

Expression and immune response analysis of *Schistosoma japonicum* VAL-1, a homologue of vespid venom allergens

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Abstract Many parasites such as trematodes and nematodes have been found to express members of a gene family variously termed as venom allergen-like protein (VAL) or sperm-coating protein (SCP)-like protein. The molecular functions of these proteins remain unclear. We isolated the corresponding gene from *Schistosoma japonicum*, which we designated as *Sj-VAL-1*. The cDNA of *Sj-VAL-1* contains an open reading frame encoding 221 amino acids, the first 25 residues being a putative secretion signal. RT-PCR analysis confirmed that *Sj-VAL-1* was transcribed mainly in cercariae and eggs stages. Western blot analysis indicated that *Sj-VAL-1* protein was an egg excretory–secretory products (ES products). Immunofluorescence showed it was secreted by eggs, head gland, and penetration glands of cercariae. In *S. japonicum*-infected mice, *Sj-VAL-1*-specific antibody significantly increased 6 weeks after infection and a higher level of IgG1 antibody contrast to IgG2a antibody indicated that a polarized Th2 immune response could be induced by *Sj-VAL-1*. These findings suggest that *Sj-VAL-1* must play a role in the interaction between parasite and host. Its role as a potential modulator of immune function or as a pathogenic factor of granuloma formation in schistosomiasis needs further study.

Introduction

Schistosomiasis, caused by blood fluke of the genus *Schistosoma*, remains a major global public health problem, affecting more than 200 million people living in the endemic areas of 76 countries and territories (Engels et al. 2002). Schistosomiasis is recognized as the most important human helminth infection in terms of morbidity and mortality. *Schistosoma japonicum* is one of the three schistosome species that spread greatly in China. Large-scale sequencing projects have created extensive *S. japonicum* expressed sequence tag (EST) and genomic databases, leading to the identification of thousands of new genes, as well as providing a repository of information useful for post-genomic activities (Hu et al. 2003).

The sperm-coating protein (SCP)-like extracellular protein family, also called the SCP/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS) family, consists of a large group of proteins that are found in many eukaryotes. Each protein contains a characteristic SCP/TAPS domain (Pfam accession No. PF00188) (Kovalick and Griffin 2005). Most characterized SCP/TAPS proteins are secreted and function extracellularly. The molecular functions of these proteins remain unclear. Various functions have been ascribed to individual SCP/TAPS proteins, including immune responses (Murphy et al. 1995), testis/sperm development (Fitzpatrick et al. 2005), envenomation (Milne et al. 2003), and parasitic nematode invasion of definitive hosts (Murray et al. 2001). In *Schistosoma mansoni*, there are two distinct groups within the SmVAL gene family, and some of them are developmentally regulated expression, and these protein products may be linked to the establishment of chronic host/parasite interactions (Chalmers et al. 2008). In the present study, we cloned, expressed, and characterized a SCP/TAPS gene in *S. japonicum* and analyzed its interaction with host immune system preliminarily.

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Materials and methods

Database searching

To identify *S. japonicum* SCP/TAPS domain-containing family members, the *Vespula vulgaris* Wasp Venom Allergen 5 (GenBank accession No. AAA30333) protein sequence was used in a tBLASTn search of *S. japonicum* ESTs. After PCR confirmation of the sequences from parasite-derived cDNA, AY812797 was selected for further study. The transcript was named *S. japonicum* Venom allergen-like transcript (*Sj-VAL-1*).

Parasites and animals

Schistosoma japonicum cercariae of Chinese mainland strain were obtained from the infected *Oncomelania hupensis* (Jiangsu Institute for Schistosomiasis control, Wuxi, China). Adult worms were obtained by perfusion of New Zealand rabbit 6 weeks after infection, and schistosome eggs were extracted from liver tissues. Soluble egg antigen (SEA), egg ES products, and soluble male and female adult worm antigens were prepared as previously described (Abdel-Hafeez et al. 2009; Schramm et al. 2009). Six-week-old BALB/c mice were provided by the Center of Experimental Animals (Sun Yat-sen University, Guangzhou, China) and challenged percutaneously with 30 ± 2 *S. japonicum* cercariae by the cover glass method. Serum samples were collected by tail bleeding before infection and thereafter at 1-week intervals. Serums were stored at -20°C until use.

