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REVIEW ARTICLE

Evaluating the Performance of PPE44, HSPX, ESAT-6 and CFP-10 Factors in Tuberculosis Subunit Vaccines

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Abstract

Mycobacterium tuberculosis (*M. tuberculosis*) is an intracellular pathogen causing long-term infection in humans that mainly attacks macrophages and can escape from the immune system with the various mechanisms. The only FDA-approved vaccine against *M. tuberculosis* (MTB) is *Mycobacterium bovis bacillus Calmette-Guérin* (BCG). The protection of this vaccine typically lasts 10–15 years. Due to the increasing number of people becoming ill with MTB each year worldwide, the need to develop a new effective treatment against the disease has been increased. During the past two decades, the research budget for TB vaccine has quadrupled to over half a billion dollars. Most of these research projects were based on amplifying and stimulating the response of T-cells and developing the subunit vaccines. Additionally, these studies have demonstrated that secretory and immunogenic proteins of MTB play a key role in the pathogenesis of the bacteria. Therefore, these proteins were used to develop the new subunit vaccines. In this review, based on the use of these proteins in the successful new subunit vaccines, the PPE44, HSPX, CFP-10 and ESAT-6 antigens were selected and the role of these antigens in designing and developing new subunit vaccines against TB and for the prevention of TB were investigated.

Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) as a human pathogen is the causative agent of pulmonary Tuberculosis (TB). TB is considered as an urgent disease according to the World Health Organization (WHO) [1, 2]. The WHO global TB report 2020 shows that, approximately, 10 million people fell ill with TB worldwide and 1.5 million people died from TB in the same year [3]. Additionally, the WHO report demonstrates that approximately one-third of the world's population infected with TB bacteria [4]. Despite the identification of the bacterium responsible for the development of the disease, as well as excellent therapeutic methods and scientific advances, TB still remains an important health-threatening problem. *M. tuberculosis* (MTB) grows slowly, having a very flexible cell wall, has a highly contagious nature and has several strategies to escape the immune system [1, 2]. Notwithstanding, directly observed treatment, short-course (DOTS, also known as TB-DOTS) control strategy and vaccination with BCG, TB still has more pathogenic factors than other infectious agents [3, 4]. In addition, one of the most alarming factors in TB patients, also found in patients with HIV, is the emergence of multi-drug resistant species [5]. Many new methods against TB have been developed within the last 25 years. Today, the principles of the molecular invasion, factors involved in the severity of the disease and the activation of TB are also identified which are essential for the survival and proliferation of the causative agents within macrophage cells [5, 6]. Nowadays, vaccination is necessary to control infectious diseases and have drastically reduced the rate of mortality caused by them throughout the world such as measles, mumps, polio and diphtheria. The WHO estimates that 80% of all infectious diseases in the world are related to the deaths of more than 20 million people globally [5]. However, vaccines play a key role in controlling infectious diseases, which are

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cost-effective. One of the most successful vaccines produced is inactivated/killed vaccines that contain whole bacteria or viruses that are killed or functionally altered so they cannot reproduce. As it is known, inactivated/killed vaccines do not contain any live bacteria or viruses to cause disease, even in people with extremely weakened immune systems. The killed vaccines have some advantages over the live attenuated vaccines; the most important benefits are as follows: (1) producing high-level protection against the specific diseases; (2) unlike attenuated vaccines, there is no risk for reversion and (3) there is no need to store these vaccines at low temperatures. However, inactivated vaccines have some distinct disadvantages including (1) not always elicit strong or long-lasting immune responses and for this reason, there is a need for regular booster injections; (2) the treatment processes involved in inactivation of a vaccine is quite expensive comparing to other vaccines [7, 8], therefore, no promising killed vaccine against TB is developed.

Up till now, old or first-generation vaccines against TB were synthesized from live and attenuated microorganisms, which had several problems. Some ongoing research projects have designed cost-effective vaccines with higher viability that can stimulate the immune system against a specific pathogen. Currently, the attenuated strain of *Mycobacterium bovis* (*M. bovis*) bacillus *Calmette-Guérin* (BCG) is used in many countries to generate immunity. This vaccine is the only approved vaccine for humans. The efficacy of this vaccine varies widely throughout the world and has shown efficacy ranges from 0 to 80% [9, 10].

Additionally, the BCG vaccine induces immunity in children, while it has low effects on the prevention of adult pulmonary TB. For this reason, the production of new vaccines that are more beneficial than BCG is highly required for preventing TB. Vaccines, such as viral vectors, DNA vaccines, subunit vaccines, attenuated *M. tuberculosis* and the recombinant BCG are among the most important newly designed vaccines in the last 20 years [9–12] (Table 1 and Fig. 1). Nowadays, bioinformatics and immunoinformatics have accelerated the development of novel vaccine candidates against infectious disease [11–13] as well as against TB [14, 15].

M. tuberculosis is a facultative intracellular bacterium and the main way that bacterium enters the body is the respiratory tract. The primary cells that fight this pathogen are the alveolar macrophages and different types of T-cells must be activated to overcome bacterial resistance against host defense system. $CD4^+$ T-cells have a role in the production of various types of cytokines, such as IFN-γ and TNF-α. $CD8^+$ T-cells play an important role in inducing appropriate immune responses against *M. tuberculosis* through cytotoxic activity and induction of programmed death in infected cells. Cellular immunity is the basis of host responses against TB infection. Recent studies have also highlighted the importance of the innate and humoral immune systems in controlling TB infection. By providing accurate knowledge about immune systems, novel strategies for the design and the development of a new generation of vaccines and drugs are attained [26, 27].

