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Zinc finger protein TTP interacts with CCL3 mRNA and regulates tissue inflammation*

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Abstract

Zinc finger protein tristetraprolin (TTP) modulates macrophage inflammatory activity by destabilizing cytokine mRNAs. Here, through a screen of TTP-bound mRNAs in activated human macrophages, we have identified CC chemokine ligand 3 (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.

Introduction

The temporal regulation of cytokine and chemokine mRNA levels, through post-transcriptional 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* (*ZFP36*) gene, as an essential factor that binds to AU-rich elements (AREs) and destablizes 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

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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 to 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 (macrophage inflammatory protein-1α, 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. Herein, 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 NHLBI Animal Care and Use Committee. *TTP*−/− mice were of *C57BL/6* background as described (4, 21). *CCL3*−/− mice and *APOE*−/− mice were also of *C57BL/6* background and obtained from Jackson Laboratories.

Antibodies

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

siRNAs and plasmids

Non-specific and TTP-specific siRNA duplexes were obtained from Dharmacon Research. TTP (ORF 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 Inc.) according to the manufacturer's protocol and activated with 2 nM phorbol 12-myristate 13-acetate (PMA) as previously described (8). Bone marrow-derived macrophages (BMDM) were isolated from mouse femora and tibia and cultured for 6 d in Dulbecco's minimal essential medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (Invitrogen) and 10% L929 cell-conditioned medium. LPS (10 ng/ml, L2880, Sigma) 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 Lipofectamin 2000 (Invitrogen) according to the manufacture's protocol.

TTP immunoprecipitation and SAGE analysis

TTP- RNA complex immunoprecipitation was performed as previously described (8). Briefly, 10^8 THP1 cells were stimulated for 4 h with 2 nM phorbol 12-myristate 13-acetate (PMA) and used for cytoplasmic extract preparation to which anti-TTP antibody (sc-14030, Santa Cruz Biotech) or control rabbit IgG (sc-2027, Santa Cruz Biotech) were added and incubated overnight at 4°C. RNA isolated from the immunoprecipitation was used to generate SAGE libraries (22). From each library, 40,000 tags were sequenced and analyzed using SAGE 2000 software (8, 22). Specific fold-enrichment was determined by the ratio of the SAGE tag counts in the TTP versus nonspecific IgG libraries. P values for 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 (QAIGEN). mRNA from tissue total RNA or cultured cell lysates was purified by binding to poly(dT) magnetic beads (Invitrogen), reverse transcribed using Superscript II (Invitrogen), and quantified by real-time RT-PCR using SYBR green fluorescence on the 7900 HT Sequence Detection System (Applied Biosystems) as previously described (8). Cycle threshold (Ct) values were normalized to the housekeeping gene eukaryotic translation initiation factor EIF35S (TIF). Primer sequences are provided in Table S2.

Blood count, 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 USA, Inc.) on a ChemWell 2910 analyzer (Awareness Technology, Inc).

Body mass composition

Body composition (fat and muscle) was measured in non-anesthetized mice using the Bruker Minispec NMR 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% bufferd formalin, decalcified in 30% formic acid, embedded in paraffin, sectioned and stained with hematoxulin and eosin (H&E). Aortas were fixed by perfusion (4% paraformaldehyde, 5% sucrose, 20 mM EDTA, pH 7.4), dissected from their origin in the heart to the ileal bifurcation, stained with Sudan IV solution, destained in 80% ethanol, and washed in water. Aortas 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, Inc.) with blinding of sample identity and expressed as a fraction of total aortic surface.

Statistical analysis

Two-tailed Student's 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 serial analysis of gene expression (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 non-specific antibody and the co-immunoprecipitated RNAs were extracted and used to synthesize SAGE libraries.

We performed a preliminary screen by looking for the presence of ARE motifs amongst the mRNA species that were two-fold enriched in the anti-TTP antibody fraction ($P < 0.05$) (27). From this list of putative candidates, 15 were confirmed by RT-PCR to be increased in the anti-TTP antibody versus non-specific (control IgG) antibody immunoprecipitated RNA (Table S1). 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 (Table S1). Our previous observation that CCL3 and TTP are highly co-expressed 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 antibody 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 while 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, bone marrrow-derived macrophages (BMDM) were prepared from *TTP*+/+ and *TTP*−/− mice. CCL3 mRNA expression levels peaked 3 to 4 h after lipopolysaccharide (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 post-transcriptional 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 half-life 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.

AU-rich elements 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 TNF (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 (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 (mutant 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 hematopoiesis (4, 29). One prominent feature of *TTP*−/− mice is the development of polyarticular arthritis characterized by paw swelling at 8 to 10 weeks of age. This has been likened to human rheumatoid arthritis although the serologies are more consistent with systemic lupus erthematosus (SLE).

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 to *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 and Fig. S1)(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 erosions of *TTP* −/− mice were markedly reduced by deleting *CCL3* (*CCL3* −/− *TTP* −/− genotype) (Fig. 4B).

