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

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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 (InsP₆) (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

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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.* 2011a). 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.* 2011a). 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.* 2000a,b; Marco *et al.* 2006). This has led to observations of general interest for granuloma biology being made initially in the *E. granulosus* system (Díaz *et al.* 2000b).

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 extra-cellular 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 (Irigoin *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.* 2011a), with 2 M NaCl, or sequentially with both agents, as indicated. The protease inhibitors PMSF (2 mM), iodoacetamide (2 mM), pepstatin A (2 $\mu\text{g}/\text{ml}$), and E-64 (100 μM) were added in each step. Extracts were concentrated

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.* 1999a). 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), cgtcgacaagcttctactctttgtgatatct (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 ($P < 0·05$) 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 μ m; 75 μ m ID \times 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 MS² 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 $P < 0·05$.

Immunohistochemistry

Microtome sections (0·5 mm thick) were dewaxed, treated with proteinase K as an antigen retrieval procedure and with 1% H₂O₂ 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

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.* 1999b; 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

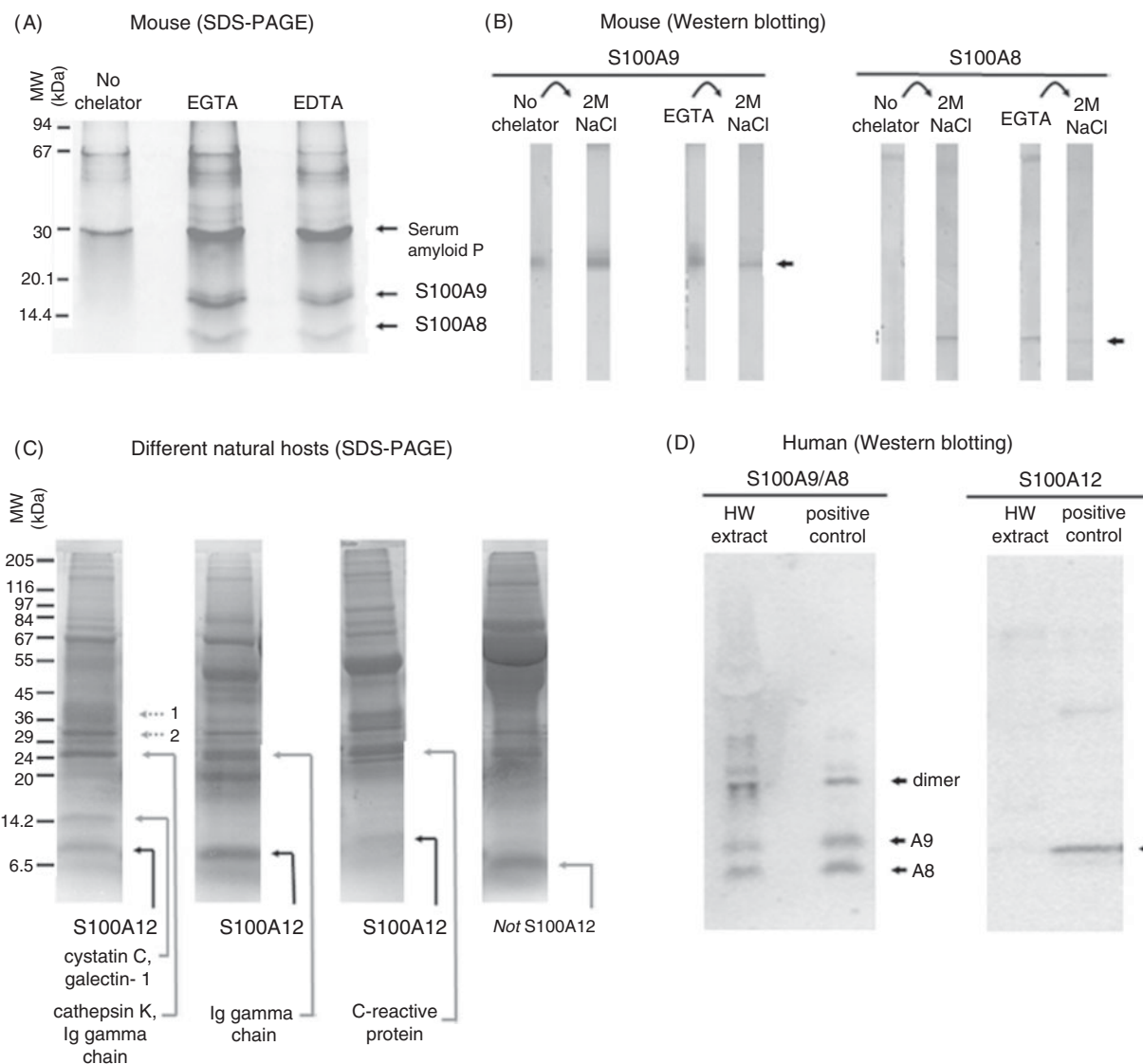


Fig. 1. Association of S100A8, S100A9 and S100A12 with the *Echinococcus granulosus* metacestode, in different hosts. (A) Intact mouse peritoneal hydatids were extracted externally with buffers without or with calcium chelators as indicated, and solubilized proteins were run on SDS-PAGE and selected 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

