Advanced Enzymology, Expression Profile and Immune Response of *Clonorchis sinensis* Hexokinase Show Its Application Potential for Prevention and Control of Clonorchiasis

Tingjin Chen¹,²☯, Jinyun Yu¹,²☯, Zeli Tang¹,², Zhizhi Xie¹,², Zhipeng Lin¹,², Hengchang Sun¹,², Shuo Wan¹,², Xuerong Li¹,², Yan Huang¹,²*, Xinbing Yu¹,²*

¹ Department of Parasitology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, Guangdong, China, ² Key Laboratory for Tropical Disease Control, Sun Yat-sen University, Ministry of Education, Guangzhou, Guangdong, China

☯ These authors contributed equally to this work.

* huang66@mail.sysu.edu.cn (YH); yuxb@mail.sysu.edu.cn (XY); xujin@mail.sysu.edu.cn (JX)

Abstract

Background

Approximately 35 million people are infected with *Clonorchis sinensis* (*C. sinensis*) globally, of whom 15 million are in China. Glycolytic enzymes are recognized as crucial molecules for trematode survival and have been targeted for vaccine and drug development. Hexokinase of *C. sinensis* (*CsHK*), as the first key regulatory enzyme of the glycolytic pathway, was investigated in the current study.

Principal Findings

There were differences in spatial structure and affinities for hexoses and phosphate donors between *CsHK* and HKs from humans or rats, the definitive hosts of *C. sinensis*. Effectors (AMP, PEP, and citrate) and a small molecular inhibitor regulated the enzymatic activity of r*CsHK*, and various allosteric systems were detected. *CsHK* was distributed in the worm extensively as well as in liver tissue and serum from *C. sinensis* infected rats. Furthermore, high-level specific IgG1 and IgG2a were induced in rats by immunization with r*CsHK*. The enzymatic activity of *CsHK* was suppressed by the antibody in vitro. Additionally, the survival of *C. sinensis* was inhibited by the antibody in vivo and in vitro.

Conclusions/Significance

Due to differences in putative spatial structure and enzymology between *CsHK* and HK from the host, its extensive distribution in adult worms, and its expression profile as a component of excretory/secretory products, together with its good immunogenicity and immunoreactivity, as a key glycolytic enzyme, *CsHK* shows potential as a vaccine and as a promising drug target for Clonorchiasis.
Author Summary

Clonorchiasis, caused by *Clonorchis sinensis* (*C. sinensis*) infection, is a kind of neglected tropical disease. There are still few effective measures to prevent clonorchiasis. As in other helminthes, hexokinase (HK) has been well characterized as a target for vaccine and drug development. In the current study, we identified differences in spatial structure between CsHK and HKs from the definitive *C. sinensis* hosts, humans and rats. We also characterized the substrate specificity and allosteric regulation of rCsHK in detail. The distribution of CsHK in the worm and in the liver tissue and serum from *C. sinensis* infected rats were confirmed. Furthermore, a high-level specific antibody in rat was induced by immunization with rCsHK. The enzymatic activity of CsHK was suppressed by the antibody in vitro. Additionally, the survival of *C. sinensis* was inhibited by the antibody in vivo and in vitro. Our study shows that CsHK has vaccine potential and is a promising drug target for Clonorchiasis.

Introduction

Clonorchiasis, induced by *Clonorchis sinensis* (*C. sinensis*) infection, is a major public health problem in Southeast Asian countries including China, Korea, Taiwan, and Vietnam. Approximately 35 million people are infected with this neglected fluke globally, of whom 15 millions are in China [1]. The World Health Organization (WHO) announced in 2009 that *C. sinensis* infection is one of the biological agents that can induce cholangiocarcinoma [2]. In spite of its public health threat, there are still few effective measures to prevent this neglected tropical disease. Humans can be infected with *C. sinensis* by ingestion of raw or undercooked freshwater fish with metacercariae. The metacercariae of *C. sinensis* excyst in the duodenum, then migrate into hepatic bile ducts where the flukes mature into adult worms [3]. During the long term of parasitism, the worms continuously release excretory/secretory products (ESPs), a cocktail of hundreds to thousands of bioactive proteins. As molecules involved in the interaction between the parasite and host, ESPs have been well characterized to be targets for vaccine and drug development [4–7].

Glycolytic enzymes such as enolase [4, 8] and phosphoglycerate kinase [9, 10] are recognized as crucial molecules for trematode survival, and they have been targeted for vaccine and drug development. Hexokinase (HK) (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1) is the first key regulatory enzyme of the glycolytic pathway [11]. In other helminthes such as *Brugia malayi* (*B. malayi*) [12], *Haemonchus contortus* [13], and *Schistosoma mansoni* (*S. mansoni*) [14–16] HKs have been well characterized as potential targets for vaccine and drug development. In our previous study, the sequence, structure, and enzymatic properties of HK from *C. sinensis* (GsHK) were confirmed, and its molecular characteristics including molecular mass, mRNA and protein levels during different life stages of *C. sinensis* were determined [17]. These studies are cornerstones for our current study.

In the present study, we compared the putative spatial structure of CsHK with HKs from definitive hosts of *C. sinensis*. The effects of a small molecule inhibitor on the enzyme kinetics of recombinant CsHK (rCsHK) and the immunological characteristics and immune protective efficacy of rCsHK were investigated in detail. Our results indicate that CsHK may be a promising candidate for development of vaccines and drugs against *C. sinensis* infection.
Methods

Ethics statement
All animals used in the present study were purchased from the animal center of Sun Yat-sen University and raised carefully in accordance with National Institutes of Health animal care and ethical guidelines. All experimental procedures were approved by the Animal Care and Use Committee of Sun Yat-sen University (Permit Numbers: SCXK (Guangdong) 2009–0011). The ethical approval for human sera was granted from the Centers for Disease Control and Prevention of Guangxi Zhuang Autonomous Region, China. All human serum samples used in this study were anonymized.

Preparation of parasites, ESPs of *C. sinensis* (CsESPs) and antiserum against CsESPs/rCsHK

Metacercariae of *C. sinensis* were isolated from experimentally infected freshwater *Ctenopharyngodon idellus* fish in our laboratory pool [18]. Each Sprague-Dawley (SD) rat was orally infected with 50 metacercariae. At 8 weeks after infection, the rats were sacrificed and *C. sinensis* adults were recovered from the livers.