RNA isolation and RT-PCR

Total RNA were isolated from eggs, cercariae, and adult male and female worms, respectively, using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized using reverse transcriptase XL (AMV) and oligo(dT)18 primer (Takara Biotechnology, Dalian, China). For PCR, *Sj-VAL-1*-specific primers were used. The sense primer was 5'-GCT GGA TCC TGG AAA GAT AAT AAA TCA CC-3' and antisense was 5'-ACG CTC GAG TTT GAT TAG GTT GAT GAC-3'. The *S. japonicum* β -actin (GenBank accession No. AF223400) specific primers (5'-TCC ACT GAA TCC GAA AGC CAA TC-3', and 5'-TCA AAG TCC AGA GCC ACA TAA CAC-3') were used to amplify a 348-bp PCR product that served as a transcribed control.

Cloning, expression, and purification of recombinant *Sj-VAL-1*

The coding region (not including the signal peptide) of *Sj-VAL-1* was amplified by PCR using *S. japonicum* eggs cDNA as template. Specific primers with BamH I/XhoI

restriction enzyme sites, respectively, were used. Conditions for PCR were as follows: 10 min at 95°C followed by 32 cycles of 60 s at 95°C , 60 s at 54°C , and 60 s at 72°C , and then a final extension of 10 min at 72°C . The purified PCR product was cloned into the expression vector pET28a (+) (Novagen), then the recombinant plasmid was transformed into *E. coli* BL21(DE3) (Promega). The recombinant protein expression was induced with 1 mM IPTG for 5 h at 37°C as insoluble inclusion body. The insoluble fraction was solubilized in 8 M urea, 500 mM NaCl, 20 mM Tris/HCl, pH 8.0. The r*Sj-VAL-1* was purified by His.Bind Purification kit (Novagen) under denatured conditions according to the manufacturer's protocol. Eluted protein was renatured by stepwise elution of urea and dialyzed against PBS at last in a final concentration of 1 mg ml^{-1} .

Preparation of mouse antiserum to r*Sj-VAL-1*

BALB/c mice were injected with s.c. 50 μg of r*Sj-VAL-1* in 50 μl PBS formulated with an equal volume of complete Freund's adjuvant (CFA, SIGMA), followed by two booster injections of 25 μg antigen mixtures with equal volume of incomplete Freund's adjuvant at 2-week intervals. Serums were collected 2 weeks after the last immunization. Enzyme-linked immunosorbent assay (ELISA) was performed to detect the levels of total IgG against r*Sj-VAL-1* and the antiserum titer to r*Sj-VAL-1* was about 1:128,000.

Western blot analysis

SEA, egg ES products, and adult male and female antigens were separated by 12% SDS-PAGE and transferred to PVDF membrane. The PVDF membrane was blocked with 5% skim milk powder in PBS at 4°C overnight, and subsequently incubated with r*Sj-VAL-1* antiserum (1:1,000 dilution) at room temperature for 2 h. After washing, the membrane was incubated with HRP-conjugated goat anti-mouse IgG (Boster, Wuhan, China) for 1 h at room temperature. Subsequently, the HRP was visualized by diaminobenzidine (DAB) substrate solution.

Immunohistological detection of *Sj-VAL-1*

The *S. japonicum*-infected rabbit livers were fixed in 4% paraformaldehyde and processed to make paraffin blocks. The blocks were cut into 5- μm sections. Living cercariae were collected into PBS-0.3% TritonX-100 for 90 min at room temperature and then fixed in glass slides by cold acetone. The slides were blocked overnight at 4°C with normal goat serum (liver sections) or 10% bovine serum albumin (cercariae), respectively. Subsequently, the slides were incubated for 2 h at room temperature with r*Sj-VAL-1*

antiserum (diluted 1:200 for liver, 1:100 for cercariae). Negative controls were performed under omission of the primary antibody. After washing, the samples were incubated with FITC-conjugated goat anti-mouse IgG for liver (1:200, Boster, Wuhan, China) or Alexa Fluor 555 goat anti-mouse IgG for cercariae (1:100, Invitrogen). Images were acquired with a fluorescence microscope.