Nowadays, different antigens are used to develop various vaccines against TB. Among all, subunit vaccines are one of the most prominent vaccines to be used here and most recent research has focused on the main marker antigens of *M. tuberculosis* such as PPE44, HSPX, ESAT-6 and CFP-10 for designing these vaccines [23, 28]. This paper investigated the role and performance of these antigens in designing vaccines against TB.

Selection of the Antigens

Several studies have demonstrated that secretory and immunogenic proteins of *M. tuberculosis*, such as RV2660c, RV1813c, PPE42, PepA, PPE18, EspD, EspC, EspF, EspR, Ag85, RV2608, Rv3619, RV1813, RV3620, PPE44, HSPX, ESAT-6 and CFP-10 play a key role in the pathogenesis of *M. tuberculosis* [21, 24, 28]. Furthermore, the activation of T-cells by these antigens suggests the suitability of these antigens as candidates for vaccine production against *M. tuberculosis*. Some of these antigens are used alone or in combination to develop subunit vaccines. Although a number of these vaccines are currently being evaluated in clinical trials, investigation for producing new vaccines is useful against this pathogen. Therefore, in this study, we have tried to evaluate these antigens (PPE44, HSPX, ESAT-6 and CFP-10) as far as possible.

An Overview of Antigens

PPE44 is one of the important antigens in vaccination of MTB. This protein is a member of PPE (Pro-Pro-Glu) protein family which is unique to *mycobacteria*. Named after the conserved proline (P) and glutamic acid (E) residues in their N-terminal domains, these proteins are suggested to perform wide-ranging roles in virulence and immune modulation [29, 30]. PPE44 with the Pro-Pro-Glu epitope at the N-terminus of the protein that is exclusively detectable by MHC I and MHC II and its nucleotide sequence found only in MTB complex (MTBC) defined this protein as a potential candidate agent for MTB vaccination [31].

Another protein that is suitable for candidate subunit vaccines is heat shock protein X (HSPX). Under the stress condition such as nutrient scarcity, the presence of nitric oxide and hypoxia and during the lag phase the produced HSPX can reach to 25% of the total bacterial protein [32]. This protein is able to escape from the host innate immune system

Table 1 New vaccine against *M. tuberculosis*

No	Name of vaccine	Mechanism	Type	Advantage	Disadvantage	Development	References
1	BCG	Inducing a strong Th1 response	Attenuated vaccine	Cheap, safe and protects children efficiently against the early manifestations of TB	Protection typically lasts 10 to 15 years	FDA approved	[10]
2	VPM1002	Inducing the Th1 responses	Attenuated vaccine	Safety and immunogenicity in healthy infants and adults	Weak T-cell response in infants	Phase II/III trial that is slated to conclude in 2020	[10, 16, 17]
3	AERAS 422	Inducing the Th1 responses	Attenuated vaccine	Induce a broad and relatively enduring immune response	Not investigated in infants	Phase I clinical trials	[18]
4	MTBVAC	Inducing poly-functional $CD4^+$ and $CD8^+$ T-cell responses	Attenuated vaccine	Improved protection in mice relative to BCG	Not significant $CD8^+$ T-cell responses are induced relative to BCG	Phase II clinical trials in South Africa	[19]
5	H4:IC31	Signals generation through Toll-like receptor 9	Subunit vaccine	Reduced the rate of sustained infection, which might indicate the ability to control infection	The primary endpoint of vaccine is the inefficacy	Clinical trial is completed	[20]
6	M72:AS01	Not specified	Subunit vaccine	Reduce the pulmonary TB in adults	The protection against *M. tuberculosis* is not clear	Phase II clinical trials	[21]
7	MVA-85A	Induction of IFN-γ and trigger a Th1-dominated immune response	Subunit vaccine	Highly immunogenic in humans	Side effects are relatively mild but not protection in infants	Undergoing multiple phase I/II trials in Africa	[22]
8	ID93:GLA-SE	Inducing the Th1 responses	Subunit vaccine	High level of IgG antibodies	Not investigated in infants	Phase II clinical trials	[23]
9	TB/FLU-04L	Delivered by the intranasal route	Viral vectors	Safe and immunogenic in healthy BCG-vaccinated	Cost-effective experimental medicine	Phase II clinical trials	[24]
10	Aeras-402-Ad35	Increasing T-cell-mediated immunity	Viral vectors	Safe in healthy infants previously vaccinated with BCG	Weak immunogen-specific T-cell response in infants	Phase I clinical trials was completed	[25]

