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Induction of cytotoxic T lymphocyte response against Mycobacterial antigen using domain I of anthrax edema factor as antigen delivery system

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Abstract

We have investigated the efficiency of N-terminal 1–260 residues of Edema factor (EFn) as a delivery system for ESAT-6, an antigenic protein of *Mycobacterium tuberculosis* $H_{37}R_v$, into the cytosol of mammalian cells. The EFn.ESAT-6 recombinant protein was obtained by genetic fusion of EFn and ESAT-6 DNA. Our data shows that in the presence of PA, EFn.ESAT-6 fusion protein is internalized into the cytosol of antigen presenting cells, and the splenocytes produced both Th1 and Th2 cytokines *in vitro*. Further, EFn.ESAT-6 elicited effective cytotoxic T lymphocyte (CTL) response in an *in vitro* CTL assay. This study for the first time demonstrates that EFn can be used as a vehicle to deliver heterologous proteins of therapeutic importance.

Keywords: Edema factor; ESAT-6; Fusion protein; CTL; Cytokines

Humoral and cellular responses constitute the two main arms of immune system. One of the key functions of cellular immunity is to generate cytotoxic T lymphocytes (CTLs) for destruction of cells expressing intracellularly processed antigens on their surface. The CTLs recognize and kill tumor and other diseased cells, which display non-self peptides on their surface [1,2]. These peptides arise from various sources, such as infectious agents or aberrant expression of self-proteins, and mark defective cells for CTL recognition. Proteins within the cytosol are processed by multi-catalytic proteosome to generate small peptides, which are then displayed by class I major histocompatibility molecules (MHC-I) on the cell surface. Recognition of foreign peptide–MHC-I complexes by $CD8^+$ cells leads to activation of specific CTLs, which clear the defective cells expressing foreign peptides or harboring pathogen [1,2–5]. Activated CTLs lyse infected cell, secrete cytokines, proliferate and differentiate. Vaccines that prime such memory CTLs, provide protection to the host, upon subsequent exposure to similar antigen displaying cells [5].

Development of vaccines with the ability to generate specific CTLs is hindered due to paucity of delivery systems of antigenic CTL epitopes into the cytosol of host cells. Several approaches to this problem have been reported [1] including the use of attenuated viruses, intracellular bacteria, bacterial toxins, naked DNA, electroporation, heat shock protein, polycationic peptides, non-ionic triblock copolymer, and adjuvants [6–11]. Each of these methods have inherent problem of safety and/or efficiency. Non-infectious, non-toxic, modified bacterial toxins for delivery of heterologous proteins have been reported in the past [6,8,12–17].

Bacillus anthracis produces a bipartite exotoxin comprising of two toxins namely edema toxin (ET) and lethal toxin (LT). Both ET and LT contain a protective antigen (PA) component along with either edema factor (EF) or lethal factor (LF), respectively. Individually, PA, EF, and LF are non-toxic. PA mediates entry of EF and LF into the

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cytosol by specific receptor on the susceptible cells [18,19]. EF acts as Ca^{2+}/calmodulin dependent adenylate cyclase that causes edema by elevating cyclic amp levels abnormally in the host cells; [20] and LF, which has zinc metalloprotease activity, causes death by inactivating key molecules like MAPK kinase of signaling pathway [21,22]. Mutational studies in our laboratory have demonstrated that in the N-terminal half of EF, residues 136 VYYEIGK142 are critical for binding to PA and subsequent translocation into cytosol [23]. Additionally, the N-terminal domain I (EFn), (1–260 residues) lacks cytotoxicity, associated with the carboxyl terminal domain of EF, and therefore can be used safely for intracellular delivery of heterologous proteins for generation of CTL response.

Here, we report for the first time, utilization of EFn for delivery of ESAT-6, an antigen of *Mycobacterium tuberculosis* $H_{37}R_v$ into the cytosol of macrophages for induction of cell mediated immune response against ESAT-6.

