# *Schistosoma mansoni* Tetraspanning Orphan Receptor - SmTOR

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**This thesis is dedicated to my parents and my brother.**

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## GENERAL SUMMARY

Schistosome parasites need to adapt to survive at various points of their complex life cycle. In the vertebrate definitive host, this involves the avoidance of the host immune system in general and the battle against the host complement system participating in the first line of immune defenses against the invading parasite in particular. There are several complement regulators found on schistosomes. We are interested in complement regulation at the level of the formation of the C3 convertase by a complement C2 binding receptor. This C2 binding receptor was first characterised in one of the human schistosoma species *S. haematobium* and denominated ShTOR for trispanning orphan receptor. After the *S. mansoni* genome had been sequenced, we had a closer look at the ShTOR receptor homologue in this species.

The aims of the project were:

1. To characterise the *S. haematobium* TOR receptor homologue SmTOR in *S. mansoni* (PART I)

2. To test if the first extracellular domain 1 of SmTOR binds C2 and to define its antigenicity (PART II)

3. To define the immunogenicity of SmTORed1 in mice and test it as a vaccine candidate against schistosomiasis in a murine vaccination and challenge model (PART III)

In line with the structure of the project outline presented above, the work presented in this thesis can be summarised as follows:

1. We found that SmTOR is a tetraspanning receptor expressed in the tegumental membranes of schistsosomes. As compared to the truncated receptor versions described before, it possesses a longer extracellular domain 1 still comprising the C2 binding motif. Its expression was highest in

the infectious stage of the parasite, in the *S. mansoni* cercariae. SmTOR might play an important role in skin penetration of *S. mansoni* larvae and it is an interesting target for vaccination since it is an early antigen on schistosoma cercariae and has a complement inhibitory activity.

2. We overexpressed SmTOR extracellular domain 1 (rSmTORed1) in *E. coli*, which was purified by immobilised metal affinity chromatography. We were able to show that full length recombinant SmTORed1 binds C2. We moreover detected specific antibodies against rSmTORed1 in sera of patients infected with *S. mansoni* and also in some normal human sera. Specificity of antibody to rSmTORed1 was ensured by pre-incubation of sera with the Halo-tagged version of SmTORed1 immobilised on a solid support.
SmTORed1 N-terminally fused to HaloTag and the corresponding control constructs were produced in *E. coli* as well.

3. We tested the immunogenicity of rSmTORed1 in two different mouse strains, BALB/c and C57BL/6 using as adjuvants muramyl dipeptide (MDP) and Complete/Incomplete Freund's adjuvant (CFA/IFA). BALB/c mice immunised with rSmTORed1 in CFA/IFA generated the highest titer of specific antibodies to rSmTORed1 and were subsequently tested in an immunisation challenge experimental setup. For this, immunised mice were infected with *S. mansoni* cercariae and status of infection assessed by adult worm count. Immunised mice showed a 60 % reduction of worm burden when compared to the two control groups.

## GENERAL INTRODUCTION

Helminths are multicellular eukaryotic parasites that are able to ensconce themselves in the human host for decades (*1*). Parasitic helminth infections account for disability and morbidity amongst the 1.2 billion people affected by these type of neglected tropical diseases (NTDs) (*2*). In fact, NTDs seem to not only be neglected by the majority of people not at risk due to their privileged economic status, but rather forgotten besides high mortality diseases such as HIV/AIDS, malaria and tuberculosis. NTDs primarily do affect the population of developing countries, where they have a non-negligible impact on child development, pregnancy outcome, worker productivity and malaria and HIV/AIDS co-infections (*3*). NTDs include chronic parasitic and bacterial infections. Schistosomes are parasitic helminths belonging to the phylum of platyhelminths (*4*). They were first discovered by Theodor Bilharz in 1815 (*5*), but must have infected their human hosts during thousands of years, as calcified eggs had been discovered already in mummies (*6*). Their persistent existence over thousands of years might be one of the reasons, why during co-evolution with their human host, schistosomes developed into well-adopted parasites very well capable to escape the host immune response and to settle down in such an unfriendly environment as the human venous system. Schistosomiasis affects more than 207 million people worldwide with an estimated number of 700 million people at risk in 74 endemic countries (*7*). Although the infection can be treated by using chemotherapeutics, it is hardly possible to influence strongly the risk of re-infection due to the fact that it is difficult to control the frequency of cercariae in contaminated waterbodies (*8*).

### *Life Cycle of Schistosomes*

The infectious stage of *Schistosoma*, the so called cercariae, rest more or less transiently in a fresh-water environment depending on the strain and are only prompted to move in response to certain stimuli (*9*). These presumably include the light shade play and water turbulence, as well as chemical cues and a thermostatic gradient that cercariae are responding

to (*10*). Medium-chain free fatty acids such as linoleic acid in the human skin act as signal for skin invasion and the cercariae start to secrete gland contents from the acetabular gland complex (*11*). The post-acetabular glands facilitate the attachement of the cercariae to the skin surface by releasing a mucous-like substance (*12*). In addition to the acetabular glands, the head gland and the sub-tegumental cell bodies play a role in the process of host invasion and subsequent parasite transformation, that is the transformation of cercariae into schistosomulae (*10*). The outermost layer of the skin though, the stratum corneum, represents no real barrier to the cercariae as in a hydrated environment the lipid lipid interactions are lost. Gland secretions containing cercarial proteases help to degrade the desmosome-linked cells of the stratum spinosum, the basement membrane contents and the subjacent dermal layers (*11, 13*). Schistosome cercariae infecting humans penetrate wrinkles, smooth skin and hair follicles with 60 percent of them still having the tails attached to the cercarial bodies (*14*). Transformation of cercariae into schistosomulae involves loss of the cercarial bifurcated tails, shedding of the glycocalyx and the re-organisation of the trilaminate surface membrane into a heptalaminate membrane (*12*). Few infective larvae are capable of reaching the dermis of human skin within 10 minutes (*15*), but the majority of the transformed schistosomulae that do not die during skin penetration reach the dermis within 48 hours (*9*). After migration through the extracellular dermis, schistosomulae enter the vascular space via a small venule or a lymphatic vessel and make their way to the lungs. They further migrate in the blood to the portal venous system. Schistosomulae mature in the portal vein for 4 - 6 weeks whereon females mate with their male counterpart in order to form the typical worm pairs (*8*). The longer thinner female is held in a groove of the male's body. As such, they migrate to superior mesenteric veins (*S. mansoni*), the inferior mesenteric and superior hemorrhoidal veins (*S. japonicum*) or the vesical plexus and the veins draining the ureters (in the case of *S. haematobium*) (*16*). The female starts to produce hundreds and thousands of eggs that penetrate the surrounding tissues and end up in the intestine or the bladder. Eggs are excreted in the urine or feces and the ciliated miracidium larvae hatch when the eggs are in contact with water. The miracidiae thereafter infect the intermediate host, specific freshwater snails of

the *Biomphalaria* (*S. mansoni*), *Bulinus* (*S. haematobium*) or *Oncomelania* genus (*S. japonicum*) and undergo asexual reproduction that generates mother and then daughter sporocysts. The daughter sporocysts give rise to cercariae that are released into the water. With this step, the schistosomes complete their life cycle.

*Immunopathology*

The pathology of schistosomiasis is manifesting in two different phases of the disease, in the acute phase and during the chronic phase.

Skin reactions at the site of penetration by the cercariae develop within a few hours after infection and especially after primary infections. The dermatitis is similar to the so-called swimmer's itch that occurs in sensitised persons infected with animal trematodes (*17*).

Acute schistosomiasis is a febrile illness occuring a few weeks to months after infections and is also referred to as Katayama fever (*8*). Symptoms at initial presentation include myalgia, headache, cough, abdominal pain and fever (*18*). Patients typically suffer from eosinophilia due to a systemic hypersensitivity reaction to antigens appearing visible to the host immune system at the time point of oviposition.

Chronic schistosomiasis is caused by the schistosome eggs that get trapped in the host tissue during perivesical or periintestinal migration. It is characterised by formation of granulomatous lesions at the site of egg accumulation resulting in hepatic and intestinal schistosomiasis, urinary disease or ectopic schistosomiasis such as pulmonary, genital and neuro-schistosomiasis (*8*).

Gastrointestinal and liver disease cause persinusoidal inflammation in the liver and hyperplasia, ulceration, microabcess formation and polyposis in the gut wall in line with the granulomatous inflammatory response due to eggs in the liver (*S. mansoni* and *S. japonicum*) and the genitourinary tract (*S. haematobium*). More severe pathology such as occlusion of the portal veins, portal hypertension and gastrointestinal bleeding develops in the course of fibrotic or chronic hepatic schistosomiasis (*8*). Excess of extracellular matrix deposited in periovular granuloma and periportal fibrosis

are the two main presentations of fibrosis in hepatic schisotomiasis. Life threatening conditions involve the emergence of portal hypertension as a consequence of the altered vascular architecture of the liver, splenomegaly and porto-systemic collateral circulation and subsequently the rupture of oesophageal varices where the host dyes of acute anemia (*19*).

Urinary schistosomiasis is caused in response to infection with *S. haematobium*. Symptoms include dysuria and hematuria, later on proteinuria and disease progression is accompanied with calcifications in the bladder and obstruction of the ureter (*16*). There is evidence of environmental factors promoting bladder cancer association infection including schistosomiasis as a major risk factor (*20*).

*Host Parasite Interactions*

Schistosomes at various stages in their life cycle have to defend themselves either against attack by the innate or adaptive immune system. When schistosome larvae penetrate the human skin, they coat themselves in blood group antigens supposably as a protective measure (*21*). Cercariae in contrast to schistosomulae are susceptible to complement attack and activation of complement was dependent on the alternative pathway but independent of antibody. Cercariae loose their glycocalyx and their tail when transforming into schistosomulae, which renders them less susceptible to complement attack (*22*). On the other hand, schistosomulae are vulnerable to oxidative stress generated by host phagocytes (*23*). Adult worms however have the capacity for oxidant detoxification due to the higher level of antioxidant enzymes and glutathione within their tegument and gut epithelium (*24, 25*).

Other than defending themselves against host immunity parasites are also capable circumventing damage and counterattack the host by immunomodulation. For example cercariae might inhibit T-cell activation by blocking potassium channel activity (*26*), interfere with TLR signaling (*27*) or modify dermal immunity by secreting sperm coat domain proteins that have similarities with host chemokines (19, 24). *S. mansoni* egg translocation from the portal capillaries to the gut lumen depends on the host immune response

(*28*), female worms produce eggs in a host TNF dependent manner (*29*) and adult worm growth and development might depend on host TGFβ (*30*).

One other important aspect beside the worm's attack and defense strategies is the subversion of immune attack on the parasite surface. There are antibodies and complement factors on the adult parasite surface (*31-33*) and complement factors are found in the human skin after invasion of cercariae (*34*), which suggests there might be a fight going on against the human complement system at the host parasite interface.

*The Complement System*

Adult parasites are constantly facing attack by the complement, a system of more than 30 glycoproteins in plasma and on cell membranes that form a proteolytic cascade once activated by one of the three possible ways; the classical pathway (CP), alternative pathway (AP) or mannose-binding lectin pathway (MBL-P) of complement activation (*16*). All three pathways converge at the level of the C3 convertase formation and subsequent proteolytic cleavages lead to the formation of a membrane attack complex C5b-C9 leading to the direct killing of pathogens. Elimination of pathogens is also triggered by the generation of covalently bound cleavage products of C3 and C4 in a process called opsonisation, marking pathogens for recognition by macrophages and neutrophils thereby facilitating their phagocytosis (*35*). Furthermore, complement plays a bridging function in linking the innate and adaptive immune system by instructing and stimulating the acquired immune response (*36*).

CP is initiated by the binding of C1q to IgG or IgM bearing immune complexes or to a variety of ligands such as a C-reactive protein, pentraxin and a variety of structurally different target molecules including parasites (*37*). An activation signal is then transmitted to C1s-C1r-C1r-C1s tetramer assembled of two serine proteases C1r and C1s (*38*), more precisely to C1r that undergoes autoactivation and cleaves C1s. Activated C1s cleaves C4 into C4b and C4a and then C2 bound to C4b generating the C4bC2a enzyme complex, the C3 convertase of the CP (*39*).

MBL-P is activated in an analogous way, when MBL or ficolins in

complex with their serine proteases MASP-1/MASP-2 get activated by binding to carbohydrates on microorganisms and auto-activated MASP-2 so cleaves its downstream targets C4 and C2.

The AP of complement is initiated when C3 gets activated to form C3($H_2O$) with a internal thioester of C3 hydrolysed by water in a so-called tick-over process (*40*). C3($H_2O$) has an altered conformation that allows the binding of factor B (FB), its cleavage and the formation of the fluid phase AP convertase C3($H_2O$)Bb (*41*). Formation of AP convertase bound to cell surface macromolecules happens when activated C3b with its fully exposed thioester is generated in an unknown process (*42, 43*). This thioester bond is displaced by nucleophilic attack by an amine or hydroxyl group on the pathogen surface thereby covalently attaching C3b to its surface (*43*). Bound C3b allows the binding of FB that undergoes a conformational change rendering it susceptible for cleavage by factor D (FD). FD cleavage of C3b bound FB generates the AP C3 convertase C3bBb.

The complement system is regulated in a tight way by membrane-bound and soluble complement regulators (all reviewed in (*44*)). The list of membrane-bound complement regulators comprise complement receptor 1 (CR1, CD35), decay-accelarating factor (DAF, CD55), membrane cofactor protein (MCP, CD46) and protectin (CD59). Fluid-phase complement regulators can be grouped according to their point of action: One group consisting of factor H (FH), factor H like protein 1 (FHL1) and properdin regulate the AP. Regulators of the CP and MBL pathways are C4b binding protein (C4BP) and C1 inhibitor (C1INH). Complement factor H related protein 1 (CFHR1), clusterin and vitronectin all interfere with the terminal pathway of the complement cascade.

CR1, DAF and MCP all act as decay accelerating factors of C3 and C5 convertases. In addition, CR1 and MCP are cofactors for the serum protease I, which degrades C3b and C4b to fragments that are no more functional. CD59 regulates inhibits terminal complement complex formation.

C1INH is a serine protease inhibitor targeting C1r, C1s and MASP2. FH, FHL and C4BP act on the AP or CP C3 convertase decay and all of them as cofactor for FI. Properdin stabilises the AP convertases. CFHR1 inhibits the C5 convertase and membrane attack complex formation. Clusterin and

vitronectin as well prevent MAC insertion into the membrane.

*Schistosomes and Complement*

It is likely, that all three pathways of complement activation are activated on the schistosome surface (*45*): multiple isoforms of IgG and IgM and IgA have been detected on the adult tegument (*46, 47*) and MBL pathway was observed to be activated *in vitro* (*48*). Worms and schistosomes that are insensitive against complement attack got vulnerable after trypsin treatment (*49, 50*). This suggests the existence of complement regulatory molecules on the parasite as a defense mechanism.

Paramyosin (a homologue of human CD59) has been assigned with a multiple role in complement regulation on the surface of schistosomes. An Fc receptor has been postulated to be at the parasite surface (*51*) that captures host IgG while loosen its capacity to activate complement. At the same time, the receptor has been suggested to function as C1INH (*52*) and blocking the complement cascade at a late stage by binding complement C8 and C9 (*53, 54*). However, there is a debate about paramyosin even being present or not on the schistosome surface (*45*).

A C3 receptor has been characterised on the *S. mansoni* tegument regulating all arms of the complement system (*55*) and adult schistosomes and schistosomulae somehow are capable of acquiring GPI-anchored host DAF (*56, 57*).

We were interested in a C2 receptor highest expressed on the tegument of complement-sensitive *S. mansoni* cercariae (*58*), interfering with the formation of the C3 convertase (*59, 60*).

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## PART I: *Schistosoma mansoni* TOR is a tetraspanning orphan receptor on the parasite surface

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ABSTRACT

A trispanning orphan receptor (TOR) has been described in *Schistosoma haematobium* and *S. mansoni*. Here we report the complete molecular organisation of the *S. mansoni* TOR gene, also known as SmCRIT (complement C2 receptor inhibitor trispanning). The SmTOR gene consists of four exons and three introns as shown by cloning the single exons from *S. mansoni* genomic DNA and the corresponding cDNA from the larval stage (cercaria) and the adult worm. The SmTOR ORF consists of 1260 bp and is longer than previously reported, with a fourth trans-membrane domain (proposed new name: Tetraspanning Orphan Receptor), and with, however, an unchanged C2-binding domain on the extracellular domain 1 (ed1). This domain differs in *S. japonicum*. A protein at the approximate expected molecular weight (55 kDa) was detected in adult worm extracts with polyclonal and monoclonal antibodies, and found to be expressed on the tegumental surface of cercariae.

# INTRODUCTION

Schistosomiasis is a parasitic infection also known as bilharzia named after Theodor Bilharz who first described it (*1*). There are five species of schistosoma that are known to infect humans by contact with its larval stage, the cercariae: *Schistosoma mekongi, S. intercalatum, S. mansoni, S. japonicum* and *S. haematobium*, the latter three being the main schistosome species that affect humans (*2*). Adult worm pairs reside in host veins and produce eggs which penetrate the tissue. They end up excreted with feces where they complete the schistosoma life cycle by hatching and re-infecting fresh-water snails as intermediate hosts (*1*). Between the mid-1990s and 2003 the estimated number of people at risk from schistosomiasis increased from 702 million to 779 million and the estimated number of individuals infected increased from 193 millions to 207 million (*3*). The treatment of choice is the chemotherapeutic agent praziquantel. However, drug treatment alone might not be sufficient and a vaccine-linked chemotherapy to control schisotomiasis is recommended (*4*). Naturally acquired immunity or vaccination in animals strongly diminishes the pathology associated with schistosome infection. There are efforts to develop a vaccine against schistosomiasis. Radiation-attenuated cercariae induced a high level of protection in animal models (*5, 6*) and naturally resistant population groups exist (*7, 8*), which suggest that development of an effective vaccine is likely to be possible (*9*). There is a list of recombinant proteins that correlate with resistance in human studies and/or have shown efficacy in animal models (*9*). Among these promising vaccine candidates are the tetraspanins SmTSP-1 and SmTSP-2, that are both recognised by IgG1 and IgG3 from putatively resistant individuals, SmTSP-2 providing high level of protection in the mouse vaccination model in addition (*10*). Another candidate, Fatty Acid-Binding Protein (FABP)-Sm14 is at the stage of planned clinical trials after scale-up and industrial production processes have been put in place (*11*).

The host complement system participates in the first line of immune defenses against the invading parasite. Of the three pathways of complement activation, the alternative pathway was shown to attack schistosomula (*12, 13*) and *S. mansoni* adult worms (*14, 15*). Schistosomes are sensitive to

killing by complement, but lose their sensitivity with loss of the glycocalyx and maturation. Partial tryptic digestion of adult worm tegumental proteins rendered them sensitive to complement attack as shown for *S. mansoni* by Fishelson's group (*16*). The mannose binding lectin pathway has also been shown to be activated (*17*), but there has been no consensus regarding the activation of the classical pathway. Some researchers find IgG (different subtypes) and IgM deposits at the parasite surface and some do not (*18-20*). Identification of host IgG1, IgG3, IgM, C3 degradation products (*21*) and C4 (*22*), but no known proteins with homology to Fc receptors by proteomic analysis of the adult worm tegument, provide a strong argument for the activation of classical pathway (*22*). In addition, there have been reports of complement regulators being present on schistosomes including C1q binding proteins, surface C3 receptor, host acquired DAF and SCIP-1/paramyosin (*23*), which might regulate the terminal membrane attack complex insertion, although this remains a matter of debate (*24*).

A specific surface receptor for C2, the Schistosoma trispanning orphan receptor (TOR) was described by Inal some time ago (*25*). In *S. haematobium* and *S. mansoni* TOR was found to be a 32 kDa trans-membrane protein located at the tegumental surface of adult worms and was also expressed in the larval stage (cercaria). Inal showed that a short amino acid sequence of the ShTOR extracellular domain 1 (ed1) binds C2 resulting in a competitive inhibition of the binding of C2 to C4b. In addition it inhibits the cleavage of C2 by C1s. Further experiments indicated that this sequence was a strong inhibitor of classical pathway activation. This sequence, corresponding to the C-terminal 11 amino acids of ed1 (termed H17 peptide), has homologies with a specific sequence of the beta chain of C4, which may explain the competitive binding between H17 and C4 for C2 (*26, 27*). Hui et al. (*28*) defined more precisely the binding site of H17 to be the vWFA domain of C2. Therefore, TOR was renamed CRIT for "complement C2 receptor inhibitor trispanning" (*29, 30*). Recently, H17 was shown to interfere also with the formation of the alternative pathway C3 convertase by binding to FB (*28, 31*). Evidently, TOR might be a central element for schistosomes to escape innate immunity.

Here we report the exon/intron structure of the SmTOR gene. Four exons were amplified and sequenced from *S. mansoni* genomic DNA based on database analysis of the *S. mansoni* genome and comparing it with *S. japonicum* TOR (SjTOR) cDNA. The full-length construct was amplified from the adult worm and cercaria cDNA preparations. Further evidence for expression of the TOR protein was gained by Western blotting with *S. mansoni* proteins and probing these with antibodies directed against parts of its extracellular domains. Based on these results we propose a new structure of SmTOR/CRIT.

## MATERIALS AND METHODS

### *Alignment of* Schistosoma japonicum *cDNA with* Schistosoma mansoni *genome: derivation of SmTOR exon/intron gene organisation*

SjTOR cDNA (PubMed accession number AY814912; http:// www.ncbi.nlm.nih.gov/) was aligned with *S. mansoni* GeneDB database entry Smp_093840 (http://www.genedb.org/genedb/smansoni/) designated as a putative trispanning orphan receptor gene. The resulting overlapping sequences served as a basis to define SmTOR exon/intron boundaries.

