


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

F. Abebe ^{*}, M. Belay,^{†‡}
M. Legesse,[‡] A. Mihret[§] and
K. S. Franken[¶]

^{*}University of Oslo, Faculty of Medicine, Institute of Health and Society, Oslo, Norway, [†]Center for Immunobiology, Blizard Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, UK, [‡]Addis Ababa University, Aklilu Lemma Institute of Pathobiology, Addis Ababa, Ethiopia, [§]Armauer Hansen Research Institute, Addis Ababa, Ethiopia, and [¶]Department of Infectious Diseases, Leiden University Medical Center, Leiden, the Netherlands

Accepted for publication 24 March 2017

Correspondence: F. Abebe, University of Oslo, Faculty of Medicine, Institute of Health and Society, 1130 Blindern, Oslo, 317, Norway.
E-mail: Fekadu.abebe@medisin.uio.no and f.a.worke@gmail.com

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 (IFN)- γ and tumour necrosis factor (TNF)- α are protective during *Mtb* 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)- β

Summary

Mycobacterium tuberculosis (*Mtb*) 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 (IFN)- γ production], while others report that these proteins as virulent factors involved in pathogenicity of *Mtb* infection. However, the role of ESAT-6/CFP-10 during natural *Mtb* infections in humans has not been established. In this paper we present results of a longitudinal study from an *Mtb*-infected human population from an endemic setting. Whole blood assay was used to determine levels of IFN- γ , tumour necrosis factor (TNF)- α and interleukin (IL)-10 against rESAT-6/CFP-10 in TB patients, household contacts and community controls. The levels of IFN- γ , TNF- α 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- γ , TNF- α and IL-10 against rESAT-6/CFP-10 are depressed during *Mtb* infection or exposure but are elevated during clinical TB. Our findings from a study of naturally infected human population suggest that IFN- γ , TNF- α and IL-10 against rESAT-6/CFP-10 are markers for clinical TB but not for protective immunity.

Keywords: CFP-10, ESAT-6, IFN- γ , IL-10, immunity, TNF- α , tuberculosis

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 *Mtb* 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 *Mtb* and its products to escape phagosome [21], and promoting intracellular dissemination of *Mtb* [20,22]. It has also been shown that ESAT-6 directly inhibits human T cell IFN- γ 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, Kazanchis, Bole, Shiro Meda, Woroda 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)P 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 μ g/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 μ g/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. Friedman'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>).

