International Journal for Parasitology 49 (2019) 257–265

Contents lists available at ScienceDirect

International Journal for Parasitology

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

The rational simplification of a recombinant cocktail vaccine to control the parasitic nematode *Teladorsagia circumcincta*

Alasdair J. Nisbet [a,*,1], Tom N. McNeilly [a,1], Daniel R.G. Price [a], E. Margaret Oliver [a], Yvonne Bartley [a], Mairi Mitchell [a], Javier Palarea-Albaladejo [b], Jacqueline B. Matthews [a]

[a] *Moredun Research Institute, Pentlands Science Park, Edinburgh EH26 0PZ, United Kingdom*
[b] *Biomathematics & Statistics Scotland, JCMB, The King's Buildings, Edinburgh EH9 3FD, United Kingdom*

ARTICLE INFO

Article history:
Received 21 August 2018
Received in revised form 23 October 2018
Accepted 28 October 2018
Available online 26 January 2019

Keywords:
Nematodes
Vaccination
Apyrase
Metalloproteinase
Teladorsagia

ABSTRACT

Using data from five independent vaccine trials, which employed a subunit cocktail vaccine containing eight recombinant proteins to protect sheep against *Teladorsagia circumcincta*, a strategy was developed to simplify antigen complexity of the vaccine. A meta-analysis of data from these five trials demonstrated statistically significant reductions in cumulative faecal egg count and worm burden in vaccinated sheep when compared with those which had received adjuvant only ($P = 0.009$ and $P < 0.0001$, respectively). Relationships between antigen-specific antibody levels, antibody avidity and parasitological parameters of efficacy were analysed for each of the eight proteins in these trials. Of these, the strongest correlations between percentage reduction in cumulative faecal egg count and avidity were obtained for the vaccine antigen *T. circumcincta* apyrase-1 (Tci-APY-1) in relation to either total antigen-specific IgG or IgG1 in sera ($P = 0.019$ and $P = 0.030$, respectively). In addition, IgG and IgA within the serum and abomasal mucus of control (parasite challenged) lambs strongly recognised Tci-APY-1 and *T. circumcincta* metalloproteinase-1 (Tci-MEP-1) but only weakly bound the other six antigens, indicating Tci-APY-1 and Tci-MEP-1 are most effectively recognised by the parasite-induced antibody response. On the basis of these findings, a two-protein vaccine comprising Tci-APY-1 and Tci-MEP-1 was tested in a direct comparison with the original eight-component vaccine. A further group was immunised with Tci-MEP-1 in combination with a mutated form of Tci-APY-1 (mTci-APY-1), which had no enzymatic activity. Across the trial, the mean faecal egg count levels of the eight-antigen recipients were lower than those of the adjuvant only control group ($P = 0.013$) and the mean FEC of the mTci-APY-1 and Tci-MEP-1 recipients was lower, although not statistically significantly, than that of the adjuvant-only control group ($P = 0.093$). Mean cumulative faecal egg count levels were reduced by 43% in lambs immunised with mTci-APY-1 plus Tci-MEP-1 compared with the controls ($P = 0.079$).

1. Introduction

The development of recombinant subunit vaccines for the protection of small ruminants against parasitic nematodes has been, until recently, relatively unsuccessful (Matthews et al., 2016; Nisbet et al., 2016a; Stutzer et al., 2018). We developed a rational approach to selecting antigens for inclusion in a vaccine to control *Teladorsagia circumcincta*, the primary cause of parasitic gastroenteritis (PGE) in small ruminants in temperate regions worldwide (Nisbet et al., 2013). First, given previous understanding of protective immune mechanisms against this parasite (Smith et al., 1985, 1986, 1987; Stear et al., 2004), we selected four proteins by examining larval antigens that were targets of local IgA responses in sheep rendered immune to re-infection. Second, via in silico analysis of stage-specific transcripts of *T. circumcincta*, we selected an immunogenic homologue of a protective antigen of the canine hookworm, *Ancylostoma caninum* (see Nisbet et al., 2009). Finally, we identified three potentially immunosuppressive molecules released by intra-host larval stages (Nisbet et al., 2013). We combined these eight antigens in a cocktail to assess whether this vaccine would induce protection against parasite challenge when administered to 6–7 month old lambs in two separate trials. In these studies, this prototype reduced cumulative faecal egg output on average by 70% and 58%, respectively, over a 6 or 10 week period after challenge (Nisbet et al., 2013). During the period of peak worm egg shedding, vaccinated lambs shed 92% and 73% fewer

* Corresponding author. Fax: +44 1314456111.
E-mail address: Alasdair.Nisbet@moredun.ac.uk (A.J. Nisbet).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ijpara.2018.10.006
0020-7519/

eggs than did Quil A adjuvant-only recipient lambs, respectively. At post-mortem, vaccinates had 75% and 56% lower mean adult nematode burdens than adjuvant only controls, respectively (Nisbet et al., 2013). In a subsequent experiment in lambing ewes, which displayed a periparturient relaxation in immunity, vaccination with this eight-protein cocktail resulted in a 44% reduction in mean cumulative faecal egg count (cFEC) levels in recipient ewes compared with adjuvant only control animals (Nisbet et al., 2016b). The vaccine prototype used in these experiments comprised eight proteins expressed separately in *Escherichia coli* or *Pichia pastoris* systems. The complexity of the expression and purification steps made this vaccine unattractive for commercial exploitation, particularly considering current anthelmintic treatment options which, in most cases, are relatively inexpensive (Charlier et al., 2017). Here, we explored the previous trial data gathered over 7 years from two previously published (Trials 1 and 2) and three unpublished (Trials 3–5) trials using the eight-protein vaccine to inform a strategy to simplify this vaccine. Based on this analysis, two two-component prototypes were tested in a direct comparison with the original eight-protein vaccine (Trial 6). The simplification strategy was guided by relationships between antigen-specific antibody levels, avidity measurements and parasitological parameters of efficacy analysed for each of the eight proteins in the previous trials and this led to the testing of a vaccine comprising *T. circumcincta* apyrase-1 (Tci-APY-1) and *T. circumcincta* metalloproteinase-1 (Tci-MEP-1). We also tested Tci-MEP-1 in combination with a mutated version of the apyrase, abolishing its enzyme activity and potential immunomodulatory capacity through site directed mutagenesis (Dai et al., 2004), to ablate any possible negative effect on vaccine efficacy.

2. Materials and methods

2.1. Ethics statement

All experimental procedures described here were approved by the Moredun Research Institute Experiments and Ethics Committee, UK, and were conducted under the legislation of UK Home Office Project Licenses (references PPL 60/4238 and 70/8870) in accordance with the Animals (Scientific Procedures) Act of 1986.

