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Patterns of intracellular cytokines in CD4 and CD8 T cells from patients with mycobacterial infections

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Abstract

Using a short-term bulk culture protocol designed for an intracellular-staining method based on a flow cytometry approach to the frequencies of cytokine-producing cells from tuberculosis and leprosy patients, we found distinct patterns of T cell subset expression. The method also reveals the profile of peak cytokine production and can provide simultaneous information about the phenotype of cytokine-producing cells, providing a reliable assay for monitoring the immunity of these patients. The immune response of *Mycobacterium leprae* and purified protein derivative (PPD) *in vitro* to a panel of mycobacteria-infected patients from an endemic area was assessed in primary mononuclear cell cultures. The kinetics and source of the cytokine pattern were measured at the single-cell level. IFN-γ-, TNF-α-, IL-4- and IL-10-secreting T cells were intracytoplasmic evaluated in an attempt to identify *M. leprae*- and PPD-specific cells directly from the peripheral blood. The analysis by this approach indicated that TNF-α was the first (8 h) to be produced, followed by IFN-γ (16 h), IL-10 (20 h) and IL-4 (24 h), and double-staining experiments confirmed that CD4+ were a greater source of TNF-α than of CD8+ T cells ($P < 0.05$). Both T cell subsets secreted similar amounts of IFN-γ. We conclude that the protocol permits rapid evaluation of cytokine production by different T cell populations. The method can also be used to define immune status in non-infected and contact individuals.

Key words
- Tuberculosis
- Leprosy
- IFN-γ
- TNF-α
- Anti-hCD28 mAb

Introduction

Cytokines are important mediators that play a significant role in the immunoregulation of leukocyte responses (for a review, see Kroemer et al., Ref. 1). Several strategies for studying cytokine production have been proposed (for a review, see Carter and Swain, Ref. 2). Some of the techniques currently employed may interfere with the detection of cytokine production and secretion. Among them are ELISA and ELISPOT, which are highly specific but only detect secreted cytokines. They reflect the net outcome of production, absorption and degradation of cytokines. Therefore, they are not measuring only the production of cytokines at the single-cell level.

Recently, powerful immunological methods for the assessment of cytokine-produc-

ing T cells have also been described for human samples. Intracellular staining for cytokines is a relatively novel method that allows simultaneous staining of several cytokines and surface markers on cells, thus permitting the identification of subpopulations and their cytokine production. A number of these methodologies are based on the count of cytokine-producing cells by flow cytometry (3-5). Sander et al. (6) first demonstrated the detection of intracellular cytokines by fixation (paraformaldehyde), permeabilization (saponin) and immunofluorescent staining using cytokine-specific monoclonal antibodies. Later, a modified method developed in order to improve specific intracellular staining permitted the detection of cytokine-producing cells by single-laser flow cytometry (3). This methodology is currently being used to study the possible role of specific cytokines in several human diseases (7-9), with a reduction of the time needed for analysis and an improved precision in the detection of cytokines, mainly those produced by CD4+ and CD8+ T cells. However, none of the cited reports has described the kinetics of fast production of major T cell cytokines using mycobacterial antigens. Only Kemp et al. (10) described a method for long-term purified protein derivative (PPD)-stimulated cultures, since no second signal to trigger the cytokine production had been used.

Cell-mediated immunity plays a major role in limiting the spread of tuberculosis and leprosy in immunocompetent hosts, and many pathological features are the result of cellular immune responses to the presence of *Mycobacterium tuberculosis* and *M. leprae*, respectively (11). Recent studies on immunity to mycobacteria have reported that some T cell functions may play an important role in controlling the infection (12). IFN-γ and TNF-α are believed to be essential for the activation of macrophages and for the control of the mycobacterial replication and granuloma formation, respectively, in humans (for a review, see Stenger and Modlin, Ref. 13). On the other hand, IL-4 and IL-10 are believed to increase the bacterial load (12).

Several methods for assessing cytokine expression and production at the single-cell level have been revisited (14); however, only a few laboratories have studied this characterization in depth and, to our knowledge, the present paper is the first to describe a standardized procedure using short-term bulk cultures plus the addition of anti-CD28 mAb for the detection of specific T cell subsets producing cytokines in leprosy and tuberculosis patients who are *M. leprae* and PPD responders.

This protocol for intracytoplasmic staining was first validated to examine the frequencies of cytokine-producing cells from Brazilian tuberculosis patients (15, and Antas P, Cardoso F, Pereira K, Oliveira E, Sarno E, Franken K, Milagres A, Klatser P, Ottenhoff T and Sampaio E, unpublished data). The method provides the profile of *in vitro* production of various cytokines by a population of peripheral blood mononuclear cells (PBMC). Also, intracellular staining can provide information similar to that obtained by ELISA and can be used to determine the frequency and immunophenotyping of cytokine-producing cells, representing a reliable assay for the definition of the immune status of these patients.

