ULLA JALONEN

# Regulation and Inflammatory Effects of Tristetraprolin in Macrophages

■

UNIVERSITY OF TAMPERE

# CONTENTS

# List of original communications

This thesis is based on the following original communications, referred to in the text by their Roman numerals I-IV. In addition, some unpublished results are presented.

I        Jalonen U, Lahti A, Korhonen R, Kankaanranta H and Moilanen E (2005): Inhibition of tristetraprolin expression by dexamethasone in activated macrophages. Biochem Pharmacol 69:733-740.

II       Jalonen U, Leppänen T, Kankaanranta H and Moilanen E (2007): Salbutamol increases tristetraprolin expression in macrophages. Life Sci 81:1651-1658.

III      Jalonen U, Paukkeri EL and Moilanen E (2008): Compounds that increase or mimic cyclic adenosine monophosphate enhance tristetraprolin degradation in lipopolysaccharide-treated murine J774 macrophages. J Pharmacol Exp Ther 326:514-522.

IV       Jalonen U, Nieminen R, Vuolteenaho K, Kankaanranta H and Moilanen E (2006): Down-regulation of tristetraprolin expression results in enhanced IL-12 and MIP-2 production and reduced MIP-3α synthesis in activated macrophages. Mediators Inflamm 2006 (Article ID 40691):1-8.

# Abbreviations

AP-2        activator protein 2
ARE         adenosine-uridine (AU)-rich element
8-Br-cAMP   8-bromoadenosine $3',5'$-cyclic monophosphate sodium salt
BMM         bone marrow-derived macrophage
cAMP        cyclic adenosine 3',5'-monophosphate
CCCH        cysteine-cysteine-cysteine-histidine
CM          cytokine mixture (IFN-$\gamma$, IL-1$\beta$, and TNF-$\alpha$)
COX-2       cyclooxygenase-2
db-cAMP     $N^6,2'$-O-dibutyryladenosine $3',5'$-cyclic monophosphate sodium salt
EGF         epidermal growth factor
ERK1/2      extracellular signal-regulated kinase 1 and 2
FAM         6-carboxy-fluoroscein, dye used in quantitative PCR
FGF         fibroblast growth factor
GAPDH       glyceraldehyde-3-phosphate dehydrogenase
GM-CSF      granulocyte-macrophage colony-stimulating factor
IFN         interferon
IL          interleukin
iNOS        inducible nitric oxide synthase
JAK         Janus kinase
JNK         c-Jun N-terminal kinase
KO          knockout, gene deletion
KSRP        heterogeneous nuclear ribonucleoprotein K homology domain-type splicing regulatory protein
LIF         leukaemia inhibitory factor
LPS         lipopolysaccharide
MAPK        mitogen-activated protein kinase
MG132       $N$-[(phenylmethoxy)carbonyl]-L-leucyl-$N$-[(1$S$)-1-formyl-3-methylbutyl]-L-leucinamide, proteasome inhibitor
MK          MAPK-activated protein kinase (also called MAPKAPK)
MIP         macrophage inflammatory protein
NF-$\kappa$B   nuclear factor $\kappa$B
NGF         nerve growth factor
PD98059     2-(2-amino-3-methoxyphenyl)-4$H$-1-benzopyran-4-one, ERK1/2 inhibitor
PKC         protein kinase C
PPAR        peroxisome proliferator-activated receptor
RIE         rat intestinal epithelial (cells)

SB203580   4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)-1*H*-imidazole, p38 inhibitor

SEM        standard error of mean

Smad       mediators of TGF-β family signals. The term is a merger of the *Sma* genes in Caenorhabditis elegans and the Drosophila gene *Mad* (*mother against decapentaplegic*) (Derynck et al. 1996)

SP600125   anthra[1,9-*cd*]pyrazol-6(2*H*)-one, JNK inhibitor

STAT       signal transducer and activator of transcription

TAMRA      6-carboxy-tetramethyl-rhodamine, dye used in quantitative PCR

TGF        transforming growth factor

TIS        TPA-induced sequence

TNF-α      tumor necrosis factor α

TPA        12-*O*-tetradecanoylphorbol-13-acetate ester

TSA        trichostatin A, inhibitor of histone deacetylases

TTP        tristetraprolin

UTR        untranslated region

WT         wildtype

ZFP        zinc finger protein

# Abstract

Inflammation is a host defence reaction against pathogens and tissue injury. However, if inflammation is dysregulated, prolonged, or inappropriately focussed, it can become harmful for the host and cause diseases such as rheumatoid arthritis and asthma. At the site of inflammation, the tissue and immune cells secrete cytokines and other inflammatory modulators. The expression of these factors is regulated at both the transcriptional and post-transcriptional levels. Recently, the post-transcriptional regulation of gene expression has become a topic of interest since it may represent a potential therapeutic target.

One of the post-transcriptional regulators is tristetraprolin (TTP). TTP can bind to the AU-rich elements (AREs) in the 3'-untranslated region (3'-UTR) of many transiently expressed genes, e.g. tumor necrosis factor $\alpha$ (TNF-$\alpha$). The bound TTP induces deadenylation and increases degradation of the target mRNA, thereby reducing the translation and expression of the target gene.

The aim of the present study was to investigate the regulation of TTP expression and the effects of TTP knockdown in macrophages. Dexamethasone, and the dissociated steroid, RU24858, both inhibited lipopolysaccharide (LPS)-induced TTP mRNA and protein expression in a glucocorticoid receptor dependent and a glucocorticoid response element-independent manner, possibly through histone deacetylation and transcriptional silencing.

cAMP-elevating agents increased the expression of TTP mRNA both in resting cells and in macrophages activated by bacterial LPS. In resting cells, cAMP-elevating agents increased TTP protein expression, with this effect likely being mediated by activation of transcription factor activator protein 2 (AP-2). In contrast, cAMP-elevating agents decreased TTP protein levels in cells activated by LPS, probably by increasing TTP degradation through the proteasome.

TTP expression was down-regulated in J774 macrophages by siRNA technique. The cytokine antibody array revealed interleukin (IL)-12, macrophage inflammatory protein 2 (MIP-2, a homologue to human IL-8), and MIP-3$\alpha$ as novel cytokine targets for TTP-regulated mRNA decay, and confirmed the earlier findings that TNF-$\alpha$ and IL-6 expression is regulated by TTP.

In the present study, novel mechanisms regulating the expression of TTP in macrophages were discovered. Glucocorticoids and cAMP-elevating agents (e.g. salbutamol) were found to decrease TTP expression in activated macrophages, and their mechanisms of action were clarified in greater detail. In addition, novel targets of TTP-mediated mRNA decay were discovered by cytokine antibody array after down-regulation of TTP by shRNA. The findings of the present study can be applied in the development of novel anti-inflammatory drugs.

# Tiivistelmä

Tulehdus on elimistön puolustusreaktio taudinaiheuttajaa tai kudosvaurioita vastaan. Tulehtuneen kudoksen solut sekä tulehdusalueelle kertyneet valkosolut erittävät tulehdusta muokkaavia välittäjäaineita, kuten sytokiineja. Jos puolustusmekanismien sammuttaminen on häiriintynyt tai reaktio kohdistuu väärin, seurauksena voi olla tulehdustauti, kuten reuma tai astma. Tulehdusgeenien aktivoitumista säätelevät mm. solun pintareseptorien määrä ja aktivoituminen, solunsisäisten signaalivälitysketjujen aktivoituminen ja transkriptiota aktivoivien transkriptiotekijöiden siirtyminen tumaan. Transkriptiossa syntynyttä lähetti-RNA:ta muokataan eri tavoin ja sen stabiiliutta ja translaatiota säädellään useilla eri mekanismeilla.

Tristetraproliini (TTP) on yksi lähetti-RNA:n stabiiliutta säätelevä tekijä. TTP voi sitoutua tiettyjen lähetti-RNA-molekyylien 3'-päässä olevaan adenosiini-uridiini (AU) -rikkaaseen proteiinia koodaamattomaan säätelyalueeseen. Tunnetuin sytokiini, jonka ilmenemistä TTP säätelee, on tuumorinekroositekijä alfa (TNF-$\alpha$). TTP voi sitoutua lähetti-RNA-molekyylien 3'-pään säätelyalueelle, edistää deadenylaatiota ja nopeuttaa lähetti-RNA:n hajoamista, jolloin näiden koodaamien proteiinien synteesi vähenee.

Tämän väitöskirjatutkimuksen tarkoituksena oli tutkia TTP:n ilmenemisen säätelyä ja TTP:n poiston vaikutuksia makrofageissa. Pääasiallinen mielenkiinto kohdistui glukokortikoidien sekä $\beta_2$-agonistien ja muiden syklisen adenosiinimonofosfaatin (AMP) tasoja nostavien yhdisteiden TTP:n ilmenemistä säätelevään vaikutukseen. TTP:n lähetti-RNA:n ja proteiinin tuoton esto glukokortikoideilla välittyi glukokortikoidireseptorin kautta, todennäköisesti histonien deasetylaation kautta.

Syklisen AMP:n määrää nostavat yhdisteet lisäsivät TTP:n lähetti-RNA:n määrää sekä stimuloimattomissa että LPS:llä aktivoiduissa makrofageissa. Samat yhdisteet lisäsivät TTP-proteiinin ilmentymistä vain stimuloimattomissa soluissa ja todennäköisesti transkriptiotekijä AP-2-välitteisesti. Sen sijaan LPS:llä käsitellyissä makrofageissa forskoliini nopeutti TTP-proteiinin hajoamista, koska TTP-proteiinin proteasomivälitteinen hajoaminen nopeutui forskoliinin vaikutuksesta.

TTP:n vaikutuksia tulehdustekijöiden tuottoon makrofageissa tutkittiin vaimentamalla TTP:n ilmentymistä siRNA-menetelmällä. Pienten inhiboivien RNA-molekyylien avulla saatiin TTP:n tuotto aktivoiduissa makrofageissa vähenemään merkittävästi. Sytokiinivasta-ainearraylla selvitettiin mahdollisia uusia proteiineja, joiden ilmenemistä TTP voisi säädellä. Menetelmällä todennettiin TTP:n tunnetut kohteet TNF-$\alpha$ ja interleukiini-6 (IL-6). Näiden

lisäksi uusiksi mahdollisiksi kohteiksi ilmenivät IL-12, makrofagitulehdusproteiini 2 (MIP-2, humaani IL-8:n homologi) ja MIP-3α.

Väitöskirjatutkimuksessa löydettiin useita uusia mekanismeja säädellä TTP:n ilmenemistä tulehdusreaktion kannalta tärkeissä soluissa, makrofageissa. Glukokortikoidit ja syklisen AMP:n määrää nostavat yhdisteet (mm. salbutamoli) vähentävät TTP:n ilmentymistä aktivoituneissa makrofageissa. Kun TTP:n ilmeneminen vaimennettiin siRNA-tekniikalla, sytokiinivasta-ainearraylla löydettiin uusia sytokiineja, joiden ilmenemistä TTP mahdollisesti säätelee. Väitöskirjatyön tuloksia voidaan hyödyntää uusien anti-inflammatoristen lääkkeiden kehittämisessä.

# Introduction

The expression of proinflammatory cytokines is upregulated in inflammation. During the stage of ongoing inflammation, the post-transcriptional regulation of gene expression might provide possible drug targets to dampen the expression of these genes. One of the mechanisms controlling gene expression at the post-transcriptional level is the regulation of mRNA stability through *cis*-acting AU-rich elements (AREs) in the 3'-untranslated region (3'-UTR) of mRNAs of many inflammatory and other transiently expressed genes. Several *trans*-acting factors have been found to bind to AREs (Hollams et al. 2002, Khabar 2005).

Tristetraprolin (TTP, also known as TIS11, Nup475, G0S24, ZFP36) is one of the *trans*-acting factors known to destabilize the mRNAs of e.g. tumor necrosis factor $\alpha$ (TNF-$\alpha$) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Blackshear 2002). A direct connection between TTP and inflammation was demonstrated in mice with a disrupted TTP gene (Taylor et al. 1996). The TTP knockout (KO) mice developed a severe inflammatory syndrome, including arthritis, autoimmunity, and myeloid hyperplasia, which was associated with elevated levels of circulating TNF-$\alpha$, and which could be alleviated by administration of TNF-$\alpha$ antibody. TTP KO mice produce excess amounts of proinflammatory cytokines, TNF-$\alpha$ and GM-CSF, due to the increased stability of their mRNAs (Carballo et al. 1998, Carballo et al. 2000). TTP can bind to the ARE of these cytokine mRNAs and induce their deadenylation and degradation (Lai et al. 1999).

The expression of TTP can be induced by several factors including serum, insulin, and 12-*O*-tetradecanoylphorbol-13-acetate ester (TPA) (Lai et al. 1990). The expression of TTP has been observed in several tissues including lung, liver, and spleen (Lai et al. 1990, Cao et al. 2004). Generally, TTP has been regarded as a factor that destabilizes mRNAs of transiently expressed genes. As an exception, TTP has been reported to stabilize inducible nitric oxide synthase mRNA by interfering with the function of other destabilizing factors (Fechir et al. 2005).

Since TTP can destabilize the mRNAs of proinflammatory genes, it down-regulates their expression and in that way tilts the equilibrium of the inflammatory responses towards an anti-inflammatory and immunosuppressive direction. The aim of the present study was to investigate the regulation of TTP expression and the effects of TTP knockdown by siRNA on cytokine production in macrophages.

# Review of the literature

## Inflammation

The inflammatory reaction is generally protective and directed against pathogens and other noxious agents. Redness, swelling, heat, pain, and loss of function are the classical signs of inflammation. Sometimes the defensive responses are focussed inappropriately, causing diseases such as rheumatoid arthritis and asthma. Several types of cells are involved in the inflammatory reaction. Some of these cells are present in tissues (e.g. mast cells and tissue macrophages) while others gain access from the blood (e.g. neutrophils, monocytes/macrophages, and lymphocytes). (Rang et al. 2007a)

Inflammation is mediated and regulated by soluble modulators. Cytokines such as interleukins (IL), tumor necrosis factors (TNF), interferons (IFN), chemokines, and colony-stimulating factors can be either proinflammatory or anti-inflammatory (Rang et al. 2007a). The eicosanoids are another group of inflammatory modulators and the expression of prostanoid producing enzyme, cyclooxygenase-2 (COX-2), is significantly upregulated during inflammation (Rajakariar et al. 2006). Another modulator and regulator of inflammation is the nitric oxide, which is produced by inducible nitric oxide synthase (iNOS) (Moncada et al. 1991, MacMicking et al. 1997, Korhonen et al. 2005, Vuolteenaho et al. 2007)

Many of the cytokines are expressed transiently in inflammation. Their induction is mediated by ligands binding to receptors, a multitude of signalling pathways as well as transcription factors. The signalling pathways regulating cytokine biosynthesis include the mitogen-activated protein kinases (MAPK) p38, c-jun N-terminal kinase (JNK), and extracellular signal-regulated kinase 1 and 2 (ERK1/2). The p38 MAPK pathway has been shown to regulate the biosynthesis of proinflammatory cytokines, to regulate the function of proteins involved in post-transcriptional regulation, and the dysregulation of p38 has been shown to be associated with inflammation and inflammatory diseases such as rheumatoid arthritis (Cuenda and Rousseau 2007). Gene expression can be regulated also by post-transcriptional mechanisms e.g. via the regulation of the rate of mRNA decay (Wilusz et al. 2001). In inflammation, the post-transcriptional regulation of gene expression represents a meaningful drug target since targeting the transcription of a gene, once it has been triggered to function or ceased, no longer provides a way to regulate gene expression. Inhibitors of the p38 MAPK pathway, which also affect post-transcriptional regulation, have been developed for the therapy of inflammatory diseases (Saklatvala 2004). For

example, inhibitors of the p38 and JNK pathways have been shown to regulate the stability of iNOS mRNA (Lahti et al. 2003, Lahti et al. 2006). In addition, some anti-inflammatory drugs appear to be able to destabilize the mRNAs of certain proinflammatory modulators and via that mechanism they can reduce inflammation. For example, the glucocorticoid, dexamethasone, has been shown to destabilize the mRNA of iNOS (Korhonen et al. 2002). It has been claimed that p38 and dexamethasone exert a combined action on the mRNA destabilization of COX-2 (Lasa et al. 2001).

# Messenger RNA decay

Aberrant control of mRNA stability has been implicated in various disease states, including inflammation. The expression of many cytokines, growth factors, and proto-oncogenes is regulated also at the level of mRNA stability i.e. not simply through the rate of gene transcription. Modifying the stability of mRNA is a flexible and rapid way to regulate mRNA expression. One good example is the regulation of iNOS expression. It has been noted that there is basal transcription of iNOS mRNA although no mRNA can be detected in resting cells, indicative of a continuous degradation of the mRNA as it is produced. Without any major changes in the transcription rate of iNOS mRNA, the expression of this gene can be markedly increased by the combination of IL-1β and interferon (IFN)-γ (Linn et al. 1997).

There are several ways of controlling and triggering the decay of mRNAs (Wilusz et al. 2001, Garneau et al. 2007). The decay of mRNA can be deadenylation-dependent, deadenylation-independent, endoribonucleolytic, or nonsense (premature stop codon)-mediated, and regulated by *trans*-acting factors on *cis*-acting elements. One of the best-characterized *cis*-acting elements is the AU-rich element (ARE) in the 3'-untranslated region (3'-UTR) of mRNAs of many inflammatory and other transiently expressed genes (Chen and Shyu 1995). Several *trans*-acting factors have been investigated and several of them have been shown to bind to AREs (Table 1). These proteins affect the stability of mRNA and may play a role in many serious diseases such as cancer, inflammatory diseases, and Alzheimer's disease (Guhaniyogi and Brewer 2001, Khabar 2005). The mechanisms, how these factors trigger mRNA decay and where the mRNA decay takes place, are not understood in detail. The proteasome (Laroia et al. 1999, Laroia et al. 2002), exosome (Chen et al. 2001, Mukherjee et al. 2002), and RNA processing body (PB) (Franks and Lykke-Andersen 2007, Stoecklin and Anderson 2007) have been suggested as sites of mRNA decay, while stress granules (SGs) have been proposed to store mRNAs during stress (Kedersha and Anderson 2002). In addition, TTP has been claimed to be a component of the miRNA and siRNA pathways, which may also play a role in ARE-mediated mRNA decay (Jing et al. 2005, Dorner et al. 2006). Efforts have been made in recent years to identify the factors binding to the ARE and TTP is recognized as one of the ARE-binding factors. TTP expression has

been shown to be induced by inflammatory stimuli and TTP KO mice develop arthritis-like symptoms (Taylor et al. 1996, Carballo et al. 1998). Therefore the present study has focused on the regulation of TTP expression in macrophages.

**Table 1.** *Examples of ARE-binding proteins.*

| RNA-Binding protein | Function (if known) | Reference |
|---|---|---|
| **ELAV family** | | |
| HuB | Stabilizing, translational enhancing | Levine et al. 1993 |
| HuC | Stabilizing | Sakai et al. 1994 |
| HuD | Stabilizing | Chung et al. 1996 |
| HuR (HuA) | Stabilizing | Ma et al. 1996 |
| **Tristetraprolin family** | | Blackshear 2002 |
| TTP (TIS11, G0S24, Nup475, ZFP36) | Destabilizing | Carballo et al. 1998 |
| BRF1 (TIS11B, ZFP36L1, cMG1, ERF1, Berg36) | Destabilizing | Lai et al. 2000 |
| BRF2 (TIS11D, ZFP36L2, ERF2) | Destabilizing | Lai et al. 2000 |
| **Others** | | |
| AUF1 (isoforms p37, p40, p42, p45) | Destabilizing, stabilizing | Zhang et al. 1993 |
| KSRP (FBP2) | Destabilizing | Chen et al. 2001 |
| TIA-1 | Translational silencing | Piecyk et al. 2000 |
| TIAR | Translational silencing | Gueydan et al. 1999 |

*AUF1, ARE- and poly(U)-binding and degradation factor-1; BRF, butyrate-response factor; ELAV, embryonic lethal abnormal vision; ERF, epidermal growth factor response factor; FBP, far upstream sequence element binding protein; HuA/B/C/D/R, Hu antigen A/B/C/D/R; KSRP, heterogeneous nuclear ribonucleoprotein K homology domain-type splicing regulatory protein; Nup, nuclear protein; TIA-1, T cell-restricted intracellular antigen-1; TIAR, TIA-1-related; TIS, TPA-induced sequence; ZFP, zinc finger protein.*

# Tristetraprolin (TTP)

Tristetraprolin (TTP) belongs to a family of the cysteine-cysteine-cysteine-histidine (CCCH) tandem zinc finger proteins (Blackshear 2002). TTP was first found to be induced by insulin in mouse NIH 3T3 HIR 3.5 cells (Lai et al. 1990). It was named tristetraprolin due to the three groups of four consecutive prolines in the mouse TTP protein. Soon the sequence of a human analogue was revealed (Taylor et al. 1991). HUGO Gene Nomenclature Committee named the gene as zinc finger protein 36 (*Zfp36* in mice, *ZFP36* in humans). TTP was also found to be induced by 12-*O*-tetradecanoylphorbol-13-acetate ester (TPA) in mouse Swiss 3T3 cells and named as TPA-induced sequence 11 (TIS11) (Varnum et al. 1989b). The original sequence reported there was later corrected in its 5' and 3' portions (Ma and Herschman 1991). Nuclear protein 475 (Nup475), an mRNA induced by growth factors in mouse 3T3 fibroblasts (DuBois et al. 1990)), and G0S24 in human lymphocytes (Heximer and Forsdyke 1993) were also found to have the same sequence as TTP. TTP has also been found in rat (Kaneda et al. 1992, Kaneda et al. 2000) and bovine cells (Lai et al. 1995), and the *Xenopus* protein XC3H-1 seems to be related to TTP protein (De et al. 1999).

Other family members of CCCH tandem zinc finger proteins include ZFP36-like 1 (ZFP36L1), ZFP36-like 2 (ZFP36L2), and Zfp36-like 3 (ZFP36L3). All of these share similar ARE-binding, deadenylation, and RNA degradation promoting properties as TTP (Lai et al. 2000, Chinn et al. 2002, Lai et al. 2003, Hudson et al. 2004, Blackshear et al. 2005).

TTP is an RNA-binding protein that affects the stability and expression of certain mRNAs and its role in the post-transcriptional regulation of transiently expressed genes has become clearer in recent years. Studies on TTP knockout (KO) mice revealed a direct connection between TTP and inflammation, i.e. the mice developed a systemic inflammatory syndrome with arthritis, autoimmunity, and myeloid hyperplasia (Taylor et al. 1996). The best-studied target of TTP-mediated mRNA instability derived from TTP KO mice is tumor necrosis factor-$\alpha$ (TNF-$\alpha$). TTP can promote the deadenylation and decay of TNF-$\alpha$ mRNA by binding to the conserved AU-rich element (ARE) in the 3'-untranslated region (3'-UTR) of TNF-$\alpha$ mRNA (Lai et al. 1999, Lai and Blackshear 2001), thereby decreasing the production of TNF-$\alpha$ protein. Another well-studied target of TTP is granulocyte-macrophage colony-stimulating factor (GM-CSF) (Carballo et al. 2000). In addition, inflammatory modulators such as cyclooxygenase-2 (COX-2), interleukin 3 (IL-3), and IL-6 have been found to be destabilized by TTP (using TTP overexpression and gene silencing methods) (Stoecklin et al. 2000, Stoecklin et al. 2001, Sawaoka et al. 2003). TTP has been shown to destabilize the proinflammatory mRNAs, down-regulating their expression, i.e. the inflammatory responses are shifted towards an anti-inflammatory and immunosuppressive direction.

TTP expression has been detected in various tissues including spleen, lymph nodes, and thymus (DuBois et al. 1990, Lai et al. 1990, Cao et al. 2004, Lu and Schneider 2004, Stumpo et al. 2004, Smoak and Cidlowski 2006) and its

expression can be induced by inflammatory and related stimuli including bacterial products, growth factors, serum, and tumor promoters (Arenander et al. 1989b, Arenander et al. 1989c, DuBois et al. 1990, Lai et al. 1990, Beauchamp et al. 1994). A better understanding of the regulation of TTP expression and its actions in inflammation may help identify new potential drug targets.

TTP is coded by a gene with one intron. Information on the sequences of the mouse (*Zfp36*) and human (*ZFP36*) TTP genes and proteins are given in Table 2. The calculated molecular weight of TTP is 36 kDa, but the observed molecular weight in gel electrophoresis is somewhat higher due to post-translational modifications such as phosphorylation (Mahtani et al. 2001, Zhu et al. 2001, Cao et al. 2004, Lai et al. 2006). TTP can bind to AU-rich sequences through its two conserved CCCH zinc finger domains and promote deadenylation and degradation (Lai et al. 1999), but additional sequences in the N- and C-terminal regions are required for full activity of TTP (Rigby et al. 2005).

**Table 2.** *Information on mouse and human TTP gene and protein sequences.*

| | Gene name | cDNA RefSeq | Chromosome | Protein RefSeq | Amino acids |
|---|---|---|---|---|---|
| Mouse TTP | *Zfp36* | NM_011756 | 7 | NP_035885 | 319 |
| Human TTP | *ZFP36* | NM_003407 | 19 | NP_003398 | 326 |

## *Transcription factor binding sites and the mRNA 3' untranslated region in TTP gene*

Several different transcription factor binding sites have been identified by computer analysis in the mouse (DuBois et al. 1990, Lai et al. 1995), human (Heximer and Forsdyke 1993, Lai et al. 1995, Smoak and Cidlowski 2006), rat (Kaneda et al. 2000, Murata et al. 2000), and bovine (Lai et al. 1995) TTP promoter regions (Figure 1) and in the mouse, human, and bovine TTP intron (Lai et al. 1998). One common feature of the promoter regions of mouse, human, rat, and bovine TTP seems to be consensus sequences of activator protein 2 (AP-2) and Sp1 (DuBois et al. 1990, Heximer and Forsdyke 1993, Lai et al. 1995, Kaneda et al. 2000). The intron regions also seem to contain these sequences (Lai et al. 1998). One other common transcription factor consensus site in mouse and human TTP promoter region is that for EGR1 (DuBois et al. 1990, Heximer and Forsdyke 1993, Lai et al. 1995). The involvement of EGR1, AP-2, and TTP promoter element 1 (TPE1) in serum inducibility of mouse TTP has been confirmed by mutation analysis and gel mobility shift assays (Lai et al. 1995). The mouse TTP promoter region was also sensitive to mutations at the

Sp1 site but direct binding of Sp1 to the binding site in TTP promoter was not demonstrated (Lai et al. 1995). In the same study, the TTP intron was also found to be important for full serum inducibility. The intron indeed may be an important binding site for Sp1 and contribute to full serum inducibility of mouse TTP (Lai et al. 1998). The mouse, human, and bovine intron and human promoter region of TTP have been shown to contain a nuclear factor κB (NF-κB)-like binding site and NF-κB consensus binding sites, respectively (Lai et al. 1998, Smoak and Cidlowski 2006).

IL-4 has been shown to stimulate TTP expression through signal transducer and activator of transcription 6 (STAT6) in mice (Suzuki et al. 2003), while another cytokine, transforming growth factor β (TGF-β), has been shown to upregulate TTP expression through Smad3 and Smad4 (Ogawa et al. 2003). Binding sites for STATs and Smads have also been putatively detected by promoter analysis (Smoak and Cidlowski 2006). A glucocorticoid-like response element has also been suggested to reside in the mouse TTP promoter region (DuBois et al. 1990) as well as a single hexameric half glucocorticoid response element sequence in the human TTP 3'-flanking region (Smoak and Cidlowski 2006). A functional GAS element that binds STAT1 has also been reported to mediate the increases in TTP expression with interferons with co-stimulatory stress signal mediated by p38 MAPK in mice (Sauer et al. 2006).
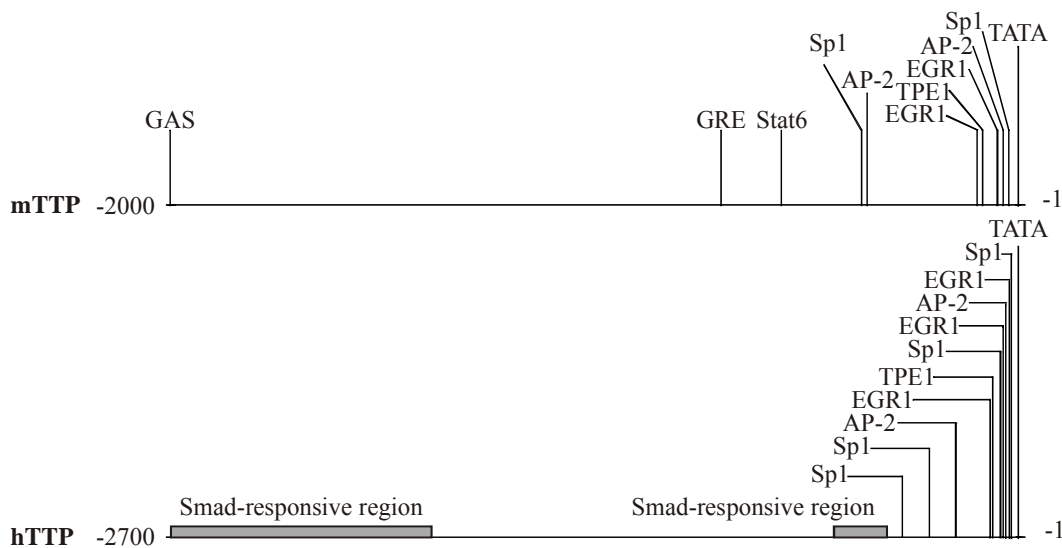


**Figure 1.** *Suggested transcription factor binding sites in mouse (mTTP) and human (hTTP) TTP promoter.*

The 3' untranslated region of mouse, human, and rat TTP mRNA contains several AUUUA or related sequences associated with unstable mRNA (DuBois et al. 1990, Lai et al. 1990, Kaneda et al. 2000, Brooks et al. 2004, Tchen et al. 2004). This has lead to the hypothesis that TTP itself regulates the stability of its own mRNA (Heximer et al. 1998, Brooks et al. 2004, Tchen et al. 2004, Lin et al. 2007). On the other hand, no difference in mRNA stability was seen between TTP mRNA and the TTP protein-disrupting TTP-neo fusion mRNA in wildtype (WT) and TTP KO cells, respectively, indicating that the regulation of TTP mRNA stability is identical in TTP WT and KO fibroblasts irrespective of TTP expression (Lai et al. 2006).


*Protein sequence of TTP and CCCH zinc finger domains*

Mouse and human TTP proteins share 84-87% identity (Taylor et al. 1991, Rigby et al. 2005), while mouse and rat TTP are 97% homologous at the protein level (Kaneda et al. 1992). The N-terminal domain of mouse TTP is 75% identical to human TTP, 16% to the mouse ZFP36L1, and 15% to the mouse ZFP36L2. The percentages for the C-terminal region are 82%, 22%, and 19%, respectively. The most striking similarity is between the CCCH zinc finger domains, where the similarity is 99%, 70%, and 72%, respectively (Johnson and Blackwell 2002). Overall the protein sequences of the TTP-related CCCH zinc finger proteins appear to be quite well conserved, especially in the region surrounding the zinc fingers (Lai et al. 2000). The amino acid sequences of the zinc finger structures are described in Figure 2. From the sequence alignments, it can be concluded that ZFP36L1 and ZFP36L2 zinc finger regions are more extensively conserved among species than these regions in TTP proteins, whereas the ZFP36L3 has the least well-conserved zinc finger region. In fact, the human, rat, and mouse ZFP36L1 coding sequences are strongly conserved (94.5% and 93.7%, respectively, at the nucleic acid level, and 99% at the protein level) (Barnard et al. 1993). ZFP36L3 found in mouse and rat X chromosome are 67% identical at the protein level, and at the zinc finger domain the mouse ZFP36L3 shares 76% amino acid homology with mouse TTP (Blackshear et al. 2005).

The two CCCH zinc fingers are separated by 18 amino acids. In all of the species listed in Figure 2, there seems to be a conserved lead-in sequence before the zinc finger domains, RYKTEL before the first one, and HPKYKTEL before the second one, with the exception of the mouse and rat ZFP36L3, where the last leucine is replaced by a proline residue in the second lead-in sequence. These sequences are underlined in Figure 2. With respect to ZFP36L2, it has been claimed that amino acids from these sequences represent a critical part of the RNA-binding surface of TTP (Hudson et al. 2004, Brown 2005).

The structure of the first zinc finger of murine TTP has been determined by multidimensional nuclear magnetic resonance spectroscopy (Worthington et al. 1996, Amann et al. 2003). Studies on the ZFP36L2 zinc finger domain later

```
Mouse TTP (NP_035886)         SSRYKTELCRTYSESGRCRYGAKCQFAH GLGELRQANRHPKYKTEL CHKFYLQGRCPYGSRCHFIHN
Human TTP (NP_003398)         PSRYKTELCRTFSESGRCRYGAKCQFAH GLGELRQANRHPKYKTEL CHKFYLQGRCPYGSRCHFIHN
Rat TTP (P47973)              SSRYKTELCRTYSESGRCRYGAKCQFAH GPGELRQANRHPKYKTEL CHKFYLQGRCPYGSRCHFIHN
Bovine TTP (P53781)           SSRYKTELCRTFSESGRCRYGAKCQFAH GLGELRQASRHPKYKTEL CHKFYLQGRCPYGSRCHFIHN
Xenopus C3H-1 (AAD24207)      SPRYKTELCRTFSETGTCKYGAKCQFAH GKIELREPNRHPKYKTEL CHKFYLYGECPYGSRCNFIHH
TTP consensus                --RYKTELCRT-SE-G-C-YGAKCQFAH G--ELR---RHPKYKTEL CHKFYL-G-CPYGSRC-FIH-

Mouse ZFP36L1 (P23950)        SSRYKTELCRPFEENGACKYGDKCQFAH GIHELRSLTRHPKYKTEL CRTFHTIGFCPYGPRCHFIHN
Human ZFP36L1 (X79066)        SSRYKTELCRPFEENGACKYGDKCQFAH GIHELRSLTRHPKYKTEL CRTFHTIGFCPYGPRCHFIHN
Rat ZFP36L1 (X52590)          SSRYKTELCRPFEENGACKYGDKCQFAH GIHELRSLTRHPKYKTEL CRTFHTIGFCPYGPRCHFIHN
Xenopus C3H-2 (AAD24208)      SSRYKTELCRPFEENGSCKYGDKCQFAH GIHELRSLTRHPKYKTEL CRTFHTIGFCPYGPRCHFIHN
ZFP36L1 consensus             SSRYKTELCRPFEENG-CKYGDKCQFAH GIHELRSLTRHPKYKTEL CRTFHTIGFCPYGPRCHFIHN

Mouse ZFP36L2 (P23949)        STRYKTELCRPFEESGTCKYGEKCQFAH GFHELRSLTRHPKYKTEL CRTFHTIGFCPYGPRCHFIHN
Human ZFP36L2 (X78992)        STRYKTELCRPFEESGTCKYGEKCQFAH GFHELRSLTRHPKYKTEL CRTFHTIGFCPYGPRCHFIHN
Xenopus C3H-3 (AAD24209)      STRYKTELCRPFEENGACKYGEKCQFAH GFHELRSLTRHPKYKTEL CRTFHTIGFCPYGPRCHFIHN
ZFP36L2 consensus             STRYKTELCRPFEE-G-CKYGEKCQFAH GFHELRSLTRHPKYKTEL CRTFHTIGFCPEGPRCHFIHN

Mouse ZFP36L3 (NM_001009549)  SERYKTELCRPFEESGICKYGHKCQFAH GYRELRTLSRHPKYKTEP CRTFHSVGFCPYGTRCHFIHN
Rat ZFP36L3 (XP_228661)       SERYKTELCRPFEENGTCRYGNKCQFAH GYHELRTLSRHPKYKTEP CRTFHSIGYCPYGSRCHFIHN
Xenopus C3H-4 (AAD24210)      SLRYKTELCSRYAESGFCAYRNRCQFAH GLSELRPPVQHPKYKTEL CRSFHVLGTCNYGLRCLFIHS
ZFP36L3 consensus             S-RYKTELC----E-G-C-Y---CQFAH G--ELR----HPKYKTE- CRTFH--G-C--G-RC-FIH-


CCCH consensus                --RYKTELC----E-G-C-Y---CQFAH G--ELR----HPKYKTE- C--F---G-C--G-RC-FIH-
```

**Figure 2.** *Comparison of the tandem zinc finger regions of the CCCH proteins. The cysteine and histidine residues of the putative zinc finger domains are marked in bold. Arginines important for nuclear import are shaded in gray. The lead-in sequences are underlined.*

```
                              N-terminal           C-terminal
     Mouse TTP (NP_035886)    MDLSAIYESLQSMSHD      RRLPIFNRISVSE
     Human TTP (NP_003398)    MDLTAIYESLLSLSPD      RRLPIFNRISVSE
     Rat TTP (P47973)         MDLSAIYESLMSMSHD      RRLPIFNRISVSE
     Bovine TTP (P53781)      MDLAAIYKSLLSLSPE      RRLPIFNRISVSE
     TTP consensus            MDL-AIY-SL-S-S--      RRLPIFNRISVSE
```

**Figure 3.** *Comparison of the N- and C-terminal regions of the mammalian TTP proteins. Hydrophobic residues of the nuclear export signal are shaded in gray. Modified from Phillips et al. (2002).*

revealed that the structure described by Amann et al. (2003) may not be completely correct (Hudson et al. 2004). The zinc fingers bind one zinc molecule per finger (Worthington et al. 1996) and it has been determined that both of the fingers are involved in the binding of RNA sequence UUAUUUAUU (Michel et al. 2003). It has also been suggested that iron as Fe(II) and Fe(III) can substitute zinc in TTP zinc fingers (diTargiani et al. 2006).

