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Evaluation of the Immunogenicity of Recombinant Espb, Espc Proteins from *Mycobacterium Tuberculosis* and the Fusion Espc/Espb Protein in *BALB/C* Mice

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Abstract

Background: Two newly identified proteins, EspB and EspC are involved in the pathogenesis of *Mycobacterium tuberculosis*. The objective of the present study was to evaluate the immunogenicity of recombinant EspC, EspB, and EspC/EspB fusion proteins in mice.

Methods: BALB/c mice were immunized subcutaneously with recombinant EspC, EspB, and fusion EspC/EspB proteins, three times with along with Quil-A as an adjuvant. The cellular and humoral immune responses were evaluated by quantifying IFN-γ, IL-4, IgG, IgG1, and IgG2a antibodies against the antigens.

Results: The results showed that the mice immunized with recombinant EspC, EspB, and EspC/EspB proteins did not produce IL-4, whereas IFN-γ was secreted in response to all three proteins. EspC/EspB group produced significant amounts of IFN-γ in response to stimulation with all the three recombinant proteins ($P<0.001$). In mice immunized with EspC, high levels of IFN-γ were detected in response to EspC/EspB, and EspC ($P<0.0001$); while mice immunized with EspB produced lower levels of IFN- γ in response to EspC/EspB, and EspB ($P<0.05$).

Mice immunized with recombinant EspC, EspB, and EspC/EspB proteins exhibited significantly high levels of IgG and IgG2a/IgG1 ratio ($P< 0.001$). Moreover, high levels of IgG and IgG2a were detected in the sera of mice immunized with EspC/EspB fusion protein.

Conclusions: All the three recombinant proteins induced Th1-type immune responses in mice against EspB and EspC; however, EspC/EspB protein is more desirable due to the presence of epitopes from both EspC and EspB proteins and the production of immune responses against both.

Keywords: EspB, EspC, ESX-1, *Mycobacterium tuberculosis*.

Introduction

Tuberculosis (TB) is a chronic infectious disease that is prevalent in many parts of the world. One-third of the world's population is infected with *Mycobacterium tuberculosis* (MTB), the causative agent of the disease. Although many of these infections are latent, about one-tenth of them eventually turns into TB disease, and more than half of the cases can lead to death if left untreated (1-3). Prevention, case-finding, definitive rapid diagnosis, and treatment are the best ways to combat the disease. The bacillus Calmette- Guérin vaccine (BCG), is the only vaccine against TB obtained from the attenuated *M. bovis* bacillus. BCG is

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widely used in many countries, including Iran. The vaccine is known to protect children efficiently against the early manifestations of TB; however, its effectiveness against adult pulmonary TB is uncertain. Moreover, reactivity to tuberculin that occurs after vaccination interferes with the management of people who are possibly infected with MTB (4). The BCG vaccine is also among the most reactogenic vaccines in use today.

One approach to overcome the short comes of the current BCG vaccine was to use subunit vaccines. Various proteins of MTB have been nominated; however, the secreted ones particularly the proteins which are absent in BCG are more attractive. *Mycobacteria* secrete proteins that manipulate host cellular processes, thereby creating an environment favorable for intracellular survival and replication (2, 5). *Mycobacteria* have at least four secretory systems, of which the Type VII secretion system (T7SS) is more specific to *Mycobacteria*. T7SS is responsible for the secretion of several proteins that lack classical signal peptides in the bacilli. *Mycobacterial* genomes contain five loci that construct the T7SS, named ESAT-6 secretion system-1 (ESX)- 1 to 5 (6). ESX-1 plays an important role in MTB virulence. The Pathogenic strains of MTB use the ESX-1 specific secretory system to transmit their major virulence factors through the abnormal bacterial cell wall. Deletion of ESX-1 locus - known as the region of difference (RD1)- from bacterium reduces its pathogenicity (7, 8). MTB uses the ESX-1 secretory system proteins to disable host defense and to optimize cell to cell spread of the bacilli. ESAT-6 and CFP10 are the most important secretory proteins of the ESAT-6 family and the potent stimulants of IFN-γ induction. These proteins are not present in the BCG vaccine and have been reported to lead to a lack of complete immunity in people vaccinated with BCG (9). ESX-1 also secretes other proteins such as ESX-1 substrate protein (Esp) A, EspB and, EspC (10).

