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].

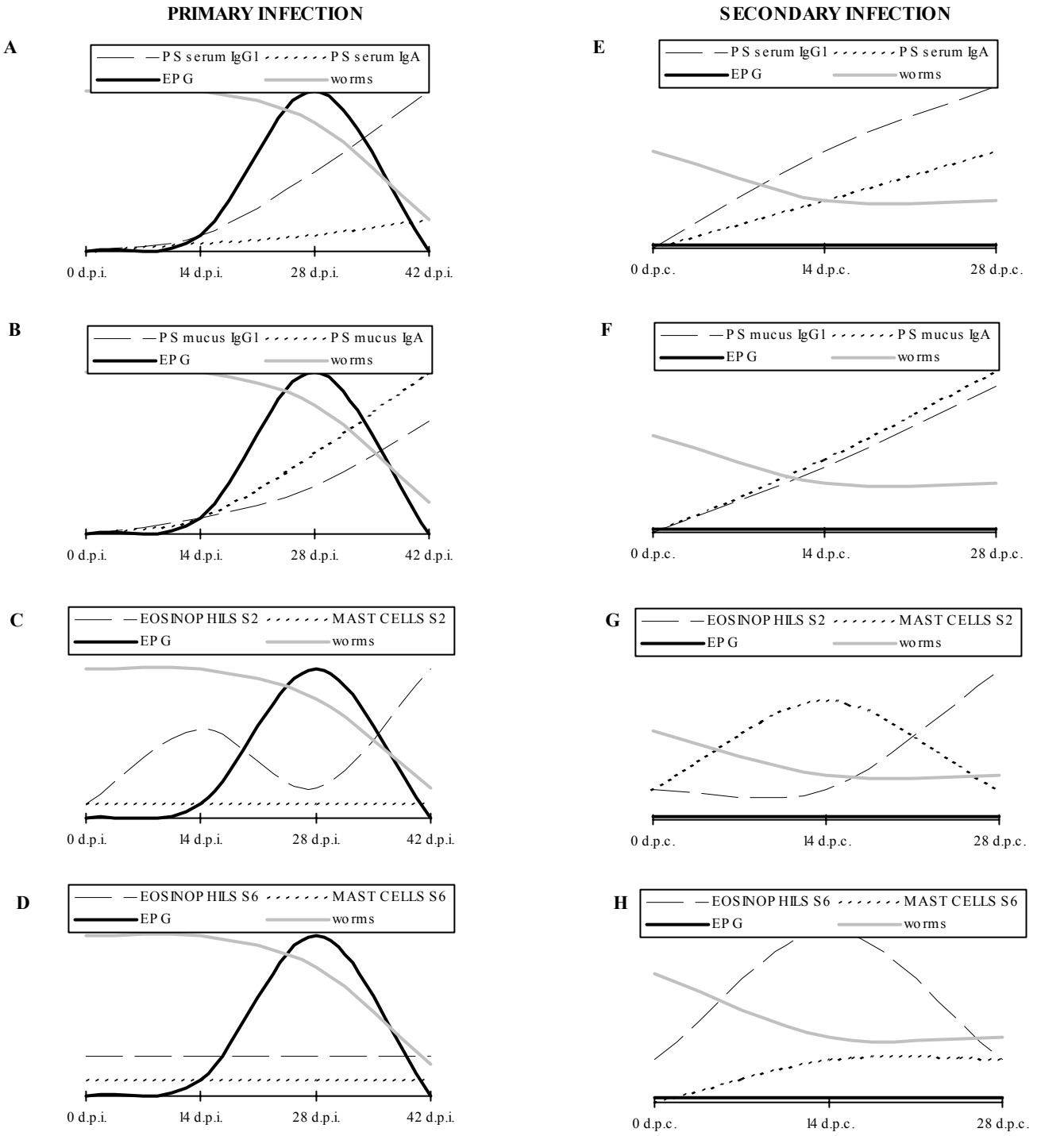

FIGURE 1. Schematic representation of effectors involved in primary and secondary infections with *Cooperia oncophora*. (A, B, C, D): during primary infection. (E, F, G, H): during secondary infection. PS=parasite specific; EPG=egg output; S2=proximal gut=jejunum; S6=distal gut=ileum. (days after infection=d.p.i.,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

