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ORIGINAL ARTICLE

Additive effect of recombinant *Mycobacterium tuberculosis* ESAT-6 protein and ESAT-6/CFP-10 fusion protein in adhesion of macrophages through fibronectin receptors

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Background/purpose: Tuberculous granulomas are the sites of interaction between the T cells, macrophages, and extracellular matrix (ECM) to control the infection caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). A predominant role of RD-1-encoded secretory proteins, early secreted antigenic target-6 (ESAT-6), and culture filtrate protein-10 (CFP-10) in the formation of granulomas has recently been emphasized. However, the precise molecular events that induce the formation of these granulomatous structures are yet to be elucidated. Macrophages use integrins to adhere to fibronectin (FN) as a major component of the ECM. The major

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Mycobacterium tuberculosis

goal of this study was to investigate whether recombinant *M. tuberculosis* antigens can modulate integrin-mediated macrophage adhesion.
Methods: Differentiated THP-1 cell line was stimulated with recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins and evaluated for alterations in the expression levels of $\alpha_5\beta_1$ and $\alpha_4\beta_1$ by semiquantitative real-time polymerase chain reaction. The role of these recombinant antigens in the cytoskeleton rearrangement was determined by adhesion assay and immunofluorescent microscopy.
Results: Our data showed that ESAT-6 and ESAT-6/CFP-10 fusion proteins could induce adhesion of macrophages to FN through $\alpha_4\beta_1$ integrin. An increased expression level of $\alpha_4\beta_1$ integrin in comparison with $\alpha_5\beta_1$ integrin in differentiated THP-1 cells was also observed. Results of immunofluorescence studies showed that recombinant proteins-treated THP-1 cells form well-organized stress fibers and focal contacts containing vinculin compared with untreated THP-1 cells.
Conclusion: Increased expression level of $\alpha_4\beta_1$ in differentiated THP-1 cells could suggest the important role of $\alpha_4\beta_1$ integrin in adhesion and focal contact formation of macrophages exposed to *M. tuberculosis* antigens.

Introduction

Tuberculosis remains a major health problem throughout the world.[1,2] The proteins secreted by *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agents of tuberculosis, have gained increased attention in recent years as both vaccine candidates and virulence factors.[3,4] Some of the proteins such as early secreted antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) are encoded by the RD-1 region of the *M. tuberculosis* genome.[5,6] The pathological hallmark of the host response to *M. tuberculosis* is the granuloma, where T cells and macrophages interact with the extracellular matrix (ECM) to control the infection.[7,8] Dedicated studies to identify the mycobacterial compounds that enable the formation of these granulomatous structures and subsequent modulation of their activities would certainly lead to a better understanding of this persistent bacilli.[9] However, the precise molecular events that are triggered by the mycobacteria, which induce the formation of granulomatous structures, remain to be clearly defined. Moreover, the relative contribution of host mechanisms, the migration and aggregation of macrophages, and the role of ECM proteins such as fibronectin (FN) in the formation of granulomas are also poorly understood.[10] Recruitment of immune cells including macrophages within granuloma depends on their adhesion to the ECM. Macrophages use integrins to adhere to FN as a major ECM component.[11] Integrins comprise a large group of heterodimeric transmembrane molecules, which consist of noncovalently linked α and β subunits. Among the 24 pairs of integrins identified in mammals so far, monocytes/macrophages are believed to express three integrins of the $\beta1$ family, including FN receptors $\alpha_4\beta_1$ and $\alpha_5\beta_1$ as well as the laminin receptor $\alpha6\beta1$. It has been proposed that ligation and clustering of integrins in phagocytic cells activate cytoplasmic tyrosine kinase, which in turn leads to activation of the downstream signaling pathways involving phosphatidylinositol-3-kinase, phospholipase C, D, and protein kinase C. These collective and highly regulated events control cytoskeletal rearrangements, focal contact formation, cell mobility, cell survival, and synthesis of the inflammatory mediators by phagocytic cells.[12] Human acute monocytic leukemia cell line (THP-1 cells) expresses $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins.[13] Because these two integrins are expressed on activated macrophages, they may play a key role in the aggregation of macrophages and subsequently in the granuloma formation process. To achieve a better understanding of macrophage aggregation and granuloma formation as a result of macrophage adhesion, we examined the response of macrophages to the specific *M. tuberculosis* antigens, ESAT-6, CFP-10, and fusion of ESAT-6/CFP-10, by adhesion and immunofluorescence assays and evaluated the expression levels of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins by real-time polymerase chain reaction (RT-PCR). The three recombinant proteins used in this study were cloned, expressed, and purified as described in our previous study.[14]