In the present study, the *M. leprae* and PPD *in vitro* immune responses were assessed in 32 leprosy and tuberculosis patients from an endemic area in Brazil. The kinetics and source of cytokines were estimated at the single-cell level in primary PBMC cultures. IFN-γ-, TNF-α-, IL-4- and IL-10-secreting T cells were determined in short-term bulk cultures in an attempt to identify *Mycobacterium*-specific cells present in peripheral blood. The analysis of cytokine production by intracellular flow cytometry allows rapid identification of functional subsets within heterogeneous T cell populations.

Material and Methods

Study population

A total of 15 pulmonary tuberculosis and 17 leprosy patients were recruited from the outpatient unit of the District hospital Raphael de Paula e Souza, and Souza Araújo Outpatient Clinic, FIOCRUZ, Rio de Janeiro, RJ, Brazil, respectively. Pulmonary tuberculosis and leprosy were diagnosed according to the criteria established by the World Health Organization (WHO) and the Brazilian Ministry of Health. The detailed methodology for the screening and diagnosis of the tuberculosis and leprosy patients has been described elsewhere (15,16). The patients were treated according to the recommendations of the Ministry of Health. All patients included were HIV negative when tested by two specific ELISA procedures. Patients with clinical conditions such as pregnancy, cancer, diabetes, AIDS, liver or kidney disease, and treatment failure were excluded. All patients provided informed written consent for blood collection and the study was approved by the Ethics Committee of the Institution, and according to resolution 196/96 of the National Health Council of the Ministry of Health.

Antigens

PPD (RT-46) was purchased from the Statens Serum Institute (Copenhagen, Denmark), and killed *M. leprae* was purchased from the WHO (Geneva, Switzerland). The antigen concentrations used for PBMC stimulation were 10 µg/ml, according to previous titration. The mitogen phytohemagglutinin (PHA, Gibco-BRL, Gaithersburg, MD, USA) was used as a positive control in most of the experiments. The endotoxic potencies (lipopolysaccharide content) in the antigenic preparations were checked using a chromogenic *Limulus* amebocyte lysate assay kit (Biowhittaker, East Rutherford, NJ, USA), and the final concentrations were found to have no influence on cellular activity.

Short-term cultures and stimulation

Heparinized venous blood was obtained and PBMC were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ, USA) density gradient centrifugation. A total of 1.0 x 10^6 cells/well were cultivated in 24-well flat-bottom plates (Costar Corp., Cambridge, MA, USA) in 1 ml complete RPMI 1640 (medium supplemented with 20% autologous plasma, antibiotics and L-glutamine) and incubated at 37ºC in a humidified 5% CO_2 incubator. *M. leprae* or PPD antigen was added to the wells after 4, 8, 10, 12, 16, 20, 22, 24, and 48 h. Four hours before the end of the cultures, either 50 ng/ml PMA plus 1 µg/ml ionomycin (Sigma Immunochemicals, St. Louis, MO, USA) for single staining experiments, or 3 µg/ml purified anti-human CD28 antibody (Pharmingen Inc., San Diego, CA, USA) for double staining experiments were added to the cultures. The inclusion of a protein transport inhibitor during the *in vitro* stimulation has been reported to enhance the levels of cytokines detected (3). Thus, 4 h before the end of incubation, 10 µg/ml of brefeldin A (Sigma) was added to the cultures. The cells and supernatants were then recovered separately. The supernatants were kept at -20ºC for further assays, and the cells were harvested by washing the wells with 1 ml of cold PBS.

Intracellular cytokine measurements by flow cytometry

Cells were prepared for intracytoplasmic cytokine analysis according to the Pharmingen Inc. protocol, with modifications (15). After a first PBS washing, the resulting cells were resuspended in cold PBS-FACS (PBS: 0.1% BSA plus 0.01% sodium azide). After the determination of cellularity, the amount of cells was adjusted to 0.5 x 10^6 cells/ml.

