Trends in Parasitology

CellPress REVIEWS

Review

Schistosomiasis from a Snail's Perspective: Advances in Snail Immunity

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The snail's immune response is an important determinant of schistosome infection success, acting in concert with host, parasite, and environmental factors. Coordinated by haemocytes and humoral factors, it possesses immunological hallmarks such as pattern recognition receptors, and predicted gastropod-unique factors like the immunoglobulin superfamily domain-containing fibrinogen-related proteins. Investigations into mechanisms that underpin snail–schistosome compatibility have advanced quickly, contributing functional insight to many observational studies. While the snail's immune response is important to continue studying from the perspective of evolutionary immunology, as the foundational determinants of snail–schistosome compatibility continue to be discovered, the possibility of exploiting the snail for schistosomiasis control moves closer into reach. Here, we review the current understanding of immune mechanisms that influence compatibility between *Schistosoma mansoni* and *Biomphalaria glabrata.*

The Integral Role of Snails to Schistosome Biology and Schistosomiasis

Snails and schistosomes have a coevolutionary relationship spanning more than 200 million years [1]. Schistosomes are parasitic, with a life cycle alternating between a gastropod mollusc and vertebrate hosts (Figure 1). They have strong host specificity, particularly for the gastropod first intermediate host, in which they undergo their larval development [2,3], and which also dictates the geographical distribution of **schistosomiasis** (see Glossary) [4–6]. Yet, not every encounter between a schistosome and a snail species deemed compatible results in the infection of the latter [7]. Understanding the basis of this compatibility polymorphism is the core interest of many studies investigating snail–schistosome interactions. Such knowledge is expected to be applicable in curtailing diseases caused by schistosomes through transmission blockade at the level of the snail host [2].

Several species of *Schistosoma* infect humans, but three are responsible for most of the infections: *Schistosoma mansoni* (infects *Biomphalaria* snails), *Schistosoma haematobium* (infects *Bulinus* snails), and *Schistosoma japonicum* (infects *Oncomelania* snails) [8]. Most studies of snail–schistosome interactions utilize the *Biomphalaria glabrata*–*S. mansoni* model [9]. From studies of this model, the snail's immune response (Figure 2, Key Figure) has emerged as an important determinant of the success of *S. mansoni* infection, acting in concert with host and parasite genetics and epigenetics, proteomic and transcriptomic regulation, and environmental factors [10]. Stress-inducing environmental factors, particularly temperature, have been shown to influence *B. glabrata* susceptibility to *S. mansoni* [11–13] by interfering with the capacity of the snail's immune response to prevent parasite infection [11,12]. This review

Trends

From the first evidence implicating specific humoral factors in innate immune memory to the first induction of antischistosome resistance, steady progress into mechanistic and functional studies is yielding great immunobiological insights into snail–schistosome interactions.

The recent publication of the first genome annotation project for a medically important gastropod – the freshwater snail *Biomphalaria glabrata* – is an important resource for further advances in studying snail–schistosome immunobiology.

Snail–schistosome (in)compatibility likely reflects the balance of multiple determinants that include both the snail host and invading schistosome. Moving beyond the classic model that focuses on specific strains of *B. glabrata* and *S. mansoni* is critical to expand our understanding of how these determinants work in concert.

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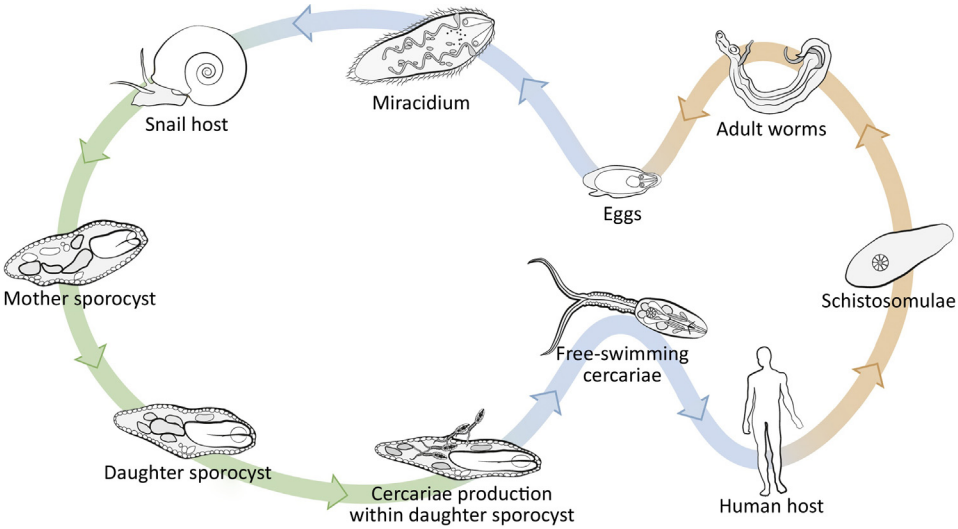

