Increased susceptibility to *Haemonchus contortus* infection by interleukin 5 modulation of eosinophil responses in sheep

Running Title: IL-5 vaccination and *H. contortus* infections in sheep

Julia N. Hernández[1]; Els Meeusen[2]; Francisco Rodríguez[1]; David Piedrafita[2,*]; Jorge F. González[1,*]

[1] Instituto Universitario de Sanidad Animal y Seguridad Alimentaria, Veterinary Faculty, Universidad de Las Palmas de Gran Canaria, Trasmontaña S/N, Arucas 35413, Spain

[2] School of Health and Life Sciences, Federation University, VIC 3842, Australia

*DP and JFG should be considered joint senior authors

Corresponding authors:

E-mail: david.piedrafita@federation.edu.au and jorgefrancisco.gonzalez@ulpgc.es

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ABSTRACT

Eosinophils are prominent effector cells in immune responses against gastrointestinal nematode infections in ruminants, but their *in vivo* role has been hard to establish in large animals. Interleukin-5 is a key cytokine in the induction and stimulation of anti-parasitic eosinophil responses. This study attempted to modulate the eosinophil response in sheep through vaccination with recombinant interleukin-5 (rIL-5), and determine the effect on subsequent *Haemonchus contortus* infection. Nematode resistant Canaria Hair Breed (CHB) sheep vaccinated with rIL-5 in Quil-A adjuvant, had lower blood eosinophil counts and higher mean worm burdens than control sheep vaccinated with Quil-A adjuvant alone. In addition, adult worms in IL-5 vaccinated sheep were significantly longer with higher eggs *in utero* in female worms, supporting an active role of eosinophils against adult parasites in CHB sheep. These results confirm that eosinophils can play a direct role in effective control of *H. contortus* infection in sheep and offer a new approach to study immune responses in ruminants.

Keywords

Interleukin 5; *Haemonchus contortus*; eosinophils; gastrointestinal nematodes; Canaria Hair Breed sheep; cytokine vaccination; helminth immunity

1. INTRODUCTION

Haemonchus contortus is a geographically widespread hematophagous parasite, infecting the abomasum and causing significant production losses in sheep. In general, sheep can develop resistance with repeated infection and eosinophils are implicated as a major resistance mechanism (1, 2). *In vitro* assays have demonstrated that eosinophils can inhibit the motility of L3 larvae with visible lesions appearing in the cuticle of the L3 (3) and reduced infectivity (4). In immunized animals, a negative association between eosinophils and faecal egg count (FEC) has been demonstrated (5) and eosinophils are observed surrounding the *H. contortus* larvae in abomasal tissues (6). In a resistant breed of sheep, the Canaria Hair Breed (CHB) sheep, immunity manifests after a single infection with *H. contortus* (7) and eosinophil numbers have been correlated with reduction in adult worm burden and fecundity (8). These data suggest an important role for eosinophils in immune mechanisms against both larvae and adult *H. contortus*, but do not provide direct evidence for their involvement in immune protection.

Interleukin-5 is a key cytokine in the differentiation and activation of anti-parasitic eosinophil responses, and has been specifically targeted in mice to demonstrate the *in vivo* role of eosinophils in helminth parasite infections (reviewed in (9, 10)). The reagents and knock-out models used in these rodent studies are however not available or suitable to large animal experimentation. Several studies have shown that mice vaccinated with recombinant cytokines, can induce autoantibodies that specifically abrogate the activity of the native cytokine *in vivo* (11-13), and this approach may provide an alternative for the *in vivo* study of immune responses in large animals. In this study, we attempted to impair the eosinophil response following experimental *H. contortus* infection in sheep, by prior immunization with recombinant IL-5 (rIL5).

2. MATERIAL AND METHODS

2.1 Animals and experimental design

Twelve CHB male lambs were acquired at weaning from farms on Gran Canaria Island (Canary Islands, Spain), dewormed with fenbendazole (2.5 Panacur®, Intervet) following the manufacturer's recommendations (5 mg/kg body weight), and housed at the facilities of the Faculty of Veterinary Medicine, *Universidad de Las Palmas de Gran Canaria* until they were approximately one year old. The animals were fed a granulated commercial ration for sheep, rye grass and water *ad libitum* and animals were confirmed free from

nematode parasites via monthly FEC until the beginning of the experiment. All animals were inoculated intraruminally with 10,000 L3 of *H. contortus* and were slaughtered at 28 days post-infection (dpi).

