Proteomic Analysis of *Biomphalaria glabrata* Hemocytes During *in vitro* Encapsulation of *Schistosoma mansoni* Sporocysts

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Circulating hemocytes of the snail *Biomphalaria glabrata*, a major intermediate host for the blood fluke *Schistosoma mansoni*, represent the primary immune effector cells comprising the host’s internal defense system. Within hours of miracidial entry into resistant *B. glabrata* strains, hemocytes infiltrate around developing sporocysts forming multi-layered cellular capsules that results in larval death, typically within 24–48 h post-infection. Using an *in vitro* model of hemocyte-sporocyst encapsulation that recapitulates *in vivo* events, we conducted a comparative proteomic analysis on the responses of hemocytes from inbred *B. glabrata* strains during the encapsulation of *S. mansoni* primary sporocysts. This was accomplished by a combination of Laser-capture microdissection (LCM) to isolate sections of hemocyte capsules both in the presence and absence of sporocysts, in conjunction with mass spectrometric analyses to establish protein expression profiles. Comparison of susceptible NMRI snail hemocytes in the presence and absence of sporocysts revealed a dramatic downregulation of proteins in during larval encapsulation, especially those involved in protein/CHO metabolism, immune-related, redox and signaling pathways. One of 4 upregulated proteins was arginase, competitor of nitric oxide synthetase and inhibitor of larval-killing NO production. By contrast, when compared to control capsules, sporocyst-encapsulating hemocytes of resistant BS-90 *B. glabrata* exhibited a more balanced profile with enhanced expression of shared proteins involved in protein synthesis/processing, immunity, and redox, and unique expression of anti-microbial/anti-parasite proteins. A final comparison of NMRI and BS-90 host hemocyte responses to co-cultured sporocysts demonstrated a decrease or downregulation of 77% of shared proteins by NMRI cells during encapsulation compared to those of the BS-90 strain, including lipopolysaccharide-binding protein, thioredoxin reductase 1 and hemoglobins 1 and 2. Overall, using this *in vitro* model, results of our proteomic analyses demonstrate striking differences in proteins expressed by susceptible NMRI and resistant BS-90 snail hemocytes to *S. mansoni* sporocysts during active encapsulation, with NMRI hemocytes exhibiting extensive downregulation of protein expression and a lower level
of constitutively expressed immune-relevant proteins (e.g., FREP2) compared to BS-90. Our data suggest that snail strain differences in hemocyte protein expression during the encapsulation process account for observed differences in their cytotoxic capacity to interact with and kill sporocysts.

Keywords: innate immunity, mollusk, Biomphalaria glabrata, Schistosoma mansoni, hemocytic encapsulation, sporocyst, proteomic response, in vitro cytotoxicity assay

INTRODUCTION

Circulating hemocytes of Biomphalaria snail species represent the primary immune effector cells of the host, and, when encountering primary sporocysts of incompatible strains of the human blood fluke Schistosoma mansoni, respond by rapid encapsulation and destruction of early developing larvae (1–5). Cellular encapsulation responses typically are characterized by infiltration of circulating hemocytes around the newly transforming primary sporocysts, commencing usually within 2–3 h post-infection, followed by adherence and spreading of hemocytes in multiple layers around the parasite that result in the killing of encapsulated larvae within 24–48 h post-infection (6, 7). An in vitro model of larval encapsulation has been developed for the B. glabrata-S. mansoni system that closely parallels the events observed under natural in vivo infection conditions (8). In this cell-mediated cytotoxicity (CMC) assay, live in vitro transformed primary sporocysts (9) were co-incubated in 1-mL tubes with freshly extracted hemolymph isolated from inbred susceptible and resistant strains of B. glabrata, resulting in the formation hemocytic capsules around sporocysts. Although hemocytes of both susceptible and resistant snail strains formed cellular encapsulations, significantly enhanced killing/damage of sporocysts was observed in reactions with resistant snail cells when compared to those involving hemocytes from susceptible snails. Follow-up experiments showed that components from plasma (cell-free hemolymph) alone also may be involved in triggering the CMC response, although the humoral factor(s) and their functional role in hemocyte reactivity have yet to be identified and described (7, 10). Additional support that hemocytes are the primary mediators of anti-schistosome resistance recently has been demonstrated in vivo (11) using a resistant Guadeloupean stain of B. glabrata exhibiting allelic variation in a gene complex designated the Guadeloupe resistance complex (GRC) (12, 13). Notably, RNAi knockdown of a novel transmembrane protein encoded within the GRC appears to influence the resistance phenotype (no development of S. mansoni) (13) but the mechanism(s) by which this is accomplished remains unknown.

Although comparative transcriptomic analyses have been performed on whole snails or circulating hemocytes from different B. glabrata strains (14–17) and/or on snails in response to schistosome infection (18–22), evaluation of parasite-induced changes in hemocyte protein expression, specifically in hemocytes actively participating in sporocyst encapsulation reactions, have not been examined in detail (23). Targeted investigations of specific hemolymph proteins, such as Cu/Zn superoxide dismutase SOD1 (24), HSP 70 and 90 (25, 26), fibrinogen-related proteins (27, 28), thioester-containing proteins (29), MIF (30), biophalysin protease (31), Toll-like receptor (TLR) (32), hemopoietic factor granulin (33), and an unknown protein encoded by gene Grctm6 (13) have demonstrated functional associations with snail immunity to schistosome infection. The diversity of protein mediators identified in these studies suggest that the molecular events transpiring prior to and during the hemocyte encapsulation reactions likely are complex, involving multiple protein components that differ depending on the specific snail-schistosome strain combination under investigation (3, 34, 35).

In the present study we performed a comparative proteomic analysis on hemocytes from inbred schistosome-susceptible (NMRI) and—resistant (BS-90) strains of B. glabrata during their active participation in in vitro encapsulation reactions with primary sporocysts of S. mansoni (NMRI strain). Specifically, we addressed the questions: What proteins are expressed by hemocytes directly participating in encapsulation reactions? Do susceptible and resistant snail hemocytes exhibit differential protein expression responses in the presence of encapsulated sporocysts? In order to address these questions we combined two powerful analytical methods, laser-capture microdissection (LCM) microscopy and nano-LC tandem mass spectrometry, to provide the first comparative proteomic analysis of schistosome-susceptible and—resistant hemocytes during active in vitro encapsulation of S. mansoni sporocysts.

MATERIALS AND METHODS

Ethics Statement

Mice were maintained in an AAALAC-approved animal housing facility (Charmany Instructional Facility, University of Wisconsin-Madison) using standard-of-care cage housing and feeding. All animal handling and experimental protocols were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC) under Animal Welfare Assurance No. A3368-01.

Maintenance of B. glabrata Snails

Two inbred laboratory strains of B. glabrata snails, the S. mansoni-susceptible NMRI and—resistant BS-90 strains, were used in the present study. Snails were maintained in 20-gal aquaria at 26°C under a 12 h light:12 h dark photoperiod and fed leaf-lettuce ad libitum supplemented with Tetramin® fish food and chalk.
Isolation of Miracidia and Sporocyst Preparation
Mice, infected with the NMRI strain of *S. mansoni*, were provided by the Biomedical Research Institute (Rockville, MD) under a NIAID contract to BEI Resources (Manassas, VA). At 7 weeks post-infection, mice were euthanized and necropsied for removal of livers and axenic egg isolation (36).

Miracidia were isolated under axenic conditions as previously described (36, 37) and placed into *in vitro* culture in 24-well tissue culture plates (Corning Costar, NY) containing Chernin’s balanced salt solution (CBSS) (38) under normoxic conditions at 26°C. The CBSS was supplemented with penicillin (100 U/mL), streptomycin sulfate (0.1 mg/mL) and 1 g/L each of glucose and trehalose. Following miracidial transformation (24 h), primary sporocysts were maintained in culture for an additional 24 h at 26°C, after which they were washed 5 times in CBSS by gravity sedimentation to remove the shed epidermal plates and other cellular debris.

Hemolymph (HL) from 120 *B. glabrata* snails (12–15 mm shell diameter) of each strain was removed by the headfoot retraction method (39). Briefly, prior to bleeding, snail shells were wiped with cotton swabs soaked in 70% ethanol and air dried, followed by carefully peeling back the shell to expose the headfoot region. A rapid poking of the tissue by forceps forced the headfoot to retract while extruding HL from the hemal pore. The HL was then collected by micropipettor using siliconized, sterilized tips, and dispensed onto a cold sterile glass plate for 15–20 s to allow for remove any shell debris or mucus before transfer to a sterile 15-mL conical centrifuge tube containing cold CBSS. The tube was kept on ice during HL collection, with the final HL: CBSS ratio after collection equaling ~1:1. All procedures outlined above for HL extraction were performed in a biosafety cabinet to minimize microbial contamination. Hemocyte concentrations of pooled samples were determined by Neubauer hemocytometer (318 ± 131 and 304 ± 56 cells/μL in BS-90 and NMRI, respectively).