Materials and methods

Reagents and antibodies

Rhodamine-conjugated phalloidin and other fine products were obtained from Sigma—Aldrich (Dorset, UK). Mouse antihuman vinculin and fluorescein isothiocyanate (FITC)-conjugated donkey antimouse immunoglobulin G (IgG) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The THP-1 cells were obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Islamic Republic of Iran). All tissue culture medium and supplements were purchased from Biosera (East Sussex, UK). Recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins were cloned and purified in the Recombinant Protein Laboratory in Shiraz University of Medical Sciences (Shiraz, Islamic Republic of Iran).

Cell culture

The cells were cultured (cell density, $1-4 \times 10^6$ cells/mL) in Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, and maintained at 37°C under 5% CO_2. In all experiments, THP-1 monocytic cells (1×10^6 cells) were differentiated into macrophages in T25 cell-culture flasks containing RPMI 1640 medium (3 mL) for over 48 hours. The RPMI 1640 medium contains 5 ng/mL phorbol 12-myristate 13-acetate (PMA).

In vitro cytotoxicity assay

The cytotoxic effects of recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 on THP-1 cells were measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, a tetrazole; Sigma–Aldrich) assay. The differentiated THP-1 cells (1×10^5 cells/mL) were seeded into a 96-well plate for 24 hours. Different concentrations of the recombinant proteins were added to the cells followed by incubation at 37°C for 24 hours under humidified air containing 5% CO_2. Cell survival was evaluated by adding 100 μL tetrazolium salt solution [1 mg MTT/mL in phosphate-buffered saline (PBS)] to each well for 4 hours. The supernatant was removed by aspiration, and 150 μL of dimethyl sulfoxide was added to the wells in order to dissolve any precipitate. The absorbance was determined at 570 nm in a multiwell scanning spectrophotometer. The THP-1 cells were stimulated by treating them with prespecified concentrations of the recombinant proteins (2.5 μg/mL, 5 μg/mL, and 10 μg/mL for ESAT-6 or CFP-10; 1.25 μg/mL, 2.5 μg/mL, and 5 μg/mL for ESAT-6/CFP-10). All experiments were performed in triplicate, independently, and the results were expressed as the mean ± standard deviation (SD).

Cell attachment assay

The adhesion assay for FN was performed as described previously.[15] In brief, 96-well plates were entirely coated with FN (10 μg/mL) at room temperature (25 °C) for 1 hour. After washing with PBS, nonspecific binding sites were blocked with 1% heat-denatured bovine serum albumin. Differentiated THP-1 cells (2×10^5) were added to the wells, and treated with recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins at 37°C for 20 minutes. The optimal concentration for each recombinant protein was set using the dose concentration assay. Based on the results of this assay, the final concentrations of recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins used were 2.5 μg/mL, 2.5 μg/mL, and 1.25 μg/mL, respectively. After removing nonadherent cells, the attached cells were fixed with 5% glutaraldehyde for 30 minutes, stained with 0.1% (w/v) crystal violet, dissolved in 10% (v/v) acetic acid, and their optical density at a wavelength of 570 nm was then read to calculate the adhesion of the cells.

Immunofluorescence assay

Filamentous actin stress fiber formation in THP-1 cells was evaluated according to our previous study.[16] In brief, differentiated THP-1 cells (10^5/mL) were treated with recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins for 3 hours at 37°C in a humidified atmosphere of 5% (v/v) CO_2. Using dose concentration assay, the optimal concentration for each recombinant protein was set and recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins were used at concentrations of 2.5 μg/mL, 2.5 μg/mL, and 1.25 μg/mL, respectively. Cells were fixed with 4% paraformaldehyde for 5 minutes, and permeabilized with 0.5% Triton X-100. Integrin complex components including vinculin were detected using antivinculin monoclonal antibody. After washing, the primary antibody was detected using FITC-conjugated goat antimouse IgG as a secondary antibody. Filamentous actin was detected using rhodamine-conjugated phalloidin. Cover slips were mounted face down onto microscope slides using VECTASHIELD solution and cells were viewed with a fluorescent microscope. Immunofluorescent images were then taken with FITC (green channels) and rhodamine (red channels) using a charge-coupled device camera. Basic image acquisition and analysis were performed using Dpx View Pro.

