High IL-35 Pleural Expression in Patients with Tuberculous Pleural Effusion

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Background: IL-35 is a novel anti-inflammatory and immunosuppressive cytokine primarily produced by Treg cells, and is involved in inflammatory diseases and autoimmune diseases. However, its roles in tuberculous pleural effusion (TPE) remain unknown. We aimed to investigate the potential involvement of IL-35 in TPE.

Material/Methods: Thirty TPE patients and 20 lung cancer patients with malignant pleural effusion (MPE) were recruited. Samples of pleural effusion (100 mL) were collected after traditional pleurocentesis. Blood was sampled from TPE patients. Mononuclear cells were isolated by Ficoll-Hypaque gradient. Proportions of Th1, Th17, and IL-35-producing cells were analyzed by flow cytometry. IL-35 was assessed by real-time RT-PCR, ELISA, and immunofluorescence. An ELISPOT assay was used to assess the effect of IL-35 on pleural effusion mononuclear cells (PEMCs).

Results: Proportions of IL-35-producing cells were higher in TPE compared with MPE (49.4±6.0 vs. 15.8±5.4%, \(P<0.001\)) and blood from TPE patients (49.4±6.0% vs. 16.6±3.1, \(P<0.001\)). IL-35, IL-17 and IFN-\(\gamma\) were elevated in TPE compared with MPE (all \(P<0.01\)). ELISPOT assay showed that IL-35 reduced the proportion of IFN-\(\gamma\)-producing CD4\(^+\) T cells in TPE. IL-35 mRNA expression was higher in TPE compared with MPE (\(P<0.001\)). Immunofluorescence showed that IL-35-positive cells were present in pleural tissues from TPE patients.

Conclusions: Results suggest that there is an imbalance in IL-35 metabolism in TPE. However, further studies are required to assess the exact relationship with the immune system response to tuberculosis. IL-35 might play a role in TPE and might be targeted as a treatment for TPE.

MeSH Keywords: Interleukin-17 • Interleukin-18 • Interleukins • Natural Killer T-Cells • Pleural Effusion • Tuberculosis

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Background

Tuberculosis is a serious public health problem and an important cause of death from infectious diseases around the globe, with 8.6 million new and relapse cases in 2012, 50% of them being in Bangladesh, China, India, Indonesia and Nigeria [1]. Tuberculosis is responsible for 1.3 million deaths each year [1]. Although the pulmonary form is the most common manifestation of tuberculosis, the disease may also affect the pleural lymph nodes. The pleural form accounts for 4–10% of all tuberculosis cases, depending upon the country [2,3]. In some areas of the world, tuberculosis is the main cause of pleural effusion [4]. Although tuberculous pleural effusion (TPE) is a self-limited disease, 50% of untreated patients will develop an active form of tuberculosis [5,6]. Therefore, a better understanding of this disease is necessary to correctly guide the treatments.

TPE results from the leakage of pleural lymph node that releases its content into the pleural space. Consequently, CD4+ T-cells recruited at the site induce an acute inflammatory hypersensitivity reaction [5,7]. Mycobacteria induce IL-12 to drive the production of IFN-γ via a Th1 response in tuberculosis patients [8,9]. IFN-γ-producing Th1 cells contribute to the control of mycobacterial replication [5,10]. However, this pathogenic mechanism fails to explain resistance/susceptibility to infection and disease [8,11].

A number of cytokines are produced by T cells to interact with other immune cells. At the initial stages, neutrophils are the main actors in the pleural cavity, but T lymphocytes are predominant in the later stages. This inflammatory reaction leads to impaired fluid clearance from the pleural space [5]. Levels of CD4+CD25+ T cells are higher in tuberculous pleural effusion compared with peripheral blood in TPE patients [12-15]. The high expression of CD4+CD25+ Foxp3+ T cells in TPE is thought to contribute to the suppression of the CD4+CD25+ T cells proliferation in TPE [16,17]. Treg can inhibit the generation and differentiation of the Th17 cells [13,18]. Both malignant pleural effusion (MPE) and late-stage TPE are lymphocytic effusions, but TPE is a Th1-dominant disease, while MPE is Th2-dominant [19–22].

