Original Article
Schistosoma japonicum serine protease inhibitor increases endothelial barrier function

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Abstract: Schistosomiasis remains the second most prevalent zoonotic disease after malaria in veterinary medicine. The egg lodgement in target host tissue plays important roles in pathogenesis of this disease, but the process prior to egg-laying is still elusive. Surely, investigation of how this parasite invades and moves inside corresponding host will probably improve our understanding of homeostasis and maintenance of animal health, further, of related pathogenesis and thus potential intervention against schistosomiasis. TNT-coupled transcription/translation-expressed Sj serpin was employed for the protease inhibition assay. Transendothelial resistance (TER), its charge selectivity and size selectivity, were measured by the ussing chamber technique in serpin-transfected or recombinant serpin-treated HUVEC monolayer. The expressions of junction proteins were assayed using real-time PCR, Western blot and immunostaining. Sj serpin blocks the protease activity of elastase in a time-dependent manner; and Sj serpin can increase TER ofendothelial monolayer by decreasing its paracellular size selectivity, but not by interfere with the charge selectivity. Altered expression of tight junction protein claudin-2 was not observed at either RNA or protein levels; however, we found a marked increase in the expression of occludin, ZO-1,VE-cadherin and beta-catenin. Sj serpin can increase the transendothelial barrier function by decreasing the transendothelial permeability, implying serpin as a potential target to limit the invasion of schistosome into animal blood vessel.

Keywords: *Schistosoma japonicum*, serpin, endothelial barrier function, tight junction, size selectivity

Introduction

Among zoonotic diseases, schistosomiasis remainsthe second most prevalent parasitic disease after malaria, affecting more than 200 million people and countless animals in the developing world [1, 2], and is mainly caused by three distinct blood-dwelling worms feeding on blood in the veins surrounding the host's intestine, liver (*S. mansoni*-occurring in Africa and South America, *S. japonicum*-South and East Asia) or bladder (*S. haematobium*-Africa). These worms produce hundreds to thousands of eggs per day, many of which lodge in host tissues and induce local inflammatory responses (Th1 response initially, then progressing to a more dominant Th2 response), which then lead to a diverse range of pathologies, including hepatic fibrosis, splenomegaly, vasculitis, granulomas and, in some cases, perhaps cancer [3-5]. But the pathophysiology caused by the schistosome prior to egg-laying is still elusive. Further

investigation of how this parasite migrates within the host will probably improve our understanding of the relevant pathogenesis and eventually facilitate potential intervention.

Once in the skin, *Schistosoma* larvae (cercariae) transform into schistosomula, whose fate depends on the balances between factors facilitating or hampering its penetration, degrading the host extracellular matrix by secreting and transferring proteolytic enzymes to facilitate their entry into the host dermal blood vessels [6]. The vascular endothelium lining the inner surface of all kinds of different blood vessels functions as the first interface for circulating blood components (nutrients and microorganisms), to interact with vascular endothelial cells and surrounding extravascular tissues. In addition to regulating blood delivery and perfusion, a major function of vascular endothelium is to provide a semipermeable barrier that controls blood-tissue exchange of fluids, nutrients, and

metabolic wastes while preventing pathogens or harmful materials in the circulation from entering into tissues [7]. Under certain stressed conditions, such as viral or bacterial infections, the endothelial barrier function may become compromised with severe consequences. On the other hand, if the barrier is enhanced it may limit further metabolic waste penetration, even bacterial or viral infection. In the endothelial barrier system, tight junctions (TJs) and adherens junctions (AJs), located between adjacent endothelial cells, serve as the principal paracellular barrier component inpolarized endothelial monolayers, contributing to the maintenance of apical-basolateral cell polarity, regulating the interaction between circulating blood and extracellular matrix through the paracellular pathway, and providing a niche for cell-cell signaling [8, 9]. The TJs and AJs complexes are composed of both transmembrane and intracellular cytosolic proteins [8, 10]. Similar to host blood vessel endothelium, the tegument in schistosome functions as a dynamic host-schistosome interaction layer involved in nutrition uptake, immune regulation, osmosis regulation, and signal transduction [11]. Some tegument proteins, such as TSP-2 [12], Sm29 [13] and Smteg [14], have shown some potential to induce high levels of protection against the attack from cercariae in the murine model, indicating that the schistosomes tegument represents an obvious target for the development of new control strategies.

