Experimental Parasitology 248 (2023) 108512

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Immunization with thiol-binding proteins from *Haemonchus contortus* adult worms partially protects goats against infection during prepatency

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ARTICLE INFO

Keywords:
Haemonchus contortus
Vaccination
Thiol-binding proteins
Immune response
Prepatency
Goat

ABSTRACT

To contribute of the knowledge of the immune mechanisms underlying the response to the immunization of goats with thiol-binding proteins fractions (PBS-TSBP) from *Haemonchus contortus* (*H. contortus*) adult worms, this study analyzed the degree of protection and the immune responses developed against the parasite after vaccination with this antigenic complex during the time-elapsing between challenge with L3 of the parasite and the development of adult worms, evidenced by the appearance of first faecal eggs (prepatent period or prepatency).

Goat kids immunized with PBS-TBSP generated an immune response during the prepatency which translates into a reduction in the number of worms, as well as a lower reduction on packed cell volume and plasma protein levels in relation to the non-vaccinated animals.

As previously described in other studies carried out after the prepatent period, this protection was associated with a systemic humoral response. At the local level, a specific humoral response was also observed, together with an immune-inflammatory infiltrate in the gastric mucosa of MCH-II + cells and $CD4^+$ lymphocytes, whose number was associated with a reduction in the number of worms and an increase in plasma proteins. A high peripheral eosinophilia was detected, but no corresponding increased infiltration of the gastric mucosa by eosinophils or globular leukocytes was observed. In agreement with previous data on the immunolocalization of the antigens used here, the results obtained contribute to the idea that these may be excretion/secretion (E/S) products necessary for parasite survival, whose inactivation during the larval and/or pre-adult stages may have contributed to immunoprotection.

1. Introduction

H. contortus is one of the most important and more widely distributed gastrointestinal nematodes (GIN) affecting small ruminants in extensive and semi-extensive production systems. Its high pathogenic potential is related to its haematophagous activity, resulting in a disease characterized by anemia and hypoproteinemia, which translates into a reduction of wool and milk production, decrease of the carcass quality, reproductive disorders and even death in the most susceptible animals (Josiah et al., 2015; Taylor et al., 2016).

Although the control of GIN infections is currently mainly based on the strategic use of anthelmintic drugs, in recent years many studies have been carried out to find alternatives to avoid some disadvantages associated with the pharmacological control of these infections, such as the increasing emergence of resistance to anthelmintics (Wolstenholme et al., 2004; Jackson et al., 2012; Charlier et al., 2020), or the presence of chemical residues in livestock products (European Food Safety Authority, 2012).

One of these alternatives has been the use of vaccines. Various immunisation strategies against GIN have been tested so far, including DNA (Han et al., 2012) and recombinant/subunit vaccines (Redmond and Knox, 2006; Nisbet et al., 2013; González-Sánchez et al., 2019), or vaccines based on the use of native antigens obtained from different parasite stages (adult worms, L3s) (Knox et al., 1999, 2005; Jacobs et al., 1999) and their excretory/secretory (E/S) products (Schallig et al., 1997; Bakker et al., 2004). Among the results achieved by immunisation with natural antigens, it is worth highlighting those obtained by using hidden antigens presented in a complex of membrane glycoproteins

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https://doi.org/10.1016/j.exppara.2023.108512
Received 29 September 2022; Received in revised form 25 November 2022; Accepted 16 March 2023
Available online 24 March 2023

from the intestine of the parasite (Smith and Smith 1996; Smith et al., 1999), which have resulted in the first commercial vaccine against *H. contortus* (Smith, 2014).

In general, the protection reported in all those trails is highly variable, probably related to several factors such as nature/composition of the antigen, the immunization strategy tested in each study, and other host and parasite-related factors. In relation to the host, the age of immunized animals seems to be a relevant factor (Martín et al., 2011). Furthermore, the genetics behind the native and acquired immune response to GIN determines different degrees of protection between animals from different breeds, even when similar immunization protocols are used (González et al., 2019). This immunoprotection heterogeneity induced by vaccination is also observed within the same host population, in a similar manner to what occurs under natural conditions, a phenomenon related to the genetics diversity associated with the immune mechanisms generated against GIN (Bambou et al., 2009; Ekroth et al., 2019; Brahma et al., 2022).

Vaccination against *H. contortus* is a struggle against a very complex parasite that also has a high genetic variability in the genes encoding target antigens (Ruiz et al., 2004; Laing et al., 2013; Doyle et al., 2020). Consequently, there could be a risk of vaccine failure against certain strains when the target molecules are natural antigens and are not hidden from immune recognition mechanisms (Martín et al., 2015; Sallé et al., 2018).

This scenario, together with the incomplete understanding of parasite-host interactions in *H. contortus* infections, suggests that the search for an immunological control of haemonchosis, or other infections caused by GIN, requires the gathering of all possible information on the immunological mechanisms underlying different vaccine candidates. This would include the use of various delivery vehicles or adjuvants, and vaccination protocols that could promote effective responses, not only in terms of the level of protection against the parasite, but also in terms of food safety and cost/benefit ratio for the development of future commercial vaccines (Piedrafita et al., 2013; Ehsan et al., 2020).

Our research group has obtained promising results on the immunoprotection conferred in goats by an antigenic complex obtained from adult *H. contortus* worms by affinity chromatography on thiol-sepharose (Ruiz et al., 2004; Molina et al., 2012; Martín et al., 2015). Analysis of the immune response generated by this immunogen revealed that protection obtained against *H. contortus* was associated with CD4 lymphocytes and increase of local and systemic specific antibodies. The immunolocalization of the antigens used in these vaccination assays, as well as other immunological parameters, suggested that this immunogen could be derived from excretory/secretory products (E/S) (Molina et al., 2018).

