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Original Article

Immunogenicity of Chitosan-Based Non-invasive Vaccine Strategy Against *Mycobacterium tuberculosis*

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Abstract

***Background*:** In spite of Bacillus Calmette-Guerin (BCG) vaccination, still tuberculosis caused by *Mycobacterium tuberculosis* remains a problematic impediment that requires perspective management. The aim of this study was to evaluate the immunogenicity of chitosan nanoparticles containing recombinant mycobacterial proteins and adjuvant in the Balb/C mice through a non-invasive nasal inhalation delivery route and measure the level of cytokines interferon-gamma (IFN-γ), interleukin-4 (IL-4), and IL-17.

Methods: Thirty mice in five different groups were vaccinated through inhalation with compounds set in different combinations. Two weeks after the last nasal delivery, IFN-γ, IL-4, and IL-17 were measured in spleen cell culture supernatants.

Results: The IFN-γ and IL-17 concentrations were found to increase in the groups that received chitosan nanoparticles containing protein and adjuvant alone or as a BCG booster. Our study showed that the chitosan nanoparticle containing protein and adjuvant induced a Th1 response. However, the groups that first received BCG and then chitosan nanoparticles containing protein and adjuvant had the greatest Th1 response in terms of IFN-γ and IL-17 production in all the groups.

Conclusion: Our findings showed that the vaccine designed to be administered through the nasal mucosa well stimulates cellular immunity and enhances the BCG vaccine's effectiveness.

Introduction

Despite many diagnostic immunoassays and well-established treatment protocols, tuberculosis (TB) still remains an important health issue that has surpassed even human-acquired immunodeficiency syndrome, reaching the first place in death numbers caused by an infectious contagious disease.[1] The persuasive evidence for the protective effect of Bacillus-Calmette Guerin (BCG) vaccination against *Mycobacterium tuberculosis* infection, as opposed to against disease, in various clinical settings has been the subject of numerous efficacy trials and, epidemiological studies conducted over several decades.[2] The last decade has given much importance to the development of novel strategies and/or efficient vaccines to build potent immunity and confine the infection.

The family of PPE proteins includes 69 proteins. PPE 44 is a part of this family that is less expressed in the attenuated strain H37Rv (H37Ra).[3] Strong humoral and cellular immunity is observed against this antigen in mice vaccinated with BCG.[4] ESAT-6-like protein (ESAT-6-like protein) or EsxV is a 94 amino acid protein that has recently been shown to have the ability to strongly stimulate T-cell immune responses in the host.[5] HSPX protein is expressed during the dormant phase of infection, especially under hypoxic conditions, and plays an important role during infection.[6,7] It plays a role in the survival of bacteria inside phagosomes and also causes the slow growth of bacteria. In addition, in patients infected with *M. tuberculosis*, it causes strong immune responses, especially interferon gamma production.[8]

Currently, a broad range of adjuvant substances is being utilized for the efficient stimulation of antigen-specific

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immune cells. As TB is primarily a pulmonary infection and nasal mucosa is the initial site of exposure. Moreover, the unique way of acquiring *M. tuberculosis* is through coughing or sneezing in patients with the active form of TB thus, conventional vaccine delivery technologies that are based on the administration of bacteria into the body, and often lack enough stimulation, it is predicted that if the vaccine is given through mucosal surfaces, it may be more effective as there are specialized cells in mucosal surfaces that are capable of antigen uptake and presenting it to the professional antigen-presenting cells (APCs) that can stimulate both local and systemic immune responses.[9,10] Researchers are on track to meet the deadlines by using biodegradable polymer nanoparticles as vaccine adjuvants and drug delivery systems.[9] Among many nanoparticles utilized for antigen delivery, chitosan nanoparticles have been found to possess the immunological activity and mucoadhesive properties. They have been used as a mucosal vaccine delivery system for many antigens.[10] On one hand, *M. tuberculosis* is an intracellular bacterium therefore elimination of TB infection requires potent cellular immunity, especially Th1 responses.[11] Among various polymeric carriers with the natural or synthetic origin being evaluated for anti-TB drug delivery or vaccination, protein-based natural polymeric nanoparticles, the potential of albumin polymer particles as vaccine delivery systems/adjuvants against TB infection was found to induce mucosal and systemic immune responses.[11] The most common natural carbohydrate polymer used for TB vaccine development is a derivative of chitin, chitosan, composed of repeating units of d-glucosamine and N-acetyl-glucosamine. This hydrophilic linear polysaccharide showed several benefits for TB vaccine delivery such as enhancing the immune responses and improving BCG-primed immunity.[12] Chitosan vaccine delivery possesses biodegradable and biocompatible characteristics, is less toxic, has a high affinity to mucosal surfaces of epithelial cells due to mucoadhesive characteristics and positive surface charge.[11] The paracellular transport via penetrating tight intercellular connections, controlled release properties, potent stimulation of the immune system due to efficient uptake by APCs, and protection from in vivo antigen degradation make the most of its usage.[11] However, the effectiveness of most of this new generation of vaccine candidates is faced with challenges due to naturally low immunogenicity, inherent inability to deliver antigens to appropriate sites in order to stimulate both innate and adaptive types of the immune system, require multiple doses and conversion to a previous virulent form.[11,12] Thus, much more research is required to study the capability of these polysaccharides-based nanoparticles. Considering that resiquimod has been introduced as a safe and suitable adjuvant in various studies and has been able to stimulate cellular immunity well,[13] in the present study we used this adjuvant along with recombinant proteins of mycobacterium in chitosan nanoparticles. So far, this adjuvant has not been used in any of the candidate tuberculosis vaccines. Secondly, we attempted to assess the potentiality of chitosan nanoparticles as recombinant vaccine carriers and adjuvant *in vivo* using BALB/c mice for effective immunization through a non-invasive nasal route.

