Phagocyte-specific S100 proteins in the local response to the *Echinococcus granulosus* larva

TATIANA BASIKA[1]†, NATALIA MUÑOZ[1]‡, CECILIA CASARAVILLA[1], FLORENCIA IRIGOÍN[1]§, CARLOS BATTHYÁNY[2], MARIANA BONILLA[1], GUSTAVO SALINAS[1], JOSÉ PEDRO PACHECO[3], JOHANESS ROTH[4], ROSARIO DURÁN[2] *and* ALVARO DÍAZ[1]*

[1] *Cátedra de Inmunología, Departamento de Biociencias (Facultad de Química) e Instituto de Química Biológica (Facultad de Ciencias), Universidad de la República, Montevideo, Uruguay*
[2] *Unidad de Bioquímica y Proteómica Analíticas, Instituto Pasteur de Montevideo, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay*
[3] *Departamento de Patología, Facultad de Veterinaria, Universidad de la República, Montevideo, Uruguay*
[4] *Department of Dermatology, University of Münster, Münster, Germany*

(*Received 8 April 2011; revised 16 August 2011; accepted 6 September 2011; first published online 5 January 2012*)

SUMMARY

Infection by larval *Echinococcus granulosus* is usually characterized by tight inflammatory control. However, various degrees of chronic granulomatous inflammation are also observed, reaching a high point in infection of cattle by the most prevalent parasite strain worldwide, which is not well adapted to this host species. In this context, epithelioid and multinucleated giant macrophages surround the parasite, and the secreted products of these cells often associate with the larval wall. The phagocyte-specific S100 proteins, S100A8, S100A9 and S100A12, are important non-conventionally secreted amplifiers of inflammatory responses. We have analysed by proteomics and immunohistochemistry the presence of these proteins at the *E. granulosus* larva-host interface. We found that, in the context of inflammatory control as observed in human infections, the S100 proteins are not abundant, but S100A9 and S100A8 can be expressed by eosinophils distal to the parasite. In the granulomatous inflammation context as observed in cattle infections, we found that S100A12 is one of the most abundant host-derived, parasite-associated proteins, while S100A9 and S100A8 are not present at similarly high levels. As expected, S100A12 derives mostly from the epithelioid and multinucleated giant cells. S100A12, as well as cathepsin K and matrix metalloproteinase-9, also expressed by *E. granulosus*-elicited epithelioid cells, are connected to the Th17 arm of immunity, which may therefore be involved in this granulomatous response.

Key words: *Echinococcus granulosus*, S100 proteins, granuloma, cathepsin K, metalloproteinase-9.

INTRODUCTION

The larval stage (metacestode) of the taeniid cestode *Echinococcus granulosus* causes cystic echinococcosis, also called hydatid disease, in a variety of livestock species as well as in humans (reviewed by Thompson (1995)). The *E. granulosus* metacestode is a bladder-like structure (hydatid) that dwells in the parenchymas of internal organs, most commonly liver and lungs, and can reach up to tens of cm in diameter. The hydatid is defined by the hydatid wall (HW), a structure comprising a thin inner layer of cells (germinal layer, GL) and the massive outer laminated layer (LL). The LL (reviewed by Díaz *et al.* (2011*a*, *b*)) is a peculiar extracellular structure formed by a meshwork of mucins bearing galactose-rich glycans. Additionally, in *E. granulosus* but not other species of the genus, it contains nano-deposits of calcium inositol hexakisphosphate ($InsP_6$) (Irigoín *et al.* 2004). Being up to 3 mm thick, and permeable to macromolecules, the LL represents a very large area for the adsorption of diffusible proteins (Coltorti and Varela-Díaz, 1974; Casaravilla *et al.* 2006).

Whereas establishing larval *E. granulosus* elicits local inflammatory responses, these responses normally resolve upon parasite deployment of the LL. Thus established hydatids normally grow surrounded by a non-infiltrated or minimally infiltrated host-derived collagen capsule (reviewed by Díaz *et al.* 2011*a*). This is readily seen in both sheep and human infections (Yamashita *et al.* 1961; Mufarrij *et al.* 1990). Although certainly a key aspect of this parasite's survival strategy, inflammatory

* Corresponding author: Cátedra de Inmunología, Instituto de Higiene, Avenida Alfredo Navarro 3051, Montevideo, CP 11600, Uruguay. Tel/Fax: +59824874320. E-mail: adiaz@fq.edu.uy.
† Current address: Unidad de Biología Parasitaria, Instituto de Higiene y Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay.
‡ Current address: Departamento de Produccion y Desarrollo Biotecnológico, Instituto de Higiene, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay.
§ Current address: Departamento de Histología y Embriología, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay.

doi:10.1017/S003118201100179X

resolution does not always take place. Chronic inflammation can often be seen, even in host species (sheep, humans) considered suitable for the G1 strain of the parasite, the most prevalent worldwide (Jenkins *et al*. 2005). The prototypical case of lack of inflammatory control is infection of cattle by this parasite strain. As a result of an undetermined host-parasite mismatch, chronic local inflammation is the rule, and parasite vitality is accordingly compromised (Rao and Mohiyuddin, 1974; Bortoletti and Ferretti, 1978; Sakamoto and Cabrera, 2003; Díaz *et al*. 2011*a*). Intermediate and variable degrees of inflammation are observed in pig infections (Slais and Vanek, 1980).

When present, the chronic inflammatory response to *E. granulosus* is typically granulomatous. A layer of palisading epithelioid and multinucleated giant macrophages is directly apposed to the parasite's LL (Nieberle and Cohrs, 1967; Slais and Vanek, 1980). Behind this first layer is a mononuclear cell infiltrate, featuring lymphocytes, plasmocytes, conventional macrophages and some eosinophils. More externally and/or intermixed with the mononuclear cell infiltrate is a layer of fibroblasts and collagen. A similar granulomatous response is (invariably) elicited by the highly invasive larval stage of *Echinococcus multilocularis*. In this context it has been demonstrated that the granuloma is T-cell dependent, and that is it damaging to the parasite (Gottstein and Hemphill, 1997; Dai *et al*. 2004).

The large capacity of the parasite's LL to adsorb proteins implies that the secreted products of the local host reaction tend to accumulate in the HW. Additional host proteins in HW extracts can derive from remnants of epithelioid cells that adhere tightly to the LL. By far the major parasite-derived macromolecules in HW extracts are the LL structural mucins (since the LL is quantitatively very dominant over the GL). As these mucins are highly insoluble, conventionally prepared HW extracts are dominated by host proteins (Casaravilla and Díaz, 2010; Díaz *et al*. 2011*a*). When HW derive from a non-resolutive (granulomatous) context, these proteins include prominently the products of the epithelioid and multinucleated giant cells (MGC) (Díaz *et al*. 2000*a*, *b*; Marco *et al*. 2006). This has led to observations of general interest for granuloma biology being made initially in the *E. granulosus* sytem (Díaz *et al*. 2000*b*).