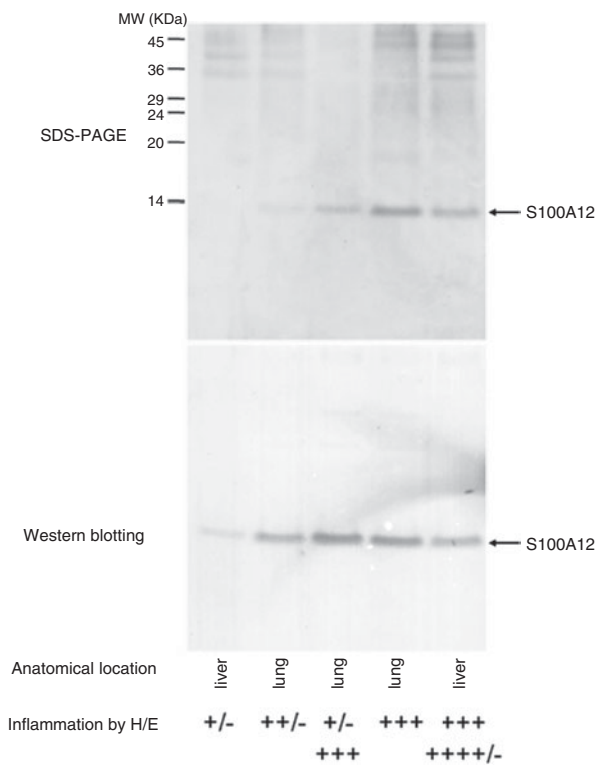


Fig. 2. Association of S100A12 with the reaction to the *Echinococcus granulosus* metacestode in representative samples from the bovine host. HW were extracted with 2 M NaCl, solubilized proteins run on SDS-PAGE and either silver stained (A), or transferred to nitrocellulose and probed with antibodies 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.* 2011b)), 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 S100A12 respectively, the number of observable peptides for S100A8 and S100A9 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.* 2000a,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 Ibarregui, 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

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 $P < 0.05$ 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.* 2011a), 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.* 2000a; 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

