HENRIQUE KRAMBECK ROFATTO

Caracterização molecular das nucleotídeo pirofosfatases/ fosfodiesterases de *Schistosoma mansoni* e investigação como antígenos vacinais

Tese apresentada ao Programa de Pós-Graduação Interunidades em Biotecnologia USP/Instituto Butantan/IPT, para obtenção do Título de Doutor em Biotecnologia.

Área de concentração: Biotecnologia

São Paulo
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Orientador: Profa. Dra. Luciana Cezar de Cerqueira Leite

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Universidade de São Paulo, Instituto Butantan, Instituto de Pesquisas Tecnológicas

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Candidato(a): Henrique Krambeck Rofatto.

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Orientador(a): Profa. Dra. Luciana Cézar de Cerqueira Leite.

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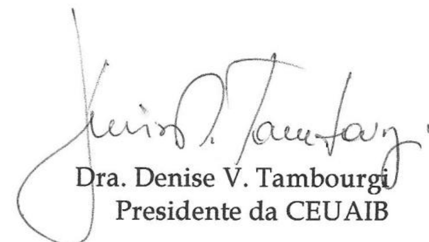
CERTIFICADO

Certificamos que o Projeto intitulado "Caracterização molecular e bioquímica das nucleotídeo pirofosfatases/fosfodiesterases (NPPs) de *Schistosoma mansoni* e investigação como antígenos vacinais" **protocolo n° 595/09** sob a responsabilidade de Luciana C. C. Leite e Henrique Krambeck Rofatto, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS DO INSTITUTO BUTANTAN (CEUAIB) em reunião de **11/02/2010.**

We certify that the research entitled "Biochemical and molecular characterization of the nucleotide pyrophosphtases/phosphosdiesterases (NPPs) from *Schistosoma mansoni*", **protocol n° 595/09**, under the responsability of Luciana C. C. Leite and Henrique Krambeck Rofatto, is in agreement with the Ethical Principles in Animal Research, adopted by the Brazilian College of Animal Experimentation, and was approved by the ETHICAL COMMITTEE FOR ANIMAL RESEARCH of BUTANTAN INSTITUTE in the meeting of 02/11/2010

Vigência do Projeto: 02/2010 – 05/2012	**N° de animais/espécie**
Centro de Biotecnologia	420/camundongos C57BL/6 (Fêmeas) 10/ ratos Wistar (Fêmeas) 150/hamster (Fêmeas)

São Paulo, 12 de fevereiro de 2010.



Dra. Denise V. Tambourgi
Presidente da CEUAIB

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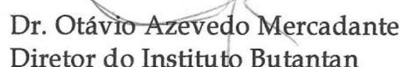

Dr. Otávio Azevedo Mercadante
Diretor do Instituto Butantan

À Layla, por tornar-se uma parte de mim (aquela formada de esperança, quando o horizonte era uma incógnita completa) e pelo amor, compartilhando os momentos felizes e apoio incondicional nos perídos intempestivos; aqui expresso um pouco da minha gratidão e do meu sentimento que, de um jeito muito especial, que ela conquistou inteiramente.

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Este trabalho foi realizado com o apoio financeiro da Fundação de Amparo à Pesquisa do Estado de São Paulo (Doutorado direto - Processo FAPESP nº: 2007/07685-8).

"É muito melhor ousar coisas grandiosas, alcançar triunfos e glórias, mesmo expondo-se ao fracasso, do que formar fila com os pobres de espírito que nem gozam muito nem sofrem muito, porque vivem nessa penumbra cinzenta que não conhece a vitória nem a derrota."

— Theodore Roosevelt

"O professor que caminha na sombra do templo, entre os seus discípulos, não dá a sua sabedoria, mas antes a sua fé e amor. Se for realmente sábio, não vos convida a entrar na casa da sua sabedoria, mas antes vos conduz ao limiar do vosso próprio espírito. [...] E aquele que é versado na ciência dos números, pode falar-vos de pesos e medidas, mas não pode levar-vos até lá. Pois a visão de um homem não empresta as suas asas a outro homem."

— Khalil Gibram

RESUMO

ROFATTO, H. K. **Caracterização molecular das nucleotídeo pirofosfatases/ fosfodiesterases de *Schistosoma mansoni* e investigação como antígenos vacinais.** 2013. 247 f. Tese (Doutorado em Biotecnologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2013.

A esquistossomose é uma doença com particularidades como a endemicidade, a transmissão, a patologia, a morbidade e a cronicidade, as quais devem ser consideradas no planejamento e na implementação de políticas que almejam a erradicação desse problema de saúde pública mundial. Como a quimioterapia não atua sobre todos esses fatores, o desenvolvimento de uma vacina auxiliaria na eliminação dessa doença. Neste trabalho foi caracterizada a família das nucleotídeo pirofosfatases/ fosfodiesterases (NPP) de *S. mansoni,* visando sua avaliação como antígenos vacinais. As enzimas desta família são associadas à membrana, hidrolisam ligações pirofosfato e ligações fosfodiester e são relacionadas a diversos processos biológicos como agregação plaquetária e modulação da sinalização purinérgica e da resposta imune. O *S. mansoni* possui quatro proteínas distintas pertencentes a esta família (SmNPP-5a, SmNPP-5b, SmNPP-5c e SmNPP-6), sendo que para duas delas verificamos maior expressão gênica nos estágios do parasita que infectam o homem. Os genes foram clonados a partir do mRNA de vermes adultos e as proteínas foram expressas de modo heterólogo em *E. coli e* purificadas por cromatografia de afinidade. As proteínas foram utilizadas para produção de anticorpos policlonais, porém apenas os anticorpos anti-SmNPP-5a apresentaram especificidade para a proteína nativa. Foi demonstrado que a SmNPP-5a é uma glicoproteína, cuja expressão aumenta após a infecção do homem e que está associada às membranas do tegumento dos vermes adultos do parasita. Também se verificou que os anticorpos anti-SmNPP-5a eram capazes de inibir parcialmente a atividade da enzima em parasitas vivos. Assim avaliamos a SmNPP-5a como antígeno vacinal juntamente com uma apirase (SmATPDase) e a fosfatase alcalina (SmAP), outras duas nucleotidases envolvidas no metabolismo de nucleotídeos e presentes no tegumento de parasitas adultos. Ambas as proteínas, SmNPP-5 e a SmNTDPase, apresentaram menor imunogenicidade que a SmAP. A SmNPP-5 induziu uma resposta imune celular, porém neutralizada por uma resposta regulatória; enquanto a SmNTDPase induziu uma resposta imune humoral, predominantemente Th2. A SmAP foi a proteína mais imunogênica das três estudadas, induzindo maior expressão de TNF-α e de IL-17 e alto níveis de IgG, especialmente IgG1. Porém só verificamos a redução da carga parasitária em camundongos imunizados com a SmAP, apenas quando os animais também receberam tratamento com doses subcurativas de praziquantel. Não foi observado nenhum efeito protetor devido a imunização com a SmNPP-5a ou com a SmATPDase, associadas ou não com a quimioterapia subcurativa.

Palavras-chave: *Schistosoma mansoni.* Vacina. Tegumento. Nucleotídeo pirofosfatase/ fosfodiesterase. Fosfatase alcalina. Apirase. Praziquantel.

ABSTRACT

ROFATTO, H. K. **Molecular characterization of nucleotide pyrophosphatases/ phosphodiesterases of *Schistosoma mansoni* and their investigation as vaccine antigens.** 2013. 247 p. Ph. D. thesis (Biotechnology) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2013.

Schistosomiasis is a disease with features such as endemicity, transmission, pathology, morbidity and chronicity, which should be considered in the planning and implementation of policies that aim to eradicate this worldwide public health problem. As chemotherapy does not act on all these factors, the development of a vaccine would be helpful to eliminate this disease. Herein the family of nucleotide pyrophosphatases/ phosphodiesterases (NPP) from *S. mansoni* was characterized and their potential as vaccine antigens was evaluated. Enzymes of this family are membrane associated and hydrolyze pyrophosphate and phosphodiester bonds; they are associated with several biological processes such as platelet aggregation and modulation of purinergic signaling and immune responses. *S. mansoni* has four distinct proteins belonging to this family (SmNPP-5a, SmNPP-5b, SmNPP-5c and SmNPP-6), whereas two of them present higher gene expression in stages of parasites that infect humans. The genes were cloned from adult worms mRNA and the proteins were expressed in *E. coli* and purified by affinity chromatography. The proteins were used for polyclonal antibody production, although only the anti-SmNPP-5a showed specificity for the native protein. It was shown that SmNPP-5a is a glycoprotein whose expression increases after infection of humans and it is associated with the tegument membranes of adult worms. It was also found that anti-SmNPP-5a antibodies were able to partially inhibit the activity of the enzyme in live parasites. Thus we evaluated SmNPP-5a as vaccine antigen together with an apyrase (SmATPDase) and alkaline phosphatase (SmAP), two other nucleotidases involved in nucleotide metabolism and present in the tegument of adult parasites. Both proteins, SmNTDPase and SmNPP-5 were less immunogenic than SmAP. The SmNPP-5a induced a cellular immune response counterbalanced by an immunoregulatory response; while SmNTDPase induced a predominantly Th2 humoral immune response. SmAP is the most immunogenic protein of the three studied, inducing increased expression of TNF-α and IL-17 and high levels of IgG, especially IgG1. However, we only verified the reduction of parasite burden in mice immunized with SmAP, when the animals also received a subcurative treatment with praziquantel. We did not observe a protective effect due to immunization with SmNPP-5a or with SmATPDase, associated or not with subcurative chemotherapy.

Keywords: *Schistosoma mansoni.* Vaccine. Tegument. Nucleotide pyrophosphatase/ phosphodiesterase. Alkaline phosphatase. Apyrase. Praziquantel.

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LISTA DE ABREVIATURAS E SIGLAS

ATP – adenosina trifosfato

ATPDase – adenosina trifosfato difosfohidrolase

cDNA – ácido desoxirribonucleico complementar

DIG – digoxigenina

DNA – ácido desoxirribonucleico

E. coli – *Escherichia coli*

ELISA – ensaio imunoenzimático

EST – seqüêcias expressas etiquetadas

FA – fosfatase alcalina

GST – glutationa S-tranferase

H. sapiens – *Homo sapiens*

HsNPP – nucleotídeo pirofostatase/fosfodiesterase de *Homo sapiens*

IFN-γ – interferon gama

IgG – imunoglobulina G

IL – interleucina

IPTG – isopropil-β-D-1-tiogalactopiranosídeo

M. mulatta – *Macaca mulatta*

M. musculus – *Mus musculus*

MmNPP – nucleotídeo pirofostatase/fosfodiesterase de *Mus musculus*

mRNA – ácido ribonucleico mesageiro

Myd88 – gene de resposta primária de diferenciação mielóide 88

NfκB – fator de transcrição nuclear kappa B

NPP – nucleotídeo pirofostatase/fosfodiesterase

OMS – Organização Mundial de Saúde

OPD – orto-fenilendiamina dicloridrato

ORF – quadro aberto de leitura

P. pastoris – *Pichia pastoris*

PBS – solução salina tamponada

PCR – reação em cadeia da polimerase

PVDF – polivinil-difluorado

R. norvergicus – *Rattus norvergicus*

RNA – ácido ribonucleico

RnNPP – nucleotídeo pirofostatase/fosfodiesterase de *Rattus norvergicus*

RT-PCR – reação de transcrição reversa seguida de reação em cadeia da polimerase

S. haematobium – *Schistosoma haematobium*

S. japonicum – *Schistosoma japonicum*

S. mansoni – *Schistosoma mansoni*

SBF – soro bovino fetal

SDS – dodecil sulfato de sódio

SDS-PAGE – eletroforese em gel de poliacrilamida e dodecil sulfato de sódio

SjNPP – nucleotídeo pirofostatase/fosfodiesterase de *Schistosoma japonicum*

SmNPP – nucleotídeo pirofostatase/fosfodiesterase de *Schistosoma mansoni*

SUCEN – Superintendência de Controle de Endemias

TGF-β – fator beta de transformação do crescimento

Th1 – célula T auxiliar tipo 1

Th2 – célula T auxiliar tipo 2

TNF-α – fator alfa de necrose tumoral

LISTA DE SÍMBOLOS

AMINOÁCIDOS

Ácido aspártico	D	Isoleucina	I
Ácido glutâmico	E	Leucina	L
Alanina	A	Lisina	K
Arginina	R	Metionina	M
Asparagina	N	Prolina	P
Cisteína	C	Serina	S
Fenilalanina	F	Tirosina	Y
Glicina	G	Treonina	T
Glutamina	Q	Triptófano	W
Histidina	H	Valina	V

BASES NITROGENADAS DOS NUCLEOTÍDEOS

Adenina	a	Guanina	g
Citosina	c	Timina	t

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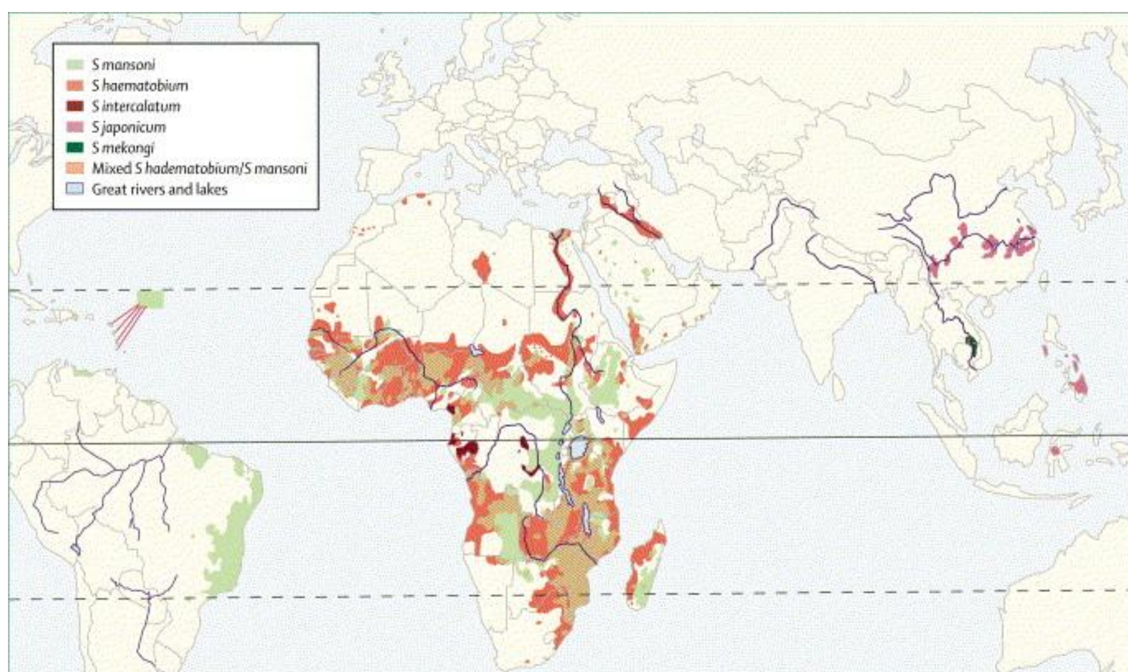
1 INTRODUÇÃO

1.1 Esquistossomose

A esquistossomose é a doença helmíntica mais grave no mundo devido aos seus índices de morbidade e mortalidade. Essa doença é um problema de saúde pública mundial, pois é endêmica em 74 países, afetando mais de 200 milhões de pessoas, principalmente indivíduos de baixas condições socioeconômicas, na África, Oriente Médio, Sudeste Asiático e América do Sul (Figura 1). Além das pessoas infectadas, estima-se que aproximadamente 800 milhões de pessoas vivam em áreas com alto risco de infecção (BERGQUIST, 2002; KING; DICKMAN; TISCH, 2005; ROSS et al., 2002; STEINMANN et al., 2006). No entanto, as áreas endêmicas têm aumentado nos países em desenvolvimento, devido à criação de novos habitats para o caramujo, através de projetos de irrigação e construções de barragens, e se tornado mais povoadas por causa do crescimento e da migração populacional (AL-SHERBINY et al., 2003).

Figura 1 – Distribuição global da esquistossomose e seus principais agentes etiológicos.



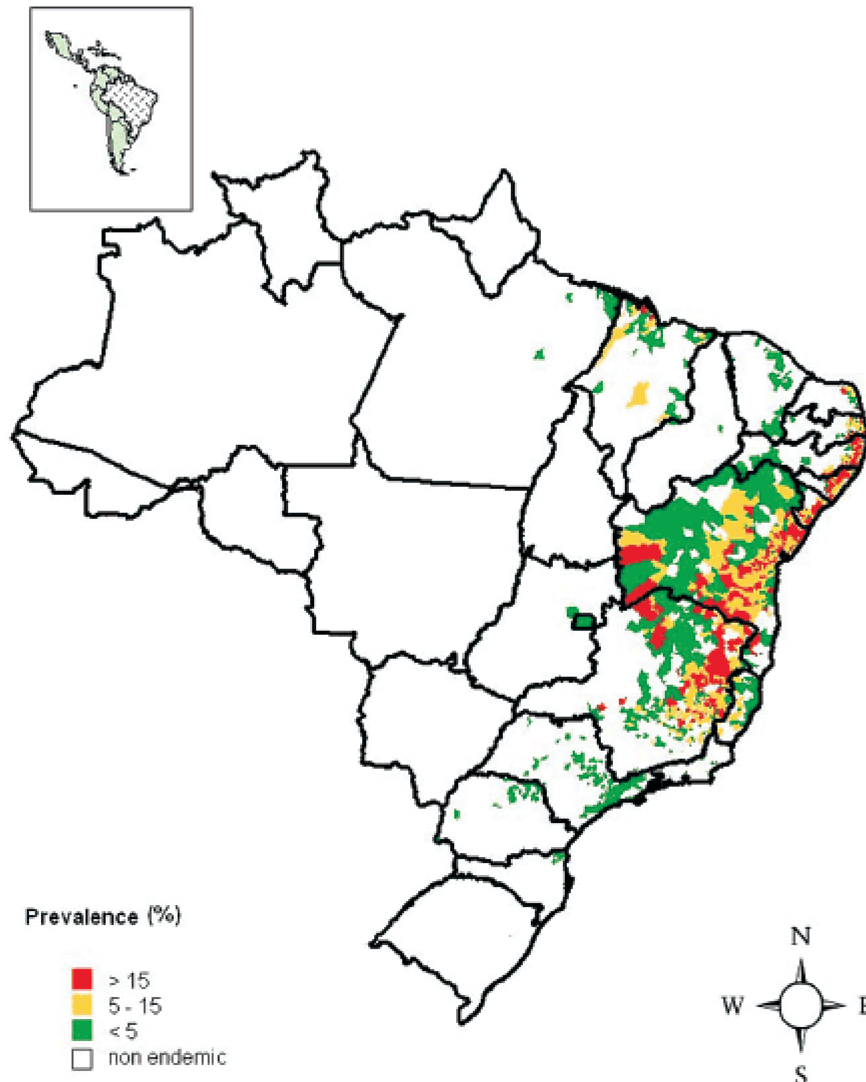
Fonte: (GRYSEELS et al., 2006).

Aproximadamente 10% dos indivíduos infectados apresentam sintomatologia severa da doença, sendo que ela é responsável por 200.000 mortes por ano no mundo (BERGQUIST,

2002). Porém, é consenso entre especialistas que a mortalidade causada por esta doença seja uma parcela pequena do problema, quando comparada aos anos de vida perdidos devido à incapacidade que ela causa (BERGQUIST, 2002). Principalmente se considerarmos que a doença atinge uma grande parcela de crianças abaixo dos 14 anos de idade, causando anemia e prejuízo no desenvolvimento cognitivo e físico, e por ser pouco reconhecida nos estágios iniciais, incapacita os indivíduos na fase mais produtiva de suas vidas (ENGELS et al., 2002). Avalia-se que o índice de DALYs ("Disability-Adjusted Life Years") para a esquistossomose seja de 1,76 milhões. O DALY é uma medida desenvolvida pela Organização Mundial da Saúde (OMS) para quantificar o impacto global de uma doença sobre a saúde da população. Este índice combina em um único parâmetro os anos de vida perdidos por uma morte prematura e os anos de vida vividos com incapacidade, ou seja, um DALY pode ser considerado como um ano de vida saudável perdido (MICHAUD; GORDON; REICH, 2003). Esse quadro fez com que a esquistossomose fosse selecionada pelo Programa Especial de Treinamento em Doenças Tropicais, da Organização das Nações Unidas, Banco Mundial e OMS como uma das dez doenças tropicais mais importantes para controle (MOREL, 2000).

A esquistossomose é causada por trematódeos hematófagos do gênero *Schistosoma*. As principais espécies patogênicas para o ser humano são: *S. mansoni*, *S. japonicum* e *S. haematobium* (Figura 1). No Brasil, ela é causada exclusivamente pelo *S. mansoni,* afetando mais de seis milhões de indivíduos, em especial nas regiões Nordeste, principalmente no estado da Bahia e na região litorânea, e Sudeste, a maior parte em Minas Gerais (Figura 2; AMARAL et al., 2006). No estado de São Paulo, foram notificados mais de duzentos mil casos nos últimos dez anos, sendo que apenas 10% desse total foram classificados como autóctones (Divisão de Orientação Técnica – SUCEN, 2002).

Figura 2 – Prevalência da esquistossomose no Brasil.

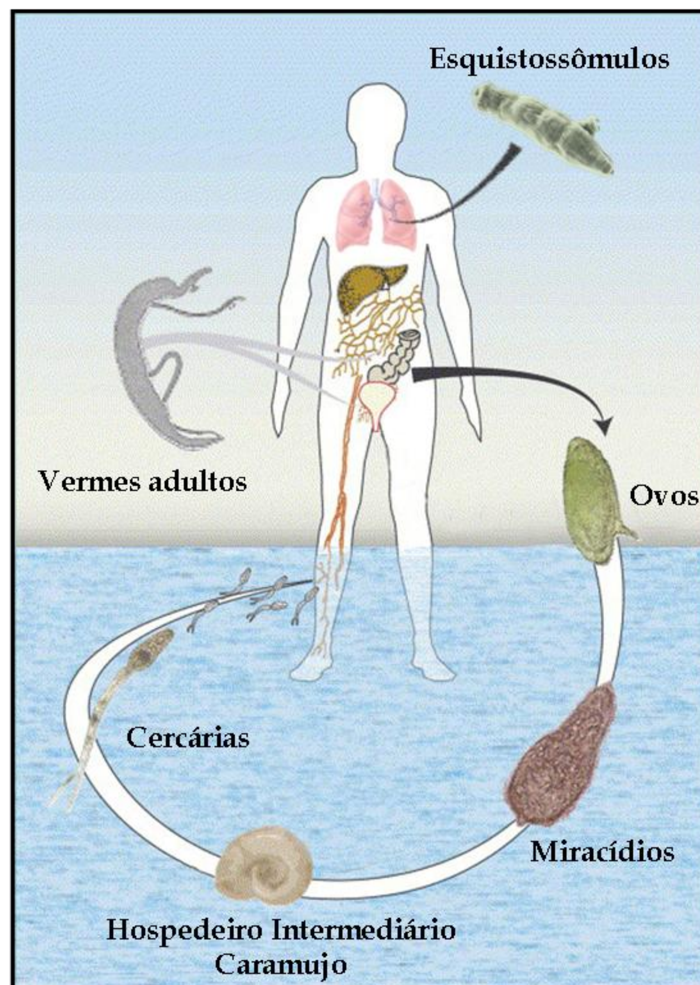


Fonte: (AMARAL et al., 2006).

O *S. mansoni* apresenta um ciclo de vida complexo compreendendo uma fase assexuada de diferenciação e multiplicação por pedogênese no caramujo hospedeiro do gênero Biomphalaria e uma fase de reprodução sexuada e oviposição no hospedeiro definitivo, o homem. O ciclo de vida envolve também dois estágios larvais infectantes, o miracídio e a cercária, que são importantes na transferência bem-sucedida do parasita de um hospedeiro para o outro (REY, 2001). Os vermes adultos residem nos vasos mesentéricos do ser humano onde realizam a oviposição. Cada fêmea produz cerca de 400 ovos por dia, os quais podem atingir a luz intestinal e serem excretados com as fezes. Uma vez na água, os miracídios eclodem e infectam o hospedeiro intermediário, o caramujo. No caramujo, cada miracídio se diferencia em esporocistos que originarão uma população clonal de cercárias.

Após serem liberadas na água, as cercárias infectam o homem penetrando na sua pele. Enquanto a cercária atravessa as camadas da pele, se diferencia em esquistossômulo, os quais penetram nos vasos, atingindo a corrente sanguínea. Na corrente sanguínea, os esquistossômulos migram para o pulmão e posteriormente para o sistema porta-hepático, onde eles amadurecem e se diferenciam em vermes adultos machos e fêmeas. Após o amadurecimento sexual, os vermes adultos formam casais, migram para os vasos mesentéricos e começam a produzir os ovos, reiniciando o ciclo (Figura 3; KING, 2009; KING, 2001; MOUNTFORD; HARROP, 1998; RIBEIRO-DOS-SANTOS; VERJOVSKI-ALMEIDA; LEITE, 2006)

Figura 3 – Ciclo de vida do Schistosoma mansoni.



Fonte: (adaptado de GRYSEELS et al., 2006).

A patologia da esquistossomose mansônica é decorrente da resposta inflamatória granulomatosa do hospedeiro induzida pelos ovos que não são eliminados com as fezes,

normalmente alojados no fígado e intestino. Posteriormente, essa resposta inflamatória evolui para fibrose e calcificação dos tecidos. A gravidade da doença está relacionada com a notável fecundidade do parasita associada à infecção duradoura, acarretando na deposição de milhões de ovos na mucosa e nos tecidos (CAPRON; CAPRON; RIVEAU, 2002).

Na fase aguda da doença, a reação inflamatória é exacerbada e o granuloma apresenta dimensão superior a 100 vezes o volume do ovo, sendo a sintomatologia variada, desde indivíduos assintomáticos, até os que apresentam febre, mal-estar, tosse, dores musculares, quadro de hepatite aguda, enterocolite aguda e hepatoesplenomegalia discreta. Na fase crônica, a resposta inflamatória é regulada e o granuloma apresenta dimensões bem menores, porém os granulomas hepáticos bloqueiam o sistema porta-hepático levando ao desenvolvimento da circulação colateral do plexo venoso mesentérico e de hipertensão portal. Em infestações severas, o acúmulo dos granulomas hepáticos e sua fibrose contínua, associada à pressão portal elevada, resultam no desenvolvimento de hepatoesplenomegalia. Esse quadro pode evoluir para ascite com o desenvolvimento de varizes esofágicas, as quais podem romper desencadeando hemorragias. Apesar da imensa maioria dos ovos ficarem retidos no sistema venoso portal, alguns podem ser levados pela corrente sanguínea, causando lesões ectópicas em diversos órgãos, como pulmão, miocárdio e sistema nervoso central (BOROS, 1989; CAPRON; CAPRON; RIVEAU, 2002; KING, 2001; REY, 2001).

O tratamento padrão da esquistossomose é a quimioterapia com o praziquantel, cujo desenvolvimento e a redução de seu custo de produção foram determinantes para a diminuição significativa da morbidade em áreas endêmicas (BERGQUIST, 2002; RIBEIRO-DOS-SANTOS; VERJOVSKI-ALMEIDA; LEITE, 2006). Entretanto, a quimioterapia não é eficaz contra os parasitas jovens e também não previne a reinfecção, comum em áreas endêmicas, resultando na necessidade de sucessivos tratamentos, tornando esse método terapêutico dispendioso e pouco eficiente na erradicação da doença, além de evidenciar a necessidade de uma abordagem mais duradoura (BERGQUIST; COLLEY, 1998). Outra desvantagem dessa abordagem terapêutica é que ela não reverte o quadro patológico, portanto, a quimioterapia elimina o parasita, mas não cura o paciente, apenas evita uma progressão do seu quadro clínico. Esse fator é agravado, pois a manifestação da esquistossomose antes da hepatoesplenomegalia tornar-se aparente é negligenciada ou indeterminada (WILSON; COULSON, 1999). Por último, deve-se ressaltar que o tratamento em massa e por um período indefinido das regiões endêmicas aumenta o risco da seleção de parasitas resistentes à droga, já tendo sido reportados baixos índices de cura no Egito e Senegal (FALLON et al., 1995; ISMAIL et al., 1999).

Assim, diante da ineficácia da quimioterapia sob o aspecto da saúde pública, das dificuldades políticas e da falta de recursos para investimento em saúde e em medidas de saneamento básico nos países em desenvolvimento, a estratégia mais eficiente e de menor custo para prevenir doenças severas e mortes seria vacinar os indivíduos susceptíveis à infecção (HOTA-MITCHELL et al., 1999; KATZ, 1999).

1.2 Fundamentos para uma vacina

Uma vacina eficaz contra a esquistossomose preveniria o desenvolvimento e a evolução da patologia, haja vista que os parasitas seriam eliminados antes de iniciarem a oviposição. A vacinação também acabaria com o círculo vicioso de sucessivas infecções, uma vez que interromperia o ciclo de vida do parasita. Modelos matemáticos demonstraram que a vacina não precisaria apresentar imunidade esterilizante e que uma redução de pelo menos 40% na carga parasitária reduziria a morbidade e as taxas de transmissão significativamente (CHAN; BUNDY, 1997). Desse modo, o combate à doença visando sua erradicação seria feita com abordagens complementares: a quimioterapia seria responsável pela redução da carga parasitária em curto prazo, enquanto a imunização proveria proteção à população em longo prazo (BERGQUIST, 1998; BERGQUIST; COLLEY, 1998).

A plausibilidade do desenvolvimento de uma vacina eficaz contra a esquistossomose está baseada principalmente na imunização de modelos experimentais, tanto roedores quanto primatas, com cercárias atenuadas por irradiação. Nestes experimentos verificou-se que a imunização com cercárias irradiadas foi capaz de induzir até 80% de proteção contra uma infecção subsequente (COULSON, 1997; HEWITSON; HAMBLIN; MOUNTFORD, 2005). Também já foi demonstrada proteção parcial pela imunização com extrato antigênico de diferentes estágios do ciclo de vida do parasita (JAMES, 1986; SMITH; CLEGG, 1985; SMITHERS et al., 1989). Porém, uma vacina para uso em seres humanos baseada em cercárias irradiadas e/ou extratos antigênicos do parasita apresentaria problemas, devido à evidência de que seu uso levaria a um nível significativo de efeitos colaterais e reações alérgicas associadas à mesma. Além da dificuldade para produção de quantidades suficientes de cercárias e antígenos em escala comercial (HAGAN; SHARAF, 2003).

Os diversos relatos de pessoas que vivem em áreas endêmicas e são naturalmente refratárias à infecção e outras que desenvolvem resistência a reinfecção após a quimioterapia também contribuem para fundamentar o desenvolvimento de uma vacina (BUTTERWORTH et al., 1985; CORREA-OLIVEIRA; CALDAS; GAZZINELLI, 2000; DESSEIN et al., 1988;

HAGAN et al., 1991; WILKINS et al., 1987). Assim como os modelos de autocura de *Rattus norvergicus* e *Macaca mulata,* nos quais o parasita é eliminado pelo sistema imune do hospedeiro. A capacidade de *R. norvergicus* em eliminar os parasitas foi associada aos elevados níveis de IgE, induzindo a ativação e degranulação de mastócitos. Enquanto na *M. mulata*, altos níveis de IgG por um período prolongado foram considerados primordiais para inibir o metabolismo dos parasitas, levando-os a inanição e morte (WILSON; COULSON, 2009; WILSON et al., 2008). Há de se considerar ainda as vacinas recombinantes antiparasitárias de uso veterinário que estão sendo desenvolvidas com sucesso, como as vacinas contra *Taenia ovis* e *Echinococcus granulosis* (DALTON; MULCAHY, 2001); e recentemente foi divulgado o início dos testes de avaliação vacinal da proteína Sm14 de *S. mansoni* contra a fasciolose em ovinos (TENDLER; SIMPSON, 2008).

O desenvolvimento de uma vacina contra a esquistossomose é um difícil desafio em função da complexidade do ciclo de vida do parasita, no qual o homem interage com quatro estágios diferentes de desenvolvimento do *S. mansoni* (cercárias, esquistossômulos, vermes adultos e ovos), desenvolvendo respostas imunes diversas e ineficazes (DUPRE et al., 2001). Por isso o principal desafio no desenvolvimento de uma vacina contra esquistossomose é a identificação de antígenos que estimulem no indivíduo uma resposta imune apropriada, acarretando na resistência contra a infecção (YANG et al., 2000). Assim, para avaliar diversos antígenos como candidatos vacinais contra esquistossomose, a OMS em 1995 estabeleceu o desenvolvimento de uma vacina contra a esquistossomose, como uma meta prioritária e organizou testes independentes. Foram avaliados seis antígenos de *S. mansoni*, reflexo dos avanços na área de biologia molecular a partir dos anos 80: uma proteína homóloga à miosina com 63 kDa, uma paramiosina de 97 kDa, uma triose-fosfato-isomerase de 28 kDa, uma proteína integral de membrana com 23 kDa (Sm23), uma proteína ligante de ácidos graxos com 14 kDa (Sm14) e uma glutationa-S-transferase com 28 kDa (Sm28GST). Os resultados destes testes nunca se tornaram públicos, mas a OMS divulgou em nota oficial que nenhum dos antígenos atingiu o objetivo, que era induzir uma redução da carga parasitária igual ou superior a 40% (WILSON; COULSON, 2006). Uma proteína ortóloga a Sm28GST, porém proveniente de *S. haematobium* (Sh28GST), atualmente está sendo avaliada em ensaios clínicos de fase II, devido a sua ação antifecundidade, apesar dos resultados dos testes de fase I nunca terem sido reportados (CAPRON et al., 2001).

Apesar dos resultados controversos desses ensaios, diversos autores defendem a continuidade dos estudos para seleção de novos antígenos vacinais, pois as moléculas selecionadas pela OMS não foram identificadas como secretadas ou expostas na superfície do

parasita, com exceção da Sm23, e atualmente acredita-se que para os antígenos induzirem uma resposta imune protetora eles devem participar da interação parasita-hospedeiro e estar acessíveis aos mecanismos efetores da resposta imune (BERGQUIST, 1998; DOENHOFF, 1998; WILSON; COULSON, 2006).

1.3 Transcriptoma, genoma, vacinologia reversa e proteoma

Em 2003, foi publicado o transcriptoma de *S. mansoni,* aumentando significativamente as informações sobre sua expressão gênica. Foram geradas 163 mil sequências expressas marcadas (EST) de seis estágios do ciclo de vida do parasita (vermes adultos, ovos, miracídios, esporocistos, cercárias e esquistossômulos), que possibilitaram sequenciar fragmentos de 92% dos 14.000 genes expressos preditos. Analisando esses dados, verificou-se que 77% representavam novos fragmentos gênicos do parasita, sendo que 1% deles era de novos parálogos, 20% de novos ortólogos e 55% dos genes codificam para proteínas sem função conhecida (VERJOVSKI-ALMEIDA et al., 2003). Esses novos dados criaram a oportunidade de identificar potenciais candidatos vacinais e alvos para drogas esquistossomicidas (MCMANUS et al., 2004; VERJOVSKI-ALMEIDA et al., 2004).

O genoma do parasita foi sequenciado em 2009, complementando e expandindo as informações disponibilizadas pelo transcriptoma. O genoma do *S. mansoni* possui 363 milhões de bases dispostas em oito pares de cromossomos, sete pares autossômicos e um par de cromossomos sexuais. O sexo é determinado no zigoto por um mecanismo cromossomal, sendo a fêmea heterogamética (ZW) e o macho, homogamético (ZZ). Foram identificados 11.809 genes, cujo tamanho médio é 4,7 kilobases com grandes íntrons e éxons pequenos. O estudo revelou o déficit do parasita no metabolismo de lipídios e identificou receptores de membrana, canais iônicos e proteases do verme como possíveis alvos quimioterápicos. Também foram identificados os gargalos metabólicos do parasita sobre os quais drogas aprovadas para outras aplicações possam ser eficazes (BERRIMAN et al., 2009).

A publicação desses bancos de dados moleculares contendo, teoricamente, toda informação gênica do parasita abriu a perspectiva da abordagem de vacinologia reversa, que consiste na seleção de candidatos vacinais através de programas de bioinformática seguida por uma triagem em larga escala destes antígenos em ensaios de imunização (RAPPUOLI, 2000). Essa técnica foi bem sucedida para propor alvos vacinais promissores para procariotos, como *Neisseria meningites* e *Streptococcus agalactiae* (MAIONE et al., 2005; PIZZA et al., 2000). Porém na seleção de candidatos para o Schistosoma e outros patógenos eucariotos

mais complexos, esta abordagem apresenta alguns inconvenientes: I – eles possuem ciclo de vida complexo com diferentes estágios, de modo que nem todos eles infectam o homem, sendo esperado que muitos genes sejam estágio-específico e nunca entrem em contato com o hospedeiro definitivo; II – ao contrário de patógenos unicelulares, o parasita é multicelular e nem todas as proteínas secretadas ou de superfície seriam apresentadas na interface parasita-hospedeiro, dificultando a seleção *in silico*; III – apesar da dedução do quadro aberto de leitura (ORF – *open reading frame*) ser útil, os programas tendem a acumular muitos erros, especialmente nas extremidades de ORFs que geralmente codificam sinais de secreção, devido à *splicings* alternativos do RNA (DEMARCO; VERJOVSKI-ALMEIDA, 2009).

Portanto, a seleção de candidatos vacinais para esquistossomose necessita de uma melhor caracterização pós-genômica destes antígenos, para isso podemos comparar o nível de expressão de muitos genes simultaneamente pela técnica de microarranjo, utilizando o mRNA dos diversos estágios do parasita. Utilizando esta abordagem já foram identificados genes diferencialmente expressos no estágio de esquistossômulos (DILLON et al., 2006), que é considerado o principal alvo do sistema imune no modelo de cercárias irradiadas; os genes com diferentes níveis de expressão em vermes adultos machos e fêmeas (FITZPATRICK et al., 2005; HOFFMANN; JOHNSTON; DUNNE, 2002) e os genes envolvidos no desenvolvimento dos esporocistos (VERMEIRE et al., 2006). Ainda baseada nesta técnica foi desenvolvida uma plataforma que explora todo o transcriptoma de vermes adultos visando avaliar sua expressão gênica quando submetidos a diferentes condições experimentais (VERJOVSKI-ALMEIDA et al., 2007).

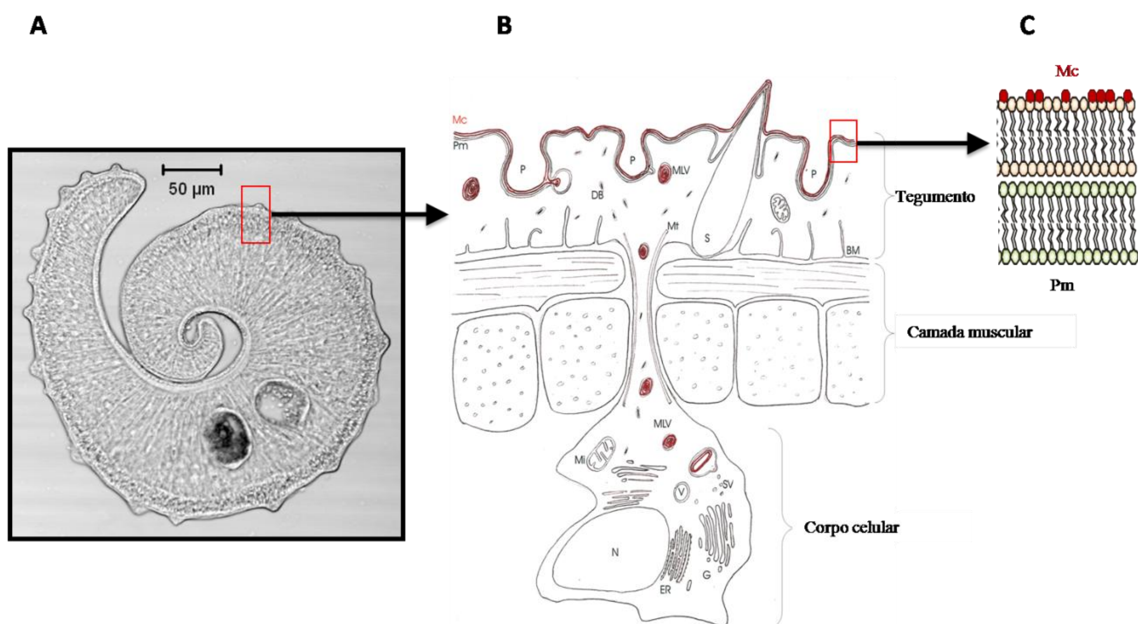
Outra forma para caracterizar e selecionar antígenos é através dos estudos de proteoma que identificam as proteínas mais abundantes em uma amostra por espectometria de massa. Esses estudos reconheceram as proteínas secretadas por cercárias envolvidas no mecanismo de penetração através da pele e evasão ao sistema imune (CURWEN et al., 2006; HANSELL et al., 2008; KNUDSEN et al., 2005) e as proteínas secretadas por ovos, buscando correlacioná-las com o desenvolvimento da patologia da doença (CASS et al., 2007). Além desses antígenos secretados, utilizando essa técnica, estudou-se a composição protéica do tegumento de vermes adultos com diferentes abordagens: proteínas exclusivas de tegumento e aquelas presentes em vermes nos quais esta estrutura foi retirada (VAN BALKOM et al., 2005), proteínas das membranas do tegumento com relação a sua solubilidade em diferentes agentes caotrópicos. (BRASCHI et al., 2006), as proteínas do tegumento acessíveis a biotinilação, portanto mais expostas em sua superfície (BRASCHI; WILSON, 2006), e as

proteínas liberadas pela digestão enzimática da superfície do tegumento de vermes adultos vivos (CASTRO-BORGES et al., 2011a).

1.4 Tegumento do *Schistosoma mansoni*

Os parasitas adultos são recobertos por uma camada sincicial, denominada tegumento, a qual é a principal interface parasita hospedeiro. Esse sincício está interligado aos corpos celulares por estreitas conexões citoplasmáticas que atravessam as camadas musculares. Nos núcleos está situada toda a maquinaria de produção e exportação protéica, composta por ribossomos, retículo endoplasmático e complexo de Golgi, que produzem e secretam para o sincício dois tipos de vesículas, corpos discóides e vesículas multilaminadas (Figura 4; SKELLY; ALAN WILSON, 2006).

Figura 4 – Tegumento do Schistosoma mansoni.



A – Secção transversal de *S. mansoni* macho adulto. B – Representação do tegumento do parasita associado ao corpo celular (sem escala). C – Detalhe das membranas da superfície externa do tegumento. BM: membrana basal; DB: corpos discóides; ER: reticulo endoplasmático; G: complexo de Golgi; Mc: membranocálice; Mi: mitocôndria; MLV: vesícula multilaminada; Mt: microtúbulo; N: núcleo; P: covas; Pm: membrana plasmática; S: espinho; SV: vesículas de transporte; V:vacúolo.
Fonte: (adaptado de BRASCHI; BORGES; WILSON, 2006).

A superfície externa do tegumento apresenta uma estrutura “heptalaminada”, considerada uma importante adaptação para a sobrevivência na corrente sanguínea. Esta estrutura é composta por duas bicamadas lipídicas: uma membrana plasmática mais interna e

um membranocálice secretado mais externo. O membranocálice seria formado pela fusão das vesículas multilaminadas, produzidas nos corpos celulares, com a membrana plasmática do tegumento. Essa fusão liberaria o conteúdo das vesículas multilaminadas na interface parasita-hospedeiro e renovaria constantemente a superfície do parasita, seu tempo de meia vida *in vivo* seria de cinco dias (SKELLY; ALAN WILSON, 2006; VAN HELLEMOND et al., 2006).

O tegumento está relacionado aos principais mecanismos de evasão ao sistema imune do hospedeiro como a digestão dos fatores do complemento (FISHELSON, 1995) e aquisição de antígenos do hospedeiro para sua mimetização (SKELLY; ALAN WILSON, 2006). Mas essa estrutura também é importante para outros processos fisiológicos do parasita, tais como nutrição, modulação da excreção, osmoregulação, recepção sensorial e transdução de sinais, demonstrando sua importância para a adaptação e sobrevivência no hospedeiro definitvo e constituindo-se, portanto, em uma importante fonte de potenciais candidatos vacinais (LOUKAS; TRAN; PEARSON, 2007).

1.5 Ectonucleotidases e nucleotídeo pirofosfatases/fosfosdiesterases

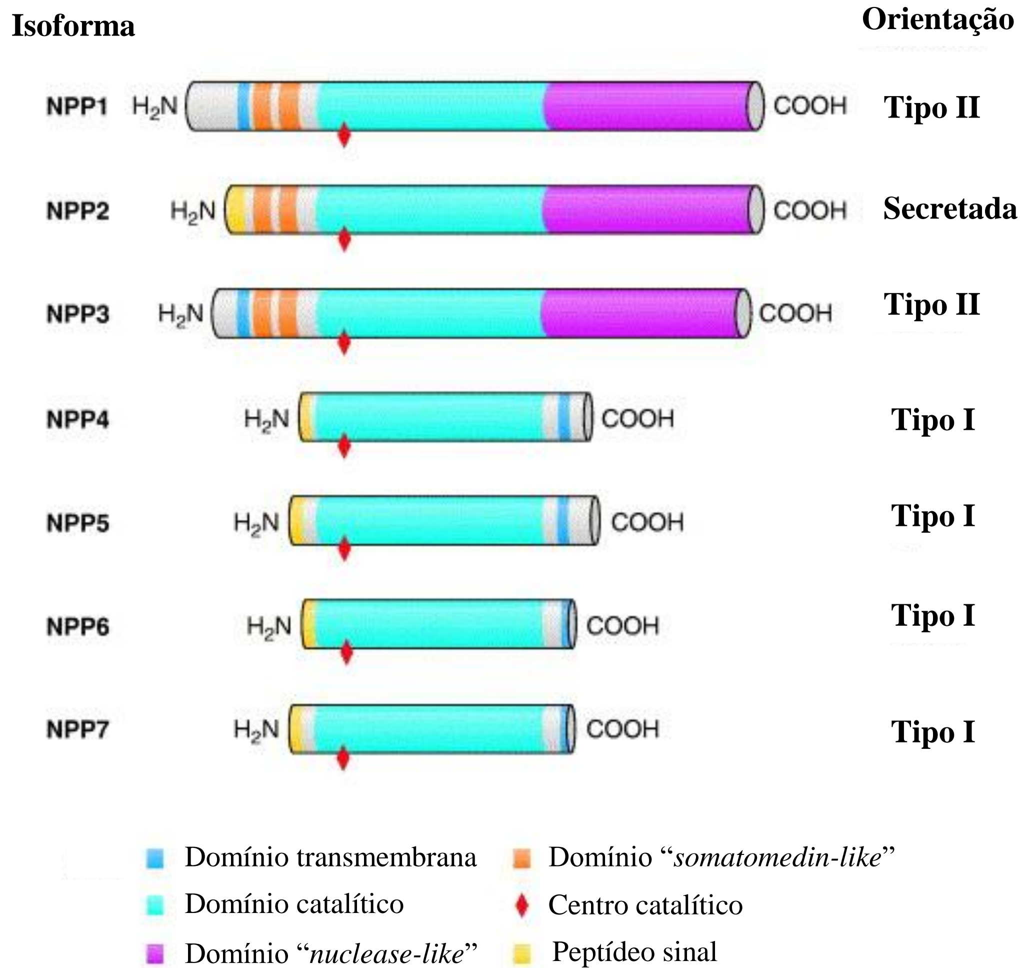
As ectonucleotidases são enzimas que metabolizam nucleotídeos, na maioria das vezes elas estão associadas à membrana com seu sítio catalítico voltado para o meio extracelular. Mas isoformas solúveis existem, sendo denominadas exonucleotidases. A máxima eficiência da atividade catalítica destas proteínas é adaptada para as condições do ambiente extracelular, requerendo a presença de cátions divalentes, como cálcio ou magnésio, e pH alcalino. As ectonucleotidases atuam no complexo metabolismo nucleotídico de modo orquestrado: várias famílias de proteínas, cada uma contendo várias espécies de enzimas semelhantes, hidrolisam diversos nucleotídeos extracelulares. Portanto, o mesmo nucleotídeo pode ser hidrolisado por diversas enzimas, dependendo do padrão de expressão gênica do tecido ou célula. É provável que enzimas pertencentes a diferentes famílias estejam colocalizadas em células individuais ou nas superfícies teciduais refutando a antiga idéia de que uma única enzima era responsável pela hidrólise de um único substrato (Zimmermann, 2000).

As ectonucleotidases conhecidas incluem membros das famílias E-NTPDase ("ecto-nucleosídeo trifosfato difosfohidrolase"), E-NPP ("ectonucleotídeo pirofosfatase/ fosfodiesterase"), fosfatases alcalinas e ecto-5'-nucleotidases (ZIMMERMANN, 2000). A presença de ectonucleotidases no tegumento de *S. mansoni* foi demonstrada há muito tempo por meio dos estudos de atividade enzimática (CESARI; SIMPSON; EVANS, 1981) e

confirmada recentemente pelos estudos de proteoma que identificaram três proteínas: uma ATPDase, uma fosfatase alcalina e uma NPP (BRASCHI et al., 2006; BRASCHI; WILSON, 2006; VAN BALKOM et al., 2005). A fosfatase alcalina talvez seja a enzima mais estudada de *S. mansoni* (BALLEN et al., 2002; BHARDWAJ; SKELLY, 2011; CESARI, 1974; DUSANIC, 1959; NIMMO-SMITH; STANDEN, 1963; PAYARES; SMITHERS; EVANS, 1984), sendo inclusive utilizada como marcador para extração do tegumento (ROBERTS et al., 1983). Duas isoformas de NTPDase, denominadas SmATPDase 1 e 2, foram caracterizadas como tegumentares (DEMARCO et al., 2003; LEVANO-GARCIA et al., 2007) e epítopos comuns entre elas e a apirase de batata já foram descritos (FARIA-PINTO et al., 2010; FARIA-PINTO et al., 2008), porém, não há uma melhor caracterização da NPP afora sua identificação.

As NPPs (do inglês, *nucleotide pyrophosphatase/phosphodiesterase*) são ectoenzimas estruturalmente relacionadas, que *in vitro* são capazes de hidrolisar ligações pirofosfato, como a do ATP, e ligações fosfodiéster, como a de oligonucleotídeos, resultando na liberação de nucleosídeos 5'monofosfatados (BOLLEN et al., 2000). Atualmente estão descritas sete isoformas de NPPs em seres humanos, numeradas conforme sua ordem de descoberta; todas apresentam um domínio catalítico extracelular com aproximadamente 400 resíduos de aminoácidos. Além do domínio catalítico, as isoformas NPP4-7 apresentam um peptídeo sinal putativo N-terminal e um domínio transmembrana C-terminal. Portanto, são proteínas transmembranas do tipo I. As isoformas NPP1 e NPP3 são proteínas transmembranas do tipo II, apresentando um domínio transmembrana N-terminal, dois domínios "*somatomedin-B like*" consecutivos, o domínio catalítico e um domínio "*nuclease-like*" C-terminal. A isoforma NPP2 apresenta quase todos os domínios das isoformas NPP1 e NPP3; porém, ao invés do domínio transmembrana, apresenta um peptídeo sinal, sendo uma proteína secretada e não transmembrana (Figura 5; STEFAN; JANSEN; BOLLEN, 2005).

Figura 5 – Estrutura das sete NPPs de seres humanos.



Fonte: (adaptado de STEFAN; JANSEN; BOLLEN, 2005).

O sítio catalítico das NPPs possui uma similaridade estrutural muito grande com a superfamília das "*phospho- or sulfo-coordinating metalloenzymes*", que inclui também as fosfatases alcalinas, apresentando estruturas conservadas como resíduos de aminoácidos que coordenam a interação com dois metais divalentes essenciais à atividade enzimática e a disposição espacial relativa desses metais a importantes resíduos de aminoácidos do sítio catalítico. Baseando-se nestas informações foi proposta uma teoria para o mecanismo de catálise das NPPs, que aconteceria em duas etapas. Na primeira etapa da catálise, o grupamento hidroxila do sítio catalítico treonina, ativado por um dos metais divalente, atacaria o grupo fosfato do substrato, resultando na formação de uma estrutura covalente intermediária. No segundo passo, uma molécula de água ativada pelo outro metal divalente atacaria essa estrutura intermediária, restaurando o sítio catalítico da treonina e liberaria um nucleosídeo 5'-monofosfato (GIJSBERS et al., 2001).

As NPPs atuam na reciclagem de nucleotídeos, no controle dos níveis extracelulares de pirofosfato, na estimulação da motilidade celular, na modulação da sinalização de receptores purinégicos e possivelmente estão envolvidas no controle da sinalização dos receptores de insulina e na atividade de ecto-quinases. Essas proteínas já foram relacionadas a diversos processos biológicos: neurotransmissão, neuroproteção à isquemia e hipóxia, regulação das funções cardiovasculares, agregação plaquetária, contração da musculatura lisa, secreção de hormônios, modulação da resposta imune e controle da apoptose, da proliferação e da diferenciação celular (GODING; GROBBEN; SLEGERS, 2003).

1.6 Sinalização purinérgica

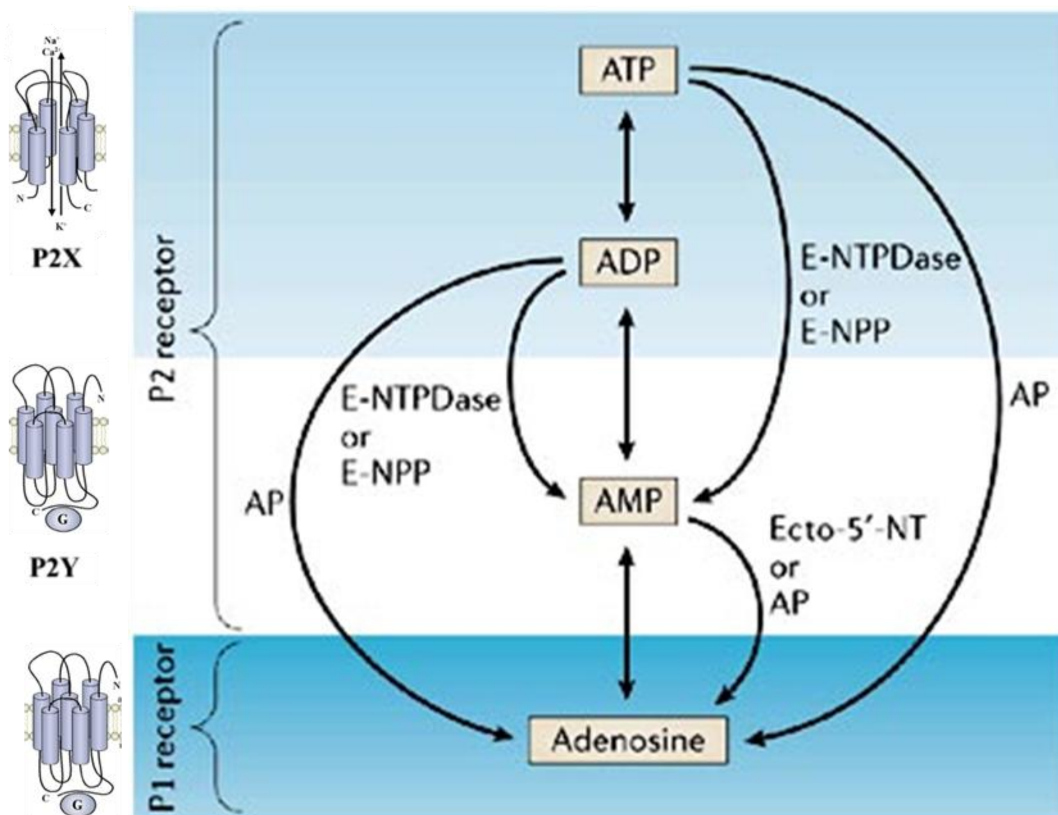
Em 1972, o termo “purinérgico” foi utilizado pela primeira vez por Burnstock para descrever a sinalização na qual o ATP era a molécula mensageira extracelular; naquela época essa foi uma idéia bastante radical. Desde então, a importância da sinalização purinérgica envolvendo não apenas ATP, mas outros nucleotídeos trifosfatados e bifosfatados, tornou-se cada vez mais evidente (ABBRACCHIO; BURNSTOCK, 1998). Atualmente, nucleotídeos extracelulares são considerados moléculas sinalizadoras autócrinas e parácrinas que modulam uma grande variedade de respostas fisiológicas em diversos tecidos de mamíferos. Essa sinalização consiste na liberação de nucleotídeos para o meio extracelular, que interagem de modo seletivo a receptores específicos; essa ligação desencadeia uma cascata de reações bioquímicas variáveis resultando em diversos efeitos fisiológicos (GOUNARIS; SELKIRK, 2005).

Os nucleotídeos sinalizadores podem ser secretados de modo regular, ou podem ser liberados por estimulação mecânica, haja vista que danos teciduais resultam em liberação massiva para os fluidos extracelulares; desse modo, nucleotídeos extracelulares são ativadores arquetípicos do sistema imune inato. Nas células hematopoiéticas, em específico, os receptores nucleotídicos estimulam diversas ações, incluindo: agregação plaquetária e liberação de mediadores; degranulação de mastócitos, neutrófilos e eosinófilos; produção de citocinas por células T, monócitos e macrófagos; ativação e diferenciação de células dendríticas (GOUNARIS; SELKIRK, 2005).

Os receptores da sinalização purinérgica são específicos e podem ser de dois tipos, P1 ou P2. Os receptores P1 são acoplados a proteína G e apresentam quatro subtipos que são ativados pela adenosina. Enquanto os receptores P2 apresentam 2 subtipos: receptores P2X, específico para ATP, e receptores P2Y ativados por ATP, ADP, UTP, UDP, ITP e açucares

nucleotídicos. Os receptores P2X são canais iônicos regulados pelo nucleotídeo ligante, enquanto receptores P2Y são receptores acoplados a proteína G (SANSOM; ROBSON; HARTLAND, 2008). Regulando a concentração extracelular dos nucleotídeos extracelulares por meio de sua hidrólise, as ectonucleotidases são capazes de modular a sinalização punérgica. As NTPDases e as NPPs hidrolizam nucleosídeos 5'-trifosfatados e 5'-difosfatados liberando um grupamento fosfato inorgânico, enquanto as fosfatases alcalinas e as 5'-nucleotidases convertem o nucleotídeo em nucleosídeo. No entanto, enquanto as 5'-nucleotidases hidrolisam apenas nucleotídeos monofosfatados, as fosfatases alcalinas produzem nucleosídeos a partir de nucleotídeos mono, di e trifosfatados (Figura 6. ZIMMERMANN, 2000)

Figura 6 – Produtos da hidrólise do ATP pelas ectonucleotidases e seus respectivos receptores purinérgicos.



E-NTPDase – ecto-nucleosídeo trifosfato difosfohidrolase; E-NPP – ectonucleotídeo pirofosfatase/fosfodiesterase; Ecto-5'-NT – ecto-5'-nucleotidase; AP – fosfatase alcalina.
Fonte: (adaptado de FIELDS; BURNSTOCK, 2006).

Já foi hipotetizado que a expressão destas ectonucleotidases na interface parasita-hospedeiro foi provavelmente uma adaptação evolutiva dos parasitas selecionada para alterar

a disponibilidade e a concentração local de nucleotídeos extracelulares, visando regular a ativação de receptores purinérgicos e a resposta inflamatória subsequente. Essa estratégia seria vantajosa para parasitas hematófagos, patógenos que causam danos aos tecidos ou espécies que residam no sistema vascular, pois inibiria a agregação plaquetária e a subsequente liberação de nucleotídeos pró-inflamatórios. Esta seria, portanto, uma característica conservada evolutivamente e subestimada em diversos organismos infecciosos, como por exemplo, o *Schistosoma* (GOUNARIS; SELKIRK, 2005).

2 OBJETIVOS

2.1 Objetivo geral

Identificar e caracterizar as nucleotídeo pirofosfatases/ fosfosdieterases de *S. mansoni* (SmNPPs) e avaliá-las como potenciais candidatos vacinais.

2.2 Objetivos específicos

- Identificar e selecionar as SmNPPs sequenciadas no genoma do parasita;
- caracterizar e clonar os genes *smnpps* e avaliar seu perfil de expressão transcricional;
- expressar e purificar essas proteínas de modo heterólogo;
- caracterizar o padrão de expressão protéico das SmNPPs;
- avaliar o perfil da resposta imune induzida pela imunização de camundongos com as SmNPPs recombinantes;
- avaliar o potencial das SmNPPs recombinantes como candidatos vacinais contra a esquistossomose.

3 MATERIAIS E MÉTODOS

3.1 Seleção dos genes *smnpps*

Foi feita uma busca no banco de dados do genoma de *S. mansoni* (BERRIMAN et al., 2009 – http://www.genedb.org/Homepage/Smansoni) e de *S. japonicum* (ZHOU et al., 2009 – http://www.genedb.org/Homepage/Sjaponicum), utilizando como palavras chave: "*ectonucleotide pyrophosphatase/phosphodiesterase*". As sequências gênicas traduzidas destas NPPs foram alinhadas duas a duas para descartar possíveis sequências redundantes, utilizando o programa bl2seq (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Essa análise também forneceu a identidade e a similaridade entre as proteínas preditas.

3.2 Análise *in silico* da estrutura primária e cladograma das SmNPPs

As sequências preditas de aminoácidos das *smnpps* e das *sjnpps* foram analisadas com o programa SMART (http://smart.embl-heidelberg.de/) para avaliar a presença de domínios protéicos significativos. O programa SignalP (http://www.cbs.dtu.dk/services/SignalP/) foi utilizado para averiguar a predição de sequências sinais de exportação, enquanto o programa TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) foi empregado para avaliação da presença de porções transmembranas.

As proteínas preditas de *S. mansoni* foram submetidas à análise com a ferramenta Compute pI/Mw (http://www.expasy.org/tools/pi_tool.html) para predizer o ponto isoelétrico (pI) e a massa molecular. A partir de suas estruturas primárias ainda foram analisados sítios putativos para modificações pós-transducionais como miristilação, acetilação, C-manosilação, glicação do ε aminogrupo de lisina, glicosilações do tipo N e O, fosforilação e sulfatação de resíduos de tirosina; utilizando as respectivas ferramentas de bioinformática: http://mendel.imp.ac.at/myristate/SUPLpredictor.htm; http://www.cbs.dtu.dk/services/NetAcet/; http://www.cbs.dtu.dk/services/NetCGlyc/; http://www.cbs.dtu.dk/services/NetGlycate/; http://www.cbs.dtu.dk/services/NetNGlyc/; http://www.cbs.dtu.dk/services/NetOGlyc/; http://www.cbs.dtu.dk/services/NetPhos/ e http://www.expasy.ch/tools/sulfinator/.

Utilizando o banco genômico do "*National Center for Biotechnology Information*" (NCBI – http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), comparou-se a identidade e a similaridade das SmNPPs e das SjNPPs com seus respectivos ortólogos mais próximos de

Homo sapiens. A partir do alinhamento múltiplo feito com o programa CLUSTALW (http://www.ebi.ac.uk/clustalw/) das NPPs de *S. mansoni* com as de *S. japonicum*, de *H. sapiens*, de *R. norvergicus* e de *Mus musculus* [números de acesso no NCBI: HsNPP-1 (NP_006199.1), HsNPP-2 (NP_006200.3), HsNPP-3 (NP_005012.2), HsNPP-4 (NP_055751.1), HsNPP-5 (NP_067547.1), HsNPP-6 (NP_699174.1), HsNPP-7 (NP_848638.2), MmNPP-1 (NP_032839.3), MmNPP-2 (NP_056559.2), MmNPP-3 (NP_598766.2), MmNPP-4 (NP_950181.2), MmNPP-5 (NP_114392.1), MmNPP-6 (NP_796278.1), MmNPP-7 (NP_001025462.1), RnNPP-1 (AAL26912.1), RnNPP-2 (NP_476445.2), RnNPP-3 (NP_062243.2), RnNPP-4 (NP_001100362.1), RnNPP-5 (NP_001012762.1), RnNPP-6 (XP_224853.3), RnNPP-7 (NP_001012484.1)], foi produzida um cladograma, visualizado pelo programa TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Também utilizando o programa CLUSTALW foi realizado um alinhamento múltiplo com a estrutura primária das SmNPPs com as SjNPPs e as NPPs-5 e 6 de *H. sapiens*. Após edição manual, sítios putativos de ligação a metal e o provável centro catalítico foram identificados por comparação (BOLLEN et al., 2000; SAKAGAMI et al., 2005).

3.3 Manutenção do ciclo de *Schistosoma mansoni*

O ciclo do *S. mansoni*, linhagem BH, é mantido no Laboratório de Parasitologia do Instituto Butantan sob a supervisão da Dra. Toshie Kawano (*in memoriam*) e Dra. Eliana Nakano pela Msc. Patrícia Aoki, utilizando caramujos *Biomphalaria glabrata* e hamsters como hospedeiros.

3.3.1 Vermes adultos

Os vermes adultos e os vermes adultos jovens foram obtidos pela perfusão a partir da artéria aorta de hamsters infectados com *S. mansoni*, 45 e 21 dias após a infecção, respectivamente. Os parasitas presentes nas veias mesentéricas foram expelidos por uma incisão da veia porta e coletados. Os animais foram perfundidos com meio RPMI 1640 (Sigma) e heparina (2,5 unidades/ml – Cristália). O sobrenadante foi descartado após a sedimentação dos vermes, que foram lavados três vezes com meio RPMI 1640.

3.3.2 Ovos

Para obtenção dos ovos, três fígados de hamsters foram coletados e dilacerados com lâminas de bisturi após 45 dias da infecção com 100 cercárias. Os fígados foram digeridos com 20 mg de colagenase II (Gibco), 50 µg/mL de gentamicina (Gibco), 2 µg/mL de ampicilina (Sigma) e q.s.p. 50 mL de tampão fosfato de sódio (PBS) por 16 h a 37 ºC sob 250 rpm de agitação. Essa mistura foi centrifugada a 400 g por 5 min e o sobrenadante foi descartado, sendo o precipitado ressuspendido em q.s.p. 50 mL de PBS. Essa lavagem foi repetida mais duas vezes; porém, após a última lavagem, o precipitado foi ressuspendido em apenas q.s.p. 25 mL de PBS. A suspensão foi filtrada, primeiro em peneira de 250 µm e em seguida em peneira de 150 µm, com auxílio de uma bomba a vácuo. Após o material peneirado ser centrifugado a 400 g por 5 min e o sobrenadante ser descartado, o precipitado foi ressuspendido em 3 mL de PBS. Em um tubo de 50 mL, foi preparada um gradiente de Percoll (8 mL de Percoll [Ge Healthcare] + 32 mL de 0,25 M Sacarose [Sigma]), sobre a qual foram aplicados os 3 mL do precipitado peneirado. Esse material foi centrifugado a 800 g por 10 min; o sobrenadante, que continha as células de fígado, foi descartado e o precipitado foi ressuspendido em tampão PBS com 2 mM de EDTA (Invitrogen), centrifugados e lavados novamente mais duas vezes, para inativar a enzima colagenase. Na última lavagem, o precipitado foi ressupendido no menor volume possível e aplicado sobre uma segunda coluna de Percoll (2 mL de Percoll + 8 mL de 0,25 M Sacarose), montada em um tubo de 15 mL. A coluna foi centrifugada a 800 g por 15 min e o sobrenadante descartado. O precipitado de ovos foi lavado mais três vezes com PBS, assegurando a ausência de células do fígado (DALTON et al., 1997).

3.3.3 Miracídios

Os ovos purificados foram ressuspendidos em água destilada e expostos a uma luz artificial para eclodirem, liberando os miracídios. O sobrenadante, contendo os miracídios, foi coletado e resfriado em gelo por 30 min para que os parasitas perdessem mobilidade. Os miracídios foram então centrifugados a 10.000 g a 4 ºC por 2 min para sua concentração sendo o sobrenadante descartado.

3.3.4 Cercárias

As cercárias foram obtidas expondo-se caramujos infectados há 35-40 dias com 10 miracídios à luz artificial por 2 h em béquer com água destilada. Após a exposição, a água contendo as cercárias foi distribuída em tubos de 50 mL que foram resfriados no gelo por 30 min para que os parasitas perdessem mobilidade. As cercárias foram então centrifugadas a 800 g a 4 ºC por 2 min, sendo a maior parte do sobrenadante descartado.

3.3.5 Esquistossômulos

Os esquistossômulos foram obtidos pela transformação *in vitro* das cercárias. Após a última centrifugação, as cercárias foram ressuspendidas em 2 mL de meio ELAC (Meio MEM [Gibco], suplementado com lactoalbumina 0,1%, glicose 0,1%, MEM vitaminas 0,5% e Hepes 10 mM) estéril a 37 ºC, para sofrerem o choque térmico. Em seguida, as cercárias foram submetidas ao choque mecânico, no vórtex em velocidade máxima por 90 s para a ruptura das caudas, e incubadas por 3 h na estufa de CO_2 a 37 ºC para sua transformação em esquistossômulos. Após essa incubação foram realizadas diversas lavagens para remoção das caudas; no fluxo laminar, os tubos foram deixados por 5 min para a sedimentação dos esquistossômulos e o sobrenadante contendo as caudas foi descartado, sendo os esquistossômulos ressuspendidos em 5 mL de meio ELAC e visualizados no microscópio óptico para averiguação, esse procedimento foi realizado tantas vezes quantas foram necessárias. No final, os esquistossômulos foram transferidos para uma garrafa de cultura contendo 20 mL de meio M-169 (Meio ELAC suplementado com hipoxantina $5x10^{-7}$ M, serotonina $1x10^{-6}$ M, hidrocortisona $1x10^{-6}$ M, triiodotironina $2x10^{-7}$ M, meio Schneider 5%, soro bovino fetal 5%, penicilina/estreptomicina 200 U/mL e gentamicina 50 mg/mL) e deixados na estufa de CO_2 a 37 ºC por 7 dias. Após esse período, os esquistossômulos foram centrifugados por 3 min a 200 g, lavados em 5 mL de PBS e centrifugados novamente (adaptado de BASCH, 1981).

3.4 PCR quantitativo

3.4.1 Extração de RNA e síntese do cDNA

Miracídios, cercárias, esquistossômulos e vermes adultos, após serem obtidos quando destinados à extração de RNA foram conservados em *RNA latter* (Ambion) a -80 ºC conforme instruções do fabricante. Os ovos quando destinados à extração de RNA eram submetidos à extração de RNA imediatamente após sua purificação. Para extração do RNA total dos diversos estágios do parasita, foi utilizado o Kit RNAspin mini (GE Healthcare) conforme as instruções do fabricante. Após a extração, as amostras de RNA foram quantificadas e analisadas quanto à pureza no espectrofotômetro ND-1000 (NanoDrop Techonologies) em comprimentos de onda de 230, 260 e 280 nm. A integridade do RNA extraído foi avaliada através de eletroforese por capilaridade no RNA LABChip do Bioanalyzer 2100 (Agilent Technologies), resultando num gel representativo virtual.

O RNA extraído (3 µg) de cada estágio foi tratado com DNAse livre de RNAse RQ1 (Promega) por 1 h à 37 ºC para eliminar uma possível contaminação com DNA genômico. O volume da reação foi dividido em duas frações. Em uma fração como controle não foi adicionada a enzima transcriptase reversa, para se averiguar posteriormente a possível amplificação de DNA genômico contaminate. Na outra fração, a enzima transcriptase reversa Superscript III (Invitrogen) foi usada na reação de transcrição reversa para a síntese da primeira fita de DNA complementar aos mRNA de cada estágio (cDNAs). A reação seguiu as indicações do fabricante, utilizando oligonucleotídeos randômicos para a síntese de cDNA.

3.4.2 Reação de PCR

Em placas de 96 poços (Applied Biosystems) foram preparadas as reações para os genes e os controles. Foram realizadas três reações para um mesmo gene de cada estágio, para se verificar a variabilidade experimental e sua reprodutibilidade. Em cada poço foram adicionados 6 µl de amostra (contendo 0,15 µl do cDNA e 5,85 µl de água, para facilitar a pipetagem), 2 µl de cada oligonucleotídeo específico do gene (10 mM) e 10 µl de SYBR Green (Applied Biosystems). A amplificação dos genes a partir das amostras para cada estágio de RNA não transcrito foi utilizado como controle de contaminação por DNA genômico. Como controle da amplificação de contaminantes inespecíficos e da dimerização dos oligonucleotídeos foi realizada uma reação sem cDNA, ou seja, apenas com água e

oligonucleotídeos . As quantidades de cDNA e oligonucleotídeos utilizadas foram avaliadas de modo que não fossem limitantes para a reação de amplificação. Os oligonucleotídeos "*forward*" e "*reverse*" foram desenhados pelo software Primer Express (Applied Biosystems) para que pareassem entre regiões de éxons diferentes, evitando assim a amplificação de DNA genômico. As placas foram revestidas por adesivos ópticos (Applied Biosystems) e colocadas no termociclador 7300 Real time PCR System para a seguinte reação: 10 min a 95 ºC e 40 ciclos de 10 s a 95 ºC seguido por 1 min a 60 ºC. Ao final dessa reação foi feita uma curva de dissociação do fragmento amplificado.

3.4.3 Análise dos dados

A primeira análise realizada foi a avaliação da curva de dissociação de cada poço realizada ao final da reação de amplificação. Foi monitorado o nível de fluorescência emitido em função da variação da temperatura e resultou em um gráfico. O nível de fluorescência se correlaciona a dissociação da dupla fita de DNA amplificada pela reação de PCR devido ao aumento da temperatura e dessa forma avalia-se a especificidade da reação.

Para análise da quantificação relativa utilizou-se a fórmula, $\text{"}Expressão\ relativa\text{"} = 2^{-\Delta\Delta Ct}$, para demonstrar quantas vezes um gene foi mais expresso em um estágio em relação a outro (LIVAK; SCHMITTGEN, 2001). Basicamente, as emissões de fluorescência a cada ciclo foram coletadas, analisadas pelo *software* 7300 *System* SDS e os resultados foram apresentados em gráficos de nível de fluorescência (ΔRn) *vs* número de ciclos. Determinou-se arbitrariamente um nível limiar de fluorescência, tendo como única exigência que este nível de fluorescência correspondesse à fase exponencial de amplificação das reações. Foi averiguado em qual ciclo cada reação atingiu o nível de fluorescência limiar (Ct, do inglês *cycle threshold*). O valor de Ct do gene de interesse foi comparado ao valor de Ct do controle endógeno, α-tubulina (Número de acesso NCBI: M80214), do mesmo estágio para normalização dos dados, determinando o ΔCt. O estágio que apresentava o maior valor de ΔCt foi utilizado como calibrador, por apresentar o menor nível de expressão gênica. Assim subtraiu-se o ΔCt de cada estágio do ΔCt calibrador, gerando o ΔΔCt. Por último, os dados foram colocados em uma escala exponencial, uma vez que os dados gerados pela reação de PCR estão em escala linear, e os gráficos foram apresentados como Expressão relativa *vs* Estágios. As diferenças de expressão foram analisadas estatisticamente por ANOVA e, posteriormente, pelo Teste de Tukey. Um ρ value <0,05 foi considerado estatisticamente significativo.

3.5 Clonagem dos genes *smnpps*

Os genes *smnpps* foram amplificados a partir do RNA de vermes adultos, que foi purificado pelo Kit RNAspin mini (GE Healthcare), segundo as orientações do fabricante. Após a extração, as amostras de RNA foram quantificadas e analisadas quanto à pureza no espectrofotômetro ND-1000 (NanoDrop Techonologies) em comprimentos de onda de 230, 260 e 280 nm. Para evitar a contaminação do material com DNA genômico, 1,5 µg de RNA extraído foi tratado com DNAse livre de RNAse RQ1 (Promega), por 1 h à 37 ºC, para cada gene a ser amplificado. A fita de cDNA específica para cada gene foi sintetizada com a enzima transcriptase reversa Superscript III (Invitrogen), conforme a orientação do fabricante, utilizando oligonucleotídeos específicos de cada gene *smnpp* (APÊNDICE). A fita de cDNA específica sintetizada (5µL) foi utilizada como molde para amplificação com Taq DNA Polimerase (Invitrogen) e os respectivos oligonucleotídeos específicos (APÊNDICE) na reação: 1 min a 94 ºC, seguido por 40 ciclos de 30 s a 94 ºC, 1 min a 45 ºC e 3 min 72 ºC. O tamanho dos fragmentos gênicos amplificados foi confirmado em gel de agarose, sendo as bandas, com os respectivos tamanhos esperados, purificadas com o kit GFX PCR DNA and Gel Band purification (GE HealthCare). Os genes foram inseridos em vetor pGEM-T easy (Promega), conforme as orientações do fabricante, e esses plasmídeos foram utilizados para transformar células competentes de *Escherichia coli* DH5α. As bactérias foram cultivadas e utilizadas para purificação dos plasmídeos com o kit plasmidPrep Mini Spin (GE HealthCare). Os plasmídeos purificados foram triados quanto à presença e orientação dos insertos por PCR. Posteriormente a sequência gênica foi confirmada por sequenciamento automático.

3.6 Expressão das SmNPPs em *Escherichia coli,* purificação e renovelamento

Para expressão das proteínas recombinantes em *E. coli* os genes foram inseridos no vetor pAE-6His (RAMOS et al., 2004). Utilizando como molde os genes clonados em pGEM-T Easy, amplificou-se o fragmento do gene, que codifica o domínio NPP e exclui-se a região transmembrana e o peptídeo sinal predito, com a enzima Taq DNA Polimerase e oligonucleotídeos específicos (APÊNDICE), que adicionaram sítios de restrição, Xho I e Kpn I, às sequências nas regiões 5´e 3´, respectivamente. Os fragmentos foram purificados com o kit GFX PCR DNA and Gel Band purification (GE Healthcare) e digeridos com as respectivas enzimas de restrição (Invitrogen), que também foram utilizadas para digerir o vetor pAE-

6His. Os fragmentos e o vetor foram novamente purificados e utilizados para ligação com a enzima T4 DNA ligase (Promega), na proporção molar de inserto:vetor, 3:1. Os produtos das reações de ligação foram utilizados para transformar células competentes de *E. coli* DH5α. As bactérias foram cultivadas na presença de ampicilina (100 µg/mL) e os clones transformados foram utilizados para purificação dos plasmídeos, os quais foram avaliados quanto à presença de inserto por PCR e posteriormente por sequenciamento automático.

Os plasmídeos pAE-6His contendo os insertos das *smnpps* foram utilizados para transformar bactérias *E.coli* da cepa BL21 Star (DE3) plysS. A expressão das proteínas foi analisada cultivando-se em meio 2YT (triptona 1,6%, extrato de levedura 1% e NaCl 0,5%) com ampicilina (100 µg/mL) a 37 ^{o}C três colônias transformadas até atingir a densidade ótica (D.O.) de 0,6-0,8. Quando o inóculo atingiu a densidade óptica desejada, foi adicionado IPTG (1 mM) para induzir a expressão da proteína recombinante pelas bactérias por 4 h. Após a indução, o cultivo das células de *E.coli* transformadas com o plasmídeo recombinante foi centrifugado por 10 min a 12.000 g. O precipitado foi ressuspendido em 1 mL de tampão de lise (Tris,50 mM e NaCl 100 mM; pH 8,8,) e analisado por eletroforese de gel de poliacrilamida e SDS (SDS-PAGE). Como controle, foi analisada a cepa transformada com o vetor vazio e induzida nas mesmas condições. Outro parâmetro avaliado foi o escape de expressão da proteína recombinante antes da indução; para tanto 1 mL de cultivo foi retirado para análise antes de adicionar o indutor.

Após se averiguar que as bactérias expressavam as proteínas recombinantes, foi analisada a solubilidade das mesmas. Para isso, expressaram-se as proteínas recombinantes como anteriromente descrito, porém em 50 mL de cultura. Depois da indução da expressão, as bactérias foram centrifugadas, ressuspendidas em 20 mL de tampão de lise e lisadas no aparelho French Press (1500 psi – 3 passagens). Em seguida, o lisado foi centrifugado por 30 min a 10.000 rpm, a fração solúvel foi coletada e armazenada. O precipitado foi ressupendido em 20 mL de tampão de lise e 1 M uréia e centrifugado novamente, sendo a fração solúvel coletada. Esse processo foi repetido diversas vezes com concentrações crescentes de uréia (2 M, 4 M, 6 M e 8 M). O *pellet* final foi ressupendido em solução de lise e 2% SDS. Todas as frações foram analisadas por SDS-PAGE.

Para a purificação das SmNPPs recombinantes, foi cultivado um clone expressando cada uma das proteínas em volumes maiores de meio de cultura (200 mL). Após atingirem a D.O. 0,6-0,8, foi induzida a expressão das proteínas recombinantes com IPTG 0,5 mM a 18 ^{o}C por 20 h visando aumentar sua solubilidade (adaptado de ZALATAN et al., 2006). Após a expressão, as bactérias foram centrifugadas, ressuspendidas em 30 mL de tampão de lise e

lisadas no aparelho French Press (1500 psi – 3 passagens). Em seguida, o lisado foi centrifugado por 30 min a 10.000 rpm, sendo o precipitado ressupendido em solução de lise com2 M uréia. Após essa lavagem, a solução foi novamente centrifugada e os corpúsculos de inclusão solubilizados em 8 M uréia. A solução foi novamente centrifugada e o precipitado foi descartado antes dacromatografia de afinidade ao níquel em colunas HisTrap HP 5 mL (GE). Esta purificação se baseia na afinidade do metal a cauda de histidina N-terminal, inserida à proteína pelo plasmídeo pAE-6His. Utilizou-se um gradiente de concentração crescente de imidazol para a eluição das proteínas, devido a competição do imidazol com a histidina pela ligação ao metal. Esse processo foi realizado com o auxílio do aparelho Äkta Prime (Amersham Pharmacia). Após a cromatografia, as frações da eluição correspondentes ao pico de absorbância de 280 nm foram analisadas por SDS-PAGE. Aquelas que apresentaram a banda da proteína recombinante expressa foram submetidas à diálise lenta em membranas *SnakeSkin* (Pierce) com poros de 3 kDa para renovelamento, na proporção de proteína e tampão de 1:100, com concentrações decrescentes de uréia.

Foram avaliadas ainda mais quatro metodologias de renovelamento das proteínas recombinantes além da diálise lenta. Primeiramente foi avaliado o renovelamento rápido por diluição em solução tampão sem uréia, no qual a proteína é diluída em tampão na proporção 1:100 antes da cromatografia de afinidade ao níquel. Também foi testado o renovelamento da proteína na coluna, que se baseia em realizar o renovelamento da proteína enquanto a mesma está ligada a coluna, para isso diminui-se gradualmente a concentração de uréia até sua retirada completa para depois eluir a proteína purificada e renovelada.

Outra metodologia de renovelamento testada foi o renovelamento das proteínas antes de sua purificação em alta pressão e temperatura (CHURA-CHAMBI et al., 2008). Basicamente os corpúsculos de inclusão foram ressuspendidos em tampão de renaturação contendo 1 M de guanidina e concentrações inversamente proporcionais de glutationa oxidada e reduzida. Amostras com 1 mL foram acondicionadas em sacos plásticos selados e submetidas a uma prensa, com uma mistura de água e óleo como fluído de transmissão de pressão. Aplicou-se uma pressão de 29.000 psi durante 16 h. Posteriormente, as amostras foram centrifugadas a 12.000 g por 15 min para remover os agregados insolúveis restantes, dialisadas e centrifugadas novamente. A fração solúvel foi submetida à análise por SDS-PAGE e à avaliação da atividade enzimática, utilizando-se o substrato ρ-Nph-5´-TMP (Sigma), para verificar a presença de proteína solúvel.

