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Successful immunization against a parasitic nematode by vaccination with recombinant proteins☆

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ABSTRACT

Infection of humans and livestock with parasitic nematodes can have devastating effects on health and production, affecting food security in both developed and developing regions. Despite decades of research, the development of recombinant sub-unit vaccines against these pathogens has been largely unsuccessful. We have developed a strategy to identify protective antigens from *Teladorsagia circumcincta*, the major pathogen causing parasitic gastroenteritis in small ruminants in temperate regions, by studying IgA responses directed at proteins specific to post-infective larvae. Antigens were also selected on the basis of their potential immunomodulatory role at the host/parasite interface. Recombinant versions of eight molecules identified by immunoproteomics, homology with vaccine candidates in other nematodes and/or with potential immunoregulatory activities, were therefore administered to sheep in a single vaccine formulation. The vaccine was administered three times with Quil A adjuvant and the animals subsequently subjected to a repeated challenge infection designed to mimic field conditions. Levels of protection in the vaccinates were compared to those obtained in sheep administered with Quil A alone. The trial was performed on two occasions. In both trials, vaccinates had significantly lower mean fecal worm egg counts (FWECs) over the sampling period, with a mean reduction in egg output of 70% (Trial 1) and 58% (Trial 2). During the period of peak worm egg shedding, vaccinates shed 92% and 73% fewer eggs than did controls in Trials 1 and 2, respectively. At post mortem, vaccinates had 75% (Trial 1) and 56% (Trial 2) lower adult nematode burdens than the controls. These levels of protection are the highest observed in any system using a nematode recombinant sub-unit vaccine in the definitive ruminant host and indicate that control of parasitic helminths via vaccination with recombinant subunit vaccine cocktails is indeed an alternative option in the face of multi-drug resistance.

1. Introduction

Infection of livestock and humans with parasitic nematodes has devastating effects on health and production, affecting food security worldwide [1,2]. Despite decades of research, the development of recombinant vaccines against these pathogens has been unsuccessful [3,4]. The primary cause of parasitic gastroenteritis (PGE) in small ruminants in temperate regions worldwide is the nematode *Teladorsagia circumcincta*. Infection is acquired via ingestion of third stage larvae (L3) from pasture. Thereafter, immature (xL3 and L4) and adult worms reside in the host's abomasum causing substantial production losses [5]. Currently, *T. circumcincta* is controlled using anthelmintics; however, multi-drug resistance is widespread [6,7] and development of a vaccine is now a priority. Protective immunity against *T. circumcincta* develops after prolonged continual exposure [8] and the degree of immunity acquired depends on the level of parasite challenge, animal age and genotype [9]. Immunity is associated with decreased establishment and development rate of nematode larvae in the abomasal glands and reduced egg output from adults [8,10–13]. The mechanisms responsible for these effects are complex: immediate hypersensitivity reactions, cellular effectors and humoral responses have all been indicated [12,14–22]. The successful adoptive transfer of immunity, using gastric lymph of previously-infected lambs, to

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Table 1
Recombinant proteins used in *Teladorsagia circumcincta* vaccine trial.

Name	Accession number	Function[a]	Expression system/solubility	Reference
Tci-SAA-1	CAQ43040	L3-enriched surface associated antigen	pET22b(+) *E. coli* BL21 (DE3)-RIL Soluble	[26]
Tci-MIF-1	CBI68362	L3-enriched macrophage migration inhibitory factor	pET22b(+) *E. coli* BL21 (DE3)-RIL Soluble	[29]
Tci-ASP-1	CBJ15404	L4-enriched activation-associated secretory protein	pET SUMO *E. coli* BL21 (DE3)-RIL Soluble	[31]
Tci-TGH-2	ACR27078	Transforming growth protein 2-like protein	pET SUMO *E. coli* BL21 (DE3)-RIL Soluble	[28]
Tci-CF-1	ABA01328[b]	L4-enriched Secreted cathepsin F	pPICZαC *Pichia pastoris* X33 strain Soluble	[23]
Tci-ES20	HF677587	Excretory/secretory (ES) protein	pPICZαC *Pichia pastoris* X33 strain Soluble	[21]
Tci-MEP-1	HF677586	Astacin-like ES metalloproteinase	pET SUMO *E. coli* BL21 (DE3)-RIL Insoluble	[21]
Tci-APY-1	CBW38507	L4-enriched ES calcium-activated apyrase	pSUMO *E. coli* BL21 (DE3)-RIL Soluble	[30]

[a] Putative or inferred function.
[b] Tci-CF-1 is highly polymorphic, the clone used for vaccine production had the following amino acid substitutions compared to published sequence. In each case the amino acid in the published sequence is in italics, that in the vaccine isoform sequence is in normal font and the amino acid position in the published sequence is in subscript: $I_{44} \Rightarrow T_{44}$, $N_{101} \Rightarrow D_{101}$, $T_{129} \Rightarrow A_{129}$, $R_{137} \Rightarrow Q_{137}$, $R_{305} \Rightarrow K_{305}$, $L_{306} \Rightarrow P_{306}$, $S_{307} \Rightarrow Y_{307}$.

helminth-free monozygotic twins highlighted the importance of local immune responses in protection against re-infection [12] and larval antigen-specific IgA in gastric secretions has been implicated as playing a crucial role [12,14–16,19,21].