Material and methods

Construction, expression, and purification of EFn.ESAT-6 fusion protein. EFn fusion protein containing ESAT-6 antigen was produced using expression vector pET23a (Novagen). The DNA sequence encoding EFn was amplified by PCR and cloned in pET23a vector using *Bam*HI and *Sac*I restriction sites. A *Kpn*I site was added by PCR amplification at 3′ end, just before *Sac*I site. A DNA sequence encoding ESAT-6 was amplified by PCR with *Kpn*I and *Sal*I sites at the 5′ and 3′ ends, respectively, and ligated to the above construct at *Kpn*1 site, such that ESAT-6 was fused at the C-terminal of EFn. This construct was sequenced to confirm the sequence.

pET23a construct containing EFn.ESAT-6 was transformed into competent cells of BL21 (DE3), Codon Plus strain of *Escherichia coli* and recombinant protein was obtained as follows. In brief, cultures were grown in LB medium containing ampicillin (100 μg/ml) and chloramphenicol (60 μg/ml) to an OD_{600} of 0.8. Protein expression was induced by 1 mM isopropyl α-D-thiogalactoside for 4 h. Cells were pelleted and EFn.ESAT-6 protein was purified using Ni–NTA affinity chromatography under denaturing conditions. The cell lysate containing the denaturant was mixed with 3 ml Ni–NTA slurry and loaded onto a column. Ni–NTA matrix was washed with 50 ml of denaturing buffer containing 8 M urea, followed by on-column renaturation of the protein using 8–0 M urea gradient. The protein was eluted with elution buffer containing 250 mM imidazole (pH 8.0). Purified fusion protein was analyzed on 12% SDS–PAGE and then dialyzed against 10 mM Hepes buffer containing 50 mM NaCl.

Purification of PA and LF. Recombinant PA and LF proteins were purified from culture supernatant of *E. coli* M15 cells as described previously [24,25].

Construction, expression, and purification of ESAT-6 protein. ESAT-6 protein was produced using the expression vector pQE30 (Qiagen). To produce ESAT-6, PCR amplified ESAT-6 gene was cloned using *Kpn*I and *Hin*dIII restriction sites at the 5′ and 3′ ends, respectively. The plasmid pQE30.ESAT-6 was transformed into *E. coli* M15 cells, and transformants were selected on plates containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml). *E. coli* M-15 culture containing plasmid pQE30.ESAT-6 was induced with 1 mM IPTG and ESAT-6 was purified as described above for EFn.ESAT-6.

Competitive inhibition of lethal toxin activity by EFn.ESAT-6. Binding of EFn.ESAT-6 to PA was tested on J774A.1 macrophage cell line by adding increasing concentrations of fusion protein in combination with PA (1 μg/ml) and LF (1 μg/ml) and incubating at 37 °C for 3 h. At the end of incubation period, cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye assay. MTT dissolved in RPMI 1640 medium was added to the cells at a final concentration of 0.5 mg/ml. Incubation was continued for additional 45 min to allow uptake and oxidation of the dye by viable cells. The medium was replaced by 100 μl of 0.5% SDS/25 mM HCl in 90% isopropanol, and plates were vortexed. Absorbance was read at 540 nm using a Microplate Reader (Bio-Rad).

Mice immunization. All the proteins used for immunization were tested for LPS, which was below 0.3 ng/μg of protein. Six- to eight-week-old female BALB/c (H-2^d) mice were obtained from Panacea Biotec Ltd. and maintained in pathogen free environment. A group of 10 BALB/c mice were injected intraperitoneally on day 0 and on day 14 with 2 μg of ESAT-6, EFn.ESAT-6, and PA+EFn.ESAT-6 in Phosphate buffer saline (PBS).