The infective stage of the parasite (cercariae) and the newly penetrated schistosomula are sensitive to killing by complement, but 48-h later they become invisible to the complement system and remain so throughout the rest of their development. Sensitivity to complement can be restored in adult worms of *S. mansoni* by incubation with protease [15], implying that there is a surface protein responsible for this parasite protection and maybe the involvement of proteases in the complement system for host resistance. In mammals, serine protease inhibitors (serpins) are involved in the regulation of proteases in blood coagulation, fibrinolysis, apoptosis, development, and inflammation. Recently, studies found that serpin also plays an important role in the immune response of insects and parasites to the external environment. Spn43Ac in *D. melanogaster* regulates the immune response to fungal infection by inhibiting the cleavage of the Toll ligand Spaeztle [16]. SRPN6 of the malaria mosquito vector *Anopheles gambiae* is important for parasite clearance and/or lysis [17].

Praziquantel is the drug of choice to treat schistosome infection, but it has limitations, including potential drug-resistance like many other drugs [18]. Endeavors to develop a vaccine against schistosome infection have been haunted by a lack of efficacy. As a result, an urgent need exists to identify novel therapeutic and/or preventive agents to combat these parasites.

In the current study, we demonstrated that *Schistosoma japonicum* (Sj) serpin plays important roles in the regulation of endothelial barrier function in both Sj serpin transfected and recombinant Sj serpin-treated endothelial cells, by decreasing the transendothelial permeability but not interfering with the charge selectivity. Tight junction proteins are involved in the regulation of these altered barrier functions.

Materials and methods

Plasmid construction

Total RNA from HUVEC cells were reverse-transcribed using the Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA) and purified with the RNeasy Mini Kit (Qiagen, Germantown, MD). Sj serpin was cloned by the PCR method with specific primers (GenBank gi: 28331989: forward 5' AATATGTGTTTGAAAGTAGCACC 3'; reverse 5' TTGGTTTACTGACAATTTCAAATC 3') and subcloned into vector pcDNA3. Site-directed mutagenesis was performed on a wild-type Sj serpin/pcDNA3 construct with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA) with specific primers (forward 5' GGAATTGAAGAAGCA<u>G</u>CTGTCACTTCACCT 3', reverse 5' AGGTGAAGTGACAG<u>C</u>TGCTTCTTCAATTCC 3'). An alanine residue at position P10 of the hinge region of the reactive loop of Sj serpin (AP10T mutant) was changed into threonine.

Cell culture

Human umbilical vein endothelialcells (HUVEC) (ATCC, Manassas, VA, USA) between passages 20 and 30 were cultured according to the standard protocol and transfected with relevant plasmids (wild-type and mutant Sj serpin/

pcDNA3, pcDNA3 vector) using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

TNT-based in vitro transcription/translation of Sj serpin

The TNT T7 Quick Coupled Transcription/ Translation System (Promega, Madison, WI) was used for *in vitro* transcription and translation of Sj serpin and the function-dead mutant. Briefly, 40 μl of TNT T7 Quick Master Mix, 2 μl of methionine and 1 μg of the appropriate plasmids were mixed in a total volume of 50 μl, and the reactions were incubated at 30°C for 70 min. The transcribed/translated proteins could be used directly for the following experiments.

Elastase inhibition assay

The determination of the anti-elastase activity of Sj serpin was performed according to protocol described previously [14]. Briefly, in the presence of 2 mM pNaMAAPV (Sigma-Aldrich, St. Louis, MO)-a synthetic substrate in working solution (Tris-HCl, pH7.5), wild type or mutant Sj serpin (40 μg/ml) was incubated with elastase (0.1 μg/ml, Sigma-Aldrich, St. Louis, MO) at different points in time (from 0 minute up to 300 minutes with 20 minutes as an interval). The resulting color reaction was measured at an optical density of 405 nm.

