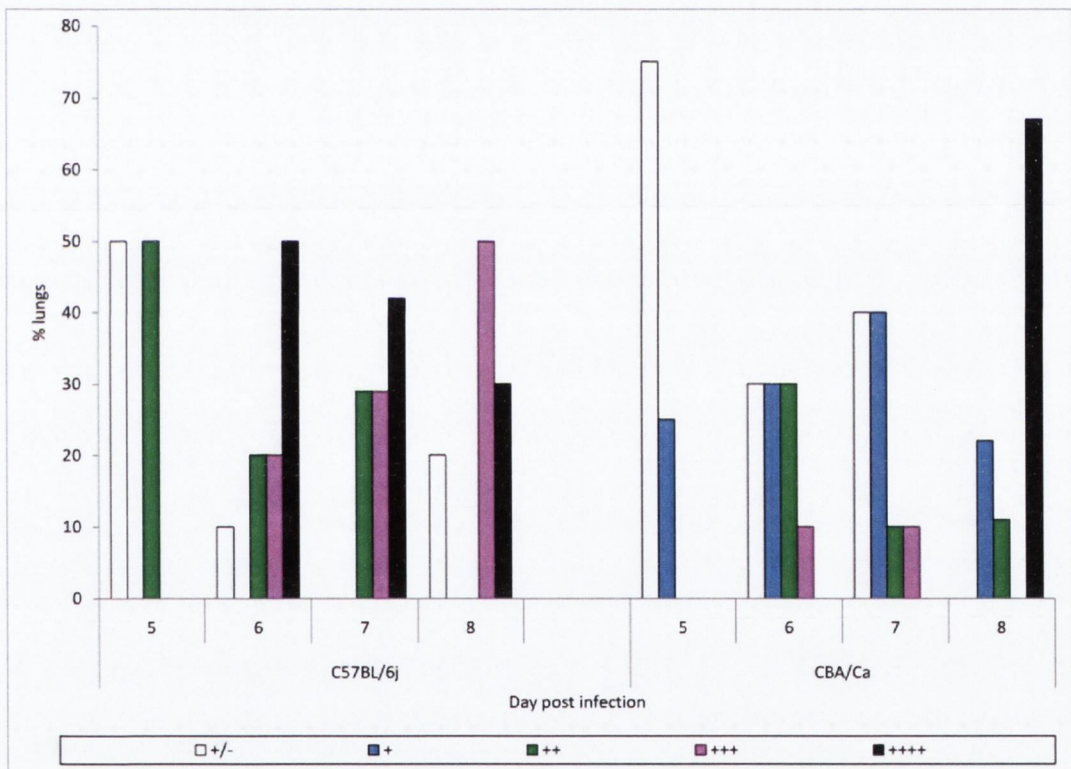


Figure 4.9. Changes in the percentage of lung sections allotted each score based on examination of one tissue section

When the left lung sections were examined separately (Fig. 4.10 (A)), a similar pattern was observed as in the total lung section scores (Fig. 4.9). Very high levels of mucus (grade ++++) were not observed on day 8 p.i. in C57BL/6j left lungs (Fig. 4.10 (A)) but were observed in a large proportion of C57BL/6j right lungs on days 6-8p.i., peaking on

day 6 p.i. (Fig. 4.10 (B)). In CBA/Ca left and right lungs, very high levels of mucus (grade +++) were not observed in left and right lungs until day 8 p.i. (Fig. 4.10 (A) and (B)). A relatively low level of mucus was observed in CBA/Ca mice on days 5-7 p.i. with a slightly higher level observed in the left lung on day 6 p.i. (Fig. 4.10 (A)) and in the right lung on day 7 p.i. (Fig. 4.10 (B))

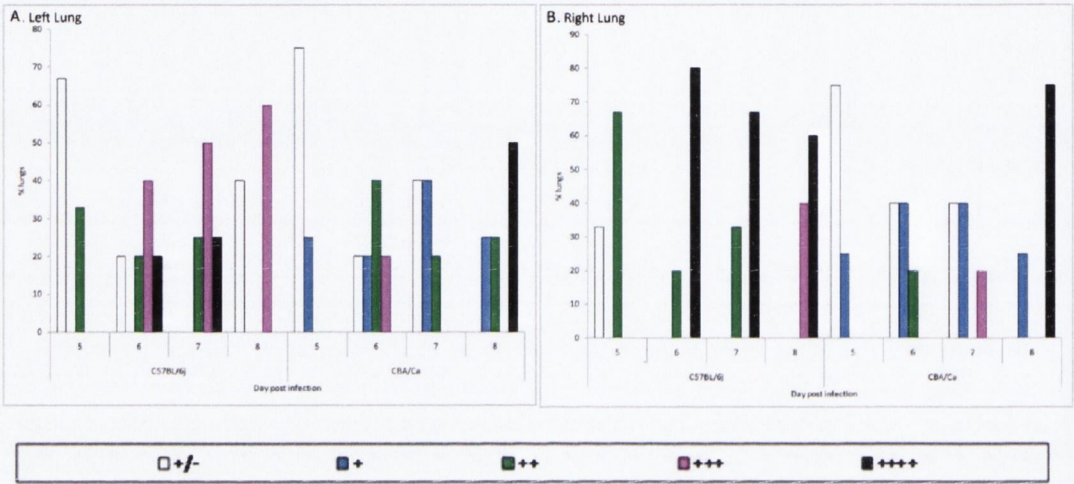


Figure 4.10. Changes in the percentage of the left (A) and right (B) lungs of C57BL/6j and CBA/Ca mice allotted each score based on examination of one tissue section

4.4. Discussion

In the present study sections of liver and lung were examined in susceptible and resistant mouse strains to assess and compare the host response to *A. suum* larval migration.

A similar type of granulocyte-macrophage rich inflammation was induced by larval migration in both strains so that there was no qualitative difference in this response between the two mouse strains. Foci of necrosis and attendant inflammation were observed in both and the inflammatory response was comparable to that previously described (Bindseil, 1981). Heavy infiltration of granulocytes and macrophages as well as necrosis was observed in both strains but the kinetics of this process in the liver between the susceptible and resistant hosts differed. The earlier more severe hepatic inflammation on day 4 p.i. in the resistant CBA/Ca mice coincided with the low numbers of larvae successfully migrating from the liver to the lungs (chapter 3, section 3.3.1.3) whereas the more delayed severe inflammatory reaction on day 6 p.i. in the susceptible C57BL/6j mice occurred when the majority of the larvae had already migrated to the lungs. In the CBA/Ca mice, the resolution of the necrosis and inflammation was more rapid following the reduction in larval numbers.

Therefore, it would appear that CBA/Ca mice dealt with the parasitic insult more effectively and economically in terms of responding earlier to restrict the same degree of larval invasion, and effecting more rapid tissue recovery without triggering excessive additional inflammation. Day 4 p.i. has played a prominent role in earlier work on *Ascaris* infections. Larvae were encapsulated in the liver from day 4 p.i. onwards by inflammatory cells in guinea pigs immunised against *A. lumbricoides* (Soulsby, 1957) with consequent reduced larval numbers noted in the lungs (Soulsby, 1957; Khoury *et al.*, 1977). Furthermore, neither little inhibition in *A. suum*-infected host growth, nor increased systemic eosinophilia was evident before this time in guinea pigs (Fallis, 1948; Soulsby, 1957). Therefore, day 4 p.i. has previously been considered a key time-point in effecting protection against *Ascaris* infection.

While it has been proposed that the day 4 p.i. inflammatory reaction in the liver of guinea pigs is induced by a larval moult in this organ (Taffs, 1968), L₃ larvae have been observed in *Ascaris ova* (Geenen *et al.*, 1999) and ecdysis has not been demonstrated in the liver (Fagerholm *et al.*, 2000). Excretory-secretory (ES) products capable of inducing a response are continuously produced by the metabolic activity of larvae during migration (Guerrero and Silverman, 1969; Stromberg, 1979) and have been shown to differ depending on their developmental stage (Kennedy and Qureshi, 1986). The ES-antigens are considered targets for responses during larval migration and the temporal difference observed in hepatic inflammation in the current study may be due to restricted responses in the susceptible C57BL/6j mice to particular ES antigens secreted early following hepatic invasion. The *in vitro* response of previously infected NIH mice, to *A. suum* ES-antigens (Kennedy and Qureshi, 1986) lacks responsiveness to the 14,000 MW component of both L₂ and L₃/L₄ ES. Interestingly, the NIH inbred strain has since been assigned to the intermediate susceptibility category when infected with *A. suum* (Lewis *et al.*, 2006). However whether CBA/Ca mice respond to this particular ES antigen has yet to be investigated and further investigation of the mechanism(s) in the liver responsible for impeding migration is required.

Statistical analysis of serial sections of the hepatic lobes revealed that a similar inflammatory reaction occurred over time between lobes within strains. Therefore, it appears that the innate cellular response, marked by the inflammatory cell infiltrate, is similar throughout the liver in both distribution and severity. Although the significant change in the inflammatory reaction over time appeared to follow a similar pattern within each lobe in each strain, this response did not reflect the contemporaneous larval burden (chapter 3, section 3.3.1.2). This was particularly evident when severe inflammation was recorded on day 4 p.i. in CBA/Ca mice and on day 6 p.i. in C57BL/6j mice as peak larval burdens were not recorded at these time-points and in the latter case, the majority of larvae had migrated to the lungs. Therefore it appears that the influx of inflammatory cells in the liver does not reflect larval burden, and may thus indicate that this event has a protective role.

Examination of lung sections indicated that the observed inflammation was similar to that previously described (Eriksen, 1981; Lewis *et al.*, 2007). The micro-emphysema observed in a proportion of sections has been reported previously (Taffs, 1965; Lewis *et al.*, 2007) and is likely the result of forced expiratory efforts through airways partially obstructed by larvae and associated inflammatory exudate (Caswell and Williams, 2007)

Inflammation to *A. suum* infection was not particularly severe until 7-8 days p.i., which is in accordance with Eriksen (1981). The lack of a noteworthy reaction at the earlier time-points reinforces the view that this response, even at the onset of pulmonary migration, is not protective (Lewis *et al.*, 2007). Furthermore, the comparatively greater response in the susceptible strain, also previously reported later in pulmonary invasion (Lewis *et al.*, 2007), coupled with the kinetics of the response in relation to the larval burden, demonstrates that the inflammatory reaction is linked to the contemporaneous larval burden. However, in light of the level of disparity in larval numbers in the pulmonary tissue, the response in the resistant CBA/Ca mice is comparatively greater in relation to larval burden than is that of the susceptible strain.

The separation of the pulmonary tissue into lobes further illustrated that the inflammatory reaction was a response to the larval burden as the intensity of the inflammation appeared to broadly reflect larval numbers in the tissue. This was particularly evident on analysis of data from the accessory lobe where, as anticipated, the highest semi-quantitative score (grade +++) was not observed in C57BL/6j mice, as the lowest larval burden was recorded in this lobe.

A greater frequency of the highest semi-quantitative scores was recorded in the lungs of C57BL/6j mice while examining and grading mucus secretion. This appeared to be a function of infection intensity. Levels of mucus reflecting larval burden was particularly evident in CBA/Ca mice as the highest level of mucus secretion (grade +++) was observed on day 8 p.i. only, at which point larval numbers in the lungs peaked in the resistant strain. Furthermore, mucus did not infiltrate the airways invaded by larvae to encapsulate the parasites. Therefore the present study indicates that mucus does not appear to facilitate larval expulsion from the lungs. Mucus production is part of the

catarrhal inflammatory response to parasites in contact with epithelial surfaces (Poynter, 1966).

The inter-relationship between the numbers of migrating larvae and the mechanical injury (necrotic larval tracts and haemorrhage) triggered is complex. It is not always possible to conclude if the cellular infiltrate is acting to solely restrict larvae or is also mobilised to resolve the attendant tissue damage. In the case of haemosiderophages, however, these cells function in removing and breaking down erythrocytes released from ruptured blood vessels. If early inflammation against larvae restricts migration, it will reduce consequent necrosis and inflammation. Since the early severe inflammation in CBA/Ca mice coincides with a distinct reduction in pulmonary larval burden and rapid tissue recovery, it would appear that inflammation is acting to restrict larval activity. In C57BL/6j mice, widespread necrosis in the liver was observed at early post-mortem time-points with no evident cellular infiltrate. Therefore, it is conceivable that the absence of migration-restricting early inflammation allows larvae to move throughout the liver more freely, creating more necrosis. The resultant increased tissue damage would lead to the mobilisation of leucocytes to phagocytose and repair the damage and thus may explain the more severe inflammation observed on day 6 p.i. in C57BL/6j mice by which time the majority of larvae have left the liver.

Previous authors have documented innate cellular responses in the liver of *Ascaris*-infected animals and have concluded that the hepatic response is key to impeding further larval migration (Kerr, 1938; Fallis, 1948; Sprent, 1949; Taffs, 1968; Copeman and Gaafar, 1972; Eriksen *et al.*, 1980). Furthermore, the temporal difference in the inflammatory response between resistant and susceptible strains observed in this study, indicates that the innate liver response plays a significant role in larval attrition. The delayed inflammatory reaction in the livers of C57BL/6j mice may be related to differing levels of cytokines, involved in the recruitment and activation of inflammation. In consequence we will further investigate the local hepatic cytokine *milieu* in infected mice in chapter 6.

5. Investigating the association between the Intelectin-2 gene and the response to *Ascaris suum* infection in C57BL/6j and CBA/Ca mice

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5.1. Introduction

There are several factors that contribute to intraspecific variation to parasitic infection. Exposure to a pathogen is regulated by ecological and behavioural factors (Wakelin, 1978), however the ability of hosts to regulate parasites through immune responses is a very important determinant that affects levels of infection. Immune responses to infection, either innate or acquired, are under well-defined genetic control (Wakelin, 1978; 1985; Wassom *et al.*, 1986; Hill, 1998; Quinnell, 2003). Furthermore, it is probable that resistance to infection would have several components so would therefore be under polygenic control (Wakelin, 1985; Quinnell, 2003).

Epidemiological studies of *A. lumbricoides* infection undertaken as early as the 1930s documented the occurrence of familial patterning of infection (Cort and Otto, 1933; Otto and Cort, 1934). Ascariasis has been referred to as a familial disease by Williams *et al.* (1974), as 82% of siblings of children infected with *A. lumbricoides* also harboured worms. Furthermore, in a Mexican study, where age-related intensity was corrected for, heavily infected individuals were found to be clustered in households and a high number of households were mainly composed of heavily infected individuals (Forrester *et al.*, 1988). Familial patterning to *Ascaris* infection has been noted in several further studies (Chai *et al.*, 1983; Forrester *et al.*, 1990; Chan *et al.*, 1994a).

Familial aggregation does not prove genetic control. A range of studies have also highlighted the contribution of environmental factors to *Ascaris* infection (Holland *et al.*, 1988; Wong *et al.*, 1988; Bundy and Blumenthal, 1990; Kightlinger *et al.*, 1998). Conflicting conclusions have been drawn regarding the relative role of host genetics and the influence of environmental and behavioural factors of familial aggregation. For example, in a study which examined intra-familial associations of infection intensity, a stronger association was recorded between unrelated parents than between parents and their offspring (Chan *et al.*, 1994b). Therefore, Chan *et al.* (1994b) concluded that if host genetics are a determinant of infection status, they are overwhelmed by environmental or behavioural factors.

As discussed by Quinnell (2003), convincing evidence of predisposition requires pedigree studies, which facilitate the separation of the effects of genetic relatedness and the shared household environment. Family-based linkage and association studies have been undertaken on a single Jirel pedigree population in the Jiri region of eastern Nepal (Williams-Blangero *et al.*, 1999; 2002; 2008). In their first study on *A. lumbricoides* infection in this pedigree Williams-Blangero *et al.* (1999) showed that 30-50% of variation in worm burdens is attributable to genetic factors. Later studies described the use of genome scans to identify three significant and three suggestive quantitative trait loci (QTL) which influenced infection intensity (Williams-Blangero *et al.*, 2002; 2008). Similarly, Nejsun *et al.* (2009a) undertook a genome scan in the *A. suum* pig model, which indicated that the heritability measure found for the intensity of *A. suum* infection in pigs (0.44) is similar to that for *A. lumbricoides* infection in humans (0.3-0.5) (Williams-Blangero *et al.*, 1999).

Kennedy *et al.* (1986) showed that mice with differing MHC haplotypes varied in their response to *Ascaris* antigens, indicating that inbred mice provide a model for genetic control to *Ascaris* infection. Furthermore, Holland *et al.* (1992) conducted a study in which the frequency of HLA class I A30/31 antigens was significantly higher in Nigerian children who were predisposed to *A. lumbricoides* infection. Therefore it appears the MHC genes contribute to host variation in response to *Ascaris* infection (Kennedy *et al.*, 1986; Holland *et al.*, 1992).

More detailed genetic mapping is required to investigate associations between specific alleles at the identified loci and infection intensity. Candidate genes for further investigation have been identified within the QTLS identified in the Jirel pedigree population (Williams-Blangero *et al.*, 2008). Furthermore, the STAT 6 gene, which promotes expression of Th2-associated genes, has been associated with low levels of *A. lumbricoides* infection (Peisong *et al.*, 2004) while the β_2 -adrenoreceptor has been associated with heavy worm burdens (Ramsay *et al.*, 1999). While work with a single candidate gene does not reveal the polygenic control of resistance to helminth infection, it may show evidence for a major causative gene of host variation. Segregation analysis allows one to determine the number of progeny that have

inherited distinct and mutually exclusive phenotypes. Therefore, in candidate gene studies, segregation analysis can then be undertaken to examine evidence for major gene effects and to determine the mode of inheritance.

The ability to manipulate the genetic background of inbred mice is beneficial to studies in which the association between candidate genes and a particular trait of interest are being studied. Furthermore, as stated by Dietrich (2001), studying mouse genetics could lead to the identification of human homologs which are likely to affect the outcome of infections in man. Neither genome scans nor candidate gene studies have been conducted in mice infected with *Ascaris*. Contrasting host intensity phenotypes in C57BL/6j and CBA/Ca mice provides a model in which the association between candidate genes and *Ascaris* infection intensity can be investigated.

Resistance to helminthic infection is multifactorial but a key question posed in a review by Quinnell (2003) is whether the same genes are involved in controlling different helminth species. Immune response genes, particularly those shown to play a role in resistance to other parasitic infections are likely candidates for future studies. The candidate gene of interest in the present study was intelectin-2 (Itln-2). Intelectins (Itlns) are a family of galactose-binding lectins with homologs documented in sea squirts, fish, frogs and mammals (Chang *et al.*, 2004; Chang and Nie, 2007). Komiya *et al.* (1998) first described mammalian intelectin, now known as mouse intelectin-1 (Itln-1), expressed by the Paneth cells of the small intestine.

Lectins play a role in the innate immune response as they act as binding enhancers for the process of phagocytosis (opsonins) and also facilitate the binding of antigens (agglutinins). Human Itln-1 is also referred to as the receptor for human lactoferrin (Suzuki *et al.*, 2005), which has antimicrobial, immunomodulatory and anti-inflammatory properties (Ward *et al.*, 2005). Murine Itln-1 has been hypothesised to function in early defence against bacterial infections by recognising the carbohydrates on the pathogen's surface (Komiya *et al.*, 1998) and so possibly recognises a range of pathogen-associated glycans. Whilst working with *Trichinella spiralis* in a mouse model, Pemberton *et al.* (2004a) found that a novel lectin, now referred to as Itln-2, is highly

upregulated in small intestinal Paneth and goblet cells in relatively resistant BALB/c mice, which expel *T. spiralis* more rapidly than C57BL/6 mice. Early upregulation of Itln-2 was observed, indicating that it is a component of the innate response to *T. spiralis* infection. Itln-2 expression levels remained high through to day 14 p.i. at which point levels peaked, coinciding with worm expulsion. In addition, Datta *et al.* (2005) also recorded significant upregulation of Itlins in the caecum of BALB/c mice but not susceptible AKR mice when infected with *Trichuris muris*. Furthermore, no transcripts for Itln were detected in uninfected mice of either strain. Artis (2006) conducted further work on the *T. muris* mouse model (resistant BALB/c and susceptible AKR mice) and confirmed that both Itln-1 and Itln-2 are upregulated in the intestines of BALB/c mice at the time of worm expulsion. Similarly to Pemberton *et al.*, (2004b) Artis (2006) demonstrated that the induced Itln was immunolocalised to the goblet cells.

Pemberton *et al.* (2004a) detected that the inbred strain, C57BL/10 naturally lacks expression of the Itln-2 gene. While the Itln-1 gene is present on mouse chromosome 1H2, the Itln-2 gene is also absent in the closely related inbred strain, C57BL/6j, which was confirmed using RT-PCR (Pemberton *et al.*, 2004a). Later work conducted by Voehringer *et al.* (2007) in a *N. brasiliensis* mouse model demonstrated that Itln-1 and 2 are STAT-6 dependant genes.

The dominant expression of Itlins following *T. spiralis* and *T. muris* infections in mouse strains resistant to infection led to the suggestion that Itlins may play an anti-nematode role (Pemberton *et al.*, 2004a; 2004b; Artis, 2006). Therefore, given the knowledge on differential expression of Itln-2 in susceptible and resistant mice in other mouse models of helminth infection and the natural deletion of the Itln-2 gene in the susceptible C57BL/6j strain, Itln-2 was considered to be a plausible candidate gene for investigation in the *A. suum* mouse model. Therefore, a F₂ backcross experiment was conducted with C57BL/6j and CBA/Ca mice to establish whether resistance is attributable to the Itln-2 gene. Backcrossing is the optimal approach to map a quantitative trait in a laboratory animal model. A backcross programme is a breeding scheme in which two parental mouse strains (the donor and recurrent or backcross parental strains) are mated to yield a F₁ progeny with the desirable characteristic. The recurrent parent is then crossed with

the F₁ progeny of the initial cross. Crossing the F₁ generation progeny with the recurrent parent ensures that the genetic contribution of the donor parent halves in the second generation mice and so, as Hospital (2005) describes, isolates a character of interest in the genetic background of the recurrent parent.

In order to maximise the efficiency of the breeding colony, both male and female F₂ progeny were included in the experiment. In prior work on the *A. suum* mouse model (Lewis *et al.*, 2006; 2007), only male mice were used in *Ascaris* infection experiments. Therefore, it was necessary to investigate whether there is a difference in the phenotypic response to infection between male and female mice of both parental strains. Therefore, a pilot experiment was undertaken in which the effect of gender on infection intensity in both strains was investigated.

5.2. Materials and Methods

5.2.1. Pilot Gender Experiment

60 (30 C57BL/6j and 30 CBA/Ca, 1:1 sex ratio) inbred mice were infected with 1,000 *A. suum* ova for this experiment. On days 6, 7 and 8 p.i., five female and five male mice of each strain were sacrificed and the total lung larval burden was enumerated from each individual by means of the modified Baermann technique (see chapter 2, section 2.4).

5.2.2. Backcross breeding experiment

The protocol undertaken for breeding mice in this study was based on information published by Wolfensohn and Lloyd (2003) and personal communication with the chief technician of the Bioresources unit, Trinity College Dublin. In order to set up breeding colonies, 50 female and 15 male mice of each strain (C57BL/6j and CBA/Ca) were purchased from Harlan UK. All female mice were separated into colonies of 10 individuals in large standard plastic cages (41 x 24 x 13 cm). Male mice were randomly assigned to groups of three individuals per small standard plastic cage (35 x 15 x 13 cm). Mice were allowed to acclimatise for one week prior to breeding, which was conducted for five days. On each day at 4pm, three male mice were placed into each female colony so that the two different strains of mice were in each cage overnight (Table 5.1). Male mice were removed from each female colony at 7am the following morning and returned to their homecages.

Female mice were individually examined for the presence of a vaginal mucus plug, which is indicative of mating. Each mated female mouse was placed in a clean separate small standard plastic cage. If an enlarged abdomen was not evident in separated females two weeks post-mating they were returned to their homecage. Litters were monitored daily and were weaned at approximately 21 days of age, at which point males and females were separated and maintained in groups of three and ten respectively. At 8 weeks old, F₁ progeny were placed in breeding colonies with parental

mouse strains (Table 5.1). Parental strains were bred simultaneously to the F₂ colonies (Table 5.1). F₂ progeny and parental strains (Plate 5.1) were also weaned at approximately 21 days old.

Table 5.1. Colonies involved in backcross breeding experiment

	Female strain (x10 individuals)	Male strain (x3 individuals)	No. of colonies
Initial cross	C57BL/6j	CBA/Ca	5
	CBA/Ca	C57BL/6j	5
Backcross	F ₁ offspring	C57BL/6j	2
	F ₁ offspring	CBA/Ca	2
	C57BL/6j	F ₁ offspring	2
	CBA/Ca	F ₁ offspring	2
Parental strains	C57BL/6j	C57BL/6j	3
	CBA/Ca	CBA/Ca	3



Plate 5.1. Parental strains (C57BL/6j (A) and CBA/Ca (B)) and F_2 progeny (C57BL/6j $\times$ F_1 (C) and CBA/Ca $\times$ F_1 (D) parental strains)

5.2.3. Infection of F_2 and parental strain mice

At 7 weeks old, 120 F_2 offspring and 60 parental strain mice (Table 5.2) were transferred to the infection unit, where they were infected one week later.

Table 5.2. Number of F₂ and parental strain progeny infected with 1,000 *Ascaris suum* ova

Female parental strain	Male parental strain	Number of progeny	
		Male	Female
C57BL/6j	C57BL/6j	15	15
CBA/Ca	CBA/Ca	15	15
C57BL/6j	F ₁	15	15
CBA/Ca	F ₁	15	15
F ₁	C57BL/6j	15	15
F ₁	CBA/Ca	15	15

5.2.4. Collection of tissue at post-mortem

All mice were euthanised on day 7 p.i. At post-mortem a tail snip (approximately 2cm of the animal's tail) was removed with a scalpel and placed in 1.5ml of 20% dimethyl sulfoxide (DMSO) and stored at -80°C. The whole lungs from each mouse were placed in an individual Modified Baermann apparatus and larval counts were undertaken (see chapter 2, section 2.4).

5.2.5. Intelectin-2 gene screening

5.2.5.1. Extracting genomic DNA from tail snips

DNA was extracted from tail snips of all F₂ mice and four reference parental mice (two C57BL/6j and CBA/Ca mice). Each tail snip was defrosted on ice and removed from the DMSO. Approximately 0.5cm was removed from each snip and excess DMSO was blotted off with tissue paper. The QIAamp Tissue Kit (Qiagen) was used to extract DNA and the manufacturer's instructions were followed.

Briefly, all tail snip samples were brought to room temperature in sterile 1.5ml eppendorf tubes and 180µl of buffer ATL and 20µl of Proteinase K was added to each

sample. All tubes were then individually placed on the Camlab Rotamixer, to ensure even digestion of the tail sample, and incubated for at least four hours in a waterbath at 55°C. Once removed from the waterbath, 400µl of buffer AL:ethanol buffer (1:1 ratio) was added to each tube and agitated thoroughly on the vortex. A 600µl aliquot of the solution was transferred into clean labelled QIAamp spin columns placed in collection tubes, leaving behind any gelatinous debris and hair. The spin columns in collection tubes were centrifuged for one minute at 8000rpm. The filtrate from the collection tubes was removed and discarded. The DNA was retained in the spin column. A 500µl aliquot of buffer AW2 was added to each spin column and the samples were centrifuged for three minutes at 8000rpm.