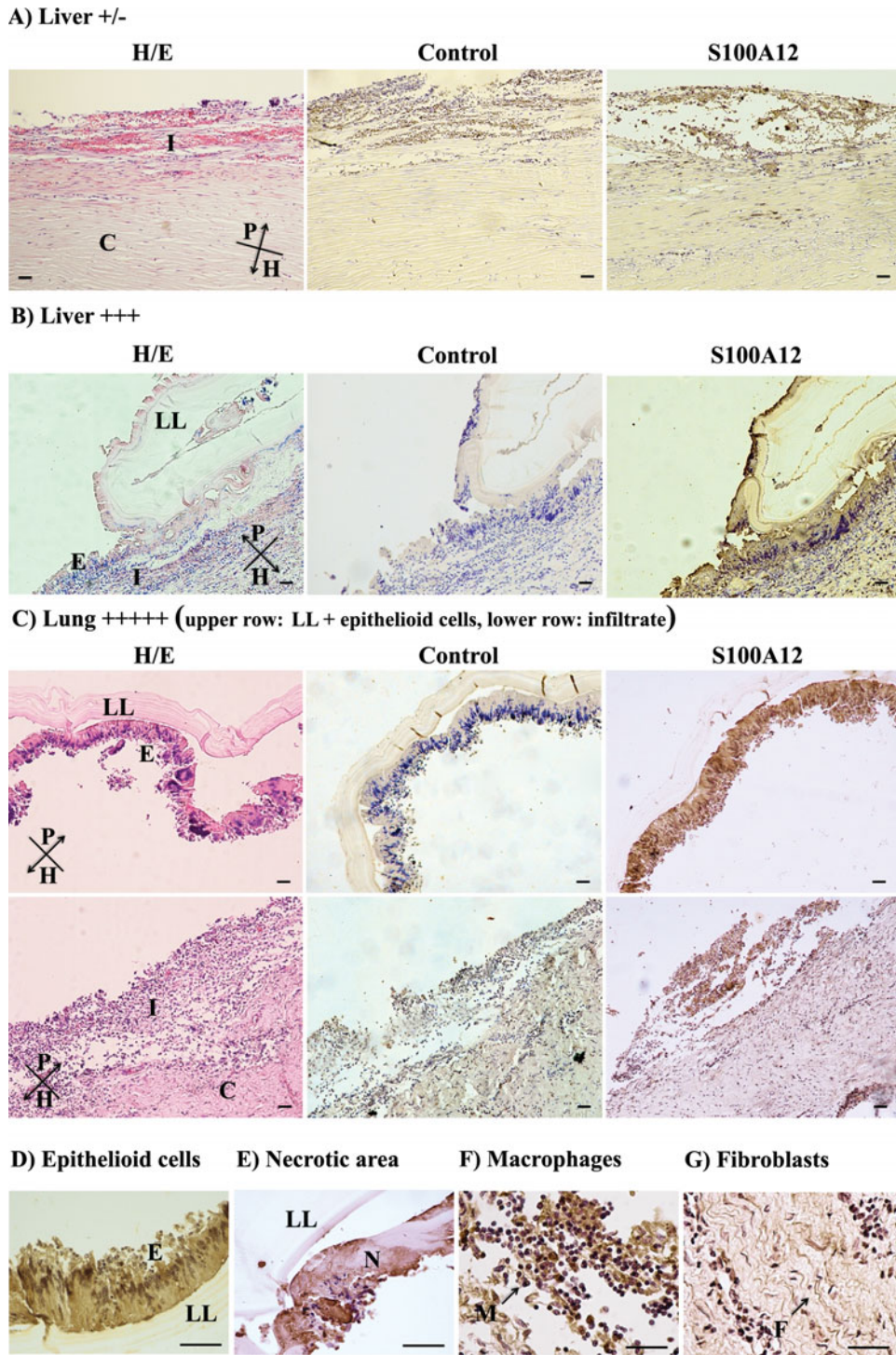


Fig. 3. Immunohistochemical detection of S100A12 in the host-hydatid interface in the bovine host. Paraffin-embedded sections of the local reaction to hydatids, including whenever possible the HW itself, were stained with haematoxylin-eosin (H/E), or subjected to immunohistochemical detection using affinity-purified 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 μ m throughout.

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/plasmacytes (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.* 2011a). 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.* 2000a,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 (Irigoin *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 (Irigoin *et al.* 2008), we speculate that this structure may bear additional anionic sites independent of InsP_6 , perhaps on mucin backbones (Díaz *et al.* 2011a).

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.* 2011a). 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.* 2011a). We thus found that S100A12, in particular, was a very abundant host protein in cattle infections, while S100A9 and S100A8 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.* 1999a; Pietzsch and

