

Comparison of Intracellular Cytokine Flow Cytometry and an Enzyme Immunoassay for Evaluation of Cellular Immune Response to Active Tuberculosis[∇]

Wai Lin Leung,¹ Kai Leung Law,¹ Veronica Sui Shan Leung,¹ Chi Wai Yip,¹
 Chi Chiu Leung,² Cheuk Ming Tam,² and Kai Man Kam^{1*}

Public Health Laboratory Services Branch, Centre for Health Protection, Department of Health,¹ and TB and Chest Service, Public Health Services Branch, Centre for Health Protection, Department of Health,² Hong Kong

Received 4 May 2008/Returned for modification 17 July 2008/Accepted 29 December 2008

A prospective cross-sectional blinded study of 28 patients (21 male and 7 female patients; mean age, 44 years) with suspected active tuberculosis (TB) attending a TB and chest clinic is described. Blood was taken for immune cell enumeration, a whole-blood enzyme-linked immunosorbent assay (ELISA) for the detection of gamma interferon (IFN- γ) by the QuantiFERON-TB Gold (QFT-G) assay, and intracellular cytokine flow cytometry (ICC) analysis; and sputum was simultaneously taken for bacteriological culture for *Mycobacterium tuberculosis*. Twelve healthy subjects were included as controls. The performance characteristics of the QFT-G and ICC assays for the detection of active TB were compared. Among the patients with active TB, we found (i) normal to slightly elevated peripheral CD4⁺ and CD8⁺ T-cell counts but a significant reduction in the number of NK cells; (ii) CD4⁺ T cells were the major cell type producing IFN- γ , a type 1 cytokine; (iii) small percentages of CD8⁺ T cells were also primed for IFN- γ production; (iv) the production of interleukin-4 (IL-4), a type 2 cytokine, was not prominent; and (v) the sensitivity and the specificity of the QFT-G assay were 88.2% and 18%, respectively, and those of the ICC assay were 94.1% and 36.4%, respectively. The specificities of the blood tests were likely underestimated due to cross-reaction to a non-*M. tuberculosis* mycobacterial infection and the lack of a confirmatory test that could be used to diagnose latent *M. tuberculosis* infection. Flow cytometry accurately locates the pool of immunological effector cells responsible for cytokine production during active TB. The ICC assay is an additional useful tool for the diagnosis of active TB.

Tuberculosis (TB) is a disease caused by an intracellular bacterium in which protective immunity and pathological hypersensitivity coexist and in which the lesions are mainly caused by the host response (8, 15, 27). TB is still one of the most important infectious diseases in the world. Therefore, an understanding of the protective immune response against TB in humans will give important clues to the future design of effective vaccines, new treatment strategies, and early detection of the disease.

To evaluate the immune response in the clinical laboratory, arrays of tests that are able to evaluate almost every component of the immune system are available. There are presently two broad approaches to the quantitative evaluation of the cellular immune response: enumeration of different cellular elements, like T-cell subsets, and determination of the functional competence of each cellular element, such as the ability of T cells to proliferate in response to a particular recall antigen. In the study described here, we undertook both tests for the enumeration of cellular elements and functional quality-controlled flow cytometry assays.

T cells are the major regulatory component of the human immune system, and much of this regulatory function is medi-

ated by the elaboration of cytokines. A naïve T cell will differentiate into a memory effector cell upon activation and acquire the ability to express cytokines of this memory state (27, 29). Cells other than CD4⁺ T cells, including CD8⁺ T cells, B cells, and monocytes/macrophages, can produce regulatory cytokines, including interleukin-4 (IL-4) and gamma interferon (IFN- γ) (4). Those that provide cellular responses are named type 1 cytokines; and these include IFN- γ , IL-2, IL-12, and IL-15. Type 2 cytokines, however, mainly enhance humoral responses; and these include IL-4, IL-5, IL-10, and IL-13 (8). Previous studies demonstrated that a minority of patients with *Mycobacterium tuberculosis* infection may express Th2 responses (33). In this study, we used IFN- γ and IL-4 as representative type 1 and type 2 cytokines, respectively, to demonstrate the T-cell-specific immune response during active *M. tuberculosis* infection.

Recently, early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) have been proven to be antigens secreted by *M. tuberculosis* but absent from *M. bovis* bacillus Calmette-Guérin (BCG), and cell-mediated responses to these specific antigens have shown a good association with *M. tuberculosis* infection (2, 3, 36). We attempted to quantify whole-blood cytokine production in vitro by two methods after stimulation with CFP-10 and ESAT-6 in a group of patients with suspected active TB. One of the ways in which we measured these responses focused on measurement of the level of IFN- γ production in plasma by using an enzyme-linked immunosorbent assay (ELISA) (1). Concurrently, we employed a single-cell intracellular cytokine flow cytometry (ICC) assay to

* Corresponding author. Mailing address: Public Health Laboratory Services Branch, Centre for Health Protection, Department of Health, Rm. 731, 7/F, Public Health Laboratory Centre, 382 Nam Cheong Street, Shek Kip Mei, Kowloon, Hong Kong. Phone: (852) 2319-8303. Fax: (852) 2776-1446. E-mail: kmkam@dh.gov.hk.

[∇] Published ahead of print on 7 January 2009.

locate and quantify the frequency of cytokine production and the type of cytokine produced by T-cell subsets when the same stimuli were used.

MATERIALS AND METHODS

Study population. This prospective cross-sectional blinded study was conducted from December 2006 to February 2007 and had 28 patients (21 male and 7 female patients; mean age, 44 years) who were human immunodeficiency virus (HIV) negative, had suspected active TB, and attended TB and chest clinics which provide free public medical service to all TB patients in Hong Kong. Blood samples were taken for lymphocyte immunophenotyping and whole-blood assays for the detection of IFN- γ , and sputum was taken for bacteriological culture for *M. tuberculosis*.

Twenty-eight consecutive patients were finally categorized as belonging to either the active TB group or the nonactive TB group. Of these 28 patients, 17 (11 male and 6 female patients; mean age, 43 years; age range, 18 to 79 years) were subsequently classified as having active TB by either culture (10/17 patients were culture positive), histological confirmation, and/or radiological confirmation and were placed in the TB group. The other 11 patients were categorized in the nonactive TB group and included patients with inactive *M. tuberculosis* infection, nontuberculous mycobacterial (NTM) infection, posttreatment TB, and other related respiratory conditions. Inactive TB cases had chest radiographic evidence suggestive of TB, cultures of their sputa for acid-fast bacilli were negative, and their chest radiographic findings did not change significantly or remained static with anti-TB treatment on follow-up or when no anti-TB treatment was started. Patients with NTM infections were culture positive for NTM, including both colonization and NTM lung disease, and did not respond to standard anti-TB treatment. Posttreatment TB cases had received anti-TB treatment for more than 3 months. Other related respiratory conditions were those in which a diagnosis of TB could not be established by bacteriological culture or other investigations, including histological analysis or testing by PCR, or an alternate diagnosis was made.

