European Journal of Immunology

Mycobacterium tuberculosis Rv1419 encodes a secreted 13 kDa lectin with immunological reactivity during human tuberculosis

Lucas Nogueira[1,2], Fernanda C. Cardoso[3], Ana M. Mattos[4], Juliano Bordignon[5], Cláudia P. Figueiredo[6], Pedro Dahlstrom[1], Cristiane C. Frota[7], Cláudia N. Duarte dos Santos[5], Marcelo Chalhoub[8], Benildo S. Cavada[7], Henrique C. Teixeira[4], Sérgio C. Oliveira[3], Manoel Barral-Netto[2] and André Báfica[1]

[1] Laboratório de Imunologia e Doenças Infecciosas, Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Catarina-SC, Brazil
[2] LIMI, Centro de Pesquisas Gonçalo Moniz, FIOCRUZ-BA, Brazil
[3] Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais-MG, Brazil
[4] Laboratório de Imunologia, Universidade Federal de Juiz de Fora-MG, Brazil
[5] Instituto Carlos Chagas, FIOCRUZ-PR, Brazil
[6] Departamento de Farmacologia, UFSC, Brazil
[7] BioMol-Lab, Universidade Federal do Ceará-CE, Brazil
[8] Hospital Octávio Mangabeira, Salvador, BA, Brazil

In this study, we have identified a secreted 13 kDa lectin from *Mtb* (*Mtb*, *Mycobacterium tuberculosis*; sMTL-13) by homology search of a non-redundant lectin database. Bioinformatic analysis revealed that sMTL-13 belongs to the ricin-type β-trefoil family of proteins containing a Sec-type signal peptide present in *Mtb* complex species, but not in non-tuberculous mycobacteria. Following heterologous expression of sMTL-13 and generation of an mAb (clone 276.B7/IgG1κ), we confirmed that this lectin is present in culture filtrate proteins from *Mtb* H37Rv, but not in non-tuberculous mycobacteria-derived culture filtrate proteins. In addition, sMTL-13 leads to an increased IFN-γ production by PBMC from active tuberculosis (ATB) patients. Furthermore, sera from ATB patients displayed high titers of IgG Ab against sMTL-13, a response found to be decreased following successful anti-tuberculosis therapy. Together, our findings reveal a secreted 13 kDa ricin-like lectin from *Mtb*, which is immunologically recognized during ATB and could serve as a biomarker of disease treatment.

Key words: Biomarkers · Lectin · *Mycobacterium tuberculosis*

Introduction

Tuberculosis (TB) remains a major public health problem in both developing and industrialized countries [1, 2]. *Mycobacterium tuberculosis* (*Mtb*), the etiologic agent of TB, is one of the most successful human pathogens and epidemiological studies estimated that one-third of the world population is infected with the bacterium [1, 2]. Although *Mtb* remains viable in the majority of the infected subjects, only 5–10% of individuals develop active disease later in life [1, 2]. However, the mechanisms for the breakdown of latency are largely unknown [3]. Evidence suggests that both humoral and cellular immune responses are implicated

Correspondence: Professor André Báfica
e-mail: abafica@ccb.ufsc.br

in host resistance against *Mtb* and cell-mediated immunity is thought to be the major component for protection [1, 4–7]. While effective immune responses are critical to control *Mtb* growth inside macrophages, it has been demonstrated that mycobacteria-associated factors play an important role in TB immunopathogenesis [8–10]. Thus, secreted molecules are amongst the possible candidates that influence pathogen–host interactions *in vivo*.

Secretion of proteins is a critical process for bacterial virulence. *Mtb* possesses a specialized secretion system to transport virulence factors across their unique cell envelope [11, 12]. Although the study of culture filtrate protein (CFP) preparations from *Mtb* has revealed a myriad of proteins, there remain several other molecules annotated as having "unknown function" [13, 14]. For example, Malen *et al*. using a proteomic approach, have recently detected 257 secreted proteins in CFP fractions from the laboratory strain *Mtb* H37Rv [13]. However, no function has yet been ascribed to 23% of those molecules. Polypeptides secreted by mycobacteria may modulate inflammatory processes and could serve as targets for immune protection. For example, the 19 kDa lipoprotein has been shown to induce proinflammatory responses by antigen-presenting cells [15] and ESAT-6, a major immunodominant *Mtb* antigen utilized in IFN-γ-based diagnosis assays, was found to modulate innate immune function [16]. These data suggest that mediators synthesized by the pathogen during infection regulate both protective as well as detrimental responses to the host. Thus, discovery and characterization of *Mtb*-secreted proteins could be an approach to identify novel therapeutic and diagnosis targets as well as biomarkers of disease.

Lectins are classically defined as a family of proteins with the ability to specifically bind carbohydrate moieties. A number of pathogens have been demonstrated to express such molecules, which are involved in recognition and invasion processes [17, 18]. For example, *Pseudomonas aeruginosa* produces several membrane-associated lectins that promote attachment to epithelial cells and contribute to its virulence [19]. In addition, bacterial lectins could be released into the extracellular milieu and play an important role during infection as demonstrated by experiments using *Bordetella* [18]. These data suggest that both membrane-expressed and secreted lectins participate in host–microbial interactions. In the case of *Mtb*, the heparin-binding hemagglutinin adhesin (HBHA) is one of the most studied cell surface-expressed lectins and it has been shown to be critical for bacterial dissemination *in vivo* [20]. Moreover, the existence of at least 11 hypothetical lectins from *Mtb* [21] suggests that these molecules may be an important component of the host–mycobacteria interplay. Consistent with this, active TB (ATB) patients have been found to display increased levels of anti-HBHA Ab during active disease [22, 23], suggesting that mycobacterial lectins may elicit specific immune responses.

We have utilized a previously generated non-redundant lectin data bank [24] in order to identify lectins from *Mtb*, a major human pathogen. In the present study, we have demonstrated a secreted 13 kDa ricin-like lectin from *Mtb* (sMTL-13). sMTL-13 was detected in pleural biopsies from ATB patients and led to an increased IFN-γ production by PBMC from patients during active disease. Importantly, ATB patients display high titers of serum IgG against sMTL-13, a response found to be rapidly decreased following successful treatment. These data report a secreted *Mtb* lectin with antigenic activity in human TB and suggest it may be useful as a biomarker of disease therapy.

