Acta Microbiologica et Immunologica Hungarica 66 (2), pp. 219–234 (2019)
DOI: 10.1556/030.65.2018.048
First published online November 28, 2018

IMMUNE RESPONSES ELICITED BY THE RECOMBINANT ERP, HSPR, LPPX, MMAA4, AND OMPA PROTEINS FROM *MYCOBACTERIUM TUBERCULOSIS* IN MICE

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(Received: 27 September 2018; accepted: 22 October 2018)

Immunogenic potency of the recombinant Erp, HspR, LppX, MmaA4, and OmpA proteins from *Mycobacterium tuberculosis* (MTB), formulated with Montanide ISA 720 VG adjuvant, was evaluated in BALB/c mice for the first time in this study. The five vaccine formulations, adjuvant, and BCG vaccine were subcutaneously injected into mice, and the sera were collected at days 0, 15, 30, 41, and 66. The humoral and cellular immune responses against vaccine formulations were determined by measuring serum IgG and serum interferon-gamma (IFN-γ) and interleukin-12 (IL-12) levels, respectively. All formulations significantly increased IgG levels post-vaccination. The highest increase in IFN-γ level was provided by MmaA4 formulation. The Erp, HspR, and LppX formulations were as effective as BCG in enhancement of IFN-γ level. The most efficient vaccine boosting the IL-12 level was HspR formulation, especially at day 66. Erp formulation also increased the IL-12 level more than BCG at days 15 and 30. The IL-12 level boosted by MmaA4 formulation was found to be similar to that by BCG. OmpA formulation was inefficient in enhancement of cellular immune responses. This study showed that MmaA4, HspR, and Erp proteins from MTB are successful in eliciting both humoral and cellular immune responses in mice.

Keywords: BCG, immune response, infectious disease, recombinant vaccines, tuberculosis

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Introduction

Mycobacterium tuberculosis (MTB) is the causative agent of the infectious disease tuberculosis (TB), and characterized with its slow growth, dormancy, complex cell envelope, and intracellular pathogenesis [1]. TB is at the ninth rank among the global causes of death, and at the first place to be a single infectious agent causing death. In 2016, 6.3 million new TB cases including 476,774 HIV-positive patients have been reported. In addition, 1.3 million HIV-negative and 374,000 HIV-positive people had died due to TB [2]. The death rate is unacceptably high as the most of them could be preventable with early diagnosis and appropriate treatment. Due to this reality, the World Health Organization declared "The End TB Strategy" with a first step of reducing the TB deaths and incidence (compared with 2015 levels) by 35% and 20%, respectively, until 2020 [2].

The only vaccine used worldwide against TB is Bacillus Calmette-Guérin (BCG), which is an attenuated *Mycobacterium bovis* strain. Its protection capacity ranges between 80% and 0% in human, and it does not protect especially the adults against pulmonary TB [3, 4]. Therefore, there is a need for vaccines more efficient than BCG. Although a potential protection equal or better than BCG was detected in earlier studies using some cell wall components or inactivated preparations of MTB, this potential was questioned due to a non-specific inflammation response [3]. Later, several studies have been conducted on the development of recombinant subunit vaccines against TB, with a single or a couple of specific antigens, and having specific and prolonged protection potential. Horwitz and Harth [5] evaluated the rBCG30 vaccine, a *M. bovis* BCG strain expressing the 30-kDa major secretory protein of MTB in the guinea pig model, and obtained better protection than the commercial BCG vaccine. The complementation of BCG strain with ESAT-6 protein from MTB also increased the protection potential [6]. The mice vaccinated with *Mycobacterium smegmatis* expressing heparin-binding hemagglutinin from MTB and human IL-12 fusion were reported to be protected against MTB infection [7]. In addition, *Bacillus subtilis* spores expressing MPT64 antigen from MTB protected the mice against TB [8]. Liu et al. [9] showed that the fusion of Mtb8.4 and HspX proteins from MTB elicited strong immune responses in mice.