Methods

Preparation chitosan nanoparticles(C) containing Hspx–PPE44–EsxV (HPE) and resiquimod(R) (HPERC)

Chitosan nanoparticles containing recombinant mycobacterial proteins and resiquimod adjuvant were synthesized. Briefly, tripolyphosphate (TPP) dissolved in deionized water with a concentration of 1 mg/mL was added to chitosan dissolved in 1% acetic acid with a concentration of 2 mg/mL, holding on the stirrer at 200 rpm. The release of recombinant proteins from chitosan nanoparticles was investigated at times 6, 12, 24, 48, 72, and 96 hours. Full descriptions of the methods and their results have been published previously.[14]

Animals

Thirty male Balb/C mice (6–8 weeks old) were bought from the Pasteur Institute of Iran (Tehran, Iran). All animal experiments were approved and performed according to the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1397.358). The experiments conformed to the international guidelines[15] for the ethical use and care of animals.

Immunization procedures

The animals were separated into 5 groups each comprising six mice. Table 1 depicts the formulations for each group. Each mouse received 30μL designed vaccine containing 50 μg of antigen on days 0, 14, and 28 as described earlier.[16] One group of mice was primed with BCG on day 0 and then boosted with HPERC on days 14 and 28. The amount of resiquimod adjuvant used was 10 μg at each nasal administration. The animals administrated with PBS were considered as controls. All mice were euthanized by cervical dislocation samples after two weeks of the last injection. The spleens were removed under an aseptic conditions ion for assessing immune responses and transferred to the cell lab in the RPMI medium to prepare the splenocytes, as defined in the following section.

The spleens collected were dispersed in 1 mL of cold

Table 1. Formulations of various antigens for nasal delivery route

Group name	Received compounds
A	PBS
B	HPERC
C	BCG
D	HPE
E	Prim BCG/boosting HPERC

PBS: phosphate-buffered saline; HPE: Hspx–PPE44–EsxV; HPERC: Hspx–PPE44–EsxV/resiquimod/chitosan; BCG: Bacillus Calmette-Guerin.

RPMI (Bioidea, Iran) medium without antibiotics under safety cabinet. The spleens were crushed using a cell strainer and the cells were inserted into falcon tubes with 4–5 ml of cold RPMI medium. The spleen cells were centrifuged (Biocen 22 R, Orto Alresa, Spain) at 500 × g for 10 minutes at 4 °C, the supernatants were removed, and 10 mL of cold ammonium chloride buffer was added and slowly mixed with the cell pellet to lyse the red blood cells. The cell pellet was resuspended in the RPMI supplemented with penicillin (100 U/mL) and streptomycin (100 mg/mL) to reach a final level of 1×10^7 cells/mL. The cells were cultured in a sterile flat-bottom 24-well plates containing RPMI supplemented with FBS (10%) and antibiotics. Three hundred microliters of the cell suspension were added into each well and incubated at 37˚C in a humidified CO_2 incubator (Memmert, ICO, Germany), with either 10^6 CFU attenuated BCG as a stimulator or 3% phytohemagglutinin as a positive control. The supernatants from three separate wells were collected after 72 hours and pooled and stored at -80°C until more analysis.