## TTP localization in the cell

The localization of TTP protein has been primarily described as cytosolic or largely nonnuclear (Carballo et al. 1998, Lai et al. 1999, Brooks et al. 2002, Fairhurst et al. 2003, Twizere et al. 2003, Cao et al. 2004). In mouse fibroblasts, constitutively expressed TTP remained mostly in the nucleus until the cells were stimulated by mitogens, and then TTP was translocated into the cytoplasm (Taylor et al. 1996). On the contrary, in resting human umbilical vein endothelial cells, TTP was predominantly located in the cytoplasm and TNF-α treatment sequestrated TTP into the nucleus (Gringhuis et al. 2005). In cells transiently transfected with TTP, its expression has been reported both in the cytoplasm and in the nucleus (Stoecklin et al. 2002). Mutations of the zinc fingers reduced the cytoplasmic localization of TTP (Johnson and Blackwell 2002). Indeed, the zinc finger regions have been shown to be important for nuclear localization, especially the two arginine residues between the two zinc fingers (shaded in grey in Figure 2) (Murata et al. 2002, Phillips et al. 2002). These arginine residues are quite well conserved in the CCCH family proteins. One of these arginines is missing from the *Xenopus* C3H-4, which may affect the localization of this protein. The N-terminal and C-terminal portions of TTP protein contain leucine rich hydrophobic nuclear export signals (Murata et al. 2002, Phillips et al. 2002), which are shown in Figure 3. In addition to the localization signals, the N- and C-terminal domains include sequences required for activation of target mRNA degradation (Lykke-Andersen and Wagner 2005).

TTP localization is also dependent on 14-3-3 proteins. The 14-3-3 family of proteins regulates many processes (such as inhibition, activation, structural stabilization, or translocation) in the cell via interactions with other cellular components (Aitken 2006). The individual isoforms of 14-3-3 bind to differentially phosphorylated forms of TTP protein and binding of 14-3-3

proteins promotes the cytoplasmic localization of TTP (Johnson et al. 2002). The phosphorylation of Ser176 of murine TTP downstream of the zinc fingers by MAPK-activated protein kinase 2 (MK2) has been shown to create a functional 14-3-3 binding site (Chrestensen et al. 2004). Nucleoporin CAN/Nup214 also interacted with TTP and this translocated TTP to the cytosol (Carman and Nadler 2004). The expression of a truncated mutant of CAN/Nup214 has resulted in a nuclear localization of TTP (Phillips et al. 2002).

There are several types of cytoplasmic RNA granules, which play an important role in the posttranscriptional regulation of gene expression by modulating the localization, stability, and translation of mRNA (Anderson and Kedersha 2006). It has been postulated that stress granules (SGs) serve as storage sites for mRNAs that are not translated during stress (Kedersha and Anderson 2002). mRNA decay and translational repression takes place in processing bodies (PBs) (Stoecklin and Anderson 2007). These granules contain the enzymes associated with decapping, deadenylation, and nonsense-mediated decay as well as exonucleases and ARE-binding proteins (Eulalio et al. 2007). Both of these granules are induced by stress and they interact with each other and also share some components (Kedersha et al. 2005, Kedersha and Anderson 2007).

TTP is one of the factors that is common to both SGs and PBs. Overexpression of TTP increases the number of SGs but arsenite-induced phosphorylation of Ser176 and the subsequent 14-3-3 binding prevent stress granule entry of TTP and therefore the stability of TNF-α mRNA increases (Stoecklin et al. 2004). Heat shock and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)-induced energy deprivation are capable of promoting zinc finger-dependent localization of TTP into SGs, while arsenite excludes TTP from the SGs by inducing 14-3-3 binding (Stoecklin et al. 2004, Murata et al. 2005). TTP and 14-3-3 interactions do not seem to take place on the polysomes (Rigby et al. 2005). TTP promotes the interactions of SGs with PBs and may direct mRNAs destined for decay from SGs to be degraded in the PBs (Kedersha et al. 2005). In TTP and ZFP36L1-depleted cells, the localization of reporter ARE-mRNAs to PBs was observed to be impaired (Franks and Lykke-Andersen 2007). The N- and C-terminal domains of TTP function as activation domains and form a link between the mRNA about to be degraded and the mRNA degradation machinery (Lykke-Andersen and Wagner 2005). TTP has been reported to be associated with many components of the PBs, for example it has been shown to interact with decapping complex subunits and activate decapping (Fenger-Grøn et al. 2005). The binding of TTP to the ARE of the target mRNA provides a route, either through SGs or via direct PB formation, for target mRNA degradation by the mRNA decay machinery at the PBs.

*TTP polymorphisms*

Several polymorphisms have been identified in the human *ZFP36* gene promoter, intron, protein-coding region, and 3'-UTR, some of which result in amino acid changes (Blackshear et al. 2003). One of the polymorphisms has been identified to be situated in the strictly conserved lead-in sequence RYKTEL and this has revealed an association to rheumatoid arthritis in African-Americans (Carrick et al. 2006). The *ZFP36* polymorphisms have also been associated to obesity-related metabolic complications (Bouchard et al. 2007a). More extensive studies are required before to the role of TTP polymorphism in inflammatory and other diseases will be fully understood.


# Regulation of TTP expression

A diverse set of compounds has been reported to induce or inhibit TTP mRNA expression. A much more limited number of reagents has been shown to induce or inhibit TTP protein levels. TTP mRNA is induced quite rapidly and transiently by epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin, LPS, and TPA (Arenander et al. 1989b, DuBois et al. 1990, Lai et al. 1990, Cao et al. 2004), and the mRNA half-life of TTP is relatively short, i.e. 15-45 min depending on cell type and treatment (Lai et al. 1990, Taylor and Blackshear 1995, Brooks et al. 2004, Lin et al. 2007). TTP protein, on the other hand, is regarded as being rather stable (Cao et al. 2004).


*Growth factors*

Growth factors were some of the first substances found to induce TTP mRNA expression (Table 3). Growth factors are cytokines that are capable of stimulating cell proliferation and differentiation.

In some cell types or conditions, the growth factors listed in Table 3 did not influence TTP mRNA expression. For instance, FGF did not induce TTP mRNA expression in mouse BaF3 pro-B cells expressing FGF-receptor-4 or wild type cells, but did so in cells expressing FGF-1, indicating that FGF-1 but not FGF-4 receptor is mediating the FGF signalling to induce TTP mRNA expression (Wang et al. 1994). In secondary rat astrocytes, nerve growth factor (NGF) did not induce TTP mRNA expression (Arenander et al. 1989b). In mouse TV-1 *plcg1* null cells (phospholipase C-γ1 KO) platelet-derived growth factor (PDGF) did not induce TTP mRNA expression, suggesting that phospholipase C-γ1 is required for TTP mRNA induction by PDGF, but it is not required for EGF inducibility, since EGF was able to induce TTP expression in cells lacking phospholipase C-γ1 (Liao et al. 2006).

**Table 3.** *Growth factors reported to induce TTP mRNA expression.*

| Growth factor | Cell type used | Reference |
|---|---|---|
| **Activin** | Human primary T cells, HuT78, and THP-1 cells | Ogawa et al. 2003 |
| **CNTF** | Rat muscle and brain | DiStefano et al. 1996, Kelly et al. 2004 |
| **EGF** | Mouse 3T3 fibroblasts | Lim et al. 1987, Lai et al. 1990, Varnum et al. 1991 |
| | Mouse TV-1 *plcg1* null and null+ cells | Liao et al. 2006 |
| | Rat PC12 cells[a] | Kujubu et al. 1987, Kaneda et al. 1992 |
| | Rat RIE-1 cells | Corps and Brown 1995 |
| | Secondary rat astrocytes | Arenander et al. 1989b, Arenander et al. 1989c |
| | Human MCF10A cells | Amit et al. 2007 |
| **FGF** | Mouse 3T3 fibroblasts | Lau and Nathans 1987, Lim et al. 1987, Lai et al. 1990, Varnum et al. 1991 |
| | Mouse BaF3 pro-B cells expressing FGF receptor-1 | Wang et al. 1994 |
| | Rat PC12 cells[a] | Kaneda et al. 1992 |
| | Secondary rat astrocytes | Arenander et al. 1989b, Arenander et al. 1989c |
| **GM-CSF** | Mouse myeloid cell line | Varnum et al. 1989b |
| | Human neutrophils | Varnum et al. 1989b |
| **NGF** | Mouse 3T3 fibroblasts | Varnum et al. 1991 |
| | Rat PC12 cells[a] | Kujubu et al. 1987, Varnum et al. 1991, Batistatou et al. 1992, Kaneda et al. 1992, Peng et al. 1995 |
| **NGF withdrawal** | Rat PC12 cells[a] | Mesner et al. 1995 |
| **PDGF** | Mouse 3T3 fibroblasts | Lau and Nathans 1987, Lai et al. 1990 |
| | Mouse TV-1 *plcg1* null+ cells | Liao et al. 2006 |
| **TGF-α** | Rat RIE-1 cells | DuBois et al. 1995 |
| **TGF-β** | Human primary T cells, HuT78, and THP-1 cells | Ogawa et al. 2003 |

[a] *pheochromocytoma cell line*
*NGF, nerve growth factor; PDGF, platelet-derived growth factor; RIE, rat intestinal epithelial.*

*Interleukins, interferons, and TNF-α*

Cytokines other than growth factors that induce TTP expression include interleukins, interferons (IFNs), and TNF-α. Interleukins 4, 6, and 11 have been shown to induce TTP mRNA expression in mouse bone marrow-derived macrophages (BMMs) and mouse B cell hybridomas (Nakajima and Wall 1991, Yin and Yang 1993, Yang and Yin 1995, Suzuki et al. 2003). IL-4 has also been used in combination with LPS or anti-CD40 to induce TTP mRNA expression in young and old BALB/c mice primary splenic B cells, but it was not able to elevate TTP mRNA levels on its own (Frasca et al. 2007). Furthermore, IL-4 did not induce TTP mRNA expression in mouse STAT6$^{-/-}$ BMMs (Suzuki et al. 2003), indicating that IL-4 acts through the activation of STAT6. Also leukaemia inhibitory factor (LIF), an IL-6 class cytokine, has been observed to induce TTP mRNA expression in male Sprague-Dawley rats (DiStefano et al. 1996). A pre-dose of LIF evoked desensitisation to a test dose of LIF as well as to ciliary neurotrophic factor (CNTF), while CNTF did not induce desensitisation towards LIF, only towards CNTF (DiStefano et al. 1996). IL-4 is anti-inflammatory, IL-6 proinflammatory, and IL-11 is a cytokine belonging to the IL-6 family, which has both pro- and anti-inflammatory effects. IL-6 is also one of the mRNAs that TTP destabilizes and it could be that induction of TTP expression by IL-6 may provide a means to down-regulate IL-6 expression. The induction of TTP expression by anti-inflammatory interleukins on the other hand could explain some of their anti-inflammatory actions.

IFN-β, in combination with the antibiotic anisomycin, which inhibits protein synthesis, anisomycin alone, as well as IFN-γ with or without anisomycin, all induce TTP mRNA expression in mouse embryonic fibroblasts (MEFs). Based on studies with STAT1 and p38 deficient MEFs, it was concluded that IFNs induce TTP expression through STAT1 in the presence of the costimulatory stress signal provided by p38 (Sauer et al. 2006).

TNF-α can induce both TTP mRNA and protein expression. TNF-α induced the expression of TTP mRNA in wildtype (WT) and TNF receptor 2 KO mouse BMMs (Carballo and Blackshear 2001) as well as in human primary bronchial epithelial cells (Cooper et al. 2001). In TNF receptor 1 and 2 double KO, and TNF receptor 1 KO mouse BMMs, TNF-α did not seem to be able to induce TTP mRNA expression, indicating that the TNF receptor 1 is crucial for TNF-α signalling to induce TTP expression (Carballo and Blackshear 2001). TTP protein expression was shown to be induced by TNF-α in primary mouse macrophages (Carballo et al. 1998) and human THP-1 cells (Fairhurst et al. 2003) as well as in human glioma cell lines (Suswam et al. 2008).

A mixture of cytokines (IFN-γ, IL-1β, and TNF-α) was reported to induce both TTP mRNA and protein expression in human DLD-1 cells (Fechir et al. 2005) and in human A549/8 alveolar epithelial cells (Korhonen et al. 2007).

*Hormones and related compounds*

Insulin was one of the first hormones found to induce TTP mRNA expression in mouse 3T3 fibroblasts (Lai et al. 1990). In the same cell type, a mixture of insulin, dexamethasone, methylisobutylxanthine, and foetal bovine serum induced both TTP mRNA and protein expression (Inuzuka et al. 1999, Lin et al. 2007). In contrast, in rat intestinal epithelial (RIE)-1 cells, insulin or insulin-like growth factor 1 did not induce TTP mRNA expression (Corps and Brown 1995).

Endogenous and synthetic glucocorticoids, e.g. dexamethasone, are anti-inflammatory agents used to prevent or inhibit the inflammatory symptoms in asthma (Rang et al. 2007c). In addition to asthma treatment, glucocorticoids are used to treat a wide range of other inflammatory diseases such as rheumatoid arthritis, and they function as immunosuppressants after organ transplantation (Rang et al. 2007b). Dexamethasone alone was reported to induce TTP mRNA and protein expression in resting human A549 alveolar epithelial cells and rat tissues such as liver, lung, spleen, and thymus (Smoak and Cidlowski 2006). In an earlier study, dexamethasone decreased TTP mRNA and protein expression in J774 mouse macrophages exposed to inflammatory stimuli (bacterial endotoxin, LPS) (Jalonen et al. 2005). Dexamethasone also inhibited TTP mRNA expression in $CD4^+CD8^+$ double-positive thymocytes from C3H/HeN mice (Bianchini et al. 2006). Dexamethasone did not affect TTP mRNA expression in rat PC12 pheochromocytoma cells (Kujubu et al. 1987) nor on TTP protein expression in TPA + phytohemaglutinin or anti-CD3 + anti-CD28-treated human peripheral blood mononuclear cells (Bergmann et al. 2004).

Other hormones or their synthetic analogues that have been reported to induce TTP mRNA expression include angiotensin II in rat RIE-1 cells (Corps and Brown 1995), gonadotropin-releasing hormone (GnRH) in mouse LβT2 gonadotrope cell line (Yuen et al. 2002), medroxyprogesterone in combination with 17β-estradiol in a human T47D breast cancer cell line (Mrusek et al. 2005), and leptin has been shown to weakly induce TTP mRNA expression in certain parts of male Sprague-Dawley rat brain (Kelly et al. 2004). Dihydrotestosterone on the other hand did not change TTP protein levels in human Jurkat T cells (Sheflin et al. 2004).

Arachidonic acid and its metabolite eikosatetraynoic acid have been reported to induce TTP mRNA expression in mouse liver cell lines (Ledwith et al. 1996).

Serum, which contains a multitude of cytokines, hormones, and other biologically active compounds, has been shown to stimulate TTP expression in several mouse (Lau and Nathans 1987, Lim et al. 1987, DuBois et al. 1990, Lai et al. 1990, Taylor et al. 1995, Ledwith et al. 1996, Taylor et al. 1996, Lai et al. 2006) and some human cell lines (Gubits et al. 1993b). Furthermore, serum withdrawal has been shown to induce TTP mRNA expression in rat PC12 pheochromocytoma and Rat-1 cells (Mesner et al. 1995).

The diverse quantity of cytokines and hormones that can induce TTP expression suggests that TTP is a crucial regulator of gene expression and it may have a function in many physiological responses.

*Tumor promoters*

One of the first compounds reported to induce TTP mRNA expression was the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate ester (TPA) (Kujubu et al. 1987, Lim et al. 1987). The effects of TPA on TTP mRNA levels in many mouse, human, and rat cells are summarized in Table 4. In addition to the treatments listed in Table 4, in human T lymphocytes, TPA + ionomycin-treatment was reported to induced both TTP mRNA and protein expression (Raghavan et al. 2001). The same treatment combination also induced TTP protein expression in *Herpesvirus saimiri*-transfected marmoset T cells (Cook et al. 2004). TPA + phytohemaglutinin in human peripheral blood mononuclear cells induced TTP protein expression (Bergmann et al. 2004). A mouse myeloma cell line J558L was unable to produce TTP mRNA when exposed to TPA, while another myeloma cell line S107 did activate TTP mRNA expression in response to TPA (Anderson et al. 1991). These cell lines also differed in the functionality of NF-κB. Another phorbol ester, phorbol dibutyrate, has induced TTP mRNA expression in resting mouse splenic cells and in B lymphoma cell lines (Mittelstadt and DeFranco 1993).

**Table 4.** *Studies and cell lines, in which TPA has been shown to increase TTP mRNA levels.*

| Cell type used | Reference |
| --- | --- |
| Mouse 3T3 fibroblasts | Lim et al. 1987, DuBois et al. 1990, Lai et al. 1990, Varnum et al. 1991 |
| Mouse myeloid cell line | Varnum et al. 1989a |
| Mouse B cell hybridomas | Nakajima and Wall 1991, Yin and Yang 1993 |
| Mouse J774 macrophages | Leppänen et al. 2008 |
| Mouse liver cell lines | Ledwith et al. 1996 |
| Human neutrophils | Varnum et al. 1989a |
| Human blood mononuclear cells | Heximer et al. 1998 |
| Human astrocytoma cell line G18 | Gubits et al. 1993b |
| Rat PC12 cells[a] | Kujubu et al. 1987, Varnum et al. 1991, Kaneda et al. 1992 |
| Rat RIE-1 cells | Corps and Brown 1995, DuBois et al. 1995 |
| Rat secondary astrocytes | Arenander et al. 1989b, Arenander et al. 1989c |
| Rat astrocytes | Priller et al. 1998b |
| Rat microglial cells | Priller et al. 1995 |

[a] *pheochromocytoma cell line*

Many tumor promoters including the nongenotoxic peroxisome proliferators, e.g. WY14643, mono-ethylhexyl phthalate, clofibrate, and ciprofibrate ethylester, but not dehydroepiandrosteronesulfate, could induce TTP mRNA expression comparable to that observed with another tumor promoter thapsigargin in mouse liver cell lines (Ledwith et al. 1996).

Benzene has induced TTP protein expression in male Sprague-Dawley rats (Lee et al. 2004) and nongenotoxic carcinogens elevated TTP mRNA levels in human HepG2 cells (van Delft et al. 2005). A tumor-promoting toxin, okadaic acid, has been reported to induce TTP expression in mouse liver cell lines as well as in rat RIE-1 cells (DuBois et al. 1995, Ledwith et al. 1996).

## *Lipopolysaccharide (LPS)*

LPS is one of the substances that has been extensively used in studies on TTP, and it has been consistently shown to increase TTP protein levels. LPS is a component of the outer membrane of Gram-negative bacteria, and it acts as an endotoxin. LPS activates Toll-like receptor 4 and promotes the secretion of proinflammatory cytokines, especially in macrophages (Fujihara et al. 2003, Jerala 2007). The induction of anti-inflammatory factor TTP by LPS is believed to be one of the feedback mechanisms intended to prevent overwhelming inflammation and to initiate the resolution of the process. The diversity of cell types in which LPS has been shown to induce TTP expression is described in Table 5.

## *Agonists of adrenergic receptors, cAMP analogues, and related compounds*

Adrenoceptor agonists, also known as sympathomimetics, mimic the actions of adrenaline and noradrenaline on β-receptors. $β_2$-agonists are widely used as bronchodilatators in the treatment of asthma and chronic obstructive pulmonary disease. Activation of $β_2$-adrenoceptors triggers a series of events that lead to airway smooth muscle relaxation but it may also evoke some anti-inflammatory effects (Bissonnette and Befus 1997, Johnson 2002, Sitkauskiene and Sakalauskas 2005, Broadley 2006, Giembycz and Newton 2006, Johnson 2006). $β_2$-adrenoceptors activate adenylate cyclase resulting in increased intracellular cyclic adenosine 3',5'-monophosphate (cAMP) levels and activation of protein kinase A (PKA), which in turn regulates phosphorylation (activation) of target molecules and gene expression. The effects of adrenoceptor agonists on TTP expression are summarized in Table 6.

**Table 5.** *Studies and cell lines, in which LPS has been shown to increase TTP levels.*

| Cell type used | Enhances TTP mRNA and/or protein expression | Reference |
|---|---|---|
| Mouse RAW264.7 macrophages | mRNA, protein | Mahtani et al. 2001, Zhu et al. 2001, Cao et al. 2004, Tchen et al. 2004, Brook et al. 2006, Chen et al. 2006, Deleault et al. 2008 |
| Mouse J774 macrophages | mRNA, protein | Jalonen et al. 2005, Jalonen et al. 2008, Leppänen et al. 2008 |
| Primary mouse macrophages | protein | Carballo et al. 1998 |
| Mouse BMMs and MEFs | protein | Cao et al. 2004, Hitti et al. 2006 |
| Mice liver | mRNA | Beauchamp et al. 1994 |
| Mouse primary splenic B cells | mRNA, protein | Frasca et al. 2007 |
| Human THP-1 cells | mRNA, protein | Baseggio et al. 2002, Fairhurst et al. 2003, Brooks et al. 2004 |
| Human peripheral blood cells | mRNA, protein | Baseggio et al. 2002, Fairhurst et al. 2003, Fabris et al. 2005 |
| Human primary monocytes | mRNA | Carrick and Blackshear 2007 |
| Human HCA-7 cell line[a] | mRNA | Sawaoka et al. 2003 |
| Rat brain | mRNA | Kelly et al. 2004 |

[a] *colorectal adenocarcinoma*
*MEF, mouse embryonic fibroblast.*

**Table 6.** *The effects of adrenoceptor agonists on TTP expression. Selectivity towards α- or β-receptors is indicated in parenthesis.* ↑ *Upregulation,* ↓ *down-regulation.*

| Adrenoceptor agonist (selectivity) | Cell type used | Effects on TTP expression | Reference |
|---|---|---|---|
| **Formoterol** (β) | Mouse J774 macrophages | mRNA ↑ | Jalonen et al. 2007 |
| **Isoprenaline** (β) | Human astrocytoma cell line G18 | mRNA ↑ | Gubits et al. 1993b |
| | Rat secondary astrocytes | mRNA ↑ | Arenander et al. 1989a |
| **Noradrenaline** (α and β) | Rat secondary astrocytes | mRNA ↑ | Arenander et al. 1989a |
| **Phenylephrine** (α) | Rat secondary astrocytes | mRNA ↑ | Arenander et al. 1989a |
| **Salbutamol** (β) | Mouse J774 macrophages | mRNA, protein ↑ | Jalonen et al. 2007 |
| **Salbutamol** (β) **with LPS** | Mouse J774 macrophages | mRNA ↑, protein ↓ | Jalonen et al. 2008 |
| **Terbutaline** (β) | Mouse J774 macrophages | mRNA ↑ | Jalonen et al. 2007 |
| **Terbutaline** (β) **with LPS** | Mouse J774 macrophages | protein ↓ | Jalonen et al. 2008 |

cAMP analogs such as $N^6$,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (db-cAMP) and 8-bromoadenosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cAMP) can also be used to mimic the effects of adrenoceptor agonists as can forskolin, an activator of adenylate cyclase, that increases intracellular cAMP levels. The effects of cAMP analogs and forskolin are shown in Table 7. In addition to cAMP analogs, the effects of the cGMP analog, 8-Br-cGMP, have been tested but it seems to exert no effect on TTP mRNA levels in two mouse B cell hybridoma cell lines (Nakajima and Wall 1991, Yin and Yang 1993).

**Table 7.** *The effects of cAMP agonists and forskolin on TTP expression. ↑ Upregulation, ↓ down-regulation, — no effect.*

| Compound | Cell type used | Effects on TTP expression | Reference |
|---|---|---|---|
| **8-Br-cAMP** | Mouse J774 macrophages | mRNA, protein ↑ | Jalonen et al. 2007 |
| **8-Br-cAMP with LPS** | Mouse J774 macrophages | mRNA ↑, protein ↓ | Jalonen et al. 2008 |
| **db-cAMP** | Mouse J774 macrophages | mRNA, protein ↑ | Jalonen et al. 2007 |
| | Mouse B cell hybridomas | mRNA — | Nakajima and Wall 1991, Yin and Yang 1993 |
| | Rat PC12 cells[a] | mRNA ↑ | Kaneda et al. 1992 |
| | Rat secondary astrocytes | mRNA ↑ | Arenander et al. 1989b |
| **db-cAMP with LPS** | Mouse J774 macrophages | mRNA ↑, protein ↓ | Jalonen et al. 2008 |
| **forskolin** | Mouse 3T3 fibroblasts | mRNA ↑ | DuBois et al. 1990 |
| | Mouse J774 macrophages | mRNA, protein ↑ | Jalonen et al. 2007 |
| | Rat secondary astrocytes | mRNA ↑ | Arenander et al. 1989b |
| | Rat microglial cells | mRNA — | Priller et al. 1995 |
| **forskolin with LPS** | Mouse J774 macrophages | mRNA ↑, protein ↓ | Jalonen et al. 2008 |

[a] *pheochromocytoma cell line*

Phosphodiesterase inhibitors of cyclic nucleotides can potentiate the effects of cAMP-elevating agents due to the inhibition of the breakdown of cAMP. A non-specific inhibitor of cyclic nucleotide phosphodiesterases, 3-isobutyl-1-methylxanthine (IBMX), and a type IV phosphodiesterase inhibitor, rolipram, increased salbutamol-induced TTP mRNA levels and forskolin-induced TTP mRNA and protein levels in mouse J774 macrophages (Jalonen et al. 2007).

Carbachol, a cholinergic agonist, has been reported to induce TTP mRNA expression in secondary rat astrocytes (Arenander et al. 1989a) and in a mouse B lymphoma cell line transfected with muscarinic receptor type 1 (Mittelstadt and DeFranco 1993). In rat secondary astrocytes, TTP mRNA induction by carbachol was potentiated by lithium and in both cell types atropine blocked the induction.

Nucleosides, like adenosine, and nucleotides like AMP, ADP, and ATP also function as neurotransmitters. Adenosine and AMP did not induce TTP mRNA expression in rat microglial cells, unlike ADP and ATP (Priller et al. 1995). Adenosine-5'-O-(2-thiodiphosphate) increased TTP mRNA levels in rat

astrocytes (Priller et al. 1998b). In rat PC12 pheochromocytoma cells, treatment with 2-aminopurine reduced NGF-induced TTP mRNA levels (Batistatou et al. 1992).

Ganglioside GM1 as well as neuropeptide galanin could induce TTP mRNA expression in rat astrocytes but not in rat PC12 pheochromocytoma cells (Arenander et al. 1989b, Priller et al. 1998a). Substance P (a short-chain neuropeptide), calcitonin gene-related peptide (a strong inducer of vasodilatation), and cholecystokinin (a stimulator of digestion and a hunger suppressant), did not seem to have any effect on TTP mRNA levels in rat astrocytes (Priller et al. 1998a).

## Inhibitors of signalling pathways

Several signalling pathways are involved in the upregulation of TTP expression as shown by studies with inhibitors of signalling pathways. Tyrosine kinases were involved in TTP mRNA upregulation by IL-6 and IL-11 in mouse B cell hybridomas as demonstrated by the use of inhibitors like tyrphostin, genistein, and herbimycin A (Nakajima and Wall 1991, Yin and Yang 1993). Genistein had no statistically significant effect on LPS-induced TTP mRNA accumulation in mouse macrophages (Jalonen et al. 2005). Tyrphostin AG 490 is a specific and potent Janus kinase 2 (JAK2) protein tyrosine kinase inhibitor, which did not inhibit LPS-induced TTP mRNA expression in mouse J774 macrophages (Jalonen et al. 2005). These results would appear to indicate that tyrosine phosphorylation is required in the mediation of some but not all signalling pathways.

H7, an inhibitor of several protein kinases (PKC, PKA, PKG), has been reported to inhibit IL-6 and IL-11-induced TTP mRNA expression in mouse B cell hybridomas (Nakajima and Wall 1991, Yin and Yang 1993) as well as TTP mRNA expression in mouse liver cell lines (Ledwith et al. 1996), and mouse 3T3-L1 cells (Yang and Yin 1995). On the other hand, H8, a potent inhibitor of cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA), was unable to influence TTP mRNA expression in mouse liver cell lines (Ledwith et al. 1996), suggesting that of these three protein kinases, PKC might be the principal mediator of signalling aimed at TTP mRNA transcription (see below).

The phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002, did not inhibit LPS-induced TTP protein expression in mouse RAW264.7 macrophages (Brook et al. 2006) or TTP mRNA expression in mouse 3T3 preadipocytes induced by adipogenic hormones (combination of insulin, dexamethasone, methylisobutylxanthine, and foetal bovine serum, Inuzuka et al. 1999). Furthermore, another PI3K inhibitor, Wortmannin, did not prevent TTP mRNA induction in rat RIE-1 cells treated with EGF, angiotensin II, or TPA (Corps and Brown 1995). TTP mRNA expression induced by several stimuli in mouse liver and B cells was not inhibited by W7, an inhibitor of calmodulin, a calcium sensor protein that activates the serine/threonine phosphatase calcineurin

(Nakajima and Wall 1991, Yin and Yang 1993, Ledwith et al. 1996). Inhibition of calcineurin, a downstream target of PI3K, did not block TTP mRNA accumulation in mouse J774 macrophages (Jalonen et al. 2005). These data suggest that PI3K signalling or the activation of nuclear factor of activated T cells (NFAT) is not important for TTP mRNA or protein expression.


*Protein kinase C (PKC)*

The protein kinase C (PKC) pathway, which consists of several different isoenzymes, is a major signal transduction pathway in inflammation (Way et al. 2000, Tan and Parker 2003). Pre-treatment with tumor promoter TPA can be used to deplete cells from classical and novel protein kinase C isoforms (Chen 1993). When cells were pre-treated to down-regulate PKC expression, TPA no longer could induce TTP mRNA expression in mouse 3T3 fibroblasts (Lai et al. 1990), mouse B cell hybridoma (Yin and Yang 1993), and rat HIRc-B and H4IIEC3 cells (Stumpo et al. 1994). In LPS+TPA-stimulated mouse J774 macrophages, both TTP mRNA and protein were inhibited by TPA pre-treatment (Leppänen et al. 2008). On the other hand, TPA pre-treatment did not inhibit induction of TTP mRNA by insulin in mouse 3T3 fibroblasts (Lai et al. 1990), and rat HIRc-B and H4IIEC3 cells (Stumpo et al. 1994), or by IL-11 in mouse B cell hybridoma (Yin and Yang 1993), indicating that TPA, but not insulin and IL-11, require PKC isoforms for induction of TTP mRNA expression.

The effects of several PKC inhibitors are listed on Table 8. The regulation of TTP expression by PKC isoforms is not yet fully understood and the results are somewhat conflicting. Clarification of the role of PKC in TTP expression will require further studies though the best-characterized candidate is PKC βII (Leppänen et al. 2008).


*Mitogen-activated protein kinases (MAPKs)*

The three better-characterized serine/threonine kinases of the mitogen-activated protein kinase (MAPK) family, p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase 1 and 2 (ERK1/2) play an essential role in signal transduction via phosphorylation (Figure 4) (Turjanski et al. 2007). MAPK p38 is involved in mRNA stabilization, but the ARE-binding proteins involved are still unknown (Dean et al. 2004). Also the role of TTP in this stabilization process is still unclear. It appears likely that p38, as well as other MAPKs, is involved in TTP upregulation, and this may serve as a negative feedback loop, inducing TTP expression to turn off the expression of inflammatory genes. The effects of p38, ERK1/2, and JNK inhibition on TTP expression are collated in Table 9.

