Role of $\gamma/\delta$ T-cells in the Peripheral Blood of Patients with Pulmonary Tuberculosis

NOBUKUNI YOSHIDA

Department of Medicine, Kurume University School of Medicine, Kurume 830-0011, Japan

Summary: Although $\gamma/\delta$ T-cells are known to contain the highest frequency of mycobacteria-reactive cells in humans, and recent studies have suggested that they play an important role in the initial immune response to Mycobacterium tuberculosis (Mtb), very few studies have attempted to analyze these cells in patients with active pulmonary tuberculosis (TB). The aim of the present study was therefore to evaluate the $\gamma/\delta$ T-cell populations present in the peripheral blood and the IFN-$\gamma$ production of $\gamma/\delta$ T-cells stimulated by PMA before and after anti-TB chemotherapy in patients in the initial treatment stage for primary active pulmonary TB. Cell populations were measured by three-color flow cytometry of peripheral blood mononuclear cells. We compared the population of $\gamma/\delta$ T-cells and the production of IFN-$\gamma$ between normal healthy controls and TB patients. Absolute numbers of $\gamma/\delta$ T-cells remained constant in the peripheral blood of TB patients. However, production of IFN-$\gamma$ in $\gamma/\delta$ T-cells was dramatically suppressed prior to anti-TB chemotherapy in comparison with healthy control subjects, and further reduced following anti-TB chemotherapy. We also examined the influence of isoniazid (INH) in anti-TB chemotherapy. INH suppressed IFN-$\gamma$ production of $\gamma/\delta$ and $\alpha/\beta$ T-cells. The findings demonstrated a strong correlation between the production of IFN-$\gamma$ in $\gamma/\delta$ T-cells and manifestation of primary active pulmonary TB, which was consistent with the hypothesized role for $\gamma/\delta$ T-cells in the protective immune response to Mtb infection.

Key words pulmonary tuberculosis, $\gamma/\delta$ T-cell, $\alpha/\beta$ T-cell, IFN-$\gamma$

INTRODUCTION

Mycobacterium tuberculosis (Mtb) continues to kill about 3 million people every year. It is the etiologic agent of tuberculosis (TB) and a major cause of morbidity and mortality worldwide [1]. Mtb remains the leading infectious disease in numerous developing countries and is on the rise in Japan, several European nations, and the United States. Acquired resistance against Mtb is T-cell dependent. The protective cellular immune response to Mtb is initiated in the lung and consists primarily of alveolar macrophages and activated T-cells [2,3]. However, little is known about the mechanisms of this initial immune response to inhaled Mtb. CD3-positive T-cells are comprised of 1-5% $\gamma/\delta$ T-cells, and approximately 95% of all T-cells are $\alpha/\beta$ T-cells. Traditionally, $\alpha/\beta$ T-cells (CD3-positive T-cells) have been considered the major T-cell subset regulating this protective cellular immune response [4-6]. However, several recent studies have found that $\gamma/\delta$ T-cells (CD3-positive T-cells) may play an important role in the immune response to Mtb. Studies in humans and animal models have demonstrated that other T-cell subsets, and in particular $\gamma/\delta$ T-cells, are activated by mycobacteria in vitro and accumulate in sites of mycobacterial infection in vivo [7-9].

In normal healthy individuals, $\gamma/\delta$ T-cells contain the highest frequency of Mtb-reactive T-cells in the peripheral blood [10,11]. Some studies have reported an increase in $\gamma/\delta$ T-cells in the peripheral blood of TB patients [12,13], whereas other studies have found that $\gamma/\delta$ T-cell numbers remain constant in the peripheral blood of TB patients [14-17]. These
contrasting results may be a consequence of analyzing \( \gamma/\delta \) T-cells from patients at different stages of disease progression.

In the present study, we analyzed the \( \gamma/\delta \) T-cell populations present in the peripheral blood and the IFN-\( \gamma \) production of \( \gamma/\delta \) T-cells stimulated by PMA before and after anti-TB chemotherapy in patients in the initial treatment stages for primary active pulmonary TB. We compared the population of \( \gamma/\delta \) T-cells and the production of IFN-\( \gamma \) between normal healthy controls and TB patients. In addition, we also examined the influence of anti-TB chemotherapy.