To contribute of the knowledge of the mechanisms underlying the response to this antigenic complex, this study analyzes the degree of protection generated in goats by thiol-binding proteins from *H. contortus* adult worms and related immunological mechanisms developed against the parasite during the prepatent period after challenge with L3 of the parasite.

2. Materials and methods

2.1. Parasites and immunogens

H. contortus adult worms used in this study were obtained from a strain isolated from naturally infected goats in Gran Canaria (Spain) and maintained experimentally in goats at the Faculty of Veterinary of the University of Las Palmas de Gran Canaria (Spain). Adult worms from slaughtered donors were homogenized in 1 x PBS, centrifuged at 10 000 g at 4 °C and the supernatant was filtered (0.22 μm) (Ruiz et al., 2004).

Afterwards, PBS soluble protein extracts were buffer exchanged to Tris-NaCl buffer pH 7.4 (10 mM Tris, 0.5 M NaCl) on a HiTrap-Desalting column (Amersham Pharmacia Biotech, Upsala, Sweden) at 5 mL/min, and then applied to a Thiol-Sepharose column (Amersham Pharmacia Biotech, Upsala, Sweden) (Knox et al., 1999). Bound material was eluted using the same buffer, but containing 25 mM L-cysteine (Merck, Darmstadt, Germany). The peak fractions were pooled and the L-cysteine was removed by passage through the desalting column. The proteins present in these elutions are referred as PBS soluble Thiol Sepharose binding proteins (PBS-TSBP) and their concentration were estimated using Pierce BCA kit (Protein Assay Kit, Thermo Scientific). The activity and characterization of the proteases present in the eluates were demonstrated by assays described previously (Ruiz et al., 2004).

2.2. Experimental animals and immunization trial

Fifteen healthy 9-month-old male (Majorera breed) goats were randomly allocated into the following experimental groups: group 1 (n = 6): goats immunized with PBS-TSBP and challenged with *H. contortus*; group 2 (control group) (n = 6): goat non-immunized and experimentally infected with *H. contortus*; group 3 (n = 3): non-immunized and uninfected goats. All animals were kept in nematode-free conditions since they were purchased (6 months–old) from local producers. No evidence of nematode infections was observed prior to the start of the experiment.

Group 1 was immunized on five consecutive weeks by intramuscular injections of 50, 75, 100, 100 and 300 μg of PBS-TSBP fractions, respectively. Freund complete adjuvant (FCA) was used for the first immunization (50 μg), and Freund incomplete adjuvant (FIA) from second week onwards (Ruiz et al., 2004). The animals from control group 2 were subjected to the same protocol of inoculation using FCA, FIA and elution buffer instead PBS-TSBP. Goats from group 3 (n = 3) were uninfected/unimmunized animals and served as controls for serological analysis. On week 7 of the experiment (two weeks after the last immunization), animals from groups 1 and 2 were experimentally infected with 7000 infective *H. contortus* L3, and on week 10 (when faecal eggs started to be observed) all the animals were slaughtered.

For haematological and serological analysis, all goats were weekly bled by jugular vein puncture during the whole experimental period. At slaughter, the abomasa were removed and opened for parasitological and mucosa analysis, including the collection of samples of mucus and gastric tissue from the fundic region.

The experiments with animals were supervised and approved by the University of Las Palmas de Gran Canaria Animal Welfare Committee, and carried out in accordance with the guidelines adopted in the European Communities Council on September 22, 2010 (Directive 2010/63/EU).

2.3. Parasitological and haematological analysis

At necropsy, the abomasa of slaughtered animals were opened and thoroughly washed with dH_2O. Resulting washings were placed into graduated flasks to determine the total volume, and 200 ml samples were preserved in 5% formalin for further estimation of the total number of worms by standard procedures. Larval burden was determined by digestion of mucosal scrapings with pepsin-HCl and immature worms were expressed as number of larvae per gram of mucosa (MAFF, 1989).

Blood samples used for haematological determinations were collected in test tubes using EDTA (7.5%) as anticoagulant. Plasma protein (PP) levels were estimated with a refractometer (Comecta S.A.) and packed cell volume (PCV) was determined using a micro-haematocrit. Total leukocyte and differential leukocyte counts of blood smears were determined using standard procedures.

2.4. ELISA tests

To confirm the presence of specific antibodies (IgG and IgA isotypes) against PBS-TSBP fractions, two indirect ELISA on serum and mucus samples were performed using a similar method to that described by

Molina et al. (1999). The immunogen (PBS-TSBP fractions) was used as the antigen at a final concentration of 1 (IgG) or 2.5 (IgA) μg/mL. Serum samples were analyzed at a 1:50 (IgA) or 1:200 (IgG) dilution, and conjugate (anti-goat IgG-peroxidase, or anti-goat IgGA-peroxidase; Sigma Aldrich Inc., USA) was employed at a 1:4000 dilutions. To avoid day-to-day variations, results for serum samples were expressed in relative units according to the optical density (OD at 492 nm) observed in a positive pool of samples used as a reference. ELISA results for mucus samples were expressed in OD at 492 nm.

