Association of ESAT-6/CFP-10-induced IFN-γ, TNF-α and IL-10 with clinical tuberculosis: evidence from cohorts of pulmonary tuberculosis patients, household contacts and community controls in an endemic setting

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Summary

Mycobacterium tuberculosis (Mtbc) early secreted protein antigen 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) are among candidate vaccines against tuberculosis (TB). Results of experimental animal models show that these antigens are associated with induction of strong T cell immunity [interferon-gamma production], while others report that these proteins as virulent factors involved in pathogenicity of Mtbc infection. However, the role of ESAT-6/CFP-10 during natural Mtbc infections in humans has not been established. In this paper we present results of a longitudinal study from an Mtbc-infected human population from an endemic setting. Whole blood assay was used to determine levels of IFN-gamma, tumour necrosis factor (TNF)-alpha and interleukin (IL)-10 against rESAT-6/CFP-10 in TB patients, household contacts and community controls. The levels of IFN-gamma, TNF-alpha and IL-10 against rESAT-6/CFP-10 at baseline were significantly higher in patients and community controls than in household contacts. In patients, no significant difference was observed in the level of these cytokines before and after chemotherapy whereas, in contacts, the level of these cytokines increased significantly and progressively over time. The study shows that the levels of IFN-gamma, TNF-alpha and IL-10 against rESAT-6/CFP-10 are depressed during Mtbc infection or exposure but are elevated during clinical TB. Our findings from a study of naturally infected human population suggest that IFN-gamma, TNF-alpha and IL-10 against rESAT-6/CFP-10 are markers for clinical TB but not for protective immunity.

Keywords: CFP-10, ESAT-6, IFN-gamma, IL-10, immunity, TNF-alpha, tuberculosis

Introduction

Tuberculosis (TB) remains one of the most important infectious diseases, with a mortality of 1·8 million people in 2015 [1]. The only licensed TB vaccine currently in use, bacille Calmette–Guérin (BCG), does not control TB transmission. Therefore, there is extensive research to develop an efficacious vaccine to replace or augment BCG vaccine, and some of these vaccine candidates are in clinical trials [2–4].

It is generally agreed that cell-mediated immunity with the production of proinflammatory cytokines such as interferon-gamma and tumour necrosis factor (TNF)-alpha are protective during Mtbc infection [5], as shown previously in animals [6] and in humans [7]. Conversely, immune regulatory cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-beta inhibit excessive production of proinflammatory cytokines, thereby mitigating immunopathology and maintaining homeostasis [8–10].

Mycobacterium tuberculosis early secreted antigen 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) are among candidate TB vaccines because they induce strong T cell immunity in animal models [11–15]. However, there is also substantial evidence implicating these antigenic proteins as virulent factors during Mtbc infection [16,17]. It has been shown that ESAT-6 inhibits antigen-presenting cell function by reducing IL-12 production by macrophages [18] by lysing epithelial cells and macrophages [19,20] and destabilizing phagolysosome, allowing Mtbc and its products to escape phagosome [21], and promoting intracellular dissemination of Mtbc [20,22]. It has also been shown that ESAT-6 directly inhibits human T cell IFN-gamma production [23].
ESAT-6 has also been shown to inhibit T cell production of IL-10 and IL-17 [24], TNF-α and IL-17 but not IL-2 [18]. However, reports on immune responses to recombinant ESAT-6/CFP-10 are limited to studies on experimental animal models and from non-endemic settings. Information is lacking on proinflammatory (IFN-γ and TNF-α) and anti-inflammatory (IL-10) cytokine responses against rESAT-6/CFP-10 from a naturally infected human population in an endemic setting. The current study was conducted as part of a major project to investigate protective immune markers during *Mtb* infection in a human population in an endemic setting.

**Materials and methods**

**Study setting and population**

This study was conducted in an endemic setting in Addis Ababa, Ethiopia, with a population of 2.6 million. Of 24 health centres with established Directly Observed Treatment, Short-Course (DOTS) services, seven health centres (Kotebe, Akaki, Kazachnis, Bole, Shiro Meda, Woreda 7 and Teklehaymanot) were selected for the large cohort study. Smear-positive pulmonary TB (PTB) patients were recruited from the selected health centres. Their household contacts (HHCs) were invited to be screened for TB and participate in the study. Similarly, community controls (CCs) were included from localities around health centres. Household contacts and CCs were screened for TB using clinical history, QuantiFERON-TB gold test (QFT-G) and chest X-ray. Acid-fast bacilli and culture were performed for those with productive cough. Patients were treated with anti-TB drugs for 6 months according to the national guideline [25]. All participants were HIV-negative adults (≥18 years). Pregnant women and anaemic patients were not included into the study. A questionnaire was used to collect information on sociodemographic characteristics of the participants, BCG vaccination and previous history of TB and other chronic diseases.

