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Evaluation of Gamma Interferon Immune Response Elicited by the Newly Constructed PstS-1(285-374):CFP10 Fusion Protein To Detect *Mycobacterium tuberculosis* Infection

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The PstS1 antigen is highly immunogenic, principally when combined with CFP10 during both latent and active TB infection. In the present study, a selected *pstS1* gene fragment was cloned, fused with CFP10, and expressed in *Escherichia coli*. The product [PstS-1(285-374):CFP10] was compared to the recombinant fused RD1 (region of deletion 1) protein (ESAT-6:CFP10) in detecting *Mycobacterium tuberculosis* infection in 108 recent contacts of pulmonary tuberculosis (TB) cases, considering a positive tuberculin skin test (TST) to be the baseline. The release of gamma interferon (IFN-γ) in 22-h whole-blood and 5-day lymphocyte stimulation assays primed with each antigen was determined. All contacts were clinically followed for up to 1 year, and 87% of the tuberculin skin test-positive ($TST^{positive}$) patients accepted preventative treatment. Concerning the IFN-γ response to PstS-1(285-374):CFP10 in the 22-h and 5-day assays, a slight increase in contact-$TST^{positive}$ detection was observed (23/54 and 26/54) compared to the level seen with the RD1 protein (18/54 and 24/54) whereas in the $TST^{negative}$ group, similarly lower numbers (≤5/48) of responders were achieved for both antigens, except for RD1 in the 5-day assay (8/48). By combining the IFN-γ responders to both antigens in the 5-day assays, slightly higher increases in positivity were found in the $TST^{positive}$ (32/54) and $TST^{negative}$ (10/48) groups. Two of 12 untreated $TST^{positive}$ contacts progressed to active TB and were concordantly positive in all assays, except for one contact who lacked positivity in the RD1 5-day assay. We demonstrated for the first time that PstS-1(285-374):CFP10 slightly increased contact positivity and detection of active disease progression, suggesting its potential application as a TB infection marker.

Mycobacterium tuberculosis, a highly successful parasite, is the cause of tuberculosis (TB). A major health care concern, the disease results in approximately 1.4 million annual deaths (990,000 among HIV-negative individuals), infecting roughly one-third of the world's population. Among the infected, 5% to 10% will develop active disease in their lifetimes and 90% will harbor the latent form. According to a recent mathematical projection of TB eradication, the treatment of latent TB infection (LTBI) and active TB is urgently required in order to lower and ultimately prevent the further spread of the disease at its present rate (1, 2).

Two principal approaches based on the adaptive immune responses elicited by *M. tuberculosis* infection are currently used to identify TBI (3): the *in vivo* tuberculin skin test (TST) and the *ex vivo* gamma interferon (IFN-γ) release assay (IGRA).

Developed in 1908, TST became the standard means of assessing the presence of TB infection. Via TST, prior TB exposure is measured by a type 4 delayed-type hypersensitivity reaction when a purified protein derivative (PPD) of *M. tuberculosis* is injected intradermally. Although TST has some biological limitations such as the occurrence of anergies in one-third of active TB cases, cross-reactivity with *M. bovis* bacillus Calmette-Guérin (BCG) and non-TB mycobacteria does not, according to some authors, result in major sensibility differences in IGRAs (4).

The IGRA was recently developed using antigens that are expressed in *M. tuberculosis* but not in BCG or *M. bovis* strains as stimuli. These antigens measure the production of IFN-γ in peripheral blood mononuclear cells (3, 5). According to a recent World Health Organization (WHO) report (1), the two currently commercially available IGRAs have yet to generate sufficient data or enough high-quality evidence regarding their performance in the low- and middle-income countries that typically have a high TB and/or HIV burden and where the coverage of BCG vaccination may cause some interference (5, 6).

Both IGRAs are based on *Mycobacterium tuberculosis*-specific antigens, namely, early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP10) (5). These antigens must be validated in different scenarios, but other antigen combinations also need to be evaluated to improve IGRA coverage.

The 38-kDa *M. tuberculosis* antigen, also known as phosphate-specific transporter-1 (PstS-1), coded by a *pstS1* gene that composes one of the 3 putative *pst* operons, is a lipoprotein phosphate transport receptor on the cell surface. These operons probably constitute a subtle biochemical adaptation of *M. tuberculosis*, enabling it to grow and survive under different phosphate-limiting conditions during its infectious cycle. The PstS-1 protein is ac-

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tively secreted by phosphate-starved mycobacteria (7); the 38-kDa protein is highly immunogenic because it contains *M. tuberculosis*-specific epitopes and is antigenic for human T cells. A proliferative response to 2 peptides within the 38-kDa antigen among vaccinated controls and pulmonary TB patients that is lower than the responses to the PPD, 16-kDa, and 19-kDa antigens has been previously described (8).

The aim of the present study was to evaluate specific IFN-γ production, in the whole-blood stimulation assay (WBA) and the long-term stimulation assay (LSA), in response to the newly synthesized PstS-1(285-374):CFP10 fusion protein based on the 38-kDa peptide and compare the resulting data with that determined with the known ESAT-6:CFP10 protein in recent close contacts (rCt) of active TB patients by utilizing the TST results for infection classification. We were successful in demonstrating the usefulness of the newly engineered PstS-1(285-374):CFP10 protein in assessing TBI in a TB endemicity scenario in which infant BCG vaccination is mandatory.