2.2. Animals

Texel crossbred lambs, reared under conditions to exclude helminth infection (confirmed by FEC analysis), were grouped in separate pens in all trials. Ages and characteristics of the animals are shown in Table 1. In Trial 4, two different ages of lambs were used; 3 month old and 6 month old lambs. Here, all animals were derived from the same lambing, with the trial in 6 month old lambs (Trial 4b) performed 3 months after the trial comprising the 3 month old lambs (Trial 4a).

2.3. Recombinant protein production for vaccines

The eight-antigen prototype vaccine used in Trials 1–6 was produced and formulated exactly as described previously (Nisbet et al., 2013). To produce Tci-APY-1 and mTci-APY-1 for Trial 6, oligonucleotide primers were designed to exclude the first 42 nucleotide bases of the open reading frame (ORF, NCBI Accession number FR671368) encoding the putative signal peptide and the remaining ORF was amplified by PCR and sub-cloned into the expression vector pET-11a (Novagen USA). Site directed mutagenesis was used to replace the nucleotide-binding residue D^{118} with N^{118} for the production of mTci-APY-1. Expression vector pET-11a containing an insert encoding either Tci-APY-1 or mTci-APY-1 was used to transform *E. coli* BL21 (DE3) competent cells (Stratagene USA). Recombinant protein expression was induced in the presence of 1 mM isopropyl β-D-1-thiogalactopyranoside. Soluble Tci-APY-1 or mTci-APY-1 was purified from cell lysate by nickel column affinity chromatography using HisTrap™ HP columns (GE Healthcare UK). Recombinant Tci-APY-1 or mTci-APY-1 was eluted in 350 mM imidazole and dialysed overnight against Tris buffer (50 mM Tris, 0.5 M NaCl, pH 7.4) at 4 °C. Apyrase activity of Tci-APY-1 and lack of activity of mTci-APY-1 was confirmed by monitoring inorganic phosphate release from soluble nucleotides using the malachite green assay as described previously (Nisbet et al., 2011). Tci-MEP-1 for Trial 6 was produced as described previously (Nisbet et al., 2013).

Table 1
Characteristics of the animals and experimental groups in each of the trials described.

Trial	Average age of lambs at first vaccination (months)	Number of lambs in each experimental group	Vaccine(s) received
1	7	7	Group 1: 8-antigen vaccine Group 2: Adjuvant only
2	6	14[a]	Group 1: 8-antigen vaccine Group 2: Adjuvant only
3	5	12	Group 1: 8-antigen vaccine Group 2: Adjuvant only
4a	3	16[b]	Group 1: 8-antigen vaccine Group 2: Adjuvant only
4b	6	16[b]	Group 1: 8-antigen vaccine Group 2: Adjuvant only
5	6	12	Group 1: 8-antigen vaccine Group 2: Adjuvant only
6	6	10[b]	Group 1: 8-antigen vaccine Group 2: Adjuvant only Group 3: Tci-APY-1 plus Tci-MEP-1 Group 4: mTci-APY-1 plus Tci-MEP-1

[a] $n = 14$ until day 84 of experiment, seven thereafter.
[b] In the eight-antigen recipient groups in each trial, a single lamb was removed from the experiment due to unrelated health issues; thus, the number of replicates in Group 1 in these trials is one fewer than in the control groups.

2.4. Efficacy trials of experimental vaccines

Six vaccine trials are described here. The protocols and procedures for Trials 1 and 2 have been published (Nisbet et al., 2013) and these were repeated in Trials 3–5 (any small amendments to the protocol are detailed in Table 1). Briefly, 50 μg of each of the seven PBS-soluble proteins (Tci-ASP-1; Tci-MIF-1; Tci-TGH-2; Tci-APY-1; Tci-SAA-1; Tci-CF-1 and Tci-ES20) were administered to lambs as a mixture in a single injection with 5 mg of Quil A in PBS. Tci-MEP-1, which is insoluble in PBS, was formulated with 2 M urea in PBS plus 5 mg of Quil A and the preparations of seven soluble proteins or one insoluble protein (Tci-MEP-1) were injected separately, one immediately following the other, at two sites on the neck. Three immunisations were administered, 3 weeks apart (2 weeks apart in Trial 5 for logistical reasons). Lambs in all control groups received three immunisations with urea/PBS/10 mg of Quil A at the same time as the lambs immunised with the recombinant proteins. In Trial 6, as well as a group of lambs immunised with all eight recombinant proteins, two additional groups were immunised with the two proteins selected from the original cocktail; Tci-APY-1 plus Tci-MEP-1 in one group and mTci-APY-1 plus Tci-MEP-1 in a second group. Each recombinant protein was formulated as individual injections with 5 mg of Quil A and three immunisations administered 3 weeks apart as described above. In all trials, following the final immunisation, each lamb was administered orally with 2,000 *T. circumcincta* L3s, three times per week for 4 weeks. FEC analysis was performed three times per week in Trials 1–5 or two times per week in Trial 6 from 14 days after the start of the larval challenge period until the end of the experiment. cFEC values were estimated using the trapezoidal method for calculation (Taylor et al., 1997). The percentage reduction in cFEC afforded by vaccination was calculated as follows:

$$\%\,\text{cFEC reduction} = [1 - (\text{cFECv}/\text{mean cFECc})] \times 100$$

where cFECv = cFEC for an individual vaccinated animal in that trial and cFECc = mean cFEC for the control animals in the same trial.

Abomasal nematode burdens were classified and enumerated following standard techniques (Jackson et al., 1984).

2.5. Antibody level and avidity analyses

Blood samples were taken from every lamb prior to each immunisation and periodically from the third immunisation onwards. Abomasal swab samples were collected from every lamb at post-mortem. ELISA was performed to determine levels of antigen-specific IgA and IgG in sera and abomasal mucus as described previously (Nisbet et al., 2013, 2016b). Serum IgG, IgG1 and IgG2 avidity specific for each vaccine antigen was also assessed in all animals immunised with the eight-antigen prototype (Group 1 in each trial) in samples collected 2–3 weeks after the final (third) immunisation. Antibody avidity was determined in sera from each animal using a standard ELISA as described previously (Nisbet et al., 2013, 2016a,b), with an additional potassium thiocyanate elution step as described by Pullen et al. (1986). Briefly, after antigen/sera incubation, microtiter plates were washed six times in PBST (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1% v/v Tween20, pH 7.4), then duplicate wells for each animal incubated with 0, 0.25, 0.5, 1, 2, 3, 4 and 5 M potassium thiocyanate solution for 10 min at room temperature. Plates were washed a further six times with PBST, then residual antibody binding detected according to the standard ELISA protocol. The absorbance readings from wells with no potassium thiocyanate represented total antibody binding and absorbance readings in the presence of increasing concentrations of potassium thiocyanate were converted to a percentage of total binding. Data were fitted to a graph of $\log_{10}$ molar concentration of potassium thiocyanate versus normalised response by non-linear regression to estimate half maximal inhibitory concentration (IC_{50}) values.