Isolation of splenocytes. Splenic effector cells were prepared by grinding spleens between frosted slides, followed by aspiration through 22-gauge needle to prepare single cell suspensions. Erythrocytes were lysed by ammonium chloride treatment. Remaining spleen cells were washed twice with complete RPMI medium and viability was determined by trypan blue exclusion. Splenocytes were suspended in complete RPMI medium containing 10% heat-inactivated FBS (Hyclone) and kept in CO_2 incubator for further use.

Cytokine ELISA. Mice were injected with PA+EFn.ESAT-6 and splenocytes were isolated after 7 days. Total splenocytes were cultured and stimulated *in vitro* by different antigens. Quantification of murine cytokines; IL-2, IL-12, IL-4, and IFN-γ present in the culture supernatant of activated splenocytes was determined using BD Opt EIA™ kits according to manufacture's protocol (Pharmingen).

Preparation of stimulator cells. For the preparation of stimulator cells, BALB/c (H-2^d) mice derived macrophage J774.1 cells were treated with the same antigen (1 μg/ml) as used for immunization [26]. ESAT-6 or EFn.ESAT-6 were either incubated with or delivered into J774A.1 cells by osmotic shock as described by Okada and Rechsteiner [27]. PA mediated delivery of EFn.ESAT-6 fusion was carried out as follows. Confluent J774A.1 macrophages in 75-cm^2-tissue culture flask were incubated with PA and EFn.ESAT-6 in RPMI-1640 glutamine minus medium for 4–5 h. The medium was removed, and extracellular protein/antigens were rinsed with complete RPMI 1640 medium. Macrophages were scraped off with a sterile rubber policeman and centrifuged at 200*g* for 5 min. Washed cells were suspended in 5 ml of complete RPMI 1640 medium with mitomycin C (35 μg/ml) and incubated at 37 °C for 45 min with 5% CO_2. Stimulator cells were washed by centrifugation four times with complete RPMI-1640 supplemented with 5% heat-inactivated fetal bovine serum. Trypan blue exclusion test was used to count macrophage cell numbers and to determine viability. As control of internalization, LF was also introduced by osmotic shock.

Preparation of effector cells. To obtain effector T cells, splenocytes from immunized mice and stimulator cells prepared with homologous antigen were mixed in 1:1 ratio and incubated in the presence of 30 U/ml murine recombinant IL-2 for 5 days at 37 °C with 5% CO_2. After 5-day incubation, cells were collected and live effector cells were obtained by removing dead cells by density gradient centrifugation over histopaque®-1119 (Sigma–Aldrich). Cell number and viability were determined by the trypan blue exclusion test.

Preparation of target cells. The protocol used to prepare different target cells was same as that for stimulator cells, except the mitomycin C step was omitted. J774A.1 cells were treated with 1 μg/ml each of PA, EFn.ESAT-6, and ESAT-6, PA+EFn.ESAT-6 for 4 h and washed twice with RPMI-1640 medium.

Colorimetric CTL assay. A previously described non-radioactive assay procedure [26,28] was followed with some modifications. This was preferred over the radioactive ^{51}Cr release CTL assay as being more sensitive and safe. Different combinations of effector and target cells were mixed at 10:1 ratio, respectively, and incubated for 16 h at 37 °C. After one wash with warm PBS (pH 7.2–7.4), 200 μl of 0.036% neutral red solution (prepared by diluting a 1% (wt/vol) stock solution to 0.036% in warm PBS (pH 7.2) just before use) was added to stain unlysed target cells. After 30 min, the cells were thoroughly washed and then lysed with 0.22 ml of 0.05 M acetic acid-0.05% sodium dodecyl sulfate solution. The amount of dye released was measured by taking optical density (OD) readings at

570 nm. The percentage of specific lysis was established by applying the formula specific lysis = (OD of control − OD of experimental group)/OD of control × 100.

