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Original Article

Elevated serum IL-35 and increased expression of IL-35-p35 or -EBI3 in $CD4^+CD25^+$ T cells in patients with active tuberculosis

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Abstract: Despite the recent appreciation of interleukin 35 (IL-35) function in inflammatory diseases, little is known for IL-35 response in patients with active tuberculosis (ATB). In the current study, we demonstrated that ATB patients exhibited increases in serum IL-35 and in mRNA expression of both subunits of IL-35 (*p35* and *EBI3*) in white blood cells and peripheral blood mononuclear cells. Consistently, anti-TB drug treatment led to reduction in serum IL-35 level and p35 or EBI3 expression. TB infection was associated with expression of p35 or EBI3 protein in $CD4^+$ but not $CD8^+$ T cells. Most $p35^+CD4^+$ T cells and $EBI3^+CD4^+$ T cells expressed Treg-associated marker CD25. Our findings may be important in understanding immune pathogenesis of TB. IL-35 in the blood may potentially serve as a biomarker for immune status and prognosis in TB.

Keywords: Human active pulmonary tuberculosis, interleukin-35, p35, EBI3, $CD4^+CD25^+$ T cells

Introduction

Tuberculosis (TB) has been becoming a top killer among infectious diseases (WHO data in 2014) despite the availability of anti-TB drugs and BCG vaccine [1]. *Mycobacterium tuberculosis* (Mtb) infection of humans induces immune responses of various T cell subpopulations including Th1, Th17 cells, Treg, $CD8^+$ CTL and TB-reactive Vγ2Vδ2 T subset [2-5]. Nevertheless, whether T-cell immune responses in TB involve interleukin 35 (IL-35) remains poorly characterized.

IL-35, a member of the IL-12 family, was discovered by Stern, et al. in 1990, and initially named as cytotoxic lymphocyte maturation factor (CLMF) [6]. IL-35 is composed of the p35 subunit of IL-12 and the Epstein-Barr Virus (EBV)-induced gene 3 (EBI3) subunit of IL-27. The anti-inflammatory role of IL-35 was described in 2007 by two separate research groups, Collison, et al., in the USA and Niedbala, et al., in the UK [7, 8]. IL-35 appears to be produced by Treg cells, DC cells, B cells, macrophages, human placental trophoblasts, and various tumor cells of lung cancer, colon cancer, esophageal carcinoma, hepatocellular carcinoma, cervical carcinoma, melanoma, colorectal cancer and B cell lymphoma [8-22]. Like TGF-β and IL-10, IL-35 can induce development and expansion of a subset of Treg, named iTr35 [7, 23]. Treg, a subset of $CD4^+$ T cells, plays a key role in controlling autoimmunity and excessive immune responses in microbial infections by means of both cell-cell contact and secretion of inhibitory cytokines (TGF-β, IL-10, and IL-35) [23]. IL-10 and TGF-β produced by mononuclear

phagocytes have been shown to suppress T-cell responses during Mtb infection [24-31]. TGF-β increases the apoptosis of IFN-γ-producing T cells in both blood and Mtb infection sites [30, 31]. A recent study has reported an increased expression of IL-35 elevated in tuberculosis pleural effusion [32]. However, a role of IL-35 in ATB and its exact relation with T cell subsets in TB remain unknown.

$CD4^+CD25^+$ $Foxp3^+$ Treg cells have been shown to suppress T cell responses during infections through both IL-10-dependent and -independent pathways [33-35]. Treg cells may also contribute to attenuation of allergic airway inflammation in a TGF-β-dependent manner during both chronic and acute phases of *Trichinella spiralis* infection [36]. Furthermore, an increased frequency of $CD4^+CD25^+$ Treg cells in TB has been prostituted to suppress immune responses of IFN-γ-producing T cells [37, 38]. However, it is unclear how IL-35 coordinates the immune-regulatory properties of Treg in infectious diseases, although IL-35 can expand $CD4^+CD25^+$ Treg cells and enhance Treg suppressive function in inflammatory diseases and autoimmune diseases [7, 8, 39-41].

In this study, we examined the serum IL-35 level and the expression of IL-35 subsets (p35 and EBI3) in peripheral αβ T cell from patients with active tuberculosis (ATB). Our data suggest that IL-35 is involved in TB pathogenesis, and IL-35 level in the blood may be a valuable biomarker of immune status and prognosis in ATB.

