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Recombinant Adenovirus Delivery of Calreticulin–ESAT-6 Produces an Antigen-Specific Immune Response but no Protection Against a Mycobacterium Tuberculosis Challenge

S. C. Esparza-González^{*,†}, A. Troy[†], J. Troudt[†], M. J. Loera-Arias^{*}, J. Villatoro-Hernández[‡], E. Torres-López^{*}, J. Ancer-Rodríguez^{*}, Y. Gutiérrez-Puente^{*}, G. Muñoz-Maldonado^{*}, O. Saucedo-Cárdenas^{*,§}, R. Montes-de-Oca-Luna^{*}, and A. Izzo[†]

^{*}Departamento de Histología, Facultad de Medicina, Universidad Autónoma de Nuevo León (UANL), Monterrey N. L., México [†]Department of Microbiology, Immunology & Pathology, Colorado State University, Fort Collins, CO, USA [‡]Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands [§]División de Genética, Centro de Investigación Biomédica del Noreste, Instituto Mexicano del Seguro Social (IMSS), Monterrey N. L., México

Abstract

Bacillus Calmette–Guerin (BCG) has failed to efficaciously control the worldwide spread of the disease. New vaccine development targets virulence antigens of *Mycobacterium tuberculosis* that are deleted in *Mycobacterium bovis* BCG. Immunization with ESAT-6 and CFP10 provides protection against *M. tuberculosis* in a murine infection model. Further, previous studies have shown that calreticulin increases the cell-mediated immune responses to antigens. Therefore, to test whether calreticulin enhances the immune response against *M. tuberculosis* antigens, we fused ESAT-6 to calreticulin and constructed a recombinant replication-deficient adenovirus to express the resulting fusion protein (AdCRT–ESAT-6). The adjuvant effect of calreticulin was assayed by measuring cytokine responses specific to ESAT-6. Recombinant adenovirus expressing the fusion protein produced higher levels of interferon- γ and tumour necrosis factor- α in response to ESAT-6. This immune response was not improved by the addition of CFP-10 to the CRT-ESAT-6 fusion protein (AdCRT–ESAT-6–CFP10). Mice immunized with these recombinant adenoviruses did not decrease the mycobacterial burden after low-dose aerosol infection with *M. tuberculosis*. We conclude that calreticulin can be used as an adjuvant to enhance the immune response against mycobacterial antigens, but it is not enough to protect against tuberculosis.

Introduction

Tuberculosis (TB) is one of the most prevalent infectious diseases in adults, and there are 8–9 million new cases and 2 million deaths from TB annually [1, 2]. The WHO has estimated

Correspondence to: A. Izzo, Department of Microbiology, Immunology & Pathology, Colorado State University, 1682 Campus Delivery, Fort Collins CO 80523-1682, USA. Angelo.Izzo@ColoState.EDU and R. Montes-de-Oca-Luna, Facultad de Medicina, Universidad Autónoma de Nuevo León. Madero y Aguirre Pequeño s/n, Col. Mitras Centro, Monterrey, Nuevo León, 64460 México. rrrmontes@yahoo.com.

