Elevated expression of T-bet in mycobacterial antigen-specific CD4+ T cells from patients with tuberculosis

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ARTICLE INFO

Article history:
Received 11 January 2015
Revised 19 July 2015
Accepted 11 August 2015
Available online 12 August 2015

Keywords:
Tuberculosis
T-bet
CD4 T cells

ABSTRACT

T-bet is a T-box transcriptional factor that controls the differentiation and effector functions of CD4 T cells. In this study, we studied the role of T-bet in regulating CD4+ T cell immunity against tuberculosis (TB). T-bet expression in Mycobacterium tuberculosis antigen-specific CD4+ T cells was significantly higher in patients with active TB than in individuals with latent TB infection (p < 0.0001). Comparison of T-bet expression in TCM and TEM subsets showed that CD4+ T-bet+ M. tuberculosis antigen-specific CD4+ T cells had significantly lower frequency of TCM (p = 0.003) and higher frequency of TEM (p = 0.003) than CD4+ T-bet+ cells. The expression of PD-1 in antigen-specific CD4+ T cells was significantly higher in patients with TB than in individuals with latent TB infection (p = 0.006). CD4+CD154+T-bet+ T cells had significantly higher expression of PD-1 than CD4+CD154+T-bet+ T cells (p = 0.0028). It is concluded that T-bet expression might be associated with differentiation into effector memory cells and PD-1 expression in mycobacterial antigen-specific CD4+ T cells.

1. Introduction

Tuberculosis (TB) is the second leading cause of death from an infectious disease worldwide. It is estimated that 8.8 million cases of TB occurred in 2010 and 2.6 million were smear-positive. In 2012 alone, there were estimated 1.3 million deaths from TB [1].

Despite high rate of Mycobacterium tuberculosis infection in humans, especially in developing countries, only 5–10% of infected people develop active TB in their lifetime [2,3]. Therefore, host-pathogen interactions largely determine the outcome of human M. tuberculosis infection. It has been proven that CD4+ and CD8+ T cells, γδ T cells and CD1-restricted T cells have important role in protection against M. tuberculosis infection [4–8]. CD4 knockout mice demonstrated increased susceptibility to M. tuberculosis infection compared with wild-type mice [4]. AIDS patients have severe defects in CD4+ T cells and are highly susceptible to development of active TB [1].

T-bet, a member of the T-box family of transcription factors, was originally discovered as master regulator of Th1 differentiation [9,10]. It is now recognized that T-bet plays an important role in both adaptive and innate immune systems [10]. T-bet is expressed in many immune cells, such as CD4+ and CD8+ T cells, B cells, γδ T cells, regulatory T (Treg) cells, dendritic cells (DCs), NK cells, NK T cells and innate lymphoid cells (ILCs), and it regulates development and/or effector functions of these immune cells [10].

The role of T-bet in animal models of M. tuberculosis infection has been investigated previously [11]. T-bet-deficient mice are susceptible to M. tuberculosis infection, and exhibited increased bacterial burden, diminished IFN-γ production, and elevated production of IL-10 [11]. However, the role of T-bet in CD4+ T cell function in patients with active TB needs further investigation.

In previous study, we found that patients with active TB had significantly lower frequency of antigen-specific central memory T cells (Tcm) and higher frequency of effector memory T cells (Tem) compared with tuberculin-positive healthy controls, and effective treatment of TB patients with standard antibiotic regimens led to recovery of TCM [12]. In this study, we investigated the role of T-bet in memory cell differentiation and also PD-1 expression in M. tuberculosis antigen-specific CD4+ T cells from patients with active TB and individuals with latent TB infection.

2. Materials and methods

2.1. Human subjects

43 patients (24 male and 19 female, mean age of 33.40 ± 1.93) with active pulmonary TB were recruited from the TB Clinical
Center of the Institute of Tuberculosis, 309th Hospital, Beijing, China (Table 1). Diagnosis of pulmonary TB was based on positive sputum smears and/or culture results and chest radiography. Individuals with malignant tumor, HIV infection or in-take immunosuppressive agents were excluded.

Healthy individuals (n = 120, 47 male and 73 female, mean age of 34.63 ± 0.94) were randomly recruited from individuals undergoing annual health check-up at the clinics of the 309th Hospital, with following inclusion criteria: (1) no fever, cough or other signs of active TB; (2) with normal physical examination result and

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<td>53/53</td>
<td>0/67</td>
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* ND: not determined.

