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Vaccination with *Strongyloides ratti* heat shock protein 60 increases susceptibility to challenge infection by induction of Th1 response

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ABSTRACT

The control of strongyloidiasis affecting approximately 100 million people – caused by the gastrointestinal nematode *Strongyloides stercoralis* – is still based on anti-helminthic treatment. In the current study we analysed the immune response to *Strongyloides ratti* heat shock protein 60 (*sr*HSP60) as a possible vaccine candidate in the murine system. We show that *sr*HSP60 is a target of both, humoral and cellular response in *S. ratti*-infected mice. Strikingly, vaccination with *sr*HSP60 without adjuvant or with CFA induced a *S. ratti*-specific Th1 response *in vivo* that did not confer protection but slightly increased larval output during challenge infection. Using *in vitro* T cell stimulation assays we provide further evidence that *sr*HSP60 skewed activated T cells towards a Th1 response that interfered with efficient clearance of *S. ratti* infection. Vaccination with alum-precipitated *sr*HSP60, in contrast, overruled the Th1-inducing activity intrinsic to *sr*HSP60, induced a Th2 response, and conferred partial protection against a challenge infection. As *sr*HSP60 is actively secreted by *S. ratti* during all life stages, our findings strongly suggest that *sr*HSP60 induced polarization towards a Th1 response reflects a mechanism of immune evasion by this pathogenic nematode.

1. Introduction

Strongyloides stercoralis is a human gastrointestinal nematode that afflicts approximately 100 million people of the world population with high prevalence in tropical and subtropical regions [1,2]. *S. stercoralis* infection is generally asymptomatic in normal healthy individuals and can remain undetected for decades. A rare but severe complication in immune compromised individuals is the hyperinfection syndrome that is caused by uncontrolled dissemination of infective larvae and is mortal in 87% of the cases [3–6]. As the majority of *S. stercoralis* infections do not induce pathology and thus are never diagnosed, hyperinfection syndrome is often induced accidentally by immune suppressive treatment of patients receiving organ transplantation [7]. Moreover, helminth infections often change the immunological status of their host and therefore the efficient response to vaccinations can be compromised also by mostly asymptomatic infections [8,9]. Thus, establishment of a vaccination conferring protection to *S. stercoralis* infection is desirable despite the asymptomatic nature of the majority of infections.

Because *S. stercoralis* does not reproduce within rodents, the closely related *Strongyloides ratti* is employed to model this infection in mice and rats [10,11]. Infective third stage larvae (iL3) actively penetrate the skin of their mammalian host, migrate percutaneously to the mouth, are swallowed and reach the gut. Here, they moult via the fourth larval stage (L4) to parasitic female adults that reproduce by parthenogenesis. Eggs as well as hatched first stage larvae (L1) leave the host with the faeces by day six post infection (p.i.). Female larvae may directly develop to iL3 stage or moult to free-living adults that mate with free-living male adults [12]. The infection is cleared spontaneously within three weeks in the context of a T helper 2 (Th2) response that is characterized by production of *S. ratti*-specific IgG1, IL-4, IL-5, and IL-13 [10,13]. Mice and rats that had cleared a primary infection remain semi-resistant to subsequent infections, displaying considerably lower worm burden and larval output [11,14].

Abbreviations: iL3, infective third stage larvae; L4, fourth stage larvae; L1, first stage larvae; p.i., post infection; E/S, excretory/secretory; HSP, heat shock protein; qPCR, quantitative PCR; mLN, mesenteric lymph node.

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It was shown before that the immunization of mice with soluble protein antigens derived from *S. ratti* or *Strongyloides venezuelensis* iL3 conferred partial protection to challenge infection [15–17]. Although *S. stercoralis* never proceed beyond the iL3 in mice [18], vaccination with iL3 extract [19,20] or DNA encoding Na+–K+ ATPase protein (*Sseat-6*) [21] induced accelerated killing of *S. stercoralis* iL3 in vaccinated mice.

Searching for more potent vaccine candidates, we analysed the E/S proteins released by *S. ratti* because they are present at the interface between parasite and host. A recent study of *S. ratti* derived E/S proteins revealed over 600 proteins produced by different free-living and infective stages [22]. The proteomic characterization by LC–MS/MS mass spectrometry identified these products amongst others as galectins, proteases, and heat shock proteins. Here, heat shock protein 60 (*sr*HSP60) was identified as an abundant constituent of E/S products derived from *in vitro*-cultured iL3 and parasitic females [23]. Further analysis showed that relative expression of *sr*HSP60 was twofold higher in parasitic females in comparison to free-living females.

Heat shock proteins (HSPs), preferentially called stress proteins, are highly conserved and ubiquitously distributed proteins [24,25]. In addition to their original role as molecular chaperones, HSPs play a central role in host–parasite interaction as they were described as major immune dominant antigens in several infections [26,27]. Vaccination with HSP in helminth infection has been recently used. Immunization of rats with *sr*HSP10 against *S. ratti* infection markedly reduced the larval output in the faeces after challenge infection [23].

Here we analysed the role of *sr*HSP60 in the immune response against *S. ratti* in mice. Although *sr*HSP60 is a target of both the humoral and cellular response in *S. ratti*-infected mice, vaccination with *sr*HSP60 induced a Th1 response that aggravated challenge infection with *S. ratti* iL3. We provide evidence that *sr*HSP60 itself skewed activated T cells towards a Th1 response that interfered with efficient expulsion of *Strongyloides* parasitic adults. Alum precipitation of *sr*HSP60 in contrast, overruled the Th1-inducing activity intrinsic to *sr*HSP60 and induced protection against challenge infections in the context of a Th2 response.