MATERIALS AND METHODS

Study set and participants. The recent close contacts (rCt) of active TB cases involved in the present study were recruited from the Clementino Fraga Filho University Hospital (CFFUH), Rio de Janeiro, RJ, Brazil. This hospital runs a well-established nationally renowned TB control program. Routine procedures require that after an index case (IC) has been confirmed, close contacts are recruited for clinical examination, a chest X-ray, and TST monitoring. A total of 108 close contacts of 51 patients diagnosed with pulmonary TB (index case) were enrolled from March 2010 through January 2012. All rCt except one tested negatively for the human immunodeficiency virus (HIV). Eligible IC were 18 years of age or older, had pulmonary TB with at least one acid-fast bacillus (AFB)-positive sputum sample for *M. tuberculosis*, a positive sputum culture, or compatible clinical evolution. Only 2 IC were infected with drug-resistant strains. Close contacts of a TB-IC were deemed eligible if they currently resided in the same dwelling as the IC or had spent more than 6 h/day in the company of the IC for 2 consecutive months prior to diagnosis. Participants underwent a TST on the day of blood sampling. The TST was administered via the Mantoux procedure using 5 IU of tuberculin RT23 (Statens Serum Institut, Copenhagen, Denmark). Results were read after 72 h; induration of at least 5 mm was considered representative of a TST-positive response.

The criteria for TB diagnosis and treatment for rCt and TB cases were in accordance with those of the Brazilian Ministry of Health (9). Treatment was provided free of charge by the National Tuberculosis Control Program and administered under direct supervision of qualified health care workers. Upon enrollment, demographic as well as clinical and laboratory characteristics, including information concerning the presence of a BCG scar and smoking habits, were determined via a structured questionnaire (Table 1). Blood samples were taken only after written informed consent of all participants was obtained. All the rCt were clinically followed for 12 consecutive months. As a first step, the study protocol was approved by the Ethics Committee of the Oswaldo Cruz Foundation (560-10).

Antigens. ESAT-6:CFP10, the *M. tuberculosis* recombinant fusion protein in *Escherichia coli*, was produced at Leiden University Medical Center, as previously described (10), and kindly provided by Tom Ottenhoff. The recombinant antigens were freeze-dried and shipped at ambient temperature, while the endotoxin contents were kept below 50 IU/mg. Upon arrival, the antigens were solubilized to a concentration of 100 μg/ml, using the supplied protocol, and then divided into aliquots and stored at −20°C until further use.

The construction of the new PstS-1(285-374):CFP10 fusion protein was done on the basis of the 267-bp sequence at the end [PstS-1(285-374); AGFASKTPANQAISMIDGPAPDGYPIINYEYAIVNNRQKDAATAQTL QAFLHWAITDGNKASFLDQVHFQPLPPAVVKLSDALIATISS] of the *Rv0934* gene (GenBank accession no. CCP43682.1) and the entire sequence of the *Rv3874* gene (GenBank accession no. CCP46703.1), which codes CFP10 antigens with 300 bp, by amplifying them from H37Rv genomic DNA via PCR using two pair of primers. Respectively, the forward primers (5′-GGATCCGCTGGCTTCGCATCG-3′ and 5′-GAGCTCATGGCAGAGATGAAGAC-3′) contained BamHI and SacI restriction enzyme recognition sites (underlined) and the reverse primers (5′-GAGCTCGCTGGAAATCGTCGC-3′ and 5′-AAGCTTTCAGAAGCCCATTTGC-3′) contained SacI and HindIII restriction enzyme recognition sites. The PCR amplification protocol consisted of 25 cycles of denaturation at 94°C for 60 s, primer annealing at 60°C for 60 s, and extension at 72°C for 60 s followed by a final extension at 72°C for 5 min. PCR products were digested by the respective restrictive enzymes. The resulting fragments were ligated with T4 DNA ligase into BamHI-, SacI-, and HindIII-digested pQE80L containing a region with an N-terminal histidine tag and an ampicillin resistance gene (Qiagen Inc., Valencia, CA).

Expression and purification of the recombinant plasmid PstS-1(285-374):CFP10-pQE80L were performed as previously described (11) but with minor modifications. Briefly, the plasmid from transformed *E. coli* BL21(DE3) cells was sequenced by Sanger's method (sequencing platform of the Oswaldo Cruz Foundation in Rio de Janeiro, RJ, Brazil), using the same primers as those used for PCR amplification whereas the external

TABLE 1 Demographic, clinical, and laboratory characteristics of the 108 recent close contacts enrolled in the study

Characteristic[a]	No. of subjects	%
Age in yrs (mean ± SD, 41.1 ± 14.4)		
18–44	59	54.6
≥45	49	45.4
Sex		
Male	35	32.4
Female	73	67.6
Previous TB	5	4.6
Smoker	43	39.8
Comorbidity	37	34.3
Degree of contact		
Close household	50	46.3
Close nonhousehold	58	53.7
BCG vaccinated	80	74.1
TST (mean ± SD)		
Positive (12 ± 6.6 mm)	54	50
Negative (0.4 ± 1.2 mm)	48	44.4
Missing	6	5.6
Chest X-ray abnormalities	10	9.3
Health care workers	9	8.3
Other reported TB contact	21	19.4
Index TB case (no. of rCt)		
Culture		
Negative	8	7.4
Positive	88	81.5
Missing	12	11.1
Sputum		
AFB negative	23	21.3
AFB positive (1+)	59	54.6
AFB positive (≥2+)	16	14.8
Missing	10	9.3

[a] Induration cutoff, 5 mm. rCt, recent close contacts; SD, standard deviation; AFB, acid-fast bacilli; BCG, Bacillus Calmette-Guérin; TB, tuberculosis; TST, tuberculin skin test.

primers were based on the flanked sequence of the pQE80L plasmid multiple-cloning site. Sequences were compared with those of the *Rv0934* and *Rv3874* genes registered in the GenBank database by Basic Local Alignment Search Tool (BLAST) analysis. After overnight growth, cells containing the recombinant plasmid were harvested by centrifugation at 3,000 × *g* for 10 min at 4°C, suspended with 5 ml/g of urea lysis buffer (10 mM Tris [pH 8], 100 mM $NaH_2Po_4{\cdot}H_2O$, 8 M urea), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 20 mM ß-mercaptoethanol, and, lastly, incubated via gentle agitation at room temperature for 1 h. The lysate was centrifuged at 10,000 × *g* for 30 min at 4°C, after which a supernatant was separated for purification. Purification of the tagged PstS-1(285-374):CFP10 fusion protein was performed using a pH gradient in a metal chelate column (HisLink protein purification resin; Promega, Madison, WI) containing nickel-nitrilotriacetic acid (Ni-NTA) affinity resin in accordance with the manufacturer's instructions. The resin was incubated with the supernatant for 30 min at 4°C with stirring, transferred to a column, and washed with wash buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 mM urea; pH 6.3). The bound protein was then eluted with pH gradient elution buffers (100 mM NaH_2PO_4, 10 mM Tris, 8 mM urea [pH 5.9, 4.5, and 4.0, respectively). The purified fractions were dialyzed overnight followed by analysis of the recombinant protein and determination of the protein concentration.