CsESPs and rat anti-CsESPs serum were obtained as described before [4]. Purified rCsHK was obtained in our previous study [17]. Purified rCsHK (200 μg) emulsified with an equal volume of complete Freund’s adjuvant (Sigma, USA) was injected subcutaneously into SD rats. Two boosters of 100 μg rCsHK with an equal volume of incomplete Freund’s adjuvant (Sigma, USA) were given at 2-week intervals. The pre-immune sera were collected prior to the first injection. The immune sera were collected at 2-week intervals from 0 to 12 weeks.

Comparison of putative spatial structure of CsHK with HKs from definitive hosts of *C. sinensis*, human and rat

As the amino acid sequence of CsHK shares 69% identical residues with the *S. mansoni* sequence [17], the putative tertiary structure of CsHK was constructed based on that of HK from *S. mansoni* (SmHK, Protein Data Bank, PDB: 1BDG_A) using SWISS-MODEL and viewed by Swiss-Pdb Viewer [17, 19]. Structural models of CsHK were superposed with closed-form human glucokinase (hHK-IV, PDB: 1V4S_A) [20] and the N-terminal half of closed-form rat hexokinase-1 (rHK-In, PDB: 1BG3_B) [21]. The allosteric sites in closed-form hHK-IV [20] and CsHK were compared. The glucose 6-phosphate (G6P) binding sites in CsHK were compared to that of rHK-In [21]. The accession numbers/ID numbers for genes and proteins mentioned in the text are listed in S1 Table.

Effects of phosphate donors, effectors and a small molecule inhibitor on the enzyme kinetics of rCsHK

The enzymatic activity of HK was assayed as described using a coupled reaction [17, 22]. A 200-μL aliquot of reaction mixture included 3 mM glucose, 3 mM ATP, 15 mM MgCl₂, 0.5 mM nicotinamide adenine dinucleotide phosphate (NADP), 0.3 U of yeast glucose 6-phosphate dehydrogenase (G6PD) Type VII, and 100 mM Tris-HCl (pH 8.5). Reduced NADP (NADPH) formation by G6P dehydrogenation was monitored at 340 nm in a microplate reader (SpectraMax M5, Molecular Devices, USA). All enzymatic reagents were purchased from Sigma-Aldrich (USA).

To determine the kinetic parameters of rCsHK, the substrate (ATP, CTP, GTP, ITP, TTP, UTP, or glucose) concentrations were varied from 0.05 to 3 mM. Effectors such as AMP (0–5 mM), phosphoenolpyruvate (PEP, 0–10 mM), and citrate (0–10 mM) were added to the
reaction mixture to investigate their effects on enzymatic activity of rCsHK, as was 2-phenyl-1, 2-benzisoselenazol-3(2H)-one (EbSe, a small molecular inhibitor, 0–100 μM). Note that EbSe was found to be ineffective in a counterscreen for inhibition of G6PD [23].

Western blotting analysis

Purified rCsHK protein (2 μg) or CsESPs (30 μg) was subjected to 12% SDS-PAGE and then electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Whatman, UK) at 100 V for 60 min in a Trans-Blot transfer cell (Bio-Rad, USA). The PVDF membranes were blocked with 5% (w/v) skimmed milk in phosphate buffer saline (PBS, pH 7.4) overnight at 4°C and then probed with serum from C. sinensis infected humans/rats, healthy people, rCsHK immunized rats or pre-immune rats for 2 h at room temperature (RT). All the sera were at the same dilution of 1:200. After washing with PBS three times, the membranes were then incubated in horseradish peroxidase (HRP)-conjugated goat anti-human/rat IgG (1:2,000 dilution, Protein tech., USA) for 1 h at RT. Both the primary and secondary antibodies were diluted with 0.1% BSA in PBS (pH 7.4). After washing five times, the membranes were developed with diaminobenzidine (DAB, Boster, China) reagent according to the manufacturer’s instructions.

Immunolocalization of CsHK in C. sinensis and in liver tissue from infected rats

Adult worms and metacercariae of C. sinensis and liver tissue from infected rats were fixed with formalin, embedded with paraffin wax and sliced into 4 μm-thick sections. The sections of adult worms and metacercariae were deparaffinized in xylene, hydrated in gradient alcohol and then blocked with normal goat serum for 2 h at RT. The sections were incubated in mouse anti-rCsHK serum (1:100 dilution) previously obtained [17] in a humid chamber at 4°C overnight. Serum from a pre-immune mouse was employed as a negative control. After successively washing three times with PBS containing 0.05% Tween-20 (PBST, pH 7.4) and two times with PBS, the sections were incubated with Cy3-conjugated goat anti-mouse IgG (1:400 dilution, Molecular Probe, USA) for 1 h at RT. The primary and secondary antibodies were diluted with 0.1% BSA in PBS (pH 7.4). After washing five times, the membranes were developed with diaminobenzidine (DAB, Boster, China) reagent according to the manufacturer’s instructions.

Enzyme-linked immunosorbent assay (ELISA) of antibody titers and isotype of IgG induced by rCsHK

Microplates were coated with 2 μg/well purified rCsHK in coating buffer (0.1 M carbonate-bicarbonate, pH 9.6) and incubated at 4°C overnight. Subsequently, the plates were blocked with 5% skimmed milk in PBST for 2 h at 37°C. After washing, the wells were incubated with different dilutions of the immune serum (6 weeks after the first immunization) raised by
CsHK. Serum from rats immunized with PBS was measured as a negative control. HRP-conjugated goat anti-rat IgG (1:20,000 dilution in 0.1% BSA-PBST, Protein tech., USA) was used as the secondary antibody. After incubation for 1 h and washing three times with PBST, the reactions were developed by adding 100 μl of substrate solution (TMB, BD biosciences, San Diego, USA) followed by 10 min in darkness. The absorbance was measured at 450 nm after adding 2 M H₂SO₄ to stop the reaction. The levels of total IgG and IgG isotype in serum collected at different time points (0, 2, 4, 6, 8, 10, 12 weeks after the first immunization) were determined by the aforementioned process. The dilutions of the serum were 1:400. HRP-conjugated goat anti-rat IgG (1:20,000 dilution)/IgG1/IgG2a (1:10,000 dilution, Bethyl, Texas, USA) were employed as secondary antibodies.

