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Trypanosoma cruzi paraflagellar rod proteins 2 and 3 contain immunodominant CD8$^+$ T-cell epitopes that are recognized by cytotoxic T cells from Chagas disease patients

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ABSTRACT

The protozoan parasite *Trypanosoma cruzi* is the etiological agent of Chagas disease. To date, no vaccine is available for protection against *T. cruzi* infection. The CD8$^+$ T cells immune response against specific antigens has shown to efficiently control the spread of the parasite in murine experimental infection. However, data concerning CD8$^+$ response in Chagas patients are still restricted to a few epitopes. We have studied the existence of immunodominant CD8$^+$ T cell epitopes in the paraflagellar rod proteins 2 and 3 (PFR2 and PFR3) from *T. cruzi* in a mouse model, and analyzed their recognition by cytotoxic T lymphocytes from Chagas disease patients. Immunization of C57BL/6-A2/K^b transgenic mice with plasmids coding for the fusion proteins PFR2-HSP70 and PFR3-HSP70 induced a specific CTL response against two PFRs epitopes ($PFR2_{449–457}$ and $PFR3_{481–489}$), and showed specific lysis percentages of 24 and 12, respectively. Moreover, the $PFR2_{19–28}$, $PFR2_{156–163}$, $PFR2_{449–457}$, $PFR3_{428–436}$, $PFR3_{475–482}$ and $PFR3_{481–489}$ peptides were observed to have a high binding affinity to the HLA-A*02:01 molecule. Remarkably, these HLA-A*02:01-binding peptides are successfully processed and presented during natural infection by *T. cruzi* in the context of MHC class I as evidenced by using peptide-pulsed K562-A2 cells as antigen presenting cells. The T cells from Chagas disease chronic patients specific for PFR2/PFR3 selected CD8$^+$ epitopes showed a pro-inflammatory cytokine secretion profile (IFN-γ, TNF-α and IL-6). A positive Granzime B secretion was observed in three out of 16 patients in response to $PFR2_{156–163}$ and $PFR2_{449–457}$ peptides, two out of 11 patients in response to $PFR2_{19–28}$ peptide and one out of 14 and 11 patients in response to $PFR3_{428–436}$ and $PFR3_{481–489}$ peptides, respectively. The PFRs-specific cytotoxic activity in purified PBMC was only detected in patients in the indeterminate phase of the disease.

Abbreviations: PFR, paraflagellar rod protein; HSP, heat shock protein; KMP, kinetoplastid membrane protein; PBMC, peripheral blood mononuclear cells; TAP, transporters associated with antigen-processing; APC, antigen-presenting cell; SFC, spot forming cell; CTL, cytotoxic T lymphocytes; K562, human immortalised myelogenous leukemia line; MHC, major histocompatibility complex; HLA, human leukocyte antigen; IFI, immunofluorescence indirect; DMSO, dimethyl sulfoxide; FSCi, inactivated fetal calf serum; SDS, sodium dodecyl sulfate; PAGE, polyacrilamide gel electrophoresis; PHA, phytohaemaglutinin; HEPES, N-[2-hydroxyethyl]piperazine-N′[2-ethane-sulfonic acid]; PMSF, phenylmethylsulfonyl fluoride; SPPS, standard t-Boc solid-phase-peptide synthesis; DAPI, 4′,6-diamidino-2-phenylindole; IFA, incomplete Freud adjuvant; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DMEN, Dulbecco's modified eagle medium; GzB, granzyme B.

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1. Introduction

The protozoan parasite *Trypanosoma cruzi* is the etiological agent of Chagas disease which affects almost 10 million people in Latin America with 75–90 million exposed to this infection (Coura and Dias, 2009). The disease courses with different clinical stages. The acute phase appears shortly after infection and in most cases presents unspecific symptoms that makes its diagnosis to be extremely difficult. In the absence of treatment, the acute phase is followed by a chronic indeterminate stage in which the parasites persist into specific tissues (Prata, 2001). In about one third of these patients the infection leads to a symptomatic chronic phase, characterized by cardiac and/or digestive implications, and less often alterations in the central nervous system (Matsuda et al., 2009; Tanowitz et al., 2009; Rassi and Rassi, 2010). Clinical manifestations are associated to morbidity and cardiac involvement is the main cause of death in the chronic stage of the disease (Prata, 2001; Dutra et al., 2005; Punukollu et al., 2007). To date, a protective vaccine against this infection is inexistent and the efficacy of the current and rather toxic anti-parasitic chemotherapy is under consideration in patients in the chronic phase of the disease (Marin-Neto et al., 2009). Given the importance of the disease, finding accurate and safe methods to control the infection is extremely urgent.

Previous studies have shown that the immune control of *T. cruzi* requires the activation of both $CD4^+$ and $CD8^+$ T cells (Cazorla et al., 2009). It is clear that natural immune response is not strong enough to reach sterility and consequently, the parasites persist into the patient's tissues. Several parasite class I-restricted antigens have been characterized in murine experimental models where it has been reported that the $CD8^+$ T cell immune response against *T. cruzi* specific antigens is sufficient to control the spread of the parasite (Miyahira, 2008). However, data concerning $CD8^+$ response in Chagas patients are still restricted to a few epitopes (Diez et al., 2006; Wizel et al., 1997; Fonseca et al., 2004; Maranon et al., 2011).

