Experimental *Cooperia oncophora* infections in calves: an insight into host-parasite interactions

Experimentele *Cooperia oncophora* infecties in kalveren:
een inzicht in gastheer-parasiet interacties
(met een samenvatting in het Nederlands)

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Chapter 1

General introduction

Relativeren moet je leren.

In het beste geval word je door een wetenschappelijke studie minder dom. (C. Palmen)

INTRODUCTION

Parasitic nematode infections are still one of the major causes of production losses in grazing cattle in temperate regions of the world, with the majority of these infections involving the intestinal lumen dwelling nematode *Cooperia oncophora* and its abomasal counterpart *Ostertagia ostertagi.* Although nematode parasitism is common in animals of all age classes, calves, entering the first grazing season, are the most susceptible to nematode infections. Therefore, most work on nematode infections in cattle refer to calves.

In general*, C. oncophora* has not received much attention compared to other nematodes such as *Dictyocaulus viviparus*, *O. ostertagi* and others. This is mainly caused by the mild pathogenicity of the worm. In natural conditions, clinical parasitism as caused by *C. oncophora* is rarely seen. Infection has been associated with production losses [179, 183] but the use of effective anthelmintics (anti worm drugs) during the last decades has reduced its clinical importance. Increasing concerns over the presence of drug residues in food animals, the incidence of anthelmintic resistance and the escalating costs of the development of new anthelmintics all suggest that alternative control strategies must be developed. The most likely alternative for anthelmintics would be a vaccine, but this requires an improved understanding of the immune response, together with a more detailed knowledge of the parasites themselves.

In the first part of this introduction, the life cycle, diagnosis and control of *C. oncophora* will be discussed. The second part will focus on the host and more specifically on the host-parasite interactions as observed during infection. Features of host immunity will be discussed from the immunological and the parasitological point of view.

1. THE PARASITE: *C. ONCOPHORA*

C. oncophora belongs to the trichostrongylid nematodes of ruminants (Phylum Nemathelminthes, Class Nematode). It is a small (10-12 mm), pink worm that preferentially resides in the small intestine of cattle but sheep and goats are also susceptible. Female worms lay eggs of the strongyle type: being oval, thin-shelled, colourless and medium sized (+/-50 µm).

1.1. Life cycle

A first detailed life cycle of *C. oncophora* was only described in the early sixties [121]. Under natural conditions, animals are infected by intake of infective third-stage larvae (L3) with the grass. The L3 exsheath in the abomasum and, subsequently moult to fourth stage larvae (L4) in the small intestine, i.e. the final habitat of the worm. A last moult takes place 10 days after ingestion, resulting in fifth-stage larvae (L5) that are the young adult stage. At this time point, male and female worms can clearly be differentiated based on their

morphology. The first gravid females occur around day 14 after infection and a few days thereafter eggs can be detected in the faeces. Eggs pass to the pasture with the faeces and develop into first stage larvae (L1) which hatch and moult to become second stage (L2) and L3. The time-span for this development merely depends on the weather conditions. Moist conditions are also necessary for L3 to migrate actively from the faecal pat to the grass where they are eaten by the host. L3 are very resistant to weather conditions that prevail in Western-European countries; they can overwinter on pasture and by doing so give rise to new generations of this species the next year.

1.2. Diagnosis, Pathogenesis and Epidemiology

1.2.1. Diagnosis

Accurate non-invasive diagnosis of infection and differentiation of infection among different trichostrongylids is an important component of proper management and efficient, economical drug treatment. The available diagnostic techniques and their usefulness for estimating nematode exposure has been reviewed recently [75].

Until now, *in vivo* diagnosis of gastro-intestinal (GI) nematode infections relies mainly on the detection of parasite eggs in the faeces of infected animals. The most widely applied method is the McMaster method [100]. The advantage of this method is that it is an easily applicable and simple technology method but the sensitivity and reproducibility are rather low. The detection limit of the McMaster assay is often between 25 and 50 eggs per gram faeces (EPG) and in cattle values less than 10 EPG are often reported [39]. The Wisconsin sugar flotation [50] technique has a higher sensitivity but is more labour intensive. Recently, a new method that uses both salt- and sugar solutions for flotation of nematode eggs was described [159].

Since eggs of many common genera are indistinguishable, differentiation of eggs in mixed infections must be performed by faecal egg counts followed by faecal cultures (LPG) for the recovery and identification of infective larvae. LPG has a higher sensitivity than EPG and allows differentiation of the species but a disadvantage of larval cultures is that yields are never 100% and, particularly when for some reasons the yield is low, the proportion of larvae developing may differ between species [75].

The golden standard of diagnosis of nematode parasitism remains the enumeration and differentiation of worms at necropsy. This method provides an accurate estimate of the level of infection and simultaneously allows differentiation of the involved species. An obvious drawback of this method is that animals have to be killed and this is far too expensive for diagnostic purposes at farm level. There is no continuous reliable correlation between EPG/LPG and the number of adult worms. Whereas in animals with a first nematode-

exposure, EPG is a good value to estimate worm burden, upon challenge this relationship decreases; immunity resulting in a reduced fecundity might affect this relationship and in *Teladorsagia circumcincta* infected sheep worm fecundity declines as worm number increases [212]. Furthermore, various nematode species greatly differ in egg-laying capacity [57] and this might disturb the relationship between egg-counts and the performance of the animals. This implies that whatever test is used, one should be aware of the limitations involved.

Beside parasitological diagnosis, there is a growing field of other means of diagnosis based on molecular or immunological tools. A PCR-based technique was developed that allows the identification and relative quantification of *O. ostertagi* eggs in a mixed faeces culture [248]. The *C. oncophora* 14.2 kDa ELISA has shown to be a sensitive tool to detect the exposure level in both naturally [185, 99] and experimentally infected calves [247]. These are only a few of the available examples but despite their relative success, new methods are not widely applied and commercially available and people stick to the conventional way of diagnosis.

1.2.2. Pathogenesis

Clinical parasitism following *C. oncophora* is very rare under natural conditions and calves, entering their first grazing season form the age class that is most susceptible to nematode infections. Obvious signs of disease are loss of appetite, dull hair coat, diarrhoea, and weight gain depression. Experimental trickle infections during six weeks with a daily dose of 10,000 *C oncophora* L3 in calves at 3 months of age revealed that despite the development of a good immunity, the damage to the intestinal mucosa resulted in considerable pathophysiological changes [9]. This was evidenced by increased plasma protein losses and stunting and fusion of the villi, together with an excessive production of mucus. However, by week 12 after infection there was substantial repair which might account for the somehow contradictory results of Coop et al. [47] who found little pathological damage following *C. oncophora* infections and no larval penetration of the mucosa. In the latter experiment, calves were necropsied at a much later time point after infection and it is likely that full recovery of disease had occurred by then.

1.2.3. Epidemiology and Prevalence

The most important GI nematodes of cattle in Western Europe are *O. ostertagi* and *C. oncophora*. Calves infect themselves after turnout with overwintered larvae and faecal egg output starts 3 weeks thereafter. Between approximately 5 and 12 weeks after turnout a correlation exists between faecal egg output and initial infection levels but, due to the onset of developing immunity this correlation is lost in the later phase of the grazing period [176].

Following the initial infections a midsummer increase of pasture infectivity occurs from two months after turnout onwards. Because overwintered pasture infectivity diminishes rapidly in spring, a delay in turnout diminishes the level of initial infection and, this effect is enhanced when the pasture is mown before turnout [31]. *C. oncophora* is far more profilic than *O. ostertagi*. Hence, it dominates the faecal egg output in the first grazing season [31]. However, the generation of immunity against *C. oncophora* is faster, and therefore in older animals *O. ostertagi* is the predominant type isolated from larval cultures [31].
Recently, our lab performed a large survey to investigate the presence of nematode eggs in faeces of grazing cows in the Netherlands (± 1,400 faeces samples collected between June and September 2000 and ± 4,000 in the same period in 2002). In 70% of the investigated animals in 2000 eggs were found in the faeces, while in 2002 50% of faecs samples contained eggs (Ted Mes, personal communication). Based on coprocultures, it was shown that only 15% of the infected animals were infected with *C. oncophora*. These results are consistent with abattoir surveys in the Netherlands [33] and Belgium [2] that revealed a prevalence of nematode eggs in faeces in more than 80% of the animals. In these surveys abomasa, blood and faeces were examined from +/- 110 dairy cows with known grazing history. Based on larval identification *Ostertagia* spp. and *Trichostrongylus* spp. were the most prevalent, with only 16% of the larvae being *Cooperia* spp. in Belgium and 4% in the Netherlands. These prevalence data only apply to adult cows and give no information on the importance of *C. oncophora* in young animals as all FGS calves (if not treated) pick up *Cooperia* [127]. The conclusions are two-fold: (i) the dominant genera in adult cows are still *Trichostrongylus* spp. and *Ostertagia* spp. as has been described in earlier reports [34] and, (ii) the observations support the more effective built up of acquired immunity against *C. oncophora* compared to *Ostertagia* ssp. and *Trichostrongylus* spp..

1.3. Parasite control

Many parasite control strategies aim at minimizing pasture infestation levels during the first grazing season (FGS). Not many farmers used to combat GI parasitism in the second year or in adult cattle, because in these age categories clinical disease does not often occur and strategic measures are more difficult to implement. Minimizing larval challenge during the FGS is beneficial for growth performance but it may also result in slower built up of immunity thereby affecting production parameters in the second year [179, 233]. The advent of anthelmintic resistance has led to renewed interest in non-chemical means of controlling helminth infections of livestock [238]. Among the methods under investigation are: genetic host resistance, improved nutrition, biological control, grazing management, and vaccination. Correctly integrated combinations of these approaches, along with occasional use of anthelmintics may provide the best approach for sustainable helminth control. This

combination of control techniques is termed integrated parasite management (IPM). All factors that might contribute to IPM will be briefly reviewed below.

1.3.1. Treatment

Nowadays in Europe, most parasite control is protective in its orientation, based on a regular and suppressive use of anthelmintics (99% protection) in the form of programs or sustained release. Liver flukes and GI nematodes are nearly always controlled by a combination of anthelmintic drugs and pasture management. Although this practice can be extremely effective, it is not sustainable as the situation is threatened by the increased evidence of anthelmintic resistance in worms and by societal concerns over the presence of drug residues in food animals. Resistance is a major concern in the sheep trichostrongylids, but it appears to be also spreading in cattle nematodes (reviewed in [228]). Anthelmintic resistance in *Cooperia spp.* has been reported against benzimidazoles (BZ, thiabendazole (TBZ), oxibendazole, oxfendazole) and macrolide lactone (ML) e.g. ivermectin (IV) and moxidectin (MD)) [238]. Most concerns surround IV resistance and the reports on this type of resistance are associated with frequent treatment of young cattle crowded on moist pastures [80]. One approach to reduce treatment frequency would be to measure infection or the risk of infection and treat tactically when the infection level reaches a threshold.

1.3.2. Vaccination

Anti-parasitic drugs are very effective, relatively cheap, and easy to administer (e.g. oral dosing and pour-on) and have accordingly set very high benchmarks by which vaccines will be judged. Conventional vaccine approaches in viral or bacterial diseases are aimed as a weapon to abolish infection completely. Regarding GI nematodes of ruminants, it is more appropriate to consider a vaccine as an epidemiological tool to maintain low-level pasture contamination [249]. Using this approach, minor infections would boost immunity and avoid the development of clinical symptoms or production losses. Mathematical models suggest that parasite vaccines may not have to achieve the efficacy of anti-parasitic drugs to offer substantial benefit to their users but could be used to prevent clinical pathology in animals and/or reduce the build up of parasites on pasture, either applied alone or along-side biological control or grazing management [21].

There are no commercially available vaccines for the control of helminth infections in ruminants, with the notable exception of that for the lungworms *D. viviparus* and *D. filaria*. The vaccine against *D. viviparus* was developed following the discovery of Jarret et al. [124] that two doses of 1,000 larvae, attenuated by irradiation, induced up to 98% protection against challenge with the parasite. Based on this success, attempts were made to develop a similar vaccine for other nematode infections such as *Ancylostomum caninum* in dogs [164]

and *Haemonchus contortus* in sheep [205]. The sheer number of larvae required, and disadvantages such as the cost of production, quality control and limited shelf life have precluded other irradiated nematode vaccines from commercial availability.
There are two types of antigens associated with nematode parasites: (1) soluble excretory and/or secretory products (ESP); and (2) those fixed at external surfaces or within the parasite (the so-called somatic antigens). Some of the ESP and exposed somatic antigens induce an immune response in the host during the course of infection and are designated 'natural antigens', while antigens that do not induce an immune response during infection are designated 'hidden antigens' (reviewed in [170]). Vaccination trials against *H. contortus*, *T. circumcincta*, *T. colubriformis* with cuticular collagens, ESP, and other putative natural antigens have resulted in substantial reduction in egg output and worm burden (reviewed in [69]). Considerable effort has also been applied to developing strategies based on the use of hidden antigens, especially gut molecules, as vaccine (reviewed in [167, 170]). Very few of these attempts are approaching a commercial product for GI nematodes, for reasons ranging from difficulties in reproducing the effect with recombinant proteins to consolidation in the animal health industry. One of the problems encountered is that all of the so far putative candidates contain glycoconjugate moieties that may carry essential protective (and parasite-specific) epitopes that cannot be properly synthesized in these protein expression systems [230].

1.3.3. Alternatives

High quality and equilibrated nutrition of cattle has shown to significantly reduce their susceptibility of animals to GI nematode infection [48]. Attempts to use biological control agents such as nematophagous fungi to limit larval population on herbage have yielded encouraging laboratory results but have not yet been commercially applicable [144, 218]. Furthermore, limiting pasture contamination can also be achieved effectively by alternate or rotational grazing [10, 76, 77]. The effectiveness of the latter method is highly influenced by factors such as the interval between grazing periods on one pasture and by mowing of the pastures [76, 77]. However, given the limited amount of grazing space for cattle in many countries, alternate grazing is not always feasible.

1.3.4. Genetic resistance and responder types

An increasing attractive adjunct for control of GI nematodes would be the identification of host genes that influence acquired or innate resistance to the parasites. The genetic background of animals has been reported to be a significant factor in how a host responds immunologically to infection and several studies demonstrated that EPG values of pastured cattle are strongly influenced by host genetics [146, 91, 134]. In addition, EPG values are not

normally distributed and within a herd, only a small percentage is responsible for the majority of parasite transmission [7, 94]. This pattern strongly suggests that genetic management of a small percentage of a herd could considerably reduce overall parasite transmission. This over-dispersion of EPG is used as one of the major characteristics to define responder types following *C. oncophora* infection.

Primary infection of 3-month-old calves with 100,000 L3 *C. oncophora* infection (which are followed during 6-7 weeks) have been used to detect genetic differences among cattle in their resistance to GI nematode infections. The choice of parasite species, age of calves and larval dose was made to simultaneously minimize environmental effects on infection and maximize host response differences. It was found that at the age of 6 months calves are less susceptible to infection with *C. oncophora* [135] which results in a lower among calves variability in response than at 3 months of age. Younger animals might be even more susceptible but under natural conditions calves are not put on pasture before 3 months of age. The larval dose was selected following the observation that with a lower (e.g. 10,000 or 20,000 L3) or higher dose (e.g. 200,000 or 500,000 L3) the variation between calves appeared to be much smaller. These experimental infections have the potential to discriminate between three major responder types based on parasitological variables (EPG, worm counts) (fig. 1) and the speed by which the parasite is expelled from the host [231].

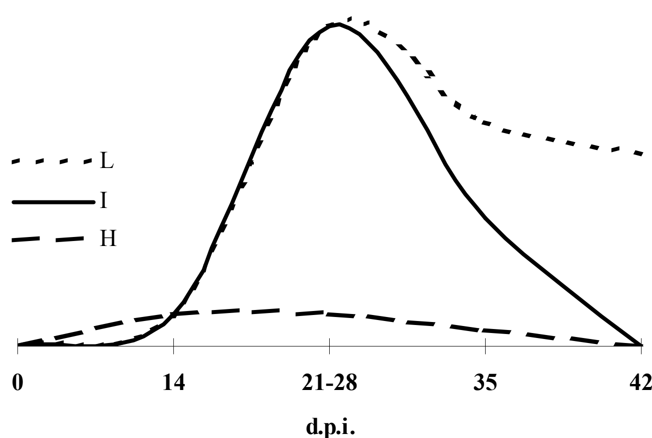

FIGURE 1 SCHEMATIC REPRESENTATION OF EGG OUTPUT PATTERN OF HOST RESPONDER TYPES FOLLOWING PRIMARY *COOPERIA ONCOPHORA* INFECTION IN 3-MONTH-OLD CALVES. Days after infection (d.p.i.) are depicted on the X-axis. L=low responders (± 40% of the population), I=intermediate responders (± 60% of the population) and H=high responders (± 2% of the population).

High responders, a small proportion of the host population, seem refractory to infection and have very low or no egg output and the worm burden at necropsy is low. *Low* responders show high EPG which is continued for weeks, and hence they have high worm burdens at necropsy. In *Intermediate* responders the EPG is initially similar to that in *Low* responders but 4 to 5 weeks after infection, the EPG starts to decline rapidly. These animals usually

show intermediate worm burdens at necropsy, but these can still range from low to high numbers.
Although a classification into responder types is to some extent an oversimplification of reality, it may be useful in studying and identifying relevant immune mechanisms. In addition, the existence of three major types of responder is not restricted to *C. oncophora*. Based on the result from an extensive breeding program, Gasbarre et al. [89] demonstrated that calves on pasture could also be separated into three types related to their EPG values: (i) *Type I* never demonstrated EPG values and is comparable to the *High* responders following experimental *C. oncophora* infection. (ii) *Type 2* showed rises in EPG values through the first two months of the test and then the EPG fell and remained at very low level, hence their EPG pattern is similar to our *Intermediate* responder animals (iii) *Type 3* maintained high EPG levels throughout the test, and resembles the *Low* responder animals. Although these pasture infections were mixed infections, the predominant species involved was *O. ostertagi*. A secondary infection experiment of these three types revealed that based on the EPG values, type 1 and type 2 animals were immune, while type 3 animals continued to shed eggs in their faeces [89]. Based on these results, the authors suggested the following categorization of the animals: innately immune (type 1 and *High* responders), acquired immune (type 2 and *Intermediate* responders) and immunologically non responsive (type 3 and *Low* responders).
The similarity in responses in a group of animals, despite the difference in nematode species and experimental versus natural infection, suggests that the occurrence of responder types is not restricted to one parasite species or one cattle breed and is promising in view of the identification of genes involved in resistance to GI nematodes. But we have to remain aware that resistance of a host to one species cannot always be extrapolated to another species. In addition, the genetic trait for resistance might compete with traits for maximal production.

2. THE HOST AND ITS DEFENCE

The development of well-defined rodent laboratory models of GI nematode infections has made significant contributions to the understanding of immunity to infection at three levels: (i) the type of T cell responses that controls and regulates the effector response (ii) the events in initiating a particular type of T cell response and (iii) the effector mechanisms responsible for worm expulsion. However, rodents are often not the natural host of the parasites studied and each host-parasite system has its own specificities. Hence, extrapolation of these studies to infection in the natural large animal host might be misleading. In the following sections, the emphasis will lay on results obtained from GI nematode infections in ruminants and data gathered from rodent models will be briefly outlined where needed.

2.1. Intestinal immune system

The gut is the major route of entry for a variety of antigenic materials, including infectious organisms, environmental contaminants, and food. Equilibrium has to be reached between tolerance towards the extensive range of nutritional antigens and the generation of effective immunological protective mechanisms against potentially harmful micro-organisms such as parasites. The intestine is protected by gut associated lymphoid tissues (GALT) consisting of Peyer's patches (PP), isolated follicles, lymphocytes within the epithelium (IEL) and within the lamina propria (LPL) of the mucosa.

The PP are lymphoid aggregates in the jejunum, ileum and cecum. In young ruminants, ileal PP consist predominantly of B cells and 1% T cells. In mature animals more αβ T cells are present, but still 70% of the cells are B cells. The majority of these T cells are CD4+ Th cells (twice as much CD4+ than CD8+ cells) with an equal Th1: Th2 distribution. In contrast the population of IEL consists mainly of CD8+ αβ T cells and other lymphocytes are rare, resulting in a CD4:CD8 ratio of 1:8. Half of the LPL are αβ T cells, with a CD4:CD8 ratio of 2:1 and the T-helper cell populations appear to be skewed towards a Th2 phenotype.

The third major T cell subpopulation is the γδ T cells, which are very prominent in ruminants as compared to other species [111]. The general principles that apply to activation of αβ T cells through their TCR are profoundly different than those that apply to γδ T cells. They do not see antigens presented on autologous MHC molecules nor do they respond specifically to protein peptides (reviewed in [109]).

The role of γδ T cells in infected ruminants is still not very clear, however, they might serve as regulatory cells in intestinal immune responses. It has been suggested that food proteins are presented to villous epithelial cells, rather than via M cells to organized lymphoid tissues [140] and that this alternate antigen presentation pathway induces suppressive T lymphocytes in the intestinal mucosa [29]. Suppresive γδ T cells have been demonstrated in cattle, both in blood and in mammary gland secretions [45, 175]. Depletion of γδ T cells in sheep infected with *T. colubriformis* reduced egg excretion and worm counts, suggesting that also in helminth infected ruminants γδ T cells might be suppressive [153]. The authors suggested that protection was affected by loss of cytokine production, leaving other cytokines free to induce different mechanisms. This is consistent with the recent observation that by early secretion of IFN-γ, bovine γδ T cells might contribute to an inflammatory response and establish a Th1 cytokine environment, which is important for the clearance of bacteria and other intracellular pathogens [13]. In nematode infections however, this would ablate the induction of a protective Th2 response.

2.1.1. Antigen presentation

The first step in the induction of the immune response is the uptake and the presentation of antigens. Both the adult and larval stages of nematodes secrete antigens in their environment. These are referred to as excretory/secretory products (ESP) some of which are likely to interact with antigen presenting cells (APC) initializing the immune response. Four major cell types are thought to present antigen within gut associated lymphoid tissues: dendritic cells (DC), macrophages, B cells and epithelial cells [172]. In ruminants the classical presentation of antigen by epithelial cells may not occur since these cells do not express MHC class II molecules [101, 186].

DC may contribute to the Th1 and Th2 dichotomy in the immune response as different types of DC capable of inducing specifically Th1 or Th2 cells have been described [43]. However, recent reports suggest that the 'DC1-DC2' idea is likely oversimplified, indicating that DC remain largely plastic and that the direction of Th1 or Th2 responses is determined by factors such as the state of DC maturation, the antigen dose and stimulation of DC by pathogen-derived products [30]. There is as yet no information available on the main cell type involved in nematode antigen presentation *in vivo*. *In vitro* assays demonstrated the ability of schistosome egg glycolipid to elicit cytokine responses by human monocytes [229]. *In vivo*, the glycan determinants can probably also instruct DC to induce Th2-polarized responses [155].

At least two types of bovine DC (called afferent lymph veiled cells) with distinct antigen presenting capacities have been described [114] but their role and contribution in *C. oncophora* infections remains unexplored.

2.1.2. Involvement of cell mediated immune response (CMI)

Attempts to define the inductive requirements for parasite immunity have come from the *in vivo* use of monoclonal antibodies (Mabs) in rodent models. These studies demonstrated the overall importance of T-lymphocytes for protection against GI nematodes in rodents infected with *Trichinella spiralis* [106], *Nippostrongylus brasiliensis* [129], *Heligmosomoides polygyrus* [222] and *Trichuris muris* [139]. In these models, the T cell subset to which protection has been ascribed is CD4+ cells. Similar studies involving treatment with anti-CD4 Mab have been attempted in ruminants when reagents became available, but the most convincing studies regarding the role of CD4+ cells in protection against GI nematodes in ruminants have come from passive transfer experiments. Transfer of gastric lymph lymphocytes from resistant lambs to their genetically identical uninfected twin conferred protection against subsequent infection with *H. contortus* [207] and *O. circumcincta* [206]. Studies involving prolonged administration of Mabs are limited in cattle by the development of anaphylactic

reactions to murine Ig within 1 week [113]. Consequently, most studies report on the kinetics of cellular changes, in peripheral blood, tissues and draining lymph nodes.
Marked differences in frequencies of lymphocyte subpopulations have been observed in the peripheral blood, draining lymph nodes and tissues following GI nematode infections in ruminants (reviewed in [15]). Reactions taking place during larval development and adult infections significantly differ, as well as reactions induced by distinct nematode species (reviewed in [15]) emphasizing that the effector mechanisms of parasite expulsion are very complex and that the contribution of a specific lymphocyte subpopulation might differ depending on the host-parasite system, and the infection and resistance level of the animals.

2.1.3. Th1-Th2 dichotomy
The seminal observation that murine and human CD4+ cells could be segregated in T-helper 1 (Th1) and T-helper 2 (Th2) based on the cytokines they secrete [165, 189], has provided a basis for understanding the underlying cell regulatory mechanisms controlling resistance to infection. Th1 cells produce IFN-γ, lymphotoxin and interleukin 2 (IL-2) whilst Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. A naïve T cell can differentiate into either a Th1 or Th2 cell type, passing through an intermediate stage where it has an unrestricted cytokine profile (often referred to as Th0). The differentiation pathway is influenced by a number of factors, the most potent of which seems to be the immediate cytokine environment a T cell experiences at the time of antigen presentation. Thus, IL-12 promotes the development of Th1 cells whilst Th2 cells can develop in the presence of IL-4.
Cytokines released by Th1 cells promote macrophage activation, which results in delayed-type hypersensitivity and promotes the production of opsonizing antibodies. These mechanisms are particularly important in the clearance of intracellular organisms. In contrast, the characteristics of helminth infections such as eosinophilia, mastocytosis, IgG1 and IgE production are all mainly controlled by Th2 cytokines [81]. The induction of mastocytosis is regulated by a variety of cytokines including IL-3, IL-4, IL-9 and the growth factor stem cell factor [149]. IL-5 is a key factor in the growth and differentiation of eosinophils [193] whereas IL-4 and IL-13 are involved in isotype switching to IgG1 and IgE [216].
Although not as clear cut as in mice, Th1 and Th2 responses have also been described in cattle [36]. Studies conducted by Brown et al. [37, 35] revealed that the majority of Th cell clones specific for *Babesia bovis* and *Fasciola hepatica* showed a less restricted cytokine profile and more resembled the Th0 like profile. Regarding GI nematodes in cattle, the immune response to *O. ostertagi* has been studied most extensively. Soon after infection with *O. ostertagi*, primed lymphocytes leave the draining lymph nodes, enter the peripheral circulation and home to the tissues immediately surrounding the parasite [4]. The immune

response in the abomasum is in many ways similar to that seen in other nematode infected mammalian hosts, with high levels of expression of IL-4 in lymphocytes from the draining lymph nodes and in lymphocytes isolated from the mucosa [5; 41]. But unlike other models, the immune response also elicits the expression of IFN-γ, implying that in *O. ostertagi* infected calves the immune response is not stereotypic Th2 like.

2.2. Effectors

2.2.1. B cells and Antibodies

B lymphocytes are ultimately responsible for the generation of protective antibodies following infection and hence both components of the humoral immune response are intimately linked. Beside their role as antibody secreting cells (ASC), B cells also act as antigen presenting cells and might contribute to the priming and maintenance of T cell responses.
While the antibody response to GI nematode infections in ruminants has been studied extensively, there is only very little information concerning other functional contributions of B cells during infection. Therefore, both aspects will be discussed in separate sections.

2.2.1.1. B cells

A redundant role for B cells and antibodies in the expulsion of *T. muris* was suggested following the observation that transfer experiments with immune CD4+ cells from infected BALB/c mice to SCID mice (which lack both T and B cells) conferred resistance [66]. However, infection of B cell deficient μMT mice (which have a disrupted immunoglobulin μ chain gene) with *T. muris* revealed that B cells are required for resistance to infection, i.e. during a primary *T. muris* infection the B cells appear to be important for the development of a protective Th2 immune response (by cytokine action and costimulation) [28]. A critical role for B lymphocytes in primary and memory anti-filarial immunity was also demonstrated in *Brugia pahangi* infection in mice [173, 174].
Infection with *H. contortus* in sheep and *O. ostertagi* in calves is associated with an increase in abomasal lymph node weight and a concurrent increase in the proportion of B cells [16, 90]. Concurrent herewith, the frequency of B cells in the abomasal mucosa increases. Although related to infection, the functional properties of these B cells have not yet been clarified.

2.2.1.2. Antibodies

Nematode infections constitutively induce the production of parasite-specific antibodies both in the peripheral blood and at mucosal surfaces and the elevation of antibody titres is more pronounced after a secondary infection than after a primary infection (reviewed in [161,

[15]). To date, most studies examining the kinetics of the antibody responses during nematode infections have used crude worm preparations of different nematode developmental stages. This makes it difficult to compare results from different laboratories, as the antigen constitution of crude worm preparations highly differ depending on the different extraction methods used.

i) IgG1 and IgG2

A functional distinction can be made between IFN-γ dependent Th1 antibody isotypes and IL-4 dependent Th2 related isotypes [1]. For cattle, it has been shown that IgG1 and IgA can be classified as Th2 associated isotypes, as opposed to IgM and IgG2 that are associated with a Th1 response [73, 36].

Consistent with the induction of a Th2 response in all infection models examined IgG1 was the predominant serum antibody isotype over IgG2 [40, 97, 112, 176]. In the same studies IgA antibodies were generally low in the serum of infected animals but IgA is a mucosae-associated Ig and peripheral titres might not have a predictive value for its role in infection.

The role of increased systemic antibody titres during *C. oncophora* infection is not entirely clear, but antibody titres correlate with various parasitological parameters of resistance [135]. Primary infection with *C. oncophora* induced significant increases in IgG1 titres against *C. oncophora* adult crude worm antigens whereas no IgG2 titres and a minor increase in IgA titres are observed [176]. In addition, western blot analysis revealed that the *C. oncophora* specific antibody response was mainly directed against low molecular weight antigens [55, 176, 232] and that the recognition of these antigens could be used to distinguish susceptible and resistant calves [231].

ii) IgA

IgA responses are typically associated with GI nematode infections and have been more observed in the local mucosal tissues, mucus, draining lymph nodes and in lymph than in serum (reviewed in [15]). The number of IgA secreting cells is generally increased in the mucosae of animals bred for resistance to GI nematodes [98]. Although the exact function of IgA is not defined, there is a strong negative correlation between worm length and IgA levels in the gastric lymph of *T. circumcincta* infected sheep, suggesting that mucosal IgA could interfere with the feeding processes of parasites [204]. In addition to a direct effect on the worms, IgA might also interact with eosinophils [60]. IgA/antigen complexes can bind through the Fc-α receptor on eosinophils and provoke the release of anti-inflammatory mediators which have a detrimental effect on the worm population.

iii) IgE

The production of IgE against worm allergens and parasite non-specific IgE during GI nematode infections in rodents and man is well documented [123]. Strikingly, in many individuals the total serum IgE level correlates roughly with both disease severity and protective immunity to parasites.

IL-4 is the most important cytokine mediating IgE synthesis but human and murine B cells also synthesize IgE in response to the closely related cytokine IL-13 [216]. IgE synthesis has also been observed in the absence of IL-4; cross-linking of CD40 (expressed on the surface of B cells) alone is sufficient to elicit polyclonal IgE responses, but when combined with signals from the B cell antigen receptor the response becomes antigen specific [78]. Although the exact mechanisms are not entirely elucidated, the polyclonal generation of IgE following parasite infection suggests that nematode products might have some activities or structure homology with cytokines responsible for the induction of IgE [187]. Recent reports demonstrated the ability of parasite products to potentiate IgE synthesis independently of infection [61, 217].

The development of different Mabs which specifically recognizes ovine and/or bovine IgE has allowed study of the role of IgE responses in ruminants [201, 136, 137]. Sheep IgE levels increased after infection with *T. colubriformis* [200], *H. contortus* [136], and *T. circumcincta* [119]. One of these studies revealed a correlation between total serum IgE and the decrease in worm counts following primary infection with *H. contortus* in sheep [136]. Data of *O. ostertagi* infections in cattle were less consistent but generally indicated a slight increase in serum IgE levels following infection [219, 11]. Recently, a correlation between total serum IgE levels and protection was demonstrated in calves re-infected with *D. viviparus* [137].

2.2.2. Eosinophils

Eosinophils and mast cells are the major effector cells typically associated with helminth infection. Eosinophils develop in the bone marrow and are constitutively released at a low rate in the blood circulation; they normally comprise only a small fraction of circulating leucocytes (<1-5%). Most of the eosinophils are found in tissues, predominantly those at surfaces of the body that interact with the external environment e.g the gut. Their half-life in blood is about 18 hours, but in tissues they are believed to survive for several days [125]. During helminth infections eosinophils are released more rapidly from the bone marrow, their survival in tissue is enhanced [202] and the rate of bone marrow eosinophilopoeisis increases substantially.

The early stages of differentiation are controlled by the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3, while the later stages of differentiation and maturation are under the influence of IL-5, which is predominantly produced by activated

T-cells and mast cells. The high conservation in both sequence [158] and biological activity [214, 215] of IL-5 between rodent, human and ruminant species supports its unique role in the generation of eosinophilia. However there is a minor population of IL-5 independent eosinophils that develops and functions in the absence of functional receptors for IL-5, GMCSF and IL-3.
The primary function of eosinophils is considered to be the defense against organisms that are to large to be phagocytosed, such as parasitic helminths. Under the influence of a Th2 environment they respond to chemoattractants and other signals by homing into inflammatory or helminth-infected sites, where they become activated and degranulate. Degranulation of eosinophils is generally initiated by cross-linking of surface receptors. Eosinophils have receptors for various components of the complement pathway and immunoglobulin isotypes, including IgG and IgA and low affinity receptors for IgE. Of particular importance for GI nematode infections, is the expression of receptors for the secretory component of IgA on the surface of eosinophils. Binding of secretory IgA provides the most potent stimulation for degranulation of eosinophils [143, 166]. In addition, eosinophils might bind and respond to carbohydrate ligands expressed on the parasite surface, such as the Lewis^{x}-related molecules and cell adhesion molecules similar to selectins that have, for example, been demonstrated on schistosomula.
The ability of eosinophils to kill a variety of parasites *in vitro* (reviewed in [157]) has led to the suggestion that eosinophils are anti-parasite effector cells. Despite an increased eosinophilia during intestinal nematode infection, a protective role for this population *in vivo* has not yet been identified. Treatment of mice with anti-IL-5 Mabs to ablate eosinophilia does not prevent the expulsion of *N. brasiliensis, T. spiralis, T. muris* or *H. polygyrus* (reviewed in [63]) which suggests that eosinophils are not the only effectors involved in the expulsion of helminths from the gut. In contrast, there is some evidence for an eosinophil-mediated protective immune response operating against the migratory tissue dwelling larval stages of two other nematodes, *Angiostrongylus cantonensis* [194] and *Strongyloides venezuelensis* [138].
Similarly, eosinophilia occurs in nematode infected ruminants [38, 15] but a functional role for this cell population is not well defined. Indirect evidence for the contribution of eosinophils in protection was provided by the significant correlations that exist between genetic variations in susceptibility to infection and the magnitude of the eosinophil response. Independently selected lines of sheep, bred for an increased resistance to nematode infections showed greater eosinophil responses after infection compared to random-bred or low-responder flocks [54, 95, 38]. This correlation was only observed after priming of sheep with natural or experimental infection. Similarly, the presence of eosinophil-potentiating activity (EPA) in the gastric lymph of sheep infected with *O. circumcincta* was inversely correlated with worm burden, but only early after secondary and not after primary infections [213]. In

contrast, an increase in eosinophil numbers was observed after primary infection of sheep with *Nematodirus battus* and this concurred with the rejection of adult worms [245]. Simultaneous infections with *O. ostertagi* and *C. oncophora* evoked a small rise in blood eosinophils [133], but the distinct contribution of both nematodes to the eosinophilia was not investigated.

2.2.3. Mast cells

Tissue mast cell hyperplasia is one of the characteristics of the mammalian host response to nematode infections [161, 82]. In rodents, the recruitment of mast cells is dependent upon the release of T-cell derived cytokines such as IL-3, IL-4, IL-9 and IL-10. The growth of mast cells is mainly regulated by stem cell factor (SCF). SCF is also known as *kit*-ligand because it binds to the c-*kit* receptor, a tyrosine kinase which is abundantly expressed on the mast cell surface [85].

The effect of mast cell activation might be directly anti-parasitic by the secretion of low molecular weight granule mediators e.g. histamine, 5-hydroxytryptamine, mast cell proteinase, prostaglandins, and leukotriens that have a negative effect on worm survival [192]. Indirectly, the granule chymases that are secreted in the gut lumen can contribute to an increased mucosal permeability and thus facilitate the translocation of plasma proteins such as humoral parasite-specific antibodies into the gut lumen [22]. Activation of mast cells and the consequent release of mediators are commonly associated with protection [192, 162] but these processes might also have detrimental effects on the host [86].

The most convincing data on the protective role of mucosal mast cells have been generated from mast cell-deficient mice that have a mutation affecting tyrosine kinase activity of the *c-kit* protein (reviewed in [162]). Data using this experimental model suggested that expulsion of *T. spiralis* or *Strongyloides ratti* is to some extent mast cell dependent [168, 169]. Treatment of *T. spiralis* infected mice with a monoclonal antibody for the receptor of SCF depleted intestinal mast cell populations and completely abrogated the protective immune response [104]. However, worm expulsion might also occur in the absence or with very low numbers of mast cells (reviewed in [192]). For examples, the expulsion of *N. brasiliensis* occurs without a significant contribution of mast cells and while *T. spiralis* infection in mice requires mast cells for adult worm expulsion, the rejection of *T. spiralis* in rats seems to be mast-cell independent.

Similarly several studies suggest that mast cells are specifically activated during the rejection of nematodes in sheep [116, 72], whereas other observations show that sheep can express high levels of resistance without any obvious increase in mast cell numbers [118]. Hence, the functional contribution of this cell population to protective immunity against nematodes is not very clear. The number of mast cells and globule leucocytes were

determined in calves primary and secondary infected with *C. oncophora*, but results were not conclusive and no clear effect of infection on these populations was observed [84].

2.2.4. Mucus and motility of the gut

Increased mucus production, increased fluidity in the gut lumen and increased motility of the gut are often associated with the host's response to GI nematodes in both rodents and ruminants (reviewed in [192] and [162]). Direct effect of mucus on nematodes was demonstrated using an in vitro larval migration assay [59]. The intestinal mucus of sheep rendered immune to *T. colubriformis* contained significantly more larval migratory inhibitory (LMI) activity in comparison to mucus derived from nematode-free sheep. These mechanisms appeared to be not species-specific as in the same experiment it was demonstrated that the 'immune' mucus exhibited LMI also against *H. contortus, Nematodirus spatigher* and *T. circumcincta* larvae [59]. Similarly, mucus from calves immunized with *O. ostertagi* showed in vitro LMI activity [46].

Although hyperplasia of goblet cells and mucus secretion are often considered as being immunological non-specific mechanisms there is growing evidence that during enteric infections the host develops an integrated response involving the coordinated actions of all tissues in the gastro-intestinal tract (reviewed in [225]). Infection with *T. spiralis* results in increased fluid secretion into the lumen of the small bowel as well as increased intestinal propulsive activity and more rapid intestinal transit. A specific recruitment of the immune response in these events was demonstrated by the attenuation of these processes in athymic rats [108, 234]. Recently, a putative role for the parasite specific response including CD4+ cells, IL-5 and eosinophils as well as *c-kit* dependent cells in the regulation of motility disturbance associated with *T. spiralis* infection was proposed [223, 224, 226, 227].

3. EFFECTS OF THE IMMUNE RESPONSES ON *C. ONCOPHORA*

There are a range of parasitological parameters that can be used to assess host responsiveness, and within each host-parasite system the effect of the host's immune response on the worms might differ. Furthermore, terms such as establishment, fecundity or survival can be defined in many ways making it difficult to compare data from one research group to another. Under the traditional view of the host immune response, all effects on the worms are a direct result of attack by effector cells and molecules of the host immune response. Based on the observation of *S. ratti* infection in rats, an alternative was proposed for the way in which worm survival and/or fecundity might be limited [236]. The author proposed that the reduction in the fecundity and survival of worms in immune hosts was at least partly a result of the energy expended by a parasite to protect itself against immune attack. Which extreme reflects the reality is not known, but as parasites have evolved to an

obligatory parasitic phase within a host, it is likely that both alternatives should be considered when interpreting the altered morphology of survival of the worms within an infected host.
A brief overview is given of the parasitological variables which are used throughout this thesis. In view of the clarity, this section will focus only on *C. oncophora* and a short definition of each of the variables will be included.

3.1. EPG and ratio EPG
EPG is defined as the number of eggs per gram faeces as measured by a modified McMaster method with a sensitivity of 50 EPG. The cumulative EPG is defined as the sum of the EPG values during a specified experimental period and within this period EPG was determined every two days.
Ratio EPG is a parameter derived from the mean EPG pattern observed in intermediate responders after primary infection with 100,000 L3 *C. oncophora* (fig. 1). Following infection a peak in egg output occurs around day 21-28 p.i.. Shortly thereafter, the EPG decreases as a consequence of acquired immunity and around day 35-42 p.i. the egg output is very low or zero. We defined ratio EPG as being the ratio: mean EPG day 35-42 p.i./mean EPG day 21-28 p.i. This parameter reflects the effect of the host immune response on both the expulsion of the worm population and worm fecundity (see section 3.2. and 3.3). The reduction in egg output at day 42 p.i. is less pronounced in low responders than in intermediate responders and this will consequently result in a distinct ratio EPG. A threshold in ratio EPG is set at 0.4 which indicates a reduction in egg output of at least 60 % as observed in intermediate responders. Animals with a ratio EPG >0.4 and a high cumulative EPG are considered as low responders and animals with a ratio EPG <0.4 are considered as intermediate responders. High responders may show a ratio EPG taking any value, but show a significantly lower peak in egg excretion by day 21-28 p.i., concurrent with a low cumulative EPG.

3.2. Establishment
The parasite must establish itself and there is ample evidence to show that the success of establishment is lower in immune as opposed to naïve hosts (reviewed in [161, 192, 15]). In the current study we defined establishment as the ability of the worms to establish as (juvenile) worms beyond the L4 stage. It is important to differentiate this way of defining with a definition of establishment based on the larval stages. Indeed, measurement of establishment based on the larval stages might result in a higher outcome as compared to our definition. Immunity can affect both, and while the infective larvae might reach their niche, their development can be hampered or delayed. The arrested development of the larvae obviously

will result in a lower adult worm burden. We chose the definition based on the adult stages because all the other parameters used also refer to the adult worm population.

3.3. Sex ratio, Worm length and Worm fecundity

Sex ratio is defined as the percentage male worms (number of male worms/sum of male and female worms). In animals primary infected with *C. oncophora* before the development of acquired immunity an equal proportion of male and female worms prevails (50% of each sex). As a result of immunity male worms are commonly expelled first [3, 51] and the sex ratio decreases. Hence, this parameter can be used to assess worm expulsion.
Worm length and fecundity can both be affected as manifestations of resistance to adult nematode parasites (reviewed in [15]). Worm length is commonly used to ascertain effects on growth and development of a parasite population while an effect on the reproduction capacity of the worms is measured by worm fecundity. Worm fecundity is defined in this thesis as the number of eggs per female worm. Such a definition allows differentiation among effects on the production of the eggs and effects on the egg-laying machinery of the worms which is not feasible with a definition of worm fecundity based on the egg output in the faeces.

4. Scope of the study

GI nematode infections in ruminants are commonly treated with effective anti-parasitic drugs, but in view of the development of resistance, there is a need for alternative means of parasite control. A likely alternative for anthelmintics would be a vaccine, but this requires an improved understanding of the immune response, together with a more detailed knowledge of the host-parasite interactions. We used *C. oncophora* infections in calves as a model to investigate the mechanisms involved in the immune response against GI nematodes.
Development of acquired immunity following *C. oncophora* infection in calves is relatively fast. Using experimental infections with 100,000 L3 in 3-month-old calves, animals can be differentiated in *High*, *Intermediate* and *Low* responders. Until now, this differentiation relied solely on parasitological variables as EPG and worm counts at necropsy. In **Chapter 2** the systemic immune response following a primary infection with 100,000 L3 *C. oncophora* was characterized. We investigated whether calves classified into different responder types based on parasitological variables also feature different immune responses. **Chapter 3** reports on the local immune responses in these primary infected animals. A detailed analysis of the immunological and parasitological events which coincided with worm expulsion was performed to identify possible effectors. The comparison of

Intermediate and *Low* responder animals allowed us to identify putative mechanisms involved in worm expulsion.

Based on the data from primary infected animals, we knew that acquired immunity is developed in *Intermediate* but not in *Low* responders. Under natural conditions, animals are re-infected after turn out during the second grazing season. Hence, it is important to know whether the immunity generated during primary infection is long-lasting and protective against re-infection. To address this question, animals primed with 30,000 or 100,000 L3 larvae were re-infected with 100,000 L3. The housing period was mimicked by keeping the animals worm-free during a period of 2.5 months. The parasitological features of the re-infection experiment are described in **Chapter 4.** We investigated two different issues; the effect of the infection dose on the development of immunity against re-infection and whether the more resistant phenotypes are sustained after re-infection. Until now responder types can only be distinguished following infection. Hence, at some time points only a few animals per group were analysed.

The immune response was compared between animals that were primary and secondary infected. The role of B cells and antibodies in the peripheral blood and in small intestine is described in **Chapter 5.** Based on the results from primary infections (**Chapter 2**), a more detailed phenotypic and functional analysis of the B cell population was done at day 28 p.i.. **Chapter 6** describes the distinct kinetics in the T cell and effector responses among primary and secondary infected animals.

The findings described in this thesis are discussed in **Chapter 7**.

Chapter 2

Characterisation of host responder types after a single *Cooperia oncophora* infection: kinetics of the systemic immune response

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Er is hier. Er is tijd
Om overmorgen, iets te hebben achtergelaten,
Daar moet je vandaag voor zorgen,
Voor sterfelijkheid.
(H. de Coninck)

ABSTRACT

After primary infection with 100,000 third stage larvae of the intestinal nematode *C. oncophora* in 3-month-old calves a high variability in egg output and worm counts is observed. Based on this variability infected animals can be divided in different responder types. The three major phenotypes can be classified as high, intermediate and low responder animals. We investigated whether calves classified into different responder types show different immune responses during infection. Peripheral blood eosinophil counts and flow cytometric analysis of different lymphocyte subsets of the blood did not reveal major differences between infected and control animals, nor between responder types. However, the levels of *Cooperia*-specific IgG1 and IgA during primary infection are significantly higher in intermediate responders than in low responders. In the intermediate responders, isotype specific responses were negatively correlated with parasitological parameters expressing worm expulsion and influence on worm fecundity. Total serum IgE levels were elevated in most of the infected animals. A quantitative positive relationship between worm counts and total serum IgE levels was observed. Based on the observed correlations, we propose a role for the humoral response against the maintenance of the infection in the gut.

INTRODUCTION

Variation in immune responses to nematodes in cattle has been reported to depend on the infection dose [3, 130], age [8, 135] and sex [146]. Apart from these, genetic background has been reported to be a significant factor in how a host responds immunologically to infection [3, 146, 132]. For *Cooperia oncophora* a dose of 100,000 infective third stage larvae (L3) at a host age of three months has proven to result in large differences in parasitological variables (eg. faecal egg output and worm burdens) between calves.

With the primary infection protocol for *C. oncophora* it is possible to distinguish between three major responder types within the wide variation in response. This is based on cluster analysis of the faecal egg output and the necropsy results such as total worm burden and the sex ratio of the harvested worms, at days 35-42 post infection (p.i.) [231]. These major phenotypes can be classified as high, intermediate and low responder animals. High responders, a small proportion of the host population, show almost no egg output (EPG) and the worm burden at necropsy at day 42 p.i. is low. Low responders show high egg output which is continued for weeks and hence have high worm burdens at necropsy. In intermediate responders egg output at first is similar to that in low responders, but around day 28-35 p.i., EPG's start to decline rapidly. These animals usually show intermediate worm burdens at necropsy, but these can still range from low to high numbers. It is likely that differences between the host responder types are at least partly immunologically based. For example, Albers [3] showed that calves showing high faecal egg counts had lower antibody titres against crude worm antigens than calves showing low faecal egg counts. Although a classification of hosts into distinct responder types is to some extent an oversimplification of reality, it may be useful in studying and identifying relevant immune mechanisms. The utility of having different responder types to study immune mechanisms in a parasite-host model has been demonstrated extensively in laboratory animal models [25, 68, 65, 169] and in sheep [96]. These studies have provided much information on immune mechanisms involved in parasite rejection and expulsion. However, various immune mechanisms are frequently linked to a specific parasite-host system. Hence, results cannot always be easily extrapolated to parasite infections in other animals. We started to investigate whether calves classified into different responder types based on parasitological variables feature different immune responses during a primary *C. oncophora* infection. The present study reports on the systemic responses observed.