Results

Plasmid construction

The amino terminal domain of EF required for binding to PA and translocation into the cell cytosol was genetically fused to ESAT-6. The DNA construct expressed a fusion protein in which 1–260 residues of EF are fused to 95 residues of ESAT-6 and 6× His tag. The addition of residues at amino and carboxyl terminal ends of EFn (1–260) do not appear to affect functioning of the fusion protein with respect to binding to PA and protein translocation. The resulting fusion protein has 382 amino acids corresponding to molecular mass of 43.07 kDa (Fig. 1A) and calculated pI of 5.25. The yield of fusion protein was about 40 mg/l.

The molecular mass of recombinant ESAT-6 protein corresponded to12 kDa based on electrophoretic mobility on SDS–PAGE (Fig. 1B) and calculated pI of 6.12. The refolded ESAT-6 protein exhibited multimer formation (Fig. 1B), as already described in the literature [29]. The yield of ESAT-6 protein was about 7 mg/l.

Quantification of fusion protein activity by competitive inhibition of lethal activity of LF

Since EFn.ESAT-6 has no toxic activity on cells, binding and internalization of the fusion protein was determined by indirect competitive inhibition of killing activity of LF. The assay is based on the fact that N-terminal 1–260 amino acids of EF and LF are very similar (56% similarity and 37% homology) and contain an identical PA binding motif VYYEIGK, used for binding to PA and subsequent internalization. Increase in concentration of the fusion protein competitively inhibits binding of LF to PA, resulting in decrease in cytolysis of J774A.1 cells. Thus, progressive inhibition of toxicity of LF+PA by the EFn.ESAT-6 gives an indirect measurement of the EFn.ESAT-6 binding and internalization into the J774A.1 cells. Our results show that about 90% protection of the cells occurred when the fusion protein was used in 2.25-fold excess over LF (Fig. 2), indicating that the fusion is able to compete with LF for binding to PA on the cells.

Cytokine ELISA

T helper cells (Th1/Th2) play an important role in eliciting both humoral and cellular responses via expansion of antigen-stimulated B cells and $CD8^+$ T cells or CTLs, respectively. Therefore, the levels of Th1 cytokines (IL-2, IL-12, and IFN-γ) and Th2 cytokines (IL-4) were measured as parameters of polarization of immune response. The Th1 specific marker cytokine IFN-γ production by splenocytes from mice immunized with ESAT-6 alone is known to be very poor [30]. Hence, we examined the comparative cytokine production profiles of the splenocytes isolated from PA+EFn.ESAT-6 immunized mice, after stimulating with different antigens *in vitro*. The time course of synthesis of cytokines by the splenocytes from immunized mice was

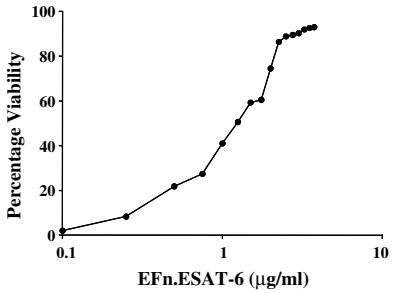

Fig. 2. Competitive inhibition of lethal toxin activity by EFn.ESAT-6. J774A.1 macrophage cells were incubated with PA (1 μg /ml) and LF (1 μg /ml) and increasing concentrations of EFn.ESAT-6 (0–4 μg /ml) for 3 h at 37 °C. Toxicity was determined by MTT assay.

