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Ovine IgA-reactive proteins from *Teladorsagia circumcincta* infective larvae

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ABSTRACT

Infection of small ruminants with *Teladorsagia circumcincta* has, until now, been controlled using a combination of pasture management and frequent anthelmintic treatments. Resistance to the commonly used anthelmintics has driven research into the development of a subunit vaccine, encouraged by the demonstration of development of protective immunity in sheep following exposure to this parasite. Local immune effectors in the abomasum, in particular IgA, are thought to play important roles in naturally- and experimentally-acquired immunity. L3s represent the first contact of this pathogen with the host immune system and, herein, the presence of L3 antigen-specific IgA was demonstrated in abomasal mucus from immune sheep. This antibody source was used to immunoaffinity purify and identify IgA-reactive molecules present in L3s. We identified 155 different proteins in this way, including a number of activation-associated secretory proteins, venom allergen-like-type proteins, detoxifying enzymes, galectins and a suite of other potential vaccine candidate molecules. Levels of immunoaffinity-enriched L3 antigen-specific IgA in gastric lymph from previously-infected sheep were statistically significantly higher ($P = 0.004$) than those measured in helminth-free sheep and a statistically significant negative correlation ($P = 0.005$, $r_s = -0.565$) was identified between immunoaffinity-enriched L3 antigen-specific IgA levels in efferent gastric lymph and total *T. circumcincta* burden measured at necropsy. In addition, a statistically significant positive correlation ($P = 0.007$, $r_s = 0.534$) was measured between immunoaffinity-enriched L3 antigen-specific IgA levels in efferent gastric lymph and the percentage of inhibited L4s enumerated at necropsy. These results indicate that the purified antigens contain components that could be strongly considered as vaccine candidates.

1. Introduction

In temperate regions, *Teladorsagia circumcincta* is the most prevalent gastrointestinal nematode of small ruminants (e.g. Bartley et al., 2003). This parasite is currently controlled using a combination of pasture management and anthelmintic treatments; however, anthelmintic resistance, including multi-class resistance, to Class I, II and III anthelmintics is now widespread (Bartley et al., 2003, 2004; Sargison et al., 2007). Protective immunity against *T. circumcincta* can be induced after repeated exposure of sheep through natural exposure or experimental infection (Seaton et al., 1989; Singleton et al., 2011) and this has driven research into the development of a subunit vaccine (Nisbet et al., 2013). From experimental studies, it is evident that protective responses act at various points in the parasite's lifecycle to decrease larval establishment in the abomasal mucosa, slow larval development at this site and diminish female worm fecundity (Smith et al., 1985, 1986; Seaton et al., 1989; Stear et al., 2004a,b). Local immune effectors in the abomasum, in particular IgA, are thought to play important roles in immunity (Smith et al., 1985; Strain et al., 2002) and abomasal IgA has been used as a tool to identify potential vaccine candidates from *T. circumcincta* (e.g. Smith et al., 2009). Subsequently recombinant protein versions of eight molecules, identified primarily in L4s through a combination of immunoproteomics, bioinformatics and functional analysis (Redmond et al., 2006; Smith et al., 2009; Nisbet et al., 2010), were shown to induce statistically significant levels of immunity against

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challenge in two independent trials when administered to lambs as a cocktail (Nisbet et al., 2013). In these trials, statistically significant reductions in faecal egg shedding (up to 70%) and abomasal worm burdens (up to 75%) were observed in vaccinated compared with adjuvant-only recipients.

To further enhance efficacy of this vaccine, candidate antigen discovery should encompass host/parasite interactions beyond the L4 stage. L3s represent the first contact of this pathogen with the host immune system and immunisation of sheep with L3 surface antigens stripped from the epicuticle using detergent and formulated with beryllium hydroxide adjuvant has been shown to induce 72% mean reductions in worm burdens compared with adjuvant-only recipients following challenge (Wedrychowicz et al., 1992, 1995). In sheep rendered immune through continual L3 infection, an immediate type hypersensitivity reaction has been identified against incoming L3s and has been proposed as responsible, in part, for preventing parasite establishment in the gastric wall (Smith et al., 1984; Seaton et al., 1989; Stear et al., 1995). An additional mechanism of protection which focused on L3 stages was highlighted in subsequent experiments in which levels of L3/early L4 excretory/secretory (ES) antigen-specific IgA in the abomasal mucus of immune ewes were demonstrated to display an inverse relationship with worm burden following challenge (Smith et al., 2009). In the current study, the objective was to identify which L3 antigens might be generating protective IgA responses in *T. circumcincta*-infected sheep. To do this, IgA-reactive molecules from L3s were immunoaffinity purified in their native state and then subjected to proteomics analysis. Next, local IgA levels to the purified antigens were measured in a cohort of sheep and the strength of the relationships between L3 antigen-specific IgA and parasitological correlates of immunity were assessed to inform their potential as vaccine candidates.

