Effective expansion of forkhead box P3$^+$ regulatory T cells via early secreted antigenic target 6 and antigen 85 complex B from *Mycobacterium tuberculosis*

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Abstract. The expansion of CD4$^+$ CD25$^+$ forkhead box (FOX)P3$^+$ regulatory T (T_{reg}) cells has been observed in patients with *Mycobacterium* (*M.*) *tuberculosis*; however, the mechanism of expansion remains to be elucidated. The aim of the present study was to examine the role of the early secreted antigenic target 6(ESAT-6) and antigen 85 complex B (Ag85B) from *M. tuberculosis* on T_{reg} cell expansion. To investigate the sensitivity of peripheral blood cultures to the *M. tuberculosis* ESAT-6 and Ag85B antigens, the proportion of circulating CD4$^+$ CD25$^+$ FOXP3$^+$ T_{reg} cells was determined using flow cytometry and the levels of FOXP3 mRNA were determined using reverse transcription quantitative polymerase chain reaction. The mRNA levels of FOXP3 and the proportion of circulating CD4$^+$ CD25$^+$ FOXP3$^+$ T_{reg} cells were increased in multiplicitous drug-resistant tuberculosis patients compared with those in healthy controls and patients with latent tuberculosis (TB) infection (LTBI) ($P<0.001$). The mycobacterial antigens ESAT-6 and Ag85B increased the expansion of the CD4$^+$ CD25$^+$ FOXP3$^+$ T_{reg} cells and the mRNA levels of FOXP3 in healthy controls and LTBI patients compared with the effect of Bacillus Calmette-Guerin ($P<0.05$). Additionally, the mRNA levels of FOXP3 were elevated in the LTBI patients following stimulations with the mycobacterial antigens ($P=0.012$). Therefore, the *M. tuberculosis* antigens ESAT-6 and Ag85B induced CD4$^+$ CD25$^+$ FOXP3$^+$ T_{reg}-cell expansion, particularly in patients with LTBI. These findings indicated that CD4$^+$ CD25$^+$ FOXP3$^+$ T_{reg} cells may have a primary role in the failure of the host immune system to eradicate *M. tuberculosis*.

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Introduction

Mycobacterium (*M.*) *tuberculosis*, the causative agent of tuberculosis (TB), was responsible for ~8.7 million novel cases of TB worldwide in 2007 (1). Tuberculosis is considered to be a sustained immune response, which is induced by chronic, persistent antigen stimulation, results in immune suppression and generally fails to eradicate the *M. tuberculosis* infection in part. The host response, which involves several branches of the cellular immune system, predominantly consists of *M. tuberculosis*-specific T helper type 1 (Th)1 interferon (IFN)-γ-secreting CD4$^+$ and CD8$^+$ effector T cells (2-4). CD4$^+$ CD25$^+$ regulatory T cells (T_{reg}), expressing the lineage marker forkhead box P3 (FOXP3), are important in controlling the immune response and in maintaining T-cell homeostasis (5,6). Several previous studies have found that the number of FOXP3$^+$ T_{reg} cells is increased in patients with active TB and are expanded at sites of active disease, where containment of inflammation and immune-mediated pathology is required most (7,8). A previous study by our group demonstrated that elevated levels of FOXP3$^+$ T_{reg} cells decreased following pulmonary resection in patients with pulmonary multiplicitous drug resistant tuberculosis (MDR-TB) (9). This suggested that FOXP3$^+$ cells may be essential during TB development; however, the mechanism underlying the increased levels of FOXP3$^+$ cells in active TB patients remains to be elucidated. A variety of mycobacterial proteins and lipids are secreted into the cytoplasm of *M. tuberculosis*-infected macrophages, where they may be important in inhibiting the ability of macrophages to eradicate the bacterium (10-12). *M. tuberculosis* infection

or treatment with mycobacterial proteins alone induces the secretion of several cytokines, including interleukin (IL)-1, -2, -10 and -12 and tumor necrosis factor-alpha (TNF-α), by monocytes/macrophages (13-15). The 6 kDa early secreted antigenic target 6 (ESAT-6) is among the proteins secreted by *M. tuberculosis* and is encoded by region of difference 1 (RD1). Comparative genomics of the *M. tuberculosis* family have revealed that overlapping portions of RD1 are absent from the attenuated or avirulent strain *M. bovis* Bacillus Calmette-Guerin (BCG) and environmental mycobacteria (16,17). Antigen 85 complex B (Ag85B), a fibronectin-binding protein with mycolyl transferase activity, is the major secretory protein in actively replicating *M. tuberculosis* (18). Ag85B is highly immunogenic, as demonstrated by the ease of detection of specific humoral and cell-mediated immune responses in latently and actively infected TB patients (19,20).

Based on the immunogenicity of proteins secreted by *M. tuberculosis*, the present study hypothesized that T_{reg} elevation may be induced by these proteins, including ESAT-6 and Ag85B, and that T_{reg} activation may be important in the failure of the host immune response to eradicate *M. tuberculosis*.

