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Passive immunization with a monoclonal IgM antibody specific for *Strongyloides ratti* HSP60 protects mice against challenge infection

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ABSTRACT

It is estimated that 30–100 million people are infected with the pathogenic nematode *Strongyloides stercoralis* worldwide but parasite control is still based on anti-helminthic treatment. To develop protective vaccination strategies, we use the murine model of *Strongyloides ratti* infection. We have shown recently that vaccination with alum-precipitated, but not with native or CFA-emulsified *S. ratti* heat shock protein 60 (*sr*HSP60) conferred protection to challenge infection. Here we describe the generation of a monoclonal IgM specific for *sr*HSP60. Anti-*sr*HSP60 detected human and *sr*HSP60 and stained *S. ratti* infective larvae *in vitro*. Passive immunization of mice with monoclonal anti-*sr*HSP60 IgM led to reduced numbers of migrating larvae in lung and head, reduced numbers of parasitic adults in the small intestine and reduced larval output upon *S. ratti* challenge infection. Taken together, our findings highlight the relevance of *sr*HSP60 as vaccine candidate for the induction of antibody-mediated protection against *Strongyloides* infection.

1. Introduction

The human gastrointestinal nematode *Strongyloides stercoralis* infects approximately 30–100 million people worldwide [1]. As Strongyloidiasis is often asymptomatic, an infection may last undiagnosed for decades. Immune suppressive treatment, however, can lead to the severe hyperinfection syndrome that is lethal in more than 80% of cases [2]. Therefore, the establishment of protective *Strongyloides* vaccines is desirable despite the usually benign course of disease. Both, the human pathogen *S. stercoralis* and the closely related rodent pathogen *Strongyloides ratti* have been used to infect mice as model organisms [3,4]. Although *S. stercoralis* does not proceed beyond the third stage larvae (L3) in immune competent mice, the mechanisms of immune mediated larval eradication have been analyzed using implanted *S. stercoralis* iL3 [3,5]. *S. ratti*, in contrast, reproduces within mice and thus allows the analysis of immune mediated expulsion of both: migrating larvae and parasitic adults that reside in the small intestine [4,6]. Searching for possible vaccine candidates, proteins that are actively secreted by helminths (ESPs) and thus are present at the interface between parasite and host have been analyzed in many infection systems [7].

We have shown recently that *S. ratti* heat shock protein 60 (*sr*HSP60) represents a major component of *S. ratti* ESPs [8,9] and functions as a target of the humoral and cellular immune response during natural *S. ratti* infection in mice [10]. Despite being a dominant target of the immune response, the application of *sr*HSP60 as vaccine candidate was problematic. Vaccination of mice with recombinant *sr*HSP60 protein without any adjuvant or with complete Freund's adjuvant (CFA) did not confer protection to subsequent challenge infection but even increased susceptibility of "vaccinated" mice. Increased susceptibility occurred in the context of a type-I immune response characterized by production of *sr*HSP60-specific IFN-γ, IgG2b and IgG2c and the absence of *sr*HSP60- or *S. ratti*-specific IL-13 release. Vaccination with a complete *S. ratti* antigen lysate either without adjuvant or emulsified with CFA, in contrast, conferred protection in the context of a type-II immune response. This protective response was characterized by production of *S. ratti*-specific IL-13 and IgG1 and the absence of IFN-γ [10]. Using alum as adjuvant during *sr*HSP60 vaccination, we overruled the proinflammatory Th1-inducing capacity intrinsic to HSP60 in general [11] and induced protection against challenge infection. The shifted immune response was characterized by the presence of both, *sr*HSP60- and *S. ratti*-specific IL-13 production, and reciprocal absence of IFN-γ production in *sr*HSP60/alum vaccinated mice. Interestingly, the induction of the humoral type-I response upon *sr*HSP60 vaccination,

Abbreviations: CFA, complete Freund's adjuvant; ESP, excretory secretory protein; iL3, infective third stage larvae; nd, not detectable; *sr*HSP60, *Strongyloides ratti* heat shock protein 60.

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*i.e. sr*HSP60-specific IgG2b and IgG2c, in addition to the type-II associated IgG1 and IgM, was not changed during the protective *sr*HSP60/alum vaccination [10].

Here we dissect the protective *srHSP60*-specific immune response from the immune modulating and thus potentially deleterious effect intrinsic to the *sr*HSP60 protein by generating monoclonal antibodies (mAbs) to *sr*HSP60. Adoptive transfer of anti-*sr*HSP60 mAb led to reduced numbers of migrating larvae in head and lung, reduced worm burden in the small intestine and reduced larval output with the feces in otherwise naïve mice after challenge infection. Taken together, we provide evidence that a humoral response to *sr*HSP60 is protective if it is uncoupled from the Th1-inducing capacity that is intrinsic to the heat shock protein itself.

2. Materials and methods

2.1. Animals, parasites and infection

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). Mice were kept in individually ventilated cages and used at the age of 6–8 weeks. Maintenance of *S. ratti* cycle by serial passage through rats and production of iL3 for infection was performed as described [6,12]. Mice were infected by s.c. injection of 1000 iL3 into the hind footpad. To count the migrating larvae, mice were sacrificed at day 2 p.i., lung and head were prepared and cut into small pieces. After 3 h incubation in tap water at 37 °C, migrating larvae were harvested and counted. To count the number of adult nematodes in the gut, mice were sacrificed at day 7 p.i. The small intestine was sliced longitudinally and incubated at 37 °C for 3 h in a petri dish with tap water. The released adult females were collected by centrifugation for 5 min at 1200 rpm at RT and counted. Larval output was measured as *S. ratti* DNA within total DNA from 200 mg representative stool samples collected over 24 h using quantitative PCR as described [6].

