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The intestinal immune response against the porcine nematode *Ascaris suum*

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Door

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“Research is the act of going up alleys to see if they are blind”

-Plutarch

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

DALY	Disability Adjusted Life Years
DPI	Days post infection
DPT	Days post transfer
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram faeces
EPO	Eosinophil peroxidase
ES	Excretory-secretory material
IELs	Intra-epithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
L3	Third stage larvae
L4	Fourth stage larvae
L5	Fifth stage larvae
MTT	3-(4,5 dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide
NO	Nitric oxide
PMA	Phorbol 12-myristate 13-acetate
RELM-ß	Resistin Like Molecule beta
RNA	Ribonucleic acid
SIN-1	3-Morpholinosydnonimine hydrochloride
STAT	Signal transducer and activator of transcription
Th	T helper
WHO	World Health Organization

Chapter 1:
Review on Ascariosis and helminth immunity

To this day, most animals and people have hosted at least one helminth at some point. It is amazing how these parasites with their often very fascinating life cycles are able to adapt so well to their hosts and their environments. Once these parasites have established themselves they can even survive for several years within their host. *Ascaris suum* and *A. lumbricoides* are some of the most remarkable helminth species that infect pigs and people, respectively. They are both the largest and the most common nematode in their host and reside most of their life in the small intestine. *A. suum* causes serious economic losses in the most important meat-producing livestock species worldwide and it is estimated that close to 1 billion people are currently infected with *A. lumbricoides*. However, despite having anthelmintics that are 100% effective against *Ascaris* worms, it remains difficult to keep this nematode under control. Due to high reinfection rates, lack of adequate sanitary standards and lack of effective vaccines, eradication of *A. suum* or *A. lumbricoides* is not for the foreseeable future. A better understanding of the protective immunity to these helminths should help in the development of new strategies of control. Research into *A. suum* in pigs offers the benefit of an investigation of the immune response against all life stages. However, not much focus has been put on elucidating the protective immune response. This thesis aims to improve our knowledge of the protective immune response in the hope of providing a basis for immunological control strategies.

This chapter provides a general overview of ascariosis in pigs and humans and concentrates on the effective immune response against this parasite. We have a very basic understanding of the dynamics of infection, but the role of the anthelmintic immune response remains elusive. In light of this, the immune responses against other helminth species is reviewed. Finally, some important consequences of the anthelmintic immune response, more specifically their link with allergies and the use of helminth infections as anti-inflammatory therapies are highlighted.

1.1 Ascariosis

1.1.1 Life cycle of *Ascaris suum*

The lifecycle of *Ascaris suum* is relatively simple since no intermediate hosts are required to complete the lifecycle, see Figure 1.1. Pigs transmit the infection by passing *Ascaris* eggs with the faeces. These eggs develop in the soil to fully embryonated eggs within 4–6 weeks at temperatures between 18 and 20 °C [1,2]. Ingestion of fully embryonated eggs through contaminated food or soil will trigger the eggs to hatch and release the L3 inside. The larvae penetrate the intestine at the caecum or proximal colon and are carried to the liver. They migrate from the liver and carried by the blood stream they will reach the lungs around 5-7 days post infection (DPI). The L3 will penetrate the alveoli, get coughed up and swallowed back in around 10 DPI. Shortly after their journey to the small intestine is concluded, they molt to L4. There is another molt to L5 around 28 DPI and ultimately they will grow into sexually mature adults of 20-40 cm in the small intestine. As soon as 50 days after infection, inseminated females will lay thousands of eggs, completing the life cycle.

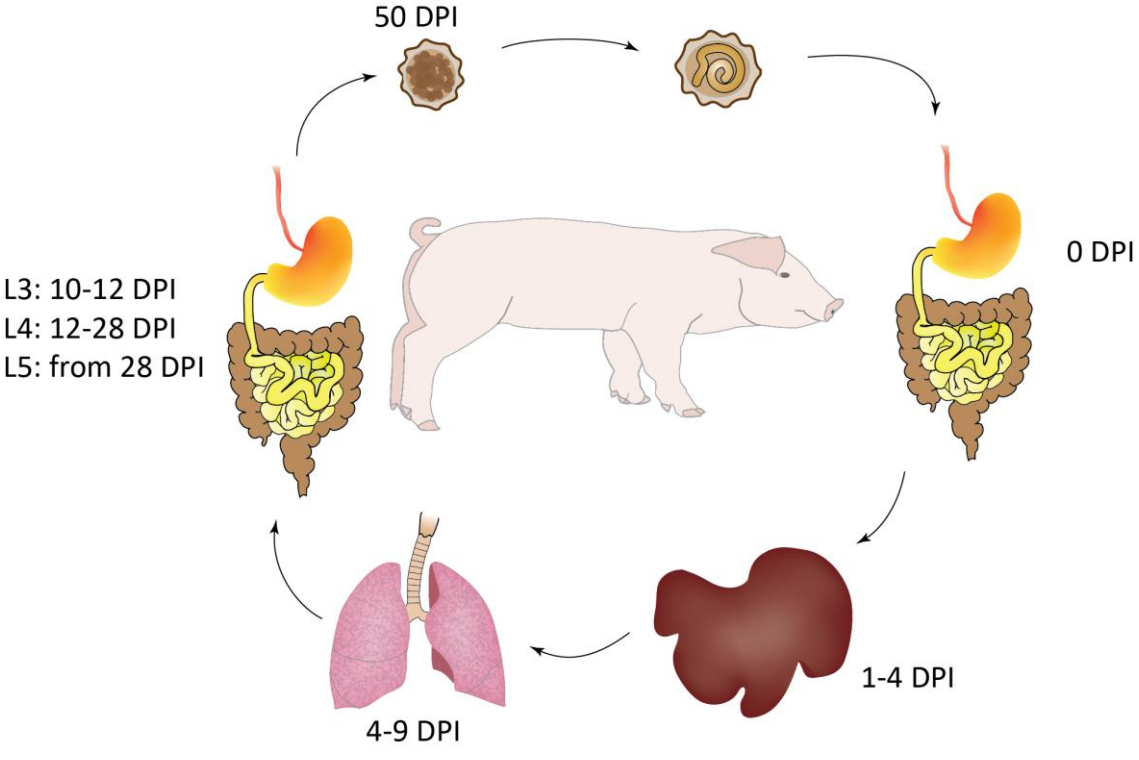


Figure 1.1: The life cycle of *A. suum*. Embryonated eggs, ingested by the host, will release the infective L3 in the small intestine. These larvae will penetrate the caecum or colon and through the portal vein they will reach the liver between 1-4 days. From the liver they will go to the lungs, around 7DPI, and after penetrating the alveoli they will be coughed up and swallowed back in. The L3 will molt to an L4 in the small intestine and eventually become adult. After sexual reproduction, the females will release eggs in the environment with the faeces. In the environment, the eggs will embryonate over the course of a few weeks and become infective.

1.1.2 Clinical and economic importance of *A. suum*

A. suum infections in pigs are highly prevalent and show a global distribution. In Belgium it is estimated that around 50% of pig farms is affected by *A. suum* [3,4]. It appears that *Ascaris* worms have developed very effective transmission mechanisms that resulted in their high prevalence. There is no need for intermediate hosts and adult worms can survive for more than a year in the small intestine. In addition, female adults can produce 200.000 eggs per day and these eggs have a protective coating that ensures their persistence in a contaminated environment.

Infections with *A. suum* are mostly subclinical, which explains why they are often overlooked by the farmer. Nevertheless this parasite has important consequences for the pig production industry. *A. suum* has a relatively high economic impact, as infected animals have a higher feed conversion rate, lower daily weight gain and lower meat quality [5,6]. In addition, passage of larvae through the liver causes a host response that leads to white spot lesions and livers with too many white spots cannot be used for consumption [5]. The most obvious clinical symptom of ascariosis is the wheezing and coughing associated with the pulmonary migration of the L3 around 7 DPI [7]. Furthermore, the hepato-tracheal migration is associated with increased susceptibility to bacterial pathogens such as *Escherichia coli, Pasteurella multocida* and *Salmonella* spp. [8-11]. Finally, an important sequella of *A. suum* infection is a reduced efficacy of vaccines that target other pathogens, such as Mycoplasma hypopneumoniae [12].

Although it is difficult to calculate the economic loss caused by subclinical infections, it has been estimated that strategic deworming can result in an increase in profit of between 3 and 12€ per average present finisher pig per year [13-15]. However, production losses are related to worm burden and may vary accordingly.

Often overlooked in pig farms however is the zoonotic potential of *A. suum*. It is clear from several studies in industrialized countries that an important source of ascariosis in humans is cross-infections of *A. suum* [16-19]. In addition, cross-infections of pigs with *A. lumbricoides* is also possible [20], and as a consequence, pigs can be reservoir hosts for *A. lumbricoides* in endemic countries. This may have important consequences for the control of ascariosis, but has so far received little attention

1.1.3 Protective immunity to *A. suum*.

By following the dynamic distribution of *A. suum* infections in pigs, essential observations in the defense against primary and secondary *Ascaris* infections have been made. The most important are undoubtedly the formation of the pre-hepatic barrier and the self-cure reaction.

Pre-hepatic barrier

When pigs are continuously exposed to low doses of infective eggs for 14 weeks, no larvae are able to penetrate the intestine and reach the liver [21-23]. When larvae are *in vitro* hatched and injected into the mesenteric veins of these immune animals, the larvae are able to reach the liver and induce white spots [24]. Since the immune barrier is situated before the liver, this type of resistance was termed the pre-hepatic barrier. Although some level of protection already establishes after a single infection [25], it is not directed at the invading larvae. It is still unclear why such a long time is required to establish protection at the gut, but it is not related to the presence of adult worms because the removal of worms did not affect protective immunity [26]. What effector mechanism prevents larval penetration remains to be determined.

Self-cure

It has always been difficult to predict the number of adult worms that will be present after giving a known infection dose. This is because there is a self-cure reaction or expulsion that eliminates most of the L4 from the small intestine between 14 and 21 days post infection (DPI) and it is independent of the inoculation dose [27]. Before this time the number of larvae in the small intestine is roughly 30-60% of the infection dose. After 21 DPI, however, the number of larvae is greatly aggregated, with the majority harboring low numbers of worms and a small proportion having the majority of worms. This overdispersion is also seen in humans infected with *A. lumbricoides* [28] and is therefore likely caused by a similar reaction there. Passage through the liver might be important as a sensitization for the later expulsion, as a previous study by Jungersen *et al*. found a higher percentage of animals harboring adult *A. suum* at 70 DPI than what is usually observed when the liver is bypassed by injecting *in vitro* hatched L3 intravenously in pigs, while at 14 DPI there were comparable numbers of L4 between intravenously and orally infected animals [29]. Unfortunately, not enough time

points and control groups were included to confirm if previous priming in the liver was indeed required to eliminate the larvae from the small intestine. Also here the effector mechanism driving the expulsion is unknown, but IgA production has been associated with the expulsion, as are increased fluid secretion and muscle contractibility [30,31].

These studies performed with pigs illustrate the importance of the intestine in natural and acquired resistance against *Ascaris*. There are also inflammatory reactions in the liver and lungs, characterized by infiltration of granulocytes, mostly eosinophils, and lymphocytes; however they are only induced after the larvae have already moved on from these organs [27,32] and therefore are not likely to have a major impact on the larvae during primary infections. They may still be important for protection against reinfections, although little research has been performed on this topic. The inflammatory response in the liver causing the white spots suggests a role of the liver in the defense against larval *A. suum* [33]. There are two main types of white spots observed after *Ascaris* infections. The first is the granulation type white spots that form along the larval migration route and consist mostly of eosinophils, neutrophils and macrophages [34]. The second is the lymphonodular white spots that appear later and thought to arise from the granulation type white spots and contain more lymphoid cells [34]. However, very little larvae are actually killed at this stage, which may suggest these reactions are part of a wound healing process.

In the acquired immunity against *A. suum* there is an important role for antibody production, since genes involved in antibody production and class switching, such as Ligase–IV, and B cell activating factor have been linked with resistance or susceptibility to this parasite [35] and transfer of protection has been achieved by giving colostrum [36,37].

1.1.4 Control of *A. suum* and vaccination studies

The most widely used drugs for treatment of *A. suum* are the benzimidazoles and to lesser extent macrocyclic lactones. Despite the availability of these drugs that are 100% efficacious against *A. suum*, it remains highly prevalent because the period between treatments is longer than the prepatent period of 7 weeks and the transmission is therefore only temporarily halted. Moreover, since the environment remains contaminated and the anthelmintic drugs used have little to no remnant effect, pigs will quickly re-infect themselves after treatment.

The rise in popularity of organic farming, with different management practices such as loose housing of sows in dynamic groups, outdoor access and no preventive anthelmintic treatment may have a negative impact on the control of *A. suum* because these systems allow for more transmission of *A. suum* [38,39]. As a consequence, the prevalence of *A. suum* is not likely to decrease anytime soon.

Vaccination against *Ascaris* would offer better means of control than anthelmintic treatment since it would interfere with the infection, disease and transmission on a long-term basis. Because there is naturally acquired immunity against *A. suum*, vaccination should be feasible. Several researchers have developed experimental vaccines based on extracts, antigens or even UV-irradiated eggs of *A. suum*, with parasite reductions ranging from 58 to 99% [40-55]. What we can conclude from these vaccination studies is that immunity against *A. suum* is inducible, which raises hope that one day vaccination against *A. lumbricoides* can also be achieved. However, most of these studies only evaluated the reduction of larvae in the lungs, while the ideal vaccine against *Ascaris* should mimic natural immunity and prevent larvae from penetrating the intestine and damage the internal organs. Especially for pigs this type of immunity is required to prevent liver white spots and make an economically more profitable vaccine. The investigation into the mechanisms of the pre-hepatic barrier may provide clues on how to induce this type of pre-hepatic immunity.

1.1.5 *Ascaris lumbricoides*

A. lumbricoides belongs to the same genus as *A. suum*, but infects humans. Due to the faeco-oral transmission of eggs, wherever hygienic standards are inadequate, *A. lumbricoides* can be found. Not surprisingly then, *A. lumbricoides* is most prevalent in rural areas or shanty towns in (sub)tropical countries. The highest prevalence is in children aged 5-15 years. Children are more playful and thus more likely to come into contact with contaminated soil. Adults usually have lower worm burdens, due to a combination of better sanitary habits and acquired immunity. *Ascaris* transmission is highest when there is a lack of sanitation, access to safe water, health education and medical treatments. As a consequence, ascariosis is a disease of the poor.

Although *A. lumbricoides* is the largest of man's nematode parasites, many cases of ascariosis go by undetected, similar as in pigs. However, symptoms can vary from decreased food intake to bowel obstruction to reduced cognitive performances. The most obvious damage comes from the early stages of infection, as the migratory route through the host

inevitably damages the affected organs. The presence of larvae in the liver induces a host response that leads to white spot lesions and the passage through the lungs causes coughing, dyspnea and pneumonitis [56]. Adult *Ascaris* worms present in the small intestine can cause abdominal obstruction, vomiting, malabsorption and in rare cases they may penetrate the small intestine or get stuck in the bile or pancreatic ducts, creating life threatening situations [56]. Nevertheless, helminth infections have a low mortality and morbidity is generally related to worm burden. Therefore disease burden is usually expressed as Disability Adjusted Life Years (DALY), which takes into account not just life years lost due to premature death but also morbidity. Although *Ascaris* infections are often subclinical, their high prevalence assures that the total loss of Disability Adjusted Life Years is close to 2 million [57].

Because of the identical life cycle, the high genetic similarity between *A. suum* and *A. lumbricoides* [58], and because *A. suum* is a zoonosis [19,59], *A. suum* infections in pigs make an ideal model for *A. lumbricoides* infections in humans. Cross infections and gene flow between the 2 species also occurs [60,61], which led to the debate whether or not they belong to the same species [62,63]. As a result, much of the findings about *A. suum* have been confirmed in humans. For example, genetic factors account for 30-50% for the variation of *Ascaris* infection load in both pigs and humans [64,65].

Although in humans there is little information what effector mechanisms confer protection against *A. lumbricoides*, there is indirect evidence that resistance or immunity against *A. lumbricoides* is acquired. First of all, in hyperendemic areas there are children who remain uninfected even though they are undoubtedly exposed to the parasite. Secondly, there is a high degree of predisposition to high or low worm burdens [66], and this predisposition declines with increased age [67]. Predisposition towards *A. lumbricoides* infections can largely be explained by host genetic factors, which may account for up to 50% of the observed variability in worm burdens [65]. Other factors are exposure, socio-economic behavior and parasite genetics. Although behavior factors are important in the age pattern of *A. lumbricoides* infections, it also suggests that adults are less susceptible than children due to the establishment of immunity. It is also striking that despite being exposed to infective *A. lumbricoides* eggs in the environment over a prolonged period, people rarely suffer from lethal hyperinfectons. Infected individuals appear to be immune against newly arriving larvae, but the adult worms that are present are not affected. The term concomitant immunity has been put forward to describe this phenomenon. When the adults are removed by anthelmintic treatment, the host is again susceptible to new infections. Concomitant immunity has mostly

been described in the context of schistosomes or tapeworm infections, but the mechanisms behind it are unclear [68,69].

As a result of the high similarities between *A. suum* and *A. lumbricoides*, findings in *A. suum* may also be of interest for research into *A. lumbricoides*. It should be noted that, although *A. lumbricoides* and *A. suum* are practically identical, there are import differences between humans and pigs in terms of their immune systems (for details, see next section).

1.2 The protective immune response against helminth infections

We now know that effective responses against *A. suum* are elicited in the intestines and that they are directed against different life stages. The effector mechanisms that drive these responses or what triggers them, however, are still unexplored. By looking at the immune response against other helminths, we might get an understanding of what effector mechanisms could be protective against *A. lumbricoides* or *A. suum* infections. The immune response against helminths is complex in nature, because helminths infect their host in a multitude of ways, affect different organs and have developed immune evasive strategies to avoid being killed. Moreover, the constant presence of helminths in the evolution of our immune system has led to a dynamic interplay between the two. How the anthelmintic response is able to create an unfavorable environment for helminths to reside in has long been abstruse. In recent years however, researchers have identified several effector mechanisms responsible for parasite clearing. It has become clear that our immune system possesses a remarkable versatility when it comes to dealing with helminth infections. What follows here is a general description of immune responses directed against helminths in general, with special attention to findings with *A. lumbricoides* and *A. suum* infections.

1.2.1 The Th2 paradigm

T helper 2 cells

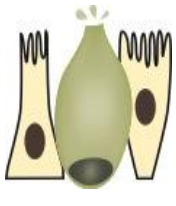
Helminth infections have classically been associated with a T helper 2 (Th2) type immune response, which is characterized by high levels of interleukin (IL) 4, IL-5, IL-9 and IL-13. The main transcription factor in the Th2 response is signal transducer and activator of transcription 6 (STAT6). Interestingly, this type of immune response is also associated with allergic diseases such as asthma and allergic rhinitis. As a consequence allergic reactions towards helminth antigens occur [24,70]. The Th2 cell orchestrates much of the anthelmintic immune response. It produces many of the cytokines involved in the recruitment and activation of effector cells such as basophils, mast cells, goblet cells and eosinophils and induces IgE class switching in B-cells (Figure 1.2). When these cells are ablated, immunity against helminths is often compromised [71,72]. Especially the signaling through IL-4 and IL-13 is essential in the defense against gastro-intestinal nematodes because it affects all cells

involved in parasite clearance [73]. Th2 cytokines also have a regulatory function, as they dampen Th1 cytokines, such as interferon-γ (IFNγ) and IL-12.

There is not much data for cytokine responses in *A. lumbricoides* infected humans, particularly localized responses, but peripheral blood lymphocytes from infected people do express more IL-4 and IL-5 [74], and Th2 cytokines seem to predispose to resistance [75]. Pigs infected with *A. suum* show in general a more Th2-associated cytokine pattern, based on analyzed cytokine responses in the jejunum, ileum, spleen, liver and bronchoalveolar lavage fluid during the intestinal expulsion of *A. suum* [31]. How these cytokines create an unfavorable environment for *A. suum* or what triggers them, however, remain enigmatic.

Of special interest to the anthelmintic immune response is the difference in function of IL-4 between humans and pigs. In humans and mice, IL-4 is one of the hallmarks of a Th2 response and often associated with helminth infections. In pigs however, IL-4 does not stimulate T cell proliferation and antibody production [76]. Still, it is induced in response to parasite infection [77], so its role in the defense against parasites is quite puzzling.

The mucosal barrier



Before any ingested pathogen can invade the intestine, it has to find a way to get past the mucosal barrier. The gastro-intestinal mucus layer forms the first line of defense by forming a barrier between the lumen and the intestinal epithelium. The most important component of this layer are the secreted mucins. Mucins are goblet cell-secreted large glycoproteins. Although the mucus layer is also part of the innate defense, the adaptive immune response can induce goblet cell proliferation and changes in the mucus composition [78]. These alterations can be very important, for example Mucin 5AC is not expressed in the caecum or colon in healthy mice, but it is induced in *Trichuris muris* infections and Mucin 5AC-deficient mice have impaired *T. muris* expulsion [79]. Increased mucus can make it more difficult for the parasite to attach to it or degrade it and changes in acidity may disorient the parasite. Furthermore, the increased mucus production can help to close the gap in the mucosal barrier caused by the parasite and thereby prevent the concomitant infiltration of bacteria. Apart from mucins, goblet cells also secrete proteins with anti-microbial functions in the lumen of the intestine. One such protein of particular interest to the anthelmintic immune response is Resistin Like Molecule beta (Relmß). Relmß is induced by IL-13 and it binds directly to parasitic worms to disrupt their chemotactic sensors [80,81].

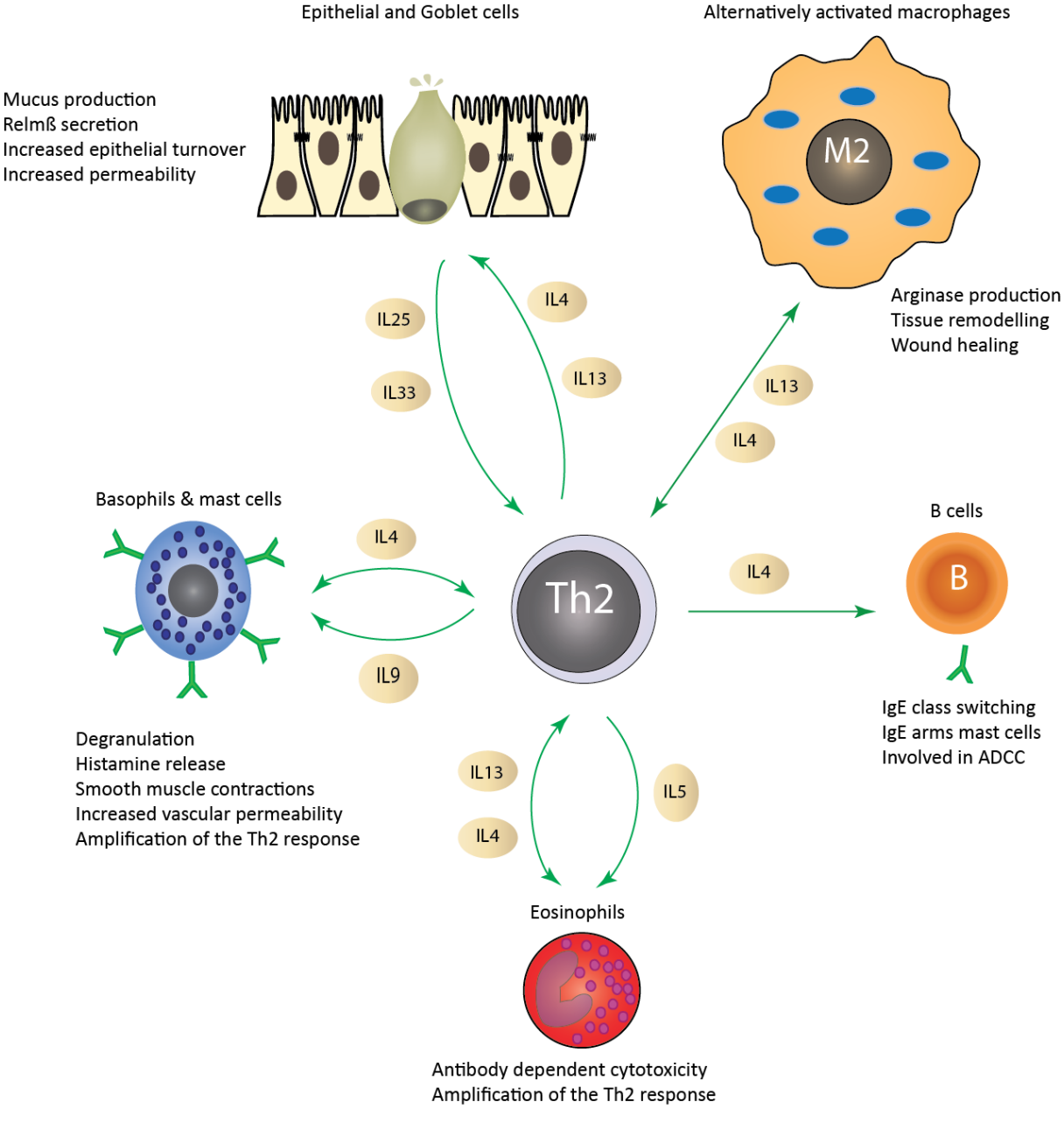


Figure 1.2: The Th2 effector response against helminths. Early danger signals such as IL-25 and IL-33 induce the proliferation and differentiation of Th2 cells that in turn produce cytokines such as IL-4, IL-5, IL-9 and IL-13. Together, these cytokines activate effector cells such as basophils, mast cells, eosinophils, B- cells, macrophages and goblet cells, which act together to eliminate the parasite.