Hemocyte Preparation

Hemocyte/sporocyst and hemocyte only cultures were incubated undisturbed for 18 h at 26°C under normoxic conditions. Following incubation, cell clumps (Figure 1A) were washed 5x with CBSS, transferred to cryomolds, and allowed to settle (Figure 1B). CBSS was then removed and replaced by the cryosectioning embedding medium, OCT (Lab-Tek Products, Naperville, IL) (Figure 1C), followed immediately by freezing on dry ice and storage at −80°C until sectioning. Frozen sections (10 μm thickness) of OTC-embedded NMRI and BS-90 hemocyte aggregates were cut using a Leica CM 1900 cryostat (Leica) at −28°C, placed on membrane-coated glass slides (Molecular Machines & Industries-MMI microslides, Haslett, MI) and maintained at −80°C before LCM. Slide-mounted cryosections were fixed in 70% EtOH for 30 sec, stained with Mayer’s hematoxylin solution (30 s) (Sigma-Aldrich), wash 2x in Milli-Q water (15 s), and dehydrated in a graded EtOH series consisting of 70% (10 s), 2 × 95% (10 s), and 2 × 100% (10 and 30 s) absolute EtOH. The stained tissue sections were air-dried in a laminar flow hood for 15 min and then subjected to LCM.

Sample Collection and Cryosectioning
Hemocyte/sporocyst and hemocyte only cultures were incubated undisturbed for 18 h at 26°C under normoxic conditions. Following incubation, cell clumps (Figure 1A) were washed 5x with CBSS, transferred to cryomolds, and allowed to settle (Figure 1B). CBSS was then removed and replaced by the cryosectioning embedding medium, OCT (Lab-Tek Products, Naperville, IL) (Figure 1C), followed immediately by freezing on dry ice and storage at −80°C until sectioning. Frozen sections (10 μm thickness) of OTC-embedded NMRI and BS-90 hemocyte aggregates were cut using a Leica CM 1900 cryostat (Leica) at −28°C, placed on membrane-coated glass slides (Molecular Machines & Industries-MMI microslides, Haslett, MI) and maintained at −80°C before LCM. Slide-mounted cryosections were fixed in 70% EtOH for 30 sec, stained with Mayer’s hematoxylin solution (30 s) (Sigma-Aldrich), wash 2x in Milli-Q water (15 s), and dehydrated in a graded EtOH series consisting of 70% (10 s), 2 × 95% (10 s), and 2 × 100% (10 and 30 s) absolute EtOH. The stained tissue sections were air-dried in a laminar flow hood for 15 min and then subjected to LCM.

LCM was used to separate and isolate hemocytes (Hc) from encapsulated sporocysts in order to enrich for Hc-associated proteins in Hc-sporocyst co-cultivation samples. The MMI CellCut® Nikon microscope (Molecular Machines & Industries, Haslett, MI) was used to image and microdissect encapsulating tissue. Using the MMI CellCut® software, regions of interest were selected in the tissue images (Figure 2A) and microdissected with a laser (Figures 2B, C). The dissected tissue sections were then collected in MMI IsolationCap® tubes for subsequent proteomic analysis. Approximately 3 × 10⁶ μm² of sectioned tissue per sample were collected with one experiment consisting of four sample collections: BS-90 Hc only (BS90), BS-90 Hc + sporocysts (BS90-SP), NMRI Hc only (NMRI), NMRI Hc + sporocysts (NMRI-SP). Two independent experiments were conducted, each consisting of fresh pooled hemolymph and a new batch of transformed sporocysts.

Proteomic Analysis
Sectioned microdissected tissue in microcap tubes were solubilized and protein denatured in 12 μL of a chaotropic detergent containing solution [6M Urea/0.5% ProteaseMAX™ surfactant (Promega Corp., Madison, WI) in 50 mM NH₄HCO₃ (pH 8.5)] for 1 min at 24°C then switched to 42°C for 10 min and subsequently diluted to 60 μL in a reduction step by addition of 2.5 μL of 25 mM dithiothreitol (DTT), 5 μL MeOH and 40.5 μL of 25 mM NH₄HCO₃ (pH 8.5). Samples were then incubated at 52°C for 15 min, cooled on ice to 22°C, followed by addition of 3 μL of 55 mM iodoacetamide for alkylation in the dark for 15 min at 22°C. Reactions were quenched by adding 6 μL of 25 mM DTT. Protease digestion was initiated by adding 4 μL of Tryptsin/LysC solution [10 ng/μL Tryptsin/LysC mix (Promega) in 25 mM NH₄HCO₃] and 27 μL of 25 mM NH₄HCO₃ (pH 8.5) to each sample tube for 2 h at 42°C, followed by the addition of 3 μL more of Tryptsin/LysC solution and continued overnight digestion at 37°C. Reactions were terminated by acidification.
with 2.5\% trifluoroacetic acid (TFA, Sigma-Aldrich) to 0.3\% final concentration.

Digests were cleaned up using OMIX C18 SPE cartridges (Agilent Technologies, Palo Alto, CA) per the manufacturer's protocol and eluted in 10 \( \mu \text{L} \) of 70/30/0.1\% acetonitrile (ACN)/\( \text{H}_2\text{O} \)/TFA, dried to completion in a speed-vac centrifuge and finally reconstituted to 20 \( \mu \text{L} \) in 0.1\% formic acid. Peptides were analyzed by nanoLC-MS/MS using an Agilent 1100 nanoflow system connected to a hybrid linear ion trap-orbitrap mass spectrometer (LTQ-Orbitrap XL, Thermo Fisher Scientific) equipped with a nanoelectrospray ion source. Chromatography of peptides prior to mass spectral analysis was accomplished using a C18 reverse-phase HPLC trap column (Zorbax 300SB-C18, 5 \( \mu \text{M} \), 5 \( \times \) 0.3 mm, Agilent Technologies) and capillary emitter column (in-house packed with MAGIC C18, 3-\( \mu \text{M} \), 150 \( \times \) 0.075 mm; Michrom Bioresources, Inc., Sacramento, CA) onto which 8 \( \mu \text{L} \) of digest was automatically loaded. A nanoHPLC system delivered solvents A (0.1\% (v/v) formic acid) and B (95\% (v/v) ACN, 0.1\% (v/v) formic acid) at a rate of 10 \( \mu \text{L/min} \) to load the peptides (over 45 min) and 0.2 \( \mu \text{L/min} \) to elute peptides directly into the nano-electrospray with a continuous gradient from 0\% (v/v) B to 40\% (v/v) B over the next 145 min. This was followed by a fast gradient elution from 40\% (v/v) B to 60\% (v/v) B for 20 min and finally a 5 min flash-out from 50 to 95\% (v/v) B.

As peptides eluted from the HPLC-column/electrospray source, survey MS scans were acquired in the Orbitrap with a resolution of 100,000, and up to 5 most intense peptides per scan were fragmented and detected in the ion trap over the 300–2,000 m/z, with redundancy being limited by dynamic exclusion.

