Diminished adherence of Biomphalaria glabrata embryonic cell line to sporocysts of Schistosoma mansoni following programmed knockout of the allograft inflammatory factor

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Research

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Abstract

Background: Larval development in an intermediate host gastropod snail of the genus Biomphalaria is an obligatory component of the life cycle of Schistosoma mansoni. Understanding of the mechanism(s) of host defense may hasten the development of tools that block transmission of schistosomiasis. The allograft inflammatory factor 1, AIF, which is evolutionarily conserved and expressed in phagocytes, is a marker of macrophage activation in both mammals and invertebrates. AIF enhances cell proliferation and migration. The embryonic cell line, termed Bge, from Biomphalaria glabrata is a versatile resource for investigation of the snail-schistosome relationship since Bge exhibits a hemocyte-like phenotype. Hemocytes perform central roles in innate and cellular immunity in gastropods and in some cases can kill the parasite. However, the Bge cells do not kill the parasite in vitro.

Methods: Bge cells were transfected by electroporation with plasmid pCas-BgAIFx4, encoding the Cas9 nuclease and a guide RNA specific for exon 4 of the B. glabrata AIF (BgAIF) gene. Transcript levels for Cas9 and for BgAIF were monitored by quantitative reverse-transcription-PCR and, in parallel, adhesion of gene-edited Bge cells during co-culture with of schistosome sporocysts was assessed.

Results: Gene knockout manipulation induced gene-disrupting indels, frequently 1-2 bp insertions and/or 8-30 bp deletions, at the programmed target site; a range from 9 to 17% of the copies of the BgAIF gene in the Bge population of cells were mutated. Transcript levels for BgAIF were reduced by up to 73% (49.5±20.2% S.D, P ≤ 0.05, n =12). Adherence by BgAIF gene-edited (ΔBgAIF) Bge to sporocysts diminished in comparison to wild type cells, although cell morphology did not change. Specifically, as scored by a semi-quantitative cell adherence index (CAI), fewer ΔBgAIF than control wild type cells adhered to sporocysts; control CAI, 2.66±0.10, ΔBgAIF, 2.30±0.22 (P ≤ 0.01).

Conclusion: The findings supported the hypothesis that BgAIF plays a role in the adherence of B. glabrata hemocytes to sporocysts during schistosome infection in vitro. This demonstration of the activity of programmed gene editing will enable functional genomics approaches using CRISPR/Cas9 to investigate additional components of the snail-schistosome host-parasite relationship.

Background

Evolution endowed the schistosomes with a complex life cycle that includes both a freshwater gastropod intermediate host and a definitive mammalian host. Several species of the freshwater snail genus Biomphalaria are the intermediate host for Schistosoma mansoni. The neotropical species, Biomphalaria glabrata has been studied extensively with respect to host-parasite relationship and coevolution with S. mansoni especially on mechanisms of susceptibility and/or resistance to the compatible parasites (1, 2). Genetic variation is evident among isolates and strains of B. glabrata, both in the laboratory and in the field, resulting in a spectrum of the susceptibility of infection with S. mansoni (3). Considerable advances have been made in the exploration and characterization of mechanisms of the internal defenses system (IDS) of the snail that determine susceptibility and resistance to schistosome (4-11). The resistance
phenotype is underpinned by a complex genetic trait, where the schistosome larva fails to develop as the consequence of innate and cellular immune responses. Hemocytes of resistant snails encapsulate and destroy the sporocyst (11-18).

*B. glabrata* embryonic cell line (Bge) (19) remains to date the only established cell line from any mollusk. The cell line originates from five-day-old embryos of *B. glabrata* susceptible to infection with *S. mansoni*. The Bge cell line has been studied extensively to interrogate the host-parasite relationship because the Bge cell exhibits a hemocyte-like behavior that includes encapsulation of the larval parasite, but does not kill the parasites (20-28).

The genome sequence of *B. glabrata* has been reported (29), along with ongoing transcriptome and proteome catalogues that include factors participating in immunological surveillance, phagocytosis, cytokine responses, and pathogen recognition receptor elements including Toll-like receptors and fibrinogen-related proteins (30-36). An orthologue of the evolutionary conserved allograft inflammatory factor (AIF) is an evolutionary conserved protein typically expressed in phagocytes and granular leukocytes in both vertebrate and invertebrate. Functions demonstrated for AIF include macrophage activation, enhancement of cellular proliferation and of migration in mammalian and invertebrate cells; protostomes and deuterostomes (37-41). AIF also plays a key role in the protective response by *B. glabrata* to invasion by schistosomes (8, 9). BgAIF, the orthologue in *B. glabrata* is expressed in hemocytes, which participate in phagocytosis, cellular proliferation, and cellular migration. Elevated expression of *BgAIF* is a characteristic of the resistance of *B. glabrata* to schistosome infection and has been considered as a marker of hemocyte activation (8, 9).