Por último foi avaliado o renovelamento rápido por diluição da SmNPP-5a purificada em um protocolo adaptado do kit iFOLD 2 Protein Refolding System (Novagen). A proteína

SmNPP-5a purificada e precipitada foi denaturada com 6M guanidina (Novagen), 50 µg de proteína foram submetidos ao renovelamento por diluição em 78 tampões diferentes na proporção, 1:50. Após 18 h de incubação, 2,5 µg de proteínas foram utilizados para avaliação da atividade enzimática NPP com o substrato ρ-Nph-5´-TMP (Sigma). Os tampões utilizados foram:

1 – MOPS 50 mM (pH 7,0 – Sigma), ciclodextrina 10 mM (Sigma), EDTA 1mM (USB), glutationa reduzida 9 mM (Sigma), glutationa oxidada 1 mM (Sigma), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

2 – MOPS 50 mM (pH 7,0 – Sigma), L-arginina 0,5 M (Synth), glutationa reduzida 9 mM (Sigma), glutationa oxidada 1 mM (Sigma), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

3 – MOPS 50 mM (pH 7,0 – Sigma), PEG 3350 0,06 % (Sigma), EDTA 1mM (USB), glutationa reduzida 9 mM (Sigma) e glutationa oxidada 1 mM (Sigma);

4 – MOPS 50 mM (pH 7,0 – Sigma), EDTA 1mM (USB), glutationa reduzida 9 mM (Sigma), glutationa oxidada 1 mM (Sigma), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

5 – MOPS 50 mM (pH 7,0 – Sigma), trealose 0,58 M (Sigma), glutationa reduzida 9 mM (Sigma) e glutationa oxidada 1 mM (Sigma);

6 – MOPS 50 mM (pH 7,0 – Sigma), sorbitol 1,5 M (USB), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma) e $ZnCl_2$ 0,25 mM (Sigma);

7 – MOPS 50 mM (pH 7,0 – Sigma), trealose 0,58 M (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

8 – MOPS 50 mM (pH 7,0 – Sigma);

9 – MOPS 50 mM (pH 7,0 – Sigma), L-arginina 0,5 M (Synth), EDTA 1mM (USB), glutationa reduzida 6 mM (Sigma), glutationa oxidada 4 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

10 – MOPS 50 mM (pH 7,0 – Sigma), ciclodextrina 10 mM (Sigma), EDTA 1mM (USB), glutationa reduzida 6 mM (Sigma), glutationa oxidada 4 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

11 – MOPS 50 mM (pH 7,0 – Sigma), NDSB-201 0,5 M (Calbiochem), glutationa reduzida 6 mM (Sigma) e glutationa oxidada 4 mM (Sigma);

12 – MOPS 50 mM (pH 7,0 – Sigma), NDSB-201 1 M (Calbiochem), glutationa reduzida 6 mM (Sigma) e glutationa oxidada 4 mM (Sigma);

13 – MOPS 50 mM (pH 7,0 – Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

14 – MOPS 50 mM (pH 7,0 – Sigma), NDSB-201 0,5 M (Calbiochem), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

15 – MOPS 50 mM (pH 7,0 – Sigma), NDSB-201 1 M (Calbiochem), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma) e TCEP 1 mM (Novagen);

16 – HEPES 50 mM (pH 7,5 – Calbiochem), sorbitol 1,5 M (USB), TCEP 1 mM (Novagen), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

17 – HEPES 50 mM (pH 7,5 – Calbiochem), NDSB-201 0,5 M (Calbiochem), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), TCEP 1 mM (Novagen), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

18 – HEPES 50 mM (pH 7,5 – Calbiochem), ciclodextrina 10 mM (Sigma) e TCEP 1 mM (Novagen);

19 – HEPES 50 mM (pH 7,5 – Calbiochem), trealose 0,58 M (Sigma), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), TCEP 1 mM (Novagen), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

20 – HEPES 50 mM (pH 7,5 – Calbiochem), PEG 3350 0,06 % (Sigma), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

21 – HEPES 50 mM (pH 7,5 – Calbiochem), trealose 0,58 M (Sigma), EDTA 1mM (USB), glutationa reduzida 9 mM (Sigma), glutationa oxidada 1 mM (Sigma), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

22 – HEPES 50 mM (pH 7,5 – Calbiochem), NDSB-201 0,5 M (Calbiochem), glutationa reduzida 9 mM (Sigma), glutationa oxidada 1 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

23 – HEPES 50 mM (pH 7,5 – Calbiochem), L-arginina 0,5 M (Synth), glutationa reduzida 9 mM (Sigma), glutationa oxidada 1 mM (Sigma), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

24 – HEPES 50 mM (pH 7,5 – Calbiochem), NDSB-201 1 M (Calbiochem), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

25 – HEPES 50 mM (pH 7,5 – Calbiochem), NDSB-201 1 M (Calbiochem), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

26 – HEPES 50 mM (pH 7,5 – Calbiochem) e ciclodextrina 10 mM (Sigma);

27 – HEPES 50 mM (pH 7,5 – Calbiochem), glutationa reduzida 6 mM (Sigma), glutationa oxidada 4 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

28 – HEPES 50 mM (pH 7,5 – Calbiochem), EDTA 1mM (USB), glutationa reduzida 6 mM (Sigma) e glutationa oxidada 4 mM (Sigma);

29 – HEPES 50 mM (pH 7,5 – Calbiochem), PEG 3350 0,06 % (Sigma), glutationa reduzida 6 mM (Sigma) e glutationa oxidada 4 mM (Sigma);

30 – HEPES 50 mM (pH 7,5 – Calbiochem) e L-arginina 0,5 M (Synth);

31 – HEPES 50 mM (pH 7,5 – Calbiochem);

32 – EPPS 50 mM (pH 8,0 – Sigma), NDSB-201 1 M (Calbiochem), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

33 – EPPS 50 mM (pH 8,0 – Sigma);

34 – EPPS 50 mM (pH 8,0 – Sigma), sorbitol 1,5 M (USB), EDTA 1mM (USB), glutationa reduzida 9 mM (Sigma) e glutationa oxidada 1 mM (Sigma);

35 – EPPS 50 mM (pH 8,0 – Sigma), ciclodextrina 10 mM (Sigma), glutationa reduzida 9 mM (Sigma), glutationa oxidada 1 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

36 – EPPS 50 mM (pH 8,0 – Sigma), PEG 3350 0,06 % (Sigma), glutationa reduzida 9 mM (Sigma), glutationa oxidada 1 mM (Sigma), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

37 – EPPS 50 mM (pH 8,0 – Sigma), PEG 3350 0,06 % (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

38 – EPPS 50 mM (pH 8,0 – Sigma), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

39 – EPPS 50 mM (pH 8,0 – Sigma), NDSB-201 0,5 M (Calbiochem), EDTA 1mM (USB), glutationa reduzida 6 mM (Sigma) e glutationa oxidada 4 mM (Sigma);

40 – EPPS 50 mM (pH 8,0 – Sigma), trealose 0,58 M (Sigma), EDTA 1mM (USB), glutationa reduzida 6 mM (Sigma) e glutationa oxidada 4 mM (Sigma);

41 – EPPS 50 mM (pH 8,0 – Sigma), NDSB-201 1 M (Calbiochem), EDTA 1mM (USB), glutationa reduzida 6 mM (Sigma), glutationa oxidada 4 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

42 – EPPS 50 mM (pH 8,0 – Sigma), trealose 0,58 M (Sigma), glutationa reduzida 6 mM (Sigma), glutationa oxidada 4 mM (Sigma), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

43 – EPPS 50 mM (pH 8,0 – Sigma), sorbitol 1,5 M (USB), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), TCEP 1 mM (Novagen), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

44 – EPPS 50 mM (pH 8,0 – Sigma), L-arginina 0,5 M (Synth), TCEP 1 mM (Novagen), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

45 – EPPS 50 mM (pH 8,0 – Sigma), ciclodextrina 10 mM (Sigma), TCEP 1 mM (Novagen), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

46 – EPPS 50 mM (pH 8,0 – Sigma), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), TCEP 1 mM (Novagen), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

47 – TAPS 50 mM (pH 8,5 – Sigma), L-arginina 0,5 M (Synth), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), TCEP 1 mM (Novagen), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

48 – TAPS 50 mM (pH 8,5 – Sigma), trealose 0,58 M (Sigma) e TCEP 1 mM (Novagen);

49 – TAPS 50 mM (pH 8,5 – Sigma), sorbitol 1,5 M (USB), TCEP 1 mM (Novagen), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

50 – TAPS 50 mM (pH 8,5 – Sigma), ciclodextrina 10 mM (Sigma), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma) e TCEP 1 mM (Novagen);

51 – TAPS 50 mM (pH 8,5 – Sigma), PEG 3350 0,06 % (Sigma) e TCEP 1 mM (Novagen);

52 – TAPS 50 mM (pH 8,5 – Sigma), NDSB-201 0,5 M (Calbiochem), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

53 – TAPS 50 mM (pH 8,5 – Sigma), glutationa reduzida 9 mM (Sigma) e glutationa oxidada 1 mM (Sigma);

54 – TAPS 50 mM (pH 8,5 – Sigma), NDSB-201 1 M (Calbiochem), EDTA 1 mM (USB), glutationa reduzida 9 mM (Sigma) e glutationa oxidada 1 mM (Sigma);

55 – TAPS 50 mM (pH 8,5 – Sigma), NDSB-201 1 M (Calbiochem), glutationa reduzida 9 mM (Sigma), glutationa oxidada 1 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

56 – TAPS 50 mM (pH 8,5 – Sigma), L-arginina 0,5 M (Synth), EDTA 1mM (USB), glutationa reduzida 9 mM (Sigma), glutationa oxidada 1 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

57 – TAPS 50 mM (pH 8,5 – Sigma), ciclodextrina 10 mM (Sigma), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

58 – TAPS 50 mM (pH 8,5 – Sigma), trealose 0,58 M (Sigma), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

59 – TAPS 50 mM (pH 8,5 – Sigma);

60 – TAPS 50 mM (pH 8,5 – Sigma), sorbitol 1,5 M (USB), glutationa reduzida 6 mM (Sigma), glutationa oxidada 4 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

61 – TAPS 50 mM (pH 8,5 – Sigma), PEG 3350 0,06 % (Sigma), EDTA 1mM (USB), glutationa reduzida 6 mM (Sigma), glutationa oxidada 4 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

62 – TAPS 50 mM (pH 8,5 – Sigma), NDSB-201 0,5 M (Calbiochem), glutationa reduzida 6 mM (Sigma) e glutationa oxidada 4 mM (Sigma);

63 – CHES 50 mM (pH 9,0 – Sigma), sorbitol 1,5 M (USB), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

64 – CHES 50 mM (pH 9,0 – Sigma), trealose 0,58 M (Sigma), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

65 – CHES 50 mM (pH 9,0 – Sigma;

66 – CHES 50 mM (pH 9,0 – Sigma), sorbitol 1,5 M (USB), glutationa reduzida 9 mM (Sigma), glutationa oxidada 1 mM (Sigma), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

67 – CHES 50 mM (pH 9,0 – Sigma), NDSB-201 0,5 M (Calbiochem), EDTA 1mM (USB), glutationa reduzida 9 mM (Sigma), glutationa oxidada 1 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

68 – CHES 50 mM (pH 9,0 – Sigma), NDSB-201 0,5 M (Calbiochem), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma), TCEP 1 mM (Novagen), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

69 – CHES 50 mM (pH 9,0 – Sigma), NDSB-201 1 M (Calbiochem), TCEP 1 mM (Novagen), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

70 – CHES 50 mM (pH 9,0 – Sigma), TCEP 1 mM (Novagen), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

71 – CHES 50 mM (pH 9,0 – Sigma), sorbitol 1,5 M (USB), EDTA 1mM (USB), glutationa reduzida 6 mM (Sigma), glutationa oxidada 4 mM (Sigma), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

72 – CHES 50 mM (pH 9,0 – Sigma), L-arginina 0,5 M (Synth), EDTA 1mM (USB), glutationa reduzida 6 mM (Sigma), glutationa oxidada 4 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

73 – CHES 50 mM (pH 9,0 – Sigma), ciclodextrina 10 mM (Sigma), glutationa reduzida 6 mM (Sigma), glutationa oxidada 4 mM (Sigma), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

74 – CHES 50 mM (pH 9,0 – Sigma), ciclodextrina 10 mM (Sigma), glutationa reduzida 6 mM (Sigma), glutationa oxidada 4 mM (Sigma), NaCl 240 mM (Dinâmica) e KCl 10 mM (Merck);

75 – CHES 50 mM (pH 9,0 – Sigma), PEG 3350 0,06 % (Sigma), glutationa reduzida 6 mM (Sigma), glutationa oxidada 4 mM (Sigma), NaCl 24 mM (Dinâmica) e KCl 1 mM (Merck);

76 – CHES 50 mM (pH 9,0 – Sigma), L-arginina 0,5 M (Synth), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma) e TCEP 1 mM (Novagen);

77 – CHES 50 mM (pH 9,0 – Sigma), PEG 3350 0,06 % (Sigma), $CaCl_2$ 0,25 mM (Sigma), $MgCl_2$ 0,25 mM (Synth), $MnCl_2$ 0,25 mM (Sigma), $ZnCl_2$ 0,25 mM (Sigma) e TCEP 1 mM (Novagen);

78 – Água.

3.7 Expressão das SmNPPs em *Pichia pastoris*

Para expressão das proteínas recombinantes em *P. pastoris*, os genes *smnpps* foram inseridos no vetor pPICZαA (Invitrogen) e posteriormente no vetor pINT-A (SILVA, 2010). Para a clonagem no vetor pPICZαA, foi utilizada uma estratégia semelhante a clonagem em pAE-6His, porém os oligonucleotídeos específicos (APÊNDICE) adicionaram sítios de restrição, EcoRI e NotI, às sequências amplificadas nas regiões 5´ e 3´, respectivamente. Para

a clonagem das *smnpps* no vetor pINT-A, os fragmentos gênicos foram amplificados a partir do vetor pPICZαA com oligonucleotídeos específicos (APÊNDICE) que adicionaram sítios de restrição, AvrII e XmnI, nas extremidades 5´ e 3´, respectivamente, dos fragmentos amplificados. Os fragmentos e os vetores foram digeridos com as respectivas enzimas de restrição, purificados com o kit GFX PCR DNA and Gel Band purification (GE Healthcare) e utilizados na reação de ligação com a enzima T4 DNA ligase (Promega). Os plasmídeos foram produzidos em *E. coli* DH5α, avaliados quanto à presença de inserto por PCR, purificados com o kit plasmidPrep Mini Spin (GE HealthCare) e posteriormente avaliados por sequenciamento automático.

As leveduras (*Pichia pastoris* cepa GS115) foram transformadas por eletroporação (1,5 kV de voltagem, 25 MF de capacitância e 200 Ω de resistência) com 5 µg dos vetores pPICZαA ou pINT-A, contendo os genes *smnpps*, linearizados com a enzima Sac I (Invitrogen) ou Sca I (Invitrogen), respectivamente. As leveduras foram plaqueadas em meio YPDS (extrato de levedura 1%, peptona 2%, dextrose 2%, sorbitol 1 M, ágar 2%), acrescido de 100 µg/mL de zeocina. Destas, foram selecionados 25 clones de cada gene *smnpp* para se avaliar a possível presença de múltiplas cópias integradas ao genoma da levedura; para tanto, os clones foram plaqueados em concentrações crescentes de Zeocina (100, 250, 500, e 1000 µg/mL). Foram selecionados para se testar a capacidade de expressão da proteína recombinante os 5 clones que melhor cresceram nas maiores concentrações do antibiótico e 3 clones que cresceram muito bem apenas até a concentração de 250 µg/mL para cada SmNPP.

Para avaliar a capacidade dos clones selecionados em expressar as SmNPPs recombinantes, uma colônia isolada de cada clone, 8 clones de cada proteína, foi cultivada em 10 mL de meio BMGY (extrato de levedura 1%, peptona 2%, fosfato de potássio 100 mM, pH 6, Biotina 4x10-5%, glicerol 1%) em tubos de 50 mL. Após 20 h de cultivo a 28 ^{o}C e agitação a 300 rpm, as culturas foram centrifugadas a 3000 rpm por 5 min. O sobrenadante foi descartado e o *pellet* ressuspendido em 10 mL de meio de indução BMMY (extrato de levedura 1%, peptona 2%, fosfato de potássio 100 mM (pH 6), Biotina 5%, metanol 0,5%). A indução foi mantida através da adição de metanol 100% para uma concentração final de 0,5% a cada 24 h até 96 horas de expressão. Alíquotas de 1 mL da cultura foram retiradas a cada 24 h, centrifugadas 3 min a 10.000 rpm e o sobrenadante e o precipitado de leveduras foram mantidos em frezer à -80 ºC para análises posteriores.

Inicialmente foram analisados os sobrenadantes das culturas (24 h, 48 h, 72 h e 96 h), as proteínas destes foram precipitadas por ácido tricloroacético e ressuspendidas em 1/10 do volume original para serem analisadas por *immunoblotting*. Basicamente, foram aplicados 20

μL de cada amostra de sobrenadante fervidos por 15 min com 5 μL de tampão de eletroforese (SDS 4%, glicerol 10%, Tris-HCl (pH 6.8) 0,1 M e 50 mg/ml de azul de bromofenol) em um gel de poliacrilamida 12%. Após a eletroforese, as proteínas foram transferidas para uma membrana de PVDF (GE Healthcare), conforme as orientações do fabricante. Para verificar a eficiência da transferência, a membrana foi corada com Ponceau. Após descorar a membrana com três lavagens com PBS, ela foi incubada em solução de bloqueio (leite em pó 5%, Tris 50 mM (pH 7,5), Tween 20 0,05%) por 16 h a 4 ºC.

Posteriormente, incubou-se a membrana em solução de incubação (leite em pó 5%, Tris 50 mM (pH 7,5), NaCl 150 mM) com o anticorpo anti-His conjugado com peroxidade (Sigma), em duas concentrações diferentes 1:1000 e 1:3000, ou com anticorpo policlonal anti-SmNPP específico, diluído 1:2000, por 4 h a temperatura ambiente. Nas membranas incubadas com anticorpos anti-His, após três lavagens com PBS e Tween 20 0,05%, foi realizada a detecção da proteína utilizando o kit quimioluminescente ECL plus (GE Healthcare). Enquanto nas membranas incubadas com anti-SmNPP, após três lavagens com PBS e Tween 20 0,05%, elas foram incubadas com anticorpo anti-IgG de camundongo conjugado com peroxidase (Sigma), diluído na proporção 1: 3.000 por 1 h à temperatura ambiente. Após três lavagens com PBS e Tween 20 0,05%, foi realizada a detecção da proteína utilizando o reagente quimioluminescente ECL plus (GE Healthcare).

Posteriormente, foram analisados os precipitados de leveduras. Primeiro eles foram ressupendidos em 1 mL de tampão de lise (fosfato de sódio 50 mM (pH 7,4), EDTA 1 mM, glicerol 5%, guanidina 8 M, inibidor de protease), sendo em seguida adicionado o mesmo volume de grânulos de vidro (0,5 mm). Essa mistura foi submetida a 10 ciclos de vórtex por 30 s, seguido por uma incubação no gelo de 30 s também. Após a lise, a mistura foi centrifugada por 12.000 rpm por 5 min. O pellet foi descartado e o sobrenadante foi coletado, precipitado com TCA e ressuspendido em 1/10 do volume original, sendo essa solução final submetida a análise por *immunoblotting*, conforme já descrito.

3.8 Expressão de ATPDase e Fosfatase alcalina em *Escherichia coli* e purificação

As proteínas recombinantes ATPDase e Fosfatase alcalina foram expressas em *E. coli* para os ensaios de vacinação. Para a expressão da SmATPDase foi utilizado o plasmídeo pET21-b com o gene já clonado (DEMARCO et al., 2003), gentilmente cedido pelo Dr. Júlio Levano-Garcia, enquanto para a expressão da Fosfatase alcalina, foi utilizado o plasmídeo

pAE-6His também com o gene já clonado (ARAUJO-MONTOYA et al., 2011), gentilmente cedido pelo Dr. Bogar Omar Araujo Montoya.

Os respectivos plasmídeos foram utilizados para transformar bactérias *E. coli* da cepa BL21 Star (DE3) plysS (Invitrogen) competentes por choque térmico. A expressão das proteínas foi analisada cultivando-se uma colônia transformada em meio 2YT (triptona 1,6%, extrato de levedura 1% e NaCl 0,5%) com ampicilina (100 µg/mL) e cloranfenicol (34 µg/mL) a 37 ^{o}C até atingir a D.O. de 0,6-0,8. Quando o inóculo atingiu a D.O. desejada foi adicionado IPTG (1 mM) para induzir a expressão da proteína recombinante pelas bactérias por 4 h. Após a indução, o cultivo das células de *E. coli* transformadas com o plasmídeo recombinante foi centrifugado por 10 min a 12.000 g. O precipitado foi ressuspendido em 1 mL de tampão de lise e analisado por SDS-PAGE. Como controle, foi analisada a cepa transformada com o vetor vazio e induzida nas mesmas condições que a cepa transformada com o vetor com inserto clonado. Após a confirmação da expressão, um clone expressando cada uma das proteínas foi cultivado em 200 mL de meio de cultura a 37 ^{o}C e, após atingirem a D.O. 0,6-0,8, a expressão das proteínas recombinantes foi induzida pela adição de IPTG 0,5 mM por 4 horas.

Após a expressão, as bactérias foram centrifugadas, ressuspendidas em 30 ml de tampão de lise e lisadas no aparelho French Press (1500 psi – 3 passagens). Em seguida, o lisado foi centrifugado por 30 min a 10.000 rpm, o sobrenadante foi descartado e o precipitado lavado com 2 M uréia. Após nova centrifugação o precipitado foi ressupendido com 8 M uréia e submetida à purificação em cromatografia de afinidade ao níquel, utilizando colunas HisTrap HP 5 mL (GE Healthcare) com o auxílio do aparelho Äkta Prime (Amersham Pharmacia).

Após a cromatografia, as frações da eluição correspondentes ao pico de absorbância de 280 nm foram analisadas por SDS-PAGE e as que apresentaram a banda da proteína recombinante expressa foram selecionadas. As frações foram condicionadas em membranas *SnakeSkin* (Pierce) com poros de 3 kDa, para renovelamento por diálise lenta com concentrações decrescentes de uréia, na proporção 1:100 de amostra para tampão.

3.9 Produção de anticorpos anti-SmNPPs em camundongos e ratos

Ratas Wistar com seis semanas de idade foram utilizadas para produção de anticorpos policlonais. Foram realizadas quatro imunizações subcutâneas com 100 µg de proteína recombinante por animal em intervalos de 15 dias. Foi utilizado Titermax (CytRx

Corporation) como adjuvante na primeira imunização e apenas as proteína nas imunizações subsequentes. O sangue foi coletado por punção cardíaca 15 dias após a última imunização e acondicionado a 4 ºC por 16 h para formar um coágulo, o qual foi descartado. O soro foi centrifugado a 4.000 rpm por 10 min e o sobrenadante coletado.

Também foram utilizadas camundongas BALB/c com seis semanas de idade para produção de anticorpos policlonais. Foram realizadas três imunizações por animal em intervalos de 15 dias, subcutaneamente, contendo 25 µg de proteína mais o adjuvante Titermax (CytRx Corporation) na primeira imunização e apenas proteína nas imunizações subsequentes. O sangue foi coletado por punção cardíaca, 15 dias após a última imunização e processado como descrito anteriormente.

Os soros dos animais foi usado individualmente no ensaio de ELISA para verificar a concentração de IgG total específico para cada proteína. Placas com 96 poços foram incubadas com 0,1 µg/poço de proteína recombinante em tampão carbonato - bicarbonato 0,05 M (pH 9.6) com 0,02% de SDS (LECHTZIER et al., 2002), durante 16 h a 4 ºC. As placas foram lavadas 3 vezes com PBS contendo 0,05% de Tween 20, e em seguida, bloqueadas com 100 µL de uma solução com 10% de SBF (Soro bovino fetal) em PBS, por 1 h a 37 °C. Depois de lavadas com PBS Tween 20 0,05%, 100 µl de uma diluição seriada do soro dos animais imunizados ou controles em tampão PBS contendo 5% de SBF foram adicionados em cada poço, sendo a diluição inicial 1:16. As placas foram incubadas a 37 °C durante 1 h e depois lavadas. A seguir, 100 µL de anticorpos monoclonais anti-IgG (Sigma) na diluição 1: 10.000 foram adicionados a todos os poços das placas que foram novamente incubadas a 37 °C por 1 h. Depois de lavadas, 100 µL de anticorpos conjugados com peroxidase foram adicionados às placas, incubando-as novamente a 37 °C durante 1 h. As placas foram então lavadas seis vezes com PBS Tween 20 0,05% e em seguida, 100 µL do tampão citrato (pH 5.0) contendo o substrato orto-fenilendiamina dicloridrato (OPD [Sigma]) 0,5 mg/ml e 5 µL de peróxido de hidrogênio para cada 10 mL, foram adicionados em cada poço. Após aproximadamente 15 min, a reação colorimétrica com OPD foi encerrada, utilizando uma solução 4 N de H_2SO_4 e a placa foi lida a 492 nm em um leitor de ELISA (Labsystem).

3.10 *Immunoblotting* das SmNPPs nos diversos estágios do parasita

Ovos, miracídios, cercárias, esquistossômulos e vermes adultos foram ressuspendidos em 1 mL de Tris 40 mM, pH 7,4, acrescido de inibidor de proteases (Sigma) e SDS 2%. Os

parasitas foram sonicados (5 vezes durante 2 min, a 40 Hz e com pulsos de 0,75 s), sendo resfriadas em gelo para evitar a degradação das proteínas. As soluções foram centrifugadas a 12.000 rpm por 30 min a 4 ºC, sendo os sobrenadantes coletados e denominados extratos protéicos totais enquanto os precipitados foram descartados. Os extratos de tegumento e vermes desnudos foram obtidos pelo método de congelamento/descongelamento/vortex (ROBERTS et al., 1983). Parasitas adultos congelados foram descongelados a 4 ºC e vortexados com 10 pulsos de 1 s para o desprendimento do tegumento do parasita. Após os vermes decantarem, o sobrenadante que contêm o extrato de tegumento é coletado e os vermes desnudos, sem tegumento, são lavados duas vezes. O tegumento de vermes adultos também foi separado em frações de membrana e do sincício, assim o tegumento recém-extraído foi centrifugado a 100 g por 30 min a 4 ºC. O sobrenadante que continha as proteínas sinciciais foi coletado enquanto o *pellet* foi lavado duas vezes. Todas essas frações foram sonicadas conforme o protocolo descrito acima para os outros extratos. Para quantificar as proteínas de cada extrato, utilizou-se o kit DC Protein Assay (BioRad), baseado no método de Lowry, seguindo o protocolo do fabricante.

Foram aplicados 20 µg de cada extrato protéico fervidos por 5 min com 5 µL de tampão de eletroforese, contendo SDS 4%, glicerol 10%, Tris-HCl 0,1 M (pH 6.8) e 50 mg/mL de azul de bromofenol em um gel de SDS-PAGE 12%. Após a eletroforese, as proteínas foram transferidas para uma membrana de PVDF (Amersham). Para verificar o êxito da transferência, a membrana foi corada com Ponceau. Após descorar a membrana com diversas lavagens com PBS, ela foi incubada em solução de leite em pó 5%, Tris 50 mM (pH 7,5), Tween 20 0,05% para bloqueio, por 16 h a 4 ºC. No dia seguinte, incubou-se a membrana em solução de leite 5%, Tris 50 mM (pH 7,5), Tween 20 0,05%, NaCl 150 mM (solução de incubação) com o anticorpo específico contra a proteína a ser analisada em diferentes diluições (ver resultados), por 4 h à temperatura ambiente. Após três lavagens com PBS, a membrana foi incubada em solução de incubação com anticorpos anti-IgG de rato ou camundongo conjugado com peroxidase, diluído na proporção 1:3.000 por 1 h à temperatura ambiente. Após três lavagens com PBS, a detecção da proteína foi realizada, utilizando o kit quimioluminescente ECL (Amersham) em filmes de radiograma.

3.11 Deglicosilação da SmNPP-5a

O extrato de tegumento de vermes adultos foi N-deglicosilado com a enzima PNGase F (New England Biolabs). Duas frações de extrato de tegumento, 20 µg cada, foram

incubadas com tampão de denaturação a 100 ºC por 10 min. Após a denaturação foi adicionado o tampão de reação G7 e NP-40 1%, porém a enzima PNGaseF foi adicionada a apenas uma das alíquotas enquanto a outra foi utilizada como controle. Após serem incubados por 6 h a 37 ºC, os extratos foram submetidos à SDS-PAGE, transferidos para membrana de PVDF e analisados por *immunoblotting*, como descrito anteriormente.

Para avaliação das O-glicosilações foi utilizado o kit EDEGLY (Sigma) seguindo o protocolo do fabricante. Duas frações de extrato de tegumento de vermes adultos, 20 µg cada, foram N-deglicosiladas e posteriormente uma das frações foi incubada com as enzimas α-(2-3,6,8,9)-Neuroaminidase, O-glicosidase, β-(1-4)-Galactosidase e β-N-Acetilglucosaminidase por 6 h a 37 ºC, enquanto a outra fração foi incubada sem a presença das enzimas para controle. Os extratos O-deglicosilados também foram submetidos à SDS-PAGE, transferidos para membrana de PVDF e analisados por *immunoblotting*.

3.12 Imunolocalização da SmNPP-5a

Vermes adultos recém perfundidos em meio RPMI foram fixados com paraformoldeído 4% a 4 ºC por 16 h. Os parasitas fixados foram incubados em solução de recuperação antigênica (citrato de sódio 10 mM, pH 6,0) a 4 ºC por 16 h. Posteriormente os parasitas foram incubados por 5 min em solução de recuperação antigênica em ebulição, seguido pela imersão em sacarose 30% gelada e incubado a 4 ºC até os parasitas decantarem (adaptado de INO, 2003). Os vermes adultos foram emblocados em líquido de inclusão O.C.T. (Sakura), com o auxílio de tubos plásticos (3 mm de diâmetro e 7 mm de comprimento), e congelados em isopentano arrefecido em nitrogênio líquido. Os blocos foram armazenados a -80 ºC até o momento de fazer as secções. Os cortes histológicos com 8 µm de espessura foram realizados em um criostato Leica CM1900 e as secções foram aderidas em lâminas silanizadas, secas por 2 h e reidratadas com PBS a 4 ºC por 16 h.

As secções foram tratadas com PBS e SDS 1% por 10 min, lavadas três vezes com PBS e incubadas com solução de bloqueio com PBS, soro de coelho 10% e Tween 20 0,1% por 4 h. As secções foram então incubadas com soro anti-SmNPP-5a produzido em camundongo na diluição 1/50 por 3 h, como controle foi utilizado soro de camundongos naive. Após cinco lavagens com PBS com Tween 20 0,1%, as secções foram incubadas com solução de bloqueio e anticorpo de coelho anti-IgG de camundongo conjugado com o fluoróforo Alexa 647 (Invitrogen) na diluição 1:200 por 1 h. Após três lavagens as lâminas

foram montadas com meio de montagem para fluorescência (Dako) e analisadas em um microscópio confocal LSM-510 META (Zeiss).

3.13 Avaliação e inibição da atividade nucleotídeo pirofosfatase/ fosfodiesterase em parasitas vivos

Vermes adultos machos e vermes com 21 dias recém perfundidos (três espécimes de cada estágio) ou aproximadamente 2000 cercárias vivas foram incubadas com "ρ-nitrophenyl 5´-thymidine monophosphate (ρ-Nph-5´-TMP)", um substrato específico para a atividade nucleotídeo pirofosfatase/ fosfodiesterase (Sigma). Os parasitas foram incubados em tampão de reação Tris-HCl 50 mM (pH 8,9), NaCl 120 mM, KCl 5 mM, glicose 60 mM e $CaCl_2$ 5 mM em um volume final de 200 μL (adaptado de FURSTENAU et al., 2006). A reação foi iniciada pela adição do ρ-Nph-5´-TMP numa concentração de 0,5 mM. Após 20 min de incubação à 37 ºC, 100 μL de NaOH 0,4 M foi adicionada ao tampão para parar a reação, o tampão de reação foi recuperado e absorbância em 405 nm foi medida em um leitor de microplaca (Labsystem). Para os ensaios de inibição mediada por anticorpos, três vermes machos adultos ou três vermes de 21 dias foram previamente incubados em 100 μL de soro de camundongo contendo anticorpos policlonais anti-rSmNPP-5 por 1 h a 37 ºC, antes de ser avaliada a atividade enzimática. Para avaliar a especificidade da hidrólise do substrato, foram feitos os controles incubando o substrato sem parasitas ou os parasitas sem susbtrato, apenas com solução de reação. Também foi feito em paralelo a avaliação da atividade fosfatase alcalina (substrato "ρ-nitrophenyl phosphate disodium salt" [Sigma]). Todas as amostras foram feitas em triplicatas e as diferenças estatísticas foram analisadas pelo teste-t de Student ou por ANOVA seguida pelo teste de Tukey. Um ρ value <0,05 foi considerado estatisticamente significativo.

3.14 Hibridização *in situ* em espécimes inteiros

3.14.1 *Síntese da sonda de RNA*

Para os ensaios de hibridização foram produzidas sondas *antisense* de RNA marcadas com digoxigenina (DIG). As sequências dos domínios NPPS amplificadas por PCR foram clonadas no vetor pGEM-T Easy seguindo as recomendações do fabricante e, após confirmação por sequenciamento, os plasmídios foram linearizados com enzima de restrição

Apa I (Invitrogen). A síntese das sondas marcadas com DIG foi realizada a partir da transcrição dos plasmídios com os insertos linearizados (2,5 µg) pela SP6 RNA polimerase 150 U (Promega) numa reação com tampão de transcrição 1x (Promega), mix de marcação de RNA-DIG 0,5x (Roche), inibidor de RNAse 50 U (Promega), DTT 100 mM (Promega) em um volume final de 50 µL. A reação foi incubada a 37 ºC por 2 h; após esse período foi adicionado mais 50 U de SP6 RNA polimerase e incubado por mais 2 h. Em seguida, 2 U de DNAse I livre de atividade de RNAse (Promega) foi adicionada e incubada por 20 min a 37 ºC.

As sondas foram então precipitadas pela adição de 1 volume de reação de água, 7 volumes de etanol e 100 mM de NaOAc e essa mistura foi incubada por 16 h a -80 ºC. Após serem centrifugadas por 15 min em velocidade máxima, as sondas foram lavadas com etanol 70%, centrifugadas novamente e secas. Por possuírem mais que 600 bp, todas as sondas foram hidrolisadas. A hidrólise foi realizada através da ressuspensão das sondas precipitadas em $NaHCO_3$ 40 mM e Na_2CO_3 60 mM e incubadas a 60 ºC pelo tempo em minutos determinado pela fórmula:

$$Tempo\ (min) = \frac{tamanho\ inicial\ (kb) - \ tamanho\ final\ (kb)}{0{,}11 * tamanho\ inicial\ (kb) * tamanho\ final\ (kb)}$$

O tamanho final desejado foi de 300 pb. Após a hidrólise, as sondas foram novamente precipitadas e lavadas, mas agora ressuspendidas em água livre de RNase e guardadas a -80 ºC até seu uso. Como controle negativo, foi sintetizada uma sonda sense do gene *smnpp5a*.

3.14.2 *Hibridizações in situ*

Os ensaios de hibridização *in situ* em espécimes inteiros foram realizados conforme descrito por Dillon, Illes et al. (2007). Os parasitas foram fixados em Carnoy (etanol 60%, clorofórmio 30%, acido acético glacial 10%) agitando a 4 ºC por 2 h, lavados 2 vezes com etanol e incubados por 1 h em MEMFA (MOPS 0,1 M (pH 7,4), EGTA 2 mM, MgSO4 1 mM, formaldeído 3,7%) à temperatura ambiente. Após duas lavagens com etanol novamente, os espécimes foram armazenados em etanol a -20 ºC até serem utilizados.

As hibridizações foram conduzidas segundo o método descrito por Harland (1991), com as modificações descritas por Pownall, Tucker et al. (1996). Os vermes foram trazidos à temperatura ambiente e reidratados. Primeiro por 2 lavagens de 5 min, a primeira em etanol 75% com PBS contendo 0,1% de Tween 20 (PBS-T) e a segunda em etanol 50% com PBS-T e posteriormente por 3 lavagens em PBS-T. Os parasitas foram então parcialmente digeridos

com Proteinase K (Roche) 10 μg/mL por 35 min. A digestão foi parada com 2 lavagens de 5 min cada de trietanolamina (Sigma) 0,1 M (pH 7,8). Após a segunda lavagem, para cada 1 mL de solução, 2,5 μL de anidrido acético (Sigma) foram adicionados à solução de trietonolamina e incubou-se por 5 min, seguidos da adição da mesma quantidade novamente e mais 5 min de incubação. Então os parasitas foram lavados mais 2 vezes por 5 min cada em PBS-T para serem refixados por 20 min em 4% formaldeído.

Após a fixação, os parasitas foram novamente lavados por 5 vezes por 5 min cada em PBS-T. Os vermes permeabilizados foram incubados a 60 ºC por 2 h em tampão de hibridização (formamida 50%, SSC 5x, heparina 100 μg/ml, solução de Denhardt 1x, Tween 20 0,1%, CHAPS 0,1% e EDTA 5 mM) com 1 mg/mL de RNA total de levedura para bloquear hibridizações não específicas. Após 2 h, a solução de hibridização foi substituída por uma nova já aquecida contendo 1 μg/mL da sonda sintetizada marcada com DIG, por 16 h a 60 ºC.

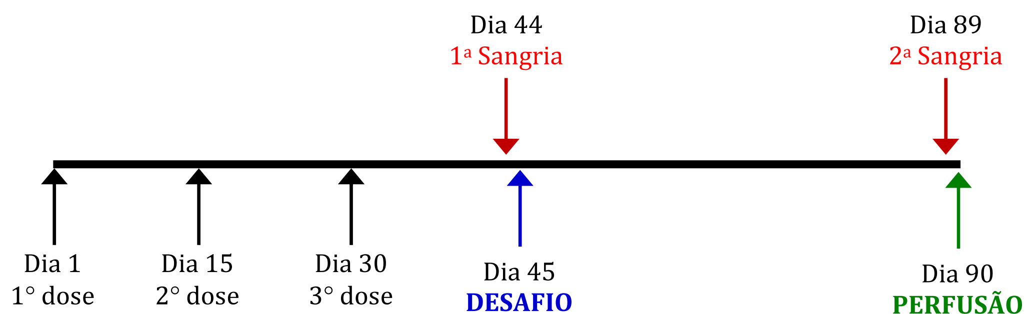
Após a hibridização, os parasitas passaram por um processo de diversas lavagens: 2 vezes em tampão de hibridização a 60 ºC por 10 min cada; 3 vezes em tampão SSC (Sigma) 2x com Tween 20 0,1% a 60 ºC por 20 min cada; 3 vezes com tampão SSC 0,2x com Tween 20 0,1% a 60 ºC por 30 min cada; 2 vezes em tampão MAB (ácido maleico 0,1 M, NaCl 0,15 M, Tween 20 0,1%, pH 7,8) a temperatura ambiente por 15 min cada. Após as lavagens, os parasitas foram incubados em tampão de bloqueio (MAB, Reagente de Bloqueio [Roche] 2% e soro de carneiro 20%) por 2 h a temperatura ambiente. Após o bloqueio, os parasitas foram incubados em tampão de bloqueio com anticorpo anti-DIG conjugado com fosfatase alcalina (Roche) em uma diluição 1:2.000 por 16 h a 4 ºC. O excesso de anticorpo foi removido por lavagens extensivas com MAB a temperatura ambiente, primeiramente 3 vezes de 5 min cada e posteriormente 3 lavagens de 1 h cada. Os espécimes foram então incubados por 3 vezes de 10 min cada em um tampão (Tris 100 mM, pH 9,5, $MgCl_2$, 100 NaCl, Tween 0,1% e levamisol 2 mM) para inibir atividade fosfatase alcalina inespecífica.

As sondas hibridizadas ligadas aos anticorpos anti-DIG conjugados a fosfatase alcalina foram detectadas pela adição do substrato BM Purple (Roche), utilizado com 1 mM de levamisol de acordo com as recomendações do fabricante; a atividade da fosfatase alcalina resulta na deposição de um precipitado azul insolúvel. A revelação foi acompanhada visualmente levando de 30 min até diversas horas, dependendo da abundância do transcrito. A revelação foi interrompida com duas lavagens dos vermes em PBS-T por 15 min cada, seguida da refixação e armazenagem em tampão formaldeído 3,7%. As imagens foram adquiridas com uma câmera 18.2 Color Mosaic (Diagnostic Instruments).

3.15 Imunização, desafio e avaliação da carga parasitária

Para os ensaios de vacinação foram utilizadas camundongos fêmeas C57BL/6 de cinco a seis semanas distribuídos em 10 animais por grupo. Os animais foram imunizados pela via subcutânea com as proteínas recombinantes na formulação de 25 µg de cada proteína para cada camundongo em 100 µL de formulação. Sendo utilizada a proporção 1:1 de volume de proteína para volume de adjuvante. Na primeira dose foi utilizado o adjuvante completo de Freund (Sigma), enquanto nas outras duas imunizações foi utilizado o adjuvante incompleto de Freund (Sigma). Os animais foram desafiados com os parasitas quinze dias após a última imunização; para tanto, eles foram tricotomizados com o aparelho Pro-Cord Trimmer (Oster) e anestesiados com cetamina (10 mg/mL) e xilasina (20 mg/mL). Após a anestesia os animais foram alocados em canaletas de acrílico com o abdomên para cima, no qual foi preso um anel de metal com fita adesiva. No orifício do anel foram colocadas aproximadamente 100 cercárias em 1 mL de volume para infecção por penetração pela pele durante 30 min. Após 45 dias da infecção, os animais foram eutaniziados com uma dose letal de uretano (150 mg/mL) e perfundidos pela artéria aorta com solução salina heparinizada (Figura 7).

Figura 7 – Regime de imunizações, sangrias, desafio e perfusão dos ensaios de vacinação.



Os vermes adultos foram removidos das veias do mesentério pela perfusão através de uma incisão da veia porta. Os parasitas foram coletados e contados com o auxílio de um estereomicroscópio. A porcentagem da redução do número de vermes em animais imunizados *vs* controle foi calculada pela fórmula:

$$\%\text{ de proteção} = \frac{\text{n° vermes do grupo controle} - \text{n° de vermes do grupo imunizado}}{\text{n° de vermes do grupo controle} \text{ x } 100}$$

Após a perfusão, um pedaço do fígado de cada camundongo foi coletado, pesado e digerido em 5 mL de hidróxido de potássio 5% a 37 °C, por 18 h. Essa solução foi homogeneizada e uma alíquota utilizada para contagem dos ovos, com auxílio de um microscópio. A porcentagem de redução do número de ovos em animais imunizados *vs* controle foi calculada por fórmula semelhante a da redução do número de vermes. Os dados do número de vermes recuperados pela perfusão e do número de ovos por grama de fígado foram analisados quanto à sua normalidade e homogeneidade de variância, e comparados por ANOVA. Um ρ value <0,05 foi considerado estatisticamente significativo.

3.16 Avaliação da resposta imune induzida

3.16.1 Avaliação da resposta imune humoral

A resposta imune humoral foi analisada em dois momentos: antes do desafio e antes da perfusão. Desse modo foi coletado sangue pela via retro-orbital dos animais um dia antes do desafio, dia 44 – 1ª sangria, e um dia antes da perfusão, dia 89 – 2ª sangria (Figura 7). Os soros dos animais foram processados conforme descrito na seção 3.9 e utilizados em ensaios de ELISA para verificar a concentração de IgG total, IgG1 e IgG2a específicos para cada proteína. Placas de 96 poços foram incubadas com 0,5 µg/poço de proteína recombinante ou com uma curva de concentração de IgG (Southern Biotech), IgG1 (Southern Biotech) ou IgG2a (Southern Biotech) recombinantes em tampão carbonato - bicarbonato 0,05 M (pH 9.6), durante 16 h a 4 ºC. As placas foram lavadas 3 vezes com PBS contendo 0,05% de Tween 20 (PBS-T), e em seguida, bloqueadas com 100 µL de uma solução de soro fetal bovino 10% (SFB – Gibco) em PBS, por 1 h a 37 °C. Depois de lavadas 3 vezes com PBS-T, 100 µL de uma diluição seriada do soro dos animais imunizados ou controles em tampão PBS contendo SFB 5% foram adicionados em cada poço, sendo a diluição inicial 1:32. Na curva de concentração, foi adicionado apenas tampão PBS contendo SFB 5%. As placas foram incubadas a 37 °C durante 1 h e depois lavadas.

A seguir, 100 µL de anticorpo monoclonal de cabra anti-IgG total (Southern Biotech), anti-IgG1 (Southern Biotech) ou anti-IgG2a (Southern Biotech) de camundongo nas foram adicionados a todos os poços das placas, nas respectivas diluições 1:10.000, 1:2.000 e 1:1.000. Depois de lavadas novamente, 100 µL de anticorpo anti-IgG de cabra conjugado com peroxidase (Southern Biotech), na diluição 1:20.000, foram adicionados às placas. As placas

foram então lavadas seis vezes com PBS-T e em seguida, 100 μL de tampão citrato (pH 5.0) contendo o substrato OPD (0,5 mg/ml) e 0,005 μL de peróxido de hidrogênio foram adicionados em cada poço. Após aproximadamente 15 min, a reação colorimétrica com OPD foi encerrada, utilizando uma solução de H_2SO_4 4 N. A absorbância foi aferida em 492 nm em um leitor de microplaca (Labsystem). A concentração das amostras foi obtida através da regressão linear da curva padrão e as diferenças estatísticas foram averiguadas por ANOVA seguida pelo teste de Tukey. Um ρ value <0,05 foi considerado estatisticamente significativo.

3.16.2 Avaliação da resposta imune celular

A resposta imune celular foi analisada apenas no momento anterior ao desafio, ou seja, após três imunizações. Para tanto, grupos de 5 camundongos fêmeas C57Bl/6 foram submetidas ao mesmo regime de imunização descrito no seção 3.14.1. Porém, 15 dias após a terceira imunização, os animais não foram desafiados, mas eutanaziados com uma dose letal de uretano (150 mg/ml) e seu baço foi coletado. Os órgão foram macerados individualmente com o auxílio de uma peneira (BD Biosciences) e suspensas em meio RPMI 1640 suplementado com SFB 10%, L-glutamina 2 mM (Merck), sulfato de gentamicina 50 μg/mL (Schering-Plough), polimixina B 10 μg/mL (Sigma) e tampão HEPES 10 mM (Sigma). As células ($1x10^6$ células) foram re-estimuladas com as proteínas recombinantes (5 μg/mL) e cultivadas por 48 h em estufa humidifica a 37 ºC e atmosfera saturada a 5% de CO_2.

Após o período de incubação, as células foram coletadas e o RNA total foi extraído com Trizol (Invitrogen) conforme as intruções do fabricante. Após a extração, as amostras de RNA foram quantificadas e analisadas quanto à pureza no espectrofotômetro ND-1000 (NanoDrop Techonologies) em comprimentos de onda de 230, 260 e 280 nm. O RNA extraído (1 μg) foi tratado com DNAse livre de RNAse RQ1 (Promega) por 1 h à 37 ºC, para eliminar uma possível contaminação com DNA genômico. O RNA tratado foi utilizado para a síntese do DNA complementar ao mRNA feito na reação de transcrição reversa utilizando-se de oligonucleotídeos randômicos e da enzima transcriptase reversa Superscript III (Invitrogen), segundo as indicações do fabricante.

Foram feitas duplicatas das reações de PCR em tempo real utilizando 50 ng de cDNA, Taqman Master Mix 1x (Invitrogen) e sonda e oligonucleotídeos 1x em um volume final de reação de 20 μl no termociclador 7300 Real time PCR System com os parâmetros: 10 min a 95 ºC e 40 ciclos de 10 s a 95 ºC seguido por 1 min a 60 ºC. Foram utilizados conjuntos de sondas e olinucleotídeos comerciais Taqman para avaliar a expressão dos seguintes genes:

Myd88, NfκB1, NfκB2, IFN-γ, TNF-α, TGF- β, IL-4, IL-5, IL-10, IL-12p40, IL-13 e IL-17. O gene da β-actina foi utilizado como controle endógeno e a análise de dados foi segundo a seção 3.4.4, salvo que não houve análise da curva de dissociação, por se utilizar a tecnologia Taqman ao invés de SYBR Green.

3.17 Avaliação da administração *in vivo* da dose subcurativa de praziquantel

Foram utilizados 11 camundongos fêmeas C57BL/6 de cinco a seis semanas por grupo para avaliar os efeitos da quimioterapia subcurativa sobres os parasitas. Os animais foram divididos aleatoriamente em grupos (controle e praziquantel) e infectados com 100 cercárias cada por penetração pela pele de maneira semelhante ao desafio dos ensaios de vacinação (seção 3.15). Aos animais do grupo praziquantel, foram administrados 150 mg/Kg de praziquantel (Roche) por *gavage* 35 dias após infecção, sendo uma segunda dose administrada 48 h após a primeira. Os animais do grupo controle receberam solução salina ao invés da droga (DOENHOFF; MODHA; LAMBERTUCCI, 1988).

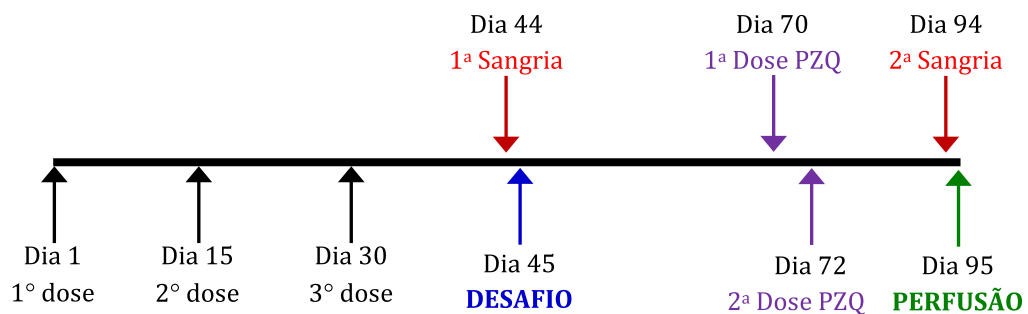
Dois animais de cada grupo foram perfundidos 2 h depois da administração da segunda dose de praziquantel pela artéria aorta com solução salina heparinizada, após eutanásia com uma dose letal de uretano (150 mg/mL). Os vermes adultos foram removidos das veias do mesentério pela perfusão através de uma incisão da veia porta. Os parasitas foram coletados, fixados em solução AFA (Etanol 30%, formaldeído 4% e ácido acético glacial 1,5%) e avaliados em um microscópio confocal a laser, Zeiss LSM 5120 META, utilizando 488 nm para excitação e captando a fluorescência em filtro de 500-550 nm. O número de tubérculos dos vermes adultos machos foi contado em 20.000 μ2 de área utilizando-se o programa Zeiss LSM Image Browser (MORAES et al., 2011) e comparados por ANOVA.

Os animais restantes de cada grupo foram perfundidos conforme descrito na seção 3.15, mas apenas 50 dias após a infecção. Nestes animais, os vermes adultos coletados foram contados com o auxílio de uma lupa para avaliação da carga parasitária, as quais foram analisadas quanto à sua normalidade e homogeneidade de variância e comparadas por ANOVA. Um ρ value $<0,05$ foi considerado estatisticamente significativo

3.18 Ensaio de vacinação associado à quimioterapia subcurativa

Para estes ensaios foram combinados os protocolos de vacinação com o do tratamento subcurativo com praziquantel. Desse modo os animais foram imunizados e desafiados como nos ensaios de vacinação e receberam as duas doses subcurativas de praziquantel antes da perfusão. O regime de imunização, as doses da droga administrada e as sangrias dos animais são os mesmos descritos previamente (seções 3.15 e 3.17). Os animais foram perfundidos 50 dias após a infecção (Figura 8) e tanto a carga parasitária quanto a fecundidade dos parasitas foram avaliados quanto à sua normalidade e homogeneidade de variância e comparados por ANOVA e pelo teste de Tukey. Um ρ value <0,05 foi considerado estatisticamente significativo.

Figura 8 – Regime de imunizações, sangrias, desafio e perfusão dos ensaios de vacinção associados à quimioterapia subcurativa com praziquantel.



4 RESULTADOS

4.1 Caracterização molecular e cladograma das NPPs de *Schistosoma*

Analisando o banco genômico de *S. mansoni* "Gene DB", foram feitas buscas por sequências que apresentavam o domínio protéico nucleotídeo pirofosfatase/fosfodiesterase. Foram encontradas quatro isoformas distintas identificadas pelos *ESTs*: Sm03458 (ou pelos números de acesso, Smp_153390.1 e Smp_153390.2); Sm03409 (Smp_104270 e Smp_106700); Sm04918 (Smp_153340.1 e Smp_153340.2) e Sm06826 (Smp_084890). Ao analisar o genoma de *S. japonicum* em busca de NPPs, foram identificadas quatro isoformas ortólogas no genoma de *S. japonicum*: Sjp_0121850.1, Sjp_0086960.1, Sjp_0012070.1 e Sjp_0027190.1.

A partir de buscas feitas no banco genômico NCBI comparando as proteínas de *S. mansoni* e seus respectivos ortólogos principais de *Homo sapiens*, verificou-se que as Sm03458, Sm03409 e Sm04918 possuem maior grau de identidade a NPP-5, enquanto a Sm06826 é mais semelhante à NPP-6 humana. Assim decidiu-se nomear a Sm03458, a Sm03409, a Sm04918 e a Sm06826, como SmNPP-5a, SmNPP-5b, SmNPP-5c e SmNPP-6, respectivamente. Devido a redundância das sequências de *S. mansoni*, 7 números de acesso para 4 genes, analisamos as sequências para verificarmos se tratavam-se de possíveis erros de anotação gênica, *splicings* alternativos ou genes distintos. Verificou-se que a SmNPP-5a apresentou a mesma sequência gênica para os dois números de acesso, porém ao analisarmos a sequência de aminoácidos verificamos mudanças significativas na porção C-terminal (Figura 9), desse modo acreditamos que seja caso de *splicing* alternativo, e denominamos as isoformas como SmNPP-5a.1 (gene ID no banco genômico – Smp_153390.1) e SmNPP-5a.2 (Smp_153390.2). Para a SmNPP-5c (Smp_153340.1 e Smp_153340.2) não foi identificada nenhuma diferença nas sua sequência gênica, por isso acreditamos que trata-se apenas de um erro de anotação gênica, enquanto a SmNPP-5b (Smp_104270 e Smp_106700) apresenta em um dos seus números de acesso, Smp_106700, uma sequência redundante pequena de aminoácidos que sequer apresenta o domínio NPP, sendo provavelmente fruto de outro erro de anotação gênica.

As isoformas de *S. mansoni*, de *S. japonicum,* a HsNPP-5 e a HsNPP-6 foram alinhadas duas a duas para determinar o grau de similaridade e identidade entre elas (Tabela 1). Comparando as SmNPPs com as NPPs encontradas no genoma de *S. japonicum* verificou-se que cada NPPs encontrada correspondia a um ortólogo de *S. mansoni* e baseando-se nessa

similaridade/identidade elas foram denominadas: SjNPP-5a (Sjp_0121850.1), SjNPP-5b (Sjp_0086960.1), SjNPP-5c (Sjp_0012070.1) e SjNPP-6 (Sjp_0027190.1).

Foi realizado também o alinhamento múltiplo destas proteínas baseado na estrutura primária delas para identificação dos aminoácidos que compunham o domínio protéico característico (caixa), os prováveis domínios transmembrana (azul) e peptídeos sinal (vermelho), os sítios putativos de ligação a metal co-fator da enzima (verde) e o provável centro catalítico (amarelo - Figura 9). Analisando o alinhamento múltiplo verifica-se que SjNPP-5a provavelmente está incompleta pois não apresenta nem o domínio NPP completo, perdendo três sítios putativos de ligação a metal. A SmNPP-5 a.1 não apresenta um dos sítios de ligação a metal no domínio NPP e também não apresenta o domínio transmembrana C-terminal, presentes na SmNPP-5a.2. Afora essas duas exceções, todas NPPs apresentaram conservados os 6 sítios de ligação a metal e todas as NPP-5 apresentaram conservado o resíduo de treonina correspondente ao provável centro catalítico. Ressalta-se que os ortólogos de *S. japonicum* apresentam conservados a mesma disposição de peptídeos sinais e domínios transmembranas que os respectivos ortólogos de *S. mansoni*; por exemplo, tanto a SmNPP-5b quanto a SjNPP-5b possuem dois domínios transmembranas, um N e outro C-terminal.

Tabela 1 - Níveis de identidade e similaridade entre as SmNPPs, SjNPPs e NPP-5 e 6 de *H. sapiens*.

		SIMILARIDADE										
		SmNPP-5a.1	**SmNPP-5a.2**	**SmNPP-5b**	**SmNPP-5c**	**SmNPP-6**	**SjNPP-5a**	**SjNPP-5b**	**SjNPP-5c**	**SjNPP-6**	**HsNPP-5**	**Hs NPP-6**
Identidade	**SmNPP-5a.1**		98%	52%	67%	46%	82%	54%	64%	47%	59%	55%
	SmNPP-5a.2	97%		54%	65%	45%	82%	54%	62%	45%	56%	52%
	SmNPP-5b	31%	32%		57%	43%	51%	88%	54%	43%	57%	49%
	SmNPP-5c	47%	46%	37%		41%	67%	56%	86%	43%	55%	49%
	SmNPP-6	27%	25%	24%	25%		45%	40%	40%	83%	41%	45%
	SjNPP-5a	69%	69%	32%	49%	27%		52%	68%	45%	58%	55%
	SjNPP-5b	32%	32%	80%	36%	25%	34%		54%	40%	55%	47%
	SjNPP-5c	45%	43%	32%	75%	23%	49%	33%		42%	58%	51%
	SjNPP-6	27%	25%	24%	25%	73%	27%	23%	22%		41%	45%
	HsNPP-5	40%	37%	36%	36%	23%	41%	35%	39%%	23%		54%
	HsNPP-6	31%	31%	30%	30%	28%%	31%	28%	30%	30%	34%	

Figura 9 – Alinhamento múltiplo das NPPs de *S. mansoni, S. japonicum* e das NPP-5 e NPP-6 de *H. sapiens*.

```
SmNPP-5a.1 : ---------------------------------------------MYCIETMQKMIILLLICFFPYIERIYASGVVGKEQF SKVILISLDGFRYDYFDMAKQRN--INMSAFDKIINQGVYIRRIENEFPILTFPSHFSIVTGLHPGSHGIVDNVF : 109
SmNPP-5a.2 : ---------------------------------------------MYCIETMQKMIILLLICFFPYIERIYASGVVGKEQF SKVILISLDGFRYDYFDMAKQRN--INMSAFDKIINQGVYIRRIENEFPILTFPSHFSIVTGLHPGSHGIVDNVF : 109
SjNPP-5a   : -----------------------------------------------MSEEMRRNFVLILLLTLLIYFKTILSSGRILNEIN PKVLLISLDGFRYDYIAEAKARN--INISAFEKIKNRGIYIESIDNEFPILTFPSHFSMVTGLHPENHGIVDNIF : 107
SmNPP-5b   : MSTEQIIQDDSDLPPPYYQINHECIRRYKRRTHCTYFTLIISGIVMMLFGALILVILIPQEINLYRWNIKGPLSRFFNHG KTVLLISMDGFRHDYIELAKNHLGSNALPNFDRIFISEGVRAAKSINVYPITTLPNHRTLITGLYPENHGVVGNSL : 155
SjNPP-5b   : MSTEQIMPKDDTLPPPYYQINHECSRRYKRRTRCTYIVLIISGIIMMLFGVFILMILIPQEINTFRWNIKTPLSSLFDRD STVLLISMDGFRHDYIELAERHLGPDSLPNFIRIFTANGVRAMESINVYPIVTLPNHRTLITGLYPENHGVVGNSL : 155
SmNPP-5c   : ----------------------------------------------MTMECFCHCFTWISLFILLQGICEA-------YKQ QKLLVISLDGFRHDYLDHAKLRN--INISSFERIWKTGVRVMKVHNEFITRTGPNHLSLVTGMHEESHGIVDNMF : 101
SjNPP-5c   : ------------------------------------------------MECFWHYVTCILVYTFFQAFCKA--------GQ QKLLMISLDGFRHDYIDRAKVRN--VNISAFEKIWRSGIRVMKVHNEFITRTAPNHLSLVTGMHEESHGIVDNTF :  98
HsNPP-5    : ------------------------------------------------MTSKFLLVSFILAALSLSTTFS-----LQPDQ QKVLLVSFDGFRWDYLYKVP-------TPHFHYIMKYGVHVKQVTNVFITKTYPNHYTLVTGLFAENHGIVANDM :  95
HsNPP-6    : -------------------------------------------------MAVKLGTLLLALALGLAQPASA--------R RKLLVFLLDGFRSDYISDEALES----LPGFKEIVSRGVKVDYLTPDFPSLSYPNYYTLMTGRHCEVHQMIGNYM :  94
SmNPP-6    : ----------------------------------------------MNIYTFLYILVYLFNTNSSTVTTTILDNDSSG-SS SRLLVILIDGLRWDVIAGHLENN--TNRFGFKRLQKNGAYLQRFTPVFPAECYPNIYSLFTGRHPVDHGVILPTT : 107
SjNPP-6    : ------------------------------------------------------------------MPNLDYDLSNSTT SRLLVILIDGLRWDVIASHLENN--TSEFGFKRLQKHGAYLERVTPVFPAECYPNIYSLFTGRHPIDHGVILPTT :  86

SmNPP-5a.1 : YDPII-NATFSSRNQSTATDSRFYDVGAEPIWVTNQFHGHKSGVTFWIGSEAIIKGERPTH------YLTPYNESITFTQRIDILMDWFEHEN--INLGLMYYHQPDRAGHIHGAAS-DEVFKAIEEINHGLEYLLTSIEMRPSLSCCLNLIITS : 254
SmNPP-5a.2 : YDPII-NATFSSRNQSTATDSRFYDVGAEPIWVTNQFHGHKSGVTFWIGSEAIIKGERPTH------YLTPYNESITFTQRIDILMDWFEHEN--INLGLMYYHQPDRAGHIHGAAS-DEVFKAIEEINHGLEYLLTSIEMRPSLSCCLNLIITS : 254
SjNPP-5a   : FDFKL-NATFISKNQSGASDSRFYDVGAEPIWVTNQLQGYKSGVAFWIGSEARIKGIRPTH------YLAPYNQNFTFNHRVDILMEWFDHKN--INLGLMYYHQPDRAGHIYGAGS-DEVF------------------------------------ : 219
SmNPP-5b   : LDKKWPNKIFSIYDQESLNHAPWLTDWPEPIWVTLQQNGGYAGSILWPLTDQFVSDDLPFQRVSQYALLNDYEHRYAYDQRIRDILWWLKNPKYHLNLILAYFEEPDETGHSYGPNS-KHVAKVVQTLDETLGHLLDGIKK-RGLTDKVDIILTA : 308
SjNPP-5b   : LDEKWPNATFRIDDQESLNHAPWLTDWPEPIWVTLQKKGGYAGSVLWPLTDQFVGGDLPFQRVSQYTLLNDYEHRYAYDQRVRDILWWLNNPKFRLNLILAYFDEPDETGHAYGPNS-KHVATTIQNLDGTLGRLLDGIEK-RGLTDKVDIILTA : 308
SmNPP-5c   : YDPKL-NDTFDLSNTRHLSQSKWFDVGSEPIWVTNQRHGHKSAVTFWPGSNTPFKGQLPSY------YYSQYNHQLPLVDRINQTIKLLDLDR--VTLGLIYFHEPDSQGHLTGPDS-IEIYNVIEKLNDDIGYLLSLIDTRPSLKSSLNIILTS : 246
SjNPP-5c   : YDPEL-NDTFDLINS---SESKWFDVGSEPIWVTNQRHGHKSAVSFWPGSNTQFKGQLPSF------YYPHYSNDVPLVDLINQTIEWLSLDE--VTLGLLYFHEPDSQGHLTGPDS-PEIFNVIEKLNNDIGFLLSLIDTKPSLKSILNVILTS : 240
HsNPP-5    : FDPIR-NKSFS-LDHMNIYDSKFWEEAT-PIWITNQRAGHTSGAAMWPGTDVKIHKRFPTH------YMP-YNESVSFEDRVAKIIEWFTSKEP-INLGLLYWEDPDDMGHHLGPDS-PLMGPVISDIDKKLGYLIQMLKK-AKLWNTLNLIITS : 237
HsNPP-6    : WDPTT--NKSFDIGVNKDSLMPLWWNGSEPLWVTLTKAKRKVYMYYWPGCEVEILGVRPTY----CLEYKNVPTDINFANAVSDALDSFKSGR--ADLAAIYHERIDVEGHHYGPAS-PQRKDALKAVDTVLKYMTKWIQE-RGLQDRLNVIIFS : 239
SmNPP-6    : FSEHT-----------TINGKPIRSQVEGLWETGVKQNKGVHLYHLPICSTEAGTENSWYCEP---YSQEVMNPMHLNATIQKAVDGLKNGS--ANLAVVYYDELDRIGHRYGPLSNELVHKHLVYLDHVLDYALNIIESIPNLN----LMLTS : 241
SjNPP-6    : FNGLT-----------TVNNKPIRSRAESIWETGVKQNKGVHLYHLPICSAETDSESSWYCEP---YNQKFMNPIDLNFTIQKAVTGLQNGS--ANLAVVYYDELDRIGHRYGPLSDQLIHKHLVYLDHVVNYMLNIVESIPNLN----VLLTS : 220

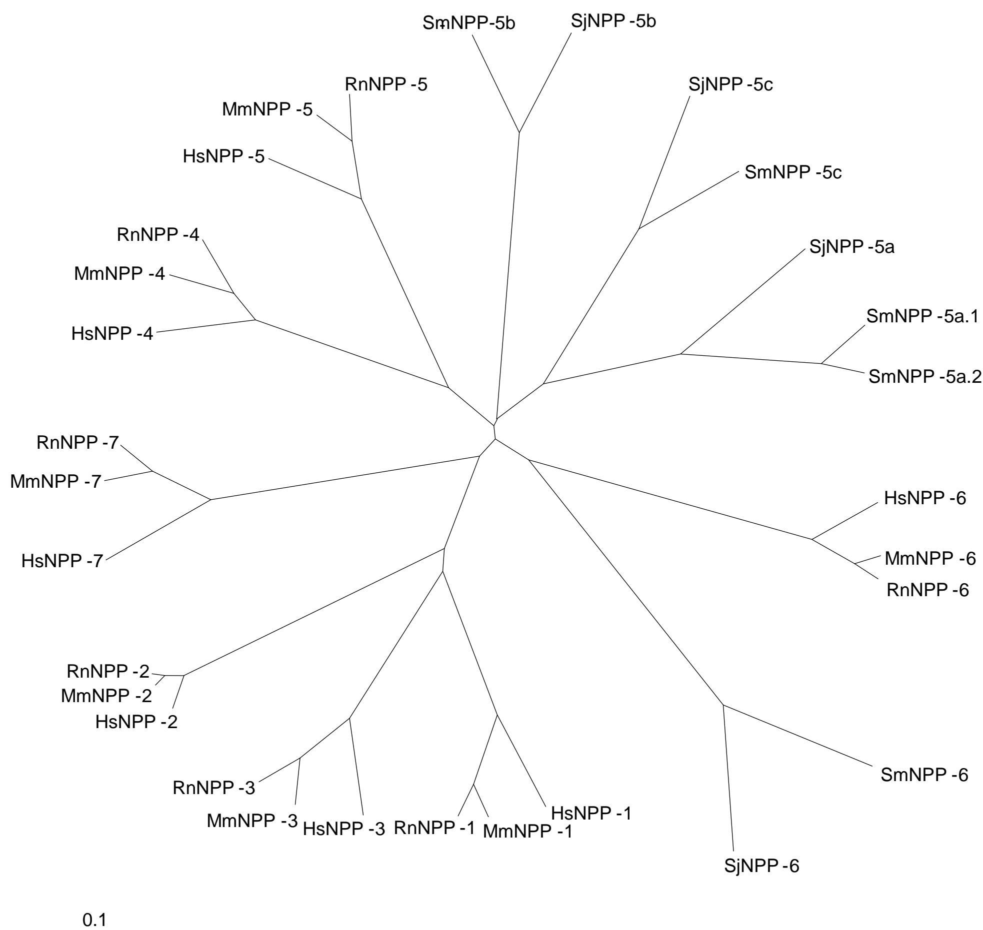
SmNPP-5a.1 : DHGMT-NISSDRVIYLHDYIHPNEYISAPKKSAEIWTLWPKQG-----------------KSYYQRSLKPRLYC--------------------AIIIQQIERSTLQVKCLFEGGTA------- ------------------------------ : 325
SmNPP-5a.2 : DHGMT-NISSDRVIYLHDYIHPNEYISAPKKSAEIWTLWPKQGYTVRSLYNKLKDRHFRLNVYLKEELPTRFFYGSSD----RVGPVVVYADIGWTIIAD---------RTSGITLKNKGAHGYD PDYKEMSPFLMAMGPQIAK--SQPTELKES : 393
SjNPP-5a   : ------------------------------------------------------------------------------------------------------------------------------ ------------------------------ : ---
SmNPP-5b   : DHGMS-ETSNTRLITLDKYIDNSLYN--YTQLSTMGFLYPAPG-KFEEVYKRLKSAHPKLDVYRRDEVPAYLNFNITS----SRMPPLILIAKPRWKIVK---------NTSQPYANVSGDHGYP TDFSEMYPFFIARGPSFKIA-----ESVPT : 441
SjNPP-5b   : DHGMS-ETSNTRLIQLDNYVDNSWYN--YTQLSVMGFLYPASG-KFEKVYKRLKNAHQYLEVYRRDEVPAYLNFNISN----SRMPPLILIAKPRWKIVT---------NASRPYANISGDHGYV TNFSEMHPFFIASGPSFKTG-----ETVPK : 441
SmNPP-5c   : DHGMS-GVDTKRIIILHDYINESMYYSPGSEDRIFWSLWPKDGSSSIDLYKKLVGKHPKMNVFLKDNIPIHLHYSNSR----RIGPVVVYSEPGWTIVRS---------QKSVAKFTKRGDHGYD PDHDEMSPFFLATGPGFRSAIVGGGQIINS : 387
SjNPP-5c   : DHGMS-SVDTKRTIVLNDYIDDNTYYSPGPEHRIVWLLWPKDGVSSVDLYEKLIRKHPKMNVFLKNNIPARLHYSENR----RISPIVVYSEPGWMIVKS---------QESVASYTKHGDHGYD PDYDEMAPFFLAMGPGFRS--TSGGRTINS : 379
HsNPP-5    : DHGMT-QCSEERLIELDQYLDKDHYTLIDQSP--VAAILPKEG-KFDEVYEALTHAHPNLTVYKKEDVPERWHYKYNS----RIQPIIAVADEGWHILQN---------KSDDFLL---GNHGYD NALADMHPIFLAHGPAFRKN-----FSKEA : 367
HsNPP-6    : DHGMTDIFWMDKVIELNKYISLNDLQQVKDRGPVVSLWPAPGKHSEIYN---KLSTVEHMTVYEKEAIPSRFYYKKGK----FVSPLTLVADEGWFITENREMLPFWMNSTGRREGWQRGWHGYD NELMDMRGIFLAFGPDFKSN-----FRAAP : 382
SmNPP-6    : DHGMA-TVS--HQAHADRYFKGTELDRVLNRGSTLWIWPKPEFENNVYNRLLIQQSKSPFTVYNSSTILSHWEIMTNNNNNNSLLPPVLLIASPNYIFHSK---AWPLNMSHYNLIDPKGMHGYD PELPDMHIPLFIYGPNINRG--VQLNFTKE : 388
SjNPP-6    : DHGMS-TVS--HEAHADRYLKGTELSRVLNRGSTLWIWPKPEYEANVYNRLLSQQSRSPFTVYNVSTIPSHWEIATTNNNSISLFPPILLIASPDYIFEST---VWPLNMSHYNLIKPKGMHGYD PELSDMQIPLFIYGPNSNRG--IRLNFTKN : 367

SmNPP-5a.1 : ---------------------------------------------NTFLLWFK------------------------------------------------------------------------------------------------- : 341
SmNPP-5a.2 : IKLIDIYSLICLMLDL-EPAPNNGSVCRVLPLLSQGSFANINRLSIIFIIKFIILSIFMVHNGLYY------------------------------------------------------------------------------------- : 458
SjNPP-5a   : ---------------------------------------------------------------------------------------------------------------------------------------------------------- : ---
SmNPP-5b   : VHAVDVYPLMCALLNIQPNPNNGSLERISNILKPDVANRLLNWNSWSYLWKWIISDLRFILFISIICVSFTILLCSIVVVMHRKETNEPFIRLPIDNDKEFSDYNT-------------------------------------------- : 547
SjNPP-5b   : VHAVDIYPLMCALLNIQPNPNNGSLERISNILKPDVANRLLNLTYWSYLWKWLTFDLRFILFVSIICVFLMVLLCSIAIIMHRKEVSDTFIRLPIDNNXXXX------------------------------------------------- : 543
SmNPP-5c   : IYLIDIYPLMCALLNLNKPAPNNGSLSRVMSLLRHSSGINVYFSDFLDMFTEV--------------------------------------------------------------------------------------------------- : 440
SjNPP-5c   : IRLIDIYPLMCTLLDLTKPAPNNGSLSRVIHLLH---GHNVYFSSFLDMFTVGVV--------LICCITLSIVYMICTCISSIQYPYLFPNNTSNRRKSHMNQPNSFLLQRRFRHSSRKQQQQPDVKLNNIKSLKMKNINHYKNKLYEVCVKDKPPIESD: 528
HsNPP-5    : MNSTDLYPLLCHLLNITAMPHNGSFWNVQDLLNSAMPRVVPYTQSTILLPGSVKP--------AEYDQEGSYPYFIGVSLGSIIVIVFFVIFIKHLIHSQIPALQDMHAEIAQPLLQA---------------------------------- : 477
HsNPP-6    : IRSVDVYNVMCNVVGITPLPNNGSWSRVMCMLKGRASTAPPVW-------------------------PSHCALALILLFLLA------------------------------------------------------------------------ : 440
SmNPP-6    : IRSVHIHSLMAYLASINLPDFRSLDLFKPLLSHNYYHHQLSPVLNNFYWPLNDKSSSSLSFSSTLYGDRFMVNFLIVAAALTCFFVLICAFIILAYVRCRISLTT-TRVFGYQDDLDKELVKSEVLSEA------------------------ : 516
SjNPP-6    : IRPIHIHSLIAYLASINLPDFDSLNLFTPLLLP--HHHQPLPSSSYLHWSWNENSSFPL---LSFHGDRFMLSIIIGIVVLSCFFVLAIGLLIIVYIRCRFALIMNTRVITCDDELDKEGASPMPNPSP------------------------ : 491

SmNPP-5a.1 : -------------------------------------------------- : ---
SmNPP-5a.2 : -------------------------------------------------- : ---
SjNPP-5a   : -------------------------------------------------- : ---
SmNPP-5b   : -------------------------------------------------- : ---
SjNPP-5b   : -------------------------------------------------- : ---
SmNPP-5c   : -------------------------------------------------- : ---
SjNPP-5c   : KQHLLNYNMSNNHVDDNDDSEEYEEYNRFSVRHIDDETFLNLIRQPNNKYP : 579
HsNPP-5    : -------------------------------------------------- : ---
HsNPP-6    : -------------------------------------------------- : ---
SmNPP-6    : -------------------------------------------------- : ---
SjNPP-6    : -------------------------------------------------- : ---
```

A partir do alinhamento múltiplo das NPPs de *S. mansoni, S. japonicum, H. sapiens, M. musculus,* e *R. norvergicus*, foi desenhada um cladograma que demonstra visualmente três ramificações: uma com as NPPs 1, 2, 3 e 7, outra com as NPPs 4 e 5 e a terceira com as NPPs 6 (Figura 10). A Figura 10 sugere também a ortologia entre as SmNPPs e as SjNPPs pois estes genes sempre aparecem aos pares. Ressalta-se ainda que as SmNPPs-5 e as SjNPPs-5 estão equidistantes na ramificação das NPPs 4 e 5 de mamíferos, sendo denominadas 5 simplesmente por apresentarem maior similaridade e identidade ao segundo grupo.

Figura 10 – Cladograma das NPPs de *S. mansoni, S. japonicum, H. sapiens, M. musculus e R. norvergicus.*



4.1.1 *SmNPP-5a*

O gene da SmNPP-5a apresentaria 5.286 pares de nucleotídeos em sua sequência genômica completa (íntrons e éxons). O mRNA transcrito e processado da SmNPP-5a.1 apresentaria 1023 nucleotídeos e sua proteína predita possuiria 341 aminoácidose,

apresentando apenas um peptídeo sinal N-terminal e o domínio catalítico (E-value = $2,9x10^{-53}$; Figuras 9 e 11). Sua massa molecular predita é de 39,5 kDa enquanto o ponto isoelétirco (pI) predito é de 6,27. A SmNPP-5a.2 apresentaria 1.377 nucleotídeos em seu mRNA transcrito e processado. A proteína possuiria 458 aminoácidos, um peptídeo sinal N-terminal, o domínio catalítico NPP (E-value = $5,9x10^{-82}$; Figuras 9 e 11; Anexo A) e um domínio transmembrana C-terminal. A massa molecular e o pI preditos para a proteína são, respectivamente, 52,5 kDa e 6,28. Como prováveis mudanças pós-traducionais, a proteína apresentaria sítios putativos para N-glicosilação, para glicação dos ε amino grupos e para fosforilação.

4.1.2 SmNPP-5b

O gene da SmNPP-5b apresentaria 2.709 pares de nucleotídeos em sua sequência gênica completa e 1.644 nucleotídeos em seu mRNA transcrito e processado. A proteína possuiria 547 aminoácidos, dois domínios transmembrana, um N-terminal e outro C-terminal, e o domínio catalítico NPP (E-value = $2,3x10^{-81}$; Figuras 9 e 11; ANEXO A). A massa molecular e o pI preditos para a proteína são, respectivamente, 63,3 kDa e 7,01. Essa proteína também apresentaria as seguintes prováveis mudanças pós-transducionais: glicação dos ε amino grupos, N-glicosilação, fosforilação e sulfatação.

4.1.3 SmNPP-5c

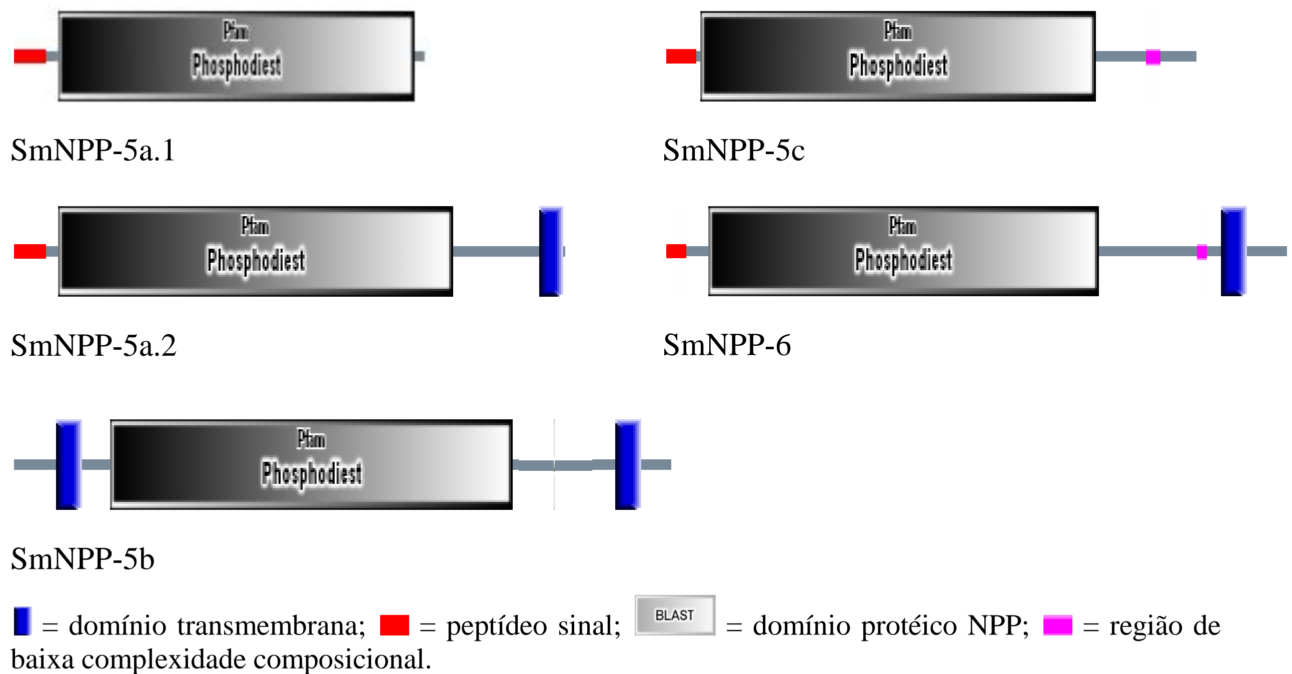
O gene da SmNPP-5c apresentaria 3.611 pares de nucleotídeos em sua sequência gênica completa e 1.323 nucleotídeos em seu mRNA trancrito e processado. A proteína possuiria 440 aminoácidos, um peptídeo sinal N-terminal e o domínio catalítico NPP (E-value = $8,6x10^{-86}$). A proteína não apresentaria domínio transmembrana (Figuras 9 e 11; ANEXO A). A massa molecular e o pI preditos para a proteína são, respectivamente, 50,3 kDa e 6,72. As mudanças pós-transducionais preditas para essa proteína são: glicação dos ε amino, N-glicosilação, fosforilação e sulfatação.

4.1.4 SmNPP-6

O gene SmNPP-6 apresentaria 22.580 pares de nucleotídeos em sua sequência genômica completa e 1.551 nucleotídeos em mRNA transcrito e processado. A proteína

possuiria 516 aminoácidos, um peptídeo sinal N-terminal, o domínio catalítico NPP (E-value = $7x10^{-25}$) e o domínio transmembrana C-terminal (Figuras 9 e 11; Anexo A). A massa molecular e o pI preditos para a proteína são, respectivamente, 58,9 kDa e 6,46. Como prováveis mudanças pós-transducionais a proteína também apresentaria: glicação dos ε amino grupos, N-glicosilação, fosforilação e sulfatação.

Figura 11 – Representação esquemática dos domínios protéicos preditos das SmNPPs.

