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Vaccine potential of PE/PPE peptide conjugated-ESAT-6 proteins against *Mycobacterium tuberculosis*

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ABSTRACT

Vaccine potential of PE/PPE peptide conjugated-ESAT-6 proteins against *Mycobacterium tuberculosis*

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Mycobacterium tuberculosis (Mtb) is a highly pathogenic bacterium, which has a unique feature of a thick, lipid-rich cell wall. Many mechanisms are associated with this lipid layer, and which is a very important factor in the pathogenicity of Mtb. In addition to the early secreted antigen target 6 (ESAT-6), a representative vaccine candidate and a typical antigen of type VII secretion systems, the Proline-Glutamate and Proline-Proline-Glutamate (PE/PPE) family proteins are virulence factors involved in the formation of the cell wall of Mtb. Although ESAT-6 is a typical T cell antigen, the immune response induced by it against Mtb is limited due to its N-terminal immunodominant epitope. To overcome this disadvantage, I prepared an ESAT-6 fusion protein conjugated with peptides in the PE/PPE family proteins selected using an IFN-γ response assay. PE/PPE peptides were fused to the front part of ESAT-6 and the vaccine efficacy was compared with that of ESAT-6 subunit

vaccine only. Mice immunized with the fused proteins exhibited greater secretion of multifunctional T cell cytokines than those immunized with ESAT-6 only, especially the group immunized with multiple fused peptides (ESAT-6: 0.55-0.92% ± 0.17-0.4, PE/PPE+ESAT-6: 0.55-1.35% ± 0.09-0.4 in lung $CD4^+$ T cells). After challenge with the hyper virulent Mtb Beijing strain HN878, increased secretion of double positive IL-2^+/IFN-γ^+ cytokines, which are known to be associated with the improvement of tuberculosis treatment and latent tuberculosis infection (LTBI), was observed in the group immunized with the fused proteins (ESAT-6: 15.1% ± 1.3, PE/PPE+ESAT-6: 19.87% ± 2.15, in lung $CD4^+$ T cells). Additionally, secretion of triple positive IFN-γ, TNF-α and IL-2 cytokines, suggested to be the main factors that contribute to protection, was maintained after 15 weeks of infection. *Ex vivo* stimulation with the PE/PPE peptide resulted in higher secretion of the triple positive T cells in the group immunized with the fusion proteins only, confirming the immunogenicity of the fusion proteins and the peptide-induced immune response. The bacterial burden for the group immunized with the protein fused with one peptide was slightly lower than that for the ESAT-6 group, while the two-peptide immunization group showed a more significant decrease in the spleen. Therefore, the effect of one peptide fusion can be expected to be greater than that of ESAT-6 alone and the PE/PPE peptide fusion protein is anticipated to be sufficient as a BCG booster since PE/PPE exists in BCG. Understanding the immunological properties of PE/PPE proteins and utilizing them effectively will lead to the development of improved vaccine candidates.

Key words: *Mycobacterium tuberculosis*, Tuberculosis, PE/PPE protein, Multifunctional T cell, Type VII secretion system, ESAT-6.

Chapter I

Vaccine potential of PE/PPE peptide conjugated ESAT-6 proteins against *Mycobacterium tuberculosis*

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I. OVERVIEW

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is one of the top 10 causes of death worldwide. The number of new and relapsed TB cases reported as well as the reporting rate per 100,000 people is increasing globally. One third of the world population is infected with Mtb and new infections occur at a rate of one every second[1,2]. Most Mtb infections remain dormant until the appearance of active TB, and only 5-10% have the potential to develop into full-blown TB during an individual's

lifetime[2]. WHO reported that 10.4 million new TB cases, and an estimated 480,000 people developing multidrug-resistant TB (MDR-TB)[3]. *Bacille* Calmette-Guérin (BCG), the first vaccine developed for TB prevention, gradually loses efficiency and can develop into active TB rather than preventing latent infection[4], therefore, a new treatment strategy is necessary.

1. History of tuberculosis (TB)

TB was believed to be hereditary in the mid-1800s and was demonstrated to be contagious in 1865[5,6]. Mtb, an obligate aerobe and the etiologic agent of TB in humans, is a pathogenic bacterial species in the family *Mycobacteriaceae* and was discovered by Robert Koch in 1882[7]. TB caused many deaths in Europe during the 19th century and was known as the "White Plague"[8]. Nearly 100% of people were infected with Mtb, and one-quarter of deaths during this period were caused by the disease[9]. Europe was the epicenter of many TB epidemics beginning in the 16th and 17th centuries. The disease peaked in Europe in the first half of the 19th century, and it is estimated that one quarter of Europeans died of TB[9]. Although immigrants from Europe brought the disease to the New World, the mortality rate did not reach the same level as in Europe. Metropolitan centers such as Boston and New York had one death due to TB per 1,000 people in 1800, increasing to 4-6 deaths per 1,000 people from 1860 to 1870. Public health measures may also have affected mortality[6]. TB and morbidity have steadily declined in the developed world in the 20th century due to improved public health practices as well as the development of antibiotics in the 1950s and widespread use of the *M. bovis* BCG vaccine[10]. Development of the BCG, which began in 1907, was completed in 1921 and BCG was the first vaccine introduced for TB treatment although its efficacy is variable in adults. Following the initial decline in the number of TB cases, new cases began to increase in the mid-1980s. The main causes were homelessness and poverty in developed countries, the

emergence of AIDS, and the lack of cell mediated-immune responses in people with the disease. Underdeveloped countries are overwhelmingly affected by TB, with an incidence ranging from less than 10 per 100,000 in North America to 300 per 300,000 and 100,000 in Asia and Western Russia, respectively. TB deaths occur every 15 seconds (more than 2 million per year) and 8 million people suffer from TB. Without treatment, 60% of people with the disease will die[11].

For centuries, doctors and scientists have attempted to describe TB in different ways and to understand the origin of the disease with the goal of better diagnosis, prevention, and treatment. Hippocrates thought that the disease was largely inherited. Aristotle (4th century BC), on the other hand, emphasized the contagious aspect. Uncertainty regarding the source of TB reappeared in the second half of the 17th century despite Galen's insistence that TB was contagious[12]. In 1865, Jean-Antoine Villemin reported that TB could be administered to laboratory rabbits by inoculating TB tissue from a dead body. However, Herman Pidoux strongly maintained that there had to be more "modern" and more social solutions to TB. A report by Robert Koch 17 years later conclusively showed that TB was indeed caused by a bacterium[13]. However, the belief in a social cause for TB continued into the French revolutionary syndicalism movement, and lasted until the early 20th century. Beginning with the work of Edward Trudeau in the late 19th and early 20th centuries, the cause of TB was eventually unraveled. Trudeau showed that TB could be induced in rabbits through a bacterium culture of tubercle bacilli, but the environmental conditions in which the animals were maintained greatly affected the course of the disease[9,14]. In the study, five TB-infected rabbits were kept in a crowded dark cage with minimal food. Four of them died of TB within three months, and the remaining rabbit was seriously ill with the disease. When five similarly infected animals were fed outdoors, one rabbit died within a month, while the remaining four survived six months later with no signs of the disease. The control group, five uninfected rabbits kept in darkness, experienced food shortages and malnutrition, but TB was definitely not observed. This simple

experiment gave scientific validity to the treatment of TB (fresh air and sufficient food), which was the basis of the movement for the control of TB initiated by European doctors in the mid-1800s[9]. The approach was also used by Trudeau at the Saranac Lake Tuberculosis Center[15]. TB is therefore caused by bacteria, but environmental factors play an important role, and pure medical technology alone is not effective in treating and preventing the disease[9]. The antibiotic era began with the introduction of TB treatment as well as treatment for many other diseases following the discovery of streptomycin by Schatz and Waksman in the 1940s[16]. However, antibiotics such as isoniazid, rifampin, and pyrazinamide, which are useful for the treatment of TB, have not eliminated the disease[17]. Similarly, widespread use of BCG, an attenuated vaccine from the *M. bovis* strain developed by Calmette and Guerin in Paris in the 1920s, has not lowered the incidence of TB in recent years[18], and TB is currently more prevalent than ever[19]. In the present study, I attempted to provide a solution by designing an improved vaccine.

2. *Mycobacterium tuberculosis* and genetic feature

The main cause of TB is Mtb, an obligate pathogenic bacterial species in the family *Mycobacteriaceae* with a thick lipid layer[7]. This aerobic bacillus is a long, slender, straight, or curved rod and is about 3.0 × 0.3 μm in size, with unusually waxy walls. The bacterium grows slowly and infection is difficult to treat. It divides every 16 to 20 hours, which is very slow compared to other bacteria that usually divide within an hour[20]. Mtb has a unique and waxy coating on its cell surface due to the presence of mycolic acid[21]. The high lipid content of this pathogen contributes to a large part of its unique clinical characteristics. The thick lipid layer on the cell surface is a very important factor in the pathogenicity of Mtb. The cell wall contains many factors including those for lipid metabolism[21], which is an important energy source, and as well as the pathogenesis of Mtb. In addition, proteins that contribute to the formation

of the complex lipid cell wall are also important virulence factors[21,22]. Therefore, the lipid layer of cell wall is a hallmark of Mtb. The coating prevents the cells from being affected by gram staining and consequently, Mtb may be gram-negative or gram-positive[23]. Acid stains such as Ziehl-Neelsen or fluorescent stains, such as auramine, are used instead to identify TB via microscopy. Mtb is highly aerobic and requires a high level of oxygen. It is primarily a pathogen of the mammalian respiratory tract that infects the lungs. The most common diagnostic methods for TB are tuberculin skin tests, culture, acid-staining, and polymerase chain reaction[21,24]. Since its isolation in 1905, the H37Rv strain of Mtb has been used in a wide range of biomedical studies because it remains fully virulent in TB animal models. It is susceptible to drugs and is also feasible for genetic engineering[21,25].

The H37Rv strain of Mtb contains 3,959 genes encoded by 4,411,529 base pairs; 40% of the genes have been characterized by function, and another 44% of the genes have putative functions[21]. There are also six pseudogenes in the genome. The proportion of G + C is 65%, which is the second largest after *E. coli* (Figure 1). Additionally, 3,924 open reading frames were identified in the genome, which accounts for 91% of the potential coding capacity. Over 200 genes are involved in encoding enzymes for the metabolism of fatty acids, comprising 6% of the total genes. Thirty-nine of these genes are involved in polyketide metabolism to produce the waxy coating[26]. About 100 genes are speculated encode enzymes that are active in the oxidation of fatty acids. In contrast, *E. coli* has only 50 enzymes involved in fatty acid metabolism[21]. This large number of conserved genes shows the evolutionary importance of the wax coating for pathogenic survival. The coat is related to the ability of this pathogen to grow in the tissues of the infected host, where fatty acids can be a major carbon source. Experimental studies have demonstrated the importance of lipid metabolism to Mtb comprising host-derived lipids such as TB and cholesterol[27].