**Table 8.** *The effects of PKC inhibitors on TTP expression. Specificity towards certain PKC isoenzymes is given in parenthesis, > describing the descending specificity towards different isoenzymes over others. ↓ Down-regulation and — no effect.*

| Compound | Cell type used | Effects on TTP expression | Reference |
|---|---|---|---|
| **CGP53353** (βII) | Mouse J774 macrophages | LPS+TPA-induced mRNA and protein ↓ | Leppänen et al. 2008 |
| **HBDDE** (α) | Mouse J774 macrophages | LPS+TPA-induced mRNA and protein — | Leppänen et al. 2008 |
| **GÖ6976** (α, β, γ) | Mouse J774 macrophages | LPS+TPA-induced mRNA and protein ↓ | Leppänen et al. 2008 |
| **LY333531** (βI, βII) | Mouse J774 macrophages | LPS+TPA-induced mRNA and protein ↓ | Leppänen et al. 2008 |
| **Ro-31-8220** (α, β, γ, ε) | Mouse J774 macrophages | LPS+TPA-induced mRNA and protein ↓ | Leppänen et al. 2008 |
| **Ro-32-0432** (α > βI > ε) | Mouse 3T3 preadipocytes | Adipogenic hormones[a]-induced mRNA ↓ | Inuzuka et al. 1999 |
| **Rottlerin** (δ > α, β, γ > ε, η, ζ) | Mouse RAW264.7 macrophages | LPS-induced protein — | Brook et al. 2006 |
| **Sphingosine** | Mouse B cell hybridoma | TPA-induced mRNA ↓ | Nakajima and Wall 1991 |
| | Mouse B cell hybridoma | IL-6-induced mRNA — | Nakajima and Wall 1991 |
| | Mouse B cell hybridoma | IL-11-induced mRNA — | Yin and Yang 1993 |
| **Staurosporine analogue compound 3** | Mouse B lymphoma cell lines | Phorbol dibutyrate-, ionomycin-, and anti-IgG-induced mRNA ↓ | Mittelstadt and DeFranco 1993 |

[a] *combination of insulin, dexamethasone, methylisobutylxanthine, and foetal bovine serum*
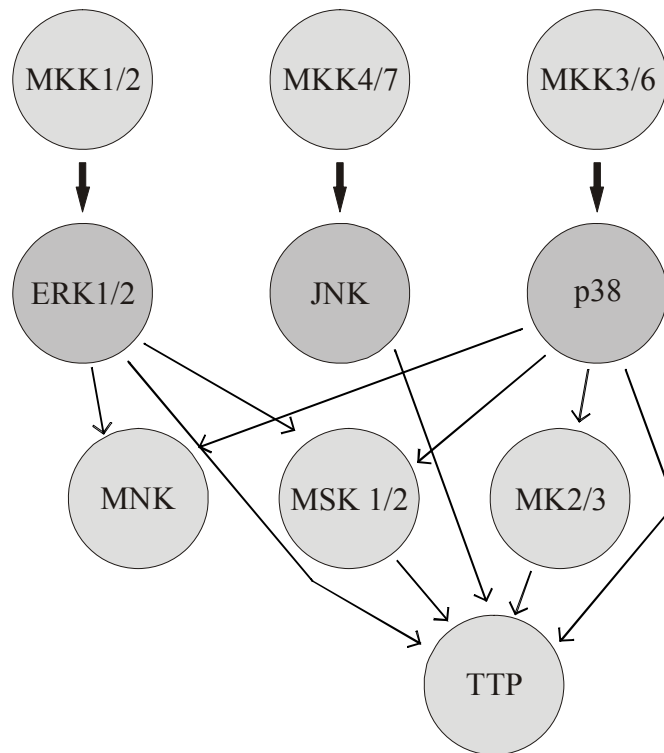
**Figure 4.** *A simplified scheme of the MAPK activation pathways including the MAPKs that have been reported to have an effect on TTP expression or phosphorylation. MKK, MAPK kinase; MK, MAPK-activated protein kinase; MNK, MAPK-interacting kinase; MSK, mitogen- and stress-activated kinase.*

There is very strong evidence for the involvement of p38 in the upregulation of LPS-induced TTP mRNA and protein expression in several human and mouse cell lines. On the other hand, the role of ERK1/2 seems to depend on the inhibitor being used and clarification will require further studies with both mouse and human cells. Tamoxifen, a selective estrogen receptor modulator used for the treatment of breast cancer, was shown to induce ERK1/2 activation and TTP mRNA expression (Essafi-Benkhadir et al. 2007). In one study, phosphorylation of TTP by ERK1/2 appeared to stabilize TTP protein but inhibited the destabilizing effect of TTP on vascular endothelial growth factor (VEGF) mRNA (Essafi-Benkhadir et al. 2007).

**Table 9.** *The effects of p38, ERK1/2, and JNK inhibition on TTP expression.* ↓ *Down-regulation,* ─── *no effect.*

| Compound | Cell type used | Effects on TTP expression | Reference |
|---|---|---|---|
| **p38 inhibition** | | | |
| **SB202190** | Mouse primary macrophages | LPS-induced protein ↓ | Brook et al. 2006 |
| | Mouse RAW264.7 macrophages | LPS-induced mRNA, protein ↓ | Brook et al. 2006 |
| | Human monocytes | LPS-induced protein ↓ | Brook et al. 2006 |
| | Human THP-1 cells | mRNA stability ─── , LPS-induced protein ↓ | Brooks et al. 2004 |
| **SB203580** | Mouse RAW264.7 macrophages | LPS-induced mRNA, protein ↓ | Mahtani et al. 2001, Chen et al. 2006 |
| | Mouse J774 macrophages | LPS-induced mRNA ↓ | Jalonen et al. 2005 |
| | Mouse 3T3 preadipocytes | Adipogenic hormones[a] - induced mRNA ─── | Inuzuka et al. 1999 |
| | Human DLD-1 cells | CM-induced mRNA, protein ↓ | Fechir et al. 2005 |
| **ERK1/2 inhibition** | | | |
| **PD98059** | Mouse J774 macrophages | LPS-induced mRNA ↓ | Jalonen et al. 2005 |
| | Mouse 3T3 preadipocytes | Adipogenic hormones[a] - induced mRNA ↓ | Inuzuka et al. 1999 |
| | Human THP-1 cells | mRNA stability ↓, LPS-induced protein ↓ | Brooks et al. 2004 |
| **U0126** | Mouse RAW264.7 macrophages | LPS-induced mRNA, protein ─── | Tchen et al. 2004, Brook et al. 2006 |
| **JNK inhibition** | | | |
| **JNK siRNA** | Human A549/8 alveolar epithelial cells | CM-induced mRNA ─── , CM-induced protein ↓ | Korhonen et al. 2007 |
| **SP600125** | Mouse J774 macrophages | LPS-induced mRNA ↓ | Jalonen et al. 2005 |
| | Mouse RAW264.7 macrophages | LPS-induced protein ─── | Brook et al. 2006 |
| | Human THP-1 cells | mRNA stability ─── , LPS-induced protein ↓ | Brooks et al. 2004 |
| | Human A549/8 alveolar epithelial cells | CM-induced mRNA ─── , CM-induced protein ↓ | Korhonen et al. 2007 |

[a] *combination of insulin, dexamethasone, methylisobutylxanthine, and foetal bovine serum*
*CM, cytokine mixture (IFN-γ, IL-1β, and TNF-α)*

There is not much known about the effects of the JNK pathway on mouse TTP expression. In human cells, JNK has been claimed to regulate TTP expression posttranscriptionally, independent of p38 (Korhonen et al. 2007). Cycloheximide (CHX), which is used as an inhibitor of protein synthesis, is also a potent activator of JNK (Newton et al. 1997, Lahti et al. 2006), which may have contributed to its effects on TTP expression. In general, cycloheximide has been reported to increase TTP mRNA expression in mouse, human and rat cells though there are a few exceptions (Lim et al. 1987, Arenander et al. 1989c, Varnum et al. 1989a, Lai et al. 1990, Nakajima and Wall 1991, Varnum et al. 1991, Mittelstadt and DeFranco 1993, Heximer et al. 1998, Maclean et al. 1998).

In addition to the three well-characterized members of MAPKs, the roles of MAPK-activated protein kinases 2 and 3 (MK2/3), MAPK-interacting kinases (MNKs), and mitogen- and stress-activated kinases (MSKs) in the regulation of TTP expression have been studied, pointing to a possible role for MK2 and MSKs in the upregulation of TTP expression in mouse macrophages (Brook et al. 2006, Hitti et al. 2006, Ronkina et al. 2007).

It is possible that MAPKs do not only induce TTP mRNA expression but they may also phosphorylate TTP protein (Cao et al. 2007). Both mouse and human TTP are extensively phosphorylated, and this has been reported be reduced by treatment with alkaline phosphatase that also increased the electrophoretic mobility of TTP in Western blot (Cao 2004, Cao et al. 2004). The stability of TNF-$\alpha$ mRNA via the ARE sites depended on p38 and JNK MAPKs (Kontoyiannis et al. 1999), with this regulation being impaired in the TTP KO mice (Carballo et al. 2001). In TTP protein, several sites, which can be phosphorylated by several kinases at least *in vitro*, have been identified, and MAPKs p38, JNK, ERK1/2, and MK2 have been implicated (Taylor et al. 1995, Mahtani et al. 2001, Cao et al. 2003, Chrestensen et al. 2004, Brook et al. 2006, Cao et al. 2006, Cao et al. 2007, Suswam et al. 2008).

The function of TTP may be influenced by phosphorylation. Some phosphorylation sites, like Ser178 of mouse TTP, create a binding site for 14-3-3 proteins and are involved in translocating TTP to the cytosol (Johnson et al. 2002, Chrestensen et al. 2004). It has been claimed that arsenite-induced phosphorylation of Ser176 induced 14-3-3 binding and prevented the entry of TTP to stress granules and therefore the stability of TNF-$\alpha$ mRNA became increased (Stoecklin et al. 2004). This binding protected phosphorylated TTP from dephosphorylation by protein phosphatase 2a thereby increasing TNF-$\alpha$ mRNA stability (Sun et al. 2007). There is conflicting evidence about whether the phosphorylation status affects TTP binding to the ARE. Cao et al. (2003) and Chrestensen et al. (2004) have shown that phosphorylation of TTP did not affect mRNA binding while others have reported that the phosphorylation of TTP reduced its binding, possibly through an interaction with 14-3-3 proteins (Carballo et al. 2001, Chen et al. 2006, Hitti et al. 2006). Phosphorylation of Ser176 by MK2 and the subsequent 14-3-3 binding prevents stress granule entry of TTP and therefore the stability of TNF-$\alpha$ mRNA increases (Stoecklin et al. 2004). Phosphorylation of TTP or activation of MAPK pathways may also reduce the function of TTP i.e. decrease the mRNA degradation rate

(Marderosian et al. 2006, Ehlting et al. 2007, Essafi-Benkhadir et al. 2007, Frasca et al. 2007), and in the presence of the p38 inhibitor, SB203580, mouse chemokine CXCL1 (KC) mRNA is degraded more rapidly (Datta et al. 2008), providing further evidence that the phosphorylation status of TTP protein represents a feasible way to control mRNA stability.


## Other conditions and compounds

Several cell manipulations and some surgical procedures in experimental animals have been shown to induce TTP expression. Overexpression studies have indicated that the protein encoded by the breast cancer susceptibility gene *BRCA1* (Harkin et al. 1999) and EGF receptors (Edman et al. 1997) positively regulate TTP mRNA expression, while STAT5 may be a negative regulator of TTP protein expression (Barnstein et al. 2006).

Cell culturing conditions such as cell confluence (Sawaoka et al. 2003) and cell cycle activation (Sachidanandan et al. 2002) seemed to increase TTP mRNA expression, whereas monocyte/macrophage differentiation reduced TTP mRNA expression (MacKenzie et al. 2002).

Ultraviolet A radiation has been shown to increase TTP mRNA in normal human melanocytes (Jean et al. 2001). Hemodynamic pressure overloading (Baumgarten et al. 2002), hypoxia-ischemia (Gubits et al. 1993a), pneumonectomy (Landesberg et al. 2001), muscle injury (Sachidanandan et al. 2002), myocardial ischemic preconditioning (Zubakov et al. 2003), as well as resections of the small bowel (Ehrenfried et al. 1995, Sacks et al. 1995) have been shown to increase TTP mRNA expression in experimental animals suggesting a role for TTP in the regulation of the healing processes.

According to these results, TTP mRNA is upregulated in several organisms in different tissues in response to very diverse kinds of treatment. It would be useful to have the same data on TTP protein in order to gain a better insight into in which tissues and procedures TTP may play a role.

The effect of several reagents has been tested on TTP mRNA and protein expression. Reagents that have been shown to have an effect on TTP expression are listed in Table 10. Several of the tested compounds did not influence TTP mRNA expression. Such compounds include purine analogues (Batistatou et al. 1992), several short-chain fatty acids (Barnard and Warwick 1993, Maclean et al. 1998, Fukae et al. 2005), calcium channel agonists (Arenander et al. 1989b), benzodiazepine without other stimulus (Arenander et al. 1989c), cholera toxin (Nakajima and Wall 1991, Yin and Yang 1993), cyclooxygenase inhibitor indomethacin and lipoxygenase inhibitor nordihydroquiaretic acid (Ledwith et al. 1996), taxol (Gubits et al. 1993b), and veratridine (Arenander et al. 1989b).

Elevated levels of cations, such as $Ag^+$, $Cd^{2+}$, $Cu^{2+}$, $K^+$, and $Zn^{2+}$, are capable of increasing TTP mRNA levels (Kujubu et al. 1987, DuBois et al. 1990, Taylor and Blackshear 1995, Muramatsu et al. 2006), whereas zinc depletion can down-regulate TTP mRNA expression (Cousins et al. 2003). The cations are most

likely coordinated to the zinc fingers and are required for the proper function of the zinc fingers.

In conclusion, a diverse group of compounds increase TTP mRNA levels but not many have been shown to increase TTP protein levels. There is still much research to be done to determine whether these substances have physiological effects, i.e. can they increase TTP protein levels or affect the binding and functional activity of TTP.

**Table 10.** *Examples of other reagents investigated in relation to TTP expression. ↑ Upregulation.*

| Compound | Cell type used | Effects on TTP expression | Reference |
|---|---|---|---|
| **Benzodiazepine Ro-3351** (with NGF) | Rat PC12 cells[a] | mRNA ↑ | Kujubu et al. 1987 |
| **Benzodiazepine Ro5-4864** (with TPA) | Rat secondary astrocytes | mRNA ↑ | Arenander et al. 1989c |
| **Calyculin A** (with LPS) | Mouse RAW264.7 macrophages | mRNA, protein ↑ | Brook et al. 2006 |
| **Capsaicin** | Male Sprague-Dawley rat brains | mRNA ↑ | Honkaniemi et al. 1994 |
| **Cinnamon extract and polyphenols** | Mouse 3T3-L1 adipocytes | mRNA, protein ↑ | Cao et al. 2007b |
| **Concanavalin A (lectin)** | Human mononuclear cells | mRNA ↑ | Heximer et al. 1998 |
| **Green tea** | Rat liver and muscle | mRNA, protein ↑ | Cao et al. 2007a |
| **Ricin** (lethal dose) | BALB/c mice | mRNA ↑ | DaSilva et al. 2003 |
| **Simvastatin** | Human monocytes | mRNA, protein ↑ | Patino et al. 2006 |

[a] *pheochromocytoma cell line*

# TTP in inflammation

Several mammalian mRNAs are destabilized by TTP and thereby their expression in the cells becomes decreased. The strongest evidence is available for some mouse target genes, which have been confirmed in cells derived from WT and KO mice. The evidence of TTP involvement in some cases relies only on actinomycin D or other mRNA stability studies of synthetic ARE probes, when TTP is over- or underexpressed, with or without evidence of TTP binding, or on the fact that in the absence of TTP or in the presence of overexpressed TTP, the expression patterns of certain genes have been altered. Mouse mRNAs proposed to be stabilized by TTP are listed in Table 11.

The best characterized target mRNA is TNF-α, which is a physiological target of TTP in intact animals and has been extensively studied. In a large microarray analysis on fibroblasts from TTP KO and WT mice, novel TTP targets were discovered, of which Ier3 was further characterized (Lai et al. 2006). In a recent study, Stoecklin et al. (2008) used a microarray to identify mRNAs to which TTP possibly binds with the binding of TTP to a smaller set of mRNAs being confirmed by Northern blot experiments.

Other mRNAs suggested to be destabilized by TTP are listed in Table 12. These include many human as well as rat mRNAs, the altered decay of which has not been confirmed in KO organisms. mRNA species that have been shown to be destabilized by TTP in both mouse and human cells include IL-2, TNF-α, and TTP itself.

There may be cell type-specific differences in the regulation of mRNA stability since it involves such a complex network of several proteins operating through AREs. In HeLa cells, a reporter gene with a part of COX-2 ARE was destabilized although it was not possible to detect TTP expression in HeLa cells, indicating that factors other than TTP were capable of destabilizing COX-2 mRNA in that cell line (Sully et al. 2004). Although TTP has been shown to destabilize mRNAs in overexpression studies, this is not definitive evidence that TTP is the sole and indispensable regulator of mRNA stability of these genes. This was the case of suppressor of cytokine signalling 3 (SOCS3), i.e. the SOCS3 mRNA stability was affected in cell transfection assays but unaffected in cells derived from TTP KO mice compared to cells derived from WT mice (Ehlting et al. 2007). TTP has also been shown to stabilize iNOS mRNA possibly through an interaction and inhibition of another ARE-binding protein, KSRP (Fechir et al. 2005, Linker et al. 2005).

There are also reports, where TTP binding to an ARE has been examined, but the effect on mRNA stability was not studied or confirmed. For example, in some studies it has been shown that TTP can bind to AREs of c-*fos*, IL-3, TNF-α, and GM-CSF, with much weaker affinity for the AREs of c-*jun* and c-*myc* but the effect on mRNA stability was not studied (Mahtani et al. 2001, Raghavan et al. 2001). In a study comparing TTP WT and KO cells, it was concluded that the stability of c-*fos* was not altered in the absence of TTP (Lai et

**Table 11.** *Mouse mRNAs suggested to be destabilized by TTP. Target mRNAs that have been shown to be bound by TTP have been specified in the mRNA binding column by +. If the stability of the target mRNA has been studied on WT and TTP KO mice, the result is described by + (significant difference between WT and TTP KO cells in the expression of the target gene), ++ (decay rates of the target mRNAs affected), or —(no difference between WT and TTP KO cells).*

| Target | mRNA binding | WT vs. KO | Reference |
|---|---|---|---|
| Bdp1 | | ++ | Lai et al. 2006 |
| Ccl2 | | + | Sauer et al. 2006 |
| Ccl3 | | + | Sauer et al. 2006 |
| CXCL1 (KC) | + | ++ | Datta et al. 2008 |
| E47 | + | | Frasca et al. 2007 |
| GM-CSF | | ++ | Carballo et al. 2000, Stoecklin et al. 2001 |
| Ier3 | + | ++ | Lai et al. 2006 |
| IL-2 | + | + | Stoecklin et al. 2001, Ogilvie et al. 2005 |
| IL-3 | + | | Ming et al. 2001, Stoecklin et al. 2001, toecklin et al. 2003 |
| IL-6 | | + | Stoecklin et al. 2001, Sauer et al. 2006 |
| IL-10 | + | + | Stoecklin et al. 2008 |
| IL-13 | | | Barnstein et al. 2006 |
| IL-15 | + | — | Stoecklin et al. 2008 |
| LIF | + | ++ | Lai et al. 2006, Stoecklin et al. 2008 |
| Mllt11 | | ++ | Lai et al. 2006 |
| Pim3 | | ++ | Lai et al. 2006 |
| Plk2 | | ++ | Lai et al. 2006 |
| Plk3 | | ++ | Lai et al. 2006 |
| Rusc2 | | ++ | Lai et al. 2006 |
| SOCS3 | | — | Ehlting et al. 2007 |
| TGF-β | | | Chang et al. 2007 |
| TNF-α | + | + | Carballo et al. 1998, Lai et al. 2000, Stoecklin et al. 2001, Chen et al. 2006 |
| TTP | + | — | Brooks et al. 2004, Tchen et al. 2004, Lai et al. 2006, Lin et al. 2007 |

*Bdp1, B double-prime 1; Mllt11, myeloid/lymphoid or mixed-lineage leukaemia (trirhorax homolog, Drosophila, translocated to 11); Pim3, proviral integration site 3; Plk, polo-like kinase; Rusc2, RUN- and SH3 domain-containing 2.*

**Table 12.** Other *mRNAs suggested to be destabilized by TTP. Target mRNAs that have been shown to be bound by TTP have been specified in the mRNA binding column by +.*

| Target | mRNA binding | Reference |
|---|---|---|
| hCOX-2 | + | Sawaoka et al. 2003 |
| Cyclin D1 | + | Briata et al. 2003, Marderosian et al. 2006 |
| hFOS | + | Patino et al. 2006, Amit et al. 2007 |
| hIL-2 | + | Ogilvie et al. 2005 |
| hIL-8 | + | Suswam et al. 2008 |
| c-Jun | | Briata et al. 2003 |
| c-*myc* | + | Marderosian et al. 2006 |
| hPAI-2 | | Yu et al. 2003 |
| Pitx2 | + | Briata et al. 2003 |
| hTNF-α | + | Lai et al. 2000, Brooks et al. 2002, Brooks et al. 2004 |
| hTTP | + | Brooks et al. 2004 |
| hVEGF | + | Essafi-Benkhadir et al. 2007, Suswam et al. 2008 |
| rVEGF | | Ciais et al. 2004 |

*h, human; PAI-2, plasminogen activator inhibitor type 2; Pitx2, paired-like homeodomain transcription factor 2; r, rat; VEGF, vascular endothelial growth factor.*

al. 2006) The involvement of TTP in the regulation of IL-3 expression was first described in a mutant cell line, where the IL-3 mRNA decay pathway was defective but expression of exogenous TTP could revert the phenotype (Stoecklin et al. 2000). In this cell line it was then established that also AREs of other mRNAs were sensitive to expression of TTP (Stoecklin et al. 2001). Later it was claimed that the mutated protein in this cell line was the butyrate response factor (BRF1, ZFP36L1), a TTP family member (Stoecklin et al. 2002). ZFP36L1 and ZFP36L2 were also described as possible targets of TTP in a large microarray study (Stoecklin et al. 2008). This may represent a negative feedback loop to down-regulate the expression of these RNA-binding proteins.

TTP expression may be implicated in many pathological conditions e.g. rheumatoid arthritis (Taylor et al. 1996, Phillips et al. 2004, Tsutsumi et al. 2004, Fabris et al. 2005, Suzuki et al. 2006b, Sugihara et al. 2007), obesity-related metabolic complications (Bouchard et al. 2007a), glycemic control, insulin resistance, and diabetes (Bouchard et al. 2007b), allergic diseases or parasitic infections (Suzuki et al. 2003), celiac disease (Benahmed et al. 2007), cardiac dysfunction (Hikoso et al. 2004), and cancers, involving IL-3 (Stoecklin et al. 2003) and COX-2 (Boutaud et al. 2003, Dixon 2003). Therefore, more research into the regulation of TTP expression and function is needed to gain more

knowledge on how to use TTP-related therapies to treat these conditions in the future.

TTP may also be involved in the outcome of viral infections. TTP expression and cytoplasmic localization were induced in studies where cells were infected with herpes simplex virus 1, (Esclatine et al. 2004a, Esclatine et al. 2004b, Taddeo et al. 2004). One report claimed that, TTP accumulation in the cells was dependent on an interaction with virion host shutoff (Vhs) protein, which mediates the degradation of both viral and cellular RNAs (Esclatine et al. 2004b). The Vhs protein also affects the stability of TTP mRNA, with TTP being more stable in WT virus-infected cells than in mutant Vhs-infected cells (Esclatine et al. 2004c). The Vhs protein appeared to be recruited to intracellular granules but it is not known whether these structures are SGs or other granules, where TTP is also colocalized (Taddeo et al. 2006).

The interaction of TTP with cellular Tax oncoproteins, transcriptional regulators of viral expression, through their C-terminal regions has been reported to inhibit TTP-induced TNF-$\alpha$ destabilization leading to increased TNF-$\alpha$ levels, which may increase the pathogenic capabilities of leukomogenic retroviruses (Twizere et al. 2003).

TTP also binds to and enhances the degradation of human immunodeficiency virus type 1 (HIV-1) but does not seem to affect the stability of HIV-1 RNA. In one experiment, cells were transfected with siRNA against TTP, and this elevated HIV-1 production, thereby suggesting a role for TTP in the regulation of HIV progression (Maeda et al. 2006).

TTP has also been proposed to have a role in transcriptional activation. TTP was first described as a transcription factor due to the presence of the zinc finger structures and its nuclear localization (DuBois et al. 1990, Taylor et al. 1995). There is evidence that TTP possesses a transcriptional activation capability (Murata et al. 2000) although no functional binding sites on DNA for TTP have been identified. In *in vitro* studies, TTP was found to bind a UUUAUUU RNA probe tighter than the corresponding DNA probe (Michel et al. 2003). The ability to activate transcription has also been shown for yeast TIS11 (Ma and Herschman 1995). Due to the lack of evidence about potential DNA targets, it is not possible to state conclusively whether or not TTP is a transcription factor.

Continuous expression of TTP induces apoptosis, and TTP, but not its family members ZFP36L1 and ZFP36L2, sensitises several cell types to undergo programmed cell death triggered by TNF-$\alpha$ (Johnson et al. 2000, Suzuki et al. 2006a). On the other hand, ZFP36L1 and ZFP36L2 induce apoptosis independently of TNF-$\alpha$, and all the family members make use of the mitochondrial pathway (Johnson and Blackwell 2002). The zinc finger domains alone are not sufficient enough to induce apoptosis, both N- and C-terminal domains are required for full pro-apoptotic effects (Johnson and Blackwell 2002). Apoptosis does not seem to involve sequestering of anti-apoptotic 14-3-3 proteins to phosphorylated TTP, but to promote TTP localization to the cytosol (Johnson et al. 2002). It still remains unclear whether this phenomenon is related to overexpression of TTP or whether it has any a physiological relevance.

*Target sequence of TTP*

There are several kinds of AREs, which can be divided into three categories (Chen and Shyu 1995, Peng et al. 1996). One set of AREs contains one to three scattered copies of AUUUA in a U-rich region. The second set contains overlapping copies of a nonamer UUAUUUA(U/A)U/A) in a U-rich region. The last set lacks the AUUUA sequences and is described only as a U-rich region. The overall minimal *cis*-acting ARE sequence that mediates mRNA instability has been suggested to be a nonamer UUAUUUAUU (Zubiaga et al. 1995). This nonamer is also believed to be the preferred sequence for binding of TTP (Worthington et al. 2002, Blackshear et al. 2003). On the other hand, poly(U) sequences have not been shown to serve as a binding site for TTP (Michel et al. 2003, Park-Lee et al. 2003), and substitutions of the As or Us of the nonamer to Cs or Gs were reported to reduce the binding affinity of TTP towards the probe (Worthington et al. 2002, Michel et al. 2003, Lai et al. 2005). The 73-amino acid zinc finger domain of human TTP required only about 9 bases on a TNF-α ARE probe to bind and two zinc finger domains have been claimed to bind to a probe as short as 19 nucleotides (Blackshear et al. 2003). The same 73-amino acid domain also demonstrated high affinity towards sequences, where two As were separated by 3-6 U-rich stretches, such as AUUUA and AUUUUA (Brewer et al. 2004). Binding of the N-terminal zinc-finger domain has induced conformational changes in both of the zinc fingers and this increased binding to the probe (Brewer et al. 2006). It has been reported that one nonamer is sufficient to induce at least a partial increase in mRNA turnover, but two binding sites increase mRNA turnover markedly (Lai et al. 2005). Due to the differences in ARE sequences, TTP can bind TNF-α ARE with higher affinity than IL-1β ARE, which may be one reason why IL-1β mRNA is more stable than TNF-α mRNA (Chen et al. 2006). A recent microarray study provided further evidence that TTP prefers binding to mRNAs with AUUUA pentamers and especially UUAUUUAUU nonamers (Stoecklin et al. 2008).

The affinity of TTP towards TNF-α ARE depends not just on sequence but also on TTP protein levels and possibly the phosphorylation status of TTP. It seems that relatively low levels of TTP protein have a greater destabilizing effect on TNF-α mRNA (Lai et al. 1999, Chen et al. 2006) because an excess of TTP may prevent other factors necessary for degradation from gaining access to the mRNA. The impact of phosphorylation status of TTP was discussed earlier in the context of MAPK in the regulation of TTP expression. There also seems to be competition between different ARE-binding proteins and TTP for the same sequence (Ming et al. 2001, Raghavan et al. 2001) and better understanding of the conditions where TTP binds to and destabilizes mRNAs in humans may provide important information for drug design purposes. The ARE-binding proteins represent one part of a network of stabilizing and destabilizing effects but a greater understanding of the mechanisms that regulate mRNA stability is needed before these mechanisms can be exploited in drug design.

*Mechanisms of action of TTP*

The function of TTP has been studied mostly on TNF-α ARE with mutated probes and mutated TTP protein. After the initial finding that TTP could destabilize TNF-α mRNA (Carballo et al. 1998) it was discovered that TTP could bind to TNF-α mRNA and thereafter promote deadenylation and degradation of TNF-α mRNA (Lai et al. 1999) by stimulating poly(A) ribonuclease (PARN) (Lai et al. 2003). Other zinc finger protein family members have similar actions on TNF-α ARE probe (Lai et al. 2000, Lai et al. 2003). The poly(A) tail is not required for TNF-α destabilization, indicating that ARE is sufficient to promote destabilization, even in the presence of a distinct 3'-end-processing sequence such as a histone stem loop structure (Lai and Blackshear 2001). The zinc finger domains of TTP are not enough to promote TNF-α ARE mediated degradation. Additional sequences in the N- and C-terminal regions are required for full activity of TTP (Rigby et al. 2005). In addition, mutations in the zinc finger domains or in the ARE sequence have been reported to prevent transcript deadenylation (Lai et al. 2003).

Non-binding zinc finger domain mutants of TTP inhibited ARE mRNA degradation and increased mRNA stability (Lai et al. 2002). This may be due to an interaction with proteins that are needed in the degradation of mRNA. In the presence of excess non-binding mutants, these factors bind to the free mutants and therefore they are not available to the ARE to promote degradation. TTP recruits several components of mRNA decay pathways to these ARE sequences. These enzymes include the components required for deadenylation, decapping, as well as 3'-to-5' and 5'-to-3' exonucleolytic activity (Fenger-Grøn et al. 2005, Lykke-Andersen and Wagner 2005, Hau et al. 2007, Stoecklin and Anderson 2007).

The processes, which occur after the binding of TTP to an ARE, are still not fully understood. Different RNA granules seem to be involved, though the roles of SGs and PBs are now well established (Kedersha and Anderson 2002, Anderson and Kedersha 2006, Stoecklin and Anderson 2007). Both of these granules are induced by stress, interact with each other and both contain TTP (Kedersha et al. 2005, Kedersha and Anderson 2007). The entry of TTP into the stress granules can be inhibited by phosphorylation of TTP by MK2 and the subsequent interaction with 14-3-3 proteins (Stoecklin et al. 2004). Either the interaction with 14-3-3 proteins does not occur on polysome-bound TTP, or else any TTP bound by 14-3-3 proteins is quickly removed from the polysomes (Rigby et al. 2005). It has been postulated that TTP promotes the interactions of SGs with PBs and may direct mRNAs destined for decay from SGs to be degraded in the PBs (Kedersha et al. 2005).

A model for the inflammatory process involving MAPK, TTP, TNF-α, and 14-3-3 is described in Figure 5. In resting cells, the expression of TNF-α is low, but in response to inflammatory stimuli, the MAPK cascade becomes activated, leading to inactivation of TTP, stabilization of TNF-α mRNA and increased TNF-α levels. In normal situations, MAPK activity is down-regulated and the activity of TTP is restored and the expression of inflammatory cytokines is

down-regulated. When this control is dysfunctional, for example in TTP KO mice, high levels of stabilized TNF-α mRNA remain and induce the inflammatory symptoms encountered in TTP KO mice. It is important to regulate the expression of TTP tightly to avoid problems that may arise if TTP is overexpressed or underexpressed but still to allow an adequate response to pathogens and injuries. Understanding the regulation of TTP expression in detail may provide an opportunity to apply this knowledge in the design of novel treatments for inflammatory diseases, in which there is TTP involvement.
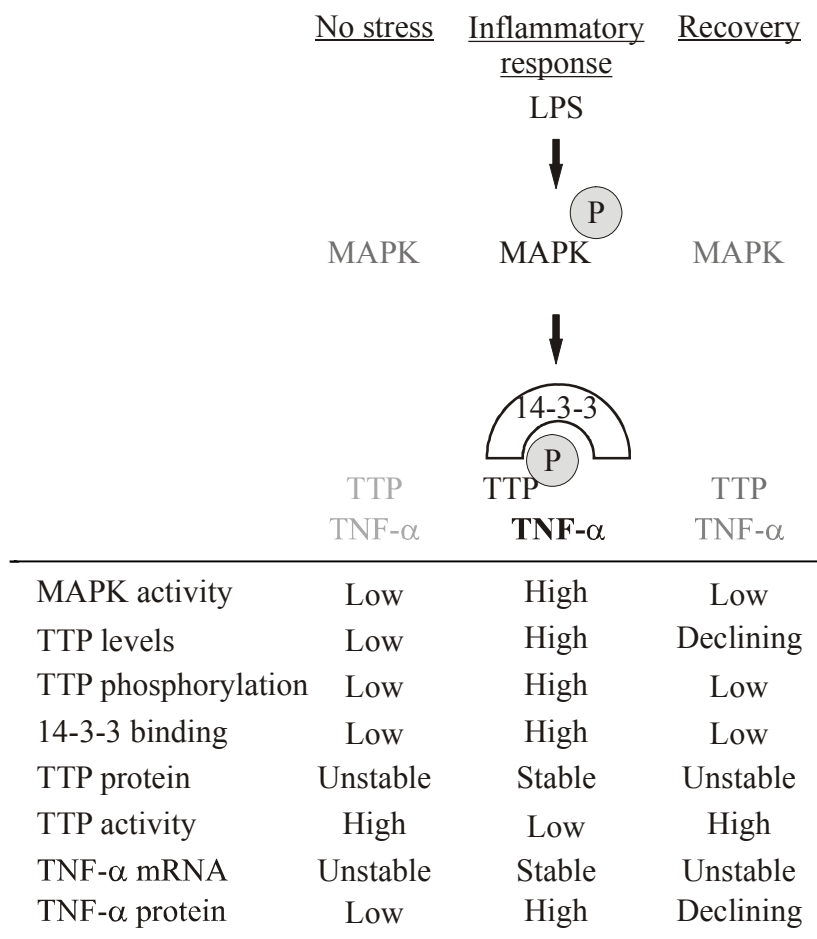
|  | No stress | Inflammatory response | Recovery |
|---|---|---|---|
| MAPK activity | Low | High | Low |
| TTP levels | Low | High | Declining |
| TTP phosphorylation | Low | High | Low |
| 14-3-3 binding | Low | High | Low |
| TTP protein | Unstable | Stable | Unstable |
| TTP activity | High | Low | High |
| TNF-α mRNA | Unstable | Stable | Unstable |
| TNF-α protein | Low | High | Declining |

**Figure 5.** *A simplified model for the inflammatory process involving MAPK, TTP, TNF-α, and 14-3-3 proteins. In resting cells, MAPK expression and activation is low. TTP expression is low as well, but unphosphorylated TTP is highly active in destabilizing TNF-α mRNA. In response to inflammatory stimuli, the MAPK cascade is activated by phosphorylation. Phosphorylation of TTP induces 14-3-3 binding and low activity of TTP. High levels of TNF-α can be detected. During the resolution of inflammation, MAPK activity becomes reduced, and TTP is dephosphorylated, restoring the mRNA destabilizing activity. Both the level of TTP protein and TNF-α mRNA begin to decline. Modified from Brook et al. (2006) and Hitti et al. (2006).*

# Aims of the study

TTP is an anti-inflammatory factor that has been shown to bind to the AU-rich elements in the 3'-UTR of TNF-α and some other inflammatory genes. TTP binding results in the degradation of the target mRNAs, thereby reducing their expression. Down-regulation of inflammatory genes at the post-transcriptional level, e.g. by mechanisms and factors related to the regulation of mRNA stability, is an important therapeutic target. Therefore it is important to gain knowledge on the mechanisms, which regulate these factors, especially TTP.

