RNA BIOLOGY
2019, VOL. 16, NO. 3, 309–319
https://doi.org/10.1080/15476286.2019.1572437

RESEARCH PAPER

OPEN ACCESS

Bi-phased regulation of the post-transcriptional inflammatory response by Tristetraprolin levels

Linah Mahmoud*, Walid Moghrabi*, Khalid S. A. Khabar, and Edward G. Hitti

Molecular BioMedicine Program, King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia

ABSTRACT

AU-rich elements (AREs) are cis-acting instability and translation inhibition elements that are present in the 3′UTR of most inducible inflammatory mRNAs such as TNF and Cxcl2. mRNAs that contain AREs are, by default, repressed and only transiently expressed in response to stimuli. They are targeted by the inducible RNA-binding protein Tristetraprolin (TTP) which blocks their translation and facilitates their decay, thereby contributing to the quick termination of their expression. The exogenous over-expression of TTP in HEK293 cells can unexpectedly lead to the upregulation and extended expression of a nanoLuciferase reporter that contains the ARE of TNF. Here we show that, a moderate downregulation of the highly expressed endogenous TTP after LPS induction by siRNA in macrophages can lead to a reduction in the release of TNF and Cxcl2. We propose that, in contrast to their canonical function, very high levels of induced TTP at the onset of the inflammatory response can enhance the expression of ARE-mRNAs at the post-transcriptional level, independently of phosphorylation status. As the inflammatory response progresses, TTP levels diminish but they continuously regain their ability to reduce the expression of ARE-mRNAs to reach a turning point of 'optimal TTP level' with a maximum ability to repress ARE-mRNA expression. Below this level, a further reduction in TTP levels now leads to the loss of canonical-TTP function resulting in increased ARE-mRNA expression. These novel findings should contribute to the understanding of feedback loops that control the kinetics of the inflammatory response.

ARTICLE HISTORY
Received 3 October 2018
Revised 18 December 2018
Accepted 14 January 2019

KEYWORDS
AU-rich elements; post-transcriptional; TNF; Tristetraprolin; ZFP36

Introduction

The innate inflammatory response is a cell-autonomous process that involves the induction of the expression of hundreds of genes in a highly regulated manner, both at the amplitude and temporal levels. Many of the induced pro-inflammatory genes are cytokines that are expressed only transiently and this is achieved by an orchestrated network of cellular signaling events that target the regulatory machineries of transcription and post-transcription [1–3]. At the post-transcriptional level, mRNA instability and translation is of particular importance for transient expression, and this is often ensured by the activity of so called AU-rich elements (AREs) that are located in the 3′Untranslated Regions (3′UTR) in the mRNAs of many inflammatory cytokines including TNF and Cxcl2 [4–6]. By default, many AREs destabilize and repress the translation of *cis*-mRNAs, however, they can also respond to inflammatory stimuli, by temporary stabilization of the mRNA and 'de-repressing' its translation [7]. The signal is mainly relayed by the p38 MAPK signaling module which can be triggered by stress, inflammatory cytokines or molecular patterns of common microbes such as LPS. This phosphorylation-dependent signaling cascade leads to the activation of the central element of the pathway, the p38 kinase [8–10]. In turn, p38 can phosphorylate and activate a number of downstream kinases including MK2 and MK3 [11–13]. The link between MK2 and target mRNAs is the inducible ARE-binding and mRNA destabilizing protein Tristetraprolin (TTP, also called ZFP36); it is suggested that p38 and MK2 are both critical for efficient expression but also the inhibition of TTP function by direct phosphorylation [14–18]. The end result is the amplification and prolongation of the inflammatory response by stabilizing and enhancing the translation of many cytokine and other ARE-mRNAs [6,19,20]

TTP is an immediate early gene and its own mRNA contains an ARE which contributes to its low basal steady state level, but can be strongly and transiently induced by inflammatory stimuli [21,22]. MK2 phosphorylates TTP at two serine residues, S52 and S178, resulting in increased protein stability but also its inhibition and accumulation in the cell [14,15,23]. Phosphorylation can also control the cytoplasmic and nuclear localization of TTP [23] and its binding to 14–3-3 proteins which regulates its association with stress granules [24]. TTP itself does not possess nuclease activity; but, it acts as a scaffold that links deadenylation, decapping, and exonucleolytic decay complexes to ARE-containing mRNA [25]. The association of TTP with the scaffolding complex Not1-Caf1 in the cytoplasm is also critical for its mRNA degrading activity [10,26,27].

Lai *et al* have shown that exogenous overexpression of TTP in HEK293 cells can, surprisingly, lead to the upregulation of

CONTACT Edward G. Hitti ehitti@kfshrc.edu.sa King Faisal Specialist Hospital and Research Centre, P3354, MBC-03, Takhasusi Street, Riyadh 11211, Saudi Arabia
*Co-first authors

© 2019 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (http://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way.

TNF mRNA, a phenomenon that was attributed to an overexpression artefact [28]. Here, and by comparing the levels and effects of transfected and induced TTP in HEK293 and RAW264.7 cells respectively, as well as by down-regulating induced TTP in RAW264.7, we report that this observation can be a physiological mechanism that regulates TTP activity.

Materials and methods

Vectors, antibodies, cells, taqman real-time PCR

The pcDNA3.1 vector was purchased from Life Technologies. TTP expressing pCR3.1 vector was used previously [29], TTP-AA mutant expressing vector was a kind gift from George Stoecklein and was also used previously [15,24]. The nanoluciferase and firefly reporter vectors were prepared by replacing the GFP gene in RPS30-GFP with nanoluciferase and firefly genes that were amplified by PCR (Promega) [30], the ARE of TNF was inserted in the 3'UTR of RPS30-nLuc as described previously [31]. Anti-TTP antibody was a kind gift from Gilles Pages [32], β-actin antibody was purchased from Sigma. TTP-Immunoprecipitation experiments and western blots were performed using custom made affinity purified TTP polyclonal antibody and against C-terminal end of TTP PRRLPIFNRISVSE with Genscript. The specificity of the antibody was tested with TTP transfected lysates or LPS induced lysates against pre-immune serum (Data not shown). HEK-293 and RAW264.7 cell lines were purchased from ATCC. Knock-out bone marrow derived macrophages were prepared as described previously [15]. Taqman Real-time PCR probes were ordered from applied Biosystems, the cycler used was CFX60 from Biorad, the 2ˆ(–delta delta CT) relative quantification method was used. The house keeping gene β-Actin was Vic labelled and used for normalization and the taqman probes for TNF, Cxcl2 and GAPDH were FAM labelled.

Transfection and nano-luciferase assays

0.5 × 10^6 HEK293 cells were seeded in 6 well culture plates. The cells were transfected using Lipofectamine 2000 (Themofisher) with total amount of 700 ng of DNA that contains 400 ng of the RPS30-nanoLuciferase reporter vectors and increasing levels of TTP expressing vectors. Empty pcDNA3.1 vector was added to keep total transfected DNA at 700 ng for all transfection. Nanoluciferase assays were performed using the kit and protocol of Promega. The levels of transfected TTP in HEK293 cells were compared with induced TTP in RAW 264.7 cells on western blots. Since TTP should be induced by LPS in all RAW264.7 cells, the transfection in HEK293 cells should be very high for a good comparison. Therefore, we tested the efficiency of the transfection protocol by transfecting 700 ng of gWIZ-GFP vector (genlantis) into HEK293 cells and performed flow cytometry to assess the transfection efficiency and it turned out to be near complete (>96%).

For RAW264.7, 5 × 10^6 cells were seeded in a 10 cm plate and transfected using Lipfectamine LTX with 4 µg nano-Luciferase reporters along with 4 µg Firefly (FF) reporter and 50 nM siRNAs that target TTP overnight (see below). Next, the cells were split into 6 well plates and left for one more day followed by treatment with 0.5 µg/ml LPS. Dual Nano Luciferase assays were performed using the kit and protocol of Promega.

