Enhanced stability of tristetraprolin mRNA protects mice against immune-mediated inflammatory pathologies

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Tristetraprolin (TTP) is an inducible, tandem zinc-finger mRNA binding protein that binds to adenylate-uridylate-rich elements (AREs) in the 3′-untranslated regions (3′ UTRs) of specific mRNAs, such as that encoding TNF, and increases their rates of deadenylation and turnover. Stabilization of Tnf mRNA and other cytokine transcripts in TTP-deficient mice results in the development of a profound, chronic inflammatory syndrome characterized by polyarticular arthritis, dermatitis, myeloid hyperplasia, and autoimmunity. To address the hypothesis that increasing endogenous levels of TTP in an intact animal might be beneficial in the treatment of inflammatory diseases, we generated a mouse model (TTPΔARE) in which a 136-base instability motif in the 3′ UTR of TTP mRNA was deleted in the endogenous genetic locus. These mice appeared normal, but cultured fibroblasts and macrophages derived from them exhibited increased stability of the otherwise highly labile TTP mRNA. This resulted in increased TTP protein expression in LPS-stimulated macrophages and increased levels of TTP protein in mouse tissues. TTPΔARE mice were protected from collagen antibody-induced arthritis, exhibited significantly reduced inflammation in imiquimod-induced dermatitis, and were resistant to induction of experimental autoimmune encephalomyelitis, presumably by dampening the excessive production of proinflammatory mediators in all cases. These data suggest that increased systemic levels of TTP, secondary to increased stability of its mRNA throughout the body, can be protective against inflammatory disease in certain models and might be viewed as an attractive therapeutic target for the treatment of human inflammatory diseases.

AU-rich elements | mRNA stability | inflammation | deadenylation

Tristetraprolin (TTP) is the prototype of a small family of RNA binding proteins that can bind to adenylate-uridylate (AU)–rich elements (AREs) in the 3′-UTR (3′ UTR) of its target mRNAs and promote their rapid turnover (1, 2). TTP-deficient mice developed a chronic systemic inflammatory syndrome (3) that was prevented by interfering with the action of TNF (3–5). Tnf mRNA was then identified as a direct target of TTP-mediated destabilization (4, 6); its increase in stability in the TTP KO mice leads to the crosstalk between overproduction of TNF protein (4, 5).

TTP mRNA expression exhibits a pattern characteristic of immediate-early response genes in several cell types, with low-to-undetectable levels of expression under basal conditions, and a rapid and transient induction upon stimulation (4, 7, 8). The transient nature of this induction is largely due to the instability of the TTP mRNA itself, part of which is thought to be due to AREs located within the 3′ UTR of TTP mRNA (4, 8, 9). Indeed, TTP has been suggested to bind to its own AREs and autoregulate its expression through a negative feedback loop (9). Although expression of TTP protein in these systems is also rapidly inducible, the protein is more stable than the mRNA after induction, often persisting at high levels for several hours (7, 10).

The severe systemic inflammatory phenotype of the TTP KO mice identified TTP as an endogenous antiinflammatory protein. We have long wondered whether increasing endogenous levels of TTP in an intact animal might protect against the development of immune and inflammatory diseases. Early attempts to accomplish this using transgenic delivery of TTP resulted in embryonic lethality, presumably due to unregulated overexpression. To test our hypothesis in a different way, we generated a novel knock-in mouse model in which an instability motif in the 3′ UTR of TTP mRNA was deleted in the mouse genome. We anticipated that, by deleting these instability elements, TTP mRNA would be stabilized under physiological conditions, and this would result in modest increases in TTP mRNA and protein levels that were still under the control of the endogenous genetic locus.

These mice, termed TTPΔARE mice, appear normal but exhibit increased TTP mRNA stability, as well as increased levels of TTP protein in their tissues. They were strikingly resistant to the development of three models of experimental immune-mediated inflammatory diseases. These data suggest that treatments leading to increased endogenous TTP levels could be a promising therapeutic approach in immune and inflammatory human diseases.