Cellular stimulation assays with the ESAT-6:CFP10 and PstS-1(285-374):CFP10 antigens. (i) Heparinized whole-blood assay (WBA). Cultures were grown in the presence or absence (control) of each stimulus, using 3 μg/ml of the fusion proteins. Concanavalin A (ConA; Sigma, St. Louis, MO) mitogen was used at 5 μg/ml (optimal concentrations were established in previous pilot experiments) to 1 ml of whole blood in 48-well microtiter plates (Corning Costar, Corning Incorporated, NY) in duplicate experiments. After 22 h at 37°C in a humidified incubator, the plates were centrifuged (913 × *g* for 15 min at 25°C), after which the plasma was harvested and stored at −20°C.

(ii) Long-term stimulation assay (LSA). Peripheral blood mononuclear cells (PBMCs) of the participants were isolated as previously described (12) with minor modifications. PBMCs were obtained from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (GE Healthcare, Uppsala, Sweden). Cells were then resuspended and washed twice with RPMI 1640-HEPES supplemented with penicillin, streptomycin, 2 mM L-glutamine, and 2 g/liter of sodium bicarbonate (LGC Biotecnologia, SP, Brazil). For the cell culture, 1×10^5 cells/ml were plated in triplicate in 96-well, flat-bottom microtiter plates (Nunc, Swedesboro, NJ) in 300 μl of RPMI 1640-HEPES enriched with 20% autologous serum and 100 U/ml penicillin and streptomycin (Gibco, Paisley, United Kingdom) in the presence or absence of each stimulus. The mitogen and antigen concentrations were the same as for the whole-blood culture. The plates were placed at 37°C in a humidified 5% CO_2 incubator for 5 days. Culture supernatants (200 μl/well for each triplicate) were pooled and then stored at −20°C for further IFN-γ quantification.

IFN-γ detection. IFN-γ secretion was measured in the culture supernatants in the WBA stimulated with antigens for a short time (22 h) and in the LSA stimulated for 5 consecutive days, using a commercial enzyme-linked immunosorbent assay (ELISA) (DuoSet IFN-γ kit; R&D), according to the manufacturer's recommendations. Results were expressed in picograms/milliliter after subtraction of the appropriate unstimulated control wells.

Data analysis. Prism5 (GraphPad Software) software was used to generate plots in the stored databank. Statistical analysis was performed using SPSS 17.0 (IBM); a difference with $P < 0.05$ was considered significant. A nonparametric test was utilized to analyze significant differences in group comparisons (Kruskal Wallis) and in pairwise comparisons (Mann-Whitney). Concordance among the results was assessed using kappa (κ) coefficients, with $\kappa > 0.79$ defined as excellent, $\kappa < 0.19$ as poor, κ between 0.20 and 0.39 as fair, κ between 0.40 and 0.59 as moderate, and κ between 0.60 and 0.79 representing substantial agreement. The odds ratios (OR) and 95% confidence intervals (CI) were calculated. Since the LSA cutoff had been established in previous studies (12), a receiver operating characteristic (ROC) curve using TST (cutoff of 5 mm) as a standard was done to establish the WBA cutoff points.

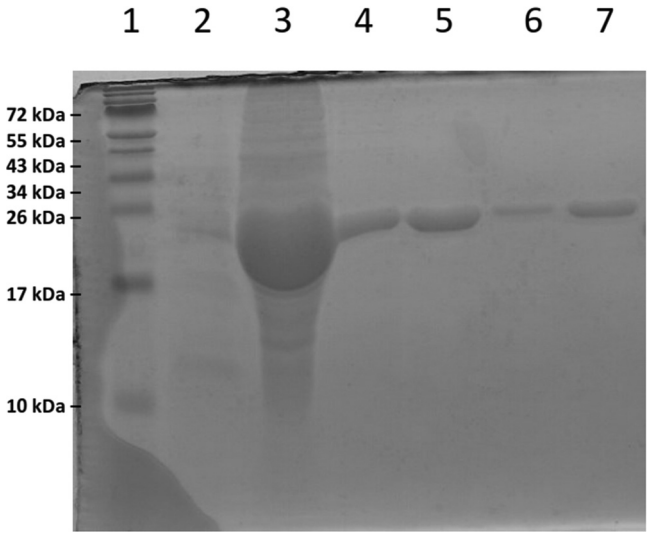

FIG 1 Coomassie-stained 12% SDS-PAGE of the purified PstS-1(285-374):CFP10 fusion protein. Lane 1, molecular mass marker; lane 2, resin; lane 3, supernatant; lanes 4 to 7, eluates collected sequentially. Unless otherwise noted, all reagents and chemicals were purchased from Sigma-Aldrich.

RESULTS

Expression and purification of the newly constructed PstS-1(285-374):CFP10 fusion protein. Except for a minor modification facilitating fusion protein construction, the nucleotide sequence encoding PstS-1(285-374):CFP10 was 100% homologous to that of the 38-kDa target peptide and to the entire *Rv3874* sequence (see the supplemental material). The molecular mass of the PstS-1(285-374):CFP10 fusion protein was approximately 22 kDa, as determined by 12% SDS-PAGE (Fig. 1). The PstS-1(285-374):CFP10 fusion protein was obtained in soluble form and at maximum concentration when the induction took place using 1.0 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C for 18 h.