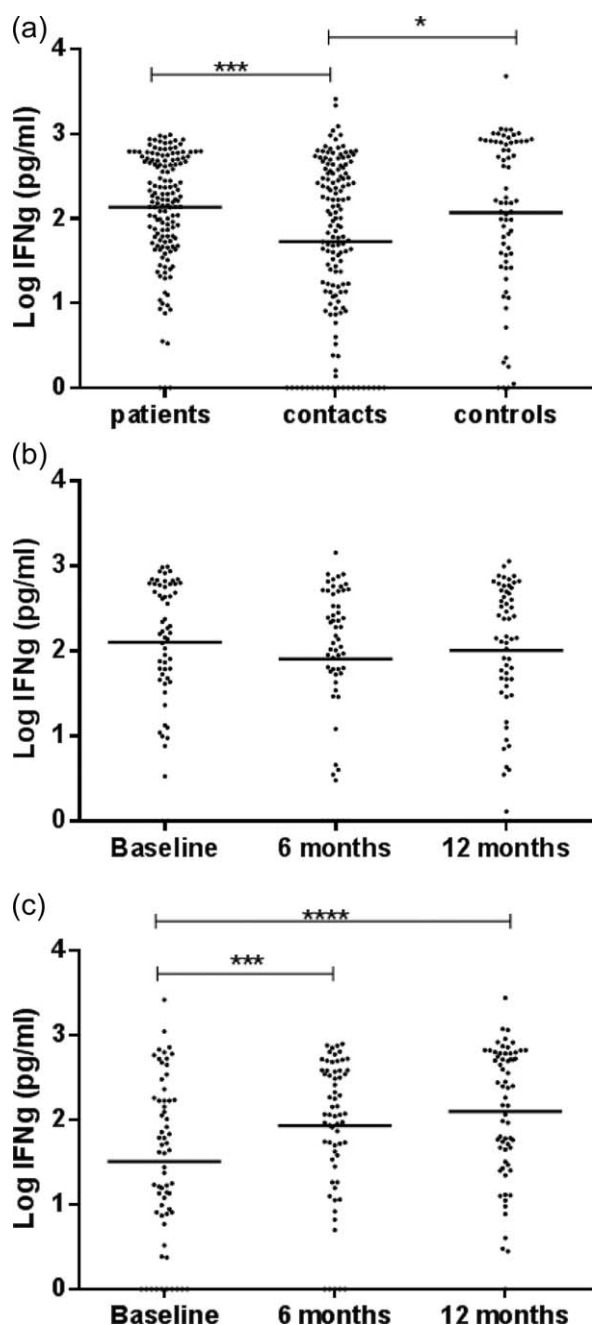
Ethical clearance

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. 4a).

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

Fig. 1. (a) The level of interferon (IFN)- γ against ESAT-6/CFP-10 in the three cohorts at baseline. Whole blood stimulation was performed with early secreted protein antigen 6 (ESAT-6)/culture filtrate protein 10 (CFP-10) for 48 h. Results are individual responses and cytokine levels are expressed as \log_{10} pg/ml. Horizontal bars are medians. Kruskal–Wallis test with Dunn's multiple comparisons was used to compare groups. *** $P < 0.001$; * $P < 0.05$. (b) The levels of IFN- γ against ESAT-6/CFP-10 in patients at baseline, 6 and 12 months post-chemotherapy. Whole blood stimulation was performed with ESAT-6/CFP-10 for 48 h. Results are individual responses and cytokine levels are expressed as \log_{10} pg/ml. Horizontal bars are medians. Friedman's test with Dunn's multiple comparisons was used to compare IFN- γ over time. (c) Levels of IFN- γ against ESAT-6/CFP-10 in contacts at baseline, 6 and 12 months. Whole blood stimulation was performed with ESAT-6/CFP-10 for 48 h. Results are individual responses and cytokine levels are expressed as \log_{10} pg/ml. Horizontal bars are medians. Friedman's test with Dunn's multiple comparisons was used to compare IFN- γ responses over time. **** $P < 0.0001$; *** $P < 0.005$.

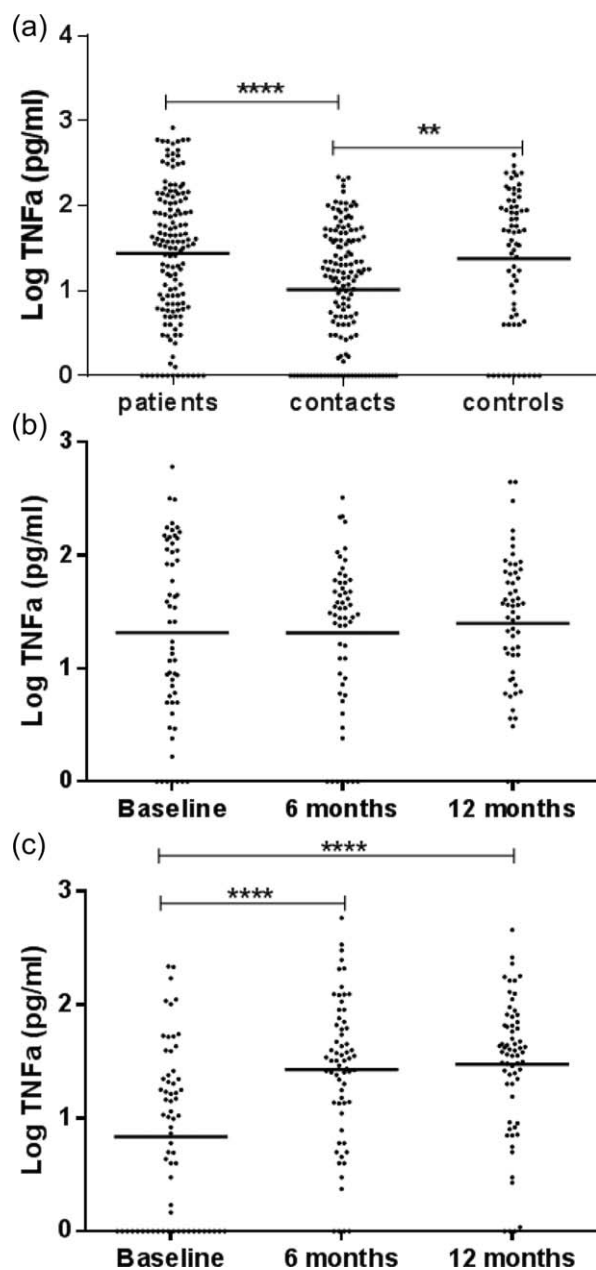
HHCs. No other significant difference was observed either between cohorts or between measurements.