A healthy control group included 12 healthy HIV-negative volunteers (3 male and 9 female volunteers; mean age, 43 years; age range, 24 to 55 years) with no known history of *M. tuberculosis* infection, as confirmed by all tests conducted (mycobacterial culture, lymphocyte immunophenotyping, and whole-blood assays for IFN- γ). Informed consent was obtained from all patients and controls prior to the study.

ELISA of whole blood for IFN- γ . The QuantiFERON-TB Gold (QFT-G) in-tube assay (Cellestis Ltd., Victoria, Australia) was performed according to the manufacturer's recommendations. In brief, 1 ml of blood was collected and placed into three blood collection tubes with the following stimulants: no stimulant (antigen-free control), TB antigen (which contained the ESAT-6, CFP-10, and TB7.7 peptide cocktail), and mitogen (which contained phytohemagglutinin). Following incubation for 16 to 24 h at 37°C, 50 μ l plasma was removed from each tube and the concentration of IFN- γ was determined by the ELISA and by use of the QFT-G (in-tube) analysis software. The cutoff value for a positive response was taken to be 0.35 IU/ml. Indeterminate results were reported if the IFN- γ level in the tube with mitogen minus that in the tube with no antigen was less than 0.5 IU/ml.

Lymphocyte immunophenotyping. Venous heparinized blood was collected and processed for flow cytometry within 6 h of venipuncture. A standardized method with a recommended four-color panel (6) of combinations of fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), and allophycocyanin (APC) conjugated to monoclonal antibody in TruCOUNT tubes obtained from a single manufacturer (Becton Dickinson, San Jose, CA) was used to detect the expression of the surface antigens. After the red blood cells were stained in the dark at room temperature for 15 min, they were lysed with 450 μ l of 1 \times fluorescent-activated cells sorter analysis lysing solution for another 15 min. Samples were acquired within 1 h on a FACSCalibur cytometer (Becton Dickinson) and analyzed with MultiSET software (Becton Dickinson). The daily instrument setup and instrument control were performed with CaliBRITE beads 3, CaliBRITE APC beads, and FACSCOMP software. The data were analyzed and were within the recommended analytic reliability checks (6).

ICC technique. The ICC technique is based on the stimulation of T cells to produce cytokine in the presence of a pharmacologic inhibitor of secretion, followed by cell fixation and permeabilization and then intracytoplasmic staining of the accumulated cytokines (12, 18, 22, 29, 32, 34). The use of the drug brefeldin A disrupts the intracellular Golgi apparatus-mediated transport system and allows the cytokines to accumulate and then be detected by flow cytometry.

In vitro lymphocyte activation. Venous heparinized blood was collected and processed within 6 h of venipuncture: three sterilized Falcon tubes (12 by 75 mm) were labeled as the unstimulated control, TB antigen, and mitogen control tubes, respectively. To each tube, 0.5 ml heparinized whole blood with 5 μ g brefeldin A was mixed with 0.5 ml supplemented RPMI 1640 medium containing 2 mmol/liter L-glutamine, 10% (vol/vol) heat-inactivated fetal bovine serum, and 100 IU penicillin-streptomycin. Activation was done in the presence of brefeldin A, which inhibited intracellular transport and allowed the antigens and the cytokines produced during the activation to be retained inside the cell. To the TB antigen tube, 5 μ g each recombinant ESAT-6 and CFP-10 (ImmunoDiagnostic Inc.) was added; to the mitogen control tube, 25 ng phorbol 12-myristate 13-acetate (PMA) and 1 μ g ionomycin were added. Costimulatory antibodies CD28 and CD49d were added to all tubes. The cells were then stained with monoclonal antibodies after 16 to 24 h of incubation at 37°C in the presence of 7% CO₂.

Monoclonal antibodies and staining. FITC-labeled anti-IFN- γ , PE-labeled anti-IL-4, APC-labeled anti-CD69, PE-Cy7-labeled anti-CD8, peridinin-chlorophyll-Cy5.5-labeled anti-CD3, and APC-Cy7-labeled anti-CD4 were obtained from the manufacturer (Becton Dickinson). Surface staining was done on ice in the dark, followed by the addition of 0.5 ml of BD 1 \times permeabilizing solution. Subsequent intracellular staining was performed, and the cells were fixed in 2% (vol/vol) paraformaldehyde in phosphate-buffered saline for flow cytometric analysis. Isotype controls were used as the corresponding clones, in order to determine the level of background staining.

Flow cytometry data acquisition and analysis. Six-color flow cytometry was performed on a FACSCanto flow cytometer (Becton Dickinson) with FACSDiva (version 4.0) software (Becton Dickinson). The instrument was controlled daily by monitoring the optical alignment with Sphero rainbow fluorescent particles of 3.0 to 3.4 μ m in diameter (Spherotech Inc.), and the stability of the optical alignment was verified when the optical alignment was within 2 standard deviations (SDs) of the preestablished values. The lymphocyte gating was set by forward and side light scatter (Fig. 1a) and was acquired with 30,000 to 100,000 CD3⁺ CD4⁺ lymphocytes events (Fig. 1b).

For data analysis, a sequential gating strategy was employed to detect cytokine-producing cells among the CD3⁺ CD4⁺ and CD3⁺ CD8⁺ lymphocytes. Briefly, a gate was set on viable lymphocytes (forward versus side scatter), CD3⁺ lymphocytes were then gated in a CD3-versus-side scatter histogram. CD3⁺ CD4⁺ and CD3⁺ CD8⁺ cells were then displayed and further gated to determine the expression of CD69⁺ and IFN- γ -producing (IFN- γ ⁺) cells and CD69⁺ IL-4-producing (IL-4⁺) cells.

Assay controls. The following controls were included in the assay. (i) Fluorescein-conjugated isotype control antibodies (isotype control) were included in all samples with the isotype-matched antibodies in order to determine the amount of nonspecific staining present. (ii) An unstimulated control served as a means of determination of the background level of in vivo residual cytokine production for each sample. (iii) Treatment with mitogen, a potent stimulus, served as a positive control to ensure the immune status of the patient cells and also as an intracellular staining control with sufficient activation when the proportion of CD69⁺ T cells was >90%.

