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Specific humoral response of hosts with variable schistosomiasis susceptibility

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Title

Specific humoral response of hosts with variable schistosomiasis susceptibility

Running Title

Humoral response and schistosomiasis susceptibility

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Abstract

The schistosome blood flukes are one of the largest global causes of parasitic morbidity. Further study of the specific antibody response during schistosomiasis may yield the vaccines and diagnostics needed to combat this disease. Therefore, for the purposes of antigen discovery, sera and antibody secreting cell (ASC) probes from semi-permissive rats and sera from susceptible mice were used to screen a schistosome protein microarray. Following *Schistosoma japonicum* infection, rats had reduced pathology, increased antibody responses and broader antigen recognition profiles compared with mice. With successive infections, rat global serological reactivity and the number of recognized antigens increased. The local antibody response in rat skin and lung, measured with ASC probes, increased after parasite migration and contributed antigen-specific antibodies to the multivalent serological response. In addition, the temporal variation of anti-parasite serum antibodies after infection and reinfection followed patterns that appear related to the antigen driving the response. Among the 29 antigens differentially recognized by the infected hosts, were numerous known vaccine candidates, drug targets, and several *S. japonicum* homologues of human schistosomiasis resistance markers - the tegument allergen-like (TAL) proteins. From this set we prioritized eight proteins that may prove to be novel schistosome vaccine and diagnostic antigens.

Introduction

Schistosomiasis, a neglected tropical parasitic disease, affects over 250 million people globally.¹ Despite extensive chemotherapy programs, endemic populations remain at high risk of reinfection and eradication of the trematode worms require a combined approach incorporating public health measures, continued drug treatment and an effective vaccine.²⁻⁴ In addition, current serological and parasitological methods of diagnosis are insensitive and/or labor intensive and new diagnostic antibody tests are required.⁴⁻⁶ In contrast to the African forms, schistosomiasis japonica, caused by *Schistosoma japonicum*, is a true zoonosis, infecting a broad range of mammalian hosts; 46 species in total.⁷ Notably, immunity and infection severity varies considerably among these hosts allowing for comparison between susceptible and resistance traits. Of the laboratory animals, the mouse is fully permissive, passing viable schistosome eggs, whereas the rat (*Rattus norvegicus*) is semi-permissive to both *S. japonicum*^{7,8} and *S. mansoni*⁹⁻¹¹ infections. The resistant characteristics of the laboratory rat have been used for several decades in vaccine research^{2,10} and resulted in the identification of the only schistosome vaccine antigen, Sh28GST, currently in phase III trials.^{4,12,13} Schistosomiasis-resistance increases with subsequent infections and is immunological in nature,

primarily due to antibody-dependent cell-mediated cytotoxicity (ADCC).^{8,9,11,14} The migration path of the schistosome parasite from skin to lung is critical for both immune priming and parasite elimination.^{9,15,16} Following infection with *S. japonicum* or *S. mansoni* cercariae the rat eliminates the parasites over the course of a few weeks, in the lungs and the liver; with the majority of the schistosomules never progressing past the lung.¹⁶⁻¹⁸ The few worms found in the liver are stunted, produce few fertile eggs and these worms are eventually eliminated by a self-cure phenomenon.^{7,9,14,18} Upon reinfection the challenge parasites are rapidly eliminated at or prior to the lung stage.^{9,14,19,20} Four to six weeks after a primary infection schistosome-specific immunoglobulin is produced that, together with complement, eosinophils, macrophages, platelets and mast cells, are able to form complexes and attack schistosomules;^{9,21} blocking of this antibody response reduces the resistance to reinfection.^{11,15,21} The predominantly humoral immunity of schistosomiasis resistance in the rat, compared to the susceptible mouse, makes this animal model ideal for antigen discovery. The specificity of the protective antibodies used by the rat are, however, largely unknown.

As opposed to conventional serological screening approaches - such as probing of display libraries or parasite extracts - parallel screening with protein microarray technology,^{22,23} particularly when combined with resistance models in experimental animal hosts, may result in the discovery of more parasite-specific antigens. Ultimately these novel antigens may prove to include new and effective vaccine and diagnostic candidates. An additional consideration is that experimental infections have controlled infection parameters and thus avoid confounding variables found in endemic human field studies, such as incomplete clinical and drug treatment histories.²⁴

In this study we describe the use of a schistosome-specific protein microarray we have developed^{23,25} to: 1) compare the specific antibody response between resistant and susceptible animal hosts; 2) examine the longitudinal serum antibody response in the resistant rat host during successive infections; 3) define the tissue-specific local antibody response using antibody secreting cell (ASC) probes;^{26,27} and 4) discover new potential vaccine and diagnostic antigens. To achieve these objectives time courses of rats and mice experimentally infected with *S. japonicum* were completed to collect antibody samples for microarray probing and to characterize the parasitological resistance of these two animal models, particularly the striking resistance to reinfection intrinsic to the rat. In addition, antibodies generated from rat skin- and lung-draining lymph nodes were used to identify antigens specifically recognized at these important immunological sites of infection and parasite elimination.

Rats have reduced pathology and less viable parasites compared to mice

Two separate time courses with rats and experimental infections with mice were completed. The first rat time course (TC1) measured serum responses after two successive infections with treatment groups having intervals of four (T₄), six (T₆) and eight (T₈) weeks between infections. The second rat time course (TC2) measured the serum and local tissue responses following a primary infection (Group I), and five (Group I₅) and nine days (Group I₉) after a secondary challenge infection. Amongst the groups within TC1 and TC2 there was high variability for liver egg burdens (Supplementary Table 1) but worm numbers and worm lengths were similar. As expected the semi-permissive rat host had few adult worms, the length of the perfused worms was reduced and few eggs were present in the liver (Supplementary Table 1). Indeed, less than 1% of the total cercarial challenge load was perfused as adult worms from the rat. Liver histology further highlighted the reduced pathology in the rat (Supplementary Figure 1A). Infected livers had low pathology and this was reflected in a low granuloma density ($3\% \pm 0.6\%$; average $\pm$ SEM for all rat groups) despite the high cercarial challenge and the long duration of the infection. In addition, few of the eggs were viable; miracidial hatch testing resulted in very few hatched miracidia (1.5 miracidia hatched per 1000 liver eggs for Group T₄; data not shown). Notably, sections of rat lung contained schistosome larval stages nine days after a secondary cercarial challenge (Supplementary Figure 1C). It could not be determined if these parasites were under immune attack but the lung is recognized as an important site for the elimination of schistosomes.^{9,17,19,28}

To provide a standardized model to compare infection in the rat a CBA mouse infection, commonly used in schistosomiasis pathology studies,²⁹⁻³¹ was also completed alongside the Swiss mouse strain used for serological analysis. Both mouse strains had similar burdens of infection (data not shown) but the CBA infection was used for detailed parasitological and histological comparisons. The permissive mouse had a higher parasite burden and pathology than the rat. Six weeks post-infection, 47% of cercariae were perfused as adults and the male and female worms were approximately two- and three-fold longer, respectively, than those from the rat (Supplementary Table 1). Liver pathology was also higher in the mouse infection (Supplementary Figure 1B). Granuloma density was four times higher in infected mouse livers ($14.7\% \pm 2.1\%$) compared with infected rat liver. Additionally, egg viability was much higher (43.7 miracidia hatched per 1000 liver eggs). Despite some differences in the experimental infections used for the rat and mouse models (e.g. number of

infections and cercariae per infection) these data clearly highlight the variation of susceptibility of the two rodent hosts to schistosome infection.