EspB protein, also called RV3881c, is secreted by ESX-1 simultaneously with other ESAT-6 family proteins (11). The protein is encoded by a 2004 bp gene within the RD1 locus. The 50 kDa protein is obtained after partial cleavage of the 61 kDa fragment. EspB is an alanine- and glycine-rich protein. Previous observations have shown that EspB plays an important role in cytolysis, bacterial diffusion, ESAT-6 secretion, and most importantly, it is essential to inhibit phagosome maturation (10, 12).

The EspC or Rv3615c protein is another protein secreted by the ESX-1 system; however, its gene is located outside the RD1 region. In terms of size and sequence, it is homologous to CFP10 and ESAT-6. The molecular weight of EspC is about 12 kDa and consists of 103 amino acids. Since EspC is secreted through the ESX-1 system, the protein is not present in strains whose RD1 region has been deleted (13-15). EspC protein is specifically detected in people with mycobacterial infections but is not detected in people vaccinated with BCG (14, 16).

Due to the role of EspB protein as a major virulence factor in MTB and the role of EspC protein in stimulating the immune responses, in the present study the immune responses against recombinant EspB, EspC, and fusion EspC/EspB proteins in BALB / c mice have been evaluated.

Materials and Methods

Mice immunization

Female BALB/ c mice aged 6 to 8 weeks were purchased from the animal facility of the Production Complex of the Pasteur Institute of Iran (Karaj, Iran). Mice were randomly divided into the five groups (n=5 in each group): EspB, EspC, EspC/EspB, Quil-A, and PBS. Mice were immunized subcutaneously at the base of the tail with 15 μg of recombinant protein along with 15 μg of Quil-A as an adjuvant. As controls, the other two groups of mice received only Quil-A or PBS. Immunizations were repeated 3 times at two-week intervals. Recombinant EspB, EspC, or EspC/EspB fusion proteins were prepared as previously described (17).

Maintenance and care of experimental animals complied with guidelines of ethical committee of Pasteur Institute of Iran.

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Cytokine measurements

Inguinal lymph nodes were removed, and a single-cell suspension was prepared and cultured in RPMI 1640 medium (Biowest, Paris, France)) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Paisley, UK), 2 mM L-glutamine, and 10 % FCS (Sigma, Vienna, Austria; complete medium). The cells were added to 96-well plates (Nunc, Denmark) in triplicate at a density of 4×10^5 cells per well and cultured with the recombinant proteins or with medium alone. Cell culture supernatants from triplicate wells were pooled after 72 h incubation and stored at -70 °C for cytokine assay. Cytokines were assessed by IFN-γ and IL-4 capture ELISA kits (Bendermed system, Austria) as recommended by the manufacturer.

Evaluation of antigen-specific antibody responses

Two weeks after the last injection, serum samples were prepared and analyzed by ELISA for total IgG, IgG1, and IgG2a responses against recombinant EspB, EspC, and EspC/EspB proteins. In brief, the 96-well microtiter plates (Greiner, Germany) were coated overnight at 4 ℃ with each protein. The plates were blocked with 3% skim milk in PBS (blocking buffer). Sera diluted in blocking buffer were added and the plates were incubated at 37 °C for 1 h. After washing, goat anti-mouse IgG-HRP, goat anti-mouse IgG1, and IgG2a (All from Sigma, Darmstadt, Germany) were added and incubated at 37 °C for 1 h. For IgG1 and IgG2a detection, HRP-conjugated rabbit anti-goat antibody (Sigma, Germany) was added for 1 h. Then, the plates were washed, and tetramethylbenzidine (TMB, Sigma, Germany) substrate was added. Absorbance was read at 450 nm using an ELISA plate reader (Anthos 2020, Austria).

Statistical Analysis

GraphPad Prism software (Prism 8.0.2., 2019, San Diego, CA, USA) was used for statistical analysis. ANOVA method was used to evaluate the statistical significance of differences between control (Quil-A) and experimental groups. The $P< 0.05$ was considered to be significant, and data were represented as mean + SD.