Materials and methods

Subjects

Eighty cases of active pulmonary tuberculosis (ATB) in Dongguan 6st People's Hospital (Dongguan, China) were evaluated in this study, and ATB was diagnosed as described previously [21, 42]. Fourty healthy volunteers (HVs), without bacteriological and clinical evidence of TB, served as controls. Subjects with HIV infection, diabetes, cancer, autoimmune diseases, immunosuppressive treatment, or history of pulmonary TB were excluded from the study. All subjects were tested for sputum Ziehl-Neelsen acid fast staining and Lowenstein-Jensen slant culture according to the standard method. The individualized treatment of ATB patients was previously described. Patients with ATB were given initial anti-tubercular drug (ATD) treatments, namely M0 phase (duration, 0 to 4 days) and M1 phase treatment (duration, 20 to 40 days) as manifested by the absence of TB-relapsed symptoms. No significant differences in terms of age and gender were noted between patients and HVs. The study was approved by the Internal Review and the Ethics Boards of Guangdong Medical University and Dongguan 6st People's Hospital, and informed consent was obtained from all study subjects.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were collected after centrifugation and stored at -80°C until use. Serum IL-35 levels were detected using a human IL-35 ELISA kit (BlueGene, Shanghai, China) as described [21].

Quantitative real-time polymerase chain reaction PCR analysis (qRT-PCR)

White blood cells (WBC) were harvested after lysis of red blood cells and peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll (GE Health, Fairfield, USA) density gradient centrifugation as previously described [21, 42]. Total RNAs were isolated from PBMC using TRizol reagent (Takara, Dalian, China) according to the manufacturer's instruction. RNA was reverse-transcribed to cDNA using a PrimeScript™RT Master Mix kit (Takara, Dalian, China) and amplified for p35 and EBI3 using a SYBR® Premix Ex Taq TM II kit (Takara, Dalian, China). β-actin was used as an internal control. Primer sequences were as follows, *EBI3*: 5'-CAGCTTCGTGCCTTTCATAA-3' (sense) and 5'-CTCCCACTGCACCTGTAGC-3' (antisense); *p35*: 5'-CTTGTGGCTACCCTGGTCCTC-3' (sense) and 5'-AGGTTTTGGGAGTGGTGAAGG-3' (antisense); β-*actin*: 5'-TTGCCGACAGGATGCAGAA-3' (sense) and 5'-GCCGATCCACACGGAGTACT-3' (antisense). Quantitative real-time polymerase chain reaction (PCR) was performed on an ABI-7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR reaction conditions were as follows: 30 s at 94°C, 5 s at 95°C, and 34 s at 60°C for 40 cycles, then for dissociation stage.

Flow cytometric analysis (FCM)

For flow cytometric detection of IL-35, 3~10×10^5 lymphocytes were surface stained with anti-human CD3, CD4 (BD Pharmingen, San Jose, CA, USA), and then intracellular stained with