Antigen-specific antibody detection in the serum of *S. japonicum*-infected mice

The antibody assay was conducted on mice serum collected at different times after *S. japonicum* infection. ELISA was performed to determine antigen-specific total IgG, IgG1, and IgG2a antibody. The 96-well microtiter plates were coated with purified r*Sj-VAL*-1 protein (2.5 µg/ml) overnight at 4°C in 0.2 M carbonate buffer (pH 9.6). They were washed three times with PBS-containing 0.05% Tween-20 (PBS-T) and were then blocked with 1% of BSA in PBS-T at 37°C for 2 h. The plates were again washed three times and incubated with 100 µL/well of infected mouse serum in 1:100 dilution at 37°C for 2 h. Following three further washes with PBS-T, 100 µL/well of HRP-conjugated, goat anti-mouse IgG (1:30,000 dilution), IgG1 and IgG2a (1:3,000 dilution, Santa Cruz) were added and incubated for 1 h at 37°C. After washing, the plates were developed with tetramethylbenzidine (TMB) substrate (BD Biosciences Pharmingen) for 10 min. The reactions were stopped by 2 M H₂SO₄ and the absorbance was measured at 450 nm.

Results

Sequence analysis of the *Sj-VAL*-1 gene

The full-length cDNA of *Sj-VAL*-1 contained 666 bp encoding an ORF of 221 amino acids with a theoretical molecular mass of 24.34 kDa and a pI of 9.38, predicted by ProtParam. A short signal peptide predicted by the SignalP program, terminated between amino acids Ala25 and Trp26. Protein homology search of *Sj-VAL*-1 in the data-banks using BLAST_P showed 58% identities to venom allergen-like (VAL) 15 protein of *S. mansoni*, and 35% identities to Venom allergen 5 of a *Vespa crabro*, and 32% identities to GLIPR1-like protein 1 of *Homo sapiens*, and 32% identities to allergen of *Aedes aegypti*. Multiple sequence alignment of *Sj-VAL*-1 with the selected homologues and proteins identified as characteristic of the SCP family partly showed sequence conservation (Fig. 1). *Sj-VAL*-1 contained most of the invariant cysteine residues and the conserved motif HYTQ (residues136–139), which is shared in most members of VAL protein that is a hallmark

of VAL proteins described from plant-parasitic nematodes up to now (Hawdon et al. 1996).

Expression and immune recognition of r*Sj-VAL*-1

The recombinant pET-28a(+) plasmid contained the *Sj-VAL*-1 coding region was confirmed by sequencing and the r*Sj-VAL*-1 was expressed as a fusion protein with a 6 × His tag at the N-terminus induced by IPTG. The purified recombinant protein showed a single band in 12% SDS-PAGE with an approximate 26-kDa molecular weight (Fig. 2a). Western blotting indicated that r*Sj-VAL*-1 could be recognized by serum from *S. japonicum* naturally infected mouse, r*Sj-VAL*-1 antiserum, and mAb-His, while not recognized by pre-immunized mouse serum (Fig. 2b).

Stage specificity of *Sj-VAL*-1 expression

RT-PCR analysis revealed that the *Sj-VAL*-1 transcripts were expressed in eggs, cercariae, while not in male and female adult worms (Fig. 3a). The expression of *Sj-VAL*-1 protein was also examined by Western blotting. *Sj-VAL*-1 protein could be detected in eggs and egg ES products, also not in adult male and female worms. The band detected was about 34 kDa, which was a little larger than its theoretical molecular weight, indicating that the native *Sj-VAL*-1 may be glycosylated like most mammalian tetraspanins as reported previously (Boucheix and Rubinstein 2001). Confusingly, *Sj-VAL*-1 protein could not be detected in cercariae (data not shown). Maybe its expression in cercariae was too little to be detected by Western blotting.