Results

In silico analysis of the Rv1419 gene

We have previously generated a non-redundant lectin database for searching lectin domains from *Arabidopsis thaliana* genome [24]. To further evaluate the presence of such domains in an important human pathogen, *Mtb*, we have adapted this database and identified a single hypothetical lectin encoded by the Rv1419 gene. Figure 1A shows the bioinformatics characterization of the Rv1419 gene. Its open reading frame (ORF) contains 474 nucleotides and the aa sequence encodes a hypothetical protein of 157 residues containing a signal peptide and a predicted molecular mass of 16.8 kDa. The primary aa sequence analysis of the Rv1419-encoded protein reveals a 33aa residues-signal peptide, with a type I signal cleavage site between Ala33 and Asp34. Analysis of the mature protein predicted a molecular weight mass of 13.6 kDa. Positions 31 and 32 of the precursor protein contain the sequence Ala-x-Ala, a motif commonly found preceding the cleavage site [25]. The Rv1419p contains a carbohydrate-binding B-chain ricin domain and belongs to the ricin-type β-trefoil family of proteins, which is composed of three homologous subdomains as well as the presence of a Q-W pattern [26]. B-chain ricin domains have been demonstrated to bind cell surface glycolipids and glycoproteins bearing β-1,4-linked galactose and mannose moieties [27]. In addition, database searching has shown that the Rv1419 ORF displays 100% identity with its homologue from the clinical strain *Mtb* CDC1551 (GenBank accession number: AE000516.2) as well as *M. bovis* BCG (GenBank accession number: AM408590.1) and 78% identity to *M. marinum* (GenBank accession number: CP000854.1) as well as *M. ulcerans* (Genbank accession number: CP000325.1) homologous gene (Table 1). These data suggest the existence of a previously uncharacterized secreted carbohydrate-binding protein from *Mtb* and related sequences in other mycobacteria.

To further study possible functions of Rv1419 gene product, we have produced a recombinant protein as described in the *Materials and methods* section. A DNA fragment of 496 bp was obtained (Fig. 1B), purified, and cloned into the vector pMOS-Blue. Sequencing procedure confirmed that cloning and amplification experiments generated an unaltered Rv1419 sequence (data not shown). The fragment was then inserted in-frame with the start codon present at *Nde*I cleavage site into the plasmid pET15b enabling full production of Rv1419-gene product using *Escherichia coli*. Figure 1C shows a typical SDS-PAGE experiment of the obtained recombinant Rv1419p demonstrating a single band with molecular weight of ∼17 kDa. Additionally, we have

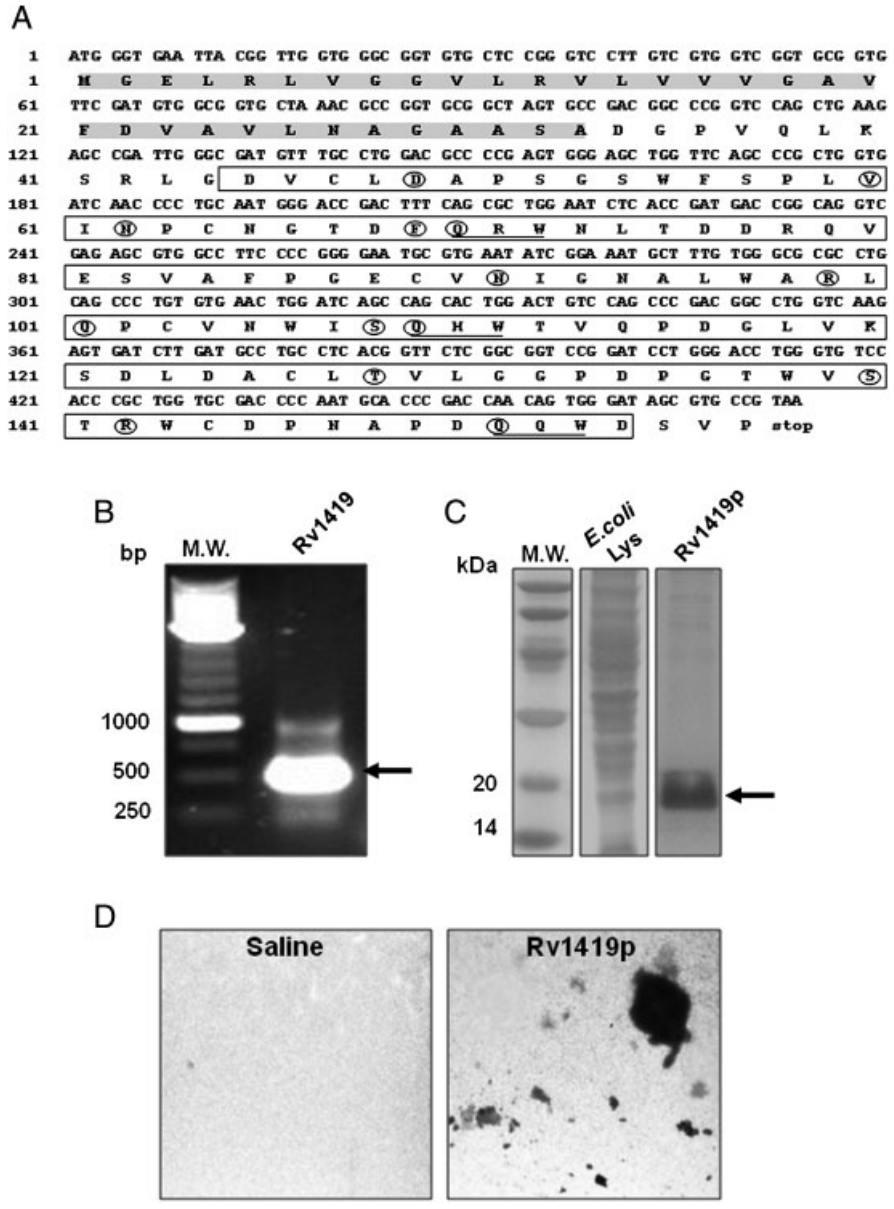

Figure 1. *In silico* analysis and molecular cloning of the *Mtb* Rv1419 gene. (A) Predicted Rv1419p is composed of a 33 aa signal peptide (gray bar), with the signal cleavage site between Ala33 and Asp34. The region between residues Asp45 and Asp154 (box) represents the ricin-like lectin domain. Putative sugar binding sites are present in the mature sequence as indicated by circles. The presence of a predicted Q-W pattern is underlined. (B) PCR amplification of *Mtb* Rv1419 gene using specific primers and H37Rv DNA as template demonstrated on a 1% agarose gel. Depicted is the isolation of Rv1419 gene. (C) Following heterologous expression, *E. coli* lysate or chromatography-purified recombinant protein (see the *Materials and methods*) was analyzed through SDS-PAGE (12%) and revealed by Coomassie staining. (D) Purified recombinant Rv1419-encoded protein induces hemagglutination. Red blood cells from rabbit (2%) were incubated with saline or Rv1419p (10 μg/mL) for 16 h. Images were generated in a digital camera (10× objective). Data shown are representative of two experiments performed.

confirmed that Rv1419p possesses lectin activity based on classical erythrocyte agglutination assays (Fig. 1D).