Analysis of integrin messenger RNA expression

The messenger RNA (mRNA) abundance of α5, α4, and β1 integrins were measured by RT-PCR in the THP-1 cells treated with the recombinant proteins for 24 hours at the same concentration used in cell attachment assay. The specific primers used for PCR were as follows: Forward: 5′-CATTTCCGAGTCTGGGCCAA-3′ and Reverse: 5′-TGGAGGCTTGAGCTGAGCTT-3′; for α5 integrin amplifying a 324-bp product, Forward: 5′-CTCGCCAACGCTTCAGTGATC-3′ and Reverse: 5′-TCGTAAATCAGGGGGCACTCC-3′; for α4 integrin amplifying a 291-bp product, Forward: 5′-AATGAAGGGCGTGTTGGTAG-3′ and Reverse: 5′-CGTTGCTGGCTTCACAAGTA-3′ for β1 integrin amplifying a 337-bp product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an internal standard, was also amplified and analyzed under identical conditions using specific primers (Forward: 5′-ATCTTCCAGGAGCGAGATCC-3′ and Reverse: 5′-ACCACTGACACGTTGGCAGT-3′ for amplifying a 509-bp product). The PCR products were electrophoresed on ethidium bromide containing agarose gels, and the band intensity was measured by densitometric scanning and analyzed with Gene tool software version 7.9 (Syngene, cambridge, UK).

Statistical analysis

All experiments were repeated independently three times. The results were expressed as mean ± SD. The data were analyzed by one-way analysis of variance using SPSS version 16 software (SPSS Inc., Chicago, IL, USA). A p value < 0.001 was considered statistically significant.

Results

Differentiated THP-1 macrophages have been widely used as an *in vitro* model of human macrophages. There are some reports about optimizing the PMA concentrations required for differentiating monocytes while minimizing

gene upregulation.[17,18] According to these reports, treatment of THP-1 cells with PMA at 2.5—100 ng/mL concentration for 48 hours can differentiate monocytes from macrophages; however, the minimal concentration of PMA required for stable differentiation without being overwhelmed by undesirable gene upregulation is 5 ng/mL. Therefore, the same concentration of PMA was used in all experiments prior to treating the cells with recombinant proteins.

Cytotoxicity of the recombinant proteins

The results of MTT assay indicated that $>$ 80% of THP-1 cells are viable and metabolically active in the presence of recombinant proteins. In other words, the viability of THP-1 cells in the presence of different concentrations of recombinant proteins remained unchanged (Fig. 1).

THP-1 cell attachment on FN

To determine the role of integrins in macrophage adhesion to the ECM, FN was tested for its ability to support macrophage cell attachment. In addition, to examine the relative involvement of the recombinant proteins in the adhesion of THP-1 cells to FN, cell attachment assay in the presence of recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins was performed. The values of attachment for ESAT-6, CFP-10, and ESAT-6/CFP-10 were 80%, 74%, and 90%, respectively, in comparison with 52% attachment on FN alone. As shown in Fig. 2, the higher level of adhesion was observed in the presence of recombinant ESAT-6/CFP-10 fusion protein, showing the additive effect of ESAT-6 and CFP-10.

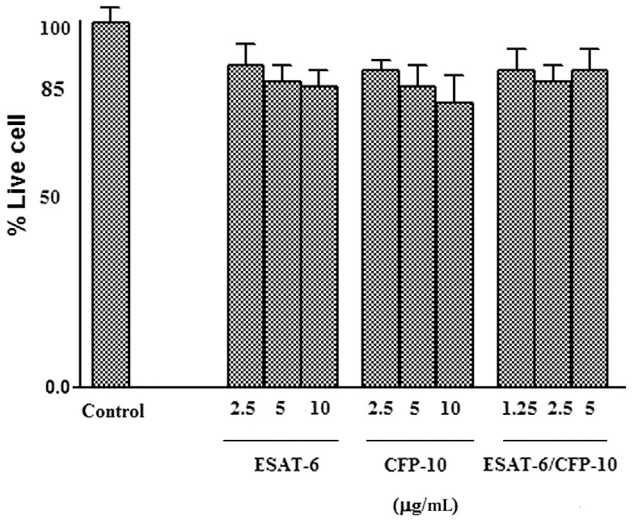

Figure 1. Viability of differentiated THP-1 cells in the absence and presence of recombinant proteins evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Values have been normalized for the control (THP-1 cells in the absence of recombinant proteins), considered as 100%, and are the mean of three independent experiments done in quintet. CFP-10 = culture filtrate protein-10; ESAT-6 = early secreted antigenic target-6.