In this study, we investigated the association of phagocyte-specific S100 proteins with the granulomatous response to the *E. granulosus* metacestode. S100 is a large family of cytosolic calcium-binding proteins thought to regulate cytoskeletal function and other calcium-dependent cellular responses. Three S100 proteins, namely S100A8, S100A9 and S100A12 are expressed prominently by myeloid cells, and therefore referred to as the phagocyte-specific S100 protein subfamily (reviewed by Ehrchen *et al*. 2009 and Pietzsch and Hoppmann, 2009). They form Ca^{2+} and Zn^{2+}-dependent dimers and higher oligomers; while S100A9 and S100A8 most commonly heterodimerize (oligomerize), S100A12 associates only with itself. These proteins are actively secreted by a non-conventional mechanism and fulfil extracellular functions. The clearest of these is as amplifiers of the inflammatory response, i.e. as endogenous danger-associated signals (DAMPs). S100A8 and also the S100A9/S100A8 heterodimer are agonists of TLR4, and this interaction has a strong impact in inflammatory disorders (Vogl *et al*. 2007; Loser *et al*. 2010). S100A12 is thought to be an agonist of the receptor for advanced glycation end-products, RAGE (Hofmann *et al*. 1999). Functions are not necessarily well conserved across mammalian species: S100A12 is absent in rodents, and rodent S100A8 has been proposed to be the functional homologue of human S100A12 (Pietzsch and Hoppmann, 2009). We reasoned that the analysis of the phagocyte-specific S100 proteins across the inflammation-resolution spectrum in hydatid disease may contribute a valuable element towards understanding the regulation of local inflammation in this infection.

MATERIALS AND METHODS

Parasite materials

Hydatids from mouse experimental infections were retrieved 8–12 months after intraperitoneal inoculation of protoscoleces obtained from natural bovine infections. Human hydatid surgical samples (fresh and/or paraffin-embedded) were obtained from the Clínica de Cirugía Pediátrica, Hospital Pereira-Rossel (Dr G. Giannini), and the Laboratorio de Anatomía Patológica, Hospital Maciel (Dr M. Roldán), both in Montevideo, Uruguay. Bovine, sheep and pig hydatid material was from natural livestock infections in Uruguay. For the bovine host, a panel of paired fresh (for protein extracts) and paraffin-embedded (for inflammatory scoring and immunohistochemistry) samples was set up.

Hydatid wall protein extracts

Hydatid walls (HW; comprising LL and GL) were retrieved from fresh samples as described by (Irigoín *et al*. 2002). HW were washed with PBS containing 0·5 mM $CaCl_2$ to remove loosely bound proteins. They were then extracted using PBS containing the calcium chelators EGTA or EDTA (in excess of the molar amount needed to solubilize the calcium InsP_6 deposits (Díaz *et al*. 2011*a*), with 2 M NaCl, or sequentially with both agents, as indicated. The protease inhibitors PMSF (2 mM), iodoacetamide (2 mM), pepstatin A (2 μg/ml), and E-64 (100 μM) were added in each step. Extracts were concentrated

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prior to analysis by precipitation with 10% (w/v) trichloroacetic acid.

Antibodies

Rabbit polyclonal antibodies against human and mouse S100A9 and S100A8, and human S100A12 were raised as previously described (Zwadlo *et al*. 1988; Roth *et al*. 1993; Vogl *et al*. 1999*a*). A monoclonal antibody against human S100A9 (clone S36·48) was purchased in biotinylated form from BMA Biomedicals AG (Switzerland); this antibody works in paraffin-embedded sections but does not react with the denatured protein in Western blotting. A rabbit polyclonal antibody was raised against bovine S100A12 recombinantly expressed in *E. coli*. Total RNA was obtained from bovine peripheral blood leukocytes (buffy coat), reverse-transcribed using an oligodT primer, and the coding sequence for S100A12 amplified by nested PCR using the primers: tctcctgaaggtgaacgtagt (outer forward), cggatccatgactaagctggaagat (inner forward), cgtcgacaagcttctactctttgtggatatct (inner reverse) and cgtcgaccgggtaaggcagcctcaggg (outer reverse). The amplicon was ligated into pGEM-T Easy (Promega), and the insert then excised using *Bam*HI and *Sal*I and introduced into the pET28a vector (Novagen) for expression in *E. coli*. The fusion protein was purified by nickel affinity chromatography from the soluble fraction of bacterial lysates. The recombinant protein was also bound to CNBr-activated Sepharose (Sigma) for affinity purification of the rabbit antibodies obtained.

SDS-PAGE and Western blotting

HW extracts were run under reducing conditions, either in conventional SDS-PAGE (10% (w/v) acrylamide) or in the Tris-tricine system (16·5% (w/v) acrylamide), and Coomassie blue- or silver-stained as indicated. Alternatively, proteins were transferred to nitrocellulose membranes for Western blotting. Membranes were probed with the specific rabbit antisera, followed by alkaline phosphatase goat anti-rabbit IgG (Calbiochem), and developed with nitro-blue tetrazolium/ 5-bromo 4-chloro 3-indolyl phosphate (NBT/BCIP; Sigma) substrate. Control membranes in which normal rabbit serum was used in the first probing step gave no staining.

One dimensional SDS-PAGE and MALDI-TOF-based proteomics

Selected Coomassie blue-stained protein bands were digested with sequencing-grade trypsin, and peptides analysed by MS and MS/MS in the Applied Biosystems 4800 Analyzer. Proteins were then identified by searching the NCBI nr database (2010) using the MASCOT program in the 'sequence query' mode. The following search parameters were employed: monoisotopic mass tolerance 0·08–0·10 Da; fragment mass tolerance 0·2–0·6 Da; methionine oxidation, and in cases also propionamide addition to cysteine residues and/or acetylated protein N-terminus, as variable modifications; 1 missed tryptic cleavage allowed. Significant peptide and/or protein scores ($P < 0{\cdot}05$) were used as criteria for positive protein identification. In initial experiments using equipment without MS/MS capabilities (Voyager DE-Pro also from Applied Biosystems), a tryptic peptide fingerprinting approach was taken, leading to presumptive identifications that were later confirmed by MS/MS as above and/or by Western blotting using specific antibodies.

