Chapter 5

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

K. Kanobana[1], A. Koets[2], F. N. J. Kooyman[1], N. Bakker[1], H.W. Ploeger[1], L. Vervelde[1]

[1] Division of Parasitology and Tropical Veterinary Medicine, Department of Infectious diseases and Immunology, Utrecht University, PO Box 80.165, 3508 TD Utrecht, The Netherlands
[2] Division of Immunology, Department of Infectious diseases and Immunology, Utrecht University, PO Box 80.165, 3508 TD Utrecht, The Netherlands

International Journal for Parasitology, in press

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

ABSTRACT

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Animal experiments

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

TABLE 1 Experimental design

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

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

Parasitology

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

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

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

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

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

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

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

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

Flow cytometry

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

ELISPOT assay

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

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

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

Statistics

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

RESULTS

Parasitology

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

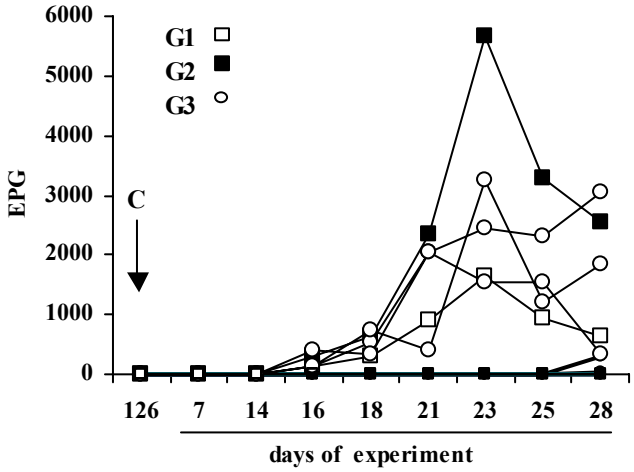

FIGURE 1 The individual EPG (mean number of eggs/gram faeces) of the animals slaughtered at day 28 p.c.. The time of challenge (C) is indicated by the arrow. Animals from G1 (30,000 L3/100,000 L3; open squares, n=4), G2 (100,000 L3/100,000 L3, closed squares, n=4) and G3 (0 L3/100,000 L3; open circles,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

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

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

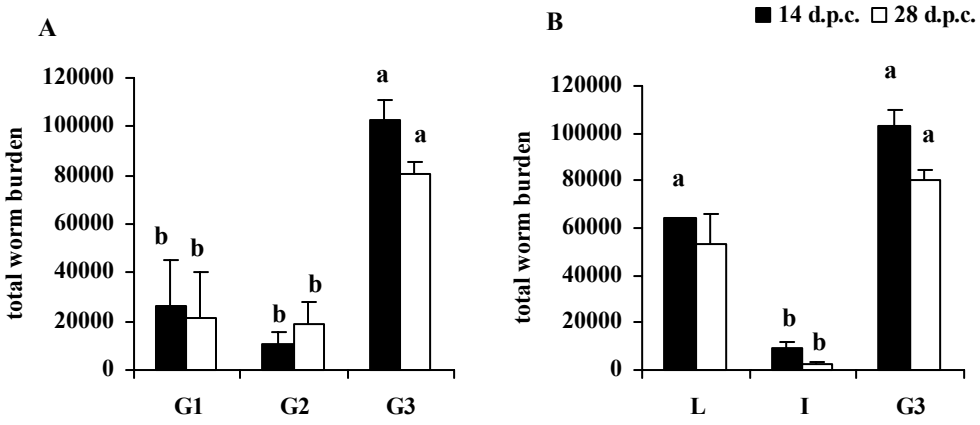

FIGURE 2 (A) Worm burdens of the animals of G1 (30,000 L3/100,000 L3), G2 (100,000 L3/100,000 L3) and G3 (0 L3/100,000 L3) at day 14 and day 28 p.c.. (B) Worm burden of low (L, n=1 at day 14 p.c. and n=4 at day 28 p.c.) and intermediate (I, n=3 at day 14 p.c. and n=4 at day 28 p.c.) responders are compared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

Cooperia 14.2 kDa-specific serum IgG responses

The 14.2 kDa is a sensitive tool for monitoring infections levels within a herd. We currently used this assay to investigate whether it can be applied to discriminate between experimental infections with 30,000 and 100,000 L3 C. *oncophora*. The 14.2 kDa titres did not differ between G1 and G2 during the primary infection (fig. 3A).