Interleukin (IL)-35 is a novel member of the IL-12 family and is composed of 2 subunits: Ebi3 (IL-27b) and p35 (IL-12a) [23]. In human, IL-35 secretion has been detected in Foxp3+ Treg stimulated by CD4+CD25- effector cells, but not in unstimulated Treg [24,25]. IL-35 is an anti-inflammatory and immunosuppressive cytokine considered to enhance the suppressive activity of Treg [26,27]. IL-35 regulates the activity of naïve T cells and is required for maximal suppressive activity [27]. IL-35 inhibits the inflammatory response in a number of autoimmune diseases, such as experimental colitis [27,28], collagen-induced arthritis [29], central nervous system demyelination [30] and Th2-mediated allergic asthma [31]. p35-knockout mice are more susceptible to Helicobacter-induced colitis, Leishmania infections and autoimmune diseases [27]. IL-35 is also necessary to maintain the testicular immune privilege [32].

CD4+CD25+Foxp3+ cells are increased at sites of infection in tuberculosis patients and were considered to suppress Th1-type immune responses via the down-regulation of IFN-γ production in T cells [33,34]. However, the molecules mediating the suppressive activity of these cells remain largely unknown, and no study explored the specific role of IL-35 in TPE. Therefore, the aim of the present study was to investigate whether IL-35 is involved in the immune response in TPE. We hypothesized that IL-35 participates in the immune response in patients with TPE and can affect Th1-type immune responses in TPE via inhibiting CD4+ T cells from releasing IFN-γ, which is required for the immunological characteristics of these cells. This preliminary information will provide the basis for understanding the role IL-35 plays in TPE and may help future investigations devise new treatments for TPE, or determine whether IL-35 could be used to diagnose, monitor or enhance the prognosis of TPE.

Material and Methods

Subjects

Thirty patients with TPE and 20 lung cancer patients diagnosed with MPE were selected from the inpatient department of Wuhan Medical Center and from the Zhongnan Hospital of Wuhan University from April 2013 to December 2013. The medical ethics committees of the Wuhan Medical Treatment Center and Zhongnan Hospital approved the study, and written informed consent was obtained from each patient.

TPE was diagnosed based on: 1) typical clinical symptoms (fever and chest pain) and B-mode ultrasound revealing pleural effusion; 2) adenosine deaminase levels in the pleural effusion of 40–80 U/L; 3) strongly positive tuberculin test result; 4) positive histopathological examination of a pleural biopsy specimen; and/or 5) clinical symptoms were rapidly relieved after 1 month of anti-tuberculosis chemotherapy [5,6].

MPE was confirmed in lung cancer patients using pathological examinations, including pleural biopsy, and cytological examination of exfoliated cells in the effusion.

Exclusion criteria were: 1) autoimmune disease; 2) human immunodeficiency virus (HIV) infection; 3) cancer for TPE patients, cancer other than lung cancer for MPE patients; 4) pregnancy; 5) ongoing infection other than pleural tuberculosis for TPE patients; or 6) any systemic disease involving immunity.
Sample collection and processing

We collected 100 ml of pleural effusion from each patient after they were treated with traditional pleurocentesis. Blood (10 ml) was collected from antecubital vein of each patient. Samples were centrifuged for 15 min at 2000 rpm at room temperature. Peripheral blood mononuclear cells (PBMCs) and pleural effusion mononuclear cells (PEMCs) were isolated by Ficoll-Hypaque gradient centrifugation (Dakewe, Beijing, China) and resuspended in 4 ml of PBS. PBMCs and PEMCs used for IL-35 detection were stimulated with phorbol-12-myristate-13-acetate (PMA; Sigma, St Louis, MI, USA) and 250 ng/ml of ionomycin (Sigma, St Louis, MI, USA) for 5 h at 37°C in a 5% CO₂ atmosphere. Mononuclear cells used for IL-17 and INF-γ detection were stimulated with PMA and ionomycin for 2 h at 37°C in a 5% CO₂ atmosphere. After stimulation with 11 μg/ml of Brefeldin A (Sigma, St Louis, MI, USA) for 4 h, the cells were harvested for intracellular staining, ELISPOT assay and quantitative real-time RT-PCR. We also harvested the pleural effusion and blood supernatants and preserved them at −20°C for cytokines measurement by ELISA.