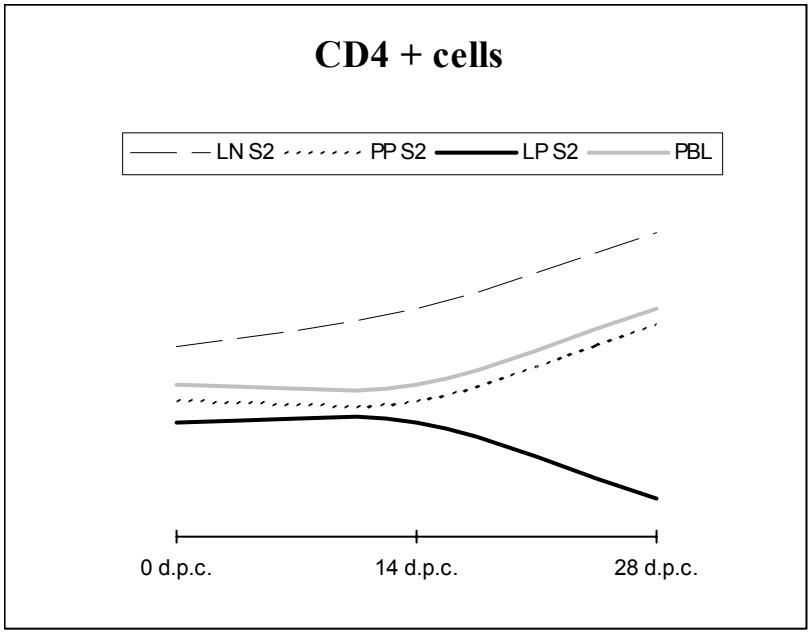

FIGURE 2. Schematic view on kinetics of CD4+ cells in different anatomical locations of *C. oncophora* re-infected calves. The observed changes are similar in primary infected animals, but occur at a slower rate. (days after challenge=d.p.c.)

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.

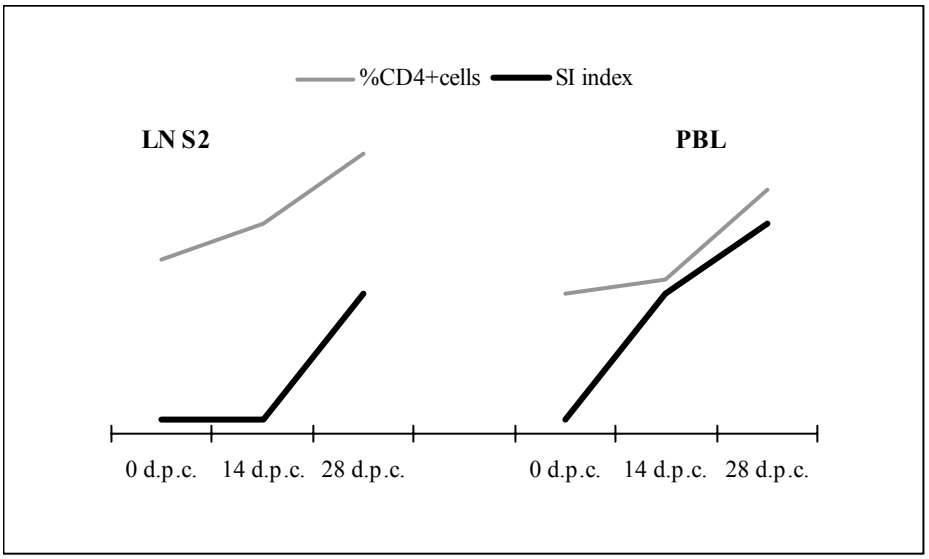

FIGURE 3. The increased frequency in CD4+ cells in draining lymph node (LN S2) and peripheral blood (PBL) of infected animals concurs with an increased number of *Cooperia*-specific cells. (days after challenge=d.p.c.)

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.