The mucus samples obtained from the abomasa were used to determine the levels of specific local mIgGs and mIgAs. Samples were collected by superficial scraping of the abomasal mucosa, before they were washed for parasitological determinations, and diluted in a buffer containing proteinase inhibitors (0.1M sodium phosphate, 0.05M sodium chloride, 3 mM sodium acid, 1 mM PMSF and 5 mM EDTA; pH 7.1) at a rate of 2.5 mL buffer/gram of mucus. Samples were centrifuged at 18 000 g for 30 min at 4 °C and supernatant filtered before used in serological tests (Molina et al., 2018). Samples were diluted (1:100 -mIgG- or 1:25 -mIgA-) in PBS and conjugate (anti-goat IgG-peroxidase or anti-goat IgA-peroxidase) (Sigma Aldrich Inc., USA) was use at a 1:1000 dilution. Positive and negative controls mucus, obtained from pooled samples of Group 1 and Group 3 respectively, were used for the development of the test, and results were expressed as optical density (OD) at 492 nm (adapted from Amarante et al., 2005).

2.5. Histology and immunohistochemistry

Tissue samples from the abomasa were formalin fixed, paraffin-wax embedded, cut (5 μm thick) and stained with hematoxylin-eosin to determine the number of eosinophils and globule leukocytes. The counts of these cell populations were carried out using an optic microscope (Leica Laborlux S) at 400× magnification in 40 randomly selected fields of 0.237 mm^2, at the upper and lower third of the mucosa with an optic microscope (Leica Laborlux S). The results were expressed as cells/mm^2 (González et al., 2011).

For immunohistochemical analyses, tissue samples were embedded in OCTM solution (Optimal Cutting Temperature, Tissue Tek, Sakura Finetek, Europe B.V. Zoeterwoude, The Netherlands), followed by immersion in 2-methilbutane (Merk, Germany) at −80 °C until processed. Tissue sections were cut with a cryostat (Reichert-Jung, 2800 Frigocut N, Germany) at −20 °C and transferred to poly-L-lysine hydrobromide (Sigma-Aldrich Inc. USA) covered slides. Primary monoclonal antibodies raised against CD4, CD8, CD45R, γδ-T lymphocyte subsets, and $MHCII^+$ cells (Balic et al., 2000) were diluted at 1:15, 1:15, 1:5, 1:10 and 1:20, respectively, in 20% v/v foetal bovine serum in PBS. The dilutions (200 μl) were incubated with each section in a humidity chamber for 1–2 h at room temperature. Positive reactions were revealed by incubation with biotinylated rabbit anti-mouse immunoglobulins (Agilent Technologies, USA) diluted 1:20 in RPMI medium; a solution of avidin-biotin peroxidase (ABC) complex, at a 1:100 dilution in PBS, was applied a third reagent. Finally, tissue sections were reacted with 0.035% (w/v) 3-3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) containing 0.01% (v/v) hydrogen peroxide. Counterstaining was performed using Harris' hematoxylin stain, and cells were counted in 40 fields located in upper and lower third of the mucosa (Molina et al., 2018) (See supplementary figure).

2.6. Statistical methods

The statistical analysis of the obtained results was carried out using IBM SPSS Statistics software for Windows (IBM Corp. USA). The statistical differences between immunized and control animals in single day parameters were determined using the non-parametric Mann-Whitney U. On the other hand, the data obtained from samples collected weekly were compared using the General Linear Model for Repeated Measures. Previously, all data were uniformly distributed according to Kolmogorov–Smirnov's Normality. Finally, the Spearman correlation test was used to evaluate the associations between different parameters assessed in this study. Probabilities of $P < 0.05$ were considered as significant.

3. Results

3.1. Parasitological and haematological data

A very low number of immature worms from the mucosal digestion were detected in immunized and control animals, and no stastically significant differences were observed between groups (immunized group: 0.5 ± 0.4; control group: 0.8 ± 0.3 larvae per gram). By contrast, as shown in Fig. 1, a 53.2% reduction in total worm burden (49.0% and 59.1% reduction in female and male worms, respectively) was observed in immunized animals (group 1) compared to control (group 2). However, the reduction in the number of worms observed in the immunized group was not statistically significant when either total, female and male worm counts were independently compared to controls.

Regarding the haematological data, PCV and total plasma protein levels showed a progressive reduction after the challenge (week 7 of the experiment) onwards. Thus, the mean PCV values at the beginning of the experiment were close to 31% in group 1 (31.4%) and group 2 (31.2%), reaching values of 25.2% and 22.8%, respectively, at the end of the study (week 10). Although the effect of the experimental infection on PCV was slightly lower in the immunized group (group 1), this PVC pattern was similar in the control (group 2), with no significant differences between both groups throughout the study.

Though, the same trend was observed in plasma protein levels, the reduction in this parameter in the immunized group (group 1) was much less evident than in the control group (group 2). Thus, the mean plasma protein concentration decreased from week 1 to week 10 of the study from 7.32 g/dL to 7.01 g/dL in group 1, while it dropped from 7.04 g/dL to 6.0 g/dL in group 2, showing significant differences at weeks 8 and 10 of the study (1 and 3 weeks after challenge, respectively). This parameter was negatively associated with the number of worms (total, male and female) after challenge, showing statistical significance the correlations with the number of male worms at weeks 8 ($r = 0.699$; $p = 0.025$) and 9 ($r = 0.738$; $p = 0.015$) of the study.

With regard to total and differential leukocyte counts, no remarkable differences were observed between the experimental groups, except for peripheral eosinophil levels (Fig. 2), which were significantly increased in the immunized group of animals after challenge. Negative associations with the number of worms were also observed in this case, but without statistical significance.

