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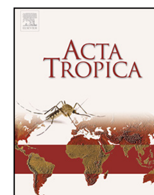

Intranasal immunisation of the recombinant *Toxoplasma gondii* receptor for activated C kinase 1 partly protects mice against *T. gondii* infection

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ABSTRACT

Nasal vaccination is an effective therapeutic regimen for preventing certain infectious diseases. The mucosal immune response is important for resistance to *Toxoplasma gondii* infection. In this study, we evaluated the immune responses elicited in BALB/c mice by nasal immunisation with recombinant *T. gondii* receptor for activated C kinase 1 (r*Tg*RACK1) and their protective efficacy against *T. gondii* RH strain during both chronic and lethal infections. Nasal vaccination with r*Tg*RACK1 increased the level of secretory IgA in nasal, intestinal and vesical washes, and the level of IFN-γ and IL-2 in intestinal washes, indicating that r*Tg*RACK1 vaccination promotes mucosal immune responses. The mice immunised with r*Tg*RACK1 also displayed increased levels of r*Tg*RACK1-specific IgA, total IgG, IgG1 and in particular IgG2a in their blood sera, increased production of IFN-γ, IL-2 and IL-4 but not IL-10 from their isolated spleen cells, and enhanced splenocyte proliferation *in vitro*. r*Tg*RACK1-vaccinated mice were effectively protected against infection with *T. gondii* RH strain, showing over 50% reduction of tachyzoite burdens in their liver and brain tissues during a chronic infection, and also a 45% increase in their survivals during a lethal challenge. These results indicate that r*Tg*RACK1 might represent an intriguing immunogen for developing a mucosal vaccine against toxoplasmosis.

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1. Introduction

Toxoplasmosis is a worldwide distributed food borne zoonosis caused by the protozoan parasite *Toxoplasma gondii*, which infects the nucleated cells of warm blooded vertebrates, including humans (Dubey and Su, 2009). Usually, *T. gondii* infection is asymptomatic in immunocompetent humans, but it could be problematic in congenitally infected and immunocompromised individuals, such as AIDS patients and organ transplant recipients (Elsheikha, 2008; Innes, 2010; Weiss and Dubey, 2009). Toxoplasmosis in animals can also be a serious threat to public health and it can cause considerable economic losses of farm animals (Dubey et al., 2005). To prevent *T. gondii* infection, vaccination represents an optimal strategy (Kur et al., 2009). In the last two decades, many live, attenuated, subunit and DNA-based vaccines against toxoplasmosis have been studied, and a degree of progress has been achieved. For example, *T. gondii* surface antigen 1 (SAG1) and dense granule antigen 2 (GRA2) have been explored as vaccine candidates to protect against toxoplasmosis (Angus et al., 2000; Golkar et al., 2007), but only limited success against chronic and acute infection has been achieved to date (Dziadek and Brzostek, 2012). Therefore, further studies are necessary to develop appropriate vaccine antigens against toxoplasmosis.

The receptor for activated C kinase 1 (RACK1) is a multifaceted scaffolding protein and serves as an integrative point for diverse

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signal transduction pathways. It links the Focal Adhesion Kinase (FAK) to the phosphodiesterase isoform PDE4D5 to control cell polarity (Serrels et al., 2011). It also interacts with the cytoplasmic tails of several receptors, including the Insulin-like Growth Factor Receptor I (IGF-IR), β-integrin receptor, the common beta-chain of the IL-5/IL-3/GM-CSF receptor (Geijsen et al., 1999) and modulates cell cycle, cell adhesion, cell spreading and cell migration (Cox et al., 2003; Mamidipudi et al., 2007). The RACK1 homologue for the parasite *Trypanosoma brucei* (TRACK) has a role in the cell cycle to ensure the mid-stage progression through cytokinesis in *T. brucei* procyclic forms (Rothberg et al., 2006). In *Leishmania major*, the RACK1 ortholog (LACK) is one of the major antigens which elicit a protective T cell response of the leishmaniasis subject (Julia et al., 1996; Mougneau et al., 1995). A RACK1 homologue expressed in the malaria parasite *Plasmodium falciparum* throughout its all asexual stages may also regulate the life cycle of malaria parasite (Madeira et al., 2003). RACK1 in *T. gondii* (*Tg*RACK1) localises to the parasite cytoplasm and nucleus, and interacts with the βCOP subunit of coatomer protein complex I involved in protein secretion and parasite invasion (Moran et al., 2007; Smith et al., 2007). *Tg*RACK1 was later identified from soluble tachyzoite antigens by a rabbit anti-*T. gondii* serum and proteomic analyses, and was proposed to be a potential vaccine candidate against toxoplasmosis (Ma et al., 2009).

T. gondii is an intracellular protozoan parasite that usually infects hosts through an oral route. The innate immune response of mucosal epithelial cells during pathogen invasion plays a central role in immune regulation in the gut. To evaluate the immune responses and protective efficacy of *Tg*RACK1 against *T. gondii*, we cloned the *Tg*RACK1 gene, prepared its recombinant protein, and investigated the effectiveness of the recombinant protein as a mucosal vaccine against *T. gondii* infection.

