# Interferon-γ release assay in HIV-infected patients with active tuberculosis: impact of antituberculous drugs on host immune response

**Ilaria Sauzullo[1], Fabio Mengoni[1], Angela Ermocida[1], Anna P. Massetti[1], Claudia D'Agostino[1], Gianluca Russo[1], Alessandra Salotti[1], Mario Falciano[1], Vincenzo Vullo[1], Claudio M. Mastroianni[2]**

*[1]Department of Public Health and Infectious Diseases, 'Sapienza' University, Rome, Italy;*
*[2]Infectious Diseases Unit, Fondazione Eleonora Lorillard Spencer Cenci, Sapienza University, Latina, Italy*

**SUMMARY**

The objective of the study was to:
1) investigate the performance of QuantiFERON-TB Gold In-Tube (QFT-GIT) in HIV-infected patients with active tuberculosis (TB);
2) evaluate the sequential changes in QFT-GIT assay during the treatment response;
3) investigate the direct in vitro effects of antituberculous drugs on both secretion of IFN-γ and apoptosis of T cells.
Forty-four HIV-patients with active TB were enrolled and tested with QFT-GIT.
Thirteen of them were followed longitudinally by QFT-GIT, performed at baseline and six and nine months after TB-treatment onset. For in vitro experiments, cells from healthy donors and HIV-naive subjects were pretreated with four antituberculous-drugs, and then examined for IFN-γ secretion and apoptosis of T-cells. The QFT-GIT was positive in 66%, negative in 11.3% and indeterminate in 22.7%. Longitudinal analysis in 13 HIV-TB subjects showed that at therapy completion a reversion to negative response was found only in 38.4% of patients, but in 30.7% the QFT-GIT remained positive. Overall, during the anti-TB treatment no significant decrease in average IFN-γ response was observed in these patients ($p<0.001$). In vitro experiments showed that the four antituberculous-drugs, within the range of therapeutically achievable concentrations, did not exert any down-regulatory effect on IFN-γ production and did not have any effect on apoptosis of T cells from HIV naïve subjects. Despite the high rate of indeterminate results, QFT-GIT assay may represent a good tool in the diagnostic workup for active TB in HIV-patients. Although the antituberculous drugs do not have any direct effect on host immune response to mycobacterial antigen, changes in longitudinal IGRA response have been found during in vivo anti-TB treatment.

*KEY WORDS:* Tuberculosis, HIV, QFT-GIT, anti-TB treatment.

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## INTRODUCTION

The World Health Organization (WHO) estimates that there were 8.7 million new cases of tuberculosis (TB) in 2011, including 1.1 million cases (13%) among human immunodeficiency virus (HIV)-infected people, and an additional 0.4 million deaths among HIV-positive TB cases (WHO, 2012).

Prevention and treatment of TB in people living with HIV is an urgent priority for both HIV/AIDS and TB programmers. HIV infection substantially increases the risk of developing TB once infected with the bacillus, shortens the time to development of the active disease (Corbett *et al*., 2003; Shafer *et al*., 1996) and also increases the risk of multidrug-resistant tuberculosis compared to people not infected with HIV (Narain *et al*., 2004; Pelly *et al*., 2004). Tuber-

*Corresponding author*
Ilaria Sauzullo, PhD
Department of Public Health and Infectious Diseases
Sapienza University, Rome
Piazzale Aldo Moro, 1 - 00185 Rome, Italy
E-mail: ilariasauzullo@libero.it

culosis in HIV-infected individuals may have unusual features, such as atypical pulmonary manifestations, low bacterial load, difficult sample acquisition, and false-negative microbiological results raising diagnostic difficulties (Shafer *et al.*, 1996).

The tuberculin skin test (TST) was the most widely used tool worldwide for detecting TB infection, but its interpretation is hampered by possibility of an anergic response that worsens with increasing immunosuppression (Mazurek *et al.*, 2010), and by false positive results frequently ascribed to cross reactivity with Bacillus Calmette-Guerin (BCG) vaccine strains and with several nontuberculous mycobacteria (American Thoracic Society, 2000; Huebner *et al.*, 1993).