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.

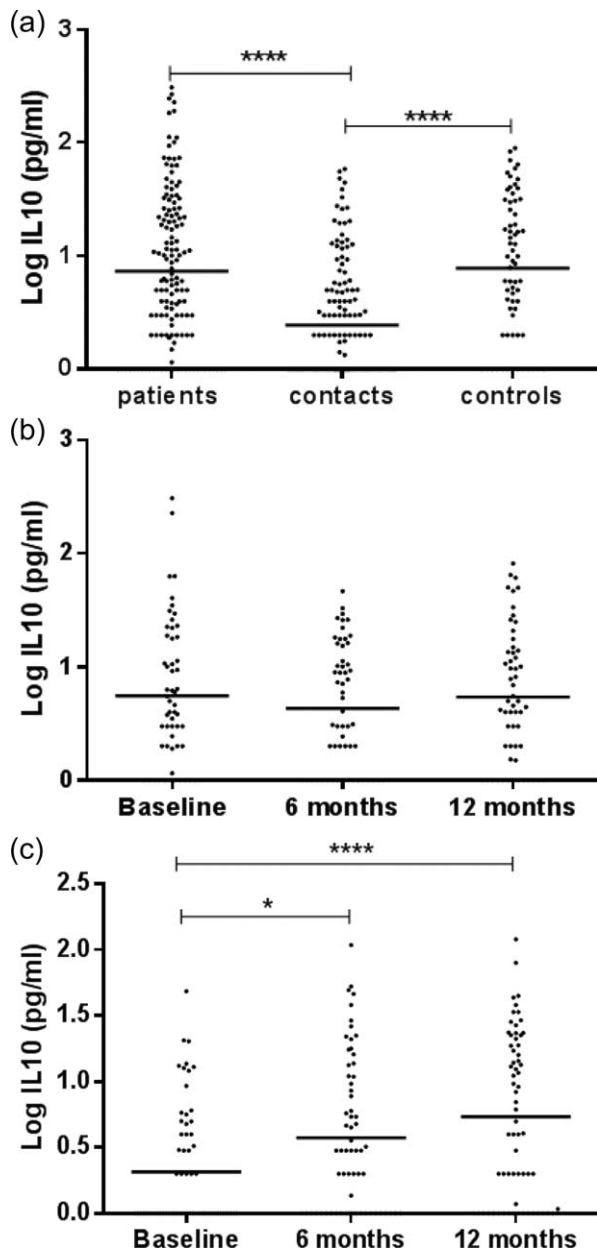
Our results support previous reports that ESAT-6/CFP-10 is associated with virulence and pathogenicity of *Mtb* based on results of a murine model of TB [23,35–38]. Some of these studies have shown not only that ESAT-6 or CFP-10 is a virulent factor promoting pathogenicity of *Mtb*, but also mechanisms 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

Fig. 2. (a) Tumour necrosis factor (TNF)- α level against early secreted protein antigen 6 (ESAT-6)/culture filtrate protein 10 (CFP-10) in the three cohorts at baseline. Whole blood stimulation was performed with ESAT-6/CFP-10 for 48 h. Results are individual responses and cytokine levels are expressed as log₁₀ pg/ml. Horizontal bars are medians. Kruskal–Wallis test with Dunn's multiple comparisons was used to compare groups. **** P < 0.001; ** P < 0.01. (b) Levels of TNF- α against ESAT-6/CFP-10 in patients at baseline, 6 and 12 months post-chemotherapy. Whole blood stimulation was performed with ESAT-6/CFP-10 for 48 h. Results are individual responses and cytokine levels are expressed as log₁₀ pg/ml. Horizontal bars are medians. Friedman's test with Dunn's multiple comparisons was used to compare TNF- α over time. (c) Levels of TNF- α against ESAT-6/CFP-10 in contacts at baseline, 6 and 12 months. Whole blood stimulation was performed with ESAT-6/CFP-10 for 48 h. Results are individual responses and cytokine levels are expressed as log₁₀ pg/ml. Horizontal bars are medians. Friedman's test with Dunn's multiple comparisons was used to compare TNF- α responses over time. **** P < 0.001.