MATERIALS AND METHODS

Infective larvae

C. oncophora L3 larvae were obtained by coprocultures using standard procedures [32] and kept in water at 10°C until use. Larvae used for infection were less than 3 months old. The larvae used

were from the Wageningen laboratory strain which has been maintained by regular passage through donor calves since 1967. This strain is known to have a shorter prepatent period (14 days) than field strains and contains 30% *C. surnabada* [3, 84.]

Animals and experimental design 1
Twelve female Holstein-Friesian calves were raised under helminth-free conditions on a commercial farm and purchased at 3 months of age. Throughout the experiment the animals were kept indoors at the animal facility of the Faculty of Veterinary Medicine in Utrecht. Animals were housed in one group, and were fed hay and water ad lib and concentrates at a maximum of 500 gr per day. Prior to infection animals were checked for general health conditions and faeces were collected to test the worm-free status of the animals.
Eight calves were infected orally with a single dose of 100,000 *C. oncophora* L3 larvae and four calves were kept as non-infected controls. Faeces were collected from the rectum once a week during the first two weeks of the infection and thereafter three times every week. Two blood samples, one for serum and one on heparine, were collected from the jugular vein one week before infection and weekly thereafter from day 7 to 42 p.i.. At day 42 p.i. calves were necropsied.
This experiment was part of a larger one with 36 animals. Twenty-four animals were infected and 12 animals served as uninfected controls. Necropsy of 8 infected and 4 non-infected calves was performed at days 14, 28 and 42 p.i.. Because the calves slaughtered at the earlier time points could not yet be allocated to a certain responder type, immunological data are not included in this paper.

Animals and experimental design 2
In order to support some of the results a supplementary group of 24 animals from a previous experiment was included in the analysis of the humoral response. Those animals were from an experiment carried out in 1993 at the University of Wageningen (Ploeger et al., unpublished results). Twenty–four, male Holstein-Friesian calves were infected with a single dose of 100,000 L3 *C. oncophora* larvae at 3 months of age. Before infection the animals were reared under helminth-free conditions at the animal husbandry facility of the Wageningen University. Faeces was collected from the rectum once a week from days 0 to 14 p.i., and two times a week thereafter. Blood samples for serum were collected two times a week from the jugular vein. Serum had been stored at - 20°C until use in this experiment. Necropsy of the infected animals was performed 42 days after infection. Only the serological data (isotype specific ELISA's) from this experiment were carried out by us, all parasitological data were gathered independently at the University of Wageningen were.

Parasitology
The numbers of eggs per gram faeces (EPG) were determined with a modified Mc Master technique (with a sensitivity of 50 EPG). After slaughter of the animals, intestinal washings were done according to the methods described by Eysker and Kooyman [74]. To obtain a more detailed insight in the localisation of the worm burden in the intestine in relation to the population dynamics (expulsion process) the small intestine was divided into six different segments in experiment 1 (table 1). All segments were processed separately. Worms were counted in 2% aliquots of the intestinal washings. In experiment 2 intestinal washings were done similarly, however the small intestine was not subdivided in different segments. Parasitological data from this experiment always refer to the entire small intestine starting from behind the pylorus to the ileo-caecal junction.

Isolation of peripheral blood lymphocytes
Peripheral blood monomorphonuclear cells (PBMC) were isolated using a density gradient technique with Ficoll-Paque (Pharmacia Biotech, Germany). Cells were resuspended in RPMI-1640

(Gibco BRL, Paisley, UK) supplemented with penicilline (500 units/ml), streptomycin (0.5 mg/ml) (P/S); 10% foetal calf serum (FCS) and glutamine (2mM) (complete medium) at a concentration of $2*10^6$ cells/ml and kept on ice until staining.

TABLE 1. Identification of the different segments in which the small intestine was divided at necropsy

Segment	Localisation	anatomical nomenclature
S1	first meter after the pylorus	duodenum
S2	next 5 meters	proximal jejunum
S3	next 5 meters	middle jejunum 1
S4	next 5 meters	middle jejunum 2
S5	next 5 meters	distal jejunum
S6	last part of the small intestine	ileum

Flow cytometric analysis of lymphocyte subset in the peripheral blood
Monoclonal antibodies to different bovine surface antigens used in this experiment are described in table 2. Irrelevant mouse serum was used as negative control.
Hundred microliter (µl) of cells at a concentration of $2*10^6$ cells/ml were incubated with the optimal concentrations of antibodies. After washing twice, goat-anti-mouse-FITC (Becton-Dickinson, San Jose, USA) was added to the cells. All incubations were performed on ice, in the dark, for 30 minutes with a total volume of 50 µl. All dilutions and washing steps were done in ice cold phosphate buffered saline (PBS) supplemented with 4% BSA (fraction V, Sigma) and 0.1% NaN_3 (FACS buffer).
After the final incubation step cells were washed twice and fixed for 10 minutes on ice with 100 µl of 0.1% paraformaldehyde in PBS. Cells were washed and resuspended in 100 µl FACS buffer and stored at 4°C in the dark until FACS analysis. Analysis was done on a flow cytometer (FACS-Calibur, Becton-Dickinson) either on the same day or one day after staining.

TABLE 2. List of monoclonal antibodies used in this experiment

Antigen identified	Mab (Clone)	Reference	Application
Bovine CD3	MM1A	Vet Immunol Immunopathol 1993; **39**: 85	FACS analysis
Bovine CD2	ILA42	Vet Immunol Immunopathol 1993; **27:** 43	FACS analysis
Bovine CD4	ILA11	J immunol 1986; **136**: 4385	FACS analysis
Bovine CD8	ILA105	ECACC (http://fuseii.star.co.uk)	FACS analysis
Bovine TCR1-N12	CACT61A	Vet Immunol Immunopathol 1993; **39**:161	FACS analysis
Ovine MHC II	VPM54	VMRD, Pullman, WA, USA	FACS analysis
Bovine BB-2	BAQ44A	Immunology 1989; **68**: 365	FACS analysis
Bovine IgG1	K372g6	Sigma, St Louis, MO, USA	ELISA
Bovine IgG2	BG-27	Sigma, St Louis, MO, USA	ELISA
Bovine IgA	K842F9	Serotec, Oxford, UK	ELISA
Ovine IgE	IE7	Parasitology 1997; **114**:395	ELISA

Leucocyte differentiation
A bloodsmear on a glass slide was made once a week from blood collected on heparine. The slides were Giemsa stained. Of each calf 2 bloodsmears were made and 200 cells were counted on each slide.
The total number of leucocytes per ml blood was counted with an automatic cell counter (Celltrac) after lysis of the red blood cells with 4% saponin. Each count was performed at least in duplicate. Results were expressed as follows: Absolute number of eosinophils = number of eosinophils counted/200 x number of leucocytes/ml or as the % of cells: e.g. % of eosinophils = number of eosinophils counted/ 200.

Isolation and preparation of *Cooperia*-specific antigens
C. oncophora L3 were exsheated by incubation in 0.1% hypochlorite solution in water under continuous shaking at 37°C. Exsheathment was monitored microscopically. Exsheated larvae were washed twice in PBS (3:1 v/w) and subsequently centrifuged at 2,000 x g for 10 minutes. Then the larvae were homogenised using a mortar and pestle which were kept in liquid nitrogen for 5 minutes prior to use. Droplets of the larval suspension were pelleted into powder which was collected and kept on ice until fluid again. The extract was centrifuged at 15,000 x g at 4°C for 20 minutes and the supernatant containing the L3 antigen extract was collected for further use.
Adult *C. oncophora* worms were obtained from donor calves at day 18 p.i. by a modified Baermann technique. Worms were washed at least three times in PBS supplemented with 2% P/S and stored at -80°C until antigen preparation. Worms were grinded with a dounce homogenizer and ice cold PBS (2:1) was added. The extract was centrifuged at 15,000 x g at 4°C for 20 minutes. Supernatant was collected and one volume of a stock protease inhibitor Complete Mini solution (1 tablet in 10 ml MilliQ) (Promega, Madison, USA) was added to 7 volumes of supernatant.
Cooperia adult excretory/secretory products (ES) were obtained as has been described before for *Haemonchus contortus* [16].
All supernatants were sterilised by filtration (low protein binding membrane, 0.45 µm, Corning) and checked for bacterial contamination. Protein concentration was determined by the Bradford assay. The antigen extracts were stored at -80°C until further use.

ELISA

Cooperia-*specific antibody isotype specific ELISA*
The *Cooperia*-specific elisa as used by Poot et al. [178] was modified to be used for the detection of IgA, IgG1 and IgG2 isotype specific antibodies against *Cooperia* L3, Ad and ES antigen. Elisa plates (Greiner high binding, cat 655093) were coated overnight at room temperature with 100 µl/well of antigen (5 µg/ml) diluted in carbonate-bicarbonate buffer (pH=9.6). All subsequent incubations were done with 100 µl for 1 hour at 37°C and between each step plates were washed at least three times with MilliQ containing 0.05% Tween. After coating, wells were blocked with 100 µl of PBS containing 0.1% gelatine (blocking buffer). All sera were diluted in blocking buffer (1:100) and incubated in duplicate. On each plate a standard positive and standard negative serum control were included. The standard positive serum was obtained from a calf repeatedly infected with *C. oncophora*. Following incubation with monoclonal mouse anti bovine IgG1 (1:1,000), IgG2 (1:2,000) and IgA (1:100) antibodies (table 2), plates were incubated with alkaline phosphatase conjugated goat-anti-mouse antibodies (DAKO, Denmark). Finally 100 µl P-nitrophenyl phosphate disodiumsalt substrate solution (PNPP, Pierce, USA) was added to each well. The enzymatic reaction was allowed to proceed for 30 minutes at room temperature after which plates were further incubated at 4°C. Plates were read on an automated ELISA reader

(CERES UV 900 C) at a density of 405 nm after 1½ hour total incubation (IgG1) or after overnight (14-16 hours) incubation (IgG2 and IgA). Optical densities were recalculated to a percentage of the OD value of the positive reference serum (PROD).

$$\text{PROD} = \frac{\text{Average of (OD sample-OD blanco)}}{\text{Average of (OD standard positive serum-OD blanco)}}$$

The blanco is the average of the OD of wells with no serum incubation.

Total IgE ELISA

The total serum IgE was measured using the monoclonal anti-ovine IgE (IE7) in a sandwich ELISA as described for *H. contortus* [136]. The cross-reactivity and specificity of this monoclonal for bovine IgE has been demonstrated recently [137].

Analysis

Data were analysed by comparing between different groups using the Kruskal-Wallis and the Mann-Whitney test. Significant results are depicted as follows: * (P<0.05), ** (P<0.01) and *** (P<0.001). A value of P<0.1 is defined as a tendency and marked with $^{+}$.

The following parasitological variables were used as a measure of host responsiveness. The sex ratio was defined as the percentage male worms (= (number of male worms/sum of male and female worms) x 100). As male worms are expelled first [3, 48, 229], this parameter is useful as an indicator of worm expulsion. Based on the distribution of worms in the gut at days 14, 28 and 42 p.i. (data not shown) we used the sex ratio in S2, which is the predilection site of the adult worm population. For experiment 2 we used the sex ratio in the worm burden of the whole small intestine.

The ratio EPG was defined as the ratio: mean EPG of week 5-6 p.i. (end in the egg excretion pattern)/mean EPG week 3-4 p.i. (peak of egg excretion pattern). This parameter features the effect of the host immune response on both the expulsion of the worm population and worm fecundity. Analysis revealed that the parameter ratio EPG is significantly positively correlated with the number of eggs per female worm (exp 1: R=0.82 (n=8 and P<0.01); exp 2: R=0.67 (n=24, P<0.001)).

Finally, to confirm our hypothesis that the humoral immune response in intermediate responders has an effect on the worm infection in contrast to what we expect in the low responders, we correlated the individual antibody titres with the corresponding EPG values during the patent phase of the infection (day 21-day 42 p.i.). For this analysis weekly serological data and corresponding EPG values from all the animals were included. This resulted in a correlation analysis with n=8 for the low responders and n=20 for the intermediate responders in experiment 1 and n=51 for the low responders and n=144 for the intermediate responders in experiment 2. All correlations were evaluated with Pearson's correlation coefficient.

RESULTS

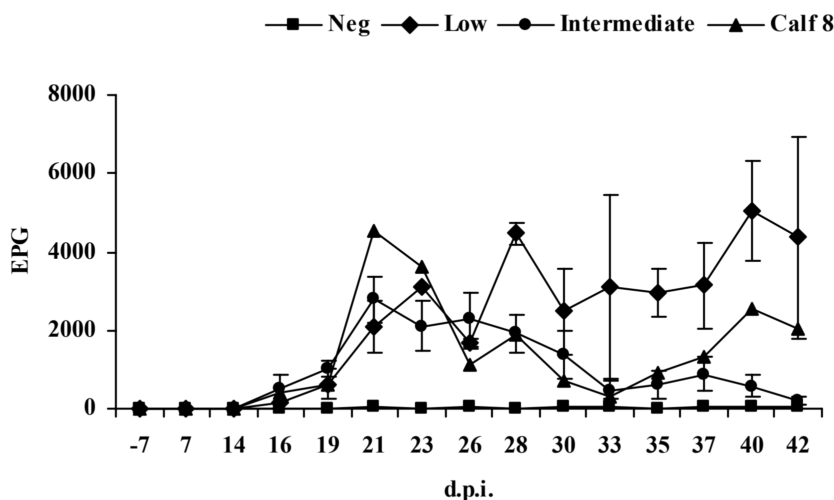
Classification into responder types

Experiment 1

Based on the faecal egg output (fig. 1), the necropsy results and the ratio EPG (table 3) we could classify the animals of experiment 1 into two low and five intermediate responders in this experiment. None of the infected calves could be classified as a high responder. In all

animals the egg output started between days 14 and 16 p.i., and a peak was reached from day 21 to day 28 p.i.. The low responders maintained this high EPG throughout the infection. In the intermediate responders a decrease in egg output was observed, with an individual variation in the rate of decrease. In those animals the egg output was reduced by 90% of the peak at day 42 p.i.. In calf 8, classified neither as a low nor as an intermediate responder, initially a 90% reduction in EPG occurred but thereafter the egg output increased again.

FIGURE 1 The EPG pattern (+/- SD) of the different responder types after infection with 100,000 L3 *Cooperia.*: (■) control animals, (◆) low responders, (●) intermediate responders, (▲) calf 8. The classification into responder types was done based on the EPG and the necropsy results as depicted in table 3.



Necropsy of *Cooperia* infected calves at day 14 and day 28 p.i. demonstrated that in the early phase of the infection *Cooperia* preferentially homes in the proximal jejunum (fig. 2) with a 1:1 ratio of male and female worms.

TABLE 3. Individual necropsy results including the percentage of the worm burden in the different segments. The corresponding sex ratio for each segment is written between brackets. The ratio EPG of the infected animals is included.

		% of total burden in each segment (% males)						Ratio EPG
Respondertype	Calf	S1	S2	S3	S4	S5	S6	
Low	2	5 (90)	65 (50)	11 (29)	4 (28)	8 (21)	7 (13)	1
	4	0.1(100)	40 (60)	54 (41)	2 (67)	1 (40)	3 (47)	1.7
Intermediate	3	13 (53)	38 (44)	4 (37)	26 (54)	10 (54)	9 (55)	0.3
	5	21 (45)	65 (45)	10 (23)	2 (73)	1 (86)	1 (100)	0.4
	9	26 (60)	52 (42)	5 (48)	4 (33)	3 (17)	10 (37)	0.3
	10	14 (32)	10 (28)	3 (35)	10 (27)	33 (54)	30 (68)	0.1
	11	37 (5)	51 (7)	3 (0)	0 (0)	0 (0)	7 (47)	0
Calf 8	8	12 (68)	47 (56)	10 (32)	13 (33)	6 (24)	11 (35)	0.6

Almost no worms were detected in the distal gut. The necropsy results of day 42 p.i. (table 3, fig. 2) showed a relocation of the worm population with a shift to the distal gut.

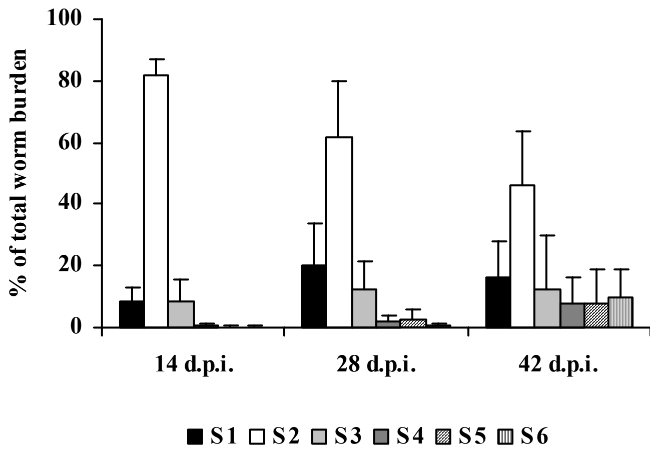
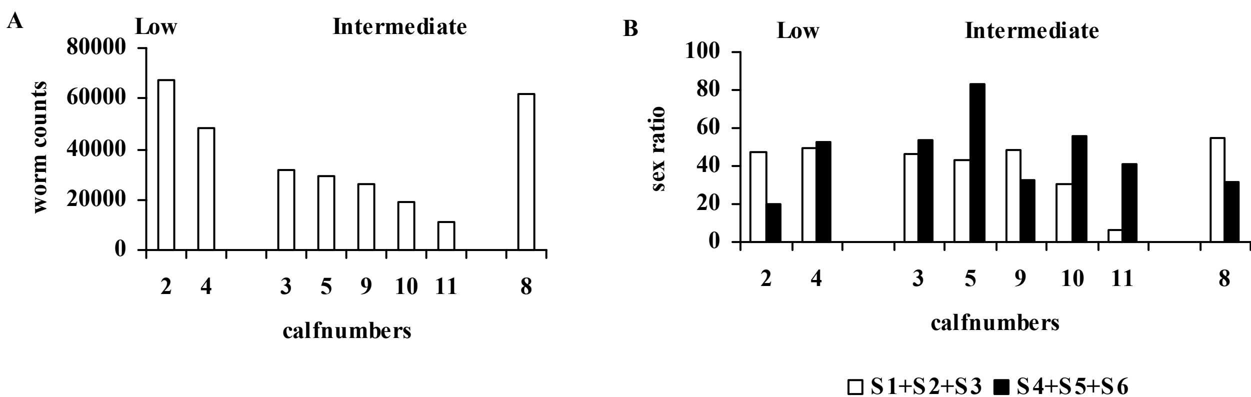


FIGURE 2 The segmental distribution of the worm population throughout the small intestine at days 14, 28 and 42 p.i. is given (n=8 at each time point).

In the intermediate responders the worm counts in the proximal half of the small intestine (S1+S2+S3) at day 42 p.i. showed a reduction of at least 25% compared to the low responders (fig. 3A). The worm burden in the proximal gut of calf 8 was higher than that of the low responders.

Both responder types also differed in the worm sex ratio in the different segments of the small intestine. Whereas in the low responders the sex ratio in the distal gut was lower or equal to the sex ratio in the proximal gut, four out of five intermediate responders showed a higher sex ratio in the distal gut, indicating a shift of the male worm population to the distal gut (fig. 3B). Together, the necropsy data indicated an ongoing expulsion of the worm population in the intermediate responders but not in the low responders.

FIGURE 3 The cumulative worm counts of the proximal segments S1, S2 and S3 at 42 DPI are depicted for the different responder types (A). The difference in sex ratio in the proximal (S1+S2+S3 □) and the distal gut (S4+S5+S6 ■) is illustrated for the different responder types (B).



Low worm numbers were recovered from the control animals slaughtered at day 42 p.i. (range: 200-1750) due to cross contamination during the experiment. Because the number of worms was low, egg output was minimal (50-150) and infection only could have occurred late in the experiment, this finding is considered inconsequential for comparison between control and infected calves.

Experiment 2

This experiment comprised a group of 24 calves which were all infected with 100,000 *C. oncophora* larvae. Classification in low and intermediate responders was done based on the EPG pattern, the ratio EPG and the necropsy results (total worm counts and sex ratio at day 42 p.i.). A threshold for the ratio EPG was set at 0.4, which implied that animals which had a reduction in their egg output of at least 60% by day 35-42 p.i. were classified as intermediate responders. Animals with a ratio EPG higher than 0.4, were classified as low responders. This classification resulted in 6 low and 16 intermediate responders. Analysis of the necropsy results revealed significantly lower worm counts in the intermediate responders (mean +/- SD= 23,976+/-25,745) compared to the low responders (mean +/- SD= 61,810+/-34,279) (P=0.015). Also the sex ratio in the intermediate responders was lower than in the low responders (29+/-13% versus 47+/-1%; P=0.05), emphasizing the ongoing expulsion of the worm population in the intermediate responders. Two animals had a similar EPG pattern as calf 8 from experiment 1 and could not be classified as any of the responder types (calf 34 and 39). Therefore, they were analysed separately.

Leucocyte differentiations

Throughout the infection peripheral eosinophil counts were highly variable both in infected (range absolute numbers 13 – $272*10^{3}$ eosinophils/ml) and non infected animals (range absolute numbers 19- $250*10^{3}$ eosinophils/ml) which did not allow us to interprete the results in view of the infection. Before infection calf 1 (control animal) had the highest number of peripheral blood eosinophils. This dropped to basal level ($23*10^{3}$ eosinophils/ml) by week 2. Twenty-eight days after infection we found that the percentage of eosinophils in four infected animals (calf 4, 5, 8 and 11) was higher than in negative control animals. At day 35 and day 42 p.i. a higher percentage was found in 5 (2, 4, 5, 8 and 11) and 6 infected animals (2, 4, 5, 8, 9 and 11), respectively. However, this pattern was not found for the absolute number of eosinophils. The course of the absolute number of eosinophils during the infection is depicted for the individual animals in fig. 4A.

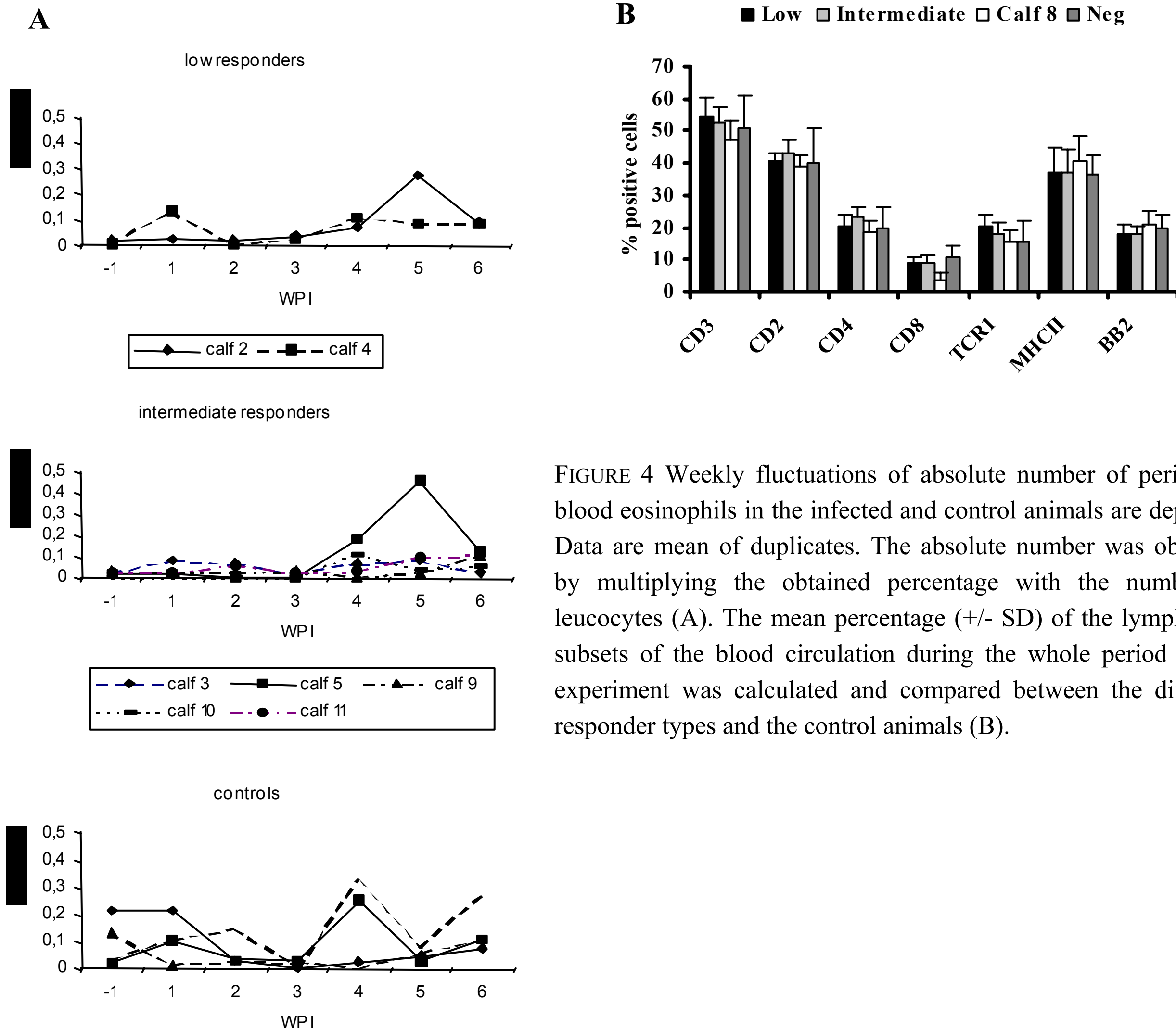


FIGURE 4 Weekly fluctuations of absolute number of peripheral blood eosinophils in the infected and control animals are depicted. Data are mean of duplicates. The absolute number was obtained by multiplying the obtained percentage with the number of leucocytes (A). The mean percentage (+/- SD) of the lymphocyte subsets of the blood circulation during the whole period of the experiment was calculated and compared between the different responder types and the control animals (B).

Flow cytometric analysis of lymphocyte subsets in the peripheral blood

Figure 4B represents the mean % of lymphocyte subsets obtained by flow cytometric analyses throughout the infection. The results depicted were obtained by calculating the mean value of the weekly average % for the different responder types. In all animals CD3+ cells accounted for the largest population with no significant differences between infected and control animals. In both control and infected animals the percentage of CD4+ cells remained within the normal range (infected: 13.8-27.3%; control: 14.9-23.4%) throughout the infection. The number of CD4+ and CD8+ cells together was continuously lower than the number of CD2+ cells which indicates the presence of a distinct CD2+ population which is negative for CD4 and CD8 (data not shown). During the whole course of the experiment

the number of CD8+ cells in the control animals was consistently lower compared to the infected animals.

Infection did not appear to affect the MHCII+ and BB2+ subsets of the PBMC's. In the control animals the BB2+ subset ranged from 12.8 to 21.9% compared to 11.8 to 22.9% in the infected animals. Similarly, the MHCII+ subsets in the control animals ranged from 27.3 to 51.5 % whereas the range in the infected animals was from 24.2 to 46.6%.

ELISA

Cooperia-*specific antibody isotype specific ELISA experiment 1*

Exposure to a primary *C. oncophora* infection with 100,000 L3 resulted in high levels of ES-specific IgG1 in all infected animals (fig. 5C). The L3 and Ad-IgG1 response was different for both responder types (fig. 5A and 5B). The *Cooperia* Ad-specific IgG1 in the intermediate responders was significantly higher compared to the low responders from day 35 p.i. onwards (P=0.05). The *Cooperia* L3-specific response revealed a higher variability in response, albeit 42 days after infection in the low responders we did measure lower levels of IgG1 (PROD 15.5 and 37.7 for calf 2 and 4 respectively) compared to the intermediate responders (PROD range 41.9-90.6).

Throughout the infection no anti-parasite IgG2 antibodies were demonstrable in the infected animals (data not shown).

TABLE 4. Pearsons correlation coefficient between the serum *Cooperia*-specific IgG1, IgA, total IgE and parasitological parameters expressing worm expulsion (sex ratio S2 and ratio EPG), exposure to the parasite (total worm burden and worm burden S2), and immunity against worm fecundity (EPG patent infection (day14- day 42 p.i.) and ratio EPG). Significant results are written as follows: *: P<0.05; **: P<0.01; ***: P<0.001. A tendency is defined as P<0.1 and depicted with no superscript. "none" is defined as R value corresponding to P>0.1.

					EPG patent infection		Wormburden	
	Isotype	Antigen	sex ratio	ratio EPG	Low	ntermediate	Total	S2
Experiment 1	IgG1	L3	-0.67	-0.65	+0.77*	-0.61**	none	none
		Ad	-0.56	-0.85*	none	-0.67**	none	none
		ES	-0.62	none	+0.49*	-0.72**	none	none
	IgA	L3	none	none	none	none	none	none
		Ad	none	none	+0.86*	-0.57**	none	none
		ES	none	none	+0.77	-0.72**	none	none
	tot IgE		none	none	none	none	+0.57	+0.6
Experiment 2	IgG1	Ad	-0.36	-0.48*	none	-0.31**	none	ND
	IgA	Ad	none	none	0.2	0.11	none	ND

During the patent phase of the infection a significant negative correlation between the *Cooperia*-specific IgG1 titres and the EPG was found in the intermediate responders (table 4). On the contrary, this correlation was positive in the low responders and calf 8. For calf 8, this relationship included only four data points and was therefore excluded from the analysis. Moreover Ad- and L3-specific IgG1 titres at day 42 p.i. were negatively correlated with the ratio EPG ($P<0.05$ and $P<0.1$ respectively) and the sex ratio ($R=-0.56$ and $P<0.1$ respectively), both parameters related to host responsiveness to the infection. A tendency to a negative correlation between ES-specific IgG1 and the sex ratio was also observed.
A slight increase in serum *Cooperia* L3-, ES- and Ad-specific IgA levels was observed in the intermediate responders from day 21 p.i. onwards (fig. 5B). Calf 8, neither an intermediate nor a low responder showed the most pronounced increase in *Cooperia*-specific IgA, starting from day 28 p.i. onwards. Low responders did not show any *Cooperia*-specific IgA response.

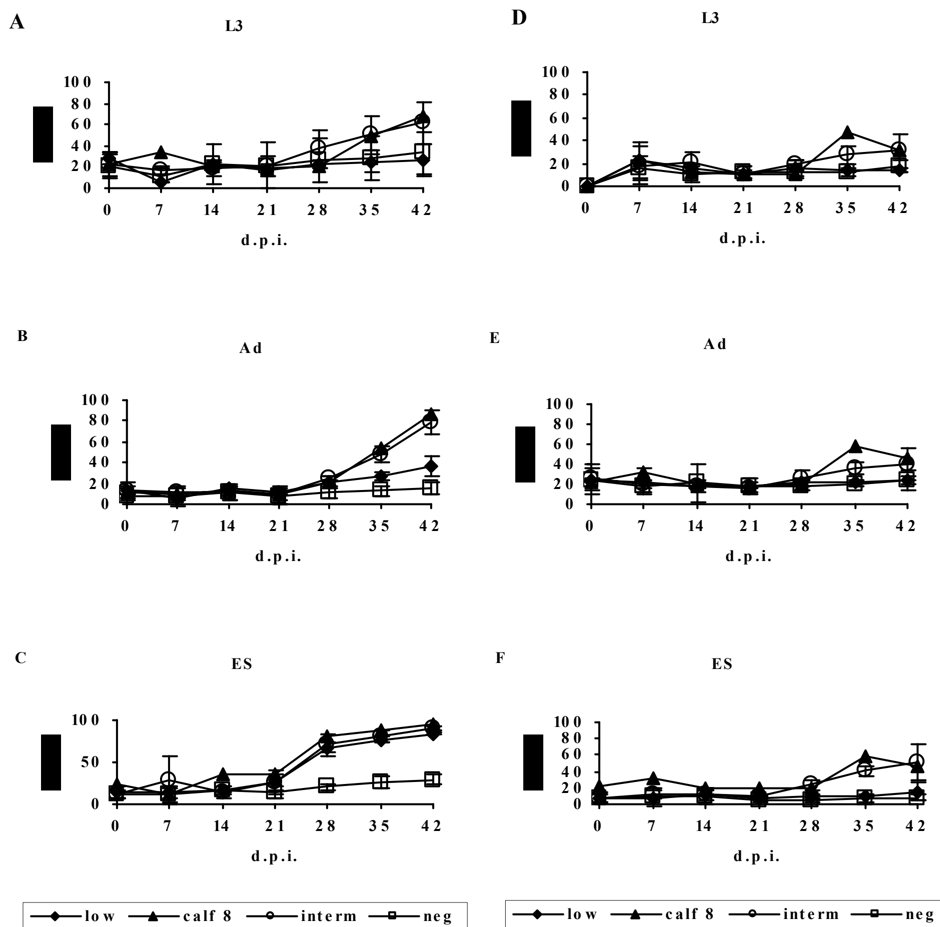


FIGURE 5 *Cooperia*-specific IgG1 (A-C) and IgA (D-F) titres against L3 crude worm antigen (L3), adult worm crude worm antigen (Ad) and adult excretory/secretory products (ES). All animals except control animals were infected with 100,000 L3 *Cooperia* at day 0. Data shown are the mean of duplicates +/- SD and given as the percentage of the OD value of the positive reference serum (PROD).

The serum IgA response of the intermediate responders to *Cooperia* ES and Ad showed the same correlation with the EPG as was seen with IgG1 (Ad (P<0.05) and ES (P<0.001)). Similar to the IgG1 titres, for both antigens a positive correlation was found in the low responders (P<0.05).

Cooperia*-specific antibody isotype specific ELISA experiment 2*

In an attempt to confirm some of the observed correlations between the *Cooperia*-specific humoral response and parasitological variables, isotype specific ELISA's were performed with sera from animals of experiment 2. Only the reaction with *Cooperia* Ad antigen was tested as this seemed to reveal the highest differences between low and intermediate responders.

In all infected animals the *Cooperia*-specific IgG1 increased from day 28 p.i. on, with the increase being higher in the intermediate than the low responders. This increase resulted in more elevated IgG1 level in the intermediate responders at day 42 p.i. (PROD range: 70-103 versus 66-95 in the low responders (P=0.22)). Similar to what we observed in experiment 1 infection did not induce the production of *Cooperia*-specific IgG2.

Compared to experiment 1 the Ad-specific IgA titres in all animals were higher (PROD exp 1 at day 0 p.i. = 13.6-45.4 versus PROD exp 2 at day 0 p.i.: 17.5-63.8). In the intermediate responders at day 39 p.i. a non-significant increase in *Cooperia* IgA was measured, whereas for the low responders this was measured at day 35 p.i.. In both responder types by day 42 p.i. the mean IgA levels dropped to baseline level (data not shown). Strikingly, calf 34 and 39, which showed a similar EPG pattern as calf 8 from experiment 1 had significantly increased *Cooperia* Ad IgA at day 42 p.i. (P<0.05, data not shown).

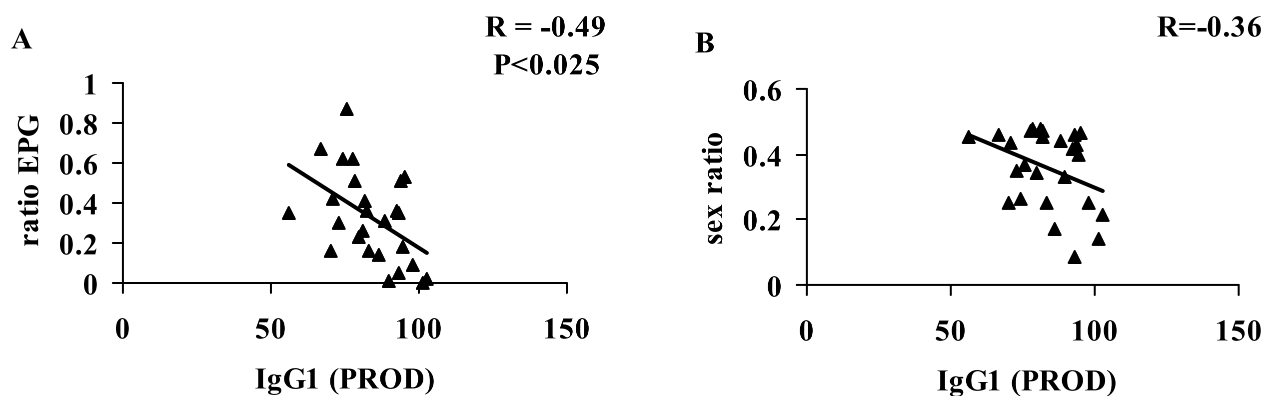


FIGURE 6 Plots of *Cooperia* Ad-specific serum IgG1 versus ratio EPG (A) and sex ratio (B) of the animals of experiment 2. Data of both intermediate and low responders are included.

The correlations between the isotype specific antibody response and parasitological variables showed a similar pattern as in experiment 1, albeit that the R values were lower (see table 4). The negative correlation between *Cooperia* Ad-specific IgG1 titres with the ratio EPG (R=-0.49, P<0.025) and with the sex ratio (R=-0.36, P=0.1) are depicted in fig. 6 A and B, respectively. Figure 7A and 7B show the relationship between the *Cooperia* Ad IgG1 and IgA titres and the EPG during the patent phase of the infection**.**

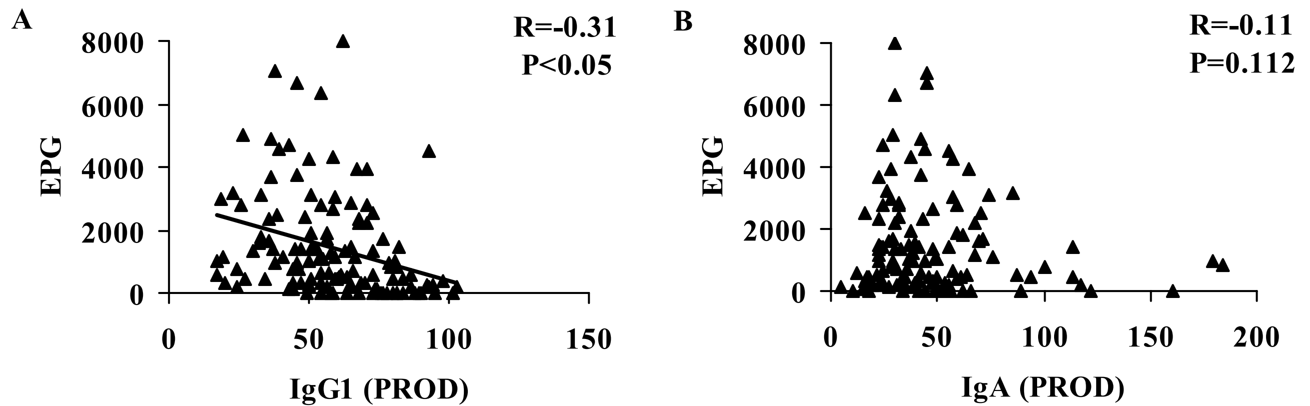


FIGURE 7 Plots of the correlations between *Cooperia* Ad-specific serum IgG1 (A) and IgA (B) with the EPG during the patent phase of the infection (day 14 to 42 p.i.). Data are derived from animals of experiment 2.

Total IgE titres

In all but two infected animals (calf 5 and 9) a marked rise in serum total IgE level was found during infection from day 14 p.i. onwards (fig. 8).

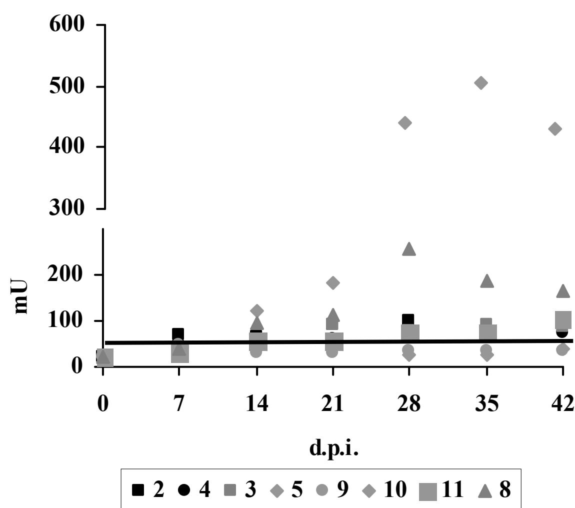


FIGURE 8 Individual values of total serum IgE levels for all infected calves. Data were transformed as described previously [136] and are expressed as mU. The cut off value (—) was set at: overall mean + 1 x SD of the serum total IgE of the four control animal

The difference in total IgE levels was individual and not related to the responder types. Forty-two days after infection, in the infected animals a positive linear correlation was present between the total worm burden as well as the worm burden in S2 and the total serum IgE level ($R=0.57$ and $P<0.1$, respectively) (table 4). No correlation was present with the sex ratio and the ratio EPG.

DISCUSSION

After primary infection of 3-month-old calves with *C. oncophora* we could distinguish three major responder types based on parasitological variables [231]. In this study, the systemic immune response of two of these responder types was investigated. Based on the EPG and the necropsy results we classified the infected calves of experiment 1 in two low and five intermediate responders. Calf 8 did not fit the classification and was analysed separately. The intermediate responders expelled their worm population before or around time of necropsy (day 42 p.i.), whereas the low responders and calf 8 did not. This was evidenced by a shift from the proximal to the distal gut in the adult worm population of the intermediate responders, as well as a higher sex ratio in the distal gut. This is similar to laboratory animal models in which it has been demonstrated that worms are redistributed proximal and distal to their predilection site just before they are expelled [161]. We classified the animals of experiment 2 in 6 low and 16 intermediate responders based on both their necropsy results and the EPG. We could not monitor the dynamics of the worm population throughout the small intestine as no segmental division had been made. However, the lower worm burden and sex ratio in the intermediate responders are an indication for an ongoing expulsion as found in experiment 1.

A slight increase was present in the number of peripheral blood eosinophils in six out of eight infected animals (experiment 1). However the same fluctuations were observed in the non infected controls. In all animals a weekly fluctuation in this cell population occurred. No evidence of a different quantitative peripheral blood eosinophil response between the intermediate and low responder types was present. Although eosinophils are known to be associated with helminth infections the different reports are not conclusive concerning their role during a parasite infection [157, 24]. To get more conclusive data on the role of eosinophils during a *Cooperia* infection we have to look at the local eosinophil response as well as the functional activity of the detected eosinophils.

The present study showed no major changes in different lymphocyte subsets in peripheral blood due to the infection based on analysis of the different subpopulation over time, (data not shown). During the entire course of the experiment the control animals showed a lower percentage of CD8+ cells. However, this was also observed prior to infection and can be due either to biological differences between the animals or to a haplotype dependent affinity

of the anti-bovine CD8 monoclonal used in this study. The cyclic pattern in the percentage of lymphocyte subsets has been described before and was attributed to physiological effects [243]. No changes were observed in the B-cell population nor in the MHCII+ and the CD4+ populations. The higher number of CD2+ cells compared to the sum of CD4+ and CD8+ cell could be attributed to a subset of the TCR1+ γδ T cells which is CD2+WC1- and occurs in the blood circulation [246]. Previous studies of gastrointestinal nematode infections in ruminants reported an increase in CD4+ cells, B cells and γδ T cells early after infection [5, 101, 154]. However, those cellular changes occurred in local tissues and draining lymph nodes. Data from experiments in which the systemic cellular immune response after gastrointestinal nematode infections was studied, lack consistency. This suggests that parasite-specific cells in blood are recirculating from the mucosal sites where the parasites are located. Thus, monitoring the local events during infection might yield more evidence of a role for the cellular immune response.

Cooperia-specific IgG1 and IgA titres were measured in the infected animals of experiment 1 from day 21 p.i. onwards. Except for the ES-specific IgG1 levels, the intermediate responders consistently produced higher titres of parasite specific antibodies than the low responders. This was also true for experiment 2, although the quantitative difference was not as big. The induction of *Cooperia*-specific IgG1 after a primary infection has been described before [176]. Similar findings were reported for other gastrointestinal nematodes in ruminants such as *O. ostertagi* in calves [150, 112, 88] or *H. contortus* in sheep [97, 195]. However, the finding that IgG1 levels after a primary *Cooperia* infection enable to differentiate between responder types is new.

In both experiments the parasite-specific humoral immune response was significantly correlated to parasitological parameters of infection. At day 42 p.i. a negative correlation is present between the *Cooperia*-specific IgG1 titres and the sex ratio in S2, and also with the ratio EPG, parameters linked to the expulsion and the fecundity of the worms. Furthermore, as can be expected based on their different EPG pattern, both specific IgG1 and IgA titres of the intermediate responders were significantly negatively correlated with the EPG during the patent infection while a positive correlation was found for the low responders. Those correlations were all significant in experiment 1 ($P<0.05$). In experiment 2 the *Cooperia* specific IgA showed the same tendency although not significant ($P=0.1$). Our results suggest an effector role for L3 and Ad specific serum IgG1 during a primary *Cooperia* infection. The role of ES-specific IgG1 seems to be more related to the level of the infection as in all infected animals similar levels were detected.

In both experiments we could not measure a significant correlation between serum IgA titres and parasitological parameters expressing worm expulsion. Throughout the infection the IgA levels remained low or were absent. However, a role for IgA is not excluded as it is

known to be a mucosae-associated Ig and peripheral titres might not have a predictive value. Moreover, both IgG1 and IgA seem to have an association with the egg output and hence the fecundity of the worms. A negative correlation between IgA and fecundity has also been reported in sheep infected with *Teladorsagia circumcincta* [212]. The higher levels of IgG1 and IgA in intermediate responders than in low responders emphasises a possible role for the humoral response in the host protective mechanisms.
In this study we could not detect *Cooperia*-specific IgE using an isotype specific Elisa with *Cooperia* Ad and ES antigen (data not shown). The difficulty to measure parasite specific IgE has been reported before [188]. This can be attributed to a lack of sensitivity of our assay or to competition of the low % of serum IgE with the more abundant isotypes such as IgG1. Measurement of total serum IgE revealed an increase in some of the infected animals. No difference was seen between the intermediate and low responders and in two infected animals (one intermediate responder and one low responder) we could not measure any serum IgE. Surprisingly, the rise in total serum IgE started as early as day 14 p.i., 7 to 14 days before the induction of *Cooperia*-specific antibodies. The knowledge that a Th2 skewed response leads to the transcription of epsilon and gamma mRNA by a common IL-4 dependent mechanism, implies that the increase of IgE early after infection is induced by a different mechanism [78]. However, regulation of IgE responses does not always require antigen specific responses, as crosslinking of the CD40 molecule on the B cell alone is sufficient to elicit a polyclonal IgE response [49]. This could be an explanation for the early onset of IgE production in the infected animals. The total serum IgE level was positively correlated with the total worm burden as well as the worm burden in S2 of the infected animals. On the contrary, no correlation was present with parasitological parameters expressing host responsiveness. Therefore, our data suggest rather a quantitative than a qualitative relation between IgE and the level of parasitosis in the infected animals. Similarly, in a study in which calves were infected with different doses of the intestinal nematode *Cooperia punctata*, the total serum IgE levels were positively correlated to the level of exposure of the animals (F. N. J. Kooyman, unpublished observation).
Taken together, our results showed that a primary infection with *Cooperia* in calves induced a type 2 shift in the immune response. Although the Th1/Th2 paradigm is not so clear in ruminants [36], the bias of the serological immune response towards IgG1 and IgE is commonly associated with a Th2 profile. Moreover, the results suggest that intermediate and low responder calves differ in their ability to initiate a Th2-type response. Based on the observed correlations, we propose an effector role for the humoral response against *Cooperia* infections in the gut. A study of the local responses is ongoing to address the possible mechanisms involved in the regulation of an effective local response against *C. oncophora*. The finding that the different responder types based on parasitological variables

also feature a different immune response is very interesting as this also provides the opportunity to study the influence of genetic components of the host response.

