Chapter 6

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

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I would never die for my beliefs, because I might be wrong.
(B. Russel)

ABSTRACT

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

INTRODUCTION

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

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

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

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

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

MATERIAL AND METHODS

Animals and experimental design

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

Parasitology

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

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

TABLE 1. Experimental design

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

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

Fractionation of *C. oncophora* excretory/secretory products (ESP)

C. oncophora adult excretory/secretory products (ESP) were obtained using a standard procedure as described before for *Haemonchus contortus* [197]. The obtained supernatant was filter sterilized (0.2 μm) and supplemented with protease inhibitors (Complete®, Roche). ESP were used in lymphocyte proliferation tests (LPT) at a concentration of 5 μg/ml in complete medium (RPMI-1640, Gibco BRL, Paisly, UK) supplemented with penicilline (500 U/ml), streptomycin (0.5 mg/ml), 10% foetal calf serum (FCS), 2 mM glutamine, and 2 mM β-mercaptoethanol).

Cooperia ESP were partially purified by ion exchange chromatography using a low pressure chromatography system (ECONO system, BIORAD, Richmond, USA). All steps were performed at room temperature, however, buffers and protein solutions were kept on ice. The supernatant containing the soluble fraction of ESP was concentrated and desalted (10 mM Tris, 0.9% NaCl pH 7.4) with 3 kDa filters (Centriprep, YM-3, Millipore). After concentration, 5 ml of ESP (2 mg/ml) was applied at a flow rate of 0.2 ml/min onto a Mono S cation exchange column (High S cartridge, 1 ml, ECONO-PAC® CARTRIDGES, BIORAD) previously equilibrated with buffer A (10 mM NaH_2PO_4/Na_2HPO_4, 1 mM EDTA, pH 7.2). Unbound proteins were removed by washing of the column with a 5 bed volume of buffer A and proteins were subsequently eluted with a flow rate of 0.3 ml/min with a 50 bed volume linear gradient (10-500 mM KCl) in buffer A and 1 ml fractions were collected. The unbound fractions of the ESP were pooled, concentrated with 3 kDa filters and subsequently applied onto a Mono Q anion exchange column (High Q cartridge, 1 ml, ECONO-PAC® CARTRIDGES, BIORAD) previously

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

Isolation of lymphocytes

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

Lymphocyte proliferation tests (LPT)

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

Flow cytometric analysis

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

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

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

RESULTS

Parasitology

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

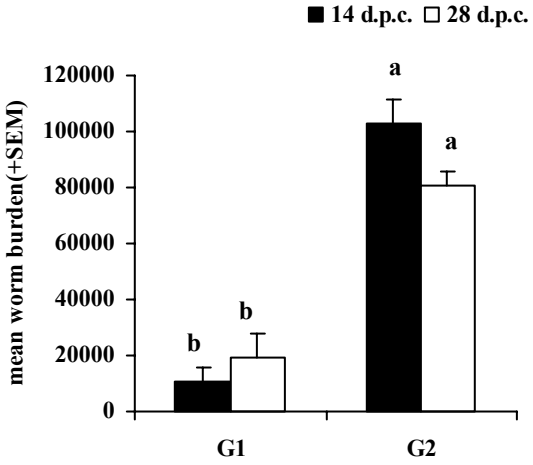

FIGURE 1 Mean worm burden (+SEM) for the animals from G1 (100,000 L3/100,000 L3) and the animals from G2 (0 L3/100,000 L3) at day 14 and 28 p.c..