Results

Cytokine response

Lymphocytes were recovered and analyzed for IFN- γ and IL-4 production two weeks after immunization. No cytokine responses were detected for mice in PBS and Quil-A control groups. Stimulation of lymphocytes in all three vaccinated groups with EspC/EspB resulted in significant production of IFN- γ compared to the Quil-A group (Fig. 1). The highest level was found in mice immunized with EspC followed by those immunized EspC/EspB, and EspB. In response to EspC, the two EspC/EspB, and EspC groups produced significant amounts of IFN- γ compared to the control group ($P< 0.0001$). Besides, Esp/B resulted in a low but significant increase in IFN- γ levels in Esp/B ($P<0.05$) and EspC/EspB ($P<0.001$) groups in comparison with the Quil-A group.

No significant levels of IL-4 were observed after stimulation of lymphocytes from none of the groups.

Antigen-specific antibody responses

Specific serum IgG, IgG1, and IgG2a responses were assayed by ELISA two weeks after the last immunization. A significant reaction between sera from groups immunized with EspC ($P<0.0001$), EspC/EspB ($P<0.0001$), and EspB ($P<0.05$) with EspC/EspB protein as antigen were documented (Fig. 2). Likewise, significant amounts of anti-EspC were detected in EspC, and EspC/EspB groups ($P<0.0001$), compared to the control (Quil-A) group. No significant anti-EspC was present in mice immunized with EspB. In sera from mice immunized with EspB, the level of anti-EspB antibody was significantly higher than that of the Quil-A group ($P<0.05$). In response to EspB antigen, high levels of IgG were also detected in EspC/EspB-immunized mice ($P<0.0001$).

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Significant levels of specific IgG1, and IgG2a were induced in response to the EspC/EspB antigen in all the three experimental groups (P<0.001) (Fig. 3). No detectable anti-EspC IgG1 and IgG2a in the EspB-immunized group were detected. However, in EspC- and EspC/EspB-immunized mice, there were significant amounts of IgG1 and IgG2a against the EspC antigen (P<0.0001). Likewise, in EspB and EspC/EspB groups, both IgG1 and IgG2a levels against EspB protein were significant (P<0.0001), however no significant anti-EspB IgG1 and IgG2a were present in the EspC-immunized group.

The IgG2a/IgG1 ratios as an indicator of a Th1 or a Th2 response were evaluated. The highest proportion belongs to the group of mice immunized with EspC/EspB and then the group of EspC. In the EspB group, the highest IgG2a / IgG1 ratio was produced in response to the EspB antigen (Fig. 4).

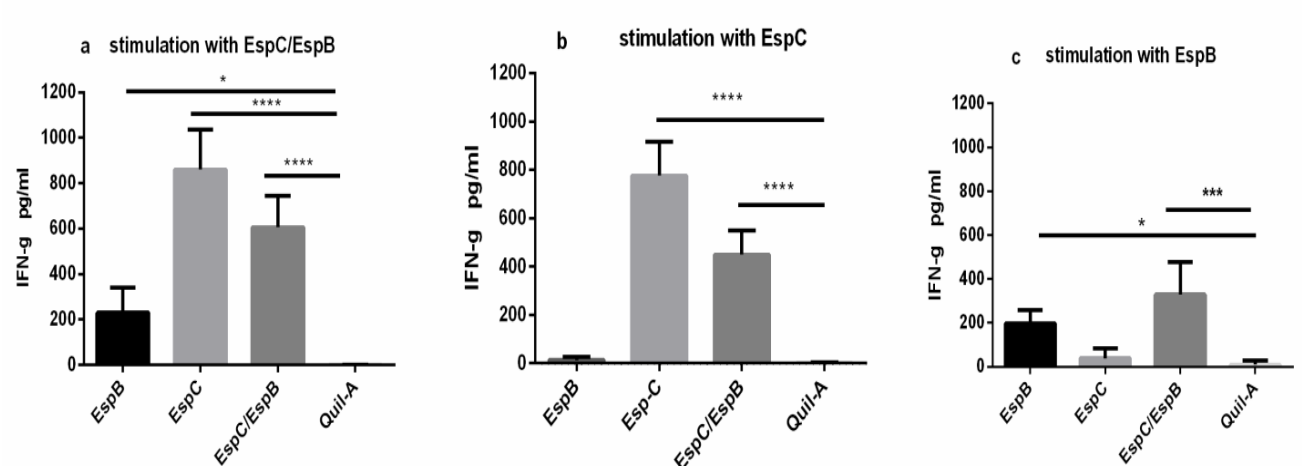

Fig. 1. IFN-γ production by splenocytes from mice immunized with EspC/EspB, EspC, and EspB stimulated with recombinant EspC/EspB (a), EspC (b), and EspB (c).