Calculation of specific response to TB antigen. The CD69 antigen is activated early, and its expression is induced during in vitro antigen stimulation. Upon activation, the level of CD69 antigen expression increases on lymphocytes (31, 35). The CD69 antibody is used to allow the better clustering of cytokine-positive cells and to ensure that cells defined as antigen responsive have been stimulated to express this activation marker. The specific response of the cells to the stimulus was obtained by subtracting the percentage of positive events for the unstimulated control cells (isotype-corrected response) from the percentage of positive events for the TB antigen-activated cells (isotype-corrected response). The antigen-reactive and cytokine-producing cells are expressed as CD69⁺ IFN- γ ⁺ CD4⁺ T cells or CD69⁺ IL-4⁺ CD4⁺ T cells. The cutoff was determined by computing the mean and 2 SDs as the reference range for the healthy control group.

Bacteriological cultures. Sputum samples were processed according to standard operating procedures within the TB Reference Laboratory, Public Health Laboratory Centre, Hong Kong, as published previously (16). Briefly, sputum was first decontaminated with an equal volume of 3% NaOH-N-acetyl-L-cysteine for 20 min, neutralized with 0.067 M phosphate buffer (pH 6.8), and centrifuged at 3,000 \times g for 15 min. After inoculation of the deposit onto two Lowenstein-Jensen slopes, the slopes were incubated at 37°C. When growth was observed, all isolates were identified by standard identification procedures (16, 38).

Statistical analysis. The performance characteristics of each test for the diagnosis of active *M. tuberculosis* infection were compared and are expressed statistically as sensitivity and specificity. Statistical analysis was performed with SPSS (version 15.0) software (SPSS Inc.). The results are presented as the mean

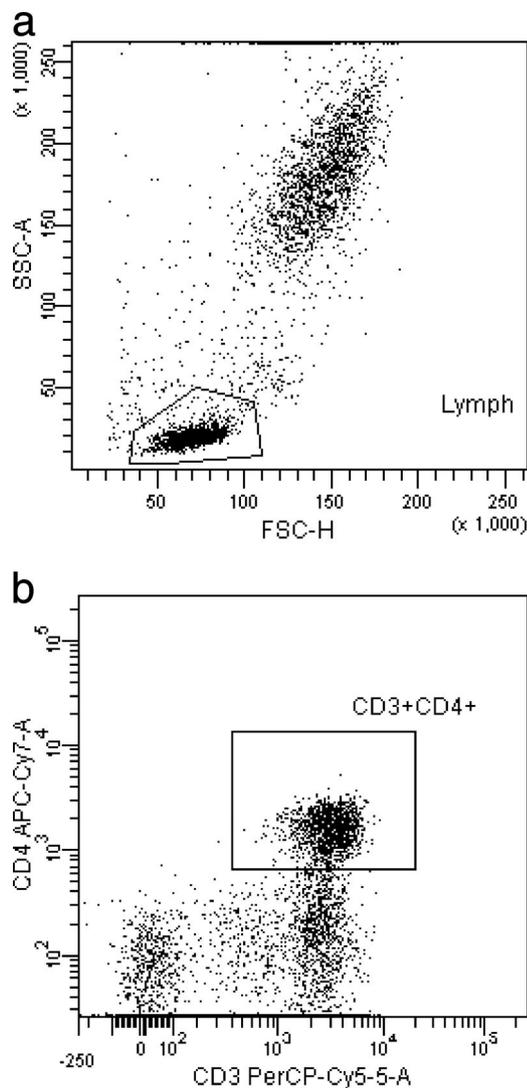


FIG. 1. Lymphocyte gating. (a) The lymphocyte gate was set by forward light scatter (FSC-H) and side light scatter (SSC-A). (b) Acquisition was performed with 30,000 to 100,000 CD3⁺ CD4⁺ lymphocyte events.

values \pm SDs of the means. Comparisons between groups with normal distributions were performed by Student's *t* test. Correlation analysis was performed with Pearson's correlation coefficient between two variables. Two-sided *P* values of less than 0.05 were considered significant.

RESULTS

T-cell subset characterization. Lymphocyte immunophenotyping was performed, and the results of the determinations for the main T-cell populations in the peripheral blood of the 17 TB patients and the 12 healthy controls are depicted in Table 1. The mean levels for all members of the healthy group fell within the reference ranges for our healthy population determined previously (14), and no significant differences were observed. For the TB group, generally slightly elevated levels of CD4⁺ T cells, CD8⁺ T cells, and B cells were detected, but they were not significantly higher, while a significant reduction in the numbers of peripheral natural killer (NK) cells (20% reduction; *P* < 0.05) was demonstrated.

Specific response by using ex vivo stimulation with PMA plus ionomycin. PMA plus ionomycin is a potent external stimulus for the activation of lymphocytes to produce cytokines. Dot plots (Fig. 2d) showed the activation results for the control assay, which utilized the surface expression of CD69 to assess whether activation had been achieved. Only activated cell populations expressed cytokines, while resting lymphocytes (T, B, or NK cells) from healthy individuals did not constitutively express cytokines (23, 28). If the expected level of CD69 expression of >90% was not seen, the whole set of cytokine data was invalid and the data were excluded from analysis.

The general immune profiles of cytokine expression between the groups are shown in Table 2. It can be seen that the trend was for the activation of CD8⁺ IFN- γ ⁺ T cells to be about three times higher than that of CD4⁺ IFN- γ ⁺ T cells in all groups. Very low to undetectable levels of IL-4-producing cells were seen.

Specific response of mitogen control in QFT-G assay. The concentration of IFN- γ production by the mitogen control in the QFT-G assay was also determined (Table 2) to validate the activation process for all patients studied. A significant difference in the mean values for the TB group compared with those for the healthy control group was observed (16.61 IU/ml and 10.73 IU/ml, respectively; *P* < 0.05).