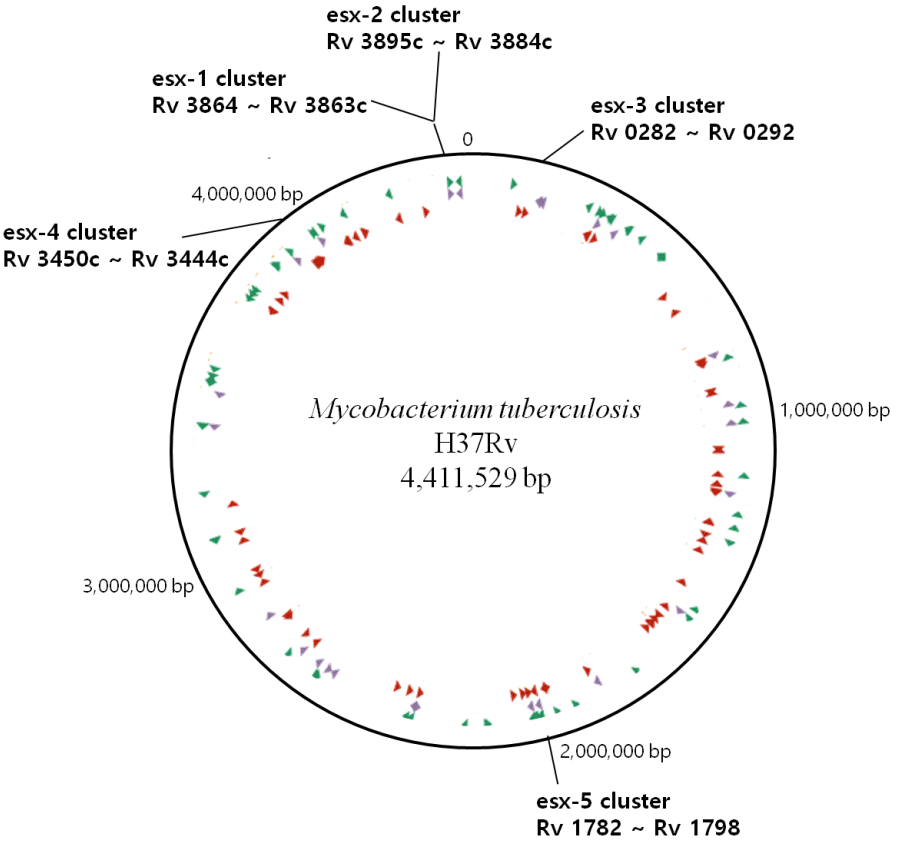

Figure 1. The genome of the *Mycobacterium tuberculosis*. Its size is 4 million base pairs, with about 4,000 genes. The PE/PPE family protein accounts for approximately 10% of the total coding capacity of the Mtb gene and encodes an acidic glycine-rich protein. In circular map, the green color shows the positions of the PPE family members, the purple shows the PE family members and the red shows the positions of the PGRS sequences.

* Modified from Nature 393, 11 June 1998[21]

Mtb can use lipids as the sole carbon source and genes involved in the cholesterol pathway have been demonstrated to be important at different stages of the infection cycle, especially when there are no other nutrient available during the chronic stage of infection[28]. The thick lipid layer of Mtb plays a role in withstanding acidity, preventing the penetration of drugs, and increasing drug resistance[21] . The cell wall contains many proteins and lipid components, among which mycolic acid is the most important constituent and plays a very important role in its pathogenesis[21,29]. Mtb forms a long chain structure by forming branches of β-hydroxyl fatty acids. When the synthesis of mycolic acid is inhibited or the chain structure is not properly formed, the pathogenicity is reduced[30]. In addition, the outer membrane of Mtb contains many diverse and unique lipids and glycolipids that are non-covalently linked, resulting in an extremely hydrophobic cell wall structure[29]. Thus, the cell wall structure of Mtb enables its survival in a host and is simultaneously responsible for the pathogenesis of the disease. Many compounds in the cell wall have potent biological activities and functions. The components in the cell wall structure, including mycolic acid, peptidoglycan, arabinogalactan, lipoarabinomannan (LAM), lipomannan, phthiocerol dimycocerosate, dimycolyl trehalose, sulfolipids and phosphatidylinositol mannosides are associated with the outer membrane, granulomatous inflammation and/or granulomatous inflammation inhibition of the lymphocyte response[29,31].

In addition to lipids, PE/PPE family proteins, which account for a large portion of the Mtb genome in relation to the unique structure of the cell wall, are important virulence factors. These proteins comprise 10% of Mtb and have a unique structure in which the protein sequences are repeated. The proteins have high G + C ratios, and play a role in providing immunological specificity and various types of antigenicity[32]. They increase the pathogenicity of Mtb by forming various antigens, and differences in the sequence of these proteins further increase the diversity of the antigen. Understanding the characteristics of Mtb that survives in the host as a pathogen can greatly improve the understanding of the pathogen mechanism and virulence factors

of TB. Furthermore, developing a new treatment technique that targets proteins can aid in controling TB efficiently.

3. Type VII secretion system (T7SS)

As mentioned above, the thick lipid structure of the cell wall is a very important factor in the pathogenicity and survival of Mtb. There are several mechanisms associated with the formation of cell wall. In addition to the lipid metabolism of Mtb, protein secretion is a very important mechanism for adaptation to the environment and a functional means to survive[33]. These secretions are essential for interacting with the host by expressing virulent proteins, and allow different bacterial species to cause pathology. Thus, exported proteins are very important to pathogenesis and most virulent factors are transported from the cytoplasm to the bacterial surface or secreted into the environment[34]. The bacterial proteins essential for pathogenesis include cell wall-associated proteins (including lipoprotein and secretion systems), inhibiting proteins for antimicrobial effectors, proteases, and proteins of unknown function (including PE/PPE protein)[33,35]. The T7SS, a specialized secretion system, produces pathogenic bacterial proteins across the complex cell envelope[22]. Mtb has five chromosomal *esx* clusters that code for specialized T7SS proteins, from *esx*-1 to *esx*-5 (Figure 2)[36,37]. This cluster is conserved among various pathogenic mycobacteria[38]. It is also known as the ESX secretion system for mycobacteria and forms a major secretion pathway for effector proteins[39]. The best characterized of these systems is ESX-1, which is responsible for the secretion of ESAT-6 (early secreted antigen target, 6 kDa) and CFP-10 (culture filtrate protein, 10 kDa), and is required for full virulence of Mtb[24]. ESX-1 has been studied extensively because it has important effects on the virulence of pathogenic mycobacteria.

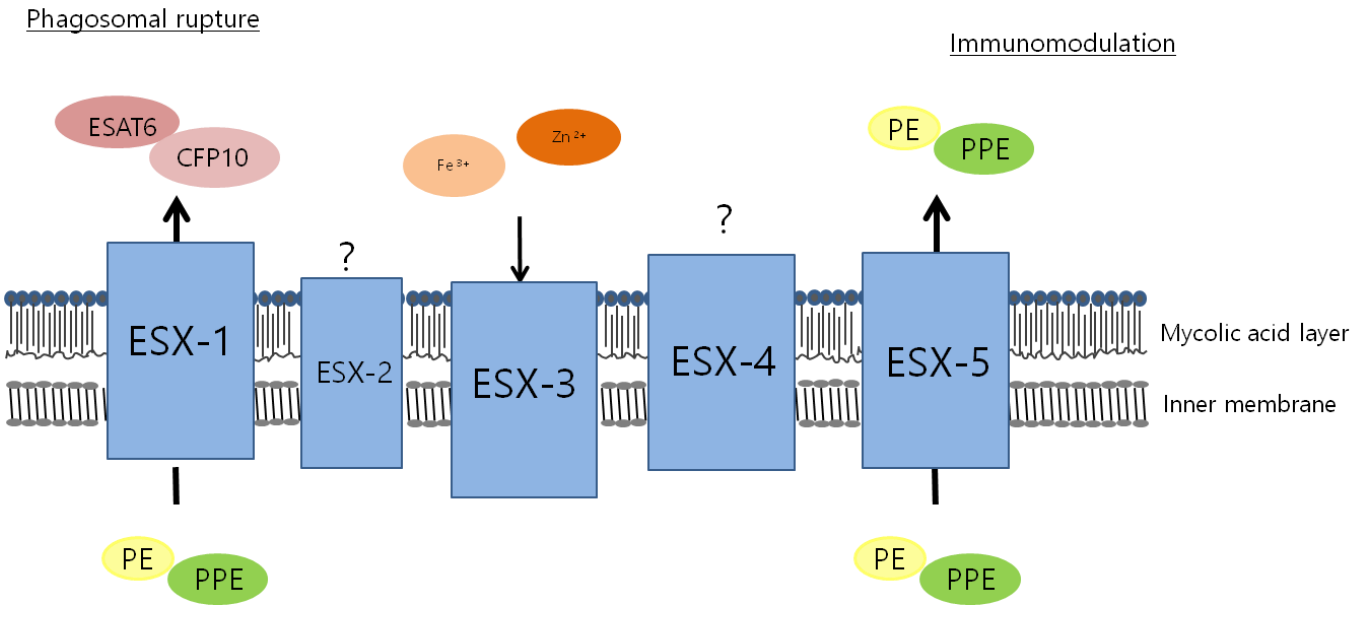

Figure 2. Protein secretion systems (Type VII secretion system-T7SS). Mtb uses sophisticated secretion systems, type VII secretion systems, to export a set of effector proteins that helps the pathogen to resist or evade the host immune response. Five different secretion systems have been identified in Mtb, from ESX-1 to ESX-5. ESX-1 and ESX-5 secrete different proteins involved in the virulence of Mtb: ESX-1 is need for Mtb's translocation from the phagosome into the cytosol inside host cells. ESX-5 exists only in slowly-growing mycobacteria and it is considered to be responsible for the secretion of PE/PPE proteins with immunomodulatory properties. However, except for ESX-4, all are known to secrete one or more PE/PPE proteins. ESX-3 may be associated with the acquisition of several metals such as iron and zinc by Mtb. The role of ESX-2 and ESX-4 remain still unknown.

* Modified from Mediterranean journal of hematology and infectious diseases 2013[20].

It also secretes EspA and other related proteins in addition to ESAT-6 and CFP-10[28]. The ESX-1 locus contains the region of difference 1 (RD1) deleted in the BCG strain. ESAT-6 and CFP-10 are secreted by ESX-1, which regulates TLR2 signaling and cytokine suppression[32]. ESAT-6 is a strong inducer of IFN-γ and is used in conjunction with CFP-10 to diagnose TB such as with Interferon Gamma Release Assays (IGRA). An active ESX-1 system that secretes ESAT-6 and CFP-10 is required for the escape of pathogenic mycobacteria from the phagocytic vacuole to the cytosol, and for intercellular diffusion of the bacilli[20]. Therefore, ESAT-6 is the most representative virulence factor and is a typical T-cell antigen involved in genetic deletions of BCG. The deleted region included RD1, which is crucial for the attenuated virulence of BCG[38]. RD1 is about 9.8 kb[37,38] and contains 9 genes including genes that encode secreted proteins ESAT-6 and CFP-10, both of which are important T cell antigens and are essential for the virulence of Mtb. These ESX systems are found in mycobacteria and a variety of other genus and gram-positive bacteria[22]. The system is encoded by RD1 and is an example of T7SS.

