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Comparison of ppe44 + HSPX + FC and PPE44 + HSPX complexes in MTB

ABSTRACT

One of the challenges in controlling tuberculosis is the many defects in the BCG vaccine. It is necessary to design and produce an effective and safe recombinant complex against this disease that covers the shortcomings of BCG.

For our study entitled: Comparison of ppe44 + HSPX + FC and PPE44 + HSPX complexes in MTB

It was extracted from NCBI, PPE44 and HSPX gene and mouse IgG2a Fc.The design was done according to a previous study by the same authors.

Cloning in pet28a and vector transfer in E. coli Bl2, purification and SDS-PAGE were performed. 35 BALB/c mice were divided into 5 groups, injected on days 0 and 21 and blood sampling on days 14 and 45. Total antibodies and IgG1 and IgG2 subclasses were investigated and the production level of cytokines IFN-γ, IL-2 and IL-. 4, TNF-α, and TGF-β.

Findings: A significant correlation was observed in the amount of total antibody in the second stage of the assay at a concentration of 1.20 between the groups receiving PHF and PH. ($p<0.05$.

There was a significant increase in the amount of IgG1 in the group with PHF and IgG2 in IL+ PHF compared to other groups. ($p < 0.05$)

There was a significant increase in the level of IFN-γ, TNF-α, TGF-β, IL-2 and IL-4 in PHF ($p<0.05$).

Conclusion: Recombinant proteins were designed to strengthen the immune system and the addition of IL-22 improved the level of antibody production. Covered one of the most important defects in the common BCG vaccine.

Keywords: *Subunit vaccine, PPE44, HSPX, IL-22*

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Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis in humans and is known as a pervasive threat to human societies (1). Leads to death. The association of tuberculosis with HIV has increased mortality (1, 2). In Iran, from 142 cases per 100,000 population in 1343 to 14.4 per 100,000 population in 1391 has been reached. Out of 10987 cases of tuberculosis reported in the country in 1390, 50% of cases are sick women and 12% of cases are non-Iranian patients (Afghans) and the highest incidence of tuberculosis was in the age group of 65 years and above. (3) In 1921, the Anti-TB vaccine, derived from the bacterium "Mycobacterium Bovis", was developed by two scientists named "Calmet" and "Green" and was introduced to the world. The protective effect of this vaccine (Bacille Calmette-Guérin (BCG was studied. Currently, BCG live-attenuated strains are used to provide immunity in many countries (4). This vaccine is the only approved vaccine for humans. The effectiveness of this vaccine varies in different parts of the world. The findings showed that the BCG vaccine has several drawbacks, the most important of which, according to studies, are the following: 1- The protective effect of this vaccine against pulmonary tuberculosis in adults is very variable and its protective spectrum is estimated between 0 to 80%; its stability effect decreases 6 to 12 months after vaccination. The largest BCG vaccine clinical trial in southern India found that the vaccine did not have a protective effect against tuberculosis in adults. Studies show that re-vaccination and booster injection have not been able to prevent the disease in adults (5). 2- The efficiency and effectiveness of this vaccine vary in different parts of the world depending on race and sex (6). 3. The BCG vaccine has been reported to increase immunity in children. Studies have shown that the BCG vaccine only protects against tuberculous meningitis and tuberculosis in children (5). 4- It does not cause high safety memory. 5- BCG contamination with environmental mycobacteria, especially in tropical and temperate regions, has been proven in various studies (7). 6. Existence of genotypic diversity in host populations, as well as the deletion of many genes in BCG strains, are other disadvantages (7). Due to the issues raised, global control of tuberculosis has faced many problems. Lack of timely diagnosis, lack of effective vaccines, concomitant tuberculosis-AIDS infection, long-term chemotherapy, and MDR and XDR resistance have increased the number of problems in controlling this disease (8). Studies have shown that a vaccine that is more effective than the existing vaccine is very important in controlling tuberculosis (5). Due to the existing challenges in the use of common vaccines, new studies have been started using the design of new effective vaccines in this regard. Various researches and tests are being carried out in this direction, many of which are in the clinical trial stage and some of them have completed the clinical trial (1). The production of new vaccines with more capabilities and effectiveness at older ages is important in clinical research. But, the use of new vaccines is necessary to reduce 8 to 10 million new cases of tuberculosis, especially in reducing latent infections each year (4, 6).