IL-4, IL-17 and IFN-γ detection

Enzyme-linked immunosorbent assays (ELISAs) were performed to determine interferon-gamma (IFN γ), interleukin-4 (IL-4), and IL-17 cytokine levels according to the manufacturer's instructions and results read on an ELISA reader (eBioscience, San Diego, CA) according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS) version 16 (SPSS Inc., Chicago, IL) statistics software. The one-way ANOVA test was applied to analyze the difference in cytokine production among different immunized groups. Statistical significance was designated as P value < 0.05.

Results

To characterize the effect of the combination of mycobacterial antigens with adjuvant Hspx–PPE44–EsxV/resiquimod/chitosan (HPERC) on cellular immune responses, concentrations of cytokines were measured. The concentrations of IFN-γ, IL-17, and IL-4 released in the supernatant of cultured splenocytes were specified by ELISA. The amount of IL-17 and IFN-γ were increased in the mice that received the BCG vaccine and the designed vaccine alone (HPERC) or as a booster of BCG, and all these groups showed a significant difference with the control group and the group that received recombinant protein (P < 0.001) (Figures 1 and 2). The booster group also showed a significant difference in the increase in the amount of these cytokines compared to the group that received BCG (P < 0.01). In all the immunized groups, a high and significant level of IL-4 was not observed (Figure 3). The group that received the designed vaccine as a booster showed the highest amount of interleukin-4

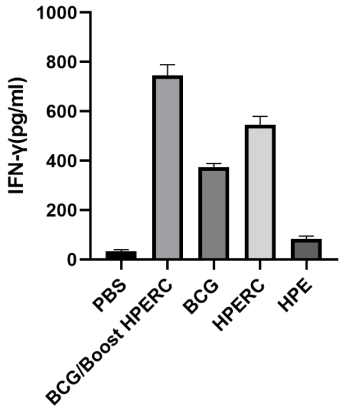

Figure 1. INF-ɣ assay in vaccinated groups. IFN-γ concentrations in immunized mice two weeks after the last nasal inhalation. Mice were immunized 3 times at two-week intervals. IFN-γ concentrations in splenocyte supernatants were measured by sandwich ELISAs after 72 h of in vitro incubation. IFN-γ: Interferon gamma, PBS: Phosphate buffered saline, BCG: Bacillus Calmette-Guerin, HPERC: Hspx–PPE44–EsxV/Resiquimod/Chitosan.

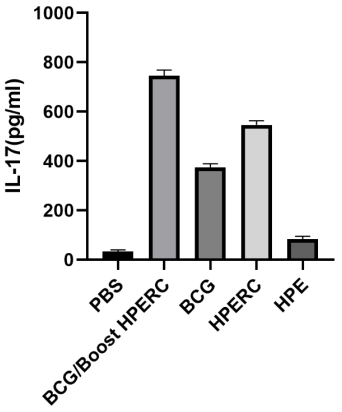

Figure 2. IL-17 assay in vaccinated groups. IL-17 concentrations in immunized mice two weeks after the last nasal inhalation. Mice were immunized 3 times at two-week intervals. IL-17 concentrations in splenocyte supernatants were measured by sandwich ELISAs after 72 h of in vitro incubation. IL-17: interleukin-17, PBS: Phosphate buffered saline, BCG: Bacillus Calmette-Guerin, HPERC: Hspx–PPE44–EsxV/Resiquimod/Chitosan.

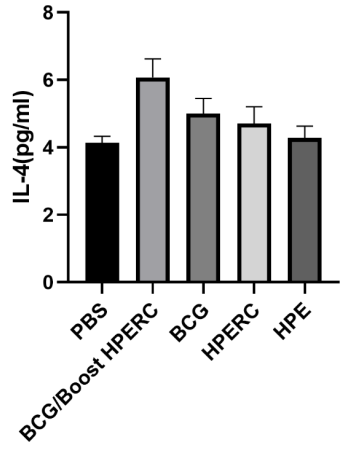

Figure 3. IL-4 assay in various immunized mice. IL-4 concentrations in immunized mice two weeks after the last nasal inhalation. Mice were immunized 3 times at two-week intervals. IL-4 concentrations in splenocyte supernatants were measured by sandwich ELISAs after 72 h of in vitro incubation. IL-4: interleukin-4, PBS: Phosphate buffered saline, BCG: Bacillus Calmette-Guerin, HPERC: Hspx–PPE44–EsxV/Resiquimod/Chitosan.

but did not show any significant difference from the control group.

