Original article

Haemonchus contortus (Nematoda: Trichostrongylidae) infection in lambs elicits an unequivocal Th2 immune response

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Abstract – Selection of resistant animals and host immunization have been proposed as alternative methods for the control of gastrointestinal nematode parasites. However, a better knowledge of the mechanisms involved in protective immunity against these parasites is required for the development of optimal strategies. In this study, 3 month old INRA 401 lambs ($n = 81$) were allocated into three groups: uninfected control, challenged either once (primary-infected animals) or twice (previously-infected animals) with 10 000 *Haemonchus contortus* L_3. Uninfected control and challenged animals were sequentially sacrificed at 3, 7, 15 and 28 days post challenge. In both challenged groups, a clear Th2-oriented immune response was observed in the abomasal lymph node and mucosa. IL-4 and IL-13 mRNA over-expression, recruitment of eosinophils, mast cells and globule leukocytes and production of specific systemic IgG and mucosal IgA were observed earlier in previously-infected animals than in primary-infected ones. At 28 days post infection, no differences between intensities of these responses were observed between the challenged groups. Worm establishment rates were similar in previously-infected and primary-infected lambs. However, reductions of worm development, female fecundity and fecal egg output were observed in previously-infected sheep. We conclude that *H. contortus* infection in young INRA 401 lambs induced an unequivocal Th2 immune response resulting in the regulation of worm egg production without affecting their establishment.

Haemonchus contortus / sheep / Th2 immune response / quantitative PCR / antibody and cellular response

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1. INTRODUCTION

Haemonchus contortus is a common nematode parasite of small ruminants that causes important losses of milk, meat and wool production and death in young animals [33, 37]. Due to the emergence of anthelmintic resistance [29, 39] and public concern about chemical residues in animal products, alternative control strategies are needed. Vaccination and genetic resistance of hosts have been viewed as sustainable methods of parasite control. Substantial progress and results have been obtained by using several *H. contortus* antigens, such as native H11 and H-gal-GP, to stimulate efficient levels of protective immunity in sheep [20]. Nevertheless, no commercial vaccine is yet available. Between and within breed differences in host resistance are currently reported throughout the world [22, 38] but the mechanisms underlying this resistance are not well known. The immune response against gastrointestinal nematodes has been extensively studied in rodents infected with *Trichinella spiralis, Nippostrongylus brasiliensis, Heligmosomoïdes polygyrus* and *Trichuris muris*. Protective immunity against these parasites is largely dependant on the activation of CD4+ T helper cells and on the Th_2 polarization of the immune response [35], while a Th_1 response is associated with the survival of these parasites and increased host susceptibility to infection [13, 14]. In humans, the protective immune response observed in natural *Ascaris lumbricoïdes* or *Necator americanus* infections is similarly associated with a high Th_2-type cytokine polarization [11, 26]. In ruminants, the nature of the immune response towards gastrointestinal helminths is less clear and the existence of a true Th_1/Th_2 dichotomy was questioned in the late nineties [9, 15, 24]. Few studies on the polarization of the adaptive immune response in *H. contortus* infected sheep are available and the results have failed to prove an unequivocal Th_2 response [31]. Nevertheless, a Th_2 polarization has been recently demonstrated in *Trichostrongylus colubriformis* infections in sheep [25].

In rodent models, Th_2 immune responses are almost invariably accompanied by local and systemic eosinophilia and mucosal mast cell hyperplasia, but also with local and general specific antibody responses (mainly IgA, IgG1 and IgE) [23]. The same features are also encountered in sheep infected with *H. contortus* [31], *Teladorsagia circumcincta* [32] and *Trichostrongylus colubriformis* [8]. The impact of these effectors and their relative importance in host protection are still unclear. Moreover, sequences of the immune response and mobilization of the antibody and cellular effectors have not been systematically characterized in *H. contortus* infections. Therefore, the objectives of this study were (i) to define, using a quantitative reverse transcriptase (RT) PCR, the polarization of the immune response in lambs infected with *H. contortus,* (ii) to investigate the kinetics of Th_2-type-associated effectors in the abomasal mucosa and (iii) to characterize the effects of this immune response on the nematode populations within the host.

2. MATERIALS AND METHODS

2.1. Experimental design

2.1.1. Animals

Eighty-one 3-month old female INRA 401 lambs were randomly allocated into three experimental groups: Group A: previously-infected, anthelmintic-treated and then challenged sheep ($n = 29$), Group B: primary-infected sheep ($n = 28$), and Group C: uninfected control sheep ($n = 24$). All sheep were born, reared and maintained worm-free in three separate pens before the experiments. Limited infections with coccidia occurred in all groups despite diclazuril treatments (Vecoxan, Janssen-Cilag, Issy-les-Moulineaux, France, 1 mg/kg BW) before the commencement of the experiment,

Table I. Schematic description of the experimental design.

Treatment and group	Initial infection*	Levamisole	Challenge infection*	Number of sheep killed			
Days from challenge	–35	–7	0	3	7	15	28
Previously-infected (A)	+	+	+	6	6	6	11
Primary-infected (B)	–	+	+	6	6	6	10
Uninfected control (C)	–	+	–	6	6	6	6

*10 000 *H. contortus* L$_3$.

but no clinical signs of coccidiosis were observed. Experimental lambs received granulated feed adapted to their age, as well as forage and tap water ad libitum. The animals were reared following European Union recommendations for animal welfare and under the supervision of the local INRA ethics committee.