Materials and methods

Study population. The study procedure was approved by the ethics committee of Shantou University Medical College (Shantou, China). Written informed consent was obtained from all individuals involved in the present study. Peripheral blood was obtained from 18 patients (10 males) with a median age of 51 years (range, 16-79 years), who had been diagnosed with MDR-TB at The Third People's Hospital of Shantou City (Shantou, China). The TB diagnosis was based on smear positivity and/or *M. tuberculosis* culture. The indication (therapeutic vs. diagnostic) and the main clinical pathologies are listed in Table I and the bacterial susceptibility assessment results are shown in Table II. A total of 18 uninfected volunteers (10 males) with a median age of 47 years (range, 25-73 years) were also included, who were tuberculin skin test-negative, had not been vaccinated and had no known exposure to *M. tuberculosis*. In addition, 18 patients with latent tuberculosis (TB) infection (LTBI; 9 males) with a median age of 35 years (range, 23-53 years) were selected according to the following recommended criteria (21): Tuberculin skin test scores between 2+ and 3+, including a risk-stratified induration 72 h after intradermal injection of 2 units tuberculin (PPD-RT23 SST; Statens Serum Institute, Copenhagen, Denmark), chest radiographs were normal and the patients exhibited no clinical signs of active TB. All individuals were human immunodeficiency virus seronegative. No patients suffered from an immunodepressive illness or received immunosuppressive treatment.

Cell preparation and culture. Heparinized peripheral blood (600 µl) was suspended in 200 µl RPMI-1640 (HyClone, Thermo Fisher Scientific, Logan, UT, USA) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal calf serum (Invitrogen Life Technologies, Carlsbad, CA, USA), termed complete medium, and was stimulated *in vitro* with recombinant ESAT-6 (cat. no. PRO-291; ProSpec-Tany, TechnoGene, Ltd., Rehovot, Israel), Ag85B (cat. no. PRO-589; ProSpec-Tany, Israel) or BCG (dead strain; cat. no 2009110102; Shanghai Institute of Biological Products, Shanghai, China). The final culture concentration of the secretory proteins was 4 µg/ml. The positive control used was phytohemagglutinin (PHA) and pure medium was used for the negative control. The blood was then incubated at 37˚C in 5% CO_2 for 72 h to separate the culture supernatant fluid from the cells. In the blood samples from six individuals (n=2 from each group) , multiple different antigen concentrations (0, 1 and 4 µg/ml) were used.

Flow cytometry. The whole blood was aliquoted (100 µl/tube) with 20 µl of the appropriate test antibody (1:5) or respective isotype control for three staining procedures: Fluorescein isothiocyanate (FITC), monoclonal anti-FOXP3 (clone PCH101; cat.no. 11-4766; eBioscience, San Diego, CA, USA); phycoerythrin peridinin chlorophyll protein (PerCP), mouse monoclonal anti-CD4 (clone RPA-T4; cat.no. 300528; Biolegend, San Diego, CA, USA); and phycoerythrin (PE), monoclonal anti-CD25 (clone B1.49.9; cat. no. IM0479u, Beckman Coulter, Los Angeles, CA, USA). The following isotype control antibodies were used: FITC, rat monoclonal immunoglobulin (Ig)G2b (clone κ; cat. no. 11-4031; eBioscience); PerCP, mouse monoclonal IgG1 (clone MOPC-21; cat.no. 400145; Biolegend); and PE, mouse monoclonal IgG1 (clone 679.1 Mc7; cat.no. IM06700u; Beckman Coulter). Following surface staining for 30 min in the dark at room temperature, the erythrocytes were lysed and the cells were fix with ImmunoPrep Reagents Systmem (PN7546946; Beckman Coulter) using a Q-prep Immunology workstation (Beckman Coulter). The surface-stained cells then underwent intracellular FOXP3 staining using the anti-FOXP3 staining kit according to the manufacturer's instructions.

The listmode data were acquired using an Epics XL flow cytometer (Beckman Coulter) and were analyzed using EXPO32 ADC analysis system (Expo 32v1.2 Analysis 1.1C; Beckman Coulter). The lymphocyte gate was generated by use of forward and side-angle scattered light window leukogating to analyze the CD4 and CD25 cell-surface antigens and to determine the proportion of T_{reg} cells. The $CD4^+$ T cells were gated by plotting forward, vs. side scatter to analyze FOXP3.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). For RT-qPCR analysis of the mRNA expression of the forkhead transcription factor FOXP3, total RNA was isolated from leukocytes using a total RNA extraction kit for mammalian RNA (TRIzol reagent; cat.no. 15596-026; Invitrogen Life Technologies) according to the manufacturer's instructions. RT-PCR was performed in duplicate in a total volume of 20 µl in a LightCycler (Prism® 7500; Applied Biosystems Life Technologies, Foster City, CA, USA), according to the manufacturer's instructions, using the following cycling conditions: 30 sec at 95˚C and 5 sec at 95˚C for 40 cycles followed by 31 sec at 60˚C. The normalized expression data were obtained by dividing the relative expression level for each sample by the relative expression level of β-actin for the same sample. The primer sequences used were as follows: β-actin forward, 5'-GGCGGCACCACCATGTACCCT-3' and reverse, 5'-AGGGGCCGGACTCGTCATACT-3'; and FOXP3 forward, 5'-ACCTGGAAGAACGCCATC-3' and reverse, 5'-TGTTCGTCCATCCTCCTTTC-3'. The results (FOXP3 / β-actin $=2^{-(CTFOXP3 - CTβ\text{-}actin)}$) were presented as the relative expression level for each gene (Fig. 1).