Culture of *C. sinensis* adults with rat anti-rCsHK serum

Adult worms newly recovered from infected rats were washed three to four times with sterilized PBS with 1% antibiotics (penicillin 100 μg/ml and streptomycin 100 U/ml). They were then transferred to 12-well plates with 20 adults per well and incubated in 2 ml of low glucose DMEM with 1% antibiotics. Serum from rCsHK immunized rats or pre-immune rats was added to the medium at dilutions of 1:160–1:40. Low glucose DMEM was used as a blank control. The worms were monitored under a microscope (Leica, Germany) for 5 min, and intact alive worms were counted at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 18, 20, 22, 24, 26, and 28 days after the incubation. Worms with no muscle contraction or no pumping after 5 consecutive shots were considered to be dead [24].

Parasites incubated in medium with diluted rat anti-rCsHK serum for 1, 3, 5, and 6 days were collected. The worms were suspended and then homogenized in RIPA lysis buffer (containing 1 mM proteinase inhibitor PMSF, Bioteke, China). The supernatant was collected after centrifugation for 15 min at 10,000 × g at 4°C and the concentration of total protein was determined using a BCA protein assay kit (Novagen, USA). The enzymatic activity of native CsHK in the samples was assayed as described above. The enzymatic activity of secreted phospholipase A2 from *C. sinensis* (CsPLA₂) was assayed as a control with the sPLA₂ assay kit (Cayman Chemical, USA) according to the manufacturer’s instructions.

Immune protective efficacy of rCsHK

Thirty-two 6-week-old SD rats were randomly divided into four equal groups: infection group, adjuvant group, PBS group, and rCsHK group. rCsHK (200 μg) or an equivalent volume of PBS was emulsified with complete Freund’s adjuvant and subcutaneously injected into SD rats in the rCsHK group and PBS group. rCsHK (100 μg) emulsified with incomplete Freund’s adjuvant was given for the next two boosters at 2-week intervals. An equivalent volume of adjuvant was injected subcutaneously into SD rats in the adjuvant group.

After the measurement of antibody titers at week 6 post immunization, the rats (n = 8 in each group) were anesthetized with ether and intragastrically challenged with 80 live metacercariae of *C. sinensis*. The eggs per gram feces (EPG) was counted with a previous method [25] at 6 weeks after the infection. All rats were sacrificed at week 8 post infection to recover adult worms from their livers for worm burden evaluation. All rats were kept under the same conditions until sacrifice. EPG and worm burden were counted blindly. Reduction rates in parasite burden were calculated as follows. Worm reduction rate (%) = [(average worm burden of control group—average worm burden of experimental group) / average worm burden of control group] × 100%. Egg reduction rate (%) = [(average EPG of control group—average EPG of experimental group) / average EPG of control group] × 100%.
Statistical analysis

All of the experiments were repeated at least three times in triplicate. SPSS version 13.0 software was used for statistical analysis. Student’s t test was used to analyze IgG isotypes and immune protective efficacy among the groups. The survival rates of cultured worms were determined using the Kaplan-Meier method, and differences between the groups were identified through log-rank analysis. The results are presented as mean ± SD, and p < 0.05 was classified as statistically significant.

Results

Spatial structure differences between CsHK and HKs from definitive hosts of C. sinensis, humans and rats

CsHK is composed of a large domain (green) and a small domain (light green). The two domains are linked by connecting regions I-III (light green). In closed-form CsHK, the α 13 helix is included in the small domain. The lengths and amino acid residues of α 13 helix and connecting region I are different from those of hHK-IV and rHK-In (Fig. 1A-1B). The allosteric sites in closed-form hHK-IV are ARG63, MET210, TYR214, TYR215, VAL452, VAL455, and ALA456. In CsHK, the corresponding sites are SER59, LEU202, ALA206, LEU207, ILE443, ALA446, and SER447 (Fig. 1C). As for G6P binding sites of rHK-In, SER88, ARG174, and THR449 are replaced by THR78, GLY163, and SER436 in CsHK (Fig. 1D).

Effects of phosphate donors, effectors and a small molecule inhibitor (EbSe) on the enzyme kinetics of rCsHK

rCsHK catalyzed the phosphorylation of a series of hexoses at the following relative velocity (Table 1): D(+)-glucose (100%) congruent to D(+)-mannose (97.13%) greater than D(-)-fructose (16.60%) greater than D(+)-galactose (0.23%). With respect to phosphate donors, rCsHK could use ATP, CTP, GTP, ITP, TTP, and UTP, and rCsHK was less specific for ATP. Very little or no dephosphorylating activity was found for ADP, AMP, and inorganic pyrophosphate (PPi). ATP was able to be replaced by other nucleotides with moderate relative velocity. ATP, GTP, ITP and TTP homotropically and allosterically activated the enzyme (Hill coefficients, h > 1), whereas CTP homotropically and allosterically inhibited the enzyme (h < 1). UTP has no allosteric effect on the enzyme (h = 1) (Fig. 2A, S2 Table). rCsHK was inhibited by high concentrations of ATP. At physiological concentration (5 mM) [14, 26], ATP showed 8% inhibition of rCsHK, whereas other nucleotides showed no inhibition of rCsHK. ATP, CTP, and TTP showed 13.8, 8.6, and 14.3% inhibition of rCsHK at 10 mM concentration, respectively, whereas other nucleotides showed no inhibition of rCsHK.

AMP exhibited a mixed allosteric K+V+ effect [27] on rCsHK by decreasing its K_{0.5} and increasing V_{max} with respect to ATP (Fig. 2B, S2 Table). PEP displayed allosteric activation of rCsHK with respect to ATP with mixed K+V+ allosteric effects in a dose-independent manner (Fig. 2C, S2 Table).