2.1.2. Experimental infections and necropsies

Lambs of group A (previously-infected) were orally infected with 10 000 *H. contortus* third-stage larvae (L$_3$) of the Humeau strain (kindly provided by L. Gruner, INRA BASE, Tours-Nouzilly, France) 35 days before challenge. All lambs were treated with an oral administration of Levamisole (7.5 mg/kg) at day –7. The lambs of groups A (previously-infected) and B (primary-infected) were orally infected with 10 000 *H. contortus* L$_3$ at D$_0$. Six animals of each group were humanely killed at each of days 3, 7 and 15 post challenge (dpc) by exsanguination following intravenous injection of 10 mg/kg pentobarbital sodium. At day 28, eleven lambs from group A, ten from group B and six from group C were killed (Tab. I). Necropsy days were chosen to conform with the expected development of the parasite within the host: only early fourth-stage larvae (L$_4$) at 3 dpc, majority of late L$_4$ at 7 dpc, immature worms at 15 dpc, and adult worms at 28 dpc [10].

2.2. Parasitological examinations

Fecal samples from animals necropsied at 28 dpc were taken on days 21 and 28 post challenge to measure the nematode egg excretion. Fecal egg counts were performed according to the modified McMaster technique [28]. After necropsies, the contents and washings of the abomasum were collected and passed through a 40 μm sieve to eliminate coarse materials. The whole abomasum was digested in pepsin-hydrochloric acid solution (37 °C, 6 h) to collect the tissue dwelling nematode stages. The contents, washings and digested materials, were preserved in absolute alcohol. The volumes of these materials were then adjusted to 1 L and worms were counted in a 10% aliquot and classified as adult male or female, immature male or female, or early and late L$_4$. Twenty adult female worms were randomly picked from each 28 dpc sample for total length measurement and egg counts in utero. For this purpose, individual female worms were allowed to disintegrate in sodium hypochloride 4% solution [19] and all eggs liberated from the uterus were counted for each worm.

2.3. Cytokine expression in abomasal lymph node and fundic mucosa

2.3.1. mRNA extraction and RT-PCR

About 300 mg of the abomasal lymph node or fundic mucosa were snap frozen in

Table II. Forward and reverse primer sequences for quantitative reverse-transcriptase (RT) PCR.

Cytokine	Forward sequence	Reverse sequence	Tm amplicon (°C)
Ovine Beta-actin	ACCAGTTCGCCATGGATGAT	AGCCGTTGTCAACCACGAG	78
Ovine IFN-γ	ATCTCTTTCGAGGCCGGAGA	ATTGCAGGCAGGAGAACCAT	77
Ovine IL-4	GGAGCTGCCTGTAGCAGACG	TTCTCAGTTGCGTTCTTTGGG	79
Ovine IL-10	CTGAGAACCATGGGCCTGAC	TCTCCCCCAGCGAGTTCAC	80
Ovine TNF-α	CCCGTCTGGACTTGGATCCT	TGCTTTTGGTGCTCATGGTG	75
Ovine IL-12p40	GAATTCTCGGCAGGTGGAAG	GTGCTCCACGTGTCAGGGTA	80
Ovine IL-13	AGAACCAGAAGGTGCCGCT	GGTTGAGGCTCCACACCATG	80
Ovine Eotaxin	ACAAGAAAATCTGTGTTGATCCCC	CCATGGCATTCTGGACCC	75
Ovine IL-5	CTGCTGATAGGTGATGGGAACTT	GGTGATTTGTATGCTGAGGAGTAGG	74
Ovine IL-3	AAGAATATCCTGGCGAATAAGAGC	GAACGCTTTCAGGTTTGCCT	75

1 mL of Trizol Reagent (Invitrogen, Cergy-Pontoise, France, reference 15596-018), and stored at –70 °C before use. Samples were homogenized using a Hybaid RiboLyser™ (two cycles of 30 s each at a speed of 4.5). Fifty µL of the obtained homogenate were mixed with 950 µL of Trizol before adding 200 µL of chloroform at room temperature for 10 min. The samples were then centrifuged for 15 min at 20 000 *g* at 4 °C. The supernatants were placed on an RNeasy Column (RNeasy Mini Kit, Qiagen, Courtabœuf, France) and then processed using the Qiagen protocol for RNA clean-up. This complete protocol allows the recovery of highly pure RNA. The RNA concentration was measured by spectrophotometry at 260 nm and RNA quality was assessed by electrophoresis on 1.2% agarose gels stained with ethidium bromide. Two µg of total RNA were digested with 1U of RNase-free DNAse (Promega, Charbonnières, France) for 1 h at 37 °C to remove any trace of genomic DNA, and then incubated 10 min at 65 °C with DNase Stop Solution. Treated RNA was used as the template for single-stranded cDNA synthesis. They were incubated 10 min at 65 °C with Random Primer oligonucleotides (Invitrogen) and chilled on ice; a mixture containing M-MLV transcriptase (400 IU/sample), 5× first-strand buffer (Life Technologies, Cergy-Pontoise, France), dithiothreitol (DTT) 0.1 M (Life Technologies), 40 U of RNase Out Ribonuclease Inhibitor (Invitrogen), 10 mM of each four dNTP (Promega) was then added and the mixture was incubated for 1 h at 42 °C. The reaction was heat-inactivated at 95 °C for 5 min and kept at –20 °C until use. For quantitative PCR, cDNA were used at dilutions of 1:100.