Despite the improvement in the inflammatory arthritis of *TTP* −/− mice by eliminating CCL3, the systemic effect of TTP-deficiency was unaltered as reflected by their low body weight (cachexia) and fat content (Fig. 5, A and B). This observation was further confirmed by the continued presence of splenomegaly and other signs of myeloid hyperplasia in *CCL3* −/− *TTP* −/− mice as previously reported in *TTP* −/− mice (Fig. 5C and Fig. S2) (4). Using female *TTP* −/− mice, we next measured the expression levels of TTP target genes in both plasma and joint as markers of inflammatory activity (Fig. 5, D and E). The elevation of CCL3 protein in *TTP* −/− plasma, along with TNF and IL-1β serving as positive control targets of TTP (31), confirmed that TTP negatively regulates CCL3 *in vivo*. This pattern of increased plasma TNF and IL-1β levels was also maintained in *CCL3* −/− *TTP* −/− mice providing additional evidence that CCL3 deficiency does not affect systemic inflammation. In contrast, the levels of TNF and IL-1β as well as macrophage infiltration in joint tissue were significantly reduced by deleting *CCL3* (Fig. 5E and Fig. S2 C). These data were consistent with increased TNF and IL-1β found in the synovial fluid of patients with autoimmune arthritis and with the established function of CCL3 as a chemokine serving to attract inflammatory cells in tissues (16).

CCL3 mediates the increased atherosclerosis of TTP−/− APOE−/− mice

Atherosclerosis is an inflammatory vascular disease that involves various cells 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 *TTP* −/− mice into an *APOE* −/− background, a well-established mouse model of hyperlipidemia and atherosclerosis (33). Here again, we used female *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, *TTP* −/− *APOE* −/− mice developed more severe atherosclerosis as measured by the total surface area of aortic plaques compared to *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 *TTP* −/− *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 *CCL3* −/− *TTP* −/− *APOE* −/− (triple knockout) mice. Although the plasma lipid level and body weight of triple knockout mice remained decreased as with *TTP* −/− mice (Fig. 6B and C), the marked increase in atherosclerosis caused by TTP deficiency was prevented by the deletion of *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 post-transcriptional regulation of CCL3 mRNA by TTP is important as the autoimmune arthritis and vascular inflammation observed in *TTP* −/− mice were both markedly reduced 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 antibody-induced model of arthritis (36). Together, these results provide *in vivo* genetic evidence underscoring the importance of both the spatial and temporal post-transcriptional 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 (Fig. S2 D). 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 antibody (4), *TTP*−/− mice continue to display myeloid hyperplasia even in the absence of TNF receptors (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 antibodies does not affect CCL3 expression while reducing 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 athritis 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 compared to *APOE*−/− mice and its prevention by the absence of CCL3 demonstrate the importance of TTP in atherosclerosis via the regulation of CCL3 mRNA homeostasis. We believe that this observation 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 to *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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Anderson P. Post-transcriptional regulons coordinate the initiation and resolution of inflammation. Nat Rev Immunol. 2010; 10:24–35. [PubMed: 20029446]
2. Stumpo DJ, Lai WS, Blackshear PJ. Inflammation: cytokines and RNA-based regulation. WIREs RNA. 2010; 1:60–80.
3. Carballo E, Lai WS, Blackshear PJ. Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science. 1998; 281:1001–1005. [PubMed: 9703499]
4. Taylor GA, Carballo E, Lee DM, Lai WS, Thompson MJ, Patel DD, Schenkman DI, Gilkeson GS, Broxmeyer HE, Haynes BF, Blackshear PJ. A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. Immunity. 1996; 4:445–454. [PubMed: 8630730]
5. Carrick DM, Lai WS, Blackshear PJ. The tandem CCCH zinc finger protein tristetraprolin and its relevance to cytokine mRNA turnover and arthritis. Arthritis Res Ther. 2004; 6:248–264. [PubMed: 15535838]
6. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med. 2005; 352:1685–1695. [PubMed: 15843671]
7. Libby P, Ridker PM, Hansson GK. Inflammation in atherosclerosis: from pathophysiology to practice. J Am Coll Cardiol. 2009; 54:2129–2138. [PubMed: 19942084]
8. Patino WD, Kang JG, Matoba S, Mian OY, Gochuico BR, Hwang PM. Atherosclerotic plaque macrophage transcriptional regulators are expressed in blood and modulated by tristetraprolin. Circ Res. 2006; 98:1282–1289. [PubMed: 16614304]
9. Maurer M, von Stebut E. Macrophage inflammatory protein-1. Int J Biochem Cell Biol. 2004; 36:1882–1886. [PubMed: 15203102]
10. Menten P, Wuyts A, Van Damme J. Macrophage inflammatory protein-1. Cytokine Growth Factor Rev. 2002; 13:455–481. [PubMed: 12401480]
11. Gerard C, Rollins BJ. Chemokines and disease. Nat Immunol. 2001; 2:108–115. [PubMed: 11175802]
12. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med. 2006; 354:610–621. [PubMed: 16467548]
13. Gladue RP, Brown MF, Zwillich SH. CCR1 antagonists: what have we learned from clinical trials. Curr Top Med Chem. 2010; 10:1268–1277. [PubMed: 20536425]
14. Hayes IM, Jordan NJ, Towers S, Smith G, Paterson JR, Earnshaw JJ, Roach AG, Westwick J, Williams RJ. Human vascular smooth muscle cells express receptors for CC chemokines. Arterioscler Thromb Vasc Biol. 1998; 18:397–403. [PubMed: 9514408]
15. Moos MP, John N, Grabner R, Nossmann S, Gunther B, Vollandt R, Funk CD, Kaiser B, Habenicht AJ. The lamina adventitia is the major site of immune cell accumulation in standard

chow-fed apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2005; 25:2386–2391. [PubMed: 16179593]