Fractionation of *Cooperia* excretory/secretory products

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

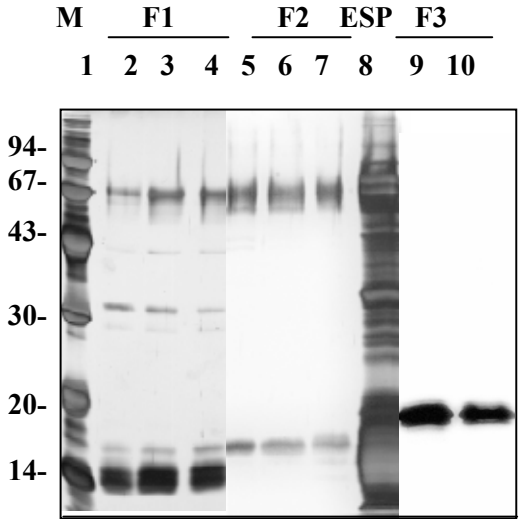

FIGURE 2 Protein pattern of ESP fractions (1 μg/lane) are shown after separation by SDS-PAGE under reducing conditions on a 15% polyacrylamide gel. A low molecular weight marker is given in lane 1. F1 (lane 2-4) and F2 (lane 5-6) are fractions obtained after exchange chromatography with a Mono S cation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

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

***Cooperia*-specific lymphocyte proliferation**

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

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

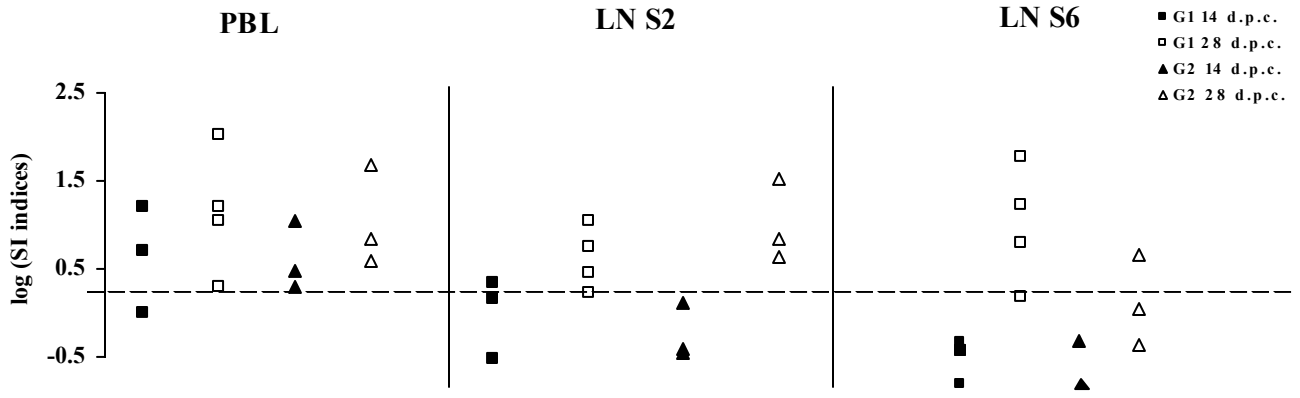

FIGURE 3 Stimulation indices (SI) after stimulation of lymphocytes from the peripheral blood (PBL) and lymph nodes of the proximal (LN S2) and the distal (LN S6) gut with total ESP (5 μg/ml). Data are shown in a logarithmic scale for individual values of the animals from G1 (100,000 L3/100,000 L3) and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

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

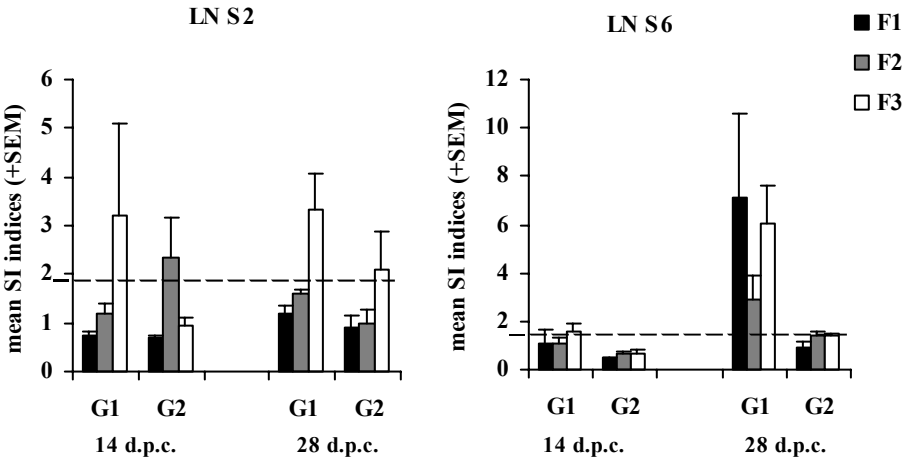

FIGURE 4 Mean SI (+SEM) after stimulation of lymphocytes from the lymph nodes of the proximal (LN S2) and the distal (LN S6) gut with the partially purified ESP fractions F1, F2 and F3 (1 μg/ml). Based on the response of non infected animals, a threshold for stimulation was set at an SI=2 (dotted line).