Underneath the mucus layer, epithelial cells line the intestine. Together with dendritic cells, they act as sentinel cells by releasing the first danger signals upon contact with pathogens. These danger signals include IL-25, IL-33 and thymic stromal lymphopoietin that promote the differentiation and activation of Th2 cells. There is also important cross-talk between epithelial cells and inflammatory cells. For example, epithelial cells release eosinophil chemoattractants when damaged and eosinophils respond by secreting growth factors such as fibroblast growth factor-2 and transforming growth factor ß (TGF-ß) [82]. Epithelial cells are kept close together by tight junctions. These junctions can be broken by mast cell proteases. As a result, the permeability of the epithelial layer increases and more fluid leaks in the lumen as part of a 'weep and sweep' response. Certain parasites, such as *Trichuris* species, burrow their way in the epithelial layer. One host defense mechanism against these parasites is to increase the epithelial turnover in such a way that the parasite is shed in the lumen along with the desquamated epithelial cells [83].

Eosinophils

Eosinophilia is a common observation in blood and tissue of helminth infected individuals and ever since the demonstration that eosinophils could kill schistosomula *in vitro*, these cells were considered to be the most important effector cells during helminth infections [84]. Follow-up studies with nematodes showed similar results and that coating of the parasite with IgE or IgG or complement factors is necessary for eosinophils to be efficient killers of these parasites. Crosslinking of IgE or IgG receptors triggers the release of the granules stored in the cytoplasm. These granules contain pre-formed cytotoxic molecules, cytokines, chemokines and growth factors. Because these molecules are pre-formed, eosinophils have a quick effect on their surroundings after their activation. The major cytotoxic proteins are eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and major basic protein (MBP). MBP is the most abundant granular protein. It interferes with the plasma membrane, thereby increasing permeability. ECP and EDN are basic proteins that belong to the ribonuclease A family. EPO forms reactive oxygen and nitrogen species, causing toxic oxidative stress. Toxicity of these eosinophil granule proteins against nematodes has been shown for *Toxocara canis*, *Trichinella spiralis, Onchocerca volvulus* and *Brugia malayi*, mostly against juvenile stages [85]. The toxicity of these proteins is not limited to parasites and uncontrolled release in the

environment causes tissue damage. As a consequence, eosinophils are sometimes associated with pathology in chronic infections.

Eosinophils have multiple ways to find their target. Tissue invaded by parasites releases eotaxins that recruit eosinophils from the blood through CCR3 binding. In addition, eosinophils carry receptors for complement factors such as C5a, which is released when complement binds to pathogens [86]. Furthermore, they can be directly recruited by parasite-secreted factors [87,88].

Apart from a direct toxic effect on the parasite, eosinophils also amplify the inflammatory response by releasing Th2 cytokines and chemokines, which leads to lymphocyte and further eosinophil recruitment and mast cell activation. Because eosinophils are depending on IL-5 for their development in bone marrow and recruitment to the blood, this cytokine makes an interesting target to study with respect to predisposition to helminth infections. These studies have shown that IL-5 levels correlate with the resistance against reinfections of schistosomes, hookworms and *A. lumbricoides* [75,89,90]. Interestingly, a population study in a hyperendemic region in Nigeria also showed that *A. lumbricoides* immune children had higher levels of eosinophil cationic protein in blood than susceptible children [91].

Despite these findings, *in vivo* studies with eosinophil ablation and transgenic models have been inconclusive in determining if they are essential in the defense against helminths. For example intestinal *Strongyloides stercoralis* infections are not affected by anti-IL-5 treatment during primary infections, yet lung stage larvae during reinfections were higher in the treated group [92]. Eosinophil-mediated resistance also depends on the susceptibility of the parasite species. IL-5 transgenic mice are resistant to *Nippostrongylus brasiliensis*, *S. stercoralis* and *Angiostrongylus costaricensis* [93,94], while resistance to *T. canis* is not affected [95]. Interestingly, the excretory and secretory material of *T. canis* (TES) contains proteins that inhibit the adherence of eosinophils to the larvae, even in such a manner that resistant IL-5 transgenic mice become susceptible to *N. brasiliensis* L3 when TES is given together with *N. brasiliensis* L3 [95]. In other studies there was no link between eosinophils and helminth resistance [96,97]. Unfortunately, functional studies in humans are scarce and studies with mice are complicated by the fact that mice are not natural hosts to many of the helminth infections studied and researchers often have to use artificial ways of infecting them. There are also important differences between eosinophils from humans and mice. While human eosinophils degranulate in response to a variety of allergens, eosinophils form mice are

less easily activated [98-100], possibly caused by the absence of certain receptors and effector proteins in mice [101].

From these findings, it appears that eosinophils are only essential in the killing of larvae during the tissue phases of helminth infections and during reinfections. It is therefore likely that eosinophils only recognize antibody or complement-coated parasites and that direct contact is required to induce eosinophil degranulation. Furthermore, differences in surface or secreted proteins between different life stages may explain why certain adult stages are not targeted by eosinophils.

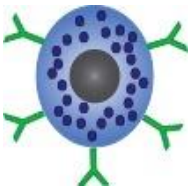
In humans and mice, eosinophils are assumed to only appear in the intestine of people suffering from diseases such as parasite infections, allergic diseases or inflammatory diseases. In pigs, eosinophils are present along the whole length of the intestine, also in healthy individuals [102]. However, recent reports could indicate that the level of eosinophils in the gut in healthy humans and mice are higher than previously recognized and that this is due to technical shortcomings in distinguishing these cells from others [103].

IgE

In healthy individuals, IgE produced by B cells is only present in small amounts in the blood and it has a short half-life of only 2 days. High IgE blood levels are often seen in parasitized patients. IgE functions in arming eosinophils, mast cells and basophils, giving these cells the ability to recognize helminth antigens. IgE and IgG are essential for eosinophils to attach to worms and crosslinking of IgE on mast cells and basophils leads to the release of histamine. IgE class switching requires two signals: CD40 binding on B cells by CD40 ligand present on T cells and signaling through IL-4 or IL-13 [104].

Although all isotypes are usually induced during helminth infections, high levels of specific IgE are often associated with protection against reinfection, for example in *A. lumbricoides* infected people [91,105]. The importance of IgE in the defense against *A. lumbricoides* is further supported by the detection of a genetic locus that is related to *A. lumbricoides* susceptibility containing a set of 3 genes involved in antibody production and IgE class switching [35].

Basophils & mast cells

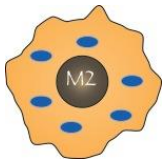


Basophils and mast cells are another cell type often associated with helminth infections. They are hard to differentiate from one another: they stain with toluidine blue, are filled with granules containing histamine and have similar functions. It was long assumed that basophils were mast cell precursors in the blood and that mast cells are the activated basophils that were recruited to the tissue. Recent work however showed that they do have distinct properties and that basophils are more than just precursor mast cells [106].

Mast cells and basophils add to the general inflammation by producing Th2 type cytokines such as IL-4, IL-5 and IL-13. They also respond to Th2 cytokines by proliferating and producing the high affinity IgE receptor. Crosslinking of IgE on mast cells and basophils is a potent trigger for degranulation, but they are also activated by complement factors C3a and C5a and IgG crosslinking [104]. Their granules are filled with histamine, which works as a chemoattractant for eosinophils and induces smooth muscle contractions and vascular permeabilization [107]. Additionally, mast cell proteases can break tight junctions, leading to increased intestinal fluid secretion. Mast cell proteases may also activate receptors on enteric nerves in order to increase smooth muscle contractility in nematode infected mice [108]. Fluid secretion and muscle contractions are part of a 'weep and sweep' response that is often seen in gastro-intestinal infections [109].

Mast cells and basophils might contribute to the resistance against *Ascaris*. Dawson *et al.* also demonstrated a histamine-dependent increase in epithelial cell secretion and muscle contractibility during *A. suum* infection between 14 and 21 DPI, which corresponds to the timing of the self-cure reaction [31]. It was previously shown that mast cells and basophils released histamine after contact with *Ascaris* secretory antigens, but only in repeatedly infected animals [110,111]. This would indicate that *A. suum* specific IgE or IgG binding on these cells is essential in their activation.

Alternatively activated macrophages



Macrophages can be divided into 2 categories: classically activated or alternatively activated. Classically activated macrophages were first discovered in the defense against bacteria. They are activated in a Th1 context and exert their bactericidal function through inducible nitric oxide synthesis (iNOS). iNOS converts arginine into nitric oxide (NO), which is toxic for most bacteria. In a Th2 type setting, however, macrophages express more arginase, which competes with iNOS for available arginine. These Th2 type macrophages are referred to as alternatively activated or M2 macrophages. These cells are further characterized by mannose receptor and Resistin like molecule alpha in mice. The exact role of AAM during helminth infections is still disputed, but depending on the setting, AAM can have immune-regulatory, wound healing or protective functions. Through arginase expression, arginine is metabolized and ultimately leads to increased proline, which is required for collagen deposition and explains why fibrosis is often associated with AAM [112]. They also promote angiogenesis and release growth factors, keeping the helminth-induced damage under control.

To date, AAM have been shown to be essential in the clearance of secondary *Heligmosomoides. polygyrus* infections in mice [113] and the ablation of AAM blocked smooth muscle hypercontractility and impaired the expulsion of *N. brasiliensis* in primary infections [114]. Studies are yet to be performed to evaluate the role of AAM in the immunological defense against *A. suum* or *A. lumbricoides*.

Classically activated macrophages and AAM have been well defined in mice but information on this subtype of cell in other mammals is scarce. Murine AAM markers RELM-α, YM-1 and arginase for example are either not present or not induced in macrophages in Th2 polarizing conditions in humans [115] and there is inconsistency whether or not human macrophages produce NO through iNOS [116-118]. There is also very little information concerning the polarization of porcine macrophages. Porcine macrophages seem to resemble more closely human macrophages than murine macrophages, in terms of their response to bacterial lipopolysaccharide and individual gene homology [119,120]. Furthermore, porcine macrophages constitutively transcribe iNOS mRNA in lymphocytes [121]. This raises the question whether this classification is applicable to non-murine macrophages.

Innate type-2 cells

Although Th2 cells are the major producers of type-2 cytokines during helminth infections, they are only induced some time after infection and do not explain the high type-2 cytokine levels sometimes observed shortly after contact with helminths. Researchers have recently identified several other, non T-cells that play an important role in producing these cytokines early in infection. They consist of nuocytes, natural helper cells and multipotent progenitor type-2 cells and respond to the early release of IL-25 and IL-33 by stressed epithelial cells [122-124]. By expressing type-2 cytokines such as IL-4 and IL-13, they aid in initiating the immune effecter response against helminths. To what extent these findings obtained in mice studies can be extrapolated to pigs is unclear.

1.2.2 The Th2 paradigm taken with a pinch of salt

Although the Th2 paradigm is widely accepted and explains many phenomena, it does not hold true for all helminth infections. The anthelmintic host response is much more dynamic than previously imagined. Over the past years, a considerable body of evidence has been collected that shows that Th1 responses are equally important for resistance against certain helminths. A nice illustration of the importance of Th1 responses is cysticercosis in rodents caused by *Taenia crassiceps*. STAT-4 is the transcription factor required for Th1 responses and when STAT4-deficient mice were infected with *T. crassiceps*, although these animals had high levels of IL-4, IL-10 and IgE, they were unable to eliminate the parasite [125]. In contrast, STAT6-deficient animals are highly resistant to *T. crassiceps* [126]. Although little information is available in humans, people who are putatively immune to *Onchocerca volvulus* do show strong responses characterized by high IFNγ levels, a Th1 cytokine, when blood lymphocytes are challenged with *O. volvulus* antigens [127].

One product that deserves special attention in the context of a Th1 response is nitric oxide. iNOS is induced in many cell types as a defense mechanism in response to pro-inflammatory cytokines such as IFNγ and TNFα and is regulated by others such as IL-4, IL-10 and IL-13 [128]. iNOS catalyzes the formation of NO out of L-arginine. Nitric oxide reacts with oxygen to form unstable radicals and peroxynitrate anions ($ONOO^-$). These molecules exert their toxicity by oxidizing protein, lipids and sulfhydryls. The role of iNOS during helminth infections is still unclear. Although nitric oxide is typically associated with bacterial infections,

it also has a detrimental effect on several helminth parasites. It has also been shown that macrophage-derived NO is toxic for *Brugia malayi*, especially the microfilariae [129]. NO production is also important for protection against *T. crassiceps* cysticercosis [130]. Both in cattle infected with *Cooperia oncophora* and in sheep infected with *Haemonchus contortus* or *Trichostrongylus colubriformis,* the iNOS encoding gene expression was associated with resistance [131,132]. In contrast, the NO produced during infection with *T. canis* is associated with pathology and not with protection [133]. Similar results are obtained with *T. spiralis* infections [134]. Interestingly *T. spiralis* excretory-secretory (ES) antigen from larvae induces iNOS, while ES antigen from adults decreases iNOS production [135]. This suggests that certain parasites have developed mechanisms to deal with iNOS. The effect of NO on *Ascaris* has not been investigated, although *A. suum* antigens are capable of inducing NO production in macrophages *in vitro* [136]. In addition to direct effects on helminths, NO can exert an anthelminthic effect indirectly by enhancing the inflammatory response [137], decreasing intestinal transit time [138] or increasing fluid secretion in the lumen [139].

Mixed Th1/Th2 responses appear more efficient against tissue residing helminths in which the Th1 response generates a pro-inflammatory environment that is toxic for the parasite and where the Th2 response helps to control excessive tissue damage to the surrounding tissue. For example, schistosome cercaria initially induce a Th1 type response and it is only once eggs are produced that the immune response will shift towards Th2 [140]. The filarial nematode *Brugia malayi* induces a similar pattern, with microfilaria that skew towards Th1 and adults towards Th2 [141]. Studies in cattle have put further emphasis on the interplay between Th1 and Th2 cytokines during the course of helminth infections, where general Th2 responses are present with intermittent peaks of IFNγ [142].

These examples illustrate that the anthelmintic immune response is not a static one: it evolves over time and is dependent on the parasite species and the tissue affected. The responses can different for juvenile and adult worms. The result of the combined effects of all these cells is reduced helminth fitness and elimination. Juveniles are often killed in situ while adults usually persist, but have reduced fecundity, impaired feeding or reduced activity and are sometimes subsequently slowly driven out of the gastro-intestinal tract [81,143,144]. It appears the Th2 paradigm is best suited to describe immune responses against gastro-intestinal nematodes, but that especially for tissue residing helminth infections, the Th2 paradigm falls short and other T helper responses are equally essential for immunity. As for *Ascaris*, apart from some observations in infected people and pigs, we still know very little

about the immune response directed against *A. lumbricoides or A. suum*. Especially the mucosal immune response has been relatively unexplored, which is surprising, since this is where this nematode resides most of its life.

1.2.3 Th2 in tissue repair

When nematodes like *Ascaris* or *Necator* spp. penetrate the intestinal wall or skin and migrate through vital organs, they leave behind a trail of destruction. Tissue damage is also evident for tissue residing helminths such as filarial nematodes and schistosomes or worms that feed of the mucosa such as whipworms and hookworms. Consequently, rapid tissue repair is needed in order to avoid impaired organ function. Part of the type-2 response is designed to deal with the acute tissue damage associated with helminth infections. For example, the Th2 response in patent schistosome infections serves to quickly repair the intestinal damage caused by the eggs, preventing infiltration of bacteria and subsequent sepsis [145].

Several factors that aid in wound repair, such as insulin-like growth factor 1 (IGF1) and arginase 1 (ARG1) are controlled by Th2 cytokines [146]. As a consequence, IL-4Rα knockouts have reduced expression of IGF1 and ARG1 in helminth infection models [147]. IGF1 is involved in wound healing and functions in the proliferation of fibroblast, collagen synthesis and control of apoptosis [148,149]. ARG1, produced by alternatively activated macrophages, also promotes cell proliferation and collagen deposition. Resistin like molecule alpha and lectin Ym-1, both expressed by murine alternatively activated macrophages, have been implicated both in tissue remodeling and immunomodulation [150]. Amphiregulin, a member of the epidermal growth factor family, is produced by T cells and eosinophils and induces epithelial cell proliferation [151,152]. In the absence of amphiregulin, the expulsion of *T. muris* is delayed, possibly because increased proliferation and shedding of epithelial cells make it harder for *T. muris* to invade the mucosal layer [153].

Although wound repair is initiated very quickly and insures organ integrity during helminth infections, it comes at a price. Healing wounds properly takes time that is not available during helminth infections. There is a quick, but hardly clean repair with scar tissue as a consequence [146]. In acute infections, the type-2 response prevents excessive organ damage, but in chronic infections, uncontrolled wound healing leads to fibrosis, as is seen in the liver during schistosome infections [140]. The liver contains resident macrophages called Kupffer cells. Activation of Kupffer cells by toxic agents results in the release of inflammatory

cytokines as well as growth factors. As such they play a role in the initiation of liver fibrosis and inflammation. When they are activated by hepatic tissue damage in a Th2 type environment such as during helminth infections, they express the alternative activated macrophage markers and trigger fibrosis without inflammation [150], illustrating that Th2 cytokines can have both anti-inflammatory and wound healing functions at the same time. As such, they play a crucial role in schistosome infections, as they ensure the eggs in the liver are walled off by forming granulomas around them, but they are also responsible for fibrotic scarring [112].

A. suum larvae migrating through the liver leave a necrotic trail behind and cause the formation of white spots [154]. White spot granulomas consist primarily out of macrophages, granulocytes and lymphocytes. Older lesions consist mostly out of fibrotic tissue [34]. Since few larvae are actually stopped in the liver, and more intense white spot formations do not necessarily impair larval migration [155], it seems likely that the infiltration of these immune cells also serves a different purpose than to attack the larvae. Although little research on the topic has been performed, the white spots may be the result of a quick wound healing response, initiated to prevent excessive hepatic tissue damage. This would help to explain the fibrosis and scar tissue on the livers of chronically infected pigs.

1.3 Immune modulation and evasion strategies of helminths

It is a fascinating concept how parasites, which are by definition detrimental to the host, can survive for several years in their hosts without triggering massive inflammation or anaphylactic shock. Helminths can even flourish in immunologically very active sites such as the lymphatic system or bloodstream. Hence, they have acquired immune-modulatory properties that prevent deregulated immune responses. The reason that helminths are so efficient in immune modulation is probably because it can be beneficial to host and parasite alike: the parasite survives longer without being eliminated from the host, but at the same time the host is protected against severe tissue damage by uncontrolled inflammation. The observation that auto-immune diseases occur much less frequent in poorer areas of the world led to the formulation of the hygiene hypothesis, which simplified, states that immunological diseases may be caused by extreme hygiene measurements that deregulate the immune system and that especially helminth infections seem to protect against uncontrolled auto-immune disorders[156].

Apart from inducing Th2 responses, thereby leading the host response away from more pathology associated Th1 and Th17 responses; helminths are actively involved in immune suppression [157]. Probably the most important immunosuppression comes from the induction of CD25+ regulatory T cells (Treg). These cells create a regulatory environment that suppresses inflammation. IL-10 and TGF-ß promote the activation and differentiation of Treg cells. Through TGF-ß signaling, Treg cells are recruited by *H. polygyrus* secreted products [158]. Immune modulation is also apparent from the antigen unresponsiveness that follows filarial infections [159-161]. Two prominent cell types in the regulatory setting of helminth infections are the alternatively activated macrophages and myeloid-derived suppressor cells (MDSCs). MDSCs are induced in both protozoan and helminth infections and inhibit both Th2 and Th1 responses [162]. Alternatively activated macrophages play a crucial role in the regulation of the immune response, as they limit arginine in environment, which is needed by proliferating T cells and the pro-inflammatory iNOS.

Apart from the general immune-regulation, there is a plethora of examples in the helminth world that show that helminths have developed very specific mechanisms in order to evade the host immune response (see Table 1.1). Many parasitic worms come into contact with blood, either by following the blood stream or by invading tissues. Apart from antibodies, blood contains complement factors that contribute to the attachment of eosinophils, the opsonization of parasites and the recruitment of inflammatory cells. It is therefore not surprising that many of the immune evasion products identified involve the inhibition of the complement cascade.

Although several *Ascaris* antigens are known allergens, adult worm extracts also contain compounds that reduce airway eosinophilia and hyper-responsiveness in asthmatic mice, most likely by suppressing IL-4 and IL-5 levels [163,164]. McConchie et al further show that pseudoceulomic fluid of adult worms also inhibits allergic responses, possibly by interfering with the activation of dendritic cells [165]. In *A. lumbricoides* infected people, an increase in CD25+ lymphocytes has been demonstrated, indicating a role for Treg cells in the immunomodulation [166]. There are however strikingly different effects on allergic diseases seen when animals are suffering from acute versus chronic *Ascaris* infections. Mice that were infected with *A. suum* at the same time of sensitization showed exacerbated allergic responses, while animals that were infected chronically before sensitization showed ameliorated responses to challenge [167]. These findings might help to explain some of the incongruent observations of allergic diseases in people suffering from *Ascaris* infections (see 1.4.1).

Table 1.1: helminth immune evasion products

Product	Function	Helminth	Reference
Cathepsin	Cleaves IgE and IgG	*F. hepatica*	[168]
Complement C2 receptor trispanning (CRIT)	Complement formation inhibition	*Schistosoma, Trypanosoma cruzi*	[169]
Cystatins	Inhibits antigen processing	*H. contortus, N. brasiliensis*	[170,171]
Cystein protease	Inhibits IgG induced eosinophil degranulation	*Paragonius westermani*	[172]
Eotaxin cleaving protease	Digests eotaxin	*N. americanus*	[173]
Glyceraldehyde 3 phosphate dehydrogenase	Complement C3 inhibition	*H. contortus*	[174]
Neutrophil inhibitory factor	Blocks neutrophil adhesion and degranulation	*A. caninum*	[175]
Paramyosin	Complement C1, C8 and C9 inhibition	*T. solium, S. Mansoni, T. spiralis*	[176]
Phosphorylcholine	Suppresses lymphocyte proliferation	*Ascaris*	[177]
Serine protease	Inactivation of the complement anaphylatoxin C5a	*Brugia malayi*	[178]
Superoxide dismutase	Neutralizes superoxide radicals	*Fasciola spp, H. contortus, O. volvulus*	[179-181]

1.4 Important consequences of the anthelmintic response

The effects that helminth species have on the immune system carry implications that stretch far beyond just the elimination or conservation of that species. When strong Th2 responses are elicited and Th1 responses are repressed, pathogens that are susceptible to Th1 responses can be harder to eliminate. *S. mansoni* for example induces strong Th2 responses that have a negative impact on the development of resistance against *Toxoplasma gondii* and *Plasmodium* spp. [182,183]. Conversely, *S. mansoni* helps to protect susceptible mice against *T. muris* infections, by inducing stringer Th2 responses [184]. This relationship is not always that clear however. Although filarial nematodes modulate cytokine responses to *Mycobacteria* spp *in vitro* [185], there is no evidence of *in vivo* modulation [186].

Helminth induced immunosuppression can also have beneficial effects by suppressing inflammation induced pathology. *A. lumbricoides* for example is negatively associated with cerebral malaria [187]. The anthelmintic response may also have important consequences for vaccination programs. The immunosuppressive properties of helminths may compromise the response that is required with vaccination. Pigs infected with *A. suum* at the time of vaccination against *Mycoplasma hypopneumoniae* showed a delay in seroconversion and a decrease in the total number of pigs with seroconversion [12]. Furthermore, animals with *A. suum* had more lung pathology in response to *M. hypopneumoniae* infections than healthy pigs. Similar results were obtained in humans where it was shown that deworming had a positive effect on the antigen specific response after bacille Calmette-Guerin (BCG) and cholera vaccination [188,189]. Finally, diagnostic tests may be compromised by helminth infections, since the diagnostic predictive value of the tuberculosis test decreased when cows were infected with *F. hepatica* [190] and preliminary results indicate that *A. lumbricoides* infection might influence the predictive value of the tuberculosis test as well [191].

These examples illustrate how widespread the repercussions are that helminth infections can have and stress the importance of identifying the helminth infection status in vaccination and diagnostic studies in endemic countries.

1.4.1 *Ascaris* and allergies

The helminth immune response and the allergic response show striking similarities: both induce eosinophilia, mastocytosis and IgE and are associated with a Th2 response. As a consequence, researchers have tried to elucidate the relationship between helminth infection and allergic diseases such as asthma and skin hypersensitivity. In general, allergic diseases are more prevalent in more developed and urban regions, while helminth infections are low in these settings. It was therefore assumed that helminth infections protect against allergic diseases. There is indeed a considerable body of evidence showing the negative correlation of helminth infections and allergic diseases [192-195]. The immune-modulatory properties of helminths can explain why helminth infected people have fewer allergic symptoms even in the presence of high allergen specific IgE levels [195].

