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Efficacy of light chain 3-fused protein multi epitope in protection of mice challenged with *Mycobacterium tuberculosis*

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Abstract

The new strategy for vaccine development such as the fused protein multi-epitope capable of preventing the reactivation of latent tuberculosis infection (LTBi) can be an effective strategy for controlling tuberculosis (TB) worldwide. This study was conducted to evaluate the immunity of experimentally infected BALB/c mice with *Mycobacterium tuberculosis* after injection of DNA construct. Nineteen female BALB/c mice were divided into three groups and injected with 0.50 mL of *M. tuberculosis*. After 3 weeks, lung and spleen samples from the infected mice were examined. The protective effects of light chain 3-fused protein multi-epitope against TB were evaluated for post-exposure and therapeutic exposure. The lungs and spleens of the mice were aseptically removed after death for histopathology analysis. The bacterial colonies were counted, and the cells were stained after 3 weeks of incubation. No significant differences were observed between the post-exposure and therapeutic exposure groups. The pathological changes in the lung tissue of mice in these groups included an increase in the thickness of interalveolar septa, hyperemia, and intraparenchymal pulmonary hemorrhage centers (positive control), scattered hyperemic areas (negative control), and hyperemia in the interstitial tissue, scattered hyperemic areas in the lung parenchyma and lymphocytic infiltration centers (experimental group). Flow cytometry of the post-exposure and therapeutic exposure models showed insignificant changes in all three groups. It seems necessary to develop a post-exposure and therapeutic exposure vaccine strategy that focuses on LTBi to prevent the progression of the active disease. In this regard, multi-epitope vaccines should be designed to induce both cellular and humoral immunity.

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Introduction

Tuberculosis (TB) is one of the major causes of death worldwide.[1] Extensive research has shown that in over 90.00% of healthy individual *Mycobacterium tuberculosis* (Mtb), the causative agent of TB, is inhibited by the acquired immune system after entering the body.[2]

The $CD4^+$ T cells are the most important component of the acquired immune system in fighting against TB. The $CD8^+$ T, T helper lymphocyte 17 (Th17), and B cells play a crucial role in combating Mtb. Macrophages and dendric cells present bacterial peptides to the non-stimulated $CD4^+$ T cells through Major Histocompatibility Complex II (MHCII), while the membrane lipids are presented through MHCI. The $CD4^+$ T cells then present the antigen to antigen-presenting cells via MHC I.[3,4]

The studies on mice have shown that a mutation in β-microglobulin, which is the main component of MHC I, increases sensitivity to Mtb infection.[5] The Mtb-infected macrophages induce apoptosis in macrophage and present antigens to $CD8^+$ T cells through cross-presentation of MHCI-associated antigens. The $CD8^+$ T cells also play a role in protective immunity during the latent phase.[6]

The $CD8^+$ T cells that are activated against Mtb have various ways of disintegrating Mtb-infected cells. They produce tumor necrosis factor α (TNFα) and interferon gamma (IFN-γ), as well as perforin and granzyme, and directly kill the infected cells by secreting granulysin.[3,6]

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The incidence of TB and its mortality rate have recently increased substantially due to the emergence of multi-drug resistant and extensively drug resistant strains and more importantly, totally drug resistant strains.[7] Consequently, stimulating the immune response by vaccination is the best way to prevent and reduce TB incidence. Bacillus Calmette-Guerin (BCG) is currently the only effective and available vaccine to prevent TB and provide immunity against its meningitis and disseminated forms in infants.[8] However; it only provides variable immunity (ranging from 0.00 to 80.00%) against respiratory TB in adults. In addition, this vaccine is contraindicated for immuno-compromised individuals.[9]

It is necessary to utilize novel methods and develop a new, effective vaccine due to the emergence of drug-resistant strains, the ineffectiveness of the BCG vaccine (especially in adults and individuals with immune system defects) and the high mortality rate of TB. Among the new vaccines, DNA-based vaccines have shown effectiveness in generating immunogenicity and providing immunity against intracellular pathogens such as Mtb. These vaccines work by stimulating the immune response through Th1 lymphocytes and cytotoxic T lymphocytes.[9,10] Studies have demonstrated that this type of vaccine can be effective as a pre-exposure vaccine (for those not yet infected with TB), a post-exposure vaccine (for individuals not infected with TB), and a therapeutic vaccine (for individuals infected with acute forms of TB in conjunction with antibiotic treatment). Additionally, DNA-based vaccines have shown potential in expediting the treatment of chronic and recurrent TB infections.[11,12]

After designing and producing the DNA-vaccines, the mouse model must be used to evaluate the designed vaccine and compare it with BCG. This is necessary due to the urgent need for a new effective vaccine and concerns regarding the effectiveness of this type of vaccines.[13]

This study investigates the post-exposure and therapeutic exposure effects of the light chain 3 (LC3)-fused multi-epitope DNA vaccine candidate in the mouse model.

