Flow-cytometric assessment of lymphocyte cytokine production in tuberculosis

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Summary We assessed by flow-cytometry the Th1/Th2 profiles in peripheral blood lymphocytes (PBL) from patients with active tuberculosis (TB), before and after antituberculostherapy, and from healthy tuberculin-positive and -negative reactors. PBL from patients showed a reduced potential for Th1-cytokine (notably IFN-γ) production after culture with a polyclonal stimulus. When these PBL from patients were cultured with a M. tuberculosis (MTB)-specific antigen such as PPD (10 μg/ml), there was no detectable production of Th1 cytokines. Only the Th2 cytokine IL10 was detected in PBL from patients but not from controls. However, at the site of the tuberculosis disease, T lymphocytes from bronchoalveolar lavage, after culture with PPD, produced IFN-γ. After completion of tuberculosis therapy, PBL did not produce IL10. These data indicate that the immunosuppression observed in PBL during active tuberculosis infection may be related to IL10 production, and to the compartmentalization of the antigen-Th1 response to sites of active MTB infection. © 2002, Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Mechanisms of protective immunity against M. tuberculosis (MTB) in humans have not been totally clarified. Cell-mediated immune response plays an essential role. Most people who become infected with MTB mount a protective immune response and remain clinically well, the only evidence of infection being a positive tuberculin skin test. A minority develop tuberculosis disease within the first year after infection (primary tuberculosis) or thereafter (reactivation tuberculosis). The balance of cytokines produced by lymphocytes in response to this infection is believed to have a profound effect on clinical outcome. Previous studies have demonstrated during active tuberculosis disease, a relative depression in peripheral blood of the T-helper 1 (Th1) cytokine responses, interleukin-2 (IL2) and interferon-γ (IFN-γ). The role of the T-helper 2 (Th2) immunosuppressive cytokines, interleukin-4 (IL4) and interleukin-10, (IL10) is less clear. Healthy tuberculin reactors seem to have protective immunity with an enhancement of Th1 responses.

The present study was undertaken to assess by flow-cytometry the Th1/Th2 profiles in peripheral blood and pulmonary lymphocytes from patients with active tuberculosis and from healthy tuberculin-positive (PPD-positive) and -negative (PPD-negative) controls. We studied the intracellular levels of Th1 (IL2 and IFN-γ) and Th2 (IL4 and IL10) cytokines from T CD4 and CD8 lymphocytes. Cells were stimulated with PMA-ionomycin to reveal the full cytokine potential of these cells and with PPD to demonstrate the antigen-specific cytokine production in tuberculosis disease.

PATIENTS AND METHODS

Patients and control subjects

We studied 15 HIV-negative patients with newly diagnosed reactivation tuberculosis. None of them had a serious medical illness. Mean age of the patients was 42.5 years (range 21–78), 10 patients were male. In each patient, the diagnosis was suggested by demonstration of acid-fast bacilli in sputum (9 patients), bronchoalveolar lavage (BAL) (4 patients), pleural fluid (one case) and peritoneal biopsy (one case), and confirmed by a positive culture for Mycobacterium tuberculosis. We assumed all of them had reactivation tuberculosis because the
radiological findings showing affection of the superior pulmonary lobules (11 patients), occasionally associated with pleural effusion (2 patients) or with caverns (5 patients, in two cases the cavern was located in the medium-lobe), or a miliary pulmonary tuberculosis (1 patient) or peritoneal (1 patient) affection; two of them had a positive tuberculin skin test done months before. A PPD skin test was positive at diagnosis in 11 patients, and negative in two patients. In two patients it was not done. Bronchoalveolar lymphocytes (BAL) from two of the patients with active TB were evaluated for PPD-induced cytokine production. Seven of the patients were re-evaluated after chemotherapy. Five HIV-negative, PPD-positive and 9 PPD-negative healthy controls were also studied, as well as one HIV-negative, PPD-positive patient with bacterial pneumonia (mean age: 41 years, range: 19–49 years).

METHODS

Cell preparation and in vitro culture

PBL (peripheral blood lymphocyte) were isolated from sterile heparinized venous blood from patients by differential centrifugation on Ficoll-Hypaque (Lymphoprep) (Nyegard). BAL were obtained by bronchoalveolar lavage, 200 ml of isotonic saline were instilled into the affected pulmonary segment by a flexible fiberoptic bronchoscope. The BAL was centrifuged, and the mucus was decanted. Cells from PBMC and from BAL were then cultured at 2 x 10⁶ cells/ml in complete medium (RPMI 1640, supplemented with 10% heat-inactivated fetal-calf serum (FCS) Bio-Whittaker) alone or in presence of PPD (10 μg/ml, Statens Serum Institut, Copenhagen, Denmark), PMA (phorbol 12-myristate acetate, 100 μg/ml, Sigma) Ionomycine (1 μg/ml, Sigma), the most robust Ionomycine (1 μg/ml, Sigma), the most robust stimulus for most lymphokines. Cultures incubated with medium alone served as negative controls. These cultures were incubated for 24 and 72 hours when the stimulus was PPD and for 4–6 hours when the stimulus was PMA-Ionomycin, at 37° in a 5% CO₂–95% air atmosphere. Brefeldine-A (10 μg/ml, Sigma) was also added for the final four hours of activation to inhibit intracellular transport and to allow the cytokines produced during activation to be retained inside the cell. Activated cultures were subsequently aliquoted for staining. Since cells numbers were limited, both PMA-Ionomycin and PPD stimuli could not be tested for all subjects in every experiment.

