

Macrophage Immune Response Suppression by Recombinant *Mycobacterium tuberculosis* Antigens, the ESAT-6, CFP-10, and ESAT-6/CFP-10 Fusion Proteins

Atefeh Seghatoleslam^{1,2}, PhD;
Mina Hemmati^{1,3}, PhD;
Saeedeh Ebadat¹, MS;
Bahram Movahedi¹, MS;
Zohreh Mostafavi-Pour^{1,4}, PhD

¹Recombinant Protein Laboratory, Biochemistry Department, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran;

²Histomorphometry and Stereology Research Center, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran;

³Department of Biochemistry, Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran;

⁴Maternal-Fetal Medicine Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Correspondence:

Zohreh Mostafavi-Pour, PhD;
Department of Biochemistry, Fifth Floor,
Building No. 3, Medical School, Zand
Street, Shiraz, Iran
Tel/Fax: +98 71 32303029
Email: zmostafavipour88@yahoo.co.uk
Received: 03 January 2016
Revised: 25 January 2016
Accepted: 31 January 2016

What's Known

- *Mycobacterium tuberculosis* (*M. tb*) pathogenesis is dependent on T cells and macrophage response to mycobacterium antigens. ESAT-6 and CFP-10 are 2 secretory antigens affecting the host's immune response. Immune response against *M. tb* plays a key role in the outcome of TB infection.

What's New

- ESAT-6 and CFP-10 inhibited 2 important arms of immune response in macrophages, ROS and NO production, through direct effects on iNOS enzyme activity.
- This effect was notable in the ESAT-6/CFP-10 protein in comparison with the antigens alone. Very low concentrations of ESAT-6/CFP-10 suppressed macrophage immune response without any side effect on cell viability.

Abstract

Background: Macrophage immune responses are affected by the secretory proteins of *Mycobacterium tuberculosis* (*Mtb*). This study aimed to examine the immune responses of macrophages to *Mtb* secretory antigens, namely ESAT-6, CFP-10, and ESAT-6/CFP-10.

Methods: THP-1 cells (a human monocytic cell line) were cultured and differentiated to macrophages by phorbol 12-myristate 13-acetate. The cytotoxicity of the recombinant *Mtb* proteins was assessed using the MTT assay. Two important immune responses of macrophages, namely NO and ROS production, were measured in response to the ESAT-6, CFP-10, and ESAT-6/CFP-10 antigens. The data were analyzed using one-way ANOVA with SPSS, version 16, and considered significant at $P < 0.05$.

Results: The results showed that the ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins markedly reduced macrophage immune response. The treatment of the THP-1-differentiated cells with ESAT-6, CFP-10, and ESAT-6/CFP-10 reduced NO and ROS production. The treated THP-1-differentiated cells exhibited less inducible NO synthase activity than did the untreated cells. No toxic effect on macrophage viability was observed for the applied proteins at the different concentrations.

Conclusion: It seems that the decline in macrophage immune response is due to the suppression of NO and ROS production pathways without any effect on cell viability.

Please cite this article as: Seghatoleslam A, Hemmati M, Ebadat S, Movahedi B, Mostafavi-Pour Z. Macrophage Immune Response Suppression by Recombinant *Mycobacterium tuberculosis* Antigens, the ESAT-6, CFP-10, and ESAT-6/CFP-10 Fusion Proteins. Iran J Med Sci. 2016;41(4):296-304.

Keywords • *Mycobacterium Tuberculosis* • Recombinant Proteins • ESAT-6 protein • CFP-10 protein • Reactive Oxygen Species

Introduction

Tuberculosis (TB) is a public health challenge that contributes to the illness and death of 2-3 million people every year around the world. About one-third of the world's population has latent TB. Individuals with latent TB are defined as those who possess TB bacteria but have yet to demonstrate the clinical signs of the disease. The state of being infected by TB bacteria brings about a 10% lifetime risk of illness, more so in those with immune defects,

HIV infection, malnutrition, diabetes, or smoking habits.¹ TB accounts for about 3 million deaths, with 9 million new cases of the disease diagnosed each year. According to the reports by the World Health Organization (WHO), 95% of the cases occur in developing countries.¹ In Iran, the trend of TB is decreasing and in 2013, the incidence rate was 13.7 per 100,000 population.²