Of the five *esx* clusters in Mtb, at least three, ESX-1, 3, and 5, are associated with full virulence. ESX-1 encodes a small secreted protein consisting of about 100 amino acids with conserved Trp-X-Gly (WXG) motifs that contribute to the formation of a helix-turn-helix structure[40]. The most well- known protein that contains the WXG motif is EsxA (ESAT-6) in Mtb as well as EsxB (CFP-10), its adjacently decoded heterodimerization partner[22]. In addition to being essential for resistance and evasion of host reactions, one of the key functions of ESX-1 is its role in inducing phagosomal rupture, causing the release of bacteria and/or bacterial products into the cytoplasmic compartments of host cells[20]. This secretion system is used to move the bacteria and/or bacterial products from the phagosome to the cytoplasm of infected macrophages to create a protective environment for the bacteria. ESAT-6 (EsxA) and CFP-10 (EsxB) are small highly immunogenic proteins that are secreted here and are the basis for the immunologic diagnosis of TB infection. In addition to ESX-1 (RD1),

ESX-3 is known to be a secretory system involved in the acquisition of mycobactin-mediated iron[22,41]. Finally, ESX-5 plays a role in the secretory system for two protein families, PE and PPE proteins, which are present in pathogenic mycobacteria. Similar to other ESX clusters, the ESX-5 locus consists of a pair of *esx* genes encoding EsxM and EsxN proteins (also immunodominant antigens) that induce strong $CD4^+$ T cell responses[42,43], and harbors the *ppe*25-*pe*19 gene cluster coding for PE/PPE proteins[43]. However, the role and function of ESX-2 and ESX-4 have not yet been deeply studies. The ESX-5 system has evolved recently, and only exists in pathogenic mycobacterial species that grow slowly[37], such as Mtb, *M. marinum*, and *M. ulcerans*. This may be a representative secretory system specifically differentiated to interact with complex immune systems such as mammals. The PE/PPE proteins secreted in this region are known to play an immunomodulatory role and are involved in cell-to-cell transmission[37]. However, all systems except ESX-4 are known to secrete one or more PE/PPE proteins[37]. In addition, ESX-5 has been reported to be genetically related to ESX-1. The genetic locus of ESX-1/RD1 is associated with pathogenicity has been shown to promote mycobacterial infection through expansion and the spread of pathogenicity[44]. The *esx* region is the region encoding the Type VII or ESX secretion system, thus, when the ESX system is first replicated, the *pe/ppe* genes are believed to be inserted and replicated together and expanded until these genes are amplified[38]. Thus, some PE/PPE proteins are structurally similar to ESAT-6. For example, some of the functions of the PE/PPE proteins have been revealed based on the three-dimensional structure or homology of PE/PPE. Complexes of PE25 and PPE41 proteins have been determined to be very similar to CFP-10 and ESAT-6 complexes[38]. Therefore, two of the T7SS systems, ESX-1 and ESX-5, are known to be most related to virulence factors, and to have evolutionary and functional associations with each other. The secreted proteins from these regions are very important factors that can cause pathogenicity. Consequently, ESX-1 and ESX-5 play important roles in the secretion of PE/PPE proteins and in the virulence of other pathogenic bacteria.

4. The major proteins secreted by secretion systems of *Mycobacterium tuberculosis*

The structure of the mycobacterial cell wall is very complex, and protein secretion plays a very important role in synthesizing the various cell surfaces and in adaptation for survival[9,33]. Some of the representative antigens associated with secretion systems are described below.

4.1 ESAT-6/CFP-10

A number of ideas have been proposed and developed as candidates for TB control. The proteins secreted by Mtb are very important due to mechanisms that disrupt the host immune network system. One of these proteins is ESAT-6. The low molecular weight secretory antigen ESAT-6 is known as an early and predominant T cell target during TB infection in experimental animals[45]. The secreted Mtb protein ESAT-6 has been extensively studied in pathogenicity and vaccine studies[46]. ESAT-6 is useful for cost-effective diagnosis and for protection as the strongest vaccine candidate. ESAT-6 and its chaperon CFP-10 are members of the ESAT-6 family and are small, secreted proteins found in the Mtb culture filtrate[47]. Both proteins are immunodominant antigens capable of detecting a majority of TB patients. In addition, these proteins are located in the deletion region for RD1, the first deletion found when comparing the genomes of wild-type (WT) *M. bovis* strains and those of BCG. This region is present in all pathogenic Mtb and *M. bovis* strains, but is lacking in all *M. bovis* BCG strains[48]. Evidence that this region is involved in virulence was found in several studies in which the RD1 region or the ESAT-6 / CFP-10 gene was mutated in the Mtb genome[49]. In those studies, RD1-deleted strains were remarkably attenuated in both immunocompetent and immunocompromised animals compared to the parent WT strain[50,51]. ESAT-6 is a potent T cell antigen as well as a typical putative virulence factor along with CFP-10[52,53]. Therefore, it has been a strong focus on TB vaccines and is used in the IGRA QuantiFERON-TB Gold kit in conjunction with CFP-10 and

TB 7.7[53]. Mtb secretes these proteins as an ESAT-6/CFP-10, a heterodimer complex stabilized by hydrophobic interaction. The complex forms a rigid 1:1 crossing, so the two proteins adopt a more stable, folded configuration[54,55]. The complex secreted from Mtb provides protection from apoptosis in macrophages[56]. This system, which is involved in the synthesis and release of these ESAT-6 and CFP-10 proteins, appears to be specific to Mtb and is called the ESX type VII secretion system. ESAT-6 has been reported to mediate mycobacterial virulence and TB, and targets host cell signaling pathways in a number of ways. More detailed investigations on the role of ESAT-6 in controlling immunity against TB have been conducted amid increased interest in the antigen with potential vaccine candidates and certain diagnostic reagents. However, the biological functions associated with ESAT-6 remain controversial. ESAT-6 can normally protect Mtb by limiting macrophage activation and host immune responses leading to antibacterial activity. Another way to suppress the immune response is through ESAT-6, which suppresses signals from Toll-like receptor 2 (TLR2)[57]. TLR2 is known to be stimulated by Mtb to produce an immunostimulant as a pattern recognition receptor on the surface of host cells[57]. Another possibility is that ESAT-6 is important for solubilizing host cell membranes to promote bacterial secretion into neighboring cells[58]. However, apoptosis is probably the greatest feature of ESAT-6. In other studies, the pore-forming activity of ESAT-6 induces apoptosis[46,58]. Previous studies have shown that ESAT-6 protein is a potent immunomodulator that can induce cell lysis by destroying membrane bilayers[59]. Thus, there is evidence suggesting that ESAT-6 may induce apoptosis through cell membrane pore formation[46]. Caspase-8 is a leading initiator caspase-associated with receptor-induced apoptosis[60]. Activation of caspase-8 suggests that ESAT-6 treatment may induce apoptosis through receptor-mediated extrinsic pathways. Caspase-3 and -7 are effector molecules that are stimulated by active caspase-8 in the exogenous process[46]. Importantly, ESAT-6 mediated apoptosis does not consistently induce the expression of caspase-9 which is involved in the mitochondrial pathway of apoptosis[46]. This suggests that ESAT-6 mediates apoptosis through an extrinsic pathway rather than an endogenous

mitochondrial apoptotic pathway. This apoptosis does not occur through TLR signaling. However, the purified ESAT-6 forms a structure similar to the amyloid-producing protein in solution and some of the proteins exhibit pore-forming properties similar to bacterial toxins[46,59]. Nonetheless, ESAT-6 is related to membrane rupture. However, this phenomenon can be observed with ESAT-6 alone or in a complex with CFP-10, but not with CFP-10 alone[58]. Thus, ESAT-6 is associated with cell membrane lysis. There have been studies demonstrating that the TB strain, which includes a complete deletion of the RD1 genomic region, is not cytolytic and reduces tissue invasiveness and cell-to-cell spread[59]. In a similar situation, the RD1 mutant of *M. marinum* lost cytolytic activity and lacked cell spreading ability. Cell lysis was restored when the RD1 mutant was supplemented with Mtb RD1[61]. Therefore, RD1 is required for cytolysis and bacterial spreading, including ESAT-6 secretion, and Mtb RD1 mutation does not induce high levels of apoptosis in THP-1 macrophages[46]. Membrane lysis is also a potential method for ESAT-6 to destroy the phagosomal membrane to release bacteria or bacterial products into the cytoplasm. In the short term, ESAT-6-mediated apoptosis may contribute to disease progression by isolating mycobacteria from the internal host; however, in the long term, induction of apoptosis by the Mtb component may be beneficial to the host[46]. Apoptotic events, including those induced by ESAT-6, may actually increase the adaptive anti-tuberculous immune response. Furthermore, a better understanding of the role of apoptosis in generating a highly effective anti-TB prophylactic immunity should provide insight into the studying tuberculosis vaccines. Therefore, ESAT-6 is an important virulence factor.

4.2 The operon *espACD*

EspA is a protein secreted by ESX-1 and is located outside the RD1 region in BCG[33]. EspC and EspD show significant homology to Rv3865 and Rv3867, respectively[62]. EspC is also secreted by ESX-1, but unlike EspA and ESXA, EspD does not exclusively require ESX-1 systems. Evidence of EspA and EspC

stabilization by EspD has been reported as well as the lack of EspD following the loss of ESXA secretion[33]. EspA and EspC secretion requires EspD, but EspD secretion does not require EspA, EspC or ESX-1[63]. EspC is a potent antigen in both active and latent TB infections, and the T cell response to EspC is highly specific (93%) for Mtb infection[64]. Because the immunodominance of EspC is equivalent to that of ESAT-6 and CFP10, and EspC is highly antigen specific, this protein is a promising TB vaccine candidate and potential T cell antigen[33].

4.3. PE/PPE proteins

The sequenced genome of Mtb revealed that PE/PPE family proteins can be associated with pathogenicity in addition to ESAT-6[21]. PE/PPE proteins are present in both pathogenic bacteria and their by-products, and play a more central role in mycobacterial metabolism than previously thought[65,66]. Non-pathogenic mycobacteria tend to have fewer *pe/ppe* genes with normal proliferation within sublineage V of the gene family of pathogenic species[66]. Pathogenic mycobacterial species other than the *M. tuberculosis* complex, namely *Mycobacterium leprae*, *Mycobacterium marinum*, *Mycobacterium ulcerans* and *Mycobacterium avium*, possess the highest number of *pe/ppe* genes[67]. PE/PPE proteins comprise about 10% of the total coding capacity of the Mtb genome and consist of a family of two genes encoding an acidic glycine-rich protein[32]. They have repeated sequences of PE and PPE as motifs conserved in the N-terminal region[67]. Although the function and characteristics of PE/PPE proteins are not yet clear, studies have shown that these antigens are important components of the pathogenicity of Mtb as constitute the cell walls or secrete proteins[68]. Thus, understanding how, where, and why PE/PPE proteins are trafficked in mycobacterial cells can provide important clues about their function. More than 35 PE/PPE proteins were also detected on the mycobacterial membranes and/or cell wall[66]. Culture filtrate, cell wall or membrane-associated PE/PPE family proteins have been identified in all PE/PPE sublineages, suggesting that although the PE/PPE function can vary, the function is often dependent on the cell wall or extracellular localization[66]. Notably,

several PE polymorphic GC-rich sequence proteins (PE_PGRS), a subgroup of PE proteins, localize to the extracellular surface of Mtb and has important effects on the virulence of pathogenic mycobacteria, are able to actively traffic out of the phagolysosome as similar to other cell wall components[69]. These proteins contain a highly conserved domain in the N-terminal region, followed by a C-terminal consisting of variable sequences with different size and repeat structure[21,29] (Figure 3). In Mtb, the PE and PPE family is composed of about 100 and 70 proteins, which can be divided into two groups according to their C-terminals[70]. The 29 members of the first PE group contain only the PE domain and the eight members of the second group have the PE domain and unique sequences that lack significant homology with other PE members[71]. The third and the largest group, consisting of 67 proteins, contains a multiple tandem repeat region termed the PGRS domain located after the PE domain[71]. These proteins have domains with constant amino acids conserved in the N-terminal region, and the C-terminus consists of sequences of very different sizes and repeats. Proteins can be categorized into subgroups with very diverse length and sequence characteristics. The PE_PGRS proteins contains multiple tandem repeats of Gly-Gly-Ala, and the PPE_MPTR (major polymorphic tandem repeat), a subgroup of PPE proteins, possesses polypeptides that are rich in repeats with the signature Asn-X-Gly-X-Gly-Asn-X-Gly[70]. Another member of the PPE family includes a motif called 'SVP' near the 350th amino acid residue[70]. They all encode proteins with high G + C ratios and are particularly abundant in *Mycobacterium* species. In addition, approximately 200 *pe/ppe* genes are found in both Mtb and *M. bovis*[21,72].

