Tristetraproline Mediates Interferon-γ mRNA Decay*

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Tristetraproline (TTP) regulates expression at the level of mRNA decay of several cytokines, including the T cell-specific cytokine, interleukin-2. We performed experiments to determine whether another T cell-specific cytokine, interferon-γ (IFN-γ), is also regulated by TTP and found that T cell receptor-activated T cells from TTP knock-out mice overproduced IFN-γ mRNA and protein compared with activated T cells from wild-type mice. The half-life of IFN-γ mRNA was 23 min in anti-CD3-stimulated T cells from wild-type mice, whereas it was 51 min in anti-CD3-stimulated T cells from TTP knock-out mice, suggesting that the overexpression of IFN-γ mRNA in TTP knock-out mice was due to stabilization of IFN-γ mRNA. Insertion of a 70-nucleotide AU-rich sequence from the murine IFN-γ 3′-untranslated region, which contained a high affinity binding site for TTP, into the 3′-untranslated region of a β-globin reporter transcript conferred TTP-dependent destabilization on the β-globin transcript. Together these results suggest that TTP binds to a functional AU-rich element in the 3′-untranslated region of IFN-γ mRNA and mediates rapid degradation of the IFN-γ transcript. Thus, TTP plays an important role in turning off IFN-γ expression at the appropriate time during an immune response.

Interferon-γ (IFN-γ),2 a critical T cell-derived cytokine, plays a key role in immune responses by inducing differentiation, activation, and proliferation of T cells, B cells, natural killer cells, and macrophages (1). It is predominantly secreted by activated CD4 T helper 1 lymphocytes, cytotoxic T lymphocytes, and natural killer cells, and it promotes effective innate and adaptive immune responses by inducing phagocytic activity of macrophages, up-regulating class I and class II major histocompatibility complex molecules, inducing expression of adhesion molecules to facilitate leukocyte-endothelium interactions, inducing the complement cascade, and initiating apoptosis (1). IFN-γ plays an essential role in host protection against intracellular pathogens and a variety of viral infections (2–6).

T cell receptor-mediated activation and co-receptor signaling trigger IFN-γ transcription, leading to protein production and secretion (7–9). Secreted IFN-γ regulates the activation of other types of immune cells and functions in autocrine or paracrine pathways to further promote the activation of T cells. Binding by IFN-γ to IFN-γ receptors 1 and 2 activates the JAK 1 and 2 and the STAT1 signaling pathway (10–15). STAT1 forms a homodimer that enters the nucleus and binds to promoter elements, activating or repressing transcription of IFN-γ regulated genes including genes involved in cellular proliferation, apoptosis, bacterial killing, immunomodulation, cellular differentiation, and leukocyte trafficking (3, 16). Thus, proper IFN-γ signaling is vital to the development of an immune response, and the expression of IFN-γ needs to be precisely regulated at multiple levels. Although transcriptional regulation of IFN-γ expression is important, post-transcriptional regulation at the level of mRNA decay is also vital for a normal immune response (for a review, see Ref. 17). For example, CD28 co-stimulation induces increased IFN-γ expression through stabilization of IFN-γ mRNA (18, 19). The rapid decay of IFN-γ mRNA also provides a potential mechanism for turning off the expression of IFN-γ at the appropriate time during an immune response.