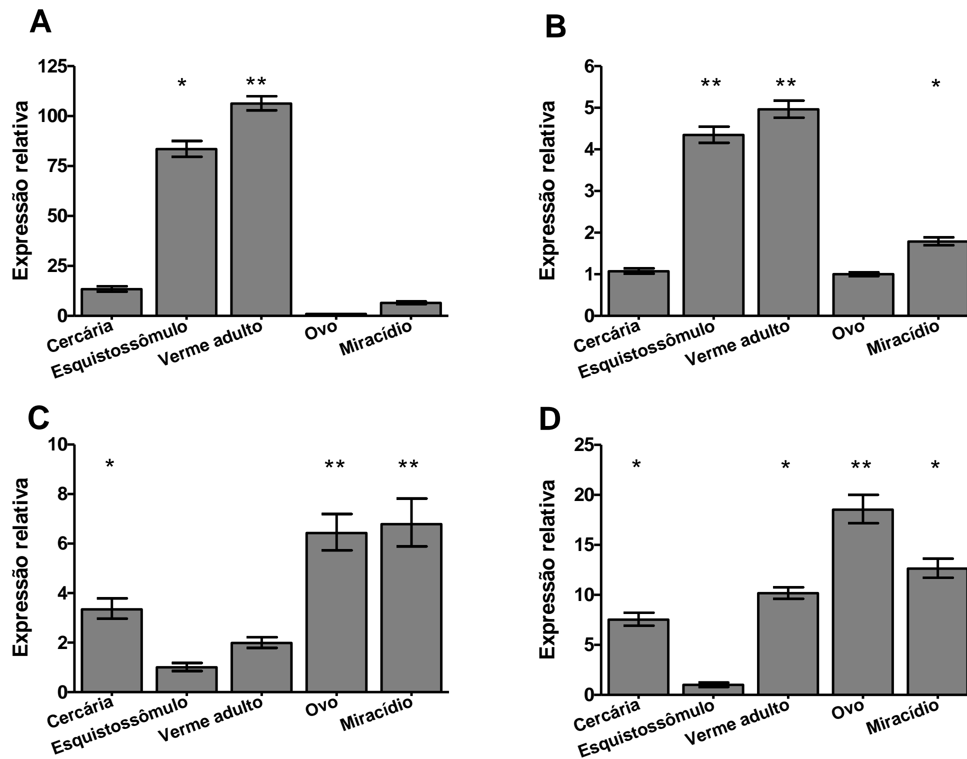


= domínio transmembrana; = peptídeo sinal; = domínio protéico NPP; = região de baixa complexidade composicional.

4.2 Perfil de expressão do mRNA das SmNPPs

Analisamos o perfil de expressão das SmNPPs, quantificando os níveis relativos de mRNA nos diferentes estágios de desenvolvimento do parasita e também em vermes adulto macho e fêmeas por meio de RT-PCR em tempo real. Quando comparados os estágios de desenvolvimento, verificou-se que a SmNPP-5a apresentou um aumento significativo da expressão em esquistossômulos e vermes adultos (Figura 12A) assim como a SmNPP-5b, porém os níveis de expressão relativa aos outros estágio foi muito menor nesse caso (Figura 12B). Diferentemente, a SmNPP-5c apresentou maiores níveis de expressão em ovos e miracídios (Figura 12C), numa amplitude semelhante a da SmNPP-5b, enquanto a SmNPP-6 demonstrou-se menos expressa no estágio de esquistossômulos, apresentando seu máximo de expressão no estágio de ovos e níveis semelhantes nos outros estágios (Figura 12D).

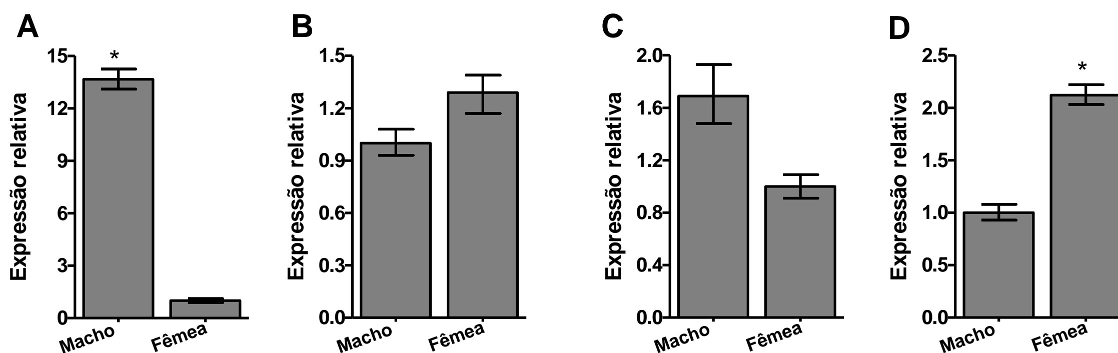
Figura 12 – Análise do perfil de expressão transcricional das SmNPPs ao longo do ciclo do parasita.



A – SmNPP-5a. B - SmNPP-5b. C - SmNPP-5c. D - SmNPP-6. Média ± valores máximos e mínimos de expressão relativa. *, ** representam diferenças estatisticamente significativas.

Ao analisarmos o nível de expressão relativo das SmNPPs comparando vermes adultos machos e fêmeas, foi observado que a SmNPP5a é cerca de dez vezes mais expressa em machos que em fêmeas (Figura 13A). Por outro lado, a SmNPP-6 apresentou maior nível de expressão em fêmeas, aproximadamente duas vezes (Figura 13D). A SmNPP-5b e a SmNPP-5c não apresentaram diferenças de expressão estatisticamente significativas em relação ao gênero dos vermes adultos (Figura 13B e C).

Figura 13 – Análise do perfil de expressão transcricional das SmNPPs em relação ao gênero de vermes adultos.



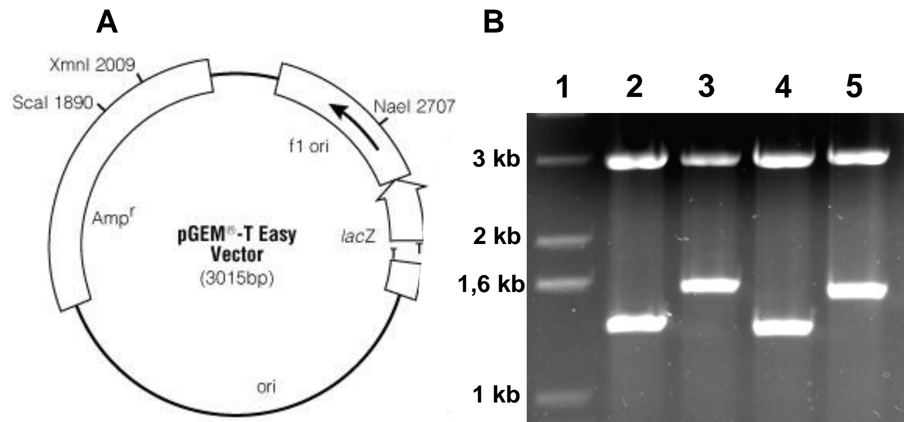
A – SmNPP-5a. B – SmNPP-5b. C – SmNPP-5c. D – SmNPP-6. Média ± valores máximos e mínimos de expressão relativa. * representa diferenças estatisticamente significativas.

4.3 Clonagem dos genes *smnpps*

Apesar de não serem todas as SmNPPs que apresentavam seu pico de expressão em vermes adultos, decidimos iniciar as clonagens utilizando mRNA desse estágio por ser um material mais fácil de se obter e apresentar um bom rendimento de extração. Desse modo, a sequência do cDNA completo das diferentes SmNPPs foi amplificada por RT-PCR, utilizando mRNA de vermes adultos e oligonucleotídeos específicos (APÊNDICE). A SmNPP-5b e a SmNPP-6 apresentaram fragmentos com aproximadamente 1.650 pb, enquanto a SmNPP-5a e a SmNPP-5c revelaram fragmentos entre 1.000 e 1.650 pb. Esses fragmentos foram clonados no vetor pGEM-Teasy (Figura 14); esse vetor apresenta-se linearizado com timidinas 3´terminais nas suas extremidades. Essas extremidades 3´-T no sítio de inserção aumentam a eficiência de ligação de produtos de PCR, prevenindo a recircularização do vetor e providenciando uma extremidade compatível para produtos de PCR gerados por diversas polimerases termoestáveis. As clonagens foram confirmadas por sequenciamento.

Como durante o desenvolvimento do projeto, o genoma do parasita ainda não estava completo, inicialmente só encontramos a sequência gênica para SmNPP-5a.2; portanto, apenas esta isoforma foi clonada, como confirmado pelo sequenciamento. Desse modo, toda referência a SmNPP-5a refere-se a esta isoforma.

Figura 14 – Clonagem das smnpps no vetor pGEM-T Easy.

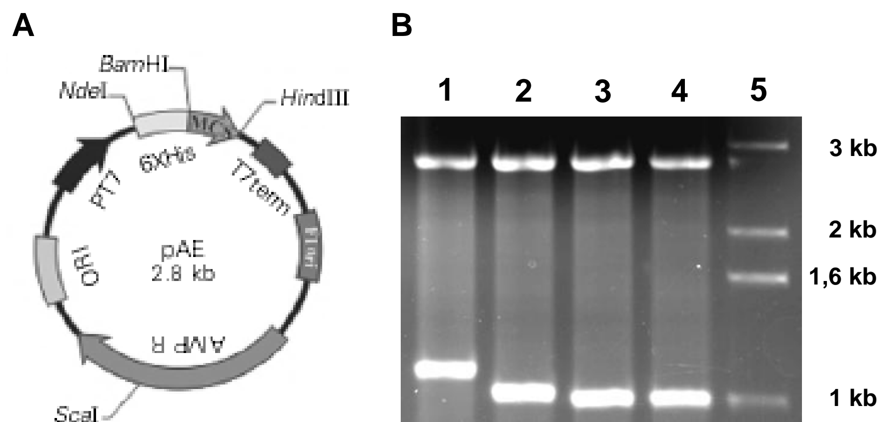


A – Mapa do vetor pGEM-T Easy. B – Análise de restrição das *smnpp*s clonadas no vetor pGEM-T Easy; 1 – padrão de tamanho molecular; 2 – *smnpp-5a*; 3 – *smnpp-5b*; 4 – *smnpp-5c*; 5 – *smnpp-6*.

4.4 Expressão em *Escherichia coli* e purificação das SmNPPs recombinantes

Os genes das SmNPPs foram clonados inteiros no vetor pGEM-T Easy e posteriormente foram reamplificadas apenas as regiões de interesse dos genes SmNPPs, contendo somente o domínio NPP e poucos aminoácidos adjacentes. Essa reamplificação foi realizada com olinucleotídeos específicos (APÊNDICE) que adicionaram sítios de restrição aos fragmentos gênicos para posterior clonagem no plasmídeo pAE-6His (Figura 15). O pAE-6His, um vetor de expressão em *E. coli,* é um plasmídeo pequeno com aproximadamente 2,8 Kb e de alta cópia (200-500). Esse vetor caracteriza-se por adicionar uma cadeia polipeptídica mínima na porção N-terminal, incluindo uma cauda de histidina (RAMOS et al., 2004).

Figura 15 – Clonagem das smnpps no vetor pAE-6His.



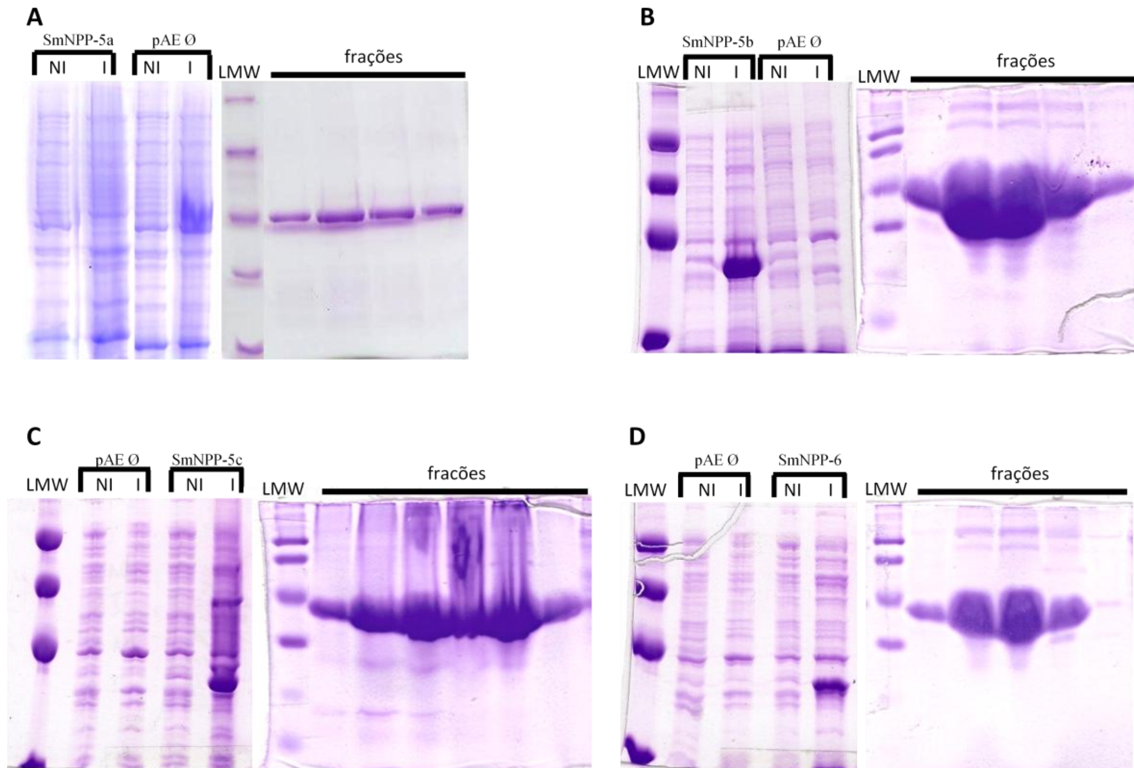
A – Mapa do vetor pAE-6His. B – Análise de restrição das *smnpp*s clonadas no vetor pAE-6His; 1 – *smnpp-5a*; 2 – *smnpp-5b*; 3 – *smnpp-5c*; 4 – *smnpp-6*; 5 – padrão de tamanho molecular.

Os plamídeos pAE-6His contendo os insertos *smnnps* foram utilizados para transformar bactérias *E. coli* BL21 Star (DE3) plysS. As quatro proteínas foram expressas e nenhuma delas apresentou escape de expressão antes da indução com IPTG (Figura 16). As proteínas foram expressas sob a forma de corpúsculos de inclusão os quais foram lavados com uréia 2 M e solubilizados com uréia 8 M. Assim testamos a expressão lenta das proteínas, a 18 ºC, que apesar de aumentar consideravelmente a expressão das proteínas recombinantes (Figura 16B, C e D), não aumentou a sua solubilidade quando analisamos a fração solúvel do lisado bacteriano (dados não apresentados).

As proteínas foram purificadas denaturadas por cromatografia de afinidade ao Ni^{2+} (Figura 16) e posteriormente dialisadas. Durante a diálise as quatro proteínas precipitaram, desse modo buscamos realizar o enovelamento por outros métodos, primeiramente por diluição lenta. Nessa metodologia ocorreu uma grande perda de proteína no processo de renovelamento devido à precipitação das proteínas e logo após a purificação verificou-se que as proteínas continuavam a precipitar. Assim fomos avaliar o renovelamento na coluna que também apresentou um rendimento muito baixo devido em grande parte a precipitação das proteínas dentro das colunas, sendo que as proteínas continuaram a precipitar após sua eluição.

Tentamos ainda realizar o *refolding* das proteínas sob alta pressão; para tal, utilizamos os corpúsculos de inclusão, ao invés da proteína purificada, os quais foram submetidos à alta pressão, porém após a diálise e separação da fração solúvel não foi possível detectar a proteína em SDS-PAGE ou mesmo a atividade enzimática nucleotídeo pirofosfatase/ fosfodiesterase. Por fim, testamos o refolding da SmNPP-5a por diluição rápida em 78 tampões com diferentes concentrações de sal, agentes redox e aditivos de renovelamento, mas também não observamos a atividade enzimática nucleotídeo pirofosfatase/ fosfodiesterase (dados não apresentados).

Figura 16 – Expressão em *E.coli* e purificação das SmNPPs.

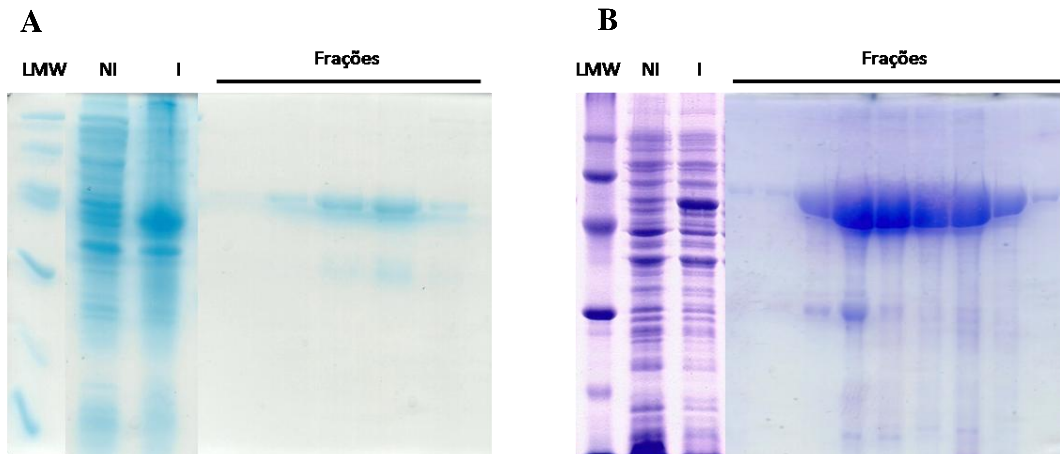


A – SmNPP-5a; B – SmNPP-5b; C – SmNPP-5c; D – SmNPP-6. LMW – padrão de peso molecular (97, 66, 45, 30 e 20 kDa, respectivamente); NI – cultura sem indução da expressão da proteína recombinante por IPTG; I – cultura com indução da expressão da proteína recombinante por IPTG; Frações – frações eluídas da cromatografia por afinidade ao níquel coletadas.

4.5 Expressão da Apirase (SmATPDase) e Fosfatase alcalina (SmFA) em *Escherichia coli* e purificação das proteínas recombinantes

Os plamídeos pET-21B-SmATPDase e pAE-6His-SmFA foram utilizados para transformar bactérias *E. coli* BL21 Star (DE3) plysS. As proteínas recombinantes foram expressas sob a forma de corpúsculos de inclusão após a indução por IPTG. As bactérias foram lisadas, centrifugadas e os corpúsculos de inclusão foram ressuspendidos e lavados com uréia 2 M. Os corpúsculos foram então centrifugados e solubilizados com uréia 8 M. As proteínas foram purificadas denaturadas por cromatografia de afinidade ao níquel (Figura 17) e posteriormente dialisadas lentamente em concentrações decrescentes de uréia. As proteínas dialisadas foram utilizadas para os experimentos de vacinação.

Figura 17 – Expressão em *E. coli* e purificação da SmATPDase e da SmFA.

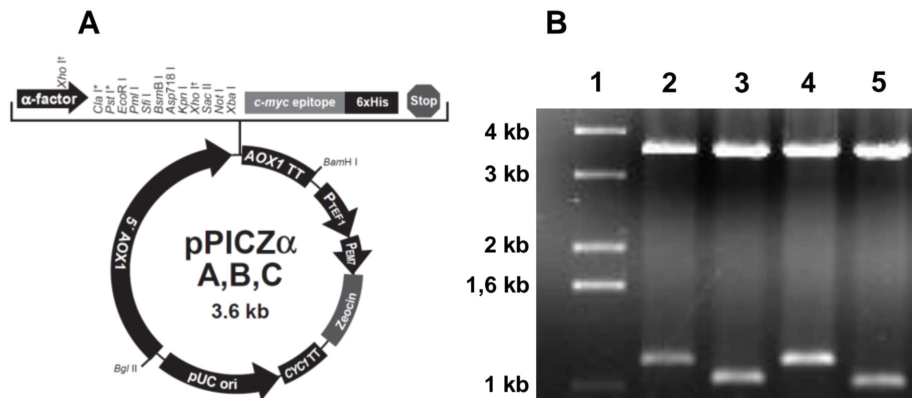


A – SmATPDase; B – SmFA. LMW – padrão de peso molecular (97, 66, 45, 30, 20 e 14 kDa, respectivamente); NI – cultura sem indução da expressão da proteína recombinante por IPTG; I – cultura com indução da expressão da proteína recombinante por IPTG; Frações – frações eluídas da cromatografia por afinidade ao níquel e coletadas.

4.6 Clonagem em vetor pPicZαA e pINT-A para expressão em *Pichia pastoris*

Uma vez que através da expressão das proteínas SmNPPs recombinantes em *E. coli* não havia sido possível obtê-las reenoveladas com estrutura, tentamos obtê-las através de expressão em *Pichia pastoris*. Os domínios NPPs das SmNPPs foram clonados no vetor de expressão eucarioto pPICZαA (Figura 18), sendo confirmado por sequenciamento. Esses plasmídios foram linearizados e utilizados para transformar leveduras *P. pastoris*. O vetor pPICZαA é utilizado para expressar proteínas recombinantes fusionadas a um peptídeo N-terminal que codifica para o sinal de secreção fator-α de *Saccharomyces cerevisiae* e desse modo as secreta para o meio de fermentação.

Figura 18 – Clonagem das smnpps no vetor pPICZαA.



A – Mapa do vetor pPICZαA. B – Análise de restrição das *smnpps* clonadas no vetor pPICZα A. 1 – padrão de tamanho molecular ; 2 –*smnpp-5a*; 3 – *smnpp-5b*; 4 – *smnpp-5c*; 5 – *smnpp-6*.

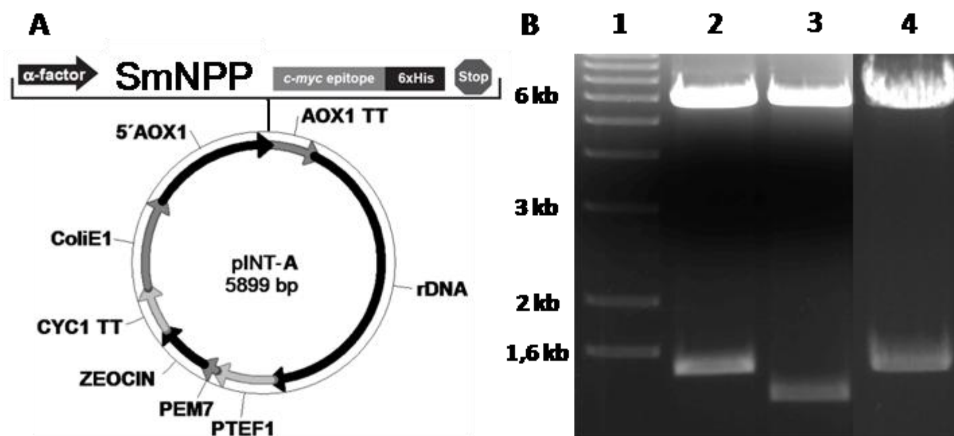
Utilizando-se esse vetor frequentemente se obtêm altos níveis de expressão induzida pelo metanol. Apesar de seu manual recomendar a análise apenas dos clones com alto número de cópias do plasmídio e por isso resistente a maiores concentrações de Zeocina, nós decidimos, baseados em resultados anteriores do laboratório, analisar também alguns clones com baixo número de cópias do vetor. Nós analisamos 5 clones com alto número de cópias do plasmídio e 3 clones com baixo número de cópias para cada proteína, totalizando 32 clones analisados. Foram analisados também 4 períodos de expressão 24, 48, 72 e 96 h, completando 128 amostras para análise.

Nenhuma das 128 amostras apresentou níveis detectáveis de proteínas recombinantes no meio de cultura. Desse modo, baseados na experiência da expressão em *E. coli* no qual as proteínas apesar de serem expressas eram altamente insolúveis, analisamos as frações intracelulares de *P. pastoris* para verificar se as proteínas não estavam sendo expressas sob a forma de corpúsculos de inclusão. Porém, também não conseguimos detectar as proteínas recombinantes nas 128 amostras analisadas (dados não mostrados).

Como não foram verificadas as expressões das SmNPPs pela *P. pastoris* utilizando-se o pPICZαA, decidiu-se utilizar um novo vetor, o pINT-A. O pINT-A é um vetor que se integra ao genoma da levedura através de um fragmento do DNA ribossomal de *Hansenula polymorpha* e que apresenta origem de replicação bacteriana, o gene de resistência a Zeocina e o promotor de expressão AOX1, (Figura 19 – SILVA, 2010). Para a clonagem dos fragmentos dos genes *smnpps* no vetor pINT-A, eles foram amplificados a partir dos vetores pPICZαA transformados com as *smnpps*. Oligonucleotídeos específicos (APÊNDICE) foram projetadas para amplificar a partir do fator-α de secreção do vetor pPICZαA até o codón sinalizador de final de transcrição e adicionar os sítios de restrição necessários para inserção

no vetor pINT-A. Assim os fragmentos *smnpps* contendo o fator-α de secreção e a cauda de histidina provenientes do vetor pPICZαA foram amplificados digeridos e inseridos no vetor pINT-A (Figura 19B). Porém não conseguimos realizar a inserção da *smnpp6*, pois este gene apresentou um sítio para XmnI ao longo de sua seqüência gênica.

Figura 19 – Clonagem das smnpps no vetor pINT-A.



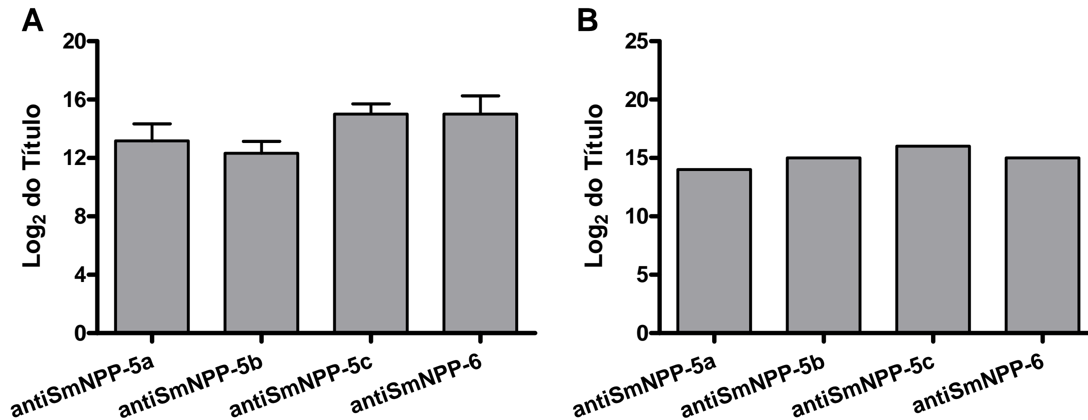
A – Mapa do vetor pINT-A com o fragmento *smnpp* amplificado a partir do pPICZαA. B – Análise de restrição das *smnpps* clonadas no vetor pINT-A. 1 – padrão de tamanho molecular; 2 – *smnpp-5a*; 3 – *smnpp-5b*; 4 – *smnpp-5c*.

O ensaio de expressão foi realizado da mesma forma que havia sido feita com *P. pastoris* transformadas com pPICZαA, sendo analisados 8 clones para cada construção em 4 períodos de tempo diferente (24, 48, 72 e 96 h), totalizando 96 amostras. Todavia, também não conseguimos detectar a expressão das proteínas recombinantes utilizando o vetor pINT-A, nem sendo secretada para o meio de cultura, nem sendo expressa intracelularmente.

4.7 Produção de anticorpos anti-SmNPPs em ratos e camundongos

Foram produzidos anticorpos anti-SmNPPs em camundongos fêmeas BALB/c e ratas Wistar imunizadas com as proteínas recombinantes produzidas em *E. coli* precipitadas e formuladas com o adjuvante Titermax. Os anticorpos específicos anti-SmNPP-5a, anti-SmNPP-5b, anti-SmNPP-5c e anti-SmNPP-6 foram quantificados por ELISA, demonstrando alta imunogenicidade com a indução de quantidades comparáveis de anticorpos (Figura 20).

Figura 20 – Produção de anticorpos policlonais em camundongos e ratos.

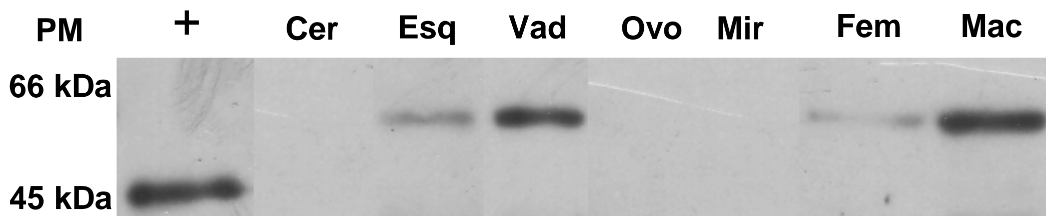


A – camundongos; B – ratos.

4.8 Perfil de expressão protéico da SmNPP-5a

Foram realizados ensaios de *immunoblotting* para analisar a expressão das SmNPPs nos diferentes estágios do parasita, utilizando os anticorpos policlonais produzidos em camundongos. O perfil de expressão protéico da SmNPP-5a ao longo do ciclo de vida do parasita caracterizou-se por um aumento de expressão após a infecção do hospedeiro definitivo. A SmNPP-5a não foi detectada nos estágio de ovos, miracídio e cercária, pouca proteína foi detectada em esquistossômulos de 7 dias e o pico de expressão foi observado em vermes adultos. Também se demonstrou que a proteína é mais expressa em vermes adultos machos que em fêmeas (Figura 21).

Figura 21 – Análise do perfil de expressão protéico das SmNPPs ao longo do ciclo de vida do parasita.

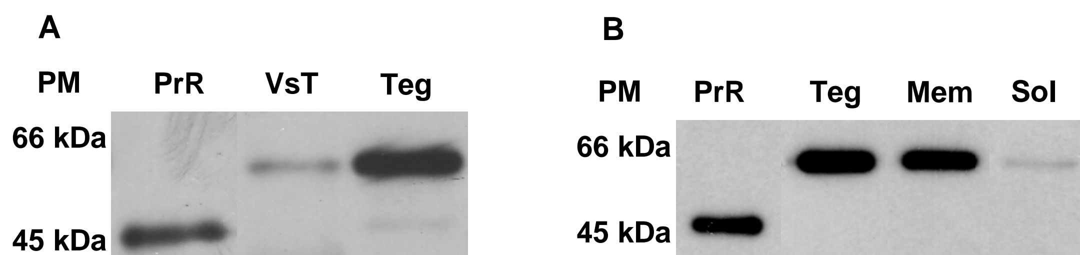


PM – padrão de massa molecular; + – controle positivo; Cer – cercárias; Esq – esquistossômulos; Vad – vermes adultos; Ovo – ovos; Mir – miracídios; Fem – vermes adultos fêmeas; Mac – vermes adultos machos.

Tentando localizar a expressão da proteína no parasita, foi realizada a extração do tegumento de vermes adultos e foi analisada a fração do tegumento e de vermes adultos sem

tegumento. Verificou-se que a fração de tegumento mostrou-se enriquecida em SmNPP-5a (Figura 22A). Buscando um refinamento dessa localização, a fração de tegumento foi dividida em duas frações: sincicial e de membranas. Nessa análise podemos constatar que a SmNPP-5a apresenta-se associada as membranas do tegumento (Figura 22B).

Figura 22 – Análise do perfil de expressão protéico das SmNPPs no tegumento de vermes adultos.

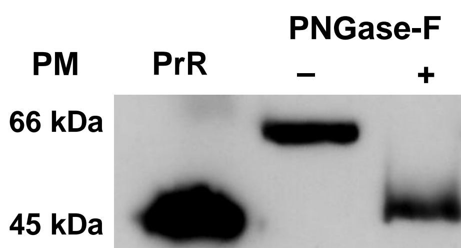


(A) PM – padrão de massa molecular; PrR – proteína recombinante, controle positivo; Vst – vermes adultos sem tegumento; Teg – tegumento extraídos de vermes adultos. (B) PM – padrão de massa molecular; + – controle positivo; Teg – tegumento extraído de vermes adultos; Mem – fração de membranas do tegumento;Sol – fração de proteínas solúveis do tegumento.

4.9 SmNPP-5a é uma enzima N-glicosilada

Como a SmNPP-5a nativa de *S. mansoni* apresentou um tamanho molecular próximo aos 66 kDa, enquanto a predição de seu tamanho era de 52,5 kDa, foi analisada a possibilidade desta proteína estar glicosilada. Para isto, o extrato de tegumento foi submetido a tratamento enzimático para avaliar a presença de possíveis N- e O- glicosilações. Após a digestão com PNGase-F, uma N-deglicosidase, verificamos que a proteína migra com tamanho menor (Figura 23), indicando que a proteína é N-glicosilada, como havia sido predito pela bioinformática.

Figura 23 – Deglicosilção da SmNPP-5a nativa.



PM – padrão de massa molecular; P – proteína recombinante; PNGase-F (–) – fração de tegumento não digerida com a enzima; PNGase-F (+) – fração de tegumento tratada com PNGase F.

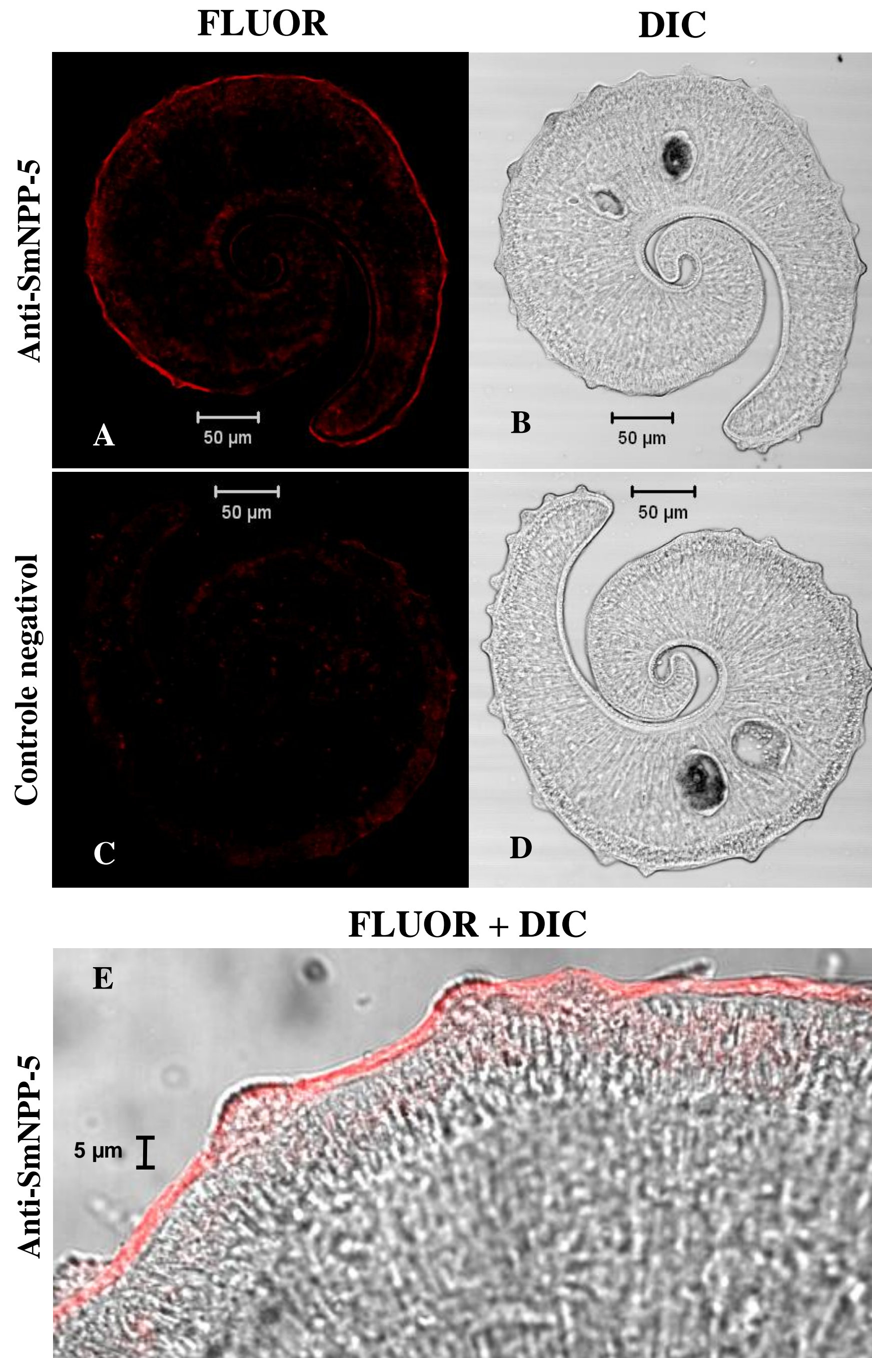
Foi analisada também a possibilidade da proteína ser O-glicosilada; porém, apesar de utilizar diversas enzimas para O-deglicosilação não foi notada nenhuma mudança na sua massa molecular (dados não mostrados).

4.10 Imunolocalização da SmNPP-5a

Para confirmar a localização da SmNPP-5a no tegumento de vermes adultos, foi realizada a imunolocalização da proteína em secções de vermes adultos machos, usando a fluorescência através de anticorpos marcados. Os resultados demonstraram que a SmNPP-5a é majoritariamente distribuída ao longo do tegumento de vermes adulto machos (Figura 24).

Um sinal de menor intensidade de fluorescência pode ser detectado nos tecidos internos do parasita, inclusive em parasitas incubados com soro *naive*, configurando-se assim como uma provável fluorescência inespecífica.

Figura 24 – Imunolocalização da SmNPP-5a.



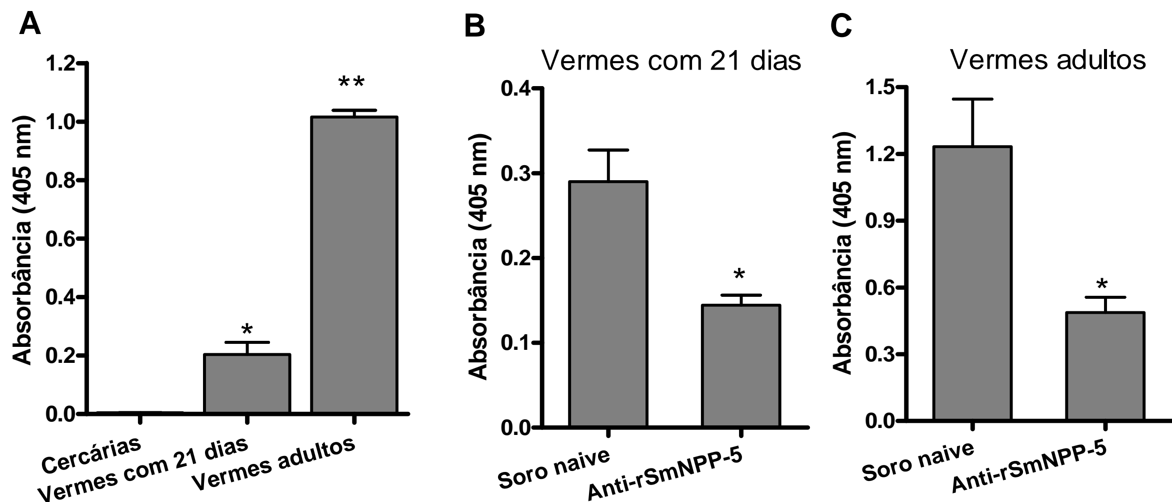
A e C – secções de vermes adultos marcados com anticorpos antiSmNPP-5a ou soro naive, respectivamente. B e D – respectivas imagens de contraste por interferência diferencial (DIC). E – Detalhe de imagem DIC sobreposta a imagem de fluorescência.

4.11 Detecção e inibição da atividade fosfodiesterase em parasitas vivos

Para demonstrar que a SmNPP-5a é uma proteína exposta na superfície do parasita, foi avaliada a capacidade de vermes vivos hidrolisarem um substrato específico, o ρ-Nph-5´-TMP. Esquistossômulos de 21 dias e vermes machos adultos demonstraram ter atividade de fosfodiesterase significativamente diferentes, enquanto as cercárias não promoveram a hidrólise do substrato (Figura 25A).

Também foi avaliada a capacidade de anticorpos anti-SmNPP-5a inibirem essa atividade enzimática. Assim os parasitas foram incubados com os anticorpos para depois se verificar a atividade enzimática superficial. Verificou-se que esses anticorpos foram capazes de reduzir a atividade enzimática em 50% e 60% aproximadamente em vermes com 21 dias e vermes adultos vivos, respectivamente (Figura 25B e C).

Figura 25 – Detecção e inibição da atividade fosfodiesterase em parasitas vivos.



A – Atividade de fosfodiesterase na superfície de parasites vivos. B – Inibição da atividade fosfodiestease superficial devido à pré-incubação de parasitas vivos com anticorpos anti-SmNPP-5a em vermes de 21 dias. C – Inibição da atividade fosfodiestease superficial devido à pré-incubação de parasitas vivos com anticorpos anti-SmNPP-5a em vermes adultos. Média ± DP. *, ** representam diferenças estatisticamente significativas.

Para avaliar a especificidade do substrato, foi feito em paralelo um experimento no qual os parasitas foram incubados na presença de um substrato para a fosfatase alcalina ("*ρ-nitrophenyl phosphate disodium salt*"). Esse dado confirmou a especificidade da reação devido aos diferentes padrões nos níveis de atividade enzimática observados em relação à atividade fosfodiesterase (dados não mostrados - ARAUJO-MONTOYA et al., 2011).

4.12 Hibridização *in situ* das SmNPPs em espécimes inteiros

Através da técnica de hibridização *in situ* em espécimes inteiros (*whole-mount in situ hibridization* – WISH), pudemos localizar em quais tecidos do parasita o mRNA de cada *smnpp* estaria sendo expresso. Utilizando o reagente BM Purple, os tecidos nos quais é detectado o mRNA específico apresenta uma coloração azul. A *smnpp-5a* é expressa nos testículos de vermes adultos machos (Figura 26A) e no sistema reprodutor feminino de vermes adultos fêmeas, em especial no útero e no oótipo (Figura 26C e E).

A *smnpp-5b* é expressa no útero e ovário de vermes adultos fêmeas (Figura 27A, B e E), enquanto nos vermes adulto machos ela aparece transcrita nos testículos (Figura 27D).

Também se pode observar a expressão da *smnpp-5c* nos testículos de vermes adultos machos (Figura 28A), sendo que em vermes adultos fêmeas ela é expressa no ovário e no oótipo (Figura 28B e C). Padrão semelhante à *smnpp-6* que também é expressa nos testículos de vermes adultos machos (Figura 29B) e no ovário de vermes adultos fêmeas (Figura 29A).

Figura 26 – Hibridização *in situ* da *smnpp-5a* em vermes adultos.

A – *smnpp-5a* expressa no testículo de verme adulto machos. B – controle negativo de verme adulto macho. C – *smnpp-5a* expressa útero e oótipo de verme adulto fêmea. D – controle negativo de verme adulto fêmea. E – detalhe da expressão da *smnpp-5a* no útero e oótipo de fêmea. Barras = 100 µm.

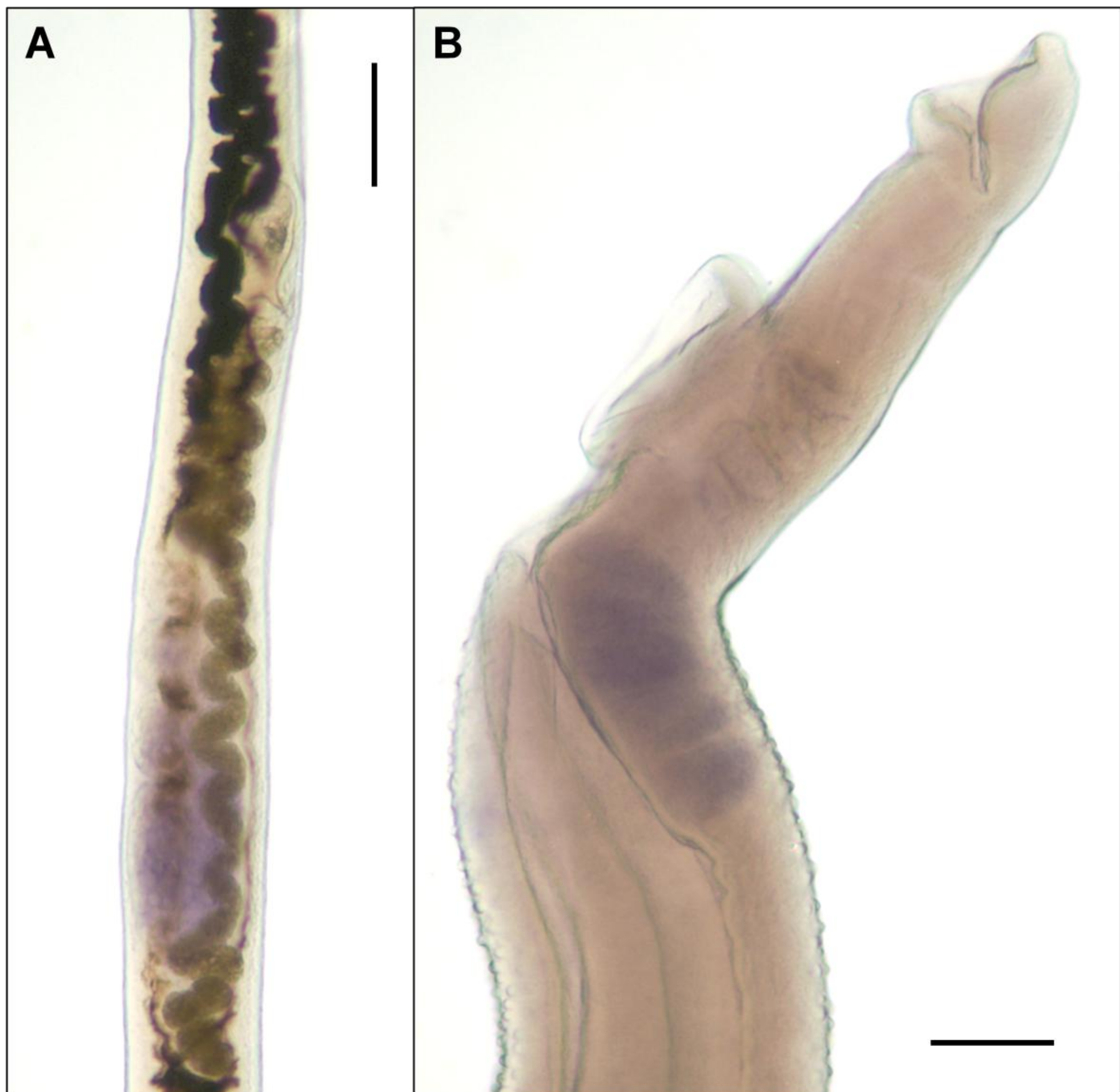
Figura 27 – Hibridização *in situ* da *smnpp-5b* em vermes adultos.

A – *smnpp-5b*expressa no útero de verme adulto fêmea. B – *smnpp-5b*expressa ao longo do sistema genital feminino, útero e ovário. C – controle negativo de fêmea. D – *smnpp-5b* expressa nos testículos de vermes adultas machos. E – Detalhe do ovário. Barras = 100 µm.

Figura 28 – Hibridização *in situ* da *smnpp-5c* em vermes adultos.

A – *smnpp-5c* expressa nos testículos de verme adulto macho.B – Detalhe da *smnpp-5c* expressa no ovário de verme fêmea. C – Detalhe da expressão no oótipo de fêmea. D – detalhe do controle negativo do oótipo de fêmea. Barras = 100 µm.

Figura 29 – Hibridização *in situ* da *smnpp-6* em vermes adultos.



A – *smnpp-6* expressa no ovário de verme adulto fêmea. B – *smnpp-6* expressa nos testículos de verme adulto macho. Barras = 100 µm.

4.13 Avaliação das ectonucleotidases do tegumento de *Schistosoma mansoni* como candidatos vacinais

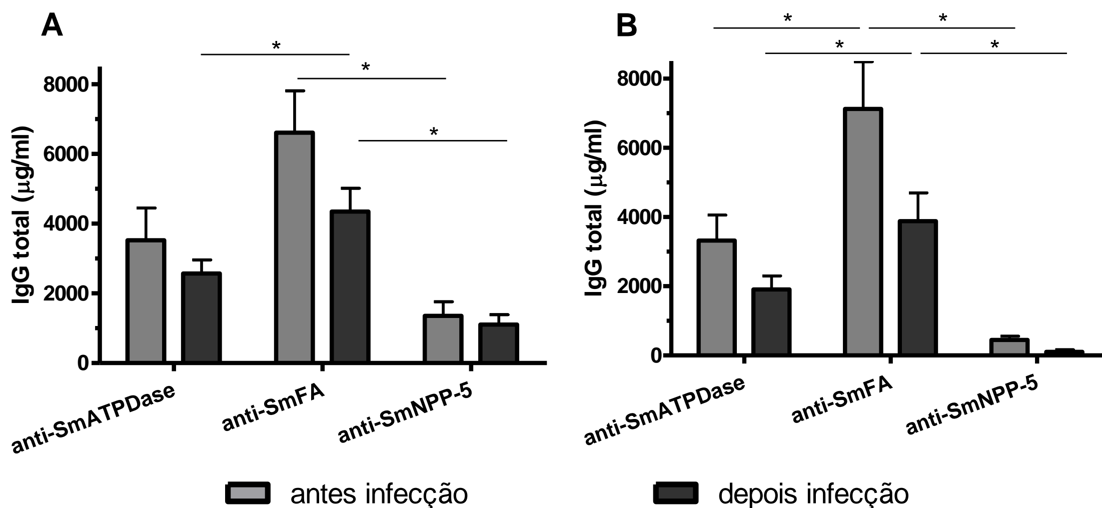
Como o parasita apresenta em seu tegumento três ectoncucleotidases: uma nucleotídeo pirofosfatase/ fosfodiesterase (SmNPP-5a), uma fosfatase alcalina (SmFA) e uma apirase (SmATPDase), decidiu-se avaliar o potencial protetor conjunto destas proteínas. Analisou-se grupos de camundongos imunizados com as proteínas recombinantes isoladamente, um grupo imunizado com as 3 proteínas conjuntamente, além do grupo controle imunizado apenas com o adjuvante.

4.13.1 Avaliação da resposta imune humoral induzida

Para avaliação da resposta imune humoral, o sangue dos animais imunizados foi coletado antes do desafio (dia 44) e antes da perfusão (dia 89), isto é, antes e depois da infecção por *S. mansoni,* respectivamente. Foram mensurados no soro dos animais os níveis de IgG total, de IgG 1 e de IgG2a específicos contra as proteínas recombinantes.

Todas as proteínas formuladas isoladas ou combinadas induziram níveis significativamente maiores de IgG total, quando comparados aos animais imunizados apenas com adjuvante (dados não mostrados). Apesar de haver diferenças antes e após a infecção por *S. mansoni* nos níveis de IgG total induzido em todos os grupos, não observamos diferenças estatisticamente significativas em nenhum deles (Figura 30).Também observou-se que a SmFA foi a proteína mais imunogênica, induzindo os maiores níveis de IgG total e que SmNPP-5a foi a proteína menos imunogênica (Figura 30).Verificamos ainda, de modo geral, níveis um pouco menores de anticorpos no grupo imunizado com as três proteínas conjuntamente, que nos grupos imunizados com as proteínas isoladas, mas essas diferenças também não foram estatisticamente significativas (Figura 30B).

Figura 30 – IgG total produzido pela imunização dos camundongos com proteínas recombinantes antes do desafio com cercárias de *S. mansoni* e antes da perfusão dos animais.

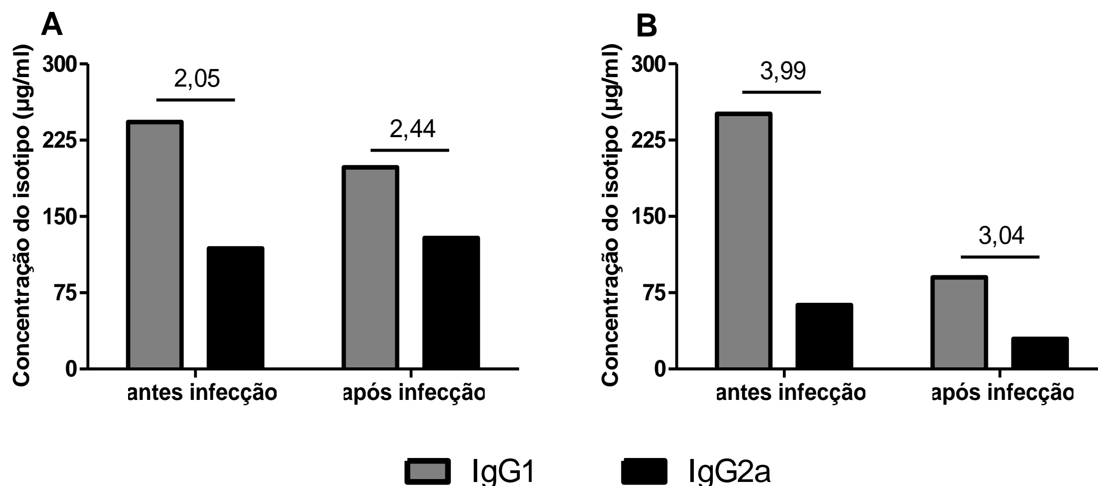


A – Níveis de IgG total específico antes e depois da infecção com S. mansoni induzidos pela imunização com as nucleotidases recombinantes isoladas. B – Níveis de IgG total específico antes e depois da infecção com S. mansoni induzidos pela imunização conjunta com as nucleotidases recombinantes. Média ± EP. * representa diferenças estatisticamente significativas.

Também foram avaliadas as concentrações dos isotipos IgG1 e IgG2a dos animais imunizados com as nucleotidases recombinantes. Nos gráficos não foram apresentandos os dados do grupo controle, pois neste grupo os anticorpos IgG1 e IgG2a não foram detectados.

Os animais imunizados apenas com a SmATPDase apresentaram antes da infecção aproximadamente 2 vezes mais IgG1 que IgG2a. Após a infecção com o parasita, a razão entre os dois isotipos de anticorpos apresentou um aumento sutil, devido a uma diminuição da concentração de IgG1 (Figura 31A). Nos animais imunizados conjuntamente com as três nucleotidases, observa-se que os níves de anticorpos IgG1 anti-SmATPDase foram comparáveis aos animais imunizados com a proteína isolada antes da infecção pelo parasita, enquanto a concentração de IgG2a anti-SmATPDase foi um pouco menor; por isso a razão entre os isotipos praticamente dobrou. Nestes animais após a infecção pelo parasita, tanto os níveis de IgG1 quanto os de IgG2a anti-SmATPDase diminuíram (Figura 31). Há de se ressaltar que, contrapondo-se aos dados de IgG total, verificou-se que os menores níveis de IgG1 e IgG2a foram induzidos pela imunização com a SmATPDase, ao invés da SmNPP-5a (Figuras 30, 31, 32 e 33).

Figura 31 – Quantificação dos níveis de IgG1 e IgG2a anti-SmATPDase antes e depois da infecção por *S. mansoni*.

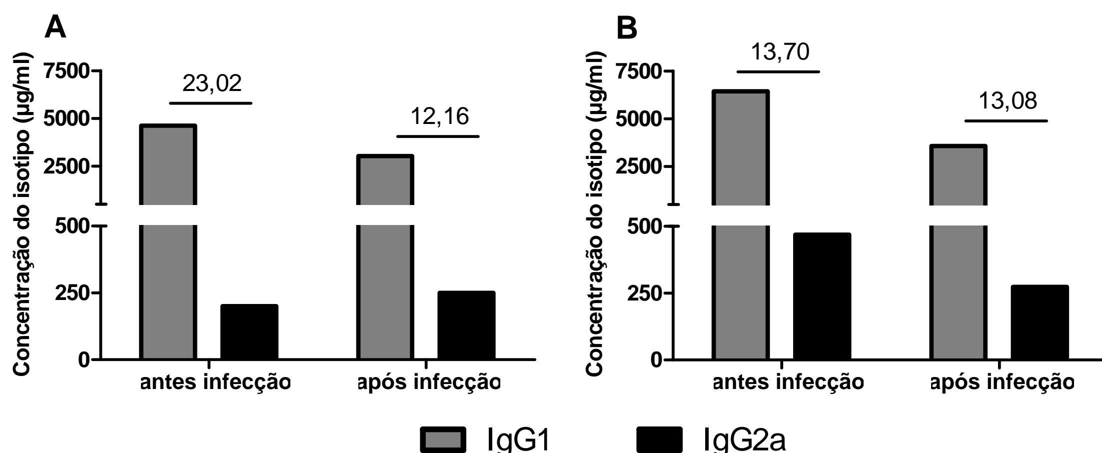


A – animais imunizados apenas com SmATPDase; B – animais imunizados com as três nucleotidases. Média ± EP; os números sobre as barras representam as razões IgG1/IgG2a.

Os animais imunizados apenas com a SmFA apresentaram níveis maiores de IgG1 ante os níveis de IgG2a anti-SmFA tanto antes quanto após a infecção pelo *S. mansoni.* Porém antes da infecção pelo parasita a razão entre os isotipos de anticorpos foi da ordem de 23 vezes, enquanto após a infecção essa razãodiminui para 12 vezes. Essa redução da razão entre

os isotipos dos anticorpos foi ocasionada pela redução de aproximadamente um quarto na concentração de IgG1 e por um aumento na mesma proporção no nível de IgG2a (Figura 32A). Os animais imunizados com as três nucleotidases combinadas apresentaram aproximadamente duas vezes mais IgG2a anti-SmFA antes da infecção por *S. mansoni*, se comparados aos animais imunizados apenas com a SmFA. Após a infecção, tanto os níveis de IgG1 quanto os de IgG2a anti-SmFA dos animais imunizados com as proteínas conjuntas diminuíram proporcionalmente, de modo que sua concentração atingiu patamares muito próximos aos dos animais imunizados com a proteína isolada. Essa redução porporcional também manteve a razão entre os dois isotipos de anticorpos bem próxima a observada antes da infecção (Figura 32). Destacamos ainda que em concordância com os dados de IgG total, os níveis de IgG1 e IgG2a anti-SmFA foram notavelmente maiores que os níveis de IgG1 e IgG2a anti-SmATPDase e anti-SmNPP-5a, tanto para os animais imunizados com as proteínas isoladas, quanto para os animais imunizados com as proteínas combinadas (Figuras 30, 31, 32 e 33).

Figura 32 – Quantificação dos níveis de IgG1 e IgG2a anti-SmFA antes e depois da infecção por *S. mansoni*.

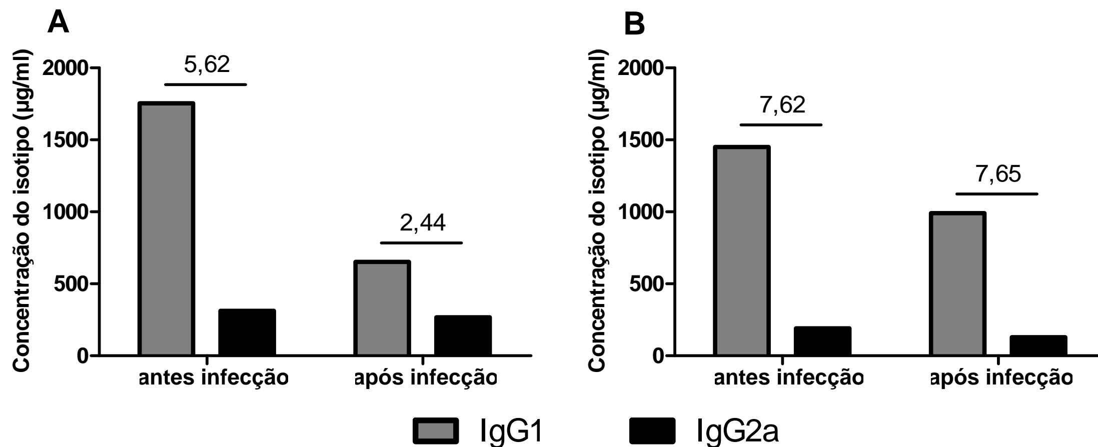


A – animais imunizados apenas com SmFA; B – animais imunizados com as três nucleotidases. Média ± EP; os números sobre as barras representam as razões IgG1/IgG2a.

Finalmente verificou-se que os animais imunizados apenas com a SmNPP-5a apresentaram 5,6 vezes mais IgG1 que IgG2a antes da infecção pelo parasita, após a infecção por *S. mansoni* a queda de IgG1 foi mais acentuada diminuindo a proporcção entre os isotipos para 2,4 vezes (Figura 33A). Quando comparado ao grupo imunizado com apenas a SmNPP-5a recombinante, os animais imunizados com as três nucleotidases combinadas apresentaram uma diminuição maior no nível de IgG2a anti-SmNPP-5a antes da infeção pelo parasita. Após

a infecção, houve uma redução proporcional das concentrações de IgG1 e IgG2a anti SmNPP-5a, mantendo a razão IgG1:IgG2a constante (Figura 33B).

Figura 33 – Quantificação dos níveis de IgG1 e IgG2a anti-SmNPP-5a antes e depois da infecção por *S. mansoni.*



A – animais imunizados com SmNPP-5a; B – animais imunizados com as três nucleotidases. Média ± EP; os números sobre as barras representam as razões IgG1/IgG2a.

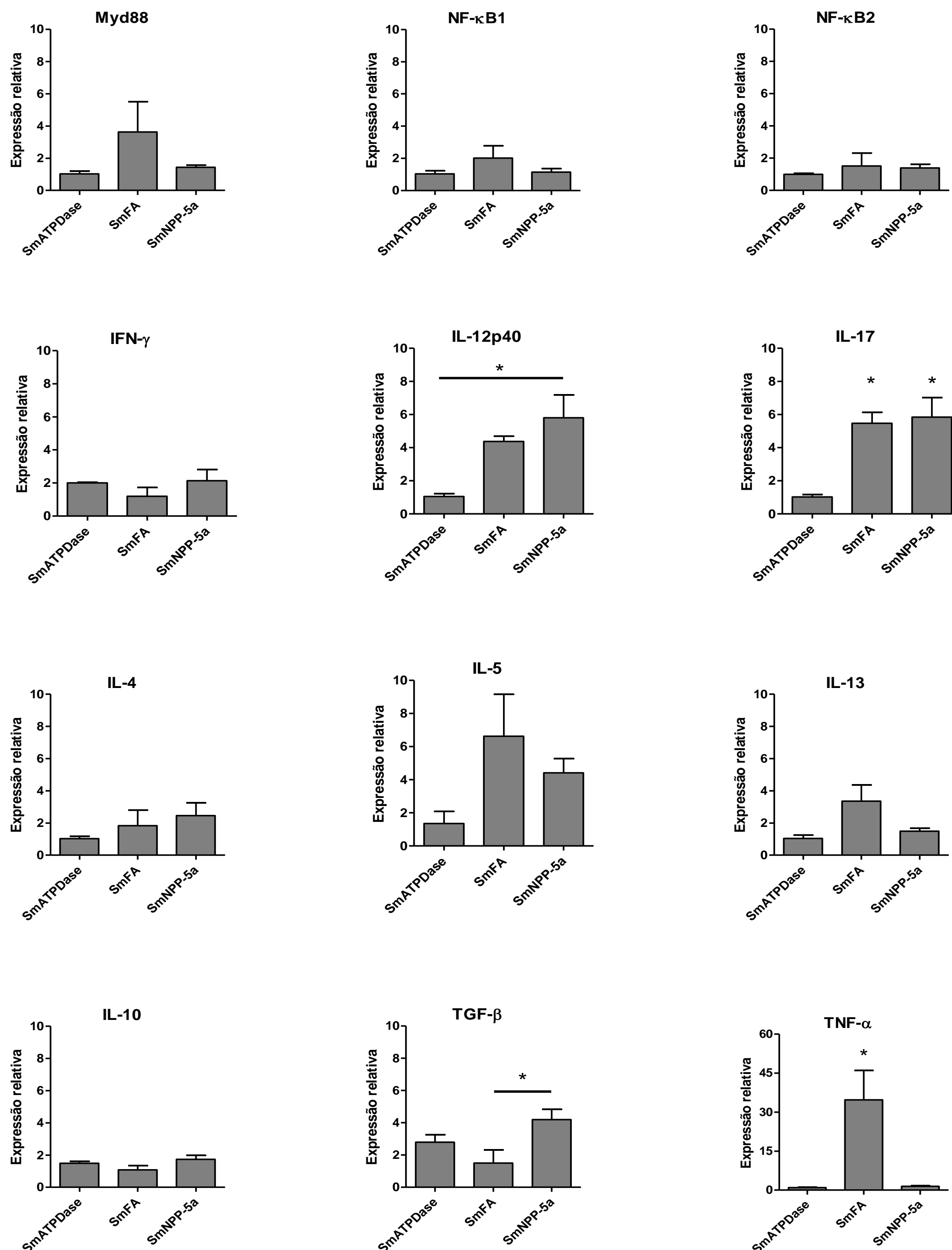
4.13.2 Avaliação da resposta imune celular induzida

Para avaliação da resposta imune celular, esplenócitos provenientes dos baços dos animais imunizados com as nucleotidases recombinantes isoladas ou combinadas foram re-estimulados *in vitro* com as respectivas proteínas para análise da expressão relativa das citocinas IFN-γ, TNF-α, TGF-β IL-4, IL-5, IL-10, IL-12p40, IL-13 e IL-17, do adaptador Myd88 e dos fatores de transcrição NF-κB1 e NF-κB2.

Os esplenócitos dos animais imunizados com a SmNPP-5a e re-estimulados com a mesma *in vitro* apresentaram uma expressão 6 vezes maior de IL-12p40 e de IL-17, quando comparados aos esplenócitos dos animais imunizados com a SmATPDase. Esse grupo também apresentou uma expressão 4 vezes maior de TGF-β, quando comparado aos esplenócitos dos animais imunizados com a SmFA. Por outro lado, o grupo dos animais imunizados com a SmFA apresentou o mesmo nível de expressão de IL-17 do grupo imunizado com SmNPP-5a, seis vezes maior que o nível de expressão dos animais imunizados com a SmATPDase. Os animais imunizados com a SmFA apresentaram ainda uma expressão 35 vezes maior de TNF-α, quando comparados tanto ao grupo imunizado com a SmATPDase quanto ao grupo imunizado com a SmNPP-5a (Figura 34). Não foram verificadas diferenças significativas nos níveis de expressão de Myd88, NF-κB1, NF-κB2,

IFN-γ, IL-4, IL-5, IL-10 e IL-13 pelos esplenócitos dos animais imunizados com as proteínas recombinantes isoladas e re-estimulados *in vitro* com as respectivas proteínas.

Figura 34 – Avaliação da expressão gênica relativa do adaptador Myd88, dos fatores de transcrição NFκB1, NFκB2 e das citocinas IFN-γ, TNF-α, TGF-β IL-4, IL-5, IL-10, IL-12p40, IL-13 e IL-17 nos esplenócitos de animais imunizados com as nucleotidases recombinantes isoladas e re-estimulados *in vitro* com a respectiva proteína.



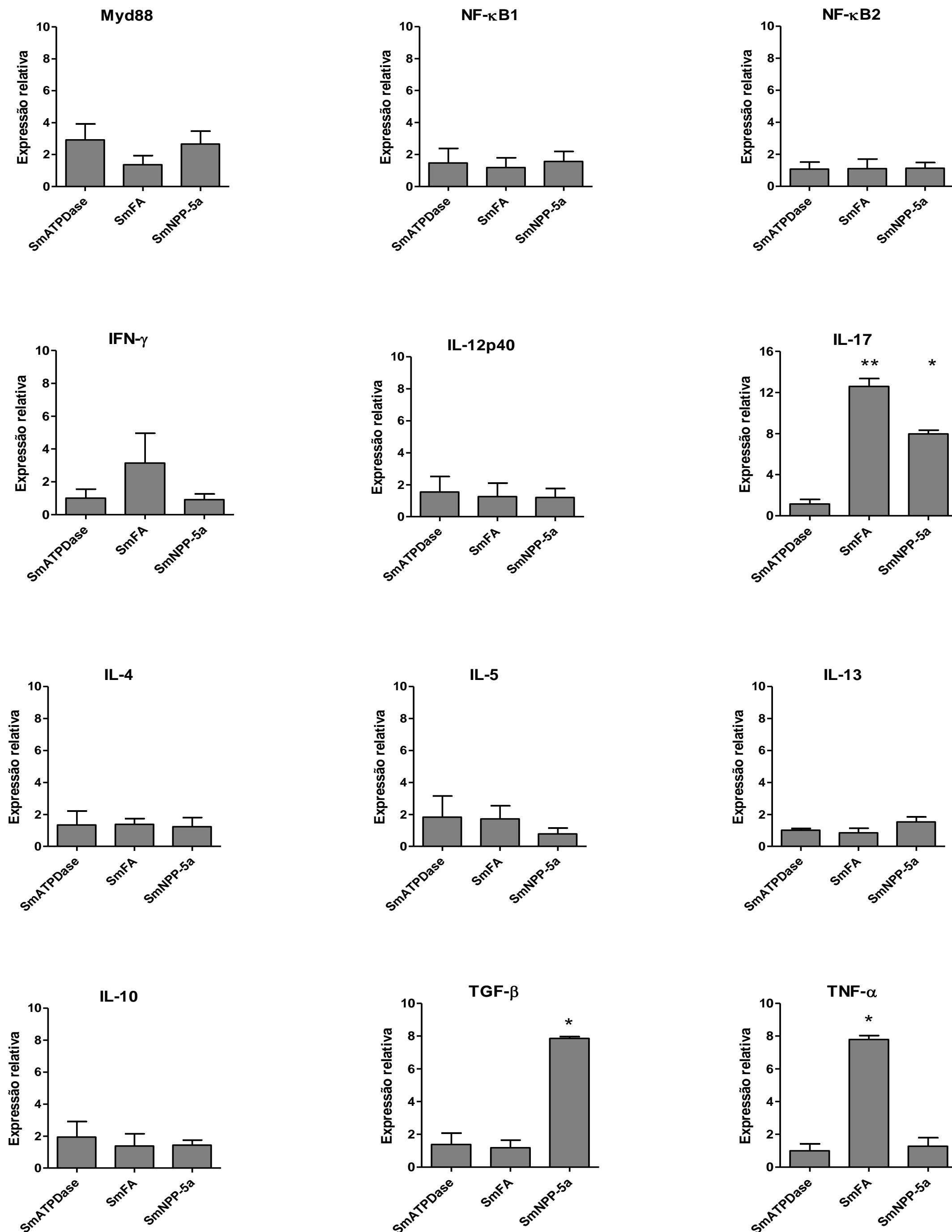
Média ± EP. * representa diferenças estatisticamente significativas.

Também avaliamos a expressão relativa das citocinas IFN-γ, TNF-α, TGF-β IL-4, IL-5, IL-10, IL-12p40, IL-13 e IL-17, do adaptador Myd88 e dos fatores de transcrição NFκB1 e NFκB2 pelos esplenócitos dos animais imunizados com as três nucleotidases simultaneamente, mas re-estimulados *in vitro* com apenas uma das proteínas de cada vez. Não foram verificadas diferenças significativas nos níveis de expressão de Myd88, NF-κB1, NF-κB2, IFN-γ, IL-4, IL-5, IL-10, IL-12p40 e IL-13 pelos esplenócitos re-estimulados com as diferentes proteínas.

Os esplenócitos reestimulados *in vitro* com a SmNPP-5a apresentaram expressão 8 vezes maior de TGF-β, quando comparado aos esplenócitos re-estimulados *in vitro* com a SmATPDase ou com SmFA. Enquanto os esplenócitos re-estimulados *in vitro* com a SmFA apresentaram expressão 8 vezes maior de TNF-α, quando comparado aos esplenócitos re-estimulados *in vitro* com a SmATPDase ou com SmNPP-5a. A re-estimulação *in vitro* dos esplenócitos com a SmNPP-5a e da SmFA induziram ainda uma maior expressão de IL-17, de 8 e 12 vezes respectivamente, quando comparados aos esplenócitos re-estimulados *in vitro* com a SmATPDase (Figura 35).

Os níveis de expressão gênica relativa do adaptador Myd88, dos fatores de transcrição e das citocinas, excetuando-se a IL-12p40, nos esplenócitos dos camundongos apresentaram uma manutenção de seu padrão dependente da proteína utilizada para realizar a re-estimulação *in vitro.* Porém não verificamos grandes influências na expressão relativa desses genes devido a ter-se utilizado as proteínas recombinantes isoladas ou em conjunto para imunizar os animais.

Figura 35 – Avaliação da expressão gênica relativa do adaptador Myd88, dos fatores de transcrição NFκB1, NFκB2 e das citocinas IFN-γ, TNF-α, TGF-β IL-4, IL-5, IL-10, IL-12p40, IL-13 e IL-17 nos esplenócitos de animais imunizados com as três nucleotidases recombinantes simultaneamente e re-estimulados *in vitro* com apenas uma das três proteínas.

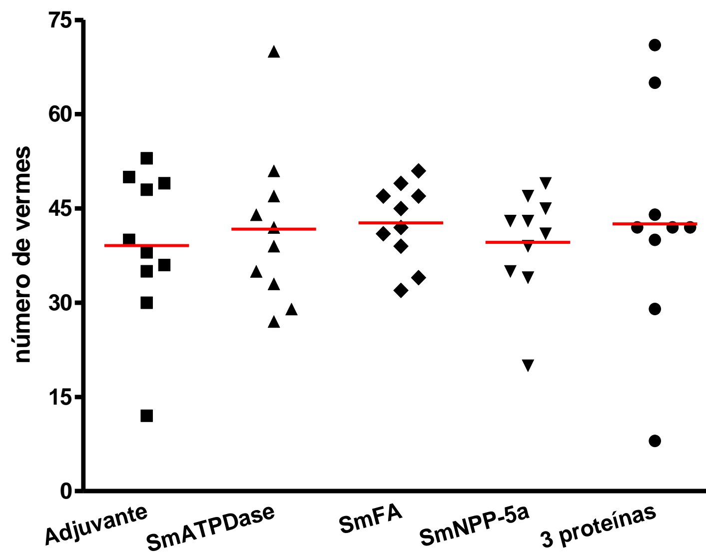


Média ± EP. * e ** representam diferenças estatisticamente significativas.

4.13.3 Avaliação da carga parasitária e oviposição

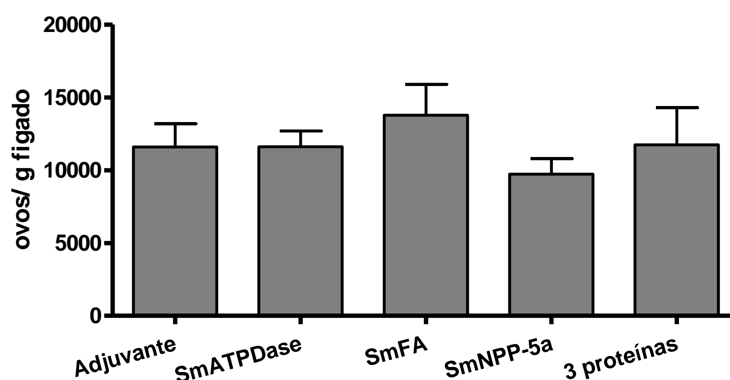
Após a perfusão dos camundongos imunizados com as proteínas recombinantes e desafiados com *S. mansoni* para retirada dos parasitas presentes nos vasos mesentéricos, a carga parasitária foi averiguada para avaliação do potencial protetor das nucleotidases do tegumento. Não foi verificada redução da carga parasitária, mesmo no grupo imunizado com as três proteínas juntas (Figura 36 – representativo de dois experimentos).

Figura 36 – Dispersão e média (linha) do número de vermes recuperados em camundongos C57BL/6 imunizados com as proteínas recombinantes.



Também foi retirado um pedaço do fígado dos camundongos imunizados e desafiados para quantificar a oviposição dos parasitas. Assim como observado para a carga parasitária também não foi observada nenhuma redução significativa na fecundidade dos parasitas (Figura 37 – representativo de dois experimentos).

Figura 37 – Número de ovos normalizados pelo peso do fígado e pelo número de vermes recuperados de camundongos imunizados e desafiados.

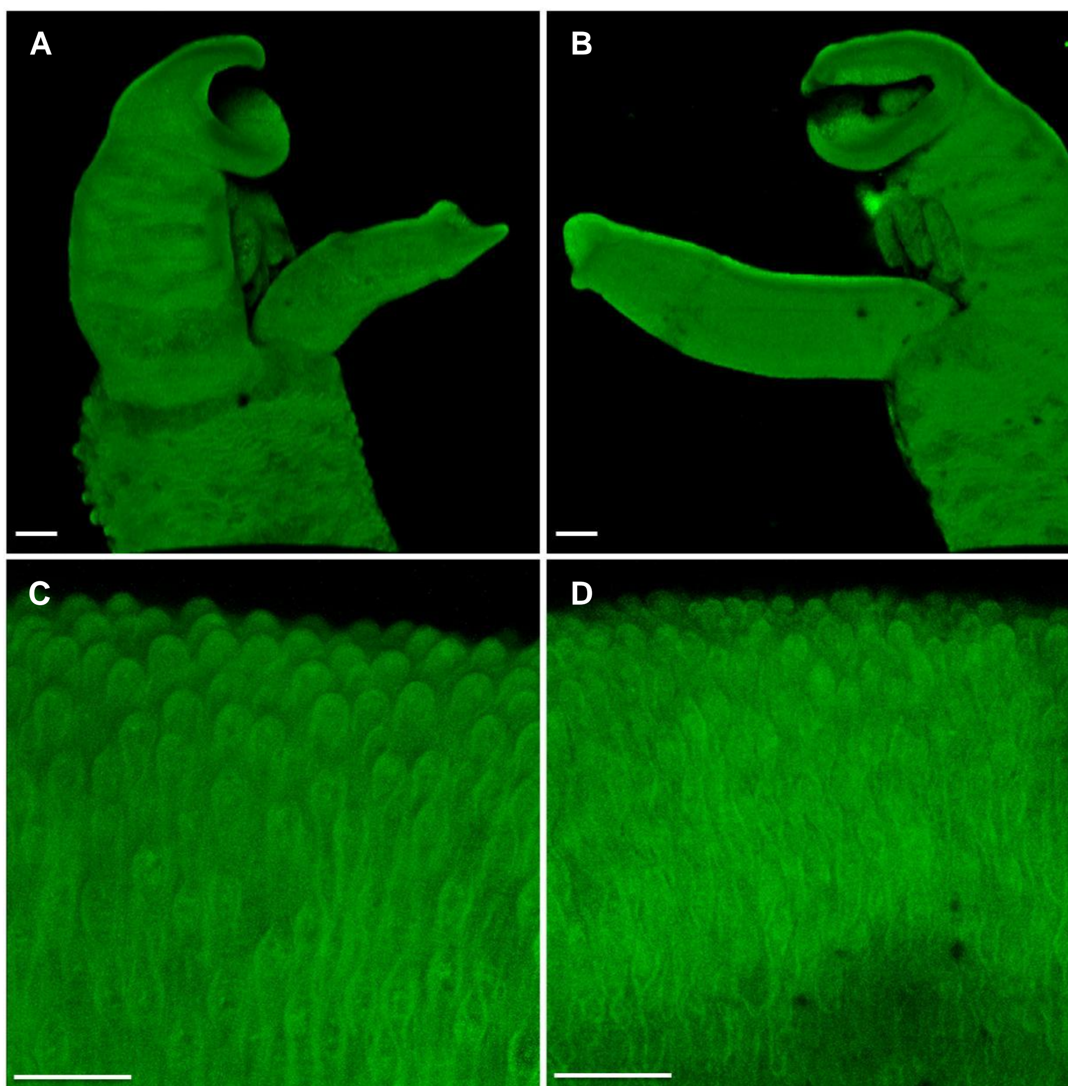


Média ± EP.

4.14 Avaliação da quimioterapia subcurativa *in vivo*

Buscando avaliar os efeitos do tratamento subcurativo com praziquantel, camundongos foram infectados com cercárias e receberam por *gavage* duas doses da droga. Alguns animais foram sacrificados pouco tempo após a segunda administração do fármaco, seus parasitas foram coletados, fixados e avaliados por microscopia confocal a laser. Não foram verificadas significativas alterações morfológicas nos parasitas do grupo de animais tratados com o fármaco, em especial nas ventosas, quando comparados aos do grupo controle (Figura 38). Visando aprimorar essa avaliação, os tubérculos do tegumento de vermes adultos machos foram contados, mas também não foram observadas variações estatisticamente significativas (Figuras 38C e D e 39B).

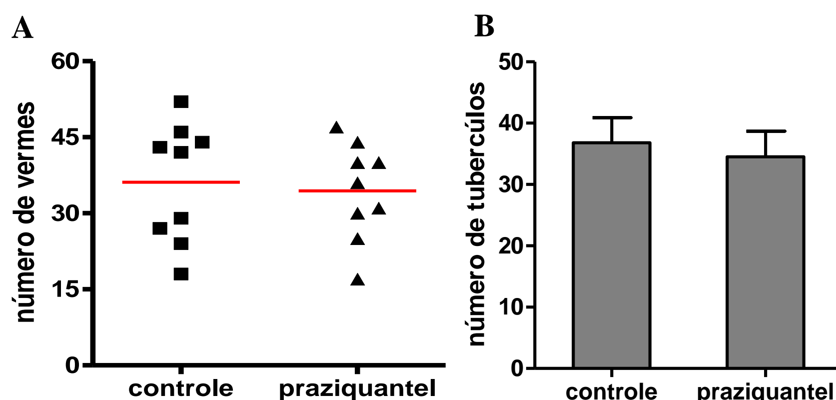
Figura 38 – Avaliação morfológica dos parasitas submetidos à quimioterapia subcurativa com praziquantel.



A e B – parte anterior de casal de vermes adultos não tratados e tratados com praziquantel, respectivamente. C e D – Tubérculos do tegumento de vermes adultos machos não tratados e tratados com praziquantel, respectivamente. Barras = 50 µm.

Outros grupos de animais foram perfundido apenas 50 dias após o desafio para análise da carga parasitária, a qual não apresentou diferenças significativas entre o grupo tratado com praziquantel e o grupo controle (Figura 39A).

Figura 39 – Avaliação da quimioterapia subcurativa com praziquantel.



A – Dispersão e média (linha) da carga parasitária de camundongos C57BL/6 tratados praziquantel após a infecção com cercárias. B – Média ± DP do número de tubéculos do tegumento de vermes adultos machos em 20.000 μ^2.

4.15 Efeito sinérgico da imunização com as nucleotidases associada à quimioterapia subcurativa com praziquantel

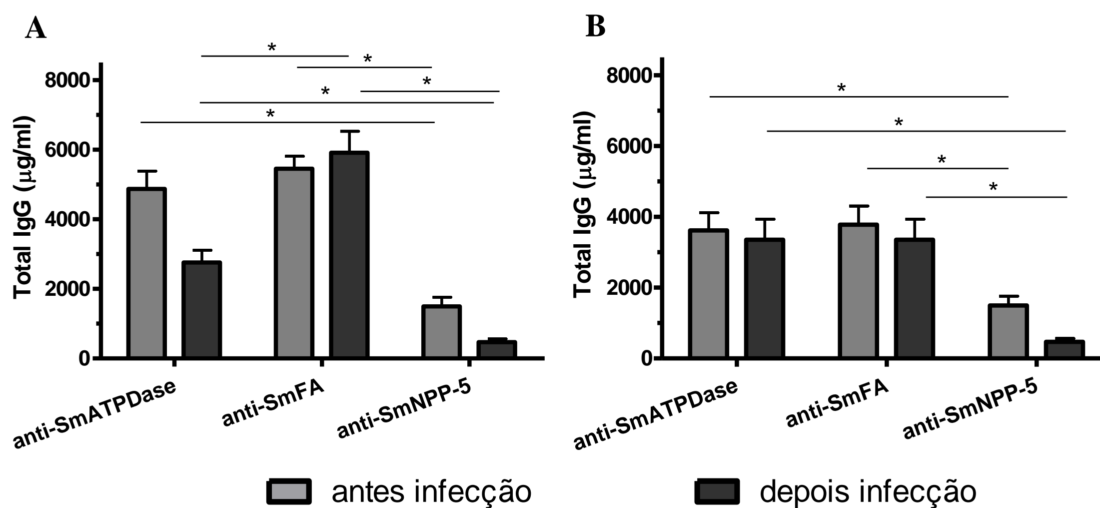
4.15.1 Avaliação da resposta imune humoral induzida

No ensaio de vacinação associado ao tratamento subcurativo com praziquantel também foi avaliada a resposta imune humoral dos camundongos. Desse modo coletou-se o soro dos animais antes do desafio (dia 44) e antes da perfusão (dia 94), isto é, antes da infecção por *S. mansoni* e após a infecção e ao tratamento quimioterápico, respectivamente. Foram medidos os níveis séricos de IgG total e de IgG 1 e IgG2a específicos contra as proteínas recombinantes.