Identification of the virulence factors playing role in the pathogenesis of MTB is important for the development of recombinant vaccines. Smith [10] grouped the virulence factors as the ones related to (a) cell secretion and cell envelope, (b) enzymes involved in the cell metabolism, and (c) transcriptional regulators. The cell surface components Erp (Rv3810), MmaA4 (Rv0642c), and OmpA (Rv0899), and transcriptional regulator HspR (Rv0353) are among the virulence factors of MTB [10]. The lipoprotein LppX (Rv2945c) is involved in the

virulence of MTB [11]. To our knowledge, the immune responses provided by these proteins have not been evaluated.

Th1-type cellular immunity, especially interferon-gamma (IFN-γ) and interleukin 12 (IL-12) cytokines, plays an important role in the resistance against TB [12, 13]. Adjuvants used for the development of subunit vaccines can direct the type of immune response. Utilization of the oil-based adjuvant, Montanide ISA 720 (Seppic, France), in human vaccines was approved, and it provides strong IFN-γ and IL-12 responses [14]. In this study, humoral and cellular immune responses elicited by the recombinant Erp, HspR, LppX, MmaA4, and OmpA proteins from a clinical isolate of MTB were first evaluated in mice as vaccine formulations prepared with Montanide ISA 720 adjuvant. One of the two control groups of mice received adjuvant only, and the other group received commercial BCG vaccine. The serum IgG, IFN-γ, and IL-12 levels were compared between the control and vaccine formulation groups.

Materials and Methods

Bacterial strain and genomic DNA isolation

A clinical isolate of MTB strain 14/1649, obtained from a TB patient in Turkey, was used. The isolate was verified using GenoType MTBC (Hain Lifescience, Germany) test and *16s rRNA* analysis (GenBank accession number: KY810766). The MTB isolate was grown in Löwenstein–Jensen medium (Salubris Inc., Turkey) at 37 °C for 2 weeks. The genomic DNA of the MTB isolate was isolated using GeneJET Genomic DNA Purification Kit (Thermo Scientific, Lithuania, EU) according to the manufacturer's recommendations. The quality of purified genomic DNA was examined using NanoDrop (Thermo Scientific) and agarose gel electrophoresis, and the genomic DNA was stored at −20 °C.

Amplification of erp, hspR, lppX, mmaA4*, and* ompA *genes*

The genomic DNA of MTB 14/1649 was used as the template for amplification of *erp, hspR, lppX, mmaA4*, and *ompA* genes. The primers listed in Supplementary Table I were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0) and BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) tools. The polymerase chain reaction (PCR) mix was prepared using 1U *Taq* DNA polymerase, 1X *Taq* buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, all from Thermo Scientific, 0.2 pmol forward and reverse primers, and 1 ng template DNA, in a final volume of 50 µl. The PCR program was set as initial denaturation at

94 °C for 10 min, 35 cycles of amplification (denaturation at 94 °C for 1 min, annealing at 52 °C for 30 s, and elongation at 72 °C for 1 min), and final extension at 72 °C for 10 min. The PCR products were run on 1% agarose gel, and the related bands were purified using GeneJET Gel Extraction Kit (Thermo Scientific) according to the manufacturer's recommendations.

Cloning of erp, hspR, lppX, mmaA4*, and* ompA *genes*

The PCR products were first ligated to pGEM-T Easy Vector System (Promega, WI, USA) according to manufacturer's recommendations, and introduced into *E. coli* DH5α; later, the clones were verified using Sanger sequencing (BGI, Denmark, Europe). Next, *erp, hspR, mmaA4*, and *ompA* genes were cloned in *Bam*HI site, and *lppX* gene was cloned in *Eco*RI site of pET-28a(+) (Novagen, Germany) in *E. coli* DH5α. The clones were verified by plasmid isolation and restriction enzyme digestion. The recombinant pET-28a(+) vectors carrying *erp, hspR, lppX, mmaA4*, and *ompA* genes were introduced into *E. coli* BL21(DE3) competent cells (Novagen).