Characteristics of the study population. A total of 108 recent close contacts of a newly diagnosed pulmonary TB case (TB-IC) treated at CFFUH-Federal University of Rio de Janeiro (CFFUH-UFRJ), RJ, Brazil, signed their informed consent to participate in the present study. All rCt underwent detailed clinical and radiological examinations and a TST to determine the presence or absence of active TB for the purpose of exclusion, if necessary. Six rCt did not return for a TST reading, and 54 (50%) were TST positive ($TST^{positive}$). Twenty-four rCt had $TST^{5–10\ mm}$, and 30 had $TST^{>10\ mm}$; of the latter, 14 had $TST^{\geq 15\ mm}$. The remaining 48 rCt were $TST^{negative}$; 42 had $TST^{=0\ mm}$, and 6 had $TST^{1–4\ mm}$. In accordance with Brazilian Ministry of Health standards (9), all rCt with TST ≥ 5 mm were offered isoniazid preventive therapy (IPT); however, 12 (13%) declined. Table 1 summarizes the demographic, clinical, and laboratory characteristics of the 108 recent close contacts of 51 pulmonary TB-IC, whose mean age was 41.1 (±14.4). The majority were female (66.7%), had a BCG scar (69.4%), and were contacts of a TB-IC with an AFB-positive sputum smear (74.1%). Only 2 individuals refused to

answer questions about smoking, and 10 did not agree to a chest X ray. Two of the rCt who declined IPT developed active pulmonary tuberculosis approximately 4 months after enrollment in the study.

IFN-γ responses to the ESAT-6:CFP10 and PstS-1(285-374):CFP10 proteins by *in vitro* WBA and LSA. All enrolled rCt responded under conditions of mitogen stimulation, with no significant differences among the TST groups. Under conditions of antigen stimulation, the mean level of increased IFN-γ production was directly proportional to the increase in the TST level in both tests. In an overall comparison of the TST groups, a significant difference was found for WBA ESAT-6:CFP10 ($P = 0.004$) and for both antigens ($P \geq 0.001$) by LSA. However, in the pairwise WBA analyses, a significant difference was observed only in the TST$^{0-4\ mm}$ versus the TST$^{>10\ mm}$ rCt groups ($P \leq 0.028$) for both antigens. For LSA, a significant difference was found regarding the TST$^{5-10\ mm}$ groups versus TST$^{>10\ mm}$ rCt groups ($P \leq 0.008$) for both antigens. For both antigens, a significant WBA difference was observed in the TST$^{0-4\ mm\ rCt}$ group ($P = 0.030$) (Fig. 2).

ROC curve analysis results using TST as a standard determined best cutoff points of 10 pg/ml for WBA ESAT-6:CFP10 and of 29.5 pg/ml for PstS-1(285-374):CFP10, demonstrating values for the area under the curve (AUC) of 0.66 and 0.61, respectively. Positive concordant results for both antigens in the WBA and at LSA scored 5 (20.8%) and 3 (12.4%), respectively, among the 24 rCt-TST$^{5-10\ mm}$ group members; 4 (25%) and 5 (32.3%) among the 16 rCt-TST$^{11-14\ mm}$ group members; and 7 (50%) and 10 (71.4%) among the 14 rCt-TST$^{\geq 15\ mm}$ group members. Highly negative concordant results for both antigens scored from 50% to 62.5% in both tests in the stratified rCt-TST groups, with the exception of LSA among the rCt-TST$^{\geq 15\ mm}$ group members (1/14; 7.2%). Note that among the rCt-TST^{negative} group members, major WBA (85.4%) and LSA (79.2%) negative concordant results were achieved (Table 2).

Discordant results were mainly observed among the rCt-TST$^{5-10\ mm}$ group members in both tests with ESAT-6:CFP10^{negative}/PstS-1(285-374):CFP10^{positive} (WBA = 20.8% and LSA = 29.2%) and among the rCt-TST^{negative} group members for LSA with ESAT-6:CFP10^{positive}/PstS-1(285-374):CFP10^{negative} (10.4%) (Table 2). In the overall analyses of rCt TST^{positive}, which showed a negative results in the WBA but a positive one in the LSA, a higher frequency was found for ESAT-6:CFP10 (11/54; 20.4%) than for PstS-1(285-374):CFP10 (5/54; 9.3%).

In summary, for WBA and LSA, a slight increase in rCt-TST^{positive} detection was observed with respect to the new PstS-1(285-374):CFP10 fusion protein (23/54 [42.6%] and 26/54 [48.2%]) compared to ESAT-6:CFP10 stimulation (18/54 [33.3%] and 24/54 [44.4%]). In contrast, the TST^{negative} group showed a lower number of responders (≤5/48 [10.4%]), with the exception of the LSA performed with ESAT-6:CFP10 (8/48 [16.7%]) (Table 2). Combining the results of the two antigens, an extra rCt in each of the groups with TST < 14 mm was detected (24/54 [44.4%]) for WBA whereas a slight increase in positivity was achieved for LSA, with 32/54 (59.3%) among the TST^{positive} and 10/48 (20.8%) among the TST^{negative} group members. Relative to the 6 rCt without a TST reading, only one was detected by LSA for each antigen tested.

While the overall levels of agreement of WBA and LSA in comparisons between the stimuli were, on the one hand, substantial ($\kappa = 0.71$) and, on the other, moderate ($\kappa = 0.50$), respectively, these tests scored only fair agreement for ESAT-6:CFP10 or PstS-1(285-374):CFP10 and TST ($\kappa \leq 0.37$) (see Table S1 in the supplemental material). However, after excluding the BCG-vaccinated rCt from the analysis, a change to moderate agreement ($\kappa = 0.46$ to 0.54) of the WBA and LSA results determined with TST was seen among those 26 rCt, with the lone exception of LSA ESAT-6:CFP10 ($\kappa = 0.32$). Nevertheless, vaccine status did not seem to affect agreement between the results determined for ESAT-6:CFP10 and those determined for PstS-1(285-374):CFP10 in the WBA ($\kappa = 0.69$ to 0.75) or LSA ($\kappa = 0.50$ to 0.48). The levels of agreement of both assays (WBA and LSA) with respect to ESAT-6:CFP10 ($\kappa = 0.32$ to 0.39) and PstS-1(285-374):CFP10 ($\kappa = 0.45$ to 0.51) were similar (see Table S1).