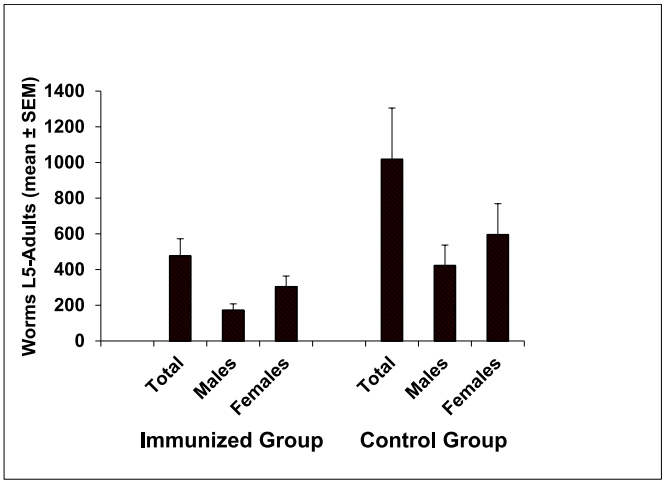

Fig. 1. Mean worm counts in goats from immunized and control groups at the end of the experiment (week 10). Results are mean ± SEM.

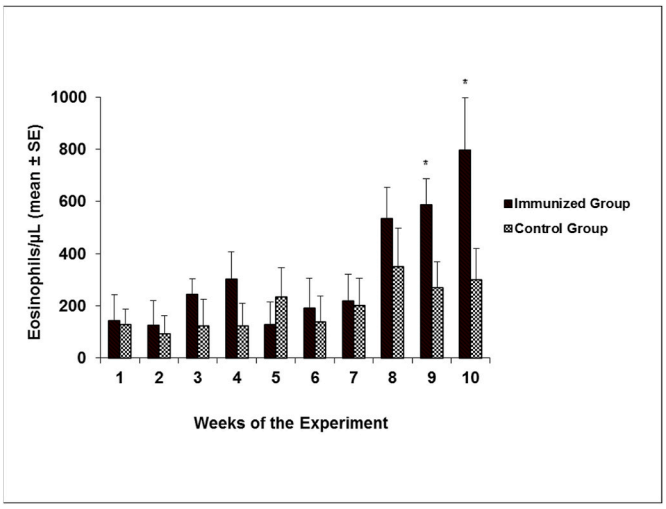

Fig. 2. Mean peripheral eosinophils in goats from immunized and control groups. Sampling was performed weekly from week 1 until the end of experiment (week 10). Results are mean ± SEM. *$P < 0.05$.

3.2. ELISAs

A significant increase in serum specific IgG levels was observed during immunization in group 1, reaching statistical significance from week 3 till the end of the trial (week 10), peaking at week 5 of the experiment (69.47 ± 10.50 R.U.). After challenge, there was not a further increase in specific antibody levels, but these remained significantly higher than in group 2 (non-immunized control) and in group 3 (uninfected) (Fig. 3A).

Similarly, the immunized group (group 1) showed a progressive increase in systemic anti-TPSP IgA antibody titers during immunization, with significantly higher levels than in the non-immunized groups (groups 2 and 3) from week 4 onwards. Moreover, an increase in specific antibodies was also observed after the challenge, from week 8 till week 10. Thus, in group 1, serum specific IgA were enhanced from week 8 of the experiment (1-week post-infection), while in group 2, the mean level of these antibody isotypes was also significantly higher than in group 3 at 3-weeks post-infection (Fig. 3B).

The analysis of correlations between systemic antibody titers of either IgG or IgA and parasitological data (total, male and female worm counts) at the end of the study showed that both antibody isotypes were negatively but not significantly related with the number of worms showing r values ranging from −0.248 to −0.455 for IgA anti-TSBP levels and from −0.382 to −0.624 for IgG anti-TSBP levels. Furthermore, at the time when immunization generated a peak of specific IgG antibodies (week 5 of the experiment), a significant negative correlation was established with the number of male worms ($r = -0.685$; $p = 0.029$). This association between IgG-anti TSBP levels and the number of males was close to statistical significance at the end of the study (week 10 of the experiment) ($r = -0.624$; $p = 0.054$).

Certain relationships could also be established between serum anti-TSBP antibody levels and some biopathological parameters (Table 1). Thus, it was observed that when the levels of these antibodies peaked during immunization at week 4 (specific IgA) or week 5 (specific IgG) of the experiment, these levels were positively associated with the concentration of plasma proteins after challenge, reaching statistical significance at week 8 and 10 of the experiment. At weeks 10 of the experiment, the same positive correlations were found when serum antibody levels of both isotypes (IgA- and IgG-anti TSBP) were compared with plasma protein concentration.

The maximum titers of specific IgG antibodies induced by immunization (week 5 of the experiment) was also related with an increase in peripheral eosinophils after challenge. Therefore, a positive correlation