Specific response to ex vivo stimulation of *M. tuberculosis* antigens ESAT-6 and CFP-10. In 12 healthy individuals with no history of exposure to TB infection, the mean proportion of CD4⁺ CD69⁺ IFN- γ ⁺ T cells was 0.0035 \pm 0.0033% (Table 3). We then established a responder frequency of the mean \pm 2 SDs, by which >0.01% activated CD4⁺ IFN- γ ⁺ T cells was considered a positive response to *M. tuberculosis* infection. The

TABLE 1. Comparison of mean lymphocyte antigen expression values for healthy controls and TB patients with the expected values for healthy individuals

Lymphocyte phenotypic marker	Mean absolute count/ μ l \pm SD		<i>P</i> value (95% CI) ^a	
	Healthy controls (<i>n</i> = 12)	TB group (<i>n</i> = 17)	Healthy controls	TB group
Lymphocytes	1,942 \pm 246	1,941 \pm 536	NS (-156-156)	NS (-276-275)
CD3 ⁺ cells	1,335 \pm 271	1,349 \pm 419	NS (-207-138)	NS (-237-194)
CD3 ⁺ CD4 ⁺ cells	703 \pm 111	751 \pm 204	NS (-92-49)	NS (-79-131)
CD3 ⁺ CD8 ⁺ cells	491 \pm 156	541 \pm 297	NS (-197-1)	NS (-200-105)
CD3-CD19 ⁺ B cells	238 \pm 62	276 \pm 132	NS (-22-57)	NS (-13-123)
CD3-CD16 ⁺ CD56 ⁺ NK cells	355 \pm 140	306 \pm 151	NS (-128-50)	0.029 (-166--10)

^a *P* values are for comparison of the data for each group and the expected levels for healthy individuals, which were based on previously published data (14). CI, confidence interval; NS, statistically not significant.

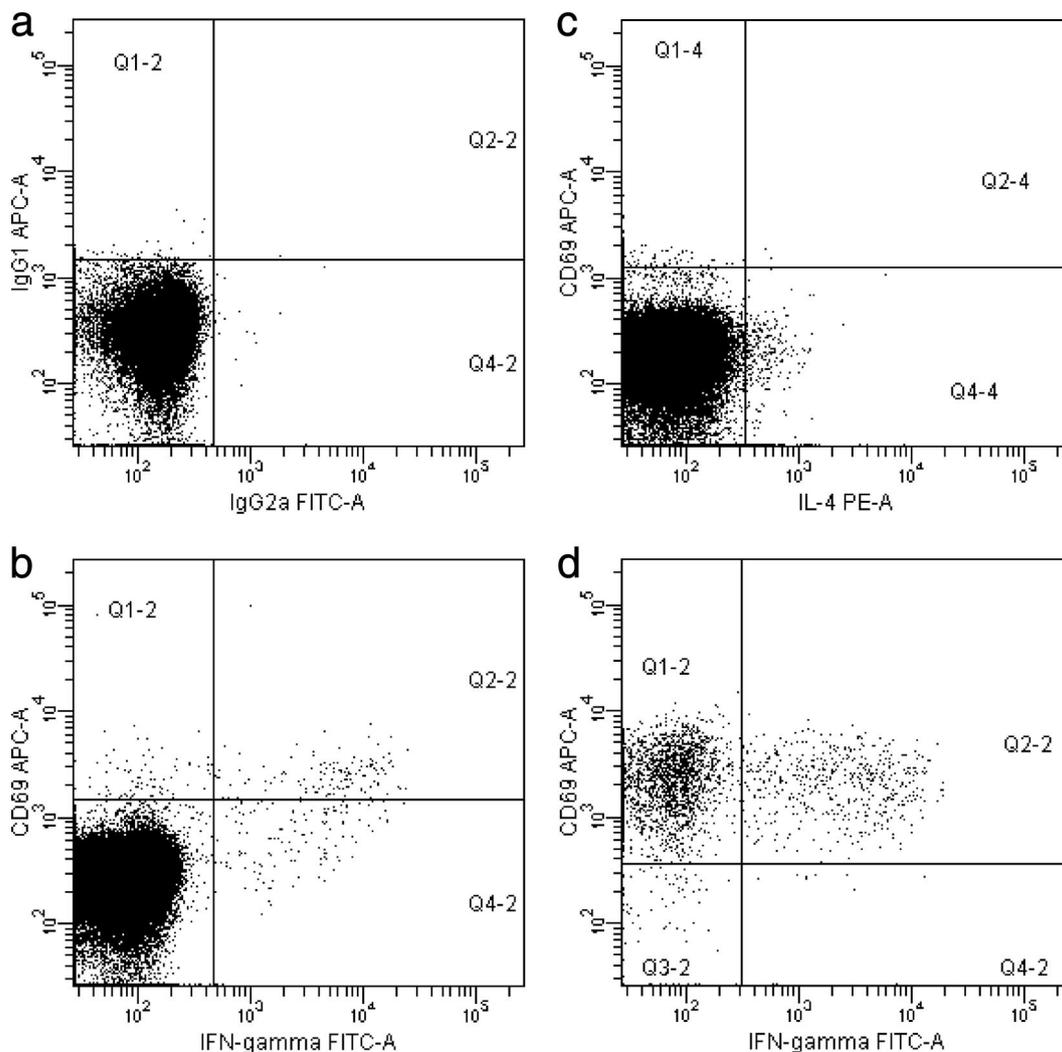


FIG. 2. Cytokine activation assay results. (a) IFN- γ production for TB case by isotype control, 0.002% (Q2-2); (b) IFN- γ production for TB case by CD3⁺ CD4⁺ CD69⁺ cells, 0.096% (Q2-2); (c) IL-4 production for TB case by CD3⁺ CD4⁺ CD69⁺ cells, 0.002% (Q2-4); (d) CD69 production for TB case with PMA-ionomycin stimulus control by CD3⁺ CD4⁺ cells, >90% (Q1-2, 77.77%, Q2-2, 18.86%; CD3⁺ CD4⁺ CD69⁺, 96.63%). IgG1, immunoglobulin G1.

pattern of the specific immune response by a healthy subject is illustrated in Fig. 3.

A significant increase ($P < 0.05$) in the mean proportion of activated CD4⁺ IFN- γ ⁺ T cells of 0.054% was seen in patients with active TB, whereas in the healthy controls the mean pro-

portion was 0.0035% (Fig. 2a to d). There was also some contribution of CD69⁺ CD8⁺ IFN- γ ⁺ T cells, the mean level of activation of which was 0.044%, although this level was not significantly greater than that for the healthy controls

TABLE 2. Analysis of mitogen control by ICC and QFT-G assays

Phenotype	Mean % cells \pm SD (10^{-2}) ^a	
	Healthy group (n = 12)	TB group (n = 17)
CD3 ⁺ CD4 ⁺ CD69 ⁺	93.80 \pm 2.50	93.47 \pm 2.06
CD3 ⁺ CD8 ⁺ CD69 ⁺	91.01 \pm 0.61	91.49 \pm 1.63
CD3 ⁺ CD4 ⁺ CD69 ⁺ IFN- γ ⁺	7.75 \pm 2.49	9.56 \pm 4.18
CD3 ⁺ CD8 ⁺ CD69 ⁺ IFN- γ ⁺	29.68 \pm 7.33	26.1 \pm 15.67

^a The mitogen levels determined by the QFT-G assay were 16.61 \pm 5.76 IU/ml for the healthy group and 10.73 \pm 7.26 IU/ml for the TB group ($P < 0.05$ compared with the results for the healthy control group).