**Bioinformatic Analyses**

Raw MS/MS data were converted to mgf file format using the Trans-Proteomic Pipeline (http://tools.proteomecenter.org/software.php; Seattle Proteome Center, Seattle, WA). Resulting mgf files were used to search against concatenated \textit{B. glabrata} and \textit{S. mansoni} protein databases consisting of RefSeq non-redundant entries plus common lab contaminants and decoy entries for false discover rate (FDR) calculations (96,790 total entries) with cysteine carbamidomethylation as fixed modification plus methionine oxidation and asparagine/glutamine deamidation.
as variable modifications. Peptide mass tolerance was set at 15 ppm and fragment mass at 0.6 Da. Thermo Proteome Discoverer version 1.4.1.14 was used to integrate results from two search engines (Sequest and Mascot) and Scaffold (version Scaffold_4.3.2, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >84.0% probability to achieve an FDR <1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they achieved an FDR <1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (41). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The complete raw dataset used in bioinformatics analyses of the hemocyte proteomic data are available as project #1492 available at chorusproject.org SMART (Simple Modular Architecture Research Tool) was employed to identify protein domains using normal mode, including outlier homologs and PFAM domains (http://smart.embl-heidelberg.de/smart/set_mode.cgi; NORMAL=1). Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (42) was used for multiple-sequence alignments. Unknown/uncharacterized/hypothetical proteins were subjected to BLASTp searches of the nrNCBI db (as of May 2018) to acquire related protein identifications. The entire experiment was performed twice on different dates using different pools of hemolymph and in vitro cultured sporocysts. The results generated in both experiments were combined into a single dataset to provide an overall assessment of the proteomic response of encapsulating hemocyte in the presence and absence of larval S. mansoni. The combined dataset from each snail strains was then subjected to pair-wise comparison (NMRI vs. NMRI-SP, BS-90 vs. BS-90-SP, and BS-90-SP vs. NMRI-SP) of unique and commonly-occurring proteins identified in each combination. All proteins identified as S. mansoni were removed from the analyses. Unique proteins (identified in only one of the pair) were further trimmed by removing all proteins with a total normalized spectral counts of <2. The remaining unique proteins were then identified and gene ontology analyses were performed using Uniprot. Similarly, proteins shared in common in each paired comparison were further processed by removing proteins with total normalized spectral counts of <4. In anticipation of analyzing these common proteins for potential differential expression, we verified that each experiment had comparable protein sample sizes using normalized spectral counts of actin (XP_013070997.1, XP_013083901.1, XP_013083930.1, XP_013083929.1) as a housekeeping protein. By comparing the average actin spectral counts identified within each proteomic database, we observed count differences ranging from 1.2 to 18% between paired samples (BS-90 vs. BS-90-SP: 18%; NMRI vs. NMRI-SP: 1.2%; and BS-90-SP vs. NMRI-SP: 7%). Given this range of sampling, for proteins shared in common between groups, a fourfold difference in total normalized spectral counts was imposed as a conservative cut-off for determining differential protein expression in pairwise treatment comparisons.

RESULTS AND DISCUSSION

In an effort to better understand the molecular events associated with the differences observed at the host-parasite interface of B. glabrata and S. mansoni, a proteomic analysis of hemocytes actively engaged in the encapsulation of S. mansoni primary sporocyst was conducted. A MALDI-MS/MS approach was undertaken to analyze the expressed protein profile of hemocytes of inbred resistant BS-90 and susceptible NMRI B. glabrata snail strains involved in the encapsulation of S. mansoni (NMRI strain) primary sporocyst. The main goal of the study was to show the differential expression pattern of proteins expressed in hemocytes during the encapsulation of S. mansoni sporocyst, and to compare the proteins expressed in hemocytes of these snail strains in order to provide a better understanding of the molecular events associated with the innate immune response of B. glabrata snails to early developing sporocysts. Using this comparative proteomics approach, a total of 1061 B. glabrata hemocyte proteins were identified from cellular capsules in the presence and/or absence (control) of in vitro co-cultured S. mansoni sporocysts. Although an additional 155 S. mansoni proteins were identified, the current study will focus on hemocyte responses during parasitic encapsulation. All identified B. glabrata and S. mansoni proteins are listed in

![Figure 3](image.png)
Supplementary Table 1 and include normalized spectral counts per protein for each treatment. Raw MS proteomics data can be downloaded at the website address:
https://chorusproject.org/anonymous/download/experiment/defa50bf549a41dda7a156928ca0a03f under project reference number 1492.

Proteomic Response of Hemocytes to Encapsulated Sporocysts

Hemocytes of both the NMRI and BS-90 B. glabrata strains readily encapsulated S. mansoni sporocysts (SP) in vitro forming cellular aggregation of various sizes (Figures 1A,B). In the absence of sporocysts (controls), hemocytes of both snail strains also spontaneously formed cellular capsules of similar size and number as corresponding co-cultures. This design permitted simultaneous comparisons of expressed hemocyte proteins between snail strains in the presence and absence of sporocysts. As shown in Venn diagrams (Figure 3), following application of filtering criteria, the numbers of unique and common hemocyte proteins identified in control/sporocyst (NMRI/NMRI-SP, BS90/BS90-SP) and sporocyst/sporocyst (NMRI-SP/BS90-SP) combinations for each snail strain are presented. Table 1 highlights a list of selected identified unique proteins of particular interest in this study and are designated as presence (+), absence (−) or presence, but below the defined threshold cutoff (+/−) for each host/parasite comparison listed above. Similarly, proteins found to be shared in common among all compared treatments and strains, and presenting with a 4-fold difference in total normalized spectral counts, are listed in Tables 2–4.

Proteomic Response of Susceptible (NMRI) Snail Hemocytes to Encapsulated Sporocysts

In the susceptible NMRI strain of B. glabrata, 680 hemocyte proteins were shared between the control and sporocyst-encapsulated groups, while 94 proteins were unique to the NMRI-control group and 13 to the NMRI-SP group (Figure 3A). Of the hemocyte proteins shared in common, 47 showed at least a 4-fold difference between control and sporocyst encapsulations. Of these 47 common proteins, the vast majority (43/47; 91%) exhibited a striking reduction in specific proteins in the presence of sporocysts, suggesting a downregulation of expression in susceptible snail cells in response to parasite exposure (Table 2). This parasite-associated decrease in NMRI hemocyte protein expression is also mirrored in the number of unique proteins identified in control (94 proteins) compared to sporocyst (13 proteins) encapsulations. The majority of proteins (4-fold and unique) observed to be down-regulated in presence of parasites appears to involve protein synthesis/trafficking, metabolism, cell signaling, or oxidation-reduction (redox) activities (Table 2), all of which may have implications for an impaired immune capability. Among the down-regulated NMRI hemocyte proteins identified as potentially involved in immune-related functions in the presence of encapsulated sporocysts include the following: LPS binding protein (LBP), an antimicrobial protein capable of interacting with the larval tegument and secretions (43); apolipophorin, an immune-stimulating molecule in insects (44), found to be downregulated in snails exposed to microbial antigens (LPS, FCN, PGN) (45); and sequestosome 1/p62, a protein involved in various mechanisms, including apoptosis, innate immunity and autophagy (46). In addition to binding ubiquitinated proteins for basal cellular autophagy, p62 may also target intracellular pathogens for degradation through a p62-dependent selective autophagy mechanisms and participate in host defense. For example, in murine macrophages, activation of this selective autophagic mechanism by LPS-triggered TLR4 requires upregulation of p62 expression, suggesting a role of p62 in innate immune responses to pathogens (47). Of relevance here, TLRs have been implicated in B. glabrata-S. mansoni interactions as demonstrated by a differential increase in BgTLR protein levels associated with snail strains exhibiting resistance to S. mansoni (32). Our finding that p62/sequestosome1 is dramatically decreased in NMRI-SP capsules, suggests an impairment of hemocyte p62-mediated selective autophagy by a parasite-induced targeting of p62. A similar disruption of p62 has been postulated as a survival mechanism by some pathogens (46).

By contrast, 4 immune-related NMRI hemocyte proteins exhibited an increase in expression in the presence of parasites: An uncharacterized protein, containing a N-terminal death domain of interleukin-1 receptor-associated kinases (IRAK) followed by a catalytic domain of the serine/threonine kinases, argonaute 18, bacterialicidal permeability increasing protein (BPI), and arginase 1. Although uncharacterized, the presence of the death-domain of IRAK and serine/threonine kinase suggests this protein belongs to the IRAK superfamly, with a potential involvement in the Toll pathway (48). The presence of argonaute 18 suggests the involvement of small RNAs, possibly in gene expression regulation via RISC (RNA-induced silencing complex). B. glabrata snails (Belo Horizonte strain) exposed to S. mansoni exhibited an upregulation of argonaute gene expression at 24h post-infection, consistent with our current proteomic data (49). Our observed increase in BPI, a pattern recognition protein known to bind to lipopolysaccharides of Gram-negative bacteria (50), in hemocytes of both NMRI and BS-90 snails participating in larval encapsulations is in contrast to a reported decrease in BPI transcript response to the echinostome Echinostoma caproni in resistant B. glabrata (51), suggesting a potential pathogen specificity for BPI expression between S. mansoni-resistant B. glabrata strains. Finally, the finding that arginase1 expression is highly increased in the presence of sporocysts in susceptible host capsules may be of functional significance. Arginase is known to inhibit the production of NO by directly competing with nitric oxide synthase (NOS) for L-arginine (substrate), resulting in reduced levels of larval-killing NO production (52). Therefore, a parasite-induced triggering of hemocyte arginase production, as shown in the present study, may be serving as an anti-immune survival mechanism by creating a detoxified cellular microenvironment for encapsulated sporocysts.