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.

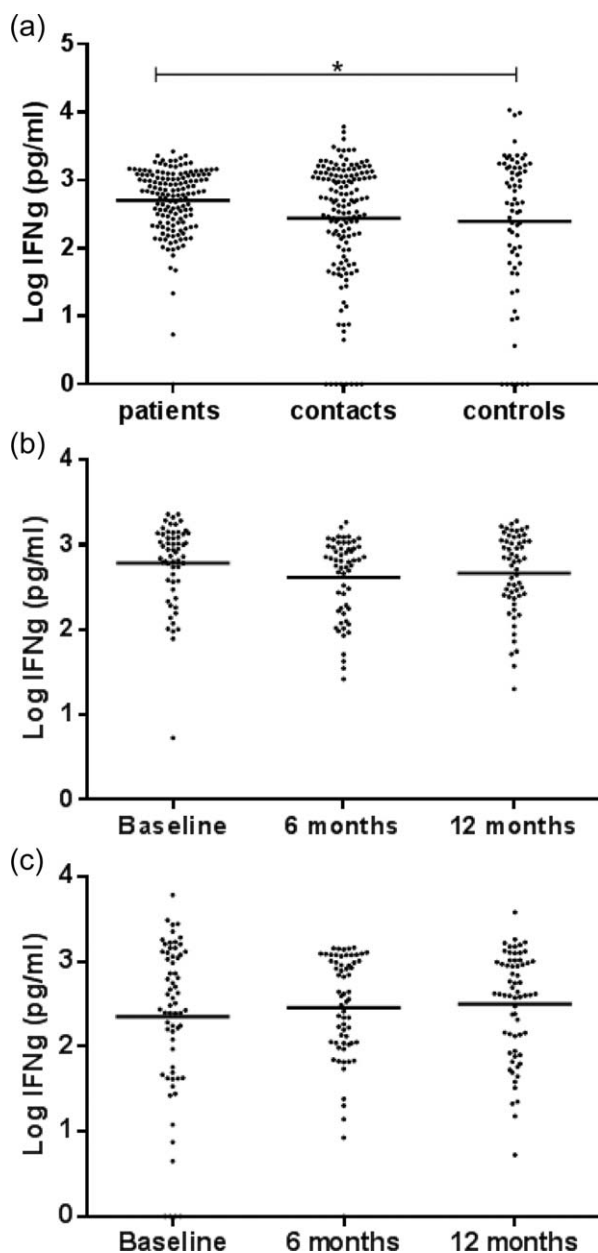
However, reports on the production of IL-10 during *Mtb* infection, based mainly on experimental animal models,

Fig. 3. (a) The level of interleukin (IL)-10 against early secreted protein antigen 6 (ESAT-6)/culture filtrate protein 10 (CFP-10) in the three cohorts at baseline. Whole blood stimulation was performed with ESAT-6/CFP-10 for 48 h. Results are individual responses and cytokine levels are expressed as \log_{10} pg/ml. Horizontal bars are medians. Kruskal–Wallis test with Dunn's multiple comparisons was used to compare groups. **** $P < 0.001$. (b) Levels of IL-10 against ESAT-6/CFP-10 in patients at baseline, 6 and 12 months post-chemotherapy. Whole blood stimulation was performed with ESAT-6/CFP-10 for 48 h. Results are individual responses and cytokine levels are expressed as \log_{10} pg/ml. Horizontal bars are medians. Friedman's test with Dunn's multiple comparisons was used to compare IL-10 over time. (c) Levels of IL-10 against ESAT-6/CFP-10 in baseline contacts at 12 months. Whole blood stimulation was performed with ESAT-6/CFP-10 for 48 h. Results are individual responses and cytokine levels are expressed as \log_{10} pg/ml. Horizontal bars are medians. Friedman's test with Dunn's multiple comparisons was used to compare IL-10 responses over time. **** $P < 0.0001$; * $P < 0.05$.