However, no simple conclusion can be drawn from the interaction between *Ascaris* and allergies, as *Ascaris* may have both a modulating and exacerbating effect on atopic diseases [196-198]. A likely explanation is that *Ascaris* extracts contain both allergens, such as tropomyosin and ABA-1 [199] and antigens that modulate immune responses [163,164]. There is a fine balance between the stimulation and the regulation of the immune response and depending on the infection intensity, deworming can have beneficial or adverse effects on people with atopy [195,200].

The similarities in immune response between allergies and helminth also led to the speculation that people with atopic disease are better protected against helminth infection. However, for *Ascaris* no evidence exists to support this theory, probably because *Ascaris* specific IgE that is associated with protection does not cross-react with allergens [35,91].

1.4.2 Helminth therapy

The immune-modulatory properties of helminths inspired researchers to investigate the use of these worms or their by-products as a means of treatment for immune disorders such as autoimmune and allergic diseases. Although deliberately infecting humans with helminths is a somewhat unconventional approach, with some precautions it is reasonably safe.

The two helminth species currently under investigation as medical treatments are the human hookworm *Necator americanus* and the pig whipworm *Trichuris suis.* Although the

trials have variable outcomes, the most promising results are obtained using *T. suis* ova (TSO), see Table 1.2. This porcine nematode is closely related to the human nematode *T. trichiura* but it is spontaneously cleared by humans after several weeks. The safety of TSO was recently confirmed in phase 1 trials with people suffering from Crohn's disease [201,202]. It is not surprising that the best results are achieved with TSO and Crohn's disease, as the parasite and the immune disorder are co-localized and *T. suis* is less pathogenic than *N. americanus*. Some adverse effects of TSO were recorded in allergic rhinitis patients, such as flatulence, diarrhea and abdominal pain [203]. The difference in adverse effects with Crohn's disease patients may be that the positive effects of TSO on the intestine in these patients outweigh the negative effects of the intestinal nematode infection. Still, trials with TSO are currently planned or already in action for multiple sclerosis, Crohn's disease, ulcerative colitis, autism, food allergy and psoriasis (clinicaltrials.gov) and are needed to confirm the preliminary trials. Nevertheless, the future of helminth therapy probably lies in the isolation of immune-modulatory molecules in order to avoid unnecessary side effects of the infection and achieve a more consistent and long-term outcome.

The use of *Ascaris* eggs as treatment for immunomodulatory diseases is obviously not advised because of the damage caused by the hepato-tracheal migration and the presence of allergens. Unless *Ascaris* immunomodulatory molecules could be isolated and produced on a large scale, the potential harmful effects on human welfare would be too great. In that respect, the recent acquisition of the genome of *A. suum* raises the hope that these modulatory molecules will be easier to identify [204].

Table 1.2: Helminth therapy studies

Disease	Helminth	Outcome	Reference
Inflammatory Bowel Disease	*T. suis*	No adverse effects Temporary remission in 6 out of 7 patients with CD or UC	[205]
		Remission in 72%, clinical improvement in 79% of CD patients	[202]
		Clinical improvement in 43% of UC patients compared to 17% with placebo No differences in remission rate	[206]
	N. americanus	Pilot study with trend towards reduced disease at 20 weeks p.i. Adverse events recorded include anemia, transient enteropathy and peripheral eosinophilia	[207]
	T. trichiura	Case report. Remission after infection	[208]
Multiple Sclerosis	*T. suis*	Phase I study with trend towards reduced disease No adverse effects	[209]
Allergic rhinitis	*T. suis*	No effect on clinical disease or cytokine response	[210,211]
	N. americanus	No effect on clinical disease	[212,213]
Celiac Disease	*N. americanus*	No effect on clinical disease. Adverse effects include reactions at the injection site and enteritis	[214]

CD: Crohn's disease; UC: ulcerative colitis

1.5 Conclusion

Ascariosis remains the most common helminth infection and an important health problem both in humans and in pigs, despite frequent treatments. Given the important consequences of the *Ascaris* immune response, and the potential benefit of vaccines for the control of this parasite, there is a need for a better understanding of the immunological changes contributing towards protection. What knowledge we have about protective responses against helminths mostly comes from studies with rodent models and may not be applicable to the situation in natural hosts. Therefore immunological studies in pigs are required. Protective immunity against invading larvae is a common observation after chronic exposure of pigs with *A. suum*, yet there is no information on how these larvae are prevented from penetrating the intestine. In addition, the distribution of *A. suum* in pigs becomes greatly aggregated after a process of expulsion or self-cure in the small intestine. Unraveling these two processes might hold the key to the development of immunological control strategies against *Ascaris* and could help to explain epidemiological phenomena in *A. lumbricoides* infected people as well.

Objectives

In order to design and evaluate potential vaccine candidates against *Ascaris suum* , it is essential to understand the immunological basis of protection. The literature review showed that the strongest levels of protection are situated at the level of the gut. However, the knowledge of the effector mechanisms or how they are induced is currently insufficient. It is important to assess the immune response where it is localized. Therefore, in this thesis the goal was to unravel the effector mechanisms responsible for the intestinal protection against *A. suum*.

The self-cure reaction forms the first strong defense against *A. suum* in primary infections and is directed against the L4. The first objective was therefore to identify the expulsion mechanism during primary infections with *A. suum* and to assess the contribution of the hepato-tracheal migration to the expulsion.

Chronic infection with *A. suum* induces strong pre-hepatic immunity. The second objective of this PhD was to investigate the immunological changes in immune pigs and the effector mechanism(s) that prevent larval penetration of the intestine after chronic exposure.

Functional studies in pigs are limited due to a lack of species-specific immunological reagents, such as antibodies and recombinant proteins. Therefore, the third objective was to determine, based on the findings of the pre-hepatic barrier in pigs, if mice also build up a similar pre-hepatic immunity and could be used as a model for the pre-hepatic barrier in pigs.

The final objective was to assess if the results obtained here can be applied to vaccine development.

Chapter 2:

The mucosal immune response during the expulsion of *A. suum*

Based on:

Masure D, Wang T, Vlaminck J, Claerhoudt S, Chiers K, Van den Broeck W, Saunders J, Vercruysse J, Geldhof P. (2013) The Intestinal Expulsion of the Roundworm *Ascaris suum* is Associated with Eosinophils, Intra-Epithelial T Cells and Decreased Intestinal Transit Time. PLoS Negl Trop Dis 7(12): e2588

2.1 INTRODUCTION

After ingestion, the *A. suum* eggs hatch and release third stage larvae in the intestine. The larvae will penetrate the caecal or colonic wall, reach the lungs via the liver, after which they will be coughed up and swallowed back in. Once back in the small intestine, they will develop first into L4 and then into adults, preferentially inhabiting the proximal half of the small intestine [27]. However, before the *A. suum* L4 can become adults, an expulsion mechanism, termed self-cure, causes the elimination of most of the L4 from the small intestine between 14 and 21 DPI. This self-cure occurs in primary infection, is independent of the inoculation dose and is the cause of the overdispersion of *A. suum* in pigs [27]. The effector mechanisms driving this elimination are largely unknown. The aim of this study was to investigate in more detail the gastro-intestinal immune response leading to the elimination of *A. suum* L4 from the small intestine and the contribution of the hepato-tracheal migration to the expulsion of the parasite.

2.2 MATERIALS AND METHODS

2.2.1 Animals and parasites

All animal experiments were conducted in accordance with the E.U. Animal Welfare Directives and VICH Guidelines for Good Clinical Practice, and ethical approval to conduct the studies were obtained from the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2011/086, EC2009/145 and EC 2013/51).

Helminth naive Rattlerow Seghers hybrid piglets of 10 weeks old were used. The animals were routinely checked for *A. suum* by coprological examination and at the start of the experiment 2 sentinel animals from the same pen were euthanized to confirm absence of larval stages. The animals had access to feed and water *ad libitum.*

A. suum eggs were obtained from gravid female *A. suum* collected at the local abattoir from pigs that were being processed as part of the normal work of the abattoir. Embryonation was confirmed by way of light microscopy after incubation in 0,1% KCr_2O_7 for 2 months.

2.2.2 Experimental design

Experimental infection with *A. suum* eggs

Four groups of 5 pigs were used. The animals of 3 groups were infected via oral intubation with 2000 embryonated *A. suum* eggs each and euthanized 10, 17 and 28 days post

infection (DPI), respectively. One group was left uninfected and served as the negative control group.

Lung stage larvae transfer experiment

Three donor animals were infected with 200.000 embryonated *A. suum* eggs and euthanized with a captive bolt pistol 9 days post infection. This time was chosen to avoid the chance that larvae would not have developed enough to be ready for the change in environment. The lungs of the animals were collected, minced and the homogenate put on a Baermann funnel incubated at 37 °C to collect the lung stage larvae. Preliminary trials showed that the transfer of larvae resulted in the manifestation of around 50% after 2 days. Within 2 hours after necropsy of the donor animals, 15 naive pigs were orally infected with 1000 lung stage larvae each, in order to have a similar number of larvae in the small intestine as in the *A. suum* egg infected animals. Five animals were killed each at day 2, 7 and 18 post transfer (DPT) respectively.

L4 intestinal larvae transfer experiment

Three donor animals were infected with 25.000 embryonated *A. suum* eggs and euthanized 14 days post infection with a captive bolt pistol. The content of the small intestines of the animals were collected and the small intestine was washed with 37°C PBS to collect any remaining larvae. The content of the small intestine and the washing was sieved with a 122 µm sieve and put on a Baermann funnel with PBS at 37°C to collect the intestinal L4. Within 2 hours after necropsy of the donor animals, the L4 were collected from the Baermann funnel and 15 naive pigs were orally infected with 1000 L4 each. Five animals were killed each at day 2, 7 and 18 DPT respectively.

2.2.3 Post mortem procedure

All animals were fasted before necropsy and then killed with a captive bolt pistol, exsanguinated and the intestines were removed. Samples for RNA extraction and histological analysis of the jejunum were taken 3 meter caudal to the pylorus. The small intestine was further divided in duodenum, jejunum and ileum. The contents of the 3 parts of the small intestine were collected separately and the intestines were rinsed twice with water. The washing was added to the corresponding content. The content plus washing was passed through a 122 µm sieve and *A. suum* larvae were counted under a microscope.

2.2.4 RNA extraction, cDNA synthesis and real time PCR assays.

Jejunal tissue was immediately snap frozen in liquid nitrogen and stored at -80 until RNA extraction. RNA extraction was performed using Trizol reagent (Invitrogen), combined with an RNeasy mini kit (Qiagen). A DNase treatment was included to prevent genomic contamination. RNA integrity was assessed using a Biorad Experion with a standard sensitivity chip. cDNA was synthesized with a Biorad cDNA synthesis kit, starting from 1 µg of RNA.

Primers for the real time PCR reactions were designed with the Primer3 software [215] and are listed in the Appendix. PCRs were run using Fast SYBR Green Master Mix (Applied Biosystems) on an AB StepOnePlus Real-Time PCR System. Primer specificity was confirmed by observing the melting curve and by sequencing PCR products. Gene expression levels were normalized based on housekeeping genes selected using Genorm [216]. Housekeeping genes tested were: *b2m*, *gapdh, hmbs, rpl4, tbp1* and *ywhaz*. The genes selected for normalization were *hmbs* and *tbp1*. Gene transcription levels are expressed as fold change compared to uninfected controls.

2.2.5 Histological analysis

Tissue samples of the jejunum without apparent peyer's patches taken 3 meter caudal to the pylorus were washed in PBS, processed with the Swiss roll technique [217] and fixed in either 10% formaldehyde or Carnoy's fixative for 24h. Carnoy's fixative was used for mucosal mast cells because this fixative leads to the best staining of these cells [218]. After fixation, the tissues were dehydrated by passage through a series of graded alcohol dilutions, followed by embedment in paraffin. Tissue samples were cut in 4 µm sections. To assess general histopathological damage and the accumulation of eosinophils, formaldehyde fixed samples were routinely stained with haematoxylin-eosin. The length of the villi and depth of the crypts in the jejunum were measured for 20 villi and their corresponding crypts under a microscope using a calibrated micrometer at 100x magnification. Mucosal eosinophils were counted at 400x magnification on 10 fields corresponding to 0.162 mm^2. Mast cells were counted on carnoy fixed, toluidine blue stained slides at 200x magnification using a weibel2 graticule [219]. For immunohistochemistry: formaldehyde fixed, paraffin embedded sections were rehydrated and an antigen retrieval step with citrate buffer was included. Endogenous peroxidase activity was blocked using 1% hydrogen peroxide. Sections were stained with rabbit anti-human CD3 (Dakocytomation A/S) to detect intra-epithelial lymphocytes (IELs) or mouse anti-human MAC387 (Serotec) to stain macrophages. Biotinylated secondary antibodies (Dakocytomation A/S) were added and staining was performed using the peroxidase streptavidine complex (Dakocytomation A/S), diaminobenzidine tetrahydrochloride (DAB,

Sigma–Aldrich) and H2O2. Sections were counterstained with haematoxylin. Macrophages were counted at 200x magnification using a weibel2 graticule [219] while IELs were counted for 5 villi randomly and expressed as number of IELs per 100µm villus epithelium.

2.2.6 ELISA

The *Ascaris*-specific IgA, IgG, IgE and IgM levels in the serum against the L4 were determined using an indirect ELISA. L4 were collected from the small intestines of animals at 14 DPI. The larvae were ground in liquid nitrogen to a fine powder and subsequently dissolved in PBS to which a 1:1000 dilution of protease inhibitor cocktail (Sigma-Aldrich) was added. After incubating for 2 hours at 4°C, the extract was centrifuged at 10.000 g for 10 minutes. The supernatant was passed through a 0.22 µm filter and stored at –70°C until use. This extract is being referred to as AsL4.

Plates were coated overnight at 4°C with 5 µg/ml AsL4 in 0.05M sodium bicarbonate buffer (pH 9,6). Serum was added at a concentration of 1/100 and HRP-conjugated goat anti-pig IgM (Thermo Scientific), IgG and IgA (Bethyl laboratories) were used as conjugate at a dilution of 1:50000, 1:10000 and 1:5000, respectively. For the detection of pig IgE antibodies, a cross-reacting mouse anti-human IgE antibody [220](Sigma-Aldrich) at 1:5000 and HRP-conjugated rabbit anti-mouse IgG at 1: 10000 were used. All measurements were performed in duplicate.

2.2.7 Eosinophil degranulation assay

The purification of circulating eosinophils and the degranulation assay were performed as previously described [221]. Reactive oxygen species production was measured using a chemiluminescence assay with PMA 5µg/ml as positive control, HBSS with Ca^{2+}/Mg^{+} as negative control or 1mM SIN-1 as a ROS donor. Eosinophils from 1 pig were seeded in a 96-well plate at 2×10^5 cells/well in 100 µl luminol (1 mM) in HBSS with Ca^{2+}/Mg^{+}. After 5 min of background measurement at 37 °C, 10,20 or 50 *A. suum* L4 collected from infected pigs at 14 DPI were added in 100 µl HBSS, as well as the control agents. To test if there was antibody or complement dependent degranulation, serum from 5 uninfected and 5 animals at 17 DPI was pooled and added at 1/100 dilution. Heat inactivation of serum was done at 58°C for 30 minutes. ROS-production was measured during 120 min in the integration mode. Each condition was performed in triplicate and ROS-production was expressed as the fold change in

relative light units (RLU) compared to negative controls (HBSS). The experiment was performed 3 times independent from each other.

2.2.8 Small intestinal transit time

Eleven pigs were infected with 3000 *A. suum* eggs. Ten days after infection, 2 animals were euthanized to confirm batch infectivity. The small intestinal worm counts in these two pigs were 2019 and 2315. Small intestinal transit time was measured in the remaining 9 pigs at 5 days before infection and at 9, 17 and 35 days after *A. suum* infection. The pigs were starved for 12 hours before barium sulfate was given through gastric intubation at a dosis of 4 ml/kg bodyweight. Lateral and dorso-ventral radiographs were taken every half hour until barium sulfate was located in the colon. If a radiograph was inconclusive about the presence of contrast material in the colon, it was repeated after 10 minutes. The time it took for the barium to reach the colon was recorded as the small intestinal transit time. After the last transit time measurement, the animals were euthanized and worms were collected.

2.2.9 Statistical analysis

For statistical analysis, GraphPad Prism software (v5.0c) was used. Because we could not assume Gaussian distribution, differences between the infected groups and uninfected animals were tested using a nonparametric Kruskal-Wallis test with Dunn's multiple comparison post hoc tests. For the transit time measurements, a repeated measures Friedman test was used with a Dunn's multiple comparison post hoc test.

2.3 RESULTS

2.3.1 Bypassing the hepato-tracheal migration does not impair the self-cure reaction

Animals were either orally infected with 2000 *A. suum* eggs or with 1000 9-day-old L3 that were collected from the lungs of donor animals. The worm counts are summarized in Table 2.1. For egg infected pigs, the average total worm count at 10 DPI was 312, with 19% of larvae present in the duodenum, 73% in the jejunum and 9% in the ileum. At 17 DPI, the average total number of larvae present was reduced to 19, most of which were present in the ileum. By 28 DPI, all animals were negative for *A. suum*. When animals were orally infected with lung larvae obtained from donor animals, they were still able to eliminate the larvae. At 2 DPT, 38% of transferred larvae were recovered, almost exclusively from the jejunum, indicating a successful transfer. At 7 DPT, although the total number of worms was similar to that of 2 DPT, 50 % of the larvae were now present in the ileum. At 18 DPT, no larvae could be recovered from the animals.

Table 2.1: Worm counts in the small intestine during an infection with 2000 A. *suum* eggs or 1000 L3 lung stage larvae. Numbers shown are the average (SD) of 5 animals.

		A. suum* age	**Duodenum**	**Jejunum**	**Ileum**	**Total**
Egg infection	10 DPI	10	58 (64)	227 (82)	27 (48)	312 (90)
	17 DPI	17	0 (0)	5 (6)	14 (20)	19 (26)
	28 DPI	28	0 (0)	0 (0)	0 (0)	0 (0)
L3 transfer	2 DPT	11	1 (1)	384 (35)	26 (23)	411 (35)
	7 DPT	18	0 (0)	220 (374)	267 (337)	487 (376)
	18 DPT	29	0 (0)	0 (0)	0 (0)	0 (0)

* in days; hatching out of the egg = 0 days

2.3.2 *A. suum* specific antibodies are not essential in the self-cure response

Ascaris L4 specific IgA, IgE, IgG and IgM antibody levels in serum of *A. suum* egg or lungs stage infected animals were measured using an indirect ELISA (Figure 2.1). During infections with eggs, AsL4 specific IgA, IgM and IgG levels were increased from 10 DPI onwards, whereas AsL4 specific IgE levels were only detectable in serum at 17 DPI. Although the self-cure reaction occurs 7 days after L3 lung stage larvae are transferred, no statistically significant

increases of AsL4 specific IgA, IgM, IgG and IgE antibodies could be detected at this time. IgM, IgG and IgE levels were significantly increased only at 18 DPT whereas no change in serum IgA levels was observed.

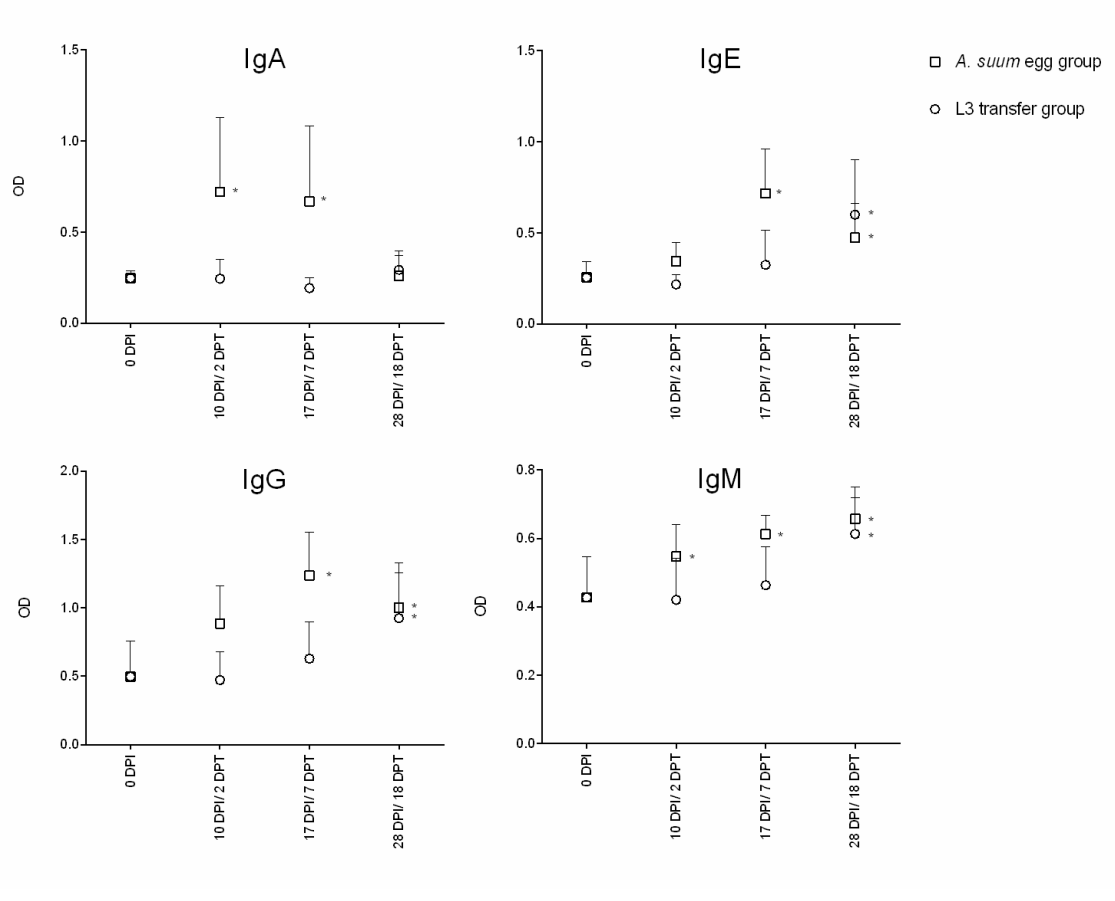


Figure 2.1: Serum antibodies are present during expulsion in infections with A. *suum* eggs, but not when larvae are transferred. Values represent the mean + SD of 5 animals. * p< 0.05 compared to uninfected controls

2.3.3 L4 transferred larvae are driven distally in the small intestine, but counteract this effect by 18 DPT

We examined whether the release of antigens during the molt from L3 to L4 that occurs around D12 is necessary to trigger the expulsion of the larvae. Therefore we collected 14-day-old L4 intestinal larvae from donor animals and transferred 1000 larvae orally into naïve animals. The number of larvae in each section of the small intestine was counted at 2, 7 and 18 days post transfer. The larvae counts are summarized in Table 2.2. At 2 DPT around 60% of the transferred larvae could be recovered and 87% of the recovered larvae are present in the jejunum. Five days later the total number of larvae in the small intestine is similar to that at 2

DPT, but most larvae are present in the terminal part of the small intestine. At 18 DPT the total number of larvae has not decreased compared to 7 DPT, but 90% of larvae are now present again in the jejunum, indicating that they could counteract the peristaltic movement to inhabit the proximal region of the small intestine.

Table 2.2: Worm counts in the small intestine after transfer of 1000 A. *suum* L4. Numbers shown are the average (SD) of 5 animals. Table 2.1 is incorporated in grey for reference.

		A. suum* age	**Duodenum**	**Jejunum**	**Ileum**	**Total**
Egg infection	10 DPI	10	58 (64)	227 (82)	27 (48)	312 (90)
	17 DPI	17	0 (0)	5 (6)	14 (20)	19 (26)
	28 DPI	28	0 (0)	0 (0)	0 (0)	0 (0)
L3 transfer	2 DPT	11	1 (1)	384 (35)	26 (23)	411 (35)
	7 DPT	18	0 (0)	220 (374)	267 (337)	487 (376)
	18 DPT	29	0 (0)	0 (0)	0 (0)	0 (0)
L4 transfer	2 DPT	16	1 (1)	521 (208)	73 (54)	595 (201)
	7 DPT	21	0.2 (0.4)	102 (148)	317 (262)	419 (369)
	18 DPT	32	24 (53)	409 (175)	19 (43)	452 (199)

* in days; hatching out of the egg = 0 days

2.3.4 Self-cure is associated with eosinophilia and intra-epithelial T cells

The results of the histological parameters investigated are shown in Figure 2.2. To assess general histopathological changes, villus length and crypt depth were measured. Villus/crypt ratios decreased shortly after contact with *A. suum* larvae, due to a blunting of the villi. Although this effect was observed in both infections with eggs and L3 and L4, it was only temporary, as the villi recovered by 17 DPI/7 DPT. At 17 DPI, coinciding with the expulsion of the parasite, there was a significant increase in mucosal eosinophils. After elimination of the larvae, i.e. 28 DPI, the number of eosinophils decreased to a level similar to that before the infection. A similar pattern was observed following transfer of L3, with a peak in eosinophil counts at 7 DPT. The transfer of L4 resulted in high eosinophil numbers at 7 DPT and 18 DPT.