Nano-LC-MS-based proteomics

HW 2 M NaCl extracts were dialysed using 3500 kDa cut-off membranes against 10 mM ammonium bicarbonate, 0·5 mM EDTA and protein content in them measured by absorbance at 280 nm. Proteins in the extracts were reduced, carbamidomethylated, and digested with sequencing-grade trypsin (1:10 enzyme to total protein ratio, 24 h at 37 °C) in the presence of 1 M guanidine hydrochloride. Samples were then injected into a nano-HPLC system (Proxeon easy-nLC, Thermo Scientific) fitted with a reverse-phase column (*easy* C18 column, 3 μm; 75 μm ID × 10 cm; Proxeon, Thermo Scientific) and separated using a 0·1% (v/v) formic acid in water – 0·1% (v/v) formic acid in acetonitrile gradient (0–60% acetonitrile in 60 min; flow 400 nl/min). Online MS detection/ analysis was carried out in the LTQ Velos nano-ESI-linear ion trap instrument (Thermo Scientific) in the data-dependent triple play MS^2 mode (full scan followed by zoom scan and MS/MS of the top 5 peaks in each segment). Proteins were identified by searching the IPI database (bovine, 2010) using the following parameters in the Mascot software in the MS/MS ion search mode: peptide tolerance 400 ppm, MS/MS tolerance 0·8 Da, and cysteine carbamidomethylation, protein N-terminus acetylation, methionine oxidation and asparagine/glutamine deamidation as these allowed variable modifications. The significance limit for protein identification was set at $P < 0{\cdot}05$.

Immunohistochemistry

Microtome sections (0·5 mm thick) were dewaxed, treated with proteinase K as an antigen retrieval procedure and with 1% H_2O_2 in methanol to inhibit endogenous peroxidase activity, and blocked using 10% (v/v) goat serum. Bovine S100A12 was detected using affinity-purified rabbit antibodies; the purification flow-through was used as negative control, and

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gave no staining. Human S100A9 was detected with the biotinylated S36·48 monoclonal antibody followed by streptavidin-peroxidase (Sigma). Human S100A8 was detected with the rabbit polyclonal antiserum; normal rabbit serum used at the same concentration as control gave no staining. Sections probed with the rabbit primary antibodies were then incubated with peroxidase-conjugated goat IgG against rabbit IgG (Calbiochem). All sections were finally developed using diaminobenzidine substrate and counterstained with Mayer's haematoxylin. In parallel, sections from each sample were stained only with haematoxylin-eosin and scored for inflammatory status on an arbitrary scale from '−' (no inflammation, only collagen present) to '+++++' (intense granulomatous inflammation featuring a full rim of epithelioid cells surrounding the parasite).

RESULTS

Phagocyte-specific S100 proteins are associated with the E. granulosus *hydatid in different host species*

Treating *E. granulosus* HW with calcium chelators dissolves the calcium InsP_6 deposits (Irigoín *et al*. 2002, 2004). Being initially interested in proteins associated with these deposits, we prepared extracts using EGTA- or EDTA-containing buffer from intact hydatids obtained by experimental infection of mice. The extracts featured 3 major protein bands (Fig. 1 A). For the bands with apparent molecular masses 14 and 8 KDa, tryptic peptide fingerprinting suggested that they corresponded to host-derived S100A9 and S100A8. This was confirmed by MS/MS (Table S1 online version only) and by Western blotting (Fig. 1 B). Immunoblotting also showed that the association of mouse S100A8 with the HW was strictly Ca^{2+}-dependent, while that of S100A9 was less strictly so. Both proteins could be extracted even in the presence of Ca^{2+}, by high ionic strength.

Hydatids, developing after intraperitoneal infection of mice, grow loose in the peritoneal cavity, while those arising from natural infections are embedded within organ parenchymas. Also, the spectrum of inflammatory conditions observed in hydatid disease is not reproduced in this model, in which resolution is always observed (Richards *et al*. 1983; Breijo *et al*. 1998). In addition, as mentioned, rodents do not encode S100A12 in their genomes. We therefore analysed the presence of host S100 proteins in parasite samples from natural infections in cattle, pig, sheep, and humans. A limited proteomic screening was carried out, using one-dimensional gels and focusing on the apparent molecular mass of the monomeric S100 proteins (6–14 kDa) and of the S100A9/S100A8 dimer (24 kDa), which for unknown reasons can run as such even after denaturation and reduction (see for example Fig. 1 D, left-hand panel). This analysis showed that prominent 8 kDa bands in the extracts studied from cattle, pig and sheep origins corresponded to S100A12 (Fig. 1 C). Part of the S100A12 molecules from all 3 species appeared to be N-terminally acetylated (Tables S1 and S2, online version only), a modification previously observed for human S100A9 but not reported for S100A12 proteins (Ilg *et al*. 1996; Vogl *et al*. 1999*b*; McMorran *et al*. 2007). S100A12 was undetectable both by proteomic methods and by more sensitive Western blotting in the human sample analysed which, however, did contain immunochemically detectable S100A9 and S100A8 (Fig. 1 D). An immunochemical assessment of the presence of S100A9 and S100A8 in the non-human samples was precluded by the lack of suitable antibodies. In sum, the initial analysis suggested that association of host phagocyte-specific S100 proteins with the HW may be a general phenomenon but differences probably exist in terms of individual S100 proteins across different host species and/or inflammatory status of individual hydatids.

Host proteins unrelated to S100 that were identified in the limited proteomic studies described above included the pentraxin-family acute-phase proteins serum amyloid P (in mouse-derived samples; Fig. 1A; GeneBank Accession no. P12246) and C-reactive protein (in sheep-derived samples; Fig. 1C; GeneBank Accession no. EE780797, identified by TBLASTN search of mammalian ESTs). Non-S100 host proteins identified in the bovine sample will be discussed in the context of similar findings by nano-LC-MS-based proteomics described in the next section.

S100A12 is consistently associated with the E. granulosus *larva in the bovine host*

The cross-host species studies described in the previous section were hampered by the limited number of samples available and the lack of a histological assessment of the local inflammatory status for each sample. We therefore chose to focus on (readily available to us) bovine-derived samples, scoring individual samples for local inflammatory status. Although complete inflammatory resolution was never found, cattle hydatid samples displayed a wide range of intensities in inflammation, thus allowing the assessment of S100 proteins across different biological conditions.