### *PCR amplification and sequencing of SmTOR fragments from* S. mansoni *genomic DNA*

*S. mansoni* genomic DNA was prepared from cercariae-infected water. Cercariae in suspension were washed by pelleting 5 min at 3000 rpm and resuspending in 1 x PBS and an additional centrifugation step as before. The pellet was resuspended in 1 x TE (Fluka 86377)/100 mM NaCl and snap frozen in liquid nitrogen. After thawing, 20 % SDS (Fluka 05030) and Proteinase K (Fluka 82456) were added to final concentration of 1 % and 1 mg/ml respectively. The mixture was incubated at 60 °C over night. One volume of TE saturated phenol/chloroform was added to the sample and mixed by inversion for 15 min. After spinning at full speed in a microfuge, the aqueous supernatant was transferred to a new tube and the extraction step repeated twice, but using chloroform only in the cycle. DNA in the aqueous phase was then precipitated by adding 1/10 v/v NaOAc pH 5.5 overlaid with 2.5 volumes of ethanol and incubating at -20 °C over night. DNA was pelleted at full speed, air-dried and resuspended in 1 x TE. Primers (Microsynth) flanking the putative SmTOR exons were designed according to the exon/intron map (Table 1). PCR was performed with Taq PCR core kit (Quiagen) using 0.75 mM specific primers and 30 ng *S. mansoni* genomic DNA as template. The PCR program was 95 °C for 5 min, then 35 cycles of 95 °C for 45 s, 59 °C for 1 min, 72 °C for 1 min followed by a final extension step of 72 °C for 15 min. Reaction products were separated by 1% agarose

gel electrophoresis, excised bands were purified (QIAquick® PCR purification kit) and cloned into a TOPO vector (Invitrogen) for sequencing. Blanks for each PCR reaction using water only were negative (not shown). All PCR products were sequenced in both directions, using plasmid preparations from three different clones respectively.

Table 1. Primer list used for amplification of single exons of SmTOR and amplifications from cDNA. Expected fragment lengths are indicated for primer pairs used to amplify single exons. Numbers used in the primer names indicate its 5' annealing position within the exon or the flanking intron, preceded by a positive or negative sign respectively, if not annealing at the ends of the exons of interest due to issues of melting temperature when designing.

| primer | sequence 5' → 3' | expecte product (bp) |
|---|---|---|
| SmTOR_ex1_-15_fwd | GTCTCGTTAACTGTCGTTGTTGAATAATTG | 246 bp |
| SmTOR_ex1_+12_rev | TCTTGTCCTCTGATGGGTCTGTATTTCCAT | 246 bp |
| SmTOR_ex2_-12_fwd | TTCTACCCTAGGTTTTTATGTTTTCTCGAC | 538 bp |
| SmTOR_ex2_rev | TTTTGTGTGAATCATCAAGCGTAGATCTGA | 538 bp |
| SmTOR_ex3_fwd | ACGGGGCCTATTTACATCAAATCTACA | 325 bp |
| SmTOR_ex3_rev | CTCATACTTTGGTAGATCGTTAGCTGG | 325 bp |
| SmTOR_exon4_7_fwd | TATTGAAAATTCCGGCAAATGCCTACGCTC | 248 bp |
| SmTOR_exon4_rev | TTAGCAAGAAGAGTGAGCATTCGATGGTGC | 248 bp |
| SmTPI_fwd | GTTGGGGGGAACTGGAAAATGAA | 219 bp |
| SmTPI_rev | TTCTCCGGTGAATGCACCCTTTG | 219 bp |
| SmTOR_ex1_-15_fwd | GTCTCGTTAACTGTCGTTGTTGAATAATTG | 291 bp |
| SmTOR_ex2_+33_rev | ACAAGACGAAAGAGAGTCGAGAAAACATAA | 291 bp |

*RNA isolation from* S. mansoni *adult worms or cercariae and full length cDNA preparation*

*S. mansoni* RNA was isolated from adult worm preparations of *S. mansoni* (Liberian strain, kindly donated by Dr. J. Chollet, STI Basel) isolated from NMRI mice or cercariae. Worm pairs were briefly rinsed with 1 x PBS and preserved in RNA*later*® reagent (Ambion). Worm tissue or cercariae were homogenised by mechanical disruption with *Molecular* Grinding Resin™ (G-Biosciences) resuspended in lysis buffer RLT (AllPrep™ DNA/RNA/Protein extraction kit, Quiagen). After removal of resin and cell debris by

centrifugation at 10 000 rpm, for 5 min at 4 °C, the homogenate was applied on a Quiagen AllPrep column and RNA extracted according to the manufacturers protocol.

*RT-PCR and cDNA alignment SmTOR/SjTOR*

Single strand cDNA synthesis was performed using random hexamers, oligo dT primers or gene specific primer SmTOR_exon4_rev (Table 1). 50 ng of RNA was reverse transcribed per reaction with SuperScript$^{TM}$ III First-Strand Synthesis System for RT-PCR (Invitrogen). Using the transcribed single stranded cDNAs as a template, the SmTOR transcribed sequence was amplified with gene specific primer pair SmTOR_ex1_-15_fwd and SmTOR_exon4_rev (Table 1) using the same cycling conditions described above. Reaction mixtures were run on a 1% agarose gel and the single bands purified, cloned and analysed as described above. SmTOR ORF on sequenced cDNA was aligned with SjTOR ORF on cDNA entry (AY814912) using EMBOSS pairwise alignment algorithm (http://www.ebi.ac.uk/emboss/align/).

*Real-time quantitative PCR analysis (qPCR)*

Total RNA was isolated from *S. mansoni* cercaria, schistosomula, adult worm pairs, eggs and miracidia as described above. Eggs and miracidia were isolated as reported before (*32*). Schistosomula were generated by *in vitro* transformation (*33*). cDNA was generated as described above and 1 ml per reaction was used performing qPCR on a ABI 7900 (Applied Biosystems) instrument using SYBR® green as a fluorescence dye (*Power* SYBR® Green PCR Master Mix, Applied Biosystems). Primers targeting a 219 bp region of the constitutively expressed SmTPI (triose phosphate isomerase) (*34*) and primers for used for SmTOR (SmTOR_ex1_-15_fwd and SmTOR_ex2_+33_rev) are listed in Table 1. Amplification of contaminating genomic DNA was avoided by using sets of primers located in different exons. Results were evaluated using the $2^{-\Delta\Delta C}{}_T$ method (*35*).

*Protein sequence alignment* S. japonicum *and* S. mansoni *TOR and secondary structure prediction of SmTOR*

Molecular weight analysis was performed using the EMBOSS Pepstats program at the European Bioinformatics Institute web site (http:// www.ebi.ac.uk/emboss/pepinfo). Proteins were aligned using EMBOSS pairwise alignment algorithm (http://www.ebi.ac.uk/emboss/align/). TopPred was used for transmembrane prediction analysis (*36*) using upper cutoff hydrophobicity values. Secondary structure prediction analysis was performed using PSIPREDView (*37, 38*). The signal sequence prediction was performed using SignalP 3.0 server (http://cbs.dtu.dk/services/SignalP).

*Adult worm tegument membrane preparations and Western blot analysis*

Tegument surface membranes of *S. mansoni* adult worms were prepared as done before applying freeze thaw method followed by vortex pulses in order to strip the parasites (*39, 40*). Samples were run on a standard 12% SDS PAGE acrylamide/bisacrylamide (30%/ 0.8%, BioRad) gel and proteins then transferred to a nitrocellulose membrane (162-0115, BioRad). After blotting, the membrane was stained with Ponceau red. Subsequently, the membrane was blocked in 1 x PBS /0.05% Tween20 (Sigma)/ 5% milk (170-6404 BioRad) for an hour at RT. Incubations with primary and secondary antibodies described below were done in 1 x PBS/Tween 0.05%/ 1% blotting milk (BioRad) with 3 washing steps of 5 min shaking at RT and before developing the blot. Two polyclonal antibodies generated respectively against SmTOR peptide ed1 $NH_2$-MSPSLVSYTQKNERGSHEVKIKHFSP-COOH (*27*) and against ShCRIT peptide ed2 $NH_2$-SSTSDIRLVIHTKTGPIYIKST-COOH 1:1000 were used in PBS/T 1:1000 (*30*), followed by goat anti-rabbit IgG HRP coupled (BioRad, #170-6515). The blot was developed using the ECL™ Western blotting detection system (Amersham Biosciences). Alternatively, a human monoclonal antibody against SmTOR peptide ed1 isolated from a human monoclonal antibody library (HuCAL) was used at a dilution of 1:1000 (2.52 mg/ml stock) in PBS/T (AbyD04644.1, AbD Serotec, Martinsried). Mouse anti Histidine-tag:HRP (MCA1396P, Serotec) 1:3000 in PBS/T was

used as secondary antibody and the blot revealed as described above. The antibody was blocked by preincubation of AbyD0644.1 with a 100-fold molar excess of ed1 peptide in PBS/T for 60 min, RT and subsequent steps were performed as described above.

*Electron microscopy*

Cercariae were fixed in 3% parafolmadehyde and 0.5% glutaraldehyde in 10 mM PBS (pH 7.4). After washing with PBS, they were treated with 0.5% $OsO_4$ for 30 min followed by dehydration in a series of graded ethanol solutions and embedding in LR White resin at 60 °C. Ultrathin sections (60 nm) were cut on an UltracutE Leica ultramicrotome and collected on copper 200 mesh grids. Grids were blocked in PBS / 2% BSA for 2 x 5 min. Polyclonal anti-ed1 or anti-ed2 antibodies described before were diluted in blocking buffer (1:50) and pre-immune serum was used as control. After incubation with primary antibodies for 2 h, grids were washed twice for 5 min with blocking buffer and incubated for 1 h with goat anti-rabbit IgG (EM-GAHL10, British BioCell Laboratories) diluted 1:20 in blocking buffer. Final washings were performed followed by staining with 6% uranyl acetate, for 1 h, and then Millonigs lead acetate for 2 min. The sections were then dried, examined and photographed using a Philips Morgani transmission electron microscope.

*Cryosections and immunolocalisation*

Thin sections (9 mM) of OCT embedded frozen *S. mansoni* cercariae were cut on a cryostat (Microm HM 560) and fixed in ice-cold methanol for 10 min. After blocking in 1 x PBS/ 3% BSA sections were stained with monoclonal antibodies against ed1 (AbyD04644.1 described above) 1:50 in 1 x PBS/ 1% BSA or anti-GFP antibody (AbyD04652, AbD Serotec, Martinsried, 1.14 mg/ml stock) diluted to the according concentration for 2.5 h at RT. A fluorescein labeled goat anti-human IgG $F(ab')_2$ specific secondary antibody (# 109-095-006, Jackson Immuno Research Laboratories) was used at a dilution of 1:100 in PBS/BSA, 30 min, RT. Slides were mounted with

Vectashield fluorescence mounting medium (Vector Laboratories) and examined using LSM 510 META confocal laser scanning microscopy system (Carl Zeiss, Feldbach, Switzerland) with a Zeiss Plan Neofluar 63 x/1.25 numeric aperture oil (∞/0.17) objective.

RESULTS

S. mansoni *TOR gene comprises four exons*

Thanks to *S. mansoni* genome sequence published on GeneDB website, we performed an alignment with *S. japonicum* mRNA sequence available in NCBI database in order to define exon/intron boundaries of the SmTOR gene. The analysis suggested the SmTOR gene comprised four exons, instead of the three suggested in GeneDB. Figure 1 represents the exon localisations along the Sm genome, showing that the gene contains three introns in the range of 2500 to 3500 bp, resulting in a 9721 bp length gene. The hatched boxes in Sj mRNA represent the 5' and 3' untranslated regions. The resulting transcript and protein would thus be longer than observed before (Inal, 1999). Thanks to this alignment, we were able to locate the ATG start codon of the SmTOR gene Smp_093840 at position 60992 in scaffold Smp_scaff000547.

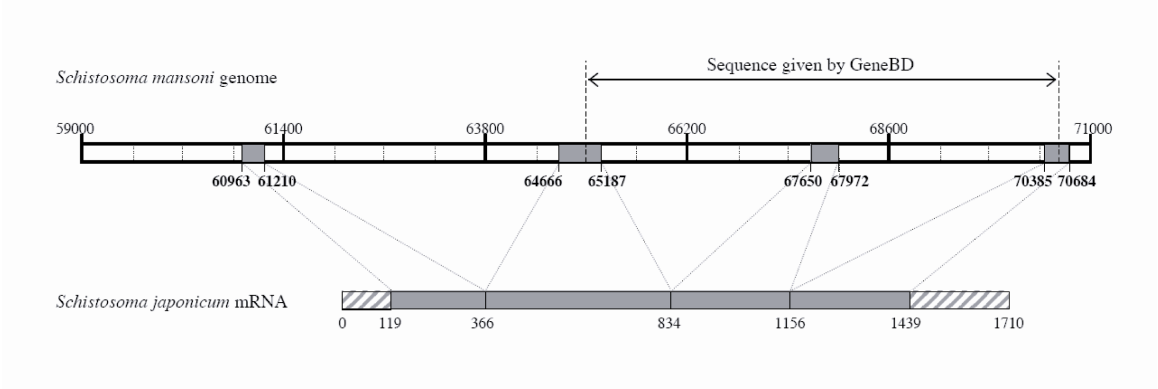


Figure 1. Alignment of Sm (*Schistosoma mansoni*) genome (Smp_093840) and Sj (*Schistosoma japonicum)* mRNA of CRIT. Representation of the gene structure of SmCRIT exons and introns. The grey boxes represent the exons, the hatched boxes represent the sequences not found in Sm genome. Numbers indicate positions on scaffold Smp_scaff000547.

*Cloning and sequencing of SmTOR exons*

We cloned the single exons from genomic DNA extracted from *S. mansoni* cercariae using primers flanking the exons (Table 1). Separation of the PCR products by agarose gel electrophoresis showed bands at the expected size (Fig. 2A). The product for the amplification of exon 2 was expected to be 538 bp long but appears to be about 50 bp smaller. After gel extraction, cloning and sequencing of the single bands, the PCR product of exon 2 was found to be 481 bp long. The difference of 50 bp is because part of this region in the *S. mansoni* GeneDB is unknown and designated as multiple NNN. The length and sequencing results of the other 3 PCR products did match the information deposited at the *S. mansoni* GeneDB database. All the fragments were cloned at least three times and sequenced in both directions. Sequencing results of the single exons are in complete concordance with the cDNA sequence shown below in an alignment with SjTOR cDNA (Fig. 3). This delineation of sequencing information represents the merged information of single exon sequencing results. How the gained information is related to the GeneDB entry is listed in the next section.

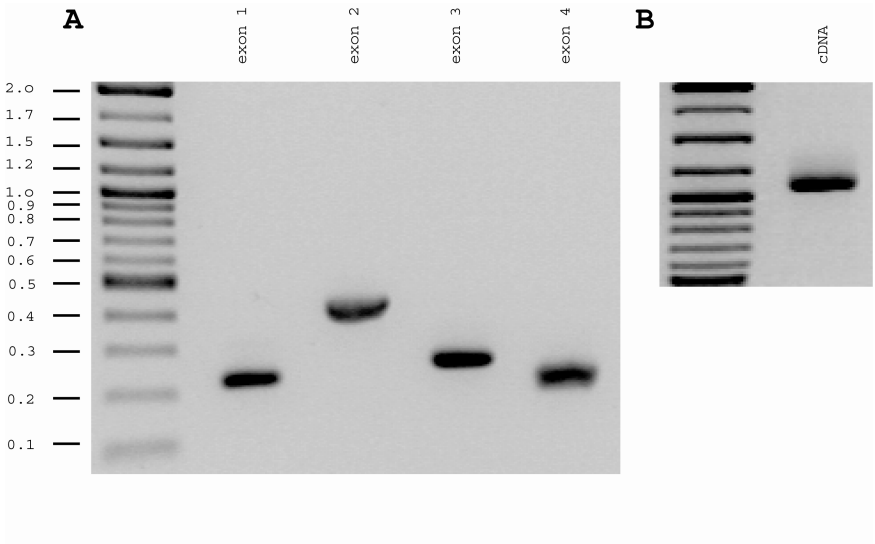


Figure 2. Electrophoretic separation of PCR amplification products from *S. mansoni* genomic DNA or adult worm cDNA. (A) lanes 1 - 4 correspond to PCR amplification of exon 1 - 4 respectively, primers used Table 1. (B) SmTOR ORF amplified from adult worm cDNA, primers: SmTOR_ex1_-15_fwd, SmTOR_exon4_rev (Table 1).

*Expression of full-length SmTOR*

PCR amplification of SmTOR ORF from cDNA preparations from *S. mansoni* adult worms and cercariae generated with different primers (as described above) yielded the same result for all the reactions. Size-separation of reaction products amplified with smTOR_ex1_-15_fwd and smTOR_exon4_rev showed a single band at the expected size (Fig. 2B). The gel-purified and cloned fragments all held the 1260 bp sequence coding for full length SmTOR. The results were identical using different clones and performing sequencing reactions. The sequencing result for SmTOR cDNA is shown in alignment with SjTOR cDNA (AY814912) starting at the translation initiation codon (Fig. 3). When aligning the results from the single exons described in the previous section with the sequenced SmTOR cDNA the correlation was 100%. Splicing sites are marked with black arrows on the SmTOR cDNA (Fig. 3).

Sequencing data for SmTOR cDNA perfectly match the corresponding sequences in the *S. mansoni* GeneDB database (positions 60991 to 70636, scaffold Smp_scaff000547) on *S. mansoni* genome (starting at ATG on exon 1) for exons 1, 3 and 4 (single mutations differing between the clones and occuring at a frequency of 2 per 1260 bp were not taken into account). The gap in the *S. mansoni* GeneDB database lying within SmTOR exon 2 (position 368 - 400 on SmTOR cDNA, 64816 to 64908, Smp_scaff000547 on *S. mansoni* GeneDB entry Smp_093840) could be filled in (Fig. 3). Furthermore we found 13 single nuclotide differences or gaps (indicated with stars in Fig. 3) within exon 2 in the range of position 348 to 400 when comparing *S. mansoni* GeneDB sequence and SmTOR cDNA. The newly sequenced SmTOR cDNA compared with the shorter SmTOR mRNA database entry (AF051138) of Inal (*25*) was nearly identical (18 single bases differed; the ATG is marked with a opened arrow (Fig. 3)). The former 5' UTR region published at the same time for *S. haematobium* TOR (64 base pairs upstream of the former ATG) matches the sequence we now find to be part of exon 2 of the longer SmTOR version, with only 5 bases being altered (not shown).

