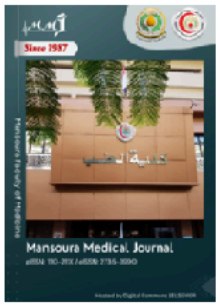
MANSOURA MEDICAL JOURNAL

ISSN - Print: 1110-211X - Online: 2735-3990

journal homepage: mmj.mans.edu.eg



Volume 45 | Issue 1 Article 3

Schistosoma mansoni alkaline phosphatase modulate platelet derived sphingosine-1-phosphate

Manal Elzoheiry

1Department of Infectious Disease, Cummings School of Veterinary Medicine, Tufts University, North Grafton, MA, USA; 2Department of Medical Parasitology, Faculty of Medicine, Mansoura University, Egypt

Samar El-Beshbishi

Department of Medical Parasitology, Faculty of Medicine, Mansoura University, Egypt, selbeshbishi@mans.edu.eg

El-Saeed El-Kholy

Department of Medical Parasitology, Faculty of Medicine, Mansoura University, Egypt

Manar Azab

Department of Medical Parasitology, Faculty of Medicine, Mansoura University, Egypt

Patrick Skelly

Department of Infectious Disease, Cummings School of Veterinary Medicine, Tufts University, North Grafton, MA, USA;

Schistosoma mansoni alkaline phosphatase modulate platelet derived sphingosine-1-phosphate

Manal Elzoheiry[1,2], Samar N. El-Beshbishi[2] , El-Saeed I. El-Kholy[2], Manar S. Azab[2], Patrick J. Skelly[1]

[1]Department of Infectious Disease, Cummings School of Veterinary Medicine, Tufts University, North Grafton, MA, USA; [2]Department of Medical Parasitology, Faculty of Medicine, Mansoura University, Egypt

Background

Schistosoma mansoni is one of Digenea that infect human causing chronic debilitating disease, schistomiasis. Although S. mansoni adults are living in the blood stream (inferior mesenteric venous system), the cut section of these vessels with living worms show no obvious immunological (inflammatory cells) or hematological (clot) attack around parasites. It was hypothesized that tegumental surface of schistosomes express proteins modulating the local environment around them, preventing host defense response. Here we have expressed an active schistosoma tegumental alkaline phosphatase (SmAP) in Chinese hamster ovary (CHO). SmAP is a ~60 kDa ecto-nucleotidase metalloenzyme- need divalent ions for fully efficiency at pH 9. . We previously reported that living worms can cleave exogenous adenosine monophosphate (AMP) to generate adenosine acting as a local anti-inflammatory mediator. We show here that rSmAP can cleave AMP to generate adenosine. In addition, it can catalyse nucleoside monophsphate (CMP, GMP, and TMP) using the products as nutrients. Also, we show that live schistosomes as well as rSmAP can cleave sphingosine-1-phosphate (S1P) - lipid mediator released by platelet regulates lymphocyte trafficking, and control platelet aggregationThis is the first demonstration of any pathogen cleaving this key control molecule. This work reveals that the worms using its tegumental SmAP to make less hostile surrounding area.

Key words:

Sphingosine -1- phosphate, Schistosoma mansoni, schistosoma tegumental alkaline phosphatase.

Introduction

Schistosoma mansoni is one of the three most important Schistosoma genus worms that cause chronic debilitating disease in human, schistosomiasis. According to WHO, over 200 million people are infected with these worms around the world and more than 800 million live at risk of infection (1). Human could catch infection after direct contact with fresh water contains infective stage, cercariae, that transform into migratory stage, schistosomula, after host skin penetration. The schistosomulae migrate through several tissues and stages to their residence in the portal vein, where maturate into adult male and female. Schistosoma mansoni paired worms migrate to inferior mesenteric venules, where lay mature eggs that shed in stool. Eggs hatch in fresh water releasing miracidia that penetrate intermediate snail host- the host of asexual cycle that produce the infective cercaiae (2).

Invasion of host blood by relatively large pathogen, like adult S. mansoni (~0.5 mm in diameter and up to 10 mm long) compared to the mesenteric veins diameter (1–4 mm), should provoke both thrombus formation and immune attack to eradicate the pathogen (3). On the other hand, schistosomes adult can live in blood stream for years up to decades, indicating that schistosomes interfere with the haemostatic and immune defense systems of their host (4, 5).

The tegument – syncytial cytoplasmic layer that covers the entire intravascular schistosomes – is a major interface between parasite and its host. Molecular analysis of this tegument described

many proteins, carbohydrates and lipids. Surface reported Proteins are belonging to several classes, such as: enzymes; receptors; and nutrient transporter [6]. Some of tegumental enzymes play an important role against normal heamostasis, such as: Sm22.6[7] inhibits thrombin (main platelet agonist); enolase [8]activates plasminogen (fibrinolytic agent); SmATPDase1 [9] hydrolyses ATP (pro- pro-thrombotic nucleotides); and calpains [10] cleaves fibronectin (blood clotting protein). Also, proteomics have contributed much of the new knowledge about the surface proteins that have localized immunosuppressive effects, such as : SmATPDase; SmNPP-5; and SmAP [11].

Schistosoma mansoni alkaline phosphatase (SmAP), is a tegumental enzyme (~60 kDa) which is highly expressed in adult tegument intravascular parasite life stages, enable parasite to cleave AMP releasing immunosuppressant adenosine. This ability was abolished after treating parasites with RNAi treatment targeting SmAP gene which suppress its expression [12]. We hypothized that Schistosoma mansoni alkaline phosphatase (SmAP) has a role in intravascular schistosomal survival through dephosphrylation of phosphate containing compounds which involved in host defense mechanisms like Sphingosine- 1-phosphate (S1P). Sphingosine- 1-phosphate is a highly active lysophospholipid, synthesized by the phosphorylation of sphingosine by sphingosine kinase in various cells of circulation, mainly platelets and erythrocytes. S1P release from activated platelets can be stimulated by clotting factors such as, thrombin and activated Factor X. Released S1P can synergistically enhance thrombin-induced tissue factor (TF) expression in endothelial cells initiating blood coagulation[13]. In addition, S1P S1P alone can initiate platelet aggregation via sphingosine 1-phosphate receptor 1 S1PR facilitating protease-activated receptor 4-peptide (PAR4-P) and adenosine diphosphate (ADP)–induced platelet activation[14].

Material and Methods

Parasites

S. mansoni cercariae were obtained from infected Biomphalaria glabrata snails at the Molecular Helminthology Laboratory at Cummings School of Veterinary Medicine, Tufts University, USA. After exposing of 6 week infected snails to light for 1.5h to promote shedding of parasites, cercariae numbers and viability were determined using a light microscope prior to infection of Swiss Webster mice with approximately 100 cercariae. Seven weeks after infection, adult worms were obtained by perfusion of mice according to(15) except: using of PBS containing 1.5% sodium citrate as a perfusion; and CO2 for euthanasia. Parasites were collected in RPMI medium, counted and immediately cultured in complete DMEM/F12 medium (supplemented with 10% heat-inactivated fetal bovine serum (Gibco# 1600), 200 U/ml penicillin and 200 μm/ml streptomycin,0.2 iM Triiodo-L-thyronine, 1.0 ?M serotonin, and 8 ig/ml human insulin) and were maintained at 37°C,in an atmosphere of 5%CO2.