TTP knockdown, LPS treatment, western blotting, ELISA, reverse transcription

Three different siRNA reagents were used to target and down regulate TTP expression. siTTP1, a single siRNA, was ordered from Metabion and was used in a previous report CCUG AGAAUCCUGGUGCU CAA [33]. siTTP2 and siTTP(4xPool) were ordered from Dharmacon (Cat. Nb. A-041045–14-0005 and E-041045–00-0005). siTTP 2 is also a single siRNA with the following sequence CUUUGAGGCAGGGGUGUUU) while siTTP(4xPool) contains 4 pooled siRNAs (1. UCCCCAU CUUCAAUCGUAU, 2. CUUUGAGGCAGGGGUGUUU, 3. CCCAAGUCUUCUGUUGUUU, and 4. GUAUUAAGAUU UUAUAGUA). Two siRNA controls (scramble) were used: SCR1; scramble siRNA that was previously used was ordered from Metabion [34]. SCR2 siGENOME Non-Targeting siRNA #1 from Dharmacon (Cat. Nb. D-001210–01-20). RAW264.7 cells were transfected with up to 100 nM siRNA using lipofectamine LTX (Thermofisher) for 48 hours. For time course experiments, equal number of cells (5 × 10^6) were seeded in 10 cm plates and transfected for ~24 hours. Equal number of cells (5 × 10^5) were then reseeded in 6 well plates and left overnight. Next day, the cells were treated with 0.5 µg/ml LPS for different time points. Aliquots from the supernatants were taken for ELISA and the cells were directly lysed in 2xSDS sample buffer (Invitrogen). Lysate were sonicated to shear DNA and equivalent levels were loaded onto SDS PAGE gel, blotted onto nylon membrane and screened with TTP and β-Actin antibodies. TNF and Cxcl2 ELISA were performed using Kits from Thermo Scientific following manufacturer protocol.

For mRNA time course and mRNA stability experiments, total mRNA was prepared using TRI reagent (Sigma-Aldrich). Reverse transcription was performed using Superscript II and Oligo-dT (Invitrogen). For mRNA stability experiments, cells were treated with Actinomycin-D (10 µg/ml) to shut-off transcription. After real time PCR, decay curves were plotted using GraphPad Prism software.

mRNA immunopreciptation

Protein G–Sepharose beads (GE Health Science) were washed 4 times with NT2 buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM MgCl2 and 0.05% NP-40] then pre-coated with 30 µg anti-TTP for 2 hours at 4°C in 100 µl NT2. The beads were washed with NT2 buffer and incubated (1 h, 4°C) with 300 µl lysates supplemented with 1 mM DTT and RNAseOut (Invitrogen). Negative control IP was performed similarly with nonspecific IgG mouse antibody. Total RNA was isolated with TRI reagent followed by reverse transcription and real time PCR. The level of bound ARE containing mRNA was normalized against background levels of un-specifically bound β-Actin mRNA.

Results

Moderate expression levels of TTP upregulate the expression of a TNF-ARE containing luciferase reporter

Overexpression of TTP can lead to the unexpected upregulation of the ARE containing mRNA of TNF [28]. To determine the minimal level of transfected TTP that loses the ability to down regulate and becomes capable of upregulating the expression of a TNF ARE containing mRNA, we performed TTP transfection/titration experiments in HEK293 cells. 400 ng of Nanoluciferase (nLuc) reporter that contains the ARE of TNF in its 3′UTR (nLuc+ARE) were co-transfected with increasing levels of a TTP expressing vector (0, 1, 3, 5, 10, 20, 50 and 100 ng) (Fig. 1A, B). All transfection mixes contained a total of 700 ng DNA by addition of appropriate levels of empty pcDNA3.1 vector. Transfected TTP led to a reduction in the expression of the nLuci+ARE construct starting at even 1 ng of transfected vector (~70% of level in control cells with no transfected TTP), 3 ng led to significant further reduction (~37% of control) and maximum reduction was at only 5 ng of transfected TTP (~28% of control), at 10 ng the levels of Luciferase started increasing but were still lower than in the cells with no transfected TTP, ~50%. At 20 ng of transfected TTP, the level of the Luciferase reporter was comparable to cells with no exogenous TTP transfection, at 50 and 100 ng of transfected TTP, reporter levels started to increase to ~180 and 280% of control (Fig. 1B). TTP had no effect on the expression of a non-ARE-reporter (nLuc) in a parallel experiment indicating that the observed effect is ARE-specific and thus post-transcriptional (Fig. 1A, B). To test if physiologically induced TTP could have a similar effect, we compared the transfected TTP levels from the reporter assay in HEK293 cells with LPS induced TTP levels in RAW264.7 mouse macrophages. Surprisingly, transfected levels that were capable of enhancing ARE-reporter expression (50 ng of transfected TTP vector) were significantly lower than induced TTP two and four

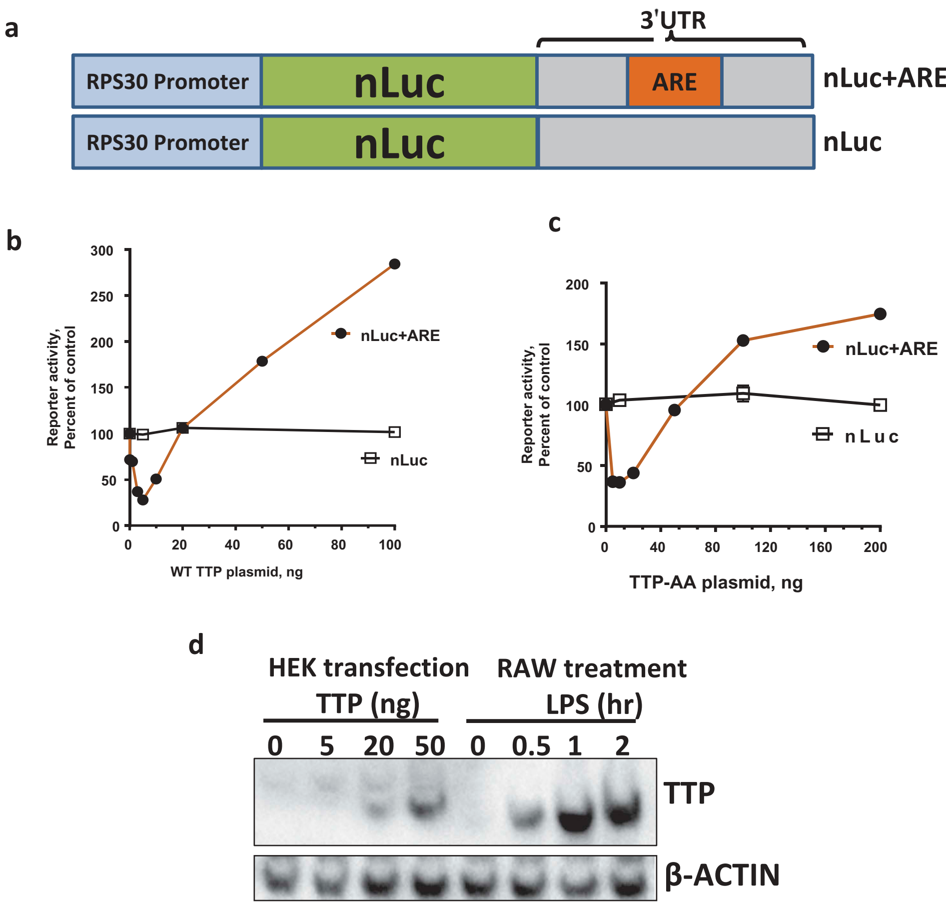

Figure 1. Moderate cellular levels of TTP and TTP-AA can upregulate the expression of an ARE containing reporter. (A) Schematic representation of reporter vectors used in this study. The RPS30 promoter is constitutively active but non-inducible and suitable for the investigation of post-transcription; it drives the expression of the nanoLuciferase reporter (nLuc). The control nLuc vector lack any ARE in its 3′UTR and in the nLuc+ARE vector has the ARE of TNF inserted in its 3′UTR. (B) In six-well culture plates 5×10^5 HEK293 cells were co-transfected with 400 ng of nanoLuciferase reporter vector that contains the ARE of TNF its 3′UTR (Luc+ARE) along with the increasing levels of a vector expressing WT TTP (0, 1, 3, 5, 10, 20, 50 and 100 ng) or (C) TTP phosphorylation mutant TTP-AA (0, 5, 10, 20, 50, 100 and 200 ng). The total amount of the DNA transfection mix was kept at 700 ng for all transfections by adding empty pcDNA3.1 vector. Transfected cell were left over night, collected, and nanoLuciferase assays were performed to quantify the expression of the reporters. The non-ARE reporter (nLuc) was used as negative control. In the y-axis the percent nLuc activity relative to the control without TTP transfection is shown. (D) TTP Western blot: an aliquot of the cells collected in A that were transfected with 0, 5, 20 and 50 ng of TTP vector were directly lysed in SDS sample buffer for western blotting. In parallel 5×10^5 RAW264.7 mouse macrophages were treated for the indicated time points with 0.5 μg/ml LPS and lysed in SDS sample buffer. Equivalent levels of lysates from both cell lines were loaded on the same gel and blotted. After probing with TTP antibody the gel was stripped and re-probed with β-actin antibody.

hours after LPS treatment suggesting that the induced TTP in RAW264.7 cells may lead to the upregulation of the expression of ARE containing mRNAs (Fig. 1D). Overall, the results indicate that there is a very narrow range of low TTP levels in HEK293 cells that is capable of reducing ARE mRNA expression and a wide range of moderate and high expression levels that does the opposite. A single low 'optimal TTP level', in this case, 5 ng of transfected TTP in HEK293 cells, has maximum TTP activity.