16. Iwamoto T, Okamoto H, Toyama Y, Momohara S. Molecular aspects of rheumatoid arthritis: chemokines in the joints of patients. Febs J. 2008; 275:4448–4455. [PubMed: 18662305]
17. Jones KL, Maguire JJ, Davenport AP. Chemokine receptor CCR5: From AIDS to atherosclerosis. Br J Pharmacol. 2010
18. Ku IA, Imboden JB, Hsue PY, Ganz P. Rheumatoid arthritis: model of systemic inflammation driving atherosclerosis. Circ J. 2009; 73:977–985. [PubMed: 19430165]
19. Zipfel PF, Balke J, Irving SG, Kelly K, Siebenlist U. Mitogenic activation of human T cells induces two closely related genes which share structural similarities with a new family of secreted factors. J Immunol. 1989; 142:1582–1590. [PubMed: 2521882]
20. Sauer I, Schaljo B, Vogl C, Gattermeier I, Kolbe T, Muller M, Blackshear PJ, Kovarik P. Interferons limit inflammatory responses by induction of tristetraprolin. Blood. 2006; 107:4790–4797. [PubMed: 16514065]
21. Ghosh S, Hoenerhoff MJ, Clayton N, Myers P, Stumpo DJ, Maronpot RR, Blackshear PJ. Left-sided cardiac valvulitis in tristetraprolin-deficient mice: the role of tumor necrosis factor alpha. Am J Pathol. 2010; 176:1484–1493. [PubMed: 20093488]
22. Saha S, Sparks AB, Rago C, Akmaev V, Wang CJ, Vogelstein B, Kinzler KW, Velculescu VE. Using the transcriptome to annotate the genome. Nat Biotechnol. 2002; 20:508–512. [PubMed: 11981567]
23. Audic S, Claverie JM. The significance of digital gene expression profiles. Genome Res. 1997; 7:986–995. [PubMed: 9331369]
24. Heron-Milhavet L, Haluzik M, Yakar S, Gavrilova O, Pack S, Jou WC, Ibrahimi A, Kim H, Hunt D, Yau D, Asghar Z, Joseph J, Wheeler MB, Abumrad NA, LeRoith D. Muscle-specific overexpression of CD36 reverses the insulin resistance and diabetes of MKR mice. Endocrinology. 2004; 145:4667–4676. [PubMed: 15231693]
25. Sakuma S, Nishigaki F, Magari K, Ogawa T, Miyata S, Ohkubo Y, Goto T. FK506 is superior to methotrexate in therapeutic effects on advanced stage of rat adjuvant-induced arthritis. Inflamm Res. 2001; 50:509–514. [PubMed: 11713905]
26. Park JY, Wang PY, Matsumoto T, Sung HJ, Ma W, Choi JW, Anderson SA, Leary SC, Balaban RS, Kang JG, Hwang PM. p53 improves aerobic exercise capacity and augments skeletal muscle mitochondrial DNA content. Circ Res. 2009; 105:705–712. 711, 712. [PubMed: 19696408]
27. Bakheet T, Williams BR, Khabar KS. ARED 2.0: an update of AU-rich element mRNA database. Nucleic Acids Res. 2003; 31:421–423. [PubMed: 12520039]
28. Lai WS, Carballo E, Strum JR, Kennington EA, Phillips RS, Blackshear PJ. Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. Mol Cell Biol. 1999; 19:4311–4323. [PubMed: 10330172]
29. Carballo E, Blackshear PJ. Roles of tumor necrosis factor-alpha receptor subtypes in the pathogenesis of the tristetraprolin-deficiency syndrome. Blood. 2001; 98:2389–2395. [PubMed: 11588035]
30. Whitacre CC. Sex differences in autoimmune disease. Nat Immunol. 2001; 2:777–780. [PubMed: 11526384]
31. Chen YL, Huang YL, Lin NY, Chen HC, Chiu WC, Chang CJ. Differential regulation of ARE-mediated TNFalpha and IL-1beta mRNA stability by lipopolysaccharide in RAW264.7 cells. Biochem Biophys Res Commun. 2006; 346:160–168. [PubMed: 16759646]
32. Hansson GK, Hermansson A. The immune system in atherosclerosis. Nat Immunol. 2011; 12:204–212. [PubMed: 21321594]
33. Plump A. Atherosclerosis and the mouse: a decade of experience. Ann Med. 1997; 29:193–198. [PubMed: 9240624]
34. Paigen B, Holmes PA, Mitchell D, Albee D. Comparison of atherosclerotic lesions and HDL-lipid levels in male, female, and testosterone-treated female mice from strains C57BL/6, BALB/c, and C3H. Atherosclerosis. 1987; 64:215–221. [PubMed: 3606719]
35. Daugherty A. Mouse models of atherosclerosis. Am J Med Sci. 2002; 323:3–10. [PubMed: 11814139]

36. Chintalacharuvu SR, Wang JX, Giaconia JM, Venkataraman C. An essential role for CCL3 in the development of collagen antibody-induced arthritis. Immunol Lett. 2005; 100:202–204. [PubMed: 15878203]

37. Fan J, Heller NM, Gorospe M, Atasoy U, Stellato C. The role of post-transcriptional regulation in chemokine gene expression in inflammation and allergy. Eur Respir J. 2005; 26:933–947. [PubMed: 16264057]

