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CREB, ATF, and AP-1 Transcription Factors Regulate IFN-γ Secretion by Human T Cells in Response to Mycobacterial Antigen

Buka Samten,* James C. Townsend,*† Steven E. Weis,§ Anindita Bhomik,¶ Peter Klucar,3§† Homayoun Shams,*† and Peter F. Barnes*†‡

IFN-γ production by T cells is pivotal for defense against many pathogens, and the proximal promoter of IFN-γ, −73 to −48 bp upstream of the transcription start site, is essential for its expression. However, transcriptional regulation mechanisms through this promoter in primary human cells remain unclear. We studied the effects of cAMP response element binding protein/activating transcription factor (CREB/ATF) and AP-1 transcription factors on the proximal promoter of IFN-γ in human T cells stimulated with Mycobacterium tuberculosis. Using EMSA, supershift assays, and promoter pulldown assays, we demonstrated that CREB, ATF-2, and c-Jun, but not cyclic AMP response element modulator, ATF-1, or c-Fos, bind to the proximal promoter of IFN-γ upon stimulation, and chromatin immunoprecipitation indicated the possibility of interaction among these transcription factors. Chromatin immunoprecipitation confirmed the recruitment of these transcription factors to the IFN-γ proximal promoter in live Ag-activated T cells. Inhibition of ATF-2 activity in T cells with a dominant-negative ATF-2 peptide or with small interfering RNA markedly reduced the expression of IFN-γ and decreased the expression of CREB and c-Jun. These findings suggest that CREB, ATF-2, and c-Jun are recruited to the IFN-γ proximal promoter and that they up-regulate IFN-γ transcription in response to microbial Ag. Additionally, ATF-2 controls expression of CREB and c-Jun during T cell activation. The Journal of Immunology, 2008, 181: 2056–2064.

IFN-γ, produced primarily by T cells and NK cells in response to microbial infection, plays an important role in protection against many pathogens, including intracellular bacteria such as Mycobacterium tuberculosis (1, 2), viruses (3), fungi (4), and protozoa (5) and in immune surveillance to prevent development of cancer (6). IFN-γ has also been used to treat patients with idiopathic pulmonary fibrosis (7). On the other hand, IFN-γ is associated with inflammatory diseases, including sarcoidosis (8) and multiple sclerosis (9). Although expression of IFN-γ is controlled to some extent by posttranscriptional processes (10), the dominant mechanism for regulating IFN-γ gene expression is through coordinated, cell-specific interactions between transcription factors and regulatory elements of the IFN-γ gene (11).

Among the IFN-γ regulatory elements, the proximal promoter of IFN-γ, located −73 to −48 bp upstream of the transcription start site, is necessary and sufficient for IFN-γ gene expression by activated T cells (12). The proximal promoter of IFN-γ is highly conserved in mammals (13), and methylation of the CpG motif at −53 bp is a major epigenetic regulatory mechanism that is thought to render the promoter region inaccessible to transcription factor binding in Th2 cells that do not produce IFN-γ, whereas this site is unmethylated in Th1 cells that express IFN-γ (14–17). Despite the central importance of the proximal promoter in controlling IFN-γ transcription, limited information is available on the detailed mechanisms by which transcription factors interact with this promoter. The cAMP response element binding protein (CREB)4 is known to bind to this promoter, and we found that CREB enhances transcription of IFN-γ by primary human T cells that are stimulated with M. tuberculosis (18, 19). Activating transcription factor (ATF) and AP-1 also bind to the proximal promoter, and glucocorticoids inhibit this binding and reduce IFN-γ gene expression in Jurkat T cells (13). However, both ATF and AP-1 are composed of several transcription factors, and their individual effects on IFN-γ transcription remain unclear. Furthermore, most studies of transcription factors that control IFN-γ expression in T cells have been performed in cell lines and in transgenic mice, and limited information is available on this subject in primary human T cells.

In the present study, we identified the transcription factors of the CREB/ATF/AP-1 family that bind to the proximal promoter of IFN-γ in primary human T cells during a physiologically relevant response to Ags from M. tuberculosis, an intracellular pathogen for which immunologic control requires IFN-γ-mediated responses. We found that CREB, ATF-2, and c-Jun are recruited

4 Abbreviations used in this paper: CREB, cAMP response element binding protein; ATF, activating transcription factor; wt-CRE, wild-type CREB consensus binding site; mt-CRE, mutant CREB consensus binding site; CREM, cAMP response element modulator; CRE, cAMP response element; AP-1, activating protein 1.
to bind the proximal promoter of IFN-γ and enhance IFN-γ gene expression in response to antigenic stimulation of T cell. Additionally, ATF-2 regulates expression of CREB and c-Jun during T cell activation.

Materials and Methods
Isolation of PBL and cell culture
Heparinized blood samples were collected from 18 tuberculin skin test-positive subjects and 8 tuberculin skin test-negative subjects under protocols approved by the institutional review boards of University of Texas Health Center (Tyler, TX) and the University of North Texas Health Science Center (Fort Worth, TX). Cells from tuberculin reactors were used in all studies that involved culture of cells with M. tuberculosis Ags, as cells from tuberculin-negative donors would not respond to M. tuberculosis. Cells from tuberculin-negative donors and tuberculin reactors were used for experiments in which cells were cultured with PMA or anti-CD3 and anti-CD28.

