

Original article

CD4⁺CD25⁺ regulatory T lymphocytes in tuberculous pleural effusion

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Keywords: CD4⁺CD25⁺ T cells; pleural effusion; regulatory T cells; tuberculosis

Background Active suppression by CD4⁺CD25⁺ regulatory T lymphocytes plays an important role in the down-regulation of T cell responses to foreign and self-antigens. This study was conducted to analyze whether the CD4⁺CD25⁺ regulatory T cells exist and function normally in tuberculous pleural effusion.

Methods The percentages of CD4⁺CD25⁺ T cells in pleural effusion and peripheral blood from patients with tuberculous pleurisy and peripheral blood from healthy control subjects were determined by flow cytometry. The expression of forkhead transcription factor Foxp3 was also examined. CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells from pleural effusion and blood were isolated, and were cultured to observe the effects of CD4⁺CD25⁺ T cells on proliferation response of CD4⁺CD25⁻ T cells *in vitro*.

Results There were increased numbers of CD4⁺CD25⁺ T cells in tuberculous pleural effusion compared with peripheral blood from both patients with tuberculous pleurisy and normal subjects, and these cells demonstrated a constitutive high-level expression of Foxp3. Moreover, CD4⁺CD25⁺ T cells mediated potent inhibition of proliferation response of CD4⁺CD25⁻ T cells.

Conclusion The increased CD4⁺CD25⁺ T cells in tuberculous pleural effusion express a high level of Foxp3 transcription factor, while potentially suppressing the proliferation of CD4⁺CD25⁻ T cells.

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Tuberculosis is the leading cause of death from a curable infectious disease. In China, the prevalence of active pulmonary tuberculosis in 2000 was 367/100 000, the prevalence of smear positive pulmonary tuberculosis was 122/100 000 and the prevalence of bacteriological positive pulmonary tuberculosis was 160/100 000.¹ The surveys of the prevalence of infection and disease, assessments of the effectiveness of surveillance systems, and death registrations revealed approximately 8.9 million new cases of tuberculosis in 2004, fewer than half of which were reported to public-health authorities and world health organization.² Tuberculous pleural effusion (TPE) is caused by a severe delayed-type hypersensitivity reaction in response to the rupture of a subpleural focus of *Mycobacterium tuberculosis* (MTB) infection. Although TPE occurs in about 10% of untreated individuals who are tested positive by the tuberculin test, it may also develop as a complication of primary pulmonary tuberculosis.³ Actually, tuberculosis is the major cause of pleural effusions in areas of high tuberculosis prevalence, and TPE usually manifests as lymphocytic exudative pleural effusion.^{4,5} Compared with peripheral blood, TPE is enriched with CD4⁺ lymphocytes.^{6,7} Selective enrichment of memory T cells with defined surface phenotype and type 1 helper T cytokine profile has been demonstrated in TPE.⁸ High levels of interferon (IFN)- γ in TPE have been reported⁹ and measurement of IFN- γ in pleural fluid is likely to be a useful diagnostic tool for TPE.¹⁰

Studies over a decade have provided firm evidence for the

existence of a unique CD4⁺CD25⁺ population of "professional" regulatory/suppressor T cells that actively and dominantly prevent both the activation and the effector function of autoreactive T cells that have escaped other mechanisms of tolerance.^{11,12} Recently, we have reported that the increased CD4⁺CD25⁺ T cells in malignant pleural effusion express a high level of Foxp3 transcription factor and potentially suppress the proliferation of CD4⁺CD25⁻ T cells, and that cytotoxic lymphocyte associated antigen-4 is involved in the suppressive activity of pleural CD4⁺CD25⁺ T cells.¹³ The involvement of such CD4⁺CD25⁺ regulatory T cells in human tuberculosis has been documented recently.^{14,15} A significantly higher percentage of CD4⁺CD25⁺ T cells was found in peripheral blood from patients with tuberculosis compared with healthy controls. In the present study, we investigated CD4⁺CD25⁺ T cells that could be involved in the control of local immune response to TPE. Our data provide the direct evidence that CD4⁺CD25⁺ T cells infiltrating into human TPE behave as regulatory T cells.

