UNIVERSITAT AUTÒNOMA DE BARCELONA

Facultat de Medicina i Cirurgia
Departament de Medicina

APLICABILITAT CLÍNICA DE LES TÈCNIQUES DE DETECCIÓ *IN VITRO* DE L'INTERFERÓ-GAMMA EN LA INFECCIÓ I LA MALALTIA TUBERCULOSA

Maria Luiza de Souza Galvão

2014

ANNEX III

Available online at www.sciencedirect.com
ScienceDirect
Diagnostic Microbiology and Infectious Disease 65 (2009) 236–246
DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE
www.elsevier.com/locate/diagmicrobio

Quantitative evaluation of T-cell response after specific antigen stimulation in active and latent tuberculosis infection in adults and children☆

Irene Latorre[a,c,d], Malú De Souza-Galvão[c,e], Juan Ruiz-Manzano[b,c,d], Alicia Lacoma[a,c,d], Cristina Prat[a,c,d], Loreto Fuenzalida[a,c], Neus Altet[e], Vicente Ausina[a,c,d], Jose Domínguez[a,c,d,*]

[a]*Servei de Microbiologia, Hospital Universitari "Germans Trias i Pujol", Fundació Institut en Ciències de la Salut Germans Trias i Pujol, Badalona, Spain*
[b]*Servei de Pneumologia, Hospital Universitari "Germans Trias i Pujol", Fundació Institut en Ciències de la Salut Germans Trias i Pujol, Badalona, Spain*
[c]*Universitat Autònoma de Barcelona, Bellaterra, Spain*
[d]*Ciber Enfermedades Respiratorias, Badalona, Spain*
[e]*Unidad de Prevención y Control de la Tuberculosis de Barcelona, Spain*

Received 27 February 2009; accepted 10 July 2009

Abstract

We have evaluated the quantitative T-cell response after specific *Mycobacterium tuberculosis* antigen stimulation in active tuberculosis (TB) and latent TB infection (LTBI) patients. In adults, the median number of T cells after RD1 antigen stimulation was significantly higher in active TB patients than in LTBI patients. In children, the number of responder T cells against the specific antigens was higher in active TB than in LTBI patients, although the differences were not significant. In summary, in patients with suspected clinical TB, although there is overlapping in the number of responder T cells between both groups, a T-cell count above the described threshold could suggest active TB, especially in patients with a high probability of having active TB and low probability of having LTBI. In addition, the results are consistent with the current evidence that T-cell response may indicate mycobacterial burden and disease activity.

Keywords: IFN-γ tests; Active TB; LTBI; Children; Mycobacterial burden

1. Background

Tuberculosis (TB) remains a serious public health problem. Approximately 9 million people develop active TB, mostly pulmonary TB (pTB), annually (Dye et al., 2006). The basis of TB control programs consists of diagnosing and correcting the treatment of patients with active TB. An essential factor for controlling the spread of this disease is the ability to diagnose it in its early stages, especially in the pediatric population. The traditional tools for diagnosing clinical TB are still clinical and radiologic examination, combined with direct microscopic examination of sputum samples and culture of bacteria. Patients with pTB may be smear-negative for acid-fast bacilli, and mycobacterial culture may take several weeks. Therefore, diagnosis often occurs in an advanced stage of the disease (Dinnes et al., 2007). Although in vitro amplification of mycobacterial targeted DNA via polymerase chain reaction-based methods can provide a rapid answer, the technology is not fully standardized and is often unsuitable for routine clinical practice. Moreover, appropriate specimens are difficult to obtain in young children because of the fact that they rarely produce sputum. In addition, there is a high proportion of extrapulmonary TB that often requires invasive diagnostic methods (Liebeschuetz et al., 2004; Migliori et al., 2006; Valdés et al., 1998).

The tuberculin skin test (TST) has been, until now, the only tool available for the diagnosis of latent TB infection (LTBI) and is commonly used as a complementary test in the diagnosis of active TB (Huebner et al., 1993). Unfortunately, the disadvantage of this test is that its poor specificity can lead to false-positive results by means of a cross-reaction

☆ None of the investigators have relevant financial interest in or a financial conflict with the subject matter or materials discussed in this manuscript.

* Corresponding author. Servei de Microbiologia, Fundación Institut en Ciències de la Salut "Germans Trias i Pujol", Carretera del Canyet s/n, 08916 Badalona, Barcelona, Spain. Tel.: +34-93-497-88-94; fax: +34-93-497-88-95.
E-mail address: jadomb@gmail.com (J. Domínguez).

0732-8893/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.diagmicrobio.2009.07.015

with the *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) vaccine strain and non-tuberculous mycobacteria (NTM) (Latorre et al., 2009). Moreover, TST has a low sensitivity in high-risk groups with impaired cellular immunity, such as children younger than 5 years, giving false-negative results (Horsburgh, 2004).

In the last years, 2 *Mycobacterium tuberculosis* (MTB) region of difference (RD) 1 antigens have been explained. These antigens are early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), which are absent in all BCG and in the majority of NTM strains (Andersen et al., 2000). Recently, a third specific MTB antigen, TB7.7 (Rv2654 [only peptide 4]), encoded in RD11 has also been studied (Brock et al., 2004). In vitro assays for the diagnosis of LTBI, based on the detection of interferon-γ (IFN-γ) secreted by effector T cells stimulated with these specific antigens, have been evaluated by several authors in the diagnosis of LTBI and active TB (Domínguez et al., 2009b; Ewer et al., 2003; Lalvani, 2007; Lalvani et al., 2001; Menzies et al., 2007; Mori et al., 2004; Pai et al., 2008; Richeldi, 2006).

Three commercialized in vitro assays based on this technology have been developed: *QuantiFERON-TB GOLD* (QFT-G), *QuantiFERON-TB GOLD In-Tube* (QFT-G-IT) (Cellestis, Carnegie, Australia), and T-SPOT.TB (Oxford Immunotec, Oxford, UK). QFT-G and QFT-G-IT detect IFN-γ from T cells stimulated with the specific antigens in whole blood. On the contrary, T-SPOT.TB stimulates the specific mononuclear cells isolated from peripheral blood (PBMCs). QFT-G-IT is the last version of QFT-G. The main difference between these 2 tests is that QFT-G-IT incorporates the 2 RD1-specific antigens and the TB7.7 antigen inside the same blood collection tube.

Nowadays, the IFN-γ assays for the diagnosis of LTBI cannot distinguish between active TB and LTBI. However, it has been hypothesized that the magnitude of the response may provide information such as the likelihood of transition to latency versus disease (Doherty et al., 2002). In this regard, there are 2 studies that have suggested the variation cutoff values of spot-forming units (SFUs) on T-SPOT.TB and the concentration of IFN-γ (IU/mL) on QFT-G. These studies improve the diagnostic accuracy of LTBI to distinguish active TB from LTBI (Janssens et al., 2007; Soysal et al., 2008).

The aim of this study is to evaluate the quantitative difference of *M. tuberculosis*-specific antigen T-cell response in the adult and pediatric populations by T-SPOT.TB and QFT-G-IT, to differentiate between subjects with active TB and those with LTBI and, secondly, to correlate the quantitative response of T cells with TB immunodiagnosis, mycobacterial burden, and disease activity.

2. Materials and methods

2.1. Patients and inclusion criteria

We prospectively recruited adult and pediatric patients between September 2004 and January 2008 who attended the *Hospital Universitari Germans Trias i Pujol* (Badalona, Spain) and the TB Control and Prevention Unit of Barcelona (CAP Drassanes, Barcelona, Spain) with suspected active TB or LTBI. An ethics approval for this study was provided by the Hospital Universitari *Germans Trias i Pujol* and the Fundació Gol i Gurina (Barcelona, Spain) Ethics Committees. We obtained written informed consent from all patients before taking blood samples, and in the case of the pediatric population, parents signed the written consent. A detailed questionnaire from all patients was collected to indicate the results of any previous TSTs, as well as information on BCG vaccination status, details of any contact with a person that has active TB, history of prior active TB, LTBI, and chest radiography, and other medical conditions (i.e., immunosuppression status). In our study, only participants with a BCG scar were considered BCG vaccinated. Patients diagnosed with active TB and those diagnosed with LTBI were included. Of the LTBI patients, we established 2 groups: individuals with a high risk of recent exposure (contact-tracing studies group) and individuals infected but with unknown exposure to an active TB patient. In this last group, it is not possible to eliminate the possibility that some of them were recently infected. In the case of children, the infection, by definition, should be considered as recent. In LTBI patients, the active TB was excluded by clinical and radiologic examination. None of the patients included in this study were HIV seropositive.

The inclusion criteria for all patients were a positive TST, at least 1 positive result for one of the IFN-γ tests studied, and no more than 2 weeks of therapy or chemoprophylaxis when taking blood samples.

We enrolled a total of 175 adult and 162 pediatric patients with a positive TST. Of these patients, we excluded 44 adults and 79 children from the analysis because of negative and/or indeterminate results for both IFN-γ tests. The patients included in the study were classified into 3 groups:

Group 1. Forty adult and 13 pediatric patients diagnosed with active pulmonary and extrapulmonary TB. The inclusion criteria were a positive culture for MTB for adult patients and, for pediatric population, a positive culture for MTB or suspected active clinical TB related to close contact with a smear-positive pTB patient, radiography compatible with TB, and a positive TST.

Group 2. Fifty-six adults and 52 children enrolled during contact-tracing studies as close contacts of a smear-positive pTB case.

Group 3. Thirty-five adults and 18 children examined for LTBI screening because of recent immigration from countries with a high prevalence of TB infection, homelessness, school teachers enrolled during preemployment examinations, or children enrolled during routine TB screening at school.

2.2. Tuberculin skin test

In all subjects studied for contact-tracing studies or screened for LTBI, a TST was performed. Two

intradermal tuberculin units of PPD RT23 (Statens Serum Institut, Copenhagen, Denmark) were used to perform the TST. The tuberculin was administered using the Mantoux method, and the induration size was interpreted after 48 to 72 h by trained personnel. In this study, according to the Spanish Society of Pneumology and Thoracic Surgery guidelines, TST indurations ≥5 mm were classified as positive in patients diagnosed with active TB and contacts. On the contrary, indurations ≥10 mm were considered positive in patients screened for LTBI (Ruiz-Manzano et al., 2008). All individuals with a positive TST induration were referred for chest radiography, and chemoprophylaxis was indicated in asymptomatic patients with normal chest radiography.

2.3. QuantiFERON-TB GOLD In-Tube

The assay is based on the detection of IFN-γ in whole-blood supernatant by an enzyme-linked immunosorbent assay. The test was performed according to the manufacturer's instructions. A total of 3 tubes of 1 mL each (nil control, positive control [phytohemagglutinin], and TB-specific antigens) were drawn by venipuncture from each patient.

Raw optical densities were interpreted by using specific software provided by the manufacturer. The result obtained by the nil control had to be subtracted from the positive control and the antigen-stimulated samples. The cutoff value for a positive test was at least 0.35 IU/mL of IFN-γ in the sample after stimulation with the specific antigens, regardless of the result of the positive control. The result of the test was considered indeterminate if an antigen-stimulated sample was negative and if the value of the positive control was less than 0.5 IU/mL after subtraction of the value of the nil control.

2.4. T-SPOT.TB

The assay detects IFN-γ produced by PBMCs by means of an enzyme-linked immunospot assay (ELISPOT), stimulated with ESAT-6 and CFP-10 individually. The test was performed according to the manufacturer's instructions. Eight milliliters of blood were drawn for the isolation of PBMC in a vacutainer CPT tube (Becton Dickinson Diagnostics, Franklin Lakes, NJ).

On T-SPOT.TB, spots were scored using an automated AID ELISPOT plate reader (Lector AID Elispots; Autoimmun Diagnostiks, Germany). All readings were also manually verified. Subjects were considered positive if there was a positive response to 1 or both of the antigen panels. Test wells were scored as positive if they contained at least 6 SFU cells more than the nil control well and if this number was at least twice the number of the nil control well. The assay result was considered indeterminate if the number of spots in the positive control was less than 20 and if the response to both of the antigen panels was negative. We also studied the overall RD1 response as the sum of ESAT-6 and CFP-10 T-cell enumeration.

2.5. Statistical methods

The comparison of the number of spots and the IFN-γ released was performed using the Mann–Whitney U test analysis. Data were expressed as mean and SD, median, and 5th and 95th percentiles. Differences were considered significant when P values were less than 0.05. Receiver operating characteristic (ROC) analysis was performed to determine a threshold level for differentiating between active TB and LTBI. The best threshold of SFU enumeration by T-SPOT.TB and international units per milliliter of IFN-γ by QFT-G-IT was given with an optimal specificity equal to or higher than 80%. All analyses were made with SPSS statistical software for Windows (SPSS version 15.0; SPSS, Chicago, IL). Graphical representation is based on GraphPad Prism version 4 (GraphPad Software, San Diego, CA).

3. Results

The main demographic characteristics of adult and pediatric patients and the positive percentages of T-SPOT.TB and QFT-G-IT of the populations included in the study are shown in Table 1. The percentages of positive results in these patients were higher for T-SPOT.TB than for QFT-G-IT, except for contact-tracing studies in the pediatric population where QFT-G-IT showed a slightly higher percentage of positive results than T-SPOT-TB. Moreover, in contact-tracing studies of adult individuals and active TB pediatric patients, the differences between T-SPOT.TB and QFT-G-IT positive results were significant ($P < 0.001$ and $P = 0.046$, respectively).

3.1. Adult population results

The median numbers of T cells after ESAT-6, CFP-10, and RD1 stimulation in T-SPOT.TB were higher in active TB patients than in contact-tracing studies individuals or those screened for LTBI. In contrast, the median IFN-γ release after antigen stimulation in QFT-G-IT was higher in contacts than in the other group of patients (Table 2 and Fig. 1).

Differences in the number of T cells stimulated with ESAT-6 by T-SPOT.TB in active TB patients, contact-tracing studies, and screening of LTBI individuals were only significant when comparing active TB patients with individuals screened for LTBI ($P = 0.039$). In contrast, the differences in the number of T cells stimulated with CFP-10 were significant between active TB patients and contact-tracing individuals and also those screened for LTBI ($P = 0.018$ and $P = 0.01$, respectively). Finally, when we evaluated both antigens together (RD1 response), the differences for active TB patients compared with contact patients and patients screened for LTBI were also significant ($P = 0.042$ and $P < 0.001$) (Fig. 1).

Table 1
Demographic characteristics of adult and pediatric patients studied

	Adult population			Pediatric population		
	Active TB, *n* = 40 (%)	Contact tracing, *n* = 56 (%)	LTBI screening, *n* = 35 (%)	Active TB, *n* = 13 (%)	Contact tracing, *n* = 52 (%)	LTBI screening, *n* = 18 (%)
Sex						
Male	27 (67.5)	29 (51.8)	21 (60)	10 (76.9)	26 (50)	9 (50)
Female	13 (32.5)	27 (48.2)	14 (40)	3 (23.1)	26 (50)	9 (50)
Age, mean ± SD	37.60 ± 15.93	36.20 ± 17.38	32.39 ± 6.16	10.85 ± 3.69	9.37 ± 3.63	10.67 ± 4.19
BCG vaccinated						
Yes	8 (20)	22 (39.3)	18 (51.4)	4 (30.8)	34 (65.4)	12 (66.7)
No	32 (80)	34 (60.7)	17 (48.6)	9 (69.2)	18 (34.6)	6 (33.3)
Immunosuppression						
Yes	0 (0)	0 (0)	2 (5.7)	0 (0)	0 (0)	0 (0)
No	40 (100)	56 (100)	33 (94.3)	13 (100)	52 (100)	18 (100)
Birth country						
Immigrants from countries with high prevalence of TB infection	20 (50)	23 (41.1)	24 (68.6)	4 (30.8)	35 (67.3)	14 (77.8)
Residents in a non-epidemic TB country	20 (50)	33 (58.9)	11 (31.4)	9 (69.2)	17 (32.7)	4 (22.2)
IFN-γ tests						
Positive T-SPOT.TB	37/39 (94.9)	54/56 (96.4)	31/34 (91.2)	12/13 (92.3)	46/52 (88.5)	15/17 (88.2)
Positive QFT-G-IT	34/40 (85)	34/54 (63)	27/35 (77.1)	9/12 (75)	43/48 (89.6)	13/16 (81.3)

There were no significant differences for the IFN-γ released in QFT-G-IT between the different groups of patients (Fig. 1).