Materials and Methods

Animals. Nineteen female BALB/c mice, aged 4 - 6 weeks and weighing 25.00 – 35.00 g, were purchased from the Animal Care Center at Razi Vaccine and Serum Research Institute (RVSRI). The mice were transferred to an isolator room with standard conditions, including a 12/12 light-dark cycle, standardized pellet mouse feed, a temperature of 22.00 ± 2.00 ˚C, and a relative humidity of 52.00%. The mice were divided into three groups (n = 6): the positive control group using BCG, the negative control group using phosphate-buffered saline (PBS), and the experimental group (LC3-fused multi- epitope protein). After one week of adaptation to the new environment, the experiments were conducted. All animal tests were conducted in accordance with the protocols of Iran Drug and Food Administration. The experimental protocols were reviewed and approved by the Ethics Committee and the Animal Care and Use Committee (Code of ethics committee: IR.RVSRI.REC; date: 29 Nov. 2020). All *in vivo* tests were performed under specific conditions in the Animal Biosafety Level-3 Facility at RVSRI. This study was performed to diagnose latent tuberculosis infection (LTBi) at RVSRI (Karaj, Iran) from January 2020 to October 2020.

Bacteria. The Mtb H37Rv strain was selected from the RVSRI microbial archives and cultured in Middlebrook 7H9 broth (BD Difco, Franklin, USA). The plates were then incubated at 37.00 ˚C for 7 days.

Immunizations and infections. All three groups of mice were peritoneally infected by injecting 0.50 mL of Mtb (0.50 McFarland). The dilution prepared from Mtb was cultured in several tubes containing Lowenstein-Jensen medium, (LJ; BD Difco) to examine colony count and infection. The mice were studied for 3 weeks to observe any changes in their biological conditions and body temperature. After 3 weeks, the infected mice were placed inside the cabinet class III at RVSRI, euthanized using CO_2 chamber and their lungs and spleens were examined for Mtb infection.[14]

Post-exposure and therapeutic exposure experiments. In the post-exposure model, the mice were given an antibiotic chemotherapy treatment (100 mg L^{-1} rifampin, 100 mg L^{-1} isoniazid) in their drinking water from week three to week five after infection. The antibiotic was purchased from Sigma (St. Louis, USA). This treatment was meant to reduce the bacterial load. In the therapeutic exposure model, the mice received the antibiotic chemotherapy treatment from week three to week eight after infection. After chemotherapy, bacterial count was undetectable in the lungs. After 8 weeks of infection, the mice were immunized three times at 3-week intervals. In the positive control group, each lung received a subcutaneous injection of 50.00 µL of BCG into the flank muscle. In the negative control group, each mouse received a 50.00 µL injection of PBS into the quadriceps. In the LC3 group, each mouse received a 50.00 µL injection of LC3-fused multi-epitope protein into the quadriceps. Three weeks after the last immunization, the mice were euthanized with CO_2 and their lungs were removed, and prepared for bacterial culture, histopathology and flow cytometry analysis.[15]

Histopathological analysis. For histopathological analysis, the lungs and spleen of the mice were aseptically removed post-mortem. The right upper lobe of each mouse was then fixed by immersing in 10.00% formalin and processed for histopathological examination. The sections were stained with Hematoxylin and Eosin (H & E) for subsequent evaluation.[16]

Bacterial colony count. The lung specimens were placed into the pounder and a solvent, 1.00 N NaOH (Merck,

Darmstadt, Germany), was added for decontamination. The specimens were completely dissolved inside the pellet, transferred into the pipes, centrifuged for 1 - 2 min at 1,900 *g*, and the liquid was discarded. Serial dilutions in a base of 10 were prepared from the homogenized sample using the obtained sediment. Each 0.15 mL dilution was incubated (model 7H10 Middlebrook; BD Difco) at approximately 37.00 ˚C. After 3 weeks of incubation, the colony was counted. For cell culture, the mouse spleen was first placed onto a sterile plate, and 1.00 mL of PBS was added and completely squeezed. The crushed liquid was collected from the bottom of the plate using a syringe, and transferred to the falcon. Then, 7.00 mL of lysis buffer was added to each tube. After 10 min, 5.00 mL of PBS was added to neutralize the solution. The liquid was then centrifuged for 5 min to settle the cells. The white cells, which were lymphocytes, circulated in the superficial fluid. The cells were washed again with 10.00 mL of PBS and centrifuged for 5 min. The cells were counted using a Neubauer slide to determine the appropriate proportions. Each well contained 3.00×10^6 cells. The cells were poured into a 24-well plate, and then 1.50 mL of the medium was added to each well. In this step, the antigens were removed from the cells. The 5.00 µL of antigens was added for every 3.00×10^6 cells. Finally, the plate was placed in a CO_2 incubator for up to 72 hr to allow for cell reproduction. The culture medium was checked for propagation and contamination during this period.[17]