Each spin column was transferred to a new clean collection tube and 200µl of RNase/DNase free water was added to each column. The samples were allowed to stand for one minute at room temperature and then centrifuged for one minute at 8000rpm. The DNA was eluted into the collection tube. The eluate in each collection tube was transferred to clean labelled 0.5ml centrifuge tubes. Samples were stored at -20°C.

5.2.5.2. Quantifying DNA samples

The concentration of DNA was quantified (ng/µl) in each sample using the NanoDrop™ ND1000 (Thermo Scientific). Prior to analysis, the machine was cleaned and a standard was set with RNase/DNase free water. For each sample the sample apparatus was opened and 1µl of sample solution was placed on the measurement pedestal (Plate 5.2 A). The top arm of the sample apparatus was lowered and once activated the top arm compresses the droplet, forming a sample column that was held in place by surface tension (Plate 5.2 B). A spectral measurement was made at 260nm. The concentration of DNA was calculated by a modified Beer-Lambert equation:

$$C = (A \cdot e) / b$$

where C is nucleic acid concentration in ng/µl, A is the absorbance at 260nm, e is the wavelength-dependent extinction coefficient in ng-cm/µl and b is the path length in cm,

which is normalised to 1cm. Double stranded DNA has an extinction coefficient of 50ng-cm/ μ l.

The sample arm and pedestal were cleaned thoroughly in between each sample with lab tissue. The quantity of DNA was recorded in each sample and all sample volumes were adjusted with RNase/DNase free water so that each had a similar concentration.

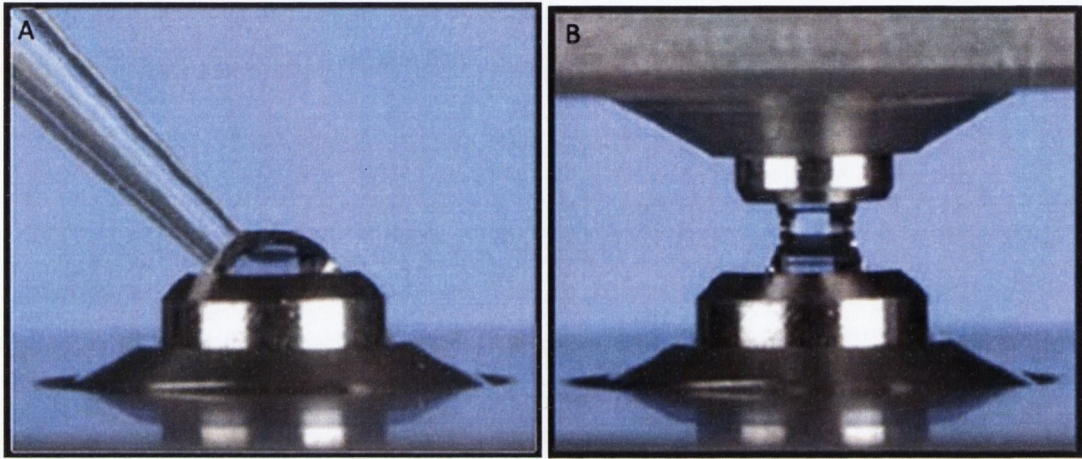


Plate 5.2. Loading sample onto pedestal of Nanodrop ND 1000 and sample arm compression causing column formation
(www.media.wiley.com)

5.2.5.3. Differential primers for *Itln* genes

Since the *Itln*-2 gene is absent in C57BL/6j mice, an *Itln*-2 specific marker was not used to establish the numbers of copies of the *Itln*-2 gene in each F₂ mouse. A differential marker and subsequent primers for the *Itln* locus were previously designed by means of comparing the locus of C57BL/6j, sequenced by Pemberton *et al.* (2004a) and 129S7 (Pemberton, unpublished sequence data) mice with the software Primer 3. When comparing the *Itln* locus, a unique region of the neighbouring gene, *SLAMF7* was shown to differ in length in C57BL/6j and 129S7 mice. Furthermore, this region was found to be a similar length in CBA/Ca mice as in 129S7 mice, when *SLAMF7* primers were tested on CBA/Ca genomic DNA (Steven Wright, unpublished data) (Fig. 5.1). Therefore the

SLAMF7 gene was considered a suitable marker to distinguish the *Itln* locus in the two mouse strains in the present study and primers were designed for this region using Primer 3 and their position is illustrated in Fig. 5.1).

The sequence of the SLAMF7 primers used were as follows:

Forward primer: TG TAGAGGTGGATGGTGCTG

Reverse primer: TGGGGTTCCATTCTGAGTTT

Using SLAMF7 marker primers, C57BL/6j mouse DNA gives a 200bp product, whereas 129S7 and CBA/Ca mouse DNA gives a 218bp product. In F₂ mice these primers yielded a 200bp and 218bp PCR fragment depending on the grandparental origin of the genome and inferred from this the inheritance of zero (both regions from C57BL/6j), one (one region from C57BL/6j, one from CBA/Ca) or two (both regions from CBA/CA) copies of the *Itln-2* gene.

The common forward primer for *Itln-1* and *Itln-2* gene, MITLNComf1 (5'-GGTTCCTGCCATTACTCAGC-3'), and the *Itln-2* specific reverse primer, MITLN2r1-r (5'-TTTATCATGATTGCCACGAGAGT-3'), were used to confirm SLAMF7 marker analysis and thus the presence of the *Itln-2* gene in mice. These two primers span intron 1, giving a 300bp product for *Itln-2* whereas the paralogous sequence for *Itln-1*, gives no product.

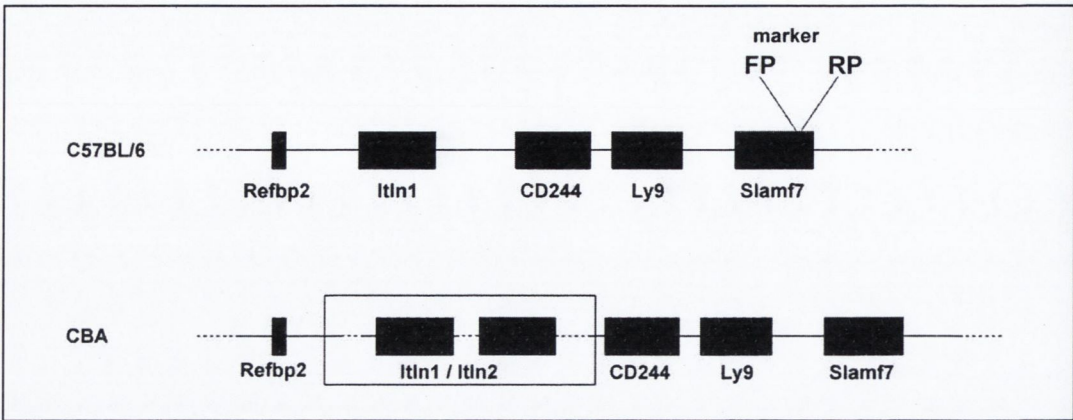


Figure 5.1. Schematic diagram (not to scale) of *Itln* locus and neighbouring genes (*Refbp2*, *CD244*, *Ly9*, *Slamf7*) on chromosome 1 of C57BL/6j and CBA/Ca mice. The forward (FP) and reverse (RP) primers used in the SLAMF7 marker analysis are also indicated (original illustration by A. Pemberton)

5.2.5.4. Polymerase Chain Reaction (PCR)

To infer the presence or absence of the intelectin-2 sequence in the genome of the F₂ mice, Polymerase Chain Reaction (PCR) using the SLAMF7 marker analysis was undertaken on all extracted DNA samples. A mastermix solution was made up in sterile 10ml tube prior to each PCR reaction. The following quantities of reagents were added to the mastermix per sample analysed:

Table 5.3. Quantity of reagents added to mastermix

Reagent	Quantity per tube (µl)
10x buffer	5
Taq polymerase	0.2
Nuclease free water	19.8
2mM dNTPs	5
2µM primers	10

10µl of each individual DNA sample was added to individual strip PCR tubes. In each set of PCR reactions, two CBA/Ca and two C57BL/6j mice samples were used as positive

controls while a sample of 10µl nuclease free water was added to one PCR tube as a negative control. As shown in Table 5.3., 40µl of mastermix was made up for each sample PCR reaction in each individual tube and was then added to each PCR tube. Caps were placed on the strips of PCR tubes and placed in the PCR machine.

Table 5.4. PCR reaction conditions (53°C, 35 cycles)

Temperature (°C)	Time	Number of cycles
94	2 minutes	1
94	40 seconds	35
53	20 seconds	
72	2 minutes	
72	10 minutes	1

5.2.5.5. Two-dimensional gel electrophoresis

Samples which were tested using ItIn specific primers were loaded on agarose gels, while those tested using SLAMF7 primers were loaded on polyacrylamide gels.

5.2.5.6. Agarose gel electrophoresis

In order to make 1.6% flatbed agarose gels, 1.6g of agarose was added to 100ml of 1x Tris-Borate EDTA (TBE) in a 500ml glass screw top bottle. The cap was loosely placed on the bottle, which was then placed in the microwave for two minutes. Once removed from the microwave, the bottle was swirled to ensure that the solution was clear indicating that the agar had dissolved. 10µl of the nucleic acid gel stain, GelRed™, was added to the solution and the bottle was swirled. The solution was allowed to cool for approximately 2-3 minutes at room temperature and poured into the gel tray placed in a stand. Two 20-well combs were secured in the tray prior to the addition of gel solution. Once the gel was set, the well combs were removed and 6µl of DNA ladder (Qiagen) was added to the first well in each row. 1µl of loading dye was added to a measure of 5 µl of each sample and the mixture was pipetted into individual wells. Gels

were resolved for 45 minutes at 100V. Gels were removed carefully from the tray and placed on the scanner tray (Bio-Rad Molecular Imager FX). Resultant PCR products were imaged using Quantity One® software.

5.2.5.7. Polyacrylamide gel electrophoresis

Polyacrylamide gels give higher resolution than standard agarose gels, which was necessary for optimal resolution of the 218 and 200bp bands resulting from the SLAMF7 marker PCRs. Therefore, samples were loaded on BIORAD Ready Gel® precast 10% TBE polyacrylamide gels with fifteen wells (15µl). Each gel was secured in a clamping frame, which was placed vertically in the tank. The inside and outside chambers were filled with approximately 125ml and 200ml of 1x TBE respectively. DNA ladder (6µl) and samples (5µl) were pipetted into individual wells as described above. A current was set for 80 minutes at 80V. Gels were removed from the clamping frame and placed in a staining solution (0.01% GelRed™ in 1x TBE) for 30 minutes in a weighboat. The weighboats were gently rocked for the duration of staining to ensure that all areas of the gel were equally exposed to the solution. Images of PAGE gels were obtained in a similar manner to flatbed gels as described above.

5.2.6. Statistical analysis

Statistical analysis was carried out at the 95% confidence interval. All lung larval burden data was assessed for normality and if necessary log+1 transformed.

The effects of gender, time and strain on the *A. suum* lung larval burdens in male and female C57BL/6j and CBA/Ca mice were analysed using a general linear model (GLM) in the pilot gender experiment. Since phenotyping of parental strains and F₂ generation mice was undertaken on day 7 p.i. in the second experiment, further analysis was separately undertaken on larval burdens from each day in the pilot gender experiment by means of a two-way GLM.

Day 7 p.i. lung larval burdens observed in mice euthanised for Itln-2 screening were compared between parental reference strains and also between F₂ offspring and parental reference strains. A two-way GLM was undertaken in which the influence of strain and sex on parental control strains lung larval burdens was assessed. A three-way GLM was conducted in order to determine the influence of the parental ancestry and gender of F₂ mice on lung larval burdens.

The relationship between the number of copies of the Itln-2 gene and lung larval burden was also investigated using a one-way GLM. Furthermore, the effect of gender and the number of copies of the Itln-2 gene on lung larval burdens was assessed using a two-way GLM. Least significant difference (LSD) post-hoc tests were carried out to further investigate the correlation between different copy numbers of the Itln-2 gene on larval burden.

5.3. Results

5.3.1. Pilot gender experiment

Mean lung larval burdens were recorded on days 6, 7 and 8 p.i. in male and female C57BL/6j and CBA/Ca mice (Fig. 5.2). Table 5.5 shows the results obtained when a three-way GLM analysing the effect of strain, gender and day p.i. was conducted on all larval burdens recovered from male and female C57BL/6j and CBA/Ca mice. Further analysis (two-way GLM) on the effect of strain and gender on larval burdens recorded at individual post-mortem time-points is given in Table 5.6.

A significant difference between the larval burdens in the two mouse strains was recorded regardless of gender over days 6-8 p.i. ($P < 0.001$) (Table 5.5). Strain was significant on days 6 p.i. ($P = 0.016$) and 7 p.i. ($P = 0.001$) but no significant difference was recorded on day 8 p.i. ($P = 0.731$) (Table 5.6), at which point larval numbers declined in both male and female C57BL/6j mice (Fig. 5.2).

In the CBA/Ca mouse strain similar parasitic burdens were recorded in male and female individuals, particularly on day 6 p.i. (Fig. 5.2). In relation to previous work (chapter 3, section 3.3.1.3), the numbers of larvae recorded in both genders of the resistant strain were similar in both genders on day 6 p.i. yet lower in this study on days 7 and 8 p.i. A higher mean larval burden was observed in male C57BL/6j mice than that previously recorded (chapter 3, section 3.3.1.3). As indicated by the large error bars in Fig. 5.2, the high S.E.M. calculated for the pulmonary parasitic burden in male C57BL/6j mice (day 6: 217 ± 70.2) indicated a wide range (day 6: 5-420 larvae) of larval numbers in the susceptible strain. The mean lung larval burden in female C57BL/6j mice (day 6: 118 ± 95.92) was lower than that of male C57BL/6j mice and comparable to that illustrated in male C57BL/6j mice in previous experiments (chapter 3, section 3.3.1.3). However, the range of larval numbers is large (day 6: 0-500 larvae). On day 7 p.i. slightly lower larval numbers were recorded in male and female C57BL/6j mice (male: 191 ± 46.62 , female: 114 ± 43.9). Similar low burdens were observed in the male and female susceptible strain on day 8 p.i.

Despite higher larval numbers in male C57BL/6j individuals on days 6 and 7 p.i., gender did not have a significant influence on larval burdens ($P=0.363$) (Table 5.5). While no significant effect of gender was detected (Table 5.5 and 5.6), a significant gender*strain interaction was observed ($P=0.043$). When the effects of gender and strain on larval burdens was investigated at individual post-mortem time-points, a gender*strain interaction was only detected on day 7p.i. (Table 5.6). A very high (365 larvae) and very low (10 larvae) larval burden was recorded in single male and female C57BL/6j individuals respectively on day 7 p.i., which were thought to account for the gender*strain interaction at this post-mortem time-point.

A significant change in larval burden over time was not recorded ($P=0.4298$) and even though male and female CBA/Ca mice larval burdens peaked on different days a significant gender*day interaction was not recorded ($P=0.5146$) (Table 5.5, Fig. 5.2).

Table 5.5. Results from the three-way GLM of the effect of strain, gender and time on the observed *Ascaris suum* larval burden in the lungs

Function	df	F-ratio	P value
strain	1	26.532	≤0.001
gender	1	0.842	0.363
gender*strain	1	4.130	0.043
day	2	0.859	0.430
strain*day	2	2.310	0.110
gender*day	2	0.673	0.515
strain*gender*day	2	0.543	0.584
error	48		

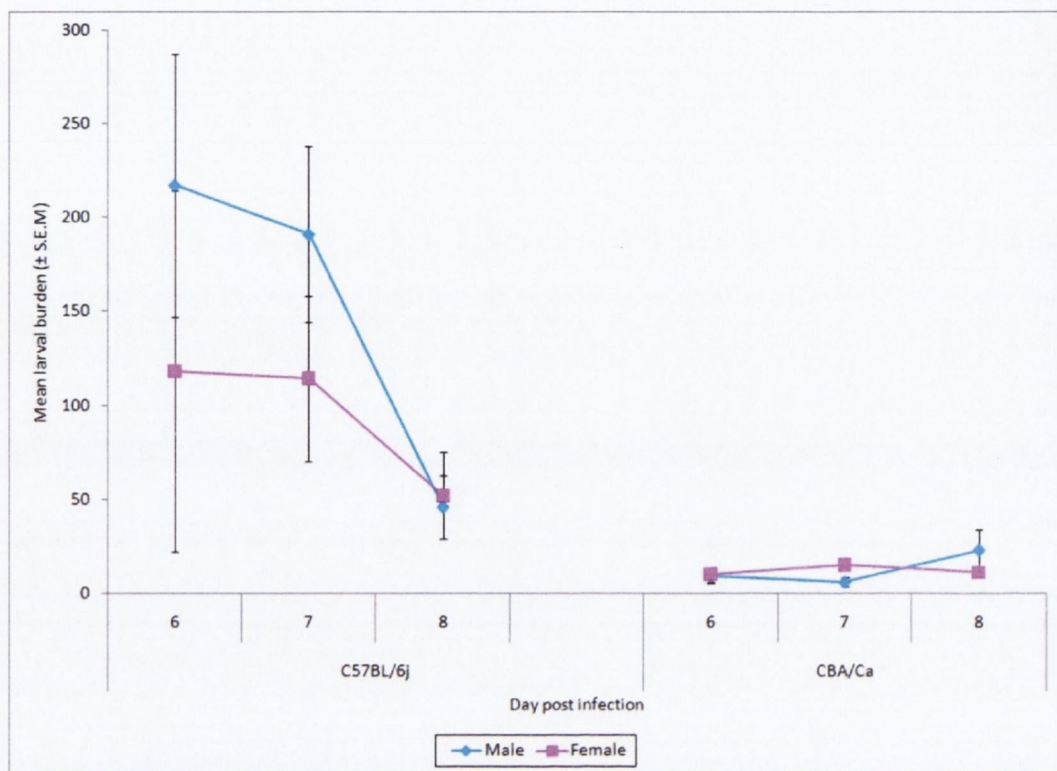


Figure 5.2. Changes in mean larval burden ($\pm$ S.E.M.) in the lungs of male and female C57BL/6j and CBA/Ca mice, following inoculation with 1000 *Ascaris suum* ova

Table 5.6. Results from the two-way GLM of the effect of strain and gender on the observed *Ascaris suum* larval burden in the lungs on days 6, 7 and 8 p.i.

Day p.i.	Source	df	F-ratio	P value
6	strain	1	7.270	0.016
	gender	1	1.140	0.301
	gender*strain	1	1.300	0.270
7	strain	1	37.710	0.001
	gender	1	0.210	0.652
	gender*strain	1	6.390	0.022
8	strain	1	1.720	0.208
	gender	1	0.353	0.560
	gender*strain	1	0.122	0.731
	error	16		

5.3.2. Backcross breeding experiment

5.3.2.1. Lung larval burdens

Pulmonary larval burdens were recorded on day 7 p.i. in male and female parental reference strains, C57BL/6j and CBA/Ca, and F₂ mice. As illustrated in Fig. 5.3, a two-way GLM indicated that larval numbers differed significantly between parental reference strains ($P<0.001$). A significant sex effect was also detected ($P=0.003$), which was particularly evident in C57BL/6j mice (Fig. 5.3).

The effect of gender was also recorded in the F₂ progeny ($P<0.001$) (Table 5.7). The most noteworthy difference in mean lung larval burdens between sexes within strains was evident in C57BL/6j mice and F₂ progeny with a C57BL/6j parent, as males harboured heavier pulmonary burdens than females (Fig. 5.3).

The influence of paternal strain of the F₂ progeny on larval burdens was significant ($P=0.012$) (Table 5.7) as the mean larval burden in F₂ individuals was markedly higher in mice with C57BL/6j fathers (199 ± 57.93) in comparison to those with F₁ (59.33 ± 8.60 , 27.50 ± 5.33) and CBA/Ca fathers (36.33 ± 6.38) (Fig. 5.3). The highly significant effect of maternal strain ($P<0.001$) was particularly apparent among F₂ offspring with F₁ fathers as those with C57BL/6j mothers were more heavily infected (59.33 ± 8.60) than those with CBA/Ca mothers (27.50 ± 5.33).

The variability between male and female F₂ offspring with a C57BL/6j parental strain was most apparent in individuals with a C57BL/6j father (male: 330.67 ± 104.58 , female: 67.67 ± 22.30) (paternal strain*gender $P=0.027$).

Table 5.7. Results from the three-way GLM of the effect of parental strain and gender on the observed *Ascaris suum* larval burden in the lungs on day 7 p.i.

Source	df	F-ratio	P value
paternal strain	2	4.517	0.012
maternal strain	2	8.235	<0.001
gender	1	14.706	<0.001
maternal*paternal strain	1	0.906	0.342
paternal strain*gender	2	3.695	0.027
error	171		

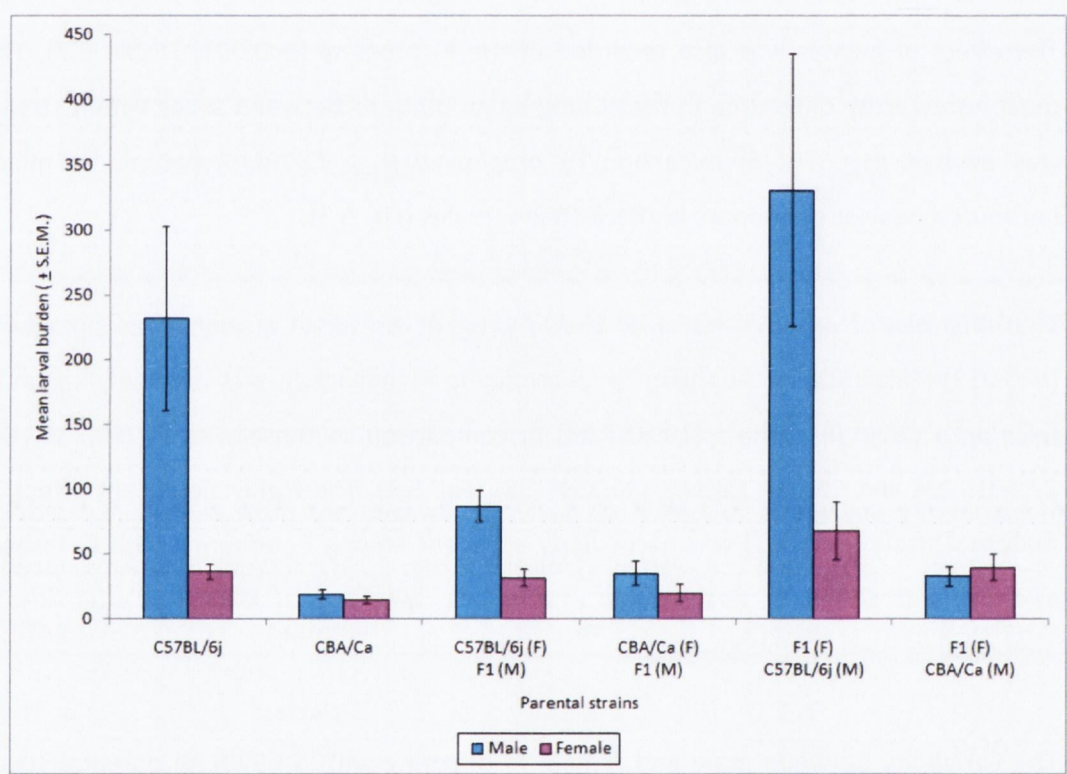


Figure 5.3. Mean lung larval burdens ($\pm$ S.E.M.) in parental strains and F₂ mice euthanised on day 7 p.i. The gender (M=male, F=female) of the parental strains is indicated.

As illustrated by the large error bars in Fig 5.3 and in the frequency distributions in Fig. 5.4, a high degree of variability in the larval burdens was recorded in individual mice, which was particularly evident in male C57BL/6j mice (10-990 larvae) (Fig. 5.4 E) and male F₂ mice with male C57BL/6j parental strains (0-980 larvae) (Fig. 5.4 A). Larval

burdens documented in female mice of these two strains were comparatively uniform in their frequency distribution. The variation in individual larval burdens was relatively low in F₂ offspring bred with male CBA/Ca mice with the exception of 6.7% of this strain, which had a lung larval burden of 151-200 on day 7 p.i. (Fig. 5.4 C). Individual larval burdens were most consistent in the CBA/Ca parental reference strain (Fig 5.4 F).

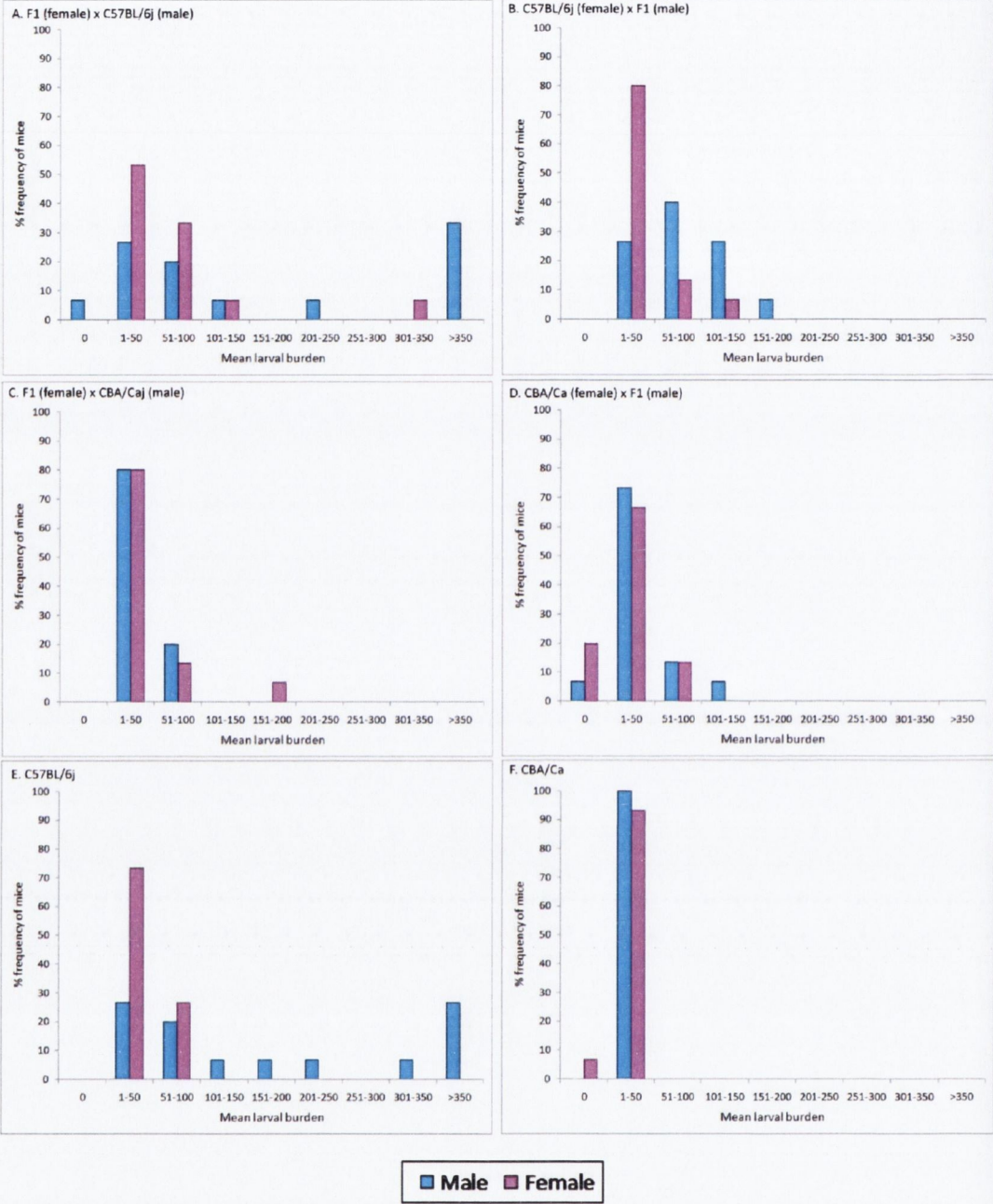


Figure 5.4. Frequency distributions of mean lung larval burdens on day 7 p.i. in F_2 mice ((A) F1 (female) x C57BL/6j (male), (B) C57BL/6j (female) x F1 (male), (C) F1 (female) x CBA/Ca (male), (D) CBA/Ca (female) x F1 (male) and reference parental strains, (E) C57BL6j and (F) CBA/Ca

5.3.2.2. Intelectin screening

All F₂ mice were screened using the SLAMF7 marker analysis. PCR products (Fig. 5.5), yielded using SLAMF7 primers were assessed and the number of copies of the Itln-2 gene present in each mouse was then inferred based on the product size. Using SLAMF7 marker primers, C57BL/6j mouse DNA gives a 200bp product, whereas CBA/Ca mouse DNA gives a 218bp product. Since the Itln genes and SLAMF7 are closely linked, the presence of Itln genes from either parental strain was inferred from the marker sizes following PCR of F₂ mouse genomic DNA. F₂ mice with a C57BL/6j parent, determined to be homozygous were inferred to have zero copies of the Itln-2 gene, if there is no evidence of the 218bp SLAMF7 product. Similarly, F₂ mice with a C57BL/6j parent, determined to be heterozygous were inferred to have one copy of the Itln-2 gene, if both 200bp and 218bp products were detected. F₂ mice with a CBA/Ca parent were determined to be homozygous, so have two copies of the Itln-2 gene, if only the higher 218bp product was observed, or heterozygous, so have one copy of the Itln-2 gene, if both 200bp and 218bp products were detected (Fig. 5.5).