Results

Generation of TTPΔARE Mice. TTPΔARE mice were generated as described in SI Materials and Methods. Briefly, 136 bases (bases 1564–1699 of GenBank accession no. NM_011756) (depicted in Fig. 1A) were deleted in the endogenous human TTP gene on chromosome 3 (11). TTPΔARE mice were protected from collagen-induced arthritis, exhibited significantly decreased inflammation in imiquimod-induced dermatitis, and were resistant to induction of experimental autoimmune encephalomyelitis (EAE), presumably by dampening the excessive production of proinflammatory mediators in all cases. These data suggest that increased systemic levels of TTP, secondary to increased stability of its mRNA throughout the body, can be protective against inflammatory disease in certain models and might be viewed as an attractive therapeutic target for the treatment of human inflammatory diseases.
The TTP mRNA expression and stability, and TTP protein expression in primary cells and tissues derived from homozygous TTPΔARE mice. (A) Relative levels of TTP mRNA under basal (i.e., unstimulated) conditions in serum-deprived BMDMs (n = 3–4). (B) Time course of expression of TTP mRNA before and after stimulation (LPS; 1 μg/mL) in BMDMs. Data are expressed as a percentage of WT at 1 h (n = 3–4). (C) TTP mRNA decay in BMDMs and (D) MEFs. BMDMs or MEFs were stimulated with LPS (1 μg/mL) or 10% FBS (vol/vol), respectively, for 1 h, followed by treatment with actinomycin D. Percent remaining mRNA was measured by real-time RT-PCR (n = 4). The insets show semilogarithmic decay plots of the same data, analyzed by nonlinear regression. The approximate half-lives were: WT (BMDMs), ~32 min; TTPΔARE (BMDMs), ~80 min; WT (MEFs), ~28 min; TTPΔARE (MEFs), ~54 min. (E) TTP protein levels in BMDMs under basal and LPS (1 μg/mL) stimulated conditions. Tubulin was used as a loading control. (F) TTP protein expression in mouse tissues. The lane labeled TTP KO contains an equal amount of protein from the respective TTP KO mouse tissue, included as a negative control. Actin was used as a loading control. Statistical analysis was performed by two-tailed Student’s t test for A, C, and D and by two-way ANOVA for B. Error bars represent SEM; *P < 0.05, **P < 0.01, ***P < 0.001.

Effect of the TTPΔARE Mutation on TTP mRNA Expression, TTP mRNA Stability, and TTP Protein Expression in Cells and Mouse Tissues. The effect of the homozygous TTPΔARE mutation on TTP mRNA expression was examined in cultured primary bone marrow-derived macrophages (BMDM). Under unstimulated conditions, TTP mRNA levels were increased approximately threefold in the TTPΔARE cells (Fig. 1E). They increased dramatically in cells of both genotypes after LPS stimulation, but TTP mRNA levels were significantly elevated in the TTPΔARE cells compared with WT cells at 3 h (Fig. 1B). To test TTP mRNA stability, we stimulated TTP gene transcription in BMDM with LPS, or mouse embryonic fibroblasts (MEF) with 10% (vol/vol) FBS, for 1 h in each case, followed by treatment with actinomycin D to inhibit transcription, and quantitated the remaining RNA at various times. Under these conditions, TTP mRNA degraded significantly more slowly in both the TTPΔARE BMDM (Fig. 1C) and the MEF (Fig. 1D). These data demonstrate the increased stability of the mutant TTP mRNA in these cell types.

TTP protein was readily detectable in unstimulated conditions in the TTPΔARE BMDM but not in the WT BMDM (Fig. 1E). Furthermore, TTP protein levels were elevated compared with WT at all times after LPS stimulation in the TTPΔARE cells (Fig. 1E). Most importantly, TTP protein levels were increased in liver, spleen, and thymus from TTPΔARE mice (Fig. 1F).