MATERIALS AND METHODS

Patients and participants

The present study analyzed 14 pulmonary TB patients (7 males and 7 females; mean age range 28-93 years, mean \( \pm \) SD 63.8 \( \pm \) 20.5) and 12 healthy volunteers (controls). All patients were hospitalized at Kurume University Hospital, and this was the first time for them to undergo anti-TB chemotherapy. The presence of Mtb in the sputum or BALF was confirmed by polymerase chain reaction (PCR) technique at admission. Table 1 summarizes the clinical profiles of the TB patients. Prior to treatment, 11 of the 14 TB patients tested positive on the Gaffky test, which is a confirmation of the presence of Mtb in the sputum smear (Table 2). All 14 patients also tested positive in the Mtb sputum culture for 4 weeks. In two of 3 patients in whom the Gaffky test was negative prior to treatment, a positive test was found in the Mtb sputum culture at 4 weeks. In the remaining patient, the Mtb sputum culture was positive at 8 weeks. Seven of the 14 patients had basal disease, 5 had diabetes mellitus, one had chronic hepatitis type-C, and one had alcoholic hepatitis. In terms of anti-TB chemotherapy, all patients received Isoniazid (INH) and Rifampicin (RFP), and in proportion to patient’s condition and appearance of the drug side effect, Ethambutol (EB), Pyrazinamide (PZA), Streptomycin Sulfate (SM), Kanamycin (KM), and Sparfloxacin (SPFX) were added or omitted from the prescription as appropriate. After 3 months, anti-TB chemotherapy was initiated. All patients were Gaffky test-negative and negative in the Mtb sputum culture for 8 weeks. The patient characteristics are summarized in Table 1. Blood samples were collected into a heparinized tube from each of the patients before the start of anti-TB chemotherapy and after 3 months of this therapy.

The healthy volunteers were comprised of 6 males and 6 females with no history of pulmonary disease, no abnormalities on chest radiography, and all were tuberculin reaction-positive. Blood samples were drawn from the healthy subjects prior to the start of the study. Informed consent was obtained from each subject, and the study was approved by the Committee of the Kurume University Hospital for

<table>
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<th>Case number</th>
<th>Sex</th>
<th>Age (years)</th>
<th>WBC (mm³)</th>
<th>Lymphocytes (mm³)</th>
<th>Gaffky score</th>
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<td>(−)</td>
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<td>(−)</td>
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Data shown were obtained at hospital admission. TB: tuberculosis; WBC: white blood-count; INH: isoniazid; RFP: rifampicin; EB: Ethambutol; PZA: Pyrazinamide; SM: Streptomycin Sulfate; KM: Kanamycin; SPFX: Sparfloxacin

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**Cell preparations**

The collected heparinized whole blood was diluted twofold with phosphate-buffered saline (PBS) containing 137 mM NaCl, 8.10 mM Na2HPO4, 2.68 mM KCl, and 1.47 mM KH2PO4. Peripheral blood mononuclear cells (PBMC) were isolated by the Ficoll-Conrey density centrifugation method and washed twice with PBS, and then stored at -80 °C until use.

**Analysis of cell surface makers**

The PBMC were thawed and suspended into RPMI 1640 supplemented with 10% fetal calf serum (FCS), and the cells were prepared at a concentration of 1.0×10^6/mm³. For simultaneous two-color flow cytometry, fluorescein isothiocyanate (FITC) conjugated anti-TCR-γ/δ monoclonal antibody (Mab) (Endogen, Woburn, MA, USA), and peridinin chlorophyll protein (Per-CP) conjugated Mab against CD3 antigen (Becton-Dickinson, San Jose, CA, USA) were added to the suspensions. In the analysis of α/β T-cells, FITC-conjugated anti-TCR-α/β Mab (Becton-Dickinson, San Jose, CA, USA) was used instead. After incubation at 4 °C for 20 min in the dark, the cells were washed once with PBS. As isotype controls, cell samples stained with FITC-, and PerCP-labeled mouse IgG1 antibody (Becton-Dickinson, San Jose, USA) were used.

**Flow cytometry**

All samples were analyzed for surface markers using a flow cytometer (FACScan, Becton-Dickinson). They were gated on lymphocytes with forward and side scatters. The percentage of T-cells was calculated by measuring the fluorescent strength of 1×10^5 cells. On the cytogram, CD3-positive T-cells were gated, and the population of γ/δ TCR-positive cells or α/β TCR-positive cells was analyzed. Their absolute numbers were calculated with WBC (white blood-count), lymphocytes, and percent of CD3-positive γ/δ T-cells or α/β T-cells.