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 anti-mycobacterial 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 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

Fig. 4. (a) Interferon (IFN)- γ against purified protein derivative (PPD) stimulation in the three cohorts at baseline. Whole blood stimulation was performed with PPD for 48 h. Results are individual responses and cytokine levels are expressed as log₁₀ pg/ml. Horizontal bars are median values. Kruskal–Wallis test with Dunn’s multiple comparisons was used to compare groups. * $P < 0.05$. Figure (b) shows the levels of IFN- γ against PPD in patients at baseline, 6 and 12 months post-chemotherapy. Whole blood stimulation was performed with PPD for 48 h. Results are individual responses and cytokine levels are expressed as log₁₀ pg/ml. Horizontal bars are medians. Freidman’s test with Dunn’s multiple comparisons was used to compare interferon (IFN)- γ over time. (c) Levels of IFN- γ against PPD in contacts at baseline, 6 and 12 months post-chemotherapy. Whole blood stimulation was performed with PPD for 48 h. Results are individual responses and cytokine levels are expressed as log₁₀ pg/ml. Horizontal bars are medians. Freidman’s test with Dunn’s multiple comparisons was used to compare IFN- γ over time.

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. szelgii*, *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.

Acknowledgements

The study was supported financially by the Research Council of Norway (GLOBVAC: project S50/196397). F. A. received the funding. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. Laboratory analysis was performed at the Armauer Hansen Research Institute, Addis Ababa, Ethiopia. We would like to thank nurses at the DOTS clinics for data collection. We would also like to thank Miss Mahlet Chanyalew at Akililu Lemma Institute of Pathobiology, Addis Ababa University for assisting in the laboratory work. Our gratitude goes to participants who took part in the study.

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.

References

- World Health Organization (WHO). Global tuberculosis report, 20th edn. Geneva, Switzerland: WHO, 2016.
- Delogu G, Proveddi R, Sali M, Manganelli R. *Mycobacterium tuberculosis* virulence: insights and impact on vaccine development. *Future Microbiol* 2015; **10**:1177–94.
- Orme IM. Vaccine development for tuberculosis: current progress. *Drugs* 2013; **73**:1015–24.
- Kaufmann SH. Novel approaches to tuberculosis vaccine development. *Int J Infect Dis* 2016; **2749**:1–5.
- Keane J, Gershon S, Wise RP *et al.* Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med* 2001; **345**:1098–104.

- Jouanguy E, Doffinger R, Dupuis S, Pallier A, Altare F, Casanova JL. IL-12 and IFN- γ in host defense against mycobacteria and salmonella in mice and men. *Curr Opin Immunol* 1999; **11**:346–51.
- Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role of interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med* 1993; **178**:2249–54.
- Garib FY, Rizpulu AP. T-regulatory cells as part of strategy of immune evasion by pathogens. *Biochemistry (Moscow)* 2015; **80**:1141–59.
- Boer MC, Joosten SA, Ottenhoff TH. Regulatory T cells at the interface between human host and pathogens in infectious diseases. *Front Immunol* 2015; **6**:1–15.
- Abdalla AE, Lambert N, Duan X, Xie J. Interleukin-10 family and tuberculosis: an old story renewed. *Int J Biol Sci* 2016; **12**: 710–6.
- Uvarova E, Belavin PA, Permyakova NV *et al.* Oral immunogenicity of plant-made *Mycobacterium tuberculosis* ESAT-6 and CFP10. *Biomed Res Int* 2013; **2013**:8.
- Maue AC, Waters WR, Palmer MV *et al.* An ESAT-6:CFP10 DNA vaccine administered in conjunction with *Mycobacterium bovis* BCG confers protection to cattle challenged with virulent *M bovis*. *Vaccine* 2007; **25**:4735–46.
- Olsen AW, William A, Okkels LM, Hatch G, Andersen P. Protective effect of a tuberculosis subunit vaccine based on a fusion of antigen 85B and ESAT-6 in the aerosol Guinea pig model. *Infect Immun* 2004; **72**:6148–50.
- Pym AS, Brodin P, Majlessi L *et al.* Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nat Med* 2003; **9**:533–9.
- Gao H, Yue L, Xu W *et al.* A novel DNA vaccine containing multiple TB-specific epitopes casted in a natural structure (ECANS) confers protective immunity against pulmonary mycobacterial challenge. *Vaccine* 2009; **27**:5313–9.
- Harboe M, Oettinger T, Wiker HG, Rosenkrands I, Andersen P. Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. *Infect Immun* 1996; **64**: 16–22.
- Cole ST, Brosch R, Parkhill J *et al.* Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 1998; **393**:537–44.
- Samten B, Wang X, Barnes PF. *Mycobacterium tuberculosis* ESX-1 system-secreted protein ESAT-6 but not CFP10 inhibits human T cell immune responses. *Tuberculosis* 2009; **51**:574–6.
- Hsu T, Hingley-Wilson M, Chen B *et al.* The primary mechanism of attenuation of bacillus Calmette–Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. *Proc Natl Acad Sci USA* 2003; **100**:12420–5.
- Gao LY, Guo S, McLaughlin B, Morisaki H, Engel JL, Brown JE. A mycobacteria virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. *Mol Microbiol* 2004; **53**:1677–93.
- De Jonge ML, Pehau-Amaudet G, Fretz MM *et al.* ESAT-6 from *Mycobacterium* dissociates from its putative chaperone CFP-10 under acidic condition and exhibits membrane-lysing activity. *J Bacteriol* 2007; **189**:6028–34.
- Van der Wel N, Hava D, Houben D *et al.* *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* 2007; **129**:1287–98.