The immune response against TB plays a fundamental role in the outcome of *Mycobacterium tuberculosis* (*Mtb*) infection. The *Mtb* genome (H37Rv) has 4,019 genes coding for various proteins (*Mtb* genes), from which more than a quarter have been categorized as hypothetical in resources such as TubercuList.^{3,4} Two secreted proteins, namely 6-kDa early secreted antigen target (ESAT-6) and 10-kDa culture filtrate protein (CFP-10), encoded from the region of difference 1 (RD1) in the *Mtb* genome, play important roles in the pathogenesis of TB in primary pulmonary infection.⁵ ESAT-6 and CFP-10 are recognized as important stimulators of T cells both *in vitro* and *in vivo*.⁶ During the early stages of human TB infection, *Mtb* induces an immune response leading to lung granulomas consisting of macrophages, T cells, B cells, and fibroblasts.⁷ *Mtb* mainly targets macrophages as hosts, and the following immune dysfunction of the infected macrophages is pathognomonic to TB.⁸ Phagocytic cells such as macrophages are among the most important components of the innate immune response, which is the first line of host defense. Reactive oxygen species (ROS) and nitric oxide (NO), generated from the nicotinamide adenine dinucleotide phosphate (NADPH) phagocyte oxidase and inducible nitric oxide synthase (iNOS) pathways, respectively, are 2 of the most important antimicrobial systems of phagocytic cells.^{9,10} Macrophages are the main cells playing a role in phagocytosis and presenting mycobacterial antigens to T cells. Specific receptors on T lymphocytes recognize the antigens and release activation signals that induce the production of cytokines such as interferon gamma (IFN- γ).¹¹ This cytokine acts with some mycobacterial compounds to promote macrophage activation.¹² The first interaction between macrophages and *Mtb* is thought to play a key role in determining the outcome of infection. Bacterial products are recognized by receptors on macrophages. These receptors include CD14 and Toll-like receptors (TLRs), which rapidly signal the presence of the pathogen through the transcription nuclear factor-kB (NF-kB), resulting in the transcription of macrophage genes responsible for an effective immune response.¹³ NADPH phagocyte oxidase and iNOS are expressed in both polymorphonuclear

and mononuclear phagocytes, although the amount of ROS produced is greater in neutrophils than that in macrophages, and macrophages generally produce considerably more reactive nitrogen species (RNS) than do neutrophils.^{10,14} RNS such as NO are required for the destruction of intracellular *Mtb* bacilli in a murine model.^{15,16} The effector mechanisms involved in direct *Mtb* killing or infected cell lysis by CD4⁺ T and CD8⁺ T lymphocytes have been previously described.^{17,18} However, *Mtb* can still survive in macrophages. Despite progress in understanding the pathogen-host relationship, the mechanisms of the protection against and the pathogenesis of this disease remains unclear. This study aimed to investigate NO and ROS production in response to the recombinant secretory antigens of *Mtb*: ESAT-6, CFP-10, and ESAT-6/CFP-10.

Materials and Methods

Cell Culture

The cells were cultured at $1-4 \times 10^6$ cells/mL in RPMI 1640 (Biosera, Kansas City, USA), containing 10% heat-inactivated FBS, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin, and maintained at 37°C under 5% CO₂. In all the experiments, THP-1 (Pasteur Institute, Tehran, Iran) monocytic cells (1×10^6 cells) were differentiated into macrophages in a T25 Cell Culture Flask containing 3 mL of an RPMI 1640 medium with 5 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma, Dorset, P8139, UK) over 48 hours.

In vitro Experiments

Differentiated THP-1 macrophages have previously been advocated as an *in vitro* model of human macrophages. In accordance with the instruction manual by Park et al.¹⁹ (protocol), the minimal PMA concentration of 5 ng/mL was applied for stable differentiation without undesirable gene upregulation. The ESAT-6, CFP-10, and ESAT-6/CFP-10 recombinant proteins used in this study were cloned in our laboratory.^{20,21}

Cytotoxicity Assay: Reduction in Formazan Dye (3-[4,5-dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide; MTT)

Cytotoxicity was assessed by the MTT assay. The differentiated THP-1 cells (1×10^5 cells/mL) were seeded in a 96-well plate in triplicate and preincubated for 12 hours for cell attachment. Then, 100 μ L of a fresh medium containing various concentrations of ESAT-6 (2.5, 5, and 10 μ g/mL), CFP-10 (2.5, 5, and 10 μ g/mL), or

ESAT-6/CFP-10 (1.25, 2.5, and 5 $\mu\text{g/mL}$) was added to the flat-bottomed 96-well microtiter tissue culture plates. The cells were incubated with each compound at 37°C for 24 hours under humidified air containing 5% CO_2 . Cell survival was evaluated by adding 100 μL of tetrazolium salt solution (1 μg of MTT/ μL in PBS). After 4 hours of incubation at 37°C, 100 μL of DMSO was added to dissolve the precipitates of the reduced MTT. The microplate was shaken for 15 minutes, and absorbance was determined at 570 nm in a multi-well plate reader (FLUOstar OPTIMA spectrofluorometer, BioTek Synergy HT, USA). The cytotoxic effects of the recombinant proteins, namely ESAT-6, CFP-10, and ESAT-6/CFP-10, were examined for the study via the application of different concentrations of each of the recombinant proteins. The THP-1 cells were treated with the recombinant proteins at concentrations of 2.5, 5, and 10 $\mu\text{g/mL}$ for ESAT-6 or CFP-10; 1.25, 2.5, and 5 $\mu\text{g/mL}$ for ESAT-6/CFP-10; and 5 $\mu\text{g/mL}$ for lipopolysaccharide (LPS, Sigma, Dorset, L3024, UK) overnight.

