Quantiation of IL-4, IL-10 and IFN-γ Genes Expression after Immunization of Mice with CFP-10 and ESAT-6 Containing Vectors

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ABSTRACT

Background: Tuberculosis is a disease with high morbidity, caused mainly by *Mycobaterium tuberculosis (M.tb.)*. DNA vaccines show a promising future due to their unique advantages over conventional methods. The early-secreted antigen target (ESAT)-6 and culture filtrate protein (CFP)-10 of *M.tb.* antigens have been identified as vaccine candidates against Mycobacteria and used as subunit vaccines, DNA or protein, in different studies. **Objective:** To investigate the potential of pcDNA3.1+ plasmid containing CFP-10 and ESAT-6 genes in induction of local immune responses after intramuscular injection in BALB/c mice. **Methods:** pcDNA 3.1+ CFP-10 and pcDNA3.1+ ESAT-6 plasmids were prepared and defined groups of mice were injected intramuscularly with the plasmids both separately and in combination. The RNA was extracted from muscles after one month and cDNA was made using RT-PCR. The expressions of IL-4, IL-10 and IFN-γ genes cytokines were evaluated using comparative real time PCR. **Results:** Expression of IL-4 and IL-10 increased in the injection site of the mice groups which received plasmids encoding ESAT-6 and CFP-10 individually or together. More than 10-fold increase in IFN-γ expression was found in samples taken from mice groups inoculated by plasmids encoding ESAT-6 and CFP-10 individually or together. **Conclusion:** pcDNA 3.1+ESAT-6 and pcDNA3.1+CFP-10 plasmids can increase the expression of IFN-γ in mice after immunization.

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Keywords: CFP-10, DNA Vaccine, ESAT-6, Mycobacterium, PPD, Real Time PCR

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INTRODUCTION

Tuberculosis outbreaks cause 2-3 million deaths in the world annually (1). Pathogenic *Mycobacteria*, including *Mycobacterium (M.) tuberculosis* and *M. bovis*, cause significant morbidity and mortality worldwide. 10% of human tuberculosis has been considered as a zoonotic disease caused by it (2). The protection by bacillus Calmette-Guerin (BCG) vaccine has been found inconsistent and controversial (3).

The pathogenic *Mycobacteria* can survive inside the host macrophages. Recent studies are looking for antigens that are shared in pathogenic *Mycobacteria*. The secretory proteins of *M. tuberculosis and M. bovis* have gained interest in recent years both as vaccine candidates and diagnostic tools; they may also be involved in the clinical symptoms of the disease and evoking protective responses (4).

In *Mycobacterium* infections, as intracellular diseases type, a TH1 immune response confers protection, while a shift to TH2 response is unfavorable to the host and is pathogenic (5). To skew the immune responses to a favorite response, the nature of antigens in vaccine must be well investigated. The 10-kDa culture filtrate protein (CFP-10) and 6-kDa early-secreted antigen target (ESAT-6) of Mycobacterium are two T cell antigens, present in virulent *M. tuberculosis* and *M. bovis* but absent in bacillus *Calmette-Guerin* (BCG) vaccine strain (6). These proteins forms a 1:1 heterodimeric complex, known as CE protein (7). The heterodimer plays a key role in tuberculosis pathogenesis and in the stimulation of immunity (8).

One of challenging component of vaccine design is the delivery of vaccine antigens to the host by DNA vaccine. Once the DNA construct is injected the host cells take up the foreign DNA, expressing the gene and producing the corresponding viral protein inside the cell. This form of antigen presentation and processing induces both MHC class I and class II restricted cellular responses as well as humoral immune responses (9).

Measurement of cytokine levels in tissue samples after antigens inoculation can provide information regarding induced immune responses. It is proposed that after injection of naked DNA encoding genes of interest to tissues (muscle), local expression of proteins; induce a local response which is followed by systemic immune responses. Quantitation of local expression of cytokines in tissues is a key objective of induced primary immune responses (10).

In several recent reports, the patterns of cytokines expression have been described in blood, spleen and lymph nodes peripheral mononuclear cells (PBMCs) of infected or immunized animals and human with *Mycobacterium* antigens (11-15). These studies did not provide an overview of cytokine gene expression in injected tissues with DNA constructs at first steps of gene expression.