TABLE 3. Comparison of mean levels of IFN- γ and IL-4 production by group within CD3⁺ CD69⁺ cells

T-cell phenotype	Mean % cells \pm SD (10^{-2}) ^a		P value ^b
	TB group (n = 17)	Healthy controls (n = 12)	
CD4 ⁺ IFN- γ ⁺	5.41 \pm 3.72	0.35 \pm 0.33	<0.05
CD8 ⁺ IFN- γ ⁺	4.45 \pm 5.53	1.73 \pm 3.17	NS
IFN- γ ⁺	5.67 \pm 3.8	1.13 \pm 1.59	<0.05
IL-4 ⁺	0.03 \pm 0.08	0.05 \pm 0.08	NS

^a The concentrations of IFN- γ released, as determined by the QFT-G assay, were 9.68 \pm 8.04 IU/ml for the TB group and 0.11 \pm 0.13 IU/ml for the healthy controls ($P < 0.05$).

^b P values were determined by the Student *t* test of the mean difference. NS, not statistically significant.

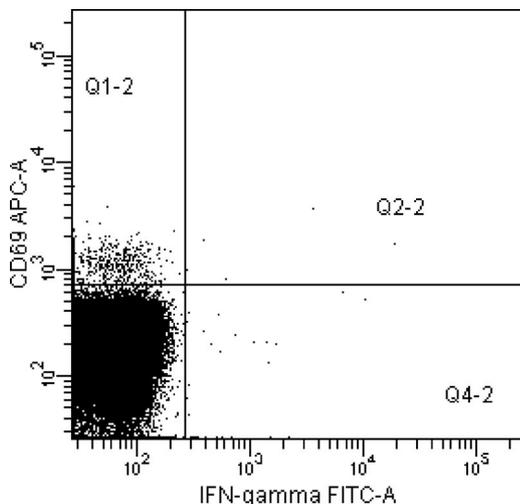


FIG. 3. IFN- γ production by CD3⁺ CD4⁺ CD69⁺ cells in a healthy control (0.002%; Q2-2).

(0.017%). The distribution of activated CD4⁺ and CD8⁺ IFN- γ ⁺ T cells for each group is illustrated in the box plots in Fig. 4. A very low to undetectable level of IL-4 expression could be found. From this, a clear pattern of type 1 cytokine production with a Th1-cell-mediated response in the TB group was demonstrated.

Comparative performances of two diagnostic tests. Of the 17 confirmed cases of active TB, 15 (88.2%) were found to be positive by the QFT-G assay and 16 (94.1%) were found to be positive by the ICC assay (Table 4), whereas 10 (58.8%) were found to be positive for *M. tuberculosis* by culture. Of the 11

cases with nonactive TB, false-positive results were found for 9 (82%) cases by the QFT-G assay and 7 (64%) cases by the ICC assay. The sensitivities of the QFT-G and ICC assays were found to be 88.2% and 94.1%, respectively, and the specificities were 18% and 36.4%, respectively. Three cases of culture-positive non-TB infections occurred: two (one *M. kansasii* infection, one *M. chelonae* infection) showed false-positive responses by both the QFT-G and the ICC assays, while one (a Runyon group II mycobacterium infection) did not respond by either test. Both the QFT-G and the ICC assays showed negative results for the healthy control group.

Correlation of IFN- γ release detected by QFT-G and ICC assays with T-cell subsets. The concentration of IFN- γ released, as detected by the QFT-G assay, was significantly higher for the TB group ($P < 0.05$) than for the healthy controls (9.68 IU/ml and 0.11 IU/ml, respectively). There was also a good correlation between the quantity of IFN- γ produced, as detected by the QFT-G assay, and the CD4⁺ T-cell count in the TB group ($r = 0.57, P < 0.05$) (Table 5), while no significant association was found for the other lymphocyte subsets enumerated by flow cytometry.

When the ICC assay was used as the detection test, there was no significant correlation between the frequency of CD4⁺ CD69⁺ IFN- γ ⁺ T-cell expression and the cell count.

DISCUSSION

Very few lymphocyte immunophenotyping data are available for patients with active TB in countries with a high prevalence of TB. In this study, we demonstrated a general increase in the CD4⁺ and CD8⁺ T-cell counts and a concomitant significant reduction in peripheral NK-cell levels during active TB. Since

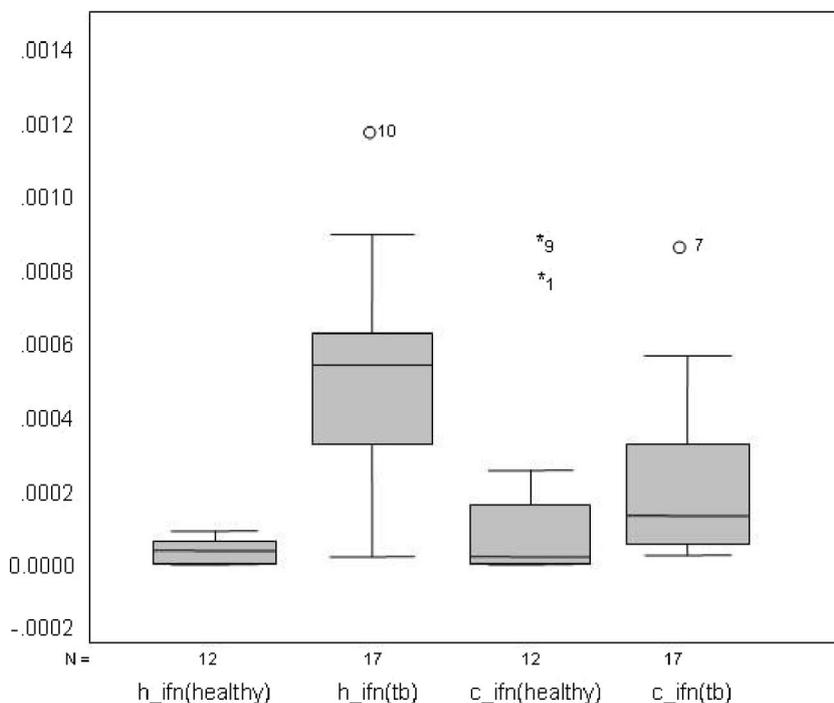


FIG. 4. Distribution of IFN- γ -producing cells among healthy controls and active TB group. The median, interquartile range, and 95% confidence interval are shown on the box plot. h_ifn, CD3⁺ CD4⁺ IFN- γ production; c_ifn, CD3⁺ CD8⁺ IFN- γ production.