Citrate exhibited an unusual mixed allosteric effect on rCsHK with respect to ATP. At 5 mM and 10 mM citrate behaved as a mixed K+V+ activator, whereas at 2 mM citrate behaved as a V activator and a K inhibitor (antiergistic or crossed mixed K–V+ effect) [28] (Fig. 2D, S2 Table). Under these conditions, V activation contributed less to the effective reaction rate compared to K inhibition. The resulting effect was a net inhibition by 2 mM citrate with a reduction of h from 1.935 ± 0.271 to 1.267 ± 0.242.
Fig 1. Comparison of putative spatial structure of CsHK with hHK-IV or rHK-In. (A) Ribbon drawing of superposed structure models of CsHK (green and light green) and closed-form hHK-IV (red and light red, PDB: 1V4S_A), which structures are complexed with glucose (blue ball) and MRK (N-thiazol-2-yl-2-amino-4-fluoro-5-(1-methylimidazol-2-yl) thiobenzamide, an allosteric activator, yellow ball). The α13 helix (magenta and light green) is included in the small domain of the closed-form. (B) Ribbon drawing of superposed structure models of CsHK (green and light green) and rHK-In (yellow and light yellow, PDB: 1BG3_B), which structures are complexed with glucose (blue ball) and G6P (red ball). The α13 helix (magenta and light green) is included in the small domain of the closed form. The structures of the α13 helix and connecting region I (brown and light green) are different. (C) Stereo view of the allosteric sites in closed-form hHK-IV (left) and CsHK (right). In the left panel, the allosteric sites are located below connecting region I (brown, ribbon model). MRK (yellow stick) forms hydrogen bonds with ARG63 and TYR215 (red stick) and hydrophobically interacts with MET210, TYR214 (red stick) of α5 helix (red ribbon) and V452, V455 (magenta stick) of α13 helix (magenta ribbon). The supposed corresponding structure of CsHK is shown in the right panel. (D) Stereo view of G6P binding sites in rHK-In (left) and CsHK (right). Interactions of G6P (red stick) with the large (yellow) and small (light yellow) domain of the rHK-In binding cleft are shown in the left panel. SER/THR residues are colored light blue (stick), and ASP residues are orange (stick). Glucose (blue stick) is bound at an adjacent position in the cleft. The ARG174 side chain unique to rHK-In is shown in magenta (stick). The supposed corresponding structure of CsHK is shown in the right panel.

doi:10.1371/journal.pntd.0003641.g001
At 0.5 μM EbSe behaved as a mixed K+V+ allosteric activator of rCsHK with respect to ATP and glucose, whereas at 5 μM, 25 μM or 100 μM EbSe displayed net allosteric inhibition of rCsHK with mixed K−V+ effects with respect to ATP and glucose in a dose-independent manner (Fig. 2E-2F, S2 Table). rCsHK was not inhibited by 2 mM of D-fructose 6-phosphate or D-fructose 1,6-diphosphate.

Western blotting analysis

Purified rCsHK was probed with serum from C. sinensis infected humans/rats and rat anti-CsESP serum yielding a cross-reactive band of approximately 54.8 kDa (including molecular mass of a His-tag) [17], but it was not recognized by serum from healthy people or from a pre-immune rat. In addition, CsESP blotted with rat anti-rCsHK serum, but not with serum from a pre-immune rat, yielded a band at approximately 50.0 kDa (Fig. 3).

Immunolocalization of CsHK in C. sinensis and in liver tissue from infected rats

In adult worms (Fig. 4), strong fluorescence of CsHK was detected in the vitellarium, tegument, intestine, spermatheca, testicle, pharynx, uterus and egg in uterus, but not in the negative control. In metacercariae, strong fluorescence was distributed in the tegument and vitellarium. In slides of liver from infected rats incubated with mouse anti-rCsHK serum, strong fluorescence was detected in the vitellarium, tegument, intestine, spermatheca, testicle, ovary, ventral sucker, uterus and egg in uterus of the worms inside the bile duct. In addition, specific fluorescence was also observed in the intrahepatic biliary epithelium and lumen of the biliary tract near the parasites. No specific fluorescence was detected in the negative control incubated with serum from a pre-immune mouse.

In slides of infected liver developed for color by DAB reagent, specific brown staining was detected in the intrahepatic bile ducts with adenomatoid hyperplasia, but it was not observed

| Substrate                 | Relative velocity (%) |
|---------------------------|-----------------------|
| Hexose^a                  | 100.00                |
| D(+) glucose              | 100.00                |
| D(+) mannose              | 97.13                 |
| D(-) fructose             | 16.60                 |
| D(+) galactose            | 0.23                  |
| Phosphate donor           |                       |
| ATP                       | 100.00                |
| CTP                       | 17.73                 |
| GTP                       | 14.22                 |
| ITP                       | 35.28                 |
| TTP                       | 21.40                 |
| UTP                       | 13.10                 |
| ADP                       | 1.65                  |
| AMP                       | 0.21                  |
| PPI                       | 0.04                  |

^a from reference [17].

doi:10.1371/journal.pntd.0003641.t001

Table 1. Substrate specificity of rCsHK.
Fig 2. Effects of phosphate donors, effectors and EbSe on the enzyme kinetics of rCsHK. The effect of 0~3 mM phosphate donors (ATP, CTP, GTP, ITP, TTP, and UTP) and fixed 3 mM glucose (A). The effect of 0~5 mM AMP (B), 0~10 mM PEP (C), 0~10 mM citrate (D), or 0~100 μM EbSe (E) and fixed 3 mM glucose with respect to ATP. The effect of 0~100 μM EbSe and fixed 3 mM ATP with respect to glucose (F).

doi:10.1371/journal.pntd.0003641.g002
Rat anti-rCsHK serum affects *C. sinensis* adult survival in vitro

The titer of anti-rCsHK IgG was up to 1:409,600 at 6 weeks after the immunization, showing the high immunogenicity of rCsHK (Fig. 5A). In serum from rCsHK immunized rats, IgG1 and IgG2a levels increased at 2 weeks and reached their peak at 6 and 8 weeks, respectively. From 2 to 8 weeks, the IgG1 level was statistically higher than IgG2a, but it was lower at 10 and 12 weeks (Fig. 5B).

The median survival time of *C. sinensis* adults in the blank control group, 1:40 pre-immune serum group, 1:80 pre-immune serum group, 1:160 pre-immune serum group, 1:40 anti-rCsHK serum group, 1:80 anti-rCsHK serum group, and 1:160 anti-rCsHK serum group was 15, 8, 8, 9, 2, 3, and 3 days, respectively (Fig. 6A). There was no significant difference in survival rate among the pre-immune serum groups at any dilution (*p* > 0.05). Significant differences were observed in the survival rates among all other groups (*p* < 0.05).

The enzymatic activity of CsHK in adult worms incubated in medium with different dilutions of anti-rCsHK serum declined significantly in a dose- and time-dependent manner (Fig. 6B). As a control, there was no obvious change in the enzymatic activity of CsPLA2 in the worms (Fig. 6C).