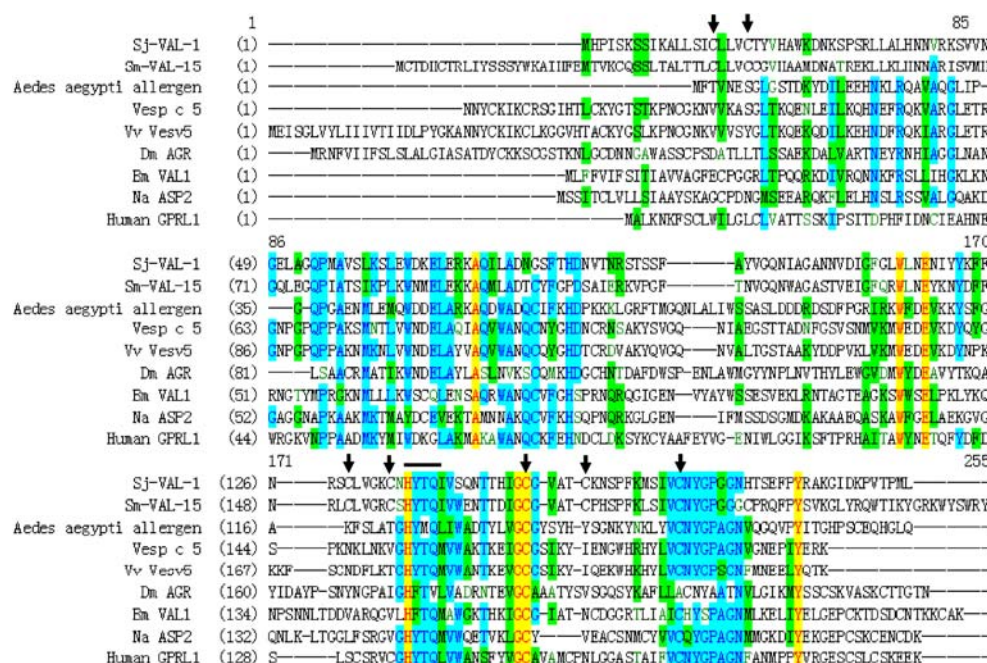
Immunolocalization of *Sj-VAL*-1 protein in *S. japonicum* eggs and cercariae

In immunofluorescence assays, *Sj-VAL*-1 protein was detectable in the fixed infected rabbit liver section and live cercariae (Fig. 4). *Sj-VAL*-1 was secreted from the eggs (Fig. 4d) and head gland and penetration glands of cercariae (Fig. 4h). No positive reaction was detected with the serum sample prior to immunization (Fig. 4b, f).

Immune responses of *S. japonicum* infected mice to *Sj-VAL*-1

Specific antibody levels in *S. japonicum*-infected mice were determined over time by ELISA using the r*Sj-VAL*-1 as antigen. As shown in Fig. 5, until 6 weeks after infection, a significant increase of antigen specific IgG emerged. And Th2-associated IgG1 antibody maintained within a higher levels contrast to Th1-associated IgG2a antibody. These results showed that *Sj-VAL*-1 could induce a predominated Th2 immune response in *S. japonicum*-infected mice.

Fig. 1 Multiple sequence alignment of *S. japonicum* *Sj-VAL-1* amino acid sequences with homologues from other trematode, nematodes, etc. Accession numbers of the sequences presented are as follows: SM-VAL-15 (accession No. XP_002582174), *Aedes aegypti* allergen (accession No. XP_001655867), Vesp c 5 (accession No. P35781), Vv Vesv5 (accession No. AAA30333), Dm AGR (accession No. AAB92563), Bm VAL1 (accession No. AAK12274), Na ASP2 (accession No. AAP41952), Human GPRL1 (accession No. Q6UWM5)



Discussion

The SCP family has been found in many parasites (Anand et al. 2007; Chalmers et al. 2008; Hawdon et al. 1996). The widespread occurrence of SCP domain-containing proteins suggests that they may be involved in many important biological processes. It has been reported that they are involved in infection and transition to parasitism, as they are very abundant in the excretory–secretory (ES) material of L3 worms (Hawdon et al. 1996, 1999); in pathogenesis based on the angiogenic capacity of *O. volvulus* ASP (Tawe et al. 2000) and the homology with venom allergens from vespid wasps (Ag5) and fire ants (Ag3), which are potent

allergens (Lu et al. 1993), in reproduction for their male enrichment (Reinke et al. 2004; Visser et al. 2008) and in the establishment and survival of the parasite in its host (Hawdon et al. 1995). However, the precise function of this large protein family remains unclear.