Each rCt participant was specifically interviewed to gather data regarding a greater or lesser risk of TB infection or development. Chest X-ray findings are considered among the principal risk factors associated with TB infection and disease. As such, in OR analyses, chest X-ray abnormalities were significantly (≥5.9-fold) associated with positivity in WBAs, LSAs, and TST, the only exception being a LSA–PstS-1(285-374):CFP10 OR of 3.4 (95% CI, 0.9 to 12.9; $P = 0.072$) (Table 3). Furthermore, LSA of ESAT-6:CFP10 and PstS-1(285-374):CFP10 reached maximum positivity among rCt with abnormal chest X-rays (90% each) followed by WBA (60% each) (data not shown).

Participants who were 45 years of age or older showed a significant tendency toward a positive response in WBA–ESAT-6:CFP10 and WBA/LSA–PstS-1(285-374):CFP10 (Table 3). The rCt with an occupational risk of exposure to TB more predictably had positive TST and PstS-1(285-374):CFP10 results in both assays. Nonetheless, interestingly, the rCt who reported previous exposure to other TB patients while showing a significant positive association by the WBA after stimuli of both antigens did not show a significant positive association by the LSA.

Variables such as a BCG scar, smoking habits, and degree of contact (household/nonhousehold) did not demonstrate a significant association in any of the assays (data not shown). All rCt were clinically followed for 12 months, and 12 TST^{positive} subjects declined IPT. Of the 4 of 12 who were WBA positive for both antigens tested, 2 progressed to active disease approximately 4 months after enrollment. One of them had a TST of 10 mm accompanied by a strong WBA IFN-γ response [121 and 92 pg/ml for ESAT-6:CFP10 and PstS-1(285-374):CFP10, respectively], while the other responded mildly (21 and 60 pg/ml, respectively) and had a TST of 15 mm. However, regarding LSA, the 2 responded differently in that the first tested positively for PstS-1(285-374):CFP10 (108 pg/ml) alone while the second yielded a higher response to both fused antigens (200 and 365 pg/ml, respectively). The two patients presented an abnormal chest X-ray result, absence of a BCG scar, and a mean age of 61.5 years. Thus, the positive predictive value (PPV) of active disease development was 3.7% for TST. For WBA, the PPVs were very similar at 7.4% and 7.7% for ESAT-6:CFP10 and PstS-1(285-374):CFP10, respectively, while, regarding LSA, the PPV was higher for PstS-1(285-374):CFP10 (6.1%) than for ESAT-6:CFP10 (2.9%). The negative predictive value (NPV) of active disease progression obtained in the present study was 100% for TST, WBA, and LSA, an exception being that for LSA–ESAT-6:CFP10 (99%).