Assim como nos ensaios de vacinação sem o tratamento subcurativo com praziquantel, todas as proteínas recombinantes, independente se administradas isoladas ou combinadas, induziram níveis significativamente maiores de IgG total, quando comparado aos animais imunizados apenas com adjuvante (dados não mostrados). Neste ensaio também verificamos diferenças antes e após a infecção por *S. mansoni* nos níveis de IgG total induzido em todos os grupos, porém sem diferenças estatisticamente significativas em nenhum deles. Neste ensaio a imunogenicidade da SmFA não foi tão acentuada em relação a SmATPDase, porém os níveis de IgG total induzidos por estas duas proteínas foram superiores ao induzidos pela SmNPP-5a (Figura 40). Observou-se ainda uma indução ligeiramente menor de anticorpos para as duas proteínas mais imunogênicas no grupo imunizado com as três proteínas conjuntamente, em relação aos grupos imunizados com as proteínas isoladas, mas essas

diferenças também não foram estatisticamente significativas. Além disso, houve pouca diferença na concentração de anticorpos anti-SmATPDase e anti-SmFA antes e aopós a infecção pelo parasita (Figura 41B).

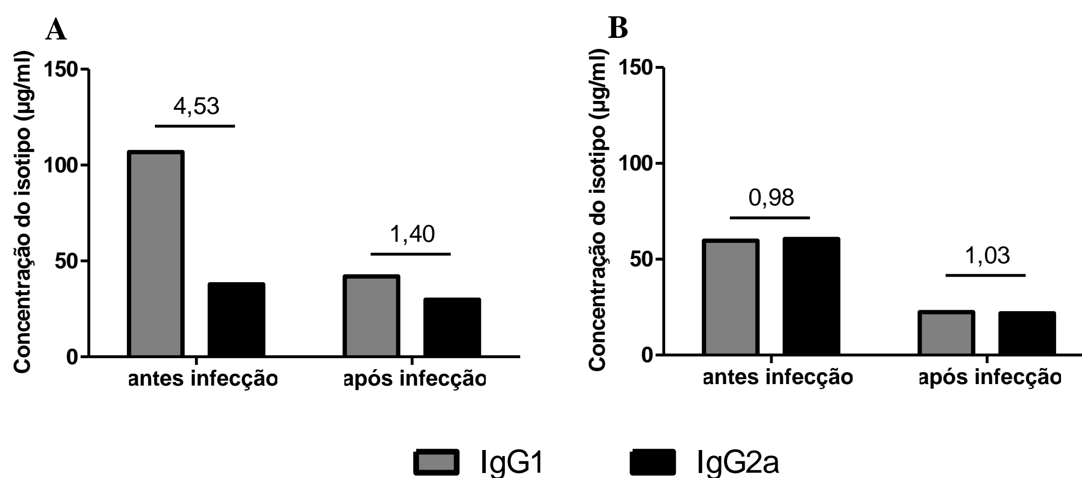
Figura 40 – IgG total produzido pela imunização dos camundongos com proteínas recombinantes antes do desafio com cercárias de *S. mansoni* e posterior à infecção com o parasita e ao tratamento subcurativo com praziquantel.



A – Níveis de IgG total específico induzidos pela imunização com as nucleotidases recombinantes isoladas B – Níveis de IgG total específico induzidos pela imunização conjunta com as nucleotidases recombinantes antes da infecção com *S. mansoni* e depois da infecção e do tratamento com praziquantel. Média ± EP. * representa diferenças estatisticamente significativas.

Assim como feito anteriormente, as concentrações dos isotipos IgG1 e IgG2a dos animais imunizados também foram avaliadas. Os animais imunizados com SmATPDase apresentaram antes da infecção aproximadamente 4 vezes mais IgG1 que IgG2a, porém após a infecção o nível de IgG1 caiu a patamares muito próximos do nível de IgG2a (Figura 41A). Nos animais imunizados conjuntamente com as três nucleotidases os níves de anticorpos IgG1 e IgG2a anti-SmATPDase foram semelhantes aos de antes da infecção, apesar dos níveis de anticorpos continuarem semelhantes entre si após a infecção, eles apresentam um redução da ordem de 3 vezes (Figura 41 B). Contrapondo-se ao dado de IgG total, verificou-se que os menores níveis de IgG1 e IgG2a foram induzidos pela imunização com aSmATPDase (Figuras 40, 41, 42 e 43). Este fenômeno já havia sido observado nos ensaios de imunização sem o tratamento com praziquantel (Figura 31).

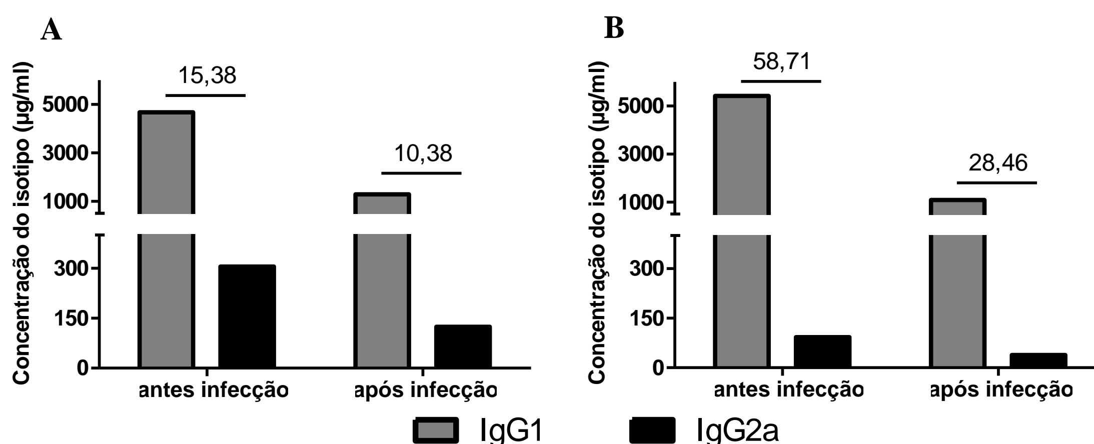
Figura 41 – Quantificação dos níveis de IgG1 e IgG2a anti-SmATPDase antes do desafio com cercárias de *S. mansoni* e posterior à infecção com o parasita e ao tratamento subcurativo com praziquantel.



A – animais imunizados apenas com SmATPDase; B – animais imunizados com as três nucleotidases. Média ± EP; os números sobre as barras representam as razões IgG1/IgG2a.

Os animais imunizados apenas com a SmFA apresentaram níveis maiores de IgG1 que de IgG2a antes e depois da infecção pelo *S. mansoni,* da ordem de 15 e 10 vezes respectivamente. Os níveis de IgG1 apresentaram um redução da ordem de 3,5 vezes após a infecção enquanto os níveis de IgG2a reduziram apenas 2,5 vezes (Figura 42A). Os animais imunizados com as três proteínas apresentaram menores níveis de IgG2a anti-SmFA quando comparados aos animais imunizados apenas com a SmFA, elevando a relação de IgG1:IgG2a para 58 antes da infecção. Após a infecção essa razão passou para 25, devido principalmente a uma diminuição maior dos níveis de IgG1 que a dos níveis de IgG2a (Figura 42B). Como já havia sido reportado nos ensaios sem a quimioterapia com praziquantel os níveis de IgG1 e IgG2a anti-SmFA foram notavelmente maiores que os níveis de IgG1 e IgG2a anti-SmATPDase e anti-SmNPP-5a, em concordância com os dados de IgG total (Figuras 40, e 41, 42, 43).

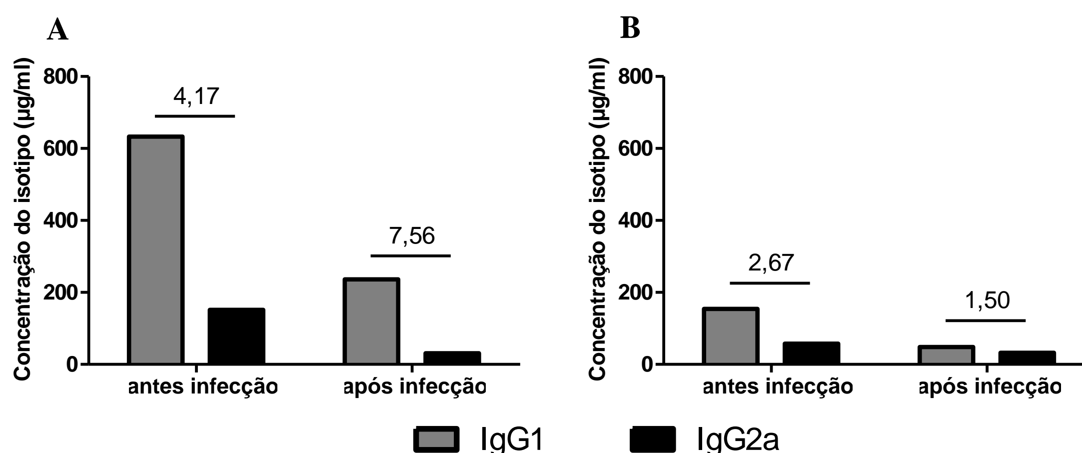
Figura 42 – Quantificação dos níveis de IgG1 e IgG2a anti-SmFA antes do desafio com cercárias de *S. mansoni* e posterior à infecção com o parasita e ao tratamento subcurativo com praziquantel.



A – animais imunizados apenas com SmFA; B – animais imunizados com as três nucleotidases. Média ± EP; os números sobre as barras representam as razões IgG1/IgG2a.

Os animais imunizados apenas com a SmNPP-5a apresentaram 4 vezes mais IgG1 que IgG2a antes da infecção pelo parasita, após a infecção por *S. mansoni* a queda de IgG2a foi mais acentuada aumentando a diferenças entre os isotipos para 7,5 vezes (Figura 43A). Quando comparado ao grupo imunizado apenas com a SmNPP-5a recombinante, os animais imunizados com as três nucleotidases apresentaram uma queda significativa no nível de IgG1 anti-SmNPP-5a. Nesse grupo a razão IgG1:IgG2a antes da infecção era 3, sendo que após a infecção essa razão passou a ser 1,5 , devido a uma maior diminuição dos níveis de IgG1 (Figura 43B).

Figura 43 – Quantificação dos níveis de IgG1 e IgG2a anti-SmNPP- antes do desafio com cercárias de *S. mansoni* e posterior à infecção com o parasita e ao tratamento subcurativo com praziquantel.



A – animais imunizados com SmNPP-5a; B – animais imunizados com as três nucleotidases. Média ± EP; os números sobre as barras representam as razões IgG1/IgG2a.

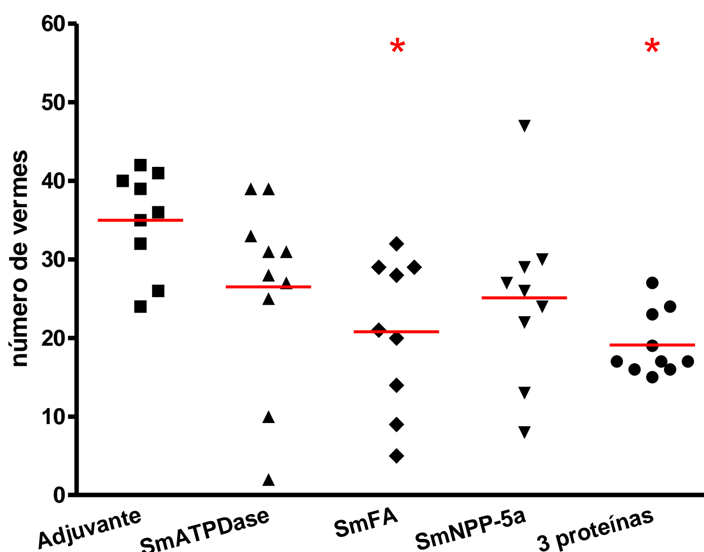
4.15.2 Avaliação da carga parasitária e oviposição

Uma vez que nenhum dos experimentos com o regime padrão de vacinação apresentou resultados promissores, decidimos avaliar se as imunizações com as proteínas recombinantes associadas ao tratamento com as doses subcurativas de praziquantel eram capazes de reduzir a carga parasitária dos animais. Os animais foram imunizados três vezes com as proteínas recombinantes com intervalos de 15 dias entre elas, usando CFA/IFA como adjuvante e 15 dias após a última imunização os animais foram desafiados com 100 cercárias por penetração pela pele. Foram administradas duas doses de praziquantel a todos os animais, 35 e 37 dias após a infecção. A perfusão dos camundongos infectados para retirada dos parasitas presentes nos vasos mesentéricos foi feita 50 dias após a infecção (Figura 8). Os parasitas recuperados foram contados para análise da carga parasitária e um pedaço do fígado foi digerido para contagem do número de ovos.

Os animais imunizados com a SmATPDase isolada apresentaram uma redução de 26% da carga parasitária quando comparados ao grupo controle, enquanto nos imunizados apenas com a SmNPP-5a verificou-se uma redução de 28%, porém essas diferenças não foram estatisticamente significativas. Os animais imunizados com a SmFA isolada apresentaram uma redução estatisticamente significativa da carga parasitária de 41% quando comparados aos do grupo controle. A maior redução da carga parasitária foi observada nos animais imunizados com as 3 nucleotidases simultaneamente, 46% de diminuição no número de

parasitas. A diferença entre os animais imunizados apenas com SmFA e os imunizados com as três proteínas combinadas não foi estatisticamente significativa (Figura 44).

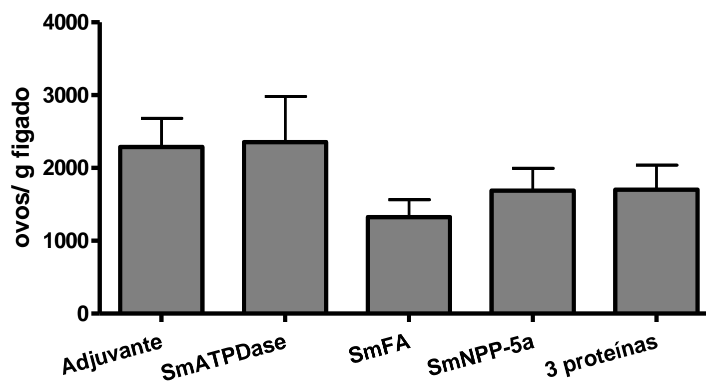
Figura 44 – Dispersão e média (linha) do número de vermes recuperados em camundongos C57BL/6 imunizados com as proteínas recombinantes e tratados com dose subcurativa de praziquantel.



* representa diferenças estatisticamente significativas.

Quando analisamos o número de ovos normalizados pelo peso do fígado dos camundongos imunizados, desafiados e tratados com o praziquantel verificamos uma redução da oviposição de 26% no grupo imunizado apenas com SmNPP-5a e nos animais imunizados simultaneamente com as 3 nucletidases e de 42% no grupo imunizado com a SmFA isolada, quando comparados com o grupo controle. Porém essas diferenças não foram estatisticamente significativas devido a grande variabilidade dos dados (Figura 45).

Figura 45 – Número de ovos normalizados pelo peso do fígado e pelo número de vermes recuperados de camundongos imunizados, tratados com dose subcurativa de praziquantel e desafiados com *S. mansoni*.



Média ± EP. * representa diferenças estatisticamente significativas.

5 DISCUSSÃO

5.1 Caracterização das SmNPPs

A publicação do transcriptoma e do genoma de *S. mansoni* abriu uma nova perspectiva para seleção de candidatos vacinais. No entanto, como o *S. mansoni* é um metazoário complexo, a seleção de candidatos vacinais baseada exclusivamente em análises *in silico* não se demonstrou muito produtiva. Assim é necessário que as proteínas, que apresentam potencial como antígenos vacinais, sejam caracterizadas por uma abordagem pós-genômica e sua avaliação experimental continua imprescindível.

O antígeno vacinal hipotético ideal deve codificar uma proteína imunogênica, não causar reações cruzadas com proteínas do hospedeiro e ser importante para o estabelecimento da infecção assim como para a sobrevivência do parasita (HOTA-MITCHELL et al., 1999). Assim, um bom candidato vacinal precisa ser expresso nos estágios que infectam o hospedeiro definitivo e adicionalmente precisa estar acessível ao sistema imune para que esse possa ser efetivo no combate ao parasita (MOUNTFORD; HARROP, 1998; WILSON et al., 2004). Como as duas maiores interfaces parasita-hospedeiro são o tegumento e o tubo digestivo do parasita, estes constituem as maiores fontes de potenciais antígenos (LOUKAS; TRAN; PEARSON, 2007). Desse modo, selecionamos para este estudo uma família de proteínas, as nucleotídeos pirofosfatases/fosfodiesterases, da qual um representante (SmNPP-5a) já havia sido identificado como presente no tegumento (BRASCHI et al., 2006; BRASCHI; WILSON, 2006; VAN BALKOM et al., 2005) e outro (SmNPP-5b) cuja expressão gênica havia sido associada a infecção do hospedeiro definitivo (DILLON et al., 2006).

Analisando o genoma do *S. mansoni,* encontramos quatro genes que codificam para as NPPs, entre os quais um deles apresenta um possível *splicing* alternativo. Esses genes foram nomeados com base em sua similaridade e identidade aos respectivos ortólogos humanos. Quando foi analisado o genoma do *S. japonicum,* também foram encontrados quatro genes ortólogos aos de *S. mansoni*. Através do alinhamento múltiplo das sequências protéicas preditas das SmNPPs com seus ortólogos humanos, verificou-se que sítios putatvos de ligação a metal estão conservados, assim como o possível centro catalítico. Baseados em suas sequências primárias, fizemos a predição da estrutura molecular das SmNPPs e verificamos que assim como as HsNPP-5 e 6, a SmNPP-5a.2 e a SmNPP-6 são proteínas transmembranas do tipo I, enquanto a SmNPP-5b apresenta dois domínios transmembrana, um N e outro C

terminal. A SmNPP-5a.1 e a SmNPP-5c não apresentaram domínio transmembrana, sugerindo a possibilidade dessas proteínas serem secretadas para o meio extracelular, tal como ocorre com a HsNPP-2 (STEFAN; JANSEN; BOLLEN, 2005).

Após a identificação dos genes, foi realizada a caracterização do perfil de expressão transcricional das *smnpps.* Verificamos que dois deles apresentam maior expressão em esquistossômulos e vermes adultos, *smnpp-5a* e *smnpp-5b*, enquanto as outras duas, *smnpp-5c* e *smnpp-6* apresentam máxima expressão em ovos e miracídios. Essa análise confirmou a identificação da *smnpp-5b* em uma análise de expressão gênica global, como um gene cuja expressão aumenta durante a infecção do hospedeiro definitivo (DILLON et al., 2006). Porém o dado mais notável desse resultado foi a ordem de grandeza do aumento da expressão da *smnpp-5a* após a infecção do hospedeiro definitivo, de aproximadamente 100 vezes. Ressaltando a importância da proteína na adaptação do parasita ao novo ambiente, assim como uma provável localização em estruturas específicas fundamentais para a sobrevivência do parasita em seu novo hábitat. O mesmo ocorre quando analisamos o perfil de expressão relacionado ao gênero dos vermes adultos; a *smnpp-5a* é quase 10 vezes mais expressa em machos que em fêmeas, confirmando uma análise de expressão gênica por *microarray* que avaliou a expressão de genes associados ao gênero em *Schistosoma mansoni* (FITZPATRICK et al., 2005).

Como a correlação entre a abundância de transcritos nem sempre possue uma correlação direta a uma grande expressão proteica devido a diversos processos metabólicos e regulatórios da expressão gênica (HOKKE; FITZPATRICK; HOFFMANN, 2007), averiguamos se o perfil de expressão transcricional corresponderia ao perfil de expressão das proteínas SmNPPs. Porém esta análise só foi possível para SmNPP-5a, uma vez que os anticorpos específicos produzidos para reconhecer as outras SmNPPs não foram capazes de reconhecer as proteínas nativas ou elas são expressas em níveis ínfimos, abaixo de nosso limite de detecção. O perfil de expressão proteica da SmNPP-5a confirmou os padrões de expressão apresentados pela analise transcricional; observou-se um aumento da expressão proteica na tranformação de cercária infectante para esquistossômulos que amadurecem em vermes adultos, com o ápice da expressão sendo verificada neste último estágio, em especial em machos.

Também averiguamos por *immunoblotting* e por imunolocalização que a SmNPP-5a é mais abundante no tegumento que em outras partes do parasita, confirmando os dados de proteoma de van Balkom et al. (2005). Verificamos ainda que a SmNPP-5a está majoritariamente associada as membranas de superfície do tegumento de vermes adultos e

não a sua fração sincicial, ratificando os dados de Braschi e Wilson (2006) e de Braschi et al. (2006) e reforçando a predição da enzima como uma proteína transmembrana do tipo I. Tanto o perfil de expressão quanto a abundância proteica no tegumento sugerem que a expressão do gene *smnpp-5a* é ativado durante ou imediatamente após o processo de tranformação cercariano. Nesse processo o parasita deixa de ser sensível ao ataque do sistema imune devido à substituição e modificação das membranas da superfície de seu tegumento sincicial e torna-se adaptado para sobreviver na corrente sanguínea (SKELLY; SHOEMAKER, 2000). Corroborando nossa hipótese sobre a importância da SmNPP-5a para adapatação e sobrevivência do parasita no hospedeiro definitivo, Bhardwaj et al. (2011) demonstraram que a inibição de sua expressão no momento da infecção do hospedeiro definitivo comprometia a capacidade do parasita em se estabelecer em seu novo hábitat e, por isso, classificaram-na como um fator de virulência do Schistosoma.

Quando tratamos as proteínas nativas do extrato de tegumento de vermes adultos com uma deglicosidade F, observamos por *immunoblotting* uma mudança na mobilidade da SmNPP-5a no SDS-PAGE. Esse dado confirmou que essa enzima é uma glicoproteína, conforme havia sido predito pela ferramenta de bioinformática, assim como seu ortólogo de rato (OHE et al., 2003). Esse resultado sugere que a atividade enzimática fosfodiesterase detectada por Pujol e Cesari (1993) em frações de membranas superficiais de vermes adultos deve-se a SmNPP-5a, pois nestes experimentos os pesquisadores utilizaram pérolas revestidas com lectinas para isolar componentes glicosilados. Nós detectamos a atividade enzimática da SmNPP-5a em parasitas vivos adultos e vermes com 21 dias, porém não em cercárias, corroborando os dados do padrão de expressão transcricional e proteico. Esse dado ratifica nossa hipótese que a expressão da SmNPP-5a é de alguma forma associada a formação e manutenção do tegumento após a transformação cercariana e que sua função é relacionada a adaptação e sobrevivência do parasita na corrente sanguínea.

A detecção da atividade NPP e sua inibição parcial mediada por anticorpos em parasitas vivos corrobora a exposição e a acessibilidade da SmNPP-5a na superfície do tegumento. A localização dessa proteína no tegumento foi inferida como sendo no folheto externo da membrana plasmática do tegumento, abaixo do membranocálice (BRASCHI et al., 2006). Somando nossos dados a esse modelo, possuímos uma forte evidência da presença de poros no membranocálice, que permitiriam a passagem do substrato e dos anticorpos, cuja existência é discutida na literatura (BRASCHI; BORGES; WILSON, 2006; BRASCHI et al., 2006; BRASCHI; WILSON, 2006). A descrição de que pessoas infectadas com *S. mansoni* apresentam anticorpos circulantes capazes de inibir parcialmente a atividade fosfodieterase

em extratos proteicos de vermes adultos (CESARI et al., 1992; PUJOL; CESARI, 1990) também reforça essa hipótese. No entanto, não foi determinado o mecanismo de inibição da atividade enzimática mediada pelos anticorpos. Presume-se que possa ser pela competição entre o substrato e o anticorpo para se ligar à região do centro catalítico da enzima ou devido a mudanças conformacionais da proteína desencadeadas pela sua ligação com o anticorpo, que afetariam a interação entre a enzima e o substrato.

Uma vez que não foi possível utilizar os anticorpos específicos para realizar uma melhor caracterização de todas as SmNPPs, foi utilizada a técnica de hibridização *in situ* em espécimes inteiros como uma alternativa para se averiguar em quais estruturas do parasita as SmNPPs eram expressas. Surpreendentemente, todos os genes estão expressos em órgãos ligados ao sistema reprodutor masculino e feminino. Inclusive a SmNPP-5a, cuja associação ao tegumento já foi demonstrada pelos nossos dados assim como na extensa literatura citada, para a qual esperávamos observar um padrão de expressão gênica associado aos corpos nucleares do tegumento, semelhante ao demonstrado pela proteína Sm29 (DILLON et al., 2007). Devido a essa discrepância, especulamos sobre a possibilidade dessas poucas células reprodutoras apresentarem uma maior expressão das SmNPPs e encobrirem uma expressão menor de outras estruturas do parasita. No caso da SmNPP-5a, postulamos que essa menor expressão dos corpos celulares poderia ser contrabalanceada pelo seu número de células, resultando numa maior expressão proteica no tegumento.

5.2 Avaliação das ectonucleotidases de tegumento como potenciais antígenos

Considerando que o *S. mansoni* apresenta três proteínas do tegumento envolvidas no metabolismo de nucleotídeos: SmNPP-5a, SmATPDase e SmAP; decidimos avaliar o potencial destas enzimas como antígenos vacinais. As proteínas recombinantes foram administradas isolamentente ou combinadas caso houvesse um possível efeito sinérgico devido à indução de uma resposta imune simultânea contra as ectonucleotidases do tegumento do parasita.

Apesar de Bhardwaj e Skelly (2009) hipotetizarem que a função das nucleotidases do tegumento seria inibir a via de sinalização purinérgica do hospedeiro por meio da produção de moléculas anti-inflamatórias, deve-se salientar que essas proteínas podem também desempenhar um papel fundamental no reaproveitamento dessas biomoléculas (LEVY; READ, 1975b); haja vista que schistosomas não sintetizam purinas *de novo* (LEVY; READ, 1975a). Porém os substratos biológicos específicos de cada nucleotidase, assim como suas

funções fisiológicas permanecem desconhecidas e uma vez que não observamos nenhum efeito sinergístico devido à imunização com as nucleotidades combinadas, torna-se admissíveis que talvez estas enzimas não partilhem funções/substratos ou que não sejam fisiologicamente correlacionadas.

Como não foi observada redução da oviposição dos parasitas ou da caraga parasitária nos camundongos imunizados com nucleotidases do tegumento isoladas ou combinadas, aventamos a possibilidade de combinar o protocolo de vacinação a um tratamento subcurativo com praziquantel. Por já ter sido demonstrado que esse tipo de tratamento era capaz de causar alterações no tegumento do parasita (LIANG et al., 2002), primeiramente observamos os efeitos da quimioterapia *in vivo* subcurativa nos parasitas e verificamos que, nas condições testadas, ela não altera drasticamente a morfologia do tegumento do parasita e também não afeta a sua sobrevivência.

O protocolo de imunização e desafio associado ao tratamento subcurativo foi realizado na esperança da quimioterapia aumentar a eficácia das imunizações, pois já foi demonstrado que a ação conjunta entre a resposta imune do hospedeiro com o tratamento com praziquantel faz com que a eliminação do parasita seja mais eficiente (BRINDLEY; SHER, 1987; FALLON; DOENHOFF, 1995). Esse efeito pôde ser observado nos animais imunizados com a SmAP isolada que apresentaram um redução de aproximdamente 40% da carga parasitária, mesmo nível da redução observada no animais imunizados com as três proteínas combinadas. Esse resultado de proteção foi corroborado pela oviposição dos parasitas, apesar desses dados não apresentarem diferenças estatísticas significativas.

No modelo murino da esquistossomose, a imunização com proteínas recombinantes ou com cercárias irradiadas apresentam melhores resultados quando associados a uma resposta imune predominantemente Th1 (CARDOSO et al., 2008; HOFFMANN et al., 1999; TRAN et al., 2006). Apesar de ter sido observado proteção relacionada a uma resposta imune do tipo Th_2 utilizando extratos proteicos como antígenos vacinais (EL RIDI; TALLIMA, 2012; MARTINS et al., 2012) ou devido a múltiplas imunizações com cercárias irradiadas (CAULADA-BENEDETTI et al., 1991; HOFFMANN et al., 1999). No entanto, também já foi argumentado que mais importante do que o tipo de resposta imune, provavelmente é a magnitude da mesma (HOFFMANN et al., 1999). Por isso, avaliamos a resposta imune humoral dos animais apenas imunizados e infectados com aqueles imunizados, infectados e tratados com praziquantel e verificamos que a SmFA e SmATPDase induziram níveis maiores de IgG total que a SmNPP-5a, independente do tratamento com praziquantel ou da adminstração isolada ou conjunta das proteínas recombinantes. Verificamos também no

protocolo de vacinação convencional, a redução dos níveis de IgG total após a infecção pelo parasita, em especial para os anti-SmFA, apesar de não haver diferenças estatisticamente significativas. Entretanto quando o desafio com cercárias foi associado ao tratamento com a droga, independente da adminitração isolada ou combinada das proteínas, os níveis de IgG total anti-SmFA foram mais homogêneos. Esse dado sugere que a proteína poderia estar mais acessível ao sistema imune após a quimioterapia como já foi reportado por Fallon et al. (1994).

Nestes ensaios também caracterizamos o perfil da resposta imune induzida pelas imunizações através da averiguação das concentrações dos isotiopos IgG1 e IgG2a. Esses dois isotipos foram utilizados como indicadores do tipo de resposta imune induzida, pois uma resposta Th2 está associada a altos níveis de IgG1, enquanto uma resposta Th1 foi correlacionada a níveis maiores de IgG2a. Essa correlação é baseada na indução da produção de IgG1 por citocinas secretadas por células Th2 e sua inibição por citocinas secretadas por células Th1; apesar das células Th1 não serem boas iniciadoras de resposta de anticorpos, as citocinas secretadas por elas induzem a troca do isotipodo dos anticorpos, preferencialmente para IgG2a (MURPHY; TRAVERS; WALPORT, 2008). Ao analisarmos a razão da concentração dos isotipos IgG1/IgG2a nos soros dos animais imunizados antes de serem infectados com *S. mansoni* verificamos uma predominância de uma resposta imune do tipo Th2, com maiores níveis de IgG1. Após a infecção pelo parasita, independente do tratamento com praziquantel, observamos de modo geral uma redução da razão IgG1/IgG2a. Essa redução seria indicativa de uma mudança da resposta imune para um perfil mais Th1, porém ela não resultou do aumento da concentração de IgG2a, mas de uma diminuição maior dos níveis de IgG1 que de IgG2a.

Visando caracterizar melhor o perfil da resposta imune induzida pelas imunizações, também foi avaliada a expressão relativa de diversos genes relacionados a ela em esplenócitos de camundongos re-estimulados *in vitro* com as proteínas recombinantes. Apesar do caráter mais Th2, indicado pelas razões dos isotipos e da evidente maior imunogenicicdade da SmFA, não foi observado que essa proteína induza uma expressão maior de citocinas caraterísticas de células Th2 como IL-4, IL-5 ou IL-13. Ao contrário verificou-se que a SmFA estimula uma maior expressão de IL-17 associada a níveis maiores de expressão da citocina próinflamatória TNF-α. Talvez essa combinação esteja envolvida no mecanismo responsável pela eliminação do parasita, haja vista que a SmNPP-5, que não foi capaz de induzir proteção contra a infecção pelo parasita, também induziu a expressão maior de IL-17, porém associada a expressão da citocina anti-inflamatória TGF-β.

A IL-17 é uma citocina muito importante na defesa do hospdeiro contra patógenos extracelulares (YE et al., 2001), que não são eliminados pela imunidade com perfil Th1 ou Th2. Porém esta citocina também está relacionada à imunopatogênese da esquistossomose no processo inflamatório da formação do granuloma ao redor dos ovos produzidos pelos vermes adultos, tendo sido proposta uma correlação direta entre a severidade da esquistossomose com o nível de IL-17 do hospedeiro no modelo murino (RUTITZKY; LOPES DA ROSA; STADECKER, 2005). Nossos dados da relação entre a proteção contra a infecção pelo schistosoma com a indução da expressão da IL-17 devido a imunização com SmFA confrontam-se com os de Wen et al. (2011) que demonstraram que a administraçção de anticorpos neutralizantes anti-IL-17 durante a passagem de esquistossômulos no pulmão foi capaz de reduzir a carga parasitária de animais infectados e que o desenvolvimento do parasita não era afetado pela adminitraão de IL-17 recombinante.

Apesar de não verificarmos nenhum efeito protetor devido a imunização com SmNPP-5a, já foi observado que a imunzação com a NPP-5 ortóloga de *S. japonicum* possui a capacidade de reduzir a carga parasitária em uma infecção subsequente (ZHANG et al., 2011). Além de haver sido demonstrado que a SmNPP-5a recombinante induzia a produção de anticorpos capazes de inibir a atividade enzimática de parasitas vivos, mesmo com a proteina recombinante não apresentado sua conformação nativa. A ineficiência da SmNPP-5a em induzir proteção pode ser associada a fraca resposta imune humoral e a alta expressão da citocina regulatória TGF-β que provavelmente contrabalanceou a resposta celular, desse modo talvez um uma proteína enovelada da mesma forma que a nativa, proveria um resultado diferente. Não obstante a SmATPDase induziu maiores níveis de IgG total que a SmNPP-5a, esses ainda ficaram aquém dos induzidos pela SmFA e, ao compararmos os isotipos, verificamos que a SmATPDase induziu os menores níveis observados; além de induzir os menores níveis de expressão das citocinas avaliadas. Considerando todos esses parâmetros acreditamos que a resposta imune induzida pelas imunizações com as nucleotidases pode justificar as difererentes eficiências observadas nos ensaios de vacinação.

Ademais, a supressão da expressão da SmNPP-5a compromete a capacidade do parsita em estabelecer a infecção (BHARDWAJ et al., 2011) enquanto a inibição da expressão da SmFA não apresentou nenhum efeito em parasitas cultivados *in vitro* (BHARDWAJ; SKELLY, 2011). Esses dados sugrerem que a SmNPP-5a seja uma proteína com uma função mais importante ou vital para o parasita que a SmFA. Entretanto, nossos resultados demonstraram que apenas a imunização com a SmFA associada com o tratamento subcurativo com praziquantel foi capaz de reduzir a carga parasitária de animais infectados. Assim

baseados nesses resultados, concluímos que a eficácia da imunização com a SmFA não deve estar relacionada a uma função desta proteína mais relevante biologicamente para o parasita.

Embora mais imunogênica, a imunização com a SmFA foi efetiva em prover proteção aos animais infectado apenas quando foi associada ao tratamento com praziquantel; isto pode ser devido a algum dano causado ao parasita, que de alguma maneira prejudicou seus mecanismos de evasão ao sistema imune do hospedeiro (BRINDLEY; SHER, 1987). Porém uma vez que a quimioterapia subcurativa tornou apenas a imunizaçaõ com a SmFA eficaz, esse efeito deve, de algum modo, ser seletivo para algumas proteínas, uma vez que já foi demonstrado a maior exposição de alguns poucos antígenos do tegumento após o tratamento com praziquantel, entre eles a SmFA (BRINDLEY et al., 1989; FALLON et al., 1994). Além disso, das três nucloetidases estudadas, é provável que a SmFA seja naturalmente mais exposta na superfície do parasita e consequentemente ao sistema immune do hospedeiro, pois foi a única da três enzimas a ser identificada por proteoma em frações de proteínas liberadas após o tratamento de vermes adultos vivo com “phosphatidylinositol-phospholipase C” (CASTRO-BORGES et al., 2011a; CASTRO-BORGES et al., 2011b). Desse modo, o nível de exposição do antigeno ao sistema immune pode ser outra possivel explicação para o efeito sinergístico entre as imunizações com a proteína recombinante e o tratamento subcurativo com praziquantel.

Pessoas infectadas apresentam naturalmente anticorpos capazes de inibir duas dessas proteínas, SmNPP-5a e SmFA (CESARI et al., 1992; PUJOL; CESARI, 1990) e como seus parasita são aparentemente imunes a esses anticorpos, poderímaos concluir que esses antígenos não seriam efetivos em induzir uma resposta imune eficaz contra a infecção. Porém é importante lembrar-se que existem indivíduos de áreas endêmicas naturalmente resistentes e que tanto essas pessoas quanto aquelas infectadas apresentam anticorpos contra antígenos, que quando usados como vacina são eficazes em diminuir a taxa de infecção do schistosoma. Entretanto os níveis e os perfis de isotipos são diferentes nesses dois grupos, apresentando uma maior proporção de anticorpos opsonizantes e ativadores do sistema complemento nas pessoas resistentes (CARDOSO et al., 2008; CARDOSO et al., 2006; TRAN et al., 2006). Portanto a questão central para a indução da proteção em seres humanos parece ser os níveis e o perfil dos anticorpos contra os antígenos do schistosoma e não simplemente sua presença ou ausência.

Nossos resultados reiteram a perspectiva de combinar diferentes estratégias para combater a doença associando a imunização com o tratamento quimioterápico. Embora bons candidatos vacinais devam ser capazes de sozinhos induzirem proteção, ainda que parcial,

contra a infecção; nossos experimentos foram desenhados para permitir a visualização do efeito sinergístico da imunização com as nucleotidases com a quimioterapia subcurativa sobre os parasitas. Esta abordagem não é inédita, já tendo sido utilizada no passado com extratos proteicos do parasita (FALLON; DOENHOFF, 1995) e foi proposta recentemente de novo para outras proteínas do tegumento de *S. japonicum* (ZHANG et al., 2012). Mesmo que estas condições não se reproduzam no combate a doença em seres humanos, é provável que a administração da vacina ocorra concomintante ao tratamento quimioterápico sendo, portanto interessante verificar a interação entre as duas abordagens para confrontar e avaliar diferentes regimes de imunização com diferentes tratamentos quimioterápicos, visando selecionar os mais apropriados para serem aplicados em testes clínicos.

6 CONCLUSÃO

Nesse trabalho foram identificadas quatro nucleotídeo pirofosfatases/fosfodiesterases no genoma do *S. manoni*. Verificamos que duas dessas proteínas apresentavam-se mais expressas nos estágios intramamíferos, sendo provavelmente importantes para adaptação e sobrevivência do parasita. As proteínas foram expressas de modo heterólogo em *E. coli,* mas não apresentaram atividade enzimática. Conseguimos produzir um anticorpo específico contra a SmNPP-5a, o qual foi utilizado para realizar a caracterização desta enzima como uma glicoproteína associada as membranas do tegumento do parasita, que são produzidas e secretadas para sua superfície durante a transformação cercariana no processo de infecção. Também demonstramos que essa proteína estaria acessível ao sistema imune do hospedeiro, pois conseguimos inibir parcialmente sua atividade enzimática em parasitas vivos incubando-os com anticorpos. Essas características fizeram da SmNPP-5a um promissor antígeno vacinal. Por último foram feitos ensaiosde hibridização *in situ* e observado que as *smnpps* têm sua expressão associada aos tecidos reprodutores de parasitas adultos. Esses dados abriram novas perspepctivas de estudos visando elucidar melhor a relação e a função destas proteínas com a reprodução do *S. mansoni* para se entender melhor o papel destas enzimas na biologia do parasita.

Em uma segunda parte do projeto nós avaliamos como antígenos vacinais, não apenas a SmNPP-5a que apresentou características tidas como importantes para um candidato vacinal, mas também as outras duas nucleotidades do tegumento previamente caracterizadas e extensivamente propostas na literatura com alvos vacinais, a SmFA e SmATPDAse. A SmFA foi a proteína mais imunogênica das três, induzindo uma resposta predominantemente Th2, com altos níveis de IgG1 associados a uma maior expresão das citocinas pró-inflamatórias TNF-α e IL-17. A despeito de somente a imunização desta proteína na formulação utilizada não ser capaz de induzir proteção nos camundongos infectados, sua associação a um tratamento com doses subcurativas de praziquantel tornou-a efetiva. Tanto a SmNPP-5a quanto a SmNTPDase apresentraram baixa imunogenicidade na formulação utilizadas e não foram capazes de conferir proteção aos animais imunizados, mesmo quando associados ao tratamento com praziquantel. A SmNPP-5a apresentou uma resposta imune caracterizada pela maior expressão relativa da citocina reguladora TGF-β, enquanto a SmATPDase induziu uma resposta predominantmente Th2. Esses dados demonstram que a SmFA é a nucleotidase do tegumento mais promissora como candidato vacinal e advoga por novos estudos, visando

otimizar sua expressão e tornar seu enovelamento mais próximo da proteína nativa, para sua avaliação como antígeno vacinal com diferentes adjuvantes e protocolos de imunização.

Os resultados indicaram que a SmFA possa vir a ser utilizada em uma vacina composta por um coquetel de antígenos, principalmente se as outras proteínas que compõe o coquetel induzirem uma resposta imune que possui como alvo o tegumento do parasita. Esses dados destacam ainda a importância de investigar diferentes regimes de imunização associados com agentes quimioterápicos.

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APÊNDICE A – Oligonucleotídeos utilizados neste projeto

- Oligonucletídeos para clonagem dos genes *smnpps* completos:

```
SmNPP-5a F: ATG TAT TGC ATT GAA ACT ATG CAA AAA ATG
SmNPP-5a R: TCA ATA ATA TAA TCC ATT GTG TAC CAT GAA

SmNPP-5b F: ATG TCA ACG GAA CAA ATA ATA CAG GA
SmNPP-5b R: TCA TGT ATT ATA ATC TGA AAA CTC TTT ATC A

SmNPP-5c F: ATG ACT ATG GAG TGT TTC TGC CAC TG
SmNPP-5c R: TCA AAC TTC AGT GAA CAT ATC CAG AAA A

SmNPP-6 F: ATG AAC ATT TAC ACA TTT CTG TAC ATC TTA G
SmNPP-6 R: TTA AGC TTC AGA AAG TAC CTC AGA TTT AAC
```

- Oligonucleotídeos para RT-PCR em tempo real dos genes *smnpps*:

```
SmNPP-5a F: TTC ATG ATT ACA TAC ATC CAA ATG AG
SmNPP-5a R: CGC ACA GTA TAG CCT TGC TTT

SmNPP-5b F: CTC TCG TAT GCC TCC ATT GAT
SmNPP-5b R: CAT GAT CAC CGG AGA CAT TG

SmNPP-5c F: AAA GAT GGT AGC TCA TCT ATT GAT
SmNPP-5c R: TGC AAA TGA ATA GGT ATA TTA TCC TT

SmNPP-6 F: TAT TTA CAG GTC GTC ATC CAG
SmNPP-6 R: TTG TGA ACG AAT AGG CTT ACC
```

- Oligonucleotídeos para clonagem dos fragmentos *smnpps* em vetor pAE-6His:

```
SmNPP-5a F: TTC TCG AGG AAC AGT TTT CTA AAG T
SmNPP-5a R: TTG GTA CCT CAT TCC AAA TCA AG

SmNPP-5b F: TTC TCG AGC ACG GCA AAA CTG TT
SmNPP-5b R: TTG GTA CCT CAT TCA GAG AAA TCT G

SmNPP-5c F: TTC TCG AGC AAC AAA AAT TAC TTG TG
SmNPP-5c R: TTG GTA CCT CAC ATT TCA TCA TGG TC
```

```
SmNPP-6 F: TTC TCG AGT CTC GTT TAC TGG TTC T
SmNPP-6 R: TTG GTA CCT CAT AAT GGA ATA TGC AT
```

- Oligonucleotídeos para clonagem dos fragmentos *smnpps* em vetor pPicZαA:

```
SmNPP-5a F: TT GAA TTC GAA CAG TTT TCT AAA GT
SmNPP-5a R: TT GC GGC CGC TTC CAA ATC AAG

SmNPP-5b F: TT GAA TTC CAC GGC AAA ACT GTT
SmNPP-5b R: TT GC GGC CGC TTC AGA GAA ATC TG

SmNPP-5c F: TT GAA TTC CAA CAA AAA TTA CTT GTG
SmNPP-5c R: TT GC GGC CGC CAT TTC ATC ATG GTC

SmNPP-6 F: TT GAA TTC T CTC GTT TAC TGG TTC T
SmNPP-6 R: TT GC GGC CGC TAA TGG AAT ATG CAT
```

- Oligonucleotídeos para clonagem dos fragmentos *smnpps* em vetor pPINT-A:

```
F: TAC CCT AGG AAC GAT GAG ATT TCC TTC AAT T
R: TAC GAA CGA TTT CTC AAT GAT GAT GAT GAT GAT GG
```