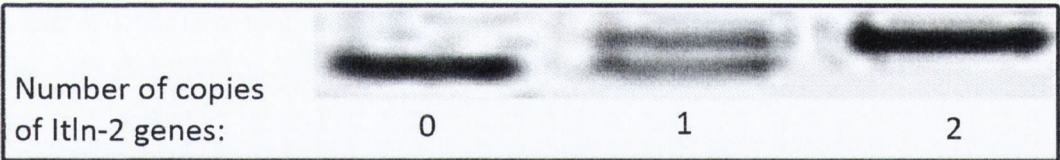


Figure 5.5. PAGE illustrating parental strain and F₂ mice tested using SLAMF7 primers. F₂ mice with C57BL/6j parent (homozygous (200bp product detected only so has 0 copies of Itln-2 gene) or heterozygous (1 copy of Itln-2 gene)) or a CBA/Ca parent (heterozygous (200bp and 218bp products detected indicating 1 copy of Itln-2 gene) or homozygous (218bp product detected so the individual has 2 copies of Itln-2 gene))

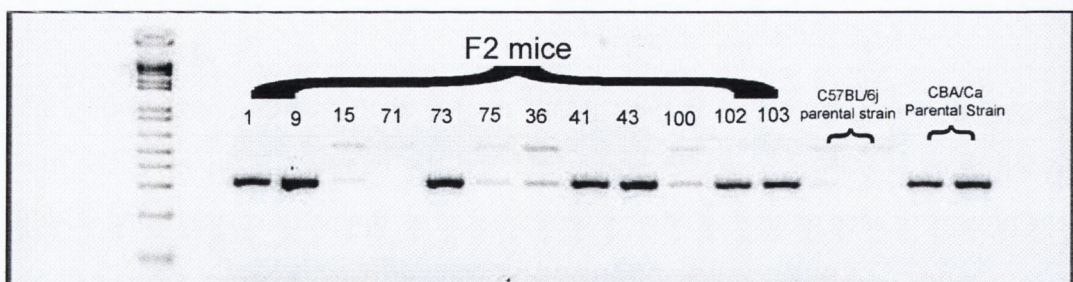


Figure 5.6. Agarose gel illustrating parental strain and F₂ mice tested using Itln specific primers

A one-way GLM, investigating the influence of the number of copies of the Itln-2 gene on larval burdens (detailed in Appendix 2), was conducted and results are illustrated in Table 5.8. Results indicated that there was no significant difference in larval burdens between F₂ mice with varying Itln-2 gene copy numbers ($P=0.092$).

Table 5.8. Results from the one-way GLM of the effect of number of copies of the Itln-2 gene on pulmonary larval burdens in F₂ mice

Source	df	F-ratio	P value
number of copies of Itln-2 gene	2	2.431	0.092
error	117		

Table 5.9. Post-hoc analysis of the one-way GLM of the effect of number of copies of the Itln-2 gene on pulmonary larval burdens in F₂ mice

Source	Mean difference	P value
zero and one copy of Itln-2 gene	0.163	0.203
zero and two copies of Itln-2 gene	0.3198	0.029
one and two copies of Itln-2 gene	0.1569	0.230

Further post-hoc analysis (least significant difference) (Table 5.8) demonstrated that a similar pulmonary larval burden was observed in F₂ mice with zero or one copy of the Itln-2 gene ($P=0.203$). As is also illustrated in Fig. 5.7 A, a comparatively lower larval burden was recorded for F₂ mice with two copies of the Itln-2 gene (Fig. 5.7 A) and larval burdens observed in F₂ mice with two copies of the Itln-2 gene significantly

differed from mice lacking the *Itln-2* gene ($P=0.029$) but not with mice with one copy of the *Itln-2* gene ($P=0.230$) (Table 5.9).

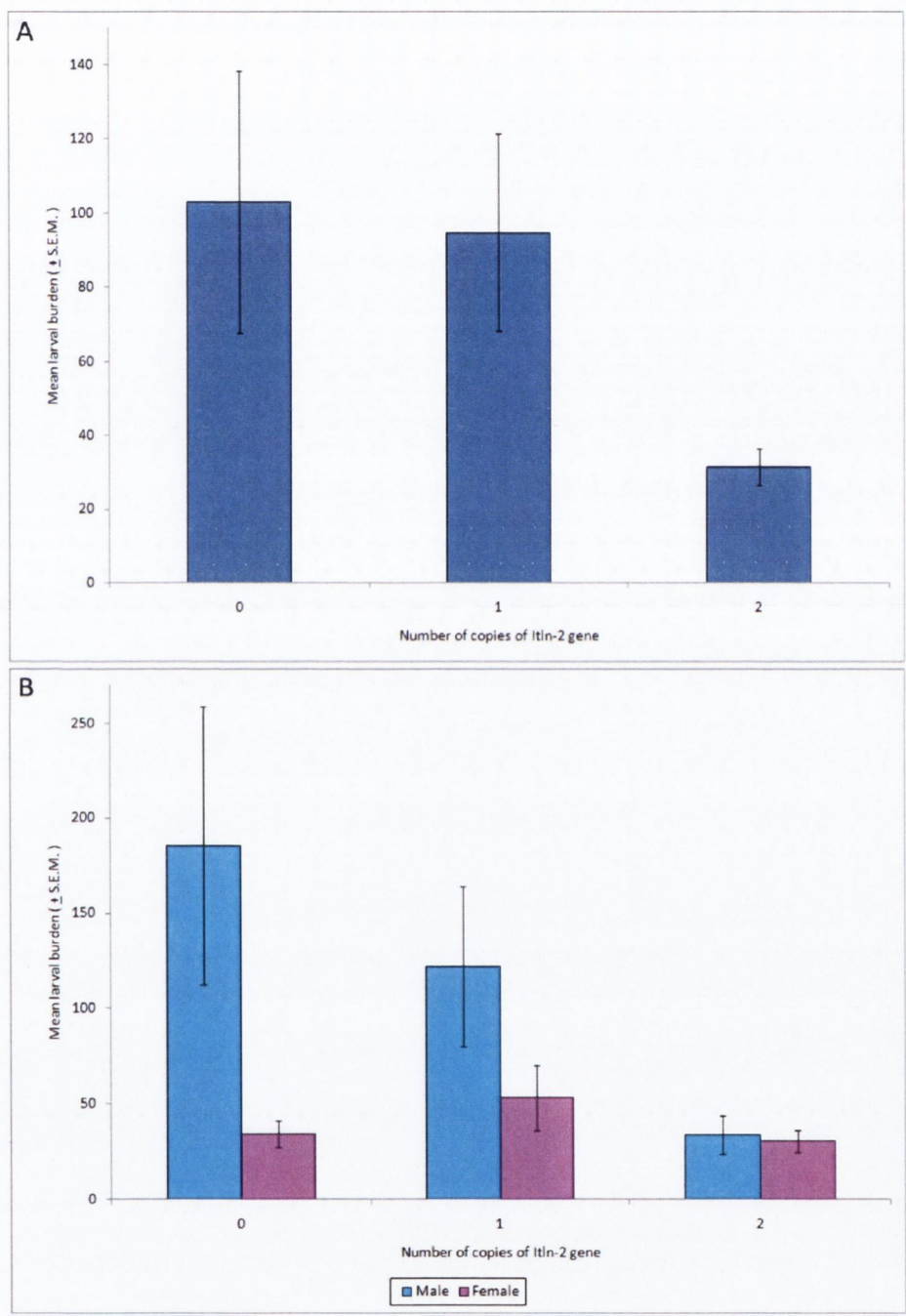


Figure 5.7. Number of copies of *Itln-2* gene detected and the mean larval burden (± S.E.M.) in (A) all F₂ mice and (B) in male and female F₂ mice

Table 5.10. Results of a two-way GLM of the effects of number of copies of the Itln-2 gene and gender on pulmonary larval burdens in F₂ mice

Source	df	F-ratio	P value
number of copies of Itln-2 gene	2	2.764	0.067
gender	1	5.343	0.023
number of copies of Itln-2 gene*gender	2	1.529	0.221
error	114		

Larval burdens recorded in mice with varying Itln-2 gene copies were further segregated into male and female mice (Fig 5.7 B). A two-way GLM was conducted to examine the influence of gender and number of copies of the Itln-2 gene on pulmonary larval burdens and results are illustrated in Table 5.10. The analysis indicated that the influence of gender on parasitic burden was significant ($P=0.023$) yet no gender*number of copies of Itln-2 gene interaction was detected ($P=0.221$).

5.4. Discussion

The present study involved assessing the contribution of a candidate gene, *Itln-2*, to host variation in the *A. suum* mouse model. In the backcross experiment undertaken, the F_1 progeny were divided in half and crossed with either the susceptible C57BL/6j or resistant CBA/Ca mouse strains as recurrent parents, thus yielding F_2 mice with zero (F_1 x C57BL/6j), one (F_1 x C57BL/6j and F_1 x CBA/Ca) or two (F_1 x CBA/Ca) copies of the *Itln-2* gene. Difficulties arose in defining high and low responders to infection among the parental reference strains bred simultaneously to the F_2 progeny due to unexpected variation in larval burdens. However, despite the aforementioned variation it was concluded that there was no association between the *Itln-2* gene and resistance to *A. suum* infection. This was particularly evident in female F_2 individuals, who did not display as high level of intra-strain variation in larval burdens, as female larval burdens were similar despite the number of copies of *Itln-2* gene.

The lack of association between the candidate gene of interest and resistance to *A. suum* infection contrasts with studies in which expulsion of *T. spiralis* and *T. muris* coincides with upregulation of *Itln-2* expression (Pemberton *et al.*, 2004a; 2004b; Datta *et al.*, 2005; Artis, 2006). Immunolocalisation of *Itln-2* protein in Paneth and goblet cells may explain the common role of *Itln-2* in resistance against *T. spiralis* and *T. muris* as both parasites share the intestinal environment at the time of the host immune response, which results in expulsion. However, even though expression of *Itln-1* and *Itln-2* in the small intestine were evident during *Nippostrongylus brasiliensis* infection (Voehringer *et al.*, 2007), *Itlns* do not mediate worm expulsion as C57BL/6j mice expel the parasite with the same kinetics as BALB/c mice (Ishiwata *et al.*, 2002) despite lacking the *Itln-2* gene (Pemberton *et al.*, 2004a). Furthermore, Voehringer *et al.* (2007) demonstrated that high *Itln* levels in the lung of transgenic mice did not enhance parasite clearance. Therefore, it could be that intelectins are responsible for parasite expulsion manifested in intestinal tissues. The mechanism of resistance to *A. suum* infection appears to manifest itself in the liver of CBA/Ca mice (chapter 3, section

3.3.1.3, chapter 4, section 4.3.1.1.3) and *Itln-2* has not yet been documented to contribute to an immune response against a murine liver infection.

Murine *Itln* genes are not direct homologues of human *Itln* genes (Takano *et al.*, 2008). However it is unlikely that human *Itln*s play a significant role in contributing to observed variation in genetic susceptibility/resistance to *A. lumbricoides* as, even though the location of human *Itln-2* (1q22-23) is on the same chromosome as quantitative trait loci which approached significance in a recent pedigree study on resistance to heavy worm burdens (Williams-Blangero *et al.*, 2008), the loci were recorded on a separate arm of chromosome 1 (1p36 and 1p34). The contribution of the data yielded from this study and previous work on humans and mice would therefore lead to the conclusion that it is unlikely the *Itln-2* gene is directly involved in the mechanism of resistance to *Ascaris* infection.

The well documented outcome of F_2 breeding experiments (Iraqi *et al.*, 2003; Menge *et al.*, 2003) yielded from two parental reference mouse strains, which represent the extremes of resistance and susceptibility while the response in the F_2 progeny would be intermediate, was not completely evident in this experiment. The influence of gender did not have an overall significant effect on larval burdens in the pilot gender experiment. In addition, the heightened larval burdens in the susceptible strain were not considered to be a concern as the data still indicated a large divergence between pulmonary burdens of the two mouse strains of the model. Coupled with this, it was more practical to work with both male and female progeny in the backcross experiment for financial, ethical and time issues.

The variability in larval burdens among susceptible individuals was magnified in the larger infection study of F_2 and parental reference strain mice. Despite this, divergence of the susceptible C57BL/6j and resistant CBA/Ca mice strains was still evident. Nevertheless, the unexpected variability in larval numbers led to difficulties in deciding on cut-off mean larval burden values to represent a level of infection which described resistance and susceptibility for responder and non-responder mice in the F_2 progeny.

This was particularly problematic in the case of the female C57BL/6j mice, eight of which had larval burdens which overlapped with female CBA/Ca mice.

The dramatically increased larval burdens in a selection of male C57BL/6j mice and male F₂ offspring bred using male C57BL/6j mice is of particular interest as previous attempts at immunosuppression, using the steroid hydrocortisone, did not enhance *Ascaris* larval burdens in the susceptible mouse strain (Lewis *et al.*, 2007).

The mechanism behind the larval burden intra-strain variability, particularly of the susceptible male individuals, when housed with females, is not fully understood and requires further investigation.

Sex differences in immune function are mediated, in part, by steroid hormones (Grossman, 1985; Klein, 2000; 2004). Many studies have investigated the complex interactions between the endocrine and immune system. The androgen testosterone is known to have suppressive effects on the immune system (Ahmed *et al.*, 1985; Alexander and Stimson, 1988; Hillgarth and Wingfield, 1997). Increased host testosterone levels have led to higher levels of *Strongyloides ratti* (Kiyota *et al.*, 1984), *N. brasiliensis* (Solomon, 1966; Bone and Bottjer, 1986), *N. muris* (Haley, 1958) and *Trichinella spiralis* (Charniga *et al.*, 1981) and *Heterakis spumosa*, a parasitic nematode that inhabits the colon of the rat, (Harder *et al.*, 1992), demonstrating that immunosuppression by testosterone is responsible for increased susceptibility to infection. The glucocorticoid corticosterone is another steroid hormone known to suppress immune responses to infection (Klein, 2000). Contrary to this, oestrogens are known to enhance host immunocompetence (Klein, 2000) and mice treated with oestrogen show a reduction in active suppressor cells (Ahmed *et al.*, 1985), which further accounts for sex differences in immune responses to infection.

As discussed by Bohus and Koolhaas (1991), exposure to environmental and social stressors can lead to immunosuppression and therefore increased susceptibility to infection. In nature, breeding season is associated with a number of stressors such as energetically expensive courtship displays and aggression when competing for

territories and mates. In captivity, aggression in male rodents can be triggered by female scent alone (Barnard *et al.*, 1997). Therefore, it is possible that even though the male mice in this study were not housed in cages with females, the scent of the females may have increased aggression and investment in courtship behaviours among the male mice. Therefore, the stressors associated with the presence of female mice may have also led to increases in gonadal steroids in males. As discussed by Laudenslager and Kennedy (2008), every organism does not respond to an environmental challenge in a similar manner and each individual's response may be influenced by social organisation. Differing responses to infection in dominance hierarchies have been documented previously in response to the protozoan *Babesia microti*. Dominant individuals became more susceptible to infection and had heightened levels of testosterone and corticosterone, while subordinate individuals did not show evidence of hormonal changes yet corticosterone levels were positively associated with the number of animals per cage (Barnard *et al.*, 1994).

It is difficult to elucidate if a mouse was dominant, midranking or subordinate as dominance ranking was not an initial objective in this study so the appropriate monitoring of behaviour was not undertaken. Furthermore, one cannot assess dominance based on the body weights of individuals recorded at post-mortems as one cannot definitely conclude whether weight loss observed during infection was due to heightened larval burdens or social organisation. Tamashiro *et al.* (2004) demonstrated that subordinate rodents experience loss of 15% body weight over a two week period during establishment of a social hierarchy. However, mice involved in this study were separated if aggressive behaviour was observed so it is likely that weight loss is due to intensity of infection as recorded in previous studies (Lewis *et al.*, 2009), rather than investment in wound healing. Weight loss may have been heightened by investment in secondary sexual characteristics, in response to female urinal odours, which leads to increased weight loss during experimental infection (Zala *et al.*, 2008).

The effect of corticosterone on susceptibility to early *Ascaris* infection has been investigated previously (Lewis *et al.*, 2007) and the intensity of infection was not affected by experimental administration of the hormone. Therefore, it is probable that

the stressor of female presence is inducing heightened testosterone levels in dominant male individuals, which leads to immunosuppression.

In this study, only C57BL/6j mice or their progeny, when crossed with F₁ mice, were affected by the presence of females. Lung larval burdens in male and female CBA/Ca mice and F₂ mice with CBA/Ca parental history were similar to burdens recorded in previous studies (Lewis *et al.*, 2006; 2007). In general, male C57BL/6j mice are known to be very aggressive (personal observations, personal communication with the chief technician of the Bioresources unit, Trinity College Dublin) but their aggressive encounters in previous studies have not led to varied or increased susceptibility. It is possible that the susceptible mice are more sensitive to female pheromones and therefore are the only mice investing in secondary sexual characteristics and not immunocompetence. However, it is more likely that genetic variation and differences in expression of sex steroid related immunosuppression account for the varied and increased susceptibility in C57BL/6j but not CBA/Ca individuals. Mice with different MHC haplotypes have significantly different responses to *Heterakis spumosa* infection, which only become apparent after treatment with testosterone (Harder *et al.*, 1994). Results obtained by Harder *et al.* (1994) demonstrated that an immune mechanism which responds to nematode infection in C57BL/10 mice (H-2^b) is influenced by testosterone, highlighting the possibility that a hormone may be influencing the immune response of susceptible C57BL/6j mice to *A. suum* infection in the susceptible C57BL/6j mice in the current study.

One must be wary of conclusions drawn from rodent studies in which differences in susceptibility are likely to be associated with social organisation. Behavioural observations in captivity do not always mirror responses to stimuli in nature. Rodents do not typically have repeated social interaction with the same dominant individual, as a subordinate mouse would flee an aggressive encounter.

In human populations *A. lumbricoides* host gender-associated patterns have been observed in epidemiological studies (Elkins *et al.*, 1988). However sex-bias in infection may be a result of socio-cultural factors, which lead one gender to be exposed to

infection more so than the other. The genetic uniformity of inbred mice, coupled with the ability to manipulate the environment in which they are held, will facilitate future investigations in attempting to uncover the influence of co-habiting female mice on increased *A. suum* infection in male mice.

While larval burdens were confounded by an unexpected effect of co-habitation of male and female mice, this study revealed that *Itln-2* is not an important mediator in preventing the accumulation of *A. suum* larvae in the lungs. The breeding and subsequent post-mortems involved in the present experiment has generated a bank of tissues that may be utilised in future candidate gene experiments. However, since it was difficult to define high and low responders due to the variation in larval burdens, the tissues could be used for pilot studies in which an association between multiple possible genes detected in a microarray is tested. Therefore, the pool of F₂ DNA can be later used to better target genes that may influence resistance to *A. suum* infection.

6. Variations in cytokine production in the hepatic tissue of naïve and *Ascaris suum*-infected mice

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6.1. Introduction

The liver is considered a major site of *Ascaris* larval migration. In experimental infections the majority of *A. suum* larvae are known to occupy the hepatic tissue for three days (days 3-6 p.i.) (Roepstorff *et al.*, 1997). Larvae were detected in the liver at the first post-mortem time-point, 6 hours post infection (p.i.) (chapter 3, section 3.3.1.2). Furthermore, it was at this time-point that Keittivuti (1974) documented *A. suum* larvae in murine hepatic tissue while investigating the intestinal-hepatic migratory pattern in mice. However, Slotved *et al.* (1998) recovered *A. suum* larvae in mouse livers as early as 4 hours p.i. Prior to onward migration to the lungs, larvae have been shown to travel through murine hepatic tissue inducing cell damage, necrosis and an inflammatory response marked by an infiltration of immune effector cells (Bindseil, 1981; Eriksen, 1981; Chapter 4, section 4.3.1.1.2).

The liver is an important regulator of the systemic innate immune response. Hepatocytes are the primary synthesisers of all acute phase proteins (APPs), numerous soluble pathogen recognition receptors (PRRs) and 80-90% of complement components (reviewed by Gao *et al.*, 2008). While hepatocytes play a key role in the biosynthesis of mediators of the innate immune system, principal cells of innate immunity are also resident in the liver (Doherty and O'Farrelly, 2000) and as a result the liver has been recently referred to as "an organ with predominant innate immunity" (Gao *et al.*, 2008). The liver's resident macrophages, known as Kupffer cells account for 80-90% of the total population of fixed tissue macrophages in the body. Kupffer cells are localised in liver sinusoids so therefore are the first hepatic cells exposed to the antigens carried to the liver from the GI tract. A normal murine liver has 2-5 times the quantity of dendritic cells found in other parenchymal organs such as the kidney and heart (Steptoe *et al.*, 2000). Intrahepatic lymphocytes are distinct in both function and phenotype from their counterparts in any other organ. Conventional CD4⁺ and CD8⁺ αβ T cell receptor (TCR)⁺ T cells and B cells are found in the liver as well as non-conventional natural killer (NK) T cells, γδ TCR⁺ T cells, and CD4⁺CD8⁻ T cells, with CD8⁺ cells predominating (Nemeth *et al.*, 2009). The liver is one of the richest sources of innate immune cells. For example, in the

mouse NK and (NK) T cells account for 5-10% and 20-30% of liver lymphocytes respectively. Furthermore $\gamma\delta$ TCR⁺ T cells account for 3-5% of murine intrahepatic lymphocytes and 15-25% of the liver's total T cell population (Gao *et al.*, 2008). Granulocytes are rarely found in a healthy liver but, as shown in chapter 4 (section 4.3.1.1.2), are attracted to the liver in response to infection and hepatic cell damage.

The difference in *A. suum* pulmonary burdens is observed in C57BL/6j and CBA/Ca mice as early as day 5 p.i. although the peak difference occurs on day 7 p.i. (Lewis *et al.*, 2006). Furthermore, congenitally athymic mice and mice with a defect in B-lymphocytes are no more susceptible to *A. suum* infection than to their intact counterparts (Mitchell *et al.*, 1976; Brown *et al.*, 1977). Therefore, while many studies of the immunological response to *Ascaris* infection have focused upon the humoral response to the parasite (Haswell-Elkins *et al.*, 1989b), in light of the above experiments the present study centred on the hepatic innate cytokine response early in infection.

Ascaris suum larvae inhabit the liver, an immune organ, and induce a notable hepatic inflammatory reaction in pigs in the form of granulomatous lesions referred to as "white spots" (Schwartz and Alicata, 1932; Ronéus, 1966; Copeman and Gaafar, 1972; Pérez *et al.*, 2001). Ethical constraints impede research on the local immune response in the migratory hepatic stages of *A. lumbricoides* infection in humans. The mouse model is advantageous in this regard as one can sample the hepatic tissue at a range of different time-points at which larvae inhabit the liver and thus examine the local immune response to hepatic infection. A comparative study of the degree of inflammatory cells in the liver of susceptible C57BL/6j and resistant CBA/Ca mice (reported in chapter 4) indicated that CBA/Ca mice respond earlier and more effectively to hepatic larval invasion. Furthermore, the timing of a strong inflammatory reaction in resistant mice coincided with a reduction of larval numbers. A similar strong inflammatory reaction was not detected in susceptible mice until the majority of the larvae were accumulating in the lungs. Therefore, it is likely that a hepatic factor varies between the mouse strains leading to the divergence in pulmonary larval numbers.

As detailed by Knolle & Gerken (2000), the hepatic immune response is regulated locally in the liver. Furthermore, the phenotype of the immune response is a result of the cytokine milieu produced in early phases of infection (Borish and Steinke, 2003). Jungersen *et al.* (2001) recognised the importance of assessing the immunological response to *A. suum* infection at the site at which immunity is manifested. The lymphocyte population which recognised antigens was dependent on the draining area of the lymph nodes assessed (Jungersen *et al.*, 2001), which may be a result of the stage-specific antigens (Kennedy and Qureshi, 1986) the surrounding organs were exposed to during infection or may be dependent on the effector mechanisms of the immune response in the particular organ. Therefore, the aim of the present study is to observe the local production of a selection of candidate cytokines (IL-6, IL-10 and IL-13) at the early stages (6 hours, days 1 and 2 p.i.) of *A. suum* hepatic infection in C57BL/6j and CBA/Ca mice.

IL-6 is considered the most important inducer of the synthesis of APPs by hepatocytes (Borish and Steinke, 2003). Furthermore, macrophages secrete IL-6 in response to tissue damage. The APP, C-reactive protein (CRP) was detected in children putatively immune to ascariasis, which was proposed to be indicative of ongoing inflammatory response as an antiparasite effector mechanism (McSharry *et al.*, 1999). Conversely, IL-6 also has anti-inflammatory potential as it inhibits the pro-inflammatory cytokines, IL-1 and TNF- α .

The anti-inflammatory cytokine with immunoregulatory potential, IL-10 is produced by monocytes and also to a lesser extent by lymphocytes and down-regulates Th1 cytokines. Furthermore, IL-10 has potent effects on Kupffer cells and liver sinusoidal endothelial cells (LSEC). IL-10 down-regulates CD4⁺ T cell activation by antigen-presenting Kupffer cells and LSEC (Knolle *et al.*, 1998).