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CHAPTER 3

Immune expulsion of the trichostrongylid *Cooperia oncophora* is associated with increased eosinophilia and mucosal IgA

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What is the truth of all we know?
To hear the crap that comes out of the mouth…
The silent can say more….waiting and watching to see.
(G. Friday)

ABSTRACT

Previous experiments have shown that a primary infection with 100,000 infective larvae of the trichostrongylid *Cooperia oncophora* allows discrimination between different type of responder animals based on the speed by which the parasite is expelled from the host. In most of the animals (intermediate responders) the expulsion occurs 35-42 days after infection. This experiment was carried out to investigate which mechanisms contribute to the clearance of the parasite from the intestine. Sequential necropsy of the animals 14, 28 and 42 days after infection together with a segmental division of the small intestine, allowed us to characterise essential components associated with development of immunity and expulsion of the parasite from its niche. The results show that during the patent phase of the infection the parasite preferentially resides in the proximal gut. At day 42 after infection ongoing expulsion is characterized by a migration of the worms to the more distal part of the intestine. Expulsion of the adult worm population appears to be mast-cell independent and is associated with a significant increase in parasite-specific mucus IgA and IgG1 as well as with an influx of eosinophils in the intestinal lamina propria. Although we did not observe a specific lymphocyte recruitment into the intestinal mucosa, the accumulation of eosinophils seems to be mediated by CD4+ cells. We measured significant negative correlations between the number of eosinophils and the expulsion rate of the parasite expressed by sex ratio and ratio EPG. Parasite-specific mucosal IgA levels were negatively correlated to the fecundity of the worms, expressed as number of eggs per female worm. Our results describe the involvement of both eosinophils and mucosal IgA in the regulation of *C. oncophora* expulsion and suggest the development of a Th2 effector immune response.

INTRODUCTION

The trichostrongylid *Cooperia oncophora* is a lumen dwelling nematode of cattle. Experimental infections with 100,000 infective larvae in 3-month-old calves are highly reproducible and have the potential to allow discrimination between different responder animals based on parasitological variables and the speed by which the parasite is expelled from the host. [3, 84, 176, 231]. High responders, a small proportion of the host population, show almost no egg output and their worm burden at necropsy on day 42 after infection (p.i.) is low. Low responders show high egg output which is continued for weeks and results in high worm burdens at necropsy. In intermediate responders the egg output is initially similar to the low responders but between days 35-42 p.i, it declines rapidly. The worm burdens at necropsy vary from low to high numbers.

Previously, we demonstrated that intermediate and low responders also develop a distinct systemic immune response [127]. Regardless of their responder type, in all animals a Th2-biased immune response was developed. However, *C. oncophora*-specific serum IgG1 was significantly higher in intermediate responders than in low responders. Moreover, based on correlations between the systemic immune response and parasitological data, we proposed an effector role for the parasite-specific humoral response in the intermediate responders.

Here, we aimed to elucidate the immune effectors associated with the rejection of the parasite and the decrease in egg output observed in the intermediate responders. To this end, we performed a detailed study of the events occurring in the gut of infected animals in the course of the infection. Following a single primary infection with 100,000 infective larvae calves were necropsied sequentially at days 14, 28 and 42 p.i., corresponding with onset of egg excretion, peak egg output, and ongoing expulsion of adult worms, respectively, in most of the infected animals. Animals slaughtered at day 42 p.i. were subdivided in different responder types. The availability of those responder types provided the opportunity to compare the development of a successfull immune response in the intermediate responders with a non effective one in the low responders.

MATERIALS AND METHODS

Infective larvae

Third stage larvae (L3) from *C. oncophora* were obtained by coprocultures using standard procedures [30] and kept in water at 10°C until use. Larvae used for infection were less than 3 months old and originated from the Wageningen laboratory strain which has been maintained by regular passage through donor calves since 1967.

Animals and experimental design

Thirty-six female Holstein Friesian calves were raised under helminth free conditions on commercial farms and purchased at 3 months of age. Throughout the experiment the animals were

kept indoors at the animal facility of the Faculty of Veterinary Medicine in Utrecht. Animals were housed in one group and fed hay and water ad lib, as well as concentrates at a maximum of 500 g/day. Prior to infection animals were checked for general health condition and faeces was collected to test the worm-free status of the animals.
Twenty-four calves were infected orally with a single dose of 100,000 *C. oncophora* L3 and twelve calves were kept as controls. Faeces were collected from the rectum once a week during the first two weeks of the infection and thereafter three times a week. The number of eggs per gram faeces (EPG) was determined using a modified McMaster technique. A blood sample for serum was collected from the jugular vein one week before infection and weekly thereafter from day 7 to day 42 p.i.. At day 14, 28 and 42 p.i., 8 infected and 4 control animals were necropsied. The groups were named group A (necropsied at day 14 p.i.), group B (necropsied at day 28 p.i.) and group C (necropsied at day 42 p.i.).

Necropsy procedure and parasitology
To obtain a more detailed insight in the localisation of the worm burden in the small intestine in relation to the population dynamics, the small intestine was divided in six segments (table 1). All individual segments were sampled for histological examinations and mucus isolation. Additional sampling of S1, S3 and S6 was done for the isolation of lamina propria lymphocytes.

TABLE 1. Segmental division of the small intestine

Segment	Localisation	anatomical nomenclature
S1	first meter after the pylorus	duodenum
S2	next 5 meters	proximal jejunum
S3	next 5 meters	middle jejunum 1
S4	next 5 meters	middle jejunum 2
S5	next 5 meters	distal jejunum
S6	last part of the small intestine	ileum

The remaining of the intestinal segments was used for worm counts. Intestinal washings were done according to the methods described by Eysker and Kooyman [74]. All segments were processed separately. Two percent of the worm burden of each segment was counted and sex and developmental stages were determined. Worm fecundity was assessed by counting the number of eggs per female worm. Twenty worms per segment were enumerated. Worms were fixed in 70% alcohol and, at time of egg counting rinsed in tap water to remove the excess of alcohol. Individual female worms were placed in a 96-well flat-bottom plate (Greiner high binding, cat 655093; Greiner Labortechnik, Frickenhausen, Germany) and lysed to release the eggs using a 100 µl of a 0.4% sodium hypochlorite solution. Eggs were enumerated using an inverted microscope 5 to 10 minutes after lysis of the adult females (magnification 16 x).

Isolation of intestinal lamina propria lymphocytes
Lamina propria lymphocytes (LPL) were isolated using an enzymatic method according to Van den Broeck et al. [228] with minor modifications. Sections of approximately 5 cm were removed from the proximal part of the duodenum (S1), the proximal jejunum (S3) and the ileum (S6) and checked for the absence of Peyers patches before processing. Erythrocytes were removed from the final cell suspension by an erythrocyte shock lysing solution and viability of the cells was assessed by

Trypan blue exclusion. After each incubation step, a sample of the gut tissue was taken for histological examination as control for the isolation procedure.

Isolation of mesenterial lymphnode lymphocytes (MLNL)

One mesenterial lymphnode at the beginning of S3 was collected aseptically in ice cold sampling medium. MLNL were isolated as described by Schallig et al. [190]. One part of the cells was resuspended in RPMI supplemented with P/S, 10% FCS, 2 mM glutamine and 2 mM β-mercapthoethanol at a concentration of $2*10^6$ cells/ml until further use in lymphocyte proliferation tests (LPT). The remaining cells were resuspended at a concentration of $1*10^7$ cells/ml in RPMI supplemented with P/S. The latter cell suspension was used for flow cytometric analysis.

Phenotyping of lymphocyte subpopulations

Flow cytometric analysis of LPL and MLNL was performed using the same panel of monoclonal antibodies and staining method as described before [127]. Interference with other cells was excluded by gating for lymphocytes, based on forward and side scatter pattern of the cells. Results are expressed as percentage positive cells after subtraction of the isotype control percentages. Percentages of the lymphocyte subpopulations were based on a total of 10,000 events within the gate set for lymphocytes.

In vitro lymphocyte response to *C. oncophora* antigen extracts

C. oncophora L3 crude worm antigen (L3), Adult crude worm antigen (Ad) and Adult excretory/secretory products (ESP) were prepared as described previously [127]. *C. oncophora*-specific proliferation was measured after stimulation of MLNL with 10 µg/ml L3, Ad or ES antigen. The LPA was performed as described by Schallig et al. [197] with minor modifications. Cells were incubated for 5 days at 37°C and 5% CO2. Twenty-four hours before harvesting a final pulse with 0.5 µCi (methyl) ^{3}H-thymidine was added to each well.

Detection of eosinophils and mast cells

Tissues were fixed in 4% paraformaldehyde in PBS (pH 7.4) or Carnoy's fluid for 24 hours. Histochemical quantification of eosinophils has been described by Vervelde et al. [235]. Eosinophils were only present in the lamina propria and cells were counted on a minimum of 25 graticule fields (0.0676 mm^2) throughout two or three sections. For the histochemical quantification of mast cells, Carnoy's fixed tissues were stained overnight with toluidine blue at pH 0.1 [118] and counterstained with light green. The counts were made separately in the different layers of the mucosae and a minimum of 10 graticule fields (0.1764 mm^2) were counted in the lamina propria of 2 or 3 sections of the tissue. A mininum of 5 graticule fields were counted in the lamina submucosa as well as in the lamina muscularis interna and externa. The total number of mast cells was obtained after summation of the number of cells in the different layers. Results are expressed as number of cells/mm^2.

Detection of *Cooperia* specific mucus Ig

A 3 to 5 cm piece of each intestinal segment was sampled for the extraction of intestinal mucus. Until storage at –20°C the samples were kept on ice to avoid proteolytic breakdown. Mucus was isolated based on the method described by McClure et al. [154] with modifications. Tissues were thawed and mucus was scraped off with a glass slide. Best results were obtained when the tissue was not completely defrosted. The scrapings were collected in a falcontube on ice. Three ml ice cold PBS supplemented with protease inhibitors (1 tablet for 10 ml; Complete®, Roche) was added to each sample. The samples were shaken for 1 hour at 4°C and centrifuged for 30 minutes at 4°C

and 3,000 x g. Finally, the supernatant was spun down for 30 minutes, 4°C at 15,000 x g. Protein concentration was determined with the Bradford assay. ELISA's were performed with samples adjusted to a final concentration of 5 mg protein per ml supernatant. *Cooperia* Ad IgG1, IgG2 and IgA specific ELISA's were performed as described before [127].
Total IgE levels were measured in the mucus using a sandwich ELISA as described before [133]. Mucus samples at a protein concentration of 10 mg/ml were used. All samples were inactivated for 1 hour at 56°C to increase the affinity of the monoclonal antibody [136].

Statistical analysis
Statistical analysis was carried out using SPSS statistical package (version 10.0). Differences between groups were tested with the non-parametric Friedman or with the Wilcoxon test for dependent samples. Independent samples were analysed with Kruskas-Wallis. Immunologial data were correlated with parasitological variables expressing host responsiveness, which were sex ratio, ratio EPG, number of eggs per female worm and the worm burden. The sex ratio was defined as the % male worms in the total adult worm burden. This parameter is useful to monitor worm expulsion because male worms are expelled first [3, 48]. Based on the worm distribution at day 14, day 28 and day 42 p.i. we used the sex ratio in S2, which is the predilection site of the adult worm population. The ratio EPG was defined as: mean EPG between day 35 and day 42 p.i. (end of egg excretion pattern)/mean EPG between day 21 and day 28 p.i. (peak of egg excretion pattern). This parameter features the effect of the host immune response on both worm survival and fecundity. Previously we demonstrated that this parameter is significantly positively correlated with the number of eggs per female worm [123]. The eight infected animals slaughtered at day 42 p.i. were classified into different responder types based on ratio EPG and worm counts as described previously [127]. All correlations between immunological and parasitological parameters were evaluated with the Pearson's correlation coefficient.

RESULTS

Parasitology

The egg excretion pattern of all the infected animals was comparable with previous experiments and within normal values. Worm counts at days 14, 28 and 42 p.i. showed that *C. oncophora* preferentially homed in the proximal jejunum (S2) (fig. 1).

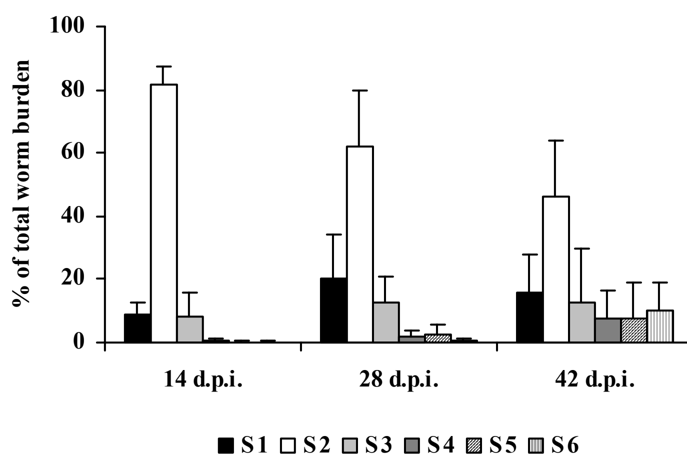

FIGURE 1 Worm distribution in the small intestine after a single-dose infection of 100,000 L3 *C. oncophora* at days 14, 28 and 42 p.i.. Data shown are mean values of 8 infected animals (+ SD).

At day 14 p.i., worms were only recovered from the proximal gut (S1, S2 and S3). The percentage recovery of the infection dose ranged between 56 and 117% at day 14 p.i., between 52 and 116% at day 28 p.i., and between 12 and 88% at day 42 p.i.. The sex ratio in the whole small intestine was consistently in the range of 46-51% with the exception of one animal which had almost no worm burden and a sex ratio of 9% at day 42 p.i.. Comparison of the sex ratio in the proximal and distal gut showed that at day 28 p.i. the % male worms was higher in the proximal gut than in the distal gut (49 versus 40%) while at day 42 p.i. the opposite was found (41 versus 45%).
Based on the faecal egg output and the necopsy results the animals slaughtered at day 42 p.i. were classified into 2 low and 5 intermediate responders [123]. None of the calves could be classified as a high responder and one calf showed a distinct EPG pattern which did not fit any of the classifications. Figure 2A shows that worm fecundity was highest at day 28 p.i., which coincided with the peak in egg output. Forty two days after infection, the worm fecundity decreased significantly compared with day 28 p.i. (P<0.01) but remained higher than day 14 p.i. (P<0.01). Analysis of the different responders showed a significantly lower fecundity in the intermediate responders compared with the low responders.

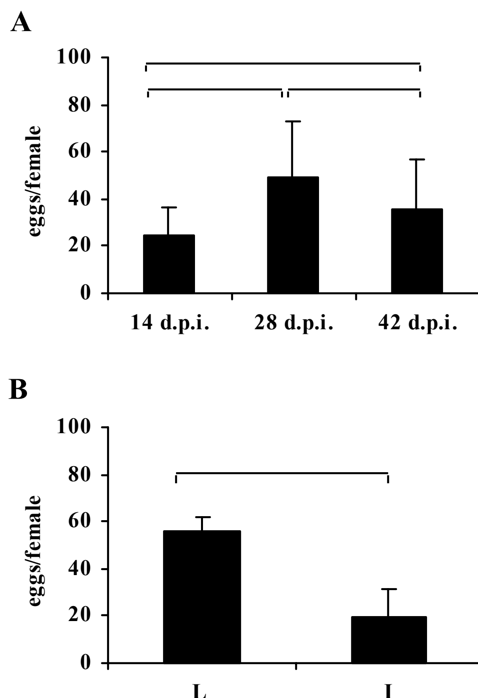

FIGURE 2 Mean number of eggs/female worm (+SD) for infected animals at day 14, 28 and 42 p.i. (A) and for low (L) and intermediate (I) responders (B). Data shown are mean number of 20 individual worms per calf. Lines indicate significant differences between groups (P<0.01).

Phenotypic characterisation of duodenal, jejunal and ileal LPL

In fig. 3 the mean percentage of the lymphocyte subsets in S1 is depicted for day 14 and day 28 p.i.. The results at day 42 p.i. were similar to those at day 28 p.i. and therefore omitted

from the figure. The %CD3+ cells was significantly higher in S3 than in S1 (P<0.01) and S6 (ileum) (P<0.05) (data not shown). This holds for both infected and control animals. The same pattern was observed for the %CD2+, CD4+ and CD8+ cells. On the contrary, the %TCR1+ cells was comparable in S1 and S3 (9.8+/-5.4 and 11.1+/-5.6 respectively) but significantly lower in S6 (7.2+/-4.5; P<0.05).

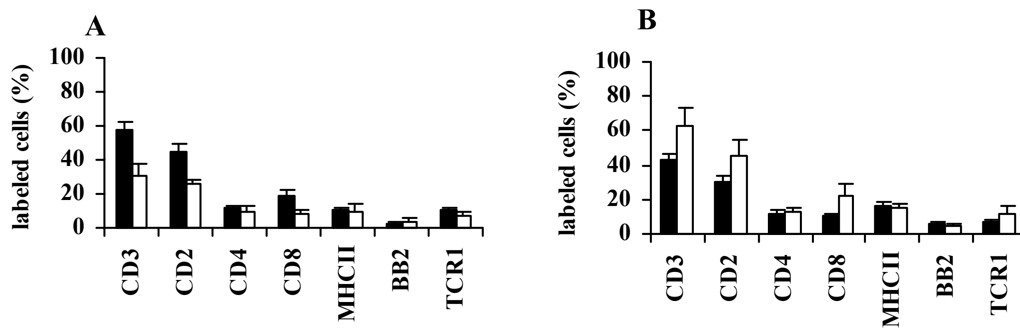

FIGURE 3 Phenotypic analysis of lymphocyte subsets of the lamina propria of the small intestinal segment S1 (duodenum) at day14 (A) and day 28 p.i. (B). Data are shown as mean % positive cells of 8 infected animals + SD (black bars) and of 4 control calves + SD (open bars).

Although no quantitative differences were measured, a negative correlation was present between the %TCR1+ cells and CD4+ cells in S1 (R=-0.62; P<0.1) and in S3 and S6 (R=-0.79 and R=-0.81 respectively; P<0.05) at day 42 p.i. in the infected animals. This was not found for the control animals (data not shown).

Phenotypic characterisation of MLNL

Subset analysis of lymphocytes from the draining lymphnode of S3 revealed no major differences in the T-cell populations. The TCR1+ population was expanded in the infected animals at day 14 p.i. (11+/-4 compared to 7+/-5 and 8+/-3 at day 28 and 42 p.i. respectively).

Comparison of infected and control animals at the different time points revealed significant changes in the BB2+ population and the MHCII+ (P<0.05) (fig. 4). Analysis of the infected animals showed a marked increase in %BB2+ cells at day 28 p.i. compared with day 14 (P<0.1) and day 42 p.i. (P<0.05). At day 28 p.i. the %BB2+ cells in the lymphnode was negatively correlated with the total worm burden (R=-0.79; P<0.05).

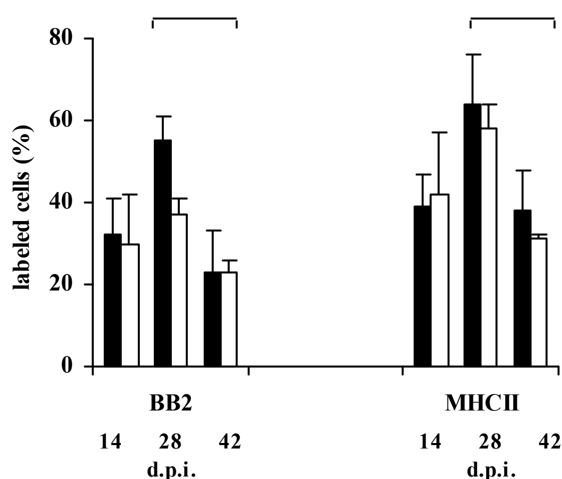

FIGURE 4 Phenotypic analysis of lymphocyte subsets of mesenterial lymph node lymphocytes (jejunum, S3) at day 14, 28, and 42 p.i.. Data are shown as mean % positive cells of 8 infected animals + SD (black bars) and of 4 control calves + SD (open bars). Lines indicate significant differences between groups ($P<0.05$).

In vitro lymphocyte proliferation to *C. oncophora* antigens

C. oncophora Ad and ESP-specific proliferation was observed in mesenterial lymphnode lymphocytes at day 14, day 28 and day 42 p.i., whereas proliferation in response to L3 was only detected at day 28 p.i.. Stimulation indices of the response to *C. oncophora* Ad are depicted in table 2. An increased SI was measured at day 28 p.i. with ESP and Ad in all infected animals, while 6 out of the 8 infected animals showed an increased response to *Cooperia* L3. At day 42 p.i. only 2 infected animals had an increased response to Ad antigen and 3 animals to ESP. Proliferation was always higher in response to ESP (range SI: 14-12,238) compared with Ad (range SI: 5-330) and L3 antigen (range SI: 4-2,053) (data not shown).

TABLE 2 Stimulation indices (cpm experimental/cpm medium) after stimulation of mesenterial lymphnode lymphocytes with *C. oncophora* adult antigen (10 µg/ml). At each time point data from 8 infected and 4 control animals are depicted (nd=not done).

Calves	Days post infection		
	14	28	42
Infected	2	76	0
	1	112	2
	1	86	1
	5	73	1
	1	330	1
	1	8	1
	2	77	11
	1	28	5
Control	3	2	1
	1	1	1
	1	18	nd
	1	9	2

Quantification of eosinophils

The number of eosinophils was counted in the proximal jejunum (S2) at the different time points after infection. At day 42 p.i. the infected animals had a significantly higher number of eosinophils compared with the control animals (P<0.01) (fig. 5A). Moreover, the number of eosinophils of infected animals necropsied at day 42 p.i. were significantly higher than in the infected animals necropsied at day 14 and day 28 p.i. (P<0.05).

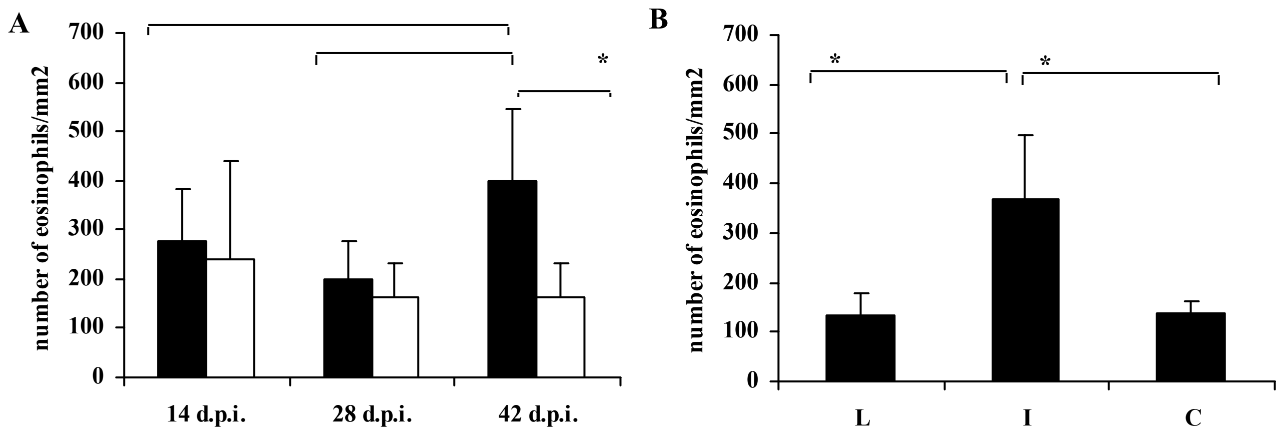

FIGURE 5 The mean number of eosinophils (+SD) in infected (n=8, black bars) and control (n=4, open bars) animals is depicted for day 14, 28 and 42 p.i.(A) and for low responders (L), intermediate responders (I) and control animals (C) (B). Lines indicate significant differences between groups (P<0.05, * P<0.01).

Analysis of the eosinophil numbers of the different responder types (day 42 p.i.) revealed that the increased number of eosinophils was caused by a higher number in the intermediate responders, while in the low responders the number of eosinophils was comparable with the control animals (fig. 5B).

At day 42 p.i. the increased number of eosinophils was negatively correlated with the sex ratio in S2 (R=-0.69, n=8, P<0.1) and the ratio EPG (R=-0.77, n=8, P<0.05) (fig. 6). Moreover, a positive correlation was observed with the % of CD4+ cells in the corresponding mesenterial lymphnode (R=0.76, P<0.05) (data not shown).

In order to investigate whether changes were induced in the distal gut during expulsion, we assessed the number of eosinophils in S6. Overall the number of eosinophils was higher compared with the proximal gut but there were no significant differences between infected and control animals. Nor were there differences between the low and intermediate responders (mean +/-SD: 1,008+/-340 and 969+/-336 respectively).

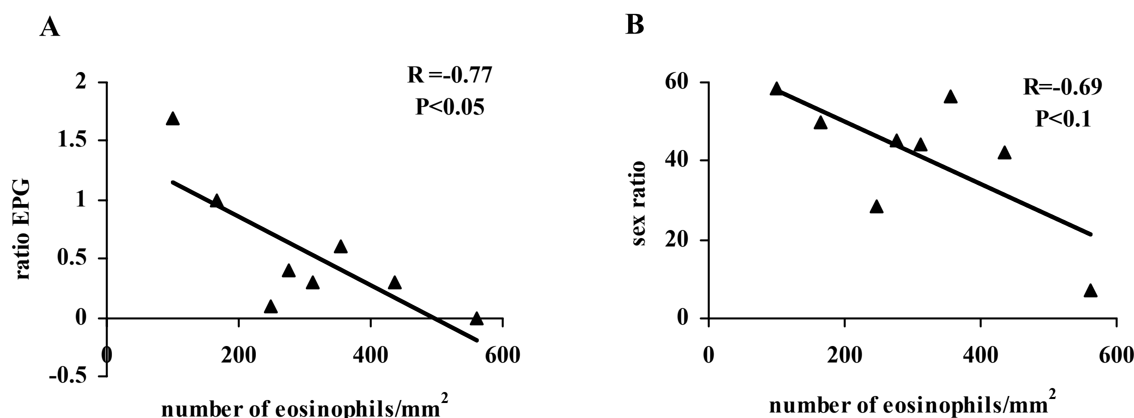

FIGURE 6 Correlations between the number of eosinophils/mm^2 in S2 and ratio EPG (A) and sex ratio in S2 (B) for the infected animals at day 42 p.i..

Quantification of mast cells

There were no significant differences between infected and control calves at any time point, and the total number of mast cells between the responder types did not differ. A significant positive correlation was measured between the number of mast cells in the LP and the total serum IgE response [123] at day 42 p.i. (R=0.71, n=10, P<0.05) (data not shown). The same trend was present with the total number of mast cells (R=0.43 and n=9) and the number of mast cells in the SM (R=0.45 and n=10), though not significant.

C. oncophora-specific Ig responses in the intestinal mucus

Exposure to a primary *C. oncophora* infection with 100,000 L3 larvae resulted in a gradual increase in *C. oncophora* Ad-specific mucus IgA and IgG1 in S2. The *C. oncophora*-specific IgG1 and IgA titres at day 42 p.i. were significantly higher than at day 28 and at day 14 p.i. (both P<0.05). Only a slight increase in mucus IgG2 was observed in some infected animals. In order to assess the Th2 phenotype of the local humoral response the IgG1/IgG2 ratio was calculated. At day 42 p.i. this ratio was higher than at day 14 and day 28 p.i. (9.3+/-6 versus 2+/-0.3 and 3+/-0.2). Low responders had a maximal ratio of 2, while in the intermediate responders the ratio was always higher than 5.

For both parasite-specific mucus IgA and IgG1 the intermediate responders had significantly elevated antibody levels compared with the low responders and the control animals (P<0.05) (fig. 7).

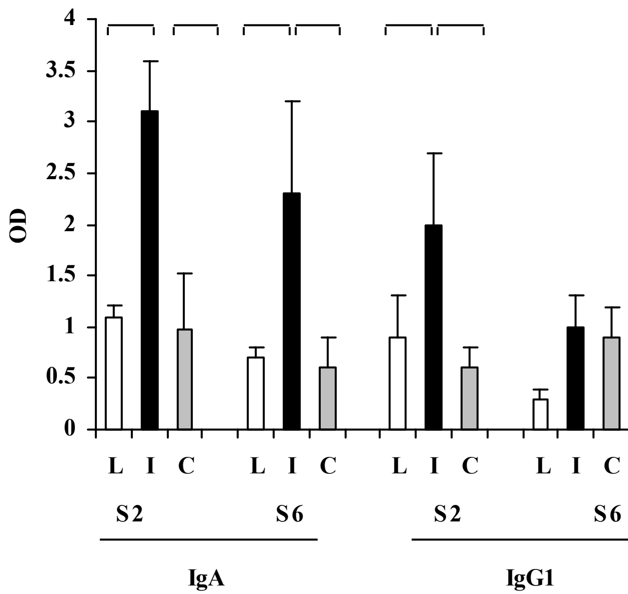

FIGURE 7 Mean OD (+SD) of *C. oncophora* Ad mucus IgA and IgG1 in S2 and S6. Low responders (L; open bars), intermediate responders (I; black bars) and control calves (shaded bars). Lines indicate significant differences between groups (P<0.05).

No correlations were observed between *C. oncophora*-specific mucus IgG1 or IgG2 and parameters suggesting worm expulsion. However, the mucus IgA in the infected calves was negatively correlated to the ratio EPG (R=-0.85, n=8, P<0.05) and the number of eggs per female worm in S2 (R=-0.85, n=8, P<0.05) (fig. 8). A similar negative trend was observed between *C. oncophora*-specific mucus IgA and the sex ratio (R=-0.56, n=8) as well as the worm burden in S2 (R=-0.56, n=8). In S2 the increase in IgA but not in IgG1 concurred with the rise in eosinophil numbers (R=0.65, n=12, P<0.05).

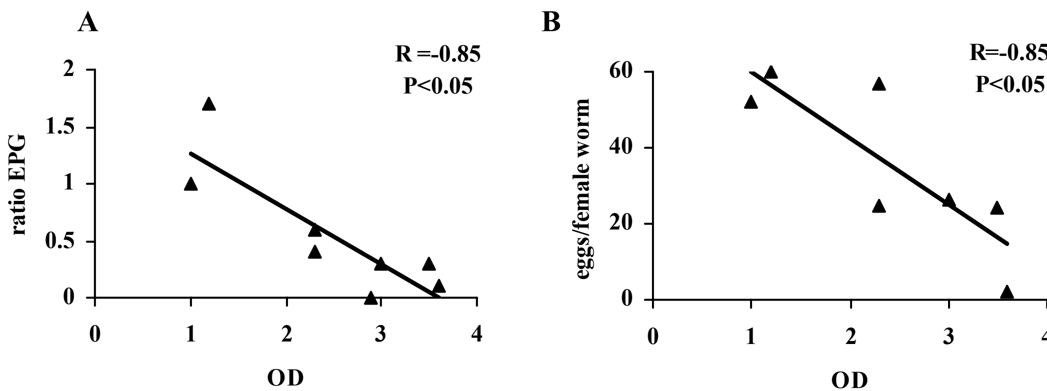

FIGURE 8 Correlations between *C. oncophora* Ad-specific mucus IgA in S2 and ratio EPG (A) and number of eggs/female worm (B) for the infected animals at day 42 p.i..

Both *C. oncophora* Ad-specific mucosal IgA and IgG1 levels during the infection were significantly positively correlated with the *C. oncophora* Ad serum IgA and IgG1 response, respectively (IgA: R=0.63, n=36, P<0.001 and IgG1: R=0.69, n=36, P<0.001).
The humoral response in S6 was assessed to evaluate whether the response in the proximal and the distal segment would be similar and whether the presence of a higher worm burden in the distal segment in intermediate responders would induce a rise in *C. oncophora* specific titres. Both *Cooperia*-specific IgA and IgG1 were increased in S6 in intermediate responders compared to low responders though at a lower level than in S2 (fig. 8). The *Cooperia*-specific mucus IgA levels in both segments were positively correlated. This did not hold for the *Cooperia*-specific mucus IgG1 levels. No difference was found in the mucus IgG2 in S6 between infected and control animals. A significant negative correlation was found between mucus IgA in S6 and the ratio EPG (R=-0.71; P<0.05). A trend was also observed for IgG1 (R=-0.68; P<0.1). No correlations were observed with the total and segmental worm burden. Analysis of variance revealed no relationship between the antibody titres and the eosinophil numbers in the distal gut.

Total IgE in mucus

We could detect total IgE in the mucus of S2 of the infected animals, however within-group variation was high. At day 14 p.i. total IgE levels ranged from 44 to 2464 mU/ml, at day 28 p.i. from 46 to 2169 mU/ml and at day 42 p.i. from 38 to 3847 mU. The observed variability could not be related to any parameter of infection and no differences were measured between the different responder types. Moreover, in some of the control animals high IgE levels were detected.

DISCUSSION

This experiment was carried out to study the dynamics of both parasitological and immunological events occurring in the gut of *C. oncophora* infected calves and which eventually result in clearance of the parasite from the gut. An infection of 100,000 L3 larvae was chosen as this protocol has been studied most extensively [3, 84, 176, 231] and induces protective immune responses. Moreover, based on the speed by which the parasite is expelled from the host, infected animals can be subdivided in high, intermediate and low responders [127]. The difference between susceptible and resistant calves is further emphasised by a different recognition of *Cooperia*-specific proteins after Western blot analysis [231]. We investigated the contribution of both the cellullar and humoral component of the immune response to the rejection of the parasite and analysed the results in view of the parasitological response to the infection.

Segmental division of the small intestine revealed that during the patent phase of the infection the parasite preferentially resides in the first 6 meters of the intestine. Generation of immunity in intermediate responder animals induced migration of the adult worm population to the distal part of the intestine. Armour et al. [9] described a similar phenomenom in animals trickle infected with *C. oncophora* during twelve weeks, however, their infection protocol did not allow discrimination between susceptible and resistant calves. At day 42 p.i. a decreased fecundity was found in the worms of the proximal gut which was caused by a lower fecundity in the intermediate responders. In the other animals the fecundity was comparable to day 28 p.i., the peak in egg output. Interestingly, the fecundity of the worms in the distal segment was not reduced and always higher than the fecundity of the worms in the proximal segment. Reduced fecundity has been implicated as a major regulatory force for gastrointestinal populations in sheep but this was a derivative of worm length [204, 209, 211]. In our study no differences in worm length were observed (data not shown). Our fecundity and localisation data suggest that part of the effector mechanisms/immune responses act on the egg laying machinery and part on the expulsion of the worms from their niche. On the other hand, vital worms which are still producing eggs, could escape the detrimental environment of the immune proximal gut.

The results presented in this study demonstrated that infection did not induce the recruitment of a specific lymphocyte subset in the intestinal mucosa. We could possibly have missed a transient early increase or decrease in one of the lymphocyte subsets as our analysis was restricted to a few time points after infection. Infection of 4-month-old calves with *Ostertagia ostertagi* did induce an influx of Ig- bearing cells, TCR1+ cells and IL-2R+ cells in both the abomasal mucosa and the draining lymphnodes [4]. Similar findings were done by Balic et al. [14] after infection of sheep with *Haemonchus contortus,* however, in this experiment the increase was not sustained until the adult infection. This can possibly be explained by the observation that both nematodes are more pathogenic and invasive. We do not exclude that an incoming *Cooperia* infection is associated with the same kinetics early after infection, but, based on our results, we predict that the patent phase of the infection as well as the expulsion of *C. oncophora* are not associated with a specific recruitment of lymphocytes in the intestinal mucosa.

Similarly, calves exposed to a primary *C. oncophora* infection showed no major changes in the T lymphocytes subsets of the mesenterial lymphnode. The increased number in TCR1+ cells in the infected animals at day 14 and day 28 p.i. supports the hypothesised role attributed to γδ T-cells in directing the immune profiles during a primary infection [79]. An exact role for this cell population in nematode infections is still not clarified. It has been proposed that expansion of this subset is associated with the rejection of *Trichostrongylus colubriformis* in sheep [154]. This is not likely in our experimental model as by day 42 p.i. the

expansion of this population is gone. Twenty-eight days after infection we observed a significant increase in both BB2+ and MHCII+ cells. Recent data have shown that B cells are recquired for a succesfull priming of a rapid Th2 response in *T. muris* infected mice [28]. In that model the B cell involvment in protective immunity could be through the role of accessory antigen presenting cells or via antibody production. In the current study the increase in B cell numbers coincided with the onset of antibody production both in the serum and the mucus of the infected animals.

Histological analysis of S2 revealed a significant influx of eosinophils in the lamina propria of the infected animals at day 42 p.i.. Eosinophil recruitment is known to be dependent on cytokines secreted by CD4+ Th2 cells. The positive correlation observed with the percentage CD4+ cells in the mesenterial lymphnode lymphocytes and the eosinophil numbers in the gut, fits within this hypothesis. The eosinophilia was more pronounced in the intermediate responders and significantly negatively correlated with the sex ratio and with the ratio EPG which suggests a role for eosinophils in the rejection of the adult worm population from the gut. The finding is surprising in that until now during primary nematode infections eosinophils have mainly been associated with expulsion of larval stages rather than adults. Increased number of eosinophils was seen during primary infection of sheep with *H. contortus* [45], *T. circumcincta* [213] and *N. battus* [245]. Only during a primary infection with *N. battus* the increased number of eosinophils concurred with the time of adult nematode expulsion [245]. In our study, at day 14 p.i. the number of eosinophils was slightly but not significantly higher in the infected animals compared to the control animals. This moderate eosinophil response might have been in response to the larvae. Analysis of S6 in the animals slaughtered at day 42 p.i. showed only a slight increase in the infected calves compared to the controls. This indicates that the immune reaction of the host was not disseminated throughout the whole small intestine and that the eosinophil response was specifically located in the proximal gut, at the site of infection.

Mast cell numbers showed a high individual variability which could not be related to the infection status of the animals. Presumably this cell population is not involved in the regulation of a primary *C. oncophora* infection. Although in this study the analysis was restricted to enumaration of the number of cells without investigation of their activation status, several studies in sheep [114, 116, 72] and rodents [104, 168] demonstrate that resistance to nematode infection and expulsion of parasites can be both mast-cell dependent or independent, depending on the host-parasite system studied.

Exposure to a single dose infection of 100,000 L3 *C. oncophora* larvae resulted in a gradual increase in *C. oncophora* Ad-specific IgA and IgG1 and an increased IgG1/IgG2 ratio in the infected animals. At day 42 p.i. the intermediate responders had significantly higher *C. oncophora* mucus IgG1 and IgA than the low responders. This was also found in serum [127],

but in all animals mucus IgA was higher than IgG1, while in serum we observed the opposite. The possible effector role of IgA at mucosal surfaces seemed confirmed by the significant negative correlation with the ratio EPG and the number of eggs/female. The rise in mucus IgA but not IgG1 concurred with the increased number of eosinophils in S2. A possible interaction between both has been postulated previously [60]. IgA/antigen complexes could bind through the Fc-alpha receptor on eosinophils and provoke the release of anti-inflammatory mediators and cytokines which have a detrimental effect on the worm population. Upregulation of Fc receptors and complement receptors on the eosinophil membrane occurs after activation. Moreover, it has been demonstrated that sIgA at mucosal surfaces has increased ability to activate eosinophils compared to serum IgA [166]. Based on our results, we hypothesise that both eosinophils and mucus IgA are involved in an effective immune response against adult *C. oncophora*. The efficacy of the immune response in reducing the level and the fecundity of the parasite burden is clear in the intermediate responders. The parasitological phenotype of these animals can be expanded with the immunological features which are characterised by high numbers of eosinophils and high parasite-specific IgA titres at the time of parasite rejection.

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Chapter 4

Priming dose level and host responder type differentially affect establishment, length and fecundity of *Cooperia oncophora* in re-infected calves

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Parasitology, in press

L'identité n' est pas donnée une fois pour toutes, elle se construit et se transforme tout au long de l'existence. (A. Maalouf)

ABSTRACT

We investigated the effect of bovine amnestic immunity on a range of parasitological variables. To this end, calves were primed with a single oral dose of 30,000 or 100,000 infective larvae (L3) of *Cooperia oncophora*, drenched with anthelmintic, maintained worm free in the following 2.5 months and subsequently re-infected with 100,000 L3. Parasitological profiles of low, intermediate, and high responders were compared. The results indicate that immunity to a secondary infection with *C. oncophora* has both dose-dependent and dose-independent mechanisms. The reduction in establishment of the worms, as shown by a lower worm burden and increased percentage of fourth-stage (L4) larvae, occurred in a dose-independent way but was associated with the responder type of the animals. Worm length and fecundity were similarly reduced by both priming doses but the speed by which the effect occurred was dose-dependent. We conclude that the memory responses to *C. oncophora* consist of both dose-dependent and dose-independent immunological processes and that different components of parasite fitness are affected differently by prior exposure. The difference in establishment between the responder types demonstrates that the ability of intermediate responders to mount a more effective and faster immune response compared to low responders is sustained after secondary infection.

INTRODUCTION

Cooperia oncophora is the most common intestinal nematode in cattle in temperate regions. Although natural infections rarely cause clinical parasitism, *C. oncophora* has been implicated in negative effects of gastrointestinal nematode infections on productivity [181, 180, 179]. In view of the development of improved control strategies against infection, there is a need for a more detailed knowledge on how immunity develops in infected animals. In natural field situations, not only development of immunity is important, but also persistence of immunity and the level of protection against re-infection. A recent survey among Dutch farms revealed that on almost 50% of the farms animals are treated with anthelmintics after housing [178]. Consequently, they remain worm-free during the housing period and re-infection of yearlings occurs after turnout for the second grazing season. This implies that long-term memory is required for protection.

Until now, we and previous authors merely focused on primary infections with 100,000 L3 larvae in 3-month-old calves [176, 231, 127, 126]. With this experimental protocol, animals can be subdivided in different responder types based on their ability to develop an effective immune response resulting in a decrease in egg output and expulsion of the worm population from the host [231, 127]. Recently, Gasbarre et al. [89] described three types of responders in a herd based on the egg excretion following a natural infection with predominantly *Ostertagia ostertagi*. For both *C. oncophora* and *O. ostertagi* it has been shown that host genetics account for a substantial amount of variation in susceptibility to infection [3, 146, 134, 89]. This seems to be the case for primary and secondary infections, as calves resistant to a single primary infection with *Cooperia* also showed a lower susceptibility to a homologous challenge infection [3].

Although we have no data on the genetic constitution of the animals we use, previous experiments demonstrated that immunity is generated in intermediate but not in low responders with a single oral dose of 100,000 L3 [127, 126]. High responders seem to be resistant to infection resulting in low establishment of the worm population and low egg output during primary infection. Despite the different level of acquired and innate immunity, anthelmintic treatment followed by a single dose challenge infection shortly thereafter, resulted in no egg excretion in most of the primed animals (unpublished data). This occurred irrespective of their responder type. In infection trials, in which animals were primed with 100,000 or 30,000 L3 for a period of 35 days, followed by anthelmintic treatment and challenge infection, we observed that the low dose did not induce protection in all animals (unpublished data). These data suggested that priming with 30,000 or 100,000 L3 induced a different level of immunity and that a lower infection dose would enable us to discriminate between levels of resistance in primed animals.

In view of these data the current experiment was set up, in which we studied the amnestic immunity after re-infection of animals that had been primed with *C. oncophora*. Low, intermediate and high responders were analysed separately to investigate whether the differentiation in responder types was sustained after a secondary infection. Furthermore, we compared a low (30,000 L3) and a high (100,000 L3) priming dose to study the dose-dependency of the generation and persistence of immunity against *C. oncophora* and its effect on the establishment and the survival of the worms. A detailed analysis of parasitological and immunological parameters was done. This paper reports on the parasitological aspects of immunity observed in calves after re-infection with *C. oncophora.* The influence of the infection dose and the responder type on the resistance to a re-infection is highlighted.

MATERIALS AND METHODS

Animals and experimental design

Thirty-four female Holstein-Friesian calves were raised under helminth-free conditions on a commercial farm and purchased at 3 months of age. Animals were housed indoors during the whole period of the experiment at the animal facility of the Faculty of Veterinary Medicine in Utrecht. The animals were fed hay and water ad lib as well as concentrate at a maximum of 500 g per day. Prior to infection animals were examined for general health condition and faeces were collected to check the worm free status of the animals. A detailed scheme of the experimental protocol is given in table 1. The animals were drenched with oxfendazole (Systamex 2.65% suspension, 2 ml/10 kg, Scheringh Plough, Kenilworth, New Jersey, USA) at day 49 after infection (p.i.). All animals were around 6 months of age at the time of challenge. All experimental procedures were approved by the ethical committee on animal experimentation of the University Utrecht.

TABLE 1 Experimental design

		Group			
TREATMENT	day	G1 (n=11)	G2 (n=11)	G3 (n=6)	GC (n=6)
primary infection	0 d.p.i.	30,000 L3	100,000 L3	none	none
anthelmintic drench	49 d.p.i.	yes	yes	yes	yes
challenge infection	126 d.p.i.	100,000 L3	100,000 L3	100,000 L3	none
necropsy	0 d.p.c.*	n=4	n=4	nd	n=2
	14 d.p.c.	n=3	n=3	n=3	n=2
	28 d.p.c.	n=4	n=4	n=3	n=2

n=number of calves; d.p.i.=days post infection; d.p.c.= days post challenge; nd= not done
* these animals did not receive a challenge infection

Parasitology
The numbers of eggs per gram faeces (EPG) were determined with a modified McMaster technique. Faeces were analysed once a week from day 0 to day 14 p.i. and during the period between treatment and secondary infection. In the remaining period, faecal samples were collected and analysed every two days. After slaughter the small intestine was subdivided in six different segments that were all processed separately as described previously [123]. Briefly, the first segment consisted of the first meter starting from the pylorus and each following segment was five meters. Results are shown from the second (S2, proximal gut) and the last (S6, distal gut) segment. Two percent aliquots of the total worm burden were counted. A differentiation was made between male and female worms and fourth-stage larvae (L4). The L4 were predominantly (>95%) early stages and therefore considered as inhibited larvae. Worm lengths were measured from 20 individual male and female worms per segment if available. From each worm an individual picture was taken and length was determined using the software package Accuroute® version 1.1 (Critical path software, http://www.accuroute.co.uk). For each picture a calibration unit of 2 mm was set. Fecundity of the female worms was estimated by counting the number of eggs in 20 individual females per segment after lysis of the worms in 0.4% sodium hypochlorite solution [122].

Responder types
Based on the egg output, the ratio EPG and the serology during the course of the primary infection animals were subdivided in different responder types [127]. Briefly, a threshold in ratio EPG (ratio mean EPG day 35-42 p.i./mean EPG day 21-28 p.i) was set at 0.4 which indicates a decrease in egg output of at least 60% by the end of the primary infection. Animals with a ratio EPG >0.4 and a high cumulative EPG (total egg output during the primary infection) were considered as low responders. Animals with ratio EPG <0.4 were considered as intermediate responders. High responders may show a ratio EPG taking any value, but show a significantly lower peak in egg excretion by day 21-28 p.i., concurrent with a low cumulative EPG. The discrimination in responder types was confirmed with the *Cooperia* Adult-specific IgG1 ELISA [127]. Low (L), intermediate (I) and high (H) responders were mutually compared during the primary infection and after the secondary infection and compared with the challenge control animals (G3).

Statistics
Statistical analysis was carried out using SPSS statistical package (version 10.0). Normally distributed data were analysed with ANOVA. Data that were not normally distributed were analysed with the non-parametric Mann-Whitney test for two independent samples and the non-parametric Kruskal-Wallis test for more independent samples. Pair-wise comparison of three or more independent samples was done by post-hoc analysis as advised for Kruskal-Wallis. Correlations between parameters were measured with the Pearson's correlation coefficient for linear correlation on normally distributed data and with the Spearman's rank correlation coefficient for non linear correlations on normally distributed data. The confidence level was set at $P<0.05$. The use of the sex ratio (% male worms) and the ratio EPG to assess host responsiveness has been described previously [126].

RESULTS

Egg output

Figure 1A shows the egg output during the course of the primary infection (day 0 to 49 p.i) in G1 and G2. In both G1 and G2 the egg excretion started around day 14 p.i. and reached a

peak at day 21 p.i. but the rise in egg output was faster in G2 and the peak significantly higher (P<0.01). Although throughout the infection the egg output in G2 remained higher than in G1, the difference was not significant after day 21 p.i.. Figure 1B shows the egg output of the different responder animals within G1 and G2.
The distribution of the responder types was as follows: in G1, 3 low responders and 7 intermediate responders were found, and in G2, 4 low and 6 intermediate responders were differentiated. Each group had only 1 high responder. In all responder types the onset of egg output was similar. Whereas the intermediate responders had no or a very low EPG at day 42 p.i, low responders still shed eggs in the faeces. Both high responders had a low egg output from day 21 p.i. onwards and no egg output at day 42 p.i..