The interferon-gamma release assays (IGRAs) which measure the release of interferon-γ (IFN-γ) following stimulation of T cells with "*Mycobacterium tuberculosis antigens*" have been introduced for detection of TB infection, showing superior diagnostic accuracy in comparison to TST (Richeldi, 2006). Overall, increasing evidence has suggested that IGRAs have a higher specificity (92%-97%) than TST (56%-95%), a better correlation with surrogate measures of exposure to *M. tuberculosis*, and are less affected by BCG vaccination than the TST (Lalvani, 2007).

Although the performance of IGRAs is less affected by T-cell anergy than TST, the role of these assays in detecting TB infection in HIV-infected patients and their use for treatment monitoring has not yet been fully clarified (Chapman *et al.*, 2002; Liebeschuetz *et al.*, 2004; Hoffmann *et al.*, 2007; Karam *et al.*, 2008; Lawn *et al.*, 2007; Rangaka *et al.*, 2007).

To better define the role of IFN-γ assay in patients with HIV-TB co-infection, we undertook the present study with the following objectives:

1) to evaluate the performance of QuantiFERON-TB Gold In-Tube (QFT-GIT), one of the commercially IGRA tests, in HIV-infected patients with active TB;
2) to evaluate the sequential changes of QFT-GIT assay for the monitoring the treatment response;
3) to investigate the direct in vitro effects of antituberculous drugs on both secretion of IFN-γ and apoptosis of T cells.

## MATERIALS AND METHODS

### Study population

The study population included 44 HIV-infected patients with active TB admitted to the Department of Public Health and Infectious Diseases, Sapienza University, Rome, between September 2008-2012 and the follow-up was completed in March 2013. Baseline information for all patients is shown in Table 1.

Diagnosis of TB was made on the basis of clinical and radiological findings and was confirmed by identification of *M. tuberculosis* with microbiological methods and/or histological examination of affected tissues. All patients were treated with the four classical antituberculous drugs (rifampicin, isoniazid, pyrazinamide and ethambutol). In all patients the TST and QFT-GIT was performed at enrollment. For 13 participants venous blood samples were also collected six months after the beginning of treatment and at completion of specific treat-

TABLE 1 - *Demographic and clinical characteristics of the subjects*

| *Characteristics* | *Patients, n (%)* |
|---|---|
| N° of patients | 44 |
| Median age: years (range) | 42 (31-62) |
| Sex | |
| Male | 29 (66%) |
| Female | 15 (34%) |
| Immigrant | 30 (68.1%) |
| CD4 Median cells/μl (range) | 319 (12-1060) |
| Patients with QFT-GIT positive | 181 (100-600) |
| Patients with QFT-GIT negative | 201 (98-1060) |
| Patients with QFT-GIT indeterminate | 97 (12-327) |
| Median Viral load copies/ml (range) | 358 (50-750,000) |
| Antiretroviral treatment* | 20 (45.4%) |
| TB disease | |
| pulmonary | 27 (61.4) |
| extrapulmonary | 17 (38.6) |

Definition of abbreviations: QFT-GIT = QuantiFERON-TB Gold In-Tube; TB = tuberculosis. *These patients receiving antiretroviral treatment at TB diagnosis

ment (9 months) and were tested for specific IFN-γ responses.

The reference standard for active TB (culture positivity for *M. tuberculosis* and/or histology) and IGRA results was blinded. A blinded interpretation of TST and QFT-GIT results was also performed. For in vitro experiments with antituberculous drugs, blood samples were also collected from healthy donors and antiretroviral-naïve infected subjects. The study was approved by the institutional review board of the Department of Public Health and Infectious Diseases, Sapienza University, Rome; informed consent was obtained from all participants before blood samples were taken.

**Tuberculin skin test and QuantiFERON TB Gold-In Tube**

After blood was drawn for the QFT-GIT assay, a TST (Biocine Test PPD, Chiron, Siena, Italy) was performed according to the Mantoux method, and an induration ≥5 mm was considered positive at 72 hours.