We developed a tripartite approach to identifying antigens for a *T. circumcincta* vaccine. First, we selected proteins [cathepsin F-1 (Tci-CF-1), astacin-like metalloproteinase-1 (Tci-MEP-1), a 20 kDa protein of unknown function (Tci-ES20) and activation-associated secretory protein-1 (Tci-ASP-1)] [21,23,31] by examining larval antigens that are targets of IgA in immune sheep [21,23], focusing on proteins excreted or secreted during the critical phase of worm growth and development [24]. We also selected Tci-SAA-1, an immunogenic homologue of a protective antigen from canine hookworm, *Ancylostoma caninum* (*Ac*-SAA-1 [25,26]) by bioinformatic analysis of *T. circumcincta* transcripts [26,27]. Finally, we identified potentially immunosuppressive molecules released by the larvae [macrophage migration inhibitory factor-1 (Tci-MIF-1), calcium-dependent apyrase-1 (Tci-APY-1) and a TGFβ homologue (Tci-TGH-2)] [28–30]. Here, we combined these antigens into a vaccine which could, in theory, nullify the immunomodulatory functions of parasite-derived molecules and concurrently allow enhanced immune responses against the immunostimulatory components. We tested the vaccine in two independent trials in which sheep were administered a sustained larval challenge after vaccination.

2. Materials and methods

2.1. Recombinant protein production

Eight recombinant proteins (Table 1) were produced. Production of recombinant Tci-SAA-1, Tci-MIF-1 and Tci-APY-1 has been described previously [26,29,30]. Tci-MEP-1 cDNA was amplified from *T. circumcincta* RNA (L4, prepared as in [27]) using the SMART™ RACE kit (Clontech) and sub-cloned into pET SUMO (Invitrogen) (omitting bases 1–48 encoding the signal peptide). The coding sequences (CDS) of *Tci-tgh-2* and *Tci-asp-1* (omitting signal peptide bases [31]) were each sub-cloned into pET SUMO (Invitrogen). Expression of recombinant proteins (Tci-SAA-1, Tci-MIF-1, Tci-APY-1, Tci-MEP-1, Tci-TGH-2 and Tci-ASP-1) in *Escherichia coli* BL21-CodonPlus® (DE3)-RIL competent cells (Stratagene) was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The CDS of *Tci-cf-1*, (omitting signal peptide bases 1–42) and the CDS of *Tci-ES20* (PCR-amplified from a *T. circumcincta* L4 SMART™ cDNA library), were each sub-cloned into pPICZαC (Invitrogen) and used to transform *P. pastoris* [X-33 (Mut$^+$) (Invitrogen)]. Tci-CF-1 and Tci-ES20 protein expression was induced with 0.5% methanol [32]. Soluble recombinant proteins were purified from cell lysates (*E. coli*) or culture supernatant (*P. pastoris*) using HisTrap™ HP columns (GE Healthcare), then dialysed against 20 mM phosphate buffer, 0.5 M NaCl, pH 7.6. Insoluble recombinant Tci-MEP-1 was purified via nickel column affinity chromatography in the presence of 8 M urea, then dialysed against 2 M urea in 20 mM phosphate buffer, 0.5 M NaCl, pH 7.6. Protein concentrations were determined using the Pierce BCA™ (bicinchoninic acid) assay (Thermo Scientific) with bovine serum albumin (BSA) standards and the integrity of each recombinant protein monitored via SDS-PAGE.

2.2. Immunization experiments

2.2.1. Trial 1

Fourteen Texel crossbred lambs, 204–206 days old, reared under conditions to exclude helminth infection (confirmed by fecal worm egg counts (FWECs), [33]), were housed in two groups of 7 animals in separate pens. Sheep in Group 1 were injected subcutaneously with 400 μg recombinant protein mix (incorporating 50 μg each of Tci-ASP-1; Tci-MIF-1; Tci-TGH-2; Tci-APY-1; Tci-SAA-1; Tci-CF-1; Tci-ES20; Tci-MEP-1) plus 10 mg Quil A (Brenntag Biosector). PBS-soluble proteins were administered as a mixture in a single injection with 5 mg Quil A in PBS. Tci-MEP-1 was formulated with 2 M urea in PBS plus 5 mg Quil A. The preparations were injected separately at two sites on the neck. Three immunizations were administered, 3 weeks apart. Sheep in Group 2 received three immunizations with urea/PBS/10 mg Quil A, at the same time as Group 1. Following the final immunization each sheep was administered orally with 2000 *T. circumcincta* L3, three times per week for 4 weeks. FWECs were performed three times per week from 14 days after the start of the L3 challenge period until the end of the experiment. Cumulative FWEC values were estimated using the trapezoidal method for calculation [34]). Abomasal nematode burdens were classified and enumerated following standard techniques [20,24,35]. Blood samples were taken prior to each immunization and weekly from the third immunization. Abomasal swab samples were collected at post-mortem [21] to determine levels of antigen-specific IgA and IgG in abomasal mucus.

2.2.2. Trial 2

Twenty-eight Texel crossbred lambs, 172–178 days old, were raised as described for Trial 1 and housed in four groups of 7 animals. Groups 1 and 3 were immunized with recombinant protein mix as described for Trial 1. Sheep in Groups 2 and 4 received immunizations with urea/PBS/Quil A, at the same time as Groups 1 and 3. At the final immunization, oral L3 challenge commenced in all Groups for 4 weeks. All biological samples were obtained as described above. Sheep in Groups 1 and 2 were euthanized 7 weeks after the start of the infection period and those in Groups 3 and 4 were euthanized 4 weeks later. Trials were performed under the regulations of a UK Home Office Project Licence; experimental design was ratified by MRI's Experiments and Ethics Committee.