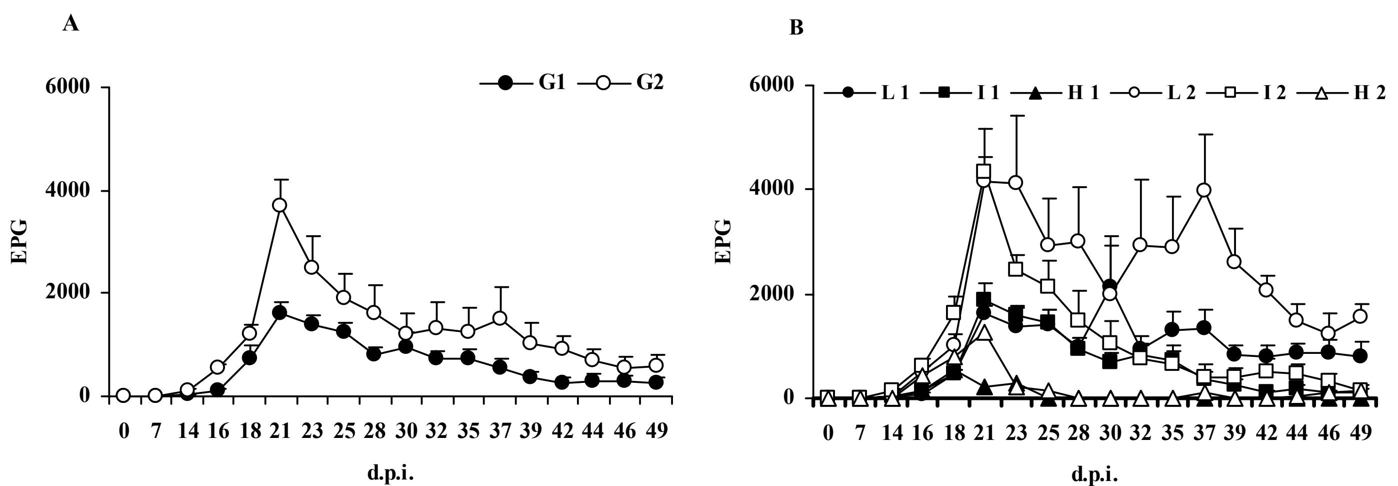

FIGURE 1 (A) Mean EPG (+SEM) for G1 (primary infected with 30,000 L3) and G2 (primary infected with 100,000 L3). (B) Mean EPG (+SEM) for the low (L), intermediate (I) and high (H) responders after infection with 30,000 L3 (1) or 100,000 L3 (2) is depicted.

Analysis of the EPG after challenge infection revealed that none of the animals were excreting eggs at day 14 after challenge (p.c.). At day 28 p.c. only 2 out of the 8 primed animals (1 animal of G1 and 1 animal of G2) were excreting eggs (cumulative EPG: 1350 and 3300 respectively) compared to the 3 challenge controls which all had EPG (cumulative EPG: 1300, 2750 and 2800).

Worm counts and differentiation

The influence of immunity on establishment of the worm population is illustrated in fig. 2. No worms were recovered from the non-infected control animals. A significant reduction in worm burden was found in the primed animals (G1 and G2) compared to G3 at day 14 and 28 p.c. (P<0.05 for all, fig. 2A). The magnitude of this reduction was similar in both primed groups. Analysis of the responder types revealed a significantly decreased number of worms

in the intermediate responders compared to the challenge controls (G3) at day 14 p.c. (fig. 2B). At day 28 p.c. a further decrease in worm burden of the intermediate responders was observed and the worm burden differed significantly from low responders and G3 (P<0.05 for both).

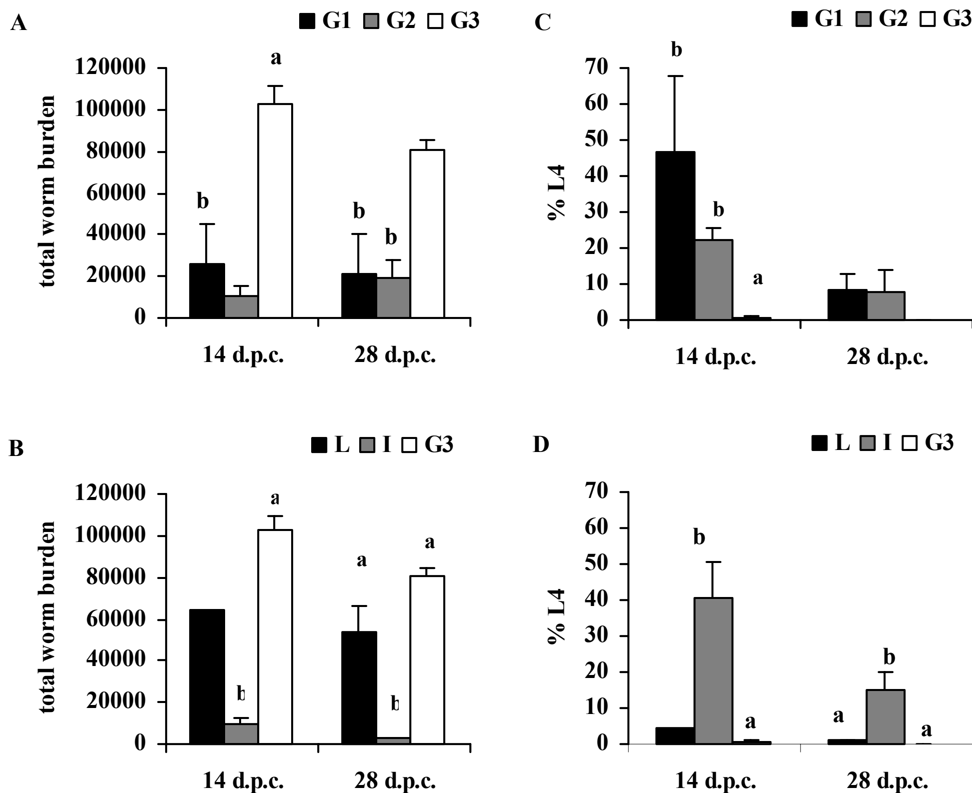

FIGURE 2 (A) The mean worm burden (+ SEM) for the animals of G1 (30,000 L3/100,000 L3), G2 (100,000 L3/100,000 L3) and G3 (0 L3/100,000 L3) at day 14 and 28 p.c.. (B) Mean arithmetic worm burden (+SEM) of low (L) and intermediate (I) responders compared with G3. (C) The mean %L4 larvae (+SEM) recovered from the intestine of the animals from G1, G2 and G3 and (D) from L and I responders and G3. Different letters indicate significant differences within time points. The confidence level was set at P<0.05

The levels of the %L4 were complementary to the kinetics of the worm burden and a significant negative correlation was measured between both parameters (P<0.01, data not shown). At day 14 p.c. the %L4 was significantly higher in G1 and G2 compared to G3 (P<0.05 for both, fig. 2C). A subsequent drop in %L4 at day 28 p.c. resulted in a still higher but not significantly different %L4 in the primed animals compared to the challenge controls. In the intermediate responders the %L4 was significantly elevated compared to low responders and G3 at both time points. In both responder types a drop in %L4 was observed at day 28 p.c. compared to day 14 p.c. (fig. 2D).

The mean sex ratio (% male worms) in S2 and S6 for the 3 infected groups is shown in fig. 3. At day 14 p.c. the sex ratio in the animals of G2 was significantly reduced compared to G3 (P<0.05) in S2. At this time point no worms were recovered from S6 of G3, hence this resulted in a sex ratio of 0. At day 28 p.c. the sex ratio in G1 and G2 was lower than in G3 in both the proximal and the distal gut

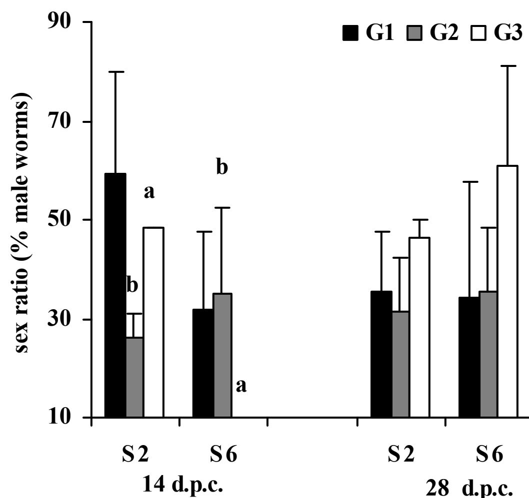

FIGURE 3 Mean sex ratio (% male worms, +SEM) in the proximal (S2) and the distal (S6) gut for the animals from G1 (30,000 L3/100,000 L3), G2 (100,000 L3/100,000 L3) and G3 (0 L3/100,000 L3) at day 14 and 28 p.c.. If no worms were recovered, a sex ratio of 0 was assumed. Different letters indicate significant differences within S2 or S6. The confidence level was set at P<0.05.

Distribution of the worm population in the small intestine

A decreased establishment often coincides with a relocation of the worms from their predilection site. Therefore, we analysed whether the decreased establishment in the intermediate responders also resulted in an altered distribution of the worm population throughout the gut. In the challenge control group (G3) the worms preferentially resided in the proximal gut at day 14 p.c. (fig. 4A). At day 28 p.c. the number of worms in the proximal gut decreased compared to day 14 p.c. and a higher number of worms were recovered from the distal gut (fig. 4A). In the intermediate responders we observed that besides a significant reduction in the worm burden, at day 14 p.c. worms were equally distributed throughout the entire gut (fig. 4B). This seemed to be a feature of a secondary challenge since at this time point worms were also recovered from the distal gut in the low responder animal (fig. 4C). However, at day 28 p.c., in both low and intermediate responders, more worms were recovered from the proximal than from the distal gut.

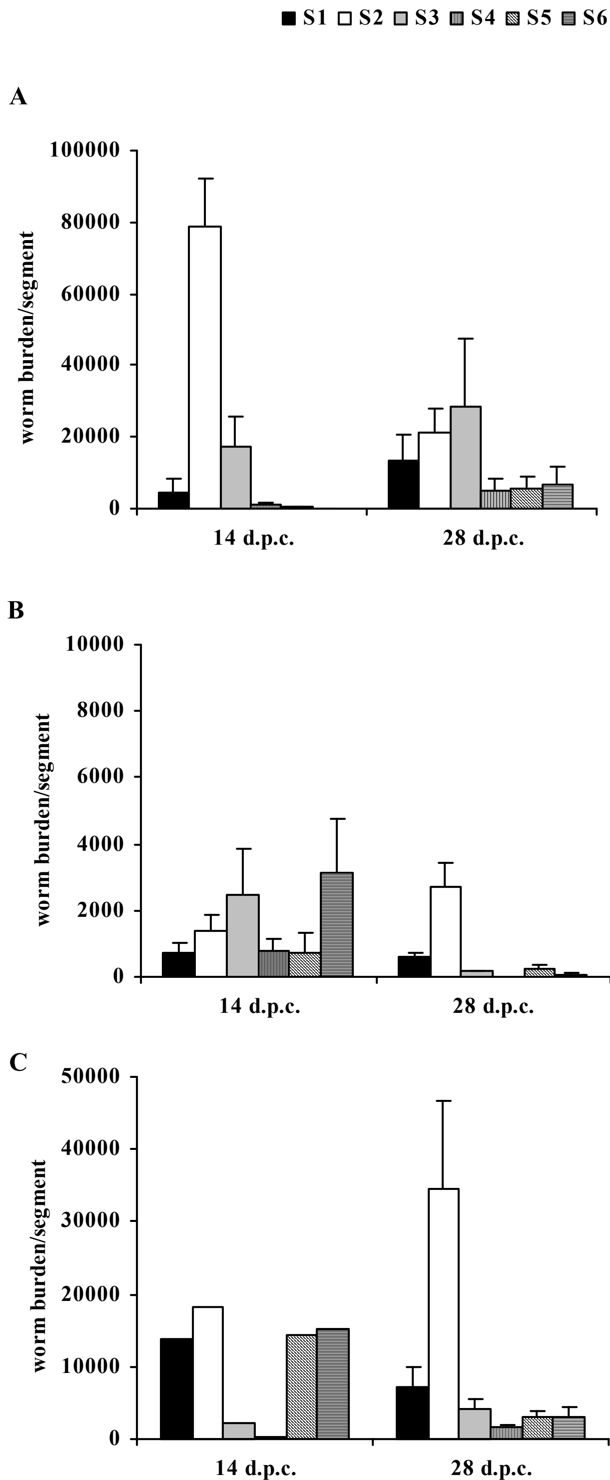

FIGURE 4 The distribution of the worm burden (+SEM) throughout the different segments of the small intestine is shown for challenge control animals (G3, A), intermediate (B) and low responders (C) at day 14 and 28 p.c.. For G3, each bar is the mean (+SEM) of 3 animals at day 14 and 28 p.c.. At day 14 p.c. 1 low responder animal and 5 intermediate responders were analysed. At day 28 p.c. 4 low and 4 intermediate responders were analysed.

Worm length and fecundity

To assess if besides reduced establishment, immunity to *C. oncophora* also resulted in a stunted development of the adult worms, worm length and fecundity were measured. Worm lengths of both male and female worms were significantly correlated in the proximal gut

(R=0.81, n=9, P<0.01 with n=number of animals from which both male and female worms were recovered), which suggested that immunity resulting in a reduced development acted similarly on male and female worms. This correlation could not be assessed in the distal gut as we did not find male worms in this segment at day 14 p.c.. Therefore only the results of the female worms will be discussed. Figure 5 shows the mean worm lengths in the proximal and the distal gut of animals in G1, G2 and G3 at day 14 and 28 p.c..

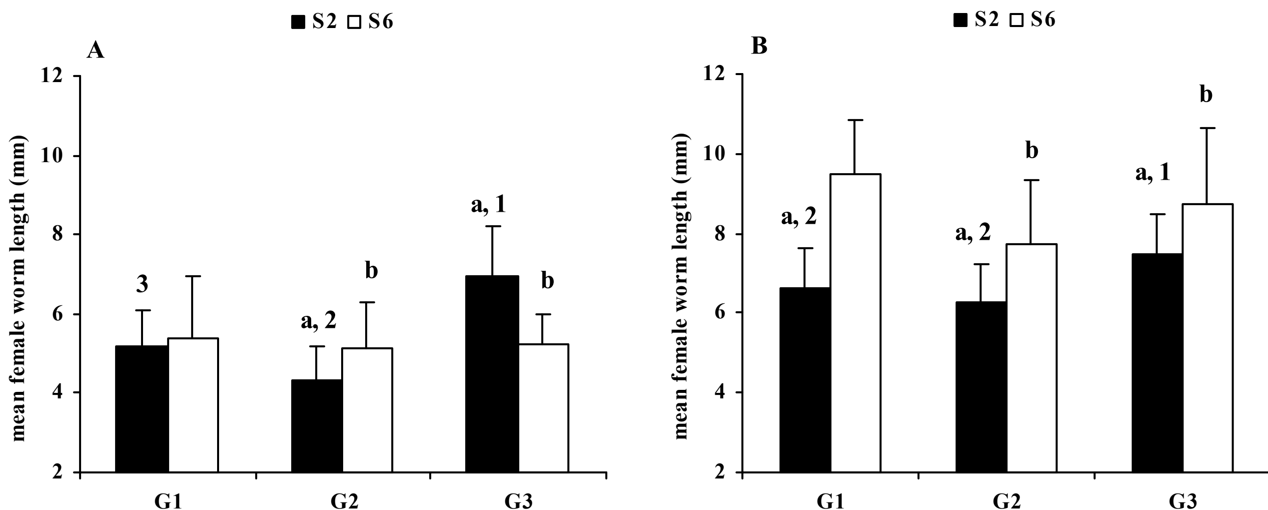

FIGURE 5 Mean length of female worms (+SEM) recovered from the proximal (S2) and the distal gut (S6) of animals of G1 (30,000 L3/100,000 L3), G2 (100,000 L3/100,000 L3) and G3 (0 L3/100,000 L3) at day 14 (A) and 28 p.c. (B). If present, 20 female worms per segment and per calf were measured. Different letters are used for significant differences within group but between intestinal segments. Different numbers indicate significant differences between groups but within intestinal segment. The confidence level was set at P<0.05.

At day 14 p.c. worms of the proximal gut were significantly shorter in the primed animals compared to the challenge control animals (P<0.001) and the worms of G2 were significantly shorter than G1 (P<0.01; fig. 5A). Comparison of the proximal and the distal gut revealed that at this time point worms of G3 were longer in the proximal gut than in the distal gut (P<0.001). The opposite was found for G2 (P<0.01) and the worm length of G1 did not differ between the proximal and the distal gut. At day 28 p.c. the worm length in the proximal gut of both G1 and G2 was reduced compared to G3, but the difference between G1 and G2 did not prevail. Independent of the primary infection dose, at this time point worms residing in the proximal gut were significantly shorter compared to worms recovered from the distal gut (P<0.001, fig. 5B).
An univariate analysis of variance was performed on the worm length of the females in order to investigate the influence of the time point (day 14 p.c., n= number of female

worms=39 versus day 28 p.c., n=184) and the infection dose (G1, n=143 versus G2, n=80). The variable number of worms analysed in each group was caused by the fact that worms were not always present in both segments in all animals. The overall analysis confirmed that the length of the worms was significantly shorter at day 14 p.c. compared to day 28 p.c. ($P<0.001$), which was expected since at day 14 p.c. the worms are not yet fully developed. Furthermore, independent of the time point, priming with 100,000 L3 larvae resulted in worms that were significantly shorter than after priming with 30,000 L3 larvae ($P<0.001$, mean worm length G1=8.047 mm versus G2=7.144 mm).
A similar analysis was performed on the number of eggs recovered from the same individual female worms. As expected an effect of the time point on the worm fecundity was found ($P<0.01$) as day 14 p.c. coincides with the onset of the egg output, while at day 28 p.c. the peak in egg excretion is reached. The fecundity of the worms of the different groups at day 28 p.c. is shown in fig. 6. The mean worm fecundity of the proximal gut was significantly lower in the primed (G1 and G2) animals than in the challenge control group ($P<0.05$). Comparison of the proximal and the distal gut within group revealed that in all groups the fecundity in S6 was significantly lower than in S2 ($P<0.05$ for all). In addition, comparison of the fecundity in S6 between G1, G2 and G3, revealed that the number of eggs per female worm was significantly lower in worms from G2 compared to G1 and G3 ($P<0.05$).

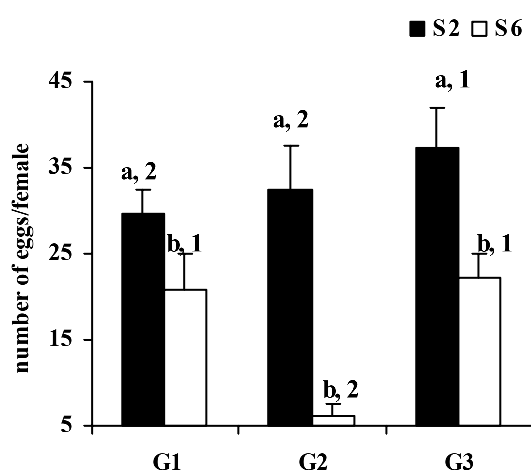

FIGURE 6 The mean number of eggs per female worm (+SEM) for G1 (30,000 L3/100,000 L3), G2 (100,000 L3/100,000 L3) and G3 (0 L3/100,000 L3) at day 28 p.c.. If present, the fecundity of 20 female worms per segment and per calf was measured. Different letters are used for significant differences within groups but between intestinal segment (S2 or S6). Different numbers are used for significant differences between groups but within intestinal segment. The confidence level was set at $P<0.05$.

Worm length and fecundity did not significantly differ in between responder types. But in the 2 low responders that were excreting eggs at day 28 p.c., worm length and fecundity were comparable to the challenge control animals and significantly higher compared to the other primed animals ($P<0.001$ for both). Figure 7 shows the relationship between worm

length and fecundity in the proximal and the distal gut of the primed animals. In both cases a significant positive correlation was found but the relationship was relatively weak.

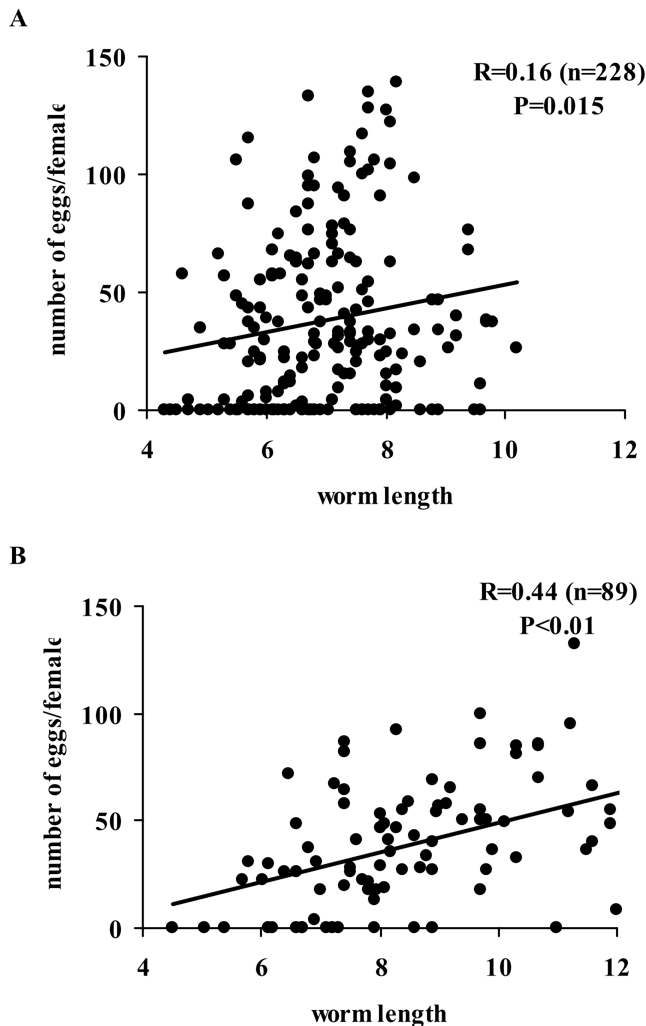

FIGURE 7 The correlation between worm length and number of eggs per individual female recovered from the proximal (S2, A) and the distal gut (S6, B) in the primed animals is given. The Pearson's correlation coefficient (R) and the number of worms analysed (n) are shown in the graph.

DISCUSSION

Immunity to gastrointestinal nematodes can be defined in different ways. From the epidemiological point of view a reduced egg output is important as this will diminish further contamination of the field. In the ideal situation the absence of egg output is concurrent with no worm burden. An intermediate outcome would be that the worm burden persists but nematode survival and fecundity are reduced.

This experiment was performed to get more knowledge about mechanisms involved in immunity against re-infection with *C. oncophora*, more specifically the amnestic immunity and the dose-dependency of the immunological processes involved. In this paper we report on the different effects of immunity on a range of parasitological variables. Moreover, we

investigated whether the more resistant phenotypes of high and intermediate responder animals compared to low responders, is sustained during a secondary infection. As we have to deal with the disadvantage that differentiation between responder animals can only be done after infection, a low number of animals within one group had to be analysed at certain time points.

Distinction of responder types with a single oral dose of 30,000 L3

A priori, it was expected that an infection with 30,000 L3 would induce less protective immunity than with 100,000 L3 and we expected a reduced ability to distinguish between the responder types with this lower dose. Priming with 100,000 L3 resulted in a significantly higher egg output at day 21 p.i. but the significant difference in egg excretion between both doses did not prevail after the peak. The differences between the infection doses in the current experiment resembled more what was described for the comparison of 50,000 and 100,000 L3 [84], than for comparison of 20,000 and 100,000 L3 [171]. However, a specific problem in all these experiments was the high variation between the animals, which cannot easily be overcome in an outbred population. An advantage of this variability lays in the occurrence of different responder types. The capacity to discriminate between low, intermediate and high responders after a single oral infection with 30,000 L3 suggested that the antigenic threshold required to provoke the development of acquired immunity in intermediate responder animals was reached with this infection dose. Analysis of the egg output in the responder animals revealed that independent of the infection dose, low responders were still excreting eggs at day 49 p.i., while intermediate and high responders were not.

Worm establishment is predominantly influenced by host responder type

Expulsion of adult nematodes in ruminants is a common manifestation of acquired immunity as a consequence of repeated infection and was reported for sheep infected with *Haemonchus contortus* [19, 16], *Teladorsagia circumcincta* [199], *Trichostrongylus colubriformis* [20] and calves after continuous larval exposure to *O. ostertagi* [160]. Alternatively, immunity can alter establishment by an effect on the larval stages. This can be by rapid expulsion of the incoming L3 in the first days after the challenge or by a delayed effect on L4 and L5 stages (reviewed in [117] and [192]). Depending on the host-parasite system resistance to adult and larval stages develops concurrently [199] or independently [160, 71] but expulsion of adult nematodes might also occur as a result of immune mechanisms triggered by larval stages [192]. The results of these studies indicate that the term “reduced establishment” can refer to both larval and adult stages and that the distinction is not always very clear. To avoid ambiguity we currently defined reduced establishment as the ability to establish as (juvenile) worms beyond the early L4 stage. In contrast to studies which demonstrated that reduced establishment and survival of worms depended on the density of the infection or the rate of

incoming larvae [6, 19, 20], the reduced establishment in the current study appeared to be particularly dependent on the responder type of the animals and not on the priming dose. The adult worm burden in the intermediate but not in the low responders was significantly reduced compared to the challenge control animals. Moreover, irrespective of the infection group, a low worm burden concurred with a relatively higher %L4. These findings indicated that animals which were more resistant to a primary infection remained more resistant after re-infection as indicated by reduced establishment. Nevertheless, the small reduction in worm burden of the low responders clearly suggested an effect of priming. Thus low responders showed an enhanced resistance as a result of the primary infection, but this occurred at a lower rate or with less efficiency than in intermediate responders.
A common mechanism of immunity is that, upon re-infection worms are redistributed proximal or distal from their normal niche [161]. Worm expulsion in the course of a primary infection with *C. oncophora* was characterized by a distal shift in the worm population at day 42 p.i. [127]. In the current experiment worms were recovered from the distal gut of low and intermediate responders as early as day 14 p.c., which denoted that expulsion was ongoing in these animals. In contrast, at this time point the establishment in challenge control animals was still around 100% and all worms resided in their predilection site, the proximal gut. At day 28 p.c. the proportion of worms recovered from the distal gut of the primed animals was lower than at day 14 p.c.. Interpretation of the results of the low responders is difficult as we had only one low responder at day 14 p.c. but, the altered distribution and the lower total worm burden in intermediate responders at day 28 p.c., suggested that a high proportion of worms was already expelled. Logically, the expelled worms were residing distally from their usual niche at day 14 p.c.. The accelerated expulsion of the adult worms in primed animals was further supported by the variation in sex ratio in the proximal and the distal gut of these animals.
In summary, the results discussed above demonstrated that the main effects of priming on worm establishment occurred irrespective of the dose, but differed between low and intermediate responders. Hence, we postulate that immune mechanisms associated with a reduced establishment are regulated by the host responder types and not by the infection dose when doses of 30,000 and 100,000 L3 are compared.
Worm length is predominantly influenced by priming infection dose
Whereas with the current model immunological processes influencing establishment seemed to be largely dose-independent, analysis of worm length revealed a significant effect of the priming dose but not of the host responder type. At day 14 p.c. a gradual decrease in worm length was observed when comparing worms of the proximal gut of challenge control animals with animals primed with 30,000 and 100,000 L3, suggesting that the growth rate of the worms (and hence their length) was influenced in a dose-dependent way.

Interestingly, at day 28 p.c. no differences in length were observed between both primed groups. This suggests that the effect of the priming dose relates more to the temporal kinetics of worm development than to the attainable adult worm length as such. This conclusion only applied for the proximal gut of the animals, as in the distal gut, worm length was always comparable between the challenge control animals and both primed groups.

Comparison of the proximal and the distal gut among primed and challenge control animals revealed interesting differences. Although in the early part of the infection only a few worms were recovered from the distal gut of the challenge control animals, we assume that the observation that worms in the proximal gut were longer than worms in the distal gut reflected the normal situation. By day 28 p.c. the situation was reversed in all infection groups, however, this was already observed in the animals primed with 100,000 L3 at day 14 p.c.. Although it remains uncertain whether such a distribution is mediated by the host's immune response, the seminal observations that the morphology of the worms was altered (longer but thinner, data not shown), underscores an immune mediated mechanism. It is likely that these worms were damaged by the detrimental immune environment of the proximal gut and on their way to be expelled. The observation that this occurred earlier in animals primed with 100,000 L3 is consistent with our previous hypothesis i.e. the effect of the priming dose relates more to the temporal kinetics of worm survival than to the attainable adult worm length/morphology as such.

Is there a link among effects on worm length and fecundity in C. oncophora *infected calves?*

There have been extensive studies on worm length and fecundity of *T. circumcincta* in sheep [209, 212]. In these studies worm length and fecundity were very well correlated and particularly worm length was affected by acquired immunity in a density-dependent manner [210]. In contrast, our results indicate that in case of *C. oncophora* worm length and fecundity are differentially affected by host responses. Though priming caused a reduced fecundity, there was no effect of the priming dose at day 14 p.c. whereas length was affected dose-dependently. At day 28 p.c. no significant differences in length were observed between both primed groups nor with the challenge control groups, whereas now fecundity of the distally located worms was strongly affected by the priming dose. Consequently, worm length and fecundity were weakly correlated. The reasons for the differences between our findings and the observation in *T. circumcincta* infected sheep are not very clear but can be addressed with the following questions: Does immunity to *C. oncophora* influences both worm length and worm fecundity? And, if yes, is the effect on both parameters comparable (both in kinetics and in strength)? A positive answer to the first question is supported by evidence found earlier for *C. oncophora* [84] and other GI nematodes (reviewed in [161] and [15]). Based on our current observation e.g. longer worms with low fecundity were found in the distal gut

compared to shorter worms with high fecundity in the proximal gut, the answer to the second question would be negative; i.e. we propose that at least part of the mechanisms involved in the regulation of worm length and worm fecundity differ, and consequently also their effect.
The fecundity of the worms of the primed animals was surprisingly high given the fact that only two animals excreted eggs in their faeces. Beside factors such as an unaltered sexual behaviour, spermatogenesis and oögenesis, faecal egg output also requires a functional egg laying machinery of the worms. This merely depends on contractions of uterine and somatic muscles which make the eggs go down the uterus [26]. The lack of relationship between egg output and worm fecundity in the primed animals suggest that, in contrast to our observations in primary infected animals [127] during the development of resistance to re-infection to *C. oncophora* these mechanisms may be influenced independently.
Conclusions
The data presented here demonstrate that the memory responses to *C. oncophora* consist of both dose-dependent and dose-independent immunological processes and that different components of parasite fitness are affected differently by prior exposure. In the current model, reduction in establishment in primed animals occurred in a dose-independent way, yet, abrogation of development resulting in a stunted growth and reduced fecundity seemed to be dose-dependent. These observations seem contradictory to a recent report which described dose-dependent effects of prior exposure to *Strongyloides ratti* on worm survival and establishment [177]. The level of prior exposure quantitatively reduced the establishment after subsequent challenge, but the authors did not find an effect of protective immunity on the fecundity or length of *S. ratti*. Similar observations were done following infection with *Ostertagia* spp. in calves [183]. But as the dose-dependency in the current experiment mainly referred to the kinetics of an effect on worm length and fecundity, the different conclusions among the experiments might result from the analysis of variable time points after infection or from variable kinetics inherent to each specific host-parasite system. Similarly, we currently only compared infections with 30,000 or 100,000 L3, and we do not exclude a different outcome with more, or less divergent dose levels.
Finally, the dose-independent effects seemed to be regulated by the host's responder types. Based on the differences in worm establishment and distribution it is clear that the ability of intermediate responder animals to mount a more effective and faster immune response as compared to low responders is sustained after a secondary infection. A better distinction and understanding of the mechanisms which are regulated by an effect of the dose and the mechanisms which depend on the responder types, and likely the genetic background of the animals, will contribute to improved preventive control strategies.

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Chapter 5

B cells and antibodies differentially influence immunity to *Cooperia oncophora* depending on priming dose level and host responder type

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The most exciting phrase to hear in science, the one that heralds new discoveries,
is not "Eureka" but " That's funny…."
(I. Asimov)

ABSTRACT

We investigated whether the generation of protective memory humoral immunity in *Cooperia oncophora* infected calves occurs in a dose-dependent way and whether it depends on the animal responder types. To this end, serum and mucus antibody responses were measured in animals primary infected with 30,000 or 100,000 L3, treated with anthelmintics and subsequently challenged with 100,000 L3. A detailed phenotypic and functional analysis of B cells was done in animals infected once or twice with 100,000 L3. Based on the similarity in parasitological variables of animals primed with 30,000 or 100,000 L3, we concluded that with these doses priming conferred protection in a dose-independent way. Upon challenge significant increases in *Cooperia*-specific serum and mucus IgG1 and IgA and total serum IgE titres were induced in primed animals in a dose-independent way. In contrast, intermediate and low responders differed in the onset of the production of *Cooperia*-specific serum IgG1. Furthermore, not only the onset but also the level of total serum IgE significantly differed between intermediate and low responders. Phenotypic and functional analysis of B lymphocytes made us conclude that i) priming induced the generation of memory B cells which upon challenge readily differentiated into antibody secreting cells ii) sensitized B cells were more efficiently recruited to the intestinal effector sites iii) based on the expression of CD62L and CD86 two distinct B cell subpopulation could be differentiated. CD62L+CD86- B cells that were likely not yet activated lymphocytes with an enhanced recirculation capacity, and CD62L-CD86+ B cells that were activated B cells with a reduced recirculation ability and finally, iv) the increased expression of CD86 and subsequent correlations with parameters of the type 2 immune response induced by *C. oncophora*, suggested for the first time that CD86-interactions are involved in the generation of protective immunity against *Cooperia.*

INTRODUCTION

Cooperia oncophora is the most prevalent small intestinal nematode of cattle in Western Europe. Natural infections are mostly subclinical. However, the economic importance remains high and a negative effect on the productivity of first and second year calves has been reported [179, 184]. The serological response to *C. oncophora* has been the subject of many studies in which animals were primary infected with 100,000 L3. These experiments revealed that infection preferentially induced the development of *C. oncophora*-specific IgG1 and IgA [176, 171, 231, 127]. Although the dichotomy in Th1 and Th2 responses is not as clear cut in cattle as in mice, it has been shown that IgG1 and IgA are associated with a type 2 response, as opposed to IgG2 and IgM which are associated with a type 1 response (reviewed in [36]).

Following experimental infection with *C. oncophora* animals can be subdivided in different responder types based on the speed by which the worms are expelled from the host [222, 123]. Intermediate responders develop an effective immune response resulting in a decrease in egg output concurrent with worm expulsion around day 42 after infection (p.i.). At this time point in low responders the egg excretion is still ongoing and worm burdens at necropsy are high. High responders seem to withstand infection with *C. oncophora* and show very low or no egg excretion with low worm burden at necropsy. Recently, we demonstrated that in addition to a distinct parasitological phenotype, the different responder types also develop a distinct humoral response during primary infection [127]. The increase in *C. oncophora*-specific IgG1 and IgA in the serum and mucus was significantly higher in intermediate responders compared to low responders and could be correlated to parasitological parameters that are indicative of an ongoing worm expulsion [127, 126]. In the same experiment, we showed that the peak in antibody titres was preceded by a significant increase in the frequency of B cells and MHCII+ cells in the draining lymph nodes. These findings highlighted the putative role of the antibody response and B cells in the development of acquired immunity against *Cooperia*. However, to get an insight in the contribution of the humoral response in protection against infection, it is important to study the B cell and antibody response in animals during re-infection.

The main contribution of B cells to adaptive immunity is the production of antibodies in response to infection, but B cells also have an important role as antigen presenting cells (APC) in the development of CD4 responses [42, 190]. Stimulation of T cells occurs via B7-1 (CD80) and B7-2 (CD86) on B cells and other APC's interacting with CD28 on the surface of T lymphocytes and results in subsequent maturation of naïve CD4+ cells into Th2 IL-4 producing cells [147]. The CD28 homologue, CTLA-4 (CD152), also interacts with B7 molecules but signaling through CTLA-4 can provide a negative signal that down-regulates T cell activation [240, 220]. The influence of B7-interactions on the development of a primary or

a secondary immune response has been investigated extensively in mice infected with the intestinal nematode *Heligmosomoides polygyrus* [92, 102, 103, 62]. Although one has to be careful in extrapolating from rodents to ruminants, the in vivo type 2 response induced after infection of mice with *H. polygyrus* resembles the response as seen in *Cooperia* infected calves. Both nematodes have a solely enteric life cycle, and common features of infection are eosinophilia, increase in CD4+ lymphocytes, B cell proliferation and differentiation that results in a marked increase of serum IgG1 and IgE [221, 25, 127, 126]. The studies with *H. polygyrus* showed that B7-1/B7-2 interactions are required for the generation of an effective primary immune response against *H. polygyrus,* but not for the generation of protective memory response [103, 62]. Until now, the role of these interactions in type 2 responses in ruminants has not been investigated but the similarity in pathogenesis of *H. polygyrus* infection in mice and *C. oncophora* infection in calves leads to the hypothesis that B7-interactions might contribute to development of immunity against *Cooperia*. The availability of cross-reacting monoclonal antibodies gave us the opportunity to perform studies on the role of CD86 expression on B cells in *Cooperia* infected animals. In addition, the recirculation ability of B lymphocytes was investigated by comparing the CD62L expression on B cells of primed and challenge control animals. In cattle CD62L expression cannot be used to differentiate between memory and naïve lymphocytes [115] but it is useful to examine the main function of CD62L, i.e. facilitation of lymphocyte recirculation between blood and lymph nodes as part of immune surveillance function.

The current experiment was designed with two objectives. First, we investigated the involvement of *Cooperia*-specific antibodies in protection against re-infection. The dose-dependency of the generation of protective antibodies was evaluated by comparing the antibody titres of animals primed with a low (30,000 L3) or a high (100,000 L3) infection dose with challenge control animals. We further investigated whether the ability of intermediate responders to develop a more effective humoral response than low responders, was sustained after challenge. Secondly, we studied the activation state and effector function of B cells. For this we focused on the animals that were infected once or twice with 100,000 L3. The occurrence of *Cooperia*-specific antibody secreting cells and the expression of CD86 and CD62L on B cells isolated from the mesenterial lymph nodes, the Peyer's patches and peripheral blood were parameters used to evaluate the recirculation capacity, activation state, and effector function of B lymphocytes in calves primary or secondary infected with *C. oncophora*.

MATERIALS AND METHODS

Animal experiments

Thirty-four female calves were raised under helminth-free conditions on a commercial farm and purchased at 3 months of age. The animals were housed at the animal facility of the Faculty of Veterinary Medicine in Utrecht. They were kept indoors per infection group and fed hay and water ad lib, as well as concentrates at a maximum of 500 g per day. Prior to infection animals were checked for general health condition and faeces were collected to confirm the worm free status of the animals. A detailed scheme of the infection protocol is given in table 1. Seven weeks after the primary infection all animals were drenched with oxfendazole (Systamex 2.65 % suspension, 2 ml/10 kg, Scheringh Plough, Kenilworth, New Jersey, USA). For dose-independent results, animals from G1 and G2 were pooled and designated as primed (P) animals and compared to the challenge control animals (G3) and the non infected control animals (GC). All animals were around 6 months of age at the time of challenge. All experimental procedures were approved by the ethical committee on animal experimentation of the Utrecht University.

TABLE 1 Experimental design

		Group (number of calves)			
	day	G1 (n=11)	G2 (n=11)	G3 (n=6)	GC (n=6)
primary infection	0 d.p.i.	30,000 L3	100,000 L3	none	none
anthelmintic drench	49 d.p.i.	yes	yes	yes	yes
challenge infection	126 d.p.i.	100,000 L3	100,000 L3	100,000 L3	none
necropsy	0 d.p.c.	n=4*	n=4*		n=2
	14 d.p.c.	n=3	n=3	n=3	n=2
	28 d.p.c.	n=4	n=4	n=3	n=2

(n=number of calves; d.p.i.=days post infection; d.p.c.= days post challenge; * those animals were not challenged)

Parasitology

The numbers of eggs per gram faeces (EPG) were determined with a modified McMaster technique (with a sensitivity of 50 EPG). Faeces were analysed once a week from day 0 to day 14 p.i. and during the period between treatment and secondary infection. In the remaining period, faeces samples were collected and analysed every two days. After slaughter, the small intestine was subdivided in six different segments which were all analysed separately [127]. Briefly, the first segment was the first meter starting from the pylorus and each following segment was five meters. Results are shown from the second (S2, proximal gut, jejunum) and the last (S6, distal gut, ileum) segment. Intestinal washings were done according to Eysker and Kooyman [74] and 2% aliquots of the intestinal washings were examined for worms. Parasitological variables used to asses host responsiveness were: worm fecundity, worm length and percentage protection. The fecundity of the female worms was assessed by individual counts of the number of eggs recovered from 20 female worms from each segment [127, 126]. Worm length was assessed by measuring the length of 20 individual worms per segment and mean values were used in the analysis. Since female and male

worm lengths were positively correlated ($R=0.81$, $n=9$, $P<0.01$ with n= number of animals from which both male and female worms were recovered) only female worm lengths were used in the analysis. Percentage protection was defined as: 100-(worm burden*100/mean worm burden in challenge control animals).

Serum samples and mucus isolation
Serum samples were taken from the jugular vein twice a week during the primary infection and after challenge. In the period between treatment and challenge infection, samples were taken only once a week. After centrifugation the serum was kept at -20ºC until use in ELISA. Mucus was isolated from the proximal gut (S2) and the distal gut (S6) as described previously [126]. Protein concentrations were determined with the Bradford assay and samples were kept at -20ºC until use in ELISA. Mucus samples were used at a protein concentration of 1.25 mg/ml in the *Cooperia*-specific ELISA and of 2.5 mg/ml in the total IgE ELISA.

***Cooperia*-specific ELISA**
The *Cooperia*-specific ELISA was performed as described before[127] with some minor modifications. *Cooperia* adult crude worm antigen (Ad) was used at a concentration of 5 µg/ml. Sera were used at a dilution of 1/1,000, and mucus samples at a concentration of 1.25 mg/ml for all ELISA. For the *Cooperia*-specific IgG1 titres after challenge a serial dilution was performed as all sera reached a plateau level with a 1/1,000 dilution. An optimal dilution of 1/20,000 was chosen for those data points. Sheep anti bovine IgA-HRP, sheep anti bovine IgG1-HRP or Sheep anti bovine IgG2-HRP (Serotec, United Kingdom) were used as conjugate. After 1 hour incubation at 37ºC, binding of the conjugate was evaluated by adding the chromogen substrate SURE BLUE (KPL, Maryland, USA). All sera and mucus samples were tested in duplicate and on all plates two standard positive and two standard negative sera were included. Absorbance was measured at 450 nm and results are expressed as mean (+SEM) of the duplicate (OD-blank) values. The blank is the average OD of the wells with no serum incubation.

14.2 kDa ELISA
The 14.2 kDa ELISA was performed as described by Poot et al. [185]. This *Cooperia* ELISA is a tool for estimating exposure levels to *Cooperia* in cattle and was used in this experiment to evaluate the difference between a single infection dose of 30,000 L3 and 100,000 L3. All sera were tested in duplicate and on all plates one standard positive and one standard negative serum were included. Optical densities were recalculated to a percentage of the OD value of the positive reference serum (PROD). PROD = mean (OD sample-OD blank)/mean (OD standard positive serum-OD blank). The blank is the average OD of the wells with no serum incubation.

Total IgE ELISA
The total IgE ELISA was performed as described previously [133] and only the effect of the challenge infection on total IgE titres was analysed. Sera were used at a dilution of 1/5 and mucus was used at a protein concentration of 2.5 mg/ml. The OD values of the test sera and mucus samples were transformed by to arbitrary units IgE (U) on a linear scale [137]. The value of the blank (no serum incubation) was set at 0 U and the OD-blank value of the undiluted standard positive serum was set at 1 U IgE. Results were expressed as mU/ml.

Isolation of lymphocytes
No challenge control animals infected with 30,000 L3 were included in the experiment and therefore only animals infected once (G3) or twice with 100,000 L3 (G2) were used in the

phenotypic and functional analysis of B lymphocytes. Lymphocytes were isolated from blood (PBL), mesenterial lymph nodes from the proximal (LN S2) and the distal gut (LN S6) and from the lamina propria of the proximal (LP S2) and distal gut (LP S6) as described previously [126]. Samples were taken from the beginning of the intestinal segments. Lymphocytes from Peyer's patches from the proximal (PP S2) and the distal gut (PP S6) were isolated based on the method described by Sopp and Howard [208]. Based on previous observations [126] the analysis was restricted to animals slaughtered at day 28 p.c.. Cells were used at a concentration of $1*10^7$cells/ml for the flow cytometric analysis. A total of 100 µl of cell suspension was used to stain the lymphocytes. For the ELISPOT assay cells were resuspended in complete medium (RPMI supplemented with penicillin/streptomycin, 10% FCS, 2 mM glutamine and 2 mM β-mercaptoethanol) and used at a concentration of $5*10^6$ cells/ml.

Flow cytometry

The following MoAbs were used to stain the lymphocytes: mouse-anti human CD86-FITC (clone 2331 (Fun-1), BD Pharmingen) (chart BD pharmingen, cross reacting antibodies) and mouse-anti human CD62L-PE (DREG 56, BD Pharmingen) [237] both cross reacting with the bovine molecule. Bovine B cells were stained with MoAb IL-A59 specific for immunoglobulin light chains [242], either labelled with biotin or PE. The labelling with biotin (D-biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester, ROCHE) was done according to the manufacturer's instructions. The labelling with PE was done by Serotec (UK). MHC class II (MHCII) expression was measured with the MoAb IL-A21 [12] labelled with biotin. For the biotin labelled antibodies, the second incubation was done with Streptavidin-APC (BD Pharmingen). After washing, cells were fixed with 1% paraformaldehyde and analysed by flow cytometry using a FACS-CALIBUR (Becton Dickinson). A minimum of 10,000 cells were analysed within a gate for lymphocytes based on the forward and the side scatter pattern of the cells. Results are presented as mean %positive cells (+SEM) within the gate for lymphocytes for expression of IL-A59+ and MHCII+ cells. Analysis of CD62L and CD86 was performed by gating on the IL-A59+ population and results are expressed as mean% + cells (+SEM) within the IL-A59+ gate. The %B cells in the lamina propria was estimated by subtraction of the %CD4+ cells (anti-bovine CD4-FITC (Serotec, UK)), %CD8+ cells (clone CC30, ECCAC), and %TCR1-N24+ cells (clone GB21A, VMRD, USA) from the total lymphocyte population (=100%).

ELISPOT assay

The ELISPOT assay for the detection of *C. oncophora*-specific antibody secreting cells (ASC) was performed using Multiscreen ™ 96 well microtiter plates (Millipore Corporation, Badford, USA). Plates were coated overnight with 20 µg/ml *Cooperia* Ad [127] or 5 µg/ml *Cooperia* 14.2 kDa recombinant protein [185] using 50 µl of antigen per well. All subsequent incubations were performed with 100 µl per well at 37ºC for 1 ½ hour, except if stated differently, and after each incubation step plates were washed manually.

After overnight incubation plates were washed three times with PBS, and wells were blocked for 1 hour at 37ºC with 100 µl complete medium. After blocking, plates were washed and 100 µl of lymphocyte suspension was added to each well. Plates were incubated overnight at 37ºC in a CO_2 incubator. Following incubation, the cell suspension was removed and plates were washed 5 times with PBS supplemented with 0.05% Tween (PBS-T). Mouse anti-bovine IgA (Serotec, clone MCA 650) at a dilution of 1/500, or mouse anti-bovine IgG1 (Serotec, clone MCA 627) at a dilution of 1/1,000 were applied in each well except the control wells; all dilutions were done in PBS-T. In the next step, goat anti-mouse Ig coupled to AP (DAKO, Danmark) diluted 1/2,000 in PBS-T was applied to the plates. ASC were detected after washing and subsequent incubation with the substrate

solution BCIP/NBT. Staining was done for 1 hour at room temperature. The substrate solution was removed and plates were left to dry until analysis of the spots. Spots were counted in each well, and results are given as mean of two duplicate wells after subtractions of background spots (wells with all reagents except the MoAb). Results are expressed as mean number of ASC/10^6cells (+SEM).

Statistics

Statistical analysis was done using SPSS statistical package (version 10.0). Normally distributed data were analysed with ANOVA. Data that were not normally distributed were analysed with the non-parametric Mann-Whitney test for 2 independent samples and the non parametric Kruskal-Wallis test for more independent samples. Pair-wise comparison of 3 or more independent samples was done by post-hoc analysis as advised for Kruskal-Wallis. Correlations between parameters were measured with the Pearson's correlation coefficient for linear correlation on normally distributed data and with the Spearman's rank correlation coefficient for data that were not normally distributed. The confidence level was set at P<0.05.

RESULTS

Parasitology

Egg excretion was only found at day 28 post challenge (p.c.) and occurred in all 3 challenge control animals, but only in 1 animal of G1 and 1 animal of G2 (fig. 1).

