Zinc Finger Protein Tristetraprolin Interacts with CCL3 mRNA and Regulates Tissue Inflammation

Ju-Gyeong Kang, Marcelo J. Amar, Alan T. Remaley, Jaeyul Kwon, Perry J. Blackshear, Ping-yuan Wang and Paul M. Hwang

_J Immunol_ 2011; 187:2696-2701; Prepublished online 22 July 2011;
doi: 10.4049/jimmunol.1101149
http://www.jimmunol.org/content/187/5/2696

Supplementary Material  http://www.jimmunol.org/content/suppl/2011/08/02/jimmunol.1101149.DC1
References  This article cites 42 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/187/5/2696.full#ref-list-1

Why The _JI_? Submit online.
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication
  *average

Subscription  Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription
Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html
Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The _Journal of Immunology_ is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Zinc Finger Protein Tristetraprolin Interacts with CCL3 mRNA and Regulates Tissue Inflammation

Ju-Gyeong Kang,* Marcelo J. Amar,† Alan T. Remaley,† Jaeyul Kwon,‡ Perry J. Blackshear,§ Ping-yuan Wang,* and Paul M. Hwang*

Zinc finger protein tristetraprolin (TTP) modulates macrophage inflammatory activity by destabilizing cytokine mRNAs. In this study, through a screen of TTP-bound mRNAs in activated human macrophages, we have identified CCL3 mRNA as the most abundantly bound TTP target mRNA and have characterized this interaction via conserved AU-rich elements. Compared to the wild-type cells, TTP−/− macrophages produced higher levels of LPS-induced CCL3. In addition, the plasma level of CCL3 in TTP−/− mice was markedly higher than that in wild-type mice. To determine the in vivo significance of TTP-regulated CCL3, we generated CCL3−/−/TTP−/− double-knockout mice. Along with decreased proinflammatory cytokines in their paw joints, there were significant functional and histologic improvements in the inflammatory arthritis of TTP−/− mice when CCL3 was absent, although cachexia, reflecting systemic inflammation, was notably unaffected. Furthermore, the marked exacerbation of aortic plaque formation caused by TTP deficiency in the APOE−/− mouse model of atherosclerosis was also rescued by disrupting CCL3. Taken together, our data indicate that the interaction between TTP and CCL3 mRNA plays an important role in modulating localized inflammatory processes in tissues that are dissociated from the systemic manifestations of chronic inflammation. The Journal of Immunology, 2011, 187: 2696–2701.

The temporal regulation of cytokine and chemokine mRNA levels, through posttranscriptional mechanisms, plays a critical role in regulating the cascades of inflammatory signaling events that when deranged can result in diseases such as autoimmune arthritis (1, 2). The identification of tristetraprolin (TTP), encoded by the zinc finger protein 36 gene, as an essential factor that binds to AU-rich elements (AREs) and destabilizes TNF mRNA provided a novel mechanism for modulating a central inflammatory cytokine (3). Mice with genetic disruption of TTP display an inflammatory syndrome comprised of cachexia, erosive arthritis, myeloid hyperplasia, and serologies consistent with autoimmune disorders (4). Since this seminal report, the expanding number of cytokines with ARE sequences that bind to TTP and other similar proteins have underscored the importance of this mechanism in modulating various immune responses (2, 5).

Identifying the molecular components involved in activating monocytes into effector macrophages may provide new insights into inflammatory diseases such as atherosclerosis (6, 7). Through gene expression analysis, we previously identified TTP as one of the most highly expressed transcriptional regulatory genes in macrophages purified from human atherosclerotic plaques compared with circulating monocytes (8). To further elucidate how TTP might regulate these inflammatory cells, we screened for TTP-interacting mRNA species in activated human macrophages by combining TTP immunoprecipitation with a sequencing-based gene expression technique. One mRNA species bound to TTP at high levels was CCL3 (MIP-1α), a cytokine belonging to the CC motif subfamily of chemokines that is involved in both acute and chronic inflammation (9).