Today, various studies use vaccines such as DNA vaccines, subunit vaccines, viral vector vaccines, recombinant Rbcg or BCG, and attenuated Mycobacterium, each of which is one of the most important vaccines of the last twenty years (5). In recent years, subunit vaccines have gained attention due to their safety and ease of production. These vaccines are based on secreted and immunogenic proteins of bacteria (9-10). The development of immune responses by DNA of vaccines and recombinant proteins and subunits has been observed. These include plasmids containing ESAT-6, IL-12N220L, Ag85A, Hsp, and PPE44 genes, which reduced the number of bacteria in the mouse model (11-10). Many subunit vaccines against Mycobacterium tuberculosis are being studied and developed. Subunit vaccines have several benefits. Thus, increasing the antigen diversity in subunit vaccines increases the efficacy of the TB vaccine and ensures that the selected antigens are detected by T cells in different human populations. 11) In recent years, various studies to use these vaccines have been pursued more, so to increase their immunization in humans, new methods have been used, which include: the use of various genetic adjuvants (such as cloning: genes encoding cytokines Along with the gene encoding antigen), electroporation, and the prime-boost method. The results of these methods have shown an increase in the cellular immune response of TH1 and TCD8 (12). Hsp60, Hsp70, ESAT-6, PPE44, and HspX are new candidates for vaccine or TB diagnostic agents (13). Using several immunogenic antigens together creates stronger immunity than one antigen (14). In this study, two antigens of PPE44 and HspX were used. PPE44 antigen is a virulence factor of Mycobacterium tuberculosis and is a member of the PPE (Pro – Pro – Glu) N-terminal family of proteins. In various studies, the PPE44 antigen has been introduced as a new and suitable candidate for vaccine production and is produced more in other pathogenic strains and the Beijing type. The immune system's response to this antigen can be detected in the early and late stages of the mouse's body. This antigen has specific epitopes that are only detectable by MHCI and MHCII and can provide adequate protection if used as a subunit vaccine or DNA vaccine (6-15).

Newer vaccines have been developed against Mycobacterium tuberculosis, which also stimulates the immune system alone, and has also increased the effectiveness of the BCG vaccine

in prim boost. But, about two billion people in the world have tuberculosis and are considered carriers of tuberculosis, so it makes sense to use latent phase antigens to design a vaccine against tuberculosis. These include HspX (Heat shock proteins) antigen. He noted that in vivo studies protected mice exposed to Mycobacterium tuberculosis aerosols. HspX is an immune-stimulating antigen that can protect the host against primary TB and secondary activated infection (16, 17, and 18). The use of adjuvants in recombinant and subunit vaccines can also increase immunogenicity. interleukin-22 adjuvants were used in this study. Interleukin-22 belongs to the IL-10 family (19). Immunogenicity and adjuvants enhancing the route of delivery of subunit vaccines can affect the manner and type of immune responses. It seems that by attaching these proteins to the Fc region of IgG antibodies, the number of IgG antibodies can be removed and the supply of these proteins to T lymphocytes can be increased. Antigen-supplying cells, such as macrophages and dendritic cells, use two mechanisms to remove extracellular antigens: a) nonspecific pinocytosis and b) specific receptors on dendritic cells that bind to several types of FcγR-binding receptors. Antigens that are in the form of immune complexes (Ab-(Ag- and delivered more and by dendritic cells), in other words, induce stimulus signals that activate FcγR and increase the effective uptake of receptor-dependent antigens. Due to the above explanations and the importance of the subject under study, in this study, two dominant antigens of Mycobacterium tuberculosis; HspX and PPE44, were used as adjuvants in association with or without FC and combination with IL-22. A new recombinant subunit protein was designed against tuberculosis.

Step One: Bioinformatics Studies (In-silico)

v **Gene Sequence Extraction from GeneBank**

The DNA sequences of (ppe44 (RV2770C) and (HSPX) genes are based on the Mycobacterium tuberculosis H37Rv genome and CH2 hinge and CH3 fragments of mouse FcIgG2a molecule. We can refer to the GeneBank website for this (NCBI National Center for Biotechnology): ncbi.nlm.nih.gov/protein). With the help of genetic engineering software, DNA sequences are evaluated by the associated software. Thus, the required sequences for expression in the bacterial system are optimized. Due to the presence of non-coding regions in the DNA of the Fcγ2a fragment, its mRNA sequence comprising Hinge, CH2, and CH3 fragments of the mouse IgG2a heavy chain was used. Then the next step is to examine the sequence of these genes using the correct reading frame (ORF) and amino acid by making use of software named GeneRunner. The GeneRunner software (http://www.generunner.net) provides the necessary information about amino acids. And this data is also used for protein analysis functions. In addition, all information about these elements can be edited.

v **Step Two: Alignment**

At this stage, software like a program (http://web.expasy.org/protparam), after which the protein's physical and chemical properties and solubility are used to check the stability and drag coefficient, along with another software called (https://www.ebi.ac.uk/Tools/msa/Cluster omega/) Cluster omega was used which would examine the nucleotide and protein sequences together(11). Physicochemical properties include amino acid number, molecular weight, theoretical isoelectric point (PI), amino acid composition, atomic composition, instability coefficients, half-life, instability index, aliphatic index, and average hydropath(12). Molecular weight and PI are calculated from user-entered sequences. A protein's instability coefficient was assessed based on information about its amino acid composition. The instability index of a protein showed protein stability. If the protein's calculated instability index was < 40, it was considered a stable protein, while values > 40 were considered unstable. The in vivo evaluation of proteins was based on the "n-end law" principle. Also, it is important to measure the hydrophobic nature of the protein, which is calculated by dividing the total hydropathicity across all amino acids by the number of amino acids remaining in the protein.

v **Step 3: extraction of protein sequence**

At this stage, we will use clc and expasy software (http://web.expasy.org) to extract the protein sequences. Protein sequence properties such as protein similarity analysis and prediction of protein function or interactions need to be studied. With this program, you can extract a specific gene sequence and organize its information in data trees. Also, the toolbox enables the conversion of DNA into RNA and vice versa. These features were measured by this software.