Schistosomula were prepared as described [16]

Ethics statement

All protocols involving animals were approved by the Institutional Animal Care and Use Committees (IACUC) of Tufts University under protocol G2012-150. All animals were fed, housed and handled in strict agreement with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cloning, Expression and Purification

Based on the complete coding region of the SmAP genes (accession number HM045783), the commercially synthetized coding sequence of the S. mansoni Alkaline Phosphatase (SmAP) was first amplified by PCR with the primers: SmAP Fw (5` CAC CTC GAG AAA TCG TCC TTA TTG AAT 3`), and SmAP Rv (5` CAT GGT ACC TCT ATC GAG ATC CAT TGT TTC C 3`). These primers

contain, respectively, AscI and XhoI restriction site to assist subsequent cloning into the expression vector pSecTag2A (Invitrogen # V90020). Then, amplified plasmid inserted fragments were confirmed Automated DNA sequencers (http://www.tucf.org/dnasequencing). The resulting plasmid was transformed into Escherichia coli TOP10 competent cells (Invirogen# C404003) that were grown in growth medium (LB broth, Miller # BP 1426-500) as described in [17]. Suspension-adapted FreeStyle CHO-S cells (Invitrogen) were grown in FreeStyle CHO Expression Medium supplemented to 8 mM L-glutamine (Thermo Fisher Scientific# 25030081) at densities between 0.2 x 106 and 2 x 106 cells/ml, and kept on the shaker platform (Thermo Scientific MaxQ SHKE2000 CO2) rotating at 120–135 rpm, at 37°C and. Cell density and viability were determined by Bright-line Metallized Hemacytometer (Hausser Scientific# 3110). The maximal cell densities were achieved while maintaining a dispersed single cell suspension and an overall viability of more than 98%.One day prior to transfection, cell culture was diluted with fresh medium to a density of 0.6-0.8x10^6 cells/ml resulting in a density of 1.2–1.8 x 10^6 cells/ml on the day of transfection. Just prior to transfection, culture was counted and cell density adjusted to 1.2 x 10^6 cells/ml by addition of fresh medium according to experimental requirements. Transfection was performed in 30 ml culture in 125 ml Erlenmeyer tissue culture flasks (VWR# 89095-258).

Transfection complex was made by diluting 40ul of FreeStyle MAX reagent (Invitrogen# 16-447-100) into 0.6 ml of OptiPRO TM SFM (gibco# 12309-050) and adding that immediately to 40ug plasmid DNA diluted into 0.6 ml of OptiPRO TM SFM. The 1.2 ml DNA-transfection reagent complex solution was then incubated at room temperature for 10 min and slowly added to the suspension culture while slowly swirling the flask. Transfected cells were placed back on the shaker and samples were harvested at various time points post-transfection for analysis. No medium changes or additions were made after transfection. The transfection efficiency, post-transfection cell growth rate, and cell viability were assessed during a period of 3 days after transfection by addition of Trypan blue solution (Sigma# T8154) to the culture sample and determination of the number of Trypan blue-stained (dead) cells versus the total cell population. As described in (18)Use the equilibrated 1ml HisTrap excel (GE Healthcare # 17-3712-06) column to load the filtered cell culture medium that contains secreted SmAP at a flow rate of 3 ml/min, then His Buffer Kit (GE Healthcare# 11-0034-00) was used to prepare wash and elution buffer. After complete running of cell culture supernatant, the column was washed with 10 column volumes of wash buffer at a flow rate of 3 ml/ min, then was eluted with elution buffer using 5 column volumes of elution buffer at flow rate of 1ml/ min. the elute was dialyzed overnight at 4°C against 5 L of 50 mM Tris-HCL (pH 7.4), 150 mM NaCl, then concentrated by ultracentrifugation into 1ml volume (Pierce Protein Concentrator PES, 30K MWCO# 88522). The eluted protein was detect by 4–20% Mini-PROTEAN TGX Precast Protein Gel (BIO RAD# 4561096), in which transferred PVDF membrane (Merck millipore# IPVH00010) probed with Anti-myc-HRP Antibody (Invitrogen# R950-25) and Coomassie Stain (BIO RAD# 1610786). The purified recombinant protein was quantified using Pierce BCA Protein Assay Kit (Thermo Scientific# 23227).

Deglycosylation of rSmAP and native protein

According to the manufacturer's instructions (NEW ENGLAND BioLabs# P0704S) Deglycosylation was performed by adding PNGase F. Briefly, 5ug of rSmAP (in 9 µl adjusted volume with dH_2O) or 9 µL of male worm lysate (separate tube) was combined with 2 µl Glyco Buffer denaturation buffer and 2 µl of PNGase F (each reac-

tion was adjusted to 20 μL using dH2O). Samples were incubated overnight at 37°C, followed by 4-20% polyacrylamide analysis SDS-PAGE gel, transferred to activated PVDF membrane and blocked with TBST (Tris-buffered saline pH 7.5 and 0.05% Tween 20) containing 5% dry non-fat milk powder. The blot was incubated for 1h with polyclonal rabbit Anti-SmAP(1:500) at room temperature, followed by 5 times washing with TBST, and another another 1h in Anti-rabbit IgG, peroxidase-linked species-specific whole (from donkey) at 1:5000(GE Healthcare# NA934). Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare# RPN2016) and ChemiDoc Touch Imaging system (BioRAD) were used to develop and image the blot respectively.

SmAP activity

The catalytic of SmAP activity in intravascular schistosomes was determined by measuring absorbance of generated p-Nitrophenol over time at 405nm after incubation of parasites (Living and lysate), 1000 schistosomula or individual adult male or female schitosomes, in p-Nirophenyl phosphate (p-NPP, Sigma# N1891) containing buffer. The assay buffer (200 il) contained 20 mM Tris-HCL pH 9 (Sigma# T2819), 135 mM NaCl (Sigma# S7653), 5 mM KCl (Sigma-P9333), 10 mM glucose (Sigma# G8270), 1mM CaCl2 (Sigma# C3306), 10mM MgCl2 (Sigma# M1028), and 2 mM enzyme substrates p-NPP. Also, rSmAP was used at 0.15ig/assay.

Different rSmAP quantities (0.15, 0.3, and 1 μg/assay) were tested to determine a range of protein showing a complete hydrolysis of p-NPP. Synergy HT spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) was used to monitor absorbance of generated p-Nitrophenol.

Biochemical characters of SmAP activity

To determine optimal pH, hydrolysis of p-NPP (synthetic substrate) or Adenosine 5'-monophosphate (AMP, natural substrate) was measured over pH range from 5.5 to12 using different buffer with different pKa: MES (Sigma# 3058); MOPS (Sigma# M1254); Tris (Sigma# T2444, T3038 and T2819); Glycine (Sigma# G8898) and adjusted to the same salt concentration. The reaction mixture (200ul) contained: 50mM of pH buffer; 10mM MgCl2; and 2mM of p-NPP or AMP. The amount of inorganic phosphate (Pi) released by rSmAP was determine using a Phosphate Colorimetric Assay Kit (BioVision# K410-500) according to the manufacturer's instructions.

We Modified the standard assay buffer (50mM Tris-HCL pH 9) with different concentrations (0, 5, 10mM) divalent metals [Mg+2 (Sigma# M1028), Ca+2 (Sigma# C3306), Zn+2 (Sigma# 229997), Cu+2, and EDTA (Sigma# E7889)] to determine the activity of rSmAP in different ions. Reactions started by adding of 2mM p-NPP to modified buffer containing 0.15 μg rSmAP.

