Vaccination with *Schistosoma mansoni* cholinesterases reduces parasite burden and egg viability in a mouse model of schistosomiasis

Bemnet A. Tedla, Darren Pickering, Luke Becker, Alex Loukas and Mark S. Pearson[#].

Centre for Molecular Therapeutics, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, Queensland, 4878, Australia.

[#]Corresponding author

Dr Mark Pearson (mark.pearson@jcu.edu.au)

Abstract

Schistosomiasis is a neglected tropical disease which kills 300,000 people every year in developing countries and there is no vaccine. Recently, we have shown that cholinesterases (ChEs) - enzymes that regulate neurotransmission - from *Schistosoma mansoni* are expressed on the tegument and present in the excretory/secretory products of schistosomula and adult worms, and are essential for parasite survival in the definitive host, highlighting their utility as potential schistosomiasis vaccine targets. When treated *in vitro* with anti-*Sm*ChE IgG, both schistosomula and adult worms displayed significantly decreased ChE activity, which eventually resulted in parasite death. Vaccination with individual SmChEs, or a combination of all three SmChEs, significantly reduced worm burdens in two independent trials compared to controls. Liver egg burdens were significantly decreased for all vaccinated mice across both trials (13% - 46%) except for those vaccinated with *Sm*AChE1 in trial 1. Egg viability, as determined by egg hatching from liver homogenates, was significantly reduced in the groups vaccinated with the *Sm*ChE cocktail (40%) and *Sm*AChE2 (46%). Further, surviving worms from each vaccinated group were significantly stunted and depleted of glycogen stores, compared to controls. These results suggest that *Sm*ChEs could be incorporated into a vaccine against schistosomiasis to reduce the pathology and transmission of this debilitating disease.

Introduction

Despite decades of concentrated research, there is still no effective and practical vaccine against schistosomiasis [1]. Further, mass chemotherapy using praziquantel (PZQ), the only effective anti-schistosomal drug, is complicated by rapid and frequent reinfection [2]. There is also evidence that resistance to PZQ is emerging [3]. So far, a considerable number of schistosome antigens have been identified and tested as vaccines and, although a number of these vaccine candidates (for example *Sm*TSP-2, *Sm*14, *Sm*29, *Sm*CB1 and *Sm*p80) have shown promising efficacy in animals models and are in various stages of pre-clinical or clinical development, none has been approved for licensure [reviewed in [1]].

Due to the fundamental roles they play in parasite biology (reviewed in [4], schistosome cholinesterases (*Sm*ChEs) have been posited as intervention targets against schistosomiasis and there are several indications to support the feasibility of their use as vaccines. Firstly, *Sm*ChEs have been localized to the tegument of schistosomula and adult worms [5, 6] and anti-*Sm*ChE antibodies have been shown to bind to and kill schistosomula [7], suggesting that the enzymes are accessible to immune attack. Anti-*Sm*ChE antibodies also showed no cross-reactivity against human AChE [5], indicating that a vaccine safe for human use could be designed. Thirdly, protein array studies have detected significantly high levels of antibodies to *Sm*ChEs in humans exhibiting resistance and low pathology to schistosomiasis, suggesting an involvement of these antibodies in a protective anti-schistosomal response [8, 9]. Most of these studies have employed the use of anti-*Sm*ChE antibodies raised against biochemically purified material and so the vaccine efficacy of any one *Sm*ChE paralog remains to be elucidated.

A recent study by us [6] has documented the existence of three *Sm*ChE paralogs (two acetylcholinesterases – *smache1* and *smache2* – and one butyrylcholinesterase - *smbche1*) and

we showed that each molecule localized to the tegument of adults and schistosomula and demonstrated, through RNAi-mediated suppression *in vitro* and *in vivo*, that each paralog was essential to parasite survival. We also reported a significant reduction in the glucose-scavenging ability of silenced parasites, providing evidence for the involvement of tegumental AChE in the mediation of exogenous glucose uptake, which has also been documented by other studies [10-12]. Despite the fundamental roles that SmChEs appear to play in parasitism, it remains to be determined which *Sm*ChE paralogs are effective vaccine targets in *S. mansoni*.

Herein, we demonstrate that purified IgG against each of the three *Sm*ChE paralogs inhibit ChE activity in both larval and adult worms *in vitro*, which results in eventual parasite death. Further, we document the efficacy of these *Sm*ChEs, when administered as recombinant vaccines in isolation or as a triple combination, in reducing parasite burden, stunting worm growth and decreasing egg viability.

Materials and Methods

Ethics statement

All experimental procedures reported in the study was approved by the James Cook University (JCU) animal ethics (Ethics approval numbers A2391). Mice were maintained in cages in the university's quarantine facility (Q2152) for the duration of the experiments. The study protocols were in accordance with the 2007 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the 2001 Queensland Animal Care and Protection Act.

Parasites

Biomphalaria glabrata snails infected with *S. mansoni* (NMRI strain) were obtained from the Biomedical Research Institute (BRI). Cercariae were shed by exposure to light at 28°C for 1.5

h and mechanically transformed to obtain schistosomula [13]. To obtain adult worms, 6-8 week old male BALB/c mice (Animal Resource Centre, WA) were infected with 120 cercariae via abdominal penetration and parasites harvested by vascular perfusion at 7-8 weeks post-infection [14].

Recombinant protein expression and purification

Complete ORFs for *smache1*, *smbche1* and *smache2* were synthesized by Genewiz. Attempts to express full-length sequences in *E. coli* were unsuccessful, so primer sets incorporating *Nde*I (forward primer) and *Xho*I restriction enzyme sites (reverse primer) were designed to amplify partial, non-conserved regions of each *smche* [6], which might prove more amenable to expression. Sequences (containing *Nde*I/ *Xho*I sites) for each *Sm*ChE were amplified from each full-length template by PCR and cloned into the pET41a expression vector (Novagen) such that the N-terminal GST tag was removed. Protein expression was induced for 24 h in *E. coli* BL21(DE3) by addition of 1 mM Isopropyl beta-D-1-thiogalactopyranoside (IPTG) using standard methods. Cultures were harvested by centrifugation (8,000 *g* for 20 min at 4°C), re-suspended in 50 ml lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 40 mM imidazole) and stored at -80°C. Cell pellets were lysed by three freeze-thaw cycles at -80°C and 42°C followed by sonication on ice (10 × 5 s pulses [70% amplitude] with 30 s rest periods between each pulse) with a Qsonica Sonicator. Triton X-100 was added to each lysate at a final concentration of 3% and incubated for 1 h at 4°C with end-over-end mixing. Insoluble material (containing *Sm*ChEs) was pelleted by centrifugation at 20,000 *g* for 20 min at 4°C. The supernatant was discarded, and inclusion bodies (IBs) were washed twice by resuspension in 30 ml of lysis buffer followed by centrifugation at 20,000 *g* for 20 min at 4°C. IBs were then solubilized sequentially by resuspension in 25 ml lysis buffers containing either 2 M, 4 M or 8 M urea, end-over-end mixing overnight at 4°C and centrifugation at 20,000 *g* for 20 min at 4°C.