**Cell cultures**

Cells stored at -80 °C were thawed and washed once, and the pellet was suspended in RPMI 1640 containing 10% FCS to make a cell suspension of 1.0×10^6 cells/mm³. The cells were incubated at 37 °C and 5% CO₂ for 4 hrs in the presence of phorbol myristate acetate (PMA 20 ng/ml; Sigma Chemical Co., St. Louis, MO, USA), ionomycin (0.5 μM, Sigma), and monensin (4 μM, Wako, Osaka, Japan) solution. The cells were washed with PBS and FITC conjugated anti-TCR-γ/δ Mab or FITC conjugated anti-TCR-α/β Mab was added by the above method. Then, the cells were fixed for 10 min on ice by the addition of 4% parafomaldehyde solution at a final concentration of 2%, and then washed once with PBS. The cells were immersed in a permeabilization buffer (0.1% saponin, 0.001% NaN₃ containing PBS).

**Cell preculture with INH**

Cells stored at -80 °C were thawed and washed once, and the pellet was suspended in RPMI 1640 containing 10% FCS to produce a cell suspension of 1.0×10^6 cells/mm³. The cells were incubated at 37 °C and 5% CO₂ for 12 hrs with 0.2 μg/ml INH (Daichiseiyaku, Tokyo, Japan), and were washed once. The pellet was resuspended in RPMI 1640 containing 10% FCS to make a cell suspension of 1.0×10^6 cells/mm³, followed by the above cell culture method.

**Determination of intracellular IFN-γ**

R-phycoerythrin-conjugated mouse anti-human IFN-γ Mab (0.2 μg/test, Pharmingen) was added to the suspension of permeabilized cells. After incubation at 4 °C for 20 min in the dark, the cells were washed once with the permeabilizing buffer and resuspended in PBS. Surface markers and intracellular IFN-γ were analyzed using a flow cytometer. Cells were gated on lymphocytes with forward and side scatters. The percentage of T-cells was calculated by measuring the fluorescent strength of 1×10^5 cells.

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**Table 2.**

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<tr>
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<td>0 per every view</td>
</tr>
<tr>
<td>1</td>
<td>1-4 per every view</td>
</tr>
<tr>
<td>2</td>
<td>1 in several views examined</td>
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<tr>
<td>3</td>
<td>1 per one view on average</td>
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<tr>
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<td>2-3 per one view on average</td>
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<tr>
<td>5</td>
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<tr>
<td>9</td>
<td>51-100 per one view on average</td>
</tr>
<tr>
<td>10</td>
<td>101 or more per one view on average</td>
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</tbody>
</table>

Gaffky score. It expresses average number of *Mycobacterium tuberculosis* (Mtb) counted in one ×500 view.
cells. On the cytogram, CD3-positive T-cells were gated, the area of \( \gamma/\delta \) TCR-positive cells or \( \alpha/\beta \) TCR-positive cells was analyzed, and this area was gated again for the reanalysis of IFN-\( \gamma \)-positive cells. The absolute number of IFN-\( \gamma \) producing cells was calculated with WBC, lymphocytes, percent of IFN-\( \gamma \) producing CD3-positive \( \gamma/\delta \) or \( \alpha/\beta \) T-cells. And we made the index as a means of comparing IFN-\( \gamma \) production. This index applies the ratio of \( \gamma/\delta \) and \( \alpha/\beta \) T-cells in CD3-positive T-cells to the mean fluorescence intensity of IFN-\( \gamma \) produced within \( \gamma/\delta \) and \( \alpha/\beta \) T-cells.

Additional examinations

At admission and 3 months after the start of anti-TB chemotherapy, patients were tested for WBC (\(/mm^3\) ) and lymphocytes (\(/mm^3\) ) counts, and the number of Mtb in the sputum smear (in terms of Gaffky score). The Gaffky score is commonly used in Japan, and an explanation of this test is given in Table 2.

Statistical analysis

Group differences were assessed by Student's t-test, and p-values less than 0.05 were considered statistically significant.