2. Materials and methods

2.1. Ovine abomasal mucus and gastric lymph

Abomasal mucus was derived from samples from a previous study (described in Knight et al., 2011; Halliday et al., 2012). Briefly, 8 month-old Scotch Mule (Blackface ewe X Blue-faced Leicester ram) sheep, raised under conditions designed to minimise the risk of helminth exposure, were divided into two groups. Group M-PI (mucus-previously infected) sheep were trickle infected with 2,000 *T. circumcincta* L3s, administered orally, three times per week for 8 weeks. Group M-CO (mucus-control) sheep were maintained under helminth-free conditions. At the end of the trickle-infection, both groups were administered fenbendazole (5 mg kg^{-1} orally). Seven days later, both groups were challenged with a single bolus dose of 50,000 *T. circumcincta* L3s, administered orally. All animals were euthansed 2 days later, the abomasum removed and mucus collected from the lumenal surface (Smith et al., 2009), and stored at −20 °C until use.

Efferent gastric lymph was also used in the current study. These latter samples had been collected following cannulation of the common gastric lymph duct of yearling Suffolk–Dorset cross or Scotch Mule sheep (Halliday et al., 2007). The animals had been experimentally infected with *T. circumcincta* using the protocol described above, to provide material from two groups – GL-PI (Gastric lymph, previously infected, $n = 13$) and GL-CO (Gastric lymph, control, $n = 10$) (Halliday et al., 2007). Finally, efferent gastric lymph was collected from an additional group of sheep, GL-HF, which had been reared under conditions designed to exclude infection with helminths (gastric lymph helminth-free $n = 6$). All studies described herein involving sheep were performed under the regulations of a UK Home Office Project Licence; experimental design was ratified by Moredun Research Institue Experiments and Ethics Committee.

2.2. Determination of immunoreactivity of T. circumcincta antigen extracts

For ELISA and immunoblot experiments, soluble extracts of *T. circumcincta* L3s were prepared as described previously (Nisbet et al., 2009). Briefly, *T. circumcincta* L3s were harvested from coproculture and homogenised in ice-cold PBS using a Hybaid RiboLyser™ with Q-Biogene lysing matrix D (4 × 20 s lysis at power setting 6), followed by centrifugation at 13,000*g* for 10 min at 4 °C. Protein concentrations were determined using the bicinchoninic acid (BCA) protocol (Pierce, USA) with BSA standards. For quantitative assessment of antigen-specific antibodies in abomasal mucus and gastric lymph by ELISA, microtitre plates (Greiner Bio-One, UK, flat-bottomed, high binding) were coated with 50 µl of 5 µg ml^{-1} *T. circumcincta* extract from L3s or with IgA-affinity-purified *T. circumcincta* L3 extract (see Section 2.3), in 50 mM sodium bicarbonate, pH 9.6, and incubated at 4 °C overnight. Plates were washed six times with PBS containing 0.05% (v/v) Tween-20 (PBST), then incubated with blocking buffer (10% soya milk powder (Infasoy, Cow and Gate, UK) in PBST) for 2 h at room temperature. After re-washing, plates were incubated with 50 µl of primary antibody (pooled abomasal mucus (derived from four M-PI sheep or from two M-CO sheep) or individual efferent gastric lymph samples, diluted at 1:5 and 1:20 in TNTT, (10 mM Tris, 0.5 M NaCl, 0.05% Tween-20, 0.01% thiomersal, pH 7.4), respectively), and incubated for 2 h at room temperature. After washing, 50 µl of secondary antibody (mouse monoclonal anti-bovine/ovine IgA Serotec, UK, MCA628) diluted in TNTT (1:1000) were added and incubated for 1 h at room temperature. Following washing, 50 µl of tertiary antibody (rabbit anti-mouse immunoglobulins horseradish peroxidase (HRP)-conjugate, Dako, UK, P0260) diluted 1:1000 in TNTT, were added to each well and incubated for 1 h at room temperature. Plates were washed six times in PBST and 50 µl of *O*-phenylenediamine dihydrochloride (OPD) substrate (Sigma Fast™, Sigma–Aldrich, UK) were added. Plates were incubated in the dark for 20 min, the reaction stopped by addition of 25 µl of 2.5 M H_2SO_4 per well and the O.D. read at 492 nm on a spectrometer. All tests were conducted in triplicate on each plate and each plate was repeated on two independent occasions. Negative controls, omitting the primary antibody incubation step, were included on each plate. A pool of efferent gastric lymph collected from GL-PI sheep at the time point corresponding to the peak total IgA concentration, 6–10 days post challenge (pc) (Halliday et al., 2007), was used as a positive reference sample on all plates.

Two-dimensional (2-D) immunoblots of *T. circumcincta* L3 extracts were performed as described previously (Huntley et al., 2004; Nisbet et al., 2009). Proteins separated by 2-D electrophoresis were transferred onto polyvinylidene fluoride (PVDF) membranes according to the manufacturer's instructions (Invitrogen, UK). For sodium periodate-treated 2-D blots, following electroblotting, blots were incubated with 50 mM sodium acetate for 1 h at room temperature in the dark, washed twice in TNTT and incubated in 50 mM sodium borohydride/TNTT for 30 min at room temperature, before three final washes in TNTT prior to incubation with the primary antibody. All antibody incubation steps were as described previously (Nisbet et al., 2013) using primary antibody (abomasal mucus diluted 1:5 in TNTT) with the appropriate secondary and conjugated tertiary antibody at the same dilutions used for ELISA (see above). After application of the final HRP-conjugated antibody, blots were re-washed in TNTT, then incubated in 3,3′-diaminobenzidine (DAB, Sigma Fast™, Sigma–Aldrich).