ANEXO A – Sequências de nucleotídeos e de aminoácidos das SmNPPs

SmNPP-5a

Sequência gênica completa (éxons em preto; íntrons em vermelho).

```
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AACATGTCAG CATTTGATAA GATTATAAAT CAAGGAGTTT ATATAAGACG TATAGAAAAT
GAATTTCCTA CTTTAACATT CCCATCACAT TTTTCAATTG TAACAGGACT ACATCCTGGA
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TATTGA

SmNPP-5a
Sequência gênica, apenas éxons.

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ATCATCAAAT TTATTATTCT AAGTATTTTC ATGGTACACA ATGGATTATA TTATTGA
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SmNPP-5a

Sequência de aminoácidos.

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RNQSTATDSR FYDVGAEPIW VTNQFHGHKS GVTFWIGSEA IIKGERPTHY LTPYNESITF
TQRIDILMDW FEHENINLGL MYYHQPDRAG HIHGAASDEV FKAIEEINHG LEYLLTSIEM
RPSLSCCLNL IITSDHGMTN ISSDRVIYLH DYIHPNEYIS APKKSAEIWT LWPKQGYTVR
SLYNKLKDRH FRLNVYLKEE LPTRFFYGSS DRVGPVVVYA DIGWTIIADR TSGITLKNKG
AHGYDPDYKE MSPFLMAMGP QIAKSQPTEL KESIKLIDIY SLICLMLDLE PAPNNGSVCR
VLPLLSQGSF ANINRLSIIF IIKFIILSIF MVHNGLYY
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SmNPP-5b

Sequência gênica completa (éxons em preto; íntrons em vermelho).

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ATGTCAACGG AACAAATAAT ACAGGATGAT AGTGACTTAC CTCCTCCATA CTATCAAATA
AATCACGAAT GTATTCGTCG TTACAAACGT AGAACTCATT GTACTTACTT CACATTAATT
ATATCAGGCA TTGTAATGAT GTTATTCGGT GCCCTTATTC TCGTGATTTT GATACCACAA
GAAATCAATT TATATCGATG GAATATAAAA GGACCCTTAT CAAGATTTTT TAATCACGGC
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GCTGCGAAAT CGATCAATGT TTATCCTACA ATTACTCTAC CTAATCATCG TACATTAATT
ACCGGTTTGT ATCCAGAAAA TCATGGAGTT GTTGGAAATA GTCTATTGGA TAAGAAATGG
CCAAATAAGA TTTTCAGTAT ATATGACCAA GAAAGTCTAA ATCATGCTCC GTGGTTAACT
GACTGGCCTG AACCTATATG GGTTACATTA CAACAAAATG GTGGCTATGC TGGTTCAATT
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CATTTACTCA CTGCAATAAA CTTTACGTTC TCAGATTTTC TTCAGTACAA AATTATCTCA
AGACGCATTA AAAACCAAGA CTTATGCAGC TACTGTTTCA TCCCGTTTTA TAAATCAAAC
GACACTGTAA ACAGTTCGTG TGGAATCAAA CTCAGGAACT GCTGATTCGC ACTAGACTCA
CTTTAGATTC CTAGATCTGA TTCAAACACT AATGATTTGC AAACTGAAAG AAATTTGTTT
CCATCCTCAC TAGCAGTGCA CTTTCTCTAA TCAACCAACT GATTGTTATC TGGCTTCGAT
TTCAGCTGAT TTTTTCCAGC TTAACAAATC TAACCCCAGA AAACTCAGTC TAATTTGATT
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AATACATGA

SmNPP-5b
Sequência gênica, apenas éxons.

ATGTCAACGG AACAAATAAT ACAGGATGAT AGTGACTTAC CTCCTCCATA CTATCAAATA
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ATTTTATTTA TTTCAATTAT ATGCGTTTCA TTTACGATTT TACTGTGTTC AATCGTAGTT
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SmNPP-5b
Sequência de aminoácidos.

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AAKSINVYPT ITLPNHRTLI TGLYPENHGV VGNSLLDKKW PNKIFSIYDQ ESLNHAPWLT
DWPEPIWVTL QQNGGYAGSI LWPLTDQFVS DDLPFQRVSQ YALLNDYEHR YAYDQRIRDI
LWWLKNPKYH LNLILAYFEE PDETGHSYGP NSKHVAKVVQ TLDETLGHLL DGIKKRGLTD
KVDIILTADH GMSETSNTRL ITLDKYIDNS LYNYTQLSTM GFLYPAPGKF EEVYKRLKSA
HPKLDVYRRD EVPAYLNFNI TSSRMPPLIL IAKPRWKIVK NTSQPYANVS GDHGYPTDFS
EMYPFFIARG PSFKIAESVP TVHAVDVYPL MCALLNIQPN PNNGSLERIS NILKPDVANR
LLNWNSWSYL WKWIISDLRF ILFISIICVS FTILLCSIVV VMHRKETNEP FIRLPIDNDK
EFSDYNT
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SmNPP-5c
Sequência gênica completa (éxons em preto; íntrons em vermelho).

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CACTGTTTTA AACAAGTGGG TCACAACTTC GAACTGTGCA TCATCTGTTG AGTCTTGGAC
AGTCATTTGG CGTTAATAAC CTTCATGGGA AGTATGGATC ATGGGTTATA GTCTCCGTGT
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TGGATATGTT CACTGGTTAG TTTACTCAAT AGTTTTTTCG AAGTTATTTG TTACTTACCG
TGCACATACA ATGTTGACAG ATTTTTGTCT ACTGCTTTGT AATGGTTACA TTTTTCCCTG
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CAGAAGTTTG A

SmNPP-5c
Sequência gênica, apenas éxons.

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AATCACTTAT CACTGGTAAC TGGGATGCAT GAAGAGAGTC ATGGTATTGT TGATAACATG
TTTTATGATC CTAAGCTTAA TGATACTTTT GACTTGAGTA ATACAAGGCA TTTATCACAG
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TATTATTATT CACAATACAA CCATCAACTT CCTTTAGTTG ATCGAATTAA TCAAACTATT
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GGTGGTCAAA TAATTAATTC TATTTATCTG ATTGATATTT ATCCACTGAT GTGTGCCTTA
CTCAATTTAA ATAAACCAGC TCCAAACAAT GGCAGTTTAT CACGTGTGAT GTCACTCCTT
CGTCATAGTA GTGGTATTAA TGTCTACTTC TCAGATTTTC TGGATATGTT CACTGAAGTT
TGA

SmNPP-5c
Sequência de aminoácidos.

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MTMECFCHCF TWISLFILLQ GICEAYKQQK LLVISLDGFR HDYLDHAKLR NINISSFERI
WKTGVRVMKV HNEFITRTGP NHLSLVTGMH EESHGIVDNM FYDPKLNDTF DLSNTRHLSQ
SKWFDVGSEP IWVTNQRHGH KSAVTFWPGS NTPFKGQLPS YYYSQYNHQL PLVDRINQTI
KLLDLDRVTL GLIYFHEPDS QGHLTGPDSI EIYNVIEKLN DDIGYLLSLI DTRPSLKSSL
NIILTSDHGM SGVDTKRIII LHDYINESMY YSPGSEDRIF WSLWPKDGSS SIDLYKKLVG
KHPKMNVFLK DNIPIHLHYS NSRRIGPVVV YSEPGWTIVR SQKSVAKFTK RGDHGYDPDH
DEMSPFFLAT GPGFRSAIVG GGQIINSIYL IDIYPLMCAL LNLNKPAPNN GSLSRVMSLL
RHSSGINVYF SDFLDMFTEV
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SmNPP-6
Sequência gênica completa (éxons em preto; íntrons em vermelho).

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CCTGCAGAGT  GTTATCCGAA  TATTTACTCA  CTATTTACAG:GTAGGTACAC  AGTATTTATG
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AATTCTTAAA ACCATTTATT GACATTATTT TTTCTTCATT ATTTAATGAC TTCAGATTGT
CACGGTTGAA CTCATGAGTC TATCTAAGCT AGACCACCGG AAAACGTTTT CACCACAGTA
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GGTCTCGCGC GCGAACGCTT AACCTCTAGG CCGACATCTG GACGCGTTAA TATCCGACTT
CAATCAATCC ATAATCTTGC TCAACCATCA TCCGTTGCCT ACTCTGGATA TCTGTTTCAC
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CATCTCAAAG CAAGTCACTA GTGAGCACAT GAATATTAGT AGTATAGGAG ATTTGTGGAG
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CCTGTTGTAC AATCACTCAT CAATTTTTAA AGATAATATA TTTTTTGTTG TTACCTTTCC
TTGTTTGTAG GACAGAATTA GATAGAGTAC TGAATCGTGG AAGTACATTG TGGATCTGGC
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TGCTTATCTG TCATTGCGTT TAACAATTTA ATCCCCATTT ATCTAGTTTG TCTTCTTATT
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GCTTACATTT ATACCAGTCT TTACATTAAT TATGGTGAAC TGATTTGTTC ATCAACCNNN
NNNNNNNACT GTACTTCCAA TTAGTAGGTT ATTGCTTCCA ATCCAACTTC TCAGGGAGTA
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GGTAACTGTT ACGTCTCGCC GGTTAGAACG CTATATTCGC TTCCCTAAAC TAGTTCCGTA
CCCCCGAGCT AGAACTACTC AGCGACTTGA AGACCAGAGA AAAGGTACAA AGTGTGAGGG
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CACCGAATTA TATATTTCAT TCCAAGGCAT GGCCATTAAA TATGAGTCAT TACAATTTAA
TCGATCCCAA AGGAATGCAT GGCTATGATC CAGAACTACC TGATATGCAT ATTCCATTAT
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CAGTTCATAT ACATAGTTTA ATGGCTTATT TAGCATCGAT TAATCTGCCA GATTTTCGTT
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TGTTGAATAA TTTCTATTGG CCATTGAATG ACAAATCATC ATCATCATTA TCATTTTCTT
CAACATTATA CGGAGATCGT TTTATGGTGA ATTTTCTAAT CGTAGCAGCA GCGTTAACCT

GTTTCTTTGT GCTAATTTGC GCTTTCATCA TATTAGCCTA TGTTAGATGT CGTATTTCAT
TGACGACTAC ACGTGTATTC GGTTATCAAG ATGATTTAGA CAAGGAATTA GTTAAATCTG
AGGTACTTTC TGAAGCTTAA

SmNPP-6
Sequência gênica, apenas éxons.

ATGAACATTT ACACATTTCT GTACATCTTA GTTTATTTAT TCAATACGAA CAGTTCAACA
GTAACAACGA CAATCTTAGA TAATGACTCG TCAGGTTCTT CATCTCGTTT ACTGGTTCTT
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TTCGGATTTA AACGACTACA GAAAAATGGT GCGTATTTGC AGCGTTTCAC TCCTGTATTT
CCTGCAGAGT GTTATCCGAA TATTTACTCA CTATTTACAG GTCGTCATCC AGTCGATCAT
GGAGTTATTC TGCCAACAAC ATTTAGTGAA CATACAACTA TTAATGGTAA GCCTATTCGT
TCACAAGTTG AAGGTCTTTG GGAAACAGGA GTAAAACAAA ATAAAGGTGT TCATCTTTAT
CATTTGCCTA TATGTTCTAC TGAAGCTGGC ACTGAAAATA GTTGGTATTG TGAACCGTAT
AGTCAAGAAG TTATGAATCC TATGCATTTG AATGCTACAA TACAAAAAGC TGTGGATGGG
CTAAAAAACG GCAGTGCGAA TTTGGCAGTG GTATACTACG ATGAATTAGA TCGTATCGGA
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GTTTTAGACT ATGCATTGAA CATCATAGAA TCTATACCAA ATTTAAATTT AATGCTAACT
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TTTGAAAACA ATGTGTATAA TCGTTTACTA ATTCAACAAA GTAAATCTCC TTTCACTGTA
TACAATTCAT CGACCATTCT TTCACATTGG GAAATTATGA CAAATAACAA TAATAATAAT
TCATTATTGC CACCGGTATT GTTAATCGCT TCACCGAATT ATATATTTCA TTCCAAGGCA
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CACAATTATT ATCATCATCA GTTGTCACCT GTGTTGAATA ATTTCTATTG GCCATTGAAT
GACAAATCAT CATCATCATT ATCATTTTCT TCAACATTAT ACGGAGATCG TTTTATGGTG
AATTTTCTAA TCGTAGCAGC AGCGTTAACC TGTTTCTTTG TGCTAATTTG CGCTTTCATC
ATATTAGCCT ATGTTAGATG TCGTATTTCA TTGACGACTA CACGTGTATT CGGTTATCAA
GATGATTTAG ACAAGGAATT AGTTAAATCT GAGGTACTTT CTGAAGCTTA A

SmNPP-6
Sequência de aminoácidos.

MNIYTFLYIL VYLFNTNSST VTTTILDNDS SGSSSRLLVL LIDGLRWDVI AGHLENNTNR
FGFKRLQKNG AYLQRFTPVF PAECYPNIYS LFTGRHPVDH GVILPTTFSE HTTINGKPIR
SQVEGLWETG VKQNKGVHLY HLPICSTEAG TENSWYCEPY SQEVMNPMHL NATIQKAVDG
LKNGSANLAV VYYDELDRIG HRYGPLSNEL VHKHLVYLDH VLDYALNIIE SIPNLNLMLT
SDHGMATVSH QAHADRYFKG TELDRVLNRG STLWIWPKPE FENNVYNRLL IQQSKSPFTV
YNSSTILSHW EIMTNNNNNN SLLPPVLLIA SPNYIFHSKA WPLNMSHYNL IDPKGMHGYD
PELPDMHIPL FIYGPNINRG VQLNFTKEIR SVHIHSLMAY LASINLPDFR SLDLFKPLLS
HNYYHHQLSP VLNNFYWPLN DKSSSSLSFS STLYGDRFMV NFLIVAAALT CFFVLICAFI
ILAYVRCRIS LTTTRVFGYQ DDLDKELVKS EVLSEA

ANEXO B – Artigos publicados

1. **Rofatto, H.K.;** Araujo-Montoya, B.O.; Miyasato, P.A.; Levano-Garcia, J.; Nakano, E.; Verjovski-Almeida, S.; Farias, L.P.; Leite, L.C.C.. **Immunization with tegument nucleotidases associated with a subcurative praziquantel treatment reduces worm burden following *Schistosoma mansoni* challenge**. *PeerJ*. Artigo submetido para publicação. 2012.

2. **Rofatto, H.K.**; Parker-Manuel, S.J.; Barbosa, T.C.; Tararam, C.A.; Wilson, R.A.; Leite L.C.C.; Farias, L.P.. **Tissue expression patterns of *Schistosoma mansoni* Venom Allergen-Like proteins 6 and 7.** International Journal for Parasitology. v. 42, p.613-620, 2012.

3. Farias, L.P.; Rodrigues, D.; Cunna, V.; **Rofatto, H.K.;** Faquim-Mauro, E.L.; Leite, L.C.C.. ***Schistosoma mansoni* Venom Allergen like Proteins present differential allergic responses in a murine model of airway inflammation.** *PLOS Neglect Tropical Diseases*. v. 6, p. e1510, 2012.

4. Araujo-Montoya, B.O.; **Rofatto, H.K.;** Tararam, C.A.; Farias, L.P.; Oliveira, K.C.; Verjovski-Almeida, S.; Wilson, R.A.; Leite, L.C.C.. ***Schistosoma mansoni*: Molecular characterization of Alkaline Phosphatase and expression patterns across life cycle stages.** *Experimental Parasitology*, v. 129, p. 284-291, 2011.

5. **Rofatto, H.K.;** Leite, L.C.C.; Tararam, C.A.; Kanno, A.I.; Araujo-Montoya, B.O.; Farias, L.P.. **Antígenos vacinais contra esquistossomose mansônica: passado e presente.** *Revista da Biologia*, v. 6b, p. 54-59, 2011.

6. Farias, L.P.; Cardoso, F.C.; Miyasato, P.A.; Araujo-Montoya, B.O.; Tararam, C.A ; **Rofatto, H.K.;** Kawano, T.; Gazzinelli, A.; Correa-Oliveira, R.; Coulson, P.S.; Wilson, R.A.; Oliveira, S.C.; Leite, L.C.C.. ***Schistosoma mansoni* Stomatin Like Protein-2 Is Located in the Tegument and Induces Partial Protection against Challenge Infection.** *Plos Neglected Tropical Diseases*, v. 4, p. e597, 2010.

7. **Rofatto, H.K.;** Tararam, C.A.; Borges, W.C.; Wilson, R.A.; Leite, L.C.C.; Farias, L.P. . **Characterization of phosphodiesterase-5 as a surface protein in the tegument of *Schistosoma mansoni***. *Molecular and Biochemical Parasitology*, v. 166, p. 32-41, 2009.

Immunization with Tegument Nucleotidases associated with a Subcurative Praziquantel Treatment reduces worm burden following *Schistosoma mansoni* challenge

Schistosomiasis is a debilitating disease caused by flatworm parasites of *Schistosoma* genus and still a high public health impact disease widespread around the world, although effective treatment with Praziquantel (PZQ) is available since 1970s. Control of this disease would be greatly improved by the development of a vaccine, which could be combined with chemotherapy. The sequencing of *Schistosoma mansoni* transcriptome and genome allowed the identification of a range of potential vaccine antigens by proteomics and their molecular characterization. Among them, three nucleotidases from the tegument of the parasite, presumably involved in purinergic signaling and nucleotide metabolism were proposed as promising vaccine candidates: an alkaline phosphatase (SmAP), a phosphodiesterase (SmNPP-5) and a diphosphohydrolase (SmNTPDase). Herein, we evaluated the potential of these enzymes as vaccine antigens, associated or not with a subcurative PZQ treatment. Immunization of mice with isolated or combined recombinant proteins demonstrated that SmAP is the most immunogenic one. It induced the highest antibody levels, particularly IgG1, associated with an inflammatory cellular immune response characterized by high TNF-α and a Th17 response, with high IL-17 expression levels. Despite the specific immune response induced, immunizations with the isolated or combined proteins did not reduce the worm burden of challenged mice. Nonetheless, immunizations with the three proteins combined or with SmAP alone associated with the subcurative PZQ chemotherapy were able to reduce the worm burden by around 40%. The immunogenicity and relative exposure of SmAP to the host immune system are discussed, as the key factors for the higher efficacy in protection by the synergistic effect of SmAP immunization associated with the subcurative PZQ treatment.

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45
46

47 **1. Introduction**

48

49 Schistosomes are parasitic blood flukes that cause schistosomiasis, a tropical
50 disease that has a major public health impact in endemic countries. It affects over 200
51 million individuals worldwide causing more than 200,000 deaths per year, with
52 almost 800 million people at risk of infection (Bergquist 2002; Engels *et al.* 2002;
53 Steinmann *et al.* 2006). Chemotherapy with Praziquantel (PZQ) is the main control
54 strategy used; however, mass treatment does not prevent reinfection and its
55 cumulative effects (Wilson & Coulson 1999). Furthermore, selection of drug resistant
56 parasites is of concern (Fallon *et al.* 1995; Ismail *et al.* 1999). Thus, the development
57 of a defined vaccine against schistosomiasis that could be associated with
58 chemotherapy would contribute to the current control strategy (Bergquist 2002).

59 The *Schistosoma mansoni* and *Schistosoma japonicum* transcriptomes (Hu *et*
60 *al.* 2003; Verjovski-Almeida *et al.* 2003) and genomic sequencing projects (Berriman
61 *et al.* 2009; Zhou *et al.* 2009), together with data from proteomics studies (Curwen *et*
62 *al.* 2004; van Balkom *et al.* 2005; Braschi *et al.* 2006; Braschi & Wilson 2006;
63 Castro-Borges *et al.* 2011a; Castro-Borges *et al.* 2011b) have opened new
64 opportunities for diagnosis, drug discovery and vaccine research. In several proteomic
65 studies, three different enzymes involved in nucleotide metabolism were identified:
66 alkaline phosphatase (SmAP), phosphodiesterase (SmNPP-5) and diphosphohydrolase
67 (SmNTPDase). These enzymes were determined to be components of the tegument
68 (van Balkom *et al.* 2005), surface membrane-associated (Braschi *et al.* 2006) and
69 surface-exposed proteins accessible to biotin labeling (Braschi & Wilson 2006). The
70 tegument is a thin syncytial layer that covers the whole parasite, limited by a

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71 multilaminate surface membrane complex, which constitutes the major host–parasite

72 interface (Skelly & Alan Wilson 2006).

73 In spite of the fact that SmAP has long been used as a marker for tegument

74 membranes (Roberts *et al.* 1983), characterization of Alkaline Phosphatase from *S.*

75 *mansoni* at the molecular level was performed only recently (Araujo-Montoya *et al.*

76 2011; Bhardwaj & Skelly 2011). Both studies concluded that the enzyme was

77 expressed along the parasite life cycle and showed a widespread distribution in adult

78 worms. One study determined the surface activity of the enzyme, which was not

79 inhibited by antibodies (Araujo-Montoya *et al.* 2011). The other study knocked down

80 its expression by RNAi, which did not alter the parasite's morphology or behavior

81 (Bhardwaj & Skelly 2011).

82 We have performed the molecular characterization of Phosphodiesterase-5

83 (SmNPP-5), showing its increased expression levels in the transition to intra-host

84 stages. The surface enzymatic activity of the protein was demonstrated in living adult

85 worms as well as its inhibition by anti-SmNPP-5 antibodies (Rofatto *et al.* 2009).

86 Furthermore it was demonstrated that parasites, whose expression of the SmNPP-5

87 gene was suppressed at the time of host infection, were greatly impaired in their

88 ability to establish infection (Bhardwaj *et al.* 2011). Additionally, the SmNTPDase

89 enzyme has been shown to have 2 isoforms, both in the tegument, one secreted and

90 more abundant in the syncytium and the other detected on the tegument basal and

91 apical membranes (DeMarco *et al.* 2003; Levano-Garcia *et al.* 2007).

92 Surface localization is an important characteristic for vaccine candidates, to

93 allow interaction with the host immune system. Therefore, immunization using a

94 combination of these 3 enzymes has been proposed as a potential vaccine strategy

95 (Braschi *et al.* 2006). Furthermore, it has been proposed that these enzymes may be

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96 involved in the purinergic signaling at the endothelial tissue. Since parasite movement
97 and oviposition may cause injury and release DAMPs (damage-associated molecular
98 pattern molecules) such as ATP, these proteins may modulate host inflammatory
99 response by regulating the levels of ATP and ADP. Based on this hypothesis, these
100 molecules were proposed as promising drug targets and vaccine candidates (Bhardwaj
101 & Skelly 2009).

102 Altogether these data suggest that the three tegument nucleotidases are likely
103 to be located on the host-parasite interface, performing overlapping functions relevant
104 for parasite survival, so the use of a cocktail of these antigens in vaccine experiments
105 may elicit a synergistic effect. In this study, we evaluated the potential of these
106 enzymes involved in nucleotide metabolism as vaccine candidates with and without a
107 subcurative PZQ treatment.

108

109 **2. Materials and Methods**

110

111 **2.1. Ethics Statement**

112

113 Animal experiments were conducted in accordance with the Brazilian Federal
114 Law number 11.794, which regulates the scientific use of animals. All animals were
115 handled in strict agreement with good animal practice according to the institutions
116 guidelines for animal husbandry and all protocols were approved by the Committee of
117 Ethics on the Use of Animals from Instituto Butantan (CEUAIB) under license
118 595-09.

119

120

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121 **2.2. Parasite maintenance**

122 The *S. mansoni* (BH strain) entire life cycle is maintained routinely on
123 *Biomphalaria glabrata* snails and hamsters at Laboratório de Parasitologia – Instituto
124 Butantan. Cercariae were prepared for mice infection or challenge by exposing
125 infected snails to light for 2 h to induce shedding; their number and viability were
126 determined using a light microscope prior to infection.

127

128 **2.3. Expression and purification of recombinant tegument nucleotidases**

129

130 To produce the recombinant tegument nucleotidases we used hexahistidine-tag
131 expression plasmids in which the respective cDNA sequences were previously
132 directionally cloned into pAE-6His vector for SmAP and SmNPP-5 (Rofatto *et al.*
133 2009; Araujo-Montoya *et al.* 2011) and pET 21-b vector (Novagen) for SmNTPDase
134 (DeMarco *et al.* 2003). These plasmids were used to transform *E. coli* BL21 Star
135 (DE3) pLys (Invitrogen), which were grown in LB plus ampicillin (100 µg/mL) until
136 reaching OD_{600} 0.6. Isopropyl-β-D-thiogalactopyranoside (Invitrogen) was added to
137 the culture to a final concentration of 1 mM and cells were incubated for 4 h at 37 °C.
138 Then cells were harvested by centrifugation and resuspended in 50 ml of lysis buffer
139 (50 mM sodium phosphate pH 8.5, 0.3 M NaCl). The cell suspension was passed
140 three times (2000 psi each) through a French press and the crude homogenate was
141 centrifuged at 20,000×g for 30 min. The pelleted inclusion bodies were washed twice
142 with wash buffer (lysis buffer plus 2 M urea) and finally resuspended in solubilization
143 buffer (lysis buffer, 5 mM β-mercaptoethanol, 20 mM imidazole, 8 M urea).

144 The recombinant proteins were then purified by immobilized metal affinity
145 chromatography using the Äkta Prime system (GE Healthcare) under denaturing

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146 conditions. Briefly, the samples were loaded onto a 5 mL bed volume Ni^{2+}-NTA
147 column (GE Healthcare) pre-equilibrated with the same buffer. The columns were
148 washed with 10 bed volumes of the equilibration buffer and then eluted with a 20–500
149 mM imidazole linear gradient. The main peak was pooled and the protein purity of
150 fractions was assessed using sodium dodecyl sulfate – polyacrylamide gel
151 electrophoresis (SDS-PAGE). Further, the elution fraction was exchanged with
152 Phosphate Buffer Saline pH 7.4 (PBS) prior to use of these proteins.
153

154 **2.4. Immunization, challenge and worm recovery**

155

156 Six to eight week-old female C57BL/6 mice from the Faculdade de Medicina
157 – USP animal facility were supplied with food and water *ad libitum*. The animals
158 were divided into five groups with 10 mice each: Control, SmAP, SmNPP-5,
159 SmNTPDase and the 3 combined proteins (3Teg-Nucl). The animals were lightly
160 anaesthetized (45 mg/kg of Ketamine and 10 mg/kg of Xylazine) before they were
161 injected subcutaneously in the nape of the neck with 3 doses, at 15-day intervals. The
162 SmAP, SmNPP-5 and the SmNTPDase animals were immunized with 25 µg of the
163 respective recombinant protein mixed with Freund's Complete Adjuvant in the first
164 dose (Sigma) or Freund's Incomplete Adjuvant (Sigma) in the subsequent doses. The
165 animals immunized with 3Teg-Nucl received 25 µg of each recombinant protein with
166 Freund's adjuvant, while the Control group was inoculated with PBS with Freund's
167 adjuvant using the same immunization protocol.
168 Fifteen days after the last dose, mice were challenged with cercariae. The
169 animals were anaesthetized with Ketamine (90 mg/kg) and Xylazine (10 mg/kg) and
170 exposed percutaneously for 30 min to 100 cercariae in water on their shaven

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171 abdomens by the ring method. Forty five days after percutaneous challenge, animals
172 were euthanized with a lethal dose of urethane solution (150 mg/mL) (Sigma).
173 Perfusion fluid (Saline solution plus 500 units/L of Heparin) was pumped into the
174 aorta artery, and perfused worms were collected from the hepatic portal vein and
175 counted using a stereomicroscope.

176 The protection was calculated by comparing the number of worms recovered
177 from each vaccinated group with the control group, using the formula:

178 **Protection level (%) = [(WRCG-WREG/WRCG)]*100,**

179 where WRCG = worms recovered from control group and WREG = worms recovered
180 from experimental group. To evaluate the liver-trapped egg burden, a piece of the
181 liver from each mouse was removed, weighed, digested and homogenized for 1 h at
182 37 °C in 5 mL of 10% KOH. The number of eggs per gram of liver was compared to
183 the Control group (Cardoso *et al.* 2008). All these data were statistically compared by
184 ANOVA followed by Dunnett's test and a $\rho \leq 0.05$ was considered significant.

185

186 **2.5. Measurement of specific anti-nucleotidases antibody levels**

187

188 Mice were bled from the retro orbital plexus one day before cercariae
189 challenge (day 44) and one day before mouse perfusion (day 89). The blood was
190 processed and the sera collected were used to perform indirect ELISA assays to
191 confirm the levels of specific anti-nucleotidases total IgG, IgG1 and IgG2a. Maxisorp
192 96-well microtiter plates (Nunc) were coated with 5 µg/mL of recombinant SmAP,
193 SmNPP-5 or SmNTPDase in carbonate-bicarbonate buffer, pH 9.6 for 18 h at 4 °C,
194 then blocked for 1 h at 37 °C with 200 µL/well phosphate buffer saline, pH 7.2 with
195 0.05% Tween-20 plus 10% fetal bovine sera (PBS-T). The serum of each animal was

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196 serially diluted starting at 1:50, and incubated for 1 h at 37 °C. Plate-bound antibodies
197 were detected by goat anti-mouse IgG, IgG1 or IgG2a (Southern Biotech) diluted in
198 PBS-T 1:10,000, 1:1000 or 1:1000, respectively. Finally the plates were incubated
199 with peroxidase-conjugated rabbit anti-goat IgG diluted 1:20,000 for 1 h at 37 °C and
200 color reaction was developed by incubation of OPD (Sigma) in citrate buffer, pH 5.0
201 plus 0.04% H_2O_2 for 15 min and stopped with 4 M sulfuric acid. The plates were read
202 at 492 nm in an ELISA plate reader (Labsystems). The standard curves were
203 generated using mouse IgG, IgG1, and IgG2a (Southern Biotech). Statistical
204 comparisons were performed with ANOVA followed by a Tukey's pairwise
205 comparison. A ρ value ≤0.05 was considered statistically significant.
206
207 **2.6. Cytokine Analysis**
208
209 Cytokine experiments were performed using splenocyte cultures from
210 individual mice (4 animals for each group) immunized with isolated or combined
211 tegument nucleotidases as described above. Splenocytes were isolated from macerated
212 spleens 15 days after the third immunization, and washed twice with sterile PBS.
213 After washing, the cells were adjusted to $1x10^6$ cells in 1 ml of RPMI 1640 medium
214 (Invitrogen) supplemented with 10% fetal bovine sera, 100 U/mL of penicillin G
215 sodium, 100 µg/mL of streptomycin sulfate, 250 ng/mL of amphotericin B and
216 polymyxin B (30 µg/ml). Splenocytes were restimulated in culture with each
217 recombinant nucleotidase (5 µg/mL) for 48 h in an incubator at 37 °C with 5% CO_2.
218 RNA from splenocytes was extracted and purified using Trizol (Invitrogen)
219 and cDNA was synthesized using the Superscript III Reverse Transcriptase kit
220 (Invitrogen). RT-PCR of the sample was performed in a thermal cycler under the

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221 manufacturer instructions using random hexamers. The following genes were
222 analyzed using Taqman Gene Expression Assays for gene quantification: β-actin
223 (4352933E) as a housekeeping gene, MyD88 (Mm00440338_m1), NF-κB1
224 (Mm00476361_m1), NF-κB2 (Mm00479807_m1), IL-4 (Mm00445259_m1), IL-5
225 (Mm00439645_m1), IL-10 (Mm00439616_m1), IL-12p40 (Mm01288992_m1),
226 IL-13 (Mm00434206_g1), IL-17 (Mm00439619_m1), IFN-γ (Mm00801778_m1),
227 TNF-α (Mm00443258_m1) and TGF-β (Mm00441724_m1). The level of β-actin was
228 used to normalize the amounts of assayable RNA in each sample and quantitation of
229 relative differences in expression were finally calculated using the comparative ΔΔCt
230 method (Livak & Schmittgen 2001). Statistical comparisons were performed with
231 ANOVA followed by a Tukey's pairwise comparison. A ρ value ≤0.05 was considered
232 statistically significant.
233
234 **2.7. Analysis of Worm morphology after subcurative Praziquantel chemotherapy**
235
236 Six to eight week-old female C57BL/6 mice were divided into control and
237 subcurative PZQ treated groups containing 4 animals each and mice were infected
238 with cercariae as described above. The subcurative PZQ treated animals received two
239 doses of 150 mg/kg of PZQ by gavage, 35 and 37 days after infection respectively;
240 saline solution was administered for control animals (adapted from Doenhoff, Modha
241 & Lambertucci 1988). Two hours after the second PZQ dose, animals were perfused
242 to recover parasites as described above. Recovered worms were fixed in
243 Formalin–Acetic acid–Ethanol solution and analyzed with a laser confocal
244 microscope (LSM 510 META, Zeiss) at 488 nm wavelength of excitation and 500-550

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245 nm wavelength of emission. The tubercle numbers from control and treated groups
246 were compared with Student's T-test (based on Moraes *et al.* 2011).
247
248 **3. Results and Discussion**
249
250 **3.1. Humoral immune response induced by Immunization with Tegument**
251 **Nucleotidases**
252
253 In order to investigate the immunogenicity of the tegument nucleotidases,
254 groups of C57BL/6 mice were immunized with either the isolated proteins or with a
255 combination of the 3 tegument nucleotidases (3Teg-Nucl), in a 3-dose schedule with 2
256 weeks intervals, including the respective controls. The animals were challenged 2
257 weeks after the last dose and perfused after 45 days. Sera were collected before
258 challenge (day 44) and before perfusion (day 89) (Figure 1A). Total IgG, IgG1 and
259 IgG2a antibody levels against SmAP, SmNPP-5 and SmNTPDase were determined.
260 Anti-SmAP total IgG levels were higher than anti-SmNPP-5 and anti-SmNTPDase
261 levels before and after cercaria challenge, irrespective of whether they were
262 administered isolated or combined (Figure 1B and C). No difference was observed in
263 Total IgG levels of the groups before and after challenge (Figure 1B and C) and all
264 groups differ from the Control group (data not shown).
265 Immunization of mice with SmAP induced the highest IgG1/IgG2a ratio,
266 indicating a Th2 predominance, which decreased by half following challenge.
267 Anti-SmNPP-5 IgG1/IgG2a ratio also decreased following challenge, but was higher
268 than SmNTPDase before challenge. On the other hand, SmNTPDase ratio was the
269 lowest and was not altered by challenge (Figure 2A-C). When the proteins were

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270 combined, the IgG1/IgG2a ratio of most groups seemed higher and not to be altered
271 by challenge (Supplementary Figure 1A-C).
272
273 **3.2. Cellular immune response induced by Immunization with Tegument**
274 **Nucleotidases**
275
276 The cellular immune response induced by the immunization with the tegument
277 nucleotidases was evaluated in groups of animals immunized with either the isolated
278 proteins or with a combination of the 3 tegument nucleotidases (3Teg-Nucl) in a
279 3-dose schedule with 2-week intervals. After 2 weeks, the splenocytes were collected
280 and the cellular immune response was evaluated by Real-Time RT-PCR after *in vitro*
281 stimulation with each protein. When the animals were immunized with the isolated
282 proteins, SmAP induced extremely high expression levels of the inflammatory
283 cytokine, TNF-α, and SmNPP-5 induced a higher level of the Th1 cytokine,
284 IL-12p40, and both showed higher expression of the Th17 cytokine, IL-17. However,
285 the cellular response induced by SmNPP-5 was also characterized by expression of
286 the regulatory cytokine, TGF-β, (Figure 3). SmNTPDase showed induction of
287 relatively lower levels of cellular immune responses. When the proteins were
288 combined, similar results were obtained, with SmAP inducing high levels of TNF-α,
289 associated with increased levels of IL-17. SmNPP-5 in combination with the other
290 nucleotidases also produced increased levels of IL-17, however, with a lower
291 expression of IL12p40 and this was again counter-balanced by significant levels of
292 TGF-β (Supplementary Figure 2). Interestingly, no differences in expression of
293 characteristic Th2 cytokines, such as IL-4, IL-5 and IL-13 were observed, either

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294 induced by the immunization with isolated or combined proteins, although the
295 antibody isotype profiles indicated otherwise.
296
297 **3.3. Evaluation of Worm Burden and Liver-Trapped Eggs induced by Standard**
298 **Immunization Protocol with Tegument Nucleotidases**
299
300 The protective potential of the tegument nucleotidases was evaluated by
301 immunization of the animals with either the isolated proteins or with a combination of
302 the 3 proteins (3Teg-Nucl). Mice were challenged 2 weeks after the last dose and after
303 45 days the worms were recovered from the mesenteric vein of immunized and
304 control animals. A piece of liver was also collected to evaluate liver-trapped egg
305 counts. It can be observed that immunization with either the isolated or combined
306 proteins did not reduce the worm burden of immunized mice nor did it reduce the
307 amount of liver-trapped eggs (Figure 4 A and B). This is one representative of two
308 independent experiments.
309
310 **3.4. Humoral immune response induced by Immunization with Tegument**
311 **Nucleotidases associated with Praziquantel subcurative treatment**
312
313 Since it has been shown that treatment of *S. mansoni*-infected mice with
314 subcurative doses of PZQ would induce alterations in the parasites' tegument (Liang
315 *et al.* 2002), we initially evaluated the integrity of the parasites surfaces by
316 autofluorescence and their survival following the subcurative doses of PZQ. Under
317 the conditions used in our experiments, parasites' teguments were not significantly
318 altered and their survival was not impaired (Supplementary Figure 3). Therefore, we

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319 evaluated the combined effect of immunization with tegument nucleotidases
320 associated with subcurative doses of PZQ (Figure 5A). As in the previous experiment,
321 SmAP and SmNTPDase showed comparable levels of Total IgG, higher than
322 SmNPP-5, either as isolated proteins or administered as a combination, 3Teg-Nucl
323 (Figure 5B and C). It is interesting to note that, the total anti-SmAP IgG before
324 challenge was 6,600 µg/mL in the first experiment, which was reduced to 4,350
325 µg/mL before perfusion (a reduction of 34%, although not statistically significant,
326 Figure 1B). When the proteins were combined the reduction was even larger (44%,
327 although still not significant, Figure 1C). However, when the challenge was
328 performed in the presence of PZQ, either with the isolated proteins or combined, the
329 level of anti-SmAP was comparable (Figure 5B and C). This data may indicate that
330 the protein could be more accessible to the immune system after PZQ treatment, as
331 previously reported (Fallon *et al.* 1994).
332 The sera of mice immunized with tegument nucleotidases and treated with
333 subcurative doses of PZQ were also evaluated as to the IgG1/IgG2a ratio, confirming
334 the Th2 predominance of the immune response before challenge and administration of
335 the PZQ doses at day 44, as previously determined (Figure 6A-C). Following the
336 challenge and PZQ treatments, the immune response for most of the groups displayed
337 a reduction in the IgG1/IgG2a ratio, indicating a shift towards Th1 characteristics
338 (Figures 6A-C, and Supplementary Figure 4). In both experiments with and without
339 PZQ treatment, it is clear that before challenge, SmAP and SmNTPDase were more
340 immunogenic than SmNPP-5, inducing higher levels of antibodies, mostly IgG1, and
341 after challenge, there is a general decrease in the levels of IgG1 and IgG2a against all
342 antigens.
343

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344 **3.5. Reduction in Worm burden and Liver-Trapped Eggs induced by**
345 **Immunization with Tegument Nucleotidases associated with Praziquantel**
346 **subcurative treatment**

347

348 It has been demonstrated that chemotherapy with PZQ interacts synergistically
349 with host immune responses for parasite elimination (Brindley & Sher 1987; Fallon &
350 Doenhoff 1995). Therefore we combined immunization and challenge protocols with
351 a subcurative treatment, expecting that chemotherapy would enhance the efficacy of
352 the immunizations. Groups of animals immunized with tegument nucleotidases were
353 challenged with live cercaria and received two subcurative doses of PZQ after 35 and
354 37 days. Adult worms were recovered from the mesenteric veins of perfused mice, 45
355 days after challenge (Figure 5A). In the first experiment, immunization with the
356 combined proteins, 3Teg-Nucl, followed by PZQ treatment, induced a 41% reduction
357 in worm burden, as compared to the Control (Figure 7A). Furthermore, this protocol
358 also induced a 54% reduction in egg deposition (Figure 7B).
359 In order to determine the contribution of each nucleotidase to the observed
360 protection, we performed a second challenge experiment, also including additional
361 groups of animals immunized with the isolated proteins. In this experiment, it was
362 possible to confirm the protective effect of 3Teg-Nucl, which displayed a worm
363 burden reduction of 46%, and to establish that the main protein contributing to worm
364 burden reduction was SmAP, which conferred a reduction of 41% (Figure 7C). The
365 liver-trapped egg count also indicated its contribution, although statistical significance
366 was not reached (Figure 7D).
367 SmAP is clearly more immunogenic than the other tegument nucleotidases, as
368 suggested by the higher antibody and cytokine expression levels induced, which could

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369 be one of the factors leading to its higher protective potential. The high IgG1/IgG2a
370 ratio induced by immunization with SmAP indicates the induction of a
371 Th2-predominant immune response. However, the cytokine profile, with low
372 expression of Th2 cytokines, and higher expression of pro-inflammatory cytokines,
373 suggests a more mixed response. The results indicate that the inflammatory cytokine,
374 TNF-α, associated with increased levels of IL-17, could also be involved in the
375 protective mechanism. Despite the evidence that induction of a Th1 immune response
376 would be more effective in preventing schistosomiasis in mice (Hoffmann *et al.* 1999;
377 Cardoso *et al.* 2008; Teixeira de Melo *et al.* 2010), it is well documented that a Th2 or
378 a mixed immune response can also reduce parasite worm burden following infection,
379 if it is strong enough (Hoffmann *et al.* 1999; El Ridi & Tallima 2012; Martins *et al.*
380 2012).
381 Although we have previously shown that anti-SmNPP-5 antibodies induced by
382 this recombinant protein inhibit the activity of SmNPP-5 in live parasites (Rofatto *et*
383 *al.* 2009), the recombinant protein was not in the native conformation, i.e., not
384 properly folded. Furthermore, our results showed that immunization of mice with
385 SmNPP-5 induced lower humoral immune responses. On the other hand, the T-cell
386 immune response that was induced was counter-balanced by a strong regulatory
387 response, characterized by TGF-β. Moreover, it has been demonstrated that
388 immunization with NPP-5 from *S. japonicum*, resulted in a partial worm burden
389 reduction following a subsequent challenge (Zhang *et al.* 2011). Therefore it is
390 possible that a properly folded protein would show a different result. SmNTPDase
391 was more immunogenic than SmNPP-5, displaying higher levels of antibodies, but
392 showed lower induction of cellular immune responses as determined by the
393 expression levels of the evaluated cytokines. Hence, the immune responses induced

394 by the different nucleotidases may justify the different protective efficacies against
395 challenge.

396 Although Bhardwaj & Skelly (2009) hypothesized that the schistosoma
397 tegument nucleotidases function in the human bloodstream, their real physiological
398 functions and biological substrates remain uncertain. As we could not verify any
399 synergistic effect following immunization with the combined tegument nucleotidases,
400 it is possible that these proteins may not share functions/substrates or be
401 physiologically correlated. Furthermore, suppression of SmNPP-5 expression impairs
402 parasite infection (Bhardwaj *et al.* 2011), while suppression of SmAP expression has
403 no effect on cultured parasites (Bhardwaj & Skelly 2011); these data suggest a more
404 vital function for SmNPP-5 than for SmAP on parasite survival. However, our results
405 showed that only immunization with SmAP associated with PZQ subcurative
406 treatment leads to worm burden reduction. Based on these data it seems that the
407 efficacy of SmAP immunization may not be related to a more relevant function of this
408 protein.

409 Even though more immunogenic, immunization with SmAP was only effective
410 in reducing worm burden when associated with PZQ treatment; this may be due to a
411 non-specific parasite injury, which involves some kind of alteration of the parasite's
412 immune evasion mechanisms (Brindley & Sher 1987). Alternatively, this effect could
413 be specific for SmAP in a synergistic way, since it has been demonstrated that PZQ
414 treatment promotes exposure of a few tegument antigens, among them SmAP
415 (Brindley *et al.* 1989; Fallon *et al.* 1994). Furthermore, of the three proteins, it is
416 probably the most exposed on the surface of the parasite, since it is the only protein
417 identified by proteomics in the fraction of proteins released following treatment of
418 live parasites with phosphatidylinositol-phospholipase C (Castro-Borges *et al.* 2011a;

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419 Castro-Borges *et al.* 2011b). Therefore, the level of exposure of the protein to the
420 immune system would be another explanation for the synergistically protective
421 efficacy of SmAP. In this case, the use of subcurative PZQ treatment was a valuable
422 tool to make this important characteristic more evident.

423 These results reinforce the perspective of combining immunization strategies
424 with chemotherapeutic treatments. Despite the fact that good vaccine candidates
425 should reduce worm burden alone, our experiments were designed to allow
426 visualization of the synergistic effect of immunization and chemotherapy on the
427 parasites. Similar approaches have been tested in the past, using parasite extract as
428 antigens (Fallon & Doenhoff 1995) and have recently been proposed again for other
429 recombinant tegument proteins for *S. japonicum* (Zhang *et al.* 2012). Although these
430 conditions will not be reproduced in human treatment, it is probable that, vaccine
431 administration will occur concurrent to chemotherapy treatment. Therefore, it is
432 interesting to envisage and evaluate different schedules of immunizations and
433 chemotherapeutic treatments, aiming to select the more appropriate to be employed in
434 human trials.

435

436 **4. Conclusion**

437

438 We evaluated three recombinant tegument nucleotidases from *S. mansoni* that
439 have been extensively proposed as vaccine antigens in the literature. SmAP was the
440 most immunogenic of them, inducing a mixed Th2 humoral immune response with an
441 inflammatory cellular immune response, characterized by high TNF-α and IL-17
442 production. Although it was not protective alone in the formulation used, it effectively
443 reduced worm burden, when associated with a subcurative PZQ treatment. Both

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444 SmNPP-5 and SmNTDPase showed lower immunogenicity in the presentation forms
445 used; SmNPP-5 displayed a Th1 cellular response counter-balanced by a regulatory
446 immune response, while SmNTDPase induced a Th2-predominant humoral immune
447 response. Our data suggested that SmAP is the more promising tegument nucleotidase
448 as vaccine candidate and advocates for additional investigations, from improving its
449 expression and folding, through to its evaluation with different adjuvants and
450 immunization protocols. We also consider that SmAP may be useful in a combination
451 with other protective antigens, particularly if the other proteins induce an immune
452 response that targets the forming tegument after infection. Finally, our studies
453 highlight the importance of investigating vaccine candidates in different immunization
454 schedules combined with chemotherapeutic agents.

455

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457

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460

461 **6. References**

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Figure 1

Standard immunization schedule and total IgG levels induced by immunization with tegument nucleotidases.

A – Immunization, bleeding, challenge and mouse perfusion schedule. B – Specific total IgG induced by immunization with isolated nucleotidases before and after challenge. C – Specific total IgG induced by immunization with combined nucleotidases (3Teg-Nucl) before and after challenge. The bars are Mean±SEM; * = $\rho \leq 0.05$.

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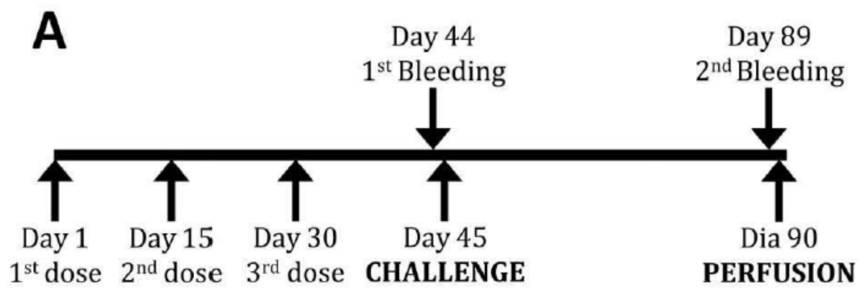

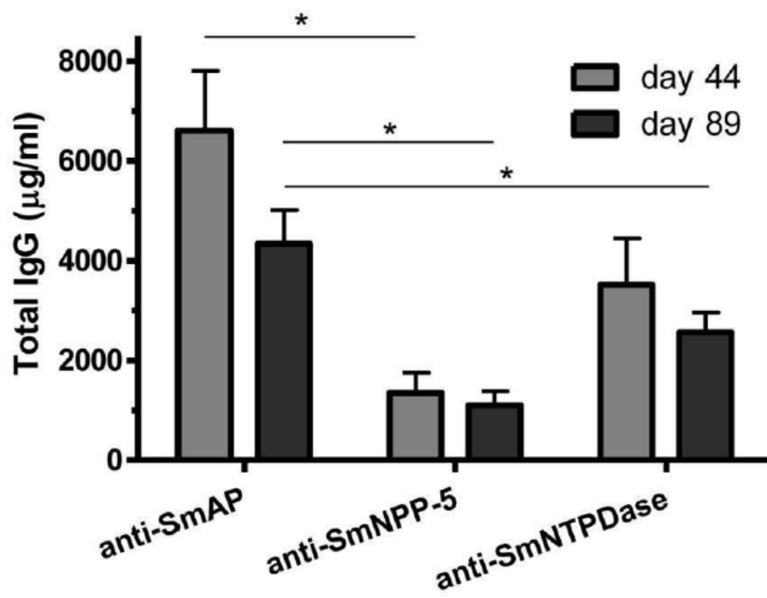

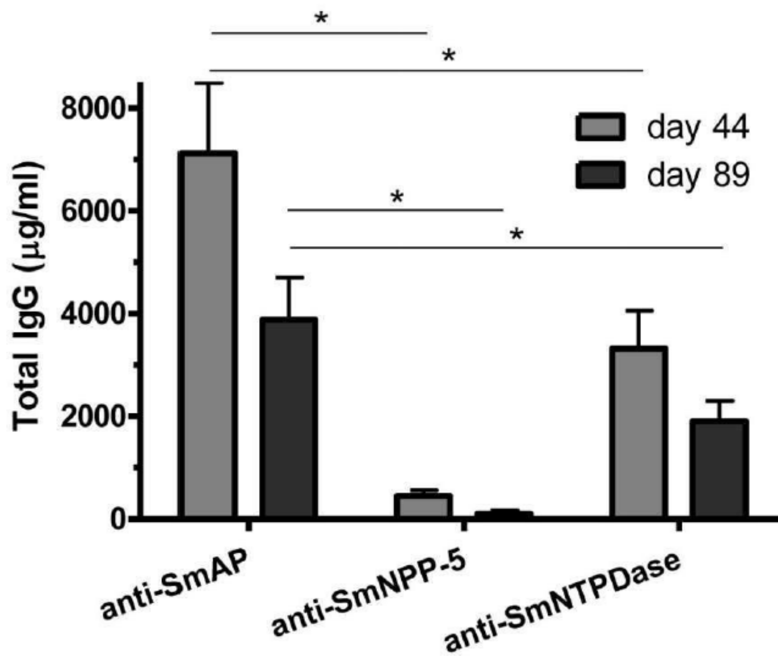

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Figure 2

IgG1 and IgG2a levels induced by immunization with isolated tegument nucleotidases.

A – Specific IgG1 and IgG2a levels induced by immunization with SmAP before and after challenge. B – Specific IgG1 and IgG2a levels induced by immunization with SmNPP-5 before and after challenge. C – Specific IgG1 and IgG2a levels induced by immunization with SmNTPDse before and after challenge. The numbers over the bars are the IgG1/IgG2a ratios.

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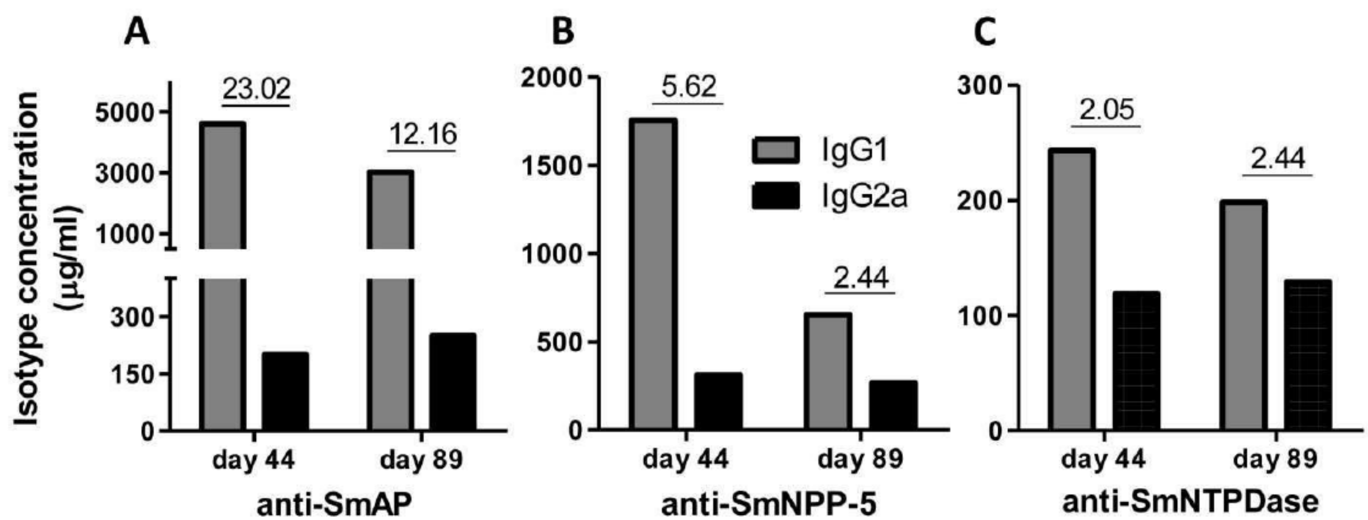

Figure 3

Cellular immune response induced by immunization with isolated tegument nucleotidases and *in vitro* restimulation with each protein evaluated by qPCR.

The bars are Mean±SEM; * = $\rho \leq 0.05$.

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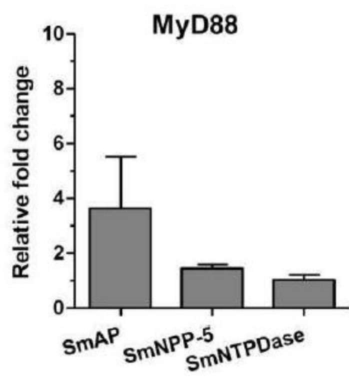

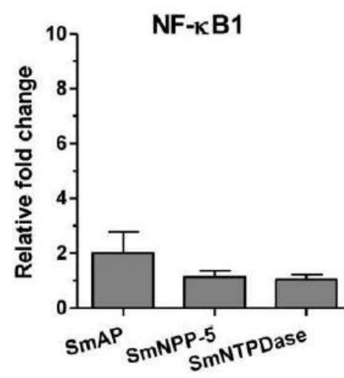

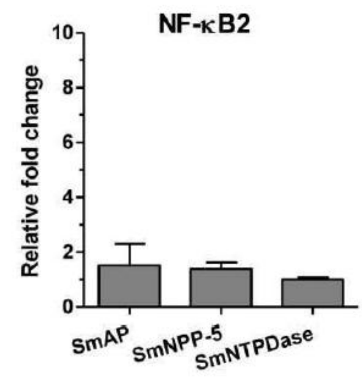

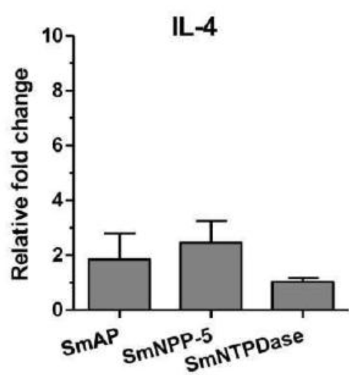

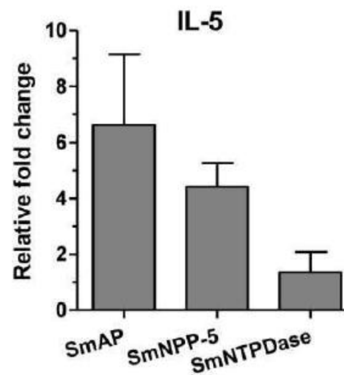

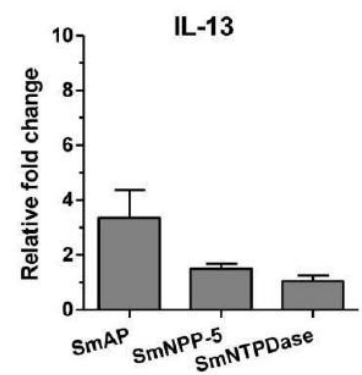

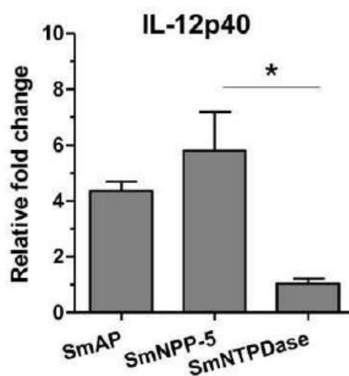

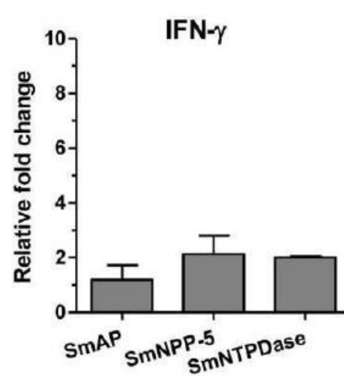

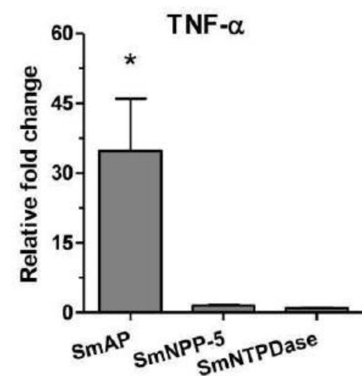

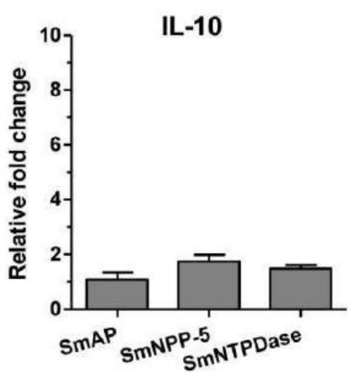

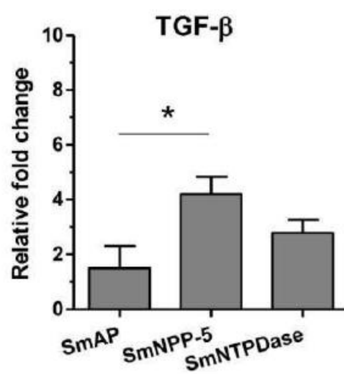

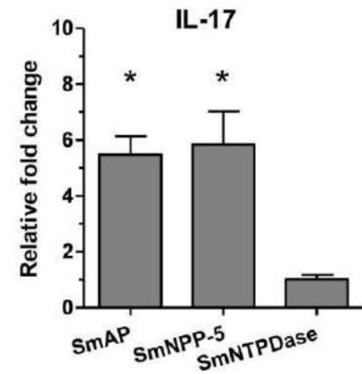

Figure 4

Evaluation of tegument nucleotidases as vaccine candidates by standard immunization protocol.

A - Worm burden dispersion of mice immunized with tegument nucleotidaes and challenged with live cercariae; the lines represent the Means. B - Liver-Trapped Eggs from mice immunized with tegument nucleotidases, the bars are the Mean±SEM.

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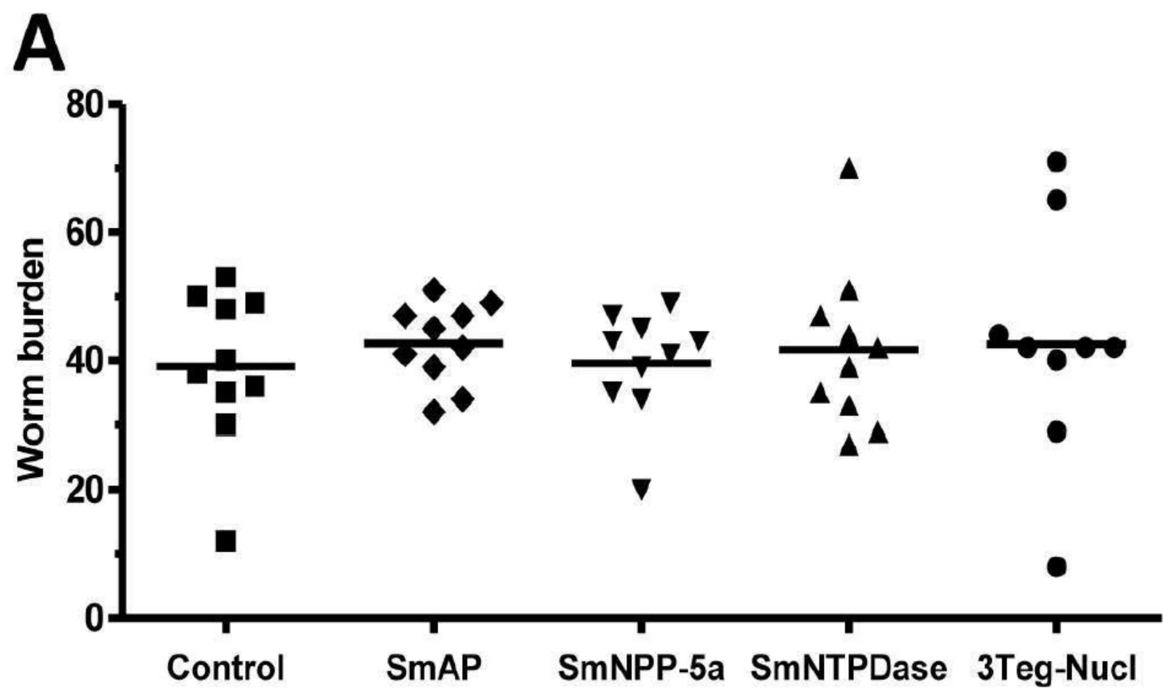

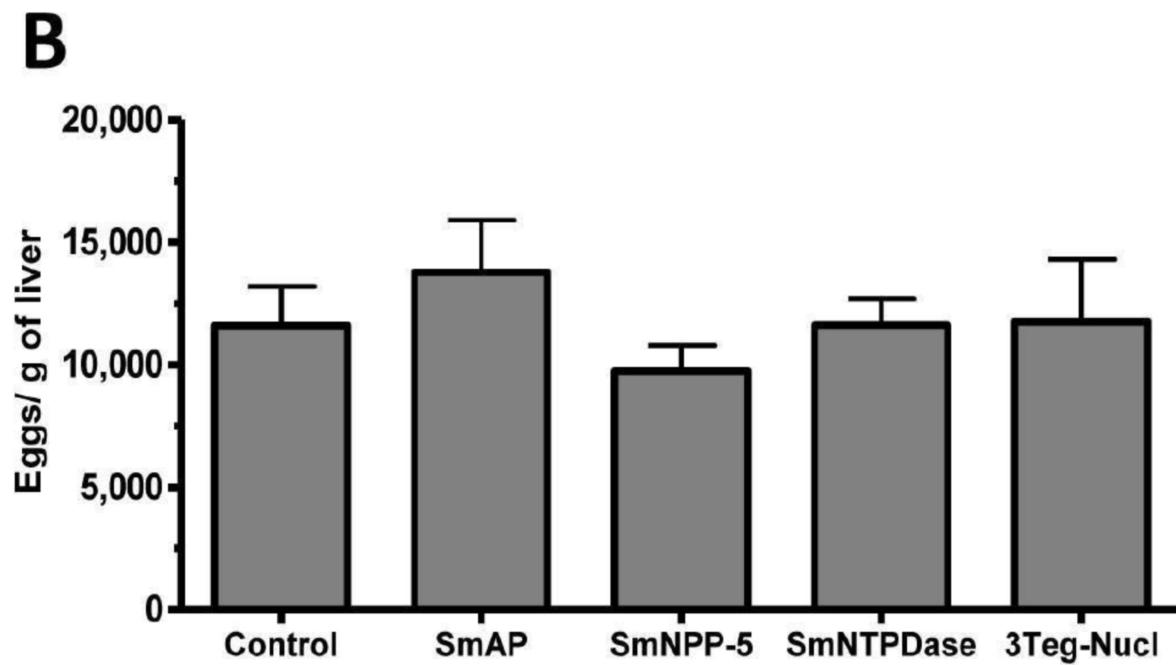

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Figure 5

Schedule for immunization with tegument nucleotidases associated with praziquantel subcurative treatment and induction of total IgG.

A – Immunization, bleeding, challenge, subcurative praziquantel treatment and perfusion schedule. B – Specific total IgG induced by immunization with isolated nucleotidase before and after challenge and praziquantel therapy. C – Specific total IgG induced by immunization with combined nucleotidases (3Teg-Nucl) before and after challenge and praziquantel therapy. The bars are Mean±SEM; * = ρ≤0.05.Mean±SEM.

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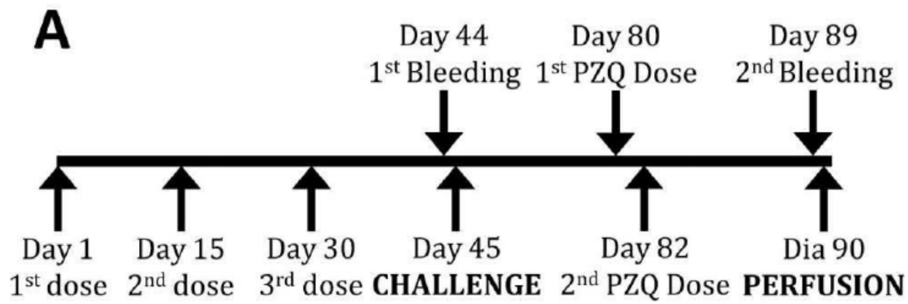

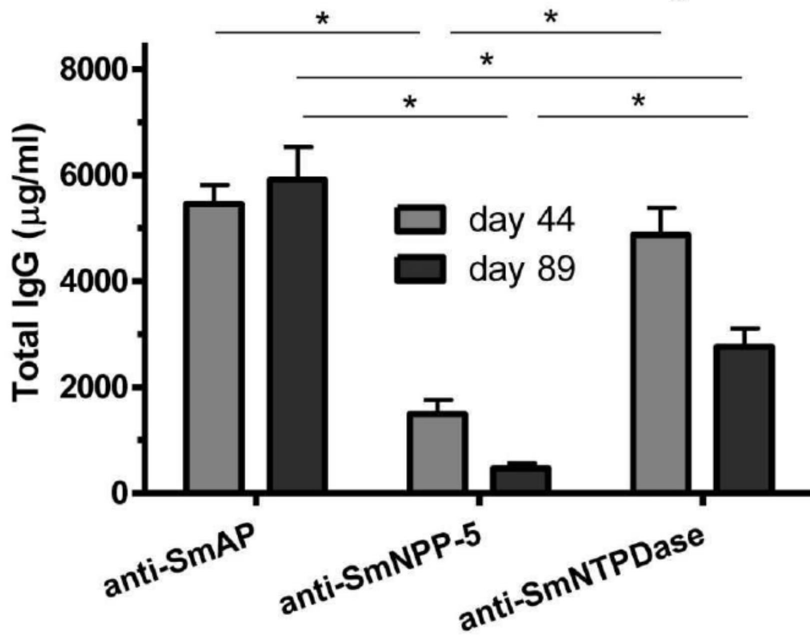

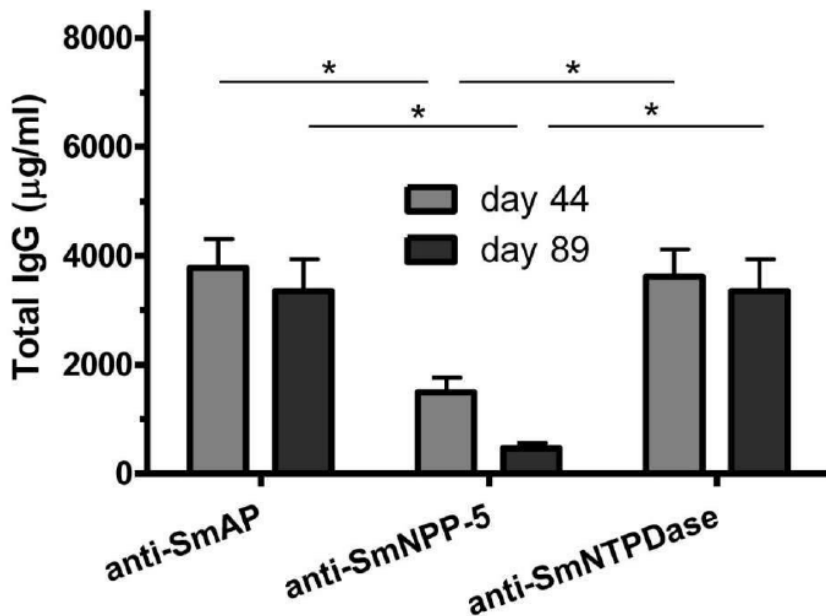

Figure 6

IgG1 and IgG2a levels induced by immunization with isolated tegument nucleotidases associated with praziquantel subcurative treatment.

A – Specific IgG1 and IgG2a levels induced by immunization with SmAP before challenge and after praziquantel treatment. B – Specific IgG1 and IgG2a levels induced by immunization with SmNPP-5 after praziquantel treatment and before challenge. C – IgG1 and IgG2a levels induced by immunization with SmNTPDse before challenge and after praziquantel treatment. The numbers over the bars are the IgG1/IgG2a ratios.

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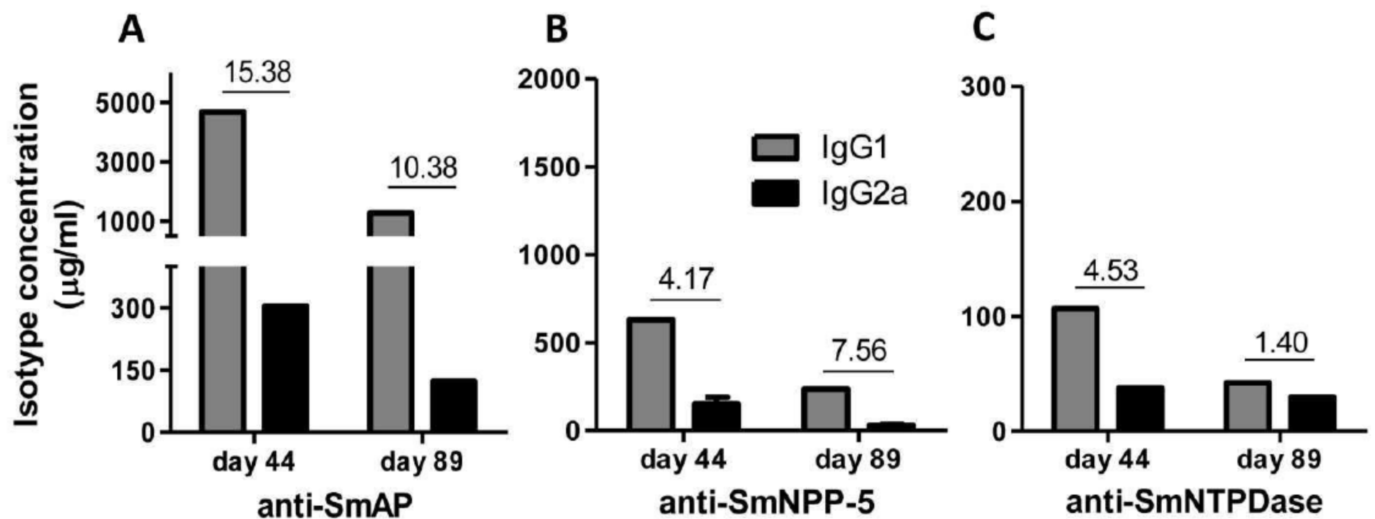
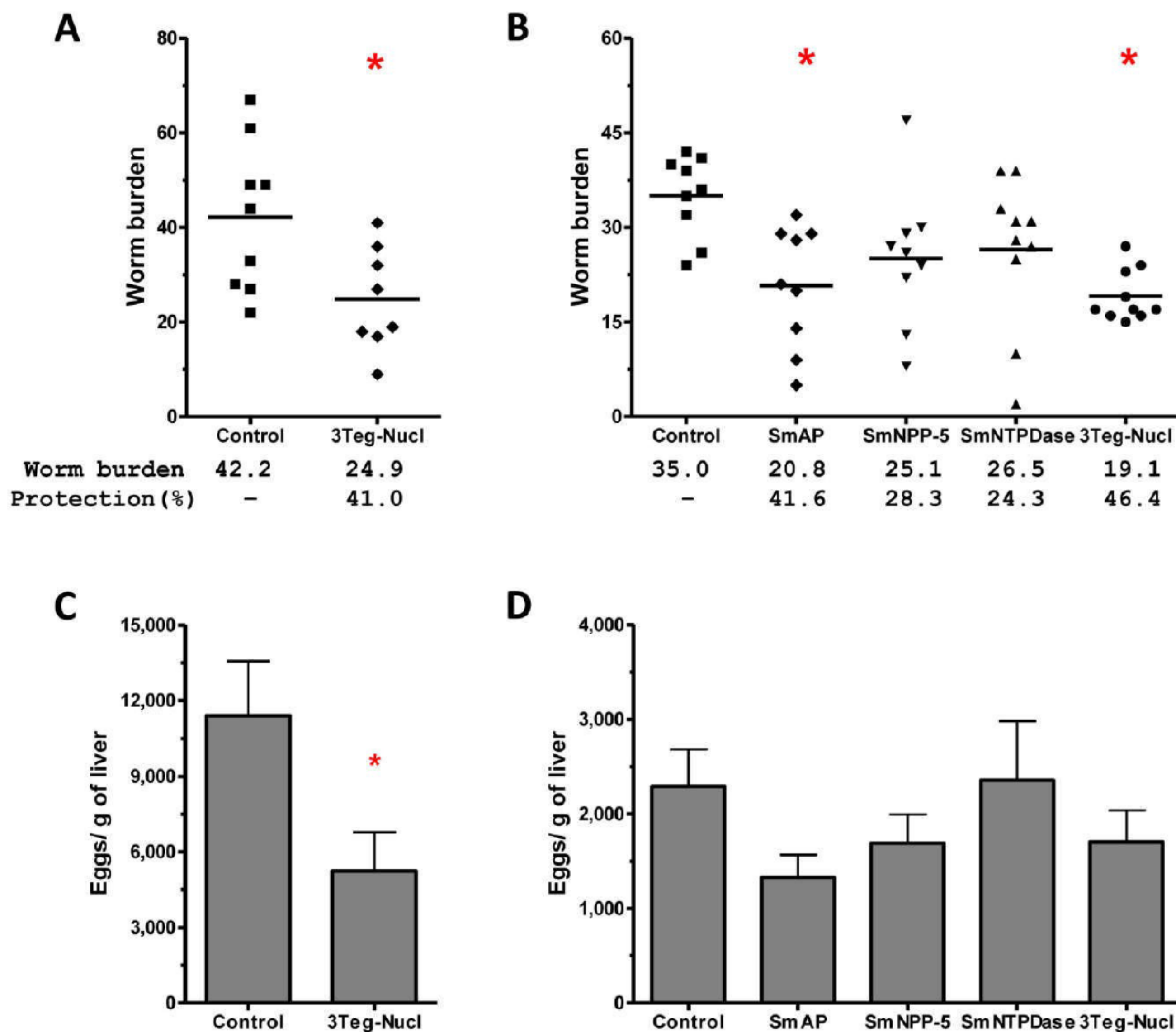


Figure 7

Evaluation of the protective potential of tegument nucleotidases associated with praziquantel subcurative treatment.

A and B – Worm burden dispersion and percentage of protection from mice immunized with tegument nucleotidases and treated with subcurative chemotherapy; the lines represent the Means. C and D – Liver-Trapped Eggs reduction induced by immunization with tegument nucleotidases associated with praziquantel subcurative treatment; the bars are the Mean±SEM. * = ρ≤0.05.



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Succinctus

Tissue expression patterns of *Schistosoma mansoni* Venom Allergen-Like proteins 6 and 7

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ABSTRACT

The *Schistosoma mansoni* Venom Allergen-Like proteins (*Sm*VALs) are members of the SCP/TAPS (Sperm-Coating Protein/Tpx-1/Ag5/PR-1/Sc7) protein superfamily, which may be important in host–pathogen interactions. Whole mount in situ hybridisation demonstrated a distinct expression pattern in oral and ventral suckers of adult worms for *Sm*VAL6 and in the oesophageal gland for *Sm*VAL7 transcripts, respectively. Additionally, immunocytochemistry analysis corroborated *Sm*VAL7 expression in the oesophageal gland. Analysis of protein expression across the parasite's life cycle revealed that the *Sm*VAL6 protein is upregulated in cercariae and adult male worms. Furthermore, *Sm*VAL6 protein was identified by mass spectrometry in tegument fractions of adult worms. Finally, we speculate on possible functions of these two *Sm*VALs at the host–parasite interface.

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Schistosomes are parasitic blood flukes that cause schistosomiasis, a tropical disease with a major public health impact in endemic countries. It affects approximately 200 million individuals worldwide causing more than 200,000 deaths per year, with more than 600 million people at risk of infection (Bergquist, 2002). After the sequencing, assembly and release of the *Schistosoma mansoni* genome (Berriman et al., 2009), supported by ongoing transcriptome and proteome analyses, schistosome research is now moving into its post-genomic phase. In our search for novel potential vaccine targets in the transcriptome database, four members of a family of wasp venom allergen orthologues were identified (Verjovski-Almeida et al., 2003). Later, this large gene family was formally named *Schistosoma mansoni* Venom Allergen-Like proteins (*Sm*VALs) and revealed to contain 28 members. These can be divided into: group 1, those with a signal peptide that may be secreted to interact with their immediate environment; and group 2, those without a signal peptide that may play an intracellular role (Chalmers et al., 2008). The *Sm*VALs are members of the SCP/TAPS (Sperm-Coating Protein/Tpx-1/Ag5/PR-1/Sc7) protein superfamily, with a domain ranging between 135 and 153 amino acids in length. The sequence identity between the different *Sm*VALs' SCP/TAPS domains averages approximately 34%, showing an *E*-value ranging from 7.51×10^{-41} to 2.5×10^{-7}. The strong conservation of the tertiary structure (three-layer α-β-α sandwich) and between particular residues within the domain suggest that all SCP/TAPS domain-containing proteins share a common biological activity (Chalmers et al., 2008), which may be important in host–pathogen interactions (He et al., 2009), but the precise biological function remains unclear.

One of the first steps towards understanding the function of a gene is to determine its tissue localisation. Dillon et al. (2007) highlighted the use of whole-mount in situ hybridisation (WISH) to establish gene expression patterns in schistosomes. Herein we applied this approach to obtain insights into possible functions of two *Sm*VAL members, namely, *Sm*VAL6 and *Sm*VAL7. Our rationale was to choose one member from each *Sm*VAL group, *Sm*VAL6 from group 2 and *Sm*VAL7 from group 1, predicted to be intracellular and secreted proteins, respectively. Another criterion for selection was the high expression levels of these transcripts in parasite stages that could interact with the definitive host, i.e., cercariae, 7-day-old schistosomula and adult worms (Chalmers et al., 2008; Farias et al., 2010b). Additionally, apart from its SCP/TAPS domain, *Sm*VAL6 possesses the characteristics of a Micro-Exon-Gene (MEG) in its C-terminal region (DeMarco et al., 2010). It is encoded by 38 exons (17 of which are smaller than 20 bp in length) with at least 35 different mRNA isoforms detected (Chalmers et al., 2008), which could create variant proteins.

The parasite life cycle was maintained in hamsters and experimental protocols received prior approval from the Ethical Committee for Animal Research of the Butantan Institute, Brazil, under license number 604/09. *Schistosoma mansoni* adult worms (BH strain) were obtained by perfusion of hamsters, 7–8 weeks p.i. with cercariae. Eggs, miracidia, cercariae and schistosomula were obtained as previously described (Verjovski-Almeida et al., 2003).

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The cDNA for *Sm*VAL6 was obtained by reverse transcriptase (RT) PCR from adult worm mRNA with specific oligonucleotides that were designed using the cDNA GenBank sequence AY953433.1. Surprisingly, the resulting amplified cDNA proved to be a new *Sm*VAL6 isoform (composed by exons 1–4, 11–25, 27–28, 30–32, 34, 37 and 38; GenBank Accession No. JN122282), not previously identified (Chalmers et al., 2008). The new *Sm*VAL6 isoform displayed an open reading frame of 1,209 bp, encoding a protein of 402 amino acids with a predicted molecular mass of approximately 46.0 kDa and a pI of 8.5. The cDNA encoding *Sm*VAL7 was derived from the previously described Dif 9 clone (Farias et al., 2010b).

To investigate the tissue distribution of *Sm*VAL6 and *Sm*VAL7 transcripts, we performed WISH on whole adult worms. The production of digoxigenin (DIG)-labelled probes, fixation, permeabilisation, in situ hybridisation and staining techniques were performed strictly according to Dillon et al. (2007). Briefly, cercariae, cultured 10-day-old schistosomula and adult worms were fixed, first in Carnoy's solution, second in MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4$ and 3.7% formaldehyde) and then stored in ethanol at −20 °C until use. After rehydration, parasites were permeabilised by proteinase K treatment and refixed with formaldehyde. Antisense RNA probes were synthesised in vitro incorporating DIG-labelled dUTP (Roche, Germany) with T7 or SP6 RNA polymerase (Promega, USA) from the cDNAs previously cloned into a pGEM-T easy vector (Promega). Before hybridisation, the probes were hydrolysed to produce 300 bp fragments. As a negative control we used the sense transcript of the chorion gene and as a positive control we used the antisense transcript of *Sm*29, a tegument-specific protein (Dillon et al., 2007). The parasites and probes were hybridised overnight at 60 °C. After several washes, parasites were incubated with alkaline phosphatase-conjugated anti-DIG antibody (Roche) overnight at 4 °C. After more washes, colour was developed using either BM-Purple (Roche) or Fast Red TR (Sigma, USA) as the alkaline phosphatase substrates. Parasites incubated with BM-Purple substrate were observed and photographed with a 18.2 Colour Mosaic camera (Diagnostic Instruments Inc., USA) and SPOT software with a Leica DM2500 microscope (Germany). Those incubated with Fast Red TR substrate were counterstained with Phalloidin-FITC (Sigma), which labels actin filaments, and imaged in a Zeiss LSM 510 Meta confocal system, attached to a Zeiss Axiovert 100 microscope (Germany).

Despite the existence of several isoforms for *Sm*VAL6 at the transcriptional level, a very conspicuous signal was observed only in the ventral and oral suckers of adult male worms (Fig. 1D–F). No staining was observed in any tissue of female adult worms (data not shown), although higher transcriptional levels of *Sm*VAL6 have been reported in the anterior region of adult female worms in a microarray study (Nawaratna et al., 2011). The staining only allowed delineation of expression to the suckers, but not to a specific group of cells within them. Analysing earlier stages in the ontogenetic development, we observed expression in the regions of the cercariae and schistosomula that would give rise to the oral and ventral suckers (Fig. 1A and B). We believe this pattern of expression is representative of all *Sm*VAL6 isoforms, since our probe is complementary to exons 1–4, 11–25, 27–28, 30–32, 34, 37 and 38, which contains 80% of all identified exons. Additionally, exons 1–4, which contain the conserved SCP/TAPS domain, are present in all *Sm*VAL6 isoforms, as described by Chalmers et al. (2008).

*Sm*VAL7 expression was localised solely to the oesophageal gland of adult worms (Fig. 2E, F, H and I), a bi-lobed structure that lies around the posterior oesophagus. Analysis of cercariae and 10-day-old schistosomula using the BM-purple substrate, revealed *Sm*VAL7 expression in a discrete area approximating the gut primordium (Fig. 2A and B). Further experiments with confocal microscopy using a fluorescent substrate and counterstaining of the musculature delimited a structure that corresponds to the nascent oesophageal gland (Fig. 2C, D and Supplementary Movie S1). No staining was observed in any tissue of cercariae, 10-day-old schistosomula (data not shown), or female and male adult worms probed with the negative control (Figs. 1C and 2J). The specificity of in situ hybridisation was validated by the tissue localisation revealed by the *Sm*29 positive control (Fig. 1G and H) and the patterns of *Sm*VAL6 and *Sm*VAL7 probes that reacted in entirely different structures.

In order to characterise the *Sm*VAL6 and *Sm*VAL7 protein expression profiles across the parasite life cycle, we produced polyclonal antibodies against the recombinant proteins. The cDNAs were cloned into the pAE vector for protein expression in *Escherichia coli* BL21 (DE3) strain upon induction with 1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma). For both proteins, the inclusion bodies were shown to contain the majority of the recombinant proteins. Solubilisation of recombinant *Sm*VAL6 (r*Sm*VAL6) was obtained by extraction with 8 M urea (Sigma) and of r*Sm*VAL7 with 6 M guanidine (Sigma). The proteins were purified under denaturing conditions by affinity chromatography on nickel-charged columns (GE Healthcare, Sweden) through an imidazole (Sigma) linear gradient from 20 to 500 mM (Supplementary Fig. S1A and B). Concerning the r*Sm*VAL6 purification, an additional product of ~29 kDa was co-purified with the expected r*Sm*VAL6 protein (~47 kDa). This is probably caused by a premature translation termination due to the presence of a rare codon for expression in *E. coli*, between positions 230–255. Eluted fractions containing the recombinant proteins (as determined by SDS–PAGE) were pooled and submitted to refolding by dialysis before formulation with TiterMax adjuvant (Sigma), as per the manufacturer's recommendation, and used to generate polyclonal antibodies in mice (Supplementary Fig. S1C). The antibodies were purified against the recombinant proteins immobilised on a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, UK) as previously described (Sajid et al., 2003). Briefly, 500 μg of r*Sm*VAL6 or r*Sm*VAL7 expressed in *E. coli* were subjected to SDS–PAGE. Following electrophoresis, proteins were electrotransferred to PVDF membranes. The transferred proteins were visualised using Ponceau-Red (Sigma) and the specific bands excised. The excised PVDF membranes were blocked with 0.02 M Tris (pH 7.5), 0.3% Tween 20 and 5% dry milk for 16 h at 4 °C, and incubated with anti-r*Sm*VAL6 or anti-r*Sm*VAL7 antibodies for 8 h at room temperature. After several washes, the bound antibodies were eluted with 100 mM glycine–HCl (pH 2.7), and the preparation immediately adjusted to pH 7.2 using 1.0 M phosphate buffer (pH 8.8). The eluted antibodies were used for immunocytochemistry and Western blot analysis.

Immunocytochemistry on whole adult worms followed a previously described protocol (Mair et al., 2000). Briefly, adult worms were fixed in 4% paraformaldehyde for 4 h, washed in PBS (0.1 M, pH 7.4) for 1 h and then transferred to a fresh fixative for another 3 h. After permeabilisation with 1% Triton X-100, 0.1% SDS, 10% rabbit serum, 0.1% NaN_3 in PBS overnight at 4 °C, the worms were incubated with primary antibody, diluted 1:200, for 96 h at 4 °C. After extensive washes, the worms were incubated for 48 h with 100 ng/mL of Phalloidin-FITC, to stain the musculature of the parasite, and with Alexa-fluor 647-labelled rabbit-anti mouse antibody (1:200, Molecular Probes, USA) in PBS containing 0.1% Triton X-100, 1% BSA, 0.1% NaN3 and 10% rabbit serum at 4 °C. After several rinses, the worms were visualised with a LSM 510 Meta confocal microscope (Zeiss), attached to a Zeiss Axiovert 100 microscope. The immunocytochemistry for *Sm*VAL7 showed a remarkable association of the protein with the oesophageal gland, as well as some staining in the oesophageal lumen of adult male and female worms (Fig. 2K, L and Supplementary Movies S2 and S3), thereby confirming the WISH data. The *Sm*VAL6 immunocytochemistry data were inconclusive since the fluorescence patterns revealed by the parasites incubated with anti-r*Sm*Val6 were very similar to the controls (data not shown).

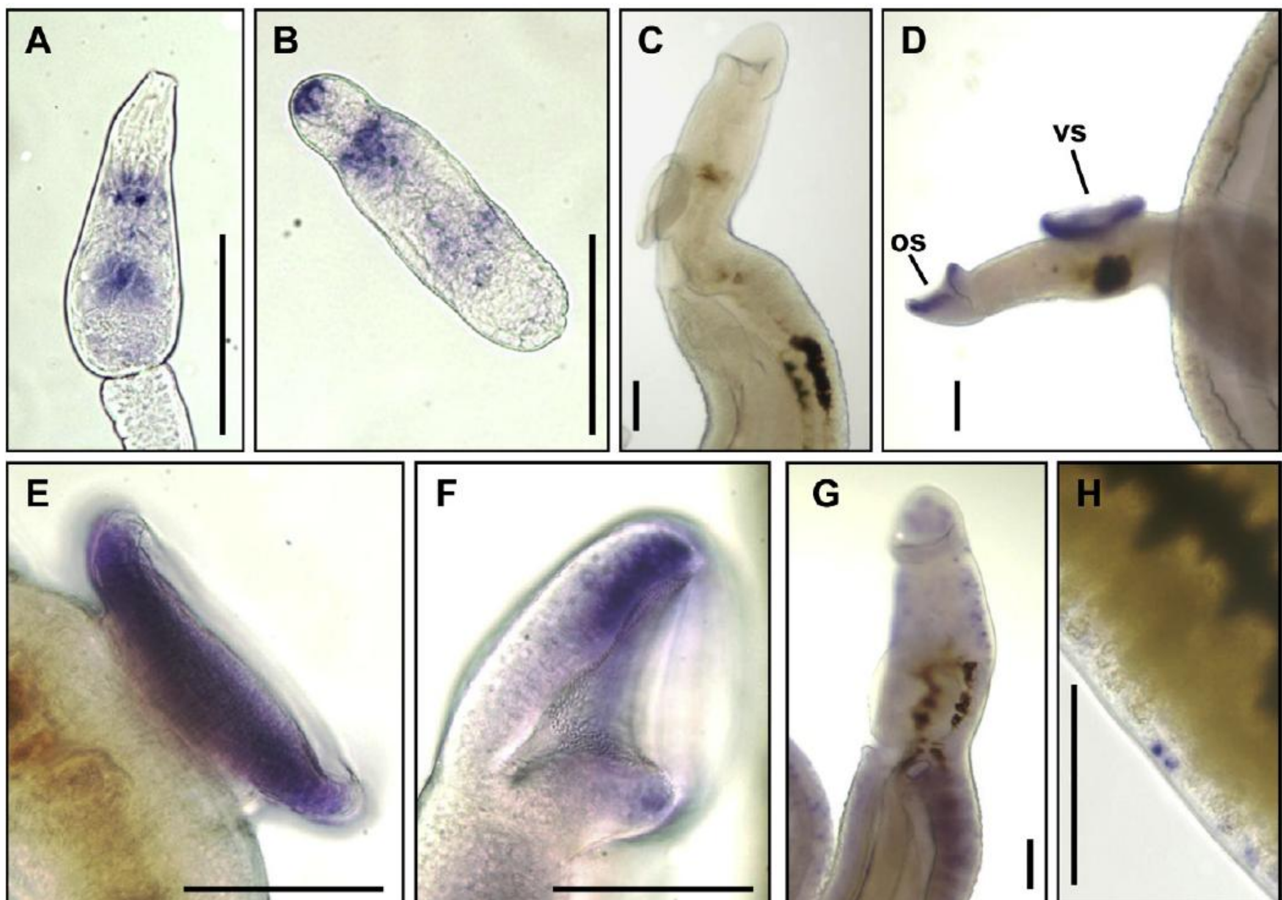


Fig. 1. Whole-mount in situ hybridisation (WISH) for the *Schistosoma mansoni* Venom Allergen-Like protein 6 (*Sm*VAL6) transcript. (A) Focal expression associated with the cells in the non-feeding infective cercaria that will become the oral and ventral suckers; (B) 10 day-old schistosomulum; (C) adult male worm probed with the sense probe of chorion gene (negative control); (D) expression of *Sm*VAL6 in the adult male oral (os) and ventral (vs) suckers; (E and F) higher magnification of an adult male showing *Sm*VAL6 expressed in the oral and ventral suckers, respectively; (G) *Sm*29 expression in an adult male worm (positive control); (H) higher magnification of an adult female worm showing *Sm*29 expressed in the tegument cell bodies. Scale bars: 100 μm.

For immunoblotting experiments, the tegument and schistosome life cycle protein extracts were prepared as previously described (Rofatto et al., 2009). Briefly, eggs, miracidia, cercariae, schistosomula, male and female adult worms were sonicated in 2% SDS-buffered solution plus protease inhibitor and the total protein extracts were recovered after centrifugation, while the tegument extract was obtained from adult worms by a freeze/thaw/vortex procedure (Roberts et al., 1983). The protein extract concentrations were measured with a DC Protein Assay kit (Bio-Rad, USA), subjected to SDS–PAGE and electroblotted onto a PVDF membrane. After blocking with 0.02 M Tris (pH 7.5), 0.3% Tween 20 and 5% dry milk powder for 16 h at 4 °C, the membrane was incubated at a 1:3,000 dilution with anti-r*Sm*VAL antibodies in blocking buffer plus 150 mM NaCl for 3 h at room temperature. After three washes using 10 mM Tris (pH 7.5), the membrane was incubated at a 1:3,000 dilution with secondary goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma) for 1 h with another three washes using the same buffer. The peroxidase activity was detected with ECL Plus reagent (GE Healthcare) according to the manufacturer's instructions and imaged using Image Quant LAS 4000 (GE Healthcare, USA).

Native *Sm*VAL6, observed in schistosome extracts, migrates with a molecular mass comparable to r*Sm*VAL6 (~47 kDa, Fig. 3). The profile of *Sm*VAL6 expression throughout the life cycle revealed very low levels of protein in females and eggs, some in schistosomula and miracidia, and the highest levels in cercariae, adult male worms, and in the tegument fraction. The protein expression profile generally correlated with the Real Time RT-PCR data described by Chalmers et al. (2008), except for detection of the protein in the miracidial stage (Fig. 3A). Additionally, the high level of *Sm*VAL6 protein in adult male worms compared with females agrees with the WISH results. In an attempt to investigate possible cross reactivity between anti-r*Sm*VAL6 and other *Sm*VALs, we performed Western blotting assays using anti-r*Sm*VAL6 antibody and three different recombinant *Sm*VALs: r*Sm*VAL4, r*Sm*VAL26 (Farias et al., 2012) and r*Sm*VAL7 (Supplementary Fig. S1D and E). Although we cannot guarantee that the anti-r*Sm*VAL6 antibody is specific to *Sm*VAL6 protein, our data did not reveal cross reactivity with other members of the *Sm*VAL family assayed. We attempted to characterise the *Sm*VAL7 protein expression profile across the parasite life cycle, but in spite of the fact that the anti-r*Sm*VAL7 antibody recognised the native protein in the immunolocalisation assay and had a similar titre to that of the anti-r*Sm*VAL6 antibody (Supplementary Fig. S1C), the protein was not recognised on Western blots of schistosome extracts, tegument or larval secretions (0–3 h proteins released by schistosomula) (data not shown).

It is remarkable that several isoforms of *Sm*VAL6, distinguished by their different molecular masses, could be detected in cercariae, adult males and tegument extracts, suggesting that the diversity of mRNA isoforms was translated to the protein level (Fig. 3A). Additionally, the tegument fraction seems to present more isoforms than other protein extracts analysed. In an attempt to visualise

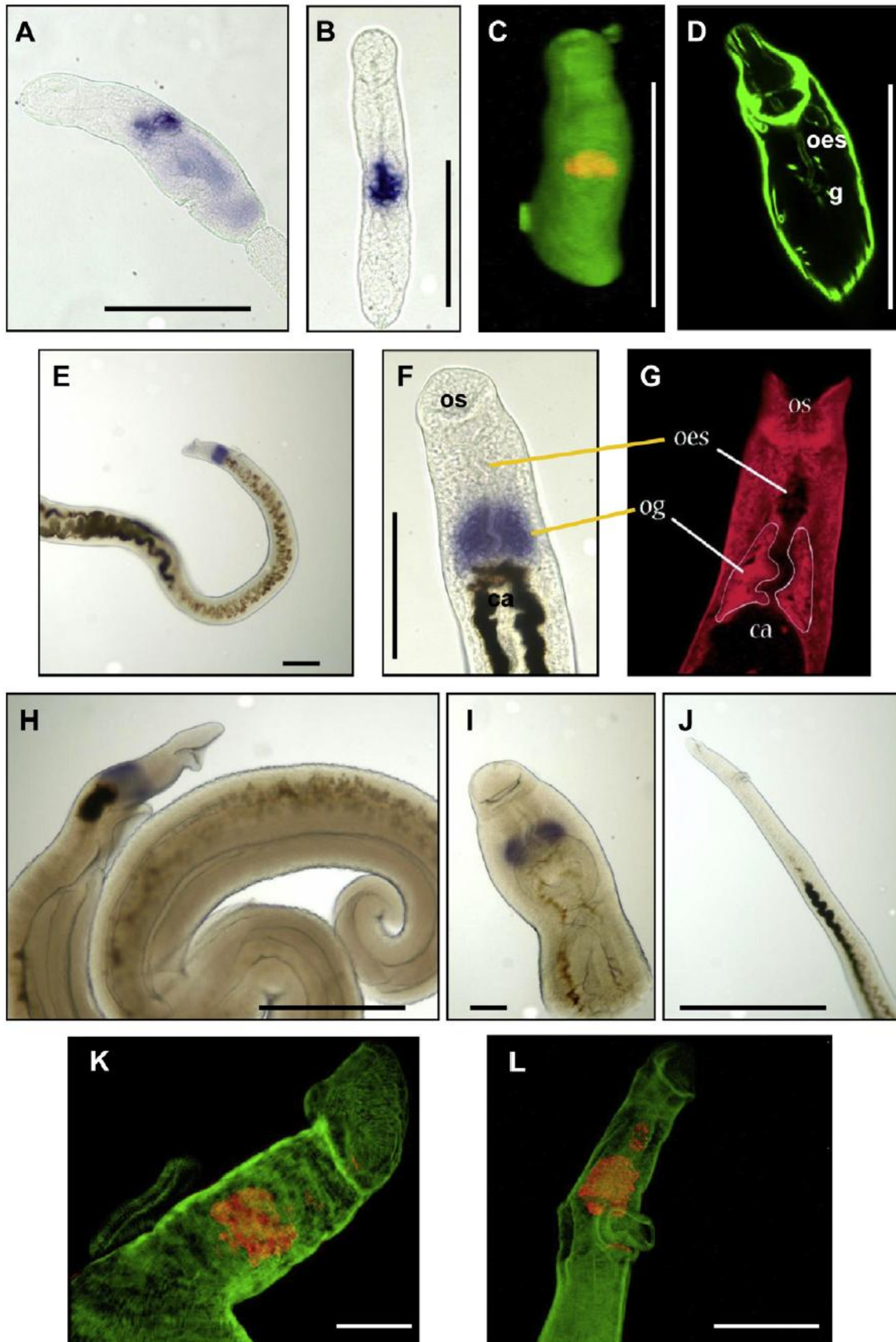


Fig. 2. Whole-mount in situ hybridisation (WISH) for the *Schistosoma mansoni* Venom Allergen-Like protein 7 (*Sm*VAL7) transcript (A–C, E, F, H and I) and immunolocalisation of the *Sm*Val7 protein (K and L). (A) Focal expression in the non-feeding infective cercaria and (B) 10-day-old schistosomulum associated with the gut primordium; (C) three-dimensional projection showing conspicuous *Sm*VAL7 expression localised to the gut primordium by Fast Red TR substrate; (D) the position of the embryonic oesophagus (oes) and gut (g) in a cercaria with similar orientation revealed by Phalloidin-FITC; (E, F, H and I) female and male expression of *Sm*VAL7 in the oesophageal gland; (G) female adult worm stained with Langeron's Carmine showing that the oesophageal gland (og) envelops the ventral aspect of the oesophagus (oes), oesophageal-caecal junction (ca), oral sucker (os). Reprinted with permission from Dillon et al. (2007). Copyright 2007 Cambridge University Press; (J) female adult worm negative control. Confocal projection of *Sm*Val7 protein visualised in the oesophageal gland of adult male (K) and female (L) worms counterstained with Phalloidin-FITC. Scale bars: 100 μm.

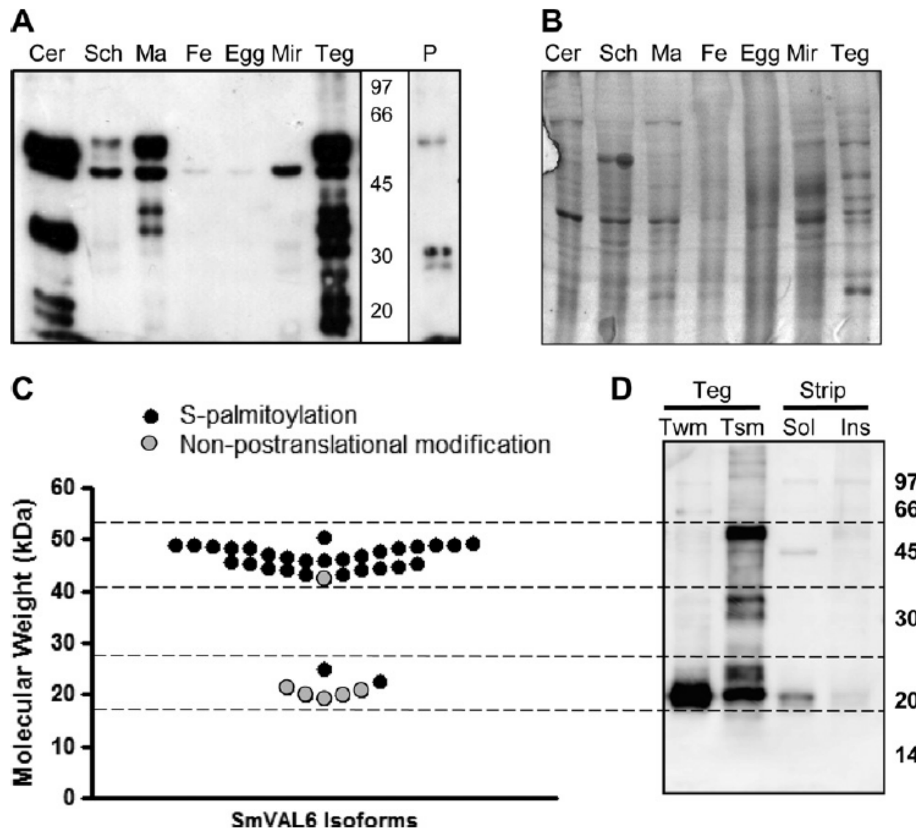
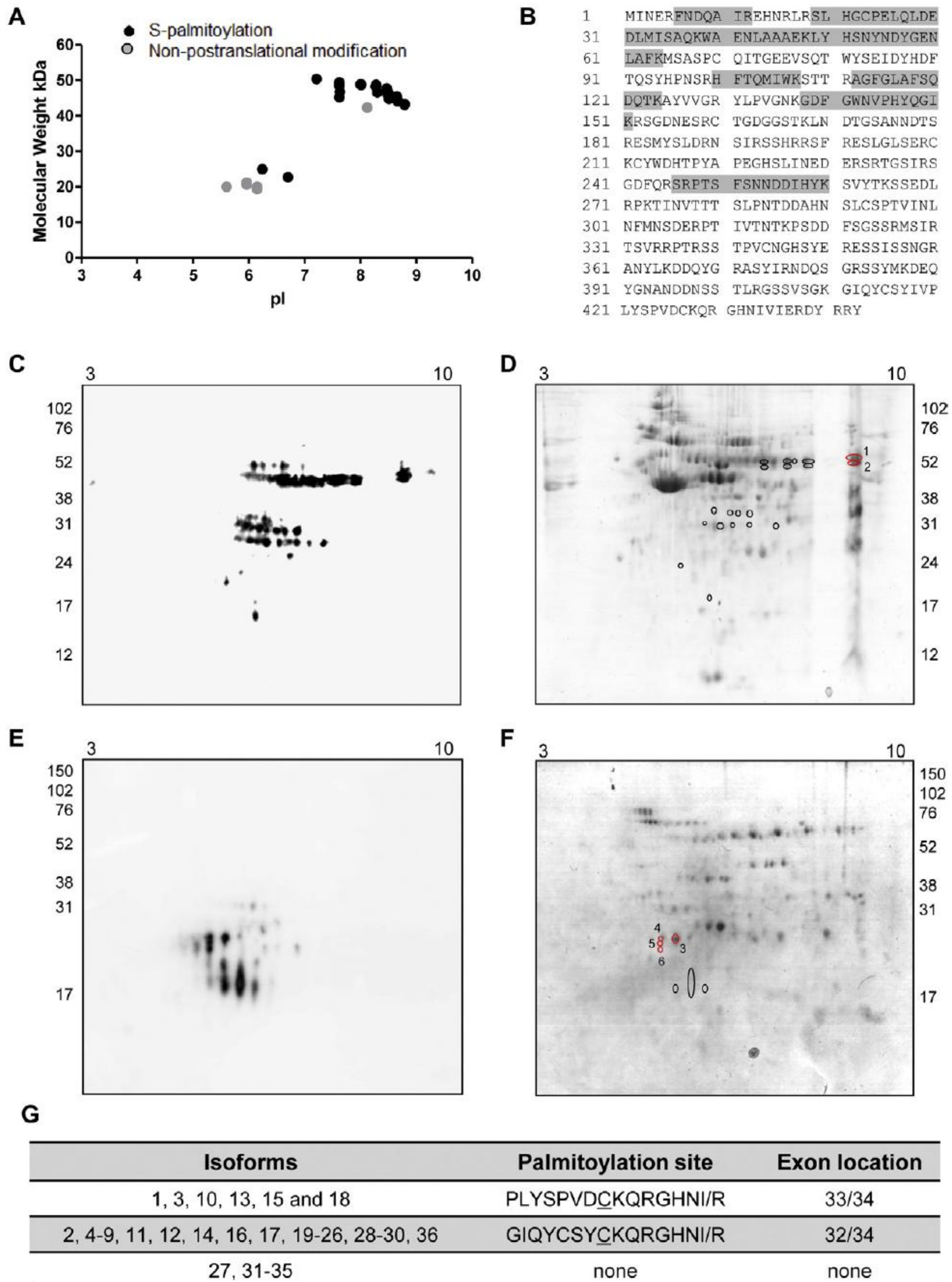


Fig. 3. *Schistosoma mansoni* Venom Allergen-Like protein 6 (*Sm*VAL6) protein expression profile across the parasite life cycle and its distribution in tegument fractions. (A) Western blot of protein extracts from *S. mansoni* stages and tegument using anti-recombinant *S. mansoni* Venom Allergen-Like protein 6 (anti-r*Sm*VAL6) polyclonal antibodies. Lanes: Cer, protein extracts of cercariae; Sch, schistosomula in vitro cultivated for 7 days; Ma, adult male; Fe, adult female; Egg, eggs; Mir, miracidia; Teg, tegument fraction (20 μg of protein were loaded in each lane); P, positive control, r*Sm*VAL6 (20 ng). (B) Corresponding Coomassie-stained gel as a loading control for the amount of protein. (C) Scatter plot graph of *Sm*VAL6 isoforms based on their molecular weight; (●) isoforms containing a putative S-palmitoylation centred on the exon junctions 32/34 or 33/34, (●) isoforms without this predicted post-translational modification. (D) Western blot of tegument (Teg) and stripped (Strip) worm extracts of adult worms. Lanes: Tsm, enriched tegument surface membranes fraction; Twm, tegument extract without surface membranes; soluble (Sol) and insoluble (Ins) protein extracts of stripped worms. Positions of molecular mass standards (kDa) are indicated on the right. It is important to note the differences in the scales between the scatter plot graph (linear) and the experimental SDS–PAGE gel (non-linear).

the putative *Sm*VAL6 isoforms distribution on a SDS–PAGE gel, we calculated the predicted molecular weight for the 36 different cDNA isoforms described by Chalmers et al. (2008). These data were summarised as scatter plot graph in Fig. 3C, revealing that the isoforms were distributed over two major ranges of molecular weight, namely 42–50 and 19–24 kDa. In order to further investigate the presence and distribution of these isoforms in the tegument of adult worms, after the freeze/thaw/vortex procedure, we separated the tegument extract by low speed centrifugation (100*g*, 30 min) generating two fractions: tegument surface membranes (Tsm) and tegument-extract without-surface membranes (Twm) (adapted from Roberts et al., 1983). Additionally, soluble (Sol) and insoluble (Ins) fractions of stripped worms after tegument removal were prepared as previously described (Farias et al., 2010a). Bands around 47–60 and 20–25 kDa were detected in the Tsm, but only of 20–25 kDa in the Twm (Fig. 3D). These data roughly correlated with the predicted molecular weight distribution of *Sm*VAL6 isoforms (Fig. 3C and D).

In order to better investigate the distribution of putative *Sm*VAL6 isoforms we performed two-dimensional electrophoretic (2-DE) analysis of Tsm and Twm fractions, followed by Western blot with anti-r*Sm*VAL6 antibody and mass spectrometric analysis. For these assays the Twm fraction was subjected to ultrafiltration in a 30 kDa cutoff centrifuge filter device (Millipore, Ireland) to enrich the proteins of <30 kDa mol. wt. Proteins were fractionated by standard 2-DE techniques (Braschi et al., 2006). Briefly, 100 μg of Tsm and 50 μg of Twm (<30 kDa) were added to 125 μL of DeStreak rehydration solution (GE Healthcare) containing 0.8% carrier ampholytes 3–10 (GE Healthcare) and used to rehydrate 7 cm, pH 3–10 linear, immobilised pH gradient (IPG) strips (Immobiline™ DryStrip Gels, GE Healthcare) for 16 h at 25 °C. Isoeletric focusing (IEF) was carried out as per the manufacturer's recommendations for a total of 5,952 Vh at 25 °C in an Ettan™ IPGphor™ 3 IEF System (GE Healthcare). During equilibration between the first and second dimension separations, proteins were reduced and alkylated. SDS–PAGE was performed across 12.5% gels in a Mini-Protean 3 Cell (Bio-Rad). Gels were electroblotted for a short period (30 min) onto PVDF membranes, which after blocking were incubated in a 1:5,000 dilution of anti-*Sm*VAL6 antibody for Tsm extract and 1:2,500 for Twm extract, and revealed as described earlier. After transfer, the gels were stained with colloidal Coomassie, documented in Image Scanner III (GE Healthcare), and aligned with the Western blot image for spot identification. Those spots visible by Coomassie staining were excised for tryptic digestion 'in gel'. Protein digestion and LC–MS/MS analysis are described in detail in Supplementary Data S1.

The 2-DE Western blot analysis revealed that *Sm*VAL6 in the Tsm fraction showed a pI ranging from 5 to 9 and three ranges of molecular weight (around 50, 30 and 20 kDa). On the other hand, in the Twm fraction *Sm*VAL6 showed a pI ranging from 5 to 7 and a narrow molecular weight distribution, from 17 to 30 kDa. The vertical and horizontal smears on the gels are suggestive of a



Isoforms	Palmitoylation site	Exon location
1, 3, 10, 13, 15 and 18	PLYSPVDCKQRGHNI/R	33/34
2, 4-9, 11, 12, 14, 16, 17, 19-26, 28-30, 36	GIQYCSYCKQRGHNI/R	32/34
27, 31-35	none	none

Fig. 4. Two-dimensional electrophoresis (2-DE) analysis of *Schistosoma mansoni* Venom Allergen-Like protein 6 (*Sm*VAL6) isoforms. (A) In silico 2-DE of *Sm*VAL6 isoforms based on their pI and on mol. wt.; (●) isoforms containing a putative S-palmitoylation centred on the exon junctions 32/34 or 33/34, (●) isoforms without this predicted post-translational modification. (B) Predicted amino acid sequence of *Sm*VAL6 isoform 1 showing the location of all mass spectrometry peptide hits (shaded amino acids). (C) Western blot of enriched tegument surface membranes fraction using anti-recombinant *Sm*VAL6 polyclonal antibodies. (D) Corresponding 2-DE Coomassie-stained gel. (E) Western blot of tegument extract without surface membranes enriched in <30 kDa proteins. (F) Corresponding 2-DE Coomassie-stained gel. Spots 1–6 were submitted to LC–MS/MS analysis and revealed the *Sm*VAL6 peptides represented in (B). Positions of molecular mass standards (kDa) are indicated on the sides of the gels. (G) Putative sites for palmitoylation identified in *Sm*VAL6 isoforms by in silico analysis and their exon junction location.

diversity of isoforms. This pattern roughly correlated with the pI/mol. wt. from the in silico predictions (Figs. 4A, C and E). The main divergence was the presence of several proteins around 30 kDa in the Western blot of Tsm (Fig. 4C), which may correspond to undescribed splice variants. Unfortunately, the proteins corresponding to this signal were in low concentrations in the Coomassie stained gel, and this impaired their visualisation, excision and mass spectrometric analysis. The LC–MS/MS analysis of the more prominent Coomassie stained Spots (1–6), corresponding to the anti-*Sm*VAL6 signal, conclusively identified *Sm*VAL6 protein (Fig. 4D and F). The six distinct peptide hits were distributed throughout exons 1, 2, 4 and 12 (Fig. 4B), common to almost all variants, so isoform discrimination was not possible. However, the identification of *Sm*VAL6 protein with different predicted molecular weights strongly suggests that the mRNA variants are translated to the protein level. These data also reinforce the specificity of the antibody as no other *Sm*VAL was recognised by this analysis.