This table was sorted based on the type of vaccines

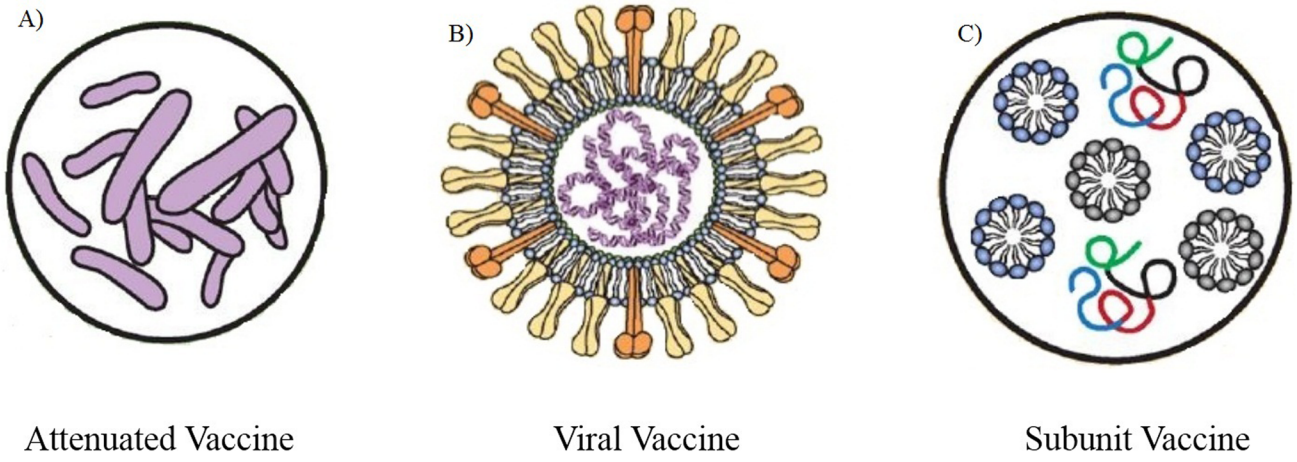

Fig. 1 The schematic of attenuated (**A**), viral (**B**) and subunit vaccines (**C**) of *M. tuberculosis*

by increasing the stability of the proteins. On the other hand, the cells overexpressing this gene grow more slowly than wild-type cells and are less susceptible to autolysis following saturation of the culture in vitro, suggesting that HSPX may slow down the growth rate of *M. tuberculosis* in culture and contribute to the spread of TB during macrophage infection [33, 34]. Due to the properties of HSPX to activate both cellular and humoral immune responses and strong induction of Th1 cytokines such as TNF-α and IFN-γ [35, 36], this protein is a good candidate for vaccination against *M. tuberculosis.*

The 6-kDa early-secreted antigenic target (ESAT-6) of *M. tuberculosis* is one of the important antigens presented in this study. This protein was first identified as a potent T-cell antigen and now is recognized as a pore-forming toxin that is essential for virulence of *M. tuberculosis*. ESAT-6 is secreted through the ESX-1 secretion system (Type VII) of *M. tuberculosis.* It has been implicated in mediating mycobacterial cytosolic translocation within the host macrophages by rupturing the phagosomal membranes. ESAT-6 is an abundantly secreted protein of *M. tuberculosis* which considered as an important virulence factor, deactivation of which results in lower virulence of MBT. ESAT-6 alone or in complex with its chaperone culture filtrate protein-10 (CFP-10), is known to modulate host immune responses. ESAT-6/ESAT-6: CFP-10 can enter the endoplasmic reticulum (ER) where it dissociates Beta-2 microglobulin (β2M) from pMHC-I complexes to inhibit cell surface expression of MHC-I-β2M complexes, resulting in downregulation of class I-mediated antigen presentation [37, 38]. The other secreted antigen that is important in vaccination of *M. tuberculosis* is Cyan Fluorescent Protein-10 (CFP-10). This protein forms a 1:1 heterodimeric complex with ESAT-6. CFP-10 has been described as a chaperone protein for ESAT-6. The ESAT-6: CFP-10 complex activates the human neutrophils and transiently induces the release of Ca^{2+} [39, 40].

The ESAT-6 and CFP-10 are expressed by ESXA (RV3875) and ESXB (RV3874) genes, respectively. The genes encoding these proteins are adjacent to each other and are located in the RD1 (Region of Difference1) locus [5, 21].

The ESX family in *M. tuberculosis* consists of 23 members and based on high sequence homology divided into three distinct subfamilies. The ESX protein contain about 100 amino acids [41] and are potently recognized by both $CD4^+$ and $CD8^+$ that are very attractive target against TB. In addition, as ESAT-6 and CFP-10 seem to be essential for the growth and pathogenicity of *M. tuberculosis* and since some of these proteins are omitted in BCG, therefore, this vaccine has low efficacy in immunization of animals and humans. As, immunizations with a single ESX antigen preparation have been inadequate for controlling TB, many studies have described that levels of protection and pathogen-specific cellular immunity can be enhanced by combining ESX antigens with other TB-associated antigens [41, 42]. The RD1 locus is a 9.5-Kbp molecular weight region of the *M. tuberculosis* genome consisting of nine genes (RV3871-RV3879) encoding protective antigens or virulence antigens of the bacterium [5, 21, 43]. The complex of these proteins is degraded in the acidic pH of the macrophage phagosome and then each of these proteins is coupled to the phagosomal membrane causing lysis. Consequently, it appears that these proteins trigger the entry of bacteria from the phagosome into the cytosol and eventually to $CD8^+$ T-cells [22, 44].

In addition to ESAT-6 and CFP-10, several studies have demonstrated that other secretory proteins, such as PPE44 and HSPX play a key role in the pathogenesis of *M. tuberculosis* and are also involved in activation of T-cells, suggesting the suitability of these antigens as candidates for vaccine preparation.

To increase and stimulate the response of T-cells, several subunit vaccines against *M. tuberculosis* have been studied and developed [23]. The advantages of subunit vaccines are their safeness, having well-known protein components, their ability to be standardized and the applicability of the protective antigens of *M. tuberculosis*, leading to the increased response of T-cells. Thus, the increase of the antigen diversity in subunit vaccines enhances the vaccine performance against TB, it must also ensure that the selected antigens are detected by T-cells in different human populations [45].