Expression of AIF also is seen during hemocyte activation in oysters (36, 38, 42, 43) and during hepatic inflammation during murine schistosomiasis (44, 45). We hypothesized that BgAIF was involved in cell mediated immune response(s) by *B. glabrata* through activation of hemocyte cell adhesion and/or migration after the schistosome miracidium has penetrated into the tissues of the snail. We addressed this hypothesis by using CRISPR/Cas9-based programmed genome editing to interrupt the *BgAIF* gene of *B. glabrata* in the Bge cell line, following reports that indicated the utility of using CRISPR-based programmed gene knockout approach in other mollusks including the Pacific oyster, *Crassostrea gigas* and the slipper limpet, *Crepidula fornicata* and the gastropod, *Lymnaea stagnalis* (46-48). As detailed below, we demonstrated the activity of programmed genome editing in Bge cells, with gene knockout at the *BgAIF* locus.

**Methods**

**Gene editing construct**
The gene encoding the allograft inflammatory factor of *B. glabrata, BgAIF* (2,226 bp; accession number BGLB005061, https://www.vectorbase.org/) includes five exons interrupted by four introns (Fig. 1a). A guide RNA (gRNA) for Cas9-catalyzed gene editing specific for the target *B. glabrata* gene locus, *BgAIF*, was identified in the BGLB005061 sequence using the 'CHOPCHOP' v3 tool, https://chopchop.cbu.uib.no/, with default parameters compatible for the protospacer adjacent motif, NGG, of Cas9 from *Streptococcus pyogenes* (49-51) and screened for off-target sites against the *Biomphalaria glabrata* genome (29).

Based on the guidance from the CHOPCHOP analysis, we chose the top ranked guide RNA (gRNA), AGACTTTGTTAGGATGATGC, specific for exon 4 of the AIF gene, with predicted high CRISPR/Cas9 efficiency for double-stranded cleavage in tandem with an absence of off-target activity in the genome of *B. glabrata* (Fig. 1a). A CRISPR/Cas9 vector encoding the gRNA targeting exon 4 of *BgAIF* under the control of the mammalian U6 promoter and encoding Cas 9, with nuclear localization signals 1 and 2, driven by the human cytomegalovirus (CMV) immediate early enhancer and promoter was assembled using the GeneArt CRISPR Nuclease Vector system (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer’s protocol. Briefly, the 20 nt of either target (including ‘gtttt’ on the 3’ end) or complementary to target (including ‘cggtg’ on the 3’end) sequences were synthesized commercially, and annealed according to manufacturer’s protocol. The annealed double strand DNA (dsDNA) was ligated into the linearized GeneArt® CRISPR Nuclease vector via *BamHI* and *BsmBI* restriction sites, respectively, and the construct was termed pCas-BgAIFx4 (Fig. 1b). (The sequence of GeneArt CRISPR nuclease vector backbone is available at https://www.thermosher.com/order/catalog/product/A21174#/A21174).

Chemically competent TOP10, *E. coli* cells (Invitrogen, Thermo Fisher Scientific) were transformed with pCas-BgAIFx4 by the heat shock method and cultured on LB-agar supplemented with ampicillin at 100 µg/ml. Subsequently, the integrity of the recombinant plasmids from several single colonies of ampicillin-resistant *E. coli* transformants was confirmed by amplicon PCR-based Sanger direct nucleotide sequence analysis using a U6 gene-specific primer for gRNA ligation and orientation (Fig. 1b).

*Biomphalaria glabrata* embryonic (Bge) cell line culture

The Bge cell line was provided by the Schistosomiasis Resource Center (SRC), Biomedical Research Institute (BRI), Rockville, MD. Historically, the Bge cell line was sourced by the SRC from the American Type Culture Collection (Manassas, VA), catalog no. ATCC CRL 1494, and thereafter maintained at BRI for >10 years. Bge cells were maintained at 26°C in air in ‘Bge medium’, which is comprised of 22% (v/v) Schneider’s *Drosophila* medium, 0.13% galactose, 0.45% lactalbumin hydrolysate, 0.5% (v/v) phenol red solution, 20 µg/ml gentamycin, and supplemented with 10% heat-inactivated fetal bovine serum (24, 52). Bge cells were grown to 80% confluence before transfection by electroporation with pCas-BgAIFx4. The Bge cells were free of contamination with *Mycoplasma*, as established with a PCR-based test (LookOut® Mycoplasma PCR Detection kit, Sigma-Aldrich, St. Louis, MO).