Protein microarray reactivity varied between hosts and antibody source

Protein microarray production was characterized by high cloning and protein expression efficiencies and quality control (QC) measures.^{25,32} For the primary purpose of vaccine and diagnostic antigen discovery, the 289 feature schistosome protein microarray (Supplementary Table 2) was probed with 72 sera, ASC probes and bronchoalveolar lavage (BAL) fluid samples from the rat experimental infections. The pre-infection time point samples were compared with primary and secondary infection time points to identify differentially reactive proteins. In the case of the ASC probes, the skin and lung samples were compared with pooled BAL fluid and liver and spleen and uninfected ASC probes to identify antigens recognized in the skin- and lung-draining lymph nodes. A time course was completed for nine individual Swiss mice (MTC). Pre-infection and post-infection mouse serum samples (18 in total) were collected as a susceptible host comparison for the semi-permissive rat samples. The raw protein microarray signal intensity (SI) (Supplementary Table 3) for all 90 biological samples was normalized (Supplementary Table 4) and heatmaps created for: (1) the individual samples for all the protein microarray features (Figure 1); (2) group averaged SI for the most reactive antigens in TC1 and TC2 samples (Figure 2); and (3) individual SI for the most reactive antigens in the MTC and ASC probe samples (Figure 3).

Within all the probed and normalization microarrays, the “No DNA” negative controls had low overall reactivity and were, on average, 2-3 times lower than the remaining protein microarray features, and at least 10-fold less than the IgG positive control (Figures 1-3). Uninfected serum reactive against the schistosome protein microarray was weak; however, reactivity increased with parasite challenge (Figures 1A-C and Supplementary Figure 2), and this was also confirmed by anti-adult parasite extract (SWAP) antibodies measured by ELISA (data not shown). In both rat time courses there was no significant difference (ANOVA, $P > 0.05$) in global average protein microarray SI between groups within the uninfected and infected timepoints. Accordingly, to show the change in average antigen recognition between the uninfected and infected timepoints, the groups were pooled; all other analyses used unpooled data. For TC1 the average global protein microarray SI was: 1010 ± 21 (average $\pm$ SEM) for uninfected rat sera; 1632 ± 119 after the primary infection, a significant increase (Tukey HSD, $P < 0.001$); and, 2025 ± 113 after the secondary challenge infection (Tukey HSD, $P < 0.001$) (Figure 1B). The average number of highly reactive antigens, i.e. features with SI $> 50\%$ of the most reactive IgG positive control, was 2.8 ± 1.2 , $8.7 \pm$

1.1 and 26.6 ± 5.9 for the pre-, primary and secondary infection groups, respectively. TC2 (Figure 1) had a similar pattern but with a lower overall SI, reflecting the raw SI (Supplementary Table 2) and shorter duration of this experiment. Uninfected samples had an average SI of 658 ± 13 compared with 1275 ± 41 for infected sample groups (Figure 1C; *t*-test, $P < 0.0001$). The average number of highly reactive antigens was 1.5 ± 0.2 with pre-infection sera, and 9.2 ± 1.9 , 7.4 ± 1.7 and 11.2 ± 1.8 for I, I₅ and I₉ infection sera, respectively.

To investigate the local tissue antibody response to schistosome antigens in the important sites of parasite elimination, the skin and lung, protein microarrays were screened with ASC probes and BAL fluid. Infected host skin and lung ASC probes were compared with control samples that included: other tissue antibodies (infected host liver and spleen ASC probes); the background lung response (infected host BAL fluid); and uninfected samples (uninfected host lung ASC probes) (Figures 1 and 3). The lower antibody titer ASC probes, as expected, had weaker SI compared to serum. The average SI for skin ASC probes was 490 ± 46 compared with 951 ± 121 for lung ASC probes and 550 ± 54 for the ASC control samples; the average number of highly reactive antigens was 1.4 ± 0.3 , 5.8 ± 1.2 and 4 ± 2.4 , respectively. Mouse sera following a 6 week schistosome infection were weakly reactive with nearly identical SI; 1068 ± 98 and 1064 ± 70 , and averaged highly reactive antigens, 1.1 ± 0.1 and 3.2 ± 0.9 , for the pre- and post-infection time points (Figures 1 and 3, and Supplementary Figure 2).

Antigens recognized by antibodies from infected hosts

The top 40 rapid translation system (RTS) cell-free expressed recombinant protein antigens for both TC1 and TC2 (Figure 2), in order of the average SI for all the infection timepoints, included four *S. mansoni* and 36 *S. japonicum* proteins. Compared with rat samples, the SI for RTS antigens was much lower for the ASC probes and mouse samples and only the top 20 antigens are displayed in the heatmap (Figure 3); 18 *S. japonicum* and two *S. mansoni* RTS proteins were in the top 20. The top five TC1 and TC2 (Figure 2) and top ten ASC and MTC (Figure 3) *in vivo* expressed recombinant proteins and controls included dilutions of *S. japonicum* SjTSP2 (ABQ44513) expressed in yeast and bacteria, Sj phosphatase (AAP06500) and Sj silencer (AAP06461). These proteins were strongly recognized by all post-infection samples although there was reactivity in some pre-infection samples for Sj silencer (Figures 3 and 4). The *in vivo* *S. mansoni* expressed recombinants were less reactive but Sm annexin 1 (AAC79802) and Sm29 (AAC98911) were recognized by rodent sera following a primary or secondary infection. Sj silencer and Sj phosphatase were the only *in vivo* expressed recombinants proteins (Supplementary Table 5) that

164 strongly correlated with their RTS equivalents (Pearson's $r = 0.24$ and 0.89 , $p = 0.025$ and $P <$
 165 0.0001 ; $n = 90$).

166 The top 25 RTS antigens for TC1 (Figure 4A) and TC2 (Figure 4B) were ranked using average SI
 167 and Benjamini-Hochberg adjusted (BH) P -values. Purely for graphing purposes, groups were
 168 pooled within timepoints for TC1 (Figure 4A). MTC (Figure 4C) and ASC probed (Figure 4D)
 169 microarrays were less reactive and therefore only 10 and 11 features, respectively, were selected,
 170 including *in vivo* recombinant proteins. In fact, only three RTS proteins were present within the
 171 reactive mouse set. There was considerable overlap between the samples with 38 proteins
 172 representing all the detected RTS antigens. Putative vaccine and diagnostic antigens were identified
 173 from the 38 detected RTS proteins by first excluding antigens if there was: less than 20% frequency
 174 of recognition within a sample group (8 proteins; Figure 4); or stronger recognition in pre-infection
 175 than post-infection sera (only Smp_194920, 46CF). The resulting 29 proteins were then categorized
 176 as putative novel vaccine and diagnostic candidate antigens (Table 1) or as associated with rodent
 177 schistosomiasis infection (Supplementary Tables 6 and 7). Vaccine antigens were selected by
 178 including antigens with high selection parameters (see Methods) that were not recognized by the
 179 mouse serum or had previously been tested for protective efficacy.