The level-dependent increase in ARE-reporter expression in HEK293 cells can occur in the absence of TTP phosphorylation

Several reports indicate that the phosphorylation of TTP at two particular sites by MK2, serines 52 and 178, can significantly reduce its function but unexpectedly also increase its level by stabilizing its protein and mRNA [14,15,17,22–24]. To test if phosphorylation at the two MK2 sites is necessary for the observed ability of TTP to increase reporter activity at high expression levels in HEK293 cells, we repeated the ARE reporter assay with a serine to alanine TTP mutant at the critical MK2 phosphorylation sites, TTP-AA (Fig. 1C) [24]. Like the wild-type, TTP-AA was capable of reducing nLuc +ARE expression at low levels but, more importantly, it also led to increased expression at high levels indicating that phosphorylation is not directly necessary for the observed 'level-dependent' upregulation of ARE-reporter expression, at least in this experimental setup. It is interesting to note, that overall the TTP-AA transfection levels necessary for the upregulation of the nLuc+ARE reporter were higher than in WT TTP, suggesting that a possible phosphorylation of WT TTP is amplifying the expression of the nLuc+ARE reporter (Fig. 1B, C). In conclusion, our observation indicates that the high levels of TTP can reduce its activity even in the absence of phosphorylation at the serines 52 and 178 sites. However, this finding does not rule out a direct inhibitory effect of phosphorylation in reducing TTP activity which we believe it is essential and can act at low expression levels.

Down regulation of LPS-induced TTP can reduce TNF and Cxcl2 release

The canonical function of TTP is to block the translation and to facilitate the decay of ARE-containing mRNA decay. However, the levels of TTP often directly correlate with its targets at the onset of the inflammatory response. For instance, in LPS treated RAW264.7 mouse macrophages, both TTP protein and the mRNAs of TNF and Cxcl2 peak at four to six hours post LPS treatment (Fig. 2). To investigate whether or not the high levels of LPS-induced TTP in RAW264.7 cells may be enhancing TNF or Cxcl2 expression,

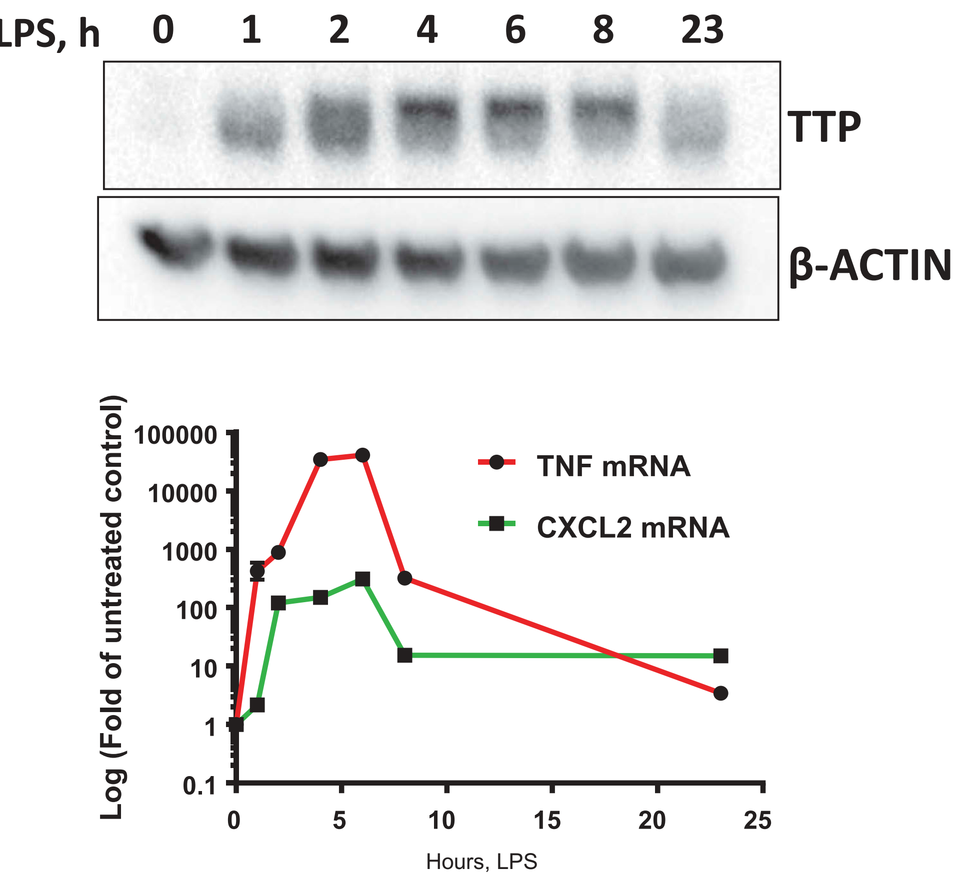

Figure 2. Direct correlation between cellular TTP and ARE-containing mRNAs levels. 5×10^5 RAW264.7 mouse macrophages were treated for the indicated time points with 0.5 μg/ml LPS. The cell were split in two on half was lysed in SDS sample buffer and a western blot was performed to assess TTP and β-actin levels. From the remaining cells total RNA was prepared and real time PCR was performed to quantify the relative levels of TNF and Cxcl2 mRNAs.

we knocked down TTP expression in LPS treated RAW264.7 macrophages using three independent siRNA procedures that target TTP. siTTP1 and siTTP2 are made of single siRNAs that target TTP mRNA at different locations. siTTP(4xPool), which contains a pool of 4 other siRNAs that co-target TTP at 4 different locations. Two non-specific scramble control siRNAs were used. 48 hours post transfection the cells were treated for 2, 4, and 6 hours with LPS. TTP levels were assessed by western blotting: the knockdowns with siTTP1 and siTTP2 were moderate, while the knockdown with siTTP (4xPool) was very strong (Fig. 3A, B and C right panels). TNF release was measured by ELISA. Interestingly, the moderate knockdowns with siTTP1 and siTTP2 led to a parallel reduction in TNF release (Fig. 3A and B left panels) suggesting that the high TTP levels induced in the SCR controls are reducing TNF expression. The dramatic knockdown with siTTP (4xPool) (TTP levels were only slightly higher than in LPS free background), led to increased levels of TNF production, similar to the well-established TTP knockout effect [35] (Fig. 3A). A similar effect was observed with Cxcl2 release; after 4 h of induction, the release of Cxcl2 was lower with siTTP1 and 2 knockdowns and higher with the strong siTTP (4xPool) knockdown. (Fig. 3A and B middle panels).

The results again, suggest the existence of an 'optimal TTP level' with a maximum ability to reduce ARE-mRNA expression. Below and above this level canonical TTP activity decreases and TNF and Cxcl2 levels increase. The knockdown with siTTP(4xPool), reduced TTP levels below the 'optimal