Figure 1. Life Cycle of a Human-Infective Schistosome. Adult schistosome worms living in the blood venules of a human host lay about 300 eggs per day [100,101], some of which become trapped in the tissues while others are released through faeces or urine, depending on the schistosome species. Upon contact with freshwater, the eggs hatch into free-swimming miracidia which seek and infect specific species of snail. The intramolluscan development takes about 4–6 weeks [102]. Within the snail, the miracidia transform into mother sporocysts that undergo asexual replication to produce daughter sporocysts. Each daughter sporocyst undergoes further replication, ultimately yielding cercariae – a second free-swimming form of the parasite infective to humans. Cercariae lose their tails during penetration of the skin and become schistosomulae. These migrate via the blood circulation to the portal veins of the liver where they differentiate and mature into adult worms. Adult male and female worms pair up, mate, and then migrate to the mesenteric venules of the bowel and rectum or the venous plexus of the bladder to complete the life cycle. It takes about 6–8 weeks from cercariae penetration to the maturation of worms and laying of eggs [103]. Coloured arrows indicate the intramolluscan (green), free-swimming (blue), and human host (gold) stages.

focuses on the current understanding of immune mechanisms that influence compatibility between *S. mansoni* and *B. glabrata.* It is divided into three sections that focus on the snail's immune cells (**haemocytes**), the humoral immune effectors, and the fibrinogen-related proteins, a gastropod-specific family of immune factors known to be important in the antischistosome immune response.

Cellular Immunity

The study of haemocytes, particularly their development and mechanisms of engagement and activation, only recently advanced to the point of specific functional investigations. In *B. glabrata,* most studies have focused on identifying and characterizing soluble immune factors; however, the haemocytes are critical in a snail's immune response to an invading schistosome. Without the haemocyte-driven **encapsulation** response, the parasite often survives and establishes infection.

Haemocyte Morphotypes Found in Gastropods and Their Role in the Immune Response

Gastropod haemocytes are often classified into two basic morphotypes based on size and granularity, namely **granulocytes** and **hyalinocytes** [14–16] (Figure 2A). Some studies have described three haemocyte morphotypes using different criteria [17–19]. For recent reviews, see [17,20,21]. Some earlier studies characterized haemocyte subsets based on specific membrane antigenic differences [22,23]. The proportion of hyalinocytes and granulocytes differs between snail species [14,16], and it fluctuates in response to various pathogens [14,24,25]. The roles of these cells in an immune response include phagocytosis [14,17], cytotoxicity [26,27], and encapsulation [28] (Figure 2B). These responses are

Glossary

***Biomphalaria glabrata* fibrinogen-related protein (*Bg*FREP):** FREPs are antigen-reactive plasma lectins of the innate immune response of invertebrates. They are defined by the presence of a fibrinogen-related domain (FReD). A family of gastropod unique FREPs (e.g., *Bg*FREP) is characterized by a fibrinogen domain coupled to one or two immunoglobulin superfamily domains.

Chemokines: a family of cytokines (small proteins that mediate signaling or communication in target cells) with the ability to induce attraction of target cells to sites of infection or injury.

Encapsulation: an innate immune response of invertebrates against pathogens that are too large to be engulfed by individual immune cells, which is characterized by cellular recruitment to infected tissue, followed by formation of cellular multilayers around, and killing of, the pathogen through production of cytotoxic molecules.

Granulocyte: a haemocyte morphotype with a low nucleus-to-cytoplasm ratio, with the cytoplasm rich in granules. These cells produce extensive filopodia and have a cellular area in the range of 174–384 μm^2.

Haemocytes: circulating phagocytic cells of invertebrates representing the primary effector cells involved in antipathogen host reactions. They are usually suspended in a soluble component called plasma but can migrate into the host tissues in response to invading pathogens.

Haemolymph: 'haemolymph' refers to haemocytes plus the cell-free soluble component or plasma in which they are suspended, which also serves as the transportation fluid. Haemolymph is essentially the equivalent of blood in vertebrates.

Hyalinocyte: a haemocyte morphotype either without granules or sparsely granular, spherical or slightly oval, smaller in size (~45 μm^2) compared to granulocytes and spread on the substrate to a lesser degree.

Larval transformation product (LTP): previously referred to as secretory/excretory products or protein (SEP or ESP), LTPs are a complex group of proteins produced