In vitro transendothelial resistance (TER) assay

As transendothelial barrier dysfunction is necessary for migration of S. japonicum larvae schistosomula into blood vessels, we studied the effects of Sj serpin expression on endothelial barrier function *in vitro*. For the *in vitro* assay, HUVEC cells grew confluent on snapwell filters (Corning Costar, Corning, NY, USA), and relative TER was measured with ussing chambers (Physiologic Instruments, San Diego, CA, USA). In parallel, confluent HUVEC cells on snapwell filters were treated with 100 ng/ml of recombinant Sj serpin expressed by TNT-transcription/translation system for 12 hours, the corresponding TER was measured with ussing chambers (Physiologic Instruments, San Diego, CA, USA).

In vitro ion selectivity assays

The ion selectivity of tight junctions was determined by measurement of dilution potentials (HUVEC endothelial monolayer) by replacing either the apical or basolateral solution, while keeping the other side (basolateral or apical) bathed in Krebs' solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 4.2 mM NaHCO3, 2 mM CaCl2, 10 mM glucose, 200 mM sulphinpyrazone and 10 mM HEPES, pH 7.4). For 2:1 NaCl dilution potentials/short-circuit current, the 118 mM NaCl solution was replaced with 60 mM NaCl in Krebs' solution, and osmolarity was maintained with mannitol.

In vitro permeability assays

In vitro permeability assays were performed using a fluorescein isothiocyanate (FITC)-labeled dextran method to assess barrier function. For the *in vitro* permeability assay, confluent and polarized HUVEC cells grown on filters were treated with FITC-labeled dextrans (4-kDa, Sigma-Aldrich, St. Louis, MO, USA) in the upper chamber for 2h at 37°C. The medium in the lower chamber was collected, the fluorescence intensity of each sample was measured (485-Ex/520Em, Cytofluor 2300; Millipore, Waters Chromatography) and FITC-dextran concentrations were determined from standard curves generated by serial dilution of FITC-dextran.

Western blot

All Western blots were performed based on standard protocols relevant to the specific antibodies. In detail, cells, transfected with Sj serpin plasmid or treated with recombinant Sj serpin protein (100 ng/ml) for 16 hours, were resuspended in lysis buffer containing 1% (wt/vol) Triton X-100, 20 mM Tris (pH 8.0), 50 mM NaCl, 5 mM EDTA, 0.2% (wt/vol) BSA, and protease inhibitors (One Tablet Complete, Mini/10 ml lysate buffer, Roche Diagnostic, Penzberg, Germany). Cell lysates with 30 μg of total protein were then processed in denaturing buffer containing 2% SDS and 20% glycerol with 10 mM β-mercaptoethanol at 100°C for 2 min. Samples were resolved by 7.5% SDS-PAGE and transferred to 0.4-μm nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked for 1 h in blocking buffer (5% nonfat dry milk, Tris-buffered saline, and 0.1% Tween 20), washed with blocking buffer, and then incubated for 1 h overnight at 4°C with Sj serpin antibody or other specific antibodies including occludin antibody (Cell Signaling Technology, Boston, MA, USA), clau-

din-2 antibody (Cell Signaling Technology, Boston, MA, USA), beta-catenin (Cell Signaling Technology, Boston, MA, USA), ZO-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and VE-cadherin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were then washed three times for 30 min in blocking buffer and further incubated for 1 h at room temperature with the appropriated peroxidase-conjugated secondary antibodies. Finally, blots were washed twice for 30 min in blocking buffer and then visualized using an ECL system (GE Healthcare Life Science, Pittsburg, PA, USA). Here, western blot with GAPDH primary antibody (Cell Signaling Technology, Boston, MA, USA) functions as an internal control.

Real-time PCR

Total RNA from HUVEC cells were reverse-transcribed using the Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA) and purified with the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). Real-time PCR was performed using the iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA) with the iCycler sequence detection system (Bio-Rad, Hercules, CA) with specific primers for Sj serpin (forward 5' TGTGTTTGAAAGTAGCACC-AAA 3'; reverse 5' ACACCAAGTGGTGATGCTAAA 3') or primers described previously [19]. Real-time PCR data were presented using the delta-delta C_t ($\Delta\Delta C_t$) method [19] with the GAPDH gene level (for 5' GTCGGAGTCAACGGATTTGG 3'; reverse 5' AAGCTTCCCGTTCTCAGCCT 3') serving as the internal standard.
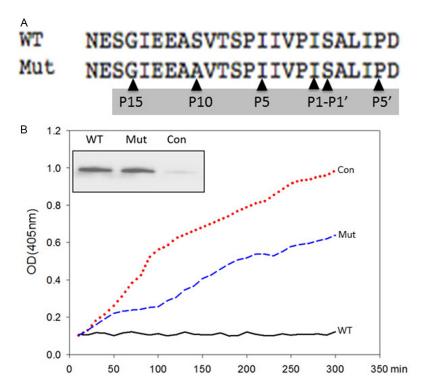
Sj serpin antibody generation