Immune protective efficacy of rCsHK

The number of worms recovered in the PBS group, infection group, adjuvant group, and rCsHK group was 25.1 ± 4.8, 26.1 ± 5.1, 24.8 ± 5.3, and 12.5 ± 2.4, respectively. The EPG values in the four groups were 3983.3 ± 386.7, 3895.8 ± 424.1, 4075.0 ± 473.0, and 1991.7 ± 245.4, respectively (Table 2). The worm burden and EPG were significantly lower in the rCsHK group compared to the control groups (*p* < 0.01). The worm reduction rate and egg reduction rate were 50.20% and 50.00%, respectively. There was no significant difference in worm burden or EPG among the infection, adjuvant, and PBS groups.

Discussion

In the current study, we identified differences in spatial structure between CsHK and HKs from the definitive hosts of *C. sinensis*, humans and rats. We also characterized the substrate specificity and allosteric regulation of rCsHK in detail. The distribution of CsHK in worms and in liver tissue and serum from *C. sinensis* infected rats was confirmed. Furthermore, a high-
Fig 4. Immunolocalization of CsHK in C. sinensis and in liver from infected rats. Mouse anti-rCsHK serum and anti-mouse IgG were applied as primary antibody and secondary antibody, respectively. Serum from pre-immune mice was employed as primary antibody for a negative control. Panels H, L, P, R, U, V, W, and X are negative controls. Panels B, D, F, H, J, L, N, P, and R are under fluorescence microscope and the same parts (panels A, C, E, G, I, K, M, O, and Q) are under white light. Panels B, D, and F, localization of CsHK in adult worms; panel J, localization of CsHK in metacercariae. Panels S and T, localization of CsHK in intrahepatic bile ducts of a C. sinensis infected rat. In panels S, T, U, V, W, and X, peroxidase staining shows as a yellow/rust colored deposit and Mayer’s hematoxylin counterstains the nuclei in light purple. White arrows highlight the regions of intrahepatic bile duct tissue and the tissue that stained positive for CsHK. Original magnification: × 50 for panels M, N, O, P, Q and R; × 100 for panels A, B, C, D, E, F, G, H, S, U, and W; × 400 for panels I, J, K, L, T, V, and X. Bar = 800 μm. v, vitellarium; e, egg; vs, ventral sucker; tg, tegument; i, intestine; u, uterus; ts, testicle; o, ovary; p, pharynx; s, spermatheca; l, lumen; w, within the cells; Cs, C. sinensis; BE, biliary epithelium.
level specific antibody was induced in rats by immunization with rCsHK. The enzymatic activity of CsHK was suppressed by the antibody in vitro. Additionally, the survival of *C. sinensis* was inhibited by the antibody in vivo and in vitro.

The length and amino acid composition of the α13 helix and of connecting region I were found to differ among CsHK, hHK-IV and rHK-In. ATP-binding sites, allosteric sites, G6P binding sites and B-cell epitopes are included in these regions [17, 20, 29]. Taken together, these data suggest that the subtle structural differences between CsHK and HKs from definitive hosts of *C. sinensis*, humans and rats, may result in remarkable changes in their enzymatic behavior.

The 100-kDa HK-I, HK-II, and HK-III of mammalian hosts have high affinity for glucose ($K_m = 7–200 \mu M$) and are strongly inhibited by G6P. The 50-kDa HK-IV, also called glucokinase, has low affinity for glucose ($K_m = 5–12 \text{ mM}$) and is not regulated by G6P [30–32].
Fig 6. Rat anti-rCsHK serum affects *C. sinensis* adult survival in vitro. (A) The median survival of *C. sinensis* adults in the blank control group, 1:40 pre-immune serum group, 1:80 pre-immune serum group, 1:160 pre-immune serum group, 1:40 anti-rCsHK serum group, 1:80 anti-rCsHK serum group, and 1:160 anti-rCsHK serum group was 15, 8, 8, 9, 2, 3, and 3 days, respectively. There was no significant difference in survival rates among pre-immune serum groups at any dilution (p > 0.05). Significant differences were observed in the survival rates among the other groups (p < 0.05). (B) The enzymatic activity of CsHK in homogenate of parasites collected from each group at 1, 3, 5, and 6 days of incubation. The enzymatic activity of CsHK in adult worms incubated in medium with different dilutions of anti-rCsHK serum declined significantly in a dose- and time-dependent manner. (C) As a control, there was no obvious change of the enzymatic activity of CsPLA₂ in the worms.

doi:10.1371/journal.pntd.0003641.g006
HK-IV, which phosphorylates glucose in liver and pancreatic islets, plays a critical role as a glucose-sensing device due to its specific regulatory properties, mainly low affinity for glucose, a sigmoidal saturation curve for this substrate, and a lack of inhibition by G6P [33–36]. Our present and previous studies [17] confirmed that rCsHK is a 50-kDa G6P-sensitive allosterically modulated HK, sharing some characteristics with HKs from mammals. Vertebrate HKs, including HK-IV, typically act on mannose, fructose and 2-deoxyglucose as well as glucose, the preferred substrate. In the rat, the four isoenzymes have essentially the same relative specificity for glucose and fructose [37]. Our results demonstrated that rCsHK could use glucose, fructose, and mannose as substrates, although it preferred to use glucose and mannose. Galactose was a much poorer substrate than glucose, mannose, or fructose, in accordance with observations of HK from Toxoplasma gondii (TgHK, a 50-kDa HK) [22]. Similarly to TgHK [22], the $k_{\text{cat}}$ values of rCsHK for glucose (4.639 ± 0.174) and ATP (4.113 ± 0.076) were almost the same. This suggests that consumption of glucose and ATP are stoichiometrically even. However, TgHK is not an allosteric enzyme [22].

Eukaryotic HKs prefer ATP as the nucleotide substrate, and TgHK is no exception. rCsHK showed less specificity and other nucleotides were relatively good substrates. For example, rCsHK had $K_{0.5}$ values of 0.315 ± 0.026 mM for ATP and 1.335 ± 0.253 mM for ITP with similar $V_{\text{max}}$ values. ITP yielded 35.28% velocity relative to ATP. As for TgHK, ITP yields 2.6% velocity relative to ATP [38]. With the other isoenzymes ITP also appears to be a poor substrate [32]. When ATP, the normal phosphate donor for rat HK-IV, is replaced by ITP, the positive cooperativity with respect to glucose disappears [38]. However, both ATP (h = 1.935 ± 0.271) and ITP (h = 1.191 ± 0.109) homotropically and allosterically activated rCsHK.