In order to characterize the mechanisms by which plasmids encoding CFP-10 and ESAT-6 antigens, as potential DNA vaccine candidates against *Mycobacterium* infection, we evaluated the local induced immune responses. To do this, the eukaryotic plasmids encoding CFP-10 and ESAT-6 were prepared and BALB/c mice were inoculated intramuscularly with these both or individually and relative real time PCR was used to measure the expression of IL-4, IL-10 and IFN-γ genes in muscle samples from injection sites.

MATERIALS AND METHODS

Female BALB/c mice, 6-8 weeks of age, were provided by the animal house of RVSRI. All chemicals were purchased from Merck Company, Inc. (Germany).

Plasmid Preparation. pcDNA 3.1+ESAT-6 and pcDNA3.1+CFP-10 plasmids were purified from transformed *E. coli* Top 10 cells by alkaline lysis method and were dissolved in sterile PBS, pH 7.2 and stored at -20°C (25). The plasmids concentrations were measured by Nanodrop at 260 nm.

Inoculation of Mice with Plasmids Encoding ESAT-6 and CFP-10 Genes and Muscle Sampling. 100 µg of DNA resuspended in 100 µl of PBS was injected in right quadriceps muscle by 30 G insulin syringes. The 28 mice, 4 in each group, were set as was shown in Table 1. After 30 days, the tissue sampling was performed from right quadriceps muscle of all mice. The tissue samples were kept in -70°C.

Table 1. Description of inoculation protocol.

Group	Explanation	Abbreviation
I	pcDNA3.1+/ESAT-6	ES
II	pcDNA3.1+/ESAT-6 + pcDNA3.1+/CFP-10	ES+CF
III	pcDNA3.1+/CFP-10	CF
IV	pcDNA3.1+	pcDNA
V	PPD	PPD
VI	Control (no injection)	CNT-
VII	Control (PBS injection)	PBS

RNA Extraction and Reverse-Transcription (RT)-PCR. Total RNA extraction from muscles was performed using Trizol (Invitrogen), treated with RNase-free DNase (Ambion, Austin, TX) to remove any residual genomic DNA that may be present in the RNA. Then RNA reverse transcribed as following:

Reverse transcription of target RNA (1–5 µg) was carried out in a 12.5 µl final volume using 1.5 µl of 10X MULV Buffer, 0.5 µl of 0.2 mM dNTP mix (Fermentas), 0.25 µl of 40 U/µl ribonuclease inhibitor (Fermentas), 0.5 µl of 0.5 µg Oligo dT primers (Fermentas), 0.25 µl of 200 U/µl MULV reverse transcriptase (Life Technologies) and 8 µl of Diethylpyrocarbonate (DEPC)-treated water.

For every reaction set, one RNA sample was performed without MULV reverse transcriptase to provide a negative control in subsequent PCR reactions. The reverse transcription reactions were performed using a PCR thermal cycler with incubation in 25°C for 10 min and 42°C for 60 min.

Primers were designed with *Primer Premier* Software on the basis of mRNA sequences available on NCBI and previously published studies for the relative quantification of target genes expression (IFN-γ, IL-4 and IL-10), and glyceraldehyde-3-phosphate

dehydrogenase gene (GAPDH), was used as the reference gene. The primers were synthesized by Bioneer Company (Korea). A brief description of the target genes and primers are listed in Table 2.

Table 2. Used primers for quantitative real time PCR.

Target Gene		Sequence (5′-3′)	Product Size (bp)
GAPDH	*Forward* *Reverse*	TTCACCACCATGGAGAAGGC GGCATGGACTGTGGTCATGA	236 bp
IL-4	*Forward* *Reverse*	ACAGGAGAAGGGACGCCAT GAAGCCCTACAGACGAGCTCA	95 bp
IFN-γ	*Forward* *Reverse*	TCAAGTGGCATAGATGTGGAAGAA TGGCTCTGCAGGATTTTCATG	92 bp
IL-10	*Forward* *Reverse*	GGTTGCCAAGCCTTATCGGA ACCTGCTCCACTGCCTTGCT	190 bp

