Immune Cell Responses to the Cattle Lungworm, *Dictyocaulus viviparus*

Malin Hagberg
Faculty of Veterinary Medicine and Animal Sciences
Department of Biomedical Sciences and Veterinary Public Health
Uppsala

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Cover: Haematoxylin-eosin stained leukocytes in a bronchoalveolar lavage sample from a *Dictyocaulus viviparus* infected animal. Photo: Malin Hagberg.

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Abstract

The cattle lungworm, *Dictyocaulus viviparus*, is a parasitic nematode, which can cause severe pulmonary disease. Repeated natural infections result in protective immunity, and a live attenuated vaccine exists. However, live vaccines have several disadvantages, and alternative control methods are needed. Awareness of increasing anthelmintic resistance makes parasite control with reduced reliance on chemotherapeutics preferable, and an effective modern vaccine to *D. viviparus* based on recombinant antigens would provide a valuable alternative. To enable the design of such a vaccine, identification of the immune mechanisms behind resistance to *D. viviparus* is fundamental.

The overall aim of this thesis was to study cellular immune responses to *D. viviparus*. In order to identify cells involved in development of protective immunity, mononuclear cell populations in the lungs of experimentally infected calves were studied during two subsequent infections. A large influx of activated γδ T cells was observed in the lungs both after primary infection and reinfection, which suggests a role for γδ T cells in the immune response to *D. viviparus*. *In vitro* analyses of lymphocyte responses to *D. viviparus* revealed that cells from naïve animals were induced to proliferate. Cells expressing CD4, CD8 as well as γδ TCR responded, suggesting that the worms contain substances with a broad mitogenic effect. When investigating responses of cells collected from immune animals, no clear acquired responses were detected. Investigations into the nature of the mitogenic components in *D. viviparus* indicated that they are low molecular mass immunogenic proteins with so far unknown identities.

Keywords: cattle, nematodes, *Dictyocaulus viviparus*, bronchoalveolar lavage, cellular immunity, PBMC, T cell responses, proliferation, γδ TCR, mitogen, CFSE, antigen separation

Author's address: Malin Hagberg, Department of Biomedical Sciences and Veterinary Public Health, SLU
Box 7036, 750 07 Uppsala, Sweden
E-mail: Malin.Hagberg@bvf.slu.se

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I M. Hagberg, E. Wattrang, R. Niskanen, M. Tråvén, J. Höglund & A. Lundén (2005). Mononuclear cell subsets in bronchoalveolar lavage fluid during *Dictyocaulus viviparus* infection of calves: a potential role for γ/δ TCR expressing cells in airway immune responses? Parasite *Immunology* 27, 151-161.

II M. Hagberg, A. Lundén, J. Höglund, D. A. Morrison, K. Persson Waller & E. Wattrang (2008). Characterisation of bovine lymphocytes stimulated *in vitro* by *Dictyocaulus viviparus* homogenate. *Parasite Immunology,* accepted for publication.

III M. Hagberg, J. Johansson, D. Morrison, E. Wattrang & A. Lundén (2008). Initial characterisation of proteinaceous mitogenic components in *D. viviparus* homogenate. Manuscript.

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Abbreviations

^{3}H	Tritium
BALF	Bronchoalveolar lavage fluid
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence interval
Con A	Concanavalin A
ES	Excretory/secretory
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
L_{1-4}	First-fourth stage larvae
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
PBMC	Peripheral blood mononuclear cells
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
TCR	T cell receptor
Th	T helper
WC1	Workshop cluster 1

1 Introduction

The cattle lungworm, *Dictyocaulus viviparus*, is a parasitic nematode that can cause severe pulmonary disease. Repeated natural infections result in protective immunity, and a live vaccine consisting of attenuated larvae was developed in the 1950's. However, development of immunity is sometimes reduced when the live vaccine is used together with prophylactic anthelmintic treatment against other nematodes, due to lack of boosting by natural infections. As a consequence there is a need for alternative control methods. Development of a vaccine based on recombinant antigens and modern vaccine technology might provide such an alternative. To enable the design of such a vaccine, identification of the immune mechanisms behind resistance to *D. viviparus* is fundamental. However, despite the long history of immunoprophylaxis, the understanding of the underlying immune reactions is poor and the studies published so far have mainly focused on the humoral immune responses. The overall aim of this thesis was to study the cell mediated immune responses to *D. viviparus*, in order to map immune events involved in the induction of protective immunity, and the mechanisms behind the pathological changes caused by the infection. Such knowledge would contribute substantially to the development of a new efficient vaccine produced with modern molecular techniques.

This thesis includes three parts: a study of mononuclear cell subsets in bronchoalveolar lavage fluid collected during experimental *D. viviparus* infection; characterisation of lymphocytes responding to *in vitro* stimulation with *D. viviparus*; and a study characterising mitogenic compounds found in *D. viviparus* homogenate. Below, background information to these studies is provided.

2 Background

2.1 *Dictyocaulus viviparus*

D. viviparus is a host specific nematode parasite that belongs to the super-family Trichostrongyloidea (Höglund *et al.*, 2003b). The parasite generates a serious respiratory disease in cattle called parasitic bronchitis, husk or dictyocaulosis, which is endemic in temperate areas with high rainfall such as northern Europe (Taylor *et al.*, 2007). In Sweden, 40% of the farms investigated in a national survey harboured the infection (Höglund *et al.*, 2004), and in another study as many as 80% of the organic farms were found positive for *D. viviparus* (Höglund *et al.*, 2001). These numbers of affected farms correspond well with studies from Germany (Schnieder *et al.*, 1993) and the Netherlands (Ploeger *et al.*, 2000) and dictyocaulosis is considered to be a potentially increasing (Ploeger, 2002), and costly (Woolley, 1997) problem.

The infection is contracted through ingestion of contaminated grass and, since protective immunity develops after infection, the disease primarily affects young animals during their first grazing season. Major outbreaks are seen from July to September, when sensitive calves have been on pasture 2-5 months and the parasites have had time to reproduce (Taylor *et al.*, 2007). Older animals are usually resistant, but they can act as carriers and spread the infection without showing any symptoms of the disease. Moreover, under favourable conditions *D. viviparus* larvae can overwinter on pasture (Saatkamp *et al.*, 1994).

2.1.1 Life cycle

D. viviparus has a direct life-cycle (Figure 1), in which infective stage three larvae (L_3) are ingested, penetrates the intestinal mucosa of the small intestine

or the upper part of the colon (Jarrett *et al.*, 1957), and travel to the mesenteric lymph nodes, where they moult into stage four larvae (L_4) (Taylor *et al.*, 2007). The L_4 then continue to the lungs via lymph and blood, where they break into the alveoli approximately one week after ingestion with moderate infection doses, and as early as after 24 hours at very high infection doses (Poynter *et al.*, 1960). In the lungs the larvae moult and develop to young adults, which migrate up through the bronchial tree as they mature. Mature adult worms are macroscopic, up to 8 cm long, and the females produce eggs that hatch almost immediately into first stage larvae (L_1). The L_1 are coughed up from the lungs, swallowed down, and can be detected in faeces from approximately day 24. On pasture, the larvae moult into the second stage (L_2) and further to L_3. The time needed for the development of the external larval stages is dependent on weather conditions but under optimal conditions L_3 can be reached within five days. The L_3 are spread over the pasture both by their own motility and through the aid of a microfungus called *Pilobolus*. The larvae climb onto the fungus and are discharged with the sporangia (Taylor *et al.*, 2007).