Key Figure

Defined Elements of *Biomphalaria glabrata* Immune Response

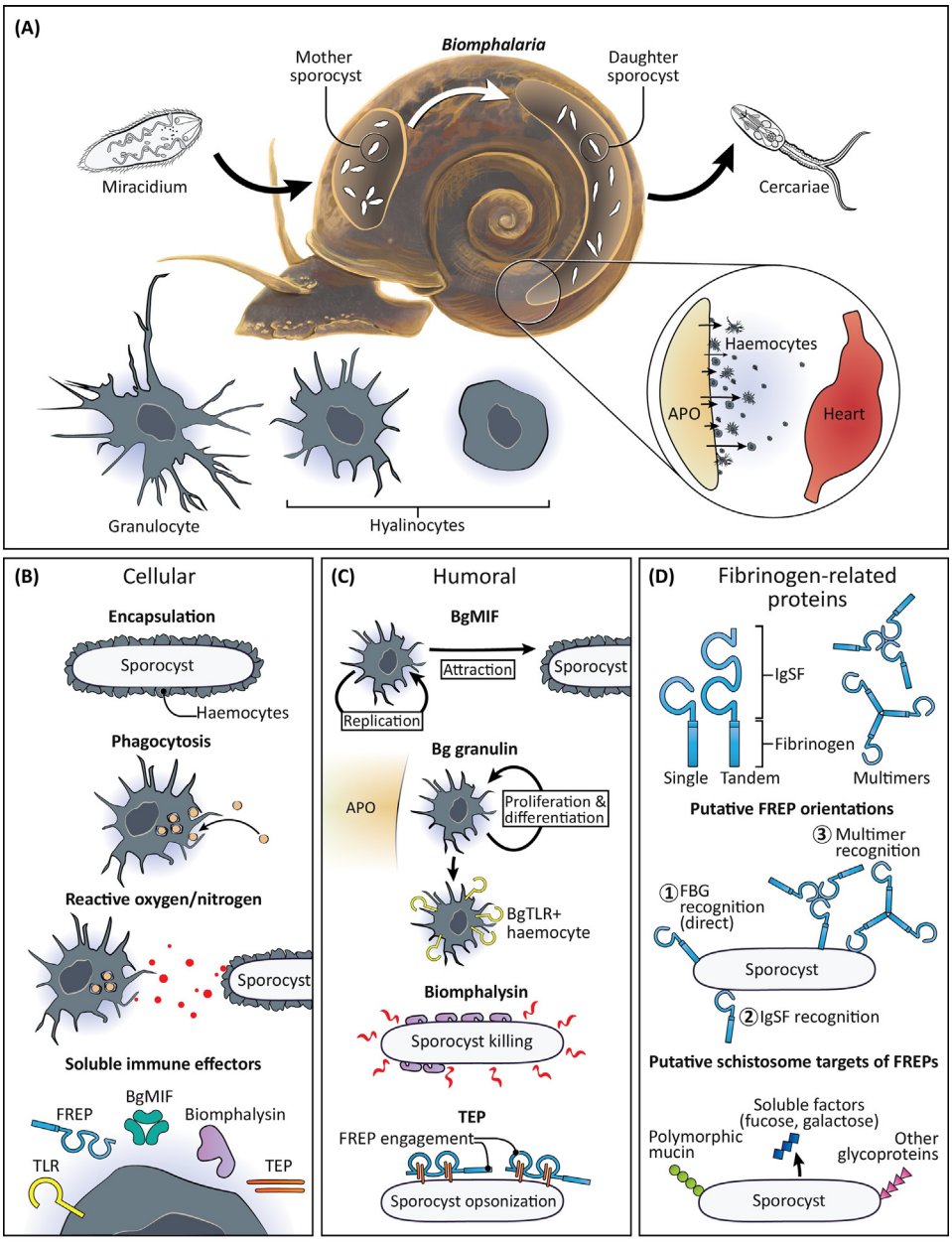

Figure 2. (A) Locations of intramolluscan schistosome stages and the amoebocyte-producing organ (APO). Miracidia often penetrate the snail at the head–foot region where they shed their ciliated epidermal plates and transform into mother sporocysts. Mother sporocysts migrate to the digestive gland and replicate into many daughter sporocysts which further replicate and eventually produce the cercariae that emerge from the snail. The APO is a flat sheet of tissue in the anterior pericardial wall which produces haemocyte precursors that mature into granulocytes and hyalinocytes. (B) Cellular immunity. Haemocyte-mediated immune responses include encapsulation, phagocytosis, and production of cytotoxic molecules (reactive oxygen/nitrogen species). Haemocytes also produce soluble immune effectors. (C) Humoral factors.

(See figure legend on the bottom of the next page.)

by miracidia during the transformation to mother sporocyst.

Macrophage migration-inhibitory factor (MIF): a constitutively expressed pleiotropic cytokine involved in the immune response to invading pathogens. It has strong proinflammatory properties and functions largely in ensuring gene expression, proliferation, and survival of macrophages during infections and apoptosis. First discovered in mammals, MIF homologues have also been characterized in various invertebrates, including *B. glabrata*.

Pattern-recognition receptors (PRRs): membrane-bound or soluble receptors that recognize and bind to fixed chemical structures on pathogens known as pathogen-associated molecular patterns (PAMPS) or molecules indicative of cellular damage or death known as damage-associated molecular patterns (DAMPs).

Plasma: the cell-free soluble component of the haemolymph.

Reactive oxygen species (ROS): oxidative intermediates of oxygen best characterized in mammals. They are produced by phagocytes such as neutrophils and macrophages. ROS have schistosomicidal and antimicrobial properties through oxidative stress as well as signaling functions as second messengers.

Ribonucleic acid interference (RNAi): an experimental technique for temporal disruption of transcript expression in a cell or whole organism by introduction of complementary double-stranded RNA or oligonucleotides called short interfering RNA, siRNA.

Schistosomiasis: a human and animal disease resulting from infection with certain schistosomes. Human schistosomiasis is a debilitating disease that affects at least 260 million people globally. Other mammalian or avian schistosomes that do not normally infect humans can also cause an allergic skin reaction and rash in humans known as cercaria dermatitis or swimmer's itch.

Superoxide dismutase (SOD): an enzyme that catalyzes the conversion of superoxide radical into hydrogen peroxide.

β-Pore-forming toxins (β-PFTs): β-PFTs constitute a category of molecules (including aerolysins and biomphalysin) with high tertiary structure and functional

enabled by intracellular synthesis of relevant proteins such as inducible immune receptors and effectors, cell adhesion molecules, and recruitment factors [9,29,30]. For most of these factors, their putative functional roles in *B. glabrata* are based on inferences from transcript expression and predicted amino acid similarity to well characterized immune molecules of other organisms.