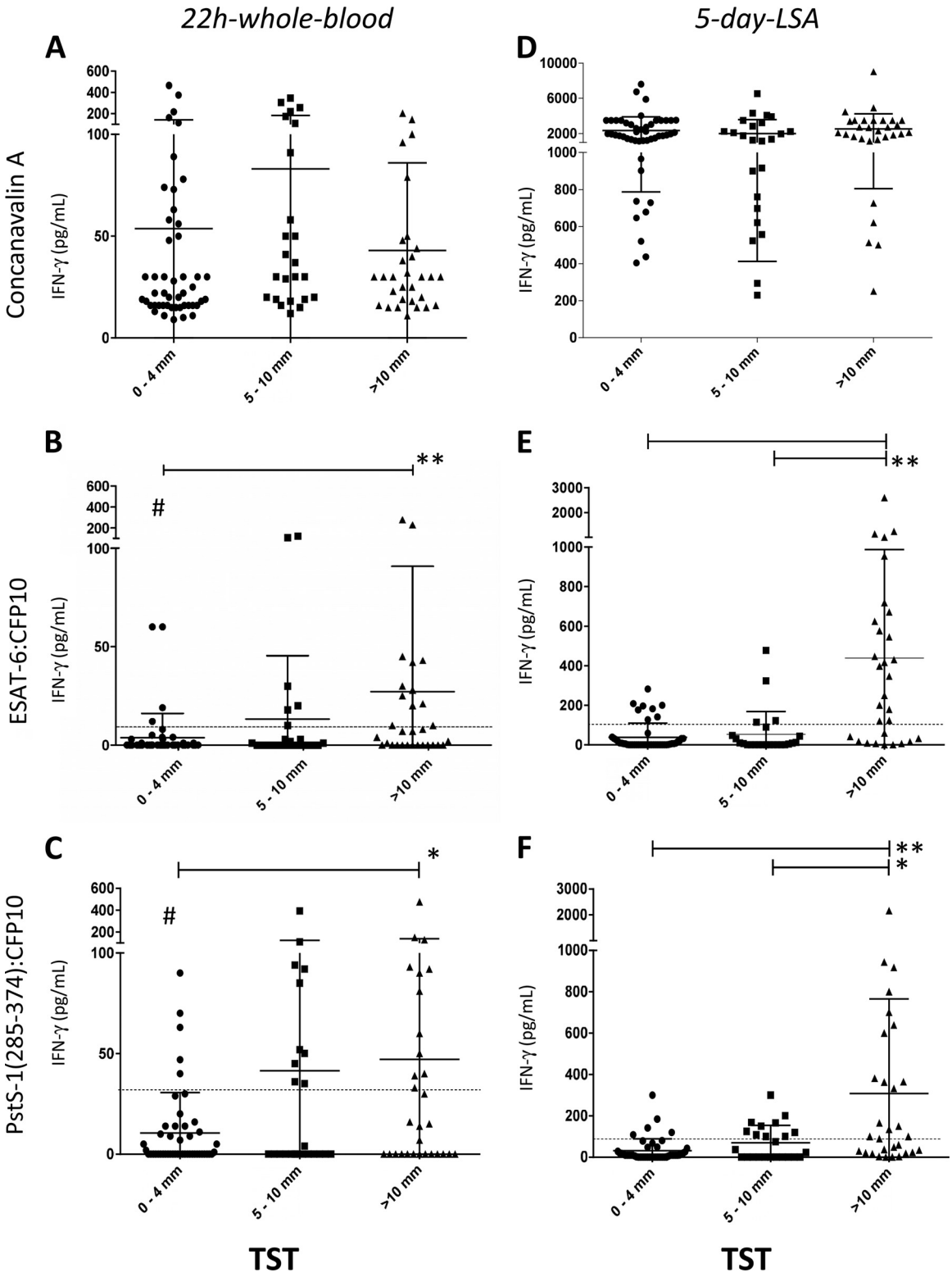

FIG 2 Gamma interferon profiling by whole-blood assays (WBA) and long-term stimulation assays (LSA) of recent close TB contacts stratified by TST size. IFN-γ levels were measured in pg/ml, and the results are represented by means ± standard deviations for each stimulus and TST group. Concanavalin A data (WBA, 53.7 ± 87.5, 83 ± 99.7, and 43 ± 43 pg/ml for induration of 0 to 4 mm, 5 to 10 mm, and more than 10 mm, respectively; LSA, 2,342.6 ± 1,554.8, 2,000.4 ± 1,588.7, and 2,530.8 ± 1,725.6 pg/ml) (A and D), ESAT-6:CFP10 data (WBA, 3.8 ± 12.4, 13.2 ± 32.2, and 27.1 ± 63.8 pg/ml; LSA, 38.7 ± 71.6, 53.8 ± 115, and 439.2 ± 548.6 pg/ml) (B and E), and PstS-1(285-374):CFP10 data (WBA, 10.5 ± 20.1, 41.5 ± 83.1, and 47.2 ± 91.5 pg/ml; LSA, 31 ± 56.9, 69.6 ± 83.7, and 307.8 ± 457.5 pg/ml) (C and F) were corrected for background levels. Dashed bars indicate the cutoff point(s) for each antigen (WBA, 10 pg/ml and 29.5 pg/ml; LSA, 100 pg/ml, respectively). *, $P < 0.05$; **, $P < 0.001$ (at the mean level of IFN-γ elicited for these antigens for each $TST^{0-4\ mm}$ group). #, $P = 0.030$ [at the mean level of IFN-γ elicited by ESAT-6:CFP10 × PstS-1(285-374):CFP10 in $TST^{0-4\ mm}$ group].

TABLE 2 Number and frequency of the concordant and discordant released gamma interferon results in WBAs and LSAs[a]

Antigens	$TST^{5–10\ mm}$ ($n = 24$) (%)		$TST^{11–14\ mm}$ ($n = 16$) (%)		$TST^{\geq 15\ mm}$ ($n = 14$) (%)		$TST^{negative}$ ($n = 48$) (%)	
	WBA	LSA	WBA	LSA	WBA	LSA	WBA	LSA
ESAT-6:CFP10^{negative}, PstS-1(285–374):CFP10^{negative}	13 (54.2)	13 (54.2)	10 (62.5)	8 (50)	7 (50)	1 (7.2)	42 (87.5)	38 (79.2)
ESAT-6:CFP10^{positive}, PstS-1(285–374):CFP10^{negative}	1 (4.2)	1 (4.2)	0 (0)	2 (12.5)		3 (21.4)	1 (2)	5 (10.4)
ESAT-6:CFP10^{positive}, PstS-1(285–374):CFP10^{positive}	5 (20.8)	3 (12.4)	5 (31.3)	5 (31.3)	7 (50)	10 (71.4)	3 (6.3)	3 (6.2)
ESAT-6:CFP10^{negative}, PstS-1(285–374):CFP10^{positive}	5 (20.8)	7 (29.2)	1 (6.2)	1 (6.2)			2 (4.2)	2 (4.2)

[a] Data represent results determined with both ESAT-6:CFP10 and PstS-1(285–374):CFP10 antigens, according to the tuberculin skin test (TST) stratification results for recent close contacts (rCt) of a TB index case.

DISCUSSION

The present report describes the evaluation of the newly constructed *M. tuberculosis*-derived PstS-1(285-374):CFP10 fusion protein by *in vitro* short- and long-term assays for IFN-γ production in a cohort of individuals recently exposed to a contagious TB index case in a tropical area of TB endemicity. Remarkable differences in the responses of the antigens were found in the TST results, including those determined with the commercially available IGRAs (ESAT-6 and CFP10), but also in the WBA and LSA results, with regard to our newly developed fusion protein. Importantly, however, the findings of our in-house assays demonstrated that the results determined for our new PstS-1(285-374):CFP10 fusion protein showed moderate-to-substantial agreement with the ESAT-6:CFP10 results along with a higher sensitivity in rCt $TST^{5–10\ mm}$ results in conjunction with detection of the participants that later developed active TB, all of which confirms the promise of PstS-1(285-374):CFP10 for *M. tuberculosis* infection immunodiagnostics.

Based on TST, several contact-tracking studies showed TBI incidence rates of around 20% to 50% (13–15). In the present population, including all recent close household and nonhousehold contacts, 54/102 (52.9%) were $TST^{positive}$. In WBAs, a lower positive frequency was observed for the antigens tested. In a study performed in The Gambia with similar characteristics but with a larger sample, 41% (300/735) tested positively in the TST whereas 30% did so in relation to an ESAT-6/CFP10 enzyme-linked immunosorbent spot (ELISPOT) assay (16). In our in-house study, similar results regarding ESAT-6:CFP10 (22% and 31.4%) and PstS-1(285-374):CFP10 (26.4% and 30.3%) for WBA and LSA, respectively, were observed.