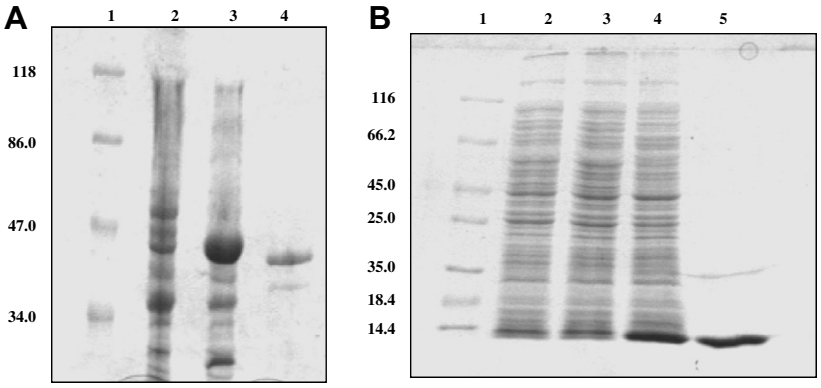

Fig. 1. Expression and purification of recombinant proteins; EFn.ESAT-6 (A) and ESAT-6 (B) in *E. coli* BL-21 (DE3) Codon Plus and *E. coli* M-15 cells respectively. Proteins were analyzed on 12% SDS–PAGE and stained with Coomassie brilliant blue; (A) Lane 1, molecular weight marker; Lane 2, Uninduced pET23a.EFn.ESAT-6; Lane 3, induced pET23a.EFn.ESAT-6; Lane 4, Purified EFn.ESAT-6. (B) Lane 1, molecular weight marker; Lane 2, *E. coli* M-15 host cells, Lane 3, Uninduced pQE30.ESAT-6; Lane 4, induced pQE30.ESAT-6, Lane 5, Purified recombinant ESAT-6 (showing both monomeric and dimeric form).

determined at 24, 48, and 72 h. The mean variation of levels of Th1 cytokines (IL-2, IL-12, and IFN-γ) and Th2 cytokine (IL-4) with time are presented in Fig. 3A, B, C, and D, respectively. A substantial increase in the levels of IL-2, IL-12, and INF-γ compared to the control (unstimulated) splenocytes indicated antigen presentation by MHC I pathway and activation of Th1 response. Further, cytokine production by cultured splenocytes was time and antigen dependent. The pattern of cytokine secretion by splenocytes activated with all the antigens was essentially same. An increase of about 2000 pg/ml of IFN-γ was observed in the presence of PA+EFn.ESAT-6 compared to ESAT-6 alone at 72 h, while IL-2 and IL-12 levels were not significantly different in the presence of various antigens. The cytokine levels measured in the supernatant represent the cumulative amounts of each type secreted by different subsets of immune cells present in the splenocytes mixture, therefore it may not accurately reflect the efficiency of antigen delivery. The IL-4 levels were suppressed in the presence of PA+EFn.ESAT-6 as compared to control and also EFn.ESAT-6 fusion, supporting Th1 mediated response in the former. Based on the results it can be inferred that antigens were Th1 response dominant.

CTL assay

The degree of cytolysis caused by effector T cells obtained from mice immunized with either ESAT-6 or EFn.ESAT-6 alone was not more than 20% irrespective of whether the target cells were obtained by incubating the J774A.1 cells in presence of different antigens or by introducing ESAT-6 or EFn.ESAT-6 into cytosol of J774A.1 cells through osmotic shock (data not shown); this was similar to the basal level of cytolysis observed with non-primed target cells (data not shown).The splenocytes form mice immunized with PA+EFn.ESAT-6 produced highest T cell mediated cytotoxicity, when tested in an *in vitro* CTL assay. Maximum cytolysis (60–70%) ($P < 0.05$) occurred when effector T cells from PA+EFn.ESAT-6 immunized mice were incubated with target cells prepared by identical method of antigen delivery i.e PA+EFn.ESAT-6 (Fig. 4-bar 6 A, B, and C). The positive target cells which were prepared by introducing the ESAT-6 or EFn.ESAT-6 into cytosol of J774A.1 through osmotic shock showed 38–45% ($P < 0.05$) level of lysis and, the equivalent lysis showed by both target cells also confirmed that CTLs generated by immunizing with PA+EFn.ESAT-