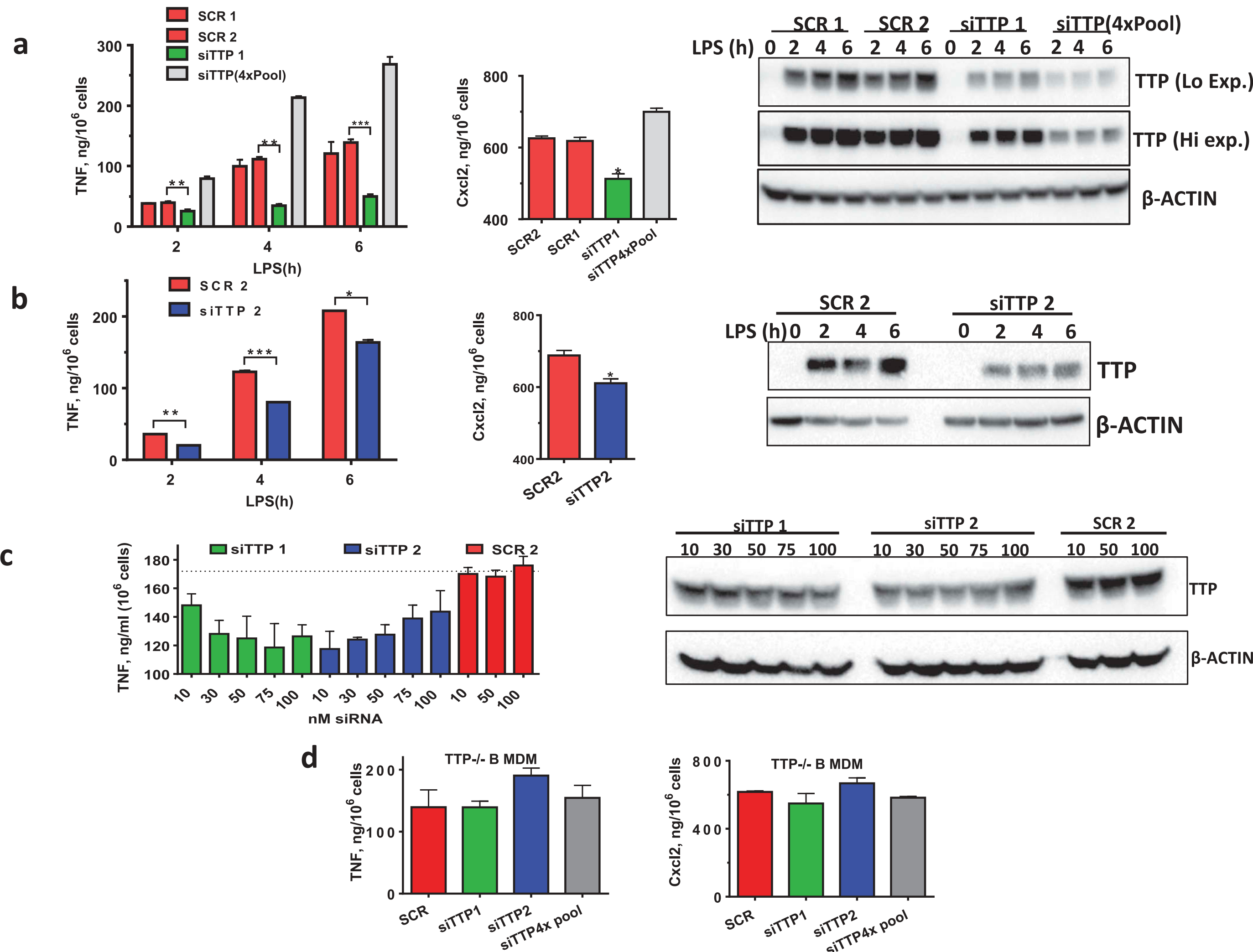

Figure 3. Moderate knockdown of LPS-induced TTP in RAW264.7 can lead to the down regulation of TNF and Cxcl2 release. (A) in 10 cm culture plates 5×10^6 RAW264.7 cells were transfected independently with two siRNA scramble controls (SCR1 and SCR2) or the single siRNA that targets TTP: siTTP1 or with siTTP (4xPool) which contains 4 pooled siRNAs (from Dharmacon). The cells were split into six well plates and after 48 hours, 0.5×10^6 cells were treated with LPS for 2, 4, and 6 hours. Supernatants were recovered and TNF release was quantified by ELISA (Left Panel). The 4 h time point was also quantified for Cxcl2 release (Middle panel). The cells were directly lysed in SDS sample buffer and equivalent levels were used for western blotting. The blots were probed with anti-TTP and β-actin antibodies (Right Panel). To highlight the difference between the knockdowns with siTTP1 and siTTP(4xPool) a low (Lo exp.) and high (Hi exp.) exposures of the anti-TTP blot are shown. (P values of paired t-test (SCR2 with siTTP1) from three independent experiments *<0.05, **<0.005, one representative experiment is shown with SEM). (B) Same experiment as in A was repeated with another single siRNA that targets TTP (siTTP2) and SCR2 was used as control (P values of paired t-tests (SCR2 with siTTP1) from three independent experiments **<0.005, ***<0.0005. *<0.05, one representative experiment is shown with SEM). (C) Same experiment as in A and B, siTTP1 and siTTP2 knockdowns were performed in parallel and with several concentrations of the siRNAs and controls as indicated and four hours LPS treatment, the dotted line represents the average of the three controls, TNF release levels were quantified. (D) TTP-knockout bone marrow derived macrophages were transfected with SCR2, siTTP1, siTTP2 and siTTP(4xPool) and treated for 4 hours with LPS. Supernatants were tested for TNF and Cxcl2 release by ELISA.

TTP level' leading to a loss in canonical TTP function and an increase in TNF release, while in the knockdowns with siTTP1 and siTTP2, TTP levels were reduced but remained above the 'optimal TTP level' leading to a gain in canonical TTP function and to a decrease in TNF release.

In Fig. 3C, we performed parallel TTP knockdowns with siTTP1 and siTTP2 at different siRNA concentrations (10, 30, 50, 75, 100 nM). We assume that in all those knockdowns TTP levels remained above the 'optimal TTP level' and in all of them a reduction in TNF release was observed. We attempted a comparable experiment with siTTP(4xPool) but the mix of the siRNAs always led to dose independent dramatic TTP knockdowns with a parallel increase in TNF levels (data not shown). Transfecting TTP-/- (knockout) bone marrow derived macrophages with siTTP1, 2 and 4XPool did not impact TNF and Cxcl2 release implying that the effect found in RAW264.7 is specific to TTP (Fig. 3D).

The mild knockdowns of TTP do not affect the steady state levels, stability and binding to target mRNAs

To investigate if the mild knockdown with siTTP1 and 2 affect ARE-containing mRNA levels and stability we performed mRNA time course and Actinomycin-D chase experiments after LPS stimulation. The levels and stability of TNF mRNA were not significantly affected by the mild knockdowns. On the other hand, the strong knockdown with siTTP(4xpool) led to a significant upregulation in TNF mRNA levels and stability (Fig. 4A). The level of Cxcl2 mRNA was not significantly affected by all knockdowns (Fig. 4B). Its stability 4 hours after LPS