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

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

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

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

*data from one animal only

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

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

A higher frequency of CD4+ cells was found in LN S2, PP S2, and PBL of the primed animals (G1) compared to the non-infected controls (GC). Concurrent with the elevation in number of CD4+ cells in these locations, we observed a drop in the frequency of CD4+ cells in the lamina propria of G1. This was found both in LP S2 and LP S6 ($P<0.05$ for both). The frequency of CD4+ cells in the proximal gut of the challenge control group (G2) only differed significantly from GC in LN S2.

The higher proportion of CD4+ cells in LN S2 of G1 and G2 coincided with a relative decrease in CD8+ and TCR1-N24+ cells at the same location. No further significant differences in the frequency of CD8+ cells were observed between the different groups or the different anatomical locations. The frequency of TCR1-N24+ cells differed between the infected and non infected animals, resulting in a significant increase in LP S2 and LP S6 in the primed animals compared to GC. In the challenge control animals, the frequency of TCR1-N24+ cells was markedly higher in LP S2 ($P<0.1$) and significantly higher in LP S6 ($P<0.05$) compared to the frequency in GC.

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

As a measure of lymphocyte recirculation capacity the co-expression of CD62L was investigated on CD4+, CD8+ and TCR1-N24+ cells of LN S2, LN S6, and peripheral blood. At day 14 p.c., the only differences in CD62L co-expression were an increased percentage CD8+ CD62L+ cells in LN S2 of G1 and a decreased percentage TCR1-N24+CD62L+ cells in LN S2 and LN S6 of G2 (fig. 5A and fig. 5B, $P<0.05$ for both). At day 28 p.c. the CD62L expression was reduced on the CD4+ and TCR1-N24+ cells of both G1 and G2 in LN S6 ($P<0.1$ for both; fig. 5B). In contrast, we observed a marked increase in CD4+CD62L+ cells in PBL of G1 and G2 compared to the non-infected control group (GC; $P<0.1$; fig. 5C). Simultaneously, a higher frequency of CD8+CD62L+ cells was found in PBL ($P<0.1$). All the observed differences on day 28 p.c. were concurrent in primed and challenge control animals and could consequently not be related to the immune status of the animals.

If CD4+ cells are involved in the immune response to *C. oncophora*, they will likely have a more activated phenotype as compared to other T cell subsets. In order to investigate this, the co-expression of MHCII on CD4+ and CD8+ cells was compared in PBL, LN S2 and LN S6 of primed and challenge control animals. Despite a relative decrease in LN S6, we observed no significant differences between the different groups, neither between the proximal and the