Both granulocytes and hyalinocytes can phagocytose microbes or experimental microspheres [14,17]. Against the larger larval schistosomes, phagocytosis is limited to stripping of sporocyst microvilli and small tegumental pieces [31], and is perhaps more important for clearing debris from dead/dying parasites [28,31,32]. Larval digenean surfaces contain complex carbohydrates and glycoproteins [33], some of which can be released during miracidium-to-sporocyst transformation. Monosaccharides (such as α-d-mannose and α-d-galactose), known components of these glycoproteins, initiate high rates of phagocytosis in haemocytes of *B. glabrata* when conjugated to fluorescent microspheres [34]. This increased phagocytic response, mediated by *B. glabrata* fibrinogen-related protein 3 **(*Bg*FREP3)**, provided evidence for one means through which haemocyte engagement with the parasite might occur. However, mechanistic details such as which domains of *Bg*FREP3 interact with parasite and haemocyte, and the specific haemocyte receptor involved, are not known (Figure 2D).

Regarding the antischistosome immune response, granulocytes seem to be most relevant because of their involvement in encapsulation, which typically results in parasite elimination. Ultrastructure and biochemical labeling of haemocyte capsules suggest that granulocytes are the primary haemocyte type in contact with the sporocyst [31,35]. Also, increased abundance of circulating haemocytes observed during schistosome infections is primarily attributed to additional circulating granulocytes [24,34,36]. High granulocytic numbers (>230 cells/μl) in adult *B. glabrata* associate with resistance to *S. mansoni*, and the majority of circulating cells in snails with high haemocyte numbers are granulocytes [37]. In uninfected adult snails, the average granulocytic cell numbers range from 84 to 172 cells/μl [24,37].

Granulocyte recruitment to the site of an invading *S. mansoni* is observable as early as 1 h in resistant snails [28]. Formation of a complete haemocyte multilayer around the sporocyst leads to its killing within 10–72 h [28,31]. Chemotactic factors produced by the snail and parasite are known to mediate granulocyte recruitment. The pleiotropic cytokine macrophage migration-inhibitory factor (**MIF**) [38,39] has been well characterized as a haemocyte **chemokine**. Another potential recruitment factor is allograft inflammatory factor (*aif*) which displays increased transcript abundance during immune challenge [40] and higher basal expression in *S. mansoni*-resistant snails [30,37]. It is also likely that haemocytes respond to exogenous cues directly from the sporocysts [28,41]; however, the identity of these factors remains unknown.

Several hypotheses have been advanced to explain the differential responses generated by individual *B. glabrata* snails to the same *S. mansoni* strain. Prominent among these is that haemocytes of compatible snails are unable to recognize the parasite and thus fail to become

conservation, which bind to their target and heptamerize to form a β-barrel pore on the cell membrane leading to cell lysis and death. β-PFTs are structurally characterized by a large lobe involved in binding glycophosphatidylinositol (GPI)-anchored receptors and oligomerization, and a small lobe involved in recognition of carbohydrates on the surface of target cells and transmembrane domains necessary for pore formation.

These include chemotactic (*Biomphalaria glabrata* migration inhibitory factor, *Bg*MIF), proliferation and differentiation (granulin) signals, and molecules capable of direct killing of sporocysts (biomphalysin) or opsonization (thioester-containing protein, TEP, and fibrinogen-related protein, FREP). (D) Fibrinogen-related proteins. Single or tandem-immunoglobulin superfamily (IgSF) FREPs may directly recognize targets through the fibrinogen (FBG) or IgSF domains. FREP recognition may also be dependent or enhanced by multimer formations, perhaps through the FBG or IgSF domains. The various FREP configurations may then directly recognize sporocyst targets (soluble factors like galactose, polymorphic mucins, or other glycoproteins) or via mediators like the snail TEP.

activated. Consequently, recruitment of more haemocytes, production of proteins required for cell-to-cell interaction, haemocyte activation, and cytotoxic factors that would kill the parasite, also fail. Supporting this hypothesis is the pattern of polymorphisms found in *B. glabrata* FREPs and *S. mansoni* polymorphic mucins (*Sm*PoMucs) which fulfil several criteria as molecular determinants of snail–schistosome compatibility. For a literature review on compatibility polymorphisms, and the FREPs-*Sm*PoMucs system, see [10]. Another explanation is that haemocytes of susceptible snails are functionally inhibited by factors in the larval transformation products (**LTPs**), which interfere with their motility and spreading [41], intracellular protein synthesis [42], and capacity to produce reactive oxygen species (**ROS**) [43]. Candidate molecules in this category include calcium-binding proteins such as calreticulin [33]. LTPs have also been implicated in modulation of proton channel proteins [44], which regulate ion balance and intracellular pH by allowing hydrogen ions to cross cellular membranes. The involvement of these channels in ROS production not only suggests a possible regulation of snail cell ROS production but also an anti-immune mechanism that could be used by sporocysts to counter snail ROS-mediated effector responses [44]. Other hypotheses include parasite deployment of molecular mimicry to dampen snail immune recognition [45] and differences in effector/anti-effector systems such as host ROS countered by parasite ROS scavengers [46].