Numerous genes involved in T cell activation, including genes encoding cytokines, signal transduction regulators, cell cycle regulators, and regulators of apoptosis, are regulated at the level of mRNA decay (19). Most mRNA transcripts that are induced following T cell activation exhibit rapid decay (19), and the rapid decay of these transcripts may provide a mechanism to turn off their expression at the appropriate time following T cell activation. Many of the labile transcripts expressed in T cells contain AU-rich elements (AREs), conserved sequence elements found in their 3′-untranslated region (UTR) that function as mediators of mRNA decay (19). ARE-dependent mRNA turnover is mediated by trans-acting proteins that bind to AREs and regulate transcript decay. Numerous ARE-binding proteins have been identified; some promote transcript degradation, whereas others promote transcript stabilization (20–25).
illustrated by the phenotype of TTP knock-out mice. These mice are normal at birth but develop a systemic inflammatory syndrome characterized by cachexia, dermatitis, erosive arthritis, and myeloid hyperplasia (28). The syndrome is caused by the overproduction of cytokines, especially tumor necrosis factor-α (TNF-α) and granulocyte macrophage colony-stimulating factor (GM-CSF), due to a lack of TTP-mediated mRNA decay (28–30). In addition to TNF-α and GM-CSF, several additional targets of TTP have been identified in macrophages and fibroblasts using genome-wide screens (31, 32). Our research group has previously shown that TTP is induced in T cells following T cell receptor-mediated activation and that the T cell-derived cytokine, interleukin-2 (IL-2), is overproduced in activated T cells from TTP knock-out mice due to stabilization of IL-2 mRNA (33). In the current work, we performed experiments to determine whether another T cell-specific cytokine, IFN-γ, is also regulated by TTP. We found dramatic overexpression of the IFN-γ transcript in activated T cells from TTP knock-out mice and showed that the overexpression of IFN-γ was due to stabilization of IFN-γ mRNA. Insertion of a 70-nucleotide AU-rich sequence, which contained an 18-nucleotide TTP-binding site, from the murine IFN-γ 3′-UTR into the 3′-UTR of a β-globin reporter transcript conferred TTP-dependent destabilization on the β-globin transcript. Together these results demonstrate that TTP mediates degradation of IFN-γ mRNA.

EXPERIMENTAL PROCEDURES

Purification and Stimulation of Primary Murine and Human T Cells—Generation of homozygous TTP knock-out mice has been described previously (28). Purification of T lymphocytes from wild-type or TTP knock-out C57BL/6 mice was performed as described previously (33). The purified T cell populations from wild-type and TTP knock-out mice were similar with respect to cell surface CD4 and CD8 expression, and both populations consisted of cells that were 87–90% CD3-positive, 46–49% CD4-positive, and 35–39% CD8-positive. Purified murine T cells were unstimulated (medium) or stimulated with an anti-CD3 antibody (R&D Systems) for 6 h as described previously (33). Protocols for animal experimentation were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee.

Primary human T cell populations from peripheral blood were purified from human buffy coat packs obtained from the American Red Cross as described previously (33) and consisted of 90–98% CD3-positive T cells. These cells were stimulated with anti-CD3 antibodies as described previously (33).

Measurement of Murine IFN-γ mRNA by Real Time Polymerase Chain Reaction—Total cellular RNA was isolated from purified T cells from wild-type and TTP knock-out mice using TRIzol reagent (Invitrogen) according to the manufacturer’s directions. RNA was additionally purified using the Qiagen RNeasy cleanup kit, including DNase treatment, according to the manufacturer’s instructions (Qiagen). cDNA used for real time PCR analysis was synthesized from total cellular RNA using Stratascript reverse transcriptase (Stratagene) and transcript-specific primers. PCR amplifications were performed using the QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer’s instructions. Commercially purchased (Integrated DNA Technologies) transcript-specific oligonucleotide primers used for the PCR amplifications are listed as sense followed by antisense: IFN-γ, 5′-CTTCTTCAGCAACA-GCAAGG-3′, 5′-TGAGCTATGTTGCTTG-3′; hypoxanthine phosphoribosyltransferase, 5′-GTTGAAAGGACC-TCTCAGA-3′, 5′-AGTCAGGGGATCCCAACA-3′. PCR amplifications were performed using a Bio-Rad iCycler thermocycler, and the data were analyzed using iCycler software. IFN-γ mRNA levels were normalized to hypoxanthine phosphoribosyltransferase mRNA levels.

To assay IFN-γ mRNA decay rates, purified T cells from wild-type or TTP homozygous knock-out mice were stimulated with anti-CD3 antibodies for 6 h and then treated with 10 μg/ml of actinomycin D, and total RNA was harvested after 0, 15, 30, and 45 min. IFN-γ mRNA levels at each time point were normalized to the corresponding level of the hypoxanthine phosphoribosyltransferase transcript. GraphPad Prism software version 4.00 was used to calculate transcript half-lives based on a one-phase exponential decay model.