Sj serpin was cloned into a PET-24a vector (EMD Millipore, Billerica, MA, USA) and expressed in *Escherichia coli* (*E. coli*). The proteins were expressed in *E. coli* strain BL21(DE3) and purified by affinity chromatography using Ni-NTA His Bind Resins (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Female BALB/c mice were injected subcutaneously with 50 μg of purified recombinant protein emulsified in complete Freund's adjuvant. Two additional injections of 50 μg of antigen emulsified in incomplete Freund's adjuvant were followed at bi-weekly intervals starting four weeks after the first immunization. Ten days after the second boost, the serum antibody titer was tested using

ELISA. Two weeks after the second boost, the mice were given a final booster injection intraperitoneally with 50-μg proteins. Three days after the last injection, spleen cells from the immunized mice were fused with myeloma Sp2/0 cells [20]. ELISA was employed for screening antigen-specific monoclonal antibodies.

Immunofluorescence

Immunostaining assays were performed according to the standard protocol with relevant primary antibodies (Sj serpin and beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA)) and Alexa Fluor® 488 secondary antibody (Molecular Probes, Carlsbad, CA, USA), and rhodamine phalloidin (Molecular Probes, Carlsbad, CA, USA) as described previously [19] to visualize actin. HUVEC cells grown on coverslip were washed twice with PBS (pH 7.4) (Invitrogen, Carlsbad, CA, USA) supplemented with 0.1 mM CaCl2 and 1 mM MgCl2 (PBS-Ca/Mg), and fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Washington, PA, USA) in PBS-Ca/Mg for 15 min at room temperature. After three washes with PBS-Ca/Mg, cells were pre-incubated at room temperature for 30 min in immunostaining buffer (PBS-Ca/Mg with 3% BSA and 0.1% Triton X-100) and incubated for 40 min at room temperature with Alexa Fluo 568-conjugated phalloidin (1 U/filter; Molecular Probes, Eugene, OR, USA) diluted in immune-staining buffer. Cells were then washed twice in PBS and exposed to Sj serpin, occludin, claudin-2, ZO-1, beta-catenin, VE-cadeherin antibody or irrelevant anti-IgG isotype for 1 h at room temperature. After washing twice in PBS, cells were incubated with a secondary antibodies conjugated to Alexa Fluo 488 (1:200, Molecular Probes), for 1 h at room temperature. After washing, samples were mounted in Prolong Gold Antifade Reagent and analyzed by ZEISS AXIOSKOP 2 PLUS Microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY, USA).

Statistical analysis

Values in the current study were expressed as means ± SE. Unpaired two-tailed Student's t test by InStat v3.06 (GraphPad) software was employed for the statistical analysis. $P < 0.05$ was considered statistically significant.

Figure 1. Antiprotease activity assay of Schistosoma japonicum serpin. A: Alignment of wild type Sj serpin with mutant Sjserpin, in which amino acid serine is replaced with amino acid alanine, black solid triangles represent the position of corresponding amino acids labeled by P5', P1', P1, P5, P10 and P15. B: 40 µg/ml of Recombinant proteins produced by theTNT® Quick Coupled Transcription/Translation Systemwere incubated with elastase (0.1 µg/ml) in the presence of a 2 mM concentration of a synthetic substrate, pNaMAAPVat different points in time (from 0 minute up to 300 minutes with 20 minutes as an interval). The resulting colored reaction was measured (OD, optical density) at 405 nm. The blackcolor represents the wild type (WT) of Sj serpin, the blue color represents the Sj serpin mutant, and the red color functions as a control in which elastase and its substrate were incubated alone.