Rv1419p is expressed on CFP fraction from *Mtb*, but not from non-tuberculous mycobacteria

Following bidimensional gel analysis and mass spectrometry of CFP from *Mtb* H37Rv, Malen *et al*. have recently detected a spot corresponding to the Rv1419 gene product [13]. To further investigate whether Rv1419p is secreted and/or expressed in other *Mtb* compartments, we have generated a mAb (clone 276.B7/IgG1Kappa) against this protein. Figure 2A reveals a single ~13 kDa band in CFP preparations from *Mtb* H37Rv, but not in the CFP fractions obtained from the non-tuberculous mycobacteria species *M. avium*, *M. kansasii*, *M. fortuitum*. Compared with the *Mtb* CFP, the whole cell lysate, cell wall, or membrane preparations presented lower amounts of a similar

Table 1. Distribution of Rv1419 homologous in Mycobacterial species

Strain	GenBank[a)]	ORF length (bp)	%Identity[b)]	Product
Mtb H37Rv	BX842576.1	474	–	Hypothetical protein
Mtb H37Ra	CP000611.1	474	100	Hypothetical protein
Mtb CDC1551	AE000516.2	474	100	Hypothetical protein
M. bovis BCG	AM408590.1	474	100	Hypothetical protein
M. bovis AF2122/97	BX248338.1	474	100	Hypothetical protein
M. marinum M	CP000854.1	477	78	Conserved hypothetical membrane protein
M. ulcerans Agy99	CP000325.1	477	78	Conserved hypothetical membrane protein

[a)] GenBank accession numbers.
[b)] Relative to Rv1419 ORF from *Mtb* H37Rv.

~13 kDa band following incubation with the mAb (Fig. 2B). In contrast, as previously demonstrated [28], high levels of the 19 kDa lipoprotein corresponding band from *Mtb* H37Rv were observed in the studied fractions incubated with an anti-19 kDa lipoprotein mAb (Fig. 2B). Together, these data suggest that CFP from *Mtb* H37Rv contains reasonable amounts of Rv1419p; therefore we named this protein secreted *Mtb* Lectin (sMTL-13). Furthermore, to investigate whether sMTL-13 is expressed during active infection *in vivo*, we have performed immuno-staining in pleural biopsies from ATB patients. Figure 2C shows positive staining for sMTL-13 in tissue granulomas from ATB patients. In contrast, as expected no staining was observed in biopsies from negative IgG1 isotype control (Fig. 2D), skin biopsies from *M. leprae*-infected patients (Fig. 2E), or in tissue granulomas associated with fungal infection (Fig. 2F and data not shown).

sMTL-13-driven PBMC IFN-γ and serum IgG responses from ATB patients

A hallmark of mycobacterial infection is the generation of a strong immune response against secreted antigens. A number of antigens secreted by *Mtb* have been proposed to function as virulence factors and may influence the clinical outcome of TB [11, 12, 29]. We therefore investigated whether sMTL-13 is recognized by TB patients during active disease. First, we measured recall responses by means of IFN-γ production of PBMC following exposure to sMTL-13 *in vitro*. As demonstrated in Fig. 3A, sMTL-13-stimulated PBMC from active TB patients ($n = 11$) display increased production of IFN-γ when compared with BCG-vaccinated purified protein derivative (PPD)-negative control subjects ($n = 6$). In addition, we have performed ELISA in serum samples from 34 diseased individuals as well as 38 control subjects. As shown in Fig. 3B, recently diagnosed TB patients (either naive of treatment or up to 15 days undergoing early chemotherapy; ATB group) presented high titers of anti-sMTL-13 total IgG Ab. Importantly, anti-sMTL-13 IgG titers rapidly decreased during the first months (1–2) of treatment and reached background levels as compared with those from endemic or non-endemic subjects. Moreover, anti-sMTL-13 IgG Ab titers remained at background levels following successful anti-TB chemotherapy (6 months). Furthermore, receiver operating characteristic (ROC) curves analysis at the optimal cutoff point revealed that anti-sMTL-13 IgG titers display high specificity (90%) as well as sensitivity (93%) for TB diagnosis (Fig. 3C). There was no significant difference between the areas for ESAT-6 (AUC = 0.956 (AUC, area under the curve), CI 95%: 0.865–0.985) and sMTL-13 (AUC = 0.943, CI 95%: 0.855–0.981). Together, these data suggest that TB patients display adaptive immune responses against sMTL-13 during active disease and anti-sMTL-13 Ab are decreased following therapeutic control of *Mtb in vivo*.

Discussion

Proteins actively secreted during the *in vitro* early growth phase of *Mtb* have been the subject of intensive investigation for their ability to elicit immune responses either *in vitro* or *in vivo* [30–34]. In support of this concept, mice immunized with live but not dead bacilli can induce a protective T-cell response, reinforcing the notion that secreted proteins are among the antigens encountered and presented by the host immune system [35]. In this study, we have identified a novel secreted 13 kDa ricin-like lectin in *Mtb*, namely sMTL-13. This protein's ORF corresponds to Rv1419, a single-copy gene, as defined in the sequenced *Mtb* H37Rv genome [36]. *In silico* analysis of the Rv1419 gene suggests that sMTL-13 is initially synthesized as a 16.8 kDa precursor containing a 33-aa hydrophobic leader sequence (signal peptide). The mature form is predicted to be exported/secreted and has a molecular mass of 13.6 kDa. In line with these observations, Western blot analysis of *Mtb* CFP preparations revealed that the sMTL-13 is at least as abundant as the 19 kDa lipoprotein, a well-known component of CFP [28]. The presence of a consensus Sec-type signal sequence at the N terminus and its removal from the mature form confirm that sMTL-13 is targeted to the extracellular space by *Mtb*. This result is consistent with a recent report in which the Rv1419-encoded product was detected in CFP by a proteomic approach [13]. Taken together, these data suggest that this protein appears to be actively secreted. However, it is not clear from this analysis whether the sMTL13 is released directly into the culture medium or expressed as a surface protein otherwise secreted by membrane turnover. Although we have not directly addressed this hypothesis, lower amounts of sMTL-13 were detected in either cell wall or membrane fractions, thus raising the possibility that sMTL-13 is anchored in the mycobacterial