The detection of some isoforms associated with the tegument surface membranes and others in the syncytial fraction, raises questions about the existence of post-translational modifications that could explain this pattern. Since we could not predict transmembrane helices in any of the 36 isoforms using analysis with TMHMM version 2.0 software (http://www.cbs.dtu.dk/services/TMHMM-2.0/), we searched the sequences for palmitoylation, prenylation and myristoylation sites using the CSS-Palm 3.0 tool (http://csspalm.biocuckoo.org/), the PrePS – Prenylation Prediction Suite (http://mendel.imp.ac.at/sat/PrePS/index.html) and the MYR predictor (http://mendel.imp.ac.at/myristate/SUPLpredictor.htm), respectively. The in silico analyses revealed no prenylation or myristoylation sites in any of the isoforms. However, the isoforms between 42 and 50 kDa, with only one exception, displayed a consensus sequence for palmitoylation centred on a cysteine located between exon junctions 32/34 or 33/34 (Figs. 3C and 4A and G). In contrast, most of the isoforms located in the region between 20 and 25 kDa, with only two exceptions, did not show this palmitoylation site due either to the skipping of exons 32/33/34 or the presence of a premature stop codon in exons 8 or 29. Thus, it is tempting to hypothesise that the occurrence of palmitoylation in some protein isoforms enhances their hydrophobicity, contributing to their membrane association, whereas its absence in other isoforms would explain why we could also detect some *Sm*VAL6 that was not associated with tegument membranes.

Some functional hypotheses can be developed for the two *Sm*VALs, based on the tissues or structures expressing these genes. Regarding *Sm*VAL6, the suckers are used primarily for attachment and locomotion by the adult male worm, which clasps the female in its gynecophoric canal and, bracing its body against the vessel walls, moves against the blood flow to the oviposition site employing the contact-anchor-extend method of movement (Sturrock, 2001). These suckers are highly muscularised, comprising circular, longitudinal and diagonal fibres. They also have a specific class of muscle fibres, the radial fibres, that run between the inner and outer faces (Mair et al., 2000). Additionally, the rims of the suckers bear numerous domed uniciliated sensory endings.

Searching the literature for proteins identified in the tegument, we noted that *Sm*VAL6 was detected in the tegument and not in stripped worms, as described by a proteomic study that aimed to identify tegument-specific proteins of adult worms (van Balkom et al., 2005). Moreover the *Schistosoma japonicum* orthologue (AY813515), sharing 81% of identity, was also detected in the tegument as described in a study integrating the transcriptome and proteomic data from *S. japonicum* (Liu et al., 2006). These data agree with our Western blot analyses, which suggest that the protein is enriched in the tegument fraction. Additionally, our 2-DE analyses indicate that *Sm*VAL6 is not a very common protein in the tegument, which could explain why it was not detected in the proteomics studies of tegument surface membranes performed by Braschi et al. (2006). However, the staining pattern revealed by the WISH assay is not typical of tegument expressed genes, characterised by a sharply punctate pattern in the tegument cell bodies, as observed for *Sm*29 (Fig. 1G and H). The staining pattern would fit better with transcripts associated with cells from the muscle layer. Thus we believe that the tissue localisation of *Sm*VAL6 protein is not a closed issue and deserves further investigation. Concerning the multiple isoforms of *Sm*VAL6, the fact that the parasite acquires and maintains such a complex mechanism of gene expression and protein variation suggests some adaptive advantage. It is important to note that the variation resides on the protein C-terminal region, while the N terminal SCP domain remains conserved. Our hypothesis, in the context of modular evolution of genes and proteins, is that *Sm*VAL6 arose from the fusion of a SCP gene domain with a MEG family member.

The presence of a signal peptide reveals that the *Sm*VAL7 gene encodes a secreted protein. Its expression solely in the oesophageal gland strongly suggests a role for the protein in the feeding process, from the lysis of cells after their ingestion by the parasite up to regurgitation of digested products. Gene expression is detected very early in development, being present in the primordium of the gland in the cercariae, and in the 10-day-old schistosomula. There is some evidence that the larval gut actively ingests fluids long before feeding on blood cells begins in the portal vein (Thornhill et al., 2009), therefore the protein could be directly involved in blood feeding or protection against the host, since the gut is the second major parasite–host interface. The localisation of *Sm*VAL orthologues from *Onchocerca volvulus* (*Ov*-ASP-1) and *Necator americanus* (Na-ASP-2) in secretory granules of the glandular oesophagus in the infective L3 suggests a common role for this class of molecules in this structure (MacDonald et al., 2004; Bethony et al., 2005). In a vaccine context, surface exposed and/or secreted proteins expressed in the intra-mammalian host stages are a source of potential antigens; several orthologues of *Sm*VALs have been identified as promising targets for protective immunity in nematode models (Goud et al., 2004; MacDonald et al., 2004; Bethony et al., 2005). Such data argue for testing *Sm*VAL7 as a vaccine candidate for schistosomiasis.

After the sequencing and release of the schistosome genome, research should evolve into the functional genomics phase. We believe this is the first report describing the tissue localisation of *Sm*VALs and data presented here can be taken as a first step towards discovering where these gene products exert their function. Since the *Sm*VALs comprise a multi-gene family with a range of distinct expression patterns across the schistosome life cycle, it is possible that more than one biological function could be involved. Therefore, we believe that the construction of an atlas of tissue localisation for several *Sm*VALs expressed in different life cycle stages, together with RNA interference (RNAi) and two-hybrid system studies could help us establish the functions of this complex gene family at the host–parasite interface.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2012.04.008.

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PLoS NEGLECTED TROPICAL DISEASES

Schistosoma mansoni Venom Allergen Like Proteins Present Differential Allergic Responses in a Murine Model of Airway Inflammation

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Abstract

Background: The *Schistosoma mansoni* Venom-Allergen-Like proteins (SmVALs) are members of the SCP/TAPS (Sperm-coating protein/Tpx-1/Ag5/PR-1/Sc7) protein superfamily, which may be important in the host-pathogen interaction. Some of these molecules were suggested by us and others as potential immunomodulators and vaccine candidates, due to their functional classification, expression profile and predicted localization. From a vaccine perspective, one of the concerns is the potential allergic effect of these molecules.

Methodology/Principal Findings: Herein, we characterized the putative secreted proteins SmVAL4 and SmVAL26 and explored the mouse model of airway inflammation to investigate their potential allergenic properties. The respective recombinant proteins were obtained in the *Pichia pastoris* system and the purified proteins used to produce specific antibodies. SmVAL4 protein was revealed to be present only in the cercarial stage, increasing from 0–6 h in the secretions of newly transformed schistosomulum. SmVAL26 was identified only in the egg stage, mainly in the hatched eggs' fluid and also in the secretions of cultured eggs. Concerning the investigation of the allergic properties of these proteins in the mouse model of airway inflammation, SmVAL4 induced a significant increase in total cells in the bronchoalveolar lavage fluid, mostly due to an increase in eosinophils and macrophages, which correlated with increases in IgG1, IgE and IL-5, characterizing a typical allergic airway inflammation response. High titers of anaphylactic IgG1 were revealed by the Passive Cutaneous Anaphylactic (PCA) hypersensitivity assay. Additionally, in a more conventional protocol of immunization for vaccine trials, rSmVAL4 still induced high levels of IgG1 and IgE.

Conclusions: Our results suggest that members of the SmVAL family do present allergic properties; however, this varies significantly and therefore should be considered in the design of a schistosomiasis vaccine. Additionally, the murine model of airway inflammation proved to be useful in the investigation of allergic properties of potential vaccine candidates.

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Introduction

Schistosomiasis is an important parasitic disease, caused by trematode worms of the genus *Schistosoma*, affecting more than 200 million people worldwide, with a further 650 million individuals living at risk of infection, remaining a major public health problem in many developing countries [1]. Transmission occurs through human contact with water containing the cercariae, the infective larval stage. These penetrate the skin, maturing into schistosomula, which reach the lungs via the systemic circulation. In the lungs, the young parasites undergo morphological transformations, gathering in the portal system, where they mature into adult worms. After pairing, the onset of egg deposition in the intestinal lumen, leads to a range of morbidities, such as granulomatous inflammation and periportal fibrosis [2]. A fraction of the eggs is eliminated with excreta, reaching the fresh water supply, where the miracidia hatch, infecting *Biomphalaria* snails. From these intermediate hosts the cercariae are released into the water to infect the definitive human host, closing the cycle [3].

Within the publication of the transcriptome data for *Schistosoma mansoni*, a series of novel genes/proteins were selected as potential vaccine candidates based on their functional classification by Gene Ontology functions, which would indicate their surface exposure to allow interaction with the host immune system [4]. Among them, four members of a family of wasp venom allergen orthologs were identified, raising the question of what benefits would there be to the parasite in amplifying allergic or other inflammatory responses in the host interface. Recently, this gene family was formally named as *Schistosoma mansoni* Venom Allergen-Like proteins (SmVALs) and its individual members analyzed concerning the phylogenetic relationships, genomic organization and mRNA expression profile across the life cycle [5]. This work

Author Summary

The *Schistosoma mansoni* Venom Allergen Like proteins (SmVALs) have been identified in the Transcriptome and Post-Genomic studies as targets for immune interventions. Two secreted members of the family were obtained as recombinant proteins in the native conformation. Antibodies produced against them showed that SmVAL4 was present mostly in cercarial secretions and SmVAL26 in egg secretions and that only the native SmVAL4 contained carbohydrate moieties. Due to concerns with potential allergic characteristics of this class of molecules, we have explored the mouse model of airway inflammation in order to investigate these properties in a more confined system. Sensitization and challenge with rSmVAL4, but not rSmVAL26, induced extensive migration of cells to the lungs, mostly eosinophils and macrophages; moreover, immunological parameters were also characteristic of an allergic inflammatory response. Our results showed that the allergic potential of this class of proteins can be variable and that the vaccine candidates should be characterized; the mouse model of airway inflammation can be useful to evaluate these properties.

revealed that it is a large family of genes composed by 28 members, with at least 24 members transcriptionally active, which can be divided into two groups; those with a signal peptide that may be released and interact with their immediate environment (group 1), and those without a secretion signal that should play an intracellular role (group 2) [5]. Since it was the first article to deal specifically with this schistosome gene family, herein we will follow their proposed numbering and nomenclature.

Following the transcriptome work, a series of proteomic studies describing different aspects of schistosome life cycle and biology were reported [6,7,8,9,10,11,12,13,14], mostly using the sequence database of the *S. mansoni* and *S. japonicum* transcriptomes [4,15], as well as the recently published genome databases [16,17]. Noteworthy, were the studies on the released proteins (RP) into the skin during the transition from cercariae to schistosomula [7,10], since these proteins could be the first ones to be accessible to the immune system. In one of these studies, three different members of the previously described wasp venom allergen orthologs family (SmVAL4, 10 and 18) were identified as potential immunomodulators [7] and, more recently, SmVAL10 and 18 were characterized as glycosylated secreted proteins after cercarial transformation [9]. Moreover, in a report using a more accurate model to mimic cercariae penetrating human skin, SmVAL4 was detected in the forming tunnels as a secreted protein, 2 hours post cercariae invasion [8]. In a study integrating the transcriptome and proteomic data from *S. japonicum*, several orthologs of this protein family were identified [11]; worthy of notice, was an SjVAL ortholog detected in the tegument of schistosomula, sharing 73% of identity with SmVAL26. Also of interest, SmVAL6, a group 2 family member, was identified as a tegument-exclusive protein in a sub-proteome analysis of *S. mansoni* [13] (Figure S1).

In schistosomiasis, morbidity and mortality have been associated with egg deposition, therefore identifying the components of Egg Secreted Proteins (ESP) is important to understand how these antigens can regulate the surrounding cytokine environment. Using the proteomics approach, four different members of the SmVAL family (SmVALs 2, 3, 5 and 9) were identified as ESP [6]. However, in marked contrast, more recently, no one of these proteins were identified in egg secretions by another proteomic study [12], emphasizing how methodological differences can result in diverse conclusions. An additional proteomic study reinforced the wide-spread distribution of this family along the life cycle, by the identification of several different SmVALs (2, 3/23, 9, 15, 26/28, and 27) released during *in vitro* miracidium-to-sporocyst transformation [14]; most of this data are summarized in Figure S1.

A natural question that emerged from all these studies is the biological function of these genes in the host-parasite interface. Some of these molecules were suggested by us and by other groups as potential vaccine candidates or immunomodulators, due to their functional classification, expression profile and predicted localization [4,5,7,8,9]. Additionally, SmVALs members present sequence similarity to the hookworm lead vaccine candidate NaASP-2 [18,19,20]. From a vaccine perspective, a major concern is the potential allergic effects of these molecules. Herein, we tried to investigate the immunomodulatory properties of some SmVALs by exploring the murine model of airway inflammation [21]. The investigation of localized inflammation in tissues is often difficult because it is hard to isolate the immune response against a particular stimulus. Therefore, the utilization of an inflammatory model in a confined location can be useful to monitor changes in cell population and to identify regulatory mechanisms.

We selected SmVALs from group 1, which would be putatively expressed in intra-host stages preferably exposed to interaction with the immune system. Therefore, we selected SmVAL4, which would be released in the transition between cercariae and schistosomula [7,8] and SmVAL26, which would probably be in the tegument of schistosomula due to its *S. japonicum* ortholog identification in the tegument [11]. The respective recombinant proteins were obtained in an eukaryotic expression system and the purified proteins used to produce specific antibodies. The protein expression profile was characterized across the life cycle stages. The allergic properties of SmVAL4 and SmVAL26 proteins were investigated in the murine model of airway inflammation. Our results show that the allergic properties of these molecules vary significantly and this should be considered in the putative design of a schistosomiasis vaccine.

Materials and Methods

Parasite maintenance

Schistosoma mansoni adult worms (BH strain) were obtained by perfusion of hamsters, 6 weeks after infection with 200 cercariae; eggs were extracted from infected hamster liver by maceration and partial digestion with collagenase followed by washes and passage through sieves and percoll gradients as previously described [22]; miracidia were obtained by exposing purified eggs to a bright light; cercariae were harvested from infected *B. glabrata* snails exposed to light. Following *in vitro* transformation of cercariae, schistosomula were cultured for 0–6 hours or 7 days prior to recovery [23].

Ethics statement

The procedures involving animals were carried out in accordance with the Brazilian legislation (11790/2008). All animals were handled in strict accordance with good animal practice and protocols were previously approved by the Ethical Committee for Animal Research of Butantan Institute, under the license number 604/09.

DNA constructs

Genes redesigned, optimized and synthesized. The published DNA sequence of SmVAL4 and SmVAL26 encoding the predicted proteins [5], were redesigned excluding the signal

peptide sequences and manufactured by DNA 2.0, Inc. USA (https://www.dna20.com/) using DNA2.0 optimization algorithms for expression in *Pichia pastoris* (Table S1). The fragments corresponding to the mature protein sequences for SmVAL4 (from K22 to E181) and SmVAL26 (from K25 to K176) were digested with *Eco*RI and *Xba*I to generate inserts with overhang ends that were purified and cloned into the same sites for the expression vector pPICZαA (Invitrogen), to produce a protein that contained a C-terminal hexa-Histidine tag. The resulting constructs were sequenced to confirm their identity.

Post-translational modification prediction

The signal peptide prediction was performed using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), N-glycosylation sites were analyzed using the NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc/), O-glycosylation sites were analyzed using the OGPET (http://ogpet.utep.edu/OGPET/), and transmembrane helices were analyzed by TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Molecular weight (MW) and isoelectric point (pI) were calculated with the Compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html).

Expression of rSmVAL4 and rSmVAL26 in *Pichia pastoris*

The plasmids containing SmVAL4 (optimized sequence) and SmVAL26 (optimized sequence) were linearized with *Sac*I and the *P. pastoris* strain GS115 (Invitrogen) was transformed by electroporation following the instructions of the manufacturer. Twenty colonies were first isolated and purified in YPDS plates containing 100 µg/mL Zeocin, to select putative multi-copy recombinants in YPDS plates containing 500, 1000, and 2000 µg/mL Zeocin. To verify production of the relevant proteins, initial studies were done in small-scale expression conditions, followed by Western blot with anti-His-tag antibody (GE).

Fermentation conditions were carried out as per manufacturer's recommendations. Briefly, selected *P. pastoris* cells were grown in 15 mL of BMGY 28–30°C in a shaking incubator (250–300 rpm) until cultures reached an $OD_{600} = 2.0$ (approximately 16–18 h). The cells were harvested by centrifuging at 3000× g for 5 min at room temperature, the supernatant was decanted and cells resuspended in 20 mL of BMMY medium to induce expression. Methanol was added to a final concentration of 0.5% methanol every 24 h to maintain induction; expression was monitored at 48 and 96 h time points. The supernatants and cell pellets for 15 colonies of each SmVAL was analyzed for protein expression by Western blot. Those colonies that presented the highest expression level were selected for scale-up fermentation.

Purification of rSmVAL4 and rSmVAL26 from *P. pastoris*

For protein expression and purification, selected clones for SmVAL4 and SmVAL26 were grown (28°C, 250 rpm) in 25 mL of BMGY in a 250 mL baffled flask until $OD_{600} = 2.0$ (approximately 18 h), then inoculated in 300 mL of BMGY in a 2.0 L baffled flask and grown in the same conditions until culture reached $OD_{600} = 2-6$. The cells were harvested (3000× g, 5 min at room temperature) and resuspended in 600 mL of BMMY to start induction. Methanol was added to a final concentration of 0.5% every 24 h to maintain the induction. Cells were harvested after 48 h (SmVAL26) and 96 h (SmVAL4) by centrifugation. The culture medium containing the secreted proteins (rSmVAL4 and rSmVAL26) were filtered through a 0.22 µm membrane, and diluted with 3 volumes of equilibration buffer (50 mM sodium phosphate pH 5.8 (for rSmVAL4) and pH 7.2 (for rSmVAL26), 150 mM NaCl, 20 mM imidazole). The recombinant proteins were then purified by metal affinity chromatography using the Akta Prime system (GE Healthcare) under native conditions. Briefly, the sample was loaded onto a Ni^{2+}-NTA column (5 mL bed volume) pre-equilibrated with the same buffer. The column was washed with 30 bed volumes of the equilibration buffer and then eluted with 20–500 mM imidazole linear gradient. Fractions encompassing the main peak and the purity of the preparation were assessed by SDS-PAGE. Before its use the proteins were dialyzed in Phosphate Buffer Saline pH 7.4 (PBS).

Circular dichroism (CD) measurements

CD measurements were carried out on a Jasco J-810 Spectropolarimeter at 20°C equipped with a Peltier unit for temperature control. Far-UV CD spectrum was acquired using a 1 mm path length cell at 0.5 nm intervals over the wavelength range from 190 to 260 nm. Five scans were averaged for each sample and subtracted from the blank average spectra. The protein concentration was kept at 10 µM in 10 mM sodium phosphate buffer pH 7.4.

Protein expression profile and N and O-deglycosylation assays

Total protein extracts from whole parasite stages (eggs, miracidia, cercariae, *in vitro* 7-day-old schistosomula and adult worms) were prepared in 40 mM Tris, pH 7.4, 2% SDS, plus protease inhibitor cocktail (Sigma) by sonication (4 cycles of 2 min, with pulses of 0.75 s, 40% amplitude). The samples were centrifuged at 20,000× g for 30 min at 4°C and the supernatant was recovered and used for the assays. The tegument extract was obtained by a freeze/thaw/vortex procedure, as previously described [24]. Their protein concentrations were determined with a DC Protein Assay Kit (Bio-Rad, CA, USA). Purified rSmVALs (50 ng), total parasite protein extracts (20 µg), and total tegument extract (20 µg) were subjected to SDS-PAGE. The gel was electroblotted onto a PVDF membrane, which was blocked with 0.02 M Tris (pH 7.5) and 0.3% Tween 20 containing 5% dry milk for 16 h at 4°C. Subsequently, the membrane was incubated in 1:4000 or 1:3000 dilution of anti-rSmVAL4 and anti-rSmVAL26 primary antibody, respectively, in blocking buffer plus 150 mM NaCl for 3 h at room temperature. After three washes using 150 mL of 10 mM Tris (pH 7.5), the membrane was incubated in a 1:2000 dilution with secondary goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma) for 1 h, followed by another three washes using the same buffer. Antibody reactivity was developed with ECL reagent (GE Healthcare) according to the manufacturer's instructions and imaged using Hyperfilm (GE Healthcare).

N-deglycosylation of native and recombinant proteins was carried out as previously described [25]. Briefly, 20 µg of parasite extracts (in 40 mM Tris, pH 7.4, 0.7% SDS, 1% 2-mercaptoethanol) or 10 µg of recombinant proteins (in PBS, pH 7.2, 0.7% SDS, 1% 2-mercaptoethanol) were denatured by boiling for 10 min. NP-40 (Sigma) was added (1% final concentration), 2 µL of recombinant N-glycosidase F (500 U/µL) (New England Biolabs), 20 mM sodium phosphate (pH 7.5) for a final volume of 20 µL, and incubated overnight at 37°C. For subsequent O-deglycosylation of native proteins the following enzymes were used α(2→3,6,8,9) Neuraminidase, O-glycosidase, β(1→4)-Galactosidase and β-N-Acetylglucosaminidase, and the reactions were carried out as per the manufacturer's recommendations (Sigma). Samples of purified rSmVALs and protein parasite extracts digested and non-digested were submitted to SDS-PAGE. The gels containing the recombinant proteins before and after treatment with glycosidases were stained with Schiff's reagent

(Sigma) for detection of glycoproteins as per the manufacturer's recommendations; as control for the specificity of the reaction, we used Bovine Serum Albumin (Bio-Rad) at the same concentration of the recombinant proteins. To analyze the glycosylation pattern of native SmVAL proteins, the protein parasite extracts treated with glycosidases were electroblotted onto a PVDF membrane and Western blot developed as described above.

Processing cercariae released proteins

Shedding of cercariae from snails was stimulated by exposure to bright light. Mechanical transformation by vortexing was used to stimulate the release of gland cell contents of 2000 parasites during a time course of 0–6 h culture period in 2 mL of RPMI 1640 medium (Invitrogen) containing 300 units/mL penicillin and 300 g/mL streptomycin at 37°C in 5% CO_2. Parasites were collected by centrifugation at 200× g at 4°C for 5 min and processed as previously described; the supernatant (medium containing released proteins) was stored at −20°C with the addition of 2 µL of 10× general use protease inhibitor mixture (Sigma). This soluble preparation, termed the 0–6 h released proteins (RP), was precipitated with trichloroacetic acid (TCA) and used for Western blot analysis.

Collection of egg secreted proteins (ESP) and the hatched fluid (Hf)

For egg released protein collection, eggs were isolated as previously described [22], followed by a separation of mature and immature eggs as previously described [26]. ESP was collected by incubating 3.0 million mature eggs in 10 mL of serum-free RPMI (Invitrogen) for 72 h at 37°C in 5% CO_2. Post-culture viability of eggs was >85%, as assessed by observation of muscular and flame cell activity in unhatched miracidia. Culture medium containing ESP was precipitated with TCA and resuspended in 40 mM Tris, pH 7.4, 2% SDS, plus protease inhibitor cocktail (Sigma) for Western blot analyses. The Hf was obtained by hatching mature eggs through exposure to bright light for 1 h in pound water. Miracidium and egg shell were pelleted by centrifugation at 200× g at 4°C for 10 min, and the protein water-soluble content was carefully collected to avoid turbulence, the sample (Hf) was filtered through a 0.22 µm polyethersulfone filter (Millipore) and concentrated through precipitation with TCA.

Pronase protein digestion

For elimination of the protein moiety, 12 µg of rSmVAL4 was incubated with 0.05% of pronase (Calbiochem, San Diego, CA) for 2 h at 37°C. A separate sample of rSmVAL4 was incubated for 2 h at 37°C without pronase to provide an undigested control. Successful pronase digestion was confirmed by gel electrophoresis followed by Coomassie staining, which revealed the absence of any remaining protein (data not shown).

rSmVALs sensitization and challenge

Female BALB/c mice (6–8 wk old) were weight matched and used throughout this study. To test whether rSmVALs could induce an inflammatory reaction in the lungs, we adapted the well-established murine model of asthma based on Ovalbumin (OVA)/alum sensitizations and challenges [21]. Briefly, mice were sensitized on days 0, 7 and 14 by subcutaneous (s.c.) injection (0.4 mL total volume) in the nape of the neck with 10 µg rSmVALs adsorbed to 2.0 mg of aluminum hydroxide (Alhydrogel-Brenntag Biosector). On days 21 and 28, mice were challenged i.n. with 10 µg of rSmVALs in 50 µL of PBS. Two additional groups were used: Control group received only the intranasal challenge with rSmVAL and naïve mice received only PBS. It is important to mention that mice were anesthetized intramuscularly with 100 µL of a solution containing ketamine (Ketamina Agener, Uniao Quimica Farmaceutica Nacional) and xylazine (Bayer) before any immunization or challenge, to ensure a complete instillation to the lungs, as previously described [27].

Bronchoalveolar lavage (BAL)

Twenty four hours after the last challenge, mice were deeply anesthetized by an i.p. injection of urethane (Sigma-Aldrich) at 15 mg/10 g body weight, the abdominal cavity was opened, and blood samples from the inferior cava vein were collected for serum antibody determinations. The trachea was cannulated and lungs were lavaged twice with 0.5 and 1.0 mL of cold PBS. After total cell counting, cytospin preparations of bronchoalveolar lavage (BAL) cells were stained with Instant-Prov (Newprov) and differential cell counts were performed on 200 cells on the basis of morphology and staining characteristics. Supernatants from BAL were collected and frozen at −80°C for cytokines measurements.

Measurement of rSmVALs-specific IgG1, IgG2a and IgE antibodies

Anti-SmVALs antibodies were assayed by sandwich ELISA, as previously described [21]. Briefly, serum samples were titrated for optimal dilutions for testing different isotypes. For SmVALs-specific IgE determinations, plates were coated with goat anti-mouse unlabelled IgE (1:250; BD Bioscience) following the manufacturer's recommendations; serum samples were incubated (1/10 dilution) for 2 h at room temperature and subsequently biotin-labeled rSmVALs (5 µg/mL) were added to the wells. The biotinylated rSmVALs were prepared by reacting 1 mL rSmVALs (1 mg/mL) in PBS with 100 µL of N-hydroxysuccinimidobiotin in dimethyl sulfoxide (DMSO) (4 mg/mL) for 4 h at room temperature, followed by overnight dialysis against PBS at 4°C. The bound rSmVALs-biotin was coupled to streptavidin-peroxidase 1:250, for 15 min incubation at room temperature and revealed as per the manufacturer's recommendations. SmVAL-specific IgE levels of samples were expressed by OD. For SmVALs-specific IgG1 and IgG2a antibodies, serum samples were plated on 96-well plates previously coated with rSmVALs (0.5 µg/well). The bound antibodies were revealed with goat anti-mouse IgG1 or IgG2a followed by peroxidase-labeled rabbit anti-goat antibodies (all from Southern Biotech). The concentration of each specific isotype was estimated by comparison with IgG1 and IgG2a standards run in parallel and expressed as the mean ± SEM of the antibody concentration of 4 mice per group.

Cytokine measurements

The cytokine concentration in the BAL fluid was quantified by ELISA kits specific for IL-5 and IL-10 (BD Biosciences PharMingen) and for IFN-γ (Peprotech INC.). The values are expressed as picograms per milliliter deduced from standards, run in parallel with the recombinant cytokines. The limit of detection values were 10 pg/mL for IL-5 and IL-10 and 16 pg/mL for IFN-γ.

Passive cutaneous anaphylaxis (PCA)

The anaphylactic activity of reactogenic antibodies was evaluated by passive cutaneous anaphylactic reaction in mice as described by Ovary et al. [28]. Previously shaved mice were injected intradermally with 50 µL of three serial dilutions of serum in each side of the dorsal skin. After 2 h, they were challenged intravenously with 250 µg of rSmVAL4, rSmVAL4-Pro (Pronase-

digested) or rSmVAL26, all plus 0.25% of Evans blue solution. All determinations were made in triplicate and the PCA titers were expressed as the reciprocal of the highest dilution that gave a lesion of >5 mm in diameter. The detection threshold of the technique was established at 1/5 dilutions.

Immunization of mice and polyclonal antibody production

Polyclonal mouse sera were produced against preparations of rSmVAL4 and rSmVAL26. BALB/c mice were immunized three times, subcutaneously in the nape of the neck, at 14-day intervals with 25 µg of rSmVAL4 or rSmVAL26 formulated with TiterMax adjuvant (CytRx Corporation; first dose) or PBS 1x (in subsequent doses). Fifteen days after the last inoculation, rodents were exsanguinated. The sera were used at a dilution of 1:4000 (anti-rSmVAL4) and 1:3000 (anti-rSmVAL26) in Western blots.

Statistical analysis

Student's *t*-test was used to compare experimental and control groups on antibody and cytokine levels. For cellular migration assays and analysis involving more than two groups, statistical comparisons were performed with one-way ANOVA followed by a Bonferroni pairwise comparison. A ρ value<0.05 was considered statistically significant.

Results

Construction, expression and purification of recombinant SmVAL4 and SmVAL26

In order to analyze SmVALs that could interact with cells in the definitive host, we chose 2 members of group 1. SmVAL4 would be released in the transition between cercaria and schistosomula. A phylogenetic analysis of SmVALs and SjVALs (Figure S2), revealed that SmVAL26 branched together with a SjVAL ortholog, with 73% of identity detected in the tegument of hepatic schistosomula (Gene Bank accession AAW27353.1)(Figure S3).

SmVALs contain the sperm-coating protein (SCP) signature sequence (outlined by a dark grey box in Figure 1A) and are recognized as part of the Pfam SCP family (PF00188) with an E-value ranging from 6.66×10^{-24} to 8.20×10^{-31}. The SmVALs under investigation, once belonging to group 1, also contain a putative N-terminal signal peptide (outlined by a grey box in Figure 1A). Putative N and O-glycosylation sites were identified and investigated (identified by * and #, respectively in Figure 1A). The predicted molecular mass and isoelectric points of these proteins are presented in Table 1.

rSmVAL4 and rSmVAL26

The recombinant proteins rSmVAL4 and rSmVAL26 were expressed using codon optimization in *P. pastoris* GS115 strain and secreted into the culture supernatant (products around 30 kDa and 20 kDa, respectively) (Figure 1B and 1C). rSmVAL4 and rSmVAL26 were purified by affinity chromatography on nickel-charged columns eliminating the main contaminant from both samples (around 66 kDa) in the flow through (Figure 1B and 1C). The eluted fractions of rSmVAL4 showed two main bands at ~30 and 34 kDa (Figure 1B lanes 5–8), while rSmVAL26 eluted fractions presents only one product (~20 kDa) (Figure 1C, lanes 4–7). Eluted fractions were pooled and submitted to extensive dialysis in PBS pH 7.2; protein yield after dialysis were estimated to be around 10.0 mg of rSmVAL4/L and 6.0 mg of rSmVAL26/L of culture. These samples were used in the sensitization and challenge assays and to generate polyclonal antibodies in mice.

rSmVAL4 and rSmVAL 26 are expressed as glycosylated proteins

Both *Pichia*-secreted proteins migrate with a higher molecular mass than that predicted (~30.0 kDa for rSmVAL4 and ~20 kDa for rSmVAL26) (Table 1), which could reflect a likely product of post-translational glycosylation. To test this hypothesis, we digested the purified proteins with a recombinant N-glycosidase F, and also stained the gels with Schiff's reagent to reveal the presence of glycans. The SDS-PAGE showed that, after digestion, rSmVAL4 migrated to a lower MW (~25 kDa), demonstrating the removal of N-glycans (Figure 1D), while rSmVAL26 does not show any mobility shift (Figure 1F), suggesting that the protein was not N-glycosylated. Additionally, for both proteins, the Schiff's reagent staining procedure revealed the presence of glycans after treatment with PNGase F, suggesting the presence of a PNGase F insensitive N-linked glycan site or an O-linked glycosylation site (Figure 1E and G).

rSmVAL4 and rSmVAL26 present an ordered secondary structure

Circular dichroism spectra revealed that rSmVAL4 and rSmVAL26 display an ordered secondary structure, which resembles the NaASP-2 protein (structure resolved – three layer α-β-α-sandwich) (Figure 1H), although the proportions of secondary structure elements (α-helix and β-sheet) were not calculated.

SmVALs protein expression profile across the parasite life cycle

Samples prepared from cercariae, schistosomula, adult worms, eggs and miracidia stages of *S. mansoni*, and tegument isolated by the freeze/thaw method, were all separated by SDS-PAGE. Immunoblotting was performed using mouse anti-rSmVAL4 and rSmVAL26 antisera. The protein expression profile of SmVALs revealed a very specific stage associated expression. Briefly, the expression of SmVAL4 seems to be restricted to the cercariae stage. In the case of SmVAL26, although we expected it to be in the tegument due to its similarity with the *S. japonicum* ortholog, it is actually detected in the egg, but not in the miracidium stage (Figure 2A and B). It is important to note that in the assessed experimental conditions, no sign of cross reactivity was observed with SmVALs in other stages or in the tegument fraction.

Native SmVAL4 is an N-glycosylated protein

Noteworthy, the SmVAL4 and SmVAL26 native proteins detected in schistosome extracts migrate with a higher molecular mass than that predicted (Table 1), which again could reflect a likely product of post-translational glycosylation. To test this hypothesis, we digested total cercariae and egg extracts with the recombinant N-glycosidase F. The immunoblot showed that, after digestion, the native proteins displayed a shift in the migration, demonstrating that native SmVAL4 is N-glycosylated, whereas SmVAL26 is not (Figure 2C and D). In order to investigate possible O-glycosylations, the extracts were treated with neuraminidase, O-glycosidase, beta (1–4) galactosidase, or N-acetylglucosaminidase, all of which had no effect on the proteins' mobility on SDS-PAGE (data not shown).

SmVAL4 is released by cercariae and SmVAL26 is released by eggs

To investigate the presence of SmVAL4 in the secretions of newly transformed schistosomula, we collected the proteins released by 2000 parasites in a time course manner. We were

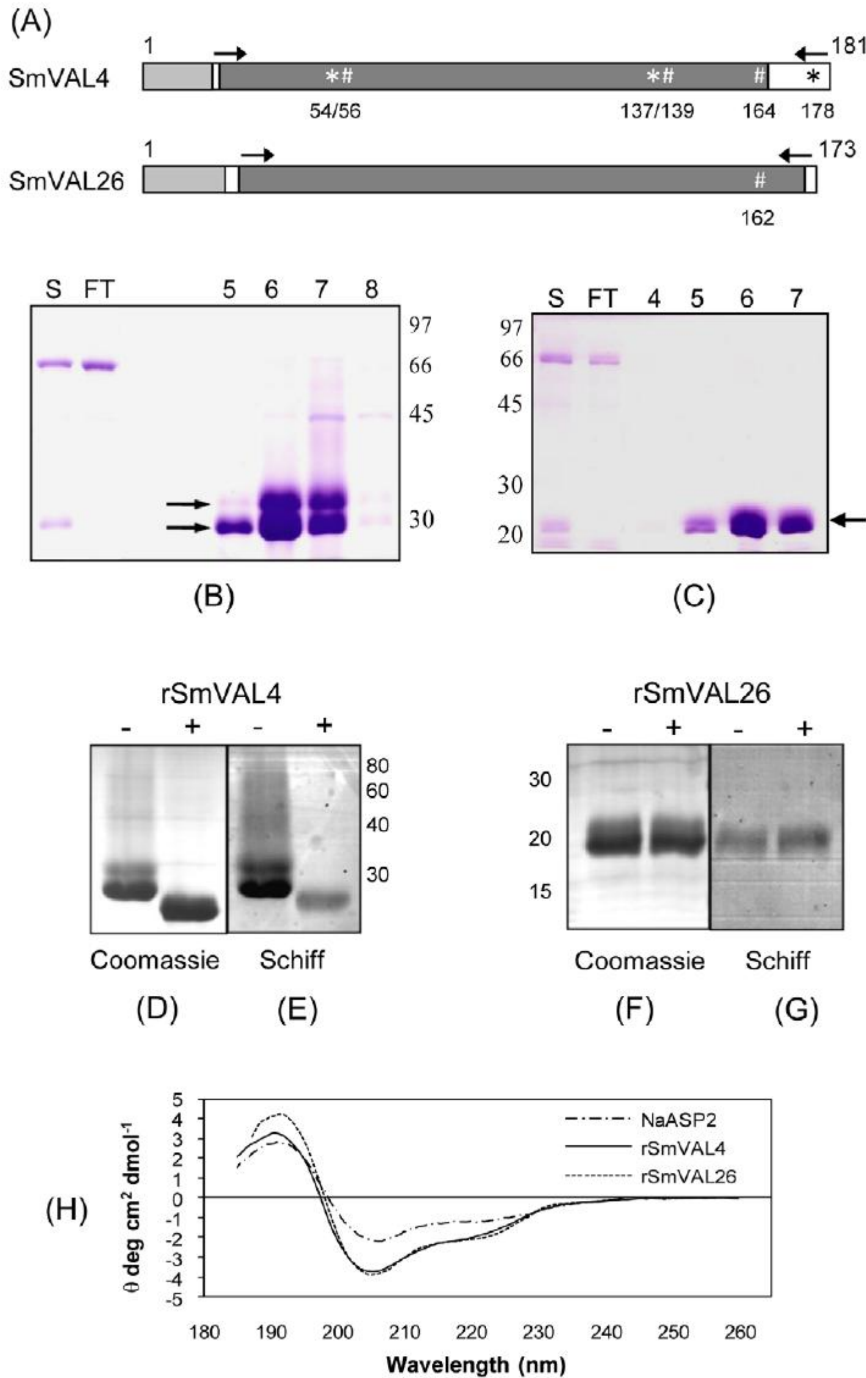

Figure 1. Expression and purification of rSmVAL4 and rSmVAL26, and characterization of their glycosylations. (A) Schematic representation of SmVAL proteins used in this study. Highlighted are the putative signal peptide (grey box), the SCP signature sequence (dark grey box), potential sites for N-glycosylation (*) and O-glycosylation (#), and the cloned region expressed in *Pichia pastoris* (→ ←). Numbers correspond to the amino acid sequence position deduced from the cDNA sequence. (B) SDS-PAGE analysis of fractions from Ni^{2+}-charged column chromatography of rSmVAL4 and (C) rSmVAL26; Lanes 5–8 and 4–7, fractions containing the main peak of rSmVAL4 and 26 respectively, eluted by linear gradient of imidazole (20–500 mM); FT - Flow-through; S – sample. (D) SDS-PAGE of rSmVAL4 and (F) rSmVAL26 stained with Coomassie before treatment with PNGase F (−) and after digestion with the enzyme (+); (E and G) the same gel was stained with Schiff's reagent. Positions of molecular mass standards (kDa) are indicated on the side, 20 μg of protein was loaded in each lane. (H) Circular Dichroism spectra of rSmVAL4 and rSmVAL26 are compared to that of the rNaASP-2 spectra. The CD spectra presented are the averages of five measurements.
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Table 1. Molecular weight and isoeletric point of SmVALs investigated in this study.

	SmVAL	^{a}Exp Mw	^{b}Obs Mw	pI
Native	SmVAL4	18.5	29.0	8.68
	SmVAL26	17.3	19.0	5.26
Recombinant	SmVAL4	21.4	30.0	7.21
	SmVAL26	20.2	20.0	5.35

^{a}Exp Mw = Expected Molecular weight.
^{b}Obs Mw = Approximate Observed Molecular weight.
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able to detect the secretion of SmVAL4 as early as 30 min after transformation and its increasing secretion in the medium of cultured schistosomula from 0–6 h (Figure 3A). However, after this period, there are still significant amounts of protein within the parasite or associated to its surface (Figure 3A).

We also evaluated the presence of SmVAL26 in the egg secretions and in the hatched fluid, using the respective antibody. SmVAL26 was detected in both extracts (Figure 3B and C).

rSmVAL4 is able to induce airway inflammatory responses

In order to investigate a putative immunomodulatory effect of rSmVALs, we explored the well-established murine model of airway inflammation induced by OVA/Alum sensitization and OVA intranasal challenge, replacing OVA by the rSmVAL4 and rSmVAL26 proteins. Our data revealed that mice sensitized and challenged with rSmVAL4 present an increased number of total cells in the bronchoalveolar lavage (BAL), when compared with the control group. This effect comprises mainly an increase in eosinophils (55%) and macrophages (32%), which resembles an allergic airway inflammatory response. Mice that received rSmVAL26 show a discrete increase in total cell counting, mostly macrophages (Figure 4A and B). When mice received up to 6 doses of rSmVAL26, this profile does not change significantly. The inflammatory response induced by rSmVALs was also evaluated in the lungs of mice by histopathology. A dense mixed-cellular infiltrate surrounding the airway (peribronchovascular inflammation) was evident only in the group treated with rSmVAL4 (Figure 4D).

Since the allergic effect could be due either to the proteic or the carbohydrate moieties of rSmVAL4 produced in *Pichia pastoris*, we eliminated the protein moiety by pronase treatment and performed the sensitization step with the pronase-treated rSmVAL4 (rSmVAL4-Pro). As shown in Figure 4A and 4E, pronase treatment of rSmVAL4 totally abolished airway inflammation.

rSmVALs induce specific IgG1, but only rSmVAL4 induces IgE production

The serum levels of SmVALs-specific IgG1, IgG2a and IgE were measured in sensitized and challenged mice and in those only challenged with different rSmVAL proteins. The production of SmVAL-specific IgG1, a Th2-affiliated antibody isotype, was significantly higher in all groups analyzed as compared to the control challenge group (Figure 5A). Additionally, rSmVAL4 showed higher levels of IgG1, and no antibody production was observed in response to immunization with pronase-treated rSmVAL4. Concerning the production of SmVALs-specific IgG2a antibodies, very low levels and no significant differences were

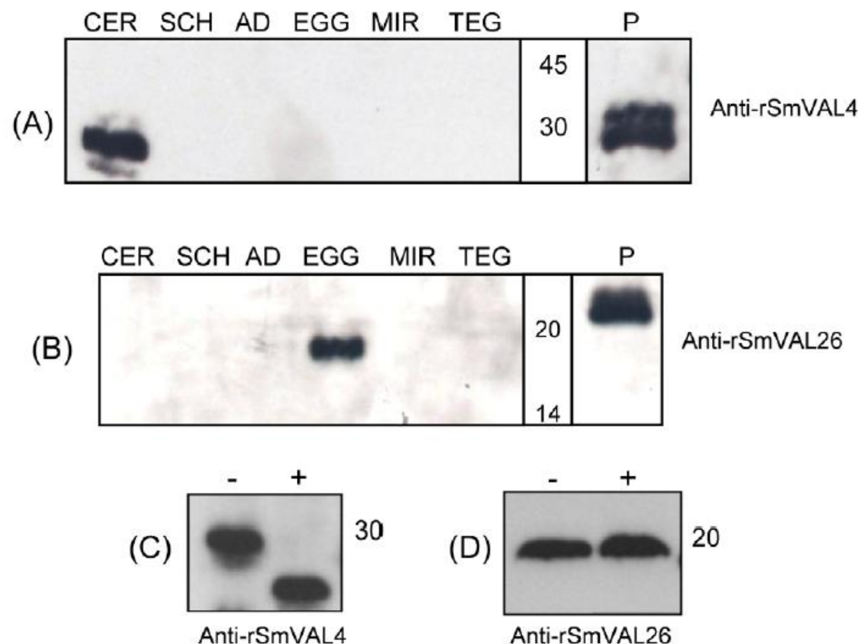

Figure 2. Immunoblotting of protein extracts from *S. mansoni* stages and tegument fraction using anti-rSmVALs polyclonal antibodies. (A) Anti-rSmVAL4, and (B) anti-rSmVAL26. CER, cercariae; EGG, eggs; MIR, miracidia; AD, adult worms (female and male); SCH, 7-day old schistosomula; TEG, tegument; (C) Immunoblotting of Cercariae extracts before (−) and after (+) treatment with PNGase F using anti-rSmVAL4; and (D) Immunoblot of Egg extracts before (−) and after (+) treatment with PNGase F using anti-rSmVAL26 (20 µg of protein was loaded in each lane); P, positive control rSmVALs (50 ng). Positions of molecular mass standards (kDa) are indicated inside or on the side the autoradiogram film.
doi:10.1371/journal.pntd.0001510.g002

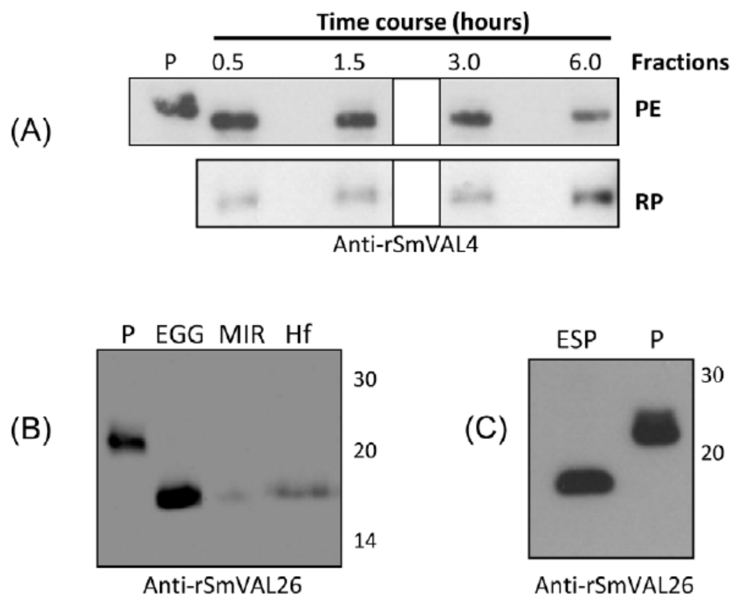

Figure 3. Immunoblotting of released proteins using anti-rSmVALs polyclonal antibodies. (A) Released proteins by newly transformed schistosomulum (RP) cultured 0–6 h or correspondent parasite extract (PE) hybridized with anti-rSmVAL4 (RP - total released proteins by 1000 parasites was loaded in each lane, PE - 10 µg of protein extract was loaded in each lane). (B) EGG, eggs; MIR, miracidia; Hf, hatched fluid containing released proteins by hatching eggs, hybridized with anti-rSmVAL26; (C) ESP, secreted proteins by 72 h cultured viable mature eggs, hybridized with anti-rSmVAL26, (20 µg of protein was loaded in each lane); P, positive control rSmVALs (50 ng).
doi:10.1371/journal.pntd.0001510.g003

detected between experimental and control groups (data not shown). The serum levels of SmVALs-specific IgE, a Th2 allergic associated isotype, were also measured, revealing significantly higher levels only in the rSmVAL4 group as compared to all other groups (Figure 5B).

rSmVAL4 induces high levels of IL-5 but does not induce IFN-γ or IL-10

We evaluated the secretion of IL-5, IL-10 and IFN-γ in the BAL fluid. The levels of IFN-γ were below detectable levels (50 pg/mL) in all analyzed groups, while the levels of IL-10 did not differ significantly from the sensitized and challenged groups to those only challenged (data not shown). The levels of IL-5 were significantly higher in the rSmVAL4 immunized mice as compared to the control groups (naïve or only challenged). SmVAL4-Pro group revealed intermediate levels of this cytokine, not differing statistically from control or rSmVAL4 (Figure 6A). It is important to mention that this IL-5 secretion observed in SmVAL4-Pro was not sufficient to induce/mediate the eosinophil infiltration or other parameters of the allergic response.

rSmVAL4 serum is able to cause passive cutaneous anaphylactic hypersensitivity

Taking into account the eosinophil migration, the presence of IL-5 in the BAL and the high levels of systemic IgE, we performed Passive Cutaneous Anaphylactic (PCA) assays to evaluate the production of anaphylactic IgG1 in heat-inactivated serum of mice sensitized and challenged with rSmVALs. Our results demonstrated that IgG1 antibodies produced by the rSmVAL4 group exhibited strong PCA activity with a very high titer of 1:1250, whereas in the other groups, including the pronase-treated rSmVAL4 or the control groups, low levels of anaphylactic IgG1 antibodies were observed (Figure 6B).

rSmVAL4 induces IgE in a conventional protocol of immunization

The induction of high levels of systemic IgE by rSmVAL4 in the model of airway inflammation led us to evaluate the use of a less Th2-prone adjuvant in a conventional protocol of immunization. Therefore, mice were immunized with rSmVALs formulated in TiterMax Gold, which is described to produce considerable levels of IgG2a in addition to IgG1. The rSmVAL4 group showed higher levels of IgG1 antibody in relation to rSmVAL26. Concerning the production of SmVALs-specific IgG2a antibodies, significant levels were detected in both immunized groups as compared to the control, with no differences between them. The levels of specific IgG1 and IgG2a and the IgG1/IgG2a ratio indicate that immunization with TiterMax Gold induced a predominant Th2 immune response to rSmVAL4 and a more balanced (Th1/Th2) response to rSmVAL26 (Figure 7A). The serum levels of SmVALs-specific IgE, were also measured, revealing significantly higher levels only in the rSmVAL4 group as compared to all other groups (Figure 7B).

Animals sensitized with rSmVAL4 and rSmVAL26 formulated in Titermax were also challenged intranasally and airway inflammation evaluated. It was observed that only the group receiving rSmVAL4 developed airway eosinophilic inflammation (data not shown).

Discussion

Recently, SmVALs have emerged from transcriptoma, microarray and proteomic studies as potential targets for immune intervention. In the present manuscript, we extend the previous molecular characterization performed by Chalmers et al. (2008), focusing on the protein products of SmVAL4 and SmVAL26, which may play different roles in the parasite-host interface.

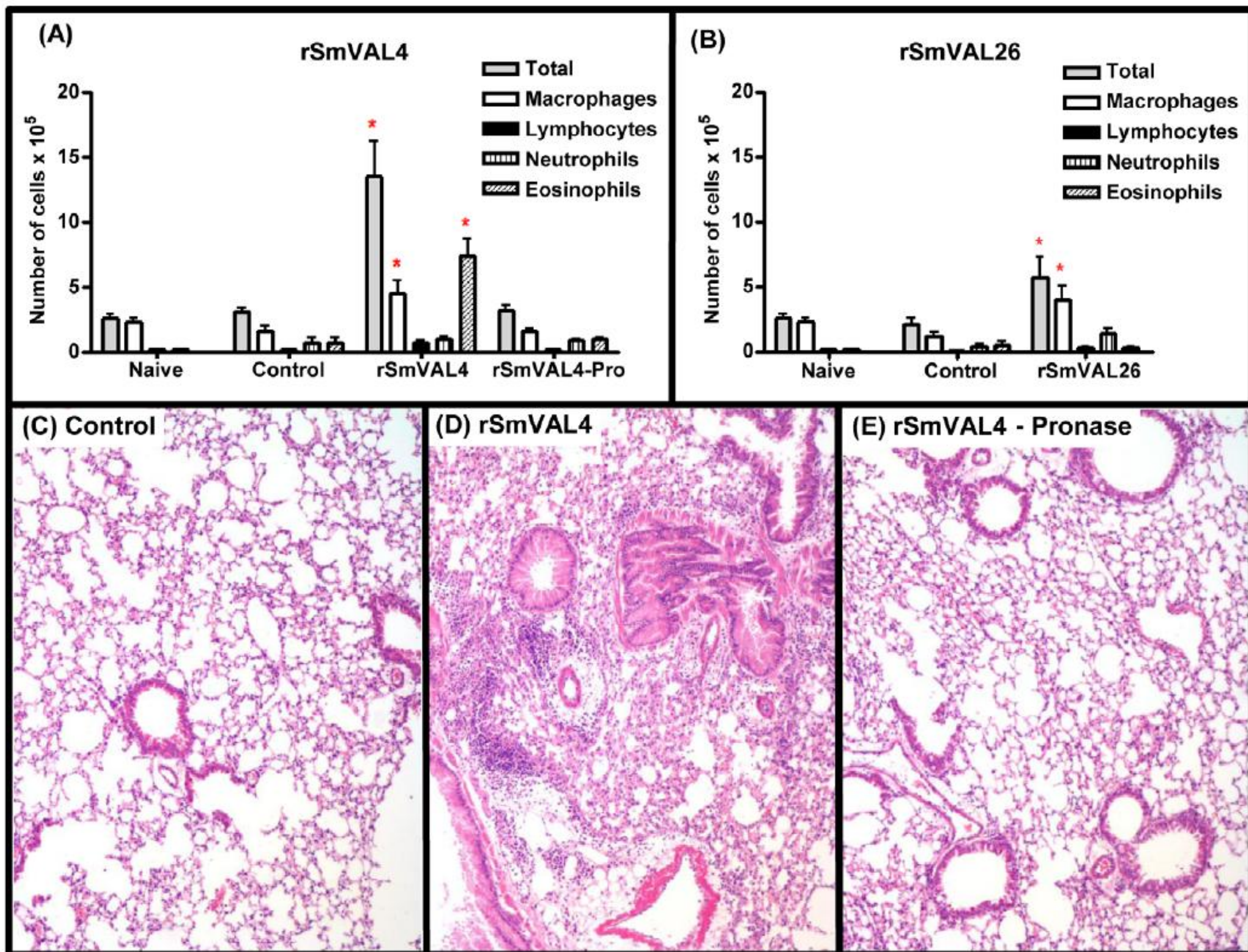

Figure 4. Evaluation of rSmVALs induction of airway inflammation and lung histopathology. (A) BAL total and differential cell counts for animals immunized with rSmVAL4 or rSmVAL4-Pro (rSmVAL4 protein after treatment with Pronase) and (B) rSmVAL26. Control group received only the intranasal challenge with rSmVAL and naïve mice received only PBS. Results are expressed as means ± SEM for groups of four mice and are representative of two experiments. *Significant differences ($p<0.05$) when compared to Control (mice that were only challenged with the respective proteins). (C) Lung sections of mice that were only challenged. (D) Representative lung sections of rSmVAL4-induced allergic airway inflammation in BALB/c mice, revealing the marked infiltration of inflammatory cells in the peribronchiolar space. (E) Lung sections of mice that received rSmVAL4-Pro, showing patterns similar to the control group; pictures at 10× of magnification.
doi:10.1371/journal.pntd.0001510.g004

Our results describe the expression of the codon-optimized versions of SmVAL4 and SmVAL26 in *Pichia pastoris*. The secretion of these proteins in the yeast expression system allowed purification of the proteins in soluble form. Maybe the most relevant feature when producing a recombinant protein for functional assays is its correct folding. Our CD data indicated that the soluble secreted forms of rSmVAL4 and rSmVAL26 contained an ordered secondary structure, with similar proportions of structural elements (α-helix and β-sheet) as determined for NaASP-2, which presents a three-layer (α-β-α) sandwich flanked by an N-terminal loop and a short cystein-rich C terminus [18].

The presence of glycans in such a class of secreted molecules is somewhat expected. Interestingly, both rSmVAL4 and rSmVAL26 were revealed to be glycosylated, which could help the proper folding and stabilization of the protein. It is important to note though, that the profile of glycosylation obtained in the *Pichia* system will not be equivalent to the native pattern in schistosomes. Concerning the native SmVAL4 protein, we confirmed the *in silico* predictions of its N-glycosylation by mobility shift. On the other hand, the native SmVAL26 was not N-glycosylated, which is also in conformity with the N-glycan *in silico* predictions. We did not detect the presence of O-glycans in the native SmVALs analyzed. However, we cannot completely exclude the presence of this class of carbohydrate, since the deglycosidases used were not specific for schistosomes, which could have modifications of the core structure, impairing or blocking enzymatic digestion.

The previously determined mRNA expression profile across the life cycle suggested that SmVAL4 could be involved in the invasion of the definitive host [5]; this stage associated expression was confirmed at the protein level by our Western blot analysis. SmVAL4 protein was revealed to be present only in the cercarial stage, increasing in the secretions of newly transformed schistosomulum in a time course manner. This data could reflect the fact that not all parasites were at the same metabolic stage during transformation or that the protein continues to be released even after 6 hours post-transformation. The identification of SmVAL4 in cercariae secretions by proteomics suggested that it could be

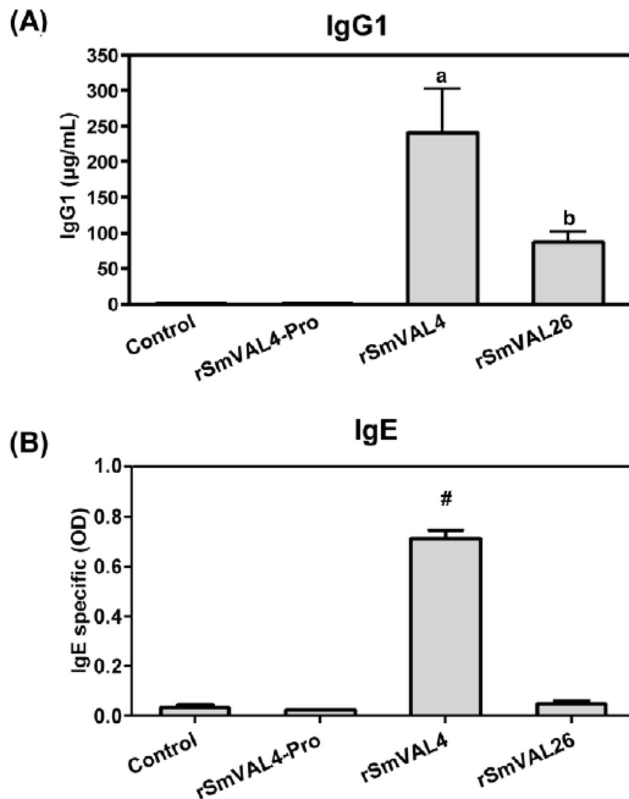

Figure 5. rSmVALs specific antibody production. BALB/c were sensitized s.c. with rSmVALs/Alum, rSmVAL4-Pro/Alum or PBS/Alum (Control) and then challenged i.n. with the proteins. The experiments were performed 24 h after the last challenge. (A) rSmVALs-specific IgG1 and (B) rSmVALs-specific IgE were determined in the sera by sandwich ELISA. Results are expressed as means ± SEM for groups of four mice and are representative of two experiments. a, b or #, significant differences ($p<0.05$) when compared to Control group (mice that were challenged with protein only).
doi:10.1371/journal.pntd.0001510.g005

localized in the acetabular glands. However, the detection of a significant amount of protein in 6 h schistosomula implies another localization, which is being investigated by imunohistochemistry and whole mount *in situ* hybridization.

Based on its similarity to a *S. japonicum* ortholog, we predicted that SmVAL26 could be in the tegument of young adults or hepatic schistosomula (14 day-old). However, contrary to our expectations, SmVAL26 was identified in the egg stage and in the hatched eggs' fluid. This is in accord with the proteomic study of Mathieson and Wilson (2010), which compared the contents of egg, miracidium, egg secretions and hatch fluid. Interestingly, we also detected SmVAL26 in the secretions of cultured eggs. Based on our data, we hypothesized that SmVAL26 could be localized in the egg envelope, or as proposed by Mathieson and Wilson (2010), in the protective fluid located between the miracidium and the envelope.

The biological function of SmVALs and orthologs belonging to the SCP/TAPS domain super-family remains unclear. The NaASP-2 protein from *N. americanus*, secreted by the infective larvae, was described to induce neutrophil recruitment *in vivo* in the air pouch model of acute inflammation [29]. Two additional hookworm SCP/TAPS proteins were reported to have immuno-modulatory activities. *Ancylostoma caninum* hookworm platelet inhibitor (*Ac*-HPI) exhibits an inhibitory effect on platelet aggregation, acting via glycoprotein Ia/IIa [30], and *A. caninum* neutrophil inhibitory factor (*Ac*-NIF) showed ability to inhibit CD11b/18-dependant leukocyte function [31,32]. Concerning the SCP orthologs from filarial nematodes, the rOv-ASP-1 from *Onchocerca volvulus*, showed striking features ranging from angiogenic activity in mouse corneas, to being proposed as an adjuvant for bystander proteins due to its ability to bind to APCs and trigger Th1 proinflammatory cytokines [33]. It is clear that no common biological function or activity has been associated with this protein family. And it is possible, as proposed by Hewitson et al. [34], that the SCP domain operates as an adaptable protein framework facilitating the evolution of various specialized functions.

In principle, schistosome proteins implicated in the tissue invasion process, are particularly good candidate antigens for the development of vaccines and drugs. One major concern on the use of these and other SmVALs as vaccine candidates is the potential allergic effects of these molecules. Herein, we have explored the murine model of airway inflammation, substituting OVA for the SmVALs, to investigate the putative allergic responses induced by these proteins. Our results demonstrate that following sensitization and challenge with the different proteins, they present varying properties in regards to the recruitment of inflammatory cells to the BAL fluid. SmVAL4 was shown to induce a marked increase in total cells in the BAL fluid, mostly due to an increase in eosinophil and macrophages, which correlated with increases in IgG1, IgE and IL-5, characterizing a typical allergic response, while SmVAL26 showed no alterations in the allergic parameters in the lungs. Furthermore, the use of a Pronase-treated SmVAL4, strongly supports the conclusion that the allergic properties are due to the protein itself and not to the carbohydrate moiety. We also demonstrated that anti-rSmVAL4 sera presented high titers of anaphylactic IgG1 antibody.

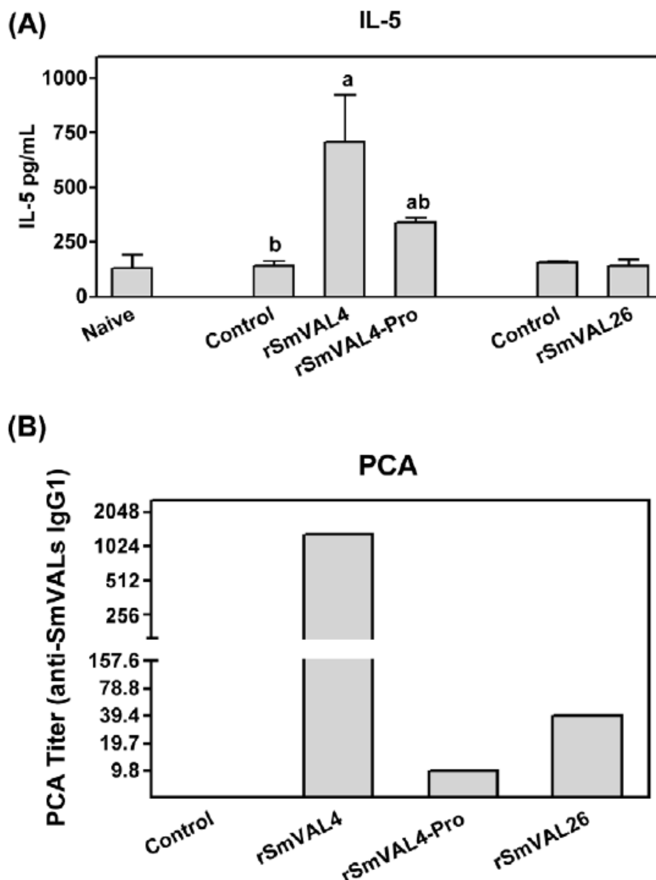

Figure 6. IL-5 and anaphylactic IgG1 detection. (A) IL-5 production in BAL fluid after sensitization and challenge with different rSmVALs. Results are expressed as means ± SEM for groups of four mice and are representative of two experiments. a and b represent statistically significant differences ($p<0.05$) between groups. (B) Anaphylactic IgG1 antibodies produced after sensitization and challenge with different rSmVALs; PCA titers represent the reciprocal of the highest dilution of heat-inactivated plasma that gave a lesion of 0.5 mm in diameter.
doi:10.1371/journal.pntd.0001510.g006

Human Schistosoma infections have been associated with the inhibition of allergies. In this context, the murine model of ovalbumin induced airway inflammation has been used to demonstrate that Schistosome infection, egg extracts or some purified antigens modulate negatively the allergic response induced by OVA treatment by a mechanism mediated by T regulatory cells [35,36]. Intrinsic properties of the antigens must be important for this modulation, since not all molecules modulate equally the allergic response. It is important to note that our model is quite different, since we have investigated the allergenic properties of the antigens per se.

On the other hand, in the course of a *S. mansoni* infection, two phases of immune responses have been recognized. In the first 3–5 weeks, during which the host is exposed to immature parasites, the immune response has been shown to be Th1-predominant. As the parasites mature, mate and begin to produce eggs at weeks 5–6, the immune response alters markedly to a strong Th2 profile [37]. However, there are reasons to believe that responses to schistosome worms during established infections are more complex. For example, immune responses classically mediated by the Th2 cytokine IL-5 (eosinophilia and eosinopoiesis) were reported in the first weeks of infection [38]. Moreover, the induction of $CD4^{+}$ T cells, specific IgE and basophils that produce IL-4 in response to worm antigens (e.g. SmCB1 – Cathepsin B) have also been described in this phase [39]. Finally, there is increasing evidence that excretory/secretory molecules from schistosome larvae can stimulate a mixed T helper response in the skin, with evidence of both Th1 and Th2 skewed responses at the site of infection [40]. Therefore, it is possible that SmVAL4 could be an antigen involved in the initial stage of the infection inducing a Th2-predominant response, whereas SmVAL26 may be more important in the late phase of infection.

In a vaccine context, the immunization of mice with rSmVAL4 and TiterMax Gold (a more balanced adjuvant) produced high levels of IgE in a conventional immunization protocol. It is interesting to note, that when challenged i.n. after this protocol, only rSmVAL4 induced airway inflammation (data not shown). This in itself poses risks, since a vaccination regime that promotes IgE production may well elicit undesirable side-effects such as exacerbation of allergy. Furthermore, in a recent report of a second Phase I trial, adult volunteers did experience allergic responses following immunization with the *Necator* ortholog, NaASP-2 [41]; this data advocates for the presence of IgE epitopes in this class of molecules. Therefore, it would be interesting to evaluate the levels of specific IgE antibodies for SmVAL4 in the sera of individuals resident in a schistosomiasis endemic area. The IgE epitopes for the SmVAL family remain to be determined, but it is tempting to conclude that the SmVAL4 protein contains some IgE epitopes absent on SmVAL26. The functional evaluation of other cercariae secreted SmVALs closely

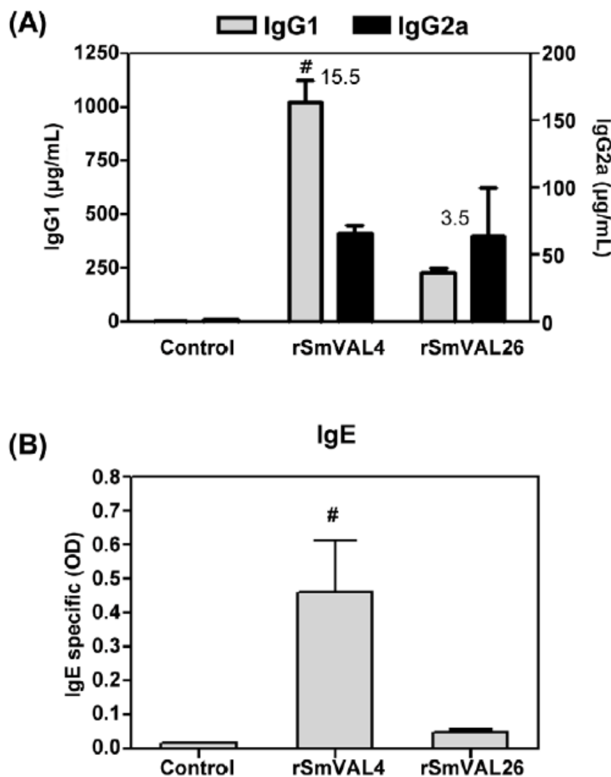

Figure 7. IgG1 and IgG2a immune profile induced by immunization of mice with rSmVALs formulated with TiterMax. BALB/c mice were immunized s.c. with 3 doses of recombinant SmVALs formulated with TiterMax adjuvant (first dose) or PBS 1x (subsequent doses). (A) rSmVALs-specific IgG1 and IgG2a. (B) rSmVALs-specific IgE were determined in the sera by sandwich ELISA. Results are expressed as means ± SEM for groups of four mice. # Significant differences ($p<0.05$), Control group (pre-bleed serum), ratios between IgG1/IgG2a are presented above the bars.
doi:10.1371/journal.pntd.0001510.g007

related to SmVAL4 (e.g. SmVAL10 and SmVAL18) (Figure S4) in this model of airway inflammation could help the identification and mapping of such IgE epitopes.

After the sequencing and assembly of the *Schistosoma* genome, efforts in schistosome research should shift from the simple identification of genes to the characterization of their functions and interactions with host cells. From this perspective, data presented here should be taken as a first insight on possible functions for members of the SmVAL family. They could have an immunomodulatory role that may be important during parasite penetration. One could hypothesize that SmVAL4 would be involved in the recruitment of mast cells and basophils, inducing secretion of histamine, which could facilitate parasite invasion through vessel dilatation. Additional studies, such as *in situ* hybridization and the evaluation of native proteins should further elucidate the localization and the role of these proteins.

Finally, we believe that, although the airway inflammation model explored here presents some divergences from physiological conditions, it reveals and differentiates the allergic properties of molecules, proving to be useful for studying molecules with allergic potential. In selecting SmVAL molecules to be further investigated as vaccine candidates, we can eliminate those that display allergic potential in this model, such as SmVAL4.

Supporting Information

Figure S1 Summary of some SmVALs developmental stage associated mRNA and proteins based on recent data from *S. mansoni* and *S. japonicum*. (A) Upper panel: the developmental stages of the life cycle of schistosomes; (B) SmVALs (mRNA or protein) expressed in these developmental stages. The arrow (↑) indicates up-regulation in a particular stage by Real-time RT-PCR [5,42] or Microarray data [43,44,45]; (•) Identification of the correspondent SmVAL protein or (•[J]) SjVAL by proteomics data [6,7,8,11,12,13,14]. M and F – male and female adult worms, * previously reported on the *Schistosoma* transcriptome analysis [4], (panel (A) was extracted and modified from [4]).
(PDF)

Figure S2 Phylogenetic analysis of *S. mansoni* and *S. japonicum* VALs protein family, demonstrating that SjVAL (AAW27353.1) branches with SmVAL26/28 and 27. Phylogenetic trees were inferred by ClustalX 1.83 and illustrated by Treeview as described in Methods. The *S. japonicum* protein GenBank accession numbers are indicated in the tree, whereas the *S. mansoni* are the following: SmVAL1 (AAY43180.1), SmVAL2 (XP_002571733.1), SmVAL3 (AAZ04923.2), SmVAL4 (XP_002571676.1), SmVAL5 (ABB88846.2), SmVAL6 (AAY-28955.1), SmVAL7 (AAZ04924.1), SmVAL8 (ABW98681.1), Sm-VAL9 (XP_002582201.1), SmVAL10 (ABO09814.2), SmVAL11 (ABA54555.1), SmVAL12 (XP_002571731.1), SmVAL13 (ABB-88843.1), SmVAL14 (XP_002569793.1), SmVAL15 (XP_002582174.1), SmVAL16 (XP_002571817.1), SmVAL17 (XP_002578833.1), SmVAL18 (XP_002571658.1), SmVAL19 (XP_002571657.1), SmVAL20 (CAZ28636.1), SmVAL21 (XP_002578075.1), SmVAL22 (XP_002574629.1), SmVAL23 (XP_002582175.1), SmVAL24 (XP_002574962.1), SmVAL25 (XP_002574963.1), SmVAL26 (XP_002577262.1), SmVAL27 (XP_002577271.1), SmVAL28 (XP_002582199.1) and SmVAL29 (XP_002571340.1).
(PDF)

Figure S3 Alignment of the derived amino acid sequence of SmVAL26 and SjVAL26 (AAW27353.1). Highlighted are the SCP domain (continuous box). The regions with high identity and similarity between sequences are shown as black and gray columns, according to the Clustal X algorithm.
(PDF)

Figure S4 Alignment of the derived amino acid sequence of SmVAL4, 5, 10, 18, 26, 27 and 28. Demonstration that SmVAL4, 10 and 18 (all secreted during the cercaria-schistosomulum transformation process) are more closely related, whereas SmVAL5, 26, 27 and 28 (all detected in the egg stage) are more related to each other. The regions with high identity and similarity between SmVALs are shown as black and gray columns, according to the Clustal X algorithm.
(PDF)

Table S1 Synthetic genes used in this study. [a]Redesigned sequence using DNA2.0 codon optimization algorithms for expression in *Pichia pastoris*.
(PDF)

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Author Contributions

Conceived and designed the experiments: LPF DR VC LCCL. Performed the experiments: LPF DR VC HKR ELF-M. Analyzed the data: LPF DR VC HKR ELF-M LCCL. Contributed reagents/materials/analysis tools: LPF LCCL ELF-M. Wrote the paper: LPF HKR DR ELF-M LCCL.

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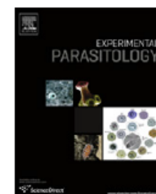

Schistosoma mansoni: Molecular characterization of Alkaline Phosphatase and expression patterns across life cycle stages

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ABSTRACT

Here we describe the cloning and characterization of the *Schistosoma mansoni* Alkaline Phosphatase (SmAP), previously identified in the tegument of adult worms. SmAP encodes a complete sequence composed of 536 amino acids containing an *N*-terminal signal peptide, five *N*-glycosylation sites, and a GPI anchor signal, similar to that described for mammalian orthologs. Real-time RT-PCR and Western blot experiments suggest a rapid translation as soon as cercariae are transformed into schistosomula. Immunolocalization analysis shows that the protein is widely distributed in the worm tissues, with increased concentration in the vitelline glands of female parasites. Furthermore, the surface localization of this enzyme was quantitatively supported by its enzymatic activity in live *ex vivo* or cultured parasites throughout the life cycle stages. The fact that cercariae accumulate large amounts of SmAP mRNA, which rapidly translates into protein upon schistosomula transformation, indicates it may have an important role in host invasion.

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1. Introduction

Schistosomiasis is considered a neglected tropical disease by WHO and the search for a vaccine is one of the main goals of Tropical Disease Research (Morel, 2000). The currently used anti-schistosomal drugs (e.g. praziquantel and/or oxamniquine) do not prevent reinfection (Bergquist et al., 2002) and potentially increase the likeliness for the parasite to acquire resistance (Al-Sherbiny et al., 2003). The *Schistosoma mansoni* and *Schistosoma japonicum* transcriptomes (Hu et al., 2003; Verjovski-Almeida et al., 2003) and genomic sequencing projects (Berriman et al., 2009; Zhou et al., 2009) opened new opportunities for diagnosis, drug discovery, and vaccine research.

The Alkaline Phosphatase from *S. mansoni* (SmAP) is so far one of the most studied molecules on the parasite–host interface (Ballen et al., 2002; Bhardwaj and Skelly, 2011; Cesari, 1974; Dusanic, 1959; Nimmo-Smith and Standen, 1963; Payares et al., 1984), although it has not been well characterized at the molecular level. It is a glycosylated enzyme (Payares et al., 1984) associated with the cell membrane (Cesari, 1974). It seems to have a fundamental role in the parasite's physiology, removing phosphate groups from organic molecules (Cesari et al., 1981). This protein may be important in the context of immune evasion within the host, by metabolizing the intrinsic host's molecular signals of danger released during skin and blood vein invasion (Bhardwaj and Skelly, 2009, 2011). The Alkaline Phosphatase activity was initially detected in different tissues of the adult parasite's body (Cesari, 1974). It was used as a marker for tegument surface plasma membrane enrichment (Braschi et al., 2006; Roberts et al., 1983). Additionally, it has been characterized as a surface-exposed tegument protein by proteomic studies (Braschi et al., 2006; Braschi and Wilson, 2006). This protein becomes even more exposed on the parasite's surface after treatment with praziquantel and the co-administration of an antisera that inhibits Alkaline Phosphatase activity increases the killing of female worms *in vivo* (Fallon et al., 1994). A more recent study showed that SmAP, among others, is recognized by antisera from infected *Rhesus* macaques which succeeded in eliminating adult worms. The concentration of antibodies was considered as a remarkable feature for worm elimination in high-responder individuals (Wilson et al., 2008). It was assumed that these protein targets should be continuously recognized and functionally inhibited by neutralizing antibodies. A study has been recently published in which the role of SmAP in degrading AMP was demonstrated (Bhardwaj and Skelly, 2011). With these and other pieces of evidence, SmAP has been proposed as a vaccine candidate (Braschi and Wilson, 2006; Verjovski-Almeida et al., 2003). In this context, we have characterized the molecular traits,

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produced the recombinant protein and evaluated its expression during the parasite's life cycle. We have also investigated the surface enzymatic activity in different parasite stages and its putative inhibition by antisera.

2. Materials and methods

2.1. Biological material

The *S. mansoni* adult worms (BH strain) were obtained from perfusion of infected hamsters. Eggs, miracidia, cercariae, and schistosomula were obtained as previously described (Verjovski-Almeida et al., 2003). Experimental animal protocols were approved by the Animal Use Ethics Committee of Instituto Butantan (CEUAIB Protocol N° 597/09, São Paulo, Brazil).

2.2. Cloning and sequence analysis

Total RNA was isolated from adult worms using TRIzol reagent (Life Technologies), followed by mRNA purification with oligo(dT)-cellulose columns according to the manufacturer's instructions (GE Healthcare). Specific oligonucleotide was designed based on SmAE 607243 contig sequence (Verjovski-Almeida et al., 2003) to perform rapid amplification of 3′ cDNA End (RACE) (Life Technologies) according to manufacturer's instructions. After the determination of the 3′ end, the SuperScriptTM First-Strand Synthesis System for RT-PCR (Life Technologies) was used for SmAP full-length cDNA generation following the manufacturer's protocol. The 5′ ATG CTT CCA ACT GTC TTA TCG AC 3′ forward and 5′ GAT TAC ATA CTT TCA CAT GTT TTA TTA TG 3′ reverse primers were used to amplify the complete open reading frame of SmAP. The obtained PCR fragment was cloned into pGEM-T easy vector (Promega) and sequenced to confirm its identity. Sequence alignments were done using the ClustalW software (Higgins, 1994); determination of the molecular weight and pI were done with the Compute pI/Mw Tool algorithm available at the Swiss Institute of Bioinformatics (http://www.expasy.org/cgi-bin/pi_tool); detection of the signal peptide and transmembrane region were performed with the SignalP algorithm, (Bendtsen et al., 2004) and TMHMM version 2.0 (Sonnhammer et al., 1998) respectively, and *N*- and *O*-glycosylation sites with the NetNGlyc version 1.0 and YingOYang algorithms (Gupta and Brunak, 2002). Homology modeling was done using Swiss-PDB Viewer 4.0.1 software (Guex and Peitsch, 1997) and putative conformational B-cell epitope prediction was done with the Discotope 1.2 algorithm (Haste Andersen et al., 2006).

2.3. Real-time RT-PCR

Total RNA from eggs, miracidia, cercariae, 7-day old cultured schistosomula, adult worms, male and female were extracted using TRIzol (Life Technologies) according to manufacturer's protocol. RNA quantitation was carried out with ND-1000 spectrophotometer (NanoDrop Technologies) and its quality verified with an Agilent 2100 Bioanalyzer. For the cDNA synthesis, RNA samples from at least three different extractions, were first treated with RNase-free DNase RQ1 (Promega), and then the Superscript III reverse transcriptase was added for the cDNA synthesis, using randomic primers, following instructions from the manufacturer (Life Technologies).

For quantitative PCR (qPCR), primers were designed using the Primer Express algorithm annealing right above splicing sites to avoid genomic DNA amplification. The primers designed this way were: forward, 5′ CGC CTC TAA AGC AGG ATT TTC TAC 3′, and reverse, 5′ GTC AAA AGT TCC ATC AAA CCA GC 3′, which together with the synthesized cDNA and SYBR Green (Applied Biosystems) were used for amplification following 10 min at 95 °C, 40 cycles of 10 s at 95 °C, followed by 1 min at 60 °C and monitored for fluorescence emission from SYBR Green only when intercalated in dsDNA in a 7300 Real Time PCR System (Applied Biosystems).

Each cycle's fluorescence was collected, analyzed and results shown as fluorescence (ΔRn) vs. cycles. The amplification curves were used to determine the number of cycles that reached the threshold (Ct). With these values we could compare the results obtained with SmAP expression and the control housekeeping gene, α-tubulin (Accession: M80214) for all the stages analyzed, using the following formula: fold change = $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001; Pfaffl, 2001). Graphics are shown as relative fold change vs. life cycle stages, in order to show how many times a gene is more expressed in a stage compared to any other stage. These differences were statistically analyzed by ANOVA and later, by Tukey's with help of the statistical analysis software GraphPad Prism 4.

2.4. Expression and purification of recombinant Alkaline Phosphatase

The coding sequence of the *S. mansoni* Alkaline Phosphatase (SmAP) was first amplified from a cDNA obtained from total mRNA from *S. mansoni* adult worms by PCR with the primers P1: 5′ CAC CTC GAG AAA TCG TCC TTA TTG AAT 3′, and P2: 5′ CAT GGT ACC TCT ATC GAG ATC CAT TGT TTC C 3′. The amplified fragments were inserted directionally into the pAE expression vector (Ramos et al., 2004) and confirmed by automatic sequencing (ABI 377, PE Applied Biosystems). *Escherichia coli* BL21 Star (DE3) pLysS strain transformed with pAE-smAP was grown overnight as a pre-inoculum in 10 mL of 2YT medium. This culture was then transferred to a 1 L flask containing 600 mL of 2YT medium, and allowed to grow until log phase and then induced with 1 mM IPTG. After induction, cells were collected by centrifugation and resuspended in lysis buffer (50 mM Tris–HCl, pH 8.5 plus Protease Inhibitor Cocktail 1× (Sigma)), and sonicated in an ice bath (40 Hz, 1s pulse, 10 min). After centrifugation at 20,000*g*, inclusion bodies were separated and resuspended in washing buffer (50 mM Tris–HCl pH 8.8, 10 mM EDTA, 2% Triton X-100) and centrifuged again at 20,000*g*, and after two more washing steps, the resulting inclusion bodies were finally solubilized with 8 M urea. These were then refolded overnight by pulsed dilution (100×) in a refolding buffer (Tris 50 mM pH 8.5 and β-mercaptoethanol 5 mM), filtered through 0.8 μm-pore filters (Millipore), and finally nickel-affinity purified using a 5 mL HisTrap™ HP column (GE Healthcare). Protein concentrations were determined by Lowry's method (Bio-Rad DC Protein Assay) using bovine serum albumin as a standard. In order to monitor the purity of the recombinant protein along the whole purification process, equal amount of samples (20 μg) were electrophoresed in 12% SDS–PAGE and stained afterwards with Coomassie Brilliant Blue R-250 (Sigma). The protein purity was determined by densitometric analysis using ImageJ software (http://rsweb.nih.gov/ij/).

2.5. Polyclonal antibody production