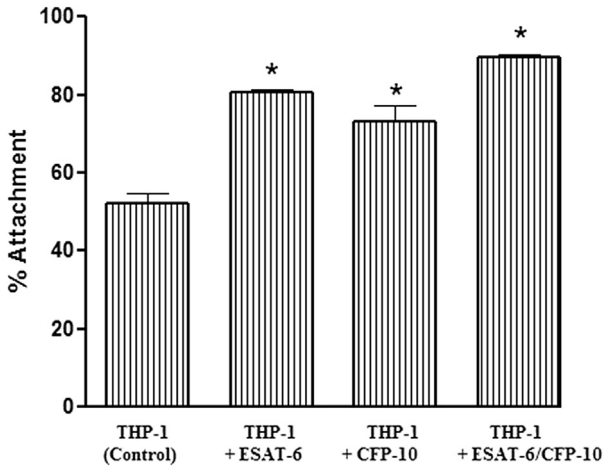

Figure 2. Attachment of THP-1 cells treated with the three recombinant proteins in their optimal concentration (as described in the cell attachment assay, see the "Materials and methods" section) on fibronectin compared with control (nontreated cells). The level of nonspecific binding, determined from cell attachment to wells coated with bovine serum albumin alone, was subtracted. Values are shown as mean $\pm$ standard deviation of triplicate wells. *Significant ($p < 0.05$) difference between treated THP-1 cells and untreated cells. CFP-10 = culture filtrate protein-10; ESAT-6 = early secreted antigenic target-6.

Organization of actin stress fibers and vinculin localization of THP-1 cells on FN in the absence or presence of recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins

The ability of FN to support focal adhesion formation and microfilament polymerization in untreated and treated differentiated THP-1 cells was assessed by double immunofluorescence microscopy using antivinculin antibody and rhodamine-conjugated phalloidin. As shown in Fig. 3, recombinant proteins-treated THP-1 cells form well-organized stress fibers and focal contacts containing vinculin compared with untreated THP-1 cells. This suggests the important role of recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins in induction of adhesion and focal contact formation of macrophages on FN through integrins $\alpha_5\beta_1$ and $\alpha_4\beta_1$. Recombinant proteins can increase formation of focal contacts either by increasing of integrin $\alpha_5\beta_1$ or $\alpha_4\beta_1$ or by raising the capacity for formation of normal focal contacts. They also reorganize actin filaments along with recruitment of integrins in focal contacts.

Expression of integrins mRNA in THP-1 cell line treated with different recombinant proteins

Fig. 4 shows the RT-PCR products corresponding to α5, α4, and β1 integrins (with GAPDH as the internal control) and mRNA expression in the cells treated with the optimal concentration of each recombinant protein compared with control untreated differentiated THP-1 cells. Using gel document densitometry and SynGene software, the level of