Staining the cells. For cell staining, the specimens were transferred to a falcon tube and 7.00 - 8.00 mL of fluorescence-activated cell sorting (FACS; including PBS with 2.00% fetal calf serum) was added. The tube was then centrifuged at 300 - 400 *g* for 5 min at 4.00 ˚C. The overcoat was disposed of, and 1.00 mL of FACS buffer (PBS, 1.00 % bovine serum albumin or 10.00 % fetal bovine serum, and 1.00 % NaN_3) was added. 1.00×10^6 cells from each sample were added to a flowcytometric tube. There were two flow tubes for each sample, one for the CD4 assay and another for the CD8 assay. The volume was raised to 100 µL using FACS buffer. Next, 1.00 – 2.00 µL of the CD4 and CD8 antibodies (Thermo Fisher, Waltham, USA) were added to their respective tubes. The tubes were vortexed to mix the contents and then placed at 2.00 - 8.00 ˚C for 30 min. Throughout the process, the tubes were kept in the foil. Afterward, 1.00 mL of FACS was added and centrifuged at 300 - 400 *g* for 5 min. This step could be repeated twice. Then, and 100 µL FACS, 100 µL of fixation buffer, and 100 µL of 1.00 X ice fixation were added. This mixture was vortexed and kept at ambient temperature in the dark for 20 min. Next, 2.00 mL of 1.00X permeabilization buffer (Thermo Fisher) was added and centrifuged at 300 to 400 *g* for 5 min. The supernatant was discarded, and another 2.00 mL of 1.00X permeabilization buffer was added. This step was repeated and then 100 µL of 1.00X permeabilization buffer, 1.00 - 3.00 µL of inter-leukin-2, IFN-γ, and TNFα (Thermo Fisher) were added. This mixture was kept in the dark for 12 min. Afterward, 2.00 mL of 1.00X permeabilization buffer was added and centrifuged at 300 - 400 *g* for 5 min. The supernatant was discarded, and 2.00 mL of FACS was added. The mixture was centrifuged again at 300 - 400 *g* for 5 min. The supernatant was discarded, and 1.00 mL of FACS was added. Finally, 1.00 mL of paraformaldehyde was added to preserve the cells. Flow cytometry was performed using the FACSCanto II (BD Biosciences, Franklin Lakes, USA), and a total of 200,000 events were acquired. The collected data were analyzed using Flow Jo software, version 7.6 (TreeStar Inc., Ashland, USA).[18]

Statistical analysis. All data were expressed as mean ± SD. The data were statistically analyzed using one-way analysis of variance (ANOVA) with Tukey´s post hoc multiple comparison test using SPSS software (version 22.0; IBM Corp., Armonk, USA). A p value less than 0.05 was considered as significant.

Results

Our study demonstrated the typical results of post-exposure and therapeutic exposure to TB vaccine models specifically regarding the LC3-fused protein vaccine candidate. Overall, no significant differences were found between the groups in both the post-exposure and therapeutic exposure settings ($p > 0.05$).

In the pre-exposure lung culture, the positive control, negative control, and experimental groups exhibited growth in LJ medium after 7, 4, and 6 weeks, respectively. Similarly, in the therapeutic exposure lung culture, uprose in the positive control, negative control, and experimental groups showed growth in LJ medium after 6, 4, and 8 weeks, respectively.

The lungs of each mouse were dissected and stained using H & E to observe the presence of granulomatosis, fibrosis, calcification, necrosis, and lymphatic infiltration. The observed group displayed typical characteristics, which of are presented in Figure 1. In all groups a specific lesion of lymphocytic foci or internal granuloma was observed.

Post-exposure models. In BCG group, an increase in the interalveolar cell wall, hyper-anemia, and focal bleeding were observed in the lung parenchymal tissue (Fig. 1A). In PBS group, symptoms such as focal hyperemia, and bleeding were seen (Fig. 1B), while hyperemia could be seen in the intercellular region and lung parenchymal cells of test group. Lymphocytic infiltration was also observed (Fig. 1C).