Stain cell surface

Following activation, cells were stained with TC-fluorochrome-conjugated anti-CD3 and FITC-anti-CD8 antibody for lymphocytes and then fixed. We assumed that the CD3-positive but CD8-negative subsets were equivalent to the CD4-positive subset, because CD4 cannot be used as a phenotypical marker after stimulation as it is down-regulated by PMA. To assess lymphocyte activation induced by the two different stimuli, PE-fluorochrome-conjugated anti-CD69 and its control were added to two separate tubes. Cell-surface expression of CD69 is a very early activation marker.

Intracellular stain

Subsequently, FACS Lysing Solution (BDIS) was added to each tube. After a further short incubation, samples were centrifuged and combined with FACS Permeabilizing solution (BDIS), for 10 min at 25 °C. Samples were washed in phosphate-buffered saline (PBS) and incubated with Phycoerytrine (PE)-labeled anti-cytokine antibodies for 30 min. The sample tubes were washed once more and resuspended in 1% paraformaldehyde prior to analysis with a flow cytometer.

Three-color flow cytometric analysis

It was performed on a FACSsort flow cytometer (BDIS) Data were acquired using CELL-Quest software (BDIS), typically collecting 10,000 gated CD3+ events for lymphocytes using FL3(Tc) as a fluorescent trigger. Data were displayed as two-color dot-plots in a PAINFA-GATE software (BDIS). The cut-off point between positively and negatively stained cells is the upper limit of the negative control. We always determined the specific production of a cytokine as the difference between the production detected in stimulated and unstimulated cells. Spontaneous release of any cytokine was always <1%.

Statistical analysis was performed using a non-parametric test. The data from the groups were compared using the Mann–Whitney U test for unpaired samples. Differences were considered significant at P<0.05. Data are given as median and interquartile range (percentile 25–percentile 75).

RESULTS

I. Cytokines expressed by lymphocytes stimulated with PMA-Ionomycine

We assessed PMA-Ionomycine-stimulated production of cytokines by lymphocytes from 11 patients with recently diagnosed tuberculosis and 14 controls (6 PPD-positive and 8 PPD-negative). Cell activation with the polyclonal stimulus PMA-Ionomycin was assessed with the CD69 membrane antigen, which was always expressed in >80% of the T lymphocytes (CD3+). The percentages of cytokine production detected after this pharmacological stimulus in both PPD-positive and PPD-negative control groups were similar (data not shown) and considered in a
single group; the number of cells expressing the Th1 cytokines IL2 and IFN-γ markedly exceeded the proportion of Th2 cytokines IL4 and IL10. The lymphocyte CD4 production of the Th1 cytokine IFN-γ in tuberculosis patients was depressed compared with that produced in the group of healthy tuberculin-positive and -negative controls ($P=0.002$). For IFN-γ produced by CD8 T lymphocytes, there was a trend to a depressed production in patients, without reaching statistical significance. There were no other significant differences in cytokine production between these groups (Table 1).

2-Cytokines expressed by lymphocytes stimulated with PPD (10 μg/ml)

We studied the expression of cytokines in lymphocytes in response to PPD in PBMC samples from 13 patients and from 6 PPD-positive healthy controls (Table 2); 3 PPD negative controls were also assessed. The median of CD69 expression after this stimulus in lymphocytes T

| Table 2 | Percentages of lymphocytes producing cytokines from patients with newly diagnosed tuberculosis infection and from PPD-positive healthy controls, following stimulation of the PBL with PPD (24 hours). Results are expressed as median and interquartile range (percentile 25–percentile 75). |
|------|------|------|------|------|------|------|------|------|------|
|       | PPD (10 μg/ml) | Controls (n=6) | P |
| IL2CD8 | 0.0 (0.0–0.0) | 0.0 (0.0–0.0) | ns |
| IL2CD4 | 0.0 (0.0–0.0) | 0.0 (0.0–0.0) | ns |
| IL4CD8 | 0.0 (0.0–0.0) | 0.0 (0.0–0.0) | ns |
| IL4CD4 | 0.0 (0.0–0.0) | 0.0 (0.0–0.0) | ns |
| IFNCD8 | 0.0 (0.0–0.0) | 0.0 (0.0–0.0) | ns |
| IFNCD4 | 0.0 (0.0–0.0) | 0.0 (0.0–0.0) | ns |
| IL10CD8 | 0.7 (0–3.5) | 0.0 (0.0–0.0) | 0.015 |
| IL10CD4 | 1.0 (0–3.9) | 0.0 (0.0–0.0) | 0.015 |

PMA-ion: PMA-ionomycine; controls: PPD-positive controls; IFN-IFNγ; ns: no statistical significance.

