INSTITUT PASTEUR

Microbes and Infection 10 (2008) 241–250

www.elsevier.com/locate/micinf

Original article

Critical contribution of immunoproteasomes in the induction of protective immunity against *Trypanosoma cruzi* in mice vaccinated with a plasmid encoding a CTL epitope fused to green fluorescence protein

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Received 15 September 2007; accepted 24 November 2007
Available online 5 December 2007

Abstract

Acquired immunity against infection with *Trypanosoma cruzi* is dependent on $CD8^+$T cells. Here, to develop a vaccine strategy taking advantage of activated $CD8^+$T cells, we constructed a DNA vaccine, designated pGFP-TSA1, encoding a fusion protein linking GFP to a single CTL epitope of TSA1, a leading candidate for vaccine against *T. cruzi*. C57BL/6 mice vaccinated with this plasmid showed suppressed parasitemia and prolonged survival. Vaccination with pGFP-TSA1 enhanced epitope-specific cytotoxicity and IFN-γ secretion by $CD8^+$T cells. Furthermore, the depletion of $CD8^+$T cells prior to challenge infection with *T. cruzi* completely abolished this protection, indicating that $CD8^+$T cells are the principal effector T cells involved. When mice deficient in the proteasome activator PA28α/β or the immunoproteasome subunits LMP2 and LMP7 were used, the protective immunity against infection was profoundly attenuated. Our findings clearly demonstrate that vaccination with pGFP-TSA1 successfully induces protection dependent on $CD8^+$T cell activation, in which immunoproteasomes play a crucial role. It is noteworthy to document that physical binding of the epitope and GFP is required for induction of this protection, since mice vaccinated with pTSA1-IRES-GFP failed to acquire resistance, probably because the epitope and GFP are separately expressed in the antigen-presenting cells.

Keywords: Antigen presentation; UPS; DNA vaccine; *Trypanosoma cruzi*; TSA1; GFP

1. Introduction

More than 16 million inhabitants of Latin America are infected with *T. cruzi* and more than 90 million people are at risk of infection. These protozoa are hemoflagellate parasites of humans and many other mammals, and are the etiological pathogen of Chagas' disease. More than 300,000 new patients become infected with *T. cruzi* every year, and approximately 21,000 people die annually as a result of *T. cruzi* infection. Chagas' disease has two, successive phases: acute and chronic. The acute phase lasts 6–8 weeks. Several years after the start of the chronic phase, 20%–35% of infected individuals suffer from irreversible lesions of the autonomous nervous system, in the heart, oesophagus, colon and/or the peripheral nervous system [1]. Initially, drugs are used to treat infected people, but most of these are not effective in chronically infected individuals. Furthermore, parasites that are naturally resistant to chemotherapy have been reported in various regions of Latin America [2]. Therefore, it is necessary to apply alternative approaches for prophylaxis or therapy of Chagas' disease.

T cell-mediated immunity, especially immunity mediated by $CD8^+$ CTL, has been demonstrated to play a crucial role

Abbreviations: APC, antigen-presenting cell; DC, dentritic cell; MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; UPS, ubiquitin-proteasome system; GFP, green fluorescent protein; *T. cruzi*, *Trypanosome cruzi*; TSA1, trypomastigote surface antigen-1; IRES, internal ribosome entry site.

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doi:10.1016/j.micinf.2007.11.010

in the infection of humans and mice with *T. cruzi* [3,4]. Antigen presentation to $CD8^+$T cells is mediated by MHC class I molecules expressed on APC/DC. Primarily, $CD8^+$T cells recognize MHC class I-associated peptides derived from endogenous antigens located in the cytosol. Prior to antigen presentation by MHC class I molecules, antigens must be ubiquitinated and processed into antigenic peptides by the proteasome. This antigen processing system is termed the UPS [5–7]. However, conventional vaccine strategies using parasite proteins/peptides or DNA do not make use of the UPS and often fail to induce a protective immune response, which is mostly mediated by $CD8^+$T cells [8,9]. Ubiquitin binds to target proteins via lysine residues, and then these proteins are polyubiquitinated [6]. GFP, which contains 19 lysine residues, is readily targeted and degraded by the UPS [10]. This chemical characteristic of GFP is likely to have been very helpful for the polyubiquitination and antigen processing of the fusion protein in the proteasome. Therefore, Vaccination with naked DNA encoding endogenous antigens fused to GFP is an efficient means for the induction of antigen-specific immunity, particularly activation of $CD8^+$T cells, since intracellularly expressed antigenic proteins are exclusively processed through the UPS and the resultant MHC class I-binding epitopes efficiently activate specific $CD8^+$T cells.