Table I. Diagnostic indicators and main clinical therapeutical pathologic features.

Subject	Gender	Age	Chest CT	TST	Sputum		Clinical features			Antitubercular drug therapy (years)
					Smear	Culture	Fever (˚C)	Cough (years)	Hemoptysis	
1	M	32	LLL cavity concurrent infection; both side pleural thickening accretio	2+	3+	+	38.0-40.0	3 Intermittently	–	2.5
2	M	79	Both pulmonary tuberculosis, concurrent crinosity cavity form Both U. local pleural thickening accretio	3+	1+	+	37.5-39.0	10 Intermittently	+	9.5
3	M	48	L.apex of lung tuberculosis; RLL cavity concurrent aspergilloma	3+	1+	+	38.5-39.5	4 Intermittently	–	3
4	F	59	Both pulmonary tuberculosis; both side pleural thickening accretio	3+	1+	+	37.2-39.5	2.5 Intermittently	–	2
5	M	69	L.apex of lung tuberculosis LUL cavity concurrent aspergilloma	2+	3+	+	37.0-38.5	3 Intermittently	–	2.5
6	F	52	RUL cavity concurrent aspergilloma; both U. local pleural thickening accretio	2+	Q	+	37.6-39.2	3.8 Intermittently	+	3.5
7	F	42	Both lobus superior pulmonis tuberculosis; Both side pleural thickening accretio	2+	1+	+	37.0-39.0	5 Intermittently	–	4.5
8	F	34	R.apex of lung tuberculosis RUL cavity concurrent aspergilloma	3+	1+	+	37.8-38.9	2 Intermittently	–	1.5
9	M	29	LUL cavity concurrent aspergilloma; RCW abscess	3+	Q	+	37.0-39.5	2 Intermittently	–	2
10	M	38	L.apex of lung tuberculosis LLL tuberculoma	3+	Q	+	37.5-39.0	3 Intermittently	–	2.5

Table I. (Continued).

Subject	Gender	Age	Chest CT	TST	Sputum		Clinical features			Antitubercular drug therapy (years)
					Smear	Culture	Fever (°C)	Cough (years)	Hemoptysis	
11	M	72	RLL cavity concurrent infection; both side pleural thickening accretio	2+	3+	+	37.2-39.0	7 Intermittently	–	7
12	F	59	Both pulmonary tuberculosis; RUL cavity concurrent infection	2+	2+	+	37.1-38.8	4 Intermittently	+	3.5
13	M	67	LUL cavity concurrent aspergilloma; LCW abscess	2+	1+	+	37.1-39.2	6 Intermittently	+	6
14	M	16	Both lobus superior pulmonis tuberculosis; both side pleural; thickening accretio	3+	2+	+	37.5-39.0	1 Intermittently	–	0.5
15	F	46	R. apex of lung tuberculosis; RLL cavity concurrent aspergilloma	3+	3+	+	37.2-39.0	3 Intermittently	-	3
16	F	72	Both pulmonary tuberculosis; RUL cavity concurrent aspergilloma	2+	Q	+	37.0-38.8	6 Intermittently	+	5.5
17	F	59	Both pulmonary tuberculosis; both side pleural thickening accretio	3+	1+	+	37.4-39.2	3 Intermittently	-	3
18	M	43	Both pulmonary tuberculosis; RUL cavity concurrent aspergilloma; RCW abscess	2+	1+	+	37.1-39.5	5 Intermittently	-	4.5

CT, computerized tomographic scanning; TST, tuberculin skin test; LLL, left lower lobe; RLL, right lower lobe; LUL, left upper lobe; RUL, right upper lobe; RCW, right chest wall; LCW, left chest wall; RL, right lobe; LL, left lobe; +, positive; -, negative; TST, size of red spot 72 h following injection of purified protein derivative tuberculin (-, <5 mm; 1+, 5-9 mm; 2+, 10-19 mm; 3+, ≥20 mm); M, male; F, female.

Table II. Preoperative bacterial susceptibility assessment results of patients.