Detection of ROS Production by Spectrofluorimetry

The intracellular production of ROS was assessed using 2',7' dichlorofluorescein diacetate (DCF-DA, Sigma, Dorset, D6883, UK) in 96-well plates. The optimum concentrations of the recombinant proteins, namely ESAT-6, CFP-10, and ESAT-6/CFP-10, for this study were determined by the application of different concentrations of each of the recombinant proteins. The differentiated THP-1 cells (3×10^5 cells/well) were treated with different concentrations of ESAT-6 (10 $\mu\text{g/mL}$), CFP-10 (2.5 $\mu\text{g/mL}$), and ESAT-6/CFP-10 (1.25 $\mu\text{g/mL}$). LPS with a concentration of 5 $\mu\text{g/mL}$ was used as a positive control. Subsequently, LPS plus ESAT-6, CFP-10, and ESAT-6/CFP-10 were incubated for 60 minutes at 37°C. Then, they were exposed to 10 μM of DCFDA in PBS⁻ (phosphate-buffered saline without calcium and magnesium) for 15 minutes at 37°C in the dark. The cells were washed with PBS⁻, and fluorescence was measured with a multi-well plate reader (FLUOstar OPTIMA Spectrofluorometer, BioTek, Synergy HT, USA), using filters with an excitation spectrum of 480 nm and emission of 520 nm.

NO Production Assessment

After incubating the differentiated THP-1 cells with ESAT-6 (2.5 $\mu\text{g/mL}$), CFP-10 (2.5 $\mu\text{g/mL}$), ESAT-6/CFP-10 (1.25 $\mu\text{g/mL}$), LPS (as a positive control, 5 $\mu\text{g/mL}$), and LPS

plus ESAT-6, CFP-10, and ESAT-6/CFP-10 for 24 hours, the supernatants were removed from the cultures. Nitrite accumulation in the supernatant was measured as a sign of NO production, based on the Griess reaction.²⁰ A 50- μL aliquot of each supernatant was mixed with the same volume of the Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water), incubated at room temperature for 10 minutes, and absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay reader. The amount of nitrite produced was determined via a sodium nitrite standard curve.

iNOS Enzyme Assay

The THP-1 cells were differentiated in a 24-well plate (1×10^6 cells) with PMA for 24 hours. Differentiation was verified by observing morphological changes in the cells before treating them with the recombinant proteins, namely ESAT-6 (2.5 $\mu\text{g/mL}$), CFP-10 (2.5 $\mu\text{g/mL}$), ESAT-6/CFP-10 (1.25 $\mu\text{g/mL}$), and LPS (5 $\mu\text{g/mL}$), for 24 hours. The cells were scraped into cold PBS⁻ and centrifuged. The cell pellets were resuspended in 0.5 mL of 40-mM Tris-buffer (pH=8.0) containing 5 $\mu\text{g/mL}$ of pepstatin A, 5 $\mu\text{g/mL}$ of aprotinin, and 100 mM of phenylmethylsulfonyl fluoride and were lysed by 3 freeze-thaw cycles. The cell lysate proteins were incubated in 20-mM Tris-HCl, containing FAD, tetrahydrobiopterin, and NADPH. The reaction was performed in duplicate for 180 minutes at 37°C in 96-well plates. iNOS activity was blocked using EDTA, and the Griess assay was performed. iNOS activity in the THP-1 cell lysate in the presence or absence of the recombinant proteins and LPS was determined and expressed as $\mu\text{mol sulfanilamide oxidized/minute/mg protein}$. The extracted protein was quantified using a commercially available kit (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, USA) following the manufacturer's instructions. Absorbance was measured at 560 nm on a multi-well plate reader (FLUOstar OPTIMA spectrofluorometer, BioTek, Synergy HT, USA), and the total protein per sample was calculated in micrograms (μg).

Statistical Analysis

All the experiments were repeated independently 3 times. The results are expressed as mean \pm SD. The data were analyzed using one-way ANOVA with SPSS, version 16 (SPSS Inc., Chicago, IL, USA). The statistical significance of differences in the mean levels of ROS, NO, etc. between the control and treatment groups was

evaluated using the Student *t*-test. *P* values ≤ 0.05 were considered significant. Graphs were drawn with GraphPad Prism, version 5 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Cytotoxic Effect of Recombinant Secretory Proteins on THP-1 Cells

The results from the MTT assay showed that more than 80% of the THP-1 cells were viable and metabolically active in the presence of the recombinant proteins at the used concentrations. In other words, THP-1 cell viability in the presence of the different concentrations of the recombinant proteins remained unchanged (figure 1).

As is shown in figure 1, the viability of the differentiated THP-1 cells in the presence of the recombinant proteins and LPS was more than 85%, demonstrating that these proteins had no significant toxic effect on cell viability.

Effect of Different Recombinant Secretory Antigens on ROS Production in Macrophages

Figure 2 shows that the optimum concentrations for the recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins were 10, 5, and 1.25 $\mu\text{g}/\text{mL}$, respectively. The recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins downregulated ROS production in the differentiated THP-1 cells in less than 60 minutes in all the concentrations applied. Downregulation by recombinant ESAT-6/CFP-10 in its optimum concentration (60%) was greater than that observed for ESAT-6 (50%) or CFP-10 (40%) alone. ESAT-6 and CFP-10 showed similar effects on the downregulation of ROS production.

The effect of these recombinant proteins on LPS-induced ROS was also assayed. As is shown in figure 3, the ESAT-6 protein (10 $\mu\text{g}/\text{mL}$) inhibited LPS-induced ROS production in the THP-1 cells by 60%, CFP-10 protein (5 $\mu\text{g}/\text{mL}$) by 50%, and ESAT-6/CFP-10 protein (1.25 $\mu\text{g}/\text{mL}$) by 70%. Therefore, the ESAT-6/CFP-10 proteins showed higher downregulating effects of LPS-induced ROS production at a lower concentration (1.25 $\mu\text{g}/\text{mL}$) than that of ESAT-6 or CFP-10 individually.