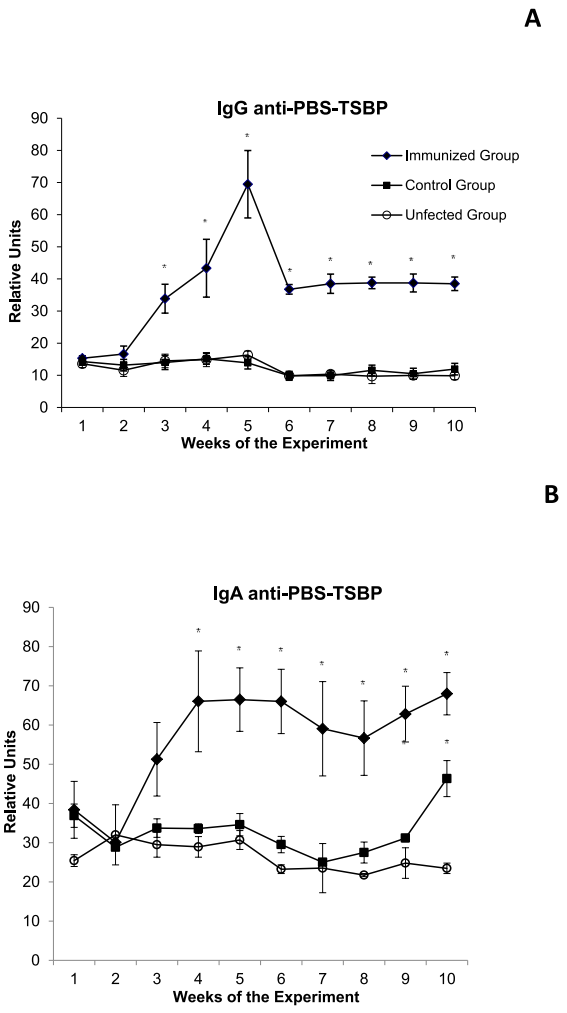

Fig. 3. Evolution of levels of specific IgGs (A) and IgAs (B) against *H. contortus* PBS-TSBP used as immunogen in immunized, non-immunized and uninfected goats from week 1 to the end of the experiment (week 10). Results are mean ± SEM. *$P < 0.05$.

was detected between anti-TSBP IgG levels at 5 weeks and peripheral eosinophil counts at weeks 8 and 10 of the experiment (Table 1). On the contrary, the comparative analysis of the systemic humoral response was not related to other indicators of parasite pathological activity, such as PCV.

Local isotype-specific antibody responses against *H. contortus*-TSBP antigens (OD values) are shown in Fig. 4. Specific mIgG titers were higher (≈6-fold higher) in the mucus of immunized animals, compared with unimmunized or uninfected control groups. These values showed statistical differences between immunized animals and control or uninfected groups. In contrast, the levels of anti-TSBP mIgA were lower, with no differences being observed between the three experimental groups.

The higher values of specific IgG in the mucus of immunized animals showed an association with parasitological and biopathological data, like those detected when correlation analysis of the humoral systemic response was carried out. Thus, mIgG levels showed a significant negative correlation with the number of male worms ($r = -0.754$; $p = 0.012$), as well as a positive correlation with plasma protein concentration at week 10 of the study. A positive association with peripheral eosinophil counts at weeks 9 ($r = 0.683$; $p = 0.03$) and 10 ($r = 0.687$; $p = 0.028$) was also observed.

Table 1
Spearman correlations between biopathological data after challenge (weeks 7, 8, 9 and 10) and level of immunoglobulims (Igs) anti-TSBP. Analysis were performed when the maximum levels of antibodies were observed during immunization (IgA: week 4, IgG week 5) or at the end of the experiment (week 10). Significant correlations are shown in bold (*$P < 0.05$; **$P < 0.01$). PCV: packed cell volume; PP: plasmatic proteins; EOS: peripheral eosinophils.

Igs Level	PCV7	PCV8	PCV9	PCV10	PP7	PP8	PP9	PP10	EOS7	EOS8	EOS9	EOS10
IgA W4	−0.215	0.000	0.158	0.196	0.417	**0.699***	0,604	**0.702***	0.394	0.624	0.292	0.503
IgA W10	−0.277	−0,314	−0.419	0.430	0.448	0.585	0.220	**0.788****	0.091	0.333	0.535	0.527
IgG W5	−0.400	−0.620	−0.170	−0.306	0.503	**0.717***	0,628	**0.917****	0.442	**0.782****	0.565	**0.709***
IgG W10	−0437	0.074	−0.128	0.208	0.380	**0.774****	0.598	**0.800****	0.309	0.576	0.413	0.612

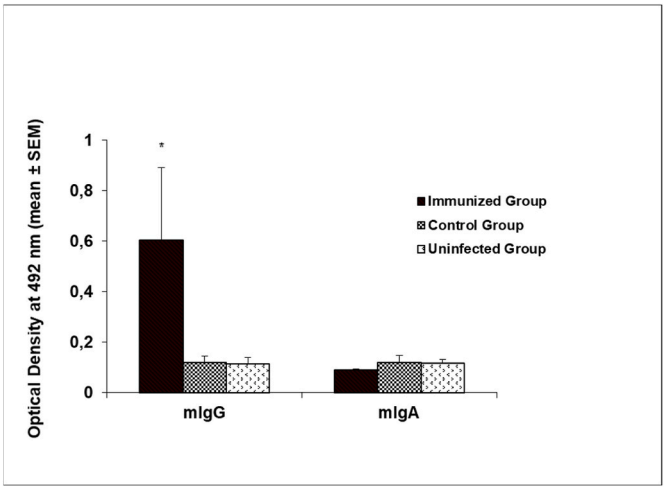

Fig. 4. Mean mucosal IgG (mIgG) and IgA (mIgA) against *H. contortus* PBS-TSBP used as immunogen in immunized, non-immunized and uninfected goats from week 1 to the end of the experiment (week 10). Results are mean ± SEM. *$P < 0.05$.