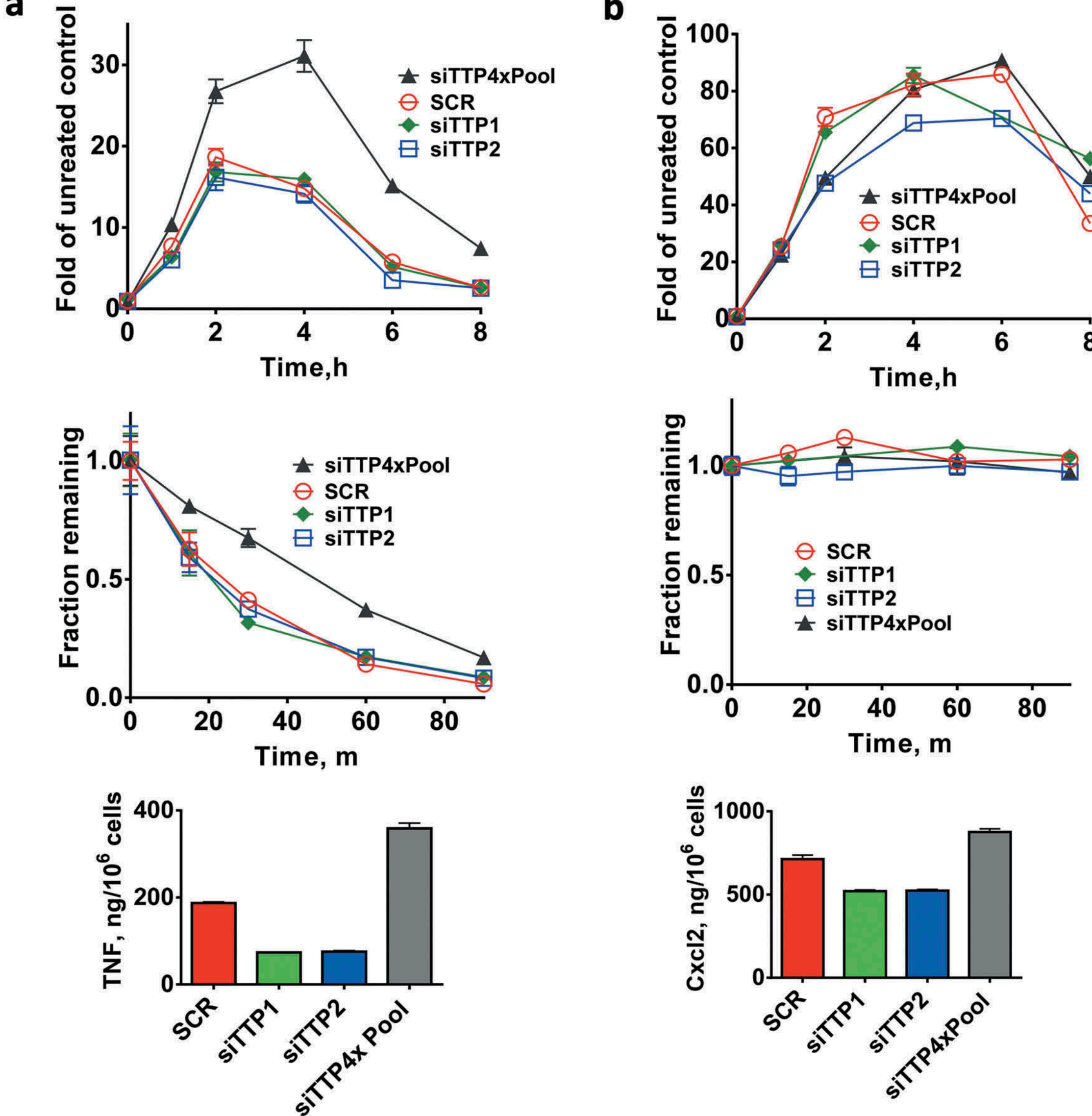

Figure 4. Steady state levels and stability of ARE containing mRNAs after TTP knockdowns. RAW 264.7 cells were transfected with the indicated siRNAs in a 10 cm plate overnight and next day seeded in two 6 well plates for LPS time course and mRNA stability experiments. For the time course the cells were treated for the indicated time point with LPS, TNF and Cxcl2 mRNAs were measured by real time PCR. The data is shown in fold of untreated SCR control (A and B upper panel). The same transfected cells were used to investigate mRNA stability of the same mRNAs by Actinomycin D chase experiments after 4 h LPS treatment (A and B middle panel). The release of TNF and Cxcl2 was monitored by ELISA (A and B lower panel).

stimulation remained very high and was not significantly affected by the neither the mild nor the strong knockdowns similar to a previously observation in LPS induced WT and TTP knockout macrophages [6] (Fig. 4B). In this experiment, we also monitored the release of TNF and Cxcl2 and again the mild knockdowns with siTTP 1 and 2 led to a reduction in the release of TNF and Cxcl2 while the strong knock down led to an increase in the release of both cytokines (Fig. 4A and B lower Panel).

To compare the binding of TTP to target mRNAs in the mild and strong knockdown conditions to SCR control, we performed mRNA/TTP co-immnunoprecipitation experiments in RAW264.7 cells that were treated with LPS for 4 h. The enrichment of the ARE-containing mRNAs with the TTP-IP was compared against a nonspecific IgG control. Typically, both cytokines were strongly enriched with the TTP-IP; 7–8 fold more than in IgG control, while the GAPDH mRNA that lacks AREs was not enriched at all (Fig. 5). Interestingly, the lower levels of immunoprecipitated TTP in the mild knockdowns (siTTP1 and 2) led to a comparable enrichment of ARE-mRNAs as SCR control. This suggests that free excess unbound TTP molecules exist in the SCR control and lower TTP levels are sufficient for full binding. Although steady state TNF mRNA levels are higher in the strong siTTP(4xPool) knockdown (Fig. 4A upper panel)), the enrichment with the TTP pulldown was weakest probably due to the very low levels of TTP. Also, the enrichment of Cxcl2 mRNA was lower in strong knockdown compared to control and the mild knockdowns. The IP of TTP was confirmed in a western blot (Fig. 5 lower panel).

Down regulation of LPS-induced TTP reduces the expression of an ARE-luciferase reporter

Next, we investigated if the observed TNF-reducing moderate TTP knockdown effect in RAW264.7 is ARE-dependent and thus post-transcriptional. Therefore, Nano-luciferase reporter experiments were performed using the same constructs as above (Fig. 1A). RAW264.7 cells were co-transfected with either the nLuc or nLuc+ARE reporter and with either SCR control or siTTP1 or siTTP2 or siTTP(4xPool). The cells were split, left untreated or treated for six hours with LPS before measuring Nano-Luciferase activity and performing western blots to assess TTP levels. The expression of the negative control reporter that lacks an ARE (nLuc) did not respond to LPS in all four transfection sets (Fig. 6A, left panel, up). Its expression was also not affected by the knockdowns with siTTP1, siTTP2 and siTTP(4xPool) after LPS treatment. The nLuc+ARE reporter on the other hand, responded to LPS and its expression increased in all four sets of transfection Fig. 6B. Before LPS treatment, the knockdowns of the background TTP levels by siTTP1, siTTP2 and siTTP(4xPool) led to upregulations of the nLuc+ARE reporter levels that negatively correlate to the level of TTP (Fig. 6B upper and lower panels). After LPS treatment the strong knockdown with siTTP (4xPool) led to the strongest upregulation of the reporter (9.8 nLuc/FF units) similar to the well-known knockout situation [35]. Most importantly, the mild knockdowns of LPS-induced TTP with siTTP1 and 2 led to a weaker upregulation of the nLuc+ARE reporter compared to SCR control (3.7 and 4.1 nLuc/FF units compared to 4.8 in the control). The TTP knockdown experiment with the nLuc+ARE was repeated five times and the LPS-dependent upregulation of reporter levels were consistently lower in the mild knockdown of siTTP1 and 2 compared to SCR control indicating that the very high level of TTP in the SCR control is reducing the upregulation of the ARE reporter after LPS stimulation (Fig. 6C). These results indicate that mild reductions in the levels of LPS induced TTP reduce the expression of a reporter gene in an ARE-dependent post-transcriptional manner (Fig. 6).

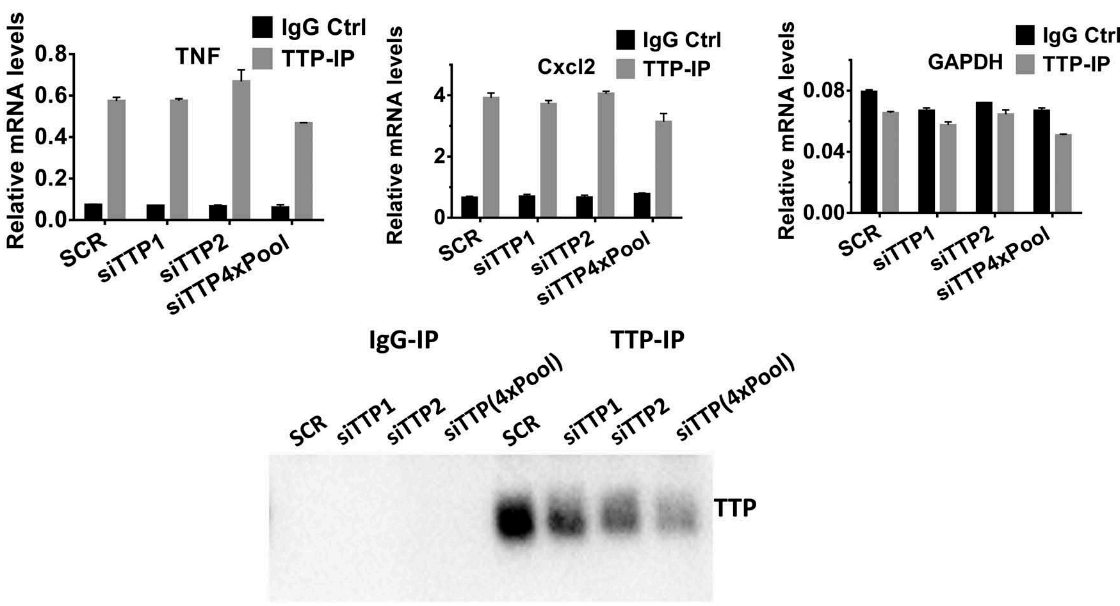

Figure 5. TTP/mRNA immunoprecipitation with mild and strong TTP knockdowns in LPS treated RAW 264.7. RAW 264.7 were transfected with SCR, siTTP1, 2 and 4XPool siRNAs and treated with LPS for 4 hours. The cells were lysed and TTP was immunoprecipitated with anti-TTP coupled beads. Negative control precipitation was performed with unspecific IgG antibody. Upper Panel; total RNA was prepared and the levels of bound TNF, Cxcl2 and GAPDH mRNA were quantified by real-time PCR and normalized against background β-Actin mRNA. Lower panel, TTP IP was monitored by western blotting; equivalent levels of immunoprecipitated beads were heated in SDS loading buffer and loaded onto western blot.