Results

Anti-elastase activity of Sj serpin

Online software analysis predicts that Sj serpin has a potential to inhibit the proteolytic activity of elastase. In order to investigate this prediction, we introduced a single amino acid mutation into the hinge region of Sj serpin (**Figure 1A**). This mutation resulted in the replacement of a bigger polar serine with a small non-charged alanine (SP10A). This transforms the Sj serpin interaction with its substrate from tight binding to competitive inhibition.

Recombinant proteins were produced using the TNT T7 Quick Coupled Transcription/Translation System (**Figure 1B**). Wild type or mutant Sj serpin (black and blue lines) was incubated with elastase (0.1 µg/ml) in the presence of a 2 mM concentration of a synthetic substrate, pNa-

MAAPV. The resulting colored reaction was measured (OD, optical density) at 405 nm. As a control, elastase and its substrate were incubated alone (red line). **Figure 1** demonstrated that wild type Sj serpin can inhibit the protease activity of elastase over time (black line) in comparison with a control (red line). However, in the SP10A mutant, the antiprotease activity is strongly affected compared to that of wild-type Sj serpin (blue line).

Overexpression of Sj serpin increases endothelial barrier function

By real-time PCR and Western blot analyses, we found significant increases in Sj serpin expression in Sj serpin/pcDNA3-transfected cells at both mRNA and protein levels in comparison with controls (**Figure 2A** and **2B**). We noticed that there are obvious band for western blot results (**Figure 2B**) because of the high similarity of amino acid sequence between serpin in schistosoma and serpin in Homo sapiens [21]. Furthermore, immunostaining showed increased Sj serpin expression in both in nuclear and cytosolic pools (**Figure 2C**).

We hypothesized that Sj serpin may play a role in endothelial barrier function. As shown in **Figure 3A**, Sj serpin overexpression significantly increased the TER to 192.03±14.81 ohms. cm^2, compared to the TER (69.51±26.72 ohms. cm^2) in vector-transfected HUVEC cellline, indicating that Sj serpin can positively regulate HUVEC barrier function. It is known that schistosomes are not intracellular; transfection of Sj serpin in endothelial cell line HUVEC doesnot exactly reflect the life functionality extracellularly. To solve this problem, recombinant Sj serpin protein was generated with TNT-coupled transcription/translation system, then subjected to treat HUVEC cells, similar results were
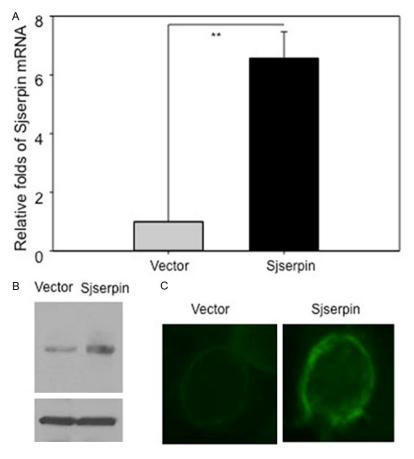
Figure 2. Constructs of Sj serpin-overexpressed HUVEC cellular model. HUVEC cells were transiently transfected with 1 μg of Sj serpin/pcDNA3 and vector pcDNA3 using lipofectamine 2000 method. The expression of Sj serpin is up-regulated in HUVEC cells transiently transfected with Sj serpin/pcDNA3 in comparison with those transiently transfected withvector pcDNA3 demonstrated by real-time PCR (A), Western blot (B), and immunofluorescence (C) (Sj serpin, green). **: $P<0.01$.

reduced by half (**Figure 3C**) at either the basolateral side (-7.1476±2.4315 μA vs. -7.0201±2.7612 μA) or the apical side (-18.6114± 4.0356 μA vs. -18.1804± 5.1329 μA). These results indicate that ion charge selectivity does not significantly affect the Sj serpin-mediated increase in endothelial barrier function. To further investigate whether the increase in TER caused by Sj serpin may be attributable to alteration of tight junction size selectivity, we examined transendothelial permeability using the 4-kDa (**Figure 3C**) FITC-dextran method. As shown in **Figure 3D**, vector-transfected cells showed an FITC-dextran flux (ng/ml/min) of 83.5697±10.6706. In comparison, a ~6-fold decrease in FITC-dextran flux was observed in Sj serpin-transfected cells (15.4542± 4.3176).