Finally, supernatant containing solubilized IBs was diluted 1:4 in lysis buffer containing 8M urea and filtered through a 0.22 µm membrane (Millipore). Solubilized IBs were purified by immobilized metal affinity chromatography (IMAC) by loading onto a prepacked 1 ml His-Trap HP column (GE Healthcare) equilibrated with lysis buffer containing 8M urea at a flow rate of 1 ml/min using an AKTA-pure-25 FPLC (GE Healthcare). After washing with 20 ml lysis buffer containing 8M urea, bound His-tagged proteins were eluted using the same buffer with a stepwise gradient of 50-250 mM imidazole (50 mM steps). Fractions containing *Sm*ChEs (as determined by SDS-PAGE) were pooled and concentrated using Amicon Ultra-15 centrifugal devices with a 3 kDa MWCO and quantified using the Pierce BCA Protein Assay kit. The final concentration of each *Sm*ChE was adjusted to 1 mg/ml and proteins were aliquoted and stored at -80°C.

Generation of anti-SmChE antisera and purification of IgG

Three groups of five male BALB/c mice (6-week-old) were intraperitoneally immunized with either *Sm*AChE1, *Sm*BChE1 or *Sm*AChE2 subunits (50 µg/mouse). Antigens were mixed with an equal volume of Imject alum adjuvant (Thermofisher) and administered three times, two weeks apart. Two weeks after the final immunization, mice were sacrificed and blood was collected via cardiac puncture. Blood from all mice in each group was pooled and serum was separated by centrifugation after clotting and stored at −20°C. Polyclonal antibodies were purified from mouse sera using Protein A Sepharose-4B (Thermofisher) according to the manufacturer's instructions. Serum from naïve mice was similarly processed.

Effect of polyclonal anti-SmChE IgG on larval worms

Newly transformed schistosomula (1000/ml) were cultured in DMEM (supplemented with 4x AA) at 37°C and 5% CO_2 in the presence of 50 µg of either anti-*Sm*AChE1, *Sm*BChE1 or

*Sm*AChE2 polyclonal IgG (section 3.2.8) or a combination of all three antibodies (equal amounts - 50 µg total). Separate sets of parasites were similarly incubated with 50 µg of naïve mouse IgG, which served as a control. After 2 and 14 h (separate experiments were conducted for each timepoint), 300 schistosomula from each experiment were removed and assessed for viability using Trypan Blue exclusion (100 parasites in triplicate) [15]. Surface and secreted ChE activity was measured using the remaining parasites by incubating them in 0.5 ml of assay buffer (0.1M sodium phosphate, pH 7.4, 2 mM acetylthiocholine [AcSCh], or 2 mM butyrylthiocholine [BcSCh], and 0.5 mM 5, 5'-dithio-bis 2-nitrobenzoc acid [DTNB]) and monitoring the absorbance increase (AcSCh conversion or BcSCh conversion) over 1 hour at 405 nm in a Polarstar Omega microplate reader (BMG Labtech). Parasites cultured with naïve IgG served as a negative control. Data are presented as the average of two biological and three technical replicates ± SEM.

Effect of polyclonal anti-SmChE IgG on adult worms

Enzyme inhibitory effects 24 h after addition of IgG (including naïve IgG) were measured as for schistosomula using 5 pairs of freshly perfused adult worms in 1 ml of media. Data are presented as the average of two biological and two technical (four total) replicates ± SEM. To investigate the effects of polyclonal anti-*Sm*ChE IgG on worm viability, ten pairs of worms were similarly incubated with antibodies for 10 days, monitored every 24 h for motility by microscopic examination and considered dead if no movement was seen. To measure the effect of polyclonal anti-*Sm*ChE IgG on glucose uptake, ten pairs of worms were similarly incubated with antibodies for 24 h, transferred to DMEM (1000 mg/l) and media glucose concentration was measured over a 24 h period using a glucose assay kit (Sigma). Glucose levels were expressed relative to media collected from worms which received naïve IgG (negative control). Data are presented as the average of two biological replicates ± SEM.

Anti-SmChE IgG responses in S. mansoni-infected mice during infection and before and after PZQ treatment

Sera from *S. mansoni* infected male BALB/c mice (6-8 weeks) (n=5) was collected at day 3, 14, 28, 42 and 56 post infection (p.i.) to assess anti-*Sm*ChE responses during the course of parasite infection. In a separate experiment, sera from *S. mansoni* infected male BALB/c mice (6-8 weeks) (n=11) was collected at 5 weeks p.i. and then mice were treated orally with PZQ (100 mg/kg) at 35, 37 and 39 days p.i. Sera was again collected at day 49 p.i. (2 weeks post-PZQ treatment). Anti-*Sm*ChE responses during infection and before and after PZQ treatment were screened by ELISA with plated *Sm*ChEs (100 ng/well) using standard methods. The cutoff value for each dilution was established as three times the mean OD of the naïve sera for that dilution and the endpoint was defined as the highest dilution above the cutoff value.

Vaccine trials

Five groups of 10 male BALB/c mice (6-8 weeks) were immunized intraperitoneally on day 1 (50 µg/mouse) with either *Sm*AChE1, *Sm*BChE1, *Sm*AChE2, a combination of all three *Sm*ChEs (17 µg each - 50 µg total) or PBS, each formulated with an equal volume of Imject alum adjuvant (Thermofisher) and 5 µg of CpG ODN1826 (InvivoGen). Immunizations were repeated on day 15 and 29 and each mouse was challenged (abdominal penetration) with 120 *S. mansoni* cercariae on day 43. Two independent trials were performed to ensure reproducibility. Blood was sampled at day 28 and 42 and on the day of a necropsy to determine pre- and post-challenge antibody titers.

Mouse necropsy and estimation of worm and egg burden

Mice were necropsied at day 91 (7 weeks p.i.) and worms harvested by vascular perfusion and counted. Worms from the mice in each group were pooled and a random sample of each pool was photographed and measured using ImageJ software. Livers were removed and halved, with one half weighed and digested for 5 h with 5% KOH at 37^0C with shaking. Schistosome eggs from digested livers were concentrated by centrifugation at 1,000 g for 10 minutes and re-suspended in 1 ml of 10% formalin. The number of eggs in a 5 µl aliquot was counted in triplicate and the number of eggs per gram (EPG) of the liver was calculated. Small intestines were removed and cleaned of debris before being weighed and digested as per the liver halves. Eggs were also similarly concentrated and counted to calculate intestinal EPG.

Egg viability assays

The other half of each liver was pooled according to the group, homogenized in H_2O and placed in identical foil-covered volumetric flasks under bright light to hatch eggs released from the livers. After 1 h, the number of miracidia in 10 x 50 µl aliquots of H_2O (sampled from the extreme top of each flask) were counted. The number of eggs in each flask at the start of the hatching experiment was determined by liver EPG calculations, allowing the egg hatching index of each group to be calculated by expressing the hatched eggs (miracidia) as a percentage of the total eggs.