2.3.2. PCR primers

Specific primers and amplicon sizes are listed in Table II. For each target cDNA, a primer pair was determined using Primer Express Software (Applied Biosystems, Courtabœuf, France) for SYBR Green real-time PCR assay on a GeneAmp 5700 Sequence Detection System (Applied Biosystems) and based on known ovine gene sequences (β-actin, IFN-γ, TNF-α, IL-3, IL-4, IL-5, IL-10, IL-12p40). Oligonucleotides were designed to amplify a product with a size of 51 bp, with a melting temperature (Tm) of 58–60 °C. When the ovine

gene sequence was not known (Eotaxin, IL-13), a consensus sequence was created, based on a minimum of three known sequences in other mammalian species. A first primer pair was then designed using Primer 3 Software in order to amplify the largest possible mRNA in all aligned sequences. PCR amplification on reverse transcript RNA obtained from ovine peripheral blood mononuclear cells (PBMC) stimulated with concanavalin A (ConA, 10 µg/mL, 24 h in a 5% CO_2 and 37 °C atmosphere) was then performed. Amplicons were purified before cloning in the TopoCloning system (Invitrogen) and sequenced (using M13 universal primers). The sequences thus obtained were then compared with the GenBank database using blast to validate the identity of the amplified product. After validation, a new set of primers suitable for quantitative PCR was designed using the obtained sequence. For IL-13, the primers used were those published by Hein et al. [17].

2.3.3. Quantitative PCR

Quantitative PCR was performed on a GeneAmp 5700 (Applied Biosystems), using the double-stranded DNA binding dye SYBR Green I. PCR products corresponding to the different amplicons (see Tab. I) of target cytokines were cloned in PBR 2.1 plasmid (TopoClonA, Invitrogen) and sequenced (M13 universal primers). The plasmids harboring the cloned sequence were then quantified by spectrometry to establish the number of target copy/µL. Ten-fold dilution series were prepared from the stock plasmid solution corresponding to 10^7 to 10^1 copies of the target sequence. Serial dilution of the plasmid was then used as an external standard, allowing the determination of the number of copies of the target cDNA in each assay. Each sample for each investigated cytokine was performed in duplicate. Ovine Beta-actin was used as a housekeeping gene to normalize expression between different samples. The results obtained for each cytokine are expressed as a ratio of the number of cytokine mRNA copies/Beta-actin mRNA copies. A non-template control reaction (NTC) was included in each run. Finally, the specificity of the amplification reaction was assessed by acquisition of a dissociation curve. Data was obtained by slowly increasing the temperature of the PCR reaction from 60 to 95 °C; denaturation of non-specific PCR products is followed by a faster decrease of fluorescence than for specific products. The melting profile of the distinct PCR product was obtained by plotting the first derivative (-dF/dT) of fluorescence (F) versus temperature (T).

2.4. Detection of antibodies in mucus and serum by indirect ELISA

A blood sample from each animal was recovered just before euthanasia and centrifuged for 5 min at 5 000 rpm. Serum was then frozen at −20 °C until analysis. A mucus sample from each animal was collected during necropsy using PBS impregnated filter paper strips of 4 cm^2 each deposited between the folds of the abomasal fundic mucosa for 5 min. The strips were then gently agitated in 2 mL of PBS for 2 h at room temperature to dilute the mucus. The liquid was centrifuged and stored frozen at −70 °C until analysis.

2.4.1. Worm antigen preparation

A donor sheep infected with 50 000 L$_3$ of *H. contortus* (Humeau strain) was sacrificed and adult worms were harvested from the abomasum. These worms were thoroughly washed in PBS (pH 7.4) containing penicillin (100 IU/mL) and streptomycin (1 mg/mL). Fifty adult worms per milliliter of the same buffer were maintained in a 5% CO_2 atmosphere at 37 °C overnight. Next, the supernatant (containing excretory-secretory products) was collected, filtered (0.2 µm) and stored at −70 °C until further use. A crude extract of *H. contortus* L$_3$ (CEL$_3$) was prepared after three cycles of freezing and thawing (−70 °C − +25 °C),

Table III. Reagents and procedures used for antigen ELISA detection in serum and mucus.

Isotype	Serum				Mucus			
	IgG		IgA		IgG		IgA	
Type of antigen	ESP Ad	CEL3	ESP Ad	CEL3	ESP Ad	CEL3	ESP Ad	CEL3
Concentration of antigen (µg/mL)	2	2	5	2	5	0.5	5	2
Dilution of serum or mucus	1:100	1:100	1:5	1:5	1:1	1:1	1:1	1:1
Dilution of secondary antibody	1:1000	1:1000	1:100	1:100	1:1000	1:1000	1:250	1:250
Dilution of conjugate	–	–	1:500	1:500	–	–	1:500	1:500

Type of antigens: ESP Ad: Excretory-secretory products from *H. contortus* adult worms; CEL_3: crude extract of *H. contortus* infective third-stage larvae. For IgG determination, the secondary antibody is a Donkey anti-sheep IgG HRP-conjugated. For IgA determination, an anti-IgA bovine / sheep mouse IgG_1 is first applied before a goat anti-mouse IgG1 HRP-conjugated.

homogenization at 4 °C and centrifugation at 30 000 *g* for 30 min at 4 °C. The supernatant was used as the crude extract of the L_3 antigen. The protein concentration of both antigenic preparations was determined with the method of Lowry et al. [21].