To determine an ESAT-6, CFP-10, and RD1 responder T-cell enumeration threshold in T-SPOT.TB, and an IFN-γ release level threshold in QFT-G-IT to differentiate between active TB and LTBI, we identified the areas under the ROC curve in adult patients as 0.599 (95% confidence interval [CI], 0.489–0.710), 0.677 (95% CI, 0.571–0.783), 0.668 (95% CI, 0.563–0.772), and 0.504 (95% CI, 0.387–0.621), respectively (Fig. 2). Although there was overlapping, we could define a threshold to differentiate between active TB and LTBI (Table 3). The optimal threshold was 69 CFP-10 T cells, with a sensitivity of 45.9% and a specificity of 81.2%. In contrast, the best threshold point was 116 RD1 T cells with a sensitivity of 43.2% and a specificity of 81.2%.

3.2. Pediatric population results

In the pediatric population, the median numbers of ESAT-6, CFP-10, and RD1 T cells in T-SPOT.TB and the median IFN-γ release in QFT-G-IT were higher in active TB individuals than in contact-tracing studies or children screened for LTBI (Table 2 and Fig. 3).

The differences in the number of ESAT-6, CFP-10, and RD1 T cells stimulated in T-SPOT.TB and the IFN-γ released in QFT-G-IT between the 3 groups of pediatric patients were not significant (Fig. 3).

Table 2
T-cell enumeration for *T-SPOT.TB* after antigen stimulation and IFN-γ release determined by *QFT-G-IT* in the different groups of patients

Group of patients	Adult population					Pediatric population				
	n	Mean	SD	Median	5–95 percentile	*n*	Mean	SD	Median	5–95 percentile
T-cell enumeration after ESAT-6 stimulation										
Active TB	37	37.95	35.62	36.00	0.90–17.50	12	50.25	52.81	32.00	1.00–152.40
Contact tracing	54	35.31	47.42	11.50	1.00–149.00	46	37.78	41.04	18.00	1.00–128.05
LTBI screening	31	20.13	23.00	12.00	0.20–79.20	15	27.87	31.52	20.00	5.00–87.40
T-cell enumeration after CFP-10 stimulation										
Active TB	37	65.14	53.00	54.00	1.80–192.80	12	45.33	31.64	40.50	4.00–85.10
Contact tracing	54	42.03	42.02	25.00	1.00–144.75	46	46.09	41.66	29.50	2.35–140.40
LTBI screening	31	27.26	27.26	15.00	2.00–91.80	15	41.00	36.12	22.00	4.00–103.40
T-cell enumeration after RD1 stimulation										
Active TB	37	103.08	70.85	87.00	10.70–247.70	12	95.58	65.26	91.50	17.00–213.10
Contact tracing	54	77.33	76.45	50.50	9.75–269.50	46	83.87	72.05	62.00	8.35–224.65
LTBI screening	31	47.39	44.89	26.00	8.00–154.00	15	68.87	42.08	66.00	13.00–136.80
IFN-γ release after antigen stimulation in QFT-G-IT										
Active TB	34	7.74	11.43	3.99	0.66–2.15	9	13.04	18.68	5.92	0.36–21.34
Contact tracing	34	17.71	48.95	6.12	0.37–105.09	43	10.43	21.11	3.55	0.45–35.86
LTBI screening	27	6.56	13.41	2.11	0.42–49.78	13	7.69	8.11	4.68	0.47–23.71

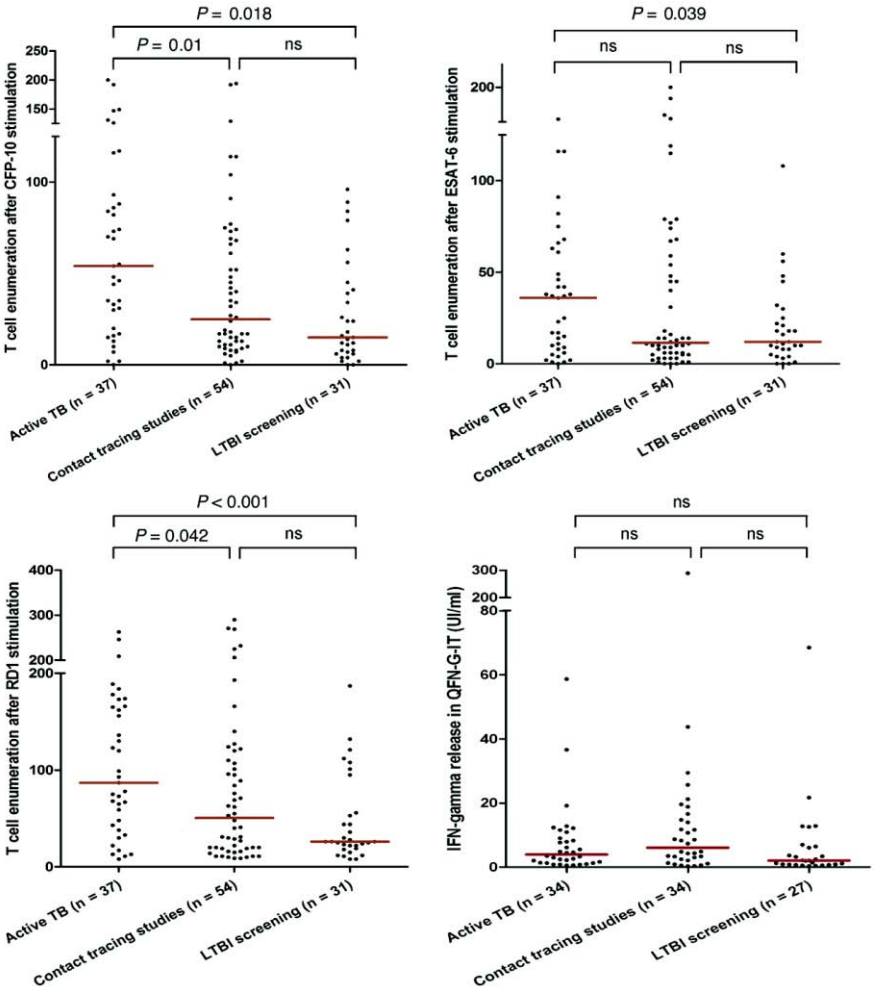

Fig. 1. T-cell enumeration and IFN-γ release after antigen stimulation in the different groups of adults.

The areas under the ROC curve for ESAT-6, CFP-10, RD1, and IFN-γ release were 0.555 (95% CI, 0.355–0.755), 0.534 (95% CI, 0.371–0.697), 0.596 (95% CI, 0.436–0.757), and 0.517 (95% CI, 0.282–0.752), respectively (Fig. 4).

The best threshold point was 65 ESAT-6 T cells, with a sensitivity of 33.3% and a specificity of 80%. However, when we studied RD1 response, the best threshold point was 145 RD1 T cells with a sensitivity of 25% and a specificity of 82%. The cutoff point determined for CFP-10 had a low sensitivity (16.7%) in this population. For QFT-G-IT, we determined 17.10 IU/mL of IFN-γ released with a sensitivity of 33.3% and a specificity of 83.9% (Table 3).

4. Discussion

Although the new generation of IFN-γ immune-based assays have been designed to diagnose LTBI, they have also

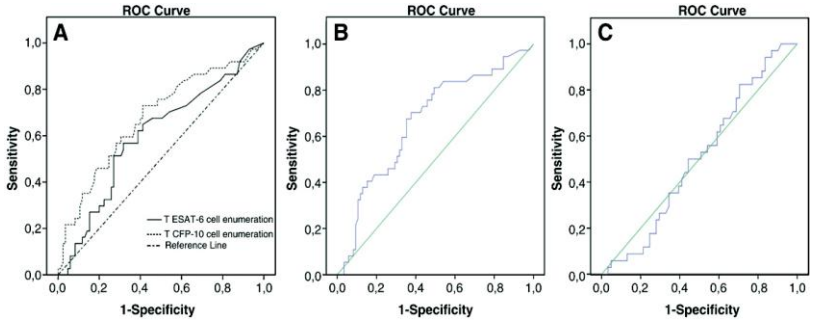

Fig. 2. ROC curve for (A) ESAT-6, CFP-10, (B) RD1 T-cells enumeration, and (C) IFN-γ release between adults with active TB and LTBI.

been explored for use in the diagnosis of active TB, reporting sensitivities between 70% and 90% (Arend et al., 2007; Detjen et al., 2007; Dominguez et al., 2008; Domínguez et al., 2009a; Ferrara et al., 2006; Kang et al., 2007; Lee et al., 2006; Rangaka et al., 2007). Nevertheless, using the cutoff point defined by the manufacturers, IFN-γ tests cannot distinguish between LTBI and active TB when they are performed on blood. We present the results of a study that was designed to evaluate the quantitative specific ESAT-6, CFP-10, and TB7.7 T-cell response in the adult and children populations by means of T-SPOT.TB and QFT-G-IT.

To establish a cutoff point to differentiate between active TB and LTBI, we have assessed the best sensitivity for ROC curve in each case, considering an optimal specificity equal to or higher than 80%. Given that the potential use of these tests would be as a complementary tool for diagnosing active TB patients without any microbiological findings, it is necessary to define a cutoff point that makes the test more specific to avoid false-positive results. However, this consideration could be adjusted according to the prevalence of the *M. tuberculosis* infection in each area. The main problem with using IFN-γ assays to diagnose active TB is their poor specificity for disease. Therefore, when active TB is suspected in adults from countries with a high incidence of TB, as well as high-risk populations in countries with a low incidence of TB (foreign-born or close contacts), the specificity of the tests is very low. Therefore, the positive predictive value will also be low. On the contrary, in patients at very high risk of disease (suspected clinical and radiologic active TB), but with a low probability of latent infection (low-risk populations from low-incidence countries), the positive predictive value is high. Positive IFN-γ assays are very useful in the diagnosis of active TB, especially the positive results above the defined thresholds (Menzies, 2008). In our study, the 14.3%, 61.9%, and 42.9% of adult patients with smear-negative active TB obtained a T-cell count above the ESAT-6, CFP-10, and RD1 described thresholds, respectively.

The overall results show a higher number of positive results for T-SPOT.TB than QFT-G-IT. This is consistent with the results reported previously (Chee et al., 2008b; Domínguez et al., 2009a; Ferrara et al., 2006; Goletti et al., 2006; Kang et al., 2007; Lee et al., 2006). In our experience, in adult patients, the number of responder T cells after RD1 antigens stimulation was significantly higher in active TB than in LTBI patients. However, there is a large amount of overlapping that makes it difficult to distinguish active TB from LTBI.

Fox et al. (2007) studied ESAT-6 and CFP-10 responses for T-SPOT.TB in 183 smear-positive TB cases and 1673 household contacts, finding a higher CFP-10 response in TB cases, indicating that CFP-10 could be an indicator of active TB. They hypothesized that CFP-10 could reflect the number and nature of T-cell epitopes and that CFP-10 responses in

Table 3
Optimal threshold level for ROC curve analysis, sensitivity and specificity in adult and pediatric patients

	Adult population			Pediatric population		
	Threshold[a]	Sensitivity (%)	Specificity (%)	Threshold[a]	Sensitivity (%)	Specificity (%)
T-SPOT.TB						
ESAT-6	61	27	84.7	65	33.3	80
CFP-10	69	45.9	81.2	82.5	16.7	80.3
RD1	116	43.2	81.2	145	25	82
QFT-G-IT						
IFN-γ released	19.04	8.8	86.9	17.10	33.3	83.9

[a] SFU enumeration for T-SPOT.TB and international unit per milliliter of IFN-γ for QFT-G-IT giving an optimal specificity equal to or higher than 80%.

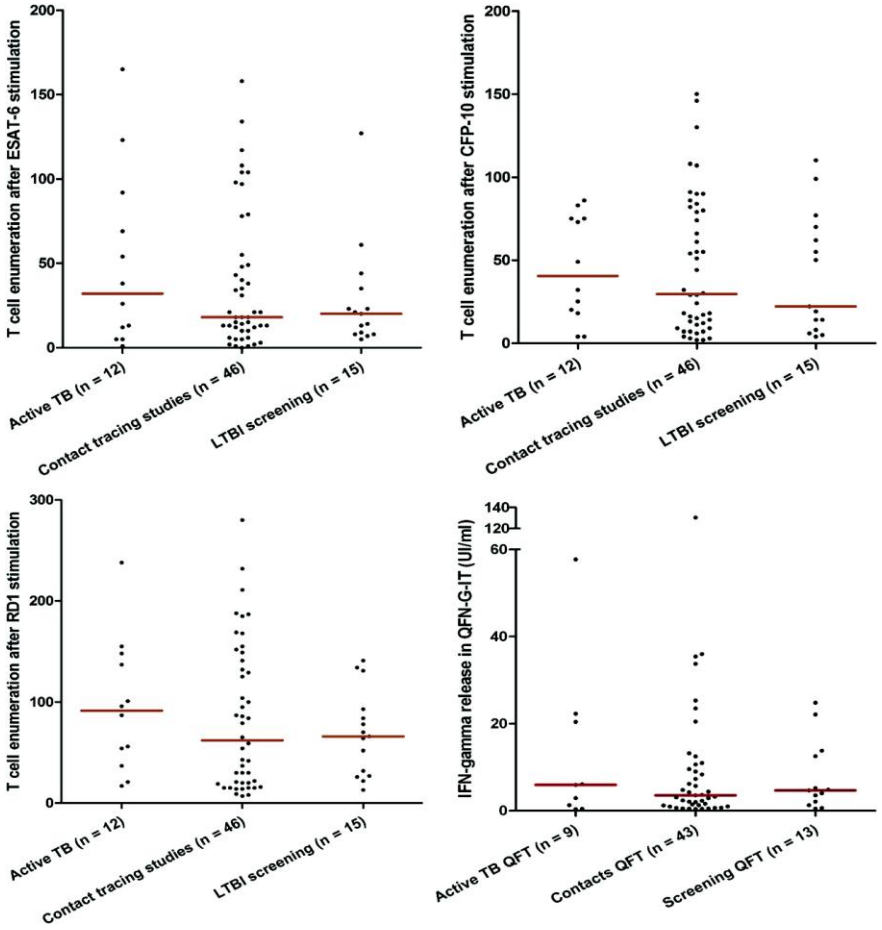

Fig. 3. T-cell enumeration and IFN-γ release after antigen stimulation in the different groups of children.

active TB patients could be more reliable than ESAT-6 responses in CD8−T cells as regards contacts. In our study, the enumeration of responder T cells after stimulation with CFP-10 yielded significant differences between active TB and the other 2 groups of LTBI patients, whereas responder T cells after stimulation with ESAT-6 only yielded differences between active TB and screened LTBI patients. In our experience, a cutoff point of 69 CFP-10 T cells provides a sensitivity of 45.9% with a specificity of 81.2%.