CCL3 is secreted by activated macrophages and other inflammatory cells for diverse functions such as chemotaxis, phagocytosis, and mediator release (9, 10). Thus, as a chemokine present at high tissue concentrations, CCL3 promotes inflammation and has been proposed to be involved in a spectrum of diseases from asthma to multiple sclerosis (11–13). CCL3 is also known to be highly expressed both in the synovial fluid of patients with rheumatoid arthritis and in the plaque tissues of patients with atherosclerosis (14–17). As there is a well-established association between rheumatoid arthritis and atherosclerosis (18), CCL3 could serve as a common chemokine for the recruitment of inflammatory cells at disparate disease sites. Given our previous observation of high TTP expression in atherosclerotic plaque macrophages (8), we speculated that regulation of CCL3 mRNA by TTP may play an important role in atherosclerosis pathogenesis. Although CCL3 mRNA has been reported to have ARE sequences and can be inhibited by TTP (19, 20), the molecular mechanism and functional consequences of the interaction between CCL3 mRNA and TTP have not been well studied. In this study, we characterize the interaction between CCL3 mRNA and TTP. We provide genetic evidence that the loss of this interaction significantly contributes to the increase in inflammatory arthritis and atherosclerosis of TTP-deficient mice, highlighting CCL3 as a pathogenically significant target of TTP in these two common diseases.
Materials and Methods

Animals

All mice were maintained and handled in accordance with National Heart, Lung, and Blood Institute Animal Care and Use Committee standards. TTP−/− mice were of the C57BL/6 background as described (4, 21). CCL3−/− mice and APOE−/− mice were also of the C57BL/6 background and obtained from The Jackson Laboratory.

Abs

Abs used for the following proteins were: human TTP rabbit polyclonal (sc-14030; Santa Cruz Biotechnology); mouse TTP goat polyclonal (sc-8458; Santa Cruz Biotechnology); β-actin monoclonal (AC-15; Sigma-Aldrich); and negative control rabbit IgG (sc-2027; Santa Cruz Biotechnology).

Small interfering RNAs and plasmids

Non-specific and TTP-specific small interfering RNA (siRNA) duplexes were obtained from Dharmacon Research. TTP (open reading frame only), full-length CCL3, and GAPDH cDNA in pcMV plasmids were obtained from Genecopoeia. Point mutations in the ARE region of CCL3 were made using the QuikChange II kit (Stratagene) according to the manufacturer’s protocol.

Cell culture and transfection

THP1 human monocytic cell line was obtained from the American Type Culture Collection and maintained as recommended. THP1 cells were transfected with siRNAs using Nucleofector (Amaxa) according to the manufacturer’s protocol and activated with 2 nM PMA as previously described (9). Bone marrow-derived macrophages (BMDM) were isolated from mouse femora and tibia and cultured for 6 d in DMEM (Invitrogen) supplemented with 10% FBS, penicillin/streptomycin (Invitrogen), and 10% 1,929 cell-conditioned medium. LPS (10 μg/ml, L2880; Sigma-Aldrich) was added to stimulate BMDM for the indicated times. For the mRNA decay assay, BMDM cultures were treated for 90 min with LPS prior to blocking transcription initiation with actinomycin D (5 μg/ml) and harvested at the indicated times. HEK293 cells were transiently transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

TTP immunoprecipitation and serial analysis of gene expression

TTP–RNA complex immunoprecipitation was performed as previously described (8). Briefly, 106 THP1 cells were stimulated for 4 h with 2 nM PMA and used for cytoplasmic extract preparation, to which anti-TTP Ab (sc-14030; Santa Cruz Biotechnology) or control rabbit IgG (sc-2027; Santa Cruz Biotechnology) was added and incubated overnight at 4°C. RNA isolated from the immunoprecipitation was used to generate serial analysis of gene expression (SAGE) libraries (22). From each library, 40,000 tags were sequenced and analyzed using SAGE2000 software (8, 22). Specific fold enrichment was determined by the ratio of the SAGE tag counts were calculated as described (23).

mRNA quantification by real-time PCR

Candidate mRNAs identified by TTP immunoprecipitation-SAGE analysis were confirmed using RNA extracted for SAGE library synthesis. For joint tissue mRNA measurements, total RNA was isolated using the RNeasy Kit (Qiagen). mRNA from tissue total RNA or cultured cell lysates was purified by binding to polyoxyethylenehexylamide magnetic beads (Invitrogen), reverse transcribed using Superscript II (Invitrogen), and quantified by real-time RT-PCR using SYBR Green fluorescence on the 7900HT Sequence Detection System (Applied Biosystems) as previously described (8). Cycle threshold values were normalized to the housekeeping gene eukaryotic translation initiation factor EIF35S. Primer sequences are provided in Supplemental Table II.