PPE44 Members and their Role

The PPE family has 69 members with conserved N-terminal and variable C-terminal. PE44 is a member of the MTB PPE proteins that has consecutive repeated sequences [46]. This family contains approximately 10% of the MTB genome [47]. The comparative genomic assay reveals that the PPE protein is largely restricted to *Mycobacterium* genus [48], particularly in virulent species of *Mycobacterium* (TB, *M. bovis*, *M. ulcerans*, *M. marinum* and *M. canis*), playing a unique role in the virulence-associated sequence and the survival of *Mycobacterium.* Based on the number of repetitions of C regions, the PPE family can be divided into four subfamilies:

1 The PPE-SV family has 24 members containing the conventional motifs of GLY-XX-SER and XX-TRP between 300 and 350 positions.
2 The PPE-MPRT family has a repeated sequence of ASN-X-GLY-X-GLY-ASN-X-GLY and a repeated sequence of GCCGGTGTTG in the C-terminal separated by 5 bp spacers.
3 The PPE-PPW family has 44 conserved amino acids with a conserved sequence of GLY-Phe-GLY-X-TR and X-XPRO-X-X-TRP in the C-terminal [49].
4 The fourth subfamily has 12 members, which has a low consistency in the C-terminal region.

It should be noted that the C-terminal region of PPE has 225 amino acids. The PPE-MPRT protein, which is similar to the PE-PGRS gene, contains a motif in N-terminal and an amino acid serine A/B for hydrolysis [50]. These hydrolyses are required for cell protection and to make cell walls impermeable and to enhance the virulence of the bacteria. Each type of PPE has a specific alpha helix or random coil [51]. PPE family proteins have played an important role in the development of antigenic diversity. Immunoassays on PPE almost showed the same response as purified protein derivative (PPD) to patients' cell lines [52].

Nowadays, new sciences such as systems biology are very influential in designing and developing different purposes. One of the methods of systems biology is to find the protein–protein network (PPI) which can be useful for anticipating the role of the targeted protein in biology such as determining the biomarker, subunit vaccine, cancers and etc. In the PPI network, one of the proteins that are extremely important and can interact with other proteins in the network is PPE. Understanding the function of this protein is very important in the development of the new vaccine such as subunit vaccines [53]. PPE44 is a member of the *M. tuberculosis* PPE family that has interaction with ten members of this family (Fig. 2).

The Mechanism of the Secretion and Molecular Structure of PPE Genes

Proving the existence of a large family of PE/PPE proteins was an important discovery in the molecular biology. PE/PPE family genes are commonly considered as a gene complex and have important effects on each other since they are cell surface components [54, 55]. Studies have shown that PE and PPE genes are transmitted as part of the ESX gene secretion system (the details will be discussed later). Further evidence is available to determine the PPE position, the composition of amino acids and their sequences, indicating the composition of amino acids, which will help to predict their position on the cell surface [52].

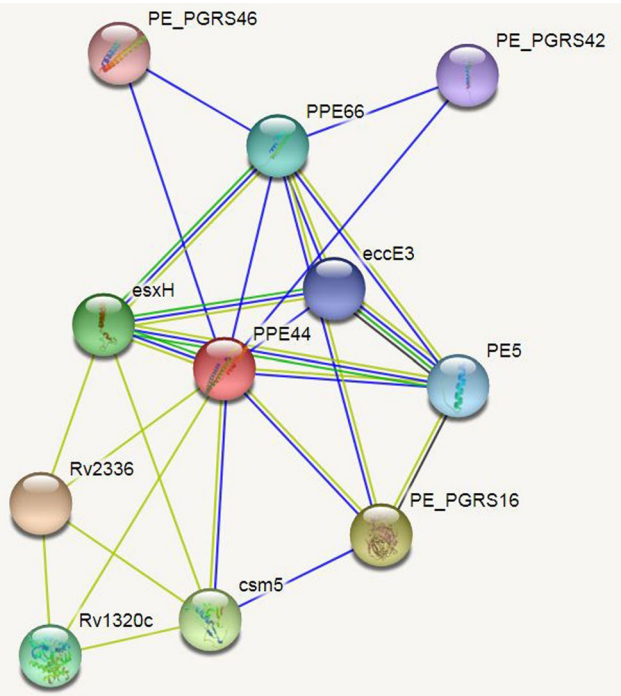

Fig. 2 Protein–protein networks between PPE44 and PPE families analyzed with STRING database (https://string-db.org)

Pajon et al. predicted that the beta proteins in the MTB genome included forty PPE proteins with a beta structure [56]. Beta proteins (beta-barrel or beta-sheet) are a group of surface proteins harbored in the outer membrane of bacteria. They are creating a channel by forming anti-parallel beta strands which cover all over the outer membrane. These proteins have various functions such as transport of ions, enzymes, siderophores and structural proteins and mediating flux of metabolites. As they play an essential role in the bacterial virulence, it would be of great interest to use them for the development of the vaccines [56, 57]. These observations support the hypothesis that PPE proteins associated with PE proteins are possibly translated on the cell surface. The following section in this study regarding evolutionary genetic topics noted that the evolution of PPE genes and associated genes such as PE was in regions proximate to ESX [58].

Genes such as PPE68 are located in an area within the ESX-1 region [59]. The PPE68 protein in *M. tuberculosis* has been reported to be strongly associated with the cell wall [52, 60].

Additionally, research on PPE68 reported its confrontation with the ESX1 secretion system [60, 61]. Other studies suggested that PPE68 acts as an ESX-1 regulator [62] and the ESX-1 has the potential role in the secretion of ESX-5; moreover, the presence of PE/PPE and the secretion of PPE by ESX-5 is demonstrated, while some believe that PPE is used by the ESX secretion system [63, 64]. The complex structure of PPE demonstrates the potential role of this compound in signal transduction and the PPE protein in this set has structural homology with the serine chemoreceptor [65]. These findings reveal the possible role of the PPE protein and its associated gene, PE, in host immunity and signaling [66].