Recently, studying the T-cell response, Kobashi et al. (2008) explained that the quantitative responses to CFP-10 decrease during the treatment of active TB, and that it might be useful as a monitoring marker of clinical efficacy. We have also stated (Domínguez et al., 2009a) that the number of CFP-10 responder T cells decreases during the treatment when compared with the beginning of the therapy, although in our experience, the difference is not significant. However, the response against CFP-10 at the end of the treatment increases, achieving similar values to the one obtained before treatment.

Janssens et al. (2007) evaluated the quantification of RD1 antigen responder T cells for T-SPOT.TB to differentiate active TB from LTBI. They studied contacts with a positive T-SPOT.TB and patients diagnosed with active TB. By ROC

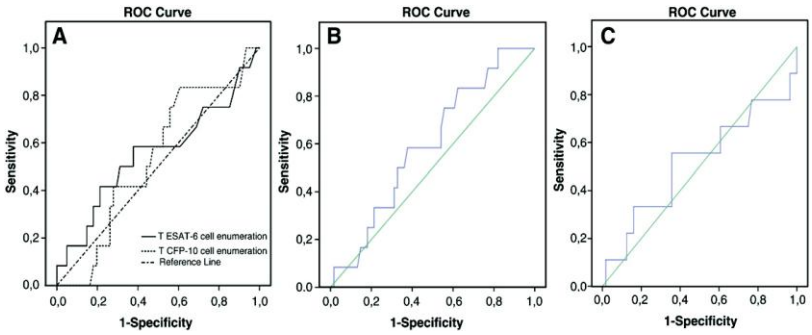

Fig. 4. ROC curve for (A) ESAT-6, CFP-10, (B) RD1 T-cells enumeration, and (C) IFN-γ release between children with active TB and LTBI.

curve analysis, they could establish a threshold value of 49.5 SFUs, with a sensitivity of 83% and a specificity of 74%. Nevertheless, they concluded that the test could not be recommended for diagnosing active TB. The difference in the threshold value between our result and the study by Janssens et al. could be explained by differences in the study population. To calculate the threshold value, Janssens et al. used patients from contact-tracing studies with both positive and negative TST results. As the authors reported, the mean SFU number obtained by negative TST contact patients was lower than that obtained by positive TST contact patients. Therefore, when both groups of patients (negative and positive TST) are analyzed together, the threshold value is lower than if only the positive TST patients are considered. We have proposed as optimum a threshold of 116 RD1 spots with a sensitivity of 43% and a specificity of 81% in adult patients. In our study, using a threshold of 50 SFUs, the sensitivity (70.3%) and the specificity (56.5%) were slightly lower than in the study by Janssens et al.

Recently, Chee et al. (2008a) also compared the quantitative T-cell response measured by the 2 available tests to distinguish active TB from LTBI. They reported that the median number of SFU in response to ESAT-6 and CFP-10 was significantly higher in active TB patients than in LTBI patients; in contrast, the median IFN-γ level of the QFT-G-IT showed no significant differences between the 2 groups. The authors noticed a large degree of overlapping in the range of values obtained by patients diagnosed with active TB or LTBI.

In the adult population, the IFN-γ released by QFT-G-IT did not show significant differences between active TB and LTBI patients. Indeed, the IFN-γ released was higher in contacts than in active TB patients. This result is probably related to the fact that patients with active TB have a low frequency of circulating specific IFN-γ–secreting T cells on peripheral blood (Pathan et al., 2001) because of a sequestration of specific T cells to the site of active TB (Barnes et al., 1993; Hirsch et al., 1999) and because of the fact that IFN-γ released in the QFT-G-IT methodology is diluted in the overall volume of blood. In contrast, T-SPOT.TB is always performed with a standardized number of T cells.

In the pediatric population, both tests have a higher number of responder T cells against the specific antigens and a higher level of IFN-γ released in active TB cases than in LTBI cases, although the differences were not significant. In all cases, the ROC curve analysis offers a very low sensitivity. The absence of significant differences in the response between active TB and LTBI could be explained by the fact that pediatric infection is usually recent. Therefore, the response is still strong, being similar to the one obtained in an active TB. This could explain the slight differences between contacts and screened children. This observation is consistent with the current evidence that T-cell response may indicate mycobacterial burden and disease activity (Chee et al., 2008a). In 1052 healthy household contacts of smear-positive pTB index patients, Hill et al. (2005) reported a high quantitative ELISPOT response in contacts with recent exposure, reflecting *M. tuberculosis* infectious load.

Although the IFN-γ tests were not able to distinguish between active TB and LTBI, given that in childhood, especially in young children, the risk of progression to active TB is high, a rapid and specific diagnosis of LTBI is essential in preventing progression to disease. Moreover, IFN-γ tests may provide useful information in children being evaluated for suspected TB. In a recent study (Connell et al., 2008) on the pediatric population, in the evaluation of 9 children with suspected active TB, QFT-G-IT and T-SPOT.TB were positive in 8 and 9 cases, respectively. In our experience (Dominguez et al., 2008), both tests were also positive in 6 (66.7%) of the 9 children examined with active TB.

Alternative approaches have been developed to use IFN-γ tests as diagnostic tools for active TB. The use of RD1 selected peptides to distinguish patients with active TB from infected individuals has been evaluated. These peptides are a selection of Human Leukocyte Antigen (HLA) class II-

restricted RD1 region from ESAT-6 and CFP-10 MTB proteins. An association between the response to these selected peptides and active TB disease has been described (Goletti et al., 2005, 2006, 2008a; Vincenti et al., 2003). Furthermore, Goletti et al. (2006) evaluated the response to RD1 selected peptides in 23 pTB cases and 32 control patients, obtaining a sensitivity and a specificity of 70% and 91%, respectively. In contrast, the sensitivity and specificity of the 2 commercially available tests were 91% and 59% for T-SPOT.TB and 83% and 59% for QFT-G, respectively.

Recently, Dosanjh et al. (2008) have developed a new IFN-γ test (ELISpot^{PLUS}) that contains ESAT-6 and CFP-10 used in T-SPOT.TB and includes a third new antigen encoded in the genomic segment RD1 called Rv3879c. They studied 389 adults with a moderate to high probability of having active TB. The sensitivity for patients with a culture confirmed result was 85% with T-SPOT.TB and 89% with ELISpot^{PLUS}. This new assay had 4% higher diagnostic sensitivity than the standard T-SPOT.TB and improved the diagnosis of suspected TB to a sensitivity of 99% in combination with the TST. Moreover, the combined use of TST with the ELISpot^{PLUS} assay conferred a rapid exclusion of TB when both tests were negative and the pretest probability was low.

In this regard, in a recent multicenter Tuberculosis Network European Trialsgroup (TBNET) study (Goletti et al., 2008b), 2 commercial IFN-γ tests and the new assay based on RD1 selected peptides were evaluated, obtaining a similar accuracy for diagnosing active TB. They found that the combination of any of the IFN-γ tests evaluated with the TST might allow exclusion of TB.

Another approach is to investigate the use of alternative markers such as CXCL-10, also named IFN-γ inducible protein 10 (IP-10), and interleukin 2 (IL-2) to improve the diagnosis of active TB (Djoba Siawaya et al., 2007; Ruhwald et al., 2007). Interestingly, Ruhwald et al. (2007) studied IP-10, IL-2, and IFN-γ on 12 positive QFT-G-IT patients with confirmed TB and 11 negative QFT-G-IT healthy controls. The results suggested that the expression of IP-10 in active TB patients was 5.6 times higher than in healthy controls. In contrast, in the pediatric population, Whittaker et al. (2008) found that the baseline levels of IP-10 are increased in active TB and in LTBI, but there is no significant difference of stimulated levels of IP-10 between active TB and LTBI. Therefore, they concluded that IP-10 does not distinguish between active TB and LTBI. This observation is consistent with the idea that in the pediatric population, no significant difference in responses between LTBI and active TB children occurs because the infection is always recent, reinforcing the theory that quantitative T-cell assay reflects infectious load. Further studies are needed to establish the use of IP-10 cytokine alone or in combination with IFN-γ for the diagnosis of active TB and LTBI in adult and pediatric patients.

A new open strategy to diagnose the disease is based on detecting the MTB-specific T cell recruited in the site of the infection by means of ELISPOT techniques (Jafari et al., 2008; Losi et al., 2007; Wilkinson et al., 2005). This strategy could be a good alternative for the diagnosis of smear-negative pTB and also some extrapulmonary TB diseases.

In summary, T-cell enumeration after ESAT-6, CFP-10, and RD1 stimulation is high in patients with active TB. Our results demonstrate that the specific antigen CFP-10 response could be a more specific marker of active TB. Moreover, although there is overlapping, in patients with clinically suspected TB, a T-cell count above the described threshold could suggest active TB, especially in patients with a high probability of having active TB and low probability of having LTBI. Further research evaluating the quantitative T-cell response in contact patients and their subsequent development of active TB is required.

Acknowledgments

The authors thank the nurse staff of the TB Control and Prevention Unit of Barcelona and C. Ramil, L. Haba, M.A. Cuesta, M. Pérez, J. Maldonado, and J.M. Pina for technical assistance and helpful discussions. I. Latorre is Formación de Profesorado Universitario (FPU) predoctoral student and is the recipient of a grant from the "Ministerio de Educación y Ciencia" (Madrid, Spain).

This work was supported by a grant from the Sociedad Española de Neumología y Cirugía Torácica (SEPAR) (Barcelona, Spain), the Societat Catalana de Pneumologia (SOCAP) (Barcelona, Spain), the Fundació Catalana de Pneumologia (FUCAP) (Barcelona, Spain), and Instituto de Salud Carlos III (Madrid, Spain) (RETIC RD06/0018). *QuantiFERON-TB GOLD In-Tube* kits were provided by Inverness Medical Ibérica SAU, Cellestis' distributor in Spain, and T.SPOT-TB was provided by Oxford Immunotec by means of a Material Transfer Agreement.

The funders had no role in the study design, conduct, collection, management, analysis, interpretation of the data, or preparation, review, or approval of the manuscript.

References

Andersen P, Munk ME, Pollock JM, Doherty TM (2000) Specific immune-based diagnosis of tuberculosis. *Lancet* 356:1099–1104.

Arend SM, Thijsen SF, Leyten EM, Bouwman JJ, Franken WP, Koster BF, Cobelens FG, van Houte AJ, Bossink AW (2007) Comparison of two interferon-gamma assays and tuberculin skin test for tracing TB contacts. *Am J Respir Crit Care Med* 175:618–627.

Barnes PF, Lu S, Abrams JS, Wang E, Yamamura M, Modlin RL (1993) Cytokine production at the site of disease in human tuberculosis. *Infect Immun* 61:3482–3489.

Brock I, Weldingh K, Leyten EM, Arend SM, Ravn P, Andersen P (2004) Specific T-cell epitopes for immunoassay-based diagnosis of *Mycobacterium tuberculosis* infection. *J Clin Microbiol* 42: 2379–2387.

Connell TG, Ritz N, Paxton GA, Buttery JP, Curtis N, Ranganathan SC (2008) A three-way comparison of tuberculin skin testing, QuantiFERON-TB gold and T-SPOT.TB in children. *PLoS ONE* e2624:3.

Chee CB, Barkham TM, Khinmar KW, Gan SH, Wang YT (2008a) Quantitative T-cell interferon-gamma responses to *Mycobacterium*

tuberculosis-specific antigens in active and latent tuberculosis. *Eur J Clin Microbiol Infect Dis* 28:667–670.

Chee CB, Gan SH, Khinmar KW, Barkham TM, Koh CK, Liang S, Wang YT (2008b) Comparison of sensitivities of two commercial gamma interferon release assays for pulmonary tuberculosis. *J Clin Microbiol* 46:1935–1940.

Detjen AK, Keil T, Roll S, Hauer B, Mauch H, Wahn U, Magdorf K (2007) Interferon-gamma release assays improve the diagnosis of tuberculosis and nontuberculous mycobacterial disease in children in a country with a low incidence of tuberculosis. *Clin Infect Dis* 45:322–328.

Dinnes J, Deeks J, Kunst H, Gibson A, Cummins E, Waugh N, Drobniewski F, Lalvani A (2007) A systematic review of rapid diagnostic tests for the detection of tuberculosis infection. *Health Technol Assess* 11:1-196.

Djoba Siawaya JF, Ruhwald M, Eugen-Olsen J, Walzl G (2007) Correlates for disease progression and prognosis during concurrent HIV/TB infection. *Int J Infect Dis* 11:289–299.

Doherty TM, Demissie A, Olobo J, Wolday D, Britton S, Eguale T, Ravn P, Andersen P (2002) Immune responses to the *Mycobacterium tuberculosis*-specific antigen ESAT-6 signal subclinical infection among contacts of tuberculosis patients. *J Clin Microbiol* 40:704–706.

Dominguez J, Ruiz-Manzano J, De Souza-Galvao M, Latorre I, Mila C, Blanco S, Jimenez MA, Prat C, Lacoma A, Altet N, Ausina V (2008) Comparison of two commercially available gamma interferon blood tests for immunodiagnosis of tuberculosis. *Clin Vaccine Immunol* 15:168–171.

Domínguez J, De Souza-Galvao M, Ruiz-Manzano J, Latorre I, Prat C, Lacoma A, Milà C, Jiménez MA, Blanco S, Maldonado J, Altet N, Ausina V (2009a) T-cell responses to the *Mycobacterium tuberculosis*-specific antigens in active tuberculosis patients at the beginning, during, and after antituberculosis treatment. *Diagn Microbiol Infect Dis* 63:43–51.

Domínguez J, Latorre I, Altet N, Mateo L, De Souza-Galvao M, Ruiz-Manzano J, Ausina V (2009b) Interferon-gamma–release assays to diagnose TB infection in immunocompromised individual. *Expert Rev Respir Med* 3:309–327.

Dosanjh DP, Hinks TS, Innes JA, Deeks JJ, Pasvol G, Hackforth S, Varia H, Millington KA, Gunatheesan R, Guyot-Revol V, Lalvani A (2008) Improved diagnostic evaluation of suspected tuberculosis. *Ann Intern Med* 148:325–336.

Dye C, Maher D, Weil D, Espinal M, Raviglione M (2006) Targets for global tuberculosis control. *Int J Tuberc Lung Dis* 10:460–462.

Ewer K, Deeks J, Alvarez L, Bryant G, Waller S, Andersen P, Monk P, Lalvani A (2003) Comparison of T-cell–based assay with tuberculin skin test for diagnosis of *Mycobacterium tuberculosis* infection in a school tuberculosis outbreak. *Lancet* 361:1168–1173.

Ferrara G, Losi M, D'Amico R, Roversi P, Piro R, Meacci M, Meccugni B, Dori IM, Andreani A, Bergamini BM, Mussini C, Rumpianesi F, Fabbri LM, Richeldi L (2006) Use in routine clinical practice of two commercial blood tests for diagnosis of infection with *Mycobacterium tuberculosis*: a prospective study. *Lancet* 367:1328–1334.

Fox A, Jeffries DJ, Hill PC, Hammond AS, Lugos MD, Jackson-Sillah D, Donkor SA, Owiafe PK, McAdam KP, Brookes RH (2007) ESAT-6 and CFP-10 can be combined to reduce the cost of testing for *Mycobacterium tuberculosis* infection, but CFP-10 responses associate with active disease. *Trans R Soc Trop Med Hyg* 101:691–698.

Goletti D, Vincenti D, Carrara S, Butera O, Bizzoni F, Bernardini G, Amicosante M, Girardi E (2005) Selected RD1 peptides for active tuberculosis diagnosis: comparison of a gamma interferon whole-blood enzyme-linked immunosorbent assay and an enzyme-linked immunospot assay. *Clin Diagn Lab Immunol* 12:1311–1316.