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2.3. Measurement of antibody responses

Recombinant antigen-specific IgG and IgA levels in serum and abomasal mucus from each animal were assessed by ELISA as described previously [31]. In addition, the binding of serum IgG to native *T. circumcincta* antigens was also assessed as follows: Native antigens (L4 ES products, prepared from ex vivo L4 nematodes maintained in culture for 48 h, as described in [21]) were used to coat ELISA plates and antigen-specific IgG levels were assessed in sera from all animals in Trial 1 and from four, randomly selected, animals from Groups 1 and 2 of Trial 2. Each sample was assayed in triplicate. OD values were corrected against a reagent blank and all test plates had a positive and negative serum control to account for plate to plate variation.

2.4. Immunoblotting of nematode somatic extracts

Somatic extracts of *T. circumcincta* L3, L4 and adult worms [26], along with L4 ES products, [21], were subjected to immunoblotting to determine serum IgG and IgA binding to components of each extract, as described previously [26], using pools of sera from 7 immunized (Group 3) and 7 control sheep (Group 4) collected on the date of the third (final) immunization before infection.

2.5. Statistical analysis

For longitudinal FWEC analysis, a generalised additive mixed model (GAMM) model on log(FWEC + 1) was specified (Gaussian error structure and identity link function, group as fixed effect, animal effects as random). The model included separate smoothing curves for the nonlinear relationship of the response with time by group, non-homogenous within-group variances were allowed. A first order autoregressive residual correlation structure was incorporated. Antibody responses were modeled using linear mixed models (LMMs) (group as fixed effect, animal as random effect). For serum antibody data, repeated measures over time were modeled by random intercept and slope LMMs, including time and its interaction with group as fixed effect. Heterogeneous within-group variances were allowed. Linear contrasts compared subsets of responses in abomasal mucus.

In Trial 2, the 28 animals were housed in 4 separate groups of 7 animals. No statistically significant pen effects were found between Groups 1 and 3 or Groups 2 and 4, so Groups 1 and 3 were combined and Groups 2 and 4 were combined for data modeling. For analyses of worm burden data, generalised linear models (GLMs) were used. Data overdispersion was generally accounted for by specifying a negative binomial error distribution. Where necessary, overdispersion was incorporated using Poisson GLMs correcting the standard errors by specifying the mean and variance relationship.

Model selection was based on the Akaike's information criterion (AIC) and likelihood ratio tests (LRT); optimal mixed models were fitted by residual maximum likelihood (REML). Comparisons between groups were made by evaluating marginal statistical significances of the associated fixed effect by conditional *t*-tests. Cook's distance with a $4/n$ cut-off was used to support decisions for outliers. Statistical significance was assessed at the 5% level. Statistical analyses were conducted using R version 2.13.

3. Results

3.1. FWEC analysis

In Trial 1, sheep in Groups 1 (immunized) and 2 (control, adjuvant only) began to excrete *T. circumcincta* eggs in feces from 16 to 19 days after the start of challenge (Fig. 1A). At the final time point, Group 1 sheep were producing a mean (±SEM) of 8.7 (±5.5) eggs per gram (EPG) of feces, whereas sheep in Group 2 were producing 107.6 (±50.8) EPG, representing a 92% reduction in mean FWEC. GAMM analysis identified a statistically significant effect of immunization on mean FWEC over the timecourse of the experiment ($P < 0.001$); mean cumulative FWECs were 595 (±316) EPG in Group 1 and 1975 (±532) EPG in Group 2, representing an overall reduction of 70% in the immunized versus the control (adjuvant only) group (Fig. 1B).

In Trial 2, sheep began to excrete nematode eggs from 14 to 16 days after challenge (Fig. 1C). At peak egg shedding (day 86) mean FWECs in the extant immunized group (Group 3) were 251 ± 75 EPG, whereas in the control group (Group 4) they were 908 ± 158 EPG, representing a 73% reduction in mean FWEC. Mean cumulative FWECs in Trial 2 were 4998 (±) 2233 EPG in Group 1 (immunized) and 4127 (±) 803 EPG in Group 2 (adjuvant only, Fig. 1D). Using Cook's distance criterion, sheep 675 J was regarded as a "highly influential" case ($D = 0.3129$ based on a LMM model) on the high mean FWECs and associated SEM for Group 1. For Groups 3 and 4, which were necropsied 4 weeks after Groups 1 and 2, mean cumulative FWECs were 7005 (±) 681 EPG in Group 3 (immunized) and 16,727 (±) 2699 EPG in Group 4 (control, adjuvant only), representing an overall mean FWEC reduction of 58% in the immunized versus the control group (Fig. 1D). GAMM analysis indicated a statistically-significant effect of immunization (data from Groups 1 and 3 combined versus Groups 2 and 4 combined) on mean FWEC over the course of the experiment ($P = 0.024$).

3.2. Abomasal nematode burdens

3.2.1. Trial 1

On average, immunized sheep (Group 1) harbored 55% fewer *T. circumcincta* (total of adults and juveniles) at necropsy than control, adjuvant only (Group 2) sheep ($P = 0.011$, Fig. 2A). Group 1 sheep had statistically-significantly lower mean adult nematode burdens than sheep in Group 2 (75% reduction, $P = 0.007$, Fig. 2A). Comparison of mean juvenile nematode burdens in the abomasum indicated no significant differences between the groups (Fig. 2A). No significant differences were observed in the mean length of worms recovered from the different groups.

3.2.2. Trial 2

3.2.2.1. Groups 1 and 2 (post mortem at day 84). The mean total abomasal nematode burdens (adults and juveniles) in immunized sheep were not statistically significantly different to those from the control, adjuvant only group (mean total nematode burdens ± SEM: Group 1; 6843 ± 1144, Group 2; 6250 ± 966).