Mucosal macrophages followed a similar pattern as eosinophils in *A. suum* egg infected pigs, with a 9-fold increase in the number of macrophages per mm^2 mucosa at 17 DPI that returned to baseline level at 28 DPI. In contrast to normal infections, in both L3 of L4 transfer

infections, no increase in the number of macrophages was observed at any of the time points investigated. No statistically significant changes were observed in the number of intestinal mast cells in any of the infection experiments. Finally, intra-epithelial T cells were significantly elevated in all infection experiments at the time when larvae were being driven towards the distal end of the small intestine, i.e. at 17 DPI/7DPT. In the *A. suum* egg infections and in the L3 transfer experiment, IELs were still elevated even after the worms were eliminated, while in the L4 transfer experiment the IELs returned to normal levels at 18 DPT.

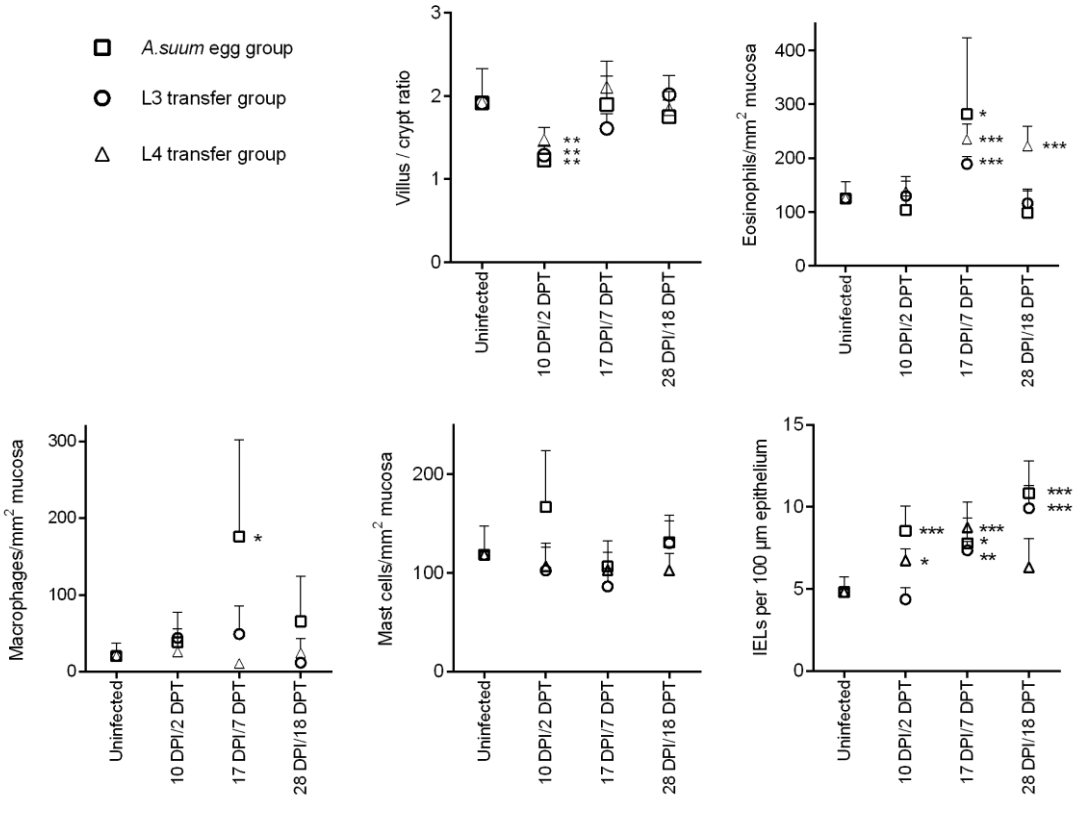


Figure 2.2: Histopathological findings during infections with A. *suum* eggs and infections with L3 or L4 transferred larvae. Values are mean + SD of 5 animals. * p<0.05 versus control group; ** p<0.01 versus control group; *** p<0.001 versus control group

The results of the quantitative PCR analysis on a set of 25 genes for egg infected, L3 and L4 transferred animals are summarized in Table 2.3 With egg infections, the gene expression pattern was polarized towards a Th1-like response, with significant upregulations observed for *ifng, il12a, il12b, stat4* and *nos2a.* In contrast, none of the Th2 related genes were significantly impacted during infection with *A. suum* eggs. In the L3 and L4 transfer experiments, more mixed responses were measured. In addition to some Th1 markers, an increase in the typical Th2 transcripts *il4* and *il13*, together with increases in regulatory transcripts, such as *foxp3,* and *tgfb* were observed.

For all infection experiments there was an upregulation of genes associated with cytotoxic cells, mainly granzyme A and B, perforin 1 and NKG2D. Additionally, several eosinophil-associated genes were induced, such as those encoding for eosinophil peroxidase, eotaxin 1, eotaxin receptor and IL-5 receptor alpha.

Table 2.3: RNA transcription profile of A. *suum* egg infected animals and L3 and L4 infected animals.

Results are shown as average fold change versus uninfected controls.

Gene	Description	Egg infection			L3 transfer			L4 transfer		
		10 DPI	17 DPI	28 DPI	2 DPT	7 DPT	18 DPT	2 DPT	7 DPT	18 DPT
	Th1 associated									
il12a	Interleukin 12 subunit p35	2.29**	1.28	1.48	1.15	1.98	1.17	1.45	0.84	0.56
il12b	Interleukin 12 subunit p40	2.28*	2.63	2.12*	2.05*	2.11**	1.98	1.26	1.09	1.26
nos2a	Nitric oxide synthase 2a, inducible	2.11	5.12*	8.81**	1.19	1.24	0.97	2.41	1.35	1.43
ifng	Interferon γ	4.30**	2.91*	3.49*	1.31	2.27	1.02	2.65*	1.44	1.03
tbx21	T-Box 21, T-bet	2.28*	1.63	2.05	2.35*	1.59	1.83	1.37	1.04	1.06
stat4	Signal transducer and activator of transcription 4	1.66*	0.96	1.70*	1.31	1.15	1.33	6.49*	3.84*	3.42*
	Th2 associated									
il4	Interleukin 4	1.59	0.76	1.16	1.24*	0.91	0.92	1.04	0.93	1.35
il5	Interleukin 5	1.06	0.92	0.84	0.79	0.91	0.80	0.78	0.59*	0.53*
il13	Interleukin 13	0.52	1.19	1.32	3.36	1.50*	1.60*	1.38	2.66	40.25**
stat6	Signal transducer and activator of transcription 6	0.74	0.77	0.86	0.69	1.39	0.89	0.76	0.74	0.81
il25	Interleukin 25	1.36	1.20	1.95	1.27	0.83	0.89	0.41*	0.92	1.59
il33	Interleukin 33	1.24	1.07	1.05	0.73	0.50	0.54	0.69	0.71	1.11
cma1	Mast cell chymase 1	0.87	0.68	0.94	0.80	0.54*	1.04	0.89	0.68	0.68*
	Treg									
foxp3	Forkhead box P3	0.89	0.98	1.47	1.55	1.64*	1.95*	1.54	2.10	2.05*
tgfb	transforming growth factor β	0.99	0.99	1.15	1.33	1.89*	1.32	1.76**	1.92	1.04
il10	Interleukin 10	1.13	1.74	1.33	0.84	1.25	0.59**	1.84	1.65	1.33
pparg	peroxisome proliferator-activated receptor gamma	0.42*	0.81	1.35	2.60*	0.83	1.11	0.74	0.89	1.53
	Cytotoxic cell associated									
nkl	NK-lysin	0.52	1.57	1.16	0.54	0.71	0.92	1.97	1.90	0.97
gzma	Granzyme A	1.61**	1.46	2.74**	2.03	2.05*	2.25	1.59*	1.46	0.24**
gzmb	Granzyme B	4.14*	2.66	3.69	2.58**	1.32	2.34	3.74**	2.11*	1.04
prf1	Perforin 1	1.50	0.83	1.61	1.67*	1.68**	1.70*	2.61*	1.86	1.37
klrk	killer cell lectin-like receptor subfamily K, NKG2D	2.71**	1.11	2.28*	1.25	1.02	1.42	0.73	0.41	0.44*
	Eosinophil associated									
epx	Eosinophil peroxidase	2.64*	0.33	0.41*	0.48	0.44	0.68	0.43	0.73	3.47**
ccl11	Chemokine (C-C motif) ligand 11, Eotaxin 1	1.03	0.79	0.98	1.25	2.17*	1.38	0.88	0.79	0.87
ccr3	Eotaxin receptor	2.28*	1.32	0.79	0.86	0.83	1.06	0.82	0.88	0.94
il5ra	Interleukin 5 Receptor, alpha	1.21	0.59	1.09	1.72	1.19	2.67*	1.42	1.32	1.33

* $p < 0.05$ compared to uninfected controls

** $p < 0.01$ compared to uninfected controls

2.3.5 Eosinophils do not degranulate in response to L4

Results of the eosinophil degranulation assay are shown in Figure 2.3. Measurement of the reactive oxygen species (ROS) indicated that the eosinophils did not degranulate after incubation with *A. suum* L4, even in the presence of serum from infected animals. To exclude the possibility that L4 would capture ROS released in the medium, *A. suum* L4 were cultured together with SIN-1, a molecule that releases NO and ROS. *A. suum* L4 together with SIN-1 in medium gave no significant differences in measured ROS compared to SIN-1 without L4 (1636 ± 704 RLU versus 977 ± 344 RLU, respectively).

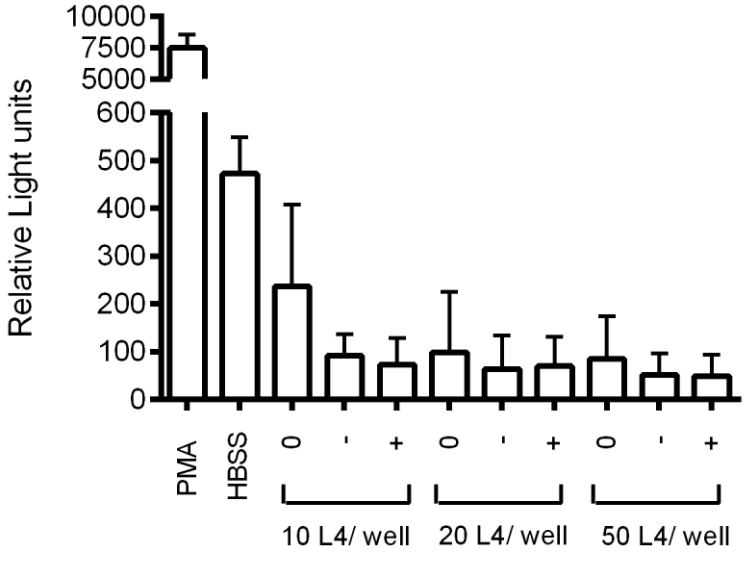


Figure 2.3: No ROS release by eosinophils in response to A. *suum* L4. Data are shown as the mean RLU ± SEM of three independent experiments. PMA (5 µg/ml) and HBSS were used as a positive and negative control, respectively. 0: no serum added to the wells; -: serum from uninfected animals added to the wells; +: serum from 17 DPI animals added to the wells. ROS: Reactive oxygen species.

2.3.6 Small intestinal transit time is decreased during self-cure

The small intestinal transit time was measured by following barium sulfate passage through the small intestine before infection and at 9, 17 and 31 days post infection with 3000 *A. suum* eggs (Figure 2.4). There was a small, non-significant increase in the small intestinal transit time at 9 DPI compared to their pre-infection transit time. At 17 DPI the small intestinal transit time was significantly lower than before the infection. By 35 DPI, 8 out of 9 animals were *A. suum* negative and one pig had 29 *A. suum* worms. At this time, the intestinal transit time was still somewhat lower than before infection, but not significantly.

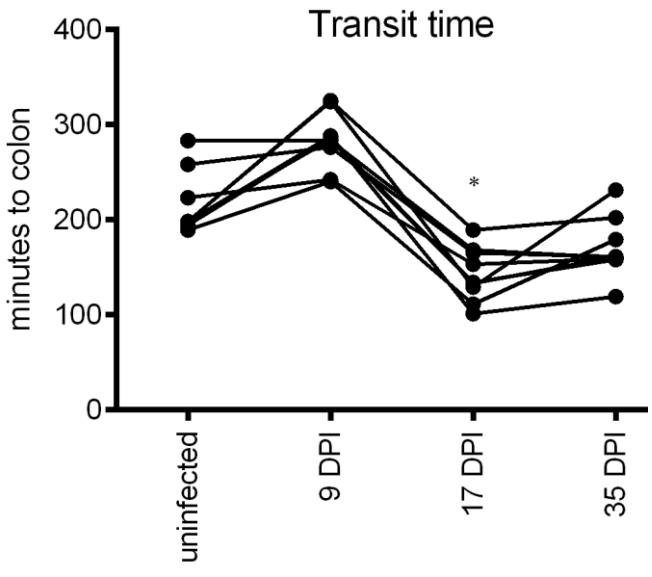


Figure 2.4: The small intestinal transit time decreases during self-cure. The time for the barium solution to reach the caecum or colon after gastric intubation was recorded in 9 animals before and during A. *suum* infection. * Transit time significantly different ($p<0.05$) than uninfected control group.

2.4 DISCUSSION

Here we investigated the immunological basis of the self-cure reaction during primary *A. suum* infections. In addition, we studied the influence of the migration of the larvae through the body on the self-cure reaction. By transferring lung stage *A. suum* larvae from one animal to another, we have a simple model to study the effect of tissue migration on the initiation of the self-cure response. In animals bypassing the passage through the liver and lungs, the self-cure reaction occurred with the same kinetics as animals receiving infectious eggs, i.e. around 7 days after contact with the small intestine. Furthermore, both in infections with eggs and with lung stage larvae all larvae were expelled by 18 days of exposure to the small intestine. A previous study by Jungersen *et al*. led to the speculation that the expulsion of *A. suum* might be affected when the liver is bypassed. They injected *in vitro* hatched L3 intravenously in pigs and found a higher percentage of animals harboring adult *A. suum* at 70 DPI than what is usually observed, even though at 14 DPI there were comparable numbers of L4 between intravenously and orally infected animals [29]. Unfortunately, not enough time points and control groups were included to confirm if previous priming in the liver was indeed required to eliminate the larvae from the small intestine. The results presented here now unequivocally

show that the self-cure mechanism is a locally triggered phenomenon, independent of previous passage through the liver or lungs.

Additionally we sought to determine whether antibodies play an important role during self-cure. Since in normal infections there are already *A. suum* specific antibodies present at 10 DPI, it was previously suggested that antibodies played an important role in the expulsion of the parasite [30]. Although we confirm the presence of antibodies during self-cure in egg infected pigs, the absence of *A. suum* specific antibodies when larvae were being expelled in animals that received lung stage larvae would indicate that *A. suum* specific antibodies do not have a major role in the early self-cure against *A. suum*. This is further supported by the observation that when L4 are transferred, most larvae were being driven to the distal end of the small intestine around 7 DPT. Therefore also the release of antigens during the molt from L3 to L4 prior to the self-cure does not appear to be an essential trigger of the expulsion. However, one of the drawbacks of this study is that we measured antibody levels solely in the serum. It would be interesting in future studies to also include the mucosal antibody response. Furthermore it remains possible that non-specific antibodies present in the mucosa could contribute to *A. suum* expulsion [222]. In addition, although it is not clear to what extent maternal antibodies would still be present in pigs of 12 weeks age, the passive transfer of antibodies has been shown to contribute to parasite expulsion [223].

Remarkably, and in contrast to the transfer of lung stage larvae, the L4 transferred larvae were able to return to the jejunum by 18 DPT. By this time, the larvae are already 32 days old, i.e. an age at which in natural infections they are also not affected by the self-cure response anymore. It appears that these larvae, once they have developed to a certain stage, are able to counteract the self-cure response. This is in agreement with a microarray study on larvae in the jejunum and ileum during self-cure, where they found that only the more metabolically active larvae could remain in the jejunum [224]. However, from our results it is clear that the larvae present in the ileum are still alive and can return to the jejunum if they are active enough. This also contradicts the suggestion that self-cure is a parasite-driven suicide phenomenon based on the density of the parasites [225]. It indicates that there is a fine balance between the host that is trying to drive the parasite out and the parasite's ability to counteract this effort. This also explains why adults can remain in the small intestine for months or years without being driven out.

The histological and RNA transcription analysis showed some common characteristics associated with the expulsion of larvae in all the experiments performed here. The peak of expulsion coincided with a peak in mucosal eosinophils and IELs, suggesting an important role

for these cells in the innate defense against *A. suum*. Eosinophils can directly respond to a broad spectrum of pathogens through signaling via Toll like receptors, complement receptors and immunoglobulin receptors. In order to investigate whether eosinophils responded directly to *A. suum* L4, we monitored the release of reactive oxygen species from the eosinophilic granules after co-incubating the cells with the larvae. In contrast to results obtained with freshly hatched L3, where eosinophil degranulation occurred quickly after contact with L3 in the presence of serum of either infected or uninfected animals ([221], see chapter 3), eosinophils did not respond directly to *Ascaris* L4, even in the presence of serum. In addition, the larvae in the L4 transferred animals at 18 DPT were seemingly unharmed, even though eosinophil numbers remained high. These results may indicate that the L4 are expressing inhibitory factors that prevent eosinophil degranulation and that eosinophils are better equipped to deal with tissue-residing larvae, rather than lumen dwelling ones. This seems indeed the case for many helminth infections [226]. The function of eosinophils in the defense against L4 might also be of an indirect nature. Since the eosinophils were located deep in the mucosa, this assumption seems indeed likely. Through the release of preformed cytokines, chemokines, lipid mediators and cytotoxic molecules, eosinophils could quickly initiate a potent immune response after recognition of pathogen-associated molecular patterns, which in turn may lead to the initiation of the expulsion of *A. suum*.

Another important finding was a clear increase in the number of intra-epithelial T cells during the course of the infection. Although the IELs were not phenotyped, RNA transcription data would suggest that it was the cytotoxic T cell subset that was the most impacted, as there was an overall induction of molecules associated with cytotoxicity such as granzymes, perforin and NKG2D, all of which have been found to be expressed by IELs [227]. One of the functions of IELs is epithelial repair [227]. IELs may be activated in response to damage caused by the larvae. For example, Granzyme B has been found to be correlated with villus damage in helminth infections [228]. Our findings support this, as in all our experiments villous blunting and granzyme B upregulation were observed shortly after contact with *A. suum* larvae. The negative effect of *A. suum* on the intestinal structure might have important consequences for humans suffering from *A. lumbricoides* as well, as it might help to explain the malabsorption often associated with these infections. Whether there is a direct effect of the IELs on the expulsion of the parasite deserves further attention, since resistance against helminth infections in sheep has been associated with genes involved in cytotoxicity [229]. Increased epithelial turnover and shedding caused by cytotoxic cells might make it harder for the small L4 to stick to the mucosa. Interestingly, IELs were lower in the L4 transferred group at 18 DPT, which may indicate an active regulation of the immune response by these larvae.

Mast cells and basophils have previously been associated with *A. suum* infections [110,111,221]. Repeated infections induced blood basophilia and intestinal mastocytosis, and these cells responded to stimulation with L3 or L4 secretory antigens by releasing histamine [110,111]. The maximum histamine release occurred between 14 and 21 days after daily exposure, therefore it has been suggested that these cells played an important role during self-cure [230]. However, only basophils or mast cells that had previously been exposed to *Ascaris* released histamine following contact with L3 or L4 secretory antigens [110,111]. We also show here that in contrast to experiments with repeated infections, mast cells were not induced in the small intestine after primary infections, suggesting that basophils or mast cells may only play a role in protection against secondary infections.

Interestingly, the local cytokine response in the jejunum seemed to be greatly impacted by the initial migration through the body. Naturally infected animals were more biased towards a Th1 type response with macrophages, while in both the L3 and L4 transfer experiments there was a much more mixed Th1/Th2 response and no recruitment of macrophages. Especially the animals infected with L4 showed high *il13* transcription at 18 DPT, which may indicate that the initial Th1 bias shifts towards a Th2 response as the infection progresses.

Together, these results suggest that the expulsion mechanism does not target the *A. suum* larvae directly. One possible mechanism by which larvae could be eliminated from the small intestine is increased gut movement. We show here that animals infected with *A. suum* indeed have decreased transit time around 17 DPI. This decrease is in agreement with a previous study showing an increase in smooth muscle contractility from 14 to 21 DPI and an increase in fluid secretion *ex vivo* [31]. Any increase in gut movement would indeed make it more difficult for the relatively small larvae to remain in the small intestine and may in fact be a universal mechanism of expulsion of intestinal lumen dwelling nematodes, as changes in intestinal smooth muscle contractility have been identified in *Cooperia oncophora* infected calves and *Trichinella spiralis* and *Nippostrongylus brasiliensis* infected mice [114,231,232]. Studies in mice have shown that the helminth induced increase in smooth muscle contractility is signaled through IL-4 or IL-13 [114,143,233], which could explain why it is a common observation with helminth infections. Of particular interest is the contribution of alternatively activated macrophages on the regulation of smooth muscle contractility [114]. While we could only detect an increase in macrophages in the A. *suum* egg infected animals, it remains possible that changes in the activation state of macrophages contribute to the change in smooth muscle contractility and deserves research.

Taken together, this study indicates that the self-cure is a locally initiated mechanism. As part of a weep and sweep response, faster gut movement will make it harder for the larvae to remain in the small intestine. This effect can probably be overcome once *A. suum* larvae have developed to a point where they are large and active enough to counteract the increased peristaltic movements. Eosinophils and intra-epithelial T cells appear to play a pivotal role since they are consistently associated with self-cure, but further research is needed to elucidate how these cells operate in order to induce the weep and sweep response.

Chapter 3:

The pre-hepatic response against *A. suum*

Based on:

Masure D, Vlaminck J, Wang T, Chiers K, Van den Broeck W, et al. (2013) A role for eosinophils in the intestinal immunity against infective *Ascaris suum* larvae. PLoS Negl Trop Dis 7(3): e2138.

3.1 INTRODUCTION

In addition to the self-cure reaction that occurs during primary infections, pigs build up a strong protective immunity after prolonged exposure to *Ascaris.* This protective immunity develops at the caecum and colon and prevents the infective larvae to penetrate the intestinal tissue and start their hepato-tracheal migration. This is the so-called pre-hepatic barrier [21-24,234]. Little is known of what immunological factors are associated with this protective immune mechanism. The purpose of this study was therefore to identify the key immunological events involved in the formation of the pre-hepatic barrier in the caecum of pigs following *Ascaris* infections.

3.2 MATERIALS AND METHODS

3.2.1 Animals and parasites

All animal experiments were conducted in accordance with the E.U. Animal Welfare Directives and VICH Guidelines for Good Clinical Practice, and ethical approval to conduct the studies were obtained from the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University. *A. suum* free, Rattlerow Seghers hybrid piglets of 10 weeks old were used. The animals had access to feed and water *ad libitum*.

A. suum eggs were obtained from gravid female *A. suum* collected at the local abattoir from pigs that were being processed as part of the normal work of the abattoir. After incubation in 0.1% KCr_2O_7 for 2 months, embryonation was confirmed by way of light microscopy.

For the *in vitro* tests, L3's were collected from embryonated eggs. The eggs were incubated in sodium hypochlorite for 1h, washed with PBS and then hatched by magnetic stirring with 2 mm diameter glass beads. To separate the larvae from unhatched eggs, the suspension was put on a baermann sieve covered with cotton cloth. After overnight incubation at 37 °C, the larvae were collected and put in DMEM medium supplemented with 50 u/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml kanamycin, 5 µg/ml amphotericin B and 2mM glutamine.

3.2.2 Infection trial

The experimental design is summarized in Table 3.1. Three groups of pigs were used. A first group of six pigs were fed 100 *A. suum* eggs per day in a small food bolus for 14 weeks. Eggs per gram feces (EPG) were monitored weekly from week 6 onwards. After 14 weeks the animals were dewormed with fenbendazole (5 mg/kg). Two weeks after deworming, these animals received a first challenge infection of 5000 eggs. Thirteen days later, a second challenge infection of 5000 eggs was administered. Twenty-four hours later, the animals were euthanized for sample collection. These animals are referred to as immune animals. A second group of 5 naïve animals received anthelmintic treatment 2 weeks before being infected with 5000 eggs and euthanized 14 days post infection (DPI). These animals served to compare larval counts between immune and naïve animals at 14 DPI. A third group of 5 animals received anthelmintic treatment 2 weeks before being infected with 5000 eggs and euthanized 24 hours later to compare the early immune response with the immune animals that received a challenge infection 24 hours prior to necropsy.

Animals were denied feed from 24 h before until necropsy and then killed with a captive bolt pistol, exsanguinated and the intestines were removed. Samples for RNA extraction and histological analysis were taken from the caecum. The small intestine was washed and the contents passed through a 220 µm sieve. *A. suum* larvae were counted under a microscope.

3.2.3 RNA extraction, cDNA synthesis and real time PCR assays.

Tissue samples from the caecum were taken from group 1 and 3 and immediately snap frozen in liquid nitrogen and stored at -80 °C until RNA extraction. RNA extraction was performed using Trizol reagent (Invitrogen), combined with an RNeasy mini kit (Qiagen). A DNase treatment was included to prevent genomic contamination. RNA integrity was assessed using a Biorad Experion with a standard sensitivity chip. cDNA was synthesized with a Biorad cDNA synthesis kit, starting from 1 µg of RNA.