**Cytokine assay**

Cytokine levels were determined using whole blood assay, as described previously [26]. Briefly, heparinized blood samples were diluted to a final concentration of 1:10 with RPMI-1640 supplemented with glutamic acid and streptomycin, and stimulated with rESAT-6/CFP-10 (E6C10) at a final concentration of 10 μg/ml. After 48 h of incubation at 37°C with 5% CO₂, supernatants were harvested and stored at −80°C until enzyme-linked immunosorbent assay (ELISA) was performed. The levels of IFN-γ, TNF-α and IL-10 were measured using eBioscience Ready-Set-Go cytokine ELISA kit (eBioscience, San Diego, CA, USA). The detection limits of the kits were 4–500 pg/ml (for IFN-γ and TNF-α and 2–300 pg/ml for IL-10). ELISA was performed according to the manufacturer’s recommendations and optical density (OD) values were interpolated with a four-parameter curve-fit after generating a standard curve. Purified protein derivative (PPD) (obtained from Statens Serum Institute, Copenhagen, Denmark) at a concentration of 10 μg/ml was used as control. ESAT-6/CFP-10 of *Mtb* for this work was supplied by Leiden University Medical Center, the Netherlands.

**Data analysis**

The Kruskal–Wallis test with Dunn’s multiple comparisons was used to compare cytokine responses among patients, contacts and community controls at baseline. The Mann–Whitney *U*-test was used to compare cytokine responses between patients and contacts at 6 and 12 months. Freidman’s test with Dunn’s multiple comparisons was used to compare cytokine levels over 12 months in patients and contacts. Analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA; http://www.graphpad.com).

**Ethical clearance**

This study was approved in Ethiopia by the National Research Ethics Review Committee and in Norway by Regional Committee for Medical and Health Research Ethics (Regional Komitee for Medisinsk og Helsefaglig Forskningsetikk, REK SØR-ØST). The objectives of the study were explained to the study participants and written informed consent was obtained from each participant.

**Results**

At baseline, 201 smear-positive PTB patients, 182 HHCs and 70 CCs were recruited. The results show that patients had significantly (*P* < 0.001) higher levels of IFN-γ against ESAT-6/CFP-10 than HHCs at baseline. The level of IFN-γ against this antigen was also significantly (*P* < 0.05) higher in CCs than in HHCs (Fig. 1a). No significant difference was observed in the level of IFN-γ against this antigen between HHCs and patients 6 and 12 months after baseline. Repeated measures in patients showed no significant difference in the level of IFN-γ before and after treatment (Fig. 1b). However, there was a significant (*P* < 0.0001) and progressive increase in the levels of IFN-γ against rESAT-6/CFP-10 in HHCs over time from entry (Fig. 1c). At baseline, patients had significantly (*P* < 0.05) higher levels IFN-γ against PPD than CCs (Fig. 1a).

At baseline, the level of TNF-α against ESAT-6/CFP-10 was significantly (*P* < 0.0001) higher in untreated patients than in HHCs. Community controls also had significantly (*P* < 0.01) higher levels of TNF-α than HHCs (Fig. 2a). There was no significant difference in the levels of TNF-α in patients before and after chemotherapy (Fig. 2b); however, there was a significant (*P* < 0.0001) and progressive
increase in the level of TNF-α from baseline to 6 months in HHCs (Fig. 2c).

At baseline, the level of IL-10 against ESAT-6/CFP-10 was significantly higher in patients and CCs ($P < 0.0001$) than in HHCs (Fig. 3a). Repeated measures did not show any significant difference in patients (Fig. 3b). However, the level of IL-10 increased significantly from baseline to 6 months ($P < 0.05$) and from 6 to 12 months ($P < 0.0001$) in HHCs (Fig. 3c). Figure 4a–c shows IFN-γ responses to PPD in the three cohorts. The level of IFN-γ against PPD was significantly ($P < 0.05$) higher in patients than in HHCs. No other significant difference was observed either between cohorts or between measurements.

**Discussion**

Several studies have suggested that ESAT-6, CFP-10 and/or ESAT-6/CFP-10, alone or in combination with other proteins or BCG, induce strong protective T cell immunity during Mtb infection, especially in a murine model of TB [11,13,14,27–29]. These reports have led to extensive research to realize the potential of these proteins as vaccine candidates [29–34]. Conversely, a substantial body of evidence indicates that these proteins are virulent factors involved in the pathogenicity of Mtb [23,35–38].