Therapeutic exposure models. An increase in the alveolar intercellular cell wall, hyperemia, and focal bleeding in the lung parenchymal tissue were visualized (Fig. 1D).

Negative control lung tissue. Dispersed hyper-emia was seen in the lung parenchymal tissue (Fig. 1E).

Lung tissue of the test group. Hyperemia was seen in the intercellular region and lung parenchymal tissue. Lymphocytic infiltration was also observed (Fig. 1F).

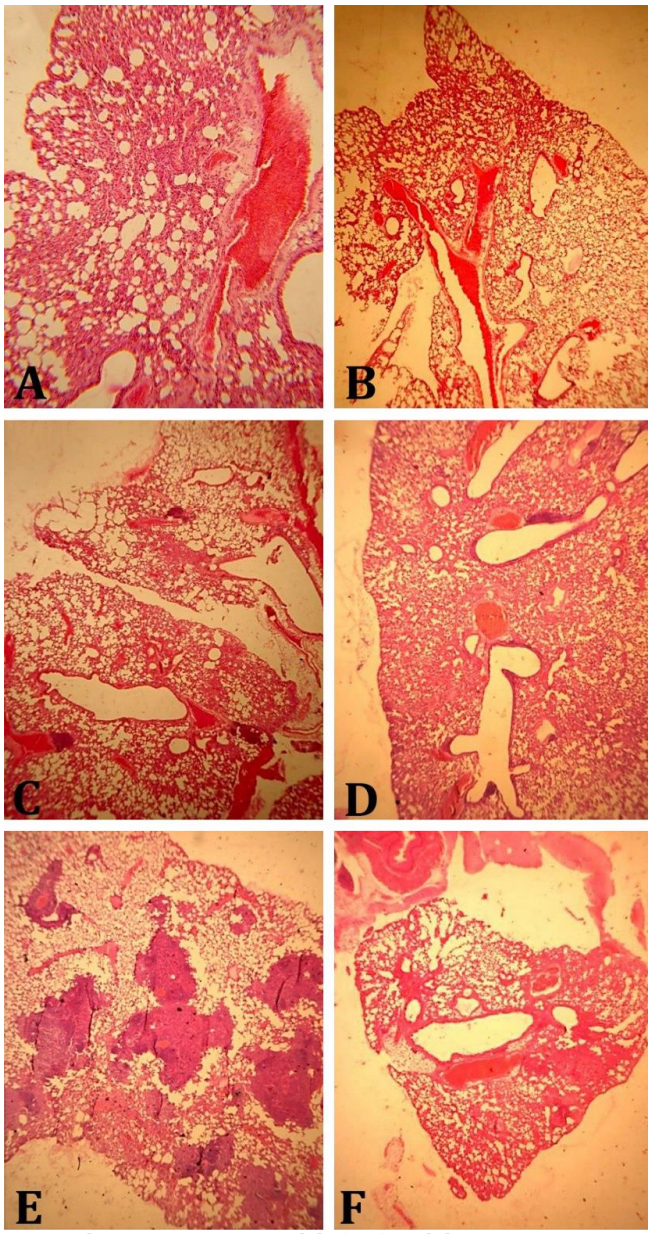

Fig. 1. The post-exposure models (A-C) and therapeutic exposure (D-F). **A)** Positive control lung tissue: Increase of inter alveolar cell wall, hyper-anemia, and focal bleeding in the lung parenchymal tissue is visualized, **B)** Negative control lung tissue: Symptoms such as, focal hyperemia, and bleeding are seen, **C)** Lung tissue of test group: Hyperemia in intercellular region and lung parenchymal tissue could be seen. Lymphocytic infiltration is also observed, **D)** Positive control lung tissue: Increased alveolar intercellular cell wall, hyperemia, and focal bleeding in the lung parenchymal tissue are visualized, **E)** Negative control lung tissue: Dispersed hyperemia is seen in lung parenchymal tissue, and **F)** Lung tissue of the test group: Hyperemia in intercellular region and lung parenchymal tissue is visualized. Lymphocytic infiltration is also observed, (H & E, 32×).

The flow cytometric analysis of the post-exposure models did not show any significant changes in the test group compared to the other groups. Additionally, no changes were observed in the therapeutic exposure group (Table 1).