Sporocyst killing is actively mediated by haemocytes. When **plasma** is replaced with culture medium, the haemocytes of *B. glabrata* 13-16-R1, which is resistant to *S. mansoni* PR-1, are still able to kill sporocysts effectively as with whole **haemolymph** [47]. Haemocyte activation triggers production of cytotoxic molecules, the best known being the ROS. Their relevance in *B. glabrata* was conclusively demonstrated by Adema and colleagues who found that interfering with haemocyte production of ROS using an antagonist of NADPH-oxidase also compromised their ability to kill sporocysts [48]. Currently, it is known that: (i) hydrogen peroxide and nitric oxide are the species that mediate the killing of *S. mansoni* sporocysts in *B. glabrata* [47,49]; (ii) of the three alleles of the *Cu/Zn* ***superoxide dismutase*** (*sod1*) gene, the B allele is associated with resistance against *S. mansoni* in *B. glabrata*, and snails with the B allele express higher levels of Sod1 [50]; and (iii) snail ROS and parasite ROS scavengers likely counter one another – compatible parasites have antioxidant capabilities matching or exceeding host oxidants [46]. However, the precise mechanisms through which hydrogen peroxide or nitric oxides kill the sporocysts have not yet been determined.

Besides ROS, snail haemocytes can also degranulate the content of their proteolytic enzymes extracellularly and into phagosomes [17,51]. The potential cytotoxic molecules include proteases and protease inhibitors which may be relevant in disrupting the sporocyst tegument or modifying the complex glycoproteins found on their surfaces [52,53]. However, as with many immune factors in *B. glabrata*, these require functional validation.

Haemocyte Growth Factors and Development

Gastropod haematopoiesis is localized to the amoebocyte-producing organ (APO) (Figure 2A), which is located in the anterior pericardial wall in *B. glabrata* [54] or anatomically equivalent regions in other species [20]. Possible haematopoietic events have also been observed in peripheral haemocytes and other locations such as the kidney and the head–foot region [20]. APO cells proliferate in response to various pathogens or pathogen products [55–58]. No parasite mitogenic factors have been identified, and it is unclear whether APO cells proliferate in a direct or indirect manner when challenged with a digenean trematode. Recent evidence implicating granulin, an endogenous growth factor from *B. glabrata* (*Bg*GRN), in the anti-*S. mansoni* immune response [24], supports an indirect response that is primarily driven by increases in the abundance of circulating growth factors, but does not exclude the possible effect of parasite mitogens *in vivo*.

The stages of haemocyte development and drivers of haematopoiesis in snails are poorly understood. The only cytokine functionally characterized in gastropods is MIF [38,39]; however, multiple homologues of MIF, interleukin-17, and tumor-necrosis factor (TNF) have been identified in the *B. glabrata* genome [59]. Growth factors identified include: granulin [24], an insulin receptor cloned from the *B. glabrata* embryonic cell line (Bge) [60], epidermal growth factor in *B. glabrata* and *Lymnaea stagnalis* [61,62] and its receptor in *L. stagnalis* [62]. Signaling molecules and pathways known to be involved in gastropod haematopoiesis include MAPKs, ERK2, and p38 [63], and from other animal models we can infer that these pathways are likely also involved in other immune processes such as cellular adhesion and spreading. Generally, our current understanding of gastropod haematopoiesis is such that answers to key questions (such as stages and sequence of haematopoietic events of proliferation, differentiation, and maturation) still await the results of future studies that should benefit greatly from the annotated *B. glabrata* genome [59].

Haemocyte Numbers versus Functional Diversity in Determining Compatibility

Maintaining sufficient haemocyte numbers is an important element of the snail immune response, but does not appear to determine the effectiveness of the encapsulation response. Doubling or quadrupling *L. stagnalis* haemocyte concentration *in vitro* increased encapsulation size but did not kill *S. mansoni* sporocysts faster [64]. It seems likely that haemocytes in the innermost layer of the encapsulation, those contacting the sporocyst, are actively involved in the killing process. Such haemocytes must have the relevant repertoire of receptors for recognition and capacity for cytotoxicity, implying a functionally heterogenous population. Emerging evidence suggests that haemocyte functional differences may be the driving force behind successful immune responses. Larson *et al*. [37] found that snails that were refractory to *S. mansoni* infection, but had low haemocyte numbers, expressed specific immune relevant genes constitutively at high abundance. Also, in one snail line that displayed high haemocyte numbers both at juvenile and adult stages, the juveniles were highly susceptible while adults were resistant; indicating that high cell numbers alone were insufficient to manifest resistance [37]. Furthermore, it was found that newly proliferated haemocytes expressed *Bg*FREP3 at a higher proportion following an immune challenge [34], suggesting that haemocyte differentiation or maturation had occurred.

Pila *et al*. recently began to characterize functionally distinct haemocyte subsets by assessing two haemocyte-associated factors known to play a role in *S. mansoni* immunity – *Bg*FREP3 [34] and *Bg*TLR [65]. They found that the proportion of *Bg*TLR^{+} haemocytes increased in snails receiving treatment with the recombinant growth factor *Bg*GRN compared to controls, whereas, the proportion of *Bg*FREP3^{+} haemocytes did not differ significantly [24]. Furthermore, *Bg*GRN treatment before challenge reduced *S. mansoni* PR-1 infection success by 54%. Conversely, the resistant phenotype of *B. glabrata* BS-90 was significantly diminished when ribonucleic acid interference (**RNAi)** was used to knockdown *Bg*TLR expression [65]. These studies suggest that *S. mansoni* challenge of *B. glabrata* leads to haemocyte proliferation and differentiation into functionally relevant subsets. As more determinants of compatibility are functionally characterized, it will become possible to define functional haemocyte subpopulations with a better resolution in the context of a specific immune response and to determine the conditions under which they are generated and the targets to which they are most relevant.