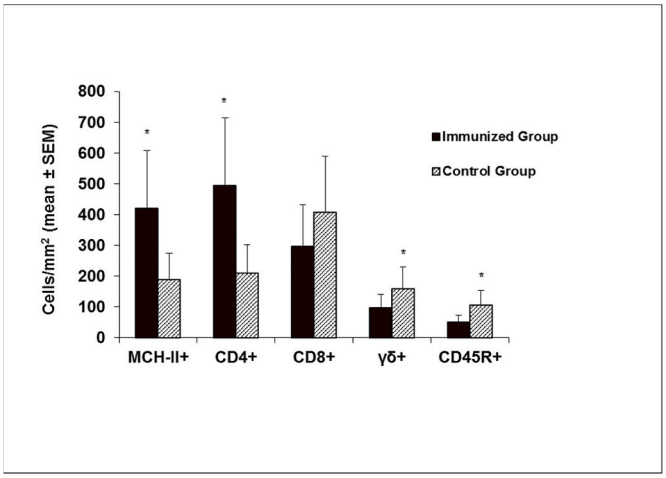

Fig. 5. Mean MHC-II+ and lymphocyte populations in abomasal tissue from immunized and control goats at the end of the experiment (week 10). Results are mean ± SEM. *$P < 0.05$.

3.3. Histopathology and immunohistochemistry

Histopathological examination revealed similar values of tissue effector cells (eosinophils and globular leukocytes) between immunized and non-immunized animals, without statistical differences between groups. When correlation analysis was performed between counts of both abomasal effector cells and worm counts or biopathological data, positive assocations were mainly observed, but no significant correlations could be proven. Similarly, not significant correlations were observed between both cellular populations of effector cells and local antibody response. Nevertheless, the globule leukocyte scores present in the gastric mucosa were associated with systemic IgG-anti-TSBP levels at week 10 of the study ($r = 0.589$; $p = 0.044$).

At the end of the study, immunohistochemical analysis of the gastric mucosa revealed a significant increase in CD4 and MCH-II$^+$ cell populations in the immunized goats (group 1) and, meanwhile in the non-immunized group (group 2) this increase was significantly detected for CDR45 and γδ-T subsets. In relation to CD8 lymphocyte subpopulation, although the non-immunized group showed a higher count, this difference was not significant (Fig. 5).

Statistical association analysis of the different cell population counts assessed by immunohistochemistry and the parasitological, biopathological and serological parameters (Table 2) highlighted that CD4 and MCH-II + antigen-presenting cell counts were negatively associated with some of these parameters, reaching statistical significance when evaluating the correlation between the number of male worms and CD4 cells. The opposite trend was observed between the number of γδ-T lymphocytes and worms, where a significant positive correlation was observed between these cells and the total number of worms as well as male and female worm counts. Consistent with these observations, a positive association was observed at the end of the study between both CD4 and MCH-II + cell counts and some biopathological parameters, such as plasma protein levels. Similarly, a positive association between the number of worms and γδ-T lymphocyte counts in the gastric mucosa and plasma protein levels. On the other hand, the presence of CD8$^+$ cells in the gastric mucosa was negatively associated with PCV and PP levels, with significant correlations being recorded at end of the experiment (week 10).

Table 2 also shows correlations between gastric mucosal cell counts and systemic antibody response (peak antibody levels and antibody values at week 10) or at local level (in gastric mucus). In general, CD4$^+$ cell counts were related to an increase in the levels of specific antibodies of both isotypes, particularly IgG, both at the systemic level (weeks 5 and 10 of the experiment) and in the gastric mucus. Specific IgA values in gastric mucus also showed a positive association with CD4$^+$ cell counts, which was close to statistical significance ($r = 0.624$; $p = 0.054$). Significantly increased MCHII$^+$ cell counts in immunized animals was associated with specific IgG levels in serum at week 5 of the study and in the gastric mucus. Finally, γδ lymphocyte and CD45R$^+$ cell counts were negatively related to isotype G-specific antibody titers, both at local and systemic levels, showing correlation coefficients ranging from −0.833 to −0.648.

4. Discussion

According to the results of the present study, the immunization of goat kids using PBS-TSBP fractions from *H. contortus* adult worms induces a 53.2% mean reduction of worm burden after a homologous challenge during the prepatent period of the experimental infection, with a higher reduction being observed in male worms (59.1%). These results seem to indicate that the extract used as immunogen, although obtained from adult worms, shares antigens with the parasitic larval stages, prior to the development of adult worms, which would be responsible for inducing these protective responses. However, the effect

Table 2
Spearman correlations between mucosal cell populations in gastric mucosa and parasitological and biopathological data at the end of the study (week 10), and between cell populations and levels of immunoglobulins (Igs) anti-TSBP in gastric mucus (mIg) or serum. Analysis in serum samples were performed when the maximum levels of antibodies were observed during immunization (IgA: week 4, IgG week 5) or at the end of the experiment (week 10). Significant correlations are shown in bold (*$P <$ 0.05; **$P < 0.01$). **PCV**: packed cell volume; **PP**: plasmatic proteins. **mIgA**: specific IgA in mucus. **mIgG**: specific IgG in mucus.

Cellular Population	Total Worms	Male Worms	Female Worms	PCV10	PP10	mIgA	mIgG	IgAW4	IgAW10	IgGW5	IgGW10
MCH-II$^+$	−0.309	−0.309	−0.345	0.495	**0.683***	0.309	**0.657***	0.467	0.515	**0.733***	0.539
CD4$^+$	−0.588	**−0,685***	−0.612	0.067	0.622	0.624	**0.863****	0.467	**0.648***	**0.673***	**0.758***
CD8$^+$	−0.176	0.006	−0.261	**−0.777***	**−0.659***	0.152	−0.322	**−0.648***	−0.333	−0.442	−0539
γδ$^+$	**0.758***	**0.733***	**0.891****	0.104	**−0.702***	0.358	**−0.833****	−0.624	**−0.673***	**−0.770***	**−0.733***
CD45R$^+$	0.588	**0.758***	0.539	−0.147	−0.591	0.236	**−0.736***	0.527	−0.382	**−0.648***	**−0.758***