			Susceptibility assessment results										Strain appraisement	
Subject	Gender	Age	INH	RFP	SM	EMB	RFT	CPM	OFX	Th1321	KM	PAS	PNB	TCH
1	M	32	R	R	S	S	R	S	R	S	S	S	S	R
2	M	79	R	R	S	R	R	S	R	S	S	S	S	R
3	M	48	R	R	S	S	R	S	S	S	S	S	S	R
4	F	59	R	R	R	S	R	R	R	S	R	S	S	R
5	M	69	R	R	R	R	R	S	R	S	S	S	S	R
6	F	52	R	R	R	S	R	S	R	S	S	S	S	R
7	F	42	S	S	R	S	S	S	R	S	S	S	S	R
8	F	34	R	R	R	R	R	R	R	S	S	S	S	R
9	M	29	R	R	R	S	R	S	R	R	R	S	S	R
10	M	38	R	R	R	S	R	S	S	S	S	R	S	R
11	M	72	R	R	R	R	R	R	R	R	R	R	R	R
12	F	59	R	R	R	R	R	R	R	R	R	R	R	R
13	M	67	R	R	R	R	R	S	R	S	S	S	S	R
14	M	16	S	R	R	S	R	R	R	S	R	S	S	R
15	F	46	R	R	S	S	R	R	R	S	R	S	S	R
16	F	72	R	R	S	R	R	S	R	S	S	S	S	R
17	F	59	S	R	R	S	R	S	R	R	R	S	S	R
18	M	43	R	R	R	S	R	S	R	R	R	S	S	R

INH, isonicotinyl hydrazide; RFP, rifampicin; SM, streptomycin; EM,: ethambutol; RFT, rifapentin; CPM, capreomycin; OFX, levofloxacin; Th1321, prothionamide; KM, kanamycin; PAS, para-aminosalicylic acid; PNB, *p*-nitrobenzoic acid; TCH, 2-thiophenecarboxylic acid hydrazide; R, resistant; S, sensitive.

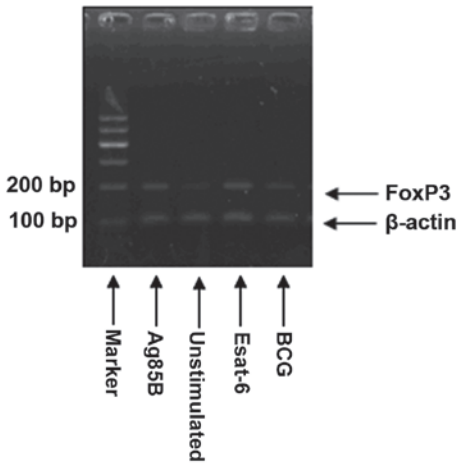

Figure 1. Representative polyacrylamide gel electrophoresis of the FOXP3 fragment (196 bp) in the blood from latent TB patients prior to and following stimulation with different antigens (4 μg/ml) for 72 h. FOXP3, forkhead box P3; Ag85B, antigen 85 complex B; Esat-6, early secreted antigenic target 6; BCG, Bacillus Calmette-Guerin.

Statistical analysis. All values are expressed as the mean ± standard deviation and all results were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Differences in the mean values between the patients and the controls were analyzed by one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Percentage of CD4$^+$ CD25$^+$ FOXP3$^+$ T_{reg} cells and the mRNA expression levels of FOXP3 in the peripheral blood of MDR-TB patients are higher than those in uninfected controls (CON) and LTBI patients. The percentage of CD4$^+$ CD25$^+$ FOXP3$^+$ T_{reg} cells in the MDR-TB patients were significantly increased (P<0.01) compared with those in the controls (CON) and the LTBI patients, whereas no significant difference (P=0.093) was observed between those in the CON and LTBI patients (MDR-TB, 0.93±0.57%; CON, 0.24±0.14% and LTBI, 0.52±0.35%). The mRNA expression levels of FOXP3 were increased in the MDR-TB patients (0.0093±0.0027) compared with those in the CON (0.0043±0.0014) and LTBI (0.0066±0.0017) groups (P<0.05); however, the levels were similar in the LTBI and control patients (P=0.080) as shown in Fig. 2.

Proportion of CD4$^+$ CD25$^+$ FOXP3$^+$ T_{reg} cells and the mRNA expression levels of FOXP3 increase depending on the concentration of ESAT-6, Ag85B or BCG. Following cell culture with different concentrations (0, 1 and 4 μg/ml) of ESAT-6, Ag85B or BCG, the proportion of CD4$^+$ CD25$^+$ FOXP3$^+$ T_{reg} cells and the mRNA levels of FOXP3 increased with increasing concentrations of the mycobacterial antigens (Fig. 3). However, due to the small sample number, no statistical analyses were performed.

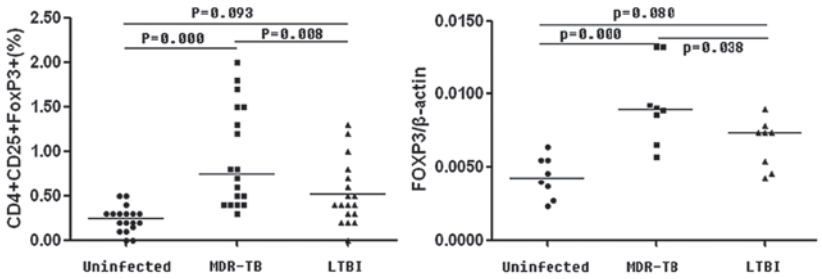

Figure 2. Percentage of $CD4^+$ $CD25^+$ $FOXP3^+$ cells in the total lymphocytes from uninfected (n=18), MDR-TB (n=18) and LTBI (n=18) patients and mRNA expression levels of FOXP3 in the peripheral blood of uninfected (n=8), MDR-TB (n=8) and LTBI (n=8) patients prior to culture with antigens. MDR-TB, multiplicitous drug resistant tuberculosis; LTBI, latent TB; FOXP3, forkhead box P3.