2.4.2. Determination of optimal assay conditions and the ELISA technique

The optimal concentrations of ELISA reagents and the optimal test conditions were determined by chequerboard titrations of *H. contortus* antigens, positive (group A at 28 dpc) and negative (group C) reference sera or mucus and specific conjugates (Tab. III). An indirect ELISA was used on serum and mucus samples, as described by Jacquiet et al. [18]. The results are expressed as optical densities (OD) for serum and mucus IgG and IgA minus the OD of PBS wells (corrected absorbance).

2.5. Histology

At necropsy, two tissue samples from the fundic area of the abomasal wall were taken; one was fixed in 10% formalin for conventional histology and immunohisto-chemistry (IHC), while the second was stored at –70 °C for frozen IHC.

2.5.1. Conventional histology and IHC with paraffin-embedded samples

Abomasal tissue was paraffin-embedded and 3 µm sections were mounted on glass slides. Eosinophils and globule leukocytes were counted on haematoxylin-eosin stained slides whereas mast cells were counted after Giemsa staining. Immunohistochemistry was performed using antibodies already described for cell phenotyping on paraffin-embedded tissues in sheep [2, 27, 36]. This antibody set allows specific labeling of macrophages/monocytes (mouse anti-human CD68 antibody, clone Ki-M6, Serotec, Cergy-Saint-Christophe, France, 1:150 dilution), B lymphocytes (mouse anti-human CD20-like antibody, clone BLA-36, Novocastra, Le-Perray-en-Yvelines, France, 1:50 dilution), or T lymphocytes (rabbit anti-human CD3 antibody, A 0542, DAKO, 1:100 dilution).

2.5.2. IHC on frozen samples

Five µm sections of abomasal tissue were obtained using a Leika cryomicrotome. Slides were fixed for 20 min in a

–20 °C acetone bath before drying. Slides were then directly processed for IHC. Monoclonal antibodies directed against CD4 (mouse anti-ovine CD4, SEROTEC, reference MCA2213, 1:2000 dilution), or CD8 (mouse anti-bovine CD8, SEROTEC, reference MCA837G, 1:800 dilution) antigens were used.

For each type, cells were counted on five randomly selected fields at high magnification (× 400). The results are expressed as the sum of the five fields.

2.6. Statistical analysis

Comparisons between the three groups (previously-infected, primary-infected and uninfected control) and between the killing times (3, 7, 15 and 28 dpc) within a group were performed using the Kruskal-Wallis and Mann-Whitney non parametric tests (SYSTAT software). $P < 0.05$ was considered significant. For the 81 observations in this study, Spearman correlation coefficients (r) were statistically significant when $r > 0.217$ ($P < 0.05$) and $r > 0.283$ ($P < 0.01$).

3. RESULTS

3.1. Efficiency of experimental *H. contortus* challenge

No worm or egg excretions were observed in uninfected control animals throughout the study (group C). All lambs in groups A and B became infected after challenge with establishment rates ranging from 4% to 28%. Highly variable individual worm counts were observed within each group and there were no significant differences between groups A and B (Tab. IV).

3.2. Cytokine mRNA transcription in abomasal lymph node and in fundic mucosa

A clear increase of IL-4 m-RNA transcription was observed in the abomasal lymph node and fundic mucosa of both challenged groups but not in uninfected control sheep (Figs. 1A and 1B). In the previously-infected group, an over-expression of IL-4 m-RNA was present as early as 3 dpc and remained significant and stable until the end of the experiment. In the primary-infected group, the IL-4 mRNA increase was not observed before 7 dpc but reached similar levels to previously-infected animals at 15 dpc. In fundic mucosa, IL-5 mRNA was higher ($P < 0.05$) in challenged lambs (groups A and B) than in uninfected control sheep at 7 dpc and 15 dpc (Fig. 1D). In the abomasal lymph node, the increase of IL-5 m-RNA was observed at 28 dpc only (data not shown). In fundic mucosa, IL-13 mRNA was higher in both challenged groups (A and B) than in uninfected control sheep (Fig. 1C) as early as 7 dpc, while no differences were recorded between the three groups in abomasal lymph node (data not shown). In both investigated tissues, no modifications of IL-3 or Eotaxin mRNA transcription were observed (data not shown). No significant group differences were recorded in the IFN-γ (Fig. 1E), IL-12 (Fig. 1F), IL-10 or TNF-α mRNA transcription profile during the whole experiment (data not shown). No cytokine mRNA transcription profiles could be directly correlated to the worm establishment rate, worm development or female fecundity.

3.3. Specific antibody detection in abomasal mucus and serum

At 7 and 15 dpc, a significant ($P < 0.05$) and specific CEL$_3$ IgA response (Fig. 2E) was observed in the mucus of previously-infected animals only. At 28 dpc, levels of specific CEL$_3$ IgA in mucus were similar in both infected groups and significantly higher than in uninfected controls ($P = 0.01$). In challenged groups, significant ($P < 0.05$) systemic IgG and IgA and local antibody responses against excretory-secretory products (ESP) of adult worms were observed at 15 and 28 dpc (Figs. 2A, 2B, 2C and 2D). No

Table IV. Egg excretion, total worm burden, length and number of eggs in utero of adult female worms, stage and sex composition of *Haemonchus contortus* populations.