3.2.2.2. Groups 3 and 4 (post mortem at day 112). On average, immunised sheep (Group 3) harbored 57% fewer *T. circumcincta* total nematodes at necropsy than did the control, adjuvant only (Group 4) recipients ($P = 0.020$, Fig. 2B). In both Groups 3 and 4, adult worms comprised 99% of the total nematode burden.

3.3. Measurement of serum antibody responses to T. circumcincta antigens

In both trials, 7–14 days after tertiary immunization, serum IgG levels against all recombinant proteins reached peak levels, then declined slowly [Supplementary Information (SI) Figs. S1, S2]. Serum IgA levels peaked after secondary immunization and, for all recombinants with the exception of Tci-MIF-1, remained relatively constant until the end of the experiment. Immunized sheep produced serum IgG, prior to parasite challenge, which bound native L4 ES components (SI, Fig. S3): IgG bound to parasite components, in L4 ES, somatic extracts of L4 and adult *T. circumcincta*, of the expected size range for Tci-CF-1 (23.9 kDa), Tci-APY-1 (38.6 kDa)

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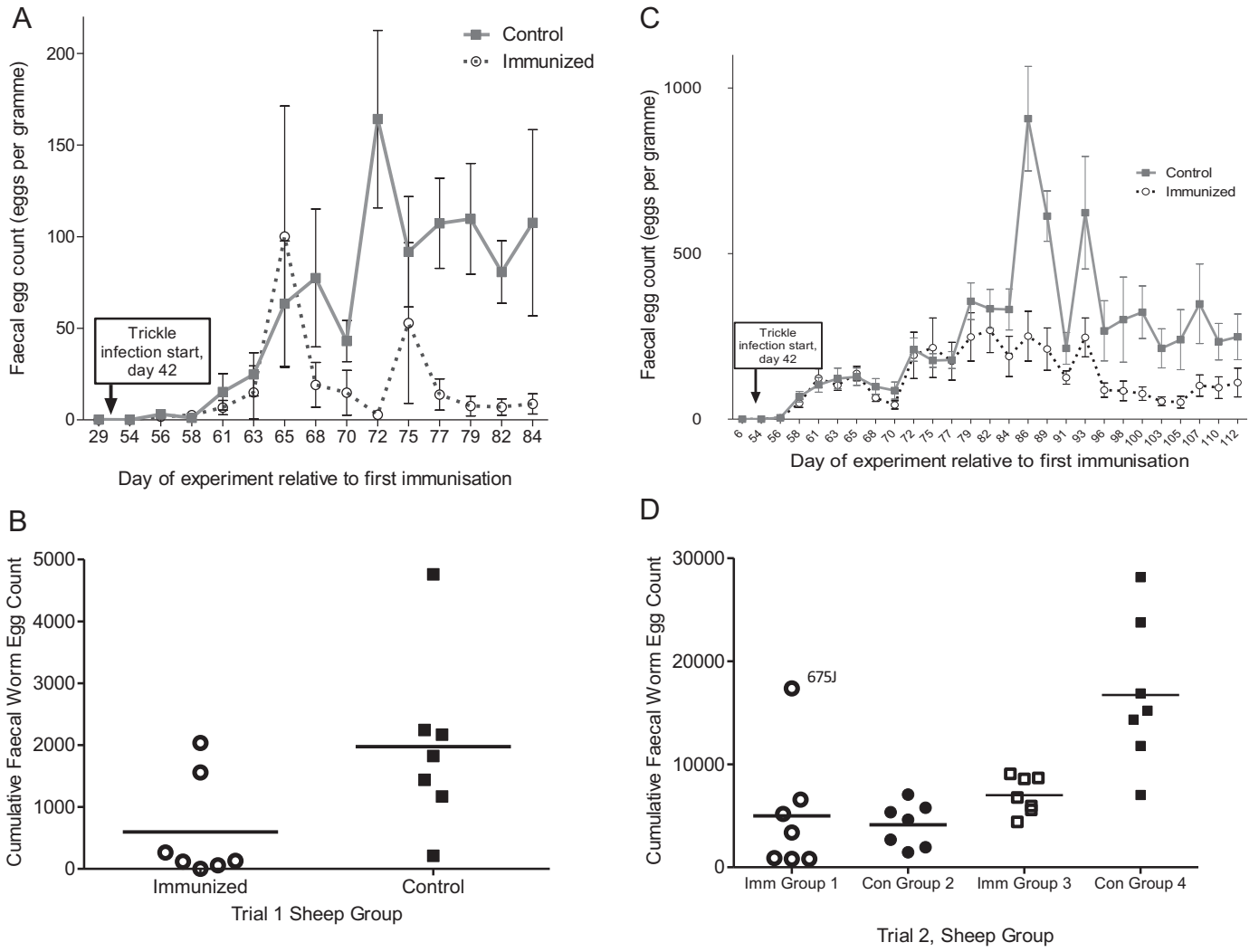

Fig. 1. Effects of immunization of sheep with recombinant antigens derived from *Teladorsagia circumcincta* on fecal worm egg counts (FWEC) after challenge infection. Panels A and C: FWECs of sheep challenged with 2000 *T. circumcincta* three times per week for 4 weeks following immunization with an 8-protein cocktail in the context of Quil A (dashed line) or with Quil A only (solid line). Each data point represents the arithmetic mean FWEC ± SEM. Panel A represents data from Trial 1; Panel C represents data from Trial 2. Panels B and D show cumulative FWECs, for each animal in each group in Trial 1 (Panel B) and Trial 2 (Panel D). "Imm" represents sheep immunized with the 8-protein cocktail; "Con" represents those administered with Quil A adjuvant only. Note that, in panel D, for Groups 1 and 2 in Trial 2, cumulative FWEC is calculated over 84 days, whereas for Groups 3 and 4 cumulative FWEC is calculated over 112 days. One "outlier" animal in Group 1 of Trial 2, sheep number 675 J, is indicated.

