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Genetic immunization based on the ubiquitin-fusion degradation pathway against *Trypanosoma cruzi*

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ABSTRACT

Cytotoxic CD8$^+$ T cells are particularly important to the development of protective immunity against the intracellular protozoan parasite, *Trypanosoma cruzi*, the etiological agent of Chagas disease. We have developed a new effective strategy of genetic immunization by activating CD8$^+$ T cells through the ubiquitin-fusion degradation (UFD) pathway. We constructed expression plasmids encoding the amastigote surface protein-2 (ASP-2) of *T. cruzi*. To induce the UFD pathway, a chimeric gene encoding ubiquitin fused to ASP-2 (pUB-ASP-2) was constructed. Mice immunized with pUB-ASP-2 presented lower parasitemia and longer survival period, compared with mice immunized with pASP-2 alone. Depletion of CD8$^+$ T cells abolished protection against *T. cruzi* in mice immunized with pUB-ASP-2 while depletion of CD4$^+$ T cells did not influence the effective immunity. Mice deficient in LMP2 or LMP7, subunits of immunoproteasomes, were not able to develop protective immunity induced. These results suggest that ubiquitin-fused antigens expressed in antigen-presenting cells were effectively degraded via the UFD pathway, and subsequently activated CD8$^+$ T cells. Consequently, immunization with pUB-ASP-2 was able to induce potent protective immunity against infection of *T. cruzi*.

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Introduction

Trypanosoma cruzi is an intracellular protozoan hemoflagellate parasite of humans and many other mammals. It is also the etiological pathogen of Chagas disease. Patients infected with *T. cruzi* have been treated with many kinds of drugs, but those therapies are hardly effective in chronically infected individuals. Furthermore, parasites that are naturally resistant to chemotherapy have been reported in various regions of Latin America [1].

T cell-mediated immunity, especially via CD8$^+$ cytotoxic T lymphocytes (CTL), has been demonstrated to play a crucial role in resolving *T. cruzi* infection in humans and mice [2]. Antigens recognized by CD8$^+$ T cells are first processed by the ubiquitin proteasome system (UPS) [3]. CD8$^+$ T cells then recognize antigenic epitopes presented by major histocompatibility complex (MHC) class I molecules on the surface of infected host cells. As a result, *T. cruzi* is cleared by cytolysis of parasite-infected host cells [4]. Activation of CD8$^+$ T cells requires antigen-processing through the UPS prior to presentation in association with MHC class I molecules [5].

Recently, it was reported that an artificially fused mono-ubiquitin and antigenic protein was readily directed to the proteasome, and those antigenic peptides are then effectively presented on antigen-presenting cells (APCs). This virtual pathway of UPS was named the ubiquitin-fusion degradation (UFD) pathway [6]. This immunization strategy is effective in maximizing CD8$^+$ T cell-responses against those antigenic peptides. We previously reported that immunization with naked DNA encoding an antigen artificially fused to a mono-ubiquitin is an efficient strategy for the induction of antigen-specific immunity mediated by CD8$^+$ T cells [7].

A number of antigens of *T. cruzi* recognized by the immune system have been defined at the molecular level in the last decade [8,9]. Genetic immunization strategies have recently become popular and attractive for prophylaxis and therapy against infection of *T. cruzi*. It has been shown that amastigote surface protein-2 (ASP-2) is one of the targets for CD8$^+$ T cells and contains CTL epitopes such as the H-2K^b restricted VNHRFTLV [10]. Based on these facts, we developed a new strategy for genetic immunization employing

Abbreviations: T. cruzi, *Trypanosoma cruzi*; UFD, ubiquitin-fusion degradation; ASP-2, amastigote surface protein-2; UPS, ubiquitin proteasome system

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the UFD pathway. Expression plasmids encoding ASP-2 fused to a mono-ubiquitin (pUB-ASP-2) were constructed. Mice immunized with pUB-ASP-2 exhibited a low parasitemia and survived longer compared with mice in the control group; while immunization with pASP-2 alone was scarcely effective. $CD8^+$ T cells exerted ASP-2 specific cytotoxic activities and IFN-γ secretion. Application of the UFD pathway for genetic immunization was confirmed by using immunoproteasome deficient mice such as PA28α/β, LMP2 or LMP7 KO mice.

Materials and methods

Animals and parasites. Female 8-week old C57BL/6 (B6) mice were purchased from Seac Yoshitomi (Fukuoka, Japan). Proteasome activator PA28 knockout (PA28α/$\beta^{-/-}$) and immunoproteasome subunit LMP2 or LMP7 knockout ($LMP2^{-/-}$ or $LMP7^{-/-}$) mice were B6 background. The Tulahuen strain of *T. cruzi* was maintained by weekly passage in B6 mice.

Cloning and sequencing. Total RNA was isolated from liver sections obtained from *T. cruzi* infected B6 mice and reverse-transcribed to cDNA. ASP-2 cDNA was amplified by PCR using sense, 5′-ATGCTCTCACGTGTTGCTGCTGTC-3′, and antisense 5′-TTA GTCGCCACCGTTTCCTTTTTTATCG-3′ primers. Specific oligonucleotides were designed on the basis of the previously published nucleotide sequence of *asp-2* [11]. The resulting amplicon was ligated into a pGEM-T easy vector (Promega, USA) and transformed into DH5α *Escherichia coli* (Invitrogen, USA). Clones containing inserts of the expected size were selected. Sequencing was initially performed using SP6 and T 7 primers, the sequences of which were present in the flanking regions of the pGEM-T easy vector. Subsequently, to complete sequencing of each clone, new oligonucleotides were designed to cover the entire sequence.