2.6. Statistical analyses

A generalised additive mixed modelling (GAMM) approach was undertaken for analysis of longitudinal FEC data from each trial. The models were fitted by restricted maximum likelihood (REML) to log(FWEC + 1), considering an identify link function and Gaussian residuals, with treatment group (either vaccinated or control) as the fixed effect and animal identity as a random effect. Separate smoothing splines for each group were used to fit their longitudinal pattern, while allowing for heterogeneous variances between groups and a temporal autoregressive correlation structure. The longitudinal FEC data were summarised for graphical representation using the median as a statistical measure of central tendency which is robust to outlying observations. cFEC and worm burden data were analysed using negative binomial generalised linear models with a logarithmic link function accounting for data overdispersion as described previously (Nisbet et al., 2013). The data generated across trials were aggregated for a meta-analysis of cFEC and worm burden by integrating the random effects of the individual trials into an overall negative binomial generalised linear mixed model (NB GLMM) with treatment group as a fixed effect. For analysis of relationships between antibody avidity and reduction in cFEC, correlation analyses were performed using Spearman's rank-order correlation. The serum antibody level was modelled using linear mixed models (LMMs) fitted by REML to rank-based inverse normal transformed data, including treatment group and time point as fixed effects and animal identity as a random effect. Post-hoc pair-wise comparisons were conducted from the LMM results, with the corresponding *P* values adjusted for the false discovery rate. Post-mortem mucosal antibody levels were modelled by fitting a one-way ANOVA model to rank-based inverse normal transformed data. Statistical test significance was assessed at the usual 5% significance level.

3. Results

3.1. Guiding vaccine refinement – vaccine efficacy in Trials 1–5

In each trial in which lambs were immunised with the eight-antigen vaccine (Table 1), the median FEC of immunised lambs across the time course demonstrated repeatable, although variable, vaccine efficacy (Fig. 1). In 85% of lambs in Trials 1–5, immunisation resulted in a reduced mean cFEC compared with that of control lambs in the same trial (Fig. 2A). Variable efficacy was observed within each trial (Fig. 2B) and this variability, together with the antibody analyses, was a key factor in undertaking simplification of the vaccine. The meta-analysis testing for differences between the cFECs of vaccinated (eight-antigen vaccine) and control animals across the five vaccine trials demonstrated an overall statistically significant effect of vaccination on cFEC reduction ($P = 0.009$) and the meta-analysis testing for differences between these groups across the five vaccine trials demonstrated an overall statistically significant effect of vaccination on worm burden reduction ($P < 0.0001$).

3.2. Guiding vaccine refinement - antibody responses in Trials 1–5

Immunisation of lambs with the eight-antigen vaccine (Group 1 in all trials) resulted in increased antigen-specific serum IgG and IgA in each trial. This reflected the levels and profile of antibody responses to these antigens observed in the initial two trials (Nisbet et al., 2013). Trial 5 data are shown in Supplementary Fig. S1 as an example. In abomasal mucus of immunised lambs in each trial for which immunoglobulin levels were assessed (Trials 1, 2, 3 and 5), average levels of Tci-APY-1- and Tci-MEP-1-specific IgG and IgA were higher than for any other recombinant protein (Trial 5 data are shown in Supplementary Fig. S1 as an example).

Control group (Group 2 in all trials) lambs in Trials 1, 2, 3 and 5, which had received injections of adjuvant prior to parasite challenge, had increased Tci-APY-1- and Tci-MEP-1-specific IgG and IgA in their sera after challenge in each trial (Trial 5 data are shown in Supplementary Fig. S2 as an example), whereas no consistent increases in antigen-specific IgG in control lambs were observed for any other recombinant protein tested. In the abomasal mucus