38. Hao S, Baltimore D. The stability of mRNA influences the temporal order of the induction of genes encoding inflammatory molecules. Nat Immunol. 2009; 10:281–288. [PubMed: 19198593]

39. Pharoah DS, Varsani H, Tatham RW, Newton KR, de Jager W, Prakken BJ, Klein N, Wedderburn LR. Expression of the inflammatory chemokines CCL5, CCL3 and CXCL10 in juvenile idiopathic arthritis, and demonstration of CCL5 production by an atypical subset of CD8+ T cells. Arthritis Res Ther. 2006; 8:R50. [PubMed: 16507178]

40. Leichtle A, Hernandez M, Ebmeyer J, Yamasaki K, Lai Y, Radek K, Choung YH, Euteneuer S, Pak K, Gallo R, Wasserman SI, Ryan AF. CC chemokine ligand 3 overcomes the bacteriocidal and phagocytic defect of macrophages and hastens recovery from experimental otitis media in TNF−/− mice. J Immunol. 2010; 184:3087–3097. [PubMed: 20164426]

41. Carballo E, Lai WS, Blackshear PJ. Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability. Blood. 2000; 95:1891–1899. [PubMed: 10706852]

42. Xia L, Lu J, Xiao W. Blockage of TNF-alpha by Infliximab Reduces CCL2 and CCR2 Levels in Patients With Rheumatoid Arthritis. J Investig Med. 2011

43. Taylor PC, Peters AM, Paleolog E, Chapman PT, Elliott MJ, McCloskey R, Feldmann M, Maini RN. Reduction of chemokine levels and leukocyte traffic to joints by tumor necrosis factor alpha blockade in patients with rheumatoid arthritis. Arthritis Rheum. 2000; 43:38–47. [PubMed: 10643698]

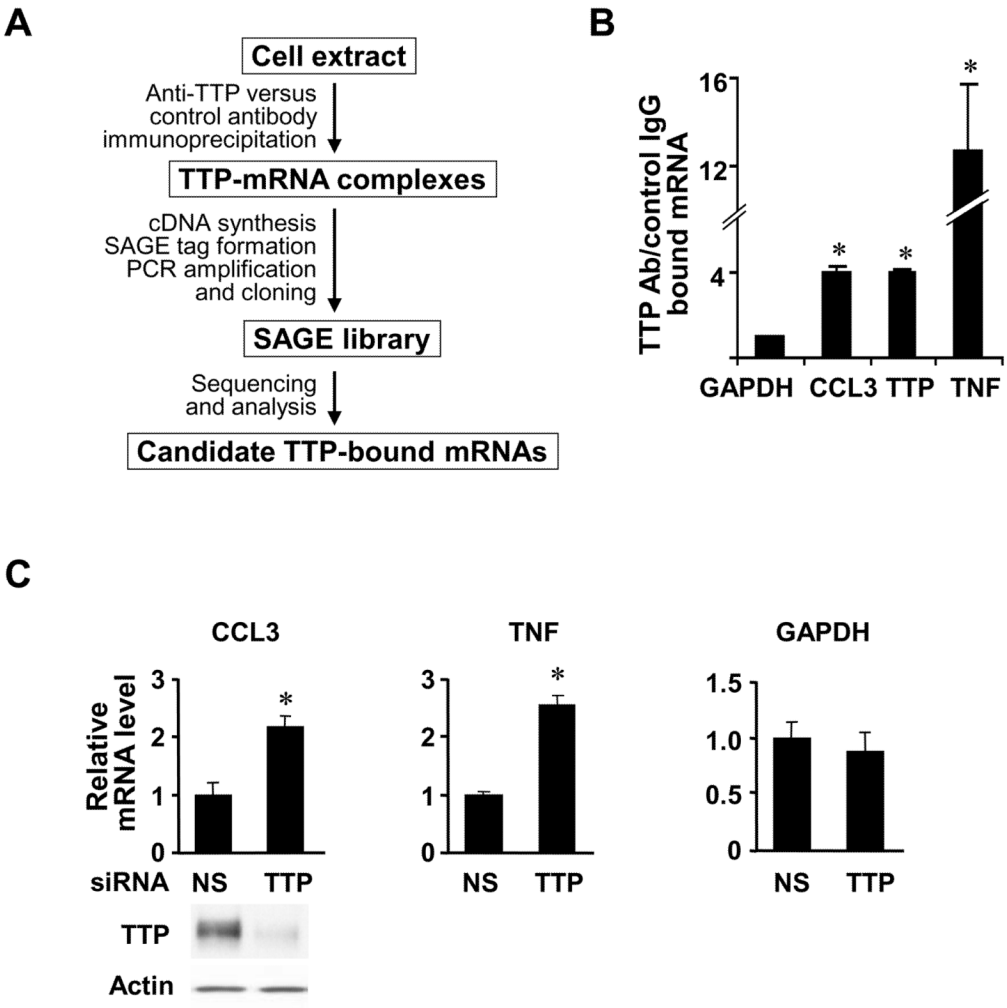
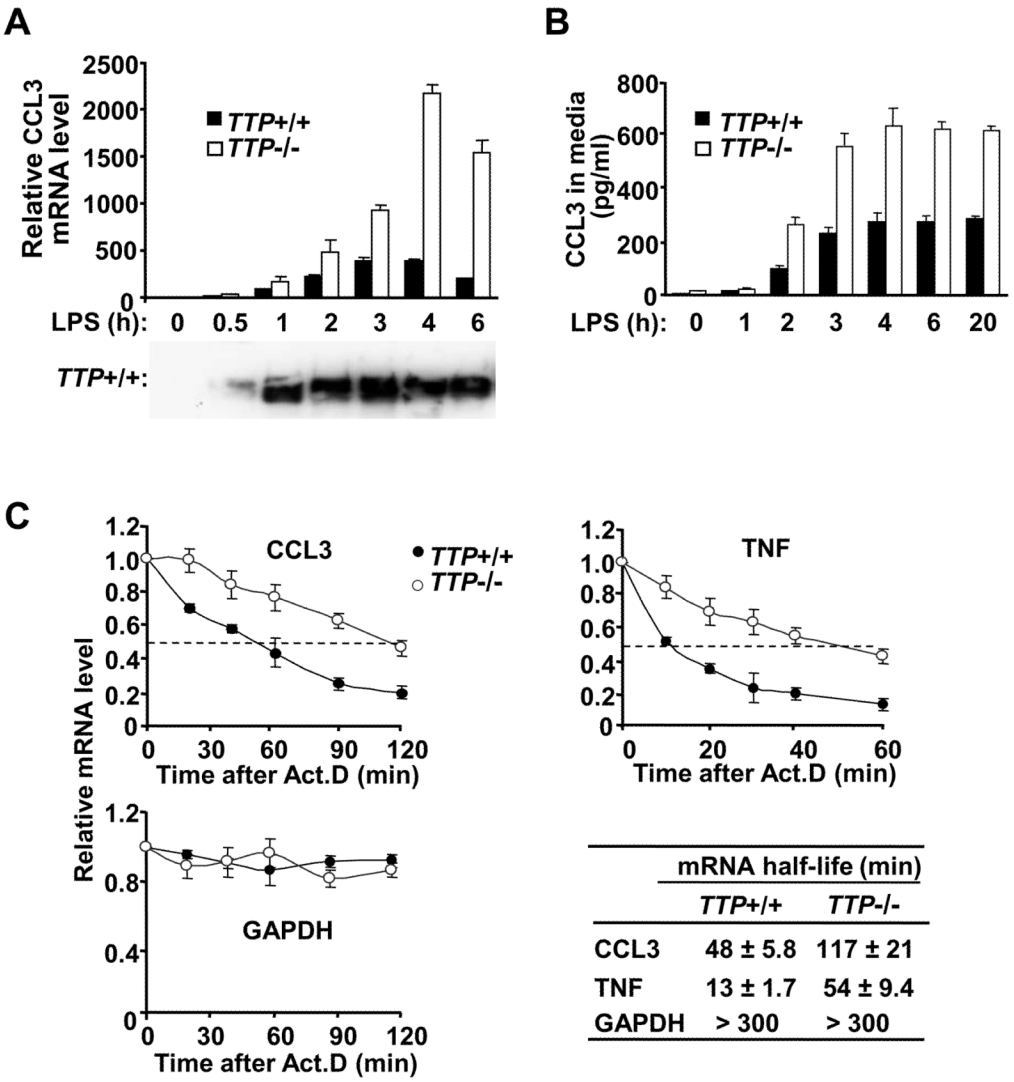