For cultures stimulated with anti-CD28, the experiments were performed with double staining: a) for cytokines and CD4+, incubated after previous titration with FITC-labeled mouse anti-human CD4 (Becton and Dickinson, San Diego, CA, USA), and b) for cytokines and CD8+ cells, incubated with FITC-labeled mouse anti-human CD8, both in PBS-FACS for 30 min at 4ºC in the dark. After two PBS-FACS washings at 4ºC, the cells were fixed in 4% paraformaldehyde (PFA) by vortexing at room temperature and the same procedure was followed for other staining. After 10 min, the cells were washed twice with PBS-FACS, and washed once with HBSS plus a 0.3% saponin solution. A total of 100 µl of saponin solution was used and cells were incubated for 30 min at room temperature with the respective PE-labeled antibodies to each cytokine: IFN-γ, TNF-α, IL-4 and IL-10 (Pharmingen). After two saponin solution washings and a subsequent PBS washing, the cells were resuspended with 1% PFA and the labeled cells were immediately analyzed by flow cytometry in a FACScalibur® instrument (Becton and Dickinson) equipped with the CellQuest® software. Cell gate regions were drawn around viable cells based on their forward scatter (FSC) versus side scatter (SSC) properties in order to exclude cell debris (inset in Figure 3A). In double-staining experiments, an extra-independent circular cell gate region was also drawn around viable blast cells. In addition to detecting the light scattered from the cells, FL-1 (FITC) and FL-2 (PE) signals were set up. A total of 50,000 events in small lymphocyte regions per sample were collected in a dot plot displaying the FSC and SSC properties of the cell. The relative fluorescence intensity of the cells was analyzed in a dot plot using a logarithmic scale for FL. Cell permeabilization may result in higher levels of non-specific staining (5). Thus, it is important to include controls for non-specific binding, and positive (PHA) and negative controls for cytokine production. We set thresholds and statistical markers for positivity using irrelevant isotype FITC- and PE-mouse IgG1 as match control. Data are reported as the percentage of single- or double-stained positive bright cells.

IFN-γ production

IFN-γ concentration in cell-free culture supernatant was determined with a commercial ELISA using specific pairs of monoclonal antibodies processed according to manufacturer specifications (Pharmingen). The detection limit of the assay was 8 pg/ml, and cytokine levels in control cultures were usually undetectable.

Statistical analysis

Results are reported as mean ± SEM and data were analyzed statistically by the Student *t*-test (GraphPad InStat V. 2.04), with the level of significance set at $P < 0.05$.

Results

Kinetics of IFN-γ and TNF-α production in response to *Mycobacterium leprae* or purified protein derivative

To our knowledge, this is the first study describing the kinetics of cytokine production in short-term bulk PBMC cultures using PPD as antigen for tuberculosis patients, and both PPD and *M. leprae* antigens for leprosy patients. Intracellular cytokine production by human lymphocytes was assessed by FACS involving staining by the permeabilization procedure. It is important to examine the kinetics of cytokine production following antigenic stimulation, since each individual cytokine may have different kinetics during expression (6). The kinetics of IFN-γ and TNF-α production by lymphocytes was determined after *in vitro* stimulation with PHA (data not shown), and PPD or *M. leprae* in 3 tuberculosis and 4

leprosy patients, respectively. The immune response to PPD and *M. leprae*, as assessed by specific ELISA, was positive (IFN-γ >100 pg/ml, and TNF-α above baseline levels) in all tuberculosis and leprosy patients (Figure 1A and data not shown).

Since non-stimulated T cells usually do not produce measurable amounts of cytokines, *in vitro* stimulation is required (17). PMA + ionomycin under the influence of brefeldin A was used to artificially stimulate production and stock of cytokines, respectively. The kinetic pattern of IFN-γ and TNF-α production by lymphocyte populations *in vitro* assessed by intracellular staining is shown in Figure 1A and 1B, respectively. The kinetics of the response to IFN-γ was monitored at 8, 12, 20, and 24 h. The peak of IFN-γ levels from one tuberculosis patient in response to PPD occurred at about 16 h. To obtain more precise data, we repeated the experiment using shorter intervals between time points at 12, 16 and 20 h. Sixteen hours was the optimum time to detect IFN-γ in this system (Figure 1A). Also, the *in vitro* kinetics of IFN-γ levels was determined in the same supernatants by ELISA, comparing the efficiency of brefeldin A in retaining the intracellular cytokine (Figure 1A). As expected, cytokine synthesis did not match the cytokine concentration in the supernatants since the peak of IFN-γ detected by FACS was observed sooner after stimulation, but the peak in the supernatant seemed to occur after 24 h.

To detect TNF-α production, we followed the same procedure as above, but at earlier times, since the production of this cytokine has been reported to precede IFN-γ (18). We monitored TNF-α at 4, 8, 10, 12, 16, 20, 22, 24, and 72 h, and the kinetics is shown in Figure 1B. Here, TNF-α was the first cytokine produced by lymphocytes in all leprosy patients. The highest TNF-α production occurred between 4 (despite high background levels) and 12 h, corroborating earlier reports (16). Therefore, the optimum response was at 8 h as the point of highest TNF-α production. At this time, the cells showed a high percentage of short-term cytokine production, as further confirmed by double-staining experiments. A positive immune response was detected in the leprosy patients tested with PHA (Table 1).