2.1.2 Clinical signs and diagnosis

Clinical signs of *D. viviparus* infection include respiratory distress, coughing, nasal and oral discharge, lacrimation, listlessness, anorexia, rough coat, malnutrition and weakness (Simpson *et al.*, 1957). The severity depends on the infection level and thus the rate of intake of infectious larvae (Jarrett *et al.*, 1957). For example, when experimentally infected with 50 000 L_3 no calves survived beyond 18 days whereas after infection with 5 000 L_3 disease was severe but not fatal (Jarrett *et al.*, 1957). Clinical signs in moderately affected animals are foremost elevation of respiratory rate, coughing and reduced growth, due to loss of appetite and increased maintenance requirements (Verstegen *et al.*, 1989).

Diagnosis can, besides clinical signs, be achieved with the help of faecal analysis using the Baermann method or with serological tests, depending on time passed after infection. The Baermann method is based on the detection of L_1 in faeces, and can therefore only be used to diagnose egg producing worms in the lungs. Two different serological tests for *D. viviparus* infection have been developed: an enzyme-linked immunosorbent assay (ELISA), and

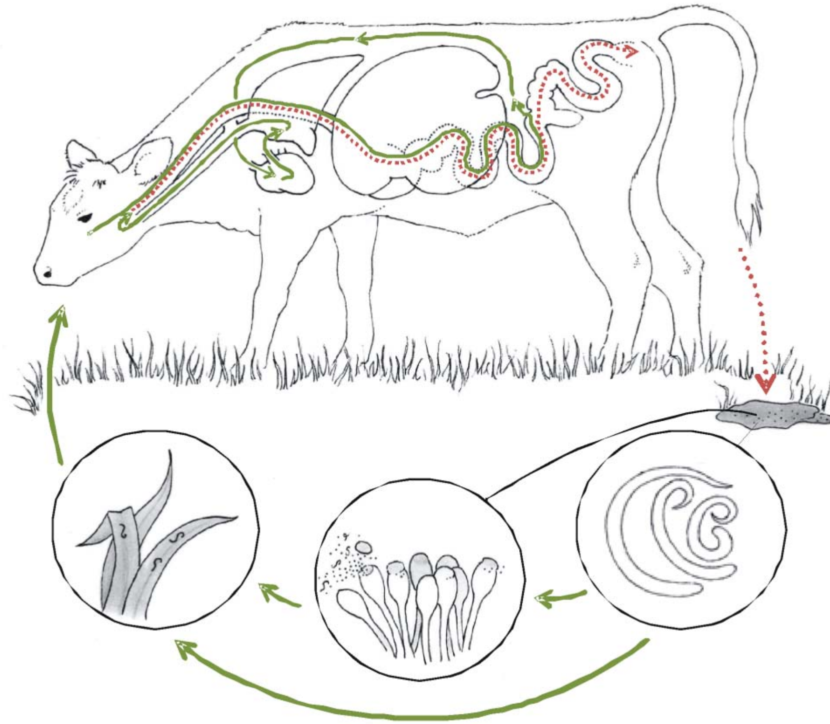

Figure 1. The lifecycle of *Dictyocaulus viviparus*. The migration route of the life stages from third stage larvae to adult worms is drawn as a green line, and the route of first stage larvae is drawn as a red dotted line for visual clarity. For a more detailed description of the lifecycle, see the text. Drawing: Katarina Näslund.

an immunoblot based dipstick test. Response time for parasite-specific serum antibodies is approximately 3-4 weeks after inoculation with L_3 and the currently used assays may detect antibodies up to 6 or 3 months after infection, for the ELISA and dipstick test respectively (Cornelissen *et al.*, 1997; Schnieder, 1993).

2.1.3 Control

There are several ways to control *D. viviparus* infections. The farm production system influences the transmission of infection, and with appropriately designed pasture rotations, transmission of pasture-borne parasitic nematodes can be partially controlled (Larsson *et al.*, 2006). Anthelmintic resistance is not yet considered to be a widespread problem of *D. viviparus*, and the infection can be treated with macrocyclic lactones, levamisole and benzimidazoles. However, decreased efficacy of using the macrocyclic lactone abamectin was recently observed in a study from Brazil (Molento *et al.*, 2006). Moreover, immunity to *D. viviparus* develops after infection, and in some parts of Europe a vaccine that is produced from L_3, attenuated by irradiation, is used. The irradiated larvae penetrate the intestine but do not reach the lungs, and are therefore not pathogenic. The vaccine however has disadvantages as infection of calves is needed for the production (McKeand, 2000), and the shelf-life is only 6 weeks (Bain, 1999). Furthermore, recurrent boosters, e.g. through grazing of infected pastures, are needed for animals to remain immune after vaccination. In some studies, prophylactic treatment with long-lasting anthelmintics against other helminths has been shown to interfere with development of protective immunity after vaccination. This has been suggested to depend on the lack of boosters (Ploeger, 2002). The disadvantages of the live vaccine have made development of a more modern recombinant vaccine a priority.

2.2 Host immune responses to helminths

Helminth infections are generally associated with eosinophilia, mastocytosis and high levels of IgE and have thereby been considered the main examples of T helper 2 (Th2) cell inducers (Maizels & Yazdanbakhsh, 2003). The protective immune response against helminths has, as a consequence, been referred to as a Th2-type response, which includes both innate and adaptive components.

The structures in helminths that trigger Th2-type responses, and the signals from dendritic cells that instruct Th2 cell development, are so far not

known (Diaz & Allen, 2007). However, it has been suggested that glycans, lipids and lipoproteins expressed by some helminths might function as pathogen-associated molecular patterns (PAMPs) (Anthony *et al.*, 2007; Diaz & Allen, 2007). Helminths are a diverse group, and it is possible that the host immune system has learnt to recognise these parasites through several different molecules but translates the signals to a single response profile (Diaz & Allen, 2007). Such a theory could explain the observation that, although the Th2-type responses involve many different populations of leukocytes that respond to most helminth infections, only certain cell types are effective in the protection against any specific helminth species (Anthony *et al.*, 2007).

2.2.1 Innate immune responses

Innate immune cells are important both during the initiation and the effector phase of Th2-type responses. Th2 cells direct and amplify innate immune cells through secretion of cytokines, and the innate cell populations in turn promote expansion of the Th2 cell population (Anthony *et al.*, 2007).