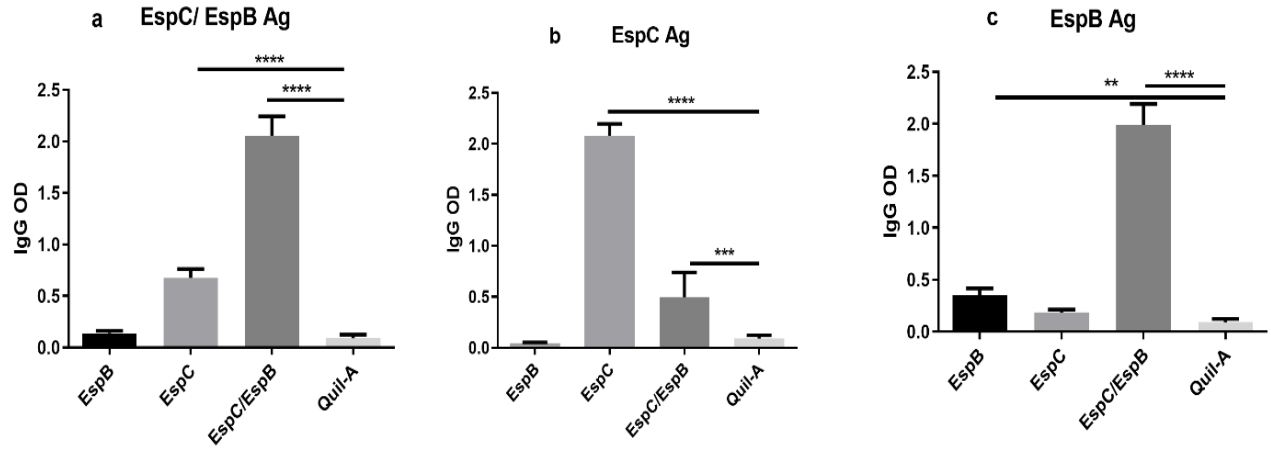

Fig. 2. Serum IgG levels in mice immunized with recombinant antigens in response to EspC/EspB (a), EspC (b), and EspB (c).

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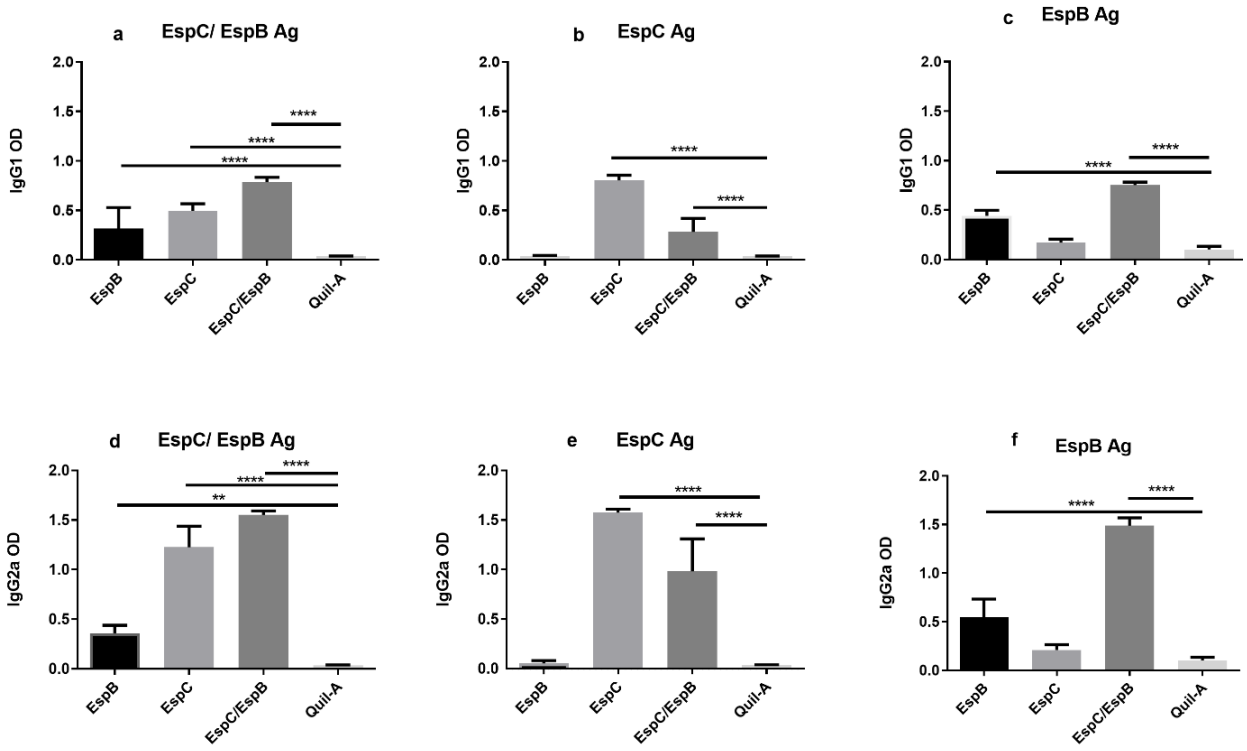