PPE Immunity Review

The PPE protein and its accompanying gene, PE, are important factors in virulence enhancement and a possible source of antigenic variation. PPE can be the first line of defense against TB owing to their role in the immune system and their ability to modulate macrophages function. Macrophages have a set of antimicrobial mechanisms trying to defend against the microbial agents by producing IFN-γ and TNF-α, nitrogen, reactive oxygen mediators and cytokines [67]. Although macrophages activate T-cells against TB to control and eliminate the infection, they can also serve as the main host cell for TB growth and survival [68, 69]. When macrophages infected with TB, some cytokines such as TNF-α, IL-12 family, IL-6, IL-1α/β, and IL-10 are secreted which are important to control MTB infection. Some proteins secreted by MTB such as CFP-10 and ESAT6, SecA 1/2 proteins and the eukaryotic like serine/threonine protein kinase G (PknG) interfere with macrophage apoptosis and phagosomal maturation. A few days after escaping from macrophages, MTB begins to multiply to spread the infection. Some studies have demonstrated that region of difference 1 (RD1) and ESAT-6 in the MTB are required to escape from phagocytosis [70, 71].

It has not yet been clarified that this PPE protein is directly related to the proliferation and intracellular survival of TB or virulence enhancement by impairing the function of the immune system via macrophages [72]. Another report showed that the inactivation of PPE46 can weaken *M. tuberculosis* in the body [73]. Moreover, a different study showed that the PPE gene deletion caused impairment of MTB growth in macrophages [72].

Various studies reinforce the view that PE/PPE genes contribute to the survival of MTB. After conducting several studies by analyzing the proteasome and considering the microarray results, Brosch et al. revealed the MTB response to nutritional stress [74]. Studies on four PPE members 24 h after infection have indicated that the presence of these proteins is necessary to maintain the long-term survival of bacteria under nutritional stress [61].

In addition, Dillon et al. indicated that the PPE protein of the RV1196 gene during the infection was better expressed in rats vaccinated with RV1196 DNA vaccine and had better function to control TB [75].

In another study, the PPE44 protein expressed by the RV2770C gene was subcutaneously or intravenously injected into BALB/C mice previously vaccinated with the BCG vaccine. The results demonstrated that the PPE44 gene-induced Th2 immune responses and IgG1 and IgG2 immunoglobulin as well as delayed sensitivity responses developed [76].

Some studies indicate that the PPE18 (RV1196) similar to PPD triggers the T-cell response which in turn stimulates T helper cells and macrophages to produce IL-10 and IL-12, respectively [73].

Other studies have revealed that PPE44 and PPE18 sometimes trigger the response of Th2 cells under certain conditions expressing IL-10, 1L-12 cytokines by modulating the levels of macrophages. The evidence refers to their role in enhancing virulence. The genes encoding polymorphic PPE proteins have been demonstrated to cause extensive antigenic changes in *Mycobacterium* [73, 77]. The frequency of variation in these proteins is very high which help them to escape from the immune system [78].

Heat shock protein X (HSPX) or *M. tuberculosis* α-Crystallin is a protein with a molecular weight of 36.1 kDa encoded by the RV2031c gene [79]. It acts as an important antigen in the latent phase of *M. tuberculosis* and is expressed mainly by non-replicating bacilli. HSPX keeps the bacteria alive in the latent period of the disease as well

as infection and is able to induce stronger immune responses in patients with latent TB [79].

HSPX, known as *M. tuberculosis* alpha-crystalline, is a protein with high immunogenicity that is a potent stimulant for Th1 responses in individuals exposed to TB [79]. This protein acts as a molecular chaperone similar to its human analog. Furthermore, the high production of this protein in hypoxia and microaerophilic conditions suggests that it also plays a role in developing TB granuloma [80]. Molecular chaperons have different functions in bacteria, including proper folding of newly synthesized proteins, protein transitions and inhibition of aggregation during thermal shock, degradation and reduction of aggregated proteins and recovery of the proteins that are damaged or have inappropriate folding due to stresses. Therefore, molecular chaperones play a pivotal role in proteome control by interacting, stabilizing and remodeling various proteins [81].

HSPX protein is expressed only in *M. tuberculosis* and is extremely important in the pathogenesis of bacteria. In vitro studies show that HSPX is expressed in hypoxia, causing bacterial stability inside the macrophages. Recently, efforts have been made to produce a vaccine containing this antigen. For example, Roupe V et al. investigated the efficiency of DNA vaccines encoding RV1733c, RV1738, RV2029c, RV2031c HSPX, RV2032 (acg), RV2626c, RV2627c and RV2628 proteins. This vaccine was injected into BALB/c and B6D2 (F1) mice. The results indicated the development of strong humoral and cell-mediated immune (CMI) responses to TB in all antigens except for RV1738. In addition, the strongest CMI response (the production of IFN-γ, IL-2) was related to RV2031c (HSPX) and RV2626c antigens. These findings suggest that latent phase proteins of *M. tuberculosis* can also be used as antigens to produce the DNA vaccine against TB [82].

Role of HSPX in Immunization

Yuan et al. designed DNA vaccine expressing the fusion protein of Ag85B-ESAT-6-HSPX and studied its performance in mice. In this study, the multi-dose vaccine was injected into BALB/C mice. Two weeks after the last injection, the concentration of IgG, IgG1 and IgG2a antibodies was measured via ELISA. The level of specific anti-HSPX IgG antibodies and the IgG2a/IgG1 ratio were significantly higher than those of antibodies of other proteins. The activation of Th1 cell ($CD4^+$ and $CD8^+$ T-cells) responses suggest that the increased production of IFN-γ and TNF-α is owing to activation of protective cell-mediated immunity [83].