and Tci-MEP-1 (55.6 kDa) (Fig. 3A). Serum IgA also bound parasite components of the expected size range for vaccine components Tci-CF-1, Tci-APY-1 and Tci-MEP-1 (Fig. 3B). In addition, IgA bound a parasite component of ca. 43 kDa in L3 somatic extract. From 14 days after initiation of challenge, control, adjuvant only recipients generated serum IgG that bound recombinant Tci-MEP-1 and Tci-APY-1 but no other recombinants (SI Fig. S4). Antigen-specific serum IgA which bound to the recombinant proteins was not observed in these control sheep.

3.4. Measurement of mucus antibody responses to recombinant antigens

Mean antigen-specific IgG levels in abomasal mucus of the immunized sheep were significantly higher than in the control sheep for each protein (SI, Figs. S5A and S6A). In Trial 1, mean Tci-APY-1-, Tci-MEP-1-, and Tci-CF-1-specific IgG levels were higher than for the other recombinants ($P < 0.0001$). In Trial 2, mean Tci-MEP-1-specific IgG levels were significantly higher than for the remaining antigens (Day 84 necropsy) while mean Tci-MEP-1- and Tci-APY-1-specific IgG levels were significantly higher at the Day 112 necropsy. Mucus Tci-APY-1- and Tci-MEP-1-specific IgA levels were significantly higher than those directed against the other six antigens (SI, Figs. S5B and S6B).

4. Discussion

Here, we demonstrate that immunization of sheep with a cocktail of eight recombinant proteins induces levels of protection against *T. circumcincta* which are higher than those observed in any other system using a recombinant vaccine against a parasitic nematode in the definitive ruminant host. Previous attempts to immunise mammalian hosts against parasitic nematodes have used:

(i) Irradiated larvae. In the case of the bovine lungworm, *Dictyocaulus viviparus* [36] and the dog hookworm, *A. caninum* [37] these have given high levels of protection and yielded commercial products. This approach was not successful for a number of other nematodes including *T. circumcincta* [38].
(ii) Native extracts. There are numerous examples of the use of extracts of dead parasites, or sub-fractions thereof, to

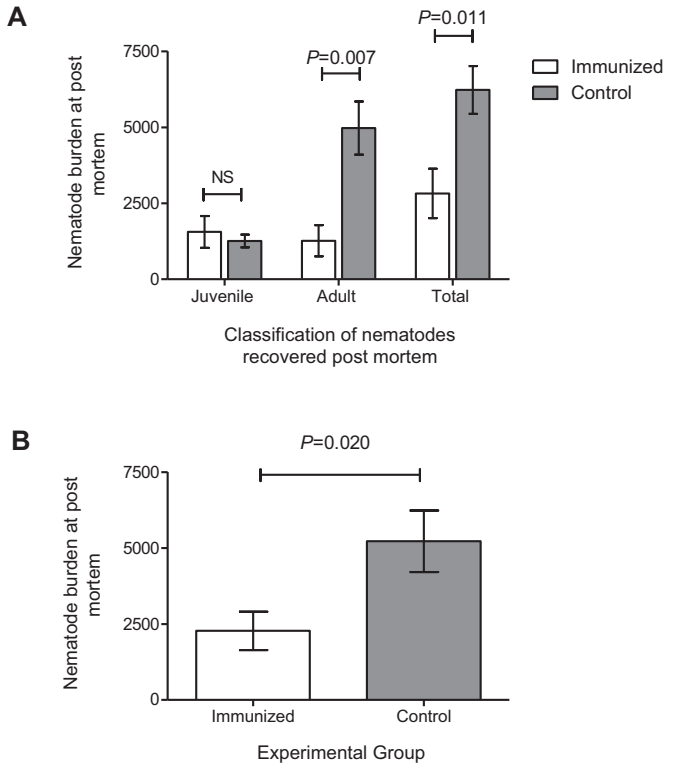

Fig. 2. Effects of immunization of sheep with recombinant antigens derived from *Teladorsagia circumcincta* on abomasal nematode burden after challenge infection. Data presented show the mean numbers of *T. circumcincta* enumerated in the abomasum ± SEM ($n = 7$). Panel A depicts the nematode burdens in Trial 1, panel B the adult nematode burden in Group 3 (immunized) or Group 4 (control, adjuvant only) of Trial 2.