Macrophages have generally been associated with T helper 1 (Th1)-type responses to bacteria and viruses. However, alternatively activated macrophages are one of the first cell populations to be recruited to sites of infection during Th2-type responses to helminths. They seem to have functions as regulators of immune responses, in tissue repair and also directly in resistance to parasite invasion (Reece *et al.*, 2006). Tissue repair is thought to be prioritised in helminth infections, since such large, often migrating, invaders have considerable potential for tissue damage (Diaz & Allen, 2007). It is thought that Th2 cell derived IL-4 provides alternative macrophage activation. Neutrophilic granulocytes are also one of the first cell populations activated and recruited to the site of infection during tissue invasion by helminths. Following rapid recruitment, neutrophils can potentially work together with other cell populations, including eosinophilic granulocytes and macrophages, to damage helminths in the tissue (Anthony *et al.*, 2006).

Eosinophil numbers increase significantly in the blood soon after infection, and the cells migrate to the site of infection, where they release their granule contents. Eosinophils have been shown to be potent in killing of helminths *in vitro*, and they are thought by some to function as a primary defence against migrating larvae (Meeusen & Balic, 2000). However, in

general, depletion of eosinophils does not seem to clearly impair the *in vivo* development of Th2-type responses to most parasitic infections. Eosinophils may instead have regulatory roles and functions in tissue remodelling and debris clearance following tissue injury. Although being a part of early, innate responses, most eosinophil activity is controlled by antigen specific activities and eosinophilia is mediated by Th2 cell secreted IL-5 (Anthony *et al.*, 2007).

Also, basophils increase in number both in blood and tissues after helminth infection. Basophils and eosinophils are thought to be a major source of IL-4 during the early stages of a Th2-type response. This suggests that they have an important role in promoting development and recruitment of Th2 cells to sites of inflammation. Mast cells share many characteristics with basophils, including high affinity Fc receptors for IgE, Toll like receptors (TLRs) TLR2 and TLR4, release of mediators and IL-4 secretion. Mast cells are present in peripheral tissue, and have the possibility to respond immediately to invading pathogens; and in affected tissues numbers of mast cells are elevated. The increase in this population is dependent on Th2-type cytokines. Most helminths induce pronounced mast cell and basophil responses, although the importance of these cells in resistance seems to vary between parasite species (Anthony *et al.*, 2007). For example in cattle, no correlation was found between mucosal mast cell numbers and repulsion of the gastrointestinal nematodes *Cooperia oncophora* (Kanobana *et al.*, 2002) or *Ostertagia ostertagi* (Claerebout *et al.*, 2005).

2.2.2 Adaptive immune responses

The cells of the adaptive immune system are the lymphocytes, mainly T and B cells. Helminths generally induce responses dominated by Th2 cells that are characterised by production of cytokines IL-4, IL-5, IL-9, IL-13 and IL-21. During Th2-type responses, B cell proliferation and antibody production are stimulated, and IL-4 mediates B cell class switching to IgE. Both basophils and mast cells have high affinity IgE Fc receptors. Cross-linking of Fc-receptor bound IgE by antigen triggers mast cells to degranulate and release soluble mediators. IgM is the primary antibody type that recognises larvae, and is thought to be produced independently of T cells (Anthony *et al.*, 2007).

2.3 Immune responses to *D. viviparus* infection

Even if many of the studies underlying the theories of host immune responses have been performed in mouse models, most of the features are also recognized in immune responses of cattle. However, in cattle, the boundaries between Th1 and Th2 are not as clear cut as in the mouse system, although evidence of Th1- and Th2-like responses has been described (Brown *et al.*, 1998).

Antibody responses in *D. viviparus* infection have been much studied, since passive transfer of serum from immune to naïve animals results in partial immunity (Jarrett *et al.*, 1955; McKeand *et al.*, 1995). More recently it has been shown that protection against *D. viviparus* is positively correlated to total IgE levels in serum (Kooyman *et al.*, 2002). Furthermore, observations of significant increases in parasite-specific IgG1 and IgA locally in the lungs of *D. viviparus* immune animals has been made (Scott *et al.*, 1996). Moreover, during *D. viviparus* infection, a strong increase in eosinophilic granulocytes is observed both locally in the lungs and in circulating blood (Schnieder & Daugschies, 1993). When studied in relation to *D. viviparus*, no positive correlation could be found between peripheral eosinophilia and protection against infection; however, it was believed that a correlation would have been found had local and not peripheral measures of eosinophilic responses been made (Kooyman *et al.*, 2002). Also, increased numbers of mast cells have been reported after *D. viviparus* infection (McKeand, 2000). Furthermore, when cytokine mRNA expression during *D. viviparus* infection was measured, a mixed response of Th2 (IL-4, IL-5 and IL-13) and Th1 (IL-12p35 and IFN-γ) type cytokines was upregulated, although Th2 cytokine mRNA expression was detected earlier (Johnson *et al.*, 2005). Finally, a study of acute phase protein responses to *D. viviparus* showed that infection could induce increases in the blood concentrations of serum amyloid A, haptoglobin and fibrinogen (Höglund *et al.*, 2003a).

2.3.1 Observations from a pilot study

Since no previous studies of specific cell mediated immune responses to *D. viviparus* infections had been reported, preliminary analyses were carried out in our laboratory. The study was performed with *in vitro* cultured bovine peripheral blood mononuclear cells (PBMC), stimulated with *D. viviparus*, using ^{3}H-thymidine incorporation as the measure of proliferation. The results revealed that homogenate of adult *D. viviparus* worms had a strong non-specific stimulatory or mitogenic effect on bovine PBMC. The proliferations from naïve and infected calves were of the same

magnitude, making it impossible to detect any parasite specific cell mediated responses (Lundén, personal communication). Similarly, mitogenic effects were also observed with excretory/secretory (ES) products from *D. viviparus* in a study by Matthews *et al.* (2001). These findings were intriguing, since antigen preparations from some other parasitic helminths have been shown to have inhibitory effects on cell proliferation (Allen & MacDonald, 1998). Such inhibitory effects have been suggested to be relevant for the ability of parasites to avoid host immune responses and cause chronic infections (Maizels *et al.*, 2004).

2.4 T cell subpopulations

It was considered possible that the non-specific proliferation observed in the pilot study originated from cell populations other than those involved in memory responses. Therefore, in the thesis, expansions in separate lymphocyte subpopulations were investigated, including B cells and several subpopulations of T cells. Of the T cell subpopulations, CD4 and CD8, as well as γδ T cell receptor (TCR) expressing cells, were analysed. Analyses of proliferation in γδ T cell subpopulations, and T cell populations expressing cell surface markers indicating activation or memory phenotypes, were also included.