that one-third of the world's population is infected with latent TB and that 5–10% of those infected will develop clinical TB. It is worth mentioning that new experimental data support that latent TB infection is a constant, endogenous reinfection process [3–5]. Mortality rates have been increasing because of coinfection with human immunodeficiency virus and other immunocompromised conditions [6]. Currently, the only approved vaccine against TB is the attenuated *Mycobacterium bovis* strain Bacillus Calmette–Guerin (BCG). BCG is highly variable in efficacy (from 0 to 80%), as evidenced by reports showing that it is efficacious in protecting children, but not adults, from TB [7, 8]. Also, emerging multidrug-resistant strains have contributed to the increase in the rate of mortality caused by TB [9]. Thus, the development of a new and more effective vaccine is needed to control TB. As a consequence, the search for a new vaccine has intensified, especially in regard to the study of using immunodominant *M. tuberculosis* antigens such as Ag85A, Ag85B, ESAT-6, CFP-10 and TB10.4 (along with fusion proteins that combine these antigens) as vaccines. Such formulations have provided effective protection against *M. tuberculosis* in animal models [10–14]. In addition, studies have demonstrated that T cell-mediated immune responses are required to control TB disease. Nevertheless, the evidence suggests that the adjuvants play an important role in stimulating these cells. Many adjuvants have been used with vaccines, including the classical adjuvanted subunit vaccines, BCG, the aluminium salts and synthetic cationic adjuvants like IC31 [15–18]. However, the recent progress in the development of novel delivery systems has allowed the fusion of *M. tuberculosis* antigens to biological molecules to couple the adjuvant with the antigen [19]. In this regard, calreticulin has been of particular interest because it allows fused antigens to be directly targeted for MHC class I presentation because it can associate with peptides delivered to the endoplasmic reticulum by transporters associated with antigen processing (TAP-1 and TAP-2) and with MHC class I β 2-microglobulin molecules [20–23]. In fact, tumour antigen linked to calreticulin can generate tumour-specific immunity and eradicate established tumours [24, 25]. Others have demonstrated that calreticulin linked to the protective antigen domain IV from *Bacillus anthracis* enhances antibody responses [26]. Here, we describe the development and characterization of a recombinant replication-deficient adenoviral vector that expresses immunogenic *M. tuberculosis* Ag ESAT-6 fused to calreticulin. Additionally, we evaluated its ability to induce the production of tumour necrosis factor (TNF)- α and interferon (IFN)- γ , two cytokines required for protective immunity, and its capacity to protect against a *M. tuberculosis* challenge. Our data demonstrate that the calreticulin–ESAT-6 and calreticulin–ESAT-6–CFP10 fusion proteins generate a specific immune response, but this response does not confer protection against pulmonary *M. tuberculosis* infection.

Materials and methods

Construction and characterization of the recombinant replication-deficient adenoviruses

The gene fusions ESAT-6–CFP10 and ESAT-6 were purchased from Invitrogen (Carlsbad, CA, USA) already cloned into pUC plasmids (pESAT-6–CFP10 and pESAT-6, respectively). A plasmid shuttle vector containing calreticulin (pSh-CRT) fused to the antigen tumour E7 (pSh-CRT-E7) was used to make the vaccine formulation constructs. Briefly, E7 was removed from pSh-CRT-E7 using the restriction sites *Mlu* I and *Not* I and

replaced with the ESAT-6–CFP10 fusion gene using the same sites to generate the plasmid pSh-CRT–ESAT-6–CFP10. This plasmid was then characterized through the use of unique sites in pShuttle such as *Bgl* II and *Eco*R V. pSh-CRT–ESAT-6 was created by deleting CFP10 from the pSh-CRT–ESAT-6–CFP10 plasmid by *Spe* I digestion. The pSh-ESAT-6–CFP10 plasmid was created by cloning the ESAT-6–CFP10 gene directly into the pShuttle using the sites *Mlu* I and *Not* I. CFP10 was removed from pSh-ESAT-6–CFP10 by *Spe* I digestion to create pSh-ESAT-6. All the pShuttle plasmids were recombined with the AdEasy plasmid (AdEasy system; Agilent Technologies, Santa Clara, CA, USA) that contained the entire type 5 human adenovirus genome except the E1, E3 and packaging regions. The recombinant adenoviral vectors were characterized with *Spe* I, *Pme* I and *Mlu* I restriction enzymes. These recombinant vectors were transfected into HEK293 cells for the generation of the adenovirus particles AdESAT-6, AdCRT–ESAT-6 and AdCRT–ESAT-6–CFP10. The recombinant adenoviruses were purified by caesium chloride.

