Biotechnology and Bioprocess Engineering 21: 653-658 (2016)
DOI 10.1007/s12257-016-0326-y

RESEARCH PAPER

TB Trifusion Antigen Adsorbed on Calcium Phosphate Nanoparticles Stimulates Strong Cellular Immunity in Mice

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Received: 9 May 2016 / Revised: 23 May 2016 / Accepted: 10 June 2016

Abstract Among various vaccine candidates, subunit vaccines play an important role in immune protection against tuberculosis (TB). Calcium phosphate (CP) is considered as a strong inorganic adjuvant due to its great potential in increasing immune responses. The purpose of this study was to evaluate specific immune responses following the administration of trifusion-CP nanoparticles. The physiochemical properties of these nanoparticles, including morphology, particle size, zeta potential and adsorption rate, were measured *in vitro*. Subcutaneous immunization was performed three times on days 0, 14, and 28. Two weeks after the last administration, IFN-gamma, IL-4, and TGF-beta levels were measured by indirect enzyme linked immunosorbent assay (ELISA). The trifusion protein was successfully adsorbed onto calcium phosphate nanoparticles. The mean sizes of the resultant trifusion-CPN and CPN were 97.84 ± 12.08 and 67 ± 11.85 nm, respectively. CPN containing trifusion had stronger ability to induce IFN-gamma than the control groups. IL-4 and TGF-beta secretions in trifusion and trifusion-CPN groups were higher than those in the PBS group. However, there was no significant ($p > 0.05$) difference in IL-4 and TGF-beta concentrations between trifusion group and trifusion-CPN group. Therefore, calcium phosphate nanoparticles are good candidates for immunization against TB because antigen can be easily adsorbed onto CPN and strong cellular immune responses against CPN-antigen can be stimulated.

Keywords: tuberculosis, HspX, Ppe44, EsxV, calcium phosphate, nanoparticle, immune response

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1. Introduction

Tuberculosis (TB), one of the major health issues worldwide, has killed millions of people in recent years. For centuries, *Mycobacterium tuberculosis* has adapted to host immune response mechanisms to successfully initiate an infection and survive in human host by modulating host immune response [1,2]. In recent years, the prevalence of multi-drug resistant TB (MDR-TB) in patients infected by human immunodeficiency virus (HIV) has increased to 9% [3]. In addition, only two new chemical drugs have been approved for use in treatment of MDR-TB patients in four decades [4,5]. Therefore, vaccination is needed to control TB. Bacillus Calmette–Guérin (BCG), the only vaccine against TB, covered 86% of the world population in 2000. However, it has shown variable protective efficacies in different countries [6,8]. Broad efforts are required to design new vaccines for controlling estimated 90% incidence of TB in 2035 [9].

Subunit vaccines based on recombinant proteins are promising new TB vaccine candidates [10]. However, most subunit TB vaccine candidates have focused on limited subunit antigens such as Ag85A and ESAT-6 [11,12]. Rv3619c is an ESAT6/CFP10 family protein found in culture filtrates of *Mycobacterium tuberculosis* (Mtb) [13]. Knudsen *et al.* have demonstrated that ESX dimer proteins

can stimulate immune response and initiate protective immune response in a mouse aerosol challenge model [11].

Despite the unknown role of PPE44 protein, these are suggestions about its important immunological roles in inhibiting antigen processing or causing antigen variations in TB infection [14]. Several studies have also shown that PPE44 has a drastic immunological potential as an effective vaccine [14,15]. In latency phase of Mtb infection, expression profiles of some genes have some fundamental changes, suggesting that prevention of Mtb reactivation needs immune responses to distinct antigens in different metabolic stages [16]. It has been suggested that HspX plays a significant role in the persistence of *M. tuberculosis* in the environment of macrophage phagosome [17]. Furthermore, these are many reports on the broad potential of HspX protein in providing protection against TB [18,19].

Despite of several advantages of subunit vaccines, they suffer from poor immunogenicity and requirement of efficient adjuvant/delivery systems [20,21]. Various groups of adjuvants including polymer-based nanoparticles and inorganic adjuvant have been used to improve the immunogenicity of different subunit vaccine candidates [22,23]. Calcium phosphate particles are among the most commonly used inorganic nano-adjuvants. They have many advantages, such as excellent biodegradability, resistance to degradation by lipase and bile salts, mostly non-toxic, and inexpensive [24,26]. Moreover, calcium phosphate has shown good adjuvant potential in stimulating immune responses against many infectious diseases. It is one of the promising candidates for preparing new vaccines [27,29].