Following anthelmintic treatment at day 49 p.i. the antibody levels were maintained for 3 more weeks, but from day 70 p.i. onwards a gradual decrease in 14.2 kDa-specific IgG antibodies was observed. At the time of challenge, antibody titres in G1 and G2 were close to control levels. Challenge infection with 100,000 L3 larvae induced a significant increase in 14.2 kDa specific-IgG, irrespective of the priming infection dose. In both primed groups the antibody response after challenge was significantly higher than during the course of the primary infection (P<0.05), and also higher as compared to both control groups (P<0.01).

Cooperia-specific serum Ig responses

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

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

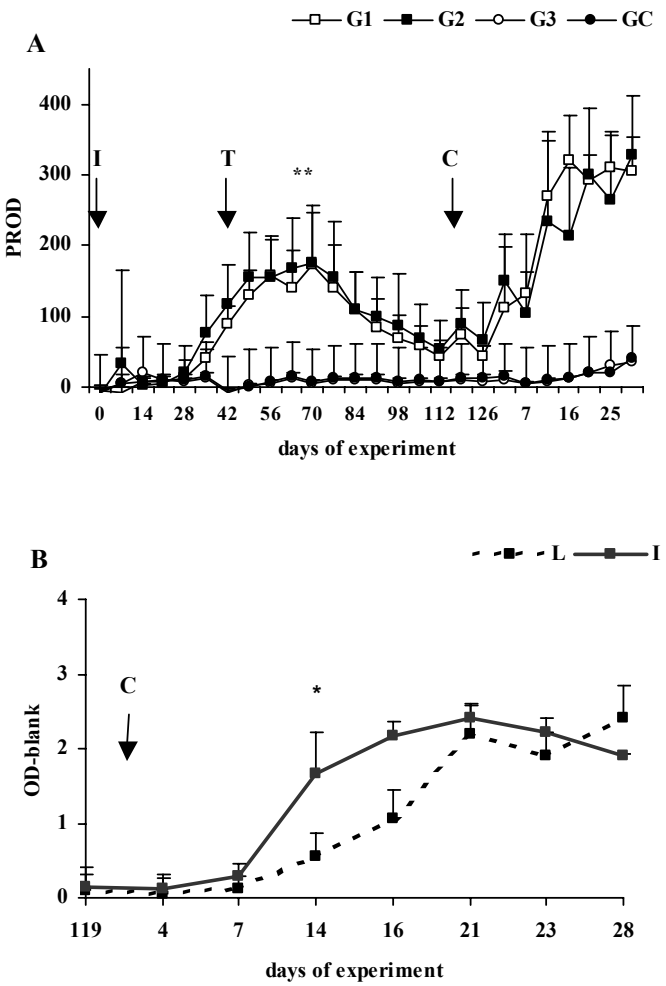

FIGURE 3 (A) The *Cooperia* 14.2 kDa IgG titres in the course of the primary and the secondary infection is given for the animals from G1 (30,000 L3/100,000 L3), G2 (100,000 L3/100,000 L3), G3 (0 L3/100,000 L3) and the non-infected control group (GC). Data are presented as mean PROD (+SEM). Time of primary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