The QFT-GIT was performed and interpreted by the same trained technicians according to the manufacturer's instructions (Cellestis Ltd, Carnegie, Australia). Based on the definitions reported by Pai *et al.* (2009) we explored one definition for QFT-GIT conversions (result changing from negative to positive: baseline IFN-γ<0.35 IU/ml, follow-up IFN-γ≥0.35 IU/ml) and one definition for QFT-GIT reversions (result changing from positive to negative: baseline IFN-γ≥0.35 IU/ml, follow-up IFN-γ<0.35 IU/ml).

**In vitro effect of antituberculous drugs on IFN-γ release**

The in vitro effect of the combination of rifampicin (RIF)/ isoniazid (INH)/ pyrazinamide (PZA)/ ethambutol (ETB) (Becton Dickinson) on IFN-γ release was determined as previously described (Sauzullo *et al.,* 2009). Briefly, aliquots of 0.5 ml heparinized blood were incubated overnight at 37°C, with 5% $CO_2$, in the presence of phytohaemagglutinin A (PHA, 5 mg/mL) and with a solution of the four antituberculous drugs at three different concentrations. On the basis of therapeutically achievable concentrations, the first concentration (C1) of combined drugs was: INH 5 mg/ml, RIF 7 mg/ml, ETB 5 mg/ml, PZA 40 mg/ml. The other concentrations were two (C2) or three (C3) times greater. All conditions were set up in triplicate wells. Positive control wells contained only PHA at 5 mg/ml. The IFN-γ release (expressed as IU/ml) in the presence or absence of the drugs was assessed by ELISA using a commercial kit according to the manufacturer's recommendations (QuantiFERON-CMI, Cellestis Ltd, Carnegie, Victoria, Australia). Results were expressed as mean [± standard deviation (S.D.)] of three different experiments using different donors.

**Effect of antituberculous drugs on apoptosis**

a) *Mononuclear cell isolation and pre-treatment with drugs:* peripheral blood mononucleated cells (PBMC) were isolated from heparinized blood using density gradient centrifugation Ficoll-Hypaque (Pharmacia Biotech). After isolation, the cells ($2\times10^6$/ml) were incubated in RPMI 1640 supplemented with 10% fetal calf serum and glutamine (2 mM) in the presence of medium alone or various concentrations of solution of four anti-TB drugs (as described above). After 18 h of incubation at 37°C under 5% $CO_2$, cell viability was measured by trypan blue exclusion.
b) *Preparation of lysates:* the cells ($2\times10^6$/ml) were centrifuged and washed twice with phosphate buffered saline (without calcium and magnesium). The pellet was resuspended in 100 ml of ice-cold lysis buffer and five cycles of freeze/thawing was done. After centrifugation at 15,000 g for 30 min at 4°C, the supernatant was then kept at -80°C until analysis.
c) *Detection of apoptosis:* Apoptosis was measured by analysis of the activity of Caspase 3 (units) in cell lysates, which was determined using the Caspase-3 Colorimetric Activity Assay Kits (Chemicon International) according to the manufacturer's recommendations. Results were expressed as mean [± standard deviation (S.D.)] of three different experiments using different donors.

**Statistical analysis**

SPSS version 13.0 for windows (SPSS Inc., *Apache Software Foundation*, Chicago, IL, USA) was used. The analysis of concordance between two tests was performed using Cohen's kappa

coefficient. The differences in values between groups were analyzed using the non-parametric Mann-Whitney *U*-test. For comparison of categorical variables or percentages, we used Fisher's exact and Chi square tests. Longitudinal analysis was evaluated with the non parametric Wilcoxon-signed-rank test. Student's t test was used for analysis of in vitro experiments. All statistical analyses were two-sided and considered significant in case of $p<0.05$.

## RESULTS

### Performance of QuantiFERON TB Gold-In Tube

Among the 44 patients with active TB, the QFT-GIT was positive in 29 (66%), negative in five (11.3%) and indeterminate in ten (22.7%) patients. The TST was positive in 24 (54.5%) and negative in 20 (45.5%) patients; the results of the two tests are shown in Figure 1.