In an analysis based on 1-D SDS-PAGE, a prominent 8 kDa band was identified proteomically as S100A12 in each of 9 independent samples studied (Fig. 2 and Table 2). The presence of S100A12 across all bovine-derived hydatid samples was confirmed by Western blotting (Fig. 2 and data not shown). S100A12 appeared to be more abundant in extracts from samples with middle to high inflammation

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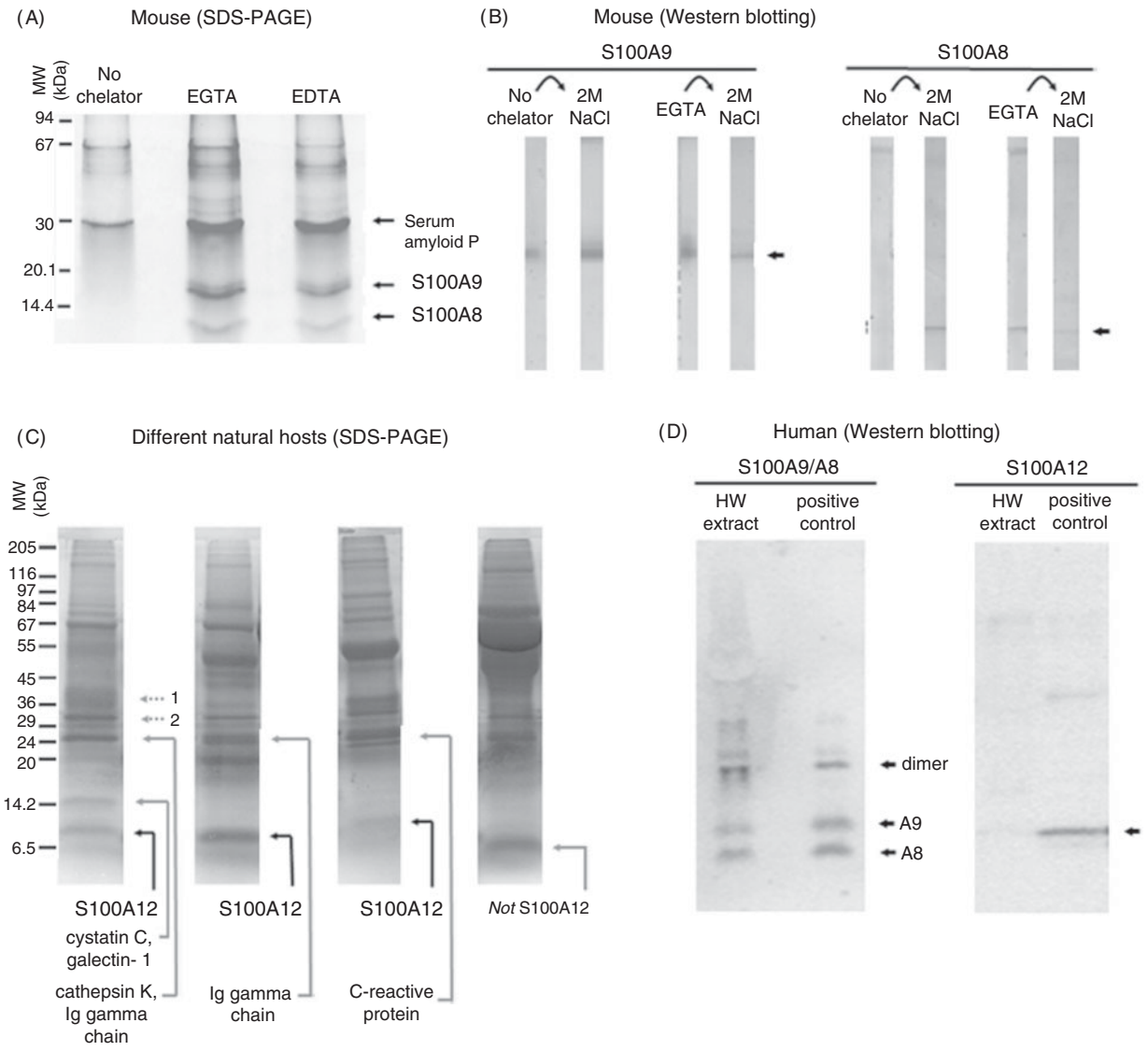

Fig. 1. Association of S100A8, S100A9 and S100A12 with the *Echinococcus granulosus* metacestode, in different hosts. (A) Intact mouse peritoneal hydatids were extracted externally with buffers without or with calcium chelators as indicated, and solubilized proteins were run on SDS-PAGE and selected Coomassie blue-stained bands studied by tryptic peptide fingerprinting; identifications were confirmed on similar samples by MS/MS-based proteomics (Table S1, online version only). (B) Mouse hydatids were extracted externally with buffer with or without calcium chelator as indicated and, in a second step, the proteins still associated with the hydatids in each case were extracted by use of 2 M NaCl. Extracted proteins were analysed by Western blotting with antibodies to mouse S100A9 or S100A8. (C) HW from natural infections in different hosts were extracted with buffers containing EDTA, solubilized proteins run on SDS-PAGE, and selected Coomassie blue-stained bands identified proteomically, as detailed in Table S1 (online version only). Bands marked as 1 and 2 correspond respectively to bovine cathepsin K pro-enzyme and truncated bovine annexin A2, on the basis of previously reported observations (Díaz *et al*. 2000*a*, *b*). The band marked 'not S100A12' could not be identified, but the possibility that it was human S100A12 was ruled out. (D) The extract of human host origin was analysed by Western blotting with antibodies to human S100A8 and S100A8 or to human S100A12. Native human S100A9/A8 dimer (100 ng) or recombinant human S100A12 (20 ng) were run as positive controls.

scores than in those samples with middle to low scores (Fig. 2). S100A9 and S100A8 were not detected, either as obvious separate bands or contaminating the S100A12 band.

In order to analyse more carefully the apparent selective abundance of S100A12 as compared to S100A9 and S100A8, we subjected several bovine host-derived samples to proteomic analysis by nano-LC-MS. In this approach, the total protein content of each sample is subjected to tryptic digestion and the resulting peptides separated and analysed, thus avoiding the bias inherent to picking bands or spots in electrophoresis gels. Also, protein abundances in the samples can be roughly estimated by emPAI (Ishihama *et al*. 2005). S100A12 was detected in all 7 samples analysed by this method (Table 1); as mentioned previously, some of the molecules were N-terminally acetylated (Table S3, online version

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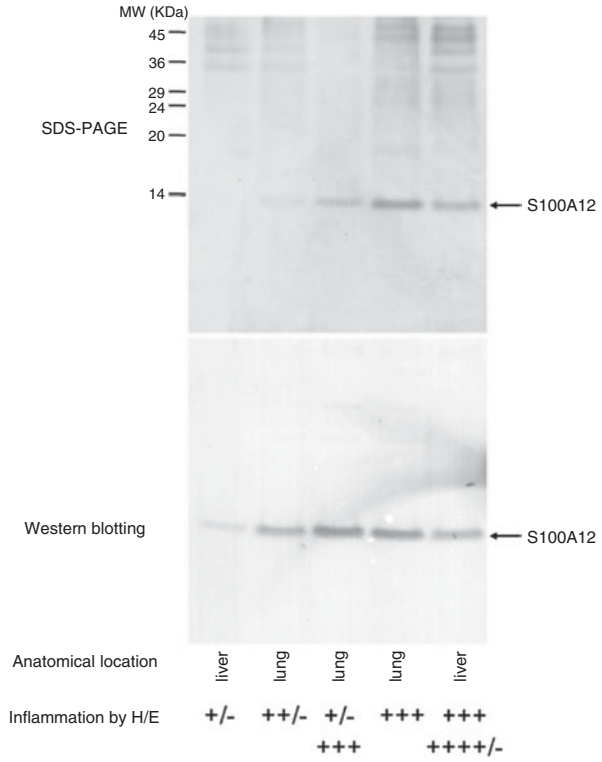