180
 181 The proteins associated with rodent infection included 17 *S. japonicum* (Supplementary Table 6)
 182 and four *S. mansoni* (Supplementary Table 7) antigens. The *S. japonicum* proteins included eight
 183 known tegument proteins, six had biological process ontologies (*microtubule-based* was the most
 184 common), and were most commonly found in the adult worm stage (8/17).³³ The *S. mansoni*
 185 proteins had transport and metabolic biological process ontologies and were mainly recognized
 186 (4/4) by sera from the primary to early secondary infection (experiment TC2). The most prominent
 187 antigen recognized by all the samples was a hypothetical protein (AY815838). This antigen
 188 generally had the highest SI of all the antigens (pre-infection 2843 ± 132 ; post-infection: $29562 \pm$
 189 2770), and was often higher than the positive controls or *in vivo* recombinant proteins. The AUC
 190 values of the ROC plots for this antigen were a perfect 1.0 for all the rat sera (Figure 5), 0.8 for the
 191 ASC probes and 0.88 for the mouse sera (Table 1), and were equivalent or higher than *in vivo*
 192 recombinant SjTSP2, the next best antigen. Accordingly, AY815838 was chosen for future testing
 193 as a potential diagnostic antigen (SjDiag).

194 ***Vaccine and therapeutic candidates recognized by rat serum and tissue antibodies***

195 Strikingly, 11 previously trialed vaccine antigens were amongst the proteins associated with rodent
 196 infection. In particular, antibodies to tegument antigens with dynein light chains, Sj21.7

(AF048759),^{34,35} Sj22.6 (L08198),^{36,37} Sj dynein light chain 1 (AF072327),^{38,39} Sm22.6 (Smp_045200),^{40,41} and the related SjTAL12 (AY815113, similar to Sm22.6; 84% aa identity with TAL12⁴²) were all present in infection rat sera and ASC probes. Other trialed vaccine candidates recognized exclusively by the rat included: SjGST-26 (M14654),⁴³ Sj serpin B6 (AF380366),^{44,45} Sjp40 major egg protein (AY814158),^{46,47} Sm filamin (Smp_000100),^{48,49} and the RTS expressed Sj phosphatase (AY223465)³ (Zhang, Li and McManus, QIMRB, unpublished data). However, the tetraspanin Sj23 (M63706),^{50,51} a known vaccine candidate, was also recognized by mouse infection sera. The remaining *S. japonicum* and *S. mansoni* antigens included hypothetical proteins, uncharacterized proteins and a potential therapeutic target (Smp_124240; Na/K transporting ATPase).^{32,52} Antigens reactive in skin and lung ASC samples were also recognized by serum samples; for example SjTSP2, SjDiag, Sj21.7 and a calponin-like protein (AY813467), closely related to the *S. japonicum* vaccine antigen,⁵³ among others (Figures 4C and 4D). These findings suggest that antibodies against these antigens are generated by cognate B-cells in tissue-specific draining lymph nodes following parasite migration through the skin and lung.²⁶ Notably, some of the antigens recognized by skin and lung ASC probes in our preliminary study²⁷ were absent, reflecting the different samples used and the more stringent analyses applied in the current study.

Putative vaccine and diagnostic candidate antigens

In total, seven putative vaccine antigens and one diagnostic antigen were chosen for future testing (Table 1): three are specific to the schistosomes (tBLASTn expected value [E] < 10⁻²⁰); half have transmembrane domains (4/8); and most have annotations or biological ontologies (6/8). Although four of the targeted antigens have not been detected in a previous shotgun proteomic study the remainder are found in the adult and/or schistosomule lifecycle stages³³. The *S. mansoni* thioredoxin peroxidase 1 (SmTPx1, Smp_059480),⁵⁴ was highly reactive with TC1 sera, and was also chosen as a putative vaccine antigen. This antigen, or potentially the *S. japonicum* homologue (tryparedoxin peroxidase, CAX71944), was included as a *S. japonicum* vaccine candidate because it had high protein microarray reactivity, is found in lung schistosomule excretory-secretory products, and is a protective *S. mansoni* vaccine.^{55,56}

Variation in antigen recognition over repeated infections in rat

The two time courses, TC1 and TC2, with their overlapping data points allowed the examination of antigen recognition (Figures 5 and 6) from pre-infection to post-primary infection (4 and 6 weeks post-primary infection), to early post-secondary infection (6 weeks 5 days and 7 weeks 2 days post-primary infection) and, finally, to late post-secondary infection (10, 12 and 14 weeks post-primary

infection). It must be noted that because of the different experimental conditions for each time course, comparisons could not be made of normalized SI between time courses; however, significant differences between pre-infection and post-infection timepoints within each time course were informative. The majority of reactive antigens belonged to three distinct profiles: consistent antigens, primary antigens and late secondary antigens.

Antigens, such as those in the EF-hand and dynein light chain family (Sj22.6, Sj21.7, Sm22.6, and similar to Sm22.6/TAL12 protein) were recognized throughout the primary and secondary infections (Figure 6A) and were characterized as consistently reactive type antigens. These consistent antigens have low *P*-values and high AUC values, high SI fold change and high frequency of recognition values for most of the infection sera and ASC probes and also included the diagnostic antigen SjDiag and the *in vivo* recombinant proteins, SjTSP2 and SmTSP3. The second reactivity profile, primary antigens, are represented by SmTPx1, heat shock protein 86 (SjHSP86, AY815164) and a hypothetical protein with thrombospondin type 1 domain (SjTHBS1, AY809526) (Figure 6B). These primary infection antigens were strongly recognized following a primary infection. In particular, recognition of SjHSP86 was highly significant 4 weeks post-primary infection ($P < 0.001$) and it had an AUC value of 0.86 with the primary infection sera (Figure 5A) but was not reactive at other time points. SmTPx1 was also strongly recognized at late timepoints but not during the early secondary infection timepoints; hence its inclusion as a primary antigen.

Late secondary antigens were recognized only 6 weeks after the secondary infection and increased with time. These proteins included Sj serpin B6 (AF380366), Sjp40 major egg protein (AY814158) and the discovered putative vaccine antigens, SjCutA (AY222951; similar to divalent cation tolerance protein) (Figure 6C), AY809178 (hypothetical guanylate kinase associated protein), Sj200 (AY810129), AY815332 (coatamer subunit beta related) and Sj annexin 4 (AY223469). Notably, the late secondary antigen and a proposed diagnostic antigen,⁴⁷ Sjp40, was an excellent indicator of late infection in this study (Figure 5B) and its recognition increased 24-27 days after infection coinciding with the onset of egg-laying.⁵⁷ Furthermore, this antigen is highly abundant in the egg; the *S. mansoni* homologue, Smp40, is the third most abundant protein released from the egg.⁵⁸ Surprisingly, recognition of the egg antigen Sjp40 at the termination of TC1 and TC2 did not correlate with liver egg or granuloma burden (TC1: Pearson's $r = -0.21$ and -0.15 , $p = 0.54$ and 0.66 , respectively, $n = 11$; TC2: Pearson's $r = 0.25$ and 0.451 , $p = 0.361$ and 0.09 , respectively, $n = 15$). All the putative vaccine candidates, with the exception of the primary antigens SjTHBS1 and

SmTPx1, were late secondary antigens (Table 1 and Figure 4). However, pending further studies, it is not clear how these profiles relate to protective immunity or the selection of vaccine antigens.