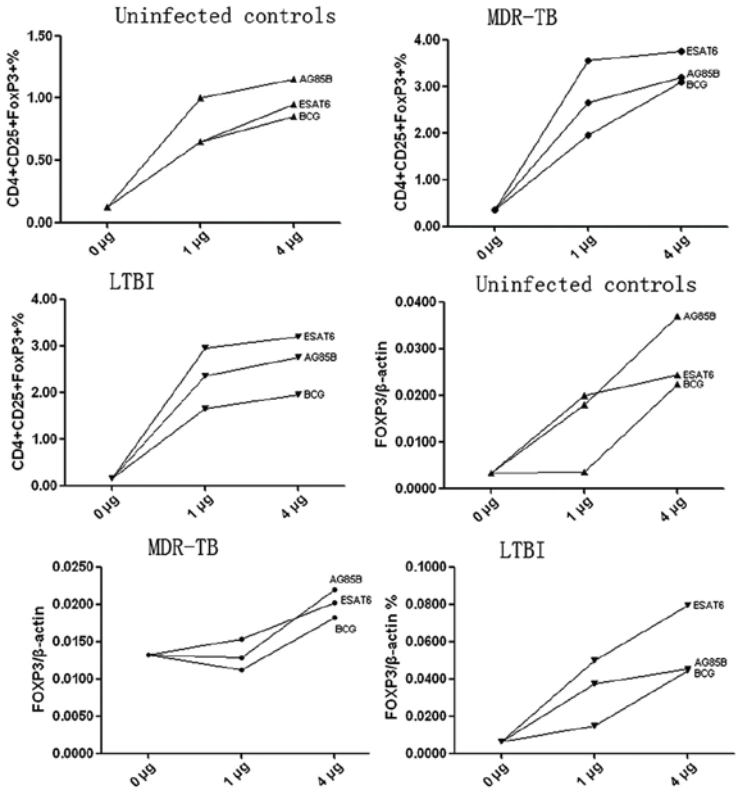

Figure 3. A total of six samples from the MDR-TB (n=2), LTBI (n=2) and uninfected controls (n=2) were stimulated with different concentrations of mycobacterial antigens. The proportion of $CD4^+$ $CD25^+$ $FOXP3^+$ cells among the total lymphocytes and the mRNA expression levels of FOXP3 increased with increasing concentrations of the mycobacterial antigens. MDR-TB, multiplicitous drug resistant tuberculosis; LTBI, latent TB; Ag85B, antigen 85 complex B; Esat-6, early secreted antigenic target 6; BCG, Bacillus Calmette-Guerin; FOXP3, forkhead box P3.

In healthy controls, the proportion of $CD4^+$ $CD25^+$ $FOXP3^+$ T_{reg} cells and the mRNA expression levels of FOXP3 increases in vitro by ESAT-6 and Ag85B. The proportion of $CD4^+$ $CD25^+$ $FOXP3^+$ T_{reg} cells increased by 2.7- and 3.2-fold following stimulation with ESAT-6 or Ag85B, respectively (ESAT-6, 0.644±0.22%; Ag85B, 0.76±0.33% and unstimulated, 0.24±0.14%; P<0.001), whereas BCG did not significantly increase the percentage of $CD4^+$ $CD25^+$ $FOXP3^+$ T_{reg} cells (P=0.104) (Fig. 4A and B). Similar results were found for the mRNA expression levels of FOXP3 following stimulation with ESAT-6 (0.0251±0.0052; P<0.01), Ag85B (0.0261±0.0115; P<0.01) or BCG (0.0174±0.0193; P=0.130) compared with those in the unstimulated controls (0.0043±0.0014). As with the expansion of the $CD4^+$ $CD25^+$ $FOXP3^+$ cells, the highest FOXP3 induction was observed following stimulation with Ag85B; however, the difference when compared with ESAT-6 or BCG was not statistically significant (P>0.05) (Fig. 4A and B).

ESAT-6 and Ag85B can expand the proportion of $CD4^+$ $CD25^+$ $FOXP3^+$ T_{reg} cells and elevate the mRNA expression levels of FOXP3 in vitro in MDR-TB. The proportion of $CD4^+$ $CD25^+$ $FOXP3^+$ T_{reg} cells increased following stimulation with ESAT-6 (3.04±0.80%) and Ag85B (2.52±0.46%) in MDR-TB (BCG, 2.19±0.70%; unstimulated, 0.93±0.57%) (Fig. 4C and D). The mRNA expression levels of FOXP3 increased 2.2-fold following Ag85B stimulation (0.0205±0.0087) compared with those in the unstimulated control (0.0093±0.0027). No statistically significant changes were observed in the mRNA expression levels of FOXP3 following stimulation with ESAT-6 (0.0182±0.0062) or BCG (0.0113±0.0079) (Fig. 4C and D).