The aim of the present study was to investigate the regulation of TTP expression and the effects of TTP knockdown in macrophages.

The detailed aims were:

1. to study the role of major signalling pathways in the regulation of TTP expression in macrophages by pharmacological means (I, II, III).

2. to study the regulation of TTP mRNA and protein expression by dexamethasone in activated macrophages (I).

3. to study the regulation of TTP mRNA and protein expression by cAMP elevating agents in resting cells (II) and in cells stimulated with LPS (III).

4. to study the effects of TTP knockdown on cytokine expression by using a cytokine antibody array (IV).

# Materials and methods

## Cell culture (I-IV)

The murine J774A.1 macrophages (I) (American Type Culture Collection, Manassas, VA, USA) and murine J774.2 macrophages (II-IV) (European Collection of Cell Cultures, Porton Down, Wiltshire, UK) were maintained in an atmosphere of 5% carbon dioxide at 37°C in Dulbecco's modified Eagle's medium with UltraGlutamine 1 (DMEM/U1, Lonza Verviers SPRL, Verviers, Belgium, formerly known as Cambrex and BioWhittaker) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Lonza Verviers SPRL), penicillin (100 units/ml), streptomycin (100 µg/ml), and amphotericin B (250 ng/ml) (Invitrogen, Paisley, UK). J774A.1 macrophages were harvested with trypsin-EDTA (Invitrogen). Cells were seeded on 6- or 24-well plates or 10 cm dishes and grown to confluence prior to the experiments.

The human THP-1 promonocytes (American Type Culture Collection) were cultured at 37°C in a humidified 5% carbon dioxide atmosphere in RPMI 1640 medium (Lonza Verviers SPRL) adjusted to contain 2 mM L-glutamine, 10 mM HEPES (Lonza Verviers SPRL), 1 mM sodium pyruvate (Lonza Verviers SPRL), 4.5 g/l glucose, and 1.5 g/l bicarbonate (Lonza Verviers SPRL) and supplemented with 10% heat-inactivated fetal bovine serum (Lonza Verviers SPRL), penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml) (Invitrogen), and 0.05 mM 2-mercaptoethanol. The cells were differentiated by adding the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA, 100 nM) 72 h before the experiments at the time of seeding of the cells on 6- or 24-well plates (Tsuchiya et al. 1982).

## Cell viability test (III)

Cell viability was tested using the Cell Proliferation Kit II (Roche Diagnostics, Mannheim, Germany), which measures the amount of an orange formazan dye produced from sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) by metabolically active cells in a cleavage reaction. Cells were incubated with LPS or LPS in combination with the tested compounds for 12 h before addition of XTT labelling reagent (final concentration 0.3 mg/ml) and N-methyl dibenzopyrazine methyl sulfate (2.5 µg/ml). Then cells were incubated for 3 h and the amount of

formazan accumulated in the growth medium was assessed spectrophotometrically. No difference in cell viability between treated and untreated cells was detected. Triton-X-treated cells were used as a positive control, resulting in 97% reduction in cell viability.

# RNA extraction and quantitative real-time reverse transcriptase polymerase chain reaction (PCR) (I-III)

Macrophages were washed with PBS and homogenized using QIAshredder (QIAGEN Inc., Valencia, CA, USA), after which total RNA was extracted with an RNeasy kit for isolation of total RNA (QIAGEN Inc.). The amount of RNA was measured with spectrophotometer and purity was confirmed by the absorbance ratio at $A_{260/280}$.

Reverse transcription to cDNA was carried out with TaqMan Reverse Transcription Reagents and random hexamers (Applied Biosystems, Foster City, CA, USA) in 10 µl reaction volume, containing 25 ng (I) or 100 ng (II-III) of purified total RNA with the following parameters: incubation at 25°C for 10 min, reverse transcription at 48°C for 30 min and inactivation of reverse transcriptase at 95°C for 5 min.

Total RNA transcribed to cDNA (1.25 ng in I, and 2.5 ng in II-III) was used in PCR, by applying primers and probes, TaqMan Universal PCR Master Mix and ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The rodent glyceraldehyde-3-phosphate dehydrogenase (rGAPDH) primers and probe were from Applied Biosystems. The rGAPDH probe contained VIC (proprietary dye, Applied Biosystems) as the 5'-reporter dye and TAMRA (6-carboxy-tetramethyl-rhodamine) as the 3'-quencher. The sequences of other primers and probes used in the present study are described in Table 13. The primers and probes were designed using Primer Express Software, version 2.0.0 (Applied Biosystems) to meet the following guidelines for primers: GC-content in the range of 20-80%, less than 5 identical nucleotides in a row, less than two G and/or C bases among the last five nucleotides at the 3'-end, no G at the 3'-end, length around 20 bases, $T_m$ of each primer 58 to 60 °C, and the $T_m$ difference between primers less than 0.5 °C; and for probes: GC-content in the range of 20-80%, less than 5 identical nucleotides in a row, no G at the 5'-end, more Cs than Gs, length less than 30 bases, $T_m$ of 68 to 70 °C. The amplicon length was kept in the range from 50 to 150 base pairs. The software also checked that the primers and probes should not base pair strongly in any combinations; especially that there should be no dimerization involving 3'-ends. A BLAST search was done to ascertain that primers and probes do not amplify any other sequences. The probes contained 6-FAM (6-carboxy-fluoroscein) as the 5'-reporter dye and TAMRA as the 3'-quencher (Metabion, Martinsried, Germany). The concentrations were optimized according to the manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C and are given in Table 13. The thermal cycling conditions were: AmpErase

uracil-N-glycosylase incubation at 50°C for 2 min, AmpliTaq Gold activation at 95°C for 10 min, thereafter 40 cycles of denaturation at 95°C for 15 sec and annealing/extension in 60°C for 1 min. Each sample was determined in duplicate.

The relative mRNA levels were quantified and compared using the relative standard curve method as described in Applied Biosystems User Bulletin #2. Total RNA was isolated from LPS-stimulated macrophages and reverse transcribed. Standard curves for all genes used in the present study were created using a dilution series of cDNA corresponding to approximately 1 pg - 50 ng of total RNA in PCR as described above. The gained threshold cycle values were plotted against the dilution factor to create a standard curve. Relative mRNA levels in test samples were then calculated using the standard curve. The relative amount of gene transcript present in samples was calculated and normalized by dividing the calculated value of TTP by the GAPDH value in each sample.

## Preparation of cell lysates for Western blot (I-IV)

After the indicated times of incubation, the cells were rapidly washed with ice cold phosphate-buffered saline (PBS) and lysed in ice-cold extraction buffer (10 mM Tris-base, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM $Na_3VO_4$, 20 µg/ml leupeptin, 50 µg/ml aprotinin, 5 mM NaF, 2 mM sodium pyrophosphate, 10 µM $n$-octyl-β-D-glucopyranoside). When preparing cell lysates for ubiquitin Western blotting (III), lysis buffer contained also ubiquitin aldehyde (20 µg/ml) and $N$-[(phenylmethoxy)carbonyl] - L- leucyl - $N$-[(1$S$) - 1- formyl - 3-methylbutyl]-L-leucinamide (MG132, 25 µM) to prevent deubiquitination of the sample. After 15 min incubation on ice and centrifugation (13400 $g$, 4°C, 10 min), the supernatant was collected and stored in sample buffer [62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulphate (SDS), 0.025% bromophenol blue, 5% β-mercaptoethanol] at -20°C. Samples were boiled for 5 min prior to Western blotting. An aliquot of the supernatant was used to determine the protein concentration by the Coomassie blue method (Bradford 1976).

**Table 13.** *Sequences of primers and probes used in quantitative real-time reverse transcriptase PCR.*

| | Sequence | Conc. | Study |
|---|---|---|---|
| **mTTP** | | | |
| forward | 5'-CTCAGAAAGCGGGCGTTGT-3' | 300 nM | I-III |
| reverse | 5'-GATTGGCTTGGCGAAGTTCA-3' | 300 nM | I-III |
| probe | 5'-FAM-CCAAGTGCCAGTTTGCTCACGGC-TAMRA-3' | 200 nM | I-III |
| | | | |
| **hTTP** | | | |
| forward | 5'-CCCCAAATACAAGACGGAACTC-3' | 300 nM | II |
| reverse | 5'-GGGCCGCCAGGTCTTC-3' | 300 nM | II |
| probe | 5'-FAM-CCCTACGGCTCTCGCTGCCACTT-TAMRA-3' | 200 nM | II |
| | | | |
| **mTNF-α** | | | |
| forward | 5'-AATGGCCTCCCTCTCATCAGTT-3' | 300 nM | III |
| reverse | 5'-TCCTCCACTTGGTGGTTTGC-3' | 300 nM | III |
| probe | 5'-FAM-CTCAAAATTCGAGTGACAAGCCTGTAGCCC-TAMRA-3' | 150 nM | III |
| | | | |
| **mGAPDH** | | | |
| forward | 5'-GCATGGCCTTCCGTGTTC-3' | 300 nM | II-III |
| reverse | 5'-GATGTCATCATACTTGGCAGGTTT-3' | 300 nM | II-III |
| probe | 5'-FAM-TCGTGGATCTGACGTGCCGCC-TAMRA-3' | 150 nM | II-III |
| | | | |
| **hGAPDH** | | | |
| forward | 5'-AAGGTCGGAGTCAACGGATTT-3' | 300 nM | II |
| reverse | 5'-GCAACAATATCCACTTTACCAGAGTTAA-3' | 300 nM | II |
| probe | 5'-FAM-CGCCTGGTCACCAGGGCTGC-TAMRA-3' | 150 nM | II |
| | | | |
| **rGAPDH** | | | |
| forward | Unknown (Applied Biosystems) | 300 nM | I |
| reverse | Unknown (Applied Biosystems) | 300 nM | I |
| probe | Unknown VIC-TAMRA (Applied Biosystems) | 50 nM | I |

## Preparation of nuclear extracts for Western blot of transcription factors activator protein 2 (AP-2) and nuclear factor κB (NF-κB) (II)

At the indicated time points, the cells were rapidly washed with ice-cold PBS and solubilized in hypotonic buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM $Na_3VO_4$, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1 mM NaF and 0.1 mM EGTA). After incubation for 10 min on ice, the cells were vortexed for 30 s and the nuclei were separated by centrifugation (21000 $g$, 4°C, 10 s). The nuclei were resuspended in buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM $Na_3VO_4$, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1 mM NaF and 0.1 mM EGTA) and incubated for 20 min on ice. The nuclei were vortexed for 30 s and nuclear extracts were obtained by centrifugation (21000 $g$, 4°C, 2 min). The protein contents of the nuclear extracts were measured by the Coomassie blue method (Bradford 1976), and samples in SDS sample buffer were boiled for 5 min prior to Western blotting.

## Western blot analysis (I-IV)

An equal amount of protein was loaded on each well on 12% SDS-polyacrylamide gels transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences UK Limited, Buckinghamshire, UK) after electrophoresis. After transfer, the membrane was blocked with TBS/T [20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20] containing 5% bovine serum albumin overnight at +4°C (I) or 5% milk powder for 1 h RT (II-IV). Thereafter the membrane was incubated with the primary antibody (Table 14) in the blocking buffer for 1 h at RT (I) or overnight at 4°C (II-IV). After the membrane was washed and incubated with the appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer for 30 min in RT, the bound antibody was detected using SuperSignal West Pico, Dura, or Femto chemiluminescent substrate for HRP detection (Pierce Chemical, Cramlington, Northumberland, UK) and FluorChem 8800 imaging system (Alpha Innotech, San Leandro, CA, USA). The chemiluminescent signal was measured with FluorChem software v. 3.1.

**Table 14.** *Antibodies used in Western blotting.*

| | | |
|---|---|---|
| Actin | Santa Cruz Biotechnology, Santa Cruz, CA, USA | II-IV |
| AP-2 | Santa Cruz Biotechnology, Santa Cruz, CA, USA | II |
| Lamin | Santa Cruz Biotechnology, Santa Cruz, CA, USA | II |
| NF-κB p65 | Cell Signaling Technology Inc., Danvers, MA, USA | II |
| TTP | Santa Cruz Biotechnology, Santa Cruz, CA, USA | I |
| TTP | A kind gift from Dr. Perry Blackshear, NIEHS, Research Triangle Park, NC, USA (Cao et al. 2004) | II-IV |
| Ubiquitin | Zymed Laboratories, South San Francisco, CA, USA | III |

# Cell lines expressing short hairpin RNAs (shRNA) against TTP or against a negative control sequence (IV)

Ambion's siRNA Target Finder was used to detect suitable 21 nucleotide sequences that begin with the AA dinucleotide, do not contain runs of 4 or more identical nucleotides, and do not detect other sequences (confirmed by BLAST search). Five sequences were selected and the Insert Design Tool for the p*Silencer* Vectors was used to design primers containing the selected sequences to be inserted into the p*Silencer* neo vector (Ambion Inc., Austin, TX, USA). The nucleotides (Metabion, Martinsried, Germany) were annealed and ligated into the p*Silencer* neo vector with T4 DNA ligase (Fermentas Inc., Burlington, Ontario, Canada). One Shot TOP10 Competent Cells (Invitrogen, Carlsbad, CA, USA) were chemically transformed according to the manufacturer's instructions. Plasmids were isolated with Plasmid Mini kit (QIAGEN Inc., Valencia, CA, USA) and the inserts were sequenced in the Department of Biology, University of Turku. The work was continued with the three sequences that were inserted correctly. The purified vectors and a negative control vector were transfected with FuGENE 6 Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, IN, USA) into J774.2 macrophages (European Collection of Cell Cultures, Porton Down, Wiltshire, UK). G418 disulfide salt was used to select and maintain the cell lines. All of the cell lines containing shRNA against TTP initially grew more slowly than the negative control cell line and only one cell line expressing shRNA against TTP (shTTP) showed decreased levels of TTP as compared to the negative control shRNA (shNEG) cell line (Table 15).

For the cytokine antibody array, shTTP and shNEG cells were plated on 6-well plates 24 hours prior the experiment. Cells were first incubated in DMEM/U1+FBS with or without LPS (100 ng/ml). After a 1 h incubation, the DMEM/U1 medium without FBS was changed to the wells and incubation was continued for 48 h. Thereafter, cell culture media were collected and stored at -20°C until assayed.

**Table 15.** *Target sequences and primers for antisense and sense siRNA oligonucleotide templates of shTTP and shNEG cell lines.*

---

**shTTP target sequence** 5'-AACAUAAACUCGGACUCCAUC-3'

| | |
|---|---|
| shTTP sense | 5'-GATCCGCATAAACTCGGACTCCATCTTCAAGAGAGATGGAGTCCGAGTTTATGTTTTTTGGAAA-3' |
| shTTP antisense | 5'-AGCTTTTCCAAAAAACATAAACTCGGACTCCATCTCTCTTGAAGATGGAGTCCGAGTTTATGCG-3' |

**shNEG target sequence** 5'-AAACUACCGUUGUUAUAGGUG-3'

| | |
|---|---|
| shNEG sense | 5'-GATCCACTACCGTTGTTATAGGTGTTCAAGAGACACCTATAACAACGGTAGTTTTTTTGGAAA-3' |
| shNEG antisense | 5'-AGCTTTTCCAAAAAAACTACCGTTGTTATAGGTGTCTCTTGAACACCTATAACAACGGTAGTG-3' |

---

For Western blot, shTTP and shNEG cells were plated on 6-well plates and grown to confluence. Cells were treated with or without LPS (100 ng/ml) for 6 h and proteins were extracted as described above.

## Cytokine antibody array (IV)

Cytokine levels in cell culture media were determined with Mouse Cytokine Antibody Array III (RayBiotech Inc., Norcross, GA, USA), which measures 62 cytokines and other inflammatory modulators (Figure 6). The array membranes were blocked with 2 ml of 1X blocking buffer for 30 min and then incubated with the sample (1 ml) for 2 h at RT. The membranes were washed three times with 2 ml of 1X wash buffer I and twice with 2 ml of 1X wash buffer II at RT. The membranes were then incubated in diluted biotin-conjugated primary antibodies overnight at 4°C. The membranes were washed and incubated with diluted HRP-conjugated streptavidin for 2 h at RT and washed. Detection buffer C and detection buffer D were combined, and applied on the membranes for 2 min. Each membrane was exposed for 1 min and images were taken with the FluorChem 8800 imaging system (Alpha Innotech, San Leandro, CA, USA) and the chemiluminescent signals for each spot were measured with FluorChem software v. 3.1. The average chemiluminescence of each cytokine and control was calculated for all the treatments separately. The average of positive controls of each treatment was set to 100 and all cytokines of the same treatment were compared to that value.

## TNF-α enzyme-linked immunosorbent assay (ELISA) (IV)

TNF-α concentrations in culture media were determined by mouse TNF-α DuoSet ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. The detection limit was 7.8 pg/ml, and intra- and inter-assay coefficients of variation were 4.6% and 5.4 %, respectively.

## Statistics

Results are expressed as the mean ± standard error of mean (SEM). The significance of differences was calculated by analysis of variance supplemented with Dunnett multiple comparisons test. A difference between treatment groups was considered significant when $P<0.05$.

## Reagents

The compounds used in the present study are listed in Table 16. Apicidin was from Alexis Corporation, Lausen, Switzerland. Tyrphostin AG-490 [α-cyano-(3,4-dihydroxy)-*N*-benzylcinnamide], cyclosporin A, PD98059 [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one], SB202474 [4-ethyl-2-(4-methoxyphenyl)-5-(4-pyridyl)-1*H*-imidazole], SB203580 [4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)-1*H*- imidazole] (I), and SP600125 {anthra[1,9-*cd*]pyrazol-6(2*H*)-one} were from Calbiochem, San Diego, CA, USA. Dexamethasone was from Orion Corporation, Espoo, Finland. Forskolin, genistein, MG132 {*N*-[(phenylmethoxy)carbonyl]-L-leucyl-*N*-[(1*S*)-1-formyl-3-methylbutyl]-L-leucinamide}, and SB203580 (III) were from Tocris Cookson Inc., Ellisville, MO, USA. RU24858 was from Aventis Pharma, Romainville Cedex, France. Ubiquitin aldehyde was from Boston Biochem, Cambridge, MA, USA. All other reagents were from Sigma-Aldrich, St. Louis, MO, USA.

|    | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | POS | POS | POS | POS | Blank | Axl | BLC | CD30 L | CD30 T | CD40 | CRG-2 | CTACK | CXCL16 | Eotaxin |
| 2 | NEG | NEG | NEG | NEG | Blank | Axl | BLC | CD30 L | CD30 T | CD40 | CRG-2 | CTACK | CXCL16 | Eotaxin |
| 3 | Eotaxin-2 | Fas Ligand | Fractalkine | GCSF | GM-CSF | IFN-γ | IGFBP-3 | IGFBP-5 | IGFBP-6 | IL-1α | IL-1β | IL-2 | IL-3 | IL-3 Rβ |
| 4 | Eotaxin-2 | Fas Ligand | Fractalkine | GCSF | GM-CSF | IFN-γ | IGFBP-3 | IGFBP-5 | IGFBP-6 | IL-1α | IL-1β | IL-2 | IL-3 | IL-3 Rβ |
| 5 | IL-4 | IL-5 | IL-6 | IL-9 | IL-10 | IL-12 p40 | IL-12 | IL-13 | IL-17 | KC | Leptin R | Leptin | LIX | L-Selectin |
| 6 | IL-4 | IL-5 | IL-6 | IL-9 | IL-10 | IL-12 p40 | IL-12 | IL-13 | IL-17 | KC | Leptin R | Leptin | LIX | L-Selectin |
| 7 | Lymphotactin | MCP-1 | MCP-5 | M-CSF | MIG | MIP-1α | MIP-1γ | MIP-2 | MIP-3β | MIP-3α | PF-4 | P-Selectin | RANTES | SCF |
| 8 | Lymphotactin | MCP-1 | MCP-5 | M-CSF | MIG | MIP-1α | MIP-1γ | MIP-2 | MIP-3β | MIP-3α | PF-4 | P-Selectin | RANTES | SCF |
| 9 | SDF-1α | TARC | TCA-3 | TECK | TIMP-1 | TNF-α | sTNF RI | sTNF RII | TPO | VCAM-1 | VEGF | Blank | Blank | Blank |
| 10 | SDF-1α | TARC | TCA-3 | TECK | TIMP-1 | TNF-α | sTNF RI | sTNF RII | TPO | VCAM-1 | VEGF | Blank | POS | POS |

**Figure 6.** *A schematic diagram of the Mouse Cytokine Antibody Array III showing the locations of controls and duplicate spots of cytokines. Axl, a protein tyrosine kinase also called UFO or ark; BLC, B-lymphocyte chemoattractant; CD, cluster of differentiation; CRG, cytokine responsive gene; CTACK, cuteaneous T cell-attracting chemokine; CXCL, CXC chemokine ligand; GCSF, granulocyte colony-stimulating factor; IGFBP, insulin-like growth factor binding protein; KC, also known as CXCL1; L, ligand; LIX, LPS-induced C-X-C chemokine; MCP, monocyte chemoattractant protein; M-CSF, macrophage colony-stimulating factor; MIG, monokine induced by IFN-γ; MIP, macrophage inflammatory protein; NEG, negative control; PF, platelet factor; POS, positive control; R, receptor; RANTES, regulated upon activation, normal T cell expressed, and presumably secreted; s, soluble; SCF, stem cell factor; SDF, stromal cell-derived factor; TARC, thymus and activation-regulated chemokine; TCA, T cell activation; TECK, thymus-expressed chemokine; TIMP, tissue inhibitor of metalloproteinases; TPO, thrombopoietin; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.*

**Table 16.** *Compounds used in the present study.*

| Compound | |
|---|---|
| AG-490 | JAK2 inhibitor |
| Apicidin | Inhibitor of histone deacetylases |
| Aurothiomalate | Disease-modifying antirheumatic drug |
| 8-Br-cAMP | Cell permeable cAMP analogue |
| Ciglitazone | Peroxisome proliferator-activated receptor (PPAR)$\gamma$ agonist |
| Cyclosporin A | Calcineurin inhibitor |
| Db-cAMP | Cell permeable cAMP analogue |
| Dexamethasone | Glucocorticoid |
| Formoterol | $\beta_2$-agonist |
| Forskolin | Activator of adenylate cyclase |
| Genistein | Inhibitor of tyrosine phosphorylation |
| GW7647 | PPAR$\alpha$ agonist |
| Hydroxychloroquine | Disease-modifying antirheumatic drug |
| IBMX | Non-specific inhibitor of cyclic nucleotide phosphodiesterases |
| Ibuprofen | Non-steroidal anti-inflammatory drug |
| Lactacystin | Proteasome inhibitor |
| Leflunomide | Disease-modifying antirheumatic drug |
| Methotrexate | Disease-modifying antirheumatic drug |
| MG132 | Proteasome inhibitor |
| Paracetamol | Centrally-acting analgesic |
| PD98059 | ERK1/2 inhibitor |
| PDTC | NF-$\kappa$B inhibitor |
| D-Penicillamine | Disease-modifying antirheumatic drug |
| 15d-PGJ$_2$ | PPAR$\gamma$ agonist |
| Rofecoxib | Non-steroidal anti-inflammatory drug |
| Rolipram | Inhibitor of type IV phosphodiesterase |
| RU24858 | Dissociated glucocorticoid |
| Salbutamol | $\beta_2$-agonist |
| SB202474 | Negative control compound for SB203580 (i.e. a structurally related compound, which does not inhibit p38) |
| SB203580 | p38 inhibitor |
| SP600125 | JNK inhibitor |
| Sulfasalazine | Disease-modifying antirheumatic drug |
| Terbutaline | $\beta_2$-agonist |
| TPA | Tumor promoter |
| Trichostatin A | Inhibitor of histone deacetylases |
| Ubiquitin aldehyde | Inhibitor of ubiquitin C-terminal hydrolases |
| WY14643 | PPAR$\alpha$ agonist |

*IBMX, 3-isobutyl-1-methylxanthine; PDTC, ammonium pyrrolidine dithiocarbamate, 15d-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$ prostaglandin J$_2$.*

# Results

## LPS enhances TTP expression. Role of MAPK pathways (I, III)

In the absence of an inflammatory stimulus, murine J774A.1 (I) and J774.2 (III) macrophages expressed low amounts of TTP mRNA and protein. When the cells were activated by adding LPS (1-1000 ng/ml), the TTP mRNA levels increased in a dose- dependent manner. LPS rapidly induced TTP mRNA expression and the maximal levels were detected after 6 h incubation, and thereafter the expression declined (Figure 7A). TTP protein appeared after 3 h induction with LPS and the expression level remained quite constant until 9 h (Figure 7B).

Pharmacological tools were used to inhibit several signalling pathways in the murine J774A.1 macrophages and TTP mRNA levels were measured (I). Inhibitors of different MAPK pathways, PD98059 (an inhibitor of ERK1/2), SP600125 (an inhibitor of JNK) and SB203580 (an inhibitor of p38), reduced LPS-induced TTP mRNA levels, evidence that these three MAPK pathways positively regulate TTP mRNA expression. Ammonium pyrrolidine dithiocarbamate (PDTC), cyclosporin A, genistein and tyrphostin AG-490 were used to assess the involvement of NF-κB, calcium/calmodulin-dependent phosphatase calcineurin, tyrosine phosphorylation and JAK2 in TTP mRNA expression. None of these inhibitors had any statistically significant effect on LPS-induced TTP mRNA expression.

In addition to the inhibitors investigated in (I), several drugs were studied for their abilities to inhibit TTP mRNA expression in LPS-treated human THP-1 cells and murine J774.2 macrophages (Table 17). In neither of the cell lines did the disease-modifying antirheumatic drugs aurothiomalate, hydroxychloroquine, methotrexate, D-penicillamine, leflunomide, and sulfasalazine have any significant effect on LPS-induced TTP mRNA expression. Non-steroidal anti-inflammatory drugs ibuprofen and rofecoxib, and paracetamol were also tested, and they had no effect on LPS-induced TTP mRNA expression.

Agonists of peroxisome proliferator-activated receptors (PPARs) ciglitazone, WY14643, 15d-PGJ$_2$, and GW7647 had no effect on TTP mRNA levels in resting THP-1 cells or in THP-1 cells stimulated with LPS (Table 18).

Inhibitors of the proteasome, lactacystin and MG132, increased LPS-induced TTP protein expression, indicating that TTP protein is degraded through the proteasome pathway (III).
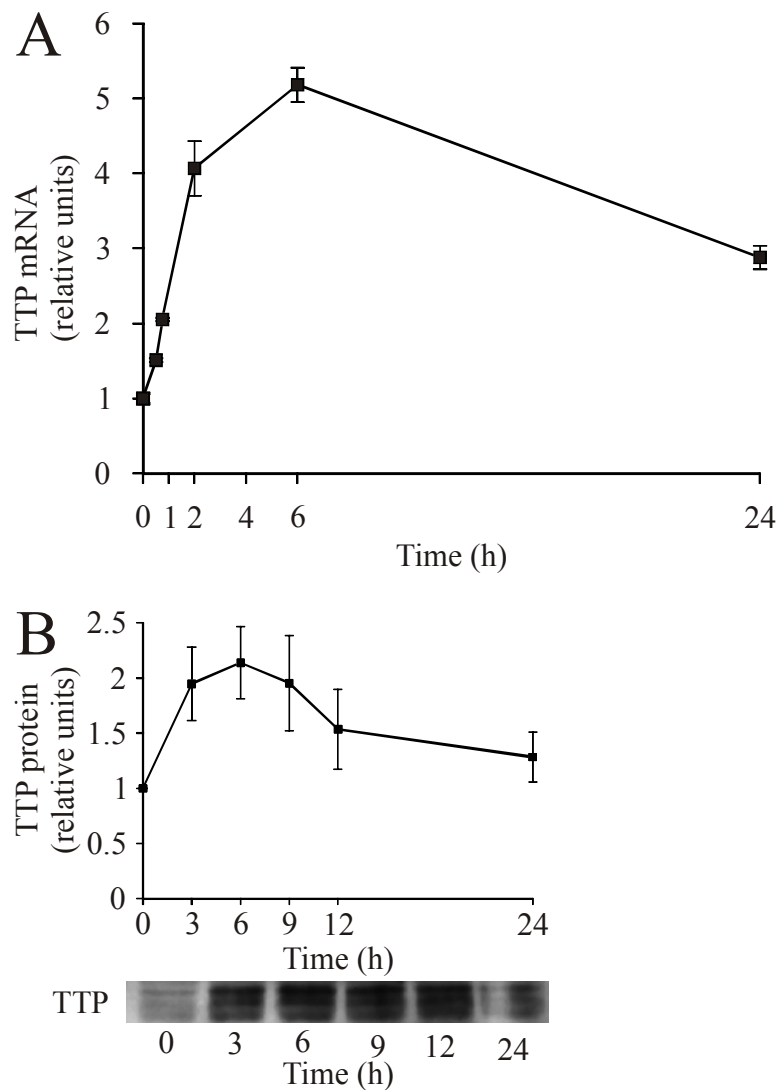
**Figure 7.** *TTP mRNA and protein expression in murine macrophages in response to LPS. (A) LPS (10 ng/ml) was used to stimulate J774A.1 macrophages and total RNA was extracted at indicated time points. Quantitative PCR was used to measure TTP mRNA and the values were normalized to GAPDH mRNA. Values are mean ± SEM (n = 3). (B) LPS (10 ng/ml) was used to stimulate J774A.1 macrophages. Proteins were extracted at the time points indicated and TTP was measured by Western blot with TTP antibody obtained from Santa Cruz Biotechnology. Values are mean ± SEM (n = 3). (Reprinted with permission from: Jalonen et al. 2005, Biochem Pharmacol, 69:733-740. © ElsevierLtd.)*

These results suggest that LPS-induced TTP expression is regulated by MAPK pathways, but is not dependent on NF-κB, calcium/calmodulin-dependent phosphatase calcineurin, tyrosine phosphorylation, JAK2, cyclooxygenase products, or PPARs.

**Table 17.** *Effects of antirheumatic and non-steroidal anti-inflammatory drugs on LPS-induced TTP mRNA expression in human (THP-1) and murine (J774.2) macrophage cell lines.*

| Treatment | TTP mRNA (% of LPS-induced levels) | |
|---|---|---|
| | J774.2 cells | THP-1 cells |
| Control | 11.1 ± 0.7 | 27.1 ± 1.4 |
| LPS (100 ng/ml) | 100 ± 7.6 | 100 ± 5.4 |
| + aurothiomalate (30 μM) | 90.1 ± 7.3 | 107.7 ± 6.6 |
| + hydroxychloroquine (30 μM) | 88.2 ± 7.1 | 117.1 ± 7.5 |
| + methotrexate (30 μM) | 83.8 ± 6.8 | 112.0 ± 11.7 |
| + D-penicillamine (30 μM) | 84.8 ± 8.1 | 118.6 ± 8.4 |
| + leflunomide (30 μM) | 79.5 ± 3.8 | 90.1 ± 2.4 |
| + sulfasalazine (30 μM) | 100.4 ± 4.6 | 97.3 ± 5.4 |
| + paracetamol (30 μM) | 97.4 ± 9.3 | 105.2 ± 4.4 |
| + ibuprofen (30 μM) | 113.2 ± 6.9 | 94.8 ± 7.1 |
| + rofecoxib (30 μM) | 112.8 ± 10.3 | 97.8 ± 4.5 |

*The cells were incubated with the drugs for 30 min before LPS (100 ng/ml) was added to induce TTP mRNA expression for 2 h in THP-1 cells and for 1 h in J774.2 macrophages. Values are mean ± SEM (n = 4).*

**Table 18.** *Effects of PPAR agonists on TTP mRNA expression in human THP-1 cell line.*

| Treatment | TTP mRNA (% of LPS-induced levels) |
|---|---|
| Control | 45.6 ± 3.6 |
| GW7647 (PPARα agonist; 30 μM) | 65.2 ± 4.4 |
| WY14643 (PPARα agonist; 30 μM) | 59.0 ± 6.1 |
| Ciglitazone (PPARγ agonist; 30 μM) | 50.2 ± 8.1 |
| 15d-PGJ$_2$ (PPARγ agonist; 30 μM) | 64.6 ± 3.6 |
| LPS (100 ng/ml) | 100 ± 7.1 |
| + GW7647 (PPARα agonist; 30 μM) | 103.7 ± 19.9 |
| + WY14643 (PPARα agonist; 30 μM) | 110.8 ± 9.0 |
| + ciglitazone (PPARγ agonist; 30 μM) | 99.3 ± 8.8 |
| + 15d-PGJ$_2$ (PPARγ agonist; 30 μM) | 96.6 ± 7.9 |

*The cells were first incubated with the drugs for 30 min and then with or without LPS (100 ng/ml) for 2 h. Values are mean ± SEM (n = 4).*

# Dexamethasone inhibits LPS-induced TTP expression. Role of histone deacetylation (I)

In an initial screening in murine J774A.1 macrophages, dexamethasone was found to decrease LPS-induced TTP mRNA levels. Therefore the aim was to investigate the effects of glucocorticoids on TTP expression in activated macrophages in more detail. Dexamethasone and the dissociated glucocorticoid, RU24858, decreased LPS-induced TTP mRNA levels in a dose-dependent manner with the maximal inhibition being reached at concentrations of 0.1-10 µM (Figure 8A, only dexamethasone shown). Both compounds were also found to decrease TTP protein expression when measured after 6 h incubation (Figure 8B). In our preliminary results on unstimulated J774A.1 macrophages, dexamethasone had no significant effect on TTP mRNA levels after 1 or 6 h incubation. At the protein level, after 6 h incubation, dexamethasone decreased TTP protein expression in unstimulated macrophages similarly as in LPS-treated macrophages. In the presence of a glucocorticoid receptor antagonist, mifepristone, neither dexamethasone nor RU24858 was able to reduce LPS-induced TTP mRNA expression, suggesting that the effects of these compounds are mediated through the glucocorticoid receptor.

When the cells were stimulated with LPS in the presence or absence of dexamethasone or RU24858 for 6 h and then treated with actinomycin D to inhibit transcription, no effect was seen on TTP mRNA decay as measured by quantitative PCR from RNAs extracted 1, 2 or 3 h after addition of actinomycin D. Instead, a difference was seen when cells were treated with LPS with or without dexamethasone and RNA was extracted after 3, 6 or 9 h incubation, suggesting that dexamethasone and RU24858 down-regulate TTP expression at the transcriptional level.