2.4.1 γδ TCR expressing cells

In cattle, especially in young animals, γδ TCR expressing cells constitute a large proportion of the peripheral blood lymphocytes (Hein & Mackay, 1991). The function of these cells is unclear, but they are different from αβ TCR expressing cells in that they do not require antigens to be presented by major histocompatibility complex (MHC) molecules (Schild *et al.*, 1994). There are, however, some similarities in function: as for the αβ T cells, the γδ T cells produce cytokines such as IL-2 and IFN-γ and activated human γδ can be cytolytic (Hanby-Flarida *et al.*, 1996). Moreover, in one study, after injecting mice with either a Th1 promoting intracellular bacteria, *Listeria monocytogenes*, or a Th2 promoting extracellular parasite, *Nippostrongylus brasiliensis*, it was concluded that cytokines produced by γδ TCR expressing cells may contribute to the cytokine environment, and thereby influence early development of αβ TCR expressing helper subsets (Ferrick *et al.*, 1995).

There is evidence of murine and human γδ TCR expressing cells playing a part in maintenance of epithelia and in the epithelial defence against

pathogens and malignancies (Boismenu *et al.*, 1996; Lahn, 2000). Also, γδ TCR expressing cells are closely linked to the recruitment of eosinophilic granulocytes and the production of Ig E, probably through production of immunoregulatory cytokines (Lahn, 2000).

2.4.2 Subpopulations of γδ TCR expressing cells

Two distinct subpopulations of bovine γδ TCR expressing cells have been described as $WC1^{+}/CD2^{-}/CD4^{-}/CD8^{-}$ and $WC1^{-}/CD2^{+}/CD8^{+/-}$ (Machugh *et al.*, 1997). The subpopulations have functional differences; for example it has been demonstrated that WC1 negative γδ TCR expressing cells are less efficient at accumulating at sites of inflammation than are WC1 positive (Blumerman *et al.*, 2006; Wilson *et al.*, 1999). Also their distribution in various tissues differs. WC1 positive cells are the predominant subset in the circulation, whereas WC1 negative γδ TCR expressing cells are more abundant in for example spleen, mammary gland, intestine, skin and uterus (Hein & Dudler, 1997; Machugh *et al.*, 1997; Meeusen *et al.*, 1993; Park *et al.*, 1992; Wilson *et al.*, 1999; Wyatt *et al.*, 1996; Wyatt *et al.*, 1994).

WC1 is a transmembrane glycoprotein that is uniquely expressed on γδ TCR expressing cells in ruminants and pigs. It occurs in three isoforms, of which WC1.1 and WC1.2 are expressed in mostly non-overlapping populations, whereas WC1.3 is expressed in a small subpopulation of WC1.1 positive cells (Rogers *et al.*, 2005a; Wijngaard *et al.*, 1994). Specifically, the WC1.1 cells are stated to serve as coordinators of inflammatory responses in young cattle, creating an environment that supports cell-mediated activities with a Th1 bias, until antigen specific CD4 expressing and CD8 expressing effector and memory cells have had time to develop (Rogers *et al.*, 2005b).

2.4.3 Activated γδ TCR expressing cells

When activated, bovine γδ T cells co-express high levels of MHC class II. They also produce co-stimulatory molecules and are known to present antigens to CD4 expressing cells. This was shown with resting CD4 expressing T cells from calves immunized with ovalbumin or respiratory syncytial virus antigen that proliferated in response to γδ T cells pulsed with antigen (Collins *et al.*, 1998). Similarly, in the human system, a subpopulation expressing the Vγ2Vδ2 TCR, which in humans make up the majority of circulating γδ TCR expressing cells, display characteristics of professional antigen presenting cells. When activated, these cells processed and displayed antigens, as well as provided co-stimulatory signals that gave

strong induction of naïve $\alpha\beta$ TCR expressing cell proliferation and differentiation. Moreover, surface versus intracellular staining of Vγ2Vδ2 TCR expressing cells proved *de novo* production of MHC class II during activation (Brandes *et al.*, 2005; Moser & Brandes, 2006).

2.4.4 Activated CD4 and CD8 expressing cells

Expression of different isoforms of CD45, also called the leukocyte common antigen, identifies T cells that have different functions, tissue distributions and recirculatory pathways (Bembridge *et al.*, 1995). Bovine CD4 expressing cells that originated from peripheral blood of immune animals, and were positive for high molecular weight (MW) CD45 isoforms, failed to proliferate in response to specific antigen. The conclusion drawn from this was that CD4 positive T cells coexpressing high MW isoforms of CD45 were naïve. The population that did not express these isoforms contained the memory cells that responded in proliferation assays and expressed a CD45 isotype denoted CD45R0 (Bembridge *et al.*, 1995). Data for CD8 expressing T cells are inconclusive, and considerable amounts of CD8 expressing memory cells have been observed also in the CD45R0 negative population (Howard *et al.*, 1991). Furthermore, although naïve T cells are CD45R0 negative, and become CD45R0 positive after exposure to antigen, expression of CD45R0 has been suggested to represent a state of activation, with some progeny reverting to a CD45R0 negative state, rather than a memory phenotype (Bembridge *et al.*, 1995).

3 Aims of the Thesis

The overall aim of this thesis was to improve the understanding of the immune events involved in the induction of protective immunity to *D. viviparus*, and the mechanisms behind the pathological changes caused by the infection. Earlier studies mainly focused on the humoral immune response, and to provide new aspects the present study was directed towards specific and non-specific cellular responses. More information on cellular responses would be of help in the search for new vaccine candidates and when choosing suitable adjuvants, and is thereby essential in the development of a new efficient vaccine against *D. viviparus*.

In more detail, the aims of the separate studies were to:

- Study local *in vivo* cellular responses to experimental *D. viviparus* infection (I)
- Identify cell populations involved in memory responses to *D. viviparus* infection through *in vitro* proliferation studies (II)
- Identify cell populations that proliferate *in vitro* after stimulation with *D. viviparus* mitogen (II)
- Identify substances in *D. viviparus* homogenate associated with mitogenic effects (III)

4 Comments on materials and methods

A general description of the materials and methods used is given here. Detailed information is available in each paper.

4.1 *In vivo* and *in vitro* analysis of immune cell responses

In the present study, immune cell responses were studied both *in vivo* and *in vitro*. These methods have their respective advantages. *In vivo* studies take into account all of the complex interactions between pathogen and host, whereas *in vitro* studies make it possible to study responses to stimulation by various antigens with limited use of laboratory animals.

In paper I, *in vivo* cell mediated responses to an experimental *D. viviparus* infection were investigated over a period of 15 weeks. Analyses of local mononuclear cell responses in the lungs were performed with indirect immunofluorescence labelling and subsequent flow cytometry analysis of cells collected from bronchoalveolar lavage fluid (BALF). Local differential leukocyte counts were determined by identification of nucleated cells on haematoxylin-eosin stained cytospin slides. Also, differential counts of peripheral blood leukocytes were performed throughout the experiment.