Fig. 3. Serum IgG1 and IgG2a levels in mice immunized with recombinant antigens in response to EspC/EspB (a, d), EspC (b, e), and EspB (c, f).

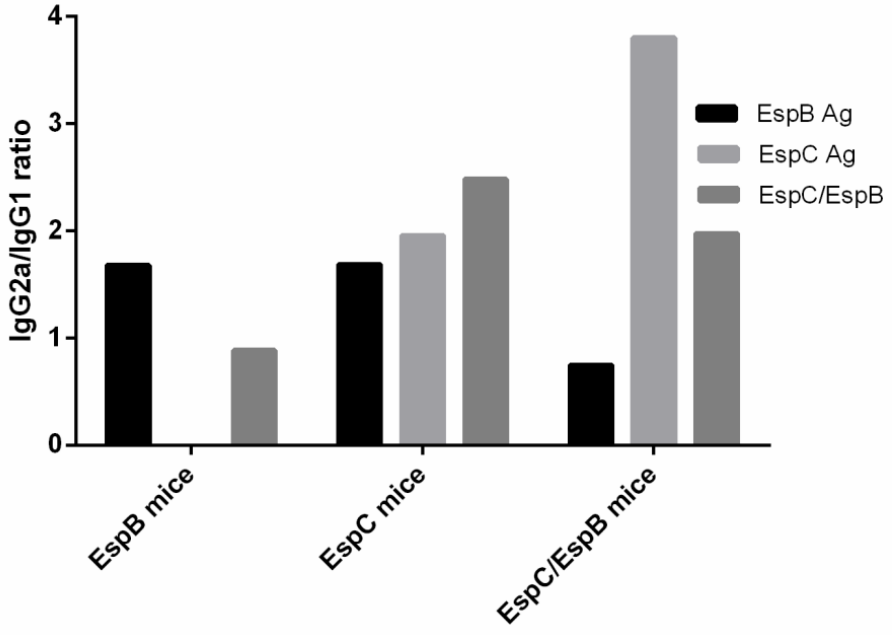

Fig. 4. IgG2a/IgG1 ratio in mice immunized with EspC/EspB, EspC, and EspB against all the three recombinant proteins.

Discussion

BCG vaccination is routinely given to infants in many parts of the world and provides considerable protection against the most severe childhood manifestations of the disease, such as TB meningitis. The BCG vaccine is valuable in preventing active TB in this age

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group since TB mortality in children under 5 years of age can be very high. However, a meta-analysis of the effect of the BCG vaccine in children has shown that the duration of protection is generally up to 10 years and the effect of the vaccine decreases over time (3, 4). On the other hand, the effectiveness of the BCG vaccine against pulmonary TB in adults is between zero and 80% (18). The reason for the variability of the effect of this vaccine is unknown and factors such as the high prevalence of non-tuberculous mycobacteria have been considered for the failure of BCG in some clinical trials. It seems that BCG alone cannot be completely effective in global TB control (3). A modified vaccine for infants and/ or a safe and effective booster vaccine in addition to BCG seems to be necessary. Different approaches are attracting attention. There is particular interest in subunit vaccines based on secretory antigens of MTB. Among the secreted proteins, the proteins secreted by the ESX-1 system have received the most attention (9), because they are absent in BCG. Due to the common antigen existing in MTB and BCG, the selection of MTB-specific antigens is critical in distinguishing the MTB infection or BCG vaccination.