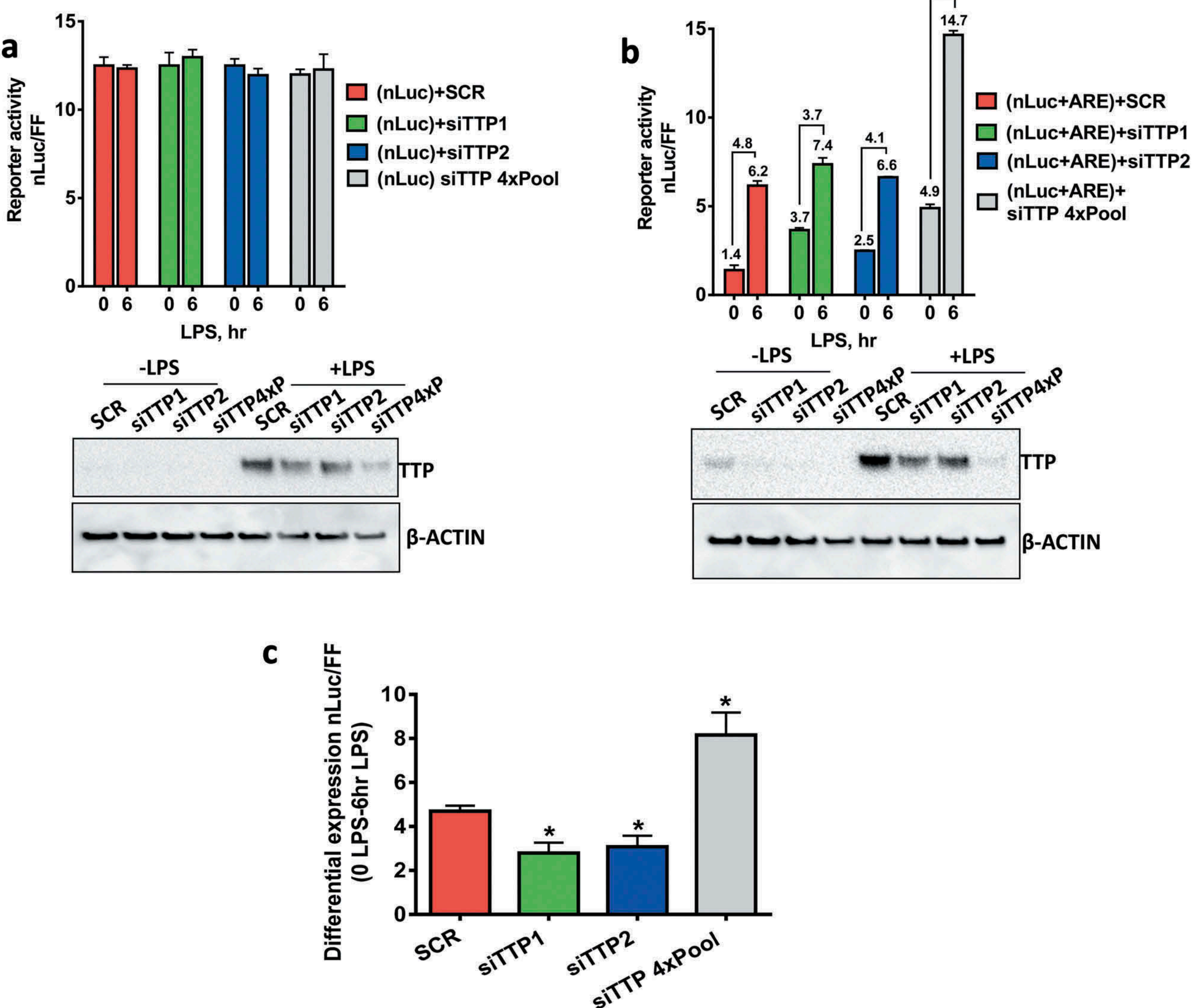

Figure 6. **Moderate knockdown of LPS-induced TTP in RAW264.7 reduce the expression of the ARE containing Nano-luciferase reporter**. In 10 cm culture plates 5×10^6 RAW264.7 cells were co-transfected with firefly-transfection-control plasmid (FF) and either with (A) a control nano-Luciferase reporter (nLuc) or (B) a reporter that contains a TNF-ARE insertion in its 3'UTR (nLuc+ARE) together with control scramble siRNA or siTTP1 or siTTP2 or siTTP (4xPool) that target TTP mRNA. Next day the transfected cells were split to two and a day later, cells were either left untreated or were treated with LPS for six hours before lysis and Nano-Luciferase and FF activity measurements. Lower panels: equivalent cell aliquots were directly lysed in SDS sample buffer and lysate levels were used for western blotting. The blots were probed with anti-TTP and β-actin antibodies. The numbers at the top of the columns in B indicate the nLuc/FF ratio of the measurements and the numbers between the columns indicate the difference in nLuc/FF units between LPS treated and untreated cells. (C) Experiment B was repeated 5 times and the normalized average and SEM of the difference in nLuc/FF units between LPS treated and untreated cells is shown (*P values of paired t-tests from three independent experiments <0.02).

Discussion

The relationship between the activity and the cellular levels of TTP during the onset of the inflammatory response is unexpected. After its induction, TTP cellular levels increase and correlate with the levels of its target mRNAs such as TNF (Fig. 2). Yet, TTP is a TNF-mRNA destabilizing protein and an indirect correlation can be expected [6,22]. Furthermore, the inhibition of the p38/MK2/TTP MAPK phosphorylation pathway either by the treatment with pharmacological inhibitors or by genetic manipulation leads to the reduction of induced TTP levels but concomitantly also to the upregulation of its activity [15,36]. The cellular abundance of TTP when TNF mRNA is most stable has been attributed to its phosphorylation by the p38/MK2 pathway, which inhibits its activity but also leads to its stabilization and accumulation [8,23,24,33]. However, no sufficient explanation has been given, to why its levels decrease while its activity increases.

Here, we provide evidence that the mere very high levels of TTP during the onset of inflammation, can lead to the reduction of its canonical activity. The expression of ARE-containing mRNAs during cellular homeostasis is, by default, repressed also due to the activity of background levels of TTP. Typically, ARE-containing gene reporters express 5 to 10% of the levels of non-ARE when transfected into cells (Fig. 6). The enhancement of the expression of ARE-containing reporter transcripts observed after the overexpression or LPS induction of TTP upregulates this expression to around 50% of the non-ARE reporter. Therefore, this observed enhancement in expression has to be due to de-repression.

Furthermore, two observations imply that this de-repression can occur independently of the phosphorylation status of TTP. First, the TTP-AA mutant that lacks the critical MK2 phosphorylation sites is still capable of upregulating the expression of the ARE-mRNA reporter when over-expressed in HEK293 cells. Second the moderate knockdown of LPS induced TTP in RAW264.7 leads to the reduction of TNF release. In the moderate knockdown experiments and after LPS treatment, the p38/MK2/TTP pathway should still be intact and active. Therefore, the increase in TTP activity and the subsequent reduction in TNF release in the siTTP transfected cells can be attributed to the reduction in TTP level and not to its phosphorylation. Importantly, the presented results do not exclude direct effects of phosphorylation in reducing TTP activity, such as the reduction in affinity to RNA or to protein factors that facilitate mRNA decay [10,25–27]. Phosphorylation due to the activity of the p38 MAPK pathway can stabilize ARE containing mRNA in the absence of TTP induction [37]. The inhibition of TTP phosphorylation by genetic disruption or with pharmacological inhibitors of the p38/MK2 pathway results in more than 90% reduction in TNF release [15,38], while the TTP knockdown effects observed here reduce TNF release in the range of 30–40% only. Therefore, the overall inhibition of TTP activity at the onset of the inflammatory response is likely to be mainly a direct result of phosphorylation complemented by high level.

Based on our data we propose, that in macrophages, TTP should be most active at a single low expression level that we call 'optimal TTP level' (OTL). In the absence of the inflammatory response, high TTP activity is not required and the expression levels are below the OTL. During the onset of the inflammatory response, TTP levels rapidly increase beyond OTL leading to increased expression ARE-mRNAs due to phosphorylation and self-inhibiting high TTP levels. Sustaining high level is also facilitated by phosphorylation and stabilization of TTP. Least TTP activity is reached at maximum expression levels, at this point p38/MK2 activity is reduced, TTP protein becomes dephosphorylated and unstable, its cellular level start to get reduced but it regains its activity and represses ARE-mRNAs expression. When the OTL is reached most induced ARE-mRNAs are already removed and the primary inflammatory response is resolved, TTP levels can return to homeostatic background (Fig. 7).