PBMC were isolated by Ficoll-Paque (Pharmacia Fine Chemicals) gradient centrifugation. In some cases, CD3+ T cells were isolated from PBMC by negative selection with the Pan T Cell Isolation Kit II (Miltenyi Biotec), with purity of >95%, as measured by flow cytometry with a FACSCalibur (BD Biosciences), using FITC anti-CD3 mAbs (eBioscience). Monocytes were prepared by culturing 10^7 PBMC per well in a 12-well plate in 1 ml RPMI 1640 with 10% pooled human AB serum at 37°C, 5% CO₂, for 1 h, followed by washing extensively with RPMI 1640 to remove nonadherent cells. Monocyte purity was >90% based on flow cytometry using FITC anti-CD14 mAbs (eBiosciences).

T cell clone
For some experiments, we utilized B9, a human CD4+ T cell clone that recognizes a 10-mer peptide from the N terminus of the 10-kDa M. tuberculosis culture filtrate protein (CFP10) of the HLA-DRB1*0401 (20).

Mycobacterial Ag and reagents to activate T cells
Heat-killed M. tuberculosis Erdman, provided by Dr. Patrick Brennan (Colorado State University), was used to stimulate PBMC or CD3+ cells and autologous monocytes. To stimulate the human T cell clone, we cultured bare lymphocyte syndrome cells expressing HLA DRB1*04, the clone, and its cognate peptide, CFP10_36-45, mAbs to CD3 (OKT3, Ortho Biotechnology) and CD28 (BD Biosciences), as well as PMA and ionomycin (both from Sigma-Aldrich), were used to stimulate purified CD3+ cells.

Preparation of nuclear and whole-cell protein extracts
Nuclear protein extracts and whole-cell protein extracts of PBMC, CD3+ cells, and T cell clones were prepared as described previously (18, 19, 21), quantified by bicinchoninic acid assay (Pierce Biotechnology), aliquoted, and stored at −70°C until use.

EMSA and supershift assay
To measure binding of transcription factors in nuclear protein extracts to the IFN-γ proximal promoter (−48 to −73 bp), EMSAs were performed as described previously, using [γ-32P]ATP-labeled proximal promoter of IFN-γ as a probe (21, 22). For EMSA competition assays, the nuclear protein extracts were incubated with labeled probe on ice for 30 min with 50 M excess of unlabeled DNA oligonucleotides of IFN-γ proximal promoter, wild-type CREB consensus binding site (wt-CRE), mutant CREB consensus binding site (mt-CRE), and NF-kB consensus binding site, respectively. For supershift assays, control IgG or Abs to CREB, cAMF response element modulator (CREM), ATF-2, c-Jun, c-Fos, JunB (all from Santa Cruz Biotechnology), and ATF-1 (Novus Biologicals) were incubated with nuclear protein extracts on ice for 30 min, then incubated with labeled probe for 25 min at room temperature. The DNA-protein complexes were resolved by electrophoresis on a 5% nondenaturing polyacrylamide gel and detected by autoradiography.

Coimmunoprecipitation and Western blotting
Coimmunoprecipitation was performed as previously described (22). Briefly, 200 μg of nuclear protein extracts were precleared by incubation with 30 μl of a 50% slurry of protein G-Sepharose beads (Sigma-Aldrich) in a total volume of 250 μl, reconstituted with coprecipitation buffer (0.1% Triton X-100, 100 mM NaCl, 15 mM EGTA, PMSF, and a proteinase inhibitor cocktail) at 4°C for 1 h with rotation. After centrifugation at 1800 × g for 3 min, the supernatants were removed and incubated with 2 μg of Abs to CREB, ATF-2, or c-Jun at 4°C overnight with rotation. Thirty microfilters of a 50% slurry of protein G-Sepharose beads was added and incubated at room temperature for 1 h with rotation. The beads were washed with wash buffer (0.1% Triton X-100, 100 mM NaCl, 5 mM EDTA) three times. SDS-PAGE sample loading buffer was added to the beads, and the samples were boiled for 5 min. Proteins were separated by SDS-PAGE, followed by Western blotting with relevant Abs, and the protein bands were visualized by chemiluminescence (GE Healthcare).

Promoter pull-down assay
Promoter pull-down assays to detect proteins bound to the proximal promoter of IFN-γ were performed according to published methods (23–25). Briefly, chemically synthesized wild-type or mutant proximal IFN-γ promoter sequences (−71 to −40 bp) were biotinylated at the 5’ end of the sense strand and annealed to the anti-sense sequence. The wild-type and mutant biotinylated IFN-γ proximal promoter sequences were 5’-Bio-AAA ACT TGT GAA AAT ACC TAA TTC GGA GA-3’ and 5’-Bio-AAA ACT TGT GAA AAT CCC TAA TTC GCA GA-3’, respectively. Two micrograms of biotinylated dsDNA was conjugated to 100 μl of UltraLink immobilized streptavidin gel (Pierce Biotechnology) in binding/washing buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 M NaCl) for 45 min at room temperature with rotation. Free unconjugated DNA was removed by extensive washing with binding/washing buffer. DNA-conjugated beads were blocked with 0.5% BSA and 5 μg/ml streptavidin in TBE buffer, then pulled down by the proximal promoter of IFN-γ (sense strand and antisense strand, 5’-GTT GAC TCA GAG GAC CCT TCA GGA GA-3’).