Effects of Different Recombinant Secretory Proteins on Macrophage NO and iNOS Enzyme Activity

Figure 4 illustrates that the concentrations of 2.5, 2.5, and 1.25 $\mu\text{g}/\text{mL}$ were optimal for the recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins, respectively. The NO production results in the differentiated THP-1 cells showed that all of these proteins downregulated NO after

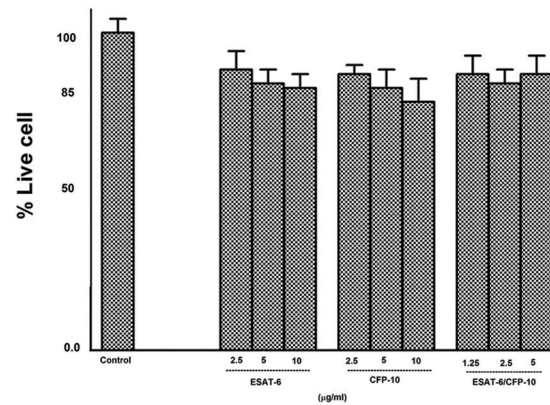


Figure 1: Effect of the recombinant antigens on the viability of the differentiated THP-1 cells. Values have been normalized to those of the control, considered as 100%, and are the mean of 2 independent experiments performed 5 times.

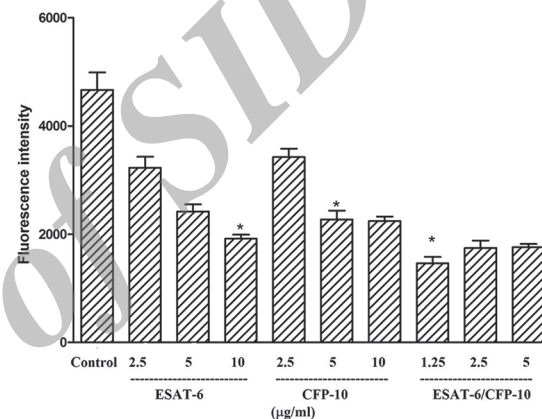


Figure 2: Effect of the different concentrations of the recombinant antigens on reactive oxygen species generation. The control was differentiated THP-1 cells without any treatment. Data are shown as mean \pm SD of 3 independent experiments. *Indicates a significant ($P < 0.05$) difference between the untreated and treated differentiated THP-1 cells.

24 hours of treatment. Although a significant difference ($P < 0.05$) was observed in the NO level between the cells treated with the recombinant proteins and the control, the NO levels for the ESAT-6, CFP-10, and ESAT-6/CFP-10 treatments at the optimal concentration were almost similar.

Figure 5 depicts the effects of these recombinant proteins on LPS-induced NO production. The ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins at their optimum concentrations inhibited LPS-induced NO production in the THP-1 cells by 50–60%. These proteins showed similar effects on LPS-induced NO generation, and no significant difference was observed among these protein treatments.

Effect of Recombinant Proteins on iNOS Enzyme Activity in Macrophages

Table 1 demonstrates the effects of the recombinant ESAT-6, CFP-10, and ESAT-6/

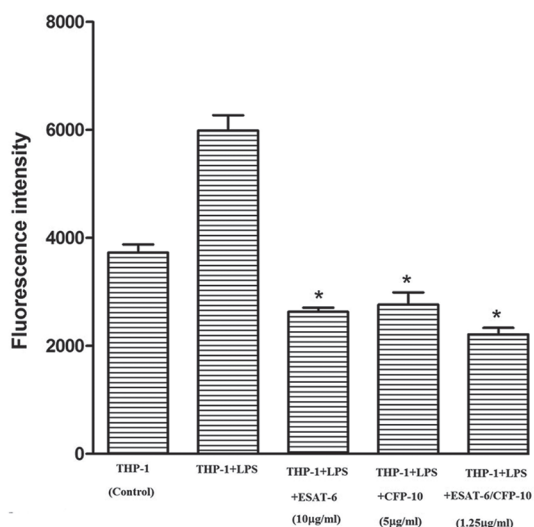


Figure 3: Recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 fusion proteins antagonized lipopolysaccharide (LPS)-induced reactive oxygen species production. The control was differentiated THP-1 cells without any treatment. Data are presented as mean±SD of 3 independent experiments. *Indicates a significant (P<0.05) difference vs. LPS-stimulated cells.

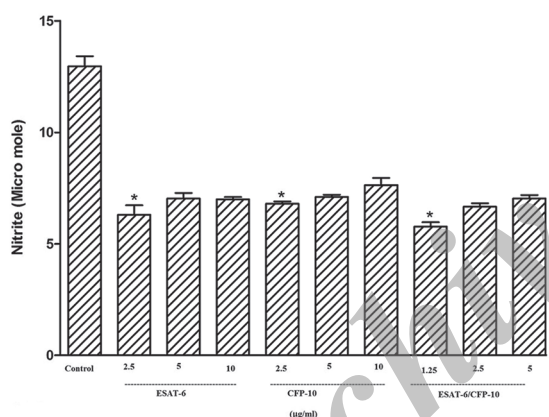


Figure 4: Effect of the different concentrations of the recombinant proteins on nitric oxide (NO) generation. Cells without any treatment were used as a control. Data are presented as mean±SD of 3 independent experiments. *Indicates a significant (P<0.05) differences between the untreated and treated differentiated THP-1 cells.