Many of the secreted proteins in the ESX-1 system are virulence factors and induce immune responses (9). The role of ESAT-6 and CFP-10 proteins from this system in the pathogenicity of MTB has been well studied. ESAT-6 and CFP-10 are also potent stimulants of cellular immunity, IFN-γ production, and humoral immune responses. The combination of ESAT-6 with other secretory proteins has resulted in a stronger response than either protein alone (19, 20). Identification of other specific proteins is important, both for developing diagnostic kits and vaccines. EspB and EspC are the other ESX-1-related proteins in MTB that are not present in BCG. MTB species that lack EspB are as weakly pathogenic as the species that lack all of the secretory apparatus (21). This protein assembles into a channel that probably transports T7SS proteins (22).

EspB can interact with the membrane and probably forms a membrane pore that facilitates phagosome permeabilization within the infected macrophages (9). Chen and his colleagues have shown that MTB without the EspB gene is not able to cause cytotoxicity in the THP-1 human macrophage cell line (23). EspC is another conserved secretory protein in the pathogenic MTB, *M. Bovis, M. marinum*, and *M. leprosy*; indicating its significant role as a virulence factor. EspC forms filamentous structures in vitro and localizes on the bacterial surface in vivo. It has been hypothesized that ESX-1 could be an injectosome system with EspC as its needle (24).

In the present study, as a first step in evaluating EspB, EspC, and a fusion of these secretory proteins (EspC/EspB) as vaccine candidates, their immunogenicity and ability to induce IFN-γ, IL-4, and antibody production in BALB/c mice were evaluated. Th1 cell responses play pivotal roles in protection against MTB infection. Identification of Th1 and Th2 responses in response to antigens plays an important role in assessing whether they are protective or not. The main characteristic of Th1 cells in the production of IFN-γ, which activates antimycobacterial action in macrophages (25). This process also leads to the formation of granulomas that can control infection (14, 26, 27). IL-4 is one of the most important Th2 cytokines that is increased in mice susceptible to TB infection. Some experimental studies have shown an association between increased IL-4 and disease progression and/ or reactivation. However, it is often believed that the absence of this cytokine does not affect susceptibility to disease (28).

In our study, evaluation of IFN-γ production by lymphocytes of immunized mice in response to the recombinant proteins indicated that EspC/EspB group produced significant amounts of IFN-γ in response to stimulation with all the three recombinant proteins compared to the Quil-A group. In mice immunized with EspC, high levels of IFN-γ were detected in response to

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EspC/EspB, and EspC; while, mice immunized with EspB produced lower levels of IFN- γ in response to EspC/EspB and EspB. To the best of our knowledge, the immunogenicity of EspB has not been investigated; however, Millington et al have shown that T cells from individuals with active TB and latent TB infection responded to the EspC by IFN-γ production (14). Moreover, it has been shown that EspC contains CTL epitopes which could improve T-cell-based diagnosis of MTB infection (29). Recombinant EspC has also been used in an ELISA-based immunoassay for the diagnosis of active TB (30). In a study similar to ours, Immunization of BALB/c mice with a DNA vaccine encoding EspC, Mtb10.4, and Rv2660c as a fusion protein-induced robust T cells mediated immune response which was more effective than that of individual antigen vaccination. No IL-4 or IL-10 was detected in response to none of the mentioned proteins (31).