Blood count and cytokine and lipid measurements

Differential cell counts from blood collected in EDTA tubes were determined using the Cell-Dyn 3700 hematology analyzer (Abbott). Plasma cytokine levels were measured by a Searchlight multiplex assay (Aushon Biosystems) or a mouse proinflammatory ultra-sensitive kit (Meso Scale Discovery) according to the manufacturer’s instructions. Plasma lipids were measured using an enzymatic assay (Wako Chemicals) on a ChemiWell 2910 analyzer (Awareness Technology).

Body mass composition

Body composition (fat and muscle) was measured in nonanesthetized mice using the Bruker Minspec nuclear magnetic resonance analyzer (Bruker Optics) (24).

Grip strength measurement

For the functional quantification of arthritis, maximum four-limb grip strength was measured by using a digital grip strength meter (Columbus Instruments) as previously described (25, 26).

Joint histology and aortic plaque analysis

Front and rear paw joints were removed and fixed in 10% buffered formalin, decalcified in 30% formic acid, embedded in paraffin, sectioned, and stained with H&E. Arthritic joints were cut longitudinally, placed on glass slides, and quantified from their origin to the ileal bifurcation, excluding branch vessels. Quantification of the atherosclerotic lesions was performed using the Image-Pro Plus version 4.1 software (Media Cybernetics) with blending of sample identity and expressed as a fraction of total aortic surface.

Statistical analysis

A two-tailed Student t test was used for statistical analyses and considered to be significant if p < 0.05.

Results

Identification and characterization of CCL3 as a TTP target

To identify new TTP-interacting mRNAs in activated human monocytes, we combined the strategy of immunoprecipitating TTP protein followed by the sequencing of all bound mRNA species using the SAGE technique (Fig. 1A) (22). An advantage of the SAGE technique is that it can provide quantitative information about the abundance of a given mRNA species by the frequency
with which the SAGE tag, corresponding to its mRNA, is encountered during sequencing. After activating THP1 human monocytic cells for 4 h with phorbol ester (PMA), cytoplasmic extracts were immunoprecipitated with either anti-TTP or nonspecific Ab, and the communoprecipitated RNAs were extracted and used to synthesize SAGE libraries.

We performed a preliminary screen by looking for the presence of ARE motifs among the mRNA species that were 2-fold enriched in the anti-TTP Ab fraction (p < 0.05) (27). From this list of putative candidates, 15 were confirmed by RT-PCR to be increased in the anti-TTP Ab versus nonspecific (control IgG) Ab immunoprecipitated RNA (Supplemental Table I). Confirming the specificity of our technique, TNF was one of the more significantly bound mRNA species to TTP. Interestingly, another cytokine mRNA encoded by CCL3 was the most abundant TTP-bound species by SAGE tag number (Supplemental Table I). Our previous observation that CCL3 and TTP are highly coexpressed in human atherosclerotic plaque macrophages provided an in vivo correlation (8).

We first verified the SAGE data by performing RT-PCR on the same RNAs used to make the libraries. CCL3 mRNA was significantly enriched in the anti-TTP Ab fraction in parallel with TTP and TNF mRNAs, which served as positive controls (Fig. 1B). TTP destabilizes its bound mRNA, and therefore, cells in which TTP protein has been depleted should have elevated levels of its target mRNAs. Indeed, siRNA knockdown of TTP, which was confirmed by Western blotting, caused a significant increase in both CCL3 and TNF mRNA levels, whereas a non-TTP target mRNA GAPDH was not affected (Fig. 1C). These results provided additional evidence that TTP can negatively regulate CCL3 mRNA by direct interaction.