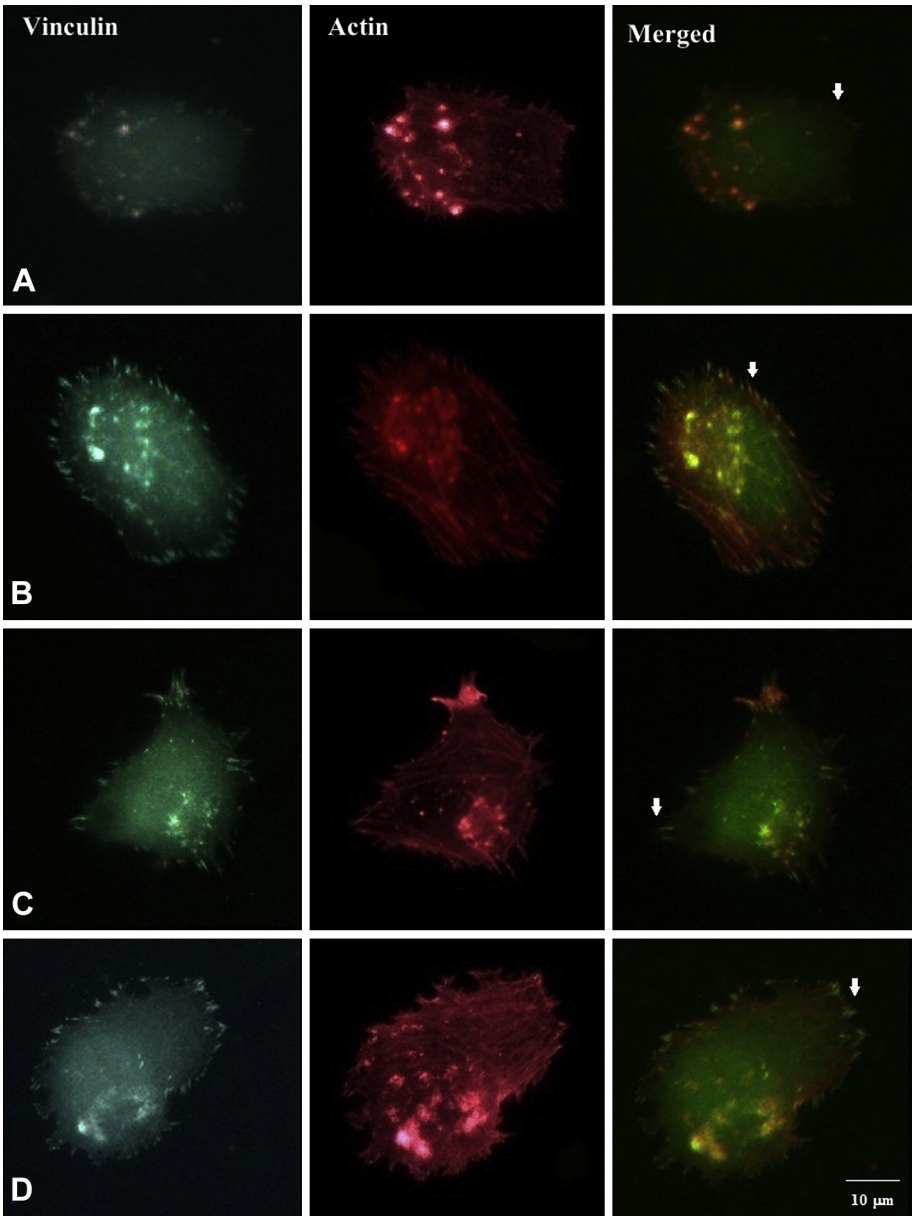

Figure 3. Focal adhesion and actin stress fiber formation in (A) untreated differentiated THP-1 cells as a control versus treated cells with different recombinant proteins, namely, (B) early secreted antigenic target-6 (ESAT-6), (C) culture filtrate protein-10 (CFP-10), and (D) ESAT-6/CFP-10. After a 4-hour incubation, cells were fixed and double stained for vinculin and actin. Arrows in the images indicate the localization of vinculin at the end of actin bundles.

the expression of β1 integrin mRNA in differentiated THP-1 cells treated with ESAT-6, CFP-10, and ESAT-6/CFP-10 recombinant proteins was estimated to be approximately 2.2, 2.5, and 3.2 times more than that in untreated cells, respectively. These amounts were 1.1, 1.4, and 1.3 times more for α5 integrin and approximately 7.2, 6.1, and 9.1 times more for α4 subunit of integrin, respectively. These data show that the levels of β1 and α4 subunits of integrin (Figs. 4A and 4C) in the differentiated THP-1 cells treated with the recombinant proteins were significantly higher than in untreated differentiated THP-1 cells ($p < 0.001$), but the level of α5 integrin (Fig. 4B) showed no significant difference compared with untreated cells. Accordingly, the fusion protein had more effect on α4 integrin expression level than ESAT-6 and CFP-10 individually.

Discussion

The data presented here show that the recombinant mycobacterial secretory proteins, ESAT-6, CFP-10, and their fusion, improve the adhesion of macrophages to ECM components, with no effect on the viability of human THP-1 cells (Fig. 1), leading to granuloma formation. We also found that $\alpha_4\beta_1$ integrin plays a key role in the adhesion of macrophages to FN. Recruitment of immune cells such as