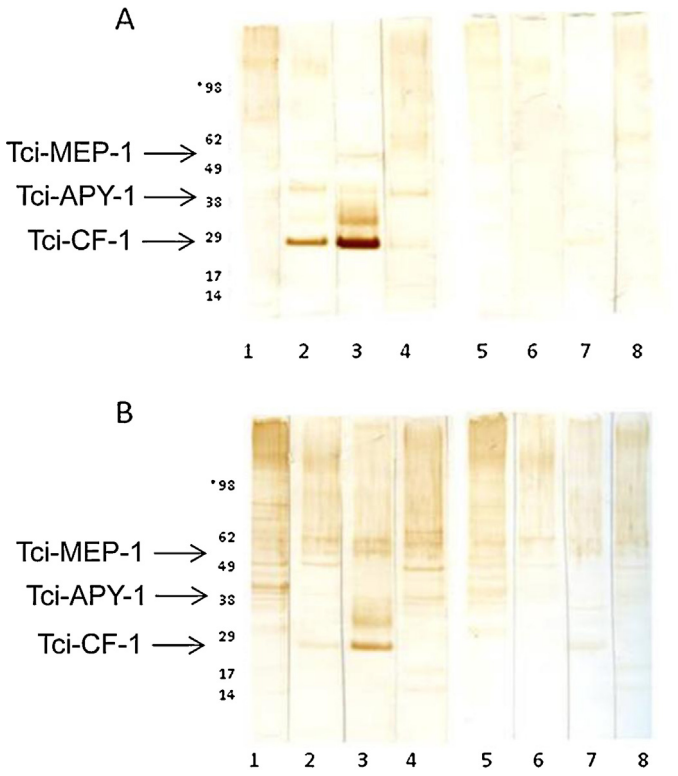

Fig. 3. Immunoblots to investigate serum IgG (Panel A) and IgA (Panel B) binding to components of somatic extracts and excretory/secretory products of *Teladorsagia circumcincta*. Lanes 1 and 5 contain L3 somatic extract, lanes 2 and 6 contain L4 somatic extract, lanes 3 and 7 contain L4 ES material and lanes 4 and 8 contain adult somatic extract. Blots were incubated with sera pooled from 7 immunized sheep (Lanes 1–4, sheep from Group 3, Trial 2) or non-immunized sheep (Lanes 5–8, sheep from Group 4, Trial 2). Sera had been collected from the animals on the date of the third immunization immediately prior to the initiation of trickle infection. Images shown are from Trial 2, Trial 1 sera reacted in a highly similar fashion. * represents molecular mass (kDa).

immunise hosts against parasitic nematodes [39]. In *T. circumcincta*, when detergent extracts of L3 were used as immunogen, anti-parasite responses were variable, but parasite burdens were significantly reduced (by up to 72%) and FWECs reduced by more than 70% [40,41]. Other attempts to protect sheep against *T. circumcincta* using native antigen preparations, for example lectin-binding integral membrane glycoproteins [42,43], were unsuccessful.

(iii) Recombinant subunit vaccines. A number of studies have reported difficulties in stimulating protective immunity with recombinant antigens [44–46]. For example, the N-type single domain ASPs, Oo-ASP-1 and Oo-ASP-2, are principal components of an ASP-enriched native extract of *Ostertagia ostertagi*, which has been used with success to immunize cattle against challenge [45,47,48]. However, immunization with a recombinant version of Oo-ASP-1 failed to induce protective immunity [44]. Other protection trials using single recombinant proteins have also failed: in *O. ostertagi*, for example, the astacin-like metalloproteinase MET-1, which shares 50% amino acid identity with Tci-MEP-1, failed to stimulate protection [49]. In addition, a recombinant version of the *Necator americanus* orthologue of Tci-SAA-1 (*Na*-SAA-1) did not induce significant protection against L3 challenge in a hamster model [50].

In contrast, our multi-component vaccine reduced FWECs and adult parasite burdens by >70% in Trial 1. It is not essential for a vaccine against ruminant parasitic nematodes to be 100% effective, and "substantial benefits" can be gained by using a vaccine that is 60% effective in 80% of the flock [51]. It has also been postulated that a vaccine which could reduce egg output by 60% in *O. ostertagi* infected calves during the first 2 months on pasture could control bovine ostertagiosis by reducing peak pasture contamination while also allowing natural immunity to develop [47,48].

The serological analyses presented here (Figs. S1–S6) demonstrate that co-administration of immunomodulatory molecules with other vaccine antigens did not prevent high antigen-specific antibody levels developing. Although the recombinant antigens elicited high serum antibody levels following immunization in both Trials, there was no antigen-specific anamnestic response following parasite challenge. Thus, if the observed protection is antibody-based, high antibody levels produced by vaccination could protect young lambs when they are most susceptible to detrimental effects of *T. circumcincta* infection [52] but, because the vaccine does not produce sterile immunity, naturally-acquired immunity (which develops as the lambs mature [11]) would be allowed to develop through exposure to the limited numbers of L3 from pasture.

Overall, both trials produced comparable effects, although the timing differed. In Trial 1, when post mortems were conducted at day 84, vaccinated sheep contained 75% fewer adult nematodes than non-vaccinated sheep. In Trial 2 however, the effects of the vaccine on both FWEC and nematode burden were not obvious by day 84, with the differences between the vaccinated animals and the control animals becoming clearer later in the experiment. This observation may have been related to the ages of the animals involved in each trial–lambs in Trial 2 were ~30 days younger than those in Trial 1 and may have been less able to control the infectious dose [11,19].

Further studies with the recombinant vaccine will focus on a reductionist approach to determine the most simple, effective

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antigen combinations; to further elucidate the mechanism of action and to determine the efficacy of the vaccine in newly-weaned lambs where infection with *T. circumcincta* has the highest economical and welfare impacts [52].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.05.026.