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METHODS

Patients and samples

The study protocol was approved by the Ethics Committee of Guangxi Medical University, Nanning, China, and all subjects provided written consent. TPE samples were collected from 16 patients (9 men) at median age of 34 years (range: 19 to 56 years) newly diagnosed with TPE, as evidenced by demonstration granulomatous pleuritis on closed pleural biopsy specimen in the absence of any evidence of other granulomatous diseases. After anti-tuberculosis chemotherapy, the resolution of pleural fluid and clinical symptoms was observed in all patients. At the time of sample collection, none of the patients had received any anti-tuberculosis treatment, corticosteroids, or other nonsteroid anti-inflammatory drugs. The pleural fluid was collected in heparin-treated tubes from each subject, using a standard thoracentesis technique within 24 hours after hospitalization. The TPE specimens were immersed in ice immediately and then centrifuged at 1200 r/min for 5 minutes. The cell pellets were resuspended in 15 ml Hanks' balanced salt solution (HBSS) for later use.

Ten milliliters of venous blood from all patients with TPE and 15 healthy volunteers (7 men) at median age 35 years (range 28–53) were drawn simultaneously for isolating mononuclear cells.

Analysis of lymphocyte subsets

Mononuclear cells in both TPE and blood were separated by centrifugation on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient and were processed for flow cytometry to determine lymphocyte phenotype. Three color flow cytometry was performed to determine phenotypes in T lymphocytes in both TPE and blood. The monoclonal antibodies (mAb) used were anti-CD3-phycoerythrin (UCHT1), anti-CD4-fluorescein isothiocyanate (RPA-T4), and anti-CD25-Cy-chrome (M-A251). Appropriate isotype controls were used for each experiment. All mAbs and controls were purchased from BD PharMingen (San Diego, CA, USA). Briefly, cells were incubated in the dark at room temperature for 30 minutes with mAbs at the concentrations recommended by the manufacturer, washed once in FACS buffer (calcium/magnesium-free HBSS containing 1 mg/ml bovine serum albumin and 0.1 mg/ml sodium azide), and fixed with 2% formaldehyde. Flow cytometry was performed on a Coulter Epics XL-MCL flow cytometer using System II software (Beckman Coulter Company, Miami, Florida, USA).

Cell isolation

CD4⁺ T cells from both TPE and blood were purified by negative selection (by depletion of CD8, CD11b, CD16, CD19, CD36, and CD56 positive cells) with the untouched CD4⁺ cell isolation kit (Miltenyi Biotec, Auburn, CA, USA). After isolation of CD4⁺ T cells,

CD25⁺ T cells were stained with phycoerythrin-coupled anti-CD25 mAb and purified after the addition of anti-phycoerythrin-coupled magnetic beads (Miltenyi Biotec). Eventually, CD4⁺CD25⁺ T cells were obtained with a purity ranging from 90% to 95%. CD4⁺CD25⁻ T cells were also collected with a purity ranging from 80% to 90%.

Quantitative real-time PCR

For quantitative real-time PCR analysis of mRNA expression of the forkhead transcription factor Foxp3, total RNA was isolated from CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells by a total RNA extraction kit for mammalian RNA (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions, and cDNA was prepared with 2.5 μmol random hexamers (Applied Biosystems Inc., Foster City, CA, USA). Real-time PCR was performed as described previously,¹³ with the following cycling conditions: 10 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C, and 1 minute at 60°C. All samples were run in triplicate, and data were expressed as normalized expression obtained by dividing the relative level for each sample by the relative level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for the same sample, where GAPDH = 1. Primer sequences were as follows: GAPDH: 5'-CCACATC-GCTCAGACACCAT-3' and 5'-GGCAACAATATCCAC-TTTACCAGAGT-3'; Foxp3: 5'-CAGCTGCCACACT-GCCCCTAG-3' and 5'-CATTGCCAGCAGTGGG-TAG-3'. The mean values from duplicates were used for calculations.

Proliferation assay

To analyze proliferation in response to polyclonal stimulation, freshly isolated CD4⁺ T cells (5×10^4 cells), CD4⁺CD25⁻ T cells (5×10^4 cells) alone or with different numbers of CD4⁺CD25⁺ T cells in a final volume of 200 μl of complete medium were incubated in 96-well round-bottomed plates in the presence of 10 μg/ml of platebound anti-CD3 plus 10 μg/ml soluble anti-CD28 mAbs. After 4 days of culture at 37°C in a 5% CO₂ humidified atmosphere in RPMI-1640 medium supplemented with 10% normal human serum (Sigma, St. Louis, MO, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L L-glutamine, and 10 mmol/L HEPES. [³H]thymidine (37 KBq/well) was added for additional 16 hours. Proliferation was measured using a liquid scintillation counter.