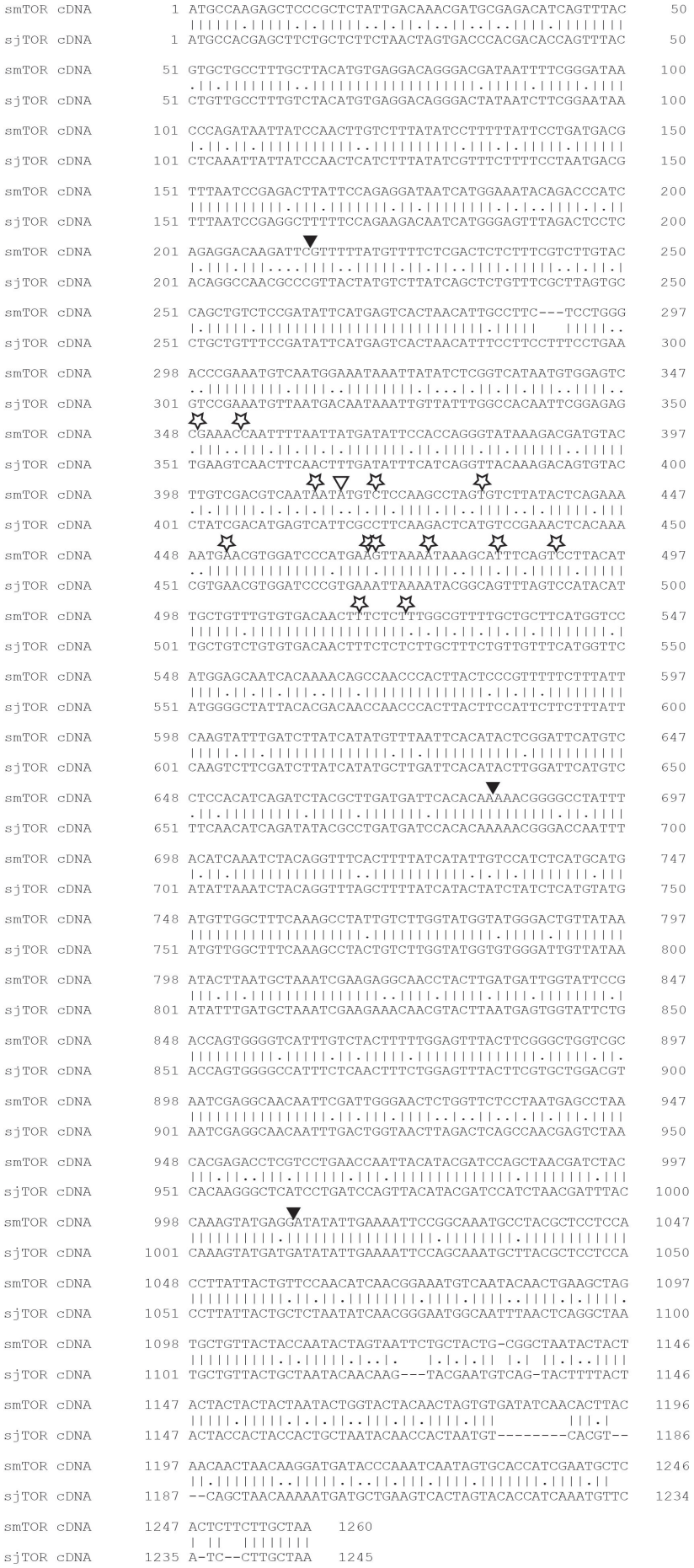
smTOR cDNA 1 ATGCCAAGAGCTCCCGCTCTATTGACAAACGATGCGAGACATCAGTTTAC 50
sjTOR cDNA 1 ATGCCACGAGCTTCTGCTCTTCTAACTAGTGACCCACGACACCAGTTTAC 50
smTOR cDNA 51 GTGCTGCCTTTGCTTACATGTGAGGACAGGGACGATAATTTTCGGGATAA 100
sjTOR cDNA 51 CTGTTGCCTTTGTCTACATGTGAGGACAGGGACTATAATCTTCGGAATAA 100
smTOR cDNA 101 CCCAGATAATTATCCAACTTGTCTTTATATCCTTTTTATTCCTGATGACG 150
sjTOR cDNA 101 CTCAAATTATTATCCAACTCATCTTTATATCGTTTCTTTTCCTAATGACG 150
smTOR cDNA 151 TTTAATCCGAGACTTATTCCAGAGGATAATCATGGAAATACAGACCCATC 200
sjTOR cDNA 151 TTTAATCCGAGGCTTTTTCCAGAAGACAATCATGGGAGTTTAGACTCCTC 200
smTOR cDNA 201 AGAGGACAAGATTCGTTTTTATGTTTTCTCGACTCTCTTTCGTCTTGTAC 250
sjTOR cDNA 201 ACAGGCCAACGCCCGTTACTATGTCTTATCAGCTCTGTTTCGCTTAGTGC 250
smTOR cDNA 251 CAGCTGTCTCCGATATTCATGAGTCACTAACATTGCCTTC---TCCTGGG 297
sjTOR cDNA 251 CTGCTGTTTCCGATATTCATGAGTCACTAACATTTCCTTCCTTTCCTGAA 300
smTOR cDNA 298 ACCCGAAATGTCAATGGAAATAAATTATATCTCGGTCATAATGTGGAGTC 347
sjTOR cDNA 301 GTCCGAAATGTTAATGACAATAAATTGTTATTTGGCCACAATTCGGAGAG 350
smTOR cDNA 348 CGAAACCAATTTTAATTATGATATTCCACCAGGGTATAAAGACGATGTAC 397
sjTOR cDNA 351 TGAAGTCAACTTCAACTTTGATATTTCATCAGGTTACAAAGACAGTGTAC 400
smTOR cDNA 398 TTGTCGACGTCAATAATATGTCTCCAAGCCTAGTGTCTTATACTCAGAAA 447
sjTOR cDNA 401 CTATCGACATGAGTCATTCGCCTTCAAGACTCATGTCCGAAACTCACAAA 450
smTOR cDNA 448 AATGAACGTGGATCCCATGAAGTTAAAATAAAGCATTTCAGTCCTTACAT 497
sjTOR cDNA 451 CGTGAACGTGGATCCCGTGAAATTAAAATACGGCAGTTTAGTCCATACAT 500
smTOR cDNA 498 TGCTGTTTGTGTGACAACTTTCTCTTTGGCGTTTTGCTGCTTCATGGTCC 547
sjTOR cDNA 501 TGCTGTCTGTGTGACAACTTTCTCTCTTGCTTTCTGTTGTTTCATGGTTC 550
smTOR cDNA 548 ATGGAGCAATCACAAAACAGCCAACCCACTTACTCCCGTTTTTCTTTATT 597
sjTOR cDNA 551 ATGGGGCTATTACACGACAACCAACCCACTTACTTCCATTCTTCTTTATT 600
smTOR cDNA 598 CAAGTATTTGATCTTATCATATGTTTAATTCACATACTCGGATTCATGTC 647
sjTOR cDNA 601 CAAGTCTTCGATCTTATCATATGCTTGATTCACATACTTGGATTCATGTC 650
smTOR cDNA 648 CTCCACATCAGATCTACGCTTGATGATTCACACAAAAACGGGGCCTATTT 697
sjTOR cDNA 651 TTCAACATCAGATATACGCCTGATGATCCACACAAAAACGGGACCAATTT 700
smTOR cDNA 698 ACATCAAATCTACAGGTTTCACTTTTATCATATTGTCCATCTCATGCATG 747
sjTOR cDNA 701 ATATTAAATCTACAGGTTTAGCTTTTATCATACTATCTATCTCATGTATG 750
smTOR cDNA 748 ATGTTGGCTTTCAAAGCCTATTGTCTTGGTATGGTATGGGACTGTTATAA 797
sjTOR cDNA 751 ATGTTGGCTTTCAAAGCCTACTGTCTTGGTATGGTGTGGGATTGTTATAA 800
smTOR cDNA 798 ATACTTAATGCTAAATCGAAGAGGCAACCTACTTGATGATTGGTATTCCG 847
sjTOR cDNA 801 ATATTTGATGCTAAATCGAAGAAACAACGTACTTAATGAGTGGTATTCTG 850
smTOR cDNA 848 ACCAGTGGGGTCATTTGTCTACTTTTTGGAGTTTACTTCGGGCTGGTCGC 897
sjTOR cDNA 851 ACCAGTGGGGCCATTTCTCAACTTTCTGGAGTTTACTTCGTGCTGGACGT 900
smTOR cDNA 898 AATCGAGGCAACAATTCGATTGGGAACTCTGGTTCTCCTAATGAGCCTAA 947
sjTOR cDNA 901 AATCGAGGCAACAATTTGACTGGTAACTTAGACTCAGCCAACGAGTCTAA 950
smTOR cDNA 948 CACGAGACCTCGTCCTGAACCAATTACATACGATCCAGCTAACGATCTAC 997
sjTOR cDNA 951 CACAAGGGCTCATCCTGATCCAGTTACATACGATCCATCTAACGATTTAC 1000
smTOR cDNA 998 CAAAGTATGAGGATATATTGAAAATTCCGGCAAATGCCTACGCTCCTCCA 1047
sjTOR cDNA 1001 CAAAGTATGATGATATATTGAAAATTCCAGCAAATGCTTACGCTCCTCCA 1050
smTOR cDNA 1048 CCTTATTACTGTTCCAACATCAACGGAAATGTCAATACAACTGAAGCTAG 1097
sjTOR cDNA 1051 CCTTATTACTGCTCTAATATCAACGGGAATGGCAATTTAACTCAGGCTAA 1100
smTOR cDNA 1098 TGCTGTTACTACCAATACTAGTAATTCTGCTACTG-CGGCTAATACTACT 1146
sjTOR cDNA 1101 TGCTGTTACTGCTAATACAACAAG---TACGAATGTCAG-TACTTTTACT 1146
smTOR cDNA 1147 ACTACTACTACTAATACTGGTACTACAACTAGTGTGATATCAACACTTAC 1196
sjTOR cDNA 1147 ACTACCACTACCACTGCTAATACAACCACTAATGT--------CACGT-- 1186
smTOR cDNA 1197 AACAACTAACAAGGATGATACCCAAATCAATAGTGCACCATCGAATGCTC 1246
sjTOR cDNA 1187 --CAGCTAACAAAAATGATGCTGAAGTCACTAGTACACCATCAAATGTTC 1234
smTOR cDNA 1247 ACTCTTCTTGCTAA 1260
sjTOR cDNA 1235 A-TC--CTTGCTAA 1245

Figure 3. SmTOR and SjTOR cDNA alignment; identical nucleotides are linked with a bar. Filled triangles indicate the positions of splice sites on SmTOR cDNA (positions 214/215, 684/685 and 1009/1010, numbering with respect to the SmTOR start codon). Stars indicate mutations or gaps comparing sequencing data and GeneDB entry data; ATG of SmTOR 0.86 kb ORF (AF051138) is indicated by a triangle. Bases 368 – 400 were newly sequenced on *S.mansoni* cDNA and from genomic DNA as described.

Developmental expression profiling of SmTOR as compared to SmTPI showed that the receptor mRNA was expressed at all the stages examined (Fig. 4). The receptor was expressed at higher level in cercariae as compared to schistosomula, adult worm, egg and miracidiae.

The cDNA sequences aligned for *S. mansoni* and *S. japonicum* TOR show 46% homology at the nucleotide level.

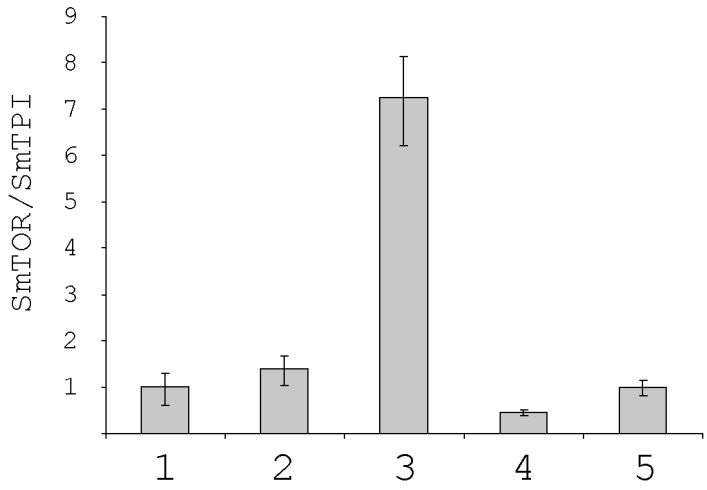


Figure 4. Quantitative RT-PCR analysis of SmTOR mRNA in different *S. mansoni* stages: (1) eggs, (2) miracidiae, (3) cercariae, (4) schistosomulae, (5) adult worm. Expression of SmTOR quantitated relatively to SmTPI as the control gene with bars representing $2^{-\Delta\Delta C}{}_T$ values.

*SmTOR translates into a protein similar in length to SjTOR and possesses four transmembrane domains*

SmTOR cDNA contains a 1260 bp open reading frame which should generate a protein of 419 amino acid length with a theoretical mass of 46.7 kDa. Its amino acid sequence is shown in alignment with SjTOR, a 414 amino acid protein based on proteomics data (*41*). Protein alignment of a possible SmTOR transcript and SjTOR shows 76.7 % homology on the protein level (Fig. 5).

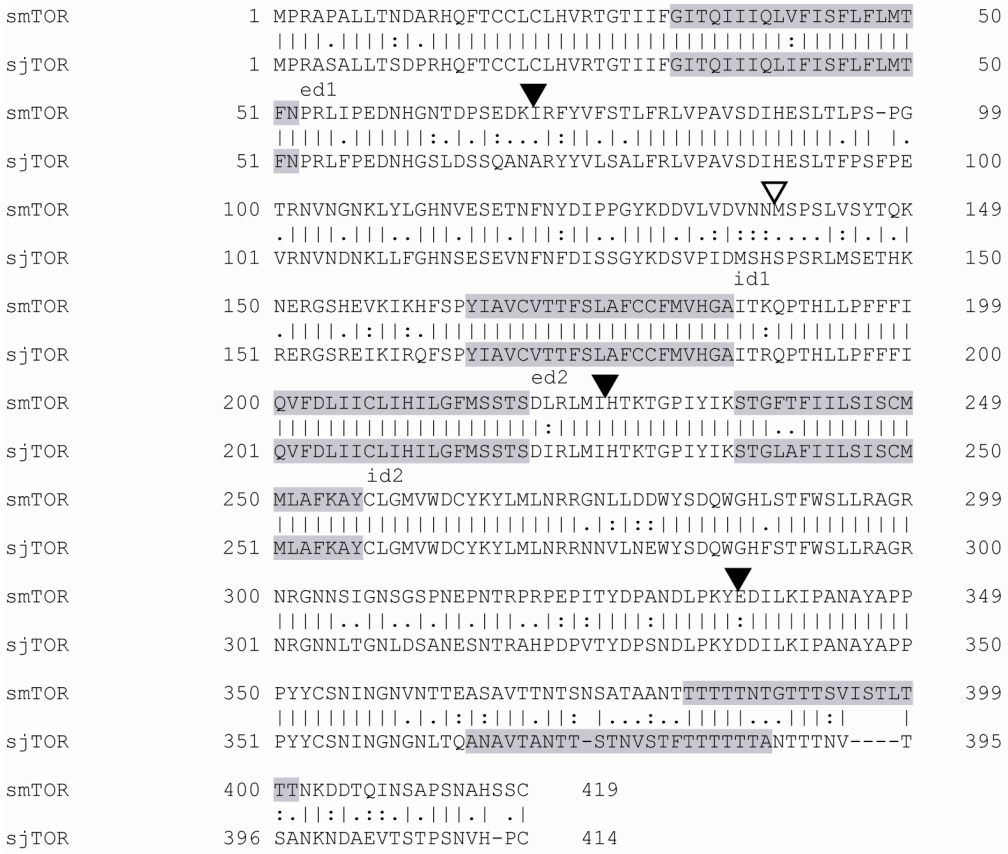


Figure 5. SjTOR and SmTOR protein sequence alignment. Identical amino acids are linked with a bar (76.7 % identity), similar amino acids are connected via ".." (84 % similarity). Exon/Intron boundaries on cDNA level for SmTOR are indicated with filled triangles. SmTOR protein (AF051138) starting with former extracellular domain 1 is marked by a triangle. Transmembrane domains 1-4 are shaded in grey as well as partially inserted helix 5. Domain designation: ed1, new extracellular domain 1; ed2, extracellular domain 2; id1, id2 intracellular domains 1 and 2.

Based on results from transmembrane prediction analysis we suggest SmTOR to have four transmembrane segments and a long C-terminal tail in homology with SjTOR (Fig. 6). In the absence of an N-terminal signal peptide, the transmembrane domain 1 (aa 31–53, Fig. 6) is most likely to function as a signal anchor sequence for ER targeting and membrane insertion, as predicted with the SignalP software. The four transmembrane segments have an alpha helical conformation predicted with high confidence, and the amphipathic alpha helix in the intracellular domain 2 is likely to be partially inserted into the plasma membrane. The domain organisation is the same for SmTOR and SjTOR. The overall transmembrane architecture remains the same as compared to the truncated protein version (UniProtKB/TrEMBL entry Q9U597) (*42, 43*).

The 11 amino acids in the C-terminal part of ed1 that have been described to bind to C2 and interfer with its cleavage are boxed (Fig. 6). This sequence is different in SmTOR and SjTOR.

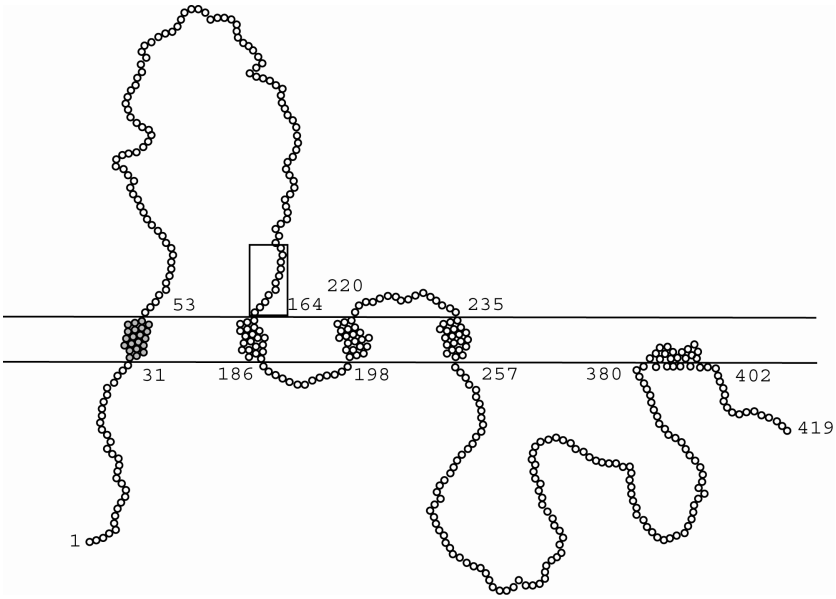


Figure 6. SmTOR protein model according to data generated with TopPred and PSIPREDView program. Numbers indicated refer to the first and last amino acid of the corresponding intra- or extracellular domain. The peptide sequence shown to bind C2 is boxed. Alphahelical transmembrane domains 1–4 are at positions 32-52, 165-185, 199-219 and 236-256 respectively. Transmembrane domain one acting as signal anchor is shaded in grey.

Performing Western blot analysis of adult worm tegument preparations we detected a protein of about 55 kDa using antibodies generated against peptide sequences of ed1 and ed2 (Fig. 7). The same result was obtained using a monoclonal antibody generated against ed1 (Fig. 8A). Staining of cryosections of cercariae with the monoclonal antibody generated against ed1 showed surface labeling, as compared to the negative control (Fig. 8B). The presence of SmTOR on the tegument surface was confirmed by electron microscopy. In sections stained with the polyclonal antibodies against SmTOR ed1 and ed2 respectively, immunogold labeling was detected at the tegument surface (Fig. 9).

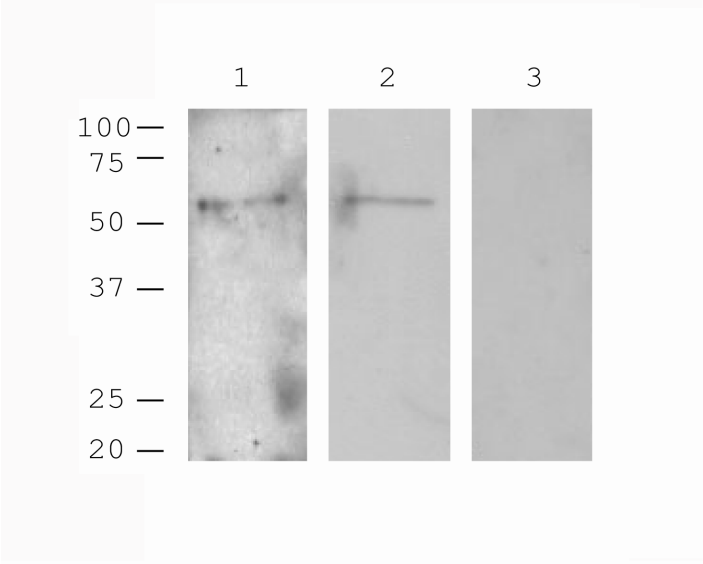


Figure 7. Western blot of adult worm tegument preparation. Polyclonal anti-ed1, anti-ed2 antibody and pre-immune serum (lanes 1, 2 and 3 respectively).

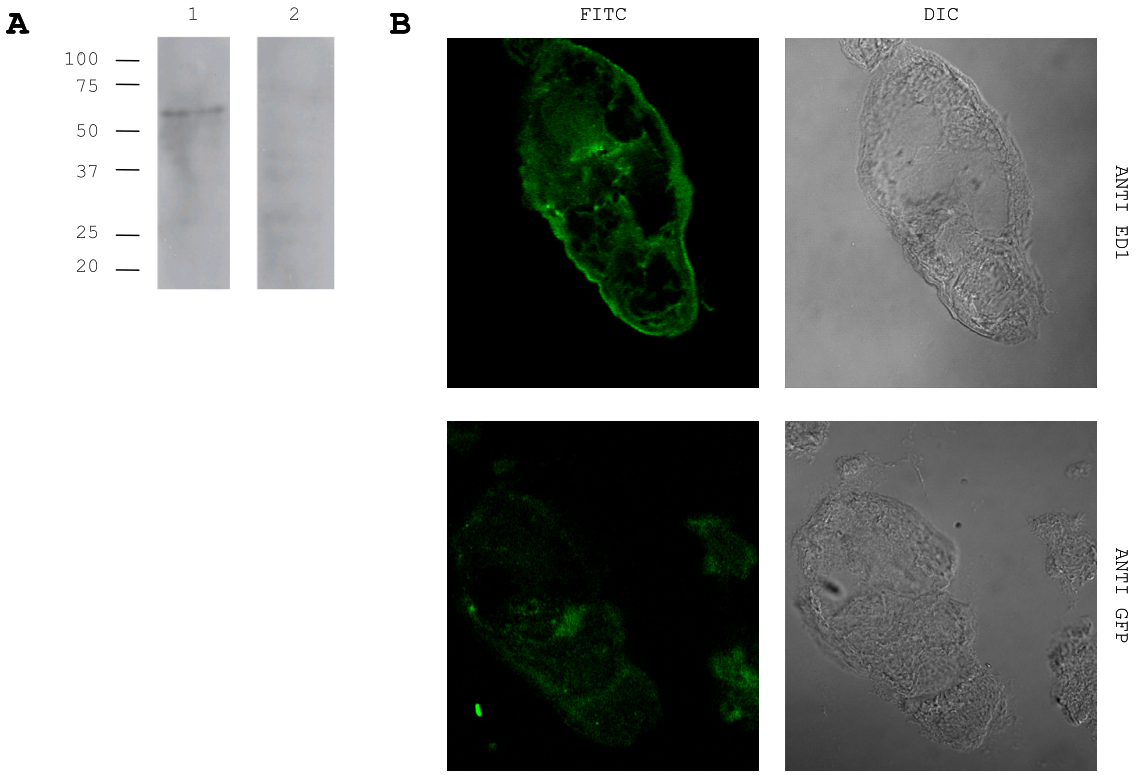


Figure 8. SmTOR detection with a monoclonal anti-ed1 antibody AbyD04644.1 (A) Adult worm tegument preparation Western blot probed with monoclonal anti-ed1 (lane 1) or anti-ed1 preincubated with 100-fold excess of ed1 peptide as control (lane 2). (B) Immunofluorescent labeling of cryosections through cercariae. Anti-ed1 labeled section top left panel and anti-GFP labeld control section bottom left panel.

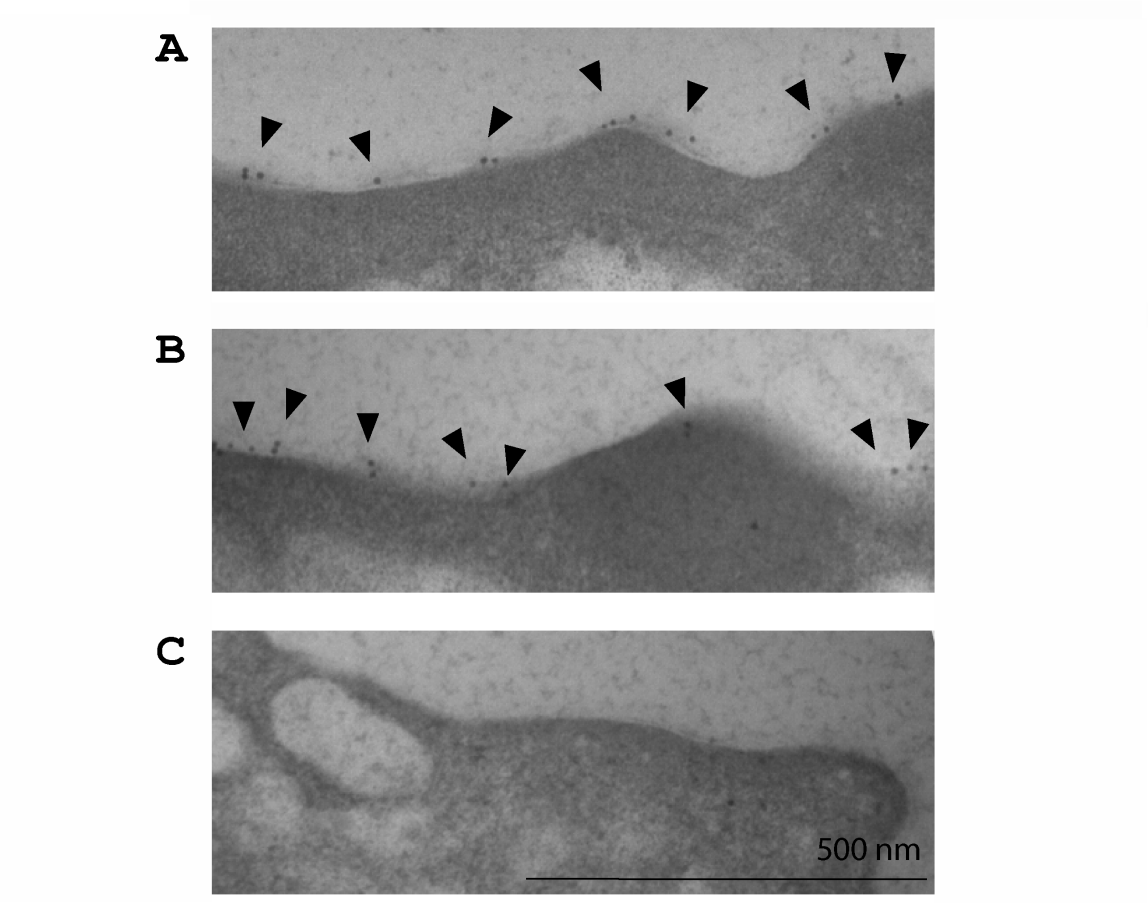


Figure 9. Localisation of SmTOR on tegument of cercariae using electron microscopy. Immunogold labelling is indicated by arrows. Sections stained with (A) polyclonal anti-ed1 antibody, (B) polyclonal anti-ed2 antibody and (C) pre-immune serum. Secondary antibody only was negative (not shown).