2.3. IgA affinity purification of L3 immunoreactive antigens

Abomasal mucus samples from four M-PI sheep were pooled and diluted 1:3 in PBS, pH 7.4, to a final volume of 20 ml and

centrifuged at 700*g* for 10 min to remove insoluble material. The supernatant was loaded onto a 1 ml HiTrap Protein G column (GE Healthcare, UK) which had been pre-equilibrated with PBS. The column was washed with 10 volumes of PBS and the unbound material collected. Unbound protein, containing IgA depleted of IgG, was concentrated to 1 ml at 4 °C using Amicon centrifugal Ultra-4 devices with a 10 kDa molecular weight cut-off (MWCO) membrane, then purified by size-exclusion chromatography using a Superose 12 HR 10/30 column (GE Healthcare) coupled to a fast protein liquid chromatography (FPLC) apparatus (AKTA™ purifier UV-900/P-900). IgA purified in this way was bound to a HiTrap NHS-activated HP column (GE Healthcare) following the manufacturer's instructions. The IgA-NHS affinity column was equilibrated prior to immunoaffinity purification by washing with 10 column volumes of PBS at a flow rate of 0.5 ml min^{-1}. L3 extracts, prepared as described in Section 2.2, were centrifuged at 14,000*g* for 10 min and 10 ml of the supernatant (~7 mg of protein) were re-circulated through the column at a flow rate of 0.5 ml min^{-1} for 16 h at 4 °C. Following sample application, unbound material was washed from the column with 10 column volume washes of PBS at a flow rate of 0.5 ml min^{-1} and bound antigens eluted with two column volumes of 0.1 M glycine–HCl, 6 M urea, pH 2.5. Eluted fractions (~10 ml) were pooled and concentrated to approximately 500 µl using Amicon Ultra-15 10 kDa MWCO centrifugal devices at 3000*g* for 20 min at 4 °C. The concentrated protein preparations were then buffer-exchanged with PBS over three washes and the remaining material transferred to Microcon YM-10 10 kDa MWCO centrifugal units. The concentrator units were centrifuged at 10,000*g* at 4 °C for 20 min, until samples were concentrated to approximately 50 µl.

2.4. Proteomic analysis of IgA affinity-purified L3 antigens

For proteomic analysis, 10 µl of concentrated affinity-purified *T. circumcincta* L3 extracts were fractionated by SDS–PAGE under reducing conditions using 4–12% gradient Bis–Tris gels with MES buffer (Invitrogen). After electrophoresis, resolved proteins were visualised with colloidal Coomassie Blue (Simply Blue Safe Stain, Invitrogen) and destained in water. Gel tracks under investigation were sliced horizontally into 26 equal gel slices of approximately 2.5 mm width and analysed as described in Smith et al. (2009). The resulting chromatography data were processed and Mascot-compatible files created using DataAnalysis™ 3.2 software (Bruker Daltonics, UK). Mascot-compatible were files generated, inserted into ProteinScape, version 2.1.0.577 (Bruker Daltonics) and were searched against five databases:

(i). Nembase Version 4 (http://www.nematodes.org/nematode-ESTs/nembase.html), July 2011 release;
(ii). A *T. circumcincta* expressed sequence tag (EST) database generated by suppressive subtractive hybridisation (SSH) (Nisbet et al., 2008);
(iii). A *T. circumcincta* L4 database generated by next generation sequencing using Roche-454 titanium technology of cDNA synthesised using total RNA extracted from *T. circumcincta* L4s (day 7 p.i.) as described in Nisbet et al. (2010);
(iv). A *T. circumcincta* L3 dataset created by 454 sequencing of cDNA derived from RNA obtained from L3s exposed to either an immune or naive ovine abomasal environment (Halliday et al., 2012);
(v). A *T. circumcincta* adult EST database generated by next generation sequencing, using 454 GS-FLX titanium technology of cDNA synthesised using RNA extracted from *T. circumcincta* adults as described in Menon et al. (2012).

Interpretation and presentation of MS data were performed in accordance with published guidelines (Taylor and Goodlett, 2005). Fixed and variable modifications selected were carbamidomethyl and oxidation, respectively, and mass tolerance values set at 1.5 and 0.5 Da for MS and MS/MS, respectively. Molecular weight search (MOWSE) scores obtained for individual protein identifications were inspected manually and considered significant only if: (i) two unique peptides were matched for each protein, and (ii) each peptide contained an unbroken "*b*" or "*y*" ion series of a minimum of four amino acid residues. Protein identifications were confirmed further with a MOWSE score of 42 or higher indicating that the match was statistically significant at the 95% confidence level when searching against the four datasets. All MS analyses were performed at the Moredun Research Institute Proteomics Facility, UK.

2.5. Statistical analysis

The data from each antigen-specific ELISA analysis using pooled abomasal mucus (Fig. 1) were statistically evaluated by linear mixed-effect modeling of OD_{490} variation between groups, with the random effect of plate added to take account of any plate effects from the sampled pools. Standard Tukey post hoc pairwise comparisons were performed if any overall differences were found. For the analysis of differences between groups in levels of binding of IgA from efferent gastric lymph to immunoaffinity-purified L3 extract standard one-way ANOVA was performed on the mean OD_{490}/individual sheep, with equivalent standard Tukey post hoc comparisons. Relationships between three immunological parameters: (i) total IgA concentration in efferent gastric lymph at 7 days pc, (ii) total nematode burden and (iii) percentage of inhibited L4s, and the level of IgA binding to the IgA-affinity purified antigens were investigated by non-parametric Spearman rank correlation coefficient analyses. All statistical analyses were carried out in R (v 3.0.0 © 2013 The R foundation for Statistical Computing, http://www.r-project.org/foundation), with the package 'nlme' (v 3.1-109) used for the linear mixed-modeling and package 'multcomp' (v-1.2-17) used for the post hoc testing, and the level of statistical significance was set at $P < 0.05$.