Measurement of Murine IFN-γ Protein—IFN-γ concentrations in culture supernatants were measured by enzyme-linked immunosorbent assay using the Pharmingen OptEIA Mouse IFN-γ Set (BD Biosciences) according to the manufacturer’s instructions.

Tet-Off mRNA Decay Assay—Measurement of the decay of β-globin reporter constructs was performed as described previously (33) with minor modifications. In some experiments, HeLa Tet-Off cells (1.6 × 10⁶ cells) were transfected with 3.0 μg of the tet-responsive reporter construct that encoded rabbit β-globin transcripts that contained the 70-nucleotide murine IFN-γ sequence or the mutated sequence shown in Fig. 3A inserted at the unique BglII restriction site in its 3′-UTR. In other experiments, cells were transfected with β-globin reporter constructs in which the β-globin 3′-UTR was replaced with the 631-bp 3′-UTR from murine IFN-γ or a mutated version that contained the mutations shown in Fig. 3A. The cells were co-transfected with either 100 ng of the pCMV.TTP.myc plasmid that expresses Myc-tagged murine TTP or the pcDNA-3 plasmid (Invitrogen) as a mock control. The pcCMV.TTP.myc plasmid was created by excising the Myc-tagged murine TTP cDNA from the plasmid hphMX-TTP (34) and cloning it into the pcDNA-3 plasmid (Invitrogen) as a mock control. The pcCMV.TTP.myc plasmid was created by excising the Myc-tagged murine TTP cDNA from the plasmid pCMV.TTP.myc plasmid that expresses Myc-tagged murine TTP and cloning it into the pcDNA-3 plasmid (Invitrogen) as a mock control. The pcCMV.TTP.myc plasmid was created by excising the Myc-tagged murine TTP cDNA from the plasmid hphMX-TTP (34) and cloning it into the pcDNA-3 plasmid (Invitrogen) as a mock control. The pcCMV.TTP.myc plasmid was created by excising the Myc-tagged murine TTP cDNA from the plasmid hphMX-TTP (34) and cloning it into the pcDNA-3 plasmid (Invitrogen) as a mock control. The pcCMV.TTP.myc plasmid was created by excising the Myc-tagged murine TTP cDNA from the plasmid hphMX-TTP (34) and cloning it into the pcDNA-3 plasmid (Invitrogen) as a mock control.
Overexpression of Murine or Human TTP in HeLa Cells—HeLa cells were grown as described previously (33). Approximately 1.1 × 10⁶ HeLa cells were transfected with 54 μg of the pCMV.TTP.myc plasmid that expresses Myc-tagged murine TTP, the pCMV.TTP.Tag plasmid that expresses hemagglutinin (HA)-tagged human TTP, or the pcDNA-3 plasmid (Invitrogen) as a mock control. After 48 h, the cells were harvested, and cytoplasmic extracts were prepared as described previously (33).

RNA Probes and Competitors—RNA oligonucleotides were purchased commercially (Dharmacon Research, Boulder, CO). The sequence for each RNA oligonucleotide was as follows: IFN-γ ARE, CUAUUUAUUAAUAUUUAA; IL-2 ARE, UAUUUAAUUAAUUUAUUUAUUUAUUU; mutated ARE, UCACUCACACUCACUCUC. The IFN-γ ARE RNA oligonucleotide probe was end-labeled with [γ-³²P]ATP (6000 Ci/mmol) using T4 polynucleotide kinase to produce a radiolabeled probe with a specific activity of ~4 × 10⁶ cpm/μg.