As previously mentioned (17), there is no gold standard for LTBI detection and the TST, traditionally used as a reference, had

TABLE 3 Clinical and epidemiological variables associated with positivity in the tuberculin skin test and whole-blood stimulation and long-term stimulation assays with the ESAT-6:CFP10 and PstS-1(285–374):CFP10 antigens[a]

Factor	*n*	Odds ratio (95% CI)[b]		
		Association with WBA/LSA positivity		Association with TST positivity (*n*)
		ESAT-6:CFP10	PstS-1(285–374):CFP10	
Gender				
Female	73	0.8 (0.3–2.1)/3.0 (1.1–8.2)*	0.5 (0.2–1.2)/0.9 (0.4–2.2)	0.76 (0.3–1.8) (69)
Male	35	1.0 (reference)		(32)
Age				
≥45 yrs	59	2.6 (0.97–6.7)†/1.9 (0.8–4.2)	3.2 (1.3–8.1)*/2.9 (1.3–6.9)*	1.7 (0.8–3.7) (57)
<45 yrs	49	1.0 (reference)		(45)
Chest X ray				
Abnormal	10	8.7 (2.1–34.9)**/5.9 (1.4–24.6)*	3.4 (0.9–12.9)/24 (2.9–199.6)**	10.4 (1.3–85.7)* (10)
Normal	88	1.0 (reference)		(84)
BCG scar				
Present	80	0.4 (0.2–1.1)/0.5 (0.2–1.2)	0.8 (0.3–2.1)/0.6 (0.2–1.4)	1.2 (0.5–2.9) (76)
Absent	28	1.0 (reference)		(26)
Occupation				
Health care worker	9	3.6 (0.9–14.8)/1.8 (0.5–7.3)	4.4 (1.1–17.7)*/5.3 (1.3–22.9)*	8.2 (1.1–68.0)* (9)
Other	99	1.0 (reference)		(93)
Reported previous TB exposure				
Yes	21	3.2 (1.1–9.2)*/1.4 (0.5–3.9)	3.7 (1.4–10.3)*/2.5 (0.9–6.7)	1.9 (0.7–5.1) (20)
No	87	1.0 (reference)		(80)

[a] BCG, Bacillus Calmette-Guérin; LSA, long-term stimulation; TB, tuberculosis; TST, tuberculin skin test; WBA, whole-blood stimulation.
[b] †, $P = 0.055$; *, $P = <0.05$; **, $P = \leq 0.001$.

the same function here. It should, however, be kept in mind that the TST is not unbiased, strictly speaking, since it is based on the intradermal inoculation of PPD, a crude mixture of 200 antigens, many of which are shared by *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG along with several species of environmental mycobacteria (18). It is interesting that, despite the fact that our rCt individuals resided in an area of TB endemicity in which infant BCG vaccination is mandatory and were recent close contacts of a known contagious TB IC, 44% were $TST^{negative}$ and the majority did not develop any reaction to the PPD antigens (42/48; 87.5%) via TST. However, 4 rCt-$TST^{0\ mm}$ subjects produced detectable IFN-γ in the WBA with both antigen stimulations whereas the LSA elicited an IFN-γ response for 8 and 5 rCt under conditions of ESAT-6:CFP10 and PstS-1(285-374):CFP10 stimulation, in that order. Furthermore, Converse et al. (19) demonstrated that reactivity to PPD was detectable by the WBA among those with a negative TST. In addition, Cobat et al. (20) reported that the absence of TST reactivity among individuals that have experienced a high level of exposure to *M. tuberculosis* has a major human genetic component. Consequently, these individuals would presumably be more resistant to *M. tuberculosis* infection. Nonetheless, the possibility that some rCt $TST^{negative}$/$IGRA^{positive}$ individuals were infected cannot be completely ruled out. Also, it is well known that conversion of the TST response after infection with *M. tuberculosis* (usually referred as the "window period" [21]) may take up to 8 weeks; thus, it is possible that, due to recent exposure, some individuals might require more time to develop a response to PPD antigens during TST and might show false-negative results at the outset. Although none of those $TST^{negative}$/$IGRA^{positive}$ subjects developed active TB during the 12-month follow-up, in another study a contact with a similar TST/IGRA-RD1 response progressed to active TB approximately 24 months after being exposed to a TB IC (22). It is hoped that prolonged clinical evaluation would clarify these types of cases.

Lower TST specificity is found in populations with a high prevalence of BCG vaccination and contact with nontuberculous mycobacteria (NTM) (17). In contrast, however, there are studies showing that the clinical effect of NTM and of BCG on TST is usually minimal, except in settings of low TB prevalence, locations in which NTM is more commonly related to a moist climate, and whenever vaccinations were performed less than 10 years prior to TST (23). In our particular setting, the worst correlations between TST and IGRAs were observed in BCG-vaccinated rCt for the tested antigens ($\kappa \leq 0.29$; see Table S1 in the supplemental material). These data may suggest that the use of PPD leads to TST false-positive results in BCG-vaccinated individuals, which is in accordance with several studies demonstrating that TST positivity was significantly associated with prior BCG vaccination (23–34), having odds ratios ranging from 3.8 (95% CI, 1.0 to 13.9) (32) to 24.7 (95% CI, 11.7 to 52.5) (34) in various epidemiological contexts. In contrast, the IGRA agreement between the 2 antigens in WBA ($\kappa = 0.69$ to 0.75) and LSA ($\kappa = 0.48$ to 0.50) was not significantly affected by vaccination status, demonstrating that the new constructed PstS-1(285-374):CFP10 fusion protein, as well as ESAT-6:CFP10, may not be cross-reactive with the BCG strains used in Brazil.