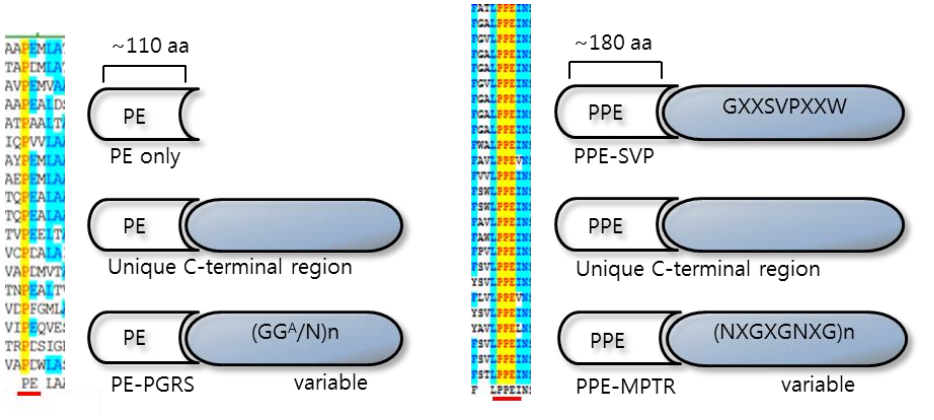

Figure 3. Structure and distinct feautres of PE/PPE family proteins of Mtb. PE and PPE proteins contain conserved N-terminal domains of about 110 aa and 180 aa, respectively. One subgroup of the PPE family incorporates a characteristic "SVP" motif at approximately 350 aa. Both PE and PPE familiesPE/PPE family proteins can be more divided into unique subgroups based on their variable C-terminal regions. The domains encoded by the Polymorphic GC Rich Sequence, called PGRS of the PE family and the Major Polymorphic Tandem Repeats, called MPTR of the PPE family are the represetatives resposible for antigenic polyporphism.

* Modified from Clin Dev Immunol 2011; **2011**: 497203[70]

The proteins produced here have immunologically relevant properties and are known to provide a variety of antigens due to various changes in the C-terminal sequence[29,44,73]. These PE/PPE proteins are secreted by ESX-1 and ESX-5, especially the ESX-5 system, and modulate the innate immune response[32]. The proteins are also associated with the ESAT-6 gene clusters, and the expansion of these proteins is linked to evolution of ESAT-6 gene clusters, which implies a co-evolution of PE/PPE proteins with the ESX system. Therefore, the proteins are functionally linked to the ESX secretion system[32,70]. The proteins have a unique structure in which sequences that have homology to each other are repeated. These repetitions either exist specifically in ESX-5 or are shared with PE/PPE homologs encoded outside of ESX-5[37]. Consequently, there exist various studies investigating the attenuation of vaccine efficacy with the deletion of this region[4]. A similar but large number of proteins suggests that the PE/PPE family can promote Mtb antigen diversity and antigen mutations are used by various microbial pathogens to evade the immune system[74,75]. Therefore, the PE/PPE protein family members have the ability to regulate the adaptive immune response for their survival. In addition, PE and PPE domains play an important role in protein localization and secretion. The PE and PPE domains have been demonstrated to contain signals necessary for the secretion of proteins in an ESX-5-dependent fashion and are secreted to the bacterial surface via the PE protein LipY[36]. Immunologically predominant PE/PPE proteins are more likely to dominate depending on how many shared epitopes are present among the family members, and this likelihood is higher when the peptide is a conserved sequence in the N-terminal than in other regions[73]. In addition, PE/PPE proteins have many potentially B cell antigenic determinants, and most of the proteins are exposed on the cell surface, so they are expected to be well-suited for humoral responses[4]. Recent studies have shown that many PE_PGRS proteins can induce immune responses *in vitro* and *in vivo*[69]. In the serum derived from patients with TB, the humoral response was found to be induced at the C-terminal of the PE_PGRS protein Wag22 and the PGRS domain of PE_PGRS51[76]. Many PE/PPE proteins have been shown to cause B-cell

responses, and it is believed that frequently repeated domains in the structure, such as in the PE-PGRS and PPE-MPTR families, are the main reason for the antibody response[77]. Peptide screening based on $CD4^{+}$ T cell responses, which is essential for effective defense immunity and inhibition of Mtb infection, revealed that approximately 45% of identified antigens were PE/PPE family proteins[44]. The PE/PPE gene is arranged in a unique pattern with the PE gene, which is always located upstream of the PPE gene and spread throughout the genome[32]. *pe*/*ppe* genes have not yet been identified in closely related bacteria such as *Nocardia farcinica* or non-mycobacterial species. Surprisingly, the PPE gene is widely present in pathogenic mycobacteria, such as *Mycobacterium avium* and *Mycobacterium marinum*. Non-pathogenic species such as *Mycobacterium smegmatis* are significantly lacking in these genes[38]. This clearly indicates that there is a strong evolutionary choice for PPE proteins in pathogenic mycobacteria. Interestingly, unlike in eukaryotes, the functions of these gene family members in bacterial genomes often need to be analyzed because large amounts of unencrypted DNA exist and the presence of the PE/PPE gene cluster is not often observed in the functional properties. Furthermore, in expression studies, different regulation of PE and PPE genes under various conditions also probably implies diverse functions. Therefore, PE/PPE proteins are very important virulence factors in relation to pathogenicity and their immunomodulatory roles.

5. Clinical application of virulent proteins

BCG is the only vaccine currently approved for tuberculosis; however, TB protection for adolescents and adults is very variable and weak[78]. It is also important to recognize that Mtb strains resistant to standard anti-TB drugs, including drug resistance (MDR) and extremely drug resistance (XDR) strains, are becoming increasingly common[79]. Most of the available and successful vaccines available today induce differentiation antibodies that provide defense immunity. However, animal

studies and several human studies suggest that a robust cellular immune response is required to protect against Mtb infection and disease[80,81]. For this reason, most current clinical TB vaccine candidates are based on various vectors, adjuvants, and antigens that induce classic Th1 cytokines such as IFN-γ or TNF-α derived from $CD4^{+}$ or $CD8^{+}$ T cells[7,81,82]. These clinical candidates are based on various vaccine approaches such as inactivated whole cell extracts, viral-vectored candidates, and fusion protein subunits with Th1-inducing adjuvants, live recombinant BCG or attenuated TB vaccine[81].

Of these, H1/H56: IC31, H4: IC31, ID93 + GLA-SE and M72 + AS01E are protein-based subunit vaccines. Dr. Peter Andersen (Statens Serum Institute, Denmark) discussed on the grounds that it supports the vaccines: 1) an excellent safety profile, 2) molecules that are well-defined, 3) the reaction is not complicated by the vector-targeted immune response so there is possibility of amplification, and 4) delivery devices that allow for slow antigen release[83]. Dr. Andersen emphasized that, unlike BCG, several vaccines in preclinical and clinical studies induce IL-2^{+} / TNF^{+} double positive $CD4^{+}$ T cells, which represent a central memory phenotype[83]. The CAF01 (cationic adjuvant)-H56 vaccine was shown to promote early protection from low-dose Mtb challenge with efficient control of bacterial growth in minimal lung inflammation, pathology, and local lymph nodes[82]. The adjuvant systems CAF01 and IC31, which are used in clinical H56 vaccines, demonstrate the ability to prevent ESAT-6-specific IFN-γ responses after infection and prevent IGRA conversion in humans[83]. In addition, the phase 1 and 2a tests are well tolerated and produce an immune profile with IL-2 and TNF-α soluble positive central memory T cells[84]. The importance of PE/PPE proteins has been recognized and applied in clinical trials. M72 + AS01E is a vaccine in which a PPE protein (PPE18, Rv1196) is fused. In a survey on QuantiFERON (QFT) + M72 / AS01E, about 50% of participants reported no serious side effects, indicating that this vaccine was found to be safe[83]. ID93+GLA-SE consists of Rv1813 (latency associated protein), Rv2608 (PPE42), Rv3619 (EsxV),

and Rv3620 (EsxW), which belong to the PE/PPE, ESX, and latency protein categories[85,86]. These vaccines were designed by combining antigens low in immunogenicity that showed protective efficacy with or without a BCG priming when formulated with GLS-SE[85]. Other vaccines include H56:IC31, a fusion recombinant subunit vaccine using ESAT-6, Antigen85, and latent-associated proteins Rv2620. The IC31 used as an adjuvant is a two-component adjuvant of an anti-microbial peptide (KLK) and oligodeoxynucleotide (ODN1a), a Toll-like receptor 9 (TLR9) agonist[84]. H56:IC31 was designed as a post-exposure vaccine for individuals infected with Mtb. According to preclinical study results, IC31 differentiates naive T cells into Th1 cells, which are thought to be important for immunity to Mtb[84,87]. The increased Rv2660c transcript in Mtb under nutritional stress suggests that Rv2660c affects intracellular survival[88]. Therefore, inclusion of Rv2660c in the fusion protein enhances vaccine-induced protection against Mtb. The H1 subunit fusion protein of ESAT-6 and Ag85B and the H4 subunit fusion protein of TB10.4 and Ag85B are used in IC31 as adjuvants and have been proven to be more effective at low doses, confirming their safety[82]. These fusion proteins were also reported to be more effective and persistent as boosters because Ag85B is also present in BCG. All of these vaccines were designed using fusion to increase the vaccine efficacy. The immune responses induced by the proteins are dominated by IFN-γ / TNF-α / IL-2 triple positive cells and IL-2 / TNF-α double positive cells. They all take advantage of the subunit vaccine and use ESAT-6, which is the most representative and important antigen, as well as another important virulence factor and PE/PPE protein. Current candidates exhibit somewhat limited immunological diversity, such as induction of traditional $CD4^+$ or $CD8^+$ T cells and induction of a restricted target antigen repertoire. The discovery effort for vaccine candidates focuses on creating candidate substances that lead to more immune mechanisms.