DNA and predicted amino acid sequences were analyzed using the Lasergene 7.1 software package (DNASTAR Inc., USA). Sequence alignments were produced using Clustal V. Analysis for potential secretory signal peptides (SP) was performed at the SignalP website (http://www.cbs.dtu.dk/services/SignalP/).

Plasmids. A plasmid encoding ASP-2 (aa65–703, signal peptide deleted) and tagged with His residues (pASP-2) was constructed by amplifying *asp-2* from clone 2 using the following primers: 5′-GCATCCTCGAGATGGCTGTGGAGGGTAAGTCCGGG-3′, 5′-GTCATCTTAAGTTAGTGATGGTGGTGATGGTGGTCGCCACCGTTTCCTTTTTTATCG-3′. PCR products were treated with enzymes and inserted into the XhoI and AflII sites of the pcDNA3.1(−) vector (Invitrogen, USA). The pUB vector we made previously was used to construct pUB-ASP-2 (aa65–703, SP deleted) and tagged with His [7]. The gene *asp-2*, was cut from pASP-2 and inserted into the XhoI and AflII sites of the pUB vector.

In vivo gene transfer and challenge of T. cruzi. As described previously, a Helios Gene Gun (BioRad, USA) was used [12]. Protocols of immunization and infection with *T. cruzi* trypomastigotes were same with previous description [12].

In vitro transfection and Western blotting. Protocols of transfection and Western immunoblotting were same as described previously [12,13].

Cytotoxicity assay, ELISA and flow cytometry. Protocols of cytotoxicity assay, ELISA and flow cytometry were same as described previously [12,13].

Statistical analysis. Data are expressed as mean ± SEM. Differences between experimental groups within each experiment were analyzed by using the unpaired Student's *t*-test and were considered significant when the *p*-value was <0.05. Survival differences between experimental groups within each experiment were analyzed by using the log-rank test and were considered significant when the *p*-value was <0.05.

Results

Construction of plasmids encoding ASP-2 or UB-ASP-2

The *asp-2* gene is polymorphic, as its sequence is different even in the same strains of *T. cruzi* [14]. In this study, we obtained four clones of the *asp-2* gene from the Tulahuen strain and picked up one particular clone (clone 2: GenBank Accession No. GU445326). Compared with the original sequence of the Brazil strain (GenBank Accession No. U77951) and the sequence of the Tulahuen strain (GenBank Accession No. EF579921), the amino acid sequence of ASP-2 used in this study contained the ASP box motif (SxDxGxTW) and the VTV box motif (VTVxNVxLYNR) (Fig. 1A). The amino acid identity of ASP-2 used in this study compared with the Brazil strain is 89.8%, and 83.1% when compared with the Tulahuen strain. The H-$2K^b$ restricted CTL epitope (aa553–560, VNHSFTLV) contains a single amino acid change when compared with the Brazil strain [15], and two amino acid changes when compared with the Tulahuen strain [14]. ASP-2 and UB-ASP-2 expression plasmids were constructed as described in the materials and methods section (Fig. 1B). Construction of pASP-2 and pUB-ASP-2 was confirmed by DNA sequencing. The expression of ASP-2 or the fusion protein UB-ASP-2 was confirmed by Western immunoblotting after transfection of COS7 cells with these plasmids; a specific band was detected in lysates from cells transfected with pASP-2 or pUB-ASP-2 (Fig. 1C).

Anti-parasite immunity against T. cruzi was induced by immunization with pUB-ASP-2

CTL epitopes are produced through the ubiquitin–proteasome pathway and we expected that antigen presentation of MHC class I-associated ASP-2 peptides to $CD8^+$ T cells would become significantly more efficient following immunization with pUB-ASP-2. To verify this, B6 mice were immunized with pcDNA, pASP-2 or pUB-ASP-2 into the abdominal skin by using a gene-gun system four times, at two-week intervals. Two weeks following the last immunization, mice were challenged with 1000 blood-derived *T. cruzi* trypomastigotes by subcutaneous injection at the base of the tail. Mice immunized with pUB-ASP-2 developed a lower parasitemia than control pcDNA immunized groups (Fig. 2A); survival was also prolonged by immunization with pUB-ASP-2 (Fig. 2B). Six out of seven pUB-ASP-2 immunized mice survived until the end of the experiment. Mice immunized with pASP-2 did not develop a protective response suggesting that artificial fusion of the gene encoding mono-ubiquitin and the ASP-2 protein was required for the induction of immunity.

Immune response following immunization with pUB-ASP-2

To investigate the mechanism of protective immunity conferred by immunization with pUB-ASP-2, spleen cells separated from mice immunized with pcDNA, pASP-2 or pUB-ASP-2, were co-cultured with the non-specific stimulator PMA. Production of intracellular IFN-γ and Granzyme b (GZM-b) by T cells was analyzed using flow cytometry. Intracellular IFN-γ and GZM-b was strongly induced in $CD8^+$ T cells of mice immunized with pUB-ASP-2 (Fig. 3A). The absolute number of both IFN-$\gamma^+$$CD8^+$ T cells and GZM-$b^+$$CD8^+$ T cells in the spleen was significantly higher in pUB-ASP-2 immunized mice (Fig. 3B). There was no significant difference in the IFN-γ and GZM-b secretion level of $CD4^+$ T cells. Production of IFN-γ and GZM-b in $CD4^+$ T cells was at almost the same level in mice treated with pcDNA, pASP-2 or pUB-ASP-2 (data not shown). These results indicate that immunization with pUB-ASP-2 promotes $CD8^+$ T cell activation, and enhances the expression level of IFN-γ and GZM-b in $CD8^+$ T cells.

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