Among 29 patients with QFT-GIT positive, 20 (69%) patients had pulmonary TB and nine (31%) extra-pulmonary TB disease. All five patients with QFT-GIT negative had extra-pulmonary TB, while among the ten patients with QFT-GIT indeterminate, seven (70%) had pulmonary TB and three (30%) extra-pulmonary TB disease.

The sensitivity of the QFT-GIT for the diagnosis of active TB in HIV-positive subjects was 85.2%, after excluding the indeterminate results, and the agreement with TST was 61.7% (k=0.04).

We found a high rate of indeterminate results (22.7%) due to an insufficient response to mitogen (PHA). The majority of these indeterminate results were in patients with a negative TST (8/10; 80%). To improve the assay sensitivity we evaluated decreasing the IFN-γ cut-off value defining a positive result to ≥0.25 IU/ml and we found that 3/10 patients with indeterminate QFT-GIT would have been reclassified as positive. Thus, lowering the cut-off we did not find an increase in the overall sensitivity.

### Longitudinal analysis of IFN-γ response during antituberculous treatment

Thirteen HIV+ patients with active TB were followed longitudinally and tested with QFT-GIT before treatment (baseline), six months after the beginning of treatment and at completion of specific treatment (9 months). At baseline the QFT-GIT was positive in nine (70%) patients and indeterminate in four (30%). Six months after anti-TB treatment onset the QFT-GIT was

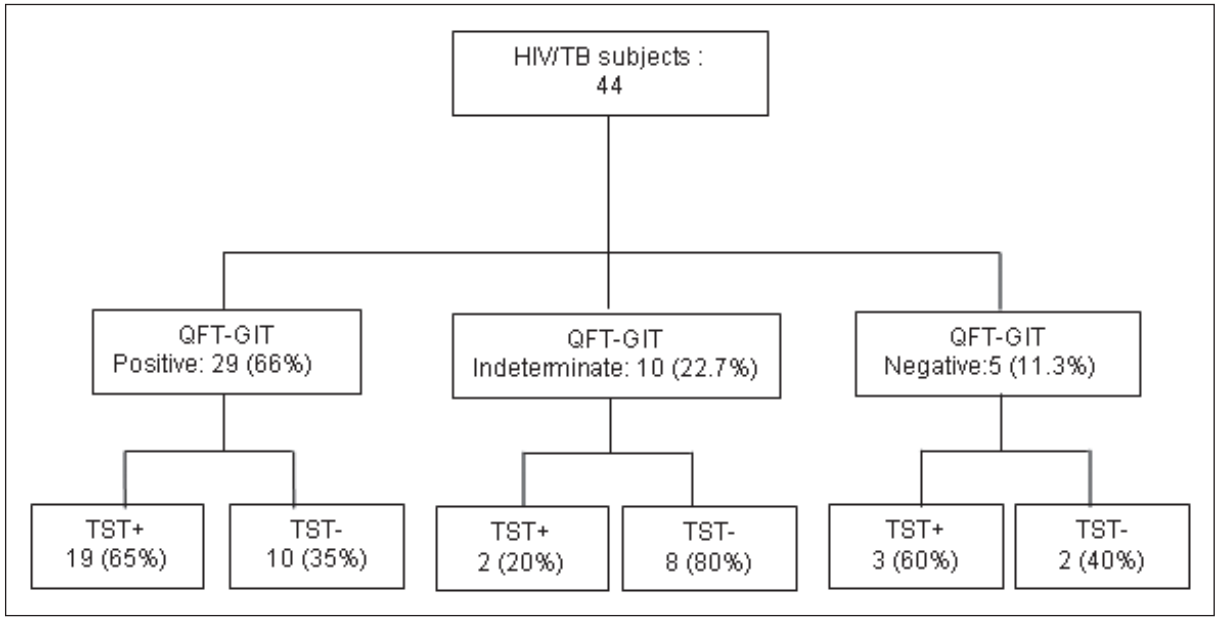


FIGURE 1 - *QFT-GIT and TST results in 44 HIV-infected patients. A response >0.35 IU/mL was considered QFT-GIT positive. QFT-GIT = QuantiFERON-TB Gold In-Tube; TST= tuberculin skin test.*

positive in eight (61.5%) patients, negative in four (30.7%) and indeterminate in one (7.8%). At the end of TB therapy a reversion to negative response was found in 5/13 (38.4%) of patients, while 4/13 (30.7%) patients remained QFT-GIT positive.