Glucose consumption and glycogen storage assays

Five pairs of freshly perfused worms from each vaccinated group were cultured in DMEM (1000 mg/l glucose). Media (50 µl) from each experiment was collected after 24 h, and the amount of glucose was quantified using a colorimetric glucose assay kit (Sigma) according to the manufacturer's instructions. Glucose levels were expressed relative to media collected from

worms recovered from PBS treated mice (negative control). To measure the glycogen content of these worms, Triton X-100-soluble extracts of each group of five pairs of worms (made by homogenizing the parasites in 1% Triton X-100, 40 mM Tris-HCl, pH 7.4, mixing overnight at 4°C and collecting the supernatant by centrifugation at 15,000 *g* for 1 h at 4°C) were assayed for glycogen in a modified procedure described by Gomez-Lechon et al. [16]. Briefly, 0.2 M sodium acetate, pH 4.8, was added to 30 µg parasite extract and 50 µl glucoamylase (10 U/ml) to make a reaction volume of 150 µl. The mixture was incubated at 40°C for 2 h with shaking at 100 rpm, 40 µl added to a new microplate with 10 µl 0.25 M NaOH and the amount of glucose quantified using the colorimetric glucose assay kit. Extracts were made from triplicate sets of parasites and assays were performed three times. Data are presented as the average of each triplicate biological and technical experiment ± SEM.

Experiments involving sera from vaccinated mice

Sera were collected from all mice in each group before cercarial challenge and at necropsy. Serum anti-*Sm*ChE IgG titres were measured by ELISA against plated *Sm*ChEs using standard methods. The cutoff value for each dilution was established as three times the mean OD of the naïve sera for that dilution and the endpoint was defined as the highest dilution above the cutoff value.

To assess whether vaccination-induced antibodies would interact with host serum AChE or BChE, AChE and BChE activity of pre-challenge sera from all vaccinated and control mice was measured by Ellman assay. Briefly, 1 µl of pre-challenge serum from each mouse was added to 200 µl assay buffer (0.1M sodium phosphate, pH 7.4, 2 mM acetylthiocholine [AcSCh], or 2 mM butyrylthiocholine [BcSCh], and 0.5 mM 5, 5'-dithio-bis 2-nitrobenzoc acid [DTNB]) and the absorbance increase (AcSCh conversion or BcSCh conversion) monitored

over 20 mins at 405 nm in a Polarstar Omega microplate reader (BMG Labtech). Data are presented as the average of three technical replicates ± SEM.

Statistical analyses

Statistical differences for all experiments in this chapter were calculated by the Student's *t* test using GraphPad Prism 7 software. Results are expressed as the mean ± standard error of the mean (SEM).

Results

Anti-SmChE polyclonal antibodies block enzyme activity and decrease viability of larval S. mansoni in vitro

To determine the ability of anti-*Sm*ChE specific polyclonal antibodies to inhibit ChE activity in *S. mansoni*, and the effect this had on parasite viability, we studied the effects of paralog-specific antibodies on schistosomula at two different timepoints. Treating schistosomula with anti-*Sm*AChE1 IgG, anti-*Sm*AChE2 IgG, or a cocktail of all three anti-*Sm*ChE IgGs caused significant inhibition ($P \leq 0.01$) of AChE activity by 56.2%, 57.1% and 59.74%, respectively, 2 h after treatment, in comparison with the naïve IgG control (Fig 1A). When schistosomula were incubated with anti-*Sm*BChE1 IgG or a cocktail of all three anti-*Sm*ChE IgGs for 2 h, BChE activity was inhibited by 37.4% ($P \leq 0.01$) and 49.3% ($P \leq 0.001$), respectively, compared to the control (Fig 1B). Schistosomula viability was not significantly affected at this timepoint (Fig 1C). Extending the treatment with anti-*Sm*AChE1 IgG, anti-*Sm*AChE2 IgG, or a cocktail of all three anti-*Sm*ChE IgGs for 14 h significantly decreased ($P \leq 0.001$) AChE activity by 66.9%, 70.5% and 72.6%, respectively, compared to the control (Fig 1D). Similarly, when schistosomula were incubated with anti-*Sm*BChE1 IgG or a cocktail of all three anti-*Sm*ChE IgGs for 14 h, BChE activity decreased significantly ($P \leq 0.01$) by 26.5%

and 35.6%, respectively, compared to the control (Fig 1E). Schistosomula viability was significantly decreased ($P \leq 0.01$) by all treatments at this timepoint, with the biggest decrease seen in the anti-*Sm*ChE cocktail IgG-treated group (Fig 1F).

Effects of anti-SmChE antibodies on adult worms

The effects of anti-*Sm*ChE antibodies on *S. mansoni* adult worms was also tested. Freshly perfused worms cultured in the presence of anti-*Sm*AChE1, anti-*Sm*AChE3, or a cocktail of all three anti-*Sm*ChEs, showed no significant inhibition of AChE or BChE activity at 2 h post-treatment, compared to controls. After 24 h treatment, however, all anti-*Sm*ChE IgG-treated groups showed significant inhibition of AChE and BChE activity with the anti-*Sm*ChE cocktail IgG-treated group displaying the greatest inhibition of AChE activity (Fig 2A and B). The rate of glucose uptake over 24 h was also measured at this timepiont and all antibody treatments significantly reduced glucose uptake in adult worms, compared with naïve IgG-treated controls, again with the anti-*Sm*ChE cocktail IgG-treated group displaying the greatest inhibition (Fig 2C). To determine if anti-*Sm*ChE antibodies can play a role in killing adult worms, the antibody experiment was repeated with 5 pairs of adult worms per treatment and worm viability post-treatment was assessed. Consistent with inhibition of AChE activity and glucose uptake, the cocktail of anti-*Sm*ChE antibodies were the most effective in killing (all worms dead at day 7 post-treatment), compared to controls (Fig 2D).

Antibody responses to SmChEs during the course of infection and following PZQ treatment in mice

Antibody responses to all *Sm*ChEs were significantly higher in infected mice than before infection and increased as infection progressed (Fig 3A). In a separate experiment, all anti-

*Sm*ChE IgG responses were shown to significantly increase ($P \leq 0.001$) after PZQ treatment (Fig 3B).

Vaccine efficacy of recombinant SmChEs in a mouse model of schistosomiasis

In both vaccine trials, all four groups of mice immunized with *Sm*ChEs, either in isolation or as a combination, showed a significant decrease in worm burden (28% - 38%), compared to controls, with the *Sm*ChE cocktail-vaccinated group displaying the highest reduction in trial 1 and 2 of 38% ($P \leq 0.001$) (Fig 4A and B). Compared to controls, significant decreases in liver egg burdens (expressed as eggs per gram – EPG) were observed for all groups across both trials (13% - 46%), except for the *Sm*AChE1-vaccinated group in trial 1. When averaged over both trials, liver egg burdens in the cocktail-vaccinated group showed the greatest reduction (Fig 4C and D). Intestinal egg burdens (expressed as EPG - only determined for trial 2) were significantly reduced for all vaccinated groups, compared to controls, with the greatest reduction seen in the group vaccinated with the *Sm*ChE cocktail (33%, $P \leq 0.001$). (Fig 4E). Egg viability (only assessed for trial 2), as determined by egg hatching from liver homogenates, was significantly reduced in the groups vaccinated with the *Sm*ChE cocktail (40%, $P \leq 0.01$) and *Sm*AChE2 (46%, $P \leq 0.01$) (Fig 4F). While there was no significant reduction in glucose uptake for worms from any of the vaccinated groups, compared to controls, the glycogen content of worms from all vaccinated groups was significantly lower (24% - 52%, $P \leq 0.001$) than worms from the control group (Fig 5A). A significant reduction in worm length (30%-50%) was also observed between worms from all vaccinated groups compared to worms from the control group (Fig 5B). As with the parasitology burden data, worms from the cocktail-vaccinated group showed the greatest decrease in glycogen content and body length. Glucose uptake, glycogen content and worm size was not significantly different between control and

vaccinated groups in trial 2. Serum AChE and BChE activity was also not significantly different between control and vaccinated mice (only measured for trial 2) (Fig S1A and B).