v **Step 4: codon usage**

At this point, the cat software was used. The aim was to use this software to optimize the desired gene codon for the desired expression host. The sequence identity of each construct was checked and optimized for codon compliance by MWG operon and JCat (version) software. Finally, after sequence optimization, the highest codon match index was obtained. For cloning, a vaccine developed to analyze codon usage in a prokaryotic organism (Escherichia coli, E. coli) must first be adapted using a tool (http://www.jcat.de/) to optimize codon usage. The optimized sequence of each recombinant protein fusion was recorded in the GeneBank NCBI database and each sequence was assigned an accession number. Finally, a pET-28a cloning vector was placed during

the codon-optimized process of the structure at the end of the C-terminus. EcoRI and HindIII enzyme cleavage sites and EcoRI/XhoI cloning sites were used for this. Gene cassette (G:HSPX: PPE44´:His´) lacks Fcγ2α. It requires a tag, thus when designing this gene cassette, His-tag and c-myc sequences are expressed before the NotI cut sequence to express these two parts during protein production. These cassettes were designed to be expressed in pTE28α since the above tags were not present in this vector, so the sequence of these two tags was included in the design of the gene sequence.

v **Step 5: stability- solubility- flaking**

The Solubility prediction server in expense was used and molecular weight, amino acid analysis, aliphatic index, alpha-helix affinity, the propensity to beta-sheet mean pI, and hydrophilicity index were examined at this stage.

Step 6: endonuclease position

In this step, the software web cutter, nebcatter, and clc were used. To examine the cut-off sites in the genes of different enzymes for each sequence, they were evaluated by online software (NEB-Cutter, http://nc2.neb.com/NEBcutter2). For this purpose, the sequence of all genes was examined for no cleavage sites for the enzymes PmeI, BstXI, BamHI, BglII, XhoI, NotI, and SacI. There is a program available through a web server named "NEBcutter version 1.0" (http://tools.neb.com/NEBcutter) that accepts an incoming DNA sequence and provides a comprehensive report of the restriction enzymes that cleave the sequence.

v **Step 7: 2d structure**

In this stage, the software 2D Structure Server2 was used. After designing the gene construct, the spatial arrangement of its recombinant protein was examined. To know the spatial structure and study the non-interfering covalent bonds between the second Fc and the attached genes of Mycobacterium tuberculosis. Gly-Gly-Ser 2) was studied by J-Cat. A linker may not be required between the Mycobacterium tuberculosis PPE44 protein and the mouse Fcγ2a fragment as the hinge portion of the IgG2a fragment acts as a linker and allows the two parts to function with flexibility. A suitable vector for expression in bacteria was then selected for the design of the gene cassette and the sequence of both gene cassettes (5HSPX: (G4S) 2: PPE44: Fcγ2a)' was designed. The gene cassette of another protein was designed with the same pattern and only by deleting the Fcγ2a gene sequence from the previous cassette design (5HSPX: (G4S) 2: PPE44': His 3').

Step 8: 3d structure laser and SWISS-MODEL software were used to predict the structure which is available via the ExPASy server.

Step 9: loop.hairpin

MFold software was used in this step. http://mfold.rna.albany.edu Predicts optimal and non-optimal secondary structures for an RNA or DNA molecule using Zucker's newest energy minimization method. Calculating the energy matrices, the secondary structure of the Mfold mRNA was predicted. Select the appropriate expression vector. The expression vector pET28 was used to express the recombinant proteins and the XhoI and NotI sites were located at the 5' and 1' ends of the gene construct.

Gene cassette synthesis and Cloning and expression and Injection into mice

According to the previous article by the same authors, it was done to the address. https://doi.org/10.30699/ijp.2022.549673.2849(1,2)

Evaluation of immune responses against recombinant proteins

1. (Th1) IL-2 and IFN-γ responses)
2. (Th2) response IL-4)
3. TGF-β response
4. IgG1 and IgG2A response levels

Method of data analysis and statistical analysis

After collecting the data, all the information was entered into the computer. Statistical indicators of mean, standard deviation, tables, and graphs were used to describe the data. In data analysis, the One Way-ANOVA statistical test is used to compare controls and groups, and then the Tukey-Kramer test is used by SPSS 13 software to compare the two groups. The difference between the groups was considered significant, with $p < 0.05$. Graphs were designed by Graphpad Prism 9.2.0 software.

Results :

This study aimed to clone and express the fusion of PPE44: HspX: Fcγ2a and PPE44: HspX: His proteins and test the immunogenicity of these fusion proteins with interleukin-22 adjuvant in a mouse model and also to compare them with each other and compare with BC vaccine. Gene structures were first designed and cloned into plasmid pET28a (Biomatik, Ontario, Canada) and then expressed in E. coli Bl21 (DE3). After their expression was confirmed by Western blotting, these proteins were the mouse model of injection and the resulting immunogenicity was compared by comparing both proteins.

Design and cloning of recombinant expression plasmids:

After synthesis in the vector, Pet28A gene constructs were transferred to the BL21 expression vector during subcloning by XhoI and NotI enzymes.