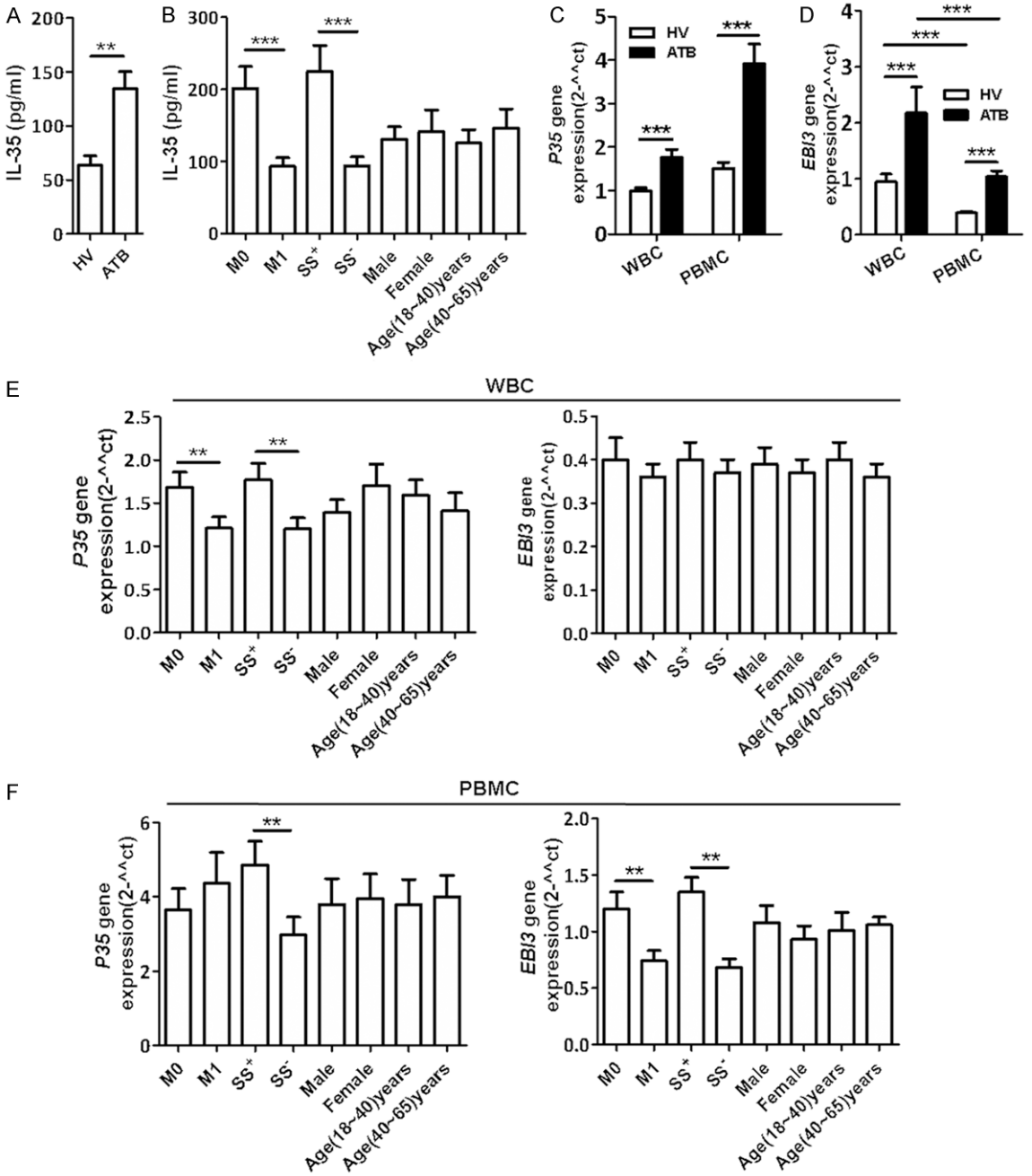

Figure 1. Mtb infection induced increases in serum IL-35 and mRNA expression of IL-35 units in leukocytes. ELISA was used to determine serum IL-35 in ATB patients (n=80) and healthy volunteers (HVs) (n=40). qRT-PCR was used to evaluate *p35* and *EBI3* mRNA expression in WBC or PBMC from ATB patients (n=30) or HVs (n=30). (A) Bar graph showing the concentration (pg/ml) of serum IL-35 in ATB patients and HVs. (B) Bar graph showing the concentration (pg/ml) of serum IL-35 in ATB patients treated with ATDs for 0 to 4 days (M0, n=37), or for 15 to 40 days (M1, n=38), patients with Mtb-positive sputum (n=40) or Mtb-negative sputum (n=40), patients with different gender (males, n=46; females, n=34) and ages (18-40 years, n=48; 40-60 years, n=32). (C and D) Bar graph showing mRNA expression of IL-35 subunits *p35* (C) and *EBI3* (D) in blood leukocytes (WBC and PBMC) from ATB patients or HVs. (E and F) Bar graph showing that the *EBI3* and *p35* gene expression in blood WBC (E) or PBMC (F) from groups of M0 (n=16), M1(n=14), sputum Mtb-positive (n=15) and sputum Mtb-negative (n=15), males (n=16), females (n=14), 18-40 years old (n=16), and 40-60 years old (n=14). **P*<0.05, ***P*<0.01, ****P*<0.0001.

anti-human p35 and anti-human EBI3 (eBioscience, San Jose, CA, USA) as described previously [21]. Matched mouse IgG isotypes were used as negative controls. All samples were