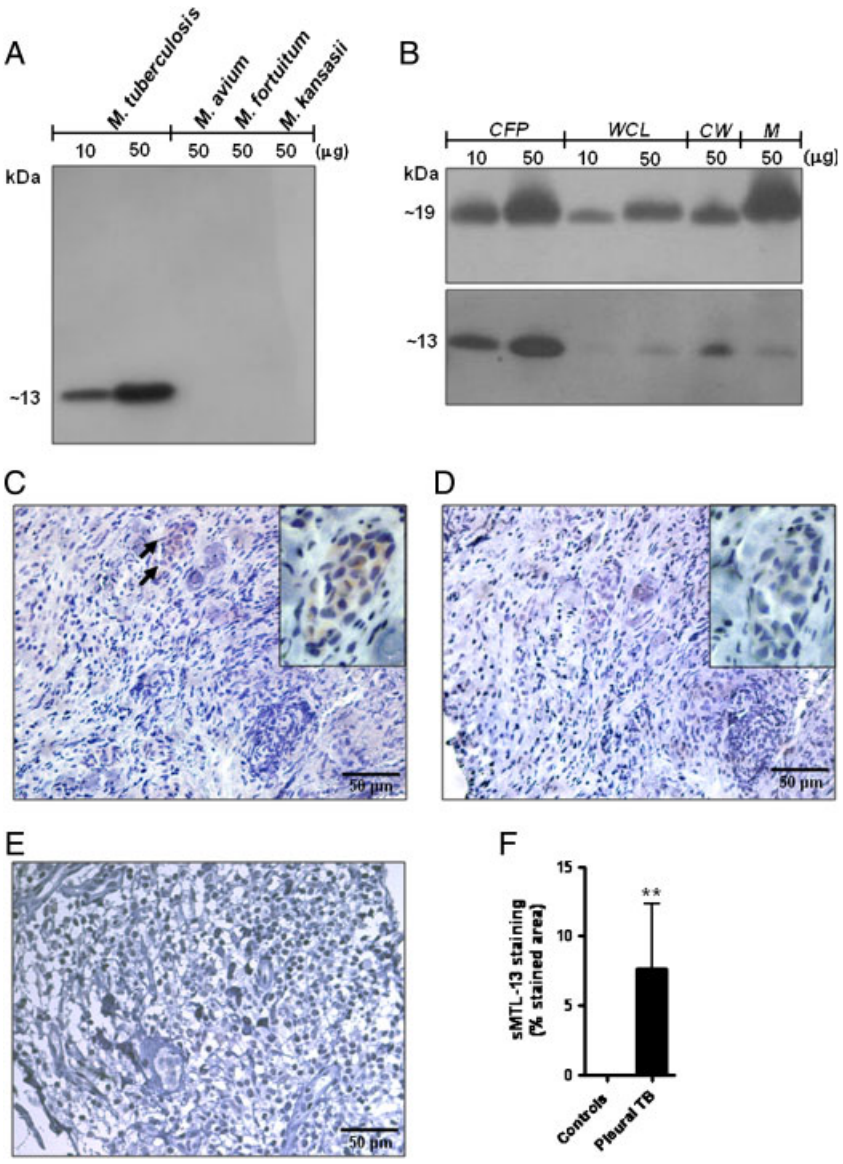

Figure 2. Rv1419-encoded protein is detected in the CFP fraction from *Mtb* H37Rv. (A) CFP fractions from *Mtb* (10 or 50 μg), *M. avium* (50 μg), *M. fortuitum* (50 μg), or *M. kansasii* (50 μg) were resolved onto an SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the monoclonal supernatant produced against the rec-Rv1419 protein (276.B7); (B) subcellular preparations from *Mtb*H37Rv were prepared as in (A). As a control, the membrane was stripped and re-probed with the mAb raised against the 19 kD lipoprotein (IT-19; 1:1000 dilution). Data shown are representative of two experiments performed. *In situ* staining of sMTL-13 in ATB patients. Immunohistochemistry of a representative pleural biopsy from three ATB patients with typical mononuclear inflammatory infiltrate (magnification × 400) is shown. (C) Anti-sMTL13 mAb-positive staining (insert, magnification × 1000); (D) negative IgG1 isotype control (insert: magnification × 1000); (E) negative staining in skin section from a leprosy patient's biopsy using the anti-sMTL-13 mAb (magnification × 400); (F) percentage of total granuloma-stained area for sMTL-13 in TB pleural biopsies or control groups. Results represent the mean ± SEM of measurements from three samples/group (five granulomas/slide). **Statistically significant difference between pleural TB patients *versus* control group (sarcoidosis, leprous, fungal infections; $p = 0.0079$, Mann–Whitney test).

cell wall. However, the high content of sMTL-13 in CFP fraction points out that this protein appears to be actively secreted.

The availability of full-length genome sequences of some mycobacterial species led us to search for Rv1419 homologies. Analysis of the database revealed that Rv1419 ORF is conserved in other strains of *Mtb* and *M. bovis*, indicating that this gene is highly conserved among members of the *Mtb* complex. In contrast, Rv1419 ORF was not detected in several other disease-inducing mycobacteria such as *M. avium, M. leprae, M. abcessus*, or *M. kansasii*. Consistent with these findings, *M. avium, M. fortuitum*, or *M. kansasii* CFP did not reveal sMTL-13 corresponding bands in immunodetection experiments. However, as expected, this lectin was found to present in *M. bovis* BCG CFP (data not shown). Database searches also revealed homology (~78%) between Rv1419 and the predicted ORFs from *M. ulcerans* and *M. marinum*, in agreement with Ben Amor *et al*, who found by Southern blotting