Days post challenge	Group	Epg at 21 dpc (mean ± SD)	Epg at 28 dpc (mean ± SD)	Total worm burden (mean ± SD)	Adult female length (mm) (mean ± SD)	Number of eggs in utero (mean ± SD)	Early L4 (%)	Late L4 (%)	Immature males (%)	Immature females (%)	Adult males (%)	Adult females (%)
3	A	–	–	438 ± 231	–	–	100	–	–	–	–	–
	B	–	–	1126 ± 500	–	–	98.8	0.2	–	–	–	–
7	A	–	–	1255 ± 1210	–	–	71	29	–	–	–	–
	B	–	–	1183 ± 750	–	–	22	78	–	–	–	–
15	A	–	–	898 ± 940	–	–	59.6	20	8	12.4	–	–
	B	–	–	955 ± 838	–	–	33.3	18.7	16.5	30.6	0.4	0.5
28	A	86 ± 197	1149 ± 2845	2169 ± 1950	15.9 ± 2.1	174 ± 160.6	8	8	11.3	20.3	29.4	23
	B	870 ± 2272	6165 ± 7485	2814 ± 2650	17.7 ± 2.7	275.4 ± 154.3	8.9	4.6	11.7	12.5	25.6	36.7

Group A: previously-infected and challenged sheep; Group B: primary-infected sheep. Six animals per group were sacrificed at 3, 7 and 15 days post challenge (dpc), and 11 and 10 sheep at 28 dpc for groups A and B respectively. Coprological examinations were performed at 21 and 28 dpc for sheep sacrificed at 28 dpc.
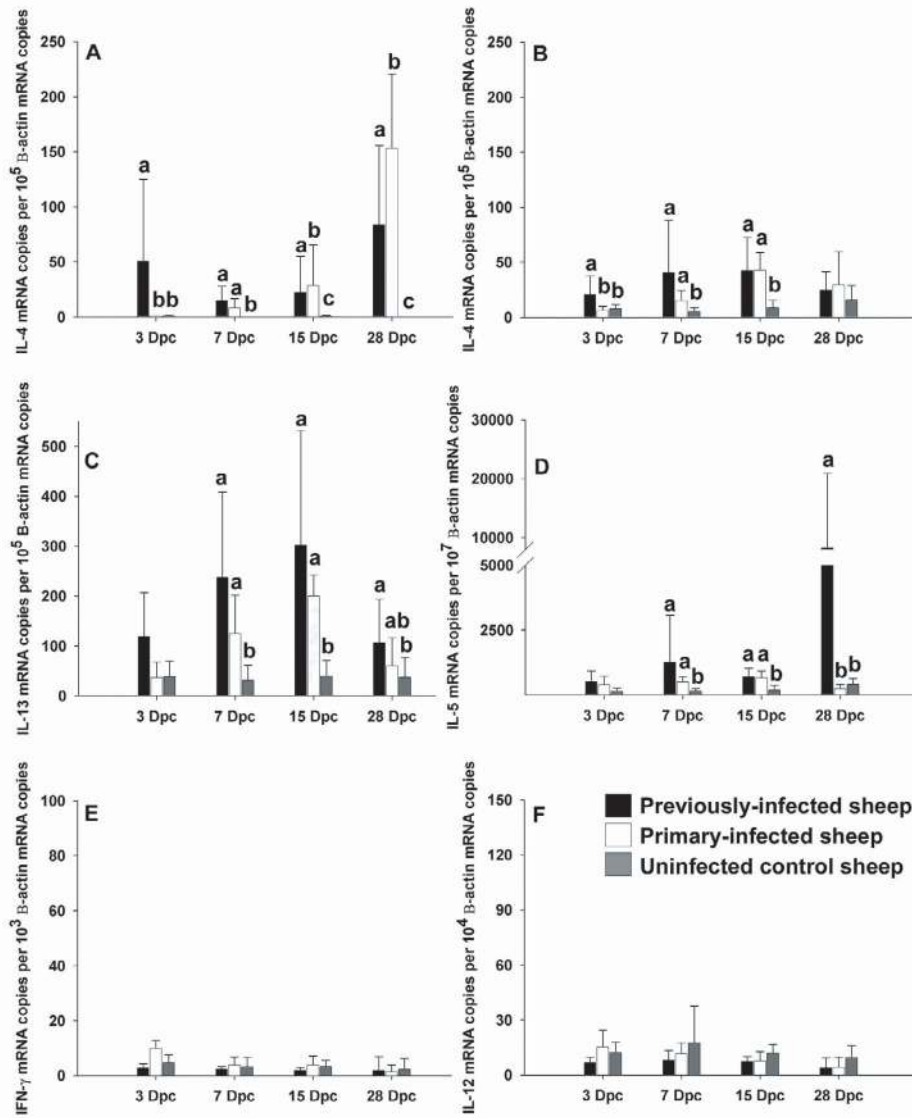