In papers II and III, *in vitro* proliferative responses to *D. viviparus* stimulation was investigated. In paper II, proliferative responses of cryopreserved PBMC collected from parasite naïve and immune animals during the experimental infection in paper I, as well as responses of freshly collected PBMC from *D. viviparus* naïve animals of different age groups, were investigated. In paper III, the mitogenic activity of *D. viviparus* homogenate that had undergone different treatments and that of separate

D. viviparus components were studied. This was done with whole blood cultures.

Two types of *in vitro* analyses of cell proliferation were used: in paper II proliferation was determined with fluorescence intensity of carboxy-fluorescein succinimidyl ester (CFSE) stained samples, analysed by flow cytometry; and in paper III proliferation was determined with ^{3}H-thymidine incorporation, quantified in a liquid scintillation counter. The major difference between the methods is that when measuring cell-associated radioactivity, the results only represent the activity during the period of ^{3}H-thymidine incorporation (in this case the last 24 h) whereas the CFSE method depicts the events throughout the whole culture period (5 days). When CFSE stained cells divide in culture, CFSE is distributed equally between daughter cells. These become half as fluorescent as their parents and so on, which gives a history of proliferative events during culture. Moreover, CFSE analyses are compatible with immunofluorescence labelling for expression of cell-surface differentiation antigens and can thereby provide information on proliferation in separate cell populations. In paper II, cell populations defined by expression of a single cell-surface differentiation antigen, or co-expression of two cell-surface differentiation antigens, were analysed for proliferative responses. In paper III, where information over all proliferative responses rather than responses of separate cell populations was investigated, measures of cell-associated radioactivity were recorded. This method provided an easier way to analyse proliferative responses to many different *D. viviparus* preparations.

4.2 Animals

The studies were performed with clinically healthy cattle of the Swedish Red and White breed. The Swedish Red and White breed is the most common breed in Sweden, and so it was natural to perform the investigations of the present study with this breed. However, in ruminants, breed has been shown to influence the sensitivity to helminths due to genetic variation (Stear & Murray, 1994).

All animals, except those experimentally infected, were free of *D. viviparus* and conventionally kept at their birth farms throughout the experiments. Calves and heifers had never been outside and only the adult cows in paper II had been on pasture. This implied that the adult cows had most likely been exposed to pasture-borne helminths, but of species other

than *D. viviparus*, and could possibly have acquired specific memory to helminth antigens consistent between species. Moreover, the animals were tested free of *D. viviparus* according to serology.

In the experimental infection performed in paper I, male calves that were approximately 2 months old at the start of the study were used. In paper II, three age groups of female cattle were included: 4-5 months old calves, 6-14 months old heifers and adult cows, 3-10 years old.

Comparing responses of cells collected from animals of different age in paper II was interesting for two main reasons. In paper I, interesting responses of γδ TCR expressing cells were observed, and these cells are found in large proportions in newborn or young calves and then decline with age (Baldwin *et al.*, 2000). Moreover, lymphocytes collected from calves were described to proliferate spontaneously *in vitro*, without stimulation by mitogens or antigens (Eisenberg *et al.*, 2008).

4.3 Parasites

The *D. viviparus* isolate used in the studies was originally obtained from Intervet, the Netherlands. In order to get highly viable larvae, the parasites were passaged through donor calves prior to the experimental infection (I). To estimate adequate infection doses for donor calves, viability of larvae was assessed by visual examination. At slaughter, adult worms were recovered from the lungs of all infected animals and later used to prepare homogenates for the subsequent *in vitro* studies (II and III).

4.4 Experimental infection and sample collection

The experimental infection (I) aimed to make comparisons between naïve and immune animals. Such a study had earlier been performed successfully by Höglund *et al.* (2003a) and the experimental design of that study was adapted to fit paper I. The calves were divided into three groups: one group was inoculated twice, 10 weeks apart, another was inoculated once, and one remained uninfected. The inoculation dose was 500 L_3 per calf, which was assumed to give a moderate infection. The animals were slaughtered and the lungs were examined at week 15. Throughout the experimental infection a clinical examination of all animals was performed once per week, and the animals were weighed every second week. Blood, faeces and BALF were collected weekly.

BALF samples provided an efficient means to study local responses to lung infections. However, the dilution factor of collected BALF samples was unknown, and samples could therefore only be compared on the basis of proportions. This implied that if, for example, the proportions of one cell population were to increase substantially, then proportions of other cell populations were reduced as a result of mathematics rather than biology. Blood cell counts of leukocytes were performed on representative samples, and therefore these results were presented as absolute numbers.

4.5 Characterisation of mitogenic components

To investigate which components of *D. viviparus* homogenate induced the non-specific proliferation (III), the homogenate was exposed to treatment with DNase and/or RNase as well as protease, and endotoxin content was estimated. Moreover, homogenate was subjected to separation by mass. This was performed using centrifuge filtration and size exclusion chromatography (SEC), and the proteins in the fractions were visualised on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The SEC fractions that induced the strongest proliferative responses of PBMC collected from parasite naïve animals were further analysed by immunoblotting. Also, amino acid sequence and glycosylation of proteins in these SEC fractions were examined. Amino acid sequences were determined using Edman degradation.

4.6 Preparation of PBMC and *in vitro* cell culture

PBMC were isolated from newly collected blood samples over a density gradient. PBMC collected during experimental infection were cryopreserved until culture. The use of cryopreserved cells was somewhat problematic. Some cultures of these cells died for unknown reasons. It was thought that small differences during the freezing procedure might be the reason, but no pattern explaining the cells' different ability to survive after thawing was found. Furthermore, analyses of cryopreserved cells required substantial preparatory work to optimise the method of analysis. Probably due to higher proportions of dead and degenerating cells in these cultures, compared to those of freshly collected cells, background signals were registered from all fluorescence detectors, which made careful adjustment of the flow cytometer settings essential.

In papers II and III, PBMC or whole blood, respectively, was suspended in culture medium and incubated in round bottomed 96-well plates for 5 days. The time of culture was determined after testing to attain maximal proliferative responses in cultures. Cultures were stimulated with *D. viviparus* homogenate in paper II; and in paper III cultures were stimulated with untreated homogenate, with homogenate that had undergone protease or DNase/RNase treatment and with separated homogenate fractions. Concanavalin A (Con A) stimulation was used as the positive control for proliferation, and unstimulated medium culture wells were included as negative controls.

To exclude specific memory responses to common helminth antigens, only animals that had never been on pasture were used in paper III. Furthermore, to comply with Swedish legislation only quite young animals could be kept stabled throughout the experiment and therefore calves and to some extent heifers were used. PBMC collected from calves, both in papers II and III, however, showed spontaneous *in vitro* proliferation without stimulation by antigens or mitogens, as has been described previously (Eisenberg *et al.*, 2008). In paper III, serum free medium was used to minimise such spontaneous proliferation. This, in combination with presentation of results as net counts per minute, where medium control proliferation was deducted from the proliferation in the sample of interest, gave a clear view of the stimulatory effect in all analyses.

4.7 Statistics

In paper I, longitudinal repeated measures ANOVA was used to detect differences in immune cell responses between groups over time. In paper II, responses from freshly collected PBMC were presented as mean ± 95% confidence intervals (CIs), where non-overlapping CIs were interpreted as statistically significant differences. However, since the results were expressed as percentages, all values were subjected to arc-sin transformation before calculation. For comparison of proliferation between PBMC collected from parasite naïve and immune animals, blood samples were collected from the same animals before infection and after reinfection. Results were analysed pair-wise using the Wilcoxon signed rank test, to avoid influence by variation in spontaneous *in vitro* proliferation between animals.