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

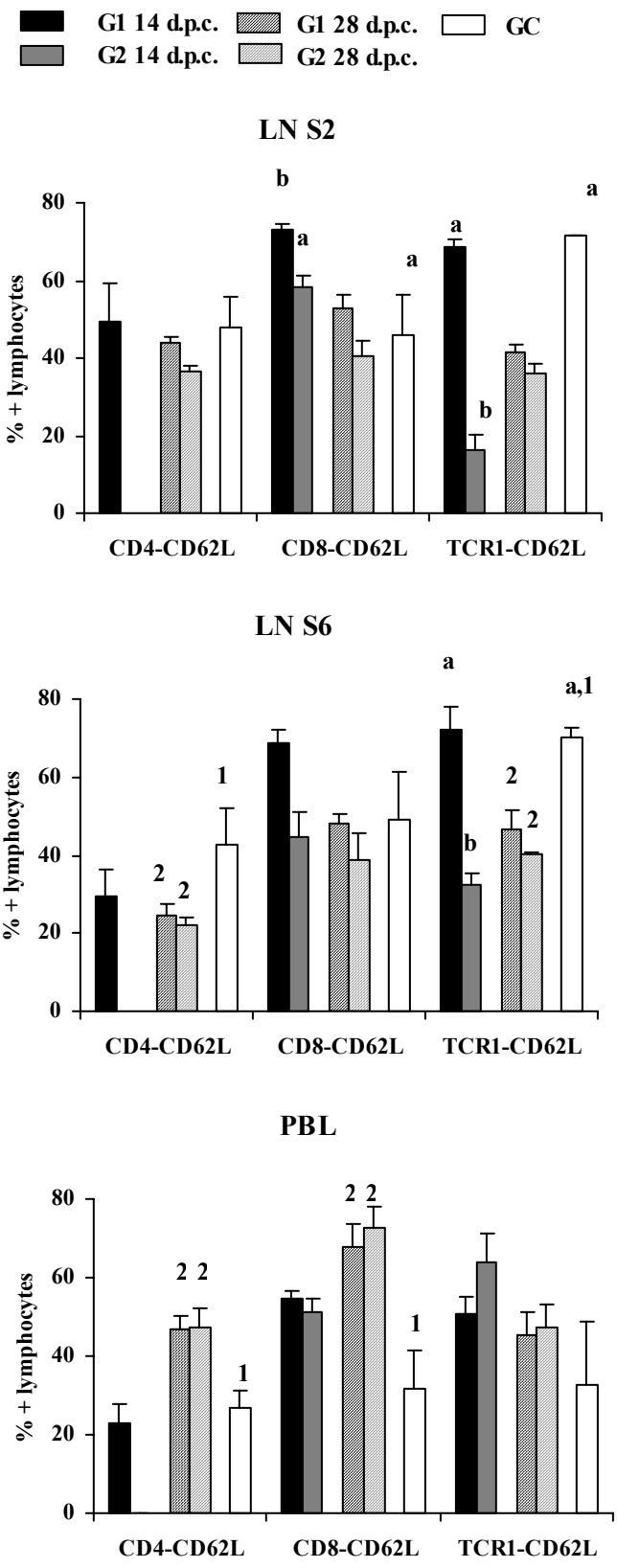

FIGURE 5 The %CD62L+ cells within the gate for CD4+, CD8+, and TCR1-N24 + cells is shown for G1 (100,000 L3/100,000 L3), G2 (0 L3/100,000 L3), and the non infected controls GC (0 L3/ 0 L3) at day 14 and day 28 p.c. in LN S2, LN S6 and the peripheral blood (PBL). Data shown are mean %lymphocytes (+SEM)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