FIGURE 1.

TTP interacts with CCL3 mRNA and its knockdown increases CCL3 mRNA level in an activated human monocytic cell line. *A*, Scheme to identify all mRNAs interacting with TTP by immunoprecipitation and subsequent expression profiling using Serial Analysis of Gene Expression (SAGE). *B*, Confirmation of CCL3 mRNA binding to TTP protein by RT-PCR in the anti-TTP antibody versus nonspecific control immunoglobulin (IgG) bound RNA fractions of PMA-activated THP-1 cells. *C*, TTP depletion using siRNA, confirmed by western blotting, increases the levels of its target mRNAs. THP1 cells were transfected with either nonspecific (NS) or TTP-specific (TTP) siRNA, activated with PMA, and the target transcripts measured at 48 h by RT-PCR. Data presented as mean ± SEM, n = 3. *P < 0.05 compared to respective nonspecific controls.



	mRNA half-life (min)	
	TTP+/+	*TTP*-/-
CCL3	48 ± 5.8	117 ± 21
TNF	13 ± 1.7	54 ± 9.4
GAPDH	> 300	> 300

FIGURE 2.

CCL3 expression is increased in *TTP*−/− mouse bone marrow-derived macrophages (BMDM). *A*, CCL3 mRNA levels after LPS (10 ng/ml) stimulation are higher in *TTP*−/− compared to *TTP*+/+ BMDM. The time course of TTP protein induction by LPS treatment is shown by western blotting. *B*, CCL3 protein in the medium was quantified by ELISA. *C*, CCL3 and TNF mRNA half-lives are increased in *TTP*−/− versus *TTP*+/+ BMDM. After stimulating with LPS for 90 min, actinomycin D (Act. D) was added to the cells. At the indicated times (Time after Act. D), mRNA was isolated from the cells and quantified by RT-PCR. Dashed line indicates 50% level. Data presented as mean ± SEM, n = 3 to 6.