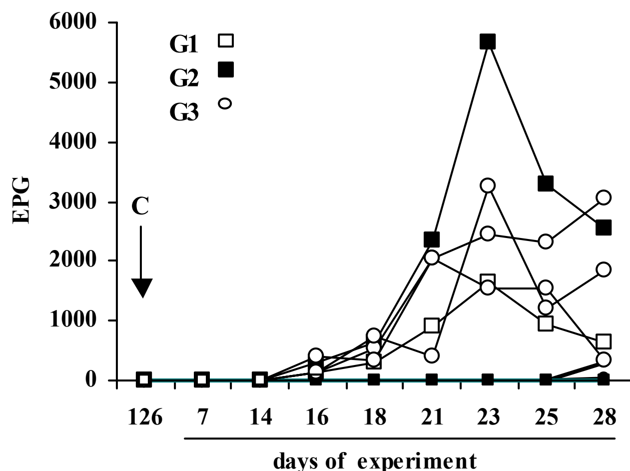

FIGURE 1 The individual EPG (mean number of eggs/gram faeces) of the animals slaughtered at day 28 p.c.. The time of challenge (C) is indicated by the arrow. Animals from G1 (30,000 L3/100,000 L3; open squares, n=4), G2 (100,000 L3/100,000 L3, closed squares, n=4) and G3 (0 L3/100,000 L3; open circles, n=3) are shown. Underlining on the X-axis indicates the days after challenge.

A significantly reduced worm burden was found in animals of G1 and G2 compared to the challenge control animals (G3) (P<0.05 for both at day 14 and day 28 p.c., fig. 2A). We measured no significant effect of the priming dose on worm burden, neither between the 2 necropsy times. Figure 2B shows that the reduced worm burden in the animals from G1 and G2 was mainly caused by a significantly reduced number of worms in intermediate responders compared to the naïve control animals (G3) (P<0.05 at day 14 and day 28 p.c.)

and at day 28 p.c. the worm burden in intermediate responders was also significantly lower than in the low responders (P<0.05).

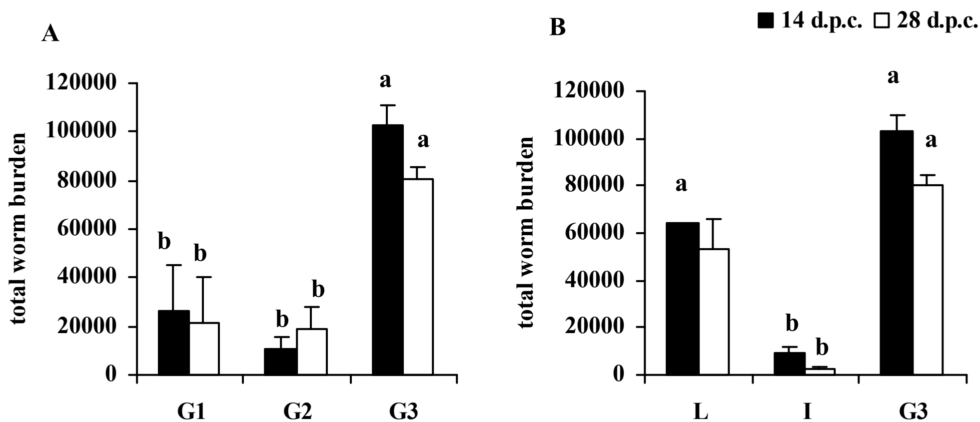

FIGURE 2 (A) Worm burdens of the animals of G1 (30,000 L3/100,000 L3), G2 (100,000 L3/100,000 L3) and G3 (0 L3/100,000 L3) at day 14 and day 28 p.c.. (B) Worm burden of low (L, n=1 at day 14 p.c. and n=4 at day 28 p.c.) and intermediate (I, n=3 at day 14 p.c. and n=4 at day 28 p.c.) responders are compared with G3. Data are presented as mean number of worms (+SEM). Different letters indicate significant differences between groups within a time point (P<0.05).

Cooperia 14.2 kDa-specific serum IgG responses

The 14.2 kDa is a sensitive tool for monitoring infections levels within a herd. We currently used this assay to investigate whether it can be applied to discriminate between experimental infections with 30,000 and 100,000 L3 C. *oncophora*. The 14.2 kDa titres did not differ between G1 and G2 during the primary infection (fig. 3A).
Following anthelmintic treatment at day 49 p.i. the antibody levels were maintained for 3 more weeks, but from day 70 p.i. onwards a gradual decrease in 14.2 kDa-specific IgG antibodies was observed. At the time of challenge, antibody titres in G1 and G2 were close to control levels. Challenge infection with 100,000 L3 larvae induced a significant increase in 14.2 kDa specific-IgG, irrespective of the priming infection dose. In both primed groups the antibody response after challenge was significantly higher than during the course of the primary infection (P<0.05), and also higher as compared to both control groups (P<0.01).

Cooperia-specific serum Ig responses

The dose-dependency and protective role of the serological response to *C. oncophora* was investigated before and after challenge using isotype specific ELISA's with *C. oncophora* adult crude worm (Ad) antigen. A primary infection dose of 100,000 L3 induced slightly higher Ad-specific IgG1 titres than a dose of 30,000 L3, though the differences were not

significant. The pattern of the Ad-specific serum IgG1 titres throughout the primary infection was comparable with the pattern of the 14.2 kDa IgG antibodies (data not shown).

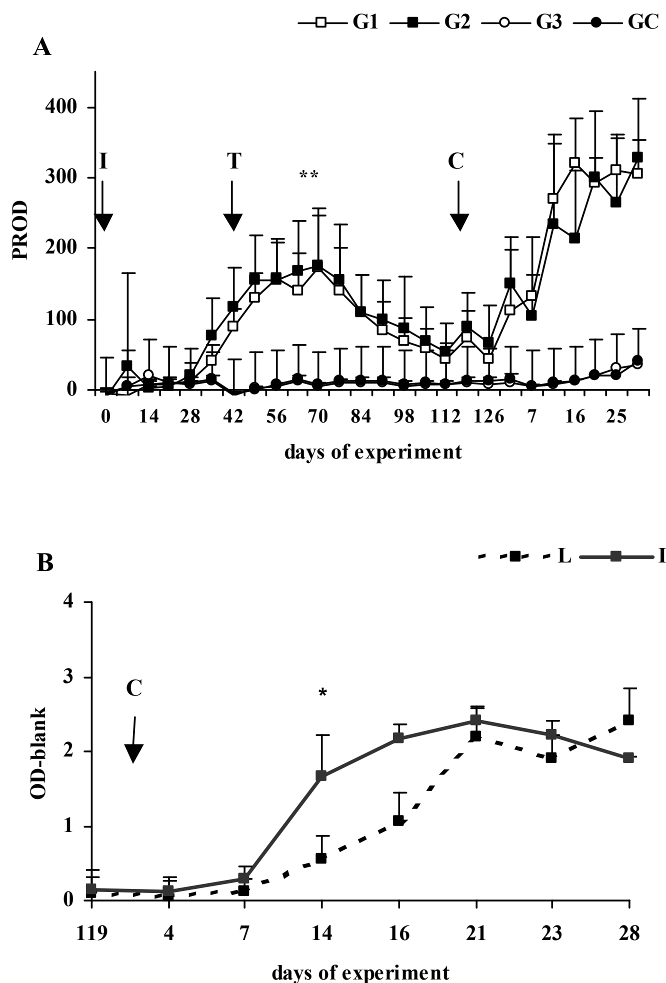

FIGURE 3 (A) The *Cooperia* 14.2 kDa IgG titres in the course of the primary and the secondary infection is given for the animals from G1 (30,000 L3/100,000 L3), G2 (100,000 L3/100,000 L3), G3 (0 L3/100,000 L3) and the non-infected control group (GC). Data are presented as mean PROD (+SEM). Time of primary infection (I), treatment (T) and challenge (C) are indicated. (B) The Ad-specific IgG1 response after challenge is given for the low (L) and intermediate (I) responders. Results are expressed as OD-blank (no serum incubation) and data are presented as mean values (+SEM). Underlining on the X-axis indicates the days after challenge. Significant differences are marked with * $P<0.05$ or ** $P<0.01$.

Challenge infection induced significantly higher antibody titres than the primary infection ($P<0.05$) and the IgG1 levels reached a plateau in our assay. A difference between individual sera after challenge was only detected with a serum dilution of 1/20,000. The onset and peak of Ad-specific IgG1 titres after challenge did not differ between the low and the high priming dose, but analysis of the different responder animals revealed a faster increase in the intermediate responders than in low responders and this resulted in significantly higher IgG1 titres in intermediate responders at day 14 p.c. ($P<0.05$, fig. 3B). At day 28 p.c. both responder types had similar Ad-specific IgG1 levels. We measured no significant correlations between serum IgG1 titres and parasitological variables.

Ad-specific IgG2 titres were not found in the serum of the animals during the whole experiment (data not shown). Both priming doses induced a minor but not significant

increase in Ad-specific IgA in the serum, which confirmed our previous observation [127]. Similarly after challenge, a small booster response was observed in primed animals compared to the challenge controls. Analysis of the Ad-specific IgA titres revealed a high individual variation after challenge and no significant differences were measured between the low and high priming dose and between the different responder animals (data not shown).

Total serum IgE levels after challenge

The correlation between protection and total or parasite specific serum IgE has been described in several nematode infections including *Dictyocaulus viviparus* [137] infection in calves and *H. contortus* infection in sheep [136]. To investigate whether a similar correlation existed in animals primed with *C. oncophora* we measured the total serum IgE levels in the animals upon challenge. Primed animals had significantly higher total serum IgE levels compared to challenge and non infected control animals (P<0.05) but this occurred irrespective of the priming dose. Analysis of the different responder animals showed a marked increase in total serum IgE in the intermediate responders compared to the low responders and both control groups (fig. 4).

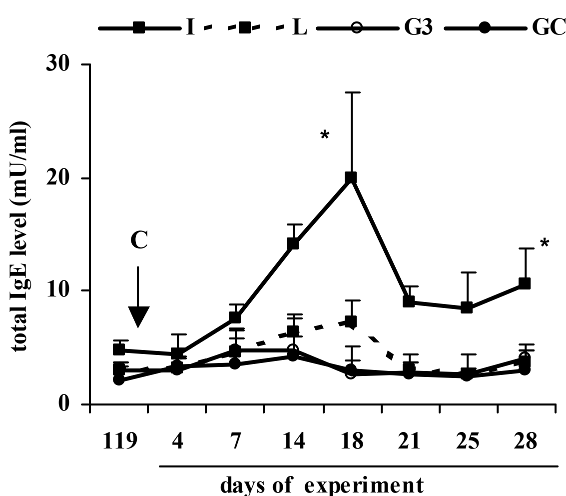

FIGURE 4 Total serum IgE titres after challenge of low (L), intermediate (I), challenge control animals (G3) and non-infected controls (GC) are shown. Time of challenge (C) is indicated. Data are presented as mean values (+SEM) of the animals per group. Underlining on the X-axis indicates the days after challenge. Significant differences are marked with * P<0.05 and ** P<0.01.

This increase was induced early after challenge and reached a peak at day 18 p.c. (P<0.05). The serum total IgE dropped from day 18 p.c. to day 21 p.c. but remained significantly higher in intermediate responders than in low responders and both control groups at day 28 p.c. (P<0.05). We further analysed whether a correlation between protection and total serum IgE levels existed in animals primed with *C. oncophora* and the results indicated that in the primed animals a maximum of 60% of the observed variation in protection could be explained by the differences in total serum IgE levels (data not shown).

Cooperia-specific mucus Ig responses

Challenge infection with 100,000 L3 larvae induced a booster in *Cooperia* Ad-specific mucus IgA and IgG1 in the primed animals compared to both control groups. We observed no significant differences between a low or high priming dose, neither between different responder animals (data not shown). Interestingly, the dynamics of the Ad-specific mucus antibody response differed between the different isotypes and between the proximal and the distal gut. Figure 5 shows the Ad-specific mucus IgA response for the primed (G1+G2) and control animals.

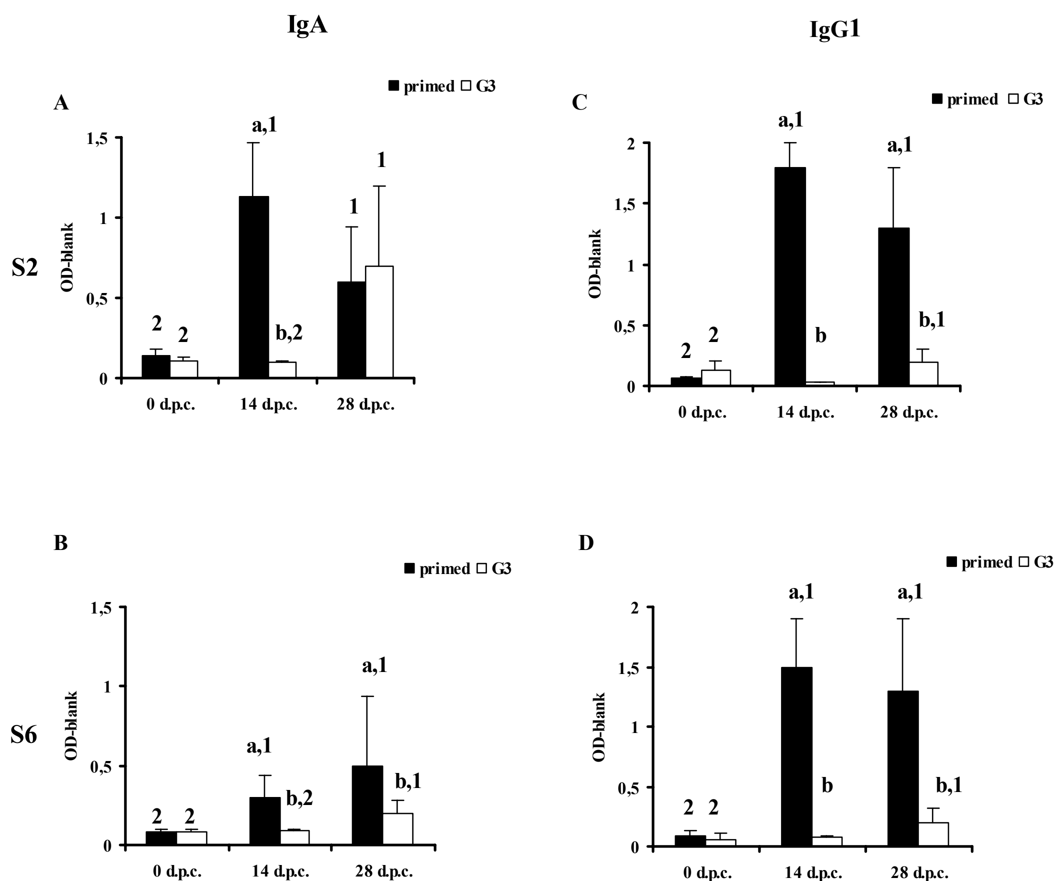

FIGURE 5 Ad-specific mucus IgA (A, B) and IgG1 (C, D) in the proximal (A, C) and the distal (B, D) gut are given for the primed (G1+G2) and the challenge control animals (G3). Results are expressed as OD-blank (no serum incubation) and for each animal the mean of duplicate wells was used. Data are presented as mean values (+SEM) of the animals per group (primed animals: n=8 at day 0 p.c., 6 at day 14 p.c. and 8 at day 28 p.c., G3: n=2 at day 0 p.c. and n=3 at day 14 and day 28 p.c.). Different letters indicate significant differences (P<0.05) between groups within time point. Different numbers indicate significant differences (P<0.05) within group between the different time points.

At day 14 p.c. a 5-fold increase in mucus Ad-specific IgA was observed in the proximal gut of the primed animals compared to G3 ($P<0.05$) and the non infected control group (day 0 p.c., $P<0.01$). At day 28 p.c. the mucus IgA response in the proximal gut of the primed animals dropped but was still significantly higher than the non infected control group ($P<0.01$). At this time point the mucus IgA titres did not differ between primed and challenge control animals. Figure 5B illustrates that the increase in Ad-specific mucus IgA in the distal gut of primed animals was not as pronounced as in the proximal gut. However, at day 14 and day 28 p.c. Ad-specific IgA titres were significantly higher in primed animals than in G3. The continuous increase in Ad-specific IgA titres in the distal gut suggested a different kinetic compared to the proximal gut. Thus, whereas primed and challenge control animals significantly differed in their kinetics of the Ad-specific mucus IgA titres in the proximal gut, in the distal gut both groups showed a similar pattern.
The kinetics of Ad-specific IgG1 in the primed and challenge control animals did not differ between the proximal and the distal gut (fig. 5C and 5D). At day 14 p.c. a significant increase in Ad-specific IgG1 titres in the primed animals was detected in both segments ($P<0.01$), and this was followed by a slight decrease at day 28 p.c.. In G3 Ad-specific mucus IgG1 increased from day 0 to day 28 p.c.. Independent of the time point or the segment, the Ad-specific mucus IgG1 titres were higher in the primed animals as compared to G3 ($P<0.01$). Throughout the infection we were not able to detect Ad-specific IgG2 in the mucus of infected animals.
Total IgE titres in the mucus of the proximal and the distal gut were low (range: 0.4-4.9 mU)) and the mucus total IgE levels of the proximal but not the distal gut were significantly correlated to the total serum IgE levels ($R=0.88$, $P<0.01$).
To get an insight in the role of the induced mucus antibody response after challenge, antibody titres were correlated with parasitological variables that reflect host responsiveness. At day 14 p.c. a significant positive correlation was measured between Ad-specific mucus IgG1 titres and the worm burden ($R=0.79$, $P<0.05$). To assess whether mucus IgG1 would be involved in immunological induced processes other than expulsion, we measured the correlation between the Ad-specific mucus IgG1 and worm length. The reduction in worm length of male and female worms separately, was negatively correlated with Ad-specific mucus IgG1 titres in the primed animals at day 28 p.c ($R=-0.94$ and $R=-0.67$ respectively, $P<0.01$) but not at day 14 p.c.. No significant correlations were found between Ad-specific mucus IgA titres or mucus total IgE titres and parasitological parameters.

Cooperia-specific ASC in the different anatomical locations

The number of *Cooperia*-specific ASC in lymph nodes (LN S2 and LN S6), Peyer's patches (PP S2 and PP S6) and peripheral blood (PBL) were counted in the animals infected once or twice with 100,000 L3 and slaughtered at day 28 p.c.. The results of Ad and 14.2 kDa-specific ASC were comparable although fewer Ad-specific cells were found (data not shown). The 14.2 kDa-specific ASC are shown in fig. 6. We observed a marked increase in the number of 14.2 kDa-specific IgG1 secreting cells in LN S2 and LN S6 of G2 ($P=0.05$ for LN S2 and $P<0.1$ for LN S6) and G3 ($P<0.05$ for both) compared to GC (fig. 6A). In the Peyer's patches the number of 14.2 kDa-specific IgG1 secreting cells remained constant in all groups whereas an increased number of 14.2 kDa-specific IgG1 secreting cells was found in PBL of G2 ($P<0.05$). Alterations in the numbers of 14.2 kDa-specific IgA secreting cells as compared to GC were observed in all anatomical locations of G2 and G3, with one major exception in PBL of G3. The number of 14.2 kDa-specific IgA secreting cells was significantly elevated in LN S2 ($P<0.1$ for G2 and $P<0.05$ for G3), LN S6 ($P<0.05$ for G2 and G3), PP S2 ($P<0.05$ for G3), PP S6 ($P<0.1$ for G2) and PBL ($P<0.1$ for G2).

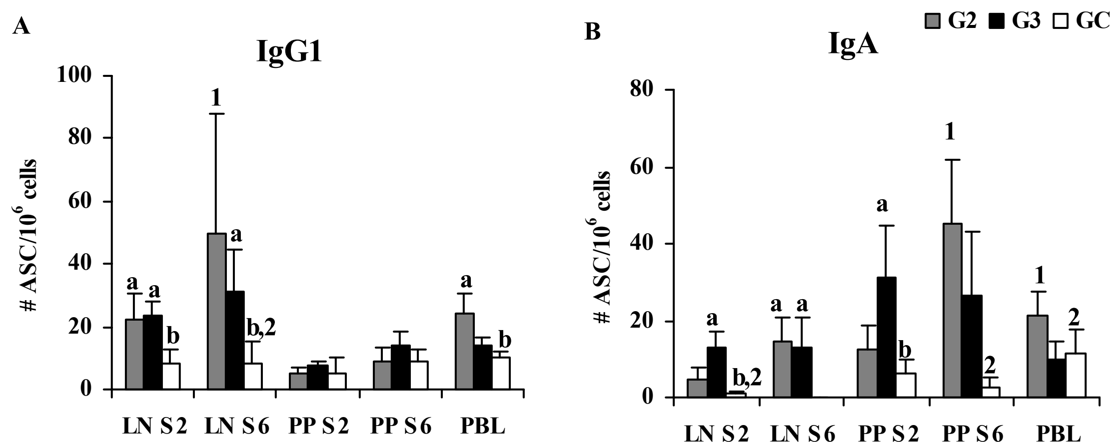

FIGURE 6 The 14.2 kDa-specific IgG1 (A) and IgA (B) secreting cells in the mesenterial lymph nodes (LN S2 and LN S6), the Peyer's patches (PP S2 and PP S6) and peripheral blood (PBL) is given for the animals from G2 (100,000 L3/100,000 L3), G3 (0 L3/100,000 L3) and the non infected control group (GC) at day 28 p.c.. Results are expressed as number of ASC/10^6cells and data are presented as mean values (+SEM) of the animals per group. Different letters indicate significant differences ($P<0.05$) between groups within location. Different numbers indicate marked differences ($P<0.1$) between groups.

Phenotypic characterization of B cells in the different anatomical locations

To measure the frequency of B cells in the different anatomical locations, we stained for Ig light chains. In figure 7A the mean frequency of B cells per group is depicted for

lymphocytes isolated from the different locations in the intestine and from peripheral blood. The frequency of B cells of G2 was significantly reduced compared to GC at all locations ($P<0.05$), except PP S2. Comparison of G2 and G3 revealed a significant decreased %B cells in lymph nodes ($P<0.05$) and peripheral blood ($P<0.1$) of G2. To evaluate whether the decreased frequency of B cells resulted from a migration of the B cell population into the lamina propria of the gut, we estimated the %B cells in the lamina propria. This was done by subtraction of the %CD4+, %CD8+ and %TCR1-N24+ cells. Although not optimal, this estimate was proven to be reliable based on data from an initial experiment (data not shown). The analysis revealed a significant increase in %B cells in the lamina propria of the proximal (LP S2) and the distal (LP S6) gut of the animals of G2 and G3 as compared to GC ($P<0.05$ for both) (fig. 8).

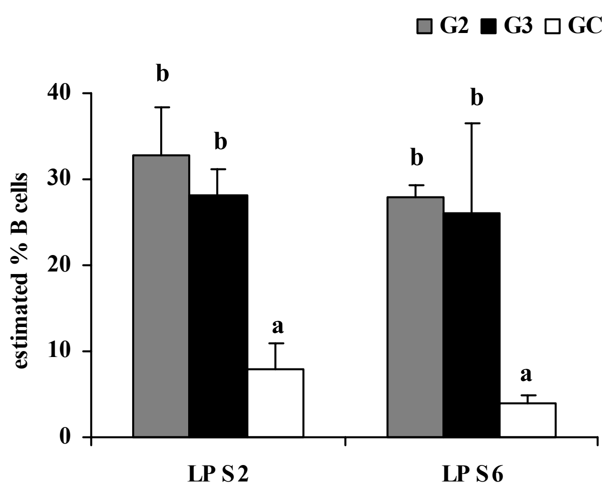

FIGURE 8 The estimated %B cells in the lamina propria of the proximal (LP S2) and the distal (LP S6) gut is given for the animals of G2 (100,000 L3/100,000 L3), G3 (0 L3/100,000 L3) and the non-infected control group (GC) at day 28 p.c.. The %B cells was estimated by subtraction of the %CD4+, the %CD8+ and the %TCR1-N24+ cells from the total population of lymphocytes (=100%). Data are presented as mean% (+SEM) per group. Different letters indicate significant differences ($P<0.05$) between groups

CD62L expression on lymphocytes is known to be involved in the recirculation capacities of the cells. Analysis of the CD62L expression on the B cells showed a significant upregulation on the B cells of G2 compared to GC in lymph nodes, Peyer's patches and PBL ($P<0.05$ for all, fig. 7B). The CD62L expression on B cells of G3 was lower than in G2 but still increased as compared to GC (LN S6: $P<0.1$, PP S2: $P<0.1$, and PP S6: $P<0.05$).
Expression of CD86 on B cells was studied to get an idea about the functional state of these cells. Comparison of the different anatomical locations revealed that the frequency of CD86+IL-A59+ cells of the non infected control group was highest in the lymph nodes (fig. 7C). The distribution was altered in the infected animals and compared to GC, an increased expression of CD86 on B cells of primed animals was found in LN S2 ($P<0.05$), PP S2 ($P<0.05$) and PP S6 ($P<0.05$). In the animals of G3 an increased %CD86+IL-A59+ was only detected in the Peyer's patches from the distal gut ($P<0.05$ for PPS6).

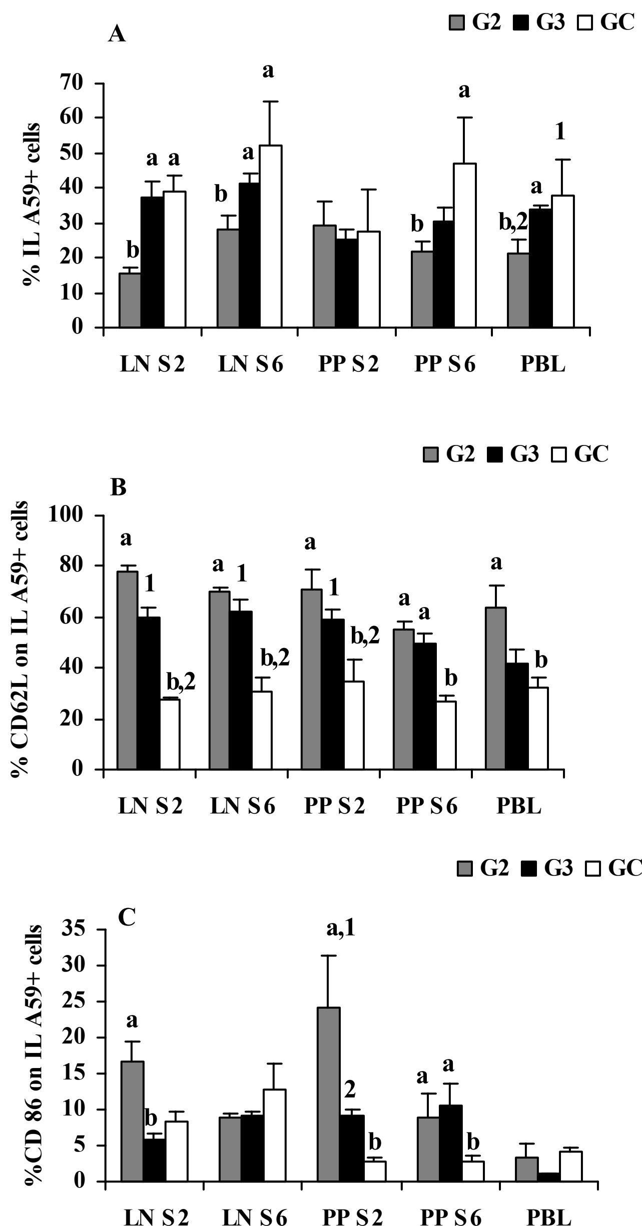

FIGURE 7 The %IL-A59+ cells (A) within the gate for lymphocytes based on the forward and side scatter of the cells, the %CD62L+ cells within the gate of IL-A59+ cells (B) and the %CD86+ cells within the gate of IL-A59+ cells (C) is given for the animals of G2 (100,000 L3/100,000 L3), G3 (0 L3/100,000 L3) and the non-infected control group (GC) at day 28 p.c.. Data are presented as mean% (+SEM) of the animals per group in the mesenterial lymph nodes (LN S2 and LN S6), the Peyer's patches (PP S2 and PP S6) and the peripheral blood (PBL). Different letters indicate significant differences (P<0.05) between groups. Different numbers indicate marked differences (P<0.1) between groups.

To assess whether the decreased %B cells in LN S2 in the primed animals was associated with an enhanced recirculation and the expression of CD62L, we calculated the correlation between the frequencies of CD62L on B cells and the %B cells in the lymph node lymphocytes. Figure 9A shows a significant negative correlation between both (P<0.05) suggesting that a relative decrease in number of B cells coincided with a higher expression of CD62L on the remaining cells.

B7-interactions have shown to be important in the Th2 induced differentiation of B cells and the resulting IgG1 and IgE secretion in *H. polygyrus* infected mice. To investigate whether a similar role could be attributed to B7-2 (CD86) in *C. oncophora* infected calves, we

measured the correlation between the distribution of CD86 and the Ad-specific IgG1 titres. We restricted the analysis to the cells of the Peyer's patches and the lymph node from the proximal gut as at these sites an increased expression of CD86 on B cells was found in the primed animals. Figure 9B shows a strong and significant positive correlation between the Ad-specific mucus IgG1 titres in the proximal gut and the frequency of CD86 on B cells from the draining lymph node (R=0.97, P<0.001). A similar correlation was found between the mucus Ad-specific IgG1 titres and the increased CD86 expression on B cells from the Peyer's patches of the proximal gut (R=0.81, P<0.01). No correlation was found with the total IgE levels in the mucus.

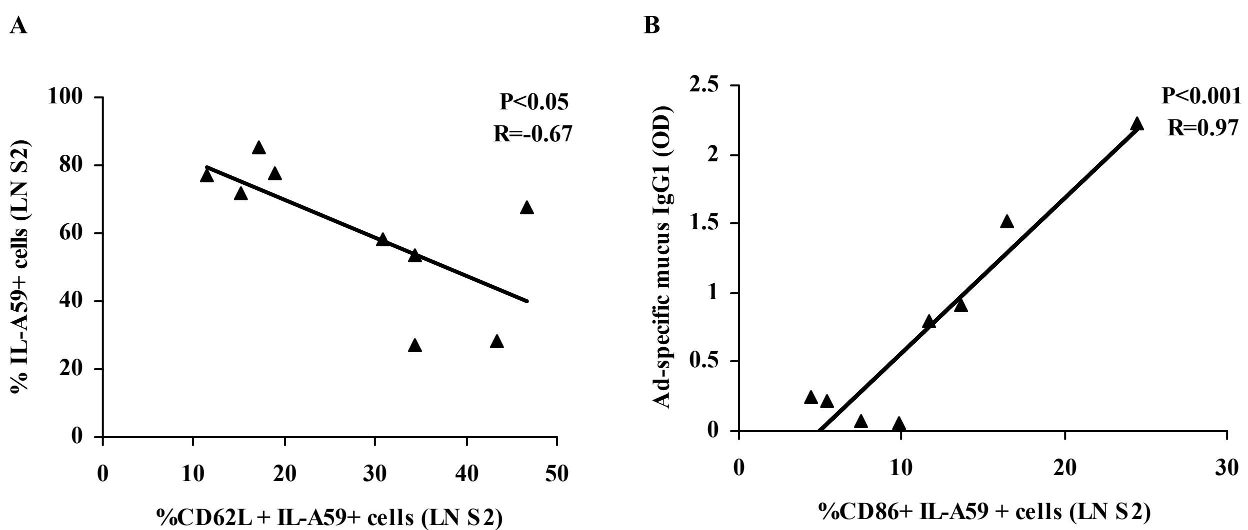

FIGURE 9 (A) The correlation between the %IL-A59+ cells and the CD62L expression on these IL-A59+ cells of the lymph node from the proximal gut (LN S2) at day 28 p.c. is shown (R=0.67). (B) The correlation between the CD86 expression on the ILA59+ cells in LN S2 and the Ad-specific IgG1 titres (OD-blank) in the mucus of the proximal gut (S2) at day 28 p.c. (R=0.97). Correlations were measured with the Pearson's correlation coefficient and all animals slaughtered at day 28 p.c. were included in the analysis.

Frequency of MHCII+ in the different anatomical locations

To assess whether changes in the frequency of B cells and MHCII+ cells were concurrent after challenge infection, we measured the expression of MHCII in lymphocytes isolated from the different anatomical locations of G2, G3 and GC at day 28 p.c.. Compared with GC a significantly decreased %MHCII+ cells was found in all anatomical locations of G2 (P<0.05 for all) (fig. 10). The frequency of MHCII+ cells was reduced only in the distal gut and the peripheral blood of G3 compared to the non infected controls (P<0.05 for PP S6 and PBL, LN S6 not significant). Furthermore, the frequency of MHCII+ cells and IL-A59+ cells throughout the different locations was correlated to investigate whether both populations had a similar distribution (data from G2, G3 and GC were pooled for this

analysis). For all anatomical sites, with the exception of PP S2, a significant positive correlation was measured between the frequency of MHCII+ cells and the frequency of IL-A59+ cells (table 2). In the distal gut (PP S6 and LN S6) the %MHCII+ cells outnumbered the% ILA-59+ cells (slope >1.0), while in the lymph node from the proximal gut and in blood the opposite was found (slope <1.0).

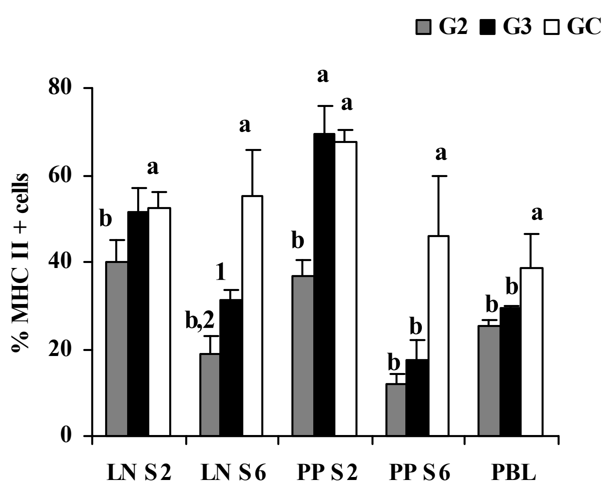

FIGURE 10 The %MHCII+ cells within a gate for lymphocytes based on the forward and the side scatter of the cells is given for the animals the animals of G2 (100,000 L3/100,000 L3), G3 (0 L3/100,000 L3) and the non infected control group (GC) at day 28 p.c.. Results are expressed as %MHCII+ cells and data are presented as mean% (+SEM) of the animals per group in the mesenterial lymph nodes (LN S2 and LN S6), the Peyer's patches (PP S2 and PP S6) and the peripheral blood (PBL). Different letters indicate significant differences (P<0.05) between groups. Different numbers indicate marked differences (P<0.1) between groups.

TABLE 2. Correlations between IL-A59 and MHC II expression on lymphocytes

	% MHCII+ (range)	% IL-A59+ (range)	R	P	slope in linear regression(±SE)
LN S2	2.7-56.9	15.1-46.6	0.68	P<0.05	0.55±0.22
LN S6	9.7-65.9	21.4-64.9	0.88	P<0.01	1.13±0.14
PP S2	29.5-77.3	15.2-49.2	0.04	NS	NS
PP S6	5.3-60.0	11.9-60.1	0.96	P<0.001	1.18±0.13
PBL	20.8-46.4	12.0-48.2	0.77	P<0.05	0.53±0.17

R=Pearson's correlation coefficient, slope= a in equation regression line: y=ax+b

DISCUSSION

The current study was performed to get an insight in the role of antibodies and B cells in the memory response to *C. oncophora*. We specifically studied whether the generation of humoral memory responses depended on the infection dose and on the responder type of the animals. To this end, animals were primary infected with 30,000 or 100,000 *Cooperia* L3, treated with anthelmintics and subsequently challenged with one oral dose of 100,000 *Cooperia* L3. Parasitological analysis at necropsy revealed that priming induced a reduction

in worm establishment as measured by a lower number of adult worms and a higher percentage of L4. Priming dose level had no influence on this reduction, but host responder type did. The intermediate responders showed a much lower establishment of the challenge infection than low responders. Based on these results we hypothesized that in the current experimental set-up, memory immune responses to *C. oncophora* were at least partly generated in a dose-independent way and that the more resistant phenotype of the intermediate responders was sustained after re-infection. Here we report on the contribution of the humoral responses in this immunity against challenge infection. Furthermore, based on the observation that B cells are specifically recruited to the infection site at the peak of a primary infection [126], we performed a more detailed phenotypic and functional analysis of B cells and compared animals primary and secondary infected with 100,000 *Cooperia* L3.
The *Cooperia* 14.2 kDa Elisa is a tool to differentiate between exposure levels to infection under natural conditions [185]. Recently, it was shown that the exposure level of animals trickle infected with different infection doses of *C. punctata* under experimental conditions correlated with the 14.2 kDa-specific serum titres [247]. The similar pattern in 14.2 kDa IgG serum titres in both primed groups in the current experiment is consistent with the parasitological findings that exposure to a single dose of 30,000 or 100,000 L3 does not significantly differ in the generation of protective immunity. In addition, the results suggest that a single infection dose uses a different mode of action to trigger the immune system compared to a trickle infection and that in our model the same mode of action can be induced by a dose of 30,000 or 100,000 L3.
Upon challenge, a booster in Ad-specific serum IgG1 and IgA was induced irrespective of the priming dose. Consistent with a previous study [126] the parasite-specific IgA response remained at a lower level than the parasite specific IgG1 response. B cells from primed animals can respond to lower doses of antigens and the antibodies they produce are generally of higher affinity, hence, this may explain the dose-independency of the secondary Ad-specific Ig response. However, intermediate and low responders did differ in the onset of Ad-specific IgG1 production. Low responders responded more slowly and reached the peak in Ad-specific serum IgG1 about two weeks after the intermediate responders. It is likely that memory B cells were more numerous in intermediate responders resulting in a faster rise in antibody titres after challenge. Plasma cells can survive and secrete antibodies for an extended period of time [203] but the observation that the primary serum antibody response was close to control levels at the time of challenge clearly suggested the extinction of short-lived plasma cells that were induced by the primary immunization. Similar to the parasite specific Ig response, the total serum IgE production was increased in a dose-independent manner in primed animals. In addition, not only the onset of total serum IgE production but also the level differed between intermediate and low responder animals. The

minor increase in total serum IgE in low responders compared to the challenge control animals suggested an effect of priming, however, it also indicated a differential sensitisation between low and intermediate responders. We previously demonstrated the ability of the systemic humoral response to affect the survival of the worm population in primary infected animals based on correlations between the serum Ig response and parasitological variables [126]. Here, we described a distinct parasitological and serological response of low and intermediate responders, however only the total serum IgE titres could be related to parasitological variables; a maximum of 60% of the variation in worm burden could be explained by the variation in total serum IgE. These results lead to the hypothesis that the involvement of the serological Ig responses in protection (as measured by a reduced worm establishment) differs between primary and secondary infected animals and that additional effector mechanisms are required during memory immune responses.

A feature of the mucosal immune system of ruminants, which sets it apart from other species, is that despite a more prominent IgA system, IgG1 is also strongly represented [145]. Hence, both isotypes might be equally involved in the mucosal immune responses. Primed animals had significantly increased Ad-specific mucus IgG1 and IgA titres; however, the level of mucus Ig did not diverge between the different responder types and between animals primed with a low or a high dose. These findings implied that memory B cells were sustained at mucosal sites and in addition, the more rapid and higher mucus IgA (and to a lesser extent mucus IgG1) response in the proximal gut as compared to the distal gut suggested an altered distribution of memory cells at both locations. This was consistent with the antigen load during primary infection, during which *Cooperia* preferentially resides in the proximal part of the intestine [126]. The protective role of the mucus antibody response was addressed by investigating to which extent it could be correlated to parasitological variables that feature an impaired establishment. Whereas at day 14 p.c. the mucus IgG1 titres were positively correlated with the worm burden, two weeks thereafter, a negative correlation with worm length was found. The latter correlation supports the view that Ad-specific mucus IgG1 titres might be of functional importance at the time of worm clearance, while in the early part of the infection Ad-specific IgG1 titres merely reflect exposure to the worm population.

Contradictory to earlier observations [126], the frequency of B cells was increased in the challenge control animals at day 14 p.c. (data not shown) but not at day 28 p.c.. The discrepancy between both experiments may be caused by two factors. First, in the previous experiment we used the MoAb BAQ44 to detect B cells. This MoAb also recognizes plasma cells [110] as opposed to the currently used MoAb IL-A59. The latter recognizes the light chain of immunoglobulins on bovine B cells [242] and therefore does not recognize plasma cells. Second, the age of the animals might affect the outcome, since the animals were 6

months of age at the time of challenge, compared to 3 months of age in the previous experiment. The earlier increase in B cell frequency is consistent with an age-related resistance and more effective development of immunity as has been reported before for *C. oncophora* infected calves [135].
The decline in frequency of B cells and MHCII+ cells in the infected animals at day 28 p.c. was more pronounced in the primed animals and concurred with an increased number of *Cooperia*-specific ASC. This suggested that at least some of the IL-A59+ cells had differentiated into plasma cells. In addition, it could have resulted from non-specific or specific recruitment of B lymphocytes to the inflamed intestinal mucosa of the infected animals. The observation that the estimated number of B cells was significantly elevated in the lamina propria of primed and challenge control animals as compared to non infected controls, raised the possibility that B cells were recruited from the Peyer's patches and the lymph nodes into the lamina propria to become effector cells. In terms of B cells, the main effector function is the secretion of antibodies, and the enhanced mucus Ig production in the primed animals is in agreement with the proposed pathway. The frequencies of MHCII+ and IL-A59+ cells were highly correlated in the infected animals at the different anatomical locations, except in PP S2. This made us hypothesize that the concurrent decline in both populations in the infected animals was caused by the same cells. However, the kinetics of the frequency of MHCII+ cells cannot solely be attributed to the kinetics of the B cell population because parasite activated T lymphocytes also upregulate MHCII expression on their surface [101, 15]; furthermore, MHCII is expressed on monocytes, macrophages, and dendritic cells . The slope of the regression line between the MHCII+ and IL-A59+ cells demonstrated that in peripheral blood and the lymph nodes from the proximal gut the MHCII+ cells outnumbered the IL-A59+ cells whereas in the distal gut the IL-A59+ cells outnumbered the MHCII+ cells. However, the analysis of challenge control and primed animals separately revealed that this divergence was caused by differences between both groups. In the challenge control animals the percentage MHCII+ and IL-A59+ cells was unaltered in LN S2 and in PBL only the frequency of MHCII+ cells decreased while both populations were decreased in LN S2 and PBL of primed animals. Based on our proposed recirculation pathway for B lymphocytes, we hypothesize that the recruitment of IL-A59+ cells and MHCII+ (being either activated T lymphocytes or antigen presenting cells) to the lamina propria of primed animals is enhanced as compared to challenge control animals. Consistent herewith a higher percentage CD62L+IL-A59+ cells were found in the primed animals. Expression of CD62L targets resting lymphocytes ($CD62^{hi}$) to areas of antigen accumulation and studies in humans revealed that CD62L is expressed by B lymphocytes stimulated subsequent to oral immunization [124]. This suggests a role for CD62L in immunity at the intestinal effector site. The lack of major differences in CD62L expression on B cells

from primed and challenge controls confirmed that CD62L expression does not correlate with memory in cattle [115] but merely mimics the recirculation capacities of lymphocytes.
Finally, we identified a possible role for B7 interactions in the anti-*C. oncophora* immune response. Compared to the non infected controls, a significantly increased expression of CD86 was found on B cells from LN S2, PP S2 and PP S6 of the primed animals while in the challenge control animals an enhanced expression of CD86 was restricted to PP S6. In our study, CD86 and CD62L expression were mutually exclusive on B cells in the different tissues. This could possibly reflect the occurrence of two distinct B cell subpopulations i.e. CD62L+CD86+ B cells that are not yet activated lymphocytes with an enhanced recirculation capacity, and CD62L-CD86+ B cells that are activated B cells with enhanced antigen presenting capacities and a reduced recirculation ability.
The influence of B7-interactions on the development of a primary or a secondary immune response has been investigated extensively in mice infected with the intestinal nematode *H. polygyrus* and revealed that combined administration of anti B7-1 and B7-2 antibodies to infected mice strongly abrogated the type 2 response. This resulted in reduced eosinophilia, an inhibited B cell activation, germinal centre formation and serum IgG1 and IgE secretion [102]. In addition, *H. polygyrus* infection in B7-1/B7-2 $^{-/-}$ mice demonstrated that the generation of parasite specific IgG1 and IgE, but not total serum IgG1 and IgE was B7 dependent [103]. The authors proposed that the distinct influences on parasite specific and total IgG1 and IgE production was due to a variation in B7-dependence of different parameters linked to a type 2 immune response [102, 103]. Based on the role of B7 interactions in *H. polygyrus* infected mice, we currently investigated the relationship between B7-2 (CD86) expression and the development of a type 2 immune response in *C. oncophora* infected animals. CD86 expression was significantly correlated with *Cooperia* Ad-specific mucus IgG1 titres but not with total serum IgE levels. In addition, we measured a significant positive correlation between the CD86 expression and eosinophilia (data not shown), another feature of the type 2 response induced by *C. oncophora* infection. Taken together, these data support the hypothesis that CD86 interactions play a role in the development of the type 2 immunity in *C. oncophora* infected calves but, that not all parameters involved in the immune response are influenced in a similar manner.
In summary, with this model we demonstrated that humoral memory immune responses to *C. oncophora* can be generated in a dose-independent way. Priming induced a humoral memory response that promoted the production of high levels of *Cooperia*-specific antibodies in serum and at mucosal surfaces, irrespective of a low or a high primary infection dose. In addition, in serum but not in mucus, the onset of the antibody secretion was earlier in intermediate responders compared to low responders. Our experimental set up does not allow us to exclude a similar kinetic in the mucus as differences in the onset of

mucosal parasite specific antibodies might have occurred before day 14 p.c.. Preliminary studies in our lab suggest that the more susceptible phenotype of low responders essentially results from the generation of antibodies to different parasite antigens.
Furthermore, from our study we hypothesize that upon challenge sensitized B cells are more efficiently recruited to the intestinal effector sites where they develop into antibody secreting plasma cells. The increased expression of CD86 and the subsequent significant correlations with parameters of the type 2 mediated immune response induced by *C. oncophora* suggest for the first time that CD86 interactions are involved in the generation of protective immunity against *Cooperia.*

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Chapter 6

T-cell mediated immune responses in calves primary infected or re-infected with *Cooperia oncophora*: similar effector cells but different timing

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I would never die for my beliefs, because I might be wrong.

(B. Russel)

ABSTRACT

Cooperia oncophora is the most prevalent intestinal nematode of cattle occurring in Western Europe. Primary infection with 100,000 third stage infective larvae (L3) induces acquired immunity in a high proportion of the animals but there is little information on immunity against re-infection. In the current experiment, the contribution of the T-cell mediated immunity in protection against re-infection with *C. oncophora* was investigated in detail. Priming elicited long-lasting protective immunity that was evidenced by a significantly decreased worm burden and egg excretion in primed animals compared to challenge control animals. Lymphocyte proliferation tests with excretory/secretory products (ESP) of *C. oncophora* and with three distinct ESP fractions indicated an enhanced reactivity in primed animals and suggested that by fractionating of ESP we selected for proteins involved in protective immunity against re-infection with *C. oncophora*. Phenotypic analysis of T cell subsets at diverse anatomical locations revealed that the enhanced reactivity of lymphocytes from peripheral blood and lymph nodes of the infected animals coincided with a significantly increased frequency of CD4+ cells at these locations but a deceased frequency of CD4+ cells in the lamina propria. These findings were independent of the immune status of the animals but more pronounced in the primed animals than in the challenge control animals. In addition we demonstrated that primary and secondary infections with *C. oncophora* were associated with two waves of eosinophils and that the kinetics of this cell population differed as a result of priming. Based on the observed correlations we propose that the early increase of eosinophils is T cell independent and merely a consequence of inflammation in the parasitized gut. In contrast, the second wave of eosinophils depends upon CD4+ cells and correlations with parasitological parameters at this time point support a role of eosinophils as effector cells against adult stages of *C. oncophora*.

INTRODUCTION

Cooperia oncophora is the most prevalent small intestinal nematode of cattle in Western Europe. Natural infections are mostly subclinical but, the economic importance remains high and a negative effect on the productivity of first and second year calves has been reported [179, 184]. While there have been many studies characterizing the immune response to a primary infection with *C. oncophora* [3, 171 176, 231], there are only a few reports on re-infection with *C. oncophora* [84; 58]. Moreover, these few experiments focused on short-term immunity in animals re-infected one or two weeks after anthelmintic treatment. Very little is known about the amnestic cell mediated immune responses (CMI) after challenge infection of previously infected animals.