Transfection of Bge cells by square wave electroporation

Bge cells were harvested using a cell scraper, washed twice in Bge medium, counted, and resuspended at 20,000 cell/µl in Opti-MEM medium (Sigma-Aldrich, St. Louis, MO). Two million cells were transferred into
0.2 mm path length electroporation cuvettes (BTX Harvard Apparatus, Hollister, MA) containing 6 µg pCas-BgAlFx4 in ~100 µl Opti-MEM. The cells were subjected to electroporation using one pulse at 125 volts for 20 milliseconds, using a square wave pulse generator (ECM 830, BTX Harvard Apparatus). Immediately thereafter, the Bge cells were maintained in 12-well plates (Greiner Bio-One) at 26°C. The mock control included Opti-MEM only for electroporation. The presence of transcripts encoding the *B. glabrata* actin and the Cas9 was monitored daily for nine days following transfection by electroporation (Fig. 1c).

**Sequential isolation of total RNA and genomic DNA**

To monitor the transfection of Bge cell by pCas9-BgAlFx4, we investigated the expression of Cas9 in Bge cells by reverse transcription PCR (RT-PCR). Both total RNA and genomic DNA were extracted sequentially from cell pellets, as described (53, 54). In brief, each sample of total RNA sample was extracted using the RNAzol® RT reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer’s protocol. Subsequently, the DNA/protein pellet retained after recovery of RNA was resuspend in DNAzol® solution (Molecular Research Center, Inc), from which total DNA was recovered. The RNAs and DNAs were dissolved in nuclease-free water and their concentration and purity established by spectrophotometry (Nanodrop 1000, Thermo Fisher Scientific).

**Expression of Cas9 in Bge cells**

To investigate transcription from the pCas-BgAlFx4 vector following transfection of Bge cells, levels of transcribed Cas9 were investigated by semi-quantitative RT-PCR. The Cas9-specific primers were Cas9-F, 5’- agcatcgcgacctgcagcag-3’ and Cas9-R, 5’- agaagctgtcgtccaccttg-3’ (Fig. 1b). Total RNA from the non-transfected, mock (Opti-MEM electroporated-), and pCas-BgAlFx4 DNA electroporated-Bge cells were treated with DNase I (Ambion, Thermo Fisher Scientific) to digest any residual vector pCas-BgAlFx4 DNA and contaminating genomic DNAs. The RNAs were reverse transcribed to cDNA using ProtoScript II reverse transcriptase with oligo dT and random primers (First Strand cDNA Synthesis kit New England Biolabs, Ipswich, MA). RT-PCRs specific for the Cas9 or actin gene of *B. glabrata, BgActin* (GenBank accession number U53348.1) were undertaken, with BgActin serving as the positive control for RNA integrity. The primer pairs used for the BgActin coding sequences were termed actin-F, 5’- aagcgacgtttttcttggtgc-3’ and actin-R, 5’- acccatacaccatcacc-3’. Amplicons and molecular size standards were separated by electrophoresis through Tris-acetate-EDTA-buffered agarose 1% stained with ethidium bromide (Fig. 1c).

**Analysis of programmed mutation of the allograft inflammatory factor gene of B. glabrata**

Genomic DNA samples from the mock-transfected and pCas-BgAlFx4-transfected cells were amplified by PCR using AIF-F (5’-gcagatttgcaattcaacactta-3’) and AIF-R (5’-tgccagctagcttactgcat-3’) primers that flank the CRISPR/Cas9 programmed double-stranded break (DSB) site (Fig. 1a). Amplicons of 568 nt in length (from residues 489 to 1,056 of the *BgAlF_BGLB0055061* gene) were obtained using the AIF-F and -R primer pair. Amplicons were isolated from the agarose gel using the PCR cleanup and gel extraction kit.
(ClonTech, Takara USA, Mountain View, CA) and the nucleotide sequence of amplicons determined by Sanger direct sequencing (GENEWIZ, South Plainfield, NJ). Chromatograms of the sequence reads from the control and experimental groups in each replicate experiment were subjected to online analysis using the TIDE algorithm, https://tide.deskgen.com/ (55, 56) and also using the Inference of CRISPR v2 Edits analysis (ICE) software, https://ice.synthego.com/#/ (Synthego Corporation, Redwood City, CA) (57). Estimates of CRISPR efficiency, insertion-deletion (INDEL)-substitution percentages, and the nucleotide sequence of mutant alleles were obtained using both the TIDE and the ICE platforms (55, 56) (Fig. 2a, 2b).