Discussion

Humoral immunity is believed to be critical for elimination of the schistosome parasite by the host.^{8,9,11,14} However, the specific antigens, that are the target of this immunological attack, are mostly unidentified. Knowledge of these antigens is not just important for the immunobiology of schistosomiasis but specifically could be exploited for the discovery and development of vaccines and diagnostics. The rat has long been used as a model of immunological resistance for both *S. japonicum*^{7,8,18,59} and *S. mansoni*^{10,11,15,60} infection. Consequently, we screened serum and tissue antibodies from the rat using a protein microarray. We have described some of the parasite proteins that are recognized by rat serum and tissue antibodies, examined the antibody profile of the schistosomiasis susceptible mouse, explored the serological recognition of parasite-specific antigens during repeated infections in the rat, and prioritized a subset of detected antigens that may be efficacious as novel vaccines or diagnostics.

In this study, and as previously observed,^{7,8,18,19,61} compared with the permissive mouse, rats had fewer adult worms, the worms present were severely stunted and egg production was drastically reduced. Furthermore, the liver egg burden, liver granuloma density and overall pathology were substantially reduced. In addition, very few viable eggs were retrieved from the liver. More importantly, the antibody profile for rats over the two time course experiments varied significantly. For example, anti-parasite serum antibody levels, determined by ELISA and the protein microarray, significantly increased following the primary and secondary challenge infections in the rat. Moreover, a similar anti-schistosome response is observed in *S. japonicum*-infected, self-curing rhesus macaques where there is a strong inverse association between the rapidity and intensity of the IgG response and the number and morphology of surviving worms.⁶² The mouse sera, on the other hand, had low protein microarray reactivity and recognized fewer features reflecting the increased susceptibility and weaker immune response in this animal model.^{8,9,11,21}

The skin- and lung-stage schistosomulum lifecycle stages are believed to be the most vulnerable to elimination by the host's immune system^{9,15,16} thereby making larval-stage specific antigens attractive vaccine targets. Our previous work²⁶ has shown that ASC probes from the skin- and lung-draining lymph nodes of the rat were induced by the passage of migrating schistosome larval stages.

In addition, our earlier preliminary protein microarray study detected some the possible antigens recognized in the skin and lung immune compartments.²⁷ We have extended this work and attempted to identify parasite protein antigens that are unique to these important sites of parasite elimination and immune priming. Compared with sera, the protein microarray SI was lower and the number of reactive antigens identified was less using the ASC probes. However, experiments with other parasites⁶³⁻⁶⁵ have similarly shown that ASC probes typically recognize a smaller and more specific set of antigens compared with serum. Reflecting the 10-fold difference in total antibody titers between the tissues,²⁶ lung ASC probes recognized more antigens and had higher SI than the skin ASC probes. This may be the consequence of the higher antigenic load resulting from parasite elimination in the lungs, compared with the skin where larvae quickly transit through and fewer are killed, or alternatively the potentially greater immunogenicity of post skin-stage schistosomules.^{21,66,67} An alternative explanation for the differences in antigen recognition between skin and lung ASC probes could be the suppressed immune response of host skin exposed to schistosome larvae excretory/secretory products.^{68,69} Notably, several specific antibodies in sera (e.g. against SjtSP2, Sjt21.7, Sjt22.6, calponin-like and putative *S. japonicum* vaccine candidate TPx1), that were highly reactive and consistently detected over repeat infections, were also present in the lung ASC probes and, to a lesser degree, in skin suggesting that these two tissues are important immune priming sites for some anti-schistosome memory responses in the rat. ASC probes provide a source of antibodies that are specific to the local tissue of origin and when used in concert with antigen discovery platforms, such as the schistosome protein microarray, are able to identify the antigens that elicit immunological responses following local parasite migration.

In an attempt to determine the temporal variation of parasite-specific antibody, rat sera were examined at several time points in the infectious cycle: prior to infection; after a primary infection; and finally after a secondary infection. As expected, antibody recognition of parasite proteins increased with repeated infections as the naïve host developed memory responses and was later re-infected. We also observed that antigens were recognized in three apparent patterns (consistent, primary and late-secondary antibody responses) that likely relate to the manner by which the antigen is exposed to the host and the immunogenicity of the antigen. For example, egg antigens, which are highly antigenic,^{4,58} were, unsurprisingly, strongly recognized late in infection and coincided with the start of egg-laying. In addition to Sjp40, Serpin B6, a serine protease inhibitor that is predominantly expressed in the egg,⁴⁵ was another late-secondary antigen. However, the current protein microarray includes only a subset of the complete schistosome proteome with a number of immunogenic proteins, such as those from the egg lifecycle stage, being absent. Future

studies using more comprehensive schistosome protein microarrays that include more egg lifecycle stage specific antigens may help to further elucidate the role of egg products in pathology and resistance in the rat and mouse.

Notably, consistently strong responses throughout the infection were found for tegument allergen-like proteins (TAL), Sj22.6, Sj21.7, Sm22.6 and SjTAL12.⁴² These immunogenic TAL proteins have conserved dynein light chain sequences with one or two EF-hand motifs and several known IgE epitopes. In particular, high anti-Sm/Sj22.6 and anti-Sm/Sj21.7 antibody levels are common in human *S. mansoni* and *S. japonicum* endemic populations.⁷⁰⁻⁷³ Dunne and colleagues have noted that age/drug-related human resistance is associated with high anti-Sm22.6 IgG1 and IgE levels. They have proposed that antibodies to Sm22.6 spike after drug treatment when the protein is released from dying worms while Sm21.7 is constantly released from eggs and becomes immunologically tolerised.^{42,70,72} Similarly, Sj21.7 is highly expressed across most lifecycles stages and is found at the protein level in all stages; Sj22.6 is transcriptionally upregulated in the adult male and has been detected proteomically in the adult and schistosomulum.^{33,74} Given the homology between these identified TAL-like proteins and the presence of conserved domains in the sequences it is not clear if rat antibodies are cross-reactive or specific for all of the detected TAL-like antigens. Nonetheless, these four *S. japonicum* and *S. mansoni* TAL-related proteins may hint at a shared resistance mechanism between rats and humans.⁹

The *Schistosoma* protein microarray proved to be an excellent screening tool for antigen discovery. Encouragingly, previously well studied antigenic and protective molecules were recognized. However, antibody recognition by the resistant rat host does not assure vaccine-induced protective immunity. For example, highly antigenic “bystander” proteins exposed during parasite death or egg-laying may also stimulate high antibody titers. In contrast, non-detection during protein microarray screening of an antigen does not imply immunological irrelevance. Indeed, the cell-free RTS proteins, used for fabricating the protein microarray, lack the translational modifications and disulfide bonds found in native proteins and therefore some epitopes may be absent.^{22,32} Yet, the relatively small number of proteins identified (29) and the high proportion of previously trialed vaccine antigens and therapeutic targets (12), including highly protective vaccines, within this set implies that there are potentially protective vaccine candidates among the untested proteins.

Potentially, the most important finding from this study was the discovery of seven new putative vaccine candidates and one potential diagnostic. These antigens include a mixture of hypothetical

and well annotated proteins. Six of the candidates (Sm/SjTPx1, AY809178, SjHSP86, Sj200, Sj Annexin 4 and SjDiag) have been detected in the proteomes of cercariae, schistosomula or adult worms, four have signal peptides and/or transmembrane domains (SjTHBS1, Sj200, SjCutA and SjDiag), and three have high sequence homology only within the schistosome species (AY809178, SjTHBS1 and Sj200). Importantly, these are novel proteins that have not been studied as *S. japonicum* vaccines or diagnostics. Further research into these proteins could include mouse vaccine trials (manuscript in preparation), diagnostic screening of endemic human populations, and, considering that most transmission of *S. japonicum* is via water buffalo,⁷⁵ bovine vaccine field trials.