- 23 Wang X, Barnes PF, Dobos-Elder KM *et al.* ESAT-6 inhibits production of IFN- γ by *Mycobacterium tuberculosis*-responsive human T cells. *J Immunol* 2009; **182**:3668–77.
- 24 Peng H, Wang X, Barnes PF *et al.* The *Mycobacterium tuberculosis* early secreted antigenic target of 6 kDa inhibits T cell interferon-g production through the p38 mitogen-activated protein kinase pathway. *J Biol Chem* 2011; **286**:24508–18.
- 25 Ministry of Health. Guidelines for HIV counselling and testing in Ethiopia. Addis Ababa: Ministry of Health, 2007.
- 26 Belay M, Legesse M, Mihret A *et al.* Pro- and anti-inflammatory cytokines against Rv2031 are elevated during latent tuberculosis: a study in cohorts of tuberculosis patients, household contacts and community controls in an endemic setting. *PLOS ONE* 2015; **10**:e0124134.
- 27 Andersen P, Andersen AB, Sorensen AL, Nagai S. Recall of long-lived immunity to *Mycobacterium tuberculosis* in mice. *J Immunol* 1995; **154**:3359–72.
- 28 Sorensen AL, Nagai S, Houen G, Andersen P, Andersen AB. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. *Infect Immun* 2005; **63**:1710–7.
- 29 Gupta N, Vedi S, Kunimoto DY, Agrawi B, Kumar R. Novel lipopeptide of ESAT-6 induce strong protective immunity against *Mycobacterium tuberculosis*: routes of immunization and TLR agonists critically impact vaccine's efficacy. *Vaccine* 2016; **34**:5677–88. doi: 10.1016/j.vaccine.2016.08.075
- 30 Bottai E, Frigui W, Rayner E *et al.* Increased protective efficacy of recombinant BCG strains expressing virulence-neutral proteins of the ESX-1 secretion system. *Vaccine* 2015; **33**:2710–8.
- 31 Yang E, Lu Y, Liang Q, Wang C, Wang H, Shen H. Recombinant BCG co-expressing Ag85B, ESAT-6 and Rv3620c elicits specific Th1 immune responses in C57BL/6 mice. *Microb Pathol* 2014; **69**:70:53–9.
- 32 Lu Y, Xu Y, Yang Y, Wang C, Wang H, Shen H. Novel recombinant BCG coexpressing Ag85B, ESAT-6 and Rv2608 elicits significantly enhanced cellular immune and antibody responses in C57BL/6 mice. *Scand J Immunol* 2012; **76**:271–7.
- 33 Tebianian M, Hoseini AZ, Ebrahimi SM *et al.* Cloning, expression, and immunogenicity of novel fusion protein of *Mycobacterium* based on ESAT-6 and truncated C-terminal fragment of HSP70. *Biologicals* 2011; **39**:143–8.
- 34 Maue AC, Waters WR, Palmer MV *et al.* An ESAT-6: CFP10DNA vaccine administered in conjunction with *Mycobacterium bovis* BCG confers protection to cattle challenged with virulent *M. bovis*. *Vaccine* 2007; **25**:4735–46.
- 35 Kumar P, Agarwal R, Siddiqui I *et al.* ESAT6 differentially inhibits IFN- γ inducible Class II transactivator isoforms in both a TLR2-dependent and independent manner. *Immunol Cell Biol* 2012; **90**:411–20.
- 36 Guo SI, Xue R, Wang SM, Ren L, Xu JJ. The CFP/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.
- 37 Wang X, Barnes PF, Huang F *et al.* Early secreted antigenic target of 6-kDa protein of *Mycobacterium tuberculosis* primes dendritic cells to stimulate Th17 and inhibit Th1 immune responses. *J Immunol* 2012; **189**:3092–103.
- 38 Samten B, Xisheng W, Peter FP *et al.* Immune regulatory activities of early secreted antigen target of 6-kDa protein of *Mycobacterium tuberculosis* and implications for tuberculosis vaccine design. *Tuberculosis* 2011; **91S1**:s114–8.