Reverse-transcription PCR quantification of cytokine gene expression was done using standard curves. mRNA levels were assessed by conventional and quantitative RT-PCR**.** Amplification was performed with Step one AB (Applied Biosystems) using SYBR Green PCR Master Mix (Fermentas) in a total volume of 12.5 µl, containing 0.5 µl cDNA sample, and 0.25 µM each primer and 5.25 µl ddH2O. Quantitative PCR amplification was performed for 40 cycles at 95°C for 10 s, specific annealing temperature for 30 s and 72°C for 30 s. Amplification specificity was checked using melting curve. The endogenous housekeeping gene, GAPDH, was used to normalize target gene expression. A no-template control cDNA was included to detect contamination or non-specific reactions.

Data analysis and calculations for relative quantification

Quantification of cytokine gene expression was calculated by the comparative *CT* method as described by Livak and Schmittgen (17,18). This method compares test samples to a comparator sample (non treated sample, control group) and uses results obtained with a uniformly expressed internal control gene (GAPDH) to correct for differences in the amounts of RNA present in the two samples being compared to generate a Δ*CT* value [Δ*CT* = (*CT* gene of interest - *CT* internal control)]. Results are expressed as the degrees of difference between Δ*CT* values of test and comparator samples [ΔΔ*CT* = Δ*CT* (test sample) - Δ*CT* (comparator sample)] and relative quantification was calculated as $2^{-\Delta\Delta CT}$. If the fold-change is greater than 1, then the result may be reported as a fold up-regulation. If the fold-change is less than 1, then the negative inverse of the result may be reported as a fold down-regulation.

Statistical analyses were performed with *GraphPad Prism*, Version 4.03 (GraphPad software, San Diego, CA). All values were expressed as mean ± standard deviation. Statistical analyses of the data were performed using a one-way analysis of variance (ANOVA). Differences in quantitative cytokine mRNA expression were analyzed using

Student's t-test. In all cases, probability p values below 0.05 were considered significant.

RESULTS

The pcDNA3.1+ESAT-6 and pcDNA3.1+CFP-10 plasmids were purified and injected to several groups of mice individually and/or combined. Three different control groups, to observe the effect of used diluent (PBS), backbone of plasmids (pcDNA3.1+) and the expression background (no injection) were incorporated. After 30 days, the RNA extracted from muscle samples were converted to cDNA and real time PCR was performed using specific primers for IL-4, IL-10 and IFN-γ genes. The GAPDH gene was used as an internal control gene to find relative expression. Calculation with the comparative *CT* method was concluded to the following results. The summaries of results are found in Tables 3 and 4.

Table 3. The comparison of fold changes between treated groups by Bonferroni's Multiple Comparison Test. Significant results are identified with bold font in shaded cell.

Bonferroni's Multiple Comparison Test	P Value		
	IL-4	IL-10	IFN-γ
ES vs ES+CF	P > 0.05	P > 0.05	P > 0.05
ES vs CF	P > 0.05	P > 0.05	P > 0.05
ES vs CNT-	P > 0.05	P > 0.05	P > 0.05
ES vs PBS	P > 0.05	P > 0.05	P > 0.05
ES vs pcDNA	P > 0.05	P > 0.05	P > 0.05
ES vs PPD	P > 0.05	P > 0.05	P < 0.01
ES+CF vs CF	P > 0.05	P > 0.05	P > 0.05
ES+CF vs CNT-	P > 0.05	P > 0.05	P < 0.05
ES+CF vs PBS	P > 0.05	P > 0.05	P < 0.05
ES+CF vs pcDNA	P > 0.05	P > 0.05	P < 0.05
ES+CF vs PPD	P > 0.05	P > 0.05	P > 0.05
CF vs CNT-	P > 0.05	P > 0.05	P > 0.05
CF vs PBS	P > 0.05	P > 0.05	P > 0.05
CF vs pcDNA	P > 0.05	P > 0.05	P > 0.05
CF vs PPD	P > 0.05	P > 0.05	P < 0.05
CNT- vs PBS	P > 0.05	P > 0.05	P > 0.05
CNT- vs pcDNA	P > 0.05	P > 0.05	P > 0.05
CNT- vs PPD	P > 0.05	P > 0.05	P < 0.001
PBS vs pcDNA	P > 0.05	P > 0.05	P > 0.05
PBS vs PPD	P > 0.05	P > 0.05	P < 0.001
pcDNA vs PPD	P > 0.05	P > 0.05	P < 0.001

Table 4. Mean of fold change (ΔΔCT) ± SD (standard deviation) between treated groups.