As the suppressive effects of anti-inflammatory steroids on certain genes are mediated through histone deacetylation (Adcock 2003), the effects of dexamethasone and RU24858 on LPS-induced TTP mRNA expression were studied in the presence or absence of two histone deacetylase inhibitors, trichostatin A (TSA) and apicidin. The inhibitory effect of dexamethasone and RU24858 on LPS-induced TTP mRNA was no longer detected after addition of TSA or apicidin (Figure 9), suggesting that the inhibitory effects of glucocorticoids on LPS-induced TTP mRNA expression are mediated by histone deacetylation and transcriptional silencing.
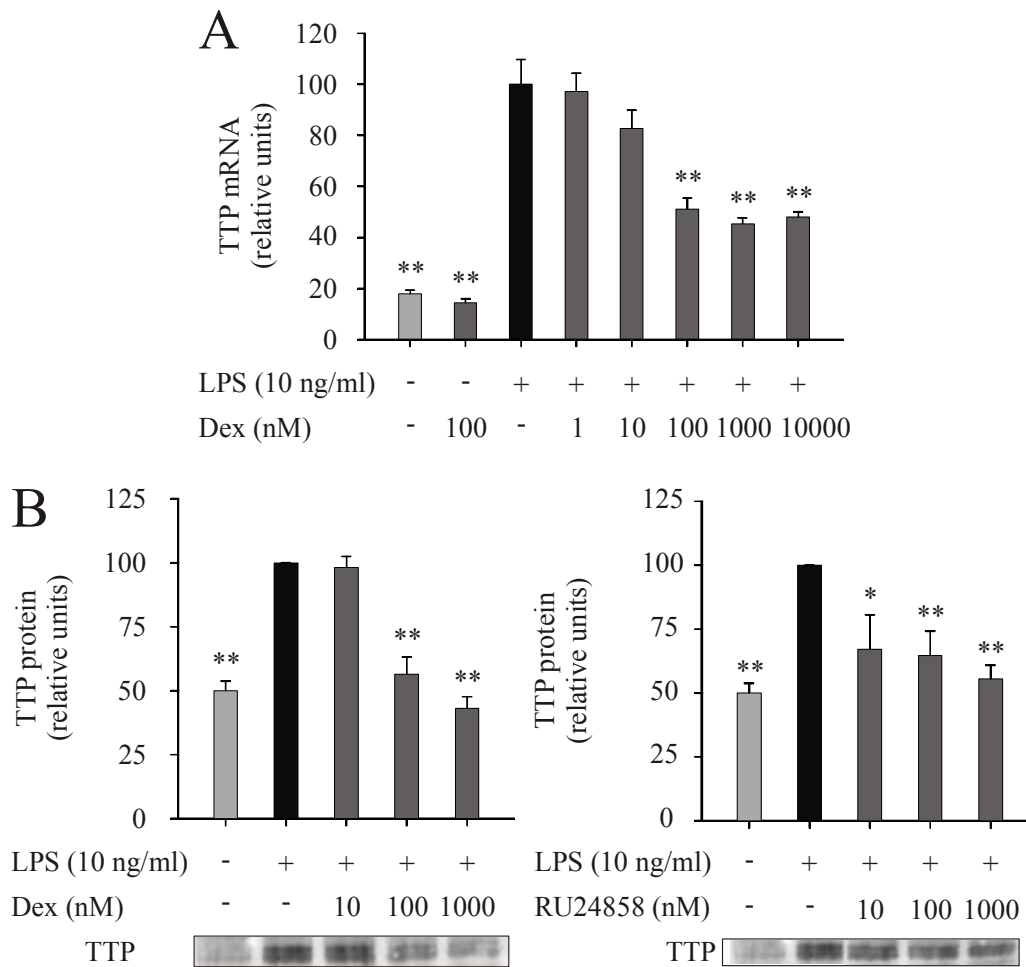
**Figure 8.** *The effects of dexamethasone (Dex) and RU24858 on LPS-induced TTP expression in J774A.1 macrophages. Cells were incubated for 6 h with or without LPS, Dex and RU24858. (A) Quantitative PCR was used to measure TTP mRNA and the values were normalized to GAPDH mRNA. Values are mean ± SEM (n = 3-6). \*\*P<0.01, when compared to samples treated with LPS only. (B) TTP protein was analysed by Western blot. The gels are representatives of four separate experiments with similar results. Density values are mean ± SEM (n = 4). \*\*P<0.01, when compared to samples treated with LPS only. (Reprinted with permission from: Jalonen et al. 2005, Biochem Pharmacol, 69:733-740. © ElsevierLtd.)*
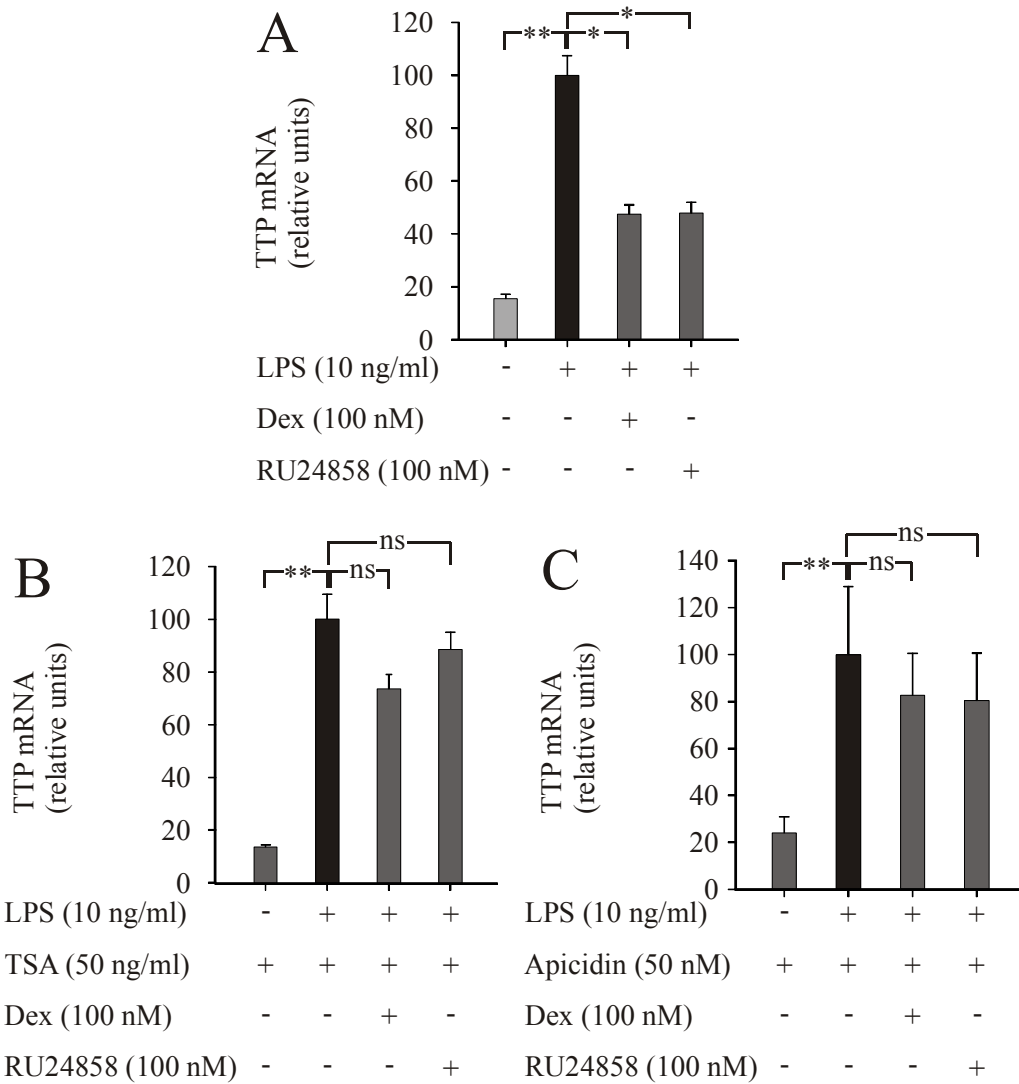
**Figure 9.** *The effect of histone deacetylase inhibitors TSA and apicidin on the suppressive effect of dexamethasone and RU24858 on LPS-induced TTP mRNA expression in J774A.1 macrophages. Cells were incubated for 6 h with the compounds tested. Quantitative PCR was used to measure TTP mRNA and the values were normalized to GAPDH mRNA. Values are mean ± SEM (n = 4). \*\*P<0.01, \*P<0.05, ns = not significant, when compared to samples treated with LPS or to samples treated with a combination of LPS and TSA or LPS and apicidin. (Reprinted with permission from: Jalonen et al. 2005, Biochem Pharmacol, 69:733-740. © ElsevierLtd.)*

# cAMP-enhancing compounds exhibit diverse effects on TTP expression. Involvement of AP-2 and proteasome. (II-III)

cAMP-enhancing compounds, i.e. $\beta_2$-agonists and forskolin, as well as cAMP analogues, all increased TTP mRNA expression in both resting and LPS-treated J774.2 macrophages (II, III). In contrast, there was a marked difference in the effects of these compounds on TTP protein expression between resting cells and cells treated with LPS. In resting cells (II), $\beta_2$-agonists, cAMP analogues (Figure 10), and forskolin induced TTP protein expression while in LPS-stimulated cells (III) these compounds reduced LPS-induced TTP protein levels.
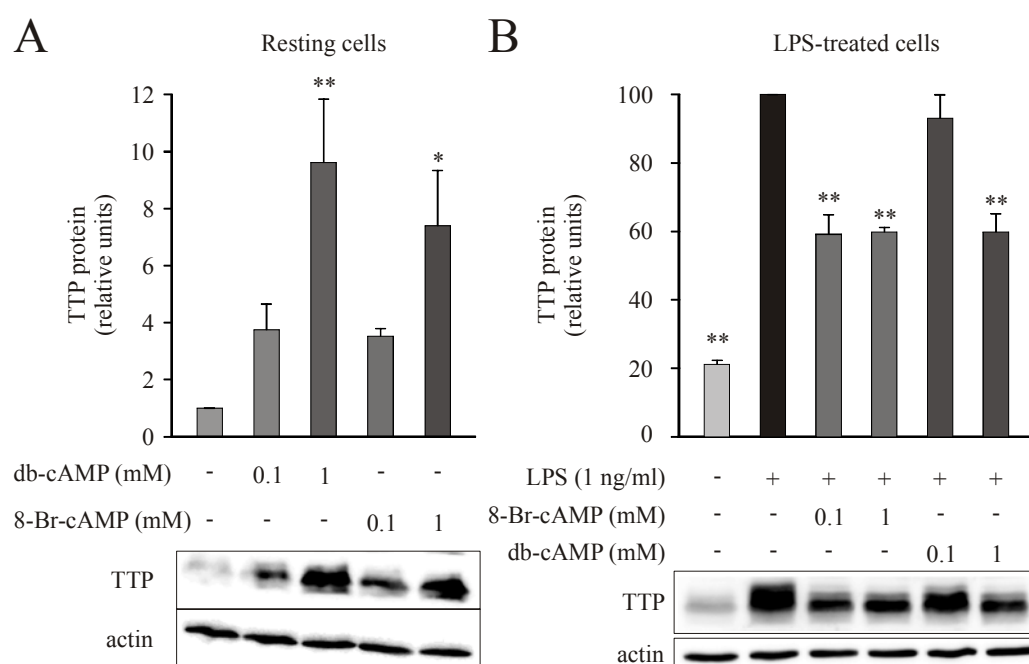


**Figure 10.** *Effects of cAMP analogues on TTP protein expression in J774.2 macrophages. (A) The cells were incubated with db-cAMP and 8-Br-cAMP (0.1 and 1 mM) for 6 h. Proteins were extracted and TTP and actin were measured by Western blot. The TTP level in the control samples was set at 1 and the other values were related to that value. The blot is a representative of three with similar results. Values are mean ± SEM (n = 3). \*\*P<0.01, \*P<0.05, when compared to the control. (B) The cells were treated with LPS (1 ng/ml) and 8-Br-cAMP or db-cAMP (0.1 and 1 mM) for 9 h. Proteins were extracted and TTP and actin proteins were measured by Western blot. The TTP level in the LPS-treated samples was set at 100 and the other values were related to that value. The blot is a representative of six with similar results. Values are mean ± SEM (n = 6). \*\*P<0.01, when compared to cells incubated with LPS only. (Reprinted with permission from: Jalonen et al. 2007, Life Sci 81:1651-1658. © ElsevierLtd. and Jalonen et al. 2008, J Pharmacol Exp Ther 326:514-522. © The American Society for Pharmacology and Experimental Therapeutics.)*

In resting cells, the phosphodiesterase inhibitors 3-isobutyl-1-methylxanthine (IBMX) and rolipram potentiated salbutamol and forskolin-induced TTP mRNA and protein expression providing evidence for the effect being mediated through cAMP (II). It could be concluded that cAMP-elevating agents induced TTP mRNA and protein expression in resting cells, possibly by activation of transcription factor activator protein 2 (AP-2) as demonstrated by the increased activation of AP-2 (measured as nuclear translocation of AP-2) by forskolin treatment (Figure 11A).
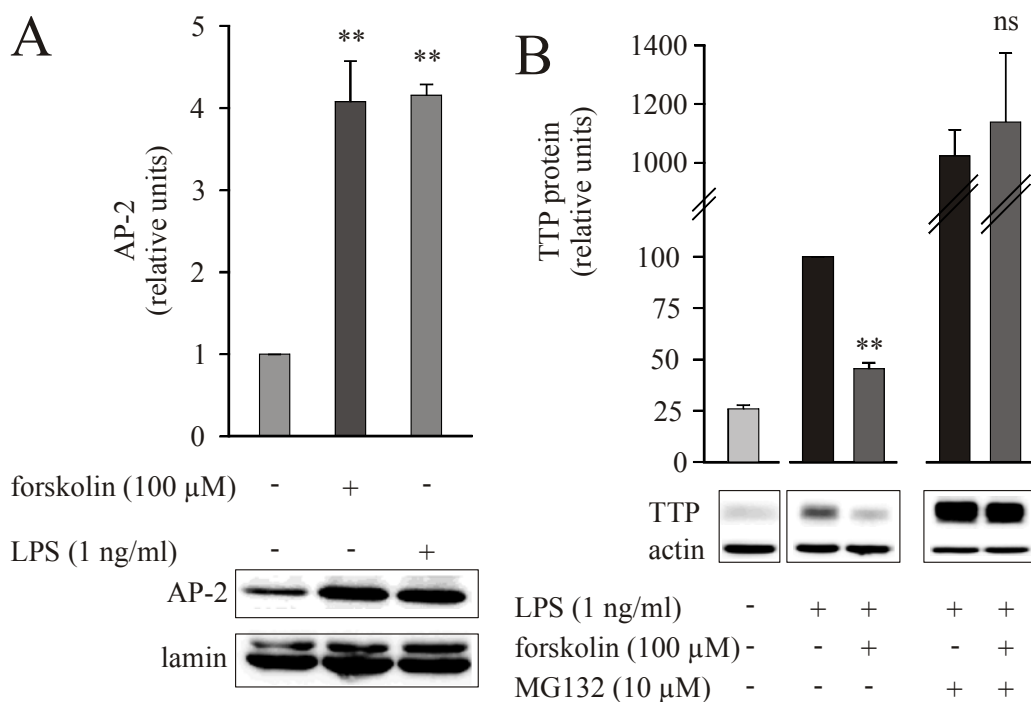


**Figure 11.** *Mechanisms how cAMP-elevating agents regulate TTP expression in J774.2. macrophages. (A) Effect of forskolin on nuclear translocation of AP-2. The cells were stimulated with forskolin (100 μM) or LPS (1 ng/ml), which was used as a control compound, for 30 min. Nuclear extracts were prepared and AP-2 and lamin were detected in nuclear extracts by Western blot. The AP-2 level in control samples was set at 1 and other values were related to that value. Values are mean ± SEM (n = 3). \*\*P<0.01, when compared to the control. (B) Effect of proteasome inhibitor MG132 on TTP protein expression. Macrophages were incubated with LPS (1 ng/ml) with or without forskolin (100 μM) for 6 h before MG132 (10 μM) was added and incubation was continued for another 6 h. Proteins were extracted and TTP and actin were detected by Western blot. The TTP protein level in the LPS-treated samples was set at 100 and the other values were related to that value. Values are mean ± SEM (n = 3). \*\*P<0.01 and not significant (ns), when compared to the LPS- and LPS+MG132-treated samples. (Reprinted with permission from: Jalonen et al. 2007, Life Sci 81:1651-1658. © ElsevierLtd. and Jalonen et al. 2008, J Pharmacol Exp Ther 326:514-522. © The American Society for Pharmacology and Experimental Therapeutics.)*

Forskolin did not affect the half-life of TTP mRNA in LPS-induced cells as measured by the actinomycin D assay but forskolin, cAMP analogues, and salbutamol decreased LPS-induced TTP protein levels in a time-dependent manner (III). The reduction in LPS-induced TTP protein levels by elevated cAMP was possibly mediated by the degradation of TTP protein through the proteasome pathway, because two proteasome inhibitors, lactacystin and MG132 (Figure 11B), reversed the effects of forskolin (III).

MAPK p38 was activated rapidly by phosphorylation when LPS was added into the culture, and it was associated with increased TTP mRNA expression (III). The increase in TTP mRNA levels was reduced in the presence of a p38 inhibitor SB203580, indicative of a role for p38 in the upregulation of TTP expression.

Since TNF-α is a target of TTP and the decay of TNF-α mRNA is increased by TTP, the actinomycin D assay was used to measure the decay of TNF-α mRNA in response to forskolin in LPS-stimulated cells. The reduction in LPS-induced TTP protein levels by forskolin at later time point was capable of increasing TNF-α mRNA stability (Figure 12).
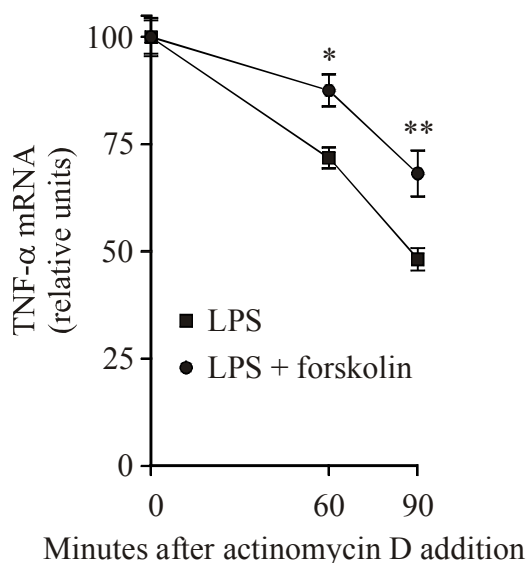


**Figure 12.** *Effects of forskolin on TNF-α mRNA decay in LPS-treated J774.2 macrophages. The cells were treated with LPS (1 ng/ml) with or without forskolin (100 μM) for 9 h before actinomycin D (0.5 μg/ml) was added to the culture. Total RNA was extracted 60 and 90 min after actinomycin D addition and quantitative PCR was used to detect TNF-α mRNA. GAPDH mRNA was measured for normalization. The mean of TNF-α mRNA levels at the time of actinomycin D addition was set at 100 and the other values were related to that. Values are mean ± SEM. (n = 4). \*\*P<0.01, \*P<0.05, when compared with the LPS-treated samples. (Reprinted with permission from: Jalonen et al. 2008, J Pharmacol Exp Ther 326:514-522. © The American Society for Pharmacology and Experimental Therapeutics.)*

# TTP knockdown by siRNA technique results in changes in cytokine expression patterns (IV)

To investigate the effects of TTP on cytokine production in macrophages, TTP expression was down-regulated by the siRNA technique. The J774.2 murine macrophages were transfected with shTTP and shNEG vectors to down-regulate TTP expression and to serve as a control, respectively. A significant reduction in TTP protein expression in the shTTP cell line was detected by Western blot and the reduction was proven to be functional by measuring the TNF-$\alpha$ levels (Figure 13). TNF-$\alpha$ levels were markedly upregulated in shTTP cells compared to shNEG cells. Cytokine antibody array was used to detect levels of 62 cytokines and other inflammatory mediators from the cell culture medium.
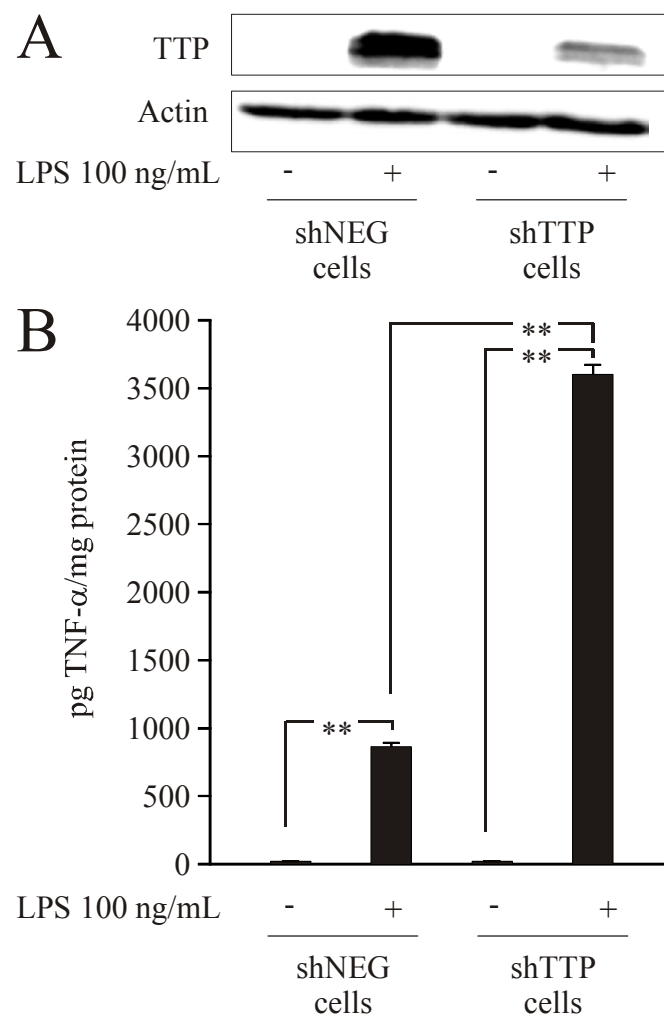


**Figure 13.** *Down-regulation of TTP expression and enhancement of TNF-$\alpha$ production in shTTP cells. (A) shNEG and shTTP cells were stimulated with LPS (100 ng/ml) and proteins were extracted after 6 h incubation. TTP and actin were detected by Western blot. The blot is a representative of three blots with similar results. (B) shNEG and shTTP cells were stimulated with LPS (100 ng/ml) for 1 h. Thereafter the medium was changed and the cells were incubated for another 48 h. TNF-$\alpha$ concentrations in the culture media were measured by ELISA. Values are mean ± SEM (n = 3). \*\*P<0.01.*

Both of the cell lines expressed five cytokines (CTACK, CXCL16, MIP-1α, MIP-1γ, and TARC) spontaneously. When LPS (100 ng/ml) was added into the cell culture, the expression of 10 cytokines (GCSF, IL-6, LIX, MCP-1, MCP-5, MIP-2, MIP-3α, RANTES, sTNF RII and the IL-12 p40 subunit) increased while the expression of CXCL16 was decreased in shNEG cells as compared to untreated shNEG cells. In the shTTP cell line, the production of 11 cytokines (GCSF, IL-6, IL-12, LIX, MCP-1, MCP-5, MIP-2, RANTES, sTNF RII, TNF-α, and the IL-12 p40 subunit) was increased following LPS treatment.

The LPS-induced production of 6 cytokines (IL-6, IL-12, IL-12 p40 subunit, MIP-2, MIP-3α, and TNF-α) was different between these two cell lines (Table 19). The production of IL-6, IL-12, MIP-2, and TNF-α was higher in shTTP cells than in shNEG cells, whereas the production of MIP-3α and IL-12p40 was lower in shTTP cells than in shNEG cells. These results indicate that IL-12, MIP-2 (a homologue of human IL-8), and MIP-3α are novel inflammatory cytokine targets for TTP-regulated mRNA decay, and confirm the earlier findings that TNF-α and IL-6 expression can be regulated by TTP.

**Table 19.** *TTP down-regulation (shTTP cell line) enhanced the production of IL-6, IL-12, MIP-2, and TNF-α and reduced the production of MIP-3 and IL-12 p40 subunit in response to LPS.*

| | shNEG cells | | shTTP cells | | |
| | without LPS | with LPS | without LPS | with LPS | *P*-value |
|---|---|---|---|---|---|
| IL-6 | $3.8 \pm 0.6$ | $172.3 \pm 18.8$ | $3.5 \pm 0.4$ | $258.0 \pm 31.1$ | * |
| IL-12 | $2.6 \pm 0.4$ | $5.3 \pm 0.6$ | $3.6 \pm 0.9$ | $44.3 \pm 4.9$ | ** |
| MIP-2 | $51.0 \pm 4.9$ | $128.1 \pm 13.5$ | $33.6 \pm 4.1$ | $182.3 \pm 20.8$ | * |
| TNF-α | $4.5 \pm 0.7$ | $9.0 \pm 1.4$ | $7.2 \pm 2.5$ | $149.0 \pm 26.4$ | ** |
| MIP-3α | $5.3 \pm 0.6$ | $18.1 \pm 2.2$ | $5.0 \pm 0.6$ | $9.6 \pm 1.2$ | ** |
| IL-12 p40 | $2.7 \pm 0.6$ | $69.0 \pm 7.7$ | $2.9 \pm 0.3$ | $41.6 \pm 5.0$ | ** |

*Cytokine antibody array membranes were incubated in media from cell cultures of TTP knockdown cell line (shTTP cells) or control cell line (shNEG cells) after treatment with or without LPS (100 ng/ml). Values are arbitrary units compared to positive controls at the antibody array membrane (100) and are presented as mean ± SEM (n = 3). \*\*P<0.01, \*P<0.05 between LPS-treated shNEG and LPS-treated shTTP cells.*

# Discussion

## Methodology

This study was conducted to investigate the regulation of TTP expression and its immunological effects in macrophages. Macrophages play a critical role in inflammatory diseases such as rheumatoid arthritis and asthma (Welte and Groneberg 2006, Sabroe et al. 2007, Szekanecz and Koch 2007). LPS has been shown to induce TTP mRNA and protein expression in mouse primary macrophages and mouse RAW264.7 macrophages (Carballo et al. 1998, Mahtani et al. 2001) as well as in many other mouse, human, and rat cell types. The present experiments were carried out by using J774 and THP-1 macrophage cell lines. The use of primary human macrophages would have strengthened the study in terms of its applicability to human pathophysiology and this possibility should be considered in future studies.

Standard molecular and cellular biology methods were used to detect mRNA (quantitative real-time reverse transcriptase PCR), protein (Western blot), and cytokine levels (ELISA). TTP, TNF-$\alpha$, and GAPDH mRNAs were detected by quantitative real-time reverse transcriptase PCR. Quantitative PCR is a simple, specific, and sensitive method to measure mRNA levels (Bustin et al. 2005), and GAPDH mRNA levels were utilized for normalization. There are some data inferring that LPS induces GAPDH expression in rat liver and lungs (Xie et al. 2006), but in the present study, the levels of GAPDH mRNA remained rather constant whereas the levels of TTP and TNF-$\alpha$ varied several cycles between treated and untreated cells.

Western blot is a standard method to measure protein levels. One of the major problems in the present study was to obtain a functional antibody against TTP. Commercial antibodies exhibited major variations in quality and most often proved worthless. Obtaining a functional antibody from the group of Prof. Blackshear substantially advanced this study. Western blot was also used to study the nuclear translocation of transcription factors. This is an indirect method to study the activation of transcription factors by detecting the cytosol to nucleus translocation of transcription factors. Another method used to study activation of transcription factors is the electrophoretic mobility shift assay (EMSA), which was not used in the present study. EMSA detects the binding of proteins to short DNA oligos thereby providing perhaps more adequate data, but does not guarantee that transcription factors actually bind to the promoter of the studied gene or that the binding induces transcription. Another method to study the activation of transcription factor consensus binding sites is to place them in front

of a reporter gene and to quantify the expression of the reporter gene. This method, on the other hand, describes the function of only one or a few binding sites, and does not reflect the situation on the intact promoter, where several factors interact with each other. Probably the best way to study transcriptional activation would be to use the whole TTP promoter region in front of a reporter gene. This also possesses defects since the TTP intron is also involved in full inducibility of TTP transcription (Lai et al. 1998).

Several inhibitors were used to inhibit signal transduction pathways in the present study. Inhibitors and drugs may also inhibit other kinases and pathways, at least if used in high concentrations (Davies et al. 2000, Bain et al. 2003). On the other hand, inhibitors of the signalling pathways were not the main research tools in the present study. In study I, inhibitors of signalling pathways were used to evaluate the possible role of these pathways in the upregulation of TTP expression. Some of these pathways were already known to be involved and were tested to confirm that the experimental procedures were appropriate. In studies I, II, and III, two chemically different inhibitors of histone deacetylases, phosphodiesterases, or proteasome, respectively, were used to strengthen the results obtained with the inhibitors. One way to confirm these results would be to use siRNA against the components of different signalling pathways.

In studies II and III, different cAMP-elevating agents and two cell-permeable cAMP analogues were used to provide evidence whether there is any involvement of cAMP-mediated effects. $\beta_2$-agonists activate adenylate cyclase and increase cAMP via the $\beta$-receptors, whereas forskolin is a direct activator of adenylate cyclase, and cAMP analogues mimic cAMP produced by the cell. Similar results were obtained with each of the three groups of compounds, convincing evidence that cAMP is involved in the upregulation of TTP mRNA expression or TTP protein down-regulation in LPS-stimulated cells. Phosphodiesterase inhibitors 3-isobutyl-1-methylxanthine (IBMX) and rolipram also increased TTP expression providing evidence that the effects are mediated through cAMP

In study IV, TTP protein expression was down-regulated by introducing a vector that produced siRNA against TTP from a short hairpin RNA. RNA interference, first described by Nobel laureates Fire and Mello (Fire et al. 1998), has proven to be a powerful method to down-regulate expression of proteins (Kong et al. 2007, Martin and Caplen 2007). Sometimes sequences with a few mismatches inhibit translation of proteins (Doench et al. 2003, Zeng et al. 2003). Therefore it is possible that the sequence against TTP also inhibited translation of ZFP36L1 and ZFP36L2, but sequence comparisons did not indicate that such a possibility was likely. Use of one or two other siRNA sequences against TTP and the possibility of obtaining similar results as well as studying the effects of TTP overexpression would have strengthened the results of study IV. In fact, the TTP overexpression vector was transfected to J774.2 macrophages, but the cells grew very poorly allowing no experimentation.

Cytokine antibody array is fast and simple method to measure expression level of several cytokines simultaneously (Templin et al. 2002, Haab 2006). At the time of the experiment, this method had been quite recently introduced and

provided an interesting tool to identify cytokines that might be regulated by TTP. The results identified a few possible targets, but the results will need to be repeated by ELISA, and the direct association of TTP with the mRNA as well as the difference in the mRNA half-life should be confirmed. One possible method to detect novel targets of TTP-mediated decay would be an actinomycin D assay on cDNA microarrays with normal cells and cells that do not express TTP (down-regulated by siRNA or cells from knockout mice) and cell that overexpress TTP. This kind of experiment was published by Lai et al. (2006) on fibroblasts from normal and TTP KO mice. This strategy has been modified by Stoecklin et al. (2008). They first conducted RNA-immunoprecipitation with TTP antibody in RAW264.7 macrophages and then used a cDNA microarray to identify the mRNAs that associated with TTP. This method on the other hand does not exclude the possibility that TTP is not bound to the mRNA directly but through some association with other proteins that bind to mRNA. However, of the mRNAs that were studied further, only the expression of IL-10 was elevated in TTP KO mice as compared to WT mice (Stoecklin et al. 2008). None of these methods alone is sufficient to provide a general view of the situation in the cells. Measuring the levels of proteins does help in elucidating the resolution of all the regulatory mechanisms and the outcome at the level of inflammatory cytokines.


## Glucocorticoids in the regulation of TTP expression

Glucocorticoids are used in the treatment of diverse inflammatory diseases. They are basic medicines for example in the treatment of asthma and rheumatoid arthritis. There were no previous studies elucidating the mechanisms how dexamethasone modifies TTP expression. Although one early study on rat PC-12 pheochromocytoma cells (Kujubu et al. 1987), and a study done in parallel with our study on human peripheral blood mononuclear cells (Bergmann et al. 2004) had shown that dexamethasone does not affect TTP expression, our hypothesis was that glucocorticoids would increase TTP expression in macrophages and down-regulate the levels of inflammatory modulators. Surprisingly, LPS-induced TTP expression was down-regulated by dexamethasone and the dissociated steroid, RU24858. In another study by Bianchini et al. (2006), treatment with dexamethasone slightly reduced TTP mRNA levels in $CD4^+CD8^+$ double-positive thymocytes from C3H/HeN mice. Later it was shown that in human A549 alveolar epithelial cells and also in rat tissues that dexamethasone in the absence of inflammatory stimuli could enhance TTP expression (Smoak and Cidlowski 2006). There seems to be discrepancy on the results about whether dexamethasone increases or decreases TTP expression. This may be a species-specific phenomenon, because based on the existing literature, TTP expression is down-regulated by dexamethasone in mice, but unaffected or upregulated in rat and human cells. Alternatively this could be a cell type-specific phenomenon, because different cell types have been used in all of these studies. The involvement of an inflammatory stimulus might also be a factor to explain the

results. In our study, LPS was used to induce inflammatory responses in macrophages and dexamethasone reduced both LPS-induced mRNA and protein levels. In the study of Smoak and Cidlowski (2006), dexamethasone was tested in resting cells. In one of their experiments, dexamethasone increased also TNF-$\alpha$-induced TTP mRNA levels, but the effects on TTP protein levels were not reported. On the other hand, in our recent preliminary experiments dexamethasone alone did not affect TTP mRNA expression in J774A.1 macrophages, but reduced TTP protein expression comparably to that in LPS-treated macrophages, suggesting that the discrepancies are not due to the stimulus used.

Dexamethasone and the dissociated steroid, RU24858, reduced LPS-induced TTP mRNA and protein levels in a dose-dependent manner. Dissociated glucocorticoids such as RU24858 are synthetic glucocorticoid ligands possessing different transactivation and transrepression profiles than the classical anti-inflammatory steroids. RU24858 has been shown to have transrepression properties similar to those of dexamethasone on AP-1 and NF-$\kappa$B-dependent gene expression, but it does not induce gene expression through glucocorticoid response element in mouse fibrosarcoma L929 and human HeLa cells (Vayssière et al. 1997, Vanden Berghe et al. 1999). Since both of the drugs showed a similar pattern of inhibition on LPS-induced TTP mRNA and protein expression, we assume that the effects are not mediated through direct binding of glucocorticoid receptor dimers to glucocorticoid response element even though a glucocorticoid-like response element has also been suggested to reside in the mouse TTP promoter region (DuBois et al. 1990) as well as a single hexameric half glucocorticoid response element sequence in human TTP 3'-flanking region (Smoak and Cidlowski 2006). Indeed the 5'- and 3'-flanking regions of TTP gene have been shown to bind glucocorticoid receptors, but no binding was observed in the intron region (Smoak and Cidlowski 2006). In the presence of mifepristone, a glucocorticoid receptor antagonist, dexamethasone and RU24858 were not able to reduce LPS-induced TTP mRNA levels, suggesting that the effects of glucocorticoids are mediated through binding to glucocorticoid receptors. In the study of Smoak and Cidlowski (2006), mifepristone inhibited the induction of TTP mRNA and protein expression by dexamethasone, suggesting that also these effects of dexamethasone do require glucocorticoid receptor binding.