Fig. 2. Association of S100A12 with the reaction to the *Echinococcus granulosus* metacestode in representative samples from the bovine host. HW were extracted with 2 M NaCl, solubilized proteins run on SDS-PAGE and either silver stained (A), or transferred to nitrocellulose and probed with antibodies to bovine S100A12 (B). Portions from the same HW were fixed, paraffin-embedded, sectioned, haematoxylin-eosin stained, and scored from the intensity of inflammation surrounding the parasite, as indicated; in some cases 2 scores were given because inflammatory status varied between regions of each sample. The anatomical locations of the different hydatids are also indicated. The identity of the S100A12 band was confirmed proteomically after Coomassie blue staining of gels similar to that shown, as detailed in Table S2 (online version only).

only). The estimated abundance of S100A12 ranged between 4 and 27% of total host protein, and again samples with lower inflammatory scores tended to display lower relative abundances of S100A12 than those with higher scores. In relation to total tissue dry mass (of which most corresponds to the LL mucins (Díaz *et al*. 2011*b*)), the emPAI data and total protein contents of extracts allowed us to estimate that S100A12 is present in the range of 0·1–1 mg per g of tissue dry mass. S100A9 was not detected in any of the samples, while S100A8 was detected in a single sample, with an emPAI that was 18-fold lower than that of S100A12 (Table 1). The emPAI method is based on the ratio between experimentally observed signals and theoretically observable peptides for each protein (Ishihama *et al*. 2005). The Mascot software estimates this second figure on the basis of protein molecular mass. As the molecular masses of bovine S100A8 and S100A9 are similar to and larger than those of S10012 respectively, the number of observable peptides for S100A8 and S1009 is at least as high as that of S100A12. Using the emPAI formula and the fact that a single peptide can allow significant protein identification, we estimate that the abundances of S100A9 and S100A8 in the bovine-origin samples must be at least an order of magnitude lower than that of S100A12. In sum, in the host species in which local inflammation against the parasite is strongest and most maintained, S100A12 is a major product at the host-parasite interface, while S100A8 and S100A9 are not present in similarly high amounts.

Non-S100 host proteins detected in the LC-MS-based proteomics search across over half the individual samples analysed (and in cases also in the SDS-PAGE-based experiments shown in Fig. 1) are listed in Table S4, online version only. These include 2 proteins previously known to be abundant in this system (Díaz *et al*. 2000*a*, *b*), namely the cysteine protease cathepsin K and the cortical (non-conventionally secreted) protein annexin A2. Abundant host proteins newly identified included cystatin C, an inhibitor of papain-family cysteine proteinases including cathepsin K (reviewed by Turk *et al*. 2008), and regakine-1, a CC chemokine present at high concentrations in bovine plasma (Struyf *et al*. 2001). They also included galectin-1, a non-conventionally secreted anti-inflammatory mediator (reviewed by Rabinovich and Ilarregui, 2009) previously observed in hydatid fluid of the same host origin (Monteiro *et al*. 2010). Other proteins found worth mentioning were the calcification inhibitor α-2-HS-glycoprotein (reviewed by Lee *et al*. 2009) and the cytotoxic T cell and NK cell cytolytic protein granulysin (reviewed by Krensky and Clayberger, 2009). The proteins mentioned, including S100A12, can be considered representative of the subset of abundant (bovine) host-derived LL-associated proteins that can be solubilized by high ionic strength and/or EDTA. Other abundant host proteins exist in this system that require more drastic treatments for extraction, namely immunoglobulins and terminal complement components (our unpublished results).

Epithelioid cells and MGC from the host granuloma adjacent to the parasite are the main source of S100A12 in the bovine host

We analysed the distribution of S100A12 in the host-parasite interface of hydatid infection in cattle by immunohistochemistry. The protein was strongly expressed in the epithelioid and MGC and present in the necrotic remnants adhering to the surface of the LL (Fig. 3 B, C, D, E). Although the strongest and most consistent staining was in the epithelioid cell and MGC rim, S100A12 was also expressed by other

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Table 1. Semi-quantitation of S100 proteins in a panel of bovine host samples by LC-MS and emPAI

(The emPAI of S100 proteins detected in each sample was normalized by the added emPAI of all host proteins identified (at the $P<0{\cdot}05$ significance level) in the sample, thus giving an indication of relative abundance with respect to total host protein. S100A9 was not detected in any of the samples tested. ND stands for not determined.)

Inflammatory score of sample	Anatomical origin of sample	S100A12 (% of total emPAI)	S100A8 (% of total emPAI)
+/−	Liver	4·0	Not detected
ND	Lung	6·5	0·35
ND	Lung	6·8	Not detected
++/−	Lung	6·8	Not detected
ND	Lung	11·9	Not detected
+/−; +++	Lung	22·1	Not detected
+++; ++++/−	Liver	26·8	Not detected

Table 2. Summary of immunohistochemical findings

(H, L and N stand for high, low and no immunoreactivity, respectively.)

Bovine	Structure/cell type	S100A12
	HW (GL)	L
	HW (LL)	N
	Epithelioid cells	H
	Necrotic area	H
	Fibroblasts	H/N
	Macrophages (in macrophage-dominated infiltrate)	H
	Macrophages (in predominantly lymphoplasmocytic infiltrate)	H
	Lymphocytes and plasmocytes	N

Human	Structure/cell type	S100A8	S100A9
	HW (GL)	H	H
	HW (LL)	N	N
	Necrotic area	H	H
	Fibroblasts	L	L
	Macrophages (in predominantly lymphoplasmocytic infiltrate)	H	H
	Lymphocytes and plasmocytes	N	N
	Eosinophils	H	H

cells of the host reaction, namely morphologically conventional macrophages present in the mononuclear cell infiltrate (Fig. 3 A, C, F), and some fibroblasts (Fig. 3 A, C, G). S100A12 was also observed to be expressed by alveolar macrophages, but this appeared to be independent of the presence of the parasite as it was also observed in samples taken from tens of cm from the infection site (not shown). As expected, when the local inflammatory reaction was weak, S100A12 staining was also weak, restricted to some macrophages infiltrating the collagenous capsule (Fig. 3 A). As for the parasite structures, we did not find clear-cut evidence of the presence of S100A12 within the LL itself, but since it is generally very difficult to stain proteins within the LL in immunohistochemistry (Stadelmann *et al*. 2010; Díaz *et al*. 2011*a*), we do not take this as evidence against the protein being also present in the interior of the LL. In contrast, the S100A12 antibodies did stain the GL (not shown). The GL is known to take up host proteins, as observed by immunohistochemistry and by proteomics (Díaz *et al*. 2000*a*; Monteiro *et al*. 2010). The immunohistochemical findings on S100A12 are summarized in Table 2. The strong expression of S100A12 by the epithelioid cells and MGC directly apposed to the HW explains the consistent presence of this protein in the extracts from bovine-origin HW samples (Fig. 2 and Table 1).