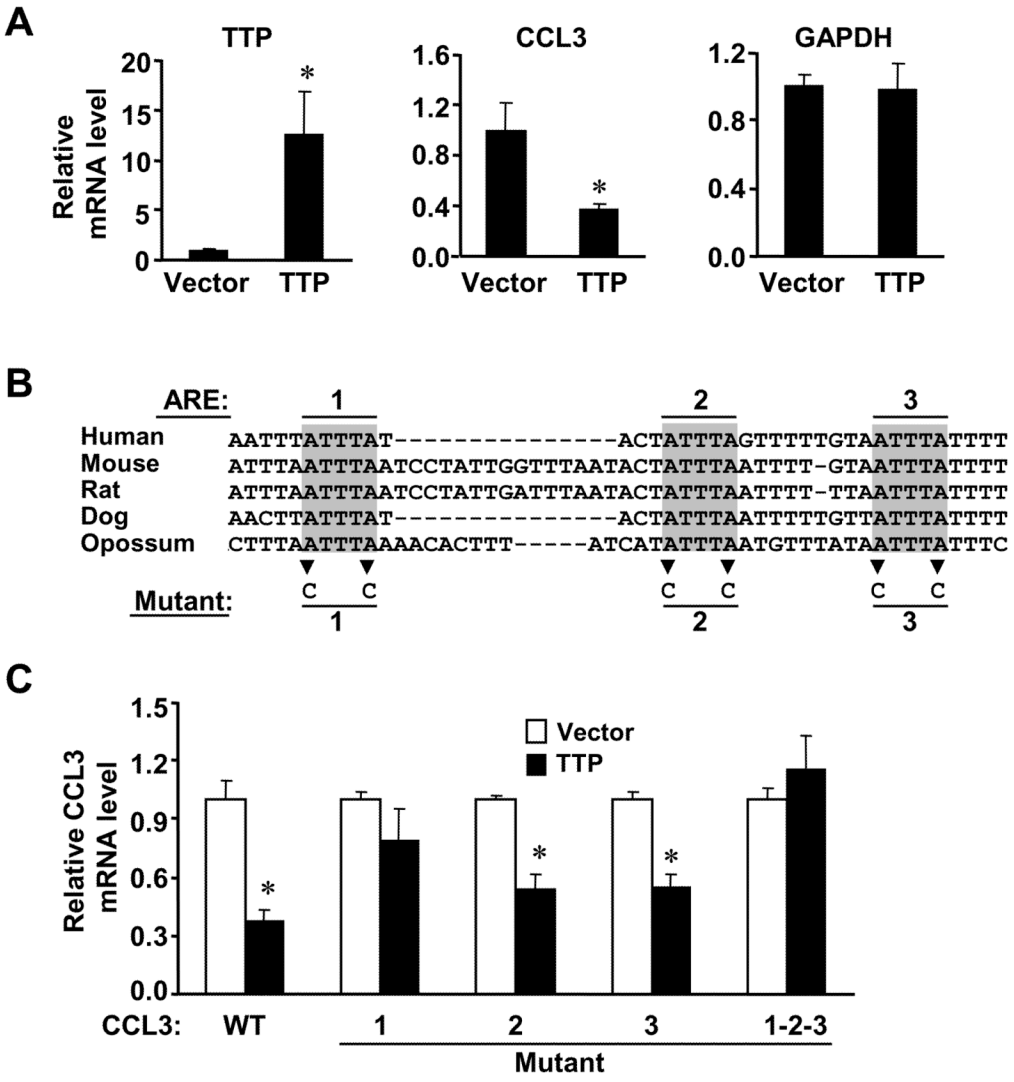


FIGURE 3.

TTP destabilizes CCL3 mRNA through conserved AU-rich elements in HEK293 cells. *A*, TTP cDNA expression specifically decreases the level of cotransfected wild-type full length CCL3 mRNA while 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.