In conclusion, screening of the schistosome protein microarray allowed for the simultaneous comparison of hundreds of parasite proteins for host antibody specificity and reactivity. This revealed patterns in parasite-host immunity, and led to the discovery of potential diagnostic and vaccine antigens. The use of ASC probes for antibody screening of protein microarrays has proven to complement serological antigen discovery and to also help elucidate immune mechanisms in experimental schistosomiasis. Importantly, using resistant and susceptible rodent antibodies from sera and tissue-specific lymph nodes, that were probed on the schistosome protein microarray, a set of 29 differentially reactive antigens were identified that were prominent in the resistant rat animal model. Within this set are well characterized antigens that have been previously trialed as schistosome vaccines and therapeutic agents, as well as seven novel vaccine candidates and one diagnostic antigen, all of which are untested and largely uncharacterized proteins.

Methods

Experimental infections and biological sample collection

For the first rat time course, TC1, four groups of 8-12 week old Wistar rats (n=4; Animal Resource Centre, Western Australia) were used. Three groups of rats (T₄, T₆ and T₈) were infected for four, six and eight weeks, respectively, with 200 cercariae per rat and challenged with an additional 200 cercariae per rat before termination six weeks later. One group, the negative control, was not infected but samples were collected for serological and histological comparison (data not shown). Similarly, during the second time course, TC2, four groups of 8-12 week old Wistar rats (n=5; Animal Resource Centre) were used. As in the previous time course, samples were collected from a negative control group for comparison with the other groups in the time course (data not shown). The three other groups (I, I₅ and I₉) were given a primary infection of 125 cercariae per rat for six weeks. After this group I was terminated while groups I₅ and I₉ were challenged with 350 cercariae

per rat and terminated five and nine days later, respectively. The time points in TC1 were based on observations that immune resistance in the rat has been shown to be highest between 4-8 weeks after re-infection.^{19,60} The time points in TC2 were selected to capture the local tissue response after schistosome larval migration through the skin and lung after the first and fourth day post-infection, respectively,¹⁹ and after allowing a further five days for an optimal lymph node response.^{26,27,63} The cercarial challenge load for TC1 was determined from previous studies^{8,19} and from preliminary experiments for TC2;²⁶ specifically the higher cercarial challenge for the second infection in TC2 ensured that skin- and lung-draining antibody secreting cells were adequately stimulated. For both TC1 and TC2 blood was collected prior to the primary and secondary infections and during termination. Equivalent time points were collected for the control groups in both rat time courses. Similar to previous studies^{8,19,59,61} we used outbred rats to better model the host heterogeneity found in schistosome-endemic animal and human populations. Blood was collected during the time course and at necropsy. A mouse time course experiment (MTC) was also completed. As with the rat, an outbred mouse strain was used for serological protein microarray probing. Eight to twelve-week old female outbred Swiss strain mice (n = 9; Animal Resource Centre) were infected with at least 70 cercariae and blood was collected the day prior to infection and 6 weeks post-infection. The Swiss mice were perfused to confirm schistosome infection and liver pathology. CBA mice (n = 10; Animal Resource Centre), one of the mouse strains typically used in schistosome immunology studies, were infected with 34 cercariae for six weeks for histological staining and parasitological burden measures.

S. japonicum (Chinese strain; Anhui province field isolate) cercariae were shed from infected *Oncomelania hupensis hupensis* snails after 3-4 hours of fluorescent light exposure. The cercariae were collected with loops, counted using a dissecting microscope, and transferred to microscope cover slips for experimental infections. Rats and mice were challenged subcutaneously with freshly shed cercariae via shaved abdominal skin for 30-40 min using the cover slip method. Rats were anaesthetized with gaseous isoflurane or IP injection of pentobarbitone (30 mg/kg) and euthanized with IP Lethobarb (Virbac, Reagents Park, Australia). Mice were anaesthetized with IP ketamine (100 mg/kg) and xylazil-20 (30 mg/kg) and euthanized by CO₂ inhalation. Blood was collected via the tail vein (for rats), tail tip (for mice) or from cardiac puncture at necropsy. The blood samples were allowed to coagulate at 4°C for 1 hour and the sera were collected by centrifuging (10 min at 16 000 x g and 4°C). Sera were stored at -80°C until use. After four weeks post-infection, mouse and rat faces were collected periodically to confirm the presence and viability of schistosome eggs. An aliquot of fresh faces was checked using the miracidial hatching test (MHT).⁷⁶ The aliquot was

homogenized in unchlorinated filter water and the sediment rinsed twice. The sediment was mixed with water and left at RT in a glass flask illuminated at the neck with a lamp. The flask was checked periodically over 24 hours for the presence of miracidia.^{77,78} At necropsy the animals were perfused through the portal vein, the worms were rinsed in perfusion buffer, fixed in neutral buffered formalin (NBF: PBS buffered with 4% (v/v) formaldehyde).^{72,73} During rat necropsies, the liver and lungs were dissected out, rinsed with perfusion buffer and a lobe of each was fixed in NBF overnight and stored in 70% (v/v) ethanol. The remainder of the liver was stored on ice for egg burden counts. Both the fixed liver lobe and remaining liver were weighed prior to storage. A sample of the fresh liver was chopped and processed as described above for the MHT. During TC2, additional samples were taken. Bronchoalveolar lavage (BAL) fluid was collected to determine the lung response, by washing the lungs with 1 ml PBS using a drawing up needle and syringe. The spleen, abdominal skin-draining (axillary and inguinal), lung-draining (mediastinal), and liver-draining (portal) lymph nodes (LN) were also dissected out. The livers of perfused mice were dissected out and processed as above. All animal experiments were approved by and performed according to the guidelines of the QIMR Animal Ethics Committee (project P288).

Parasite burden, histology and antibody secreting cell (ASC) probe preparation

The lengths of fixed worms were measured from digital images (Leica MZFL stereo microscope) using ImageJ software (version 1.45, National Institutes of Health, USA).⁷⁷ The lung lobes and one lobe of the liver were fixed in 10% formalin in neutral phosphate buffered saline, embedded in paraffin for sectioning, and stained with hematoxylin and eosin (H&E). Digital images of the stained slides were prepared (Aperio XT whole slide scanner, Aperio, USA) and the granuloma density was measured using the point counting method with ImageJ software, as previously described.⁷⁷ The average of five randomly selected snapshots was used to determine the granuloma burden. The liver egg burden was measured by digesting the remaining liver for 20 hours in 5% (w/v) potassium hydroxide at 37°C.⁷⁹ The solution was centrifuged at 1000 rpm for 10 min; the pellet was washed and resuspended in 1 ml NBF. The liver egg burden was measured by counting, at 40x magnification, numbers of eggs in three 5 µl aliquots and using the average to calculate the eggs per gram of liver (liver EPG).