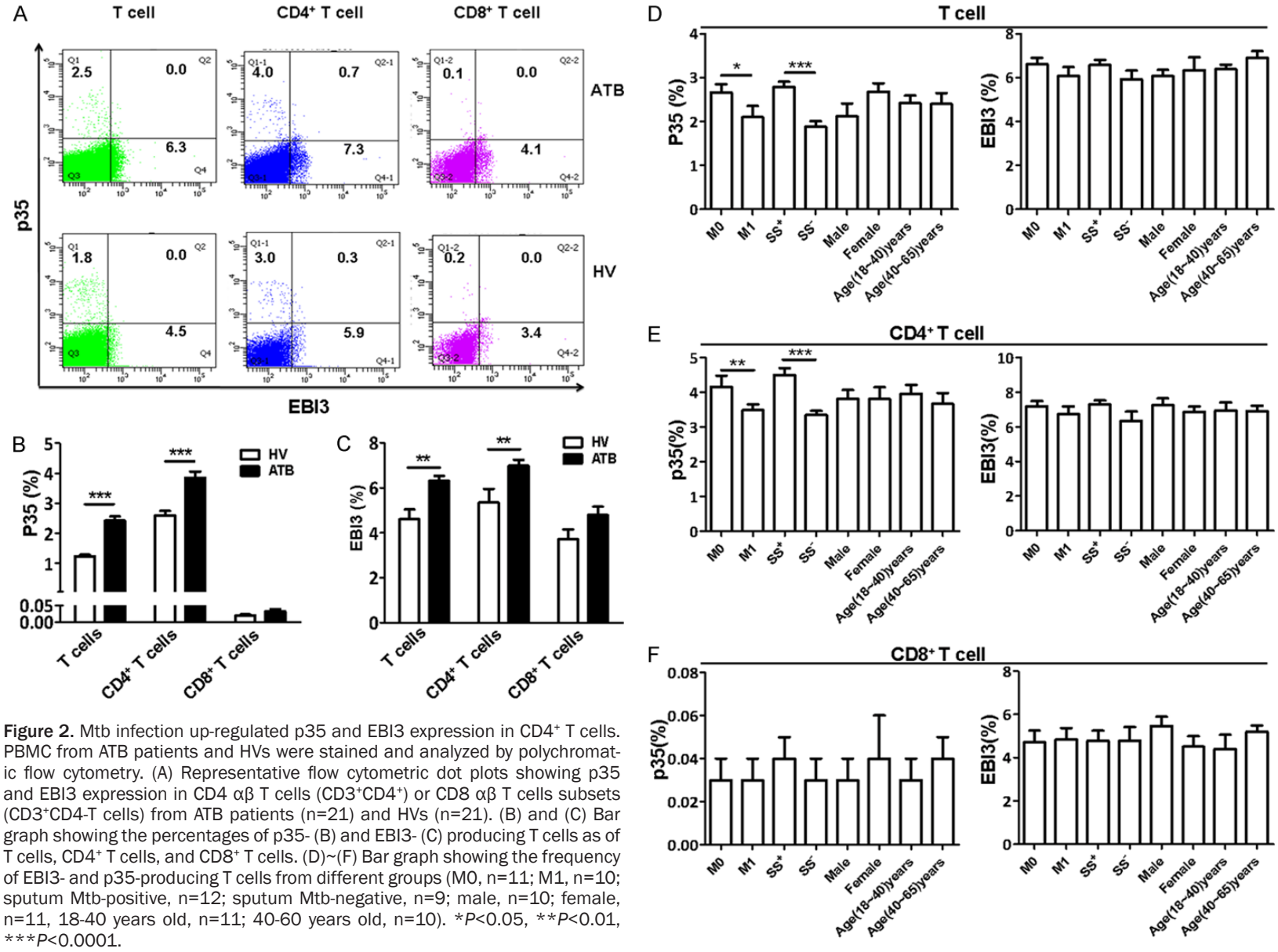

Figure 2. Mtb infection up-regulated p35 and EBI3 expression in CD4+ T cells. PBMC from ATB patients and HVs were stained and analyzed by polychromatic flow cytometry. (A) Representative flow cytometric dot plots showing p35 and EBI3 expression in CD4 αβ T cells ($CD3^+CD4^+$) or CD8 αβ T cells subsets ($CD3^+CD4$-T cells) from ATB patients (n=21) and HVs (n=21). (B) and (C) Bar graph showing the percentages of p35- (B) and EBI3- (C) producing T cells as of T cells, $CD4^+$ T cells, and $CD8^+$ T cells. (D)~(F) Bar graph showing the frequency of EBI3- and p35-producing T cells from different groups (M0, n=11; M1, n=10; sputum Mtb-positive, n=12; sputum Mtb-negative, n=9; male, n=10; female, n=11, 18-40 years old, n=11; 40-60 years old, n=10). *P<0.05, **P<0.01, ***P<0.0001.

analyzed on a BD FACSCalibur™ II flow cytometer (BD, San Jose, CA, USA).