Gel Shift, Supershift, and UV Cross-linking Assays—Gel shift, supershift, and UV cross-linking assays were performed as described previously (33). Cytoplasmic extracts (8–10 μg of protein) were incubated for 20 min with 15–35 fmol of the ³²P-labeled IFN-γ ARE RNA probe in the absence or presence of 10 pmol of unlabeled IFN-γ, IL-2, or mutated ARE RNA oligonucleotides in a total volume of 20–24 μl. For supershift assays, an anti-Myc antibody (clone 9E10, Upstate), an anti-HA antibody (Y-11, Santa Cruz Biotechnologies), an anti-TTP antibody (N-18, Santa Cruz Biotechnologies), or a control anti-Sp1 antibody (Santa Cruz Biotechnologies) was added to the reaction mixtures.

RESULTS

IFN-γ Is Overexpressed in T Cells from TTP Knock-out Mice—We have previously shown that TTP is expressed in human and murine T cells (33, 35) and is regulated following T cell receptor-mediated stimulation (35). TTP regulates the expression of numerous ARE-containing cytokines at the level of mRNA decay (29, 30, 36), including the T cell-specific cytokine IL-2 (33). To determine whether IFN-γ, another T cell-specific cytokine mRNA. Compared with wild-type mice, however, the anti-CD3-induced expression of IFN-γ mRNA was 4.7-fold higher in T cells from TTP knock-out mice. In parallel experiments, culture supernatants were collected 24 h after stimulation, and IFN-γ protein levels were measured by enzyme-linked immunosorbent assay (Fig. 1B). IFN-γ protein production was not detected in T cells from wild-type or TTP knock-out mice following stimulation with medium alone. Stimulation of wild-type mice with anti-CD3 induced no detectable IFN-γ production; however, IFN-γ production was dramatically increased following stimulation of T cells from TTP knock-out mice with anti-CD3. These data demonstrate that IFN-γ production is dramatically increased in activated T cells from TTP knock-out mice. Overall these experiments demonstrate that IFN-γ mRNA and protein are both overexpressed in activated T cells from TTP knock-out mice.

IFN-γ mRNA Is Stabilized in T Cells from TTP Knock-out Mice—Because TTP regulates the expression of other cytokines by mediating mRNA decay (29, 30, 33), we hypothesized that overexpression of IFN-γ mRNA in T cells from TTP knock-out mice could be due to mRNA stabilization as a result of TTP deficiency. To test this hypothesis, we measured IFN-γ mRNA decay rates in purified T cells from wild-type and TTP knock-out mice. T cells were stimulated for 6 h with an anti-CD3 antibody, and then actinomycin D was added to stop transcription. Total cellular RNA was harvested 0, 15, 30, and 45 min after the addition of actinomycin D, and IFN-γ mRNA levels were measured by real time PCR (Fig. 2). IFN-γ mRNA decay half-lives were calculated based on a model of first order decay to be 23 min (95% confidence interval, 21–26 min) in T cells from wild-type mice and 51 min (95% confidence interval, 42–66 min) in T cells from TTP knock-out mice. The difference in transcript half-lives comparing wild-type and TTP knock-out mice was statistically significant with a p value of less than 0.0001, indicating that IFN-γ mRNA is more stable in the absence of TTP. The increased stability of IFN-γ in T cells from TTP knock-out mice compared with wild-type mice suggests that TTP mediates IFN-γ mRNA decay.
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TTP Mediates the Decay of a β-globin Reporter Transcript That Contains an AU-rich Sequence Derived from the Murine IFN-γ 3′-UTR—TTP regulates the decay of cytokine transcripts such as the TNF-α, GM-CSF, and IL-2 transcripts by binding to conserved AU-rich sequences in their 3′-UTR (29, 30, 33). By comparing the murine and human IFN-γ 3′-UTR sequences, we identified a 70-nucleotide AU-rich sequence that was highly conserved (Fig. 3A) and hypothesized that this conserved sequence could function as a RNA decay regulator. To test this hypothesis, HeLa Tet-Off cells, which do not express detectable levels of endogenous TTP (35), were transiently transfected with a murine TTP expression construct or a pcDNA-3 control plasmid (Mock) along with tet-repressible β-globin reporter constructs in which the conserved 70-nucleotide murine IFN-γ sequence or the mutated sequence shown in Fig. 3A was inserted into the 3′-UTR. A GFP expression construct that was not tetracycline-regulated was also co-transfected to control for transfection efficiency. After 48 h, doxycycline was added to the medium to stop transcription from the tet-responsive promoter, and then β-globin and GFP mRNA levels were measured over time by Northern blot (Fig. 3, B and