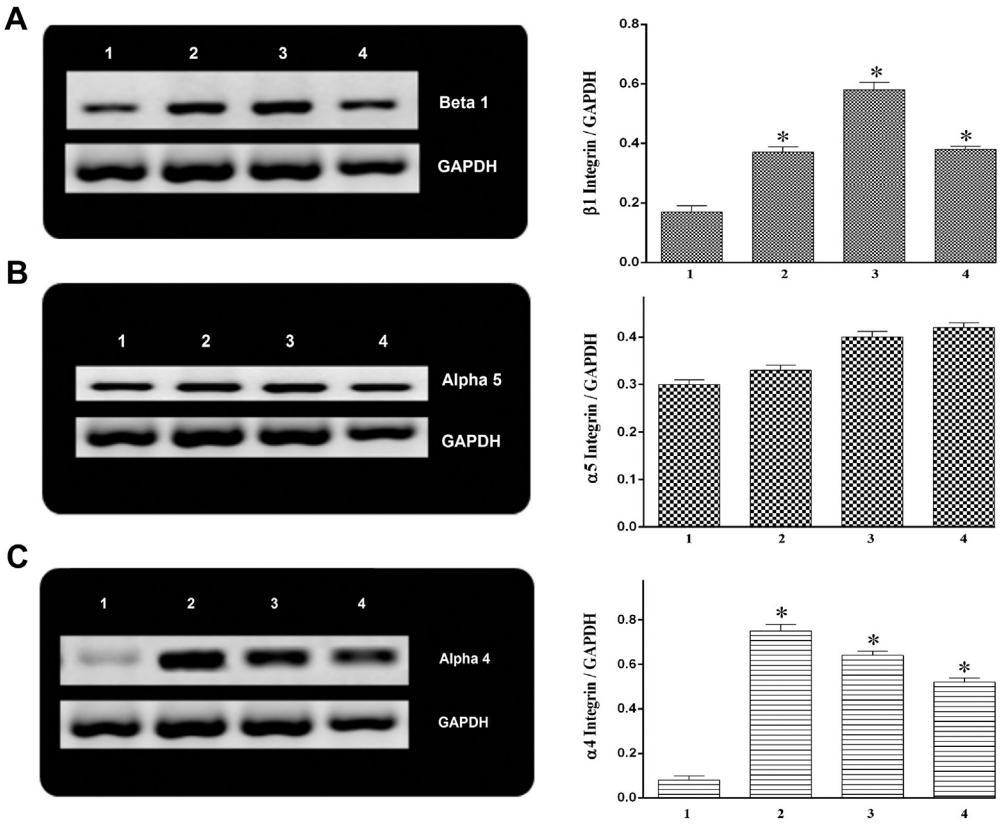

Figure 4. Ethidium bromide-stained agarose gel electrophoresis of real-time polymerase chain reaction products of (A) β1, (B) α5, and (C) α4 subunits of integrin in untreated and treated differentiated THP-1 cells. Lane 1 = untreated differentiated THP-1; lane 2 = differentiated THP-1 treated with 1.25 μg/mL ESAT-6/CFP-10; lane 3 = differentiated THP-1 treated with 2.5 μg/mL ESAT-6; lane 4 = differentiated THP-1 treated with 2.5 μg/mL CFP-10. *Significant ($P < 0.001$) difference between THP-1 cells and untreated cells. CFP-10 = culture filtrate protein-10; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; ESAT-6 = early secreted antigenic target-6.

macrophages within pulmonary granulomas depends on their adhesion to the ECM. Macrophages use integrins to adhere to FN as a major ECM component.[19] The most common receptors for FN are the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins.[19] The interaction between FN and these integrins is effective in adhesion, spreading, and focal contact formation in monocytes/macrophages.[20] Despite the good understanding of the structural organization of granulomas structure, the molecular and cellular mechanism of formation of granulomas and the role of ECM proteins in this process are not well defined.[20] A predominant role of mycobacterial glycolipids envelop in the formation of granulomas has been recently uncovered. It has been shown that adhesion and fusion of macrophages are mediated by Toll-like receptor-2 (TLR2) and is dependent on the β1 integrin. In the same study, using microarray analysis, Puissegur et al[21] showed that the expression of $\alpha_4\beta_1$ integrin has been induced in granuloma cells. Rojas et al[10] also indicated that the mycobacterial glycolipids envelop such as phosphatidylinositol mannoside binds $\alpha_5\beta_1$ on T cells and induces adhesion to FN. Using transparent zebrafish embryo for monitoring the infection process in real time,[22] it has been shown that RD1-deficient bacteria fail to elicit efficient granuloma formation, but macrophages infected with virulent mycobacteria produce an RD1-dependent signal, which directs macrophages to aggregate into granulomas. These observations indicated that the mycobacterium RD1 locus induces infected macrophages to send chemotactic signals for aggregation of macrophages, which in turn affect adhesion and other downstream events, resulting in granuloma formation. Despite the obvious inducing role of RD1 locus in macrophages aggregation, little is known about the effects of RD1 antigens, individually. In addition, the role of integrins is not clear in this process. Therefore, we aimed to investigate the role of recombinant *M. tuberculosis* antigens in the interactions and adhesion between macrophages and FN mediated by FN receptor integrins ($\alpha_4\beta_1$ and $\alpha_5\beta_1$) using a series of biochemical methods. The results obtained from cell attachment assay showed that differentiated THP-1 cell attachment to FN by 52 ± 3.08% is likely due to expression of $\alpha_5\beta_1$ and $\alpha_4\beta_1$ integrins. As shown in Fig. 2, the treatment of these cells with recombinant ESAT-6, CFP-10, and ESAT-6/CFP-10 proteins significantly increased the attachment on FN by 80 ± 1.2%, 74 ± 2.6%, and 90 ± 1.7%, respectively ($p < 0.001$). These results indicate that THP-1 cells adhere to FN and the recombinant antigens are able to increase attachment of this cell type on FN.