Statistical analysis

Values are presented as mean ± standard error of mean (SEM). Nonparametric tests were used to analyze variables of TPE and blood since these variables were not normally distributed. Comparisons of the data between different groups were made using the Mann-Whitney *U* test or Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Increased proportion of CD4⁺CD25⁺ T cells in TPE

Lymphocytes from both TPE and blood from all subjects were analyzed by flow cytometry (Figure 1). (19.2±1.5)% of CD4⁺ T cells in TPE was CD4⁺CD25⁺; in contrast, only (12.1±0.8)% of CD4⁺ T cells in the corresponding blood and (7.7±0.6)% in control blood had this phenotype (both *P*<0.001). CD4⁺CD25⁺ T cell numbers in blood from patients with TPE were much higher than those in control blood (*P*=0.003).

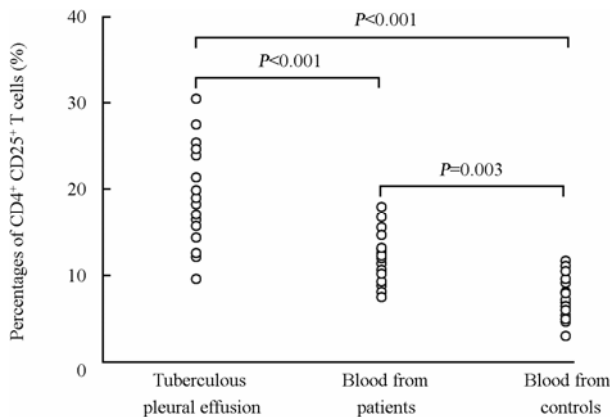


Figure 1. Percentages of CD4⁺CD25⁺ T lymphocytes in total CD4⁺ T cells in tuberculous pleural effusion and peripheral blood from patients with tuberculous pleurisy (*n*=16), and blood from healthy control subjects (*n*=15). The percentage of CD4⁺CD25⁺ T cells present in total CD4⁺ T cells was determined by flow cytometry.

Pleural CD4⁺CD25⁺ T cells express higher level of Foxp3

Foxp3 was recently described as an important transcription factor involved in the development and functions of CD4⁺CD25⁺ T cells and appears to be the most specific molecular marker available to date.¹⁶ In the present study, Foxp3 mRNA expression was analyzed by real-time PCR in both purified CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells in pleural fluid and blood from 6 patients with TPE, and in blood from 6 healthy subjects. As expected, blood CD4⁺CD25⁺ T cells from both patients with TPE and healthy subjects expressed high levels of Foxp3 while their blood CD4⁺CD25⁻ T cells expressed very low levels (Figure 2). Similarly, pleural CD4⁺CD25⁺ T cells highly expressed Foxp3 while corresponding CD4⁺CD25⁻ T cells expressed very low levels. Thus, these data revealed that CD4⁺CD25⁺ T cells infiltrating TPE express Foxp3, strongly suggesting that this population includes regulatory T cells. It was also found that Foxp3 expression in TPE CD4⁺CD25⁺ T cells was significantly higher than that in their time-matched blood CD4⁺CD25⁺ T cells (*P*=0.004), and that in control CD4⁺CD25⁺ T cells (*P*=0.002). On the other hand, there was no quantitative difference in Foxp3 expression in blood CD4⁺CD25⁺ T cells between TPE patients and normal controls (*P*=0.655).

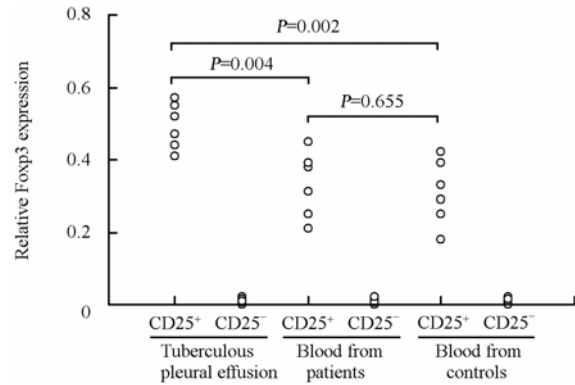


Figure 2. CD4⁺CD25⁺ T lymphocytes constitutively expressed Foxp3. cDNAs obtained from CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, isolated from tuberculous pleural effusion and peripheral blood of patients with tuberculous pleurisy (*n*=6), and from blood of healthy subjects (*n*=6), were subjected to quantitative real-time PCR for Foxp3 and GAPDH. Relative Foxp3 expression for each sample is shown after normalization of GAPDH expression, where GAPDH = 1.