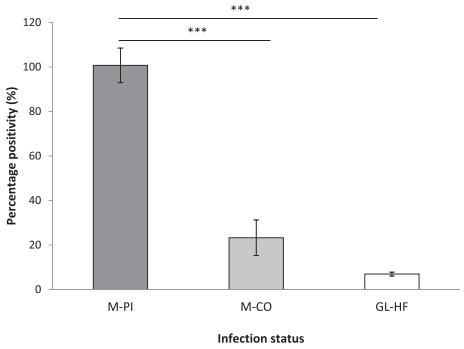

Fig. 1. Antigen-specific mucus IgA responses in previously-infected and primary-infected sheep to *Teladorsagia circumcincta* L3 antigens. Group M-PI represents abomasal mucus pooled from four previously-infected sheep and group M-CO represents abomasal mucus pooled from two helminth-naïve sheep. All animals were given a single bolus infection of 50,000 *T. circumcincta* L3s and necropsied 2 days later. Group GL-HF represents pooled efferent gastric lymph from six helminth-naïve sheep. The positive control was pooled efferent gastric lymph collected during days 6–10 post-challenge from previously-infected/bolus-challenged sheep. The mean OD_{490} values (±S.E.M.) from two sets of triplicate wells are shown as a percentage of the positive control. Mean L3 antigen-specific IgA levels of M-PI sheep were statistically significantly higher ($P < 0.001$) than M-CO and GL-HF sheep, respectively.

3. Results

3.1. Determination of immunoreactivity of T. circumcincta antigen extracts

Teladorsagia circumcincta L3-specific IgA was detected in abomasal mucus obtained from previously-infected sheep 2 days after the bolus challenge (Group M-PI, Fig. 1) and levels of L3-specific IgA in these samples were statistically significantly higher than those measured in abomasal mucus from sheep that had only received a single bolus infection 2 days previously ($P < 0.001$, Group M-CO, Fig. 1). Multiple IgA-reactive antigens were identified in L3 extracts by 2-D immunoblotting (Fig. 2). Several areas of immunoreactivity, particularly at high molecular mass (>90 kDa), were found to be periodate-sensitive (Fig. 2), demonstrating reactivity to glycan residues on those molecules.

3.2. IgA affinity purification of L3 immunoreactive antigens and proteomic analysis of the purified antigens

Mucosal IgA from sheep subjected to a trickle infection/bolus challenge protocol (M-PI) was purified and used to create an immunoaffinity column for selecting IgA-reactive L3 antigens. The enriched material contained a number of polypeptides over a broad molecular weight range with enrichment of molecules of ~30, 60 and >90 kDa (Fig. 3). The immunoaffinity-enriched L3 extract was analysed by liquid chromatography-electrospray ionisation/multi-stage mass spectrometry (LC–ESI-MS/MS). The resulting peptides demonstrated homology to 155 unique proteins (Supplementary Table S1), each with peptide mass fingerprints containing two unique peptides with a consecutive sequence of four 'b' or 'y' ions with a MOWSE score of >100. Of the total proteins identified, 28.1% were hypothetical proteins or their function has not yet been elucidated. The remaining proteins were classified according to their inferred function including: developmental, metabolic, transport, carbohydrate binding, cuticle synthesis, detoxification, lipid binding, heat shock, protein folding, cytoskeletal, actin binding, proteolytic enzymes, signalling and gut-associated (Supplementary Table S1). A selection of these proteins is presented in Table 1.

3.3. Relationships between IgA reactivity to immunoaffinity-purified T. circumcincta antigens and correlates of immunity

To determine whether immune reactivity to the immunoaffinity-enriched antigens correlated with previously measured parameters of immunity in different groups of sheep, immunoaffinity-enriched antigen-specific IgA levels were assessed. Mean levels of immunoaffinity-enriched antigen-specific IgA in GL-PI sheep were statistically significantly higher ($P = 0.004$) than those measured in sheep subjected to a single infection (GL-CO) and in GL-HF, sheep (Fig. 4) at the same time point ($P = 0.044$, 7 days pc). A statistically significant positive correlation ($P < 0.001$, $r_s = 0.865$, Fig. 5A) was observed between immunoaffinity-enriched L3 antigen-specific IgA and total IgA measured in efferent gastric lymph collected 7 days pc. Importantly, a statistically significant negative correlation ($P = 0.005$, $r_s = -0.565$) was observed between immunoaffinity-enriched L3 antigen-specific IgA levels in efferent gastric lymph and the total *T. circumcincta* burden measured at necropsy (Fig. 5B). Furthermore, a statistically significant positive correlation ($P = 0.007$, $r_s = 0.534$) also was observed between immunoaffinity-enriched L3 antigen-specific IgA levels in efferent gastric lymph and the percentage of inhibited L4s enumerated at necropsy (Fig. 5C), the latter being generally accepted as a measure of developing protective immunity in *T. circumcincta*-infected sheep (Smith et al., 1984).