Primers for the real time PCR reactions were designed with the Primer3 software [215], or taken from the PIN database (http://199.133.11.115/fmi/iwp/cgi?-db=PINdb&-loadframes). For a list of primers, see Appendix. PCRs were run using Fast SYBR Green Master Mix (Applied Biosystems) on an AB StepOnePlus Real-Time PCR System. Primer specificity was confirmed by observing the melting curve. PCR products were confirmed through sequencing. Gene expression levels were normalized based on housekeeping genes selected using Genorm [216]. Housekeeping genes tested were: *b2m, gapdh, hmbs, rpl4, tbp1* and *ywhaz*. The genes selected for normalization were *hmbs* and *tbp1*. Gene transcription levels are expressed as fold change in transcription levels of immune animals compared to naïve animals.

3.2.4 Histological analysis

Tissue samples were taken from animals in group 1 and 3 and were washed in PBS, processed with the Swiss roll technique [217] and fixed in either 10% formaldehyde or Carnoy's fixative for 24h. Carnoy's fixative was used for mucosal mast cells and goblet cells because this fixative leads to the best staining of these cells [218,235]. After fixation, the tissues were dehydrated by passage through a series of graded alcohol dilutions, followed by embedment in paraffin. Tissue samples were cut in 4 µm sections. To assess general histopathological damage and the accumulation of eosinophils, formaldehyde fixed samples were routinely stained with haematoxylin-eosin. Mucosal eosinophils were counted at 400x magnification on 10 fields corresponding to 0.162 mm^2. Mast cells were counted on toluidine blue stained slides at 200x magnification using a weibel2 graticule[219]. Goblet cells were counted on Alcian blue-periodic acid shiff's stain and expressed as number of goblet cells per 100 µm crypt length. For immunohistochemistry, formaldehyde fixed, paraffin embedded sections were rehydrated and an antigen retrieval step with citrate buffer was included. Endogenous peroxidase activity was blocked using 1% hydrogen peroxide. Sections were stained with mouse anti-human MAC387 (Serotec) to stain macrophages. Biotinylated secondary antibodies (Dakocytomation A/S) were added and staining was performed using the peroxidase streptavidine complex (Dakocytomation A/S), diaminobenzidine tetrahydrochloride (DAB, Sigma–Aldrich) and H_2O_2. Sections were subsequently counterstained with haematoxylin. Macrophages were counted at 200x magnification using a weibel2 graticule [219]

3.2.5 Isolation of circulating eosinophils

Peripheral blood was collected on EDTA from the jugular vein of pigs at 14 DPI. The blood was diluted with an equal amount of PBS and layered onto a discontinuous Percoll gradient (68% and 75%) and centrifuged (500 × *g* at 4 °C for 30 min) to separate the granulocyte fraction. After lysis of contaminating erythrocytes in 0.2% NaCl solution, eosinophils were separated by negative magnetic activated cell separation with mouse anti-pig CD16 antibody (AbD Serotec) and rat anti-mouse IgG1 microbeads (Miltenyi-Biotec). The purity of eosinophils was verified with a Giemsa stain after cytospin and was >95%. The cells were washed three times and resuspended at 10^6 cells/ml in RPMI-1640 without phenol-red.

3.2.6 Eosinophil degranulation assay

The degranulation assay was essentially performed as described by Donne *et al.* [236]. Reactive oxygen species production was measured using a chemiluminescence assay. Eosinophils from 1 pig were seeded in a 96-well plate at 2×10^5 cells/well in 200 µl of RPMI without phenol-red. The plates were incubated at 37 °C for 2 h in a humidified atmosphere with 5% CO_2, so that the cells could adhere to the plastic surface. The supernatant was removed and 100 µl luminol (1 mM) in HBSS with Ca^{2+}/Mg^{+} was added. After 5 min of background measurement at 37 °C, 100, 200 or 300 *A. suum* L3 in HBSS were added in 100 µl as well as the control agents (PMA 5µg/ml as positive control and HBSS with Ca^{2+}/Mg^{+} as negative control). To test if there was antibody or complement dependent degranulation, serum taken either from 5 uninfected naive or 5 immune animals was pooled and added at 1/100 dilution. Heat inactivation of serum was done at 58°C for 30 minutes. ROS-production was measured during 120 min in the integration mode. Each condition was performed in triplicate and ROS-production was expressed as the fold change in relative light units (RLU) compared to negative controls (HBSS). The experiment was performed 3 times independent from each other.

3.2.7 *A. suum* L3 viability assay

Eosinophils from 1 animal were seeded at 2.10^6 /ml in 100 µl in a 96 well plate in RPMI supplemented with 50 u/ml penicillin, 50 µg/ml streptomycin and 2mM glutamine. L3 were added at 100 per well, with or without serum pooled from 5 uninfected naïve or 5 immune pigs at a final concentration of 1/100. After 16 h of incubation, viability of L3 was assessed morphologically. Curled up or moving larvae were considered alive, while immobile, straight larvae were considered dead. Viability was expressed as the number of live larvae to the total number of larvae. Every condition was assessed in triplicate with eosinophils from 2 different animals. Negative control conditions consisted of medium without eosinophils.

Viability was also tested using an MTT assay as previously described [237]. Briefly, eosinophils were seeded at 2.10^6 /ml in a 96 well plate in 100 µl RPMI supplemented with 50 u/ml penicillin, 50 µg/ml streptomycin and 2mM glutamine. 100 L3 were added per well with or without serum pooled from 5 naïve or 5 immune pigs at a final concentration of 1/100. MTT was added at a final concentration of 1 mg/ml. After 3 h of incubation at 37°C and 5% CO_2, larvae were collected, washed and transferred to DMSO. After 1 hour the plate was read at 562 nm. Every condition was tested in triplicate.

3.2.8 Statistical analysis

For statistical analysis, GraphPad Prism software (v5.0c) was used. Mann-whitney tests were used to test differences between immune and naïve animals. The data collected from each group in the degranulation and viability assays were compared by analysis of variance (ANOVA) using the SPSS v20.0 software package.

3.3 RESULTS

3.3.1 Parasitological data

The infection protocol and worm counts are summarized in Table 3.1. Pigs in group 1 were immunized for 14 weeks with 100 eggs/day. The average EPG at 14 weeks was 4008 (range 50-11050). The animals were dewormed and then challenged with 5000 eggs. Worm counts at 14 days post challenge were compared to naïve animals receiving only anthelmintic treatment and the challenge infection (group 2). Immune pigs had a 99,7% reduction in the number of larvae that can migrate through the body and reach the small intestine compared to naïve pigs from group 2 (8 ± 4 in immune group versus 2333 ± 496 in naïve group).

Table 3.1: Infection protocol and worm counts

Group	N[a]	Immunized[b]	Challenge 1[c]	Challenge 2[d]	Worm counts[e]
1	6	yes	yes	yes	8 ± 4
2	5	no	yes	no	2333 ± 496
3	5	no	no	yes	N.D.

a: number of animals in the group

b: 100 *A. suum* eggs daily for 14 weeks

c: 5000 *A. suum* eggs 14 days prior to necropsy

d: 5000 *A. suum* eggs 24 hours prior to necropsy

e: worm counts determined in the small intestine

N.D. Not determined due to the early stage of infection

3.3.2 Cellular parameters associated with immunity

Caecal tissue was collected from naïve and immune animals 24 hours post challenge. Eosinophils, goblet cells, macrophages and mast cells were quantified and results are shown in Figure 3.1. The major effect was seen for eosinophils, with a significant almost 10-fold increase in mucosal eosinophils ($p<0.001$) in the immune animals. There was also a modest increase in goblet cells in immune animals ($p<0.05$). In addition, mast cells seemed to be specifically recruited to the submucosa and muscularis layers of the caecum ($p<0.05$) of immune animals. No significant difference was observed for the number of macrophages between naïve and immune animals.

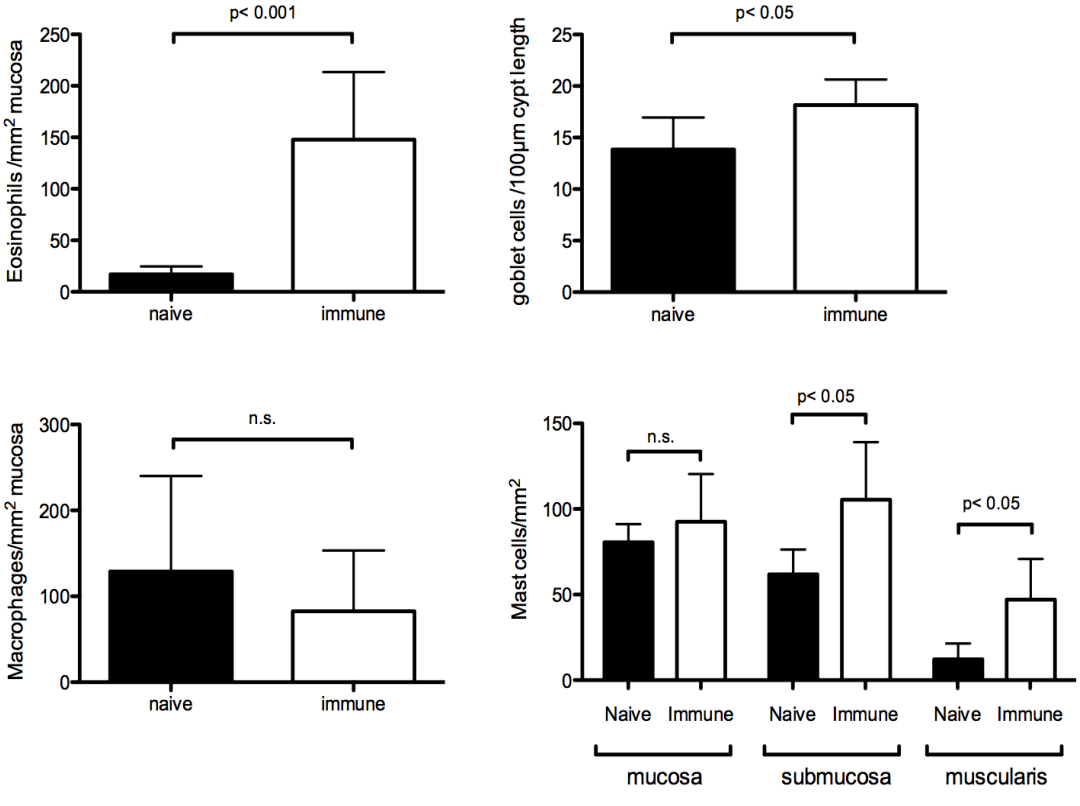


Figure 3.1: Eosinophil, macrophage, goblet cell and mast cell counts in the caecum of naïve and immune animals. Results are shown as average + SD. n.s.: not significant.

3.3.3 RNA transcription profile

The outcome of the qRT-PCR analyses is shown in Table 3.2. Significantly higher transcription levels for *c3* (complement factor 3), *ccl11* (Eotaxin), *ccr3*, *epx* (Eosinophil peroxidase), *gata3, il5, il12b, il13* and *retnlb* (Resistin Like Beta) were detected in the caecum of immune animals, whereas *muc5ac* (mucin 5AC) was significantly down regulated in immune animals compared to naïve ones. No significant differences were observed for the other genes analyzed.

Table 3.2: RNA transcription profile of the caecum.

Gene	Description	Fold change
ARG1	Arginase I	0.65
C3	Complement factor 3	1.84 *
CCL11	Chemokine (C-C motif) ligand 11, Eotaxin 1	2.50 *
CCR3	Chemokine (C-C motif) receptor 3, Eotaxin receptor	4.70 *
ELANE	Elastase, neutrophil expressed	0.89
EPX	Eosinophil peroxidase	10.2 *
FOXP3	Forkhead box P3	1.05
GATA3	GATA binding protein 3	1.62 *
IFNγ	Interferon γ	1.27
IL10	Interleukin 10	1.28
IL12A	Interleukin 12 subunit p35	0.98
IL12B	Interleukin 12 subunit p40	2.43 *
IL13	Interleukin 13	2.57 *
IL17A	Interleukin 17 A	1.87
IL33	Interleukin 33	0.71
IL4	Interleukin 4	1.12
IL5	Interleukin 5	1.65 *
ITLN2	Intelectin 2	1.70
MRC1	Mannose receptor C type 1	1.06
MUC1	Mucin 1	1.36
MUC2	Mucin 2	1.19
MUC3	Mucin 3	1.08
MUC5AC	Mucin 5 AC	0.17 *
RETNLB	Resistin-like molecule β	2.33 *
TGFB	Transforming growth factor β	0.96
TNFA	Tumour necrosis factor α	1.16

Results are shown as average fold change of transcription of immune animals versus naïve animals + SD

* $p < 0.05$

3.3.4 Eosinophil ROS production in response to *A. suum*

To investigate if eosinophils degranulated in the presence of infective L3, reactive oxygen species (ROS) release was measured in the medium for 2 hours following the addition of larvae to purified eosinophil cultures (Figure 3.2). Eosinophils or larvae alone with serum did not induce ROS release and eosinophils did not degranulate when larvae were added in the absence of serum. However, when serum from either immunized or naïve animals was added together with the L3, eosinophils released ROS in the medium. The release of ROS was proportional to the amount of larvae added. Heat-inactivation of serum reduced the amount of ROS release.

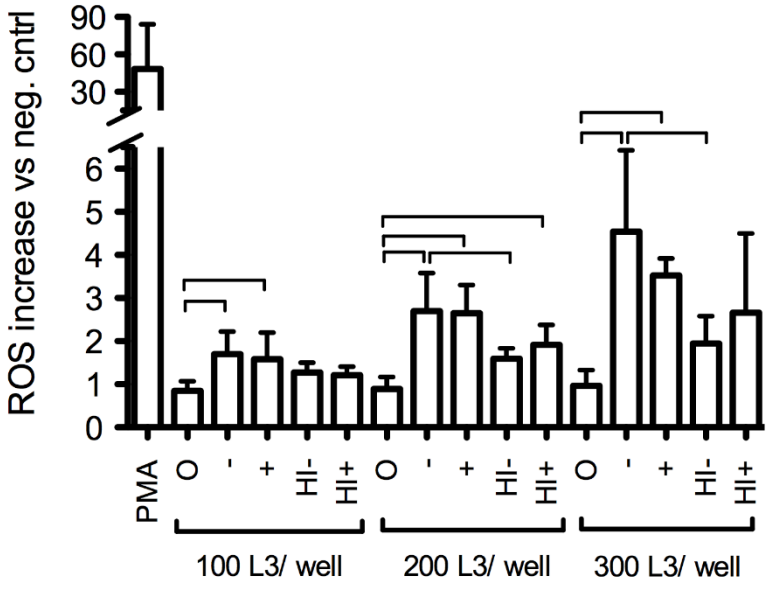


Figure 3.2: Eosinophil ROS production in response to direct contact with infective larvae. Eosinophils were purified from blood of animals at 14 DPI. 2.10^5 eosinophils from 1 animal were seeded per well in HBSS. PMA: Phorbol myristate acetate (5 µg/ml), positive control. HBSS: negative control. O: no serum added. -: Serum pooled from 5 naive animals added. +: Serum pooled from 5 immune animals added. HI-: heat inactivated serum pooled from 5 naïve animals. HI+: heat inactivated serum pooled from 5 immune animals added. Results shown are expressed as the fold increase in ROS production compared to negative control (HBSS) and are the average + SD of 3 experiments with different animals. The bars indicate statistically significant differences between groups ($p<0.05$).

3.3.5 Viability of infective *A. suum* larvae after culture with eosinophils

Eosinophils were cultured together with infective third stage *A. suum* larvae for 16 hours after which viability of the larvae was assessed (Figure 3.3). Eosinophils had a toxic effect on the L3, which was enhanced when serum from naive animals was added and was highest when serum from immune animals was added. Heat inactivation of serum led to reduced killing compared to non-heat inactivated serum. Similar results were obtained with the MTT colorimetric assay (Figure 3.4).

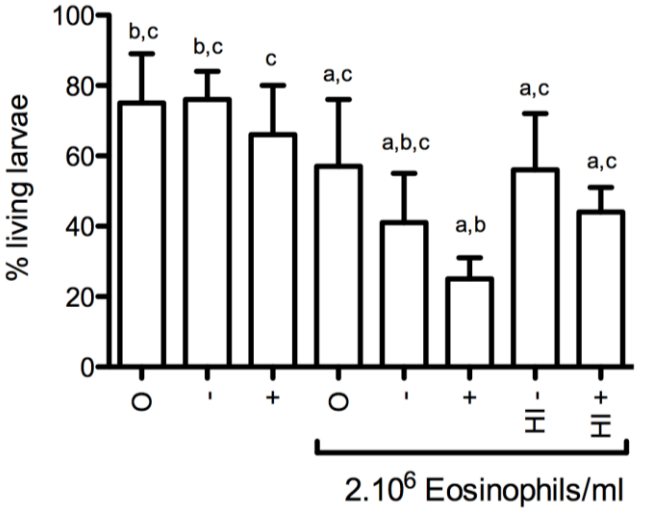


Figure 3.3: A. *suum* L3 viability after culture with eosinophils. Eosinophils were purified from blood of animals at 14 DPI. Viability was assessed visually after 16 hours of incubation with 100 L3. O: no serum added. -: Serum pooled from 5 naive animals added. +: Serum pooled from 5 immune animals added. HI-: heat inactivated serum pooled from 5 naïve animals. HI+: heat inactivated serum pooled from 5 immune animals added. Results are shown as mean + SD of two independent experiments with three incubations each. a: significantly different than L3 cultured without eosinophils or serum ($p<0.05$). b: significantly different than L3 cultured with eosinophils without serum ($p<0.05$). c: significantly different than L3 cultured with eosinophils and serum from immune animals ($p<0.05$).

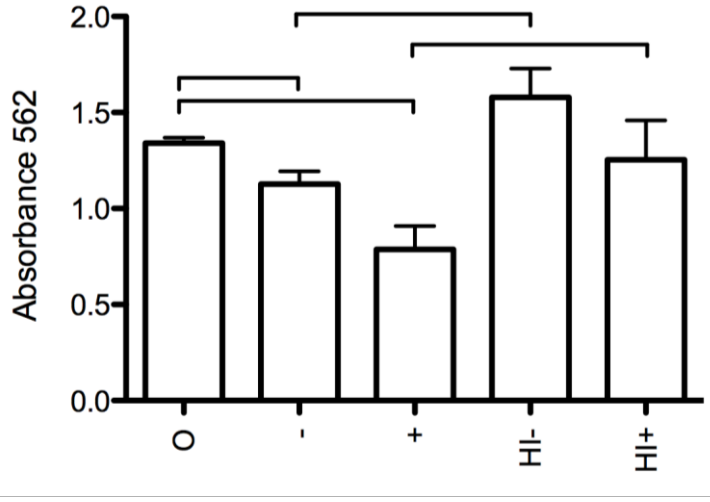


Figure 3.4: MTT assay of viability of infective larvae after culture with eosinophils. Eosinophils were purified from blood of 1 animal at 14 DPI. 2.10^6 /ml eosinophils were incubated together with 100 infective L3 A. *suum* larvae. Viability was determined by the MTT assay after 3 hours of incubation. O: no serum added. -: Serum pooled from 5 naive animals added. +: Serum pooled from 5 immune animals added. HI-: heat inactivated serum pooled from 5 naïve animals. HI+: heat inactivated serum pooled from 5 immune animals added. Results are shown as mean + SD of three incubations. The bars indicate statistically significant differences between groups ($p<0.05$).

3.4 DISCUSSION

In this study we showed that pigs continually exposed to infective *A. suum* eggs for 14 weeks developed an almost sterilizing immunity, demonstrated by a 99,7% reduction in number of larvae that were able to migrate through the host, and that this immunity was associated with eosinophilia, mastocytosis and goblet cell hyperplasia in the caecum. To our knowledge, this is the first study to describe the immunological parameters at the actual site of parasite penetration, i.e. the caecum or proximal colon. Although immunity against *A. suum* infections can occur at the different organs affected, Urban *et al*. showed that the strongest response is already at the level of the gut [21]. They reported increased mast cell and eosinophil numbers in the small intestines of animals with intestinal immunity to *A. suum.* However, since it was later discovered that in fact the caecum and proximal colon are the site of parasite entry, it was unclear whether these findings reflected the response against the adult worms residing in the small intestine, rather than the response against the invading larvae.

In our experiments, only a few larvae could complete their migration and reenter the small intestine. These few larvae would have a minimal impact on the immunological parameters observed in the caecum, since protective immunity was already present at the time of first challenge and results from another experimental infection trial performed by our research group showed that the presence of approximately 50 L4 in the small intestine at 14 DPI in a primary infection did not result in eosinophilia, mastocytosis or goblet cell hyperplasia in the caecal tissue (unpublished observations). Furthermore, it was previously shown that removal of adult *A. suum* worms before challenge did not influence immunity against invading larvae [24].

We observed an almost 10-fold increase in mucosal eosinophils in immune animals. The recruitment of eosinophils to the caecum of immune animals was further supported by increased levels of IL-5, IL-13, CCL11 and eosinophil peroxidase (EPX) transcripts in the caecal mucosa. IL-5 is one of the key cytokines involved in the development of eosinophils. It is also essential in the recruitment of eosinophils from the bone marrow to the blood [238]. CCL11, also termed Eotaxin 1, is an eosinophil specific chemoattractant and functions to home eosinophils from blood to tissue and it can be induced by IL-13 [238]. EPX is a granule protein specific for eosinophils and results in the formation of reactive oxygen species [85]. As the *A. suum* larvae penetrate the caecal mucosa to reach the liver, they are likely to come into close contact with the mucosal eosinophils. Circulating eosinophils responded *in vitro* to direct

contact with the larvae by releasing the contents of their granules. This degranulation was observed with serum from both infected and uninfected animals and the effect was diminished when serum samples were heat-inactivated, indicating that at least a part of it was complement dependent. *A. suum* specific antibodies appear to be non-essential in the degranulation, since serum from immune animals did not lead to increased degranulation compared to serum from naïve animals. However, it is important to note that the experiments were performed with circulating eosinophils and so it still has to be determined if mucosal eosinophils would respond similarly.

Previous work with guinea pigs and mice has shown that complement components can bind the surface of the *Ascaris* larvae and that leukocytes may damage larvae in the presence of serum [239,240]. In the current study, we extended this knowledge by demonstrating that the combination of purified circulating eosinophils from the natural host and serum from immune animals was highly effective in killing the infective larvae. Since the most efficient killing of the larvae was in the presence of serum from immune animals, *A. suum* specific antibodies, in addition to complement components, probably also play an important role in the toxicity towards the parasite. In humans, IgG and IgE are the predominant isotypes for the killing of schistosomula by eosinophils [241,242]. Although we did not test isotype specific responses, these isotypes might also be involved in the *Ascaris* larval killing, since these *A. suum* specific antibody isotypes were elevated from 5-6 weeks of exposure to *A. suum* eggs (data not shown).

Eosinophils have long been associated with helminth infections and antibody dependent eosinophil cytotoxicity against helminths *in vitro* was first shown for *Shistosoma* [242]. Toxicity of eosinophil granule proteins against nematodes has been shown for *Toxocara canis*, *Trichinella spiralis,* and *Brugia malayi*, mostly against juvenile stages [85]. Indeed, eosinophils appear to be essential only in the defense against juvenile, tissue-residing helminthes [243]. Our findings support this conclusion, as eosinophils only degranulated in response to the tissue dwelling L3, and not the lumen dwelling L4 (Chapter 2). It would be interesting to investigate if these differences are caused by diminished complement activation in different life stages of *Ascaris,* as is the case for *Nippostrongylus brasiliensis* [244]. To build up a high enough concentration of eosinophils, complement and antibodies at the site of parasite entry probably requires multiple infection cycles over a longer period of time. This would explain why sterilizing immunity is not established until after several weeks of exposure to infectious *A. suum* eggs.

In addition to eosinophils, mast cells were also recruited to the submucosa and muscularis layer of the caecum of the immune animals. Whether or not mast cell derived

products have direct effects on the invading larvae is unclear, but their submucosal and muscularis location would suggest that mast cells would more likely act in an indirect manner. Mast cells add to the general inflammation by producing Th2 type cytokines such as IL-4, IL-5 and IL-13. They are also the primary source of histamine. It was previously shown that mast cells and basophils from repeatedly infected animals released histamine after contact with *Ascaris* secretory antigens [110,111]. Histamine has various functions. Amongst others, it works as a chemoattractant for eosinophils and histamine release by mast cells can also induce smooth muscle contractions [107]. Additionally, mast cell proteases can break tight junctions, leading to increased intestinal fluid secretion. Although we did not measure fluid secretion and muscle contractions, they are part of a 'weep and sweep' response that is often seen in gastro-intestinal infections [109] and might contribute to the resistance against *Ascaris*.

Interestingly, Urban *et al*. previously also described eosinophilia and mast cell influx in the midgut region of the small intestine of animals with a pre-hepatic barrier [24]. Whether the influx of these immune cells is a result of the development of the pre-hepatic barrier at the level of the caecum and colon or rather caused by the exposure of the small intestinal mucosa to L4 and adults worms is still unclear.