Haemocyte-Associated Receptors Involved in Schistosome Recognition and Engagement

Many receptors have been identified in snails with putative roles in the immunobiology of snail–schistosome interactions [29,30,53,61,66]. While some of these receptors, such as peptidoglycan recognition proteins (PGRPs) and Toll-like receptors (TLRs), are canonical pattern-recognition receptors (**PRRs**), others such as variable immunoglobulin and lectin domain-containing molecules [67] and the Guadeloupe resistance complex (GRC) [68] appear

to be gastropod-specific. The broad categories of receptors include PRRs, integrin-related proteins, and growth factor/cytokine-like receptors. Our focus here is on PRRs because they are known to interact with *S. mansoni*.

B. glabrata PRRs include TLRs [59,65], GRC [68], mannan and laminarin-binding molecules, PGRP, Gram-negative bacteria-binding protein and lipopolysaccharide-binding protein [21,51], as well as variable immunoglobulin and lectin domain-containing molecules [67]. The implication of *Bg*TLR in the resistance of *B. glabrata* snails against *S. mansoni* provided functional insight into how haemocytes might engage *S. mansoni* through cell-associated receptors [65]. Signaling molecules downstream of the TLR pathway have also been identified, including the transcription factor NFκB that displays expression patterns [69] and possesses conserved binding motifs [70] that suggest involvement in the immune response as would be expected from studies of other organisms. Other signaling molecules identified include: MyD88, TRAF, IRAK, and IKK [59]. The fact that there is great diversity of TLR genes in the genome of *B. glabrata* [59] is indicative of their importance in the snail immune response, including against schistosomes, and suggests that leucine-rich repeat-containing molecules are likely involved in recognition of various pathogen types. Another putative PRR – Grctm6, a member of GRC with the hallmarks of a cell-bound receptor – has been shown to modulate the schistosome burden in *B. glabrata* [71].

Much remains to be discovered regarding haemocyte immune recognition, and integration with other elements of the snail's immune response. Most of the identified receptors still require functional characterization to confirm an immune role. For those supported by functional data, mechanistic details – such as what ligands they bind and the signaling mechanisms through which they elicit a response – remain unknown. Although haemocytes are directly involved in parasite killing, humoral factors produced by these cells can act in concert or play sentinel roles.

Humoral Factors of the *B. glabrata* Immune Response

During penetration and establishment within the snail, *S. mansoni* is exposed to host hemolymph and the bevy of soluble immune effector molecules therein (Figure 2C). Proteomic analysis of *B. glabrata* plasma revealed that an array of factors display affinity for sporocyst tegumental membrane proteins (Mem) and LTPs. These factors include PRRs such as the *B. glabrata* thioester-containing protein (*Bg*TEP), cytotoxins such as biomphalysin, and variable immunoglobulin and lectin domain-containing molecules [72]. Soluble immune effectors are involved in both the direct killing of sporocysts and preparation of haemocytes to mount a cell-mediated response.

While cell-mediated immune responses are heavily featured in primary schistosome infections, subsequent challenges seem to shift the response towards dependence on humoral effectors, highlighting their necessity and foundational role in the appearance of innate immune memory (acquired resistance) [73]. This is evident from the observations that primary infections lead to the encapsulation and destruction of sporocysts by host haemocytes, whereas sporocysts in secondary immune challenge can be killed without encapsulation [73]. Circulating humoral factors, particularly FREPs [73,74] and biomphalysin [73], have been shown to be involved in this process. Both were transcriptionally upregulated during secondary challenge [73], and the transfer of primed snail plasma to naïve snails significantly decreased the prevalence of primary *S. mansoni* infections [73]. Additionally, downregulation of several humoral immune factors, including *Bg*FREP3, has been demonstrated to result in loss of snail resistance phenotype, supporting the role of these proteins in combatting secondary trematode infections [74]. The appearance of *B. glabrata* innate immune memory in response to trematode infections is a phenomenon that has been observed for the past four decades [75–78], though the underlying mechanisms were not well understood. Recent studies [73] have investigated the molecular

mechanisms of this phenomenon and demonstrated that humoral factors are likely crucial to the appearance of innate immune memory in which invertebrates, which lack an adaptive immune system, apparently demonstrate a level of 'acquired resistance' to pathogens.

Thioester-Containing Protein

Mounting a successful immune response requires recognizing invading pathogens. This task is accomplished in large part by PRRs like the complement C3 protein in vertebrates, and thioester-containing protein homologues in invertebrates. The TEP superfamily can be separated into three families: (i) vertebrate complement proteins (C3/C4/C5), (ii) alpha2 macroglobulin (A2M) pan-protease inhibitors, and (iii) a group of TEPs unique to invertebrates. These homologous proteins share several conserved features, including their secretion as inactive forms, activation via proteolytic cleavage, and a conserved thioester motif used to covalently bind their target. Complement proteins and invertebrate TEPs use this binding to opsonize foreign targets.

*Bg*TEP displays numerous similarities to other invertebrate TEPs. It possesses a classic thioester motif, and is cleaved prior to binding *Sm*PoMucs. It features an A2M receptor-binding domain, which in other invertebrate models has been shown to bind the A2M receptor found on circulating phagocytic cells [79,80]. These features, coupled with its association with LTPs, *Sm*PoMucs, and other Mem proteins, suggest a role for *Bg*TEP in the opsonization of *S. mansoni* sporocysts [72,81]. This role is also supported by functional studies of TEPs in insect models. *Drosophila melanogaster* possesses six TEPs, which differ in their preference between opsonizing Gram-negative and Gram-positive bacteria [79]. *Anopheles gambiae* TEP1 binds *Plasmodium berghei* ookinetes, targeting them for subsequent lysis or melanization. Also, *A. gambiae* TEP polymorphisms are linked to *P. berghei*-resistant and susceptible phenotypes [80]. The observation that *Bg*FREP2 can also form complexes with *Bg*TEP and *Sm*PoMucs envisions a model where *Bg*FREPs recognize invading trematodes, before associating with *Bg*TEP (Figure 2C,D), which then kills the parasite directly or opsonizes it for encapsulation and killing by circulating haemocytes.