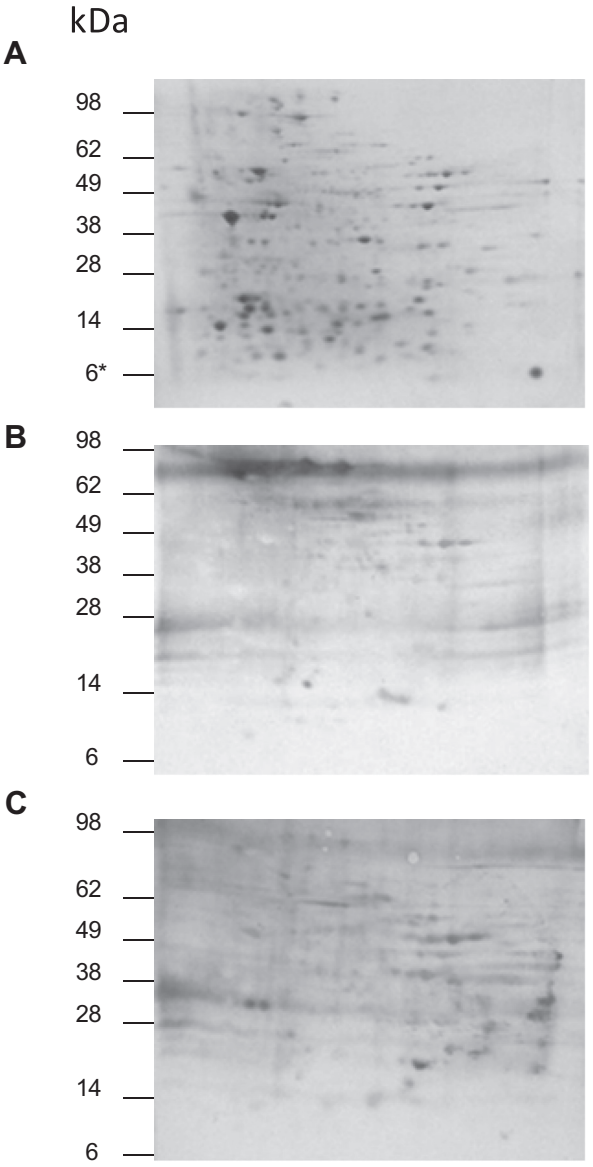

Fig. 2. Two-dimensional SDS–PAGE and IgA-immunoblot analysis of *Teladorsagia circumcincta* L3 antigens. *Teladorsagia circumcincta* L3 extract was resolved by two-dimensional gel electrophoresis and stained with colloidal Coomassie blue (A) or transferred to polyvinylidene fluoride membrane by electroblotting (B, C). Following electroblotting, antigens were either left intact (B) or treated with sodium periodate (C) prior to incubation with abomasal mucus, pooled from previously-infected sheep which were given a single challenge of 50,000 *T. circumcincta* L3s and necropsied 2 days later. Following incubation in purified IgA, all blots were developed as described previously (Nisbet et al., 2009).

4. Discussion

Here for the first known time, we obtained the identity of infective *T. circumcincta* L3 antigens bound by IgA in abomasal mucus and efferent gastric lymph of sheep which had been rendered immune to this important abomasal parasite. The profile of IgA immunoreactivity detected against *T. circumcincta* L3 antigens was similar to that identified previously when L3 antigens were probed with antibody-secreting cell probes derived from abomasal lymph node cells of sheep which had been subjected to a trickle infection of *T. circumcincta* L3s weekly for 9 weeks followed by a

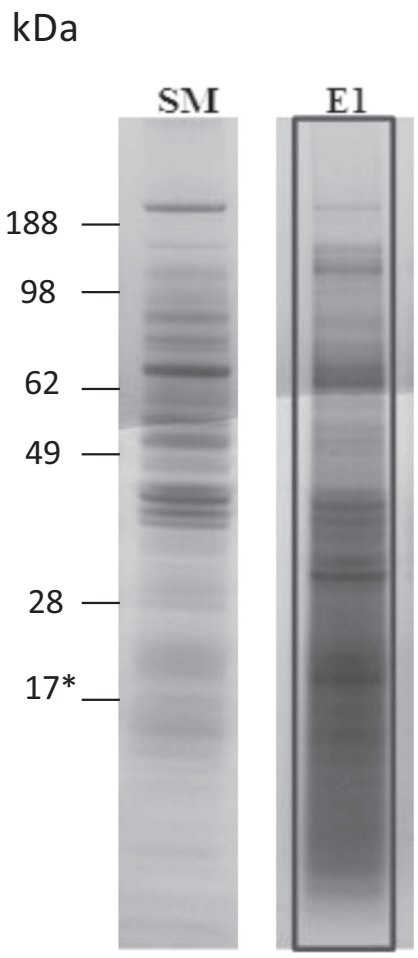

Fig. 3. SDS–PAGE gel demonstrating IgA-immunoaffinity enrichment of *Teladorsagia circumcincta* L3 extract. A sample of *T. circumcincta* L3 extract (SM) was passed through a column of immobilised IgA purified from the abomasal mucus of previously-infected sheep which had been given a single challenge of 50,000 *T. circumcincta* L3s and necropsied 2 days later. The eluted, immunoaffinity-enriched fraction (E1, framed) was collected in 0.1 M glycine–HCl, 6 M urea, pH 2.4. Proteins were stained with Simply Blue stain.

bolus challenge of L3s (Balic et al., 2003). The complexity of the immunoreactive profile of the L3 extract, coupled with the potential for SDS–PAGE to denature and disrupt conformational epitopes, led us to avoid traditional 2-D electrophoresis/immunoblotting techniques (cf. Smith et al., 2009) and here we employed immunoaffinity chromatography coupled with LC–ESI-MS/MS analysis to identify IgA-reactive antigens in the L3 extracts. The proteomic analysis identified a range of proteins including antigens identified as vaccine candidates in this and other systems; in particular, activation-associated secretory proteins (ASPs), galectins and several enzymes involved in detoxification processes.