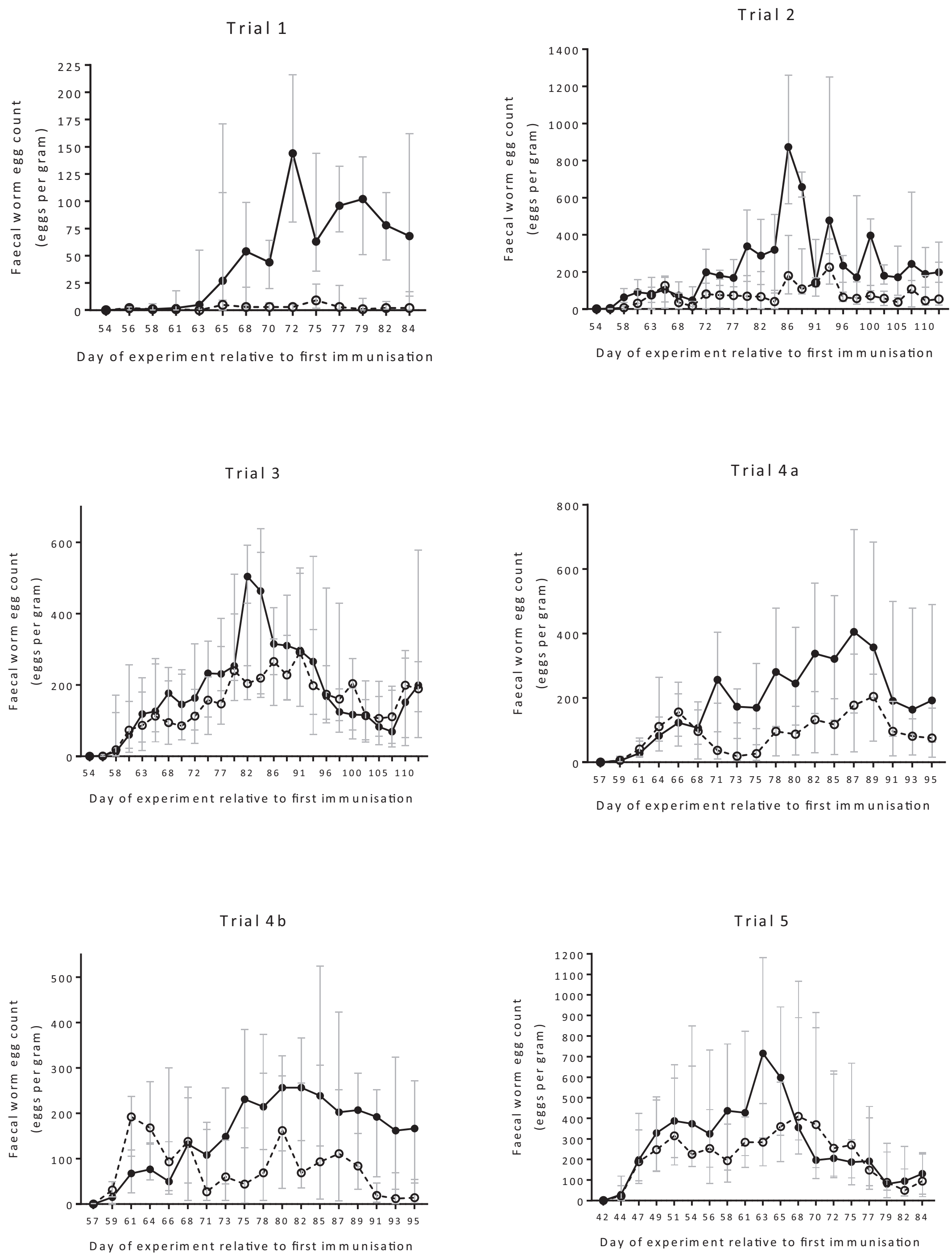

Fig. 1. Effects of immunisation of lambs with an eight-antigen cocktail from *Teladorsagia circumcincta* on faecal egg counts across the time course of infection after larval challenge. Each panel represents a separate trial and shows median faecal egg count (with interquartile range) for groups of lambs challenged with 2000 *T. circumcincta* three times per week for 4 weeks following immunisation with an eight-protein cocktail in the context of Quil A (dashed line, open circles) or with Quil A alone (solid line, closed circles). Each data point represents the median FEC derived from observations on 7, 14*, 12, 16, 16 and 12 lambs in Trials 1, 2, 3, 4a, 4b and 5 respectively. *In Trial 2, a cohort of seven lambs was euthanased at day 84, so from day 84 to day 112 each data point represents the median FEC of the remaining seven lambs.

of controls in each trial, average Tci-APY-1- and Tci-MEP-1-specific IgG and IgA levels were higher compared with levels of these antibodies against the other six recombinant proteins (Trial 5 data are shown in Supplementary Fig. S2 as an example). The two exceptions to this were: in Trial 3, levels of Tci-ES20-specific IgA were similar to those of Tci-APY-1-specific IgA and, in Trial 2, levels of Tci-SAA-1-specific IgG were similar to those of Tci-APY-1-specific IgG (data not shown).

Investigations of the relationship of antigen-specific antibody avidity with vaccine efficacy focussed on serum IgG, IgG1 and IgG2 in animals immunised with the eight-antigen vaccine (Group 1 in all trials) collected 2 weeks after the final immunisation in Trials 1–5. The analysis incorporated all lambs protected by immunisation, as determined by having a positive value for the percentage of reduction in cFEC (i.e. each animal represented by a point above the X axis in Fig. 2B). For total serum IgG, a statistically significant

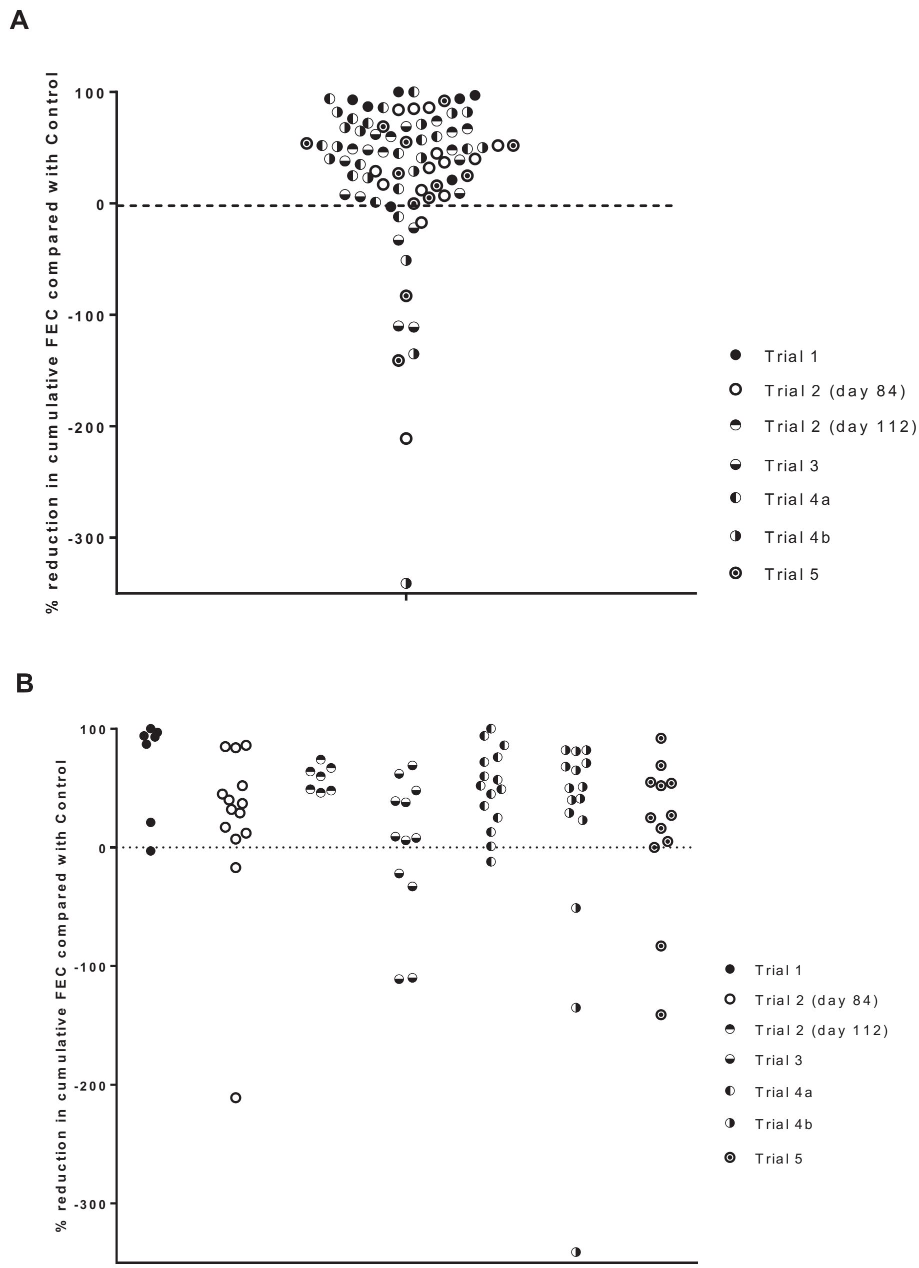

Fig. 2. Effect of an eight-antigen cocktail derived from *Teladorsagia circumcincta* on cumulative faecal egg count of lambs after challenge infection. To allow comparison across different trials, efficacy is presented as % reduction in cumulative faecal egg count, calculated as follows: % cFEC reduction = [1 − (cFECv/mean cFECc)] × 100 where cFECv = cFEC for an individual vaccinated animal in that trial and cFECc = mean cFEC for the control animals in the same trial. Each data point on each panel represents a single vaccinate. (A) All data from all trials plotted together. (B) Data from individual trials plotted separately.

positive correlation between the percentage of reduction in cFEC and antibody avidity was only observed for Tci-APY-1 ($r_s = 0.30$; $P = 0.019$). Subclass-specific analyses demonstrated statistically significant positive correlations between the percentage of reduction in cFEC and IgG1 ($r_s = 0.28$; $P = 0.030$), but not IgG2, avidity for Tci-APY-1 (Fig. 3). Based on these outcomes from the analysis of Trials 1–5, a trial (Trial 6) using a simplified prototype containing Tci-APY-1 and Tci-MEP-1 or mTci-APY-1 (i.e. the loss of function mutant for Tci-APY-1) and Tci-MEP-1 was performed (Table 1).