Flow cytometry

We detected specific cytokine-producing cells from TPE, MPE, and blood from TPE patients by flow cytometry via surface staining using anti-human PE-cy5-labeled anti-CD3 and FITC-labeled anti-CD8 (Biolegend, San Diego, CA, USA). After surface staining, intracellular staining was performed by incubating the cells with PE-labeled anti-human cytokine antibodies (IFN-γ, IL-17, and IL-35 antibodies, Biolegend, San Diego, CA, USA) at room temperature in the dark. Isotype controls were used. CD3 CD8– cells were studied as measure of CD3 CD4 cells. Cells were analyzed with using an Epics Altra flow cytometer (Beckman Coulter, Brea, CA, USA).

ELISPOT assay

A human TB IFN-γ pre-coated ELISPOT kit (Dakewe, Beijing, China) was used according to the manufacturer’s instructions. The concentration of PEMCs was adjusted using RPMI 1640. The PEMCs were left unstimulated (Blank control, 500,000 cells) or were stimulated with 10 μl of PMA (Positive control, 100,000 cells), or 10 μl of the ESAT-6 and CFP-10 peptides (Shanghai Fosun Pharmaceutical Co. Ltd.) (Negative control, 500,000 cells). The PEMCs in the experimental group (500 000 cells) were stimulated with 10 μl of the ESAT-6 and CFP-10 peptides plus 150 ng/ml IL-35 antigen (Shanghai Sino Biological Technology Co., Ltd., China). The number of the ESAT-6 and CFP-10 peptides plus 150 ng/ml IL-35 antigen in the experimental group (500 000 cells) were stimulated with 10 μl of PMA (Positive control, 100,000 cells), or 10 μl of Brefeldin A (Sigma, St Louis, MI, USA) for 4 h, the cells were harvested for intracellular staining, ELISPOT assay and quantitative real-time RT-PCR. We also harvested the pleural effusion and blood supernatants and preserved them at −20°C for cytokines measurement by ELISA.

ELISA

We detected IL-35, IL-17, and INF-γ in the supernatants from TPE, MPE, and blood by ELISA, according to the manufacturers’ instructions. The IL-17 ELISA kits were from eBioscience (San Diego, CA, USA), the IL-35 ELISA kits were from Cusabio (Wuhan, China) and the INF-γ ELISA kits were from Dakewe (Beijing, China). All samples were measured in duplicate. The minimum detectable concentrations were 12.5 pg/ml for INF-γ, 1.6 pg for IL-17 and 62.5 pg/ml for IL-35.

Real-time RT-PCR analysis

Total RNA was isolated from PEMCs from TPE and MPE using the Trizol reagent (Invitrogen Inc., Carlsbad, CA, USA). RNA purity was determined using the absorbance at 260 and 280 nm (A260/280). RNA integrity was verified by electrophoresis on formaldehyde gels. cDNAs were prepared using a first strand cDNA synthesis kit (TOYOBO Co., Ltd, Tokyo, Japan), according to the manufacturer’s instructions. PCR amplifications reactions were performed using the THUNDERBIRD™ SYBR® qPCR Mix kit (TOYOBO Co., Ltd, Tokyo, Japan) in a SLAN Real-Time PCR System (Shanghai Hongshi Medical Technology Co., Ltd., China). PCR steps were an initial denaturation for 1 min at 95°C and 40 cycles of 95°C for 15 s, 58°C for 20 s and 72°C for 20 s. 2−ΔΔCt was calculated to represent the relative mRNA expression of target genes in TPE and MPE. β-actin was used as an internal control.

The primer sequences were: β-actin, Forward: 5'-CAC CCA GCA CAA TGA AGA TCA AG T-3' and Reverse: 5’-CCA GTT TTT AAA TCC TGA GTG AAC C-3'; EBI3, Forward: 5’-GCT TCG TGC CTT TCA TAA CAG AG-3' and Reverse: 5’-TAG TCT GTG AGG TCC TGA GCC G-3' and IL-12A, Forward: 5’-CCA CTC CAG ACC CAG GAA TGT-3' and Reverse: 5’-CCT CCA CTG TGG TTT TAT CT-3'.