In addition, in another study, recombinant rBCG strains expressing HSPX and HSPX DNA vaccine were injected into mice and guinea pigs through the prime-boost technique. Immunological assessments demonstrated that the levels of IL-12, TGFβ, IL-10 cytokines were elevated and the microbial load in the lung was reduced [84].

It has been proven that the mycobacterial infection induces $CD4^+$ and $CD8^+$ T-cells responses. $CD8^+$ T-cells are activated by peptides presented by Major Histocompatibility Complex-I (MHC-I) on the surface of infected cells [85].

Shi et al. conducted one study on the recombinant vaccine expressing the HSPX protein (rBCG). According to their findings, HSPX protein epitopes in patients with TB are detected by $CD4^+$ and $CD8^+$ T-cells. Furthermore, mice immunized with the DNA vaccine containing HSPX had a strong Th1 immune response induced by this antigen. They suggested that immune responses against HSPX antigen were effective in controlling the *M. tuberculosis* infection [80]. In addition, Shi et al. in another survey detected the anti-HSPX antibodies in 77% of patients with chronic *tuberculosis* [86, 87]. They surveyed the HSPX antigen and its epitopes finding that the HSPX gene deletion reduced the virulence of the pathogenic strain of *M. tuberculosis*. They reported that attenuated strains, including strains reducing HSPX expression, could be used as anti-tuberculosis vaccines. According to their studies, HSPX (16.3 kDa) is a potentially important component ensuring the survival of *M. tuberculosis* in the latent phase of human infection [86].

In another study, Yuan et al. evaluated the immunogenic and protective effects of the fusion protein of Ag85B: ESAT-6: HSPX in mice. They found that the vaccine containing the mentioned recombinant proteins was a strong stimulant of humoral immune responses and acted as a strong T-cell inducer. They also measured IgG levels using ELISA two weeks after the last immunization of mice and observed that antibody level was significantly higher in the vaccine group than in other groups. To evaluate cell-mediated immunity, the frequency of $CD4^+$ and $CD8^+$ T-cell in peripheral blood and γδ T-cells was investigated two weeks after the last immunization. Their findings demonstrated that immunization of mice with fusion protein significantly induced $CD4^+$ and $CD8^+$ T-cells. Furthermore, an assessment of *M. tuberculosis* colony counts in the spleen of different groups of mice revealed that recombinant proteins induced Th1 response and inhibited the growth and proliferation of *M. tuberculosis* compared to BCG [83].

Martinus et al. conducted one study to evaluate the protective effect of Ag85A and HSPX on controlling TB progression. They concluded that Ag85A and HSPX antigens were able to induce IFN-γ which is the main cytokine in the development of immunity against TB. They stated that when the combination of the two above-mentioned antigens in mice was applied, the IFN-γ response was stronger than the sole antigens. They found that the combination of these antigens significantly reduced the bacterial count in the lung and spleen of the mouse. Therefore, Ag85A and HSPX are suitable candidates for vaccine production [88].

Further, Niu et al. developed and evaluated a multi-stage subunit vaccine consisted of Mtb10.4: HSPX. According to their reports, the obtained recombinant protein is able to induce higher levels of immune response. To evaluate the immunogenic effect, they injected each molecule with an emulsified adjuvant to the mice, showing that the IFN-γ and IL-17 production levels are much higher than the levels when BCG is used. To evaluate the humoral immune response, they measured the serum level of IgG antibodies against HSPX by ELISA and the IgG level was much higher than that of the BCG vaccine [89].

HSPX (HSP16.3(and its Role in Vaccine Design

HSPX (or the HSP16.3) is a latency-related antigen for multistage vaccines. Small heat shock proteins (sHSPs) are one of the five families of proteins acting as a molecular chaperone. sHSPs possess a universally conserved alpha-crystallin domain, hence, also known as the alpha-crystallin family. HSPs, play a key role in handling damaged proteins or intracellular protein accumulation and their association with virulence of bacteria, including *M. tuberculosis,* has been studied. Expression of many of them increases under stress conditions in TB disease. The role of HSP in the introduction of antigen is to activate lymphocytes and macrophages [90]. The virulence of *M. tuberculosis* depends on several genes using the macrophage system and its modification for its successful survival. Therefore, the use of HSPs can be important to treat TB [46] and HSPX is one of the most prominent HSPs in this case. HSPX in TB was primarily identified as 14-kDa and 16-kDa antigens and then classified as a molecular chaperone known as alpha-crystalline. These small HSPs prevent the accumulation and denaturation of proteins and wrong folding under stress conditions [90].

Later, Cunningham et al. investigated and reported the major role of this HSP in MTB. They stated that HSPX was able to produce a strong CMI response and delayed-type hypersensitivity (DTH) in mice and guinea pigs [90, 91]. HSPX is produced as the dominant protein in the stationary and latent phases of TB infection and in response to the increased stress [83]. These proteins are one of the most important antigens expressed by granulomas which stimulate the immune responses. HSPX is a heat-sensitive intramembrane protein expressed under the control of the transcription factor SigH. This transcription factor is responsible for some genes produced in response to heat or oxidative shock. Studies have indicated that the HSPX gene is the most important gene expressed in the latent form of the bacteria. Therefore, it is called the hypoxia reporter gene (HRG). There is a serological test using an antibody to detect the latent form of the bacterium against this protein. Accordingly, subunit vaccines appear to be suitable to be used against the latent form of the disease [33].