Proliferation in paper II was expressed as the proportion of lymphocytes positive for a specific cell-surface differentiation antigen that had undergone

cell divisions, defined by decreased CFSE fluorescence intensity, i.e. (proliferated CD4 expressing cells)/(total CD4 expressing cells). This should not be confused with the proportion that a single subpopulation constitutes of the total number of lymphocytes after culture, i.e. (total CD4 expressing cells)/(total lymphocytes). Moreover, in paper II most analyses were performed on 6 animals, but simultaneous stainings of two cell-surface differentiation antigens gave such large variation of proportions of proliferated cells that 12 animals per analysis were included.

It is worth noting that the sample sizes in paper II were sometimes affected by the exclusion of samples when less than 200 out of the 10 000 counted lymphocytes expressed the antigens of interest. This potentially affected the ability to detect significant differences between experimental treatments. Such problems could possibly be alleviated in future work by including larger number of animals in the experiment, thus allowing for potential data loss. Just as important, however, is the possibility that there were biological causes to the low proliferation in some samples, and this would not have been alleviated by larger sample sizes.

In paper III, the proliferation data were presented as mean net counts per minute (cpm), i.e. cpm values for mitogen stimulated cultures minus cpm values for corresponding medium controls, for six animals with 95% confidence intervals (CI), where non-overlapping CI indicated statistically significant differences.

5 Results and discussion

5.1 Outcome of the experimental infection (I)

The experimental infection was designed to make comparisons between immune and naïve animals possible. Results showed that reinfected animals had, indeed, developed a certain level of protective immunity to *D. viviparus*, indicated by reduced shedding of larvae, and reduced numbers and length of adult worms in the lungs at slaughter, compared to primary infected animals.

The inoculation dose used induced, as expected, a mild to moderate primary infection, indicated by coughing, elevated respiratory rate (Figure 2; paper I) and larval secretion after primary infection. Moreover, it was noted that BALF samples from reinfected animals contained more blood and debris than did samples from primarily infected animals. This indicated that the lungs had not healed between inoculations, and it could be argued that a longer inoculation interval would have been better, since the aim was to study responses to the parasite infection and not cells involved in tissue repair.

Use of specific pathogen free (SPF) animals, i.e. laboratory animals that are guaranteed free of particular pathogens, was not an option in the present study. The study was designed to investigate immune responses to *D. viviparus* in the natural host, and keeping cattle SPF was considered too awkward, not to mention too expensive. This created a difficulty: some calves seroconverted to bovine adenovirus type 3 during the early part of the experimental infection. This was considered a possible reason for observed differences in some parameters, e.g. coughing (Figure 2; I) and proportions of CD4 expressing cells (Figure 4c; I), between the two primary

infections. These differences were taken into consideration when the results were interpreted. However, many of the parameters recorded, in particular γδ TCR expressing cells (Figure 4a; I) and eosinophilic granulocytes (Figures 5a and 5c; I), responded strikingly similarly in both groups.

5.2 Immune cell responses to *D. viviparus* infection (I)

When studying the immune cell responses to *D. viviparus* infection *in vivo*, the most striking observation was the large increase in proportion and relative cell size of γδ TCR expressing cells in BALF, 2 weeks after primary infection and 3 weeks after reinfection (Figures 4a and 4b; I). The increase in γδ T cells coincided with recovery of adult worms in BALF, and was therefore thought either to be a response to *D. viviparus* proteins, similar to responses of human γδ TCR expressing cells after *in vitro* stimulation with proteins of the filarid nematode *Onchocerca volvulus* (Munk *et al.*, 1996), or an indication of tissue damage. In fact, γδ T cells have earlier been suggested to have functions in the maintenance of both normal and diseased epithelia, and to help sustain immune homeostasis in the lungs (Boismenu *et al.*, 1996; Lahn, 2000).

The increase in proportions of γδ TCR expressing cells in BALF after primary infection was observed simultaneously with significant increases in proportions of eosinophils, both in BALF and blood. γδ TCR expressing cells are known to be correlated to recruitment of eosinophils and production of IgE (Lahn, 2000), and the present results were considered a reflection of this. The recruitment of eosinophils in the present study could, however, also have been induced by other cells and/or directly by parasite antigens. Moreover, after reinfection eosinophilic responses were more pronounced than after primary infection, which was thought to be a sign of B or T cell mediated memory, or possibly due to priming by inflammatory cytokines. Only 10 weeks had passed since the first inoculation, and inflammatory processes were still ongoing in the lungs.

As for the rest of the cell populations studied, no changes in proportions were detected that could clearly be attributed to the *D. viviparus* infection, other than an increase in the proportions of neutrophilic granulocytes in BALF (Figure 4b; I). The increase in proportions of neutrophils occurred simultaneously with the increase in proportions of eosinophils after primary infection. This was in agreement with an earlier study, where increasing

proportions of neutrophils were detected after *D. viviparus* infection, making this cell population the most abundant (Schnieder & Daugschies, 1993).

5.3 Non-specific *in vitro* responses to *D. viviparus* stimulation (II)

As found in previous investigations (Lundén, personal communication), *in vitro* stimulation with *D. viviparus* homogenate induced non-specific proliferation in both naïve and parasite immune bovine PBMC. The non-specific and the specific proliferation could have originated from different cell populations; and the hypothesis at the beginning of the investigations was that analysis of proliferation in separate cell populations could make it possible to distinguish between specific memory responses and non-specific proliferation. Therefore analyses were carried out using CFSE loading combined with immunoflourescence labelling for expression of cell surface antigens, a method that clearly showed proliferation of separate cell populations.

The first set of experiments was performed on freshly collected cells from *D. viviparus* naïve animals of different ages. Results showed that stimulation with *D. viviparus* homogenate did induce non-specific proliferation in all of the T cell subpopulations investigated. CD4 and CD8 expressing cells as well as γδ TCR expressing cells, proliferated among PBMC collected from naïve animals of all three age groups (Figure 2; II). However, since experiments including cell sorting were not performed, it can not be determined how many, if not all, of the cell populations were actually induced to proliferate directly by parasite substances. Activation of a single population could have large impacts on the activation of others.

Differences in responses between age groups were observed for Ig expressing cells (B cells), which only showed significant proliferative responses in cultures of PBMC collected from adult cows (Figure 2b; II). The adult cows had been grazing, unlike the younger animals that had never been outside, and although pastures were free of *D. viviparus*, cross reactive specific memory responses to common nematode epitopes were regarded as the most probable explanation for the difference between age groups. Such cross reactive responses seemed to be the reason for the proliferation of lymphocytes from non-exposed individuals induced by *Leishmania* and *Plasmodium* antigens. Depletion of CD45R0 positive memory cells abolished

these responses, indicating that the responses were dependent on specific memory (Jones *et al.*, 1990; Kemp *et al.*, 1992).