Statistical analysis

The normality evaluation was first performed to determine whether the data set was well-modeled with a normal distribution. If data passed the normality distribution evaluation, Student's t test was used for 2-tailed comparisons; if data did not pass the normality, a Mann-Whitney U test was employed. Pearson correlation was used to measure the degree of dependency between variables by GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). A *P* value of less than 0.05 (95% confidence interval) was considered as statistically significant, as previously described [42, 43, 52].

Results

ATB patients showed increases in soluble serum IL-35 cytokine and in mRNA expressions of IL-35 units in leukocytes and PBMC

IL-35 has been reported to function as a novel anti-inflammatory cytokine, with potential therapeutic effects on autoimmune disorders (e.g., arthritis) [7, 12, 16], infectious diseases (e.g., salmonella) [16], transplantation (e.g., acute graft-versus-host disease), cancer (e.g., colorectal cancer) [21], and atherosclerosis [13, 44]. To examine if IL-35 is involved in TB infection, we comparatively measure IL-35 levels in ATB patients and healthy controls. Serum samples isolated from 80 ATB patients and 40 HVs were used to measure IL-35 using ELISA. We found a significant increase in serum IL-35 level in patients with ATB (**Figure 1A**). The IL-35 level was particularly high in the patients whose sputum was Mtb-positive and those who started ATD treatment for only 0-4 days (**Figure 1B**).

To reveal IL-35 sources in blood, total RNA was isolated from WBC and PBMC of 30 patients with ATB and 30 HVs, and used to measure expression of IL-35 subunits p35 and EBI3 using qRT-PCR. Interestingly, *p35* and *EBI3* mRNA were up-regulated in both WBC and PBMC from patients with ATB. The relative expression of *EBI3* mRNA, but not *p35* mRNA, in WBC was higher than that in PBMC (**Figure 1C** and **1D**). In addition, sputum Mtb-positive patients expressed higher *p35* and *EBI3* mRNA in WBC and higher *EBI3* mRNA in PBMC than Mtb-negative patients (**Figure 1E** and **1F**). Furthermore, *EBI3* mRNA expression in PBMC appeared be affected by ATD treatment. Patients undergoing ATD treatment for 0-4 days showed a higher *EBI3* mRNA expression than those who were treated for 20 to 40 days (**Figure 1F**). Together, these results suggest that Mtb infection increases IL-35 in both serum and leukocytes.

ATB patients exhibited increases in $p35^+CD4^+$ T cells and $EBI3^+CD4^+$ T cells, but not $p35^+$ $EBI3^+CD4^+$ T cells

αβ T cells play an important role in adaptive immune responses against TB infection. To examine a potential role of IL-35 in αβ T cell response in TB, intracellular expressions of p35 and EBI3 in αβ $CD4^+$ and $CD8^+$ T cells were measured by FCM analysis. Surprisingly, the expression of p35 or EBI3 was elevated in $CD4^+$ T cells but not $CD8^+$ T cells from patients with ATB. The numbers of $p35^+$ $CD8^+$ T cell subset and $p35^+$ $EBI3^+$ T cells were very low in both groups (**Figure 2A-C**). Additionally, p35 expression in T cells, especially in $CD4^+$ T cells, was higher in sputum Mtb-positive patients than that in patients with Mtb-negative sputum (**Figure 2D-F**). Of note, the frequency of $p35^+CD4^+$ T cells was positively correlated to that of $EBI3^+CD4^+$ T cells in patients with ATB but not in HVs (**Figure 3A** and **3B**).

Most $p35^+CD4^+$ T cells and $EBI3^+CD4^+$ T cells express CD25 marker

Given that increased Treg is one of the characteristics in TB infection, we evaluated p35 or EBI3 expression in $CD4^+CD25^+$ T cells to determine whether they possess an inhibitory phenotype. Remarkably, p35 or EBI3 expressions in $CD4^+CD25^+$ T cells were increased in TB patients (**Figure 4A** and **4B**), and the frequency of $p35^+CD4^+CD25^+$ T cells was positively correlated to that of $EBI3^+CD4^+CD25^+$ T cells in patients with ATB (**Figure 4C**). Interestingly, most $p35^+CD4^+$ T cells and $EBI3^+CD4^+$ T cells expressed CD25 (**Figure 4D**). Furthermore, the frequencies of $p35^+CD4^+CD25^+$ T cells and $EBI3^+CD4^+CD25^+$ T cells increased in sputum Mtb-positive ATB patients, when compared with sputum Mtb-negative patients (**Figure 4E**). Also, the frequency of $EBI3^+CD4^+CD25^+$ T cells increased in patients who started to receive ATD treatment for 0-4 days (**Figure 4E**).