Figure 1. Th$_1$/Th$_2$ cytokine mRNA transcription in the abomasal lymph node (ALN) and in the fundic mucosa (AFM) from previously-infected, primary-infected and uninfected control sheep. IL-4 mRNA (**A**: in ALN and **B**: in AFM), IL-13 mRNA in AFM (**C**), IL-5 mRNA in AFM (**D**), IFN-γ in ALN (**E**) and IL-12 in ALN (**F**) were quantified and measured in each duplicate sample using an external standard. The results are expressed after normalisation (ratio) with a housekeeping gene (ovine Beta-actin) (means and SD). [a, b, c] Values with no letter in common are significantly different ($P < 0.05$).
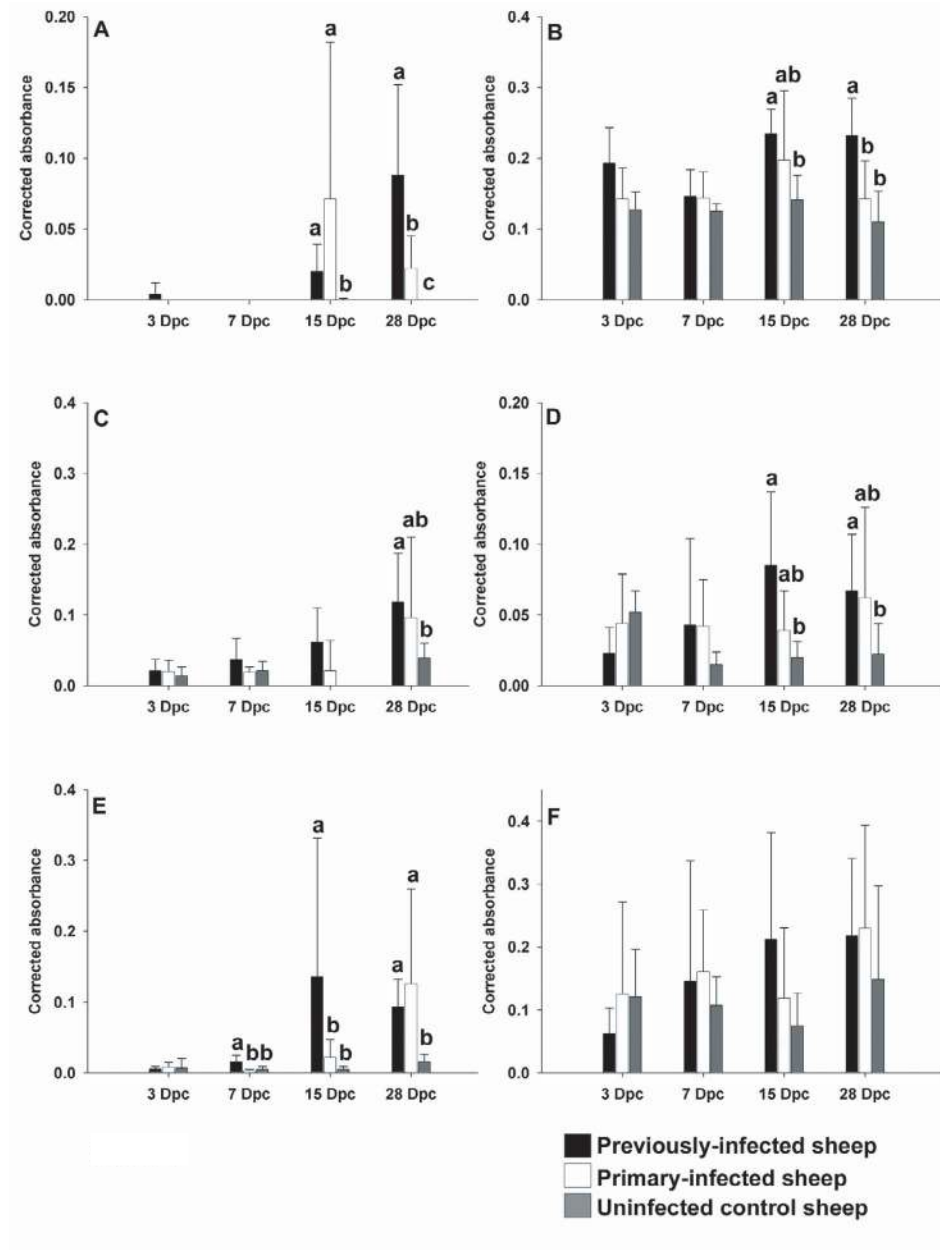
Figure 2. Local and systemic IgG and IgA responses to parasite (excretory-secretory products (ESP) of *H. contortus* adult worms or crude extract of L_3 (CEL_3)) antigens, in previously-infected, primary-infected and uninfected control sheep. **A**: Total mucus IgG against ESP; **B**: Total systemic IgG against ESP, **C**: Mucus IgA against ESP; **D**: Systemic IgA against ESP, **E**: Mucus IgA against CEL_3, **F**: Systemic IgA against CEL_3 (means and SD). [a, b, c] Values with no letter in common are significantly different ($P < 0.05$).