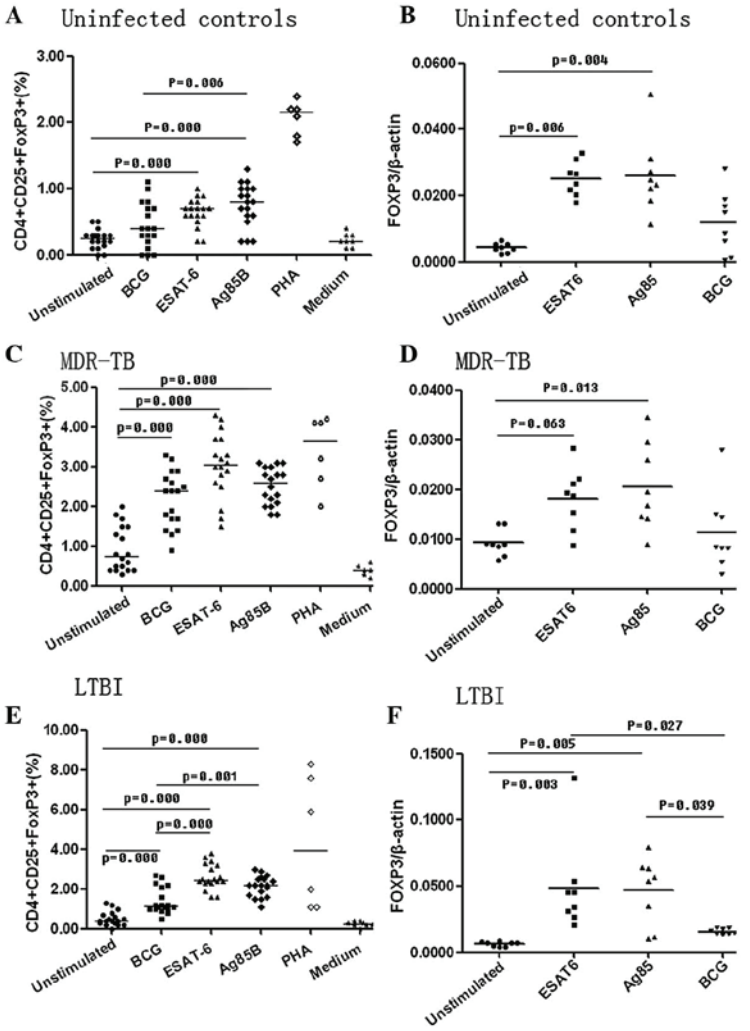

Figure 4. Proportion of $CD4^+$ $CD25^+$ $FOXP3^+$ cells among total lymphocytes in the unstimulated (n=18), BCG (n=18), ESAT-6 (n=18) and Ag85B (n=18) and the mRNA expression level of FOXP3 in the peripheral blood of unstimulated (n=8), BCG(n=8), ESAT-6 (n=8) and Ag85B (n=8) patients prior to and following stimulation with different antigens (4 μg/ml) for 72 h. The positive control was PHA (n=6) and the negative control was medium (n=8). (A and B) show the results in uninfected controls; (C and D) show the results in the MDR-TB patients; (E and F) show the results in the LTBI patients. LTBI, latent tuberculosis infection; Ag85B, antigen 85 complex B; Esat-6, early secreted antigenic target 6; BCG, Bacillus Calmette-Guerin; PHA, phytohemagglutinin; FOXP3, forkhead box P3.

ESAT-6 and Ag85B expand the proportion of $CD4^+$ $CD25^+$ $FOXP3^+$ T_{reg} cells and elevate the mRNA expression levels of FOXP3 in vitro in LTBI. Following cell culture for 72 h in the presence of ESAT-6, Ag85B or BCG, the proportion of $CD4^+$ $CD25^+$ $FOXP3^+$ T_{reg} cells and the mRNA expression levels of FOXP3 increased significantly. There was a significant difference in the proportion of $CD4^+$ $CD25^+$ $FOXP3^+$ T_{reg} cells between the simulated and unstimulated groups (P<0.001) (Fig. 4E and F). Increases of 5.0- and 4.2-fold were obtained following stimulation with ESAT-6 (2.62±0.64) or Ag85B (2.16±0.52%), respectively (BCG 1.42±0.66%; unstimulated 0.52±0.35%). As shown in Fig. 4E and F, the mRNA expression levels of FOXP3 increased following stimulation with ESAT-6 (0.0486±0.0353) or Ag85B (0.0468±0.0254) only (unstimulated 0.0066±0.0017). No statistically significant difference in mRNA levels was observed between the BCG-stimulated group and the unstimulated control (P=0.830). When comparing BCG, ESAT-6 and Ag85B, the effects of ESAT6 and Ag85B were greater compared with those of BCG (P<0.05), although no statistically significant difference was observed between ESAT-6 and Ag85B (P=0.998) (Fig. 4E and F).