CFP-10 proteins on iNOS enzyme activity in the differentiated THP-1 cells. The treatment of the differentiated THP-1 cells with LPS (5 µg/mL) significantly increased iNOS enzyme activity over 24 hours (P<0.05), while the recombinant proteins significantly suppressed the effects of LPS on iNOS enzyme activity to nearly the same level as that in the control (table 1).

Discussion

The current study aimed to investigate NO and ROS production in response to the recombinant secretory antigens of *Mtb*, namely ESAT-6,

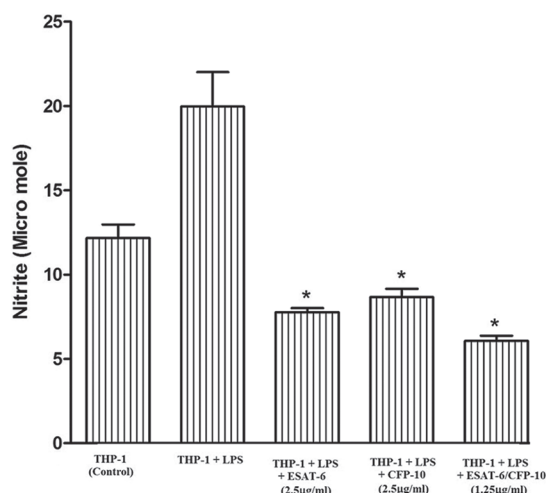


Figure 5: Recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins antagonized lipopolysaccharide (LPS)-induced nitric oxide (NO) production. Differentiated THP-1 cells without any treatment were used as a control. Data are presented as mean±SD of 3 independent experiments. *Indicates a significant (P<0.05) difference vs. LPS-stimulated cells.

CFP-10, and ESAT-6/CFP-10. Our results showed that the recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins inhibited ROS and NO production in differentiated THP-1 cells. ROS and NO are 2 key elements involved in antimicrobial macrophage-mediated host defense.¹⁰ ROS seem to play a significant role in host defense during intramacrophage infection against bacteria by oxidizing the -SH group of cysteine in proteins.²² Another important microbiocidal function of macrophages is the generation of highly reactive free radical NO by iNOS.²³ LPS-induced oxidative burst in macrophages assists in killing invading pathogens.^{24,25} Macrophages respond to LPS by phosphorylating many intracellular signaling molecules.^{24,26,27} This initiates a cascade of signaling events, leading to the secretion of inflammatory cytokines such as IL-1, IL-6, tumor necrosis factor alpha (TNF-α), and antimicrobial immune responses.²⁸

Trajkovic et al.²⁹ (2002) showed that CFP-10 was able to bind to the surface of mouse J774 macrophage-like cells and stimulate the secretion of the proinflammatory cytokine TNF-α, but it markedly reduced NO release on subsequent stimulation with LPS or *Mtb* cell lysate. That study also showed that CFP-10 blocked the shift in the cellular equilibrium toward signal activation by inhibiting ROS generation via LPS. In other words, inhibiting LPS-induced ROS by CFP-10 worked as a “control switch” that regulated consequent events.^{30,31}

ROS activate NF-κB, which leads to gene transcription.³² NF-κB plays a role in containing

Table 1: Effects of the recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins on iNOS activity

Experimental groups [†]	iNOS (U/mg protein)	P value
Differentiated THP-1	7.2±0.02 ^{‡*}	0.01
Differentiated THP-1+LPS	16.6±0.07	0.12
Differentiated THP-1+ESAT-6	4.6±0.05 [*]	0.01
Differentiated THP-1+CFP-10	4.3±0.02 [*]	0.02
Differentiated THP-1+ESAT-6/CFP-10	3.9±0.03 [*]	0.01
Differentiated THP-1+LPS+ESAT-6	7.8±0.07 [*]	0.02
Differentiated THP-1+LPS+CFP-10	8.6±0.05 [*]	0.02
Differentiated THP-1+LPS+ESAT-6/CFP-10	6.4±0.04 [*]	0.01

[†]For details of the experimental conditions, see the text; [‡]Data are expressed as mean±SD of 3 replicates in each group; ^{*}was considered significant at P<0.05 when compared with the differentiated THP-1 cells with LPS (one-way ANOVA and the Duncan test)