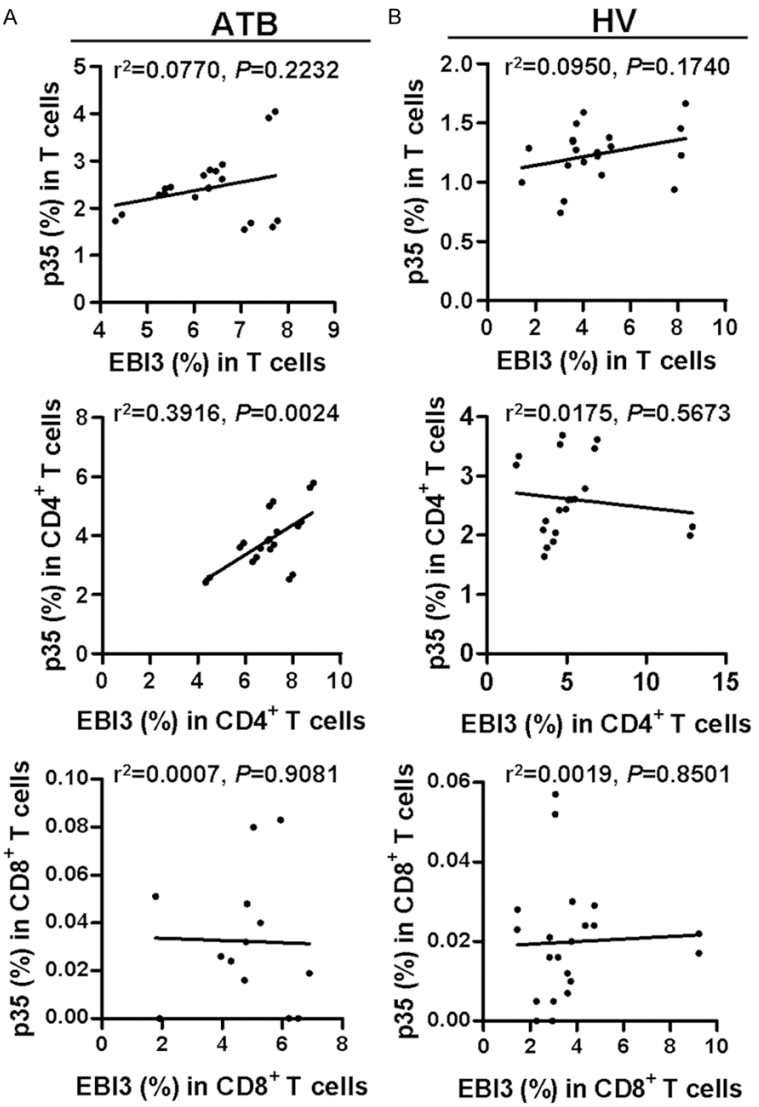

Figure 3. Associations among the percentages of p35 and EBI3 producing T cells within T cells, $CD4^+$ T cells, or $CD8^+$ T cells from ATB patients or HV individuals. Correlation of the percentages of p35- and EBI3-producing T cells within T cells, $CD4^+$ T cells, or $CD8^+$ T cells from ATB patients (A) or HVs (B) were analyzed by Pearson correlation using GraphPad Prism Version 5.0.

Discussion

To our knowledge, we demonstrated for the first time that TB patients exhibited increases in serum IL-35 cytokine and IL-35 subunits (p35 and EBI3) expression in leukocytes. In fact, TB infection is associated with increase in $CD4^+$ T subset expressing single unit of IL-35, but not those expressing double units of IL-35 or $CD8^+$ T cells expressing either of IL-35 units. However, we could not provide evidence that p35- or EBI3-expressing $CD4^+$ T cells was the source of elevated serum IL-35. Importantly, most $CD4^+$ T cells positive for either subunit of IL-35 expressed CD25. These findings suggest the expression of IL-35 subunits may be associated with immune regulation in ATB patients.