Kinetics of IL-4 and IL-10 production in response to *Mycobacterium leprae* or purified protein derivative

It has been reported that primary stimulation is required for IL-4 and IL-10 produc-

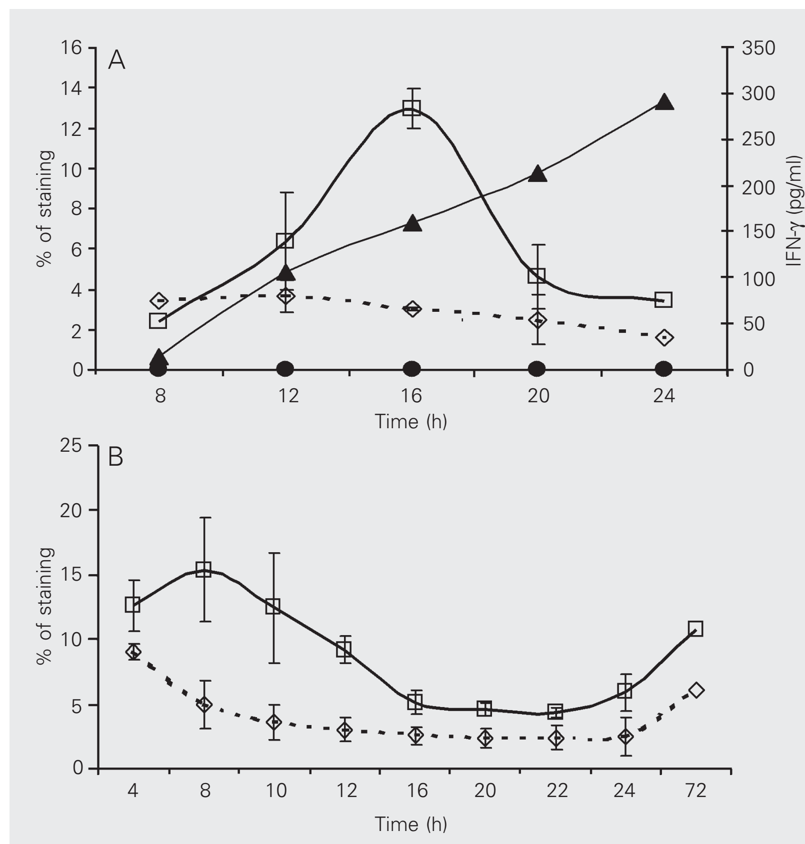

Figure 1. Kinetics of IFN-γ (A) and TNF-α (B) production by total lymphocytes from purified protein derivative- or *Mycobacterium leprae*-stimulated (squares) peripheral blood mononuclear cells of tuberculosis or leprosy patients, respectively. In A, a second axis on the right side of the figure denotes IFN-γ levels (pg/ml) from one representative tuberculosis patient culture supernatant comparing the same previous stimuli (circles) and where brefeldin A was suppressed (triangles). The dashed lines and diamonds indicate the non-stimulated cultures (medium). Data are reported as means ± SEM (N = 7) obtained from medium or *Mycobacterium*-antigens (at 10 µg/ml) plus cells additionally stimulated with PMA, ionomycin and brefeldin A.

Table 1. Percentages of IFN-γ (16 h) and TNF-α (8 h) staining in CD4+ and CD8+ T cells following *in vitro* antigen stimulation of peripheral blood mononuclear cells from tuberculosis and leprosy patients.

	Stimulus							
	Small lymphocytes				Blast lymphocytes			
	Medium	PPD	*M. leprae*	PHA	Medium	PPD	*M. leprae*	PHA
Tuberculosis patients								
CD4+/IFN-γ (10)	0.46 ± 0.07	1.67 ± 0.45*	ND	ND	2.04 ± 0.3 (8)	8.73 ± 2.2*	ND	ND
CD8+/IFN-γ (10)	0.44 ± 0.08	1.50 ± 0.38*	ND	ND	1.77 ± 0.2 (8)	7.27 ± 1.8*	ND	ND
CD4+/TNF-α (5)	0.78 ± 0.04	2.05 ± 0.63*	ND	ND	3.11 ± 0.3 (5)	9.87 ± 2.1*	ND	ND
CD8+/TNF-α (5)	0.49 ± 0.14	1.01 ± 0.24	ND	ND	2.14 ± 0.4 (5)	7.87 ± 1.2*	ND	ND
Leprosy patients								
CD4+/TNF-α (13)	1.88 ± 0.48	3.72 ± 1.24	4.39 ± 1.24	14.6 ± 4.73*	8.16 ± 2.5 (9)	11.45 ± 3.2	18.62 ± 9.0	36.47 ± 10.9*
CD8+/TNF-α (13)	0.70 ± 0.12	1.45 ± 0.33*	1.79 ± 0.38*	4.77 ± 0.75*	4.68 ± 1.3 (9)	11.91 ± 4.9	6.72 ± 1.5	14.45 ± 1.3*