## DISCUSSION

The data presented here clarify the exon/intron gene structure of SmTOR and provide evidence that *S. mansoni* expresses the corresponding protein.

We found that SmTOR gene structure contains four exons. A transcript containing the 1.26 kb SmTOR ORF showing 46% identity with the 1.25 kb SjTOR ORF was found in adult worm and cercariae cDNA preparations. The sequencing data generated not only complete the gaps present in the *S. mansoni* GeneDB for SmTOR, but also correct some likely sequencing errors present in the database entry (stars on SmTOR cDNA Fig. 3). The sequencing data are likely to be correct. SmTOR ORF presented here aligns with the previously described SmTOR ORF on mRNA entry AF0511378 from position 418 on (alignment not shown), and translates into a functional protein aligning with SjTOR.

SmTOR is expressed at various developmental stages of *S. mansoni*, as illustrated in Fig. 4. The highest expression was in cercariae, where we were able to detect it at the surface by confocal microscopy of cryosections (Fig. 8B) and electron microscopy (Fig. 9). The higher expression in cercariae as compared to adult worms has previously been described for the truncated receptor version (*25*). This observation is of interest since cercariae are the first to come into contact with the human skin, an encounter that might determine the fate of the infection. SmTOR might be essential at that specific time-point. Many complement proteins are produced by epidermal cells (*44*) and several authors have shown that complement proteins diffuse from the vascular compartment to the dermis and epidermis (*45, 46*). The complete complement cascade is active in the skin as indicated by the role of complement in many human blistering skin diseases (*47*). SmTOR might at that time help the parasite to escape complement attack.

We had previously not been able to replicate the amplification of the short version ORF of the TOR receptor homologue from *Schistosoma* genomic DNA or from vertebrate genomic DNA of various sources (*30*). This is now understandable since the gene has three large introns not described previously. In addition, we did not find the sequence or fragments of it when

performing BLASTn searches on different vertebrate genomes. DeMarco et al. (*48*) recently analysed the controversy about the origin of schistosome albumin, and demonstrated it to be of hamster origin. In his discussion, he suggested that CRIT might be an example of reverse contamination from schistosome into the vertebrate samples. Our present data are in agreement with DeMarco's comments. We must now consider our previous observations as being due to contamination and non-specific antibody stainings (*30, 49, 50*).

Previously, for both ShTOR and SmTOR, transcripts of 1.2 kb and 1.35 kb cloned from adult worm cDNA libraries had been reported to contain an open reading frame of 0.86 kb, as proteins of 31.7 and 31.2 kDa were detected in adult worm preparations (*25*). Alignment of the region published as 5' UTR of ShTOR (*25*) with SmTOR exon 2 sequenced resulted in a nearly perfect match (alignment not shown). SjTOR cDNA and protein (*41*) are of the same length as we now found in *S. mansoni*. We therefore suggest SmTOR to be longer than originally described with the former ATG lying within the second exon of the gene. The translation initiation site we suggest for SmTOR does not lie in a classical Kozak sequence (*51*), but possible variation around the translation initiation codon has been reported for invertebrates (*52*).

TOR protein had been shown on the surface of the adult worm by immunohistochemistry (*25*). We detected a band at approximately 55 kDa in adult worm tegument membrane preparation using two different polyclonal antibodies generated against ed1 and ed2 respectively (Fig. 7). The SmTOR amino acid sequence contains a potential N-linked glycosylation site at position 138. The protein detected might be the glycosylated form, as its theoretical molecular weight was calculated as 46.7 kDa. SmTOR in alignment with SjTOR protein shows 76% identity and 84% similarity (Fig. 5). TOR was found by Liu in *S. japonicum* in cercariae, schistosomula and adult worm (*41*), whereas no proteomic analysis of the schistosome tegument of adult worms identified peptides belonging to SmTOR (*21, 53, 54*). This might be due to the low abundance of the protein in the tegument of the adult worm (*24*).

Based on the secondary structure analysis described above we generated a hypothetical protein model for SmTOR (Fig. 6). SmTOR (or CRIT) was previously thought to span the membrane three times (hence the “T” for trispanning). The longer protein we found is very likely to be tetraspanning, as suggested also for SjTOR, which allows us to suggest a change in the name of the molecule, but without a change in the abbreviations used. The amphiphathic helix inserted partially in the membrane is present in SjTOR and SmTOR, although there is a high variability of amino acid sequence at this site between the two species.

SmTOR had been renamed to CRIT (complement C2 receptor inhibitor trispanning) according to its putative function to bind C2. The former N-terminal extracellular domain ed1 of ShTOR as isolated peptide had been shown to bind C2 and with its C-terminal 11 amino acids designated as H17 peptide being the active binding site (*27*). Incubation with this peptide has been shown to interfere with the binding of C2 to C4b and with C2 cleavage by complement C1s, thus blocking complement activation by the classical pathway. The new tetraspanning SmTOR protein still contains the same amino acid sequence in the extracellular domain 1 known to bind C2. Whether this sequence binds C2 *in vivo* on schistosomes remains to be explored, and it is interesting to note several differences in it between *S. mansoni* and *S. japonicum* (Fig. 5). It will be interesting to test if these differences alter C2 binding to TOR/CRIT.

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## PART II: First extracellular domain of SmTOR: overexpression, purification, binding to C2 and antigenicity

Results published in: *Clinical & Experimental Immunology,* (2012), **170(3)**, 342-357

ABSTRACT

Complement C2 had been identified as a ligand of a peptide sequence of the first extracellular domain of *S. haematobium* TOR (ShTORed1). To investigate, whether this binding would be maintained in the whole extracellular domain 1 of *S. mansoni* TOR (SmTORed1, 111 aa), we recombinantly overexpressed it as a N-terminally His-tagged fusion protein and used this 111 aa peptide purified by nickel affinity chromatography for subsequent testing. This purified rSmTORed1 bound C2 similarly to the peptide sequence of ShTORed1. We then used rSmTORed1 to see whether humans infected with *S. mansoni* develop specific antibodies to this domain. We found, that some patients with schistosomiasis (5/20), but also uninfected humans (4/40) do have antibodies against rSmTORed1. The specificity of antibody binding against SmTORed1 was evaluated by pre-incubation of serum dilutions with recombinant SmTORed1 produced as HaloTag fusion protein coupled to a solid support. The results of the competition ELISA showed, that IgG to rSmTORed1 detected were specific in 2/5 of the patients and 2/4 of uninfected individuals. These data indicate, that specific antibodies can be produced in patients, but evidently the antigen is not frequently recognised by the human immune system. That uninfected individuals have such antibodies might be related to the schistosoma that infest ducks and are responsible for swimmer's itch.

INTRODUCTION

The schistosoma TOR protein was originally identified in *S. mansoni* (SmTOR) and *S. haematobium* (ShTOR) (*1*) and later on suggested to be named complement C2 receptor inhibitor trispanning (CRIT), since the 27 amino acid long ShTOR extracellular domain 1 (ed1) was shown to bind C2 and interfere with its function in the complement cascade (*2*). This capacity was later on shown to depend the C-terminal 11 amino acids of ShTOR ed1 domain or H17 peptide that even more effectively limited the extent of the C3 convertase formation (*3*). As discussed above (Part I), Schistosoma TOR ed1 is 111 aa long and the receptor described originally represents a truncated receptor version. The long extracellular domain 1 of the *S. mansoni* TOR receptor still contains the C-terminal H17 motif that binds C2 (*2, 3*).

Schistosome membrane proteins possessing signal peptides or signal anchors that direct their trafficking through the secretory pathway to the plasma membrane of the tegument, provide interesting vaccine targets as they are predicted to be in the contact with the host (*4*). Amongst the truly "exposed" vaccine candidates are the family of tetraspanins, four-pass transmembrane-domain proteins including a short (EC-1) and a longer (EC-2) extracellular loop. Beside Sm23 which is most efficacious when delivered as DNA vaccine (*5*), TSP-1 and TSP-2 extracellular loop 2 expressed as soluble fusion proteins in *E. coli* turned out to be effective vaccine candidates (*6*). Screening of sera of either putatively resistant or chronically infected patient sera for specific antibodies against TSP-2 showed that the IgG antibody response was higher for putatively resistant individuals (IgG1 and IgG3 subclasses), whereas chronically infected individuals failed to mount a detectable antibody response (*6*). Elevated levels of IgG1, IgG2 and IgG3 to recombinant antigen correlating with the human resistance status have been observed for other vaccine candidates, such as Sm14-FABP, Sm29 and SmStoLP-2 (*7-9*). These antigens produced and injected as recombinant proteins do confer protection against infection in mice (*9-11*). We assume, that SmTOR plays a major role in protecting the worms, and mainly the cercaria against complement attack, and due to its surface localisation, antibodies against SmTOR might in addition to its general effects block the

capacity of the worm to protect itself against complement.

In order to confirm its C2 binding capacity *in vitro*, recombinant SmTORed1 was overexpressed in *E. coli* and purified rSmTORed1 was obtained. This peptide was used to measure antibodies to rSmTORed1 in *S. mansoni* infected patients and uninfected humans. In addition, we used the purified His-tagged SmTORed1 to define its immunogenicity in mice and in a murine immunisation challenge model (Part III). We furthermore produced SmTORed1 as a Halo-tagged fusion peptide with a modified bacterial haloalkane dehalogenase tag (HaloTag) attached to the N-terminus (*12*) that could be covalently immobilised on beads. We used this construct in a competition ELISA to test the specificity of the anti-rSmTORed1 antibodies.

## MATERIALS AND METHODS

### *C2 binding ELISA*

SmTORed1_27aa peptide and biotinylated (BT-) BT-SmTORed1_27aa were purchased from PT Peptides Technologies (Berlin, Germany). BT-SmH17, BT-SjH17, BT-C4beta, BT-SmH17scr and BT-SB78 were from Peptides and Elephants (Berlin, Germany). SmH17, SmH17scr and C4beta peptide sequences correspond to H17, H17S and $C4\beta^{222-232}$ sequences described before (*3*). Sequence SB78 is an irrelevant scrambled peptide sequence. Sequence alignments were performed with ClustalW (*13*) and decorated using the BoxShade server facility. Purified human complement C2 was purchased from CompTech (Tyler, TX, USA).

96-well plates (Maxisorp, Nunc) were coated overnight at 4 °C with peptide (500 nmoles/well) or NeutrAvidin (Pierce; 0.5 mg/well) in 0.1 M bicarbonate buffer, pH 9.6. Plates were the either blocked for 1 h, RT with 5 % BSA in 1 x PBS/0.05 % Tween 20 (PBST) or incubated with 500 nmoles biotinylated peptide in 1 x PBS, RT (in the dark) and then blocked with 5 % BSA/PBST. For the C2 binding assay with all the different biotinylated (BT) peptides (Fig. 1), BT-peptides were first bound to the plate (1 x PBS, 500 nmoles/well) and then free binding sites blocked free biotin for 1 h, RT (1 x PBS, 900 ng/well). After blocking, C2 was diluted in binding buffer (amounts as indicated in figures) and incubated 1 h at RT. Plates were washed 5 times with PBST (as it was done for all the washing steps), incubated with anti-C2 antibody (Calbiochem) diluted 1:5'000 in PBST for 45 min, RT. After additional washes, plates were incubated with donkey anti-goat HRP, 1: 10'000 in 1 x PBS, 30 min, RT. Plates were washed and developed by incubation with a TMB peroxidase substrate (BD Pharmigen). The absorbance was measured at 450 nm.

*Expression of recombinant extracellular domain 1, purification and solubilisation*

SmTORed1 ORF was cloned into the pET15b expression vector (Novagen) by sticky-end PCR method (*14*) using the NdeI and BamHI restriction sites and pCR2.1-TOPO SmTOR (*15*) as template. The primer pairs used were for PCR 1: 5'-TATGCCGAGACTTATTCCAGAGGATAAT-3' (forward1) and 5'-GATCCTTAGTAAGGACTGAAATGCTTTAT-3' (reverse1), for PCR 2: 5'-TGCCGAGACTTATTCCAGAGGATAAT-3' (forward2) and 5'-CGATCCTAGTAAGGACTGAAATGCTTTAT-3' (reverse2). Sticky-end PCR was performed as described in brief: two different PCR reactions were performed, reaction products were cleaned up separately by agarose gel electrophoresis and purification of the bands at the expected molecular weight (345 bp) using the QIAquick gel extraction kit (QIAGEN). The extracted DNA was then mixed in equimolar ratio (1:1), denatured at 95 °C, 5 min and then let re-anneal on ice and used for ligation into digested and cleaned-up vector. pET15b vector was prepared by double digestion with NdeI and BamHI (NEB) and purified as described above. Ligation was performed using Quick Ligase (NEB) and the mixture was transformed into TOP 10 bacteria (Invitrogen) and grown on LB agar plates containing 100 μg/ml ampicillin (Sigma). Clones were analysed by restriction enzyme digestion with SalI (NEB) cutting at position 244 of the insert sequence. Positive clones were sequenced and used for transformation into a bacterial expression strain.

SmTORed1pET15b was transformed into BL21 (DE3) bacteria for protein overexpression. Batch cultures were grown in auto-induction media as described by Studier (*16*). Pre-cultures were grown in MDAG non-inducing medium and main cultures were grown in MDA-5052 auto-inducing medium, both supplemented with 100 μg/ml carbenicillin (Sigma). Single clones of SmTORed1pET15b (rSmTORed1 purification) or pET15b (mock transfection, purification of control fraction) transformants were picked and pre-cultures were grown overnight at RT with shaking at 220 rpm and subsequently diluted 1:50 in MDA-5052 medium for protein expression. 50 ml main cultures in 250 ml Erlenmeyer flasks were shaking at 250 rpm for 18 h, RT. Bacteria

were harvested by centrifugation and pellets frozen at -20 °C till required.

Recombinant protein was purified from the insoluble cytoplasmic fraction (inclusion bodies) of SmTORed1pET15b transformed BL21 (DE3) bacteria. Bacteria were lysed using BugBuster bacterial cell lysis detergent (Novagen) supplemented with EDTA-free protease inhibitor cocktail (Roche Diagnostics) and inclusion bodies were purified by repeated centrifugation and washing steps as described in the pET system manual (Novagen). The final inclusion body pellet was resuspended in 10 ml denaturing buffer containing 10 mM imidazole (50 mM $NaH_2PO_4$, 500 mM NaCl, 6 M Guanidine hydrochloride, pH 8 + 10 mM imidazole). The recombinant protein was then purified by metal affinity chromatography using ÄKTAprime plus system (GE Healthcare Biosciences). A 5 ml Ni-NTA Superflow Cartridge was equilibrated with denaturing buffer/10 mM imidazole (buffer A) and 5 ml of solubilised sample loaded onto the column (1 batch cleaned up corresponded to 25 ml original bacterial culture). The column was then washed with 5 bed volumes of denaturing buffer and then eluted with 10 - 250 mM gradient of imidazole in denaturing buffer in V = 100 ml. Fractions containing the main protein peak were pooled and analysed by SDS-PAGE (15 %). Purified His-tagged rSmTORed1 was refolded by step-wise dialysis. First, the sample (in a volume of 20 - 25 ml denaturing buffer/imidazole) was dialysed into refolding buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 400 mM L-arginine) at 4 °C overnight using a dialysis membrane of 3500 molecular weight cutoff (Spectrum Laboratories). Two additional dialysis steps against 1 x PBS were then performed for 3 h at 4 °C each. The purity of the sample and concentration were then evaluated analysing an aliquot by 15 % SDS-PAGE and bands visualised by Coomassie staining (Instant Blue, Expedeon). Molecular weight markers used were Precision Plus Protein standards (BioRad) and BenchMark ladder (Invitrogen). Protein concentration in the sample was determined using DC protein assay (BioRad). The Coomassie stained protein band at the expected molecular weight was excised and analysed by in-gel tryptic digestion and LC-MS/MS analysis (*17*).

*Characterisation of SmTORed1 by Western Blot and ELISA*

Western blot using anti-ed1 antibody AbyD04644.1 (Serotec) was performed as described above (Part I). Protein samples were run on a standard 15 % acrylamide/bisacrylamide gel (BioRad) and transferred to a nitrocellulose membrane. The membrane was blocked in PBST/5 % milk followed by incubations with primary (AbyD04644.1, 1:1000) and secondary antibody (goat F(ab')2 anti-human IgG-HRP, Serotec, 1:3000) in PBST 1 % milk, 1 h RT each. The blot was developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

The protocol for C2 binding ELISA was the same as described in the previous paragraph. AbDy04644.1 (2.51 mg/ml stock) diluted in PBST was used at dilutions ranging from 1:1000 to 1:10'000 to detect rSmTORed1 by ELISA. The amounts of anti-GFP AbDy04652.1 corresponding to the highest and lowest dilution respectively (1.14 mg/ml stock) were used as negative controls. Peptide was immobilised as above, blocked in 5 % BSA/PBST, 1 h, RT followed by 30 min incubation with primary antibody. Secondary antibody (goat F(ab')2 anti-human IgG-HRP, Serotec) was diluted 1:10'000. Incubation time was 30 min, RT and the ELISA was then developed as done before.

*Anti-rSmTORed1 antibody measurement in human sera*

Patient sera were archived anonymised sera from *S. mansoni* infected patients (provided by the SwissTPH Institute, Basel). Sera had been tested positively for antibodies against soluble worm antigen (SWA) and soluble egg antigen (SEA) and by immuno fluorescence antibody tests (IFAT) as done at the SwissTPH Institute. Normal human serum samples were collected at the Blutspendezentrum, Basel.

Nunc Maxisorp 96-well plates were coated with 1 μmole rSmTORed1 per well diluted 0.1 M bicarbonate buffer, pH 9.6 overnight at 4 °C. Plates were washed 3 times with PBST and serum samples diluted 1:500 in PBST were added for 45 min, 37 °C. After incubation with sera, plates were washed 5 times with PBST and biotinylated secondary antibody diluted 1:10'000 was added to each well (goat F(ab')2 anti human IgG, Biosource) for 1 h, RT.

This was followed by additional 5 washes and 30 min incubation with Streptavidin-HRP (Pierce, 1:10'000 in 1 x PBS) at RT. After washing as in the previous steps, ELISA was developed using TMB peroxidase substrate and the absorbance at 450 nm was measured.

*Cloning of HaloTag fusion constructs*

All N-terminal HaloTag fusion constructs were cloned with the primers listed in Table 1 into the bacterial T7 promotor based expression vector pFN18A HaloTag T7® Flexi® Vector (Promega, Madison, WI, USA). The barnase positive selection cassette was removed by digestion of pFN18A with PvuI and PmeI and replaced by HALOfwd and HALOrev annealed oligos as described before (*18*). The construct pFN18A Halo expressing HaloTag only was used as negative control. Halo-SmTORed1 (111 aa) and Halo-C4beta (C4beta: 26 aa corresponding to $C4\beta^{206\text{-}232}$ peptide stretch) were amplified from pCR2.1-TOPO SmTOR (*15*) for the SmTORed1 constructs or Hep2G (human hepatoma cell line) cDNA for the C4beta chain peptide. Cloning was performed with the sticky-end PCR method (*14*) using iProof High-Fidelity DNA Polymerase (Bio-Rad) and the so generated inserts were ligated into PvuI/PmeI digested pFN18A vector with Quick Ligase (New England Biolabs). Plasmids were propagated in TOP10 *E.coli* (Invitrogen) and open reading frames verified by sequencing.

Table 1. Primer list used for cloning of Halo-tagged fusion constructs.

| primer/oligo | sequence 5' - 3' | restriction site |
|---|---|---|
| HALOfwd | **CG**CGTAAGGGTAG**GTTT** | PvuI/PmeI |
| HALOrev | **AAAC**CTACCCTTACG**CGAT** | PvuI/PmeI |
| HALOed1fwd1 | **CG**CGCCGAGACTTATTCCAGAGGATAAT | PvuI |
| HALOed1fwd2 | **ATCG**CGCCGAGACTTATTCCAGAGGATAAT | PmeI |
| HALOed1rev | **AAAC**TTAGTAAGGACTGAAATGCTTTAT | PmeI |
| HALOC4beta_fwd1 | **CG**CGTCCAACAGCAGCACCCAGTTTGAG | PmeI |
| HALOC4beta_fwd2 | **ATCG**CGTCCAACAGCAGCACCCAGTTTGAG | PvuI |
| HALOC4beta_rev | **AAAC**TTAGTAGGGCTTTCCAGGGGTGATCTT | PmeI |

*Over-expression and purification of Halo-tagged peptides*

Plasmids (pFN18A HALO, pFN18A HALOed1 or pFN18A HALO C4 beta) were transformed into chemically competent BL21 (Sigma). Single clones were picked from LB/Amp plates and were grown overnight at 37 °C (at 220 rpm) in 2 ml LB medium supplemented with 100 μg/ml Ampicillin (Sigma). Pre-cultures were diluted tenfold in LB/Ampicillin and when grown to an $OD_{600}$ of > 0.6 induced with 1 mM IPTG. Aliquots of the cultures were screened for expression of target gene 3 - 5 h after induction by SDS-PAGE and Coomassie staining. Total cell protein fractions were prepared using PopCulture reagent (Novagen) according to the protocol in the pET system manual (Novagen). Theoretical molecular weights of the fusion constructs were calculated as 35.4 kDa (HALO), 38.5 kDa (HALO C4 beta) and 48.2 kDa (HALO ed1).

