CREB Is a Positive Transcriptional Regulator of Gamma Interferon in Latent but Not Active Tuberculosis Infections

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Tuberculosis (TB) is a major health problem throughout the world and is responsible for more deaths than any other single infectious disease. In China, an estimated 0.16 million (range, 0.064 to 0.32 million) people died from TB in 2008 (21). TB is a disease mainly caused by Mycobacterium tuberculosis, and host interactions with this bacterium may lead to a cell-mediated protective immune response. Gamma interferon (IFN-γ) is a crucial cytokine for protection against Mycobacterium tuberculosis, but the mechanism of IFN-γ transcription is still unclear. The cyclic AMP (cAMP) responsive element binding (CREB) proteins belong to the bZip (basic leucine zipper) family of transcription factors and are essential for T-cell function and cytokine production. This study focused on the capacity of CREB proteins to regulate IFN-γ transcription in CD3+ T cells obtained from tuberculosis (TB) patients and persons with latent tuberculosis infection (LTBI) in China. The electrophoretic mobility shift assay (EMSA), chromatin immunoprecipitation (ChIP), and Western blotting were used to demonstrate the regulatory role of CREB. EMSA (in vitro) and ChIP (in vivo) experiments suggested CREB could bind to the IFN-γ proximal promoter in persons with LTBI, whereas no binding was detected in TB patients. Western blotting confirmed the expression of CREB proteins, especially serine-133-phosphorylated CREB, was markedly reduced in TB patients compared with persons with LTBI. These results suggested that CREB could promote the transcription and production of IFN-γ through binding with the IFN-γ proximal promoter, but the regulatory role of CREB was decreased in tuberculosis patients owing to diminished expression of CREB proteins, which in turn reduced the IFN-γ production.
Based on the sensitivity and specificity of the PPD TST and the high prevalence of TB in clinical settings, which increases the risk for progression to active TB, induration of >10 mm is considered positive for LTBI (1). This work was approved by the institutional review boards of the Beijing Tuberculosis and Thoracic Tumor Research Institute. Informed consent was signed by everyone involved in the study.

**Antigens.** Cells of the Mycobacterium tuberculosis strain H37Rv were harvested in the mid-exponential phase of mycobacterial growth and boiled in an 80°C water bath for 20 min. After being centrifuged at 10,000 × g for 10 min, the supernatant was used as antigens (Abs) for incubation with and stimulation of CD3⁺ T cells for 48 or 72 h.

**Preparation of T cells and nuclear proteins.** Peripheral blood mononuclear cells (PBMCs) were isolated by differential centrifugation through Ficoll-Paque. Freshly separated PBMCs were incubated with magnetic beads conjugated to monoclonal anti-CD3⁺ antibodies (Abs; Milteny Biotec, Germany), and the autoMACS magnetic cell separator (Milteny Biotec, Germany) was used to positively select CD3⁺ cells. The purity of CD3⁺ cells was 93.2% by flow cytometry analysis with FACS Calibur (BD Biosciences, United States).

After incubation of CD3⁺ cells stimulated by heat-killed M. tuberculosis Ag, the cellular pellets of CD3⁺ cells were suspended in buffer A containing 10 mM HEPES (pH 7.9), 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 5 μl of 10 μg/ml each aprotinin, leupeptin, and chymostatin (Sangon Biotech, China). After incubation on ice for 10 min, cells were centrifuged at 12,000 × g for 3 min at 4°C, and supernatants were discarded. Pellets were resuspended in buffer B containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, and 5 μl of 10 μg/ml each aprotinin, leupeptin, and chymostatin. The samples were frozen and thawed twice using liquid nitrogen and a 37°C water bath, shaken vigorously at 4°C for 30 min, and centrifuged at 14,000 × g for 10 min. The supernatants were collected and quantified using the Bradford method and were aliquoted and stored at −70°C.

**EMSA.** The target probe contained the IFN-γ proximal promoter: 5'-TCTTCTAATAGCTGAGAGCTAG-3' (wild-type CREB probe); the mutated CREB consensus binding site (mutated), AGAGATTGCCTGTGGTCAAGAGATTGCCTGACGTCAGAGAGCTA-AAGAGATTGCCTGACGTCAGAGAGCTA-3' (mutated CREB probe). The underlined nucleotides on the probes are essential for binding to CREB. The 5'-proximal promoter probe and the wild-type CREB probe but not by the NF-κB probe or the mutated CREB probe.