Effect of the TTPΔARE Mutation on Potential TTP Targets in TTPΔARE BMDM and Intact Mice. We next analyzed the expression of known or suspected TTP targets in LPS-stimulated BMDM. For Tnf, Il1b, and Cxcl2 mRNAs, the levels in the TTPΔARE cells were significantly lower than WT at 6 h, although there was a general pattern of decreased expression at 3 h as well (Fig. S4A). There
were no significant differences at any time point for IL10, IL6, and II23a mRNAs, although there was a trend toward decreased expression at 3 and 6 h in the TTP\ ARE cells (Fig. S4A). In cultures of BMDM cells stimulated by LPS, IL-10 concentrations were significantly lower in the medium of TTP\ ARE cells at both 8 and 24 h, whereas levels of IL-1B and IL-6 were unchanged (Fig. S4B). Surprisingly, levels of TNF and chemokine (C-X-C motif) ligand 2 (CXCL2) were significantly increased at both 8 and 24 h, whereas levels of IL-1B and IL-6 were unchanged.

We then injected WT and TTP\ ARE mice with three different doses of LPS and measured the serum levels of TNF, IL10, IL1B, and IL6 on day 7, but none showed any external signs of redness, marked swelling, or ankle swelling of the tarsal joints, all of which were observed in all 14 of the WT animals tested, from day 6 to day 9.

Histopathological analysis of the paws and other limb joints showed signs of disease in all seven WT mice that were assessed, with more than three or four joints affected per mouse. In contrast, only two of the seven TTP\ ARE mice showed histological signs of disease, with only one or two joints affected per mouse. The WT mice exhibited inflammatory cell infiltrates, particularly neutrophils with a few macrophages, in the joint space, as well as marked synovial hyperplasia, cartilage necrosis, bone erosion, and fibrosis; none of these lesions was present in the TTP\ ARE mice. (Fig. 2B). Of the 13 TTP\ ARE animals that received the collagen antibody, 6 showed very minimal swelling on day 7, but none showed any external signs of redness, marked swelling, or ankle swelling of the tarsal joints, all of which were observed in 14 of the WT animals tested, from day 6 to day 9.

**Effect of the TTP\ ARE Mutation on Collagen Antibody-Induced Arthritis.**

Because of the recent appreciation that TTP exerts modulatory effects on transcripts involved in several aspects of the immune system (3, 4, 12), we investigated the effect of the systemic TTP overexpression found in the TTP\ ARE mice on the severity of certain models of immune and inflammatory diseases. We first evaluated the susceptibility of the TTP\ ARE mice to collagen antibody-induced arthritis (CAIA). When the WT and TTP\ ARE mice were taken through this protocol, the body weights of animals from both genotypes decreased in parallel after LPS; although the WT mice did not recover their original body weights, the TTP\ ARE animals were able to gain back almost 90% of their preinjection body weight by day 9 (Fig. 2A). Clinical arthritis scores (13) in the same animals demonstrated rapid increases from day 6 in the WT mice, but no or minimal clinical disease in the TTP\ ARE mice (Fig. 2B). Of the 13 TTP\ ARE animals that received the collagen antibody, 6 showed very minimal swelling on day 7, but none showed any external signs of redness, marked swelling, or ankle swelling of the tarsal joints, all of which were observed in all 14 of the WT animals tested, from day 6 to day 9.

Histopathological analysis of the paws and other limb joints showed signs of disease in all seven WT mice that were assessed, with more than three or four joints affected per mouse. In contrast, only two of the seven TTP\ ARE mice showed histological signs of disease, with only one or two joints affected per mouse. The WT mice exhibited inflammatory cell infiltrates, particularly neutrophils with a few macrophages, in the joint space, as well as marked synovial hyperplasia, cartilage necrosis, bone erosion, and fibrosis; none of these lesions was present in the TTP\ ARE mice (Fig. 2C). The histopathology scores for the four joints were consistently higher in WT mice compared with TTP\ ARE mice, in which the four joints were almost entirely normal, with the exception of one joint in one mouse, which exhibited minimal signs of arthritis (Fig. 2D).