Rats were immunized with 100 μg of the purified recombinant protein with Freund's adjuvant according to the following schedule: a first immunization, and three subsequent boosts at every 14 days, and a final bleeding 2 weeks after the third boost. These sera obtained were immunoadsorbed with an extract of *E. coli* transformed with empty pAE vector and utilized in the Western blot and immunolocalization assays.

2.6. Protein expression profile along life cycle stages

Total protein extracts from eggs, miracidia, cercariae, schistosomula, and adult worms were prepared in 40 mM Tris, pH 7.4,

2% SDS plus protease inhibitor through sonication, as described above. The samples were centrifuged at 20,000g for 30 min at 4 °C and the supernatant was recovered and used for the assays. The tegument extract from adult worms was obtained by a freeze/thaw/vortex procedure, as previously described (Roberts et al., 1983). Briefly, frozen worms (1000) were thawed on ice in the presence of 1 mL ice-cold RPMI medium plus protease inhibitors (Protease inhibitor cocktail; Sigma); 10 vortex pulses at maximum speed during 1 s each, to detach the tegument. After the stripped worms had settled, the supernatant containing pieces of tegument was collected and sonicated. Protein concentrations were determined with a DC Protein Assay (Bio-Rad) using BSA as a standard. For Western blot, purified recombinant SmAP (rSmAP, 25 ng, ~80% purity) and total parasite protein extracts (5 μg each) were subjected to SDS–PAGE (12%) 1D gels under reducing conditions. After transfer onto PVDF membranes (GE Healthcare) and blocking (Tris 10 mM pH 8.0, 0.3% Tween 20, and 5% [wt/vol] skimmed milk powder), blots were incubated with immunoadsorbed rat antisera against rSmAP in a 1:5000 dilution. After three washes with Tris 10 mM pH 8.0, blots were incubated with HRP-conjugated goat anti-rat IgG antibody in a 1:6000 dilution (Pierce Biotechnology), then washed three times as before and specific antibody binding was visualized using the ECL Western Blotting Detection System (GE Healthcare).

2.7. Deglycosylation experiment

Deglycosylation of native SmAP was carried out as previously described (Plummer and Tarentino, 1991). Briefly, 20 μg of tegument or egg extract was denatured for 10 min at 100 °C with 1× glycoprotein denaturing buffer (10×: 5% SDS, 0.4 M DTT) and then incubated for 16 h at 37 °C with PNGaseF (New England Biolabs) followed or not by treatment with a set of enzymes for determining potential *O*-glycosylations (EDEGLY Kit, Sigma). Negative controls contained the same reagents as samples minus enzyme.

2.8. Immunolocalization of SmAP in adult worms

Freshly perfused adult worms were embedded in OCT medium (Sakura) in a pre-cooled beaker of isopentane, frozen in liquid N_2. Eight micrometer cryostat sections of adult worms were adhered to silanized glass slides (DakoCytomation), fixed in acetone for 30 min at −20 °C and rehydrated with PBS overnight at 4 °C. Slices were then blocked 4 h with PBS, 0.1% Tween 20 and 10% normal rabbit serum. Following this, incubations were made with the rat anti-rSmAP serum (1:200 dilution in the same blocking buffer) and rhodamine–falloidin (1:100) for 3 h at room temperature. After washing six times with PBS, 0.1% Tween 20, the slides were incubated with Alexa Fluor® 488 rabbit anti-rat IgG (H + L) (Life Techonologies) (1:200 dilution). *E. coli*-immunoadsorbed serum from naïve rats was used as negative control. Images were acquired in a Zeiss LSM 510 Meta Confocal System, attached to a Zeiss Axiovert 100 microscope using a 1.2 NA 40× PlanApochromatic objective with differential interference contrast and a Band Pass filter (BP 500-550 IR) to avoid autofluorescence.

2.9. Activity assay of Alkaline Phosphatase on live stages of the parasite

We used the synthetic substrate *p*-Nitrophenyl Phosphate (pNPP) in a adapted protocol (Cesari et al., 1981) to assess the surface activity of live parasites. We followed two criteria: the first one compared enzymatic activity per surface area of the life cycle stages and the second one compared activity per parasite number between male and female adult worms. For the first analysis, we made an estimate of the surface areas of the different stages of intact parasites, to roughly normalize them. We considered the surface area of cercaria to be 10,682–20,000 μm^2 (Crabtree and Wilson, 1980; Samuelson and Caulfield, 1985), that from eggs to be around 16,304 μm^2 using an approach to measure the surface area of a hen egg (Narushin, 2005), and male adult worms to be around 12,000,000 μm^2 without considering the tegument pits (Smith et al., 1969). Based on these calculations, we used a total number of 2000 cercariae, 4000 eggs, 1500 7-day old cultured schistosomula and three male adult worms. In the second analysis, we used three individuals of each, male and female live *ex vivo* adult parasites. All of the specimens were incubated in 100 μL of the pNPP substrate (Sigma), pH 9.5, for 30 min, after which parasites were visually inspected for viability, and finally the absorbance at 405 nm was read on a multiplate ELISA reader (Multiskan EX, LabSystems). As a control, 100 μL of pNPP were incubated with 100 μL of PBS; each sample was assessed in triplicate and statistical analysis was performed using ANOVA. For the antibody blocking assay, adult parasites were incubated in 50 μL of anti-rSmAP sera for 1 h at 37 °C, and then assayed for surface activity using the same parameters and calculations as mentioned above.

3. Results

3.1. Sequence analysis of Alkaline Phosphatase from S. mansoni

To clone the full-length sequence of the SmAP cDNA, we performed a 3′ RACE experiment based on SmAE 607243 contig (Verjovski-Almeida et al., 2003) to determine the 3′ end of the gene. With this information we designed specific oligonucleotides to amplify the full-length SmAP by RT-PCR from adult worm total mRNA. The resulting full-length cDNA (GenBank Accession HM045783) proved to be 99% identical to a previously reported SmAP cDNA [GenBank Accession EU040139, (Ndegwa et al., 2007)], with some divergences, possibly due to the differences between the two parasite strains compared (BH in this study and Puerto Rican in the former) or to sequencing errors (Supplementary Fig. 1).

Searching the genomic sequence of the corresponding cDNA at the *S. mansoni* genome database (www.schistodb.org/Homepage/Smansoni), we identified two predicted Alkaline Phosphatase proteins (Smp_155890 and Smp_145290). The sequence identified in Smp_155890 turned out to be the same sequence as SmAP, although with a shorter and divergent 3′ end, probably because its last exon was not correctly predicted (Supplementary Fig. 1). Subsequent re-alignment analysis of these two sequences on the corresponding scaffold (supercontig Smp_scaff000188) revealed a potential misassembly as being responsible for this artifact (data not shown). On the other hand, Smp_145290 (supercontig Smp_scaff000103; 881397–886893) shares 88% identity with SmAP, revealing the presence of polymorphisms all over the sequence and differing greatly in the 5th exon; however, this predicted protein could not be validated with any EST from *S. mansoni* databases (data not shown).

The full-length cloned cDNA displayed an ORF of 1698 bp, encoding a protein of 536 amino acids with a predicted molecular mass of approximately 59.3 kDa and an isoeletric point of 5.92. The SmAP protein sequence showed an overall aminoacid sequence identity of 33–37% to those of *Homo sapiens* placental Alkaline Phosphatase, *Rattus norvegicus* tissue non-specific Alkaline Phosphatase, *Mus musculus* liver Alkaline Phosphatase, and *Canis familiaris* Alkaline Phosphatase (Fig. 1). SmAP contains the AP signature sequence (residues 64-495) (outlined by a continuous box in Fig. 1), and is recognized as part of the Pfam Alkaline Phosphatase family (PF00245) with an *E*-value of 8.60×10^{-109}. SmAP also contains a putative *N*-terminal signal peptide (bold characters in

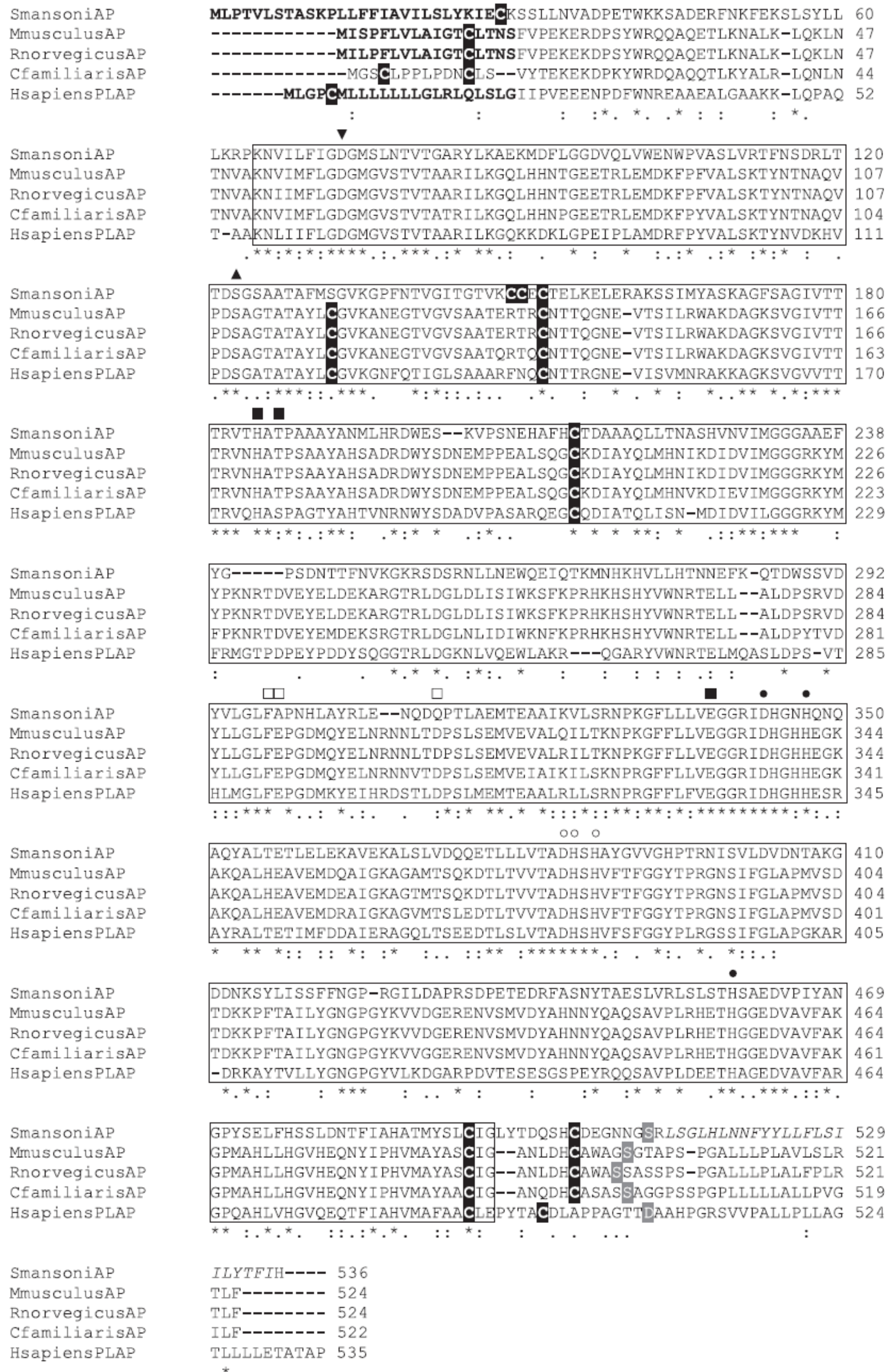

Fig. 1. Sequence alignment of Alkaline Phosphatase (AP) from *S. mansoni* compared to different mammalian APs [*Homo sapiens* placental AP (Accession Number M14169), *Rattus norvegicus* tissue non-specific AP (Accession Number BC088399), *Mus musculus* liver/bone/kidney AP (Accession Number X13409) and *Canis familiaris* liver/bone/kidney AP (Accession Number XM_535374)]. Boxed aminoacids correspond to the AP domain. Cysteins are shaded in black, putative GPI-anchor signal is shaded in grey, putative signal peptides are in bold type, putative transmembrane region is in italics. Active site is marked with (▲), residues predicted to bind to magnesium (■), to calcium (□), to zinc1 (●), to zinc2 (○) and to both magnesium and zinc2 (▼).

Fig. 1), and a putative *C*-terminal transmembrane domain (italic characters), characterizing it as a type-I, single-spanning transmembrane protein, with its catalytic domain facing the extracellular space. Examining the distribution of potential post-translational modifications, a putative signal for GPI anchor modification centered on S511 could be identified. There were also five

potential *N*-glycosylation sites (Asn223, Asn244, Asn398, Asn413, and Asn445) and a few potential *O*-glycosylation sites with low-probability. Comparing the SmAP amino acid sequence with those of mammalian AP's (Kozlenkov et al., 2002), we further identified three metal atom binding sites: one for magnesium (at positions Asp73, His185, Thr187, and Glu 338) and two for zinc (Asp 343, His 347, His 459 for Zinc1; Asp 73, Asp 374, His375, His 377 for Zinc2). In the mammalian sequence there is a calcium binding site which is not totally conserved in the SmAP (Phe 298, Ala 299, and Asp 311). The catalytic center would be a serine residue at position 123 (Kozlenkov et al., 2002) (Fig. 1).

3.2. Homology modeling of the protein and putative B-cell epitope determination

We searched the closest homolog structure already resolved for the 3D modeling of the protein. The crystal structure of human placental Alkaline Phosphatase (PDB entry: 1zed) has an identity to our sequence of 37%, with an *E*-value of 4e−88. With this template, we generated a non-glycosylated 3D model of our protein using the Swiss PDB-Viewer software (Fig. 2). Residues belonging to the active site and putative conformational B-cell epitopes are highlighted with a unique color. The aminoacid residues involved in metal binding, as well as the active site, are localized very close to each other in this model, and we can also see the number of putative B-cell epitopes predicted, a couple of them in proximity to the active site and another one elsewhere on the molecule's surface.

3.3. Transcriptional pattern along the life cycle stages

Transcriptional levels of SmAP were evaluated in all developmental stages of the life cycle by real-time RT-PCR. Our data revealed that SmAP mRNA is expressed in all stages, with the highest transcriptional activity observed in the cercariae stage (∼50-fold relative to schistosomulum stage), followed by eggs (∼20-fold) and adults (∼6-fold) (Fig. 3A), all of which had statistically significant differences between themselves and also when compared with miracidia and schistosomula stages. Miracidia and 7-day old cultured schistosomula levels were the lowest when compared to other developmental stages. We further determined that there was no statistically significant difference between the transcriptional patterns of male and female parasites (Fig. 3B).

3.4. Expression of SmAP in different parasite stages

Recombinant SmAP produced in *E. coli* BL21 Star (DE3) pLysS was nickel-affinity purified (∼80% purity, Supplementary Fig. 2B)

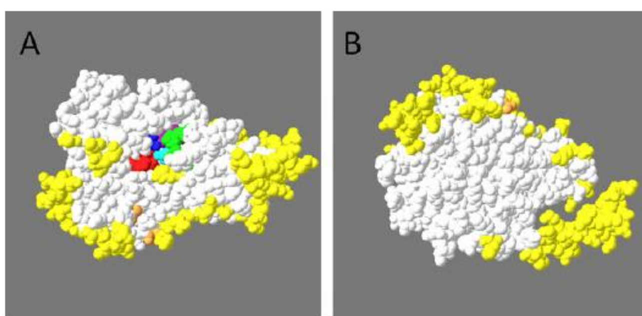

Fig. 2. Homology modeling of SmAP. A and B represent two opposite images of the protein. Active site is a serine residue colored in dark blue and putative conformational B-cell epitopes are in yellow. Aminoacidic residues predicted to bind to zinc 1 are in green, to zinc 2 in violet, to magnesium in red, to both magnesium and zinc 2 in light blue. In orange would be the corresponding calcium binding residues according to alignment analysis with mammalian sequences.

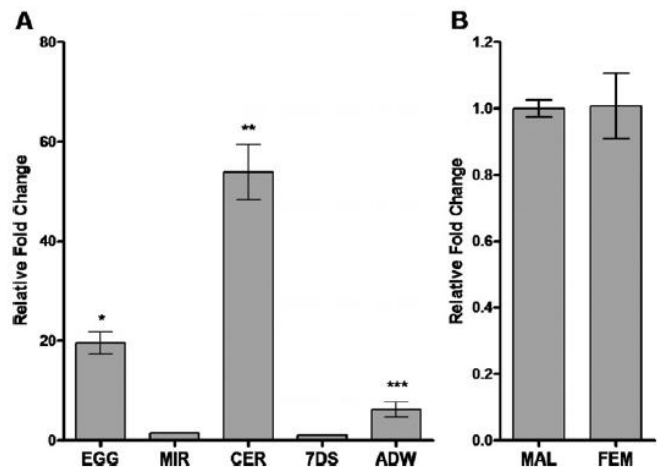

Fig. 3. (A) Transcriptional profile along parasite life cycle stages and (B) between male and female adult worms. Samples were analyzed by real-time RT-PCR and data were normalized against the housekeeping α-tubulin gene. The data were calculated according to the comparative ΔΔCt method and are shown as the fold-change in SmAP expression relative to (A) 7-day old cultured schistosomula or (B) male adult parasites. EGG, eggs; MIR, miracidia; CER, cercariae; 7DS, 7-day cultured schistosomula; ADW, Adult worms; MAL, Males; FEM, Females. *, **, ***Represent statistically significant differences, $p \leqslant 0.05$.

and then used to generate polyclonal antibodies in rats, in order to analyze the protein expression in the parasite's life cycle by Western blot. The SmAP is expressed throughout all analyzed stages of *S. mansoni* (Fig. 4A and B) with some particular features. The lowest expression level was found in cercariae and it can be observed that protein level continues increasing up to 12 h after transformation. At the 7-day old schistosomula stage it has decreased to levels comparable to that of the cercariae stage. Gender analysis of adult worms revealed higher expression in females than in males (Fig. 4A). A weaker band present in females, also observed in miracidia, is probably a product of degradation during sample processing. We compared the tegument and stripped worms fractions (Fig. 4C), showing that the protein expression level was

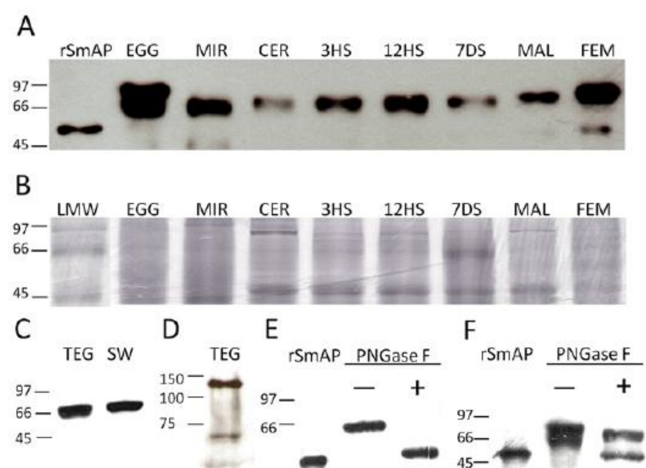

Fig. 4. (A) Western blot analysis of the expression of SmAP along different stages of the parasite's life cycle. EGG, eggs; MIR, miracidia; CER, cercariae; 3HS, 3-h cultured schistosomula; 12HS, 12-h cultured schistosomula; 7DS, 7-day cultured schistosomula; MAL, Males; FEM, Females. (B) Corresponding Coomassie-stained gel as a loading control for the amount of protein. (C) SmAP in 20 μg of tegument (TEG) and stripped worms (SW). (D) Tegument extract under non-reducing conditions. (E) Deglycosylation of native tegument SmAP; PNGase F, tegumental extract deglycosylated with PNGase F (+) or not (−). (F) Deglycosylation of eggs extract; PNGase F, eggs extract deglycosylated with PNGase F (+) or not (−).

comparable in both fractions analyzed. Noteworthy, is the presence of an additional band with a slightly higher molecular mass in eggs (Fig. 4A). This last feature could reflect a product of different stage-specific glycosylation [as previously reported for egg secreted glycoproteins (Jang-Lee et al., 2007)], since a potential second isoform was not found in amplifications done with SmAP primers using mRNA from this stage, nor in 5′ and 3′ RACE experiments (data not shown).

3.5. Characterization of SmAP as a N-glycosylated protein

The native SmAP observed in schistosome extracts migrates with a molecular mass of ~65 kDa, higher than the predicted ~56 kDa for the mature protein (Fig. 4A). We also observed that SmAP dimerizes in non-reducing conditions as demonstrated by the ~130 kDa product from tegument extracts (Fig. 4D). In order to determine if the ~65 kDa protein band observed was due to glycosylation, we digested total tegument extracts with a recombinant *N*-glycosidase F. The Western blot revealed that, after digestion, the SmAP band shifted migration to a lower molecular mass, demonstrating that SmAP present in the tegument is *N*-glycosylated (Fig. 4E). Furthermore, deglycosylation of the egg extract revealed two bands, the lower with the expected size for the studied protein and the second one still having a higher molecular mass (Fig. 4F), which could not be further deglycosylated when treated with enzymes specific for *O*-deglycosylation (data not shown).

3.6. Immunolocalization of SmAP in parasite tissues

We performed immunolocalization experiments with the purpose of determining the protein distribution in adult worm tissues. In male, SmAP is localized in almost all tissues of the parasite (Fig. 5A). It is also possible to see fluorescence in the tegument, cytoplasmic projections and cytons laying beneath the muscle layer (Fig. 5C). In females, there is a remarkable fluorescence in the region of the vitelline glands (Fig. 5D) and a lower uniform fluorescence in the parenchyma. Parasites treated with rat naïve serum showed no fluorescence under confocal microscopy observation (data not shown).

3.7. Alkaline Phosphatase activity in the different stages of live parasites

We determined the Alkaline Phosphatase activity on the surface of live parasites across the different life stages normalized by surface area. The lowest activity was observed in the 7 day-old cultured schistosomula. There was a statistically significant difference among all stages, except when comparing eggs with cercariae (Fig. 6A). In comparing genders, in this case normalized by number of parasites, females showed the highest activity levels, twice the activity of male worms. This is very significant, especially considering that females have a smaller surface area (Fig. 6B).

Antibodies raised in rats against the recombinant protein were used to investigate possible inhibition of the activity of the native enzyme. Live adult parasites were incubated with anti-rSmAP antisera and enzymatic activity of Alkaline Phosphatase evaluated. No inhibition in AP activity was detected as compared to parasites incubated with naïve sera (data not shown).

4. Discussion

In this report, we described the molecular cloning of the full-length cDNA Alkaline Phosphatase from *S. mansoni*. Based on sequence comparisons with the previously reported SmAP (Ndegwa et al., 2007) and the *S. mansoni* genome prediction (Smp_155890), we conclude that our cDNA better represents the SmAP sequence. We also believe that Smp_145290 may be a non-transcribed gene, since it was not found in either 3′ or 5′ RACE experiments, raising questions about the existence of this putative protein of the same Alkaline Phosphatase family.

The SmAP protein sequence shows a putative transmembrane region and a GPI anchor signal prediction. This could indicate that the protein may be associated with the plasma membrane by these two mechanisms, as has been described for the tetraspanin, Sm23

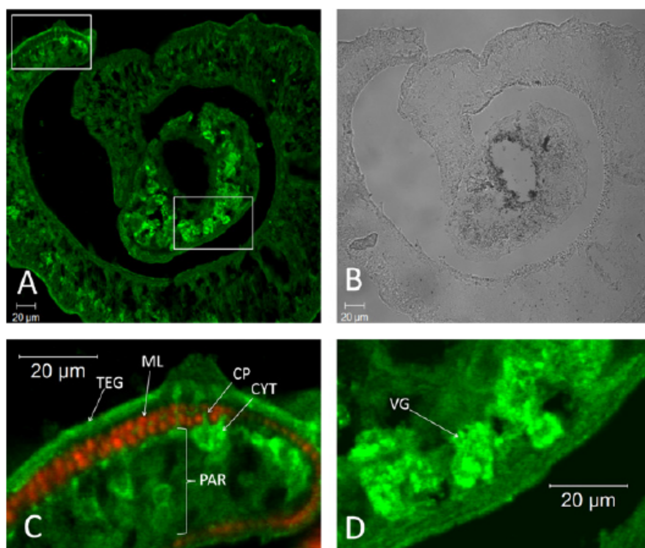

Fig. 5. Immunolocalization of SmAP in transversal section of adult worms. (A) Fluorescence confocal microscopy image of male and female sections, SmAP (green, Alexa Fluor 488). (B) Differential contrast image of (A). (C) Inset of male section, tegument (TEG), cytoplasmic projection (CP), cyton (CYT), and parenchyma (PAR); the muscle layer (ML) is labeled in red (phalloidin-rhodamin). (D) Inset of female section, vitelline gland (VG).

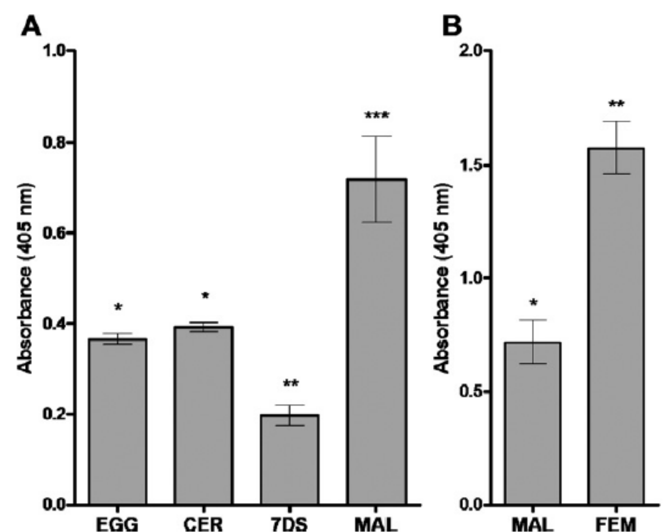

Fig. 6. Alkaline Phosphatase activity assay on live parasites. (A) Comparison between stages, normalized for surface area of each stage. (B) Comparison between genders, normalized for the number of individuals (three of each). The substrate used was a ready-to-use solution of *p*-Nitrophenyl Phosphate incubated with each stage for 30 min at 37 °C, and the absorbance read on a multiplate ELISA reader. Bars represent means ± S.D. of one experiment representative of three independent experiments. EGG, eggs; CER, cercariae; 7DS, 7-day cultured schistosomula; MAL, Males; FEM, Females. *, **, ***Represent statistically significant differences, p value < 0.05.

(Koster and Strand, 1994). The association of SmAP with the parasite's membrane has been previously observed (Cesari, 1974) and the treatment of live worms with Phosphatidylinositol-Phospholipase C removes SmAP from the tegument surface (Castro-Borges et al., 2011). The ~65 kDa predicted size of the glycosylated monomer (Ballen et al., 2002; Cesari et al., 1981; Pujol and Cesari, 1990) was confirmed by our results, as well as the ~130 kDa dimer. Our experiments with PNGaseF also support that SmAP is *N*-glycosylated, agreeing with previous observations (Payares et al., 1984). The identification of an extra SmAP protein band in eggs by Western blot should be further investigated, since it could constitute a product of post-translational modification. This higher band in eggs is strongly recognized by the anti-SmAP sera and is also *N*-glycosylated. The anti-SmAP serum was raised using a recombinant protein produced in *E. coli*, so a cross-reaction with a different protein due to oligosaccharide moieties can be excluded. Additionally, the analysis of the genome revealed the presence of an isoform (Smp_145290), which could not be amplified by either 3′ or 5′ RACE experiments using RNA from eggs, nor validated in any transcriptome study; hence, it is not likely that this isoform is present in this stage. Our hypothesis is that SmAP is *O*-glycosylated. However, *O*-deglycosylation with enzymes specific for mammalian glycoproteins failed. It is possible that the mammalian-specific enzymes do not recognize the schistosome *O*-glycosylation properly.

It is interesting to note that there is a very high transcriptional activity in the cercarial stage, which is not immediately translated into protein, since protein levels are very low in this stage. However, they rapidly increase following host penetration until at least 12 h. At 7 days, however, protein levels have decreased to levels comparable to those of cercariae, as well as the transcriptional activity. A different transcriptional profile has been recently reported for this same SmAP along the life stages of the parasite (Bhardwaj and Skelly, 2011). In this respect, it is noteworthy that we have performed qRT-PCR with three different cDNA libraries from each of these stages and observed that all three gave identical patterns of transcriptional levels among life cycle stages, with special note to cercariae and 7-day old schistosomula, where marked differences were observed here as compared to (Bhardwaj and Skelly, 2011). These differences can be ascribed to the use of different gene normalizer controls and/or due to the use of different cDNA libraries. Our results support the hypothesis that, after cercarial transformation, schistosomula may require SmAP messenger RNA that had been transcribed in the cercarial stage to be rapidly translated into protein as a means to adapt to a new hostile intra-host environment. Interestingly, a similar pattern of expression has been observed for SmTNF-alpha receptor, which is most highly expressed in cercariae and is believed to represent a way for the parasite to be able to respond to human TNF-alpha ligand when recently transformed schistosomula penetrate the human host skin (Oliveira et al., 2009).

We also observed that despite the fact that transcriptional levels of SmAP among male and female adult worms are similar, female parasites express higher protein levels of SmAP and show an increased surface enzymatic activity. The lack of correlation between the levels of messenger and its protein end-product has been related to a range of metabolic and regulatory events (Hokke et al., 2007). Although it seems strange that egg express high levels of SmAP, but display low surface activity, this probably reflects the fact that most of the activity should be inside the shells (Dusanic, 1959).

The immunolocalization of SmAP in adult male and female sections confirmed its ubiquitary tissue distribution. The SmAP antisera also recognized, with a particular intensity, the vitelline glands in the female tissue, suggesting a special function for SmAP in some processes associated with egg production, such as protein and nucleic acid synthesis (Dusanic, 1959).

SmAP has been considered a possible target for immune intervention, since it is a tegument surface protein that is postulated to be involved in worm elimination in the self-curing *Rhesus* macaque model. Furthermore, the treatment with praziquantel increases the exposure of SmAP on the parasite's surface and in this condition, SmAP antisera was able to kill female worms. Based on this information, we hypothesized that if rSmAP antisera could block/impair the worm's Alkaline Phosphatase activity, it may protect mice against parasite infection in a vaccine trial. However, the rSmAP antisera did not impair the adult worm surface enzymatic activity as postulated. This is not the first report of the inability of SmAP antisera to inhibit its enzymatic activity (Pujol et al., 1990). These studies suggested the presence of two populations of antibodies against SmAP: one inhibitory and the other non-inhibitory. Upon analysis of the hypothetical non-glycosylated 3D-model, at least two potential B-cell discontinuous epitopes can be visualized in the vicinity of the active site, and many others around the molecule. Antibodies targeting these sites would not neutralize the active or metal binding sites, being non-inhibitory antibodies. It has been hypothesized that the induction of high titers of neutralizing antibody is important for the development of an effective vaccine. If this is the case, it would be important to produce an active recombinant molecule in the eukaryotic *Pichia pastoris* system, in order to obtain a protein in the native conformation to be evaluated as a potential vaccine candidate in the schistosomiasis murine model.

Our results present the first evidence that schistosomes in the cercarial phase prepare to produce large quantities of Alkaline Phosphatase by accumulating mRNA, to be able to rapidly translate it into protein upon invasion of the host and transformation into schistosomula, indicating its important role in invasion. Putative functions as immune modulators have been suggested (Bhardwaj and Skelly, 2011). On a whole, these results support this molecule as an important target for immune intervention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2011.07.008.

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Ensaio

Antígenos vacinais contra esquistossomose mansônica: passado e presente

Vaccine antigens against schistosomiasis: past and present

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Resumo. A esquistossomose ainda constitui um grave problema de saúde publica em países tropicais. Apesar do tratamento quimioterápico em áreas endêmicas se demonstrar eficiente no controle da morbidade, ele não reduz a prevalência em função de constantes re-infecções. Desta maneira, a estratégia mais eficaz de longo prazo se daria combinando a quimioterapia com uma vacina. Este artigo revisa alguns aspectos referentes às recentes estratégias de identificação de novas moléculas como alvos vacinais. Historicamente, a área de desenvolvimento de vacinas contra parasitas tem experimentado mais fracassos do que sucessos, entretanto, dados recentes utilizando as informações derivadas do transcriptoma, genoma e proteoma do *Schistosoma mansoni* revelaram resultados encorajadores, sendo os estudos de proteoma a maior fonte de novos candidatos vacinais.
Palavras-chave. *Vacina, Esquistossomose, Schistosoma mansoni.*

Abstract. Schistosomiasis remains a serious public health problem in tropical countries. Chemotherapy treatment in endemic areas has been effective only for morbidity control. Chemotherapy does not reduce the prevalence due to re-infection in endemic area. Thus, the most effective strategy for long-term disease control would be the combination of chemotherapy with a vaccine. This article reviews some aspects of the recent strategies in the identification of new molecules as vaccine targets for schistosomiasis. Historically, the development of vaccines against parasites is frequently unsuccessful. However, recent results using the information derived from the transcriptome, genome and proteome of *Schistosoma mansoni* have shown encouraging results.
Keywords. *Vaccine, Schistosomiasis, Schistosoma mansoni.*

A doença e o parasita

A esquistossomose é uma doença crônica causada por parasitas hematófagos do gênero *Schistosoma*. As principais espécies patogênicas para o ser humano são: *S. mansoni*, o *S. japonicum* e o *S. haematobium*. É a verminose mais prevalente no mundo, sendo endêmica em 74 países e afetando mais de 200 milhões de pessoas na África, Oriente Médio, Sudeste da Ásia e América do Sul. Estima-se ainda que aproximadamente 600 milhões de pessoas vivam em áreas de risco e que o número de mortes por ano causadas por esta parasitose chegue a 200.000 (Bergquist, 2002). Porém é consenso entre especialistas que a mortalidade causada por esta doença seja uma parcela pequena do problema quando comparada aos anos de vida perdidos devido à morbidade que ela causa. Avalia-se que o índice de *DALYs* ("Disability-Adjusted Life Years") para a esquistossomose seja de 1,76 milhões. *O DALY* é uma medida desenvolvida pela Organização Mundial da Saúde (OMS) para quantificar o impacto global de uma doença sobre a saúde da população. Este índice combina em um único parâmetro os anos de vida perdidos por uma morte prematura e os anos de vida vividos com incapacidade, ou seja, um *DALY* pode ser considerado como um ano de vida saudável perdido (Michaud e col., 2003). No Brasil a doença é causada exclusivamente pelo *S. mansoni*, sendo endêmica no Nordeste, principalmente no estado da Bahia e na região litorânea, e em partes do Sudeste, em especial Minas Gerais. Admite-se existirem mais de seis milhões de indivíduos afetados e estima-se que já foram tratados no país mais de 12 milhões de casos (Amaral e col., 2006). É uma doença negligenciada típica de países em desenvolvimento, que não desperta muito interesse de grandes companhias farmacêuticas e de biotecnologia. Assim sendo, este problema médico pode ser visto como uma oportunidade para que as comunidades científicas dos países em desenvolvimento contribuam para a melhoria da situação sanitária de sua população. Esse panorama mundial fez com que a esquistossomose fosse selecionada pelo Programa Especial de Treinamento em Doenças Tropicais da Organização das Nações Unidas, Banco Mundial

e OMS como uma das dez doenças tropicais mais importantes para controle (Morel, 2000).

O *S. mansoni* é um trematódeo dióico que vive na corrente sanguínea e foi descrito por Sambon (1907) e denominado como uma espécie diferente de *S. japonicum* pelo pesquisador brasileiro Pirajá da Silva (1908) (Katz, 2008). O parasita apresenta um complexo ciclo de vida com duas partes distintas: uma fase de reprodução assexuada no caramujo hospedeiro e uma etapa com reprodução sexuada no hospedeiro definitivo. Entre essas duas fases, o ciclo de vida envolve também dois estágios larvais infectantes, a cercária e o miracídio, importantes na transferência bem-sucedida do parasita de um hospedeiro para o outro. O miracídio, guiado pela luz e por estímulos químicos, penetra no hospedeiro intermediário, o caramujo do gênero *Biomphalaria*. Após infectar o caramujo, o miracídio se multiplica de maneira assexuada, diferenciando-se em esporocisto, o qual posteriormente se desenvolve na segunda forma larval, as cercárias, cuja liberação na água é induzida pela luminosidade diurna. Quando as cercárias encontram um hospedeiro definitivo adequado, como o homem, penetram pela pele até atingir a corrente sanguínea, se transformando em esquistossômulos, os quais migram para os pulmões e em seguida para o fígado. No fígado, os parasitas jovens amadurecem, formam casais e migram até os vasos mesentéricos, iniciando a oviposição. Uma parte dos ovos postos atravessa a parede dos vasos mesentéricos e acaba atingindo a luz do intestino. Outra parte é levada pela corrente sanguínea e atinge outros órgãos do hospedeiro humano. Os ovos que atingiram o intestino são excretados juntamente com as fezes e em contato com a água, cada ovo libera um miracídio, reiniciando o ciclo (Figura 1; Gryseels e col., 2006).

A patologia da esquistossomose mansônica é causada pela resposta inflamatória granulomatosa altamente agressiva do próprio hospedeiro, em virtude dos ovos que ficam retidos no fígado e intestinos, podendo resultar em fibrose e calcificação desses tecidos. Com o avanço da patologia, a circulação colateral do plexo venoso meséntérico se desenvolve e os granulomas hepáticos acabam bloqueando o sistema porta-hepático, gerando hipertensão portal (Boros, 1989). Em infestações severas, o acúmulo dos granulomas hepáticos e sua fibrose contínua, associada à pressão portal elevada e à intensa resposta imunológica, acarretam na dilatação anormal do fígado e baço (hepatoesplenomegalia). Esse quadro evolui em algumas pessoas para um acúmulo de líquido na cavidade peritoneal, ascite, com o desenvolvimento de varizes esofágicas, as quais podem romper desencadeando hemorragias. Casos graves não tratados podem ser fatais (Boros, 1989; Capron e col., 2002). A gravidade da doença é resultante da altíssima fecundidade do verme fêmea associada à infestação duradoura; existem relatos de vermes que sobreviveram até 30 anos no hospedeiro definitivo (Harris e col., 1984).

Justificativas para o desenvolvimento de uma vacina

O tratamento padrão da esquistossomose é a quimioterapia com praziquantel, que é eficaz contra todas as espécies que infestam o homem, não apresenta efeitos colaterais severos e atualmente é barato e de fácil acesso (Ribeiro-dos-Santos e col., 2006). Entretanto, a quimioterapia em massa nas regiões endêmicas é pouco efetiva porque não age sobre o estágio de esquistossômulos e não previne à re-infecção, dificultando a erradicação da doença e sendo necessária a repetição do tratamento de tempos em tempos (Sabah e col., 1986). Além disso, já foi descrita a baixa eficácia do tratamento no Egito e Senegal, onde linhagens resistentes a droga foram isoladas (Fallon e col., 1995; Ismail e col., 1999). Desse modo, fica evidente a necessidade do desenvolvimento de novas drogas efetivas contra o parasita e de novas abordagens para erradicar a doença, como a utilização de vacinas.

Uma vacina eficaz preveniria a evolução de linhagens de parasitas resistentes ao tratamento com drogas e protegeria contra a patologia. Esta forma de prevenção pode ser considerada ainda mais relevante, pois a patologia não é revertida pela quimioterapia e o diagnóstico da doença é difícil nos estágios iniciais devido à sintomatologia inespecífica (Wilson e Coulson, 1999). A vacinação também acabaria com o circulo vicioso de sucessivas infecções, uma vez que interromperia o ciclo de vida do parasita. Modelos matemáticos indicam que a vacina não precisaria apresentar imunidade esterilizante, e que uma redução de pelo menos 40% na carga parasitária reduziria a morbidade e as taxas de transmissão significativamente (Chan e Bundy, 1997). Assim, se analisadas as dificuldades políticas e a falta de recursos para investimentos maciços em saúde pública e em medidas de saneamento básico

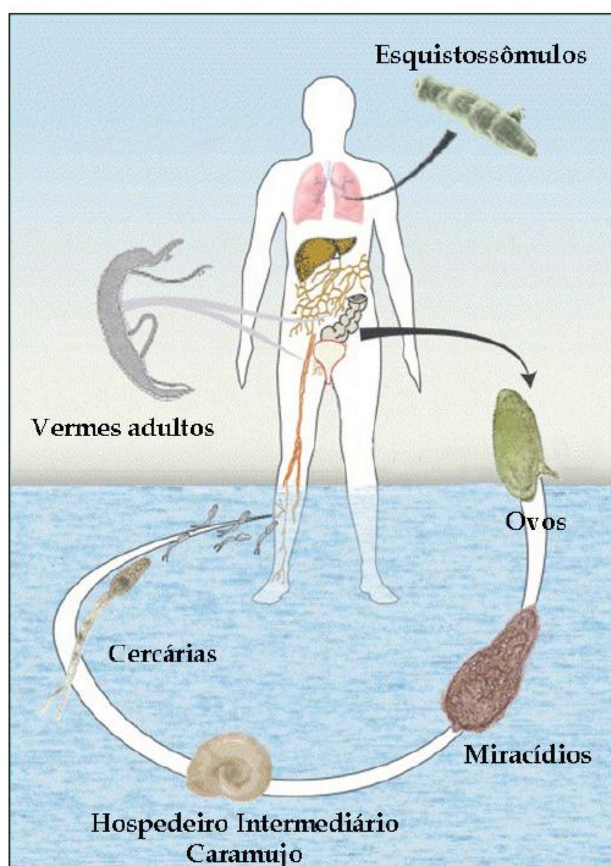

Figura 1 - Ciclo de vida do S. mansoni (adaptado de Gryseels e col., 2006).

Tabela 1. Antígenos vacinais contra a esquistossomose selecionados por estratégias pós-genômicas.

Antígeno	Função predita/ localização	Redução da carga parasitária
Tetraspaninas	Proteínas estruturais do tegumento de vermes adultos	Até 60%
Sm29	Proteína sem função conhecida, localizada no tegumento de vermes adultos e esquistossômulos	55%
SmStoLP-2	Proteína que possivelmente apresenta dupla localização em vermes adultos: nas mitocôndrias e no tegumento	31%
Anexina	Proteína estrutural de tegumento que interage com membranas	em avaliação
SmVals	Proteínas semelhantes a venenos alergênicos que podem possuir função imunomodulatória	em avaliação
SmLy6.5	Proteínas semelhantes a venenos alergênicos que podem possuir função imunomodulatória	em avaliação
Apirase, fosfodiesterase e fosfatase alcalina	Ectonucleotidases presentes na interface parasita-hospedeiro envolvidas no metabolismo de nucleotídeos	em avaliação
SmIg	Proteína presente no tegumento que apresenta um domínio de imunoglobulinas conservado	sem redução significativa
Sm21.6	Proteína similar a cadeia leve de dineína	sem redução significativa

nos países em desenvolvimento, a vacinação preventiva se apresenta como o instrumento com menor relação custo-benefício para o controle da doença. Compondo um tratamento com abordagens complementares: a quimioterapia seria responsável pela redução da carga parasitária em curto prazo, enquanto a imunização proveria proteção à população em longo prazo (Bergquist, 1998; Bergquist e Colley, 1998).

Diversos dados apóiam a plausibilidade do desenvolvimento de uma vacina eficaz contra a esquistossomose: I – pessoas residentes em áreas endêmicas desenvolvem naturalmente diferentes níveis de proteção contra a infecção (Bergquist, 2002); II – diversos modelos animais da esquistossomose apresentam autocura baseada na resposta imune, como *Macaca mulata* e *Rattus norvergicus* (Wilson e Coulson, 2009); III – diversos modelos animais, tanto primatas quanto roedores, apresentaram altos níveis de proteção quando imunizados com cercárias atenuadas por irradiação (Coulson, 1997; Hewitson e col., 2005). Assim seria necessário apenas caracterizar e mimetizar essas respostas imunes protetoras, identificando os antígenos por elas responsáveis. Entretanto, na prática essa tarefa demonstrou-se mais difícil do que inicialmente previsto.

Em função da complexidade do tema e do espaço disponível, neste artigo não abordaremos os aspectos referentes ao perfil de resposta imune mais eficaz contra este parasita. Dessa maneira, escolhemos nos centralizar nos aspectos referentes às recentes estratégias de seleção dos alvos vacinais.

Os seis candidatos vacinais da OMS

Em 1995, a OMS estabeleceu o desenvolvimento de uma vacina contra a esquistossomose como uma meta prioritária e organizou testes independentes para avaliar diversos antígenos como candidatos vacinais. Este ensaio pode ser interpretado como um reflexo dos avanços na área de biologia molecular, ocorrido durante os anos 80, que permitiu a seleção, clonagem, expressão e purificação de moléculas recombinantes do *Schistosoma*. Foram testadas uma proteína homóloga à miosina com 63 kDa, uma paramiosina de 97 kDa, uma triose-fosfato-isomerase de 28 kDa, uma proteína integral de membrana com 23 kDa (Sm23), uma proteína ligadora de ácidos graxo com 14 kDa (Sm14) e uma glutationa-S-transferase com 28 kDa (Sm28GST). Os resultados destes testes nunca se tornaram públicos, mas em nota oficial, a OMS divulgou que nenhum dos antígenos atingiu o objetivo, que era induzir uma redução da carga parasitária igual ou superior a 40% (Wilson e Coulson, 2006). O antígeno Sm14 atualmente está em fase de produção em boas práticas de fabricação para iniciar os ensaios clínicos tanto em humanos quanto em animais, devido a uma reatividade cruzada com *Fasciola hepatica* (Tendler e Simpson, 2008). A não identificação dessas moléculas como secretadas ou expostas na superfície do parasita, exceção a Sm23, e, por conseguinte, acessíveis aos mecanismos efetores da resposta imune, fizeram com que diversos autores defendessem a continuidade dos estudos para seleção de novos antígenos vacinais (Braschi e Wilson, 2006).

Transcriptoma, genoma e proteoma

Em 2003, foi publicado o transcriptoma do *S. mansoni* aumentando significativamente as informações sobre a composição gênica deste parasita. Foram gerados 163 mil sequencias expressas marcadas (EST = expressed sequence tags) de seis estágios do parasita, representando aproximadamente 92% de todo o transcriptoma e predizendo aproximadamente 14.000 genes. A análise desses dados revelou que 77% representam novos fragmentos gênicos de *S. mansoni*; sendo 1% novos parálogos; 20%

de novos ortólogos e 55% de fragmentos sem função conhecida. Neste trabalho foram descritos possíveis alvos quimioterápicos e potenciais candidatos vacinais selecionados por abordagem bioinformática (Verjovski-Almeida e col., 2003).

Em 2009, foi a vez do sequenciamento do genoma completo do parasita com 363 megabases que contém ao menos 11.809 genes, cujo tamanho médio é 4,7 kilobases com grandes íntrons e éxons pequenos. O estudou enfocou a descoberta de novos alvos quimioterápicos, revelando o déficit do parasita no metabolismo de lipídios e identificando receptores de membrana, canais iônicos e proteases do verme. Também foram identificados os gargalos metabólicos do parasita sobre os quais drogas aprovadas para outras aplicações possam ser eficazes (Berriman e col., 2009).

A publicação desses bancos de dados moleculares contendo, teoricamente, toda informação gênica do parasita abriu a perspectiva da abordagem de vacinologia reversa, que consiste na seleção de candidatos vacinais através de programas de bioinformática seguida por uma triagem em larga escala destes antígenos em ensaios de imunização (Rappuoli, 2000). Essa técnica foi bem sucedida para propor alvos vacinais promissores para procariotos, como *Neisseria meningites* e *Streptococcus agalactiae* (Maione e col., 2005; Pizza e col., 2000). Porém na seleção de candidatos para o *Schistosoma* e outros patógenos eucariotos mais complexos, esta abordagem apresenta alguns inconvenientes: I – eles possuem ciclo de vida complexo com diferentes estágios, de modo que nem todos eles infectam o homem, sendo esperado que muitos genes sejam estágio-específico e nunca entrem em contato com o hospedeiro definitivo; II – ao contrário de patógenos unicelulares, o parasita é multicelular e nem todas as proteínas secretadas ou de superfície seriam apresentadas na interface parasita-hospedeiro, dificultando a seleção *in silico*; III – apesar da dedução do quadro aberto de leitura (ORF = open reading frame) ser útil, os programas tendem a acumular muitos erros, especialmente nas extremidades de ORFs que geralmente codificam sinais de secreção, devido à *splicings* (processamento de íntrons) alternativos do RNA (DeMarco e Verjovski-Almeida, 2009).

Assim, apenas procurar dentro do genoma e do transcriptoma do parasita por possíveis alvos vacinais, foi uma tarefa árdua e não muito frutífera, sendo necessária uma abordagem pós-genômica para uma seleção mais eficaz. No caso do *S. mansoni* a abordagem mais produtiva, até o momento, foram os estudos de proteoma, os quais conseguiram determinar as proteínas mais abundantes que estariam acessíveis ao sistema imune no verme adulto (Braschi e col., 2006; Braschi e Wilson, 2006; van Balkom e col., 2005) e àquelas secretadas no momento da infecção do homem (Curwen e col., 2006; Hansell e col., 2008; Knudsen e col., 2005); desse modo, essa técnica revelou dezenas de novas proteínas que podem desempenhar funções relevantes na interface parasita-hospedeiro e constituir antígenos vacinais promissores.

Novos candidatos vacinais e futuras perspectivas

A predição de candidatos vacinais baseada apenas na seqüência gênica e na localização da proteína é em certa medida especulativa, restando à tarefa considerável da análise funcional e imunológica de cada novo gene/proteína. Algumas moléculas selecionadas segundo estas novas estratégias revelaram potencial como candidatos vacinais (Tabela 1). Imunizações com tetraespaninas (TSP1/2) presentes na superfície do *S. mansoni* reduziram a carga parasitaria de camundongos em ~40% e ~60%, respectivamente, além de reduzir a oviposição em ~60% (Tran e col., 2006). Estas proteínas integrais de membrana possuem função estrutural, sendo importantes para o desenvolvimento, amadurecimento e manutenção do tegumento do parasita (Tran e col., 2010). Outra proteína também identificada na superfície do parasita, a Sm29, demonstrou uma redução significativa da carga parasitária da ordem de 55%, porém esta proteína não apresenta domínios conhecidos dificultando predizer sua função para o parasita (Cardoso e col., 2008). Foi descrita ainda uma proteína similar a estomatina 2 (SmStoLP-2), que possivelmente apresenta uma dupla localização no parasita, nas mitocôndrias e associada às membranas do tegumento, sendo que animais imunizados com estas proteínas apresentaram uma redução da carga parasitaria mais modesta, da ordem de 31% (Farias e col., 2010). Apesar desses novos antígenos apresentarem resultados encorajadores, deve-se considerar que todos esses índices de proteção foram obtidos através de imunizações com adjuvante de Freund, que apesar de sua efetividade não possui uso liberado para humanos devido à forte resposta inflamatória induzida (Gupta e Siber, 1995). Adicionalmente, esses índices de proteção precisam ser alcançados em outros roedores e primatas não-humanos. Além disso, os mecanismos efetores que resultam na morte do parasita precisam ser melhor caracterizados e elucidados.

Outras proteínas foram identificadas como possíveis candidatos vacinais, sendo necessário avaliar sua capacidade protetora. A anexina é uma proteína estrutural presente no tegumento que provavelmente atua na organização de suas membranas de superfície (Tararam e col., 2010). A SmLy6.5 é uma proteína semelhante a CD59 humana, a qual é um potente inibidor do complexo de ataque a membrana resultante da ativação do sistema complemento (Wilson e Coulson, 2009). As SmVALs são proteínas que por apresentar similaridade com venenos alergênicos poderiam promover uma resposta inflamatória no hospedeiro mamífero através de mecanismos imunomoduladores (Chalmers e col., 2008). A apirase, a fosfodiesterase e a fosfatase alcalina são três enzimas presentes na interface parasita-hospedeiro, envolvidas no metabolismo de nucleotídeos e sinalização purinérgica que poderiam atuar na inibição da agregação de plaquetas e apoptose (Bhardwaj e Skelly, 2009; Levano-Garcia e col., 2007; Rofatto e col., 2009). No entanto, experimentalmente sabemos que nem todas as proteínas presentes na interface parasita-hospedeiro e acessíveis ao sistema imune se revelarão bons candidatos vacinais, caso da proteína SmIg, que apresenta um domínio de imunoglobulina conservado, e da Sm21.6, proteína similar a cadeia leve da dineína. Ambas não foram capazes de reduzir a carga pa-

rasitária, apesar de diminuírem a patologia no fígado de camundongos infectados (Lopes e col., 2009; Pinho e col., 2010).

Certamente o desenvolvimento de uma vacina para a esquistossomose dependerá de uma melhor compreensão da biologia do parasita, uma vez que os mecanismos de coevolução e seleção natural permitiram ao parasita desenvolver estratégias de sobrevivência ao ataque orquestrado pelo sistema imune do hospedeiro mamífero. Desse modo, as informações fornecidas pelo transcriptoma, pelo genoma e pelo proteoma do *S. mansoni* criaram novas oportunidades a serem exploradas pelos cientistas na tentativa de sobrepujar este adversário tão formidável.

Contribuição dos autores

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PLoS NEGLECTED TROPICAL DISEASES

Schistosoma mansoni Stomatin Like Protein-2 Is Located in the Tegument and Induces Partial Protection against Challenge Infection

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Abstract

Background: Schistosomiasis affects more than 200 million individuals worldwide, with a further 650 million living at risk of infection, constituting a severe health problem in developing countries. Even though an effective treatment exists, it does not prevent re-infection, and the development of an effective vaccine still remains the most desirable means of control for this disease.

Methodology/Principal Findings: Herein, we report the cloning and characterization of a *S. mansoni* Stomatin-like protein 2 (SmStoLP-2). *In silico* analysis predicts three putative sites for palmitoylation (Cys11, Cys61 and Cys330), which could contribute to protein membrane association; and a putative mitochondrial targeting sequence, similar to that described for human Stomatin-like protein 2 (HuSLP-2). The protein was detected by Western blot with comparable levels in all stages across the parasite life cycle. Fractionation by differential centrifugation of schistosome tegument suggested that SmStoLP-2 displays a dual targeting to the tegument membranes and mitochondria; additionally, immunolocalization experiments confirm its localization in the tegument of the adult worms and, more importantly, in 7-day-old schistosomula. Analysis of the antibody isotype profile to rSmStoLP-2 in the sera of patients living in endemic areas for schistosomiasis revealed that IgG1, IgG2, IgG3 and IgA antibodies were predominant in sera of individuals resistant to reinfection as compared to those susceptible. Next, immunization of mice with rSmStoLP-2 engendered a 30%–32% reduction in adult worm burden. Protective immunity in mice was associated with specific anti-rSmStoLP-2 IgG1 and IgG2a antibodies and elevated production of IFN-γ and TNF-α, while no IL-4 production was detected, suggesting a Th1-predominant immune response.

Conclusions/Significance: Data presented here demonstrate that SmStoLP-2 is a novel tegument protein located in the host-parasite interface. It is recognized by different subclasses of antibodies in patients resistant and susceptible to reinfection and, based on the data from murine studies, shows protective potential against schistosomiasis. These results indicate that SmStoLP-2 could be useful in a combination vaccine.

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Introduction

Schistosomiasis is an important parasitic disease, caused by trematode worms of the genus *Schistosoma*; it affects approximately 200 million of individuals primarily in developing countries and an estimated additional 500 to 600 million are at risk. The digenetic blood fluke, *Schistosoma mansoni*, is one of the major causative agents [1]. Parasite eggs are trapped in the liver and intestine, where they induce granuloma formation and fibrosis, the main cause of morbidity and mortality in schistosomiasis [2]. Chemotherapy is an important control strategy against this parasitic disease [3]; however, it has not reduced the endemicity [4] and rapid reinfection demands frequent treatment [5]. Therefore, it is considered that an effective vaccine combined with chemotherapy would be an efficient control mechanism [6].

Until October 2003, schistosome research suffered from limited genomic information; this situation has changed significantly with the simultaneous publication of the *S. mansoni* and *S. japonicum* transcriptomes [7,8]. These initiatives, together with the advent of entire *S. mansoni* genome sequencing, all boosted by advances in bioinformatics, have markedly changed the schistosome vaccine research field. Simultaneously with the publication of the transcriptome data, and its scrutiny for genes with functions that would indicate their surface exposure to allow interaction with the

Author Summary

Schistosomiasis is a parasitic disease causing serious chronic morbidity in tropical countries. Together with the publication of the transcriptome database, a series of new vaccine candidates were proposed based on their functional classification. However, the prediction of vaccine candidates from sequence information or even by proteomics or microarrays data is somewhat speculative and there remains the considerable task of functional analysis of each new gene/protein. In this study, we present the characterization of one of these molecules, a stomatin like protein 2 (SmStoLP-2). Sequence analysis predicts signals that could contribute to protein membrane association and mitochondrial targeting, which was confirmed by differential extractions of schistosome tegument membranes and mitochondria. Additionally, confocal microscope analysis showed SmStoLP-2 present in the tegument of 7-day-old schistosomula and adult worms. Studies in patients living in endemic areas for schistosomiasis revealed high levels of IgG1, IgG2, IgG3 and IgA anti-SmStoLP-2 antibodies in individuals resistant to reinfection. Recombinant SmStoLP-2 protein, when used as vaccine, induced significant levels of protection in mice. This reduction in worm burden was associated with a typical Th1-type immune response. These results indicate that SmStoLP-2 could be useful in association with other antigens for the composition of a vaccine against schistosomiasis.

host immune system, a series of novel genes were suggested as potential vaccine candidates based on their functional classification by Gene Ontology [8]. One of these, stomatin, was assigned a role in lipid raft formation or receptor binding by Gene Ontology categorization. Actually, it is most similar to the sub-family Stomatin Like Protein 2 (SLP-2), of which the best characterized gene is the human ortholog [9]. The protein was also proposed as a schistosome drug target [8], since the human ortholog was described as interacting with anti-malarial drugs, participating in the transfer of the drug Mefloquine to the intracellular parasite via a pathway used for the uptake of exogenous phospholipids [10].

The SLP-2 was first identified in humans (HuSLP-2); it presents, like other stomatins (e.g. Stomatin, SLP-1 and SLP-3), a central Stomatin, Prohibitin, Flotillin, HflK/C (SPFH) domain that may mediate interactions with plasma and mitochondrial membranes [11–13]. HuSLP-2 is the first member of this family that lacks an N-terminal hydrophobic domain, displaying a mitochondrial targeting sequence in this region. Additionally, a palmitoylation centered on Cys29 could not be identified [9,11]. The function of stomatins, including SLP-2, remains undetermined. In erythrocytes, it may link stomatin or other integral membrane proteins to the peripheral cytoskeleton, playing a role in the regulation of ion channel conductance or in the organization of sphingolipids and cholesterol-rich lipid rafts [9]. More recently, this gene has been investigated as a novel cancer-related gene over-expressed in certain kinds of human tumours [14,15], and in the assembly of mechanosensation receptors [16–22]. Moreover, it has been proposed to function as a link between synapse-polarized mitochondria and T-cell receptor (TCR) signalosomes, contributing to modulate TCR signalling and T cell activation [23,24].

In this work, we describe and characterize a novel *S. mansoni* stomatin like protein 2 (SmStoLP-2). Data obtained here establishes that SmStoLP-2 is present in the tegument of adult worms and schistosomula. In addition, we evaluated the reactivity of rSmStoLP-2 antigen against the sera from individuals living in endemic areas for schistosomiasis in Brazil, showing that the groups resistant and susceptible to reinfection showed different antibody profiles. We subsequently demonstrated the ability of anti-rSmStoLP-2 serum to inhibit penetration and migration of cercariae *in vivo*. Lastly, immunization of mice with rSmStoLP-2 induced a Th1-type of immune response and a significant reduction in worm burden upon challenge with cercariae.

Materials and Methods

Parasite maintenance

Schistosoma mansoni adult worms (BH strain) were obtained by perfusion of mice, 7–8 weeks after infection. Eggs, miracidia, cercariae, and schistosomula were obtained as previously described [8]. Cercaria number and viability were determined using a light microscope prior to infection.

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of Fundação Oswaldo Cruz (0083/99-CEP/FIOCRUZ). All patients or their legal guardians provided written informed consent for the collection of samples and subsequent analysis.

All animals were handled in strict accordance with good animal practice as defined by Animals Use Ethics Committee of UFMG (Universidade Federal de Minas Gerais, Brazil) and Instituto Butantan (São Paulo, Brazil), and the study was conducted adhering to the institution's guidelines for animal husbandry.

Study population

Peripheral blood was obtained from individuals with different genetic background living in three endemic areas for schistosomiasis ('Melquiades', 'Caatinga do Moura' and 'Côrrego do Onça', all in the state of Minas Gerais, Brazil). These individuals were classified in four groups according to their infection status and the selection of subjects was performed based only on the criteria for inclusion and exclusion of each group independent of previous knowledge of immune responses for each individual. Non-infected (NI) individuals are healthy people from non-endemic areas without any parasite infection or contact with contaminated water. One group was shown to be stool-negative after treatment with praziquantel and was classified as resistant to *S. mansoni* reinfection (RR) [25]. The water contact exposure was determined using previously described methods [26–28], objectively evaluated by observers and studied population had at least one contact daily. Individuals classified as susceptible to *S. mansoni* reinfection (SR) were shown to be stool-positive following treatment with praziquantel (40 mg/kg) (at 1 and 5 months). The sera from RR and SR groups were obtained six months after praziquantel treatment and these individuals were examined for *S. mansoni* infections using the Kato–Katz technique before treatment and one, 6 and 12 months after treatment to check for reinfection rates [25]. Individuals grouped as infected (INF) showed stool-positive examination and no treatment history (never received anti-helminthic treatment, as determined by survey). These infected patients had infection levels that varied from 48 to 224 epg (egg counts per gram of feces). For each time point, three independent (consecutive days) stool samples were taken and two slides were prepared from each sample. These patients or their legal guardians gave informed consent after explanation of the protocol that had been previously approved by the Ethical Committee of Fundação Oswaldo Cruz. Details regarding sex and age of the individuals included in this study are described in Table S1.

Cloning and molecular characterization of SmStoLP-2

Total RNA was isolated from adult worms (1 g) using TRIzol reagent (Invitrogen), followed by mRNA purification with oligo (dT)-cellulose columns according to the manufacturer's instructions (Amersham Biosciences). The SuperScript™ plasmid system for cDNA synthesis and cloning (Invitrogen) was used for cDNA library construction following the manufacturer's protocol. The cDNA fragments were directionally ligated into the SalI/NotI cloning sites of the pSPORT1 vector and transformed into competent *Escherichia coli* DH5α.

Specific oligonucleotides were designed using the EST assembly partial sequence from the São Paulo Transcriptome data (SmAE 606856.1, http://bioinfo.iq.usp.br/schisto6/) together with an EST from TIGR (BF936634). The 5′ and 3′ oligonucleotides, CACCATGATTCGTAGTATCATTGG and CTATTCTTG-TTTATCGCTATC, were used in a PCR reaction to amplify the complete open reading frame of SmStoLP-2 from a cDNA library made from adult worms. The PCR reaction was performed using Platinum *Pfx* enzyme (Invitrogen), and initiated with one cycle of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at 55°C, and 3 min at 68°C. PCR products were purified from agarose gel electrophoresis, cloned into pENTR/D TOPO cloning vector (Invitrogen), and sequenced to confirm its identity.

Phylogenetic and sequence analysis

Blast and PSI-Blast searches against the non-redundant protein sequence database, using SmStoLP-2 as a query, were used to identify orthologs of SmStoLP-2. Additionally, we searched the *S. mansoni* genome (GeneDB, http://www.genedb.org/genedb/smansoni/) for proteins with Pfam SPFH/Band 7 domains. Post-translational modification prediction: the signal peptide prediction was performed using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), transmembrane helices were analyzed by TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), palmitoylation sites were predicted by CSS-Palm (http://csspalm.biocuckoo.org/1.0/index.php) [29], and mitochondrial targeting sequence as predicted by the MitoProt program (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html). Molecular weight (MW) and isoelectric point (pI) were calculated with the Compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html).

For phylogenetic analyses, alignments of protein sequences were performed using the ClustalX 1.83 software. The tree was constructed using Clustal with the Neighbour Joining method, excluding positions with gaps. The numbers represent the confidence of the branches assigned by bootstrap (in 1000 samplings). The TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) was used to visualize the tree.

Expression of recombinant protein and polyclonal antibody production

To produce a recombinant SmStoLP-2, the full-length cDNA sequence was directionally cloned by recombination into pDEST17 (to produce a protein that contains an N-terminal hexahistidine tag) and transformed into BL21 (DE3) (Invitrogen). For protein expression, the transformed cells were grown in 600 ml LB plus ampicilin (OD_{600} = 0.6). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM, and cells were incubated for 3–4 h at 37°C. Cells were harvested by centrifugation and resuspended in 50 ml of lysis buffer (50 mM sodium phosphate pH 8.3, 0.3 M NaCl). The cell suspension was passed twice (1500 psi) through a French press and the crude homogenate was centrifuged at 20,000×*g* for 40 min. The pelleted inclusion bodies were washed twice with wash buffer (lysis buffer, 2% Triton X-100, 2 M urea) and finally resuspended in solubilization buffer (lysis buffer, 5 mM beta-mercaptoethanol, 20 mM imidazole, 8 M urea). The recombinant protein was refolded from the inclusion bodies by diluting 100-fold into equilibration buffer (solubilization buffer without urea).

The recombinant protein was then purified by metal affinity chromatography using the Akta Prime system (Amersham Biosciences) under native conditions. Briefly, the sample was loaded onto a Ni^{2+}-NTA column (5 ml bed volume) pre-equilibrated with the same buffer. The column was washed with 10 bed volumes of the equilibration buffer and then eluted with 20–500 mM imidazole linear gradient. The main peak was pooled and the protein purity of fractions was assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Further, the elution buffer was exchanged with Phosphate Buffer Saline pH 7.4 (PBS) before use of this protein.

Polyclonal rat serum was produced against preparations of recombinant SmStoLP-2. Rodents were inoculated four times, at 21-day intervals with 100 µg of protein mixed with TiterMax adjuvant (CytRx Corporation; first dose) or PBS (in subsequent doses). Fifteen days after the last inoculation, rodents were exsanguinated. The sera were used at a dilution of 1:10,000 (v:v) in Western blots and 1:100 in indirect immunofluorescence assays.

Circular dichroism (CD) measurements

CD measurements were carried out on a Jasco J-810 Spectropolarimeter at 20°C equipped with a Peltier unit for temperature control. Far-UV CD spectrum was acquired using a 1 mm path length cell at 0.5 nm intervals over the wavelength range from 190 to 260 nm. Five scans were averaged for each sample and subtracted from the blank average spectra. The protein concentration was kept at 10 µM in 10 mM sodium phosphate buffer pH 7.4.

Protein expression profile

Total parasite extracts from eggs, miracidia, cercariae, 10-day old schistosomula and adult worms of *S. mansoni* were prepared in 40 mM Tris, pH 7.4, 2% SDS plus protease inhibitor cocktail (Sigma) through sonication (4 cycles of 2 min, with pulses of 0.75 s, 40% amplitude). The samples were centrifuged at 20,000×*g* for 30 min at 4°C and the supernatant was quantified and used for assays. The soluble fraction of adult worms and schistosomula was obtained in a similar way, with the exception of 2% SDS in the sonication buffer. After centrifugation at 20,000×*g* for 30 min at 4°C, the supernatant was recovered, and the insoluble pellet was sonicated in the presence of 2% SDS, which after centrifugation at 20,000×*g* for 30 min at 4°C, originated the so-called insoluble fraction. Their protein concentrations were determined with a RC DC Protein Assay Kit (Bio-Rad, CA, USA). Samples of purified rSmStoLP-2 and extracts (20 µg) were submitted to SDS-PAGE. The gel was electroblotted onto a PVDF membrane, which was blocked with 0.02 M Tris (pH 7.5) and 0.3% Tween 20 containing 5% dry milk for 16 h at 4°C. Subsequently, the membrane was incubated in a 1:10,000 dilution with primary antibody in blocking buffer plus 150 mM NaCl for 3 h at room temperature. After three washes using Tris 10 mM (pH 7.5), the membrane was incubated in a 1:4000 dilution with secondary goat anti-rat IgG conjugated to horseradish peroxidase (HRP) (Pierce) for 1 h and after three washes using Tris 10 mM (pH 7.5), the membrane was treated with ECL plus (GE) reagent according to manufacturer's instructions.

Tegument removal, differential extraction and fractionation: Surface membranes and mitochondrial enrichment

The sample used in this experiment was kindly provided by Dr. Simon Braschi (University of York, England, UK). Briefly, the tegument was removed by a freeze/thaw method and surface membranes enriched by sucrose-gradient centrifugation as previously described [30,31], generating a gradient pellet. Proteins were sequentially extracted from the gradient pellet using a three-step process with reagents of increasing solubilizing power as follows: Extract 1 (soluble proteins): 40 mM Tris, pH 7.4; Extract 2 (non-covalent, but firmly bound proteins): 5 M urea (BDH, VWR International, Dorset, UK), 2 M thiourea (BDH) in 40 mM Tris, pH 7.4 (Extraction Buffer 2; EB2); Extract 3 (GPI-anchored and single membrane spanners): EB2 plus 4% CHAPS (Sigma) and 2% N-decyl-N, N-dimethyl-3-ammonio-1-propane sulphate (SB 3–10; Sigma), pH 7.4; Final pellet (multispanning membrane proteins): solubilized with 40 mM Tris, pH 7.4 plus 2% SDS.

Mitochondrial enriched fraction was prepared from adult worm tegument in isotonic mitochondrial buffer (MB) (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES pH 7.5) supplemented with complete protease inhibitor cocktail (Sigma). The tegument membranes were obtained by centrifugation at $100\times g$ for 30 min at 4°C. The resulting supernatant was centrifuged at $10{,}000\times g$ for 10 min at 4°C to purify the mitochondrial fraction. The resulting pellets were ressuspended in 40 mM Tris, pH 7.4 plus 2% SDS. The protein concentration was estimated by the method of Lowry with a RC DC Protein Assay Kit (Bio-Rad, CA, USA). Anti-Mitofusin-1 antibody (Mfn1 (H-65) (Santa Cruz Biotechnology) (1:200 dilution) was used as a mitochondrial tracker in Western blot experiments followed by incubation with secondary goat anti-rabbit IgG conjugated to HRP (Sigma).

Indirect immunofluorescence and confocal microscopy

Freshly perfused worms were embedded in OCT medium in a pre-cooled beaker of isopentene, frozen in liquid N_2. Eight-micrometer cryostat adult worm sections were adhered to silanized glass slides (DakoCytomation) and fixed in acetone for 30 min at −20°C before blocking with PNT (PBS 1x, 10% Naive rabbit serum and 0.1% Tween 20) for 4 h at room temperature. They were then incubated with anti-rSmStoLP-2 antisera diluted 1:100 in PNT for 2 h at room temperature. After washing five times with PBS 0.1% Tween 20, pH 7.4 (PBS-T), an Alexa Fluor 488 conjugated anti-rat IgG 1:200 (v:v), 20 mM DAPI (4′, 6-diamidino-2-phenylindole dihydrochloride, Molecular Probes) to visualize nuclei, and 0.1 µg/ml phalloidin rhodamine (Molecular Probes) to stain actin microfilaments, were added to PNT solution, and the samples incubated for 1 h at room temperature. Sections were washed five times with PBS-T, and then mounted in Fluorescent Mounting Medium (DakoCytomation). In order to label the whole parasite, 7-day cultured schistosomula were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h on ice, washed with PBS and kept at 4°C until use, the hybridisation conditions were the same used for adult worm sections. Rat pre-immune sera were used as negative control. Images were acquired in a Zeiss LSM 510 Meta confocal system, attached to a Zeiss Axiovert 100 microscope using a LD-Achroplan 20x/0.4 or C-Apochromatic 63x/1.2 water immersion objectives with differential interference contrast.

Measurement of human humoral response to SmStoLP-2

Sera of schistosomiasis patients living in endemic areas in Brazil were tested by ELISA as previously described [32–34] to measure the levels of immunoglobulin isoytpes to rSmStoLP-2 protein. For this assay, 96 well flat-bottom microtiter plates (Nunc) were coated overnight at 4°C with 100 µl/well of rSmStoLP-2 at a concentration of 5 µg/ml in 0.1 M carbonate bicarbonate buffer (pH 9.6). The plates were then blocked with 10% bovine fetal serum in PBS (pH 7.4) for 2 h at room temperature. Subsequently, the plates were washed three times with PBS plus 0.05% Tween-20 (PBS-T). Serum samples diluted 1:50 (IgG) and 1:40 (IgA) in PBS-T (100 µl/well) were added in duplicate and the plates incubated for 1 h at room temperature. Peroxidase-labelled anti-human IgG and anti-human IgA (Sigma) was added at dilutions of 1:10,000 and 1:1000 (100 µl/well), respectively. After 1 h at 37°C, the plates were washed and orthophenyl-diaminobenzidine plus 0.05% hydrogen peroxide in phosphate citrate buffer (pH 5) was added (100 µl/well). This mixture was than incubated for 15 min at room temperature, and the reaction was stopped by addition of 5% H_2SO_4 (50 µl/well). Absorbance was read at 492 nm using a microplate reader (Bio-Rad, Hercules. CA, USA). To measure IgG subclasses, the previous protocol was modified. The serum dilution was changed based on the isotype to be detected. Serum samples diluted 1:30 (IgG1), 1:5 (IgG2 or IgG4) and 1:80 (IgG3) were added to the plates and incubated for 2 h at 37°C, as previously described [32]. After washing, peroxidase labelled mouse anti-human antibody was added in each well at concentrations of 1:1000 (IgG1, IgG3, IgG2 or IgG4), and the plates were incubated for 16 h at 4°C. The subsequent steps were identical to those described for the other isotypes.

Cercariae penetration and migration inhibition assay

Cercariae penetration and migration inhibition assays were adapted from a previously described method for *Necator americanus* [35]. Briefly, six aliquots of 100 µl, each containing 100 cercariae of *S. mansoni*, were incubated for 1 h at 37°C with 50 µl of sera from rats immunized with rSmStoLP-2 formulated with Titer-Max. Sera from rats injected with saline was used as control. To eliminate any effect of complement, the sera were previously heated for 30 min at 56°C. After the 1h incubation period, 800 µl of pond water was added to the samples and the whole volume (950 µl) was then applied to the shaven abdomen of six anaesthetized mice (90 mg/Kg of Ketamine and 10 mg/Kg of Xylazine). The cercariae were allowed to penetrate by the ring method for 30 min at room temperature. Non-penetrating parasites were evaluated by counting those that remained on the surface of the skin, which were collected by removing the remaining liquid with a pipette and washing the skin twice with 1 ml of PBS. The mean value was considered the percentage of larval penetration inhibition by the antiserum. Six weeks after percutaneous penetration, 6 mice per group were sacrificed with a lethal dose of Ketamine/Xylazine solution. Perfusion fluid (Saline solution, 500 units/L of heparin) was pumped into the aorta artery, and perfused worms were collected from the hepatic portal vein. Adult male and female worms were counted using a stereomicroscope.

Immunization of mice, challenge infections and parasite loads

Five to six week-old female C57BL/6 from the Universidade Federal de Minas Gerais (UFMG) animal facility, were supplied with food and water *ad libitum*. Groups of C57BL/6 mice were lightly anaesthetized (with 45 mg/kg of Ketamine and 10 mg/kg of Xylazine) and injected subcutaneously in the nape of the neck with 3 doses, at 15-day intervals, of 25 µg of protein mixed with Freund's Complete Adjuvant (Sigma; first dose) or Freund's Incomplete Adjuvant (in subsequent doses). In the control group,

PBS with Freund's adjuvant was administered using the same immunization protocol. Challenge infections were performed 2 weeks following the final immunization. Mice were anaesthetized with 90 mg/Kg of Ketamine and 10 mg/Kg of Xylazine and exposed percutaneously to 100 cercariae by the ring method on their shaven abdomens. Six weeks after percutaneous challenge infections, 10 mice per group were sacrificed and perfused as described in the cercariae inhibition of penetration assay. The protection was calculated by comparing the number of worms recovered from each vaccinated group with its respective control group, in two independent experiments.

The livers were collected from the same animals fixed in 10% paraformaldehyde, processed for paraffin embedding and histopathological sections performed using microtome at 6–7 µm and stained in a slide with hematoxilin-eosin (HE). The number of granulomas was obtained from the liver sections using 10× objective in a microscope. The area from each liver section was calculated using capture in scanner followed by analysis in the KS300 software connected to a Carl Zeiss image analyzer, and the number of granulomas calculated by the area of the liver.

Humoral response in mice immunized with rSmStoLP-2

Mice were bled from the retro orbital plexus and ELISA was performed to confirm the titer of specific anti-rSmStoLP-2 IgG, IgG1 and IgG2a in the serum of immunized animals. Briefly, 96 well flat-bottom microtiter plates (Nunc) were coated overnight at 4°C with 100 µl/well of rSmStoLP-2 at a concentration of 5 µg/ml in a 0.1 M carbonate bicarbonate buffer (pH 9.6). The plate was then blocked with bovine fetal serum 10% in PBS for 2 h at room temperature. Further, the plates were washed three times with PBS plus 0.05% Tween-20 (PBS-T). One hundred microliters of each serum diluted 1:100 in PBS-T was added per well and incubated for 1 h at room temperature. Plate-bound antibody was detected by peroxidase-conjugated anti-mouse IgG, IgG1 and IgG2a (Southern Biotechnology) diluted in PBST 1:10,000, 1:5000 and 1:2000, respectively. After 1 h at 37°C, the plate was washed and orthophenyl-diaminobenzidine plus 0.05% hydrogen peroxide in phosphate citrate buffer (pH 5) was added (100 µl/well). This mixture was then incubated for 30 min at room temperature, and the reaction was stopped by addition of 5% H_2SO_4 (50 µl/well). Absorbance was read at 492 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Animals that received PBS with Freund's adjuvant were used as negative control.