Eosinophils distal to the parasite can express S100A9 and S100A8 in the human host

Human hydatid samples are, in most cases, characterized by the lack of inflammation, in particular in the tissue directly apposed to the parasite. Thus, this type of sample could complement the bovine hydatid samples, which are characterized by continuing inflammation. The non-infiltrated collagenous capsule of human hydatid samples generally did not stain

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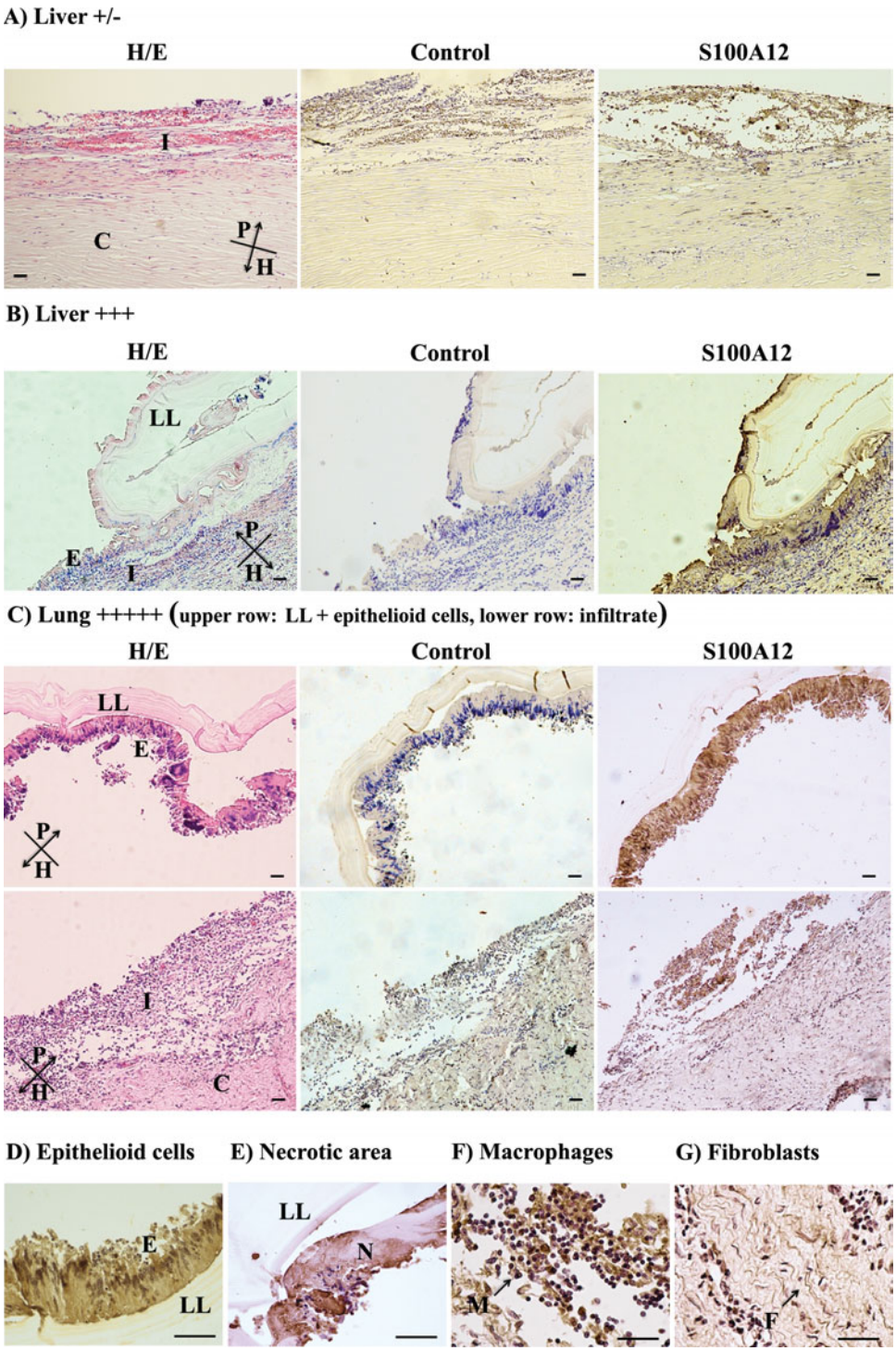

Fig. 3. Immunohistochemical detection of S100A12 in the host-hydatid interface in the bovine host. Paraffin-embedded sections of the local reaction to hydatids, including whenever possible the HW itself, were stained with haematoxylin-eosin (H/E), or subjected to immunohistochemical detection using affinity-purified antibodies to bovine S100A12 or control antibodies (corresponding to the flow-through of the affinity purification). (A–C) Panoramic views of 3 representative samples with different degrees of inflammation; inflammatory scores are given besides the hydatid localization in each case. In (C), the LL and attached host epithelioid cells have separated from the remainder of the host tissue: the 2 components are shown in separate micrographs. (D–G) Higher resolution views of the main elements showing positive reaction for S100A12: epithelioid cells (and MGC), necrotic area found between host reaction and LL, morphologically conventional inflammatory macrophages, activated fibroblasts and lung parenchyma. In each H/E stain in (A–C), the approximate orientation of the host (H)-parasite (P) interface has been indicated. LL, laminated layer; E, epithelioid and MGC; I, mononuclear cell-dominated infiltrate; C, collagen (and fibroblasts); F, fibroblast; AM, alveolar macrophage. Scale bars represent 100 μm throughout.

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for S100A9 or S100A8 (Fig. 4 A, B); however, some regions of collagen did give positive, extracellular, staining for both proteins (Fig. 4 D). Necrotic remnants adhered to the HW also stained for both proteins (Fig. 4 C). Some human samples feature inflammatory infiltrates that are distal to the parasite, so that the host structure in contact with the larva is still the collagenous capsule. In some cases, these infiltrates are dominated by lymphocytes/plasmocytes (Fig. 4 C); in this situation, macrophages interspersed in the lymphoplasmocytic infiltrate stained for S100A9 and S100A8 (not shown). Other human samples have infiltrates dominated by eosinophils, which expressed S100A9 and S100A8 strongly (Fig. 4 B, E). These observations are summarized in Table 2.

Overall, S100A9 and S100A8 are only weakly expressed at the host-parasite interface in conditions of inflammatory resolution or low inflammation in human hydatid disease. In the situation of existing (low) inflammation, they are most prominently expressed by eosinophils, and also macrophages, neither directly apposed to the parasite. These results are broadly consistent with the presence of small amounts of S100A9 and S100A8, detectable by Western blotting but not readily detected by SDS-PAGE, in an LL extract of human hydatid origin (Fig. 1 C, D).