Serum cytokine analysis revealed significantly decreased levels of granulocyte-colony stimulating factor (G-CSF) and IL6 on day 7 following CAIA induction in the TTP\ ARE mice (Fig. 2E and F). The levels of 23 other cytokines/chemokines tested were found to be very low or undetectable. NanoString gene expression profiling (14) of the whole-joint RNA showed that 49 inflammatory transcripts (of the 248 total transcripts tested) were significantly down-regulated in the joints from the TTP\ ARE mice compared with WT joints (Table S3). Of these 49, at least 5 are known or suspected TTP...
targets (2) (i.e., Cd2, Ccl20, Cxcl1, Il1β, and Il6 mRNAs), and another 11 have typical TTP binding sites within their 3'UTRs. These data demonstrate that the homozygous TTPARE animals were largely protected against CAIA, possibly due to the relative down-regulation of inflammatory mediators.

**Imiquimod-Induced Dermatitis in WT and TTPARE Mice.** We next evaluated the response to imiquimod (IMQ)-induced dermatitis, a commonly used model of human psoriasis (15). IMQ-containing cream was applied daily for five consecutive days to the shaved backs of 7- to 9-wk-old mice. Clinical signs of psoriasis-like dermatitis, particularly erythema, scaling, and thickening, were observed by approximately day 2 or 3. The increase in skinfold thickness was significantly lower in the TTPARE mice than in the WT mice (Fig. 3A).

Skin pathology resembling psoriasis, particularly thickening of the epidermis (acanthosis and hyperkeratinization), was observed in both the WT and the TTPARE mice; however, cellular infiltration into the dermis was significantly decreased in the TTPARE mice (Fig. 3B, Middle and Right). Moreover, epidermal neutrophilic abscesses/pustules were absent in the TTPARE mice (Fig. 3B, Middle). In addition, neutrophil infiltration into the dermis was markedly decreased in the TTPARE mice (Fig. 3C). Overall, the WT mice showed more severe epidermal hyperplasia, parakeratosis, and dermal inflammation, whereas the TTPARE mice showed more pronounced hyperkeratinization (Fig. 3E). NanoString gene expression profiling of day-6 skin RNA demonstrated 11 transcripts that were significantly down-regulated in TTPARE compared with WT skin (Fig. 3F). Four of the 11 transcripts are known or strongly suspected TTP targets (i.e., Cxcl1, Il1β, Il6, and Cxcl3). The remaining seven, C1qa, Cxcl3, Cxcr1, Cysltr1, Il1r1, Ptgir, and Tnfαip3, although not known to be direct TTP targets, also play critical roles in inflammation. These results suggest that the TTPARE mice, although still susceptible to IMQ-induced dermatitis, exhibited significantly decreased inflammation compared with WT mice.

