Imbalance of Th17 Cells and Regulatory T Cells in Tuberculous Pleural Effusion

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Both T helper interleukin 17 (IL-17)-producing cells (Th17 cells) and regulatory T cells (Tregs) have been found to be increased in human tuberculosis pleural effusion (TPE); however, the possible interaction between Th17 cells and Tregs in TPE remains to be elucidated. The objective of the present study was to investigate the distribution of Th17 cells in relation to Tregs, as well as the mechanism of Tregs in regulating generation and differentiation of Th17 cells in TPE. In the present study, the numbers of Th17 cells and Tregs in TPE and blood were determined by flow cytometry. The regulation and mechanism of CD39+ Tregs on generation and differentiation of Th17 cells were explored. Our data demonstrated that the numbers of Th17 cells and CD39+ Tregs were both increased in TPE compared with blood. Th17 cell numbers were correlated negatively with Tregs in TPE but not in blood. When naïve CD4+ T cells were cultured with CD39+ Tregs, Th17 cell numbers decreased as CD39+ Treg numbers increased, and the addition of the anti-latency-associated peptide monoclonal antibody to the coculture reversed the inhibitory effect exerted by CD39+ Tregs. This study shows that Th17/Treg imbalance exists in TPE and that pleural CD39+ Tregs inhibit generation and differentiation of Th17 cells via a latency-associated peptide-dependent mechanism.

One-third of the world’s population is infected with Mycobacterium tuberculosis, and in 2008, 9.4 million new active tuberculosis cases were reported with 1.8 million tuberculosis-linked deaths (32). Despite the enormous number of people infected, only about 10% of affected individuals show evidence of symptoms and develop the clinical disease. Infection with M. tuberculosis elicits humoral and cellular immune responses that normally control bacterial burden. Although immune response against tuberculosis exists, M. tuberculosis is seldom eradicated, suggesting that their immune response is not protective against active disease (31). Since the identification of T-helper type 1 (Th1) or Th2 lineage more than 2 decades ago, regulatory T cells (Tregs), and T helper interleukin 17 (IL-17)-producing cells (Th17 cells) have been added to the “portfolio” of Th cells. Tregs depress the T cell-mediated immune responses to the protective mycobacterial antigen during active tuberculosis in humans (11). Th17 cells have been reported to contribute to the adaptive immune response to M. tuberculosis in exposed persons and in patients with tuberculosis (22). Moreover, Tregs can modulate Th17 responses even in patients with latent M. tuberculosis infection (3). Tuberculous pleural effusion (TPE) is caused by a severe delayed-type hypersensitivity reaction in response to the rupture of a subpleural focus of M. tuberculosis infection (15). An accumulation of lymphocytes, especially CD4+ T cells, in TPE has been well documented (18). In previous studies, we have demonstrated that increased Tregs are found in TPE and that these Tregs are recruited into pleural space induced by chemokine CCL2 (21, 33). More recently, Wang et al. (30) demonstrated that Th17 cells were significantly increased in TPE compared with blood and that the mRNA and protein expression levels of IL-17 and IL-6 were significantly increased, whereas the expression level of transforming growth factor β (TGF-β) was decreased in TPE. 

TGF-β is a key cytokine involving in regulating the generation and differentiation of Th17 cells and Tregs. TGF-β is synthesized in cells as a pro-TGF-β precursor. Following homodimerization, pro-TGF-β is cleaved into two fragments: the C-terminal homodimer corresponds to mature TGF-β, while the N-terminal homodimer is latency-associated peptide (LAP) (9). Mature TGF-β and LAP remain noncovalently bound to each other in a complex called latent TGF-β. Latent TGF-β is inactive because LAP prevents mature TGF-β from binding to its receptor and hence from transducing a signal (14).  

In the present study, we investigated the distribution of Th17 cells in relation to CD39+ Tregs. We were also prompted to investigate whether CD39+ Tregs are capable of suppressing generation and differentiation of Th17 cells in TPE, as well as whether LAP is involved in such a possible suppression.