Characterization of apical junction proteins

To explore the underlying mechanisms of Sj serpin overexpression in the retention of blood vessel barrier function, we characterized the expression of apical junction proteins using immunofluorescence, real-time PCR and Western blots (**Figure 4**). The expression of junction protein claudin-2 did not change significantly in Sj serpin-transfected cells at either mRNA or protein levels. In contrast, expression of occludin, E-cadherin, β-catenin, and ZO-1 increased significantly in Sj serpin-transfected cells in comparison with vector-transfected cells. By real-time PCR and Western blots, similar results were noticed in recombinant Sj serpin-treated HUVEC cells (**Figure 5**).

Discussion

In the current study, we have highlighted for the first time that *S. japonicum* serpin regulates

found in recombinant Sj serpin-treated HUVEC cell line (**Figure 3B**), which the TER rose up to 136.4353±13.275 ohms.cm^2 in recombinant Sjserpin-treated HUVEC cells in comparison with untreated HUVEC cells (65.5613±10.3569 ohms.cm^2). In short, we confirm that serpin overexpression can up-regulate the barrier function in endothelial cells, which is in concert with some other published data [22, 23]. Since TER is influenced by both paracellular size selectivity and ion selectivity [24], the enhanced TER may be due to an alteration in either of these parameters. To further evaluate the effects of Sj serpin expression on endothelial barrier function, the NaCl ion selectivity was examined. We found that the transendothelial current in Sj serpin-overexpressed and vector-transfected cells did not show a significant difference when the NaCl concentration was
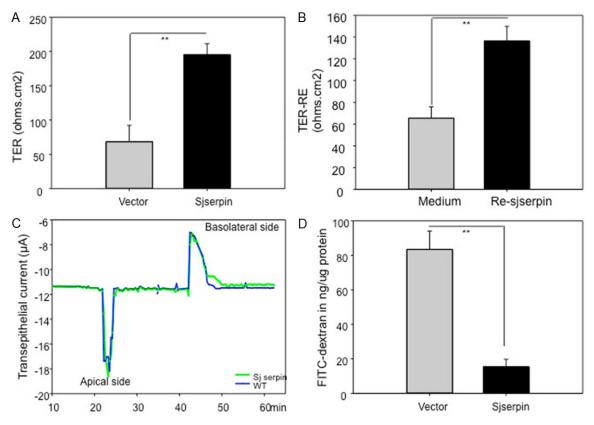
Figure 3. Sj serpin is involved in the regulation of barrier function of HUVEC *in vitro*. (A) overexpression of Sj serpin significantly increased TER in HUVEC cells in comparison with vector-transfected cells; (B) Treatment of HUVEC cells with recombinant Sj serpin protein (100 ng/ml) in culture medium significantly increased TER in comparison with treatment by only medium; (C) represents the transepithelial current on both the apical and basolateral sides of HUVEC cells as described in *Materials and Methods*. Sj serpin showed no significantly different transepithelial current on either the apical side or the basolateral side; Further, (D) overexpression of Sj serpin significantly reduces the flux of 4-kDa FITC-dextran. **: $P<0.01$.

endothelial barrier function, which gives us substantial new mechanistic insights into the pathogenesis of schistosomiasis.

Schistosomiasis has drawn tremendous attention, since the life and economic loss associated with this disease may reach levels on a par with global killers including malaria, tuberculosis, or HIV/AIDS [25, 26], thus, concerns about the pathogenesis of schistosomiasis have always remained at the forefront of medical research. Schistosomes can block neutrophil attack [27] and are seemingly invisible to the complement system 48 h post-infection [15, 28]. The underlying mechanisms of how schistosome manages to neutralize this extremely hostile environment are elusive, but it is clear that the defense-and-repair system in the schistosome, which relies on the function of proteases and their inhibitors, plays a critical role [29]. Thus, the involvement of protease

inhibitors such as a serpin in the survival and migration of schistosome is a logical conclusion. Our previous study demonstrated that Sj serpin can decrease worm burden and egg-laying [21] by stimulating a Th2 immune response and a similar result occurred in another study with cysteine protease inhibitor [30]. A serpin from *S. mansoni* cercariae can be released into the host to perform an anti-proteolytic function [31, 32].