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

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

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

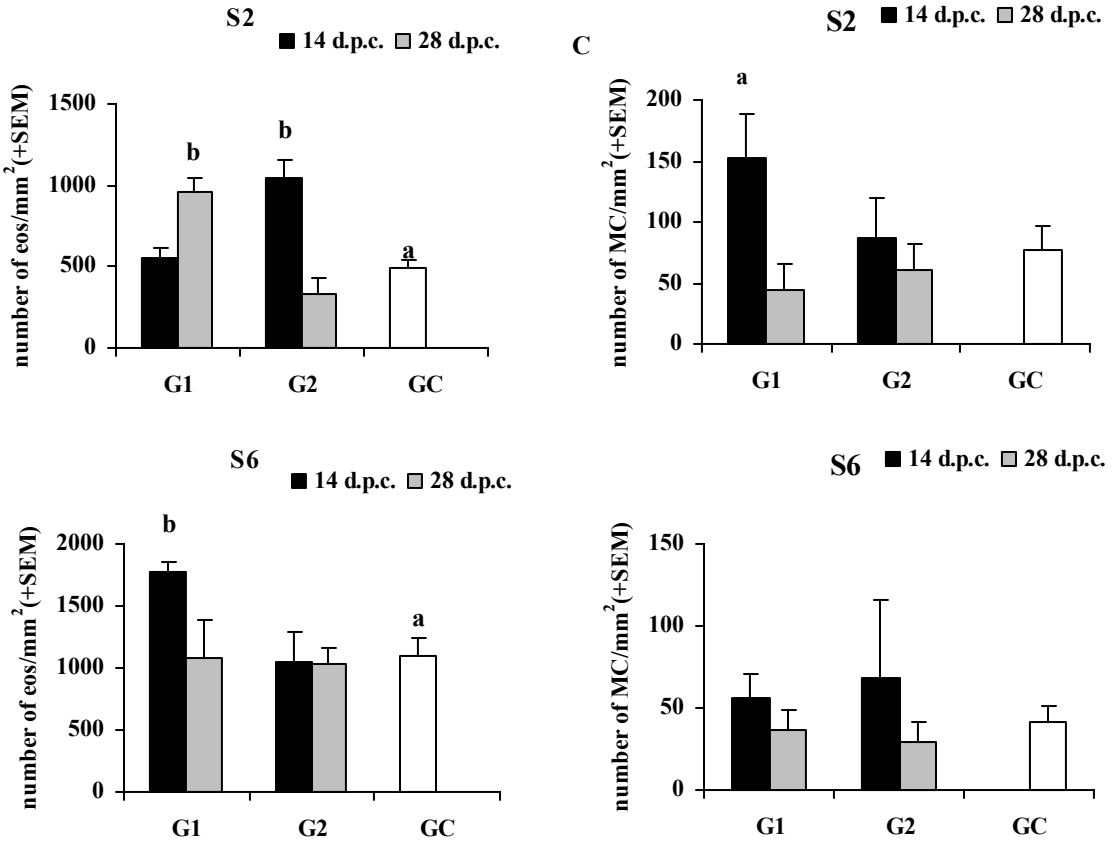

FIGURE 6 Number of eosinophils in the proximal (A) and the distal (B) gut and number of mast cells in the proximal (C) and the distal (D) gut are given for the animals of G1 (100,000 L3/100,000 L3), G2 (0 L3/100,000 L3) and the non infected controls (GC). Different letters indicate significant differences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