The purpose of this study was to determine whether subcutaneous administration of adsorbed trifusion antigen onto calcium phosphate nanoparticles could promote the inherent low immunogenicity of trifusion protein as a model of subunit vaccine.

2. Materials and Methods

2.1. Nanoparticle synthesis

Calcium phosphate nanoparticles (CPN) were synthesized according to previously described method [29]. Briefly, 12.5 mM calcium chloride (Sigma-Aldrich), 12.5 mM dibasic sodium phosphate (Sigma-Aldrich), and 15.6 mM sodium citrate (Sigma-Aldrich) were slowly mixed together and stirred for 48 h. After 30 min of sonication, nanoparticles were characterized.

2.2. Preparation of trifusion adsorbed onto calcium phosphate nanoparticles

Briefly, 1 mg of trifusion protein in 300 μL of PBS (pH 7.5) was mixed with 1 mL of nanoparticle dispersion (5 mg/mL) and shacked at 4ºC for 16 h. The mixture was then centrifuged at 8,000 g and washed with distilled water [30].

2.3. Physicochemical characterization of calcium phosphate nanoparticles

The zeta potential and the size of CPN were measured with dynamic light scattering (DLS) (Malvern 3000, UK). Morphology of the nanoparticle was studied by scanning electron microscope (SEM).

2.4. Stability of calcium phosphate nanoparticles

CPN dispersions were kept at 4°C or room temperature for 15 days. The size and zeta potential of these nanoparticles were determined daily.

2.5. Measurement of adsorption efficiency

The amount of trifusion protein adsorbed onto CPN was determined using the following equation: [26]

$$\text{asorption}=\frac{\text{Total amount of trifusion–trifusion in the supernatant}}{\text{Total amount of trifusion}}\times 100$$

The amount of free trifusion protein in the supernatant was determined by Micro Bicinchoninic Acid (BCA) method according to the manufacturer's instruction.

2.6. MTT assay

HeLa cell line was obtained from the National Cell Bank of Iran. Cells were cultured in cell culture flasks. After culturing, cells were scraped off and centrifuged at 1,100 g for 5 min. Cells were then resuspended in fresh medium and counted by Trypan Blue exclusion (99% viability). Cell concentration was adjusted to 10^5 cells/mL. A hundred μl aliquot of different concentrations of nanoparticle suspension was added to each well of 96-well cell culture plates (Corning, USA) with RPMI-1640 medium (Cambrex Bioscience) supplemented with L-glutamine (2 mM, Gibco), penicillin–streptomycin (100 IU/mL penicillin, 100 mg/mL streptomycin (Gibco), and 10% heat inactivated fetal bovine serum (FBS, Biowest). Cells were maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere. Twenty-four and forty- eight hours after the incubation, MTT solution (USB Corporation, Cleveland, USA) was added to each well and plates were incubated at 37°C for 4 h. The MTT solution was removed and DMSO was added to dissolve formazan crystals. The absorbance of each well at 540 nm was read on a 680 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). The percentage of viability was calculated as AT/AC × 100, where AT and AC were the absorbance values of treated cells and control cells, respectively.

2.7. Experimental groups and immunization

Six- to eight- weeks old female BALB/c mice were purchased from a local animal facility (Razi Vaccine and Serum Research Institute, Mashhad-Iran). Mice were maintained under controlled environment. Mice were separated into 4 groups (6 mice/group) and immunized three times in 2-week intervals with 10 μg protein in 200 μL formulation per mouse. Groups were immunized with trifusion-CPN, trifusion, CPN, or PBS by subcutaneous injection [31].

2.8. Cytokine assay

Four weeks after the last immunization, all mice were scarified and their spleens were dissected aseptically. Splenocytes were prepared as a single cell suspension and erythrocytes were lysed with lysis buffer (ammonium chloride). Isolated spleen cells were cultured in duplicate wells (2×10^5 cells/well) in RPMI-1640 medium supplemented with 10% fetal calf serum (GIBCO, UK) and penicillin/streptomycin solution (Penstrep, Biosera, UK) in the presence of 5 μg/mL of trifusion protein. Cells were incubated at 37°C for 72 h in an atmosphere of 5% CO_2. Positive controls were stimulated with phytohemagglutinin-A (PHA) (2 μg/mL) while negative controls were left unstimulated. Cell culture supernatants were harvested for measurement of IFN-γ, TGF-beta, and IL-4 levels using respective ELISA kits (eBioscience, Austria) according to manufacturer's instructions.