A large number of *T. cruzi* antigens able to activate immune responses have been identified [11–13]. Among them, transialidase is the most prominent antigenic peptide in terms of the induction of immune responses. The TSA1 is a transialidase expressed in the trypomastigotes of *T. cruzi*. Several epitopes recognized by human $CD8^+$T cells have been identified within the TSA1 protein. $TSA1_{515-522}$ (VDYNFTIV) was identified as a major CTL epitope, recognized by $H\text{-}2K^b$ MHC class I molecules [12].

In this study, we investigated the effect of DNA vaccination of mice with a gene encoding $TSA1_{515-522}$ fused to GFP (pGFP-TSA1) on protection against infection with *T. cruzi in vivo*. We also performed vaccination with the control plasmid pTSA1-IRES-GFP, which led to separate expression of the TSA1 epitope and GFP protein in APCs due to the presence of the IRES. The IRES is a nucleotide sequence that allows for translation initiation in the middle of an mRNA sequence during protein synthesis [14]. Strikingly, immunization of mice with pGFP-TSA1 induced potent protective immunity against the parasite. However, immunization with either $pTSA1_{515-522}$ alone or with pTSA1-IRES-GFP failed to induce anti-parasite immunity. A key role of the UPS in the present study was confirmed *in vitro* using the proteasome inhibitor MG-132, and *in vivo* using mice deficient in the proteasome activator PA28α/β or mice deficient in either of the immunoproteasome subunits LMP2 and LMP7.

2. Materials and methods

2.1. Animals and parasites

Eight week-old female C57BL/6 (B6) mice were purchased from Seac Yoshitomi (Fukuoka, Japan) and kept in accordance with the institutional guidelines of Kyushu University. Proteasome activator PA28 knockout ($PA28\alpha/\beta^{-/-}$) and immunoproteasome subunit LMP2 or LMP7 knockout ($LMP2^{-/-}$ or $LMP7^{-/-}$) mice on a B6 background were established by our group [15,16].

The Tulahuen strain of *T. cruzi* was maintained by weekly passage in B6 mice.

2.2. Plasmids

pGFP plasmid (Clontech, Palo Alto, CA) was used to construct the GFP-TSA1 plasmid: $GFP\text{-}TSA1_{515-522}$ DNA was amplified by PCR using sense (5′-CAGAGAATTCAGACGC CACCATGGTG-3′) and antisense (5′-CCATGGATCCTTA CACAATAGTGAAGTTGTAATCCACCTTGTACAGCTCGT C-3′) primers with pGFP plasmid DNA as a template. The $GFP\text{-}TSA1_{515-522}$ PCR product was inserted into the EcoRI and BamHI sites of the pcDNA3.1 (−) vector (Invitrogen, San Diego, CA). A pTSA1 plasmid encoding $TSA1_{515-522}$ was constructed by PCR using sense (5′-TGGCTAGCATGG TGGATTACAACTTCACTATTGTGTAAGTTTAAACGGGC CCTCTAGA-3′) and antisense (5′-ATGCTAGCCAGCTTGG GTCTCC-3′) primers with pcDNA3.1 (−) plasmid DNA as template. After treatment with NheI, the PCR product was ligated. A pTSA1-IRES-GFP plasmid expressing $TSA1_{515-522}$ and GFP separately was constructed as follows: first, a pTS A1-IRES plasmid, expressing $TSA1_{515-522}$, was constructed by PCR using sense (5′-TGGCTAGCATGGTGGATTA CAACTTCACTATTGTGTAACTCGAGAATTCACGCGT-3′) and antisense (5′-ATGCTAGCCTATAGTGAGTCG-3′) primers with pIRES (Clontech Palo Alto, CA) plasmid DNA as a template. After treatment with NheI, the PCR product was ligated. Second, DNA encoding GFP was amplified by PCR using sense (5′-TGTCTAGAAGACGCCACCATGGT G-3′) and antisense (5′-ATGCGGCCGCTTACTTGTACAGCT CGTC-3′) primers with pGFP plasmid DNA as template. The PCR product was then inserted into the XbaI and NotI sites of a pTSA1-IRES plasmid.

2.3. In vivo gene transfer and challenge of T. cruzi

We used a Helios Gene Gun (BioRad, NY, USA) as described previously [17]. B6 mice were immunized four times at two-week intervals with 6 μg of each plasmid. Two weeks after the last vaccination, mice were infected with 1000 blood-derived *T. cruzi* trypomastigotes by s.c. injection at the base of the tail. Parasitemia levels were evaluated by counting the number of parasites in 5 μl of blood from the tail vein.

2.4. In vitro transfection and Western blotting

Two million COS-7 cells were transfected with each plasmid at a dose of 2 μg using Lipofectamine (Invitrogen, Carlsbad, CA, USA). Twenty four hours after transfection, cell lysates were prepared and 15 μg proteins was used for Western blotting as described previously [18]. The proteasome inhibitor MG-132 (Bostonchem, Boston, MA) was added at a concentration of