Transformation and selection of transformed clones:

After the transformation process (electroporation method), the transformed bacterial clones were selected by culture on a YPDS agar medium containing 388 micrograms per milliliter of zeocin. Then the colonies that were large in appearance and suitable were re-cultured in the same medium

Confirmation of the transformation process by the molecular method: After transformation, the recombinant plasmids enter the bacterium according to the homologous recombination process. PCR was used for molecular confirmation of this process. This process was performed according to the instructions of the Invitrogen company using AOX1 and -α factor primers. At the end of this stage, clones whose PCR results were positive were selected for expression processes.

Optimization of expression conditions : To find the best environmental conditions to achieve the highest amount of protein production, recombinant HSPX-PPE44-Fc proteins were placed in different conditions at different concentrations of IPTG at specific temperatures and different incubation times. Finally, it was found that a concentration of 0.9 mM IPTG at 37 ° C overnight, was the best condition for achieving the highest content of recombinant protein. Recombinant proteins were labeled with the 6-His-at label at the N end to ease purification by the Ni-NTR column (Ni-NTA) (Bioneer, Daejeon, South Korea)

Evaluation of expression of recombinant SDS-PAGE and Western blot proteins

After high volume expression, the fractions obtained from the purification steps were examined for the presence of recombinant proteins. To ensure the purity of the proteins, both proteins were evaluated using SDS-PAGE, and the absence of a non-specific band indicated the purity of the protein. Specific confirmation of the presence of proteins was performed by Western blotting.

Evaluation of immune responses against recombinant proteins: After injection of recombinant proteins into mouse groups, the concentration of total antibodies and their subclasses along with the amount of cytokines IL-2, IL-4, IFN-γ, TNF-α, and TGF-B were measured. In each vaccinated group of spleen cells in cell culture, supernatant to measure the immune response pattern was measured by ELISA. Evaluation of immune responses against recombinant proteins from these cytokines as well as IgG1 and IgG2a were evaluated by spleen cell culture on day 45 after vaccination.

Check the response level of Total Ab: On (day 14) at concentrations of 1.10 and 1.20, due to the difference between the recombinant proteins PHF and PH, a significant relationship was observed between the two groups ($p < 0.05$).

In the study on the total antibody level in our study, the results showed that at concentrations of 1.10 and 1.20, the highest antibody production was assigned to the PH receiving group and there was a significant relationship between this group and the PHF receiving group. ($P < 0.05$. At this concentration, a significant relationship was observed between the groups receiving PHF and IL + PH. Fig(1)

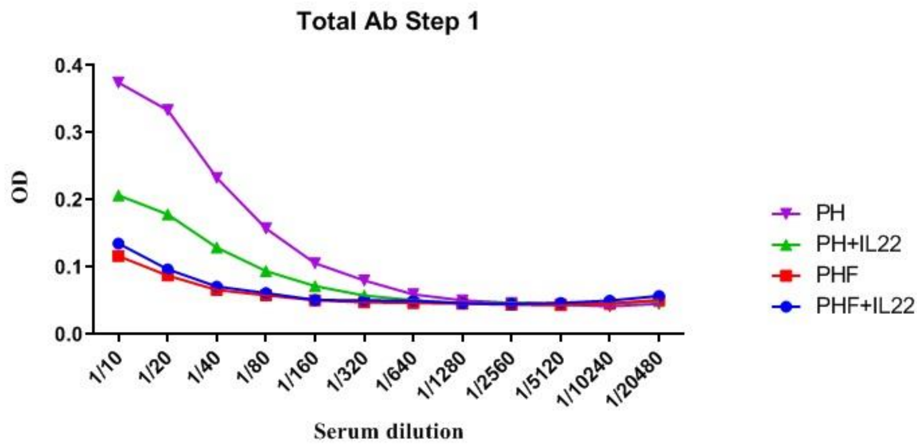

Fig1: level of Total Ab On (day 14)

Total Ab response level of stage two (day 45) at different concentrations. The results showed that the amount of total antibody in the second stage of the assay at a concentration of 1.20 was observed between the groups receiving PHF and PH with each other and a significant relationship was observed ($p < 0.05$). In the study on the number of total antibodies in the second stage of the essay, ie on day 45 in our study, the results showed that at a concentration of 1.20, the highest antibody production was in the pH group and this group with the PHF + IL group and PHF showed a significant relationship ($p < 0.05$). Fig2