Fig. 1. T-bet expression in CD4+ T cells from patients with active TB, latent TB infection and healthy controls. (A) Representative flow cytometric plot showing gating of lymphocytes. (B) Representative flow cytometric plot showing gating of total CD3+CD4+ T cells. (C) Representative flow cytometric plots showing expression of T-bet in total CD3+CD4+ T cells from patient with active TB (TB) and individual with latent TB infection (LI), or stained with isotype control antibody (ISO). (D) The expression of T-bet in total CD3+CD4+ T cells was similar among patients with active TB (TB), individuals with latent TB infection (LI) and healthy controls (HD). (E) The expression of T-bet in CD3+CD4+ T cells before (ns) and after *M. tuberculosis* H37Rv lysates stimulation (sti). Mann–Whitney test was used for statistical analysis between groups in Fig. 1D. Paired t-test analysis was used for statistical analysis between groups in Fig. 1E.
normal radiography; (3) without HIV infection. Latent TB infection individuals were defined as T-SPOT.TB-positive.

The study was approved by the Ethics Committee of the Beijing 309th Hospital, and informed consent was obtained from all participants.

2.2. Antibodies and reagents

The following antibodies were used in this study: anti-human CD4-FITC, PE-Cy5 or PE-Cy7 (clone OKT4), anti-CD154-APC (clone 24-31), anti-CD8-PE-Cy5 (clone HIT8a), anti-CD56-PE-Cy5 (clone MEM-188), anti-CD69-PE-Cy5 (clone FN50) and anti-CD197 (CCR7)-PE-Cy7 (clone G043H7) were purchased from BioLegend (San Diego, CA, USA); anti-human CD3-PE-Cy7 or -PE-CF594 (clone UCHT1), anti-T-bet-PE (clone 4B10), anti-CD4-PE-CF594 (clone RPA-T4), anti-CD8-PE-CF594 (clone RPA-T8), anti-PD-1-FITC (clone MIH4), and anti-CD45RO-FITC (clone UCHL1) were obtained from BD Biosciences (San Diego, California, USA); anti-human NKG2C-Alexa Fluor® 488 (clone 134591) and anti-IFN-γ-FITC (clone 25723) was obtained from R&D Systems (Minneapolis, MN, USA).

2.3. Preparation of human PBMCs

Peripheral blood mononuclear cells (PBMCs) were purified from peripheral whole blood obtained from patients with active TB and control individuals by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Pittsburgh, PE, USA). 1 × 10⁶ PBMCs were washed twice with RPMI 1640 (Gibco, Life Technologies, Grand Island, NY, USA) and resuspended in AIM V® Serum Free Medium (Gibco). The cells were added to 96 well tissue culture plate (Corning, New York, USA), 200 µl/well.

2.4. IFN-γ release assay

IFN-γ-release assay was performed by using T-SPOT.TB kit (Oxford Immunotec, Oxfordshire, United Kingdom) by following manufacture’s instruction. Briefly, PBMCs obtained from patients with active TB and healthy individuals were added to 96-well plates, 2.5 × 10⁵ cells/well, and were stimulated with M. tuberculosis antigen ESAT-6 and CFP-10 peptide pools for 20 h at 37 °C. Spots were counted by using CTL-ImmuNoSpot® Analyzer (Cellular Technology Ltd, Shaker Heights, OH, USA).

2.5. Surface antibody staining and flow cytometric analysis

PBMCs were incubated overnight at 37 °C in CO₂ incubator with heat-inactivated M. tuberculosis strain H37Rv lysates at a final concentration of 10 µg/ml, as estimated by BCA assay. For identification of antigen-specific CD4⁺ T cells, anti-CD154 antibody was included at the start of antigen stimulation. Monensin (BD
Biosciences), at a final concentration of 1 μg/ml, was also added to the cells. The cells were stained with fluorochrome-labeled monoclonal antibodies and analyzed by an FC-500 flow cytometer (Beckman Coulter, Brea, CA, USA).

2.6. Intracellular cytokine staining

PBMCs from patients with active TB were stimulated with heat-inactivated *M. tuberculosis* strain H37Rv lysates at a final concentration of 10 μg/ml. Brefeldin A was added for the last 4 h of antigen stimulation. After 16 h of incubation, cells were collected, washed, stained first with surface markers. After being permeabilized with cytofix/cytoperm fixation/permeabilization buffer (BD Biosciences), the cells were incubated with fluorochrome-labeled anti-IFN-γ for 30 min at 4 °C. The production of cytokines was analyzed by an FC-500 flow cytometer.

2.7. Statistical analysis

Mann–Whitney and paired t-test analysis were used for statistical analysis between groups by using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA). All tests were two-tailed and *p* < 0.05 was considered significant.