Fructose 6-phosphate, which is an inhibitor of yeast HK [39], does not affect the enzymatic activity of rCsHK or TgHK [22]. AMP exhibited a mixed allosteric $K+V+$ effect on rCsHK by decreasing its $K_{0.5}$ and increasing $V_{\text{max}}$ with respect to ATP. AMP at 2 mM reduced the $V_{\text{max}}$ value of TgHK by 15%; however, no change in the $K_{m}$ value of TgHK for either glucose or ATP was observed [22].

Glycogen is essential to C. sinensis, suggesting that enzymes involved in the pathway could be targets for drug and vaccine development [10, 40]. EbSe was identified in a screen as a potent inhibitor of Trypanosoma brucei HK1 (TbHK1) and Plasmodium falciparum HK (PfHK) by interrogating a selected small-molecule library of HK inhibitors [41, 42]. EbSe can promiscuously modify cysteine residues, and this nonspecific interaction is known to be the mechanism of its inhibition of some enzymes such as human indoleamine 2, 3-dioxygenase [43]. However, site-directed mutagenesis of cysteines in TbHK1 and PfHK did not alter their

### Table 2. Worm burden and EPG of rats in different groups.

| Group            | Worm burden | Worm reduction rate (%) | EPG       | Egg reduction rate (%) |
|------------------|-------------|-------------------------|-----------|------------------------|
| PBS (n = 8)      | 25.1 ± 4.8  | 3983.3 ± 386.7          |           |                        |
| infection (n = 8)| 26.1 ± 5.1$^a$ | 3895.8 ± 424.1$^a$     |           |                        |
| adjuvant (n = 8) | 24.8 ± 5.3$^a$ | 4075.0 ± 473.0$^a$     |           |                        |
| rCsHK (n = 8)    | 12.5 ± 2.4$^b$ | 50.20                   | 1991.7 ± 245.4$^b$ | 50.00                  |

Results of analysis represent the mean ± SD, and the recovered worm numbers and EPG in groups were compared by Student’s t-test. $^a p > 0.05$ and $^b p < 0.01$ (compared with PBS group).

doi:10.1371/journal.pntd.0003641.t002
sensitivity to EbSe inhibition, indicating that either cysteine residues are not involved in EbSe inhibition or multiple cysteines must be bound in order for inhibition to occur [41, 42]. CsHK shares limited sequence identity with TbHK1 (36%) and PfHK (31%). At 0.5 μM, 2 μM and 5 μM EbSe acts as a mixed inhibitor of TbHK1 with respect to ATP [41]. However, at 0.5 μM EbSe behaved as a mixed K+V+ allosteric activator of rCsHK with respect to ATP and glucose. At 5 μM, 25 μM or 100 μM EbSe displayed net allosteric inhibition of rCsHK with mixed K–V+ effects with respect to ATP and glucose in a dose-independent manner. The results suggest that EbSe interacts with the two enzymes differently. EbSe has no effect on mammalian cells [41], suggesting that it may hold promise for the development of new anti-clonorchiasis compounds. Comparison of the putative spatial structure between CsHK and its human and rat counterparts supports possible explanations for the significant differences in the enzymes’ allosteric behavior observed in the presence of the effectors and the small molecular inhibitor, which could be exploited in drug design.

rCsHK was recognized by rat anti-rCsHK serum in western blotting, showing the immunoreactivity of rCsHK. rCsHK recognition by serum from C. sinensis infected humans/rats suggests that CsHK might be a component of circulating antigens from C. sinensis [44, 45]. In addition, CsESPs were blotted with rat anti-rCsHK serum, yielding a band at approximately 50 kDa. Moreover, rCsHK could be recognized by rat anti-rCsESPs serum. In liver tissue from C. sinensis infected rats, immunofluorescence and immunohistochemistry showed that CsHK was distributed in the intrahepatic biliary epithelium and lumen of the biliary tract near the parasites. These results indicated that CsHK was also an ingredient of CsESPs.

In adult slides, CsHK was extensively distributed. The locations included tegument, intestine and pharynx, where ESPs usually discharge from. The wide distribution hints that as a key enzyme involved in glycolysis, CsHK is important for the worm.

CsHK was observed to be expressed in the tegument. The trematode tegument is a dynamic organ involved in host-parasite interactions in addition to participating in nutrition, immune evasion and modulation, excretion, osmoregulation and signal transduction [46]. The presence of CsHK in CsESPs was probably due to renewal and shedding of the tegument [47]. In trematodes, the intestine is not only a major source of secretory proteins but also a place for nutritive digestion and absorption [48]. Coupled with its localization in the tegument as a feeding structure, CsHK might participate in the absorption and digestion of glucose from the host for energy supply. Moreover, the distribution of CsHK in muscular tissues such as the ventral sucker and pharynx might be associated with the energy requirement for muscle contraction and adhesion behavior. Its distribution in reproductive organs such as the vitellarium, testis, spermatheca, ovary, and uterus suggests that continuous catalytic activity of CsHK for glucose metabolism might take place in these organs to meet the energy demands for growth and reproduction of the parasite. The trematode vitellarium plays a key role in egg production by supplying eggshell material, relevant enzymatic activity and nutrients to the fertilized ovum [49]. The localization of CsHK in eggs is consistent with the highest mRNA and protein levels of CsHK occurring in the egg life stage [17]. It has been speculated that CsHK plays a crucial role in maintaining glucose metabolism for the development of eggs and formation of the eggshell.