Antibody responses in vaccinated mice

Moderate to high (>1,000,000) anti-*Sm*ChE endpoint titres were seen in the pre-challenge serum of all mice in all vaccinated groups. Post-challenge titres in all groups were four- to ten-fold lower than pre-challenge titres (Table S1 and S2). There was no correlation between pre-challenge titres and worm burdens in any groups.

Discussion

Surface-exposed proteins and secreted proteins are effective targets for vaccine development in schistosomes due to their capacity for interaction with host antibodies [17, 18]. In this regard, *Sm*ChEs are promising candidates as we have shown in previous immunolocalization studies in *S. mansoni* that *Sm*AChE1, *Sm*BChE1 and *Sm*AChE2 are expressed in the tegument of adult worms and schistosomula and proteomic analysis of *S. mansoni* ES products has confirmed the presence of *Sm*AChE1 and *Sm*BChE1 [6]. Further, RNAi-mediated silencing of all 3 *Sm*ChE genes, both individually and in combination, significantly decreased schistosomula viability *in vitro* and parasite survival *in vivo* [6], implying that these genes are essential for proper worm development and function. Moreover, recent protein array studies have demonstrated high levels of circulating antibodies to *Sm*BChE1 in individuals exhibiting drug-induced resistance and a low pathology reaction to schistosomiasis, implicating these antibodies in a protective anti-schistosomal response [8, 9].

Given *Sm*ChEs are accessible to antibody attack, and are enzymatically functional [6], catalytic activity can be used to measure the effectiveness of antibody binding as the interaction between

enzymes and their corresponding antibodies generally leads to a complete or partial reduction in their enzymatic activity [19-21]. The data presented herein show that antibodies against recombinant *Sm*ChEs are capable of inhibiting surface (and, in the case of *Sm*AChE1 and *Sm*BChE1, secreted) enzymatic activities in both schistosomula and adult worms, which is similar to previous studies that have used antibodies raised against parasite-derived AChE to inhibit AChE activity on intact *S. mansoni* [22], *Necator americanus* [23], *Dictyocaulus viviparus* [24] and *Electrophorus electricus* [25]. The inhibitory effect of antibodies is potentially due to steric hindrance, potentially blocking substrate access to the peripheral anionic sites or catalytic gorge of AChE. Indeed, previous studies on rabbit [26], human [27] and bovine [28] AChE have documented the AChE-inhibitory ability of antibodies raised against epitopes other than AChE-active sites.

Consistent with the effects of RNAi-mediated *smche* gene silencing [6], antibody-mediated *Sm*ChE inhibition resulted in a significant decrease in parasite viability. We posit that this mode of enzyme inhibition was the cause of eventual parasite death as schistosomula were still viable, compared to controls, at 2 hours after antibody treatment, despite a significant decrease in ChE activity; it was not until after a much longer exposure (14 h) to anti-*Sm*ChE antibodies that parasite viability was significantly lower.

Glucose uptake in adult worms was also significantly reduced by anti-*Sm*ChE antibody treatment. The cholinergic action of surface AChE has been implicated in mediation of the glucose scavenging mechanism in schistosomes [10], AChE-inhibitory metal complexes reduce glucose import in the parasites [11] and we and others have shown that RNAi-mediated silencing of schistosome *che* genes lessens the uptake of glucose by these parasites [6, 12], so it is possible that antibody-mediated impairment of AChE involvement in the glucose uptake

pathway is the cause of this effect. It may be that there is some redundancy in the cholinergic functioning of these molecules (even BChEs, like *Sm*BChE1, can perform a cholinergic role in situations of AChE deficiency [29]) and so collective inhibition of the molecules is required to produce a functional deficit. Also, given the multiple proposed functions for parasite ChEs [4, 30], it is possible is that the neutralization of multiple enzymatic targets more profoundly interrupts varied processes of parasite biology than just cholinergic transmission.

Given the relative cytotoxic potential of antibodies against all three *Sm*ChEs as opposed to any single *Sm*ChE, we decided to test the efficacy of this antigen cocktail as a vaccine in a mouse model of schistosomiasis. The vaccine efficacy of each individual *Sm*ChE was also tested to investigate the relative anti-parasitic effects of each *Sm*ChE over the cocktail or one another. Mice vaccinated with the cocktail of *Sm*ChE antigens displayed the highest level of protection against experimental schistosomiasis, showing the greatest reductions in every parameter tested. An additive protective effect was not readily apparent, however, as protection levels were not significantly different from groups vaccinated with single antigens. Of the groups vaccinated with individual *Sm*AChEs, the *Sm*AChE2-vaccinated group engendered the highest levels of protection. Similar results were reported in a test of the vaccine efficacy of a recombinant AChE from *S. japonicum* [12]. Further, vaccine trials using purified secretory AChE from the nematodes *Trichostrongylus colubriformis* and *Dictyocaulus viviparous* have resulted in significant protection in animal models [31, 32].

Egg burdens did not concomitantly decrease with worm burdens, but there were significant reductions in egg viability in all but the *Sm*AChE1-vaccinated group, an observation we have previously reported when testing the *in vivo* anti-schistosomal efficacy of AChE-inhibitory drugs [11]. Studies in rats and honey bees have observed abdominal spasms and involuntary

muscle contractions when AChE inhibitors have been administered to these organisms [33, 34] so a possible explanation for this "less than expected" decrease in egg number but significant reduction in viability could be that ova are being prematurely released as a result of antibody-mediated AChE inhibition affecting reproductive tract motility. It could also be that ChE vaccination affected fecundity and egg maturity given the significantly smaller size of worms recovered from vaccinated groups, compared to controls. Indeed, previous studies on insects demonstrated that suppression of AChE expression considerably reduced the weight and length of surviving organisms [35-37] and severely affected the hatching ability of the eggs laid [35, 38]. These reports have suggested that dysregulated cell proliferation and apoptosis during larval growth may be reasons for such phenotypic effects attributed to the absence of AChE, although such a link in trematodes remains to be established. Finally, parasites recovered from vaccinated mice had significantly depleted glycogen stores. Reduced glycogen content and glucose uptake have been previously observed in worms treated with AChE-inhibitory drugs *in vitro* [11] and be attributed to interference with the tegumental AChE-mediated glucose scavenging pathway [10] through the inhibition of this enzyme. It could be that the same effect is being orchestrated by antibody-mediated inhibition of AChE (which would be consistent with the results of *in vitro* antibody-based experiments), forcing the parasite to rely on its glycogen stores, rather than the scavenging of exogenous glucose, for nutrition.