The fact that γδ TCR expressing cells proliferated to a large extent after *in vitro* stimulation with *D. viviparus* was considered interesting, since this cell population responded also in the *in vivo* study. This finding supported the idea of γδ T cells being directly stimulated by the nematodes, or by substances released from them, rather than the possibility that the influx observed in the *in vivo* study (I) was a response to tissue damage after invasion of the lungs. Furthermore, the finding that T cells rather than B cells proliferated made it unlikely that the proliferation originated from components that are known to have stimulatory action foremost on B cells, e.g. LPS and CpG oligodeoxynucleotides (Ulmer 2000, Klinman 2004).

T cell responses were further examined only in PBMC from 6-14 months old heifers, since no differences between age groups had been observed among these cells. It was concluded that both WC1 and CD8 expressing subpopulations of γδ T cells proliferated in response to the stimulation (Table 2; II), and when examining subtypes of WC1, mainly WC1.1 expressing cell proliferated. This was interesting, since WC1.1 has been described as the primary source of γδ T cell derived IFN-γ. Proliferative responses after stimulation with other mitogens have previously shown a bias toward responses by WC1.2 (Rogers *et al.*, 2005b).

Moreover, γδ TCR and CD4 expressing cells that were activated, as indicated by their respective expression of MHC class II and CD45R0, had proliferated in larger proportions than naïve cells. This characteristic was not observed for CD8 expressing cells (Table 2; II). These results could either indicate that the proliferative responses of γδ TCR and CD4 expressing cells were strongest among cells that were activated, or that expression of the respective activation markers was upregulated as a result of proliferation.

Furthermore, the present study showed similar results after stimulation with *D. viviparus* homogenate and Con A, although responses to *D. viviparus* were overall smaller (Figure 2a; II). In other studies, differences in proliferative responses to various mitogens have been observed. For example, Quade and Roth (1999) showed that bovine CD4 and CD8 expressing lymphocytes proliferated after stimulation with Con A, pokeweed mitogen (PWM), phytohemagglutinin (PHA) or anti-CD3 monoclonal antibody, whereas γδ T cells proliferated after stimulation with

Con A or PHA, but not in response to PWM or anti-CD3 stimulation. In another study, stimulation with staphylococcal enterotoxin C1 led to proliferation of CD8 expressing lymphocytes, but not of CD4 and γδ T cells (Ferens *et al.*, 1998). Also, homogenate of the filarial nematode *Oncocerca volvulus* preferentially stimulated the Vδ1 expressing subset among γδ T cells from parasite naïve humans and in the presence of *O. volvulus* ES-products αβ T cells, but not Vδ1 or Vδ2 expressing γδ T cells (Munk *et al.*, 1996).

5.4 *In vitro* responses of PBMC collected from immune animals (II)

Since non-specific responses evidently were extensive and observed in a wide range of lymphocyte populations, comparison between parasite naïve and immune animals, to distinguish specific memory responses, was difficult. In addition to the more refined method that could analyse proliferation of separate cell populations, pair-wise comparison of results from individual animals before infection and after reinfection was used. This step avoided influences of variation in non-specific proliferation between individual animals. However, detection of increased proliferative responses from reinfected animals was not possible in any of the cell subpopulations studied. In contrast, overall proliferative responses to *D. viviparus* homogenate were in fact reduced for reinfected animals, an effect that was accounted for by a similar reduction in responses from γδ TCR expressing cells (Figure 3; II). However, it could not be excluded that the observed reduction in reactivity possibly was related to increased age of the animals and not to the infection. Since no significant differences in proliferation of γδ TCR expressing cells from adult cows and calves was detected in the first part of the *in vitro* study, age related differences were considered unlikely. An explanation for the reduced reactivity of γδ TCR expressing cells could be down-regulation through cytokines produced by *D. viviparus* specific T cells or the previous exposure to stimulatory *D. viviparus* substance(s) *in vivo*. Down-regulating effects of mitogens on host immune responses have been suggested by earlier *in vivo* studies (Diamantstein *et al.*, 1976).

No increased proliferative responses of CD4 or CD8 cells expressing the memory marker CD45R0 were detected among PBMC collected from parasite exposed animals. It is possible that the large non-specific proliferation concealed actual differences, and it would be interesting to continue the investigation with memory markers other than CD45R0. Such alternatives could be for example CD44, which has been used as a marker

for memory T cells in the bovine system or CD25 (IL-2R), which is expressed by antigen activated T cells (Blumerman *et al.*, 2007).

5.5 Mitogenic components of *D. viviparus* homogenate (III)

When performing the *in vitro* study of immune cell responses to *D. viviparus*, a lot of questions concerning the nature of mitogen, i.e. the substance that induced the non-specific proliferation, were raised. More information on the mitogenic component(s) would hopefully give insight into host-parasite interactions and make it possible to sort out and remove the mitogen from antigen preparations. This would be a way to by-pass the problem of the non-specific proliferation in *in vitro* studies. The main object of paper III was therefore to characterise and possibly identify the mitogenic component(s) of the homogenate.

Since treatment with protease completely removed the mitogenic activity it was concluded to be caused by protein(s) (Table 1; III). Moreover, endotoxins and nucleic acid were determined not to be possible causes for the mitogenic activity (Table 2; III). An initial separation of the homogenate, using centrifuge filtration, showed that the fraction that had passed through the 10 kDa filter had no mitogenic activity. Separation of the homogenate by SEC showed that the fractions denominated 17 and 18 provided the largest non-specific proliferation. These fractions contained a few proteins with masses of approximately 13-16 kDa and one of approximately 25 kDa, determined by SDS-PAGE and silver staining. Moreover, also fraction 19, which contained only the 13-16 kDa proteins, induced proliferation (Figure 1; III).

All proteins in SEC fractions 17-18 that were detected by silver staining were also recognised by sera from *D. viviparus* immune animals (Figure 2; III). Similar results were observed also from a previous study where sera from horses, naturally infected with *S. vulgaris*, recognised *S. vulgaris* larval mitogen (Adeyefa, 1992). The immune animals in paper III had experienced the post patent phase of infection when adult worms were degraded. It would have been interesting to also investigate sera collected earlier during infection, to determine when seroconversion to these proteins occurs. Such investigations could possibly determine when during the course of infection the proteins are exposed to the host immune system.

In the homogenate, proteins in the size range of those found in fractions 17-19 were stained for glycans, but results from the SEC fractions were negative (Figure 3; III). The negative results were possibly caused by low protein amounts in the fractions or the proteins in these fractions were not glycosylated. Proteins from SEC fractions 17-19 were submitted to amino acid sequence analysis, but no significant matches to reported proteins were found, and so identification was not possible.