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bands are indicated by arrows. Three representative results are shown.

\[ \text{GATCGTCCAGCATATTACAGG-3}^/H11032-\text{TAATACGACTCACTATAGGGGA} \]

tagged ATF-2 primers (forward: \(5^/H11032\)). The lower limit of detection was 5 pg/ml.

eight healthy tuberculin reactors, cultured with medium alone for 48 h or in the presence of 2.5 \(\mu\)g/ml of heat-killed \(M.\) \(tuberculosis\) for 48 h and labeled proximal promoter of IFN-\(\gamma\), in the absence or presence of unlabeled oligonucleotides representing the IFN-\(\gamma\) proximal promoter, the NF-\(\kappa\)B consensus binding site, the wild-type CREB consensus binding site, and the mutant CREB consensus binding site, as indicated. A representative result is shown. C. Supershift analysis of the protein complexes binding to the proximal promoter of IFN-\(\gamma\) with specific Abs against CREB/ATF/AP-1 transcription factors. EMSA was performed as described in A, with nuclear protein extracts from five healthy tuberculin reactors, cultured with 2.5 \(\mu\)g/ml of heat-killed \(M.\) \(tuberculosis\) for 48 h and labeled proximal promoter of IFN-\(\gamma\), in the absence or presence of the Abs indicated. A representative result is shown.

with 10% human serum in a 96-well flat-bottom plate. Heat-killed \(M.\) \(tuberculosis\) (2.5 \(\mu\)g/ml) was added to some wells, and the cells were cultured at 37°C in 5% \(CO_2\). After an additional 72 h, supernatants were collected, counted, and cultured with autologous monocyte-derived macrophages at a ratio of 4 T cells (2 \(\times\) 10^5) to 1 macrophage (5 \(\times\) 10^4) to 1 macrophage (5 \(\times\) 10^4), with or without 2.5 \(\mu\)g/ml of heat-killed \(M.\) \(tuberculosis\) in a 96-well flat-bottom plate in triplicate. After 72 h of additional incubation, supernatants were collected for measurement of IFN-\(\gamma\), and the cells were collected for detection of ATF-2, CREB, c-Jun, and \(\beta\)-actin by Western blotting.

### Results

**Binding of CREB/ATF/AP-1 transcription factors to the IFN-\(\gamma\) proximal promoter using EMSA, supershift assays, and promoter pull-down assays**

We previously showed that stimulation of PBMC with heat-killed \(M.\) \(tuberculosis\) yields increased binding of a nuclear protein complex containing CREB to the IFN-\(\gamma\) proximal promoter (19). To elucidate the composition of these complexes, we performed EMSA with a labeled IFN-\(\gamma\) proximal promoter and nuclear protein extracts of PBMC from healthy tuberculin reactors that had been cultured in medium alone or stimulated with heat-killed \(M.\) \(tuberculosis\) (Fig. 1A). Baseline levels of DNA-binding protein complexes were present in unstimulated cells, and stimulation with \(M.\) \(tuberculosis\) induced increased binding of two major protein complexes to the IFN-\(\gamma\) proximal promoter (designated A and B for the slower and faster mobility complexes, respectively). Binding of these protein complexes after stimulation was paralleled by increased expression of mRNA and maximal secretion of IFN-\(\gamma\) (data not shown). The specificity of this binding was evaluated by competitive EMSA. Binding of these two major complexes was abrogated by excess unlabeled IFN-\(\gamma\) proximal promoter and wild-type CRE, but not by the mutated CRE or NF-\(\kappa\)B binding site (Fig. 1B). Similar results were observed when CD3^+ cells were stimulated with either PMA and ionomycin or with anti-CD3 plus anti-CD28 (data not shown), confirming the existence of these IFN-\(\gamma\) proximal promoter binding protein complexes in purified primary human T cells upon stimulation.

To dissect the protein composition of these complexes, supershift assays were performed with the labeled IFN-\(\gamma\) proximal promoter as a probe and nuclear protein extracts of \(M.\) \(tuberculosis\)-stimulated PBMC from healthy tuberculin reactors, using Abs to