The first evidence for a role of CMI to *C. oncophora* was given by Hanharan et al. [107] who compared the proliferative responses of peripheral blood and visceral lymph lymphocytes as well as delayed-type hypersensitivity responses in non-infected and infected animals. The high variability and intermittent response of the animals was attributed to a low antigenic stimulation by the parasite and the assumption that *Cooperia*-specific cells were localized elsewhere. In a previous experiment, we demonstrated the generation of *C. oncophora* reactive cells in the mesenterial lymph nodes of animals which were primary infected with 100,000 L3 [126]. Lymphocytes proliferated after stimulation with *C. oncophora* L3 and adult crude worm antigen, however the response was most pronounced to the excretory/secretory products (ESP) of adult *C. oncophora*.

ESP of nematodes are highly immunogenic in both experimental and natural infections [148, 196]. They contain a mixture of glycoproteins that has both stimulating [197, 241] and inhibitory [53, 198] capacities *in vitro* and are likely targets for immune effector mechanisms. In this study, *C. oncophora* adult ESP were partially purified and distinct fractions were used in proliferation assays to identify products that specifically elicit proliferation of naïve and sensitized lymphocytes. Based on the distinct distribution of the worm population throughout the gut of primary and secondary infected animals, the response in the proximal (jejunum) and the distal (ileum) part of the intestine was compared.

A central role for CD4+ cells in resistance to gastro-intestinal nematode infections has been demonstrated in rodents [63] and ruminants [151] and although the dichotomy in Th1 and Th2 is not as clear cut in cattle as it is in mice, effective immunity against nematodes inhabiting the gastro-intestinal tract of ruminants is commonly associated with eosinophilia, mastocytosis and immunoglobulin E production [163, 15], which strongly support a Th2 dominated host response. We have previously shown that the development of immunity following a primary infection

with *C. oncophora* was associated with a typical type 2 mucosal response *in vivo* [126]. This response included an early as well as a late increase in lamina propria eosinophils, marked increases in *C. oncophora*-specific serum IgG1 and IgE and mucosal IgG1 and IgA. No quantitative differences were observed in T cell subsets of the intestinal mucosa and peripheral blood of infected animals, but based on observed correlations we proposed a role for mesenterial lymph node CD4+ cells in the recruitment of the second wave of eosinophils [126]. In the current study we investigated whether eosinophils and mast cells are involved as effector cells in immunity against re-infection with *C. oncophora*. To assess whether the recruitment of eosinophils and mast cells is triggered by a specific T cell subset, a phenotypic analysis of CD4+, CD8+, and TCR1-N24+ cells in peripheral blood and the proximal and distal gut was performed and subsequently correlations between T cells and effector cells were measured. In addition, changes in the expression of the homing and activation markers CD62L and MHC class II on lymphocyte subsets were examined. The hypothesis that different mechanisms are involved in the development of acquired immunity during infection and in protective immunity against re-infection was tested by the comparison of these lymphocyte subsets in primary and secondary infected animals. Herein we demonstrate that despite the recruitment of similar effectors, the CMI during a primary and a memory immune response to *C. oncophora* significantly differs in the kinetics and the anatomical localization of the response.

MATERIAL AND METHODS

Animals and experimental design

Seventeen female Holstein Friesian calves were raised under helminth-free conditions on a commercial farm and purchased at 3 months of age. The animals were housed indoors at the animal facility of the Faculty of Veterinary Medicine in Utrecht during the whole period of the experiment. They were fed hay and water ad lib as well as concentrates up to 500 g per cow per day. Prior to infection, animals were checked for general health condition and faeces were collected to confirm the worm free status of the animals. A summary of the infection protocol and the experimental groups is given in table 1. At day 49 after infection (p.i.) all animals were drenched with oxfendazole (Systamex 2.65% suspension, 2 ml/10 kg, Scheringh Plough, Kenilworth, New Jersey, USA). All experimental procedures were approved by the ethical committee on animal experimentation of the Utrecht University.

Parasitology

The numbers of eggs per gram faeces (EPG) were determined with a modified McMaster technique with a sensitivity of 50 EPG. Faeces were analysed once a week from day 0 to day 14 p.i. and during the period between treatment and secondary infection. In the remaining period, faecal samples were collected and analysed every two days. After slaughter the small intestine was subdivided in six different segments that were all processed separately as described previously [126]. Briefly, the first segment consisted of the first meter starting from the pylorus and each following segment was five

meters. Results are shown from the second (S2, jejunum) and the last (S6, ileum) segment. Two percent aliquots of the total worm burden were counted. A differentiation was made between male and female worms and fourth-stage larvae (L4). The L4 were predominantly (>95%) early stages and therefore considered as inhibited larvae. Worm lengths were measured from 20 individual male and female worms per segment if available. From each worm an individual picture was taken and length was determined using the software package Accuroute® version 1.1 (Critical path software, http://www.accuroute.co.uk). For each picture a calibration unit of 2 mm was set. Fecundity of the female worms was estimated by counting the number of eggs in 20 individual females per segment after lysis of the worms in 0.4% sodium hypochlorite solution [122].

TABLE 1. Experimental design

		Group (number of calves)		
	day	G1(n=7)	G2 (n=6)	GC (n=4)
primary infection	0 d.p.i.	100,000 L3	none	none
anthelmintic drench *	49 d.p.i.	yes	yes	yes
challenge infection	126 d.p.i.	100,000 L3	100,000 L3	none
necropsy	14 d.p.c.	n=3	n=3	n=2
	28 d.p.c.	n=4	n=3	n=2

(n=number of calves; d.p.i.=days post infection; d.p.c.= days post challenge)
* oxfendazole 2 ml/10 kg (Systamex 2.65% suspension)

Fractionation of *C. oncophora* excretory/secretory products (ESP)
C. oncophora adult excretory/secretory products (ESP) were obtained using a standard procedure as described before for *Haemonchus contortus* [197]. The obtained supernatant was filter sterilized (0.2 µm) and supplemented with protease inhibitors (Complete®, Roche). ESP were used in lymphocyte proliferation tests (LPT) at a concentration of 5 µg/ml in complete medium (RPMI-1640, Gibco BRL, Paisly, UK) supplemented with penicilline (500 U/ml), streptomycin (0.5 mg/ml), 10% foetal calf serum (FCS), 2 mM glutamine, and 2 mM β-mercaptoethanol).
Cooperia ESP were partially purified by ion exchange chromatography using a low pressure chromatography system (ECONO system, BIORAD, Richmond, USA). All steps were performed at room temperature, however, buffers and protein solutions were kept on ice. The supernatant containing the soluble fraction of ESP was concentrated and desalted (10 mM Tris, 0.9% NaCl pH 7.4) with 3 kDa filters (Centriprep, YM-3, Millipore). After concentration, 5 ml of ESP (2 mg/ml) was applied at a flow rate of 0.2 ml/min onto a Mono S cation exchange column (High S cartridge, 1 ml, ECONO-PAC® CARTRIDGES, BIORAD) previously equilibrated with buffer A (10 mM NaH_2PO_4/Na_2HPO_4, 1 mM EDTA, pH 7.2). Unbound proteins were removed by washing of the column with a 5 bed volume of buffer A and proteins were subsequently eluted with a flow rate of 0.3 ml/min with a 50 bed volume linear gradient (10-500 mM KCl) in buffer A and 1 ml fractions were collected. The unbound fractions of the ESP were pooled, concentrated with 3 kDa filters and subsequently applied onto a Mono Q anion exchange column (High Q cartridge, 1 ml, ECONO-PAC® CARTRIDGES, BIORAD) previously

equilibrated with buffer B (10 mM Tris, 1 mM EDTA, pH 7.4). Unbound proteins were removed by washing of the column with a 5 bed volume of buffer B and proteins were subsequently eluted with a flow rate of 0.3 ml/min in a 50 bed volume linear gradient (10-500 mM KCl) in buffer B and 1 ml fractions were collected. The homogeneity and apparent molecular weight (MW) of the purified fractions were analysed by SDS-PAGE according to the method of Laemmli [142] under reducing conditions on a 15% polyacrylamide gel (5 µl/lane) using the Mini-Protean II system (BioRad). Proteins were subsequently visualized by silver staining. Commercial low molecular weight proteins (Pharmacia) were used as molecular weight standards. Based on the appearance on 1D gel, similar fractions were pooled and concentrated. Protein concentration was determined by the Bradford assay and fractions were used in LPT at a concentration of 1 µg/ml.

Isolation of lymphocytes

Lymphocytes were isolated from peripheral blood, mesenterial lymph nodes and lamina propria of the proximal and the distal gut as described previously [126]. Briefly, peripheral blood and lymph node lymphocytes were isolated using a density gradient technique (Ficoll Paque, Amersham Pharmacia Biotech, Freiburg, Germany) and lamina propria lymphocytes where isolated by enzymatic digestion with collagenase. Lymphocytes from Peyer's patches were harvested based on the method described by Sopp and Howard [208]. Erythrocytes were removed from the final cell suspensions by an erythrocyte shock lysing solution and viability of the cells was assessed by trypan blue exclusion. Cells were resuspended in complete medium and kept on ice until use in lymphocyte proliferation tests (LPT) or flow cytometric analysis.

Lymphocyte proliferation tests (LPT)

LPT with the incorporation of ^{3}H-thymidine were performed as described previously [197, 126]. Briefly, cells were resuspended at a concentration of $2*10^6$ cells/ml in complete medium and incubated with either ESP at a concentration of 5 µg/ml or the ESP- fractions (F1, F2 and F3) at a concentration of 1 µg/ml. Cells were incubated for 5 days at 37ºC and 5% CO_2. Twenty-four hours before harvesting a final pulse with 0.5 µCi (methyl) ^{3}H-thymidine was added to each well. Results are expressed as stimulation indices (SI= cpm antigen stimulation/cpm medium control).

Flow cytometric analysis

The following monoclonal antibodies were used for surface staining of the lymphocytes: anti-bovine CD4-FITC (Serotec, UK), anti-bovine CD8 (clone CC30, ECCAC) labelled with APC, anti bovine TCR1-24 (clone GB21A, VMRD, USA), anti-bovine MHCII (clone IL A21, [12]) labelled with biotin, and anti-human CD62L-PE (DREG 56, BD Pharmingen; [237]). The labelling with biotin (D-biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester, ROCHE) and APC (Phyconil®, APC conjugation kit, Prozyme, San Leonardo, USA) was done according to the manufacturers instructions. The second step to detect MHCII was performed with streptavidin-PerCP or streptavidin-APC (BD Bioscience) and second step to detect TCR1-N24 was done with anti-mouse IgG2b-FITC (BD Bioscience). Staining procedure was described previously[127]. After the final wash, cells were fixed with 1% paraformaldehyde and analysed by flow cytometry using a FACS-CALIBUR (Becton Dickinson, Sunnyvale, CA, USA). Analysis was done on lymphocytes within a gate based on forward and side scatter of the cells. A minimum of 10,000 events was collected for the phenotypic analysis of isolated lymphocytes.

Histological examinations
Detection of eosinophils and mast cells in the intestinal tissues was done as described before [126] with some slight modifications for the eosinophils. Briefly, tissues were fixed in 4% paraformaldehyde in PBS and eosinophils were identified on Haematoxylin and Eosin Y (Sigma) stained sections. Cells were counted on a total surface of at least 0.5 mm^2 on 2 different sections.

Statistics
Statistical analysis was carried out using SPSS statistical package (version 10.0). Normally distributed data were analysed with ANOVA. Data that were not normally distributed were analysed with the non-parametric Mann-Whitney test for 2 independent samples and the non parametric Kruskal-Wallis test for more independent samples. Pair-wise comparison of 3 or more independent samples was done by post-hoc analysis as advised for Kruskal-Wallis. Correlations between parameters were measured with the Pearson's correlation coefficient for linear correlation on normally distributed data and with the Spearman's rank correlation coefficient for non linear correlations on normally distributed data. Data from the non infected control groups (necropsied at day 14 and day 28 p.c.) were combined for comparison of T cell subsets, eosinophil and mast cell numbers with primed and challenge control animals. The level of confidence was set at P<0.05 for significant differences between groups.

RESULTS

Parasitology

Protective immunity resulting in reduced establishment of *C. oncophora* was evaluated by counting the number of worms recovered from the small intestine and by comparing the egg output between G1 and G2. Priming resulted in a significantly lower worm burden on day 14 and day 28 p.c. (P<0.05 for both) but within G1 and G2 the worm burden did not significantly differ between day 14 and day 28 p.c. (fig. 1). At day 14 p.c. none of the animals were excreting eggs whereas by day 28 p.c. all challenge control animals had an egg output. In contrast, only one of the primed animals had egg excretion by day 28 p.c. (data not shown).

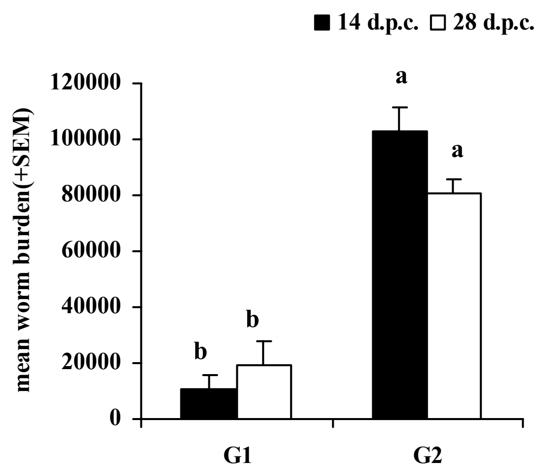

FIGURE 1 Mean worm burden (+SEM) for the animals from G1 (100,000 L3/100,000 L3) and the animals from G2 (0 L3/100,000 L3) at day 14 and 28 p.c..

Fractionation of *Cooperia* excretory/secretory products

Fractionation of ESP was achieved in 2 steps. First, the ESP were applied onto a Mono S cation exchange column. In a second step, the unbound fraction was applied onto a Mono Q anion exchange column. SDS-PAGE of the Mono S chromatography showed two distinct protein fractions.

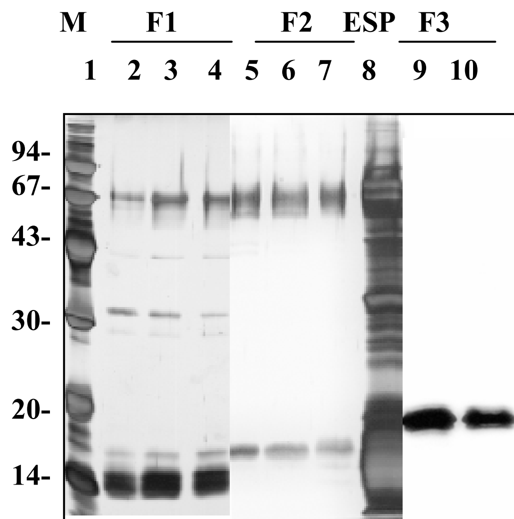

FIGURE 2 Protein pattern of ESP fractions (1 µg/lane) are shown after separation by SDS-PAGE under reducing conditions on a 15% polyacrylamide gel. A low molecular weight marker is given in lane 1. F1 (lane 2-4) and F2 (lane 5-6) are fractions obtained after exchange chromatography with a Mono S cation exchange column and F3 (lane 9-10) was obtained after a subsequent chromatography step with a Mono Q anion exchange column. Lane 8 shows the protein pattern of native ESP (5 µg/lane).

The first fraction (F1, fig. 2) consisted of a band having an apparent MW of 12-14 kDa under reducing conditions. The second fraction (F2, fig. 2) included a single band with an apparent MW of 15 kDa under reducing conditions. Although the low MW proteins were the most prominent, both fractions also contained proteins with higher MW (30 and 67 kDa for F1, and 67 kDa for F2). The subsequent anion chromatography yielded one fraction with an apparent MW of 19 kDa (F3, fig. 2) and one fraction with an apparent MW of 39 kDa (data not shown). Only very low amounts of proteins of the latter fraction were obtained and therefore this fraction was not used in the LPT. Based on 2D-gel electrophoresis of the individual fractions we estimated that the fractionation resulted in a loss of >60% of the proteins for each fraction separately as compared to total ESP (data not shown).

***Cooperia*-specific lymphocyte proliferation**

In order to detect *Cooperia*-specific reactive cells lymphocyte proliferation tests (LPT) with ESP and partially purified fractions were performed. Figure 3 shows the individual values for the ESP-specific proliferation after stimulation of lymphocytes isolated from peripheral blood (PBL) and mesenterial lymph nodes from the proximal (LN S2) and distal gut (LN S6). At day

14 p.c., ESP-specific cells were mainly detected in the PBL in both primed and challenge control animals. At day 28 p.c., ESP-specific proliferation of lymphocytes was observed in all locations. The localization of this increased reactivity however differed between primed and challenge control animals. Increased proliferation of lymphocytes from challenge control animals was observed mainly in the proximal gut ($P<0.05$) whereas the primed animals also showed increased reactivity in the distal gut ($P<0.05$).

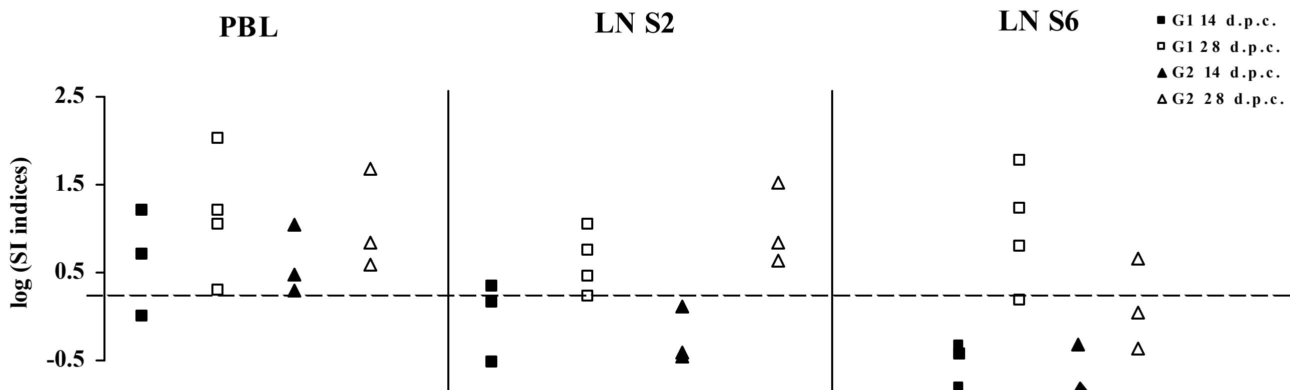

FIGURE 3 Stimulation indices (SI) after stimulation of lymphocytes from the peripheral blood (PBL) and lymph nodes of the proximal (LN S2) and the distal (LN S6) gut with total ESP (5 µg/ml). Data are shown in a logarithmic scale for individual values of the animals from G1 (100,000 L3/100,000 L3) and G2 (0 L3/100,000 L3) at day 14 and 28 p.c.. Based on the response of non infected animals, a threshold for stimulation was set at a SI=2 (log scale=0.3; dotted line). All values are means of duplicate or triplicate wells.

As a first step to identify ESP proteins involved in proliferation, we analysed the reactivity of lymphocytes from LN S2 and LN S6 to 3 different ESP fractions (fig. 4). The response to the fractions depended on the immune status of the animals and the localization in the gut. At day 14 p.c. in LN S2 the primed animals had an increased response to F3, whereas in the challenge control animals specific proliferation was restricted to F2 (fig. 4A). None of the animals showed F1-specific proliferation. At day 28 p.c. both primed and challenge control animals showed an increased F3-specific proliferation in LN S2. The F3-specific response in G1 was significantly higher than at day 14 p.c. ($P<0.05$) and also significantly elevated compared to G2 ($P<0.05$). In LN S6, the reactivity of the lymphocytes in response to the fractions was more limited (fig. 4B). At day 14 p.c. none of the animals showed an increased proliferation in response to the fractions whereas at day 28 p.c. only the primed animals had an increased proliferation that was mainly specific for F1 and F3.

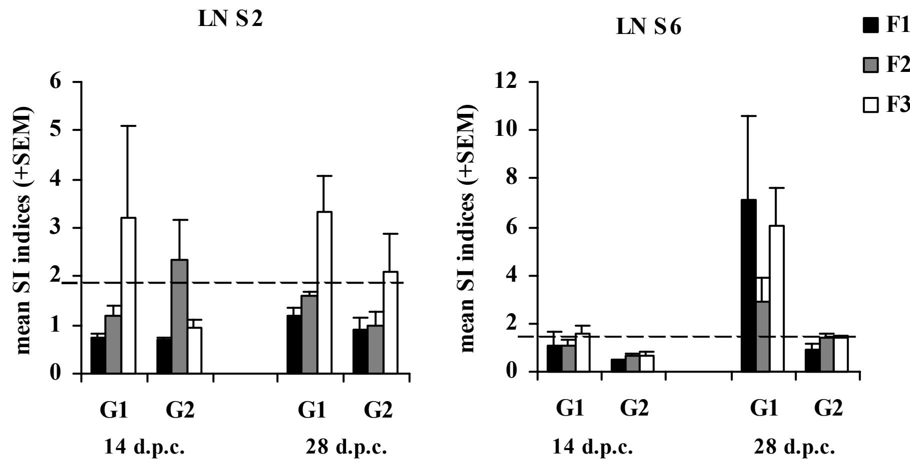

FIGURE 4 Mean SI (+SEM) after stimulation of lymphocytes from the lymph nodes of the proximal (LN S2) and the distal (LN S6) gut with the partially purified ESP fractions F1, F2 and F3 (1 µg/ml). Based on the response of non infected animals, a threshold for stimulation was set at an SI=2 (dotted line).

Distribution of T lymphocyte subsets in various anatomical locations of *C. oncophora* infected animals

A phenotypic analysis of T cell subsets in peripheral blood (PBL), mesenterial lymph nodes (LN), Peyer's patches (PP), and lamina propria (LP) was performed to distinguish between the relative role of CD4+, CD8+, and TCR1-N24+ cells in primary and secondary infected animals. No infection related differences were observed at day 14 p.c. (data not shown). The observed frequencies of the T cell subsets at day 28 p.c. are shown in table 2.

TABLE 2. Mean percentage of T lymphocyte subsets in lymph nodes, Peyer's patches, lamina propria and peripheral blood at day 28 p.c.

	CD4 (%±SEM)			CD8 (%±SEM)			TCR1-N24 (%±SEM)		
GROUP	G1	G2	GC	G1	G2	GC	G1	G2	GC
LN S2	37.4±2.7 [a]	33.6±5.2 [1]	25.3±4.1 [b,2]	10.9±3.7 [1]	11.7±4.2 [1]	22.8±8.0 [2]	3.6±0.8	4.4±0.3	15.6*
LN S6	23.1± 6.5	25.2±11.4	21.7±7.7	9.5±2.3	15.1±3.1	15±7.1	7.7±7	10.5±6.1	14.4±1.3
PP S2	27.7±8.2 [a,1]	14.0±0.6 [b]	17.2±4.2 [2]	19.9±5.5	14.2±2.6	17.6±6.1	18.3±9.0	11.4±0.6	15.1±0.3
PP S6	14.6±-5.1	9.7±1.8	13.3±6.1	15.5±3.9	18.4±2.5	11.1±2.5	14.6±4.2[b]	24.9±6.2 [a]	12.4±0.5 [b]
LP S2	6.7±3.1[a]	12.1±6.9[a,b]	13.3±4.1[b]	24.5±7.9	25.2±0.4	37.1±16.8	42.1±8.0 [a]	34.4±7.3[1]	23±0.6 [b,2]
LP S6	4.6±0.7 [a]	4.6±1.2 [a]	13.4±5.8 [b]	30.2±5.0	25.8±3.5	36±5.7	37.1±6.0 [a]	35.7±9.2[a]	19.7±2.3 [b]
PBL	30.4±0.4[1]	24.3±9.2	19.6±11.4[2]	8.8±4.0	9.1±3.4	12.1±3.1	15.2±5.9	12.9±4.0	14.6±7.5

*data from one animal only

[a,b] different letters indicate significant differences (P<0.05)

[1,2] different numbers indicate marked differences (P<0.1)

A higher frequency of CD4+ cells was found in LN S2, PP S2, and PBL of the primed animals (G1) compared to the non-infected controls (GC). Concurrent with the elevation in number of CD4+ cells in these locations, we observed a drop in the frequency of CD4+ cells in the lamina propria of G1. This was found both in LP S2 and LP S6 ($P<0.05$ for both). The frequency of CD4+ cells in the proximal gut of the challenge control group (G2) only differed significantly from GC in LN S2.
The higher proportion of CD4+ cells in LN S2 of G1 and G2 coincided with a relative decrease in CD8+ and TCR1-N24+ cells at the same location. No further significant differences in the frequency of CD8+ cells were observed between the different groups or the different anatomical locations. The frequency of TCR1-N24+ cells differed between the infected and non infected animals, resulting in a significant increase in LP S2 and LP S6 in the primed animals compared to GC. In the challenge control animals, the frequency of TCR1-N24+ cells was markedly higher in LP S2 ($P<0.1$) and significantly higher in LP S6 ($P<0.05$) compared to the frequency in GC.

CD62L and MHCII expression on T lymphocyte subsets of animals infected with *C. oncophora*

As a measure of lymphocyte recirculation capacity the co-expression of CD62L was investigated on CD4+, CD8+ and TCR1-N24+ cells of LN S2, LN S6, and peripheral blood. At day 14 p.c., the only differences in CD62L co-expression were an increased percentage CD8+ CD62L+ cells in LN S2 of G1 and a decreased percentage TCR1-N24+CD62L+ cells in LN S2 and LN S6 of G2 (fig. 5A and fig. 5B, $P<0.05$ for both). At day 28 p.c. the CD62L expression was reduced on the CD4+ and TCR1-N24+ cells of both G1 and G2 in LN S6 ($P<0.1$ for both; fig. 5B). In contrast, we observed a marked increase in CD4+CD62L+ cells in PBL of G1 and G2 compared to the non-infected control group (GC; $P<0.1$; fig. 5C). Simultaneously, a higher frequency of CD8+CD62L+ cells was found in PBL ($P<0.1$). All the observed differences on day 28 p.c. were concurrent in primed and challenge control animals and could consequently not be related to the immune status of the animals.
If CD4+ cells are involved in the immune response to *C. oncophora*, they will likely have a more activated phenotype as compared to other T cell subsets. In order to investigate this, the co-expression of MHCII on CD4+ and CD8+ cells was compared in PBL, LN S2 and LN S6 of primed and challenge control animals. Despite a relative decrease in LN S6, we observed no significant differences between the different groups, neither between the proximal and the

distal gut. Similarly, the frequency of CD4+MHCII+ and CD8+MHCII+ cells did not differ between G1, G2 and GC in PBL (data not shown).

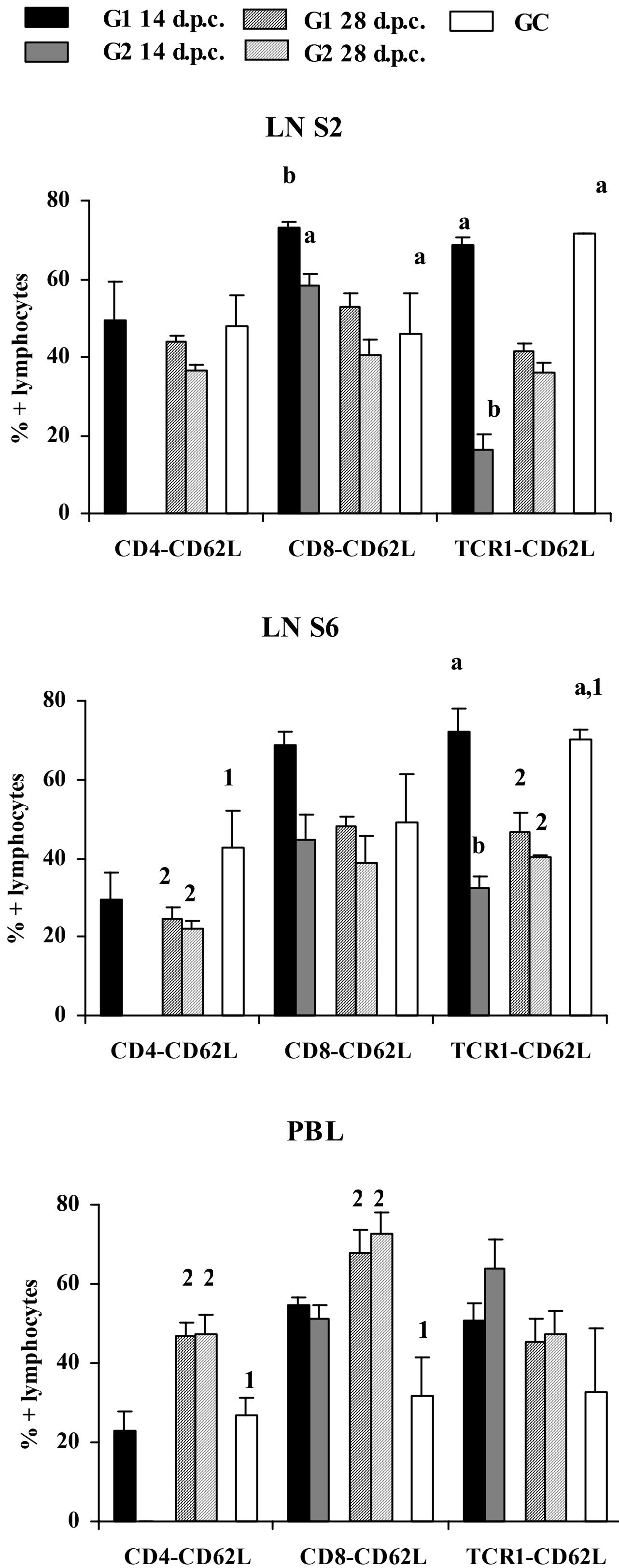

FIGURE 5 The %CD62L+ cells within the gate for CD4+, CD8+, and TCR1-N24 + cells is shown for G1 (100,000 L3/100,000 L3), G2 (0 L3/100,000 L3), and the non infected controls GC (0 L3/ 0 L3) at day 14 and day 28 p.c. in LN S2, LN S6 and the peripheral blood (PBL). Data shown are mean %lymphocytes (+SEM) of 3 animals for G1 at day 14 p.c. and for G2 at day 14 and day 28 p.c., and mean %lymphocytes (+SEM) of 4 animals for GC and for G1 at day 28 p.c.. (Exception: TCR1-N24+ cells in LN S2: only one control animal is included). Different letters indicate significant differences (P<0.05) within day 14 p.c. and different numbers indicate significant differences (P<0.05) within day 28 p.c..

Number of eosinophils and mast cells in the gut of animals infected with *C. oncophora*

In view of previous results [126] that indicated a contribution of eosinophils in the expulsion of the worms, the recruitment of this cell population was also investigated in the current experiment.

Increased eosinophil numbers were found in the proximal gut of the challenge control animals (G2) at day 14 p.c. but not at day 28 p.c. (P<0.05). In contrast, the number of eosinophils in the primed animals was increased at day 28 p.c. but not at day 14 p.c. (P<0.05) (fig. 6A). In the distal gut, the number of eosinophils were only increased in the primed animals at day 14 p.c.. In both S2 and S6 eosinophils were dispersed throughout the lamina propria of the gut but the influx of eosinophils resulted more specifically in a higher number of cells in the upper part of the villi (data not shown).

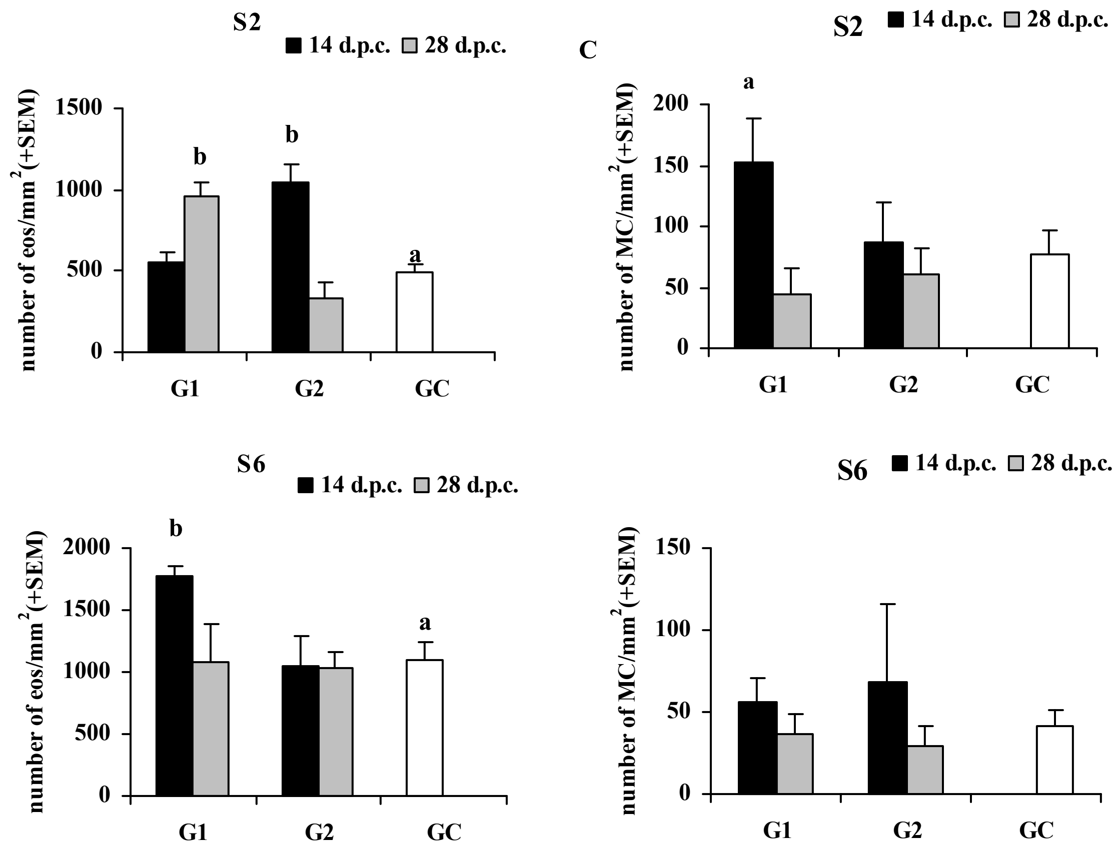

FIGURE 6 Number of eosinophils in the proximal (A) and the distal (B) gut and number of mast cells in the proximal (C) and the distal (D) gut are given for the animals of G1 (100,000 L3/100,000 L3), G2 (0 L3/100,000 L3) and the non infected controls (GC). Different letters indicate significant differences (P<0.05).

We previously demonstrated that expulsion of worms from calves which were primary infected with *C. oncophora* was not related to mast cell numbers in the gut. Similarly, in the current experiment the number of mast cells in S2 and S6 of the challenge control group (G2) did not differ with the non infected controls. However, an increased number of mast cells was found in S2 of the primed animals (G1) at day 14 p.c. (P<0.05) (fig. 6C). Mast cell numbers in the distal gut did not significantly differ among G1, G2 and GC (fig. 6D).

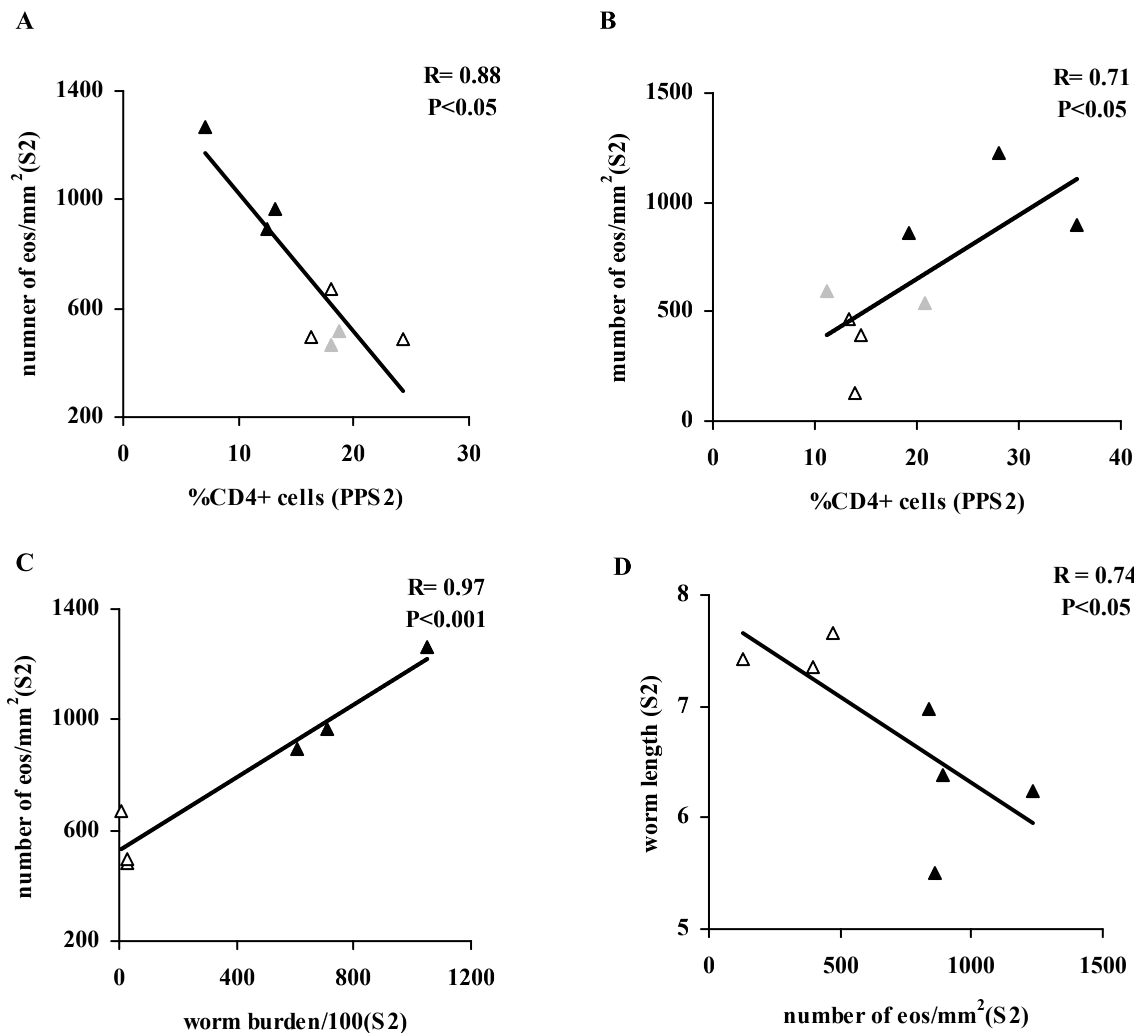

FIGURE 7 Correlation between %CD4+ cells in PP S2 and eosinophils in the proximal gut at day 14 (A) and day 28 p.c. (B). Data from animals from G1 (white), G2 (black) and GC (grey) are included (at day 28 p.c. data from 1 animal of G1 are lacking). Correlations between eosinophils and worm burden in the proximal gut at day 14 p.c. (C) and between eosinophils and worm length in the proximal gut at day 28 p.c. (D). Data from animals from G1 (white) and G2 (black) are included.

Relationships between T lymphocyte subsets and effector cells

Analysis of the proximal gut at day 14 p.c. revealed a weak and negative correlation between the frequency of CD4+ cells in the lamina propria and the peripheral blood with the number of eosinophils ($R=-0.45$ and $R=-0.5$ respectively, $n=7$). The same negative relationship, but stronger was found between eosinophilia and %CD4+ cells in LN S2 ($R=-0.81$, $P<0.05$) and PP S2 (fig. 7A). The relationship was reversed at day 28 p.c. (fig. 7B). At this time point, the relationship between eosinophils and CD4+ cells from the other locations was weak and the negative correlation with the CD4+ cells in LP S2 prevailed (LN S2: $R=0.44$, LP S2: $R=-0.48$ and PBL: $R=0.54$). In the distal gut, irrespective of the anatomical location, the frequency of CD4+ cells was always positively correlated to the eosinophil numbers at day 14 p.c., but only the correlation in the lamina propria was significant ($R=0.71$, $P<0.05$). At day 28 p.c. the frequencies of both cell populations were not related in the distal gut.

Similar analyses revealed no relationships between the frequency of CD4+ cells and the mast cell numbers. Correlation analysis with other T cell subsets revealed that at day 14 p.c. the number of mast cells in the proximal gut was significantly correlated with the frequency of TCR1-N24+ cells in LN S2 ($R=0.86$, $P<0.01$), which coincided with the higher number of mast cells found in LP S2 of primed animals at this time point.

Relationship between effector cells and parasitological parameters

In this study, a strong relationship between the number of eosinophils and the worm burden in the proximal gut was observed at day 14 p.c. ($P<0.01$, fig. 7C). This coincided with the increased number of eosinophils in the challenge control animals. At day 28 p.c. the number of eosinophils were only increased in the primed animals and at this time point, we measured a significant negative correlation between eosinophil numbers and worm length (in the proximal gut) in the infected animals ($P<0.05$, fig. 7D). No significant effect of mast cells on parasitological variables was found.

DISCUSSION

The current experiment was performed to get an insight in the participation of the T cell mediated immune response (CMI) in amnestic immunity to *C. oncophora*. The parasitological data demonstrated that priming with 100,000 L3 conferred protective immunity against a homologous re-infection and this was evidenced by a reduced worm burden, reduction in egg excretion and a decrease in worm length and fecundity (Chapter 4). Herein, we report on the kinetics of the CMI in the peripheral blood and the intestine during this rejection phase.

Expulsion of *C. oncophora* is characterized by a distal shift in the worm distribution throughout the intestine [126] and therefore, the CMI in the proximal (duodenum) and the distal (ileum) gut were analysed separately and compared between primed and the challenge control animals.
The proliferative response to *Cooperia* ESP as well as partially purified fractions revealed differences between primed and challenge control animals. Consistent with previous observation [126], the magnitude of the response was highly variable between the animals. In addition, the reactions were not disseminated throughout the intestine resulting in distinct proliferative responses in the lymph nodes of the proximal and the distal gut. The reactions in peripheral blood and the lymph node of the proximal gut were comparable between primed and challenge control animals while in the distal gut, mainly the primed animals showed ESP-specific proliferation. Throughout the challenge infection the proportion of worms residing in the distal gut was higher in the primed animals (Chapter 4). Hence, the enhanced proliferative response likely resulted from a combined effect of the higher antigenic load during secondary infection and the antigenic priming elicited by the primary infection in the distal gut of these animals.
The reactivity of lymphocytes in response to total ESP resumes the effects induced by different antigens and we hypothesized that the use of ESP fractions in LPT would be superior to total ESP in discriminating among the responses of primed and challenge control animals. However, the main conclusion of the LPT with ESP fractions was that the response of the challenge control animals was markedly reduced as compared to their ESP-specific response. Although the number of animals used in the current experiment was too low to generate any conclusive evidence, the data suggest that fractionating resulted in a pool of proteins which are specifically involved in protective immunity against re-infection with *C. oncophora*.
The potential to discriminate between levels of resistance to infection with *C. oncophora* by means of low molecular weight proteins was previously demonstrated by western blot analysis [171, 231] and suggested that resistance could be related to the recognition of distinct B cell epitopes. There is no information on a similar link with T cell epitopes of *C. oncophora* antigens, but our results suggest that protective immunity against re-infection with *C. oncophora* is linked with an enhanced reactivity to low molecular weight fractions of adult ESP. In calves immunized or primary infected with *O. ostertagi*, no difference was detected in the proliferative response to L3 antigen [56], but there are a few reports in which an enhanced and earlier parasite-specific reactivity was observed after secondary infection or immunization [90, 154, 196]. A more comprehensive analysis of the proteins in the involved ESP fractions and a

higher number of animals will be required to identify the exact role and contribution of these proteins to protective immunity.
Priming induced a minor increase in the frequency of CD4+ T lymphocytes in the mesenterial lymph node at day 14 p.c. (data not shown). Two weeks later, the frequency of CD4+ cells in primed animals was significantly increased in the peripheral blood and the lymph nodes and Peyer's Patches from the proximal gut but decreased in the lamina propria of the proximal and distal gut and this coincided with a drop in CD8+ and TCR1-N24+ cells in the mesenterial lymph nodes. In the absence of detailed studies in ruminants, the hypotheses for local sensitization by and response to gut luminal antigens are extrapolated from other species [122, 120, 191]. It is assumed that lymphocytes that have been primed in the intestine migrate via the afferent lymphatic to the draining lymph node where they further proliferate and differentiate. Thereafter, they recirculate via the efferent lymphatics and blood back to the lamina propria of the gut. Based on this recirculation pathway, we postulate that the decreased frequency of CD4+ cells in the lamina propria of the infected animals at day 28 p.c. was caused by a migration of activated cells to the lymph node and the peripheral blood. These findings were consistent with the enhanced proliferation in blood and lymph nodes in response to *C. oncophora* antigens at this time point and suggested that the CD4+ cells were the main proliferating cells. The latter observations were done irrespective of the immune status of the animals, and only the increased frequency of CD4+ cells in Peyer's Patches and peripheral blood of the primed animals supported an enhanced trafficking of lymphocytes in immune animals as compared to challenge control animals. The analysis of CD8+ and TCR1-N24+ cells and the co-expression of CD62L and MHCII on the different T cell subsets were not conclusive. It is however conceivable that substantial proportional changes in CD62L+ or MHCII+ T lymphocytes were obscured by the background of responses to other antigens to which the gut is exposed.
Eosinophils and mast cells are both hallmarks of GI nematode infections [192, 157, 162, 163]. We previously demonstrated that a primary infection with 100,000 L3 resulted in a recruitment of eosinophils in the lamina propria of infected animals at day 42 p.i. [126]. A minor increase in eosinophils was found at day 14 p.i. while no changes were observed in the mast cell population [126]. Based on the features of the infection we hypothesized that the early increase in eosinophils was caused by inflammation as a consequence of larval/worm exposure, whereas by the end of the primary infection eosinophils were involved in expulsion of the adult worm population [126]. The results from the current study confirmed these previous observations. The number of eosinophils in the lamina propria of the proximal gut of the challenge control

animals at day 14 p.c. was likely increased as a consequence of inflammation of the parasitized intestine. Considering their previous exposure, we hypothesized that the acute inflammation of the proximal gut occurred earlier in the primed animals and could not be detected any more at day 14 p.c.. Consistent herewith the more distal distribution of the worm population and the accelerated expulsion in the primed animals resulted in an accumulation of eosinophils in the distal gut at day 14 p.c. while at day 28 p.c. the number of eosinophils was elevated in the proximal gut of these animals. The positive correlation between eosinophil numbers and the worm burden at day 14 p.c. and the subsequent negative correlation with worm length and fecundity at day 28 p.c. fitted within the hypothesis that the early recruitment of eosinophils was caused by recent exposure to the parasite while in the later phase of the infection, eosinophils acted as effector cells.

The appearance of two waves of eosinophil infiltrates has been observed in other parasite host interactions [156]. A kinetic study of the inflammatory response induced by parasite products in a mammary gland model indicated the existence of two separate mechanisms of eosinophil recruitment [27]. The first response occurred early after infection and induced a recruitment of eosinophils mediated by a type 1 hypersensitivity reaction. The second response consisted of a recruitment of eosinophils mediated by a Th2 type reaction, involving the secretion of IL-5 and eosinophil specific chemotactic factors by the T cells. Similarly, experimental infection of T cell deficient mice with *Toxocara canis* revealed that the second wave of eosinophils was T cell-dependent in contrast to the first wave which did not depend on T cells [141]. The T cell dependency of the eosinophilia was currently investigated by measuring the correlation between eosinophils and CD4+ cells. The strongest correlation prevailed with CD4+ lymphocytes from the Peyer's patches but the tendency was the same in the different anatomical locations. The correlation between the CD4+ cells and eosinophils at day 14 p.c. was negative whereas at day 28 p.c. a positive correlation was found. This supports a key role for CD4+ cells in the recruitment of the second but not the first wave of eosinophils following *C. oncophora* infection.

Analysis of number of mast cells revealed no dramatic changes. Mast cells remained comparable to control levels in the challenge control animals, but were significantly increased in the primed animals at day 14 p.c. and the influx of mast cells was associated with a higher number of cells found scattered throughout the lamina propria and the upper part of the villi. In both rodents and ruminants, in situations where mast cells appear to play an important role in resistance to infection, the exact mechanism by which they do so has not yet been identified [163, 65]. Although the accumulation of mast cells in the proximal gut of the primed animals at day 14

p.c. concurred with a decreased worm burden in these animals, the lack of significant correlations underscores that mast cells do not represent an important effector mechanisms in immunity against *C. oncophora*. The observation that mast cell numbers in the proximal gut were significantly correlated with the TCR1-N24+ cells but not with CD4+ cells was surprising, considering that mastocytosis is controlled by cytokines such as IL-3, IL-4, IL-9 and IL-10 which are predominantly secreted by CD4+ Th2 cells [83]. Further investigation is required to discern the exact meaning of the positive correlation between the two cell populations.
In conclusion, with the current results we hypothesize that at least part of the protective memory response to *C. oncophora* is T-cell mediated. This is evidenced by a recruitment of CD4+ cells and an enhanced proliferative response to *C. oncophora* antigens. Proliferation induced by ESP fractions occurred predominantly in primed animals suggesting that fractionating resulted in a pool of proteins associated with protective immunity against re-infection. In addition, our data support that primary and secondary infection with *C. oncophora* induced 2 waves of eosinophils and that the kinetics of this cell population differed as a result of priming. Based on observed correlations we propose that the early increase in eosinophils is independent of T cells and a consequence of inflammation in the parasitized gut. In contrast, in the later phase of the infection eosinophils seem to be recruited by CD4+ cells and involved as effector cells against adult stages of *C. oncophora*.