References

[1] Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, et al. Soil-transmitted helminth infections: Ascariasis, trichuriasis, and hookworm. Lancet 2006;367:1521–32.
[2] Hotez PJ, Molyneux DH, Fenwick A, Ottesen E, Ehrlich Sachs S, Sachs JD. Incorporating a rapid-impact package for neglected tropical diseases with programs for HIV/AIDS, tuberculosis and malaria. PLoS Med 2006;3:e102.
[3] Geldhof P, De Maere V, Vercruysse J, Claerebout E. Recombinant expression systems: the obstacle to helminth vaccines? Trends Parasitol 2007;23:527–32.
[4] Bethony JM, Cole RN, Guo X, Kamhawi S, Lightowlers MW, Loukas A, et al. Vaccines to combat the neglected tropical diseases. Immunol Rev 2011;239:237–70.
[5] Nieuwhof GJ, Bishop SC. Costs of the major endemic diseases of sheep in Great Britain and the potential benefits of reduction in disease impact. Anim Sci 2005;81:23–9.
[6] Bartley DJ, Jackson F, Jackson E, Sargison N. Characterisation of two triple resistant field isolates of Teladorsagia from Scottish lowland sheep farms. Vet Parasitol 2004;123:189–99.
[7] Wrigley J, McArthur M, McKenna PB, Mariadass B. Resistance to a triple combination of broad-spectrum anthelmintics in naturally-acquired *Ostertagia circumcincta* infections in sheep. NZ Vet J 2006;54:47–9.
[8] Seaton DS, Jackson F, Smith WD, Angus KW. Development of immunity to incoming radiolabelled larvae in lambs continuously infected with *Ostertagia circumcincta*. Res Vet Sci 1989;46:241–6.
[9] Singleton DR, Stear MJ, Matthews L. A mechanistic model of developing immunity to *Teladorsagia circumcincta* infection in lambs. Parasitology 2011;138:322–32.
[10] Balic A, Bowles VM, Liu YS, Meeusen EN. Local immune responses in sensitized sheep following challenge infection with *Teladorsagia circumcincta*. Parasite Immunol 2003;25:371–81.
[11] Smith WD, Jackson F, Jackson E, Williams J. Age immunity to *Ostertagia circumcincta*: comparison of the local immune responses of 4 1/2- and 10-month-old lambs. J Comp Pathol 1985;95:235–45.
[12] Smith WD, Jackson F, Jackson E, Graham R, Williams J. Transfer of immunity to *Ostertagia circumcincta* and IgA memory between identical sheep by lymphocytes collected from gastric lymph. Res Vet Sci 1986;41:300–6.
[13] Stear MJ, Bairden K, Innocent GT, Mitchell S, Strain S. 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 2004;129:363–9.
[14] Smith WD, Jackson F, Graham R, Jackson E, Williams J. Mucosal IgA production and lymph cell traffic following prolonged low level infections of *Ostertagia circumcincta* in sheep. Res Vet Sci 1987;43:320–6.
[15] Stear MJ, Strain S, Bishop SC. How lambs control infection with *Ostertagia circumcincta*. Vet Immunol Immunopathol 1995;72:213–8.
[16] Stear MJ, Strain S, Bishop SC. Mechanisms underlying resistance to nematode infection. Int J Parasitol 1999;29:51–6.
[17] Stear MJ, Henderson NG, Kerr A, McKellar QA, Mitchell S, Seeley C, et al. Eosinophilia as a marker of resistance to *Teladorsagia circumcincta* in Scottish blackface lambs. Parasitology 2002;124:553–60.
[18] Stear MJ, Boag B, Cattadori I, Murphy L. Genetic variation in resistance to mixed, predominantly *Teladorsagia circumcincta* nematode infections of sheep: from heritabilites to gene identficiation. Parasite Immunol 2009;31:274–82.
[19] Halliday AM, Routledge CM, Smith SK, Matthews JB, Smith WD. Parasite loss and inhibited development of *Teladorsagia circumcincta* in relation to the kinetics of the local IgA response in sheep. Parasite Immunol 2007;29:425–34.
[20] Halliday AM, McAllister HC, Smith WD. Kinetics of the local immune response in the gastric lymph of lambs after primary and challenge infection with *Teladorsagia circumcincta*. Parasite Immunol 2010;32:81–90.
[21] Smith SK, Nisbet AJ, Meikle L, Inglis N, Sales J, Beynon RJ, et al. Proteomic analysis of excretory/secretory products released by *Teladorsagia circumcincta* larvae early post-infection. Parasite Immunol 2009;31:10–9.
[22] Williams AR. Some observations on the role of bradykinin in immunity to *Teladorsagia circumcincta* in sheep. J Parasitol Res 2013, http://dx.doi.org/10.1155/2012/569287.
[23] Redmond DL, Smith SK, Halliday A, Smith WD, Jackson F, Know DP, et al. An immunogenic cathepsin F secreted by the parasitic stages of *Teladorsagia circumcincta*. Int J Parasitol 2006;36:277–86.
[24] Denham DA. The development of *Ostertagia circumcincta* in lambs. J Helminthol 1969;43:299–310.
[25] Zhan B, Wang Y, Liu Y, Williamson A, Loukas A, Hawdon JM, et al. Ac-SAA-1, an immunodominant 16 kDa surface-associated antigen of infective larvae and adults of *Ancylostoma caninum*. Int J Parasitol 2004;34:1037–45.
[26] Nisbet AJ, Knox DP, McNair CM, Meikle LI, Smith SK, Wildblood LA, et al. Immune recognition of the surface associated antigen, Tc-SAA-1, from infective larvae of *Teladorsagia circumcincta*. Parasite Immunol 2009;31:32–40.
[27] Nisbet AJ, Redmond DL, Matthews JB, Watkins C, Yaga R, Jones JT, et al. Stage-specific gene expression in *Teladorsagia circumcincta* (Nematoda: Strongylida) infective larvae and early parasitic stages. Int J Parasitol 2008;38:829–38.