6. The immunological background of ESAT-6 and its applications in vaccine development.

ESAT-6 is a very important T cell antigen. Despite having a large number of epitopes that T cells can recognize, the immune response induced by ESAT-6 may be somewhat limited as only the immunologically most dominant epitope is recognized[89]. Vaccine candidates currently in clinical trials based on ESAT-6 do not prevent secondary infections of Mtb, and this should be noted. ESAT-6 has been shown to have a subdominant epitope that is not recognized in H-$2^{d/b}$ mice during Mtb infection[89,90]. Concentration of the immune responses of a few epitopes is associated with poor prognosis, while a broad T cell repertoire is associated with disease control[91,92]. One way to broaden the repertoire is to initiate an immune response to a secondary epitope that is generally "silent" and does not participate in the response during the infection or that is not stimulated by conventional vaccination[89]. The refocusing of these Ab responses to subdominant epitopes resulted in a more protective response[93]. Thus, strategies aimed at filling holes in T-cell repertoires with new T-cell specificity that are not recognized during natural infection have emerged. In addition, according to a number of immunization studies on viral disease, CTL responses to the lower dominant epitope can compensate for loss of the dominant epitope, provide effective protection against subsequent challenges, and potential strategies to avoid mutants that remove their dominant antigenic determinants under selective pressure[94]. The ESAT-6 vaccine molecule (Δ15ESAT-6), which cleaves 15 of the most immunologically dominant N-terminal epitopes of the ESAT-6 sequence, has been introduced[89]. Vaccination with this truncated ESAT-6 showed better protection against Mtb infection than full-length ESAT-6 immunization due to the appearance of cryptic epitope-specific T cells that recognize alternate peptides in ESAT-6[89]. In addition to understanding the characteristics of protective $CD4^+$ T cells, identifying T cell phenotypes associated with vaccine-induced protection is crucial to the control of Mtb[95]. Recent studies have shown that the protective effect of a vaccine is associated

with an increase in KLRG1^{-}CD4^{+} T cells producing IL-2. Thus, enhanced protection mediated by epitope-specific cells in the crypts was associated with an increase in KLRG1^{-} CD4^{+} T cells that maintained IL-2 production capacity. KLRG1 is often associated with terminally differentiated cells, while IL-2 is found in central memory cells[4,90]. In addition, Mtb-specific KLRG1^{+} CD4^{+} T cells are known to produce very high levels of cytokines[95,96]. Thus, enhanced vaccine effect-induced protection is associated with less differentiated CD4^{+} T cells.

As mentioned above, most virulence factors are transferred to the bacterial surface, so the exported protein is important for the pathogenesis of Mtb and most of the PE /PPE family proteins located on the cell surface are important virulence factors. Knowing these important points, several vaccine candidates containing the PPE proteins are currently in clinical trials[82]. However, researchers have tended to investigate a limited number of PE/PPE proteins, and such an approach may not improve the overall understanding of the immunogenicity of proteins. I investigated and selected sequences that were consistently conserved in all PE and PPE family proteins using the characteristic structure of the PE/PPE protein showing repeat sequences and selected the peptides from which IFN-γ was detected. Unlike the fusion of the entire antigen sequence, the unnecessary structure of the peptide was eliminated, and the side effects could be reduced accordingly. In addition, the peptide is too small to cause immunogenicity, but when combined with other proteins, augmented immune responses can be expected. In other words, the complete protein acts as a carrier to make the vaccine stable and facilitates a stronger immune response. Consequently, these peptide vaccines are safer, easier to produce, and less toxic. In this study, I attempted to fuse ESAT-6 with peptides that are homologous and repetitively shared in Mtb, rather than those present in only one antigen, and investigated the potential of the PE/PPE peptide fusion proteins as vaccine candidates.

Chapter II

Vaccine potential of ESAT-6 protein fused with consensus $CD4^+$ T-cell epitopes of PE/PPE proteins against highly pathogenic *Mycobacterium tuberculosis* strain HN878

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(Directed by Professor Sung Jae Shin)

I. INTRODUCTION

Mycobacterium tuberculosis (Mtb) is an obligate pathogenic bacterial species causing tuberculosis (TB) in humans[1,7]. Its cell wall, comprising a thick lipid layer on cell surface, is a crucial factor in Mtb's pathogenicity[21]. Also, cell wall contains various proteins that contribute to the formation of its complex as[21,22]. Among many proteins involved in the formation of cell wall complex, Pro-Glu/Pro-Pro-Glu (PE/PPE) protein family are well known as important virulence factors[21,22]. Type VII secretion system (T7SS) is a specialized secretion system that produces pathogenic bacterial proteins across Mtb's complex cell envelope[22]. Mtb has five chromosomal *esx* clusters that code for specialized T7SSs: *esx*-1–5[22,97]. The best characterized *esx*-1 is responsible for secretion of ESAT-6, which belongs to a part of genetic deletion of BCG region, and is required for full virulence of Mtb[36]. In addition, there are PE/PPE proteins that attract attention as other virulence factors. Their names are derived from their unique characteristics and conserved N-terminal PE or PPE motifs, which are two large mycobacterial-specific protein families that account for approximately 10% of the total coding capacity of Mtb genome. These protein families are secreted from ESX-5, which is the most recently evolved system, and they also play a role in immunomodulation[37]. These protein families have a unique structure in which sequences having homology to each other are repeated, and these exist specifically in ESX-5 or are shared with PE/PPE homologs encoded outside *esx-5* [37].

Thus, fusion protein vaccines containing PE/PPE protein have shown promising results in animal studies and are currently under clinical trials[82,98-100]. Therefore, two systems, ESX-1 and ESX-5, are involved in virulence, and PE/PPE proteins are an important virulence factor along with ESAT-6[38]. On the other hand, immunization using truncated ESAT-6 that lacks immunodominant epitope N-terminus provides protective effects against Mtb infection[38]. In this study, specific PE/PPE peptides, which are conserved with high homology in each protein family, were conjugated to

the front of ESAT-6, and we investigated its improved efficacy compared to that of existing ESAT-6.

II. MATERIALS AND METHODS

1. Animal experiments

The protocol for animal experiments in this study was reviewed and approved by the Institutional Animal Care and Yonsei University Health System's Use Committee. C57BL/6J female mice at age 6–7 weeks were purchased from Japan SLC, Inc. (Shizuoka, Japan) and maintained under barrier conditions in a BL-3 biohazard animal facility at the Yonsei University Avison Biomedical Research Center.

2. PE/PPE peptide selection and production

We searched for all PE/PPE proteins through Mtb whole genome. Selection of conserved PE/PPE peptides was determined by multiple comparisons of all PE and PPE protein families. Confirmed peptides were then synthesized by GenScript (Piscataway, NJ, USA) and reconstituted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA).

3. PE/PPE peptide screening by IFN-γ response analysis during Mtb infection

Spleen and lung cells from C57BL/6J mice infected with Mtb K strain at 4 weeks post-infection were stimulated with 5 μg/ml of individual peptide. Peptide-specific IFN-γ response was evaluated by ELISA for the generation of recombinant ESAT-6 fusion proteins.

4. Cloning and expression of PE/PPE peptide-conjugated ESAT-6 recombinant fusion proteins

We devised the combination of PE peptide+ESAT-6, PPE peptide+ESAT-6, and PE+PPE peptide+ESAT-6. Forward primer was based on this sequence for each peptide: PE forward 5′-cat atg tcn ttt gtg act act cag ccg gag gcg ctt gcg gcg gcg gcg gga tca ctt caa ggt att atg aca gag cag-3′, PPE forward 5′-cat atg gat ttc gga gtt ctt cca cca gag atc aat tcg ggg cgt atg tac gcc gga cct ggt ttc atg aca gag cag-3′, and PE forward 5′-cat atg tcn ttt gtg act act cag ccg gag gcg ctt gcg gcg gcg gcg gga tca ctt caa ggt att gat ttc gga gtt ctt-3′ for PE+PPE peptide fusion. Reverse primer was based on ESAT-6 C-terminal region, and it was used for all recombinant proteins: 5′-aag ctt tgc gaa cat ccc agt gac gtt-3′. *Nde*I and *Hind*III, used as restriction enzyme sites, are underlined. PCR conditions were as follows: 95℃ (5 min); followed by 30 cycles at 95℃ (30 s), 60℃ (30 s), and 72℃ (50 s); and a final extension step of 72℃ (10 min). PCR product was cloned and inserted into pET-22b vector (Novagen, WI, USA). Ligated product was transformed in DH5α and BL21, then incubated in LB media.

5. Purification of recombinant fusion proteins

The harvested cell pellets were resuspended in extraction buffer (6 M urea, 50 mM sodium phosphate buffer, and 300 mM NaCl), sonicated, and centrifuged for 30 m at 4℃. Based on affinity chromatography, we combined the cell lysate with Ni-NTA resin (Invitrogen, CA, USA) and washed it. Target protein was then eluted with

elution buffer (125 mM imidazole in extraction buffer) and dialyzed (Slide-A-Lyzer® Dialysis cassette 2000 MWCO. Thermo, MA, USA) with 0.5× PBS buffer containing 6 M urea, slowly reducing the amount of urea until it reached 0 M urea at 4℃ in cold-room conditions. We removed lipopolysaccharides (LPS) using Triton X-114 (Sigma, MO, USA) three times, performed a concentration process (Amicon® Ultra-15, 3000 NMWL, Merck Millipore. MA, USA), and the final products were then eluted in 10 mM PBS.

6. SDS-PAGE and western blotting

Purified fusion recombinant proteins were separated by electrophoresis on 15% polyacrylamide gels (Bio-Rad, 75 V for 2 h). Proteins were transferred onto PVDF membrane, and blocked at RT with 5% non-fat dried milk in Tris-buffered saline with Tween 20 (TBS-T). Then, proteins were incubated with 1:4000 diluted His-probe (H-3) mouse monoclonal IgG1 (Santa Cruz Biotechnology, TX, USA) at 4℃ overnight. The membrane was washed with TBS-T and incubated with 1:5000 diluted goat anti-mouse IgG (Thermo, MA, USA) for 1 h. The membrane was developed using a substrate system (Pico ECL Solution. GenDEPOT, TX, USA).

7. Preparation of bacteria strain and infection

Mtb Beijing K strain and HN878 were obtained from the Korean Institute of Tuberculosis (KIT, Osong, Chungchungbuk-do, Korea) and the International Tuberculosis Research Center (ITRC, Changwon, Gyeongsangnam-do, Korea), respectively. All strains were cultured in 7H9 broth supplemented with 10% OADC and prepared as described previously[101]. For PE/PPE peptide screening, C57BL/6J

mice infected subcutaneous with 1×10^6 CFU of Mtb K strain and 200 CFU of HN878 was used for challenge by aerosol at 4 weeks after final immunization.

8. Mice infection with Mtb K strain and analysis of PE/PPE peptide fusion protein-specific IFN-γ response by ELISA

PE/PPE peptide fusion protein-specific IFN-γ response was assessed in lung and spleen cells from C57BL/6J mice infected with Mtb K strain at 4 weeks post-infection by stimulating cells with conjugated fusion proteins by ELISA. For stimulation, 0.5 ug/ml of each antigen was used. Single ESAT-6 protein, $CD4^+$ T-cell peptide of ESAT-6, and control media were used as controls.

9. Immunization of mice with PE/PPE peptide fusion proteins and challenge with Mtb Beijing strain HN878

C57BL/6J mice were subcutaneously immunized with manufactured fusion proteins (2 μg) with 5 μg of glucopyranosyl lipid adjuvant (GLA-SE. IDRI, W A, USA), three times at 3-week intervals. At 4 weeks after the finalimmuniz-at ion, mice were aerogenically challenged with Mtb Beijing strain HN878. For co ntrol groups, immunization group with single ESAT-6 protein and adjuvant-injec ted group were included.