Animal experiments were approved by the University of Las Palmas de Gran Canaria Animal Welfare Committee.

2.2 Recombinant Interleukin-5 production

Fourteen mg of recombinant IL-5 (rIL-5) were produced at the Protein Production Unit from Monash University (Australia). Briefly, the bovine IL-5 DNA sequence was synthesized and cloned into an expression vector, pET28a which contained a modified precision protease cleavage site and N-terminal 6x His-Tag. The vector containing the IL-5 gene was transformed into BL21 (DE3) *Escherichia coli* expression strain and induced with 1mm IPTG (T7 - lac operon). Expressed cultures were purified via Immobilised Metal Affinity Chromatography (IMAC), using both Ni2+ and Co2+ resins and eluted using 500mM Imidazole using standard protocols and the bands identified in IL-5 SDS PAGE were confirmed by mass spectrometry.

2.3 Immunization

Recombinant interleukin-5 (rIL5) (0.7 mg) with Quil-A (Brenntag Biosector, Denmark) in saline was administered subcutaneously in six randomly selected animals (immunized IL-5 group). The other six animals were the control group and received only Quil-A in saline. Three immunizations were performed corresponding to days 28 and 14 pre-infection and on the same day of infection with the parasite.

2.4 Blood samples

Blood was collected via the jugular vein on days 32, 26, 22, 19, 15, 12, 8, 5 and 1 pre-infection and on days 2, 6, 9, 13, 16, 23 and 27 post-infection for blood eosinophil counts and serum collection.

2.5 Parasitology

Faeces were taken daily from day 16 post infection (pi) until the end of the trial to carry out the fecal egg counts using the modified McMaster technique (sensitivity: 50 eggs per gram). At postmortem on day 28 pi, the abomasum of each sheep was removed and opened by cutting along the greater curvature, followed by collection of abomasal

contents and mucosal samples. Worms were counted from aliquots (300 ml, 10% formaldehyde) obtained from each abomasal washing, adjusting the result to the total volume of the abomasum. Mature adult worms were sexually differentiated, and thirty random female worms were measured from each animal using a digital camera (ProgRes C12PLUS) coupled to an inverted microscope (Olympus CKX41). Eggs in utero in these adult female worms were counted using a microscope at 100× magnification after disruption of the parasite tissues using a coverslip.

2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of serum IgG against rIL-5 were determined by enzyme-linked immunosorbent assay (ELISA). The plates (Costar 3369, Corning) were incubated with the antigen (rIL-5) diluted (5µg/ml) in carbonate buffer (pH 9.6) for 24 h at 4ºC followed by three 5-min-washes with PBS tween 20 (0.05% v/v) (PBSt) to remove unbound antigen. Nonspecific reactions were blocked with 3% bovine serum albumin by incubating plates for 45 min at 37ºC, followed by washing with PBSt. Serum samples were diluted (1:100) in PBS sodium azide 0.02% (w/v) and incubated for one hour (37ºC), followed by three washes. HRP-conjugated donkey anti-Sheep IgG (H+L) (Thermo Fisher Scientific) in PBS (1:1000) was added and plates were incubated for 45 min at 37ºC, followed by three washes with PBSt. The substrate (12.2 ml of citric acid 2.1%, 12.8 ml of sodium phosphate 2.8%, 10 mg of OPD and 35 µl of H2O2) was added and plates incubated for 10 minutes in the dark. Sulfuric acid 2M was added to stop the reaction and after five minutes of incubation, the plates were read at 492nm on ELISA plate reader (Multiskan Ascent).