Overall, during the anti-TB treatment no significant variations of IFN-γ concentrations were observed in these patients [median (range) of IFN-γ in response to antigens: 0.4 (0.01-6.2) IU/ml at baseline; 0.68 (0.01-7.64) IU/ml at six months; 0.25 (0.02-5.66) IU/ml at nine months; p=0.1]. The individual change in IFN-γ response to specific antigens during the treatment for each participant is shown in Figure 2.

**In vitro effect of antituberculous drugs both on IFN-γ release and apoptosis**

Serial QFT-GIT testing performed during anti-TB treatment disclosed dynamic changes in IFN-γ levels. To investigate whether these fluctuations were due to the effect of the anti-TB therapy, the cells from HIV+ antiretroviral-naive subjects and healthy donors were treated in vitro with a solution of the four anti-TB agents, and then examined for IFN-γ production and apoptosis.

The levels of IFN-γ (expressed as mean ±SE, IU/ml) measured at all different drug concentrations are shown in Table 2. When the anti-TB drugs were used at concentration C1, compatible with those achieved in the serum of treated patients, the IFN-γ levels were not significantly different from controls containing only PHA for both groups of patients (p=0.9 for HIV+; p=1 for HIV-). A significant inhibitory effect was seen only at more elevated drug concentrations if compared to controls (p=0.01, for both C2 and C3 for HIV+; p=0.02, for both C2 and C3 for HIV-). No significant difference was ob-

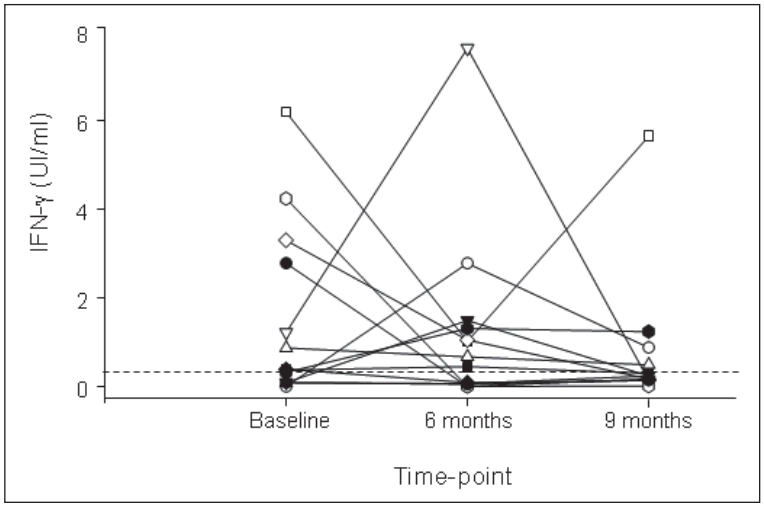


FIGURE 2 - *Changes in specific IFN-γ response in 13 HIV-TB patients following anti-TB treatment. IFN-γ response to M. tuberculosis antigens was measured by QFT-GIT before, during and at the end of antituberculous treatment. The median (range) of IFN-γ in response to antigens at each time-point was: 0.4 (0.01-6.2) IU/ml at baseline; 0.68 (0.01-7.64) IU/ml at 6 months; 0.25 (0.02-5.66) IU/ml at 9 months; p=0.1; Wilcoxon's signed-rank test. [p: for the comparison of the results at baseline vs. completion of therapy]. Dashed black line indicates the cut-off value for a positive QFT-GIT result (0.35UI/mL).*

TABLE 2 - *In vitro effect of antituberculous drugs on IFN-γ release and on apoptosis in antiretroviral-naïve HIV-infected subjects and in HIV uninfected individuals*

| *Drug concentration* | *IFN-γ release (IU/ml) (Mean + SD)* | | *P value* | *Apoptosis: activity of caspase3 (Units) (Mean +SD)* |
|---|---|---|---|---|
| | *HIV positive* | *HIV negative* | | *HIV positive* |
| Control | 28.8±7 | 25.7±11 | >0.5 | 0.162±0.03 |
| C1 | 28.1±9 | 24.4±13 | >0.5 | 0.129±0.1 |
| C2 | 9.9±2 | 4.4±3 | >0.5 | 0.086±0.002 |
| C3 | 5.3±0.9 | 2.7±1.5 | >0.5 | 0.065±0.004 |