- 39 Demissie A, Wassie L, Abebe M, Aseffa A. The 6-kilodalton early secreted antigenic target-responsive, asymptomatic contacts of tuberculosis patients express elevated levels of interleukin-4 and reduced levels of gamma interferon. *Infect Immun* 2006; **74**:2817–22.
- 40 Boss JM, Jensen PE. Transcriptional regulation of MHC class II antigen presentation pathway. *Curr Opin Immunol* 2003; **15**:105–11.
- 41 Reith W, LeibundGut-landmann S, Waldburger J. Regulation of MHC class II gene expression by the class II transactivator. *Nat Rev Immunol* 2005; **5**:793–806.
- 42 Ting JP, Trowsdale J. Genetic control of MHC class II expression. *Cell* 2002; **109**:s21–33.
- 43 Renshaw PS, Lightbody KL, Veverka V *et al.* Structure and function of the complex formed by the tuberculosis virulence factor CFP-10 and ESAT-6. *EMBO J* 2005; **24**:2491–8.
- 44 Korholz D, Banning U, Bonig H *et al.* The role of interleukin-10 (IL-10) in IL-15 mediated T cell responses. *Blood* 1997; **90**:4513–21.
- 45 Rojas RE, Balaji KN, Subramanian A, Boom WH. Regulation of human CD4+ ab T cell-receptor-positive (TCR+) and gd TCR+ T cell responses to *Mycobacterium tuberculosis* by IL-10 and transforming growth factor B. *Infect Immun* 1999; **67**:6461–72.
- 46 De Waal Malefyt R, Yssel H, de Vries JE. Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells: specific inhibition of IL-2 production and proliferation. *J Immunol* 1993; **150**:4754–65.
- 47 Fiontino DF, Ziotnik A, Vierira P *et al.* IL-10 acts on the antigen presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 1991; **146**:444–51.
- 48 Moore KW, de Waal Malefyt R, Coffman L, Garra AO. Interleukin 10 and interleukin-10 receptor. *Ann Rev Immunol* 2001; **19**:683–765.
- 49 De La Barrera S, Aleman M, Musella R *et al.* IL-10 down-regulates co-stimulatory molecules on *Mycobacterium tuberculosis*-pulsed macrophages and impairs the lytic activity of CD4 and CD8 CTL in tuberculosis patients. *Clin Exp Immunol* 2004; **138**:128–38.
- 50 O'Leary S, O'Sullivan MP, Keane J. IL-10 blocks phagosome maturation in *Mycobacterium tuberculosis*-infection in human macrophages. *Am J Respir Cell Mol Biol* 2010; **45**:172–80.
- 51 Redford PS, Murray PJ, O'Garra A. the role of IL-10 in immune regulation during *M.tuberculosis* infection. *Mucosal Immunol* 2011; **4**:261–9.
- 52 Higgins DM, Sanchez-Campillo J, Rosas-Taraco AG *et al.* IL-10 alters inflammatory and immune responses during pulmonary *Mycobacterium tuberculosis* infection. *Tuberculosis* 2009; **89**:149–57.
- 53 Shaw TC, Thomas LH, Friedland JS. Regulation of IL-10 secretion after phagocytosis of *Mycobacterium tuberculosis* by human monocytic cells. *Cytokine* 2000; **12**:483–6.
- 54 Giacomini E. Infection of human macrophages and dendritic cells with *Mycobacterium tuberculosis* induces a differential cytokine gene expression that modulates T cell responses. *J Immunol* 2001; **186**:7033–41.
- 55 Bodner KA, Serbina NV, Flynn JL. Fate of *Mycobacterium tuberculosis* within murine dendritic cells. *Infect Immun* 2001; **69**:800–9.
- 56 Hickman SP, Chan J, Salgame P. *Mycobacterium tuberculosis* induces differential cytokine production from dendritic cells