The study of humoral immune responses showed that all the three recombinant proteins i.e. EspC, EspB, and EspC/EspB stimulated the production of antibodies in mice; however, the EspB protein induced much weaker immune responses compared to the other two proteins. EspC and EspC/EspB proteins induced comparable amounts of IgG. However, EspC/EspB protein is more desirable due to the presence of epitopes from both proteins and the production of specific antibodies against both EspC and EspB proteins. Besides, fusion proteins reduce the production and purification costs. We have already shown that antibodies generated against all the three recombinant proteins can detect EspC and EspB proteins in their native state in MTB (17). This finding indicates the conservation of antigenic determinants in the recombinant proteins.

In our study, IgG2a production was significantly higher in all the three immunized groups compared to the controls. Since IFN- γ is associated with the production of IgG2a and IL-4 is associated with the production of IgG; the high ratio of IgG2a/ IgG1 can be considered as an indicator of the dominance of Th1 responses. Analysis of this ratio in different groups of this study showed that the highest ratio belongs to the group of mice immunized with EspC/ EspB.

In the present study, we demonstrated that EspC/ EspB fusion protein is a strong inducer of Th1-type response and can be further developed as a vaccine against TB.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

References

1. Bloom BR, Atun R. Back to the future: Rethinking global control of tuberculosis. Sci Transl Med. 2016;8(329):329ps7.
2. Houben RM, Dodd PJ. The Global Burden of Latent Tuberculosis Infection: A Re-estimation Using Mathematical Modelling. PLoS Med. 2016;13(10):e1002152.
3. Furin J, Cox H, Pai M. Tuberculosis. Lancet. 2019;393(10181):1642-56.
4. Abubakar I, Pimpin L, Ariti C, Beynon R, Mangtani P, Sterne JA, et al. Systematic review and meta-analysis of the current evidence on the duration of protection by bacillus Calmette-Guerin vaccination against tuberculosis. Health Technol Assess. 2013;17(37):1-372, v-vi.
5. Finlay BB, Falkow S. Common themes in microbial pathogenicity revisited. Microbiol Mol Biol Rev. 1997;61(2):136-69.
6. Simeone R, Bottai D, Frigui W, Majlessi L, Brosch R. ESX/type VII secretion systems of mycobacteria: Insights into evolution, pathogenicity and protection. Tuberculosis (Edinb). 2015;95 Suppl 1:S150-4.

[DOI: 10.52547/rbmb.11.4.590]