Purification of the recombinant Erp, HspR, LppX, MmaA4, and OmpA proteins

Purification of the recombinant proteins was performed according to Okay et al. [15]. The recombinant *E. coli* BL21(DE3) cells with pET-28a(+) vectors carrying *erp, hspR, lppX, mmaA4*, and *ompA* genes were grown in Luria Broth (Merck, Germany) containing kanamycin (30 μg/ml) at 37 °C until logarithmic growth phase. Later, isopropyl-β-D-galactopyranoside (Sigma, Germany) was added to a final concentration of 1 mM, and the culture was incubated at 37 °C for 5 h shaking at 165 rpm. After incubation, the cells were collected using centrifugation at 4,500 rpm for 10 min, resuspended in 5 ml of lysis buffer (8 M urea, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0), and disrupted using sonicator (Bandelin-Sonoplus, Germany) for 6 × 10 s at 60% amplitude. The cellular debris was separated using centrifugation at 15,000 rpm for 15 min, and the supernatants containing recombinant proteins were collected. The his-tagged recombinant proteins were purified using Protino® Ni-TED 2000 packed columns (Macherey-Nagel, Germany) according to the manufacturer's recommendations. Purity of the proteins were monitored using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [16] staining with Coomassie Blue R-250 (Merck), and the quantity of recombinant proteins was determined according to Bradford's [17] method. The purified recombinant proteins were sterilized using 0.2 μm membrane filter, and maintained at −20 °C until use.

Preparation of the vaccine formulations and immunization

Each vaccine formulation was prepared by mixing 0.1 mg of the purified recombinant protein and Montanide ISA 720 VG in 7:3 proportion [18, 19]. The mixtures were vortexed for 1 min and incubated at 4 °C for 24 h. The vaccine formulations (0.1 ml) were inoculated on Luria agar plates and incubated at 37 °C for 24 h to verify their sterility.

The animal experiments were approved by the Local Ethics Committee of Kobay DHL Inc. (Ankara, Turkey) with the protocol number of 119. Six- to eight-week-old female BALB/c mice were used. A group of five mice were subcutaneously immunized with 0.33 ml of Erp, HspR, LppX, MmaA4, and OmpA vaccine formulations as well as adjuvant only, and 1×10^5 CFU BCG vaccine (Serum Institute of India Pvt. Ltd., India) [7]. The injections were performed twice at days 0 and 15. The blood samples from tail vein were collected from mice at days 0 (before first injections), 15 (before second injections), 30, 41, and 66. After the last blood collection (day 66), all of the mice were euthanized through cervical dislocation. The sera collected from mice were pooled and maintained at −20 °C until use.

Western blot

Protein samples were run on two separate 12% SDS–polyacrylamide gels. One of the gels was stained with Coomassie Blue R-250. Proteins on the other gel was transferred to a 0.2 μm nitrocellulose membrane through Mini Trans-Blot Cell (Bio-Rad, CA, USA) using a modified procedure of Towbin et al. [20]. The membrane was cut into pieces, each containing one of the recombinant proteins, and 1:400 dilution of respective serum for each protein was applied as primary antibody. The secondary antibody, alkaline phosphatase-conjugated anti-mouse IgG (Sigma, Germany), was used at a dilution of 1:10,000. The AP Conjugate Substrate Kit (Bio-Rad, CA, USA) was used for visualization of the bands [15].

Determination of antibody response

The humoral immune response elicited by the vaccination groups was detected measuring the serum IgG titers via the enzyme-linked immunosorbent assay (ELISA) [15]. The 96-well ELISA plates were coated with 1 μg/well of purified recombinant proteins. The primary antibody, sera obtained from vaccinated mice, was applied as twofold serial dilutions ranging from 1:50 to 1:3,200 in triplicates, and alkaline phosphatase-conjugated anti-mouse IgG was applied to

each well as secondary antibody at a dilution of 1:1000. The AP Conjugate Substrate Kit was used as colorimetric reagent. Plates were read at 405 nm to determine optical density on a microtiter plate reader.

Determination of IFN-γ and IL-12 titers

The cellular immune response elicited by the vaccination groups was detected measuring the serum IFN-γ and IL-12 titers using Mouse IFN-γ ELISA Total Kit (Thermo Scientific, MD, USA) and Mouse IL-12 ELISA Total Kit (Thermo Scientific), respectively, according to manufacturer's recommendations. The sera from the vaccinated mice were used as primary antibody. The amount of IFN-γ and IL-12 in the sera was calculated as pg/ml using a standard curve.