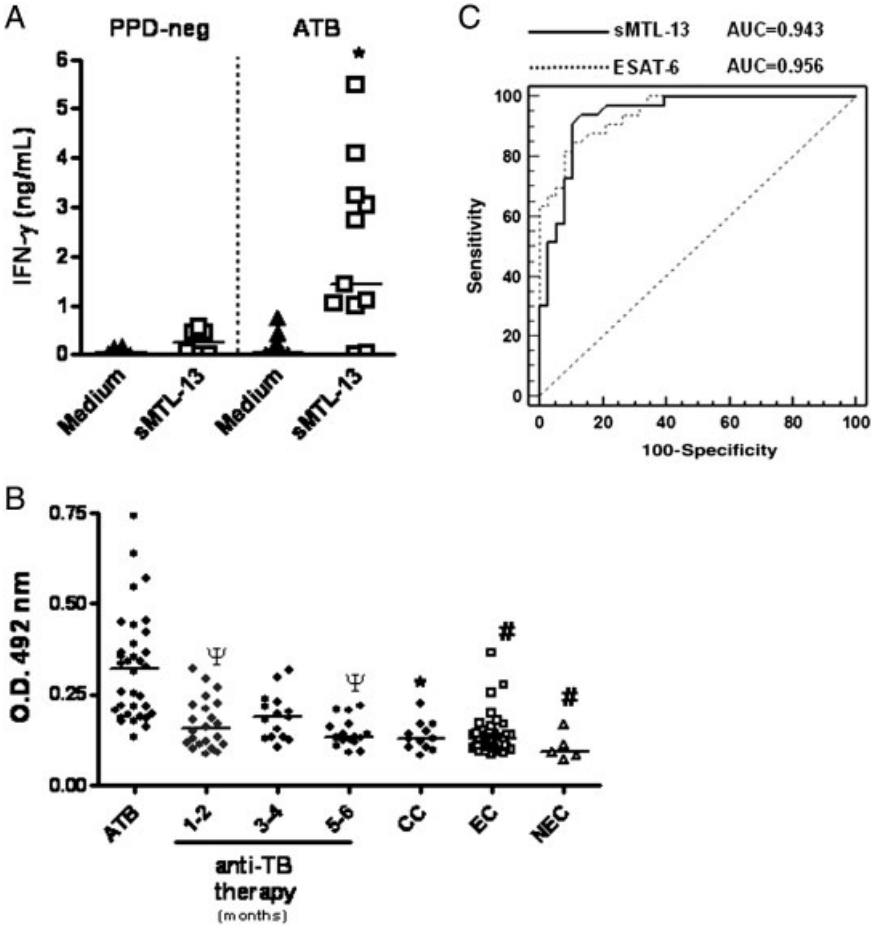

Figure 3. Adaptive immune responses against sMTL-13 during active TB. (A) PBMC from healthy BCG-vaccinated control subjects (PPD-negative, $n = 6$) or ATB patients ($n = 11$) were exposed to purified sMTL-13 (10 μg/mL) for 48 h. IFN-γ production was detected in culture supernatants by cytometric beads array (BD). *Statistically significant when comparing the sMTL-13 ATB (active TB) group *versus* the sMTL-13 PPD-negative group ($p = 0.0238$, Mann–Whitney test); (B) sera from either healthy subjects (endemic and non-EC) or TB patients (active pulmonary disease and ongoing therapy) were assayed for specific IgG anti-sMTL-13 by ELISA (see the *Materials and methods*). Results are expressed as median of individual measurement of optical density. #Statistically significant difference ($p < 0.001$, Kruskal–Wallis test) between ATB and EC or NEC (non-EC) groups. ΨStatistically significant difference ($p<0.01$, Friedman test) between ATB and ongoing therapy (1–2 and 5–6 months). *Statistically significant difference ($p < 0.001$, Friedman test) between ATB and CC (clinical cure) groups. (C) Based on anti-ESAT-6 and anti-sMTL-13 IgG titers (ATB *versus* EC), an ROC curve was utilized to compare the accuracy of indicator variables (variable 1: ESAT-6; variable 2: sMTL-13) of ATB. For each indicator, sensitivity is plotted against 100-specificity and accuracy is measured by the AUC.

analysis that Rv1419-related gene sequence may be present in species from the non-*Mtb* complex [37]. However, it remains to be determined whether non-*Mtb* complex mycobacteria express the Rv1419 homologous protein.

As determined by the bioinformatics studies, sMTL-13 possesses 14 predicted sites for carbohydrate recognition (Fig. 1A). Consistent with this, recombinant sMTL-13 (rec-sMTL-13) induced agglutination of rabbit erythrocytes *in vitro* (Fig. 1D), suggesting that this protein displays lectin activity. Several other lectins from *Mtb* have been described [38, 39]. To date, one of the most studied *Mtb* lectin is heparin binding HBHA, which is expressed on the bacterium surface and it has been shown to be important in bacterial dissemination *in vivo* [20]. These evidences suggest that lectin–host interactions are a potential target to facilitate establishment of infection. For that matter, it has been demonstrated that sera from active TB patients display high titers of IgG against HBHA [22], suggesting that lectins derived from *Mtb* could play an important role in *in vivo* infection.

It has been previously shown that active TB patients display circulating IgG Ab against several *Mtb* secreted molecules [40, 41]. In the present work, we have shown that active TB patients presented high titers of anti-sMTL-13 IgG, a response that decreased following therapy. In comparison with IgG Ab against the well-known secreted protein ESAT-6, ROC curves analysis at the optimal cutoff point revealed that anti-sMTL-13 IgG titers displayed high specificity (90%) as well as sensitivity (93%) for TB diagnosis. Interestingly, titers of anti-sMTL-13 IgG rapidly decreased within the first 2 months of treatment, suggesting that immune responses against this protein diminish following drug-induced control of *Mtb* proliferation. We therefore speculate that anti-sMTL-13 IgG titers could be utilized as a

serum biomarker of treatment efficacy. Although this subject is not directly addressed in the present article, it is possible that serum from non-successful treated TB patients display elevated serum anti-sMTL-13 IgG, as demonstrated for CFP antigens [42]. Whether sMTL-13 is a reliable antigen for diagnosis and/or therapeutic purposes remains to be determined.

In summary, our findings demonstrate the existence of a novel secreted ricin-like lectin from *Mtb* that is recognized by patients during active TB infection. These observations suggest that sMTL-13–host interaction merits further investigation as a potential biomarker of diagnosis/treatment efficacy as well as immunization target. In this regard, it should be noted that secreted antigens are utilized as diagnostic tests as well as a vaccine candidates in current clinical trials [43, 44].

Materials and methods

In silico sMTL-13 aa sequence analysis

The ORF annotated as hypothetical proteins, unknown function, or putative were filtered from the whole *Mtb* genome by using a *Perl* script [24]. The deduced aa sequence from the entire Rv1419 ORF (sMTL-13 containing the signal peptide) was structurally analyzed using ExPASy (Expert Protein Analysis System) Proteomics Server [45]. The SignalP 3.0 server was utilized to identify potential Sec-type signal peptides and cleavage sites based on several Neural Network methods and Hidden Markov models [46]. In order to compare multiple sequences, CLUSTALW and T-COFFE programs were used [47]. Finally, Blast network server at the NCBI has been utilized to identify sequences similar to sMTL-13 and conserved domains.

Expression and purification of rec-sMTL-13

The protein sMTL-13 containing the signal peptide was expressed as a (His)-tagged protein in *E. coli*. The gene Rv1419 was subcloned into pET15b expression vector (Novagen, USA) coding for six N-terminally located His-residues allowing the expression of a fusion protein. The Rv1419 PCR fragment representing the entire ORF was generated with specific primers engineered to introduce *Nde*I e *Xho*I restriction enzymes sites into the resulting PCR product, using *Mtb* H37Rv DNA as template: *Nde*I, sense (5′-GGAATTCCATATGGGTGAATTACGGTTGG-3′) and XhoI, antisense (5′-CCGCTCGAGTCATTACGGCACGCTATCCC-3′). PCR was performed (4 min at 94°C, 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C for 36 cycles) and sequence was confirmed by DNA sequencing. *E. coli* BL21(DE3) was grown at 37°C to an A_{600}(nm) of 0.6, and the expression was performed in the presence of 1 mM isopropylthiogalactoside. Following 4 h induction, cells were harvested by centrifugation and resuspended in 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.5 M NaCl, and 10 mM of imidazole (lysis buffer). Cells were lysed by sonication three times at 30% of amplitude and centrifuged at 5400 × *g*, 4°C for 20 min. rec-sMTL-13 was recovered as inclusion bodies and resuspended in lysis buffer containing 8 M urea. rec-sMTL-13 was purified by nickel affinity chromatography (GE Healthcare, Brazil) under denaturing conditions, dialyzed, and resuspended in PBS.