Data are reported as mean ± SEM, with the number of patients given in parentheses. *Mycobacterium* antigens were used at a final concentration of 10 µg/ml and 1% phytohemagglutinin (PHA) was used as mitogen. ND = not determined; PPD = purified protein derivative. *P < 0.05 compared to medium (Student *t*-test).

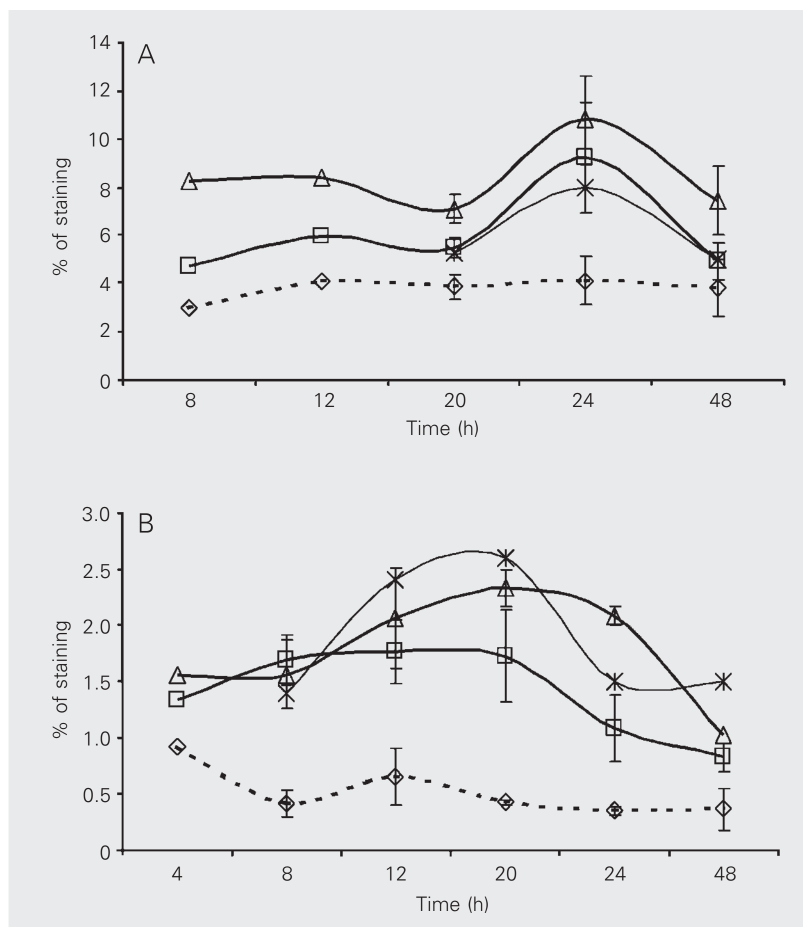

Figure 2. Kinetics of IL-4 (A) and IL-10 (B) production in total lymphocytes from phytohemagglutinin (PHA)-, purified protein derivative (PPD)- or *Mycobacterium leprae*-stimulated peripheral blood mononuclear cells of tuberculosis or leprosy patients, respectively. The dashed lines and diamonds indicate non-stimulated cultures (medium), the squares indicate PPD-stimulated cultures, the triangles indicate PHA-stimulated cultures, and the asterisks indicate *M. leprae*-stimulated cultures. Data are reported as means ± SEM (N = 3) obtained from antigens (*Mycobacterium* antigens, 10 µg/ml, and 1% PHA) plus cells additionally stimulated with PMA, ionomycin and brefeldin A.

tion, followed by restimulation with a mitogen (6). The kinetics of IL-4 and IL-10 production by lymphocytes was determined *in vitro* as described above in 2 other tuberculosis patients and in 1 leprosy patient. Again, the immune response to PPD was positive (IFN-γ levels >100 pg/ml) by ELISA in these next 3 patients (data not shown). IL-4 is also produced by mitogens-stimulated T cells (19) and, therefore we first used stimulation with PHA to induce both IL-4 and IL-10 production. With this procedure, it was possible to detect IL-4- and IL-10-producing cells following primary stimulation with PHA, *M. leprae* or PPD, and subsequent PMA + ionomycin addition, in contrast to the results obtained by others using an experimental model (20). As shown in Figure 2A, the peak of IL-4 synthesis in response to these 3 stimuli occurred after one day. At that time, the cells showed a high percentage of cytokine levels, except for the cells of one patient stimulated with PHA (12 h). Therefore, the highest IL-4 production occurred at 24 h.