**TTP regulates CCL3 mRNA in vivo**

To examine the interaction between TTP protein and CCL3 mRNA in primary cells, BMDM were prepared from *TTP*+/− and *TTP*−/− mice. CCL3 mRNA expression levels peaked 3 to 4 h after LPS treatment in *TTP*+/− BMDM (Fig. 2A). In *TTP*−/− BMDM, CCL3 mRNA induction was significantly higher with a slightly delayed peak 4 h after LPS treatment. The dampening of CCL3 mRNA levels in *TTP*+/− BMDM correlated well with TTP protein expression (Fig. 2A). The effect of altered CCL3 mRNA levels was evident from parallel changes in CCL3 protein released into the medium (Fig. 2B).

To show that the increase in CCL3 mRNA in *TTP*−/− BMDM was due to posttranscriptional stabilization, we measured the steady-state level of CCL3 mRNA after blocking transcription with actinomycin D. With TNF and GAPDH mRNAs serving as positive and negative controls, respectively, the t1/2 of CCL3 mRNA increased from 48 min in *TTP*+/− cells to 117 min in *TTP*−/− cells (Fig. 2C). The stabilization of CCL3 mRNA in *TTP*−/− cells provided in vivo genetic evidence that TTP negatively regulates it in macrophages.

**AREs mediate CCL3 mRNA binding to TTP protein**

The direct interaction between TTP protein and CCL3 mRNA was further assessed by in vitro cell transfection assays without the potential confounding effects of endogenous TNP (28). HEK293 cells were cotransfected with CCL3 and TTP cDNA in pcMV vectors (Fig. 3A). The transiently expressed TTP specifically reduced CCL3 mRNA, but not GAPDH mRNA, confirming their specific interaction in a heterologous cell system. Because important regulatory sequences are likely to be conserved, CCL3 sequences from five different species were queried, and their alignment revealed three conserved AREs (AUUUA) in the 3′ untranslated region (ARE 1, 2, and 3) (Fig. 3B). Point mutations were then introduced into each of these AREs to determine which mediated TTP regulation. The CCL3 mutants were cotransfected with either TTP cDNA or empty vector followed by CCL3 mRNA measurement (Fig. 3C). The 5′-most ARE mutant (1) had the most significant effect on abolishing the inhibitory effect of TTP on CCL3 mRNA levels (Fig. 3C). The individual effect of the other two AREs (mutants 2 and 3) was weak, but the combined mutant (mutant 1-2-3) synergistically neutralized the effect of TTP on CCL3 mRNA stability.

**Deletion of CCL3 ameliorates the inflammatory arthritis of *TTP*−/− mice**

TTP-deficient mice develop an inflammatory syndrome consisting of cachexia, arthritis, systemic autoimmunity, myeloid hyperplasia, and extramedullary hematopoesis (4, 29). One prominent feature of *TTP*−/− mice is the development of polyarticular arthritis characterized by paw swelling at 8–10 wk of age. This has been likened to human rheumatoid arthritis, although the serologies are more consistent with systemic lupus erythematosus.

To determine the effect of CCL3 mRNA regulation by TTP in vivo, we crossed *CCL3*−/− with *TTP*−/− mice to generate *CCL3*−/−*TTP*−/− (double-knockout) mice. When paw grip strength was determined as a functional measure of arthritis, we observed a significant improvement in *CCL3*−/−*TTP*−/− mice compared with *TTP*−/− mice (Fig. 4A). Notably, the improvement in female *CCL3*−/−*TTP*−/− mice appeared more significant than that observed in the male mice, consistent with the influential role gender can play in autoimmune diseases (p values for female versus male were 0.002 and 0.02, respectively) (Fig. 4A, Supplemental Fig. 1) (30). Based on this observation, we focused our study on female mice and found that, in parallel with the functional improvement, the severe synovial infiltration and bone...
CCL3 mediates the increased atherosclerosis of \( \text{TTP}^{-/-} \) \( \text{APOE}^{-/-} \) mice