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**FIGURE 1.** Binding of CREB/ATF/AP-1 transcription factors to the proximal promoter of IFN-\(\gamma\). A. Ag-induced binding of protein complexes to the proximal promoter of IFN-\(\gamma\). EMSA was performed using the [\(\gamma-32\)P]ATP-labeled proximal promoter of IFN-\(\gamma\) and nuclear protein extracts of PBMC from eight healthy tuberculin reactors, cultured with medium alone for 48 h or in the presence of 2.5 \(\mu\)g/ml of heat-killed \(M.\) \(tuberculosis\) (TB) for 48 h. Specific bands are indicated by arrows. Three representative results are shown. B. Specific binding of the protein complexes to the proximal promoter of IFN-\(\gamma\). EMSA was performed as described in A, with nuclear protein extracts of PBMC from six healthy tuberculin reactors, cultured in the presence of 2.5 \(\mu\)g/ml of heat-killed \(M.\) \(tuberculosis\) for 48 h and labeled proximal promoter of IFN-\(\gamma\), in the absence or presence of unlabeled oligonucleotides representing the IFN-\(\gamma\) proximal promoter, the NF-\(\kappa\)B consensus binding site, the wild-type CREB consensus binding site, and the mutant CREB consensus binding site, as indicated. A representative result is shown. C. Supershift analysis of the protein complexes binding to the proximal promoter of IFN-\(\gamma\) with specific Abs against CREB/ATF/AP-1 transcription factors. EMSA was performed as described in A, with nuclear protein extracts from five healthy tuberculin reactors, cultured with 2.5 \(\mu\)g/ml of heat-killed \(M.\) \(tuberculosis\) for 48 h and labeled proximal promoter of IFN-\(\gamma\), in the absence or presence of the Abs indicated. A representative result is shown.
CREB/ATF/AP-1 transcription factors (Fig. 1C). Although the effects of these Abs on protein complexes from different individuals was variable, anti-CREB consistently reduced the intensity of complex B, anti-ATF-1 reduced the intensity of complex A, and anti-ATF-2 supershifted both complexes. The effects of the other Abs were not clear.

To use a more definitive alternative technique to identify CREB/ATF/AP-1 transcription factors that bind to the IFN-γ proximal promoter, we used a promoter pull-down assay with a biotinylated IFN-γ proximal promoter, followed by Western blotting for relevant transcription factors. To optimize experimental conditions, we incubated nuclear protein extracts of M. tuberculosis-stimulated PBMC from a healthy tuberculin reactor with a biotinylated IFN-γ proximal promoter, either alone or with excess unlabeled proximal promoter of IFN-γ, wt-CRE, or mt-CRE. The eluents were subjected to immunoblotting with anti-CREB. A representative result is shown. B, Specific binding of CREB to the proximal promoter of IFN-γ. Promoter pull-down assays were performed, as described in A, with nuclear protein extracts of PBMC from three healthy tuberculin reactors, cultured with 2.5 μg/ml of heat-killed M. tuberculosis (TB). The eluents were subjected to immunoblotting with anti-CREB. A representative result is shown. C, Recruitment of CREB, ATF-2, and c-Jun to the proximal promoter of IFN-γ. Promoter pull-down assays were performed, as described in A, using a 5′-biotinylated wild-type or mutant IFN-γ proximal promoter and nuclear protein extracts of PBMC from three healthy tuberculin reactors, cultured with medium alone or with 2.5 μg/ml heat-killed M. tuberculosis (TB). The eluents were blotted for transcription factors as indicated. A representative result is shown.

FIGURE 2. Recruitment of CREB, ATF-2, and c-Jun to the proximal promoter of IFN-γ. A, Ag stimulation increases binding of CREB to the proximal promoter of IFN-γ. Promoter pull-down assays were performed with a 5′-biotinylated proximal promoter of IFN-γ and nuclear protein extracts of PBMC from three healthy tuberculin reactors, cultured in medium alone or with 2.5 μg/ml of heat-killed M. tuberculosis (TB). The eluents were subjected to immunoblotting with anti-CREB. A representative result is shown. B, Specific binding of CREB to the proximal promoter of IFN-γ. Promoter pull-down assays were performed, as described in A, with nuclear protein extracts of PBMC from three healthy tuberculin reactors, cultured with 2.5 μg/ml of heat-killed M. tuberculosis for 48 h with the 5′-biotinylated IFN-γ proximal promoter, either alone or with excess unlabeled proximal promoter of IFN-γ, wt-CRE, or mt-CRE. The eluents were subjected to immunoblotting for CREB. A representative result is shown. C, Recruitment of CREB, ATF-2, and c-Jun to the proximal promoter of IFN-γ. Promoter pull-down assays were performed, as described in A, using a 5′-biotinylated wild-type or mutant IFN-γ proximal promoter and nuclear protein extracts of PBMC from six healthy tuberculin reactors, cultured with medium alone or 2.5 μg/ml heat-killed M. tuberculosis (TB). The eluents were blotted for transcription factors as indicated. A representative result is shown.

FIGURE 3. Recruitment of CREB, ATF-2, and c-Jun to the proximal promoter of IFN-γ in Ag-stimulated live cells. A, Recruitment of CREB, ATF-2, and c-Jun to the proximal promoter of IFN-γ. Chromatin immunoprecipitation was performed on formaldehyde cross-linked chromatin supernatants of PBMC from five healthy tuberculin reactors, cultured with 2.5 μg/ml of heat-killed M. tuberculosis for 48 h, using Abs against the indicated transcription factors. PCR was performed with primers for the IFN-γ proximal promoter, the rRNA promoter, and the c-Fos promoter. A representative result is shown. B, Ag stimulation-induced recruitment of CREB, ATF-2, and c-Jun to the proximal promoter of IFN-γ in live cells. Chromatin immunoprecipitation was performed on formaldehyde cross-linked chromatin of PBMC from three healthy tuberculin reactors, cultured in medium alone or with 2.5 μg/ml of heat-killed M. tuberculosis for 48 h, using Abs against the transcription factors indicated. PCR was performed with primers for the IFN-γ proximal promoter. A representative result is shown.