**FIGURE 3.** TTP regulates the decay of mRNA containing the IFN-γ ARE. A, the top line shows a 70-nucleotide AT-rich sequence from the 3′-UTR of the murine IFN-γ CDNA (RefSeq transcript ID NM_008337) that was cloned into the 3′-UTR of the pTetBBB β-globin reporter construct. The boxed sequence indicates an 18-nucleotide AU-rich IFN-γ ribo-oligonucleotide that was identical in mouse and human sequences and was used for the binding reactions shown in Figs. 4 and 5. In B and C, HeLa Tet-Off cells were transfected with tet-responsive β-globin reporter plasmids in which the 70-nucleotide murine IFN-γ ARE sequence (IFN-γ 70 nt) or the mutated sequence shown in A (mtIFN-γ 70 nt) was inserted into the β-globin 3′-UTR. In D and E, HeLa Tet-Off cells were transfected with tet-responsive β-globin reporter plasmids in which the β-globin 3′-UTR was replaced with the entire 3′-UTR from murine IFN-γ (IFN-γ 3′-UTR) or a mutated murine IFN-γ 3′-UTR that contained the mutations shown in A (mtIFN-γ 3′-UTR). The cells were co-transfected with the pCMV.TTP.Tag plasmid (TTP) or a pcDNA3 control plasmid (Mock), and the pTracer GFP expression construct was co-transfected in each group as a control for transfection efficiency. After 72 h, doxycycline was added to stop transcription from the tet-responsive promoter, and total RNA was isolated after the indicated times. Total RNA (10 μg) from each sample was analyzed by Northern blot using a 32P-labeled rabbit β-globin probe (BG) and a 32P-labeled GFP probe (GFP). The experiments shown in B and D were both performed four times. Band intensities were measured using a phosphorimaging system. For each condition, the hybridization intensity values of β-globin mRNA were normalized to GFP mRNA levels. The normalized value for β-globin expression at time 0 was then set to 100%, and all other mRNA levels were graphed over time relative to that value. The graphs in C and E correspond to the experiments shown in B and D, respectively. Each point represents the mean and S.E. from the four experiments.

**FIGURE 2.** IFN-γ mRNA is stabilized in TTP knock-out mice. Purified murine T cells from wild-type or TTP knock-out mice were stimulated for 6 h with anti-CD3 antibodies. Actinomycin D (10 μg/ml) was then added to stop transcription, and total RNA was isolated after 0, 15, 30, and 45 min. IFN-γ mRNA was measured using real-time reverse transcription-PCR, and expression levels were normalized to hypoxanthine phosphoribosyltransferase mRNA levels. The normalized level of IFN-γ mRNA was then graphed over time relative to that value. The graphs in Fig. 3 were inserted into the 3′-UTR. A GAF expression construct that was not tetracycline-regulated was also co-transfected to control for transfection efficiency. After 48 h, doxycycline was added to the medium to stop transcription from the tet-responsive promoter, and then β-globin and GFP mRNA levels were measured over time by Northern blot (Fig. 3, B and