Early IL-10 production was also detected, since kinetic studies conducted between 4 and 48 h showed the peak of synthesis of this cytokine at about 20 h, with a rapid reduction thereafter (Figure 2B). Here, as also observed for TNF-α, a high background level was detected earlier at 4 h of culture. IL-4 and IL-10 production was markedly reduced after 48 h, while IL-10 remained relatively stable for the same period of time in only one leprosy patient. Positive responses to PHA were detected in all patients tested (data not shown).

Immunophenotyping profile for intracellular IFN-γ and TNF-α in T cell subsets among tuberculosis and leprosy patients

To validate our previous kinetic study, we performed a single study in another tuberculosis and leprosy patient panel from the same endemic region. Each protocol can be combined with surface labeling in order to determine the phenotype of cytokine-secreting cells. Thus, we carried out double-fluorescence staining experiments to detect which T cell subsets might produce the large amounts of IFN-γ and TNF-α observed previously but using different groups of patients.

A decrease of >95% of human CD4 and CD8 T cells following incubation with PMA + ionomycin has been reported (21-23). Therefore, to circumvent this problem, we slightly modified our protocol by replacing these reagents with a more physiological second signal triggering molecule, the purified anti-human CD28 antibody, after stimulation with specific antigen for the next step. The use of anti-CD28 antibody as a co-stimulatory signal for rapid cytokine production by T cells has been described recently, giving satisfactory results with our system and others (15,24,25). Accordingly, we also assayed, in parallel, some stimulated PBMC cultures in the absence of anti-CD28 antibody. There was an average 10% decrease in specific staining for IFN-γ (data not shown).

Therefore, further *in vitro* priming of T cells with purified anti-CD28 for 5 h followed by incubation of brefeldin A 1 h after was required to stimulate cells to produce the cytokines. Figure 3 shows representative experiments of the double-staining pattern using the same antibodies to IFN-γ and TNF-α as above, in PPD- or *M. leprae*-stimulated CD4+ and CD8+ T cells from tuberculosis and leprosy patients, respectively, compared to baseline (medium) in CD4+ T cells. All of the 10 tuberculosis patients studied (100%) showed positive T cell staining for IFN-γ, and 5 of 5 (100%) showed CD4+-positive staining for TNF-α production. Four of 5 (80%) showed positive staining for TNF-α production by CD8+ T cells in response to PPD ($P = 0.09$, when compared to medium in small lymphocytes). For comparison purposes, Table 1 lists the percentages of cytokine production by these T cell subset populations. In the present cohort of tuberculosis patients, the *in vitro*