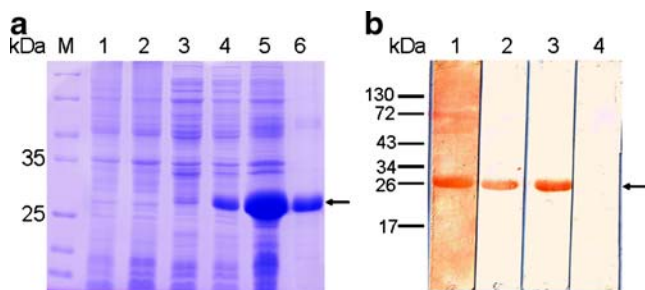


Fig. 2 Expression, purification, and immune recognition of rSj-VAL-1. **a** Expression and purification of rSj-VAL-1 by 12% SDS-PAGE. Protein molecular weight markers (M), lysate of *E. coli* with pET28a(+) before induction (lane 1) and after induction (lane 2), lysate of *E. coli* with pET28a(+)-*Sj-VAL-1* before induction (lane 3) and after induction (lane 4), precipitant of lysate of *E. coli* with pET28a(+)-*Sj-VAL-1* after induction (lane 5), rSj-VAL-1 after purification (lane 6). **b** Western blot analysis of rSj-VAL-1. rSj-VAL-1 probed with serum from mouse infected with *S. japonicum* (lane 1), with rSj-VAL-1 antiserum (lane 2), with mAb-His (lane 3), and pre-immunized mouse serum (lane 4)

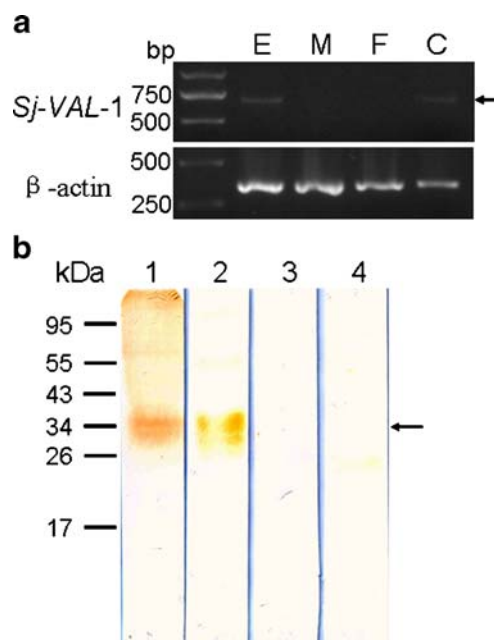


Fig. 3 Detection of transcription and expression of *Sj-VAL-1* at various developmental stages. **a** RT-PCR analysis of *Sj-VAL-1* transcription in major definitive host stages. Lane E, eggs; lane M, male adult worms; lane F, female adult worms; lane C, cercariae. **b** Western blot analysis of *Sj-VAL-1* expression. SEA (lane 1), egg ES products (lane 2), adult male (lane 3), and female (lane 4) worm antigens