Mean (Fold change) ΔΔ*CT* ± SD	ES	ES+CF	CF	CNT-	PBS	pcDNA	PPD
IFN-γ	4.217 ± 1.790 ↑	6.935 ± 2.698 ↑*	4.844 ± 2.707 ↑	0.6773 ± 0.215	0.6135 ± 0.142	0.4473 ± 0.235	10.99 ± 3.406 ↑*
IL-4	2.288 ± 0.632 ↑	1.506 ± 0.995 ↑	2.120 ± 0.914 ↑	0.6050 ± 0.158	0.5726 ± 0.144	0.6725 ± 0.151	1.455 ± 0.656 ↑
IL-10	1.490 ± 0.932 ↑	1.795 ± 0.994 ↑	1.572 ± 1.233 ↑	0.5218 ± 0.0878	0.5234 ± 0.120	0.4927 ± 0.102	0.7271 ± 0.351 ↓

IL-4 Expression in Muscle Samples. The expression of IL-4 gene was increased within muscle samples taken from groups which received plasmids encoding ESAT-6 (2.288 ± 0.632), CFP-10 (2.120 ± 0.914) individually or together (1.506 ± 0.995) and PPD group (1.455 ± 0.656). No significant changes were observed for these fold changes compared with control groups. Figure 1 shows these findings.

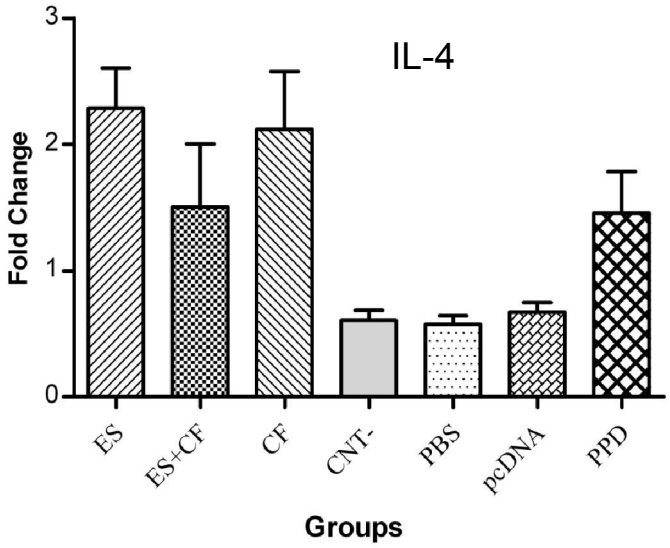

Figure 1. Fold change (ΔΔCT) of IL-4 expression in different treated groups obtained by real time PCR. None of changes was found significant compared to control groups.

IL-10 Expression in Muscle Samples. The expression of IL-10 gene was increased within muscle samples taken from groups which received plasmids encoding ESAT-6 (1.490 ± 0.932), CFP-10 (1.572 ± 1.233) individually (1.506 ± 0.995) or together (1.795 ± 0.994). In PPD group a small decrease (0.7271 ± 0.351) was observed. None of these fold changes were not significant compared with control groups. Figure 2 shows these results.