Similarly to IL-6 and IL-10, IL-13 is a Th2 cytokine which has pro- and anti-inflammatory potential. There are many lines of evidence for IL-13 being involved with protection against a range of helminth parasites including *A. lumbricoides* (Jackson *et al.*, 2004). As described by Maizels & Holland (1998), IL-13 has overlapping but not identical biological

activities to IL-4, which is also commonly associated with resistance to parasitic worms (e.g. Else and Grencis, 1996)

As stated above the aim of the present study was to examine the changes in the levels of relevant cytokines in resistant and susceptible mice in response to *A. suum* infection. Results from this study combined with the histopathological data obtained in chapter 4 will enable one to evaluate the studied cytokines' role in the progression, inhibition and the resultant pathology of *A. suum* infection in mice.

6.2. Materials and Methods

6.2.1. Collection of tissues at post-mortem

6.2.1.1. Experiments 1: Investigating the hepatic innate cytokine response to *Ascaris suum* infection

A total of 70 (35 C57BL/6j and 35 CBA/Ca) male inbred mice were purchased for this experiment. Mice were randomly assigned as experimental (n=40) and control (n=30) animals. All experimental and control mice were administered 1000 *A. suum* ova and 100µl of 0.1N H₂SO₄ respectively. Five experimental and five control mice of each strain were sacrificed at 6 hours and days 1 and 2 p.i.. Post-mortem time-points were selected based on the strong inflammatory response observed on day 4 p.i. in resistant CBA/Ca mice but absent in susceptible C57BL/6j mice until day 6 p.i. Therefore, it was of interest to observe the effector cytokines resulting in the varying abundance of inflammatory cells on day 4 p.i. in the two mouse strains. An intraperitoneal injection of 0.5 ml of Butatyl was administered to each mouse, which was returned to a separate cage until terminally anaesthetised. Reflexes were monitored and mice were not dissected until deemed unresponsive. The abdominal and thoracic cavities were exposed. The heart was held in place at the apex using a fine forceps, and a 23G needle connected to a perfusion pump (5ml/minute), was inserted approximately 5mm into the left ventricle. The vena cava was immediately cut open using a fine scissor to allow waste perfusate to flow out. The needle was held in place until the waste perfusate ran clear (approximately 5-8 minutes). During perfusion, the colour of the liver changed from red to tan.

The spleen and hepatic lobes were individually placed in 2ml cryovials and snap frozen in liquid nitrogen and subsequently stored at -80°C.

The remaining ten experimental mice (5 C57BL/6j and 5 CBA/Ca) were euthanised by means of cervical dislocation on day 7 p.i. The Lungs were removed and larvae were

isolated by means of the modified Baermann technique (Eriksen, 1981; Lewis *et al.*, 2006) (chapter 2, section 2.4) and later enumerated (chapter 2, section 2.5). This procedure was to ensure that infection was successful and that larval burdens in each strain were similar to that observed previously (Lewis *et al.*, 2006; chapter 3).

6.2.2. Liver and spleen protein extraction

Samples were defrosted on ice and weighed prior to protein extraction. A RIPA lysis buffer was prepared (appendix 1, section 1.4) and 300µl was added to the caudate lobe or spleen, in a 2ml microcentrifuge tube. The caudate lobe was selected for analysis as the remainder of the liver was digested for flow cytometry in the second experiment. Therefore, the caudate lobe was also selected in the first experiment for consistency. Furthermore, the concentration of protein extracted from hepatic tissue using the protocol detailed below plateaus at approximately 0.50g tissue and the average mass of the caudate lobes sampled was 0.41g (C. Dold, data not shown). Therefore, the caudate lobe was considered suitable for protein extraction. A handheld laboratory homogeniser (Pro Scientific PRO200) was used to homogenise the tissue. In order to avoid contamination, the homogeniser was pulsed whilst placed in five 50ml centrifuge tubes containing dH₂O, RNase Zap, dH₂O, 70% ethanol and dH₂O prior to each sample. Once the homogeniser was placed in the microcentrifuge tube containing a tissue sample, the motor was pulsed repeatedly at full speed for a duration of approximately one second, to avoid overheating and thus degradation of proteins, until the tissue was completely homogenised. Each sample was left on ice for 15 minutes and then centrifuged for 15 minutes at 13,500g at 4°C. The supernatant was then transferred to a clean 1.5ml microcentrifuge tube and stored at -80°C.

6.2.3. Quantification of protein concentration in liver and spleens

The quantity of protein in individual liver and spleen samples was determined by means of the bicinchoninic acid (BCA) assay (Thermo Scientific Pierce BCA assay reagents). A 1:50 dilution of Reagent A in Reagent B was prepared and 200µl of the mixed reagent was placed in each well of a 96 well plate (Nunc MaxiSorp Immunoplates). A 3µl aliquot

of each individual spleen and liver sample was added to a well and mixed thoroughly with the mixed reagent. The plate was placed in an incubator at 37°C for 30 minutes. Absorbance of each sample was measured at a wavelength of 562nm.

6.2.4. Standard sandwich Enzyme-linked immunosorbent assay (ELISA) protocol

All ELISA kits (Mouse DuoSets) were purchased from R&D systems. All optimisation steps, detailed below were based on the standard sandwich ELISA protocol.

The standard sandwich ELISA protocol for mouse DuoSets is as follows. On the day prior to conducting the assay, the ELISA plate is coated with capture antibody (100µl/well) (Fig. 6.1 A), sealed with Parafilm and incubated overnight at room temperature. The following day, plates are washed and aspirated using a wash buffer (PBS containing 0.05% (v/v) Tween-20). In order to block any non-specific binding sites, 300µl of the reagent diluent (1% BSA in PBS) is added to each well. Plates are sealed and incubated for a minimum of one hour at room temperature.

Standards are prepared using recombinant mouse proteins. A seven point standard curve using two-fold serial dilutions is prepared in reagent diluent with the recommended high standard concentration varying among assays. Plates are washed and 100µl of each standard and sample is added to individual wells (Fig. 6.1 B). Standards and samples are tested in triplicate. Plates are sealed, incubated for two hours at room temperature and then washed again with wash buffer.

100µl of detection antibody is added to each well (Fig. 6.1 C). Plates are sealed, incubated for two hours at room temperature and washed. 100µl of the enzyme HRP-Streptavidin (0.005% in reagent diluent) is added to each well (Fig. 6.1 D); plates are sealed and incubated for thirty minutes at room temperature. Plates are washed and 100µl of substrate solution (SureBlue™ TMB microwell peroxidase substrate) is added to each well (Fig. 6.1 E) and plates are covered with tinfoil. Plates are incubated at room temperature for twenty minutes and 50µl of 1M H₂SO₄ is added to each well to halt the enzyme reaction.

Each plate is read at 450nm and the concentration of each sample (tested in triplicate) is determined using an individual standard curve.

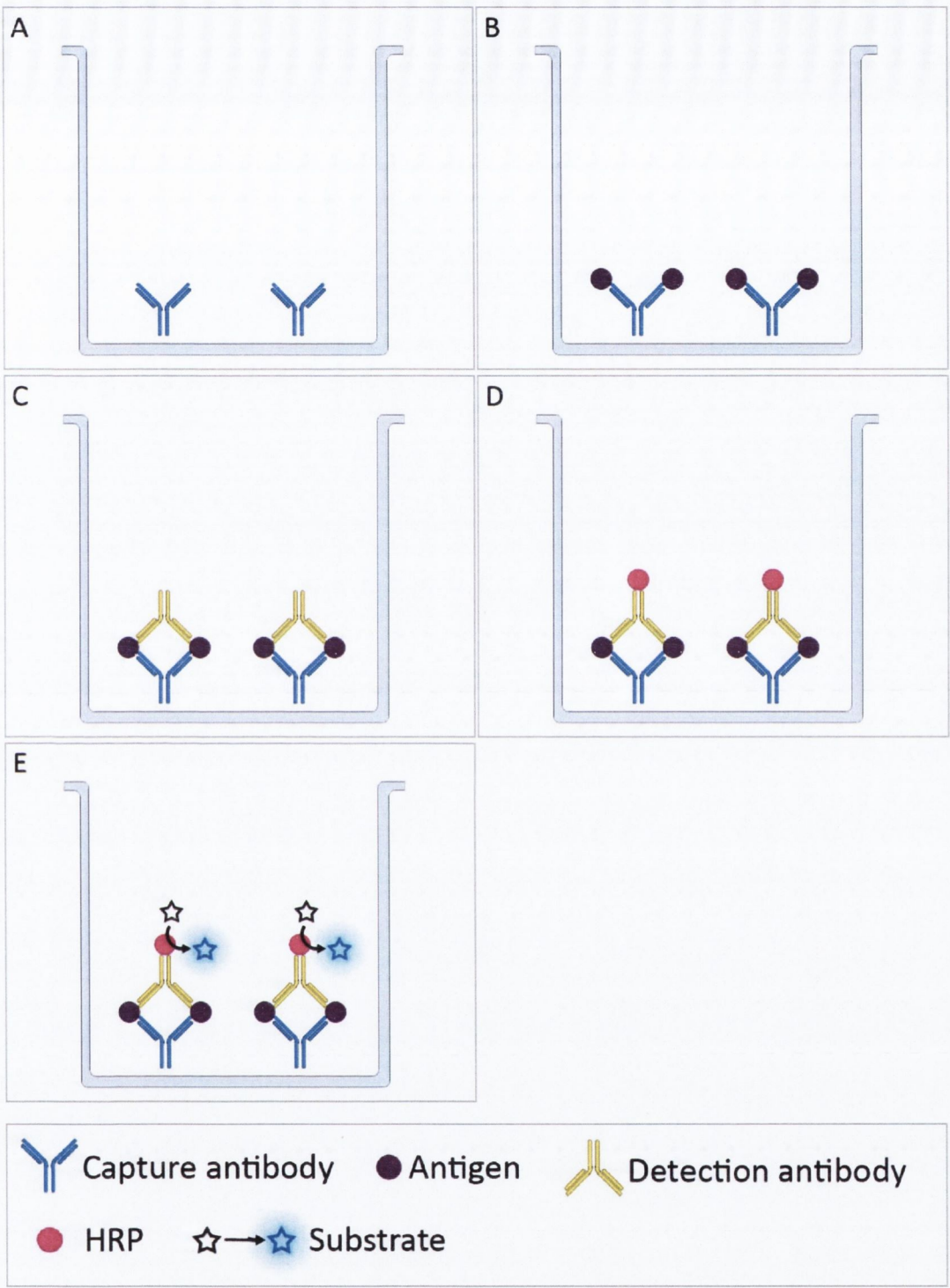


Figure 6.1. Schematics of standard sandwich ELISA protocol illustrating addition of (A) capture antibody, (B) samples containing an unknown amount of antigen, (C) detection antibody, (D) HRP and (E) substrate, which fluoresces allowing for quantification of antigen.

The steps illustrated in A-D are followed by a washing step. (Original illustration)

6.2.5. Optimisation of ELISA for liver and spleen homogenate

Optimisation of the protocol for liver and spleen homogenate was conducted using an IL-6 mouse ELISA DuoSet.

6.2.5.1. Investigating non-specific binding of hepatic tissue proteins to the ELISA plate

As discussed above, on the day prior to undertaking an ELISA, the ELISA plate is coated with capture antibody. In the present optimisation step, 36 wells of each 96-well ELISA plate were coated with 100µl capture antibody solution (2.0µg/ml). To control for non-specific binding of hepatic tissue proteins to the plate and the contribution of endogenous peroxidase and biotin to the signal generated, the remaining wells were coated with 100µl of an IgG control antibody (rat IgG) (2.0µg/ml). Plates were then covered with Parafilm and incubated overnight at room temperature.

Standards were prepared using recombinant mouse IL-6 proteins with a high standard of 1000pg/ml. Samples were diluted in reagent diluent and each sample was added to three wells coated with capture antibody and three wells coated with IgG control antibody. The detection antibody, enzyme, substrate and stop solutions steps were undertaken as described in section 6.2.4.

Each plate was read at 450nm. Values obtained for samples tested using IgG control antibody were subtracted from those tested using capture antibody and then the concentration of each sample was determined using an individual standard curve.

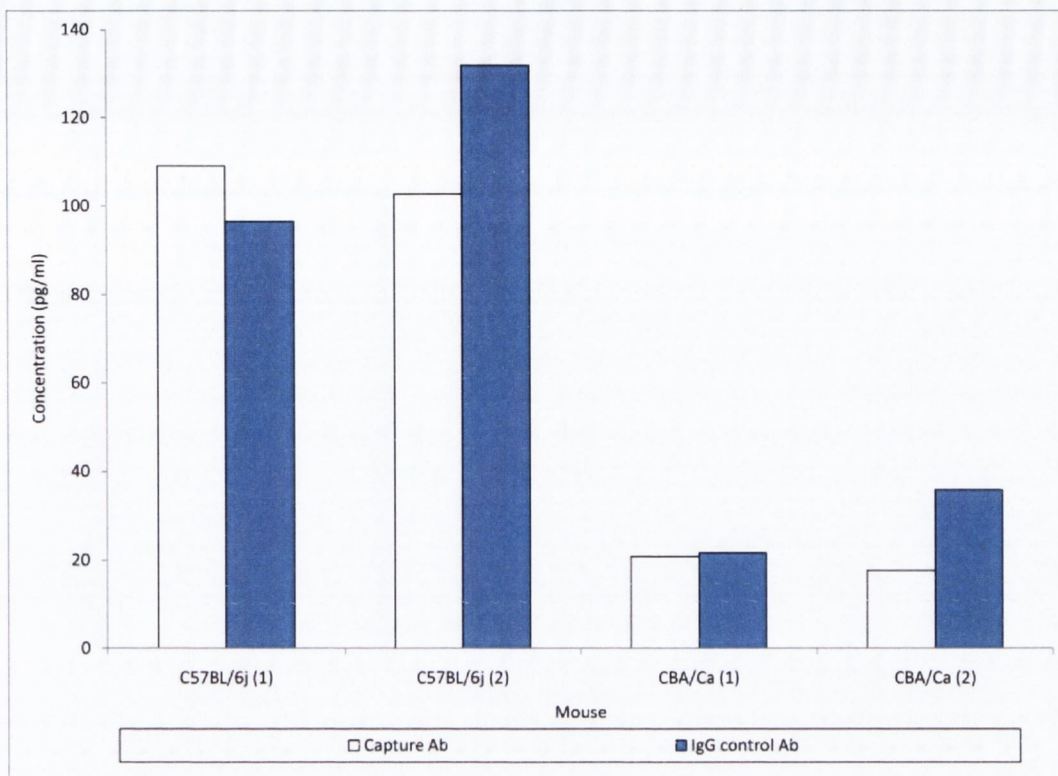


Figure 6.2. IL-6 levels in hepatic tissue from two C57BL/6j ((1) and (2)) and CBA/Ca ((1) and (2)) mice using standard ELISA protocol with capture antibody or control IgG antibody

The data shown in Fig. 6.2 indicated that the background signal was very high and on occasion the readings for samples tested on control IgG antibody wells were comparatively higher than those tested with capture antibody.

6.2.5.2. Investigating enzyme and substrate suitability for ELISA using liver and spleen tissue homogenate

As illustrated in Fig. 6.1, a substrate is used in an ELISA for the detection and quantification of the target protein in the sample. The enzyme, HRP and an appropriate peroxidase substrate (e.g. SureBlue™ TMB Microwell Peroxidase Substrate) are recommended for use with mouse DuoSets and are commonly used, as HRP amplifies a weak signal. The enzyme alkaline phosphatase (AP) exhibits a slower catalytic rate and can be used with the substrate p-Nitrophenyl Phosphate (pNPP). When pNPP is reacted

with AP, a yellow water soluble product is formed, whereupon the reaction can be stopped using 3N NaoH. The reaction product absorbs light at 405nm.

Liver samples (1,000 ng/ml) were tested using the standard ELISA protocol and a manipulated protocol in which the HRP and peroxidase substrate were substituted for AP (Sigma ExtrAvidin®) and pNPP (Sigma SIGMAFAST™ p-Nitrophenyl phosphate tablets) respectively. The manipulated protocol was read at 405nm. In both protocols samples were tested using either capture antibody or IgG control antibody.

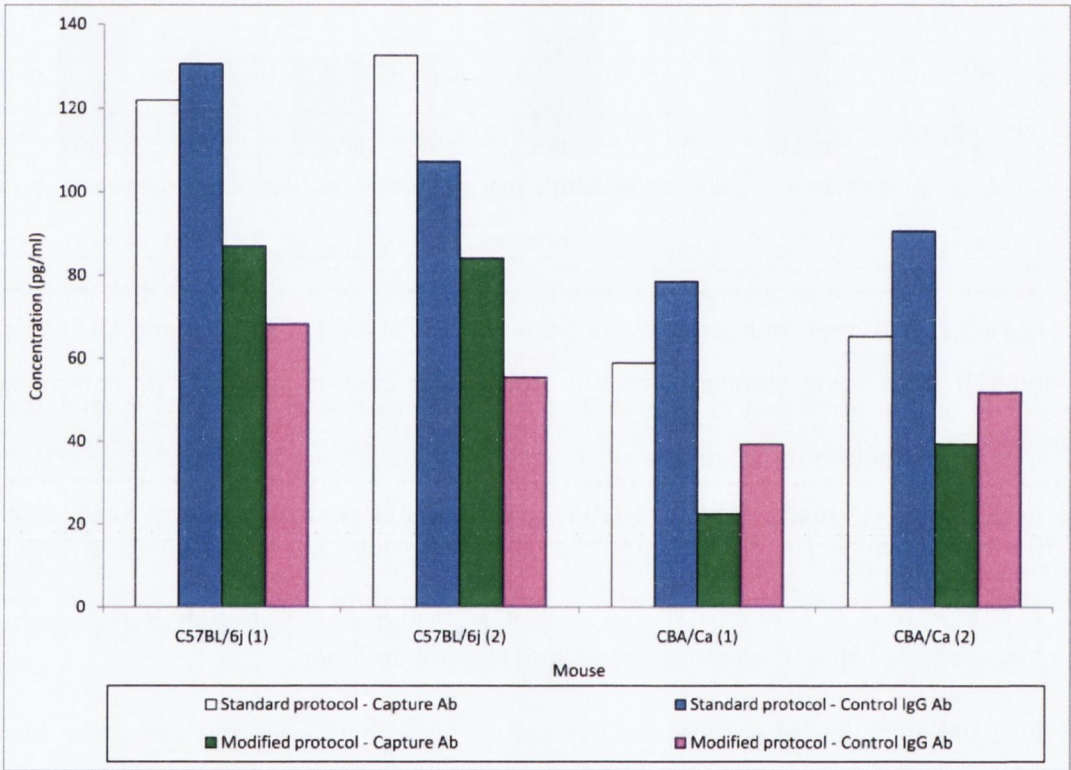


Figure 6.3. IL-6 levels in hepatic tissue (protein: 1,000 ng/ml) from two C57BL/6j ((1) and (2)) and CBA/Ca ((1) and (2)) mice using the standard and the modified substrate ELISA protocols with capture antibody or control IgG antibody

IL-6 levels were measured in hepatic tissue homogenate of C57BL/6j and CBA/Ca mice for both the standard and modified substrate ELISA protocols (Fig. 6.3). Lower levels of cytokine were quantified for the liver of both mouse strains when the AP enzyme and substrate were used. Therefore, the modified protocol appeared to reduce the

background signal generated by endogenous peroxidase and biotin, particularly in hepatic tissue.

Coating the plate with IgG to account for background signal yielded both higher and lower concentrations of IL-6 than when samples were tested on plates coated with capture antibody (Fig. 6.3).

Different concentrations of test sample protein (5,000, 10,000 and 20,000 ng/ml) of liver and spleen were tested using the modified substrate protocol. Samples were tested using the IL-6 (Fig. 6.4 A), IL-10 (Fig. 6.5 A) and IL-13 (Fig. 6.6 A) mouse DuoSet assays.

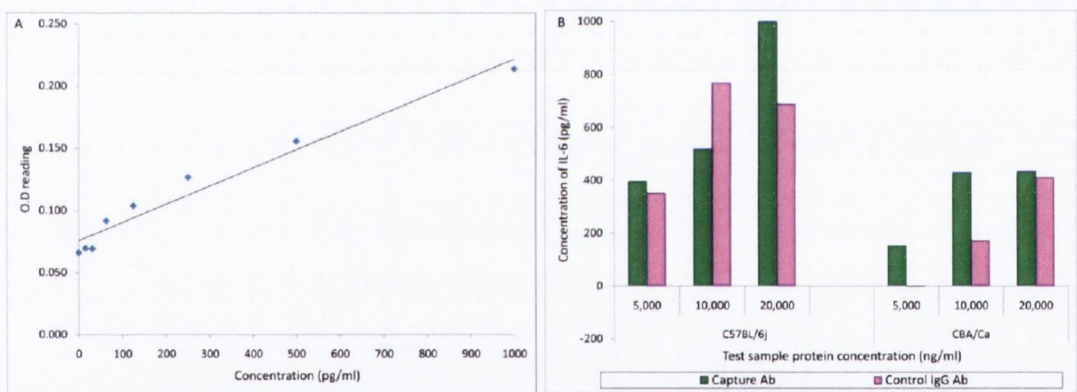


Figure 6.4. Results obtained for testing the IL-6 levels in hepatic tissue at varying protein concentrations

(A) standard curve ($y = 0.0001x + 0.0758$) (B) IL-6 levels in hepatic tissue from a C57BL/6j and CBA/Ca mouse using the modified substrate ELISA protocol with capture antibody or control IgG antibody

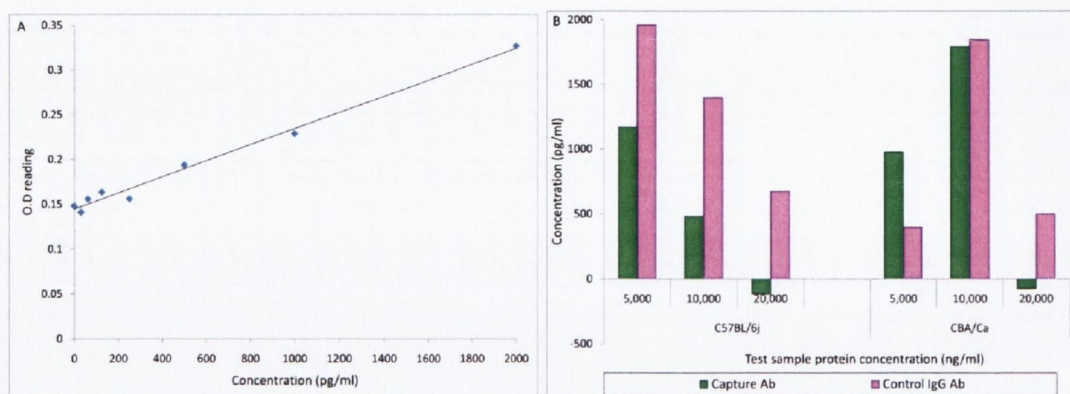


Figure 6.5. Results obtained for testing the IL-10 levels in hepatic tissue at varying protein concentrations

(A) standard curve ($y = 9E-05x + 0.1446$) (B) IL-10 levels in hepatic tissue from a C57BL/6j and CBA/Ca mouse using the modified substrate ELISA protocol with capture antibody or control IgG antibody

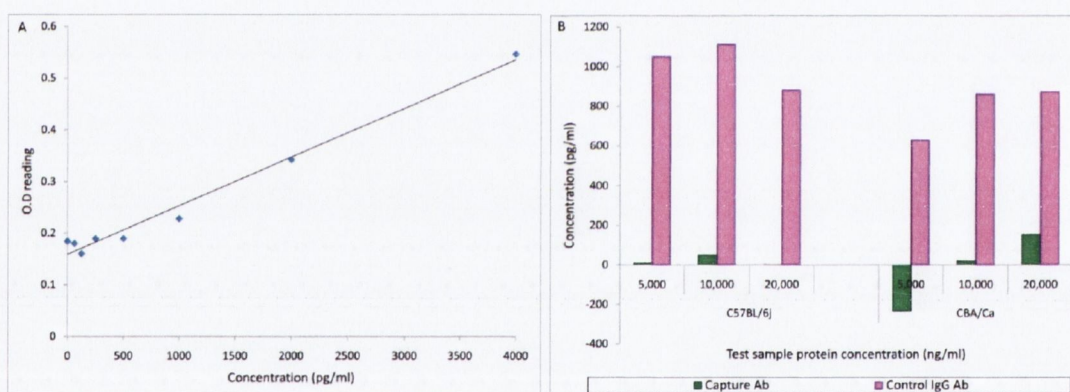


Figure 6.6. Results obtained for testing the IL-13 levels in hepatic tissue at varying protein concentrations

(A) standard curve ($y = 9E-05x + 0.1589$) (B) IL-13 levels in hepatic tissue from a C57BL/6j and CBA/Ca mouse using the modified substrate ELISA protocol with capture antibody or control IgG antibody

As the concentration of protein in the sample tested was increased, the level of IL-6 observed also increased in C57BL/6j mice in plates coated with capture antibody (Fig 6.4 B). A slight increase in IL-6 was observed in CBA/Ca test samples with 10,000 and 20,000 ng/ml protein. Similarly the levels of IL-10 increased as the test sample protein increased from 5,000 to 10,000 ng/ml but decreased when samples of 20,000 ng/ml

were tested. The decrease observed was particularly evident in samples tested on wells coated with capture antibody (Fig. 6.5 B). Fig. 6.6 (B) shows that comparatively low levels of IL-13 were detected in samples when capture antibody was used relative to those tested on wells coated with IgG control antibody. As the concentration of protein in the sample tested was increased, the level of IL-13 observed also increased in CBA/Ca mice in plates coated with capture antibody while levels appeared to plateau at 10,000 ng/ml (Fig 6.6 B).

As illustrated in Fig 6.4 (A), 6.5 (A) and 6.6 (A), the standard curves generated when using the modified substrate protocol were not optimal as the optical density (OD) readings for the concentrations of recombinant proteins were very similar. Therefore, the protocol was not very sensitive to increasing concentrations of the recombinant proteins provided. Furthermore, there was not a linear relationship at the lower detection levels, which lead to exaggerated calculated differences between the concentrations of cytokines between samples.

6.2.5.3. Discussion of optimisation of ELISA liver and spleen tissue homogenate

Spleen tissue homogenate was also tested under different capture antibody and substrate conditions (data not shown) to represent the systemic immunological response. However, cytokine levels were not successfully detected in spleen tissue even though protein was extracted from the tissue. The concentration of protein available in spleen tissue homogenate samples may have not been adequate for the cytokines tested in the current study.

During the optimisation steps, detailed above the levels of the cytokine, IL-6 were observed in liver tissue homogenate samples under different ELISA conditions. A similar concentration of rat IgG control was used to coat plates in order to control for non-specific binding to the plate. In the cases of two different enzyme and substrate conditions, the IgG controls yielded consistently varying results either higher or lower

than the corresponding control capture antibody coated plate. Higher readings for IgG coated wells were not optimal as they indicated negative levels of the cytokine.

The modified substrate protocol which involved the use of AP and pNPP reduced the background noise yet, due to low sensitivity and a slower catalytic rate in comparison to HRP, a non-linear relationship was obtained in the standard curves. Therefore, when readings for test samples were analysed with the resultant standard curves, deviations between samples appeared extremely high.

It was concluded that in order to observe the local hepatic cytokine milieu, the standard ELISA protocol would be undertaken with capture antibody only. Since this protocol did not eliminate the background signal generated by endogenous peroxidase and biotin, the values obtained were not considered to be absolute values but relative differences between strains and treatments over time could be evaluated.