3-PPD-induced cytokine production from BAL

Two of the patients were evaluated for the in-site of disease Th1-cytokine production after culturing their BAL lymphocytes with PPD (10 μg/ml) during 24-hours. A BAL from a HIV-negative patient with bacterial pneumonia and a positive tuberculin skin test was evaluated as a control. CD69 was detected in at least 75% of the BAL lymphocytes from each sample. IL2 was produced in 4 and 5.6% and IFN in 9 and 14.3% of the CD4 T lymphocytes from the tuberculous patients. These cytokines were not detected in significant amounts in the CD4 T cells from BAL from the patient with bacterial pneumonia. These results are shown in Fig. 1.

4-Cytokine production after recovery

Follow-up data are reported from 6 patients after 6 months of standard tuberculosis treatment. PPD-induced cytokine production in PBMC from these patients was evaluated at 24h and 72h. The median of PPD-induced CD69 expression in lymphocytes from this group was 40.5 (19.7–52.7). We did not detect IL2 or IL4 production at 24 hours nor IFN-γ production at 24 or 72 hours. IL10, a cytokine produced in significant amounts in acute tuberculosis patients, was not observed in this post-treatment group, and the difference reached statistical significance for IL 10 produced by CD4 lymphocytes ($P=0.046$).

**DISCUSSION**

These data illustrate the capability of cytokine flow cytometry to show a new perspective on the involvement of cytokines in the tuberculosis process. Peripheral blood
CD4 T lymphocytes and to a lesser extent CD8 T lymphocytes from tuberculosis patients showed a reduced potential for Th1-cytokine (notably IFN-γ) production after culture with a polyclonal stimulus compared with controls. When these lymphocytes from patients were cultured with a MTB-specific antigen such as PPD (10 μg/ml), there was no detectable production of the Th1 cytokines IL2 and IFN-γ. Only the suppressive Th2 cytokine IL10 was detected in PBMC from patients but not from controls. However, at the site of the tuberculosis disease, CD4 and CD8 T lymphocytes from BAL fluid, after a 24-hour culture with PPD, produced the Th1 cytokines IL2 and IFN-γ. After completion of tuberculosis therapy and recovery, peripheral blood lymphocytes did not produce any cytokines, neither did they produce the suppressive cytokine IL10.

A decreased cellular immune response from peripheral blood lymphocytes in tuberculosis has been described by others: several studies have found in PBL a depressed T cell proliferative response to MTB antigens; however, a flow-cytometric study found IFN-γ production after a PMA-Ionomycin stimulus in similar percentages in PBMC from tuberculosis patients and controls. A recent publication has demonstrated the sequestration or compartmentalization of antigen-specific CD4 T lymphocytes at the site of disease in tuberculosis, with local production of Th1 cytokines, notably IFN-γ, in BAL and pleural fluid and absence of detection in PBMC from these patients. The nature of type 2 cytokine (IL4 and IL10) involvement is less clear. IL4, an inhibitory cytokine that remains undetectable in our study, has yielded controversial results in other studies: an increase in IL4 gene expression or in IL4-secreting T cell in patients compared with controls has been demonstrated, but others have found low serum IL4 concentrations in tuberculosis patients with and without any stimulus. We found specific T-cell IL10 production in PBMC from patients and these results are in good agreement with other studies. IL10 is a suppressive cytokine that may contribute to the anergy and the absence of IFN-γ observed in human tuberculosis. Treatment of patients with active TB changed the pattern of cytokine production in response to PPD, there was a decrease in IL10 production but we did not observe an increase in IFN-γ production. A technical problem that could explain the absence of detection of IFN-γ-producing lymphocytes in PBMC, even after completion of therapy as well as in PPD-positive contacts, is not totally excluded, but IFN-γ was detected in BAL fluid in same experimental conditions. This long-lasting Th1 immunosuppression has suggested the existence of other immunosuppressive mechanisms different from IL10. MTB-induced apoptosis observed

![Fig. 1](image-url) Staining patterns of Th1-cytokines production seen after a 24 h PPD stimulation of lymphocytes from BAL in a patient with a non-tuberculous bacterial pneumonia (A) and a patient with pulmonary tuberculosis (B). (A) IL2 is produced in 1.2% of CD4 T cells (CD3+CD8-); IFN is produced in 1% of CD4 T cells (CD3+CD8-). (B) IL2 is produced in 5.6% of CD4 T cells (CD3+CD8-); IFN is produced in 14.3% of CD4 T cells (CD3+CD8-).
in PBMC may play an important role, and recently apoptosis has also been demonstrated in pleural fluid, this latter in association with IFN production. Activated T lymphocytes may not be the unique source of IFN-γ during tuberculosis infection.

Further studies are necessary to better understand the immunopathogenesis of tuberculosis. Flow cytometric intracellular detection of cytokines is a new specific technique for the assessment of complex cytokine production phenotypes in fresh ex vivo T cell subpopulations. It may enhance our understanding of the complex host-pathogen interaction in tuberculosis and facilitate development of immunomodulatory therapies.

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REFERENCE