Hydrolysis of monophosphate nucleotides by rSmAP

In this assay, the amount of Pi released following hydrolysis of AMP (Sigma# A2225), CMP (Sigma#C1131), TMP (Sigma# T7004), and GMP (Sigma# G8377) is determined using Phosphate Colorimetric Assay Kit according to the manufacturer's instructions. Briefly, the assay reactions were performed in in 96-well micro titer plates (Corning# 3997), each well contained 200 il of 20 mM Tris-HCL buffer, pH 9, 135 mM Na2Cl, 5 mM KCl, 10 mM glucose, 2mM Ca2Cl, 10mM Mg2Cl and 0.15 μg rSmAP (control reactions were incubated without rSmAP). Reactions were initiated by the addition of 2mM monophosphate nucleotide solution to assay. Reactions were incubated at 37 °C for 24h. At different time points, 10 iL aliquots were transferred to 190 iL ice-cold water and stored at -20 °C until measuring of released Pi from monophosphate nucleotides.

To determine the kinetic parameters of rSmAP, each phosphate compound was hydrolyzed at in-

creasing concentration, fixed pH [9], and temperature (room temperature) for 1 hour. The substrates dissolved at the AP buffer and final concentration of 0.15 µg rSmAP used. To set up controls, substrates were incubated without rSmAP. Released Pi was measured calorimetrically and results were plotted against substrate concentration using Mechalis- Menten to determine Km- the substrate concentration at which the reaction rate is half maximum and inverse substrate affinity for rSmAP- for each substrate.

Dephosphorylation and inactivation of Sphingosine 1-Phosphate by SmAP

To determine the catalytic activity of rSmAP for Sphingosine 1-Phosphate (S1P, Sigma# S9666), diluted 1.3 mM S1P in 95% methanol, followed by drying Nitrogen and reconstitution in 4mg/mL Bovine serum albumin (BSA). The reconstituted S1P in BSA was incubated with assay buffer at final concentration of 500 µM at 37 °C for 24h. Assay buffer contained 100 iL of 20 mM Tris-HCL buffer, pH 9, 135 mM Na2Cl, 5 mM KCl, 10 mM glucose, 2mM Ca2Cl, 10mM Mg2Cl and 1 or 5 µg rSmAP. Control was sat up with the same buffer lacking rSmAP. At different time points, 10 iL aliquots were transferred to 190 iL ce-cold water and stored at -20 °C until measuring of released Pi from reactions using using Phosphate Colorimetric Assay Kit.

Catalytic activity of tegumental SmAP of intravascular schistosomes was measured by the same way of rSmAP, but incubation of 250 µM reconstituted S1P with: ~1000 schistosomula, or individual male or female adult for 24h at 37 °C.

Immunolocalization of SmAP in S.mansoni schistosomula

Cultured schistosomula (2 weeks old) were fixed in 4% paraformaldehyde or acetone for 30min were blocked with blocking buffer -1% BSA (bovine serum albumin) in HBSS (Hank's Balanced Salt Solution blocking buffer) - for 1 hour. The samples were incubated with primary anti-AP antibody (1:50) for 1hours. After 4 times washing with blocking buffer, Schistosomula were incubated with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, USA) diluted 1:100 in blocking buffer. Samples were 4 times washed and viewed using an inverted fluorescent microscope (Eclipse Ti-E; Nikon).

Statistical analysis

All experiments were repeated at least 3 times with reproducible results. Data are presented as Mean ±SD. Means are compared by: t-test (two-tailed, unpaired) for comparison of two groups; one-way ANOVA for comparison of more than two groups; repeated- measures ANOVA for comparison of more than two matched groups; and two-way ANOVA for comparison of different groups with different factors followed by a post hoc Bonferroni multiple comparison test (GraphPadPrism, v. 5; GraphPad Software, Inc, San Diego, CA, USA). A probability value of less than 0.05 was considered significant.

Results

Expression, purification of rSmAP

According to European nucleotide Archive (https://www.ebi.ac.uk), SmAP sequence is 1,611 pb with Accession number HM045783.1. As described in methods, expressed rSmAP in CHO-S cells was purified and concentrated, then analyzed by SDS-PAGE probed with anti-myc (Fig 1A, central lane). Schistosomal lysate analysis by SDS-PAGE probed with anti-AP shows almost the same prominent band at ~60kDa (Fig 1A, left lane). The soluble rSmAP was purified using His-tag excel column and elute purity was detected by SDS- PAGE followed by Coomassie blue staining (Fig 1A, right lane). We characterized SmAP glycosylation using Peptide -N-Glycosidase F (PNGase F), is an amidase that cleaves between the innermost GlcNAc and asparagine residues of

high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins. Figure 1B shows immunoblots of expressed transiently rSmAP and native SmAP with and without PNGase F. rSmAP was observed as ~60 kDa band, and upon PNGase F the band size was reduced (unglycosylated SmAP). The latter size corresponds well to the predicted molecular mass .Similar results were seen for native SmAP.

SmAP activity in intravascular stages of S. mansoni

Figure 2A shows increase releasing of p-Nitrophenol from p-NPP over 30 min after incubation with intact living 1000 schistosomula or total lysate of equivalent number of parasites, which reveal activity of SmAP. It is observed that SmAP activity in total lysate is double activity in intact one. Also, there is no activity in media around cultured Schistosomula indicating absence of SmAP in surrounding area. In Figure 2B, adult parasites (male in blue and female in red) are releasing p-Nitrophenol after exposure to p-NPP indicating SmAP activity in both intact living and total lysate. As in schistosomula lysate, adult total lysate shows more activity than intact ones.

Biochemical characters of rSmAP

Purified concentrated rSmAP was tested to determine the phosphatase activity through releasing of terminal phosphate from synthetic substrate (p-NPP) producing yellow p-nitrophenol in alkaline medium. Figure 3A shows that increasing concentration of rSmAP releases more p-nitrophenol indicating more activity, while adding of known inhibitor of alkaline phosphatase, Imidazole, to previous reaction decreases p-nitrophenol release indicating enzyme inhibition. Recombinant SmAP shows specific activity of 7.20 µmol p-nitrophenol generated /min/mg.

Figure 3B shows relative significant increase (130-140%) of rSmAP activity after adding of Mg^{+2} to standard buffer assay, while other ions (Ca^{+2}, Zn^{+2}, and Cu^{+2}) show little or no increase in enzyme activity. On the other hand, Adding of chelating agent, EDTA, significantly abolishes rSmAP activity (-95%).

Optimal pH of rSmAP, shown in Figure3C and 3D, ranges between 9- 10.5 according to tested substrate. Also, the capability of rSmAP to remove the phosphate group in alkaline medium depends on the used substrate.

Nucleoside monophosphate catalysis by rSmAP

The capability of rSmAP to release inorganic phosphate from AMP, TMP, CMP, and GMP is shown in figure 4A. Released phosphate level, after adding of nucleotides to buffer contains rSmAP, is directly proportional to incubation time of reaction. Affinity of rSmAP for nucleotides and p-NPP was examined to detect the kinetic parameters of rSmAP, which expressed as Michaelis-Menten equation. Michaelis constant (KM) of rSmAP for each substrate is depicted in figures 4B-4F.

Impact of SmAP on S1P

The catalytic ability of rSmAP to degrade exogenously added S1P was determined through measuring of released phosphate over time. Figure 5A shows significant increase of inorganic phosphate at 6 and 24h after incubation with different concentrations of rSmAP (1 and 5 µg).

Also, intravascular schistosomes can degrade S1P over time as shown in Figure 5B. Male shows higher catalytic activity compared to females, mostly explained by larger surface area of male. In figure 5C, Adding of parasites (+) can cleave S1P releasing Pi, while Reaction lacks parasites (-) shows no released phosphate.

SmAP immunolocalization in schistosomula

In figure 7, Immunostaining of one week Schistosomula demonstrates the local distribution of that SmAP on the tegument (left panel) while, Control parasites that exposed to 2nry Antibodies only, don't show any signals (right panel).