Discussion and Conclusion

One of the preferred methods to confrontation with infectious diseases is the use of vaccination. Successful vaccines against infectious diseases such as tetanus, whooping cough, diphtheria, hepatitis, smallpox, and polio have saved the lives of millions of people. In tuberculosis disease, the BCG vaccine has been used in most parts of the world since 1921 in newborns.[17] Despite the safety and high efficiency of this vaccine in preventing infant tuberculosis, the effectiveness of this vaccine has decreased due to its inability to prevent pulmonary tuberculosis in adults.[18] In addition, BCG vaccine does not have the necessary efficiency in generating cellular responses, especially CD8 + responses, due to the deletion of parts of the genome that are effective in its innate immunity.[19] Several vaccines have been introduced to replace or increase the effectiveness of the BCG vaccine and are being studied.[8,20] One of these categories is the subunit vaccines that are used to increase the immune responses of the BCG vaccine.[4] The use of recombinant proteins is efficient to increase the cellular responses of the BCG vaccine. The most important factor to evaluate the effectiveness of a protein to use the Prime-Boost strategy in vaccination is the increase in the induction of interferon-gamma following the stimulation of T cells.[21] Studies have determined that proteins that are expressed both in the acute phase and in the chronic phase, or in the conditions of nutritional deficiency and oxygen deficiency, such as HspX and InhA, have a higher efficiency for vaccines.[22] In a study conducted by Yuan et al[8] it was observed that the recombinant BCG strain that has the ability to express HspX and Ag85 antigens has a high ability to stimulate CD4 and CD8 responses compared to the BCG vaccine. In a study conducted by Romano et al,[23] it was emphasized on the ability to stimulate high cellular and humoral immunity by this antigen. It was also observed that this antigen induces similar and comparable immunity to BCG vaccine against tuberculosis. Based on the explanations given, it can be said that HspX, Ppe44, and EsxV antigens can be effective in designing a subunit vaccine. Despite the many benefits of subunit vaccines, these vaccines alone lack adequate immunogenicity and cannot induce protective immunity. For this reason, adjuvant compounds are used to increase their immunogenicity. Nanoparticles can also be used to carry vaccines and create cellular and humoral responses.[24-26] Vaccine delivery systems designed on the basis of nanoparticles have several advantages compared to only proteins. Firstly, the nanoparticle prevents the enzymatic degradation of the protein, and secondly, it causes the antigen to be delivered to the APCs. On the other hand, as antigen storage, it causes the release of antigen in the place of accumulation of dendritic cells. Chitosan, as a natural polymer, has received attention in the fields of medicine and pharmaceuticals due to its biocompatibility, biodegradability, and biological activity.[11,27,28] The cellular immune response can play a decisive role in developing immunity to tuberculosis. It has been observed that vaccines that promote immunity towards CD4 and CD8 cell responses induce adequate immunity against this disease.[29] There are several reports on the suitability of nasal administration to induce systemic immunity. In the nasal mucus, there are various cells such as APC that can lead the immune system to a strong cellular and humoral response.[30,31] The balance between humoral immune responses and Th1 and Th17, or in other words, the level of cytokines IFN-γ, IL-4, and IL-17 can play an important role in the treatment of tuberculosis infection.[32,33] Based on the results obtained in our study, it was shown that by encapsulating the antigen and adjuvant inside chitosan nanoparticles, either alone or together with the BCG vaccine, acquired immunity, especially of the Th1 type, has been activated due to the increase of IFN-γ and IL-17 and non-increasing level of IL-4. Also, in our study, it was found that the level of IFN-γ and IL-17 were the highest in the group where the vaccine designed as a booster was used, and it can be concluded that this vaccine will have a better result if it is used as a booster. Although it is necessary that in future studies, these formulations should be investigated in an *M. tuberculosis*-infected mouse challenge model to assess these vaccine candidates.

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Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

This study was approved by the Ethical Committee of Tabriz University of Medical Sciences, Tabriz (IR.TBZMED.