**Effect of the TTPARE Mutation on Experimental Autoimmune Encephalomyelitis.** Finally, we assessed the effect of the TTPARE mutation on one type of experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis (16). We used the C57BL/6-MOG35-55 version of EAE, which has been described recently as the “gold standard” animal model for multiple sclerosis (17). In this experiment, the entire population of WT (n = 11) and homozygous TTPARE (n = 10) mice was given the encephalitogenic stimulus without knowledge of their genotype, with the genotype code broken only at the end of the experiment. WT mice exhibited progressive weight loss beginning on approximately day 12, whereas the TTPARE mice did not lose body weight, on average (Fig. 4A). The WT mice, but not the TTPARE mice, began exhibiting clinical signs of EAE on approximately day 10. By day 14, 8 of the 11 WT mice exhibited clinical signs of EAE, and an additional WT mouse was killed because of a maximal clinical score of 5.0 on day 13. In contrast, none of the 10 TTPARE mice exhibited clinical signs of EAE by day 14. By day 20, another WT mouse was killed because it reached a near-maximal clinical score, and the remaining nine of nine mice exhibited severe clinical signs (clinical scores between 2.0 and 4.0) of EAE. Four of the 10 TTPARE mice also exhibited clinical signs of EAE by day 20; however, the signs exhibited by TTPARE mice were of mild severity (clinical score between 0.5 and 2.0) (Fig. 4B). Another WT
mouse was killed on day 27. By day 30, 8 of the original 11 WT mice remained, all of which exhibited clinical signs of severe EAE, but 10 of the 10 original TTPΔARE mice remained, of which only 5 mice exhibited clinical signs of mild EAE. The mean cumulative disease scores and the mean maximal disease scores for the TTPΔARE mice were significantly lower than those of the WT mice (Fig. 4C). These data demonstrate that the TTPΔARE mice were markedly resistant to the induction of this model of EAE compared with the WT mice.

Discussion

The severe inflammatory syndrome seen in the TTP KO mice led to the identification of Tnf mRNA as a major target of TTP (4, 6). As a result, the emphasis in most follow-up studies has been on the involvement of TTP in innate immunity. More recently, several studies have highlighted the importance of other aspects of the immune response in the pathogenesis of the TTP deficiency syndrome. For example, mice in which TTP was deleted only from myeloid cells did not exhibit the TTP deficiency syndrome, although they were hyporesponsive to small amounts of injected LPS (11), suggesting the involvement of other cell types in the development of the syndrome. In another study, genetic ablation of genes in the IL17/IL23 axis protected mice against the development of the TTP deficiency syndrome, even in the setting of normal TNF pathways and responses (12). These recent studies highlight the concept that TTP deficiency in an intact animal affects many arms of the immune system and is a true systemic disease.

Since the earliest discovery of TTP as an endogenous antiinflammatory protein, we have been interested in the concept of attempting to increase levels or activities of TTP in animals, and potentially in humans, as a possible treatment for inflammatory conditions. Our initial attempts to accomplish this involved transgenic mice in which TTP was overexpressed using strong general promoters; these invariably led to embryonic lethality. In a different approach to increasing TTP expression without loss of its normal physiological regulation, we developed the knock-in TTPΔARE mouse described in this study, in which an ARE instability element was genetically removed from TTP mRNA. Our hope was that this germ-line mutation would lead to stabilization of TTP mRNA and overexpression of the protein throughout the body, without preconceived biases about which cell types should be preferentially targeted. Similar genetic approaches to the stabilization of ARE-containing mRNAs have been used in the cases of TNF (18) and IFN-γ (19), among others.

Remarkably, the TTPΔARE mice appeared phenotypically normal, despite elevated and stabilized TTP mRNA in cells derived from them, and increased levels of TTP protein in several tissues. We then tested the hypothesis that the increased “whole-body” levels of TTP might protect the mice against inflammation in four distinct experimental models that involved different aspects of the immune system. First, we tested the response of the TTPΔARE mice to LPS endotoxemia. Somewhat surprisingly, the TTPΔARE mice did not exhibit decreased levels of TNF in the serum after the i.p. injection of three different doses of LPS. However, serum levels of IL10, whose mRNA is also a known TTP target (20), were lower in the mutant mice than in the WT mice at the highest LPS dose and returned more rapidly to baseline levels in the TTPΔARE mice than in the WT mice at all LPS doses. These results suggest that the acute TNF response to LPS was not affected by further increasing endogenous levels of TTP, which are already massively induced in myeloid cells by LPS in parallel with TNF (11).