Analysis of protein expression by recombinant adenovirus

To verify the expression of the proteins by the recombinant adenoviruses, Western blotting and immunofluorescence studies were performed. HEK293 cells were infected with the recombinant adenoviruses and were monitored daily for viral cytopathic effect, at which time the proteins were extracted with lysis buffer (0.14 M NaCl, 1.5 mM MgCl₂, 10 mM Tris–HCl pH 8.0, 0.5% Nonidet P-40 and 1 mM dithiothreitol). The protein concentration of the cell lysates was determined using a Micro BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), and equal concentrations of protein (200 ng) were added to each well of a 12% Bis–Tris gel (Fermentas, Glen Burnie, MD, USA). After electrophoresis, the proteins were transferred onto nitrocellulose membrane using a transblot semidry system (BioRad, Hercules, CA, USA). Protein bands were visualized using a primary polyclonal antibody against CFP10 (Abcam, Cambridge, MA, USA) diluted 1:500 and an alkaline phosphatase-conjugated goat anti-mouse IgG diluted 1:100 as the secondary antibody. Immunofluorescence was also used to detect antigen expression (Thermo Fisher Scientific Inc.). HEK293 cells were transduced with recombinant adenovirus and monitored daily for viral cytopathic effect, after which the cells were fixed with acetone/ethanol (1:1) for 10 min at –20 °C. The cells were then incubated with a primary antibody to ESAT-6 (Abcam ab13960 rabbit polyclonal) at a dilution 1:1000 for 1 h at room temperature, followed by incubation with an anti-rabbit antibody (Invitrogen mouse polyclonal) conjugated to Alexa 488. Cells were then counterstained with 4',6-diamidino-2-phenylindole and mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Expression of the proteins was observed using a fluorescent microscope (Leica Microscopy system Inc., Buffalo Grove, IL, USA).

Immunization with recombinant adenovirus

Six- to 8- week-old female C57BL-6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA) and maintained under specific pathogen-free conditions at the animal facility at Colorado State University. For intranasal immunization (i.n.) of one dose of recombinant adenovirus, 5×10^7 PFU of recombinant adenovirus or AdLac Z was diluted with phosphate-buffered saline (PBS) to a total volume of 20 μ l and delivered into the

airway of a mouse with a fine pipette tip [12]. *Mycobacterium bovis* BCG at a dose of 5×10^5 CFU/mouse was diluted in PBS to a total volume of 100 μ l and injected subcutaneously.

Lymphocyte isolation and in vitro antigen stimulation. At 4 weeks post-vaccination, mice were humanely euthanized, and the spleens were removed. Single-cell suspensions were prepared as described previously [12] and cultured. Approximately 1×10^6 cells per well were seeded in 96-well plates in RPMI-1640 medium containing 10% foetal calf serum, 100 U/ml penicillin, 100 g/ml streptomycin and 2 mM of L-glutamine at 37 °C in 5% CO₂ with or without antigen stimulation (ESAT-6 at 5 μ g/ml). The culture supernatants were collected after 24 h and stored at -80 °C until used.

Analysis of ESAT-6-specific T cells by ELISPOT assay

Isolated splenocytes were seeded at 1×10^6 per well in a 96-well PVDF microplate (Millipore, Bedford, MA, USA) that was precoated overnight with a mouse IFN- γ capture antibody (R&D Systems, Minneapolis, MN, USA; 1:60 dilution). Cells were incubated for 24 h with or without stimulation by ESAT-6. The plate was then developed by a standardized streptavidin-conjugated alkaline phosphatase and chromogen method (R&D Systems). The number of IFN- γ -releasing cells was determined using a Cellular Technology Ltd Series 5 UV Immunospot Analyzer (Shaker Heights, OH, USA).

TNF- α measurement

The level of TNF- α in the supernatants was measured with a mouse-specific enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). Stored samples were thawed, and the manufacturer's protocol was used to assess the concentration. The sensitivity of detection was 2 pg/ml.

Low-dose aerosol infection with *Mycobacterium tuberculosis*

Animals were infected with live *M. tuberculosis* H37Rv via the aerosol route in a Glass-Col Airborne inhalation exposure system that delivered approximately 100 CFU *M. tuberculosis* bacilli per mouse. At 4 weeks post-infection, the protective efficacy was evaluated by plating serial 10-fold dilutions of lung and spleen homogenates on Middle-brook 7H11 agar plates. Plates were then incubated at 37 °C for 21 days, and colonies were counted.