Statistical analysis

The data obtained from ELISA experiments were evaluated statistically via GraphPad Prism 5 Software (GraphPad Software, Inc., CA, USA) using one-way analysis of variance (ANOVA) and a *post-hoc* test (Tukey's or Dunnett's test). The mean and the standard deviation values were calculated using Microsoft Office Excel software (Microsoft Co., WA, USA).

Results

Cloning of erp, hspR, lppX, mmaA4*, and* ompA *genes*

The *erp, hspR, lppX, mmaA4*, and *ompA* genes from MTB strain 14/1649, a clinical isolate, were successfully cloned in pGEM-T Easy vector, their sequences were verified, and submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank) having the accession numbers of KY848243, KY848244, KY848245, KY848246, and KY848247, respectively. Next, each of these genes was successfully cloned in pET-28a(+) vector.

SDS-PAGE and Western blot analysis of the recombinant proteins

The recombinant proteins Erp, HspR, LppX, MmaA4, and OmpA were expressed using pET-28a(+) vector in *E. coli* BL21(DE) cells, and purified using nickel affinity chromatography. The predicted molecular weights (MWs) of the

recombinant proteins (including his-tags) were calculated using ProtParam tool (https://web.expasy.org/protparam) as 28.5 kDa for Erp, 15 kDa for HspR, 25 kDa for LppX, 35.5 kDa for MmaA4, and 34.5 kDa for OmpA. The observed MWs of the recombinant proteins on SDS-PAGE were ca. 31 kDa for Erp, 17 kDa for HspR, 31 kDa for LppX, 40 kDa for MmaA4, and 40 kDa for OmpA (Figure 1A). However, some other bands with different MWs were also observed due to the folding, cleavage, or possible dimerization of the proteins. Therefore, additional bands together with these main bands were detected in Western blot analysis when protein-specific sera were used (Figure 1B). A strong signal was obtained for the recombinant proteins, being weakest for LppX.

Antibody response against the vaccine formulations

The humoral immune responses against recombinant Erp, HspR, LppX, MmaA4, and OmpA proteins in BALB/c mice were evaluated quantitatively via ELISA. The total IgG levels were compared between sera collected at day 0 (pre-immunization) and at days 15, 30, 41, and 66 using one-way ANOVA and Dunnett's test (Figure 2). When pre-immunization values were used as control, the highest increase in humoral immune response was obtained against Erp and OmpA formulations with a statistical confidence of 99.9%, and remained high until the day 66. Next, HspR and MmaA4 formulations increased serum IgG levels after first vaccination with a confidence of 99% and 95%, respectively, and their confidence was increased to 99% after boost injection. The LppX formulation did not increase the IgG level significantly after the first injection, whereas an increment was observed after the second vaccination with a confidence level of 95% until the day 66.

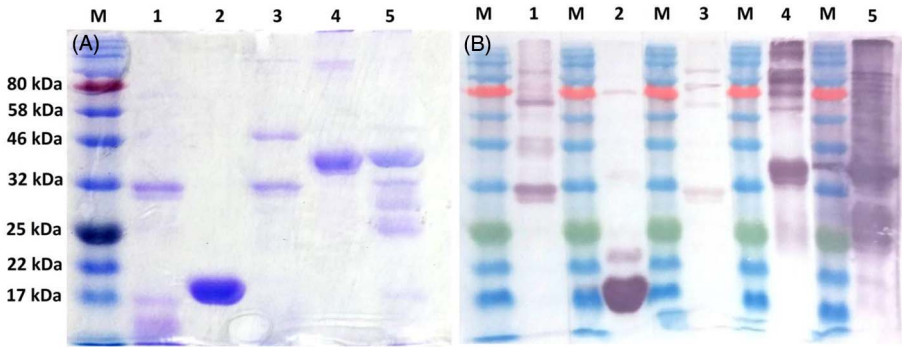

Figure 1. (A) SDS-PAGE and (B) Western blot analyses of the purified recombinant proteins. M: prestained marker (New England Biolabs P7712), 1: Erp, 2: HspR, 3: LppX, 4: MmaA4, and 5: OmpA