Goletti D, Carrara S, Vincenti D, Saltini C, Rizzi EB, Schinina V, Ippolito G, Amicosante M, Girardi E (2006) Accuracy of an immune diagnostic assay based on RD1 selected epitopes for active tuberculosis in a clinical setting: a pilot study. *Clin Microbiol Infect* 12:544–550.

Goletti D, Carrara S, Mayanja-Kizza H, Baseke J, Mugerwa MA, Girardi E, Toossi Z (2008a) Response to *M. tuberculosis* selected RD1 peptides in Ugandan HIV-infected patients with smear positive pulmonary tuberculosis: a pilot study. *BMC Infect Dis* 8:11.

Goletti D, Stefania C, Butera O, Amicosante M, Ernst M, Sauzullo I, Vullo V, Cirillo D, Borroni E, Markova R, Drenska R, Dominguez J, Latorre I, Angeletti C, Navarra A, Petrosillo N, Lauria FN, Ippolito G, Migliori GB, Lange C, Girardi E (2008b) Accuracy of immunodiagnostic tests for active tuberculosis using single and combined results: a multicenter TBNET-Study. *PLoS ONE* e3417:3.

Hill PC, Fox A, Jeffries DJ, Jackson-Sillah D, Lugos MD, Owiafe PK, Donkor SA, Hammond AS, Corrah T, Adegbola RA, McAdam KP, Brookes RH (2005) Quantitative T cell assay reflects infectious load of *Mycobacterium tuberculosis* in an endemic case contact model. *Clin Infect Dis* 40:273–278.

Hirsch CS, Toossi Z, Othieno C, Johnson JL, Schwander SK, Robertson S, Wallis RS, Edmonds K, Okwera A, Mugerwa R, Peters P, Ellner JJ (1999) Depressed T-cell interferon-gamma responses in pulmonary tuberculosis: analysis of underlying mechanisms and modulation with therapy. *J Infect Dis* 180:2069–2073.

Horsburgh Jr CR (2004) Priorities for the treatment of latent tuberculosis infection in the United States. *N Engl J Med* 350:2060–2067.

Huebner RE, Schein MF, Bass Jr JB (1993) The tuberculin skin test. *Clin Infect Dis* 17:968–975.

Jafari C, Ernst M, Strassburg A, Greinert U, Kalsdorf B, Kirsten D, Lange C (2008) Local immunodiagnosis of pulmonary tuberculosis by enzyme-linked immunospot. *Eur Respir J* 31:261–265.

Janssens JP, Roux-Lombard P, Perneger T, Metzger M, Vivien R, Rochat T (2007) Quantitative scoring of an interferon-gamma assay for differentiating active from latent tuberculosis. *Eur Respir J* 30:722–728.

Kang YA, Lee HW, Hwang SS, Um SW, Han SK, Shim YS, Yim JJ (2007) Usefulness of whole-blood interferon-{gamma} assay and interferon-{gamma} enzyme-linked immunospot assay in the diagnosis of active pulmonary tuberculosis. *Chest* 132:959–965.

Kobashi Y, Mouri K, Yagi S, Obase Y, Miyashita N, Oka M (2008) Transitional changes in T-cell responses to *Mycobacterium tuberculosis*-specific antigens during treatment. *J Infect* 58:197–204.

Lalvani A (2007) Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy. *Chest* 131:1898–1906.

Lalvani A, Pathan AA, McShane H, Wilkinson RJ, Latif M, Conlon CP, Pasvol G, Hill AV (2001) Rapid detection of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cells. *Am J Respir Crit Care Med* 163:824–828.

Latorre I, De Souza-Galvao M, Ruiz-Manzano J, Lacoma A, Prat C, Altet N, Ausina V, Dominguez J (2009) Evaluating the non-tuberculous mycobacteria effect in the tuberculosis infection diagnosis. *Eur Respir J* 34, doi:10.1183/09031936.0196608.

Lee JY, Choi HJ, Park IN, Hong SB, Oh YM, Lim CM, Lee SD, Koh Y, Kim WS, Kim DS, Kim WD, Shim TS (2006) Comparison of two commercial interferon-gamma assays for diagnosing *Mycobacterium tuberculosis* infection. *Eur Respir J* 28:24–30.

Liebeschuetz S, Bamber S, Ewer K, Deeks J, Pathan AA, Lalvani A (2004) Diagnosis of tuberculosis in South African children with a T-cell–based assay: a prospective cohort study. *Lancet* 364:2196–2203.

Losi M, Bossink A, Codecasa L, Jafari C, Ernst M, Thijsen S, Cirillo D, Ferrarese M, Greinert U, Fabbri LM, Richeldi L, Lange C (2007) Use of a T-cell interferon-{gamma} release assay for the diagnosis of tuberculous pleurisy. *Eur Respir J* 30:1173–1179.

Menzies D (2008) Using tests for latent tuberculous infection to diagnose active tuberculosis: can we eat our cake and have it too? *Ann Intern Med* 148:398–399.

Menzies D, Pai M, Comstock G (2007) Meta-analysis: new tests for the diagnosis of latent tuberculosis infection: areas of uncertainty and recommendations for research. *Ann Intern Med* 146:340–354.

Migliori GB, Hopewell PC, Blasi F, Spanevello A, Raviglione MC (2006) Improving the TB case management: the International Standards for Tuberculosis Care. *Eur Respir J* 28:687–690.

Mori T, Sakatani M, Yamagishi F, Takashima T, Kawabe Y, Nagao K, Shigeto E, Harada N, Mitarai S, Okada M, Suzuki K, Inoue Y, Tsuyuguchi K, Sasaki Y, Mazurek GH, Tsuyuguchi I (2004) Specific

detection of tuberculosis infection: an interferon-gamma–based assay using new antigens. *Am J Respir Crit Care Med* 170:59–64.

Pai M, Zwerling A, Menzies D (2008) Systematic review: T-cell–based assays for the diagnosis of latent tuberculosis infection: an update. *Ann Intern Med* 149:177–184.

Pathan AA, Wilkinson KA, Klenerman P, McShane H, Davidson RN, Pasvol G, Hill AV, Lalvani A (2001) Direct ex vivo analysis of antigen-specific IFN-gamma–secreting CD4 T cells in *Mycobacterium tuberculosis*-infected individuals: associations with clinical disease state and effect of treatment. *J Immunol* 167:5217–5225.

Rangaka MX, Wilkinson KA, Seldon R, Van Cutsem G, Meintjes GA, Morroni C, Mouton P, Diwakar L, Connell TG, Maartens G, Wilkinson RJ (2007) Effect of HIV-1 infection on T-Cell–based and skin test detection of tuberculosis infection. *Am J Respir Crit Care Med* 175:514–520.

Richeldi L (2006) An update on the diagnosis of tuberculosis infection. *Am J Respir Crit Care Med* 174:736–742.

Ruhwald M, Bjerregaard-Andersen M, Rabna P, Kofoed K, Eugen-Olsen J, Ravn P (2007) CXCL10/IP-10 release is induced by incubation of whole blood from tuberculosis patients with ESAT-6, CFP10 and TB7.7. *Microbes Infect* 9:806–812.

Ruiz-Manzano J, Blanquer R, Calpe JL, Caminero JA, Caylà J, Domínguez J, García JM, Vidal R (2008) SEPAR Guidelines. Diagnostic and treatment of tuberculosis. *Arch Bronconeumol* 44:551–566.

Soysal A, Torun T, Efe S, Gencer H, Tahaoglu K, Bakir M (2008) Evaluation of cut-off values of interferon-gamma–based assays in the diagnosis of *M. tuberculosis* infection. *Int J Tuberc Lung Dis* 12:50–56.

Valdes L, Alvarez D, San Jose E, Penela P, Valle JM, Garcia-Pazos JM, Suarez J, Pose A (1998) Tuberculous pleurisy: a study of 254 patients. *Arch Intern Med* 158:2017–2021.

Vincenti D, Carrara S, De Mori P, Pucillo LP, Petrosillo N, Palmieri F, Armignacco O, Ippolito G, Girardi E, Amicosante M, Goletti D (2003) Identification of early secretory antigen target-6 epitopes for the immunodiagnosis of active tuberculosis. *Mol Med* 9:105–111.

Whittaker E, Gordon A, Kampmann B (2008) Is IP-10 a better biomarker for active and latent tuberculosis in children than IFNgamma? *PLoS ONE* 3: e3901.

Wilkinson KA, Wilkinson RJ, Pathan A, Ewer K, Prakash M, Klenerman P, Maskell N, Davies R, Pasvol G, Lalvani A (2005) Ex vivo characterization of early secretory antigenic target 6-specific T cells at sites of active disease in pleural tuberculosis. *Clin Infect Dis* 40:184–187.

ANNEXE IV

Eur Respir J 2010; 35: 338–342
DOI: 10.1183/09031936.00196608

Evaluating the non-tuberculous mycobacteria effect in the tuberculosis infection diagnosis

I. Latorre[*,#,¶], M. De Souza-Galvão[#,+], J. Ruiz-Manzano[#,¶,§], A. Lacoma[*,#,¶], C. Prat[*,#,¶], N. Altet[+], V. Ausina[*,#,¶] and J. Domínguez[*,#,¶]

ABSTRACT: **The aim of the present study was to determine the role of previous non-tuberculous mycobacteria sensitisation in children as a factor of discordant results between tuberculin skin test (TST) and an *in vitro* T-cell based assay (T-SPOT.TB; Oxford Immunotec, Oxford, UK).**

We enrolled 21 non-bacille Calmette-Guérin-vaccinated paediatric patients for suspicious of latent tuberculosis infection (LTBI). These patients yielded a positive TST and a negative T-SPOT.TB. Cells were stimulated with *Mycobacterium avium* sensitin (having cross-reaction with *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*) and the presence of reactive T-cells was determined by an *ex vivo* ELISPOT.

From the 21 patients, in 10 cases (47.6%), we obtained a positive ELISPOT result after stimulation with *M. avium* sensitin, in six (28.6%) cases, the result was negative and in the remaining five (23.8%) cases, the result was indeterminate.

In conclusion, previous non-tuberculous mycobacteria sensitisation induces false-positive results in the TST for diagnosing LTBI and the use of γ-interferon tests could avoid unnecessary chemoprophylaxis treatment among a child population.

KEYWORDS: **Childhood, ELISPOT, interferon-γ release assays, latent tuberculosis infection, *Mycobacterium avium* sensitin, non-tuberculous mycobacteria**

The detection and treatment of active TB is a key strategy in the control of childhood tuberculosis (TB) [1]. Children have a high risk of progression to active TB [2]. Therefore, a rapid and specific diagnosis of latent TB infection (LTBI) is essential in preventing the progression to disease. The tuberculin skin test (TST) attempts to measure cell-mediated immunity in the form of a delayed-type hypersensitivity response to the purified protein derivative (PPD) [3]. The biggest drawback of TST is that individuals sensitised by previous exposure to non-tuberculous mycobacteria (NTM) or vaccinated with *Mycobacterium bovis* bacilli Calmette-Guérin (BCG) respond immunologically to PPD. Consequently, unnecessary latent tuberculosis treatments are prescribed.

In vitro assays for measuring T-cell-mediated immune responses have been developed. In these assays, infected individuals are identified by the detection of γ-interferon (IFN-γ) released by the T-cells that are sensitised after being stimulated with the specific *Mycobacterium tuberculosis* (MTB) antigens of region of deletion (RD) 1 (early-secreted antigenic target protein (ESAT)-6 and 10-kD culture filtrate protein (CFP)-10) [4, 5]. Promising results from these diagnostic tests in both adults and children have been published [6–11].

However, there are several discordant results between the IFN-γ tests and the TST [12]. One of the more challenging correct interpretations remains in the instance of positive TST and negative IFN-γ results in non-BCG vaccinated children. In our experience [7], among unvaccinated children with a positive TST, the T-SPOT.TB result was negative in 56.6% of the cases.

The aim of the present study was to determine the role of previous NTM sensitisation in children as a factor of discordant results between TST and the T-SPOT.TB test.

MATERIAL AND METHODS

Patients and inclusion criteria

We retrospectively enrolled a total of 21 paediatric patients, who attended Hospital Universitari Germans Trias i Pujol (Badalona, Spain) or TB Control and Prevention Unit of Barcelona (CAP

AFFILIATIONS
[*]Servei de Microbiologia,
[§]Servei de Pneumologia, Hospital Universitari "Germans Trias i Pujol", Fundació Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol,
[¶]Ciber Enfermedades Respiratorias, Instituto de Salud Carlos III, Badalona,
[#]Universitat Autònoma de Barcelona. Bellaterra, and
[+]Unidad de Prevención y Control de la Tuberculosis de Barcelona, Barcelona, Spain.

CORRESPONDENCE
J. Domínguez
Servei de Microbiologia
Fundació Institut d'Investigació en Ciències de la Salut "Germans Trias i Pujol"
Carretera del Canyet s/n
08916 Badalona
Barcelona
Spain
E-mail: jadomb@gmail.com

Received:
Dec 28 2008
Accepted after revision:
April 07 2009

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

Drassanes, Barcalona, Spain) for suspicion of LTBI. These patients were enrolled for contact tracing studies or for screening of LTBI. Inclusion criteria for this selected population were a positive TST, a negative T-SPOT.TB, non-BCG vaccination and no more than 2 weeks of chemoprophylaxis when blood sampling. None of the children presented lymphadenitis at the time of inclusion. We have also included control groups to validate the methodology and the results: 11 children with both TST and T-SPOT.TB negative results, and six individuals with microbiologically confirmed *M. avium* infection (four lymphadenitis and two respiratory infections). Another additional group of 10 children with both TST- and T-SPOT.TB-positive results was included in order to know the background of *M. tuberculosis* and *M. avium* sensitisation in the population. The main demographic characteristics of the groups included in the study are shown in table 1.

Ethics approval for this study was provided by the corresponding Ethics Committees. We obtained written informed consent from all parents before blood sampling. A detailed questionnaire from all patients was completed to indicate the results of any previous TST, BCG vaccination status, details of any contact with a person diagnosed of active TB, history of prior active TB, LTBI and HIV infection, chest radiography and other medical conditions.

TST

Two intradermal tuberculin units of PPD RT23 Tween 80 (Statens Serum Institut, Copenhagen, Denmark) were used to perform TST. The tuberculin was administered using Mantoux method, and the size of the induration was interpreted after 48–72 h by trained personnel. In this study, TST indurations ⩾5 mm were classified as positive [13].

Detection of T-cell sensitised against MTB specific antigens

Peripheral blood mononuclear cells (PBMCs) were stimulated with ESAT-6 and CFP-10 antigens individually. The presence of reactive antigen-specific T-cells was revealed by ELISPOT (T-SPOT.TB; Oxford Immunotec, Oxford, UK). The test was performed in accordance with the manufacturer's instructions. Unstimulated cells were washed with RPMI medium (Invitrogen, Auckland, New Zealand) and resuspended in freeze medium (80% RPMI and 20% free bovine serum (PAA Laboratories GmbH, Pasching, Austria)), adding dropwise 10% DMSO (Merck, Darmstadt, Germany) and frozen at -80°C.