Ag-induced recruitment of CREB, ATF-2, and c-Jun to the proximal promoter of IFN-γ in live cells

EMSAs and promoter pull-down assays demonstrated binding of transcription factors to a double-stranded DNA sequence in vitro. To determine whether CREB, ATF-2, and c-Jun are recruited to the IFN-γ proximal promoter of IFN-γ in live cells, we performed EMSAs and promoter pull-down assays to identify transcription factors that bind to the IFN-γ proximal promoter, using nuclear extracts from healthy tuberculin reactors. Western blotting demonstrated that CREB, ATF-2, and c-Jun bind to the proximal promoter of IFN-γ in a stimulation-dependent manner. Additionally, these transcription factors failed to bind to a probe with changes in four nucleotides that are essential for IFN-γ proximal promoter activity, further demonstrating the specificity of the transcription factor/IFN-γ proximal promoter interactions in this system. The promoter pull-down assay showed no binding of ATF-1, CREM, and c-Fos to the IFN-γ proximal promoter (Fig. 2C), although these Abs detected their respective proteins in nuclear extracts in standard Western blotting assays (data not shown).

In summary, both EMSAs and promoter pull-down assays showed that CREB, ATF-2, and c-Jun in human T cells bind to the proximal promoter of IFN-γ, and that binding is enhanced by antigenic stimulation.
transcription factors are known to bind (26). As a specificity control, PCR for the rRNA promoter, which does not have a CRE consensus site, yielded negative results after immunoprecipitation with all Abs. Next, we determined if recruitment of transcription factors to the proximal promoter of IFN-γ in live cells is stimulation-dependent. Chromatin supernatants from unstimulated and M. tuberculosis-stimulated PBMC from three healthy tuberculin reactors were immunoprecipitated with Abs against CREB, ATF-2, and c-Jun. As shown in Fig. 3, CREB, ATF-2, and c-Jun were recruited to the proximal promoter of IFN-γ in Ag-stimulated PBMC but not in PBMC cultured in medium alone. Taken together with the results of EMSAs and promoter pull-down assays, these results indicate that CREB, ATF-2, and c-Jun were recruited and bound to the proximal promoter of IFN-γ in live Ag-stimulated cells.

Activation of CREB, ATF-2, and c-Jun by stimulation with M. tuberculosis Ags

CREB/ATF/AP-1 transcription factors are constitutively expressed and become phosphorylated upon cellular activation. Phosphorylation of CREB and ATF-2 increases the affinity for their target gene promoters and facilitates recruitment of CREB-binding protein and P300, transcriptional enhancers with histone acetyltransferase activity, favoring initiation of transcription of the target genes (28). We therefore wanted to determine whether stimulation of peripheral blood T cells by Ag induces phosphorylation of these transcription factors. Using phospho-specific Abs for CREB, ATF-2, and c-Jun, we evaluated the activation of these transcription factors in PBMC from eight healthy tuberculin reactors cultured with heat-killed M. tuberculosis for different periods (Fig. 4). Stimulation of PBMC with M. tuberculosis induced phosphorylation of CREB, ATF-2, and c-Jun after 24 h, with increased expression at 48–72 h. Stimulation with M. tuberculosis markedly increased levels of total c-Jun, and this may account in part for the increase in phosphorylated c-Jun that was observed. When purified peripheral blood CD3+ cells were stimulated with PMA plus ionomycin or anti-CD3 plus anti-CD28, phosphorylation of these same transcription factors was observed, although with much faster kinetics (data not shown).

Protein-protein interactions among CREB, ATF-2, and c-Jun in response to antigenic stimulation

Our results demonstrate that T cell activation results in phosphorylation and binding of CREB, ATF-2, and c-Jun AP-1 to the proximal promoter of IFN-γ. Because CREB/ATF and AP-1 transcription factors bind to the CRE sequence by forming homo- or heterodimers (29, 30), we used coimmunoprecipitation to determine whether these transcription factors form a complex in cell extracts, and if these interactions are stimulation-dependent. When nuclear extracts from M. tuberculosis-stimulated PBMC from four healthy tuberculin reactors were immunoprecipitated with anti-CREB, both ATF-2 and c-Jun were detected by immunoblotting, and this interaction was more marked in M. tuberculosis-stimulated cells than in those cultured in medium alone (Fig. 5A). Similarly, c-Jun and CREB were detected after immunoprecipitation with anti-ATF-2, suggesting that the interaction of CREB, ATF-2, and c-Jun was stimulation-dependent.