2.7 Histology

Tissue samples (approximately 2 × 2 cm) were collected at necropsy for histological studies from the abomasal fundic region. Samples were preserved in 4% neutral-buffered formalin and embedded in paraffin-wax. Tissue sections were cut (5µm thick sections) and stained with haematoxylin and eosin for eosinophil counts. Cells were counted in at least 20 randomly selected fields of 0.0688 mm^2 at 400× magnification with an optical micro-scope (Olympus BH2).

2.8 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics software version 17 and Excell. Fecal Egg Count data (FEC) were transformed into Log_{10+1}. One animal from the immunized group was considered an outlier because its values were extremely low in comparison with the rest of the lambs of this group (Table 1). This outlier was eliminated from some of the statistical analysis of egg counts and worm burden as described in the results.

Parasitological data were analyzed using the generalized linear model (GENLIN) with SPSS, with a gamma distribution using the estimation method Newton-Raphson and Pearson Chi-square scale, adding 0.1 to all values in order to avoid zero data for statistical analysis. The length of the worms was also analyzed with a GENLIN with a normal distribution. Comparisons of blood eosinophils, FEC and serum IgG were made using the general linear model (GLM). Cumulative blood eosinophil counts were estimated for each group using the trapezoidal method for calculation of area under the curve (AUC_Eos). To examine if IL-5 vaccination resulted in the expected reduction in blood eosinophils, comparison of AUC_Eos and cell counts in the abomasum were done by one-tailed student t-test. Variances between the two groups were compared by the F-test. Shapiro-Wilk normality test was applied to data prior to analysis. Probabilities of $p \leq 0.05$ were considered statistically significant.

3. RESULTS

3.1 Induction of specific antibodies in IL-5 vaccinated sheep

A significant increase in the levels of specific serum IgG against rIL-5 was observed in the IL-5 vaccinated group compared to the control, from 10 days after the first immunization, reaching a peak at day 15 pre-infection and remaining high until the end of the experiment (Figure 1).

3.2 Effect of IL-5 vaccination on eosinophilia

Overall blood eosinophil counts were lower in the IL-5 vaccinated group compared to the control group over the experimental time period, although this was not significantly different using the Repeated Measures GLM procedure of group means (Fig. 2a). Cumulative blood eosinophil counts were considered in three distinct time periods: (1) before or (2) after peak anti-IL-5 antibody and (3) after infection (Fig. 2b). While there was no difference between the two groups before high IL-5 antibodies were generated,

cumulative eosinophil counts during the elevated antibody response pre-infection (day -19 to day -1) were significantly lower in the IL-5 vaccinated group compared to the control group (Fig. 2b). After the experimental infection, an increase in the blood eosinophil count was observed in some of the sheep in the control group (Fig. 2a). This increase after infection was not observed in any of the IL-5 vaccinated sheep and variances between groups were consequently highly significantly different (F-test, $p<0.001$).

In the abomasum, no statistical differences in eosinophil counts were observed between groups at the killing time point (Mean ±SEM: rIL5 vaccinated group: 72±22; control group: 57 ± 22), or in the number of mast cells and globular leucocytes (data not shown).

3.3 Effect of IL-5 vaccination on parasitological parameters

Faecal egg counts were positive on day 18 pi in the immunized group and two days later in the control group (Fig. 3). Faecal egg counts were always higher in the immunized group and these differences were significant from 20 days pi onwards. The worm count in the immunized group was more than double compared to the control group (Table 1). In addition, female worms in the IL-5 vaccinated group were significantly longer and harboured more eggs in their uterus than in the control group even with one outlier in the vaccinated group included (Table 1).

Table 1: Mean ± SEM of worm burden, length and eggs in uterus

Group	Worm burden	Worm Length	Eggs in uterus
Control	1937±669	14,52±0,11**	162±6**
rIL5ov (- outlier)	4785±457	15,40±0,14**	229±13**
rIL5ov (+ outlier)	4025±847	15,02±0,14**	203±12**
Outlier	223	13,13±0,24	71±6

** significantly different at $p<0.01$.

4. DISCUSSION

The interaction between the different stages of *H. contortus* and sheep is a complex phenomenon in which several components of the immune response seem to be implicated (reviewed in (1, 2)). Generally, these components have been identified because their

number/levels increase while the immune response is developing and through correlation studies with parasitological variables. For some components, it has been possible to determine more directly their functional *in vivo* role by neutralising activity through monoclonal antibody infusion. For example, the key role of CD4+ (14) and γδT cells (15) in protection of different sheep breeds. However, because of the animal size, these are generally laborious and expensive experiments, and specific reagents are not always available for livestock.