The distribution of CsHK in liver tissue from C. sinensis infected rats demonstrated the abundant secretory expression profile of CsHK in intrahepatic bile ducts of the host. This suggests that CsHK might mediate direct interactions with host cells as a component of CsESPs, and it may derive from the excoriation of parasites and excretion through the intestine or glands [4] when C. sinensis inhabits the host. The localization of CsHK on bile duct epithelial cells close to the resident worms and the surface of hyperplastic adenoma suggests that CsHK might be internalized, taken up and/or translocated from the parasite by host cells.
The rapid increase of specific antibody and titers up to 1:409,600 at 6 weeks after immunization with rCsHK by ELISA shows the strong immunogenicity of rCsHK. Bioinformatics tools indicate an abundance of putative B-cell and T-cell epitopes in CsHK [17]. The high levels of specific antibody elicited by rCsHK might result from its multiple B-cell epitopes. In serum from rCsHK immunized rats, IgG1 and IgG2a levels increased. It is well known that IgG2a and IgG1 are, respectively, induced by T helper cells (Th) 1 and Th2. Our results suggest that rCsHK induced a combined Th1/Th2 immune response. During long-term C. sinensis infections, there is a Th1 to Th2 shift, resulting in chronic liver fluke disease and long-term survival of the worm [50]. In rCsHK immunized rats, the levels of IgG1 were statistically higher than those of IgG2a from 2 to 8 weeks, but lower at 10 and 12 weeks. The rats were challenged 6 weeks after the first immunization. The worm burden and EPG in the rCsHK immunized group significantly decreased compared to the control groups at 12 weeks after the first immunization. The role of Th1 cells is to orchestrate protective proinflammatory immune responses [51]. It has been documented that protected animals elicit high levels of both IgG1 and IgG2 antibodies, whereas the magnitude of these are 10- and 100-fold lower in non-protected animals. Protection is tightly correlated with the level and avidity of the IgG2 antibodies induced [52–54]. Moreover, for successful vaccination against most bacterial and viral diseases, an efficient Th1 response is required [55]. The decrease of worm burden and EPG in the rCsHK immunized group might be related to the up-regulated immune responses, especially Th1, evoked by rCsHK at 10 weeks post immunization.

The survival rates of C. sinensis adults incubated in medium with different concentrations of rat anti-rCsHK serum statistically decreased compared to those of worms incubated in medium with pre-immune serum. The enzymatic activity of CsHK in adult worms incubated in medium with different dilutions of anti-rCsHK serum declined significantly in a dose- and time-dependent manner. The inhibition of CsHK enzymatic activity by anti-rCsHK serum might contribute to the decrease of worm burden and EPG in the rCsHK immunized group.

Collectively, we confirmed that differences exist in spatial structure and affinity for hexoses and phosphate donors between CsHK and HKs from humans or rats, the definitive hosts of C. sinensis. We found that effectors (AMP, PEP, and citrate) and a small molecular inhibitor regulate the enzymatic activity of rCsHK with various allosteric systems. CsHK was found to be extensively distributed in adult worms. It was confirmed to be a component of ESPs. rCsHK showed relatively good immunogenicity and immunoreactivity. Subcutaneous immunization with rCsHK decreased worm burden and EPG in challenged rats, which might be related to the up-regulated immune responses, especially Th1, evoked by rCsHK and to the inhibition of CsHK enzymatic activity by anti-rCsHK serum. Our study showed that CsHK has vaccine potential and is a promising drug target for Clonorchiasis, making it worthy of further investigation.

Supporting Information
S1 Table. Accession numbers/ID numbers for genes and proteins mentioned in the text. (XLS)

S2 Table. Summarized kinetic parameters of rCsHK fitting the Hill equation. (XLS)

Acknowledgments
We would like to thank the Centers for Disease Control and Prevention of Guangxi Zhuang Autonomous Region, China, for serum from humans used in this work.
Author Contributions
Conceived and designed the experiments: TC JY YH XY JX. Performed the experiments: TC JY. Analyzed the data: TC JY ZT. Contributed reagents/materials/analysis tools: TC JY ZT ZX ZL HS SW XL. Wrote the paper: TC YH.

References
1. Lun ZR, Gasser RB, Lai DH, Li AX, Zhu XQ, Yu XB, et al. Clonorchiasis: a key foodborne zoonosis in China. The Lancet infectious diseases. 2005; 5(1):31–41. PMID:15620559
2. Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, et al. A review of human carcinogens—Part B: biological agents. The lancet oncology. 2009; 10(4):321–2. PMID:19350698
3. Hong ST, Kho WG, Kim VH, Chai JY, Lee SH. Turnover of biliary epithelial cells in Clonorchis sinensis infected rats. The Korean journal of parasitology. 1993; 31(2):83–9. PMID:8343460
4. Wang X, Chen W, Hu F, Deng C, Zhou C, Lv X, et al. Clonorchis sinensis enolase: identification and biochemical characterization of a glycolytic enzyme from excretory/secretory products. Mol Biochem Parasitol. 2011; 177(2):135–42. doi:10.1016/j.molbiopara.2011.02.011 PMID: 21382423
5. Liao Q, Yuan X, Xiao H, Liu C, Lv Z, Zhao Y, et al. Identifying Schistosoma japonicum excretory/secretory proteins and their interactions with host immune system. PloS one. 2011; 6(8):e23786. doi:10.1371/journal.pone.0023786 PMID: 21887319
6. LaCourse EJ, Perally S, Morphey RM, Moxon JV, Prescott M, Dowling DJ, et al. The Sigma class glutathione transferase from the liver fluke Fasciola hepatica. PLoS neglected tropical diseases. 2012; 6(5): doi: 10.1371/journal.pntd.0001666 PMID: 22666515
7. Chen W, Wang X, Lv X, Tian Y, Xu Y, Mao Q, et al. Characterization of the secreted cathepsin B cysteine proteases family of the carcinogenic liver fluke Clonorchis sinensis. Parasitology research. 2014; 113(9):3409–18. doi: 10.1007/s00436-014-4006-6 PMID: 24985486
8. Wang X, Chen W, Tian Y, Mao Q, Lv X, Shang M, et al. Surface display of Clonorchis sinensis enolase on Bacillus subtilis spores potentializes an oral vaccine candidate. Vaccine. 2014; 32(12):1338–45. doi:10.1016/j.vaccine.2014.01.039 PMID: 24486347
9. Lee KW, Shalaby KA, Thakur A, Medhat AM, Karim AM, LoVerde PT. Cloning of the gene for phosphoglycerate kinase from Schistosoma mansoni and characterization of its gene product. Mol Biochem Parasitol. 1995; 71(2):221–31. PMID: 7477104
10. Hong SJ, Sohn WM, Song KY. Molecular cloning and immunological characterization of phosphoglycerate kinase from Clonorchis sinensis. Mol Biochem Parasitol. 2000; 108(2):207–16. PMID: 10989223
11. Phillips D, Blake C, Watson H. The enzymes of glycolysis: structure, activity and evolution. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 1981; 293(1063):1–214. PMID: 6115410
12. Singh AR, Joshi S, Arya R, Kayastha AM, Srivastava KK, Tripathi LM, et al. Molecular cloning and characterization of Brugia malayi hexokinase. Parasitology international. 2008; 57(3):354–61. doi:10.1016/ j.parint.2008.03.004 PMID: 18499611
13. Schmitt-Wrede HP, Waldraff A, Krucken J, Harder A, Wunderlich F. Characterization of a hexokinase encoding cDNA of the parasitic nematode Haemonchus contortus. Biochimica et biophysica acta. 1999; 1444(3):439–44. PMID: 10095069
14. Tielen AG, van den Heuvel JM, van Mazijk HJ, Wilson JE, Shoemaker CB. The 50-kDa glucose 6-phosphate-sensitive hexokinase of Schistosoma mansoni. The Journal of biological chemistry. 1994; 269(40):24736–41. PMID: 7929149
15. Shoemaker CB, Reynolds SR, Wei G, Tielen AG, Harn DA. Schistosoma mansoni hexokinase: cDNA cloning and immunogenicity studies. Experimental parasitology. 1995; 80(1):36–45. PMID: 7821409
16. Armstrong RL, Wilson JE, Shoemaker CB. Purification and characterization of the hexokinase from Schistosoma mansoni, expressed in Escherichia coli. Protein expression and purification. 1996; 8(3):374–80. PMID: 8936600
17. Chen T, Ning D, Sun H, Li R, Shang M, Li X, et al. Sequence analysis and molecular characterization of Clonorchis sinensis hexokinase, an unusual trimeric 50-kDa glucose-6-phosphate-sensitive allosteric enzyme. PloS one. 2014; 9(9):e107940. doi: 10.1371/journal.pone.0107940 PMID: 25232723
18. Liang C, Hu XC, Lv ZY, Wu ZD, Yu XB, Xu J, et al. [Experimental establishment of life cycle of Clonorchis sinensis]. Zhongguo ji sheng chong xue yu ji sheng chong bing za zhi = Chinese journal of parasitology & parasitic diseases. 2009; 27(2):148–50. PMID: 24818415
42. Harris MT, Walker DM, Drew ME, Mitchell WG, Dao K, Schroeder CE, et al. Interrogating a hexokinase-selected small-molecule library for inhibitors of Plasmodium falciparum hexokinase. Antimicrobial agents and chemotherapy. 2013; 57(8):3731–7. doi:10.1128/AAC.00662-13 PMID: 23716053