DISCUSSION

In this work, we detected the phagocyte-specific S100 proteins associated with the HW of *E. granulosus* developed in different host species. In experimental infection in mice (a species in which S100A12 is absent), S100A9 and S100A8 were among the major host proteins associated with the HW. In cattle, a host species whose genome does encode S100A12, this protein was instead the dominant subfamily member associated with the parasite.

We did not determine what proportion of the S100 proteins in our extracts arose from necrotic deposits and/or epithelioid cells attaching to the LL *vs* from the interior of the LL itself. The lack of staining of the LL observed by immunohistochemistry (for bovine S100A12 in particular) is not informative, as the LL is remarkably refractory to immunostaining (Díaz *et al*. 2011*a*). It should be noted that, in common with other host proteins previously found to be associated with the HW (Díaz *et al*. 1997; Díaz *et al*. 2000*a*,*b*), S100A12 and the S100A9/S100A8 dimer are known to bind heparin and related glycosaminoglycans (Robinson *et al*. 2002; Liu *et al*. 2009). This is also the case of serum amyloid P (Hamazaki, 1987; Heegaard *et al*. 2006), identified in this study as a further major HW-associated protein in mouse infections. Calcium InsP_6 may contribute anionic sites for adsorption of host proteins (Irigoín *et al*. 2008), and the calcium-dependent extraction of S100 proteins is compatible with at least part of them being adsorbed onto the calcium InsP_6 deposits. We obtained preliminary evidence indicating adsorption of exogenously added purified human S100A9/S100A8 onto the native, but not the InsP_6-depleted, HW *in vitro*. However, this result was not robust with respect to changes in the experimental conditions. As complement factor H, a well-known heparin-binding protein, associates with the LL independently of InsP_6 (Irigoín *et al*. 2008), we speculate that this structure may bear additional anionic sites independent of InsP_6, perhaps on mucin backbones (Díaz *et al*. 2011*a*).

The phagocyte-specific S100 proteins are amplifiers of inflammation (Ehrchen *et al*. 2009; Pietzsch and Hoppmann, 2009). We found them associated with a parasite characterized by controlling host inflammation (Díaz *et al*. 2011*a*). This apparent paradox is reconciled by 2 important considerations. The first of these is that the establishing parasite does elicit inflammation, the cellular remnants of which may persist after inflammatory resolution (Richards *et al*. 1983). This applies to the observations on hydatids from mouse experimental infections. Although we did not study these samples by immunohistochemistry, this type of hydatid is known not to be surrounded by active inflammation (Richards *et al*. 1983). Therefore neutrophils from the reaction against the establishing parasite (Richards *et al*. 1983; Breijo *et al*. 1998) are the most likely cellular origin of S100A8 and S100A9. The second consideration is that the inflammatory control exerted by the parasite is not absolute, and a continuum of local response types is observed across natural host species, reaching chronic granulomatous inflammation in non-permissive host species such as cattle (Díaz *et al*. 2011*a*). We thus found that S100A12, in particular, was a very abundant host protein in cattle infections, while S100A9 and S1008 were present at much lower levels if at all. S100A8, identified by us as being associated with the HW in low levels in a single sample, has previously been reported to be present in hydatid fluid, in material also from bovine host (Monteiro *et al*. 2010).

In the context of granulomatous inflammation as studied in cattle hydatids, the major cell types expressing S100A12 were epithelioid and MGC immediately adjacent to the HW. This result, together with the proteomic data, suggests that the epithelioid and MGC express high levels of S100A12 selectively with respect to S100A9 and S100A8. The possibility that it is only the adsorption of S100A12 onto the HW that is selective is essentially ruled out by the results in the mouse system showing that S100A9 and S100A8 can indeed associate with the HW. In other systems, S100A12 is known to be expressed in granulocytes but not in monocytes or resident macrophages (Vogl *et al*. 1999*a*; Pietzsch and

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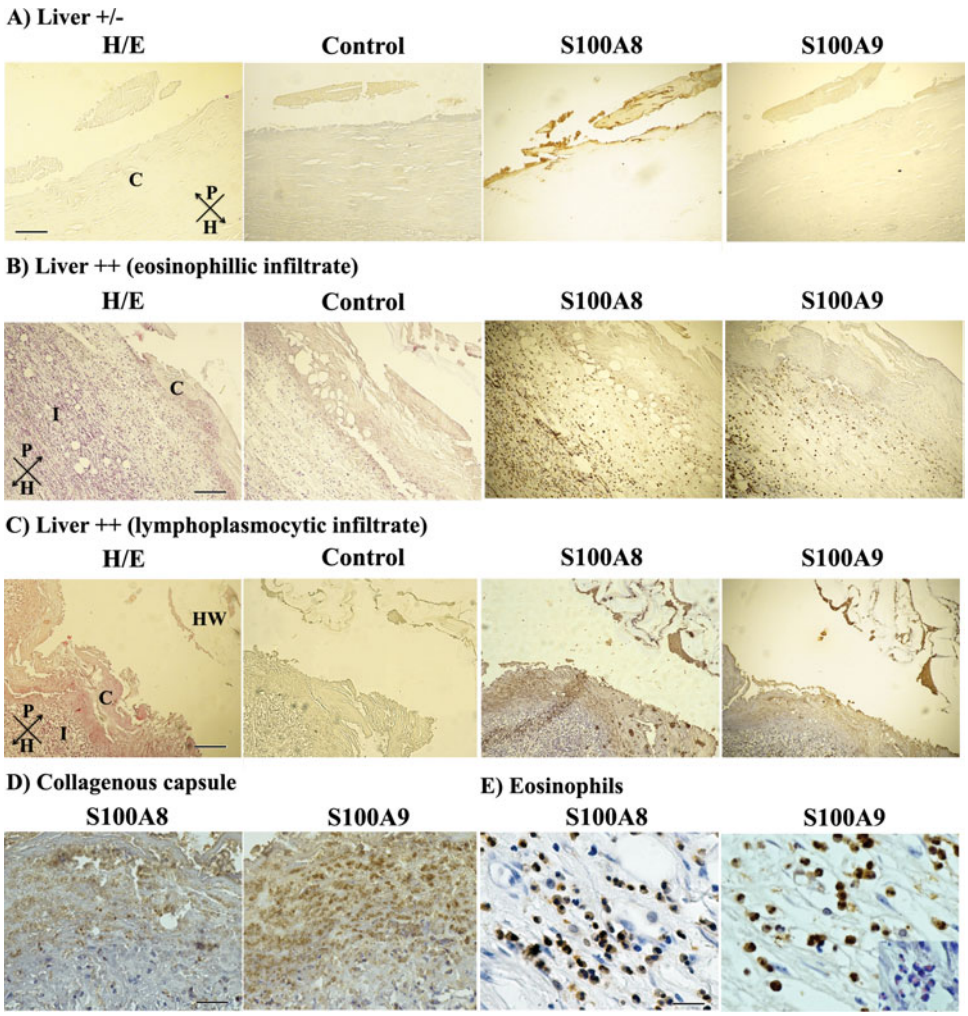