Although proteins involved in redox reactions were found to be mostly down-regulated in presence of parasites (3-oxo-5-alpha-steroid 4-dehydrogenase 1; NADPH-cytochrome P450 reductase; peroxidasins; glutaredoxin-3; ferric-chelate reductase...
### TABLE 1

**Unique hemocyte proteins of interest identified in paired comparisons of susceptible (NMRI) and resistant (BS90) strains of *B. glabrata* in the presence and absence of *S. mansoni* sporocysts (SP) (NMRI vs. NMRI-SP; BS90 vs. BS90-SP; NMRI-SP vs. BS90-SP).**

| Protein identification | NMRI | NMRI-SP | BS90 | BS90-SP | NMRI-SP | BS90-SP |
|------------------------|------|---------|------|---------|---------|---------|
| **IMMUNE RELATED**     |      |         |      |         |         |         |
| Integrin alpha-PS1-like [Bg] | +    | −       | −    | −       | −       | −       |
| Fibrinogen-related protein F precursor [Bg] | +    | −       | +/−  | +       | −       | +/−     |
| Lipopolysaccharide-binding protein-like isoform X1 [Bg] | −    | −       | +/−  | +       | −       | +       |
| Interleukin-1 receptor-associated kinase -a [Mytilus galloprovincialis] | −    | +       | −    | +       | −       | +       |
| Fibrinogen-related protein F precursor [Bg] | +    | −       | −    | +       | +       | −       |
| Lipopolysaccharide-binding protein-like isoform X1 [Bg] | −    | −       | +/−  | +       | −       | +/−     |
| Interleukin-1 receptor-associated kinase -a [Mytilus galloprovincialis] | −    | +       | −    | +       | −       | +       |
| Histone H2A.V [Bg] | +    | −       | +    | +       | +       | +       |
| Bactericidal permeability-increasing protein-like [Bg] | +    | −       | −    | +       | −       | +       |
| Bactericidal permeability-increasing protein-like [Bg] | +    | −       | −    | +       | −       | +       |
| Redox 1 [Bg] | +    | −       | +    | +       | −       | +       |
| Glutaredoxin-3-like [Bg] | +    | −       | +/−  | +       | −       | +/−     |
| Peroxidase homolog [Bg] | +    | −       | −    | +       | −       | +       |
| SH3 and PX domain-containing protein 2A-like isoform X1 [Bg] | +    | −       | −    | +       | −       | +       |
| Glutamate-semialdehyde dehydrogenase DavD-like, partial [Bg] | +    | +       | +    | −       | +       | −       |
| Microsomal glutathione S-transferase 3-like [Bg] | +    | +/−     | +    | −       | +       | −/−     |
| Ferric-chelate reductase 1 [Bg] | +    | −       | +    | +       | −       | +       |
| Glutaredoxin-3-like [Bg] | +    | −       | +/−  | +       | −       | +/−     |
| Peroxidase homolog [Bg] | +    | −       | −    | +       | −       | +       |
| SH3 and PX domain-containing protein 2A-like isoform X1 [Bg] | +    | −       | −    | +       | −       | +       |
| Glutamate-semialdehyde dehydrogenase DavD-like, partial [Bg] | +    | +       | +    | −       | +       | −       |
| Microsomal glutathione S-transferase 3-like [Bg] | +    | +/−     | +    | −       | +       | −/−     |
| D-arabinono-1,4-lactone oxidase-like [Bg] | +    | −       | +    | −       | +       | −       |
| Superoxide dismutase [Cu-Zn]-like [Bg] | +    | −       | +    | −       | +       | −       |
| Dual oxidase-like [Bg] | +    | −       | +/−  | +       | −       | +/−     |
| Glutathione S-transferase 4-like [Bg] | +    | −       | +/−  | +       | −       | +/−     |
| Nicotinamide phosphoribosyltransferase -like [Bg] | +    | −       | +    | −       | +       | −       |
| Parkin coregulated gene protein homolog isoform X1 [Bg] | +    | −       | +    | −       | +       | −       |
| NADPH−cytochrome P450 reductase-like [Bg] | +    | −       | +/−  | +       | −       | +/−     |
| Cytochrome c oxidase subunit II (mitochondrion) [Bg] | +    | +       | +    | −       | +       | −       |
| Cytochrome c oxidase subunit II (mitochondrion) [Bg] | +    | +       | +    | −       | +       | −       |
| Cytochrome P450 2J3-like [Bg] | +    | −       | +    | −       | +       | −       |
| APOTOPSIS 1 [Bg] | +    | −       | +    | −       | +       | −       |
| Death-associated protein 1-like [Bg] | −    | −       | −    | +       | −       | +       |
| Lactase-3-like isoform X1 [Bg] | −    | −       | −    | +       | −       | +       |
| Isocitrate dehydrogenase [NADP] cytoplasmic-like isoform X1 [Bg] | −    | −       | −    | +       | −       | +       |
| NADPH−cytochrome P450 reductase-like [Bg] | +    | −       | +/−  | +       | −       | +/−     |
| Cytochrome c oxidase subunit II (mitochondrion) [Bg] | +    | +       | +    | −       | +       | −       |
| Cytochrome P450 2J3-like [Bg] | +    | −       | +    | −       | +       | −       |
| STRESS RESPONSE 1 [Bg] | +    | −       | +    | −       | +       | −       |
| T-complex protein 1 subunit gamma-like, partial [Bg] | +    | −       | −    | +       | −       | +       |
| Prefoldin subunit 1-like [Bg] | +    | +       | +    | −       | +       | −       |
| Parkin coregulated gene protein homolog isoform X1 [Bg] | +    | −       | +    | −       | +       | −       |
| CDGSH iron-sulfur domain-containing protein 2-like [Bg] | −    | −       | −    | +       | −       | +       |

(Continued)
## TABLE 1 | Continued

| NCBI acc. no. | Protein identification | NMRI | NMRI-SP | BS90 | BS90-SP | NMRI-SP | BS90-SP |
|---------------|------------------------|------|---------|------|---------|---------|---------|
| **SIGNALING** |                        |      |         |      |         |         |         |
| XP_013077238.1 | Coronin-1A-like [Bg]   | +    | +       | −    | +       | −       | +       |
| XP_013070025.1 | Rap1 GTPase-activating protein 1-like isoform X4 [Bg] | + | + | − | + | − | + |
| XP_013074990.1 | COP9 signalosome complex subunit 6-like [Bg] | + | − | + | − | − | − |
| XP_013086090.1 | COP9 signalosome complex subunit 4-like isoform X1 [Bg] | + | − | + | − | + | + |
| XP_013078600.1 | SH3 domain-containing kinase-binding protein 1-like isoform X1 [Bg] | + | − | + | + | − | − |
| XP_013079149.1 | SHC-transforming protein 1-like [Bg] | − | − | + | − | − | − |
| XP_013088835.1 | */SH3 Domain-containing guanine exchange factor [Crassostrea gigas]; EKC39783.1 (48%) | + | − | + | + | − | − |
| XP_013078554.1 | */Receptor-type tyrosine-protein phosphatase T-like isoform X1 [Bg]; XP_013072072.1 (40%) | + | − | + | + | − | − |
| **CALCIUM BINDING** |                        |      |         |      |         |         |         |
| XP_013077729.1 | Calmodulin-like, partial [Bg] | − | − | + | + | − | + |
| XP_013079182.1 | */Microtubule-actin cross-linking factor 1-like isoform X13 [Bg]; XP_013079259.1 (99%) | + | − | + | + | − | − |
| XP_013083369.1 | Clathrin intermediate 1-like isoform X3 [Bg] | + | − | + | − | + | + |
| XP_013091491.1 | 16 kDa calcium-binding protein-like [Bg] | + | − | + | + | − | − |
| XP_013072348.1 | Calcineurin subunit B type 1 [Bg] | − | − | + | + | − | − |
| XP_013060775.1 | Calumenin-like [Bg] | − | + | + | + | − | − |
| XP_013066201.1 | Gelsolin-like protein 1, partial [Bg] | − | + | + | + | − | − |
| XP_013088775.1 | Gelsolin-like protein 1 [Bg] | + | − | + | + | − | − |
| XP_013068705.1 | Peflin-like [Bg] | + | − | + | + | − | − |
| XP_013087123.1 | */Tensin-1-like, partial [Aplysia californica] (49%) | + | − | + | + | − | − |
| **TRANSCRIPTION/TRANSLATION PROCESS** |                        |      |         |      |         |         |         |
| XP_013063581.1 | 60S Ribosomal protein L14-like [Bg] | +− | − | + | + | − | − |
| XP_013075095.1 | 60S Ribosomal protein L31-like [Bg] | + | − | + | + | − | − |
| XP_013089238.1 | 60S Ribosomal protein L36-like [Bg] | + | − | + | + | − | − |
| XP_013096652.1 | 60S Ribosomal protein L37a-like [Bg] | + | − | + | + | − | − |
| XP_013091387.1 | Host cell factor-like isoform X1 [Bg] | + | − | + | + | − | − |
| XP_013062003.1 | LUM and senescent cell antigen-like-containing domain protein1 isoform X1 [Bg] | + | − | + | + | − | − |
| XP_013062476.1 | Signal recognition particle subunit SRP72-like [Bg] | − | − | + | + | − | − |
| **METABOLISM** |                        |      |         |      |         |         |         |
| XP_013083732.1 | Inter-alpha-trypsin inhibitor heavy chain H3-like isoform X1 [Bg] | + | − | + | + | − | − |
| XP_013079110.1 | Alpha-N-acetylgalactosaminidase-like isoform X1 [Bg] | + | − | + | + | − | − |
| XP_013060405.1 | Gamma-glutamyl hydrolase A-like [Bg] | + | − | + | + | − | − |
| **TRAFFICKING** |                        |      |         |      |         |         |         |
| XP_01303454.1 | Intersectin-1-like [Bg] | + | − | + | + | − | − |
| XP_01304430.1 | Nuclear pore membrane glycoprotein 210-like [Bg] | + | − | + | + | − | − |
| XP_013086966.1 | AP-3 complex subunit delta-1-like isoform X1 [Bg] | + | − | + | + | − | − |
| XP_013060735.1 | Sorting nexin-33-like, partial [Bg] | + | − | + | + | − | − |
| **MISC.** |                        |      |         |      |         |         |         |
| XP_013092372.1 | Endothelin-converting enzyme 2-like [Bg] | − | − | + | + | − | − |
| XP_013063554.1 | Basement membrane-specific heparan sulfate proteoglycan core protein-like [Bg] | + | + | − | + | − | − |