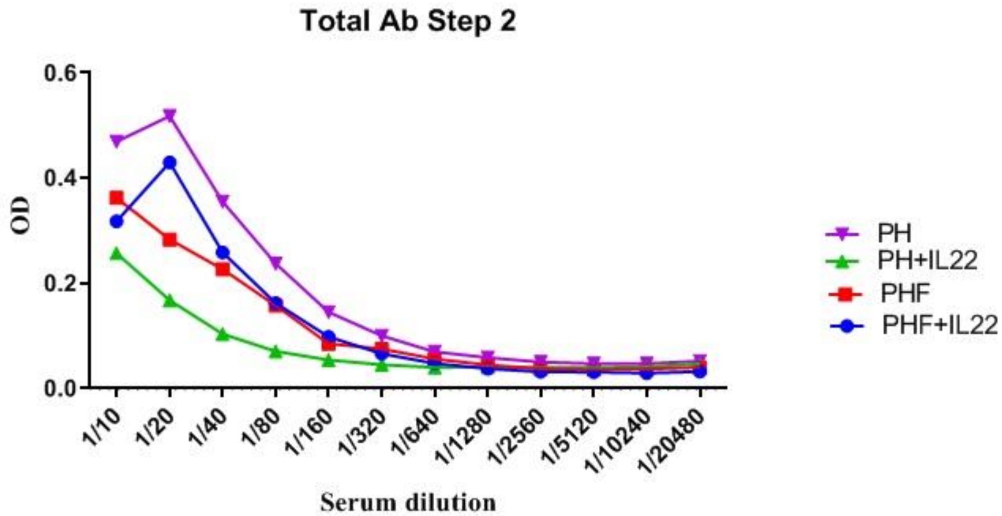

Fig2: level of stage two (day 45)

Antibody response levels in IgG1 and IgG2A subclasses: The amount of IgG1 in the groups vaccinated with PHF also showed a significant increase compared to other groups and a significant difference was observed with the group receiving PH. (p <0.05) The results showed that there was a significant relationship between PHF + IL and PH and PH + IL groups. (p <0.05). Also, a significant relationship was observed between F H P and PH and PH + IL groups (p <0.05) Fig3

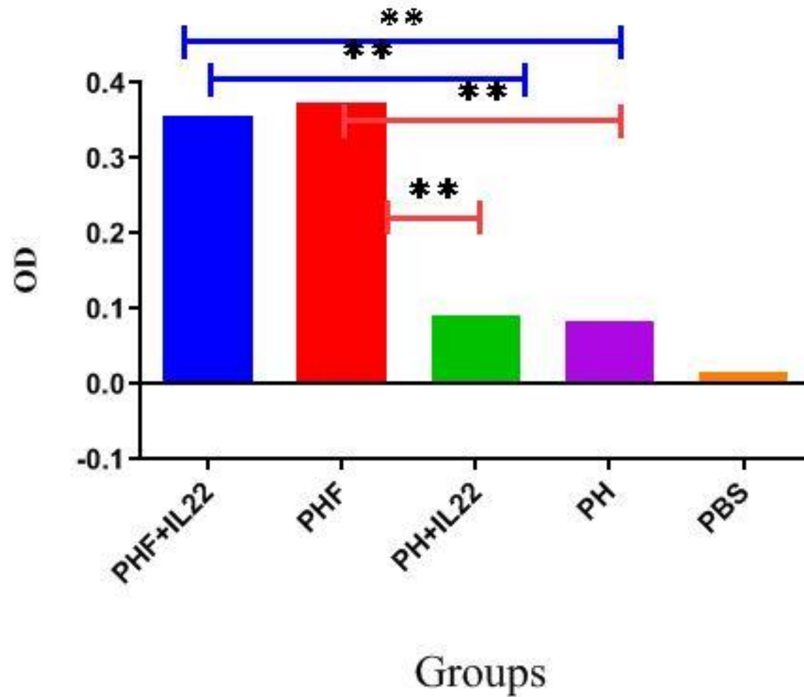

Fig3: levels in IgG1 and IgG1A subclasses

IgG2A response level: The level of IgG1 in the groups vaccinated with IL + PHF + showed a significant increase compared to other groups and a significant difference was observed with the group receiving PH. (p <0.05) The results showed that there was a significant relationship between PHF + IL and PH and PH + IL groups. (p <0.05). No significant relationship was observed between PHF and PH and PH + IL groups (p <0.05). Fig4

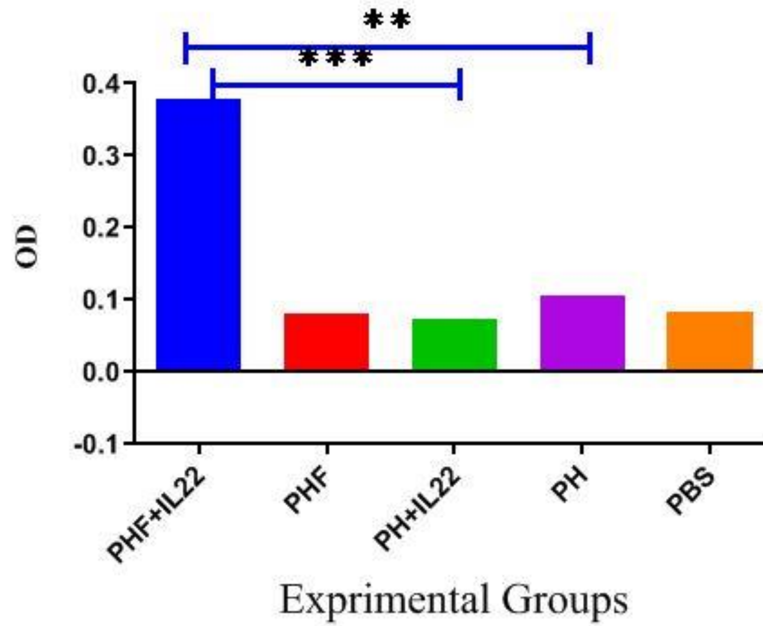

Fig4: levels in IgG1 and IgG2A subclasses

(Th1) IFN-γ: The level of IFN-γ response in the groups vaccinated with PHF showed a significant increase compared to other groups and a significant difference was observed with the group receiving PH. ($p < 0.05$) The results showed that there was a significant relationship between PHF + IL and PH groups. ($p < 0.05$). There was no significant relationship between PHF and PH + IL groups ($p < 0.05$). Fig5