Samples were tested at a concentration of 20,000 ng protein/ml as when using standard ELISA protocol (IL-6, IL-10 and IL-13 assays), the cytokine concentration positively correlated with the test sample protein concentration (data not shown). The IL-6 capture antibody was used at 2.0µg/ml, while IL-10 and IL-13 capture antibodies were used as 4.0µg/ml. Detection antibodies were used at concentrations ranging from 200ng/ml to 400ng/ml. The standard curve range differed among assays (IL-6: 0-1000 pg/ml, IL-10: 0-2000pg/ml, IL-13: 0-4000pg/ml). The concentration of cytokine was expressed in ng/100mg protein tested.

6.2.6. Statistical analysis

The pulmonary larval burden data was not normally distributed and following transformation, data remained non normal. Therefore, in order to test whether a difference existed between the day 7 p.i. pulmonary larval burdens of C57BL/6j and CBA/Ca mice, a non-parametric Mann-Whitney U test was conducted.

In infected mice, the calculated concentrations of each cytokine in the liver were compared between C57BL/6j and CBA/Ca mice. Treatment groups (experimental (infected) and control (not infected)) were compared over time by means of a three-way general linear model (GLM). Post-hoc analysis (LSD) was performed to assess whether cytokine levels differed between particular post-mortem time-points.

Similarly, the calculated concentrations of baseline cytokines in naive C57BL/6j, CBA/Ca and BALB/c mice were compared using one-way GLM for each individual cytokine. Further analysis (post-hoc LSD) was conducted to determine which strains differ at basal levels.

Prior to testing, normality was assessed and samples were log transformed if necessary. All tests were conducted at the 95% confidence interval and appropriate statistics are quoted in the text.

6.3. Results

6.3.1. Experiments 1: Investigating the hepatic innate cytokine response to *Ascaris suum* infection

6.3.1.1. Pulmonary larval burdens on day 7 p.i.

Ascaris suum larvae were recovered in the lungs of C57BL/6j and CBA/Ca mice on day 7 p.i. [mean $\pm$ S.E.M. (C57BL/6j: 135 ± 7.9 , CBA/Ca: 32 ± 2.5)]. Analysis indicated that the pulmonary larval burdens significantly differed between C57BL/6j and CBA/Ca mice on day 7 p.i. ($P=0.008$).

6.3.1.2. Relative quantification of IL-6 in the hepatic tissue of *A. suum* infected C57BL/6j and CBA/Ca mice

The cytokine, IL-6 was detected at significantly lower levels in experimental mice in comparison to control mice in both C57BL/6j and CBA/Ca mice ($P<0.001$) (Fig. 6.7, Table 6.1). Concentration levels of IL-6 differed significantly between the two mouse strains ($P<0.001$) and over the three post-mortem time-points ($P=0.030$). The differences observed between mouse strains was evident in both experimental and control mice ($P=0.027$) (Table 6.1). As illustrated in Fig 6.7, the IL-6 concentrations observed in the resistant strain were consistently higher, regardless of treatment. In control mice of the susceptible strain, levels of IL-6 increased on day 2 p.i., whereas no appreciable changes were detected in levels recorded over the three post-mortem time-points in C57BL/6j experimental mice (Fig. 6.7). The kinetics of IL-6 over time in the strains differed ($P=0.002$). In both experimental and control CBA/Ca mice, IL-6 concentration levels peaked at 6 hours p.i., decreased to a minimum on day 1 p.i. and slightly increased on day 2 p.i. (Fig. 6.7) The noteworthy decrease in IL-6 in CBA/Ca mice over the first two time-points was highlighted in the post-hoc analysis ($P=0.021$).

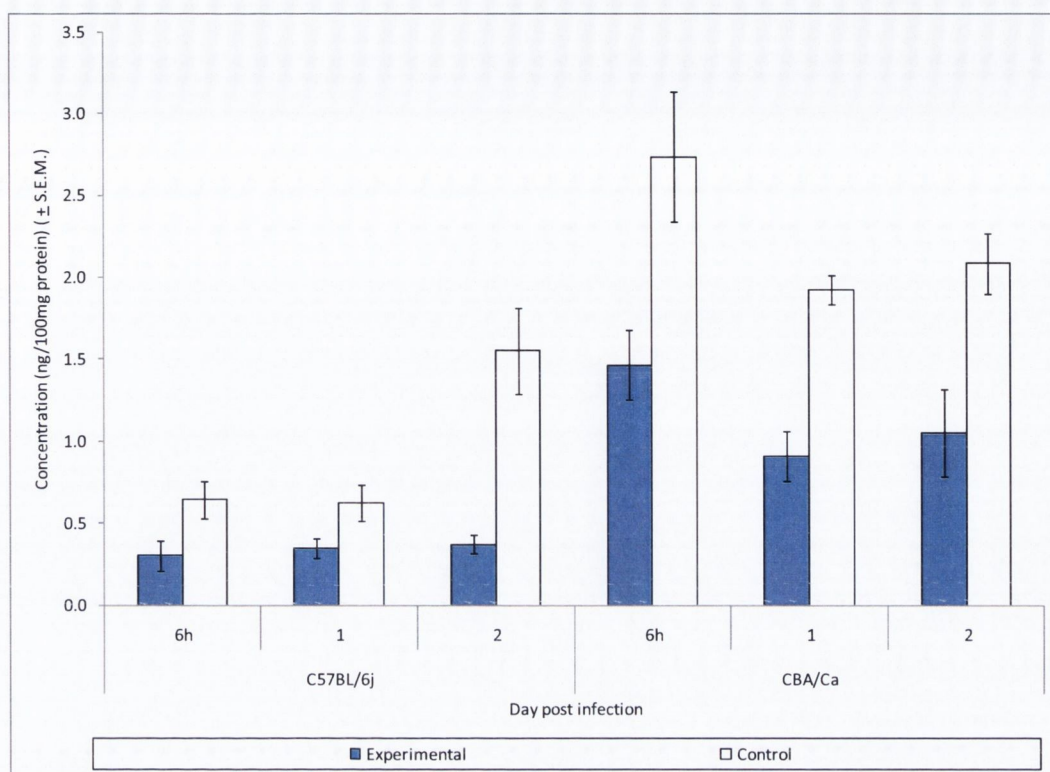


Figure 6.7. Relative changes in IL-6 ($\pm$ S.E.M.) in the hepatic tissue of *Ascaris suum* infected C57BL/6j and CBA/Ca mice

Table 6.1. Results from a three-way GLM, which examined the effects of time, strain and treatment on IL-6 levels in hepatic tissue

Source	df	F-ratio	P value
strain	1	91.921	<0.001
treatment	1	60.123	<0.001
day	2	3.852	0.030
strain*treatment	1	5.320	0.027
strain*day	2	7.504	0.002
treatment*day	2	1.498	0.237
strain*treatment*day	2	2.269	0.118
error	36		

6.3.1.3. Relative quantification of IL-10 in the hepatic tissue of *A. suum* infected C57BL/6j and CBA/Ca mice

The levels of IL-10 observed in the hepatic tissue of infected mice differed significantly between susceptible C57BL/6j and resistant CBA/Ca mice ($P=0.001$) and the main effect of day was also significant ($P=0.007$). There was no difference between experimental and control animals ($P=0.541$). However, the strain*treatment interaction was highly significant ($P=0.001$), as appreciable lower levels of IL-10 were detected in experimental C57BL/6j mice, in comparison to their control counterparts on day 1 p.i. and also in control CBA/Ca mice relative to infected CBA/Ca mice at all post-mortem time-points. (Fig. 6.8). In C57BL/6j livers, IL-10 remained consistent over three post-mortem time-points in infected mice. A considerably higher level of IL-10 in infected CBA/Ca livers relative to control CBA/Ca mice was observed at each time-point (Fig. 6.8). The level of IL-10 at this time-point was the highest recorded in infected mice at any time-point. In infected CBA/Ca mice, IL-10 levels decreased considerably on day 1 p.i. and remains at a similar level on day 2 p.i. Post-hoc analysis highlighted the significant difference between the concentrations of IL-10 recorded at 6 hour and on day 1 p.i. ($P=0.020$) and at 6 hours and on day 2 p.i. ($P=0.006$), particularly evident in CBA/Ca mice.

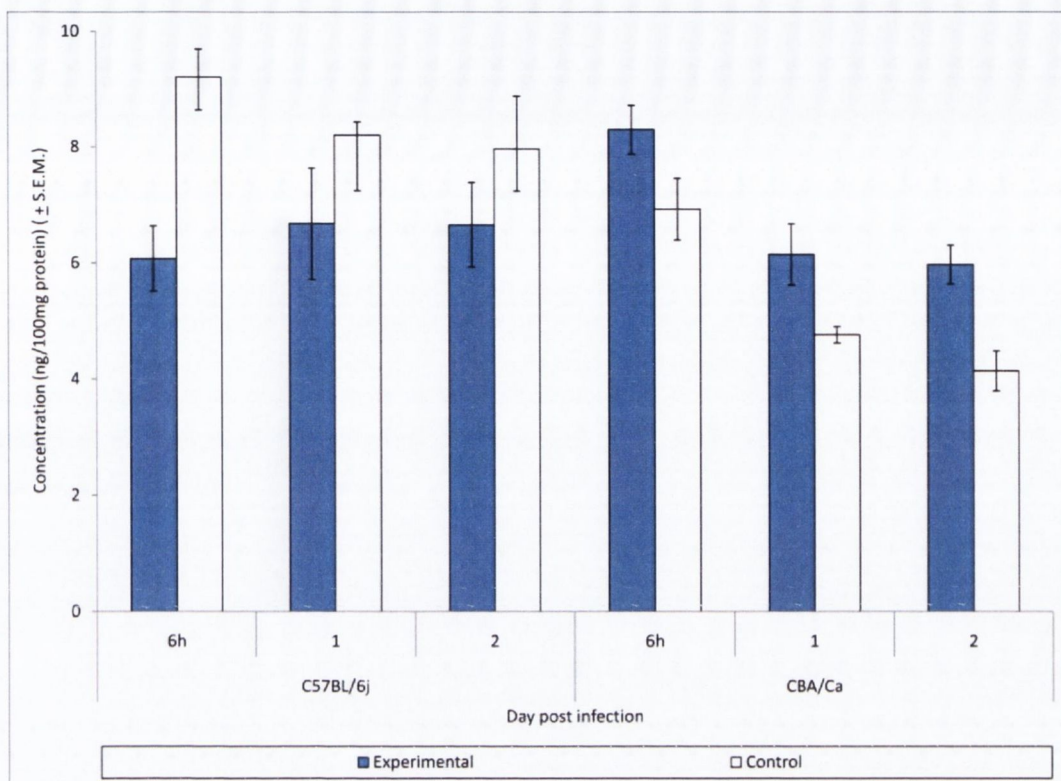


Figure 6.8. Relative changes in IL-10 ($\pm$ S.E.M.) in the hepatic tissue of *Ascaris suum* infected C57BL/6j and CBA/Ca mice

Table 6.2. Results from a three-way GLM, which examined the effects of time, strain and treatment on IL-10 levels in hepatic tissue

Source	df	F-ratio	P value
strain	1	14.618	0.001
treatment	1	0.381	0.541
day	2	5.677	0.007
strain*treatment	1	22.338	<0.001
strain*day	2	3.566	0.039
treatment*day	2	0.826	0.446
strain*treatment*day	2	0.443	0.645
error	36		

6.3.1.4. Relative quantification of IL-13 in the hepatic tissue of *A. suum* infected C57BL/6j and CBA/Ca mice

While not significant, different levels of IL-13 were observed in the two strains of mice ($P=0.613$) with no significant changes from basal levels observed in infected mice ($P=0.148$). Changes in IL-13 levels were observed in mice over the three post-mortem time-points ($P=0.031$). In experimental mice of both mouse strains, IL-13 levels decreased significantly on day 1 p.i. (post-hoc LSD: $P=0.008$) and subsequently increased on day 2 p.i. (post-hoc LSD: $P=0.103$). The change in IL-13 production on day 1 p.i. was most evident in C57BL/6j experimental mice. A different pattern of change was observed in both mouse strains ($P=0.031$), which was most evident when comparing control mice (Fig. 6.9).

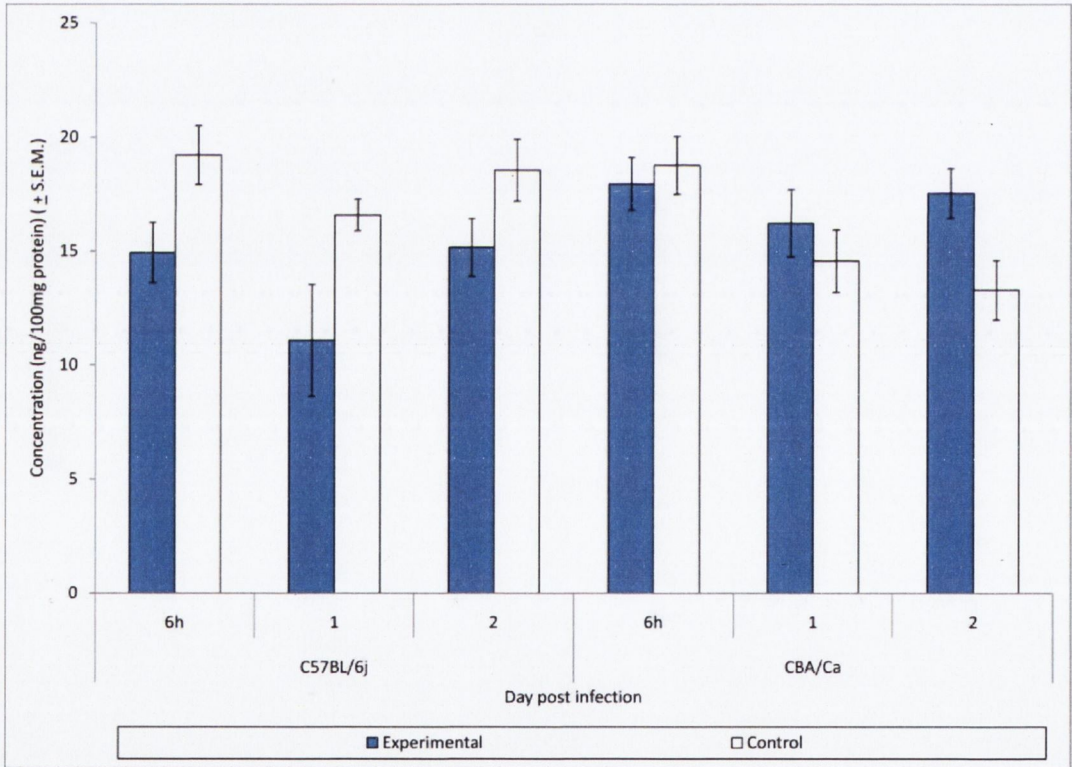


Figure 6.9. Relative changes in IL-13 ($\pm$ S.E.M.) in the hepatic tissue of *Ascaris suum* infected C57BL/6j and CBA/Ca mice

Table 6.3. Results from a three-way GLM, which examined the effects of time, strain and treatment on IL-13 levels in hepatic tissue

Source	df	F-ratio	P value
strain	1	0.261	0.613
treatment	1	2.191	0.148
day	2	3.830	0.031
strain*treatment	1	11.102	0.002
strain*day	2	1.097	0.345
treatment*day	2	0.987	0.383
strain*treatment*day	2	0.522	0.598
error	36		

6.4. Discussion

Pulmonary larval burdens indicate that *A. suum* larvae did not successfully migrate in appreciable numbers to the lungs in resistant CBA/Ca mice. The numbers of larvae recorded in the lungs were similar to observed burdens in past experiments (Lewis *et al.*, 2006; chapter 3, section 3.3.1.3). Coupled with this, relative differences were detected in the levels of a selection of intrahepatic cytokines between mouse strains.

Prior to the cytokine assays on the liver samples collected during the experiments, attempts were made to optimise the ELISA protocol in order to quantify the absolute values of cytokines present in murine hepatic tissue, which has been successfully undertaken in human liver samples (Kelly *et al.*, 2006). To the best of our knowledge, absolute values have not previously been recorded for murine intrahepatic cytokines and authors tend to quote relative values as the contribution of the complex mix of hepatic proteins is not controlled for (e.g. Mohammed *et al.*, 2004). Optimisation steps did not improve the assay to a satisfactory standard and therefore the standard ELISA sandwich protocol was undertaken to detect relative differences in intrahepatic cytokines as has been conducted previously. Subsequent to the work presented here, experiments were undertaken to optimise the ELISA protocol for mouse liver homogenate. It was determined that in order to successfully control for background signal, it is necessary to use a capture antibody not raised in rat as it is too similar to the mouse and thus results in cross-reactivity.

Pathologic conditions elicit a complex network of cytokine responses, and each cytokine might have a different function depending on the cellular source, target, and the specific phase of the immune response during which it is presented (Anthony *et al.*, 2007). Despite this, the cytokines chosen represent proinflammatory and regulatory aspects of the host immune response and therefore one can hypothesise the outcome of changes in IL-6, IL-10 and IL-13 production in the liver during *A. suum* infection

IL-6 stimulates lymphocyte differentiation, the acute phase response and Th2 cell differentiation (Diehl and Rincón, 2002). Unusually, the levels detected in control mice were consistently higher than those detected in experimental mice. This suggests a parasite or host mechanism in which the cytokine is downregulated. CBA/Ca mice appear to inherently produce higher levels of IL-6. Therefore, the downmodulation of IL-6, regardless of effector mechanism, may be comparable in both strains but lead to a difference in total IL-6 levels between strains. When comparing the experimental mice over time, the IL-6 levels remained consistently low in susceptible animals. The observed high level of intrahepatic IL-6 at 6 hours p.i. in experimental CBA/Ca mice may be indicative of the initial response to the hepatic damage induced by *A. suum* larvae entering the liver. While levels decrease in the infected resistant mice on day 1 p.i., they remain higher than those recorded in C57BL/6j mice. This is indicative of a higher degree of an inflammatory process at the earlier time-points of *A. suum* infection in resistant animals.

IL-10 levels in CBA/Ca mice were consistently higher in infected animals in comparison to control mice. As demonstrated in the two experiments conducted observing cytokines in naïve and infected mice, uninfected C57BL/6j mice appear to have higher baseline levels of hepatic IL-10 when compared to CBA/Ca mice. Therefore the higher IL-10 levels in CBA/Ca mice appear to be in response to *A. suum* infection. Contrasting IL-10 levels have been documented in endemic human populations in which some studies describe no difference in expression between the infected and uninfected groups studied (Cooper *et al.*, 2000) and others document an inverse correlation with intensity of *A. lumbricoides* infection (Turner *et al.*, 2003). As commented by Turner *et al.* (2003), the presence of IL-10 may be “reflective of a regulatory network that is induced to hold potentially damaging inflammatory responses in check.” As stated above, the heightened IL-6 levels in CBA/Ca infected mice may reflect an early inflammatory response to infection, while the IL-10 production in CBA/Ca mice may be key in minimising immunopathology as this role has previously been cited in helminth infections (Sher and Coffman, 1992). A failure to control pathological reactions has been documented in IL-10 deficient models for both *Trichuris muris* (Schopf *et al.*, 2002) and *Schistosoma mansoni* (Sadler *et al.*, 2003).

Coupled with IL-10, IL-4 has been documented to play a crucial role in controlling infections with parasitic nematodes (Else and Grencis, 1996). IL-13 is closely linked to IL-4 and has also been shown to play a role in resistance to helminth infection (eg Bancroft *et al.*, 1998). Furthermore, IL-13 levels have been shown to be negatively associated with general susceptibility (Jackson *et al.*, 2004), while the percentage of individuals who produced IL-13 to *Ascaris* antigen increased in the older age classes (Turner *et al.*, 2003), which has been postulated to correspond with acquired resistance. A notable increase in IL-13 concentration in CBA/Ca livers on day 2 p.i., relative to the uninfected control CBA/Ca mice, is indicative of a Th2 response in resistant mice. In contrast, considerably lower levels of IL-13 were observed in experimental C57BL/6j mice relative to their non-infected counterparts. It is possible that low levels of IL-6, IL-10 and IL-13 in experimental C57BL/6j relative to the control mice are linked, as IL-6 plays a role in differentiating Th2 cells (Diehl and Rincón, 2002) as well as inducing IL-1, an important mediator of the inflammatory response. The marked increase in IL-13 on day 2 p.i. in experimental CBA/CA relative to control mice may also reflect the induction of tissue repair (Lee *et al.*, 2001), as *A. suum* larvae cause mechanical injury and resultant necrosis whilst migrating through hepatic tissue. Furthermore, IL-13 plays a significant role in hepatic tissue fibrosis in schistosomiasis (Chiaramonte *et al.*, 1999; Cheever *et al.*, 2000; Fallon *et al.*, 2000).

The effector cells behind the apparent Th2 cytokine response in *A. suum* infected CBA/Ca mice are unknown. However, flow cytometry analysis revealed that a higher percentage of $\gamma\delta$ TCR⁺ cells are present in the liver of naïve CBA/Ca mice in comparison to C57BL/6j mice, which may impact on the early response to infection (E. Nemeth, personal communication). $\gamma\delta$ TCR⁺ cells are known to be capable of inducing pathogen-specific stress responses and then discriminating early in infection by producing either Th1 or Th2 cytokines as demonstrated by Ferrick *et al.* (1995). Therefore, since $\gamma\delta$ TCR⁺ cells are known to be involved in establishing primary immune responses to *Nippostrongylus brasiliensis* infection (Ferrick *et al.*, 1995), the heightened number inherently present in resistant CBA/Ca mice may lead to the Th2 response evident early in infection.

While absolute values were not obtained for cytokines in the liver, relative values indicated upregulation of the candidate Th2 cytokines, IL-10 and IL-13 (day 2 p.i.) in infected resistant CBA/Ca mice relative to control mice. Therefore, the present study suggests that variations in hepatic cytokine response to *A. suum* infection are evident early in infection and therefore may contribute to the mechanism of resistance. Consequently, it is likely that the observed cytokines play a role in *A. suum* inhibition and also in determining the pathological outcome of infection in CBA/Ca mice. The upregulation of IL-13 on day 2 p.i. in CBA/Ca infected mice relative to control animals is of particular interest as this cytokine induces eosinophilia and IgE production, both of which are characteristic of resistance to *Ascaris* infection (Eriksen, 1981; Hagel *et al.*, 1993; Lynch *et al.*, 1993; McSharry *et al.*, 1999; Pérez *et al.*, 2001; Cooper, 2002). The results are therefore in line with speculation that the heightened IgE response in *A. lumbricoides*-infected hosts is stimulated by larval migration (Radermecker *et al.*, 1974).

7. General discussion

7.1. General discussion

The body of work presented in this thesis was focused upon the mechanism(s) underlying the generation of resistance and susceptibility to *Ascaris* infection. All experiments were conducted in an optimised mouse model in which a large divergence in pulmonary larval burdens are observed between strains (Lewis *et al.*, 2006). Various explanatory mechanisms for this divergence were investigated in the putatively susceptible C57BL/6j and resistant CBA/Ca mice, encompassing host determinants of infection intensity such as genetics, and the innate inflammatory and cytokine responses.

An initial fine-detailed approach was undertaken to determine the time and location of larval attrition and accumulation in both mouse strains. As in previous studies using the mouse model of resistance/susceptibility (Lewis *et al.*, 2006; 2007), larval numbers were similar in the whole liver of C57BL/6j and CBA/Ca mice yet differed significantly in the total lung recoveries, particularly on day 7 p.i. An inconsistent pattern of larval dispersal in the liver lobes was observed in both mouse strains. One cannot conclusively determine the reason for the observed predilection for different lobes over the time-points studied due to a lack of mouse strain-specific studies on liver anatomy. Since a significant change in the proportion of larvae was detected in the right and caudate lobes, and was approaching significance in the left lobe, it is likely that the erratic pattern of dispersal over time is representative of the migratory nature of the larvae within the liver. In addition, a similar inflammatory response was documented across all liver lobes, as discussed below, which indicates that it is unlikely that the consistent migration within the liver is an immunoevasive strategy.

Larval recoveries also showed a heterogenous distribution among the lobes of the lungs, being higher in the right lung of both strains, and in the susceptible strain, larvae accumulated preferentially in two (caudal and middle) of the four lobes. Similar to the liver, there is a lack of literature detailing the migration or establishment of particular

helminthic parasites within pulmonary lobes so therefore it was difficult to explain the heterogeneous distribution of larvae observed. This result stimulated interest in the potential of a physical barrier to the onward migration of *A. suum* larvae in CBA/Ca mice. The examination of the gross anatomical structure of the pulmonary artery did not ultimately prove to be a fruitful line of enquiry as noteworthy differences in the primary branching were not evident between the two strains. This result eliminated the possibility of an underlying anatomical pulmonary mechanism accounting for diverging lung larval burdens and thus indicated a hepatic/post-hepatic factor varying between the two mouse strains.

Lewis *et al.* (2007) also did not find evidence for a pulmonary mechanism underlying resistance to *A. suum* in the mouse model. The researchers evaluated the bronchoalveolar lavage (BAL) fluid and pulmonary histopathology during days 7-10 and 8-10 p.i. respectively and concluded that the responses mirrored larval intensity. The degree of the pulmonary inflammatory response at the onset of larval migration to the lungs, conducted in the present study, confirmed that the early response to infection in the pulmonary tissues also reflects the larval burden. Furthermore, mucus production in the lungs again mirrored pulmonary larval intensities and is therefore simply a product of irritation to the epithelial surfaces rather than a mechanism of larval expulsion. Therefore, combined with previous results (Lewis *et al.*, 2007) the present study further indicates that the lungs are not prominently involved in primary protection to *A. suum* infection.

Multiple locations involved in the migratory path have been presented as sites at which the mechanism of resistance is manifested. Pre-hepatic protective immunity, marked by a reduction in hepatic WS and pulmonary larval burdens has been observed previously (Urban *et al.*, 1988; Eriksen *et al.*, 1992a). Coupled with this, WS have been linked to larval trapping in pigs (Schwartz and Alicata, 1932; Copeman and Gaafar, 1972; Eriksen *et al.*, 1980; Pérez *et al.*, 2001). Furthermore, the liver has also been suggested as a potential site for the priming of larvae for later expulsion from the intestine (Jungersen *et al.*, 1999a). The liver has previously been cited as a key site in the immobilization of migrating *Ascaris* larvae in mice (Sprent, 1949; Taffs, 1968; Mitchell *et al.*, 1976;

Johnstone *et al.*, 1978; Song *et al.*, 1985), guinea pigs (Kerr, 1938; Fallis, 1948) and rabbits (Arean and Crandall, 1962). Despite the large number of studies presenting evidence of a role for the liver in resistance to *Ascaris* infection, the experiments were undertaken in different model organisms, in some of which resistance/susceptibility was not established. However, the putatively susceptible and resistant mouse strains used in the set of experiments detailed in this thesis provide a robust model for examining the effector mechanism(s) behind the effective and efficient hepatic inflammatory response in CBA/Ca mice.

Hepatic inflammation was evaluated by means of semi-quantitative grading of histopathological sections of infected C57BL/6j and CBA/Ca mouse livers. Examination of individual liver lobes revealed that an individual section from a liver lobe was generally representative of the response throughout the liver, despite variability in the larval numbers between lobes. An intense inflammatory reaction was identified in the livers of CBA/Ca and C57BL/6j mice on days 4 and 6 p.i. respectively. The earlier intense inflammation in CBA/Ca mice coincided with reduced larval numbers in the lungs, so may function in larval trapping which has been a proposed role of white spots in pigs.