Unique proteins are organized by functional category and NCBI accession numbers and protein identification are listed. For each paired comparison, (+) indicates the presence of the protein; (−) indicates the absence of the protein and (+/−) indicates the protein presence, but the threshold of less than 2 peptides was not met. Uncharacterized protein are indicated (*), followed by their closest NCBI Blastp IDs and ID percentages.
Table 2: Proteins shared in common between control NMRI and sporocyst-encapsulating (NMRI-SP) hemocytes that present at least a 4-fold difference in total normalized spectral counts.

| NCBI acc. no. | Protein identification | NMRI | NMRI-SP | Function |
|---------------|------------------------|------|---------|----------|
| **IMMUNE RELATED** | | | | |
| XP_013070669.1 | Lipopolysaccharide-binding protein-like [Bg] | 9.57 | 1.01 | Cellular defense |
| XP_013070436.1 | Syntenin-1-like isoform X1 [Bg] | 11.96 | 0.96 | Immunomodulation |
| XP_0130682155.1 | Apolipoporphins-like isoform X1 [Bg] | 9.57 | 0.96 | Detox LPS |
| XP_013067749.1 | Lysosomal alpha-mannosidase-like, partial [Bg] | 5.30 | 0.96 | Killing of foreign material |
| XP_013066053.1 | */ Sequestosome-1-like [Limulus polyphemus] XP_013792674.1 (36%) | 18.78 | 0.96 | Autophagy |
| XP_013093897.1 | Lysosomal alpha-glucosidase-like isoform X1 [Bg] | 7.44 | 1.01 | Degradation of Phagocytosed materials |
| XP_013088376.1 | Bactericidal permeability-increasing protein-like [Bg] | 1.11 | 4.89 | Anti-microbial |
| XP_013074089.1 | Arginase-1-like isoform X3 [Bg] | 15.05 | | |
| **REDOX** | | | | |
| XP_013077724.1 | */ Carbonyl reductase [NADPH] 1-like [Bg] XP_013077725.1 (74%) | 4.27 | 0.96 | Redox |
| XP_013076465.1 | 3-Oxo-5-alpha-steroid 4-dehydrogenase 1-like [Bg] | 4.37 | 1.01 | NADPH binding/Redox |
| XP_013093038.1 | Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial-like [Bg] | 1.02 | 6.86 | Mitochondrial electron transport |
| XP_013073195.1 | Cytochrome c oxidase subunit 4 isoform 1, mitochondrial-like [Bg] | 1.11 | 4.84 | Mitochondrial electron transport |
| **STRESS RESPONSE** | | | | |
| XP_013091012.1 | Stromal cell-derived factor 2-like isoform X1 [Bg] | 4.27 | 1.01 | Regulation apoptosis/ chaperonin |
| XP_013065387.1 | Protein FAM107B-like, partial [Bg] | 4.27 | 0.96 | Similarity with heat-shock proteins |
| **SIGNALING** | | | | |
| XP_013088940.1 | G-protein coupled receptor 64-like isoform X1 [Bg] | 4.18 | 0.96 | Signaling proteins |
| XP_013077039.1 | */ Receptor-type tyrosine-protein phosphatase alpha-like [Bg] XP_013063115.1 (42%) | 4.27 | 0.96 | MAPK cascade |
| XP_013077041.1 | Receptor-type tyrosine-protein phosphatase alpha-like, partial [Bg] | 5.30 | 0.96 | MAPK cascade |
| XP_013081619.1 | Serine/threonine-protein kinase PAK 2-like [Bg] | 8.55 | 1.97 | Signaling |
| **CELL ADHESION** | | | | |
| XP_013081823.1 | Collagen alpha-1(XVIII) chain-like [Bg] | 8.55 | 1.91 | Cell adhesion |
| XP_013066335.1 | */ Neurexin-1-like [Aplysia californica] XP_012941838.1 (48%) | 38.20 | 1.91 | Cell adhesion |
| **TRANSCRIPTION/TRANSLATION PROCESS** | | | | |
| XP_013061577.1 | 40S Ribosomal protein S25-like [Bg] | 4.27 | 0.96 | Ribosomal |
| XP_013084756.1 | 60S Ribosomal protein L19-like, partial [Bg] | 4.18 | 1.01 | Ribosomal |
| XP_013071682.1 | 60S Ribosomal protein L30-like [Bg] | 4.37 | 0.96 | Ribosomal |
| XP_013073467.1 | 60S Ribosomal protein L34-like [Bg] | 5.39 | 1.01 | Ribosomal |
| XP_013063478.1 | 60S Ribosomal protein L8 [Bg] | 21.55 | 4.95 | Ribosomal |
| XP_013084934.1 | Heterogeneous nuclear ribonucleoprotein U-like protein 1 isoform X1 [Bg] | 8.28 | 1.97 | Transcription regulation |
| XP_013070062.1 | Protein LZIC-like [Bg] | 5.39 | 1.01 | B-catenin binding |
| XP_013081352.1 | Protein TFG-like isoform X1 [Bg] | 4.37 | 0.96 | Regulation of NFKB signaling |
| XP_013063363.1 | Ribonuclease UK114-like [Bg] | 5.30 | 1.01 | Translation inhibition |
| XP_013062948.1 | RNA-binding protein lark-like [Bg] | 5.39 | 0.96 | Translation regulation |
| XP_013075272.1 | Serine/arginine repetitive matrix protein 2-like [Bg] | 4.37 | 0.96 | Pre-mRNA splicing |
| XP_013069331.1 | Serine/arginine-rich splicing factor 7-like [Bg] | 4.18 | 1.01 | Pre-mRNA splicing |
| **METABOLISM PROCESS** | | | | |
| XP_013069940.1 | Alpha-galactosidase A-like isoform X1 [Bg] | 5.48 | 1.01 | Glycosidase |
| XP_013076662.1 | Succinyl-CoA-3-ketoacid coenz. A transferase 1, mitochondrial-like [Bg] | 8.37 | 1.01 | Ketone body catabolism |
| XP_013062675.1 | UTP-glucose-1-phosphate uridyltransferase-like [Bg] | 9.75 | 0.96 | Cellular metabolic pathways |
| XP_013061266.1 | 1,4-alpha-glucan-branching enzyme-like [Bg] | 4.27 | 0.96 | Glycogen biosynthesis |
| XP_013092915.1 | 2-amino-3-ketobutyrate coenzyme A ligase, mitochondrial-like [Bg] | 8.64 | 1.91 | Cellular amino acid metabolism |
| XP_013094977.1 | 3-oxoacyl-[acyl-carrier-protein] reductase FabG-like [Bg] | 5.48 | 1.01 | Fatty acid biosynthesis |