TABLE 1 Demographic characteristics of patients studied

Variable	Study group	Control groups		
		TST and T-SPOT.TB negatives	TST and T-SPOT.TB positives	Microbiologically confirmed *M. avium* infection
Subjects	21	11	10	6
Sex				
Male	10 (47.6)	3 (27.3)	6 (60)	4 (66.7)
Female	11 (52.4)	8 (72.7)	4 (40)	2 (33.3)
Age yrs	8.81±4.03	11.55±4.52	10±3.02	17.5±20.92
BCG vaccinated				
Yes	0 (0)	5 (45.5)	3 (30)	0 (0)
No	21 (100)	6 (54.5)	7 (70)	6 (100)
Immunosupression				
Yes	0 (0)	0 (0)	0 (0)	0 (0)
No	21 (100)	11 (100)	10 (100)	6 (100)
Birth country				
Immigrants from countries with high prevalence of TB infection	5 (23.8)	6 (54.5)	3(30)	0 (0)
Residents in a non-epidemic TB country	16 (76.2)	5 (45.5)	7 (70)	6 (100)
Origin				
Contact tracing studies	6 (28.6)	8 (72.7)	10 (100)	
Screening of LTBI at school	15 (71.4)	3 (27.3)	0 (0)	
T-SPOT.TB				
Positive	0	0	10	2#
Negative	21	21	0	3
Indeterminate	0	0	0	1
***Ex vivo* ELISPOT *M. avium* seneitin stimulation**				
Positive	10	0	5	4
Negative	6	11	3	1¶
Indeterminate	5	0	2	1

Data are presented as n, n (%) or mean±SD. TST: tuberculin skin test; *M. avium*: *Mycobacterium avium*; BCG: bacilli Calmette-Guérin; TB: tuberculosis; LTBI: latent TB infection. #: in one case, active TB was documented 8 yrs before; ¶: *M. avium* infection was reported 2 yrs before.

Detection of T-cell sensitised against NTM sensitin

The stimulation of the T-cells was performed using *M. avium* sensitin (Statens Serum Institute, Copenhagen, Denmark). The manufacturer informed that this sensitin has cross reaction with *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*. In order to perform *ex vivo* ELISPOT, stimulating with *M. avium* sensitin, cells were thawed and re-suspended in 10 mL of RPMI medium. Finally, cells were washed, re-suspended in AIM-V medium (Invitrogen, Auckland, New Zealand) and stimulated with medium alone (as nil control), phytohaemagglutinin (as positive control) and *M. avium* sensitin at a concentration of 10 $\mu g \cdot mL^{-1}$. Plates were incubated for 16–20 h at 37°C with 5% CO_2. Following incubation, wells were washed with PBS and incubated for 1 h at 2°C with a monoclonal antibody to IFN-γ conjugated to alkaline phosphatase. The presence of reactive antigen-specific T-cells was revealed as a spot in the well.

Interpretation of the results

Spots were scored using an automated ELISPOT plate reader (Lector AID Elispots; Autoimmun Diagnostiks GmbH, Germany). All readings were also manually verified. The results of the assays were expressed as ESAT-6, CFP-10 and *M. avium* sensitin specific responder cells per million PBMCs. Test wells were scored as positive if the number of responder cells per million PBMCs minus their number in the control negative was >24. The result of the assay was considered indeterminate if the number of positive control cells per million PBMCs was <80, and the response to both of the antigen panels was negative.

RESULTS

From the 21 children with positive TST and negative T-SPOT.TB, a positive ELISPOT result after stimulation with *M. avium* sensitin was obtained in 10 (47.6%) cases. In six (28.6%) cases the result was negative and in the remaining five (23.8%) cases the result was indeterminate. The number of responder T-cells after *M. avium* sensitin stimulation was significantly higher than the number of responder T-cells after specific MTB antigens (ESAT-6 and CFP-10) stimulation: p=0.001 and p<0.001, respectively.

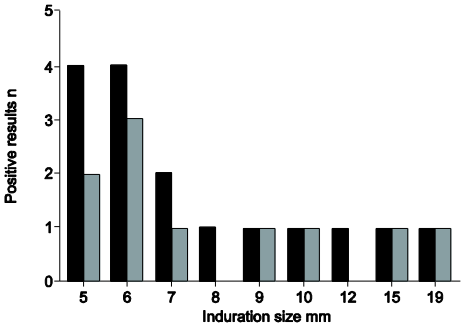

FIGURE 1. Induration size distribution of positive results of the tuberculin skin test (■) and *ex vivo Mycobacterium avium* sensitin ELISPOT (□) among the children with a valid result.

Among the 10 children that obtained a positive result after stimulation with *M. avium* sensitin, five children were aged 6–7 yrs and the other five children were aged 11–16 yrs. Additionally, eight were enrolled during LTBI screening at school and the remaining two, during a contact tracing study. Regarding the induration of the TST, eight of these children were in the range of 5–10 mm, one case was 15 mm and the other case was 19 mm (fig. 1).

In all children with both TST- and T-SPOT.TB-negative results included as controls, negative ELISPOT results after stimulation with *M. avium* sensitin were obtained. There were no significant differences between the number of responder T-cells after stimulation with ESAT-6, CFP-10 and *M. avium* sensitin. The differences in the number of responder T-cells to *M. avium* sensitin between the patients study group and this control group were significant (p=0.004) (fig. 2). In the group of individuals with microbiologically confirmed *M. avium* infection, four out of five cases with valid results, cells

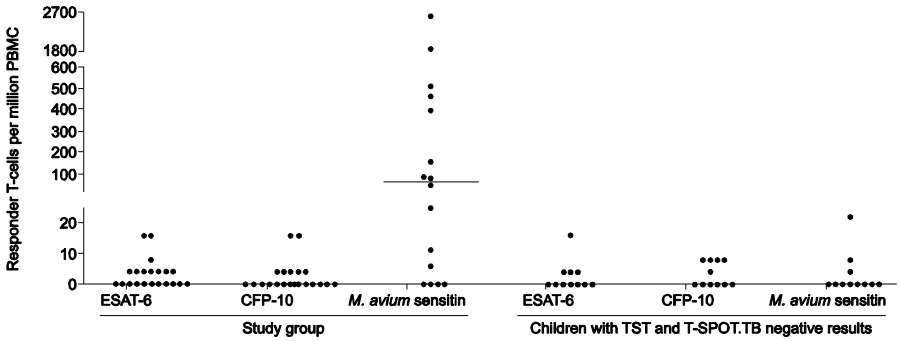

FIGURE 2. Number of responder T-cells enumerated by *ex vivo* ELISPOT after stimulation with the specific *Mycobacterium tuberculosis* antigens (early secretary antigen target (ESAT)-6 and culture filtrate protein (CFP)-10) and *Mycobacterium avium* (*M. avium*) sensitin in the study group and children with tuberculin skin test (TST)- and T-SPOT.TB-negative results. PBMC: peripheral blood mononuclear cells.

sensitised against *M. avium* were detected. The results obtained by the study and all control groups are presented in table 1. The indeterminate results were due to the low number of cells recovered after thawing.

DISCUSSION

Although specificity of IFN-γ tests is excellent because the assay is not affected by BCG vaccination [6–8], frequent discordant results with TST have been described [6, 7, 14]. In fact, it has been recommended for priority research to obtain data to understand discordant TST and IFN-γ tests results, including the role of NTM [12]. To date, the effect of NTM on IFN-γ tests results has been poorly studied. In this sense, we have studied the effect of previous NTM sensitisation to try to give an explanation for the discordant results of positive TST and negative IFN-γ results in non-BCG vaccinated children. Among the 16 children with a valid result, 10 (62.5%) children had a specific response of T-cells after stimulation with *M. avium* sensitin.

It has been described that asymptomatic infections with *M. avium* and other NTM are common [15] and probably acquired in childhood [16–19]. In our area, the estimation of NTM infection in children with a positive TST (5–10 mm) ranged 20–50% [16]. According with our results, using the *ex vivo* ELISPOT, eight (80%) of the 10 children reactive against *M. avium* sensitin had a positive TST between 5 and 10 mm, and nine (90%) of them between 5 and 15 mm. Indeed, in the children control group with TST- and T-SPOT.TB-positive results, the presence of T-cell sensitised against *M. avium* was detected in five out of the eight cases with valid result.

In our study, eight out of 10 children with a positive *M. avium* sensitin T-cell assay from our study group were enrolled from a routine screening of LTBI without known exposure to any active TB patient. Given that NTM infection affects the TST reading, it is in this group of children where IFN-γ tests could be used to confirm the diagnosis in case of a positive TST result.

Regarding the six remaining discordant results without T-cell response after *M. avium* sensitin stimulation, there are three possible explanations. First, a real LTBI not detected by the IFN-γ test. Nevertheless, the sensitivity of the IFN-γ tests is considered to be higher than the TST, or at least at the same level. Secondly, the IFN-γ test enumerates effectors T-cells that have recently been in contact with the antigen, in contrast, TST remains positive a long period after past *M. tuberculosis* infection [20]. However, in children the infection is usually recent. The third explanation is that the positive TST was due to a previous infection by a NTM without *M. avium* sensitin cross-reaction. It was impossible to test more NTM sensitins given that we didn't have more PBMCs stored from these patients.

One limitation of our study is that the skin test reactions to *M. avium* sensitin were not performed at the moment of inclusion of the children; therefore, it was not possible to correlate with the *ex vivo* result. Another limitation is that we have tested a reduced number of children. Nevertheless, despite these limitations, the results obtained are sufficiently consistent to draw some conclusions.

In summary, our results show enough evidence to state that previous NTM sensitisation in children induces false-positive results in the TST for diagnosing LTBI and that the IFN-γ tests could avoid both unnecessary chemoprophylaxis treatment among child populations and consuming resources searching the index case.

SUPPORT STATEMENT

This work was supported by a grant from Sociedad Española de Neumología y Cirugía Torácica; Societat Catalana de Pneumologia (SOCAP); Fundació Catalana de Pneumologia (FUCAP); and Instituto de Salud Carlos III (RETIC RD06/0018). I. Latorre is a FPU pre-doctoral student and is the recipient of a grant from the Ministerio de Educación y Ciencia.

STATEMENT OF INTEREST

A statement of interest for J. Domínguez can be found at www.erj.ersjournals.com/misc/statements.dtl

ACKNOWLEDGEMENTS

The authors would like to thank the nursing staff of the TB Control and Prevention Unit of Barcelona and C. Ramil, L. Haba, M. Ángel Cuesta, M. Pérez (all Servei de Microbiologia, Hospital Universitari Germans Trias i Pujol, Badalona, Spain), and J. María Pina (Programa de Tuberculosi Regió Centre, Terrassa, Spain), for technical assistance and helpful discussions. The authors are also indebted to C. Rodrigo (Servei de Pediatria, Hospital Universitari Germans Trias i Pujol, Badalona), N. Díez and A. Escribano (both Servicio de Pediatría, Hospital Clínico Universitario, Valencia, Spain) for their kind help in enrolling children for the control groups.

REFERENCES

1 Migliori GB, Hopewell PC, Blasi F, *et al*. Improving the TB case management: The International Standards for Tuberculosis Care. *Eur Respir J* 2006; 28: 687–690.
2 Lalvani A, Millington KA. T-cell-based diagnosis of childhood tuberculosis infection. *Curr Opin Infect Dis* 2007; 20: 264–271.
3 Jasmer RM, Nahid P, Hopewell PC. Clinical practice. Latent tuberculosis infection. *N Engl J Med* 2002; 347: 1860–1866.
4 Andersen P, Munk ME, Pollock JM, *et al*. Specific immune-based diagnosis of tuberculosis. *Lancet* 2000; 356: 1099–1104.
5 Brock I, Weldingh K, Leyten EM, *et al*. Specific T-cell epitopes for immunoassay-based diagnosis of *Mycobacterium tuberculosis* infection. *J Clin Microbiol* 2004; 42: 2379–2387.
6 Connell TG, Curtis N, Ranganathan SC, *et al*. Performance of a whole blood interferon gamma assay for detecting latent infection with *Mycobacterium tuberculosis* in children. *Thorax* 2006; 61: 616–620.
7 Dominguez J, Ruiz-Manzano J, De Souza-Galvao M, *et al*. Comparison of two commercially available gamma interferon blood tests for immunodiagnosis of tuberculosis. *Clin Vaccine Immunol* 2008; 15: 168–171.
8 Ewer K, Deeks J, Alvarez L, *et al*. Comparison of T-cell-based assay with tuberculin skin test for diagnosis of *Mycobacterium tuberculosis* infection in a school tuberculosis outbreak. *Lancet* 2003; 361: 1168–1173.
9 Ferrara G, Losi M, D'Amico R, *et al*. Use in routine clinical practice of two commercial blood tests for diagnosis of infection with *Mycobacterium tuberculosis*: a prospective study. *Lancet* 2006; 367: 1328–1334.
10 Goletti D, Stefania C, Butera O, *et al*. Accuracy of immunodiagnostic tests for active tuberculosis using single and combined results: a multicenter TBNET-Study. *PLoS ONE* 2008; 3: e3417.
11 Domínguez J, De Souza-Galvao M, Ruiz-Manzano J, *et al*. T-cell responses to the *Mycobacterium tuberculosis*-specific antigens in active

tuberculosis patients at the beginning, during, and after antituberculosis treatment. *Diagn Microbiol Infect Dis* 2009; 63: 43–51.
12 Menzies D, Pai M, Comstock G. Meta-analysis: new tests for the diagnosis of latent tuberculosis infection: areas of uncertainty and recommendations for research. *Ann Intern Med* 2007; 146: 340–354.
13 Ruiz-Manzano J, Blanquer R, Calpe JL, *et al*. SEPAR Guidelines. Diagnostic and treatment of tuberculosis. *Arch Bronconeumol* 2008; 44: 551–566.
14 Connell TG, Ritz N, Paxton GA, *et al*. A three-way comparison of tuberculin skin testing, QuantiFERON-TB gold and T-SPOT.TB in children. *PLoS ONE* 2008; 3: e2624.
15 von Reyn CF, Horsburgh CR, Olivier KN, *et al*. Skin test reactions to *Mycobacterium tuberculosis* purified protein derivative and *Mycobacterium avium* sensitin among health care workers and medical students in the United States. *Int J Tuberc Lung Dis* 2001; 5: 1122–1128.
16 Alcaide Megias J, Altet Gomez MN, Canela i Soler J. [Epidemiology of tuberculosis.] *An Esp Pediatr* 2000; 53: 449–457.
17 Bierrenbach AL, Floyd S, Cunha SC, *et al*. A comparison of dual skin test with mycobacterial antigens and tuberculin skin test alone in estimating prevalence of *Mycobacterium tuberculosis* infection from population surveys. *Int J Tuberc Lung Dis* 2003; 7: 312–319.
18 Fairchok MP, Rouse JH, Morris SL. Age-dependent humoral responses of children to mycobacterial antigens. *Clin Diagn Lab Immunol* 1995; 2: 443–447.
19 Larsson LO, Skoogh BE, Bentzon MW, *et al*. Sensitivity to sensitins and tuberculin in Swedish children. II. A study of preschool children. *Tubercle* 1991; 72: 37–42.
20 Leyten EM, Arend SM, Prins C, *et al*. Discrepancy between *Mycobacterium tuberculosis*-specific gamma interferon release assays using short and prolonged *in vitro* incubation. *Clin Vaccine Immunol* 2007; 14: 880–885.

Discordance between TSTs and IFN-γ release assays: the role of NTM and the relevance of mycobacterial sensitins

To the Editors:

We read with interest the recent study by LATORRE *et al.* [1] entitled "Evaluating the non-tuberculous mycobacteria effect in the tuberculosis infection diagnosis". We agree that discordance between tuberculin skin tests (TSTs) and interferon (IFN)-γ release assays (IGRAs) presents physicians with a considerable management dilemma when evaluating children for latent tuberculosis (TB) infection (LTBI) in routine clinical practice. We have previously urged caution in the interpretation of discordant results and have highlighted this area as a research priority [2, 3]. We therefore commend the authors for investigating a potential underlying cause of discordance. However, we believe that the interpretation of the data presented by LATORRE *et al.* [1] is based on erroneous assumptions, and that as a result the conclusions are overstated. We suggest that a more cautious and contextualised interpretation of the study findings is warranted.