3.3. Efficacy of the simplified vaccine – Trial 6

Lambs in all groups began to excrete *T. circumcincta* eggs from 16-19 days after the start of challenge (Fig. 4A). At peak egg shedding (day 82), the mean FEC in the eight-antigen vaccine recipients (Group 1) was 135 ± 44 eggs per gram (EPG), and in the controls (Group 2), the mean FEC was 304 ± 75 EPG. At the same time point, the mean FEC in the two-antigen vaccine group (Group 3; Tci-APY-1 plus Tci-MEP-1) was 342 ± 90 EPG, whereas in lambs that received mTci-APY-1 and Tci-MEP-1 (Group 4), the mean FEC was 186 ± 45 EPG (Table 2). The GAMM analysis identified statistically significant differences in patterns of mean FEC between the groups over the time course of the experiment ($P = 0.044$); in particular, the mean FEC of the eight-antigen recipients (Group 1) was significantly lower than that of the control (adjuvant only) group ($P = 0.013$), and the difference between the mean FEC of the mTci-APY-1 and Tci-MEP-1 recipients (Group 4) and the controls approached statistical significance ($P = 0.093$). Mean cumulative FEC levels (Fig. 4B; Table 2) were reduced by 43% in Group 4

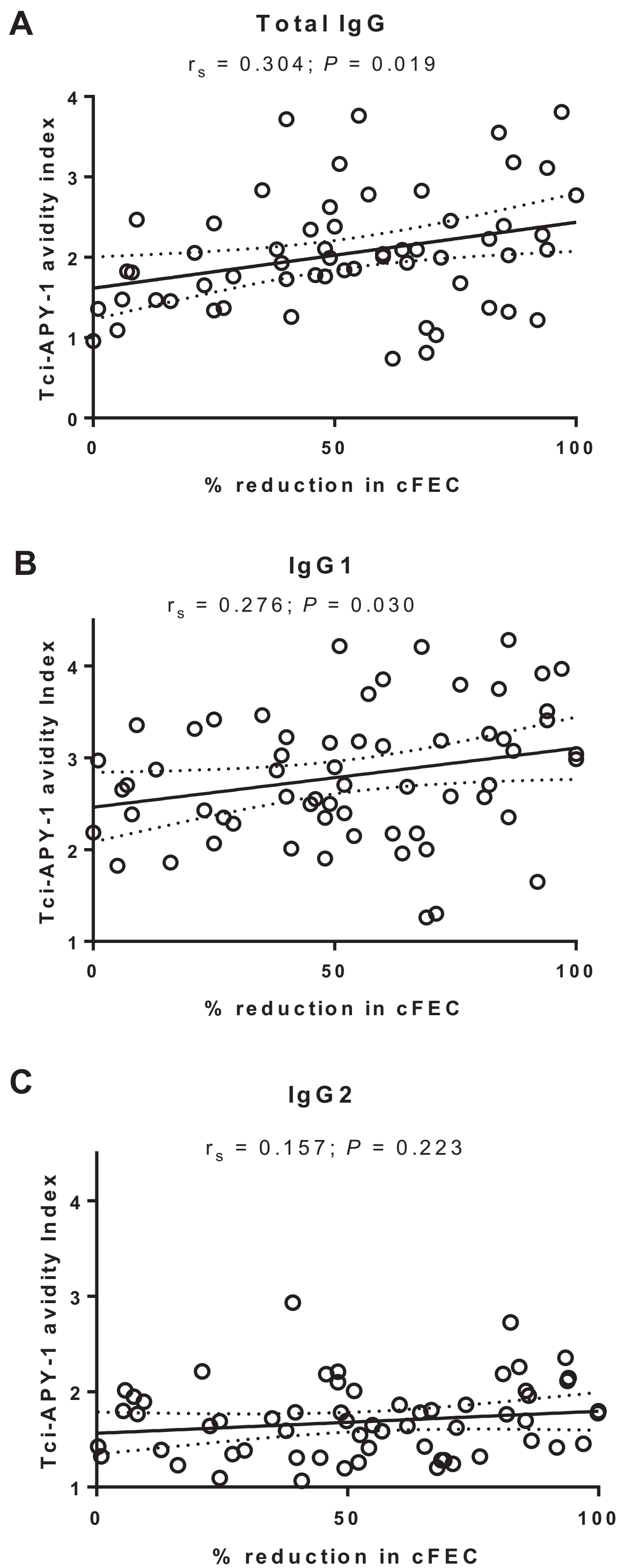

Fig. 3. The relationship between antibody avidity and efficacy of an eight-antigen cocktail derived from *Teladorsagia circumcincta* on lamb cumulative faecal egg counts after challenge infection. Avidity of the interaction between total IgG (A), IgG1 (B) and IgG2 (C) and the antigen *T. circumcincta* apyrase-1 (Tci-APY-1) was expressed as a half maximal inhibitory concentration (IC50) value, defined as the concentration of potassium thiocyanate required to reduce the maximal response (in the absence of potassium thiocyanate) by 50%, and plotted against efficacy (presented as % reduction in cumulative faecal egg count). The 95% confidence bands of the best fit line are shown and each graph is annotated with the estimated correlation (r_s) between the biological parameters together with the associated *P* value.

(mTci-APY-1 plus Tci-MEP-1) compared with the controls ($P = 0.079$), but only by 3% in lambs immunised with Tci-APY-1 and Tci-MEP-1 (Group 3).

Abomasal nematode burdens at post-mortem are shown in Fig. 5. The mean worm count in each of the groups of immunised lambs was lower than that of the controls (34–52% average reduction in worm count). Worm burdens in the immunised groups in Trial 6 were not statistically significant in comparison with each other, or with those of the control group. When the data from lambs immunised with the eight-antigen vaccine in Trial 6 were incorporated with those from all previous trials and the meta-analyses testing for differences between the cFECs and worm burdens of vaccinated and control animals across all six vaccine trials was re-performed, overall statistically significant effects of vaccination ($P = 0.006$) on cFEC reduction and worm burden reduction ($P < 0.0001$) were observed.

Antigen-specific serum and mucosal antibody levels were similar to those observed in the previous trials (Supplementary Figs. S3 and S4). Serum levels of Tci-APY-1-specific IgG were statistically significantly ($P = 0.002$) elevated in sera taken 3 weeks after the final (third) immunisation) in lambs immunised with mTci-APY-1 plus Tci-MEP-1 compared with the other immunised and control groups (Supplementary Fig. S3C). At post-mortem, levels of Tci-APY-1-specific mucosal IgG were statistically significantly ($P = 0.026$) elevated in lambs immunised with all eight antigens compared with those immunised with Tci-APY-1 plus Tci-MEP-1 (Supplementary Fig. S4A). There were no other statistically significant differences between levels of serum or mucosal IgG and IgA against Tci-APY-1 or Tci-MEP-1 or IgG avidity for Tci-APY-1 in any of the groups of vaccinates in this trial.