Mycobacterial fractions

Subcellular fractions from *Mtb* H37Rv were used. Whole cell lysate, CFP, membrane, and cell wall fractions were obtained by strain growth to a late-log phase (day 14) in GAS medium as described elsewhere [14, 48, 49].

Generation of mAb against sMTL-13 (clone 276.B7)

Balb/c mice were i.p. immunized with rec-sMTL-13 ($4 \times 20\,\mu g$) plus AluGel followed by one (20 μg) i.v. injection with the lectin at weekly intervals. Splenocytes were fused with Ag8XP3653 myeloma cells (kindly provided by Prof. Carlos Zanetti/UFSC) in a 5:1 ratio using PEG 50% as fusogen. Cells were then cultured in RPMI 1640 medium (Invitrogen, Brazil) supplemented with 20% FBS (Hyclone, USA) and hybridomas were selected using 0.1 mM hypoxanthine, 4×10^{-4} M aminopterine and 0.016 mM thymidine. Hybridoma supernatants were screened by ELISA, in which purified rec-sMTL-13 was used as the capture antigen (see *Detection of Ab against sMTL-13 by ELISA*). Out of the initial 900 clones screened, 12 positive clones were selected based on production of higher titers of Ab against the lectin. Of these, one clone was subcloned by limited dilution and Ig class and subclass were found to be IgG1κ as determined by the SBA Clonotyping System/HRP (Southern Biotech, USA). The UFPR Animal Experimental Ethics Committee has approved the study protocol (23075.031314/2008-41).

Detection of Ab against sMTL-13 by ELISA

Polystyrene microplates (Biosystems, Brazil) were coated overnight with sMTL-13 (5 μg/mL) diluted in 0.06 M carbonate buffer (pH9.6). Microplates were blocked, washed, and incubated with supernatants from hybridome cultures for 40 min at 37°C. Plates were then incubated with HRP-goat anti-mouse IgG (SC Biotechnology, USA; 1:1,200) for 40 min at 37°C. Color development was performed by adding ABTS® Peroxidase substrate (KPL, USA). Reactions were stopped with 10% SDS and A_{405} measured in an ELISA reader (Molecular Devices, USA).

Detection of sMTL-13 by Western blotting

Total proteins from *Mtb* subcellular fractions (10 or 50 μg *per* lane) were subjected to SDS-PAGE (Invitrogen, USA) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in *tris*-buffered saline containing

0.1% Tween 20 (TBS-T) and probed with the monoclonal supernatant (Clone 276.B7/IgG1κ) or anti-19 KD lipoprotein mAb (clone IT-19; kindly provided by Dr. Antônio Rothfuchs, NIH/NIAID-TVTRM Contract) at 1:1000 dilution followed by incubation with HRP-conjugated secondary Ab (1:2000). Detection was performed by ECL analysis (Pierce, USA).

Study population

Thirty-four patients with active pulmonary TB in the Division of Respiratory Diseases of the Central Public Health Clinic of Juiz de Fora, Minas Gerais State and 11 active-diseased patients from Hospital Octávio Mangabeira, Bahia, Brazil were selected. Only those patients with detectable AFB in the sputum bacilloscopy or culture-confirmed disease and who had undergone clinical and chest X-ray examinations, as prescribed by the Brazilian Ministry of Health, were included in the study. AIDS, diabetes, hepatitis, hypertension, pregnancy, and alcoholism were exclusion criteria. All patients included in the study have been confirmed to present negative bacilloscopy following treatment. Thirty-eight healthy BCG-vaccinated, which constituted the endemic control (EC) group formed by medical students and staff from UFJF, five foreign PPD-negative non-BCG-vaccinated subjects (the non-endemic group) and six PPD-negative BCG-vaccinated individuals were included in the control groups without prior history of *Mtb* infection. All patients and control subjects have been informed of the study and have given consent for blood sampling. The UFJF Medical Ethics as well as the Oswaldo Cruz Foundation Committees have approved the study protocols (UFJF-1495.186.2008; CPqGM-219 (CAAE) 2221.0.000.225-06).

Immunohistochemistry

Histological sections from pleural TB patients or control leprosy patients were deparaffinized in xylene, rehydrated in alcohol and water. Quenching of endogenous peroxidase was performed with a 1.5% hydrogen peroxide-methanol solution for 20 min. Sections were incubated with normal goat serum (30 min 37°C) and then exposed to monoclonal anti-sMTL-13 supernatant (Clone 276.B7). Incubations with biotinylated goat anti-mouse Ab with streptavidin–HRP complex (Vectastain Elite ABC reagent, Vector Laboratories, CA, USA) were performed for 30 min at 37°C. Positive reactions was detected with 3,3′-diaminobenzidine (Dako Cytomation, CA, USA), followed by Harris's hematoxylin counterstaining. Sections were examined microscopically and images were acquired using a Sight DS-5M-L1 digital camera (Nikon, Melville, NY, USA) connected to an Eclipse 50i light microscope (Nikon).

Quantification of anti-sMTL-13 IgG titers

Maxsorb plates (Nunc, Denmark) were coated with rec-sMTL-13 in carbonate buffer overnight at 4°C. Plates were washed with PBS/0.05% Tween-20. Sera from patients and individuals were diluted to 1:20 in PBS-0.05% Tween-20 plus 10% goat serum and incubated for 1 h at 37°C. Plates were then washed and incubated with HRP-conjugated anti-human IgG (Sigma, USA) at 1:3000 dilution. A substrate solution containing OPD (0.5 mg/mL) in sodium citrate buffer, pH 5.0, and 0.03% H_2O_2 was used to develop the colorimetric reaction. Reactions were then stopped with 2 M H_2SO_4 and the A_{492} was measured in an ELISA reader (Spectramax, Molecular Devices).

Recall response assay

Blood from active TB patients ($n = 11$) or PPD-negative ($n = 6$) healthy BCG-vaccinated subjects were collected and PBMC were obtained through Ficoll gradient as previously described [50]. PBMC (5×10^6 cells/mL) were exposed to purified sMTL-13 (10 μg/mL) for 48 h and IFN-γ was measured in culture supernatants by a cytometric bead assay (Bencton, Dickinson and Company, USA) following the manufacturer's instructions.

Statistical analysis

Non-parametric Mann–Whitney test, Kruskall–Wallis with Dunn's multiple comparison tests or Friedman test were used to the significance of differences between groups. Values of $p<0.05$ were considered statistically significant. The ROC curve was used for analysis of the accuracy values: area under the ROC curve, sensitivity, and specificity, obtained by using MedCalc Statistical (Version 5.00.020, Brussels, Belgium).