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

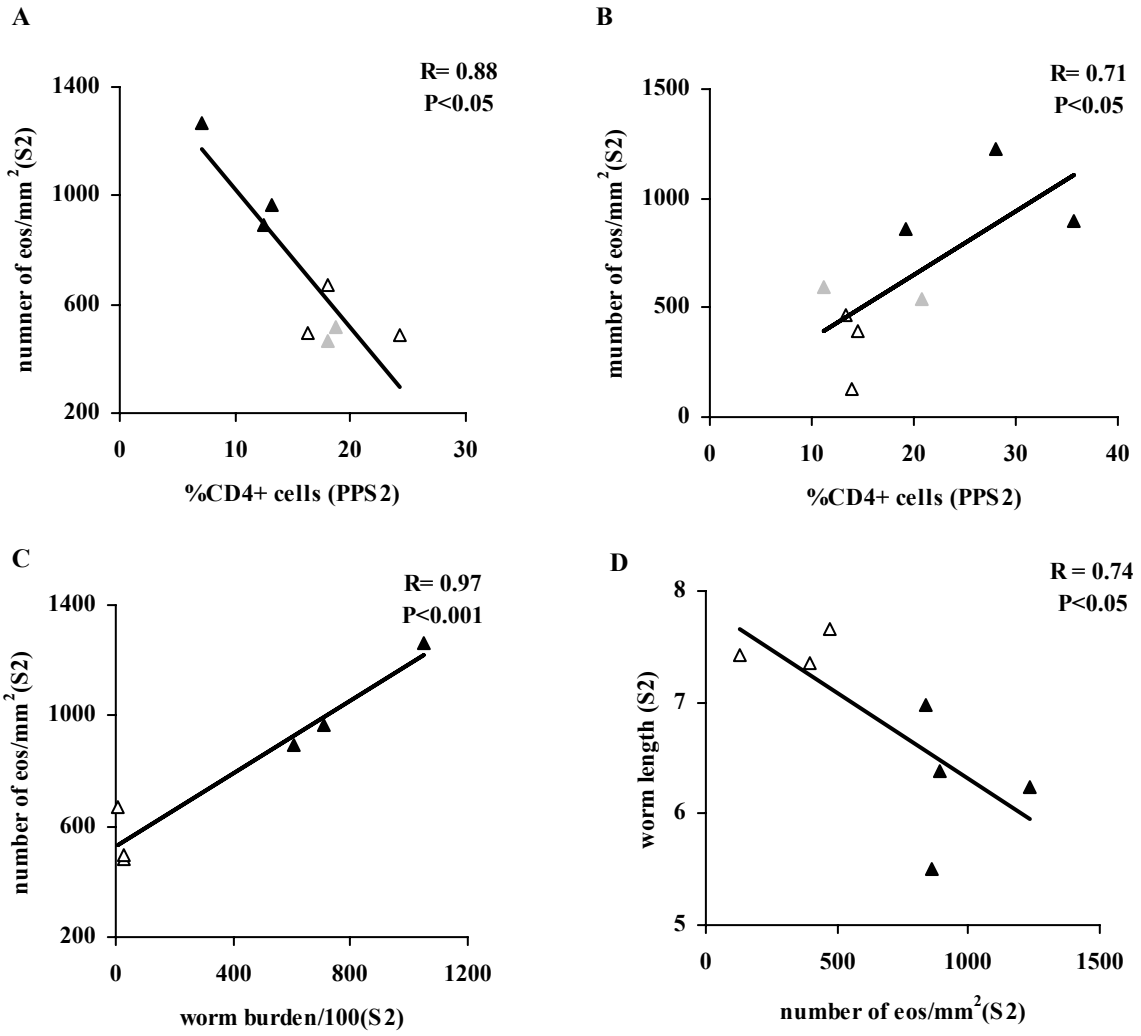

FIGURE 7 Correlation between %CD4+ cells in PP S2 and eosinophils in the proximal gut at day 14 (A) and day 28 p.c. (B). Data from animals from G1 (white), G2 (black) and GC (grey) are included (at day 28 p.c. data from 1 animal of G1 are lacking). Correlations between eosinophils and worm burden in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

Relationships between T lymphocyte subsets and effector cells

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

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

Relationship between effector cells and parasitological parameters

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

DISCUSSION

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

Expulsion of *C. oncophora* is characterized by a distal shift in the worm distribution throughout the intestine [126] and therefore, the CMI in the proximal (duodenum) and the distal (ileum) gut were analysed separately and compared between primed and the challenge control animals.

The proliferative response to *Cooperia* ESP as well as partially purified fractions revealed differences between primed and challenge control animals. Consistent with previous observation [126], the magnitude of the response was highly variable between the animals. In addition, the reactions were not disseminated throughout the intestine resulting in distinct proliferative responses in the lymph nodes of the proximal and the distal gut. The reactions in peripheral blood and the lymph node of the proximal gut were comparable between primed and challenge control animals while in the distal gut, mainly the primed animals showed ESP-specific proliferation. Throughout the challenge infection the proportion of worms residing in the distal gut was higher in the primed animals (Chapter 4). Hence, the enhanced proliferative response likely resulted from a combined effect of the higher antigenic load during secondary infection and the antigenic priming elicited by the primary infection in the distal gut of these animals.

The reactivity of lymphocytes in response to total ESP resumes the effects induced by different antigens and we hypothesized that the use of ESP fractions in LPT would be superior to total ESP in discriminating among the responses of primed and challenge control animals. However, the main conclusion of the LPT with ESP fractions was that the response of the challenge control animals was markedly reduced as compared to their ESP-specific response. Although the number of animals used in the current experiment was too low to generate any conclusive evidence, the data suggest that fractionating resulted in a pool of proteins which are specifically involved in protective immunity against re-infection with *C. oncophora*.

The potential to discriminate between levels of resistance to infection with *C. oncophora* by means of low molecular weight proteins was previously demonstrated by western blot analysis [171, 231] and suggested that resistance could be related to the recognition of distinct B cell epitopes. There is no information on a similar link with T cell epitopes of *C. oncophora* antigens, but our results suggest that protective immunity against re-infection with *C. oncophora* is linked with an enhanced reactivity to low molecular weight fractions of adult ESP. In calves immunized or primary infected with *O. ostertagi*, no difference was detected in the proliferative response to L3 antigen [56], but there are a few reports in which an enhanced and earlier parasite-specific reactivity was observed after secondary infection or immunization [90, 154, 196]. A more comprehensive analysis of the proteins in the involved ESP fractions and a

higher number of animals will be required to identify the exact role and contribution of these proteins to protective immunity.