4. Discussion

We demonstrated repeated efficacy of a prototype recombinant vaccine for *T. circumcincta*; however, notable variability between trials and between animals within trials was observed. We exploited this variability between and within trials as a tool to target the most effective antigens in the cocktail to simplify the vaccine.

The relationship between the percentage of reduction in cFEC and IgG avidity in vaccinated sheep for one antigen, Tci-APY-1, and its increased recognition by IgG and IgA in control sheep following a trickle challenge, indicated that this antigen should be considered for inclusion in a simplified prototype. In previous studies (Nisbet et al., 2013, 2016b), we used recombinant Tci-APY-1 as an active enzyme in the vaccine cocktail and the recombinant Tci-APY-1 efficiently hydrolysed the purinergic nucleotide ATP in vitro to give an indication that it retained some of the tertiary structure of native apyrase. However, a recent publication (Vono et al., 2013) addressing adjuvancy in vaccine preparations demonstrated that transient ATP release at the injection site is required for optimal efficacy of some adjuvants and that co-injection of mice with an adjuvanted influenza vaccine plus active apyrase led to inhibition of innate and adaptive responses to the vaccine compared with injection in the absence of apyrase (Vono et al., 2013). For this reason, in Trial 6, a vaccine was formulated with mTci-APY-1 using a mutagenesis strategy previously used with an apyrase from humans to produce a loss-of-function mutant enzyme with folding properties identical to the wild type version (Dai et al., 2004). This approach, of using inactive versions of recombinant parasite-derived immunomodulators, has been shown to enhance efficacy of a recombinant vaccine composed of two immunomodulatory molecules (abundant larval transcript-1 (LsALT) and cysteine proteinase inhibitor-2 (LsCPI)) when used in mice against the filarial nematode, *Litomosoides sigmodontis*, with increased efficacy in

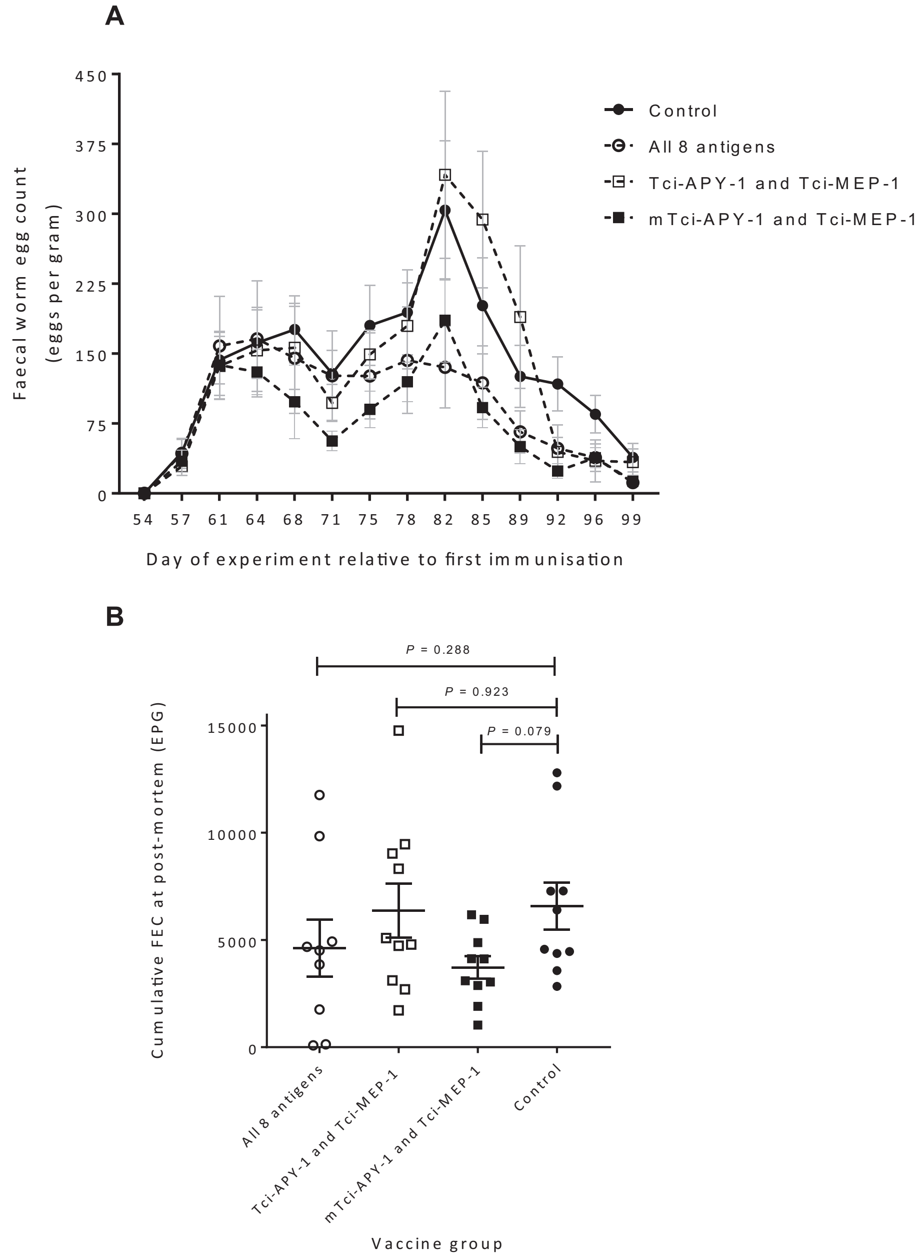

Fig. 4. Effects of the eight- and two-protein recombinant vaccines against *Teladorsagia circumcincta* on lamb faecal egg counts during and after challenge infection in Trial 6. The mean values for faecal egg counts over the period of the experiment are depicted in A (±S.E.M.) whereas in B, the cumulative faecal egg counts for each lamb, calculated at the end of the experiment, are shown, together with the mean ± S.E.M. In each group $n = 10$, with the exception of the "All 8 antigens" group where $n = 9$.

Table 2
The effect of the eight- and two-protein recombinant vaccines against *Teladorsagia circumcincta* on faecal egg counts during challenge infection and worm burdens measured at the end of the experiment.

Group	Peak egg output (FEC on Day 82, eggs per gram)[a]	% Reduction in peak egg output relative to control	Cumulative FEC at post mortem (eggs per gram)[a]	% Reduction in cumulative FEC relative to control
Group 1: 8-antigen vaccine	135 ± 44	66%	4616 ± 1332 ($P = 0.288$)[b]	30%
Group 2: Control, adjuvant only	304 ± 75	–	6573 ± 1093	–
Group 3: Tci-APY-1 plus Tci-MEP-1	342 ± 90	−12.5%	6371 ± 1264 ($P = 0.923$)	3%
Group 4: mTci-APY-1 plus Tci-MEP-1	186 ± 45	39%	3721 ± 526 ($P = 0.079$)	43%

[a] Mean ± S.E.M., $n = 10$ for each group with the exception of Group 1 where $n = 9$.
[b] Compared with Group 2 (Control, adjuvant only).