ASP-like and venom allergen-like (VAL) proteins are key vaccine candidates in a number of systems (Hawdon et al., 1996, 1999) and are highly represented in the transcriptome and proteome of *T. circumcincta* L4s (Nisbet et al., 2008, 2010). ASPs are nematode-specific members of a diverse family (SCP/Tpx-1/Ag5/PR-1/Sct, SCP/TAPS) represented in all eukaryotes (Cantacessi et al., 2009, 2012) and are thought to be involved in worm establishment in the host (Tawe et al., 2000). Here, several IgA affinity-purified proteins showed high homology to ASPs from the hookworm *Necator americanus* (Na-ASP-2) and a C-type single domain ASP (ASP-3) from *Ostertagia ostertagi* (Oo-ASP-3). Na-ASP-2 was previously the lead candidate for the human hookworm vaccine initiative (Bethony et al., 2005) and a randomised, placebo-controlled double-blind vaccination trial with Na-ASP-2 indicated that immunisation of humans with recombinant Na-ASP-2 with alhydrogel adjuvant induced antigen-specific serum IgG titres statistically significantly higher than controls (Bethony et al., 2008; Diemert et al., 2012). In *O. ostertagi*, ASPs are also prime vaccine candidates (Meyvis et al., 2007). The identification of an ASP polypeptide dominant in *T. circumcincta* L3s supports the proposed role of ASPs in nematode establishment (Hawdon et al., 1996, 1999). Here, another member of the SCP/TAPS protein family was identified; this was a cysteine-rich secretory VAL protein, which is closely related to ASPs (Mitreva et al., 2007). In *Brugia malayi*, VAL-1 is a target of IgG3 and IgG4 in 95% of microfilaraemic patients (Murray et al., 2001). Vaccination with recombinant *B. malayi* VAL-1 in jirds indicated there was a protective response with a 64% decrease in the number of parasites recovered post-challenge in immunised jirds compared with controls (Murray et al., 2001).

Immunoreactivity of structural and muscle-derived proteins, including paramyosin, myosin and calponin, during natural exposure has been observed in other proteomic studies of other helminth species (Curwen et al., 2004; Kiel et al., 2007; Bennuru et al., 2009; Murphy et al., 2010). Paramyosin has been identified as a vaccine candidate for a range of helminths, including *B. malayi* (Li et al., 1999, 2004), *Taenia solium* (Vázquez-Talavera et al., 2001), *Trichinella spiralis* (Yang et al., 2010; Wei et al., 2011) and *Schistosoma japonicum* (McManus et al., 2001). In *T. circumcincta*, Murphy et al. (2010) also identified paramyosin in L3 extracts using proteomic analysis. In those studies, immunoblotting revealed that naturally infected lambs develop a specific serum IgE response to this protein.

In the work presented here, two enzymes involved in detoxification were also identified in the IgA affinity-purified extract: extracellular superoxide dismutase and glutathione-S-transferase. Detoxifying antioxidant enzymes scavenge free radicals produced during oxidative stress (Sheehan et al., 2001) and could play a key role in parasite survival. Superoxide dismutase activity is higher in L3s than in adult stages of a number of gastrointestinal parasitic nematode species, including *T. circumcincta* (Knox and Jones, 1992; Hadas and Stankiewicz, 1998) and may be involved in counteracting the oxidative stress associated with the mucosal inflammatory response during parasite invasion (Knox and Jones, 1992).

A further protein identified in the immunoaffinity-purified *T. circumcincta* extract was thrombospondin. In *Haemonchus contortus*, thrombospondin is highly immunogenic (Kooyman et al., 2009) and is associated with the components of the galectin-containing lead vaccine candidate complex, H-gal-GP; however its transcript was previously not detected in *H. contortus* L3s (Skuce et al., 2001). Galectins were also highly represented in the *T. circumcincta* immunoaffinity purified extract here. These lectins have a high affinity for β-galactosides and have been identified in a number of helminths (Klion and Donelson, 1994; Greenhalgh et al., 1999, 2000; Newlands et al., 1999), including *T. circumcincta* L3s (Newton et al., 1997). The role of galectins is not fully understood, but these proteins may be involved in modulating the host immune responses by mimicking host galectins (Young and Meeusen, 2004; Vasta, 2009).

Although a large number of proteins were identifiable in the IgA-affinity-purified *T. circumcincta* L3 extract, there were a considerable number that were not assigned identities or functions. The genome sequencing project for *T. circumcincta* (http://www.sanger.ac.uk/research/projects/parasitegenomics) is not yet complete and it is inevitable that sequence coverage of the selected proteins will be improved once this genome project is complete.