Two of the antigens reported as vaccine candidates are the paraflagellar rod proteins (PFRs) which are located at the *T. cruzi* flagellum and specific to the kinetoplastids (Wrightsman et al., 2002; Morell et al., 2006; Vazquez-Chagoyan et al., 2011). Four different paraflagellar rod proteins (named PFR1-4) have been described in *T. cruzi* (Fouts et al., 1998; Maga et al., 1999). Mice immunization with a mixture of these PFRs as recombinant proteins has been shown to induce an immune response capable of reducing the level of circulating parasites, and to provide survival rates of 83–100% against a lethal *T. cruzi* challenge (Wrightsman et al., 2002). Furthermore, it was reported that immunization with the PFR2-HSP70 fused genes as DNA vaccine provides a protective response against a *T. cruzi* experimental infection by inducing an increase in the expression of IL-2 and IFN-γ in spleen cells, and generating antigen-specific $CD8^+$ T cells (Morell et al., 2006). In the present paper we describe the identification of two immunodominant cytotoxic T cell epitopes restricted to the HLA-A*02:01 molecule in the PFR2 and PFR3 proteins ($PFR2_{449–457}$ and $PFR3_{481–489}$), respectively, in an experimental infection model. We also describe the identification of three epitopes contained in PFR2 ($PFR2_{19–28}$, $PFR2_{156–163}$ and $PFR2_{449–457}$) and three in PFR3 ($PFR3_{428–436}$, $PFR3_{475–482}$ and $PFR3_{481–489}$) which are successfully processed and presented during natural human infection with *T. cruzi* in the context of class I MHC.

2. Materials and methods

2.1. Mice, immunization and organ extraction

Six to eight weeks old C57BL/6-A2K^b female mice (Vitiello et al., 1991) were bred in the animal facilities of the Institute of Parasitology and Biomedicine López-Neyra CSIC. The said transgenic mice express the product of the HLA-A*02:01 chimeric gene (α1 and α2 domains of human HLA-A*02:01 molecule and α3 domain of murine H-2Kb) (Vitiello et al., 1991). Groups of four mice were intramuscularly immunized with 100 μg of DNA of each one of the two constructs (*PFR2-HSP70* and *PFR3-HSP70*) (Morell et al., 2006) four times at 3-week intervals. As control two groups of mice were employed, one immunized with the empty pCMV4 vector and another with sterile saline solution (0.9% sodium chloride solution; Sigma). Six weeks after the last inoculation, mice were sacrificed by CO_2 anoxia. Spleens from four mice per group were removed and homogenized in sterile conditions. The extracts were washed twice in PBS + 10% iFCS (inactivated Fetal Calf Serum; Life Technologies) and incubated for 3 min with erythrocyte ACK lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4). Cells were then washed again with Dulbecco's Modified Eagle Medium (Gibco BRL), re-suspended and cultured in DMEM complete medium (DMEM supplemented with 10% iFCS, 2 mM L-glutamine; Gibco RBL), 50 μM 2-mercaptoethanol (Sigma), 100 μg/mL streptomycin (Sigma), 25 mM HEPES and 1 mM sodium pyruvate.

2.2. Study population

For the HLA-A*02:01 restriction assay, eight adult patients were tested from Fundación Clínica Abood Shaio (Bogota-Colombia). Peripheral blood mononuclear cells (PBMC) were purified as described in Maranon et al., 2011, stored in iFCS with 10% DMSO and cryopreserved in liquid nitrogen until use. HLA-A genotyping was carried out using the RELI™ SSO HLA-A Typing kit (invitrogen). For cytotoxicity and functional activity determinations, 30 mL of blood samples from 18 adults HLA-A*02:01$^+$ Chagas disease patients and nine healthy donors were collected in EDTA at Hospital Virgen de la Arrixaca in Murcia and Hospital Clinic of Barcelona (both in Spain). The clinical status of each patient was diagnosed as indeterminate (IND, $n = 8$) or cardiac (CARD, $n = 10$) based on Kuschnir classification of Chagas disease (Kuschnir et al., 1985). All selected individuals had never received treatment for the disease.

2.3. Cell lines

For HLA-A*02:01 binding assays the TAP-deficient T2 cell line (HLA-A*02:01$^+$) was employed and grown in DMEM complete medium. Murine EL-4 and human Jurkat (HLA-A*02:01) lines stably transfected with the A2/K^b transgene were used and maintained in DMEM complete medium (with FCS, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin) supplemented with 0.6 mg/mL of G418 (Sigma). Transient transfection of EL-4 cells was carried out by electroporation for their use as target cells in chromium release assays.

2.4. Antibody generation

Two anti-PFRs polyclonal antibodies generated in rabbits were obtained by inoculating the PFR2 and PFR3 purified recombinant proteins, respectively, emulsified in incomplete Freud adjuvant (IFA) in two doses (200 μg each) at 2-week intervals. Sera from both immunized rabbits were analyzed by ELISA and western blot assays showing that both antibodies were able to specifically recognize, respectively, the aforementioned PFR2 and PFR3 recombinant proteins (data not shown).

2.5. Protein purification and western blot

To obtain soluble protein extracts from trypomastigote and amastigote forms, monolayers of monkey LLC-MK2 fibroblasts were infected with *T. cruzi* trypomastigote forms (Y strain) obtained