The concentration C1 of combined drugs was: isoniazid 5 μg/ml, rifampicin 7 μg/ml, ethambutol 5 μg/ml, pyrazinamide 40 μg/ml. The other concentrations were two (C2) or three (C3) times greater. Results were expressed as mean [± standard deviation (S.D.)] of 3 different experiments using different donors.

served in the IFN-γ release between HIV+ and HIV- subjects when looking at each concentration of anti-TB drugs used (p>0.5 for each concentration).
To assess the apoptotic effect we monitored apoptosis by analysis of the activity of caspase 3, the main executioner of apoptosis, in cell lysates. We found that caspase 3 activity was not affected by anti-TB drugs in the range of concentrations used. The differences between control and drug-treated samples did not exceed 5% and failed to reach statistical significance (p>0.5 for each concentration). The treatment of cells with solution of the four anti-TB drugs did not exert any apoptotic effect on PBMC from HIV+ subjects (Tab. 2).
On the other hand, the four anti-TB drugs, within the range of therapeutically achievable concentrations, did not exert any down-regulatory effect on IFN-γ production and did not have any effect on apoptosis of PBMC from HIV naïve.

## DISCUSSION

The rapid and accurate diagnosis of active TB is particularly important in HIV-infected patients because of the accelerated progression of TB disease and higher mortality rate. Therefore, the availability of sensitive and specific easy to use tools is of critical relevance. Recent advances in the diagnosis of TB infection have led to the introduction of immunologic methods, such as IGRAs. It is known that these tests cannot distinguish between latent infection and active disease and their performance might be impaired by HIV-associated immune suppression. Nevertheless the role of IGRAs in the diagnosis of active TB in HIV-infected patients is still not well established. Recently, Metcalfe *et al.* (2011) performed a meta-analysis to evaluate the diagnostic value of IGRAs for active TB in low- and middle-income countries and concluded that neither the TST nor IGRAs have values for active TB diagnosis in adults, especially in the context of HIV co-infection. Although Chen *et al.* (2011), found that the pooled sensitivity for QFT-GIT in the diagnosis of active TB was 76.7% (95% CI: 71.6- 80.5%) after excluding indeterminate test results, the IGRAs were still not sensitive enough to rule out or rule in active TB in HIV-infected patients alone as they missed more than 20% of the patients.
In our HIV-infected adults, the QFT-GIT assay showed an excellent degree of sensitivity for detection of active TB (85.2%) after excluding indeterminate results, and a poor degree of agreement with TST (61.7%; k=0.04), due to a significant discordance between two tests resembling values reported in previous observations (Sauzullo *et al.*, 2010; Scrivo *et al.,* 2012; Scrivo *et al.,* 2013). These discordances were mainly related to false negative TST results caused by anergy or false negative QFT-GIT results in patients receiving antiretroviral therapy.
Another aspect that can further dampen the application of IGRAs in HIV-infected patients is the high rate of indeterminate results, ranging from 1.9% to 36% on the basis of clinical setting. A recent meta-analysis (Metcalfe *et al.,* 2011) showed that the pooled proportion of indeterminate QFT-GIT results were 15% (95% CI, 9-21) especially for high level of immunosuppression. The influence of CD4 T cell count on the performance of QFT-GIT represents a limitation of the IGRA test in HIV infection, particularly when the CD4 count is < 200 cells/μl. (Brock *et al.,* 2006; Sauzullo *et al.,* 2010). In our immunosuppressed patients the most indeterminate QFT-GIT results (22.7%) were overrepresented in TST-negative patients (80%). These findings suggest that the availability of an internal positive and negative control is a distinct advantage for considering the possibility of false-negative TST results in immunosuppressed patients. In addition, to improve the QFT-GIT assay sensitivity we evaluated decreasing the IFN-γ cut-off value defining positive result to ≥0.25 IU/ml and we found that 3/10 patients with indeterminate QFT-GIT would have been reclassified as positive. Thus, lowering the cut-off we did not find an increase in the overall sensitivity.
Literature data also show controversial results on the effect on the IFN-γ responses of anti-TB therapy. Some authors demonstrated that during and after anti-TB treatment, the IFN-γ response decreased or became negative (Carrara *et al.,* 2004; Dheda *et al.,* 2007; Pathan *et al.,* 2001), whereas others have reported persistently positive responses (Al-Attiyah *et al.,*