The function of serpins in schistosomes remains speculative, and more studies should be pursued thereafter. However, different serpins display different relative specific substrates, thus causing specific biologic functions. For example, *S. mansoni* serp_C potentially targets thrombin [31], while squamous cell carcinoma antigen 2 is regarded as a serpin and can inhibit proteolysis specifically of neutrophil elastase [33]. *S. japonicum* serpin
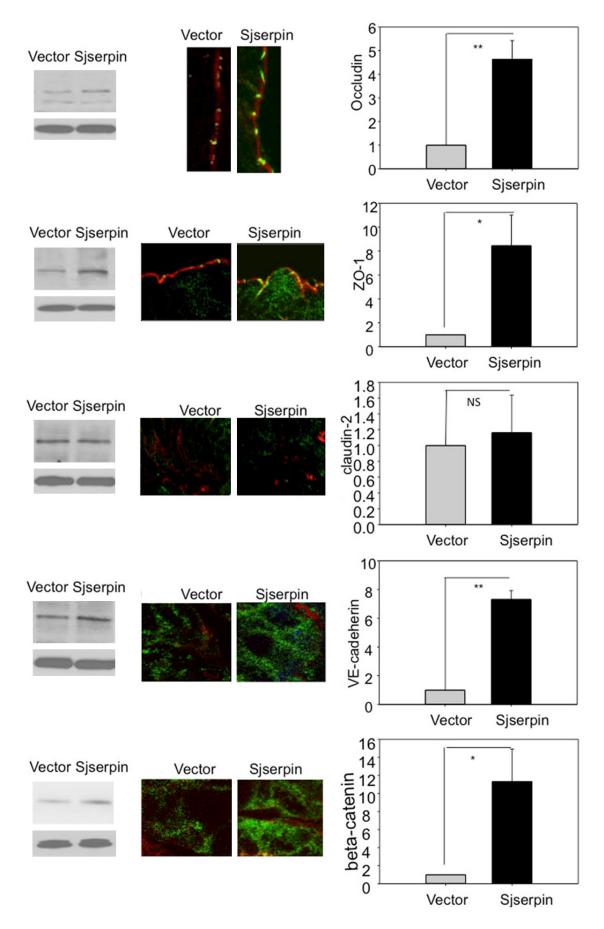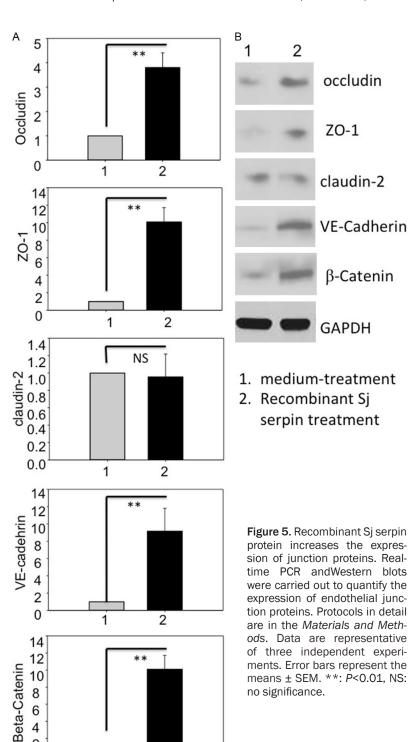
Figure 4. Overexpression of Sj serpin in HUVEC cells is involved in expression modulation of junction proteins. Immunofluorescence, Western blots and real-time PCR were performed to quantify the expression of endothelial junction proteins. Protocols in detail are in the *Materials and Methods*. For immunofluorescence, green represents actin, red represents relevant molecules, and blue represents nuclei. Data are representative of three independent experiments. Error bars represent the means ± SEM. *: $P<0.05$, **: $P<0.01$, NS: no significance.

1. medium-treatment
2. Recombinant Sj serpin treatment

Figure 5. Recombinant Sj serpin protein increases the expression of junction proteins. Real-time PCR and Western blots were carried out to quantify the expression of endothelial junction proteins. Protocols in detail are in the *Materials and Methods*. Data are representative of three independent experiments. Error bars represent the means ± SEM. **: $P<0.01$, NS: no significance.