Inhibition of proliferation of CD4⁺CD25⁻ T cells by CD4⁺CD25⁺ T cells

To assess the function of CD4⁺CD25⁺ T cells in TPE, we separated CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells from pleural fluid and blood of 5 patients with TPE, as well as blood of 5 healthy subjects, determined their proliferative capacity, and the effect of CD4⁺CD25⁺ T cells on CD4⁺CD25⁻ T cell proliferation. As shown in Figure 3A, proliferative responses to anti-CD3 and CD28 mAbs were observed in the total CD4⁺ T-cell populations from pleural fluid and their counterparts in blood from both TPE patients and healthy subjects. A significant decrease of proliferation response could be observed in TPE CD4⁺ T cells compared with that in both blood groups from TPE patients and normal controls. Upon depletion of CD4⁺CD25⁺ T cells, the proliferation of the remaining CD4⁺CD25⁻ T cells was increased significantly, while the proliferation of CD4⁺CD25⁺ T cells from all three groups was low, indicating that TPE CD4⁺CD25⁺ T cells, as those in peripheral blood, were anergic. When CD4⁺CD25⁻ T cells were cocultured with CD4⁺CD25⁺ T cells, proliferative response of CD4⁺CD25⁻ T cells decreased as the number of CD4⁺CD25⁺ T cells increased. Moreover, suppressive capacity of TPE CD4⁺CD25⁺ T cells was stronger than that of blood CD4⁺CD25⁺ T cells from both TPE patients and healthy subjects (Figure 3B).

DISCUSSION

Tuberculosis is associated with chronic, persistent antigen stimulation *in vivo* that maintains a sustained immune response that suppresses, but generally fails to eradicate MTB. The host response involves many limbs of the cellular immune system but consists predominantly of MTB-specific IFN-γ-secreting CD4⁺ and CD8⁺ effector T cells.¹⁷⁻¹⁹ Although this response helps to limit bacterial replication and dissemination *in vivo*, it also causes

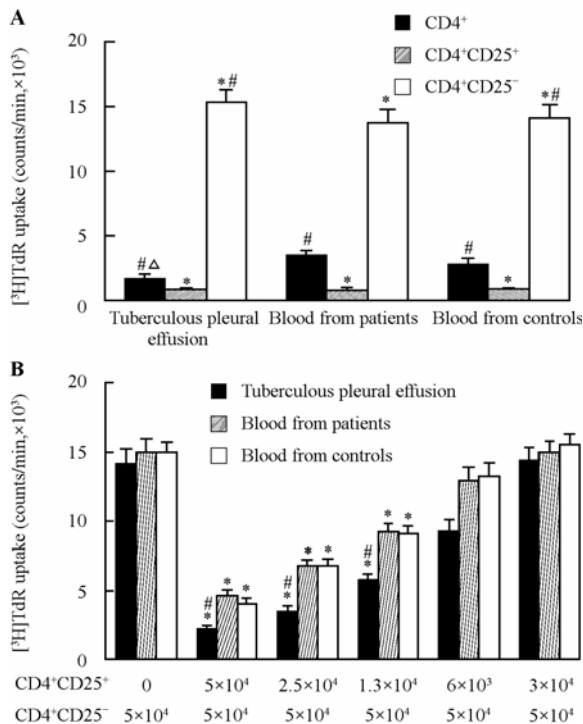


Figure 3. Inhibition of anti-CD3/CD28-induced CD4⁺CD25⁻ T cell proliferation by CD4⁺CD25⁺ T cells. **A:** CD4⁺, CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells (5×10^4 cells each), isolated from tuberculous pleural effusion and peripheral blood of patients with tuberculous pleurisy ($n=5$), and from blood of healthy subjects ($n=5$), were incubated in the presence of platebound anti-CD3 plus soluble anti-CD28 mAbs for 4 days. Proliferation of T cells was determined by [³H]thymidine incorporation. * $P < 0.01$ compared with CD4⁺ cells; # $P < 0.01$ compared with CD4⁺CD25⁺ T cells, determined by Kruskal-Wallis one-way ANOVA on ranks; $\Delta P < 0.01$ compared with blood CD4⁺ T cells from both groups, determined by Kruskal-Wallis one-way ANOVA on ranks. **B:** In order to compare difference in suppression by pleural CD4⁺CD25⁺ T cells ($n=5$) and blood CD4⁺CD25⁺ T cells from patients with tuberculous pleurisy ($n=5$), or blood CD4⁺CD25⁺ T cells from normal donors ($n=5$) on the proliferation response of CD4⁺CD25⁻ T cells, designated numbers of CD4⁺CD25⁺ T cells were added to 5×10^4 CD4⁺CD25⁻ T cells, and the cocultures were stimulated with platebound anti-CD3 plus soluble anti-CD28 mAbs. After 4 days of culture, the proliferation of T cells was determined by [³H]thymidine incorporation. * $P < 0.01$ compared with the corresponding CD4⁺CD25⁻ T cells alone, determined by Kruskal-Wallis one-way ANOVA on ranks; # $P < 0.01$ compared with blood values within the same experimental groups, determined by Kruskal-Wallis one-way ANOVA on ranks.