Several *in vitro* killing or *in vivo* histological and correlation studies have suggested that eosinophils could be relevant in sheep resistance to *H. contortus*. IL-5 is a cytokine with a defined, specific activity in eosinophil differentiation and activation and has been extensively validated as a target to reduce eosinophil-mediated inflammation using specific monoclonal antibodies or through genetic manipulation (9, 10). In the present study, we use an alternative approach to target IL-5 in sheep through vaccination with the recombinant molecule. Cytokine vaccination has been effectively used in several mouse models to study the role of eosinophils in disease, including IL-9 in helminth infections (13) and IL-5 in asthma (12). To our knowledge, this approach has not been previously reported in ruminants. One recent study reported the effectiveness of IL-5 vaccination in reducing eosinophilia and insect-bite hypersensitivity in horses (16). As only small amounts of reasonably easy to prepare reagents were needed, this approach was considered more cost-effective and feasible for large animal experimentation, such as sheep.

In the present study, vaccination with recombinant IL-5 resulted in a complete absence of blood eosinophilia in sheep after infections with *H. contortus* larvae, as well as lower worm burdens and a significant effect on worm length and fecundity. The CHB sheep used in this study have been shown to have enhanced resistance to *H. contortus* after a single infection, with resistance directed against the adult parasite stage manifested in reduced worm length and fecundity (7). Further studies have shown that this resistance correlates significantly with both γδ−T cells and eosinophils (8), and that depletion of γδ−T cells abrogates protection as well as the eosinophil correlation (15). The present study shows a similar effect on adult worms after targeting eosinophilia through recombinant-IL-5 immunization and supports the initial hypothesis that both γδ−T cells

and eosinophils, possibly in cooperation, are responsible for the effect on adult worms in the resistant CHB sheep. The exact mechanism of this immunity is still not known, however it has been shown that gastrointestinal γδ–T cells of sheep express IL-5 (17) and that IL-5 activates eosinophils (18) and enhances the *in vitro* killing of *H. contortus* larvae by eosinophils (3). This could indicate that IL-5 mediated activation of tissue eosinophils is required to negatively affect worm physiology and fecundity, possibly through the ingestion of toxic mediators released by activated eosinophils. This would require further studies to be confirmed.

In contrast to the reduction in blood eosinophilia, there was no significant reduction in tissue eosinophils after rIL-5 vaccination. This is in accordance with studies in humans and mice, where it was shown that anti-IL-5 treatment effectively reduces blood eosinophilia, but not tissue eosinophils, whose recruitment is likely controlled by chemokines such as eotaxin (18, 19). Despite their presence in the tissue, the activation of tissue eosinophils through IL-5 may be crucial in exerting their negative effect on adult worms as indicated above.

In conclusion, the present study indicates, for the first time, an *in vivo* role for eosinophils in immunity against a gastrointestinal parasite of sheep. In addition, it illustrates a new approach for studying immune responses in ruminants through vaccination with recombinant cytokines.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request

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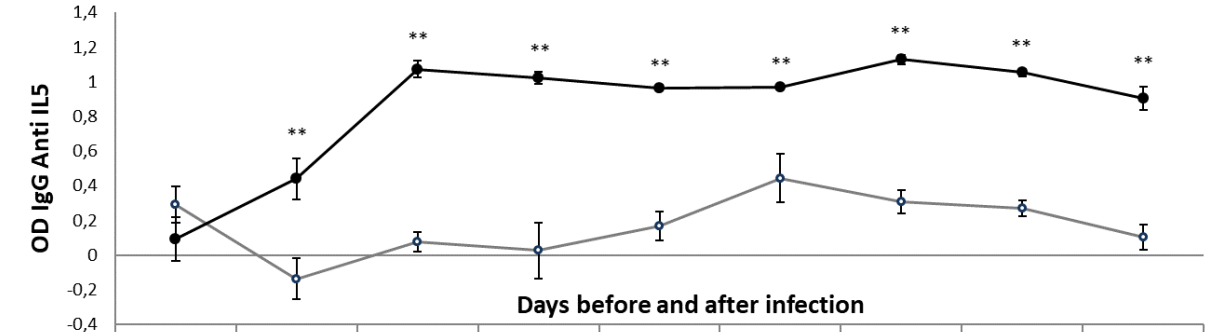
Figure 1.