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References

1. World Health Organization (WHO). Global Tuberculosis Report 2019. Geneva: WHO; 2019.
2. Roa García M, Másmela GA, Bohórquez NG, Pinilla DA. Educación e inclusión financieras en América Latina y el Caribe: programas de los bancos centrales y las superintendencias financieras. Centro de Estudios Monetarios Latinoamericanos (CEMLA); 2014.
3. Rindi L, Lari N, Garzelli C. Genes of *Mycobacterium tuberculosis* H37Rv downregulated in the attenuated strain H37Ra are restricted to *M. tuberculosis* complex species. New Microbiol. 2001;24(3):289-94.
4. Bonanni D, Rindi L, Lari N, Garzelli C. Immunogenicity of mycobacterial PPE44 (Rv2770c) in *Mycobacterium bovis* BCG-infected mice. J Med Microbiol. 2005;54(Pt 5):443-8. doi: 10.1099/jmm.0.45960-0.
5. Mahmood A, Srivastava S, Tripathi S, Ansari MA, Owais M, Arora A. Molecular characterization of secretory proteins Rv3619c and Rv3620c from *Mycobacterium tuberculosis* H37Rv. FEBS J. 2011;278(2):341-53. doi: 10.1111/j.1742-4658.2010.07958.x.
6. Geluk A, Lin MY, van Meijgaarden KE, Leyten EM, Franken KL, Ottenhoff TH, et al. T-cell recognition of the HspX protein of *Mycobacterium tuberculosis* correlates with latent *M. tuberculosis* infection but not with M. bovis BCG vaccination. Infect Immun. 2007;75(6):2914-21. doi: 10.1128/iai.01990-06.
7. Hu Y, Movahedzadeh F, Stoker NG, Coates AR. Deletion of the *Mycobacterium tuberculosis* alpha-crystallin-like hspX gene causes increased bacterial growth in vivo. Infect Immun. 2006;74(2):861-8. doi: 10.1128/iai.74.2.861-868.2006.
8. Yuan X, Teng X, Jing Y, Ma J, Tian M, Yu Q, et al. A live attenuated BCG vaccine overexpressing multistage antigens Ag85B and HspX provides superior protection against *Mycobacterium tuberculosis* infection. Appl Microbiol Biotechnol. 2015;99(24):10587-95. doi: 10.1007/s00253-015-6962-x .
9. Jin Z, Gao S, Cui X, Sun D, Zhao K. Adjuvants and delivery systems based on polymeric nanoparticles for mucosal vaccines. Int J Pharm. 2019;572:118731. doi: 10.1016/j.ijpharm.2019.118731.
10. Mehrabi M, Montazeri H, Mohamadpour Dounighi N, Rashti A, Vakili-Ghartavol R. Chitosan-based nanoparticles in mucosal vaccine delivery. Arch Razi Inst. 2018;73(3):165-76. doi: 10.22092/ari.2017.109235.1101.
11. Khademi F, Taheri RA, Yousefi Avarvand A, Vaez H, Momtazi-Borojeni AA, Soleimanpour S. Are chitosan natural polymers suitable as adjuvant/delivery system for anti-tuberculosis vaccines? Microb Pathog. 2018;121:218-23. doi: 10.1016/j.micpath.2018.05.035.
12. Tafaghodi M, Khademi F, Shiehzadeh F, Firouzi Z. Polymer-based nanoparticles as delivery systems for treatment and vaccination of tuberculosis. In: Kesharwani P, ed. Nanotechnology Based Approaches for Tuberculosis Treatment. Academic Press; 2020. p. 123-42. doi: 10.1016/b978-0-12-819811-7.00008-4.
13. Wu JJ, Huang DB, Tyring SK. Resiquimod: a new immune response modifier with potential as a vaccine adjuvant for Th1 immune responses. Antiviral Res. 2004;64(2):79-83. doi: 10.1016/j.antiviral.2004.07.002.
14. Hoseinpur R, Hasani A, Baradaran B, Abdolalizadeh J, Amini Y, Salehi R, et al. Chitosan nanoparticles containing fusion protein (Hspx-PPE44-EsxV) and resiquimod adjuvant (HPERC) as a novel booster vaccine for *Mycobacterium tuberculosis*. J Biomater Appl. 2022;37(1):40-7. doi: 10.1177/08853282221079105.
15. Dolan K. Laboratory Animal Law: Legal Control of the Use of Animals in Research. John Wiley & Sons; 2008.