Discussion

The 2015 World Health Organization report in 2015 estimated that one-third of the world's population was latently infected with Mtb. Therefore, it is necessary to implement a post-exposure vaccine strategy that focuses on preventing the progression of active diseases from latent Mtb infections.[19]

In this study, there were no significant changes observed in the production of T $CD4^+/CD8^+$ cells among the three post-exposure groups. This could be attributed to the immune system's avoidance mechanism in response to Mtb. To gain a better understanding of the host-pathogen interactions of Mtb, it would be beneficial to analyze protein-protein and metabolome interactions in order to design more effective vaccine candidates.

For the intracellular pathogen Mtb, the optimal approach is to develop multi-epitope vaccines that can stimulate both cellular and humoral immune responses. However, Mtb is a distinct pathogen, requiring a specific defense strategy known as "cheesy-necrosis" formation.[20,21] In this study, we observed that this necrosis resulted in fewer histopathological changes in lung parenchymal cells, suggesting reduced efficacy of the recombinant vaccine.

The engineered multi-epitope antigen is based on the positioning of separate epitope genes, each with its own advantages. The optimum efficacy of this vaccine candidate may increase if the final recombinant protein is combined with nanoparticles such as quantum dots, liposomes, and/or virus-like proteins. These nanoparticles are widely used in today's combined and conjugated vaccines. Therefore, it cannot be concluded which antigen, gene, or epitope combination is best for use as a vaccine candidate for Mtb. Further investigations are required to determine the potential for antibody induction by positioning other epitope genes. Moreover, the epitopes should not interfere with any other proteins or metabolic pathways in the host.[10,12]

Moradi *et al*. designed a novel post-exposure multi-epitope DNA construct based on three latency-associated antigens: Rv2029c, Rv2031c, and Rv2627c. They also included microtubule-associated protein LC3 as a hallmark protein of the autophagy system. According to their findings, the predicted epitopes for MHC I and MHC II showed a high potential for binding to human leukocyte antigen (HLA) alleles. However, when this candidate vaccine was injected into TB-infected mice, they did not show a proper immune response. This suggests that the

Table 1. Flow cytometric analysis of the post-exposure and therapeutic exposure models.

Group	CD4-IL2	CD4-IFNγ	CD4-TNFα	CD8-IL2	CD8-IFNγ	CD8-TNFα
Post-exposure						
Negative	10.84	11.20	9.04	11.92	9.30	8.52
Positive	16.18	14.50	11.68	16.10	11.86	9.74
Test	13.52	12.96	10.64	13.60	11.14	9.80
Therapeutic exposure						
Negative	9.38	10.40	10.90	10.18	8.80	9.56
Positive	10.28	11.50	11.20	12.10	10.46	10.74
Test	9.36	10.80	10.24	10.40	8.96	9.90

IL: Interleukin, IFN γ: Interferon gamma, and TNFα: Tumor necrosis factor α.

candidate vaccine may not effectively stimulate the immune system. Therefore, it is suggested that future studies focus on improving the stimulating power of this candidate vaccine by utilizing adjuvants or nanoparticles.[22]

Gao *et al*. designed a multi-epitope protein vaccine candidate consisting of thioredoxin at the N-terminal and a histidine tag at the C-terminal. The vaccine included a multi-epitope Mtb recombinant antigen with 11 B-cell epitopes from six Mtb antigens (PstS1, ESAT6, CFP10, Ag85B, Ag85A and PPE54). Serology by enzyme-linked immunoassay (ELISA) for 60 positive and negative serums showed no statistical difference between the "Gold standard" and the novel ELISA.[23]

Mohamud *et al*. created a gene construct that contained 3 epitopes for the Ag85B antigen (P1, P2, P3) fused to the Mtb 8.4 protein (rBCG018). They also used a combination of these antigens fused to B cell epitopes from ESAT-6, CFP-10, and MTP40 proteins (rBCG032). The immunological evaluation of this recombination was performed by measuring immunoglobulin G3 against the P1 and P2 B cell epitopes of MPT40. The $CD4^+$ and $CD8^+$ lymphocytes from the mice immunized with rBCG018 produced Th1 cytokines, as did those immunized with rBCG032.[24]

As mentioned in the above studies, the response to T-helper type cells 1 and 2 was similar. The same results were obtained in this study, and the Mtb LC3-fused protein multi-epitope did not induce T-helper cells as much as the BCG vaccine.

The results revealed that the novel multi-epitope DNA construct could be an effective candidate in a TB vaccine if adjuvants and nanoparticles are used to improve the stimulating power. It is necessary to investigate its potential to induce CD4 and CD8 T cell immune responses in an experimental animal model.

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Conflict of interest

The authors declare that there is no conflict of interest.

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