Although dexamethasone has been shown to destabilize several mRNAs such as iNOS (Korhonen et al. 2002) and COX-2 (Ristimäki et al. 1996, Lasa et al. 2001), dexamethasone did not affect TTP mRNA half-life in the present study as measured by the actinomycin D assay. That was not investigated in the study of Smoak and Cidlowski (2006). Since TTP has been shown to interact with KSRP and interfere with its action (Fechir et al. 2005, Linker et al. 2005), it is possible that dexamethasone, by decreasing the amount of TTP, increases the destabilizing effect of KSRP on inflammatory genes. This was not investigated in the present study, but may be worth examining in the future.

Several mechanisms have been reported to explain how glucocorticoids affect the expression of inflammatory genes. These include the regulation of

transcription by direct activation or inhibition of transcription through glucocorticoid response elements in promoter regions, activation or repression of transcription by interactions with other transcription factors, histone acetylation/deacetylation and chromatin remodelling, regulation of mRNA stability, and induction of MAPK phosphatase-1 and inactivation of JNK and p38 pathways (Barnes 1998, Newton 2000, Adcock 2003, Ito et al. 2006, Clark 2007). The present study and the study by Smoak and Cidlowski (2006) suggest that the effects of dexamethasone on TTP expression occur at the transcriptional level. Glucocorticoid receptors recruit several coactivator factors and induce changes in chromatin structure (Li et al. 2003). The histone acetylation status of chromatin reflects the availability of DNA for transcription. Histone acetylation increases transcriptional activation, whereas histone deacetylation leads to gene silencing. Glucocorticoids have been shown to cause histone deacetylation both by recruiting histone deacetylase 2 (Ito et al. 2000) and repressing the activity of histone acetyltransferase (Ito et al. 2001). In the present study, the presence of two histone deacetylase inhibitors, trichostatin A (TSA) and apicidin, abolished the inhibitory effect of dexamethasone and RU24858 on LPS-induced TTP mRNA suggesting that the inhibitory effects of glucocorticoids on LPS-induced TTP mRNA expression are mediated by histone deacetylation and transcriptional silencing

Since TTP expression may be implicated in rheumatoid arthritis (Taylor et al. 1996, Phillips et al. 2004, Tsutsumi et al. 2004, Fabris et al. 2005, Suzuki et al. 2006b, Sugihara et al. 2007) glucocorticoids may reflect the outcome of arthritic inflammation by regulation of TTP. It would be of interest to investigate the effects of glucocorticoids on TTP expression in human synoviums as well as their effects on TTP in human lung tissues since it has been shown that TTP expression is induced by TNF-α in human bronchial cells (Cooper et al. 2001) and glucocorticoids may also regulate TTP expression in asthmatic lung.

In conclusion, glucocorticoids were found to decrease TTP expression in activated macrophages in a glucocorticoid receptor-dependent and glucocorticoid response element-independent manner, possibly through histone deacetylation and transcriptional silencing. These results suggest that glucocorticoids down-regulate TTP expression in cells exposed to inflammatory stimuli and this may result in increased expression of cytokines and other genes, which are regulated by TTP, unless other destabilizing proteins are able to substitute for TTP.


## cAMP in the regulation of TTP expression


The mechanisms by which cAMP-elevating agents affect TTP expression, were studied both in resting and in LPS-activated macrophages. The results presented in this thesis show that in resting macrophages, TTP mRNA and protein expression are upregulated by β$_2$-agonists and forskolin, which increase the intracellular levels of cAMP, and by cAMP analogues. Similar results on TTP mRNA expression with these agents were also found in human THP-1 cells,

indicating that a similar phenomenon occurs in human cells, as well. α- and β-agonists were reported to increase TTP mRNA levels in only a few cell lines but the mechanisms, or the effect on TTP protein levels, have not been studied (Arenander et al. 1989a, Gubits et al. 1993b).

The effects of 8-Br-cAMP on TTP expression have not been examined previously. Db-cAMP has been shown to increase TTP mRNA levels in rat secondary astrocytes (Arenander et al. 1989b) and in PC12 pheochromocytoma cells (Kaneda et al. 1992), but it had no effect in mouse B cell hybridomas (Nakajima and Wall 1991, Yin and Yang 1993). This may reflect some kind of species-specific mechanism. However, since db-cAMP was shown to increase TTP levels in mouse macrophages in the present study, it is more likely that this is a cell type-specific phenomenon.

The TTP mRNA-increasing effect of forskolin has been shown in mouse 3T3 fibroblasts (DuBois et al. 1990) and rat secondary astrocytes (Arenander et al. 1989b), but it had no effect in rat microglial cells (Priller et al. 1995). In the present study, forskolin induced nuclear translocation of transcription factor AP-2, which may provide a mechanism to explain how cAMP-elevating agents induce TTP expression. The cAMP pathway has been shown to activate AP-2 also in human HeLa cells (Imagawa et al. 1987). AP-2 consensus sequences are found in the promoter regions of mouse, human, rat, and bovine TTP (DuBois et al. 1990, Heximer and Forsdyke 1993, Lai et al. 1995, Kaneda et al. 2000), as well as in the mouse TTP intron sequence (Lai et al. 1998). The binding of AP-2 to these sequences was not investigated in three of the studies. In the work of Lai et al. (1995) on the mouse TTP promoter region, the deletion of AP-2 site resulted a major reduction in serum-induced TTP mRNA accumulation. They also inserted the AP-2 consensus site in front of a minimal human β-globin gene and TTP cDNA and showed that TTP mRNA expression in cells transfected with this vector was increased as compared to control. In DNA mobility shift analyses, an AP-2 competitor oligo was able to inhibit binding of nuclear extracts to the AP-2 probe of mouse TTP. In studies on mouse TTP intron, competitor oligos of AP-2 were not able to affect the binding results of nuclear extracts on mouse TTP intron probes (Lai et al. 1998). These results indicate that the AP-2 site in the TTP promoter might be functional, whereas the AP-2 sites in the intron appear to be inactive. Therefore it is likely that increased AP-2 activation induced by forskolin and LPS may explain at least partly the increasing effects of forskolin and LPS on TTP mRNA expression.

The effects of $\beta_2$-agonists, forskolin, and cAMP analogues were also studied in LPS-activated J774.2 macrophages. The actions of cAMP-elevating agents in LPS-stimulated macrophages have not been studied previously. As was the case in resting cells, these agents also increased TTP mRNA levels in LPS-stimulated macrophages. However, these agents decreased TTP protein expression although mRNA levels were increased. The stability of LPS-induced TTP mRNA was not altered by forskolin. Instead it was noted that the degradation of TTP protein was increased i.e. the effects of cAMP were possibly mediated via the proteasome. Brook et al. (2006) also reported that hypophosphorylated TTP is targeted for degradation by the proteasome and these results were duplicated by Deleault et

74

al. (2008). TTP protein has been shown to contain three putative PEST domains (proline, glutamic acid, serine, and threonine) that target proteins for degradation by the proteasome, but studies with mutated domains have not yet revealed the functional activity of these domains (Rigby et al. 2005).

MAPK p38 activation was studied at an early time point before the peak of TTP mRNA and at a later time point, when LPS-induced TTP protein levels showed the greatest decline following forskolin treatment. In LPS-treated cells, the activation of p38 by phosphorylation was detected before the increase in TTP mRNA levels and the addition of p38 inhibitor SB203580 abolished the increase in TTP mRNA levels, providing evidence for a role of p38 in LPS- and LPS + forskolin-induced TTP mRNA expression. In addition, p38 is involved in the phosphorylation of TTP, which in turn regulates TTP activity. Since phosphorylation of TTP creates a binding site for 14-3-3 proteins (Johnson et al. 2002, Chrestensen et al. 2004), prevents stress granule entry, and increases TNF-$\alpha$ mRNA stability (Stoecklin et al. 2004), i.e. inhibits TTP activity, it is possible that at the early time points, TTP is phosphorylated by p38 kinase and TNF-$\alpha$ mRNA becomes stabilized. At the later time point when LPS-induced TTP protein levels showed the greatest decrease after forskolin treatment, very low levels of phosphorylated (i.e. active) p38 were detected, suggesting that the remaining TTP may be less phosphorylated and have more functional activity e.g. on TNF-$\alpha$ mRNA stability. The stability of TNF-$\alpha$ mRNA was studied at the later time point and it was shown that forskolin increased LPS-induced TNF-$\alpha$ mRNA stability along with its reducing effect on TTP expression.

The phosphorylation status of TTP was not investigated in the present study, but in resting cells it seemed that the molecular weight of the TTP bands detected by Western blot in salbutamol, 8-Br-cAMP, and forskolin-treated cells was similar to control, suggesting that the TTP protein induced by cAMP-elevating agents in resting cells might be unphosphorylated and active. Interestingly, in LPS-treated cells, cAMP-elevating agents seem to decrease especially the higher molecular weight forms of TTP, raising the possibility that the remaining TTP protein is in its active form. These results need to be confirmed with an antibody against phosphorylated TTP or alternatively determining whether treatment with the appropriate phosphatase alters the molecular weight of TTP.

These results suggest that cAMP-elevating agents (e.g. salbutamol) regulate TTP expression differently during different phases of inflammation, which is likely to induce time-dependent changes in the expression pattern of cytokines in different stages of the inflammation process.

## Other signalling pathways involved in TTP upregulation

In the present study, other signalling pathways in addition to cAMP were examined to elucidate their role, if any, on TTP mRNA expression (I). The inhibitors of NF-$\kappa$B, calcium/calmodulin-dependent phosphatase calcineurin,

cyclooxygenases, tyrosine phosphorylation, and JAK2 did not have any statistically significant effect on LPS-induced TTP mRNA expression in J774 mouse macrophages in the present study. In fact, little is known about the involvement of these factors in the regulation of TTP expression. The mouse, human, and bovine intron and human promoter region of TTP has been shown to include an NF-κB-like binding site and NF-κB consensus binding sites, respectively (Lai et al. 1998, Smoak and Cidlowski 2006). The role of the NF-κB-like binding site in TTP intron is still unclear since NF-κB probes were shown to compete with nuclear extract binding to this site and antibody against NF-κB induced a supershift in the electrophoretic mobility shift assay (EMSA), when cells treated with TNF-α were examined, but not when serum or TPA-treated cells were used (Lai et al. 1998). In the present study, an inhibitor of NF-κB, ammonium pyrrolidine dithiocarbamate (PDTC), was used and the results suggest that NF-κB is not an important transcription factor for LPS-induced TTP expression.

In the present study, inhibition of the serine/threonine phosphatase calcineurin by cyclosporin A did not inhibit LPS-induced TTP mRNA expression. Similar results have been obtained earlier with W7, an inhibitor of calmodulin (a calcium sensor protein that activates calcineurin), in mouse liver and B cells (Nakajima and Wall 1991, Yin and Yang 1993, Ledwith et al. 1996). This data suggests that calmodulin and calcineurin are not important modulators of TTP expression.

Genistein, an inhibitor of tyrosine phosphorylation, and tyrphostin AG-490, an inhibitor of JAK2 protein tyrosine kinase did not inhibit LPS-induced TTP mRNA expression in the present study. A slight decrease in TTP mRNA levels was found with genistein, but it was not statistically significant. Tyrosine kinases are involved in TTP mRNA upregulation by IL-6 and IL-11 in mouse B cell hybridomas as demonstrated by inhibitors like tyrphostin, genistein, and herbimycin A (Nakajima and Wall 1991, Yin and Yang 1993). These results may indicate that tyrosine phosphorylation is required to mediate some signalling pathways but not others or else this pathway is restricted to certain cell types.

Inhibitory compounds, PD98059, SP600125, and SB203580, were used to study the role of MAPKs ERK1/2, JNK, and p38, respectively, on TTP mRNA expression. The involvement of ERK1/2 in TTP expression is unclear since the use of another inhibitor of ERK1/2, U0126, was shown not to have effects on LPS-induced TTP mRNA or protein expression in RAW264.7 macrophages (Tchen et al. 2004, Brook et al. 2006) but PD98059 has been shown to decrease TTP mRNA levels induced by adipogenic hormones (combination of insulin, dexamethasone, methylisobutylxanthine, and foetal bovine serum) in mouse 3T3 preadipocytes (Inuzuka et al. 1999), and also to decrease LPS-induced TTP protein levels and to reduce TTP mRNA stability in human THP-1 cells (Brooks et al. 2004). In a study where the specificities of several protein kinase inhibitors were evaluated, it was noted that PD98059 and U0126 had somewhat different properties (Davies et al. 2000). Therefore the results obtained with inhibitors, must be viewed with caution and the role of ERK1/2 in the upregulation of TTP transcription needs to be confirmed with other methods. Our results suggest that

TTP mRNA expression is, at least partly, dependent on the activation of the ERK1/2 pathway.

Furthermore, the role of JNK in the upregulation of TTP expression seems somewhat ambiguous. In the present study, the JNK inhibitor, SP600125, decreased LPS-induced TTP mRNA levels in J774 murine macrophages. In human A549/8 alveolar epithelial cells, SP600125 and an siRNA against JNK did not affect CM-induced TTP mRNA levels, whereas TTP protein levels were decreased (Korhonen et al. 2007). SP600125 also inhibited TTP protein levels in LPS-induced human THP-1 cells, whereas TTP mRNA stability was unaffected (Brooks et al. 2004). In mouse RAW264.7 macrophages SP600125 did not affect TTP protein levels (Brook et al. 2006). SP600125 has been shown to inhibit some other protein kinases in addition to JNK (Bain et al. 2003), which may be a complicating factor. In addition, the effects of JNK inhibitors may be cell type and/or stimulus-specific.

The most consistent evidence lies for an involvement of p38 in the upregulation of TTP mRNA expression. In the present study, the p38 inhibitor, SB203580, decreased TTP mRNA levels in LPS-stimulated J774 macrophages. This inhibitor, as well as another p38 inhibitor SB202190, have been claimed to be quite specific p38 inhibitors (Davies et al. 2000), increasing the reliability of the data. Both of these inhibitors have been shown to decrease TTP mRNA and protein expression in mouse RAW264.7 macrophages (Mahtani et al. 2001, Brook et al. 2006, Chen et al. 2006). In primary mouse macrophages, SB202190 decreased LPS-induced TTP protein levels (Brook et al. 2006). In one study, SB203580 did not have any effect on TTP expression in mouse 3T3 preadipocytes (Inuzuka et al. 1999). The effects of these inhibitors have also been studied in human cells. SB202190 inhibited TTP protein expression in human monocytes and THP-1 cells exposed to LPS (Brooks et al. 2004, Brook et al. 2006). In CM-induced human DLD-1 cells, the levels of both TTP mRNA and protein were inhibited by SB203580 (Korhonen et al. 2007).

In addition, the effects of several antirheumatic drugs, aurothiomalate, hydroxychloroquine, methotrexate, D-penicillamine, leflunomide, and sulfasalazine, as well as those of paracetamol and nonselective and COX-2 selective non-steroidal anti-inflammatory drugs, and PPARα and PPARγ agonists on TTP mRNA expression were investigated. None of the compounds had any significant effect on TTP mRNA expression, indicating that cyclooxygenase products and PPARs are not involved in the upregulation of TTP mRNA expression, and that regulation of TTP is not involved in the mechanisms of the anti-inflammatory actions of these pharmaceuticals.

Based on the evidence reported in this thesis and in the literature, it would seem that NF-κB, serine/threonine phosphatase calcineurin, cyclooxygenase products, PPARs, and tyrosine phosphorylation are not crucial for the upregulation of TTP expression in mouse macrophages. The involvement of MAPK pathways seems evident, although the roles of JNK and ERK1/2 do need to be clarified. On the other hand, MAPK p38 has been shown to be a critical modulator of LPS-induced TTP expression in mouse macrophages.

# Effects of TTP down-regulation on cytokine production

In the present study, TTP expression was down-regulated by an siRNA against TTP. The reduction in TTP protein levels was functionally significant since it resulted in increased TNF-α production. A cytokine antibody array was used to detect the levels of 62 cytokines from the cell culture media of control and LPS-treated J774 macrophages. At the time of starting our experiments only a few cytokines had been reported to be targets of TTP, i.e. GM-CSF (Carballo et al. 2000, Stoecklin et al. 2001), IL-2 (Stoecklin et al. 2001, Ogilvie et al. 2005), IL-3 (Ming et al. 2001, Stoecklin et al. 2001, Stoecklin et al. 2003), IL-6 (Stoecklin et al. 2001, Sauer et al. 2006), TNF-α (Carballo et al. 1998, Lai et al. 2000, Stoecklin et al. 2001, Brooks et al. 2002, Brooks et al. 2004, Chen et al. 2006), and COX-2 (Sawaoka et al. 2003). After our study, also several other cytokines have been reported to be regulated by TTP, of which KC (CXCL1) (Datta et al. 2008), IL-8 (Suswam et al. 2008), IL-10 (Stoecklin et al. 2008), IL-13 (Barnstein et al. 2006), and vascular endothelial growth factor (VEGF) (Ciais et al. 2004, Essafi-Benkhadir et al. 2007, Suswam et al. 2008) were also included in our cytokine antibody array. The present study revealed that in TTP-deficient cells, IL-12 and MIP-2 expressions were enhanced, whereas MIP-3α levels were decreased, indicating that IL-12, MIP-2, and MIP-3α are novel targets of TTP.

IL-12 is a proinflammatory cytokine involved in the pathogenesis of autoimmune diseases (McInnes and Gracie 2004, Becker et al. 2005). IL-12 is a homodimer with two subunits, p35 and p40, and is designated as IL-12 p70 (Becker et al. 2005). The subunits are encoded by two distinct genes and the simultaneous expression of both genes is required to generate the biologically active heterodimer p70. Neither of the subunits is active on its own, but the p40 subunit can also form homodimers (IL-12 p80), which can function as endogenous IL-12 antagonists, as well as constituting a part of IL-23. On the other hand, the expression of the IL-12 p40 subunit was decreased when TTP expression was down-regulated. In macrophages, the p40 subunit is secreted in several-fold excess as compared to the p35-p40 heterodimer, and the formation of active IL-12 p70 is therefore regulated by the synthesis of the p35 subunit (Snijders et al. 1996). The mRNA of p35 subunit contains one copy of the preferred UUAUUUAUU nonamer, whereas none was found in the p40 mRNA. Therefore the target of TTP might be the p35 subunit, though this will have to be confirmed in further studies. Other studies have not shown IL-12 to be a target of TTP-mediated degradation.

A member of the CXC chemokine superfamily, MIP-2 (a homologue to human IL-8), is a potent chemoattractant for neutrophils (Driscoll 2000). In the present study, we found that MIP-2 production in response to LPS was higher in TTP knockdown cells than in control cells. A recent study reported that human IL-8 was a target of TTP-mediated mRNA destabilization (Suswam et al. 2008), increasing the evidence that MIP-2, a homologue to human IL-8, might also be a true target of TTP. The fact that the mRNA of MIP-2 contains three copies of the

78

preferred nonamer binding site, also increases the possibility that it may be a target of TTP-mediated degradation.

Chemokine MIP-3α has a potential role in rheumatoid arthritis (Schutyser et al. 2003) and the expression pattern of MIP-3α was different from the other targets found in the present study, as its expression was decreased in TTP knockdown cells as compared to control cells. The 3'-UTR of MIP-3α mRNA does not contain any nonamer binding sequences, suggesting that it may not be a direct target of TTP. Interestingly, at present, iNOS mRNA is the only other mRNA reported to be stabilized by TTP (Fechir et al. 2005). TTP did not bind to iNOS mRNA in bronchial epithelial cell line but it was shown to interact with another ARE-binding protein, KSRP, and by capturing KSRP and preventing its destabilizing effects, there was stabilization of iNOS mRNA (Fechir et al. 2005, Linker et al. 2005).

The cytokine antibody array represents a unique technique to identify novel targets of TTP-mediated decay. Most of the previous targets of TTP have been found by conventional methods. For example, the TTP-deficient mice were first shown to circulate elevated levels of TNF-α, and subsequently the stability of TNF-α mRNA in TTP KO mice was found to be elevated (Taylor et al. 1996, Carballo et al. 1998). Recently, microarray methods have been applied in the search of new target mRNAs (Lai et al. 2006, Stoecklin et al. 2008). In these studies, the half-life of several mRNAs was first detected by microarray from cells derived from WT or TTP KO mice, or the mRNAs that were immunoprecipitated with TTP were detected by microarray. The outcome of mRNA targets on protein level also need to be confirmed by other techniques, since other compensating mechanisms may restore the dysregulation of mRNA stability.


# Pharmacological regulation of TTP expression and the use of TTP as a target of drug development

The effects of several drugs and chemical compounds on TTP expression were investigated in the present study. Resting cells were used only in study II, when the effects of cAMP-enhancing compounds on TTP expression in macrophages were studied. The results on the regulation of TTP expression in resting macrophages are summarized in Figure 14.
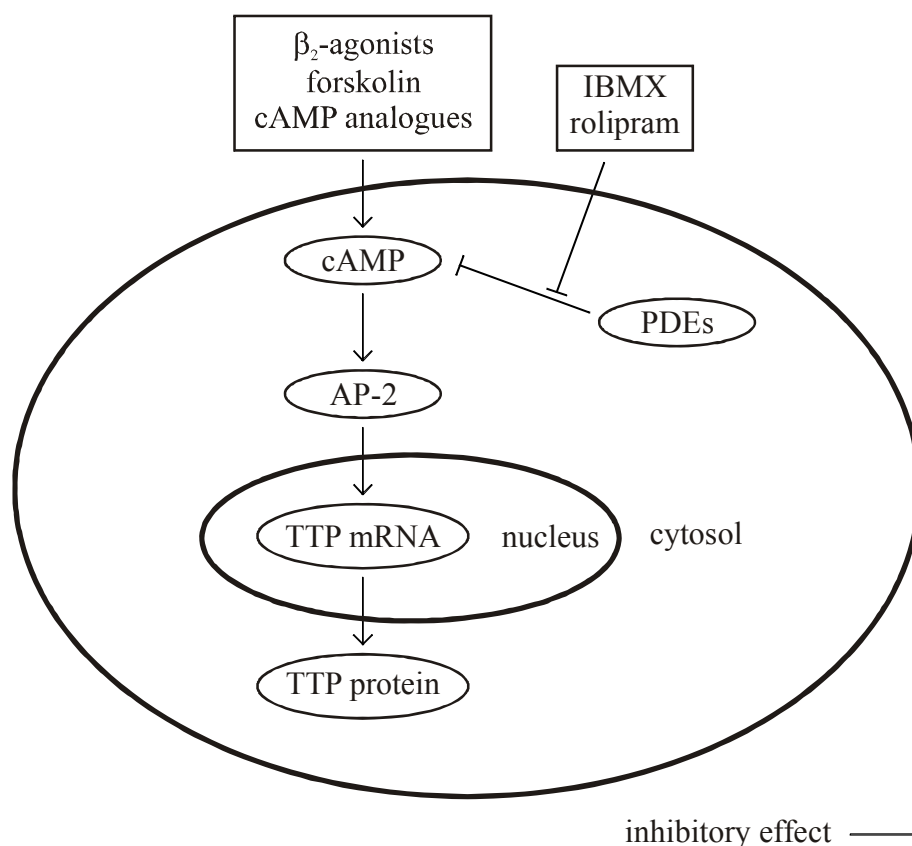
**Figure 14.** *Summary of the main results in resting macrophages (II). β₂-agonists, forskolin, and cAMP analogues increase or mimic intracellular cAMP and activate AP-2 translocation to the nucleus, where TTP mRNA transcription is initiated. The increase in TTP mRNA levels results in increased TTP protein expression. PDE, phosphodiesterase.*

In the three other studies, LPS was used to stimulate inflammatory responses in macrophages, and the effects of dexamethasone, cAMP-elevating agents, and several inhibitors were investigated. The results on the regulation of TTP expression in LPS-activated macrophages are summarized in Figure 15.

TTP is a fascinating therapeutic target. However, altered expression of TTP is likely to affect the stability of both anti-inflammatory and proinflammatory modulators. The amounts of other ARE-binding proteins and the interactions of TTP with these may also determine the outcome. The phosphorylation of TTP also reflects the functional activity of TTP. In some cases it could be beneficial to enhance TTP expression by vectors or by medicines in targeted cells, for example cancer cells overexpressing IL-3, TNF-α, or COX-2, or cells producing degenerative cytokines in rheumatic joints. The issue of the activity of phosphorylated TTP should also be examined carefully and taken into account when designing these overexpression vectors or other means to upregulate functional activity of TTP to shift the cytokine balance towards an anti-inflammatory direction.
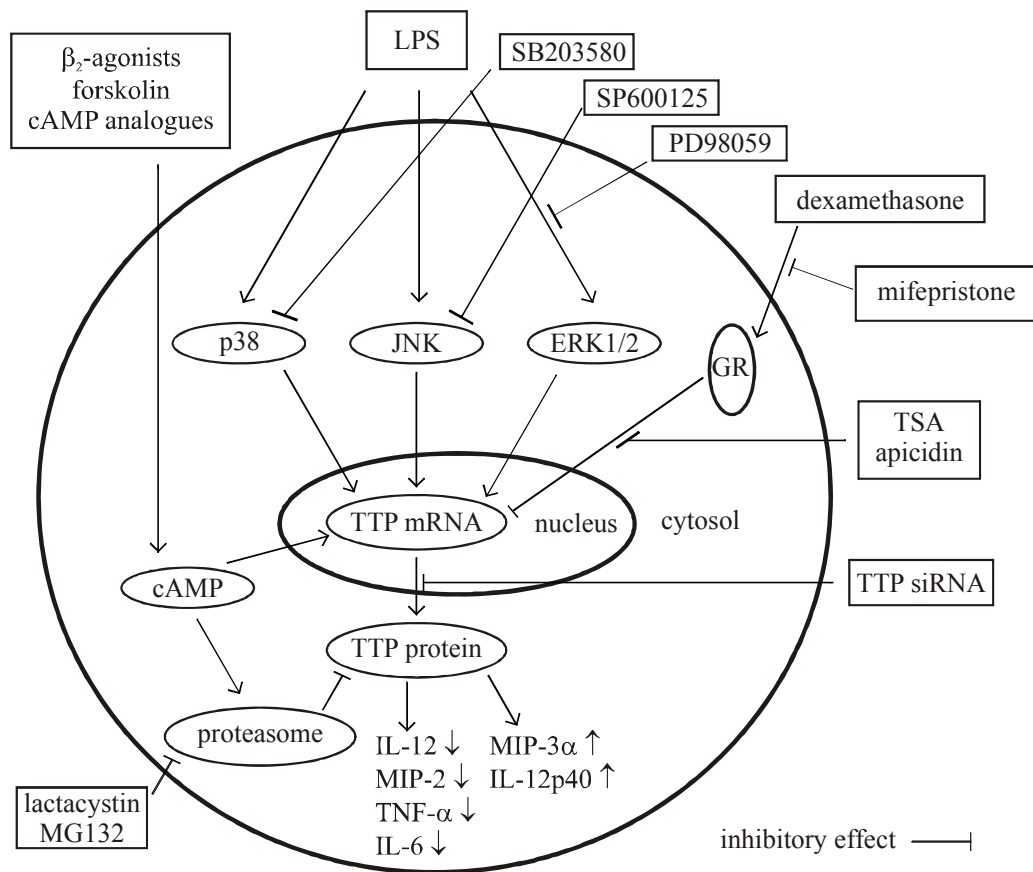
**Figure 15.** *Summary of the main results in LPS-treated macrophages (I, III, IV). Dexamethasone inhibits TTP expression through a glucocorticoid receptor (GR)-mediated mechanism possibly by histone deacetylation (I). β₂-agonists, forskolin, and cAMP analogues increase or mimic intracellular cAMP and increase TTP mRNA expression and TTP protein degradation (III). The increase in TTP protein expression may inhibit the expression of IL-12, MIP-2, TNF-α, and IL-6 as well as increase the levels of MIP-3α and IL-12p40 (IV).*

The quantification of the expression levels of ARE-binding proteins may also prove beneficial in the diagnosis of certain diseases, where cytokines produced by ARE-containing mRNAs are involved. In some cases, these expression patterns may provide data on the responsiveness to drug treatment.

Overall, TTP is a very interesting protein and a thorough understanding of the regulation of TTP expression as well as the inflammatory and immunological effects of TTP expression may lead to significant advances in drug development.

# Summary and conclusions

The present study was designed to investigate the regulation of TTP expression and the effects of TTP knockdown in macrophages. The major findings and conclusions are:

1. LPS induced TTP mRNA and protein expression as well as the expression of a number of cytokines in macrophages.

2. TTP mRNA levels were regulated by MAPK pathways and intracellular cAMP levels, but were not dependent on NF-κB, calcium/calmodulin-dependent phosphatase calcineurin, tyrosine phosphorylation, JAK2, cyclooxygenase products, or PPARs.

3. Dexamethasone and dissociated steroid RU24858 decreased LPS-induced TTP mRNA and protein expression through glucocorticoid receptors, but independently of the glucocorticoid response element. The results suggest that histone deacetylation and transcriptional silencing may be involved in the inhibition of TTP expression by dexamethasone and RU24858.

4. In resting macrophages, $\beta_2$-agonists, cAMP analogues, and forskolin enhanced TTP mRNA and protein expression. AP-2 relocation to the nucleus was detected prior to the increased TTP expression, which may serve as a mediator of cAMP-elevating agents in the upregulation of TTP expression.

5. In contrast, in macrophages activated by LPS, $\beta_2$-agonists, cAMP analogues, and forskolin increased TTP mRNA expression but protein expression was reduced. The results suggest that TTP protein undergoes a rapid degradation via the proteasome pathway, and that cAMP-enhancing compounds increase the rate of TTP protein degradation in macrophages exposed to inflammatory stimuli.

6. siRNA against TTP was used to down-regulate TTP expression. A cytokine antibody array identified IL-12, MIP-2, and MIP-3α as potential novel inflammatory cytokine targets, and regulation of TNF-α and IL-6 expression by TTP was confirmed.

There is increasing evidence that the post-transcriptional regulation of inflammatory genes, e.g. regulation of mRNA stability, is an important way to regulate their expression and represents a powerful drug target in inflammatory diseases. Therefore it is important to gain more knowledge on the mechanisms and factors, including tristetraprolin, which regulate the expression of inflammatory genes at the post-transcriptional level.

# Acknowledgements

I owe my deepest gratitude to my supervisor Professor Eeva Moilanen for all the guidance and encouragement during the years. I had not planned to do a doctoral thesis but after gaining some information on proteins that affect mRNA stability and the first experiments, I was on my way to delving deeper into the process of post-transcriptional regulation of mRNAs. There have been many moments when I have doubted my suitability for conducting research and questioned many decisions made during these years, but Eeva has always patiently listened and given me new insights such that I have grown to enjoy my work as a scientist. Working as a member of the research group has given me many opportunities to travel to national and international meetings to present my work and to meet colleagues.

I thank Professors Perry J. Blackshear and Jukka Mönkkönen for the careful review of the thesis and for their critical comments. Professor Seppo Nikkari and Docent Hannu Kankaanranta are acknowledged as members of my thesis committee and for the fruitful discussions we have had in our meetings. Dr. Ewen MacDonald and MSc Sirkka Nieminen are thanked for the careful checking of the English and Finnish language of this thesis, respectively.

I have had many memorable moments during these years in the Immunopharmacology Research Group together with the former and current members of the group. Thank you all! I would like to express my warm thanks to my co-authors Hannu Kankaanranta, Riku Korhonen, Aleksi Lahti, Tiina Leppänen, Riina Nieminen, Erja-Leena Paukkeri, and Katriina Vuolteenaho. I appreciate the technical assistance that Salla Hietakangas, Niina Ikonen, Marja Jousimies, Meiju Kukkonen, Eeva Lahtinen, Marja-Leena Lampén, Raija Pinola, and Jaana Tägtström have provided during these years. I thank Heli Määttä for all the help with computers and paper work. Time after time you have patiently helped me to solve all sorts of problems. Mari Hämäläinen and Outi Sareila are acknowledged for their enthusiasm towards sports and dragging me out for a run when I most needed the push.

Helen Cooper has proven to be a woman of great enthusiasm, which is contagious. It has been a privilege to be your deputy in TGSBB board as well as the vice president of LATTE. We have also organized several seminars and meetings together. Other organizers are also acknowledged as well as all the companies who have financially supported the meetings. I hope our co-operation continues in the future.

I wish to thank all my old and new friends, especially Heidi, Henna, Jenni, Mira, Outi, Paula, and Sanna, thanks for all the study groups while working towards MSc in Turku and all the activities we have had then and thereafter. Special thanks to Outi for also being a workmate, a guide to the wonderful world of orienteering, and for so often providing the place for our little Christmas parties and other occasions. I wish we could spend more time together.

My family as well as Mika's family are warmly thanked for all the support during these years, whether it is financial, baby-sitting, long walks and talks, cottage evenings, or help during the endless renovation of our house. Mika and Vilma - thanks for your love, support, and understanding.

Tampere, August 2008

Ulla Jalonen

# References

Adcock IM (2003): Glucocorticoids: new mechanisms and future agents. Curr Allergy Asthma Rep 3:249-257.

Aitken A (2006): 14-3-3 Proteins: a Historic Overview. Semin Cancer Biol 16:162-172.

Amann BT, Worthington MT and Berg JM (2003): A $Cys_3His$ zinc-binding domain from Nup475/tristetraprolin: a novel fold with a disklike structure. Biochemistry 42:217-221.

Amit I, Citri A, Shay T, Lu Y, Katz M, Zhang F, Tarcic G, Siwak D, Lahad J, Jacob-Hirsch J, Amariglio N, Vaisman N, Segal E, Rechavi G, Alon U, Mills GB, Domany E and Yarden Y (2007): A module of negative feedback regulators defines growth factor signaling. Nat Genet 39:503-512.

Anderson MT, Wims LA and Morrison SL (1991): The plasmacytoma J558L lacks constitutively active NF-κ B and is deficient in early response gene activation. Nucleic Acids Res 19:7235-7241.

Anderson P and Kedersha N (2006): RNA granules. J Cell Biol 172:803-808.

Arenander AT, de Vellis J and Herschman HR (1989a): Induction of c-fos and TIS genes in cultured rat astrocytes by neurotransmitters. J Neurosci Res 24:107-114.

Arenander AT, Lim RW, Varnum BC, Cole R, de Vellis J and Herschman HR (1989b): TIS gene expression in cultured rat astrocytes: induction by mitogens and stellation agents. J Neurosci Res 23:247-256.

Arenander AT, Lim RW, Varnum BC, Cole R, de Vellis J and Herschman HR (1989c): TIS gene expression in cultured rat astrocytes: multiple pathways of induction by mitogens. J Neurosci Res 23:257-265.

Bain J, McLauchlan H, Elliott M and Cohen P (2003): The specificities of protein kinase inhibitors: an update. Biochem J 371:199-204.

Barnard JA and Warwick G (1993): Butyrate rapidly induces growth inhibition and differentiation in HT-29 cells. Cell Growth Differ 4:495-501.