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Chapter 7

Summarizing Discussion

Imagination is more important than knowledge.

(A. Einstein)

INTRODUCTION

In this thesis, we focused on the study of the development of immunity to *C. oncophora* in young animals, which is the most susceptible age group. First, we characterised the events associated with the development of acquired immunity during a primary infection. Subsequently, we investigated whether the generated immune response was long lasting and protective against re-infection. The cellular and humoral components of the immune response during primary and secondary infections were analysed separately and attempts were made to link the immune response with the parasitological outcome of the infection. For this purpose we used experimental infections with 100,000 L3 infective larvae in calves at 3 months of age. For the study of the local events during infection the model was adapted; the small intestine was divided in different segments which provided us with the opportunity to perform a detailed analysis of the events occurring in the gut of the infected animals. In the following section, the term 'acquired immunity' will be used for immunity generated during a primary *C. oncophora* infection, whereas ' protective immunity' will refer to the immune responses in animals previously primed with *Cooperia.*

1. HOST RESPONDER TYPES FOLLOWING PRIMARY *COOPERIA ONCOPHORA* INFECTION

Infection of calves of 3 months of age with 100,000 L3 infective larvae of *C. oncophora* has proven to result in a large difference in parasitological variables [231]. Although variability is commonly accepted as being a major disadvantage in the study of biological processes, the advantage of this experimental scheme is that based on egg output and worm burden, animals can be subdivided in three major responder types: high, intermediate and low responder animals (see chapter 1 (fig 1) and 2). The classification of animals infected with *C. oncophora* in three responder types based on the parasitological outcome of the infection is likely an oversimplification of reality. However, it has provided an excellent framework to characterize the immune responses associated with expulsion of *Cooperia*. In chapter 2, we demonstrated that in addition to parasitological variables the systemic *Cooperia*-specific antibody response can also be used to differentiate between low and intermediate responder animals. High responders were not included in the analysis and this was caused by the fact that high responders comprise only a small proportion of the population (± 2%). Hence, a large group of animals would be required to obtain a sufficient number of this responder type for analysis. Moreover, the genetic determinants associated with the responder types are still unknown and consequently the differentiation into low, intermediate and high responders can only be done after infection.

2. PARASITOLOGICAL RESPONSES TO INFECTION WITH *C. ONCOPHORA*

Both primary and secondary infections with *C. oncophora* had a significant effect on parasitological parameters (chapter 1, 2 and 3) but some key features differed between the effect of acquired and protective immunity on the parasitological outcome of the infection. Moreover, whether an additional effect of the host responder type prevailed depended on both the parasitological parameter investigated as on the immune status (primary versus secondary infection) of the animals.

A decrease in worm survival and establishment and survival occurred as a result of acquired immunity (chapter 1 and 2) and of protective immunity (chapter 3). In both cases, the reduced establishment was related to the responder type of the animals: intermediate responders had less worms than low responders. We did not discriminate between the parasitological events involved in the decreased establishment but based on the kinetics of the infection, these were likely different in primary and secondary infected animals. In general there are three patterns of worm expulsion (reviewed in [117]): i) rapid expulsion of incoming infective larvae apparently occurs before larval establishment takes place ii) expulsion of developing larvae or pre-adults which have already established occurs before they reach adulthood and iii) expulsion of adult worms. Reduced establishment in primary infected animals occurred far beyond the development into adult worms and was caused predominantly by adult worm expulsion. In contrast, the reduced worm burden in primed animals was likely caused by a combined effect of rapid expulsion, larval and adult expulsion. The absolute numbers of L4 in primed animals suggested that inhibited development did not contribute substantially to protective immunity against *C. oncophora*. Worm length and fecundity were affected differently by acquired and protective immunity. In addition, an effect on worm length was not always related to an effect on worm fecundity. Within our experimental set up, worm length was influenced by the protective immune response, but not by acquired immunity. Primed animals had shorter worms, irrespective of their host responder type. Furthermore, the dose-dependency of the effect on worm length related more to the temporal kinetics of worm development than to the attainable adult worm length as such. Based on the observation that animals primed with a low or high dose had similar worm burdens, we propose that the effect on worm length was caused by a 'distinct' immune response induced specifically by the low or the high priming dose and that it was not a consequence of density-dependent intra-specific competition for resources within the host gut.

Worm fecundity was influenced by acquired and by protective immunity and clearly related to the host responder type in primary infected animals (chapter 3 and 4). We were not able to

confirm this responder type-dependent effect in primed animals but this might have been caused by the low number of animals involved. The observation that the two primed animals which were excreting eggs were low responders and had the highest fecundity pleads for a sustained influence of host responder type on worm fecundity in primed animals (chapter 3).
Interestingly, based on our data we would conclude that immunity to *Cooperia* resulting in altered parasitological parameters develops in two stages; firstly, animals control worm establishment and subsequently they control worm length and fecundity. This is contradictory to what has been demonstrated for sheep infected with *O. circumcincta*, and suggested for abomasal infections of *O. ostertagi* in cattle and *H. contortus* in sheep [211]. Is this the consequence of the different anatomical and immunological environment? Or does it solely depend on distinct host-parasite interactions? The observation that lambs infected with the intestinal *T. colubriformis* succeed in controlling worm numbers at relatively young age [244] supports the hypothesis that in the intestine worm burden is more easily controlled than in the abomasum. However, worm length and fecundity were not assessed in *T. colubriformis* infected lambs [244] and we can consequently not exclude an earlier effect on worm length or fecundity in these lambs. Other reports have yielded variable results [152, 70]; hence, a comparable experimental set up together with a similar definition of worm fecundity should reveal whether the regulation of immunity depends on the anatomical location.

3. Acquired and Protective immunity to *Cooperia oncophora*

It remains a challenge to elucidate at which level genetic determinants affect the immune response in different responder types, and although the understanding of the complexity of the immune response to parasites is still at a rudimentary stage, the amount of knowledge obtained from rodent models is increasing steadily. Commonly protective immunity to helminths is associated with a Th2 response [83]. The magnitude and effectiveness of the immune response of mice infected with *Heligmosomoides polygyrus* varies between mouse strains. However, irrespective of the strain, the immune response remains polarized towards a type-2 cytokine pattern (reviewed in [93]). In contrast, following infections with the whipworm *Trichuris muris*, a spectrum of responses develop in different mouse strains, ranging from a strong Th2 response associated with worm expulsion (BALB/c), a mixed Th1 and Th2 response with delayed expulsion, to finally a Th1 response resulting in chronic infection (AKR) [67, 64]. Based on these observations, one could hypothesize that the higher susceptibility and the inadequate development of acquired immunity in the low responders would be a consequence of the

development of a Th1 response, as found in *T. muris*, or, the development of an ineffective Th2 response as found with *H. polygyrus*.

By means of isotype-specific ELISAs with different *Cooperia* antigens, we demonstrated that irrespective of the responder types, a primary infection with *Cooperia* induced a type 2-shift in the immune response. Low responders had a hampered ability to initiate this type 2 response and serum IgG1 levels remained significantly lower as compared to intermediate responders. The ineffectiveness of the response was further illustrated by correlations with parasitological variables and serological findings were confirmed by analysis of the local immune response (chapter 3). Infection provoked a significant increase in eosinophils and in *Cooperia*-specific mucus IgG1 and IgA in intermediate responders and these parameters where negatively correlated with infection intensity (fig. 1). We cannot exclude that we missed an immune response in the low responders as the tools we used were biased towards the detection of a Th2 response, but the development of a Th1 response would probably have been associated with increased *Cooperia* IgG2 levels, which were not found. Therefore, we propose that the unresponsiveness of the low responders mainly results from their inability to induce an effective Th2 response. However, it still remains to be elucidated at which level the development of an effective immune response in low responders is hindered.

It is well established that multiple factors contribute to the initiation and development of an immune response, including cytokine environment, antigen presenting cell type, antigen dose and others. Dendritic cells occupy a central position in the immune system as the cells responsible for priming of naïve T cells [17]. One could hypothesize that the distinction among low and intermediate responder animals originates from an impaired antigen presenting capacity in low responders. However, there are a few observations to consider before drawing any conclusions. Experiments in which corticosteroids were administered to animals during infection revealed that the observed peak in egg excretion at day 21-28 p.i. can be enhanced as a result of immune suppression (H. W. Ploeger, personal communication). These observations suggest that the initiation of the immune response is comparable in effectiveness between low and intermediate responders but that in the later phase of the infection the level and effectiveness of this response becomes insufficient in low responders. However, treatment of hosts with corticosteroids to abrogate the parasite specific immune response, also affects the host metabolism [22]. The increased appetite and food intake induced by steroids also influences the availability of nutrients for the parasites and might thereby enhance their reproduction capacity but, in contrast, might also enhance the acquisition of immunity by the host [48].

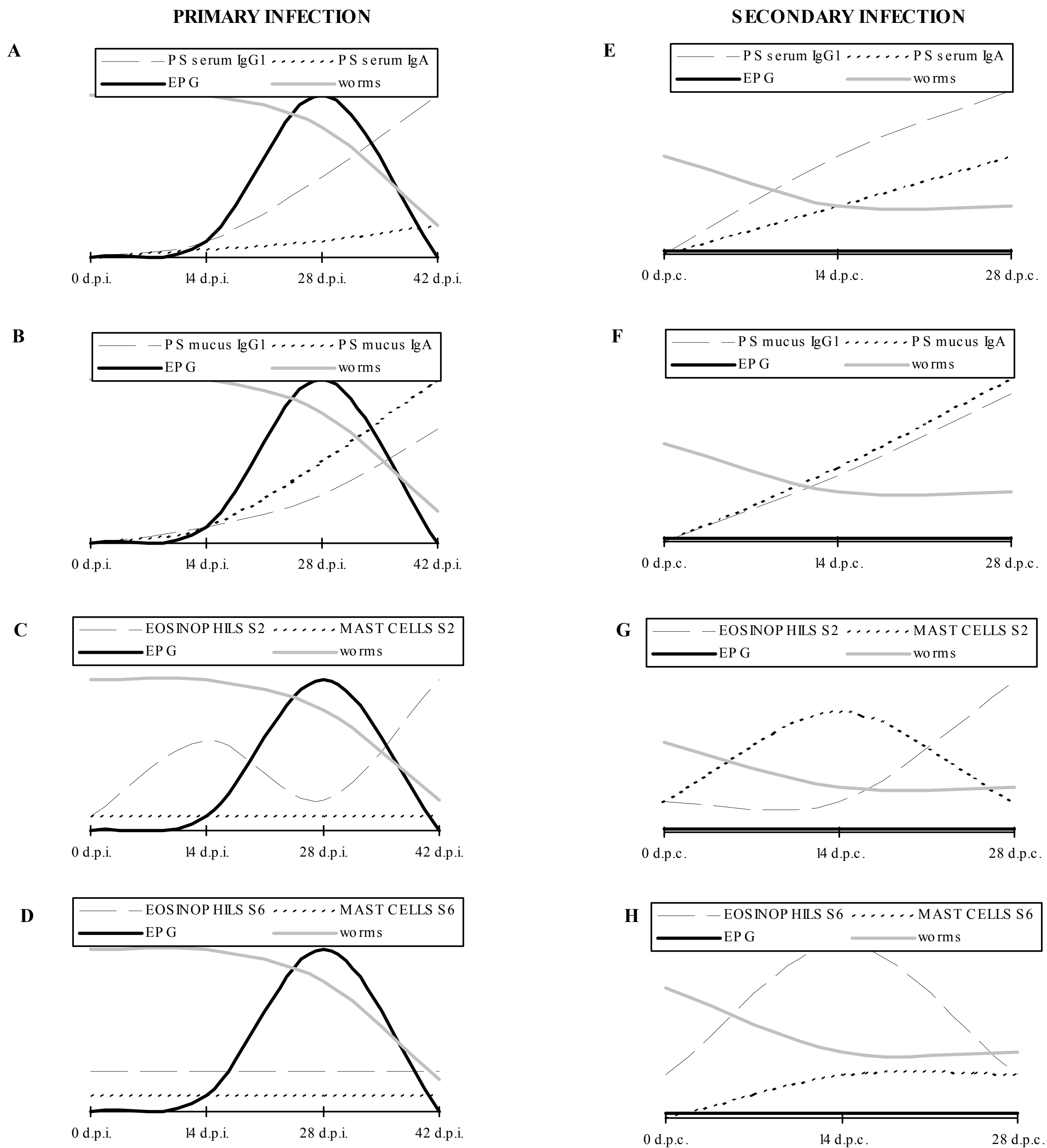

FIGURE 1. Schematic representation of effectors involved in primary and secondary infections with *Cooperia oncophora*. (A, B, C, D): during primary infection. (E, F, G, H): during secondary infection. PS=parasite specific; EPG=egg output; S2=proximal gut=jejunum; S6=distal gut=ileum. (days after infection=d.p.i., days after challenge=d.p.c.)

Nevertheless, priming had an effect on establishment of worms in low responders, implying that immunity was generated (and consequently initiated in these animals) (chapter 4). It is interesting to note that despite the distinct parasitological outcome, the serological response upon re-infection of low and intermediate responders only differed in its kinetics. Hence, maybe low responders are only 'slow' responders. Given the complexity of interactions, every conclusion needs to be taken carefully but we propose that some additional factors inherent to the genetic constitution of low responders might be suppressive to the host immune response or subvert it into producing an ineffective response.
All the above observations are done assuming that differences in the outcome of infection between low and intermediate responders are mainly driven by host factors. However, there is an alternative view which comes from the *T. muris* model [105]. In addition to differences in susceptibility to infection between inbred strains, there are also some strains which show a so called split-response phenotype i.e. differences between individuals within one strain. Animals are genetically identical and consequently they cannot differ in terms of host genes or mechanisms involved in immune responses. Furthermore, their split-response phenotype is observed following the same infection regime. Thus for the *T. muris* system at least, there is compelling evidence to suggest that the parasite itself is inducing susceptibility by redirecting the host immune response towards one which is inappropriate for mediating worm expulsion. Still, the observation that the split response phenotype in *T. muris* infected mice occurs in some inbred strains but not in all emphasize a role for the genetic constitution of the host and suggest that independent of the host-parasite system studied, the observed phenotype results from an interaction of both host and parasite-derived factors. Implemented in our model, this hypothesis implies that worms which infect low responders would succeed in subverting the immune response and this could be achieved by the secretion of products which affect the immune system.

3.1. Involvement of the humoral immune response

The induction of antibodies to *Cooperia* in primary infected animals is the most convincing evidence in our model that within the intestine the host recognizes *C. oncophora* antigens and respond vigorously to them (chapter 2, 3 and 5). The preferential induction of *Cooperia*-specific serum IgG1 titres following infection was described previously [176], but the finding that *Cooperia*-specific serum IgG1 levels enabled differentiation among responder types was novel. Antibody levels in serum and mucus increased as the egg output decreased in primary infected animals (fig. 1A and 1B). The causality of both parameters was not directly investigated and

left us with the following questions: i) Is the decrease in egg output induced by the increase in antibody levels? Or, ii) are increased antibody titres and decreased egg output the outcome of a yet non identified mechanism? The different outcome of correlation analyses in animals that mount an effective immune response and animals which do not, supported the predicted role of antibodies as effectors of the acquired immunity against adult worms. In addition, correlation analysis enabled us to distinguish between the selective effects of the different Ig isotypes.

The role of the antibody response as effector in worm expulsion was less clear in secondary infected animals (chapter 5 and fig. 1E and 1F). Upon challenge a high and fast increase in parasite specific IgG1 and IgA antibodies was observed, at a similar level in low and intermediate responder animals that was not strongly correlated to parasitological parameters associated with an effect on adult worms. The antibodies were nearly back to control levels at the time of challenge, but given the fast increase they might have affected the larval stages in the early phase of the infection. Hence, in primed animals antibodies could be partly involved in the clearance of the pre-adult stages and have less effect on the established adult worm population. This fits with the observation that upon re-infection the increase in antibody titres was slower in low responders. Thus, the less reduced establishment in these animals may be attributed to a lower level of antibodies at the time of expulsion of larval stages.

B cells seemed similarly involved in primary and secondary infected animals. The high serological and mucosal antibody titres associated with *Cooperia* infection emphasize the prominent functional role of B cells as antibody secreting cells. In addition to their role as antibody secreting cells, B cells can also act as antigen presenting cells and enhance a Th2 driven immune response. The increased CD86 expression and the observed correlations with the *Cooperia*-specific mucus IgG1 titres and eosinophilia, two hallmarks of the type 2 immune response induced in *Cooperia* infected animals, indicated for the first time that B7-interactions might be involved in the generation of a type 2 response following *C. oncophora* infection (chapter 5). In addition, the subtle difference in CD86 expression on B cells between primary and secondary infected animals, suggested that CD86-interactions have a more prominent role in protective immune responses to *Cooperia* than in acquired immunity.

3.2. Involvement of the cell mediated immune response

The role of CMI immune response has been shown in different rodent models [63]. The differentiation into an IL-4 producing T cell is an important step in the development of effective host-protective immune responses in the *H. polygyrus* and *T. muris* models In contrast, the role of IL-4 in protective immunity to *N. brasiliensis* is more complex, and the

contribution of IL-13, another Th2 cytokine, was revealed [83]. Independently of the cytokines required, the consensus of these models is that CD4+ Th2 cells drive the immune response into effectors which clear the infection. A direct role for this cell population was not demonstrated in calves infected with *C. oncophora*, but indirectly, the preferential induction of IgG1 and IgA antibodies as opposed to IgG2 antibodies, the elevation in total serum IgE levels and the involvement of eosinophils as effectors all support the contribution of CD4+ Th2 cells in acquired and protective immunity to *C. oncophora.*

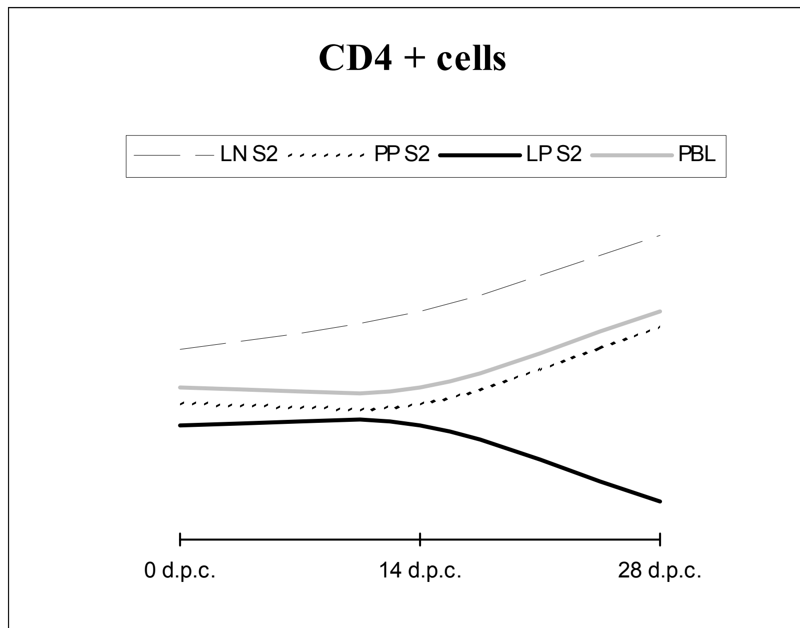

FIGURE 2. Schematic view on kinetics of CD4+ cells in different anatomical locations of *C. oncophora* re-infected calves. The observed changes are similar in primary infected animals, but occur at a slower rate. (days after challenge=d.p.c.)

Based on the phenotypic analysis of T cell subsets in different anatomical locations (fig. 2) and with the underlying assumption that *Cooperia* specific T-lymphocytes are indeed CD4+ cells we defined the following pathway. In naïve animals, upon infection parasite antigens are presented in the gut by APC which in turn activate CD4+ cells. These *Cooperia*-specific cells migrate to the draining lymph node and can be detected with *in vitro* proliferation tests (chapter 3 and fig. 3). Lymphocytes which have been primed seem to have an enhanced recirculation capacity and are recruited more efficiently to the draining lymph node upon re-infection (chapter 5 and fig. 3). Parasite specific cells will eventually migrate back to the gut and differentiate into memory CD4+ cells. Their main function in the gut is immune surveillance and upon a next encounter with the antigen, they will differentiate into effector cells and recirculate again.

3.3. A specific role for eosinophils in the expulsion of *C. oncophora*

Both primary and secondary infections with *C. oncophora* were characterised by two waves of eosinophils, the first one in the early part of the infection and the second one, which was more prominent, coinciding with adult worm expulsion (chapter 3 and 5, fig. 1 C, D, G, H).

The appearance of two waves of eosinophil infiltrates has been observed in other parasite host interactions (reviewed in [156]). A kinetic study of the inflammatory response induced by parasite products in a mammary gland model indicated the existence of two separate mechanisms of eosinophil recruitment [27]. The first response occurred early after infection and induced a recruitment of eosinophils mediated by a type 1 hypersensitivity reaction. The second response consisted of a recruitment of eosinophils mediated by a Th2 type reaction, involving the secretion of IL-5 and eosinophil specific chemotactic factors by the T cells.

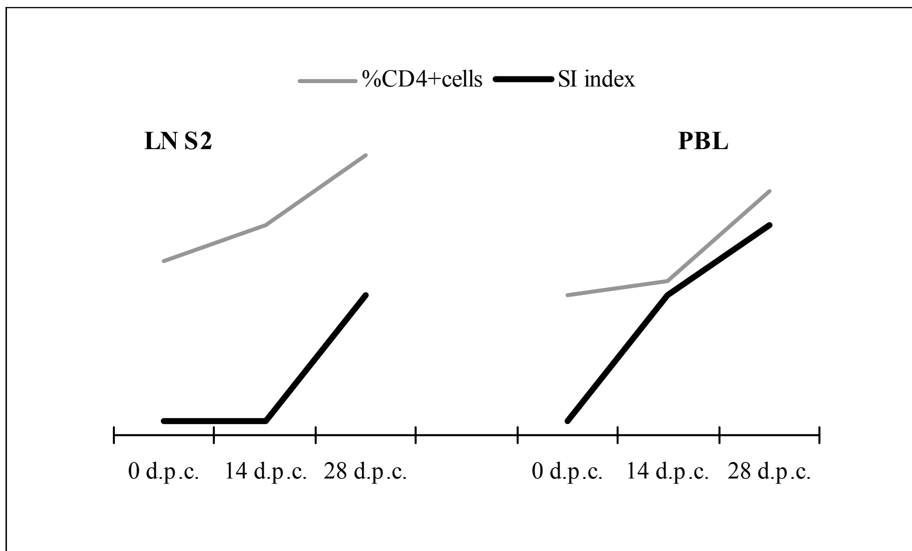

FIGURE 3. The increased frequency in CD4+ cells in draining lymph node (LN S2) and peripheral blood (PBL) of infected animals concurs with an increased number of *Cooperia*-specific cells. (days after challenge=d.p.c.)

The immediate hypersensitivity type 1 or IgE mediate hypersensitivity depends on mast cells and is assumed to occur within 30 min of sensitization. Stimulated mast cells release all kind of mediators including eosinophil chemotactic factors; thus, massive accumulations of eosinophils are characteristics of this type of hypersensitivity [192]. Eosinophils are believed to survive in tissues for several days [125] and, during helminth infection their survival in tissue is enhanced [202]. Hence, the observed eosinophilia in the early part of the infection might be a remainder of this reaction.

A potent stimulus for mast cell degranulation is provided by antigen binding to antigen-specific IgE attached to the high affinity IgE receptor (FcεRI) expressed by mast cells. This pathway would however imply that *Cooperia* induces the generation of parasite specific IgE in the early

stage of the infection. The first wave of eosinophils in primary infected animals occurred far ahead the generation of a specific immune response and if mast cells were indeed responsible for the inflammatory response and subsequent influx of eosinophils, the activation of mast cells was likely triggered by a distinct mechanism (reviewed by [52]). However, the situation in secondary infected animals differed; we did not measure *Cooperia*-specific IgE but total IgE levels increased rapidly following challenge of primed animals. Hence, IgE-dependent mast cell degranulation is plausible in primed animals.

Finally, a mechanism which may apply for both primary and secondary infected animals is a mast cell-independent innate inflammatory response which affects worm establishment and development (reviewed in [156]). In the described models, resistance was reduced by IL-5 depletion and it was suggested that some IL-5 dependent eosinophil induction might have occurred via leukocyte populations involved in innate immune responses (NK cells or γδ T-cells) [157]. Consistent herewith, it was demonstrated that a substantial proportion of γδ T-cells in sheep expressed IL-5 and thereby might act as important regulatory cells in the mechanisms involved in parasite expulsion [18].

The second wave of eosinophils in *Cooperia* infected calves occurred as a result of adaptive immune responses, seemed to be dependent on CD4+ cells and involved in the expulsion of adult *Cooperia* (chapter 4 and 6). We did not provide *in vitro* or *in vivo* evidence for an eosinophil-mediated killing of adult *Cooperia,* but the observed correlations with parasitological variables related to survival of adult worms lead us to conclude such an action. Larvae or adult worms are most efficiently affected by eosinophils in cooperation with antibodies or complement factors which bind on their cell surface, thereby inducing the release of cytotoxic products. Our observations supported a cooperative role for *Cooperia*-specific mucus IgA in primary infected animals whereas in secondary infected animals, *Cooperia*-specific mucus IgG1 antibodies seemed predominantly involved.

4. SUMMARY: A PROPOSED MECHANISM FOR EXPULSION OF *COOPERIA ONCOPHORA*

When speculating which mechanisms are involved in the expulsion of *Cooperia*, it is important to bear in mind that the observed findings were done under strict experimental conditions. Hence, the redundancy of a particular protection mechanism under a well defined experimental set-up will not necessarily reflect natural conditions. However, the comparison of the current model with natural infections should reveal the relevance of our observations. In the following section, the ideal situation will be outlined i.e. an effective acquired or protective immune

response eventually resulting in parasite expulsion. In reality, this will resemble the immune response as observed in intermediate responders.

Following infection, L3 infective larvae arrive in the anterior part of the small intestine as early as day 3 p.i. [121]. The larvae or parasite derived products generate an inflammatory reaction which results in an increased number of eosinophils into the parasitized gut. This can be mast cell-dependent or via IL-5 secreted by leukocyte populations involved in the innate immune response, such as γδ T cells or NK cells. The innate inflammatory response in primary infected animals is not effective in killing larval stages, presumably due to the lack of parasite-specific antibodies at this time point. In primed animals however, the activation of eosinophils might contribute to the rapid expulsion of the incoming larvae, resulting in a significantly reduced establishment of the worms in the gut. In addition, the inflammatory response in the proximal gut possibly influences the worms to move to the more distal part of the gut, either passively as a consequence of the inflammatory-mediated enhanced gut motility, or actively to avoid the detrimental immune environment.

Simultaneously with this non specific inflammatory response the generation of an adaptive immune response is induced. Based on the increase in L3, Ad en ES-specific Ig titres it is likely that both somatic and excretory/secretory antigens are internalised and presented to CD4+ cells which then provide help for B cells and the production of *Cooperia*-specific antibodies. Which APCs are initially involved in the activation of naïve T cells is not clear yet but a role for DCs is likely. Primed T cells subsequently activate B cells which results in upregulation of B7-2 on the surface of B cells. The functional role of B cells in *Cooperia* infected animals seems double. Their main function as antibody secreting cells is evidenced by the high amount of antibodies which are induced upon primary and secondary infection. A second function relies on the expression of B7-2 on their surface which allows them to potently enhance and maintain the generation of a Th2 effector immune response.

Activation of lymphocytes and the most prominent changes in lymphocyte population coincide with the peak in egg excretion in primary infected animals. The causality of these observations is difficult to interpret but from then on, immune effectors are induced that results in affected parasite fitness. In primary infected animals this is evidenced by a decreased egg output, decreased fecundity and reduced worm burden in the second phase of the infection. In primed animals parasite fitness is affected immediately upon challenge and features are delayed development, stunted growth, reduced fecundity, altered morphology and reduced establishment. We identified two main effectors being eosinophils and parasite-specific antibodies, more specifically serum IgG1 and mucus IgA and IgG1 antibodies. Antibodies

might directly affect the parasite or indirectly by cross-linking on the Fc receptor on the surface of eosinophils. Their contribution in adult worm expulsion is presumably more important in primary infected animals but, although we have no direct proof for this, we propose that in secondary infected animals parasite specific antibodies are predominantly involved in larval expulsion in the early phase of the infection. The role of IgE in *Cooperia* infection is less clear but seems to differ between primary and secondary infected animals. While we could not link the IgE response to generation of acquired immunity in primary infected animals, the association with host responder types upon secondary challenge indicated a role for total serum IgE in protective immunity against *Cooperia*.

5. Conclusions and Future Strategies

As shown in this report, the development of acquired and protective immunity to *C. oncophora* is fast, but remains quite complex. This is emphasized by the observations that infection does not successfully induce immunity in all animals. *Low* responders remain susceptible to infection, and therefore, these animals should be targeted if control strategies such as vaccines are to be implemented in the total population. The key to a suitable vaccine will depend on its ability to induce a protective Th2 response in *Low* responder animals. Based on the observation that in *Low* responders a Th2 response is initiated but remains at a level which is not effective to induce worm expulsion, we could speculate that the use of a suitable adjuvant enhancing Th2 responses might overcome the inefficiency of their immune response, but reality will likely be more complex. Furthermore, a comprehensive analysis of *C. oncophora* specific proteins and the identification of proteins specifically involved in immune responses would allow a more targeted analysis of the immune response and might reveal some fundamental differences between *Low* and *Intermediate* responder animals. However, vaccine trials with purified antigens or recombinant proteins still have to deal with a high variability in response within a host population (reviewed in [69]), which emphasizes the need for the identification of host genes linked with host resistance, either innate or acquired.

Given the effective immunity following natural infection and the low pathogenicity of *Cooperia*, it needs to be considered weather the cost/effectiveness and the long run ahead of the development of such a vaccine, is worthwhile for *C. oncophora* alone. But, in view of the development of a cross-reactive protective vaccine against nematodes in general, the study of *C. oncophora* could contribute to the knowledge required. The reproducibility of the induction of protective immunity in a large proportion of the animals is a great advantage as opposed for example to *O. ostertagi*. The latter does not succeed in inducing immunity upon infection and

even suppresses the development of immunity [87]. Consequently, many more aspects need to be circumvented and both the variability of the host's response and the modulation of host response by *O. ostertagi* enhance the complexity of the host-parasite interaction.

In conclusion, the identification of the genetic determinants responsible for the variability in response to infection within a population might be of great value and a breeding program excluding Low responder animals would simplify the global picture. However, with this approach one needs to investigate first whether animals more susceptible to *C. oncophora* infections are equally more susceptible to *O. ostertagi* and other nematodes. Only once genes or markers for the genes that determine parasite resistance will be identified, it will be possible to fully understand how immunity is generated and regulated, and, how to implement immunity-based control strategies against nematodes.

References

1. **Abbas, A.K., K.M. Murphy, and A. Sher, (1996).** Functional diversity of helper T lymphocytes. *Nature*, 383: p. 787-793.
2. **Agneessens, J., et al., (1997).** Epidemiological observations on gastrointestinal nematode infections in grazing cow-calf pairs in Belgium. *Vet Parasitol*, 69: p. 65-75.
3. **Albers, G., (1981).** Genetic resistance to experimental *Cooperia oncophora* infections in calves. *PhD thesis. Agricultural university, Wageningen.*
4. **Almeria, S., et al., (1997).** Isolation and phenotypic characterization of abomasal mucosal lymphocytes in the course of a primary *Ostertagia ostertagi* infection in calves. *Vet Immunol Immunopathol*, 57: p. 87-98.
5. **Almeria, S., et al., (1997).** Quantification of cytokine gene expression in lamina propria lymphocytes of cattle following infection with *Ostertagia ostertagi*. *J Parasitol*, 83: p. 1051-1055.
6. **Anderson, M.C. and J.F. Michel, (1977).** Density-dependent survival in populations of *Ostertagia ostertagi*. *Int J Parasitol*, 7: p. 321-329.
7. **Anderson, R.M. and R.M. May, (1985).** Herd immunity to helminth infection and implications for parasite control. *Nature*, 315: p. 493-496.
8. **Armour, J., (1989).** The influence of host immunity on the epidemiology of trichostrongyle infections in cattle. *Vet Parasitol*, 32: p. 5-19.
9. **Armour, J., et al., (1987).** Pathophysiological and parasitological studies on *Cooperia oncophora* infections in calves. *Res Vet Sci*, 42: p. 373-381.
10. **Axelsen, J., et al., (1986).** Grazing management and nematode parasite control in cattle in the temperate climatic zone of Australia. *Aust J Exp Agric*, 26: p. 267-273.
11. **Baker, D.G. and L.J. Gershwin, (1992).** Seasonal patterns of total and *Ostertagia*-specific IgE in grazing cattle. *Vet Parasitol*, 44: p. 211-221.
12. **Baldwin, C.L., I.W. Morrison, and J. Naessens, (1988).** Differentiation antigens and functional characteristics of bovine leucocytes. *In Z. Trnka and M. Miyasaka (Eds.) Comparative aspects of Differentiation antigens in lympho-haematopoetic tissues.*, Marcel Dekker Inc., New York, N. Y.: p. 455-465.
13. **Baldwin, C.L., et al., (2002).** Activation of bovine peripheral blood gd T cells for cell division and IFN-g production. *Vet Immunol Immunopathol*, 87: p. 251-259.
14. **Balic, A., V.M. Bowles, and E.N. Meeusen, (2000).** Cellular profiles in the abomasal mucosa and lymph node during primary infection with *Haemonchus contortus* in sheep. *Vet Immunol Immunopathol*, 75: p. 109-120.
15. **Balic, A., V.M. Bowles, and E.N. Meeusen, (2000).** The immunobiology of gastrointestinal nematode infections in ruminants. *Adv Parasitol*, 45: p. 181-241.
16. **Balic, A., V.M. Bowles, and E.N. Meeusen, (2002).** Mechanisms of immunity to *Haemonchus contortus* infection in sheep. *Parasite Immunol*, 24: p. 39-46.
17. **Banchereau, J. and R.M. Steinman, (1998).** Dendritic cells and the control of immunity. *Nature*, 392: p. 245-252.
18. **Bao, S., et al., (1996).** Interleukin-5 mRNA expressed by eosinophils and gamma/delta T cells in parasite-immune sheep. *Eur J Immunol*, 26: p. 552-556.
19. **Barger, I.A., et al., (1985).** Regulation of *Haemonchus contortus* populations in sheep exposed to continuous infection. *Int J Parasitol*, 15: p. 529-533.

20. **Barnes, E.H. and R.J. Dobson, (1990).** Population dynamics of *Trichostrongylus colubriformis* in sheep: model to predict the worm population over time as a function of infection rate and host age. *Int J Parasitol*, 20: p. 365-373.
21. **Barnes, E.H., R.J. Dobson, and I.A. Barnes, (1995).** Worm control and anthelmintic resistance: adventures with a model. *Parasitology Today*, 11: p. 56-63.
22. **Barth, E.E., W.F. Jarrett, and G.M. Urquhart, (1966).** Studies on the mechanism of self-cure reaction in rats injected with *Nippostrongylus brasiliensis*. *Immunology*, 10: p. 459-464.
23. **Baxter, E.H. and G.G. Rousseau, (1979).** Glucocorticoid hormone action: an overview. *In J.D. Baxter and G.G. Rousseau (Eds.). Glucocorticoid Hormone Action.*, Springer-Verlag, Heidelberg: p. 1-24.
24. **Behm, C.A. and K.S. Ovington, (2000).** The role of eosinophils in Parasitic Helminth infections: Insigths from genetically Modified Mice. *Parasitology Today*, 16: p. 202-209.
25. **Ben Smith, A., et al., (1999).** The relationship between circulating and intestinal *Heligmosomoides polygyrus* specific IgG1 and IgA and resistance to primary infections. *Parasite Immunol*, 21: p. 587-593.
26. **Bird, A.F. and J. Bird, (1991).** The structure of nematodes. *2nd Ed,* Academic Press limited, London.
27. **Bischof, R.J. and E.N. Meeusen, (2002).** Cellular kinetics of an allergic-type response in a sheep mammary gland model of inflammation. *Clin Exp Allergy*, 32: p. 619-626.
28. **Blackwell, N.M. and K.J. Else, (2001).** B cells and antibodies are required for resistance to the parasitic gastrointestinal nematode *Trichuris muris*. *Infect Immun*, 69: p. 3860-3868.
29. **Bland, P.W. and L.G. Warren, (1986).** Antigen presentation by epithelial cells of the rat small intestine. II. Selective induction of suppressor T cells. *Immunology*, 58: p. 9-14.
30. **Boonstra, A., et al., (2003).** Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper 1 and 2 cells development: dependency on antigen dose and differential toll-like receptor ligation. *Exp Med*, 197: p. 101-109.
31. **Borgsteede, F.H., (1977).** The epidemiology of gastro-intestinal helminth infections in young cattle in the Netherlands. *PhD thesis, Utrecht, The Netherlands*.
32. **Borgsteede, F.H. and J. Hendriks, (1979).** Experimental infections with *Cooperia oncophora* (Railliet, 1918) in calves. Results of single infections with two graded dose levels of larvae. *Parasitology*, 78: p. 331-342.
33. **Borgsteede, F.H., et al., (2000).** Nematode parasies of adult dairy cattle in the Netherlands. *Vet Parasitol*, 89: p. 287-296.
34. **Borgsteede, F.H. and W.P. van der Burg, (1982).** Worm burdens in cows. II. An analysis of the population of nematodes in the abomasum of adult dairy cows. *Vet Parasitol*, 10: p. 323-330.
35. **Brown, W.C., et al., (1994).** CD4+ T-cell clones obtained from cattle chronically infected with *Fasciola hepatica* and specific for adult worm antigen express both unrestricted and Th2 cytokine profiles. *Infect Immun*, 62: p. 818-827.
36. **Brown, W.C., A.C. Rice-Ficht, and D.M. Estes, (1998).** Bovine type 1 and type 2 responses. *Vet Immunol Immunopathol*, 63: p. 45-55.
37. **Brown, W.C., et al., (1993).** Heterogeneity in cytokine profiles of *Babesia bovis*-specific bovine CD4+ T cells clones activated in vitro. *Infect Immun*, 61: p. 3273-3281.
38. **Buddle, B.M., et al., (1992).** Association of blood eosinophilia with the expression of resistance in Romney lambs to nematodes. *Int J Parasitol*, 22: p. 955-960.

39. **Burrows, R.O., P.J. Best, and J.M. Preston, (1980).** Trychostrongylid egg output of dairy cows. *Vet Rec*, 107: p. 399-401.
40. **Canals, A. and L.C. Gasbarre, (1990).** *Ostertagia ostertagi*: isolation and partial characterization of somatic and metabolic antigens. *Int J Parasitol*, 20: p. 1047-1054.
41. **Canals, A., et al., (1997).** Cytokine profile induced by a primary infection with *Ostertagia ostertagi* in cattle. *Vet Immunol Immunopathol*, 58: p. 63-75.
42. **Cassel, D.J. and R. H. Schwartz, (1994).** A quantitative analysis of antigen presenting cell function: activated B cells stimulate naive CD4+ cells but are inferior to dendritic cells in providing costimulation. *J Exp Med*, 180: p. 1829-1840.
43. **Cella, M., F. Sallusto, and A. Lanzavecchia, (1997).** Origin, maturation and antigen presenting function of dendritic cells. *Curr Opin Immunol*, 9: p. 10-16.
44. **Charleston, W.A.G., (1965)**. Pathogenesis of experimental haemonchosis in sheep with special reference to the development of resistance. *J Comp Pathol*, 75: p. 55-67.
45. **Chiodini, R.J. and W.C. Davis, (1992).** The cellular immunology of bovine paratuberculosis: the predominant response is mediated by cytotoxic gamma/delta T cells which prevent CD4+ activity. *Microb Pathogenesis*, 13: p. 447-463.
46. **Claerebout, E., et al., (1999).** Larval migration inhibition activity in abomasal mucus and serum from calves infected with *Ostertagia ostertagi. Res Vet Sci*, 66: p. 253-257.
47. **Coop, R., A. Sykes, and K. Angus, (1979).** The pathogenicity of daily intakes of *Cooperia oncophora* in growing calves. *Vet Parasitol*, 5: p. 261-269.
48. **Coop, R.L. and I. Kyriazakis, (1999).** Nutrition-parasite interaction. *Vet Parasitol*, 84: p. 187-204.
49. **Corry, D.B. and F. Kheradmand, (1999).** Induction and regulation of the IgE response. *Nature*, 402: p. 18-23.
50. **Cox, D.D. and A.C. Todd, (1962).** Survey of gastointestinal parasitism in Wisconsin dairy cattle. *J Am Vet Med Assoc*, 141: p. 706-709.
51. **Crofton, H. and J. Withlock, (1968).** Changes in sex ratio in *Haemonchus contortus cayugensis*. *Cornell Veterinarian*, 58: p. 388-392.
52. **Crowe, S.E. and M.H. Perdue, (1992).** Gastrointestinal tract hypersensitivity: Basic mechanisms of pathophysiology. *Gastroenterology*, 103: p. 1075-1095.
53. **Dainichi, T., et al., (2001).** Nippocystatin, a cysteine protease inhibitor from *Nippostrongylus brasiliensis*, inhibits antigen processing and modulates antigen-specific immune response. *Infect Immun*, 69: p. 7380-7386.
54. **Dawkins, H.J., R.G. Windon, and G.K. Eagleson, (1989).** Eosinophil responses in sheep selected for high and low responsiveness to *Trichostrongylus colubriformis. Int J Parasitol*, 19: p. 199-205.
55. **De Graaf, D., et al., (1992).** Antigenic differences between the life cycle stages of *Cooperia oncophora*. *Res Vet Sci*, 53: p. 390-392.
56. **De Marez, T., et al., (1997).** Induction and suppression of lymphocyte proliferation by antigen extracts of *Ostertagia ostertagi*. *Vet Imm Immpath*, 57: p. 69-77.
57. **Dewhirst, L.W. and M.F. Hansen, (1961).** Methods to differentiate and estimate worm burdens in cattle. *Vet Med*, 56: p. 84-89.
58. **Dorny, P., et al., (1997).** The influence of a *Cooperia oncophora* priming on a concurrent challenge with *Ostertagia ostertagi* and *C. oncophora* in calves. *Vet Parasitol*, 70: p. 143-151.

59. **Douch, P.G., et al., (1983).** In vitro bioassay of sheep gastrointestinal mucus for nematode paralysing activity mediated by substances with some properties characteristic of SRS-A. *Int J Parasitol*, 13: p. 207-212.
60. **Dubucquoi, S., et al., (1994).** Interleukin 5 synthesis by eosinophils: association with granules and immunoglobulin-dependent secretion. *J Exp Med*, 179: p. 703-708.
61. **Ehigiator, H.N., A.W. Stadnyk, and T.D. Lee, (2000).** Extract of *Nippostrongylus brasiliensis* stimulates polyclonal type-2 immunoglobulin response by inducing de novo class switch. *Infect Immun*, 68: p. 4913-4922.
62. **Ekkens, M.J., et al., (2002).** Memory Th2 effector cells can develop in the absence of B7-1/B7-2 interactions and effector Th cells after priming with an intestinal nematode parasite. *J Immunol*, 168: p. 6344-6351.
63. **Else, K.J. and F.D. Finkelman, (1998).** Intestinal nematode parasites, cytokines and effector mechanisms. *Int J Parasitol*, 28: p. 1145-1158.
64. **Else, K.J., et al., (1994).** Cytokine mediated regulation of chronic intestinal helminth infection. *J Exp Med*, 179: p. 347-351.
65. **Else, K.J. and R.K. Grencis, (1991).** Cellullar immune responses to the murine nematode parasite *Trichuris muris*. I. Differential cytokine production during acute or chronic infection. *Immunology*, 72: p. 508-513.
66. **Else, K.J. and R.K. Grencis, (1996).** Antibody-independent effector mechanisms in resistance to the intestinal nematode parasite *Trichuris muris*. *Infect Immun*, 64: p. 2950-2954.
67. **Else, K.J., L. Hultner, and R.K. Grencis, (1992).** Modulation of cytokine production and response phenotypes in murine trichuriasis. *Parasite Immunol*, 14: p. 441-449.
68. **Else, K.J. and D. Wakelin, (1988).** The effects of H-2 and non H-2 genes on the expulsion of the nematode *Trichuris muris* from inbred and congenic mice. *Parasitology*, 96: p. 543-550.
69. **Emery, D.L., (1996).** Vaccination against worm parasites of animals. *Vet Parasitol*, 64: p. 31-45.
70. **Emery, D.L., et al., (1999).** Induction of protective immunity to *Trichostrongylus colubriformis* in neonatal Merino lambs. *Int J Parasitol*, 29: p. 1037-1046.
71. **Emery, D.L., et al., (1992).** Studies of stage-specific immunity against *Trichostrongylus colubriformis* in sheep: immunization by normal and truncated infections. *Int J Parasitol*, 22: p. 215-220.
72. **Emery, D.L., B.M. Wagland, and S.J. McClure, (1993).** Rejection of heterologous nematodes by sheep immunized with larval or adult *Trichostrongylus colubriformis*. *Int J Parasitol*, 23: p. 841-846.
73. **Estes, D.M., N.M. Closser, and G.K. Allen, (1994).** IFN-gamma stimulates IgG2 production from bovine B cells costimulated with anti-mu and mitogen. *Cell Immunol*, 154: p. 287-295.
74. **Eysker, M. and F.N. Kooyman, (1993).** Notes on necropsy and herbage processing techniques for gastrointestinal nematodes of ruminants. *Vet Parasitol*, 46: p. 205-213.
75. **Eysker, M. and H.W. Ploeger, (2000).** Value of present diagnostic methods for gastrointestinal nematode infections in ruminants. *Parasitology*, 120: p. S109-S119.
76. **Eysker M., et al., (1998)**. The efficacy of Michel's dose and move system on gastrointestinal nematode infections in dairy calves. *Vet Parasitol*. 75: p. 99-114.
77. **Eysker M., et al., (1998).** The effect of repeated moves to clean pasture on the build up of gaastrointestinal nematodes in calves. *Vet Parasitol*, 76: p. 81-94.

78. **Ferlin, W.G., et al., (1996).** CD40 signaling induces interleukin-4 independent IgE switching in vivo. *Eur J Immunol*, 26: p. 2911-2915.
79. **Ferrick, D.A., et al., (1995).** Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. *Nature*, 373: p. 255-257.
80. **Fiel, C.A., et al., (2001).** Resistance of *Cooperia* to ivermectin treatments in grazing cattle of the Humid Pampa, Argentina. *Vet Parasitol*, 97: p. 211-217.
81. **Finkelman, F.D., et al., (1991).** Regulation and biological function of helminth-induced cytokine responses. *Immunology Today*, 12: p. 62-67.
82. **Finkelman, F.D., et al., (1997).** Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. *Annu Rev Immunol*, 15: p. 505-533.
83. **Finkelman, F.D., et al., (1997).** Cytokine regulation of host defense against parasitic gastrointestinal nematodes. *Annu Rev Immunol*, 15: p. 505-533.
84. **Frankena, K., (1987).** The interaction between *Cooperia* spp. and *Ostertagia* spp. (nematoda: trichostrongylidae) in cattle. *PhD thesis. Agricultural University, Wageningen.*
85. **Galli, S.J., K.M. Zsebo, and E.N. Geissler, (1994).** The kit ligand, stem cell factor. *Adv Immunol*, 55: p. 1-96.
86. **Garside, P., et al., (2000).** Immunopathology of intestinal helminth infection. *Parasite Immunol*, 22: p. 605-612.
87. **Gasbarre, L., (1997).** Effects of gastrointestinal nematode infection on the ruminant immune system. *Vet Parasitol*, 72: p. 327-343.
88. **Gasbarre, L., E.A. Leighton, and C.J. Davies, (1993).** Influence of host genetics upon antibody responses against gastrointestinal nematode infections in cattle. *Vet Parasitol*, 46: p. 81-91.
89. **Gasbarre, L., E.A. Leighton, and T. Sonstegard, (2001).** Role of the bovine immune system and genome in resistance to gastrointestinal nematode. *Vet Parasitol*, 98: p. 51-64.
90. **Gasbarre, L.C., (1994).** *Ostertagia ostertagi*: changes in lymphoid populations in the local lymphoid tissues after primary or secondary infection. *Vet Parasitol*, 55: p. 105-114.
91. **Gasbarre, L.C., E.A. Leighton, and C.J. Davies, (1990).** Genetic control of immunity to gastrointestinal nematodes of cattle. *Vet Parasitol*, 37: p. 257-272.
92. **Gause, W.C., et al., (1996).** *H. polygyrus*: B7-independence of the secondary type 2 response. *Exp Parasitol*, 84: p. 264-273.
93. **Gause, W.C., J.F. Urban Jr, and M.J. Stadecker, (2003).** The immune response to parasitic helminths: insigths from murine models. *Trends Immunol*, 24: p. 269-277.
94. **Genchi, C., M. Madonna, and G. Traldi, (1989).** Epidemiology of *Ostertagia ostertagi* in dairy cow from different breeding systems. *Parasitologia*, 31: p. 123-132.
95. **Gill, H.S., (1991).** Genetic control of acquired resistance to haemonchosis in Merino lambs. *Parasite Immunol*, 13: p. 617-628.
96. **Gill, H.S., et al., (2000).** Induction of T-helper-1 and T-helper-2 immune responses during *Haemonchus contortus* infection in sheep. *Immunology*, 99: p. 458-463.
97. **Gill, H.S., et al., (1993).** Isotype-specific antibody responses to *Haemonchus contortus* in genetically resistant sheep. *Parasite Immunol*, 15: p. 61-67.
98. **Gill, H.S., et al., (1994).** Antibody-containing cells in the abomasal mucosa of sheep with genetic resistance to *Haemonchus contortus*. *Res Vet Sci*, 56: p. 41-47.