16. Mansury D, Ghazvini K, Amel Jamehdar S, Badiee A, Tafaghodi M, Nikpoor AR, et al. Enhancement of the effect of BCG vaccine against tuberculosis using DDA/TDB liposomes containing a fusion protein of HspX, PPE44, and EsxV. Artif Cells Nanomed Biotechnol. 2019;47(1):370-7. doi: 10.1080/21691401.2018.1557674.
17. Fletcher HA, Schrager L. TB vaccine development and the end TB strategy: importance and current status. Trans R Soc Trop Med Hyg. 2016;110(4):212-8. doi: 10.1093/trstmh/trw016.
18. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. J Exp Med. 1993;178(6):2249-54. doi: 10.1084/jem.178.6.2249.
19. Stenger S. Cytolytic T cells in the immune response to *Mycobacterium tuberculosis*. Scand J Infect Dis. 2001;33(7):483-7. doi: 10.1080/00365540110026584.
20. Schrager LK, Vekemens J, Drager N, Lewinsohn DM, Olesen OF. The status of tuberculosis vaccine development. Lancet Infect Dis. 2020;20(3):e28-e37. doi: 10.1016/s1473-3099(19)30625-5.
21. Kaufmann SH, Weiner J, von Reyn CF. Novel approaches to tuberculosis vaccine development. Int J Infect Dis. 2017;56:263-7. doi: 10.1016/j.ijid.2016.10.018.
22. Yuan Y, Crane DD, Barry CE, 3rd. Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: function of the mycobacterial alpha-crystallin homolog. J Bacteriol. 1996;178(15):4484-92. doi: 10.1128/jb.178.15.4484-4492.1996.
23. Romano M, Rindi L, Korf H, Bonanni D, Adnet PY, Jurion F, et al. Immunogenicity and protective efficacy of tuberculosis subunit vaccines expressing PPE44 (Rv2770c). Vaccine. 2008;26(48):6053-63. doi: 10.1016/j.vaccine.2008.09.025.
24. Mamo T, Poland GA. Nanovaccinology: the next generation of vaccines meets 21st century materials science and engineering. Vaccine. 2012;30(47):6609-11. doi: 10.1016/j.vaccine.2012.08.023.
25. Stewart E, Triccas JA, Petrovsky N. Adjuvant strategies for more effective tuberculosis vaccine immunity. Microorganisms. 2019;7(8):255. doi: 10.3390/microorganisms7080255.
26. Baldwin SL, Bertholet S, Reese VA, Ching LK, Reed SG, Coler RN. The importance of adjuvant formulation in the development of a tuberculosis vaccine. J Immunol. 2012;188(5):2189-97. doi: 10.4049/jimmunol.1102696.
27. Zhu B, Qie Y, Wang J, Zhang Y, Wang Q, Xu Y, et al. Chitosan microspheres enhance the immunogenicity of an Ag85B-based fusion protein containing multiple T-cell epitopes of *Mycobacterium tuberculosis*. Eur J Pharm Biopharm. 2007;66(3):318-26. doi: 10.1016/j.ejpb.2006.11.028.
28. Knudsen NP, Nørskov-Lauritsen S, Dolganov GM, Schoolnik GK, Lindenstrøm T, Andersen P, et al. Tuberculosis vaccine with high predicted population coverage and compatibility with modern diagnostics. Proc Natl Acad Sci U S A. 2014;111(3):1096-101. doi: 10.1073/pnas.1314973111.
29. Chan J, Mehta S, Bharrhan S, Chen Y, Achkar JM, Casadevall A, et al. The role of B cells and humoral immunity in *Mycobacterium tuberculosis* infection. Semin Immunol. 2014;26(6):588-600. doi: 10.1016/j.smim.2014.10.005.
30. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med. 2005;11(4 Suppl):S45-53. doi: 10.1038/nm1213.
31. Ozer AY. Alternative applications for drug delivery: nasal and pulmonary routes. In: Mozafari MR, ed. Nanomaterials

and Nanosystems for Biomedical Applications. Dordrecht: Springer; 2007. p. 99-112. doi: 10.1007/978-1-4020-6289-6_6.

32. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4+T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. Nat Immunol. 2007;8(4):369-77. doi: 10.1038/ni1449.
33. Choi HG, Kwon KW, Choi S, Back YW, Park HS, Kang SM, et al. Antigen-specific IFN-γ/IL-17-co-producing CD4(+) T-cells are the determinants for protective efficacy of tuberculosis subunit vaccine. Vaccines (Basel). 2020;8(2):300. doi: 10.3390/vaccines8020300.