In the present study, we studied IFN-γ, TNF-α and IL-10 responses against rESAT-6/CFP-10 in cohorts of PTB patients, their HHCs and CCs in an endemic setting at baseline, 6 and 12 months from entry point. We used the following assumptions to denote a marker for immune protection and susceptibility/pathogenesis: (i) an elevated level of cytokine response in untreated patients (failed to control Mtb infection) compared to HHCs and CCs is a marker for clinical TB; and (ii) an elevated level of cytokine response in HHCs and CCs (those with no apparent clinical TB) compared with TB patients is an indication of protective immunity.

In this study, in a naturally Mtb-infected population in an endemic setting, we show that the level of IFN-γ against rESAT-6/CFP-10 was significantly higher in untreated patients compared to HHCs at baseline. This implies that ESAT-6/CFP-10 is not associated with T cell-based protective immunity as measured by IFN-γ production, as suggested previously. Instead, the results show that ESAT-6/CFP-10 is associated with clinical TB or disease
progression. In agreement with our results, a study by the VACSEL study group [39] involving a naturally Mtb-infected human population in a similar endemic setting showed that the IFN-γ response against ESAT-6 was associated with risk of developing clinical disease.

Our results support previous reports that ESAT-6/CFP-10 is associated with virulence and pathogenicity of Mtb based on results of a murine model of TB [23,35–38]. Some of these studies have shown not only that ESAT-6 or CFP-10 is a virulent factor promoting pathogenicity of Mtb, but also mechanisms involved in the pathogenesis. For instance, Wang et al. [23] have reported that higher concentrations (1·6–3·3 mM) of ESAT-6 inhibit IFN-γ production by Mtb-responsive T cells in PBMC, and suppress both proliferation and IFN-γ production by purified CD3+ T cells stimulated with anti-CD3+ and anti-CD28, indicating that these effects were independent of antigen-presenting cells. In another study, Kumar and co-workers [35] have indicated that ESAT-6 differentially inhibits the IFN-γ inducible class II transactivator isoform [in both a Toll-like receptor (TLR)-2-dependent and independent manner]. Class II transactivator protein has been described as the master regulator of gene expression of major histocompatibility complex class II (MHC-II) molecules [40–42]. Moreover, Wang et al. [37] have shown that ESAT-6 primes dendritic cells to stimulate Th17 and inhibits Th1 immune responses. In this study, an important role played by ESAT-6 and CFP-10 in the pathogenicity of Mtb was shown by the fact that incorporation of RD1 region of Mtb genome (encoding these proteins) into BCG (lacking this region) has led to increased virulence and pathogenicity of this strain of M. bovis [43].

The second interesting observation from the present study is that the levels of TNF-α and IL-10 against ESAT-6/CFP-10 were also elevated in untreated patients, but were suppressed in HHCs. This is the first study, to our knowledge, from a naturally Mtb-infected human population to show that these two functionally opposing cytokines are suppressed concurrently during Mtb infection but were elevated during clinical disease or disease progression. Previously, Samten et al. [18] have shown that ESAT-6 inhibits not only the production of IFN-γ but also T cell production of TNF-α and IL-17.

The ESAT-6/CFP-10 complex has been reported to bind to monocytes through the C-terminal flexible arm of CFP-10 [18]. In another study, it was reported that ESAT-6 binds to T cells, monocytes and B cells, whereas CFP-10 binds only to monocytes and B cells but did not reduce...
viability or increase apoptosis of these cells, or did not inhibit IL-2 production [23].

Similar to IFN-γ and TNF-α (proinflammatory cytokines), the level of IL-10 (anti-inflammatory cytokine) against ESAT-6/CFP-10 was also depressed in HHCs but was elevated in clinical TB. Results of the present study from a naturally Mtb-infected human population may suggest that IL-10 may not have any apparent influence on the production of IFN-γ and TNF-α, as suggested previously [44], as both pro- and anti-inflammatory cytokines increased concurrently during clinical TB and decreased during Mtb infection or exposure to infection.

However, reports on the production of IL-10 during Mtb infection, based mainly on experimental animal models, are conflicting. Several studies have implicated IL-10 with the development of clinical TB through multiple proposed immune-suppressive mechanisms, such as inhibition of IFN-γ production [45–47], down-regulation of macrophage activation [48], inhibition of TNF-α production [44], down-regulation of the expression of co-stimulatory molecules [49] and blocking macrophage maturation [50]. However, some studies did not find any sign of lung immunopathology (including weight loss) in IL-10-deficient mice during Mtb infection [51]. Moreover, there are limited published data supporting a role for IL-10 in blocking host-mediated immunopathology during chronic Mtb infection [52]. Some studies have shown that upon exposure to Mtb, alveolar macrophages and lung dendritic cells become activated and produce cytokines such as TNF-α and IL-12 (host antimycobacterial cytokines) and IL-10, which may inhibit the production these protective cytokines [53–56].