Biomphalysin

Molecules that can directly lyse a target cell are important elements of the humoral immune response. Biomphalysin, a β-pore-forming toxin (**β-PFT**) functions as one such molecule. Biomphalysin has the typical features of β-PFTs except that the small lobe does not feature a lectin-like domain, which is required for carbohydrate binding. The lack of this lectin-like domain has led to speculation that biomphalysin could function in targeting unique sporocyst antigens, though further work is required to examine this possibility [82].

While prokaryotes utilize these proteins to attack host immune cells, *B. glabrata* employs biomphalysin for killing sporocysts [82–84]. Exposure of sporocysts to biomphalysin alone does not increase mortality unless *B. glabrata* plasma is added, suggesting the presence of a yet undetermined cofactor. *In vitro* exposure of sporocysts to combined plasma and biomphalysin results in cellular swelling, followed by sporocyst disintegration [82]. Biomphalysin transcript levels increase during secondary *S. mansoni* infections compared to snails undergoing a primary infection, suggesting that it is a key factor in the appearance of innate immune memory in *B. glabrata* [73].

Macrophage Migration-Inhibitory Factor

Preparing haemocytes to engage invading trematodes is another requirement of an effective immune response. The cytokine *B. glabrata* migration inhibitory factor (*Bg*MIF) is a mediator of this function. It is ubiquitously expressed, including in haemocytes. Work done in Bge cells shows that *Bg*MIF is secreted in response to *S. mansoni* LTPs. *Bg*MIF induces cellular

proliferation via activation of the ERK1/ERK2 pathway, aiding survival through inhibition of p53 phosphorylation and suppression of NO-induced apoptosis [39]. *In vivo* studies demonstrated that *Bg*MIF provides a migration signal to circulating granulocytes towards schistosome-invaded tissue [38,39]. RNAi-mediated knockdown of *Bg*MIF leads to higher establishment success of *S. mansoni* sporocysts within *B. glabrata* [39], demonstrating that this cytokine influences the ability of haemocytes to effectively encapsulate *S. mansoni*.

Variable Immunoglobulin and Lectin Domain-Containing Molecules

The study of *B. glabrata* RNA sequencing data from a *de novo* reference transcriptome led to identification of novel molecules that consist of one or two immunoglobulin superfamily (IgSF) domains and an interceding region (ICR), suggesting a similarity to FREPs, but that instead of a fibrinogen (FBG) domain, associate with either a C-type lectin domain or a galectin domain [67]. These were named C-type lectin-related protein (CREP) and galectin-related protein (GREP), respectively. Like the FREPs, the expression of CREPs and GREPs also associates with *S. mansoni* resistance in *B. glabrata* [59,67]. Although CREPs and GREPs await functional characterization, a possible role in pathogen recognition is supported by their similarities to perhaps the most well characterized soluble immune molecules that *B. glabrata* possesses, FREPs.

Fibrinogen-Related Proteins (FREPs)

FREPs are broadly defined by the presence of a fibrinogen-related domain. These domains are conserved throughout animal evolution, and are diverse in both form and function [85]. Gastropods possess a unique subset of FREPs which marries a C-terminal FBG domain, connected via an ICR, to one or two IgSF domains at the N-terminus [86] (Figure 2D). In 1997, Adema *et al*. identified four peptides that were obtained from precipitates evoked by LTP of *Echinostoma paraensei* sporocysts in *B. glabrata* M-line snail plasma [86]. Amino acid sequences of gel-purified proteins ~65 kDa in size were used to instruct PCR primer design that led to the discovery that the *B. glabrata* genome encoded sequences with high identity to fibrinogen-like sequences. Therefore, the 65 kDa protein was designated a FREP [86].

According to the number of IgSF domains, *Bg*FREPs can be classified as: single-IgSF and tandem-IgSF *Bg*FREPs (Figure 2D) [85], in which the tandemly arranged IgSF domains are joined by a small connecting region [87]. Whole-genome analysis of *B. glabrata* indicates that *Bg*FREPs are encoded by 24 germline genes, 20 of which belong to tandem-IgSF and 4 to single-IgSF *Bg*FREPs [59].

A unique aspect of *Bg*FREPs is their capacity for somatic diversification that is further expanded upon by alternative splicing and multimerization in snail haemolymph [88] (Figure 2D). This phenomenon was first discovered because otherwise identical genomic *Bg*FREP sequence fragments occasionally differed randomly at single nucleotide positions [87,89]. Subsequent studies on somatic diversity focused on a region of exon 2 that encodes the IgSF1 domain of *Bg*FREP3. *Bg*FREP3 was estimated to be encoded by 3 to 5 genomic loci, but up to 45 and 37 different amplification sequences could be obtained from two snail individuals, respectively [90]. The extent of diversity of *Bg*FREP3 IgSF1 sequences in individual snails was far greater than the estimated number of loci, suggesting a novel mechanism that increases the potential diversity of *Bg*FREPs [90]. Zhang *et al*. found that this diversity occurs at the somatic cell level, mainly through point mutations and gene conversion [90].