2. Materials and methods

2.1. Animals and parasites

The mice used in this study were 6-week-old female BALB/c mice acquired from the Institute of Laboratory Animals, Chinese Academy of Medical Science, Beijing, China. All mice were maintained under standard conditions and provided with rodent feed and water *ad libitum*. Animal experiments were carried out in strict accordance to the Guidelines for Animal Experiments issued by the Ethics Committee of Shanxi Medical University under the licenses of 20110320-1. *T. gondii* tachyzoites (RH strain) were obtained from the Centre for Health Sciences of Peking University (Beijing, China) and maintained by serial intraperitoneal passaging in BALB/c mice.

2.2. Expression and purification of recombinant TgRACK1

Total RNA was extracted from tachyzoites of *T. gondii* using Trizol reagent according to the manufacturer's instructions (Invitrogen, USA). First strand cDNA was synthesised using the EasyScript First-Strand cDNA Synthesis SuperMix (Transgen, China). Primers for amplification of the open reading frame (ORF) of *Tg*RACK1 gene were designed according to the gene sequence of *Tg*RACK1 from *T. gondii* RH strain (GenBank accession No: AY547291). The forward primer was 5′-ACGGAATTCATGTCGGGTGAATCTCCCCTC-3′, and the reverse primer was 5′-AAGGAAAAAAGCGGCCGCTTACGCGG-TCACTTGCTCTGAA-3′, which containing an *Eco*RI and *Not*I restriction site (underlined), respectively. The PCR conditions were as follows: 94 °C for 5 min; 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, 30 cycles; and 72 °C for 10 min.

For recombinant protein expression in *Escherichia coli*, the cDNA fragment of *Tg*RACK1, which was confirmed via DNA sequencing, was digested with *Eco*RI and *Not*I, and cloned into the vector pGEX-6P-1 (Merck Biosciences, Germany) using T_4 DNA ligase (Transgen, China). The resulting pGEX-6P-1-*Tg*RACK1 plasmid was transformed into *E. coli* BL21 (DE3) host cells (Transgen, China), and recombinant protein expression was induced with 0.1 mM IPTG at 20 °C overnight. Cultures were harvested by centrifugation, cell pellets were resuspended in lysis buffer (50 mM Tris pH 7.5, 1 mM PMSF, 1 mM DTT) and homogenised by sonication on ice. The lysate was then centrifuged to separate the cell debris from the supernatant which was subject to 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting assay to verify the expression of r*Tg*RACK1A by using anti-GST and anti-*T. gondii* polyclonal antibodies (Ma et al., 2009). The levels of r*Tg*RACK1 expression and its purity were evaluated by SDS-PAGE and Coomassie blue R-250 staining.

r*Tg*RACK1 protein was purified using a self-packaged GST-affinity column (2 ml glutathione Sepharose 6B; CoWin Biotechnology, China). Contaminant proteins were removed with a wash buffer (lysis buffer plus 200 mM NaCl) at 4 °C. The fusion protein was then digested on column with PreScission protease (GE Healthcare, USA) at 4 °C overnight, and r*Tg*RACK1 protein without the GST tag at its N-terminus was eluted with the lysis buffer. Following centrifugation with an Ultrafree 10,000 molecular weight cut-off filter unit (Millipore, USA), the protein eluent was further purified using a Superdex-75 (Pharmacia, USA) column, and the purified protein was analysed by SDS-PAGE and Coomassie blue R-250 staining. After endotoxin removal by using a ToxinEraser™ Endotoxin Removal Kit, the level of endotoxin remains in the final protein preparations was determined by using a Chromogenic End-point Endotoxin Assay Kit (Chinese Horseshoe Crab Reagent Manufactory, Xiamen, China) to be less than 0.1 EU/ml. The purified protein was then quantified by the BCA method, filtered throughout a 0.2 μm-pore membrane, and stored at −70 °C until use.

2.3. Western blotting

The products of r*Tg*RACK1 expressed in *E. coli* were boiled in the gel loading buffer at 98 °C for 5 min and were centrifuged at 12,000 × *g* for 10 min at room temperature (RT). The supernatants were separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane (GE Healthcare, USA). The membrane was blocked in 5% (w/v) skim milk for 1 h at RT and then incubated with an anti-GST antibody (1:1000) or rabbit anti-*T. gondii* serum (1:200) at 4 °C overnight. After washes, the membrane was incubated with a HRP-conjugated secondary anti-mouse or anti-rabbit antibody for 1 h at RT and developed with the ECL Western blotting analysis reagents (Engreen, China).

2.4. rTgRACK1 immunisation and samples collection

Fifty 6-week-old female BALB/c mice were randomly divided into five groups (10 per group) and were intranasally immunised with 15, 25, 35 or 45 μg of r*Tg*RACK1 dissolved in 20 μl sterile phosphate-buffered saline (PBS). Control mice received PBS alone. The mice were caught and their heads were hold in an upward position to expose their nostrils fully. To extenuate the distress of the mice, each dose of r*Tg*RACK1 was instilled into the two nostrils alternatively (10 μl/nostril) within a period of 3 min per mouse by using a micropipettor. The same protocol of nasal inoculation was performed on days 0, 14, and 21. Two weeks after the final inoculation (on day 35), the mice were deprived of food and water for 8 h to deplete the contents in the intestines for intestinal wash collections. The mice were anesthetised with sodium pentobarbital (1.5%, 0.1 ml/20 g weight, intraperitoneal injection), and blood samples from the mice in each group were collected by retro-orbital plexus puncture. The sera were separated, stored at

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