**Quantitative real time PCR analysis of transcription of BgAIF**

To evaluate the differential levels of the BgAIF transcript among the control and experimental groups, total RNAs were extracted and treated with DNase I, as above. DNase I treated-RNA (200 ng) was reverse transcribed to cDNA, followed by quantitative RT-PCR, using the ViiA7 Real Time PCR System (Applied Biosystems, Scientific), and the SSoAdvanced Universal SYBR Green Supermix reagents (Bio-Rad), according to the manufacturer’s recommendations. The following nucleotide primers used BgAIF gene-specific primers amplify 119-257 nt of BgAIF GenBank accession number EX001601.1; BgAIF-rt-F, 5’-cctgcttttaacccgacaga-3’ and BgAIF-rt-R, 5’-tgaatgaaagctcctcgtca-3’. Differential BgAIF gene expression were calculated after normalizing with BgActin (primers as above) and comparison with the non-treated (control) cells. The ΔΔCt method was used to calculate the differential gene expression (58), with assistance of the GraphPad Prism 8 software (San Diego, CA) (Fig. 2c).

**Schistosome sporocysts**

Miracidia of the NMRI strain of *S. mansoni* were hatched from eggs recovered from livers of schistosome infected mice (Schistosomiasis Resource Center, Biomedical Research Institute, Rockville, MD) under axenic conditions (28), primary sporocysts were transformed from the miracidia *in vitro*, as described (26). Briefly, miracidia were immobilized by chilling on ice for 25 min, following by pelleting using centrifugation, 500´g at 4°C, 60 sec. The miracidia were washed with ice cold Chemin’s balanced salt solution (28 mM NaCl, 0.5mM Na₂HPO₄, 2mM KCl, 1.8mM MgSO₄, 7H₂O, 0.6mM NaHCO₃, 3.6 mM CaCl₂.2H₂O) with 1 mg/ml glucose, trehalose, and antibiotic, 10 µl/ml of 100x penicillin/streptomycin (Thermo Fisher Scientific), termed CBSS+. Approximately 5,000 miracidia per well of 24-well plates were cultured in CBSS+ at 26°C for 24 hrs, after which the sporocysts were washed to remove shed ciliated epidermal plates and other debris, followed by transfer to a 1.5 ml microcentrifuge tube (26).

**Sporocyst-Bge cell binding assay and cell adhesion index (CAI)**

To investigate the if BgAIF would affect the ability of cell adhesion to *S. mansoni* sporocyst, we co-cultured the non-transfected Bge cell or non-selected-, transfected-pCas-BgAIFx4 cells (BgAIF depleted-cells named ‘ΔBgAIF-Bge’) with *in vitro* transformed sporocysts, then the cell adhesion index (CAI) were calculated as described (26, 59). With the limitations in this study, we were not be able to select or enrich for BgAIF edited-cells, and hence the ΔBgAIF-Bge cell populations can be considered to be a population of
gene mutated mixed with non-modified (wild type) cells. CAI is a semi-quantitative method of cell adhesion to primary sporocysts using the four categories of scores ranging from one to four - lower to higher numbers of cells adherent to the parasite's surface. In brief, we mixed single cell suspensions of 500,000 Bge cells with 200 freshly prepared sporocysts (total volume 200 µl of CBSS+ in sterile, siliconized tubes (Bio Plas, Thomas Scientific, Swedesboro, NJ). The Bge cell-sporocyst co-culture was maintained at 26°C for 24 hrs. Cellular morphology and adhesion of the cells to the surface of the sporocysts was monitored and recorded using an inverted microscope, at 20x magnification (Zeiss Axios Observer A1, Carl Zeiss LLC, White Plains, NY) after gently transferring the parasite-cell suspension to the tissue culture plate (Greiner Bio-One). Scoring of the adherence index was carried out in a blinded fashion to the investigator reading the score; ³50 sporocysts from each experimental group were counted each time, and triplicates of each treatment group were scored. Seven independent biological replicates of this CAI-based sporocyst-Bge cell binding assay were carried out. In total, ³400 sporocysts were examined from each treatment and control group. Averages for the CAI values were calculated from the cell adhesion scores ranging from 1 to 4 (examples presented in Fig. 3a) according to the formula, CAI = total binding value per number of sporocysts (26).