ASC probes were prepared as previously described.²⁶ Briefly, LNs were aseptically teased apart with forceps into media supplemented with fetal bovine serum [RPMI+FCS: RPMI + L-glutamine with 5% (v/v) fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco,

Carlsbad, USA)] and washed twice in cold media. Viable cells were counted with trypan blue dye and a haemocytometer, resuspended to a density of 3×10^5 cells/ml in media supplemented with 0.1 ng/ml recombinant rat interleukin-6 (IL-6; R&D Systems, Minneapolis, USA), and then incubated for five days at 37°C and 5% CO₂. Cultures were centrifuged at 500 x g for 8 min at 4°C and the supernatant stored at -20°C.

Protein microarray immunoscreening

Schistosome protein microarrays were produced as described.^{23,25,32} From published *S. japonicum* and *S. mansoni* transcriptomic and proteomic databases^{33,80,81} a subset of 214 *S. japonicum* and 63 *S. mansoni* putatively immunogenic protein-coding sequences was selected. Sequence inclusion was based on known/predicted tegument surface location, high sequence conservation between *S. japonicum* and *S. mansoni*, expression in the larval schistosomulum lifecycle stage, and low sequence homology with mammalian hosts.^{25,32} Sequences were PCR amplified, using homologous recombination, cloned into the pXi expression vector, and quality checked using PCR and plasmid sequencing.^{22,23} Two hundred and twenty two (172 *S. japonicum* and 50 *S. mansoni*) high quality sequences were selected (Supplementary Table 2) for expression in a cell-free system (RTS) (RTS 100, Roche, Germany). The unpurified protein extracts were contact printed (Omnigrid, Digilab, USA) onto nitrocellulose slides (FAST, Whatman, UK). In addition, negative controls (No DNA controls: RTS lysate containing no plasmid DNA; PBS only; and blank spots), positive controls (IgG control: purified immunoglobulin; and Epstein-Barr nuclear antigen [EBNA-1]), as well as comparison controls, consisting of recombinant bacterial- and yeast-expressed *S. japonicum* and *S. mansoni* proteins with known immunogenicity, were printed on the protein microarrays.^{25,32} The expression efficiency of printed RTS proteins was checked by probing for the presence of N-terminal 10xHis and C-terminal hemagglutinin (HA) tags; over 97% RTS proteins were detected by anti-His and/or anti-HA antibodies.

The protein microarrays were probed with mouse sera and rat BAL fluid, sera and ASC probes and IgG reactivity measured. As a consequence of variations in aims, different samples were used and a more rigorous probing and analysis strategy was applied compared to our preliminary protein microarray ASC study.²⁷ BAL fluid and ASC probes had lower antibody titers than sera with reactivity being at least 100 fold lower for the ASC probes^{26,27} and 50 fold lower for BAL fluid (data not presented), and they were therefore used at lower dilutions (1:2 and 1:10, for ASC probes and BAL fluid respectively, compared with 1:100 for sera). Moreover only ASC probes with high

antibody titers were used for the microarray experiments. As a result a total of four individual skin ASC probes (three from I₅ and one from I₉) with the highest antibody titers and one pooled sample of the remaining ASC probes were used. Likewise for lung ASC probes, four individual samples (one from I₅ and three from I₉) and a pooled sample were used. For comparison, pooled (four from I₉) liver, pooled (five from I₉) spleen and individual control lung ASC probes and pooled (five from I₉) BAL fluid were tested on the protein microarray. BAL fluid, liver, spleen and control ASC probes were used as controls to better enable selection of antigens specific to the skin and lung tissue compartments. All samples were diluted accordingly in blocking buffer (Whatman, Maidstone, UK), and mixed at room temperature (RT) with gentle rocking for 30 min with 10% (w/v) *E. coli* lysate (MC Lab, San Francisco, USA). Protein microarray slides, pre-hydrated in blocking buffer for 30 min at RT, were probed with diluted sera, BAL fluid or ASC probes overnight at 4°C with gentle rocking. After three sets of 5 min washes with 0.05% Tween 20 in TBS (TBST), IgG-detecting secondary antibody conjugated to biotin (1:1000 in blocking buffer) was incubated for 1 hour at RT. The secondary antibodies used were goat anti-rat IgG (whole molecule) biotin-conjugated (Sigma Aldrich, St Louis, MO, USA) and goat anti-mouse IgG (Fc specific) biotin-conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After washing, the protein microarrays were incubated with streptavidin Cy5 (Surelight P3, Columbia Biosciences, Columbia, USA) for 1 hour at RT. After a final washing step with TBST, TBS and water each three times for 5 min, the protein microarrays were air dried in the dark and scanned with a Genepix 4300A scanner (Molecular Devices, Sunnyvale, USA). The signal intensities (SI) for all IgG reactivity were measured at a laser setting of 100% and PMT of 600. These settings were found to provide the highest signal to noise ratio for the majority of the probed samples.

Data analysis

Protein microarray feature data were pre-processed and analyzed as described^{27,32} but with some modifications. The raw SI was generated after local background correction of protein microarray features. In addition, to account for reactivity in the RTS lysate, the mean of the “No DNA” features was subtracted from the raw SI within each microarray. These data were transformed and normalized using the variance stabilizing normalization algorithm⁸² in the *vsr* R package (R project for statistical computing, www.r-project.org; www.bioconductor.org). The normalized data were then analyzed further or retransformed (inverse log₂) to a relative SI for graphs and heat maps. The average SI across all the protein microarray features (global SI) was used to compare the change in total protein microarray reactivity during the experimental time course and between tissue regions.

Seropositive antigens were defined as having an SI greater than the average plus four standard deviations (SD) of the “No DNA” controls within each microarray. This high positivity threshold was used to more stringently identify reactive antigens for putative vaccine and diagnostic selection.^{32,83}

The *Cyber-T* package (<http://cybert.ics.uci.edu/>), also written in the R language, used extensively for analysis of protein and nucleic acid microarrays,⁸³⁻⁸⁵ was employed to generate Bayesian-adjusted *t*-test and ANOVA results and multiple hypothesis testing correction of resulting *P*-values. Bayesian standard deviation estimation corrects for low replication within treatment groups, usually inherent in microarray data, by estimating the variance from similar SI within each microarray and calculating the regularized *t*-test and ANOVA.⁸⁴ For larger datasets, typically with thousands of microarray features, non-specific filtering of unchanged features is required to control false positives following multiple comparisons.⁸⁶ However, the schistosome protein microarray contains only 289 features; therefore all the data were used without pre-filtering and the false discovery rate (FDR) was controlled using the Benjamini and Hochberg (BH) correction within the *Cyber-T* package.⁸⁴ Significant BH-corrected ANOVA *P*-values were then subjected to testing with pairwise post-hoc *t*-tests (Tukey HSD) to identify differentially reactive features between groups. To further identify differentially reactive antigens between comparison groups the area under the curve (AUC) was calculated for receiver operating characteristic curves (ROC; R package, www.bioconductor.org). ROC curves plot the false positive rate (1 - specificity) against the true positive rate (sensitivity) and measure the ability of an antigen to differentiate between two groups where a value close to 1 is a good prediction and 0.5 to 0 is poorly predictive.