METHODS AND MATERIALS

Subjects. The study protocol was approved by our institutional review boards for human studies, and informed consent was obtained from all subjects. The patients were included subsequently if the examinations of pleural fluid and/or biopsy specimens established a diagnosis of TPE. Twenty-three patients (age range, 21 to 64 years) were proven to have TPE, as evidenced by growth of M. tuberculosis from pleural fluid or by demonstration of

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granulomatous pleurisy on a closed pleural biopsy specimen in the absence of any evidence of other granulomatous diseases (Table 1). All TPE patients were anti-human immunodeficiency virus antibody (Ab) negative and were recruited from Department of Internal Medicine, Wuhan Institute of Tuberculosis Prevention and Control. After antituberculosis chemotherapy, the resolution of TPE and clinical symptoms was observed in all patients with TPE.

The patients were excluded if they had accepted any invasive procedures directed into the pleural cavity, if any chest trauma was occurred within 3 months prior to their hospitalization, or if there was a pleural effusion of origin unknown.

At the time of sample collection, none of the patients had received any antituberculosis therapy, corticosteroids, or other nonsteroid anti-inflammatory drugs.

**Sample collection and processing.** Five-hundred-milliliter TPE samples from each patient were collected in heparin-treated tubes, through a standard thoracoscopic pleural biopsy in the absence of granulomatous pleurisy on a closed pleural biopsy specimen in the absence of granulomatous pleurisy on a closed pleural biopsy specimen. TPE specimens were immersed in ice immediately after antituberculosis chemotherapy, the resolution of TPE and clinical symptoms was observed in all patients with TPE.

**Flow cytometry.** The expression markers on T cells from TPE and blood were determined by flow cytometry after surface or intracellular staining with anti-human-specific Abs conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), PEcy5.5, PEcy7, peridinin chlorophyll protein (PerCP)-cy5.5, or allophycocyanin (APC). These human Abs included anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CD45RA, anti-CD45RO, anti-CD28, anti-CD27, anti-IL-2, anti-IL-6, and anti-CD16. The purity of naïve CD4+ T cells was >98% as measured by flow cytometry.

**Histological confirmation with TPE.** Purified naïve CD4+ T cells (5 × 10^5) were cultured in 1 ml of complete medium containing human IL-2 (2 ng/ml) in 48-well plates and stimulated with plate-bound anti-CD3 (OKT3; 1 μg/ml) and soluble anti-CD28 (1 μg/ml) MAb for 7 days. The exogenous cytokines used were IL-1β (10 ng/ml), IL-6 (100 ng/ml), and TGF-β1 (5 ng/ml). Recombinant human IL-1β, IL-2, IL-6, and TGF-β1 were purchased from R&D Systems. In some experiments, designated numbers of CD39− Tregs were added into the culture. To demonstrate that LAP was responsible for the inhibitive effects of CD39−/Tregs, blocking experiments were performed by adding 500 ng/ml of anti-LAP MAb (clone 27235) or mouse IgG irrelevant isotype control (R&D Systems) into the coculture.

Additionally, purified naïve CD4+ T cells labeled with fluorescent dye CFSE (carboxyfluorescein succinimidyl ester) (Molecular Probes) by incubation in phosphate-buffered saline (PBS) containing 5 μM CFSE for 10 min at 37°C were used, and the fluorescence was detected by flow cytometry.

**Statistics.** Data are expressed as medians (25th to 75th percentiles) or mean ± standard error of the mean (SEM). Comparisons of the data between different groups were performed using one-way analysis of variance. For variables in TPE and in corresponding blood, paired data comparisons were made using a Wilcoxon signed-rank test. The correlations between variables were determined by Spearman rank correlation coefficients. Analysis was completed with SPSS Version 16.0 statistical software (Chicago, IL), and P values of <0.05 were considered to indicate statistical significance.