Results

Cas9 nuclease transcribed in transfected Bge cells

Total RNA was extracted from non-transfected cell (wild type; WT), mock control and pCas-BgAlxF4-transfected Bge cells to assess the expression of Cas9 (Fig. 1b). The cDNAs from either controls or pCas-BgAlxF4-transfected Bge cells were employed as the template in PCRs using two primer pairs, one specific for Cas9 and the other for BgActin, the actin gene of B. glabrata that served as the reference gene (Fig. 1b, c). Transcripts encoding Cas9 in transient pCas-BgAlxF4 transfected-Bge cells were detected at 24 hrs after transfection and expression was maintained for the nine days of the assay. The specific amplicon of Cas9 mRNA (231 bp) was observed from pCas-BgAlxF4 transfected cells, but was absent from the non-transfected cells (Fig. 1c). Our findings supported previous findings that revealed s CMV promoter driven luciferase in Bge cells (60). Expression of the control reference BgActin was observed at 214 bp amplicon in both controls and experimental samples (Fig. 1c).

Programmed mutation of BgAlF confirmed functional CRISPR/Cas9 activity in Bge cells

Genomic DNAs from wild type Bge, medium-transfected (mock) and pCas-BgAlxF4-transfected cells were used as the template for PCRs with the primer pair, AlF-F and AlF-R, flanking the programmed Cas9 cleavage site on BgAlF, exon 4 (Fig. 1a, green arrows; amplicon size, ~200 nt). The red arrow indicates the predicted site of the Cas9-catalyzed double-strand break (DSB) within the BgAlF locus (Fig 1a). The nucleotide sequence of the amplicons was determined by Sanger direct sequencing using the same primers. Both forward and reverse Sanger direct sequencing reads from the same amplicon were estimated for insertion-deletion (INDELs) by the ICE and the TIDE algorithms (55, 56). The reads from the Bge cells transfected with the pCas9-BgAlxF4 contained INDELs at or around the programmed
CRISPR/Cas9 cleavage site. The percentage of reads that included INDELs ranged from 8.9% to 17.1%, in the 12 biological replicates that were carried out (Fig. 2a, b). Notably, the mutation profile in the vicinity of the predicted DSB in *BgAIF* was similar among these 12 replicates, which were undertaken independently. Commonly observed INDELs at the DSBs site as revealed by the ICE analysis included deletions of 8 to 30 bp and insertions of 1 or 2 bp (Fig. 2a). These mutations were predicted to result in frameshift mutations, the consequent loss of the open reading frame, and hence and permanent knockout of *BgAIF* in the gene-edited Bge cell. The profile of the frequency of mutations observed in each biological replicate was used to plot the curve (Prism 8 software) presented in figure 2b. These findings demonstrated that programmed genome editing using CRISPR/Cas9 was active in Bge cells, and that the non-homologous end-joining (NHEJ) pathway (61) was active in *B. glabrata* for the repair of programmed double-stranded breaks, leading to targeted gene knockout.

**Programmed mutation interrupted expression of *BgAIF***

The aims of the study included the investigation of the activity or not CRISPR/Cas9 gene editing in the Bge cell line and addressing the hypothesis that AIF functions in the activation of a macrophage like phenotype by the Bge cell. Accordingly, Bge cells were transfected with pCas9-*BgAIFx4* plasmid DNA. The experimental approach did not include drug resistance and/or reporter gene markers in order to enrich for transfected Bge cells. However, even without enrichment for transfected cells, there was a highly statistically significant reduction in levels of *BgAIF* transcripts in the transfected Bge cell population. Expression of *BgAIF* transcripts were assessed using RNAs from the cells at nine days post transfection. Comparison of the experimental and control groups revealed significantly reduced levels of the *BgAIF* in the pCas-*BgAIFx4*-transfected cells, mean 49.55±20.22%, range 28.1 to 86.3% (n =12) compared to the wild type Bge (normalized sample, 100% expression), mock control cells (unpaired *t*-test and *F* test to compare variances; *F*, DFn, Dfd=294.7, 11, 11, *P* < 0.0001) (Fig. 1b). An inverse correlation between the percentage of INDELs and reduction in transcript levels was not apparent (not shown).