However, other studies have shown different results. Earlier, in B6D2F1 Bom mice challenged with H37Rv of Mtb, we have shown that gene expression of IL-10, IL-12 and IFN-γ increased significantly from infection to development of clinical TB, whereas that of TNF-α decreased significantly and progressively with progression of clinical TB [57]. Moreover, Wang et al. [23] did not find any effect of IL-10 on IFN-γ production and T cell stimulation and proliferation. In another study, it was observed that IL-10 gene-disrupted and wild-type mice generated similar and vigorous IL-12p40 and IFN-γ synthesis and a displayed identical capacity to control Mtb infection [58]. Based on the above results, it has been suggested that the production of IL-10 following phagocytosis of Mtb by macrophages may occur as a natural anti-microbial response by the host, or may be induced by the bacteria as an evasion mechanism [51].
The production of IL-10 and its influence on proinflammatory cytokines may be influenced by the type of cell producing IL-10, a type of *Mtb* strain, and exposure to non-tuberculous mycobacteria (NTM). Regarding the *Mtb* strain, it has been shown that the hypervirulent phenotype of HN878 (a member of the Beijing genotype) is associated strongly with the induction of type 1 IFNs [59,60], as well as the early induction of IL-10 by CD4^+^CD25^+^forkhead box protein 3 (FoxP3^+^)CD233^+^regulatory T cell population [61] and enhanced arginase expression [62]. This differential induction of cytokines is believed to be due to the presence of phenolic glycolipid in HN878 strain of *Mtb* [63], normally absent in other clinical isolates. Conversely, infection of human monocyte-derived macrophages with the CH strains CDC1551 and H37Rv led to the induction of IL-10, which correlated with reduced production of IL-12p40 [64].

Another possible explanation is the stage of *Mtb* infection and type of cells that produce these cytokines. It has been well established that early sources of IL-10 during infection are myeloid cells such as macrophages, whereas T cells become a major source at a later stage. As indicated earlier, there is limited information on specific cellular sources of IL-10 during *Mtb* infection and clinical TB [51].

In recent years, a number of double-positive T cells (IFN-γ/IL-4^+^ [65]; IFN-γ/IL-17^+^ [66]; IFN-γ/IL-10^+^ [67] have been added to the list of helper T cells, but their exact role during *Mtb* infection is not understood fully.

In addition, the presence of NTM infection may influence the production of these cytokines under natural infections of human population. The fact that there was a similar pattern of IFN-γ responses against rESAT-6/CFP-10 and PPD (a cocktail of antigens) in the present study may suggest that ESAT-6 may not be as specific as thought originally. Support for this view comes from a recent study that shows NTM mycobacteria (e.g. *M. avium*, *M. szelgai*, *M. kansasii* and *M. marinum*) contain ESAT-6 homologue or ESAT-6-like protein that can cross-react with sera of some TB patients [68,69]. In a study in Malawi, it has been shown that more than 45% of respondents who produced IFN-γ against *Mtb* ESAT-6 also showed IFN-γ responses to *M. avium* and *M. kansasii* ESAT-6 [70].

As indicated earlier, most of the studies involving pathogenicity and protective T cell responses of ESAT-6 or CFP-10 come from animal models. Therefore, it is difficult to extrapolate results from a mouse model to human population in an endemic setting, as exemplified by a dozen vaccine candidates (e.g. Ag85B), that worked well in the mouse model but failed to yield the desired results in human trials [71].
Conclusions

In the present study we show, from a cohort of PTB patients, their contacts and apparently healthy community controls in an endemic setting, that IFN-γ, TNF-α and IL-10 responses against rESAT-6/CFP-10 are associated with clinical TB but not with protective immunity. Our results support earlier findings that ESAT-6/CFP-10 is a virulent factor that is involved in pathogenicity of Mtb infection. Together, our results and some previous studies raise an outstanding issue regarding the candidacy of ESAT-6/CFP-10 as an effective vaccine against TB.

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Disclosure

The authors declare no conflicts of interest

Authors’ contribution

F. A. conceived and developed the study and prepared the manuscript; M. B. collected and analysed data and reviewed the manuscript; M. L. was involved in the collection of data and laboratory analysis and reviewed the manuscript; A. M. and K. S. reviewed the manuscript and K. S. provided antigens. All have read and agreed to publication of the manuscript.

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