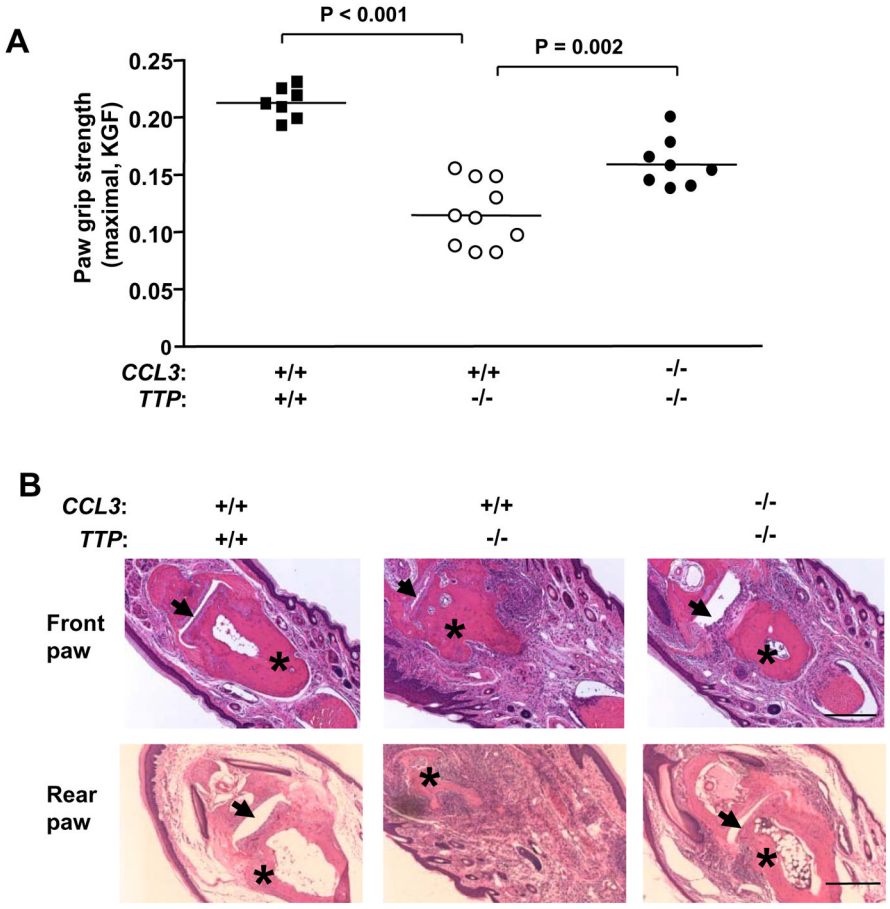


FIGURE 4.

Deletion of *CCL3* reduces the inflammatory arthritis of *TTP*−/− mice. *A*, Paw grip strength is improved in the absence of CCL3. Four-paw grip strength of 9 wk-old female mice was measured by using a digital grip strength meter. *TTP*+/+ (square), *TTP*−/− (open circle), and *CCL3*−/− *TTP*−/− (filled circle). *B*, Bone erosion and tissue inflammation of *TTP*−/− mouse paws are reduced in the absence of CCL3 (*TTP*−/− *CCL3*−/− genotype). Representative H&E stained sections of front (upper panel) and rear (lower panel) paw joints of female mice in the 22 to 35 wk range. Synovial space (arrowhead); bone (asterisk). Scale bar: 200 μm.

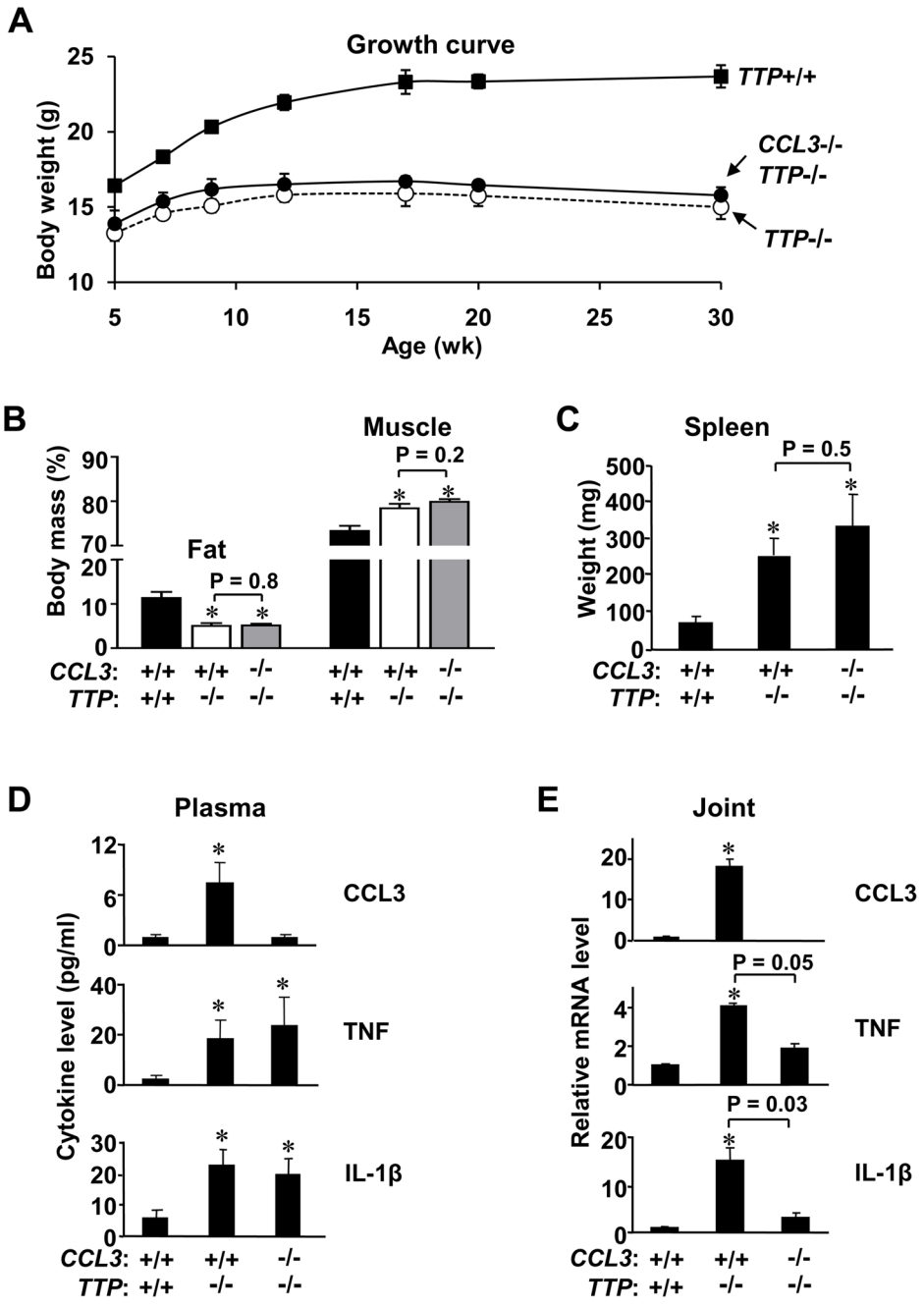


FIGURE 5.