significant immunopathology. TPE is caused by a severe delayed-type hypersensitivity reaction in response to the rupture of a subpleural focus of MTB infection. The inflammatory process results in an increased pleural vascular permeability leading to the accumulation of fluid enriched in proteins and the recruitment of specific leukocytes into the pleural space.^{5,20} TPE usually shows a lymphocytic preponderance, especially CD4⁺ T cells.^{6,7} Studies ongoing for more than a decade have provided firm evidence for the existence of a unique CD4⁺CD25⁺ population of “professional” regulatory/suppressor T cells that actively and dominantly prevent both the activation and the effector function of autoreactive T cells that have

escaped other mechanism of tolerance.^{11,12} The elimination or inactivation of CD4⁺CD25⁺ T cells resulted in severe autoimmune disease and was also found to enhance immune responses to alloantigens and even tumors.²¹⁻²³ CD4⁺CD25⁺ T cells have a unique immunological characteristic compared with other regulatory or suppressor T cells induced by certain routes of exogenous immunization or tolerance induction. For example, they do not proliferate in response to antigenic stimulation *in vitro* (that is, they are naturally anergic) and can potently suppress the activation and proliferation of CD4⁺CD25⁻ T cells in an antigen-nonspecific manner through cell-to-cell interaction.²⁴

Guyot-Revol and colleagues¹⁴ reported that a significant increase in the frequency of CD4⁺CD25⁺ T cells and a significant increase in Foxp3 expression were observed in peripheral blood from patients with tuberculosis. Increased expression of interleukin (IL)-10 and transforming growth factor- β 1 mRNA was also detected in patients with tuberculosis but did not correlate with regulatory T-cell markers. *Ex vivo* depletion of CD4⁺CD25⁺ T cells from peripheral blood mononuclear cells resulted in increased numbers of MTB antigen-specific IFN- γ -producing T cells in tuberculous patients. In another study,¹⁵ frequencies of CD4⁺CD25⁺ T cells were increased in blood from patients with pulmonary tuberculosis compared to healthy subjects, and remained elevated at completion of 6-month anti-tuberculosis therapy. A role for CD4⁺CD25⁺ T cells in depressed IFN- γ production during tuberculosis was substantiated in depletion experiments, where CD25⁻-depleted CD4 T cells produced increased amount of IFN- γ upon MTB stimulation compared to unseparated T cells. In the present study, the numbers of CD4⁺CD25⁺ T cells in TPE were much higher than those in autologous blood and blood from normal controls. Also CD4⁺CD25⁺ T cell numbers in tuberculous blood were significantly higher than those in normal control blood.

We did not study the mechanisms by which CD4⁺CD25⁺ T cells were recruited into TPE in the present study. The primary aim of this study was to explore the presence of CD4⁺CD25⁺ T cells in TPE, as well as the immunosuppressive activity of these TPE CD4⁺CD25⁺ T cells. We speculated that an increased percentage of CD4⁺CD25⁺ T cells in TPE might be due to active recruitment or local differentiation. It has been demonstrated that human CD4⁺CD25⁺ T cells preferentially move to and accumulate in ascites, but rarely enter draining lymph nodes, and the chemokine CCL22 mediates trafficking of CD4⁺CD25⁺ T cells to the ascites.²⁵ In a previous study,²⁶ direct evidence was found for the first time that the levels of IL-16 were significantly higher in TPE than in malignant effusions. Positive correlations were found between the IL-16 levels and CD4⁺ T cells, and pleural fluid was chemotactic for CD4⁺ T cells *in vitro*. Intrapleural administration of IL-16 of patients produced a marked progressive influx of CD4⁺

T cells into the pleural space. Therefore, as a subpopulation of CD4⁺ T cells, CD4⁺CD25⁺ T cells might also be recruited into TPE by local production of IL-16.