significant systemic CEL$_3$ specific IgA (Fig. 2F) or IgG (data not shown) response was observed during the study. Concentrations of mucus ESP or CEL$_3$ specific IgA or IgG antibodies were significantly correlated with those found in the serum (ESP specific IgG ($r = 0.606$, $P < 0.01$), ESP specific IgA ($r = 0.494$, $P < 0.01$) and CEL$_3$ specific IgA ($r = 0.586$, $P < 0.01$)).

3.4. Dynamics of the cellular abomasal infiltration

In the abomasal mucosa of previously-infected sheep, eosinophils, mast cells and globule leucocytes were already present in significantly higher numbers than in primary-infected or uninfected control sheep ($P < 0.01$) at 3 or 7 dpc. In both challenged groups, a gradual increase of these cell populations was observed between 7 and 28 dpc. However, the cellular recruitment was delayed in the primary-infected group when compared to the previously-infected one. Eosinophil counts were higher in primary-infected animals than those of uninfected control animals at 7 dpc, at 15 dpc for mast cells and at 28 dpc for globule leukocytes. At the end of the experiment (28 dpc), similar mucosal infiltrations (three cell types) were observed in previously-infected and primary-infected sheep (Figs. 3A, 3B and 3C). Significant correlations were established between mucosal IL-4 mRNA transcription level and recruitment of (i) eosinophils, mast cells and globule leucocytes at 3 and 7 dpc ($P < 0.01$), and (ii) mast cells and globule leucocytes at 15 dpc ($P < 0.05$). These correlations strongly suggest a relation between the Th$_2$-cytokine level response and intensity and the rapidity of mucosal cellular infiltration. Concerning CD3+-T cells, BLA 36+-B cells, and CD68+-monocyte/macrophage cells, no significant differences between group A, B and C animals were observed (data not shown). The CD4+/CD8+ ratio (approximately 3:1) remained similar among the three groups and stable during the course of the experiment (data not shown).
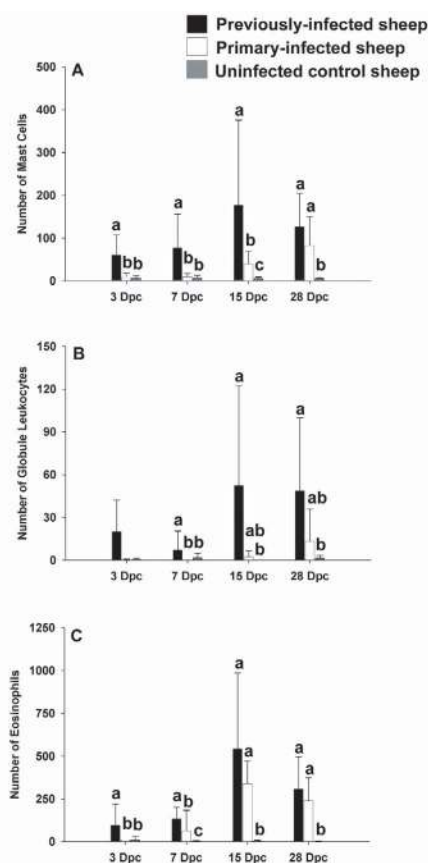

Figure 3. Cellular infiltration in the abomasal mucosa (fundus): mast cells (A), globule leukocytes (B) and eosinophils (C), in previously-infected, primary-infected and uninfected control sheep (means and SD). For each cell type, the evaluation of cell number was performed on five randomly selected fields at high magnification ($\times 400$). The results are expressed as the sum of five fields. [a, b, c] Values with no letter in common are significantly different ($P < 0.05$).

3.5. Impact of immune responses on parasite populations

3.5.1. Similar total worm burdens in previously-infected and primary-infected lambs

No significant difference in the total worm burden was observed between

Table V. Correlations between abomasal eosinophils, mast cells or globule leukocytes and worm parameters 28 days post challenge.

	Eosinophils	Mast cells	Globule leukocytes
% L$_4$ worms	0.308	0.189	0.019
% Immature worms	**0.431***	**0.439***	0.297
% Adults worms	**−0.413***	−0.332	−0.170
Total worm burden	−0.086	−0.102	−0.129
Adult female length	**−0.589****	**−0.508****	**−0.676****
Number of eggs in utero of adult females	**−0.477****	**−0.486****	**−0.592****
Fecal egg output	**−0.378***	**−0.402***	**−0.369***

* $P < 0.05$ and ** $P < 0.01$.

previously-infected and primary-infected sheep. Total worm burdens were not significantly correlated with antibody responses, or with eosinophils, mast cells or globule leukocytes (Tab. V).

3.5.2. Delayed worm development

At 3 and 7 dpc, only fourth-stage larvae (L$_4$) were observed in the challenged animals. Both early and late stage L$_4$ were observed at 7 dpc in groups A and B but the relative proportion of late L$_4$ was significantly ($P < 0.01$) increased in group B (78%) compared to group A (29%). Similarly, the mean ratio of immature worms/L$_4$ was 0.9 in primary-infected sheep and only 0.25 in previously-infected sheep ($P < 0.01$) at 15 dpc and several lambs from the primary-infected group already harbored adult worms while none were observed in the previously-infected group. At 28 dpc, the percentage of adult worms was higher in the primary-infected animals than in the previously-infected animals (Tab. IV). Taken together, these results suggest a delayed development of the parasite in previously-infected animals. The retarded development of early L$_4$ to late L$_4$ was associated with high eosinophil numbers in mucosa (7 dpc, $r = 0.661$, $P < 0.01$) and with increased IgA in the mucosa, both CEL$_3$ ($r = 0.553$, $P < 0.01$, at 7 dpc and $r = 0.881$,

$P < 0.01$ at 15 dpc) and ESP specific ($r = 0.712$, $P < 0.01$ at 15 dpc). In the late phase of the experiment (28 dpc), eosinophils and mast cell populations were positively correlated with the proportion of immature worms. Eosinophil counts were also negatively correlated with the proportion of adult worms (Tab. V). Systemic ESP specific IgG response and mucosal CEL$_3$ specific IgA response were correlated with the proportion of immature worms (respectively $r = 0.42$, $P < 0.05$ and $r = 0.59$, $P < 0.01$).

3.5.3. Reduced female worm length and number of eggs in utero

In previously-infected sheep, adult females were shorter than in the primary-infected group (15.9 ± 2.1 mm versus 17.7 ± 2.7 mm) and contained fewer eggs in their uterus (174 ± 160.6 versus 275.4 ± 154.3 eggs per female) but the differences were not significant. Worm length and number of eggs in the uterus were highly correlated ($r = 0.847$). Increased eosinophils, mast cells and globule leukocyte populations were negatively correlated with the female worm length and the in utero egg numbers ($P < 0.01$) (Tab. V). Mucus CEL$_3$ specific IgA and systemic ESP specific IgG levels were correlated with the reduction of female worm length (respectively $r = -0.36$, $P < 0.05$ and $r = -0.59$, $P < 0.01$) (Tab. III).