Even though immunization with *Sm*ChEs induced high antibody titers, these antibody levels were not sustained during the course of infection, with titers at necropsy dropping between four- and ten-fold from pre-challenge levels. This would seem to indicate that the specific antibody response induced by immunization was not augmented by natural infection, a hypothesis corroborated by the generation of modest anti-*Sm*ChE titers during the course of parasite infection in a separate experiment. That being said, the *Sm*ChEs used in this study were still

capable of inducing moderate levels of protection in the face of modest antibody titers. Treatment with PZQ has been shown to induce antibody-mediated resistance to schistosomiasis in humans through the exposure of parasite antigens to the immune system (and subsequent generation of an antibody response) as a result of tegument damage [39] and protein array studies by us have shown that antibodies to *Sm*BChE1 are significantly upregulated in resistant individuals [8]. The upregulation of *Sm*ChE immune responses following PZQ treatment has been verified in this study. Given that an effective anti-schistosomal vaccine strategy would ideally be linked with chemotherapy [8], it is possible that the vaccine efficacy of antigens such as the ones described here could be increased by vaccination after PZQ treatment due to the augmentation of an already upregulated immune response.

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Figure legends

Fig 1. Anti-*Sm*ChE antibodies inhibit ChE activity in schistosomula which leads to decreased parasite viability. Newly transformed schistosomula (1000/treatment) were incubated in DMEM in the presence of anti-*Sm*ChE IgG and incubated at 37 °C in 5% CO_2. Naïve IgG served as a negative control. (A) AChE activity 2 h after treatment (B) BChE activity 2 h after treatment (C) schistosomula viability 2 h after treatment (D) AChE activity 14 h after treatment (E) BChE activity 14 h after treatment (F) schistosomula viability 14 h after treatment. Data represents the mean ± SEM of two biological and three technical replicates. Significance (relative to the naïve IgG control) determined by the student's *t* test ** $P \leq 0.01$, *** $P \leq 0.001$.

Fig 2. Effects of anti-*Sm*ChE antibodies on adult *S. mansoni* worms. Five pairs of freshly perfused worms were incubated in the presence of anti-*Sm*ChE purified IgG in DMEM at 37 °C in 5% CO_2. Naïve IgG served as a negative control. (A) AChE activity 24 h after treatment (B) BChE activity 24 h after treatment, (C) glucose uptake over 24 h, one day after treatment (D) survivability up to 10 days after treatment. The results are the mean ± SEM of two biological and three technical replicates (A-C) or two biological replicates (D). Significance (relative to the naïve IgG control) determined by the student's *t* test *$P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Fig 3. Antibody responses to *Sm*ChEs during the course of infection and following PZQ treatment in mice. ELISAs showing anti-*Sm*ChE IgG responses in mice (A) from 3 days to 8 weeks post-infection (n=5) and (B) before (5 weeks post-infection) and 2 weeks after PZQ treatment (n=11). Data represents the mean of 2 technical replicates and significance determined by the student's *t* test ***$P \leq 0.001$.

Fig 4. Vaccine efficacy of recombinant *Sm*ChEs in a mouse model of schistosomiasis. Graphs show parasitology burdens from vaccinated and control mice. (A) trial 1 adult worms (B) trial 2 adult worms (C) trial 1 liver EPG (D) trial 2 liver EPG (E) trial 2 intestinal EPG. (F) Hatching viability of eggs obtained from the pooled livers of control and vaccinated mice from trial 2. Data are the average of ten replicate counts ± SEM of hatched miracidia. Significance and percent reductions (if any) for all parameters are measured relative to the control group. Significance determined by the student's *t* test $*P \leq 0.05$, $**P \leq 0.01$, $**P \leq 0.001$.

Fig 5. Effect of *Sm*ChE vaccination on glycogen storage in, and size of, *S. mansoni* adult worms. (A) Triton-X-100 extracts were made from 5 pairs of worms freshly perfused from each vaccinated or control group and the glycogen content in these extracts was measured. Plotted data are the average of triplicate biological and technical experiments ± SEM. (**B**) Worm sizes (mm) were assessed by randomly selecting and measuring (ImageJ) at least 20 worms from each group. Significance and percent reductions (if any) for both parameters are measured relative to the control group. Differences for both experiments were measured by the student's *t* test $**P \leq 0.01$, $***P \leq 0.001$.

Supporting information

Fig S1. ChE activity in the serum of control and vaccinated mice (trial 2). Activity was measured by Ellman assay. (A) AChE. (B) BChE. Data are the average of triplicate technical experiments ± SEM.

Table S1. Anti-*Sm*ChE IgG titers and worm burdens of vaccinated groups from trial 1.

Immunogen	Mouse	Total IgG endpoint titres		Worm burden
		Pre-challenge	Necropsy	
*Sm*AChE1 n = 10	1	1,974,491	229,723	29
	2	682,718	134,438	39
	3	866,872	186,065	30
	4	1,260,333	163,544	36
	5	2,129,001	171,513	34
	6	1,173,196	299,021	21
	7	2,096,392	113,649	17
	8	1,597,200	188,144	40
	9	1,692,421	210,666	19
	10	1,638,522	153,495	35
*Sm*BChE1 n = 10	1	516,530	106,352	30
	2	1,430,114	105,517	42
	3	540,784	120,551	19
	4	985,449	79,625	18
	5	1,617,861	75,449	29
	6	955,805	74,614	28
	7	1,609,776	28,676	39
	8	1,223,502	83,244	21
	9	1,275,604	59,579	20
	10	1,257,638	117,210	21
*Sm*AChE2 n = 9	1	485,262	120,273	37
	2	488,682	178,182	40
	3	495,869	77,676	34
	4	510,242	94,102	37
	5	495,869	143,659	30
	7	492,275	49,835	20
	8	560,547	197,392	23
	9	538,988	163,426	15
	10	510,242	96,886	20
*Sm*ChE cocktail n=10	1	919,872	154,517	40
	2	1,616,963	173,170	29
	3	1,185,773	165,096	28
	4	2,378,732	309,590	34
	5	1,381,605	172,335	37
	6	1,622,353	448,795	17
	7	1,192,959	97,165	21
	8	1,500,182	199,341	23
	9	3,171,044	449,630	19
	10	2,112,832	351,073	21

Table S2. Anti-*Sm*ChE IgG titers and worm burdens of vaccinated groups from trial 2.