**FIGURE 1.** The six single nucleotide mutations (T to C) that were introduced to create a mutated sequence. The corresponding sequence from the human IFN-γ 3′-UTR is shown on the top line to illustrate the cross-species sequence conservation. The boxed sequence indicates an 18-nucleotide AU-rich IFN-γ ribo-oligonucleotide that was identical in mouse and human sequences and was used for the binding reactions shown in Figs. 4 and 5. In B and C, HeLa Tet-Off cells were transfected with tet-responsive β-globin reporter plasmids in which the 70-nucleotide murine IFN-γ ARE sequence (IFN-γ 70 nt) or the mutated sequence shown in A (mtIFN-γ 70 nt) was inserted into the β-globin 3′-UTR. In D and E, HeLa Tet-Off cells were transfected with tet-responsive β-globin reporter plasmids in which the β-globin 3′-UTR was replaced with the entire 3′-UTR from murine IFN-γ (IFN-γ 3′-UTR) or a mutated murine IFN-γ 3′-UTR that contained the mutations shown in A (mtIFN-γ 3′-UTR). The cells were co-transfected with the pCMV.TTP.Tag plasmid (TTP) or a pcDNA3 control plasmid (Mock), and the pTracer GFP expression construct was co-transfected in each group as a control for transfection efficiency. After 72 h, doxycycline was added to stop transcription from the tet-responsive promoter, and total RNA was isolated after the indicated times. Total RNA (10 μg) from each sample was analyzed by Northern blot using a 32P-labeled rabbit β-globin probe (BG) and a 32P-labeled GFP probe (GFP). The experiments shown in B and D were both performed four times. Band intensities were measured using a phosphorimaging system. For each condition, the hybridization intensity values of β-globin mRNA were normalized to GFP mRNA levels. The normalized value for β-globin expression at time 0 was then set to 100%, and all other mRNA levels were graphed over time relative to that value. The graphs in C and E correspond to the experiments shown in B and D, respectively. Each point represents the mean and S.E. from the four experiments.
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C). In the absence of TTP, the β-globin reporter transcripts were stable with a half-life of 380 min (95% confidence interval, 307–499 min) for the transcript containing the IFN-γ insert and 441 min (95% confidence interval, 325–687 min) for the transcript containing the mutated insert. Co-transfection of TTP led to destabilization (p < 0.05) of the transcript containing the IFN-γ insert (half-life, 75 min; 95% confidence interval, 50–146 min) but not the transcript containing the mutated insert (half-life, 565 min; 95% confidence interval, 426–839 min). Thus, insertion of the 70-nucleotide AU-rich sequence from IFN-γ mRNA into the 3′-UTR of β-globin mRNA was sufficient to promote TTP-dependent mRNA decay.

We performed similar experiments using the same β-globin reporter transcript in which the entire β-globin 3′-UTR was replaced with the full-length murine IFN-γ 3′-UTR or a 3′-UTR that was identical except it contained the mutations shown in Fig. 3A. The β-globin reporter transcript containing the wild-type IFN-γ 3′-UTR decayed with a half-life of 174 min (95% confidence interval, 121–206 min) in mock-transfected cells but was destabilized (p < 0.001) in TTP-transfected cells with a half-life of 91 min (95% confidence interval, 54–114 min) (Fig. 3, D and E). In contrast, the β-globin reporter transcript that contained the mutated IFN-γ 3′-UTR was relatively stable whether or not TTP was present with a half-life of 876 min (95% confidence interval, 723–2593 min) in mock-transfected cells and a half-life of 845 min (95% confidence interval, 560–3791 min) in TTP-transfected cells. Thus, exogenous expression of TTP in HeLa cells induced a more rapid decay of the β-globin mRNA than did the wild-type IFN-γ 3′-UTR, which was stabilized (p > 0.05) of the transcript containing the mutated insert (half-life, 565 min; 95% confidence interval, 426–839 min). Thus, insertion of the 70-nucleotide AU-rich sequence from IFN-γ mRNA into the 3′-UTR of β-globin mRNA was sufficient to promote TTP-dependent mRNA decay.