Increased FOXP3 mRNA levels and proportion of $CD4^+$ $CD25^+$ $FOXP3^+$ cells following stimulation with ESAT-6, Ag85B or BCG. In the LTBI group, culture with ESAT-6 resulted in a six-fold increase in the mRNA expression of FOXP3 (Fig. 5A), which was higher compared with those in the MDR-TB group (P=0.012); however, there was no significant difference from levels in healthy controls (P=0.151). In the cultures stimulated with Ag85B, the mRNA expression of FOXP3 increased six-fold in the LTBI group (Fig. 5C), which was higher compared with that in the MDR-TB group (P=0.007), but not significantly different from that in the healthy controls (P=0.102). BCG increased the mRNA expression of FOXP3 in the healthy controls only (MDR-TB, 0.22-fold; control, three-fold, LTBI, 1.4-fold; Fig. 5E). The proportion of $CD4^+$ $CD25^+$ $FOXP3^+$ T_{reg} cells increased following stimulation and the increment was higher in the MDT-TB and LTBI groups

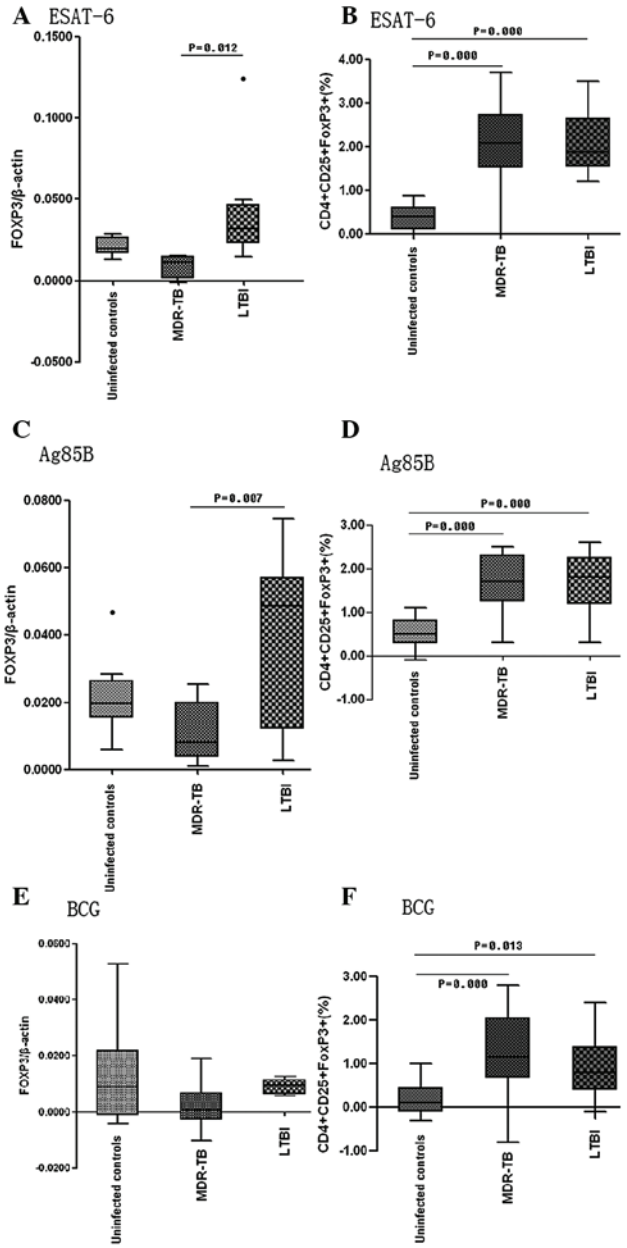

Figure 5. Comparisons between the different groups stimulated with (A and B) ESAT-6, (C and D) Ag85B or (E and F) BCG indicated that the increased incidence of $CD4^+ CD25^+ FOXP3^+$ cells was the highest in the MDR-TB patients (A, C and E) and the increase in the mRNA levels of FOXP3 was highest in the LTBI patients (B, D and F). MDR-TB, multiplicitous drug resistant tuberculosis; LTBI, latent TB; Ag85B, antigen 85 complex B; Esat-6, early secreted antigenic target 6; BCG, Bacillus Calmette-Guerin; FOP3, forkhead box P3.

following culture with ESAT-6, Ag85B and BCG compared with that in the healthy controls ($P<0.01$; Fig. 5B, D and F).