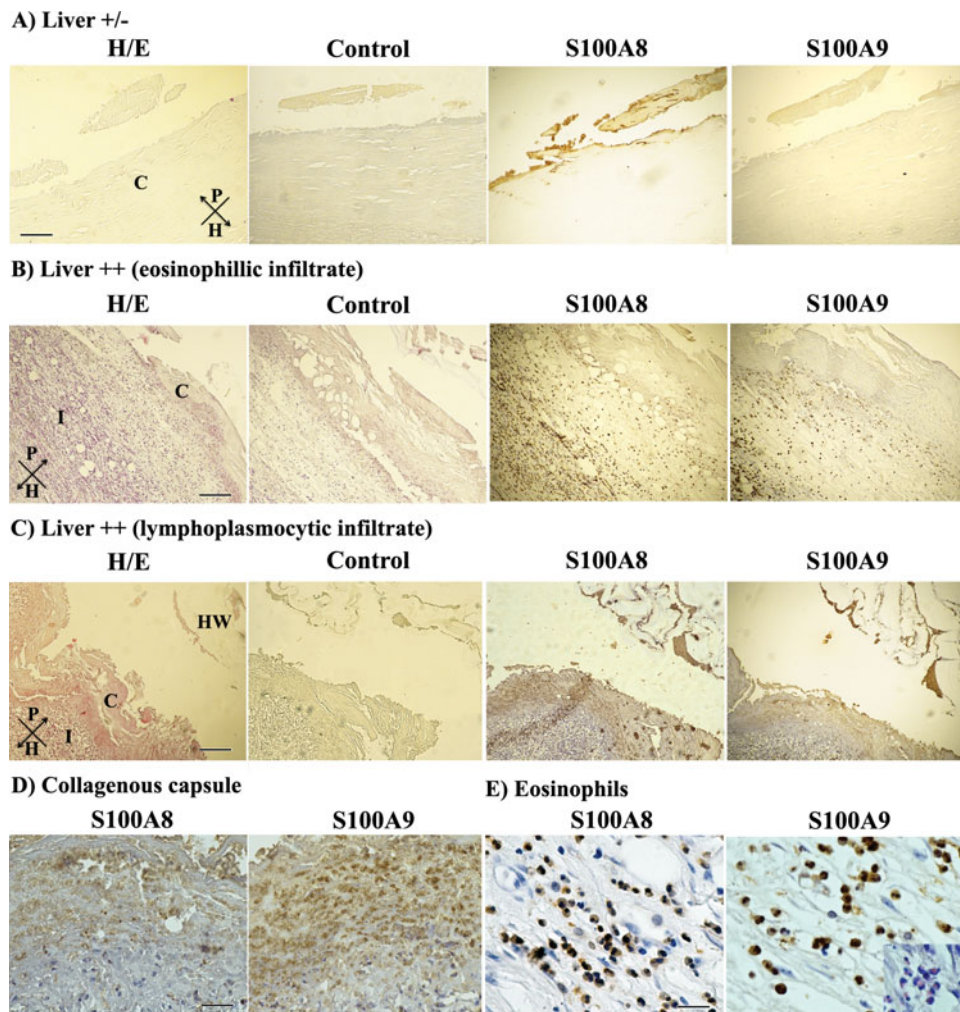


Fig. 4. Immunohistochemical detection of S100A9 and S100A8 in the host-hydatid interface in the human host. Paraffin-embedded sections of the local reaction to *Echinococcus granulosus* metacestodes were stained with haematoxylin-eosin (H/E), or subjected to immunohistochemical detection using antibodies 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 μ m 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-haematopoietic cells (keratinocytes) in response to the Th17 cytokine IL-22 (Wolk *et al.* 2006), the

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.* 2011a). 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.* 2011a). 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.* 2000a; Marco *et al.* 2006), have

reported associations with Th17 responses (Prause *et al.* 2004; Koenders *et al.* 2005a,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.

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REFERENCES

- Aguiar-Passeti, T., Postol, E., Sorg, C. and Mariano, M. (1997). Epithelioid cells from foreign-body granuloma selectively express the calcium-binding protein MRP-14, a novel down-regulatory molecule of macrophage activation. *Journal of Leukocyte Biology* **62**, 852–858.
- Akpek, E.K., Liu, S.H., Thompson, R. and Gottsch, J.D. (2002). Identification of paramyosin as a binding protein for calgranulin C in experimental helminthic keratitis. *Investigative Ophthalmology and Visual Science* **43**, 2677–2684.
- Arai, K., Mizuno, K., Yamada, T. and Nozawa, R. (1999). Immunohistochemical evaluation of MRP-14 expression in epithelioid granuloma using monoclonal antibody 60B8. *Journal of Investigative Allergology and Clinical Immunology* **9**, 21–26.
- Bortoletti, G. and Ferretti, G. (1978). Ultrastructural aspects of fertile and sterile cysts of *Echinococcus granulosus* developed in hosts of different species. *International Journal for Parasitology* **8**, 421–431.
- Breijo, M., Spinelli, P., Sim, R.B. and Ferreira, A.M. (1998). *Echinococcus granulosus*: an intraperitoneal diffusion chamber model of secondary infection in mice. *Experimental Parasitology* **90**, 270–276.
- Campo, I., Morbini, P., Zorzetto, M., Tinelli, C., Brunetta, E., Villa, C., Bombieri, C., Cuccia, M., Agostini, C., Bozzi, V., Facoetti, A., Ferrarotti, I., Mazzola, P., Scabini, R., Semenzato, G., Pignatti, P.F., Pozzi, E. and Luisetti, M. (2007). Expression of receptor for advanced glycation end products in sarcoid granulomas. *American Journal of Respiratory and Critical Care Medicine* **175**, 498–506.
- Casaravilla, C., Brearley, C., Soule, S., Fontana, C., Veiga, N., Bessio, M.I., Ferreira, F., Kremer, C. and Diaz, A. (2006). Characterization of myo-inositol hexakisphosphate deposits from larval *Echinococcus granulosus*. *FEBS Journal* **273**, 3192–3203.
- Casaravilla, C. and Diaz, A. (2010). Studies on the structural mucins of the *Echinococcus granulosus* laminated layer. *Molecular and Biochemical Parasitology* **174**, 132–136.
- Coltorti, E.A. and Varela-Díaz, V.M. (1974). *Echinococcus granulosus*: Penetration of macromolecules and their localization in the parasite membranes of cysts. *Experimental Parasitology* **35**, 225–231.
- Coury, F., Annels, N., Rivollier, A., Olsson, S., Santoro, A., Speziani, C., Azocar, O., Flacher, M., Djebali, S., Tebib, J., Brytting, M., Egeler, R.M., Rabourdin-Combe, C., Henter, J.I., Arico, M. and Delprat, C. (2008). Langerhans cell histiocytosis reveals a new IL-17A-dependent pathway of dendritic cell fusion. *Nature, Medicine* **14**, 81–87.
- Dai, W.J., Waldvogel, A., Siles-Lucas, M. and Gottstein, B. (2004). *Echinococcus multilocularis* proliferation in mice and respective parasite