infection by inducing the expression of several proinflammatory cytokines such as TNF- α , IL-12, IFN- γ , and iNOS; therefore, it is critically targeted by pathogens.³³ Ganguly et al.²² (2008) showed that ESAT-6, CFP-10, and the CFP-10/ESAT-6 complex downregulated NF- κ B-dependent reporter gene expression by inhibiting ROS production in RAW264.7 cells. It has been reported that ESAT-6 may inhibit the innate immune response through a diminished production of the IL-12 p40 chain, TNF- α , and NO.³⁴ Pathak et al.³⁵ (2007) showed that ESAT-6 or its peptide-derived diminished TLR signaling by preventing the assembly of the cytosolic MyD88-dependent signaling scaffold and led to inhibited NF- κ B activation, thereby attenuating the immune response in macrophages. Our results also showed that the recombinant ESAT-6, CFP-10, and the ESAT-6/CFP-10 proteins inhibited the production of ROS intermediates, which agreed with the results of Ganguly et al.²² (2008). Because NF- κ B induces the expression of iNOS, the suppression of ROS leads to the suppression of transcription factor NF- κ B and iNOS expression. Intracellular pathogens such as *Mtb* use a repertoire of dampening signals to subvert the innate immune response. ESAT-6 has emerged as a likely candidate for the investigation of its effect on macrophages, based on the finding that inactivating the *esat-6* gene in *Mtb* increases the innate immune response and increases the production of IL-12 p40, IL-1 α , TNF- α , and NO.^{34,36} The direct interaction between TLR2 and ESAT-6 inhibits the assembly of TLR-signaling molecules required for activating the innate immune response, but it is unclear whether ESAT-6/CFP-10 acts through a similar mechanism to suppress macrophage responses.³⁵ However, it is worthy to examine

the possible involvement of other receptors in recombinant *Mtb* antigen-induced macrophage deactivation. Gupta et al.³⁷ (2010) showed that other recombinant *Mtb* antigens such as Rv1483 and Rv3416 suppressed TLR2-induced IL-12, ROS, and iNOS synthase expression during infection. The inhibited production of NO in the present study may be the result of inhibited ROS by the recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 antigens. The downregulation of ROS by these recombinant proteins affects iNOS expression via NF- κ B.²² It seems that downregulating ROS also affects iNOS activity, as was shown in our results (table 1). Our NO level findings in the THP-1 treated cells confirmed the iNOS activity results. Thus, the decrease in the NO level in the THP-1 treated cells could be due to the inhibition of the NF- κ B signaling pathway²³ or the direct inhibition of iNOS activity (table 1). In addition, the mycobacterial secretory protein ESAT-6 inhibits the expression of c-Myc, a key factor in macrophage activation.³⁸ These findings emphasize the role of ESAT-6 in deactivating macrophages, which are the host cells for *Mtb*. It seems that intracellular pathogens such as *Mtb* can evade the host immune response by inducing granuloma formation and suppressing the macrophage immune response. A study of the interactions between *Mtb* antigens such as ESAT-6 and host proteins leads us to an understanding of the mechanism of action between these two. As it is unclear whether CFP-10 and ESAT-6/CFP-10 act in such a way, identifying the mechanism of the action of *Mtb* antigens in host cells may offer promising alternatives to control TB infection and associated immunopathology.

The results of the current study also implied that the recombinant ESAT-6 and CFP-10 antigens helped *Mtb* evade the macrophage immune response by suppressing macrophage activation (production of ROS and NO), similar to other *Mtb* antigens such as Rv1483 and Rv3416.³⁷

That *Mtb* escapes mechanisms from the host immune system in order to stay hidden is a vital topic and a subject of interest in this field. Therefore, one of the concerns of our research team was to investigate the role of TLR and signaling pathways that could play an important role. In this regard, finding and determining the role of proteins involved in the signaling of TLR on the surface of macrophages can be useful. In future studies, we will aim to determine the interactions between signaling proteins and secretory antigens, which were a subject of this research, with a view to achieving a better understanding of the functions of immune cells when exposed to *Mtb*.

Conclusion

It seems that the decline in the macrophage immune response is due to the suppression of NO and ROS production pathways by the modulation of gene expression without any effect on cell viability. The ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins suppressed iNOS enzyme activity, which is notable for the fusion protein. The ESAT-6/CFP-10 fusion protein showed more suppressive effects at a low concentration than did ESAT-6 and CFP-10 alone. The possibility of crosstalk between receptors on macrophages such as TLRs and integrins and whether *Mtb* antigens result in downstream signaling are other issues that need further investigations. The findings of this study and those from others highlight the need for more research to understand the mechanism of action of *Mtb* antigens in host cells, which in turn, will open the door to future studies.

Acknowledgement

This study was done as a part of PhD thesis of M. Hemmati and supported by grant No. 87-4289 from the office of Vice Chancellor for Research and the Committee for Advanced Biomedical Sciences, Shiraz University of Medical Sciences.

Conflict of Interest: None declared.