2. Materials and methods

2.1. Animals and parasites

All *in vivo* experiments were carried out at the animal facility of the Bernhard Nocht Institute with permission of the Federal Health Authorities of the State of Hamburg, Germany. Female C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany) and DO11.10 mice were obtained from the University Hospital Eppendorf (Hamburg, Germany). Mice were kept in individually ventilated cages and used at the age of six to eight weeks. The *S. ratti* cycle was maintained by serial passage of *S. ratti* through rats. *S. ratti* iL3 for infection of mice were purified from charcoal faeces cultures as described [1]. Previous to infection, iL3 were stored overnight in PBS supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). *Strongyloides* antigen lysate was prepared from purified iL3 as described [13].

2.2. Expression and purification of srHSP60

The gene encoding *sr*HSP60 was amplified using the primers 5′-TAAGGATCCGGCTAAAGATCTTAAATTTGGAG-3′ and 5′-ACTAAGCTTTTAGAACATTCCACCACCCATTCC-3′ that were designed according to the published nucleotide sequence of *sr*HSP60 [23] and add a 5′ *Bam*HI and a 3′ *Hind*III restriction site to the PCR product. The 1700 bp PCR product encoding for the *sr*HSP60 protein without its mitochondrial leader sequence was cloned into the *Bam*HI and *Hind*III restriction sites of the vector pRSET-B that fuses a N-terminal 6x Histidine tag to the encoded protein. The resulting expression vector pRSET-B-*sr*HSP60 was used for the transformation of competent *Escherichia coli* BL21 (DE3) (Merck, Germany). Positive clones were selected on LB agar-plates containing 50 μg/ml ampicillin. Expression of the recombinant *sr*HSP60 protein was induced by the addition of 0.4 mM IPTG (Sigma–Aldrich) to bacterial cultures in LB medium containing 2.5 mM betain and 0.5 M sorbitol for 3 h at RT. Expressed recombinant *sr*HSP60 was extracted from bacterial cells after lysis in hypotonic buffer (50 mM NaH_2PO_4, 0.5 M NaCl, 0.2% Triton X-100) for 30 min followed by sonification (3 times 1 min) in a Sonicator Sonifier 250 (Branson, Danbury, CT, USA). After ultracentrifugation at 25,000 × *g* for 1 h at 4 °C, the clarified supernatant was mixed with native purification buffer (50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0) and loaded onto a nickel-based resin column (ProBondTM Purification System, Invitrogen, Germany) to capture the His-tagged recombinant protein. The recombinant fusion protein was eluted with native purification buffer substituted with 500 mM imidazole. Eluted protein was passed via PD10 column (GE, Healthcare, Germany) to change buffer to phosphate buffered saline (PBS) pH 7.4 and stored at −20 °C until used. Protein expression and purification were analysed by SDS-PAGE and silver staining. The endotoxin contamination was measured by quantitative Chromogenic Limulus Amebocyte Lysate (LAL) assay (QCL-1000® Karlskoga, Sweden) according to manufacturer's instructions. Protein preparations with endotoxin content below 4 EU/mg protein were used.

2.3. Expression of membrane-bound srHSP60 and murine HSP60 (mHSP60) in COS1 cells

For the expression of *sr*HSP60 on the cell surface of eukaryotic cells, the *sr*HSP60 encoding DNA was amplified with the sense primer 5′-GCTACCCGGGGCTAAAGATCTTAAATTTGGAGC-3′ and antisense primer 5′-CAGTGTCGACGAACATTCCACCACCCATTCC-3′, thereby eliminating the mitochondrial leader sequence and introducing a 5′ *Xma*I and 3′ *Sal*I restriction site for cloning into the vector pDisplay. The newly generated pDisplay-*sr*HSP60-TM vector encodes for *sr*HSP60 fused to an Igκ leader sequence at the N-terminus and the platelet derived growth factor receptor (PDGFR) transmembrane region at the C terminus. Eukaryotic COS1 cells were transiently transfected using FuGENE 6 transfection reagent (Roche Applied Science, Germany) according to manufacturer's protocol. In brief, COS1 cells were plated into 6 well culture plates. 5 μg of pFM92, pFM92-mHSP60 or pDisplay-*sr*HSP60-TM vector DNA and 6 μl of FuGENE 6 reagent were added to 100 μl RPMI 1640 without foetal calf serum and incubated for 30 min. Cell culture medium was replaced by 2 ml of RPMI 1640 containing 10% foetal calf serum and the transfection mix was added for 24 h. Expression of mHSP60 or *sr*HSP60 on the surface of COS1 cells was verified by flow cytometry as described before [28]. Both proteins were expressed on the cell surface of around 30% of the transfected cells (data not shown).

2.4. Western blotting

Recombinant *sr*HSP60 protein (3 μg/slot) was separated on a 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane (GE Healthcare, Germany) and incubated for 2 h at RT with either naïve sera or sera from *S. ratti*-infected mice (d7 post re-infection) diluted at 1/200 in PBS 0.1% BSA, 0.05% Tween 20. As positive control anti-HSP60 (SPA806) (1 μg/ml) (Stressgen, BC, Canada) was used. Membranes were washed six times (PBS 0.1% BSA, 0.05% Tween 20) and incubated with horseradish