TABLE 4. Comparative performances of the QFT-G and ICC diagnostic assays for detection of active TB

Group	No. of samples with the following QFT-G assay result ^a :		No. of samples with the following ICC assay result ^b :	
	Positive	Negative	Positive	Negative
TB group (n = 17) ^c	15	2	16	1
Nonactive TB group (n = 11) ^d	9	2	7	4
Total (n = 28)	24	4	23	5
NTM culture positive (n = 3)				
Runyon group II (n = 1)	0	1	0	1
<i>M. kansasii</i> (n = 1)	1	0	1	0
<i>M. chelonae</i> (n = 1)	1	0	1	0
Total	2	1	2	1

^a The sensitivity and the specificity of the QFT-G assay were 88.2% and 18%, respectively.

^b The sensitivity and the specificity of the ICC assay were 94.1% and 36.4%, respectively.

^c The TB group included patients with definite PTB and radiological PTB (n = 16) and patients with extrapulmonary TB (n = 1); 10 of these 17 patients were TB culture positive.

^d The nonactive TB group included patients who were culture positive for NTM (n = 3), patients with inactive TB (n = 2) and other related respiratory conditions (n = 5), and a patient who had received post-TB treatment for >3 months (n = 1).

CD4⁺ and CD8⁺ T cells are needed to establish protective immunity with a homeostatic downregulation of NK cells upon *M. tuberculosis* infection (11), flow cytometric evaluation of the specific immune response in patients with *M. tuberculosis* infection provides this information, which is valuable for the design of tools for the diagnosis of active infection.

Recently, in vitro assays that measure blood IFN- γ release have been developed as new diagnostic tools for the detection of TB, including latent *M. tuberculosis* infection. A number of studies have indicated the limitations and potential usefulness of IFN- γ release assays over the existing conventional tuberculin skin test for the diagnosis of TB (2, 7, 10, 19, 20, 26).

Presently available laboratory methods that can be used to assess cytokine expression in the cellular response include ELISAs that detect the cytokines expressed by cell populations and the enzyme-linked immunospot assay, which detects the cytokines expressed by single types of cells (5, 9, 13). Commercially available kits, such as the T-Spot TB test (Oxford Immunotec, Oxford, United Kingdom), have been extensively evaluated (17, 37). However, one drawback of the enzyme-linked immunospot assay technique is that it can detect only one cytokine at a time, and careful interpretation of true rather than artifactual (nonspecific) reactions is essential when the number of spots is counted. In this study, we used a ICC assay as a tool for the analysis of multiple parameters per cell. The ICC assay allowed the accurate characterization of type 1 or type 2 cytokine-producing T-lymphocyte subsets during active infection.

We included a positive control consisting of PMA plus ionomycin as the stimulus in the ICC assay and demonstrated that the general cytokine immune profiles of the healthy control and TB groups were very similar. The pool of immunological effector CD8⁺ T cells that expressed the CD69⁺ IFN- γ ⁺ phe-

TABLE 5. Correlation of IFN- γ release detected by QFT-G and ICC assays with lymphocyte subsets from the TB group^a

Cell type	IFN- γ concn (IU/ml) determined by QFT-G assay (P value)	% IFN- γ ⁺ CD4 ⁺ T cells determined by ICC assay (P value)
Lymphocytes	0.095 (NS) ^b	0.065 (NS)
CD3 ⁺ cells	0.066 (NS)	-0.002 (NS)
CD3 ⁺ CD4 ⁺ cells	0.570 (<0.05)	0.135 (NS)
CD3 ⁺ CD8 ⁺ cells	-0.283 (NS)	-0.091 (NS)
NK cell (CD3-CD16 ⁺ CD56 ⁺)	-0.010 (NS)	-0.038 (NS)
B cell (CD3- CD19 ⁺)	0.18 (NS)	0.298 (NS)

^a n = 17 for the TB group.

^b NS, not statistically significant.

notype was generally three times higher than the pool of CD4⁺ T cells that expressed that phenotype.

There is little information regarding the types of cells that produce IFN- γ in the mitogen control of the QFT-G ELISA. Interestingly, we found a significant negative mean difference in the magnitude of IFN- γ release between the TB group and the healthy control group. Further study is required to elucidate the mechanism and the possible significance of this difference. We tried to correlate the magnitude of IFN- γ production in the mitogen control group with the sensitivity of active TB detection by ELISA and the ICC assay but did not find a significant association between them (unpublished data).

In this study, using healthy controls we established a responder frequency of >0.01% activated CD4⁺ IFN- γ ⁺ T cells as a positive cutoff response for patients with TB. This cutoff percentage is comparable to the arbitrary cutoff of 0.01% CD4⁺ T cells used as a positive response in two other studies (12, 34). Both activated CD4⁺ cells and CD8⁺ T cells (Fig. 4) are primed for IFN- γ production, while CD4⁺ T cells are responsible for the majority of the IFN- γ production during active TB. A very low to undetectable level of IL-4 production in patients with active TB was found, and thus, a significant Th2 response was not supported.

The performance characteristics of the two diagnostic methods, the QFT-G assay and the ICC assay, are shown in Table 4. The ICC assay demonstrated a higher sensitivity (94.1%) than the QFT-G assay (88.2%). Both assays showed poorer performance in terms of their specificities, which were 36.4% for the ICC assay and 18% for the QFT-G assay, when the strict criterion of a positive culture result for *M. tuberculosis* was used for comparison. Nevertheless, because the sensitivity of both whole-blood IFN- γ release assays is greater than 85% for the diagnosis of active TB, these assays provide rapid supplementary tools for screening for the disease. Furthermore, we also included one case of extrapulmonary active TB in the TB category; the conventional microbiological assay may not always detect such cases of TB, but the case was readily detected by both whole-blood assays.