- 14-3-3 gene expression is mainly controlled by an alphabeta CD4⁺T-cell-mediated immune response. *Immunology* **112**, 481–488.
- Delabie, J., De Wolf-Peeters, C., Van Den Oord, J. J. and Desmet, V. J.** (1990). Differential expression of the calcium-binding proteins MRP8 and MRP14 in granulomatous conditions: an immunohistochemical study. *Clinical and Experimental Immunology* **81**, 123–126.
- Dell'angelica, E. C., Schleicher, C. H. and Santome, J. A.** (1994). Primary structure and binding properties of calgranulin C, a novel S100-like calcium-binding protein from pig granulocytes. *Journal of Biological Chemistry* **269**, 28929–28936.
- Diaz, A. and Allen, J. E.** (2007). Mapping immune response profiles: the emerging scenario from helminth immunology. *European Journal of Immunology* **37**, 3319–3326.
- Diaz, A., Casaravilla, C., Allen, J. E., Sim, R. B. and Ferreira, A. M.** (2011a). Understanding the laminated layer of larval *Echinococcus* II: immunology. *Trends in Parasitology* **27**, 264–272.
- Diaz, A., Casaravilla, C., Irigoín, F., Lin, G., Previato, J. O. and Ferreira, F.** (2011b). Understanding the laminated layer of larval *Echinococcus* I: structure. *Trends in Parasitology* **27**, 204–213.
- Diaz, A., Ferreira, A. and Sim, R. B.** (1997). Complement evasion by *Echinococcus granulosus*: sequestration of host factor H in the hydatid cyst wall. *Journal of Immunology* **158**, 3779–3786.
- Diaz, A., Ibareguren, S., Brejijo, M., Willis, A. C. and Sim, R. B.** (2000a). Host-derived annexin II at the host-parasite interface of the *Echinococcus granulosus* hydatid cyst. *Molecular and Biochemical Parasitology* **110**, 171–176.
- Diaz, A., Willis, A. C. and Sim, R. B.** (2000b). Expression of the proteinase specialized in bone resorption, cathepsin K, in granulomatous inflammation. *Molecular Medicine* **6**, 648–659.
- Edgeworth, J. D., Abiose, A. and Jones, B. R.** (1993). An immunohistochemical analysis of onchocercal nodules: evidence for an interaction between macrophage MRP8/MRP14 and adult *Onchocerca volvulus*. *Clinical and Experimental Immunology* **92**, 84–92.
- Ehrchen, J. M., Sunderkotter, C., Foell, D., Vogl, T. and Roth, J.** (2009). The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *Journal of Leukocyte Biology* **86**, 557–566.
- Gottsch, J. D., Eisinger, S. W., Liu, S. H. and Scott, A. L.** (1999). Calgranulin C has filariacidal and filariostatic activity. *Infection and Immunity* **67**, 6631–6636.
- Gottstein, B. and Hemphill, A.** (1997). Immunopathology of *Echinococcosis*. *Chemical Immunology* **66**, 177–208.
- Haider, A. S., Lowes, M. A., Suarez-Farinas, M., Zaba, L. C., Cardinale, I., Khatcherian, A., Novitskaya, I., Wittkowski, K. M. and Krueger, J. G.** (2008). Identification of cellular pathways of “type 1,” Th17 T cells, and TNF- and inducible nitric oxide synthase-producing dendritic cells in autoimmune inflammation through pharmacogenomic study of cyclosporine A in psoriasis. *Journal of Immunology* **180**, 1913–1920.
- Hamazaki, H.** (1987). Ca²⁺-mediated association of human serum amyloid P component with heparan sulfate and dermatan sulfate. *Journal of Biological Chemistry* **262**, 1456–1460.
- Heegaard, N. H., He, X. and Blomberg, L. G.** (2006). Binding of Ca²⁺, Mg²⁺, and heparin by human serum amyloid P component in affinity capillary electrophoresis. *Electrophoresis* **27**, 2609–2615.
- Hitomi, J., Yamaguchi, K., Kikuchi, Y., Kimura, T., Maruyama, K. and Nagasaki, K.** (1996). A novel calcium-binding protein in amniotic fluid, CAAF1: its molecular cloning and tissue distribution. *Journal of Cell Science* **109**, 805–815.
- Hofmann, M. A., Drury, S., Fu, C., Qu, W., Taguchi, A., Lu, Y., Avila, C., Kambham, N., Bierhaus, A., Nawroth, P., Neurath, M. F., Slattery, T., Beach, D., McClary, J., Nagashima, M., Morser, J., Stern, D. and Schmidt, A. M.** (1999). RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell* **97**, 889–901.
- Ilg, E. C., Troxler, H., Burgisser, D. M., Kuster, T., Markert, M., Guignard, F., Hunziker, P., Birchler, N. and Heizmann, C. W.** (1996). Amino acid sequence determination of human S100A12 (P6, calgranulin C, CGRP, CAAF1) by tandem mass spectrometry. *Biochemical and Biophysical Research Communications* **225**, 146–150.
- Irigoín, F., Casaravilla, C., Iborra, F., Sim, R. B., Ferreira, F. and Diaz, A.** (2004). Unique precipitation and exocytosis of a calcium salt of myo-inositol hexakisphosphate in larval *Echinococcus granulosus*. *Journal of Cellular Biochemistry* **93**, 1272–1281.
- Irigoín, F., Ferreira, F., Fernández, C., Sim, R. B. and Diaz, A.** (2002). myo-Inositol hexakisphosphate is a major component of an extracellular structure in the parasitic cestode *Echinococcus granulosus*. *The Biochemical Journal* **362**, 297–304.