Barnard RC, Pascall JC, Brown KD, McKay IA, Williams NS and Bustin SA (1993): Coding sequence of ERF-1, the human homologue of Tis11b/cMG1, members of the Tis11 family of early response genes. Nucleic Acids Res 21:3580.

Barnes PJ (1998): Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci 94:557-572.

Barnstein BO, Li G, Wang Z, Kennedy S, Chalfant C, Nakajima H, Bunting KD and Ryan JJ (2006): Stat5 expression is required for IgE-mediated mast cell function. J Immunol 177:3421-3426.

Baseggio L, Charlot C, Bienvenu J, Felman P and Salles G (2002): Tumor necrosis factor-alpha mRNA stability in human peripheral blood cells after lipopolysaccharide stimulation. Eur Cytokine Netw 13:92-98.

Batistatou A, Volonté C and Greene LA (1992): Nerve growth factor employs multiple pathways to induce primary response genes in PC12 cells. Mol Biol Cell 3:363-371.

Baumgarten G, Knuefermann P, Kalra D, Gao F, Taffet GE, Michael L, Blackshear PJ, Carballo E, Sivasubramanian N and Mann DL (2002): Load-dependent and -independent regulation of proinflammatory cytokine and cytokine receptor gene expression in the adult mammalian heart. Circulation 105:2192-2197.

Beauchamp RD, Papaconstantinou J, Henderson AM, Sheng HM, Townsend CM Jr and Thompson JC (1994): Activation of hepatic proliferation-associated transcription factors by lipopolysaccharide. Surgery 116:367-376.

Becker C, Wirtz S and Neurath MF (2005): Stepwise regulation of $T_H1$ responses in autoimmunity: IL-12-related cytokines and their receptors. Inflamm Bowel Dis 11:755-764.

Benahmed M, Meresse B, Arnulf B, Barbe U, Mention JJ, Verkarre V, Allez M, Cellier C, Hermine O and Cerf-Bensussan N (2007): Inhibition of TGF-β signaling by IL-15: a new role for IL-15 in the loss of immune homeostasis in celiac disease. Gastroenterology 132:994-1008.

Bergmann MW, Staples KJ, Smith SJ, Barnes PJ and Newton R (2004): Glucocorticoid inhibition of granulocyte macrophage-colony-stimulating factor from T cells is independent of control by nuclear factor-κB and conserved lymphokine element 0. Am J Respir Cell Mol Biol 30:555-563.

Bianchini R, Nocentini G, Krausz LT, Fettucciari K, Coaccioli S, Ronchetti S and Riccardi C (2006): Modulation of pro- and antiapoptotic molecules in double-positive (CD4^{+}CD8^{+}) thymocytes following dexamethasone treatment. J Pharmacol Exp Ther 319:887-897.

Bissonnette EY and Befus AD (1997): Anti-inflammatory effect of $\beta_2$-agonists: inhibition of TNF-α release from human mast cells. J Allergy Clin Immunol 100:825-831.

Blackshear PJ (2002): Tristetraprolin and other CCCH tandem zinc-finger proteins in the regulation of mRNA turnover. Biochem Soc T 30:945-952.

Blackshear PJ, Lai WS, Kennington EA, Brewer G, Wilson GM, Guan X and Zhou P (2003): Characteristics of the interaction of a synthetic human tristetraprolin tandem zinc finger peptide with AU-rich element-containing RNA substrates. J Biol Chem 278:19947-19955.

Blackshear PJ, Phillips RS, Ghosh S, Ramos SBV, Richfield EK and Lai WS (2005): *Zfp36l3*, a rodent X chromosome gene encoding a placenta-specific member of the Tristetraprolin family of CCCH tandem zinc finger proteins. Biol Reprod 73:297-307.

Blackshear PJ, Phillips RS, Vazquez-Matias J and Mohrenweiser H (2003): Polymorphisms in the genes encoding members of the tristetraprolin family of human tandem CCCH zinc finger proteins. Prog Nucleic Acid Res Mol Biol 75:43-68.

Bouchard L, Tchernof A, Deshaies Y, Marceau S, Lescelleur O, Biron S and Vohl MC (2007a): *ZFP36*: a promising candidate gene for obesity-related metabolic complications identified by converging genomics. Obes Surg 17:372-382.

Bouchard L, Vohl MC, Deshaies Y, Rhéaume C, Daris M and Tchernof A (2007b): Visceral adipose tissue zinc finger protein 36 mRNA levels are correlated with insulin, insulin resistance index, and adiponectinemia in women. Eur J Endocrinol 157:451-457.

Boutaud O, Dixon DA, Oates JA and Sawaoka H (2003): Tristetraprolin binds to the COX-2 mRNA 3' untranslated region in cancer cells. Adv Exp Med Biol 525:157-160.

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Brewer BY, Ballin JD, Fialcowitz-White EJ, Blackshear PJ and Wilson GM (2006): Substrate dependence of conformational changes in the RNA-binding domain of tristetraprolin assessed by fluorescence spectroscopy of tryptophan mutants. Biochemistry 45:13807-13817.

Brewer BY, Malicka J, Blackshear PJ and Wilson GM (2004): RNA sequence elements required for high affinity binding by the zinc finger domain of tristetraprolin: conformational changes coupled to the bipartite nature of Au-rich MRNA-destabilizing motifs. J Biol Chem 279:27870-27877.

Briata P, Ilengo C, Corte G, Moroni C, Rosenfeld MG, Chen CY and Gherzi R (2003): The Wnt/β-catenin → Pitx2 pathway controls the turnover of Pitx2 and other unstable mRNAs. Mol Cell 12:1201-1211.

Broadley KJ (2006): β-adrenoceptor responses of the airways: For better or worse? Eur J Pharmacol 533:15-27.

Brook M, Tchen CR, Santalucia T, McIlrath J, Arthur JSC, Saklatvala J and Clark AR (2006): Posttranslational regulation of tristetraprolin subcellular localization and protein stability by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways. Mol Cell Biol 26:2408-2418.

Brooks SA, Connolly JE, Diegel RJ, Fava RA and Rigby WF (2002): Analysis of the function, expression, and subcellular distribution of human tristetraprolin. Arthritis Rheum 46:1362-1370.

Brooks SA, Connolly JE and Rigby WF (2004): The role of mRNA turnover in the regulation of tristetraprolin expression: evidence for an extracellular signal-regulated kinase-specific, AU-rich element-dependent, autoregulatory pathway. J Immunol 172:7263-7271.

Brown RS (2005): Zinc finger proteins: getting a grip on RNA. Curr Opin Struct Biol 15:94-98.

Bustin SA, Benes V, Nolan T and Pfaffl MW (2005): Quantitative real-time RT-PCR - a perspective. J Mol Endocrinol 34:597-601.

Cao H (2004): Expression, purification, and biochemical characterization of the antiinflammatory tristetraprolin: a zinc-dependent mRNA binding protein affected by posttranslational modifications. Biochemistry 43:13724-13738.

Cao H, Deterding LJ and Blackshear PJ (2007): Phosphorylation site analysis of the anti-inflammatory and mRNA-destabilizing protein tristetraprolin. Expert Rev Proteomics 4:711-726.

Cao H, Deterding LJ, Venable JD, Kennington EA, Yates JR,3rd, Tomer KB and Blackshear PJ (2006): Identification of the anti-inflammatory protein tristetraprolin as a hyperphosphorylated protein by mass spectrometry and site-directed mutagenesis. Biochem J 394:285-297.

Cao H, Dzineku F and Blackshear PJ (2003): Expression and purification of recombinant tristetraprolin that can bind to tumor necrosis factor-α mRNA and serve as a substrate for mitogen-activated protein kinases. Arch Biochem Biophys 412:106-120.

88

Cao H, Kelly MA, Kari F, Dawson HD, Urban JF Jr, Coves S, Roussel AM and Anderson RA (2007a): Green tea increases anti-inflammatory tristetraprolin and decreases pro-inflammatory tumor necrosis factor mRNA levels in rats. J Inflamm (Lond) 4:1.

Cao H, Polansky MM and Anderson RA (2007b): Cinnamon extract and polyphenols affect the expression of tristetraprolin, insulin receptor, and glucose transporter 4 in mouse 3T3-L1 adipocytes. Arch Biochem Biophys 459:214-222.

Cao H, Tuttle JS and Blackshear PJ (2004): Immunological characterization of tristetraprolin as a low abundance, inducible, stable cytosolic protein. J Biol Chem 279:21489-21499.

Carballo E and Blackshear PJ (2001): Roles of tumor necrosis factor-α receptor subtypes in the pathogenesis of the tristetraprolin-deficiency syndrome. Blood 98:2389-2395.

Carballo E, Cao H, Lai WS, Kennington EA, Campbell D and Blackshear PJ (2001): Decreased sensitivity of tristetraprolin-deficient cells to p38 inhibitors suggests the involvement of tristetraprolin in the p38 signaling pathway. J Biol Chem 276:42580-42587.

Carballo E, Lai WS and Blackshear PJ (1998): Feedback inhibition of macrophage tumor necrosis factor-α production by tristetraprolin. Science 281:1001-1005.

Carballo E, Lai WS and Blackshear PJ (2000): Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability. Blood 95:1891-1899.

Carman JA and Nadler SG (2004): Direct association of tristetraprolin with the nucleoporin CAN/Nup214. Biochem Bioph Res Co 315:445-449.

Carrick DM and Blackshear PJ (2007): Comparative expression of tristetraprolin (TTP) family member transcripts in normal human tissues and cancer cell lines. Arch Biochem Biophys 462:278-285.

Carrick DM, Chulada P, Donn R, Fabris M, McNicholl J, Whitworth W and Blackshear PJ (2006): Genetic variations in *ZFP36* and their possible relationship to autoimmune diseases. J Autoimmun 26:182-196.

Chang X, Fan Y, Karyala S, Schwemberger S, Tomlinson CR, Sartor MA and Puga A (2007): Ligand-independent regulation of transforming growth factor β1 expression and cell cycle progression by the aryl hydrocarbon receptor. Mol Cell Biol 27:6127-6139.

Chen CC (1993): Protein kinase C α, δ, ε and ζ in $C_6$ glioma cells. TPA induces translocation and down-regulation of conventional and new PKC isoforms but not atypical PKC ζ. FEBS Lett 332:169-173.

Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJM, Stoecklin G, Moroni C, Mann M and Karin M (2001): AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. Cell 107:451-464.

Chen CYA and Shyu AB (1995): AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem Sci 20:465-470.

Chen YL, Huang YL, Lin NY, Chen HC, Chiu WC and Chang CJ (2006): Differential regulation of ARE-mediated TNFα and IL-1β mRNA stability by lipopolysaccharide in RAW264.7 cells. Biochem Biophys Res Commun 346:160-168.

Chinn AM, Ciais D, Bailly S, Chambaz E, LaMarre J and Feige JJ (2002): Identification of two novel ACTH-responsive genes encoding manganese-dependent superoxide dismutase (SOD2) and the zinc finger protein TIS11b [tetradecanoyl phorbol acetate (TPA)-inducible sequence 11b]. Mol Endocrinol 16:1417-1427.

Chrestensen CA, Schroeder MJ, Shabanowitz J, Hunt DF, Pelo JW, Worthington MT and Sturgill TW (2004): MAPKAP kinase 2 phosphorylates tristetraprolin on *in vivo* sites including Ser[178], a site required for 14-3-3 binding. J Biol Chem 279:10176-10184.

Chung S, Jiang L, Cheng S and Furneaux H (1996): Purification and properties of HuD, a neuronal RNA-binding protein. J Biol Chem 271:11518-11524.

Ciais D, Cherradi N, Bailly S, Grenier E, Berra E, Pouyssegur J, LaMarre J and Feige JJ (2004): Destabilization of vascular endothelial growth factor mRNA by the zinc-finger protein TIS11b. Oncogene 23:8673-8680.

Clark AR (2007): Anti-inflammatory functions of glucocorticoid-induced genes. Mol Cell Endocrinol 275:79-97.

Cook HL, Mischo HE and Steitz JA (2004): The *Herpesvirus saimiri* small nuclear RNAs recruit AU-rich element-binding proteins but do not alter host AU-rich element-containing mRNA levels in virally transformed T cells. Mol Cell Biol 24:4522-4533.

Cooper P, Potter S, Mueck B, Yousefi S and Jarai G (2001): Identification of genes induced by inflammatory cytokines in airway epithelium. Am J Physiol -Lung C 280:L841-L852.

Corps AN and Brown KD (1995): Insulin and insulin-like growth factor I stimulate expression of the primary response gene cMG1/TIS11b by a wortmannin-sensitive pathway in RIE-1 cells. FEBS Lett 368:160-164.

Cousins RJ, Blanchard RK, Popp MP, Liu L, Cao J, Moore JB and Green CL (2003): A global view of the selectivity of zinc deprivation and excess on genes expressed in human THP-1 mononuclear cells. P Natl Acad Sci USA

Cuenda A and Rousseau S (2007): p38 MAP-kinases pathway regulation, function and role in human diseases. Biochim Biophys Acta 1773:1358-1375.

DaSilva L, Cote D, Roy C, Martinez M, Duniho S, Pitt MLM, Downey T and Dertzbaugh M (2003): Pulmonary gene expression profiling of inhaled ricin. Toxicon 41:813-822.

Datta S, Biswas R, Novotny M, Pavicic PG Jr, Herjan T, Mandal P and Hamilton TA (2008): Tristetraprolin Regulates CXCL1 (KC) mRNA Stability. J Immunol 180:2545-2552.

Davies SP, Reddy H, Caivano M and Cohen P (2000): Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95-105.

De J, Lai WS, Thorn JM, Goldsworthy SM, Liu X, Blackwell TK and Blackshear PJ (1999): Identification of four CCCH zinc finger proteins in *Xenopus*, including a novel vertebrate protein with four zinc fingers and severely restricted expression. Gene 228:133-145.

Dean JLE, Sully G, Clark AR and Saklatvala J (2004): The involvement of AU-rich element-binding proteins in p38 mitogen-activated protein kinase pathway-mediated mRNA stabilisation. Cell Signal 16:1113-1121.

Deleault KM, Skinner SJ and Brooks SA (2008): Tristetraprolin regulates TNF TNF-α mRNA stability via a proteasome dependent mechanism involving the combined action of the ERK and p38 pathways. Mol Immunol 45:13-24.

Derynck R, Gelbart WM, Harland RM, Heldin CH, Kern SE, Massagué J, Melton DA, Mlodzik M, Padgett RW, Roberts AB, Smith J, Thomsen GH, Vogelstein B and Wang XF (1996): Nomenclature: vertebrate mediators of TGFβ family signals. Cell 87:173.

DiStefano PS, Boulton TG, Stark JL, Zhu Y, Adryan KM, Ryan TE and Lindsay RM (1996): Ciliary neurotrophic factor induces down-regulation of its receptor and desensitization of

signal transduction pathways *in vivo*: non-equivalence with pharmacological activity. J Biol Chem 271:22839-22846.

diTargiani RC, Lee SJ, Wassink S and Michel SL (2006): Functional characterization of iron-substituted tristetraprolin-2D (TTP-2D, NUP475-2D): RNA binding affinity and selectivity. Biochemistry 45:13641-13649.

Dixon DA (2003): Regulation of COX-2 expression in human cancers. Prog Exp Tumor Res 37:52-71.

Doench JG, Petersen CP and Sharp PA (2003): siRNAs can function as miRNAs. Genes Dev 17:438-442.

Dorner S, Lum L, Kim M, Paro R, Beachy PA and Green R (2006): A genomewide screen for components of the RNAi pathway in *Drosophila* cultured cells. Proc Natl Acad Sci U S A 103:11880-11885.

Driscoll KE (2000): TNFα and MIP-2: role in particle-induced inflammation and regulation by oxidative stress. Toxicol Lett 112-113:177-183.

DuBois RN, Bishop PR, Graves-Deal R and Coffey RJ (1995): Transforming growth factor α regulation of two zinc finger-containing immediate early response genes in intestine. Cell Growth Differ 6:523-529.

DuBois RN, McLane MW, Ryder K, Lau LF and Nathans D (1990): A growth factor-inducible nuclear protein with a novel cysteine/histidine repetitive sequence. J Biol Chem 265:19185-19191.

Edman CF, Prigent SA, Schipper A and Feramisco JR (1997): Identification of ErbB3-stimulated genes using modified representational difference analysis. Biochem J 323:113-118.

Ehlting C, Lai WS, Schaper F, Brenndörfer ED, Matthes RJ, Heinrich PC, Ludwig S, Blackshear PJ, Gaestel M, Häussinger D and Bode JG (2007): Regulation of suppressor of cytokine signaling 3 (SOCS3) mRNA stability by TNF-α involves activation of the MKK6/p38$^{MAPK}$/MK2 cascade. J Immunol 178:2813-2826.

Ehrenfried JA, Townsend CM Jr, Thompson JC and Evers BM (1995): Increases in *nup475* and *c-jun* are early molecular events that precede the adaptive hyperplastic response after small bowel resection. Ann Surg 222:51-56.

Esclatine A, Taddeo B, Evans L and Roizman B (2004a): The herpes simplex virus 1 $U_L41$ gene-dependent destabilization of cellular RNAs is selective and may be sequence-specific. P Natl Acad Sci USA 101:3603-3608.

Esclatine A, Taddeo B and Roizman B (2004b): Herpes simplex virus 1 induces cytoplasmic accumulation of TIA-1/TIAR and both synthesis and cytoplasmic accumulation of tristetraprolin, two cellular proteins that bind and destabilize AU-rich RNAs. J Virol 78:8582-8592.

Esclatine A, Taddeo B and Roizman B (2004c): The $U_L41$ protein of herpes simplex virus mediates selective stabilization or degradation of cellular mRNAs. P Natl Acad Sci USA 101:18165-18170.

Essafi-Benkhadir K, Onesto C, Stebe E, Moroni C and Pagès G (2007): Tristetraprolin inhibits Ras-dependent tumor vascularization by inducing vascular endothelial growth factor mRNA degradation. Mol Biol Cell 18:4648-4658.

Eulalio A, Behm-Ansmant I and Izaurralde E (2007): P bodies: at the crossroads of post-transcriptional pathways. Nat Rev Mol Cell Biol 8:9-22.

Fabris M, Tolusso B, Di Poi E, Tomietto P, Sacco S, Gremese E and Ferraccioli G (2005): Mononuclear cell response to lipopolysaccharide in patients with rheumatoid arthritis: relationship with tristetraprolin expression. J Rheumatol 32:998-1005.

Fairhurst AM, Connolly JE, Hintz KA, Goulding NJ, Rassias AJ, Yeager MP, Rigby W and Wallace PK (2003): Regulation and localization of endogenous human tristetraprolin. Arthritis Res Ther 5:R214-R225.

Fechir M, Linker K, Pautz A, Hubrich T, Förstermann U, Rodriguez-Pascual F and Kleinert H (2005): Tristetraprolin regulates the expression of the human inducible nitric oxide synthase gene. Mol Pharmacol 67:2148-2161.

Fenger-Grøn M, Fillman C, Norrild B and Lykke-Andersen J (2005): Multiple processing body factors and the ARE binding protein TTP activate mRNA decapping. Mol Cell 20:905-915.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE and Mello CC (1998): Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806-811.

Franks TM and Lykke-Andersen J (2007): TTP and BRF proteins nucleate processing body formation to silence mRNAs with AU-rich elements. Genes Dev 21:719-735.

Frasca D, Landin AM, Alvarez JP, Blackshear PJ, Riley RL and Blomberg BB (2007): Tristetraprolin, a negative regulator of mRNA stability, is increased in old B cells and is involved in the degradation of E47 mRNA. J Immunol 179:918-927.

Fujihara M, Muroi M, Tanamoto K, Suzuki T, Azuma H and Ikeda H (2003): Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. Pharmacol Ther 100:171-194.

Fukae J, Amasaki Y, Yamashita Y, Bohgaki T, Yasuda S, Jodo S, Atsumi T and Koike T (2005): Butyrate suppresses tumor necrosis factor α production by regulating specific messenger RNA degradation mediated through a *cis*-acting AU-rich element. Arthritis Rheum 52:2697-2707.

Garneau NL, Wilusz J and Wilusz CJ (2007): The highways and byways of mRNA decay. Nat Rev Mol Cell Biol 8:113-126.

Giembycz MA and Newton R (2006): Beyond the dogma: novel $\beta_2$-adrenoceptor signalling in the airways. Eur Respir J 27:1286-1306.

Gringhuis SI, García-Vallejo JJ, van het Hof B and van Dijk W (2005): Convergent actions of IκB kinase β and protein kinase Cδ modulate mRNA stability through phosphorylation of 14-3-3 β complexed with tristetraprolin. Mol Cell Biol 25:6454-6463.

Gubits RM, Burke RE, Casey-McIntosh G, Bandele A and Munell F (1993a): Immediate early gene induction after neonatal hypoxia-ischemia. Brain Res Molecular Brain Research. 18:228-238.

Gubits RM, Geard CR and Schiff PB (1993b): Expression of immediate early genes after treatment of human astrocytoma cells with radiation and taxol. Int J Radiat Oncol 27:637-642.

Gueydan C, Droogmans L, Chalon P, Huez G, Caput D and Kruys V (1999): Identification of TIAR as a protein binding to the translational regulatory AU-rich element of tumor necrosis factor α mRNA. J Biol Chem 274:2322-2326.

Guhaniyogi J and Brewer G (2001): Regulation of mRNA stability in mammalian cells. Gene 265:11-23.

Haab BB (2006): Applications of antibody array platforms. Curr Opin Biotechnol 17:415-421.

Harkin DP, Bean JM, Miklos D, Song YH, Truong VB, Englert C, Christians FC, Ellisen LW, Maheswaran S, Oliner JD and Haber DA (1999): Induction of *GADD45* and JNK/SAPK-dependent apoptosis following inducible expression of *BRCA1* . Cell 97:575-586.

Hau HH, Walsh RJ, Ogilvie RL, Williams DA, Reilly CS and Bohjanen PR (2007): Tristetraprolin recruits functional mRNA decay complexes to ARE sequences. J Cell Biochem 100:1477-1492.

Heximer SP, Cristillo AD, Russell L and Forsdyke DR (1998): Expression and processing of $G_0/G_1$ *switch gene 24 (G0S24/TIS11/TTP/NUP475)* RNA in cultured human blood mononuclear cells. DNA Cell Biol 17:249-263.

Heximer SP and Forsdyke DR (1993): A human putative lymphocyte $G_0/G_1$ switch gene homologous to a rodent gene encoding a zinc-binding potential transcription factor. DNA Cell Biol 12:73-88.

Hikoso S, Yamaguchi O, Higuchi Y, Hirotani S, Takeda T, Kashiwase K, Watanabe T, Taniike M, Tsujimoto I, Asahi M, Matsumura Y, Nishida K, Nakajima H, Akira S, Hori M and Otsu K (2004): Pressure overload induces cardiac dysfunction and dilation in signal transducer and activator of transcription 6-deficient mice. Circulation 110:2631-2637.

Hitti E, Iakovleva T, Brook M, Deppenmeier S, Gruber AD, Radzioch D, Clark AR, Blackshear PJ, Kotlyarov A and Gaestel M (2006): 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 26:2399-2407.

Hollams EM, Giles KM, Thomson AM and Leedman PJ (2002): mRNA stability and the control of gene expression: implications for human disease. Neurochem Res 27:957-980.

Honkaniemi J, Kononen J, Kainu T, Pyykönen I and Pelto-Huikko M (1994): Induction of multiple immediate early genes in rat hypothalamic paraventricular nucleus after stress. Brain Res Molecular Brain Research. 25:234-241.

Hudson BP, Martinez-Yamout MA, Dyson HJ and Wright PE (2004): Recognition of the mRNA AU-rich element by the zinc finger domain of TIS11d. Nat Struct Mol Biol 11:257-264.

Imagawa M, Chiu R and Karin M (1987): Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell 51:251-260.

Inuzuka H, Nanbu-Wakao R, Masuho Y, Muramatsu M, Tojo H and Wakao H (1999): Differential regulation of immediate early gene expression in preadipocyte cells through multiple signaling pathways. Biochem Bioph Res Co 265:664-668.

Ito K, Barnes PJ and Adcock IM (2000): Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1β-induced histone H4 acetylation on lysines 8 and 12. Mol Cell Biol 20:6891-6903.

Ito K, Chung KF and Adcock IM (2006): Update on glucocorticoid action and resistance. J Allergy Clin Immunol 117:522-543.

Ito K, Jazrawi E, Cosio B, Barnes PJ and Adcock IM (2001): p65-activated histone acetyltransferase activity is repressed by glucocorticoids: mifepristone fails to recruit HDAC2 to the p65-HAT complex. J Biol Chem 276:30208-30215.

Jalonen U, Lahti A, Korhonen R, Kankaanranta H and Moilanen E (2005): Inhibition of tristetraprolin expression by dexamethasone in activated macrophages. Biochem Pharmacol 69:733-740.

Jalonen U, Leppänen T, Kankaanranta H and Moilanen E (2007): Salbutamol increases tristetraprolin expression in macrophages. Life Sci 81:1651-1658.

Jalonen U, Paukkeri EL and Moilanen E (2008): Compounds that increase or mimic cyclic adenosine monophosphate enhance tristetraprolin degradation in lipopolysaccharide-treated murine J774 macrophages. J Pharmacol Exp Ther 326:514-522.

Jean S, Bideau C, Bellon L, Halimi G, De Méo M, Orsière T, Dumenil G, Bergé-Lefranc JL and Botta A (2001): The expression of genes induced in melanocytes by exposure to 365-nm UVA: study by cDNA arrays and real-time quantitative RT-PCR. Biochim Biophys Acta 1522:89-96.

Jerala R (2007): Structural biology of the LPS recognition. Int J Med Microbiol 297:353-363.

Jing Q, Huang S, Guth S, Zarubin T, Motoyama A, Chen J, Di Padova F, Lin SC, Gram H and Han J (2005): Involvement of microRNA in AU-rich element-mediated mRNA instability. Cell 120:623-634.

Johnson BA and Blackwell TK (2002): Multiple tristetraprolin sequence domains required to induce apoptosis and modulate responses to TNF$\alpha$ through distinct pathways. Oncogene 21:4237-4246.

Johnson BA, Geha M and Blackwell TK (2000): Similar but distinct effects of the tristetraprolin/TIS11 immediate-early proteins on cell survival. Oncogene 19:1657-1664.

Johnson BA, Stehn JR, Yaffe MB and Blackwell TK (2002): Cytoplasmic localization of tristetraprolin involves 14-3-3-dependent and -independent mechanisms. J Biol Chem 277:18029-18036.

Johnson M (2002): Effects of $\beta_2$-agonists on resident and infiltrating inflammatory cells. J Allergy Clin Immunol 110:S282-90.

Johnson M (2006): Molecular mechanisms of $\beta_2$-adrenergic receptor function, response, and regulation. J Allergy Clin Immunol 117:18-24.

Kaneda N, Murata T, Niimi Y and Minamiyama M (2000): Cloning and characterization of the rat TIS11 gene. Mol Cell Biochem 213:119-126.

Kaneda N, Oshima M, Chung SY and Guroff G (1992): Sequence of a rat *TIS11* cDNA, an immediate early gene induced by growth factors and phorbol esters. Gene 118:289-291.

Kedersha N and Anderson P (2002): Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. Biochem Soc T 30:963-969.

Kedersha N and Anderson P (2007): Mammalian stress granules and processing bodies. Methods Enzymol 431:61-81.

Kedersha N, Stoecklin G, Ayodele M, Yacono P, Lykke-Andersen J, Fitzler MJ, Scheuner D, Kaufman RJ, Golan DE and Anderson P (2005): Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. J Cell Biol 169:871-884.

Kelly JF, Elias CF, Lee CE, Ahima RS, Seeley RJ, Bjørbæk C, Oka T, Saper CB, Flier JS and Elmquist JK (2004): Ciliary neurotrophic factor and leptin induce distinct patterns of immediate early gene expression in the brain. Diabetes 53:911-920.

Khabar KSA (2005): The AU-rich transcriptome: more than interferons and cytokines, and its role in disease. J Interferon Cytokine Res 25:1-10.

Kong Y, Ruan L, Ma L, Cui Y, Wang JM and Le Y (2007): RNA interference as a novel and powerful tool in immunopharmacological research. Int Immunopharmacol 7:417-426.

Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F and Kollias G (1999): Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. Immunity 10:387-398.

Korhonen R, Lahti A, Hämäläinen M, Kankaanranta H and Moilanen E (2002): Dexamethasone inhibits inducible nitric-oxide synthase expression and nitric oxide production by destabilizing mRNA in lipopolysaccharide-treated macrophages. Mol Pharmacol 62:698-704.

Korhonen R, Lahti A, Kankaanranta H and Moilanen E (2005): Nitric oxide production and signaling in inflammation. Curr Drug Targets Inflamm Allergy 4:471-479.

Korhonen R, Linker K, Pautz A, Förstermann U, Moilanen E and Kleinert H (2007): Post-transcriptional regulation of human inducible nitric-oxide synthase expression by the Jun N-terminal kinase. Mol Pharmacol 71:1427-1434.

Kujubu DA, Lim RW, Varnum BC and Herschman HR (1987): Induction of transiently expressed genes in PC-12 pheochromocytoma cells. Oncogene 1:257-262.

Lahti A, Jalonen U, Kankaanranta H and Moilanen E (2003): c-Jun NH$_2$-terminal kinase inhibitor anthra(1,9-*cd*)pyrazol-6(2H)-one reduces inducible nitric-oxide synthase expression by destabilizing mRNA in activated macrophages. Mol Pharmacol 64:308-315.

Lahti A, Sareila O, Kankaanranta H and Moilanen E (2006): Inhibition of p38 mitogen-activated protein kinase enhances c-Jun N-terminal kinase activity: implication in inducible nitric oxide synthase expression. BMC Pharmacol 6:5.

Lai WS and Blackshear PJ (2001): Interactions of CCCH zinc finger proteins with mRNA: tristetraprolin-mediated AU-rich element-dependent mRNA degradation can occur in the absence of a poly(A) tail. J Biol Chem 276:23144-23154.

Lai WS, Carballo E, Strum JR, Kennington EA, Phillips RS and Blackshear PJ (1999): Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. Mol Cell Biol 19:4311-4323.

Lai WS, Carballo E, Thorn JM, Kennington EA and Blackshear PJ (2000): Interactions of CCCH zinc finger proteins with mRNA. Binding of tristetraprolin-related zinc finger proteins to AU-rich elements and destabilization of mRNA. J Biol Chem 275:17827-17837.

Lai WS, Carrick DM and Blackshear PJ (2005): Influence of Nonameric AU-rich tristetraprolin-binding sites on mRNA deadenylation and turnover. J Biol Chem 280:34365-34377.

Lai WS, Kennington EA and Blackshear PJ (2002): Interactions of CCCH zinc finger proteins with mRNA: non-binding tristetraprolin mutants exert an inhibitory effect on degradation of AU-rich element-containing mRNAs. J Biol Chem 277:9606-9613.

Lai WS, Kennington EA and Blackshear PJ (2003): Tristetraprolin and its family members can promote the cell-free deadenylation of AU-rich element-containing mRNAs by poly(A) ribonuclease. Mol Cell Biol 23:3798-3812.

Lai WS, Parker JS, Grissom SF, Stumpo DJ and Blackshear PJ (2006): Novel mRNA targets for tristetraprolin (TTP) identified by global analysis of stabilized transcripts in TTP-deficient fibroblasts. Mol Cell Biol 26:9196-9208.

Lai WS, Stumpo DJ and Blackshear PJ (1990): Rapid insulin-stimulated accumulation of an mRNA encoding a proline-rich protein. J Biol Chem 265:16556-16563.

Lai WS, Thompson MJ and Blackshear PJ (1998): Characteristics of the intron involvement in the mitogen-induced expression of *Zfp-36*. J Biol Chem 273:506-517.

Lai WS, Thompson MJ, Taylor GA, Liu Y and Blackshear PJ (1995): Promoter analysis of *Zfp-36*, the mitogen-inducible gene encoding the zinc finger protein tristetraprolin. J Biol Chem 270:25266-25272.

Landesberg LJ, Ramalingam R, Lee K, Rosengart TK and Crystal RG (2001): Upregulation of transcription factors in lung in the early phase of postpneumonectomy lung growth. Am J Physiol -Lung C 281:L1138-L1149.

Laroia G, Cuesta R, Brewer G and Schneider RJ (1999): Control of mRNA decay by heat shock-ubiquitin-proteasome pathway. Science 284:499-502.

Laroia G, Sarkar B and Schneider RJ (2002): Ubiquitin-dependent mechanism regulates rapid turnover of AU-rich cytokine mRNAs. P Natl Acad Sci USA 99:1842-1846.

Lasa M, Brook M, Saklatvala J and Clark AR (2001): Dexamethasone destabilizes cyclooxygenase 2 mRNA by inhibiting mitogen-activated protein kinase p38. Mol Cell Biol 21:771-780.

Lau LF and Nathans D (1987): Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with *c-fos* or *c-myc*. P Natl Acad Sci USA 84:1182-1186.

Ledwith BJ, Johnson TE, Wagner LK, Pauley CJ, Manam S, Galloway SM and Nichols WW (1996): Growth regulation by peroxisome proliferators: opposing activities in early and late $G_1$. Cancer Res 56:3257-3264.

Lee HJ, Lee DY, Joo WA, Sul D, Lee E and Kim CW (2004): Differential expression of proteins in rat plasma exposed to benzene. Proteomics 4:3498-3504.

Leppänen T, Jalonen U, Kankaanranta H, Tuominen R and Moilanen E (2008): Inhibition of protein kinase C βII downregulates tristetraprolin expression in activated macrophages. Inflamm Res 57:230-240.

Levine TD, Gao F, King PH, Andrews LG and Keene JD (1993): Hel-N1: an autoimmune RNA-binding protein with specificity for 3' uridylate-rich untranslated regions of growth factor mRNAs. Mol Cell Biol 13:3494-3504.

Li X, Wong J, Tsai SY, Tsai MJ and O'Malley BW (2003): Progesterone and glucocorticoid receptors recruit distinct coactivator complexes and promote distinct patterns of local chromatin modification. Mol Cell Biol 23:3763-3773.

Liao HJ, de Los Santos J and Carpenter G (2006): Contrasting role of phospholipase C-γ1 in the expression of immediate early genes induced by epidermal or platelet-derived growth factors. Exp Cell Res 312:807-816.

Lim RW, Varnum BC and Herschman HR (1987): Cloning of tetradecanoyl phorbol ester-induced 'primary response' sequences and their expression in density-arrested Swiss 3T3 cells and a TPA non-proliferative variant. Oncogene 1:263-270.

Lin NY, Lin CT, Chen YL and Chang CJ (2007): Regulation of tristetraprolin during differentiation of 3T3-L1 preadipocytes. FEBS J 274:867-878.

Linker K, Pautz A, Fechir M, Hubrich T, Greeve J and Kleinert H (2005): Involvement of KSRP in the post-transcriptional regulation of human iNOS expression-complex interplay of KSRP with TTP and HuR. Nucleic Acids Res 33:4813-4827.

Linn SC, Morelli PJ, Edry I, Cottongim SE, Szabó C and Salzman AL (1997): Transcriptional regulation of human inducible nitric oxide synthase gene in an intestinal epithelial cell line. Am J Physiol 272:G1499-508.