Immunogen	Mouse	Total IgG endpoint titres		Worm burden
		Pre-challenge	Necropsy	
*Sm*AChE1 n = 10	1	547,971	168,394	32
	2	2,355,376	86,969	21
	3	923,466	124,390	20
	4	1,131,874	110,877	18
	5	2,966,229	128,548	30
	6	1,166,010	199,579	20
	7	1,947,542	135,824	21
	8	2,089,475	118,153	22
	9	1,764,286	145,180	19
	10	2,711,108	114,342	20
*Sm*BChE1 n = 10	1	284,276	71,273	9
	2	449,954	141,988	20
	3	396,539	176,790	19
	4	522,381	124,170	28
	5	536,866	102,176	19
	6	1,879,484	89,648	18
	7	451,764	36,750	22
	8	899,002	108,579	30
	9	716,123	125,005	18
	10	588,470	156,466	20
*Sm*AChE2 n = 8	1	1,130,077	430,420	17
	2	2,076,899	190,432	10
	3	1,404,961	338,545	32
	5	1,609,776	319,056	26
	7	1,814,592	249,733	19
	8	2,427,241	270,056	25
	9	1,805,609	288,153	16
	10	2,136,188	248,062	15
*Sm*ChE cocktail n = 9	1	2,567,378	364,715	28
	2	1,951,135	387,173	20
	3	1,352,859	361,122	15
	4	1,891,847	720,447	14
	5	1,245,061	363,817	16
	6	2,005,034	1,370,825	21
	7	1,478,623	293,748	19
	9	3,066,840	1,105,823	11
	10	1,999,644	893,821	24

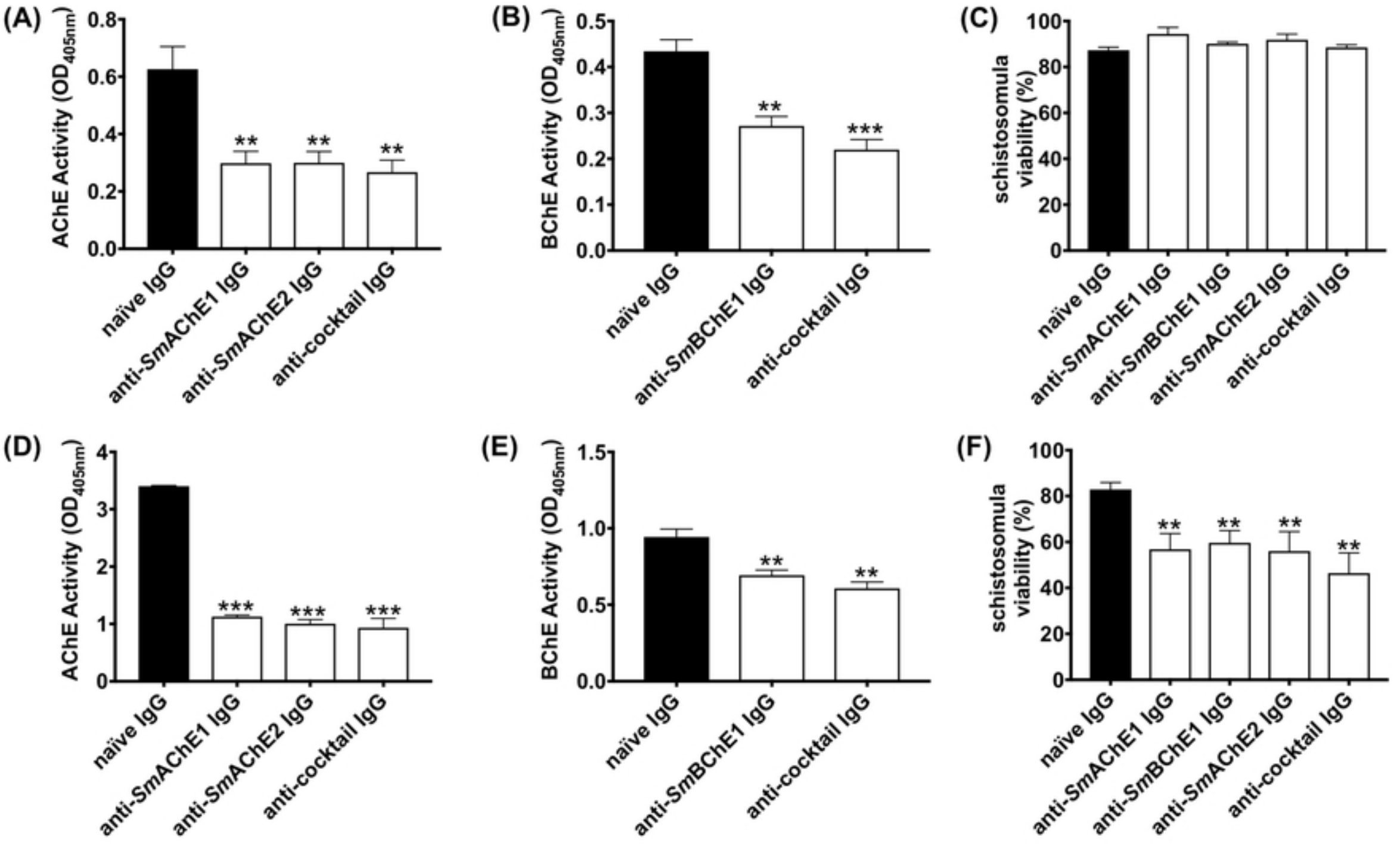
(A)
AChE Activity (OD405nm)
0.0
0.2
0.4
0.6
0.8
**
**
**
naïve IgG
anti-SmAChE1 IgG
anti-SmAChE2 IgG
anti-cocktail IgG
(B)
BChE Activity (OD405nm)
0.0
0.1
0.2
0.3
0.4
0.5
**
***
naïve IgG
anti-SmBChE1 IgG
anti-cocktail IgG
(C)
schistosomula viability (%)
0
20
40
60
80
100
naïve IgG
anti-SmAChE1 IgG
anti-SmBChE1 IgG
anti-SmAChE2 IgG
anti-cocktail IgG
(D)
AChE Activity (OD405nm)
0
1
2
3
4
***
***
***
naïve IgG
anti-SmAChE1 IgG
anti-SmAChE2 IgG
anti-cocktail IgG
(E)
BChE Activity (OD405nm)
0.0
0.5
1.0
1.5
**
**
naïve IgG
anti-SmBChE1 IgG
anti-cocktail IgG
(F)
schistosomula viability (%)
0
20
40
60
80
100
**
**
**
**
naïve IgG
anti-SmAChE1 IgG
anti-SmBChE1 IgG
anti-SmAChE2 IgG
anti-cocktail IgG