Acknowledgements: The authors thank Mr. Jorge Tolentino and Dr. Bruno Bezerril (Fiocruz/BA) for technical support and Prof. Mario Steindel for critical reading of this manuscript. They also thank Marcos L'Hotellier and the staff of the DRD-CPHC/JF for helping with the TB patients. They are indebted to Drs. Luciana Leite and Ivan Nascimento (I. Butantan) as well as Profa. Maria Luiza Bazzo (UFSC) for providing the *M. bovis* BCG CFP and non-tuberculous mycobacteria strains, respectively. L.N. received CAPES/CNPq fellowship. A.B. received funding from CNPq (472477/2007-2 and 565496/2008-5), CAPES (210/2007), FAPESC (04524/2008-1) and WHO/TDR (2008-8734-0). C.D.S., B.S.C., H.C.T., S.C.O., M.B.N., and A.B. are CNPq investigators.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **World Health Organization.** 2006. Fact Sheet No. 104. Geneva.

2 **Maartens, G. and Wilkinson, R. J.,** Tuberculosis. *Lancet* 2007. **370**: 2030–2043.

3 **Feng, C. G., Jankovic, D., Kullberg, M., Cheever, A., Scanga, C. A., Hieny, S., Caspar, P. *et al.*,** Maintenance of pulmonary Th1 effector function in chronic tuberculosis requires persistent IL-12 production. *J. Immunol.* 2005. **174**: 4185–4192.

4 **Maglione, P. J. and Chan, J.,** How B cells shape the immune response against Mycobacterium tuberculosis. *Eur. J. Immunol.* 2009. **39**: 676–686.

5 **Flynn, J. L. and Chan, J.,** Immunology of tuberculosis. *Annu. Rev. Immunol.* 2001. **19**: 93–129.

6 **Cooper, A. M. and Flynn, J. L.,** The protective immune response to Mycobacterium tuberculosis. *Curr. Opin. Immunol.* 1995. **7**: 512–516.

7 **Glatman-Freedman, A.,** The role of antibody-mediated immunity in defense against Mycobacterium tuberculosis: advances toward a novel vaccine strategy. *Tuberculosis (Edinb.)* 2006. **86**: 191–197.

8 **Stanley, S. A., Johndrow, J. E., Manzanillo, P. and Cox, J. S.,** The Type I IFN response to infection with Mycobacterium tuberculosis requires ESX-1-mediated secretion and contributes to pathogenesis. *J. Immunol.* 2007. **178**: 3143–3152.

9 **Frigui, W., Bottai, D., Majlessi, L., Monot, M., Josselin, E., Brodin, P., Garnier, T. *et al.*,** Control of M tuberculosis ESAT-6 secretion and specific T cell recognition by PhoP. *PLoS Pathog.* 2008. **4**: e33.

10 **Smith, I.,** Mycobacterium tuberculosis pathogenesis and molecular determinants of virulence. *Clin. Microbiol. Rev.* 2003. **16**: 463–496.

11 **Abdallah, A. M., Gey van Pittius, N. C., Champion, P. A., Cox, J., Luirink, J., Vandenbroucke-Grauls, C. M., Appelmelk, B. J. and Bitter, W.,** Type VII secretion--mycobacteria show the way. *Nat. Rev. Microbiol.* 2007. **5**: 883–891.

12 **Guinn, K. M., Hickey, M. J., Mathur, S. K., Zakel, K. L., Grotzke, J. E., Lewinsohn, D. M., Smith, S. and Sherman, D. R.,** Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of Mycobacterium tuberculosis. *Mol. Microbiol.* 2004. **51**: 359–370.

13 **Malen, H., Berven, F. S., Fladmark, K. E. and Wiker, H. G.,** Comprehensive analysis of exported proteins from Mycobacterium tuberculosis H37Rv. *Proteomics* 2007. **7**: 1702–1718.

14 **Sonnenberg, M. G. and Belisle, J. T.,** Definition of Mycobacterium tuberculosis culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. *Infect. Immun.* 1997. **65**: 4515–4524.

15 **Brightbill, H. D., Libraty, D. H., Krutzik, S. R., Yang, R. B., Belisle, J. T., Bleharski, J. R., Maitland, M. *et al.*,** Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science* 1999. **285**: 732–736.

16 **Pathak, S. K., Basu, S., Basu, K. K., Banerjee, A., Pathak, S., Bhattacharyya, A., Kaisho, T. *et al.*,** Direct extracellular interaction between the early secreted antigen ESAT-6 of Mycobacterium tuberculosis and TLR2 inhibits TLR signaling in macrophages. *Nat. Immunol.* 2007. **8**: 610–618.

17 **Soto, G. E. and Hultgren, S. J.,** Bacterial adhesins: common themes and variations in architecture and assembly. *J. Bacteriol.* 1999. **181**: 1059–1071.

18 **Coutte, L., Alonso, S., Reveneau, N., Willery, E., Quatannens, B., Locht, C. and Jacob-Dubuisson, F.,** Role of adhesin release for mucosal colonization by a bacterial pathogen. *J. Exp. Med.* 2003. **197**: 735–742.

19 **Prince, A.,** Adhesins and receptors of Pseudomonas aeruginosa associated with infection of the respiratory tract. *Microb. Pathog.* 1992. **13**: 251–260.

20 **Pethe, K., Alonso, S., Biet, F., Delogu, G., Brennan, M. J., Locht, C. and Menozzi, F. D.,** The heparin-binding haemagglutinin of M tuberculosis is required for extrapulmonary dissemination. *Nature* 2001. **412**: 190–194.

21 **Singh, D. D., Chandran, D., Jeyakani, J. and Chandra, N.,** Scanning the genome of Mycobacterium tuberculosis to identify potential lectins. *Protein Pept. Lett.* 2007. **14**: 683–691.

22 **Masungi, C., Temmerman, S., Van Vooren, J. P., Drowart, A., Pethe, K., Menozzi, F. D., Locht, C. and Mascart, F.,** Differential T and B cell responses against Mycobacterium tuberculosis heparin-binding hemagglutinin adhesin in infected healthy individuals and patients with tuberculosis. *J. Infect. Dis.* 2002. **185**: 513–520.

23 **Shin, A. R., Lee, K. S., Lee, J. S., Kim, S. Y., Song, C. H., Jung, S. B., Yang, C. S. *et al.*,** Mycobacterium tuberculosis HBHA protein reacts strongly with the serum immunoglobulin M of tuberculosis patients. *Clin. Vaccine Immunol.* 2006. **13**: 869–875.

24 **Moreno, F. B., Facó, F., Ceccatto, V. M., Sampaio, A. H., Costa, A. B. S., Freitas, J. L. T., Nogueira, L. L. *et al.*,** Matching carbohydrate-binding domains in Arabidopsis thaliana genome: development of a lectin database. *Online J. Bioinform.* 2003. **4**: 96–105.