of immunization on the number of worms observed here was slightly lower than that obtained in similar trials (65%), in which the infection lasted 5 additional weeks (week 15 instead of week 10) (Ruiz et al., 2004; Martín et al., 2015; Molina et al., 2018). The number of larvae in the gastric mucosa was similar between both experimental groups (groups 1 and 2), which seems to indicate that the immune response generated by immunization does not result in larval immobilization (Harrison et al., 2003). According with these results of the mucosal larvae, the partial protection induced by the immunization could be related to the rejection of infective larvae (Balic et al., 2002). Alternatively, it is also possible that defensive responses against parasitic larval stages (L4, L5) were similar to those developed against adult worms, not having an immediate effect on the parasites. This would explain why, when the effect of immunization is maintained over time, the reduction of the number of worms is higher, as has been observed in vaccination trials against other parasitic metazoans (Stutzer et al., 2018).

The protective effect during prepatency generated by previous immunization is also reflected on parameters related with pathological activity of pre-adult stages, such as PCV and PP levels (Taylor et al., 2016). Such findings have also been observed in similar trials using this immunogen, in which the development of adult worms was completed after challenge (Molina et al., 2018). However, these differences did not reach statistical significance in both studies, probably due to sample size and/or intra-group variations. A negative association between these biopathological parameters (PCV and PP) and the worm burden was only found in the present study when the correlations between PP levels and the number of male worms were analyzed.

Increased peripheral eosinophils seems to be associated with resistance against GIN nematodes (Terefe et al., 2007; Beraldi et al., 2008; Albuquerque et al., 2019). Accordingly, the levels of blood eosinophils were significantly increased in vaccinated animals when compared to control group after two weeks of the challenge. This observation has been previously found in goats immunized with PBS -TSBP (Molina et al., 2018).

Immunization induced a rapid systemic humoral response of specific antibodies against PBS-TSBP fractions, whose evolution after challenge confirms previous observations (Molina et al., 2018) showing that this extract contains natural antigens which would be recognized by immunological mechanisms. Based on immunolocalization of PBS-TSBP in internal organs of the worms (intestinal epithelium and reproductive organs), as well as the secondary responses observed after challenge, the authors stated that those fractions may contain products of excretion/secretion (E/S) (Molina et al., 2018). Accordingly, the associated immunoprotection could be related to the neutralizing effect of some of the components of these E/S products, thereby impairing the functions they could perform for the survival of the parasite (Pearson et al., 2010; McNeilly and Nisbet, 2014). The secondary response observed in the present study was particularly evident for the IgA isotype, which showed an antibody peak two weeks after the experimental infection. As for IgGs, this increase is not observed here, probably because both isotypes have a different dynamic after the challenge, so that longer time is needed for the development of secondary response of specific IgGs as previously observed in similar trials (Molina et al., 2012, 2018; Martín et al., 2015).

The systemic increase of specific antibodies, which is considered to be related to natural or induced resistance against *H. contortus* in small ruminants (Ruiz et al., 2004; De Vries et al., 2009; Albuquerque et al., 2019), usually reflects the degree of protection induced by immunization. Accordingly, we here found a negative association between antibody levels and the number of worms, particularly relevant when analyzing the antibody peak after immunization and the number of male worms. In addition, positive correlations were also established with PP levels, indicating that the immunization leads to a more benign course of the infection. All these results suggest that serum antibodies could be involved in the protection provided by immunization with these protein fractions, both against pre-adult stages and adult worms.

Despite a secondary systemic response of specific IgG had not been generated at 3 weeks after the challenge (time of slaughter), these immunoglobulins were significantly higher in the group of immunized animals at the local level. Interestingly, IgG values in gastric mucus was associated to some protective parameters, similar to those observed at the systemic level, which support the role of the humoral response in the immunoprotection mechanisms triggered by the immunization. Increased specific antibody levels in gastric mucus is usually a common finding in sheep resistant to gastric nematodes, and it is known –in contrast to our results-that IgA immunoglobulins, probably in conjunction with gastric mucosal eosinophils, play a more relevant protective role than the IgG isotype (Amarante et al., 2005; Aboshady et al., 2020). Abomasal IgA has also been found to be closely related to natural resistance to *Teladorsagia circumcincta* in the same goat breed used in this study (Ortega et al., 2022). Discrepancies may be explained by differences between experimental immunizations and immunoprotection attained under natural conditions, or even between particularities of the design of the experimental immunizations. In fact, in vaccination trials with this PBS-TSBP an enhanced IgA and IgG specific response at local level could be detected in the immunized group 8 weeks after challenge (Molina et al., 2018).

Another distinctive feature of natural resistance to gastric nematodes in small ruminants is the presence of eosinophilia in association with high mucosal infiltrating eosinophils, as well as an increase in other effector cells such as globular leukocytes (Pérez et al., 2001, 2003; Meeusen et al., 2005; González et al., 2011). However, this finding was not found in our study, so no correlation between peripheral and mucosal eosinophils was detected in vaccinated animals, nor were substantial differences in the number of infiltrating effector cells observed in relation to control animals. Accordingly, no statistical associations could be established with any of the markers of protection analyzed. That weak relationship between mucosal effector cells and the level of protection was already observed in other similar immunization trials, where even the number of mucosal infiltrating eosinophils were positively correlated to parasitological parameters such as FEC (Molina et al., 2018).