**RESULTS**

**Increased proportion of Th17 cells and Tregs in TPE.** We are not showing the cytological and biochemical characteristics in TPE here, since these data have been reported previously (13, 16), and similar results were observed in the present study.
We first used flow cytometry to identify Th17 cells in CD4+ T cells in TPE and blood. It was found that percentages of Th17 cells represented the higher values in TPE (median, 2.5%; 25th to 75th percentiles, 2.0% to 3.1%), showing significant increases in comparison with those in blood (0.4%; 0.3% to 0.7%) (P < 0.001) (Fig. 1A). We next identified CD4+CD25^{high} regulatory T cells (Tregs) with expression both FOXP3 and CD39 in TPE and blood. Consistent with our previous studies (21), Treg numbers in TPE (11.6%; 8.2% to 15.3%) were much higher than those in blood (4.2%; 3.0% to 6.3%), (P < 0.001) (Fig. 1B). We also noted that some pleural CD4+ T cells were CD39^{+}IL-17^{+} cells (~2%).

We further noted that Tregs correlated negatively with Th17 cells in TPE (r = −0.695, P < 0.001) (Fig. 1C). However, Tregs did not correlate with Th17 cells in blood (r = −0.090, P = 0.684) (Fig. 1D).

**Impacts of cytokines on Th17 cells and Tregs in TPE.** Some proinflammatory cytokines, including IL-1β, IL-6, and TGF-β, have been found in TPE (10, 12, 23, 30). To evaluate the contribution of these cytokines to the increases of pleural Th17 cells and Tregs, we isolated naïve CD4+ T cells from TPE and cultured them in the presence of IL-1β, IL-6, and/or TGF-β. When IL-2-containing medium served as the baseline control, IL-1β, or IL-6, but not TGF-β, could promote the generation and differentiation of Th17 cells from naïve CD4+ T cells (Fig. 2). The combination of IL-1β plus IL-6 significantly increased...
FIG. 2. Generation and differentiation of Th17 cells and CD39+ Tregs from tuberculous pleural effusion regulated by different cytokines. (A) The representative dot plots of freshly isolated naive CD4+ T cells from tuberculous pleural effusion were determined for expression of IL-17 and CD39 by flow cytometry (top). The representative dot plots of Th17 cells and CD39+ Tregs detected in naive CD4+ T cells after culturing in the presence of both IL-1β and IL-6 (second from top). The representative dot plots of Th17 cells and CD39+ Tregs detected in naive CD4+ T cells after culturing in the presence of TGF-β1 (second from bottom). The representative dot plots of Th17 cells and CD39+ Tregs detected in naive CD4+ T cells after culturing in the presence of IL-1β, IL-6, and TGF-β1 (bottom). (B) The mean ± SEM of Th17 cells (closed bars) and CD39+ Tregs (open bars) detected in naive CD4+ T cells from 5 independent experiments. The purified naive CD4+ T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 MAbs in the presence of the indicated cytokines, either alone or in various combinations for 7 days. *, P < 0.01 compared with their corresponding controls with no cytokines.
the percentage of Th17 cells at higher extents compared with any single one of them, whereas TGF-β could reduce the increased percentage of Th17 cells stimulated by IL-1β and/or IL-6. In contrast, TGF-β was capable of promoting the differentiation of Tregs during the 7-day culture, and IL-1β and/or IL-6 did not affect the increase in Treg numbers induced by TGF-β (Fig. 2).

Inhibition of generation and differentiation of Th17 cells by CD39+ Tregs. We purified CD39+ CD4+ CD25high T cells from both TPE and blood and found that majority of this Th subset were FOXP3 positive (92 to 97%) and CD127 negative (90 to 96%), possessing phenotypes typical of Tregs (Fig. 3A).