The predictive value of a diagnostic test is influenced by the prevalence of the disease in a given population. The specificities of both QFT-G and ICC assays were very low for the group with nonactive TB. There are several possible reasons for the high false-positive rate. First, the nonactive TB category comprised patients with inactive TB (two patients), patients

with culture-positive NTM infection (three patients), and a patient who had been receiving treatment for TB for 3 months (one patient), with the rest of the patients having other related respiratory medical conditions. It is not always possible to differentiate the immune response triggered by an old case of TB, by latent TB, or during or after TB treatment (25). Second, we used TB antigen stimulants, ESAT-6 and CFP-10, similar to those to which the blood of patients infected with other mycobacterial species may respond. Of note, patients with certain other mycobacterial infections might also be responsive to ESAT-6 and CFP-10, as the genes encoding these proteins are also present in *M. kansasii*, *M. szulgai*, and *M. marinum* (2, 21). In this study, there were three cases of culture-positive non-*M. tuberculosis* infection, and both the QFT-G and the ICC assays showed false-positive responses for samples infected with *M. kansasii* and *M. chelonae* but provided no response for the samples infected with Runyon group II mycobacteria. Third, this study was conducted in the setting of a routine clinical practice with patients with suspected active TB, and its specificity would tend to be lower than those of studies in which healthy subjects were also enrolled for the calculation of specificity.

As shown in Table 5, there was good correlation between the level of IFN- γ production determined by the QFT-G assay and the CD4 T-cell count ($r = 0.57$, $P < 0.05$), which may indicate that CD4⁺ T cells are major contributors to the release of IFN- γ . It is of concern that test performance will be compromised when the test is used with samples from HIV-infected individuals, especially those with CD4 T-cell counts lower than normal. Under such circumstances, the ICC assay is clearly the method of choice, as its performance is not affected by the CD4 count.

Although previous studies have shown promising results with the QFT-G assay (21, 30), the limitations of the ELISA would need to be carefully considered, even though it is convenient and relatively easy to perform. By this test, only the bulk production of the cytokine in the culture supernatant is detected, and the assay provides limited information regarding the exact types of cells engaged in cytokine production or the kinetics of this response. The amount of cytokine measured also reflects only the net amount of uptake or destruction as well as secretion during incubation *ex vivo*.

The ICC assay may therefore be a useful additional tool for the diagnosis of active TB, as it accurately locates the pool of immunological effector cells responsible for cytokine production. However, due to the need for technical expertise and expensive equipment, it is recommended that this assay be done only in a reference laboratory setting.

This study has several limitations. First, it was a cross-sectional study, in that we did not evaluate the kinetics of activation of the T-cell subsets and the associated changes in cytokine levels at various times postinfection. Second, the sample size was not large enough to show more subtle differences between the groups. With a larger sample size, we possibly could have further subdivided the nonactive TB group into a past TB group, an NTM infection group, and a group with other respiratory conditions. Third, the functional assay is an *in vitro* activation test and has *in vitro* artifacts and assumptions different from those of *in vivo* tests. Fourth, while the QFT-G assay used three antigens (ESAT-6, CFP-10, and TB7.7) for *in vitro*

cell stimulation, only two of those (ESAT-6 and CFP-10) were used in the ICC assay.

In summary, we determined that patients with active TB (i) have normal to slightly elevated levels of CD4⁺ and CD8⁺ T cells but a significant reduction in the numbers of NK cells; (ii) CD4⁺ T cells are the major cell type that produce IFN- γ , a type 1 cytokine; (iii) small percentages of CD8⁺ T cells are primed for IFN- γ production; (iv) type 2 cytokine (IL-4) production is not prominent; and (v) the sensitivity and specificity values are higher by the ICC method than by the QFT-G ELISA.

Further research is required to define the clinical usefulness of the IFN- γ assay and directions for future research, especially in resource-limited settings with a large population of individuals infected with HIV (24). The appropriate use of laboratory diagnostics for the early detection of active TB will definitely provide great assistance with efforts to control TB worldwide.

ACKNOWLEDGMENTS

We thank the TB and chest clinic staff who took care of the TB patients and provided necessary clinical details for the study and the laboratory staff who performed the flow cytometric tests and QFT-G assays.

REFERENCES

- Adetifa, I. M., M. D. Luos, A. Hammond, D. Jeffries, S. Donkor, R. A. Adegbola, and P. C. Hill. 2007. Comparison of two interferon gamma release assays in the diagnosis of *Mycobacterium tuberculosis* infection and disease in The Gambia. *BMC Infect. Dis.* 7:122.
- Andersen, P., M. E. Munk, J. M. Pollock, and T. M. Doherty. 2000. Specific immune-based diagnosis of tuberculosis. *Lancet* 356:1099–1104.
- Brock, I., M. E. Munk, A. Kok Jensen, and P. Anderson. 2001. Performance of whole blood IFN- γ test for tuberculosis diagnosis based on PPD or the specific antigens ESAT-6 and CFP-10. *Int. J. Tuberc. Lung Dis.* 5:462–467.
- Carter, L. L., and R. W. Dutton. 1996. Type 1 and type 2: a fundamental dichotomy for all T-cell subsets. *Curr. Opin. Immunol.* 8:336–342.
- Carter, L. L., and S. L. Swain. 1997. Single cell analyses of cytokine production. *Curr. Opin. Immunol.* 9:177–182.
- Centers for Disease Control and Prevention. 2003. Guidelines for performing single-platform absolute CD4⁺ T-cell determinations with CD45 gating for persons infected with human immunodeficiency virus. *MMWR Recomm. Rep.* 52(RR-2):1–13.
- Centers for Disease Control and Prevention. 2005. Guidelines for using the Quantiferon-TB Gold test for detecting *Mycobacterium tuberculosis* infection, United States. *MMWR Recomm. Rep.* 54(RR-15):49–55.
- Clerici, M., and G. M. Shearer. 1994. The Th1-Th2 hypothesis of HIV infection: new insights. *Immunol. Today* 15:575–581.
- Czerkinsky, C. C., L. A. Nilsson, H. Nygren, O. Ouchterlony, and A. Tarkowski. 1983. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J. Immunol. Methods* 65:109–121.
- Detjen, A. K., T. Keil, S. Roll, B. Hauer, H. Mauch, U. Wahn, and K. Magdort. 2007. Interferon γ 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.
- Flynn, J. L., and J. Chan. 2001. Immunology of tuberculosis. *Annu. Rev. Immunol.* 19:93–129.
- Hughes, A. J., P. Hutchinson, T. Gooding, N. J. Freezer, S. R. Holdsworth, and P. D. R. Johnson. 2005. Diagnosis of *Mycobacterium tuberculosis* infection using ESAT-6 and intracellular cytokine cytometry. *Clin. Exp. Immunol.* 142:132–139.
- Hutchings, P. R., G. Cambridge, J. P. Tite, T. Meager, and A. Cooke. 1989. The detection and enumeration of cytokine-secreting cells in mice and man and the clinical application of these assays. *J. Immunol. Methods* 120:1–8.
- Kam, K. M., W. L. Leung, M. Y. Kwok, M. Y. Hung, S. S. Lee, and W. P. Mak. 1996. Lymphocyte subpopulation reference ranges for monitoring HIV-infected Chinese adults. *Clin. Diagn. Lab. Immunol.* 3:326–330.
- Kaufmann, S. H. E. 1993. Immunity to intracellular bacteria. *Annu. Rev. Immunol.* 11:129–163.
- Kent, P. T., and G. P. Kubica. 1985. A guide for the level III laboratory. Centers for Disease Control, Atlanta, GA.
- Lalvani, A., A. A. Pathan, H. Durkan, et al. 2001. Enhanced contact tracing