RESULTS

Analysis of T-cell receptors before and after antituberculosis chemotherapy

The \( \gamma/\delta \) and \( \alpha/\beta \) T-cell subsets of the CD3-positive cells were measured with flow cytometry. As shown in Fig. 1a, no significant difference was noted in the absolute number of CD3-positive \( \gamma/\delta \) T-cells before treatment (90±104/mm\(^3\) ) or after treatment (109±138/mm\(^3\) ). The absolute number of \( \gamma/\delta \) T-cells of TB patients both before and after treatment was slightly less than for normal healthy individuals (152±112/mm\(^3\) ), however no statistically significant difference was noted.

As shown in Fig. 1b, no significant difference was noted in the absolute number of CD3-positive \( \alpha/\beta \) T-cells before (1249±761/mm\(^3\) ) and after (1183±481/mm\(^3\) ) treatment. The absolute number of \( \alpha/\beta \) T-cells of TB patients was less than for normal healthy individuals, however no significant difference was noted.

Intracellular IFN-\( \gamma \) in \( \gamma/\delta \) and \( \alpha/\beta \) T-cells

The absolute number of \( \gamma/\delta \) T-cells both before (40±29/mm\(^3\) ) and after (21±16/mm\(^3\) ) treatment in TB patients in whom production of IFN-\( \gamma \) had been stimulated with PMA was dramatically lower than the number of IFN-\( \gamma \) producing cells (110±74/mm\(^3\) ) (p<0.01) in normal healthy individuals, as shown in Fig. 2a.

The number of IFN-\( \gamma \) producing cells was further reduced after treatment (p<0.05). As shown in Fig. 2b, the investigation revealed that even with CD3-positive \( \alpha/\beta \) T-cells in TB patients, a comparison with the number of IFN-\( \gamma \) producing cells (482±239/mm\(^3\) ) in normal healthy individuals revealed that the number of IFN-\( \gamma \) producing cells in TB patients both before (210±201/mm\(^3\) ) and after treatment...
Fig. 2. a: Absolute number of IFN-γ producing CD3-positive γ/δ T-cells. control: healthy controls, before treatment: patients with active pulmonary TB before anti-TB chemotherapy, after treatment: patients with active pulmonary TB after anti-TB chemotherapy. * p<0.01, † p<0.05. b: Absolute number of IFN-γ producing CD3-positive αβ T-cells. control: healthy controls, before treatment: patients with active pulmonary TB before anti-TB chemotherapy, after treatment: patients with active pulmonary TB after anti-TB chemotherapy. * p<0.01.

Fig. 3. a: Effects of INH on IFN-γ production of γ/δ T-cells. control: γ/δ T-cells were cultured without isoniazid (INH). INH: γ/δ T-cells were cultured with INH. * p<0.05. b: Effects of INH on IFN-γ production of αβ T-cells. control: αβ T-cells were cultured without INH. INH: γ/δ T-cells were cultured with INH. * p<0.05.

(190±156/mm³) was lower (p<0.01), however no significant difference was apparent in IFN-γ producing αβ T-cells before or after treatment.

**Effects of INH on IFN-γ production of γ/δ and αβ T-cells**

Normal healthy individuals were divided into two groups, one in which INH was added to PMBC, and the other in which it was not. After cultivating for 12 hrs, PMA stimulation was applied and cultivation continued for a further four hours to produce IFN-γ which was then measured with a flow cytometer. The production of IFN-γ in each case was compared using the index. As shown in Fig. 3a, IFN-γ production (65750±22177) in the γ/δ T-cells of the group to which INH was added was significantly less than that for the group to which INH was not added (135451±49037) (p<0.05). As shown in Fig. 3b, as with γ/δ T-cells, IFN-γ production for both the group to which INH was added (14463±5328), and the group to which it was not added (31570±8797), was significantly less for αβ T-cells (p<0.05).
**DISCUSSION**