- and macrophages with divergent effects on naïve T cell polarization. *J Immunol* 2001; **168**:4636–42.
- 57 Abebe F, Mustafa T, Nerland AH, Bjune GA. Cytokine profile during latent and slowly progressive primary tuberculosis: a possible role for interleukin 15 in mediating clinical disease. *Clin Exp Immunol* 2006; **143**:180–92.
- 58 Ma J, Jung B-G, Yi N, Samten B. Early secreted antigenic target of 6 kDa of *Mycobacterium tuberculosis* stimulates macrophage chemo-attractant protein-1 production by macrophages and its regulation by p38 mitogen activated protein kinases and interleukin-4. *Scan J Immunol* 2016; **84**:39–48.
- 59 Manca C, Tsenova L, Bergtold A *et al.* Virulence of *Mycobacterium tuberculosis* clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha/beta. *Proc Natl Acad Sci USA* 2001; **98**:5752–7.
- 60 Manca C, Tsenova L, Freeman S *et al.* Hyper-virulent *M.tuberculosis* W/Beijing strains upregulate type 1 IFNs and increase expression of negative regulators of the Jak-Stat-pathway. *J Interferon Cytokine Res* 2005; **25**:694–701.
- 61 Ordway D, Henao-Tamayo M, Harton M *et al.* The hyper-virulent *Mycobacterium tuberculosis* strain HN878 induces a potent TH1 response followed by rapid down-regulation. *J Immunol* 2007; **179**:522–31.
- 62 Qualls GE, Neale G, Smith AM *et al.* Arginine usage in mycobacteria-infected macrophages depends on autocrine-paracrine cytokine signaling. *Sci Signal* 2010; **3**:1–15.
- 63 Reed MB, Domenech P, Manca C *et al.* A glycolipid of hyper-virulent tuberculosis strain that inhibits the innate immune response. *Nature* 2001; **431**:84–7.
- 64 Newton SM, Smith RJ, Wilkison KA *et al.* A deletion defining a common Asian lineage of *Mycobacterium tuberculosis* associated with immune subversion. *Proc Natl Acad Sci USA* 2006; **103**:15594–8.
- 65 Deaton AM, Cook PC, De Sousa D, Phythian-Adams AT, Bird A, MacDonald AS. A unique DNA methylation signature defines a population of IFN-g/IL-4 double positive T cells during helminth infection. *Eur J Immunol* 2014; **44**:1835–41.
- 66 Boniface K, Blumenschein WM, Brovont-Porth K *et al.* Human TH17 cells comprise heterogeneous subsets including IFN-gamma-producing cells with distinct properties from Th1 lineage. *J Immunol* 2010; **185**:679–87.
- 67 Gerosa F, Nissi C, Righetti S *et al.* CD4(+) T cell clones producing both interferon-gamma and interleukin-10 predominate in bronchoalveolar lavages of active pulmonary tuberculosis patients. *J Immunol* 1999; **92**:224–34.
- 68 Arend SM, van Meijgaarden KE, de Boer K *et al.* Tuberculin skin testing and *in vitro* T cell responses to ESAT-6 and culture filtrate protein 10 after infection with *Mycobacterium marinum* or *M.kansasii*. *J Infect Dis* 2002; **186**:1797–807.
- 69 Geluk A, van Meijgaarden KE, Franken KL *et al.* Identification and characterization of the ESAT-6 homologue of *Mycobacterium leprae* and T cell cross-reactivity with *Mycobacterium tuberculosis*. *Infect Immun* 2002; **70**:2544–8.
- 70 Hur Y-G, Crampin AC, Chisambo C *et al.* Identification of immunological biomarkers which may differentiate latent tuberculosis from exposure to environmental nontuberculous mycobacteria in children. *Clin Vacc Immunol* 2014; **21**:133–42.
- 71 Tameris M, Hokey DA, Nduba V *et al.* A double-blind randomized, placebo controlled, dose-finding trial of the novel tuberculosis vaccine AERAS-402, an adenovirus-vectored fusion protein, in healthy, BCG-vaccinated infants. *Vaccine* 2015; **33**:2944–54.