10. Analysis of multifunctional T-cell immune response before and after challenge with Mtb

Lung and spleen cells were collected from mice before and after challenge with Mtb strain HN878, and stimulation with 1 μg of single ESAT-6 protein or 1 μg of PE/PPE peptide, respectively. *Ex vivo*-stimulated ESAT-6 protein- or peptide-specific Th1 immune response were then assessed by flow cytometry.

11. IgG1 and IgG2c ELISA

IgG1/IgG2c responses in the serum of immunized mice were evaluated. Ninety-six-well plates were coated with 2 μg/mL of single ESAT-6 protein and then blocked with 5% fetal bovine serum (FBS), thereby binding the serum of mice, which was diluted 1,000 (for the ESAT-6 group) and 4,000 times (for the fusion protein group). Horseradish peroxidase (HRP)-linked antibody against IgG1 (BD Bioscience, San Diego, CA) or IgG2c (Southern Biotech, Birmingham, AL) was used as secondary antibody. OD was measured at 450 nm after cessation of 5-min reaction.

12. Flow Cytometry

Single-cell suspensions from lung and spleen of immunized mice were incubated in Roswell Park Memorial Institute (RPMI) 1640 media [10% FBS, 0.1% collagenase type II (Worthington, OH, USA), 1 mM $MgCl_2$, and 1 mM $CaCl_2$] at 37℃. Cell suspensions were treated with red blood cell lysis buffer for 5 min, and washed with RPMI 1640 medium. Lung and spleen cells were plated at a concentration of 1×10^5 cells/well (200 μl) and 1.5×10^6 cells/well (200 μl), respectively, then stimulated with 1 ug/ml single ESAT-6 PE/PPE peptide complex, and immediately incubated for 12 h at 37℃ in the presence of GolgiStop (Thermo, MA, USA). Cells were washed and blocked with anti-CD16/32 antibody, and surfaces were stained with fluorochrome-conjugated antibodies against CD4, CD3, CD62L, CD44, and LIVE/DEAD™ for 30 min at 4℃. Cells were washed again, and then fixed and permeabilized with

Cytofix/Cytoperm (Thermo, MA, USA). They were then washed with Perm/Wash, and intracellular staining was performed with PE-conjugated anti-IFN-γ, APC-conjugated anti-TNF-α, and PE-Cy7-conjugated anti-IL-2 (Thermo, MA, USA). Next, cells were fixed with IC Fixation Buffer and analyzed with FACS verse flow cytometer using FlowJo software.

13. Evaluation of protection efficacy

To investigate protection efficacy, homogenized organs were plated onto Middlebrook 7H11 agar plates (Becton Dickson, Franklin Lakes, NJ, USA) supplemented with 10% Oleic Albumin Dextrose Catalase (Difco Laboratories). CFU were counted after 4 weeks of incubation at 37℃. Lung samples were collected for histopathology and preserved overnight in 10% normal buffered formalin, embedded with parafilm, divided into 4–5 μm sections, and stained with hematoxylin-eosin (H&E).

14. Statistical analysis

All *in vitro* experiments were repeated at least three times, and they yielded consistent results. Data are expressed as means ± standard deviation. Data from *in vivo* experiments are reported as medians ± interquartile range (IQR). Statistical comparisons were made using an unpaired *t*-test or one-way ANOVA, followed by Tukey's multiple comparison test using Prism (GraphPad Software version 5.0, San Diego, CA).

III. RESULTS AND DISCUSSION

1. PE/PPE peptides and peptides of N-terminal region induced IFN-γ response in Mtb- infected mice

After multiple comparisons of all PE and PPE proteins (36 proteins in PE family, 62 proteins in PE-PGRS family, and 69 proteins in PPE family), we obtained 21 conserved PE/PPE peptides (Table 1). These repeated PE/PPE epitopes suggest that selected family members can be partially induced by highly immunogenic cross-reactivity[66]. The most immunogenic domain of PE/PPE proteins is a more conserved N-terminal motif (based on IFN-γ response)[72]. Thus, peptides which belong to the N-terminal portion of PE and PPE protein family induced IFN-γ Th1 response (Figure 1A).

2. Production of PE/PPE peptide fusion proteins and assessment of their capacities inducing IFN-γ Th1 response in Mtb K strain-infected mice

We developed recombinant proteins that conjugated these positive peptides to ESAT-6 (Figure 1B), and investigated whether these fusion proteins have immunobiological potential during *in vivo* infection. IFN-γ is a key $CD4^+$ T-cell-derived cytokine; and as an important component, it could serve as a marker of recognition by host immune system[102,103]. Therefore, we evaluated antigen-specific IFN-γ responses, and confirmed increased levels of IFN-γ of all PE/PPE peptide fusion protein groups compared with single ESAT-6 (Figure 1C). Thus, it was found that all fusion proteins were recognized during infection.

Table 1. PE/PPE protein peptide selection

Peptide number	Peptide class	Mer	Peptide sequence
1*	**PE peptide 1**	21	**MSFVTTQPEALAAAAGSLQGI**
2	PE peptide 2	21	AATPTTGVVPAAADEVSALTA
3*	**PPE peptide 3**	21	**MDFGVLPPEINSGRMYAGPGS**
4	PPE peptide 4	27	PMLAAAAAWDGLATELQSTAADYGSV
5	PPE peptide 5	26	LAATGYASVIAELTGAPWVGAASLSM
6	PPE peptide 6	21	WSGQSSGTMAAAAAPYVAWMS
7	PPE peptide 7	22	AGMQARAAAAAYELAFAMTVPP
8	PPE peptide 8	23	AMTVPPPVVVANRALLVALVATN
9√	PPE peptide 9	24	VVANRALLVALVATNFFGQNTPAI
10	PPE peptide 10	21	LVATNFFGQNTPAIAATEAQY
11	PPE peptide 11	21	IAATEAQYAEMWAQDAAAMYA
12	PPE peptide 12	23	SASLARANKIGALSVPPSWVKTT
13	PE-PGRS peptide 13	22	MSFVVTIPEALAAVATDLAGIG
14	PE-PGRS peptide 14	21	STIGTANAAAAVPTTTVLAAA
15	PE-PGRS peptide 15	22	VLAAAADEVSAAMAALFSGHAQ
16	PE-PGRS peptide 16	25	AYQALSAQAALFMEQFVRALTAGAG
17	PE-PGRS peptide 17	23	QFVRALTAGAGSYAAAEAASAAP
18	PE-PGRS peptide 18	20	INAPVQSLTGRPLIGDGANG
19	PE-PGRS peptide 19	23	SLTGRPLIGDGANGIDGTGQAGG
20	PE-PGRS peptide 20	21	GGNGGWLWGNGGNGGSGAPGQ
21	PE-PGRS peptide 21	17	GGAGGAAGLIGNGGAGG

*Bold peptide number and amino acid sequences indicate proven induction of IFN-γ response.

√ Not produced

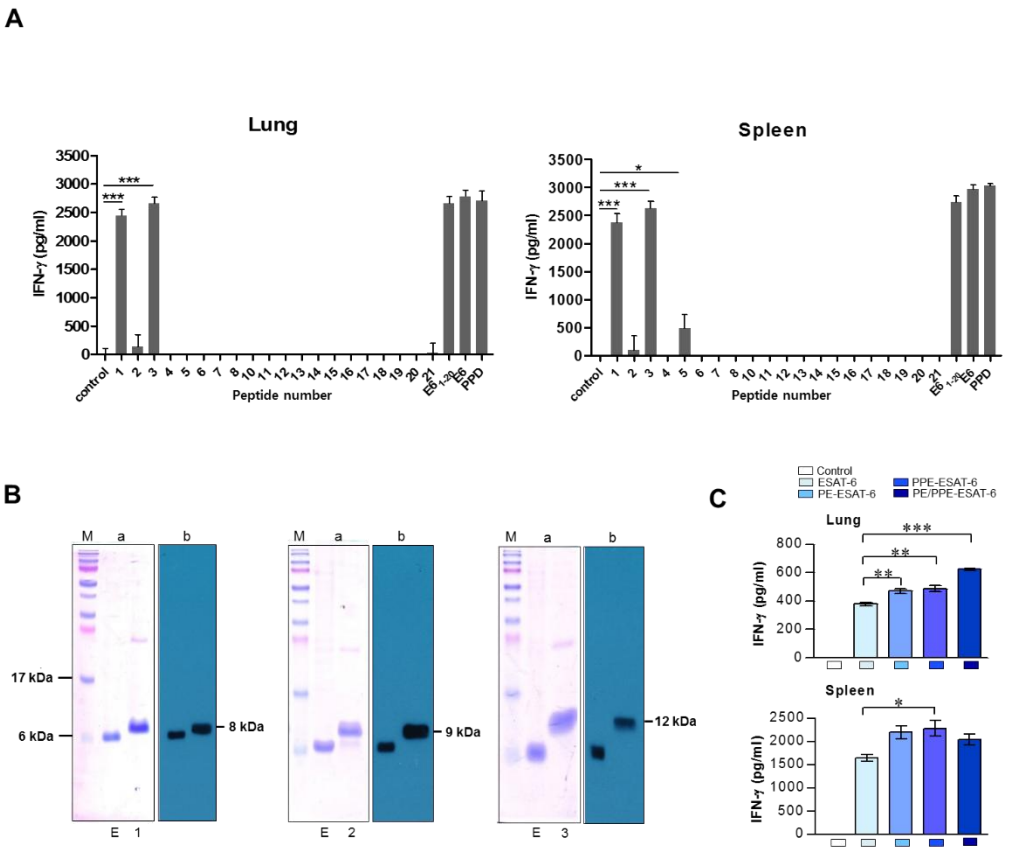

Figure 1. PE/PPE peptide screening for IFN-γ response and manufacture of fusion recombinant protein (A) IFN-γ response obtained for each peptide of PE and PPE family region (peptide numbers 1 and 3, respectively). (B) Manufacture of PE/PPE peptide fusion recombinant proteins. Selected PE and PPE peptides are conjugated to ESAT-6 protein (Lane M: Marker, lane E: full-length ESAT-6, lane 1: PE + ESAT-6, lane 2: PPE + ESAT-6, lane 3: PE/PPE + ESAT-6, a: 15 % SDS-PAGE gel, and b: western blotting). (C) Result of IFN-γ ELISA after infection of C57BL/6 mice with Mtb K strain for 4 weeks. Significance of difference was determined by unpaired *t*-test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with single ESAT-6 or other positive control groups.

3. Immunological response, including serum IgG1 antibody titers, and multifunctional T-cells were induced in PE/PPE fusion protein immunization groups prior to challenge with Mtb strain

Four weeks after final immunization, IFN-γ response showed higher levels in PPE or PE/PPE peptide+ESAT-6 immunized groups than in single ESAT-6 group (except for lung stimulation with ESAT-6, Figure 2A). In addition, these fusion protein-specific antibody titers against single ESAT-6 showed higher levels of IgG1 than IgG2c (Figure 2B). Antibody response was thought to have little immune control in the course of infection[104]; however, $CD4^+$ T-cell-mediated immunity may not be sufficient to protect against TB. Protective antibodies against Mtb may be presumed to immediately prevent infection by Mtb at an early stage[104,105]. PE/PPE family proteins can be targets of humoral immune system at the same time, as these proteins are exposed on cell surface with repetitive sequences[70,106]. Therefore, PE/PPE proteins produce strong humoral and cell-mediated response[106], and in this study, these fusion proteins are considered to have a strong effect on humoral immune response as well as cellular immune response (Figure 3).