To assess the suppressor activity of these Tregs, we separated CD39+ Tregs and naive CD4+ T cells from TPE and blood and then determined their proliferative capacity and the effect of CD39+ Tregs on CD4+ T cell proliferation. As shown in the representative suppression data (Fig. 3B), proliferative responses to anti-CD3 and -CD28 MAbs in the presence of IL-1β plus IL-6 for 7 days; CFSE cells were then analyzed by flow cytometry. The representative dot plots are from 1 of 5 independent experiments. (C) Naïve CD4+ T cells isolated from tuberculous pleural effusion (open bars) and blood (closed bars) were cultured in the above-described conditions, and Th17 cell numbers were determined by flow cytometry. The results are reported as mean ± SEM from 5 independent experiments. *, P < 0.01 compared with naïve CD4+ T cells without CD39+ Tregs.

FIG. 3. CD39+ Tregs inhibit generation and differentiation of Th17 cells. (A) The representative dot plots showing the isolated pleural CD39+ CD4+ CD25high T cells were almost entirely CD39 positive and CD127 negative. (B) CFSE-labeled naïve CD4+ T cells isolated from tuberculous pleural effusion were cultured with indicated ratios of CD39+ Tregs and stimulated with plate-bound anti-CD3 and soluble anti-CD28 MAbs in the presence of IL-1β plus IL-6 for 7 days; CFSE cells were then analyzed by flow cytometry. The representative dot plots are from 1 of 5 independent experiments. (C) Naïve CD4+ T cells isolated from tuberculous pleural effusion (open bars) and blood (closed bars) were cultured in the above-described conditions, and Th17 cell numbers were determined by flow cytometry. The results are reported as mean ± SEM from 5 independent experiments. *, P < 0.01 compared with naïve CD4+ T cells without CD39+ Tregs.
As shown in Fig. 3C, generation and differentiation of Th17 cells were observed when the purified naïve CD4⁺ T cells were cultured for 7 days in the presence of IL-1β and IL-6. When CD39⁺ Tregs were added into the coculture, Th17 cell numbers decreased as CD39⁺ Treg numbers increased. There were no differences in inhibiting effects on Th17 cell numbers between pleural CD39⁺ Tregs and blood CD39⁺ Tregs.

**LAP mediates Treg-induced inhibition of Th17 Cells.** We determined LAP expression on the cell surface of CD39⁺ Tregs by flow cytometry and found that LAP surface expressions on freshly purified pleural CD39⁺ Tregs (median, 11.6%; 25th to 75th percentiles, 9.2% to 12.3%) were much higher than those in blood CD39⁺ Tregs (median, 2.1%; 0.8% to 4.1%) (P < 0.001); when CD39⁺ Tregs were cultured with plate-bound anti-CD3 and soluble anti-CD28 MAbs in the presence of TGF-β for 7 days, the expression of LAP increased significantly (median, 71.4%; 59.0% to 74.5%) (Fig. 4A).

The nature of LAP present on the surface of CD39⁺ Tregs that were responsible for their suppressive activity was also investigated in the present study. To do this, purified naïve CD4⁺ T cells served as a control, and CD39⁺ Tregs were cultured with CFSE-labeled naïve CD4⁺ T cells in the above-described conditions. CFSE⁺ CD4⁺ T cells were gated to exclude CD39⁺ Tregs, and expression of IL-17 was examined. The representative dot plots are from 1 of 5 independent experiments. (D) Naïve CD4⁺ T cells isolated from tuberculous pleural effusion (open bars) and blood (closed bars) were cultured alone or with CD39⁺ Tregs (ratio, 1:1), an anti-LAP MAb or isotype control IgG was added into the coculture, and Th17 cell numbers were determined by flow cytometry. The results are reported as mean ± SEM from 5 independent experiments. *, P < 0.01 compared with CD4⁺ T cells alone.