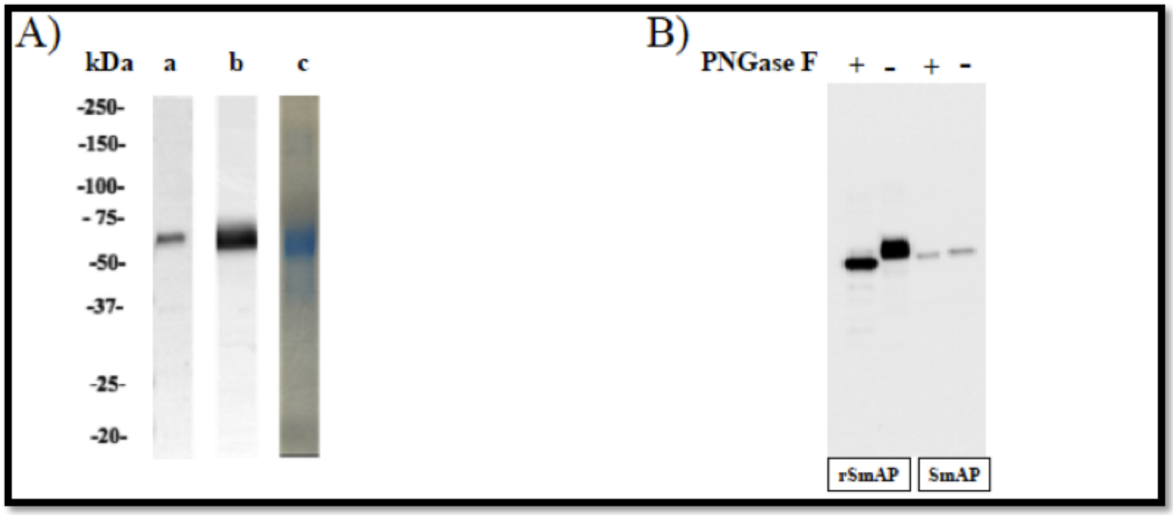


Figure 1A. Detecting both native and recombinant SmAP by SDS-PAGE. In lane (a) S. mansoni adult male lysate (~ 0.5 male per lane) was resolved by SDS-PAGE , blotted to PVDF, and probed with anti-SmAP as primary antibody then anti-rabbit IgG -HRP as secondary antibody. Lane (b) shows SDS-PAGE resolved pure concentrated CHO-S expressed SmAP (~ 0.5µg/lane) after blotting to PVDF and probing with anti-myc-HRP. The purity of CHO expressed SmAP is detected in lane (c) by staining of resolved SmAP with Coomassie Blue stain. All lanes in (A) show prominent bands at 60kDa. B. Western blot analysis of both native and recombinant SmAP. After overnight with PNGase F (+) or without (-), Both rSmAP and adult worm lysate are resolved by SDS-PAGE, blotted to PVDF, and probed with anti-SmAP as primary antibody then anti-rabbit IgG -HRP as secondary antibody. The treated samples show lower shifting compared to non-treated ones.

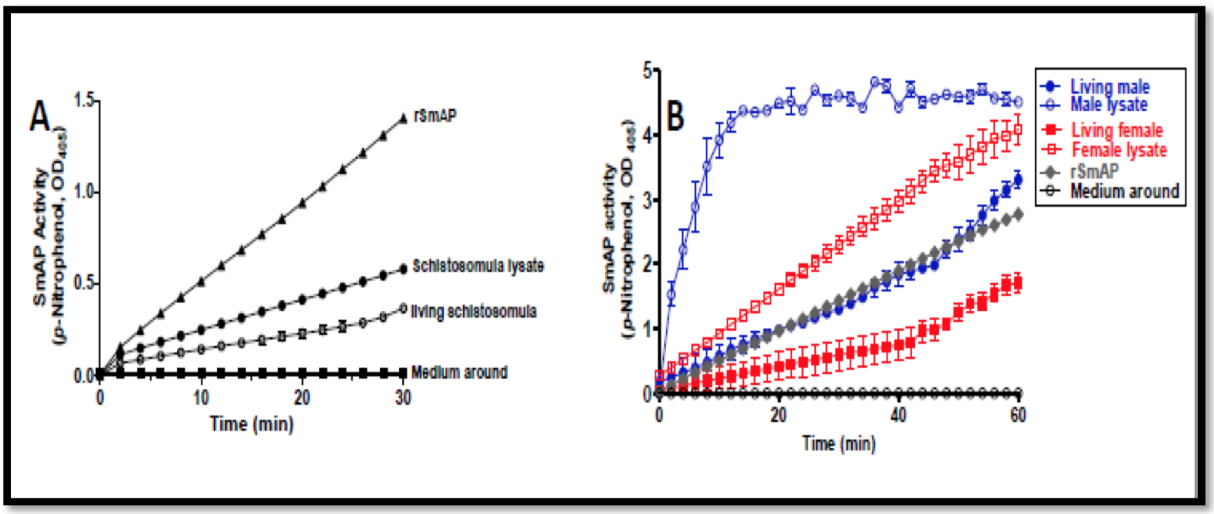


Figure 2.Activity of SmAP. A) Hydrolysis of *p*-NPP is measured over 30 min in: 1000 schistosomula (living and lysate); equivalent volume of cultured media around Schistosomula; and 0.15 µg rSmAP. B) Hydrolysis of *p*-NPP is detected at 60 min in individual male and individual female in both living and lysed conditions, compared to 0.15 µg rSmAP. Unpaired t test shows P value for comparison of SmAp activity separately in both conditions in male and female; *p <0.01;**p <0.001.