Different *Bg*FREPs exhibit functional specialization in their binding preferences for particular pathogens; for example, 65–75-kDa FREPs (mainly *Bg*FREP4) associate with *E. paraensei,* whereas *Bg*FREP3 recognizes bacteria, fungi, [91], and *S. mansoni* [72]. *Bg*FREPs are capable of direct binding to *S. mansoni* Mem and LTP [72]. As mentioned above, the highly polymorphic

and variable *Sm*PoMuc proteins may be serving as targets for some *Bg*FREPs, and certainly *Bg*FREP2 [81]. *Bg*FREPs 2, 3, and 12 are capable of binding soluble LTP [92]. However, the mechanism underpinning these complex formations and the targets for other *Bg*FREPs have yet to be explored, as has the implications of diversification on target recognition.

Transcript abundance of many *Bg*FREPs increases in response to infection of *B. glabrata* with *E. paraensei* or *S. mansoni* [61,92,93], eliciting expression profiles reflective of the immune challenge [94,95]. *Bg*FREPs display differential expression profiles in response to bacteria (Gram-negative or Gram-positive), but wounding does not appear to alter transcript expression [93]. Increased expression of *Bg*FREP3 is linked to the resistant phenotype displayed by the *S. mansoni*-resistant BS-90 strain of *B. glabrata* [30,34,74,96]. Specifically, *Bg*FREP3 expression was elevated compared to controls in three different types of resistance in *B. glabrata* to infection with *S. mansoni* or *E. paraensei*: BS-90 compared to M-line strains of *B. glabrata,* adult compared to juvenile *B. glabrata* M-line, and *B. glabrata* in which innate immune memory was induced against *E. paraensei* [34,74].

RNAi-mediated knockdown of *Bg*FREP3 in adult *B. glabrata* M-line resulted in 30% of the normally resistant snails becoming infected with *E. paraensei* [34]. In addition, knockdown of *Bg*FREP3 in *B. glabrata* BS-90, which is typically 100% refractory to *S. mansoni* challenge (PR-1 or NMRI strain), resulted in patent infections in 21% of the snails, suggesting that *Bg*FREP3 plays an important and broad role in resistance to digenean trematodes [74]. Increased *Bg*FREP transcript expression during secondary immune response of *B. glabrata* to *S. mansoni* suggests that *Bg*FREPs may also be involved in innate immune memory [73]. Knockdown of *Bg*FREP 2, 3, and 4 was found to reduce the innate immune memory phenotype by 15%, rendering primed snails more susceptible to *S. mansoni* infection [73]. Whether the diversification of *Bg*FREPs influences innate immune memory has yet to be investigated.

Concluding Remarks

Decades of investigations have significantly advanced our understanding of gastropod immunobiology, particularly with respect to digenetic trematodes. From the early studies of snail susceptibility and histological observations of infections, we are now undertaking functional and mechanistic assessments. Rapid progress of the last three decades notwithstanding, several questions remain to be addressed – see Outstanding Questions. The literature on snail–schistosome interactions is currently dominated by the *B. glabrata*–*S. mansoni* model, which may not always directly translate into natural snail–schistosome associations, or be relevant to associations of other snail species and their respective parasites. Nevertheless, knowledge gained from studies of this model is important from the standpoint of evolutionary immunology, in terms of the functional capabilities of invertebrate immune systems, and it certainly forms the foundation of further investigation using alternative snail and schistosome species/strains. The *B. glabrata* model can also provide insight when studying immunological interactions in other snail–trematode combinations. Understanding snail–schistosome compatibility determinants is a prerequisite for novel schistosomiasis control approaches that can exploit the snail. Genomic modification technology, epitomized by the CRISPR/cas9 system, has already been used to modify mosquitos for resistance to *Plasmodium* infection [97–99]. This system could theoretically be coupled with factors such as *Bg*GRN to drive increased resistance to schistosome infection in snails, or to reduce resistance to another pathogen, thereby reducing snail populations. Coupled with mass drug administration, this could provide the leverage required to achieve sustained control of human schistosomiasis.

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Outstanding Questions

How and where does haemocyte development takes place? The haematopoietic process – including the stages of haemocyte maturation, the sequence of haematopoietic events, the important signalling networks, and the number of functionally distinct haemocyte lineages that are present and the drivers of their development – all remain relatively unknown. Also poorly understood are the endogenous and exogenous mitogenic factors that initiate haemocyte development.

How do haemocytes recognize larval schistosomes? We understand very little about the schistosome-specific targets recognized by haemocyte receptors or soluble factors. The mechanisms of FREP engagement (IgSF vs FBG-domain mediated engagement) with SmPoMucs or other schistosome-specific glycoproteins, and how TEP is involved, or the basis for biomphalysin-mediated parasite killing are important areas of investigation. The encapsulation response by haemocytes, including chemotaxis to the larval schistosome and the factors/pathways that initiate encapsulation and, ultimately, parasite killing, is another area worthy of additional investigation.

What are the mechanisms and immunological implications of *Bg*FREP diversification? While it is clear that FREPs are important elements of the antischistosome immune response, the role that diversification plays in conveying resistance to infection remains unknown.

How can the knowledge gained from the study of snail immunity to schistosomes be integrated into schistosomiasis control initiatives?

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