- and spatial tracking of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cell. *Lancet* **357**:2017–2021.
18. **Maino, V. C., and L. J. Picker.** 1998. Identification of functional subsets by flow cytometry: intracellular detection of cytokine expression. *Cytometry* **34**:207–215.
 19. **Mazurek, G. H., P. LoBue, C. L. Daley, J. Bernardo, A. A. Lardizabal, W. R. Bishai, M. F. Iademarco, and J. S. Rothel.** 2001. Comparison of a whole-blood interferon- γ assay with tuberculin skin testing for detecting latent *Mycobacterium tuberculosis* infection. *JAMA* **286**:1740–1747.
 20. **Mazurek, G. H., M. J. Zajdowicz, A. L. Hankinson, et al.** 2007. Detection of *Mycobacterium tuberculosis* infection in United States Navy recruits using the tuberculin skin test or whole-blood IFN- γ release assays. *Clin. Infect. Dis.* **45**:826–836.
 21. **Mori, T., M. Sakatani, F. Yamagishi, T. Takashima, et al.** 2004. Specific detection of tuberculosis infection: an interferon-gamma-based assay using new antigens. *Am. J. Respir. Crit. Care Med.* **170**:59–64.
 22. **Nomura, L. E., J. M. Walker, and H. T. Maecker.** 2000. Optimisation of whole blood antigen-specific cytokine assays for CD4⁺ T cells. *Cytometry* **40**:60–68.
 23. **Openshaw, P., E. E. Murphy, and N. A. Hosken.** 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J. Exp. Med.* **182**:1357–1367.
 24. **Pai, M., K. Dheda, J. Cunningham, F. Scano, and R. O'Brien.** 2007. T-cell assays for the diagnosis of latent tuberculosis infection: moving the research agenda forward. *Lancet Infect. Dis.* **7**:428–438.
 25. **Pai, M., R. Joshi, M. Bandyopadhyay, P. Narang, S. Dogra, B. Taksande, and S. Kalantri.** 2007. Sensitivity of a whole-blood interferon-gamma assay among patients with pulmonary tuberculosis and variations in T-cell responses during anti-tuberculosis treatment. *Infection* **35**:98–103.
 26. **Pai, M., L. W. Riley, and J. M. Colford, Jr.** 2004. Interferon- γ assays in the immunodiagnosis of tuberculosis: a systematic review. *Lancet Infect. Dis.* **4**:761–776.
 27. **Paul, W. E., and R. A. Sedar.** 1994. Lymphocyte responses and cytokines. *Cell* **76**:241–251.
 28. **Picker, L. J., M. K. Singh, Z. Zdraveske, et al.** 1995. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood* **86**:1408–1419.
 29. **Prussin, C.** 1997. Cytokine flow cytometry: understanding cytokine biology at the single-cell level. *J. Clin. Immunol.* **17**:195–204.
 30. **Ravn, P., M. E. Munk, A. B. Anderson, et al.** 2005. Prospective evaluation of a whole-blood test for using *Mycobacterium tuberculosis*-specific antigens ESAT-6 and CFP-10 for diagnosis of active tuberculosis. *Clin. Diagn. Lab. Immunol.* **12**:491–496.
 31. **Rutella, S., C. Rumi, M. B. Lucia, T. Barberi, P. L. Puggioni, M. Lai, A. Romano, R. Cauda, and G. Leone.** 1999. Induction of CD69 antigen on normal CD4⁺ and CD8⁺ lymphocyte subsets and its relationship with the phenotype of responding T-cells. *Cytometry* **38**:95–101.
 32. **Suni, M. A., L. J. Picker, and V. C. Maino.** 1998. Detection of antigen-specific T cell cytokine expression in whole blood by flow cytometry. *J. Immunol. Methods* **212**:89–98.
 33. **Surcel, H. M., M. Troye-Blomberg, S. Paulie, et al.** 1994. Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. *Immunology* **81**:171–176.
 34. **Tesfa, L., F. W. Koch, W. Pankow, H. Volk, and F. Kern.** 2004. Confirmation of *Mycobacterium tuberculosis* infection by flow cytometry after ex vivo incubation of peripheral blood T cells with an ESAT-6-derived peptide pool. *Cytometry B Clin. Cytom.* **60**:47–53.
 35. **Testi, R., D. D'Ambrosio, R. De Maria, and A. Santoni.** 1994. The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. *Immunol. Today* **15**:479–483.
 36. **Van Pinxteren, L. A., P. Ravn, E. M. Agger, J. Pollock, and P. Andersen.** 2000. Diagnosis of tuberculosis based on the two specific antigens ESAT-6 and CFP10. *Clin. Diagn. Lab. Immunol.* **7**:155–160.
 37. **Wang, J. Y., C. H. Chou, L. N. Lee, H. L. Hsu, I. S. Jan, P. R. Hsueh, P. C. Yang, and K. T. Luh.** 2007. Diagnosis of tuberculosis by an enzyme linked immunoblot assay for interferon- γ . *Emerg. Infect. Dis.* **13**:553–557.
 38. **Wong, D. A., P. C. Yip, D. T. Cheung, and K. M. Kam.** 2001. Simple and rational approach to the identification of *Mycobacterium tuberculosis*, *Mycobacterium avium* complex species, and other commonly isolated mycobacteria. *J. Clin. Microbiol.* **39**:3768–3771.