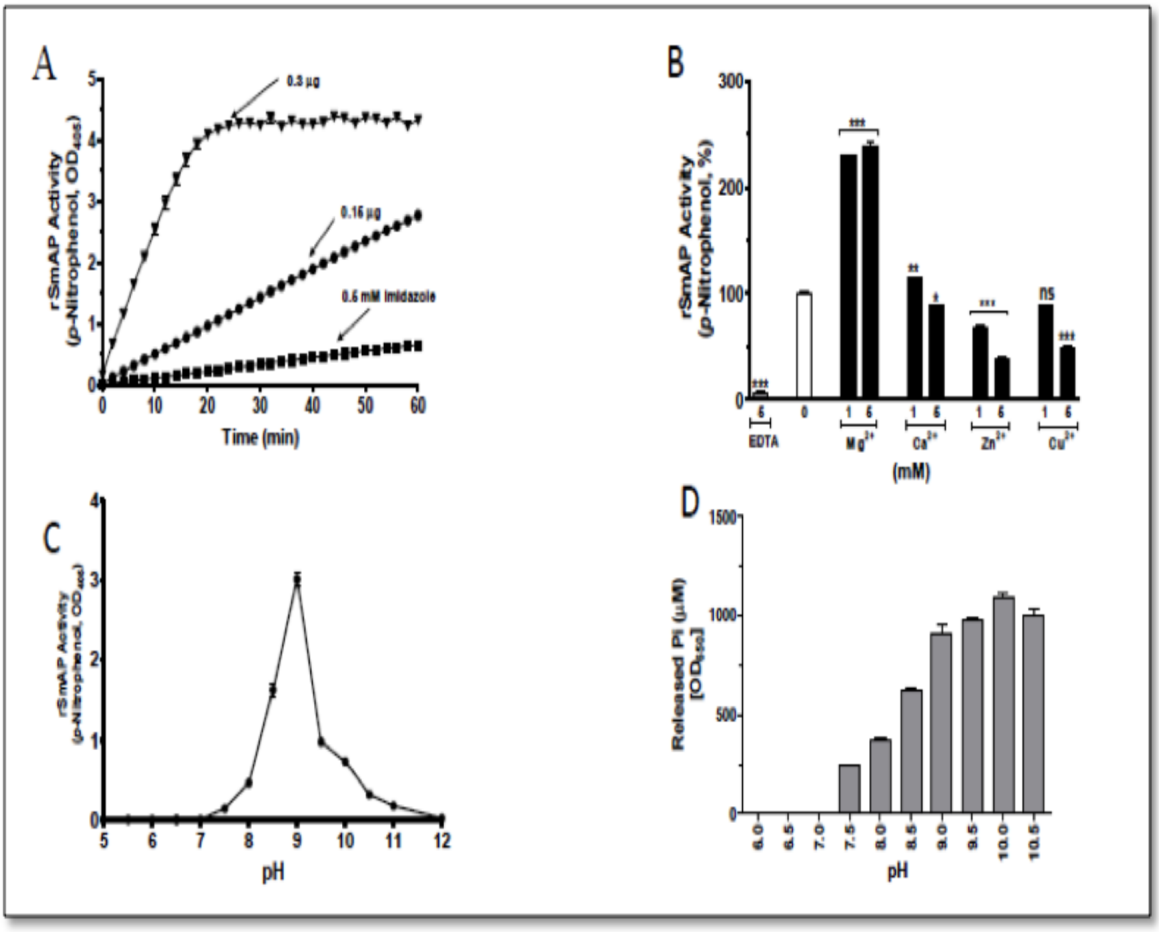


Figure 3. Characterization of rSmAP. A) Dose dependent hydrolysis of p-Nitrophenyl phosphate by active rSmAP, releasing p-Nitrophenol, at OD405, while adding of 0.5 mM imidazole (alkaline phosphatase inhibitor) to 0.15 µg r SmAP reaction inhibits release of p-Nitrophenol. B) Hydrolysis activity of rSmAP in buffer containing: 5 mM of EDTA; No metal ions; then 1 and 5 mM of (Mg+2, Ca+2, Zn+2, and Cu+2). Results expressed as relative activity compared with activity of rSmAP in buffer lacking metal ions, which was set as 100%. Repeated- measures ANOVA Shows P values for the comparison between rSmAP activity in non-treated buffer (0) and other treated buffers: *p <0.01;**p <0.001; ***p < 0.0001. C) Hydrolysis of p-NNP by r.SmAP in different pH levels. D) Dephosphorylation of AMP by rSmAP in different pH buffers.

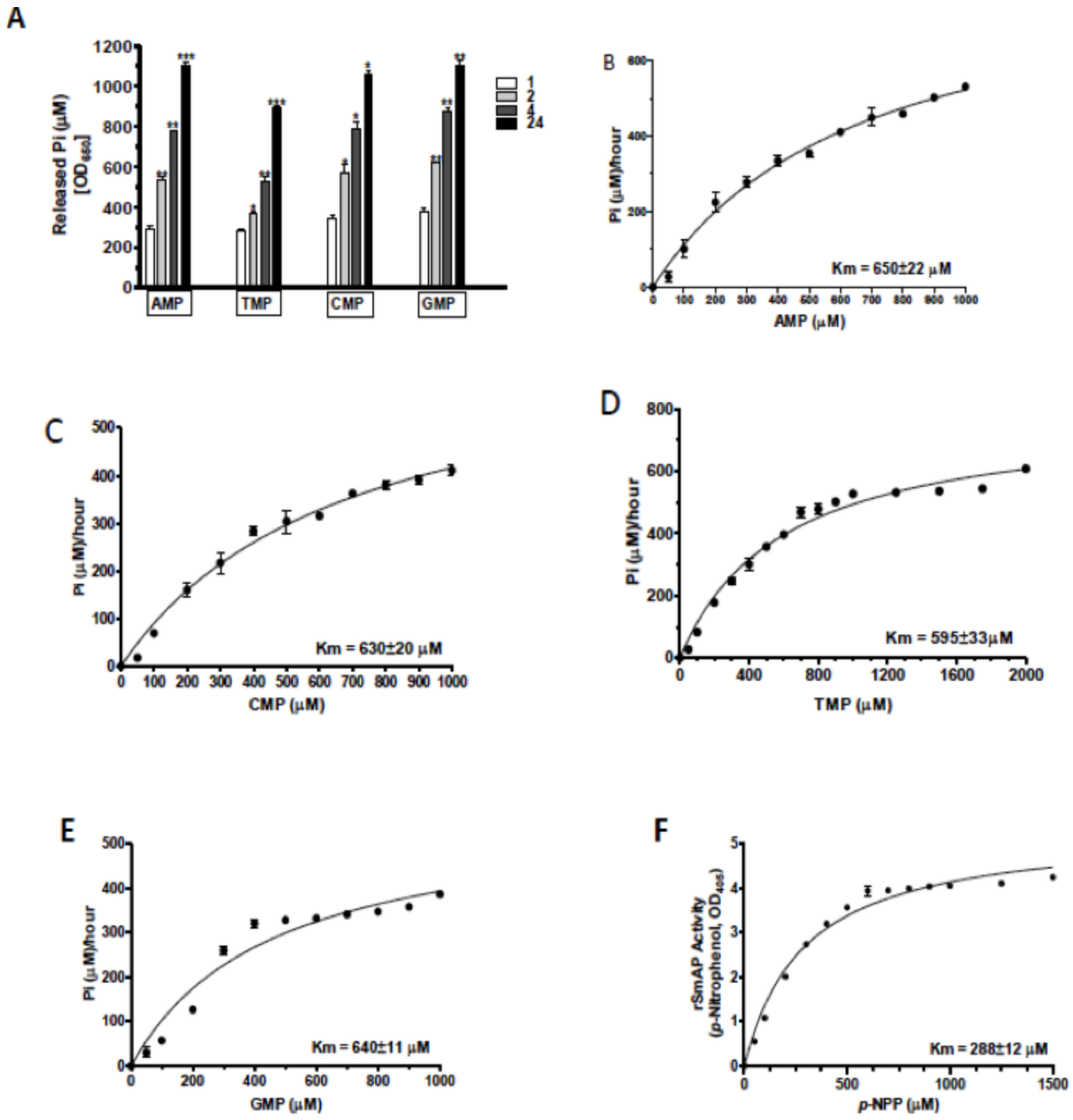


Figure 4. Figure 4. rSmAP releases phosphate from nucleoside monophosphate. A) Significant increase of released Pi level from AMP, TMP, CMP, and GMP over time after incubation with 0.15 µg rSmAP. One way ANOVA Shows P values for the comparison between released Pi level in each time point and pervious one: *p <0.01;**p <0.001; ***p < 0.0001. Figures B to F show Michaelis-Menten kinetics of rSmAP for different nucleotides and specific synthetic substrate (p-NPP). Michaelis constant KM of rSmAP for each substrate is depicted and derived from three independent experiments.