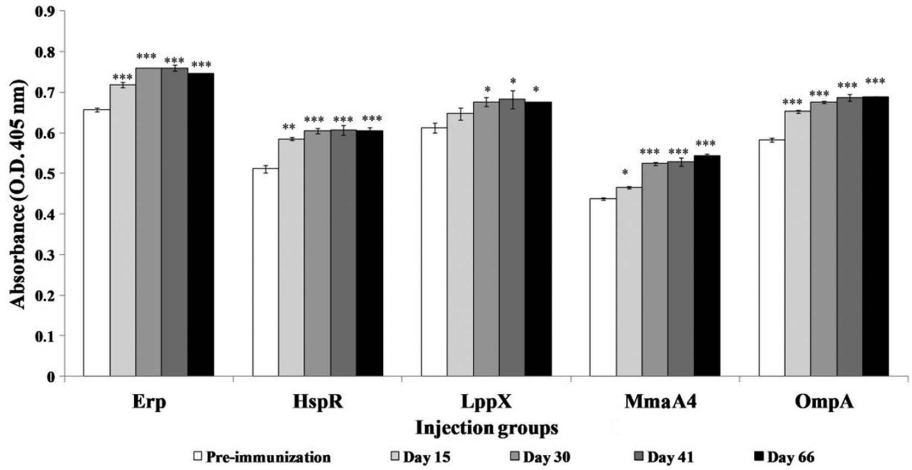

Figure 2. The serum IgG levels in BALB/c mice provided by the vaccine formulations prepared using Erp, HspR, LppX, MmaA4, and OmpA proteins together with Montanide ISA 720 VG adjuvant. 1:800 dilution values were used. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$

IFN-γ and IL-12 response against vaccine formulations

The cellular immune responses elicited by vaccine formulations in BALB/c mice were evaluated measuring the serum IFN-γ and IL-12 levels. The IFN-γ level belonging to each injection group at days 0, 15, 30, 41, and 66 (Figure 3) was compared with each other using one-way ANOVA and Tukey's test (Table I). At day 41, all vaccine groups, except OmpA, and at day 66, BCG, HspR, and MmaA4 groups increased the serum IFN-γ levels more than the adjuvant. Moreover, MmaA4 formulation elicited IFN-γ levels more than BCG after the day 30. There was no statistically significant difference between the IFN-γ levels boosted by BCG and HspR or LppX formulations. The difference in IFN-γ levels for BCG and Erp formulation was also not significant until the day 66, being higher for BCG at day 66. The best increment in serum IFN-γ levels was provided by MmaA4 formulation. Erp, HspR, and LppX formulations showed a potential as BCG in eliciting the serum IFN-γ levels. The increment in IFN-γ level provided by the OmpA formulation was found to be low.

The serum IL-12 levels boosted by the vaccination groups at days 0, 15, 30, 41, and 66 (Figure 4) were also compared with each other (Table II). The adjuvant itself increased the serum IL-12 levels as much as BCG until the day 41, and more than BCG at day 66. The highest IL-12 level was provided by HspR formulation at day 66. Erp formulation boosted IL-12 levels more than BCG at days 15 and 30. There was no significant difference in serum IL-12 levels belonging to MmaA4