Disruption of carbohydrate moieties on the *T. circumcincta* L3 antigens altered the IgA binding profile, indicating that a

Table 1
A selection of functionally-identified, potential vaccine candidate proteins identified from the proteomic analysis of an IgA-immunoaffinity purified fraction of *Teladorsagia circumcincta* L3s.

Protein description[a]	Organism	Accession number	MOWSE[b]	No. of peptides	Estimated mol. weight (kDa)
ASP-2	*Necator americanus*	AAP41952	166.1	13	26.8
ASP-3	*Ostertagi ostertagi*	CAO00416	100.0	2	79.1
Venom allergen-like (VAL) protein	*Caenorhabditis brenneri*	EGT59294	441.1	9	66.6
DVA-1 polyprotein allergen	*Dictyocaulus viviparus*	Q24702	122.2	2	78.1
Superoxide dismutase (extracellular)	*Haemonchus contortus*	P51547	401.1	6	66.7
Glutathione-S-transferase	*H. contortus*	AAF81283	614.7	9	64.1
Thrombospondin	*H. contortus*	AFO43121	468.2	9	171.8
Paramyosin	*H. contortus*	CBO16022	365.4	6	113.1
Myosin	*Caenorhabditis briggsae*	CAP36983	382.2	5	159.6
Calponin	*Caenorhabditis elegans*	O01542	201.5	5	43.2
Ferritin	*C. elegans*	CE20622	285.4	5	76.5
Beta-D-galactosidase	*Brugia malayi*	AAA27859	367.2	8	66.3
Fructose bisphosphate aldolase	*T. circumcincta*	CBO37380	190.5	3	57.1
Galectin 1	*T. circumcincta*	AAD39095	533.1	9	91.4
Galectin	*Angiostrongylus cantonensis*	AEK98127	296.4	7	56.2
Transthyretin like	*C. brenneri*	EGT36246	209.5	4	56.4
Fatty acid/retinol binding protein	*H. contortus*	CDJ96356	272.8	4	49.8
Putative HEH-1, lipid binding	*C. elegans*	O17271	238.7	4	61.4
Peptidyl prolyl cis–trans isomerase	*O. ostertagi*	P52013	219.1	3	55.0
Pterin-4-alpha carbinolamine	*C. elegans*	Q9TZH6	224.7	4	35.7
Cytochrome C	*H. contortus*	ACG69807	229.4	5	39.3
Putative ES protein F7	*O. ostertagi*	CAD20464	196.0	4	41.6
DUF148-containing, gut-associated	*T. circumcincta*	AAM45145	371.7	7	68.6
Phosphatidylethanolamine binding	*H. contortus*	O16264	247.2	3	19.2
Intermediate filament protein	*Ascaris suum*	ADY43340	52.6	2	42.5
Saponin like	*C. elegans*	NP741465	71.3	2	37.3

[a] All proteins were identified by searching MS data against publically-available and in-house transcriptomic databases. Coverage of the full-length protein sequence by the assigned peptides is shown under the percentage of sequence coverage.
[b] The protein molecular weight search (MOWSE) score is shown. All scores are above the threshold and are significant at the 95% confidence threshold.

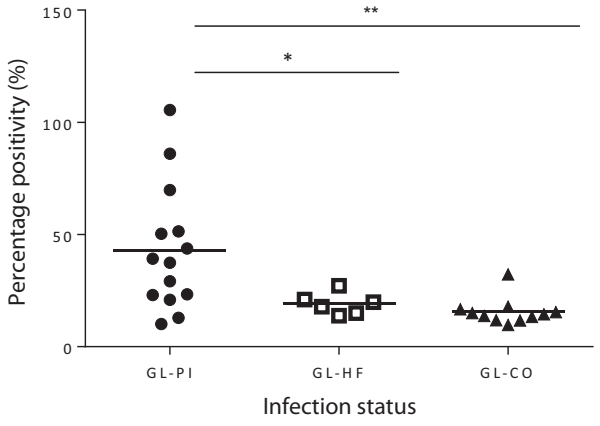

Fig. 4. Levels of binding of IgA in efferent gastric lymph to IgA-immunoaffinity purified *Teladorsagia circumcincta* L3 antigens. ELISA plates were coated with IgA-immunoaffinity purified L3 extract and incubated with efferent gastric lymph from individual sheep of different infection status: Group GL-PI, previously-infected/bolus challenge ($n = 13$); Group GL-CO, single bolus infection ($n = 10$); and Group GL-HF, helminth-free ($n = 6$). Samples from Groups GL-PI and GL-CO were from a single time-point of 7 days post-challenge with 50,000 *T. circumcincta* L3s. Results are expressed as percentages of the positive control. The positive control was efferent gastric lymph pooled from previously-infected/challenged (GL-PI) sheep from 6–10 days post-challenge. Samples were performed in triplicate with two independent repetitions. Mean IgA concentrations of GL-PI sheep were statistically significantly higher than GL-CO (**$P = 0.004$) and GL-HF (*$P = 0.044$), respectively.

proportion of the immunoreactivity was directed against glycans. Numerous nematode antigens contain unique and immunogenic glycans, some of which may play a role in immune evasion (Schallig and van Leeuwen, 1996; Vervelde et al., 2003; Nyame et al., 2004; Kooyman et al., 2007; Harrison et al., 2008; van Die and Cummings, 2010; Van Stijn et al., 2010). The three main strongylid nematodes of sheep, *T. circumcincta*, *H. contortus* and *Trichostrongylus colubriformis*, each possess three immunodominant surface molecules, two of which are thought to lack proteins (Maass et al., 2007). One of these molecules, carbohydrate larval antigen (CarLA), is an L3-specific surface antigen. High antibody levels generated against CarLA in infected animals have been associated with a reduction in larval establishment, although the molecule itself is not considered to be an effective vaccine candidate (Harrison et al., 2003a, 2003b, 2008).