We also identified goblet cell hyperplasia in animals resistant to invading *Ascaris* larvae. Increased mucus production is often part of a general Th2 type response against gastro-intestinal nematode infections [245]. It might play an important role as it could trap the hatched larvae, making it more difficult to penetrate the intestinal wall. Despite the apparent goblet cell hyperplasia, we could not demonstrate an increase in any specific mucin on transcriptional level. Although mucin 5AC has been described as a crucial mucin in the expulsion of gastro-intestinal nematodes in rodent models [79] and is up regulated in pigs infected with *Trichuris suis* [246], *muc5ac* was significantly down regulated in immune pigs compared to naïve ones. The apparent down regulation of *muc5ac* in immune animals may however reflect an early increase in transcription caused by the challenge infection in the naïve animals. In addition to mucus production, goblet cells also secrete proteins with antimicrobial properties. We demonstrated a significant increase in transcription of *retnlb*, the gene coding for Relmß. This goblet cell specific protein has shown to have direct anthelmintic properties. Relmß knockout mice are more susceptible to *N. brasiliensis* and *Heligmosomoides polygyrus* [81] and it was also shown that Relmß was able to bind the lateral alae of *Strongyloides stercoralis*, thereby disrupting chemotactic functions [80]. Whether it acts in a similar way against *A. suum* is still unclear and needs further research.

It is unclear to what extent the results obtained with *A. suum* in pigs can be extrapolated to humans and *A. lumbricoides*. However, similar infection patterns are observed in humans and because of the extremely high similarity between these two parasites on molecular level, there is even question whether or not *A. suum* and *A. lumbricoides* are the same species [62,247]. Eosinophilia is also often observed in humans infected with *A. lumbricoides*, but the link with protection against reinfection has not been made. Nevertheless, it seems likely that in humans eosinophils also play a crucial role in the defense against invading larvae, as pre-treatment levels of IL-5 in humans are also related to resistance against reinfections with *A. lumbricoides* [248]. The fact that immunity against *Ascaris* is only built up after continuous exposure over a long period of time might explain why reinfections are so common in children treated for *Ascaris*. However, it is also likely that as the immune response increases with exposure, fewer larvae will be able to penetrate the gut and as such acute morbidity due to the hepato-tracheal migration will be lower as children age.

In conclusion our results indicate that mast cells, eosinophils and goblet cells operate together to create an inhospitable environment that protects the host against invading *Ascaris* larvae. A general Th2 response, propagated by mast cells and eosinophils seems pivotal in the resistance against invading larvae.

Chapter 4:

The use of a mouse model to study the intestinal pre-hepatic barrier

Based on:

Masure D, De Keyser J, Chiers K, Van den Broeck W, Geldhof P. (2013) Long term exposure of mice to infective *Ascaris suum* larvae results in mucosal eosinophilia and goblet cell hyperplasia. *In preparation.*

4.1 INTRODUCTION

In chapter 3 we identified eosinophilia as an important factor to prevent larval invasion of the intestine. Unfortunately, we could not determine whether these cells are an absolute requirement for pre-hepatic immunity, due to disadvantages of using pigs in research. The number of immunological reagents, such as antibodies available for pigs is low and there are no knockout or inbred strains available. Although mice are not natural hosts for *A. lumbricoides* or *A. suum*, larvae from these nematodes can migrate through the liver towards the lungs. The larvae will however quickly be eliminated after the migration and will not grow into adults. As a consequence, mice have been used to study the early migratory phase of *Ascaris* infections. It was found that similar as in pigs, the migration of larvae in mice also starts in the caecum and colon [249]. Studies in mice have also put emphasis on the negative impact of larval migration on body weight [250] and differences in susceptibility to *A. suum* for the different inbred strains stress the importance of host genetics [251]. The host factors responsible have not been identified yet, but research suggests that the protective response is located before the larvae reach the lungs, but after they arrive in the liver [252].

Although the use of mice in *Ascaris* research warrants caution due to the unnatural host-parasite relationship, they could provide us the necessary tool to further elucidate the immunological basis of protection against larval migration. The mouse model has been very useful in elucidating host immune reactions during helminth infections [253]. Because pre-hepatic immunity is directed against the early migratory *A. suum* larval stage, it might be possible to use mouse models to study the pre-hepatic immunity. As such, the mouse model could be very useful to investigate the contribution of RELM-ß, identified in Chapter 3, towards the defense against invading *A. suum* larvae.

Therefore in this chapter we explored the use of a mouse model to investigate the mechanisms of the pre-hepatic barrier. More specifically, we wanted to examine whether prolonged exposure to infective larvae resulted in the induction of a protective intestinal response and to compare these results to those obtained in pigs.

4.2 MATERIALS AND METHODS

4.2.1 Animals and parasites

All animal experiments were conducted in accordance with the E.U. Animal Welfare Directives and VICH Guidelines for Good Clinical Practice, and ethical approval to conduct the studies were obtained from the Ethical Committee of the Faculty of Veterinary Medicine,

Ghent University. *A. suum* free, female BALB/c mice of 7 weeks old were used. The animals had access to feed and water ad libitum.

A. suum eggs were collected from gravid female *A. suum* collected at the local pig abattoir. After incubation in 0.1% $KCr2O_7$ for 2 months, embryonation was confirmed by way of light microscopy.

Third stage larvae were collected from embryonated eggs. After incubation in sodium hypochlorite for 1h, they were washed with PBS and then hatched by magnetic stirring with 2 mm diameter glass beads. The suspension was put on a baermann sieve covered with cotton cloth to separate the larvae from unhatched eggs. After overnight incubation at 37 °C, the larvae were collected and put in DMEM medium supplemented with 50 u/ml penicillin, 50 µg/ml streptomycin, 100 µg⁄ml kanamycin, 5 µg/ml amphotericin B and 2mM glutamine.

A preliminary trial was performed to determine the optimal dose for the chronic infection. Ten mice were infected through gastric intubation with 100 embryonated *A. suum* eggs and 10 mice were infected with 1000 *A. suum* eggs. After 7 days the lungs were minced in PBS and the larvae were counted. From the 10 mice infected with 100 eggs, only 2 mice had lung stage larvae, and just one larva. The mice infected with 1000 eggs all had between 1 and 5 larvae. Based on these results, for the chronic infection trial, we used 1000 *A. suum* eggs. Seven mice were daily infected orally with 1000 *A. suum* eggs in 15 µl for 14 weeks. A second group of 5 mice were kept uninfected. After 14 weeks, both groups received an oral challenge infection of 30000 infective eggs. Four days post challenge infection, the livers were removed, minced and larvae were counted under a binocular.

4.2.2 RNA extraction, cDNA synthesis and real time PCR assays.

Tissue samples from the caecum were taken and immediately snap frozen in liquid nitrogen and stored at -80 °C until RNA extraction. RNA extraction was performed using Trizol reagent (Invitrogen), combined with an RNeasy mini kit (Qiagen) and a DNase treatment to prevent genomic contamination. RNA integrity was assessed using a Biorad Experion with a standard sensitivity chip. cDNA was synthesized with a Biorad cDNA synthesis kit, starting from 1 µg of RNA. Primers for the real time PCR reactions were designed with the Primer3 software [215]. For a list of primers, see Appendix Table A2. PCRs were run using Fast SYBR Green Master Mix (Applied Biosystems) on an AB StepOnePlus Real-Time PCR System. Primer specificity was confirmed by observing the melting curve. Gene expression levels were normalized based on housekeeping genes selected using Genorm [216]. Housekeeping genes

tested were: *actb, gapdh, gusb, hprt1, prlp*o and *tbp1*. The genes selected for normalization were *hprt1* and *tbp1*.

4.2.3 Histological analysis

Tissue samples were processed essentially as previously described in Chapter 3. Briefly, either 10% formaldehyde or Carnoy's fixated, paraffin embedded tissue samples were cut in 4 µm sections. To assess general histopathological damage and the accumulation of eosinophils, formaldehyde fixed samples were routinely stained with haematoxylin-eosin. Mucosal eosinophils were counted for 20 crypt units with 400x magnification. Mast cells were counted on toluidine blue stained slides of Carnoy's fixed samples at 400x magnification using a weibel2 graticule [219]. For goblet cells, Alcian blue-periodic acid shiff's stain was used. For immunohistochemistry, formaldehyde fixed, paraffin-embedded sections were rehydrated and an antigen retrieval step with citrate buffer was included. Endogenous peroxidase activity was blocked using 1% hydrogen peroxide. Sections were stained overnight with 1/500 rabbit anti-mouse RELM-ß (Peprotech), 1/100 goat anti-rabbit peroxidase (Sigma-Aldrich), diamino-benzidine tetrahydrochloride (DAB, Sigma–Aldrich) and H2O2, according to [80]. Sections were subsequently counterstained with haematoxylin.

4.2.4 L3 incubations with RELM-ß

Recombinant murine RELM-ß (rmRELM-ß, Peprotech) was incubated together with 1000 live or dead (heated at 60°C for 2 minutes) L3 at a final concentration of 0.5 µg/ml in PBS. After 2 hours larvae were washed and stained with 1/100 rabbit anti-RELM-ß antibody and 1/100 anti-rabbit IgG-alexa fluor 488-conjugated antibody.

To study the effect of RELM-ß on the infectivity of the L3, larvae were incubated with 0, 0.01, 0.1 and 1 µg/ml rmRELM-ß for 2 hours before inoculation. For each concentration, 5 mice were infected with 10000 of the incubated larvae. After 4 days, worms present in the liver were counted. To test whether the acidic environment of the stomach caused degradation of the RELM-ß, mice were administered intraperitoneally with 60mg/kg body weight of acid secretion inhibitor Cimetidine one hour prior to infection with 1 µg/ml rmRELM-ß incubated larvae.

4.2.5 Statistical analysis

Results are expressed as mean + SD in the graphs. To test differences in worm counts between naïve and chronically infected mice, a one-sided Mann-Whitney test was used. The effect of different concentrations of rmRELM-ß on the infectivity of A. *suum* larvae was tested via one-way ANOVA. To test the effect of cimetidine and or RELM-ß treatment, a univariate ANOVA was performed.

4.3 RESULTS

4.3.1 Worm counts

The preliminary trial to determine the optimal dose for the chronic exposure revealed that 100 eggs per day would be too low because only 2 mice out of 10 had lung stage larvae,. The mice infected with 1000 eggs all had between 1 and 5 larvae. Based on these results, for the chronic infection trial, we used 1000 *A. suum* eggs. Compared to naïve mice, the mice chronically exposed with 1000 *A. suum* eggs daily for 14 weeks showed a 55% reduction in liver worm counts 4 days after a challenge infection of 30000 infective *A. suum* eggs (Figure 4.1).

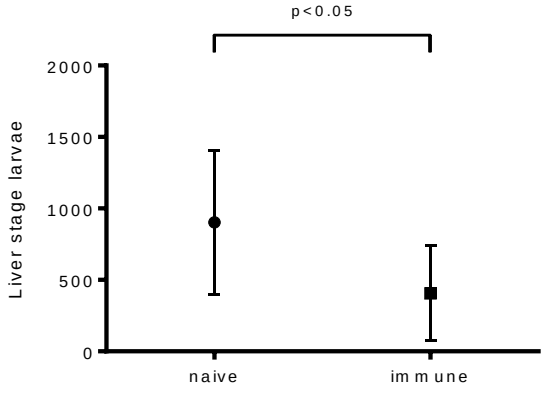


Figure 4.1: liver worm counts in naïve and chronically exposed mice.

4.3.2 RNA transcription profile

Tissue samples from the caecum were investigated for differential transcription of genes commonly associated with helminth infections. The results of the RNA transcription profiles are shown in Table 4.1. The transcription profile was skewed towards a Th2 type response, with increased transcription of typical Th2 markers such as *il4* and *il5* in immunized mice compared to naïve animals. Furthermore, no changes in *ifng* or *il17a* levels were detected. Alternatively activated macrophage markers *arg1* (arginase 1) and *mrc1* (mannose receptor 1) were also increased, while the classical activated macrophage marker *nos2a* (inducible nitric oxide synthase) was down regulated. In addition, the eosinophil specific genes eotaxin receptor (CCR3) and *rnase3* showed an increased transcription.

Other genes that have previously shown to be involved in the anthelmintic response and were also induced here include *retnlb* (resistin like molecule beta, RELM-ß) and *itln2* (intelectin 2). No differences in transcription were found for any of the mucin genes investigated.

Table 4.1: RNA transcription profiles of immune related genes in the caecum of immunized animals. Gene transcription levels are expressed as fold change in transcription levels of chronically infected animals compared to naïve animals.

Gene	Description	Fold change	
areg	Amphiregulin	0.42	*
arg1	Arginase I	6.94	*
c3	Complement factor 3	1.04	
c9	Complement factor 9	1.10	
ccl11	Chemokine (C-C motif) ligand 11, Eotaxin 1	1.09	
ccr3	Eotaxin receptor	3.41	*
cma1	Chymase 1	9.31	*
elane	Elastase, neutrophil expressed	ND	
epx	Eosinophil peroxidase	ND	
foxp3	Forkhead box P3	0.59	*
gata3	GATA binding protein 3	0.94	
gzma	Granzyme A	1.57	
gzmb	Granzyme B	1.87	
ifng	Interferon γ	1.45	
il10	Interleukin 10	1.83	
il12b	Interleukin 12 subunit p40	0.58	
il13	Interleukin 13	1.17	
il1b	Interleukin 1β	0.83	
il33	Interleukin 33	0.42	
il4	Interleukin 4	3.16	*
il5	Interleukin 5	6.05	*
itln2	Intelectin 2	14.58	*
mrc1	Mannose receptor C type 1	1.50	*
muc1	Mucin 1	0.56	
muc2	Mucin 2	1.13	
muc5ac	Mucin 5AC	ND	
nos2a	Nitric oxide synthase 2a, inducible	0.75	*
prg2	Proteoglycan 2, eosinophil major basic protein	0.89	
retnlb	Resistin-like molecule β	7.02	*
rnase3	Ear3 eosinophil-associated, ribonuclease A family, member 3	1.83	*
rorc	RAR-related orphan receptor C	1.07	
stat6	Signal transducer and activator of transcription 6	0.88	
tgfb	Transforming growth factor β	1.03	
tnfa	Tumor necrosis factor α	0.48	*
tslp	Thymic stromal lymphopoietin	0.92	

*: $p<0.05$

ND: Not deteceted

4.3.3 Histopathological findings

The caecum of both groups of mice was investigated for histopathological changes. There was no mastocytosis in naïve or immunized animals (result not depicted). There was however a modest eosinophilia in the chronically infected mice (Figure 4.2A). In addition, the number of goblet cells in the caecum of immunized mice was significantly higher than in naïve mice (Figure 4.2B). To confirm whether or not the increase in transcription of *retnlb* translated into increased protein production in the caecum, we stained the caecum with anti-RELM-ß antibody. In naive mice there was very little RELM-ß present in the intestine. In contrast, chronically infected mice showed uniform RELM-ß production, which was confined to the goblet cells (Figure 4.2C).

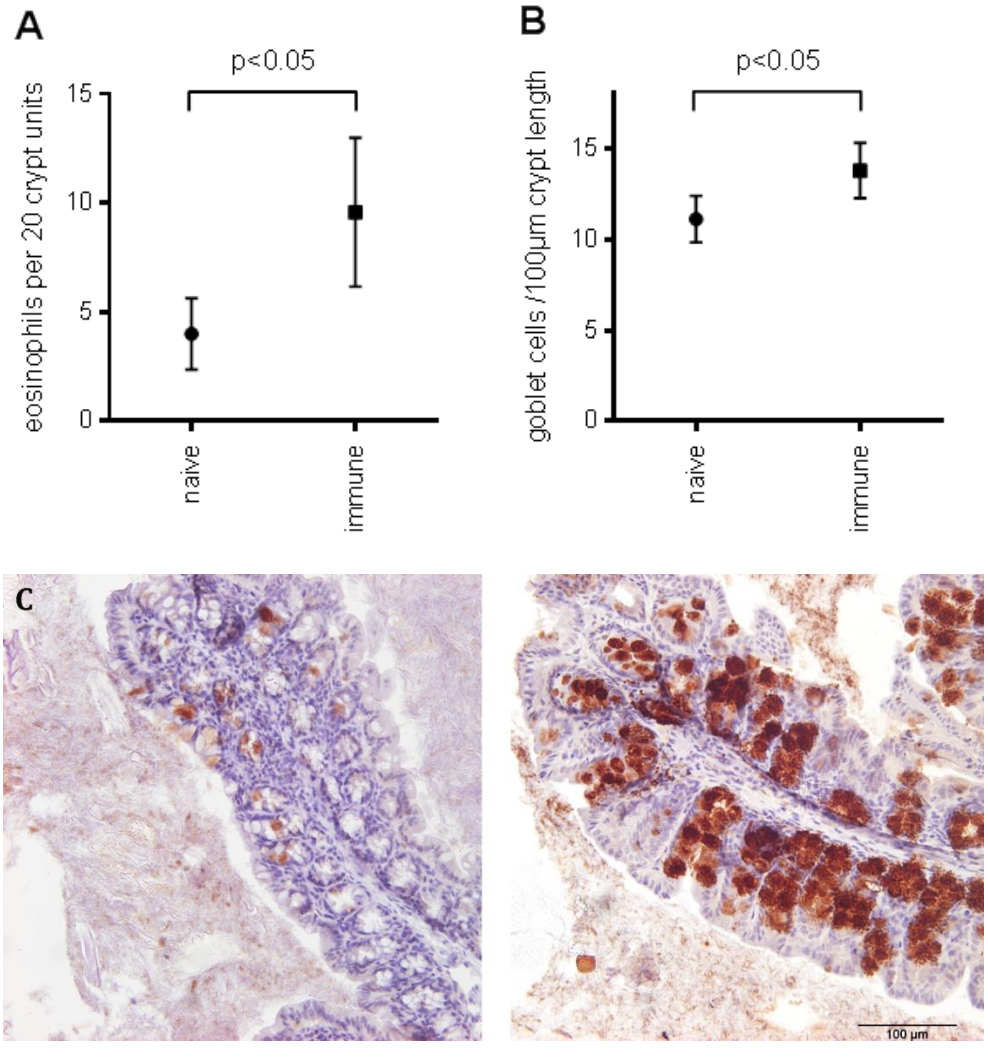


Figure 4.2: (Immuno-) histopathological changes in the caecum of naïve and immunized mice. A: mean number of eosinophils +SD per 20 crypt units. B: Number of goblet cells +SD per 100 µm crypt length. C: RELM-ß staining of caecum of naïve (left) and chronically infected (right) animals.

4.3.4 Pre-incubation of *A. suum* larvae with RELM-ß does not impair infectivity

Previous studies showed a direct anthelmintic effect of RELM-ß by binding to the nematodes [80,81]. To test whether RELM-ß had any direct effect on the larvae, *A. suum* larvae were hatched from the eggs *in vitro* and incubated with 0.5 µg/ml recombinant murine RELM-ß. After washing, binding of RELM-ß on the larvae was visualized using fluorescently labeled anti-RELM-ß antibody. No RELM-ß binding could be detected microscopically, neither on live or dead larvae (data not depicted).

To test if binding was too weak to remain attached to the larvae after washing, or whether there was any effect on the infectivity without direct binding to the larvae, infective L3 were incubated in 0, 0.01, 0.1 or 1µg/ml rmRELM-ß and after 2 hours 10000 larvae were administered orally to naïve mice. There were no significant differences in liver establishment rates between the different conditions tested (Figure 4.3A). To exclude the possibility that the acidic environment of the stomach had a detrimental effect on the RELM-ß, mice were also treated with an acidic inhibitor, cimetidine, prior to infection with RELM-ß treated larvae (Figure 4.3B). RELM-ß incubation still had no effect on the infectivity of the larvae. However, cimetidine treatment of mice had a negative inpact on the number of larvae that infiltrated the liver ($p<0.05$).

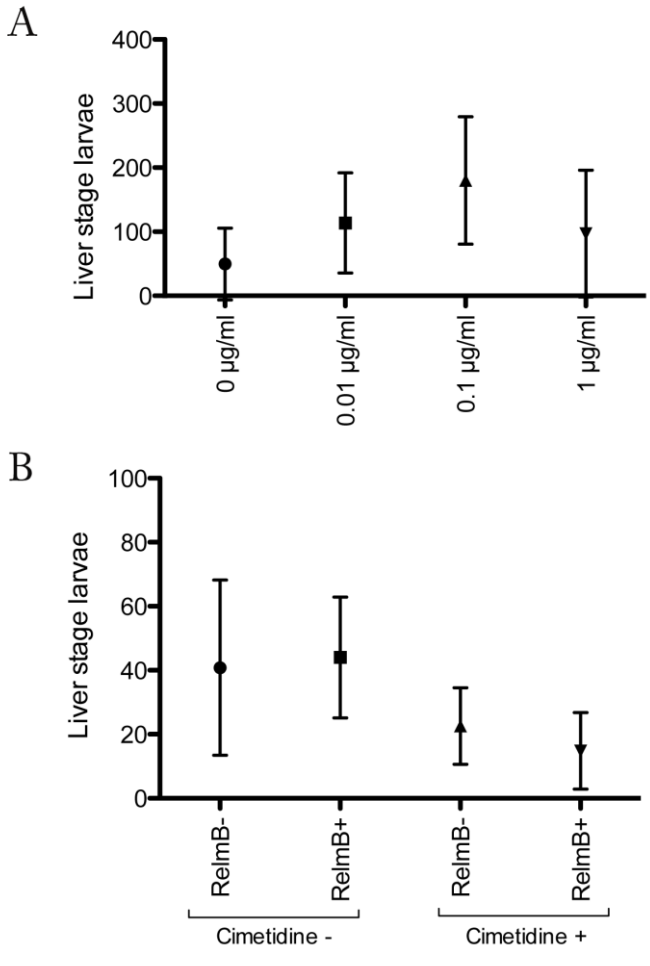


Figure 4.3: The effect of rmRELM-ß pre-incubation on the infectivity of *A. suum* larvae. A: Liver worm counts after pre-incubation of infective larvae in different concentrations of rmRELM-ß. No differences were statistically significant. B: The effect of cimetidine treatment prior to infection with larvae with or without pre-incubation in 1µg/ml rmRELM-ß.

4.4 DISCUSSION

While the mouse model has been used to study natural resistance during primary infections with *A. suum* [250-252], to our knowledge no detailed study has been performed to explore the mechanisms of acquired immunity against reinfections. We chose to use the BALB/c mouse strain with intermediate susceptibility to *A. suum* [251] because we did not want to select for high natural susceptibility or resistance to *A. suum* and because this strain has been used before in immunological studies related to *Ascaris* infections [254-256]. Furthermore, the difference in susceptibility for the different mouse strains is only apparent in the post-hepatic phase of the infection [252] and will therefore probably not be a major factor in the development of the pre-hepatic immunity. For the chronic infection model, we wanted to expose the mice to a consistent low number of migrating larvae to avoid the development of severe pathology or weight loss, as was previously described [250]. The preliminary trial showed that an infection with 100 eggs resulted in only a few mice with lung stage larvae. Therefore we chose to use 1000 eggs for the daily infection, which led to a low number of lung stage larvae in all animals. There was a general low take of infection in our study, compared to some studies [251], but low recovery for *A. suum* in mice has been observed and attributed to *Ascaris* strain variability by others [257].

Mice chronically exposed for 14 weeks showed a reduction of 55 % in the number of larvae that could reach the liver. This reduction was associated with a Th2 response, characterized by the transcriptional upregulation of typical Th2 markers such as *il4* and *il5*, eosinophilia and goblet cell hyperplasia in the caecum. Furthermore, RELM-ß was expressed in the goblet cells of immunized mice. The presence of alternatively activated macrophage markers arginase and mannose receptor 1 suggests that immune-regulatory or wound healing processes are at play here. These functions are probably essential to maintain tissue integrity, because the migration of the larvae inevitably causes damage to the tissue.

The goblet cell hyperplasia, as observed in the immunized mice, is often seen during gastro-intestinal nematode infections and mucus overproduction has been postulated to be one of the mechanisms of nematode expulsion [109]. Despite a significant increase in goblet cells, we did not detect an increase on transcription level of mucin 1, 2 or 5AC, the major mucins described in relation to helminth infections, which may indicate that these mucins are not essential in the defense against invading larvae. Goblet cells also secrete proteins with antimicrobial properties. Similar to our observations in pigs, RELM-ß was also more expressed in the goblet cells of the immunized mice. RELM-ß is a cysteine rich protein shown to have direct anthelmintic properties. Binding of RELM-ß to the worm alae hampers *Strongyloides*

stercoralis chemotaxis [80] and its expression is essential in the expulsion of *Nippostrongylus brasiliensis* [81]. However, RELM-ß does not appear to be important in the defense against all gastro-intestinal nematodes; for example, RELM-ß deficient mice are still able to expel *Trichuris muris* [258]. In line of these findings it is still unclear what role RELM-ß plays in the protective immune response against infective *A. suum* larvae. We could not demonstrate binding of rmRELM-ß on the infective larvae, nor did pre-treatment of the larvae with rmRELM-ß decrease their establishment in the liver. It remains possible however that RELM-ß works in an indirect fashion to help expel *A. suum* larvae. Other functions of RELM-ß identified include promotion of inflammation, tissue remodeling, epithelial cell proliferation and mucin production [80,259,260]. Furthermore RELM-ß might also exert its function in combination with other factors that were not present in primary infections, but are induced after chronic exposure. Future studies with RELM-ß knockout mice are therefore needed to clarify the role of RELM-ß during chronic *A. suum* infections.