- Irigoín, F., Laich, A., Ferreira, A. M., Fernández, C., Sim, R. B. and Diaz, A.** (2008). Resistance of the *Echinococcus granulosus* cyst wall to complement activation: analysis of the role of InsP₆ deposits. *Parasite Immunology* **30**, 354–364.
- Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J. and Mann, M.** (2005). Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Molecular and Cellular Proteomics* **4**, 1265–1272.
- Ivanov, S., Bozinovski, S., Bossios, A., Valadi, H., Vlahos, R., Malmhall, C., Sjostrand, M., Kolls, J. K., Anderson, G. P. and Linden, A.** (2007). Functional relevance of the IL-23-IL-17 axis in lungs in vivo. *American Journal of Respiratory Cell and Molecular Biology* **36**, 442–451.
- Jenkins, D. J., Romig, T. and Thompson, R. C.** (2005). Emergence/re-emergence of *Echinococcus* spp. – a global update. *International Journal for Parasitology* **35**, 1205–1219.
- Kim, M. H., Choi, Y. W., Choi, H. Y., Myung, K. B. and Cho, S. N.** (2006). The expression of RAGE and EN-RAGE in leprosy. *British Journal of Dermatology* **154**, 594–601.
- Koenders, M. I., Kolls, J. K., Oppers-Walgreen, B., Van Den Bersselaar, L., Joosten, L. A., Schurr, J. R., Schwarzenberger, P., Van Den Berg, W. B. and Lubberts, E.** (2005a). Interleukin-17 receptor deficiency results in impaired synovial expression of interleukin-1 and matrix metalloproteinases 3, 9, and 13 and prevents cartilage destruction during chronic reactivated streptococcal cell wall-induced arthritis. *Arthritis and Rheumatism* **52**, 3239–3247.
- Koenders, M. I., Lubberts, E., Oppers-Walgreen, B., Van Den Bersselaar, L., Helsen, M. M., Di Padova, F. E., Boots, A. M., Gram, H., Joosten, L. A. and Van Den Berg, W. B.** (2005b). Blocking of interleukin-17 during reactivation of experimental arthritis prevents joint inflammation and bone erosion by decreasing RANKL and interleukin-1. *American Journal of Pathology* **167**, 141–149.
- Krensky, A. M. and Clayberger, C.** (2009). Biology and clinical relevance of granulysin. *Tissue Antigens* **73**, 193–198.
- Kurata, A., Terado, Y., Schulz, A., Fujioka, Y. and Franke, F. E.** (2005). Inflammatory cells in the formation of tumor-related sarcoid reactions. *Human Pathology* **36**, 546–554.
- Lee, C., Bongcam-Rudloff, E., Sollner, C., Jahnen-Dechent, W. and Claesson-Welsh, L.** (2009). Type 3 cystatins; fetuins, kininogen and histidine-rich glycoprotein. *Frontiers in Bioscience* **14**, 2911–2922.
- Liu, R., Mori, S., Wake, H., Zhang, J., Liu, K., Izushi, Y., Takahashi, H. K., Peng, B. and Nishibori, M.** (2009). Establishment of in vitro binding assay of high mobility group box-1 and S100A12 to receptor for advanced glycation endproducts: heparin's effect on binding. *Acta Medica Okayama* **63**, 203–211.
- Liu, S. H. and Gottsch, J. D.** (1996). Amino acid sequence of an immunogenic corneal stromal protein. *Invest Ophthalmol Vis Sci* **37**, 944–948.
- Loser, K., Vogl, T., Voskort, M., Lueken, A., Kupas, V., Nacken, W., Klenner, L., Kuhn, A., Foell, D., Sorokin, L., Luger, T. A., Roth, J. and Beissert, S.** (2010). The Toll-like receptor 4 ligands Mrp8 and Mrp14 are crucial in the development of autoreactive CD8⁺ T cells. *Nature Medicine* **16**, 713–717.
- Marco, M., Baz, A., Fernández, C., Gonzalez, G., Hellman, U., Salinas, G. and Nieto, A.** (2006). A relevant enzyme in granulomatous reaction, active matrix metalloproteinase-9, found in bovine *Echinococcus granulosus* hydatid cyst wall and fluid. *Parasitology Research* **100**, 131–139.
- Marti, T., Erttmann, K. D. and Gallin, M. Y.** (1996). Host-parasite interaction in human onchocerciasis: identification and sequence analysis of a novel human calgranulin. *Biochemical and Biophysical Research Communications* **221**, 454–458.
- McMorran, B. J., Patat, S. A., Carlin, J. B., Grimwood, K., Jones, A., Armstrong, D. S., Galati, J. C., Cooper, P. J., Byrnes, C. A., Francis, P. W., Robertson, C. F., Hume, D. A., Borchers, C. H., Wainwright, C. E. and Wainwright, B. J.** (2007). Novel neutrophil-derived proteins in bronchoalveolar lavage fluid indicate an exaggerated inflammatory response in pediatric cystic fibrosis patients. *Clinical Chemistry* **53**, 1782–1791.
- Monteiro, K. M., De Carvalho, M. O., Zaha, A. and Ferreira, H. B.** (2010). Proteomic analysis of the *Echinococcus granulosus* metacystode during infection of its intermediate host. *Proteomics* **10**, 1985–1999.
- Morbini, P., Villa, C., Campo, I., Zorzetto, M., Inghilleri, S. and Luisetti, M.** (2006). The receptor for advanced glycation end products and its ligands: a new inflammatory pathway in lung disease? *Modern Pathology* **19**, 1437–1445.