As indicated by LATORRE *et al.* [1], previous bacille Calmette–Guérin immunisation and exposure to non-tuberculous mycobacteria (NTM) are frequently cited as the primary factors underlying discordance between TSTs and IGRAs, although convincing data to support these concepts are currently lacking. In the study by LATORRE *et al.* [1], children with suspected LTBI were assessed with a TST, a commercial IFN-γ ELISpot assay (the T.SPOT.*TB* assay, incorporating early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10)) and an in-house IFN-γ ELISpot assay using *Mycobacterium avium* sensitin (MAS) as the stimulating antigen. In the subgroup of children that had TST+/T.SPOT.*TB*- discordance, 47.6% showed a "positive" response in the IFN-γ ELISpot using MAS as the stimulant (contrasting with the absence of response to ESAT-6 and CFP-10). The authors interpret this observation as evidence that previous NTM sensitisation in these children resulted in a false-positive TST result and thereby discordance.

While we agree that this is one possible explanation, there is an alternative explanation that would equally account for these observations. Importantly, significant cross-reactivity between different mycobacterial sensitins has been previously consistently shown in animal models [4]. Furthermore, more than a decade ago, LEIN *et al.* [5] convincingly demonstrated that T cell assays incorporating MAS cannot reliably distinguish between *M. tuberculosis* and *M. avium* complex (MAC) infection in humans. In that study, the authors used the same MAS preparation as LATORRE *et al.* [1] to assess T-cell responses in adults with culture-confirmed TB (n=27) or MAC (n=10) infection. Somewhat unexpectedly, higher mean IFN-γ concentrations were observed in supernatants from peripheral blood mononuclear cells stimulated with MAS in patients with TB than those with MAC infection. In addition, MAS-sensitised T cells were detected in the majority of patients with TB. These data strongly suggest that there is considerable cross-reactivity between antigens encountered by the human immune system during *M. tuberculosis* infection and antigens contained in MAS. We also note that in the study by LATORRE *et al.* [1], in the subgroup of children that were TST+/T.SPOT.*TB*+ (and therefore highly likely to have LTBI), 50% showed a "positive" response to MAS in the in-house IFN-γ ELISpot assay, which further questions the ability of MAS-based assays to discriminate between TB and NTM infection, or alternatively exposure.

The limited ability of MAS to distinguish between different mycobacterial infections is not surprising. Unlike the well-defined peptides ESAT-6 and CFP-10, which are thought to be relatively *M. tuberculosis*-specific (despite orthologues of these proteins being present in several other mycobacterial species including *M. kansasii*, *M. marinum* and *M. szulgai*), MAS is a mixture of heterogenous mycobacterial antigens, analogous to the purified protein derivative used in the TST [6]. Cross-reactivity with other mycobacterial species is therefore likely to occur, as indicated by the manufacturer's warning mentioned by LATORRE *et al.* [1], that is particularly likely to be the case with *M. intracellulare* and *M. scrofulaceum*.

Taken together, these facts make it questionable whether the observations by LATORRE *et al.* [1] in the subgroup of children with TST+/T.SPOT.*TB*- discordance truly reflect previous NTM exposure. An alternative explanation is that the assays using MAS detected T-cell sensitisation resulting from previous *M. tuberculosis* exposure and/or LTBI (*i.e.* confirming the positive TST), while the T.SPOT.*TB* produced a false-negative result. Published data show that up to one-third of children with culture-proven active TB have negative or indeterminate T.SPOT.*TB* assay results [7], which highlights the limitations of these assays and lends support to the latter explanation. In the absence of a gold standard for LTBI, neither hypothesis can be tested with certainty. However, given these uncertainties we believe it is premature of the authors to suggest that chemoprophylaxis could be safely withheld in these patients. Contrary to the authors' assertions, we believe their study does not provide "enough evidence" to justify changes in clinical practice.

We concur with LATORRE *et al.* [1] that there remains an urgent need to explore the immunology of underlying discordance between TSTs and IGRAs in greater detail. However, in view of the comparatively poor performance of IGRAs in children and the uncertainties surrounding their interpretation we, and other researchers in this field, firmly believe that research to identify better biomarkers and immunological correlates of TB infection remains crucial [3].

M. Tebruegge[*,#,¶], T. Connell[*,#,¶], N. Ritz[*,#,¶], P.A. Bryant[*,#,¶] and N. Curtis[*,#,¶]

*Dept of Paediatrics, The University of Melbourne, #Infectious Diseases Unit, Dept of General Medicine, and ¶Murdoch Children's Research Institute, Royal Children's Hospital Melbourne, Parkville, Victoria, Australia.

Correspondence: M. Tebruegge, Dept of Paediatrics, The University of Melbourne, Royal Children's Hospital Melbourne, Parkville, VIC 3052, Australia. E-mail: marc.tebruegge@rch.org.au

Statement of Interest: None declared.

REFERENCES

1 Latorre I, De Souza-Galvao M, Ruiz-Manzano J, *et al.* Evaluating the non-tuberculous mycobacteria effect in the tuberculosis infection diagnosis. *Eur Respir J* 2010; 35: 338–342.
2 Connell T, Tebruegge M, Ritz N, *et al.* Interferon-gamma release assays for the diagnosis of tuberculosis. *Pediatr Infect Dis J* 2009; 28: 758–759.
3 Connell T, Tebruegge M, Ritz N, *et al.* Indeterminate interferon-γ release assay results in children. *Pediatr Infect Dis J* 2010; 29: 285–286.
4 Landi S, Held HR, Tseng MC. Comparative study of 14C-labeled purified protein derivative from various mycobacteria. *Appl Microbiol* 1970; 20: 696–709.
5 Lein AD, von Reyn CF, Ravn P, *et al.* Cellular immune responses to ESAT-6 discriminate between patients with pulmonary disease due to *Mycobacterium avium* complex and those with pulmonary disease due to *Mycobacterium tuberculosis*. *Clin Diagn Lab Immunol* 1999; 6: 606–609.
6 Gey van Pittius NC, Warren RM, van Helden PD. ESAT-6 and CFP-10: what is the diagnosis? *Infect Immun* 2002; 70: 6509–6510.
7 Bamford AR, Crook AM, Clark J, *et al.* Comparison of Interferon-gamma release assays and tuberculin skin test in predicting active tuberculosis (TB) in children in the UK – a Paediatric TB Network Study. *Arch Dis Child* 2010 95: 180–186.

DOI: 10.1183/09031936.00025510

From the authors:

We do appreciate the comments by M. Tebruegge and co-workers about our recently published manuscript in the *European Respiratory Journal* [1]. The aim of this reply is to clarify some points in order to interpret better the results of the study, given that we think there were some misunderstandings.

In vitro assays for measuring interferon (IFN)-γ released by the T-cells after specific *Mycobacterium tuberculosis* stimulation have demonstrated promising results in adults and also in children for diagnosing tuberculosis (TB) infection [2, 3]. However, there are discordant results between IFN-γ based assays and the tuberculin skin test (TST) that require clarification in order to assess the real utility of the *in vitro* tests in the management of patients [2, 4].

In our study we determined the potential role of non-tuberculous mycobacteria (NTM) sensitisation in children as a factor of discordant results between TST and an *in vitro* T-cell based assay (T.SPOT.*TB*; Oxford Immunotec, Oxford, UK). We enrolled 21 non-bacille Calmette–Guérin vaccinated paediatric patients for suspicion of latent TB infection (LTBI). These patients yielded a positive TST and a negative T.SPOT.*TB*. Cells were stimulated with *Mycobacterium avium* sensitin (MAS) and the presence of reactive T-cells was determined by an *ex vivo* enzyme-linked immunospot assay. From the 16 patients with a valid result, in 10 cases we obtained a positive ELISPOT result after stimulation with MAS.

Our main disagreement with the argumentation by M. Tebruegge and co-workers resides in the fact that we are not using MAS for distinguishing *M. tuberculosis* from NTM infection. For this objective, we used the specific *M. tuberculosis* RD1 antigens included in the T.SPOT.*TB* test, and, as no response against RD1 antigens was obtained, we assessed T-cell sensitisation against MAS antigens to investigate if NTM sensitisation could be responsible for TST positivity. Indeed, LEIN *et al.* [5], also referred to in the letter by M. Tebruegge and co-workers, obtained significant immune responses to ESAT-6 from 59% of pulmonary *M. tuberculosis* disease patients diagnosed, but no response was obtained from patients with *M. avium* complex pulmonary disease.

However, we agree with M. Tebruegge and co-workers that in some cases alternative explanation can also be possible. Given that MAS are not totally specific, and cross-reactions with other mycobacteria species have been described, we cannot totally exclude the possibility that we are detecting, in some cases, a response of specific T-cells against some *M. tuberculosis* antigens different from ESAT-6 and CFP-10; or a false-negative result of the T.SPOT.*TB*.

On the one hand, M. Tebruegge and co-workers have shown some concerns about our group of children with positive TST and positive T.SPOT.*TB* where 50% of children responded to the MAS. The results are in concordance with the known cross-reaction between MAS and other mycobacteria. Nevertheless, we cannot totally reject simultaneous infection of *M. tuberculosis* and NTM. Furthermore, these results are in total agreement with those obtained by LEIN *et al.* [5], where they found response against MAS in 24 out of 27 *M. tuberculosis* disease patients.

On the other hand, we want to point out that the main MAS positive results were obtained in children enrolled during LTBI screening at school with TST induration >5 mm and <10 mm. In all these children a complete medical exploration, including clinical and radiographic studies, was performed, and active TB was excluded. In the subsequent contact tracing studies no index case was found. Based on the classical studies performed by NYBOE [6], the main guidelines in this kind of child population consider as a cut-off for *M. tuberculosis* infection a TST induration ⩾10 mm, in order to avoid false-positive TST results induced by NTM immunisation [7]. Nevertheless, indurations >15 mm [8] and 20 mm [9] have been reported in children with NTM infections. Therefore, our results reinforce, in part, the guidelines in that unnecessary chemoprophylaxis treatment in this unexposed population could be avoided, and that IFN-γ based assays could help to confirm a positive TST result.

Children from contact-tracing studies truly exposed to an active TB case merit special consideration as they can develop the disease very quickly after primary infection, with the most severe forms prevailing in younger children [10]. For this child population we did not recommend withholding the chemoprophylaxis;

but we stated that, according to our results, IFN-γ based assays could reduce unnecessary chemoprophylaxis in non-*M. tuberculosis* infected children. In fact, BAKIR *et al.* [11] in a recent study concluded that a positive IFN-γ based assay result predicted the development of active TB as well as the TST, allowing more focused preventive therapy to fewer contacts.

In conclusion, we believe our results provide enough evidence that previous NTM sensitisation induces false-positive results in the TST for diagnosing LTBI; but, we also strongly agree with TEBRUEGGE *et al.* that additional studies are needed in order to clarify different issues related to the discordant IFN-γ based assay results, and to assess the real utility in the management and benefit of a child population.

I. Latorre[*,¶,+], M. De Souza-Galvão[¶,§], J. Ruiz-Manzano[#,¶,+], A. Lacoma[*,¶,+], C. Prat[*,¶,+], N. Altet[§], V. Ausina[*,¶,+] and J. Domínguez[*,¶,+]

*Servei de Microbiologia, and #Servei de Pneumologia, Hospital Universitari ''Germans Trias i Pujol'', Fundació Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, +Ciber Enfermedades Respiratorias, Instituto de Salud Carlos III, Badalona, ¶Universitat Autònoma de Barcelona, Bellaterra, and §Unidad de Prevención y Control de la Tuberculosis de Barcelona, Barcelona, Spain.

Correspondence: J. Domínguez, Servei de Microbiologia, Fundació Institut d'Investigació en Ciències de la Salut ''Germans Trias i Pujol'', Carretera del Canyet s/n, 08916 Badalona, Barcelona. Spain. E-mail: jadomb@gmail.com

Statement of Interest: A statement of interest for J. Domínguez can be found at www.erj.ersjournals.com/misc/statements.dtl

REFERENCES

1 Latorre I, De Souza-Galvao M, Ruiz-Manzano J, *et al.* Evaluating the non-tuberculous mycobacteria effect in the tuberculosis infection diagnosis. *Eur Respir J* 2010; 35: 338–342.
2 Domínguez J, Ruiz-Manzano J, De Souza-Galvao M, *et al.* Comparison of two commercially available gamma interferon blood tests for immunodiagnosis of tuberculosis. *Clin Vaccine Immunol* 2008; 15: 168–171.
3 Domínguez J, Latorre I, Altet N, *et al.* Interferon-gamma-release assays to diagnose TB infection in immunocompromised individual. *Expert Rev Respir Med* 2009; 3: 309–327.
4 Connell TG, Ritz N, Paxton GA, *et al.* A three-way comparison of tuberculin skin testing, QuantiFERON-TB gold and T-SPOT.TB in children. *PLoS ONE* 2008; 3: e2624.
5 Lein AD, von Reyn CF, Ravn P, *et al.* Cellular immune responses to ESAT-6 discriminate between patients with pulmonary disease due to *Mycobacterium avium* complex and those with pulmonary disease due to *Mycobacterium tuberculosis*. *Clin Diagn Lab Immunol* 1999; 6: 606–609.
6 Nyboe J. Interpretation of tuberculosis infection age curves. *Bull World Health Organ* 1957; 17: 319–339.
7 Ruiz-Manzano J, Blanquer R, Calpe JL, *et al.* SEPAR Guidelines. Diagnostic and treatment of tuberculosis. *Arch Bronconeumol* 2008; 44: 551–566.
8 Detjen AK, Keil T, Roll S, *et al.* Interferon-gamma release assays improve the diagnosis of tuberculosis and nontuberculous mycobacterial disease in children in a country with a low incidence of tuberculosis. *Clin Infect Dis* 2007; 45: 322–328.
9 Haimi-Cohen Y, Zeharia A, Mimouni M, *et al.* Skin indurations in response to tuberculin testing in patients with nontuberculous mycobacterial lymphadenitis. *Clin Infect Dis* 2001; 33: 1786–1788.
10 Lalvani A, Millington KA. T cell-based diagnosis of childhood tuberculosis infection. *Curr Opin Infect Dis* 2007; 20: 264–271.
11 Bakir M, Millington KA, Soysal A, *et al.* Prognostic value of a T-cell-based, interferon-gamma biomarker in children with tuberculosis contact. *Ann Intern Med* 2008; 149: 777–787.

DOI: 10.1183/09031936.00033510

ANNEX V

Scandinavian Journal of Infectious Diseases, 2012; 44: 161–167

informa
healthcare

ORIGINAL ARTICLE

Specific Mycobacterium tuberculosis T cell responses to RD1-selected peptides for the monitoring of anti-tuberculosis therapy

IRENE LATORRE[1,2,3], NEUS ALTET[4], MALÚ DE SOUZA-GALVÃO[2,4], JOAN RUIZ-MANZANO[2,3,5], ALICIA LACOMA[1,2,3], CRISTINA PRAT[1,2,3], MIGUEL PÉREZ[1], VICENTE AUSINA[1,2,3] & JOSE DOMÍNGUEZ[1,2,3]

From the [1]Servei de Microbiologia, Hospital Universitari Germans Trias i Pujol, Institut en Ciències de la Salut Germans Trias i Pujol, Badalona, [2]Universitat Autònoma de Barcelona, Bellaterra, [3]Ciber Enfermedades Respiratorias, Instituto de Salud Carlos III, Badalona, [4]Unitat de Prevenció i Control de la Tuberculosi de Barcelona, and [5]Servei de Pneumologia, Hospital Universitari Germans Trias i Pujol, Institut en Ciències de la Salut Germans Trias i Pujol, Badalona, Spain

Abstract

Background: Recently, a selection of HLA class II-restricted epitopes of ESAT-6 and CFP-10 Mycobacterium tuberculosis proteins from the region of difference (RD) 1 have been described. We have evaluated the host interferon-gamma (IFN-g) T cell response to these RD1 selected peptides at the beginning and during anti-tuberculosis therapy. *Methods:* We studied 29 pulmonary TB patients enrolled at the beginning of treatment and 24 enrolled during treatment. We performed T-SPOT.TB and ELISPOT with RD1 selected peptides. *Results:* Patients included at the beginning of treatment responded producing IFN-g after antigen stimulation in 89.7% by means of T-SPOT.TB and 79.3% by means of RD1 selected ELISPOT. In contrast, for patients included during treatment the percentages were 87.5% and 25%, respectively. Differences in sensitivities between patients evaluated at the beginning and during treatment were only significant for RD1 selected ELISPOT ($p < 0.0001$). *Conclusions:* The host immune response to RD1 selected peptides is lower than to T-SPOT.TB during therapy. Immunological assays based on RD1 selected peptides may be useful tools for studying the immune response during anti-tuberculosis therapy.