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

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

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

Total serum IgE levels after challenge

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

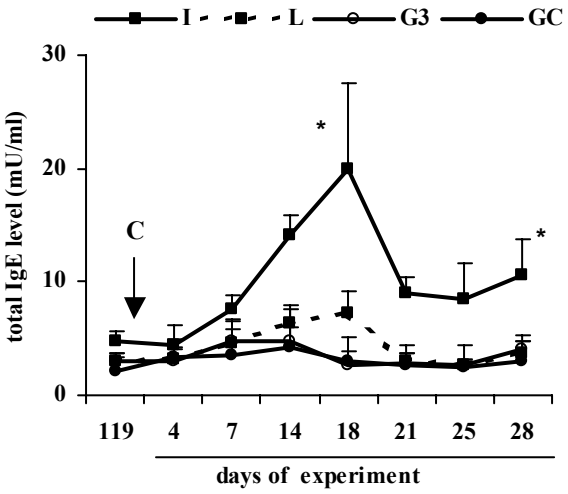

FIGURE 4 Total serum IgE titres after challenge of low (L), intermediate (I), challenge control animals (G3) and non-infected controls (GC) are shown. Time of challenge (C) is indicated. Data are presented as mean values (+SEM) of the animals per group. Underlining on the X-axis indicates the days after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

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

Cooperia-specific mucus Ig responses

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

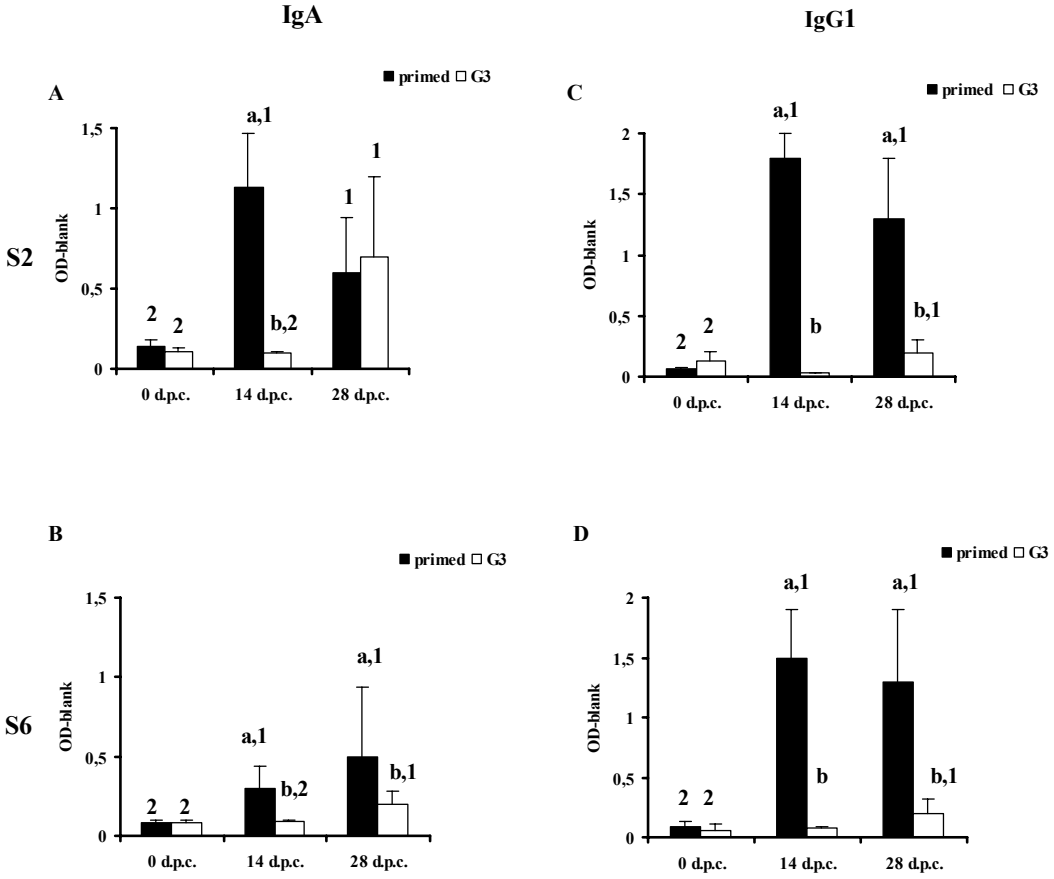

FIGURE 5 Ad-specific mucus IgA (A, B) and IgG1 (C, D) in the proximal (A, C) and the distal (B, D) gut are given for the primed (G1+G2) and the challenge control animals (G3). Results are expressed as OD-blank (no serum incubation) and for each animal the mean of duplicate wells was used. Data are presented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