(Continued)
TABLE 2 | Continued

| NCBI acc. no. | Protein identification | NMRI | NMRI-SP | Function |
|---------------|------------------------|------|---------|----------|
| PROTEIN SORTING AND TRAFFICKING | | | | |
| XP_013069878.1 | AP-1 complex subunit beta-1-like [Bg] | 9.86 | 1.91 | Clathrin binding/ transporter activity |
| XP_013096772.1 | Charged multivesicular body protein 4b-like [Bg] | 12.73 | 2.92 | Multivesicular body pathway |
| XP_013061300.1 | FK506-binding protein 15-like [Bg] | 5.39 | 1.01 | Transport of early endosomes |
| XP_013067947.1 | Huntingtin-interacting protein 1-like isoform X1 [Bg] | 4.18 | 0.96 | Trafficking |
| XP_013082360.1 | Vacular protein sorting-associated protein 27 isoform X1 [Bg] | 5.50 | 0.96 | Trafficking |
| XP_013080513.1 | Vacular protein sorting-associated protein 35-like [Bg] | 6.50 | 0.96 | Trafficking |
| MISCELLANEOUS | | | | |
| XP_013069471.1 | */Coiled-coil protein 90B, mitochondrial [Crassostrea gigas] EKC25784.1 (50%) | 6.41 | 0.96 | Transmembrane protein |
| XP_013080174.1 | */Voltage-dependent calcium channel y2 subunit-like [Bg] XP_013080170.1 (40%) | 5.39 | 1.01 | Voltage-dependent calcium channel |
| XP_013069538.1 | V-type proton ATPase 116 kDa subunit 1 [Bg] | 4.27 | 0.96 | Proton transporter |

NCBI accession numbers and protein identification are listed. Color backgrounds indicate an increased spectral count for proteins in the corresponding treatment. Uncharacterized proteins (*) are followed by their closest NCBI Blastp IDs and ID percentages.

1), two others, cytochrome c oxidase (Cyt c) and succinate dehydrogenase (ubiquinone-containing complex) exhibited increased protein expression. Cyt c and ubiquinone are part of the mitochondrial electron transport chain that, in addition to generating ATP, also are involved in the production of ROS. However, it is not known whether metabolically-generated ROS from this source may be serving a host immune effector role. More likely, the increased expression of these proteins probably reflects an enhancement of metabolic stress of host cells in response to sporocysts.

Overall, hemocytes of susceptible NMRI snails in the presence of S. mansoni sporocysts appear to have 2 distinct responses: A general down-regulation of proteomic activity across various protein groups, and a discrete increase of selected proteins, suggesting that different mechanisms potentially may be initiated either by the host or parasite. Although ROS, BPI and the death domain-IRAK containing protein increases are likely to be initiated by the host in response to a pathogen invasion, the increase of arginase, and with it, a resulting decrease in toxic NO production, or the general protein knockdown observed in NMRI hemocytes, could be a direct result of host modulation by the parasite to favor its own survival.

Proteomic Response of Resistant (BS-90) Snail Hemocytes to Encapsulated Sporocysts

As a result of proteomic analysis of BS-90 hemocyte capsules, 696 proteins were identified as shared in common between unexposed (BS90) and S. mansoni sporocyst-encapsulated (BS90-SP) hemocyte groups, while 35 and 32 unique proteins were identified in BS90 and BS90-SP hemocyte samples, respectively (Figure 3B). Similar to the NMRI treatment groups, 44 of the identified common proteins met the 4-fold minimum threshold difference (Table 3). However, in contrast to susceptible snail hemocytes, approximatively the same number of sporocyst-encapsulating BS-90 cell proteins were observed to be up and down-regulated in categories such as protein processing, metabolism, signaling, redox or immune-related proteins, with 59% of the proteins exhibiting a 4-fold decrease in presence of parasite.

Contrary to the near complete shutdown of protein processing/metabolism in larval-encapsulating NMRI hemocytes, we observed an increase in 40S ribosomal proteins and others involved in mRNA/tRNA processing in BS-90 capsules indicating a marked enhancement of cellular translation processes in response to contact with encapsulated larvae (Table 3). By contrast, members of the argonaute family proteins (piwi and argonaute2), potentially involved in gene expression regulation via mi/piRNA were decreased in presence of sporocysts, suggesting a decrease in transcription mechanisms. Apoptosis also appears to be downregulated with a decrease in apoptosis-inducing factor 3, and a concurrent increase in an anti-apoptotic protein (acidic leucine-rich nuclear phosphoprotein 32), suggesting an overall reduction of the apoptosis mechanism in presence of parasites. Host modulation of hemocyte apoptosis in response to direct contact with the parasite, would likely prevent premature cell death in effector hemocytes thereby sustaining a more effective immune response. Interestingly, like NMRI hemocytes, sequestosome 1/p62 also was sharply down-regulated in sporocyst-encapsulated BS-90 cells (Table 3) suggesting that, although clearly a response to larval contact, hemocytes apparently do not require this protein for their cytotoxic activity. Upregulation of other immune-related genes such as T-complex protein 1, BPI (Table 1) and N-acetylgalactosamine kinase, involved in GalNAc binding interactions (Table 3) would be consistent with a functioning immune system.

The snail's redox system plays a central role, not only in maintaining the host's own redox balance, but as a primary
### TABLE 3 | Proteins shared in common between control BS90 and sporocyst-encapsulating (BS90-SP) hemocytes that present at least a 4-fold difference in total normalized spectral counts.

| NCBI acc. no. | Protein identification | BS90 | BS90-SP | Function |
|---------------|------------------------|------|---------|----------|
| **IMMUNE-RELATED** |                        |      |         |          |
| XP_013095968.1 | */Proteasome subunit alpha type-5-like [Aplysia californica] XP_005110071.1 | 3.938 | 0.94133 | Proteasome |
| XP_013066054.1 | Sequestosome-1-like [Bg] | 7.447 | 1.0082  | Autophagy |
| XP_013066053.1 | */Sequestosome-1-like [Limulus polyphemus] XP_013792674.1 | 29.823 | 4.0329 | Autophagy |
| XP_013064208.1 | Reelin-like [Bg] | 20.299 | 2.8909 | Lipoprotein receptor binding |
| XP_013079231.1 | N-acetylgalactosamine kinase-like [Bg] | 0.9309 | 3.8991 | Galactose binding |
| XP_013062216.1 | 26S proteasome non-ATPase regulatory subunit 12-like [Bg] | 1.0381 | 4.7735 | Proteasome |
| **REDOX** |                        |      |         |          |
| XP_013075526.1 | Glutathione S-transferase 7 [Bg] | 8.6998 | 2.0164 | Detoxification of ROS |
| XP_0130989814.1 | Alcohol dehydrogenase class-3-like [Bg] | 3.938 | 0.94133 | Redox |
| XP_01307425.1 | Ferric-chelate reductase 1 [Bg] | 4.8689 | 1.0082 | Redox |
| XP_01307342.1 | Aldehyde dehydrogenase family 16 member A1-like [Bg] | 10.883 | 1.94953 | Redox |
| XP_013066335.1 | */Neurexin-1 [Aplysia californica] XP_012941838.1 | 20.621 | 7.7982 | Fatty acid alpha-oxidation |
| XP_013084558.1 | Dual oxidase-like [Bg] | 0.9309 | 4.0329 | Generates ROS |
| **APOPTOSIS AND REGULATION** |                        |      |         |          |
| XP_013091656.1 | Apoptosis-inducing factor 3-like isoform X1 [Bg] | 7.6617 | 1.0082 | Pro-apoptosis |
| XP_013062216.1 | 26S proteasome non-ATPase regulatory subunit 12-like [Bg] | 1.0381 | 4.7735 | Proteasome |
| **STRESS RESPONSE** |                        |      |         |          |
| XP_013094041.1 | Universal stress protein Sr1101-like [Bg] | 3.8309 | 0.94133 | Response to stress |
| **SIGNALING** |                        |      |         |          |
| XP_01309708.1 | Protein Mo25-like [Bg] | 0.9309 | 3.7653 | Calcium binding protein |
| **CELL ADHESION/MOVEMENT** |                        |      |         |          |
| XP_013073410.1 | Sushi, von Willebrand factor A, EGF /pentraxin domain-containing protein 1-like [Bg] | 4.8689 | 0.94133 | Cell attachment process |
| XP_013092731.1 | Triple functional domain protein-like, partial [Bg] | 4.1522 | 1.0082 | Actin remodeling |
| XP_013082027.1 | Myosin heavy chain, striated muscle-like isoform X1 [Bg] | 5.1903 | 1.0082 | Muscle contraction |
| **TRANSCRIPTION/TRANSLATION PROCESS** |                        |      |         |          |
| XP_01309238.1 | 60S Ribosomal protein L36-like [Bg] | 3.938 | 0.94133 | Ribosomal |
| XP_01308258.1 | Eukaryotic translation initiation factor 3 subunit K-like [Bg] | 4.9761 | 0.94133 | Translation regulation |
| XP_013096529.1 | Eukaryotic peptide chain release factor subunit 1 [Bg] | 3.8309 | 0.94133 | Translation regulation |
| XP_01309019.1 | Protein disulfide-isomerase A4-like [Bg] | 4.0451 | 1.0082 | Protein folding |
| XP_01309243.1 | */DNA ligase 1-like isoform X3 [Bg] XP_013093256.1 | 16.683 | 3.8322 | DNA binding/ repair |
| XP_013078837.1 | 40S Ribosomal protein S12-like [Bg] | 1.0381 | 5.7817 | Ribosomal |
| XP_013061577.1 | 40S Ribosomal protein S25-like [Bg] | 1.0381 | 4.8404 | Ribosomal |
| XP_013073681.1 | Cleavage and polyadenylation specificity factor subunit 5-like [Bg] | 1.969 | 9.6809 | Polyadenylation |
| XP_01308527.1 | Bifunctional glutamate/proline-tRNA ligase-like [Bg] | 1.0381 | 4.8404 | Translation regulation |
| XP_01306775.1 | Calumenin-like [Bg] | 0.9309 | 6.5893 | Calcium binding |
| **METABOLISM** |                        |      |         |          |
| XP_013093293.1 | Trifunctional nucleotide phosphoesterase protein YKA-N-like, partial [Bg] | 4.7618 | 0.94133 | Nucleotide catabolic process |
| XP_013063683.1 | Sulfortransferase family cytosolic 1B member 1-like, partial [Bg] | 4.9761 | 1.0082 | Lipid metabolism |
| XP_013096124.1 | Stomatin-like protein 2, mitochondrial [Bg] | 4.9761 | 1.0082 | Lipid binding |
| XP_013069854.1 | Phosphonoacetaldehyde hydrolase-like [Bg] | 0.9309 | 8.8064 | Phosphonate catabolism |
| XP_013070092.1 | Ethanolamine-phosphate cytidylyltransferase-like isoform X1 [Bg] | 0.9309 | 3.8991 | Lipid metabolism |