3. Results

3.1. Elevated expression of T-bet in *M. tuberculosis* antigen-specific CD4+ T cells from patients with active TB

Previous studies found that patients with active TB have altered differentiation of memory CD4+ T cells, and the regulation mechanism remains to be elucidated. Since T-bet plays critical role in differentiation and functions of Th1 cells, we studied the expression T-bet in CD4+ T cells. PBMCs from patients with active TB and healthy controls were stained with fluorochrome-labeled CD3, CD4 and T-bet antibodies and analyzed by flow cytometry (Fig. 1A–C). The frequencies of T-bet expression in total CD3+CD4+ T cells of peripheral blood were similar among patients with active TB, individuals with latent TB infection and healthy controls (Fig. 1D). The expression of T-bet in CD3+CD4+ T cells was similar before and after *M. tuberculosis* lysates stimulation (*p* = 0.37) (Fig. 1E).
We next determined the expression of T-bet in antigen-specific CD4+ T cells from patients with active TB and individuals with latent TB infection that showed immune response to M. tuberculosis antigen stimulation. Antigen-specific CD4+ T cells were identified by appearance of IFN-γ or CD154 following stimulation with M. tuberculosis antigens (Fig. 2A–F). Majority of IFN-γ-positive CD4+ T cells was CD154+, while only part of CD154+CD4+ T cells were IFN-γ+ (Fig. 2A). The frequencies of IFN-γ+CD4+ T cells and CD154+CD4+ T cells did not have significant difference between patients with active TB and individuals with latent TB infection (Fig. 2G and I). Patients with active TB had significantly higher expression of T-bet in M. tuberculosis antigen-specific CD4+IFN-γ+ T cells (p < 0.0001) and CD4+CD154+ T cells (p < 0.0001) than individuals with latent TB infection (Fig. 2H and J).

3.2. T-bet expression in memory cell subsets of antigen-specific CD4+ T cells

Based on surface expression of CD45RO and CCR7, CD4+ T cells can be divided into CD45RO+CCR7+ central memory (T_cm) and CD45RO−CCR7− effector memory (T_em) subsets. The frequencies of T_cm and T_em subsets were compared between patients with active TB and individuals with latent TB infection that showed immune response to M. tuberculosis antigen stimulation. In total CD4+ T cells, patients with TB and individuals with latent TB infection had similar frequency of both T_cm and T_em (Fig. 3A and B). However, the frequency of M. tuberculosis antigen-specific CD4+ T_cm was significantly lower in patients with active TB than in individuals with latent TB infection (p = 0.01) (Fig. 3C and D), while the frequency of M. tuberculosis antigen-specific CD4+ T_em was similar (Fig. 3C and D).

To study the role of T-bet in differentiation of T_cm and T_em in patients with active TB, we analyzed T-bet expression in CD4+ memory T cell subsets. T-bet+ M. tuberculosis antigen-specific CD4+ T cells had significantly lower frequency of T_cm (p < 0.0001) and higher frequency of T_em (p < 0.0001) than T-bet− cells (Fig. 3E and F). The study suggests that T-bet could suppress differentiation of antigen-specific CD4+ T cells into T_cm but promote differentiation into T_em in patients with active TB.

T-bet expression in CD8+ T cells was also analyzed. Frequencies of T_cm and T_em in both total CD8+ T cells and CD69-positive antigen-specific CD8+ T were similar between individuals with latent TB infection and patients with active TB (Fig. 4A–D). No difference was observed in frequencies of

**Fig. 4.** T-bet expression in CD8+ central memory and effector memory T cells. (A) Representative flow cytometric plots showing gating strategy for identification of CD45RO−CCR7− central memory T cells (T_cm) and CD45RO+CCR7+ effector memory T cells (T_em) in total CD8+ T cells from individual with latent TB infection (LI) and patient with active TB (TB). (B) Frequencies of T_cm and T_em in total CD8+ T cells. (C) Representative flow cytometric plots showing identification of CD69-positive antigen-specific CD8+ T_cm and T_em in individuals with latent TB infection (LI) and patients with active TB (TB). (E) Representative flow cytometric plots showing identification of antigen-specific CD8+ T_cm and T_em in T-bet+ and T-bet− T cells. (F) Frequency of antigen-specific T_cm and T_em in T-bet+ and T-bet− CD8+ memory cells. Mann–Whitney test was used for statistical analysis between groups.
antigen-specific CD8⁺ TCM and TEM in T-bet⁺ and T-bet⁻ cells (Fig. 4E and F).