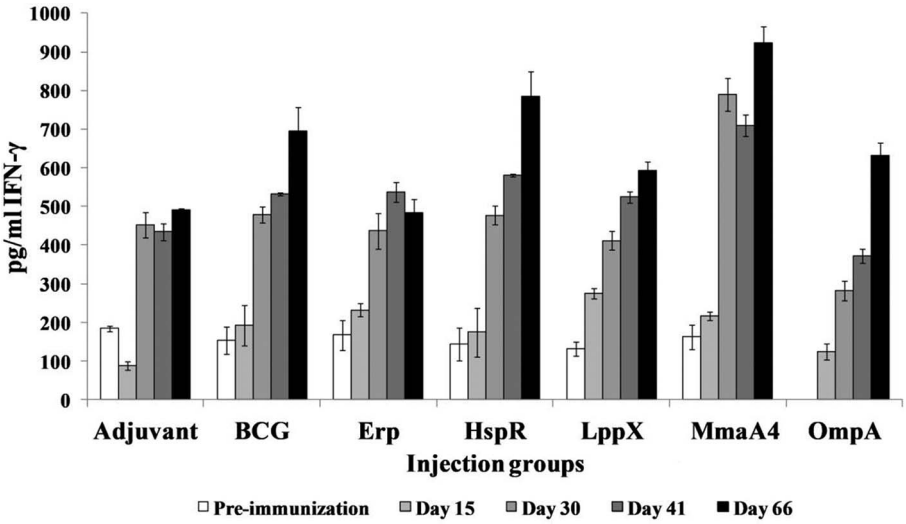

Figure 3. The serum IFN-γ levels in BALB/c mice conferred by the vaccine formulations prepared using Erp, HspR, LppX, MmaA4, and OmpA proteins together with Montanide ISA 720 VG adjuvant as well as adjuvant only and BCG vaccine

Table I. Analysis of variance for the serum IFN-γ levels in BALB/c mice, provided by the vaccine formulations

Injection groups	Pre-immunization	Day 15	Day 30	Day 41	Day 66
Adjuvant – BCG	NS	NS	NS	*	*
Adjuvant – Erp	NS	*	NS	**	NS
Adjuvant – HspR	NS	NS	NS	**	**
Adjuvant – LppX	NS	**	NS	*	NS
Adjuvant – MmaA4	NS	NS	***	***	***
Adjuvant – OmpA	**	NS	*	NS	NS
BCG – Erp	NS	NS	NS	NS	*
BCG – HspR	NS	NS	NS	NS	NS
BCG – LppX	NS	NS	NS	NS	NS
BCG – MmaA4	NS	NS	***	***	*
BCG – OmpA	*	NS	**	***	NS
Erp – HspR	NS	NS	NS	NS	**
Erp – LppX	NS	NS	NS	NS	NS
Erp – MmaA4	NS	NS	***	***	***
Erp – OmpA	**	NS	*	***	NS
HspR – LppX	NS	NS	NS	NS	*
HspR – MmaA4	NS	NS	***	**	NS
HspR – OmpA	*	NS	**	***	NS
LppX – MmaA4	NS	NS	***	***	**
LppX – OmpA	*	*	*	***	NS
MmaA4 – OmpA	**	NS	***	***	**

Note: Each injection group was compared with others to find out if there is a significant difference in IFN-γ levels. The increased or decreased levels can be seen in Figure 3. NS: not significant.
*$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

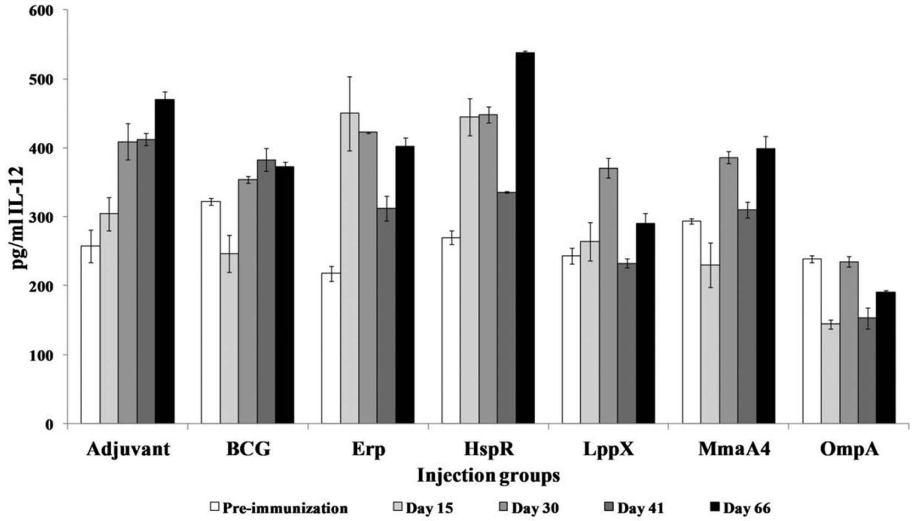

Figure 4. The serum IL-12 levels in BALB/c mice conferred by the vaccine formulations prepared using Erp, HspR, LppX, MmaA4, and OmpA proteins together with Montanide ISA 720 VG adjuvant as well as adjuvant only and BCG vaccine