(Continued)
TABLE 3 | Continued

| NCBI acc. no. | Protein identification | BS90 | BS90-SP | Function |
|---------------|------------------------|------|---------|----------|
| XP_013094998.1 | Enoyl-CoA hydratase, mitochondrial-like isoform X1 [Bg] | 1.0381 | 4.7735 | Lipid metabolism |
| XP_013075978.1 | Perilipin-2-like [Bg] | 1.0381 | 4.8404 | Lipid metabolism |

**PROTEIN SORTING AND TRAFFICKING**

| NCBI acc. no. | Protein identification | BS90 | BS90-SP | Function |
|---------------|------------------------|------|---------|----------|
| XP_013077091.1 | ATP synthase subunit g, mitochondrial-like, partial [Bg] | 4.9761 | 0.9413 | Proton transporter |
| XP_013085659.1 | Coatomer subunit beta-like [Bg] | 5.7998 | 1.0082 | Protein transporter |
| XP_013086081.1 | *Tripartite motif-containing protein 3-like isoform X1 [Pomacea canaliculata] (25%) | 4.0451 | 0.9413 | Vesicular trafficking |
| XP_013073443.1 | Dynamin-1-like [Bg] | 8.0902 | 1.0082 | Endocytosis. |

**MISC**

| NCBI acc. no. | Protein identification | BS90 | BS90-SP | Function |
|---------------|------------------------|------|---------|----------|
| XP_013062313.1 | Unch. prot. LOC106051655 [Bg] | 4.0451 | 28.369 | Unknown |
| XP_013069315.1 | */Caffeoyl-CoA O-methyltransferase 1 [Bg] XP_013087214.1 (59%) | 0.9309 | 3.96603 | Methyl transferase |

NCBI accession numbers and protein identification are listed. Color backgrounds indicate an increased spectral count for proteins in the corresponding treatment. Uncharacterized proteins (*) are followed by their closest NCBI Blastp IDs and ID percentages.

Host Strain Comparison of NMRI and BS-90 B. glabrata Hemocyte Responses to Sporocysts

Paired comparison of the proteomic responses of B. glabrata strains to the presence of S. mansoni sporocysts (NMRI-SP vs. BS-90-SP) revealed 76 unique proteins in the BS90-SP group, 29 unique proteins in NMRI-SP group and 642 commonly expressed proteins (Figure 3C). A total of 39 proteins shared between the two strains, presented a minimum of 4-fold differences. A large majority of these proteins (9/39, 77%) were found to be decreased/downregulated in NMRI-SP compared to BS90-SP suggesting either a suppressive parasite effect on the susceptible snail host and/or an enhanced response by the resistant host strain to encapsulated parasite (Table 4). As for unique proteins of interest, all of those found earlier in BS90-SP capsules when compared to BS-90 controls were also uniquely present in BS90-SP when compared to NMRI-SP, suggesting either a lack/delay in the recognition of, or response to, sporocysts by NMRI hemocytes at this time point and/or a parasite-mediated suppression exclusively in hemocyte of the NMRI snail strain. In total, we observed 30 proteins uniquely expressed in BS90-SP compared to NMRI-SP that appear to be constitutively present in hemocytes of the BS-90 B. glabrata strain, which could be an important contributing factor to earlier parasite recognition/response in resistant snails.

Among those specific proteins with reduced expression in NMRI-SP compared to BS-90-SP, a high proportion were associated with immune, redox and cell signaling functions. For immune-related proteins, those exhibiting decreased expression include LPS binding protein (LBP), syntenin1, and lysosomal alpha-mannosidase (Table 4), as well as uniquely identified perilipin 2, BPI protein, and fibrinogen-related protein A precursor (FREP A precursor) (Table 1). As previously mentioned, LBP from snail plasma has been found to interact with the tegument and secretions of S. mansoni sporocysts (43) and therefore, differences may reflect lower NMRI
TABLE 4 | Proteins shared in common between sporocyst (SP)-encapsulating hemocytes of susceptible NMRI and resistant BS90 *B. glabrata* that present at least a 4-fold difference in total normalized spectral counts.

| NCBI acc. no. | Protein identification                                      | BS90-SP  | NMRI-SP  | Function                        |
|--------------|-------------------------------------------------------------|----------|----------|---------------------------------|
| XP_013070436.1 | Syntenin-1-like isoform X1 [Bg]                             | 3.96603  | 0.95609  | Trafficking/immunomodulation     |
| XP_013067749.1 | Lysosomal alpha-mannosidase-like, partial [Bg]              | 3.8991   | 0.95609  | Oligosaccharide catabolism       |
| XP_013070669.1 | Lipopolysaccharide-binding protein-like [Bg]               | 14.923   | 1.0127   | Innate immune response          |
| XP_013078814.1 | * Proteoglycan 4-like [Aplysia californica] XP_005092461.1 (45%) | 8.6727   | 1.98879  | Immune response                 |
| XP_013079231.1 | N-acetylgalactosamine kinase-like [Bg]                     | 3.8991   | 0.95609  | Galactose binding               |
| XP_013062408.1 | Reelin-like [Bg]                                            | 2.8909   | 16.3097  | Lipoprotein receptor binding     |
| XP_013077724.1 | *Carbonyl reductase [NADPH] 1-like [Bg] XP_013077725.1 (74%) | 19.6296  | 0.95609  | Redox                           |
| XP_013068834.1 | 3-Hydroxacyl-CoA dehydrogenase type-2-like [Bg]            | 4.7735   | 0.95609  | Lipid metabolism                |
| XP_013072118.1 | Glycogen debranching enzyme-like [Bg]                      | 4.7735   | 0.95609  | Catabolic glycogen process       |
| XP_013062757.1 | UTP-glucose-1-phosphate uridylyltransferase-like [Bg]      | 7.7313   | 0.95609  | Glucose metabolism              |
| XP_013061577.1 | 40S Ribosomal protein S25-like [Bg]                        | 4.8404   | 0.95609  | Translation initiation          |
| XP_013062948.1 | RNA-binding protein lark-like [Bg]                         | 5.7148   | 0.95609  | Translation regulation          |
| XP_013063676.1 | * Nuclearin-like [Aplysia californica] XP_005109234.1 (31%) | 5.0411   | 0.95609  | Translation regulation          |
| XP_013061577.1 | 40S Ribosomal protein S25-like [Bg]                        | 4.8404   | 0.95609  | Translation initiation          |
| XP_013088283.1 | *Cartilage matrix protein-like [Aplysia californica] XP_005102985.2 (50%) | 5.98243  | 0.95609  | Collagen binding                |
| XP_013075555.1 | ATP synthase subunit I, mitochondrial [Bg]                 | 1.0062   | 6.9192   | Proton transport                |