In order to purify Halo-tagged constructs, bacteria cultures (V = 20 ml) were pelleted, resuspended in BugBuster mix (Novagen) and soluble cytoplasmic fractions were prepared according to the manufacturer protocol. Protein samples were then prepared for coupling onto magnetic beads (HaloLink, G9311, Promega). Buffer exchange into 1 x PBS was accomplished by using 3'000 MWCO Amicon Ultra Centrifugal Filter Devices (Milipore). Halo-protein concentrations were estimated in comparison with marker protein bands (BenchMark Protein Ladder Invitrogen; each protein present at a concentration of approximately 0.1 μg/μl). For the coupling reaction, samples were diluted at the appropriate concentrations in 100 mM TrisHCl, pH 7.5, 150 mM NaCl, 0.01 % NP40. HaloTag fusion proteins were immobilised on magnetic beads as described in the instruction manual (G9311, Promega). Magnetic beads were incubated with the HaloTag fusion proteins in excess in order to ensure that equal amounts of protein i.e. beads were used in the assay. This was the case because the covalent bond formation between HaloTag and chloralkane linker is specific and highly irreversible (*12*). After coupling reactions, beads were washed extensively in PBS and used for competition ELISA or peptide purification. An amount of 40 μg Halo-tagged protein was coupled onto 20 μl beads in gel slurry that was

then used in the competition ELISA.

*Competition ELISA assay*

For competition ELISA, 96 well plates were coated as described above but with 500 nmoles rSmTORed1 per well. Anti-rSmTORed1 antibody measurement in human sera was performed exactly as described above, but sera were first incubated with Halo-tagged fusion constructs as described as follows. 20 µl washed beads with immobilised HALO or HALOed1 were resuspended in 250 µl PBST. 1 µl of serum sample (resulting in a serum dilution of 1:250) was added and the mix incubated on a wheel for 20 min, RT. As a control, 1 µl of serum sample was diluted in 250 µl PBST and incubated in parallel without beads. The coated ELISA plate was washed and 50 µl of PBST added per well. Magnetic beads were removed after incubation time and 50 µl of depleted serum (or control sample) was added per well (experiments were done in triplicates), resulting in a final serum dilution of 1:500. Plates were incubated at 37 °C, 45 min and proceeded with the protocol as described above.

*Mouse Ig detection to rSmTORed1 or peptides purified from HaloTag fusion proteins*

40 µg Halo-tagged protein on beads was incubated with ProTEV protease (Promega, #V6051) in a total reaction volume of V = 200 µl in order to get cleaved peptide in solution at a concentration of 0.2 µg/µl. Cleavage was performed according to instruction manual provided. Cleavage of HaloTag fusion peptides occurs in the 16 aa linker sequence (N-terminally to the peptide sequence) EPTTEDLYFQ/SDNAIA between Q and S (TEV protease cleavage site EXXYXQ(G/S)) releasing the peptide of interest with the C-terminal 6 aa (SDNAIA) attached. After completion of cleavage, magnetic beads were removed. 96-well plates (Maxisorp, Nunc) were coated overnight at 4 °C with cleaved peptides or rSmTORed1 (500 nmoles/well) in 0.1 M carbonate bicarbonate buffer, pH 9.6. Plates were blocked in PBST/5 % BSA for 1 h, 37 °C. and washed 5 x with PBST (as all the

following washes). Mouse serum samples from BALB/c mice immunised with rSmTORed1 in Complete/Incomplete Freund's (all described in Part III) adjuvant were diluted 1:25'600 in 100 µl PBST and plates were incubated for 1 h, RT. After washes biotinylated primary antibody (Goat anti-mouse IgG+IgM+IgA, ab6005, AbCam) diluted 1:10'000 in PBST was added for another hour at RT. Bound antibody was detected with streptavidin-HRP (Pierce) diluted 1:10'000 in PBS (30 min, RT) and the ELISA was developed by incubation with a TMB peroxidase substrate. The absorbance was measured at 450 nm.

RESULTS

*The C-terminal 27 amino acids of SmTORed1 and ed1-derived peptides bind purified C2*

We compared the binding of C2 to different synthetic peptides derived from the SmTORed1 sequence measured by ELISA. The C-terminal 27 amino acids of *S. haematobium* TOR extracellular domain 1 show 92.6 % sequence identity with the corresponding sequence found in *S. mansoni* TORed1 (Fig. 1A). C2 binds in a concentration dependent manner to SmTORed_27aa peptide immobilised directly on the ELISA plate (Fig. 1B) and to the biotinylated peptide BT-SmTORed1_27aa captured on a NeutrAvidin-coated plate (Fig. 1C). In both cases, the binding buffer was 1 x PBS containing 150 mM NaCl. Binding of C2 to the uncoated plate was in the range of background OD levels. A panel of biotinylated ed1-derived peptides (Fig. 1D) was immobilised the same way to test for C2 binding. C2 in 1 x PBS buffer with salt adjusted to a final concentration of 180 mM NaCl, generating a medium salt binding buffer, did specifically bind to SmTORed1_27aa, but not specifically to the other peptides tested (Fig. 1E). This difference in C2 binding was not observed when 1 x PBS or under high salt conditions in 1 x PBS containing 250 mM NaCl were used as buffers instead (not shown). In 1 x PBS, C2 did bind unspecifically to all the peptides, in high salt buffer no binding to any of the peptides was detectable. As negative control, C2 was let to bind in the corresponding buffer on the plate and the primary antibody was omitted in detection process, which resulted in OD values as low as the blank OD values for all of the biotinylated peptides ($OD_{450}$ = 0.04 - 0.07; not shown).

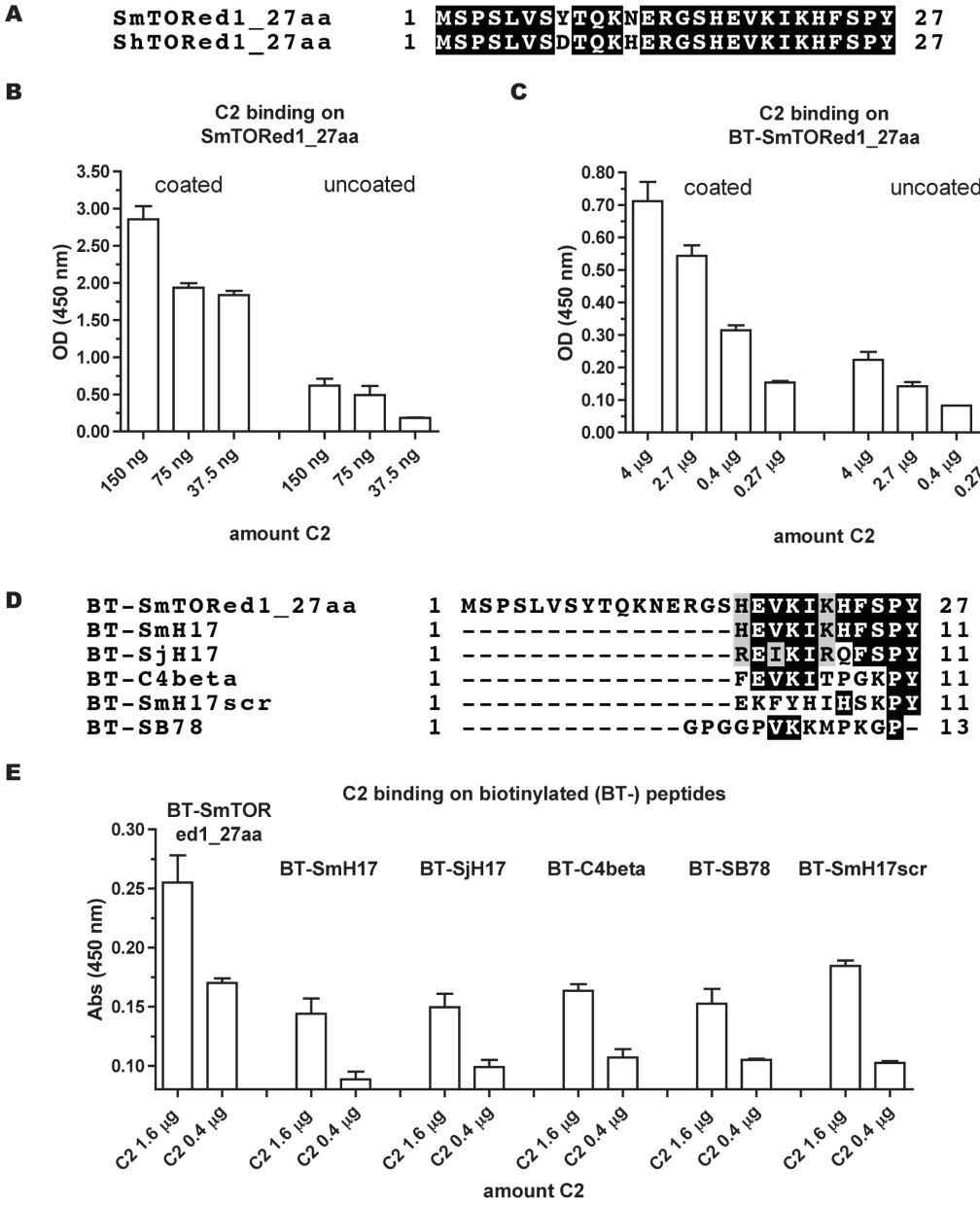


Figure 1. C2 binding on SmTORed1-derived peptides. (A) Amino acid sequence alignment of C-terminal 27 amino acids *S. mansoni* and *S. haematobium* TORed1 domains termed SmTORed1_27aa and SmTORed1_27aa respectively. Sequence alignments were performed with ClustalW2. The regions with high identity and similarity between peptide sequences are shaded in black and grey. (B, C) C2 binding (in 1 x PBS) ELISA to immobilised SmTORed1_27aa (B) or biotinylated (BT-) SmTORed1_27aa peptide (C). ELISA Results are expressed as means of individual measurements. Error bars indicate S.D. of the means. (D) Alignment of SmTORed1_27aa sequence with SmH17, SjH17 (C-terminal 11 aa of *S. mansoni* and *S. japonicum* ed1), C4beta (human C4β chain peptide $F^{222}$), scrambled peptide SmH17scr and a control peptide SB78. (E) C2 binding on different biotinylated peptides with sequences listed in (D). 1 x PBS/180 mM NaCl was used as binding buffer.

*Recombinant SmTORed1 overexpression in* E. coli*, purification and characterisation*

SmTORed1 ORF was cloned into a pET15b vector and subsequently transformed into bacteria in order to produce the N-terminally His-tagged fusion protein rSmTORed1 containing only a short linker sequence (Fig. 2A). BL21 (DE3) bacteria transformed with pET15bSmTORed1 were grown in auto-induction media and rSmTORed1 was purified from inclusion bodies by $Ni^{2+}$-affinity chromatography. Fractions eluted from the FPLC column were pooled (as described in the method section) and the denaturing buffer was exchanged by step-wise dialysis against 1 x PBS. An aliquot of the preparation was analysed by gel electrophoresis before and after the final centrifugation step and a protein band of the calculated molecular weight of 14.7 kDa was detected (Fig. 2B). The protein band was sequenced by mass spectrometry and fragments belonging to recombinant SmTORed1 were detected (Fig. 2A). The preparation did not contain any impurities that could be visualised by Coomasie blue staining and only a small portion of rSmTORed1 was not soluble in 1 x PBS. The protein concentration of total compared to soluble peptide was 145 ng/μl and 132 ng/μl respectively for the peptide batch shown. Pooled protein fractions analysed before re-folding into 1 x PBS showed an additional protein band at an approximate molecular weight of 30 kDa (Fig. 2C). Recombinant SmTOed1 as well as the 30 kDa protein band, that presumably is the peptide dimer, were detected by Western blot using monoclonal anti-ed1 antibody (Fig. 2C). The same monoclonal antibody did recognise plate-bound rSmTORed1 (Fig. 2D), which was also shown to bind C2 (Fig. 2E).

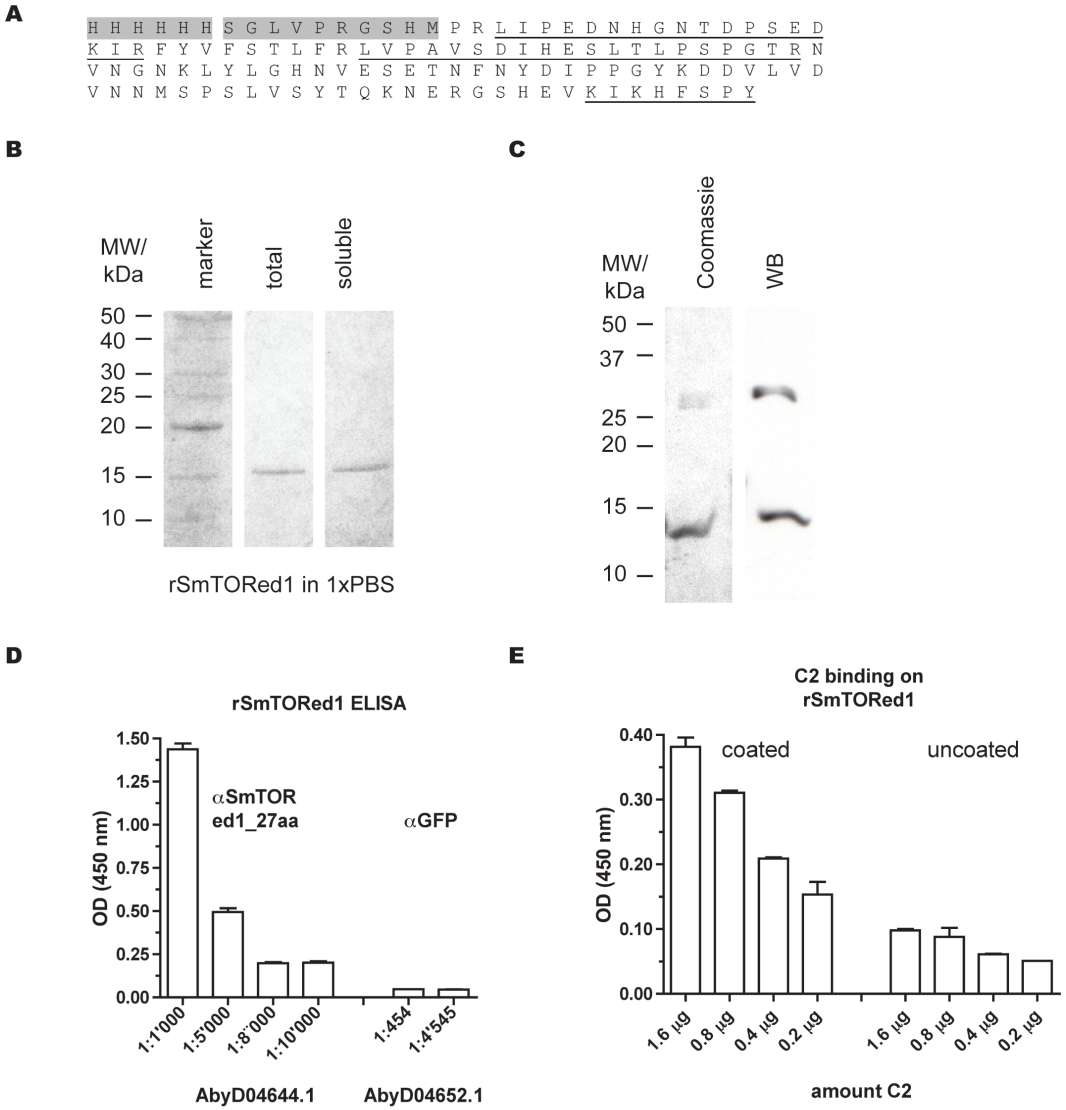


Figure 2. Production of His-tagged SmTORed1 in *E. coli* and molecular characterisation of purified peptide by mass spectrometry, Western blotting and ELISA. (A) SmTORed1 peptide sequence with the 6 x His-tag and linker sequence (10 aa) originating from the pET15b vector shaded in grey. Fragments identified by mass spectrometry are underlined. (B) SDS-PAGE analysis of purified rSmTORed1 after $Ni^{2+}$ chromatography (pooled eluates) and refolding into 1 x PBS. Total amount of peptide in buffer was analysed before (total, lane 2) and after removal of residual precipitate by centrifugation (soluble, lane 3). 4 µl of sample was loaded per lane. Lane 1 (marker): BenchMark Protein Ladder. Protein bands are visualised by Coomassie stain. (C) Pooled fractions of FPLC-purified peptide analysed before dialysis into 1 x PBS. 20 µl sample was loaded per lane. Peptides detected by Coomassie Blue (left) and by Western blot using monoclonal anti-ed1 antibody AbDy04644.1 (right). (D) Peptide ELISA showing AbDy04644.1 specifically binding purified rSmTORed1, as compared to the isotype control antibody (AbDy04652.1). (E) C2 binding on a rSmTORed1 coated ELISA plate, binding in 20 mM Tris buffer + 1 mM $MgCl_2$ + 1 mM $CaCl_2$. ELISA Results are expressed as means of individual measurements. Error bars indicate S.D. of the means.

*Human anti-rSmTORed1 antibodies detected in* S. mansoni *infected and uninfected individuals*

We evaluated by ELISA the reactivity of anti-rSmTORed1 antibodies in sera of individuals that were infected with *S. mansoni*, as classified by positive titers against soluble worm and egg antigen (SWA/SEA) and immuno-fluorescence antibody tests (IFAT), and in uninfected individuals. IgG antibodies to rSmTORed1 were detected in 5 out of 20 patients (25 %) infected with *S. mansoni*, but also in 4 out of 40 uninfected individuals (10 %) living in Switzerland (Fig. 3). The positive signal threshold was set arbitrary considering the relative fold increase values indicated.

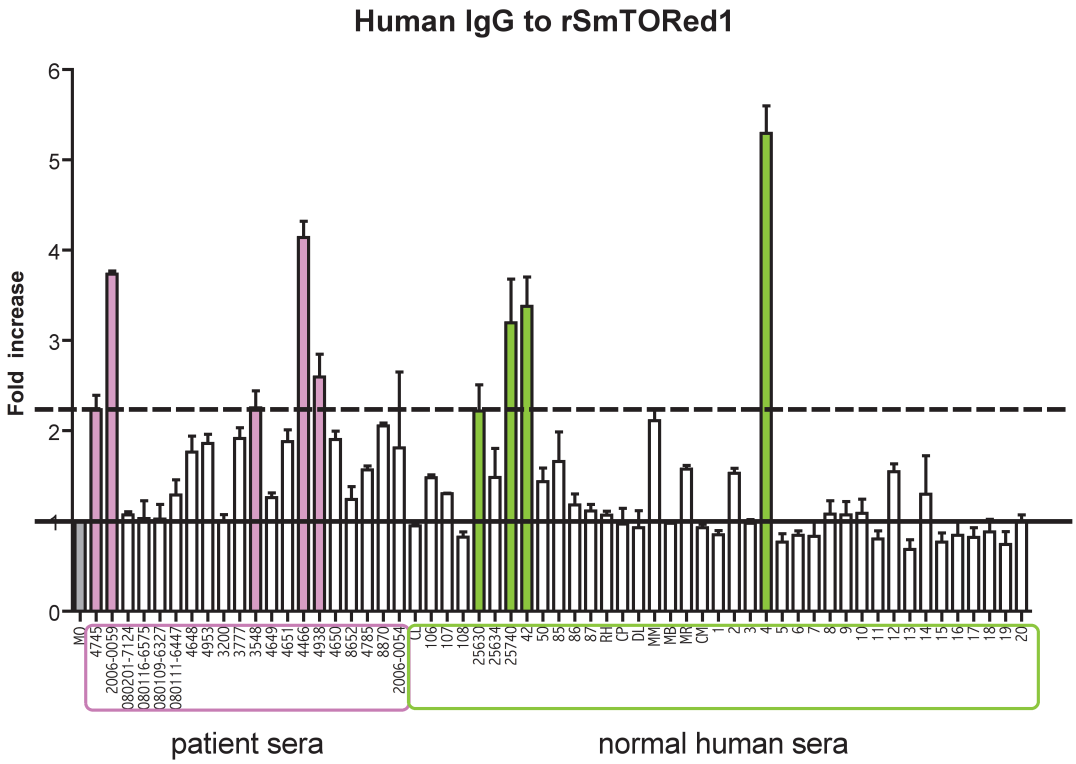


Figure 3. Measurement of antibodies to rSmTORed1 by ELISA. Total IgG levels in patients (archived anonymised sera, purple box) and NHS (green box) normalised to control serum (MO, grey bar). The black solid line set at 1 corresponds to the mean of absorbance measuring control serum MO (mean OD ± S.D (n = 9): $OD_{450\ nm}$ = 0.176 ± 0.05), that was set to 1 in order to picture patient and NHS values normalised to one control serum value. The dashed black line was set at an arbitrary treshold and marks the lower limit of a fold-increase value considered as postive signal. 5 (filled purple bars) out of 20 patients and 4 (filled green bars) out of 40 patients were tested as positive. The results of three different experiments done in duplicates are shown. Error bars indicate S.D. of the means.

*Production of HALO-tagged ed1 and C4β chain stretch as bacterial fusion proteins used for competition ELISA covalently coupled to magnetic beads*

Halo-tagged peptides can be covalently immobilised on a solid support and be used in competition ELISA assays. SmTORed1 peptide and the homologous sequence on the C4β chain peptide aligning with it were produced as N-terminal Halo-tagged fusion proteins, schematically pictured in Fig. 4A, and were used linked to magnetic beads in a competition ELISA together with HaloTag on magnetic beads alone or as an alternative method to purify recombinant peptides (described in the method sections and the following results sections). *E. coli* strains transformed with pFN18AHALO, pFN18AHALOC4beta and pFN18AHALOed1 were grown to an $OD_{600}$ > 0.5 in LB medium and were overexpressing the corresponding fusion protein upon induction with 1 mM IPTG (Fig. 4B). Aliquots of the crude soluble *E. coli* fractions were separated by SDS-PAGE 3 h after induction of protein overexpression and protein bands were visualised by Coomassie blue staining. Protein bands of 35.4 kDa, 38.5 kDa and 48.2 kDa corresponding to the theoretical molecular weights calculated for HaloTag (lane 2), HaloC4beta (lane 3) and HALOed1 (lane 4) were detected. HALOed1 but not HALOC4beta or HaloTag alone was also detected by Western blot probed with monoclonal anti-ed1 antibody AbDy04644.1 (Fig. 4C). Thus, this monoclonal antibody does not show any cross-reactivity with the C4β chain peptide homologous to the 27 C-terminal amino acids of SmTORed1.