The similarity in the hepatic inflammatory response in CBA/Ca and C57BL/6j mice on days 4 and 6 p.i. respectively indicates that both mouse strains are capable of mounting a similar response to *A. suum* infection, albeit at different stages of infection. In the *Trichuris muris* mouse model of infection, BALB/c mice expel their parasites from the gut on days 17-21 p.i., which has led to speculation that the resistance status of the host animal may be related to the speed with which the worms are expelled (Else *et al.*, 1989). In an experiment where severe combined immunodeficient (SCID) mice were transplanted with a high number of CD4⁺ cells from BALB/c mice, the acceptor mice were shown to mount a dominant Th2 response and successfully expel *T. muris* when infection and donor cells were administered simultaneously. This contrasts greatly with animals transplanted with a comparatively lower number of BALB/c CD4⁺ cells at the time of infection, which display a strong Th1 response and are unable to expel the parasites so infection develops to patency. However Else & Grencis (1996) noted that if the lower number of CD4⁺ cells are donated to SCID mice 28 days prior to infection, a

Th2 response dominates and leads to effective parasite expulsion. Therefore, kinetics of the immune response is key to the effective removal of the *T. muris* from the host. The early efficient inflammatory response to hepatic larval *A. suum* migration in CBA/Ca mice may hinge on an inherent higher number of an immune effector cell in the resistant strain, thus facilitating a rapid response to infection.

In general, the expulsion of gastrointestinal helminths is critically dependent upon the ability of the host to generate a dominant Th2 response (e.g. Grencis, 1997), which has been observed in putatively resistant human hosts (Turner *et al.*, 2003; Jackson *et al.*, 2004). The importance of the prevailing cytokine environment at the time of the generation of CD4+ Th subsets has been well documented (Fitch *et al.*, 1993; Borish and Steinke, 2003), and IL-4 appears to play a considerable role in generating a Th2 response. There is a lack of literature detailing the inter-strain variations in hepatic lymphocyte subpopulations, which leads to difficulties in speculating as to the sources of effector cytokines.

Recent work on the cellular composition of the liver in CBA/Ca and C57BL/6j mice, undertaken by means of flow cytometry, indicates a lower (though not significantly lower) number of $\gamma\delta$ T cells in C57BL/6j mice (E. Nemeth, personal communication). $\gamma\delta$ T cells are innate-like lymphocytes that are commonly associated with Th1 responses to microparasites, but are additionally capable of producing Th2 cytokines. As discussed by Kaufmann (1996), the wide use of bacteria as a stimulating agent may be the reason behind the established link between $\gamma\delta$ T cells and Th1 cytokines. It is feasible that the higher number of $\gamma\delta$ T cells, which are capable of producing IL-4 once stimulated with *Nippostrongylus brasiliensis* (Ferrick *et al.*, 1995) may therefore be responsible for a protective Th2 response in CBA/Ca mice infected with *A. suum*. A higher number of IL-4-producing $\gamma\delta$ T cells were also noted during *T. muris* infection in the intestines of resistant BALB/c mice than in susceptible AKR mice, which may influence Th2 selection (R. Lukaszewski, personal communication).

The early intrahepatic cytokine profiles recorded in the two mouse strains during *A. suum* infection were representative of the cellular inflammatory response detected in

histopathological examination of liver tissue. Higher levels of IL-6 in CBA/Ca mice relative to C57BL/6j mice shows that the resistant strain responded to the tissue damage induced by migrating larvae. The lack of stimulation in C57BL/6j livers was mirrored in the histopathology as necrotic larval tracts were evident yet accompanying cellular infiltrate was absent on the early days of infection. Therefore, the initial low levels of IL-6 may account for the delay in inflammatory stimulation and the recruitment of cellular infiltrate in the livers of susceptible mice. This result is of interest as an ongoing inflammatory response is characteristic of putatively immune children in *A. lumbricoides*-endemic Nigeria (McSharry *et al.*, 1999), which was determined by evaluating the serum levels of C-reactive protein (CRP), an acute phase reactant produced in the liver. Results presented in this thesis confirm that resistance to *Ascaris* infection is associated with a comparatively heightened inflammatory response in the liver, timed effectively to curb successful migration to the lungs in CBA/Ca mice.

The intense inflammatory response in CBA/Ca mice was effectively resolved on day 5 p.i. Therefore, the recorded patterns of heightened IL-10 in infected CBA/Ca mice, relative to their controls, are also representative of the observed inflammatory cellular reaction. Notably higher levels of IL-10 in resistant infected mice relative to their controls on days 2 and 3 p.i. may account for the highly efficient and economic inflammatory reaction, as heightened levels on these days would lead to the downmodulation of the pronounced early reaction. IL-10 suppresses Th1 responses but is also known to regulate inflammation, and observed heightened levels of IL-10 in resistant human hosts in endemic areas may play a similar role in regulating inflammatory responses to infection (Turner *et al.*, 2003). This regulatory cytokine is responsible for the control of granulomas induced by *Schistosoma mansoni* migration through the liver, and contributes to the effective protection of the host tissue during parasite expulsion. IL-10 dampens ongoing cytokine responses that can lead to tissue damage if prolonged (Wynn *et al.*, 1997; Hoffmann *et al.*, 2000). It is possible that IL-10 plays a similar role in the hepatic inflammatory response to *A. suum* infection in CBA/Ca mice, as the observed tissue damage is efficiently repaired in this mouse strain, which contrasts with a slow and prolonged build-up of inflammatory cell infiltration in the susceptible strain, peaking on day 6 p.i. when larvae are established in the lungs.

The marked reduction in inflammatory cells observed on day 5 p.i. in CBA/Ca mice may also be a product of induced IL-13, evident in CBA/Ca infected mice on day 3 p.i., as this cytokine plays a documented role in the generation of tissue repair (Wynn, 2003). In addition, IL-13 is involved in the onset and effector phase of Th2 immune responses and has previously been shown to play a role in resistance to helminth parasites (Bancroft *et al.*, 1998).

The work presented in this thesis has provided evidence for a Th2 response in the hepatic tissue of resistant mice (increased IL-10 and IL-13 relative to controls and higher levels of IL-6 in comparison to susceptible counterparts) which was not detected in susceptible C57BL/6j mice at the early time-points. However, a comprehensive model of the murine immunological response to *A. suum* infection will require investigation into the production of Th1 cytokines in both mouse strains. Based on its response to *Leishmania major*, the prototypical model of Th1/Th2 response, C57BL/6j mice are classed as Th1 responders (Watanabe *et al.*, 2004). Therefore, since this mouse strain is the susceptible strain of the mouse model used in the present study, it would be of particular interest to directly assess if its insufficient response to *A. suum* infection is Th1-driven. The liver is now regarded as an organ with predominant innate immunity (Gao *et al.*, 2008), controlled locally by means of an array of cytokines and chemokines (Knolle and Gerken, 2000). Therefore, there are many possible cytokines in the liver that could potentially mediate the early response to *A. suum* infection. However, investigation of more candidate cytokines in this study was limited by practical considerations.

The selection of *Itln-2* as a candidate gene for assessing an association with resistance to *A. suum* infection in mice was largely decided upon due to the mutation in C57BL/6j mouse chromosome 1 that encompasses the deletion of the *Itln-2* gene (Pemberton *et al.*, 2004a). Therefore, it was thought that an early pulmonary response, on earlier days than those assessed by Lewis *et al.* (2007), mediated by increased levels of *Itln-2* was a feasible explanatory mechanism for the disparity in lung larval burdens. While a lack of an association between *Itln-2* and resistance to *A. suum* was detected, the

immunosuppressant effect brought on by co-habitation of male and female mice during infection in C57BL/6j was an interesting unexpected finding.

Hormones appear to affect the immune response to *A. suum* infection in mice, but more noticeably in one mouse strain (C57BL/6j), further confirming that the underlying mechanism(s) of resistance to *A. suum* infection is not anatomical but rather a component of the immune system under defined genetic control in the two mouse strains. Future candidate immune mechanisms may be assessed for their response to direct hormone administration.

The immunosuppressant 'sex effect' encountered was observed in a laboratory situation in which dominance hierarchies were established and behaviour uncharacteristic of wild populations was observed (e.g. an inability to flee from aggressive encounters). These unexpected results highlighted the importance of careful gender selection as a consideration for future cohabitation studies.

Similar hormonal modulation of parasite-host interaction has been observed in other model systems (Haley, 1958; Solomon, 1966; Charniga *et al.*, 1981; Kiyota *et al.*, 1984; Bone and Bottjer, 1986; Harder *et al.*, 1992). While malaria is not a parasitic helminth, its hepatic stages are likely to be the most widely studied of any parasite-host relationship which involves the liver. Interestingly, testosterone affects malaria-induced changes in reticular endothelial system activity and gene expression in the liver, but not in the spleen (Krücken *et al.*, 2005). In this study, particle trapping and gene expression were assessed in C57BL/6j mice infected with *Plasmodium chabaudi* and administered testosterone. Krücken *et al.* (2005) demonstrated that testosterone impairs protective responses of the liver rather than the spleen to blood-stage malaria. Therefore, this study confirms that the liver is capable of mediating suppressive effects of testosterone during an infection.

Examination of the hepatic inflammatory response has resulted in a greater understanding of the responses to infection by *A. suum* in inbred strains of mice, but also highlighted the impact of larval migration on the host's liver, a somewhat

unappreciated topic constrained by obvious physical limitations in humans. The pathophysiological effects of *A. lumbricoides* larval migration are therefore largely unknown even though an *Ascaris*-induced inflammatory response is known to occur in infected individuals (Javid, 1999; Sakakibara *et al.*, 2002; Kakihara *et al.*, 2004). In pigs, larval migration in the liver and the resultant WS lead to liver condemnation (Stewart and Hale, 1988). The observed differences in the onset of the inflammatory response in C57BL/6j and CBA/Ca mice indicate that susceptible hosts do not initially respond to migrating *Ascaris* larvae and the associated mechanical injury to, and necrosis of, hepatic tissue. Murrell *et al.* (1997) commented that the potential for liver damage in humans may be underappreciated. Results obtained in the mouse model indicate that this may be true, particularly of susceptible hosts, as C57BL/6j mice did not appear to respond to initial hepatic infection despite the induction of mechanical injury, which was evident in the widespread necrosis devoid of cellular infiltrate indicative of a response to infection.

The evident lack of early response from C57BL/6j mice points to a varying hepatic factor between the two mouse strains that renders susceptible mice incapable of mounting an early response to infection. Both the histopathological results and cytokine profiles in the two mouse strains indicate that the mechanism of resistance is largely dependent on the kinetics of the inflammatory response, which is evident at a comparatively earlier stage of infection. This leads to effective and efficient removal of the parasite and reduced tissue damage, presumably mediated by the heightened IL-10 response on day 2 p.i. in CBA/Ca mice and a lower pathophysiological effect of larval migration in the resistant host.

The marked contrast observed in the timing of a pronounced inflammatory response in C57BL/6j and CBA/Ca mice has led us to conclude that we have a useful model of differential hepatic inflammation, which will prove amenable to further study. Future work in the mouse model, particularly investigation of the immune response to infection, will focus on the liver. The importance of directing immunological studies of *Ascaris* infection to the exploration of local responses at the site of immunity was highlighted by Jungersen and colleagues (2001), who found that the lymphocyte

recognition patterns obtained in the pig host reflect the actual antigen exposure of the draining area. Coupled with this, *Ascaris* antigens are stage-specific (Kennedy and Qureshi, 1986) and responses to infection are very much dependent on the tissues in which the infections are localised.

Future work should focus on a selection of both Th1 and Th2 cytokines so that one can conclusively assess whether the levels of IL-6, IL-10 and IL-13 observed in CBA/Ca mice are in fact representative of a dominant protective Th2 response or a component of a mixed Th1/Th2 response, which is regularly documented in reaction to invasive tissue helminths and is key in granuloma formation in the liver during *Schistosoma* infection (Infante-Duarte and Kamradt, 1999; Diaz and Allen, 2007). In particular, it would be interesting to assess the levels of IL-4 and IFN- γ during the early stages of hepatic infection as these cytokines are considered to strongly influence the Th phenotype. In addition, it would be worth noting the production of the cytokines that tend to play a role in liver infections and homeostasis such as IL-1, which is tightly linked with IL-6 and is crucial in the hepatic inflammatory response, and TGF- β , which is an important hepatic regulatory cytokine (Invernizzi *et al.*, 2007). Coupled with susceptible C57BL/6j and resistant CBA/Ca mice, the production levels of cytokines in BALB/c mice would provide information on the innate immune response in an intermediate susceptible mouse strain (Lewis *et al.*, 2006).

The highly potent orchestrated network of cytokines induced during infection may be difficult to unravel without a more comprehensive picture of the cellular response to infection. Therefore, it would be of particular interest to characterise the cells involved in the hepatic lesions associated with migrating larvae in C57BL/6j and CBA/Ca mice. Pérez *et al.* (2001) described the composition of the cellular infiltrate (T and B lymphocytes, macrophages and, IgG, IgA and IgM bearing cells) as well as the distribution of S-100 protein and MHC class II antigen in porcine hepatic WS. T lymphocytes were broadly characterised using T-cell receptor (TCR) marker CD3+.

However, a more comprehensive examination of WS cellular composition has not been conducted to date, despite a wealth of knowledge on the immunity-related functions of

the liver (Gao *et al.*, 2008). Furthermore, the use of flow cytometry rather than immunohistochemistry would facilitate a high-throughput, quantitative approach to examining the *Ascaris*-induced hepatic cellular infiltrate, thus defining the sentinel cells more accurately. The cell populations such as Kupffer cells, CD4+ and CD8+ T cells, $\gamma\delta$ T-cells, natural killer (NK) cells, NK T-cells, and regulatory T-cells would be of particular interest due to their particularly high abundance in hepatic lymphocyte populations (reviewed by Nemeth *et al.*, 2009).

In addition to fixed liver tissue immune cells, further characterisation of the recruited granulocytes could also be undertaken using flow cytometry, with particular focus on eosinophils. Eosinophils are characteristic of helminth-induced granulomas (Kaplan *et al.*, 1998) and are implicated in helminth protection (Meeusen *et al.*, 2005), particularly in relation to tissue-migrating helminths (Maizels and Holland, 1998). Furthermore, eosinophilia is repeatedly noted during *Ascaris* infection in a range of host organisms (e.g. Fallis, 1948; Kelley and Nayak, 1964; Eriksen, 1981). Additionally, there is a recently classified population of cells known as the alternatively activated macrophages, which control inflammation and effect tissue repair in Th2 responses (Anthony *et al.*, 2007; Diaz and Allen, 2007). It would be of interest to evaluate the presence or absence of these cells during the rapid tissue repair illustrated in CBA/CA mice hepatic sections on day 5 p.i. Once effector sentinel cells were characterised in the hepatic inflammatory response to *A. suum*, one could combine these results with the observed intrahepatic cytokine profile during infection, giving a more complete picture of the underlying mechanism(s) of resistance/susceptibility.

Taken in its entirety, the work presented in this thesis represents a significant and novel contribution to the body of scientific research concerning the mechanistic basis of resistance to *Ascaris* infection, with a particular focus on the kinetics of the hepatic inflammatory response, which this work has positioned as a key mediator of primary protection in the mouse model.

8. References

8.1. References

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9. Appendices

Appendix 1. Supplemental laboratory protocol

1.1 Checking inocula

On every occasion that inocula were prepared, five random samples were examined prior to infection of mice.

Table 1.1 Counts of infective ova in 10 x 10ul from individual doses of 1000 ova and estimated mean number of ova per inoculation dose in five randomly examined samples

Sample	Number of infective ova in 10μl											Estimated number of ova in inoculation dose
	1	2	3	4	5	6	7	8	9	10	Mean	
A	115	83	91	107	86	109	119	89	87	105	99.1	991
B	136	71	53	97	98	107	108	92	111	120	99.3	993
C	83	117	96	104	116	76	109	113	119	73	100.6	1006
D	108	109	106	114	99	83	94	89	113	97	101.2	1012
E	96	114	53	104	97	95	103	99	134	113	100.8	1008

Mean ± S.E.M. of infective ova in five randomly examined samples = 1002 ± 4.21

1.2 Haemotoxylin and eosin staining procedure

Slides were stained in an automatic stainer (Thermo Shandon Auto Stainer) at the following settings:

Histoclear™	1-3 minutes
Absolute alcohol	1 minute
90% alcohol	30 seconds
70% alcohol	30 seconds
Distilled water	1 minute
Haemotoxylin (0.5%)	2 minutes
Distilled water	30 seconds
Scott's tap water	15 seconds
Eosin (1% aqueous solution)	30 seconds
Distilled water	30 seconds

Slides were dehydrated in a series of alcohol dilutions (70%, 90% and absolute alcohol 1 and 2) (2 minutes each) and Histoclear™ (3-5 minutes). A coverslip was placed on each slide using DPX mountant.

1.3 Periodic acid Schiff staining procedure

Slides were placed in HistoClear™ (1-3 minutes) and a series of alcohol dilutions (absolute 1 and 2, 90% and 70%) (10 minutes) to remove wax and hydrate the tissue. Prior to staining, slides were washed in distilled water (1 minute).

In the periodic acid Schiff staining protocol a 1% Alcian blue in 3% acetic acid solution and a 0.1% eosin in 0.25% acetone staining solutions were prepared.

The staining procedure was as follows:

Alcian blue	5 min
Wash in distilled water	
1% periodic acid	7 min
Wash in distilled water	
Schiff solution	10 min
Wash in running water	10 min
Discombe's eosin	10 min
Wash in distilled water	
Harris haemotoxylin	1 min
Wash in warm water	1 min

Slides were dehydrated in a series of alcohol dilutions (70%, 90% and absolute alcohol 1 and 2) (2 minutes each) and HistoClear™ (3-5 minutes). A coverslip was placed on each slide using DPX mountant.

1.4 Lysis buffer solution for protein extraction

The lysis buffer used to extract protein from liver and spleen tissues as detailed in chapter 6 was prepared as follows:

5ml RIPA buffer (see below)

33.3 μ l aprotinin (1.7mg/ml)

5 μ l leupeptin (10mg/ml)

50 μ l phenylmethanesulphonyl fluoride (PMSF) (250mM)

10 μ l NaVO₄ (1M)

The RIPA buffer used in the lysis buffer was prepared as follows:

10ml Tris (1M, pH 7.4)

15ml NaCl (5M)

1ml EDTA (0.5M, pH 8)

5ml Triton X-100

5ml SDS (10%)

464ml ddH₂O

Appendix 2. Supplementary data for Chapter 5

Table 2.1 Mean larval burden and number of copies of *Itln-2* gene in F₂ mice with female C57BL/6j and male F₁ parental strains

Mouse	Sex	Female parental strain	Male parental strain	Day 7 lung larval burden	Number of copies of <i>Itln-2</i> gene
1	M	C57BL/6j	F ₁	80	1
2	M	C57BL/6j	F ₁	140	1
3	M	C57BL/6j	F ₁	70	0
4	M	C57BL/6j	F ₁	85	1
5	M	C57BL/6j	F ₁	50	0
6	M	C57BL/6j	F ₁	85	1
7	M	C57BL/6j	F ₁	25	0
8	M	C57BL/6j	F ₁	155	0
9	M	C57BL/6j	F ₁	95	1
10	M	C57BL/6j	F ₁	10	1
11	M	C57BL/6j	F ₁	140	0
12	M	C57BL/6j	F ₁	145	0
13	M	C57BL/6j	F ₁	15	0
14	M	C57BL/6j	F ₁	115	0
15	M	C57BL/6j	F ₁	95	0
61	F	C57BL/6j	F ₁	55	0
62	F	C57BL/6j	F ₁	25	0
63	F	C57BL/6j	F ₁	60	0
64	F	C57BL/6j	F ₁	45	1
65	F	C57BL/6j	F ₁	10	1
66	F	C57BL/6j	F ₁	110	0
67	F	C57BL/6j	F ₁	20	0
68	F	C57BL/6j	F ₁	10	0
69	F	C57BL/6j	F ₁	25	1
70	F	C57BL/6j	F ₁	35	0
71	F	C57BL/6j	F ₁	5	0
72	F	C57BL/6j	F ₁	15	0
73	F	C57BL/6j	F ₁	20	1
74	F	C57BL/6j	F ₁	20	0
75	F	C57BL/6j	F ₁	20	0

Table 2.2 Mean larval burden and number of copies of Itln-2 gene in F₂ mice with female CBA/Ca and male F₁ parental strains

Mouse	Sex	Female parental strain	Male parental strain	Day 7 lung larval burden	Number of copies of Itln-2 gene
16	M	CBA/Ca	F ₁	15	1
17	M	CBA/Ca	F ₁	25	2
18	M	CBA/Ca	F ₁	90	1
19	M	CBA/Ca	F ₁	100	1
20	M	CBA/Ca	F ₁	25	1
21	M	CBA/Ca	F ₁	10	1
22	M	CBA/Ca	F ₁	0	1
23	M	CBA/Ca	F ₁	115	2
24	M	CBA/Ca	F ₁	30	1
25	M	CBA/Ca	F ₁	20	1
26	M	CBA/Ca	F ₁	15	2
27	M	CBA/Ca	F ₁	10	1
28	M	CBA/Ca	F ₁	40	1
29	M	CBA/Ca	F ₁	25	1
30	M	CBA/Ca	F ₁	5	1
76	F	CBA/Ca	F ₁	10	2
77	F	CBA/Ca	F ₁	20	1
78	F	CBA/Ca	F ₁	15	1
79	F	CBA/Ca	F ₁	25	2
80	F	CBA/Ca	F ₁	0	1
81	F	CBA/Ca	F ₁	15	1
82	F	CBA/Ca	F ₁	20	2
83	F	CBA/Ca	F ₁	0	1
84	F	CBA/Ca	F ₁	0	2
85	F	CBA/Ca	F ₁	5	2
86	F	CBA/Ca	F ₁	5	2
87	F	CBA/Ca	F ₁	20	2
88	F	CBA/Ca	F ₁	70	2
89	F	CBA/Ca	F ₁	90	2
90	F	CBA/Ca	F ₁	5	2

Table 2.3 Mean larval burden and number of copies of Itln-2 gene in F₂ mice with female F₁ and male C57BL/6j parental strains

Mouse	Sex	Female parental strain	Male parental strain	Day 7 lung larval burden	Number of copies of Itln-2 gene
31	M	F ₁	C57BL/6j	650	1
32	M	F ₁	C57BL/6j	925	0
33	M	F ₁	C57BL/6j	980	1
34	M	F ₁	C57BL/6j	10	1
35	M	F ₁	C57BL/6j	230	1
36	M	F ₁	C57BL/6j	15	0
37	M	F ₁	C57BL/6j	0	1
38	M	F ₁	C57BL/6j	130	0
39	M	F ₁	C57BL/6j	60	1
40	M	F ₁	C57BL/6j	25	0
41	M	F ₁	C57BL/6j	10	1
42	M	F ₁	C57BL/6j	975	1
43	M	F ₁	C57BL/6j	75	1
44	M	F ₁	C57BL/6j	60	0
45	M	F ₁	C57BL/6j	815	0
91	F	F ₁	C57BL/6j	25	1
92	F	F ₁	C57BL/6j	30	0
93	F	F ₁	C57BL/6j	15	1
94	F	F ₁	C57BL/6j	20	0
95	F	F ₁	C57BL/6j	20	0
96	F	F ₁	C57BL/6j	80	1
97	F	F ₁	C57BL/6j	90	0
98	F	F ₁	C57BL/6j	25	1
99	F	F ₁	C57BL/6j	60	0
100	F	F ₁	C57BL/6j	15	0
101	F	F ₁	C57BL/6j	5	0
102	F	F ₁	C57BL/6j	350	1
103	F	F ₁	C57BL/6j	85	1
104	F	F ₁	C57BL/6j	135	1
105	F	F ₁	C57BL/6j	60	1

Table 2.4 Mean larval burden and number of copies of Itln-2 gene in F₂ mice with female F₁ and male CBA/Ca parental strains

Mouse	Sex	Female parental strain	Male parental strain	Day 7 lung larval burden	Number of copies of Itln-2 gene
46	M	F ₁	CBA/Ca	35	2
47	M	F ₁	CBA/Ca	10	1
48	M	F ₁	CBA/Ca	45	2
49	M	F ₁	CBA/Ca	70	2
50	M	F ₁	CBA/Ca	45	1
51	M	F ₁	CBA/Ca	45	1
52	M	F ₁	CBA/Ca	5	1
53	M	F ₁	CBA/Ca	20	2
54	M	F ₁	CBA/Ca	5	2
55	M	F ₁	CBA/Ca	95	1
56	M	F ₁	CBA/Ca	5	2
57	M	F ₁	CBA/Ca	5	1
58	M	F ₁	CBA/Ca	10	2
59	M	F ₁	CBA/Ca	75	1
60	M	F ₁	CBA/Ca	25	2
106	F	F ₁	CBA/Ca	25	2
107	F	F ₁	CBA/Ca	35	2
108	F	F ₁	CBA/Ca	15	1
109	F	F ₁	CBA/Ca	15	1
110	F	F ₁	CBA/Ca	50	2
111	F	F ₁	CBA/Ca	15	2
112	F	F ₁	CBA/Ca	10	2
113	F	F ₁	CBA/Ca	30	2
114	F	F ₁	CBA/Ca	55	2
115	F	F ₁	CBA/Ca	35	1
116	F	F ₁	CBA/Ca	75	2
117	F	F ₁	CBA/Ca	170	1
118	F	F ₁	CBA/Ca	5	1
119	F	F ₁	CBA/Ca	20	2
120	F	F ₁	CBA/Ca	40	2

Appendix 3. Publications

Genetic influence on the kinetics and associated pathology of the early stage (intestinal-hepatic) migration of *Ascaris suum* in mice

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SUMMARY

The generative mechanism(s) of aggregation and predisposition to *Ascaris lumbricoides* and *A. suum* infections in their host population are currently unknown and difficult to elucidate in humans and pigs for ethical/logistical reasons. A recently developed, optimized murine model based on 2 inbred strains, putatively susceptible (C57BL/6j) and resistant (CBA/Ca) to infection, was exploited to elucidate further the basis of the contrasting parasite burdens, most evident at the pulmonary stage. We explored the kinetics of early infection, focusing on the composite lobes of the liver and lung, over the first 8 days in an effort to achieve a more detailed understanding of the larval dispersal over time and the point at which worm burdens diverge. Larval recoveries showed a heterogeneous distribution among the lobes of the lungs, being higher in the right lung of both strains, and in the susceptible strain larvae accumulating preferentially in 2 (caudal and middle) of the 4 lobes. Total larval burdens in these 2 lobes were largely responsible for the higher worm burdens in the susceptible strain. While total lung larval recoveries significantly differed between mouse strains, a difference in liver larval burdens was not observed. However, an earlier intense inflammatory response coupled with more rapid tissue repair in the hepatic lobes was observed in CBA/Ca mice, in contrast to C57BL/6j mice, and it is possible that these processes are responsible for restricting onward pulmonary larval migration in the resistant genotype.

Key words: *Ascaris suum*, mouse model, C57BL/6j, CBA/Ca, susceptibility, migration, liver, lungs, lobar distribution, inflammatory reaction.