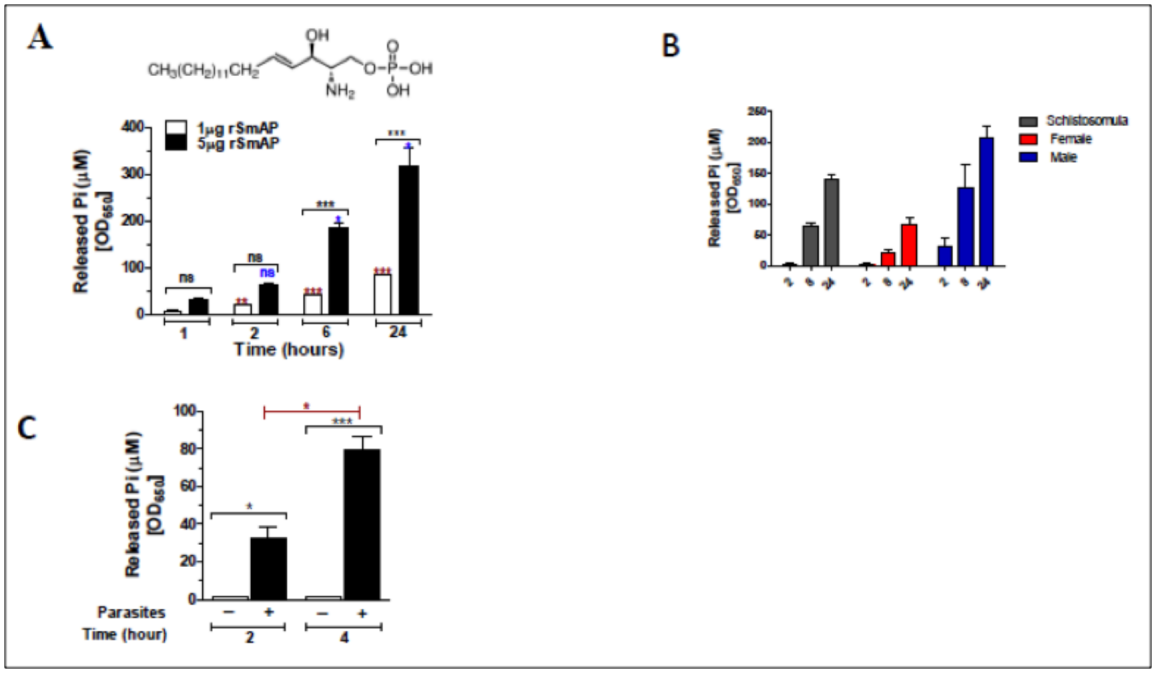


Figure 5. Dephosphorylation of S1P by SmAP. A) rSmAP capacity to degrade 500 µM S1P at different time points in presence of 1 or 5 µg. Repeated- measures ANOVA (red and blue) Shows P values for the comparison between different time point released Pi level in each concentration. Two ways ANOVA (black) shows P value for comparison between both concentrations in each time point. B) Significant increase of released Pi from 250 µM S1P after incubation with: 1000 schistosomula (gray bars); individual adult female (red bars) and male (blue bars). Repeated- measures ANOVA shows P values for comparison between different time points pi level in each group. C) Dephosphorylation of 250 µM S1P in presence (+) and absence (-) of two male parasites. Two ways ANOVA (black) shows P value for comparison between Pi level in (+/-) parasites, while unpaired t test (red) shows P value for comparison of Pi level in presence of parasites at 2 and 4 hours. ns $p>0.05$ *$p<0.01$;**$p<0.001$; ***$p < 0.0001$.

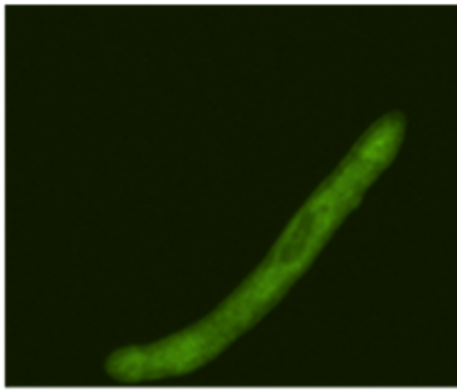


Figure 6. Immunolocalization of SmAP in 7-day cultured schistosomula

Discussion

In this work, we have generated and purified functionally active recombinant SmAP using a CHO cell expression system. SDS-PAGE and Coomassie staining reveal that the recombinant protein is of high purity and reacts with commercially generated anti-SmAP antibodies (in addition to antibodies directed to the recombinant protein's myc tag). Analysis of rSmAP by SDS-PAGE shows it to run at its predicted monomeric size: 60 kDa. Treatment of rSmAP with the N-glycan cleaving enzyme, PNGase F, results in a small but discernable protein mobility shift on SDS-gels. Similarly, treatment of the native protein in worm extracts also leads to a mobility shift showing that SmAP is a glycosylated protein [19]. This is in agreement with earlier work in which the enzyme was purified from extracts of adult parasites following lectin (Concanavalin A) agarose affinity chromatography [20]. SmAP protein sequence analysis shows it to possess several potential N-linked glycosylation sites [12].

We show here that rSmAP can cleave the artificial substrate p-nitrophenyl phosphate (p-NPP) in a reaction that requires Mg^{2+} ions. Other cations (Ca, Cu, and Zn) cannot substitute for Mg^{2+} and removing cations by treating the protein extract with the chelator EDTA eliminates enzyme activity. These data are consistent with an earlier report in which the alkaline phosphatase activity of isolated S. mansoni tegumental material was stimulated by 10mM Mg^{2+} but inhibited by >1mM Ca^{2+} [21]. Adding increasing amounts of non-Mg^{2+} cations to the reaction measured here diminishes enzyme activity, presumably by competing for efficient Mg^{2+} binding to the protein.

Like other schistosome ectoenzymes that are well expressed at the host-parasite interface (e.g. the nucleotide pyrophosphatase/phosphodiesterase SmNPP5 and the diphophodiesterase SmATPDase1 [12, 21, 22]), SmAP too displays highest activity under alkaline conditions. The enzyme's pH optimum is 9. How enzymatically efficient alkaline-loving parasite ectoenzymes like SmAP are in the host vasculature is unclear given that blood, the habitat of the worms studied here, is strongly buffered and maintains a neutral pH. Additionally, the adult worms excrete large amounts of lactate [23, 24] which would serve to acidify the parasite's local environment, potentially further dampening the activity of these tegumental ectoenzymes.

Early work revealed alkaline phosphatase activity in the parasite surface membranes [25, 26] and almost certainly the activity of SmAP was being measured. More recent tegument proteomic studies [27-29] as well as immunolocalization experiments [12, 19] confirm that SmAP is found in the parasite surface membranes. We show here that living schistosomes (schistosomula as well as adult male and female worms) can cleave the alkaline phosphatase substrate p-NPP. We have previously shown that live schistosomula whose SmAP gene is suppressed by RNAi are severely impaired in their ability to cleave this substrate [12]. In this work we provide direct evidence that rSmAP can cleave p-NPP.

Immunolocalization data reveal that SmAP, in addition to being in the tegument, is also found in the parasite's internal tissues. This is consistent with our finding that parasite lysates (schistosomula as well as adult male and female worms) display greater p-NPP cleaving ability compared to the live worms. Activity displayed by living parasites represents the action of SmAP enzyme that is located at the host-parasite interface whereas lysates contain both surface and internal SmAP. The activity measured for individual males versus individual females is comparable. So, while individual females are considerably smaller than their male counterparts, they do display higher relative expression of the SmAP gene [12]. Earlier work examining isolated adult tegumental material re-

ported that >75% of total alkaline phosphatase activity was found in these “epidermis membranes” [30]. Our experiments involving live worm alkaline phosphatase activity, suggest that tegumental activity accounts for somewhat less (~ 50%) of that measured in total parasite lysates. This is true for schistosomula as well as adult males and females. Note that medium in which schistosomula were cultured for 24 hours does not have SmAP activity showing that the protein has not been excreted or secreted from the parasites to any measurable extent during culture.