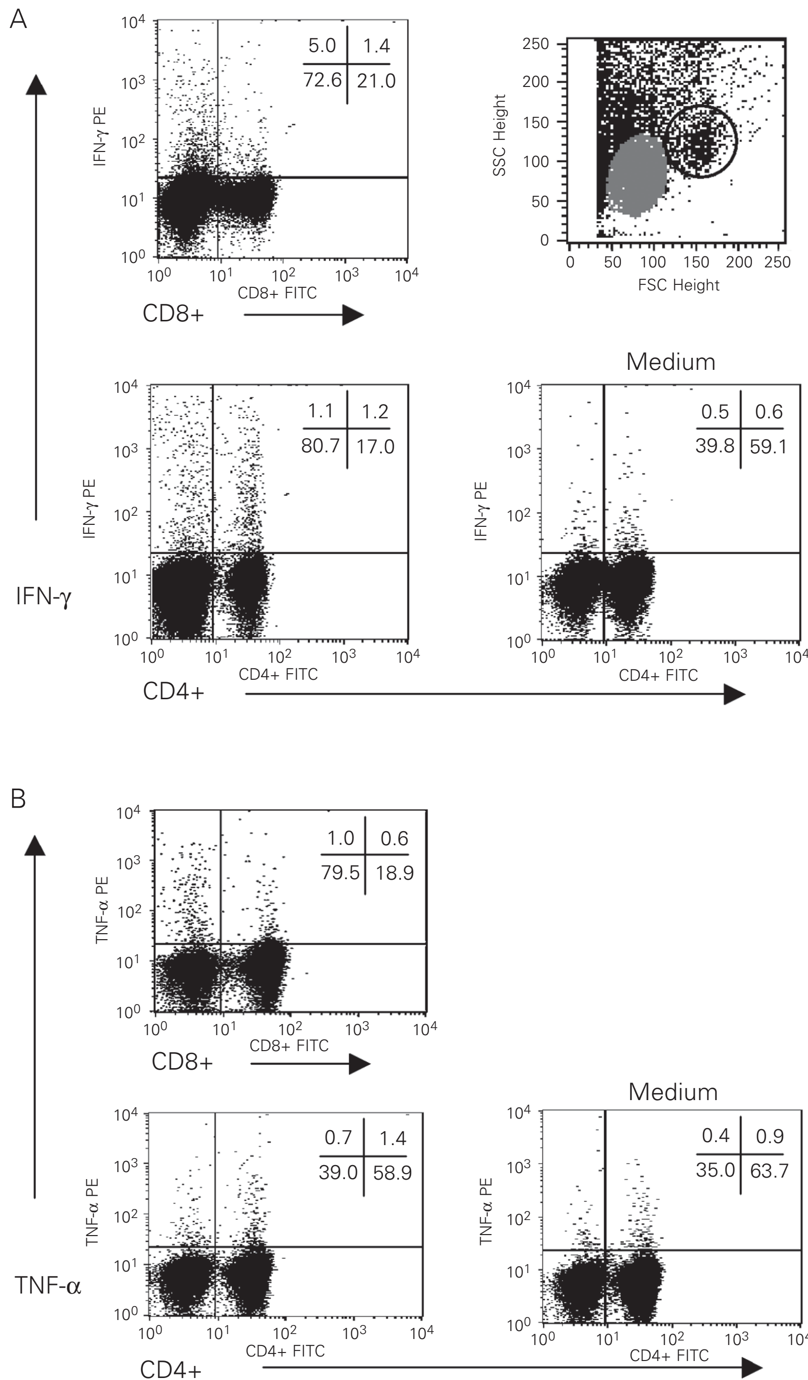

Figure 3. Typical profiles of IFN-γ (A) and TNF-α (B) intracellular expression in CD4+ and CD8+ T cells from purified protein derivative- or *Mycobacterium leprae*-stimulated peripheral blood mononuclear cells, respectively. Cytokine production after specific antigenic stimuli (10 µg/ml) was enhanced by the addition of purified anti-human CD28 antibody (at 3 µg/ml) and brefeldin A in representative tuberculosis and leprosy patients. The inset in A shows the light scattering of the two distinct populations (gray and circle regions indicating small and blast cells, respectively) gated on the basis of their properties. Percentages of cells for the respective quadrants are indicated in each plot.

IFN-γ and TNF-α response to PPD was enhanced among treated patients compared to untreated ones (Antas P, Cardoso F, Pereira K, Oliveira E, Sarno E, Franken K, Milagres A, Klatser P, Ottenhoff T and Sampaio E, unpublished data). More importantly, however, the difference between these T cell subsets in terms of IFN-γ production by PPD has not been consistent, in contrast to TNF-α which showed a 2.4-fold increase of CD4+ compared to CD8+ T cells ($P < 0.05$). Eleven of 13 leprosy patients (85%) showed mainly TNF-α production by CD4+ T cells, while all (100%) showed that CD8+ T cells primarily yielded TNF-α in response to *M. leprae*. Likewise, the data for the percentages of TNF-α production by these T cell subsets for leprosy patients are presented in Table 1. Like tuberculosis patients, leprosy patients also showed an increase in CD4+ cells ranging from 2.3- (*M. leprae*, $P < 0.05$) and 2.5- (PPD, $P < 0.05$) to 3.1-fold (PHA, $P = 0.07$) compared to CD8+ T cells.

For comparative analysis, we also attempted to detect the potential presence of IFN-γ and TNF-α in some blast populations from both T cell subsets, since there was not enough time for our short-term bulk cultures to yield a large amount of blast cells for FACS counting (inset in Figure 3A). We observed an increase of background staining in this case, and all comparative results are summarized in Table 1. Finally, the results regarding the immunological differences inherent to leprosy and tuberculosis patients can be explored in terms of TNF-α staining (Table 1).

Discussion

The detection of intracellular cytokines by flow cytometry has been used in several studies (3-5). However, to our knowledge, no report concerning the optimization of this technique to measure the frequency of human *Mycobacterium*-specific IFN-γ-, TNF-α-, IL-4- and IL-10-producing T cells by

short-term bulk culture has been reported thus far.