Figure 1

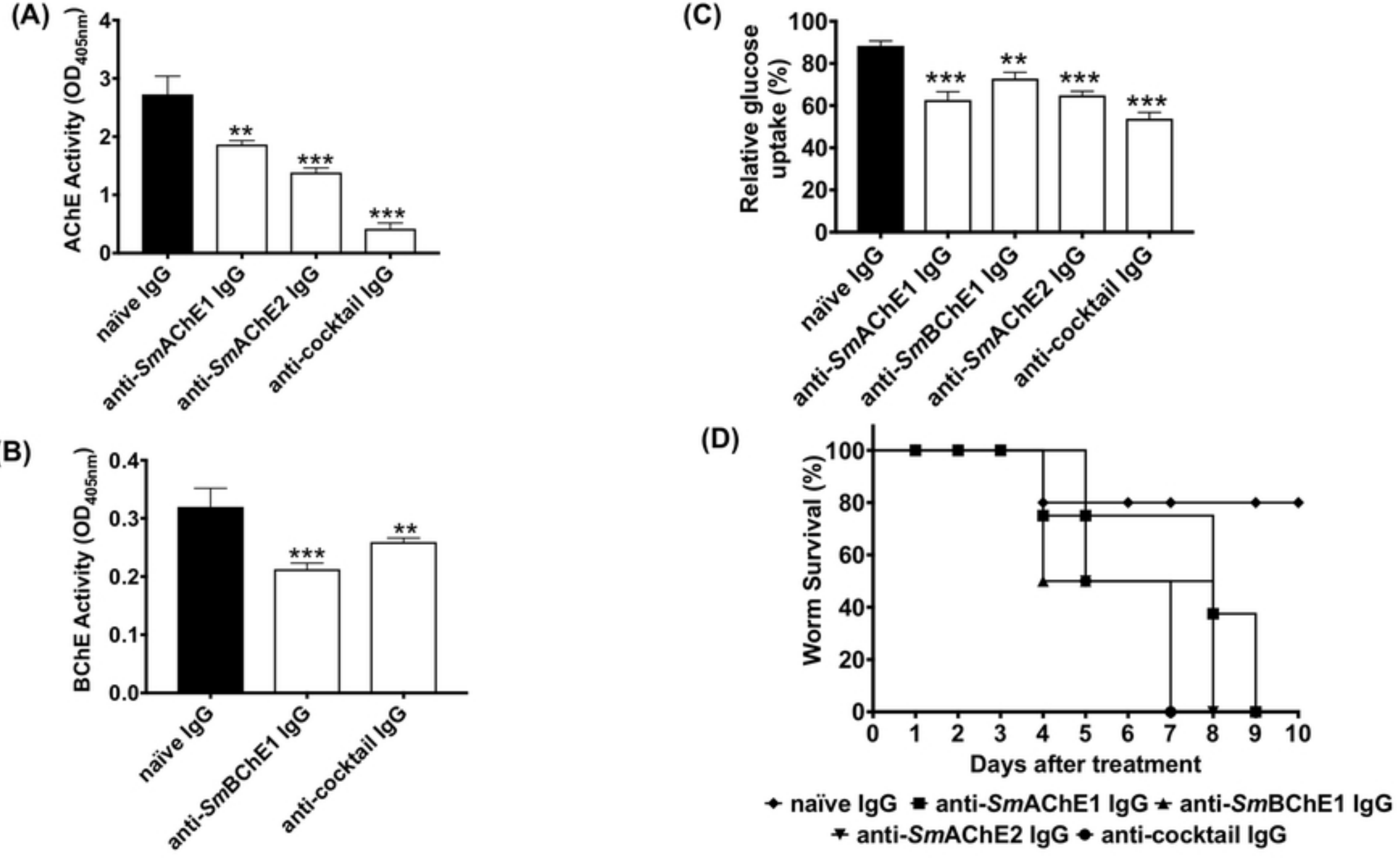
(A)
AChE Activity (OD405nm)
naïve IgG
anti-SmAChE1 IgG
anti-SmAChE2 IgG
anti-cocktail IgG
(B)
BChE Activity (OD405nm)
naïve IgG
anti-SmBChE1 IgG
anti-cocktail IgG
(C)
Relative glucose uptake (%)
naïve IgG
anti-SmAChE1 IgG
anti-SmBChE1 IgG
anti-SmAChE2 IgG
anti-cocktail IgG
(D)
Worm Survival (%)
Days after treatment
naïve IgG
anti-SmAChE1 IgG
anti-SmBChE1 IgG
anti-SmAChE2 IgG
anti-cocktail IgG

Figure 2

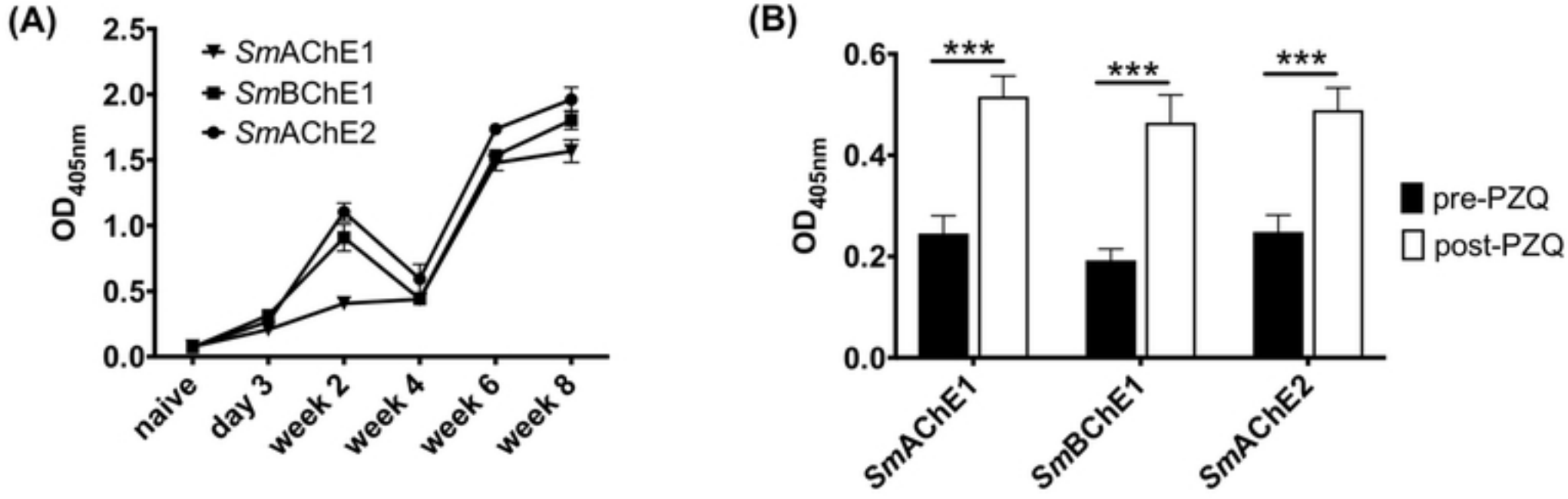
(A)
2.5
2.0
1.5
1.0
0.5
0.0
OD405nm
SmAChE1
SmBChE1
SmAChE2
naive
day 3
week 2
week 4
week 6
week 8
(B)
0.6
0.4
0.2
0.0
OD405nm
***
***
***
pre-PZQ
post-PZQ
SmAChE1
SmBChE1
SmAChE2