ELISA detection of IFN-γ, IL-4, IL-10 and TNF-α in the supernatant of spleen cell cultures from mice immunized with rSmStoLP-2

Cytokine experiments were performed using splenocyte cultures from individual mice immunized with rSmStoLP-2 plus CFA/IFA ($n = 5$ for each group). Splenocytes were isolated from macerated spleens of individual mice 10 days after the third immunization and washed twice with sterile PBS. The cells were adjusted to 1×10^6 cells per well in RPMI 1640 medium (Gibco, CA, USA) supplemented with 10% FBS, 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, 250 ng/ml amphotericin B. Splenocytes were maintained in culture with medium alone or stimulated with rSmStoLP-2 (25 µg/ml) or concanavalin A (ConA) (5µg/ml) as previously described [36]. The 96-well plates (Nunc) were maintained in an incubator at 37°C with 5% CO_2. For cytokine assays, polymyxin B (30 µg/ml) was added to the cultures and this treatment completely abrogated the cytokine response to LPS, as previously described [37]. Culture supernatants were collected after 48 h of rSmStoLP-2 stimulation for IL-4 and TNF-α analysis and 72 h of rSmStoLP-2 stimulation for IL-10 and IFN-γ. The assays for measurement of IL-4, IL-10, IFN-γ and TNF-α were performed using the Duoset ELISA kit (R&D Diagnostic) according to the manufacturer's recommendations.

Statistical analysis

Student's *t*-test was used and the two-tailed *p*-value was calculated to compare experimental and control groups on challenge infections, antibody profiles and cytokine assays in mice. For the human humoral response against rSmStoLP-2, the Kruskal–Wallis test was used to evaluate the significance of the results of all groups compared to the non-infected (NI). The Mann–Whitney test was used to evaluate the significance of antibody measurements obtained between the groups resistant to *S. mansoni* reinfection (RR) *versus* the groups susceptible to reinfection (SR).

Results

Cloning and molecular characterization of *S. mansoni* StoLP-2

The full-length sequence of the *S. mansoni* cDNA encoding Stomatin like protein-2 was obtained by PCR from an adult worm cDNA library with specific oligonucleotides. The resulting full-length cDNA (GenBank accession EU531730) displays an ORF of 1077 bp, encoding a protein of 358 amino acids with a predicted molecular mass of approximately 39.5 kDa and an isoelectric point of 5.83. BlastP comparisons of the deduced *S. mansoni* protein sequence to GenBank showed that the best match (E-value = 5×10^{-95}) was to *Danio rerio* hypothetical protein, with 58% identity and 81% similarity over 355 amino acids. The next best match was against an unknown *S. japonicum* protein (probably an incomplete SLP-2). This was followed by several other SLP-2 proteins including human (58% of identity), therefore we designated this gene as SmStoLP-2 (since there was already another gene named as Sm-SLP-2, although not related to Stomatin like proteins [38]).

SmStoLP-2 contains the stomatin signature sequence (residues 31–189) (outlined by a dashed box in Figure 1), and is recognized as part of the Pfam SPFH/Band 7 family with an E-value of 1.2×10^{-75}. Additionally, searching the *S. mansoni* genome (GeneDB) for proteins with Pfam SPFH/Band 7 domains, we found putative orthologues of *H. sapiens* stomatin (Band 7), and *C. elegans* Mec-2 (Figure 1). We identified a further five schistosome stomatin-related genes (data not shown).

Human stomatin (Band 7) may associate with membranes via a hairpin loop (continuous box) with both the N- and C- termini facing the cytoplasm (Figure 1). This domain is conserved among several members of the SPFH/Band 7 superfamily, such as *C. elegans* MEC-2 and *S. mansoni* Stomatin and Mec-2, but is absent in SLP-2 members (Figure 1). We further identify in all SLP-2 sequences putative signal peptides (ranging from 16 to 32 amino acids at the N-terminal region), which were predicted to be a mitochondrial targeting sequence (dashed - dotted box). Some previously recognized stomatin family members, such as human stomatin, have a consensus sequence for palmitoylation centered on Cys29 and Cys86 [39], which apparently increase the affinity of stomatin for the membrane. Further examining the distribution of potential post-translational modifications of SmStoLP-2, we found three putative sites for lipid modification (palmitoylation) centered on Cys11, Cys61 and Cys330 (Figure 1, underlined). Surprisingly, these palmitoylation sites were not detected in any other analysed member of the SLP-2 subfamily, except for the *S. japonicum* ortholog (data not shown).

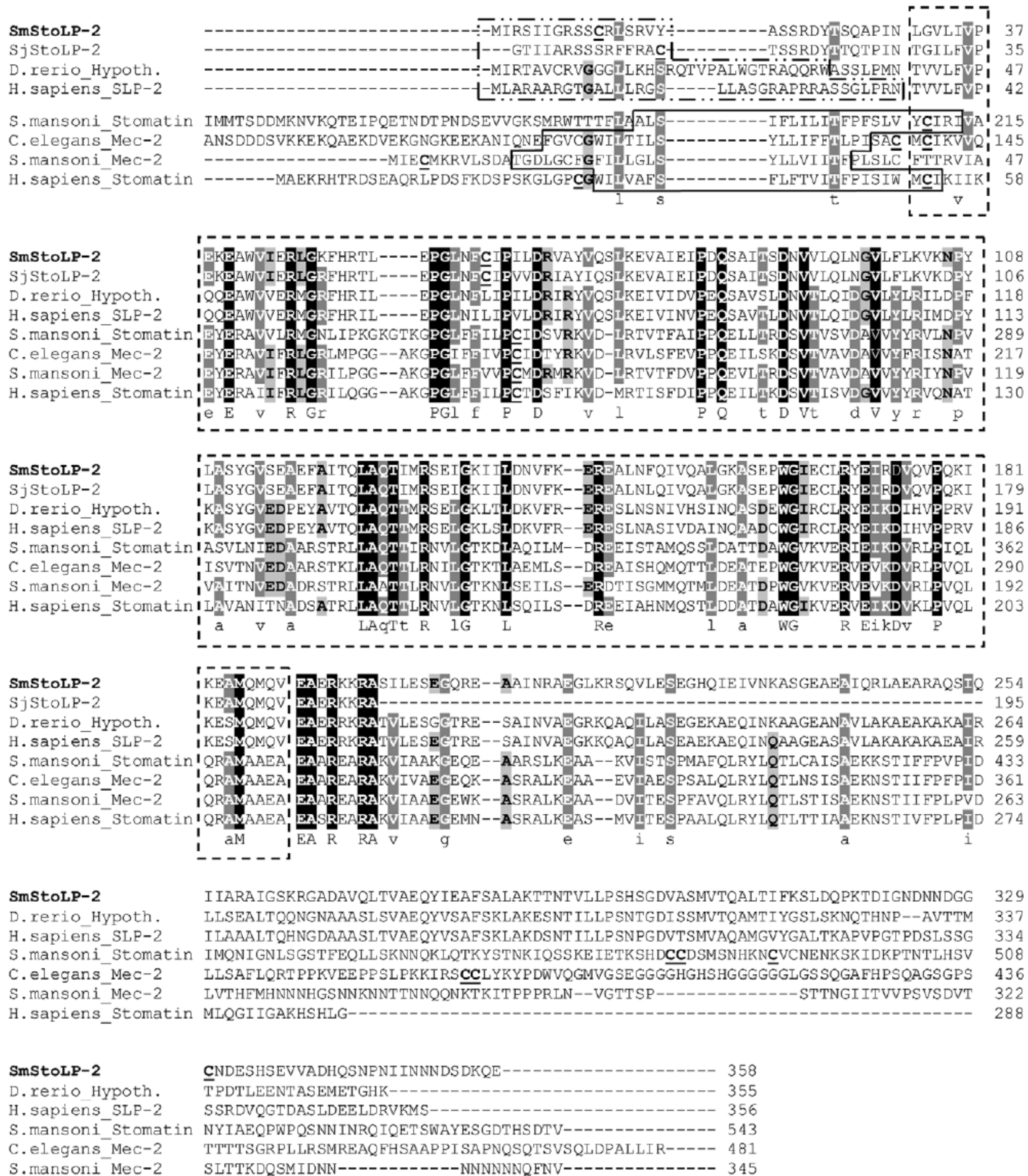

Figure 1. The complete protein sequence of SmStoLP-2 in relation to other members of the stomatin gene family. ClustalX alignment of the derived amino acid sequence of SmStoLP-2 (**EU531730**) with *S. japonicum* StoLP-2 (**AAX30477**), *D. rerio* Hypothetical (**NP_957325**), *H. sapiens* SLP-2 (**NP_038470**), *S. mansoni* stomatin excluding the first 120 amino acids (GeneDB accession no. Smp_162440), *C. elegans* Mec-2 excluding the first 50 amino acids (**NP_741797**), *S. mansoni* Mec-2 (GeneDB accession no. Smp_122810), *H. sapiens* stomatin (**NP_004090**), *H. sapiens* SLP-3 (**NP_660329**), *M. musculus* SLP-3 (**NP_694796**), *H. sapiens* SLP-1 (**NP_004800**) and *C. elegans* Unc-24 (**NP_501335**). Highlighted are the putative transmembrane domains as predicted by TMHMM (continuous box) absent in all SLP-2, the stomatin signature sequence (dashed box), mitochondrial targeting sequence as predicted by MitoProt II (dashed and dotted box), sites for palmitoylation (underlined). The regions with high identity and similarity between stomatin sequences are shown as black and gray columns, according to the Clustal X algorithm.
doi:10.1371/journal.pntd.0000597.g001

Phylogenetic analysis of the SFPH superfamily confirmed that SmStoLP-2 is a member of the stomatin family, grouping it in a branch with other SLP-2s (Figure S1). Like other stomatins [13,40,41], it is distantly related to flotillin, prohibitin, and HflK/C. Two putative flotillin and two putative prohibitins genes were identified in the *S. mansoni* genome. As expected, and probably due to its prokaryotic origin, no orthologues of HflK/C were found (Figure S1).

Production of recombinant SmStoLP-2

E. coli transformed with pDEST17-SmStoLP-2 showed a band at 45 kDa when induced with IPTG, which is slightly higher than the expected molecular mass for rSmStoLP-2 (Figure 2A). The bacteria were lysed by a French Press and separated into soluble and insoluble fractions. The insoluble fraction (inclusion bodies) was shown to contain the majority of the recombinant protein (Figure 2B, lanes 1, 2). The inclusion bodies were extracted with 8 M urea, refolded by dilution and purified by affinity chromatography on nickel-charged columns through an imidazole linear gradient from 20 to 500 mM (Figure 2B, lanes 3–8). The fractions were pooled and dialyzed to remove imidazole, yielding 8.0 mg of rSmStoLP-2/L culture. Circular Dichroism spectra indicated that the rSmStoLP-2 contains a regular secondary structure, although the proportions of secondary structure elements (α-helix and β-sheet) were not calculated (data not shown).

Analysis of protein expression across the life cycle stages

Extracts were prepared from cercariae, schistosomula, adult worms, eggs and miracidia stages of *S. mansoni* and subjected to immunoblotting with rat anti-rSmStoLP-2 serum, showing comparable levels of expression in all stages across the parasite life cycle. Native SmStoLP-2 observed in schistosome extracts, migrates with a molecular mass higher than that predicted, which was comparable to rSmStoLP-2 (~49 kDa) (Figure 2C). It is not known whether an additional smaller band (~47 kDa) could be a product of post-translational modification, alternative initiation, protein degradation or alternative mRNA splicing.

Differential tegument extraction of SmStoLP-2 from adult worms

Extracts from schistosomula and adult worms were separated into soluble and insoluble fractions and Western blot analysis revealed SmStoLP-2 to be present in the insoluble fractions in both stages (Figure 2D). A higher molecular mass band can be seen in the insoluble fraction of schistosomula, similar to that observed in the recombinant protein (P). The two most prominent bands correspond closely to the monomeric and dimeric forms of the protein at 49 and 98 kDa.

To further characterize the distribution of SmStoLP-2 in *S. mansoni* tegument, differential extractions of tegument membrane proteins were analysed. Western blot using anti-rSmStoLP-2 serum, revealed that SmStoLP-2 was recovered in the first extraction fraction solubilized with urea/thiourea (Figure 2E), suggesting SmStoLP-2 to be firmly bound, although non-covalently, to the tegument membranes.

On the other hand, SmStoLP-2 displays a mitochondrial signal sequence, which, if functional, may target it to the tegumental mitochondria. To address this issue, we isolated the tegument and performed a differential fractionation, separating the membrane and mitochondrial fractions. The anti-rSmStoLP-2 antibody recognized the protein in both fractions (Figure 2F). Mitochondrial enrichment was ascertained using a Mitofusin-1 antibody, which only detected this protein in the mitochondria-enriched fraction.

SmStoLP-2 is immunolocalized to the tegument of *S. mansoni*

Immunolocalization studies using rat serum raised against rSmStoLP-2 revealed through confocal fluorescence microscopy, that SmStoLP-2 is mainly expressed in the tegument of the adult *S. mansoni* male and female worms and seems to be expressed at lower levels in the muscle cells of male worms (Figure 3A and E). In an attempt to localize SmStoLP-2 in relation to the cytoskeletal tegument components, we used phalloidin-rhodamine as an actin marker. As shown in Figure 3P, there was some overlap staining on the muscle layers of adult male worms, revealed by the yellow signal. In contrast, the green band in the tegument, which corresponds to the main location of SmStoLP-2, did not seem to be co-localized with actin (Figure 3P). Additionally, the protein in male adult worms appears to be located more basally in the tegument, but it is interesting to note that the green band also seems to be running around and outside of their dorsal tubercles (Figure 3P).

Intact schistosomula stained with anti-rSmStoLP-2 and phalloidin-rhodamine suggested SmStoLP-2 to be external to the muscle layers in the tegument, as revealed by the green band running around and externally to the red band (Figure 3I–K, and R–T); additionally, the phalloidin-rhodamine internal labelling confirms that the parasites were well permeabilized (Figure 3J and M). No staining was observed in male and female sections or intact schistosomula incubated with naive rat serum (Figure 3C, G and L). Preliminary experiments indicate that, also in cercariae, SmStoLP-2 would be located in the evolving tegumental layer.

Immunoglobulin isotype profile of schistosomiasis patients to rSmStoLP-2

We evaluated by ELISA the specific reactivity of anti-SmStoLP-2 antibodies in sera of individuals with different status of resistance and susceptibility to *S. mansoni* reinfection. The sera of schistosomiasis patients, with the exception of the group susceptible to reinfection (SR), had significant levels of total anti-SmStoLP-2 IgG as compared to the non-infected group (Figure 4A). Furthermore, individuals from the group resistant to reinfection (RR) had increased levels of anti-SmStoLP-2 IgG when compared to individuals susceptible to reinfection (SR). Regarding IgA, statistically significant levels of antibodies to rSmStoLP-2 were observed in the INF and RR groups when compared to the NI group (Figure 4B). Once more, the RR group produced more anti-SmStoLP-2 IgA as compared to the SR individuals.

The IgG subclass profile of schistosomiasis patients was characterized predominantly by IgG1, IgG2 and IgG3 antibody responses to rSmStoLP-2. Individuals resistant to reinfection (RR) displayed at least a 2-fold higher level of IgG1, IgG2 and IgG3 anti-SmStoLP-2 antibodies as compared to those susceptible to reinfection (SR); these isotypes were also significantly higher when compared to the NI group (Figure 4C). Concerning IgG4, this antibody isotype was not detected in any of the groups studied.

Inhibition of skin penetration

In an attempt to check if the anti-rSmStoLP-2 antibodies could impair penetration of cercariae and their survival afterwards, we performed a skin penetration inhibition assay. As shown in Figure S2A, the rat anti-rSmStoLP-2 serum inhibited cercarial skin penetration by 77%, as compared with 40% inhibition by serum from rats that received saline only ($p = 0.002$). Six weeks after the infection, we assessed the parasite load in the infected mice; data revealed that only 12% of the penetrating parasites matured to adult worms in the group in which cercariae were incubated with

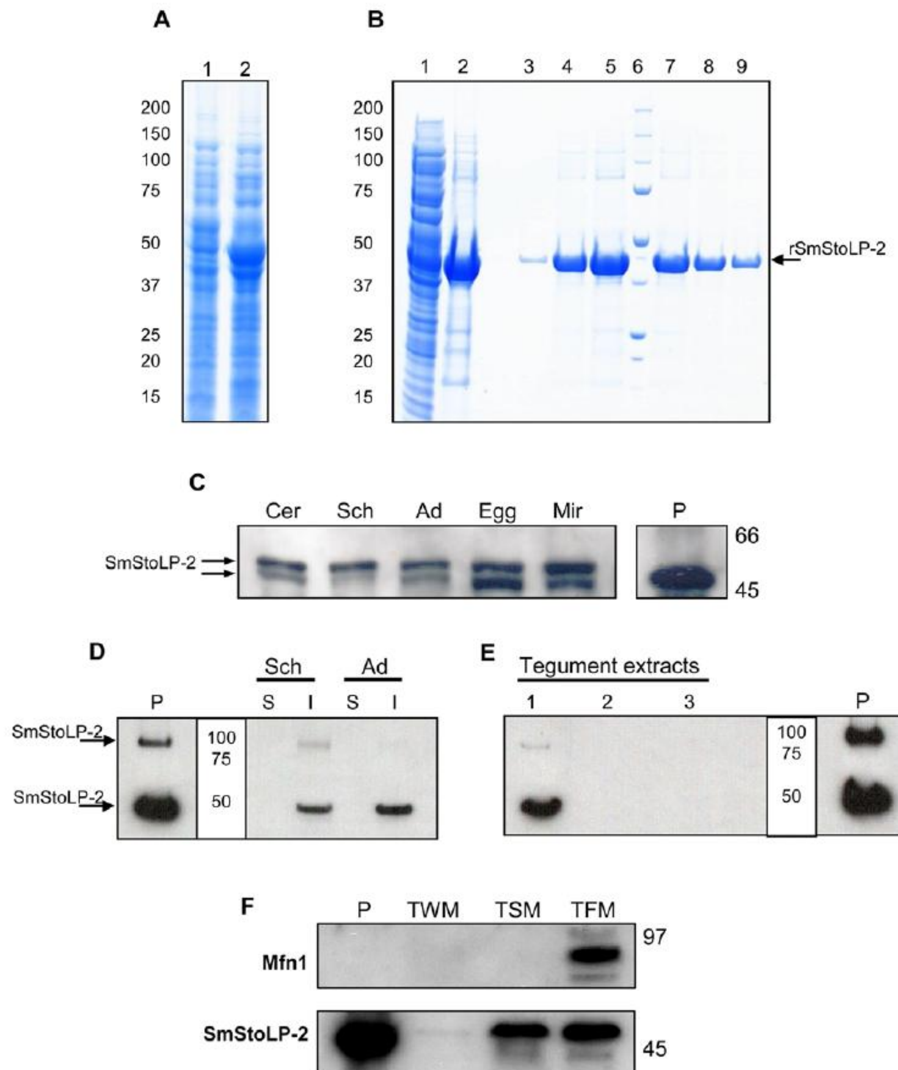

Figure 2. SDS-PAGE (4%–12%) analysis of cell extracts and fractions from *E. coli* (BL21DE3) transformed with the pDEST17-SmStoLP-2, and immunoblotting of protein extracts from *S. mansoni* stages and fractions using anti-rSmStoLP-2 polyclonal antibodies. (A) Lanes 1 and 2 represent a clone before and after induction with 1 mM IPTG, respectively; (B) Inclusion bodies were extracted with urea and denatured protein was refolded by dilution before being purified through Ni^{2+}-charged column chromatography. Lanes 1 and 2 show the soluble fraction after lysis and the inclusion bodies after solubilization with 8 M urea, respectively. Lanes 3–5 and 7–9 show the fractions of rSmStoLP-2-6xHIS-tag fusion protein eluted after Ni^{2+} chromatography, Lane 6, MW ladder (kDa); (C) Immunoblotting of *S. mansoni* extracts from different stages using anti-rSmStoLP-2 polyclonal antibodies (20 µg of protein were loaded in each lane). Cer – cercariae, Sch – 7-day-old schistosomula, Ad – adult worms, Egg – eggs, Mir – miracidia. (D) Western blot of soluble (S) and insoluble (I) protein extracts of 10-day-old schistosomula (Sch) and adult worms (Ad); (E) Detection of SmStoLP-2 in the tegument of *S. mansoni* adult worms, (1) proteins soluble in urea and thiourea, (2) proteins soluble in urea, thiourea, CHAPS and SB 3–10, (3) proteins soluble in 2% SDS. (F) Dual targeting of SmStoLP-2 to tegumental membranes and tegumental mitochondria, TWM, tegument extract without surface membranes, TSM, tegument enriched in surface membranes and TFM, tegument fraction enriched in mitochondria (20 µg of protein were loaded in each lane), Mfn-1 – is the Mitofusin-1 mitochondrial marker. Arrows indicate the rSmStoLP-2 and the most reactive bands of native SmStoLP-2 detected in each experiment. Positions of molecular mass standards (kDa) are indicated on the right or in the center. Positive control (P) contains 50–60 ng of rSmStoLP-2.
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anti-rSmStoLP-2, while 42% matured in the group incubated with control serum (Figure S2B). In a typical infection in the murine model, usually the maturation rate is around 35–40% [42].

Humoral immune response elicited by immunization with rSmStoLP-2

C57BL/6 mice were immunized with 3 doses of rSmStoLP-2 formulated with Freund's adjuvant and sera were analyzed by ELISA at 15, 30, 45, 60, 75 and 90 days for production of anti-SmStoLP-2 antibodies. Significant titers of specific anti-rSmStoLP-2 IgG antibodies were detected at all time points, showing a plateau after the third dose (data not shown). To determine the IgG isotype profile induced by immunization, specific IgG1 and IgG2a to rSmStoLP-2 were also analyzed. The levels of specific IgG1 and IgG2a and the IgG1/IgG2a ratio indicate that until the second dose there is a predominant Th2 response and after the third immunization there occurs a drift towards a more balanced or Th1-modulated immune response (Table 1).

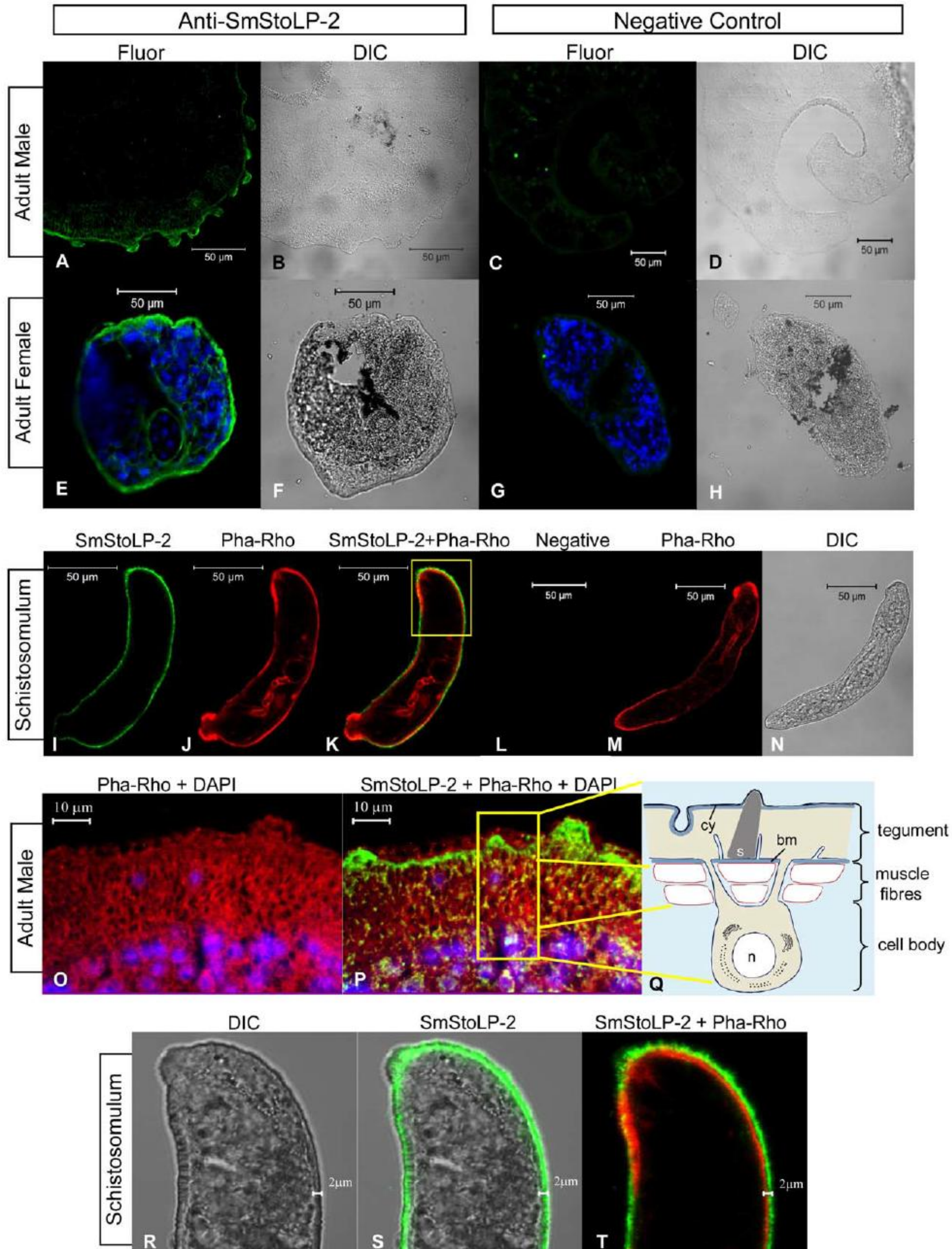

Figure 3. Immunolocalization of SmStoLP-2 in *S. mansoni* tegument. Fluorescence confocal microscopy images (Fluor) and corresponding differential interface contrast (DIC) images of male (A–D, O, and P), female adult worms (E–H), and schistosomulum (I–N and R–T) of *S. mansoni* are shown. Polyclonal anti-rSmStoLP-2 and secondary antibody coupled to Alexa 488 (green) were used for fluorescence detection of SmStoLP-2. Serum from naive rat was used as negative control for male (C), female (G) and schistosomulum (L). DAPI (blue) was used for nucleus localization (E, G, O, and P), and phalloidin rhodamine (red) was used for actin localization (J, K, M, O, P and T); (M) Diagram of the *S. mansoni* tegument and an associated cell body (not to scale). Cy: cytoskeleton; bm: basal membrane; n: nucleus; s: spine (extracted and modified from [43]).
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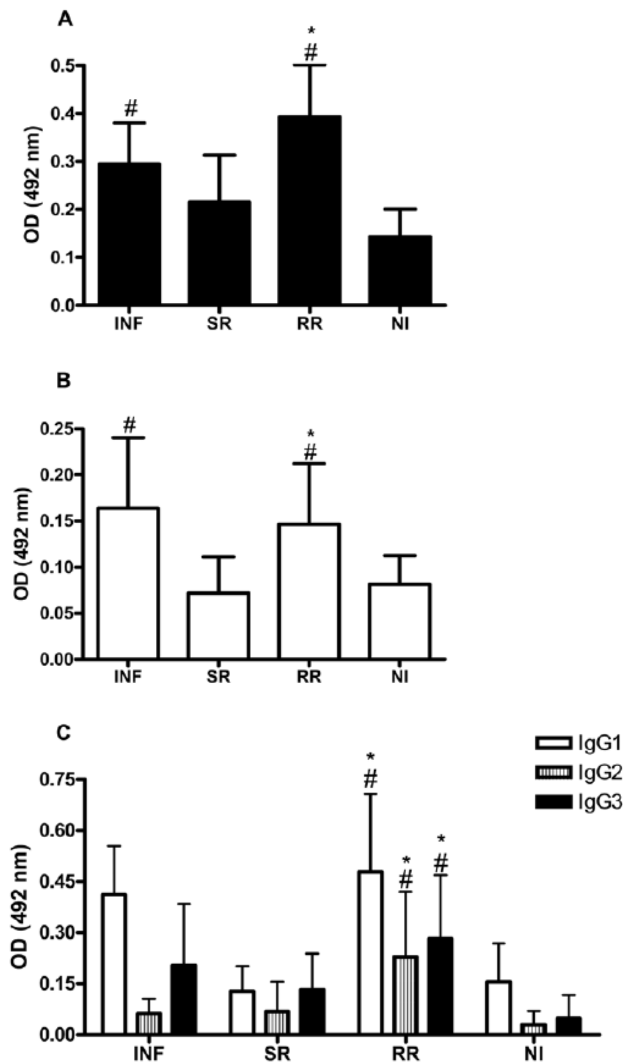


Figure 4. Isotype profile of sera from schistosomiasis patients reactive to rSmStoLP-2. Analysis of (A) IgG, (B) IgA and (C) IgG subclass antibody responses in sera of infected patients (INF), individuals susceptible to reinfection (SR), resistant to reinfection (RR) and non-infected individuals (NI). Results are expressed as means of individual measurements. Error bars indicate S.D. of the means. # Statistically significant as compared to the non-infected group ($P<0.05$). * Statistically significant as compared to the group susceptible to reinfection.
doi:10.1371/journal.pntd.0000597.g004

Cytokine secretion induced by the recombinant protein

In order to investigate the cytokine profile induced by the rSmStoLP-2 immunization regimen described above, we isolated splenocytes 10 days after the third immunization. Cytokine production (IFN-γ, TNF-α, IL-4 and IL-10) was measured in the culture supernatants from *in vitro* rSmStoLP-2-stimulated spleen cells of immunized mice. Statistically significant levels of IFN-γ, signature of Th1-type immune response, and TNF-α, a proinflammatory cytokine, were produced in the stimulated splenocytes from the rSmStoLP-2-immunized group as compared with the control (Figure 5A and B). Additionally, high levels of the modulatory cytokine, IL-10, were also observed (Figure 5C), and no secretion of IL-4, a Th2 cytokine, was detected (data not shown). These results indicate that immunization of mice with rSmStoLP-2 formulated with Freund's adjuvant induces a Th1-predominant immune response, with increased levels of IFN-γ, TNF-α and IL-10 and non-detectable levels of IL-4 secretion.

Protection against challenge with cercariae

In order to determine the protective potential of rSmStoLP-2, immunized mice were challenged with 100 cercariae. The worms were recovered by perfusion 6 weeks after challenge and results were expressed as the "mean worm burden" (mean ± S.D.) and are summarized in Figure 6. The animals immunized with rSmStoLP-2 in Freund's adjuvant showed 30 and 32% reduction in worm burden against challenge infection in two independent experiments when compared to the control group. Analysis of egg counts in the liver did not show a statistically significant reduction in oviposition.

Discussion

In this report, we have identified SmStoLP-2 as a member of the stomatin super family, displaying several properties shared with other SLP-2 proteins and some unique features. The widespread distribution of the 'conserved' SPFH domain across life kingdoms has been taken as an indication of its ancient origin, suggesting the common ancestry and functional homology of all SPFH proteins [13]. However, in a recent review, it has been proposed that SPFH grouping has little phylogenetic support, probably due to convergent evolution of its members [41]. Independently of its origin, our phylogenetic analysis of the deduced SmStoLP-2 protein has grouped it together with human SLP-2, and SLP-2 from *Danio rerio* and *Xenopus tropicalis*, and at some distance from SLP-1, stomatin (band 7) and SLP-3. We can highlight the following primary sequence features: 1) SmStoLP-2 lacks an N-terminal hydrophobic domain, similar to other SLP-2 members [9]; 2) SmStoLP-2 and SjStoLP-2 are the unique members of SLP-2 family, which show putative sites for palmitoylation, a property that could enhance the hydrophobicity

Table 1. IgG1 and IgG2a immune profile induced by immunization of mice with rSmStoLP-2.

	Days[a]					
	15	30	45	60	75	90
IgG1[b]	0.88±0.33	1.26±0.05	1.31±0.04	1.28±0.05	1.25±0.09	1.32±0.06
IgG2a[b]	0.02±0.10	0.14±0.12	0.23±0.11	0.27±0.10	0.25±0.08	0.30±0.12
IgG1/IgG2a	31.7	9.0	5.6	4.7	4.9	4.3

[a]Days after the first immunization.
[b]Absorbance 492nm.
doi:10.1371/journal.pntd.0000597.t001

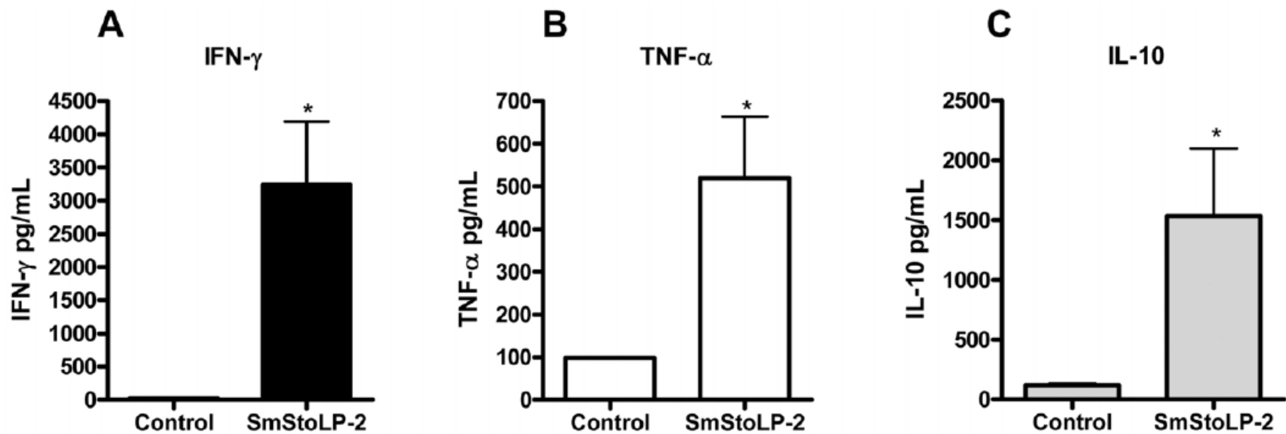
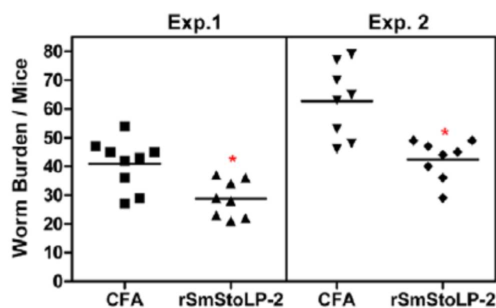


Figure 5. Cytokine profile of mice immunized with rSmStoLP-2. Ten days after the last immunization, splenocytes were isolated and assayed for (A) IFN-γ, (B) TNF-α and (C) IL-10 production in response to rSmStoLP-2 (25 μg/ml) or medium as control. The results are presented as mean ± S.D. for each group. Significant differences of secreted cytokines after rSmStoLP-2 stimulation or non-stimulated splenocytes are denoted by an asterisk ($p<0.05$).
doi:10.1371/journal.pntd.0000597.g005

of proteins and contribute to their membrane association; 3) additionally, SmStoLP-2, like all SLP-2 proteins, seems to have a mitochondrial targeting peptide in the N-terminal region.

The recovery of SmStoLP-2 in the insoluble fraction of parasite extracts suggests that the protein could be membrane-associated. Furthermore, solubilization of SmStoLP-2 from tegument membranes after the treatment with the chaotropic agents, urea and thiourea, indicates that it should be non-covalently bound to the tegument. It is interesting to note that SmStoLP-2 was not identified on Braschi's proteomic study [30], which could be explained based on the differences in sensitivity of the two methods used to detected the protein (mass spectrometry and Western blot). Moreover, albeit not with a quantitative analysis, the fact that SmStoLP-2 protein is present in the free-living (freshwater) cercariae and miracidia, as well as in the egg stage, suggests that the protein has other



Worm Burden (Mean ± S.D.)	40.8 ± 8.7	28.7 ± 6.4	62.6 ±12.6	42.3 ± 7.0
Protection (%)	-	**30**	-	**32**
(P- value)		**0.0055**		**0.0014**

Figure 6. Scattergram of worm burden from two independent experiments of mice immunized with rSmStoLP-2 and challenged with live *S. mansoni* cercariae. Statistical analyses were performed with Student's t-test, *statistically significant ($p<0.05$) compared to control group (CFA/IFA).
doi:10.1371/journal.pntd.0000597.g006

function(s), not exclusively associated with the tegument, which are common to both free-living and parasitic stages.

It has been recently proposed that HuSLP-2 may interact with actin (a cytoskeletal constituent), vav (a small GTPase that regulates cytoskeleton reorganization) and Nck (an adaptor protein that links transmembrane and scaffolding molecules to the cytoskeleton) [23]. The confocal immunofluorescence images of the parasites confirm the SmStoLP-2 tegument localization and suggest some weak co-localization with actin, only on muscle layers of adult male worms. In addition, SmStoLP-2 seems to be located externally to the muscle layers in the 7-day-old schistosomulum.

Data from the HuSLP-2 suggests that there are at least two cellular pools of this protein: one associated with the plasma membrane and the other with mitochondria [9,11,24]. It is important to note that the tegumental cytoplasmic layer lying under the surface membranes, contains small mitochondria [43]. However, confocal immunofluorescence microscopy does not have sufficient resolution to address this question with confidence. Our results on the differential fractionation of tegument extracts analyzed by Western blot addressed this issue and strongly suggested that SmStoLP-2 also displays a dual targeting, one associated to the tegument membrane and one to the mitochondria.

As a consequence of the studies in the attenuated cercaria vaccine model, the schistosomula is believed to be the target of protective immunity [42]. Given the tegument localization of SmStoLP-2 in the schistosomula suggested by our immunolocalization results, this molecule should be accessible as an immune target. In individuals putatively resistant to reinfection (RR), the antibody response mounted against SmStoLP-2, consisted mainly of the cytophilic antibodies IgG1 and IgG3, which have opsonization properties, cell dependent cytotoxicity, and the ability to activate the classical complement pathway, functions which could be involved in the resistance to *S. mansoni* reinfection. Elevated levels of IgG1, IgG2 and IgG3 have been linked to the human resistant status for several vaccine candidates, such as, Sm23, Sm28, Sm14-FABP, Sm29 and TSP-2 [32,44,45]. Concerning IgA levels, investigators have associated the increased levels of this isotype with resistance to reinfection stimulated by Sm28GST antigen [46,47], which parallels our results, where high levels of IgA antibodies to rSmStoLP-2 were observed in patients which are resistant to reinfection (RR).

Although no function has been ascribed for SmStoLP-2, our finding that anti-rSmStoLP-2 antibodies inhibits cercariae skin penetration and migration, suggests that the molecule may have an important role in larval host entry and in migration through the tissues until the lungs before reaching the portal hepatic system. This finding provides further support for testing SmStoLP-2 as a vaccine candidate against murine schistosomiasis.

In the murine model, rSmStoLP-2 induced high levels of anti-rSmStoLP-2 IgG after the second immunization and showed a reduced IgG1/IgG2a ratio at 45 days after the first immunization. Additionally, we confirmed by cytokine analysis that rSmStoLP-2 immunization elicited a Th1-predominant type of immune response characterized by production of high levels of IFN-γ and no detection of IL-4. To determine if rSmStoLP-2 conferred protection against *S. mansoni* infection, immunized mice were challenged with cercaria and worm burden analyzed. Immunization with rSmStoLP-2 induced a 30-32% worm burden reduction.

A primary obstacle in the research and development of a schistosomiasis vaccine is a lack of understanding of what type of immune response should be induced. In the irradiated cercariae vaccination model, protection can be either based on a Th1, a Th2, or a mixed Th1/Th2 immune response [48]. However, in the case of recombinant proteins, Th1 inducing antigens have been described to induce protection against infection in the mouse model [36,49–53]. The role of IFN-γ in the protective immunity to schistosomiasis is well described in mice exposed to the irradiated vaccine and there is compelling evidence that immune elimination of challenge parasites occurs in the lungs. Since IFN-γ is likely to be required to activate pulmonary macrophages which may mediate the protective response [54], we could expect that similar mechanisms would be involved in rSmStoLP-2 protective immunity.

Recently, HuSLP-2 has been proposed as a potentially useful target for immunotherapy in humans, since it modulates effector T cell responses [24]. Thus, down-regulation of HuSLP-2 expression could be valuable in the course of autoimmune disease treatment, since it decreases T cell reactivity; alternatively, enhancement of HuSLP-2 expression could be explored in vaccine development, since it would increase T cell responsiveness [24]. Given that orthologs often, but not always, have the same function, it is still unclear what is the function of SmStoLP-2; it could have an immunomodulatory role like its human ortholog [24] or could acquire a different function on the parasite-host interface, like providing structural scaffolding for the tegument or supporting the traffic of vesicles to the surface plasma membrane [9], organizing the peripheral cytoskeleton and assembly of multichain receptors, such as ion channels [11,55], or even mechanosensation receptors [18,20]. Further investigations of SmStoLP-2 will be valuable in understanding how the tegument functions as the parasite-host interface.

A critical issue in vaccine design is the use of an appropriate adjuvant and/or delivery system to induce the suitable immune response. Experiments are underway investigating rSmStoLP-2 with different adjuvant formulations, which would be suitable for use in humans. In conclusion, our study showed that SmStoLP-2 is a novel tegument protein, being recognized by different subclasses of antibodies in patients resistant and susceptible to reinfection, and in the light of data obtained from murine studies, protective properties against schistosomiasis were revealed. We believe that an ideal vaccine may require the combination of quite a few antigen targets to induce an effective protection against the parasite, and SmStoLP-2 could contribute to reach this goal.

Supporting Information

Figure S1 Phylogenetic analysis performed with protein sequences showing that SmStoLP-2 is part of the stomatin family. The sequences accession numbers are: *X. tropicalis* hypothetical (GenBank accession no. NP_001004808.1), human prohibitin (AAP36079), *S. mansoni* putative prohibitin-1 (GeneDB accession no. Smp_075210.2), Yeast prohibitin 1 (NP_011648), *S. mansoni* putative prohibitin-2 (Smp_075940), Yeast prohibitin 2 (NP_011747), human flotillin-1 (AAP36527), *S. mansoni* putative flotillin-1 (Smp_016200.1), human flotillin- 2 (NP_004466), *S. mansoni* putative flotillin-2 (Smp_033970), *Escherichia coli* HflK (NP_458799), *E. coli* HflC (NP_418596). (Accession numbers of the other members are cited in the legend of Figure 1).
Found at: doi:10.1371/journal.pntd.0000597.s001 (0.01 MB PDF)

Figure S2 Inhibition of cercariae skin penetration by rat anti-SmStoLP-2 antiserum. For these studies, 100 *S. mansoni* cercariae were applied in pond water for percutaneous infection and the number of non-penetrating parasites were counted. The percentage inhibition resulting from either rat-anti-SmStoLP-2 antiserum or antiserum obtained from control rats immunized with saline is expressed as the mean ± S.D. of one representative of three independent experiments.
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Table S1 Study population.
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Author Contributions

Conceived and designed the experiments: LPF FCC TK RCO PSC RAW SCO LCCL. Performed the experiments: LPF FCC PAM BOM CAT PSC. Analyzed the data: LPF FCC PAM BOM CAT HKR TK AG RCO PSC RAW SCO LCCL. Contributed reagents/materials/analysis tools: AG RCO RAW SCO LCCL. Wrote the paper: LPF FCC RAW SCO LCCL.

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Characterization of phosphodiesterase-5 as a surface protein in the tegument of *Schistosoma mansoni*☆

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ABSTRACT

Schistosoma mansoni is a major causative agent of schistosomiasis, an important parasitic disease that constitutes a severe health problem in developing countries. Even though an effective treatment exists, it does not prevent re-infection and the development of an effective vaccine still remains the most desirable means of control for this disease. In this work we describe the cloning and characterization of a *S. mansoni* nucleotide pyrophosphatase/phosphosdiesterase type 5 (SmNPP-5), previously identified in the tegument by proteomic studies. *In silico* analysis predicts an N-terminal signal peptide, three N-glycosylation sites and a C-terminal transmembrane domain similar to that described for mammalian isoforms. Real-time quantitative RT-PCR and Western blot analyses determined that SmNPP-5 is significantly upregulated in the transition from free-living cercaria to schistosomulum and adult worm parasitic stages; additionally, the native protein was demonstrated to be N-glycosylated. Immunolocalization experiments and tegument surface membrane preparations confirm the protein as a tegument surface protein. Furthermore, the ectolocalization of this enzyme was corroborated through the hydrolysis of the phosphodiesterase specific substrate (ρ-Nph-5′-TMP) by living adult and 21-day-old worms. Interestingly, pre-incubation of adult and 21-day-old worms with anti-rSmNPP-5 antibody was able to reduce by 50–60% the enzyme activity. These results suggest that SmNPP-5 is closely associated with the new tegument surface generation after cercarial penetration, and being located at the host–parasite interface, is a potential target for immune intervention.

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1. Introduction

Schistosomes are parasitic blood flukes that cause schistosomiasis, a tropical disease that has a major public health impact in endemic countries. It affects about 200 million individuals worldwide causing more than 200,000 deaths per year, with more than 600 million people at risk of infection [1,2]. The current strategy for schistosomiasis control aims at the reduction of morbidity through treatment with praziquantel [3]; however, chemotherapy does not prevent re-infection. Aiming at the elimination of the disease, it is clear that morbidity, transmission, and infection control are different battlefronts and that mass praziquantel chemotherapy will not be enough to reach the goal [2,4]. Thus it has been argued that vaccination would contribute enormously to the reduction of the burden of this disease.

The greatest obstacle in a vaccination context is the need for adequate amounts of the appropriate protective antigens. This is particularly important in the case of schistosomiasis, because the parasite uses many strategies to evade the immune responses of the host, resulting in a chronic debilitating infection [5]. Surface exposed and/or secreted proteins expressed in the intra-mammalian host stages are a source of potential antigens, but the parasite develops important structures immediately following invasion of the vertebrate host, which are responsible for its immune evasion mechanisms. One of the most important is the new tegument surface organisation in the transformed schistosomulum. The tegument is a thin syncytial layer that covers the whole parasite, limited by a basal membrane and a multilaminate surface membrane complex, which constitutes the major host–parasite interface [6].

The simultaneous publication of the *S. mansoni* and *S. japonicum* transcriptomes [7,8], together with the ongoing whole-genome

Abbreviations: NPP, nucleotides pyrophosphatase/phosphodiesterase; qPCR, real-time quantitative polymerase chain reaction; EST, expressed sequence tag; Ct, cycle threshold; ρ-Nph-5′-TMP, ρ-nitrophenyl 5′-thymidine monophosphate.

☆ *Note:* The nucleotide sequence reported in this paper has been deposited to GenBank and assigned an accession number of EU570984.

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sequencing project (www.SchistoDB.org), opened the avenue for parasite proteomic analysis resulting in a series of reports aiming at the characterization of different aspects of the schistosome life cycle and biology [9–16]. As a result, a remarkable understanding of the protein composition of the schistosome tegument and of its surface membranes has been achieved [17–20]. In these studies, a putative nucleotide pyrophosphatase/phosphosdiesterase (NPP) was identified, first as an exclusive tegument protein [20], then by differential extraction as a plasma membrane enzyme [17], and finally, since it was labelled with biotin, as a surface-exposed protein [19]. Its presence has been previously implied by the detection of phosphosdiesterase activity on the tegument [21].

NPPs are ubiquitous membrane-associated or secreted ecto-enzymes that have a role in regulating extracellular nucleotide metabolism; they hydrolyze pyrophosphate or phosphodiester bonds in a variety of extracellular compounds including nucleotides, (lyso) phospholipids and choline phosphate esters, releasing nucleoside 5′-monophosphate [22]. Extracellular nucleotides, and in particular ATP and adenosine, elicit a broad range of responses in biological processes [23], therefore, catalysis by NPPs could affect multiple physiological processes as diverse as platelet aggregation, apoptosis, cell proliferation, differentiation and motility. The mammalian family of NPPs is composed of seven structurally related ecto-enzymes that are numbered according to the order in which they were identified [22]. NPP-5 was predicted to be a type I-transmembrane protein, with its catalytic site in its extracellular region and a very short cytoplasmic tail [24]. The best characterized ortholog is the rat protein, that was suggested to be a neural oligomannosidic glycoprotein, which may participate in neural cell communications [25]. In the current work we characterized a putative NPP ortholog, identified in the aforementioned proteomic studies of the *S. mansoni* tegument, and one of the so-called new generation vaccine candidates.

2. Materials and methods

2.1. Parasite maintenance

Schistosoma mansoni 21-day-old and 45-day-old adult worms (BH strain) were obtained by perfusion of infected hamsters. Eggs, miracidia, cercariae, and schistosomula were obtained as previously described [8]. Animal experimental protocols were approved by Animals Use Ethics Committee of Instituto Butantan (São Paulo, Brazil).

2.2. Cloning and molecular characterization

Specific oligonucleotides were designed using the *S. mansoni* EST assembly contig (Sm03458), GeneDB, (http://www.genedb.org/genedb/smansoni/). Total RNA was isolated from adult worms using TRIzol reagent (Invitrogen), followed by mRNA purification with oligo(dT)-cellulose columns according to the manufacturer's instructions (Amersham Biosciences). The SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen) was used for SmNPP cDNA generation following the manufacturer's protocol. The 5′ and 3′ oligonucleotides, GAA TTC GGT GTT GTT GGG AAG GAA CAG TTT and CTC GAG TCA GGG AAG AAC TCG ACA AAC ACT, were used to amplify the complete open reading frame of SmNPP. The obtained PCR fragment was cloned into pGEM-T easy vector (Promega) and sequenced to confirm its identity.

Post-translational modification prediction: the signal peptide prediction was performed using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), N-glycosylation sites were analyzed using the NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc/) and transmembrane helices were analyzed by TMHMM, version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Molecular weight (MW) and isoelectric point (pI) were calculated with the Compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html).

2.3. Phylogenetic and sequence analysis

Blast and PSI-Blast searches against the NCBI non-redundant protein sequence database, using SmNPP as a query, were used to identify orthologues of SmNPP. For phylogenetic analysis, alignments of protein sequences were performed using the ClustalX 1.83 software. The tree was constructed using Clustal with the Neighbour Joining method, excluding positions with gaps. The numbers represent the confidence of the branches assigned by bootstrap (in 1000 samplings). The TreeView program (taxonomy.zoology.gla.ac.uk/rod/treeview.html) was used to visualize the tree.

2.4. Real-time RT-PCR analysis

Total RNA was extracted from 7-day schistosomula, adult parasites, eggs and miracidia using TRIzol (Invitrogen), and from cercaria using the RNeasy Protect Kit (Qiagen), as per the manufacturer's recommendations. The RNA was quantitated by spectrophotometry using a NanoDrop instrument (Wilmington, USA), and the quality was analyzed with an Agilent 2100 Bioanalyzer. Equal amounts of total RNA (3 μg) from the various developmental stages were digested with RNase-free DNase (Promega) for 1 h at 37 °C, and used for reverse-transcription (RT). The RT was performed with random hexamer primers and Superscript III reverse transcriptase (Invitrogen), according to standard protocols and the resulting cDNA was used for quantitative PCR (qPCR). The cDNA was amplified with the SYBR Green (Applied Biosystems) in a GeneAmp®PCR System 9600 (Corbett Research). Primers for qPCR were designed using Real-Time PCR Primer Design (http://www.genscript.com/ssl-bin/app/primer) and the settings were adjusted to the highest possible stringency to generate 70–100 bp amplicons, as recommended. The primers selected for SmNPP (forward: 5′ ATT ATG ATT ACA TAC ATC CAA ATG AG 3′ and reverse: 5′ CGC ACA GTA TAG CCT TGA TTT 3′) amplified a product of 94 bp. The expression levels for three housekeeping genes Actin (accession: U19945), α-Tubulin (accession: M80214) and GAPDH (accession: M92359) were assessed in the cercaria, schistosomulum and adult worms. The actin gene seems to be a slightly better internal control among life cycle stages because its Ct values were less variable (data not shown), while α-Tubulin showed less variation in the amount of male and female adult worm transcripts [26]. Thus, as an endogenous housekeeping reference for qPCR among life cycle stages we used primers targeting *S. mansoni* Actin (forward: 5′ CGT TGG ACG ACC TCG ACA T 3′and reverse: 5′ TGT CTT TCT GAC CCA TAC CAA CC 3′; product size of 53 bp), whereas for qPCR between male and female adult worms we used primers targeting α-Tubulin (forward: 5′ CCA TTT ATG ATA TTT GTC GAC GGA 3′and reverse: 5′ TTT GTG TAG GTT GGA CGC TCT ATA TCT A 3′; product size of 55 bp). Reaction conditions were as described in the SYBR green kit and the cycling protocol was as follows: 95 °C for 10 min and 40 cycles of 95 °C for 10 s, 60 °C for 1 min. The generation of PCR products was measured in real time by the GeneAmp® 5700 Sequence Detection System; specific PCR products were confirmed by dissociation curve analysis and agarose gel electrophoresis. Quantitation of relative differences in expression were finally calculated using the comparative $\Delta\Delta$Ct method [27]. Statistical comparisons were performed with t-Student or one-way ANOVA followed by a Tukey pairwise comparison, as required. A ρ value <0.05 was considered statistically significant.

2.5. Expression of recombinant protein and polyclonal antibody production

We amplified by PCR the cDNA fragments corresponding to SmNPP from G20 to K414 (with the forward, TAC TGA ATT CGG TGT TGT TGG GAA GGA ACA GTT TTC, and reverse, TAC TCT CGA GTC AGG GAA GAA CTC GAC AAA CAC TAC CAT, primers). The SmNPP cDNA fragment was purified, digested with EcoRI and XhoI to generate inserts with overhang ends that were ligated into the same sites of the expression vector pET28a (Novagen), to produce a protein that contained an N-terminal hexahistidine tag.

For protein expression, transformed BL21(DE3) cells (Invitrogen) were grown in 600 ml LB plus ampicillin (1 mg/ml) until OD600 = 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM, and cells were incubated for 3–4 h at 37 °C. Cells were harvested by centrifugation and resuspended in 50 ml of lysis buffer (50 mM Tris pH 8.0, 100 mM NaCl). The cell suspension was passed three times (1500 psi) through a French press and the crude homogenate was centrifuged at 20,000 × g for 30 min.

In order to determine the solubility of the recombinant SmNPP protein (rSmNPP), inclusion bodies were resuspended and pelleted (centrifuged at 20,000 × g) sequentially in lysis buffer with increasing urea concentrations of 2, 4, 6 and 8 M, and lastly with 6 and 8 M guanidine hydrochloride. For protein purification, the inclusion bodies of rSmNPP were rinsed twice with washing buffer I (lysis buffer, 2% Triton X-100, 2 M urea), then rinsed once with washing buffer II (lysis buffer, 2% Triton X-100, 6 M guanidine hydrochloride), and finally resuspended in solubilization buffer (lysis buffer plus 5 mM beta-mercaptoethanol, 20 mM imidazole, 8 M guanidine hydrochloride). The recombinant protein was then purified by metal affinity chromatography using the Akta Prime system (GE Healthcare), under denaturing conditions. Briefly, the sample was loaded onto a Ni^{2+}-NTA column (5 ml bed volume) pre-equilibrated with the same buffer. The column was washed with 10 bed volumes of the equilibration buffer and then eluted with 20–500 mM imidazole linear gradient. Fractions encompassing the main peak were pooled and the purity of the preparation assessed by SDS-PAGE. The protein was refolded by slow dialysis in Phosphate Buffer Saline pH 7.4 (PBS) containing decreasing concentrations of urea (6, 4, 3, 2, 1 M and no urea).

Polyclonal mouse serum was produced against preparations of rSmNPP. BALB/c mice were immunized four times, at 21-day intervals with 25 μg of protein adsorbed to 2500 μg of aluminum hydroxide. Fifteen days after the last inoculation, rodents were exsanguinated. The sera were used at a dilution of 1:1000 in Western blots and 1:50 in immunofluorescence assays.

2.6. Protein expression profile and deglycosylation assay

The tegument extract was obtained by a freeze/thaw/vortex procedure, as previously described [28]. Briefly, frozen worms (1000) were thawed on ice in the presence of 1 ml ice-cold RPMI medium plus protease inhibitors (Protease inhibitor cocktail; Sigma), with 10 vortex pulses at maximum speed duration 1 s to detach the tegument. After the stripped worms had settled, the supernatant was collected and sonicated (four cycles of 2 min, with pulses of 0.75 s, 40% amplitude).

Total protein extracts from eggs, miracidia, cercariae, schistosomula, paired and unpaired male and female adult worms were prepared in 40 mM Tris, pH 7.4, 2% SDS plus protease inhibitor by sonication as described above. The samples were centrifuged at 20,000 × g for 30 min at 4 °C and the supernatant was recovered and used for the assays. Their protein concentrations were determined with a DC Protein Assay Kit (Bio-Rad, CA, USA). Purified rSmNPP (100 ng), total parasite protein extracts (20 μg), total tegument extract (20 μg), and tegument surface membranes (2 μg) isolated from total tegument extract after low speed centrifugation (100 g, 30 min) were subjected to SDS-PAGE. The gel was electroblotted onto a PVDF membrane, which was blocked with 0.02 M Tris (pH 7.5) and 0.3% Tween 20 containing 5% dry milk for 16 h at 4 °C. Subsequently, the membrane was incubated in a 1:1000 dilution of anti-rSmNPP primary antibody in blocking buffer plus 150 mM NaCl for 3 h at room temperature. After three washes using 10 mM Tris (pH 7.5), the membrane was incubated in a 1:4000 dilution with secondary goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma) for 1 h with another three washes using the same buffer. Antibody reactivity was developed with ECL reagent (GE Healthcare) according to the manufacturer's instructions and imaged using Hyperfilm (GE Healthcare).

N-deglycosylation of native SmNPP was carried out as previously described [29]. Briefly, 200 μg of tegument extracts (in 40 mM Tris pH 7.4) were denatured by boiling for 10 min in the presence of 0.5% SDS and 1% 2-mercaptoethanol. Then, 1% NP-40 (Sigma), 1 μl of 1 M sodium phosphate (pH 7.5) and 20 μL (3.5 U) recombinant N-glycosidase F (Roche Diagnostics) were added and incubated for 4 h at 37 °C. A sample of purified rSmNPP, digested and non-digested tegument fractions were submitted to SDS-PAGE. The gel was electroblotted onto a PVDF membrane and Western blot developed as described above.

2.7. Indirect immunofluorescence and confocal microscopy

Freshly perfused adult worms from hamsters were fixed with 4% paraformaldehyde in PBS, at 4 °C overnight. The antigen retrieval was based on [30]. Briefly, worms were incubated in retrieval solution (10 mM sodium citrate buffer, pH 6.0) at 4 °C overnight. Next they were placed in boiling retrieval solution for 5 min, and followed by an immersion in cold 30% sucrose in PBS and incubated at 4 °C until they sink. Then they were recovered and embedded in OCT medium (Sakura) in a pre-cooled beaker of isopentene, frozen in liquid N_2. Eight-micrometer cryostat adult worm sections were adhered to silanized glass slides (DakoCytomation), dried for 2 h and rehydrated with PBS at 4 °C overnight. The sections were treated with PBS containing 1% SDS for 10 min at room temperature and washed with PBS [31], before blocking with PBS containing 10% naive rabbit serum and 0.1% Tween 20 for 4 h at room temperature. Sections were then incubated with anti-rSmNPP-5 antisera, 1:50 (v:v), for 3 h at room temperature. After washing five times with PBS with 0.1% Tween 20 (PBS-T), an Alexa Fluor 647 conjugated rabbit anti-mouse IgG (Invitrogen), 1:200 (v:v), was added to the blocking solution, and the samples incubated for 1 h at room temperature. Sections were washed five times and mounted in Fluorescent Mounting Medium (DakoCytomation). Mouse pre-immune serum was used as negative control. Images were acquired in a Zeiss LSM 510 Meta confocal system, attached to a Zeiss Axiovert 100 microscope using LD-Achroplan 20×/0.4 or C-Apochromatic 63×/1.2 W objectives with differential interference contrast.

2.8. Assay of NPP activity from live parasites

The NPP activity was assessed using ρ-nitrophenyl 5′-thymidine monophosphate (ρ-Nph-5′-TMP) a specific artificial marker substrate, the protocol was adapted from [32]. Freshly perfused living adult worms and 21-day-old worms (three individuals of each stage), or approximately 2000 living cercariae were incubated with ρ-Nph-5′-TMP in the reaction buffer containing 50 mM Tris–HCl buffer, pH 8.9, 120 mM NaCl, 5.0 mM KCl, 60 mM glucose and 5.0 mM $CaCl_2$ in a final volume of 200 μl. We estimated the schistosome male adult worm area to be around 12,000,000 μm^2, based on [33] and not considering the tegument pits. The cercaria surface

area was estimated as 10,682–20,000 μm^2 [34,35], so in an attempt to normalise for the area exposed to the phosphodiesterase substrate we used 2000 cercariae, equivalent to the surface of three adult male worms.

The reaction started by the addition of ρ-Nph-5′-TMP to a final concentration of 0.5 mM. After 20 min of incubation at 37 °C, 100 μl of 0.4 M NaOH was added to the buffer to stop the reaction, the reaction buffer was recovered and the amount of ρ-nitrophenol released was measured at 405 nm. For antibody blocking assays, three adult male worms or 21-day-old worms were previously incubated with 100 μl of undiluted mouse serum containing anti-rSmNPP-5 polyclonal antibodies for 2 h at 37 °C, before assessing the phosphodiesterase activity. Following the incubation time the parasites were visually inspected to assure their viability. Controls to guarantee the specificity of substrate hydrolysis were performed by incubating the substrate without worms and the worms without substrate. All samples were performed in triplicate and statistical comparisons were performed with a *t*-test. A ρ value <0.05 was considered statistically significant.

3. Results

3.1. Cloning and molecular characterization of *S. mansoni* NPP

The full-length sequence of the *S. mansoni* cDNA encoding a putative NPP was obtained by RT-PCR from adult worm mRNA with specific oligonucleotides that were designed using the corresponding EST contig, Sm03458. The resulting full-length cDNA (Gene Bank accession EU570984) displayed an ORF of 1350 bp, encoding a protein of 449 amino acids with a predicted molecular mass of approximately 51.4 kDa and an isoelectric point of 6.28. Comparing the cDNA sequence and EST contig (Sm03458), a single sequence difference was found at nucleotide position 482 (T instead of C in the genome sequence), but the amino acid sequence remains conserved. In v4.0 of the genome assembly the predicted gene (Smp_153390.2) for Sm03458 EST contig displays an extended signal peptide sequence (MYCIETMQK); at present it is still unknown which of these two predictions represents the more accurate sequence.

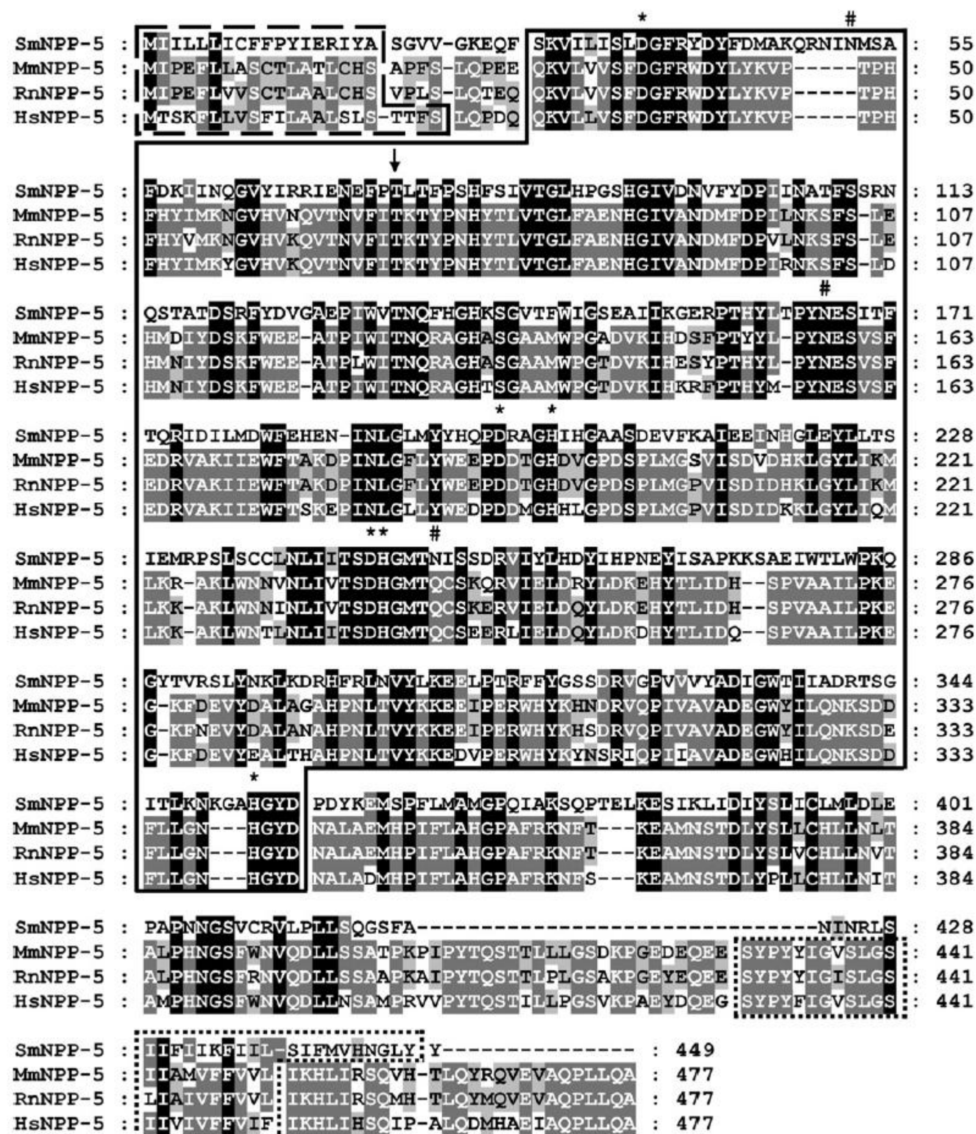

Fig. 1. The complete protein sequence of SmNPP-5 in relation to mouse, rat and human NPP-5. ClustalX alignment of the derived amino acid sequence of SmNPP-5 (EU570984), HsNPP-5 (NP_067547.1), MmNPP-5 (NP_114392.1), and RnNPP-5 (NP_001012762.1). Highlighted are the putative transmembrane domains, as predicted by the TMHMM (dotted box), the phosphodiesterase signature sequence (continuous box), signal peptide, as predicted by SignalP (dashed box), sites for metal binding (*), potential sites for N-glycosylation (#), and the putative catalytic center indicated by an arrow. The regions with high identity and similarity between phosphodiesterase sequences are shown as black and gray columns, according to the Clustal X algorithm.

BlastP comparisons of the deduced *S. mansoni* protein sequence to GenBank showed that the best match (E-value = 1×10^{-95}) was to an *S. japonicum* protein (SJCHGC05898) (probably an incomplete sequence of a NPP), with 70% identity and 82% similarity over 236 amino acids. The next closest orthologs of SmNPP were from ectonucleotide pyrophosphatase/phosphodiesterase 5 proteins from *Monodelphis domestica* (E-value = 2×10^{-75}), *Ornithorhynchus anatinus* (E-value = 9×10^{-73}) and *Rattus norvegicus* (E-value = 2×10^{-71}). These were followed by mouse, dog, horse, starlet sea anemone, zebrafish, rhesus monkey, chimpanzee, and human NPP-5 proteins (the last three with E-value of 4×10^{-67}); therefore we designated this gene as SmNPP-5.

SmNPP-5 contains the NPP signature sequence (residues 28–356) (outlined by a continuous box in Fig. 1), and is recognized as part of the Pfam Type I phosphodiesterase/nucleotide pyrophosphatase family (PF01663) with an E-value of 1.7×10^{-47}. SmNPP-5 also contains a putative N-terminal signal peptide (outlined by a dashed box in Fig. 1) and a C-terminal transmembrane domain (dotted box), characterizing it as a type-I, single spanning transmembrane protein, with its catalytic domain facing the extracellular space [22]. Searching the *S. mansoni* genome (GeneDB) for proteins with Pfam Type I phosphodiesterase/nucleotide pyrophosphatase domains, we found three other *Schistosoma* full-length phosphodiesterase-related genes (accession numbers Smp_104270 (EST Sm03904), Smp_153340.1 (EST Sm04918) and Smp_084890 (EST Sm06826 and Sm00959)), and one probably incomplete assembled EST (Sm23345).

BlastP comparisons of the deduced *S. mansoni* protein sequence to GenBank revealed Smp_104270 and Smp_153340.1 to have higher similarity (57 and 55%, respectively) with human NPP-5, whereas Smp_084890 shows higher identity to human NPP-6 (45%). Comparing the amino acid sequence of SmNPP-5 with those of humans NPPs [36], we further identify conserved putative sites for metal binding, at positions Asp36, Asp198, His202, Asp246 and His247 (*asterisk*). A probable catalytic center is indicated by an arrow, at position Thr77. High probability N-glycosylation sites were found at Asn52, Asn166 and Asn251 according to the NetNGlyc 1.0 Server.

Phylogenetic analyses of the phosphodiesterase superfamily generated the tree shown in Fig. 2. This first analysis grouped SmNPP-5 in a branch together with NPP-5 and NPP-4 sequences (Fig. 2A). To further classify the SmNPP-5 sequences, we performed a second phylogenetic analysis including only NPP-5 and NPP-4 sequences. This analysis showed that SmNPP-5 branches together with other NPP-5 family members, suggesting its classification as a NPP-5 member (Fig. 2B).

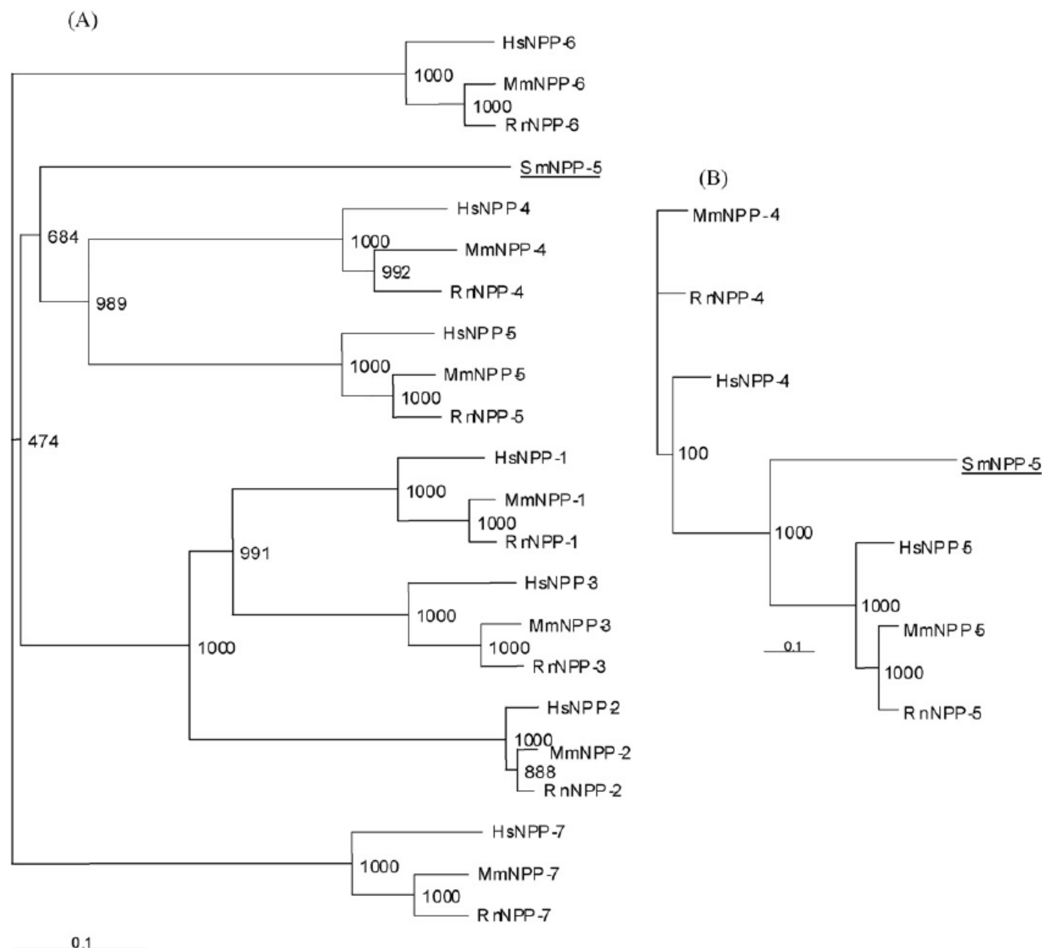

Fig. 2. Phylogenetic analysis of *Schistosoma mansoni*, *Homo sapiens*, *Mus musculus* and *Rat norvergicus* NPP family. (A) Phylogenetic tree of all seven human, mouse and rat NPP and the schistosome NPP, demonstrating that SmNPP-5 branches with NPP-5s and NPP-4s. (B) Phylogenetic tree of SmNPP-5 and NPP-5s and NPP-4s, resolving SmNPP-5 and NPP-5s in the same branch. The sequences accession numbers are HsNPP-1 (NP_006199.1), HsNPP-2 (NP_006200.3), HsNPP-3 (NP_005012.2), HsNPP-4 (NP_055751.1), HsNPP-6 (NP_699174.1), HsNPP-7 (NP_848638.2), MmNPP-1 (NP_032839.3), MmNPP-2 (NP_056559.2), MmNPP-3 (NP_598766.2), MmNPP-4 (NP_950181.2), MmNPP-6 (NP_796278.1), MmNPP-7 (NP_001025462.1), RnNPP-1 (AAL26912.1), RnNPP-2 (NP_476445.2), RnNPP-3 (NP_062243.2), RnNPP-4 (NP_001100362.1), RnNPP-6 (XP_224853.3) and RnNPP-7 (NP_001012484.1) (the accession numbers of the other members are cited in the legend of Fig. 1).

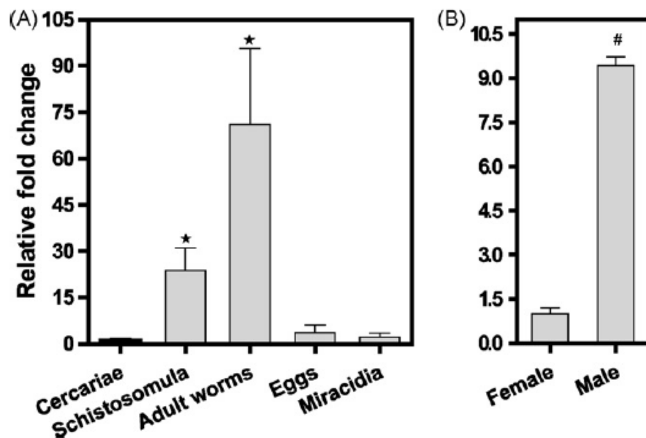

Fig. 3. Differential gene expression of SmNPP-5 (A) throughout five stages of the *S. mansoni* life cycle and (B) between male and female adult worms. Samples were analyzed as to expression of SmNPP-5 by real-time RT-PCR; data were normalised against amplification of an internal housekeeping control gene. The data were calculated according to the comparative ΔΔCt method and are shown as the fold-change in SmNPP-5 expression relative to (A) cercariae or (B) female adult worms. The data are the means ± S.D. of one representative of two independent experiments. *,# Represents statistically significant differences between groups, taking $p \leq 0.05$ or 0.001, respectively.

3.2. mRNA expression analysis by quantitative RT-PCR

Expression of SmNPP-5 was evaluated at the mRNA level in the developmental stages of *S. mansoni* using real-time quantitative RT-PCR analysis. Five stages were examined, including the free-living infective stage (cercaria), *in vitro* transformed and cultured 7-day-old schistosomula, adult worms, eggs and miracidia. Fold-changes in SmNPP-5 expression levels were calculated relative to the cercarial stage after normalisation to actin as the housekeeping gene. The results show that SmNPP-5 mRNA levels were significantly increased by 24-fold in the schistosomula and by 71-fold in the adult worms, as compared to cercariae (Fig. 3A) (Tukey pairwise; $p < 0.05$). These results demonstrate that SmNPP-5 transcription is upregulated following the transition from free-living cercaria to parasitic schistosomulum stage, and although not statistically significant, from schistosomulum to the adult worm stage. Furthermore, in eggs and miracidia, transcription of SmNPP-5 is at minimal levels, comparable to those found in the cercariae. We also evaluated the mRNA level in male and female adult worms and verified that SmNPP-5 is differentially expressed in males, being increased by 9.5-fold as compared to females (Fig. 3B) (*t*-Student; $p < 0.001$).

3.3. Production of the SmNPP-5 recombinant protein and generation of specific antibodies

The gene was cloned into pET28a expression vector, and the protein was expressed in *E. coli* BL21 (DE3) strain upon induction with IPTG. Protein extracts of transformed bacteria showed a band at ~49 kDa (Fig. 4A). When the bacteria were lysed by a French press and the lysate separated into soluble and insoluble fractions, the inclusion bodies were shown to contain the majority of the recombinant protein, which was mostly solubilized by extraction with 6 and 8 M guanidine (Fig. 4A). The protein extracted with 8 M guanidine chloride was purified under denaturing conditions by affinity chromatography on nickel-charged columns through an imidazole linear gradient from 20 to 500 mM (Fig. 4B lanes 3–8). The main contaminant present in the sample was eliminated in the flow through (Fig. 4B lane 2). Eluted fractions were pooled and submitted to refolding by dialysis, which resulted in substantial protein precipitation but not in protein degradation (data not shown). Protein yield after dialysis was estimated to be around 6.0 mg of rSmNPP-5/L of culture and this sample was used to generate polyclonal antibodies in mice.

3.4. Analysis of protein expression across the life cycle stages

Samples prepared from cercariae, schistosomula, adult worms, eggs and miracidia stages of *S. mansoni*, and tegument isolated by the freeze/thaw method, were all separated by SDS-PAGE. Immunoblotting was performed using mouse anti-rSmNPP-5 antiserum. The profile of SmNPP-5 expression throughout the life cycle revealed no protein in cercariae, eggs and miracidia, some in schistosomula, and the highest level in the adult worms. Furthermore, male worms had higher amounts in their total protein extract than females. Separation of the tegument from the worm body revealed that the protein was mostly concentrated in the former, with stripped worms showing expression levels comparable to those observed in schistosomula (Fig. 5A). Analysis of the tegument surface membrane fraction, after differential extraction, revealed that the majority of the protein remained attached, while the soluble supernatant (soluble syncytial proteins) showed a very low signal (Fig. 5B).

3.5. Characterization of SmNPP-5 as a glycosylated protein

Native SmNPP-5 observed in schistosome extracts migrates with a higher molecular mass (~66 kDa) than that predicted (~51 kDa), which could reflect a likely product of post-translational glyco-

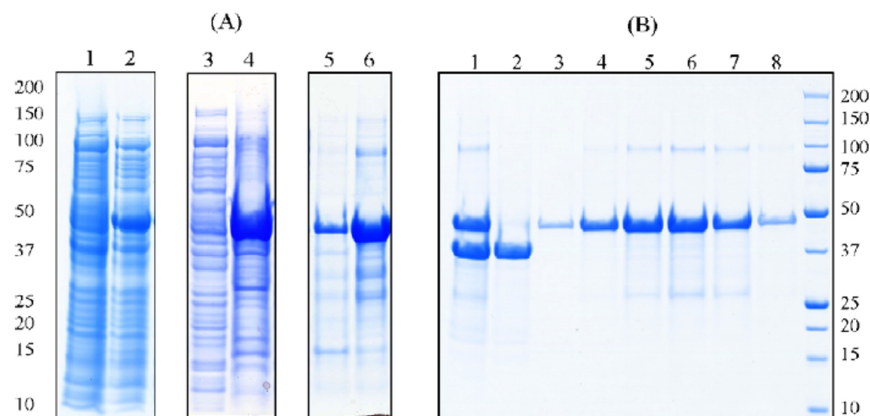

Fig. 4. SDS-PAGE (4–12%) analysis of cell extracts and fractions from *E. coli* (BL21DE3) transformed with the pET28-SmNPP-5. (A) Lanes 1 and 2, total extract of a clone before and after induction with 1 mM IPTG. Lanes 3 and 4, supernatant and inclusion bodies after lysis, respectively. Lanes 5 and 6, solubilization of inclusion bodies with 8 M urea and 6 M guanidine, respectively. (B) The protein in inclusion bodies was further extracted with 8 M guanidine (lane 1) and purified through Ni^{2+}-charged column chromatography; lane 2, flow-through; lanes 3–8, fractions of rSmNPP-5 eluted by linear gradient of imidazole (20–500 mM).

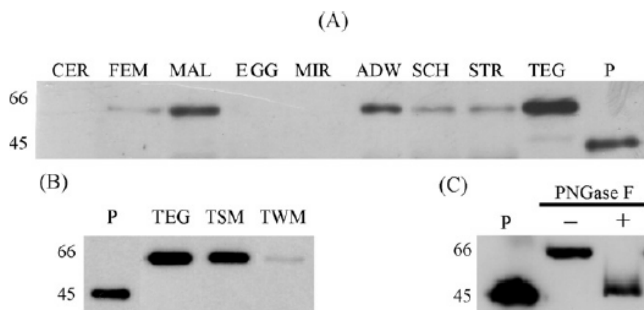

Fig. 5. Immunoblotting of protein extracts from *S. mansoni* stages and tegument fractions using anti-rSmNPP-5 polyclonal antibodies. (A) CER, cercariae; FEM, female adult worms; MAL, male adult worms; EGG, eggs; MIR, miracidia; ADW, adult worms (female and male); SCH, 7-day-old schistosomula; STR, stripped worms; TEG, tegument; (20 μg of protein was loaded in each lane); P, positive control rSmNPP-5 (100 ng). (B) P, positive control rSmNPP-5; TEG, tegument; TSM, enriched tegument surface membranes; TWM, tegument extract without surface membranes. (C) Tegument extract before treatment with PNGase F (−) and after digestion with the enzyme (+) and positive control rSmNPP-5 (P). Positions of molecular mass standards (kDa) are indicated on the left.

sylation. A very faint band with the predicted molecular weight (∼51 kDa) was observed in the tegument extract, which could represent a fraction of non-glycosylated protein (Fig. 5A). To test the hypothesis that the higher molecular mass observed for SmNPP-5 on SDS-PAGE is due to glycosylation, we digested total tegument extracts with a recombinant N-glycosidase F. The immunoblot showed that, after digestion, SmNPP-5 migrated to a MW slightly higher than the rSmNPP protein, demonstrating that native SmNPP-5 is N-glycosylated (Fig. 5C).

3.6. Immunolocalization of SmNNP-5 in adult worms

To confirm the tegument localization of SmNPP-5, immunofluorescence staining was performed on adult worm cryosections with the same antibody used in the Western blot analysis. Results showed SmNPP-5 to be mainly distributed over the tegument of male adult worms and at lower levels in the internal tissues of the parasite body (Fig. 6A, B, and E); no specific staining was observed in sections incubated with naive mouse serum (Fig. 6C and D). Additionally, Fig. 6E demonstrated that the red band running around the parasite body showed the thickness expected of the tegument syncytium (1–3 μm) [37].

3.7. NPP activity from living worms

To further characterize the surface exposed nature of SmNPP-5 protein we tested the ability of live worms (cercariae, 21-day old and adult worms) to promote the ρ-Nph-5′-TMP hydrolysis. Corroborating the RT-PCR and imunnoblotting results, enzymatic activity was detected only in the 21-day old and adult worms incubations; no activity was observed in the cercarial sample (Fig. 7A). Statistically significant differences between the three stages could be found. Although we have tried to normalise for surface area based on previous studies, the total amount of activity detected in the investigated stages can only be considered as a rough estimate. Next, we investigated if pre-incubation of live adult and 21-day-old worms with anti-rSmNPP-5 antibodies impaired the subsequent enzyme activity. As shown in Fig. 7B and C, the antibody treatment reduces the detected enzyme activity in 60 and 50%, respectively. The recombinant protein showed no enzyme activity (data not shown).

To check the substrate specificity together with cercarial viability, we performed a parallel experiment in which live cercariae were incubated in the presence of the alkaline phosphatase substrate (ρ-nitrophenyl phosphate disodium salt). Our result confirmed the cercarial viability, as judged by a significant detection of alkaline phosphatase activity associated with the infective larvae (data not shown).

4. Discussion

In the present report, we describe the molecular cloning and characterization of SmNPP-5, as a member of the nucleotide pyrophosphatases/phosphodiesterases family from *S. mansoni*. The cDNA coding for the SmNPP-5 gene was sequenced and analyzed by bioinformatic techniques. It displays an N-terminal signal peptide, three N-glycosylation sites and a hydrophobic transmembrane region. When we treated the native proteins from the tegument extracts with N-glycosidase F, we observed a mobility shift of SmNPP-5 on SDS–PAGE, revealed by Western blotting, confirming the N-glycosylation predicted by bioinformatics. This indicates that SmNPP-5, similar to its ortholog from rat [25], is a glycoprotein. These results agree with data from Pujol and Cesari, who isolated a surface membrane-bound complex from adult worms using lectin-coated beads, implying N-glicosylated components, which presented phosphodiesterase activity [38]. It is possible that SmNPP-5 is the enzyme responsible for the activity detected in those studies.

The results of RT-PCR analysis showed that the SmNPP-5 transcript was expressed at very low levels in eggs, miracidium and cercariae stages, and was significantly upregulated in the 7-day-old schistosomula and adult worms. Additionally, gender comparison of this last stage revealed male SmNPP-5 mRNA upregulation, confirming a microarray analysis that evaluated global gene gender-associated expression [39]. Since the correlation between transcript abundance and protein abundance is not always proportional, we analyzed the expression of SmNPP-5 at the protein level. Immunoblotting studies confirmed the mRNA data showing an increased expression in the transition from free-living cercaria to parasitic schistosomulum, and from this last stage to adult worms. This increase suggests that SmNPP-5 was upregulated during or shortly after the process of cercarial transformation, a period in which the parasite switches from its immune-sensitive to an immune-refractory state by completely replacing and remodeling the surface membranes of the tegument syncytium [40]. Searching the literature for differentially expressed NPPs in *S. mansoni*, we found another member of the family (Smp_104270, EST Sm03904) being identified as a preferentially expressed gene in the lung schistosomulum through microarray analysis [41], SmNPP-5 could not be identified in Dillon's study, as it was not spotted on the array. These findings reinforce the need for better investigation of the role these enzymes may play in Schistosome's physiology.

The immunoblotting result revealed male worms expressing higher amounts of SmNPP-5 as compared to females. This finding was expected due to the large tegument surface area exhibited by the male parasite, moreover it could reflect higher male exposure in the blood stream since the female resides largely in the gynaecophoric canal [42]. Furthermore, we analyzed tegument extracts, important from a vaccine perspective, because this structure is a dynamic host-interactive layer involved in nutrition, immune evasion and modulation, excretion, osmoregulation, sensory reception and signal transduction [43]. It was observed that SmNPP-5 was mostly located in the tegument, and at lower levels in the stripped worms; this complements the data obtained by van Balkom et al. [20], which characterized the protein as tegument-specific. Considering the differences in sensitivity of the two methods (mass spectrometry and Western blot) and that all tegument proteins must originate in the cell bodies where the synthetic machinery resides [6], we judged it more appropriate to describe the protein as a highly abundant tegument protein. To further characterize

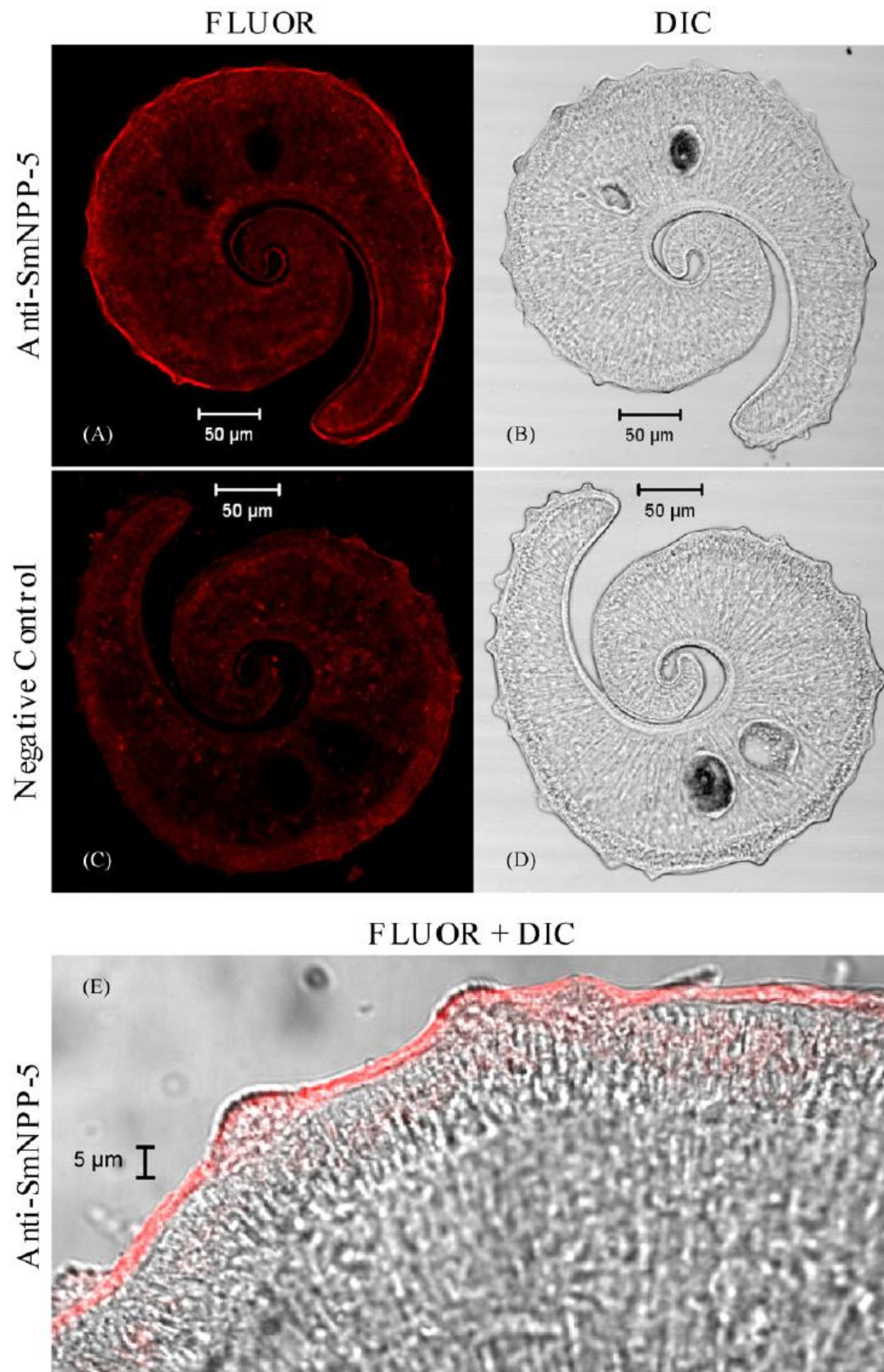

Fig. 6. Fluorescence confocal microscopy images (Fluor) with corresponding differential interface contrast (DIC) images to show immunolocalization of SmNPP-5 in transverse sections of *S. mansoni* adult worms. (A) For fluorescence detection of SmNPP-5 or (C) negative control, polyclonal anti-rSmNPP-5 antibodies or serum from naïve mice, respectively, were used with secondary antibody coupled to Alexa 647, using 20× magnification; (B and D) DIC images. (E) Detail of the merged Fluor image with corresponding DIC from panel (A) for fluorescence detection of SmNPP-5, using 63× magnification.

the SmNPP-5 distribution across the tegument we compared its presence in the tegument surface membranes with the tegument cytoplasmatic content. SmNPP-5 was mainly detected in the surface membranes and at low levels in the remaining tegument, confirming the data obtained by Braschi [18,19], which allows its characterization as a possible type I transmembrane protein.

Finally, we immunolocalized SmNPP-5 in the adult worm tegument, and it seems to be present at lower levels in internal tissues. We have detected NPP activity in living adult worms and in 21-day-old worms, but not in cercariae, which is in agreement with the results of mRNA and protein expression profiles. These data suggest that SmNPP-5 is closely associated with the generation of the new tegument surface after cercarial penetration, and hence could be involved in parasite adaptation for immune evasion and survival in the bloodstream. The enzymatic activity detected in live parasites strengthens the hypothesis that SmNPP-5 is very accessible at the tegument surface. This data agrees with the predicted site for this protein on the external leaflet of the plasma membrane, right below the membranocalyx [18]; however, such location would imply the existence of water-filled "pores" in the membranocalyx to allow the entrance of substrates, such as the ρ-Nph-5′-TMP used for the enzymatic activity assay. Whether these pores would be large

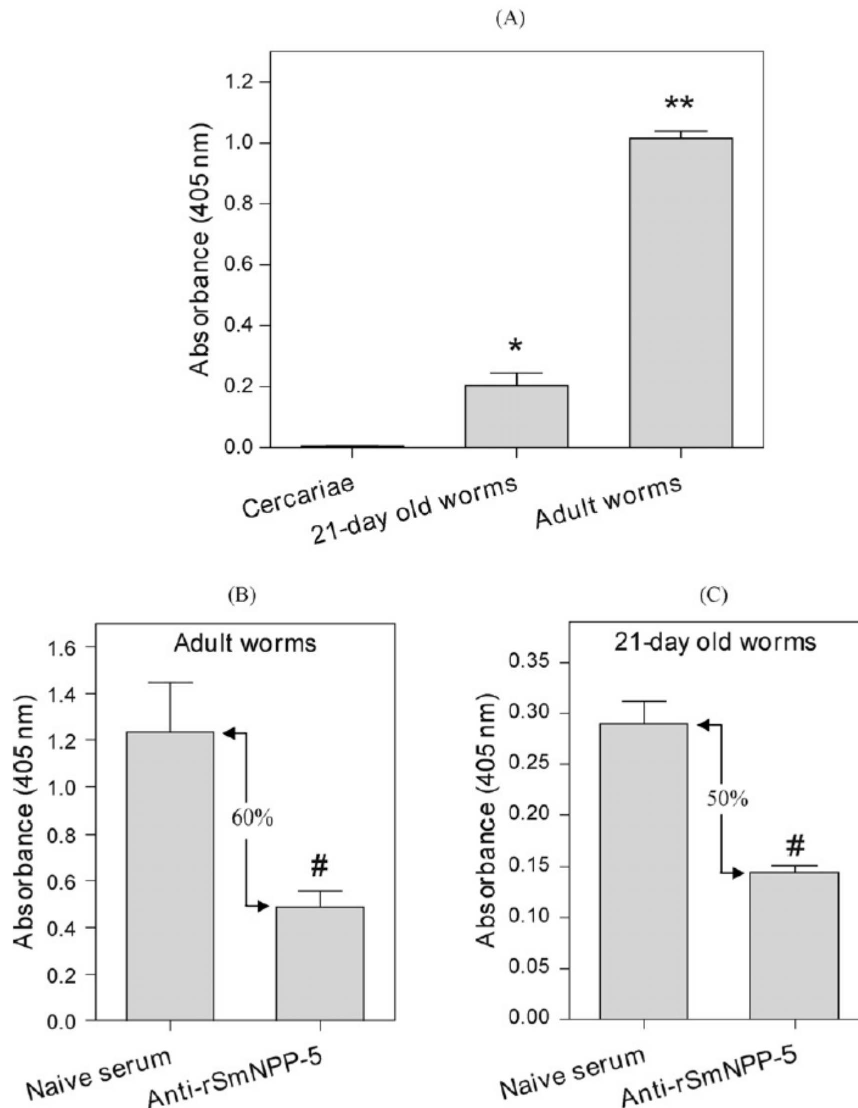

Fig. 7. (A) Surface NPP activity from living parasites. (B and C) Surface NPP activity inhibition by pre-incubation of living adult worms or 21-day-old worms with anti-rSmNPP-5 antibodies. ρ-Nph-5′-TMP hydrolysis in the presence of living worms was measured at 405 nm. Bars represent means ± S.D. of one representative of two independent experiments. *,# Represents statistically significant differences between groups, taking $p \leq 0.001$, and # taking $p \leq 0.004$.

enough to give antibodies (molecules of ∼150 kDa) access to the protein domains situated immediately external to the plasma membrane, has been a subject of debate in the literature [17–19]. We have tried to address this question using anti-rSmNPP-5 antibodies to block the enzymatic activity on the schistosome tegument. Our results showing a significant inhibition of 50–60% of the phosphodiesterase activity, suggests that the anti-rSmNPP-5 antibodies have access to the site of enzyme location. However, we cannot ascertain the mechanism of inhibition, whether by competition for the catalytic center of the enzyme or induction of conformational changes. It has been described that patients infected with *S. mansoni* present circulating antibodies capable of partially inhibiting phosphodiesterase activity from total adult worm extracts [44,45]; however, since no attempt was performed to isolate the tegument surface membranes, it is difficult to compare this study with data provided here. Additionally, the identification of several phosphodiesterase isoforms in the schistosome genome suggests that the parasite possess different enzymes of this class, whose localization and stage expression profiles remains to be established.

The localization and accessibility of the SmNPP-5 protein to the immune system reinforces the potential of this protein as a vaccine candidate. An extension of this approach could be a combined attack against enzymes involved in nucleotide metabolism, since three have been described on the tegument surface, alkaline phosphatase, diphosphohydrolase and phosphodiesterase [17–19]. These three proteins are likely to be located on the external leaflet of the plasma membrane, so the use of a cocktail of these antigens in vaccine experiments may elicit a synergistic effect. The presence of circulating antibodies against two of these enzymes (phosphodiesterase and alkaline phosphatase) in infected patients [44,45], and the fact that the parasite seems to be unaffected by these antibodies, would, at first sight indicate that it may be non-effective to induce an immune response against these antigens. On the other hand, it is important to note the occurrence of individuals, in endemic areas naturally resistant to infection. It has been described that these individuals develop antibody responses against some protective schistosome antigens (e.g. TSP-2 and Sm29); however, the levels and isotype profile are different to those observed in infected individuals, displaying a higher proportion of antibodies involved in opsonization and complement system activation [46–48]. Therefore, the key question seems to be the levels and isotype profile of human antibod-

ies against schistosoma surface antigens, and not its presence or absence.

A nucleotidase vaccine cocktail could impair worm survival by two potential mechanisms. One would be through the action of opsonizing antibodies that could mediate complement deposition and parasite death; and the other would be based on the blockade of these enzymes, which could be playing a role in immune evasion at the parasite–host interface. Experiments are under way to address these questions.

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