5.6 Biological effects of helminth mitogens

Studies on how helminths effect host immune responses have typically been focused on suppression of antibody production and/or *in vitro* T cell responses (Harnett, 2005), and it has been suggested that such suppression is relevant for the ability of parasites to avoid host immune responses and cause chronic infections (Maizels *et al.*, 2004). Moreover, suppression of responses to bovine helminths has indeed been identified. For example, *Oesophagostomum radiatum* and *Ostertagia ostertagi* antigen preparations have been observed to inhibit both antigen and mitogen induced cell proliferation *in vitro* (De Marez *et al.*, 1997; Gasbarre *et al.*, 1985).

However, researchers now consider the effects of parasite infections to be immunomodulatory rather than immunosuppressive, and it has been observed that host responses in fact sometimes include proliferation of particular T cell subsets rather than suppression. In this way the parasites, instead of suppressing, direct the host responses to benefit their continued existence (Harnett, 2005). Mitogenic microbial components have generally been suggested to inhibit development of specific immune responses. For example, when polyclonal lymphocyte responses are triggered, specific immune responses may be seriously disturbed, with lack of specificity of antibodies or inappropriate T cell responses as a result (Reina-San-Martin *et al.*, 2000).

Interestingly, non-specific *in vitro* proliferation of lymphocytes has been observed in response to nematodes other than *D. viviparus* parasitizing domestic animals. For example, adult worm extracts and larval ES products of *Strongulus vulgaris* (Adeyefa, 1992; Bailey *et al.*, 1984) and larval antigens of *Haemonchus contortus* (Torgerson & Lloyd, 1993) induced non-specific proliferation. Moreover, a number of parasitic helminths, including *Toxocara canis, Dirofilaria immitis*, *Fasciola hepatica* and *Paragonimus westermani*, have been reported to contain B cell specific mitogens (Wang *et al.*, 1995).

Furthermore, immunomodulatory components that are not strictly stimulatory or suppressive have been observed. For example, the quite extensively examined glycoprotein ES-62, secreted by filarial nematodes, stimulates B cells *in vitro* when administered at high concentrations (25-50 mg/ml) but acts as a suppressant at lower concentrations (0.2-2 mg/ml) (Harnett & Harnett, 1999). However, no down-regulating effect on host cell *in vitro* responses was observed when *D. viviparus* homogenate was added at varying concentrations to cells cultured with Con A (Gomez-Munoz *et al*., 2004).

Also, *in vivo* studies have been performed that suggested down-regulating effects of mitogens on host immune responses. For example, *in vitro* B cell mitogens, polyacrylic acid, lipopolysaccharide, dextran sulphate and purified protein derivate of tubercle bacilli, suppressed the immune response of mice, when injected 2-4 days before *in vivo* immunisation with sheep erythrocytes. On the other hand, the same components enhanced the immune response when injected 30 minutes before immunisation with a suboptimal antigen dose. The study was thought to indicate loss of B cell capacity to respond properly to antigen when stimulated with mitogen in the absence of a particular antigen (Diamantstein *et al*., 1976).

6 Concluding remarks

This thesis suggests a role for γδ TCR expressing cells in the immune response to *D. viviparus* infection. A large influx of activated γδ TCR expressing cells was observed in the lungs after both primary infection and reinfection. Also, when *in vitro* stimulations with *D. viviparus* homogenate of PBMC collected from parasite naïve animals were performed, large proportions of MHC class II expressing γδ T cells had proliferated. MHC class II expression of γδ T cells is considered to be a marker for activation. The *in vitro* responses were seen as a possible indication of γδ TCR expressing cells responding in the lungs being directly stimulated by the parasite, rather than responding to tissue damage. Moreover, when comparing *in vitro* responses of PBMC collected from parasite naïve and *D. viviparus* reinfected animals, the only observed difference was smaller proportions of proliferating cells in *D. viviparus* stimulated cultures of cells from reinfected animals. This was possibly due to down-regulation through cytokines produced by *D. viviparus* specific T cells, or to the previous exposure to mitogenic *D. viviparus* substance(s) *in vivo*. Earlier *in vivo* studies have suggested down regulating effects of mitogens on host immune responses (Diamantstein *et al.*, 1976).

Non-specific *in vitro* proliferative responses to *D. viviparus* homogenate by all of the T cell subpopulations studied suggested that the homogenate contained mitogenic substances. Mitogenic substances are known from several helminths infecting domestic animals and mitogenic effects have indeed previously been reported also of *D. viviparus* ES-products (Matthews *et al.*, 2001). Biological functions of parasite mitogens have not been established, but mitogens are supposed to inhibit development of specific immune responses and direct the host responses to facilitate survival of the parasites (Harnett, 2005; Reina-San-Martin *et al.*, 2000). Thus, to gain more

knowledge of the mitogenic components of *D. viviparus*, characterisation and identification of the components was attempted. Investigations led to the conclusion that the mitogenic components in *D. viviparus* most likely are immunogenic proteins of 13-16 kDa size.

Due to increasing levels of anthelmintic resistance, new approaches to parasite control with reduced reliance on chemotherapeutics are sought after. Effective vaccines to helminth infections would provide alternative approaches in parasite management together with, for example, carefully thought out turnout dates and pasture rotation schemes. However, to develop modern subunit vaccines to nematodes such as *D. viviparus*, further comprehension of the complex host-parasite interactions is needed. Not only identification of potentially protective antigens is crucial for vaccine development, but also knowledge about host mechanisms responsible for the protective immunity is important; for example, to understand the requirements for optimal antigen delivering and presentation. Moreover, insight to immunomodulatory properties of the parasites is required, and protocols including neutralisation of mitogenic pathogen components have been suggested to make vaccines more effective (Reina-San-Martin *et al.*, 2000). This thesis has contributed to this highly complicated issue with information on the immune cell responses to *D. viviparus* infection, and on the mitogenic components found in *D. viviparus*.

7 Future research

Below, some suggestions for future research in relation to the findings of the present study are noted:

- A study of cytokine production by immune cells collected at different time points throughout *D. viviparus* infection, for example by analysis of mRNA expression of BALF cells, would provide central information on the development of protection.

- In the present study only responses of mixed cell populations to *D. viviparus* stimulation was investigated. Stimulating cultures of separate cell populations would show if proliferation in single populations was dependent on activation of other cells. Such investigation of γδ TCR expressing cells would be especially interesting.

- Detection of increased proliferative responses from reinfected animals was not possible in any cell subpopulations studied here, probably due to extensive non-specific proliferation. Further separation of *D. viviparus* antigens, possibly using other antigen properties than size, e.g. 2-D gels including separation by isoelectric point, would hopefully give fractions that do not induce non-specific proliferation. If this was achieved, the method combining CFSE and surface marker analysis could provide information on specific responses to such separated antigens.

- Further investigations of the mitogenic component could for example include examination of contents of lipoproteins. These molecules are interesting, since it has been demonstrated that γδ

TCR expressing cells can recognize lipid antigens (Cui *et al.*, 2005). Moreover, it would be interesting to find out if the mitogenic components are present in other life stages of the parasite.

- *In situ* localisation of both mitogenic components and immunogenic antigens in the worm would provide information about their respective roles in the physiology of the worm and their impacts on the host.

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