Atherosclerosis is an inflammatory vascular disease that involves various cell types including plaque macrophages, and our previous study of human plaque macrophage transcriptome identified TTP as a potentially important regulator of gene expression (8, 32). To investigate whether TTP-regulated CCL3 is also involved in atherosclerosis, we crossed \( \text{TTP}^{-/-} \) mice into an \( \text{APOE}^{-/-} \) background, a well-established mouse model of hyperlipidemia and atherosclerosis (33). Again, we used female \( \text{APOE}^{-/-} \) mice, as they have been shown to develop larger, less lipid-laden atherosclerotic lesions than male mice when fed a normal diet (34, 35). As predicted with the increase in inflammation caused by TTP deficiency, \( \text{TTP}^{-/-}\text{APOE}^{-/-} \) mice developed more severe atherosclerosis as measured by the total surface area of aortic plaques compared with \( \text{APOE}^{-/-} \) mice (Fig. 6A).

Hyperlipidemia has been associated with systemic inflammation, but the plasma levels of triglycerides and total cholesterol were in fact reduced in \( \text{TTP}^{-/-}\text{APOE}^{-/-} \) mice, which is consistent with the cachexia caused by TTP deficiency (Fig. 6B). This suggested that inflammatory factors play a pivotal role in worsening the atherosclerosis of TTP-deficient mice. Therefore, to delineate the role of CCL3, we generated \( \text{CCL3}^{-/-}\text{TTP}^{-/-}\text{APOE}^{-/-} \) (triple-knockout) mice. Although the plasma lipid level and body weight of triple-knockout mice remained decreased as with \( \text{TTP}^{-/-} \) mice (Fig. 6B, 6C), the marked increase in atherosclerosis caused by TTP deficiency was prevented by the deletion of \( \text{CCL3} \) (Fig. 6A). This observation provided additional in vivo genetic evidence that TTP-regulated CCL3 plays an important role in inflammatory diseases beyond those involving joint tissues.

Discussion

In summary, through a global screen for transcripts binding to TTP in activated human macrophages, we have identified CCL3 mRNA as the most abundantly bound chemokine species and have characterized the nature of its destabilization by TTP through ARE sequences. The posttranscriptional regulation of CCL3 mRNA by TTP is important, as the autoimmune arthritis and vascular inflammation observed in \( \text{TTP}^{-/-} \) mice were both markedly reduced.

FIGURE 3. TTP destabilizes CCL3 mRNA through conserved AREs in HEK293 cells. A, TTP cDNA expression specifically decreases the level of cotransfected wild-type full-length CCL3 mRNA, whereas control GAPDH mRNA levels are unchanged. RT-PCR was used to measure mRNA levels 48 h after cotransfection into HEK293. B, Alignment of the CCL3 mRNA 3′ untranslated regions from the indicated species identifies conserved AREs (shaded and numbered). A-to-C point mutations were introduced into the CCL3 AREs as shown (Mutant). C, The conserved AREs either individually or in combination contribute to the destabilization of CCL3 mRNA by TTP. TTP cDNA or empty vector plasmid was cotransfected with either wild-type (WT) or mutant CCL3 into HEK293 cells. Data presented as mean ± SEM; \( n = 3 \). * \( p < 0.05 \).
in the absence of CCL3. Furthermore, the disruption of CCL3 in the TTP−/− state dissociated localized tissue inflammation from systemic disease, a finding that is consistent with the function of CCL3 as an inflammatory chemokine and its previously observed role in a collagen Ab-induced model of arthritis (36). Together, these results provide in vivo genetic evidence underscoring the importance of both the spatial and temporal posttranscriptional regulation of chemokines in modulating the outcome of inflammation (37).