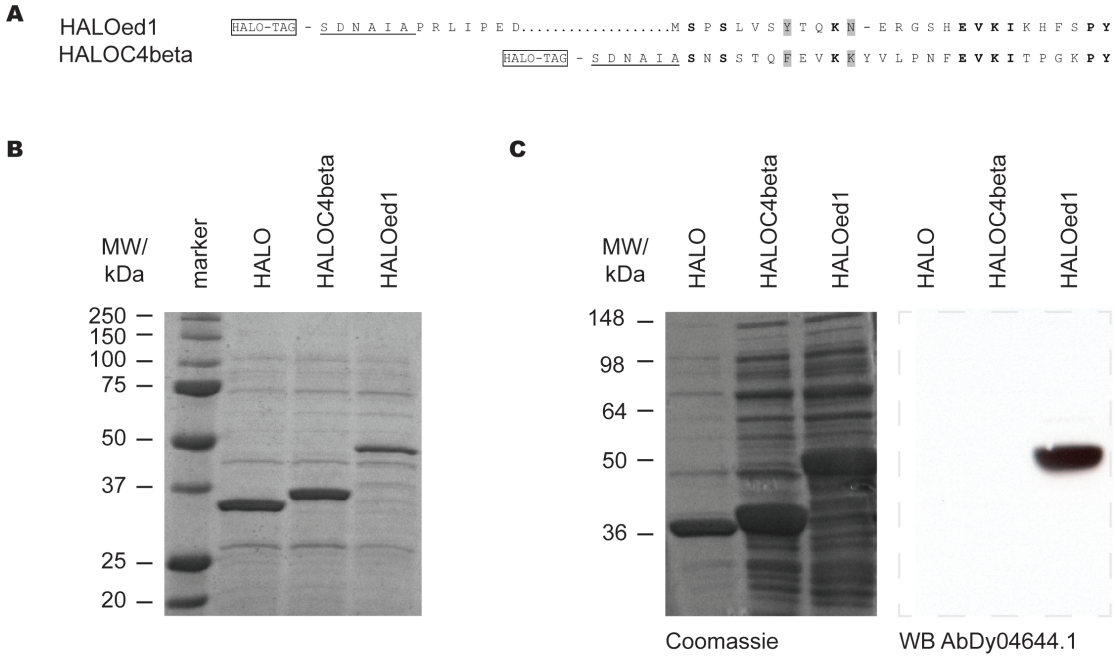


Figure 4. SDS-PAGE (10 %) analysis and immunoblotting cell extracts from *E. coli* transformed with pFN18AHALO, pFN18AHALOC4beta and pFN18AHALOed1. (A) Schematic representation of HaloTag fusion peptides generated. The HaloTag protein (boxed) is attached N-terminally to the sequences of interest via a 16 aa linker containing a TEV protease cleavage site. The 6 C-terminal amino acids of the linker sequence remaining attached to the peptides after cleavage are shown (underlined). HaloTag protein linked to SmTORed1 loop is denoted as HALOed1 and HaloTag fused to a 26 aa C4β chain stretch is termed HALOC4beta (human C4β chain peptide $S^{206}$). Local sequence alignment over the C-terminal 27 aa was performed with ClustalW2 and then manually edited (identical amino acids in bold letters, similar amino acids shaded in grey). (B) Coomassie stained gel with protein extracts from recombinant *E. coli* (BL21) overexpressing HaloTag (lane 2), HALOC4beta (lane 3) or HALOed1 (lane 4) analysed 3 h after induction with 1 mM IPTG and resolved by SDS-PAGE. Lane1: Molecular weight marker. (C) Protein samples extracted from BL21 4.5 h after induction with IPTG. HaloTag (HALO) and Halo-tagged (HALOC4beta, HALOed1) peptides in crude *E. coli* extracts visualised by Coomassie stain (left) and detected by Western blot probed with monoclonal anti-ed1 antibody AbDy04644.1 (right).

*Some patients and uninfected humans have specific antibodies against rSmTORed1*

The 5 schistosomiasis patients and 4 uninfected individuals considered to have antibodies against rSmTORed1 measured by ELISA (Fig. 3) were tested in a competition type ELISA. The specificity of antibody binding against SmTORed1 was evaluated by preincubation of diluted patient sera with Halo-tagged ed1 or HaloTag alone coupled to magnetic beads. Competition ELISA results show, that 2 out of 5 schistosomiasis patients (Fig. 5A) and 2 out of 4 uninfected individuals (Fig. 5B) have specific anti-rSmTORed1 IgG antibodies whose binding on the peptide could be abolished by preincubation of diluted sera with HALOed1, but not with HaloTag alone. This decrease in signal (indicated by an arrow) was significant for the patient sera 4466 and 2006-0059 (Fig. 5A) and uninfected human 42 and 4 sera (Fig. 5B) except one (serum 25740) that had shown the highest positive values measured before (Fig. 3). In the sera tested with positive signals just above the arbitrarily set threshold value, namely the patient sera 4745, 3548, 4938 (Fig. 5A) and uninfected human serum 25630 (Fig. 5B) there were no specific antibodies detected. In retrospective, NHS 4 and NHS 42 were confirmed to have no detectable levels of anti-SWA and anti-SEA antibodies.

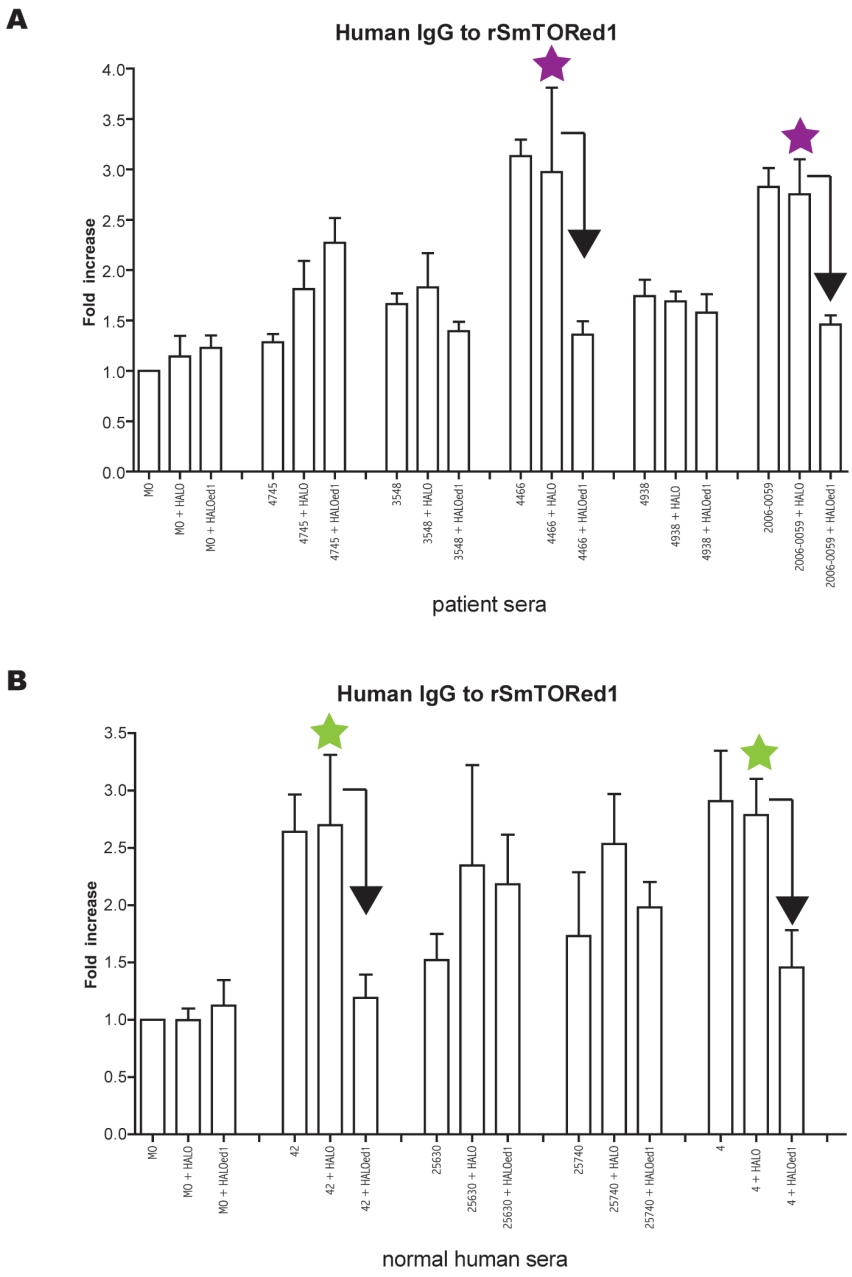


Figure 5. Measurement of antibodies to rSmTORed1 by ELISA and confirmation of specificity by competition ELISA using Halo-tagged bead-bound fusion constructs. $OD_{450}$ values measured were normalised to mean values recorded for the control sample (MO, $OD_{450\ nm}$ = 0.243 ± 0.06, n = 7) and indicated as fold increased values. Prior to IgG measurement, individual sera were preincubated with Halo constructs coupled to magnetic beads: no competition (sample + no beads), with HaloTag alone (sample + HALO) or Halo-SmTORed1 (HALOed1). Measurement of IgG specificity in schistosomiasis patients (A) and uninfected human sera (B). Stars indicate specific IgG to rSmTORed1 in the corresponding serum, tested by competition ELISA. Bars represent the mean values of three independent experiments. Error bars indicate S.D. of the means. Arrows indicate a significant decrease (> 40 %) in signal due to depletion of specific antibodies by pre-incubation with HALOed1.

*Antibodies against rSmTORed1 generated in mice do not recognise the homologous stretch on the complement C4 β chain*

Since there are described homologies between SmTORed1 and the C4beta chain, we tested for possible cross-reactions. BALB/c mice vaccinated with purified rSmTORed1 (see Part III) do generate antibodies against rSmTORed1 that do not cross-react with the 27aa region in the C4β chain aligning with the ed1 domain (Fig. 6, Fig. 4A). Antibodies to rSmTORed1 can be detected equally well when $Ni^{2+}$ purified His-tagged peptide or ed1 generated as Halo-fusion protein subsequently cleaved off in the peptide purification procedure are immobilised. Neither immunised nor control immunised mice recognise the inclusion body control fraction that is the $Ni^{2+}$ purified insoluble fraction of bacteria transformed with empty pET15b vector.

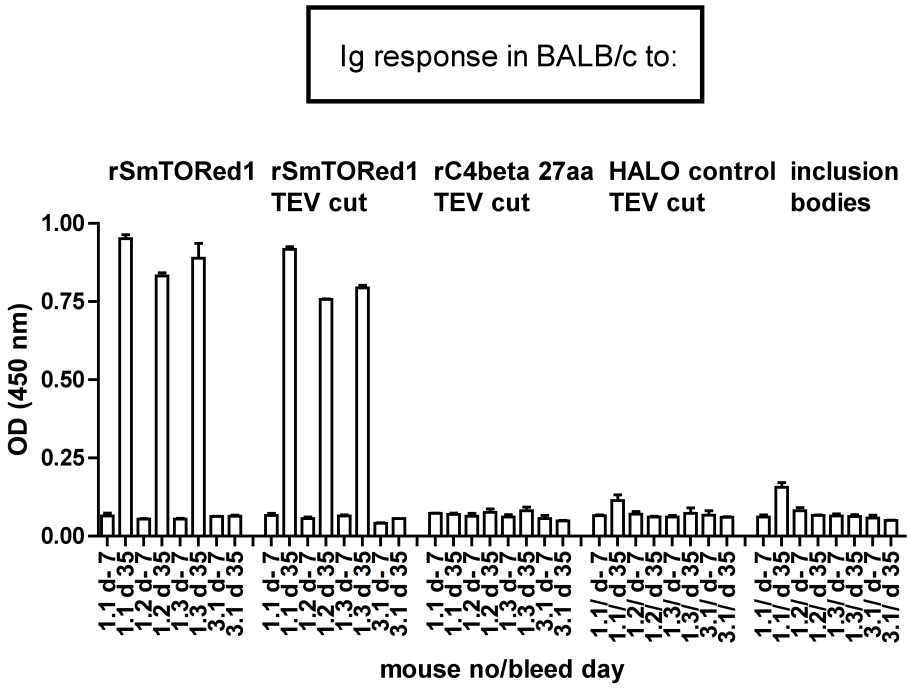


Figure 6. Total Ig detection in sera of individual BALB/c mice immunised with rSmTORed1 (n =3; mice 1.1 - 1.3) and a control mouse injected with PBS only (mouse 3.1). Pre-immune sera (bleed day -7) and sera obtained after the first boost (day 35) were diluted 1:25'600. Ig response measured to rSmTORed1, to TEV cut rSmTORed1, rC4beta 27aa and HALO control (purified as Halo-tagged peptides or Halo-tag alone subsequently cut with TEV protease- peptide sequences shown in Fig. 4A) and to *E. coli* inclusion bodies. Results are expressed as means of individual measurements. Error bars indicate S.D. of the means.

## DISCUSSION

Complement C2 had been first identified as a ligand of *S. haematobium* TOR by receptor affinity chromatography (*2*). ShTORed1_27aa was subsequently shown to bind C2 by chemical cross-linking assay, using the homologous C4β chain sequence as binding control, and to interfere with C2 cleavage by C1s and thus prevent its interaction with C4b resulting in inhibition of CP C3 convertase activity. Consequently there is a reduced hemolysis of sheep erythrocytes (SRBC) in the presence of peptide (*3*).

We were investigating the C2 binding to the C-terminal 27 amino acids of SmTORed1 (SmTORed1_27aa). The sequences of ShTORed1_27aa, that has been studied before, and SmTORed1_27aa are not identical (Fig. 1A). We show, that SmTORed1_27aa binds C2 in a concentration dependent manner in an ELISA setup when the peptide was conventionally immobilised in carbonate buffer (Fig. 1B), or to biotinylated SmTOR_27aa captured on a NeutrAvidin coated plate (Fig. 1C). This suggests, that C2 might bind to the C-terminal 27 amino acids of *S. mansoni* and *S. haematobium* TOR ed1 domain.

In previous work it was shown that the complement inhibitory characteristics of ShTORed1_27aa were also found, and even to a higher degree, in the shorter H17 peptide (11 amino acids). This sequences was very similar to a sequence found in $C4\beta^{222-232}$ which inhibited complement as well. Here we could not demonstrate in an ELISA assay any specific binding of C2 to this $C4\beta^{222-232}$ chain and the Sm/ShTOR H17 peptide (Fig. 3E).

These later results contrast with the binding of the long peptide (27 aa) of both SmTOR and ShTOR, which despite two amino acid difference had the same sequence for the 11 aa corresponding to H17. Thus it is likely, that the 11 aa are involved in C2 binding but for us not demonstrable because of non-significant binding or buffers or other interactions due to biotin for example. Of interest the binding of the biotinylated 27 aa peptides were strongly dependent on buffers. We had to increase the salt concentration to 180 mM NaCl, as compared to the one found in 1 x PBS routinely used in the lab (150 mM). In 1 x PBS we did only observe unspecific C2 binding to

the peptides, and using a 1 x PBS buffer containing 250 mM NaCl did abrogate all the C2 binding. This suggests, that specific C2 binding to the 27 aa peptide in an ELISA system only occurs under certain conditions with salt adjusted in 1 x PBS when peptide is presented in solution (bound to NeutrAvidin) and only to the full-length 27 aa sequence. In agreement with previous work (*19*), this binding was not magnesium dependent.

These data emphasise, that the interactions between C2 and SmTOR are very dependent on the physicochemical environment. However, that the 27 aa sequence might bind better than the 11 aa sequence (H17) could be related to more homologies between a larger stretch of $C4\beta^{212-231}$ chain with the 27 aa sequence of SmTOR than the homologies between the shorter $C4\beta^{222-232}$ chain sequence and H17.

Because we planned to test the immunogenicity of the SmTORed1 domain, we set up a system to overexpress and purify it. Recombinant His-tagged SmTORed1 was produced in defined rich media by auto-induction (*16*), because the overexpression of rSmTORed1, with its ORF under the control of the lac repressor on the pET15b plasmid (*20*) was not manageable when bacteria were grown in LB medium and protein expression was induced with IPTG. This was possibly due to the basal expression of rSmTORed1 that was toxic for the bacteria. By using auto-induction media for bacterial overexpression, we could circumvent this problem and in addition produce high amounts of proteins in cultures reaching high densities without detrimental effects on bacterial viability.

His-tagged SmTORed1 needed to be purified to yield a not too highly concentrated protein solution in order to avoid protein precipitation in the course of denaturant removal, that was most likely due to the formation of peptide dimers visible in highly concentrated eluates (Fig. 2C). This phenomenon was also observed to occur in the case of the vaccine candidate Sm14, where protein dimerisation and subsequent aggregation led to a reduction in vaccine efficacy (*21*). Optimised rSmTORed1 purification under denaturing conditions and refolding in to 1 x PBS under optimised conditions yielded reasonable amounts of soluble protein of up to 150 ng/µl (Fig. 2B) that could then be used in immunisation experiments (Part III) and ELISA

assays (Fig. 2D, 2E, 3, 5).

Purified rSmTORed1 was functional in binding monoclonal anti-ed1 (Fig. 2D) and did also bind C2 (Fig. 2E). We therefore screened by ELISA sera of *S. mansoni* infected patients and normal human sera for the presence of anti-rSmTORed1 antibodies (Fig. 3) and found 25 % of patients, but also 10 % of uninfected individuals to be positive. Because the positive signal threshold of fold-increase values indicated was estimated arbitrarily, we wanted to ensure antibody specificity by competing real positive signals away by pre-incubation with soluble peptide. To this end we produced SmTORed1 as a Halo-tagged fusion protein (Fig. 4). HALOed1 and the two control constructs could be purified from the soluble *E. coli* fraction were after immobilisation on magnetic beads and several washing steps readily used for different downstream applications. HALOed1 was specifically recognised by Western blot with monoclonal anti-ed1, but not HALO and HALOC4beta (Fig. 4C).

Sera of patients and uninfected humans that bound to rSmTORed1 (Fig. 3) were pre-incubated either with HALO or HALOed1 on magnetic beads and ELISA was then performed with the sera depleted of specific antibodies by removal of the beads. With this assay, we were able to verity that the antibodies detected in 2 patients and 2 uninfected individuals were specific for immobilised antigen (Fig. 5). These individuals were the ones with the highest signal measured before (Fig. 3). This does not exclude, that some specific antibodies were present in others, but with this stringent method we are in the position to produce a convincing result. Healthy individuals displaying specific antibodies against rSmTORed1 might have been in contact with different bird schistosomes (belonging to the genus of *Trichobilhariza* in most of the cases). *Trichobilharzia* presumably die in the human skin after penetration of its non-specific host thereby causing an inflammatory skin reaction also known as swimmer's itch (*22*).

SmTORed1 can be cleaved off the magnetic beads with TEV protease when produced as HALOed1, yielding a peptide with the SmTORed1 sequence with only 6 residual N-terminally attached amino acids (Fig. 4A) instead of a His-tag and a 10 aa linker (Fig. 2A) as it is the case for rSmTORed1. Both peptides, rSmTORed1 and TEV protease cut SmTORed1

were recognised by sera of mice immunised with rSmTORed1 (Fig. 6). Mouse Ig to rSmTORed1 did not cross-react with TEV cut rC4beta peptide sequence. No Ig bound on the immobilised inclusion body fraction indicating that a fairly clean preparation of rSmTORed1 had been used for immunisation.

In summary, we were able to produce purify functional recombinant SmTORed1 either as His-tagged protein, that was used for vaccination experiments (Part III), or Halo-tagged fusion protein. With this we were able to show that the long SmTORed1 loop containing the C2-binding motif of ShTORed1 also binds C2 (Fig. 1) and that some schistosomiasis patients and uninfected individuals have specific antibodies against rSmTORed1 (Fig. 5). SmTORed1 immunogenicity has been tested in mouse a murine vaccination model described below (Part III).

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**PART III: Recombinant SmTORed1 tested in a murine vaccination and challenge model confers protection against schistosomiasis**

Results published in: *Clinical & Experimental Immunology,* (2012), **170(3)**, 342-357

ABSTRACT

The search for a vaccine against schistosomiasis is a desirable goal, since there is currently only one chemotherapeutic available for the treatment of schistosomiasis. We had previously characterised SmTOR as an antigen expressed highest on the tegumental surface of *S. mansoni* cercariae. We tested its surface exposed extracellular loop 1, SmTORed1, as a potential vaccine candidate in the murine schistosomiasis model. Recombinant SmTORed1 was tested in two different mouse strains, BALB/c and C57BL/6, in the presence of either Complete/Incomplete Freund's adjuvant (CFA/IFA) or muramyl dipeptide (MDP) as adjuvant. Only BALB/c mice receiving rSmTORed1 mixed with CFA/IFA generated significantly high specific antibody titers. Vaccination with rSmTORed1 induced a Th1-Type of immune response in BALB/c mice and was protective against parasite infection. A significant decrease in worm burden in the immunised animals versus the control groups was recorded. Thus, we consider rSmTORed1 to be an interesting anti-schistosome vaccine target to be followed up in additional experiments.

## INTRODUCTION

Despite over a decade of research, praziquantel (PZQ) is the only chemotherapeutic available for treatment of schistosomiasis effective against all five schistosome species infecting humans (*1*). The concern about the emergence of developing drug resistance (*2*) and the biphasic sensitivity of the parasite to the drug (*3*) with juvenile worm stages being insensitive to drug treatment (*4*), are reasons pushing the search for new compounds with schistosomicidal activity. But chemotherapy as such offers other additional disadvantages besides the development of drug resistance, such as the high costs generated and the lack of protection against re-infection after treatment (*5*). Consequently there is still plenty of reasons to aim for the development of a vaccine against schistosomiasis, although it has been stated already more than ten years ago to be difficult, but an achievable goal (*6*).

In the mid-1990ies, the WHO suggested six vaccine candidates to be tested independently for their efficacy against *S. mansoni* infection in a murine vaccination challenge model (*7*). Of those, Sm14 or Fatty Acid-Binding Protein (FABP) has clearly run the fastest through the vaccine development process involving important aspects of scale-up and industrial production and with Phase I and II clinical trials granted (*8*). In addition, there is a list of new proteins including those discovered with the help of proteomic analysis of the adult schistosome surface (*9*) and to which, amongst other membrane proteins (*5*), members of the so-called tetraspanin family belong to, four pass trans-membrane proteins whose extracellular loops have been shown to be protective against schistosome infection in the mouse when administered as recombinant vaccine in the case of TSP-1 and TSP-2 (*10*).

Obviously, the most interesting vaccine candidates are transmembrane proteins localised on the *Schistosoma mansoni* tegument due to their accessibility to the host immune response (*11*). Additionally, proteins highly expressed in the early intra-mammalian stages of *S. mansoni* like cercariae and schistosomulae are considered as interesting vaccine targets (*12*).