According to the attachment assay results in the cells treated with different antigens, adhesion on FN was

significantly increased when compared with untreated cells ($p < 0.001$). To examine whether treating cells with recombinant *M. tuberculosis* antigens leads to changes in F-actin organization and focal contact formation, immunofluorescent assay was performed. Results showed that THP-1 cells treated with all recombinant antigens used in this study form well-organized fibers and focal contacts containing vinculin in comparison with untreated cells. Accordingly, ESAT-6 and ESAT-6/CFP-10 showed better results in increasing focal contacts than CFP-10 (Fig. 3). Because adhesion of macrophages to ECM components, especially FN, depends on the two integrins, $\alpha_4\beta_1$ and $\alpha_5\beta_1$,[23] it is suggested that the induction of stress fibers and formation of focal contacts after treatment with these recombinant antigens could be through inducing the expression of these two integrins.

Our gene expression studies using semiquantitative RT-PCR revealed that α4, α5, and β1 subunits of integrins are being expressed by differentiated THP-1 cells, which are in agreement with Wang and Fu.[13] Treatment with the recombinant proteins is accompanied with an increase in the expression levels of these subunits of integrin. The RT-PCR results were generally in congruence with the data from attachment and immunofluorescent assays. Although we expected a significant increase in the expression level of α5 compared with α4 subunits of integrin in the cells coated on FN as indicated by Mostafavi-Pour et al,[16] interestingly we observed a significant increase ($p < 0.001$) in the expression level of α4 subunit of integrin in treated cells; for example, a 9.1-fold increase in the case of ESAT-6/CFP-10. This increase was especially observed in the case of treatment with ESAT-6 and ESAT-6/CFP-10 proteins, showing both the important role of ESAT-6 in the adhesion of macrophages and the additive effect of CFP-10. This observation also emphasizes the importance of $\alpha_4\beta_1$ integrin in the adhesion of macrophages to FN in the granulomas structure, suggesting the induction of adhesion of macrophages to FN through the activation of $\alpha_4\beta_1$ integrin signaling pathways. It seems these recombinant proteins can induce adhesion in granulomas structure, considering the agreement of microarray data of Puissegur et al[21] with our RT-PCR results.

Based on our data, the *M. tuberculosis* secretory recombinant proteins can support macrophage adhesion to the ECM following granuloma formation. New strategies to combat tuberculosis require a better understanding of the host—pathogen interaction. Based on the results of recent studies, ESAT-6 can interact with host proteins such as TLR2 and syntenin-1.[24,25] Interaction with TLR2 is associated with lower innate immune responses to infection. Syntenin-1 may function as an adaptor that couples syndecans to cytoskeletal proteins, and therefore ESAT-6 may affect adhesion by interaction with syntenin-1.

It seems that more studies are needed to be carried out to uncover the protein—protein interactions of the virulence factor, ESAT-6, for better understanding of the role of ESAT-6 during the infection process. Using yeast two-hybrid system and ligand overlay, we could identify other proteins that interact with *M. tuberculosis*-secreted antigens, especially with those we studied. The possibility of cross-talk between receptors on macrophages, such as TLRs and integrins, and the *M. tuberculosis* antigens results in downstream signaling, and this is another issue that requires further investigation.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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