TTP Binds with High Affinity to a Conserved 18-nucleotide ARE from the 3′-UTR of IFN-γ mRNA—In humans, the consensus sequence WWWWUAAUUUUWW (W represents U or A) has been used to identify ARE-containing transcripts (37), and TTP binds to AU-rich sequences that are identical or similar to this consensus (26, 38, 39). Although we did not find the precise consensus ARE sequence in the 3′-UTR of IFN-γ mRNA, we identified the similar sequence CTATTTAATATTTAA, which is identical in the murine and human IFN-γ sequences (Fig. 3A), as a candidate ARE. UV cross-linking assays (Fig. 4A) were performed to determine whether TTP was able to interact with this IFN-γ sequence. We transfected HeLa cells with plasmids that expressed the murine TTP protein, the human TTP protein, or a control plasmid (mock) and prepared cytoplasmic extracts from these cells. These extracts were mixed with a radiolabeled probe containing the 18-nucleotide IFN-γ ARE sequence, and the reaction mixtures were treated with UV energy and then separated by electrophoresis on SDS-polyacrylamide gels. Although mock-transfected cells expressed minimal activity that UV cross-linked to the IFN-γ ARE probe (lane 1), transfection with murine TTP (lane 2) or human TTP (lane 6) led to the expression of a TTP-dependent RNA binding activity. This binding was competed by the addition of an excess of an unlabeled IFN-γ ARE ribo-oligonucleotide (lanes 3 and 7) or an IL-2 ARE ribo-oligonucleotide that contains a known TTP-binding site (33) (lanes 4 and 8) but was competed less efficiently with a ribo-oligonucleotide that contained a mutated ARE (lanes 5 and 9). Gel shift assays showed the same results: murine or human TTP-dependent RNA binding activities were...
FIGURE 5. TTP binds to the IFN-γ ARE with high affinity. Cytoplasmic extracts from HeLa cells (10 μg of protein) that were transfected with the murine pcMV.mTTP.myc-Tag expression plasmid (mTTP) or the human pcMV. hTTP.Tag expression plasmid (hTTP) were incubated with 20 fmol of a 32P end-labeled IFN-γ ARE probe in the presence of increasing amounts of unlabeled ARE RNA (40 fmol to 5 pmol) RNA oligonucleotides. Binding reactions were then separated by electrophoresis on 10% polyacrylamide gels under non-denaturing conditions. The bands representing the free probe and the TTP-containing RNA-protein complex were visualized and quantified using a phosphorimaging system. The position of migration of the TTP-containing RNA-protein complex was indicated with an arrow. The graph to the right of each gel presents binding data from three identical independent experiments. Percent maximal binding (y axis) was plotted against the log of the total RNA concentration in molar (x axis) using GraphPad Prism version 4.03 software. Each point represents the mean and S.E. from three experiments.

The results obtained in this study demonstrate that IFN-γ mRNA expression in T cells is regulated by TTP. In TTP knock-out mice, the absence of TTP led to increased expression of IFN-γ mRNA and protein by activated T cells. The increased expression of IFN-γ in activated T cells from TTP knock-out mice could be explained by the stabilization of IFN-γ mRNA due to the absence of TTP. The effect of TTP knock-out on IFN-γ protein levels was more profound than the effect on IFN-γ mRNA levels, implying that TTP could have an effect on IFN-γ translation in addition to its effect on mRNA degradation. We identified a functional ARE in the 3′-UTR of IFN-γ mRNA that was capable of conferring TTP-dependent destabilization when it was inserted into the 3′-UTR of an otherwise stable β-globin reporter transcript, and we showed that TTP binds with high affinity to a conserved AU-rich sequence contained within this functional ARE. Overall, these results suggest that TTP functions to mediate ARE-dependent decay of the IFN-γ transcript following T cell activation.

The overexpression of IFN-γ protein by activated T cells from TTP knock-out mice may induce further cellular activation through autocrine or paracrine mechanisms, leading to the activation of a variety of IFN-γ-responsive genes that are important for combating a variety of intracellular pathogens and viral infections (2–6). Several of these IFN-γ-responsive transcripts encode proteins that regulate important functions in the immune system, including complement activation and opsonization (41–43), chemotaxis and cellular migration (44–
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and co-stimulation for antigen-specific T cells (49). Thus, overproduction of IFN-γ in TTP knock-out mice would be expected to propagate and amplify an immune response.