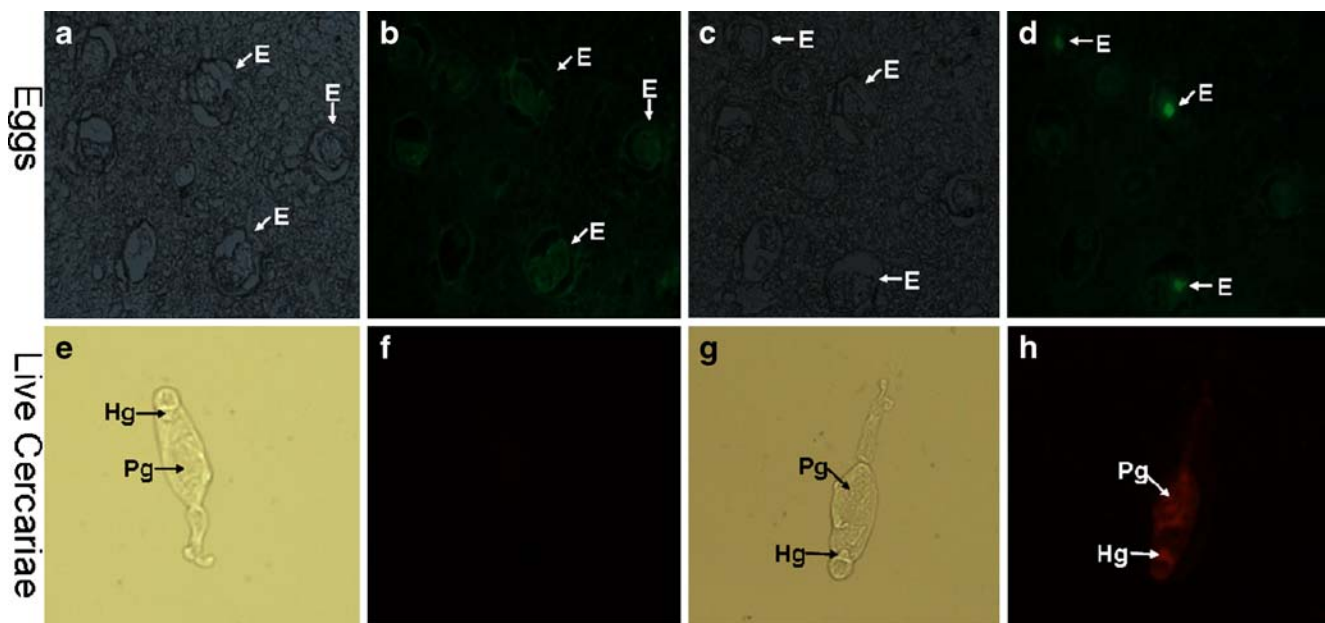


Fig. 4 Immunolocalization of *Sj-VAL-1* protein in *S. japonicum* eggs and cercariae. **a, b, e, f** Detected with pre-immunized mouse serum. **c, d, g, h** Detected with r*Sj-VAL-1* antiserum. *E*, egg; *Hg*, head gland; *Pg*, penetration glands. Magnification was 200×

For members of SCP family, sequence variations, transcription, and expression patterns differences have been observed (Hawdon et al. 1999; Zhan et al. 2003). The reason for this is unclear, but Yatsuda showed that these variations can have an effect on the immunogenicity (Yatsuda et al. 2002). In the present study, we cloned a VAL protein from *S. japonicum*. The transcription and expression patterns of *Sj-VAL-1* were also different. RT-PCR found it transcribed mainly in cercariae and eggs stages. The proteins were mainly secreted by eggs trapped in host organs and

interplayed directly with the host. Native *Sj-VAL-1* must be a glycoprotein because its molecular weight was larger than theoretical molecular weight (Fig. 3b) and as predicted by MOTIFSCAN with seven N-glycosylation sites.

Sj-VAL-1 could evoke intensive immune responses with host in the eggs stage but not in the cercariae, schistosomulum, or adult worms stages because of the lower specific IgG in the early stages of *S. japonicum* infected mouse. As was shown in Fig. 5, 6 weeks after infection, until the mix adult worms matured and began laying eggs, *Sj-VAL-1* specific IgG significantly increased and was maintained at a high level because of the continuous secretion of *Sj-VAL-1* by eggs. Furthermore, a predominantly Th2 reaction was induced for the higher IgG1 antibody contrast to IgG2a. It is undoubted that the schistosome eggs were the principal cause of pathology in schistosomiasis (Pearce and MacDonald 2002). Animal experimental findings showed that a predominantly Th1 reaction in the early stages of infection shifts to an egg-induced Th2-biased profile, and imbalances between these responses lead to severe lesions (Pearce 2005). Determining whether *Sj-VAL-1* is involved in this pathology process or has other roles during the life cycle of *S. japonicum* needs further studies.

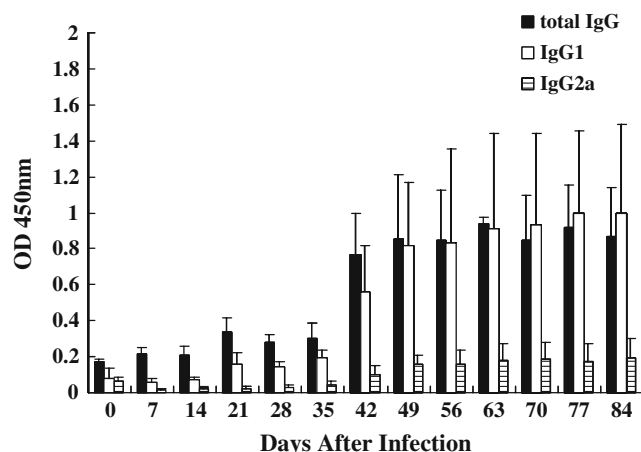


Fig. 5 Total IgG, IgG1, and IgG2a antibody responses of *S. japonicum* infected BALB/c mice to r*Sj-VAL-1*

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