At day 14 p.c. a 5-fold increase in mucus Ad-specific IgA was observed in the proximal gut of the primed animals compared to G3 ($P<0.05$) and the non infected control group (day 0 p.c., $P<0.01$). At day 28 p.c. the mucus IgA response in the proximal gut of the primed animals dropped but was still significantly higher than the non infected control group ($P<0.01$). At this time point the mucus IgA titres did not differ between primed and challenge control animals. Figure 5B illustrates that the increase in Ad-specific mucus IgA in the distal gut of primed animals was not as pronounced as in the proximal gut. However, at day 14 and day 28 p.c. Ad-specific IgA titres were significantly higher in primed animals than in G3. The continuous increase in Ad-specific IgA titres in the distal gut suggested a different kinetic compared to the proximal gut. Thus, whereas primed and challenge control animals significantly differed in their kinetics of the Ad-specific mucus IgA titres in the proximal gut, in the distal gut both groups showed a similar pattern.

The kinetics of Ad-specific IgG1 in the primed and challenge control animals did not differ between the proximal and the distal gut (fig. 5C and 5D). At day 14 p.c. a significant increase in Ad-specific IgG1 titres in the primed animals was detected in both segments ($P<0.01$), and this was followed by a slight decrease at day 28 p.c.. In G3 Ad-specific mucus IgG1 increased from day 0 to day 28 p.c.. Independent of the time point or the segment, the Ad-specific mucus IgG1 titres were higher in the primed animals as compared to G3 ($P<0.01$). Throughout the infection we were not able to detect Ad-specific IgG2 in the mucus of infected animals.

Total IgE titres in the mucus of the proximal and the distal gut were low (range: 0.4-4.9 mU)) and the mucus total IgE levels of the proximal but not the distal gut were significantly correlated to the total serum IgE levels ($R=0.88$, $P<0.01$).

To get an insight in the role of the induced mucus antibody response after challenge, antibody titres were correlated with parasitological variables that reflect host responsiveness. At day 14 p.c. a significant positive correlation was measured between Ad-specific mucus IgG1 titres and the worm burden ($R=0.79$, $P<0.05$). To assess whether mucus IgG1 would be involved in immunological induced processes other than expulsion, we measured the correlation between the Ad-specific mucus IgG1 and worm length. The reduction in worm length of male and female worms separately, was negatively correlated with Ad-specific mucus IgG1 titres in the primed animals at day 28 p.c ($R=-0.94$ and $R=-0.67$ respectively, $P<0.01$) but not at day 14 p.c.. No significant correlations were found between Ad-specific mucus IgA titres or mucus total IgE titres and parasitological parameters.