- Mufarrij, A. A., Arnaut, A., Meshefedjian, G. and Matossian, R. M. (1990). Comparative histopathological study in the hepatic and pulmonary human hydatidosis. *Helminthologia* **27**, 279–290.
- Muhschlegel, F., Sygulla, L., Frosch, P., Massetti, P. and Frosch, M. (1993). Paramyosin of *Echinococcus granulosus*: cDNA sequence and characterization of a tegumental antigen. *Parasitol Research* **79**, 660–666.
- Nieberle, K. and Cohrs, P. (1967). *Textbook of the Special Pathological Anatomy of Domestic Animals*. Pergamon Press, Oxford, UK.
- Okamoto Yoshida, Y., Umemura, M., Yahagi, A., O'Brien, R. L., Ikuta, K., Kishihara, K., Hara, H., Nakae, S., Iwakura, Y. and Matsuzaki, G. (2010). Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. *Journal of Immunology* **184**, 4414–4422.
- Pietzsch, J. and Hoppmann, S. (2009). Human S100A12: a novel key player in inflammation? *Amino Acids* **36**, 381–389.
- Prause, O., Bozinovski, S., Anderson, G. P. and Linden, A. (2004). Increased matrix metalloproteinase-9 concentration and activity after stimulation with interleukin-17 in mouse airways. *Thorax* **59**, 313–317.
- Rabinovich, G. A. and Iarregui, J. M. (2009). Conveying glycan information into T-cell homeostatic programs: a challenging role for galectin-1 in inflammatory and tumor microenvironments. *Immunological Reviews* **230**, 144–159.
- Rao, D. G. and Mohiyuddin, S. (1974). Incidence of hydatid cysts in bovines and histopathological changes of pulmonary tissue in hydatidosis. *Indian Journal of Animal Science* **44**, 437–440.
- Richards, K. S., Arme, C. and Bridges, J. F. (1983). *Echinococcus granulosus equinus*: an ultrastructural study of murine tissue response to hydatid cysts. *Parasitology* **86**, 407–417.
- Ritter, M., Gross, O., Kays, S., Ruland, J., Nimmerjahn, F., Saijo, S., Tschopp, J., Layland, L. E. and Prazeres Da Costa, C. (2010). *Schistosoma mansoni* triggers Dectin-2, which activates the Nlrp3 inflammasome and alters adaptive immune responses. *Proceedings of the National Academy of Sciences, USA* **107**, 20459–20464.
- Robinson, M. J., Tessier, P., Poulosom, R. and Hogg, N. (2002). The S100 family heterodimer, MRP-8/14, binds with high affinity to heparin and heparan sulfate glycosaminoglycans on endothelial cells. *Journal of Biological Chemistry* **277**, 3658–3665.
- Roth, J., Burwinkel, F., Van Den Bos, C., Goebeler, M., Vollmer, E. and Sorg, C. (1993). MRP8 and MRP14, S-100-like proteins associated with myeloid differentiation, are translocated to plasma membrane and intermediate filaments in a calcium-dependent manner. *Blood* **82**, 1875–1883.
- Rutitzky, L. I., Smith, P. M. and Stadecker, M. J. (2009). T-bet protects against exacerbation of schistosome egg-induced immunopathology by regulating Th17-mediated inflammation. *European Journal of Immunology* **39**, 2470–2481.
- Sakamoto, T. and Cabrera, P. A. (2003). Immunohistochemical observations on cellular response in unilocular hydatid lesions and lymph nodes of cattle. *Acta Tropica* **85**, 271–279.
- Slais, J. and Vanek, M. (1980). Tissue reaction to spherical and lobular hydatid cysts of *Echinococcus granulosus* (Batsch, 1786). *Folia Parasitologica (Praha)* **27**, 135–143.
- Sonnenberg, G. F., Fouser, L. A. and Artis, D. (2010). Functional biology of the IL-22-IL-22R pathway in regulating immunity and inflammation at barrier surfaces. *Advances in Immunology* **107**, 1–29.
- Stadelmann, B., Spiliotis, M., Muller, J., Scholl, S., Muller, N., Gottstein, B. and Hemphill, A. (2010). *Echinococcus multilocularis* phosphoglucose isomerase (EmPGI): A glycolytic enzyme involved in metacystode growth and parasite-host cell interactions. *International Journal for Parasitology* **40**, 1563–1574.