ESAT-6 and CFP-10 Antigens

The *M. tuberculosis* strain H37Rv genome comprises 4,411,529 base pairs including about 4000 genes and approximately 3900 proteins that are isolated from the bacterial culture supernatant [92]. This genome consists of 36 regions of differences RDs (region of differences) [1, 2] and among these regions, the RD1 locus plays a key role in the virulence of the bacterium. Although, this region of the gene is present in the pathogenic strains of *M. tuberculosis* and *M. bovis*, it has been deleted in the BCG vaccine strain. The RD1 locus is a 25.3-Kbp molecular weight region of the *M. tuberculosis* genome consisting of nine genes (RV3871-RV3879) encoding protective antigens or virulence antigens of the bacterium [14].

One of the proteins encoded by the RD1 region but the early secretory antigenic target-6 (ESAT-6) and CFP-10 are encoded by the RV3875 (ESXA) gene and the RV3874 (ESXB) gene, respectively (Fig. 3). These two genes form a heterodimer complex with a ratio of 1: 1 [93].

On the other hand, a number of studies suggest that the secretory proteins of ESAT-6 and CFP-10 play a crucial role in the pathogenesis of *M. tuberculosis*. Furthermore,

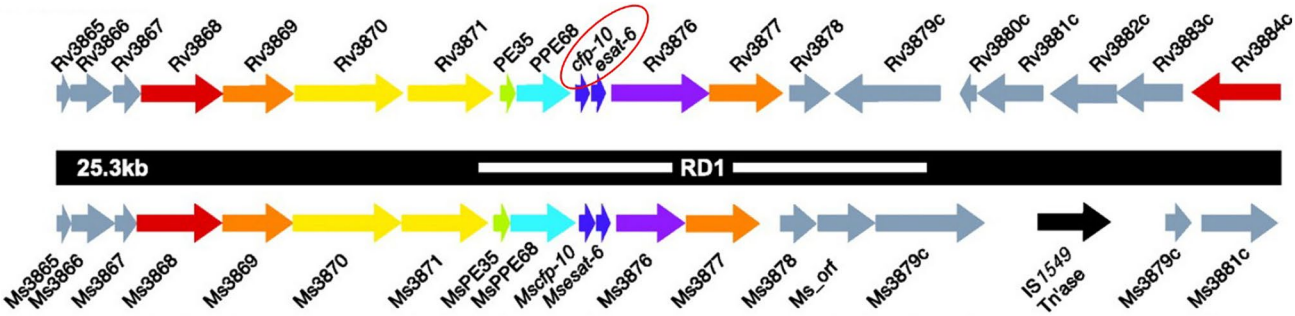

Fig. 3 The location of esat-6 and cfp-10 genes on RD1 locus

the activation of T-cells by these antigens proves their suitability for the vaccine [79].

The CFP-10 activates and aggregates cytotoxic T lymphocytes and extends the granuloma tissue in humans and mice infected with *M. tuberculosis* [83].

The secretory proteins of ESAT-6 and CFP-10 are produced only in the early stages of TB infection. Deletion of genes encoding secretory proteins of the pathogenic strains is proven to reduce bacterial virulence [80]. To date, more than 20 different immunodominant antigens have been identified in *M. tuberculosis*. The use of recombinant protein viruses and viral vector vaccines induces relative protections against TB [81].

Recently, several immunogenic regions of CFP-10 have been identified in humans. Specifically, CFP-10 peptides are capable of stimulating and producing IFN-γ, resulting in the activation of cytotoxic T-cells. The CD^{4+} T-cell plays a crucial role in developing immune responses against TB in humans. Moreover, the use of appropriate antigens from *M. tuberculosis* causing proper protective responses, leads to a better understanding of designing an optimal vaccine against TB [69, 70]. The human immune response against TB depends on several factors of which the Th1 response is of great importance. Moreover, it is essential to stimulate and produce IFN-γ [81].

Several studies indicate that *M. tuberculosis* expresses different proteins during different stages of infection that are specific to each stage. In fact, the immune system of people with active TB may identify and respond to antigens specific for the acute phase of infection and those infected with latent TB can identify and respond to latent antigens of *M. tuberculosis*. For example, ESAT-6 and CFP-10 proteins are accurately detected in individuals with active TB. Accordingly, it is desirable to use multistage latent acute phase antigens in designing a vaccine against TB [5]. Additionally, the results of recent studies suggest that new TB vaccines should contain proliferating bacteria and latency-associated multi-stage antigens [81]. Okkels et al. reported that the secretory proteins of ESAT-6 and CFP-10 were capable of producing cytotoxic T-cells in response to IFN-γ production. They also stated that the main immunogenic antigen was related to the *M. tuberculosis* detected by T-cells in individuals infected with TB. Recent studies have reported that several immunogenic regions of the CFP-10 antigen are identified for humans. For example, CFP-10 peptides are capable of stimulating and producing IFN-γ and activating cytotoxic T-cells [60]. This study did not respond to some questions posed by researchers including, do inflammatory reactions cause damage to the living creature owing to the cytotoxic T-cells stimulated by IFN-γ production? Does the inflammatory effect prevent the sustained effect of the vaccine?

Maue et al. demonstrated that administration of ESAT-6: CFP-10 DNA vaccine induced an effective immune response in calves infected with *M. bovis*. This study showed that administration of the BCG vaccine together with ESAT-6: CFP-10 DNA vaccine induced more severe immune responses compared to BCG alone [94]. The researcher suggested that the presence of ESAT-6: CFP-10 as a booster can be combined with BCG to increase the responsiveness of immune mediators in the body.