As shown in Fig. 4C, CFSE⁺ CD4⁺ T cells were gated to exclude CD39⁺ Tregs, and expression of IL-17 was examined. We noted that substantial amounts of Th17 cells could be
generated and differentiated from naïve CD4+ T cells alone. Coculturing with CD39+ Tregs (ratio, 1:1) suppressed the frequency of Th17 cells, and the addition of anti-LAP MAb, but not a mouse IgG irrelevant isotype control, to the coculture markedly reversed the inhibitory effect exerted by CD39+ Tregs. The overall results in terms of mean ± SEM from 5 independent experiments are shown in Fig. 4D. Therefore, the above results indicate that CD39+ Tregs inhibit generation and differentiation of Th17 cells via a LAP-dependent mechanism.

**DISCUSSION**

Pleural effusion is characterized by the presence of specific subsets of leukocytes which, together with pleural mesothelial cells, contribute to the local production of cytokines and chemokines (1, 3). Lymphocytic pleural effusion refers to those types in which lymphocytes account for more than 50% of total leukocytes in the pleural effusion, which are commonly seen in TPE (7). In previous studies, we and other authors have demonstrated that Treg numbers in TPE were much higher than those in blood (6, 21, 33). Although it cannot be excluded that the overrepresentation of Tregs in TPE may be due to increased local antigen stimulation, chemokine CCL22 might be capable of inducing the migration of Tregs to the pleural space of the patients with TPE (33). We therefore were prompted to investigate whether Tregs are capable of suppressing the generation and differentiation of Th17 cells in the pleural space of patients with TPE.

Tregs in human studies have been identified mostly on high expression of CD25 and FOXP3 and, in some cases, low expression of CD127 (17). However, FOXP3 mRNA expression could be induced in human CD25+ and CD8+ peripheral blood mononuclear cells, which were both negative for FOXP3 mRNA expression after isolation, indicating that FOXP3 expression in humans, unlike that in mice, may not be specific for Tregs and may be only a consequence of activation status (20). Furthermore, these markers cannot be used to identify Treg poststimulation in vitro, since their expression patterns change toward the Treg phenotype upon the activation of effector T cells. Recently, the technique of isolating human Tregs has been proven to be highly desirable (19). In the present study, we isolated Tregs from TPE- and blood-based CD39 expression and found that CD39+ Tregs could be able to inhibit the generation and differentiation of Th17 cells from naïve CD4+ T cells in a dose-dependent manner.

The mechanism by which human Tregs inhibit the generation and differentiation of Th17 cells is unknown. Fletcher et al. (8) demonstrated for the first time that human Tregs can suppress IL-17 production by responder T cells; their data suggested that the CD39 molecule might be involved in the mechanism by which Tregs suppress the generation and differentiation of Th17, since the hydrolysis of ATP by CD39 could reduce IL-17 production by CD4+ T cells, and an analog of adenosine, the final breakdown product of ATP, effectively inhibited IL-17. Our data showed that pleural CD39+ Tregs could be induced to express LAP on their surfaces; we thus explored whether LAP was involved in the generation and differentiation of Th17 cells in the pleural space of patients with TPE. For the first time, we show that Th17/Tregs are present at high frequencies in TPE compared with peripheral blood. Interestingly, we further noted that the numbers of Th17 cells and Tregs were both increased in TPE compared with peripheral blood. It was not surprising that immune response against M. tuberculosis was more intensive in a pathological site than in peripheral blood. We therefore were prompted to investigate whether Tregs are capable of suppressing the generation and differentiation of Th17 cells in the pleural space of patients with TPE.

Both Tregs and Th17 cells have been known to be involved in tuberculosis immunity (11, 22). Tregs recognizing M. tuberculosis-derived antigens specifically and potently restrict protective immune responses during tuberculosis (24, 25). It has been reported that IL-17 is an important cytokine in protective immunity against M. tuberculosis infection (26). We hypothesize that Tregs might participate in the suppression of local immune responses by inhibiting Th17 responses in TPE. This study supports earlier data that both Th17 cells and Tregs are present at high frequencies in TPE compared with the autologous blood. For the first time, we show that Th17/
Treg imbalance exists in TPE and that CD39+/ Tregs inhibit the generation and differentiation of pleural Th17 cells via a LAP-dependent mechanism.

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