Intelectin-2 gene transcription was highly increased in the chronically exposed animals. Since intelectin-2 is strongly induced in mice strains resistant to *T. muris* and *Trichinella spiralis* [261,262] and the gene is not present in *A. suum* susceptible C57B/6 mice, it was thought that intelectin might be responsible for the observed phenotype. However, it was recently shown in a cross-breeding study between susceptible and resistant mice strains that the presence of the intelectin-2 gene was not linked to resistance against *A. suum* [263]. The function of intelectin-2 is not fully understood, but it is probably involved in pathogen recognition, as it binds bacteria and chitin, a component of many nematodes, which may explain its upregulation in nematode infections [264].

In pigs, pre-hepatic immunity is associated with eosinophils, goblet cells, mast cells and transcription of RELM-ß (See chapter 3). The induction of eosinophils, goblet cells and RELM-ß in mice are therefore very similar to what we observed in pigs. Mice might therefore be a good model for a detailed immunological investigation into the immunological principles of the defense against *A. suum* larvae. However, in contrast to pigs, which develop an almost sterilizing immunity that prevents larvae from penetrating the caecum after 14 weeks of exposure [21,23], mice that were chronically exposed for the same time period showed a reduction of only 55 % of the number of larvae that could reach the liver. The difference in acquired immunity against *A. suum* in mice and pigs could be explained by several factors. First, the mucosa of the porcine caecum is several times thicker than that of mice (300-400 µm in pigs versus 100-150 µm in mice), suggesting that it probably takes longer for the larvae to penetrate the mucosa and thus increases the chance of being exposed to host immune factors. Second, the influx of eosinophils in the mucosa of chronically infected mice is rather

modest compared to the eosinophilia observed in pigs (a factor 2 versus 9, respectively), which could also be a consequence of the limited exposure in the mucosa. Furthermore, there are also important differences in biochemical, cellular, and physiologic pathways of eosinophils between mice and humans or other mammals (reviewed in [265]). Most notably, mouse eosinophils do not readily degranulate when challenged with allergens and they lack the high affinity IgE receptor [98-100]. It remains to be determined if eosinophils from mice degranulate to the same extent and under the same conditions as pig eosinophils after contact with *A. suum*.

In conclusion, this work describes the mucosal changes at the site of parasite entry in mice and illustrates that although the impacted immunological pathways are consistent between mice and pigs infected with *A. suum*, there are important host specific differences in the efficiency of pre-hepatic immunity.

Chapter 5:
Contemplation on the impact for vaccine development against *A. suum*

The objectives of this thesis were the identification of the immunological basis of protection against *Ascaris suum* during the self-cure in primary infections and the establishment of the pre-hepatic barrier and their implications for vaccine development. However, some peculiar findings surfaced during this work that also deserve extended attention in the general discussion of this work and that will be discussed before the implications on vaccine development. The first is the marked difference in eosinophil degranulation in response to contact with L3 compared to L4. The second is the implication of increased transit on the pathology and epidemiology of *Ascaris*.

5.1 Mechanism of resistance to *A. suum*: the role of eosinophils

A schematic representation of our findings concerning the pre-hepatic barrier is given in Figure 5.1. Long-term exposure of pigs to *A. suum* led to localized mastocytosis, eosinophilia and goblet cell hyperplasia. Known functions of activated mast cells during helminth infection include increased epithelial permeability and smooth muscle contractility. Together with an increase in mucus it can make it harder for the larvae to reach the epithelial border by creating a 'weep and sweep' response. Any larvae that would make it past the intestinal lining will be attacked by the combination of antibodies, complement and eosinophils, as shown in our *in vitro* assays.

While the role of eosinophils in the immune response against helminths remains somewhat controversial (see Chapter 1.2.1), eosinophils appear to play an important role in the defense against invading *A. suum* larvae by attaching to the larvae and releasing the toxic content of their granules. Toxicity towards the larvae is probably mediated through the formation of reactive oxygen species (ROS). The *in vitro* degranulation assay demonstrated that ROS are released after contact between eosinophils and L3 and incubation of L3 with a chemical compound, SIN-1, that forms ROS when dissolved was also toxic for the larvae (data not shown). Mucosal eosinophils were also consistently associated with *A. suum* expulsion from the small intestine in primary infected animals as well. Surprisingly, eosinophils degranulated in response to L3's, but not to L4. For degranulation against L3's, the presence of serum of uninfected animals was sufficient, suggesting that degranulation is complement dependent. The complement cascade has been shown to be important in the defense against nematodes [266] and nematodes have developed several mechanisms to deal with complement factors (see Chapter 1.3). The acquisition of complement inhibitory factors can be life stage dependent, as has been shown for *N. brasiliensis*, where complement inhibitory factors are acquired in the lung L4 stage [244]. A recent study by Wang *et al* shows clear

differences in ES products released by different *A. suum* larval stages [267], see Figure 1.2 and the same is true for surface antigens [268]. Therefore it is possible that *A. suum* L4 express immune evasion products, such as complement inhibitory factors, distinct from L3.

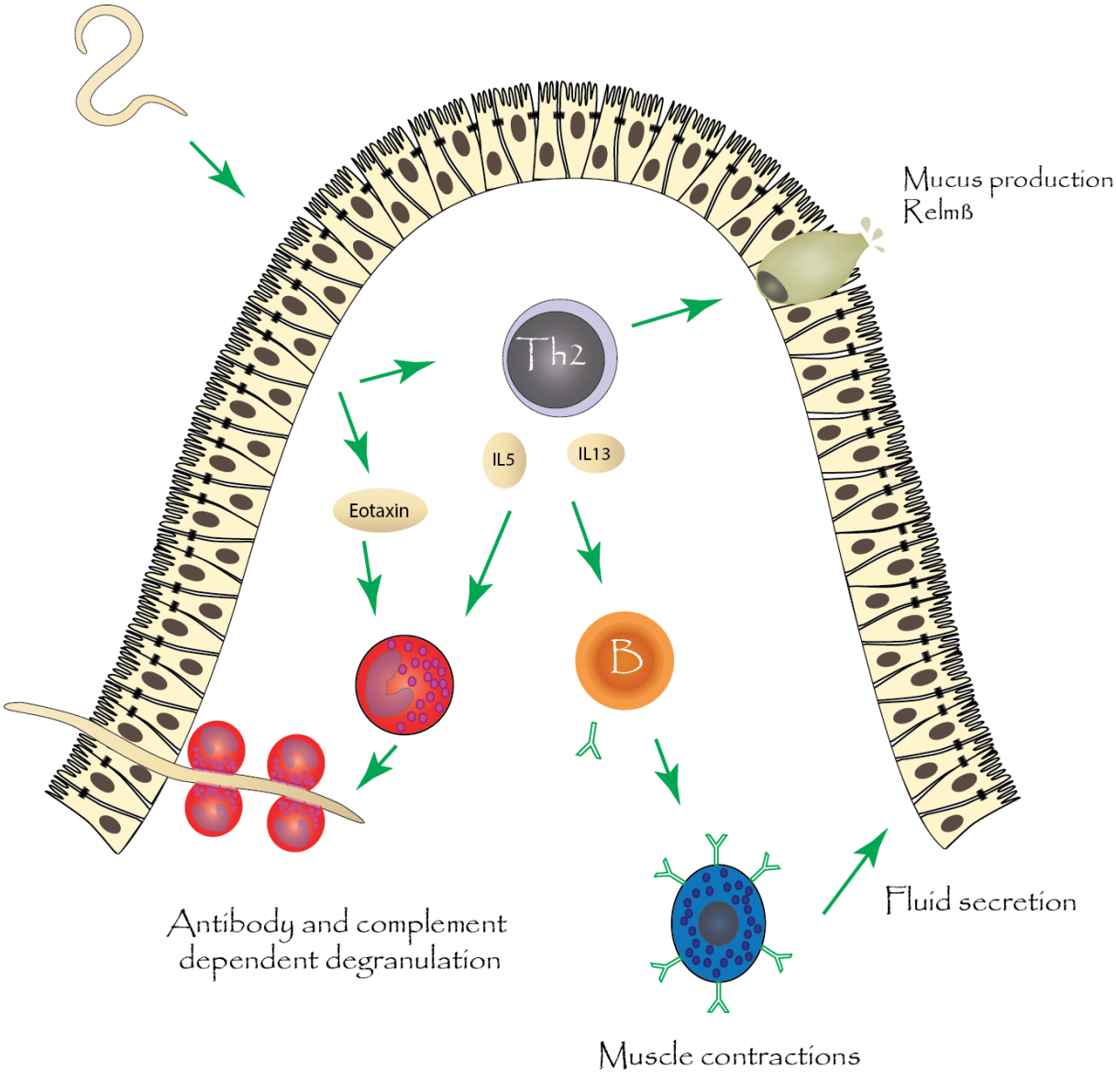


Figure 5.1: The effector mechanisms of the pre-hepatic barrier. Infective L3's penetrate the intestinal barrier and induce a type-2 response characterized by IL4, IL-5, IL-13, eosinophils, goblet cells and mast cells. Eosinophils attach to the larvae and degranulate in a complement and antibody dependent manner. Mast cells during helminth infections can induce muscle contractions and fluid secretions that, together with goblet cell produced mucus, forms a weep and sweep response, making it harder for the larvae to reach the epithelial border.

It is however not clear why *A. suum* L4 would express these factors and L3 would not, especially given that L4 worms reside in the lumen of the small intestine and would not come into close contact with the mucosal eosinophils. Further research is needed to elucidate this enigma. Hopefully the recent acquisition of the genome and stage specific transcriptomes of *A. suum* can shed more light on which factors could account for the observed findings [204,269]. One such potential candidate that can be identified this way is paramyosin. Paramyosin is a structural component of many helminth species and paramyosin in *T. spiralis* has demonstrated to play a role in the defense against host complement [176]. In the genomic *A. suum* database we can find a predicted protein (L4_05431) with >80% homology to many other helminth paramyosins and transcriptomic analysis of the different life stages shows that this protein is highly expressed in *A. suum* L4 compared to L3 and it would therefore be a possible candidate to investigate further.

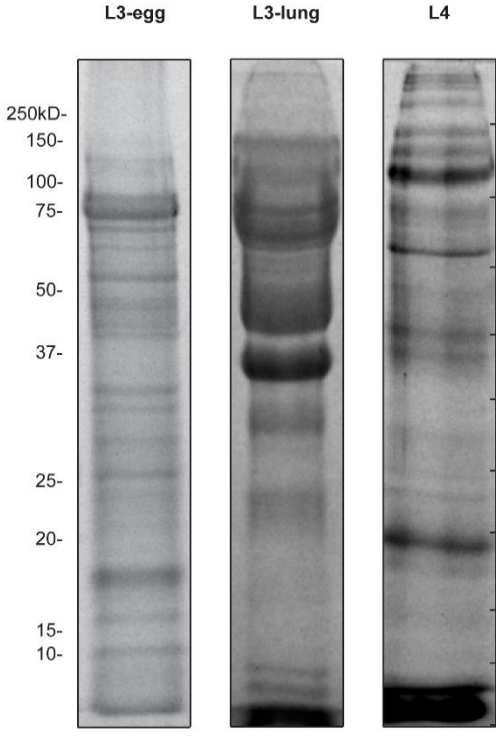


Figure 1.2: Protein profile of the A. *suum* ES products. Note the distinct profile for each life stage. Adapted from Wang *et al.* [267].

Unfortunately, because we lack the necessary immunological tools to ablate the eosinophils in pigs, we could not determine if eosinophils are an absolute requirement for protection against *A. suum in vivo*. For mice, these tools are available and for this reason much of our knowledge about the immune response against helminths has been unraveled by using mouse models. Since *A. suum* penetrates the caecum and colon of mice and reaches their lungs, mouse models could offer the necessary means of elucidating the importance of eosinophils in the defense against invading *A. suum* larvae. However, before we use these models in further research, it should be confirmed that chronic *A. suum* exposure in mice leads to pre-hepatic immunity. One of the goals of this PhD was therefore to determine if chronic *A. suum* infection in mice is indeed a relevant model for the pre-hepatic barrier in pigs. The immunological response after chronic exposure of mice to *A. suum* was very similar to that of pigs. There was a general Th2 response with influx of eosinophils and goblet cell hyperplasia. However, despite the induction of a strong inflammatory response, the reduction in larvae that reached the liver was disappointing. As a consequence, ablation studies to test the requirement of cells like eosinophils for the defense against invading larvae might not give clear-cut answers. Nevertheless, the *A. suum* mouse model can be useful for several reasons. They can help elucidate if certain host factors have a direct effect on larvae or to evaluate anthelmintic products or techniques such as RNA interference on the infectivity of the larvae. Moreover, they can be used to understand the role of inflammatory cells or products in the homeostasis of the affected tissue during helminth infections. For example, our experiment in mice did offer an interesting target for further fundamental research: RELM-ß. RELM-ß was upregulated in pigs and mice and since it has reported anthelmintic functions, most notably against lumen residing nematodes [80,81], it deserves further research in the context of an *A. suum* infection. We could not demonstrate direct binding of RELM-ß on *A. suum* and pre-incubation of *A. suum* in recombinant RELM-ß did not alter infectivity of *A. suum*. However, it is still possible that RELM-ß plays an indirect role in the defense against *A. suum*, by promoting inflammation, tissue remodeling, epithelial cell proliferation and mucin production [80,259,260]. Since there are RELM-ß knockout mice available, the mouse model could be useful to clarify the role of RELM-ß during *A. suum* and other helminth infections.

5.2 Implications of increased intestinal transit during *Ascaris* infections

A compelling finding in our study is that the expulsion of larvae was associated with a decrease in small intestinal transit time. Apart from *A. suum* in pigs, expulsion of intestinal nematodes has mostly been observed in mice with *T. spiralis, H. polygyrus, N. brasiliensis*, but also in large ruminants with *Toxocara vitulorum* [270] and *Cooperia oncophora* [271], probably because these animals are the most widely used in experimental research. Increased smooth muscle contractility has been shown for the small intestinal nematode infections in mice, *T. spiralis* and *N. brasiliensis* [231,233] and for *C. oncophora* in cattle [232]. It may therefore represent a universal reaction against intestinal nematode infections. Studies in mice have shown that the helminth induced increase in smooth muscle contractility is signaled through IL4 or IL-13 [114,143,233], which could explain why it is a common observation with helminth infections. Whether or not increased muscle contractions and faster transit will be sufficient to expel the worms will depend on the activity of these worms and their evasion mechanisms. Hookworms for example will be less or not susceptible to increased peristalsis compared to non-invasive species. But even for non-invasive species like *A. suum* there is a fine balance between being expelled and being able to remain in the small intestine, as our L4 transfer experiment illustrated.

Because all but one animal was *A. suum*-free at 35 DPI in the intestinal transit study, it is not clear whether the intestinal transit is increased as long as worms are present in the small intestine. This could nevertheless have important consequences. Would prolonged faster transit for example have an effect on the intestinal absorption of nutrients or would it be negligible? Moreover, if the transit would indeed remain higher in the presence of adult worms, it would resolve the puzzling concept of concomitant immunity. It is assumed that the adult worms induce an activated state of the immune response directed against juvenile stages in order to prevent hyperinfections that could potentially be lethal to the host and thus also the parasite. The mechanism of concomitant immunity is however unclear and some questions remain unanswered. For example, when immunity is established, why does it quickly disappear when adult worms are eliminated? If the adults would indeed induce a permanent state of increased transit, newly arriving larvae would indeed be easier eliminated from the small intestine, while the full-grown adults are large enough to counter the increased peristalsis. Furthermore, removal of adult worms would also quickly revert the changes in the intestine.

Some other essential aspects remain unanswered. We could demonstrate that self-cure

is a locally triggered response and that previous priming as part of a hypersensitivity reaction is not required to induce the expulsion. However, it is not clear how this response is initiated and whether the increased transit is the result of increased muscle contractility, fluid secretion or a combination of both. Intra-epithelial T cells and eosinophils seem pivotal player during self-cure, because they were consistently associated with self-cure in infections with *A. suum* eggs, L3 and L4 infections, but it will require further research to determine how these cells operate to induce the self-cure.

5.3 Implications for vaccine development for *A. suum* and *A. lumbricoides*

High reinfection rates after deworming, up to 55-88% within the year [272,273], create a an urgent need for a more permanent solution, such as vaccination. The prospects that our results give on the development of a vaccine against *A. suum* in the near future are however not very promising. Especially the high levels of eosinophils in the mucosa of immune animals combined with toxicity towards the invading L3, suggests that these cells are indispensable in pre-hepatic immunity. A successful vaccine would therefore have to result in a very fast influx of eosinophils in the mucosa of the caecum and colon. The question therefore remains how we could achieve this type of mucosal immunity through vaccination. The highest chance to induce mucosal immunity would be through mucosal administration, either oral or nasal vaccination, rather than systemic administration. But even then it is not likely that it would cause a long-term influx of eosinophils. Vaccination typically results in memory T and B cells which make it possible to induce a faster and strong immune response after contact with the pathogen. However, given that eosinophilia is required and because larvae can reach the liver as soon as 6 hours after ingestion of *A. suum* eggs, it is not very likely that robust pre-hepatic immunity can easily be achieved through vaccination, because even for vaccinated animals this time frame is extremely short to produce a strong immune response.

If we cannot prevent larvae from penetrating the intestinal mucosa, perhaps we can prevent these larvae from reaching adulthood. During natural infections most larvae are eliminated by the self-cure reaction between 14 and 21 days post infection and could offer a second target for intervention. However, this self-cure of *A. suum* from the small intestine appears to be antibody-independent, since no *A. suum* specific antibodies were present in the animals infected with lung stage larvae at the time of parasite expulsion. Further support for this conclusion comes from the observation that in secondary infections the expulsion does

not occur faster [25]. As a consequence, there is little hope that this reaction can be induced by means of vaccination.

Perhaps an even more important finding in light of vaccine development is that, despite the potential of sterilizing immunity, the formation of the pre-hepatic barrier takes more than 10 weeks of chronic exposure to induce a high level of protection and even then some larvae are able to complete the hepato-tracheal migration. One possibility for this phenomenon is that it takes several rounds of exposure in the gut to reach a high enough concentration of eosinophils and antibody. This does however not seem very likely, since eosinophilia and *A. suum* specific antibody is already observed after primary infections within 2 weeks. A more likely explanation is the circulation of antigenic diverse strains which has also been put forward as a possible explanation for the age-dependent resistance against *Ascaris* and other helminths [274]. Antigenic diversity or polymorphism could indeed explain the epidemiological patterns of *A. lumbricoides* and *A. suum* and helps to explain why vaccination trials against *Ascaris* or in fact any other nematode with single antigens have so far not been very successful. Antigenic diversity is supported by the highly diverse *A. suum* specific antigenic repertoire against which individuals develop antibodies, even in individuals from the same household [275]. Furthermore, antibodies elicited against *A. lumbricoides* in one individual may not bind *A. lumbricoides* from another individual [276]. Although differences in expressed proteins could cause the observed antigen repertoire, antigens in the excretory-secretory material and surface of the worms are highly glycosylated and variation in the exposed epitopes could more easily be achieved by adding, modifying or removing carbohydrate residues. Support for this can be found in a recent report that show an exceptionally high proportion of glycosidases in the *A. suum* compared to other (free-living) nematodes [267], which, apart from a role in the digestion of host nutrients, may also be involved in immune evasion.

These facts notwithstanding, since serum from immune animals had a slightly toxic effect on the larvae by itself and more so in the presence of eosinophils *in vitro*, if *A. suum* antibodies are already present before infection, it should help to mount a faster immune response directed towards these larvae, even if there is antigen polymorphism. In this regard, although worms would perhaps not be completely stopped pre-hepatically, vaccination would help to reduce worm burden, which may alleviate the high morbidity associated with *A. suum* and *A. lumbricoides*. Furthermore, larvae in secondary infections are smaller and more susceptible to the expulsion reaction [25], most likely due to contact with host antibodies and inflammatory cells during the hepato-tracheal migration through the host, which makes them

weaker on returning to the small intestine and consequently they have less chance to survive the expulsion reaction and reach adulthood. In this regard, in order to recognize the full potential of the investigated vaccine candidates, perhaps the evaluation of vaccine candidates in pigs should also take into account the possible effects on adult worms rather than just the larval stages, which is common practice today, although inevitably this comes with more variation and consequently larger samples sizes will be needed.

It was previously shown that pigs show stronger white spot reactions against larvae that manage to enter the liver if the pigs have previously been primed by vaccination [155]. A concern is that when larval infiltration cannot be prevented, that increased liver white spot reactions would make a vaccine against *A. suum* economically less profitable. This concern about increased white spot reactions in vaccinated animals is probably overrated because it is most likely the result of a secondary immune reaction because more pronounced white spots are also observed in animals with secondary infections [23]. Non-vaccinated pigs in practice will be continuously exposed to infective *A. suum* eggs in the environment and will therefore always show strong white spot reactions. Simply comparing the white spots of primary infected animals to vaccinated and challenged animals may therefore not be biologically relevant. To evaluate the economic benefit of a potential *A. suum* vaccine, it would only be relevant to look at liver white spots in pigs at the time they would normally go to the slaughterhouse, because although white spots could be more pronounced when they are initially exposed to *A. suum* early in life, the vaccine induced immune response may result in a higher level of protection at an earlier age, giving the liver enough time to heal from these white spots before they are evaluated at the slaughterhouse. Furthermore, compared to the effects of *A. suum* on weight gain and feed conversion, liver white spots are a minor contributor on the economic impact of *A. suum* infections [277]. So even if white spots may not be prevented by vaccination, when the worm burdens can be kept to a minimum, there can still be an important improvement in pig health, as low *A. suum* worm burdens have little impact on pig health and profitability. Especially for humans, where people are exposed to *A. lumbricoides* for several years, the positive effects of vaccination would outweigh potential negative effects on liver white spot reactions early after exposure.

There are still other barriers to overcome, however. Despite multiple successful vaccinations in animal models, no human vaccine against any of the STH exists today. One of the few recombinant vaccines against hookworms being tested in human clinical trials, *Necator americanus* ASP-2 protein, unfortunately had to be terminated prematurely in phase I due to unacceptable side effects [278]. Apparently, vaccination induced urticaria in people

previously exposed to *N. americanus*, whereas hookworm naïve people tolerated the vaccine. This does point out that some problems still remain and vaccine development against any helminth will probably be complicated by two factors: first the shared characteristics of Th2 responses that are induced both during allergic reactions and helminth infections and secondly the complex regulation and interplay of protective and tolerant immune responses during infection. In order to resolve these shortcomings, we will need a better understanding of the protective and regulatory framework that is associated with these helminth infections.

Another problem in vaccine development is that recombinant vaccines very often fail to induce strong protection, especially in single subunit vaccines [279-281]. To date, not a single molecularly defined vaccine against nematodes is available that gives rise to high levels of protection. Apart from potential antigenic diversity, as described earlier, the reason for the failure is most likely that the folding and post-translational modification in the recombinant expression systems (usually prokaryotic cells or yeast) do not mimic that of the native antigen or that multiple antigens are present in native protective antigen fractions. Furthermore, metazoan parasites are much more complex than other human pathogens and much better suited to manipulate the host immune response. A possible solution to deal with the complexity of helminth biology could be to combine several recombinant antigens in one cocktail. This has recently been tried with relative successes for several vaccines against parasites, such as filarial nematodes, leishmania and malaria [282-284]. However, for helminths, these vaccines rarely offer protection over 70%. Strikingly, a combination of 8 different recombinant antigens was made for the small ruminant nematode *Teladorsagia circumsincta.* Although the protection levels on worm counts were higher than any previously tested recombinant vaccine for this parasite, it is still offers only a limited protection of between 56% and 75% [285]. Attempts have also been made to express recombinant helminth antigens in more complex systems such as plants [286] or even the free-living nematode *Caenorhabditis elegans* [287] in the hope that these systems will make the appropriate modifications and folding, but also here we still have to see the first real successes.

5.4 Concluding thoughts and future prospects

The mucosal immune response against *A. suum* has not received much attention, and yet it may hold important clues for immunological strategies of control such as vaccine development and a better understanding of the pathology associated with *Ascaris* infections. In this work we focused on the 2 major phases of protection against *A. suum* in pigs: the formation of the pre-hepatic barrier and the self-cure reaction during primary infections.

Because pre-hepatic immunity is the result of long-term exposure and requires a combination of cellular and humoral factors, there is an important consideration for the epidemiology and control of *A. lumbricoides*. Although the implementation of mass drug treatments does not prevent larval reinfection because the drugs used do not have a remnant effect, the exposure to *A. lumbricoides* may decrease as a consequence of the recent mass drug treatments. Because egg-excreting adults are periodically removed, transmission will consequently decrease. Since immunity is related to exposure, it will be interesting to see if the age pattern of *A. lumbricoides* infected people shifts as a result of these treatments.

An important role for eosinophils was identified in the defense against *A. suum*, especially against the early infective L3. The acquisition of high levels of eosinophils and antibody seems pivotal in killing larvae that penetrate the mucosal border. However, the relation between eosinophils and the expulsion reaction is less clear. Although there is an influx of eosinophils during self-cure, further research will be necessary in order to understand their purpose. In addition, the expulsion of *A. suum* is associated with faster transit, which holds a lot of potential for further research into the effects of *Ascaris* infections on the intestinal physiology.

Since pigs acquire natural immunity to *A. suum*, vaccination remains feasible, but due to the nature of the immune response and the biology of *Ascaris*, we should not expect dramatic worm reductions from single antigen vaccines. Moreover, to evaluate potential vaccine candidates, we should take into account the effect of the vaccine on all life stages of the parasite, preferably over a longer period of time.