TTP does not possess nuclease or other enzymatic activity; instead it promotes repression of ARE-mRNA expression by recruiting deadenylation and decay complexes to ARE-containing mRNA [10,25–27]. Therefore, TTP can be considered a scaffold; and the cellular concentration of a scaffold needs to stoichiometrically match the concentration of the components to which it binds for optimal function. Too much scaffold protein will sequester the pathway components away from substrate and negate the purpose of the scaffold [39]. Exogenous overexpression of a scaffold has been shown to reduce its own physiological activity and has been considered as 'informative artefact' [39]. We suppose this type of explanation that was previously given for overexpressed TTP in HEK293 cells may explain the presented

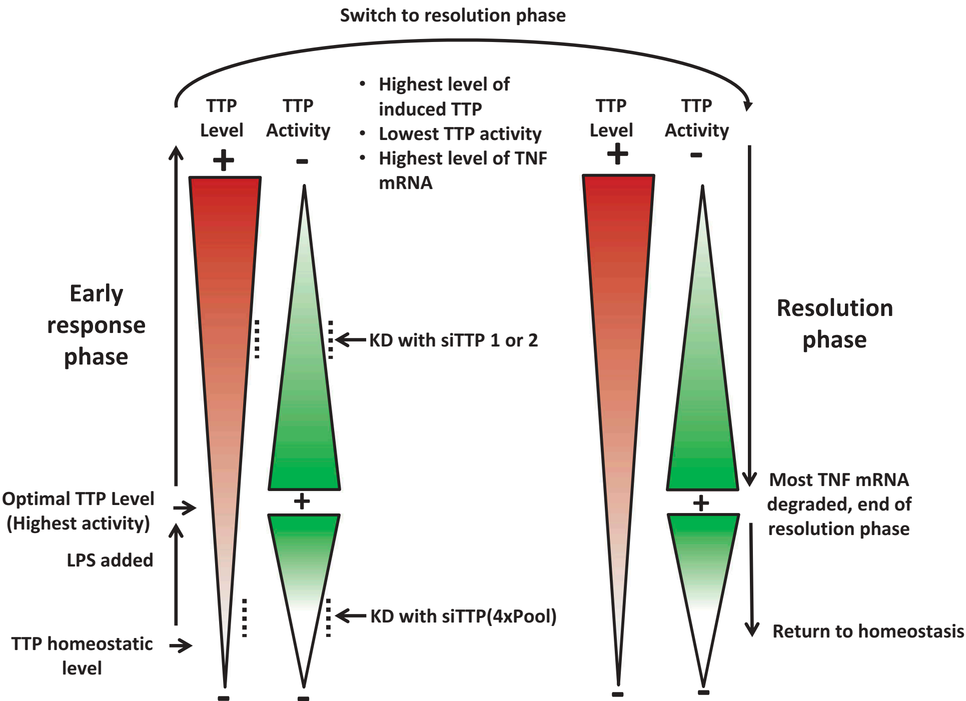

Figure 7. Schematic representation of level-dependent regulation of the mRNA decay activity of TTP. During the onset of the inflammatory response, TTP levels rapidly increase beyond an 'optimal TTP level' leading to increased expression of ARE-mRNAs due to the self-inhibiting effect of high TTP levels. This increase may be promoted by phosphorylation which leads to the stabilization of TTP protein (in addition to the direct TTP inhibiting effects of phosphorylation). Least TTP activity is reached at maximum expression levels, at this point p38/MK2 activity is reduced, TTP protein become dephosphorylated and unstable, its cellular level diminish but it regains its activity reaching an 'optimal TTP level' with maximum TTP effect. At this point most induced ARE-mRNAs are degraded and the primary inflammatory response is resolved. High TTP activity is no longer needed and TTP levels can return to homeostatic background. The estimated levels of TTP after the knockdowns (KD) with siTTP1, siTTP2 and siTTP(4xPool) are depicted.

observations [28] and suggest that this can also be a physiological phenomenon that controls TTP activity and the kinetics of the post-transcriptional inflammatory response.

Acknowledgments

This Project is supported by King Faisal Specialist Hospital and Research Center intramural funding and by King Abdulaziz City of Science and Technology (KACST) under the Long-Term Comprehensive National Science, Technology and Innovation Plan (NSTIP) (KACST Project No. 13-BIO1034-20). The authors would like to thank Mr. Pulicat S. Manogaran and Mr. Amer Al-Marzou for technical help with flow cytometry and Mrs. Maha Alghamdi and Dr. Monther Al-Alwan for critical reading of the manuscript.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by the King Abdulaziz City for Science and Technology [13-BIO1034-20].

ORCID

Khalid S. A. Khabar http://orcid.org/0000-0003-1003-9788
Edward G. Hitti http://orcid.org/0000-0003-1587-2824

References

[1] Ovstebo R, Olstad OK, Brusletto B, et al. Identification of genes particularly sensitive to lipopolysaccharide (LPS) in human monocytes induced by wild-type versus LPS-deficient Neisseria meningitidis strains. Infect Immun. 2008 Jun;76(6):2685–2695. IAI.01625-07 [pii]. PubMed PMID: 18362127; PubMed Central PMCID: PMC2423066. eng.

[2] Litvak V, Ramsey SA, Rust AG, et al. Function of C/EBPdelta in a regulatory circuit that discriminates between transient and persistent TLR4-induced signals. Nat Immunol. 2009 Apr;10 (4):437–443. ni.1721 [pii]. PubMed PMID: 19270711; PubMed Central PMCID: PMC2780024. eng.

[3] Han J, Ulevitch RJ. Limiting inflammatory responses during activation of innate immunity. Nat Immunol. 2005 Dec;6 (12):1198–1205. ni1274 [pii]. PubMed PMID: 16369559; eng.

[4] Bakheet T, Hitti E, Khabar KSA. ARED-Plus: an updated and expanded database of AU-rich element-containing mRNAs and pre-mRNAs. Nucleic Acids Res. 2018 Jan 4;46(D1):D218–D220. 4564798 [pii]. PubMed PMID: 29077946; PubMed Central PMCID: PMC5753209. eng.

[5] Vlasova-St Louis I, Bohjanen PR. Post-transcriptional regulation of cytokine signaling by AU-rich and GU-rich elements. J Interferon Cytokine Res. 2014 Apr;34(4):233–241. PubMed PMID: 24697201; PubMed Central PMCID: PMC3976587. eng.

[6] Kratochvill F, Machacek C, Vogl C, et al. Tristetraprolin-driven regulatory circuit controls quality and timing of mRNA decay in inflammation. Mol Syst Biol. 2011;7:560. msb201193 [pii]. PubMed PMID: 22186734; PubMed Central PMCID: PMC3737733. eng.

[7] Schott J, Reitter S, Philipp J, et al. Translational regulation of specific mRNAs controls feedback inhibition and survival during macrophage activation. PLoS Genet. 2014 Jun;10(6):e1004368. PGENETICS-D-14-00088 [pii]. PubMed PMID: 24945926; PubMed Central PMCID: PMC4063670. eng.

[8] Cuadrado A, Nebreda AR. Mechanisms and functions of p38 MAPK signalling. Biochem J. 2010 Aug 1;429(3):403–417. BJ20100323 [pii]. PubMed PMID: 20626350; eng.

[9] Dean JL, Sully G, Clark AR, et al. The involvement of AU-rich element-binding proteins in p38 mitogen-activated protein kinase pathway-mediated mRNA stabilisation. Cell Signal. 2004 Oct;16 (10):1113–1121. S0898656804000762 [pii]. PubMed PMID: 15240006; eng.

[10] Sandler H, Stoecklin G. Control of mRNA decay by phosphorylation of tristetraprolin. Biochem Soc Trans. 2008 Jun;36(Pt 3):491–496. BST0360491 [pii]. PubMed PMID: 18481987; eng.

[11] Ronkina N, Menon MB, Schwermann J, et al. MAPKAP kinases MK2 and MK3 in inflammation: complex regulation of TNF biosynthesis via expression and phosphorylation of tristetraprolin. Biochem Pharmacol. 2010 Dec 15;80(12):1915–1920. S0006-2952(10)00455-7 [pii]. PubMed PMID: 20599781; eng.