NK memory-like cells were defined as CD3⁻CD56⁺ NKG2C⁺ cells (Fig. 5A). Frequencies of memory-like NK cells were similar in individuals with latent TB infection and patients with active TB (Fig. 5B). There was no significant difference in frequency of memory-like cells between T-bet⁺ and T-bet⁻ NK cells (Fig. 5C and D).

3.3. The expression of T-bet was positively correlated with the expression of PD-1

PD-1 expression in total CD4⁺ T cells and M. tuberculosis antigen-specific CD4⁺ T cells was compared between patients with TB and individuals of latent TB infection. There was no significant difference on PD-1 expression in total CD4⁺ T cells between patients with TB and individuals of latent TB infection (Fig. 6A and B). However, the expression of PD-1 in antigen-specific CD4⁺ T cells was significantly higher in patients with active TB than in individuals of latent TB infection (p = 0.006) (Fig. 6C and D).

To know the relationship of between T-bet and PD-1 expression, a double staining of T-bet, PD-1 and CD154 in M. tuberculosis antigen-specific CD4⁺ T cells was performed. The expression of PD-1 was significantly higher in CD4⁺CD154⁺T-bet⁺ T cells than in CD4⁺CD154⁻T-bet⁻ T cells (p = 0.0028) (Fig. 6E and F). The result suggests that there may be a correlation between T-bet and PD-1 expression in mycobacterial antigen-specific CD4⁺ T cells.

4. Discussion

T-bet is a transcription factor that controls differentiation and function of Th1 cells. In this study, we studied the role of T-bet in regulating CD4⁺ T cell immunity against TB.

It is generally believed that cytokines produced by CD4⁺ T cells, such as IFN-γ, are critical for protection against TB infection [2]. T-bet is found to function by directly initiating IFN-γ transcription and by suppressing Th2-specific transcription factor GATA-3 [13]. T-bet deficient mice have decreased Th1 and increased numbers of Th17 cells [14]. It has been reported that T-bet sustains virus-specific CD8⁺ T cell responses during chronic viral infection [15], and it enhances immunity to Trypanosoma cruzi by promoting the expansion of T. cruzi-specific CD8⁺ T cells [16]. T-bet expression is associated with increased HIV-specific CD8⁺ T cell cytotoxicity in HIV elite controllers [17]. Our study found that patients with active TB had significantly higher expression of T-bet in M. tuberculosis antigen-specific CD4⁺ T cells than individuals with latent TB infection.

Memory T cells are hallmark of acquired immunological responses [18,19]. Memory T cells are long-lived cells that reactivate upon antigen stimulation. TCM are long-lived T cell with strong proliferative capacity and are capable of self-renewal, and TEM are memory T cells with effector phenotypes and can respond rapidly to re-infection. It is reported that patients with active TB has significantly lower frequency of antigen–specific TCM and higher frequency of TEM compared with healthy controls, and effective treatment leads to recovery of TCM [12]. We found that T-bet might promote differentiation of antigen-specific CD4⁺ T cells into TEM in patients with TB.

T cell exhaustion plays a major role in failure to control chronic infections. High expression of inhibitory receptor, such as PD-1, contributes to T cell functional defect [20]. The inhibitory receptor PD-1 is significantly increased in both CD4⁺ and CD8⁺ T cells from patients with active TB after antigen stimulation. Blocking of PD-1 receptor leads to increased frequency of IFN-γ-producing cells and degranulation of CD8 T cells [21]. Patients with active TB have significantly elevated expression of Tim-3 in both total CD8 T cells and TB antigen-specific T cells. The elevated expression of Tim-3 on CD8 T cells is significantly associated with disease severity of TB patients [22]. Our results showed that T-bet expression is positively associated with expression of PD-1, which is in contrast with viral chronic infection [15].

In summary, patients with active TB had significantly higher expression of T-bet in M. tuberculosis antigen-specific CD4⁺ T cells than individuals with latent TB infection. CD4⁺T-bet⁺ M.
tuberculosis antigen-specific CD4$^+$ T cells had significantly lower frequency of TCM and higher frequency of TEM than CD4$^+$ T-bet$^-$/C0 cells. There may be a correlation between T-bet and PD-1 expression in mycobacterial antigen-specific CD4$^+$ T cells.

Conflict of interest

None.

Acknowledgments

The study was supported by Grants from National Natural Science Foundation of China (81302537, 81201258 and 81273225) and by Grant from National Science & Technology Major Project for Infectious Diseases (#2013ZX10003006-003-001).

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