2.2. Generation of a monoclonal srHSP60-specific antibody

Female 6–8 weeks old BALB/c mice were immunized by three consecutive injections (3 weeks interval) of 50 μg pDisplay-*sr*HSP60 DNA [10] into the hind leg muscle. Three days after the last boost immunization spleen cells were harvested, fused with X63 cells and cultured in HAT selection medium. Hybridoma cell clones were obtained after 10–14 days of culture, subcloned by limiting dilution and further cultured in protein-free hybridoma medium (PFHM II supplemented with 50 μg/ml gentamycine). The supernatants of hybridoma clones were screened for antibody content by flow cytometry employing pDisplay-*sr*HSP60-TM transfected COS1 cells that transiently express membrane-bound cell surface *sr*HSP60 [10] while control COS1 cells received the empty pDisplay vector (Invitrogen, Karlsruhe, Germany). Transfection was performed with the Fugene6 reagent (Roche Diagnostics, Mannheim, German) according to the manufacturer's protocol. After 24 h transfected COS1 cells were incubated with hybridoma supernatant or with mouse anti-hemagglutinin (HA) antibody (clone 12CA5, Roche, Mannheim, Germany; 1:100 in PBS) that detects the HA tag of the recombinant cell surface *sr*HSP60 protein as a positive control. PE-labeled goat anti-mouse IgG + IgM antibody (Jackson ImmunoResearch, Suffolk, UK; 1:200 in PBS) was used as secondary antibody. Cells were analyzed by flow cytometry on a FACS Calibur employing CellQuest Pro software (BD PharMingen, Heidelberg, Germany). The isotype of the anti-*sr*HSP60 (13F1) antibody was determined to be IgM using the mouse immunoglobulin isotyping ELISA kit according to manufacturer's recommendation (Becton Dickinson, Heidelberg, Germany).

2.3. Western blot

Either 1 μg recombinant His-tagged *sr*HSP60 (*sr*HSP60-His), recombinant human HSP60 (hHsp60; Loke Diagnostics ApS, Aarhus, Denmark) or 20 μg lysate of *S. ratti* iL3 were separated on a 10–20% polyacrylamide gradient SDS PAGE gel (Anamed, Darmstadt, Germany) and transferred on an immobilon-P polyvinylidene difluoride membrane (Millipore, Schwalbach, Germany) by semi-dry western blotting. The membrane was blocked for 1 h in PBS/0.1% Tween20 with 4% milk powder. Proteins were detected by subsequent incubation with undiluted anti-*sr*HSP60 (13F1) hybridoma supernatant or anti-HSP60 (clone LK-1; 1 μg/ml PBS/1% BSA) for 2 h at RT and HRP-conjugated goat anti-mouse Ig (Dako, Hamburg, Germany; 0.5 μg/ml in PBS/0.1% Tween20) for 1 h at RT. Western blots were developed with ECL western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK) and exposed to X-ray films (CEA, Hamburg, Germany).

2.4. Immunofluorescence microscopy

S. ratti iL3 were immobilized between two poly-L-lysin-coated slides, immediately frozen in liquid nitrogen and then carefully separated to obtain slides with cracked larvae. The larvae were fixed in methanol (10 min, −20 °C) followed by incubation in acetone (10 min, −20 °C). Slides were gradually rehydrated in 96% ethanol (5 min, −20 °C), 60% ethanol (5 min, −20 °C), 30% ethanol (5 min, RT) and finally PBS (5 min, RT), blocked at RT with PBS/1% BSA and incubated with 100 μl anti-*sr*HSP60 (13F1) hybridoma supernatant overnight at 4 °C. Control larvae were incubated with 100 μl PFHM II hybridoma culture medium without antibody. Slides were washed in PBS and stained with biotinylated goat anti-mouse IgM antibody (DAKO, Glastrup, Denmark; 1:100 in PBS/1% BSA) for 1 h at RT followed by streptavidin-TRITC (1:500 in PBS/1% BSA; Jackson ImmunoResearch Laboratories, West Grove, USA) and DAPI (1:1000 in PBS/1% BSA; Sigma, Deisenhofen, Germany) for 1 h at RT. Analysis was performed using a Zeiss fluorescence microscope (Carl Zeiss GmbH, Jena, Germany) and OpenLab software (PerkinElmer, Massachusetts, USA).

2.5. Passive transfer of immunity

Supernatant from one *sr*HSP60 clone (13F1) that was cultured in protein-free PFHM II medium was collected and concentrated to 160 μg/ml protein using 10 kDa Amicon filters (Amicon, Ultra-15, Millipore, Germany). The <10 kDa filtrate was used as negative control for passive immunization experiments. To generate *S. ratti*-specific immune serum, mice were infected with 2500 iL3 and re-infected at day 28 p.i. Serum was collected 15 days later. Serum from age matched naïve mice was used as negative control. *sr*HSP60-specific and *S. ratti* lysate-specific titers were measured by ELISA as described [10] (sup. Fig. 1). Titers were: *S. ratti* lysate-specific titers: immune serum: 1:25,000; naïve serum: 1:200; 13F1: 1:400; filtrate: not detectable (nd). *sr*HSP60-specific titers: immune serum: 1:800; naïve serum: nd; 13F1: 1:400; filtrate: nd. Mice received 0.5 ml concentrated anti-*sr*HSP60 (13F1) supernatant or 10 kDa filtrate of the 13F1 supernatant i.p. 1 day before infection and another 0.5 ml 3 h before infection. For passive immunization with antiserum mice received a single 0.5 ml i.p. injection with immune or naïve serum 3 h before infection. All groups were challenged by s.c. injection of 1000 iL3 into the hind footpad.