formulation and BCG. HspR was the strongest and OmpA was the weakest formulation in boosting the serum IL-12 levels.

Discussion

The BCG is the only licensed vaccine used today against TB, a deadly disease killing millions of people each year. Although BCG protects the children against TB meningitis to some extent, the efficacy of this vaccine is variable in adults, and its usage is not recommended for people with immune deficiency [21]. Therefore, there are an increased number of studies related to the development of new vaccines against TB. Some of these studies report development of DNA vaccines. Dai et al. [21] showed that the IFN-γ, IL-2, and TNF-α levels were increased in lungs and spleen of neonatal mice injected with a DNA vaccine composed of *ag85b* gene. Similarly, Liang et al. [22] reported that DNA vaccines prepared with *rv2190c* or *ag85a* provided increased IFN-γ levels and decreased number of MTB in lungs and spleen of mice. The genes encoding PE and PE_PGRS cell surface proteins [23], Hsp65 and IL-12 combination [24], or *ag85a*/*b* chimera [25] were also used in experimental DNA vaccines. Some other studies utilized proteins, such as TFP846 fusion composed of Rv3615c, Mtb10.4,

Table II. Analysis of variance for the serum IL-12 levels in BALB/c mice, provided by the vaccine formulations

Injection groups	Pre-immunization	Day 15	Day 30	Day 41	Day 66
Adjuvant – BCG	*	NS	NS	NS	**
Adjuvant – Erp	NS	NS	NS	**	*
Adjuvant – HspR	NS	NS	NS	*	*
Adjuvant – LppX	NS	NS	NS	***	***
Adjuvant – MmaA4	NS	NS	NS	**	*
Adjuvant – OmpA	NS	*	***	***	***
BCG – Erp	**	*	*	*	NS
BCG – HspR	NS	*	**	NS	***
BCG – LppX	**	NS	NS	***	**
BCG – MmaA4	NS	NS	NS	*	NS
BCG – OmpA	**	NS	**	***	***
Erp – HspR	NS	NS	NS	NS	***
Erp – LppX	NS	*	NS	*	***
Erp – MmaA4	*	**	NS	NS	NS
Erp – OmpA	NS	**	***	***	***
HspR – LppX	NS	*	*	**	***
HspR – MmaA4	NS	**	NS	NS	***
HspR – OmpA	NS	**	***	***	***
LppX – MmaA4	NS	NS	NS	*	**
LppX – OmpA	NS	NS	***	*	**
MmaA4 – OmpA	NS	NS	***	***	***

Note: Each injection group was compared with others to find out if there is a significant difference in IL-12 levels. The increased or decreased levels can be seen in Figure 4. NS: not significant.
*$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

and Rv2660c antigens [26], AERAS-402 vaccine including 85A, 85B, and TB10.4 antigens [27], and adenovirus expressing CFP10, ESAT6, Ag85A, and Ag85B proteins [28].

In addition to these reports utilizing similar antigens, new candidate proteins and formulations have also been investigated to develop TB vaccines. The hypothetical nitroreductase Rv3131 protein in the MTB-specific DosR dormancy regulon increased TNF-α, IFN-γ, and IL-2 levels in lungs and spleen of mice, and decreased the bacterial load [29]. Larrouy-Maumus et al. [30] formulated two mycobacterial lipid antigens, Ac2SGL and PIM2, with the liposomes dimethyl-dioctadecyl-ammonium and synthetic trehalose-6, 6′-dibehenate. These formulations provided increased protection against MTB infection in the spleen of guinea pigs, whereas no statistically significant difference was found in lungs as compared to non-vaccinated animals. Xue et al. [31] reported that the recombinant RpfE protein from MTB induced immune responses in mice.