43. Terentis AC, Freewan M, Sempertegui Plaza TS, Raftery MJ, Stocker R, Thomas SR. The selenazal drug ebselen potently inhibits indoleamine 2,3-dioxygenase by targeting enzyme cysteine residues. Biochemistry. 2010; 49(3):591–600. doi:10.1021/bi901546e PMID: 20000778

44. Zhan JH, Yao JP, Liu W, Hu XC, Wu ZD, Zhou XW. Analysis of a novel cathepsin B circulating antigen and its response to drug treatment in Trichinella-infected mice. Parasitology research. 2013; 112(9):3213–22. doi:10.1007/s00436-013-3497-x PMID: 23828190

45. Huang L, Lv X, Huang Y, Hu Y, Yan H, Zheng M, et al. Identification, sequence analysis, and characterization of serine/threonine protein kinase 17A from Clonorchis sinensis. Parasitology research. 2014; 113(5):1713–23. doi:10.1007/s00436-014-3816-x PMID: 24578258

46. Mulvenna J, Moertel L, Jones MK, Nawaratna S, Lovas EM, Gobert GN, et al. Exposed proteins of the Schistosoma japonicum tegument. International journal for parasitology. 2010; 40(5):543–54. doi:10.1016/j.ijpara.2009.10.002 PMID: 19853607

47. Gomez-Arreaza A, Acosta H, Quinones W, Concepcion JL, Michels PA, Avilan L. Extracellular functions of glycolytic enzymes of parasites: unpredicted use of ancient proteins. Mol Biochem Parasitol. 2014; 193(2):75–81. doi:10.1016/j.molbiopara.2014.02.005 PMID: 24602601

48. Lv X, Chen W, Wang X, Li X, Sun J, Deng C, et al. Molecular characterization and expression of a cysteine protease from Clonorchis sinensis and its application for serodiagnosis of clonorchiasis. Parasitology research. 2012; 110(6):2211–9. doi:10.1007/s00436-011-2751-3 PMID: 22170263

49. Cai GB, Bae YA, Kim SH, Sohn WM, Lee YS, Jiang MS, et al. Vitellocyte-specific expression of phospholipid hydroperoxide glutathione peroxidases in Clonorchis sinensis. International journal for parasitology. 2008; 38(14):1613–23. doi:10.1016/j.ijpara.2008.05.011 PMID: 18588894

50. Choi YK, Yoon BI, Won YS, Lee CH, Hyun BH, Kim HC, et al. Cytokine responses in mice infected with Clonorchis sinensis. Parasitology research. 2003; 91(2):87–93. PMID: 12898229

51. Dalton JP, Robinson MW, Mulcahy G, O'Neill SM, Donnelly S. Immunomodulatory molecules of Fasciola hepatica: candidates for both vaccine and immunotherapeutic development. Veterinary parasitology. 2013; 195(3–4):272–85. doi:10.1016/j.vetpar.2013.03.016 PMID: 23582664

52. Mulcahy GO'Conner F, McGonigle S, Dowd A, Clery DG, Andrews SJ, et al. Correlation of specific antibody titre and avidity with protection in cattle immunized against Fasciola hepatica. Vaccine. 1998; 16(9–10):932–9.

53. Mulcahy GO'Conner F, Clery D, Hogan SF, Dowd AJ, Andrews SJ, et al. Immune responses of cattle to experimental anti-Fasciola hepatica vaccines. Research in veterinary science. 1999; 67(1):27–33. PMID: 10425237

54. Mulcahy G, Dalton JP. Cathepsin L proteinases as vaccines against infection with Fasciola hepatica (liver fluke) in ruminants. Research in veterinary science. 2001; 70(1):83–6. PMID: 11170858

55. van Riet E, Hartgers FC, Yazdanbakhsh M. Chronic helminth infections induce immunomodulation: consequences and mechanisms. Immunobiology. 2007; 212(6):475–90. PMID: 17544832