Living schistosomes can also cleave several nucleoside monophosphates (NMPs): AMP, CMP, GMP and TMP. We show here that parasites whose SmAP gene has been suppressed are significantly impaired in their ability to cleave these substrates. Recombinant SmAP also cleaves these NMPs and with a generally similar Km (~600-650 μM). SmAP-mediated cleavage of AMP leads to the generation of adenosine [12] which, by signalling through anti-inflammatory P1 purinergic receptors, could dampen host immune responses [31, 32]. Adenosine generated by the SmAP-mediated dephosphorylation of AMP may additionally (or instead) be taken up by the parasites as a nutrient [12]. Since schistosomes cannot synthesize purines de novo [33], salvage of such biomolecules from the host is vital. In a similar manner, SmAP-mediated dephosphorylation of CMP, GMP and TMP may generate the nutrients cytosine, guanine and thymine in the immediate environment of the parasites from where these might be conveniently and efficiently taken in.

Clues as to other biomolecules that might act as substrates for SmAP arose following our analysis of the metabolome of murine plasma in which adult schistosomes were incubated for one hour. Such a plasma sample, compared to a control, shows a diminution in sphingosine-1-phosphate (S1P) levels along with a concomitant increase in sphingosine and phosphate. This suggests that schistosomes can cleave S1P to generate its component parts. The fold increase in sphingosine and phosphate is notably greater than the fold decrease in S1P, suggesting that sources other than S1P account for much of the accumulated sphingosine and phosphate. To test the hypothesis that schistosomes can indeed hydrolyze S1P (and not merely recruit or activate a host enzyme with this function), living intravascular life-stage parasites (schistosomula and adult males and females) were each incubated with commercially obtained S1P. At selected times thereafter, S1P cleavage was examined by monitoring the level of phosphate released. In each case, this was observed. Consistent with this finding is the observation that rSmAP can, itself, cleave S1P to liberate phosphate. This is the first report of any parasite possessing the ability to cleave this important bioreactive metabolite.

S1P is a lipid signaling molecule that plays a critical role in the orchestration of immune responses. It binds specifically to a family of G-protein-coupled receptors (S1P receptors 1 to 5) leading to downstream signaling and cellular effects. S1P is enriched in blood and lymph whereas it is much lower in interstitial fluids of tissues, creating a steep S1P gradient [34] that is utilized to regulate trafficking of immune cells like lymphocytes, dendritic cells and neutrophils [35]. S1P can be secreted by monocytes and vascular endothelial cells [36, 37] and during some inflammatory reactions “a burst of S1P” is reported to become available to its receptors in the extravascular compartment likely leading to tissue responses [34]. Elevated local S1P concentrations have been postulated to play an important role in directing immune cells to sites of local injury [38]. Processes like lymphocyte circulation, leukocyte recruitment and positioning, antigen presentation, and inflammation are all influenced by local and systemic S1P levels and by S1P receptors expressed

on immune cells [36, 37]. By degrading S1P using SmAP, we hypothesize that schistosomes contain any S1P burst and dampen associated parasite-damaging host responses.

Recent studies have revealed that S1P signaling is not just involved in immune cell function but is also actively coupled with coagulation processes [38]. During vascular injury the coagulation proteases thrombin and activated factor X can enhance the synthesis and release of S1P from vascular smooth muscle cells [38]. Platelets, which contain high concentrations of S1P, can release it during coagulation [39]. In addition, S1P can directly initiate whole blood aggregation via S1P receptor 1 expressed by platelets and facilitates platelet aggregation in response to protease-activated receptor 4-peptide (PAR4-P) and ADP [40]. For schistosomes, the action of tegumental SmAP would likely diminish the local concentration of S1P to ameliorate its downstream pro-coagulant effects.

This is the first report of an ability of any pathogen to target and degrade extracellular S1P. It has been reported that some pathogenic bacteria can subvert intracellular S1P pathways to promote survival. For instance, within macrophages Burkholderia pseudomallei and B. thailandensis secrete S1P cleaving enzymes (S1P lyases) that are required for replication and virulence [41]. A Legionella pneumophila S1P lyase restrains autophagy within infected macrophages and contributes to bacterial virulence [42]. In these examples the pathogen enzymes appear to target intracellular S1P; in the case of schistosomes, it is an ectoenzyme - tegumental SmAP - degrading extracellular S1P levels that could impede S1P signalling and promote parasite survival.

We are now developing a more complete understanding of the molecular capabilities of the intravascular S. mansoni tegument. The tegument is more than a surface for the uptake and exchange of metabolites [43]. It contains a collection of proteins that can profoundly impact the biochemistry of the parasite's local environment. For instance, host interactive tegumental proteases can cleave key components of the coagulation cascade like fibronectin [44] and high molecular weight kininogen. Tegumental ATPdiphosphohydrolase SmATPDase1 can cleave the pro-inflammatory mediator ATP as well as the procoagulant ADP [22, 45]. The ectonucleotide pyrophosphatase / phosphodiesterase SmNPP5 can additionally cleave ADP and has been shown to block platelet aggregation in vitro. In addition, the parasites express proteins at their surface (e.g. SmEnolase) that can act to recruit and activate the thrombus-degrading enzyme plasminogen [46]. The tegumental ectoenzyme under study here - SmAP - contributes to these capabilities by converting AMP to its anti-inflammatory derivative adenosine [12] and, as shown in this report, by degrading the pro-inflammatory and pro-coagulant lipid mediator S1P. All of these molecular effects likely contribute to the known ability of living schistosomes to impede blood coagulation in vitro [47], to remain overtly unmolested by immune and coagulation effectors in vivo [48] and to survive for many years within the vasculature of their hosts.

In this work, we have expressed a fully active recombinant SmAP using CHO cell expression system. It was identified by commercially synthetized anti SmAP antibodies.

References

1. Steel N. Global, regional, and national age-sex specific mortality for 264 causes of death, 1980–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet. 2017; 390(10100):1151-210.
2. Gryseels B, Polman K, Clerinx J, Kestens L. Human schistosomiasis. The Lancet. 2006;368(9541):1106-18.