Since PBMC contain cells other than T cells, we performed coinmunoprecipitation to study the interaction of these transcription factors in protein extracts of purified CD3+ T cells stimulated with PMA plus ionomycin. CREB, ATF-2, and c-Jun formed a complex upon stimulation, and this interaction was increased during 60 min of stimulation (Fig. 5B). To confirm these findings at the level of single T cells, we cultured a M. tuberculosis-reactive human T cell clone with APCs and either medium alone or its cognate peptide. Nuclear extracts were then prepared, and immunoprecipitation with anti-ATF-2 revealed CREB and c-Jun (Fig. 5C), indicating that these three transcription factors formed a complex.

![FIGURE 4.](image-url) Stimulation-induced phosphorylation of CREB, ATF-2, and c-Jun. PBMC from eight healthy tuberculin reactors were cultured with 2.5 μg/ml of heat-killed M. tuberculosis. At the time points indicated, whole-cell protein extracts were subjected to Western blotting with Abs to phosphorylated CREB, ATF-2, and c-Jun. After stripping, the nitrocellulose membrane was reblotted with Abs to total CREB, ATF-2, and c-Jun. Representative results are shown.

![FIGURE 5.](image-url) CREB, ATF-2, and c-Jun form a complex in response to T cell activation. Coimmunoprecipitation was performed using Abs to CREB or ATF-2 and nuclear protein extracts of (A) PBMC from four healthy tuberculin reactors, in the presence or absence of 2.5 μg/ml of heat-killed M. tuberculosis; (B) purified CD3+ cells from three donors cultured with 50 ng/ml PMA plus 1 μM/L ionomycin for different time points; or (C) a human M. tuberculosis-reactive T cell clone cultured with Ag-presenting B lymphocyte syndrome cells, cultured with medium alone or with its cognate peptide. The experiment with the T cell clone was performed twice. For each panel, nuclear extracts were immunoprecipitated with Abs to either ATF-2 or CREB, and the immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose. Western blotting was performed with anti-c-Jun Abs. The blots were stripped and reblotted with anti-ATF-2, then stripped again and blotted with anti-CREB. A representative result is shown in each panel.
ATF-250 –100 peptide did not affect IFN-γ production by CD3+ cells from six healthy tuberculin reactors that were nucleofected with different amounts of the dominant-negative ATF-2 plasmid (DN-ATF-2), a control plasmid (2 μg), or no plasmid. Forty-eight hours postnucleofection, the cells were cocultured with autologous monocytes in the presence of 2.5 μg/ml of heat-killed M. tuberculosis. After 72 h of incubation, culture supernatants were collected and IFN-γ levels were quantified by ELISA. Mean values and SEs are shown. B, IFN-γ mRNA expression. Purified CD3+ cells from three healthy tuberculin reactors were nucleofected and cultured with autologous monocytes in the absence or presence of heat-killed M. tuberculosis, as in A. After 48 h of incubation, cells were collected and mRNA for IFN-γ was quantified by real-time PCR. A representative experiment is shown of the fold change in IFN-γ mRNA induced by stimulation with M. tuberculosis, relative to medium alone. C, Effect of the dominant-negative ATF-2 peptide on expression of CREB, ATF-2, and c-Jun. Purified CD3+ cells from six healthy tuberculin reactors were nucleofected with the plasmids shown, and cultured with autologous monocytes and heat-killed M. tuberculosis, as in A. After 72 h of incubation, cells were collected and blotted for ATF-2, CREB, c-Jun, and β-actin. A representative result is shown.

In summary, the results of coimmunoprecipitation and chromatin immunoprecipitation suggest that, upon activation of T cells, CREB, ATF-2, and c-Jun are phosphorylated and recruited to form part of a protein complex that binds to the IFN-γ proximal promoter.

**Effect of neutralization of ATF-2 on M. tuberculosis-stimulated IFN-γ secretion**

Our previous work demonstrated that binding of CREB to the IFN-γ proximal promoter enhances M. tuberculosis-induced IFN-γ transcription (19). To determine the physiologic effects of ATF-2 on IFN-γ gene expression, we used a plasmid that expresses the ATF-2\(^{50-100}\) peptide, which binds to ATF-2 and behaves as a dominant negative form of ATF-2 by blocking its transcriptional activity in melanoma cells (27). Nucleofection of this plasmid into CD3+ cells from six healthy tuberculin reactors that were cultured with autologous monocytes and heat-killed M. tuberculosis reduced IFN-γ secretion in a dose-dependent manner by up to 80%, compared with cells nucleofected with the empty vector (Fig. 6A). Nucleofection of the ATF-2\(^{50-100}\) peptide did not affect IFN-γ production by CD3+ cells cultured with monocytes and medium alone, with mean IFN-γ levels ranging from 16 to 30 pg/ml (data not shown). Inhibition of ATF-2 also reduced IFN-γ mRNA expression by up to 99% (Fig. 6B), suggesting that this effect was mediated through transcription of IFN-γ. Nucleofection of CD3+ cells with the plasmid expressing the ATF-2\(^{50-100}\) peptide significantly reduced expression, not only of ATF-2, but also of CREB and c-Jun in a dose-dependent manner, compared with cells nucleofected with the empty plasmid (Fig. 6C). To confirm the effect of the ATF-2\(^{50-100}\)-expressing plasmid with a stably transfected cell line, we used nucleofection to introduce the empty pcDNA3 plasmid or the pcDNA3 plasmid encoding ATF-2\(^{50-100}\) into the human HUT-78 T cell line. The cells expressing ATF-2\(^{50-100}\) had reduced expression of CREB, c-Jun, and ATF-2 (data not shown). These findings indicate that ATF-2 positively regulates Ag-induced IFN-γ transcription and that ATF-2 also controls the expression of CREB and c-Jun.