The aforementioned study (Molina et al., 2018) also showed an infiltrating cellular profile in the gastric mucosa closely similar to that observed in the present experiment, where a significant increase of CD4 lymphocytes and MCHII$^+$ cells in the mucosa of immunized goats with respect to the control group was noticed. Both findings are consistent with the role of these cell populations in the development of protective

responses in small ruminants against GIN (Balic et al., 2002, 2003; Halliday et al., 2010; Ortega et al., 2022). Particularly, the increase in these cell populations recorded here was associated with a reduction in the number of worms ($CD4^+$ cells) or an increase in parameters related to resilience to infection, such as PP levels ($MHCII^+$ cells).

Consistent with similar immunization trials where immnoprotection was reported (Molina et al., 2018), increased levels of CD8, γδ-T and CD45R lymphocytes were observed in gastric mucosa in challenged control (unvaccinated) animals. With respect to CD8 cells, the increase in the gastric mucosa during different GIN infection has not been associated with the development of protection (Pérez et al., 2008; González et al., 2011; Ortega et al., 2022), even more, it seems that resistance to these parasites is not modified by the depletion of this cell lymphocyte subset (Gill et al., 1993). Therefore, perhaps the increased number CD8 cells in unvaccinated goats could be related to mechanisms of evasion of the immune response generated by the parasite, given that both in the current and previous previous studies (Molina et al., 2018) vaccinated animals in which protection was observed, CD8 cells showed lower mean values in the gastric mucosa.

In relation to γδ+ lymphocytes, it is known that this cell population constitutes an important percentage of lymphocytes in goats, especially in young animals such as those used in this study (Yirsaw and Balwing, 2021). However, there is not much information available on their role in the development of protection against GIN, and different results have been found when analyzing the protective role of these cells against different GIN species (McClure et al., 1995; González et al., 2011). Perhaps this effect may be affected by the parasite species involved, host-related factors, or even the time at which the analysis is carried out, since this type of study shows a snapshot of all the events that takes place after infection/challenge. In our case, the significant increase of γδ+ lymphocytes in the gastric mucosa of unvaccinated control animals shows associations with a lower degree of protection, as indicated by positive correlations with the number of worms and negative associations with plasma protein levels.

Similarly, the increase of cells labelled with CD45R antibody was associated in unvaccinated control animals with an increase in the number of worms, despite that the presence of plasma cells in the gastric mucosa is recognized to be related with resistance against GIN (Pérez et al., 2008; González et al., 2008; Ortega et al., 2022). However, in the immunized goat kids, a lower number of this subpopulation was observed, which contrasts with the high local humoral response generated in this group, a circumstance that may be determined because the increase of CD45R cells in the mucosa is not always associated with a specific humoral response.

In conclusion, goat kids immunized with PBS-TBSP fractions obtained from adult *H. contortus* worms generate an immune response during the prepatent period of the infection, which translates into a reduction in the number of worms as well as a lower effect on PCV and PP levels, in relation with non-vaccinated animals, although these effects were only statistically significant for PP levels probably due to the high variability observed in these parameters within the experimental groups. This response is accompanied by a series of mechanisms very similar to those that take place in vaccinated goat kids after prepatency (several weeks after the development of adult worms), and characterized by an increase of specific immunoglobulins in serum (Molina et al., 2018). At the local level, in addition to the development of a specific humoral response with the IgG isotype being predominant, the gastric mucosa of immunized animals showed a cellular infiltration of CD4 lymphocytes and MHCII, whose number were associated with lower number of worms and parameters related with resilience. By contrast, peripheral eosinophilia was not related with defensive mechanisms developed by these cells in gastric mucosa nor with local infiltration of other effector cells.

This response is accompanied by a series of immunological mechanisms very similar to those occurring in vaccinated kids after prepatency, several weeks after the development of adult worms (Molina et al., 2018).

Authorship statement

Authorship contributions (Dr. JM Molina ensure that the following descriptions of activities are accurate and agreed by all authors). Conception and design of study: Molina, J.M. and Ruiz, A.-Acquisition of data: Molina, J.M. Hernández, Y.I., Ferrer, O., Conde-Felipe M[a]. M., Rodríguez, F., Ruiz, A. -Analysis and/or interpretation of data: Molina, J.M. Hernández, Y.I., Ferrer, O., Conde-Felipe M[a]. M., Rodríguez, F., Ruiz, A. -Drafting the manuscript: Molina, J.M. Hernández, Y.I., Ferrer, O., Conde-Felipe M[a]. M., Rodríguez, F., Ruiz, A. -Revising the manuscript critically for important intellectual content: Molina, J.M. Hernández, Y.I., Ferrer, O., Conde-Felipe M[a]. M., Rodríguez, F., Ruiz, A.-Approval of the version of the manuscript to be published: Molina, J.M. Hernández, Y.I., Ferrer, O., Conde-Felipe M[a]. M., Rodríguez, F., Ruiz, A.

Data availability

No data was used for the research described in the article.

Acknowledgments

We thank Drs Piedrafita and Meeusen (Federation University, Australia) and Dr González (University of Las Palmas de Gran Canaria, Spain) for kindly donation of the antisera used in this study for immunohistochemical analysis.

This study was fund by the Ministerio de Ciencia y Tecnología (MICT, projects AGL-03528/GAN-ACU and AGL-62611/GAN) and FEDER funds. Dr. Yeray I. Hernández was the recipient of a fellowship funded by "Agencia Canaria de Investigación, Innovación y Sociedad de la Información de la Consejería de Economía, Industria, Comercio y Conocimiento" (ACIISI) from "Gobierno de Canarias".

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2023.108512.

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