Cooperia-specific ASC in the different anatomical locations

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

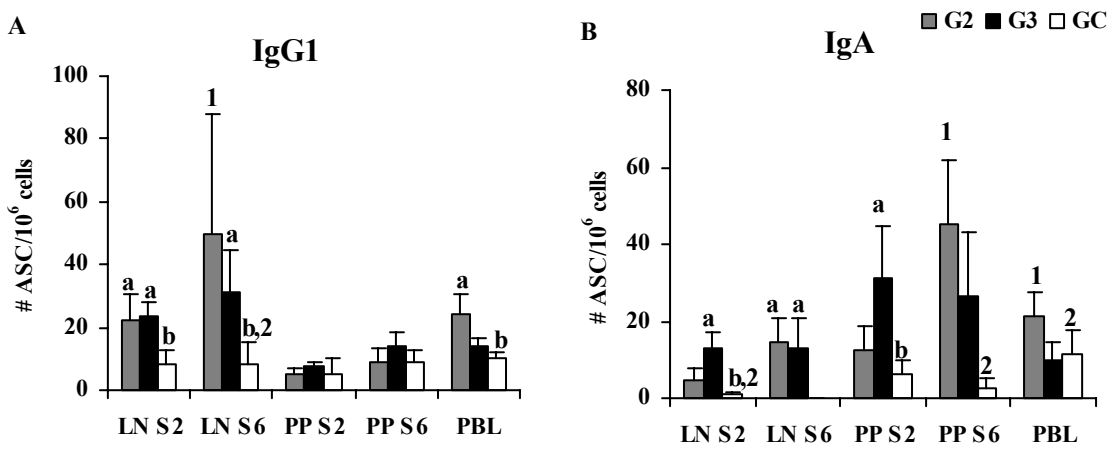

FIGURE 6 The 14.2 kDa-specific IgG1 (A) and IgA (B) secreting cells in the mesenterial lymph nodes (LN S2 and LN S6), the Peyer's patches (PP S2 and PP S6) and peripheral blood (PBL) is given for the animals from G2 (100,000 L3/100,000 L3), G3 (0 L3/100,000 L3) and the non infected control group (GC)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

Phenotypic characterization of B cells in the different anatomical locations

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

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

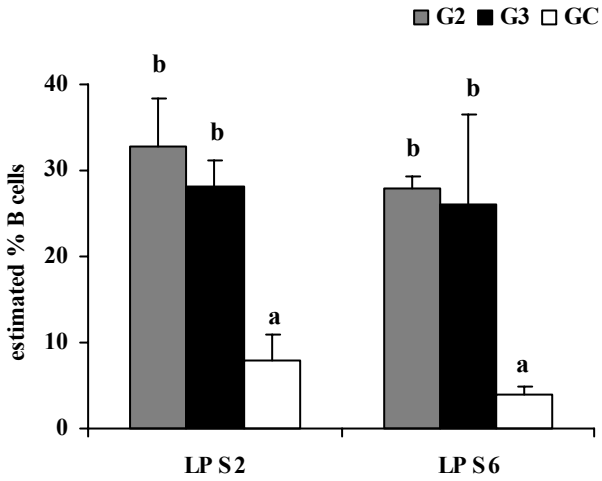

FIGURE 8 The estimated %B cells in the lamina propria of the proximal (LP S2) and the distal (LP S6) gut is given for the animals of G2 (100,000 L3/100,000 L3), G3 (0 L3/100,000 L3) and the non-infected control group (GC) at day 28 p.c.. The %B cells was estimated by subtraction of the %CD4+, the %CD8+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