Analysis of multifunctional $CD4^+$ T-cells of lung by flow cytometry, according to gating strategy, is shown in Fig. 2C. PE/PPE peptide fusion protein showed increased level of cytokine secretion for most cytokine combinations, triple- (IFN-γ, TNF-α, and IL-2) or double-positive cytokines, compared to single ESAT-6 (control group) (Fig. 2D). In spleen $CD4^+$ T-cells, increased level of bi-functional T-cells (IFN-γ^+/TNF-α^+) was induced in fusion protein group, and this phenomenon was more prominent when stimulated with PE/PPE peptide (Figure 4). These results show that immunogenic potential could be expected in fusion protein immunized group, and that immune responses were elicited by only PE/PPE peptide.

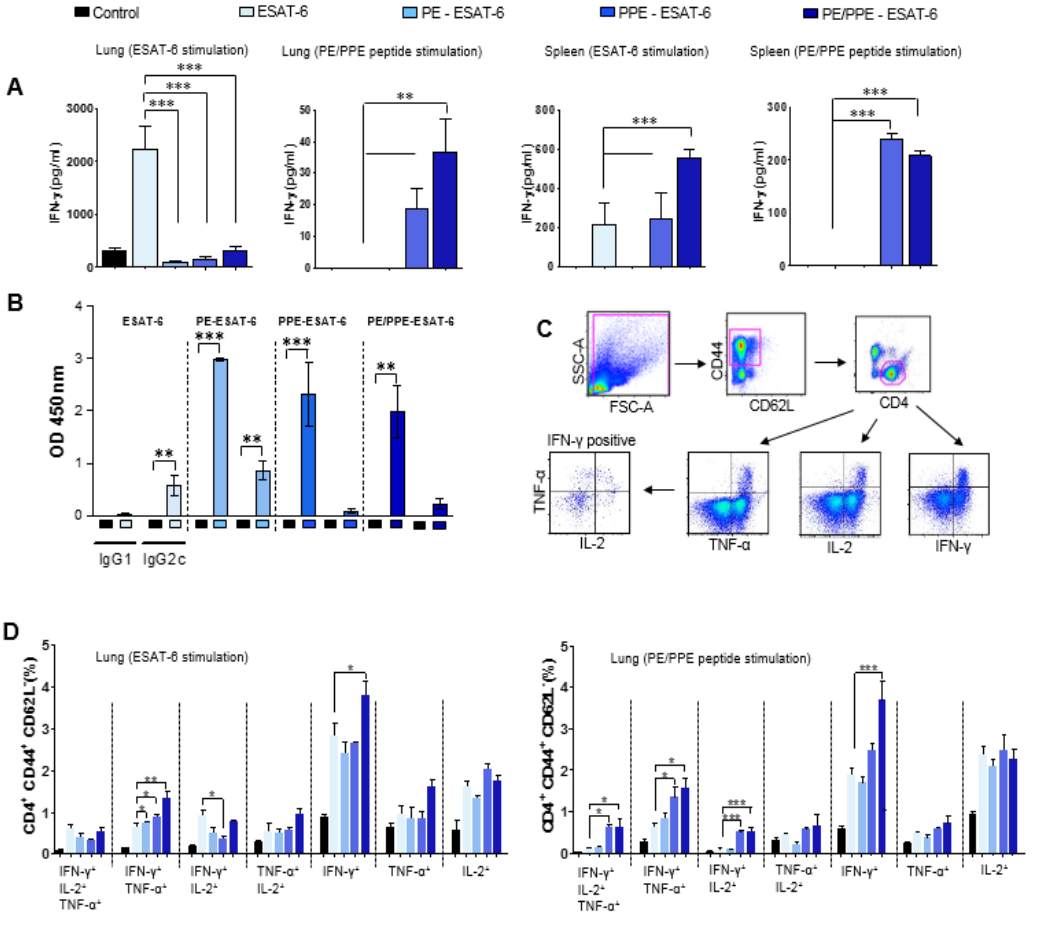

Figure 2. Immune response and antibody titer post immunization with PE/PPE peptide fusion protein (A) IFN-γ response assay by ELISA in each peptide fusion protein immunization group. (B) Serum IgG1/IgG2a antibody response with PE/PPE peptide fusion proteins against that with single ESAT-6 protein (control: only GLA-SE adjuvant). (C) Gating strategy for multifunctional $CD4^+$ T-cells from immunized mice with fusion proteins. (D) Multifunctional $CD4^+CD44^+CD62L^-$ T-cells secreting cytokines of three (IFN-γ, TNF-α, and IL-2) or double combination (co-producing IFN-γ, TNF-α, and/or IL-2) and single cytokines in each immunization group, according to stimulation with single ESAT-6 or PE/PPE peptide in lung. Control is adjuvant-only group. Data was expressed as mean ± SD. *, **, and *** = significant difference, as determined by unpaired *t*-test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with single ESAT-6 group.

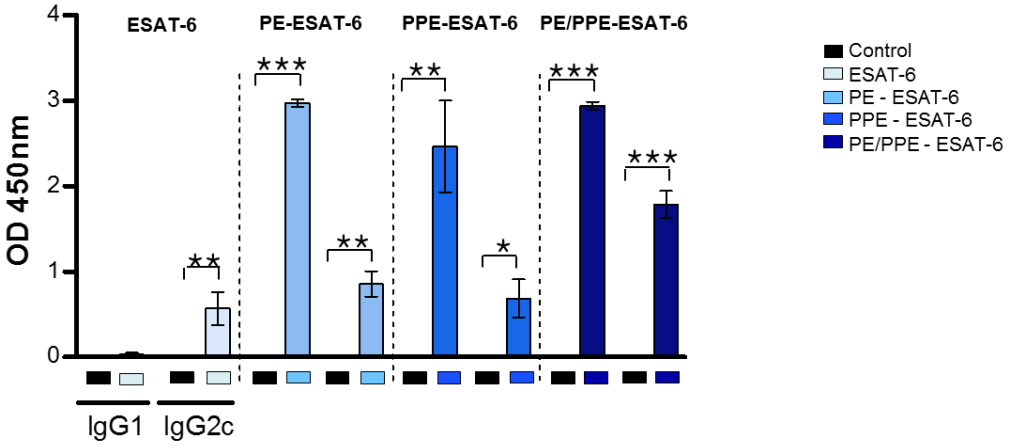

Figure 3. PE/PPE peptide fusion protein-specific IgG1 and IgG2c response in each protein immunized group. Proteins used in immuization were coated with 2 μg/ml respectively, and assessed IgG1/IgG2c responses in serum of immunized mice (control: only GLA-SE adjuvant). Data was expressed as mean ± SD. *, **, and *** = significant difference, as determined by unpaired *t*-test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with control group.

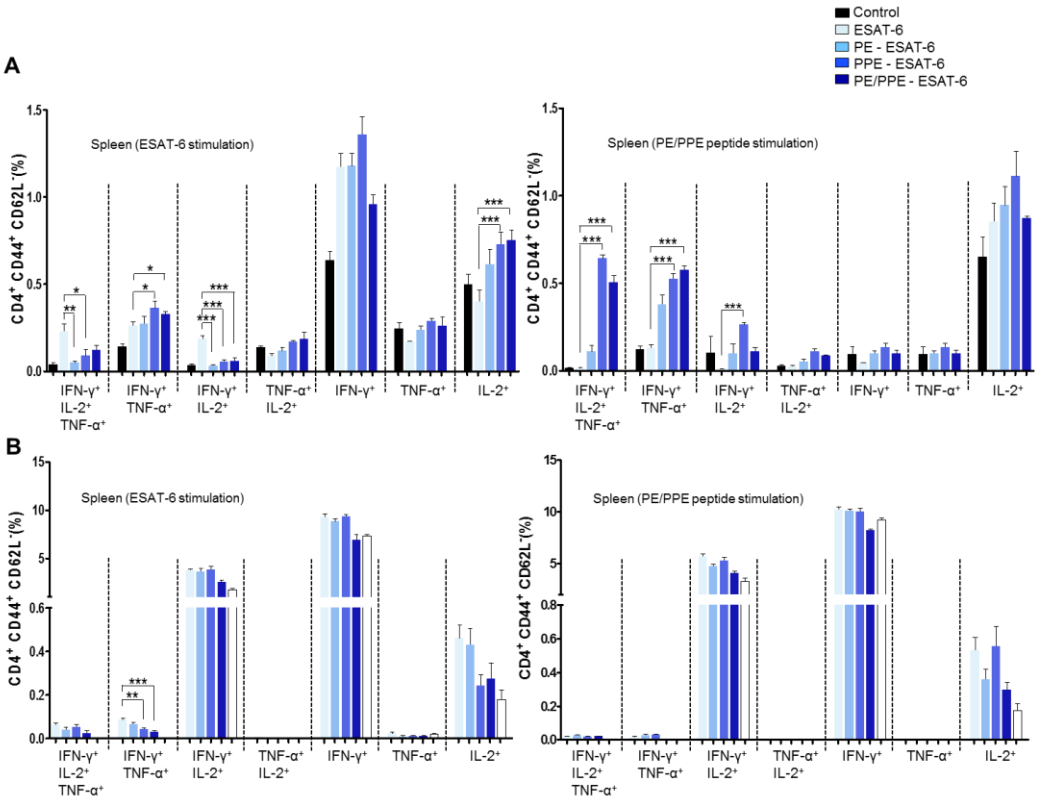

Figure 4. Multifunctional CD4$^+$ T-cell in spleen after immunization

(A) Percentage of multifunctional CD4$^+$CD44$^+$CD62L$^-$ T-cells secreting variable cytokine combination in each immunization group, according to stimulation with single ESAT-6 or PE/PPE peptide prior to infection. (B) Status of cytokine secretion after challenge with Mtb strain. Data is expressed as mean ± SD. *, **, and *** = significant difference, as determined by unpaired *t*-test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with single ESAT-6 group.

4. Increased bi-functional (IFN-γ^+/IL-2^+) $CD4^+$ T-cell immunity after challenge with Mtb Beijing strain

After 15 weeks of challenge, $CD4^+$ T-cells secreting double-positive cytokine IFN-γ^+/IL-2^+ (including single IFN-γ^+) showed increased levels in all fusion protein immunization groups in lung, compared to single ESAT-6 group (Figure 5A and 5B). With respect to spleen, results are shown in S2 Fig B. IFN-γ^+/IL-2^+ double-positive cytokines, which have been reported to be predominantly present in latent tuberculosis infection (LTBI) subjects with single IFN-γ, have also been shown to occur when treating patients with active TB[107]. Multifunctional (IFN-γ, TNF-α, and IL-2) $CD4^+$ T-cells are associated with protection[102,107]; however, they could also be indicative of the status of active TB[107]. PPD-stimulated IL-2/IFN-γ ratio is possible to only distinguish TB infection and non-infection by short-term stimulation with a TB antigen. However, when stimulated for >72 h, IL-2 levels are higher in LTBI, and are distinguishable from that observed in active TB[108,109].