IFN γ

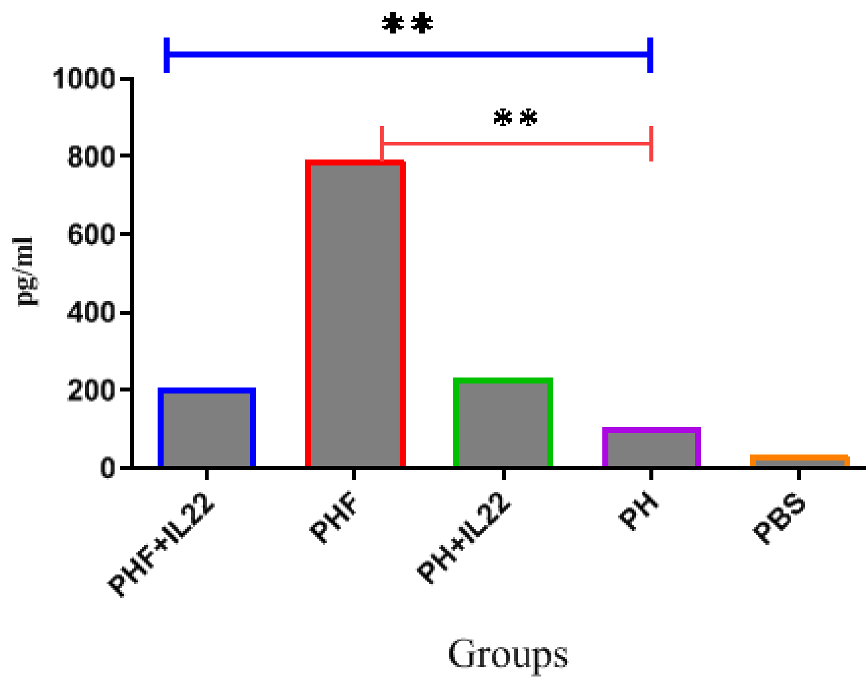

Fig5: The level of IFN-γ response

TNF-α response: The level of TNF-α response in the groups vaccinated with PHF showed a significant increase compared to other groups and a significant difference was observed with the group receiving PH. ($p < 0.05$) The results showed that there was a significant relationship between PHF + IL and PHF groups. ($p < 0.05$). There was no significant relationship between PHF and PH + IL groups ($p < 0.05$). Fig6

TNF α

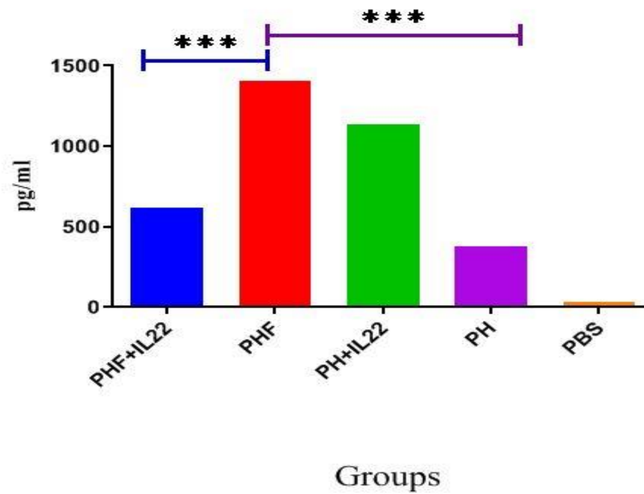

Fig6: The level of **TNF-α** response

TGF-β response: The level of TGF-β response in the groups vaccinated with PHF showed a significant increase compared to other groups and no significant difference was observed with the group receiving PH. (p <0.05) The results showed that there was a significant relationship between PHF + IL groups and the group. (p <0.05). There was no significant relationship between PHF and PH + IL groups (p <0.05). Fig7

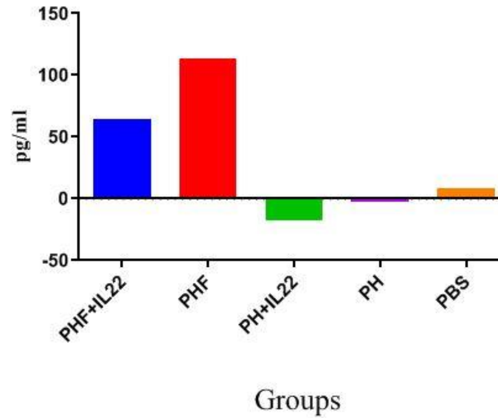

Fig7: The level of **TGF-β** response

Responses (IL-2, IL-4) Th2: The level of IL-2 response in the groups vaccinated with PHF showed a significant increase compared to other groups and a significant difference was observed with the group receiving PH. (p <0.05) No significant relationship was observed between the PHF node and PH + IL groups (p <0.05). The level of IL-4 response in the groups vaccinated with PHF + IL showed a significant increase compared to other groups and a significant difference was observed with the group receiving PH. (p <0.05) There was no significant relationship between the PHF node and PH + IL and PH groups (p <0.05). Fig8aand Fig8b