NCBI accession numbers and protein identification are listed. Color backgrounds indicate an increased spectral count for proteins in the corresponding treatment. Uncharacterized proteins (*) are followed by their closest NCBI Blastp IDs and ID percentages.
plasma LBP concentrations and/or decreased hemocyte LBP synthesis. Syntenin 1 is an adapter protein, containing PDZ (postsynaptic density, discs large, zona-occludens) domains, allowing for multiprotein complex assembly (58) involved in various mechanisms including apoptosis regulation (eukaryotic translation initiation factor 5A) and immunity (alpha-2-macroglobulin) (59, 60). Alpha-mannosidase-like is a lysosomal hydrolase that, similar to other known hydrolases in B. glabrata hemolymph (61), can be released from hemocytes to degrade pathogen-associated glycans (62), while periplin 2, a component of lipid droplets (LD), may potentially be involved in immune reactivity to microbial pathogens as shown in Aedes mosquitoes (63).

FREP A precursor protein, which presents a 96% identity with FREP 2.1, was found constitutively expressed solely in hemocytes of the BS-90 resistant strain. FREPs represent a diverse group of lectin PRRs (27, 64) that are closely linked to the snail’s immune response against larval S. mansoni (65). Consistent with our current data, FREP2 transcripts were found to be dramatically increased following S. mansoni exposure of BS-90, but not susceptible M-line, snails (66). Although FREP2 is present in the plasma of both susceptible and resistant B. glabrata strains, and is reactive with glycoproteins [e.g., polymorphic mucins (67)] of larval S. mansoni, our finding of these immune proteins in BS90/BS-90-SP hemocyte capsules, but not those of NMRI/NMRI-SP, suggests a specific interaction of a FREP2 subfamily variant with BS-90 hemocytes during cell-cell or cell-parasite aggregation.

Similarly, we observed numerous redox proteins that were reduced or downregulated in NMRI-SP capsules when compared to those of BS90-SP, including SOD1, thioredoxin reductase 1, GST4, dual oxidase, laccase 3 and hemoglobins 1 and 2. H2O2 and NO production have previously been shown to be critical to in vitro killing of S. mansoni sporocysts in the snail (53). Hemocyte SOD1, especially important as it catalyzes the conversion of O2− to H2O2 during encapsulation, has been shown to have differential activity and transcriptomic levels according to the resistance/susceptibility of selected snail strains (57). Similarly, dual oxidase and laccase 3, as described in the previous section, also are involved in H2O2 production and phenoxidase activity, respectively, thereby contributing to the innate immune defenses in the host (56). Thioredoxin1 (TRX1) and GST4 are antioxidants likely contributing to cellular redox homeostasis that would be required by the hemocytes to regulate their own production of ROS in response of the parasite, thereby preventing intracellular damage while also engaging in cytotoxic activities. In NMRI-SP encapsulations, a similar homeostatic mechanism may not be required since NMRI hemocytes were not found to release as much ROS in presence of parasites. GST was previously reported to be upregulated in snails exposed to sublethal doses of molluscicides, suggesting a stress-related response more than a specific immune response to a pathogen (68).

One explanation for the comparatively weak response of encapsulating NMRI hemocytes to sporocysts is that susceptible snail strains are, in general, immunologically inferior to the resistant strains. However, previous studies have shown that a single strain of B. glabrata can be both susceptible to one strain of S. mansoni while at the same time resistant to a different S. mansoni strain (34, 35), indicating that parasite infectivity factors are equally important in determining host-parasite compatibility as the genetic makeup of the snail host (2–4). This also holds true for snails confronted with other non-schistosome pathogens. For example, Echinostoma paraensaeci has been shown to infect both R and S strains of B. glabrata (66, 69), while bacterial pathogens Paenibacillus (70) and Vibrio parahaemolyticus (71) infect all exposed B. glabrata strains regardless of their schistosome-susceptibility status. This strongly suggests that the S and R phenotypes exhibited by these model B. glabrata/S. mansoni systems are host/parasite strain-specific, and generalizations as to their immune status or susceptibility to other pathogens cannot be made.

Finally, we demonstrated that NMRI and BS-90 snail hemocytes, irrespective of the presence or absence of sporocysts, consistently differed in their levels of hemoglobins (Hb1 and 2, with BS-90 capsules containing more protein (based on fourfold higher total normalized spectral counts) than NMRI (Supplementary Table 2). This difference is most likely due to different levels of plasma Hb binding to hemocytes (72) and/or sporocysts (73, 74), and not due to the synthesis and differential expression by encapsulating cells. The immune-relevance of hemocyte-bound Hb is presently unclear. However, it has previously been demonstrated that snail Hb was toxic to schistosome sporocysts in vitro (75) and that reactions of Hb-conjugated Fe2+ with NO can lead to the generation of peroxynitrites, a powerful RNS with potential pathogen killing capacity (76). Recently, a role for Hb2 as a parasite immune “sensing” protein has been postulated (74), thereby opening up the possibility that higher constitutive levels of cell-bound Hb (as shown in the present study) may contribute to an early response/killing by resistant BS-90 snail hemocytes in this model system. In addition to Hb, other B. glabrata plasma proteins with sporocyst membrane-binding activity (43, 67) were found to be associated with capsular hemocytes including several with known or proposed immune function: LBP, fibrinogen-like protein A (FREP 2.1), FREP F precursor, alpha-amylase and an actin2 protein (4, 50, 64, 67, 74). Although hemocytes themselves may be the source of these proteins, it is more likely that they are of plasma origin and possess hemocyte and/or sporocyst binding activity.

In summary, we have investigated the proteomic response of B. glabrata hemocytes during in vitro encapsulation of S. mansoni sporocysts by comparing the proteomic profiles of reactive hemocytes both within and between susceptible and resistant snail strains. In the susceptible NMRI B. glabrata strain, we observed a dramatic downregulation of proteins during parasite encapsulation, many of which are related to immune, redox and autophagic function. Of the few upregulated proteins, one (arginase 1) appeared to be induced by the parasite to favor its survival during encapsulation. A more balance proteomic response was exhibited by resistance BS-90 snails, supporting a more robust immune capacity. These included several mechanisms involved in parasite killing such as an upregulation of proteins associated with H2O2 production (SOD1, dual oxidase) and antioxidants to counter intracellular...
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MC and TY developed study concept and, with ND, created the experimental design. MC performed experiments. MC, ND, X-JW, and UB-W data analysis and interpretation. GS generated proteomic data. GS, ND, and UB-W bioinformatic analyses. ND, MC, GS, and TY manuscript writing and editing. ND and TY critical review and final editing. All authors approved the final submitted draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02773/full#supplementary-material

SUPPLEMENTARY Table 1 | Biomphalaria glabrata hemocyte proteins identified in two replicate experiments. Each entry lists the identified protein, NCBI accession number, molecular weight and normalized spectral counts for each treatment: control hemocyte capsules (BS90, NMRI), hemocyte capsules with sporocysts (BS90-SP, NMRI-SP). Numbers 1 and 2 (e.g., BS90 1) represent the first and second independent replicate, respectively.

SUPPLEMENTARY Table 2 | List of proteins showing high identities to Biomphalaria glabrata hemoglobins (Hb) 1 or 2. NCBI accession numbers and molecular weights are listed for each entry (NMRI, NMRI-SP, BS90, BS90-SP) with their corresponding total normalized spectral counts. Initial protein identifications were returned as “uncharacterized protein.” However, subsequent BlastP searches of these sequences revealed highest identities to B. glabrata Hb 1 and 2.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer CA declared a past co-authorship with one of the authors TY, to the handling Editor.

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