2.9. Statistical analysis

Data were reported as mean ± standard error of the mean (SEM). Differences between groups were analyzed by one way analysis of variance (ANOVA) with Tukey-Kramer post -test using SPSS 13.0 software. A difference was considered to be statistically significant if P value was less than 0.05 ($P < 0.05$).

3. Results

3.1. Characterization of antigen adsorbed onto calcium phosphate nanoparticles

Particle sizes of trifusion-CPN and CPN were determined with DLS method. Their average diameters were 97.84 ± 12.08 and 67 ± 11.85 nm, respectively (n = 5). Zeta potential of CPN nanoparticle was -20.37. It was increased to -26.71 after protein adsorption. Stability of size and zeta potential of nanoparticles were also examined. After incubation of CPN and trifusion-CPN dispersions at 4°C or room temperature, no significant change was observed during 15 days. Morphology of the trifusion-CPN nanoparticles was studied by SEM. Spherical and smooth shaped nanoparticles were found Fig. 1. The adsorption efficiency of antigen to nanoparticles was determined to be 61.26% based on BCA protein assay.

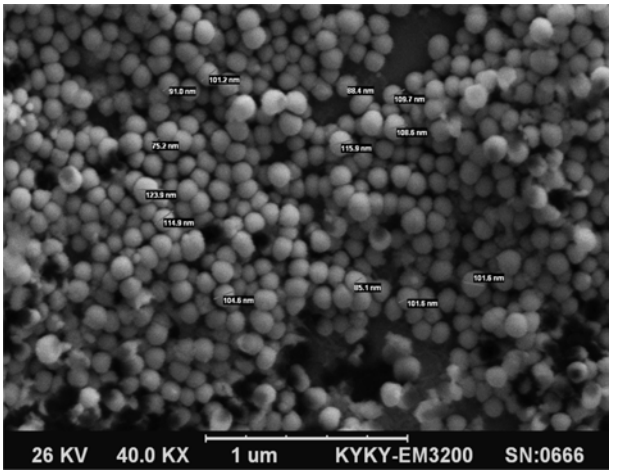

Fig. 1. Scanning electron microscope of Tri-fusion adsorbed of calcium phosphate nanoparticles.

3.2. Cytotoxicity assay

These calcium phosphate nanoparticles prepared in this study showed dose-dependent low cytotoxicities. Although cell viability was decreased with increased calcium phosphate concentration in HeLa cells, the MTT values were not significantly ($P > 0.05$) different. These results indicated that calcium phosphate nanoparticles had low toxicities (Fig. 2).

3.3. Immune response studies

To determine the effect of CPN nanoparticles on cellular immune responses, cytokine patterns were studied. The concentrations of IFN-γ, TGF-beta, and IL-4 released to the supernatant of cultured splenocytes were determined by indirect ELISA. Compared to PBS group, groups immunized with trifusion ($P < 0.01$) and trifusion-CPN ($P < 0.001$) antigens showed stronger IFN-γ responses. In compare to all groups, BCG immunized group showed significantly ($P < 0.001$) higher level of IFN-gamma. As shown in Fig. 3, trifusion-CPN group induced higher ($P < 0.001$) concentrations of IFN-γ than trifusion protein alone. Although IL-4 secretion in trifusion or trifusion-CPN group was higher ($P < 0.05$) than that in the PBS group, there was no significant ($P > 0.05$) difference in IL-4 concentration between trifusion group and trifusion-CPN group. Production of TGF-beta, a marker of T-reg, in all groups was higher ($P < 0.05$) than that in the PBS control group. While the BCG group induced higher ($P < 0.05$) TGF-beta production than trifusion group or trifusion-CPN group, there were no significant ($P > 0.05$) difference in its