We tested a new vaccine candidate, the 111 amino acid long extra-cellular loop one of SmTOR (SmTORed1), a membrane protein shown above to be expressed highest in *S. mansoni* cercariae (*13*). We did test

the recombinant peptide SmTORed1 in two different mouse strains, as suggested in standardised WHO vaccine trials before evaluating different candidate antigens for efficacy in a murine vaccination model (*7*) formulated either with Complete Freund's adjuvant (CFA) or with muramyl dipeptide (MDP) as an alternative. We did assess serum antibody levels to evaluate specific immune responses in the different experimental settings. We then performed an immunisation challenge experiment to see whether rSmTORed1 could interfere with *S. mansoni* infection in a mouse model.

MATERIALS AND METHODS

*Immunisation Procedure*

Preliminary round: C57BL/6 and BALB/c mice (Harlan) were used first round of immunisation. 10 animals were immunised with antigen and the corresponding adjuvant, 5 mice in both of the two control groups were either immunised with inclusion bodies or 1 x PBS alone plus the corresponding adjuvant. Immunisation with antigen took place on day 0 followed by boost with antigen at days 21 and 42.

10 μg purified peptide in PBS was used per injection and animal, having 10 animals in the immunised group. Inclusion bodies for injection of the first control group were generated from *E. coli* transfected with pET15b plasmid alone, purified over a $Ni^{2+}$ as done for rSmTORed1 running the same FPLC program established for the peptide purification, followed by re-folding into 1 x PBS as described under Materials and Methods in Section II. As adjuvant, either CFA and ICF (Complete Freund's adjuvant: first immunisation and Incomplete Freund's adjuvant: boosting) or MDP (muramyl dipeptide: first immunisation and boosting) were used. Mice were injected subcutaneously in the neck fold.

Bleeding to monitor antibody response was performed at days -7, 14, 35 and 63 via the tail vein (Fig. 1). Mice were terminated at day 63 and spleens were dissected for generation of spleen cell cultures.

The immunisation challenge experiment was performed using BALB/c mice (Charles River) in combination with CFA/IFA only. The experiment was performed with 10 animals in each group, receiving antigen, inclusion bodies or PBS mixed with adjuvant, as tested in the preliminary round. Challenge with infection took place at day 55 (13 days after the 2$^{nd}$ boost). Mice were infected subcutaneously with 150 *S. mansoni* cercariae via injection into the abdominal skin. At day 105 (7 weeks post infection) mice were sacrificed and parasite status was assessed by evaluation of the adult worm burden. Worms were dissected from the mesenteric veins and livers of the mice. Protection values were calculated as described elsewhere (*14*). The spleens were

removed in order to generate spleen cell cultures.

Serum samples to assess antibody response were taken at days -7, 14, 35 as in the preliminary round (pre-challenge) and between days 105 and 107 (post-challenge) (Fig. 1).

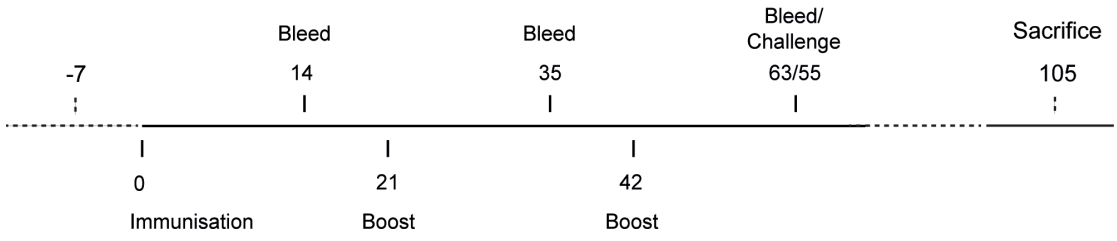


Figure 1. Schedule murine vaccination and challenge model. Numbers indicate the days at which manipulations were done. Numbering starts at day 0, at the time point of the first injection. Mice were boosted twice in 3 week intervals and bleeded at the days indicated.

*Mouse Serology*

Mouse sera obtained at different time points were collected (Fig. 1) and specific antibody titers against rSmTORed1 were measured using an ELISA assay. For this, 96 well flat-bottom microtiter plates (Nunc) were coated overnight at 4 °C with 500 nmoles rSmTORed1 peptide per well in 100µl 0.1 M carbonate bicarbonate buffer, pH 6.8. The plates were then blocked in 1 x PBS containing 0.05 % Tween 20 (PBST)/ 5 % BSA for 1 h, 37 °C. Plates were washed 5 x with PBST between the different incubation steps. Serum samples were diluted in PBST at the appropriate dilution determined by serial dilutions for individual samples of each of the possible mouse strain adjuvant combinations and the different detection antibodies (described below), and 100 µl were then added per well. Plates were incubated for 1 h at RT, and after washing the biotinylated primary antibody diluted in PBST was then added for another hour at RT. Bound antibody was detected with streptavidin-HRP (Pierce) diluted 1:10'000 in PBS (30 min, RT) and the ELISA was developed by incubation with a TMB peroxidase substrate (BD Pharmigen). The absorbance was measured at 450 nm.

Biotinylated detection antibodies used were: Goat anti-mouse

IgG+IgM+IgA (ab6005, AbCam) and goat anti-mouse IgG (ab5868, AbCam) both diluted 1:10'000. Goat anti-mouse IgM (ab5929, AbCam) was diluted 1:20'000. Serial dilutions were performed for the individual mouse strain adjuvant combinations as shown in Fig. 2 for BALB/c mice immunised with CFA/IFA. For Ig serology (IgG + IgM + IgA determination) sera of BALB/c CFA/IFA groups were diluted 1:25'600; C57BL/6 CFA/IFA sera were diluted 1:6'400 and sera of MDP vaccinated mice were diluted 1:3'200 for BALB/c and C57BL/6 mice. For IgG and IgM determination in BALB/c CFA/IFA vaccinated mice, sera were diluted 1/12'800 or 1/3'200 respectively, as determined by endpoint titrations as well (data not shown).

*Spleen Cell Culture*

Freshly removed spleen was cut into small pieces and the suspension pressed through a 70 μm nylon mesh (BD Falcon, #352350) into a 15 ml falcon tube containing RPMI medium supplemented with 5 % FCS (RPMI/FCS). Cells were pelleted, resuspended in 5 ml lysis buffer (0.15 M $NH_4Cl$, 15 mM $KHCO_3$, 0.1 mM $Na_2EDTA$, pH 7.4) and incubated 5 min at RT. Cell lysis was stopped by adding 10 ml RPMI/FCS and centrifuged 10 min, 200 x g at 4 °C. Cells were resuspended in complete medium (RPMI/FCS + 2 mM L-glutamine, 50 μM beta-mercaptoethanol, 100 U/ml Penicilin, 100 μg/ml streptomycin sulfate + 250 ng/ml amphotericin B + 30 μg/ml polymyxin B) and cell number adjusted 1 x $10^7$ cells per ml. Cells were cultured at 1 x $10^6$ cells/ml in 200 μl in a 96-well tissue culture plate, either stimulated with 10 μg/ml rSmTORed1 or 5 μg/ml Concanavalin A. Cell culture supernatants were collected after 48 h of stimulation for IL-4 and TNFα analysis and after 72 h for IL-10 and INFγ. Mouse cytokine ELISA measurements were performed using ELISA Sets (BD OptiEA™).

## RESULTS

### *Immunisation with rSmTORed1 generates a humoral immune response in BALB/c and C57BL/6 mice*

We immunised two different mouse strains with rSmTORed1 formulated with CFA/IFA or MDP as adjuvant according to the immunisation schedule in shown in Fig. 1. Serum samples were analysed before the injection and after the first boost in order to assess the amount of total Ig generated in the individual groups. Serial dilutions of individual sera of immunised versus control immunised animals (groups injected with adjuvant only) were tested for antibodies against rSmTORed1. Immunisation with CFA/IFA generated higher levels of anti-rSmTORed1 levels in C57BL/6 and BALB/c mice (Fig. 2), than immunisation with MDP. Therefore, these sera were subsequently analysed at higher dilutions. Specific antibodies in CFA/IFA BALB/c mice were detected at a dilution as high as 1:1'638'400 as compared to the control vaccinated mice (Fig. 2). MDP vaccinated mice did generate very low amounts of anti-rSmTORed1 Ig and sera were therefore tested at a low dilution, as determined experimentally (data not shown).

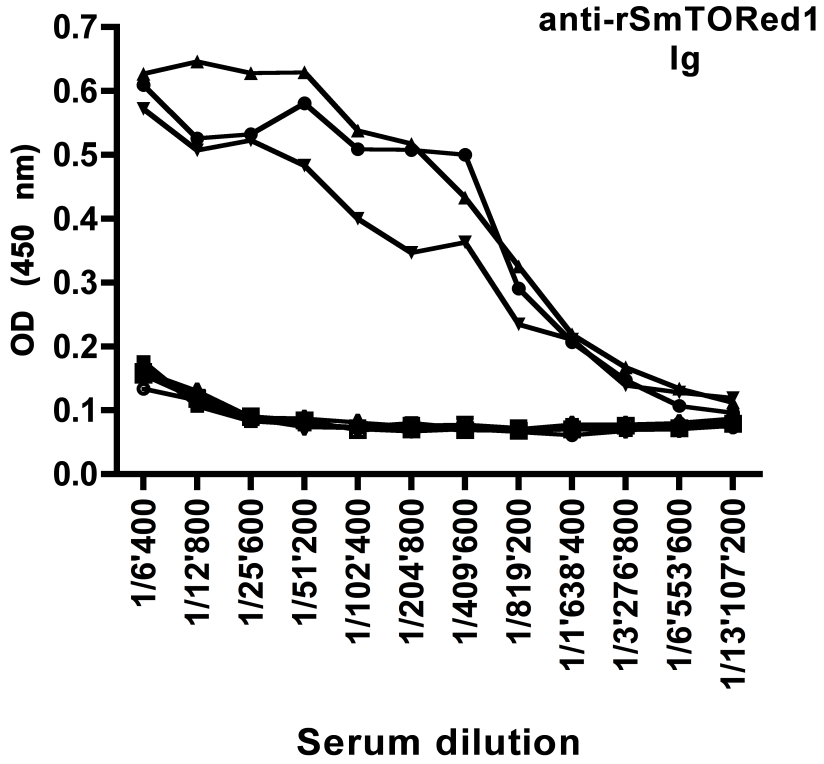


Figure 2. Endpoint titration of sera from four individual mice before (day -7, open symbols) and after (day 35, filled symbols) immunisation analysing anti-rSmTORed1 Ig (IgG, IgM, IgA) responses. Data points are represented using triangles and dots for the three BALB/c mice immunised with antigen and CFA/IFA; values for the control mouse immunised with CFA/IFA/PBS only are shown as squares.

*BALB/c but not C57BL/6 mice immunised with antigen in CFA/IFA generate significantly high antibody titers unlike the MDP immunised animals*

After two injections with antigen and the corresponding adjuvant, mice of both strains immunised with CFA and boosted with IFA as well as BALB/c mice immunised with MDP showed a significant increase in Ig levels compared to levels in pre-immune sera (Fig. 3A and 3B, 3C). In C57BL/6 mice immunised with MDP anti-rSmTORed1 Ig levels did not increase significantly (Fig. 3D). Yet, only BALB/c mice injected with antigen in CFA/IFA had significantly higher Ig levels at day 35 when compared to inclusion body ($p = 0.0002$) and PBS injected mice ($p = 0.0002$) at the same time point (Fig. 3A). Sera were diluted 1:3'200 for Ig analysis of MDP vaccinated strains (Fig. 3C and 3D) or 1:6'400 (Fig. 3B) and 1:25'600 (Fig. 3A) for CFA/IFA immunised mice. These dilutions were determined experimentally by titration of sera as shown in Fig. 2 and an appropriate dilution for screening of sera set according to OD values at 450 nm still within but near the boarder of the plateau reached at low serum dilutions. As per definition serum dilution is inversely correlated to the amount of antibody in a sample, low dilutions tested in MDP immunised mice and CFA/IFA immunised C57BL/6 mice reflect the relative weak immune response provoked (Fig. 3B, 3C and 3D) as compared to the one generated in BALB/c mice immunised with CFA/IFA (Fig. 3A).

None of the control animals, both mice receiving inclusion bodies together with adjuvant and mice receiving adjuvant mixed with buffer only, did show an increase in anti-rSmTORed1 specific immune response.

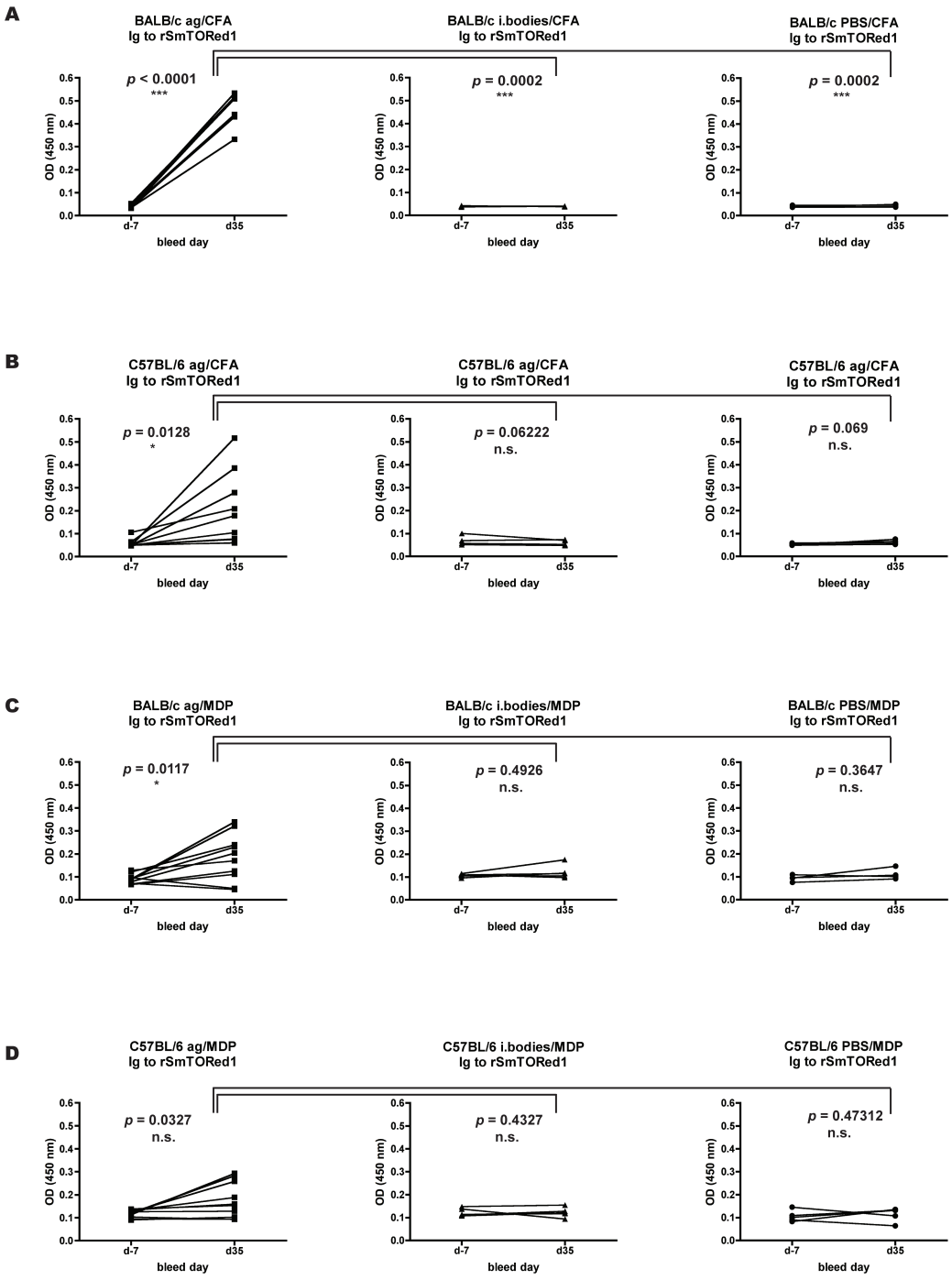


Figure 3. Total Ig amounts in sera of individual mice immunised with rSmTORed1 (antigen, ag; n = 10) and the control groups injected with inclusion bodies (i. bodies; n = 5) or PBS (n = 5) monitored at day -7 and day 35 of the trial. Sera for the ELISA assay were diluted 1:25'600 (A), 1:6'400 (B) or 1:3'200 (C and D) as determined by endpoint titrations.
Statistical analyses were performed with Student's t-test: n.s. statistically not significant; statistically significant * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

*Peptide specific immune response can be boosted in BALB/c mice in every single animal, but not consistently in C57BL/6 mice*

BALB/c mice responded to the second booster injection with peptide and IFA with a further increase in serum Ig to rSmTORed1 (Fig. 4A). Every single animal shows a slight increase of serum Ig, whereas the control Ig levels in the control animals do not increase markedly. This observation was not made in C57BL/6 mice. We didn't observe a uniform response to vaccination neither after the first nor after the second booster injection looking for anti-rSmTORed1 Ig (Fig. 4B).

An increase in anti-SmTORed1 IgG (Fig. 5A) and IgM (Fig. 5B) subclass antibodies was detectable after the first and second booster of BALB/c with CFA/IFA.

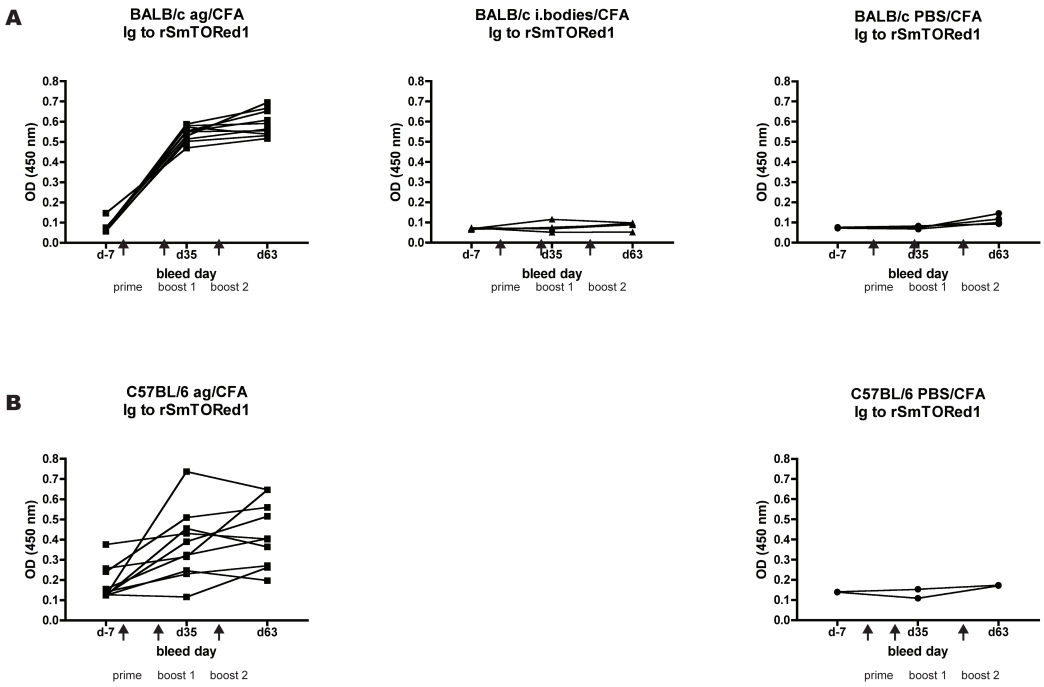


Figure 4. Ig (IgG, IgM, IgA) serology at days -7, 35 and 63 comparing (A) BALB/c with (B) C57BL/6 mice immunised with rSmTORed1 (antigen, ag; n = 10) or control groups immunised with inclusion bodies or buffer only (i. bodies and PBS; n = 5 or 2) using CFA/IFA as adjuvant. Sera for the ELISA assay were diluted 1:25'600 (A) or 1:6'400 (B). Arrows indicate injections at days 0 (prime), 21 (boost 1) and 42 (boost 2).

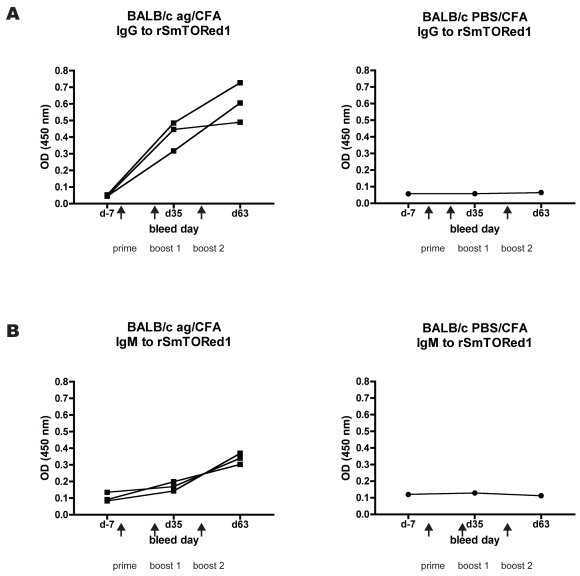


Figure 5. (A) IgG serology and (B) IgM serology at days -7, 14 and 35 of BALB/c mice immunised with rSmTORed1 (antigen, ag; n = 3) using CFA/IFA as adjuvant; serum dilutions 1:12'800 for A and B. One representative sample of a PBS immunised mouse is shown as negative control for each isotype. Arrows indicate injections at days 0 (prime), 21 (boost 1) and 42 (boost 2).

*rSmTORed1 CFA/IFA induced antibody response persists in BALB/c mice after challenge with* S. mansoni *infection*

In an immunisation challenge experiment, we repeatedly observed an increase in peptide specific Ig when rSmTORed1 was administered together with CFA/IFA. Analysis of Ig response in mouse sera before the first boost (day 14) shows, that antibody titers only start to rise after applying the second dose of antigen (Fig. 6A). Antibodies generated against rSmTORed1 persisted during the whole duration of the experiment and levels were not significantly altered in most of the animals after infection with *S. mansoni* cercariae (Fig. 6B). The infection itself did not lead to generation of anti-rSmTORed1 antibodies, as no Ig was detectable in the two control groups.