In the first step of the present study, antigenic *in vitro* stimulation for IFN-γ, TNF-α, IL-4 and IL-10 production required incubation of PBMC with PMA + ionomycin for 5 h in the presence of brefeldin A. The highest frequency of cells producing IL-4, IL-10 and TNF-α was observed at 24, 20 and 8 h, respectively, in agreement with an earlier report (26). The highest frequencies of IFN-γ cells were seen at about 16 h, as also reported by others (26,27). It is noteworthy that high background levels of TNF-α and IL-10 were detected in PBMC cultures at 4 h, even without the addition of *Mycobacterium* antigens. This may probably reflect an initial, nonspecific adhesion process of mononuclear cells to the bottom of plastic culture dishes, since both endotoxic assay and later time points ruled out any contamination (16, and DeLuca P, personal communication).

We describe here the detection of intracytoplasmic cytokines by flow cytometry in order to circumvent some technical limitations imposed by ELISA. The advantage of flow cytometry using two different fluorescent dyes is the identification of single-cells and the cytokines they produce, as well as the ability to measure the kinetics of cytokine production (28). However, detection of cytokine production by FACS intracellular staining is probably less sensitive than ELISA, as indicated by the fact that the frequencies of IL-10-producing cells after PHA and PMA plus ionomycin stimulation are usually close to the FACS detection limit (0.1 to 2.0%), whereas, with these stimuli, the quantities of secreted protein measured by ELISA are far above the detection limit of the method (data not shown). Additional experiments are required to improve the parameters for determining the amounts of IL-4 and IL-10 produced. Thus, the use of intracellular cytokine staining, expressed as percentage of positive cells, may be a more suitable, reliable and reproducible method than ELISA.

It should be emphasized that these two methods give different types of results (amount of protein *vs* frequency of cytokine-producing cells), and thus may lead to different interpretations of the data. Differences in the percentage of stained cells may reflect the net amount of secreted protein (3). Indeed, cytokine levels accumulated in culture supernatants represent the net balance between autocrine or paracrine consumption and the amount of secreted cytokine, which is itself influenced by several parameters including the rate of cytokine synthesis, intracellular transport, accumulation in granules, and secretion. A clear example is shown here by the peak in kinetics of IFN-γ, mainly between 20 and 24 h, when comparing FACS and ELISA data. By contrast, intracellular cytokines may be solely dependent upon the level of cytokine synthesis, and not secretion, since most cell cultures were performed in the presence of an inhibitor of trans-Golgi protein transport that blocks cytokine secretion and leads to intracellular cytokine accumulation.

Several groups have also reported the use of multi-parameter flow cytometry for assessment of single-cell intracellular cytokine production in human PBMC (3,28,29). The main strengths of the method are the ability to distinguish between Th1 and Th2 cells, to examine cytokine production in rare cell populations and also to analyze cytokine production at the level of individual cells (17).

We also evaluated the stimulation protocols for IFN-γ and TNF-α production by human PBMC. We used double-color flow cytometry analysis for the detection of intracellular cytokines both in PPD- and *M. leprae*-stimulated CD4+ and CD8+ T cells, for the same period of 5 h with anti-CD28 in the presence of brefeldin A. Importantly, the addition of anti-CD28 antibody increased the specific staining for IFN-γ by about 10%. As expected, lower frequencies of double-

positive cells were achieved in peripheral blood, although we confirm here that helper lymphocytes are a larger source of TNF-α than CD8+ T cells. We are now performing additional experiments using enriched T cell populations that could circumvent this problem (30). The difference between T cell subsets in terms of IFN-γ production by PPD is not consistent and fits the initial proposal of our group and others: CD8+ T cells play an important role as a source of *in vitro* IFN-γ secretion (15,30). More recently, memory CD8+ T cells have been reported to be a possible early *in vivo* source of IFN-γ in response to bacterial products (31). In addition, a study is currently in progress in our laboratory to improve the phenotyping analysis associated with intracellular IL-4 and IL-10 staining, with the initial results obtained being too preliminary to be presented here.

Overall, our data suggest that, in addition to permitting the identification of the cytokine-producing cell phenotype, intracellular cytokine staining by flow cytometry might be more reliable than ELISA for the biological follow-up of clinical samples. This method will be useful for analyzing the nature of immunity after a variety of antigenic stimuli, and allow us to improve understandings of the complex relationship between mycobacteria and immune system, which is fundamental in order to develop new prophylactic and diagnostic tools for control strategies. Further studies to determine the use of this protocol for the study of *in vitro Mycobacterium*-immune responses would be helpful also to clarify the pathogenesis of both tuberculosis and leprosy.

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