The current research team also examined the protective and immunoglobulin effects of a DNA vaccine expressing CFP-10 in mice. The results of this study proved that the CFP-10 protein stimulated cytotoxic T lymphocytes. In addition, this vaccine is able to prevent the proliferation of *M. tuberculosis* in the lung and spleen [94].

Kamath et al. reported that the secretory protein of CFP-10 was able to stimulate T lymphocytes. They also stated that some CFP-10 epitopes were capable of stimulating and producing IFN-γ and activating cytotoxic T-cells [81]. The effects of these antigens on the stimulation of B-lymphocytes need to be addressed in this paper.

Dietrich et al. showed that vaccination with the ESAT-6: Ag85B fusion protein induced highly effective immune responses. This effect has been studied in animal and non-human primate models. This fusion molecule is also effective even as a DNA vaccine. Intranasal administration of the Ag85B: ESAT-6 combination vaccine with LTK63 mucosal adjuvant was also tested. Vaccination with LTK63/Ag85B: ESAT-6 resulted in a strong Th1 response, followed by IFN-γ secretion from TCD^{+4} cells and thus sustained protection against TB infection [95].

Moradi et al. evaluated the protective effect and immunogenic activity of novel recombinant fusion protein from *M. tuberculosis* consisting of ESAT-6 and the short domain of the c-terminus of the HSP70 thermal shock protein after its expression in the mouse model. The results showed that the level of IFN-γ and titer of specific antibodies in the fusion protein was higher than that in ESAT-6 alone. Therefore, this fusion protein (E6H70c) was suggested as a candidate for vaccine preparation [96]. CFP-10 and ESAT-6 proteins are produced only in the early stages of TB infection. It is proven that deletion of the relevant genes in pathogenic strains reduces bacterial virulence.

Conclusion

Most researchers declare that complete eradication of TB is only possible with an effective vaccine, particularly in developing countries with a high incidence of TB and limited financial resources to access the treatment. During the past two decades, the research budget to develop TB vaccine has quadrupled up to over half a billion dollars. Based

Table 2 The immune responses and the protective efficacy of the PPE44, HSPX, ESAT-6 and CFP10

Antigen	Immune responses	Protective efficacy	References
PPE44	Cellular and humoral immune responses, induced Th1 immune response, IFN-γ and TNF-α	Protective efficacy is comparable to BCG	[97]
HSPX	Increased the production of IFN-γ	HSPX subunit vaccine alone provide weaker protection than BCG but in combination with another protein such as Ag85 provide stronger protection than BCG	[84, 98]
ESAT-6	Increase the level of IFN-γ/IL-4 expressing T-cells and IL-2 and CTL upon antigen-specific stimulation	Provide stronger protection than BCG	[99]
CFP-10	Increase the level of IFN-γ, IL-4 and Il-2	In combination with ESAT-6 has the protection efficacy similar to that of BCG	[100]

on the results of various studies, recombinant proteins and DNA vaccines have immunodominant characteristics and are capable of inducing a long-term immunological memory. To evaluate the immune response and protective efficacy of subunit vaccines based on PPE44, HSPX, ESAT-6 and CFP-10 several studies have been carried out. Table 2 demonstrated the results of these studies.

Various studies on recombinant vaccines against TB have demonstrated that PPE44, HSPX, ESAT-6 and CFP-10 antigens can generally play a role in the following items:

1. It has the ability to induce a Th1 lymphocytes-mediated immune response, which is an important criterion to evaluate the efficacy of vaccines against TB.
2. In designing a subunit vaccine against TB, it is important to select an immunodominant antigen that can provide appropriate protective immune responses.
3. It causes a protective immune response in animal cells during conducting various studies, such as vaccination with *Mycobacterium smegmatis* (*M. smegmatis*) and DNA vaccines containing PPE44, HSPX, ESAT-6 and CFP-10 antigens.
4. Studies have indicated that the levels of immune mediators such as INF and IL-12 in mice vaccinated with these antigens are significantly higher than those in other compared groups.
5. Activation of the immune system by the mentioned antigens demonstrates that there is always a Th1 protective response accompanied by a response with a lower level induced by Th2. This low level response is reported in various studies that can prevent immunopathological effects of a potent protective CMI system. Furthermore, it may create balance in the immune system after eliminating the infection.
6. Studies have demonstrated that the use of these antigens along with BCG is highly effective in boosting the immunological memory of BCG, suggesting the use of these recombinant proteins to enhance the efficacy of BCG.
7. Based on recent reports and studies conducted on animal models, it has been found that IFN-α is produced from Th1 cells; as a result, these antigens are the most important and effective protection against *M. tuberculosis*. The produced IFN activates macrophages to kill and eliminate the reproducing bacteria. The production of IL-12 by macrophages and dendritic cells plays a major role in the differentiation of intact T-cells to Th1 lymphocytes. Therefore, increasing the diversity of antigens in subunit vaccines leads to improved efficacy of the vaccine against TB, ensuring that selected antigens will be identified by T-cells of different human populations. Among these, ESAT-6, CFP-10, HSPX and PPE antigens have been employed as the major antigens for subunit vaccines in various studies. Various articles confirm that these antigens have been able to apply their own anti-tuberculosis effectiveness in designing new vaccines. It is hoped that in the near future, these antigens will play a role as subunit vaccines in the different phases of clinical trials to achieve a vaccine with long-term viability. Findings from this review article provide an avenue for future researchers interested in vaccine development against TB

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Declarations

Conflict of interest The authors declared that they have no conflict of interest.

Ethical Approval This is a review articles and it does not contain any with human participants or animals performed by any of the authors.

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