**Programmed knockout of *BgAIF* interfered with adherence of Bge cells to schistosome sporocysts**

Single cell suspensions of Bge cells in the mock-treated and Δ*BgAIF* groups were co-cultured for 24 hrs in siliconized tubes with primary *S. mansoni* sporocysts. At that point, the numbers of cells that had adhered to each sporocyst were scored. This was accomplished by examination of at least five discrete sites of the well of the 24-well plate with >50 sporocysts of each group. The cell adhesion index (CAI) were scored from 1 to 4, with a score of 1 indicating few or no adherent cells and a score of 4 indicating that cells or clumps of cells covered more than half the tegumental surface of the sporocyst, as defined in earlier reports (26) (Fig. 3a). Cells from mock-transfected control group mostly adhered in clumps or singly to the surface of the parasite (representative images in the upper panels of Fig. 3B), with CAI values that ranged from 2 to 4. By contrast, fewer cells adhered to the surface of the sporocysts in the Δ*BgAIF*-Bge group (representative images, lower panels in Fig. 3B), with CAI values ranging from 2 to 3. Only ~20% of the Δ*BgAIF*-Bge cells adhered to the surface of the sporocysts and most of the cells retained remained spread singly on the surface of the well of tissue culture plate (Fig. 3b). More
specifically, the mean CAI values ascertained from the seven biological replicates (≥50 parasites in each replicate (≥400 parasites scored), mean 2.66±0.10, range, 2.53 to 2.78 in the mock-treated, transfection control group was significantly higher than the ΔBgAIF group, mean 2.25±0.22, range, 2.08 to 2.55 (Fig 3c) (unpaired t-test: t=3.661 df = 12, P = 0.0033). More specifically, the CAI category-specific CAI values for mock treated cells averaged from the cell adherence to single sporocysts, were 2.5±1.58 SE, 29.28±4.06, 20.85±2.15, and 13.14±2.55 for categories 1, 2, 3 and 4, respectively. For the ΔBgAIF-Bge cells, the CAI values were 8.86±1.57, 31.42±4.07, 13.28±2.51, and 13.13±2.55 for categories 1, 2, 3 and 4, respectively. Although we observed CAI scores of 1 to 4 in both mock control cells and ΔBgAIF cells, nonetheless there were statistically significant higher numbers of sporocysts with the lowest adherence, scored as ‘1’, in the ΔBgAIF group compared with the mock control group as confirmed using a multiple t-test (t ratio = 3.98, df = 12, P = 0.001). By contrast, there were significantly higher numbers of sporocysts scored as ‘3’ in the control compared with the ΔBgAIF group (t ratio = 3.52032, df = 12, P = 0.004) (Fig. 3d). Last, morphological changes were not apparent between the ΔBgAIF and the control group Bge cells.

Discussion

This report describes a novel use of programmed genome editing by the CRISPR/Cas9 approach in the embryonic cell line from the gastropod snail, B. glabrata, an intermediate host snail of the human blood fluke, S. mansoni. The Bge cell line is an informative tool in investigation of snail-schistosome, host-parasite interactions. A key attribute of the Bge cell is its hemocyte-like phenotype, given the central role of the snail hemocyte in innate and cellular immunity. However, even though Bge cells adhere to the schistosome, the parasite is not killed by these cells in vitro. The allograft inflammatory factor 1 (AIF) is a conserved calcium-binding protein typically expressed in phagocytic and granular leukocytes and is a marker of macrophage activation (38, 41, 45, 62-65). An orthologue, termed BgAIF, is highly expressed in isolates of B. glabrata that are resistant to infection with S. mansoni and this gene may be linked to hemocyte activation (8, 9). Here, we targeted the AIF gene of B. glabrata embryonic cell line using programmed gene knockout to further interrogate its role in the intermediate host-schistosome interaction. We constructed a plasmid vector encoding the CRISPR/Cas9 nuclease and a guide RNA targeting exon 4 of BgAIF gene and the Cas9 nuclease from Streptococcus pyogenes. Bge cells were transfected with the gene-editing construct by square wave electroporation. Transcript levels of BgAIF were significantly reduced by up to 71.9% following transformation. In parallel, sequence reads of amplicons spanning the locus targeted for programmed gene knock-out revealed on-target mutation on the BgAIF gene, that had been repaired by non-homologous end joining leading to gene-inactivating insertions and deletions. In addition, the adherence of gene-edited Bge cells to sporocysts was significantly impeded in comparison to control cells, as ascertained using a semi-quantitative, cell adherence index. In our study, the %INDELs (8.9-17.1%) resulting from NHEJ after CRISPR/Cas9 gene editing on BgAIF exon 4 locus did not correlate with its transcript reduction (~50%) in all experimental samples. Nonetheless, alternative mechanisms could be used as the fusion of suppressors with a ‘dead’ Cas9 which enables gene regulation and increase the level of repression of the target gene (66).
The *B. glabrata* IDS comprises hemocytes and soluble proteins found in the hemolymph, among them the *BgAIF* (67-69). The response of resistant mollusks is given by the adherence and encapsulation of sporocysts by hemocytes, leading to the parasite destruction (70). The AIF-1 was demonstrated to be a pro-inflammatory cytokine that regulates immune-related genes of the oyster, *Crassostrea ariakensis* (36). An orthologue in the leech *Hirudo medicinalis* promotes macrophage-like migration by a chemotactic activity, in addition to being involved in the innate immune responses as also seen in other species (41). The adherence of the mixed populations of *BgAIF* gene-edited/non edited-Bge cells to sporocysts was significantly impeded in comparison to control cells, as ascertained using a semi-quantitative cell adherence index. These cells, albeit in a low percentage, are less responsive to the *S. mansoni* parasite. These data suggested that, in the presence of *S. mansoni*, the Bge cells need to secrete *BgAIF* for activating the recruitment of more adherent Bge cells. Thus, the *BgAIF* protein appears to play a role in cell recognition, migration, and/or adhesion, and to participate in the early immune response to the parasite. The AIF gene is conserved broadly among protostomes and deuterostomes, including vertebrates, and also in prebilaterian including sponges, where it likely performs similar functional roles in macrophage activation and migration (71). In humans, the *HmAIF1* is an NF-κB pathway regulator, a pathway that comprises a family of evolutionarily conserved proteins, important to the immune system by participating in the expression of other proteins related to the immune system (72, 73). Although more studies will be required to decipher the regulation of these pathways in *B. glabrata*, after the pathogen invasion, the *BgAIF* possibly acts throughout the activation of the NF-κB pathway, leading to the recruitment of hemocytes and consequent pathogen elimination (72, 74).