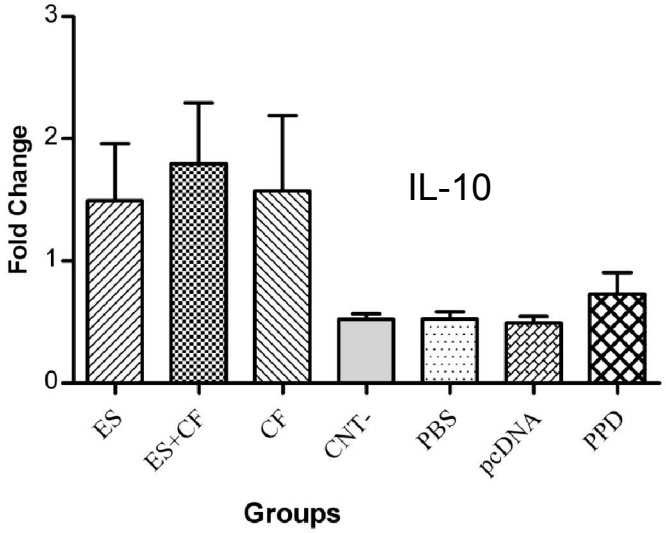

Figure 2. Fold change (ΔΔCT) of IL-10 expression in different treated groups obtained by real time PCR. None of changes was found significant compared to control groups.

IFN-γ Expression in Muscle Samples. The results (Figure 3) showed increase in IFN-γ expression within muscle samples taken from groups which received plasmids encoding ESAT-6 (4.217 ± 1.790), CFP-10 (4.844 ± 2.707) individually or together (6.935 ± 2.698) and PPD group. PPD and ES+CF groups showed significant increase compared with control groups with more than 10- fold and 6- fold changes, respectively.

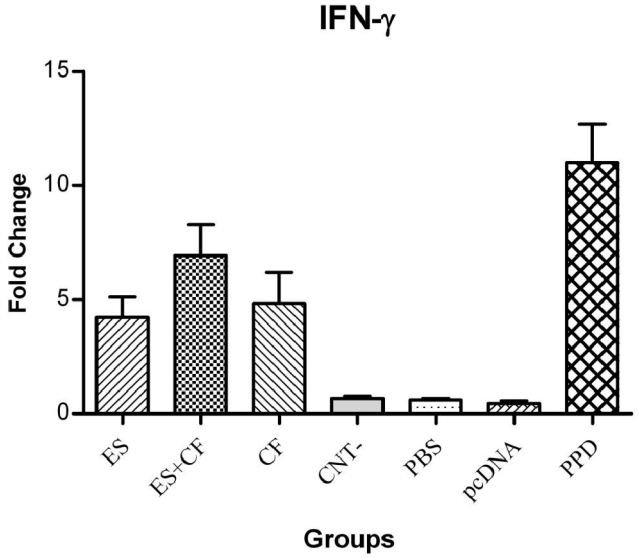

Figure 3. Fold change (ΔΔCT) of IFN-γ expression in different treated groups obtained by real time PCR. Groups that received pcDNA3.1+/ESAT-6 and pcDNA3.1+/CFP-10 and PPD showed elevated levels of IFN-γ significantly ($p<0.05$).

DISCUSSION

Tuberculosis is a contagious disease that is easily spread, caused more by *M. tuberculosis* and to a lesser extent by *M. bovis*. Control strategies of tuberculosis primarily rely on the vaccination. The only currently available vaccine is BCG; while it is effective against disseminated disease in childhood, confers inconsistent protection against contracting pulmonary TB (19). However, the immunity induced by BCG vaccination decreases after about ten years. A number of new vaccines are currently under development to make more effective and safe TB vaccines (20).

DNA vaccines, a strategy that relies on inoculation of DNA coding for immunogenic antigens rather than the immunogenic peptide, have been proposed as a possible key for the prevention of tuberculosis (21). Potential advantages of these vaccines over standard subunit peptide vaccines include diminished cost of production, increased stability and ease of use, enhanced efficacy, and improved safety. These vaccines are believed to work by introducing DNA encoding for an immunogenic peptide from a pathogen into a small number of antigen-presenting cells, which transcribe, translate, process, and present the antigen to the immune system in the context of class I and II MHC. This presentation induces both a humoral and a cellular immune response (22). Animal studies have demonstrated that DNA vaccines against tuberculosis are feasible and efficacious. There are many evidences that DNA vaccines may be superior to BCG vaccine (21).

T cell-mediated cellular immune response is a key immune response for effective protective immunity against TB. DNA vaccines have an intrinsic adjuvant effect because of the presence of immune stimulatory CpG dinucleotides in base contexts which shift the immune response to cellular immunity (23).