The second disease model tested was CAIA, often used as an experimental model of human rheumatoid arthritis (21). We found that TTPΔARE mice were essentially completely protected against the development of CAIA. The mechanism of this protection is likely to involve the decreased stability of proinflammatory cytokine mRNAs in many cell types and tissues. For example, we found that serum levels of G-CSF and IL-6 were significantly reduced in TTPΔARE mice 7 d after the start of the CAIA protocol. IL-6 mRNA has been suggested previously to be a target of TTP (22), whereas, to our knowledge, Csf1 mRNA, encoding G-CSF, has not been identified as a direct target. However, the 3′UTR of Csf1 mRNA in the mouse contains two sequence elements that form the core of ideal TTP binding sites, UAUUUAU, and several other closely related sequences; many of these, including the two core 7-mers, are widely conserved among mammals, including humans. G-CSF-encoding mRNAs have been shown to be elevated in TTP KO mouse placenta (23), and Csf1 mRNA in IL-6 transgenic mice is protected from collagen-induced arthritis. G-CSF is known for its role in maintaining homeostatic levels of granulocytes (25), and G-CSF administration results in neutrophil production (26). G-CSF also enhances neutrophil trafficking in the synovium, and neutrophil depletion arrests the progression of arthritis (27), suggesting that G-CSF plays a critical role in mediating inflammatory arthritis by promoting the trafficking of neutrophils into the synovium. It seems reasonable to suggest that, among other proinflammatory pathways affected by the increased levels of TTP, the decreased stability of G-CSF may have resulted in decreased manifestations of CAIA.

Gene expression profiling of whole-joint RNA revealed decreased levels in the levels of at least five known or suspected TTP targets in the TTPΔARE mice, whereas at least 11 other significantly down-regulated transcripts contained potential TTP binding sites and encode proteins that play critical roles in inflammation. The remaining 33 down-regulated transcripts encode components of the complement pathway, toll-like receptor signaling, cytokines/chemokines and their receptors, and enzymes that break down extracellular matrix, supporting the idea that increased levels of TTP can directly and perhaps indirectly regulate immune response transcripts and protect against inflammation.

We also explored the response of the TTPΔARE mice to IMQ-induced dermatitis. We found that the TTPΔARE mice had less severe epidermal hyperplasia and strikingly reduced neutrophil infiltration. A previous study demonstrated that neutrophil infiltration was considerably reduced in IMQ-induced dermatitis in lllr1 KO mice (28). Interestingly, neutrophil depletion has been shown to ameliorate the severity of disease in this model of psoriasis (29), and clinical studies have demonstrated disease remission by drug-induced agranulocytosis, supporting a critical pathogenic role of neutrophils in psoriasis (30). We found that lllr1 mRNA expression was significantly reduced in skin from the TTPΔARE mice, suggesting that decreased IL-1R1 signaling may have resulted in reduced dermal neutrophilic infiltration. Four other known or suspected TTP targets were also decreased in the TTPΔARE mice.
transplantation of genetically modified cells (35). Ideally, small molecules could be identified that, when administered orally, could cause elevations of TTP throughout the body. If such compounds could be identified that had minimal toxicity, it seems reasonable to suggest that they might be useful therapeutically in the treatment of certain chronic inflammatory diseases.

Materials and Methods

TTPΔARE knock-in mice with a 136-bp deletion of an AU-rich region of the Zfp36 3' UTR were generated at Ogezene using CSB8L6 embryonic stem cells and standard targeting techniques. Adult male homozygous mutant mice were injected intraperitoneally with three different doses of LPS as acute models of inflammation. They were also subjected to the following models of inflammatory disease: CAIA (13), IMQ-induced dermatitis (36), and the murine CD4spiderocyte glycoprotein MGO33-SS peptide-induced model of EAE (37). Detailed materials and methods are contained in SI Materials and Methods.

All animal procedures were conducted according to US Public Health Service policy on the humane care and use of laboratory animals. The National Institute of Environmental Health Sciences Institutional Animal Care and Use Committee approved all animal procedures used in this study. The East Carolina University Institutional Animal Care and Use Committee approved the animal protocol used to induce EAE.

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