References

- World Health Organization [Internet]. Tuberculosis. Fact sheet No. 104, 2013. Available from: <http://www.who.int/mediacentre/factsheets/fs104/en>
- Masoumi Asl HMM, Alborzi AM, Pourabbas BP, Kalani MM. QuantiFERON-TB Gold and Tuberculin Skin Test for the Diagnosis of Latent Tuberculosis Infection in Children. *Iran J Med Sci*. 2015;40:411-7. PubMed PMID: 26379347; PubMed Central PMCID: PMC4567600.
- Cao W, Tang S, Yuan H, Wang H, Zhao X, Lu H. Mycobacterium tuberculosis antigen Wag31 induces expression of C-chemokine XCL2 in macrophages. *Curr Microbiol*. 2008;57:189-94. doi: 10.1007/s00284-008-9172-2. PubMed PMID: 18618175.
- Lew JM, Kapopoulou A, Jones LM, Cole ST. TubercuList--10 years after. *Tuberculosis (Edinb)*. 2011;91:1-7. doi: 10.1016/j.tube.2010.09.008. PubMed PMID: 20980199.
- Guo S, Xue R, Li Y, Wang SM, Ren L, Xu JJ. The CFP10/ESAT6 complex of Mycobacterium tuberculosis may function as a regulator of macrophage cell death at different stages of tuberculosis infection. *Med Hypotheses*. 2012;78:389-92. doi: 10.1016/j.mehy.2011.11.022. PubMed PMID: 22192908.
- Marei A, Ghaemmaghami A, Renshaw P, Wiselka M, Barer M, Carr M, et al. Superior T cell activation by ESAT-6 as compared with the ESAT-6-CFP-10 complex. *Int Immunol*. 2005;17:1439-46. doi: 10.1093/intimm/dxh322. PubMed PMID: 16186161.
- Boom WH, Canaday DH, Fulton SA, Gehring AJ, Rojas RE, Torres M. Human immunity to *M. tuberculosis*: T cell subsets and antigen processing. *Tuberculosis (Edinb)*. 2003;83:98-106. doi: 10.1016/S1472-9792(02)00054-9. PubMed PMID: 12758197.
- Koo MS, Subbian S, Kaplan G. Strain specific transcriptional response in Mycobacterium tuberculosis infected macrophages. *Cell Commun Signal*. 2012;10:2. doi: 10.1186/1478-811X-10-2. PubMed PMID: 22280836; PubMed Central PMCID: PMC3317440.
- Ebtekar M, Yaraee R, Ahmadiani A, Sabahi F. Kinetics of Nitric Oxide Production and MTT Reduction by HSV-1 Infected Macrophages. *Iran J Med Sci*. 2006;31:9-13.
- Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol*. 2004;2:820-32. doi: 10.1038/nrmicro1004. PubMed PMID: 15378046.
- Bonato VL, Medeiros AI, Lima VM, Dias AR, Faccioliti LH, Silva CL. Downmodulation of CD18 and CD86 on macrophages and VLA-4 on lymphocytes in experimental tuberculosis. *Scand J Immunol*. 2001;54:564-73. doi: 10.1046/j.1365-3083.2001.00996.x. PubMed PMID: 11902331.
- Schlesinger LS. Role of mononuclear phagocytes in *M tuberculosis* pathogenesis. *J Investig Med*. 1996;44:312-23. PubMed PMID: 8795295.
- Brightbill HD, Libraty DH, Krutzik SR, Yang RB, Belisle JT, Bleharski JR, et al. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science*. 1999;285:732-6. doi: 10.1126/science.285.5428.732. PubMed PMID: 10426995.
- Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts

- and microbial pathogens. *Proc Natl Acad Sci U S A*. 2000;97:8841-8. doi: 10.1073/pnas.97.16.8841. PubMed PMID: 10922044; PubMed Central PMCID: PMC34021.
15. Chan J, Tanaka K, Carroll D, Flynn J, Bloom BR. Effects of nitric oxide synthase inhibitors on murine infection with *Mycobacterium tuberculosis*. *Infect Immun*. 1995;63:736-40. PubMed PMID: 7529749; PubMed Central PMCID: PMC173063.
 16. Hanano R, Kaufmann SH. Nitric oxide production and mycobacterial growth inhibition by murine alveolar macrophages: the sequence of rIFN-gamma stimulation and *Mycobacterium bovis* BCG infection determines macrophage activation. *Immunol Lett*. 1995;45:23-7. doi: 10.1016/0165-2478(94)00193-U. PubMed PMID: 7622183.
 17. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MP. The immune response in tuberculosis. *Annu Rev Immunol*. 2013;31:475-527. doi: 10.1146/annurev-immunol-032712-095939. PubMed PMID: 23516984.
 18. Silva CL, Lowrie DB. Identification and characterization of murine cytotoxic T cells that kill *Mycobacterium tuberculosis*. *Infect Immun*. 2000;68:3269-74. doi: 10.1128/IAI.68.6.3269-3274.2000. PubMed PMID: 10816472; PubMed Central PMCID: PMC97577.
 19. Park EK, Jung HS, Yang HI, Yoo MC, Kim C, Kim KS. Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflamm Res*. 2007;56:45-50. doi: 10.1007/s00011-007-6115-5. PubMed PMID: 17334670.
 20. Leoni F, Magnani JL, Miotti S, Canevari S, Pasquali M, Sonnino S, et al. The antitumor monoclonal antibody MOv2 recognizes the Lewis A hapten. *Hybridoma*. 1988;7:129-39. doi: 10.1089/hyb.1988.7.129. PubMed PMID: 3372000.
 21. Hemmati M, Seghatoleslam A, Rasti M, Ebadat S, Naghibalhossaini F, Mostafavi-Pour Z. Additive effect of recombinant *Mycobacterium tuberculosis* ESAT-6 protein and ESAT-6/CFP-10 fusion protein in adhesion of macrophages through fibronectin receptors. *J Microbiol Immunol Infect*. 2014.
 22. Ganguly N, Giang PH, Gupta C, Basu SK, Siddiqui I, Salunke DM, et al. *Mycobacterium tuberculosis* secretory proteins CFP-10, ESAT-6 and the CFP10:ESAT6 complex inhibit lipopolysaccharide-induced NF-kappaB transactivation by downregulation of reactive oxidative species (ROS) production. *Immunol Cell Biol*. 2008;86:98-106. doi: 10.1038/sj.icb.7100117. PubMed PMID: 17909563.
 23. Singh G, Singh B, Trajkovic V, Sharma P. *Mycobacterium tuberculosis* 6kDa early secreted antigenic target stimulates activation of J774 macrophages. *Immunology letters*. 2005;98:180-8. doi: 10.1016/j.imlet.2004.11.011.
 24. Ganeshan K, Chawla A. Metabolic regulation of immune responses. *Annu Rev Immunol*. 2014;32:609-34. doi: 10.1146/annurev-immunol-032713-120236. PubMed PMID: 24655299.
 25. Okugawa S, Ota Y, Kitazawa T, Nakayama K, Yanagimoto S, Tsukada K, et al. Janus kinase 2 is involved in lipopolysaccharide-induced activation of macrophages. *Am J Physiol Cell Physiol*. 2003;285:C399-408. doi: 10.1152/ajpcell.00026.2003. PubMed PMID: 12686512.
 26. Ehlting C, Häussinger D, Bode JG. Crucial role of the MAPKAP kinases 2 and 3 for pathogen-induced inflammation and their relevance for the immune response of the liver. *Eur J Med Res*. 2014;19:S24. doi: 10.1186/2047-783X-19-S1-S24.
 27. Weinstein SL, Sanghera JS, Lemke K, DeFranco AL, Pelech SL. Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages. *J Biol Chem*. 1992;267:14955-62. PubMed PMID: 1321821.
 28. Russell DG. *Mycobacterium tuberculosis*: here today, and here tomorrow. *Nat Rev Mol Cell Biol*. 2001;2:569-77. doi: 10.1038/35085034. PubMed PMID: 11483990.
 29. Trajkovic V, Singh G, Singh B, Singh S, Sharma P. Effect of *Mycobacterium tuberculosis*-specific 10-kilodalton antigen on macrophage release of tumor necrosis factor alpha and nitric oxide. *Infect Immun*. 2002;70:6558-66. doi: 10.1128/IAI.70.12.6558-6566.2002. PubMed PMID: 12438325; PubMed Central PMCID: PMC132989.
 30. Basu SK, Kumar D, Ganguly N, Rao KV, Sharma P. *Mycobacterium tuberculosis* secreted antigen (MTSA-10) inhibits macrophage response to lipopolysaccharide by redox regulation of phosphatases. *Indian J Exp Biol*. 2009;47:505-19. PubMed PMID: 19634717.
 31. Stefanova I, Corcoran ML, Horak EM, Wahl LM, Bolen JB, Horak ID. Lipopolysaccharide induces activation of