INTRODUCTION

Ascaris lumbricoides and *Ascaris suum* are important widespread parasitic nematodes that cause infections of humans and pigs respectively (O' Lorcain and Holland, 2000; Crompton, 2001). *Ascaris* exhibits an aggregated frequency distribution in its hosts (Croll and Ghadirian, 1981; Boes *et al.* 1998; Holland and Boes, 2002). Furthermore, observations of re-infection patterns post-chemotherapy have indicated that individuals display a degree of predisposition to their worm burden status (Haswell-Elkins *et al.* 1987; Holland *et al.* 1989; Keymer and Pagel, 1990; Boes *et al.* 1998). The generative mechanism(s) of these observed phenomena are currently unknown and are difficult to elucidate in humans and pigs for ethical and logistical reasons. Recent studies have proposed that host genetics play a role in predisposition although precisely which genes are involved and what they control is still

largely unknown. One possibility is that genes influencing immune mechanisms that control the intensity of infection play a key role (Holland *et al.* 1989, 1992; Williams-Blangero *et al.* 2008; Nejsun *et al.* 2009), and conceivably together with physiological factors, contribute to the processes that are responsible for predisposition (Mc Sharry *et al.* 1999; Holland and Boes, 2002). Inevitably these processes are likely to be confounded further by the influence of environmental factors such as nutrition and concurrent infections which are known to affect susceptibility to infection (Keymer and Pagel, 1990). Predisposition to *Ascaris* infection has important implications for transmission, morbidity and control, and it is therefore crucially important to identify the mechanistic basis of resistance/susceptibility (Anderson and May, 1985) and their roles in predisposition to infection.

Comparative studies on larval migration have demonstrated that the mouse is a suitable model for investigating the early phase of *A. suum* infection (Murrell *et al.* 1997; Slotved *et al.* 1998). Mitchell *et al.* (1976) reported that mouse strains vary in their susceptibility to *Ascaris* infection and therefore provide a convenient model for investigating the

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genetic basis of variation in migratory kinetics and immune responses to infection. A murine model was recently developed and optimized and 2 inbred strains were identified as putatively susceptible (C57BL/6j) and resistant (CBA/Ca) to infection (Lewis *et al.* 2006). This was reflected in the distinct, repeatable difference in *A. suum* larval burden in the lungs on day 7 post-infection (Lewis *et al.* 2006). The contrast of resistance and susceptibility between CBA and C57BL/6j mouse strains respectively represents the extremes of host phenotype displayed in an aggregated distribution.

The underlying mechanism responsible for the loss of *A. suum* larvae during primary infections and its precise location within the body are currently unknown. Recent studies undertaken by Lewis *et al.* (2006, 2007) revealed that a similar numbers of larvae were detected in the liver of both mouse strains. The majority of the larvae observed in the liver successfully migrated to the lungs in the C57BL/6j, while far fewer larvae accumulated in the lungs of CBA/Ca mice. Subsequent examination of the leukocyte population in bronchoalveolar lavage (BAL) fluid and lung histopathology indicated that whilst there were clear differences in the intensity of the response between strains, the pulmonary inflammatory reaction was unlikely to play a prominent role in explaining the difference between *Ascaris* larval burdens in these mouse strains (Lewis *et al.* 2007). The liver has previously been cited as a key site in the immobilization of migrating *Ascaris* larvae in mice (Sprent and Chen, 1949; Taffs, 1968; Mitchell *et al.* 1976; Johnstone *et al.* 1978; Song *et al.* 1985), guinea pigs (Kerr, 1938; Fallis, 1948) and rabbits (Arean and Crandall, 1962).

The white spot (WS) lesions that form as a result of mechanical injury and inflammatory response induced by migrating *Ascaris* larvae in the liver have been suggested to form along the larval migration routes or encapsulate trapped larvae (Ronéus, 1966; Copeman and Gaafar, 1972; Pérez *et al.* 2001). Therefore WS have been proposed to play a role in immunity to *A. suum* infection in pigs (Copeman and Gaafar, 1972; Eriksen *et al.* 1980). *A. suum* larval debris has been detected within WS (Schwartz and Alicata, 1932; Sprent and Chen, 1949; Copeman and Gaafar, 1972; Pérez *et al.* 2001).

There has been little focus on the larval distribution within the various lobes of the liver and lungs. McCraw and Greenway (1970) recovered *A. suum* larvae from lung lobes in calves but stated that no significant difference between the pulmonary lobes was detected. The larval and adult intestinal burden has been quantified in pigs (Eriksen *et al.* 1992; Murrell *et al.* 1997; Roepstorff *et al.* 1997; Jungersen *et al.* 1999) and in model organisms such as mice and guinea pigs (Jenkins, 1968; Keittivuti, 1974). Furthermore, larvae of *A. suum* have been observed in the wall and lumen of large intestinal segments, the

caecum, colon and rectum in mice (Jenkins, 1968; Douvres and Tromba, 1971; Keittivuti, 1974; Lewis *et al.* 2007) and pigs (Murrell *et al.* 1997).

The main aim of the present study was to determine if the liver plays a significant role in parasite attrition in resistant mice. In order to assess the precise times and locations of larval attrition and accumulation, during the tissue-migratory phase of *Ascaris* infection in C57BL/6j and CBA/Ca mice, the distribution of larval numbers was quantified in the small and large intestines and in individual hepatic and pulmonary lobes. Moreover, the role of hepatic inflammation in explaining variation in susceptibility between the two inbred mouse strains was determined by semi-quantitative scoring of the histopathological changes within the liver.

MATERIALS AND METHODS

Experimental animals

A total of 180 (90 C57BL/6j and 90 CBA/Ca) male inbred mice was purchased from Harlan UK Ltd at 7 weeks of age. They were allowed a week to acclimatize to animal house conditions before infection at 8 weeks of age. Mice were housed in an animal maintenance room in the Bioresources unit, Trinity College Dublin for the duration of the experimental procedure. The room was maintained at approximately 22 °C with a daily 12 h light/dark photoperiodicity. Water and commercial pelleted food were supplied *ad libitum*, and cages were cleaned on a regular basis. The mice were individually weighed on arrival, and randomly assigned, within each strain, to groups of 4 per cage.

Parasite

Approximately 5 000 000 embryonated *Ascaris suum* ova (batch no AP 04.04) were supplied by the Danish Centre for Experimental Parasitology (CEP), Copenhagen. Individual doses were adjusted in order to contain 1000 fully embryonated ova in 0.1 M H₂SO₄ as described previously (Lewis *et al.* 2006). Placebo doses for control mice consisted of 0.9% saline only.

Infection and post-mortem of mice

Upon commencement of infections, mice were randomly assigned as experimental (infected: 72 C57BL/6j and 72 CBA/Ca) and control (not infected: 18 C57BL/6j and 18 CBA/Ca) mice. In order to facilitate larval counts from the intestines, liver and lungs of both mouse strains, 45 experimental mice of each strain were designated for this purpose, while the remainder of the experimental mice (27 of each strain) and all control uninfected mice were assigned for histopathological examination of the

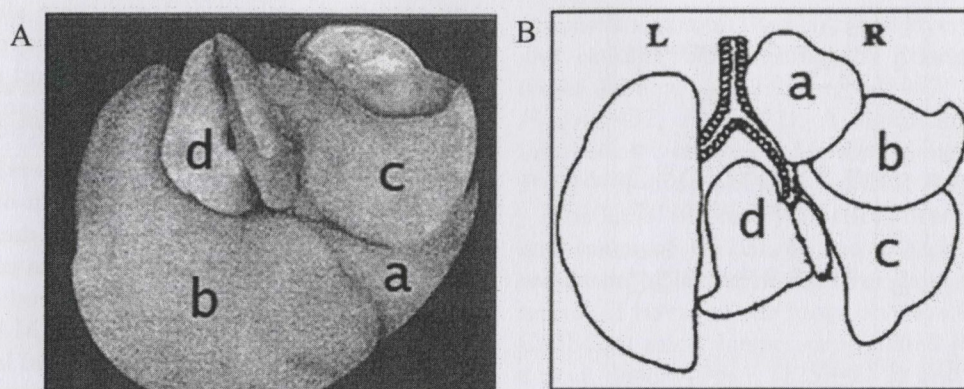


Fig. 1. The composite lobes of the (A) murine liver (a, median lobe; b, left lobe; c, right lobe; d, caudate lobe) and (B) murine left (L) and right (R) lungs (a, cranial lobe; b, middle lobe; c, caudal lobe; d, accessory lobe) (adapted from Maronpot *et al.* 1999).

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liver. Infective *A. suum* ova and placebo doses were administered by gastric intubation to experimental and control mice respectively. Post-mortems were conducted at 6 h and on days 1–8 post-infection (p.i.). At each post-mortem time-point 8 experimental mice (5 for larval counts and 3 for histopathological examination) and 2 control mice of each strain were euthanized by cervical dislocation. Prior to euthanasia, mice were individually weighed.

Larval counts

Five mice of each strain were euthanized at each post-mortem time-point and viable larvae were enumerated in the large and small intestines, liver and lungs. The small intestine was separated into 7 cm segments (1–7 cm, 8–14 cm, 15–21 cm and the remainder, 22–x cm) from the pylorus of the stomach. The large intestine was also divided into the caecum and colon. The liver was separated into its multiple lobes, the median, caudate, left and right lobes. Similarly, the right lung was separated into its composite lobes, the cranial, middle, caudal and accessory lobes. The left lung cannot be further separated (Fig. 1). All lobes and intestinal segments were evaluated as separate entities.

Living larvae were recovered from each lobe and segment by means of the modified Baermann technique (Lewis *et al.* 2006). A pellet of the isolated viable larvae was suspended in a 5 ml solution of 0.9% saline and 6% formalin. Prior to larval counts the 5 ml solutions were agitated to ensure a homogeneous distribution of larvae within the sample. Larval counts were conducted on 1 ml of each sample. In order to enumerate the live larvae in the liver, large and small intestinal samples, twenty 50 μ l, ten 100 μ l and five 200 μ l aliquots respectively were screened under $\times 40$ magnification. When recording the larval burden in the lung samples, 2 ml were pipetted into the chamber of a nematode counting

slide (Chalex Corporation). The number of larvae present in the grid area, which represents 1 ml, was counted under $\times 40$ magnification. The number of larvae in a 1 ml solution was multiplied by total volume in order to estimate the number of larvae in the tissue sample.

Histopathological examination

Three experimental and 2 control mice of each strain were euthanized at each post-mortem time-point. The liver was separated into individual lobes as outlined above, fixed in 10% buffered formalin for 24 h. Following paraffin embedding, 5 μ m sections of each lobe were cut, stained with haematoxylin and eosin, and examined. Hepatic injury and inflammation was scored semi-quantitatively ranging from +/– (no inflammation) and + (very mild inflammation) to ++++ (severe inflammation). Examining sections of each hepatic lobe from each animal reduced the margin of error in scoring that could have resulted from a localized response. Histopathological examination was performed without prior knowledge of sample identity.

To observe changes in the hepatic inflammatory reaction an initial histopathological section from each hepatic lobe from each animal sampled at 6 h and days 1–8 p.i. was examined and graded. A further 3 histopathological sections were taken at a fixed distance of 100 μ m apart from each lobe from each animal euthanised on days 2–5 p.i. The scores allotted to the 3 further serial histopathological sections examined were subjected to statistical analysis.

Statistical analysis

All statistical analysis was carried out at the 95% confidence limit. Larval recovery data were assessed for normality and were subsequently log transformed. Intestinal larval numbers were not subjected

Table 1. Mean larval numbers ($\pm$ S.E.M.) recovered at 6 h and 1–8 days p.i. in the large and small intestines in C57BL/6j and CBA/Ca mice, following inoculation with 1000 *Ascaris suum* ova

Day p.i.	Large intestine					Small intestine										Total	
	Caecum		Colon		Total	1-7 cm		8-14 cm		15-21 cm		22-x cm					
	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca		C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca		
6 h	102 ± 24.0	180 ± 45.3	7 ± 4.4	8 ± 4.9	109 ± 24.4	188 ± 45.3	0	2 ± 2.0	0	0	0	1 ± 1.0	2 ± 1.2	0	3 ± 1.2	3 ± 2.0	
1	1 ± 1.0	0		2 ± 1.2	1 ± 1.0	2 ± 1.2	0	0	0	1 ± 1.0	0	0	0	0	0	1 ± 1.0	
2	1 ± 1.0	0	3 ± 1.2	1 ± 1.0	4 ± 1.9	1 ± 1.0	0	0	0	0	0	0	0	0	0	0	
3	0	0	1 ± 1.0	1 ± 1.0	1 ± 1.0	1 ± 1.0	0	0	1 ± 1.0	0	0	0	0	0	1 ± 1.0	0	
4	0	0	0	2 ± 1.2	0	2 ± 1.2	0	0	0	0	0	0	0	0	0	0	
5	0	2 ± 1.2	1 ± 1.0	1 ± 1.0	1 ± 1.0	3 ± 1.2	1 ± 1.0	0	0	0	0	0	2 ± 1.2	0	3 ± 2.0	0	
6	2 ± 1.2	0	1 ± 1.0	3 ± 2.0	3 ± 1.2	3 ± 2.0	0	0	1 ± 1.0	0	0	0	1 ± 1.0	0	4 ± 1.9	0	
7	0	0	0	0	0	0	1 ± 1.0	0	0	0	0	0	0	0	6 ± 4.9	7 ± 5.8	
8	4 ± 2.9	1 ± 1.0	2 ± 1.2	0	6 ± 2.9	1 ± 1.0	0	0	1 ± 1.0	0	1 ± 1.0	1 ± 1.0	1 ± 1.0	0	3 ± 3.0	5 ± 2.7	

to statistical analysis as larvae were almost exclusively recovered on a single post-mortem time-point. The influence of the factors, time and strain on the mean larval recoveries from the liver and lungs were investigated using a 2-way GLM (SPSS 15.0). To investigate the influence of the factors, time, strain and the distribution of larvae within organs on the mean larval recoveries in the lobes of the liver and the lungs, a MANOVA (SPSS 15.0) was conducted. In order to gauge whether a larval predilection for a specific lobe in an organ exists, a MANOVA was undertaken on the proportion of total larvae in each mouse recovered from each hepatic and pulmonary lobe. Proportional data were arcsine, square root transformed prior to analysis. Analysis of the semi-quantitative pathological scores (converted into a scale ranging from zero (+/-), to 4 (++++) in the liver was carried out using a mixed-GLM model, with time and strain as fixed factors, mouse as a random factor nested within the 2-way interaction of time*strain and liver lobe as a fixed effect. The model was therefore time+strain+time*strain+mouse (time*strain)+liver lobe, with mouse as a random factor. We also explored models involving interactions between liver lobe and time and strain, but none of these proved significant. Appropriate statistics are reported in the text.

RESULTS

Ascaris suum migration in the intestines, liver and lungs of C57BL/6j and CBA/Ca mice at 6 h and 1 to 8 days p.i.

Larvae were detected at 6 h and on days 1–8 p.i. in the intestines, liver and lungs of both mouse strains, but larval burdens varied over time and differed between mouse strains.

The highest presence of larvae in the intestines of both mouse strains was recorded at 6 h p.i. At this time-point, the majority of larvae was recovered from the large intestine and had a strong predilection for the caecum as can be seen in Table 1. Mean larval burdens were higher in the resistant CBA mice. The mean larval numbers in the large intestine then decreased by day 1 p.i. in both strains and no consistent pattern distinguishing the two strains was evident (Table 1).

Ascaris suum larvae were recovered from the liver at every post-mortem time-point in each mouse strain. Appreciable numbers were recovered in this organ at 6 h and marked decreases were observed on days 4 and 5 p.i. in C57BL/6j and CBA/Ca mice respectively. A 2-way GLM demonstrated that the total numbers of larvae observed in the liver did not differ significantly between the two strains (main effect of strain, $F_{1,71}=3.060$, $P=0.085$). However, recoveries of larvae differed significantly over post-mortem time-points (main effect of time, $F_{8,71}=23.785$,

Table 2. Mean larval numbers ($\pm$ S.E.M) recovered at 6 h and 1–8 days p.i. in the liver of C57BL/6j and CBA/Ca mice, following inoculation with 1000 *Ascaris suum* ova

Day p.i.	C57BL/6j	CBA/Ca
6 h	32 $\pm$ 5.1	63 $\pm$ 14.2
1	98 $\pm$ 15.6	88 $\pm$ 18.5
2	161 $\pm$ 21.9	67 $\pm$ 11.9
3	97.5 $\pm$ 24.4	70 $\pm$ 18.8
4	98 $\pm$ 20.0	57 $\pm$ 10.7
5	51 $\pm$ 17.8	89 $\pm$ 16.3
6	48 $\pm$ 8.7	31 $\pm$ 7.0
7	11 $\pm$ 2.9	3 $\pm$ 2.0
8	14 $\pm$ 5.3	17 $\pm$ 3.7

$P<0.001$). A significant strain*time interaction was found ($F_{8,71}=2.745$, $P=0.011$) as the larvae recovered from each strain displayed different migratory patterns through this organ (Table 2).

Larvae were recovered in each liver lobe as early as 6 h p.i. In C57BL/6j the mean number of larvae peaked on days 1–3 p.i. but was dependent on the hepatic lobe and subsequently declined to comparatively low larval numbers on days 7 and 8 p.i. (Fig. 2). As in C57BL/6j mice, low larval numbers were observed on the later days of infection in the CBA/Ca liver lobes (Fig. 2).

The results from a 2-way MANOVA on the proportion of total larvae recovered in the 4 hepatic lobes as opposed to the absolute mean numbers revealed that a significant strain effect was detected in the median ($F_{1,71}=5.519$, $P=0.022$) and right ($F_{1,71}=4.238$, $P=0.043$) lobes. The proportion of total liver larvae recovered in the median lobe of C57BL/6j mice increased to a peak on day 2 p.i. (0.26 ± 0.07) and then fluctuated until days 7 and 8 p.i. at which point no larvae were recovered in this lobe. In CBA/Ca mice the proportion recovered in the median lobe fluctuated consistently with the lowest proportion recovered on day 4 p.i. (0.04 ± 0.03) and the highest on day 3 p.i. (0.42 ± 0.10). With the exception of days 2 and 4 p.i. a higher proportion of larvae was recorded in the median lobe of CBA/Ca mice. In the right lobe, the proportion of total liver larvae observed in C57BL/6j mice fluctuated consistently, peaking on day 8 p.i. (0.61 ± 0.42). However in CBA/Ca mice the proportion of total larvae in the liver recovered in the right lobe increased to a peak on day 4 p.i. (0.46 ± 0.10) and then decreased steadily.

The strain effect was approaching significance in the caudate lobe ($F_{1,71}=3.732$, $P=0.057$), in which the proportion of total liver larvae was seen to fluctuate slightly in both mouse strains. However, a noteworthy decrease in the proportion of larvae recovered in the caudate lobe in CBA/Ca mice was observed on day 7 p.i. (0.00) followed by a considerable increase on day 8 p.i. (0.43 ± 0.17). The

proportion of total liver larvae recovered in the right and caudate lobe significantly changed over time (right lobe: $F_{8,71}=2.950$, $P=0.007$, caudate lobe: $F_{8,71}=2.437$, $P=0.022$). A significant time*strain interaction was observed in the median ($F_{8,71}=2.091$, $P=0.048$) and right ($F_{8,71}=3.085$, $P=0.005$) lobes.

The larval migratory kinetics observed in the liver and lungs of C57BL/6j and CBA/Ca indicated a reduction of larvae in the liver coinciding with the arrival of larvae in the lungs on days 4 and 5 p.i. In C57BL/6j mice, larvae accumulated in the lungs to a peak on day 7 p.i. (125 ± 21.7) and subsequently fell by day 8 p.i. (50 ± 21.5). Larvae consistently increased to a maximum on day 8 p.i. (31 ± 7.5) in CBA/Ca mice. A 2-way GLM conducted on larval numbers in the lungs on days 5 to 8 p.i. indicated a highly significant effect of strain ($F_{1,32}=16.164$, $P<0.001$) and time ($F_{3,32}=9.025$, $P<0.001$). No strain*time interaction was detected ($F_{3,32}=2.507$, $P=0.077$) as the pattern of larval migration in this organ was relatively similar in both strains.

Appreciable numbers of larvae entered the left and right lungs on day 5 p.i. In C57BL/6j mice larvae in each lung increased to a peak on day 7 p.i. and subsequently declined on day 8 p.i. Larvae recovered in the left lung of CBA/Ca mice increased to a maximum on day 8 p.i. A similar burden was recovered on days 7 and 8 p.i. in the right lung of CBA/Ca mice (day 7 p.i.: 23 ± 4.9 , day 8 p.i.: 22 ± 6.0) (Fig. 3).

A 2-way MANOVA conducted on proportional larval burdens on days 5–8 p.i. in the left and right lungs indicated that a strain effect was not detected in either the left ($F_{1,32}=0.198$, $P=0.659$) or right ($F_{1,32}=2.124$, $P=0.155$) lungs. The proportion of larvae in the lungs, which were recovered in the left and right lungs varied over time (left lung: $F_{3,32}=4.193$, $P=0.013$, right lung: $F_{3,32}=5.994$, $P=0.002$). On some post-mortem time-points, the proportion of larvae recovered in the right lung differed significantly between C57BL/6j and CBA/Ca mice ($F_{3,32}=5.060$, $P=0.006$).

The mean larval burden recovered on days 5–8 p.i. in the right lung lobes is illustrated in Fig. 4. In C57BL/6j larvae increased to a peak on day 7 p.i. in each lobe, which was also observed in the middle lobe in CBA/Ca mice. However, in the cranial, caudal and accessory lobe of CBA/Ca mice, larvae increased to a maximum on day 8 p.i.

A 2-way MANOVA conducted on the proportional data confirmed the effects of strain ($F_{4,29}=3.576$, $P=0.014$) and time ($F_{12,77}=2.565$, $P=0.007$) on lobar distribution in the right lung. A strain*time interaction was observed ($F_{12,77}=3.285$, $P=0.001$) as the percentage of larvae in the right lung recovered in the 4 lobes varied between strains at some post-mortem time-points. From the 4 right lung lobes, only the larval burden of the middle lobe changed over time ($F_{3,32}=8.546$, $P<0.001$). The migratory pattern of larval burdens differed significantly

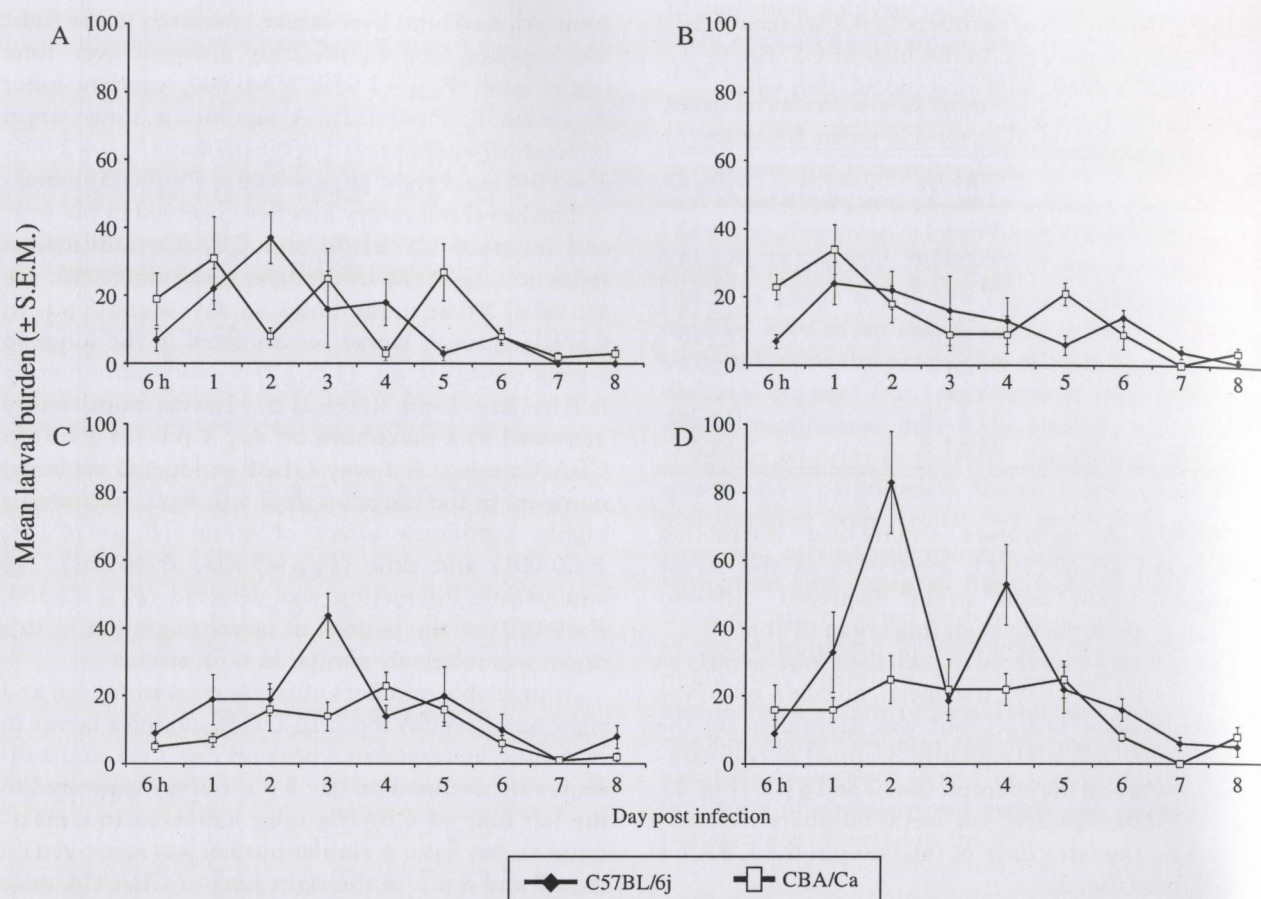


Fig. 2. Changes in total mean larval burden in the hepatic (A) median, (B) left, (C) right and (D) caudate lobes of C57BL/6j and CBA/Ca, following inoculation with 1000 *Ascaris suum* ova.

between the two mouse strains in the cranial ($F_{3,32} = 3.046$, $P = 0.043$) and middle ($F_{3,32} = 3.410$, $P = 0.029$) lobes. The proportional larval burden recovered in the caudal lobe was highly significantly different between C57BL/6j and CBA/Ca mice ($F_{1,32} = 15.331$, $P < 0.001$). The proportion of larvae in this lobe was consistently higher in C57BL/6j and the main sites for recovery in this mouse strain were the middle and caudal lobe. The disparity was most pronounced on day 6 p.i., when larvae were absent from the caudal lobe of CBA/Ca mice, and day 7 p.i., at which point 42.5% and 17.4% of the larvae were recovered from the caudal lobes of C57BL/6j and CBA/Ca mice respectively.

Histopathological examination of the liver

None of the uninfected control animals showed histopathological changes in the liver (Fig. 5A). Focal accumulations of granulocytes and macrophages were observed in infected animals with a predominant peri-portal and centrilobular distribution in each lobe but were also detected within the intervening hepatic lobular parenchyma (Fig. 5I). Necrotic tracts were widely distributed throughout the tissue with a particular association with portal and perilobular blood vessels (Fig. 5F). Longitudinal and transverse

sections of *A. suum* larvae were frequently identified within blood vessels and sinusoids (Fig. 5F–H).