Our results suggest that TTP is an important immune modulator because it functions to turn off the expression of IFN-γ and other cytokines at the appropriate time during an immune response, thereby returning the immune system to homeostasis. IFN-γ itself has been shown to induce the expression of TTP (50), creating a regulatory loop whereby expression of IFN-γ would down-modulate an immune response by promoting TTP-dependent down-regulation of cytokine expression. In addition to IFN-γ, other cytokines such as TNF-α (28, 29), GM-CSF (30), IL-2 (33), IL-10 (32), and the chemokine CXCL1 (51) are coordinately down-regulated by TTP during the course of an immune response. The importance of TTP-dependent ARE-mediated mRNA decay in maintaining homeostasis is vividly displayed in the TTP knock-out mice where lack of TTP results in a severe inflammatory syndrome due to the overproduction of cytokines (28, 29). Interestingly we have found that T cell cytokines such as IL-2 and IFN-γ are expressed at low or undetectable levels in purified T cells from TTP knock-out mice, suggesting that these T cells are not activated in vivo (Ref. 33 and Fig. 1B). We only observed overproduction of these cytokines by T cells when we activated them ex vivo. Our TTP knock-out mice, however, are maintained in a specific pathogen-free environment, and it would be interesting to determine how T cells from these mice would behave if they were confronted with an infectious challenge.

In addition to TTP, several other ARE-binding proteins can stabilize or destabilize a variety of ARE-containing transcripts including TNF-α, GM-CSF, IL-2, c-fos, and c-jun transcripts (20–25). ARE-containing transcripts are stabilized when bound by ARE-binding proteins such as HuR and NF90 (20–22). In contrast, ARE-binding proteins such as TTP, BRF1, or K homology splicing regulatory protein (KSRP) target ARE-containing transcripts for rapid degradation (23–25). TTP mediates mRNA decay by recruiting components of the mRNA decay machinery to the transcript (26, 27). TTP binds to the deadenylase Ccr4 (27) and stimulates deadenylation through the exosome, a multiprotein complex that mediates 3′-5′ mRNA decay (26, 27). In addition, it binds to the decapping enzyme Dcp1 (27) and the exonuclease Xrn1 (26, 27), components of the mammalian 5′ to 3′ mRNA decay pathway. Although TTP binds to several components of the mRNA decay machinery, the biochemical mechanisms by which TTP mediates mRNA decay are still largely unknown.

We have shown that TTP and HuR are the predominant ARE-binding proteins expressed in primary human T cells (35). The ARE-stabilizing protein, HuR, binds to newly synthesized ARE-containing mRNAs and transports them from the nucleus to the cytoplasm (53). Once in the cytoplasm, HuR transiently mediates transcript stabilization by preventing the recruitment of the mRNA decay machinery to the transcript and allowing the transcript to be translated (54, 55). We have previously shown that TTP competes with HuR for binding to several ARE-containing transcripts and proposed a model whereby TTP induced following T cell activation displaces HuR from ARE-containing transcripts and mediates transcript degradation (35). This model provides a mechanism for the transient expression and subsequent down-regulation of ARE-containing transcripts, such as cytokine transcripts, following T cell activation.

This process might be regulated by T cell activation signals. For example, the p38 MAPK signaling pathway, acting through the downstream kinase MAPK-activated protein kinase-2 (MK2), regulates the stability of many ARE-containing mRNAs. MK2 phosphorylates TTP at serines 52 and 178 (56), and phosphorylated TTP then forms a complex with the 14–3–3 adaptor and is rendered unable to mediate the decay of ARE-containing transcripts (57). Activation of the p38 MAPK pathway also promotes cytoplasmic localization and stabilization of the TTP protein (58, 59). Based on these findings, we hypothesize that TTP phosphorylation after T cell activation modulates its ability to mediate mRNA decay. CD28 co-stimulation of T cell receptor-stimulated T cells leads to stabilization of several cytokine transcripts, including IFN-γ transcripts (18, 19), and it is possible that this effect is mediated by TTP phosphorylation. Further work is needed to better understand the mechanisms by which TTP regulates T cell cytokine expression.

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