Data analysis

The comparison of data generated from the ELISA and ELISPOT studies was conducted using an unpaired, two-tailed Student's *t*-test using SIGMAPLOT 11.0 software. The difference was considered statistically significant when $P < 0.05$.

Image acquisition and manipulation

Leica Microscopy system was used to take the picture, and magnification used was 40 with numerical aperture of the objectives, at temperature room. The slides were mounted using Vectashield mounting medium (Vector laboratories), and Alexa 488 fluorochrome was used to detect the positive signal (Invitrogen).

Results

Characterization of the recombinant replication-deficient adenoviral vectors expressing *Mycobacterium tuberculosis* antigens with or without calreticulin

As a first step, we designed recombinant adenovirus vectors containing ESAT-6 with and without calreticulin to determine whether calreticulin increased the immune response to the antigen. AdESAT-6 and AdCRT-ESAT-6 were created as described in the Materials and methods. Expression of ESAT-6 in both constructs was under the control of a cytomegalovirus promoter (Fig. 1A–C). The capacity of these constructs to express ESAT-6 was first verified by immunoblot analyses of HEK293 cells transfected with one of the recombinant vectors (data not shown). ESAT-6 protein expression was also demonstrated by immunofluorescence analysis of HEK293 cells transfected with AdESAT-6, AdCRT-ESAT-6 or AdLacZ (Fig. 1D). As shown in Fig. 1D, only cells transfected with AdESAT-6 and AdCRT-ESAT-6 express ESAT-6. Therefore, our recombinant adenovirus constructs were proven to be capable of producing ESAT-6.

Evaluation of cytokine responses induced by intranasal immunization with the recombinant adenoviruses

To test the ability of AdCRT-ESAT-6 or AdESAT-6 to generate ESAT-6-specific cellular immune responses *in vivo*, mice were immunized by the intranasal route with the adenovirus constructs. At 4 weeks post-vaccination, splenocyte cultures were prepared and restimulated with ESAT-6, and the resultant cytokine responses were analysed. It was found that while splenocytes from mice immunized with the antigen alone (AdESAT-6) showed no differences in cytokine production compared to splenocytes from LacZ-immunized mice (controls), there were significant inductions of IFN- γ and TNF- α (measured by ELISPOT and ELISA, respectively) in splenocytes from mice immunized with the antigen ESAT-6 fused to calreticulin (AdCRT-ESAT-6) (Fig. 2A,B). Taken together, these data demonstrate that immunization with ESAT-6 linked to calreticulin is an effective approach to generate potent immune responses.

Comparison of cytokine responses induced by AdCRT-ESAT-6 or AdCRT-ESAT-6-CFP10 intranasal immunization

It has been previously shown that fusion of ESAT-6 with CFP-10 enhances the immune response. Hence, using the same strategy, we expressed a calreticulin-ESAT-6-CFP10 fusion protein (AdCRT-ESAT-6-CFP10) and compared its ability to induce a cytokine response against AdCRT-ESAT-6. The expression of the fusion protein was demonstrated by immunoblot analysis of lysates of cells transfected with the fusion vector using an anti-CFP10 polyclonal mouse antibody (Fig. 3A). While no reaction was observed in the uninfected HEK293 cell lysates, a single antibody-reactive band of approximately 90 kDa was detected in the AdCRT-ESAT-6-CFP10 cell lysates. The size of the reactive band correlated with the predicted size of the CRT-ESAT-6-CFP10 fusion protein. Mice were immunized with 5×10^7 PFU of either AdCRT-ESAT-6 or the AdCRT-ESAT-6-CFP10 fusion. At 4 weeks post-immunization, mice were sacrificed, and their spleens were removed. Splenocytes were restimulated with ESAT-6 protein *in vitro*, and the number of IFN- γ -secreting cells and the concentration of TNF- α in the supernatant were measured

using ELISPOT and ELISA, respectively. No significant differences in the number of IFN- γ -secreting cells or the concentration of TNF- α were observed between the two groups (Fig. 3B,C). Thus, the addition of CFP-10 to the calreticulin–ESAT-6 fusion did not provide an enhancement of the ESAT-6-specific immune response.