Teladorsagia circumcincta L3 and L4 antigen-specific IgA levels in abomasal mucus have been found previously to have an inverse relationship with the length of L4 parasites recovered from the abomasum (Stear et al., 1999,2004a,b; Strain and Stear, 1999). Here, statistically significant relationships between IgA reactivity to the purified antigens and parasitological correlates of immunity revealed that the enriched antigens could be involved in the induction of protective responses directed at regulation of total burden and inhibition of parasite development. This is consistent with findings from another study where a statistically significant positive correlation was found between levels of anti-*T. circumcincta* L3 serum IgA and increased frequency of inhibited L4s (Beraldi et al., 2008). In addition, levels of circulating serum IgA generated against *T. circumcincta* L3 extracts were statistically significant and correlated with a reduction in the number of total adult worms measured at necropsy (Beraldi et al., 2008). Taken together, these findings suggest that mucus IgA directed against L3 antigens has an important role in controlling *T. circumcincta* development. In the current study, some of the antigens involved in the induction of these responses have been identified and the next steps are to select the most appropriate antigens from this suite of proteins, so that they can be exploited to enhance efficacy of the prototype *T. circumcincta* vaccine recently demonstrated to induce statistically significant protection in lambs (Nisbet et al., 2013).

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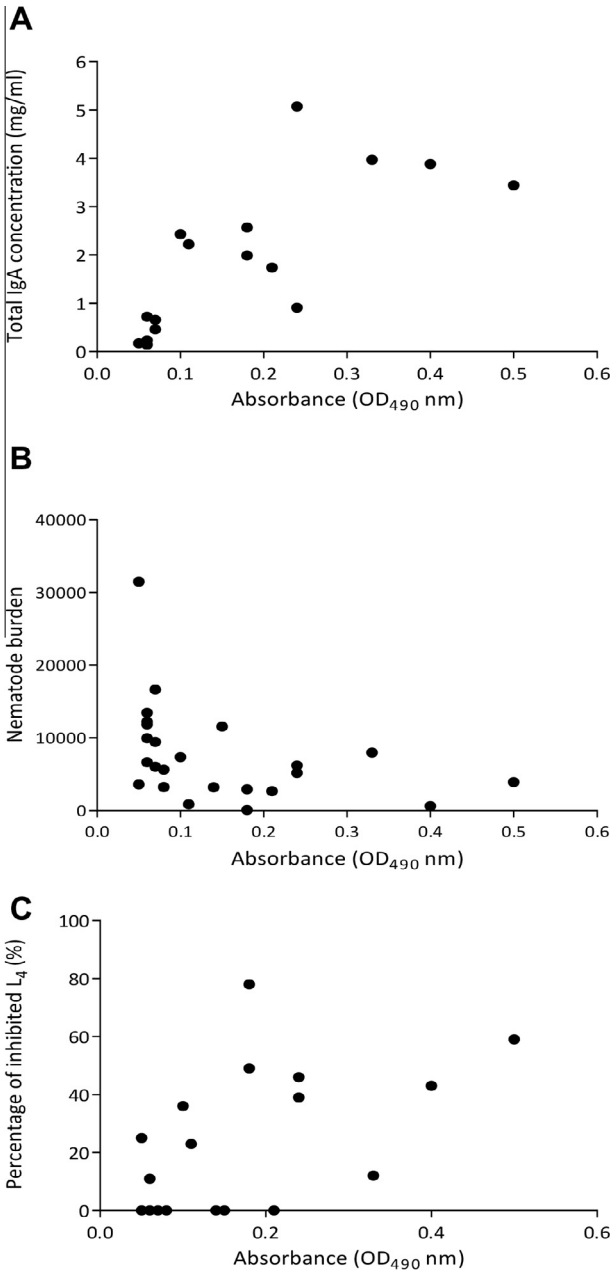

Fig. 5. Relationship between immunoaffinity-enriched L3 antigen-specific IgA levels in efferent gastric lymph and correlates of immunity to *Teladorsagia circumcincta* in experimentally-infected sheep. Relationships between immunoaffinity-purified antigen-specific IgA and total IgA levels in gastric lymph (A); worm burden at post-mortem (B); and the percentage of inhibited *T. circumcincta* L4s (C) in sheep previously infected/challenged with *T. circumcincta*. Data points represent absorbance (O.D.) values from gastric lymph samples of individual sheep which had been: (i) previouslyinfected with *T. circumcincta* and then subjected to a challenge of 50,000 L3s ($n = 12$), (ii) subjected to a single challenge of 50,000 L3s ($n = 4$) and (iii) helminth naïve ($n = 4$). The gastric lymph samples used were all from a single time-point (7 days post-challenge). The worm burdens of the sheep were assessed by counting the number of males, females and inhibited L4s in a sub-sample of the digests of the abomasal tissue and contents (Halliday et al., 2007).

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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.ijpara.2014.05.007.

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