- Struyf, S., Proost, P., Lenaerts, J. P., Stoops, G., Wuyts, A. and Van Damme, J. (2001). Identification of a blood-derived chemoattractant for neutrophils and lymphocytes as a novel CC chemokine, Regakine-1. *Blood* **97**, 2197–2204.
- Sunderkotter, C. H., Tomimori-Yamashita, J., Nix, V., Maeda, S. M., Sindrilaru, A., Mariano, M., Sorg, C. and Roth, J. (2004). High expression of myeloid-related proteins 8 and 14 characterizes an inflammationally active but ineffective response of macrophages during leprosy. *Immunology* **111**, 472–480.
- Terasaki, F., Fujita, M., Shimomura, H., Tsukada, B., Otsuka, K., Otsuka, K., Katashima, T., Ikemoto, M. and Kitaura, Y. (2007). Enhanced expression of myeloid-related protein complex (MRP8/14) in macrophages and multinucleated giant cells in granulomas of patients with active cardiac sarcoidosis. *Circulation Journal* **71**, 1545–1550.
- Thompson, R. C. A. (1995). Biology and systematics of *Echinococcus*. In *Echinococcus and Hydatid Disease* (ed. Thompson, R. C. A. and Lymbery, A. J.), pp. 1–50. CAB International, Wallingford, UK.
- Turk, V., Stoka, V. and Turk, D. (2008). Cystatins: biochemical and structural properties, and medical relevance. *Front Biosci* **13**, 5406–5420.
- Vogl, T., Propper, C., Hartmann, M., Strey, A., Strupat, K., Van Den Bos, C., Sorg, C. and Roth, J. (1999a). S100A12 is expressed exclusively by granulocytes and acts independently from MRP8 and MRP14. *Journal of Biological Chemistry* **274**, 25291–25296.
- Vogl, T., Roth, J., Sorg, C., Hillenkamp, F. and Strupat, K. (1999b). Calcium-induced noncovalently linked tetramers of MRP8 and MRP14 detected by ultraviolet matrix-assisted laser desorption/ionization mass spectrometry. *Journal of the American Society of Mass Spectrometry* **10**, 1124–1130.
- Vogl, T., Tenbrock, K., Ludwig, S., Leukert, N., Ehrhardt, C., Van Zoelen, M. A., Nacken, W., Foell, D., Van Der Poll, T., Sorg, C. and Roth, J. (2007). Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nature Medicine* **13**, 1042–1049.
- Vuitton, D. A. and Gottstein, B. (2010). *Echinococcus multilocularis* and its intermediate host: a model of parasite-host interplay. *Journal of Biomedicine and Biotechnology* **2010**, 923193.
- Wolk, K., Witte, E., Wallace, E., Docke, W. D., Kunz, S., Asadullah, K., Volk, H. D., Sterry, W. and Sabat, R. (2006). IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. *European Journal of Immunology* **36**, 1309–1323.
- Yamashita, J., Ohbayashi, M. and Sakamoto, T. (1961). Studies on echinococcosis XII. Ovine experimental cases of unilocular echinococcosis. *Japanese Journal of Veterinary Research* **9**, 23–30.
- Yang, T. H., Tzeng, S., Cheng, I., Burnett, M. G., Yoshizawa, Y., Fukuyama, K., Lee, S. C. and Epstein, W. L. (1997). Identification of the mouse calcium-binding proteins, MRP 8 and MRP 14, in *Schistosoma mansoni*-induced granulomas: biochemical and functional characterization. *Journal of Leukocyte Biology* **61**, 258–266.
- Yang, Z., Yan, W. X., Cai, H., Tedla, N., Armishaw, C., Di Girolamo, N., Wang, H. W., Hampartzoumian, T., Simpson, J. L., Gibson, P. G., Hunt, J., Hart, P., Hughes, J. M., Perry, M. A., Alewood, P. F. and Geczy, C. L. (2007). S100A12 provokes mast cell activation: a potential amplification pathway in asthma and innate immunity. *Journal of Allergy and Clinical Immunology* **119**, 106–114.
- Zwadlo, G., Bruggen, J., Gerhards, G., Schlegel, R. and Sorg, C. (1988). Two calcium-binding proteins associated with specific stages of myeloid cell differentiation are expressed by subsets of macrophages in inflammatory tissues. *Clinical and Experimental Immunology* **72**, 510–515.