Deletion of *CCL3* dissociates localized from systemic inflammation in *TTP −/−* mice. *A*, Growth curves of female *TTP+/+*, *TTP −/−*, and *CCL3 −/− TTP −/−* mice are shown. Arrowheads indicate the closely apposed growth curves of *TTP −/−* mice and *CCL3 −/− TTP −/−* mice (n= 4 to 7). *B*, Body mass composition determined by NMR (n = 3 to 6). *C*, The splenomegaly of *TTP −/−* mice is unaltered in the absence of CCL3 (n = 4). *D*, The plasma protein levels of TTP targets TNF and IL-1β continue to be elevated in the absence of CCL3 in *TTP −/−* mice (n = 3). *E*, The joint tissue mRNA levels of TTP targets TNF and IL-1β are reduced in the absence of CCL3 in *TTP −/−* mice (n = 3). Except as indicated in (A) the mice

used were in the age range of 17 to 22 wk. Data are presented as mean ± SEM. *P < 0.05 compared to *TTP*+/+ mice.

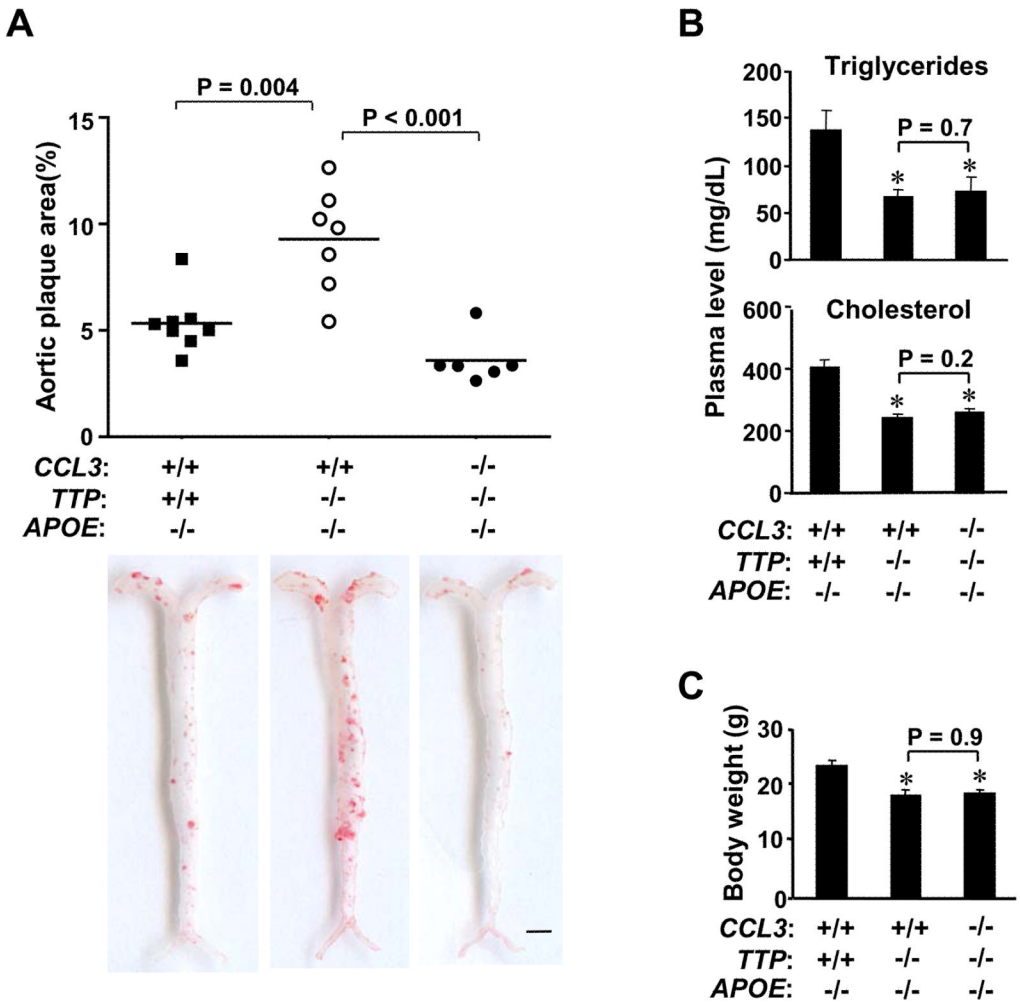


FIGURE 6.

Deletion of *CCL3* prevents the increase in aortic atherosclerosis of *TTP −/− APOE −/−* mice. *A*, The increased aortic atherosclerotic plaque area (Sudan IV stained) of female *TTP −/− APOE−/−* mice (23 to 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 to *APOE −/−* mice (n = 6 to 8). Data are presented as mean ± SEM. *P < 0.05 compared to *APOE−/−* mice. *C*, Body weights of 23 to 25 wk-old female mice of the indicated genotypes. Data shown as mean ± SEM, n = 4 to 8. *P < 0.05 compared to *APOE −/−* mice.