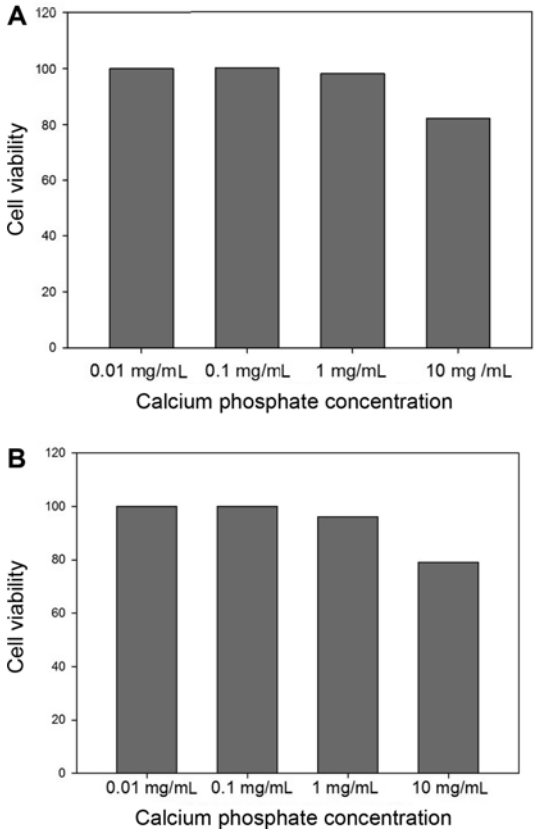

Fig. 2. Effect of calcium phosphate nanoparticle dosage on cell viability. (A) Cell viability percentage after 24 h. (B) After 48 h.

production between the trifusion immunization group and the trifusion-CPN immunization group (Fig. 5).

4. Discussion

Aluminum compounds are the most commonly used vaccine adjuvant in human vaccines [31,32]. Many adjuvant candidates such as calcium phosphate have also been evaluated for immunization against bacterial meningitis, HIV, herpes simplex type 2, and influenza [28,33-35]. Two mechanisms have been suggested to be responsible for the adjuvant effect of calcium phosphate. First, the slow release of antigen from calcium phosphate at the site of injection (depot effect) might have contributed to its adjuvant effect [36]. Second, calcium phosphate increases intracellular free Ca^{2+} ions as an intracellular signal that can stimulate the transcription of NF-AT, CREB, and NF-κB factors that can act as second messenger in T cell proliferation [37].

In this study, calcium phosphate was used as an adjuvant and carrier to promote the immunogenicity of a recombinant subunit vaccine candidate against *Mycobacterium tuberculosis*. As trifusion vaccine candidate has three immunogenic

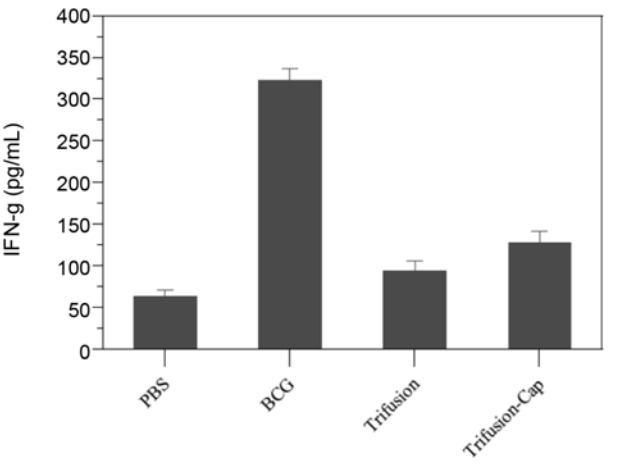

Fig. 3. IFN-γ release from splenic lymphocytes of immunized mice.

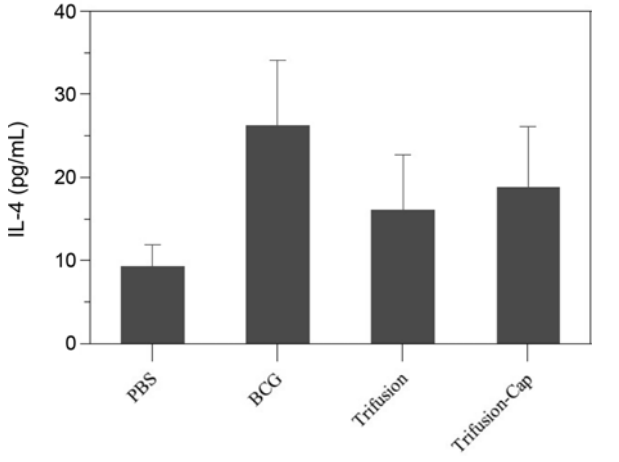

Fig. 4. IL-4 release from splenic lymphocytes of immunized mice.