25 **Tuteja, R.,** Type I signal peptidase: an overview. *Arch. Biochem. Biophys.* 2005. **441**: 107–111.

26 **Hazes, B.,** The (QxW)3 domain: a flexible lectin scaffold. *Protein Sci.* 1996. **5**: 1490–1501.

27 **Hartley, M. R. and Lord, J. M.,** Cytotoxic ribosome-inactivating lectins from plants. *Biochim. Biophys. Acta* 2004. **1701**: 1–14.

28 **Rosenkrands, I., King, A., Weldingh, K., Moniatte, M., Moertz, E. and Andersen, P.,** Towards the proteome of Mycobacterium tuberculosis. *Electrophoresis* 2000. **21**: 3740–3756.

29 **Raghavan, S., Manzanillo, P., Chan, K., Dovey, C. and Cox, J. S.,** Secreted transcription factor controls Mycobacterium tuberculosis virulence. *Nature* 2008. **454**: 717–721.

30 **Andersen, P.,** Effective vaccination of mice against Mycobacterium tuberculosis infection with a soluble mixture of secreted mycobacterial proteins. *Infect. Immun.* 1994. **62**: 2536–2544.

31 **Hubbard, R. D., Flory, C. M. and Collins, F. M.,** Immunization of mice with mycobacterial culture filtrate proteins. *Clin. Exp. Immunol.* 1992. **87**: 94–98.

32 **Boesen, H., Jensen, B. N., Wilcke, T. and Andersen, P.,** Human T-cell responses to secreted antigen fractions of Mycobacterium tuberculosis. *Infect. Immun.* 1995. **63**: 1491–1497.

33 **Coler, R. N., Skeiky, Y. A., Vedvick, T., Bement, T., Ovendale, P., Campos-Neto, A., Alderson, M. R. and Reed, S. G.,** Molecular cloning and immunologic reactivity of a novel low molecular mass antigen of Mycobacterium tuberculosis. *J. Immunol.* 1998. **161**: 2356–2364.

34 **Orme, I. M., Andersen, P. and Boom, W. H.,** T cell response to Mycobacterium tuberculosis. *J. Infect. Dis.* 1993. **167**: 1481–1497.

35 **Orme, I. M.,** Induction of nonspecific acquired resistance and delayed-type hypersensitivity, but not specific acquired resistance in mice inoculated with killed mycobacterial vaccines. *Infect. Immun.* 1988. **56**: 3310–3312.

36 **Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V. *et al.*,** Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature* 1998. **393**: 537–544.

37 **Ben Amor, Y., Shashkina, E., Johnson, S., Bifani, P. J., Kurepina, N., Kreiswirth, B., Bhattacharya, S. *et al.*,** Immunological characterization of novel secreted antigens of Mycobacterium tuberculosis. *Scand. J. Immunol.* 2005. **61**: 139–146.

38 **Menozzi, F. D., Rouse, J. H., Alavi, M., Laude-Sharp, M., Muller, J., Bischoff, R., Brennan, M. J. and Locht, C.,** Identification of a heparin-binding hemagglutinin present in mycobacteria. *J. Exp. Med.* 1996. **184**: 993–1001.

39 **Kinhikar, A. G., Vargas, D., Li, H., Mahaffey, S. B., Hinds, L., Belisle, J. T. and Laal, S.,** Mycobacterium tuberculosis malate synthase is a laminin-binding adhesin. *Mol. Microbiol.* 2006. **60**: 999–1013.

40 **Laal, S., Samanich, K. M., Sonnenberg, M. G., Zolla-Pazner, S., Phadtare, J. M. and Belisle, J. T.,** Human humoral responses to antigens of Mycobacterium tuberculosis: immunodominance of high-molecular-mass antigens. *Clin. Diagn. Lab. Immunol.* 1997. **4**: 49–56.

41 **Samanich, K. M., Belisle, J. T., Sonnenberg, M. G., Keen, M. A., Zolla-Pazner, S. and Laal, S.,** Delineation of human antibody responses to culture filtrate antigens of Mycobacterium tuberculosis. *J. Infect. Dis.* 1998. **178**: 1534–1538.

42 **Imaz, M. S. and Zerbini, E.,** Antibody response to culture filtrate antigens of Mycobacterium tuberculosis during and after treatment of tuberculosis patients. *Int. J. Tuberc. Lung Dis.* 2000. **4**: 562–569.

43 **Reece, S. T. and Kaufmann, S. H.,** Rational design of vaccines against tuberculosis directed by basic immunology. *Int. J. Med. Microbiol.* 2008. **298**: 143–150.

44 **Verma, R. K. and Jain, A.,** Antibodies to mycobacterial antigens for diagnosis of tuberculosis. *FEMS Immunol. Med. Microbiol.* 2007. **51**: 453–461.

45 **Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D. and Bairoch, A.,** ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.* 2003. **31**: 3784–3788.

46 **Emanuelsson, O., Brunak, S., von Heijne, G. and Nielsen, H.,** Locating proteins in the cell using TargetP, SignalP and related tools. *Nat. Protoc.* 2007. **2**: 953–971.

47 **Notredame, C., Higgins, D. G. and Heringa, J.,** T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.* 2000. **302**: 205–217.

48 **Hirschfield, G. R., McNeil, M. and Brennan, P. J.,** Peptidoglycan-associated polypeptides of Mycobacterium tuberculosis. *J. Bacteriol.* 1990. **172**: 1005–1013.

49 **Lee, B. Y., Hefta, S. A. and Brennan, P. J.,** Characterization of the major membrane protein of virulent Mycobacterium tuberculosis. *Infect. Immun.* 1992. **60**: 2066–2074.

50 **Van Weyenbergh, J., MP, P. S., Bafica, A., Cardoso, S., Wietzerbin, J. and Barral-Netto, M.,** IFN-beta and TGF-beta differentially regulate IL-12 activity in human peripheral blood mononuclear cells. *Immunol. Lett.* 2001. **75**: 117–122.

Abbreviations: **ATB:** active tuberculosis · **CFP:** culture filtrate proteins · **EC:** endemic control · **HBHA:** hemagglutinin adhesin · ***Mtb:*** *Mycobacterium tuberculosis* · **ORF:** open reading frame · **PPD:** purified protein derivative · **rec-sMTL-13:** recombinant sMTL-13 · **ROC:** receiver operating characteristic · **sMTL-13:** secreted 13 kDa lectin from *Mtb* · **TB:** tuberculosis

Full correspondence: Professor André Báfica, Universidade Federal de Santa Catarina, R João Pio Duarte Silva s/n, Bloco A, sala 309, Campus Trindade, 88040-900 Florianópolis, SC, Brazil
Fax: +55-48-3721-9258
e-mail: abafica@ccb.ufsc.br

Received: 29/6/2009
Revised: 19/10/2009
Accepted: 2/12/2009