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

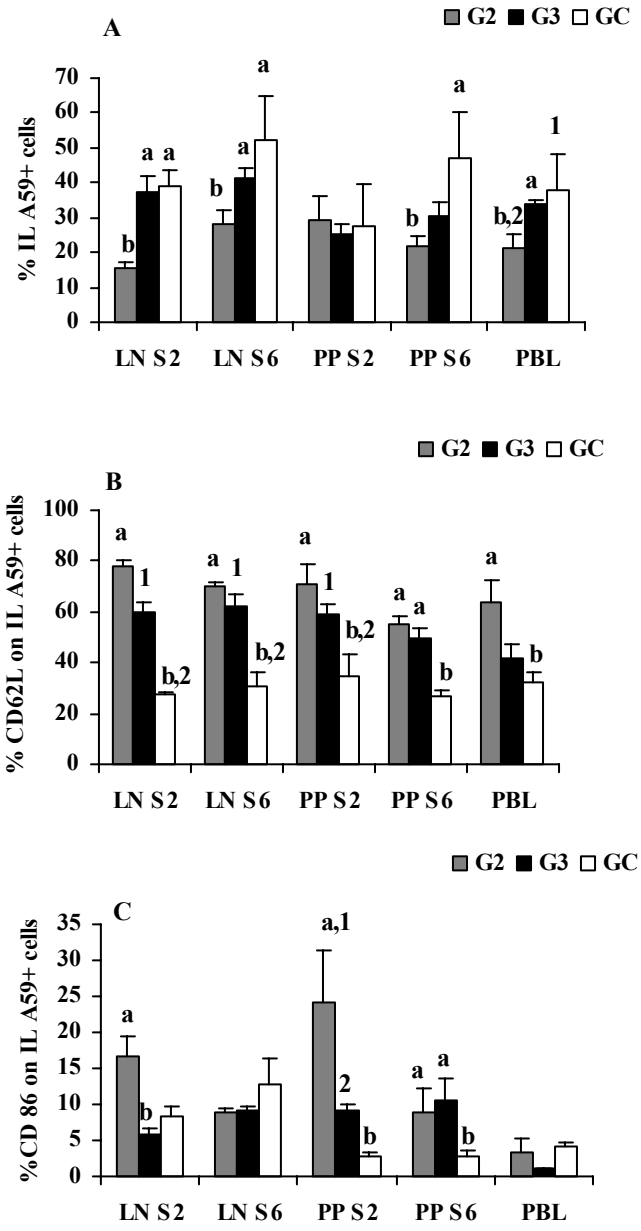

FIGURE 7 The %IL-A59+ cells (A) within the gate for lymphocytes based on the forward and side scatter of the cells, the %CD62L+ cells within the gate of IL-A59+ cells (B) and the %CD86+ cells within the gate of IL-A59+ cells (C) is given for the animals of G2 (100,000 L3/100,000 L3), G3 (0 L3/100,000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

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

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

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

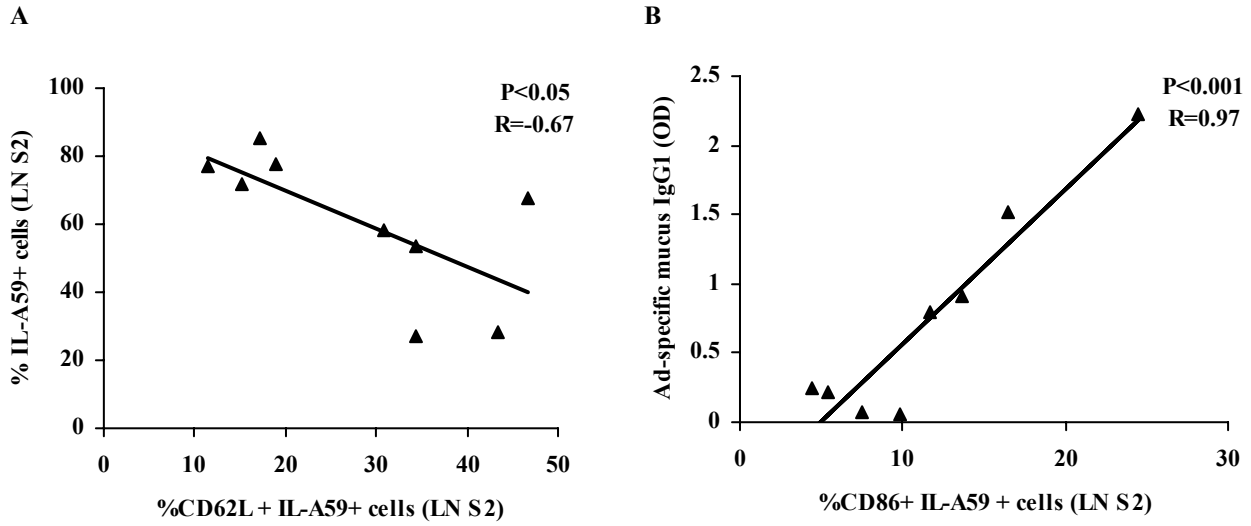

FIGURE 9 (A) The correlation between the %IL-A59+ cells and the CD62L expression on these IL-A59+ cells of the lymph node from the proximal gut (LN S2) at day 28 p.c. is shown (R=0.67). (B) The correlation between the CD86 expression on the ILA59+ cells in LN S2 and the Ad-specific IgG1 titres (OD-blank)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