5. Potential of protective efficacy observed in the fusion proteins immunization group

Lungs of immunized and Mtb-infected mice were photographed and stained with H&E, in order to evaluate protective efficacy (Figure 6A and 6B). Progress and status of lung inflammation are shown in Fig. 4C. When investigating bacterial burden, CFU were found to be lower in spleen in fusion protein immunization groups than in that of single ESAT-6 group. This result seemed synergic when two peptides were conjugated, and PPE, the shared peptide, appeared more effective than PE (*$p < 0.05$, PE/PPE-ESAT-6) (Figure 6D, panel below). However, it showed much lower CFU compared to infection control group (***$p < 0.001$, PE/PPE-ESAT-6). In the case of lung, median value of CFU measurement of fusion protein groups was lower than that in

ESAT-6 group (Figure 6D, upper panel); this value was also lower than that in infection control group (***$p < 0.001$ or **$p < 0.01$). Such visible result may be also affected by the mouse strains and administration route of the vaccine[110], thus further study is required by considering these factors.

ESX-5-specific epitope was found to have no correlation with pathogenicity, as both ESX-5-specific and shared peptides have a similar protective effect[4]. However, the shared epitopes appear to be more effective, as it has a large range of possible immune responses. In this study, wide-ranging sequences in PE/PPE proteins were applied, and this result suggests that the conjugation of PE/PPE peptide enhances protective efficacy. T-cell epitopes may significantly differ depending on host HLA haplotype, and it may be ideal to include all epitopes[89]. in development of TB vaccines. Therefore, if characteristics of these PE/PPE families are well understood and utilized appropriately, this strategy will be very promising as a vaccine component.

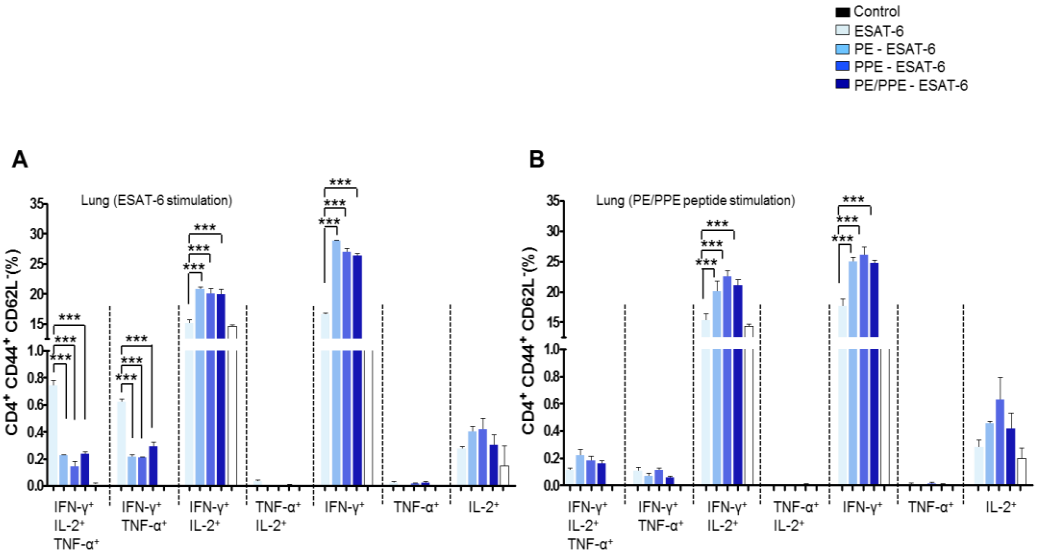

Figure 5. Cytokine secretion of T-cells in lung after challenge with Mtb strain

Lung $CD4^+$ T-cells from immunized mice stimulated with single ESAT-6 (1 μg/ml) (A) and only PE/PPE peptide (1 μg/ml) (B), respectively. Data was expressed as mean ± SD. *, **, and *** = significant difference, as determined by unpaired *t*-test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with single ESAT-6 group.

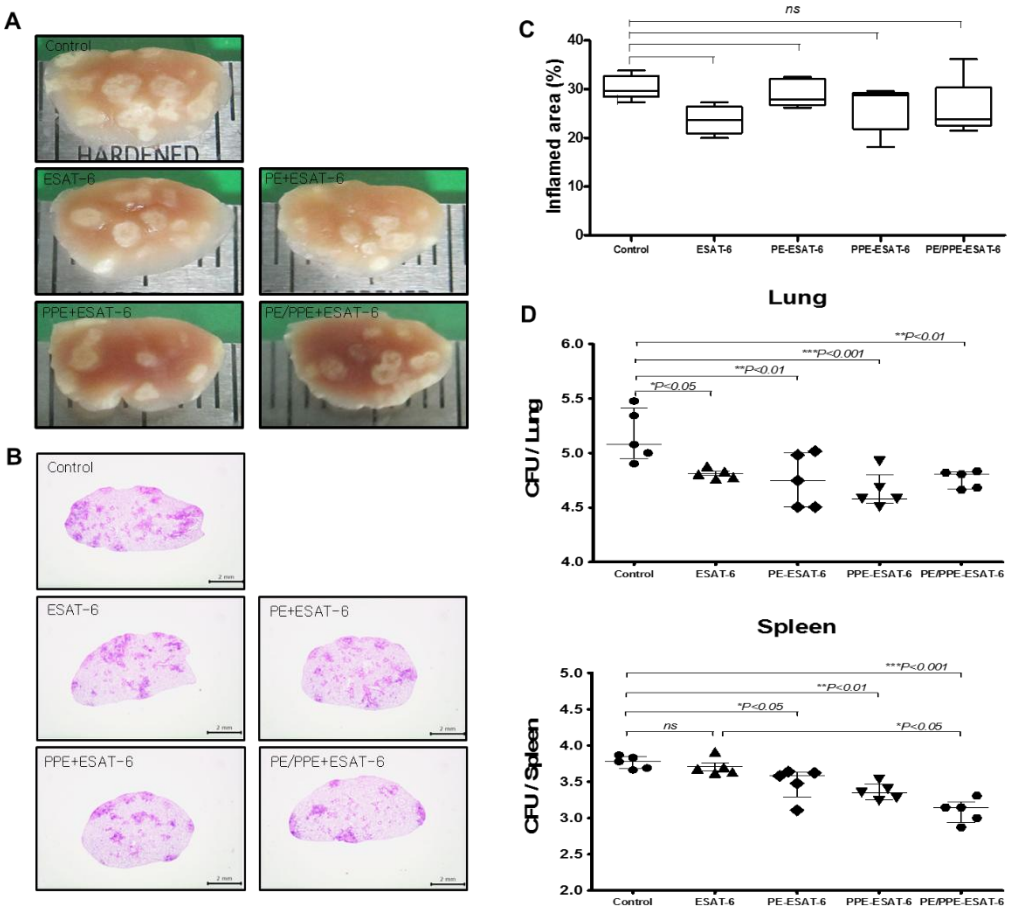

Figure 6. Protective efficacy of PE/PPE peptide fusion proteins (A) Growth pathology of lung from immunized mice with each protein, (B**)** Lung section stained with H&E at 15 weeks post challenge with Mtb. (C) Percentage of inflamed area of lung. (D) Bacterial burden of lung and spleen are shown in individual peptide fusion protein immunization and control (infection control, ESAT-6) groups (n = 5 animals/group). *ns.* = not significant; *, **, and *** = significant difference, as determined by one-way analysis of variance; $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively.

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ABSTRACT (Korean)

ESAT-6 단백질과 PE/PPE 단백질의 peptide를 융합시킨 재조합 단백질의 vaccine potential 조사

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최수영

결핵균은 두껍고 지질층이 많은 외피 구조 때문에 병원성이 높은 균이다. 이런 외피 구조를 통해 단백질을 분비하기 위한 시스템인 type VII secretion systems (T7SSs) 에서 나오는 대표적인 백신 후보 물질인 ESAT-6 외에 또 다른 virulence factor로 부각 되고 있는 것으로 PE/PPE 단백질이 있으며, 이들 역시 결핵균의 외피를 이루고 있는 단백질들이다. 현재 실제로 PE/PPE 단백질을 다른 단백질과 융합하여 제조한 subunit vaccine이 임상 실험 중에 진행되고 있으며, 본 연구에서는 특정 항원이 아닌 PE/PPE 단백질들이 공통적으로 갖고 있는 peptide를 IFN-γ assay로 선별하여 ESAT-6 항원과 융합시킨 재조합 단백질을 제조하였다. 한편 ESAT-6는 대표적인 T cell 항원임에도 불구하고 N-terminal의 immunodominant peptide 때문에 도리어

결핵균에 대한 제한적인 면역반응이 일어난다는 보고가 있다. 따라서 본 연구에서는 이런 단점을 보완하기 위하여 ESAT-6의 앞부분에 이러한 PE PPE의 peptide를 융합하여 재조합 단백질을 만들고 그 백신 효과를 단독 ESAT-6와 비교하여 조사하여 보았다. 그 결과 결핵균 감염 전, 융합 단백질의 면역화 그룹에서 ESAT-6와 peptide로 자극했을 때, 결핵균 방어 효능에 중요한 지표가 되는 multifunctional T cell의 cytokine이 더 많이 분비되는 것을 보였고 (ESAT-6: 0.55~0.92% ± 0.17~0.4, PE/PPE + ESAT-6: 0.55-1.35% ± 0.09~0.4 in the lung $CD4^+$ T cell), 또한 결핵균 감염 이후에도 결핵의 치료와 진단의 향상과 관련되어 있는 marker로 알려져 있는 $IL\text{-}2^+/IFN\text{-}\gamma^+$의 double positive cytokine의 상승된 분비 결과를 보이는 것으로 나타났다 (ESAT-6: 15.1% ± 1.3, PE/PPE+ESAT-6: 19.9% ± 2.1, in the lung $CD4^+$ T cell).

결핵의 예방에 있어 주요한 요소로 제안 된 triple cytokine인 IFN-γ, TNF-α 및 IL-2의 cytokine 분비 또한 감염 이후에 융합 단백질로 면역화시킨 그룹에서 검출되었으며, 특히 PE/PPE peptide로 자극했을 때에는 ESAT-6로 면역화 시킨 그룹보다 높게 나타난 결과를 보였다. Bacteria burden 조사에서도 spleen 세포에서 하나의 peptide를 융합시킨 항원의 immunization 그룹에서 단독 ESAT-6보다 조금씩 떨어지는 결과를 보였으며, 두 개의 peptide가 융합되었을 때는 상대적으로 더 상승된 synergy 효과를 보였다 (*$p<0.05$, unpaired t-test). 따라서 본 연구 결과, 하나의 peptide를 ESAT-6 항원에 융합시켜도 백신으로서의 더 상승된 효과를 기대할 수 있었고, 또한 더 증대된 항결핵의 Th1 type의 면역 반응을 이끌어 낼 수 있었다. 또한 BCG에서 결여되어 있는 ESAT-6와는 달리 ESX-5 system은 존재하기 때문에, PE/PPE의 epitope에 의한 면역반응을 이끌어 낼 수 있다는 것을 고려할 수 있었다. 따라서 BCG booster vaccine으로써도 충분히 가능할 것으로 사료되었다. 그러므로 이러한 PE/PPE protein의 특성을 잘 이해하고 적절한 항원

결정기를 잘 활용한다면, 이러한 peptide와 항원 결합의 전략은 백신 후보 물질의 개발에 유망할 것으로 기대된다.

핵심되는 말: 결핵균, PE/PPE 단백질, ESAT-6 단백질, 다기능 T 세포, Type VII secretion system, ESAT-6.