In the development of TB and in related initial defensive immunity, γδ T-cells are instrumental. In normal healthy individuals, γδ T-cells account for 1-5% of all T-cells, with the remainder being αβ T-cells [18-20]. In recent years the important role of γδ T-cells in the development of initial defensive immunity to TB has been understood, however the associated mechanism is as yet unclear. As apparent from the fact that the number of γδ T-cells in the peripheral blood of TB patients is variously reported as increased, the same, or reduced, many hypotheses on the subject are mutually exclusive. The results of our research have shown that the number of γδ T-cells in TB patients tends to be reduced in comparison with normal healthy individuals, however the difference is not significant. On the other hand, research conducted by Hirsch et al. indicates that both CD4-positive and CD4-non-positive T-cells in TB patients are more readily subject to apoptosis than in normal healthy individuals, and that the addition of Mtb antigens promotes apoptosis, thus reducing T-cells in peripheral blood [21-23]. The majority of γδ T-cells were CD4 and CD8 double-negative. If these cells are responsible for apoptosis in the peripheral blood of TB patients, there is no contradiction with the tendency to reduction of γδ T-cells in peripheral blood as found in our results, despite the fact that no significant difference was apparent. The question remains as to why the T-cells of TB patients promote apoptosis in the presence of the Mtb. It appears that localized areas of lungs and trachea infected with the Mtb isolate the bacillus, thus preventing its dispersion and inflammation, and resulting in apoptosis and granulation in the periphery of the lesion to produce a robust wall. Reports from Ladel et al. indicate that mice infected with Mtb, in which γδ T-cells have been knocked out to leave only αβ T-cells, are not subject to granulation in the lungs, and that Mtb spreads throughout the body of the mouse with a mortality rate of almost 100% [24].

Why are some people infected with TB and others not? It appears that this may be related to a problem with the function of the γδ T-cells responsible for the initial defensive response to tubercular infection. No reports in which production of IFN-γ in γδ T-cells has been observed directly are available, however our previous observations that the response to PPD of γδ T-cells in TB patients is weaker than that in normal healthy individuals raises doubts as to the ability of IFN-γ to produce type 1 cytokines [25].

In order to evaluate production of IFN-γ from T-cells, mitogens such as PPD, PHA, PMA, and con-A were employed for stimulation of T-cells (Data not shown). When stimulation is excessive, γδ and αβ TCR is down-regulated, and when stimulation is too weak, no IFN-γ production is apparent. PMA was therefore used for its ability to non-specifically stimulate IFN-γ production without down-regulation of TCR. Results showed that the number of IFN-γ producing cells from both γδ and αβ T-cells was lower in TB patients than in normal healthy individuals, and it is therefore concluded that the bactericidal function of T-cells of TB patients on Mtb is reduced. It is currently unclear as to whether the Mtb infection is due to, or is the cause of, the reduction in the bactericidal function of the T-cells. No significant difference was noted in the number of IFN-γ producing cells from αβ T-cells before or after anti-TB chemotherapy, and the number of IFN-γ producing cells from γδ T-cells was even lower. This is considered to be due to the fact that a healthy subject with weak γδ T-cell functions had been infected with Mtb, resulting in γδ T-cells initially increasing IFN-γ production as a defensive response to the initial Mtb, after which symptoms were relieved, thus reducing IFN-γ production. In an experiment conducted by Ladel et al., the mice in which the αβ T-cells were knocked out to leave only γδ T-cells showed increased IFN-γ production in the initial stages of the infection, associated with strong interstitial inflammation in the lung, and a reduction in γδ T-cell functions between the intermediate and late stages of the infection. When the action of the αβ T-cells was of primary importance they were not present in sufficient numbers, and the infection worsened. When the role of the αβ T-cells is considered in this light, any reduction in their ability to provide initial defensive immunity to Mtb results in an excessive inflammatory reaction which in turn brings αβ T-cells and other IFN-γ producing cells to the fore in bactericidal action against remaining Mtb in order to avoid excessive invasion of the organism.

INH was found to suppress IFN-γ production of CD3-positive T-cells. A number of anti-TB drugs, including those with RFP anti-inflammatory and steroid-like actions, have been reported [26], however this action has not been reported for other anti-TB drugs such as INH. In this experiment T-cells of healthy individuals were employed in an investigation of the influence of INH on IFN-γ production from T-cells by staining of IFN-γ within cells. Productivity of both γδ and αβ T-cells was shown
to be reduced by INH. INH has previously been used in the treatment of TB for its action in inhibiting synthesis of Mtb cell walls, however its action in suppressing IFN-γ production of T-cells as shown in this experiment suggests a role in the treatment of pulmonary TB through its anti-inflammatory action.

In light of the effects of INH on both γ/δ and α/β T-cells, it is apparent that the further reduction in the number of γ/δ T-cells producing IFN-γ in TB patients following treatment is not solely a result of anti-TB drugs, but is also related to the defensive immune reaction of the organism to Mtb.

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