Keywords: *Mycobacterium tuberculosis, IFN-g assays, RD1 selected peptides, monitoring anti-TB treatment, immune response*

Introduction

The basis for controlling the spread of tuberculosis (TB) consists of the ability to diagnose it in its early stages and to treat patients with active TB adequately [1]. However, the diagnosis of TB is still difficult in certain cases. Also, the monitoring of anti-TB treatment based solely on clinical findings is difficult in patients with TB. There is no evidence, besides clinical findings, of a specific marker for the evaluation and/or prediction of the correct progress of patients during therapy and for the assessment of an adequate treatment.

With regard to diagnosis, the tuberculin skin test (TST) has been used widely as a complementary tool for the diagnosis of TB. The TST measures a T cell-mediated delayed-type hypersensitivity to purified protein derivative (PPD). However, it is well known that the TST has poor specificity in bacille Calmette–Guérin (BCG) vaccinated individuals and in patients infected by non-tuberculous mycobacteria (NTM).

In recent years, new assays based on the in vitro detection of interferon-gamma (IFN-g) secreted by sensitized T cells to specific Mycobacterium tuberculosis antigens have been developed. IFN-g assays provide a significant improvement for the diagnosis of latent tuberculosis infection (LTBI) in adults and children [2–8], and numerous studies have evaluated the utility of these assays in diagnosing active TB [9–12]. These IFN-g immune-assays use specific

Correspondence: J. Domínguez, Servei de Microbiologia, Institut en Ciències de la Salut "Germans Trias i Pujol", Carretera del Canyet s/n, 08916 Badalona, Barcelona, Spain. Tel: +34 93 497 88 94. Fax: +34 93 497 88 95. E-mail: jadomb@gmail.com

(Received 3 January 2011; accepted 24 July 2011)

ISSN 0036-5548 print/ISSN 1651-1980 online © 2012 Informa Healthcare
DOI: 10.3109/00365548.2011.611167

M. tuberculosis antigens that are encoded in the genomic region of difference (RD) 1 (early-secreted antigenic target protein (ESAT-6) and 10-kD culture filtrate protein (CFP-10)) and in RD11 (TB7.7 (Rv2654)) [13,14]. These antigens are based on specific M. tuberculosis overlapping peptides spanning the whole ESAT-6 and CFP-10 proteins [15] and they are not present in the major ity of NTM and BCG strains. Nowadays, there are 2 commercial assays that detect IFN-g secreted by specific T cells: Quantiferon-TB Gold In-Tube (QFN-G-IT, Cellestis Ltd, Carnegie, Australia) and T-SPOT.TB (Oxford Immunotec Ltd, Abingdon, UK).

Regarding the monitoring of treatment, the short period of T cell incubation with specific M. tuberculosis antigens in conventional IFN-g assays reflects the frequencies of effector T cells that are related to the antigen load. As a result, the number of effector T cells and the amount of IFN-g released could be useful for monitoring anti-TB treatment [16]. The impact of anti-TB treatment in reducing the IFN-g response has been described elsewhere [9,17–19]. However, other studies have shown an increase in response during treatment [20–22].

In order to better control the spread of TB, it is necessary to identify new antigenic targets to improve knowledge of the host response against M. tuberculosis during active disease, and the kinetics during treatment.

Recently, RD1 selected peptides have been described [15,23]. They are a selection of HLA class II-restricted epitopes of ESAT-6 and CFP-10 M. tuberculosis proteins, which were identified by quantitative implemented HLA peptide-binding motif analysis and are associated with the response to active TB. In addition, it has also been described that the IFN-g response after RD1 selected peptides stimulation falls below the detection level in those patients responding to treatment [24].

Therefore, the aim of the present study was to determine the host IFN-gT cell responses to these RD1 selected peptides by means of an experimental ELISPOT in patients with active TB, both at the moment of diagnosis and during TB therapy, and to compare the results with a commercial IFN-g-based assay (T-SPOT.TB).

Materials and methods

Study population

We studied a total of 81 adult patients who attended the Hospital Universitari Germans Trias i Pujol and the Unitat de Prevenció i Control de la Tuberculosi of Barcelona. The main demographic characteristics of patients included in the study are summarized in Table I. Ethics committee approval was obtained from both institutions, as well as informed consent from all patients. A detailed patient questionnaire was used to collect the following data: previous TST, BCG vaccination status, history of prior TB, chest radiography, and other medical conditions. For those patients with active TB, microbiology results and details of anti-TB treatment were collected. Only patients with a BCG scar were considered vaccinated. None of the patients had immunosuppression.

Patients at different stages of anti-TB treatment – at the beginning and during treatment – were included in the study in order to evaluate the effect of antibiotic therapy on the IFN-g immune response. For logistical reasons, it was impossible to perform a longitudinal follow-up of the patients diagnosed at the beginning of therapy.

Patients were classified into 3 different groups: group 1 consisted of 29 active pulmonary TB patients at the beginning of anti-TB treatment. Patients were untreated or had received less than 2 weeks of therapy. Group 2 consisted of 24 active pulmonary TB patients during their anti-TB treatment. These patients were different from those in group 1. Ten patients had received between 2 weeks and 4 months of therapy, and the remaining 14 had received more than 5 months of therapy. In both groups, all TB cases were confirmed by culture. All patients enrolled during treatment had an appropriate clinical response to anti-TB treatment (clinical and radiological improvement, and culture negativity). Group 3 consisted of 28 healthy individuals, without any previous positive TST, who were identified by

Table I. Demographic characteristics of patients included in the study.

	Group of patients		
	Active pulmonary TB		
Variable	Beginning treatment	During treatment	Healthy controls
Gender, *n* (%)			
Male	18 (62.1)	21 (87.5)	12 (42.9)
Age median ± SD, y	35 ± 17.7	39.5 ± 15.08	28 ± 8.04
BCG-vaccinated, *n* (%)			
Yes	6 (20.7)	3 (12.5)	4 (14.3)
Birth country, *n* (%)			
Immigrants from countries with high prevalence of TB infection	16 (55.2)	11 (45.8)	4 (14.3)
History of previous active TB	0 (0)	0 (0)	0 (0)
History of prior diagnosed LTBI	0 (0)	0 (0)	0 (0)

TB, tuberculosis; SD, standard deviation; BCG, bacille Calmette–Guérin; LTBI, latent TB infection.

contact tracing studies and who had been exposed to a smear-positive TB case. These patients were enrolled as controls, with negative commercial IFN-γ assays and negative TST.

None of the patients in any of the 3 groups enrolled had a history of prior or past active TB or diagnosed LTBI.

Tuberculin skin test

In accordance with our national guidelines [25], the TST was performed using 2 intradermal tuberculin units of PPD RT23 (Statens Serum Institute, Copenhagen, Denmark). The tuberculin was administered following the Mantoux method, and the size of the induration was measured by an experienced medical doctor 48–72 h after administration. TST was performed at the beginning of treatment for active pulmonary TB patients. TST indurations ≥5 mm were classified as positive in patients diagnosed with active TB and contacts, in accordance with the Spanish Society of Pneumology and Thoracic Surgery guidelines [26].

T-SPOT.TB

Eight millilitres of blood were drawn in a vacutainer CPT tube (Beckton Dickinson Diagnostics, Franklin Lakes, NJ, USA) for the isolation of peripheral blood mononuclear cells (PBMCs). Later, PBMCs were washed twice by centrifugation with RPMI medium (Invitrogen, Auckland, New Zealand) and resuspended in AIM-V medium (Invitrogen, Auckland, New Zealand). Finally, viable cells were counted with an inverted microscope using the trypan blue method.

The IFN-γ produced by PBMCs was detected by T-SPOT.TB (Oxford Immunotec Limited, Abingdon, UK), based in an enzyme-linked immunospot assay (ELISPOT); testing was performed following the manufacturer's recommendations. Cells were incubated with medium alone (negative control), phytohemagglutinin (PHA) (positive control), ESAT-6 (panel A), and CFP-10 (panel B) in different wells pre-coated with antibodies against IFN-γ. The assay requires a total of 250,000 cells per well. The results were interpreted according to the manufacturer's recommendations, and expressed as spot-forming cells (SFCs)/million PBMCs.

Reactive antigen-specific T cells were revealed as spots on the well, and scored by naked eye with the support of an automated AID ELISPOT plate reader (Lector AID Elispots, Autoimmun Diagnostiks GmbH, Germany). Test wells were scored as positive if there were >24 SFCs/million PBMCs to 1 or both antigen panels. The result was considered indeterminate if the response to both antigen panels was negative and if the number of SFCs/million PBMCs in the positive control well was <80. In addition, the immune response was also considered indeterminate if the number of SFCs/million PBMCs in the negative control was higher than 40. The number of SFCs/million PBMCs in the negative control was subtracted from the number of SFCs/million PBMCs in the stimulated cultures.

ELISPOT with RD1 selected peptides

PBMCs from each patient were incubated overnight in an ELISPOT plate with a pool of selected ESAT-6 (at 10 μg/ml each) and CFP-10 (at 2 μg/ml each), as described previously [27]. PBMCs were also incubated with PHA (positive control, at 5 μg/ml) and with dimethyl sulfoxide (DMSO; negative control, at 10 μg/ml; peptides were diluted in this reagent). The assay requires a total of 250,000 cells per well. The RD1 selected antigens were kindly provided by D. Goletti from the National Institute for Infectious Diseases of Rome (Italy).

According to previous data [15], the results were interpreted as positive if >34 SFCs/million PBMCs on ESAT-6 and/or CFP-10 selected peptide wells were obtained. The result was considered valid if SFCs/million PBMCs were >60 when incubated with PHA and <40 when incubated with DMSO (negative control). The number of SFCs/million PBMCs in the negative control was subtracted from the number of SFCs/million PBMCs in the stimulated cultures.

Statistical analysis

Comparison of the number of spots and the IFN-γ released, and conversion to negative responses between different groups of patients was performed by Mann–Whitney U-test analysis. Differences were considered significant when *p*-values were less than 0.05.

The number of ESAT-6 and CFP-10 SFCs/million PBMCs were summed and considered as an overall RD1 response, as well as for RD1 selected peptides [28]. All analyses were undertaken using SPSS statistical software for Windows (SPSS v. 15.0; SPSS Inc., Chicago; IL, USA). Graphical representations were done with GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA).

Results

Patients at the beginning and during anti-TB treatment

The overall sensitivities of T-SPOT.TB and RD1 selected ELISPOT in patients included at the

beginning of treatment were 89.7% and 79.3%, respectively (Table II). Differences in sensitivities between RD1 selected ELISPOT and T-SPOT.TB or TST for those patients enrolled at the beginning of treatment were not significant ($p = 0.102$ and $p = 0.129$, respectively).

In contrast, when evaluating patients during treatment, the sensitivities decreased to 87.5% and 25%, respectively (Table II). Differences in sensitivity between patients evaluated at the beginning and during treatment were only significant for RD1 selected ELISPOT ($p < 0.0001$). Positive TST results were obtained for 92.3% (24/26 patients) of patients at the beginning of treatment. Only 7 patients included during treatment were TST tested at the moment of diagnosis, and all of them were positive.

The rate of indeterminate results found in active TB patients enrolled at the beginning of therapy was 6.9% (2/29) for T-SPOT.TB and 10.3% (3/29) for RD1 selected peptides ELISPOT; the difference was not significant ($p = 0.317$). Indeterminate results in active TB patients enrolled during treatment were not obtained.

In patients from group 2, a relationship between IFN-γ response on T-SPOT.TB or RD1 selected ELISPOT and the length of anti-TB treatment (more than 5 months and less than 4 months of treatment) was not observed.

Median numbers of responder T cells in TB patients at the beginning of therapy with regard to those studied during treatment were significantly different for both RD1 antigens included in T-SPOT.TB and RD1 selected peptides ($p = 0.013$ and $p < 0.0001$, respectively) (Figure 1). Furthermore, in active TB patients enrolled during treatment, responses for RD1 selected ELISPOT were significantly lower than for T-SPOT.TB ($p < 0.0001$).

Table II. Results of T-SPOT.TB and RD1 selected peptides in the different groups of patients.

	Group of patients		
	Active pulmonary TB		
	Beginning treatment	During treatment	Healthy controls
T-SPOT.TB			
Positive, *n* (%)	26 (89.7)	21 (87.5)	–
Negative, *n* (%)	1 (3.4)	3 (12.5)	28 (100)
Indeterminate, *n* (%)	2 (6.9)	–	–
RD1 selected peptides ELISPOT			
Positive, *n* (%)	23 (79.3)	6 (25)	4 (14.3)
Negative, *n* (%)	3 (10.3)	18 (75)	24 (85.7)
Indeterminate, *n* (%)	3 (10.3)	–	–

TB, tuberculosis.

Healthy controls

The proportion of negative results by RD1 selected peptides ELISPOT in those individuals with negative commercial IFN-γ assays and TST was 85.7% (Table II). In addition, in this group of patients we found 4 positive results with RD1 selected peptides. No indeterminate results were observed in this group.

Moreover, the median number of responder T cells in TB patients at the beginning of therapy in relation to healthy controls was significantly different for both in vitro assays ($p < 0.0001$ in both cases). However, when comparing TB patients during therapy with regard to healthy controls these differences were only significant for T-SPOT.TB ($p < 0.0001$) (Figure 1).

Discussion

We present the results of an experimental ELISPOT based on ESAT-6 and CFP-10 selected peptides in active pulmonary TB patients enrolled at the time of TB diagnosis and during anti-TB treatment, in order to explore the specific IFN-γ M. tuberculosis T cell responses during anti-TB therapy.

The utility of HLA class II-restricted RD1 selected peptides has been evaluated in the immunodiagnosis of active TB. Vincenti et al. [23] studied the response to ESAT-6 selected peptides, obtaining a sensitivity of 74% and a specificity of 100%. Furthermore, Goletti et al. [29] measured the diagnostic accuracy of ESAT-6 and CFP-10 selected peptides for diagnosing TB disease using ELISPOT, and they reported a sensitivity of 70% and a specificity of 91%. The associated RD1 selected response with active TB has also recently been described [30].

The sensitivity of ELISPOT based on selected peptides reported in our study was lower compared with T-SPOT.TB and TST, although the difference was not statistically significant. These differences in sensitivities among the 3 assays may be related to the amount and the composition of epitopes that are employed in the different tests. Nevertheless, in a recent multi-centre study by the Tuberculosis Network European Trials group (TBNET) [27] it was found that the combined use of negative results of commercial IFN-γ tests or novel assays based on selected peptides with TST may allow rapid exclusion of active TB.

Carrara et al. [24] studied the response to ESAT-6 selected peptides in 18 patients with microbiologically confirmed TB when they started treatment and at 3 months after therapy. All patients had positive results at the time of the diagnosis, and only 5 patients presented positive results 3 months later.