DNA vaccines encoding Mycobacterial secretory antigens, ESAT-6 and CFP-10, are found as promising future *Mycobacterium* vaccines (6,24,25).

In this study, pcDNA3.1+ESAT-6 and pcDNA3.1+CFP-10 plasmids were used as a DNA vaccine based and BALB/c mice were injected intramuscularly by those, individually or together. To the best of our knowledge in all published evaluation studies of the immune responses raised by ESAT-6 and CFP-10 antigens, serological assays and in vitro TH cytokine assays have been done on sera and/or lymphocytes in lymph nodes or blood (26,27). To find weather injection of these plasmids can induce local immune responses, cytokine expression was investigated within muscles at injection sites 1 month after plasmids injection. Due to the main role of T-cell derived cytokines in the regulation of immune responses, assessment of cytokine milieu is a principal part of study on efficacy of vaccine candidates.

IL-4 is known as a major indicator of TH2 type cells which skews immune responses to humoral immunity. IL-10 and IFN-γ have been introduced as TH1 type cells associated with cellular immunity. IFN-γ plays a major role in inducing protective immune response against Mycobacterial infections. It is proposed that an alteration in TH1/TH2 cytokine balance is happen in patients with advanced clinical tuberculosis (28).

Here, the total RNA was extracted from muscles, reverse transcribed to cDNA and expression of IL-4, IL-10 and IFN-γ genes were compared with control samples using real time PCR.

We found changes in expression of these cytokines in mice received plasmids encoding ESAT-6 and CFP-10 proteins and group that received PPD. The expression of IL-4 was elevated 2 times more in ESAT and CF groups than control groups and about 1.5 times

more in ESAT+CF and PPD groups. However, these elevated levels were not found statistically significant. It seems that using two proteins together and PPD, a mix of many proteins, have less effect on increasing the IL-4 gene expression level. The roles of IL-4, IL-10 and IFN-γ in Mycobacterial infections are well studied (29). In some reports the elevated levels of cytokines have been found while others were found decreased. This inconsistency reflects the different effects of Mycobacterial antigens. It has been reported previously that IL-4 exerts a suppressor effect on production of IFN-γ, IL-1, TNF, IL-1, IL-6 and IL-2 (30). Study of Mycobacterial infection in IL-4 knockout mice has detected that IL-4 may play a protective role in defense against Mycobacteria (31)

IL-10 expression was found about 1.5 times more in ESAT, CF and ESAT+CF groups than control groups. In contrast in PPD group less expression was observed. IL-10 is a product of TH2 clones that blocked cytokine production from TH1 clones (48). IL-10 achieved this effect by inhibiting the ability of macrophages and dendritic cells (DCs) to activate TH1 cells. IL-10 has been identified as a correlate of susceptibility for tuberculosis (TB) in both mice and humans (33).

Our results showed feasibility of pcDNA3.1+ESTA-6 and pcDNA3.1+CFP plasmids to induce IFN-γ expression within muscle samples about 4 times more than controls. These changes were found about 7 and 10 folds when used combined plasmids and PPD, respectively. IFN-γ is thought to be a principal mediator of macrophage activation and resistance to intracellular pathogens (34). Macrophages are the main host cells of *Mycobacterium*. Pathogenic Mycobacteria can survive in resting macrophages. IFN-γ activated macrophages and inhibit the growth of *Mycobacterium* maybe by production of reactive oxygen or nitrogen intermediates, the products of NADPH oxidase and nitric oxide synthase (NOS2) (35).

To sum up these finding, it seems that introducing ESAT-6 and CFP-10 genes in the pcDNA3.1+ plasmid vehicle, may be useful to induce desirable immune responses to fight against tuberculosis. Using these plasmids together has a synergic effect on change of cytokine profile. Using of control group confirmed that cDNA3.1+ plasmid and PBS as diluent do not have any effects on expression levels of cytokines.

These results support of potential of pcDNA3.1+ESAT-6 and pcDNA3.1+CFP-10 plasmids in the induction of immune response even locally in muscle cells. These data could serve as a basis for further studies on the usefulness of these constructs in development of DNA vaccine and the correlation of the level of cytokines expression in muscle with serum.

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