These findings confirmed the tractability of transfection of Bge cells by electroporation with the genome-editing construct, pCas-*BgAIFx4*, and that the CMV promoter drove transcription of Cas9 in this snail species. Whereas transformation by plasmid DNA of Bge cells by square wave electroporation appears to be novel, Bge cells have been transformed using DNA complexed with cationic lipid-based transfection reagents and with polyethyleneimine (23) Nevertheless, our study has some limitations. Thus far we have yet to enrich the transfected cells from wild type cells. Future studies using a drug selectable marker can be designed to address this issue. Other approaches to deliver the CRISPR/Cas gene-editing cargo can be tried including repeated inoculation with ribonuclear protein complexes (75), titration of the transfection chemicals (76), titration of electroporation parameters (77), and/or transduction by lentiviral virions encoding the gRNA and *S. pyogenes* Cas9 nuclease as we have demonstrated with eggs of *S. mansoni* (53, 54). Moreover, CIRCLE-Seq and like approaches can be employed to investigate the off-target mutations (78).

**Conclusions**

Here we demonstrated CRISPR/Cas-based gene editing in a cell line from a medically important taxon of freshwater gastropods that are vectors for the transmission of schistosomiasis. We showed the functional role of a *B. glabrata* allograft inflammatory factor in the recognition/attachment of *S. mansoni* sporocysts *in vitro*. The demonstration of the activity of CRISPR/Cas9 gene editing in Bge cells suggests that genome editing in the germline and somatic tissues of intact *B. glabrata* snails will also be
functional. Whereas improvements can be anticipated in these approaches, an obvious next step will be to gene edit the intact snail \textit{B. glabrata}. Transfection of germline cells within the snail using microinjection can be considered \cite{79}. These findings, together with the first application of the CRISPR/Cas technique in the genetic edition of \textit{Lymnaea stagnalis} mollusk \cite{48} are a step-change since they can favor the creation of a genetically modified \textit{Biomphalaria} line to study the biology and physiology of the snail as well the schistosome-intermediate host relationship. Functional genomics using CRISPR/Cas-based genome editing in schistosomes and other trematodes responsible for major neglected tropical diseases has been reported \cite{53, 54}. The establishment of a functional genomic protocols involving programmed gene editing to address fundamental questions in this host-parasite relationship using genetically modified snails and schistosomes now seems to be feasible.

\section*{Declarations}

\section*{Acknowledgments}

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\section*{Ethics approval and consent to participate}

The protocols and procedures to maintain \textit{S. mansoni} life cycle in mice performed at the NIAID Schistosomiasis Resource Center of the Biomedical Research Institute, Rockville were approved by the Institutional Animal Care and Use Committee (IACUC) (protocol number 18-04) and followed by United States Animal Welfare Act and George Washington University IACUC policies (assurance number A3205-01)

\section*{Consent for publication}

Not applicable

\section*{Availability of data and materials}

Data supporting the conclusions of this article are included within the article. The raw datasets used and analyzed during the present study are available from the corresponding authors upon reasonable request.

\section*{Competing interests}

The authors declare that they have no competing interests.