Semi-quantitative grading of sections from liver lobes (Fig. 5B–E) revealed a contrast in timing of the inflammatory reaction in C57BL/6j and CBA/Ca mice (Fig. 6A). A low inflammatory reaction score was recorded in both mouse strains at the initial stages of infection but was relatively greater in the CBA/Ca mice (mixed model as explained in the Materials and Methods section, main effect of strain $F_{1,16} = 5.0$, $P = 0.04$). Although C57BL/6j mice had a higher percentage of lobes with mild inflammation (grade +) than CBA/Ca mice, the C57BL/6j animals had extensive necrotic tracts radiating from blood vessels. Comparatively severe inflammation was observed in CBA/Ca mice on day 2 p.i., which became more pronounced on day 3 p.i. On day 4 p.i., more severe inflammation (grade ++++) was observed in more hepatic lobes of CBA/Ca animals. A similar response in the hepatic lobes in C57BL/6j mice was not detected until day 6 p.i. Comparatively less severe inflammation was detected on days 7 and 8 p.i. in CBA/Ca mice (Fig. 6A).

The mean semi-quantitative scores allotted to the 3 serial histopathological sections of each hepatic lobe illustrated a similar response in both mouse strains. In C57BL/6j mice the inflammatory reaction

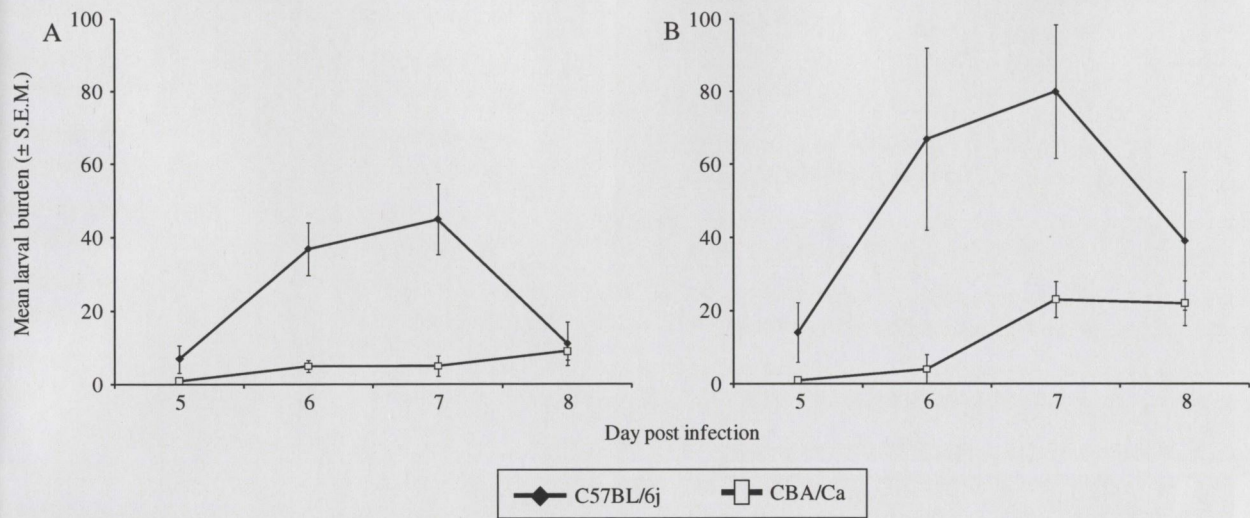


Fig. 3. Changes in total mean larval burden in the left (A) and right (B) lungs of C57BL/6j and CBA/Ca mice, following inoculation with 1000 *Ascaris suum* ova.

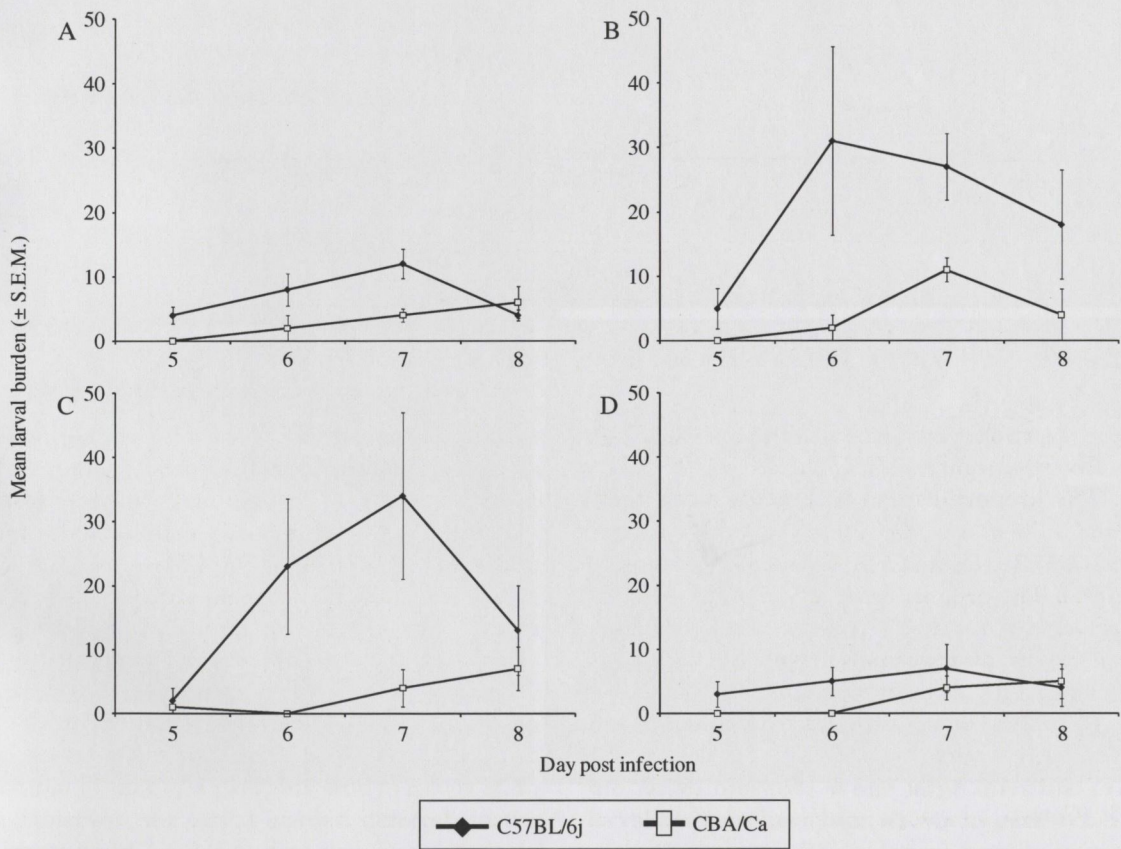


Fig. 4. Changes in total mean larval burden in the right lung (A) cranial, (B) middle, (C) caudal and (D) accessory lobes of C57BL/6j and CBA/Ca mice, following inoculation with 1000 *Ascaris suum* ova.

increased steadily over days 2–5 p.i in the left, right and caudate lobes. A similar inflammatory reaction was observed in the median lobe on days 3–5 p.i. In CBA/Ca mice the strong inflammatory reaction on day 4 p.i. and subsequent recovery on day 5 p.i. was observed in each hepatic lobe (Fig 6B). These changes over time were significant (mixed model, main effect of time $F_{3,16}=8.8$, $P=0.001$), and both strains showed a similar pattern of changes,

if not intensity, over time (2-way interaction time* strain, $F_{3,16}=2$, $P=NS$). There was no significant difference between liver lobes (main effect of liver lobe $F_{3,257}=1.9$, $P=NS$) but with time, strain, the interaction between time and strain, and the main effect of liver lobe taken into account, there was a highly significant component of variation attributed to between-mouse variation in scores (mouse (time* strain), $F_{16,257}=10.4$, $P<0.001$).

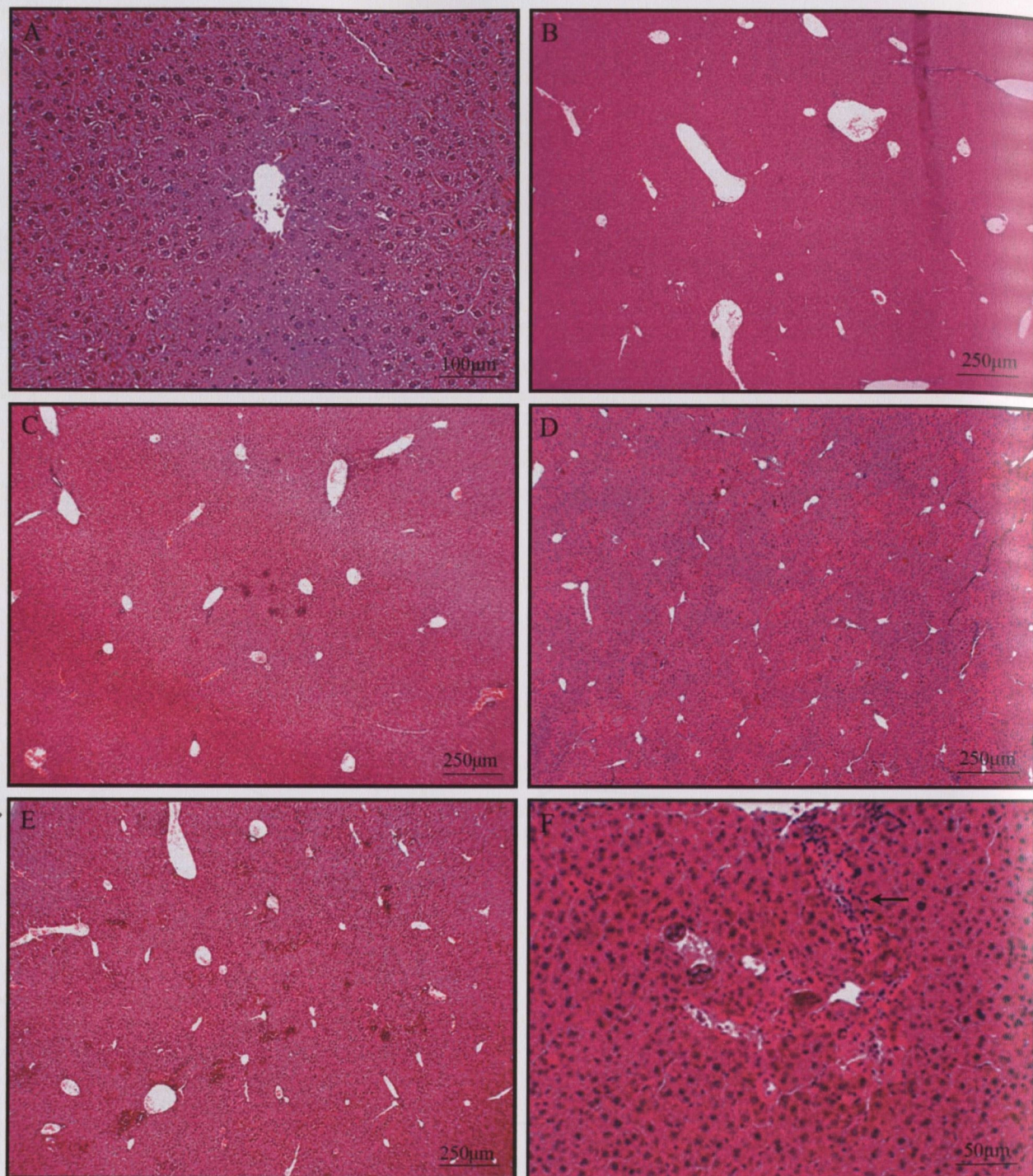


Fig. 5. (Cont.)

DISCUSSION

The results of the current study confirm and markedly extend earlier work indicating that migration of *A. suum* larvae is impeded in the liver or in between the liver and lungs in CBA/Ca mice. During migration in this study larvae were found to preferentially accumulate in particular lobes of critical organs in susceptible mice, thereby contributing to the difference in total larval burdens between strains of mice. Analysis of larval burdens in the organs

through which the parasite migrates, particularly the significant disparity in lung larval numbers between strains and histopathological examination of the liver revealed a significant differential response, adding weight to the idea that the liver of resistant CBA/Ca mice plays an important role in the impediment of onward larval migration.

The importance of the large intestine in the early stages of the tissue migratory phase in *A. suum* infection in mice has been highlighted previously (Keittivuti, 1974; Slotved *et al.* 1998; Lewis *et al.*

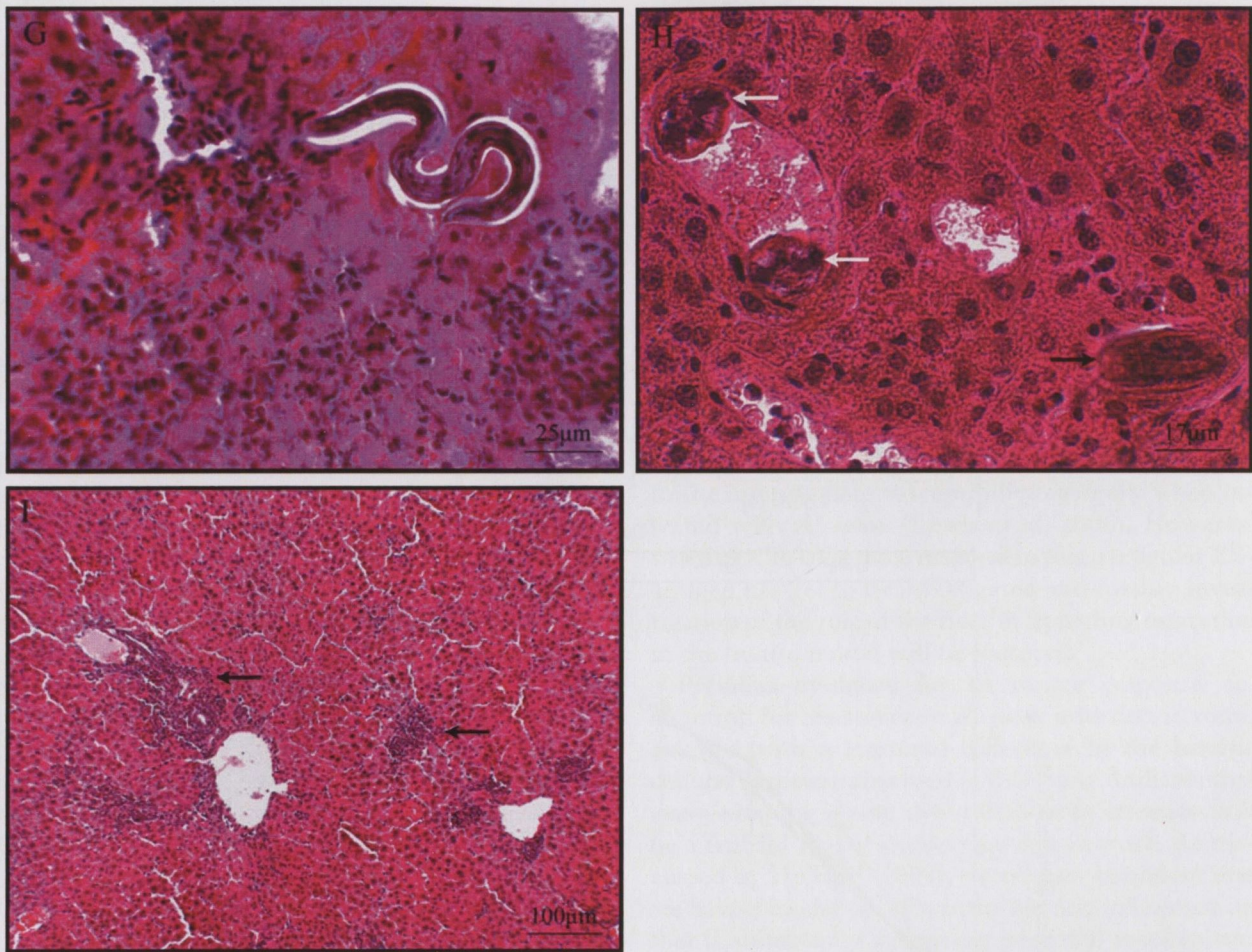


Fig. 5. Photomicrographs of haematoxylin and eosin-stained liver sections. (A) Control uninfected liver lobe (grade +/–), (B) mild inflammatory reaction (grade +), (C) moderate inflammatory reaction (grade ++), (D) strong inflammatory reaction (grade +++), (E) severe inflammatory reaction (++++), (F) Larvae with associated necrotic larval tracts (indicated by black arrow), (G) Longitudinal larva in hepatic parenchyma and nearby granulocytes, (H) Transverse (within blood vessel) (indicated by white arrows) and longitudinal (indicated by black arrow) (parenchymal) sections of larvae, (I) Focal accumulations of granulocytes in hepatic tissue (indicated by black arrows).

2007). In the current study, the number of larvae in the intestines was monitored over an 8-day period in susceptible and resistant mouse strains yet a high number of larvae was only recovered in the intestines at the 6-h p.i. time-point and was predominantly in the large intestine. The observed burden in C57BL/6j mice at this time in the large intestines was comparable to that of a previous study (Lewis *et al.* 2007). However, the larval burden detected in the large intestine of CBA/Ca mice was comparatively higher than in the C57BL/6j mice and also relative to the previously recorded larval burden in the large intestine of this strain at 6 h p.i. (Lewis *et al.* 2007). The observed high caecal numbers at the 6-h p.i. time-point was documented in mice by Keittivuti (1974) and Jenkins (1968), and in both of these studies a predilection for the caecal wall was noted. Despite higher numbers in the large intestine of CBA/Ca mice at 6 h p.i., equivalent numbers in the liver suggest that a similar number of larvae successfully migrate from the large intestine to the liver in both mouse strains. This finding is consistent with

earlier studies on our mouse model (Lewis *et al.* 2007) and in immunized and control guinea pigs (Soulsby, 1957). Timing, therefore, may account for the strain difference in the early intestinal burden as suggested by Slotved *et al.* (1998). These authors noted that individual mice of the same strain show variation in the rate at which larvae passed through the large intestine. While large intestinal *A. suum* larval burdens have been shown to peak at 6 h p.i. in mice (Keittivuti, 1974), larvae have been recorded in this tissue as early as 2 h p.i. (Keittivuti, 1974; Slotved *et al.* 1998). Furthermore, in the current study, larvae were recovered at 6 h p.i. in liver, indicating earlier hatching in the intestines. A more comprehensive examination of larval numbers in the large intestine at hourly intervals p.i. would indicate whether there is a temporal difference in hatching and larval penetration of the large intestinal wall. Nevertheless, if a temporal difference exists between the two strains, it is confined to a short period of time. This is reflected in the inconsistency displayed in the intestinal larval burdens on days

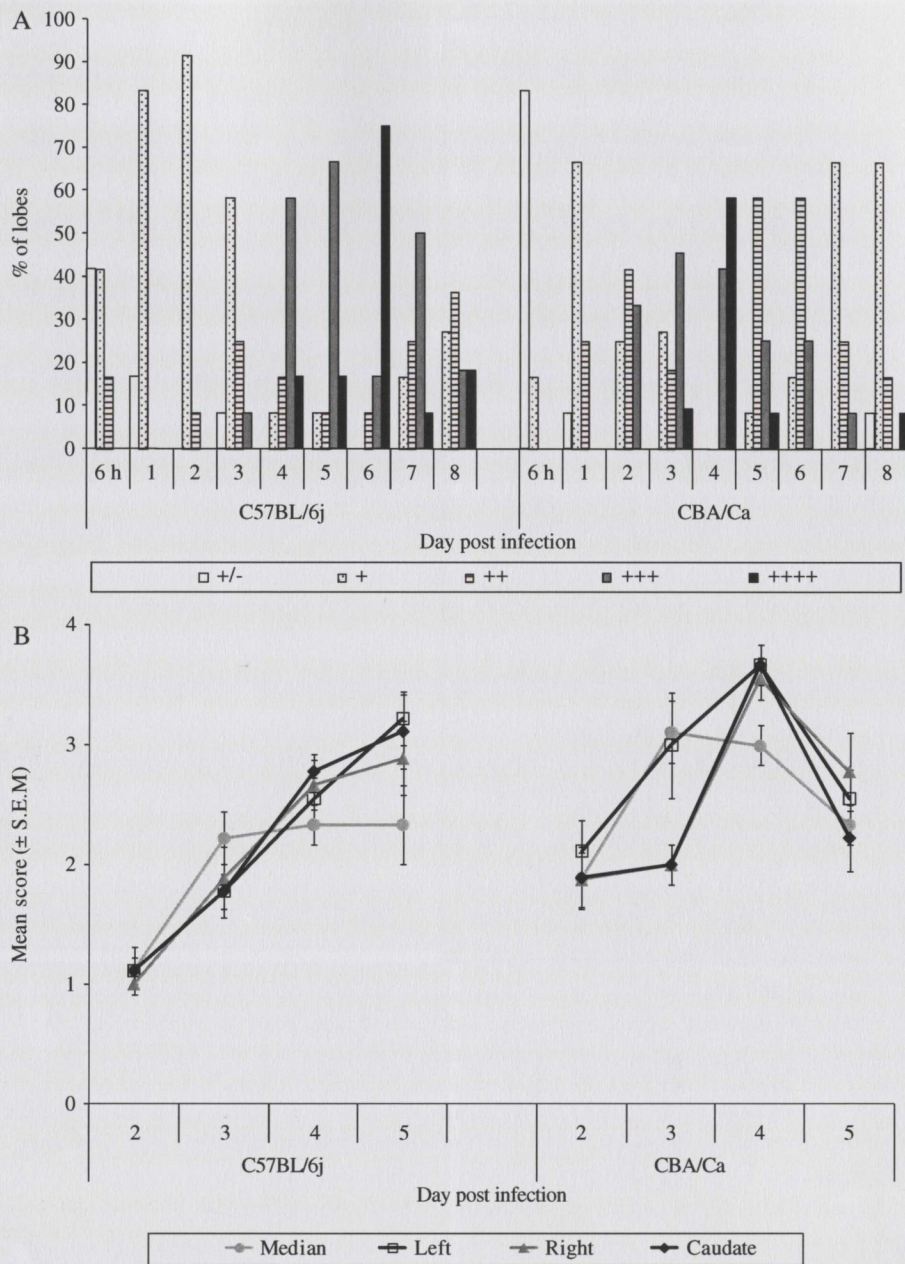


Fig. 6. Histopathological semi-quantitative scoring of inflammation in the hepatic lobes. (A) Changes in the percentage of hepatic lobes allotted each score based on examination of a single tissue section (B) Changes in the mean score allotted to each hepatic lobe based on examination of 3 serial tissue sections.

1–8 p.i. observed in our mouse model and up to 10 days p.i. in a previous study where the strain of mouse used was not specified (Keittivuti, 1974).

Ascaris suum larvae have been speculated to migrate within the liver of their host, for a period of days, in an attempt to find a suitable blood vessel for onward migration to the lungs. This was reflected in the erratic pattern of larval burden in each hepatic lobe, particularly the right and caudate lobes, in both strains. The pattern of migration within the liver was shown to differ between the two mouse strains in the median and right lobes. Furthermore, a difference in the proportion of total liver larvae was observed in the median and right lobes and was shown to be approaching significance in the caudate lobe. In an

anatomical study of the mouse's liver, the caudate lobe was found to be the smallest, accounting for $8.1 \pm 1.0\%$ of the total liver in Balb/c mice, while the left lobe was shown to be the largest ($34.4 \pm 2.0\%$) (Inderbitzin *et al.* 2006). However, while no anatomical studies have been undertaken on the lobes of C57BL/6j and CBA/Ca mice, the large size of the left lobe does not explain the lack of disparity in the larval burdens in this lobe between the two mouse strains. The higher vascular density of the caudate lobe, observed in Balb/c mice (Inderbitzin *et al.* 2006) may vary between the two mouse strains exploited in this study, accounting for the increased larval accumulation in C57BL/6j mice. However, since Balb/c mice were found to be in the intermediate susceptibility

category in previous *A. suum* experiments (Lewis *et al.* 2006), this explanation seems unlikely.

It is currently not possible to determine if the difference in larval recovery from the hepatic caudate lobe is due to anatomical differences between the two mouse strains. Furthermore, the increased successful migration of *A. suum* larvae in C57BL/6j mice from the liver to the lungs may be explained by a hepatic or post-hepatic anatomical difference between the two strains. Strain-specific imaging of the liver and lungs may indicate barriers to dispersal within organs or between organs.

The histopathological changes induced by the larvae did not differ qualitatively between the two mouse strains. Foci of necrosis and attendant inflammation were observed in both and the inflammatory response was comparable to that previously described (Bindseil, 1981). Heavy infiltration of granulocytes and macrophages as well as necrosis was observed in both strains but the kinetics of these hepatic histopathological changes differed between the susceptible and resistant hosts. The earlier severe hepatic inflammatory reaction on day 4 p.i. in the resistant CBA/Ca mice coincided with the low numbers of larvae successfully migrating from the liver to the lungs whereas the more delayed severe inflammatory reaction on day 6 p.i. in the susceptible C57BL/6j mice occurred when the majority of the larvae had already migrated to the lungs. In the CBA/Ca mice, the resolution of the necrosis and inflammation was more rapid following the reduction of larval numbers.

Therefore, it appears that CBA/Ca mice handled the parasitic insult more effectively and economically in terms of responding earlier to restrict the same degree of larval invasion, and effecting more rapid tissue recovery without triggering additional inflammation. Day 4 p.i. has played a prominent role in earlier work on *Ascaris* infections. Larvae were encapsulated on day 4 p.i. onwards by inflammatory cells in guinea pigs immunized against *A. lumbricoides* (Soulsby, 1957) with consequent reduced larval numbers noted in the lungs (Soulsby, 1957; Khoury *et al.* 1977). Furthermore, neither little inhibition in *A. suum*-infected host growth nor a rise of eosinophilia was shown to be evident before this time in guinea pigs (Fallis, 1948; Soulsby, 1957). Therefore, day 4 p.i. has previously been considered a key time-point in effecting protection against *Ascaris* infection.

While it has been proposed that the day 4 p.i. inflammatory reaction in the liver of guinea pigs is induced by a larval moult in this organ (Taffs, 1968), L3 larvae have been observed in *Ascaris* ova (Geenen *et al.* 1999) and ecdysis has been shown not to occur in the liver (Fagerholm *et al.* 2000). Excretory-secretory products capable of inducing a response are continuously produced by the metabolic activity of larvae during migration (Guerrero and Silverman,

1969; Stromberg, 1979) and have been shown to differ depending on their developmental stage (Kennedy and Qureshi, 1986). The ES-antigens are considered targets for responses during larval migration and the temporal difference observed in hepatic inflammatory response in the current study may be due to restricted responses in the susceptible C57BL/6j to particular ES-antigens secreted early in hepatic tissue invasion. The *in vitro* response of previously infected NIH mice to *A. suum* ES-antigens has been observed (Kennedy and Qureshi, 1986) and a lack of responsiveness to the 14000 MW component of both L2 and L3/L4 ES was noted. Interestingly, the NIH strain has been assigned since to the intermediate susceptibility category when infected with *A. suum* (Lewis *et al.* 2006). However, whether CBA/Ca mice respond to this particular ES-antigen has yet to be investigated and further investigation of the role of the liver in impeding migration in the mouse model will be required.

Previous evidence for an innate response accounting for resistance to *A. suum* infection in mice, coupled with a temporal difference in the hepatic cellular response observed in this study, indicate that examining the innate liver response to infection will be a fruitful line of enquiry for future work. As discussed by Holland (2009), we are now confident that we have a model of differential hepatic inflammation that is suitable for examining potential mechanisms of resistance and susceptibility in the liver. Future work will mainly focus on sentinel cells of the hepatic innate immune system and levels of cytokines and chemokines produced during infection, which may explain the temporal difference between strains in the hepatic inflammatory response observed.

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