IL-35 was first identified as an anti-inflammatory and immunosuppressive cytokine, and was mainly expressed upon stimulation [13, 45]. IL-35 induces the development of iTr35 and is responsible for the suppressive function of regulatory B-cell (Breg) and Treg [7, 12, 46, 47]. Recently, several groups have shown that IL-35 appears to play an immunomodulatory role in a wide variety of disease conditions. In a murine acute graft-versus-host disease (aGVHD) model, IL-35 overexpression suppresses $CD4^+$ T effector activation and induces expansion of $Foxp3^+$ Tregs to modulate graft-versus-leukemia immune response [48]. We and others also found IL-35 could help to promote tumor (e.g., pancreas cancer, colorectal cancer) growth and angiogenesis [11, 21, 49]. A mathematical model developed by Liao, et al. based on previous experimental data also supports these findings [22]. Furthermore, circulating levels of IL-35 are aberrant in patients with multiple sclerosis [50-52], allergic asthma [51, 52], inflammatory bowel disease [53], immune thrombocytopenia [54], portal hypertension [55], atherosclerosis [44], periodontitis [56], preeclampsia [57], hyperimmune-related diseases [20], coronary artery diseases [58], acute myeloid leukemia [17], allergic airways disease [59, 60], chronic hepatitis B [61], and autoimmune and infectious diseases [12]. Here, we found circulating level of IL-35 is also aberrant in patients with ATB and may be involved in the disease pathogenesis.