Figure 3

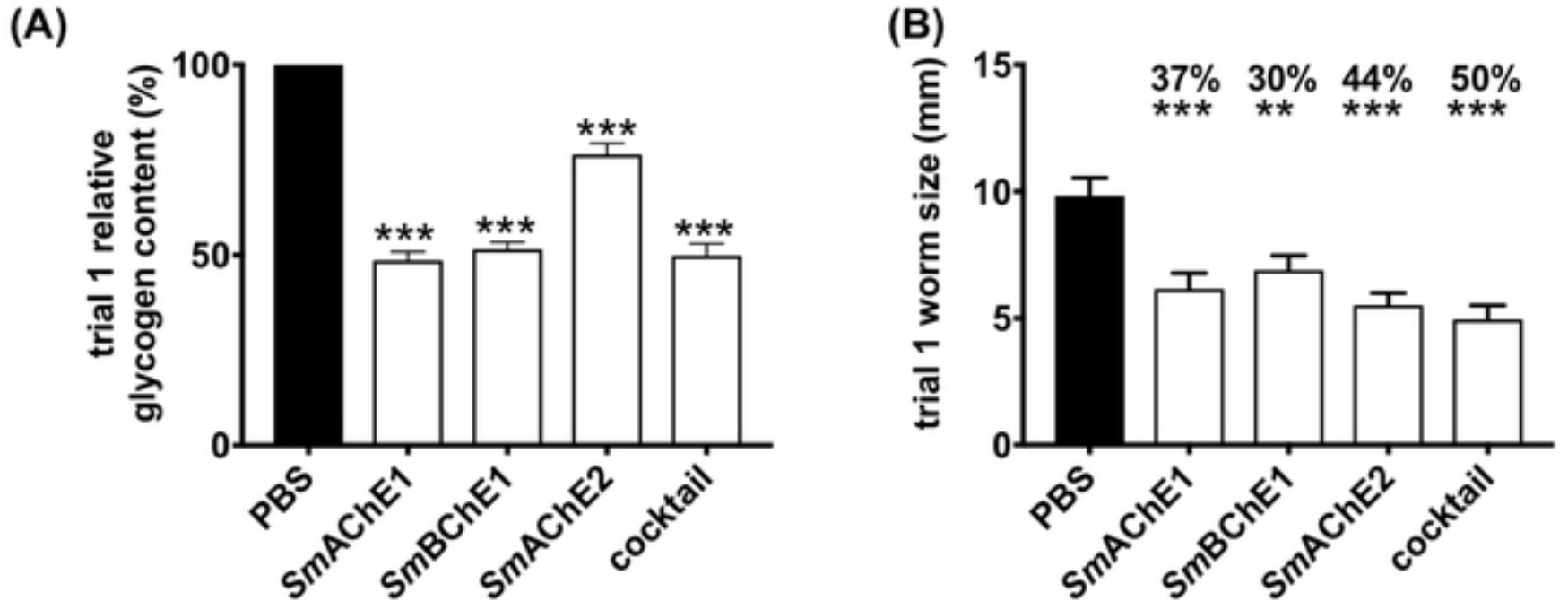
(A)
trial 1 relative glycogen content (%)
100
50
0
***
***
***
***
PBS
SmAChE1
SmBChE1
SmAChE2
cocktail
(B)
trial 1 worm size (mm)
15
10
5
0
37%
***
30%
**
44%
***
50%
***
PBS
SmAChE1
SmBChE1
SmAChE2
cocktail

Figure 5

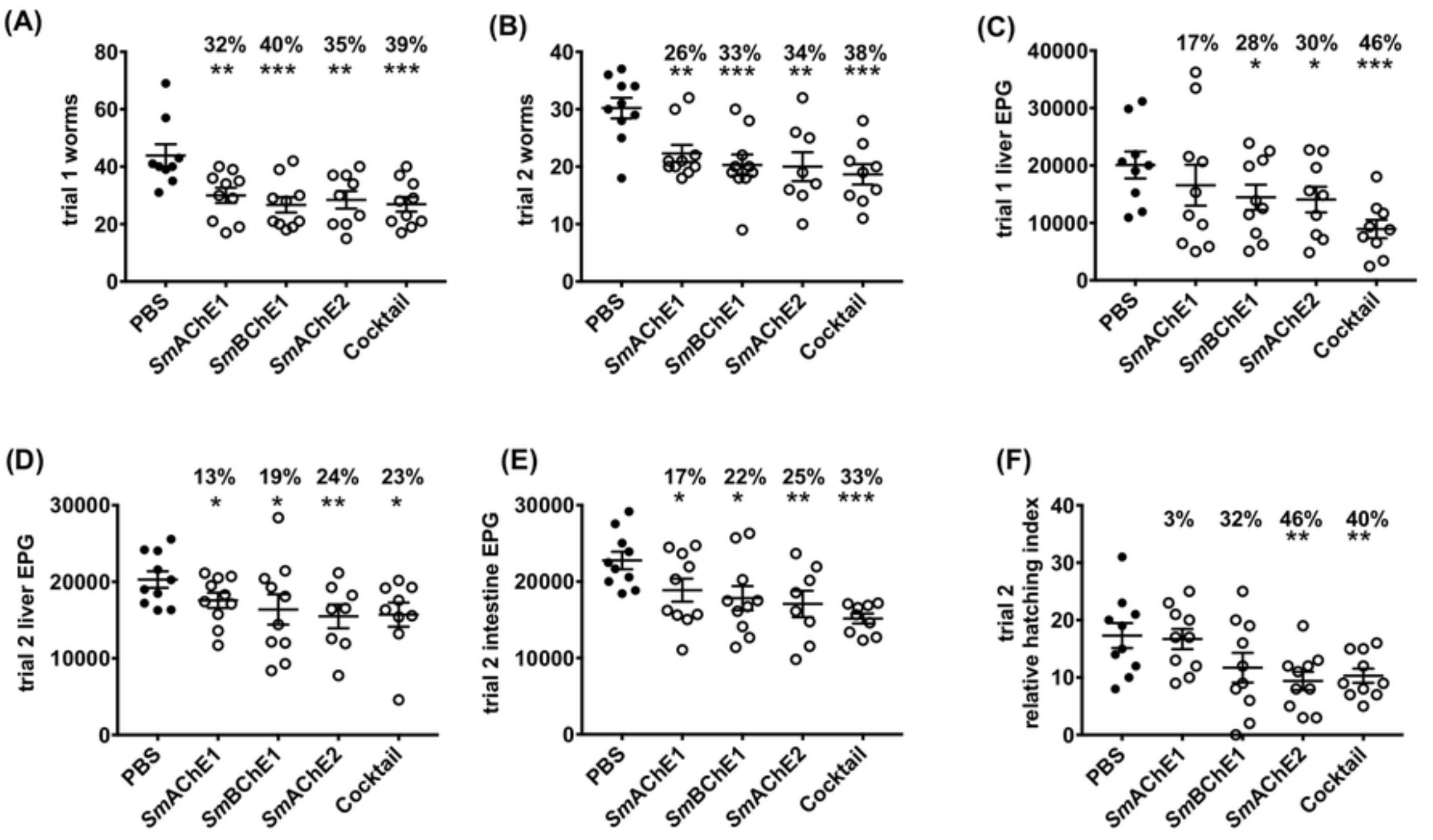
(A)
trial 1 worms
32% 40% 35% 39%
** *** ** ***
PBS
SmAChE1
SmBChE1
SmAChE2
Cocktail
(B)
trial 2 worms
26% 33% 34% 38%
** *** ** ***
(C)
trial 1 liver EPG
17% 28% 30% 46%
* * ***
(D)
trial 2 liver EPG
13% 19% 24% 23%
* * ** *
(E)
trial 2 intestine EPG
17% 22% 25% 33%
* * ** ***
(F)
trial 2
relative hatching index
3% 32% 46% 40%
** **

Figure 4