Frequency of MHCII+ in the different anatomical locations

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

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

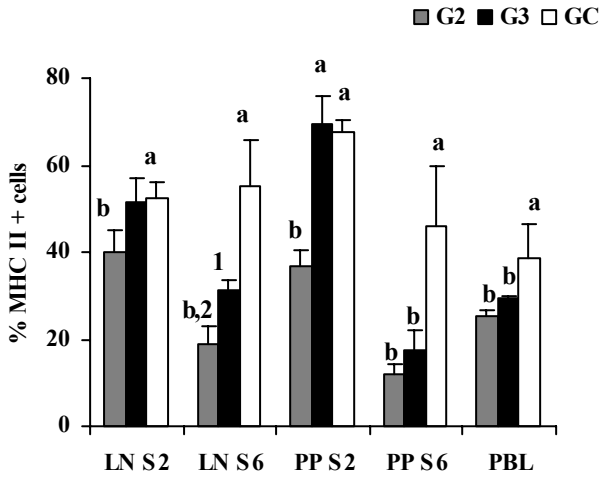

FIGURE 10 The %MHCII+ cells within a gate for lymphocytes based on the forward and the side scatter of the cells is given for the animals the animals of G2 (100,000 L3/100,000 L3), G3 (0 L3/100,000 L3) and the non infected control group (GC) at day 28 p.c.. Results are expressed as %MHCII+ cells and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

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

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

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

DISCUSSION

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

in worm establishment as measured by a lower number of adult worms and a higher percentage of L4. Priming dose level had no influence on this reduction, but host responder type did. The intermediate responders showed a much lower establishment of the challenge infection than low responders. Based on these results we hypothesized that in the current experimental set-up, memory immune responses to *C. oncophora* were at least partly generated in a dose-independent way and that the more resistant phenotype of the intermediate responders was sustained after re-infection. Here we report on the contribution of the humoral responses in this immunity against challenge infection. Furthermore, based on the observation that B cells are specifically recruited to the infection site at the peak of a primary infection [126], we performed a more detailed phenotypic and functional analysis of B cells and compared animals primary and secondary infected with 100,000 *Cooperia* L3.

The *Cooperia* 14.2 kDa Elisa is a tool to differentiate between exposure levels to infection under natural conditions [185]. Recently, it was shown that the exposure level of animals trickle infected with different infection doses of *C. punctata* under experimental conditions correlated with the 14.2 kDa-specific serum titres [247]. The similar pattern in 14.2 kDa IgG serum titres in both primed groups in the current experiment is consistent with the parasitological findings that exposure to a single dose of 30,000 or 100,000 L3 does not significantly differ in the generation of protective immunity. In addition, the results suggest that a single infection dose uses a different mode of action to trigger the immune system compared to a trickle infection and that in our model the same mode of action can be induced by a dose of 30,000 or 100,000 L3.

Upon challenge, a booster in Ad-specific serum IgG1 and IgA was induced irrespective of the priming dose. Consistent with a previous study [126] the parasite-specific IgA response remained at a lower level than the parasite specific IgG1 response. B cells from primed animals can respond to lower doses of antigens and the antibodies they produce are generally of higher affinity, hence, this may explain the dose-independency of the secondary Ad-specific Ig response. However, intermediate and low responders did differ in the onset of Ad-specific IgG1 production. Low responders responded more slowly and reached the peak in Ad-specific serum IgG1 about two weeks after the intermediate responders. It is likely that memory B cells were more numerous in intermediate responders resulting in a faster rise in antibody titres after challenge. Plasma cells can survive and secrete antibodies for an extended period of time [203] but the observation that the primary serum antibody response was close to control levels at the time of challenge clearly suggested the extinction of short-lived plasma cells that were induced by the primary immunization. Similar to the parasite specific Ig response, the total serum IgE production was increased in a dose-independent manner in primed animals. In addition, not only the onset of total serum IgE production but also the level differed between intermediate and low responder animals. The

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

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

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

months of age at the time of challenge, compared to 3 months of age in the previous experiment. The earlier increase in B cell frequency is consistent with an age-related resistance and more effective development of immunity as has been reported before for *C. oncophora* infected calves [135].

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

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

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

AKNOWLEDGEMENTS

The authors would like to thank Margreet van der Veer and Dr. Ana Yatsuda for their technical support throughout the experiment. Johan van Amerongen from the Department of Pathology and the animal caretakers of the Department Infectious Diseases and Immunology from the faculty of Veterinary Medicine in Utrecht are thanked for their help during the necropsies. Dr. Maarten Eysker and Prof. Albert Cornelissen are thanked for the helpful discussions and the critical reading of the manuscript. This research was supported by the Technology foundation STW, applied science division of NWO and the technology program of the Dutch Ministry of Economic Affairs.