**Effect of inhibiting ATF-2 in CD3+ T cell with siRNA**

Given the unexpected result that ATF-2 may contribute to expression of CREB and c-Jun, we wanted to confirm this effect by an alternative method and therefore used the RNAi technique to inhibit ATF-2 expression in human T cells. We nucleofected CD3+ cells from three healthy tuberculin reactors with ATF-2 siRNA, cultured them with autologous monocytes stimulated with heat-killed M. tuberculosis, and then measured expression of CREB, ATF-2, and c-Jun (Figs. 7, A and B). Control siRNA to the irrelevant gene encoding GFP did not affect expression of these transcription factors, whereas ATF-2 siRNA reduced expression of CREB and c-Jun by >70%. ATF-2 siRNA also decreased M. tuberculosis-induced secretion of IFN-γ by >60%, whereas the GFP control siRNA had no effect (Fig. 7C). Thus, these findings confirmed our results with the dominant-negative ATF-2 plasmid.
FIGURE 7. Effect of ATF-2 siRNA on expression of ATF-2, CREB, c-Jun, and IFN-γ. A. Effect of ATF-2 siRNA on expression of transcription factors. Purified CD3+ cells from three healthy tuberculin reactors were nucleofected with siRNA to ATF-2 or to GFP, or no siRNA. Forty-eight hours postnucleofection, the cells were cocultured with autologous monocytes in the presence of 2.5 μg/ml of heat-killed *M. tuberculosis* for 72 h. Cells were collected and blotted for ATF-2, CREB, c-Jun, and β-actin. A representative result is shown. B, Densitometry analysis of ATF-2, CREB, and c-Jun bands in A. For the experiment shown in A, band intensity was quantified by densitometry after normalization for intensity of the β-actin band. C. Effect of ATF-2 siRNA on *M. tuberculosis*-induced IFN-γ secretion. Cells from three healthy tuberculin reactors were treated as in A. After culture of CD3+ cells with autologous monocytes in the presence of *M. tuberculosis* for 72 h, supernatants were collected and IFN-γ concentrations were measured by ELISA. A representative result is shown.

Discussion

IFN-γ is pivotal for human defenses against *M. tuberculosis* and other intracellular pathogens, and the proximal promoter of IFN-γ is necessary and sufficient for its transcription in activated T cells (31). Previous studies of the molecules that bind to the IFN-γ proximal promoter have utilized IFN-γ promoter constructs in mitogen-stimulated Jurkat T cells and in transgenic mice (17, 31–34), but it is unclear if these findings reflect events in primary human T cells during a physiologic response to microbial Ag. In the present report, we studied the binding and regulatory effects of CREB/ATF and AP-1 transcription factors on the proximal promoter of IFN-γ in human T cells stimulated with *M. tuberculosis* Ags. Using EMSAs, supershift assays, and promoter pull-down assays, in combination with Western blotting, we demonstrated that CREB, ATF-2, and c-Jun, but not CREM, ATF-1, or c-Fos, bind to the proximal promoter of IFN-γ upon stimulation with *M. tuberculosis*, and chromatin immunoprecipitation indicated that these transcription factors form a complex upon stimulation in T cells. Chromatin immunoprecipitation assay confirmed these results in live Ag-activated T cells. Inhibition of ATF-2 by a dominant-negative plasmid or by siRNA reduced *M. tuberculosis*-induced expression of IFN-γ mRNA and protein and decreased levels of CREB, ATF-2, and c-Jun. We previously showed that siRNA to CREB reduced expression of IFN-γ but did not have an effect on expression of ATF-2 and c-Jun. These findings suggest that CREB, ATF-2, and c-Jun are recruited to and bind to the proximal promoter of IFN-γ as part of a protein complex, and that they positively regulate IFN-γ transcription in response to microbial Ag. Additionally, ATF-2 controls expression of CREB and c-Jun during T cell activation.

IFN-γ gene regulation is complex, involving multiple enhancer and repressor elements upstream of the transcription start site (12, 31, 33, 35–38) as well as regulatory regions in the introns (32). We focused our study on the proximal (−73 to −48 bp) IFN-γ promoter for several reasons. First, the sequence is highly conserved in mammals and is necessary and sufficient for transcription of IFN-γ in activated human T cells (31). Second, activation of T cells through the TCR and the IL-12 receptor, a process that parallels production of IFN-γ in response to *M. tuberculosis* and other intracellular pathogens, increased activation of the proximal but not the distal IFN-γ promoter element (33). Finally, methylation is a widespread mechanism used to control mammalian gene expression, and the Cpg motif at −53 bp in the proximal promoter of IFN-γ is hypermethylated in non-T cells, naive T cells, and Th2 cells that do not produce IFN-γ upon stimulation, whereas this motif is hypomethylated in Th1 cells that produce IFN-γ (14).