7. Abdallah AM, Gey van Pittius NC, Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CM, et al. Type VII secretion--mycobacteria show the way. Nat Rev Microbiol. 2007;5(11):883-91.
8. Clemmensen HS, Knudsen NPH, Rasmussen EM, Winkler J, Rosenkrands I, Ahmad A, et al. An attenuated Mycobacterium tuberculosis clinical strain with a defect in ESX-1 secretion induces minimal host immune responses and pathology. Sci Rep. 2017;7:46666.
9. Wong KW. The Role of ESX-1 in Mycobacterium tuberculosis Pathogenesis. Microbiol Spectr. 2017;5(3).
10. Solomonson M, Setiaputra D, Makepeace KAT, Lameignere E, Petrotchenko EV, Conrady DG, et al. Structure of EspB from the ESX-1 type VII secretion system and insights into its export mechanism. Structure. 2015;23(3):571-83.
11. Xu J, Laine O, Masciocchi M, Manoranjan J, Smith J, Du SJ, et al. A unique Mycobacterium ESX-1 protein co-secretes with CFP-10/ESAT-6 and is necessary for inhibiting phagosome maturation. Mol Microbiol. 2007;66(3):787-800.
12.Satchidanandam V, Amara RR, Uchil PD, Singh V. The regulatory elements of the Mycobacterium tuberculosis gene Rv3881c function efficiently in Escherichia coli. FEMS Microbiol Lett. 2003;218(2):365-70.
13. Fortune SM, Jaeger A, Sarracino DA, Chase MR, Sassetti CM, Sherman DR, et al. Mutually dependent secretion of proteins required for mycobacterial virulence. Proc Natl Acad Sci U S A. 2005;102(30):10676-81.
14. Millington KA, Fortune SM, Low J, Garces A, Hingley-Wilson SM, Wickremasinghe M, et al. Rv3615c is a highly immunodominant RD1 (Region of Difference 1)-dependent secreted antigen specific for Mycobacterium tuberculosis infection. Proc Natl Acad Sci U S A. 2011;108(14):5730-5.
15. McLaughlin B, Chon JS, MacGurn JA, Carlsson F, Cheng TL, Cox JS, et al. A mycobacterium ESX-1-secreted virulence factor with unique requirements for export. PLoS Pathog. 2007;3(8):e105.
16. Sidders B, Pirson C, Hogarth PJ, Hewinson RG, Stoker NG, Vordermeier HM, et al. Screening of highly expressed mycobacterial genes identifies Rv3615c as a useful differential diagnostic antigen for the Mycobacterium tuberculosis complex. Infect Immun. 2008;76(9):3932-9.
17. Salemi O, Noormohammadi Z, Bahrami F, Siadat SD, Ajdary S. Cloning, Expression and Purification of Espc, Espb and Espc/Espb Proteins of Mycobacterium tuberculosis ESX-1 Secretion System. Rep Biochem Mol Biol. 2020;8(4):465-72.
18. Martin C. Tuberculosis vaccines: past, present and future. Curr Opin Pulm Med. 2006;12(3):186-91.
19. Barker LF, Brennan MJ, Rosenstein PK, Sadoff JC. Tuberculosis vaccine research: the impact of immunology. Curr Opin Immunol. 2009;21(3):331-8.
20. van Dissel JT, Arend SM, Prins C, Bang P, Tingskov PN, Lingnau K, et al. Ag85B-ESAT-6 adjuvanted with IC31 promotes strong and long-lived Mycobacterium tuberculosis specific T cell responses in naive human volunteers. Vaccine. 2010;28(20):3571-81.
21. Gao LY, Guo S, McLaughlin B, Morisaki H, Engel JN, Brown EJ. A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. Mol Microbiol. 2004;53(6):1677-93.
22. Gijsbers A, Vinciauskaite V, Siroy A, Gao Y, Tria G, Mathew A, et al. Priming mycobacterial ESX-secreted protein B to form a channel-like structure. Curr Res Struct Biol. 2021;3:153-64.
23. Chen JM, Zhang M, Rybniker J, Boy-Rottger S, Dhar N, Pojer F, et al. Mycobacterium tuberculosis EspB binds phospholipids and mediates EsxA-independent virulence. Mol Microbiol. 2013;89(6):1154-66.
24. Lou Y, Rybniker J, Sala C, Cole ST. EspC

[DOI: 10.52547/rbmb.11.4.590]

forms a filamentous structure in the cell envelope of Mycobacterium tuberculosis and impacts ESX-1 secretion. Mol Microbiol. 2017;103(1):26-38.
25. Zeng G, Zhang G, Chen X. Th1 cytokines, true functional signatures for protective immunity against TB? Cell Mol Immunol. 2018;15(3):206-15.
26. Domingo-Gonzalez R, Prince O, Cooper A, Khader SA. Cytokines and Chemokines in Mycobacterium tuberculosis Infection. Microbiol Spectr. 2016;4(5).
27. Xu G, Wang J, Gao GF, Liu CH. Insights into battles between Mycobacterium tuberculosis and macrophages. Protein Cell. 2014;5(10):728-36.
28. da Silva MV, Tiburcio MG, Machado JR, Silva DA, Rodrigues DB, Rodrigues V, et al. Complexity and Controversies over the Cytokine Profiles of T Helper Cell Subpopulations in Tuberculosis. J Immunol Res. 2015;2015:639107.
29. Tan S, Lin N, Huang M, Wang Q, Tan Y, Li B, et al. CTL immunogenicity of Rv3615c antigen and diagnostic performances of an ESAT-6/CFP-10/Rv3615c antigen cocktail for Mycobacterium tuberculosis infection. Tuberculosis (Edinb). 2017;107:5-12.
30. Zhang X, Su Z, Zhang X, Hu C, Yu J, Gao Q, et al. Generation of Mycobacterium tuberculosis-specific recombinant antigens and evaluation of the clinical value of antibody detection for serological diagnosis of pulmonary tuberculosis. Int J Mol Med. 2013;31(3):751-7.
31. Kong H, Dong C, Xiong S. A novel vaccine p846 encoding Rv3615c, Mtb10.4, and Rv2660c elicits robust immune response and alleviates lung injury induced by Mycobacterium infection. Hum Vaccin Immunother. 2014;10(2):378-90.

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