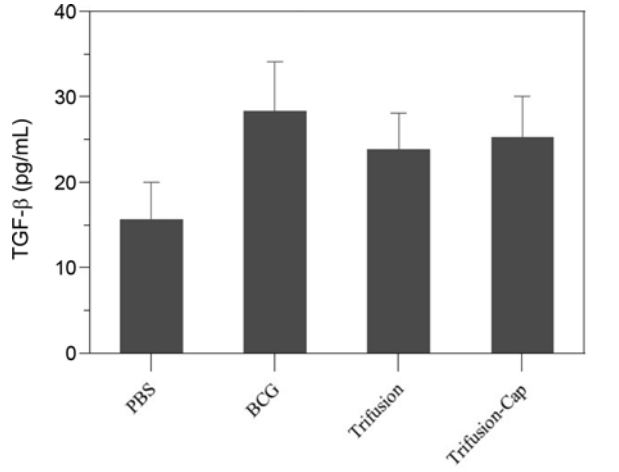

Fig. 5. TGF-beta release from splenic lymphocytes of immunized mice.

proteins (HspX, EsxV, and Ppe44) that belong to different stages of TB infection, it might be able to induce strong

immune responses to all stages of TB infection. Hspx is highly expressed in the dormant stage of bacterial life cycles. It could be a promising vaccine candidate for the prevention of TB reactivation in BCG vaccinated individuals [38]. Moreover, it has been frequently demonstrated that EsxV, one of the early secreted protein family (ESAT-6), has efficient B and T-cell epitopes to induce Th1 immune responses [11,39]. Ppe44 is a protein that is continuously presented to the immune system during all stages of TB infection. It could be introduced as a suitable candidate to induce effective immunogenic response in all stages of infection [15].

SEM image of the trifusion-CpN showed that these NPs had a particle size of 75 ~ 100 nm with spherical shape. Previous studies have suggested that NPs can traffic to lymph nodes in a size dependent manner. It has been reported that NPs with size of 30 ~ 200 nm are able to migrate freely toward LN-resident DC and macrophages [40]. In addition, compared to larger particles, virus sized NPs (20 ~ 200 nm) could be up-taken by endocytosis *via* clathrin-coated vesicles, caveolae, or their independent receptors and significantly induce Th1 immune response [41].

Different immune cell subsets such as gδ+ T cells and ab+ CD8+ can contribute to the immune protection. In addition, Th1 has an important role in preventing Tb infection and its reactivation [42,43]. In the present study, trifusion protein was found to be adsorbed onto calcium phosphate NPs and produce significantly higher levels of IFN-γ compared to antigen alone. The adjuvant effect of calcium phosphate NPs for the induction of antigen-specific CD4+ T cells response has been reported previously [44]. Sokolova *et al.* have demonstrated that CAP NPs are able to amplify and initiate the adaptive immune responses by activating dendritic cells, which may reflect promising immune responses against TB infection [44].

It is generally accepted that Th1 immune response is a significant mechanism to inhibit tubercle bacillus infection [1]. However, these are also studies that emphasize the great impact of antibodies against TB infection. In one study, serum analysis of children with tuberculosis infection has revealed that disseminated tuberculosis is associated with a decrease in IgG level against *mycobacterial* cell wall glycol-lipid lipoarabinomannan (LAM) [45]. Th2 antibody response acts in different classical and non-classical mechanisms [46]. In another study, it has been reported that mice infected with TB could not secrete antibodies, demonstrating enhanced mortality compared to healthy mice [47]. In the present study, trifusion and trifusion-CPN vaccinated groups exhibited high levels of IL-4 as a marker of Th2 immune response. Group vaccinated with trifusion-CPN secreted higher levels of IL-4 compared to the group immunized with trifusion solution alone.

5. Conclusion

The present study revealed that calcium phosphate NPs could be used as adjuvant and carrier to enhance the immunogenic properties of trifusion protein. These NPs could be used for designing new and efficient vaccines against TB. Further studies are needed to determine whether calcium phosphate NPs have other immunological potentials for immunization against Tb infection.

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