Discussion

Tuberculosis remains to be a challenging medical, social, and economic problem worldwide, especially in the third world (1). The host response to infection with *M. tuberculosis* involves the cellular immune system, specifically the Th1-type interferon-γ-secreting $CD4^+$ and $CD8^+$ effector T cells (4). This response assists in limiting bacterial replication and dissemination *in vivo* and results in important immunopathological features, including inflammation; however, it does not eradicate the infection (22). The present study hypothesized that the immune system may possess regulatory mechanisms that suppress the response to persistent antigens. It has been previously reported that patients with TB have a high proportion of circulating T_{reg} cells of the $CD4^+ CD25^+ FOXP3^+$ phenotype compared with patients with LTBI or uninfected controls (7,8). A previous study by our group revealed that the elevated numbers of $CD4^+ CD25^+ FOXP3^+$ cells observed in MDR-TB were found to decrease following removal of the *M. tuberculosis* burden by pulmonary resection (9), which demonstrated that the increase in $FOXP3^+$ T_{reg} cells is a potential mechanism by which *M. tuberculosis* evades immune eradication. In individuals with tuberculosis, these cells have been observed to suppress the T-cell response to mycobacterial antigens, whereas the $CD4^+ CD25^+ FOXP3^+$ cells from non-healthy individuals do not suppress the secretion of IFN-γ induced by protective mycobacterial antigens (8,23). The increased levels of functional $CD4^+ CD25^+ FOXP3^+$ T_{reg} cells in the peripheral blood of patients with TB may be either caused by or a result of the disease, which suggests that they are either generated or expanded during latent infection prior to the onset of disease. Although $CD4^+ CD25^+ FOXP3^+$ T_{reg} cells are naturally generated in the thymus (24), they are induced in the periphery in mice (25) and humans (26), which suggests that peripheral T_{reg} cells may arise from antigenic challenge during the immune response.

In the present study, intracellular staining combined with flow cytometry and RT-qPCR analysis was performed to detect the expression of the most accurate available T_{reg} markers, $CD25^+$ and FOXP3. The results demonstrated that there was a higher frequency of $CD4^+ CD25^+ FOXP3^+$ T_{reg} cells and elevated mRNA levels of FOXP3 in MDR-TB patients compared with the LTBI patients or the uninfected controls. No difference was observed between the LTBI patients and the uninfected controls prior to stimulation with ESAT-6 and Ag85B, the proteins secreted by *M. tuberculosis*, *in vitro*. However, when the peripheral blood from individual patients was cultured with either ESAT-6 or Ag85B *in vitro*, the results demonstrated that circulating $CD4^+ CD25^+ FOXP3^+$ T_{reg} cells were expanded by stimulation with ESAT-6 or Ag85B. This observation supported the hypothesis of the present study that the elevation of circulating $CD4^+ CD25^+ FOXP3^+$ T_{reg} cells in active TB patients was induced by the proteins secreted by *M. tuberculosis*, which may ultimately decrease anti-tuberculosis immunity and may be partly responsible for failure of the host to eradicate *M. tuberculosis*. Comparison of ESAT-6, Ag85B and BCG revealed that $CD4^+ CD25^+ FOXP3^+$ T_{reg} expansion and elevation of mRNA levels of FOXP3 was caused by ESAT-6 or Ag85B to a greater extent than by BCG. In addition, exposure to BCG did not increase the proportion of $CD4^+ CD25^+ FOXP3^+$ T_{reg} cells or the mRNA expression levels of FOXP3, which suggested that the higher levels of circulating $CD4^+ CD25^+ FOXP3^+$ T_{reg} cells in active TB patients may be associated with *M. tuberculosis* pathogenicity.

In the present study, the highest levels of elevated FOXP3 mRNA were observed in the LTBI patients compared with those in the uninfected controls or the MDR-TB patients. Therefore, the peripheral $CD4^+ CD25^+ FOXP3^+$ T_{reg} cells may be involved in the early stages of TB pathogenesis. They may be generated and expanded from the peripheral blood mononuclear cells in LTBI and have been demonstrated to

depress cellular immune responses in non-anergic patients with pulmonary TB (7,8). These results indicated that, in the majority of *M. tuberculosis* infections, a cell-mediated protective immune response controls the pathogen over several years and often for a lifetime without clinical consequences. Upon exposure of the infected individual to mycobacterial antigens, latent infection, re-exposure to *M. tuberculosis* or post-exposure BCG vaccination causes marked generation and expansion of $CD4^+$ $CD25^+$ $FOXP3^+$ T_{reg} cells. These cells then suppress the effective immune response, allowing mycobacteria to evade the control of the immune system and to proliferate and colonize vulnerable tissue, including the lungs (8). This may be the reason why patients with LTBI reacquire the disease more easily upon re-exposure to *M. tuberculosis*.

Antigen-specific γδ T cells may be involved in antimycobacterial immunity and complex patterns of γδT-cell immune responses have been observed in humans and animal models during early mycobacterial infections and chronic tuberculosis (27,28). ESAT-6, a protein secreted by the *M. tuberculosis* ESX-1 system, inhibits human T-cell immune responses and the secretion of IFN-γ, and $CD4^+$ $CD25^+$ T_{reg} cells inhibit the production of IFN-γ by human memory γδ T cells in response to ESAT-6 (29,30). The results of the present study suggested that $CD4^+$ $CD25^+$ $FOXP3^+$ T_{reg} cells are important in immunity against *M. tuberculosis* by expanding in response to proteins secreted by *M. tuberculosis*, including ESAT-6 and Ag85B, and reducing the ability of the host to eradicate *M. tuberculosis*.

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