99. **Githiori, J.B., et al., (2000).** Use of a 14.2 kDa recombinant *Cooperia oncophora* protein in an ELISA for herd health monitoring of nematode infections in first grazing season calves. *Vet Parasitol*, 91: p. 63-78.
100. **Gordon, H.M. and H.V. Whitlock, (1939).** A new technique for counting nematode eggs in sheep faeces. *J Counc Sci Ind Res*, 12: p. 50-52.
101. **Gorrell, M.D., et al., (1988).** Lymphocyte phenotypes in the intestinal mucosa of sheep infected with *Trichostrongylus colubriformis*. *Clin Exp Immunol*, 72: p. 274-279.
102. **Greenwald, R.J., et al., (1997).** Effects of blocking B7-1 and B7-2 interactions during a type 2 in vivo immune response. *J Immunol*, 158: p. 4088-4096.
103. **Greenwald, R.J., et al., (1999).** B7-2 is required for the progression but not the type 2 immune response to a gatrointestinal nematode parasite. *J Immunol*, 162: p. 4133-4139.
104. **Grencis, R.K., et al., (1993).** The in vivo role of stem cell factor (c-kit ligand) on mastocytosis and host protective immunity to the intestinal nematode *Trichinella spiralis* in mice. *Parasite Immunol*, 15: p. 55-59.
105. **Grencis, R.K. and G.M. Entwistle, (1997).** Production of an interferon-gamma homologue by an intestinal nematode: functionally significant or interesting artefact? *Parasitology*, 115: p. S101-S105.
106. **Grencis, R.K., J. Riedlinger, and D. Wakelin, (1985).** L3T4-positive T lymphoblasts are responsible for transfer of immunity to *Trichinella spiralis* in mice. *Immunology*, 56: p. 213-218.
107. **Hanrahan, L.A., G.W. Benz, and R.D. Schultz, (1984).** Experimentally induced *Cooperia oncophora* infection in calves: lymphocyte blastogenic and delayed hypersensitivity responses. *Am J Vet Res*, 45: p. 855-862.
108. **Harari, Y. and G.A. Castro, (1991).** Reconstruction of immune-mediated ion secretion in gut mucosa of the athymic rat. *Parasite Immunol*, 13: p. 313-328.
109. **Hayday, A., (2000).** gd Cells: a right time and a right way for a conserved third way of protection. *Ann Rev Immunol*, 18: p. 975-1026.
110. **Hein, W., L. Dudler, and C.R. Mackay, (1989).** Surface expression of differentiation antigens on lymphocytes in the ileal and jejunal Peyer's patches of lambs. *Immunology*, 68: p. 365-370.
111. **Hein, W.R. and C.R. Mackay, (1991).** Prominence of gd T cells in the ruminant immune system. *Immunology Today*, 12: p. 30-34.
112. **Hilderson, H., et al., (1993).** Characterisation of *Ostertagia ostertagi* antigens by the different bovine immunoglobulin isotypes. *Res Vet Sci*, 55: p. 203-208.
113. **Howard, C.J., et al., (1992).** Immunity to bovine virus diarrhoea virus in calves: the role of different T-cell subpopulations analysed by specific depletion in vivo with monoclonal antibodies. *Vet Immunol Immunopathol*, 32: p. 303-314.
114. **Howard, C.J., et al., (1997).** Identification of two distinct populations of dendritic cells in afferent lymph that vary in their ability to stimulate T cells. *J Immunol*, 159: p. 5372-5282.
115. **Howard, C.J., P. Sopp, and K.R. Parsons, (1992).** L-selectin expression differentiates T cells isolated from different lymphoid organs in cattle but does not correlate with memory. *Immunology*, 77: p. 228-234.
116. **Huntley, J.F., et al., (1987).** Systemic release of a mast cell proteinase following nematode infections in sheep. *Parasite Immunol*, 9: p. 603-614.

117. **Huntley, J.F., G. Newlands, and H.R. Miller, (1984).** The isolation and characterization of globule leucocytes: their derivation from mucosal mast cells in parasitized sheep. *Parasite Immunol*, 6: p. 371-390.
118. **Huntley, J.F., et al., (1992).** The influence of challenge dose, duration of immunity, or steroid treatment on mucosal mast cells and on the distribution of sheep mast cell proteinase in *Haemonchus*-infected sheep. *Parasite Immunol*, 14: p. 429-440.
119. **Huntley, J.F., et al., (1998).** IgE antibody during infection with the ovine abomasal nematode, *Teladorsagia circumcincta*: primary and secondary responses in serum and gastric lymph of sheep. *Parasite Immunol*, 20: p. 565-571.
120. **Hurst, S.D. and T.A. Barret, (1996).** T-cell responses to enteric antigens. *Semin Gastrointest Dis*, 7: p. 118-123.
121. **Isenstein, R.S., (1963).** The life history of *Cooperia oncophora* (Raillet, 1898) Ransom, 1907, a nematode parasite of cattle. *Parasitology*, 49: p. 235-240.
122. **James, S.P., (1993).** The gastrointestinal mucosal immune system. *Dig Dis*, 11: p. 146-156.
123. **Jarrett, E.E. and H.R. Miller, (1982).** Production and activities of IgE in helminth infection. *Progress in Allergy*, 31: p. 178-233.
124. **Jarrett, W.F., et al., (1960).** Immunological studies on *Dictyocaulus viviparus* infection. Immunity produced by the administration of irradiated larvae. *Immunology*, 3: p. 145-151.
125. **Jones, D.G., (1993).** The eosinophil. *J Comp Pathol*, 108: p. 317-335.
126. **Kanobana, K., H.W. Ploeger, and L. Vervelde, (2002).** Immune expulsion of the trichostrongylid *Cooperia oncophora* is associated with increased eosinophilia and mucosal IgA. *Int J Parasitol*, 32: p. 1389-1398.
127. **Kanobana, K., et al., (2001).** Characterization of host responder types after a single *Cooperia oncophora* infection: kinetics of the systemic immune response. *Parasite Immunol*, 23: p. 641-653.
128. **Kantele, A., et al., (1997).** Homing potentials of circulating lymphocytes in humans depend on the site of activation: oral, but not parenteral, typhoid vaccination induces circulating antibody-secreting cells that all bear homing receptors directing them to the gut. *J Immunol*, 158: p. 574-579.
129. **Katona, I.M., J.F. Urban, Jr., and F.D. Finkelman, (1988).** The role of L3T4+ and Lyt-2+ T cells in the IgE response and immunity to *Nippostrongylus brasiliensis*. *J Immunol*, 140: p. 3206-3211.
130. **Klesius, P.H., (1988).** Immunity to *Ostertagia ostertagi*. *Vet Parasitol*, 27: p. 159-167.
131. **Kloosterman, A., (1971).** Observations on the epidemiology of trichostrongylosis of calves. *PhD thesis. Agricultural university, Wageningen.*
132. **Kloosterman, A., G. Albers, and R. van den Brink, (1978).** Genetic variation among calves in resistance to nematode parasites. *Vet Parasitol*, 4: p. 353-368.
133. **Kloosterman, A. and K. Frankena, (1988).** Interactions between lungworms and gastrointestinal worms in calves. *Vet Parasitol*, 26: p. 305-320.
134. **Kloosterman, A., H.K. Parmentier, and H.W. Ploeger, (1992).** Breeding cattle and sheep for resistance to gastrointestinal nematodes. *Parasitology Today*, 8: p. 330-335.
135. **Kloosterman, A., H.W. Ploeger, and K. Frankena, (1991).** Age resistance in calves to *Ostertagia ostertagi* and *Cooperia oncophora*. *Vet Parasitol*, 39: p. 101-113.

136. **Kooyman, F.N., et al., (1997).** Production of a monoclonal antibody specific for ovine immunoglobulin E and its application to monitor serum IgE responses to *Haemonchus contortus* infection. *Parasitology*, 114: p. 395-406.
137. **Kooyman, F.N., et al., (2002).** Serum immunoglobulin E response in calves infected with the lungworm *Dictyocaulus viviparus* and its correlation with protection. *Parasite Immunol*, 24: p. 47-56.
138. **Korenaga, M., et al., (1994).** Regulatory effect of anti-interleukin-5 monoclonal antibody on intestinal worm burden in a primary infection with *Strongyloides venezuelensis* in mice. *Int J Parasitol*, 24: p. 951-957.
139. **Koyama, K., H. Tamauchi, and Y. Ito, (1995).** The role of CD4+ and CD8+ cells in the protective immunity to the murine nematode parasite *Trichuris muris*. *Parasite Immunol*, 17: p. 161-165.
140. **Kraehenbuhl, J.-P. and M.R. Neutra, (1992).** Molecular and cellullar basis of immune protection of mucosal surfaces. *Physiol Rev*, 72: p. 853-879.
141. **Kusama, Y., et al., (1995).** Mechanisms of eosinophilia in Balb/c-nu/+ and congenitally athymic Balb/C-nu/nu mice infected with *Toxocara canis*. *Immunology*, 84: p. 461-468.
142. **Laemmli, U.K., (1970).** Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature*, 227: p. 680-687.
143. **Lamkhioued, B., et al., (1995).** Human eosinophils express a receptor for secretory component. Role in secretory IgA-dependent activation. *Eur J Immunol*, 25: p. 117-125.
144. **Larsen, M., et al., (1997).** Biological control of gastro-intestinal nematodes - facts, future, or fiction? *Vet Parasitol*, 72: p. 479-492.
145. **Lascelles, A. K., et al., (1986**). The mucosal immune system with particular reference to ruminant animals. *In: The ruminant immune system in health and disease. I.W. Morrison (Ed.).* Cambridge University Press, Cambridge. p 429-457.
146. **Leighton, E.A., K.D. Murrell and L.C. Gasbarre, (1989).** Evidence for genetic control of nematode egg-shedding rates in calves. *J Parasitol*, 75: p. 498-504.
147. **Lenshow, D.J., T.L. Walunas, and J.A. Bluestone, (1996).** CD28/B7 system of costimulation. *Annu Rev Immunol*, 14: p. 233-258.
148. **Lightowlers, M.W. and M.D. Rickard, (1988).** Excretory-secretory products of helminth parasites: effects on host immune responses. *Parasitology*, 96: p. S123-166.
149. **Madden, K.B., et al., (1991).** Antibodies to IL-3 and IL-4 suppress helminth-induced intestinal mastocytosis. *J Immunol*, 147: p. 1387-1391.
150. **Mansour, M.M., et al., (1990).** Bovine immune recognition of *Ostertagia ostertagi* larval antigens. *Vet Immunol Immunopathol*, 24: p. 361-371.
151. **McClure, S.J. and D.L. Emery, (1994)**. Cell-mediated responses against gastrointestinal nematode parasites of ruminants. *In: Cell mediated immunity in Ruminants. B.M. Goddeeris and I.W. Morrison (Eds.)*, Boca Ranton, CRC Press, p 250
152. **McClure, S.J., et al., (1998).** Attempts to generate immunity against *Trichostrongylus colubriformis* and *Haemonchus contortus* in young lambs by vaccination with viable parasites. *Int J Parasitol*, 28: p. 739-746.
153. **McClure, S.J., et al., (1995).** Depletion of IFN-gamma, CD8+ or TCR gamma delta+ cells in vivo during primary infection with an enteric parasite (*Trichostrongylus colubriformis*) enhances protective immunity. *Immunol Cell Biol*, 73: p. 552-555.

154. **McClure, S.J., et al., (1992).** A serial study of rejection of *Trichostrongylus colubriformis* by immune sheep. *Int J Parasitol*, 22: p. 227-234.
155. **McDonald, A.S. and E.J. Pearce, (2002).** Cutting edge: polarized Th induction by transferred antigen pulsed dendritic cells is depedent upon IL-4 or IL-12 production by recipient cells. *J Immunol*, 168: p. 3127-3130.
156. **Meeusen, E.N., (1999).** Immunology of helminth infection with special references to immunopathology. *Vet Parasitol*, 84: p. 259-273.
157. **Meeusen, E.N. and A. Balic, (2000).** Do Eosinophils have a Role in the Killing of Helminth Parasites? *Parasitology Today*, 16: p. 95-101.
158. **Mertens, B., E. Gobright, and H.F. Seow, (1996).** The nucleotide sequence of the bovine interleukin-5-encoding cDNA. *Gene*, 176: p. 273-274.
159. **Mes, T.H., et al., (2001).** A novel method for the isolation of gastro-intestinal nematode eggs that allows automated analysis of digital images of eg preparations and high troughput screening. *Parasitology*, 123.
160. **Michel, J.F., (1963).** The phenomena of host resistance and the course of infection of *Ostertagia ostertagi* in calves. *Parasitology*, 53: p. 63-84.
161. **Miller, H.R., (1984).** The protective mucosal response against gastrointestinal nematodes in ruminants and laboratory animals. *Vet Immunol Immunopathol*, 6: p. 167-259.
162. **Miller, H.R., (1996).** Mucosal mast cells and the allergic response against nematode parasites. *Vet Immunol Immunopathol*, 54: p. 331-336.
163. **Miller, H.R., (1996).** Prospects for the immunological control of ruminant gastrointestinal nematodes: natural immunity, can it be harnessed? *Int J Parasitol*, 26: p. 801-811.
164. **Miller, T.A., (1971).** Vaccination against the canine hookworm disease. *Adv Parasitol*, 9: p. 153-183.
165. **Mosmann, T.R., et al., (1986).** Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*, 136: p. 2605-2616.
166. **Motegi, Y., et al., (2000).** Role of secretory IgA, secretory component, and eosinophils in mucosal inflammation. *Int Arch Allergy Immunol*, 122 Suppl 1: p. 25-27.
167. **Munn, E.A., (1997).** Rational design of nematode vaccines: hidden antigens. *Int J Parasitol*, 27: p. 359-366.
168. **Nawa, Y., et al., (1994).** Selective effector mechanisms for the expulsion of intestinal helminths. *Parasite Immunol*, 16: p. 333-338
169. **Nawa, Y. and H.R.P. Miller, (1979).** Adoptive transfer of the intestinal mast cell response in rats infected with *Nippostrongylus brasiliensis*. *Cell Immunol*, 42: p. 225-239.
170. **Newton, S.E. and E.A. Munn, (1999).** The development of vaccines against gastrointestinal nematode parasites, particularly *Haemonchus contortus*. *Parasitology Today*, 15: p. 116-122.
171. **Nieuwland, M.G., et al., (1995).** Systemic antibody responses of calves to low molecular weight *Cooperia oncophora* antigens. *Vet Parasitol*, 59: p. 231-239.
172. **Owen, R.L., (1994).** M cells-entryways of opportunity for enteropathogens. *J Exp Med*, 180: p. 7-9.
173. **Paciorkowski, N., L.D. Shultz, and T.V. Rajan, (2003).** Primed peritoneal B lymphocytes are sufficient to transfer protection against *Brugia pahangi* infection in mice. *Infect Immun*, 71: p. 1370-1378.

174. **Parciorkowsky, N., et al., (2000).** B1 B lymphocytes play an critical role in host protection against lymphatic filarial parasites. *J Exp Med*, 191: p. 731-736.
175. **Park, Y.H., et al., (1994).** A subset of mammary gland GD T lymphocytes downregulates BoCD4 T lymphocyte response to *Staphylococcus aureus* in cattle with intramammary infection. *Korean J Immunol*, 16: p. 19-27.
176. **Parmentier, H.K., et al., (1995).** Low molecular weight *Cooperia oncophora* antigens: characterization and humoral immune responses in calves mono-infected with 100,000 infective larvae. *Vet Parasitol*, 59: p. 219-230.
177. **Paterson, S. and M.E. Viney, (2002).** Host immune responses are necessary for density dependence in nematode infections. *Parasitology*, 125: p. 283-292.
178. **Ploeger, H., et al., (2000).** Cross-sectional serological survey on gastrointestinal and lung nematode infections in first and second-year replacement stock in the Netherlands: relation with management practices and use of anthelminthics. *Vet Parasitol*, 90: p. 285-304.
179. **Ploeger, H. and A. Kloosterman, (1993).** Gastrointestinal infections and weight gain in dairy replacement stock: first-year calves. *Vet Parasitol*, 46: p. 223-241.
180. **Ploeger, H.W., et al., (1990).** Effect of nematode infections on growth performance of calves after stabling on commercial dairy farms. *Vet Parasitol*, 36: p. 71-81.
181. **Ploeger, H.W., et al., (1990).** Effect of naturally occuring nematode infections in the first and the second grazing season on the growth performance of second year cattle. *Vet Parasitol*, 36: p. 57-70.
182. **Ploeger, H.W., et al., (1994)**. Quantitative estimation of the level of exposure to gastrointestinal nematode infection in first-year calves. *Vet Parasitol*, 55: p. 287-315
183. **Ploeger, H.W., A. Kloosterman, and F.W. Rietveld, (1995).** Acquired immunity against *Cooperia* spp. and *Ostertagia* spp. in calves: effect of level exposure and timing of the midsummer increase. *Vet Parasitol*, 58: p. 61-74.
184. **Ploeger, H.W., et al., (1996).** Production of dairy replacement stock in relation to level of exposure to gastrointestinal nematode infection in the first grazing season: second year calves and heifers. *Vet Parasitol*, 22.
185. **Poot, J., et al., (1997).** Use of cloned excretory/secretory low-molecular-weight proteins of *Cooperia oncophora* in a serological assay. *J Clin Microbiol*, 35: p. 1728-1733.
186. **Press, C., S. McClure, and T. Landsverk, (1991).** Computer-assisted morphometric analysis of absorptive and follicle- associated epithelia of Peyer's patches in sheep foetuses and lambs indicates the presence of distinct T- and B-cell components. *Immunology*, 72: p. 386-392.
187. **Pritchard, D.I., (1993).** Immunity to helminths: is too much IgE parasite- -rather than host-protective? *Parasite Immunol*, 15: p. 5-9.
188. **Pritchard, D.I., R.J. Quinnel, and E.A. Walsh, (1995).** Immunity in humans to *Necator americanus*: IgE, parasite weigth and fecundity. *Parasite Immunol*, 17: p. 71-75.
189. **Romagnani, S., (1992).** Human TH1 and TH2 subsets: regulation of differentiation and role in protection and immunopathology. *Int Arch Allergy Immunol*, 98: p. 279-285.
190. **Ron, Y. and J. Sprent, (1987).** T cell priming in vivo: a major role for B cells in presenting antigen to T cells in lymph nodes. *J Immunol*, 138: p. 2848.
191. **Rothkotter, H., R. Pabst, and M. Bailey, (1999).** Lymphocyte migration in the intestinal mucosa: entry, transit and emigration of lymphoid cells and the influence of antigen. *Vet Immunol Immunopathol*, 72: p. 157-165.

192. **Rothwell, T.L., (1989).** Immune expulsion of parasitic nematodes from the alimentary tract. *Int J Parasitol*, 19: p. 139-168.
193. **Sanderson, C.J., (1992).** Interleukin-5, eosinophils, and disease. *Blood*, 79: p. 3101-3109.
194. **Sasaki, O., et al., (1993).** Ablation of eosinophils with anti-IL-5 antibody enhances the survival of intracranial worms of *Angiostrongylus cantonensis* in the mouse. *Parasite Immunol*, 15: p. 349-354.
195. **Schallig, H.D., et al., (1994).** Serum antibody responses of Texel sheep experimentally infected with *Haemonchus contortus*. *Res Vet Sci*, 57: p. 63-68.
196. **Schallig, H.D., M.A. van Leeuwen, and A.W. Cornelissen, (1997).** Protective immunity induced by vaccination with two *Haemonchus contortus* excretory secretory proteins in sheep. *Parasite Immunol*, 118: p. 297-304.
197. **Schallig, H.D., M.A. van Leeuwen, and W.M. Hendrikx, (1994).** Immune responses of Texel sheep to excretory/secretory products of adult *Haemonchus contortus*. *Parasitology*, 108: p. 351-357.
198. **Schierack, P., et al., (2003).** Parasite-specific immunomodulatory functions of filarial cystatin. *Infect Immun*, 71: p. 2422-2429.
199. **Seaton, D.S., et al., (1989a).** Development of immunity to incoming radiolabelled larvae in lambs continuously infected with *Ostertagia circumcincta*. *Res Vet Sci*, 46: p. 241-246.
200. **Shaw, R.J., T.K. Gatehouse, and M.M. McNeill, (1998).** Serum IgE responses during primary and challenge infections of sheep with *Trichostrongylus colubriformis*. *Int J Parasitol*, 28: p. 293-302.
201. **Shaw, R.J., et al., (1997).** Quantification of total sheep IgE concentration using anti-ovine IgE monoclonal antibodies in an enzyme immunoassay. *Vet Immunol Immunopathol*, 57: p. 253-265.
202. **Simon, H.U., (1997).** Apoptosis and *in vivo* distribution and clearance of eosinophils in normal and *Trichinella spiralis*-infected rats. *J Leukocyte Biol*, 62: p. 309-317.
203. **Slifka, M.K., et al., (1998).** Humoral immunity due to long-lived plasma cells. *Immunity*, 8: p. 363-372.
204. **Smith, G. and D.T. Galligan, (1988).** Mathematical models of the population biology of *Ostertagia ostertagi* and *Teladorsagia circumcincta*, and the economic evaluation of disease control strategies. *Vet Parasitol*, 27: p. 73-83.
205. **Smith, W.D. and K.W. Angus, (1980).** *Haemonchus contortus*: attempts to immunise lambs with irradiated larvae. *Res Vet Sci*, 29: p. 45-50.
206. **Smith, W.D., et al., (1986).** Transfer of immunity to *Ostertagia circumcincta* and IgA memory between identical sheep by lymphocytes collected from gastric lymph. *Res Vet Sci*, 41: p. 300-306.
207. **Smith, W.D., et al., (1984).** Resistance to *Haemonchus contortus* transferred between genetically histocompatible sheep by immune lymphocytes. *Res Vet Sci*, 37: p. 199-204.
208. **Sopp, P. and C.J. Howard, (2001).** INF-γ and IL-4 production by CD4, CD8 and WC1 γδ TCR+ T cells from cattle lymph nodes and blood. *Vet Immunol Immunopathol*, 81: p. 85-96.
209. **Stear, M.J., et al., (1997a).** The genetic basis of resistance to *Ostertagia circumcincta* in lambs. *Vet J*, 154: p. 111-119.
210. **Stear, M.J., et al., (1997b).** How hosts control worms. *Nature*, 389: p. 27.
211. **Stear, M.J. and S.C. Bishop, (1999a).** The curvilinear relationship between worm length and fecundity of *Teladorsagia circumcincta*. *Int J Parasitol*, 29: p. 777-780.

212. **Stear, M.J., S. Strain, and S.C. Bishop, (1999).** Mechanisms underlying resistance to nematode infection. *Int J Parasitol*, 29: p. 51-56; discussion 73-55.
213. **Stevenson, L.M., et al., (1994).** Local eosinophil- and mast cell-related responses in abomasal nematode infections of lambs. *FEMS Immunol Med Microbiol*, 8: p. 167-173.
214. **Stevenson, L.M. and D.G. Jones, (1994).** Cross-reactivity amongst recombinant haematopoietic cytokines from different species for sheep bone-marrow eosinophils. *J Comp Pathol*, 111: p. 99-106.
215. **Stevenson, L.M., et al., (1998).** Eosinophil-specific biological activity of recombinant ovine interleukin-5. *Vet Immunol Immunopathol*, 66: p. 359-365.
216. **Sutton, B.J. and H.J. Gould, (1993).** The human IgE network. *Nature*, 366: p. 421-428.
217. **Tezuka, H., et al., (2002).** Recombinant *Dirofilaria immitis* polyprotein that stimulates murine B cells to produce nonspecific polyclonal immunoglobulin E antibody. *Infect Immun*, 70: p. 1235-1244.
218. **Thamsborg, S.M., A. Roepstorff, and M. Larsen, (1999).** Integrated and biological control of parasites in organic and conventional production systems. *Vet Parasitol*, 84: p. 169-186.
219. **Thatcher, E.F., L.J. Gershwin, and N.F. Baker, (1989).** Levels of serum IgE in response to gastrointestinal nematodes in cattle. *Vet Parasitol*, 32: p. 153-161.
220. **Tivol, E.A., et al., (1995).** Loss of CTLA-4 leads to massive lymphoproliferations and fetal multiorgan tisuue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity*, 3: p. 542.
221. **Urban, J.F. Jr., et al., (1992).** The importance of Th2 cytokines in protective immunity to nematodes. *Immunol rev*, 127: p. 205.
222. **Urban, J.F. Jr., I.M. Katona, and F.D. Finkelman, (1991).** *Heligmosomoides polygyrus*: CD4+ but not CD8+ T cells regulate the IgE response and protective immunity in mice. *Exp Parasitol*, 73: p. 500-511.
223. **Vallance, B.A., et al., (1999).** IL-5 contributes to worm expulsion and muscle hypercontractility in a primary *T. spiralis* infection. *Am J Physiol*, 277: p. G400-408.
224. **Vallance, B.A., et al., (2001).** Mast cell-independent impairment of host defense and muscle contraction in *T. spiralis*-infected W/W(V) mice. *Am J Physiol Gastrointest Liver Physiol*, 280: p. G640-648.
225. **Vallance, B.A. and S.M. Collins, (1998).** The effect of nematode infection upon intestinal smooth muscle function. *Parasite Immunol*, 20: p. 249-253.
226. **Vallance, B.A., et al., (1999).** CD4 T cells and major histocompatibility complex class II expression influence worm expulsion and increased intestinal muscle contraction during *Trichinella spiralis* infection. *Infect Immun*, 67: p. 6090-6097.
227. **Vallance, B.A., et al., (2000).** Interleukin-5 deficient mice exhibit impaired host defence against challenge *Trichinella spiralis* infections. *Parasite Immunol*, 22: p. 487-492.
228. **Van den Broeck, W., E. Cox, and B.M. Goddeeris, (1999).** Induction of immune responses in pigs following oral administration of purified F4 fimbriae. *Vaccine*, 17: p. 2020-2029
229. **Van der Kleij, D., et al., (2002).** Triggering of innate immune responses by schistosome egg glycolipids and their carbohydrate epitope GalNAc beta 1-4 (Fuc alpha 1-2Fuc alpha 1-3) GlcNAc. *J Infect Dis*, 185: p. 531-539.
230. **Van Die, I., et al., (1999).** Core alpha 1-3-fucose is a common modification of N-glycans in parasitic helminths and constitutes an impotant epitope of IgE from *Haemonchus* infected sheep. *Febs Lett*, 463: p. 189-193.

231. **Van Diemen, P.M., et al., (1997).** Low molecular weigth *Cooperia oncophora* antigens. Potential to discriminate between susceptible and resistant calves after infection. *Int J Parasitol*, 27: p. 587-593.
232. **Van Diemen, P.M., et al., (1996).** Recognition of low molecular weigth *Cooperia oncophora* antigens after primary and trickle infection of calves with third-stage infective larvae. *Int J Parasitol*, 26: p. 1305-1310.
233. **Vercruysse, J. and E. Claerebout, (2001).** Treatment vs non-treatment of helminth infections in cattle: defining the treshold. *Vet Parasitol*, 98: p. 195-214.
234. **Vermillion, D.L., P.B. Ernst, and S.M. Collins, (1991).** T-lymphocyte modulation of intestinal muscle function in the *Trichinella*-infected rat. *Gastroenterology*, 101: p. 31-38.
235. **Vervelde, L., et al., (2001).** Protection studies with recombinant excretory/secretory proteins of *Haemonchus contortus*. *Parasite Immunol*, 24: p. 189-201.
236. **Viney, M., (2002).** How do host immune responses affect nematode infections? *Trends Parasitol*, 18: p. 63-66.
237. **Walcheck, B. and M.A. Jutila, (1994).** Bovine gamma delta T cells express high levels of functional peripheral lymph node homing receptor (L-selectin). *Int Immunol*, 6: p. 81-91.
238. **Waller, P., (1997).** Anthelminthic resistance. *Vet Parasitol*, 72: p. 391-412.
239. **Waller, P.J., (1978).** Host-induced effects on the morphology of *Ostertagia circumcincta* in grazing sheep. *Int J Parasitol*, 8: p. 365-370.
240. **Waterhouse, P., et al., (1995).** Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. *Science*, 270: p. 932.
241. **Whelan, M., et al., (2000).** A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells. *J Immunol*, 164: p. 6453-6460.
242. **Williams, D.J., J. Newson and J. Naessens, (1990).** Quantitation of bovine immunoglobulin isotypes and allotypes using monoclonal antibodies. *Vet Immunol Immunopathol*, 24: p. 267-283.
243. **Wilson, R.A., et al., (1996).** T-cell subsets in blood and lymphoid tissues obtained from fetal calves, mature calves and adult bovine. *Vet Immunol Immunopathol*, 53: p. 49-60.
244. **Windon, R.G., J.K. Dineen, and J.D. Kelly, (1980).** The segregation of lambs into 'responders' and 'non responders': response to vaccination with irradiated *Trichostrongylus colubriformis* larvae before weaning. *Int J Parasitol*, 10: p. 65-73.
245. **Winter, M.D., C. Wright, and D.L. Lee, (1997).** The mast cell and eosinophil response of young lambs to a primary infection with *Nematodirus battus*. *Parasitology*, 114: p. 189-193.
246. **Wyatt, C.R., et al., (1994).** Differential distribution of gdT-cell receptor lymphocyte subpopulation in blood and spleen of young and adult cattle. *Vet Immunol Immunopathol*, 40: p. 187-199.
247. **Yatsuda, A.P., et al., (2002).** *Cooperia punctata* trickle infections: parasitological parameters and evaluation of a *Cooperia* recombinant 14.2 kDa protein ELISA for estimating cumulative exposure of calves. *Vet Parasitol*, 105: p. 131-138.
248. **Zarlenga, D.S., et al., (1998).** Identification and semi-quantitation of *Ostertagia ostertagi* eggs by enzymatic amplification of ITS-1 sequences. *Vet Parasitol*, 77: p. 245-257.
249. **Dalton, J.P. and G. Mulcahy, (2001).** Parasite vaccines--a reality? Vet Parasitol, 98: p. 149-167.

Appendices

Nederlandse Samenvatting
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Dankwoord

I like life. It is something to do.
(R.Shakes)

INLEIDING

Cooperia oncophora is de voornaamste dunne darm nematode bij runderen in streken met een gematigd klimaat, zoals West Europa. Dieren worden geinfecteerd door opname van infectieuse larven die zich op het grasland bevinden. In de dunne darm van het rund ontwikkelen deze larven zich tot volwassen wormen die zich sexueel voortplanten en eieren leggen. De eieren worden in de mest uitgescheiden en kunnen op die manier weer het grasland besmetten; in de mestplak ontwikkelen die eieren zich opnieuw tot larven. Deze cyclus houdt in dat als er nergens ingegrepen wordt, de runderen zich voortdurend kunnen herbesmetten door het eten van gras.

Cooperia wordt over het algemeen als een relatief weinig pathogene worm beschouwd en dit is mede te verklaren door de snelle weerstandsontwikkeling tegen *Cooperia*. Dit verhindert de opbouw van een belangrijke wormpopulatie in de darm en bijgevolg worden slechts minimale letsels veroorzaakt. Echter, ook asymptomatische infecties kunnen interfereren met de darmfysiologie en in die mate een negatieve invloed op de gastheer hebben. Herkauwers besmet met maagdarm nematoden produceren onder hun normale capaciteit en een minder goed gewichtsaanzet is waarschijnlijk het meest voorkomende effect. Daarnaast zijn er ook negatieve invloeden beschreven op de melkproductie. Om deze verliezen te compenseren worden dieren preventief behandeld met anti-worm middelen. Dit heeft als voordeel dat de productieverliezen geringer worden maar door het uitblijven van infectie wordt de kans op het ontwikkelen van weerstand eveneens kleiner, waardoor dieren in een daaropvolgende weideseizoen gevoeliger blijven voor infectie. Een bijkomend nadeel van intensief behandelen is het ontstaan van resistentie tegen anti-worm middelen. Dit houdt in dat een deel van de wormpopulatie niet meer gevoelig is voor behandeling en dus in de gastheer gehandhaafd blijft. Daarenboven is er heel wat bekommernis vanuit de voedselketen, met name betreffende het voorkomen van residuen in melk- en vleesprodukten. Mede hierdoor is er een groeiende vraag naar alternatieve behandelingsmethoden zoals bijvoorbeeld een vaccin. De ontwikkelling van een vaccin is gebaseerd op 3 belangrijke pijlers:1) het begrijpen van de mechanismen geassocieerd met de opbouw van weerstand, met name: hoe worden de wormen uit de gastheer verdreven? en, 2) hoe wordt bij herinfectie verhinderd dat de worm zich in de gastheer nestelt? 3) het identificeren van die eiwitten die worm specifiek zijn en geassocieerd met weerstandsontwikkeling. In dit proefschrift is vooral aandacht geschonken aan dit eerste aspect. Aan de hand van experimentele infecties in kalfjes, die ook de meest gevoelige populatie zijn, hebben we getracht een inzicht te krijgen in die mechanismen die geassocieerd worden met de opbouw van weerstand tegen *Cooperia.*

RESPONDER TYPEN

Een kenmerk van de experimentele infecties met *Cooperia* die wij hebben gebruikt is dat men op basis van parasitologische parameters in staat is om drie types dieren te onderkennen. '*Low responders*': dit zijn gevoelige dieren die slecht in staat zijn om weerstand te ontwikkelen. Na infectie blijven de dieren langdurig eieren uitscheiden in hun mest en op het ogenblik dat ze geslacht worden, vindt men nog steeds een groot aantal wormen in de dunne darm. '*Intermediate responders*': dit zijn dieren die op het ogenblik van infectie nog gevoelig zijn maar zij slagen erin in de loop van de infectie weerstand te ontwikkelen wat leidt tot een daling in ei-uitscheiding. Bij slachten varieert het aantal wormen in de dunne darm, zowel lage als hoge aantallen worden teruggevonden. Indien een hoog aantal wormen gepaard gaat met een lage ei-uitscheiding betekent dit dat de weerstand van de gastheer een effect heeft gehad op de vruchtbaarheid van de wormen, en minder op het uitdrijven van de wormen. Tot slot zijn er ook nog '*High responders*': dit is slechts een heel klein deel van de populatie en deze dieren blijken resistent te zijn tegen infectie met *Cooperia.* Na infectie vertonen zij geringe tot geen ei-uitscheiding en bij slachten worden ook nauwelijks wormen teruggevonden in de dunne darm. Het is nog steeds niet duidelijk of de resistentie immunologisch, fysiologisch dan wel genetisch is bepaald, vermoedelijk zal het een combinatie zijn.
Het grote voordeel van dit model is dat door het vergelijken van de immuniteitsreaktie tussen *Low* en *Intermediate responders*, men in staat wordt gesteld om die mechanismen te identificeren die geassocieerd zijn met het ontwikkelen van weerstand in de *Intermediate responders.* Immers, een dergelijk mechanisme zal in *Low responders* vermoedelijk niet ontwikkeld worden.

IMMUNITEIT TEGEN COOPERIA

Voor de bescherming tegen ziekteverwerkers die in de darm binnendringen heeft het lichaam zowel een niet specifieke afweersysteem (bijvoorbeeld het darmslijmvlies en de motiliteit van de darm) en een specifieke afweersysteem dat gericht is tegen een bepaald pathogeen. In principe zijn er twee specifieke immunologische reacties mogelijk, een antilichaam-gemedieerde reactie en een cel-gemedieerde reactie. De antilichaam-gemedieerde reactie komt op gang als B cellen, na contact met de ziekteverwekker, geactiveerd worden en antilichamen gaan produceren. Antilichamen zijn specifiek voor een bepaald ziekteverwekker en voornamelijk belangrijk tegen pathogenen die zich buiten een cel bevinden, zoals bijvoorbeeld wormen. Hiernaast bestaat de cel-gemedieerde immuunrespons, waarin voornamelijk T cellen een rol spelen. Er zijn twee types T cellen. T cellen kunnen een direct effect hebben op

geinfecteerde cellen en deze doden (cytotoxische T cellen, CD8+) of indirect, door het uitscheiden van signaalstoffen, andere cellen stimuleren tot het vernietigen van de pathogeen (helper T cellen, CD4+). Op basis van de signaalstoffen die helper T cellen secreteren en het type antilichaam dat geproduceerd wordt, zijn er twee groepen gedefinieerd, T helper 1 en T helper 2. T helper 2 cellen spelen een belangrijke rol bij worm infecties, zowel in muizen, mensen als landbouwhuisdieren. Aan de hand van hun signaal stoffen zorgen zij dat B cellen geactiveerd worden en effectiever antilichamen gaan produceren van het type IgG1, IgE en IgA, in tegenstelling tot IgM en IgG2 geassocieerd met T helper 1. Daarnaast worden ontstekingscellen gerecruteerd naar de plaats van infectie, zoals mestcellen en eosinofielen. Mestcellen en eosinofielen kunnen op hun beurt geactiveerd worden en allerhande stoffen secreteren in hun omgeving. Deze zijn toxisch voor de wormen, maar hebben ook een invloed op de fysiologie van de darm, zoals bijvoorbeeld een verhoogde secretie van darmvloeistoffen en een verhoogde darmmotiliteit, waardoor wormen gemakkelijker kunnen uitgedreven worden. Mestcellen en eosinofielen worden het beste geactiveerd door antilichamen, die op specifieke receptoren op hun oppervlakte binden, en op dit punt werken de antilichaam-gemedieerde en cel-gemedieerde immuunrespons tesamen.

In hoofdstuk 2 en 3 hebben we de weerstands ontwikkeling na een primaire infectie (dit is bij dieren die voorafgaand nooit geinfecteerd zijn geweest) gekarakteriseerd. Uit hoofdstuk 1 blijkt dat, naast een parasitologische karakterisatie ook een immunologische karakterisatie van respondertypen mogelijk is. *Intermediate responders* vertonen een duidelijke antilichamen respons na infectie (met name IgG1 en IgA), terwijl *Low responders* dat in mindere mate of niet doen. Daarenboven, werd aan de hand van correlatie analyses aangetoond dat de serologische respons een nadelig effect heeft op de parasiet. In hoofdstuk 2 hebben we de lokale respons in de darm van geïnfecteerde dieren gekarakteriseerd. Om dit gedetailleerd te kunnen doen, werd de dunne darm van het kalf (die een lengte heeft varierend van 25 tot 40 meter) in 6 segmenten onderverdeeld en elk van deze segmenten werd afzonderlijk geanalyseerd. Dit toonde aan dat weerstandsontwikkeling verschillende parasitologische en immunologische gevolgen heeft. In een niet immuun dier, vindt men *Cooperia* terug in het voorste deel van de dunne darm. In de loop van de infectie zien we dat in dieren die immuun worden de worm geleidelijk naar het achterste deel van de darm verhuist. Dit ten gevolge van een immunologische reaktie in het voorste deel van de darm, die het verblijf voor de worm waarschijnlijk onaangenaam maakt. Het verplaatsen naar het achterste deel van de darm, gaat uiteindelijk gepaard met het uitdrijven van de worm, en typisch is dat mannelijke wormen het eerst verdreven worden. Bij vrouwelijke wormen wordt een daling in vruchtbaarheid

waargenomen, waardoor minder eieren geproduceerd en uitgescheiden worden. Karakterisatie van de immunreactie toonde aan dat hierin eosinofielen en parasiet-specifieke antilichamen (IgG1 en IgA) die lokaal worden geproduceerd, een essentiele rol spelen in de verminderde vruchtbaarheid en de uitdrijving van de volwassen wormen.
Het ontwikkelen van immuniteit is een eerste stap, maar belangrijk is ook hoe lang deze immuniteit in stand wordt gehouden en of ze ook beschermend is tegen herinfectie. Om dit te onderzoeken werd een experimenten uitgevoerd waarin dieren na een eerste infectie behandeld werden en gedurende een lange periode in een worm-vrije omgeving gehouden. Tenslotte werden de dieren opnieuw geïnfecteerd en werd de mate van bescherming geevalueerd door hun reactie te vergelijken met primair geïnfecteerde dieren. Om te onderzoeken of de mate van bescherming bepaald wordt door de zwaarte van de primaire infectie, werd het effect van een hoge en een lage primaire infectie op herinfectie vergeleken. In hoofdstuk 4 worden de parasitologische aspecten van een herinfectie beschreven. In beschermende dieren werden veel minder wormen teruggevonden dan in primair geinfecteerde dieren en dit bleek onafhankelijk te zijn van de grootte van de primaire infectie. In tegenstelling hiermee bleek de invloed op de lengte en de vruchtbaarheid van de wormen wel bepaald door de grootte van de primaire infectie. In dieren die een hogere primaire infectie hadden meegemaakt, werd een nadelig effect op de worm lengte en een verminderde vruchtbaarheid op een eerder tijdstip waargenomen. Daarnaast werd duidelijk dat het verschil tussen *Low* en *Intermediate responders* ook na herinfectie behouden blijft. Hoewel de *Low responders* enigszins beschermd waren in vergelijking met primair geinfecteerde kalfjes, bleven ze gevoeliger dan *Intermediate responders.*
Hoofdstuk 5 beschrijft de rol van B cellen en antilichamen bij herinfectie. Antilichamen worden veel vroeger na infectie en in grotere hoeveelheden geproduceerd. Bij herinfectie blijken ze ook een groter effect te hebben op de larvale stadia dan op volwassen wormen. Naast hun rol als antilichaam producerende cellen, hebben we ook aangetoond dat B cellen mogelijk een bijdrage leveren tot het in stand houden van de T helper 2 respons. Dit kan door het tot expressie brengen van bepaalde moleculen op hun celoppervlak, die dan op hun beurt met moleculen op het oppervlak van T cellen kunnen interageren.
In hoofdstuk 6 werd het aandeel van de T-cel gemedieerde immuunreactie in bescherming tegen herinfectie bestudeerd. De conclusie van dit hoofdstuk is dat bij dieren met een herinfectie dezelfde mechanismen als bij primair geinfecteerde dieren een rol spelen, met dit verschil dat de reactie veel sneller en effectiever gebeurt.

In conclusie kan gesteld worden dat de onwikkeling van weerstand en bescherming tegen herinfectie met *Cooperia* deels beïnvloed wordt door de genetische achtergrond van de dieren. Low responders reageren weliswaar met een immuunreactie op de infectie maar deze blijkt niet effectief te zijn, wormen worden niet uitgedreven en bij herinfectie zijn de dieren slechts deels beschermd. De effectieve immuun reaktie, zoals die wordt waargenomen in *Intermediate responders* wordt gekenmerkt door de ontwikkelling van een T helper 2 respons. Dit gaat gepaard met de produktie van parasiet-specifieke antilichamen in het serum en in de darm van geïnfecteerde dieren. Verder blijken B cellen, T helper cellen en eosinofielen essentieel te zijn voor het uitdrijven van de worm. Al deze waarnemingen worden geïntegreerdin de discussie zoals beschreven in hoofdstuk 7.

Curriculum vitae

Kirezi Kanobana werd geboren op 4 mei 1972 in Kinshasa (toen nog Zaire, ondertussen Congo). In 1989 behaalde zij het middelbare school diploma aan het Maria-Assumpta Lyceum te Laeken. Hierna vertrok zij in het kader van een uitwissellings programma voor een jaar naar Saskatchewan (Canada). Aansluitend begon zij aan de studie diergeneeskunde, en in juli 1997 behaalde zij het diploma van dierenarts aan de Universiteit van Gent. Van juli 1997 tot december 1998 werkte zij als wetenschappelijk assistent op de Faculteit Diergeneeskunde van de Universiteit Gent, eerst op het Laboratorium voor Virologie (hoofd Prof. Dr. M. Pensaert) en daarna op het Laboratorium voor Immunologie (hoofd Prof. Dr. B. Goddeeris). In december 1998 verhuisde ze naar Leiden. Na zes maanden als dierenarts in een groepspraktijk gewerkt te hebben, startte zij, als Onderzoeker in Opleiding, met het promotieonderzoek zoals gedeeltelijk beschreven in dit proefschrift op de Afdeling Parasitologie (hoofd Prof. Dr. A.W.C.A. Cornelissen) van de Faculteit Diergeneeskunde van de Universiteit Utrecht.

Dankwoord

Een glimlach, een huilbui, een gelukt experiment, een frustrerende dag, vallen, opstaan, nog eens vallen en opstaan… vier jaar en 93 kalfjes later is het zover: ben bijna dokter….. mama, papas, broers en zusjes, familie, vriendjes en vriendinnetjes uit Belgie en Nederland, collega's, luisterende oortjes, helpende handjes, …..elk van jullie heeft op zijn eigen manier bijgedragen en dit boekje draag ik dan ook aan jullie allemaal op!

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Het begon allemaal een paar jaar eerder, toen ik in Belgie, naief en zonder het besef dat ik ooit in Nederland zou verzeild geraken, na mijn studies op het Laboratorium voor Virologie in Gent begon te werken.

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Frans K, flegmatisch en laconiek, je slaagde erin om mijn razende olifantjes weer tot kleine muisjes te herleiden….en mijn polsslag binnen fysiologische waarden, dank je wel…

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Margreet, kamergenoot, *Cooperia*-genoot, handlanger in goede en kwade dagen, ….misschien dat jij wel het beste beseft wat ik vandaag voel, thanks!

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Publication List

Kanobana K., L. Vervelde, M. Eysker, M. van der Veer and H.W. Ploeger (2001). Characterisation of host responder types after a single *Cooperia oncophora* infection: kinetics of the systemic immune response. *Parasite immunology* **23**, 641-653.

Kanobana K., Ploeger H. W., Vervelde L. (2001). Experimental *Cooperia oncophora* infections in calves: characterization of the local immune response during a primary infection. Proceedings of the Sixth International Veterinary Immunology Symposium p186 (Abstract)

Kanobana K., H. W. Ploeger and L.Vervelde (2002). Immune expulsion of the trichostrongylid *Cooperia oncophora* is associated with increased eosinophilia and mucosal IgA. *International Journal for Parasitology* **32**, 1389-1398.

Kanobana K., H. W. Ploeger and L. Vervelde (2003). Priming dose level and host responder type differentially affect establishment, length and fecundity of *Cooperia oncophora* in re-infected calves. *Parasitology,* in press.

Kanobana K., A. Koets, F. N. Kooyman, N. Bakker, H. W. Ploeger, L. Vervelde (2003). B cells and antibodies differentially influence immunity to *Cooperia oncophora* depending on priming dose level and host responder type. *International Journal for Parasitology,* in press.

Kanobana K., A. Koets, N. Bakker, H. W. Ploeger, L. Vervelde (2003). T- cell mediated immune responses in calves primary infected or re-infected with *Cooperia oncophora*: similar efector cells but different timing. *International Journal for Parasitology,* in press.

Van der Veer, M., Kanobana, K., Ploeger, H. W., De Vries, E. (2003). Cytochrome oxidase c subunit I polymorphisms show significant differences in distribution between a laboratory maintained population and a field isolate of *Cooperia oncophora*. Veterinary Parasitology, *in press.*

Als je zelf nog iets neer wil pennen……