The low-mobility band was either undetectable (18 patients; P < 0.05, chi-square test) or weakly detectable (7 patients), suggesting that in TB patients, proteins binding to the IFN-γ proximal promoter probe but not by the NF-κB probe or the mutated CREB probe.

**RESULTS**

CREB binding to the IFN-γ proximal promoter in vitro. EMSA was performed with the DIG-labeled IFN-γ proximal promoter probe. Nuclear proteins were obtained from purified CD3⁺ T cells of 18 persons with LTBI and 25 TB patients. EMSA results were comparable for all the 18 persons with LTBI such that one low-mobility band and one high-mobility band were observed after the CD3⁺ T cells were stimulated by M. tuberculosis Ags (Fig. 1). However, in 25 TB patients, the low-mobility band was either undetectable (18 patients; P < 0.05, chi-square test) or weakly detectable (7 patients), suggesting that in TB patients, proteins binding to the IFN-γ proximal promoter were absent or reduced.
Specificity of protein binding to the IFN-γ proximal promoter. Competitive EMSA was performed with an excess of unlabeled IFN-γ proximal promoter probe, which was added to the nuclear extracts derived from the T cells of persons with LTBI before incubation with labeled IFN-γ proximal promoter probe. The low-mobility complex was completely outcompeted by the excess unlabeled probe (Fig. 1B, lane 3), demonstrating the specificity of the DNA-binding proteins for the IFN-γ proximal promoter.

For identification of CREB in the binding complex, an excess of unlabeled CREB wild-type or mutated probe or the NF-κB binding site probe was added in the competitive EMSA experiments. Figure 1B shows that the low-mobility complex binding to the IFN-γ proximal promoter was eliminated by the excess of unlabeled wild-type CREB probe (lane 4) but not by the unlabeled mutated CREB probe (lane 2) or the NF-κB probe (lane 1). These results suggest that the protein complex binding to the IFN-γ proximal promoter includes CREB.

In vivo CREB binding to the IFN-γ proximal promoter. ChIP was performed using CD3+ T cells that were freshly obtained from 12 TB patients and 10 persons with LTBI. Briefly, DNA-binding proteins were cross-linked to their target DNA sequences with formaldehyde, and specific anti-CREB Abs were used in immunoprecipitation after cross-linkages were reversed. PCR with primers specific to the IFN-γ proximal promoter sequence was used to test whether CREB proteins bound to the promoter. A 204-bp product was amplified from the T cells of all persons with LTBI, but no amplicon was observed when T cells from 12 TB patients were used (P < 0.01; chi-square test), despite extending the incubation time from 48 h to 72 h (Fig. 2). The ChIP results suggest that stimulation by *M. tuberculosis* Ags results in the production of CREB proteins in CD3+ T cells from persons with LTBI but not from TB patients. CREB proteins could bind to the IFN-γ proximal promoter in vivo.

The EMSA and ChIP data suggest that the capacity of CREB protein to bind to the IFN-γ proximal promoter was reduced in TB patients compared with that in persons with LTBI. To ascertain whether the reduction was due to the decreased levels of CREB proteins in TB patients, Western blotting was performed using an anti-CREB Ab. Of 25 TB patients, CREB was not detected in 20 TB patients (Fig. 3, lanes 3 and 4); however, CREB was detected in all 18 persons with LTBI (Fig. 3, lanes 1 and 2). Anti-serine-133-phosphorylated CREB Ab also was used in Western blotting. Phosphorylated CREB was detected in all 18 persons with LTBI but in none of the 25 TB patients (Fig. 3). β-Actin Ab was used as control in Western blotting and was found in TB patients and persons with LTBI. These results indicated that the level of CREB protein, and specifically serine-133-phosphorylated CREB protein, was markedly decreased in TB patients compared with that in persons with LTBI.

**DISCUSSION**

IFN-γ-producing T cells protect against acute infection by *M. tuberculosis*, and these mechanisms may also be operative during LTBI (20). IFN-γ has been considered a key macrophage-activating cytokine during TB, results in altered trafficking of intracellularly residing mycobacteria that induce phagosome-lysosome fusion, and may be critical to containment of *M. tuberculosis* (7). The transcription of IFN-γ is controlled by regulatory regions in its distal (bp −96 to −80) and proximal (bp −73 to −40) promoters (13). The proximal promoter plays an important role in gene regulation and may contribute to the differential expression of IFN-γ. Proteins binding to the proximal promoter markedly affect the activity of the IFN-γ promoter and the transcription of IFN-γ (14).