Immunofluorescence

Double immunofluorescence labeling was performed on pleural tissues from TPE patients to confirm the expression of IL-35. After ultrasound-guided pleural biopsy, the pleural tissues were immediately fixed in 4% paraformaldehyde for 2 h and were embedded in paraffin. Sections were prepared as 4- to 5-μm thick sections. A rabbit polyclonal antibody raised against human IL-12Ap35 and a mouse polyclonal antibody raised against human Ebi3 were used as the primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cy3-conjugated goat anti-rabbit IgG and 488-Alexa-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Delaware, USA) were used to label IL-12Ap35 and Ebi3, respectively. After antigen retrieval with 0.01 mol/l EDTA, tissue sections were incubated at 4°C overnight with 1:100 dilutions of primary IL-12A and EBI-3 Abs (IL-12A 1:100; EBI-3 1:100), according to the manufacturer’s instructions. After washing, tissue sections were incubated with secondary antibodies (1:300 dilution for Cy3-conjugated goat anti-rabbit IgG, and 1:400 dilution for 488-Alexa-conjugated goat anti-mouse IgG) for 1 h at 25°C in the dark. Cell nuclei were stained with 0.1 ug/ml DAPI.
Patients' characteristics

TPE patients were 36.8±7.8 years old, including 18 males and 12 females. MPE patients were 50.8±7.8 years old, including 13 males and 7 females. All TPE and MPE patients were confirmed by pleural biopsy. In the TPE group, 19 patients presented with non-active pulmonary tuberculosis and 11 with active pulmonary tuberculosis. No patients had severe tuberculosis such as hematogenous disseminated pulmonary tuberculosis, or tuberculous meningitis. All patients were HIV-negative and no patient had been diagnosed with immunodeficiencies. Furthermore, clinical symptoms of all TPE patients were rapidly relieved after 1 month of anti-tuberculosis chemotherapy.

Proportions of Th1, Th17 and IL-35-producing cells are higher in TPE

The proportion of IL-35-producing cells in TPE was higher than in MPE (median ±IQR: 51.7±26.7% vs. 11.3±10.1%, P<0.001), and in the corresponding blood samples (51.7±26.7% vs. 19.8±11.7%, P<0.001) (Figure 1A, 1D).

Consistent with previous studies [7,10], the proportion of CD3+CD8+ IL-17+ cells was clearly higher in TPE than in MPE (3.9±1.9% vs. 1.6±0.8%, P<0.001), and compared with the
corresponding blood samples from TPE patients (3.9±1.9% vs. 0.4±0.3%, P<0.001) (Figure 1B, 1E).

Similar results were observed for IFN-γ. The proportion of CD3⁺CD8⁻ IFN-γ⁺ cells was higher in TPE than in MPE (21.6±6.9% vs. 2.1±1.7%, P<0.001), and compared with the corresponding blood samples (21.6±6.9% vs. 4.7±2.4%, P<0.001) (Figure 1C, 1F).

**TPE patients have higher levels of IFN-γ, IL-17 and IL-35**

IL-35 levels were higher in TPE compared with MPE (P<0.001), and with the corresponding blood samples from TPE patients (P<0.001) (Figure 2A). IL-17 and IFN-γ levels were higher in TPE compared with MPE (P<0.001) and with the corresponding blood samples (P<0.001) (Figure 2B, 2C).

**IL-35 reduces the frequency of IFN-γ-producing CD4⁺ T cells in TPE**

The ELISPOT assay targets the CD4⁺T cells, which produce IFN-γ. Therefore, IL-35 was added to the cell culture medium as an antigen to observe whether it inhibits CD4⁺ T cells producing IFN-γ. The results showed that IL-35 reduced the proportion of IFN-γ-producing CD4⁺ T cells in TPE (P<0.001) (Figure 3).

**Elevated levels of IL-35 genes in PEMCs from patients with TPE**

The mRNA expression of the 2 IL-35 subunits (EBi3 and IL-12Ap35) were higher in PEMCs from patients with TPE compared with that from patients with MPE (both P<0.001) (Figure 4A, 4B).
Expression of IL-35 in pleural tissue of TPE patients

Immunofluorescence showed that Ebi3 and p35 were expressed in Langerhans giant cells of pleural tissues from TPE patients (Figure 5).

Discussion

As an anti-inflammatory molecule, IL-35 contributes to the immune regulation of chronic inflammatory diseases and parasitic/bacterial diseases [27]. The aim of the present study was to investigate the potential involvement of IL-35 in TPE. Results showed that IL-35-producing cells were higher in TPE compared with MPE. IL-25, IL-17, and IFN-γ were elevated in TPE compared with MPE. ELISPOT assay showed that IL-35 reduced the proportion of IFN-γ-producing CD4+ T cells in TPE. IL-35 mRNA was higher in TPE compared with MPE. Immunofluorescence showed that IL-35-positive cells were present in pleural tissues from TPE patients.