- CD14-associated protein tyrosine kinase p53/56lyn. *J Biol Chem.* 1993;268:20725-8. PubMed PMID: 7691802.
32. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. *Curr Biol.* 2014;24:R453-62. doi: 10.1016/j.cub.2014.03.034. PubMed PMID: 24845678; PubMed Central PMCID: PMC4055301.
33. Lee SB, Schorey JS. Activation and mitogen-activated protein kinase regulation of transcription factors Ets and NF-kappaB in Mycobacterium-infected macrophages and role of these factors in tumor necrosis factor alpha and nitric oxide synthase 2 promoter function. *Infect Immun.* 2005;73:6499-507. doi: 10.1128/IAI.73.10.6499-6507.2005. PubMed PMID: 16177323; PubMed Central PMCID: PMC1230939.
34. Stanley SA, Raghavan S, Hwang WW, Cox JS. Acute infection and macrophage subversion by Mycobacterium tuberculosis require a specialized secretion system. *Proc Natl Acad Sci U S A.* 2003;100:13001-6. doi: 10.1073/pnas.2235593100. PubMed PMID: 14557536; PubMed Central PMCID: PMC240734.
35. Pathak SK, Basu S, Basu KK, Banerjee A, Pathak S, Bhattacharyya A, et al. Direct extracellular interaction between the early secreted antigen ESAT-6 of Mycobacterium tuberculosis and TLR2 inhibits TLR signaling in macrophages. *Nat Immunol.* 2007;8:610-8. doi: 10.1038/ni1468. PubMed PMID: 17486091.
36. Yang R, Xi C, Sita DR, Sakai S, Tsuchiya K, Hara H, et al. The RD1 locus in the Mycobacterium tuberculosis genome contributes to the maturation and secretion of IL-1alpha from infected macrophages through the elevation of cytoplasmic calcium levels and calpain activation. *Pathog Dis.* 2014;70:51-60. doi: 10.1111/2049-632X.12075. PubMed PMID: 23913588.
37. Gupta D, Sharma S, Singhal J, Satsangi AT, Antony C, Natarajan K. Suppression of TLR2-induced IL-12, reactive oxygen species, and inducible nitric oxide synthase expression by Mycobacterium tuberculosis antigens expressed inside macrophages during the course of infection. *J Immunol.* 2010;184:5444-55. doi: 10.4049/jimmunol.0903283. PubMed PMID: 20385877.
38. Ganguly N, Giang PH, Basu SK, Mir FA, Siddiqui I, Sharma P. Mycobacterium tuberculosis 6-kDa early secreted antigenic target (ESAT-6) protein downregulates lipopolysaccharide induced c-myc expression by modulating the extracellular signal regulated kinases 1/2. *BMC Immunol.* 2007;8:24. doi: 10.1186/1471-2172-8-24. PubMed PMID: 17915024; PubMed Central PMCID: PMC2082026.