Mean ± standard error of the mean (SEM) of anti- rIL-5 IgG levels in serum of sheep immunized with rIL-5/Quil-A (black circles) or Quil-A only controls (white circles) on various days before and after infection with *H. contortus*. Immunizations were administered on days -28, -14 and 0. ** shows significant differences at $p<0.01$.



	-32	-22	-15	-8	-1	6	13	20	27
Control	0,292	-0,138	0,074	0,025	0,169	0,444	0,305	0,268	0,103
rIL-5ov	0,091	0,44	1,072	1,022	0,965	0,969	1,127	1,052	0,904

Figure 2a: Individual blood eosinophil counts of sheep immunized with rIL-5/Quil-A (rIL-5 vaccinates, upper graph, n=6) or Quil-A only (Controls, lower graph, n=6) on various days before and after infection with *H. contortus*.

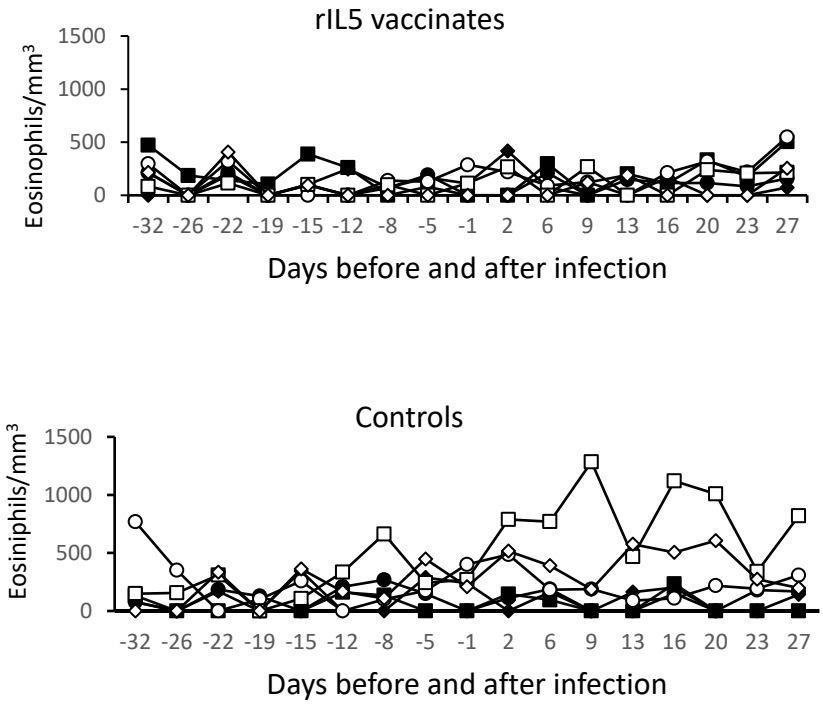


Figure 2b: Mean area under the curve (AUC) blood eosinophil counts ± SEM of sheep immunized with rIL-5/Quil-A (black bars) or Quil-A only controls (white bars) at three time periods: (1) before high anti-IL-5 IgG (days -32 to -22), (2) at the start of high anti-IL-5 IgG but before infection (days -19 to -1) and (3) at high anti-IL-5 IgG and after infection (days 2 to 27). * shows significant differences at $p<0.05$.

Figure 3:

Mean ± SEM of transformed (Log_{10}+1) faecal egg counts (FEC) of sheep immunized with rIL-5/Quil-A (black diamonds) or Quil-A only controls (grey squares) at various days post-infection. * shows significant differences at $p<0.05$.