2003; Ferrand *et al.,* 2005; Pai *et al.,* 2007). It has also been suggested that negative IFN-γ responses after active TB treatment indicate successful antibiotic-induced killing of all bacilli (Pathan *et al.,* 2001). However, it is unclear if the persistence of IFN-γ responses detected in serial IGRA assay can predict clinical and microbiological treatment failure or relapse. Serial IGRA testing, when repeated any number of times does not risk boosting or sensitization (Leyten *et al.,* 2007; Sauzullo *et al.,* 2011), but it is associated with dynamic changes in IFN-γ levels with QFT-GIT conversion and reversion (Pai *et al.,* 2006). These fluctuations do not always correlate with clinical outcome (Lange *et al.,* 2012; Garcovich *et al.,* 2011; Scrivo *et al.,* 2012; Scrivo *et al.,* 2013). Moreover, T cell responses, especially weakly positive responses, tend to fluctuate over time, even in the absence of specific treatment (Ewer *et al.,* 2006; Hill *et al.,* 2007).

The present study also evaluated the impact of anti-TB therapy on IFN-γ response in patients with HIV-TB co-infection, to assess whether changes in IFN-γ levels could allow the efficacy of pharmacologic intervention for active TB to be monitored. During the follow-up we found variations of IFN-γ levels. In particular, we observed that at the end of specific therapy only five (38.4%) patients showed a QFT-GIT reversion from positive to negative, while in four (30.7%) subjects the assay remained persistently positive during the follow-up. These data are in agreement with our previous study (Sauzullo *et al.,* 2009) on immunocompetent patients who had showed a progressive decline of IFN-γ release during treatment, changing from positive to negative IGRA after their initial diagnosis. It is interesting to note that, as reported for HIV-negative subjects, about 30% of our HIV-infected subjects with active TB remained persistently QFT-GIT positive despite TB treatment. The reasons for the persistence of positive IFN-γ response in spite of successful treatment for active TB are not yet established. Some authors suggested that a delayed drop in TB IFN-γ release could be an indicator of adverse outcome and poor response to treatment (Carrara *et al.,* 2004).

To better understand the nature of the decrease in the frequency of a positive IFN-γ response observed during follow-up in active TB-HIV co-infected patients ongoing specific treatment, we performed ex vivo experiments treating the cells from healthy donors and HIV+ antiretroviral-naive with anti-TB drugs. The direct in vitro effects of anti-TB drugs on both secretion of IFN-γ and apoptosis of T cells was investigated. We showed that the pre-treatment of the cells with four anti-TB drugs, used within the range of therapeutically achievable concentrations, did not exert any down-regulatory effect on IFN-γ release in response to specific antigens. In addition, in vitro treatment did not further enhance the rate of apoptosis of T cells from HIV-infected patients Overall, our in vitro findings suggest that the decrease of IFN-γ response to mycobacterial antigens in patients with successful treatment response is not due to a direct inhibitory of antituberculous drugs or to the variability of the IGRA, but it likely reflects the reduction in bacterial burden.

In conclusion, the present study indicates that, despite the high rate of indeterminate results, QFT-GIT assay may represent a supplemental tool in the diagnostic workup for active TB in HIV-infected patients. Although the antituberculous drugs do not have any direct effect on host immune response to mycobacterial antigen, changes in longitudinal IGRA response have been found during in vivo anti-TB treatment. Future studies on a large number of patients with HIV-TB co-infection are needed to better clarify the clinical and biological significance of the decrease or persistence of IFN-γ response during anti-TB therapy.

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