Even so, the possibility of a cross-reactive response to 38-kDa peptides, which, in contrast to the RD1 antigens, are also encoded by a BCG genome, cannot be completely ignored. Nonetheless, the immune dominancy of the 38-kDa peptides in a TB infection has been reported (12, 35–39), and comparative analyses have demonstrated that there is a quantitative difference in the levels of expression of the 380-kDa protein in *M. bovis* versus *M. tuberculosis*. It has been found, for example, that the *M. bovis* soluble extract had to be at least 10 times more concentrated than the *M. tuberculosis* soluble extract to obtain the same level of binding via Western blotting and ELISA (36). Moreover, a *M. tuberculosis* 38-kDa peptide included in the PstS-1(285-374) protein used in the present study has been found to react at a higher level in LTBI than in active TB subjects (37–39). Additionally, in our data, no significantly positive TST association was revealed for BCG-vaccinated individuals (Table 3), which may be related to some special characteristic of the population under study such as age (mean, 41.1 ± 14.4 years), resulting in spanning a longer time period from vaccination to adulthood.

According to other authors (40–42), with respect to short-term (~1 day) incubation time periods, as used with our WBAs, only circulating effector memory T cells would have enough time to produce IFN-γ since central memory and resting T cells need a more prolonged incubation period to differentiate and begin IFN-γ production. Other factors related to infection status and intrinsic host-pathogen characteristics may directly contribute to the discrepancies found in the results determined for the antigens tested in both assays.

In our data, the PstS-1(285-374):CFP10 fusion antigen revealed improved detection rates among the $TST^{5–10\ mm}$ group members irrespective of the assay used since the rCt individuals detected by WBA were not always the same as those detected by LSA–PstS-1(285-374):CFP10. It has been reported that, in a setting (as in a cohort of individuals recently exposed to a contagious TB case) in which greater TBI sensitivity is required (as in the present study), shorter- and longer-term assays should preferably be performed and their combination should improve TBI detection (40). Furthermore, it is also possible that the newly fused antigen developed here is recognized by a restricted number of circulating effector cells and other T cell populations and is, therefore, more suitable for detecting TBI among recently exposed individuals with $TST^{5–10\ mm}$. Thus, positive improvement in this group is of great value for TBI detection since it has been demonstrated that while a threshold induration size of over 5 mm does not accrue specificity for the skin test, it does lower the sensitivity of active TB detection (43).

Some clinical and epidemiological data, such as those of Kleinert et al. (44), who showed that chest X-ray lesions suggestive of latent or prior TB are associated with TST and commercial IGRA positivity, were available for analysis. The present study also found that chest X-ray abnormalities were the best predictors of WBA and LSA reactivity in both antigens tested. Older individuals were significantly associated with positive IFN-γ production in both assays for the new fusion. Since the participants were natives of an area of TB endemicity, advanced age might have been a factor in increasing their chances of encountering *M. tuberculosis* bacilli. Also, similar data were reported in a Canadian TB clinic using commercial IGRAs in immigrant screening (45).

With regard to smoking habits, existing literature has shown higher TST reactivity among smokers, which is in general agreement with our results, although no statistical significance in the odds ratio analyses was found. In a study that included HIV-infected individuals, Oni et al. (46) showed that smokers had lower IGRA reactivity. This finding may be related to their immunode-

ficiency, since, among our rCt-HIV^{negative} subjects, higher mean IFN-γ production was detected for all antigens, although a significant difference was achieved only by the TST and LSA–PstS-1(285-374):CFP10 (data not shown). Lastly, although the results have not yet been reported, our study found a significant association between female sex and LSA–ESAT-6:CFP10 positivity ($P < 0.05$), a result which may have been influenced by the high number of female participants.

Few studies have examined the positive and negative predictive values of IFN-γ production assays among LTBI for further conversion to active TB (19, 47). In our study, the TST PPV was similar to that determined in other studies but the values determined by WBA for both antigens (7.4% and 7.7%) and the value determined by LSA–PstS-1(285-374):CFP10 (6.1%) were lower in the absence of any change in NPV. Diel et al. (27) reported a remarkable PPV, in children as well as adults, using commercial IGRA (QFT-IT) in a low-burden setting of recent TB contacts (14.6%) related to TST (2.3%; cutoff = 5 mm). It is important that, in the present study, rCt-TST^{positive} individuals were more likely to accept IPT. Moreover, most (87%) completed treatment regardless of the bacillary load of their active TB index cases. As a result, it could be concluded that this pharmacological intervention contributed to jeopardizing the PPV and NPV analyses.

To our knowledge, this is the first time that a 38-kDa peptide epitope has been successfully fused to CFP10 and used to assess natural *M. tuberculosis* infection in humans. We believe we have demonstrated that the overall IFN-γ responses in the WBAs were similar with regard to the tested *M. tuberculosis* antigens. We also confirmed that the results of LSA performed with ESAT-6:CFP10, including PstS-1(285-374):CFP10, slightly improved positivity among the TST^{positive}/WBA^{negative} participants. Despite the fact that RD1 antigens have been described as more specific, they are less sensitive than TST at the moment of *M. tuberculosis* infection diagnoses. In these cases, recombinant PstS-1(285-374):CFP10 provides slightly more sensitivity, mainly with regard to TST$^{5–10\ mm}$, in both assays. Thus, there is still an urgent need to discover new antigens that add accuracy for the traditional RD1-IGRAs. Nonetheless, in the belief that the most adequate "gold standard" for LTBI remains the consecutive development of TB in infected individuals, the findings of this study need to be further evaluated in future prospective longitudinal cohort studies that may include a far larger number of recent close TB contacts.

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ERRATUM

Erratum for Araujo et al., Evaluation of Gamma Interferon Immune Response Elicited by the Newly Constructed PstS-1(285-374):CFP10 Fusion Protein To Detect *Mycobacterium tuberculosis* Infection

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Volume 21, no. 4, p. 552–560, 2014. Page 553, column 1, line 36: "5 IU" should read "2 tuberculin units."

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