PI-6 specifically targets chymotrypsin [34]. Here, we found that Sj serpin can target and inhibit the activity of elastase. Lately, the role of elastase in the regulation of epithelial/endothelial barrier function and the succeeding inflammatory response has drawn tremendous attention. For example, elastase-2 plays a key role in skin barrier function, particularly in the pathogenesis of Netherton syndrome, a rare autosomal recessive skin disorder [35]. Neutrophil elastase can trigger G-protein associated signaling through proteinase-activated receptor 1 (PAR1) to compromise endothelial barrier function [36, 37]. Neutrophil elastases are capable of rapidly degrading components of the vascular endothelial (VE)-cadherin complex during neutrophil-endothelial contact [38-40]. This can be prevented by pretreatment of the neutrophils with a combination of several recombinant elastase inhibitors [41-43].

Endothelial barrier function regulates transport and host defense mechanisms on the inner surface of the blood vessel, made up of transcellular and paracellular pathways tightly mediated by membrane pumps, ion channels, tight junctions (TJs) and adherens junctions (AJs), adapting

transendothelial permeability to physiological needs [44]. TJs are crucial in the maintenance of endothelial barrier function, being made up of intracellular and transmembrane TJ proteins. The first transmembrane TJ protein to be identified, occludin, plays a key role in the regulation of endothelium permeabilityand integrity [45]. Intracellular TJ proteins, including zonulae occludentes (ZO)-1, ZO-2, and ZO-3, harbor important scaffolding and adaptor functions [46], connecting perijunctional actin filaments with transmembrane TJ proteins, suchas occludin [47]. Here, we specifically characterized the roles of occludin and ZO-1 based on their key involvement in TJ assembly and function,thereby serving as prototypical molecules for observing theeffects of Sj serpin on these TJ proteins. It is well accepted that ion selectivity of tight junctions is important for the maintenance of epithelial/endothelial cell integrity, and thus also for TER [48, 49]. Specifically, the claudin family plays an important role in the regulation of ion selectivity to mediate TER [50, 51]. Our data showed that Sj serpin does not significantly change the expression of pore-forming claudin-2 either in endothelial size selectivity or in ion selectivity.

Besides TJs, adherens junctions (AJs), mainly composed of a plaque structure (e.g., β-catenin), and transmembrane components (e.g., VE-cadherin), make important contributions to embryogenesis and tissue homeostasis by resisting dissociating forces and transmitting forces to adjacent cells [52]. Recent reports suggested that neutrophil transmigration across the endothelium may disrupt AJs by releasing proteases [38, 39]. VE-cadherin plays a crucial role in the maintenance and restoration of endothelium integrity and in the regulation of vessel permeability [53, 54]. Proteolysis of VE-cadherin can be prevented by elastase-inhibitors to maintain the integrity of endothelial barrier function [41-43]. Except in the endothelium, elastase and its inhibitors are important in the pathogenesis of epithelial injury in inflammatory bowel disease, in which the gut lumen of patients with inflammatory bowel disease contains elevated levels of elastase and administration of anti-elastase compounds can ameliorate intestinal damage in animal models of inflammatory bowel diseases [22, 23, 55, 56].

For extracellular parasites like schistosomes, one of the most common methods to study the pathogenesis and prevention of these parasites-related disease like schistosomiasis is to clone and transfect the candidate molecules of schistosomes into different human or animal cell lines and characterize the corresponding morphologic and functional alteration, to predict, or even further, to apply to the pathogenesis and prevention of schistosomiasis [57-63]. Given the facts that schistosomes are extracellular and can secret plenty of molecules like proteolytic molecules or serpin to the surroundings during their process of penetration into EMT and endothelial cells, hence, we treated HUVEC calls with recombinant Sj serpin in the medium to mimic the penetration process in the great extent.

At molecular level, endothelial barrier function is regulated by many signaling molecules, such as protein kinase C, G protein-coupled receptors,serine-, threonine-, and tyrosine-kinases, calcium, protease-activated receptors, and cytokines [64-68] andare vulnerable to the pathological consequences of infection [69-71]. How Sj serpin is associated with these cellular signaling molecules in endothelial cells to protect against infection would be our next step for study.

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Disclosure of conflict of interest

None.

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