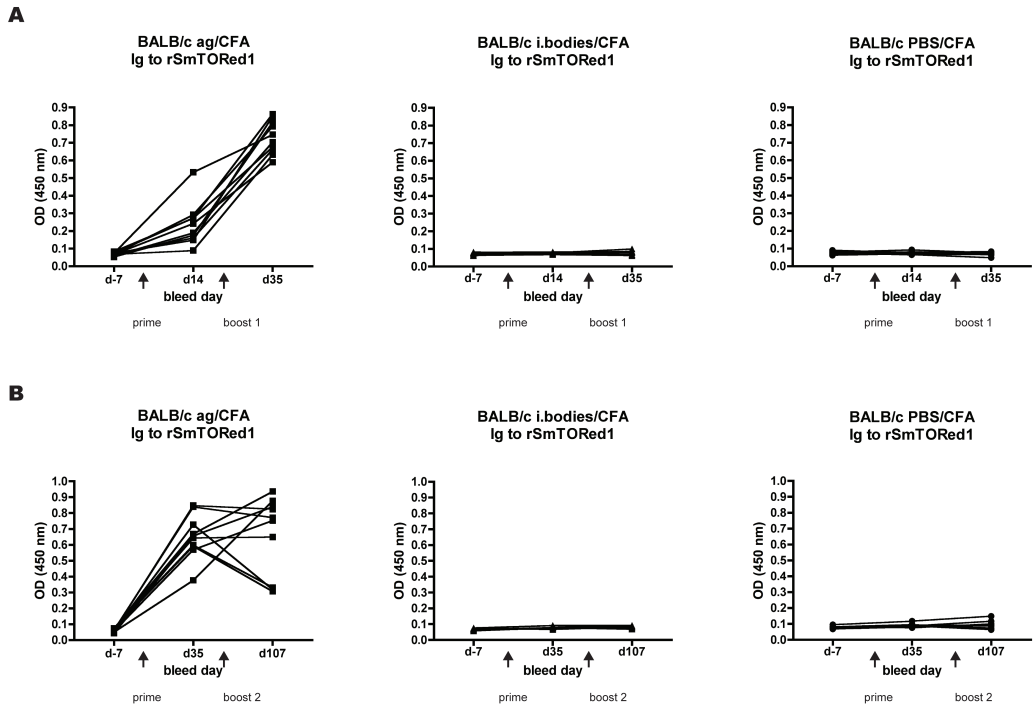


Figure 6. Ig (IgG, IgM, IgA) serology in mouse sera sampled at days -7, 14 and 35 (A) or day 107 in addition (serum dilution 1:25'600). BALB/c mice were immunised with rSmTORed1 (antigen, ag) or control groups immunised with inclusion bodies (i.bodies) or buffer only (PBS) using CFA/IFA as adjuvant. All groups were infected with *S. mansoni* cercariae at day 55. Groups consisted of n=10 animals. Arrows indicate injections at days 0 (prime), 21 (boost 1) and 42 (boost 2).

*Cytokine secretion of spleen cell cultures of immunised mice induced by rSmTORed1 does not differ in mice after immunisation and challenge*

Spleen cell cultures of BALB/c mice immunised with rSmTORed1 in CFA/IFA (Fig. 7A) or mice immunised with peptide and then challenged by infection with *S. mansoni* cercariae (Fig. 7B) were tested for cytokine release upon stimulation with rSmTORed1. Spleen cell cultures of immunised animals performed 14 days after the second booster with antigen produced significant amounts of IFN$\gamma$ and IL-10 when stimulated with rSmTORed1, as compared to the control spleen cell cultures originating from control vaccinated (PBS) animals (Fig. 7A). The IL-4 and TNF$\alpha$ levels after stimulation were not detectable. IFN$\gamma$, IL-10 and IL-4 but not TNF$\alpha$ were produced in all the spleen cell cultures tested when stimulated with Concanavalin A. This cytokine profile generated in immunised versus control animal spleen cell cultures after stimulation with purified antigen was not changed markedly after challenge with infection (Fig. 7B). The difference in the amount of cytokines detected was due to different experimental conditions, as verified by measuring an old reference sample together with the new samples. The infection itself does therefore not seem to alter the cytokine profile elicited by stimulation of the spleen cell cultures with rSmTORed1. We conclude, that vaccination with rSmTORed1 induces a Th1 type of immune response in mice that is not altered after infection.

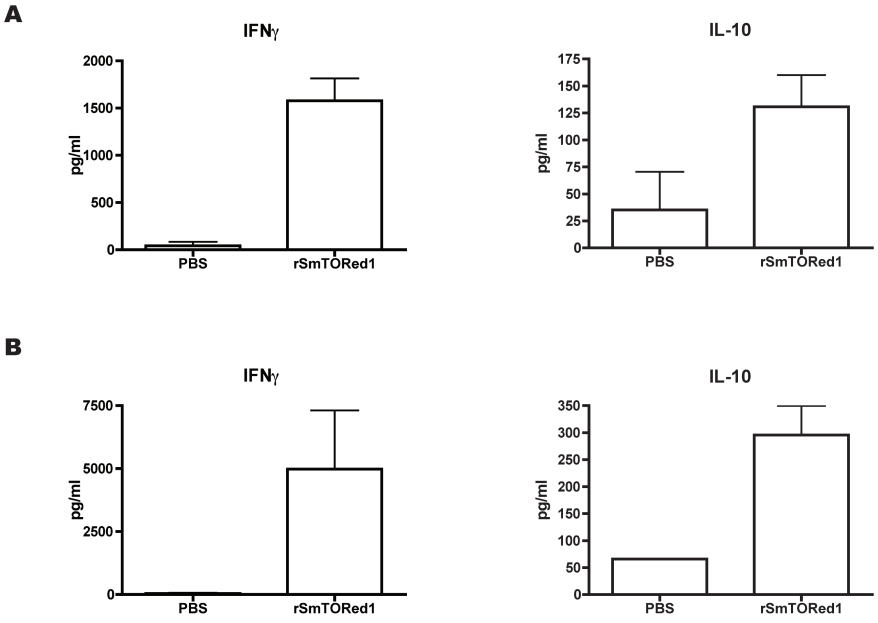


Figure 7. Measurement of cytokines in the supernatant of spleen cell cultures after stimulation with rSmTORed1 (A) 14 days after the second boost or (B) after immunisation challenge experiment after 8 weeks (d 114) of infection. Number of spleens analysed: (A) n = 3/2 (rSmTORed1/PBS), (B) n = 2/2 (rSmTORed1/PBS). The bars represent the mean values ± S.D. of either 3 or 2 (rSmTORed1/PBS) different samples measured in triplicates each.

*Vaccination of BALB/c with rSmTORed1 induces significant protection against challenge with* S. mansoni *cercariae*

BALB/c mice were vaccinated with rSmTORed1, inclusion bodies or PBS only mixed with adjuvant (CFA/IFA) according to the immunisation schedule evaluated before (Fig. 1), challenged with infection by injection of *S. mansoni* cercariae and status of infection evaluated by analysing total worm burden after the whole duration of the experiment (7 weeks of infection). We found, that mice immunised with rSmTORed1 showed a significant reduction in worm burden when compared to both the control groups (Fig. 8). Vaccination resulted in 57 % reduction of worm burden when compared to the mice injected with the inclusion body fraction and a reduction of 62 % when compared to the PBS control mice. This difference could be due to fact that the inclusion body fraction might contain some impurities even after the sample cleanup by chromatography and dialysis that are able to slightly alter the immune response to infection in mice.

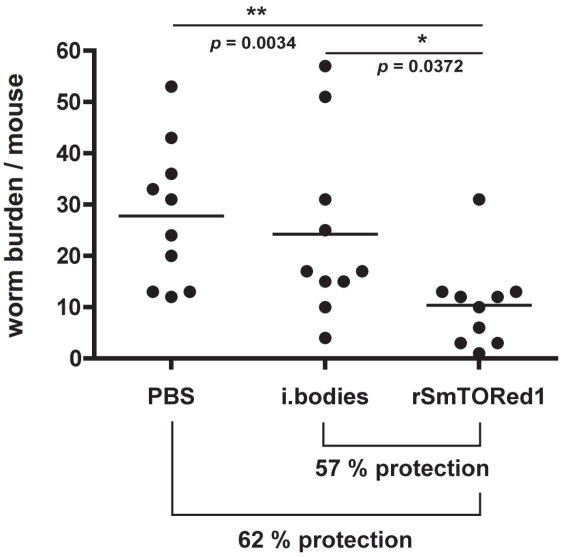


Figure 8. Scattergram of total worm burden of BALB/c mice that were immunised with adjuvanted (CFA/IFA) rSmTORed1, inclusion bodies (i.bodies) or PBS and challenged with *S. mansoni* cercariae. The number of animals used per group was n = 10. Mean worm burden ± S.D.for the different groups were 27.8 ± 13.9 (PBS), 24.2 ± 17.4 (inclusion bodies) and 10.4 ± 8.6 (rSmTORed1). Statistical analysis were performed using Student's t-test (unpaired): statistically significant * ($p < 0.05$), ** ($p < 0.01$).

## DISCUSSION

The overall humoral immune response against a peptide used for vaccination in a murine vaccination model depends on the intrinsic immunogenicity of the peptide, on the mouse strain and on the type of adjuvant used. We did evaluate that in our setting by measuring the amount of Ig generated against rSmTORed1.

BALB/c mice mounted a high response, when SmTOR extracellular domain 1 was produced as a recombinant peptide and was mixed with CFA/IFA for immunisation (Fig. 3A). Using the same adjuvant in C57BL/6 mice generated a less high antibody titer, as sera were diluted 1:6'400 as compared to 1:1:25'600 for Ig detection (Fig. 3B). When injected with MDP, rSmTORed1 is little immunogenic both in BALB/c and C57BL/6 mice (Fig. 3C and D).

It is no big surprise that murmamyl dipeptide, an isolated cell wall component from *M. tuberculosis* targeting NOD-2 receptor (*15*) is not such a strong adjuvant as Complete Freund's adjuvant, a water in oil emulsion containing the whole inactivated mycobacterium (*15*). Furthermore, one can expect a differential innate immune response in the context of varying T-helper cell polarisation induced with regards to cytokine production, in two mouse strains with different genetic background and either prototypical Th1- (C57BL/6) or Th2- (BALB/c) type mouse strains (*16*). Schistosome infection indeed induces Th1 cytokines in C57BL/6 mice at the time of parasite residency in the lung and Th2 cytokines in BALB/c mice with however no measurable specific antibody production (*17, 18*). It has been shown that exogenous administration of IL-1 and IL-6 at the that time point had an effect on the specific antibody response against the parasite (adult worm tegumental antigen) with increased antibody levels in BALB/c mice associated with a significant reduction in worm burden, and promoting the opposite effect in C57BL/6 mice (*17*). This might partially explain the difference in responsiveness in terms of generation of a specific anti-SmTORed1 response upon vaccination with a strong pro-inflammatory response provoked by CFA in BALB/c and C57BL/6 mice, but also point to the fact that opposite effects in mice of different genetic background might be a confounding factor in

immunisation challenge experiments. For example, immunisation with recombinant 3-P dehydrogenase emulsified in CFA protected BALB/c but not C57BL/6 mice from infection with *S. mansoni* (*19*).

The anti-rSmTORed1 immune response generated in BALB/c mice could be boosted with a third injection (Fig. 4A) and in these mice, specific IgG and IgM were measured in the context of a successful immunisation (Fig.5). Immunised BALB/c mice used for the infection model also generated antibody titers against rSmTORed1 (Fig. 6A), that did not vanish after the injection of cercariae in the context of the infection (Fig. 6B). The cytokine response in spleen cell cultures of immunised versus control mice, when these cells were stimulated with peptide, did not change if immunisation alone was performed (Fig. 7A) or after immunisation and infection of these mice (Fig. 7B). Immunised mice show protection against infection as compared to the two control groups, mice immunised with inclusion bodies or PBS together with adjuvant, apparent as a 57 % and 62 % reduction in worm burden respectively (Fig. 8). It is of major interest that infected mice without prior immunisation did not develop antibodies against SmTOR indicating that SmTOR is not recognised at the time of infection. This would explain, that few humans infected with schistosomes develop such antibodies as well. In addition, since our data indicate that SmTOR antibodies are protective, it goes without saying that the immunisation with this antigen might represent a real progress in the fight against schistosomes.

We assume, that the antibodies generated are protective against the infection, as an antigen expressed highest in the infectious larvae is targeted by those preventing the migration of the parasite to the lung and further on. Coating cercariae with host antibody might also prolong the dwell time in the skin, where it possibly can be attacked by host complement for a prolonged time period and binding of cytophilic antibodies might enhance the magnitude of the effect.

There are several examples of vaccine candidates that meet the target of a consistent induction of at least 40 % protection in the mouse model, as defined by the TDR/WHO program (*7*). Amongst those are original members of the six vaccine candidates proposed by the WHO to be tested independently by different labs (*12*) as well as new vaccine targets discovered

not least due to the various proteomic analysis of adult worm surface proteins (*9, 20, 21*). Recombinant Sm14 or FABP, a member of the "old guard" of vaccine candidates that induces high protection levels in Swiss outbred mice (*22, 23*), is now in the clinical trial phase (*8*). Old candidate number two mentioned here, Sm23 on the other hand did not confer protection as a recombinant protein (*24*), but only when delivered as DNA vaccine (*25*). Sm23 is a member of the so-called tetraspanin family that are present on eukaryotic cells and are also abundant in the tegument of *S. mansoni* and promising vaccine candidates (*11*). Braschi et al. confirmed the presence of four members of tetraspanins on adult worm surface by proteomic analysis (*20*), all considered as new vaccine candidates. Besides the tetraspanins TSP-1 and TSP-2, whose extracellular loops were tested in CBA mice (*10*) and showed a protective effect in this setting using a low responder mouse strain (*26, 27*), there are other membrane proteins tested in mouse. Sm29 (*14*), and most recently SmStoLP-2 (*28*), reduce worm burden by 51 % and 32 % after immunisation and infection of C57BL/6 mice respectively, both formulated with CFA/IFA. Both proteins are not only detectable in the tegument of adult worm, but more importantly also in skin-stage and lung-stage schistosomula (Sm29) and in 7-day old *in vitro* transformed schistosomula (SmStoLP-2) and therefore accessible as immune targets at an early time point after infection. It has been shown, that anti-rSmStoLP-2 antibodies could impair skin penetration of cercariae (*28*).

*S. mansoni* TOR is also a membrane bound antigen expressed highest even in the first intra-mammalian stage in the parasite cycle. Our work presented here shows, that its extracellular loop injected as recombinant protein protects BALB/c mice against infection with *S. mansoni* (Fig. 8). We therefore suggest to include rSmTORed1 as a member of potential vaccine candidates, that could be administered within a cocktail of antigens (*29*). Immunisation with a mixture of antigens tested and effective in different settings in mouse is most likely to do justice to the even bigger diversity in humans.

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## GENERAL DISCUSSION AND PERSPECTIVES

The genome of the flatworm *Schistosoma mansoni* has been sequenced completely (*1*). This remarkable achievement produced by genomics together with the flood of data generated by the various other "-omics" disciplines, that imply large-scale screens of the *S. mansoni* transcriptome, proteome and glycome (*2, 3*), pave the way for schistosomiasis vaccine research. However, high throughput approaches to identify new vaccine targets also offer disadvantages. In the case of proteomic studies, the detection limits of mass spectrometers also set a limit to the detection of low abundant proteins (*4*) only allowing detection of the most abundant and possibly least meaningful proteins (*5*). Proteins with no inferred functions will not be identified, if peptide searches are performed by using EST assemblies or gene prediction data based on the genome (*3, 4*). Previously, vaccine candidates were identified by the more classical method where highly immunogenic proteins were found by screening cDNA expression libraries (*6*) or whole protein extracts using sera from animals immunised with radiation-attenuated (RA) cercariae (*7*) and resistant versus susceptible infection sera from animal models or humans from endemic areas (*5*).

### *The SmTOR Project*

Schistosoma TOR had been identified by screening an *S. haematobium* adult worm cDNA library by phage display, using passively protective serum from vaccinated baboons (*8*). Based on the ShTOR cDNA sequence the receptor ORF sequence was also described for SmTOR (*8*). Both receptors were depicted as trispanning orphan receptors binding complement C2 and were also referred to as CRIT for complement C2 receptor inhibitor trispanning (*9*). We were therefore at the beginning of the project more interested in the role of SmTOR as a complement regulator rather than its immunogenicity in mice and humans. We then observed, while trying to clone the full-length ORF from *S. mansoni* genomic DNA, that the SmTOR gene is not intronless as stated before (*10*) but contains three large introns, and that

the ORF reported originally was truncated at its 5' end. We were able to complete the molecular organisation of the SmTOR gene and report that SmTOR is a tetraspanning orphan receptor located on the tegument of *S. mansoni* cercariae and adult worm (*11*). We noticed in the first place, that its extracellular domain 1 containing the C2 binding motif (*12*) was predicted to not only consist of 27 amino acids, but instead to be a over hundred amino acid loop reinserting into the membrane with N-terminus, and secondly, that SmTOR is expressed highest in cercariae. We thus reasoned that SmTOR extracellular domain 1 (SmTORed1) might be an interesting new vaccine target.

*Recombinant SmTORed1 Vaccine Potential in the Murine Vaccination and Challenge Model*

Regardless of the approach by which a putative vaccine candidate against schistosomiasis is identified, all vaccine candidates have to be expressed as proteins for testing of protection in an animal model (*13*). We consequently established the overexpression and purification of rSmTORed1 produced as His-tagged peptide in *E. coli*. In a first round, we tested the immunogenicity of purified rSmTORed1 in different mouse strains in combination with different adjuvants. As we saw, that highest specific antibody titers were generated in BALB/c mice immunised with antigen and CFA/IFA, we chose this mouse strain for the ensuing immunisation challenge experiment. We found the immunised mice to show 57 % and 62 % reduction in worm burden when compared to the inclusion bodies and PBS injected control groups. With this we demonstrated that rSmTOR is a potent vaccine in the murine schistosomiasis model.

We agree with others that it is unlikely that there is one magic bullet that can induce high levels of protection akin to the one provoked by immunisation with RA cercariae in the animal model (*14, 15*) and that a mixture of different peptides should be administered as antischistosomal vaccine (*13*). We wanted to evaluate in our work, if rSmTORed1 has the potential to induce protective immunity in the animal model and should be included in a list of antigens for a vaccine cocktail. Based on our preliminary

date we strongly suggest to do so, since rSmTORed1 in deed is an interesting vaccine candidate that confers significant resistance (57 - 62 %) against challenge infection. However, it is clear that the data produced in the murine schistosomiasis model cannot be extrapolated to the human condition (*16*).

Mice infected with laboratory strains of *S. mansoni* comprise of homogenous groups of animals with the same genetic background, challenged once with a defined *S. mansoni* isolate at an exactly defined dose after being immunised. Human schistosomiasis on the other hand affects a heterogeneous population, that most of the times gradually acquires schistosome infections of varying intensity (*16*). All these differences itemised contribute to the difficulty of the task to develop an effective vaccine against schistosomiasis. To date, there is no vaccine candidate against schistosome infection that is released for use in endemic areas (*17*). This situation will not change in the foreseeable future (*18*).

*SmTOR and Human Schistosomiasis*

In human schistsosomiasis, it is of substantial interest to find markers for resistance and susceptibility to re-infection. These basically depend on the polarisation of the helper T cell subsets towards a Th1 or Th2 phenotype marking the type of Ig isotypes generated. This process depends amongst other things on the nature of antigen and the genetic composition of the individual (*19*). Whereas in most of the animal models a Th1 based mechanism seems to induce protection against schistosomes, the picture is not that clear in humans where acquired resistance can also correlate with the production of specific antibodies generated in a Th2 environment (*20*). No immune mechanism or specific antigens that are strongly associated with protection have been identified. An in vitro correlate study investigating cellular and humoral immune responses to *S. mansoni* vaccine candidate antigens in fact showed, that immune response profiles correlating with protection or susceptibility were unique for each molecule tested (*20*). That of course has an influence on the type of immune reaction one aims for

vaccination, since induction of the wrong or undesired immune response might lead to enhanced susceptibility or more serious pathology. The type of immune response generated can be modulated with different adjuvants. Unfortunately, the number of adjuvants allowed for use in humans is limited complicating the generation of the desired immune response upon vaccination.

Despite the fact, that two antigens when administered as a peptide vaccine require two completely different cytokine milieus in order to induce protection against infection, we attempt to speculate that in the case of rSmTORed1 the desirable antibody response in humans and mice might be the production of complement fixing antibodies. The presence or absence of ineffective and effective antibodies targeting the migrating larvae might be important (*21*). In deed, it has recently been shown for the two surface exposed proteins TSP-2 and SmStoLP-2, that show efficacy in the murine vaccination and challenge model, that putatively resistant individuals have high IgG1 and IgG3 titers against the recombinant antigen (*22, 23*).

In order to examine the occurrence of specific anti-SmTORed1 antibodies, we were able to set up an ELISA measuring antibody response in infected versus non-infected humans. We found specific antibodies, as confirmed by a competition type ELISA performed with the Halo-tagged SmTORed1 domain, in a low percentage of infected and also in normal human sera. We did not expect the number of positive patients to be higher in infected individuals, because *S. mansoni* cercariae do not seem to die in the skin to such a high percentage that would make antigen available for the induction of an effective humoral immune response. The fact, that also uninfected individuals can have specific antibodies against rSmTORed1 we interpreted as such, that these individuals might have been in contact with bird schistosomes. *Trichobilharzia* is a member of the schistosome genera usually infecting birds.

It would of course be nice to get a grip on well-defined patient cohorts, both putatively resistant and susceptible with respect to *S. mansoni* infection or people with a history of *Trichobilharzia* infection. We could then evaluate, if the people that have antibodies against crude cercarial extract (*24*) also recognise rSmTORed1. That would hint towards the existence of a

SmTOR receptor homologue in *Trichobilharzia.* The mitochondrial genome of *T. regenti* has been sequenced (*25*), but not the whole genome. It would therefore require alternative approaches besides PCR amplification to eventually identify a new SmTOR receptor homologue in *Trichobilharzia*.

*Biological Activity of SmTOR*

We have a strong interest to follow up on the biological function of SmTOR. We were able to show that rSmTORed1 also binds C2. We were so far not able to show the whole molecule to be functional on the cell surface. Several other complement regulators have been show to protect CHO cells bearing the receptor on its surface against complement attack (*26-28*). Up to date, we were not able to generate a CHO cell line that is overexpressing SmTOR on the cell surface. We moreover observed in transient transfectant, that the C-terminally GFP tagged receptor was not properly delivered to the cell surface membrane. This was also the case when transiently transfecting HeLa or HEK cells. We are still working on the SmTOR receptor overexpression in CHO cells, where we have a established an LDH release assay to measure complement mediated lysis. We want to see whether SmTOR overexpressing cells are less susceptible to complement attack than the wild type cell line.

Future plans include to identify possible new ligands, including the functional C2 analogue FB (*29*), of rSmTORed1 by receptor affinity chromatography and to eventually identify other biological functions of SmTORed1.

Taken together, we managed to newly characterise the *S. mansoni* TOR receptor and to show that it is a potential target for vaccination against schistosomiasis.

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