Summary / Samenvatting

Summary

Ascaris suum is the most common nematode infection in pigs and has detrimental effects on pig health and well-being, and consequently meat production. Embryonated eggs, ingested by the host, will release the infective L3 in the small intestine. These larvae will penetrate the caecum or colon and through the portal vein they will reach the liver. From the liver they will go to the lungs and after penetrating the alveoli they will be coughed up and swallowed back in. The L3 will molt to an L4 in the small intestine and eventually become adult. Today, the control strategy is based on mass treatment with anthelmintics, but it is insufficient for the elimination of this parasite. A better understanding of the immune response against *Ascaris* is necessary for the development and evaluation of alternative control strategies such as vaccination. *A. suum* is almost identical to *A. lumbricoides*, that infects humans. Infections in pigs with *A. suum* can therefore be used as models for infections in humans with *A. lumbricoides*. *Ascaris lumbricoides* is the most common helminth in humans, and affects mostly children in (sub)tropical climate. The literature review demonstrated that the strongest immune responses against primary and chronic *A. suum* infections are situated in the intestine. In primary infections there is an expulsion of larvae from the small intestine, termed self-cure, that eliminates most larvae after they completed their migration through the host. After chronic exposure to *A. suum*, pigs acquire immunity at the caecum and colon that prevents infective larvae to penetrate the mucosa and reach the liver. This type of immunity is called the pre-hepatic barrier. Unfortunately, almost no research has been performed on the development of these protective responses. Knowledge about the immune response in the context of helminth infections mostly comes from murine infection models, of which it is not clear whether it is also applicable to *A. suum* infections in pigs. The objective of this project was therefore to characterize the intestinal immune response of pigs following *A. suum* infections during the self-cure reaction and the formation of the pre-hepatic barrier.

In chapter 2 we investigated the immunological changes in the small intestine during the expulsion of *A. suum* in primary infections and we determined whether the early migration through the host is required to initiate the self-cure reaction. Pigs that were infected orally with lung stage *A. suum* larvae and thereby bypassed the migration phase were able to eliminate the L4 to the same extent as pigs infected with *A. suum* eggs and also around 7 days after exposure to the small intestine, showing that the initial migration is not required to trigger the expulsion reaction. *A. suum* specific antibody is not yet present in animals that received lung stage larvae and that are driving the larvae out, which indicates that antibody is

not essential for self-cure. When pigs were orally infected with 14 day old intestinal L4, these larvae were also being driven out 7 days later. However, the L4 were subsequently able to counteract the expulsion reaction by 18 days after their transfer and to inhabit the more proximal region of the small intestine. This showed that larvae are not killed during self-cure. One possibility is that increased transit is responsible for the expulsion and that bigger and more active larvae can counteract this reaction. We therefore measured the intestinal transit prior to, during and after the expulsion reaction in pigs. The intestinal transit was indeed increased during the expulsion reaction. The RNA transcription profile showed a mixed T helper response and the histological analysis identified an influx of eosinophils and intra-epithelial T cells during the self-cure in all experiments performed here. Although it has not become clear how these cells operate, it does seem likely that they are crucial in inducing the expulsion of *A. suum* larvae.

Apart from the expulsion reaction, we studied the buildup of pre-hepatic immunity (Chapter 3). Chronic exposure of pigs to low doses of *A. suum* eggs for 14 weeks led to a reduction of 99.7% in the number of larvae after a challenge infection of 5000 *A. suum* eggs. Protection against infective larvae was associated with a general Th2 reaction with eosinophils, goblet cells and mast cells in the caecum. The RNA transcription profile demonstrated an upregulation of several eosinophil related genes. We showed that porcine eosinophils in combination with serum of infected and uninfected pigs degranulated after contact with *A. suum*, suggesting that degranulation was complement dependent. Eosinophils were the most toxic for *A. suum* L3 when used in combination with serum of the chronically exposed pigs, indicating that antibody also plays an important role in the formation of effective pre-hepatic protection. The role of the mast cells and goblet cells is less clear, but they possibly contribute to a weep and sweep response where more fluid and mucus is secreted in the lumen of the gut and larvae will be hindered in their attempt to reach the mucosa. Moreover, we also detected a higher transcription of the gene encoding for RELM-ß, a protein secreted by goblet cells with anthelmintic properties. Unfortunately, we lack the necessary immunological tools in pigs such as specific antibodies or knockout strains to elucidate the role of this protein in the defense against *A. suum*.

For mice these tools are available and therefore we examined the use of a mouse model for research into the pre-hepatic barrier in Chapter 4. Mice were exposed for 14 weeks to *A. suum* and subsequently received a challenge infection. These immunized mice did not develop strong pre-hepatic immunity, since there was a 55% reduction in the number of larvae that reached the liver. Nevertheless, chronic exposure in mice, similar to pigs, did lead to a general

Th2 response with an influx of eosinophils and goblet cell hyperplasia. Furthermore, RELM-ß was also induced in these mice. Given the anthelmintic properties of this protein we tried to unravel the function of this protein during *A. suum* infections. We could not demonstrate binding of this protein directly on the infective larvae. Next we investigated whether incubation of larvae in this protein had an effect on the infectivity of the larvae, as has been shown for other gastro-intestinal nematodes. RELM-ß did not significantly affect the infectivity of the larvae. Other possible functions of RELM-ß and further directions were considered in the discussion.

The general discussion addresses some of the most important and remarkable finding of this work. For example, eosinophils degranulated in response to contact with *A. suum* L3, but not to L4. Possible explanations for this phenomenon, such as the acquisition of complement inhibitory factors during the life cycles of *A. suum* are discussed. In addition, the increased intestinal transit during the expulsion of *A. suum* is considered as an explanation for concomitant immunity and compared to other gastro-intestinal nematode infections.

As a last point in the discussion the results are considered in the context of *A. suum* vaccine development. The presence of antigenic diversity was put forward as the most likely explanation for the necessity of long-term exposure before strong immunity is established. Because pigs acquire natural immunity to *A. suum*, vaccination remains a feasible option for control strategies, but given the need for prolonged exposure and the presence of antigenic diversity, we should not expect dramatic reductions in the larvae that reach the liver, especially when using single antigen vaccines. It is also important that the evaluation of vaccine candidates takes into account the effect on adult worms, because larvae that complete their migration in vaccinated animals may be weakened and consequently more susceptible to the expulsion reaction.

Samenvatting

Ascaris suum is de meest voorkomende wormbesmeting bij varkens, met een grote nadelige impact op de gezondheid en welzijn van de dieren, met als gevolg een slechtere vleesproductie. Varkens worden geïnfecteerd door het inslikken van geëmbryoneerde eieren die in de darm een *Ascaris* larve vrijstellen. Deze larven dringen door de dikke darm en bereiken via de portaalvene de lever. Vanuit de lever gaan ze via de bloedsomloop naar de longen, waarna ze opgehoest en terug ingeslikt worden. In de dunne darm zullen de wormen volwassen worden en zich seksueel voortplanten. De controle van deze infecties steunt voornamelijk op ontwormingsschema's, maar dit blijkt ontoereikend te zijn om deze parasiet te elimineren. Voor de ontwikkeling en evaluatie van alternatieve controlestrategieën zoals vaccinatie is een betere kennis van de immuunrespons tegen *Ascaris* nodig. *A. suum* is zeer gelijkaardig als *A. lumbricoides*, die voorkomt bij mensen. *A. lumbricoides* infecties vormen de overgrote meerderheid van de wormbesmettingen bij de mens, waarbij de hoogste prevalentie voorkomt bij kinderen in (sub)tropische gebieden. Wegens de grote gelijkenis tussen deze twee wormen kunnen we varkens geïnfecteerd met *A. suum* gebruiken als model voor *A. lumbricoides* infecties bij de mens. De literatuurstudie toont aan dat de meest beschermende immuunreacties tegen *A. suum* zich ter hoogte van de darm bevinden. Enerzijds is er tijdens een primaire infectie met *A. suum* een expulsiereactie die juveniele L4 larven uit de dunne darm verdrijft kort nadat ze hun migratie door het lichaam voltooid hebben. Anderzijds bouwen varken na langdurige blootstelling immuniteit op ter hoogte van het caecum en colon die belet dat infectieuze L3 larven de darm kunnen penetreren en de lever bereiken. Dit type van bescherming kreeg de term pre-hepatische barrière. Er is echter nauwelijks onderzoek gevoerd naar de ontwikkeling van deze beschermende reacties van de gastheer. Kennis van de immuunrespons tegen worminfecties komt voornamelijk uit studies met muismodellen, waarvan niet duidelijk is of ze ook van toepassing is op *A. suum* infecties bij het varken. Het doel van dit doctoraatsproject was dan ook om de intestinale immuunrespons bij varkens te karakteriseren tijdens de pre- en posthepatische fase van *A. Suum* infecties.

In hoofdstuk 2 werden de immunologische veranderingen ter hoogte van de darm tijdens de expulsie van *A. suum* in primaire infecties gevolgd en werd nagegaan of de initiële migratie door de gastheer een noodzakelijke trigger is voor de expulsiereactie. Varkens die oraal longstadium *A. suum* larven kregen en dus geen passage van *A. suum* larven door hun lever of longen hadden, dreven de L4 larven uit hun dunne darm in dezelfde mate als varkens

geïnfecteerd met *A. suum* eieren en ook 7 dagen na blootstelling aan de darm, waardoor duidelijk bleek dat de expulsie lokaal getriggerd wordt. *Ascaris* specifieke antilichamen zijn nog niet aanwezig in deze dieren op het moment dat de larven uitgedreven worden, wat er op wijst dat antilichaam wellicht niet essentieel is in deze fase. Wanneer varkens oraal geïnfecteerd werden met 14 dagen oude L4 wormen vanuit de darm van een ander varken, werden deze larven ook caudaal gedreven 7 dagen later. Deze larven waren echter in staat deze uitdrijvingsreactie tegen te werken tegen 18 dagen na transfectie en zich meer craniaal te begeven. Mogelijk ligt versnelde transit aan de basis van de expulsie reactie en kunnen grotere en actievere larven dit voldoende tegenwerken. Daarom werd de transittijd door de dunne darm gemeten in varkens vóór, tijdens en na de expulsiefase. Hieruit bleek dat de transit versneld was tijdens de uitdrijving van *A. suum*. Dit zou het mechanisme van expulsie tijdens primaire infecties kunnen verklaren. Het RNA transcriptiepatroon bij varkens tijdens de expulsie toonde een gemengde T helper reactie en in de verschillende experimenten in deze studie was de expulsie telkens geassocieerd met eosinofielen en intra-epitheliale T-cellen. Hoewel het niet duidelijk geworden is op welke manier deze immuuncellen fungeren, spelen ze wellicht een cruciale rol bij het tot stand brengen van de expulsie van *A. suum*.

Naast de uitdrijvingsreactie werd ook de opbouw van de pre-hepatische barrière bestudeerd (hoofdstuk 3). Chronische blootstelling van varkens gedurende 14 weken aan *A. suum* eieren leidde tot een reductie van 99.7% van de larven na een challenge infectie van 5000 *A. suum* eieren. Dit ging gepaard met een algemene Th2 reactie en een influx van eosinofielen, slijmbekercellen en mastcellen in de darm. Het RNA transcriptiepatroon demonstreerde een opregulatie van verschillende eosinofiel-gerelateerde genen. Er werd aangetoond dat eosinofielen van varkens in combinatie met serum van zowel geïnfecteerde als niet geïnfecteerde varkens degranuleren wanneer ze in contact komen met de infectieuze L3 larven. Dit wees erop dat degranulatie complement-afhankelijk is. Bovendien was de combinatie van eosinofielen en serum toxisch voor de larven. Het grootste toxische effect werd bereikt met serum van langdurig blootgestelde dieren, wat erop wijst dat antilichamen ook een belangrijke rol spelen bij het tot stand brengen van de pre-hepatische barrière. De rol van mastcellen en slijmbekercellen is minder duidelijk, maar wellicht dragen die bij tot een '*weep and sweep*' reactie waarbij meer vloeistof en mucus secretie plaatsvindt waardoor de larven moeilijker het darmepitheel kunnen bereiken. Bovendien was er meer RNA transcriptie van het gen coderend voor RELM-β, een eiwit met anti-worm eigenschappen. Helaas ontbreken de immunologische middelen zoals knockout stammen en specifieke antilichamen bij varkens om de rol van dit eiwit in de verdediging tegen *A. suum* te ontrafelen.

Voor muizen zijn deze middelen wel beschikbaar. Vandaar dat in hoofdstuk 4 het gebruik van een muismodel voor onderzoek naar de pre-hepatische barrière bestudeerd werd. Muizen werden gedurende 14 weken blootgesteld aan *A. suum* eieren en kregen vervolgens een challenge infectie. Deze muizen beschikten na de chronische blootstelling echter niet over een sterke pre-hepatische barrière, getuige de reductie van slechts 55% in het aantal larven in de lever. Niettemin leidde chronische blootstelling van muizen aan *A. suum* net zoals bij varkens tot een Th2 respons met een influx van eosinofielen en een toename van de slijmbekercellen. Bovendien nam de secretie van RELM-β door de slijmbekercellen naar het lumen van de darm toe. Gezien de anthelmintische eigenschappen van dit eiwit werd de functie ervan tijdens *A. suum* infecties nagegaan. Directe binding van RELM-β op de larven kon niet aangetoond worden. Vervolgens werd onderzocht of incubatie van infectieuze *A. suum* larven met RELM-β een negatieve impact had op de infectiviteit van de larven. Er bleek geen significant verschil in infectiviteit tussen larven geïncubeerd in RELM-β en larven geïncubeerd in controle-medium. Andere mogelijke functies van RELM-β werden besproken in de discussie.

In de algemene discussie werden een aantal opperkelijke bevindingen in meer detail besproken. Opvallend in dit onderzoek was dat eosinofielen niet degranuleren na contact met L4 larven, maar wel na contact met L3 larven van *A. suum*. Mogelijke oorzaken zoals het verwerven van complement inhiberende factoren tijdens de levenscyclus van *A. suum* worden besproken in de algemene discussie. Verder wordt de verandering in intestinale darmmobiliteit tijdens de expulsie van *A. suum* in verband gebracht met concomitante immuniteit en andere gastro-intestinale worminfecties.

Als laatste punt in de discussie worden de resultaten in het kader van vaccinonderzoek geplaatst. De aanwezigheid van antigenische diversiteit wordt als de meest valabele verklaring naar voor gebracht voor de nood aan langdurige blootstelling van varkens aan *A. suum* vooraleer sterke pre-hepatische immuniteit wordt opgebouwd. Omdat varkens natuurlijke immuniteit opbouwen tegen *A. suum*, is vaccinatie een haalbare controlestrategie, maar gezien de tijd die het immuunsysteem nodig heeft om bescherming te ontwikkelen en de aanwezigheid van antigen polymorfisme, kunnen we geen dramatische reducties verwachten in het aantal larven dat pre-hepatisch wordt tegengehouden, vooral niet wanneer gebruik gemaakt wordt van enkelvoudige antigen-vaccins. Belangrijk is ook dat tijdens vaccinonderzoek bij *A. suum* het effect op volwassen wormen wordt nagegaan, want larven die in gevaccineerde dieren toch de migratie kunnen voltooien, zijn mogelijk verzwakt en bijgevolg veel gevoeliger aan de expulsiereactie.

Appendix

Table A1: primer sequences for porcine genes

Gene	Forward primer	Reverse primer	Accession number
b2m	CACTCCTAACGCTGTGGATCAG	CCACTTAACTATCTTGGGCTTATCG	AB436775.1
arg1	GGCCACTGGCACACCAGTCC	ACTGCCGTGTTCACCGTCCG	NM_214048.2
c3	CAAGAAATGATTGGTGGCTTCAA	GACCTGTGGTTCACAGATGTCTTT	NM_214009
ccl11	CTTCTGTCGCCACCATCTG	ATTCTCTTGGGCATCAGCAC	XM_003131725.1
ccr3	ACAATGTTCTGCATCTGACCTAAAAT	AGAATGGAAAGAACCAGCTCTGTCT	NM_001001620
epx	TGGCCTCCCAGGGTACAAT	CAGGAACTTCCTCGCCAAAG	Ssc.33169
elane	CAGCTCAACAGATTTGCCTTCA	ACGCCTTGGTCCTGAGCA	FP015903.2
foxp3	GGTGCAGTCTCTGGAACAAC	GGTGCCAGTGGCTACAATAC	AY669812
gapdh	GGCATGGCCTTCCGTGT	GCCCAGGATGCCCTTGAG	DQ845173.1
gata3	TCTAGCAAATCCAAAAAGTGCAAA	GGGTTGAACGAGCTGCTCTT	NM_001044567
gzma	GGAGCTCACTCGATAACCAAGAAA	GCTTTAGAAGTTTAAGGTCACCCTCAT	NM_001198926.1
gzmb	TCTCCTATGGAAGAAAGGATGGAA	ATCCAGGGCAGGAAACTTGA	NM_001143710
hmbs	GCACGGCCATGTCTGGTAAC	CCACCACACTGTCCGTTTGTAT	NM_001097412
ifng	TGGTAGCTCTGGGAAACTGAATG	GGCTTTGCGCTGGATCTG	AY188090
il10	TGAGAACAGCTGCATCCACTTC	TCTGGTCCTTCGTTTGAAAGAAA	NM_214041
il12a	GGCCTGCTTACCACTTGAAC	GCATTCATGGCCTGGAACTC	NM_213993
il12b	CTGAAGAAGACGGCATCACG	AGGAGTGACTGGCTCAGAAC	NM_214013
il13	CTGACCACCAGCATGCAGTACT	GCTGCAGTCGGAGATGTTGA	NM_213803
il25	GAACCCACACCTTCCATTTG	ATCTCCAGAGGAGGCATGAG	XM_001926286.2
il33	AGCTTCGCTCTGGCCTTATC	GCTGACAGGCAGCAAGTACC	XM_003121912.1
il4	GCCGGGCCTCGACTGT	TCCGCTCAGGAGGCTCTTC	NM_214123
il5	TGGTGGCAGAGACCTTGACA	CCATCGCCTATCAGCAGAGTT	AJ010088
il5ra	CAAGGATGCCCCTGAGGA	TGCTGTATTCTTGGCATTCTTCA	XM_003358500.2
irln2	CCGTGTCAACATGACTTCCAA	GCCTCACAGAGAGCTGCAGAA	NM_213867
il17	GATGCTCATCCCAATTGCAA	TGACGCGTAACTCCAGGAGAA	U58142
klrk1	TCTCAAAATTCCAGTCTTCTGAAGATATA	AGGATCTGTTTGTTGGAATTTGTACTA	NM_213813
nkl	GTCTGACCCCTGAGCACTCT	CCCAGCTCCTCTCTTTGGAG	XM_003124939.1
nos2a	CGTTATGCCACCAACAATGG	AGACCCGGAAGTCGTGCTT	NM_001143690
rpl4	CAAGAGTAACTACAACCTTC	GAACTCTACGATGAATCTTC	DQ845176.1
stat4	ACCATTCGCTGACATCCTTC	TGGGAGCTGTAGTGTTTACC	XM_001924928.1
stat6	TCCCAGCTACGATCAAGATG	AGTGAGAGTGTGGTGGATAC	HM135386.1
tbp1	AACAGTTCAGTAGTTATGAGCCAGA	AGATGTTCTCAAACGCTTCG	DQ178129
tgfb	GAAGCGCATCGAGGCCATTC	GGCTCCGGTTCGACACTTTC	NM_214015
muc1	GTGGGCAGCTGGACATCTTT	GCCTGCAGAAACCTGCTCAT	NC_010446
muc2	GTGCAGGTGCAGGTCAACA	AGAGGCCGTTGTAGGAGATGAG	BX671371
muc3	AGTGGTTCGAGATCTGGGATGA	CCAAGGCCACATGGAGGTT	BP153612
muc5ac	TGCTCCTGGTCCAAGTGGTT	GGAGGATATTGCTGTAGGTCTCAAA	AF054583
retnlb	GCCTTTCTATAGGATGAAGCCAACT	ACAAGGGAGTCTAAGGAACACTGAGA	NM_001103210
mrc1	GGATGGCTCTGGTGTGGAA	AATGCTGGTCAGTGGATCTTTATTC	AY368183
tnfa	CCAATGGCAGAGTGGGTATG	TGAAGAGGACCTGGGAGTAG	X54859
ywhaz	ATGCAACCAACACATCCTATC	GCATTATTAGCGTGCTGTCTT	XM_001927228.2

Table A2: Primer sequences for murine genes

Gene	Forward Primer	Reverse Primer	Accession Number
actb	CTTCTTTGCAGCTCCTTCGTT	TTCTGACCCATTCCCACCA	NM_007393.3
arg1	TGGTGCTGGGTGGAGACCACA	AGGGTCTACGTCTCGCAAGCCA	NM_007482.4
c3	TACAGCCCCAGCTCGCCTCTG	GGTCTCTTCGCTCTCCAGCCG	NM_009778.2
c9	AGGCTGTGAACCCACCCAGGA	TCCGCTACTCGGTCACGGCA	NM_013485.1
ccl11	GGGTCCAGGATGCCACAAAGCA	TCCTTGGGCGACTGGTGCTG	NM_011330.4
ccl2	GCAGAGAGCCAGACGGGAGGA	AGTAGCAGCAGGTGAGTGGGGC	NM_011333.3
ccr3	GGGTCATGGCTCCTGCCTCCA	GCCAAAACCCCACTCATTCCACAGA	NM_009914.4
cma1	TGAGCCAGCCTCCGACACACT	ACAGCAGGGGGCTTTGCATTCC	NM_010780.2
elane	TGCTTCGGGGACTCTGGCGG	GGGGTGGGAGTGCAGACAGGT	NM_015779.2
foxp3	GCCCATGTCGCTGTGTTGGG	TGCTCACTACTAGGCAGAGCTGTT	NM_001199347.1
gapdh	TGGATACAGGCCAGACTTTGTT	TGAAGGGGTCGTTGATGGC	NM_008084.2
gata3	GCAGCGTACCAGCTACCAACATGC	CCAGGTGGCTCTCAGGACCAGG	NM_008091.4
gusb	CCGATTATCCAGAGCGAGTATG	CTCAGCGGTGACTGGTTCG	NM_010368.1
gzma	CCTGAAGGAGGCTGTGAAAG	GTTACAGTGGGCAGCAGTCA	NM_010370.2
gzmb	ACAAGGTCACAGAGCCCCCTC	CCCCGATGATCTCCCCTGCCTT	NM_013542.2
hprt1	TGGATACAGGCCAGACTTTGTT	CAGATTCAACTTGCGCTCATC	NM_013556.2
ifng	AGCTGCCATCGGCTGACCTA	CCGCAGGAGGAGAAGCCCAGA	NM_008337.3
il10	GCTAACCGACTCCTTAATGCAG	AGCTTCTCACCCAGGGAATT	NM_010548.2
il-4	TGTACCAGGAGCCATATCCAC	CACCTTGGAAGCCCTACAGA	NM_021283.2
il12b	ACCAGACCCGCCCAAGAACT	GCACGCAGACATTCCCGCCT	NM_008352.2
il13	CTCAGCCTGCACTGCCTGCC	GCTCAAGCTGCTGCCTGCCT	NM_008355.3
il17a	CTACCTCAACCGTTCCACGT	AGCTCTCAGGCTCCCTCTTC	NM_010552.3
il33	GCAGAAGGGAGAAATCACGGCA	CAAGGCGGGACCAGGGCTTC	NM_133775.2
il4	TGTACCAGGAGCCATATCCAC	CACCTTGGAAGCCCTACAGA	NM_021283.2
il5	GGCGAGGAGAGACGGAGGACG	GCCTCAGCCTTCCATTGCCCA	NM_010558.1
itln2	TGCTGGCGTGAGGGTCACTG	GACGCAAAGTCTCCACACTGCAC	NM_010584.3
mrc1	GCCAGGACGAAAGGCGGGATG	GTGGGCTCTGGTGGGCGAGT	NM_008625.2
muc5ac	GCAACTGGACCAAGTGGTTT	TGACCCAGATCCTCCATCTC	NM_010844.2
nos2	CCGTGGTCACCTACCGCACC	TCGGAAGGGAGCAATGCCCG	NM_010927.4
prg2	TCGGGGGAGCGTCTGCTCTT	GGGGCACTGAAGGTCCACGTC	NM_008920.4
prlpo	ACTGAGATTCGGGATATGCTGT	TGCCTCTGGAGATTTTCGTG	NM_007475.5
retnlb	CCGCTGCTGCCGAATGGCTTA	TTCCTGGTCGAGACCGTGGTT	NM_023881.4
rnase3	TGTCTGTAACATCACCAGTCGGAGG	CCAGTGAAGTTCTGGGATTACAGGC	NM_017388.1
rorc	GAACCAGAACAGGGTCCAGA	CGTAGAAGGTCCTCCAGTCG	NM_011281.2
stat6	GCACACGTCATCCGGGGTCA	ATCCGGTCCCCCAGTGAGCG	NM_009284.2
tbp	CAAACCCAGAATTGTTCTCCTT	ATGTGGTCTTCCTGAATCCCT	NM_013684.3
tgfb1	TTGCTTCAGCTCCACAGAGA	TGGTTGTAGAGGGCAAGGAC	NM_011577.1
tnfa	ACGGCATGGATCTCAAAGAC	GTGGGTGAGGAGCACGTAGT	NM_010548.2

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