Priming induced a minor increase in the frequency of CD4+ T lymphocytes in the mesenterial lymph node at day 14 p.c. (data not shown). Two weeks later, the frequency of CD4+ cells in primed animals was significantly increased in the peripheral blood and the lymph nodes and Peyer's Patches from the proximal gut but decreased in the lamina propria of the proximal and distal gut and this coincided with a drop in CD8+ and TCR1-N24+ cells in the mesenterial lymph nodes. In the absence of detailed studies in ruminants, the hypotheses for local sensitization by and response to gut luminal antigens are extrapolated from other species [122, 120, 191]. It is assumed that lymphocytes that have been primed in the intestine migrate via the afferent lymphatic to the draining lymph node where they further proliferate and differentiate. Thereafter, they recirculate via the efferent lymphatics and blood back to the lamina propria of the gut. Based on this recirculation pathway, we postulate that the decreased frequency of CD4+ cells in the lamina propria of the infected animals at day 28 p.c. was caused by a migration of activated cells to the lymph node and the peripheral blood. These findings were consistent with the enhanced proliferation in blood and lymph nodes in response to *C. oncophora* antigens at this time point and suggested that the CD4+ cells were the main proliferating cells. The latter observations were done irrespective of the immune status of the animals, and only the increased frequency of CD4+ cells in Peyer's Patches and peripheral blood of the primed animals supported an enhanced trafficking of lymphocytes in immune animals as compared to challenge control animals. The analysis of CD8+ and TCR1-N24+ cells and the co-expression of CD62L and MHCII on the different T cell subsets were not conclusive. It is however conceivable that substantial proportional changes in CD62L+ or MHCII+ T lymphocytes were obscured by the background of responses to other antigens to which the gut is exposed.

Eosinophils and mast cells are both hallmarks of GI nematode infections [192, 157, 162, 163]. We previously demonstrated that a primary infection with 100,000 L3 resulted in a recruitment of eosinophils in the lamina propria of infected animals at day 42 p.i. [126]. A minor increase in eosinophils was found at day 14 p.i. while no changes were observed in the mast cell population [126]. Based on the features of the infection we hypothesized that the early increase in eosinophils was caused by inflammation as a consequence of larval/worm exposure, whereas by the end of the primary infection eosinophils were involved in expulsion of the adult worm population [126]. The results from the current study confirmed these previous observations. The number of eosinophils in the lamina propria of the proximal gut of the challenge control

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

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

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

p.c. concurred with a decreased worm burden in these animals, the lack of significant correlations underscores that mast cells do not represent an important effector mechanisms in immunity against *C. oncophora*. The observation that mast cell numbers in the proximal gut were significantly correlated with the TCR1-N24+ cells but not with CD4+ cells was surprising, considering that mastocytosis is controlled by cytokines such as IL-3, IL-4, IL-9 and IL-10 which are predominantly secreted by CD4+ Th2 cells [83]. Further investigation is required to discern the exact meaning of the positive correlation between the two cell populations.

In conclusion, with the current results we hypothesize that at least part of the protective memory response to *C. oncophora* is T-cell mediated. This is evidenced by a recruitment of CD4+ cells and an enhanced proliferative response to *C. oncophora* antigens. Proliferation induced by ESP fractions occurred predominantly in primed animals suggesting that fractionating resulted in a pool of proteins associated with protective immunity against re-infection. In addition, our data support that primary and secondary infection with *C. oncophora* induced 2 waves of eosinophils and that the kinetics of this cell population differed as a result of priming. Based on observed correlations we propose that the early increase in eosinophils is independent of T cells and a consequence of inflammation in the parasitized gut. In contrast, in the later phase of the infection eosinophils seem to be recruited by CD4+ cells and involved as effector cells against adult stages of *C. oncophora*.

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