[28] McSorley HJ, Grainger JR, Harcus YM, Murray J, Nisbet AJ, Knox DP, et al. Expression of highly conserved TGF-β family members in the Trichostrongyloid nematodes *Haemonchus contortus*, *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis* and *Teladorsagia circumcincta*. Parasitology 2010;137:159–71.
[29] Nisbet AJ, Bell NEV, McNeilly TN, Knox DP, Maizels RM, Meikle LI, et al. A macrophage migration inhibitory factor-like tautomerase from *Teladorsagia circumcincta* (Nematoda: Strongylida). Parasite Immunol 2010;32:503–11.
[30] Nisbet AJ, Zarlenga DS, Knox DP, Meikle LI, Wildblood LA, Matthews JB. A calcium-activated apyrase from *Teladorsagia circumcincta*: an excretory/secretory antigen capable of modulating host immune responses? Parasite Immunol 2011;33:236–43.
[31] Nisbet AJ, Smith SK, Armstrong S, Meikle LI, Wildblood LA, Beynon RJ, et al. *Teladorsagia circumcincta*: activation-associated secreted proteins in excretory/secretory products of fourth stage larvae are targets of early IgA responses in infected sheep. Exp Parasitol 2010;125:329–37.
[32] Nisbet AJ, MacKellar A, McLean K, Brennan GP, Huntley JF. Eukaryotic expression of recombinant Pso o 1, an allergen from *Psoroptes ovis*, and its localization in the mite. Parasitology 2007;134:83–9.
[33] Christie M, Jackson F. Specific identification of strongyle eggs in small samples of sheep faeces. Res Vet Sci 1982;32:113–7.
[34] Taylor SM, Kenny J, Edgar HW, Ellison S, Ferguson L. 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 Record 1997;141:357–60.
[35] Jackson E, Jackson F, Smith WD. Comparison of saline incubation and pepsin digestion as methods for recovering *Ostertagia circumcincta* larvae from the abomasum of sheep. Res Vet Sci 1984;36:380–1.
[36] Jarrett WFH, Jennings FW, McIntyre WIM, Mulligan W, Urquart GM. Irradiated helminth larvae in vaccination. Proc R Soc Med 1958;51:743–4.
[37] Miller TA. Industrial development and field use of the canine hookworm vaccine. Adv Parasitol 1978;16:333–42.
[38] Smith WD, Jackson E, Jackson F. Attempts to immunise sheep against *Ostertagia circumcincta* with irradiated larvae. Res Vet Sci 1982;32:101–5.
[39] Smith WD, Zarlenga DS. Developments and hurdles in generating vaccines for controlling helminth parasites of grazing ruminants. Vet Parasitol 2006;139:347–59.
[40] Wedrychowicz H, Bairden K, Tait A, Holmes PH. Immune responses of sheep to surface antigens of infective larvae of *Ostertagia circumcincta*. Parasite Immunol 1992;14:249–66.
[41] Wedrychowicz H, Bairden K, Dunlop EM, Holmes PH, Tait A. Immune response of lambs to vaccination with *Ostertagia circumcincta* surface antigens eliciting bile antibody responses. Int J Parasitol 1995;25:1111–21.
[42] Smith WD, Pettit D, Smith SK. Cross-protection studies with gut membrane glycoproteins from *Haemonchus contortus* and *Teladorsagia circumcincta*. Parasite Immunol 2001;23:203–11.
[43] Halliday AM, Smith WD. Attempts to immunize sheep against *Teladorsagia circumcincta* using fourth-stage larval extracts. Parasitology 2011;33:554–60.
[44] Geldhof P, Meyvis Y, Vercruysse J, Claerebout E. Vaccine testing of a recombinant activation-associated secreted protein (ASP1) from *Ostertagia ostertagi*. Parasite Immunol 2008;30:57–60.
[45] Meyvis Y, Geldhof P, Gevaert K, Timmerman E, Vercruysse J, Claerebout E. Vaccination against *Ostertagia ostertagi* with subfractions of the protective ES-thiol fraction. Vet Parasitol 2007;149:239–45.
[46] Newton SE, Meeusen EN. Progress and new technologies for developing vaccines against gastrointestinal nematode parasites of sheep. Parasite Immunol 2003;25:283–96.
[47] Geldhof P, Claerebout E, Knox D, Vercauteren I, Looszova A, Vercruysse J. Vaccination of calves against *Ostertagia ostertagi* with cysteine proteinase enriched protein fractions. Parasite Immunol 2002;24:263–70.

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[48] Geldhof P, Vercauteren I, Vercruysse J, Knox DP, Van Den Broeck W, Claerebout E. Validation of the protective *Ostertagia ostertagi* ES-thiol antigens with different adjuvantia. Parasite Immunol 2004;26:37–43.
[49] De Maere V, Vercauteren I, Geldhof P, Gevaert K, Vercruysse J, Claerebout E. Molecular analysis of astacin-like metalloproteases of *Ostertagia ostertagi*. Parasitology 2005;130:89–98.
[50] Xiao S, Zhan B, Xue J, Goud GN, Loukas A, Liu Y, et al. The evaluation of recombinant hookworm antigens as vaccines in hamsters (*Mesocricetus auratus*) challenged with human hookworm, *Necator americanus*. Exp Parasitol 2008;118:32–40.
[51] Barnes EH, Dobson RJ, Barger IA. Worm control and anthelmintic resistance: adventures with a model. Parasitol Today 1995;11:56–63.
[52] Coop RL, Sykes AR, Angus KW. The effect of three levels of intake of *Ostertagia circumcincta* larvae on growth rate, food intake and body composition of growing lambs. J Agric Sci 1982;98:247–55.

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