Our results strongly suggest the involvement of IL-35 cells in the immunology of TPE. Higher IL-35 levels were observed in TPE than in MPE and blood from the same patients. We inferred that a stronger immune response might occur in the pleural cavity compared with other body regions. Similar observations were reported in studies of the immune roles of TH17 and TH1 in TPE [13,15,18]. Our results provide additional data about the immune effect of IL-35 in infectious diseases, particularly in tuberculosis.

IL-35 has been recently recognized as an important mediator with the ability to directly suppress effector T cell responses in inflammatory and autoimmune diseases [29]. Previous animal experiments revealed that, in mice lacking Ebi3, the expression of IL-17 was significantly higher [36]. Niedbala et al. [29] reported that IL-35 can inhibit the differentiation of Th17 cells in vitro and alleviate collagen-induced arthritis in mice. Increasing IL-35 levels in patients with systemic lupus erythematosus may protect the immune system from pathogenic factors and avoid the impairment of tissues and organs [37]. Liu et al. [38] reported that IL-35 plays a role during immunosuppression in chronic hepatitis B patients. However, relatively little is known about the occurrence of IL-35 immunosuppression in TPE. To assess the suppressor activity of IL-35 in TPE, we used an ELISPOT assay to determine whether IL-35 inhibition affects CD4+CD25− T cells in TPE. Our results showed that IL-35 reduces the proportion of IFN-γ-producing CD4+ T cells in TPE. These results are supported by those from Niedbala et al. [29]. Those authors suggested that IL-35 suppressed the proliferation of CD4+ cells in collagen-induced arthritis.

IFN-γ is a well-known marker for TPE. IFN-γ is released by activated CD4+ T cells and is a key cytokine involved in increasing the anti-mycobacteria activity of macrophages [39]. Numerous studies showed the clinical diagnostic value of IFN-γ for TPE, with a sensitivity of 100% and a specificity of 100% [40]. IFN-γ levels significantly increase in TPE, regardless of the immunological status of the patient [41]. In the present study, we
observed Th1 differentiation in TPE, as indicated by the higher proportion of IFN-γ-producing CD4+ T cells in TPE compared with MPE. Previous studies showed that the increased frequency of CD4+CD25+Foxp3+ T cells in TPE may inhibit the proliferation of CD4+CD25- T cells [16]. These previous studies support our ELISPOT results.

A new Th cell population that is distinct from Th1 and Th2 cells has been referred to as Th17 cells. The Th17 family expresses IL-17A, IL-17F, IL-21, and IL-22. Th17 cells play an important role in the induction of autoimmune tissue injury [42]. Th17 cells recruits neutrophils and monocytes to the site of inflammation. These cells exert immune effects during the early phase of host defense in many diseases [43]. Recently, an increasing number of studies indicated that IL-17 cells were involved in the immunopathology of pulmonary tuberculosis and extrapulmonary tuberculosis, especially in TPE [44,45]. The proportion of Th17 cells and the levels of the corresponding cytokines in the peripheral blood of tuberculosis patients were significantly different from the values observed in pleural effusion from the same patients. This phenomenon strongly suggests that Th17 differentiation occurs in TPE [13]. Fletcher et al. [46] reported that CD4+CD25+Foxp3+CD39+ T cells contribute to the suppression of pathogenic Th17 cells and to the reduction of these cells in multiple sclerosis patients. Ye et al. [18] demonstrated that a Th17/Treg imbalance exists in TPE and that CD39+ Tregs can inhibit the generation and differentiation of Th17 cells in TPE. These previous studies support the results from the present study.

The present study might suffer from some limitations. Indeed, it was an exploratory study that was performed in a small number of patients. Only a few cytokines were assessed. Future studies in a larger sample of patients are needed.

Conclusions

Activated PEMCs from TPE showed higher IL-35 levels compared with the blood from the same patients or with MPE patients. IL-35 may play an important role in suppressing effector T cell responses in TPE. Future studies are needed to identify the immunological mechanisms of IL-35 in TPE. Anti-tuberculosis treatment efficacy might have a relation with IL-35 expression in TPE patients. IL-35 might also be a pharmacological target, or could be used to diagnose, monitor, or enhance the prognosis of TPE. However, further studies are required to assess these points.

Conflict of interest

The authors declare that they have no conflict of interest.

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