Based on the average SI and BH *P*-values, the top RTS proteins were chosen for further analysis. From this set of top RTS proteins those with < 20% frequency of recognition (percentage of seropositive individual samples within a group) or were more strongly recognized pre-infection compared to post-infection were excluded. Selection parameters (BH *P*-value, AUC value, SI fold-change and frequency of recognition) were used to categorize RTS proteins. Putative novel *S. japonicum* vaccine candidates were selected by: including antigens with high selection parameters in post-infection rat sera and ASC probes; removing antigens that had been previously tested as vaccines or therapeutics; or were associated with infection in the mouse using the above criteria. The remaining antigens were defined as proteins associated with rodent schistosomiasis infection. Diagnostic antigens were selected on the basis of high reactivity in all infection samples. Gene ontologies (<http://www.geneontology.org>) and protein details were retrieved using the BLAST2GO

program (www.blast2go.com). Statistical analysis between groups for ELISA immunoglobulin levels and protein microarray global SI were completed using *t*-tests or ANOVA and Tukey HSD post-hoc *t*-test pair-wise testing (GraphPad Prism 5, San Diego, CA, USA).

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Appendices

Supplementary Figure 1: Rat pathology is reduced compared with that in the mouse after *S. japonicum* infection. Haematoxylin and eosin (H&E) staining of sectioned formalin-fixed

paraffin-embedded: typical appearance of infected rat liver (A); typical appearance of infected mouse liver (B); and infected rat lung (C). The rat liver has a small granuloma (arrow) following a primary infection and termination 6 weeks later (TC2 – I). The mouse liver is characterized by large granulomas with numerous eggs (arrows) following infection and termination 6 weeks later. Three lung stage schistosomules (arrows) are shown in the rat lung following primary infection, secondary challenge 6 weeks later and termination after 9 days (TC2 – I₉) (200 µm scale bar on images).

Supplementary Figure 2: Schistosome protein recognition by host antibodies increases with infection frequency and is consistent within treatment groups but varies between host species.

The schistosome protein microarray after probing with serum: from an individual rat (T₄-1) prior to infection (A), four weeks after a primary infection (B) and six weeks after a secondary infection; from a treatment group 8 weeks after a secondary infection for rats T₈-2 (D), T₈-3 (E), and T₈-4 (F); and from a representative mouse prior to infection (G) and 6 weeks after infection (H). Note the grayscale representation used here emphasizes strongly recognized protein antigens.

Supplementary Table 1: Parasitological measurements of rats from time courses 1 (TC1) and 2 (TC2). TC1 used three different intervals between the primary and secondary infection (200 cercariae/rat/infection): group T₄ with 4 weeks; T₆ with 6 weeks; and T₈ with 8 weeks. TC2 used three groups, all with a primary infection (125 cercariae/rat) for six weeks and followed by a secondary infection for groups I₅ and I₉ (350 cercariae/rat). Group I was sacrificed six weeks after the primary infection; groups I₅ and I₉ were sacrificed 5 and 9 days after the secondary infection, respectively.

Supplementary Table 2: Composition of the schistosome protein microarray (XLSX). The features of the schistosome protein microarray including control features, *in vivo* recombinant proteins and RTS expressed proteins. The dilutions, annotations, accession numbers and protein characteristics are included as applicable.

Supplementary Table 3: Raw signal intensities for protein microarrays probed with rodent antibodies (XLSX). The un-normalized signal intensities for protein microarray features probed with 90 rodent serum and tissue-specific antibodies.

Supplementary Table 4: Normalized signal intensities for protein microarrays probed with rodent antibodies (XLSX). The normalized signal intensities (SI) for protein microarray features

probed with 90 rodent serum and tissue-specific antibodies. The raw SI was VSN normalized. For visualization purposes the VSN-normalized data was inverse log₂ transformed. The VSN-normalized data were analyzed using a Bayesian regularized ANOVA with post-hoc pairwise Tukey HSD *t*-test comparisons. The *P*-values were adjusted for multiple testing using the Benjamini and Hochberg (BH) correction. The frequency of recognition within each group was calculated for each protein microarray feature.

Supplementary Table 5: Pearson correlations of rodent sample reactivity to cell-free (RST) and *in vivo* recombinant proteins (DOCX). Pearson R values and statistical significance were calculated for the correlations between RTS and *in vivo* recombinant antigens on the schistosome protein microarray after probing with 90 rodent serum and tissue-specific antibodies.

Supplementary Table 6: *S. japonicum* antigens on the schistosome protein microarray associated with rodent infection. Antigens were selected by Benjamini-Hochberg adjusted *P*-values between pre-infection and post-infection samples from rat time course one (TC1) and two (TC2), mouse (M) and ASC (A) sample probing.

Supplementary Table 7: *S. mansoni* antigens on the schistosome protein microarray associated with rodent infection. Antigens were selected by Benjamini-Hochberg adjusted *P*-values between pre-infection and post-infection samples from rat time course one (T1) and two (T2), mouse (M) and ASC (A) sample probing.

Figure Legends

Figure 1: Schistosome protein microarray probed with rat and mouse antibody samples. A: Raw signal intensities (SI) were normalized with the *vsn* R package, retransformed (inverse log₂) and displayed in a heatmap. Protein microarray features are in rows (21 Controls include IgG positive controls and “No DNA” negative controls; 46 *in vivo* recombinant proteins include dilutions of 20 proteins and 222 cell-free (RTS) proteins (Supplementary Table 2) and biological samples are in columns (TC1: rat time course 1, n = 28; TC2: rat time course 2, n = 30; ASC: antibody secreting cell probes, n = 14; MTC: mouse time course, n = 18). The sample groups are labelled (Pre: pre-infection; Inf: infected; Prim: primary infection; and Sec: secondary infection; Ctrl: control, liver, spleen ASC; Skin: skin-draining lymph node ASC; and Lung: lung-draining

ASC). The features were ranked in decreasing order by the average SI. The color scale indicates the normalized SI. Scatter plot of normalized SI for TC1 (B) and TC2 (C). ANOVA and *t*-test significance are displayed on the graph (** $P < 0.001$, *** $P < 0.0001$).

Figure 2: Heat map of the schistosome protein microarray reactive antigens and controls from rat time course 1 (TC1) and 2 (TC2). Raw signal intensities (SI) were normalized (*vsu*), retransformed (inverse $\log_2$) to a relative signal intensity and averaged within each group at each time point (Pre: pre-infection; Inf: infected; Prim: primary infection; and Sec: secondary infection). The antigens are in decreasing order by the average SI and the top 40 cell-free (RTS) proteins and top five *in vivo* recombinant proteins are displayed along with “No DNA” negative controls 1 and 2. The color scale indicates the normalized signal intensity. RTS proteins include the accession number, feature name in brackets (PD code for *S. japonicum* and CF code for *S. mansoni*), and additional annotation. *In vivo* proteins include the construct name, expression host and dilution number (see Supplementary Table 2).

Figure 3: Heat map of the schistosome protein microarray reactive antigens from rat antibody secreting cell (ASC) probes and mouse infection sera (MTC). (From previous page) Raw signal intensities were normalized with the *vsu* package and retransformed (inverse $\log_2$) to a relative signal intensity. The antigens were ranked in decreasing order by the average of the skin and lung ASC probes and infected mouse sera. The top 20 cell-free (RTS) proteins and top 15 *in vivo* recombinant proteins are displayed along with “No DNA” negative controls 1 and 2. The color scale indicates the signal intensity. RTS proteins include the accession number, feature name in brackets (PD code for *S. japonicum* and CF code for *S. mansoni*), and additional annotation. *In vivo* proteins include the construct name, expression host and dilution number (Supplementary Table 2). Probed samples: Ctrl: control samples; Neg: control rat 2 lung ASC probes; Liver: pooled I₉ liver ASC probes; Spleen: pooled I₉ spleen ASC probes; BAL: pooled I₉ BAL fluid; Skin: skin ASC probes; Lung: lung ASC probes; and MTC: Swiss mouse uninfected and after schistosome infection (6 weeks) individuals (1-9).