In this study, the Erp, HspR, LppX, MmaA4, and OmpA proteins of MTB were expressed in and purified from *E. coli* as well as their usability in development of a recombinant subunit TB vaccine was evaluated in mice, for the first time. The cell surface components Erp, MmaA4, and OmpA and the transcriptional regulator HspR are among the virulence factors of MTB. The Erp is required for the formation of cell wall structure in *M. smegmatis* [32], and plays role in the virulence of MTB [33, 34]. In addition, Martinez et al. [35] showed that the Erp can be used to differentiate the TB infection and disease in human. Another cell wall component, OmpA, is a virulence factor protecting the MTB against acidity in host phagosomes [36]. The IL-12 production in macrophages is repressed in MTB infection, and one of the repressors is MmaA4 protein responsible for modification of mycolic acid [37]. The BCG mutant with *mmaA4* gene deletion was reported to provide increased protection against TB [38]. The Hsp is also among the potent antigens [39]. Increase in the amount of Hsp may trigger the immune responses against MTB infection [40]. Bell et al. [39] reported that the LppX, a lipoprotein, was found in high amount among the culture filtrate proteins of MTB. On the other hand, a gene from *M. bovis* BCG with 98% identity to LppX was evaluated as a DNA vaccine, but a successful result could not be obtained [41]. Nevertheless, as the strong antigenic properties of lipoproteins [39] were taken into account, evaluation of the antigenic capacity of LppX from a local MTB isolate together with a suitable adjuvant was decided to be useful in this study. Here, the effect of formulations, prepared by mixing the recombinant Erp, HspR, LppX, MmaA4, or OmpA protein with Montanide ISA 720 adjuvant, on the cellular immune responses in BALB/c mice was evaluated by measuring the serum IFN-γ and IL-12 levels. The MmaA4 formulation provided the highest increment in serum IFN-γ level. The Erp, HspR, and LppX formulations were as effective as BCG vaccine in boosting the IFN-γ level. The OmpA formulation did not provide a statistically significant increase in the IFN-γ level. On the other hand, HspR formulation conferred higher IL-12 level than adjuvant and other vaccine groups at day 66, whereas Erp formulation provided higher IL-12 level than the BCG at days 15 and 30. The IL-12 level conferred by MmaA4 formulation was found to be similar to that by BCG. The most successful formulation increasing the serum IL-12 level determined was HspR, and the weakest was OmpA.

Although the importance of cellular immune responses has been mentioned in the studies related to the development of vaccines against TB, recently Prados-Rosales et al. [42] reported that the conjugate vaccine, prepared by mycobacterial capsular arabinomannan and Ag85b, provided lowered MTB count in lungs and spleen and increased lifetime together with higher antibody (IgG) response in mice as compared to control group, showing the contribution of humoral immunity in the protection against MTB infection. The vaccine formulations

prepared in this study conferred significant increment in serum IgG levels in BALB/c mice, which might be useful for the development of a TB vaccine.

Conclusions

The vaccine formulations prepared using Erp, HspR, LppX, MmaA4, and OmpA proteins from MTB and Montanide ISA 720 VG adjuvant successfully induced serum IgG levels in BALB/c mice. The MmaA4 formulation provided higher serum IFN-γ and similar IL-12 levels, and the HspR formulation provided higher IL-12 and similar IFN-γ levels as compared to commercial BCG vaccine. The Erp formulation was also prospering in induction of the cellular immune responses. Consequently, the MmaA4, HspR, and Erp proteins from MTB were shown to be successful in triggering both humoral and cellular immune responses in BALB/c mice. In future studies, the efficacy of double or triple fusions of MmaA4, HspR, and Erp proteins may be evaluated.

Acknowledgements

This study is funded by The Scientific and Technological Research Council of Turkey (TÜBİTAK) with the project number of 214S359. The authors would like to thank Dr. Erkan Özcengiz for his valuable suggestions.

Conflict of Interest

The authors declare no conflict of interest.

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