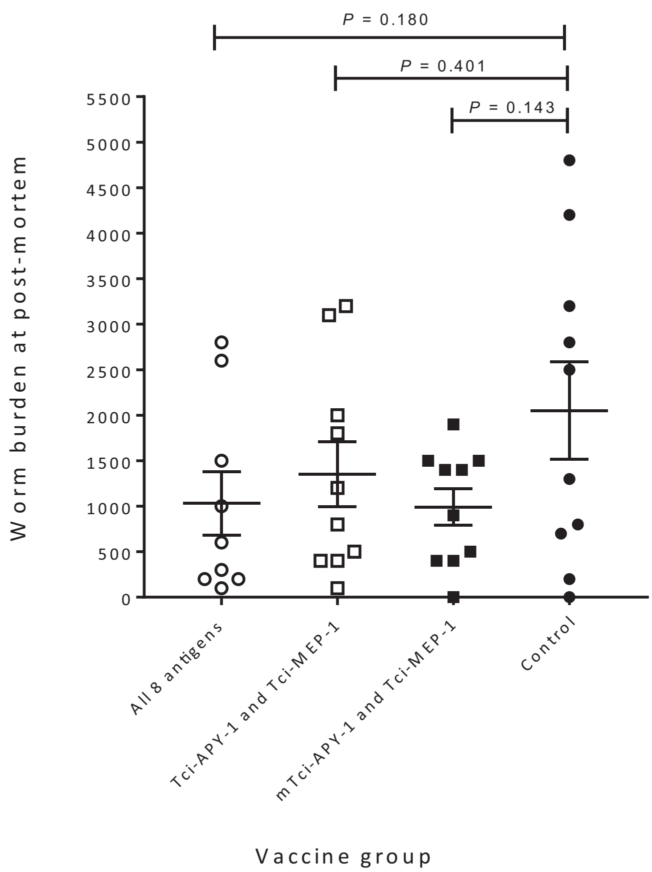

Fig. 5. Effect of the eight- and two-protein recombinant vaccines against *Teladorsagia circumcincta* on lamb abomasal worm burden after larval challenge in Trial 6. The nematode counts for each lamb are shown, together with the mean ± S.E.M. In each group *n* = 10, with the exception of the "All 8 antigens" group where *n* = 9.

mice immunised with functionally-inactivated LsALT and LcCPI being associated with enhancement of Th-2 responses (production of parasite-specific IgG1) and enhanced parasite antigen presentation by dendritic cells (Babayan et al., 2012). In Trial 6 in the work described here, lambs immunised with mTci-APY-1 and Tci-MEP-1 (Group 4) demonstrated a substantially lower mean FEC at peak egg shedding and a lower cFEC throughout the trial than those immunised with the non-mutated Tci-APY-1 and Tci-MEP-1 (Group 3) or the eight-antigen vaccine, suggesting an enhanced effect of the vaccine when the apyrase was non-functional. Mean antigen-specific levels of serum IgG against Tci-APY-1 were also higher in Group 4 lambs 2 weeks after final immunisation than in Group 3 lambs (Supplementary Fig. S3C), although cellular responses were not recorded.

In Trial 6, Group 4 lambs exhibited reductions in both mean FEC and worm burden, whereas Group 3 animals exhibited only reductions in mean worm burden. The relationship between *T. circumcincta* burden and FEC is not straightforward or linear (Stear and Bishop, 1999) and is it has been demonstrated that there is a strong density-dependent effect of parasite numbers on worm fecundity (Bishop and Stear, 2000). The relationship between FEC and worm burden is further complicated in experiments such as the ones described here where FEC is recorded longitudinally at various time points across the trial, whereas worm burden is a static measurement estimated post-mortem only. However, if the desired outcome of vaccination is to prevent parasite transmission through reduced pasture contamination, the impact of the vaccine on worm egg shedding must be considered as the most important parameter. The eight-antigen vaccine and the mTci-APY-1-containing vaccine here were able to suppress FEC but, as with most other vaccines against parasitic nematodes (reviewed in Charlier et al., 2017), levels of suppression required for the vaccine to have a significant impact on lamb production have not yet been established. The threshold for protection will be dependent on many aspects of the management system including husbandry, climate, and how the development of natural immunity is impacted by reduced exposure levels (Singleton et al., 2011); we are currently acquiring baseline field data in a range of management systems to inform models for determining the required vaccine efficacy.

The mTci-APY-1 plus Tci-MEP-1 vaccine reduced both cFEC and worm burden, with the caveat that inherent variability in FEC and worm burden, even with accurately measured doses of infective larvae, makes statistical interpretation of the data challenging (Stear and Bishop, 1999). The meta-analyses performed here clearly illustrate this variability. In the NB GLMM analyses of the effects of vaccination on cFEC across all trials, predicted mean cFECs are attributed to both the control and vaccinated groups as part of the analytical process and the S.E.M. for both groups is high, representing ±27% of the mean values. The reason for variability between and within trials is under investigation using a variety of analytical, modelling and empirical approaches, but FEC is clearly affected by the establishment rate of the infection and is, thereafter, regulated by a number of host-specific factors (McRae et al., 2015). The key factors identified by Gaba et al. (2006) as being associated with establishment rate in naïve lambs were host breed and age, infective dose, method of dose administration and the number of repeated infective doses. Of these factors, those relating to administration of infective larvae were standardised across trials here and host breed was as similar as possible when using a commercial cross-breed in trials spanning 7 years. With the exception of Trial 4a, where 3 month old lambs were used, the age of the lambs was similar across trials, but variability in FEC in both the control and vaccinated groups (see Fig. 2B) was high irrespective of lamb age. The sex ratios in the groups of lambs may contribute to variation in FEC, with castrated 1–5 month old male blackface lambs shown previously to have significantly higher worm egg counts than female lambs of the same age (Abuargob and Stear, 2014). For this reason, all groups were balanced in each trial for numbers of male and female lambs, although there was little indication in these trials of differences in FEC between male and female Texel cross lambs (data not shown).

Overall, in Trial 6, the dual antigen prototype vaccine containing mTci-APY-1 and Tci-MEP-1 performed as well as, if not better than, the proven eight-antigen vaccine in terms of reducing mean cFEC and worm burden, suggesting that further exploitation of this combination of antigens may yield an effective, simplified vaccine. The required efficacy of such a vaccine will be examined through further experimental and modelling studies.