IL-2

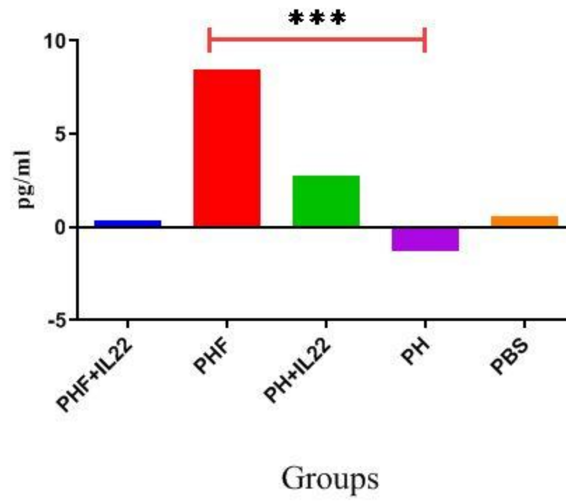

Fig8a: The level of **IL-2** response

IL- 4

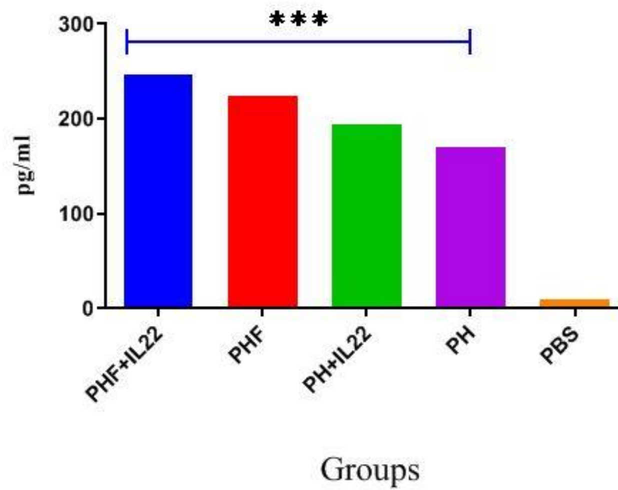

Fig8b: The level of **IL-2** response

Discussion and conclusion :

Given the challenges that the BCG vaccine faces in preventing tuberculosis, the design and production of new and effective vaccines against this disease are very important. One of the most important strategies used is to design subunit vaccines that contain one or more immunogens of Mycobacterium tuberculosis. The selection of subunit vaccine-producing antigens is based on their ability to stimulate protective immune responses. Studies have shown that PPE44 and HSPX proteins have abundant Th1 and Th2 responses against tuberculosis due to their stimulatory epitopes. PPE44 protein, which is rich in T cell epitopes and stimulates cellular immune responses as immunogens, is known as the new M. tuberculosis antigen and its role as a subunit vaccine comparable to BCG protection has been reported in the previous report (23). . It should also be noted that latent phase antigens should be used in the design of vaccines (recombinant proteins) against tuberculosis to cover one of the most important defects of the BCG vaccine. One of these antigens used in the design of subunit vaccines in previous studies is the HspX (Heat shock proteins) antigen, which in invivo studies protected mice exposed to Mycobacterium tuberculosis aerosols. HspX is an immune-stimulating antigen that can protect the host against primary tuberculosis and secondary activated infection (24). Our immune cells. The use of more bacterial antigens can induce stronger immune responses that increase the likelihood of protection, and also to this strategy, the use of a strong adjuvant can be effective by enhancing antigen immunization and modulating immune responses. So, in this study, we used two recombinant complexes, PPE44: HSPX: FC complex (PHF) and PPE44: HSPX: HIS complex (PH), which are composed of tuberculosis antigens with IL-22, which immunized the two. To increase the immunogenicity of these recombinant complexes, one of the constructs was fused with the Fc domain of immunoglobulin to bind the compound to Fc receptors at the surface of the target dendritic cells, thus activating strong signals to provide T cell proliferation and hemorrhagic immunity. The cytokine IL-22 was also used in combination with the vaccine to enhance the immunization of the recombinant complex to amplify the responses. What was obtained from the studies in this study is that at the level of 1IgG production, the highest production was in the groups receiving PHF recombinant protein compared to the group receiving PH and PH + IL, which showed a significant relationship between the PH group and the PH + IL group. . Also, a significant relationship was observed between the PHF + IL group and the PH and PH + IL groups. About the increase in 2IgG production level in this study, considering the significant relationship between PHF + IL groups and PH + IL and PH groups, it seems that the increase in production levels of both 1IgG and 2IgG antibodies in PHF and PHF groups IL +, which are complete recombinant proteins, have a higher pH than incomplete recombinant proteins, and with this recombinant protein, immunity progresses. In this regard, a significant difference was observed from previous studies that further increased cellular immunity. One of the most important points is that previous studies have shown that routine tuberculosis (BCG) vaccine stimulates the cellular immune system, while in our study we showed that the recombinant PHF protein encourages the result to increase hemolar immunity. Studies have reported that the strongest vaccines are those that also stimulate the hemorrhagic immune system and make it respond. In the study of specific immune responses, especially cellular immunity, the production level of some cytokines was measured in this study, although due to the high volume of work, the type of cells producing these cytokines, such as macrophage or DC type. It was not measured, but the study of the production of these cytokines may be a sign that the recombinant proteins designed to produce these cytokines may also induce their cellular immune responses. The production levels of some of them may elicit responses induced by Th1 and Th1 cells.