Fig. 4. Immunohistochemical detection of S100A9 and S100A8 in the host-hydatid interface in the human host. Paraffin-embedded sections of the local reaction to *Echinococcus granulosus* metacestodes were stained with haematoxylin-eosin (H/E), or subjected to immunohistochemical detection using antibodies to human S100A9 or S100A8, or control antibodies. (A–C) Panoramic views of 3 representative samples with different degrees of inflammation; inflammatory scores are given besides the hydatid localization in each case. The control stains shown correspond to normal rabbit serum. (D and E) Higher resolution views of the main elements showing positive reaction for the proteins under study, namely the collagenous capsule and an eosinophil infiltrate respectively. In (E) an H/E stain has been included in an inset to show that the infiltrating cells are eosinophils. In each H/E stain in (A–C), the approximate orientation of the host (H)-parasite (P) interface has been indicated. I, mononuclear cell-dominated infiltrate; C, collagen (and fibroblasts); HW, hydatid wall. Scale bars represent 100 μm throughout.

Hoppmann, 2009). S100A12 expression in granuloma macrophages, including epithelioid cells and MGC specifically, has been reported, in human systems, in the last few years (Kim *et al*. 2006; Morbini *et al*. 2006; Campo *et al*. 2007); the expression of S100A9 and S100A8 was not analysed in these works. Other works have reported widely different observations on the expression of S100A9 and S100A8 in these cell types; these observations include co-expression of both, expression of only S100A9, and lack of expression of either (Zwadlo *et al*. 1988; Delabie *et al*. 1990; Aguiar-Passeti *et al*. 1997; Arai *et al*. 1999; Sunderkotter *et al*. 2004; Kurata *et al*. 2005; Terasaki *et al*. 2007). Also, in a helminth-induced granuloma model in mice (patent *S. mansoni* infection), S100A9 and S100A8 were found to be expressed by macrophages at the edge of the granuloma but not by the epithelioid and MGCs at the centre of the reaction (Yang *et al*. 1997). Our results suggest that comparative analysis of all 3 phagocyte-specific S100 proteins in epithelioid and MGCs might show that, at least in some strongly inflammatory contexts, these cells express high levels of S100A12 selectively.

We also found S100A12 to be expressed by fibroblasts, although only in the samples displaying the most intense inflammatory reactions. S100A9 and S100A8 are now known to be expressed in certain non-haematopoetic cells (keratinocytes) in response to the Th17 cytokine IL-22 (Wolk *et al*. 2006), the

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receptor for which can also be expressed by fibroblasts (Sonnenberg *et al*. 2010). The possibility that S100A12 expression in our system may be an indication of a Th17 response is further discussed below.

In a human sample featuring an eosinophil infiltrate, we observed strong expression of S100A9 and S100A8 in this cell type. While eosinophil expression of S100A9 and/or S100A8 had not been reported before, S100A12 had been observed in asthma eosinophils, but not eosinophils from normal blood (Yang *et al*. 2007). Therefore all 3 phagocyte-specific S100 proteins can be expressed by eosinophils in appropriate inflammatory contexts.

An early observation on S100A12 was its association with the inflammatory reaction to a tissue-dwelling helminth. In a study with strong parallels to ours, human S100A12 was identified in extracts of the adult stage of the nematode *Onchocerca volvulus* (Marti *et al*. 1996). Noteworthy in relation to our results, S100A8 was detected in this study but at only 20% the concentration of S100A12. As the starting material had been freed of host tissue, S100A12 and S100A8 were deduced to bind the parasite. In another study (previous to the discovery of S100A12), S100A9 and S100A8 were observed to be expressed by (morphologically conventional) inflammatory macrophages adjacent to adult *O. volvulus*, and apparently secreted onto the worms (Edgeworth *et al*. 1993). *In vitro*, S100A12 has filariostatic and filaricidal activities, possibly because of binding to nematode paramyosin (Gottsch *et al*. 1999; Akpek *et al*. 2002). The possibility that S100A12 is one of the effectors through which the granulomatous response damages *Echinococcus* larvae deserves further investigation. *Echinococcus* possesses paramyosin (Muhlschlegel *et al*. 1993), and therefore binding to this protein is a conceivable anti-parasite mechanism. An inflammatory response very similar to the chronic granulomatous version of the response to *E. granulosus* is normally elicited by *E. multilocularis* (Gottstein and Hemphill, 1997; Díaz *et al*. 2011*a*). It would be worth analysing the expression of S100A12 in the human host response against *E. multilocularis*.

Although immune responses to helminths have a dominant Th2 profile, other effector response arms, including the highly inflammatory Th17 arm, can contribute to the overall response (Díaz and Allen, 2007; Ritter *et al*. 2010). It has been proposed that the granulomatous response to larval *Echinococcus* has a Th17 component (Vuitton and Gottstein, 2010; Díaz *et al*. 2011*a*). There are strong links between granulomatous responses and Th17 immunity in other contexts (Coury *et al*. 2008; Rutitzky *et al*. 2009; Okamoto Yoshida *et al*. 2010). Two previously known major products of the epithelioid and MGC elicited by *E. granulosus*, namely cathepsin K and MMP-9 (Díaz *et al*. 2000*a*; Marco *et al*. 2006), have reported associations with Th17 responses (Prause *et al*. 2004; Koenders *et al*. 2005*a*,*b*; Ivanov *et al*. 2007). More specifically, S100A12, identified in this study as produced by same epithelioid cells and MGC, is also connected to Th17 responses, as are also S100A8 and S100A9 (Wolk *et al*. 2006; Haider *et al*. 2008; Loser *et al*. 2010). It is therefore worth analysing Th17 responses in the larval *Echinococcus* system.

ACKNOWLEDGEMENTS

The authors are grateful to Madelón Portela (Unidad de Bioquímica y Proteómica Analíticas, Instituto Pasteur de Montevideo) for expert processing of protein bands for proteomics. They also acknowledge Dr Carolina Arredondo (Departamento de Patología, Facultad de Veterinaria, Uruguay) for assistance with microscopy and Dr Edgardo Berriel (Clínica Quirúrgica 1, Facultad de Medicina, Montevideo, Uruguay) for useful discussions. They are also grateful for Professor Robert B. Sim (Department of Pharmacology, University of Oxford, UK) for critical reading of the manuscript. This work was supported by the Government of Uruguay (A.D., PDT grant no. 54/078) and by the Third World Academy of Sciences (A.D., Research Grant 04-433 RG/BIO/LA).

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https://doi.org/10.1017/S003118201100179X Published online by Cambridge University Press