3.5.4. Reduced fecal egg output

At 28 dpc, the number of egg-excreting animals was the following: 8 of group A (90 to 9 650 epg) and 9 of group B (300 to 25 000 epg). Despite the variability between sheep, egg excretions were significantly different between the two groups ($P < 0.05$) with a five-fold reduction in group A compared to group B. Eosinophils, mast cells and globule leukocyte numbers as well as the systemic IgG response against ESP of adult worms ($r = -0.47$, $P < 0.05$) were negatively correlated with the intensity of egg excretion in individual sheep (Tab. V).

4. DISCUSSION

The main objectives of this work were (i) to establish the polarization of the adaptive immune response in *H. contortus* infected sheep, (ii) to describe the kinetics of immune effector mechanisms following a single *H. contortus* infection in primary-infected or previously-infected sheep and (iii) to study the effects of these responses on parasite establishment, development and females fecundity.

Few data were available on the orientation of the immune response in *H. contortus* challenged sheep. In a preliminary study using semi quantitative PCR, Schallig [31] observed a non-protective Th$_1$ response in primary-infected *H. contortus* infected sheep. A second challenge induced a protective Th$_2$ response with a high level of IL-4 mRNA expression without significant IL-5 over-expression. However, these results were obtained with a limited number of sheep. In our experiment, neither primary-infected nor previously-infected *H. contortus* infected sheep showed a Th$_1$ cytokine pattern. In contrast, a clear over transcription of the IL-4 gene was observed in the abomasal lymph node and in the mucosa in both primary-infected and previously-infected lambs. Furthermore, specific IgG and IgA antibody responses in mucus and

serum and higher mucosal recruitment of eosinophils, mast cells and globule leukocytes were observed in these two groups. Taken together, IL-4 mRNA over-transcription and immunoglobulin production/cell recruitment strongly support a Th$_2$ orientation of the immune response in lambs challenged with *H. contortus* [4]. In the present study, the over-expression of IL-5 and IL-13 in infected animals also suggested the Th$_2$ polarization. Recently, Pernthaner et al. [25] described an increased expression of Th$_2$ related cytokines, IL-5 and IL-13 (but not IL-4), in intestinal lymph cells of sheep selected for enhanced resistance to *T. colubriformis*. Data collected in these two nematode models support the hypothesis of a Th$_2$ orientation in sheep, as reported in rodent [6, 14] or in human [11] gastro-intestinal nematode infections.

The second objective of our study was to investigate the dynamics of the adaptive immune response after a single *H. contortus* infection. Repetitive or continuous infections are usually used to study the host immune response and to mimic natural infections as well as possible [1, 5, 7]. Nevertheless, single *H. contortus* infections were necessary in our experiment which was mainly designed to measure host immune response effects on worm development. IL-4 mRNA expression increased gradually from 7 dpc to 28 dpc in primary-infected sheep but remained stable and high in previously-infected sheep from 3 to 28 dpc. Eosinophil, mast cell, and globule leukocyte recruitments were apparent at 7, 15 and 28 dpc respectively in primary-infected animals while these cells were still present in previously-infected lambs at 3 dpc. Antibody responses were delayed and low in primary-infected lambs and rapid and higher in previously-infected ones as previously shown by Schallig et al. [30] and Gomez-Munoz et al. [16]. Consequently, while final response intensities were similar in both infected groups, recruitment of cells and secretion of immunoglobulins in previously-infected sheep were observed before their occurrence in primary-infected

sheep. This difference could be explained by (i) a rapid mobilization of immune mechanisms after challenge in group A compared to group B or from (ii) the presence of remaining cells and antibodies induced by the first infection. Unfortunately, our experimental design did not allow to state between these two hypotheses. Interestingly, this earlier mobilization of the immune effectors was associated with significant higher IL-4 mRNA levels at 3 dpc in previously-infected sheep.

No differences in the establishment rates of worms were observed between previously-infected and primary-infected lambs in this experiment. Our data provide evidence that the parasite development and the female fecundity were depressed in previously-infected animals compared to primary-infected ones. This delay in parasite development has been shown after repeated exposure of lambs to *H. contortus* [30] or *T. circumcincta* [32]. Here we established that alterations of parasite life traits (development of worm and female fecundity) are related to the earliness of effector mobilization rather than to the final level of the responses. Eosinophils, mast cells and globule leucocyte recruitment are typically associated with helminth infections [3]. In our study, these cells were strongly and negatively correlated with adult female length and number of eggs in utero. Moreover, positive correlations were also observed between mast cell and eosinophil numbers and the relative proportion of immature worms while a strong negative correlation was seen between eosinophils and the proportion of adult worms (Tab. V). Thus, these cells may have a role in the delayed development of the parasite and the decrease of fecal egg output as previously shown by many authors [4, 12, 34]. In the present study, two antibody responses (CEL$_3$ specific IgA response in abomasal mucus and ESP specific IgG in serum) were positively correlated to the proportion of immature worms and negatively correlated to the female length and number of eggs in utero suggesting that they could interfere with

H. contortus development within the host. The importance of the local IgA response in host protection has already been indicated in *T. circumcincta* infected sheep, where the production of local IgA against somatic or L$_4$ ESP molecules induced a reduction in the female parasite length and fecundity [32].

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