Adenovirus expressing the calreticulin–ESAT-6–CFP10 fusion protein does not protect against a pulmonary *Mycobacterium tuberculosis* challenge

We next investigated the ability of the vaccine-induced immune response to reduce the mycobacterial burden after low-dose aerosol infection in the mouse model. Mice were vaccinated with AdCRT–ESAT-6–CFP10 via the intranasal route and BCG via the subcutaneous route, only once as described in Materials and methods. At 4 weeks post-immunization, mice were infected with *M. tuberculosis*. Four weeks after challenge, the *M. tuberculosis* burden of infected animals was determined to evaluate the protective efficacy in both lung and spleen. The trends were similar in both organs (Fig. 4A,B). BCG caused a reduction in CFU in both the lungs and spleen of infected animals. However, there was no significant difference between mice vaccinated with the adenovirus constructs and the saline-treated group for both organs.

Discussion

The high incidence of TB has stimulated interest in understanding the immune response to infection, resulting in the accelerated identification of novel immunodominant mycobacterial proteins as possible vaccine candidates. Culture filtrates and RD sequences have attracted particular interest as a source of antigens. ESAT-6, TB 10.4, CFP10, MTB12, MTB39 and Ag85 A and B have all been shown to elicit protective immune responses in various animal models of TB [12, 16, 27, 28]. Even though many strategies for vaccination increase the overall immune response, this may not be the ideal solution. When multiple antigens are presented to the immune system, they will compete for presentation, and the antigens dominating the response will not necessarily be those most relevant for protection. Thus, a targeted approach may be ideal. It has been repeatedly demonstrated that calreticulin can enhance immune responses when linked to antigens in DNA and viral vaccines [23–26]. This suggests that the use of calreticulin may be broadly applicable as a strategy to enhance vaccine efficacy. In addition, several reports have suggested the efficacious use of vaccines against TB in mice using adenoviral vectors expressing different *M. tuberculosis* antigens [10]. We herein demonstrate the effects of a replication-deficient adenoviral vector that contains the *M. tuberculosis* ESAT-6 antigen fused to calreticulin and show that there is an increased immune response to this antigen as demonstrated by increased cytokine expression. We compared the IFN- γ expression because it is well established that it plays a role in protective immunity, primarily in the context of antigen-specific T cell immunity. We also evaluated TNF- α levels because TNF- α is known to play a key role in granuloma formation and induction of macrophage activation [29]. The adenoviral vector expressing the CRT-ESAT-6 fusion protein demonstrated an enhanced ability to induce both of these cytokines in comparison with ESAT-6, which generated levels of cytokines similar to those induced by control vector, Lac. These data support calreticulin being able to enhance immunity against *M. tuberculosis* antigens. The fact that ESAT-6 alone did not show a

better cytokine response than the control may be because the C57BL/6 mice do not recognize AdESAT6 epitopes or that the immune response generated in these mice is relatively small and cannot be observed above background levels. It is important to determine whether the increased response is caused by CD8 and/or CD4 T cells, and whether it is a short- or long-term response. Even though it has been demonstrated previously that intranasal vaccination with adenovirus gives rise to a better immune response in the lung versus parenteral vaccination [10, 12], it would be interesting to demonstrate how this will work in our system. Our results support those of others that also showed that calreticulin increased the production of cytokines important in the control of TB [28]. Other studies have demonstrated that using a fusion of different *M. tuberculosis* antigens provides better control of infection in a mouse model of TB [30]. Thus, we also investigated whether multivalent or mixture-based adenoviral TB vaccines expressing an ESAT-6–CFP10 fusion protein could perform better than ESAT-6 alone when both were linked to CRT. Our result demonstrated that there was no difference between the ESAT-6 and ESAT-6–CFP10 constructs when the cells were stimulated with the ESAT-6 protein. Thus, the fusion did not increase the T cell response to ESAT-6 nor did the ESAT-6 protein stimulate the T cell response to CFP10. Accordingly, with this result, none of these adenoviruses gave protection against a challenge infection, even those constructs that induce increased IFN- γ and TNF- α antigen-specific cytokine levels. Others have reported similar data: Bennekov *et al.* [31], using a recombinant adenovirus expressing Ag85B–ESAT, found no protection after vaccination with adenoviral vaccine and also demonstrated that the adenovirus vaccine induced a non-protective, CD8 T cell-targeted response. Recent evidence also demonstrated differences in the types of protective immune response between the C57BL/6 and BALB/c mouse strains. In BALB/c (H-2^d) mice, a dominant CD8 T cell response has been reported [32], whereas in C57BL/6 (H-2^b) mice, more balanced CD4/CD8 T cell responses, with a more pronounced CD4 response in the lungs, has been reported [33]. In this regard, it is possible that the adenovirus constructs used here induced immune responses that were predominantly from CD8 T cells, because adenovirus has a broad host cell tropism and preferentially targets tissue cells that express MHC class I but not MHC class II. In addition, we used calreticulin as an adjuvant, and others have demonstrated that calreticulin increases CD8 T cell responses. In conclusion, our current data demonstrate that adenoviral vectors expressing fusion proteins consisting of CRT and ESAT-6 promote a specific immune response but do not protect against TB challenge.