In the present study, proliferative responses to anti-CD3 and CD28 mAbs were observed in the total CD4⁺ T-cell population in TPE. On depletion of the CD4⁺CD25⁺ T cells, the proliferation of the remaining CD4⁺CD25⁻ T-cell population was increased significantly, proving indirectly that CD4⁺CD25⁺ T cells suppressed the proliferative response. The proliferation of CD4⁺CD25⁺ T cells alone was low, indicating that these cells were anergic. To investigate the direct suppressive capacity of CD4⁺CD25⁺ T cells, we cocultured CD4⁺CD25⁻ T cells with CD4⁺CD25⁺ T cells and stimulated them with anti-CD3 and CD28 mAbs. Of note, when CD4⁺CD25⁺ T cells were added to the coculture, suppression could be observed. More importantly, we observed an increase in inhibitory effects of TPE CD4⁺CD25⁺ T cells, but not blood CD4⁺CD25⁺ T cells from TPE patients or normal subjects. An increase in TPE CD4⁺CD25⁺ T cell number as well as a functional augments of these CD4⁺CD25⁺ T cells might thus lead to a disturbed balance between immunity and tolerance.

The mechanism by which CD4⁺CD25⁺ T cells suppress proliferation of naive T cells *in vitro* seems to be independent of soluble factors and requires cell-cell contact, but the molecular basis for this effect is not well known.^{11,12,27} Recent studies²⁸⁻³⁰ have shown that Foxp3 is specifically expressed in CD4⁺CD25⁺ T cells and is necessary for their development and functions. Foxp3 is not simply a marker of activation because CD4⁺CD25⁻ T cells do not express Foxp3 after activation.²⁸⁻³⁰ More recently, Voo and colleagues³¹ have demonstrated that the suppressive activity of these GITR⁺Foxp3⁺ regulatory CD4⁺CD25⁺ T cells is dependent on antigen specificity. Our present data revealed that CD4⁺CD25⁺ T cells infiltrating TPE express Foxp3, indicating that this population includes regulatory T cells. More importantly, we also found that Foxp3 mRNA expression in purified pleural CD4⁺CD25⁺ T cells in patients with TPE was much higher than that in autologue blood CD4⁺CD25⁺ T cells. These data implicate Foxp3 gene overexpression as a probable mechanism involving CD4⁺CD25⁺ T cells in the pathogenesis of TPE, and suggest a possible role of Foxp3 gene up-regulation in the outcome of chronic infections.

The functional hallmark of CD4⁺CD25⁺ T cells is their ability to suppress cellular immune responses, and specific depletion of CD4⁺CD25⁺ T cells should therefore lead to an increase in T-cell responses. In a recent study,³² an anti-CD25 mAb was given before a pulmonary infection with *Mycobacterium bovis* bacille calmette Guerin or MTB to inactivate the CD4⁺CD25⁺ T cells *in vivo*. The authors found that the number of CD4⁺CD25⁺ T cells was effectively reduced for up to 31 days in the lung by anti-CD25 mAb treatment and this is unaffected by

mycobacterial infection. Anti-CD25 mAb treatment before mycobacterial infection resulted in a modest increase in cytokine-producing CD4⁺ T cells in the spleen and the lung; however, pathogen clearance and pathology during the acute phase of the infection remained unaffected by the treatment. Although the magnitude of the suppressive effect of blood CD4⁺CD25⁺ T cells from patients with TPE was modest, the expansion of CD4⁺CD25⁺ T cells pleural space suggested that suppression of effector responses may be amplified in inflamed tissues. However, the fact that tissue inflammation was manifested at disease sites despite the high local concentration of CD4⁺CD25⁺ T cells with their increased regulatory T-cell activity we observed is not sufficient to prevent pleural immunopathology in the patients with TPE.

CD4⁺CD25⁺ T cell numbers in TPE were much higher than those in peripheral blood from patients with TPE and healthy subjects in this study. We also found that CD4⁺CD25⁺ T cells infiltrating the pleural space were regulatory T cells since they expressed a high level of Foxp3 transcription factor. Moreover, pleural CD4⁺CD25⁺ T cells could potentially suppress the proliferation of CD4⁺CD25⁻ T cells. Further studies are required to delineate the role of CD4⁺CD25⁺ T cells in TPE and they should be focused on identifying the mediators and mechanisms involved in the immunoregulatory properties of pleural CD4⁺CD25⁺ T cells in patients with tuberculous pleurisy.

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