Figure 4: Average signal intensity and *P*-value of the top rodent antigens on the schistosome protein microarray. The average signal intensity (columns with standard error of the mean bars) and Benjamini-Hochberg adjusted *P*-values (yellow line) for rat time course 1, TC1 (A), time course 2, TC2 (B), mouse time course MTC (C) and antibody secreting cell (ASC) probe screening (D). The rat and mouse sera samples probed were: pre-infection (Pre), primary infection (Prim),

secondary infection (Sec), group I (I), group I₅ (I₅) and group I₉ (I₉). ASC probes were derived from infected rat lung- and skin-draining lymph nodes (LN) and the control (Ctrl) samples were from infected liver, spleen-LN and uninfected control lung-LN ASC probes, and bronchoalveolar lavage (BAL) fluid from infected rats. *In vivo* expressed recombinant proteins for experiments one and two are not displayed. Previously tested (T) vaccines and putative vaccine (V) antigens are marked in the figure. BH $P = 0.05$ is displayed as a dashed line on the graph. Antigens with low frequency of recognition ($< 20\%$) within a sample group are marked (#).

Figure 5: Receiver operating characteristic (ROC) curves of antigens recognized by rat serum. ROC curves for time course 1 (TC1) primary infection (A); secondary infection (B); and time course 2 (TC2) post-infection (C) rat sera are displayed. The area under the curve (AUC) value is calculated from the ROC curve. The diagnostic antigen, SjDiag (AY815838) has a perfect AUC of 1 in all graphs. Heat shock protein 86 (SjHSP86, AY815164) and Sjp40 major egg antigen (AY814158) are good diagnostics of primary (0.89) and secondary infection (0.96), respectively. A value of AUC 0.5 is shown as reference (thin solid blue).

Figure 6: Reactivity of selected antigens during the rat time course (TC1 and TC2) experiments. The reactivity profiles of: consistently reactive type antigens, Sj22.6 (L08198), Sj21.7 (AF048759), Sm22.6 (Smp_045200) and SjTAL12/similar to Sm22.6 (AY815113) (A); primary infection reactive type antigens, SmTPx1 (Smp_059480; thioredoxin peroxidase 1), SjTHBS1 (AY809526; hypothetical, thrombospondin type 1 domain) and SjHSP86 (AY809526; heat shock protein 86, (B); and late secondary infection reactive type antigens, Sj serpin B6 (187PD257), Sjp40 major egg protein (AY814158) and SjCutA (AY222951; similar to divalent cation tolerance protein) (C). Each column (error bars are standard error of the mean) is the average normalized signal intensity of sera collected at the time points in weeks during time course 1 (TC1) and 2 (TC2). Antigens are identified in the legend. The significance values of each time point compared with pre-infection sera are marked (Tukey HSD post-hoc pairwise t-test: * $P < 0.05$; † $P < 0.001$; ‡ $P < 10^{-4}$).

Table 1: Putative vaccine and diagnostic antigens on the schistosome protein microarray selected after rodent sample probing.

Accession Number ID	Annotation (BLASTp % amino acid identity) [§]	Signal intensity fold change AUC value						Proteomic Information						Putative Diagnostic/ Vaccine
		Frequency of recognition (%) [®]						Protein/ fragment length	Signal Peptide	Transmem domains	Lifecycle Stage [#]	Schisto. Specific	Biological Process Gene Ontology (GO)	
		Rat					Mouse							
		Prim	Early Sec.	Late Sec.	ASC									
					Skin	Lung								
Smp_059480 2CF	SmTPx1 , Thioredoxin peroxidase 1, (82% aa Sj AAW27020; 72% aa Sj22.6)	7.2	1.2	16.6	0.1	2.1	1.1	185	n	-	SE [†]	n	cell redox homeostasis	Vaccine ^{25,26}
		0.89	0.84	0.94	0.70	0.66	0.51							
		55%	30%	91%	0%	40%	0%							
AY809178 54PD110	Hypothetical/ guanylate-kinase-assoc. (61% aa Sm CCD75025)	3.1	0.9	8.9	0.5	0.5	1.1	291	n	-	SAM	y	Cell-cell signalling	Vaccine
		0.89	0.56	0.93	0.65	0.65	0.54							
		27%	0%	100%	0%	0%	0%							
AY809526 80PD136	SjTHBS1, hypothetical/ thrombospondin type 1	5.9	2.7	2.1	0.9	1.2	1.2	121	n	1	S	y		Vaccine
		0.54	0.64	0.76	0.63	0.56	0.60							
		27%	40%	27%	60%	80%	0%							
AY810129 108PD169	Sj200, sim. surface protein (70% aa Sm200 GPI-anchored / 31CF)	1.0	0.6	3.1	0.8	1.1	0.9	238	n	1	A	y		Vaccine ³⁰
		0.56	0.66	0.80	0.76	0.49	0.62							
		27%	40%	64%	0%	40%	11%							
AY815332 159PD225	Coatomer subunit beta (63% aa Cs coatomer)	1.6	1.6	3.2	0.6	1.1	1.0	162	n	-	-	n	vesicle-mediated transport; protein complex assembly	Vaccine
		0.61	0.78	0.83	0.70	0.60	0.54							
		0%	10%	27%	0%	0%	0%							
AY222951 195PD269	SjCutA (85% aa Sm divalent cation tolerance Cut A XP002576196)	1.3	0.7	12.1	1.5	1.3	1.3	130	y	2	-	n	response to metal ion	Vaccine
		0.64	0.66	0.96	0.78	0.66	0.67							
		27%	40%	82%	80%	100%	22%							
AY223469* 204PD278	Sj Annexin 4 (80% aa Sm annexin XP002575877)	2.2	1.5	8.9	0.3	0.3	1.1	330	n	-	CA	n	calcium ion binding	Vaccine ^{31,32}
		0.63	0.54	0.9	0.63	0.65	0.58							
		18%	20%	55%	0%	0%	0%							
AY815838 170PD239	SjDiag, Hypothetical	15.1	13.0	15.0	0.2	2.2	3.9	120	y	2	CA	n		Diagnostic
		1.0	1.0	1.0	0.80	0.78	0.88							
		100%	100%	100%	100%	100%	100%							

Proteomic and biological ontology information from ²⁶ and BLAST2GO (www.blast2go.com). [#] Protein lifecycle stage expression for cercaria (C), schistosomulum (S), adult (A), egg (E) and miracidium (M). Homologues: [†]SJCHGC02884 (AAW27020), ^{*}SJCHGC02020 (AAW27836).

[§] *S. japonicum* or *S. mansoni* BLASTp identity with expected value < 10⁻⁵⁰. Prim: primary time points TC1 and group I / TC2; Early Sec: groups

I₅ and I₉; and Late Sec: secondary time points TC1. ©For pooled values, the average fold change and frequency of recognition and lowest AUC were used.

A **B** **C**