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Figure 1.

Construction of Adenoviral vector AdCRT–ESAT-6–CFP10 (A) AdCRT–ESAT-6 (B) and AdESAT-6 (C). A shuttle plasmid vector was designed as detailed in Materials and methods. Panels A, B and C showed the recombinant adenovirus vector constructed by multiple cloning steps. Characterization of AdESAT-6 and AdCRT–ESAT-6 by immunofluorescence (D). HEK293 cells were infected with AdESAT-6, AdCRT–ESAT-6 or control vector AdLacZ and subjected to immunofluorescence with an anti-ESAT-6 mAb.

Figure 2.

Characterization *in vivo* of the immune responses produced by AdESAT-6 and AdCRT-ESAT-6. The C57BL-6 mice were immunized i.n. with 5×10^7 PFU of AdESAT-6, AdCRT-ESAT-6 or AdLacZ and killed 4 weeks later. The splenocytes were isolated and cultured with or without the ESAT-6 protein. The level of TNF- α in the culture supernatants was measured by enzyme-linked immunosorbent assay (A). The number of interferon- γ -releasing cells was determined by ELISPOT assay. Data are the mean value $\pm$ SE of triplicate samples from three independent experiments (B).

Figure 3.

Characterization of AdCRT–ESAT-6–CFP10 (AdCRTE-C) by Western blot (A). The HEK293 cells were transfected with AdLacZ or AdCRT–ESAT-6–CFP10. The cells were monitored daily for the cytopathic effect caused by the virus, after which cells were lysed and the extracted protein was analysed by western blot analysis using an antibody against CFP10. A comparison was made of the immune response *in vivo* generated by AdCRT–ESAT-6 and AdCRT–ESAT-6–CFP10 (B and C). C57BL-6 mice were immunized i.n. with 5×10^7 PFU of AdCRT–ESAT-6 or AdCRT–ESAT-6–CFP10 and killed 4 weeks later. The splenocytes were isolated and cultured with or without ESAT-6 protein. The level of TNF- α in the culture supernatants was measured by enzyme-linked immunosorbent assay (B). The

number of interferon- γ -releasing cells was determined by ELISPOT assay (C). Data are the mean value $\pm$ SE of triplicate samples from three independent experiments.

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Figure 4.

Comparison of CFU counts in lung and spleen of Bacillus Calmette–Guerin (BCG)-, AdESAT-6-, AdCRT–ESAT-6- and AdCRT–ESAT-6–CFP10-vaccinated C57BL-6 mice after infection with *Mycobacterium tuberculosis*. C57BL-6 mice were immunized i.n. with AdESAT-6, AdCRT–ESAT-6, AdCRT–ESAT6–CFP10, AdLacZ or BCG. At 4 weeks after immunization, the mice were challenged with tuberculosis. At 4 weeks post-infection, the mice were sacrificed, and the mycobacterial burden was determined in the lung (A) and spleen (B). Results are expressed as the mean $\pm$ SE of four samples from three independent experiments.