[12] Ronkina N, Kotlyarov A, Dittrich-Breiholz O, et al. The mitogen-activated protein kinase (MAPK)-activated protein kinases MK2 and MK3 cooperate in stimulation of tumor necrosis factor biosynthesis and stabilization of p38 MAPK. Mol Cell Biol. 2007 Jan;27(1):170–181. MCB.01456-06 [pii]. PubMed PMID: 17030606; PubMed Central PMCID: PMC1800641. eng.

[13] Hitti E, Kotlyarov A. The ERK and p38MAPK pathways as targets for anti-inflammatory therapy [Review]. Antiinflamm Antiallergy Agents Med Chem. 2007 Feb;6(1):85–97.

[14] Chrestensen CA, Schroeder MJ, Shabanowitz J, et al. MAPKAP kinase 2 phosphorylates tristetraprolin on in vivo sites including Ser178, a site required for 14-3-3 binding. J Biol Chem. 2004 Mar 12;279(11):10176–10184. M310486200 [pii]. PubMed PMID: 14688255; eng.

[15] Hitti E, Iakovleva T, Brook M, et al. Mitogen-activated protein kinase-activated protein kinase 2 regulates tumor necrosis factor mRNA stability and translation mainly by altering tristetraprolin expression, stability, and binding to adenine/uridine-rich element. Mol Cell Biol. 2006 Mar;26(6):2399–2407. 26/6/2399 [pii]. PubMed PMID: 16508014; PubMed Central PMCID: PMC1430282. eng.

[16] Smallie T, Ross EA, Ammit AJ, et al. Dual-specificity phosphatase 1 and tristetraprolin cooperate to regulate macrophage responses to lipopolysaccharide. J Immunol. 2015 Jul 1;195(1):277–288. jimmunol.1402830 [pii]. PubMed PMID: 26019272; PubMed Central PMCID: PMC4472943. eng.

[17] Ross EA, Smallie T, Ding Q, et al. Dominant suppression of inflammation via targeted mutation of the mRNA destabilizing protein tristetraprolin. J Immunol. 2015 Jul 1;195(1):265–276. jimmunol.1402826 [pii]. PubMed PMID: 26002976; PubMed Central PMCID: PMC4472942. eng.

[18] O'Neil JD, Ammit AJ, Clark AR. MAPK p38 regulates inflammatory gene expression via tristetraprolin: doing good by stealth. Int J Biochem Cell Biol. 2018 Jan;94:6–9. S1357-2725(17)30287-X [pii]. PubMed PMID: 29128684; eng.

[19] Sedlyarov V, Fallmann J, Ebner F, et al. Tristetraprolin binding site atlas in the macrophage transcriptome reveals a switch for inflammation resolution. Mol Syst Biol. 2016;12(5):868. PubMed PMID: 27178967; eng.

[20] Tiedje C, Diaz-Munoz MD, Trulley P, et al. The RNA-binding protein TTP is a global post-transcriptional regulator of feedback control in inflammation. Nucleic Acids Res. 2016 May 24. DOI: 10.1093/nar/gkw474 gkw474 [pii]. PubMed PMID: 27220464; Eng.

[21] Johnson BA, Geha M, Blackwell TK. Similar but distinct effects of the tristetraprolin/TIS11 immediate-early proteins on cell survival. Oncogene. 2000 Mar 23;19(13):1657–1664. PubMed PMID: 10763822; eng.

[22] Tchen CR, Brook M, Saklatvala J, et al. The stability of tristetraprolin mRNA is regulated by mitogen-activated protein kinase p38 and by tristetraprolin itself. J Biol Chem. 2004 Jul 30;279 (31):32393–32400. M402059200 [pii]. PubMed PMID: 15187092; eng.

[23] Brook M, Tchen CR, Santalucia T, et al. Posttranslational regulation of tristetraprolin subcellular localization and protein stability by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways. Mol Cell Biol. 2006 Mar;26 (6):2408–2418. 26/6/2408 [pii]. PubMed PMID: 16508015; PubMed Central PMCID: PMC1430283. eng.

[24] Stoecklin G, Stubbs T, Kedersha N, et al. MK2-induced tristetraprolin:14-3-3 complexes prevent stress granule association and ARE-mRNA decay. Embo J. 2004 Mar 24;23(6):1313–1324. 7600163 [pii]. PubMed PMID: 15014438; PubMed Central PMCID: PMC381421. eng.

[25] Lykke-Andersen J, Wagner E. Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1. Genes Dev. 2005 Feb 1;19(3):351–361. 19/3/351 [pii]. PubMed PMID: 15687258; PubMed Central PMCID: PMC546513. eng.

[26] Blackshear PJ, Perera L. Phylogenetic distribution and evolution of the linked RNA-binding and NOT1-binding domains in the tristetraprolin family of tandem CCCH zinc finger proteins. J Interferon Cytokine Res. 2014 Apr;34(4):297–306. PubMed PMID: 24697206; PubMed Central PMCID: PMC3976581. eng.

[27] Sandler H, Kreth J, Timmers HT, et al. Not1 mediates recruitment of the deadenylase Caf1 to mRNAs targeted for degradation by tristetraprolin. Nucleic Acids Res. 2011 May;39(10):4373–4386. gkr011 [pii]. PubMed PMID: 21278420; PubMed Central PMCID: PMC3105394. eng.

[28] Lai WS, Carballo E, Strum JR, et al. Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. Mol Cell Biol. 1999 Jun;19(6):4311–4323. PubMed PMID: 10330172; PubMed Central PMCID: PMC104391. eng.

[29] Al-Souhibani N, Al-Ahmadi W, Hesketh JE, et al. The RNA-binding zinc-finger protein tristetraprolin regulates AU-rich mRNAs involved in breast cancer-related processes. Oncogene. 2010 Jul 22;29(29):4205–4215. onc2010168 [pii]. PubMed PMID: 20498646; PubMed Central PMCID: PMC3422647. eng.

[30] Hitti E, Al-Yahya S, Al-Saif M, et al. A versatile ribosomal protein promoter-based reporter system for selective assessment of RNA stability and post-transcriptional control. Rna. 2010 Jun;16(6):1245–1255. rna.2026310 [pii]. PubMed PMID: 20418359; PubMed Central PMCID: PMC2874176. eng.

[31] Mahmoud L, Al-Enezi F, Al-Saif M, et al. Sustained stabilization of Interleukin-8 mRNA in human macrophages. RNA Biol. 2014;11(2):124–133. 27863 [pii]. PubMed PMID: 24525793; PubMed Central PMCID: PMC3973731. eng.

[32] Griseri P, Bourcier C, Hieblot C, et al. A synonymous polymorphism of the Tristetraprolin (TTP) gene, an AU-rich mRNA-binding protein, affects translation efficiency and response to Herceptin treatment in breast cancer patients. Hum Mol Genet. 2011 Dec 1;20(23):4556–4568. ddr390 [pii]. PubMed PMID: 21875902; eng.

[33] Tiedje C, Ronkina N, Tehrani M, et al. The p38/MK2-driven exchange between tristetraprolin and HuR regulates AU-rich element-dependent translation. PLoS Genet. 2012 Sep;8(9):e1002977. PGENETICS-D-12-01134 [pii]. PubMed PMID: 23028373; PubMed Central PMCID: PMC3459988. eng.

[34] Al-Souhibani N, Al-Ghamdi M, Al-Ahmadi W, et al. Posttranscriptional control of the chemokine receptor CXCR4 expression in cancer cells. Carcinogenesis. 2014 Sep;35(9):1983–1992. bgu080 [pii]. PubMed PMID: 24692066; PubMed Central PMCID: PMC4146410. eng.

[35] Carballo E, Lai WS, Blackshear PJ. Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science. 1998 Aug 14;281(5379):1001–1005. PubMed PMID: 9703499; eng.

[36] Mahtani KR, Brook M, Dean JL, et al. Mitogen-activated protein kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability. Mol Cell Biol. 2001 Oct;21(19):6461–6469. PubMed PMID: 11533235; PubMed Central PMCID: PMC99793. eng.

[37] Winzen R, Kracht M, Ritter B, et al. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. Embo J. 1999 Sep 15;18(18):4969–4980. PubMed PMID: 10487749; PubMed Central PMCID: PMC1171568. eng.

[38] Kotlyarov A, Neininger A, Schubert C, et al. MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. Nat Cell Biol. 1999 Jun;1(2):94–97. PubMed PMID: 10559880; eng.

[39] Burack WR, Shaw AS. Signal transduction: hanging on a scaffold. Curr Opin Cell Biol. 2000 Apr;12(2):211–216. S0955-0674(99)00078-2 [pii]. PubMed PMID: 10712921; eng.