3. Yeaman MR. Platelets: at the nexus of antimicrobial defence. Nature Reviews Microbiology. 2014;12(6):426-37.
4. Wu YP, Lenting PJ, Tielens AGM, De Groot PG, Hellemond JJV. Differential platelet adhesion to distinct life-cycle stages of the parasitic helminth Schistosoma mansoni. Journal of Thrombosis and Haemostasis. 2007;5 (10):2146-8.
5. Wilson RA. The saga of schistosome migration and attrition. Parasitology. 2009;136 (12):1581-92.
6. Skelly PJ, Alan Wilson R. Making Sense of the Schistosome Surface. In: Baker JR, Muller R, Rollinson D, editors. Advances in Parasitology. 63: Academic Press; 2006. p. 185-284.
7. Lin Y-L, He S. Sm22. 6 antigen is an inhibitor to human thrombin. Molecular and biochemical parasitology. 2006;147(1):95-100.
8. Figueiredo BC, Da'dara AA, Oliveira SC, Skelly PJ. Schistosomes Enhance Plasminogen Activation: The Role of Tegumental Enolase. PLoS Pathogens. 2015;11(12):e1005335.
9. Da'dara AA, Bhardwaj R, Ali YBM, Skelly PJ. Schistosome tegumental ecto-apyrase (SmATPDase1) degrades exogenous pro-inflammatory and pro-thrombotic nucleotides. PeerJ. 2014;2:e316.
10. Wang Q, Da'dara AA, Skelly PJ. The human blood parasite Schistosoma mansoni expresses extracellular tegumental calpains that cleave the blood clotting protein fibronectin. Scientific Reports. 2017;7(1):12912.
11. Wilson RA. Virulence factors of schistosomes. Microbes and Infection. 2012;14 (15):1442-50.
12. Bhardwaj R, Skelly PJ. Characterization of schistosome tegumental alkaline phosphatase (SmAP). PLoS Negl Trop Dis. 2011;5 (4):e1011.
13. Takeya H, Gabazza EC, Aoki S, Ueno H, Suzuki K. Synergistic effect of sphingosine 1-phosphate on thrombin-induced tissue factor expression in endothelial cells. Blood. 2003;102(5):1693-700.
14. Urtz N, Gaertner F, von Bruehl M-L, Chandraratne S, Rahimi F, Zhang L, et al. Sphingosine 1-Phosphate Produced by Sphingosine Kinase 2 Intrinsically Controls Platelet Aggregation In Vitro And In Vivo. Circulation Research. 2015.
15. Tucker MS, Karunaratne LB, Lewis FA, Freitas TC, Liang Y-s. Schistosomiasis. Current Protocols in Immunology: John Wiley & Sons, Inc.; 2001.
16. Da'dara AA, Skelly PJ. Gene suppression in schistosomes using RNAi. Methods in molecular biology (Clifton, NJ). 2015;1201:143-64.
17. O'Sullivan LM, Patel S, Ward JM, Woodley JM, Doig SD. Large scale production of cyclohexanone monooxygenase from Escherichia coli TOP10 pQR239. Enzyme and Microbial Technology. 2001;28(2–3):265-74.
18. Jadhao SJ, Anderson LJ. Secretory Expression and Purification of Respiratory Syncytial Virus G and F Proteins in Human Cells. In: Tripp RA, Jorquera PA, editors. Human Respiratory Syncytial Virus: Methods and Protocols. New York, NY: Springer New York; 2016. p. 53-62.
19. Araujo-Montoya BO, Rofatto HK, Tararam CA, Farias LP, Oliveira KC, Verjovski-Almeida S, et al. Schistosoma mansoni: molecular characterization of Alkaline Phosphatase and expression patterns across life cycle stages. Exp Parasitol. 2011;129(3):284-91.
20. Payares G, Smithers SR, Evans WH. Purification and topographical location of tegumental alkaline phosphatase from adult Schistosoma mansoni. Mol Biochem Parasitol. 1984;13 (3):343-60.
21. Cesari IM, Simpson AJ, Evans WH. Properties of a series of tegumental membrane-bound phosphohydrolase activities of Schistosoma mansoni. Biochem J. 1981;198(3):467-73.
22. Da'dara AA, Bhardwaj R, Ali YB, Skelly PJ. Schistosome tegumental ecto-apyrase (SmATPDase1) degrades exogenous pro-

inflammatory and pro-thrombotic nucleotides. PeerJ. 2014;2:e316.

23. Githui EK, Damian RT, Aman RA. In vitro efflux of lactic acid by schistosomes cultured in varying concentrations of glucose: potential toxicity of accumulated lactic acid. J Trop Microbiol Biotechnol. 2006;2:31-6.
24. Shapiro TA, Talalay P. Schistosoma mansoni: mechanisms in regulation of glycolysis. Exp Parasitol. 1982;54(3):379-90.
25. Morris GP, Threadgold LT. Ultrastructure of the tegument of adult Schistosoma mansoni. J Parasitol. 1968;54(1):15-27.
26. Ninno-Smith RH, Standen OD. Phosphomonoesterases of Schistosoma mansoni. Exp Parasitol. 1963;13:305-22.
27. van Balkom BW, van Gestel RA, Brouwers JF, Krijgsveld J, Tielens AG, Heck AJ, et al. Mass spectrometric analysis of the Schistosoma mansoni tegumental sub-proteome. J Proteome Res. 2005;4(3):958-66.
28. Braschi S, Borges WC, Wilson RA. Proteomic analysis of the schistosome tegument and its surface membranes. Memorias do Instituto Oswaldo Cruz. 2006;101 Suppl 1:205-12.
29. Braschi S, Curwen RS, Ashton PD, Verjovski-Almeida S, Wilson A. The tegument surface membranes of the human blood parasite Schistosoma mansoni: a proteomic analysis after differential extraction. Proteomics. 2006;6 (5):1471-82.
30. Cesari IM. Schistosoma mansoni: distribution and characteristics of alkaline and acid phosphatase. Exp Parasitol. 1974;36(3):405-14.
31. Cekic C, Linden J. Purinergic regulation of the immune system. Nat Rev Immunol. 2016;16(3):177-92.
32. Bhardwaj R, Skelly PJ. Purinergic signaling and immune modulation at the schistosome surface? Trends Parasitol. 2009;25(6):256-60.
33. Levy MG, Read CP. Purine and pyrimidine transport in Schistosoma mansoni. J Parasitol. 1975;61(4):627-32.
34. Hla T, Venkataraman K, Michaud J. The vascular S1P gradient-cellular sources and biological significance. Biochim Biophys Acta. 2008;1781(9):477-82.
35. Cyster JG, Schwab SR. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. Annu Rev Immunol. 2012;30:69-94.
36. Bandhuvula P, Saba JD. Sphingosine-1-phosphate lyase in immunity and cancer: silencing the siren. Trends Mol Med. 2007;13 (5):210-7.
37. Kim RH, Takabe K, Milstien S, Spiegel S. Export and functions of sphingosine-1-phosphate. Biochim Biophys Acta. 2009;1791 (7):692-6.
38. Mahajan-Thakur S, Bohm A, Jedlitschky G, Schror K, Rauch BH. Sphingosine-1-Phosphate and Its Receptors: A Mutual Link between Blood Coagulation and Inflammation. Mediators Inflamm. 2015; 2015 :831059.
39. Obinata H, Hla T. Sphingosine 1-phosphate in coagulation and inflammation. Semin Immunopathol. 2012;34(1):73-91.
40. Urtz N, Gaertner F, von Bruehl ML, Chandraratne S, Rahimi F, Zhang L, et al. Sphingosine 1-Phosphate Produced by Sphingosine Kinase 2 Intrinsically Controls Platelet Aggregation In Vitro and In Vivo. Circ Res. 2015;117(4):376-87.
41. Custodio R, McLean CJ, Scott AE, Lowther J, Kennedy A, Clarke DJ, et al. Characterization of secreted sphingosine-1-phosphate lyases required for virulence and intracellular survival of Burkholderia pseudomallei. Mol Microbiol. 2016;102(6):1004-19.
42. Rolando M, Escoll P, Nora T, Botti J, Boitez V, Bedia C, et al. Legionella pneumophila S1P-lyase targets host sphingolipid metabolism and restrains autophagy. Proc Natl Acad Sci U S A. 2016;113(7):1901-6.
43. Da'dara A, Krautz-Peterson G, Faghiri Z, Skelly PJ. Metabolite movement across the

schistosome surface. Journal of helminthology. 2012;86(2):141-7.

44. Wang Q, Da'dara AA, Skelly PJ. The human blood parasite Schistosoma mansoni expresses extracellular tegumental calpains that cleave the blood clotting protein fibronectin. Sci Rep. 2017;7(1):12912.
45. Da'dara AA, Bhardwaj R, Skelly PJ. Schistosome apyrase SmATPDase1, but not SmATPDase2, hydrolyses exogenous ATP and ADP. Purinergic Signal. 2014;10(4):573-80.
46. Figueiredo BC, Da'dara AA, Oliveira SC, Skelly PJ. Schistosomes Enhance Plasminogen Activation: The Role of Tegumental Enolase. PLoS Pathog. 2015;11(12):e1005335.
47. Da'dara AA, de Laforcade AM, Skelly PJ. The impact of schistosomes and schistosomiasis on murine blood coagulation and fibrinolysis as determined by thromboelastography (TEG). J Thromb Thrombolysis. 2015.
48. Keating JH, Wilson RA, Skelly PJ. No overt cellular inflammation around intravascular schistosomes in vivo. The Journal of parasitology. 2006;92(6):1365-9.