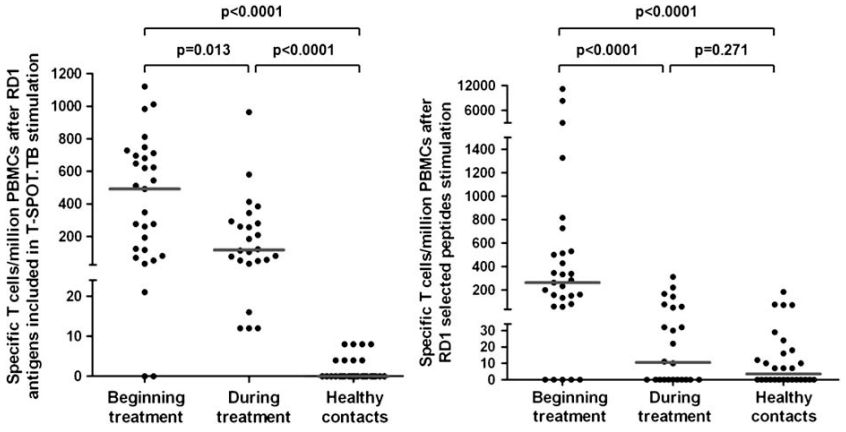

Figure 1. Number of responder T cells by RD1 antigens included in T-SPOT.TB and RD1 selected peptides in active TB patients included at the beginning and during anti-TB treatment and healthy controls. The median number of responder T cells from each group is represented by the grey line.

Additionally, Goletti et al. [31] found a significant decrease in IFN-g response to selected RD1 peptides after completion of therapy in 12 patients co-infected with HIV and M. tuberculosis. Our study confirms previous studies [24,31] with the finding that RD1 selected responder T cells are lower during anti-TB therapy than at the moment of diagnosis. Further, we also compared the results of the experimental RD1 selected ELISPOT with T-SPOT.TB in active TB patients enrolled during treatment, and the responses for RD1 selected ELISPOT were significantly lower than for T-SPOT.TB ($p < 0.0001$). Our results also show that differences in responder T cells between TB patients enrolled during therapy with regard to healthy controls were only significant for T-SPOT.TB ($p < 0.0001$), suggesting that the IFN-g response in the 2 groups mentioned above reached similar values after RD1 selected peptides stimulation.

These findings suggest that the immunological assay based on RD1 selected peptides correlates better with bacterial burden during TB treatment than T-SPOT.TB, and could also be useful for monitoring M. tuberculosis replication.

In this sense, memory T cells are able to persist over time, even after bacterial clearance, but they are not able to produce IFN-g during the short period of time that they are incubated with the specific antigens. In contrast, effector T cells are able to release IFN-g during this short exposure; therefore, their frequency could be directly proportional to antigen and bacterial load. This is probably the reason why IFN-g responses decrease during an adequate anti-TB treatment [16].

Controversially, it has recently been proposed that the single quantification of IFN-g responses to conventional antigens is insufficient as a biomarker of mycobacterial load and active TB disease status. In this regard, promising results of IFN-g measurements with other cytokines like interleukin (IL)-2, IFN-g inducible protein (IP)-10, or monocyte chemotactic protein (MCP)-2 have been obtained [32–35]. However, it has recently been described that only IFN-g responses to RD1 selected peptides are associated with active TB, and although the detection of other biomarkers like IL-2, IP-10, and MCP-2 with these selected peptides have also been associated with active TB, this fact did not improve the accuracy of the assay [36]. In fact, it has recently been described that both IP-10 and IFN-g responses to RD1 selected peptides could be useful biomarkers for monitoring the efficacy of therapy in patients with active TB. They have shown how the response significantly decreases after 6 months of treatment [37].

Furthermore, immunogenicity of selected peptides in individuals with LTBI who have recently been exposed to M. tuberculosis has been described during the monitoring of isoniazid prophylaxis. In this study, RD1 selected peptides had a higher decrease in IFN-g response compared to RD1 antigens [38]. Interestingly, we found 4 positive results with ELISPOT based on selected peptides in

healthy controls (4/28 patients) coming from contact tracing studies and who had been exposed to a smear-positive TB index case. These results may suggest that the immunological assay based on selected peptides could be useful in the diagnosis of LTBI, and, therefore, that it correlates well with bacterial burden.

The main limitations of our study are the lack of longitudinal follow-up of patients included at the beginning of treatment, and that the TST was only performed on a subgroup of the included TB patients (26/29 patients at the beginning of treatment and 7/24 patients during treatment). However, from our point of view, results observed with RD1selected peptides are consistent enough to strengthen the idea that immunological assays could be useful tools for monitoring the effect of anti-TB therapy.

Although the role of immunological IFN-g assays based on selected peptides for monitoring active TB treatment requires further research, especially in patients who do not respond to therapy, our study has shown that specific M. tuberculosis T cell responses to RD1-selected peptides are lower during anti-TB treatment than at the moment of diagnosis. In summary, immunological assays based on selected peptides correlate well with bacterial burden and could be a potential tool for studying the host immune response during anti-TB therapy.

Acknowledgements

We thank the nursing staff of the Unitat de Prevenció i Control de la Tuberculosi of Barcelona, and C. Ramil, L. Haba, and M.A. Cuesta for technical assistance. The authors are members of the TBNET group. J. Domínguez is a researcher funded by the Miguel Servet programme of the Instituto de Salud Carlos III (Spain). This work was supported by a grant from the Sociedad Española de Neumología y Cirugía Torácica; the Societat Catalana de Pneumologia (SOCAP); Fundació Catalana de Pneumologia (FUCAP); and Instituto de Salud Carlos III-RETIC (RD06/0018), Red de Actividades Preventivas y Promoción de la Salud (REDIAP).

Declaration of interest: None of the investigators have relevant financial interest in or a financial conflict with the subject matter or materials discussed in this manuscript. None of the scientific societies, neither Cellestis (Carnegie, Australia) nor Oxford Immunotec (Abingdon, UK) had a role in the study design, conduct, collection, management, analysis, or interpretation of the data, or preparation, review, or approval of the manuscript.

References

[1] World Health Organization. Global tuberculosis control—epidemiology, strategy, financing. WHO/HTM/TB/2009.411. Geneva, Switzerland: WHO; 2009.
[2] Dominguez J, Latorre I, Altet N, Mateo L, De Souza-Galvão M, Ruiz-Manzano J, et al. IFN-gamma release assays to diagnose tuberculosis infection in the immunocompromised individual. Expert Rev Resp Med 2009;3:309–27.
[3] Dominguez J, Ruiz-Manzano J, De Souza-Galvao M, Latorre I, Mila C, Blanco S, et al. Comparison of two commercially available gamma interferon blood tests for immunodiagnosis of tuberculosis. Clin Vaccine Immunol 2008;15:168–71.
[4] Ewer K, Deeks J, Alvarez L, Bryant G, Waller S, Andersen P, et al. Comparison of T-cell-based assay with tuberculin skin test for diagnosis of Mycobacterium tuberculosis infection in a school tuberculosis outbreak. Lancet 2003;361:1168–73.
[5] Casas I, Latorre I, Esteve M, Ruiz-Manzano J, Rodriguez D, Prat C, et al. Evaluation of interferon-gamma release assays in the diagnosis of recent tuberculosis infection in health care workers. PLoS One 2009;4:e6686.
[6] Lalvani A, Pathan AA, Durkan H, Wilkinson KA, Whelan A, Deeks JJ, et al. Enhanced contact tracing and spatial tracking of Mycobacterium tuberculosis infection by enumeration of antigen-specific T cells. Lancet 2001;357:2017–21.
[7] Latorre I, De Souza-Galvão M, Ruiz-Manzano J, Lacoma A, Prat C, Altet N, et al. Evaluating the non-tuberculous mycobacteria effect in the tuberculosis infection diagnosis. Eur Respir J 2010;35:338–42.
[8] Altet-Gómez N, De Souza-Galvao M, Latorre I, Milá C, Jimenez MA, Solsona J, et al. Diagnosing TB infection in children: analysis of discordances using in vitro tests and tuberculin skin test. Eur Respir J 2011;37:1166–74.
[9] Dominguez J, De Souza-Galvao M, Ruiz-Manzano J, Latorre I, Prat C, Lacoma A, et al. T-cell responses to the Mycobacterium tuberculosis-specific antigens in active tuberculosis patients at the beginning, during, and after antituberculosis treatment. Diagn Microbiol Infect Dis 2009;63:43–51.
[10] Lalvani A. Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy. Chest 2007;131:1898–906.
[11] Pai M, Menzies D. Interferon-gamma release assays: what is their role in the diagnosis of active tuberculosis? Clin Infect Dis 2007;44:74–7.
[12] Latorre I, De Souza-Galvao M, Ruiz-Manzano J, Lacoma A, Prat C, Fuenzalida L, et al. Quantitative evaluation of T-cell response after specific antigen stimulation in active and latent tuberculosis infection in adults and children. Diagn Microbiol Infect Dis 2009;65:236–46.
[13] Andersen P, Munk ME, Pollock JM, Doherty TM. Specific immune-based diagnosis of tuberculosis. Lancet 2000;356:1099–104.
[14] Brock I, Weldingh K, Leyten EM, Arend SM, Ravn P, Andersen P. Specific T-cell epitopes for immunoassay-based diagnosis of Mycobacterium tuberculosis infection. J Clin Microbiol 2004;42:2379–87.
[15] Goletti D, Vincenti D, Carrara S, Butera O, Bizzoni F, Bernardini G, et al. Selected RD1 peptides for active tuberculosis diagnosis: comparison of a gamma interferon whole-blood enzyme-linked immunosorbent assay and an enzyme-linked immunospot assay. Clin Diagn Lab Immunol 2005;12:1311–6.

[16] Lalvani A. Counting antigen-specific T cells: a new approach for monitoring response to tuberculosis treatment? Clin Infect Dis 2004;38:757–9.

[17] Pathan AA, Wilkinson KA, Klenerman P, McShane H, Davidson RN, Pasvol G, et al. Direct ex vivo analysis of antigen-specific IFN-gamma-secreting CD4 T cells in Mycobacterium tuberculosis-infected individuals: associations with clinical disease state and effect of treatment. J Immunol 2001;167:5217–25.

[18] Lalvani A, Nagvenkar P, Udwadia Z, Pathan AA, Wilkinson KA, Shastri JS, et al. Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent Mycobacterium tuberculosis infection in healthy urban Indians. J Infect Dis 2001;183:469–77.

[19] Sauzullo I, Mengoni F, Lichtner M, Massetti AP, Rossi R, Iannetta M, et al. In vivo and in vitro effects of antituberculosis treatment on mycobacterial interferon-gamma T cell response. PLoS One 2009;4:e5187.

[20] Ferrand RA, Bothamley GH, Whelan A, Dockrell HM. Interferon-gamma responses to ESAT-6 in tuberculosis patients early into and after anti-tuberculosis treatment. Int J Tuberc Lung Dis 2005;9:1034–9.

[21] Ulrichs T, Anding R, Kaufmann SH, Munk ME. Numbers of IFN-gamma-producing cells against ESAT-6 increase in tuberculosis patients during chemotherapy. Int J Tuberc Lung Dis 2000;4:1181–3.

[22] Al-Attiyah R, Mustafa AS, Abal AT, Madi NM, Andersen P. Restoration of mycobacterial antigen-induced proliferation and interferon-gamma responses in peripheral blood mononuclear cells of tuberculosis patients upon effective chemotherapy. FEMS Immunol Med Microbiol 2003;38:249–56.

[23] Vincenti D, Carrara S, De Mori P, Pucillo LP, Petrosillo N, Palmieri F, et al. Identification of early secretory antigen target-6 epitopes for the immunodiagnosis of active tuberculosis. Mol Med 2003;9:105–11.

[24] Carrara S, Vincenti D, Petrosillo N, Amicosante M, Girardi E, Goletti D. Use of a T cell-based assay for monitoring efficacy of antituberculosis therapy. Clin Infect Dis 2004;38:754–6.

[25] SEPAR (Sociedad Española de Patología Respiratoria). [Guidelines for tuberculosis prevention]. Arch Bronconeumol 2002;38:441–51.

[26] Ruiz-Manzano J, Blanquer R, Calpe JL, Caminero JA, Cayla J, Dominguez JA, et al. [Diagnosis and treatment of tuberculosis]. Arch Bronconeumol 2008;44:551–66.

[27] Goletti D, Stefania C, Butera O, Amicosante M, Ernst M, Sauzullo I, et al. Accuracy of immunodiagnostic tests for active tuberculosis using single and combined results: a multicenter TBNET-Study. PLoS One 2008;3:e3417.

[28] Ewer K, Millington KA, Deeks JJ, Alvarez L, Bryant G, Lalvani A. Dynamic antigen-specific T-cell responses after point-source exposure to Mycobacterium tuberculosis. Am J Respir Crit Care Med 2006;174:831–9.

[29] Goletti D, Carrara S, Vincenti D, Saltini C, Rizzi EB, Schinina V, et al. Accuracy of an immune diagnostic assay based on RD1 selected epitopes for active tuberculosis in a clinical setting: a pilot study. Clin Microbiol Infect 2006;12:544–50.

[30] Sauzullo I, Mengoni F, Lichtner M, Rossi R, Massetti AP, Mastroianni CM, et al. QuantiFERON-TB Gold and selected region of difference 1 peptide-based assays for the detection of Mycobacterium tuberculosis infection in a cohort of patients enrolled with suspected tuberculosis. Diagn Microbiol Infect Dis 2008;62:395–401.

[31] Goletti D, Carrara S, Mayanja-Kizza H, Baseke J, Mugerwa MA, Girardi E, et al. Response to M. tuberculosis selected RD1 peptides in Ugandan HIV-infected patients with smear positive pulmonary tuberculosis: a pilot study. BMC Infect Dis 2008;8:11.

[32] Millington KA, Innes JA, Hackforth S, Hinks TS, Deeks JJ, Dosanjh DP, et al. Dynamic relationship between IFN-gamma and IL-2 profile of Mycobacterium tuberculosis-specific T cells and antigen load. J Immunol 2007;178:5217–26.

[33] Ruhwald M, Bodmer T, Maier C, Jepsen M, Haaland MB, Eugen-Olsen J, et al. Evaluating the potential of IP-10 and MCP-2 as biomarkers for the diagnosis of tuberculosis. Eur Respir J 2008;32:1607–15.

[34] Azzurri A, Sow OY, Amedei A, Bah B, Diallo S, Peri G, et al. IFN-gamma-inducible protein 10 and pentraxin 3 plasma levels are tools for monitoring inflammation and disease activity in Mycobacterium tuberculosis infection. Microbes Infect 2005;7:1–8.

[35] Ruhwald M, Dominguez J, Latorre I, Losi M, Richeldi L, Pasticci MB, et al. A multicentre evaluation of the accuracy and performance of IP-10 for the diagnosis of infection with M. tuberculosis. Tuberculosis (Edinb) 2011;91:260–7.

[36] Goletti D, Raja A, Ahamed Kabeer BS, Rodrigues C, Sodha A, Butera O, et al. IFN-gamma, but not IP-10, MCP-2 or IL-2 response to RD1 selected peptides associates to active tuberculosis. J Infect 2010;61:133–43.

[37] Syed Ahamed Kabeer B, Raja A, Raman B, Thangaraj S, Leportier M, Ippolito G, et al. IP-10 response to RD1 antigens might be a useful biomarker for monitoring tuberculosis therapy. BMC Infect Dis 2011;11:135.

[38] Goletti D, Parracino MP, Butera O, Bizzoni F, Casetti R, Dainotto D, et al. Isoniazid prophylaxis differently modulates T-cell responses to RD1-epitopes in contacts recently exposed to Mycobacterium tuberculosis: a pilot study. Respir Res 2007;8:5.