TNF plays a central role in coordinating complex inflammatory responses through associated mediator genes. A recent study has shown that the gene expression kinetics of both TNF and TNF-induced genes are largely influenced by their respective mRNA stabilities due to differences in ARE sequences (38). Although excessive TNF signaling, by loss of its mRNA destabilization in TTP−/− mice, has been shown to cause many of the inflammatory sequelae including the recent report of left-sided cardiac valvulitis, the identity of the genes that mediate the specific disease phenotypes is less clear (4, 21, 29). Our study reveals that CCL3 is an important mediator of the autoimmune arthritis and vascular inflammatory phenotypes of TTP−/− mice, but other chemokines are also likely to be involved in pathogenesis. For example, the mRNA level of another chemokine associated with arthritis CCL5 (39) is elevated in TTP−/− joints but reduced in TTP−/− CCL3−/− joints, suggesting that CCL5 could be regulated by CCL3 (Supplemental Fig. 2D). To further understand the autoimmune arthritis of TTP−/− mice, more investigations are needed to determine the contributions of CCL3, CCL5, and possibly other chemokines, as the arthritis phenotype is not completely rescued by removing CCL3 alone.

It is possible that the expression level of CCL3 is regulated by both TTP and TNF. TNF has been shown to transcriptionally regulate CCL3 (40). Therefore, CCL3 could be a downstream messenger of TNF excess in TTP−/− mice, and our current study does not rule out the possibility that CCL3 mediates a subset of specific effects of TNF on the inflammation. Although it has been reported that the inflammatory phenotype of the TTP−/− mice can be prevented by treating with TNF Ab (4), TTP−/− mice continue to display myeloid hyperplasia even in the absence of TNFRs (29, 41). Thus, other TTP targets, which are independent of TNF signaling, appear to play a role in the pathogenesis of TTP−/− mice. In patients with rheumatoid arthritis, the blockade of TNF signaling using Abs does not affect CCL3 expression, whereas it reduces CCL2 and IL-8 levels (42, 43). Regardless of the extent of TNF involvement, our study reveals that CCL3 plays a critical role in localized tissue inflammation in TTP−/− mice, although its regulatory mechanism is likely complex and depends on various factors including cell type and species.

Our study also suggests a possible explanation for the increased prevalence of atherosclerotic disease in patients with rheumatoid arthritis by providing genetic evidence for a shared pathway in these two diseases whereby TTP regulates CCL3 mRNA stability. A growing body of work has implicated CCL3 and its receptor CCR5 in the pathogenesis of atherosclerosis (6, 7, 17). The marked increase in atherosclerotic plaque formation in TTP−/−/APOE−/− mice (23–25 wk old) is prevented by deleting CCL3. Scale bar, 2 mm. B, Plasma lipids levels are reduced both in TTP−/−APOE−/− and in CCL3−/− TTP−/−/APOE−/− mice compared with APOE−/− mice (n = 6–8). Data are presented as mean ± SEM. C, Body weights of 23–25-wk-old female mice of the indicated genotypes. Data shown as mean ± SEM; n = 4–8. *p < 0.05 compared with APOE−/− mice.
tion in a mouse model is applicable to human disease, as there is abundant expression of both TTP and CCL3 in human atherosclerotic lesions (8, 17). In addition, CCR5 is also present in arterial smooth muscle cells, which are involved in plaque formation (17), Although hyperlipidemia is thought to be a primary driver of atherosclerosis, the unregulated expression of CCL3 appears to be pivotal in worsening atherosclerosis in TTP+/−APOE−/− mice despite their significantly lower lipid levels compared with APOE−/− mice.

The CCL3 pathway may represent a potential target for site-specific modulation of autoimmune arthritis and atherosclerotic disease activity. Circulating cytokines have pleiotropic effects on multiple systems, and their chronic blockade may lead to potential complications such as the immune suppression seen with TNF antagonist treatments. As chemokines spatially and selectively target subsets of leukocytes, their blockade may reduce the likelihood of systemic side effects (11). By disrupting CCL3 chemokine signaling, the vicious cycle of recruiting more inflammatory cells to the disease site may be prevented. Given our observations using two different disease models, TTP regulation of CCL3 is likely to be important for the pathogenesis of other common inflammatory conditions and may merit therapeutic targeting.

Acknowledgments
We thank Milton Pryor, Rafael Molina, Deborah Stumpo, Cory Lago, Ho Joong Sung, Wenzhe Ma, and Toren Finkel for helpful advice and assistance.

Disclosures
The authors have no financial conflicts of interest.

References