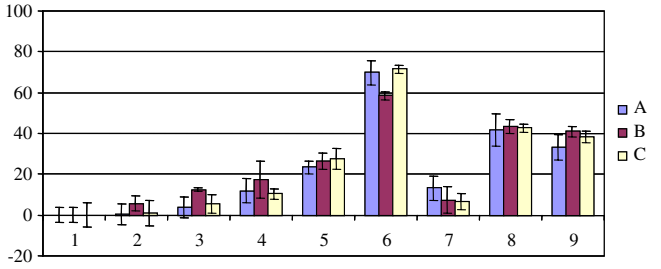

Fig. 4. CTL-mediated lysis of J774A.1 target cells. Splenocytes were isolated from mice immunized with PA+EFn.ESAT-6. Stimulated *in vitro* by stimulator cells prepared by osmotic shock with (A), ESAT-6; (B), EFn.ESAT-6 and (C) by incubating with PA+EFn.ESAT-6. Cytolytic activity of the effector T cells was determined by incubation with differently primed target cells. The effector-to-target-cell ratios (E:T ratios) examined were 10:1. (1), J774A.1 cells (no effector cells); (2), J774A.1; (3), J774A.1 treated with PA; (4), J774A.1 treated with ESAT-6; (5), J774A.1 treated with EFn.ESAT-6; (6), J774A.1 treated with PA+EFn.ESAT-6; (7), J774A.1 given osmotic shock without any antigen; (8), J774A.1 given osmotic shock with ESAT-6; (9), J774A.1 given osmotic shock with EFn.ESAT-6. Data are expressed as means ± SEM of triplicates samples.

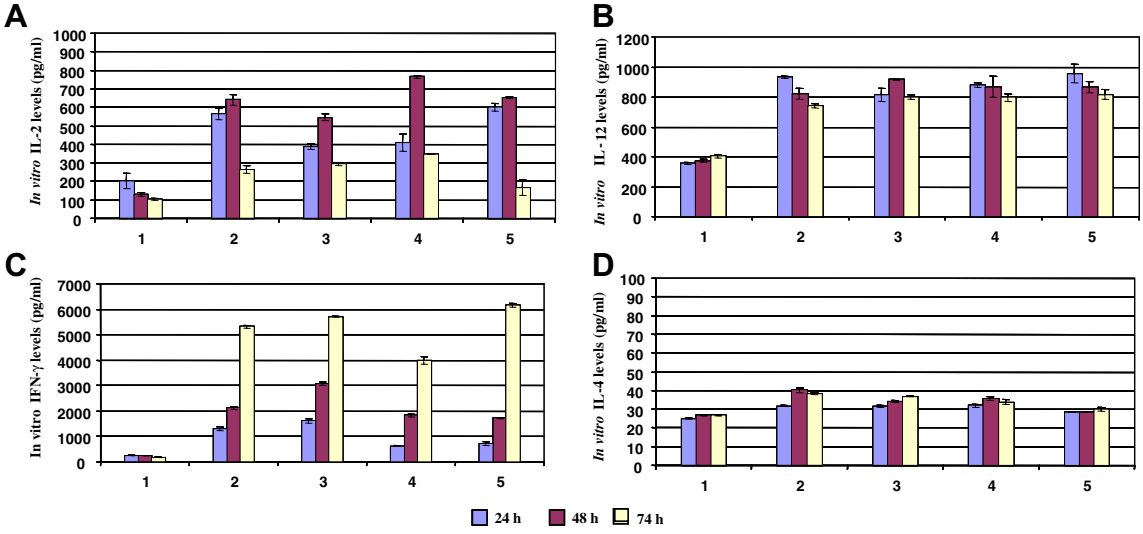

Fig. 3. Concentration of IL-2, (A); IL-12, (B); IFN-γ, (C); and IL-4, (D) in culture supernatants of BALB/c mouse splenocytes. Splenocytes (5×10^5 cells/ml) were stimulated with 10 μg/ml of PA+EFn.ESAT-6 up to 72 h, culture supernatants were collected at 1 h intervals and analyzed by capture ELISA for different cytokines. Splenocytes from four mice were included in each experiment. Data are expressed as means ± SEM of triplicates samples (1), control; (2) PA; (3), EFn.ESAT-6; (4), ESAT-6 and (5), PA+EFn.ESAT-6.