CREB protein is an essential transcription factor for T-cell differentiation and IFN-γ production. Deletion of CREB proteins induces defective T-cell differentiation and IFN-γ production in transgenic mice (2). To assess whether these events occur in human T cells, we performed experiments with CD3+ T cells from TB patients and persons with LTBI.

EMSA is a powerful technique used to quantitatively analyze sequence-specific DNA-binding proteins *in vitro*. Traditionally a radiolabeled probe is used, and when the probe and proteins form a complex, it is retarded by the gel and appears as a mobility-shifted band in comparison to the free probe. Because safety concerns are associated with the proper storage, usage, and disposal of radioactive substances and nonra-

![FIG. 2. ChIP. PCR was performed with primers specific for the IFN-γ proximal promoter probe. In lanes 1, 4, and 6, the CD 3+ T cells were extracted from a TB patient. Lane 1 was incubated without *M. tuberculosis* Ags stimulation; lanes 4 and 6 were incubated with *M. tuberculosis* Ags (8 μg/ml) for 48 h (lane 4) or 72 h (lane 6), and then 1% formaldehyde was added to the cells to cross-link DNA to protein, and immunoprecipitation was performed with anti-CREB, followed by PCR amplification. The positive control is in lanes 2 and 3. DNA was added to PCR after sonication (lane 2). Lane 3 is genomic DNA was used as template in PCR; the CD 3+ T cells extracted from persons with LTBI were incubated with *M. tuberculosis* Ags for 24 h (lane 5).](image-url)
ative results were obtained from CD3
CREB. ChIP results suggested that regardless of incubation
EMSA demonstrated that the binding complex included
These data suggest that the reduced expression of CREB pro-
patients with moderately advanced or far-advanced pulmonary
disease (18). In the current research, based on Western blotting,
the expression of CREB, and especially serine-133-phos-
T cells bound to the IFN-
72% of samples from TB patients lacked the low-mobility
EMSA results indicated that in 72% (18/25) of samples from
protein binding to the IFN-
(ChIP) DNA binding results showed good agreement in CREB
proximal promoter. However, EMSA results indicated that in 72% (18/25) of samples from TB patients, no CREB protein bound to the promoter. In comparison, ChIP results indicated no CREB protein binding in 100% (12/12) of samples from TB patients. This is probably because many different factors that can affect binding are included in the complex biological environment in vivo but are not present under the defined in vitro binding conditions used for the EMSA experiments (3).

Some studies have reported that IFN-γ levels are depressed in patients with active TB (9, 23), especially in malnourished patients with moderately advanced or far-advanced pulmonary disease (18). To our knowledge, this study was the first to analyze the function of CREB on IFN-γ transcription in Chinese individuals with LTBI or TB. Our EMSA experiments indicated that 72% of samples from TB patients lacked the low-mobility complex bound to the IFN-γ proximal promoter. Competitive EMSA demonstrated that the binding complex included CREB. ChIP results suggested that regardless of incubation time with heat-killed M. tuberculosis Ags (48 h or 72 h), negative results were obtained from CD3+ T cells from TB patients compared with positive results from individuals with LTBI. Western blotting indicated that the expression of CREB was markedly reduced in TB patients. Taken together, these results suggest that CD3+ T cells extracted from TB patients produce little or no CREB when stimulated by M. tuberculosis Ags, and the expression of CREB proteins was notably reduced in TB patients compared with persons with LTBI. These findings strongly support that CREB proteins play a positive regulatory role in transcription and production of IFN-γ in individuals with LTBI.

China is one of the 22 countries with a high burden of TB, and the population of TB patients in China is surpassed only by India. With the prevalence of HIV and multidrug-resistant TB and the potential of persons with LTBI to develop active TB, the control and treatment of TB are great challenges (5). Understanding the immune mechanisms of LTBI is very important in the control of TB. IFN-γ is a key cytokine in the control of M. tuberculosis infection. Humans defective in genes for IFN-γ or the IFN-γ receptor are prone to serious mycobacterial infections, including M. tuberculosis. The regulation of IFN-γ transcription is complex, involving multiple enhancer and repressor elements in response to mycobacterial Ags (12, 17). Further work should continue to focus on the mechanism for decreased IFN-γ production in TB patients, which will enrich our knowledge of protective immunity against M. tuberculosis and contribute to finding new strategies for the control and treatment of TB.