The proximal promoter of IFN-γ contains the sequence AGCGT, which is a noncanonical low-affinity CRE half site that forms the central portion of the full high-affinity octamer CRE site, TCGACGTCAG (39). CRE sites are bound by CREB/ATF and AP-1 transcription factors, and previous studies have shown that CREB, ATF-1, ATF-2, and c-Jun bind to the IFN-γ proximal promoter (13, 31), but the effect of binding has been reported to inhibit or enhance IFN-γ transcription. Studies in Jurkat T cells and transgenic mice suggested that CREB inhibited transcription of IFN-γ (31, 33). In contrast, chromatin immunoprecipitation demonstrated increased recruitment of CREB to the IFN-γ proximal promoter in human Th1 but not Th2 cells (14), and we found that down-regulation of CREB by siRNA and intracellular Abs reduced IFN-γ production by primary human T cells in response to *M. tuberculosis* (19), indicating that CREB enhances IFN-γ production. Additionally, some authors found that c-Jun positively regulates transcription of IFN-γ by mitogen-stimulated Jurkat T cells (31), whereas others suggested that c-Jun does not affect IFN-γ expression, because c-Jun levels were similar in Th1 and Th2 cells (33).
Using several techniques, we found that activation of peripheral blood T cells by *M. tuberculosis* increased phosphorylation of CREB, ATF-2, and c-Jun. Coimmunoprecipitation demonstrated that these three transcription factors interact with each other in stimulated T cells, and chromatin immunoprecipitation showed that these same transcription factors bind to the IFN-γ proximal promoter in live Ag-stimulated T cells. This combination of findings strongly suggests that CREB, ATF-2, and c-Jun bind as part of a complex to the IFN-γ proximal promoter during T cell activation. Our conclusions are supported by recent work demonstrating that hypermethylation of the proximal promoter of IFN-γ inhibits binding by CREB, ATF-2, and c-Jun in nuclear extracts of a Th1 cell line (14). Furthermore, the present study and our previous work (16) show that ATF-2 and CREB are both required for optimal transcription of IFN-γ, and that ATF-2 controls expression of CREB and c-Jun in primary human T cells.

Transcription of IFN-β, IL-2, and TNF-α are controlled through an enhancerome consisting of a complex of transcription factors that directly bind DNA and transcriptional coactivators that form a scaffold to stabilize interactions between DNA-binding proteins and the transcriptional machinery, such as RNA polymerase. These coactivators can also acetylate histones at the site of transcription initiation, opening up the chromatin structure and facilitating transcription, as has been demonstrated for expression of IFN-γ during Th1 development (40, 41). We speculate that CREB, ATF-2, and c-Jun form at least part of such a complex that increases the stability of binding to the IFN-γ proximal promoter and facilitates recruitment of transcriptional coactivators, such as CREB-binding protein and p300, that enhance efficient transcription of IFN-γ. Our results are consistent with genome-wide promoter analysis that demonstrated that gene transcription is not generally activated by phosphorylated CREB and transcriptional coactivators, and that additional CREB-associated partners are required to mediate this process (42). This is especially important during interactions with noncanonical CRE regulatory elements, such as that present in the IFN-γ proximal promoter.

We found that activation of T cells with *M. tuberculosis* induced phosphorylation of CREB, ATF-2, and c-Jun. Previous studies demonstrated that Ser133 phosphorylation does not affect binding of CREB to the high-affinity full CRE site, but increased binding of CREB to the noncanonical low-affinity CRE site in the IL-2Rα promoter (43). We speculate that phosphorylation of CREB may similarly enhance binding to the low-affinity CRE site in the IFN-γ proximal promoter. When T cells were stimulated with *M. tuberculosis*, reduction of ATF-2 expression by a dominant-negative plasmid or by siRNA inhibited expression of protein for CREB and c-Jun, whereas CREB siRNA did not affect levels of ATF-2 or c-Jun (19). The distal AP-1 binding site of the c-Jun promoter binds heterodimers of ATF-2 and c-Jun but not CREB (44). Additional ATF-2 regulates c-Jun expression in a teratocarcinoma cell line (41) and in murine thymocytes (45), whereas heterodimers of CREB and c-Jun do not activate transcription of target genes (29). These published data and our current findings suggest that ATF-2 and c-Jun heterodimers may increase c-Jun mRNA expression in T cells that respond to *M. tuberculosis*, whereas CREB does not.

Although we are not aware of published data directly demonstrating that heterodimers of ATF-2 and c-Jun bind to the CREB promoter, the latter contains CRE sites (46). Our results suggest that ATF-2 positively regulates transcription of CREB or increases stability of CREB mRNA and/or protein. Further studies are needed to address this question.

In summary, we found that CREB, ATF-2, and c-Jun are recruited to the proximal promoter of IFN-γ and regulate IFN-γ transcription in response to microbial Ag. Additionally, ATF-2 enhances expression of CREB and c-Jun through transcriptional or posttranscriptional mechanisms.

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Disclosures

The authors have no financial conflicts of interest.

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