6 were specific to ESAT-6. Further, the method of preparation of stimulator cells *in vitro*, target cells and CTL assay indicated that the route of delivery of ESAT-6 in the antigen presenting cells, proteosomal processing and presentation of epitopes to naïve T cells is critical factor for eliciting the CTL response. Thus, improved efficiency of antigen delivery by PA+EFn.Antigen delivery system resulted in enhanced display of T-cell epitopes on the target cell surface, leading to higher cytotoxicity. The overall results of the CTL assay indicated that CTLs generated by immunization with PA+EFn.ESAT-6 were specific to ESAT-6. In short the data demonstrates that PA mediated delivery of EFn.ESAT-6 fusion protein can elicit effective/desired CTL response.

Discussion

A number of strategies have been used experimentally to deliver an antigen into cytosol for presentation on the cell surface in association with MHC I molecule. Delivery into cytosol and presentation through MHC I molecule is a necessary step for elicitation of CTLs against the host cells expressing foreign peptide [1,10]. ESAT-6 is an important T-cell antigen of *Mycobacterium* and is being investigated as a potential subunit vaccine candidate in several studies [29–31]. Due to relatively low inherent immunogenicity of ESAT-6 [29], all the efforts are directed towards enhancing its ability to evoke a robust T cell response, crucial in the context of tuberculosis infection [30,31].

The mechanism of generation of ESAT-6 specific CTL by PA+EFn.ESAT-6 system can be rationalized as occurring through entry of ESAT-6 into the cytosol of antigen presenting cells in a manner closely resembling its delivery by mycobacterial cells during natural infection. Direct entry of ESAT-6 into the cytosol would induce macrophages and dendritic cells to secrete IL-12, causing differentiation of $TCD4^+$ into Th1 cells. The cytokines produced by the Th1 cells would eventually stimulate differentiation and proliferation of antigen primed $TCD8^+$ cells into cytotoxic T lymphocyte. Synthesis of Th1 type cytokines (IL-2, IL-12, and IFN-γ) by the splenocytes after *in vitro* stimulation supports the proposed mechanism of generation of CTL response. The results of this study demonstrate that an important T cell specific antigen ESAT-6 of *M. tuberculosis*, which is not able to generate adequate protective immunity against infection when used alone [30], worked very efficiently when introduced in the cytosol by this strategy. Thus, detoxified anthrax toxin components can be exploited to deliver protective T cell antigens not only of *M. tuberculosis* but of other intracellular pathogens also. We have experimentally shown that EFn.ESAT-6, when administered along with PA, results in generation of specific CTL response, against ESAT-6. The importance of this study lies in the fact that EFn is able to deliver a target protein into the cytosol of antigen presenting cells in the mice model, for eliciting T cell response essentially required for protection in *M. tuberculosis* infection. This is the first time EF component of anthrax toxin is successfully used as a delivery vehicle.

It is well established that ESAT-6 protein is a potent T-cell antigen [29] and is a vaccine candidate in DNA and subunit vaccines against tuberculosis [31]. In our study, the most likely mechanism of processing of the fusion protein in the mice appears to be PA mediated delivery of the latter in the cytosol followed by proteosomal processing of EF.ESAT-6 and presentation of T cell epitopes by class I MHC complex leading to elicitation of ESAT-6 specific CTL response. The immune response generated by the EFn.ESAT-6 fusion strongly supports the role of EFn as a carrier to deliver specific antigens of *M. tuberculosis*. Further, due to extensive polymorphism of MHC I molecule from individual to individual, protective T-cell epitopes of an antigen also vary from host to host. The ability of EFn to deliver a whole protein molecule into cytosol might be useful in developing vaccine for intracellular pathogens that require T cell specific protective immunity. Multiple epitopes can also be fused to develop more efficient T-cell vaccines against intracellular pathogens. The EFn fusion-mediated delivery has important implications for development of vaccines against infections requiring protective T-cell immunity.

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