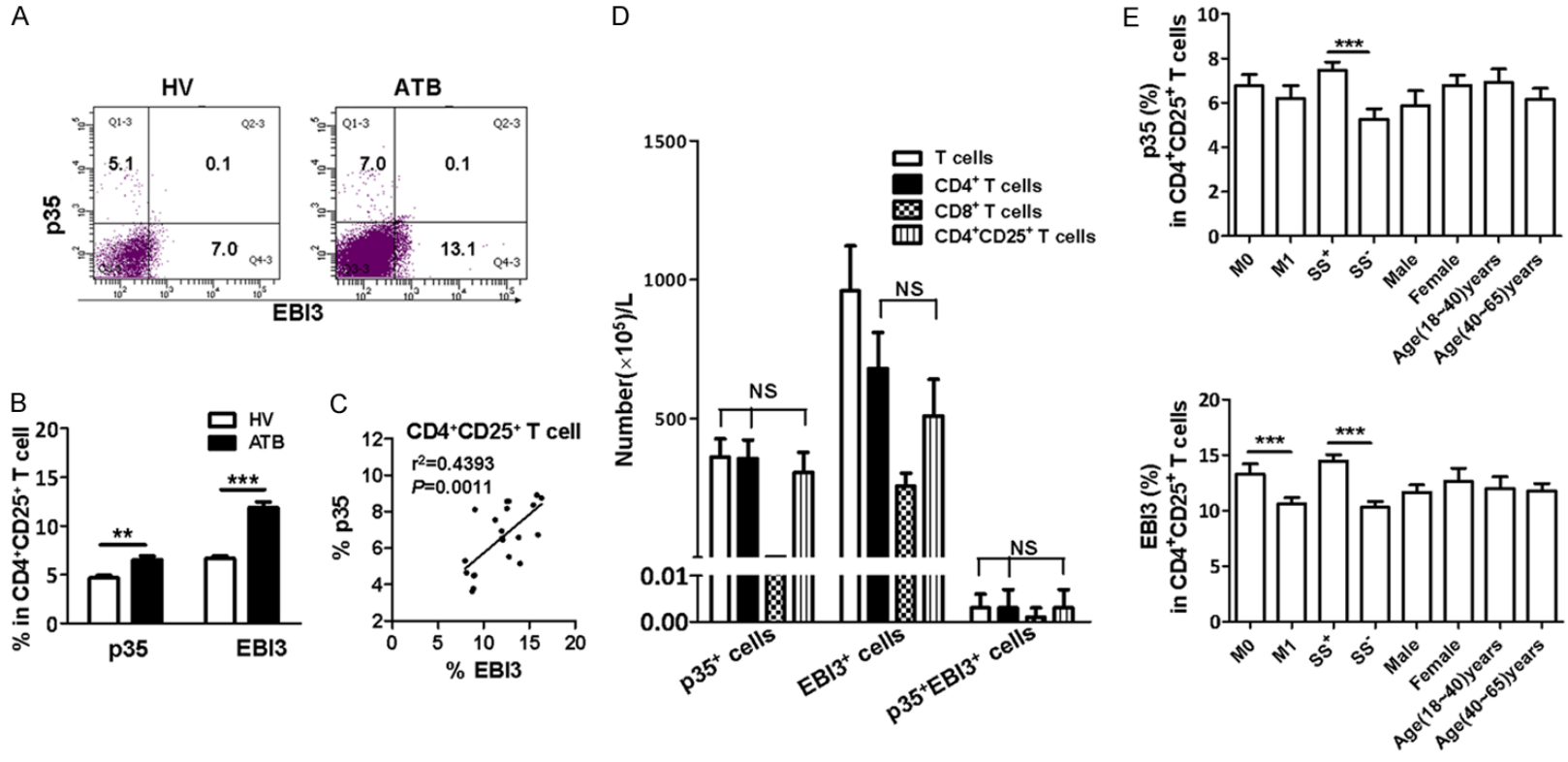

Figure 4. Most p35⁺CD4⁺ T cell and EBI3⁺CD4⁺ T cell expressed CD25. PBMC from ATB patients and HVs were stained and analyzed by polychromatic flow cytometry. (A) Representative flow cytometric dot plots showing p35 and EBI3 expression in CD4⁺CD25⁺ T cells from ATB patients (n=21) or HVs (n=21). (B) Bar graph demonstrating the percentages of p35- and EBI3-producing T cells within CD4⁺CD25⁺ T cells from ATB patients or HVs. (C) Correlation of the percentages of p35- and EBI3-producing cells within CD4⁺CD25⁺ T cells. (D) Bar graph showing the number (10^5/L) of EBI3⁺ T cells, p35⁺ T cells, and p35⁺EBI3⁺ T cells within T cells, CD4⁺ T cells, CD8⁺ T cells, and CD4⁺CD25⁺ T cells, respectively. (E) Bar graph showing the EBI3- and p35-producing CD4⁺CD25⁺ T cells from ATB patients with ATDs treatment for 0-4 days (M0, n=11), and for 15-40 days (M1, n=10), with sputum Mtb-positive (n=12) and sputum Mtb-negative (n=9), different genders (males, n=10; females, n=11), and different ages (18-40 years, n=11; 40-60 years, n=10). *P<0.05, **P<0.01, ***P<0.0001.

It has been suggested that IL-35 is secreted by αβ T cells and IL-35 plays a role in $CD4^+CD25^+$ Treg expansion [39, 41]. Here we found that p35 or EBI3 expressions in $CD4^+$ T cells but not $CD8^+$ T cells were increased in patients with ATB. Interestingly, p35 expression in $CD8^+$ T cells and p35 and EBI3 co-expressions in αβ T cell were very low in both ATB patients and healthy controls. These results suggest that TB is associated with p35 or EBI3 expression in $CD4^+$ T cells but not IL-35 (co-expression of p35 and EBI3) expression in $CD4^+$ T cells. It is noteworthy that most $p35^+CD4^+$ or $EBI3^+CD4^+$ T cells also expressed CD25, suggesting that p35 or EBI3 expression in $CD4^+$ T cells may be associated with regulatory T cell function in TB. It has been shown that p35 or EBI3 expression in $CD4^+$ T cells is important for the immunomodulatory role, as Treg cells lacking either p35 or EBI3 cannot attenuate experimental inflammatory bowel disease in mice [53]. We demonstrate that in patients with ATB, most p35+$CD4^+$ T cells and $EBI3^+CD4^+$ T cells also expressed Treg-related marker CD25. This finding may be relevant to the immunomodulatory role of IL-35 subunits in TB infection.

To examine whether IL-35 expression is associated with pathogenesis or prognosis of patients with ATB, we comparatively evaluate sputum Mtb-positive and -negative patients as well as patients who initiated ATD treatment for 0 to 4 days and those treated for 15 to 40 days. It is noted that patients with Mtb-positive sputum and patients treated for 0 to 4 days displayed higher levels of circulating IL-35, *p35* and *EBI3* mRNA in leukocytes, and p35 or EBI3 expression in $CD4^+CD25^+$ T cells. These results suggest that serum IL-35 level may have a potential prognostic value for patients with ATB.

In summary, we provided previously unreported data of IL-35 in patients with ATB. TB infection drove increases in serum IL-35 cytokine and p35 or EBI3 expression in $CD4^+CD25^+$ T cells. Consistently, bacterial control by ATD treatment reduces serum IL-35 level and p35 or EBI3 expression. $p35^+$ $CD4^+$ or $EBI3^+$ $CD4^+$ T cells express Treg-related marker CD25. Our findings may be important in understanding immune pathogenesis of TB. IL-35 in the blood may potentially serve as a biomarker for immune status and prognosis in TB.

Acknowledgements

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Disclosure of conflict of interest

None.

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