INF-γ is known as one of the most important immune factors that play a role in the activation of Th1 as the most important arm of immune responses against tuberculosis. INF-γ released by Th1 cells activates macrophages, and activated macrophages kill bacteria by secreting nitric oxide and other immune products (25). Th2 is also activated by the production of cytokines IL-4 and IL-10. It suppresses Th1 responses and plays an important role in stimulating antibody production by switching cellular immune responses to hemolar immune responses (26). An important factor that determines the type of cytokines present at the site of infection or vaccination is an antigen (27). Thus, the ability to stimulate and induce a cellular immune response by Th2 and Th1 lymphocytes is an important criterion for evaluating newer vaccines and proteins against TB. In the design of a subunit vaccine against tuberculosis, the selection of immunodominant antigens that can protect the immune response of Mena. The results of our study showed that IFN-γ levels in PHF-vaccinated mice were significantly higher than in the PH + IL and PH groups, although in other groups IFN-γ production was induced. The intramuscular injection was used intramuscularly. The production of this cytokine by injection of the PHF + IL complex also showed a significant increase. High levels of TNF-α cytokine production were observed in mice vaccinated with PHF, which were significantly associated with pH groups. The results of this study showed that the groups receiving PHF + IL had a

significant relationship with the PHF group G. The results obtained in our study were consistent with some previous studies. Previous studies have shown that TNF-α is essential for controlling M. tuberculosis infection. Deficiency in the production of the cytokine TNF-α appears to lead to uncontrolled infection (29).

In this study, high levels of IL-4 were observed in mice vaccinated with PHF + IL, which had a significant relationship with the pH group. It seems that the increase in this cytokine in this study may balance the host's immune system after the removal of the infection, and may increase the production of these responses to enhance hemolar immunity.IL-2 production level in this study was higher in PHF-receiving groups than in other groups and PHF-receiving groups showed a significant relationship with the PH-receiving group. In a study by Shi et al., high levels of IL-2 were reported after HSPX injection (30). According to the studies performed, the proteins expressed in the latent phase of tuberculosis-like infection (HSPX) induce the response and production of IL-2, which were consistent with our study of the response to recombinant proteins. In the study, TGF-β levels were higher in the PHF-receiving groups compared to the PH-receiving groups, but no significant relationship was observed between the groups receiving the two recombinant proteins. While in some similar reports the amount of this cytokine was reported higher. Based on the results obtained in the study, the recombinant PHF protein showed a higher level of responses due to the production of cytokines and antibodies compared to the PH protein. Although the study of the type of responses (Where cellular and hemolar immune responses are generated) requires investigation and ongoing research to confirm the type of cells from which these antibodies and cytokines are produced, the hypothesis is that recombinantly designed proteins can provide humoral and cellular immunity is strengthened. Previous studies have shown that targeting antigens to bind to Fc receptors (FcγRs) at the level of antigen-supplying cells not only increases the uptake and delivery of T lymphocytes but also alters the processing process and type of delivery (31). It is thought that the use of Fc biogenic proteins can simultaneously stimulate CD4 + T and CD8 + lymphocytes. This is important for establishing a protective immune response against intracellular bacteria (32).

After removal of the complex, PPE44: HSPX is separated from HSPX under acidic conditions inside the PPE44 phagosome, in which case it acquires cytolytic properties. The activated PPE44 then degrades the phagosome membrane, causing antigens to enter the cytoplasm and delivering their immunogenic epitopes through MHC class I and I I molecules to the cytotoxic T + lymphocytes (CD8 +). Dimerization due to Fc II increases the capacity and size of recombinant proteins and decreases their renal excretion. Some studies have shown that the fusion of immunogenic proteins of some viruses such as HIV, A, and Ebola to the second Fc stimulates a protective immune response against these viral infections. Fusion of proteins confirms further immune responses in stimulation (32). Therefore, it seems that more research can be performed based on Fc fusion of proteins, a new generation of z vaccines. Designed and produced a unit against tuberculosis. On the other hand, investigating the protective effect of this recombinant protein in an animal model infected with Mycobacterium tuberculosis (mice or guinea pigs) can help to better evaluate these recombinant subunit proteins. The lack of necessary conditions and facilities to perform this evaluation was the most important limitation of the present.

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Conflict of Interest

The authors declared no conflict of interest.

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