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Authors’ contributions

WI, MM and PJB designed the study. FSC and WI wrote the protocol. MMM, VMH and PJB reviewed the protocol. WI served as study team leader and director. VHM and AM obtained ethical approval for vertebrate animal use. FSC, RR, AM and SEK conducted the gene editing and cell culture experiments. FSC, RR and WI performed gene mutation analysis. FSC, OSC and RCL performed CAI analysis. WI, MMM and PJB wrote the manuscript. All authors read and approved the final manuscript.

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Abbreviations

AIF: allograft inflammatory factor 1; Bge: Biomphalaria glabrata embryonic cell line; Cas9: CRISPR associated protein 9; RNA: ribonucleic acid; PCR: polymerase chain reaction; CAI: cell adherence index; CRISPR: Clustered Regularly-Interspaced Short Palindromic Repeats; gRNA: guide RNA; CMV: cytomegalovirus; DBS: double strand break; SRC: Schistosomiasis Resource Center; BRI: Biomedical Research Institute; RT-PCR: reverse transcription PCR; DNA: deoxyribonucleic acid; cDNA: complementary deoxyribonucleic acid; IDS: internal defense system; EDTA: ethylenediaminetetraacetic acid; INDEL: insertion–deletion mutation; TIDE: Tracking of Indels by Decomposition; ICE: Inference of CRISPR Edits analysis; DNase I: deoxyribonuclease I, CBSS: Chernin’s balanced salt solution; WT: wild type; NHEJ: non-homologous end-joining; NF-κB: nuclear factor kappa-B.
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Figures

Figure 1
Schematic diagram of BgAIF gene structure, CRISPR/Cas9 vector and expression in Bge cell. Panel a. Gene structure of B. glabrata allograft inflammatory factor (BgAIF), accession number BGLB005061 and gene editing target locus (red box) on exon 4. BgAIF gene composed of 5 exons and 4 introns. The green arrows indicate the location of primers flanking expected DSBs which were used in PCR to generate the on-target amplicon for INDELs estimation. Panel b. Map of the pCas-BgAIFx4 vector which includes the Pol III-dependent mammalian U6 gene promoter (red arrow) to drive transcription of the guide RNA targeting exon 4 of BgAIF gene (red arrow) and the CMV promoter to drive expression of the S. pyogenes Cas9 nuclease (blue arrow). Primer pairs specific for the guide RNA and for Cas9 are indicated (green arrows). Panel c. Expression of Cas9 and of BgActin (as the reference gene) transcripts as established by semi-quantitative RT-PCR in pCas-BgAIF-transfected (right) and control (left) Bge cells from days one to nine following transfection. The amplicons of the expected sizes are as indicated: 23 bp for Cas9 and 214 bp for BgActin. All RNA samples were positive for the BgActin reference gene; the 214 bp band.

Figure 2

Establishment of BgAIF-knockout lines of Bge cells. Panel a. Representative examples of frequent gene insertions-deletions (1-2 bp insertions and 8-30 bp deletions, straddling the programmed CRISPR/Cas9-induced double-stranded break in exon 4, as determined by ICE software-based analysis. Panel b. TIDE algorithm-based violin plot of insertion-deletion percentages (%INDEL) computed using the amplicon sequence traces from the 12 biological replicates of pCas-BgAIF-transfected Bge cell populations. Panel c. Reduction of BgAIF transcription by about 50% following programmed genome editing of Bge cells (∆BgAIF-Bge) in comparison to control Bge cells. Mean transcript reduction, 49.55± 20.22 (S.D.) percent, P ≤ 0.0001 (****), n =12 (unpaired Student’s t-test).
Figure 3

Programmed knockout of BgAIF in Bge cells caused reduced adherence to primary sporocysts. Panel a. Representative micrographs of primary sporocysts co-cultured with Bge cells in our laboratory to profile the semi-quantitative scoring of the cell adhesion index (CAI); CAI value = 1; no cells adhering to the surface of the sporocyst; value = 2; ≤10 cells adhering to the sporocyst; value = 3; >10 cells < half of the sporocyst surface covered by cells or clumps of cells; value = 4; > half the sporocyst surface covered by Bge cells. Panel b. Representative micrographs indicate the reduced levels of ΔBgAIF-Bge cells adherence (right panel) in comparison to control, mock-transfected Bge cells (left panel) to the co-cultured sporocysts. Panel c. Bar chart to present the CAI values from control (mock-transfected) ΔBgAIF-Bge cells during co-culture with primary sporocysts at a co-culture ratio of one sporocyst to 100 Bge cells; CAI value = 2.66±0.10, mean ±SD (476 sporocysts in total scored) for the mock-transfected Bge and 2.31±0.23 for the ΔBgAIF-Bge cells (424 sporocysts in total scored); P = 0.0033, unpaired Student’s t test; n =7 biological replicates.