Acknowledgements

The authors gratefully acknowledge funding for this project from Scottish Government Rural & Environment Science & Analytical Services (RESAS), from the Biotechnology and Biological Sciences Research Council, UK, award number BB/L027186/1 and from the European Union's Horizon 2020 Research and Innovation Programme (Grant Number: 635408). We would also like to thank Leigh Andrews, Alison Morrison and Dave Bartley, Moredun Research Institute, UK, for their help and in the provision of parasite material and the Bioservices Unit, Moredun Research Institute, for expert care of the animals.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2018.10.006.

References

Abuargob, O., Stear, M.J., 2014. Differences between female and castrated male lambs in susceptibility to natural, predominantly *Teladorsagia circumcincta* infection. Vet. Parasitol. 205, 588–594.

Babayan, S.A., Luo, H., Gray, N., Taylor, D.W., Allen, J.E., 2012. Deletion of parasite immune modulatory sequences combined with immune activating signals enhances vaccine mediated protection against filarial nematodes. PLoS Negl. Trop. Dis. 6, e1968.

Bishop, S.C., Stear, M.J., 2000. The use of a gamma-type function to assess the relationship between the number of adult *Teladorsagia circumcincta* and total egg output. Parasitology 121, 435–440.

Charlier, J., Thamsborg, S.M., Bartley, D.J., Skuce, P.J., Kenyon, F., Geurden, T., Hoste, H., Williams, A.R., Sotiraki, S., Höglund, J., Chartier, C., Geldhof, P., van Dijk, J., Rinaldi, L., Morgan, E.R., von Samson-Himmelstjerna, G., Vercruysse, J., Claerebout, E., 2017. Mind the gaps in research on the control of gastrointestinal nematodes of farmed ruminants and pigs. Transbound. Emerg. Dis. https://doi.org/10.1111/tbed.12707.

Dai, J., Liu, J., Deng, Y., Smith, T.M., Lu, M., 2004. Structure and protein design of a human platelet function inhibitor. Cell 116, 649–659.

Gaba, S., Gruner, L., Cabaret, J., 2006. The establishment rate of a sheep nematode: revisiting classics using a meta-analysis of 87 experiments. Vet. Parasitol. 140, 302–311.

Jackson, E., Jackson, F., Smith, W.D., 1984. Comparison of saline incubation and pepsin digestion as methods for recovering *Ostertagia circumcincta* larvae from the abomasum of sheep. Res. Vet. Sci. 36, 380–381.

Matthews, J.B., Geldhof, P., Tzelos, T., Claerebout, E., 2016. Progress in the development of subunit vaccines for gastrointestinal nematodes of ruminants. Parasite Immunol. 38, 744–753.

McRae, K.M., Stear, M.J., Good, B., Keane, O.M., 2015. The host immune response to gastrointestinal nematode infection in sheep. Parasite Immunol. 37, 605–613.

Nisbet, A.J., Knox, D.P., McNair, C.M., Meikle, L.I., Smith, S.K., Wildblood, L.A., Matthews, J.B., 2009. Immune recognition of the surface associated antigen, Tc-SAA-1, from infective larvae of *Teladorsagia circumcincta*. Parasite Immunol. 31, 32–40.

Nisbet, A.J., Zarlenga, D.S., Knox, D.P., Meikle, L.I., Wildblood, L.A., Matthews, J.B., 2011. A calcium-activated apyrase from *Teladorsagia circumcincta*: an excretory/secretory antigen capable of modulating host immune responses? Parasite Immunol. 33, 236–243.

Nisbet, A.J., McNeilly, T.N., Wildblood, L.A., Morrison, A.A., Bartley, D.J., Bartley, Y., Longhi, C., McKendrick, I.J., Palarea-Albaladejo, J., Matthews, J.B., 2013. Successful immunization against a parasitic nematode by vaccination with recombinant proteins. Vaccine 31, 4017–4023.

Nisbet, A.J., McNeilly, T.N., Greer, A.W., Bartley, Y., Oliver, E.M., Smith, S., Palarea-Albaladejo, J., Matthews, J.B., 2016b. Protection of ewes against *Teladorsagia circumcincta* infection in the periparturient period by vaccination with recombinant antigens. Vet. Parasitol. 228, 130–136.

Nisbet, A.J., Meeusen, E.N., González, J.F., Piedrafita, D.M., 2016a. Immunity to *Haemonchus contortus* and vaccine development. Adv. Parasitol. 93, 353–396.

Pullen, G.R., Fitzgerald, M.G., Hosking, C.S., 1986. Antibody avidity determination by ELISA using thiocyanate elution. J. Immunol. Methods 86, 83–87.

Singleton, D.R., Stear, M.J., Matthews, L., 2011. A mechanistic model of developing immunity to *Teladorsagia circumcincta* infection in lambs. Parasitology 138, 322–332.

Smith, W.D., Jackson, F., Jackson, E., Williams, J., 1985. Age immunity to *Ostertagia circumcincta*: comparison of the local immune responses of 4 1/2- and 10-month-old lambs. J. Comp. Pathol. 95, 235–245.

Smith, W.D., Jackson, F., Jackson, E., Graham, R., Williams, J., 1986. Transfer of immunity to *Ostertagia circumcincta* and IgA memory between identical sheep by lymphocytes collected from gastric lymph. Res. Vet. Sci. 41, 300–306.

Smith, W.D., Jackson, F., Graham, R., Jackson, E., Williams, J., 1987. Mucosal IgA production and lymph cell traffic following prolonged low level infections of *Ostertagia circumcincta* in sheep. Res. Vet. Sci. 43, 320–326.

Stear, M.J., Bishop, S.C., 1999. The curvilinear relationship between worm length and fecundity of *Teladorsagia circumcincta*. Int. J. Parasitol. 29, 777–780.

Stear, M.J., Bairden, K., Innocent, G.T., Mitchell, S., Strain, S., 2004. The relationship between IgA activity against 4th-stage larvae and density-dependent effects on the number of 4th-stage larvae of *Teladorsagia circumcincta* in naturally infected sheep. Parasitology 129, 363–369.

Stutzer, C., Richards, S.A., Ferreira, M., Baron, S., Maritz-Olivier, C., 2018. Metazoan parasite vaccines: Present status and future prospects. Front. Cell Infect. Microbiol. 8, 67.

Taylor, S.M., Kenny, J., Edgar, H.W., Ellison, S., Ferguson, L., 1997. Efficacy of moxidectin, ivermectin and albendazole oral drenches for suppression of periparturient rise in ewe worm egg output and reduction of anthelmintic treatment for lambs. Vet. Rec. 141, 357–360.

Vono, M., Taccone, M., Caccin, P., Gallotta, M., Donvito, G., Falzoni, S., Palmieri, E., Pallaoro, M., Rappuoli, R., Di Virgilio, F., De Gregorio, E., Montecucco, C., Seubert, A., 2013. The adjuvant MF59 induces ATP release from muscle that potentiates response to vaccination. Proc. Natl. Acad. Sci. USA 110, 21095–21100.