Lu JY and Schneider RJ (2004): Tissue distribution of AU-rich mRNA-binding proteins involved in regulation of mRNA decay. J Biol Chem 279:12974-12979.

Lykke-Andersen J and Wagner E (2005): Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1. Gene Dev 19:351-361.

Ma Q and Herschman HR (1991): A corrected sequence for the predicted protein from the mitogen-inducible TIS11 primary response gene. Oncogene 6:1277-1278.

Ma Q and Herschman HR (1995): The yeast homologue *YTIS11*, of the mammalian *TIS11* gene family is a non-essential, glucose repressible gene. Oncogene 10:487-494.

Ma WJ, Cheng S, Campbell C, Wright A and Furneaux H (1996): Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. J Biol Chem 271:8144-8151.

MacKenzie S, Fernàndez-Troy N and Espel E (2002): Post-transcriptional regulation of TNF-α during in vitro differentiation of human monocytes/macrophages in primary culture. J Leukocyte Biol 71:1026-1032.

Maclean KN, McKay IA and Bustin SA (1998): Differential effects of sodium butyrate on the transcription of the human TIS11 family of early-response genes in colorectal cancer cells. Brit J Biomed Sci 55:184-191.

Maclean KN, See CG, McKay IA and Bustin SA (1995): The human immediate early gene *BRF1* maps to chromosome 14q22-q24. Genomics 30:89-90.

MacMicking J, Xie QW and Nathan C (1997): Nitric oxide and macrophage function. Annu Rev Immunol 15:323-350.

Maeda M, Sawa H, Tobiume M, Tokunaga K, Hasegawa H, Ichinohe T, Sata T, Moriyama M, Hall WW, Kurata T and Takahashi H (2006): Tristetraprolin inhibits HIV-1 production by binding to genomic RNA. Microbes Infect 8:2647-2656.

Mahtani KR, Brook M, Dean JL, Sully G, Saklatvala J and Clark AR (2001): 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 21:6461-6469.

Marderosian M, Sharma A, Funk AP, Vartanian R, Masri J, Jo OD and Gera JF (2006): Tristetraprolin regulates Cyclin D1 and c-*Myc* mRNA stability in response to rapamycin in an Akt-dependent manner via p38 MAPK signaling. Oncogene 25:6277-6290.

Martin SE and Caplen NJ (2007): Applications of RNA interference in mammalian systems. Annu Rev Genomics Hum Genet 8:81-108.

McInnes IB and Gracie JA (2004): Targeting cytokines beyond tumor necrosis factor-α and interleukin-1 in rheumatoid arthritis. Curr Rheumatol Rep 6:336-342.

Mello CC, Draper BW, Krause M, Weintraub H and Priess JR (1992): The *pie-1* and *mex-1* genes and maternal control of blastomere identity in early *C. elegans* embryos. Cell 70:163-176.

Mesner PW, Epting CL, Hegarty JL and Green SH (1995): A timetable of events during programmed cell death induced by trophic factor withdrawal from neuronal PC12 cells. J Neurosci 15:7357-7366.

Michel SL, Guerrerio AL and Berg JM (2003): Selective RNA binding by a single CCCH zinc-binding domain from Nup475 (Tristetraprolin). Biochemistry (N Y ) 42:4626-4630.

Ming XF, Stoecklin G, Lu M, Looser R and Moroni C (2001): Parallel and independent regulation of interleukin-3 mRNA turnover by phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase. Mol Cell Biol 21:5778-5789.

Mittelstadt PR and DeFranco AL (1993): Induction of early response genes by cross-linking membrane Ig on B lymphocytes. J Immunol 150:4822-4832.

Moncada S, Palmer RM and Higgs EA (1991): Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 43:109-142.

Mrusek S, Classen-Linke I, Vloet A, Beier HM and Krusche CA (2005): Estradiol and medroxyprogesterone acetate regulated genes in T47D breast cancer cells. Mol Cell Endocrinol 235:39-50.

Mukherjee D, Gao M, O'Connor JP, Raijmakers R, Pruijn G, Lutz CS and Wilusz J (2002): The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. EMBO J 21:165-174.

Muramatsu H, Welsh FA and Karikó K (2006): Cerebral preconditioning using cortical application of hypertonic salt solutions: upregulation of mRNAs encoding inhibitors of inflammation. Brain Res 1097:31-38.

Murata T, Hikita K and Kaneda N (2000): Transcriptional activation function of zinc finger protein TIS11 and its negative regulation by phorbol ester. Biochem Bioph Res Co 274:526-532.

Murata T, Morita N, Hikita K, Kiuchi K, Kiuchi K and Kaneda N (2005): Recruitment of mRNA-destabilizing protein TIS11 to stress granules is mediated by its zinc finger domain. Exp Cell Res 303:287-299.

Murata T, Niimi Y and Kaneda N (2000): Characterization of the 5'-flanking region of the rat TIS11 gene. Mol Cell Biochem 214:1-6.

Murata T, Yoshino Y, Morita N and Kaneda N (2002): Identification of nuclear import and export signals within the structure of the zinc finger protein TIS11. Biochem Bioph Res Co 293:1242-1247.

Nakajima K and Wall R (1991): Interleukin-6 signals activating *junB* and TIS11 gene transcription in a B-cell hybridoma. Mol Cell Biol 11:1409-1418.

Newton R (2000): Molecular mechanisms of glucocorticoid action: what is important? Thorax 55:603-613.

Newton R, Stevens DA, Hart LA, Lindsay M, Adcock IM and Barnes PJ (1997): Superinduction of COX-2 mRNA by cycloheximide and interleukin-1β involves increased transcription and correlates with increased NF-κB and JNK activation. FEBS Lett 418:135-138.

Ogawa K, Chen F, Kim YJ and Chen Y (2003): Transcriptional Regulation of Tristetraprolin by Transforming Growth Factor-β in Human T Cells. J Biol Chem 278:30373-30381.

Ogilvie RL, Abelson M, Hau HH, Vlasova I, Blackshear PJ and Bohjanen PR (2005): Tristetraprolin down-regulates *IL-2* gene expression through AU-rich element-mediated mRNA decay. J Immunol 174:953-961.

Park-Lee S, Kim S and Laird-Offringa IA (2003): Characterization of the interaction between neuronal RNA-binding protein HuD and AU-rich RNA. J Biol Chem 278:39801-39808.

Patino WD, Kang JG, Matoba S, Mian OY, Gochuico BR and Hwang PM (2006): Atherosclerotic plaque macrophage transcriptional regulators are expressed in blood and modulated by tristetraprolin. Circ Res 98:1282-1289.

Peng SSY, Chen CYA and Shyu AB (1996): Functional characterization of a non-AUUUA AU-rich element from the c-*jun* proto-oncogene mRNA: evidence for a novel class of AU-rich elements. Mol Cell Biol 16:1490-1499.

Peng X, Greene LA, Kaplan DR and Stephens RM (1995): Deletion of a conserved juxtamembrane sequence in Trk abolishes NGF-promoted neuritogenesis. Neuron 15:395-406.

Phillips K, Kedersha N, Shen L, Blackshear PJ and Anderson P (2004): Arthritis suppressor genes TIA-1 and TTP dampen the expression of tumor necrosis factor α, cyclooxygenase 2, and inflammatory arthritis. P Natl Acad Sci USA 101:2011-2016.

Phillips RS, Ramos SBV and Blackshear PJ (2002): Members of the tristetraprolin family of tandem CCCH zinc finger proteins exhibit CRM1-dependent nucleocytoplasmic shuttling. J Biol Chem 277:11606-11613.

Piecyk M, Wax S, Beck ARP, Kedersha N, Gupta M, Maritim B, Chen S, Gueydan C, Kruys V, Streuli M and Anderson P (2000): TIA-1 is a translational silencer that selectively regulates the expression of TNF-α. EMBO J 19:4154-4163.

Priller J, Haas CA, Reddington M and Kreutzberg GW (1995): Calcitonin gene-related peptide and ATP induce immediate early gene expression in cultured rat microglial cells. Glia 15:447-457.

Priller J, Haas CA, Reddington M and Kreutzberg GW (1998a): Cultured astrocytes express functional receptors for galanin. Glia 24:323-328.

Priller J, Reddington M, Haas CA and Kreutzberg GW (1998b): Stimulation of $P_{2Y}$-purinoceptors on astrocytes results in immediate early gene expression and potentiation of neuropeptide action. Neuroscience 85:521-525.

Raghavan A, Robison RL, McNabb J, Miller CR, Williams DA and Bohjanen PR (2001): HuA and tristetraprolin are induced following T cell activation and display distinct but overlapping RNA binding specificities. J Biol Chem 276:47958-47965.

Rajakariar R, Yaqoob MM and Gilroy DW (2006): COX-2 in inflammation and resolution. Mol Interv 6:199-207.

Rang HP, Dale MM, Ritter JM and Flower RJ (2007a): Local hormones, inflammation and immune reactions. In: Rang and Dale's pharmacology, pp. 202-225. Churchill Livingstone Elsevier Science, Philadelphia.

Rang HP, Dale MM, Ritter JM and Flower RJ (2007b): The pituitary and the adrenal cortex. In: Rang and Dale's pharmacology, pp. 420-436. Churchill Livingstone Elsevier Science, Philadelphia.

Rang HP, Dale MM, Ritter JM and Flower RJ (2007c): The respiratory system. In: Rang and Dale's pharmacology, pp. 356-367. Churchill Livingstone Elsevier Science, Philadelphia.

Rigby WFC, Roy K, Collins J, Rigby S, Connolly JE, Bloch DB and Brooks SA (2005): Structure/function analysis of tristetraprolin (TTP): p38 stress-activated protein kinase and lipopolysaccharide stimulation do not alter TTP function. J Immunol 174:7883-7893.

Ristimäki A, Narko K and Hla T (1996): Down-regulation of cytokine-induced cyclo-oxygenase-2 transcript isoforms by dexamethasone: evidence for post-transcriptional regulation. Biochem J 318:325-331.

Ronkina N, Kotlyarov A, Dittrich-Breiholz O, Kracht M, Hitti E, Milarski K, Askew R, Marusic S, Lin LL, Gaestel M and Telliez JB (2007): 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 27:170-181.

Sabroe I, Parker LC, Dockrell DH, Davies DE, Dower SK and Whyte MK (2007): Targeting the networks that underpin contiguous immunity in asthma and chronic obstructive pulmonary disease. Am J Respir Crit Care Med 175:306-311.

Sachidanandan C, Sambasivan R and Dhawan J (2002): Tristetraprolin and LPS-inducible CXC chemokine are rapidly induced in presumptive satellite cells in response to skeletal muscle injury. J Cell Sci 115:2701-2712.

Sacks AI, Warwick GJ and Barnard JA (1995): Early proliferative events following intestinal resection in the rat. J Pediatr Gastr Nutr 21:158-164.

Sakai K, Gofuku M, Kitagawa Y, Ogasawara T, Hirose G, Yamazaki M, Koh CS, Yanagisawa N and Steinman L (1994): A hippocampal protein associated with paraneoplastic neurologic syndrome and small cell lung carcinoma. Biochem Biophys Res Commun 199:1200-1208.

Saklatvala J (2004): The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. Curr Opin Pharmacol 4:372-377.

Sauer I, Schaljo B, Vogl C, Gattermeier I, Kolbe T, Müller M, Blackshear PJ and Kovarik P (2006): Interferons limit inflammatory responses by induction of tristetraprolin. Blood 107:4790-4797.

Sawaoka H, Dixon DA, Oates JA and Boutaud O (2003): Tristetraprolin binds to the 3'-untranslated region of cyclooxygenase-2 mRNA. A polyadenylation variant in a cancer cell line lacks the binding site. J Biol Chem 278:13928-13935.

Schutyser E, Struyf S and Van Damme J (2003): The CC chemokine CCL20 and its receptor CCR6. Cytokine Growth Factor Rev 14:409-426.

Sheflin LG, Zou AP and Spaulding SW (2004): Androgens regulate the binding of endogenous HuR to the AU-rich 3'UTRs of HIF-1$\alpha$ and EGF mRNA. Biochem Bioph Res Co 322:644-651.

Sitkauskiene B and Sakalauskas R (2005): The role of $\beta_2$-adrenergic receptors in inflammation and allergy. Curr Drug Targets Inflamm Allergy 4:157-162.

Smoak K and Cidlowski JA (2006): Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling. Mol Cell Biol 26:9126-9135.

Snijders A, Hilkens CMU, van der Pouw Kraan,T.C.T.M., Engel M, Aarden LA and Kapsenberg ML (1996): Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated

human monocytes is determined by the expression of the p35 subunit. J Immunol 156:1207-1212.

Stoecklin G and Anderson P (2007): In a tight spot: ARE-mRNAs at processing bodies. Genes Dev 21:627-631.

Stoecklin G, Colombi M, Raineri I, Leuenberger S, Mallaun M, Schmidlin M, Gross B, Lu M, Kitamura T and Moroni C (2002): Functional cloning of BRF1, a regulator of ARE-dependent mRNA turnover. EMBO J 21:4709-4718.

Stoecklin G, Gross B, Ming XF and Moroni C (2003): A novel mechanism of tumor suppression by destabilizing AU-rich growth factor mRNA. Oncogene 22:3554-3561.

Stoecklin G, Ming XF, Looser R and Moroni C (2000): Somatic mRNA turnover mutants implicate tristetraprolin in the interleukin-3 mRNA degradation pathway. Mol Cell Biol 20:3753-3763.

Stoecklin G, Stoeckle P, Lu M, Muehlemann O and Moroni C (2001): Cellular mutants define a common mRNA degradation pathway targeting cytokine AU-rich elements. RNA 7:1578-1588.

Stoecklin G, Stubbs T, Kedersha N, Wax S, Rigby WFC, Blackwell TK and Anderson P (2004): MK2-induced tristetraprolin:14-3-3 complexes prevent stress granule association and ARE-mRNA decay. EMBO J 23:1313-1324.

Stoecklin G, Tenenbaum SA, Mayo T, Chittur SV, George AD, Baroni TE, Blackshear PJ and Anderson P (2008): Genome-wide analysis identifies interleukin-10 mRNA as target of tristetraprolin. J Biol Chem 283:11689-11699.

Stumpo DJ, Byrd NA, Phillips RS, Ghosh S, Maronpot RR, Castranio T, Meyers EN, Mishina Y and Blackshear PJ (2004): Chorioallantoic fusion defects and embryonic lethality resulting from disruption of *Zfp36L1*, a gene encoding a CCCH tandem zinc finger protein of the Tristetraprolin family. Mol Cell Biol 24:6445-6455.

Stumpo DJ, Haupt DM and Blackshear PJ (1994): Protein kinase C isozyme distribution and down-regulation in relation to insulin-stimulated c-*fos* induction. J Biol Chem 269:21184-21190.

Sugihara M, Tsutsumi A, Suzuki E, Wakamatsu E, Suzuki T, Ogishima H, Hayashi T, Chino Y, Ishii W, Mamura M, Goto D, Matsumoto I, Ito S and Sumida T (2007): Effects of infliximab therapy on gene expression levels of tumor necrosis factor α, tristetraprolin, T cell intracellular antigen 1, and Hu antigen R in patients with rheumatoid arthritis. Arthritis Rheum 56:2160-2169.

Sully G, Dean JLE, Wait R, Rawlinson L, Santalucia T, Saklatvala J and Clark AR (2004): Structural and functional dissection of a conserved destabilizing element of cyclo-oxygenase-2 mRNA: evidence against the involvement of AUF-1 [AU-rich element/poly(U)-binding/degradation factor-1], AUF-2, tristetraprolin, HuR (Hu antigen R) or FBP1 (far-upstream-sequence-element-binding protein 1). Biochem J 377:629-639.

Sun L, Stoecklin G, Van Way S, Hinkovska-Galcheva V, Guo RF, Anderson P and Shanley TP (2007): Tristetraprolin (TTP)-14-3-3 complex formation protects TTP from dephosphorylation by protein phosphatase 2A and stabilizes tumor necrosis factor-α mRNA. J Biol Chem 282:3766-3777.

Suswam E, Li Y, Zhang X, Gillespie GY, Li X, Shacka JJ, Lu L, Zheng L and King PH (2008): Tristetraprolin down-regulates interleukin-8 and vascular endothelial growth factor in malignant glioma cells. Cancer Res 68:674-682.

Suzuki E, Tsutsumi A, Goto D, Matsumoto I, Ito S, Otsu M, Onodera M, Takahashi S, Sato Y and Sumida T (2006a): Gene transduction of tristetraprolin or its active domain reduces TNF-α production by Jurkat T cells. Int J Mol Med 17:801-809.

Suzuki E, Tsutsumi A, Sugihara M, Mamura M, Goto D, Matsumoto I, Ito S, Ikeda K, Ochiai N, Sato Y and Sumida T (2006b): Expression of TNF-α, tristetraprolin, T-cell intracellular antigen-1 and Hu antigen R genes in synovium of patients with rheumatoid arthritis. Int J Mol Med 18:273-278.

Suzuki K, Nakajima H, Ikeda K, Maezawa Y, Suto A, Takatori H, Saito Y and Iwamoto I (2003): IL-4-Stat6 signaling induces tristetraprolin expression and inhibits TNF-α production in mast cells. J Exp Med 198:1717-1727.

Szekanecz Z and Koch AE (2007): Macrophages and their products in rheumatoid arthritis. Curr Opin Rheumatol 19:289-295.

Taddeo B, Esclatine A and Roizman B (2004): Post-transcriptional processing of cellular RNAs in herpes simplex virus-infected cells. Biochem Soc T 32:697-701.

Taddeo B, Zhang W and Roizman B (2006): The $U_L$41 protein of herpes simplex virus 1 degrades RNA by endonucleolytic cleavage in absence of other cellular or viral proteins. Proc Natl Acad Sci U S A 103:2827-2832.

Tan SL and Parker PJ (2003): Emerging and diverse roles of protein kinase C in immune cell signalling. Biochem J 376:545-552.

Taylor GA and Blackshear PJ (1995): Zinc inhibits turnover of labile mRNAs in intact cells. J Cell Physiol 162:378-387.

Taylor GA, Carballo E, Lee DM, Lai WS, Thompson MJ, Patel DD, Schenkman DI, Gilkeson GS, Broxmeyer HE, Haynes BF and Blackshear PJ (1996): A pathogenetic role for TNF α in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. Immunity 4:445-454.

Taylor GA, Lai WS, Oakey RJ, Seldin MF, Shows TB, Eddy RL Jr and Blackshear PJ (1991): The human TTP protein: sequence, alignment with related proteins, and chromosomal localization of the mouse and human genes. Nucleic Acids Res 19:3454.

Taylor GA, Thompson MJ, Lai WS and Blackshear PJ (1995): Phosphorylation of tristetraprolin, a potential zinc finger transcription factor, by mitogen stimulation in intact cells and by mitogen-activated protein kinase *in vitro*. J Biol Chem 270:13341-13347.

Taylor GA, Thompson MJ, Lai WS and Blackshear PJ (1996): Mitogens stimulate the rapid nuclear to cytosolic translocation of tristetraprolin, a potential zinc-finger transcription factor. Mol Endocrinol 10:140-146.

Tchen CR, Brook M, Saklatvala J and Clark AR (2004): The stability of tristetraprolin mRNA is regulated by mitogen-activated protein kinase p38 and by tristetraprolin itself. J Biol Chem 279:32393-32400.

Templin MF, Stoll D, Schrenk M, Traub PC, Vöhringer CF and Joos TO (2002): Protein microarray technology. Drug Discov Today 7:815-822.

Tsuchiya S, Kobayashi Y, Goto Y, Okumura H, Nakae S, Konno T and Tada K (1982): Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. Cancer Res 42:1530-1536.

Tsutsumi A, Suzuki E, Adachi Y, Murata H, Goto D, Kojo S, Matsumoto I, Zhong L, Nakamura H and Sumida T (2004): Expression of tristetraprolin (G0S24) mRNA, a regulator of tumor

necrosis factor-α production, in synovial tissues of patients with rheumatoid arthritis. J Rheumatol 31:1044-1049.

Turjanski AG, Vaqué JP and Gutkind JS (2007): MAP kinases and the control of nuclear events. Oncogene 26:3240-3253.

Twizere JC, Kruys V, Lefèbvre L, Vanderplasschen A, Collete D, Debacq C, Lai WS, Jauniaux JC, Bernstein LR, Semmes OJ, Burny A, Blackshear PJ, Kettmann R and Willems L (2003): Interaction of retroviral Tax oncoproteins with tristetraprolin and regulation of tumor necrosis factor-α expression. J Natl Cancer Inst 95:1846-1859.

van Delft JHM, van Agen E, van Breda SGJ, Herwijnen MH, Staal YCM and Kleinjans JCS (2005): Comparison of supervised clustering methods to discriminate genotoxic from non-genotoxic carcinogens by gene expression profiling. Mutat Res 575:17-33.

Vanden Berghe W, Francesconi E, De Bosscher K, Resche-Rigon M and Haegeman G (1999): Dissociated glucocorticoids with anti-inflammatory potential repress interleukin-6 gene expression by a nuclear factor-κB-dependent mechanism. Mol Pharmacol 56:797-806.

Varnum BC, Lim RW, Kujubu DA, Luner SJ, Kaufman SE, Greenberger JS, Gasson JC and Herschman HR (1989a): Granulocyte-macrophage colony-stimulating factor and tetradecanoyl phorbol acetate induce a distinct, restricted subset of primary-response TIS genes in both proliferating and terminally differentiated myeloid cells. Mol Cell Biol 9:3580-3583.

Varnum BC, Lim RW, Sukhatme VP and Herschman HR (1989b): Nucleotide sequence of a cDNA encoding TIS11, a message induced in Swiss 3T3 cells by the tumor promoter tetradecanoyl phorbol acetate. Oncogene 4:119-120.

Varnum BC, Ma QF, Chi TH, Fletcher B and Herschman HR (1991): The TIS11 primary response gene is a member of a gene family that encodes proteins with a highly conserved sequence containing an unusual Cys-His repeat. Mol Cell Biol 11:1754-1758.

Vayssière BM, Dupont S, Choquart A, Petit F, Garcia T, Marchandeau C, Gronemeyer H and Resche-Rigon M (1997): Synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression exhibit antiinflammatory activity in vivo. Mol Endocrinol 11:1245-1255.

Vuolteenaho K, Moilanen T, Knowles RG and Moilanen E (2007): The role of nitric oxide in osteoarthritis. Scand J Rheumatol 36:247-258.

Wang JK, Gao G and Goldfarb M (1994): Fibroblast growth factor receptors have different signaling and mitogenic potentials. Mol Cell Biol 14:181-188.

Way KJ, Chou E and King GL (2000): Identification of PKC-isoform-specific biological actions using pharmacological approaches. Trends Pharmacol Sci 21:181-187.

Welte T and Groneberg DA (2006): Asthma and COPD. Exp Toxicol Pathol 57 Suppl 2:35-40.

Wilusz CJ, Wormington M and Peltz SW (2001): The cap-to-tail guide to mRNA turnover. Nat Rev Mol Cell Bio 2:237-246.

Worthington MT, Amann BT, Nathans D and Berg JM (1996): Metal binding properties and secondary structure of the zinc-binding domain of Nup475. P Natl Acad Sci USA 93:13754-13759.

Worthington MT, Pelo JW, Sachedina MA, Applegate JL, Arseneau KO and Pizarro TT (2002): RNA binding properties of the AU-rich element-binding recombinant Nup475/TIS11/tristetraprolin protein. J Biol Chem 277:48558-48564.

Xie W, Shao N, Ma X, Ling B, Wei Y, Ding Q, Yang G, Liu N, Wang H and Chen K (2006): Bacterial endotoxin lipopolysaccharide induces up-regulation of glyceraldehyde-3-phosphate dehydrogenase in rat liver and lungs. Life Sci 79:1820-1827.

Yang YC and Yin T (1995): Interleukin (IL)-11--mediated signal transduction. Ann N Y Acad Sci 762:31-40.

Yin T and Yang YC (1993): Protein tyrosine phosphorylation and activation of primary response genes by interleukin 11 in B9-TY1 cells. Cell Growth Differ 4:603-609.

Yu H, Stasinopoulos S, Leedman P and Medcalf RL (2003): Inherent instability of plasminogen activator inhibitor type 2 mRNA is regulated by tristetraprolin. J Biol Chem 278:13912-13918.

Yuen T, Wurmbach E, Ebersole BJ, Ruf F, Pfeffer RL and Sealfon SC (2002): Coupling of GnRH concentration and the GnRH receptor-activated gene program. Mol Endocrinol 16:1145-1153.

Zeng Y, Yi R and Cullen BR (2003): MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. Proc Natl Acad Sci U S A 100:9779-9784.

Zhang W, Wagner BJ, Ehrenman K, Schaefer AW, DeMaria CT, Crater D, DeHaven K, Long L and Brewer G (1993): Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF1. Mol Cell Biol 13:7652-7665.

Zhu W, Brauchle MA, Di Padova F, Gram H, New L, Ono K, Downey JS and Han J (2001): Gene suppression by tristetraprolin and release by the p38 pathway. Am J Physiol Lung Cell Mol Physiol 281:L499-L508.

Zubakov D, Hoheisel JD, Kluxen FW, Brändle M, Ehring T, Hentsch B and Frohme M (2003): Late ischemic preconditioning of the myocardium alters the expression of genes involved in inflammatory response. FEBS Lett 547:51-55.

Zubiaga AM, Belasco JG and Greenberg ME (1995): The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. Mol Cell Biol 15:2219-2230.

# Original communications

The author acknowledges permissions from the following copyright owners to reprint the original communications:

ELSEVIER

# Inhibition of tristetraprolin expression by dexamethasone in activated macrophages

Ulla Jalonen, Aleksi Lahti, Riku Korhonen, Hannu Kankaanranta, Eeva Moilanen[*]

*The Immunopharmacological Research Group, University of Tampere, Medical School, FIN-33014 University of Tampere, Finland, and Tampere University Hospital, Research Unit, Tampere, Finland*

## Abstract

Tristetraprolin (TTP) is a factor that regulates mRNA stability and the expression of certain inflammatory genes. In the present study, we found that TTP expression was increased in macrophages exposed to bacterial lipopolysaccharide (LPS). Dexamethasone and dissociated steroid RU24858 inhibited LPS-induced TTP protein and mRNA expression and the inhibitory effect was reversed by a glucocorticoid receptor antagonist mifepristone. Histone deacetylase inhibitors trichostatin A (TSA) and apicidin reduced the inhibitory effect of dexamethasone and RU24858 on TTP expression, but the glucocorticoids did not alter TTP mRNA half-life. These results suggest that anti-inflammatory steroids reduce TTP expression in activated macrophages by a glucocorticoid response element (GRE)-independent mechanism, possibly through histone deacetylation and transcriptional silencing.
© 2004 Elsevier Inc. All rights reserved.

*Keywords:* Glucocorticoid; Histone deacetylation; Inflammation; Mitogen-activated protein kinase; mRNA stability; Tristetraprolin

## 1. Introduction

Tristetraprolin (TTP), also known as Nup475, TIS11, G0S24 and Zfp36, was first described as an immediate early response gene in cells stimulated by growth factors and mitogens and by factors like cycloheximide, forskolin and 12-*O*-tetradecanoylphorbol-13-acetate ester [1–6]. TTP belongs to a family of CCCH tandem zinc-finger proteins together with Zfp36l1 (Zfp36-like 1, also known as Berg36, BRF1, cMG1, ERF1, TIS11b) and Zfp36l2 (Zfp36-like 2, also known as BRF2, ERF2 and TIS11d) [7]. Although these proteins are differentially expressed, they have structural similarities: they all have two Cys-Cys-Cys-His zinc-finger domains, which have RNA-binding properties [8,9]. TTP mRNA is widely expressed at particularly high levels in spleen, lymph nodes and thymus [1,3].

TTP is involved in the regulation of inflammatory responses. A direct connection between TTP and inflammation was demonstrated in mice lacking the TTP gene [10]. TTP knock-out mice were found to develop a severe inflammatory syndrome, which was associated with elevated levels of circulating tumor necrosis factor $\alpha$ (TNF-$\alpha$). Increased TNF-$\alpha$ production in these mice was found to be due to increased mRNA stability [11,12]. In subsequent studies, TTP has been shown to bind to the A + U-rich element in the TNF-$\alpha$ 3′-untranslated region which promotes deadenylation and destabilization of the TNF-$\alpha$ mRNA [13]. The mRNA of the granulocyte-macrophage colony-stimulating factor is also destabilized by TTP, which is assumed to be the reason for myeloid hyperplasia observed in TTP-deficient mice [14]. The stability of the mRNAs of interleukin-3 and cyclooxygenase-2 are also regulated by TTP [15,16].

TTP is known to be involved in the expression of certain inflammatory genes, but the regulation of the expression of TTP itself remains largely unknown. The aim of the

present study was to investigate the regulation of TTP expression in macrophages exposed to an inflammatory stimulus [lipopolysaccharide (LPS)], and especially the effect of anti-inflammatory steroids.

## 2. Materials and methods

### 2.1. Materials

Reagents were purchased as follows: Tyrphostin AG-490, 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD 98059), anthra[1,9-*cd*]pyrazol-6(2*H*)-one (SP 600125), cyclosporin A, 4-ethyl-2-(4-methoxyphenyl)-5-(4-pyridyl)-imidazole (SB 202474) and 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)-imidazole (SB 203580) from Calbiochem, apicidin from Alexis Corporation, genistein from Tocris, dexamethasone from Orion Corp., and RU24858 was received from Aventis Pharma. All other reagents were from Sigma unless otherwise stated.

### 2.2. Cell culture

J774 murine macrophages (American Type Culture Collection) were cultured at 37 °C in humidified 5% carbon dioxide atmosphere in Dulbecco's modified Eagle medium with Ultraglutamine 1 (Cambrex Bioproducts Europe) supplemented with 10% heat-inactivated fetal bovine serum (Cambrex Bioproducts Europe), penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml) (Gibco) and harvested with trypsin–EDTA (Gibco). Cells were seeded on 6- or 24-well plates and grown to confluence prior to the experiments.

### 2.3. RNA extraction and quantitative real-time PCR

Cells were homogenized using QIAshredder™ (QIAGEN Inc.) after which total RNA was extracted with RNeasy® Mini kit (QIAGEN Inc.). The amount of RNA was measured with a spectrophotometer and the purity was confirmed by the absorbance ratio at $A_{260/280}$. Reverse transcription and quantitative PCR were performed according to the manufacturer's instructions (Applied Biosystems). Reverse transcription was carried out with TaqMan Reverse Transcription reagents and random hexamers in 10 μl reaction volume containing 25 ng purified RNA. Gene transcript levels of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and mouse TTP were quantified by real-time PCR on ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). Approximately 1 ng of total RNA reverse-transcribed into cDNA was used in the polymerase chain reaction applying TaqMan® Universal PCR Master Mix and sequence detection primers and TaqMan TAMRA™ probes. Rodent GAPDH Control Reagents were

obtained from Applied Biosystems and primer and probe concentrations were optimized and primers and probes were used in the following concentrations: both forward and reverse rGAPDH primers 300 nM, rGAPDH probe 50 nM containing VIC™ (proprietary dye, Applied Biosystems) as 5′-reporter dye and TAMRA (6-carboxy-tetramethyl-rhodamine) as 3′-quencher. The mouse TTP primers and probe were designed using Primer Express® Software (Applied Biosystems) and were 5′-CTCAGAAAGCGGGCGTTGT-3′ (TTP forward, GenBank accession no. NM_011756, 343–361), 5′-GATTGGCTTGGCGAAGTTCA-3′ (TTP reverse, 400–419), 5′-CCAAGTGCCAGTTTGCTCACGGC-3′ [TTP probe containing 6-FAM (6-carboxy-fluoroscein) as 5′-reporter dye and TAMRA as 3′-quencher, 372–394] and used in concentrations of 300, 300 and 200 nM respectively (all from Metabion). Thermal cycling conditions were: incubation in 50 °C for 2 min, 95 °C for 10 min, thereafter 40 cycles of denaturation in 92 °C for 15 s and annealing/extension in 60 °C for 1 min. Each sample was determined in duplicate.

The relative mRNA levels were quantified and compared using the relative standard curve method as described in Applied Biosystems User Bulletin #2. Total RNA was isolated from LPS-stimulated J774 macrophages and reverse transcribed. Standard curves for GAPDH and TTP were created using dilution series of cDNA corresponding approximately 1 pg to 10 ng of total RNA in PCR as described above. The threshold cycle values obtained were plotted against dilution factor to create a standard curve. Relative mRNA levels in test samples were then calculated using the standard curve. The relative amount of gene transcript present was calculated and normalized by dividing the calculated value of TTP by the GAPDH value in each sample.

### 2.4. Western blotting

After the time indicated, J774 macrophages were washed with ice-cold PBS and lysed in ice-cold extraction buffer [10 mM Tris–base, 5 mM EDTA, 50 mM NaCl, 1% (w/v) Triton-X-100, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM sodiumorthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1.25 mM NaF, 1 mM sodium pyrophosphate, 10 mM *n*-octyl-β-D-glucopyranoside]. After a 15-min incubation on ice and centrifugation (13 400 × *g*, 4 °C, 10 min), supernatants were collected and stored in sample buffer [62.5 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.025% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol] at −20 °C. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method [17]. Prior to Western blotting, proteins were boiled for 5 min with sample buffer [62.5 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.025% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol] and 20 μg of protein was used per lane on 12% SDS-polyacrylamide gel and transferred to Hybond

ECL$^{TM}$ nitrocellulose membrane (Amersham). After transfer, the membrane was blocked with TBS/T [20 mM Tris–base pH 7.6, 150 mM NaCl, 0.1% (v/v) Tween-20] containing 5% (w/v) bovine serum albumin. Thereafter the membrane was incubated with TTP antibody (Santa Cruz Biotechnology) in the blocking buffer for 1 h at room temperature. The membrane was washed and incubated with the secondary antibody in TBS/T containing 5% (w/v) milk powder (30 min at room temperature), and thereafter the bound antibody was detected using Super Signal® West Pico chemiluminescent substrate for HRP detection (Pierce) and FluorChem$^{TM}$ 8800 imaging system (Alpha Innotech).

## 2.5. Statistics

Results are expressed as the mean ± standard error of mean (S.E.M.). The significance of differences was calculated by analysis of variance supported by Dunnett's adjusted significance levels. A difference between treatment groups was considered significant when $P < 0.05$.

## 3. Results

### 3.1. LPS-induced TTP expression in J774 macrophages

Low levels of TTP mRNA were found in unstimulated J774 macrophages. When the cells were exposed to LPS (10 ng/ml) the amount of TTP mRNA was doubled in less than an hour, it peaked at 6 h, and declined thereafter (Fig. 1A). LPS (1–1000 ng/ml) induced TTP mRNA expression in a dose-dependent manner (Fig. 1B). Accordingly, increased TTP protein expression was found in LPS-treated cells (Fig. 1C). TTP protein levels were doubled in 3 h after addition of LPS, peaked at 6 h and declined thereafter, showing only slightly elevated levels at 24 h. In Western blot analysis of the TTP protein three immunoreactive bands between MWs of 36 and 46 kDa were found. This concurs with previous studies and is explained by different molecular sizes resulting from post-translational modifications of the molecule [18,19].

Pharmacological inhibitors were used to evaluate signaling pathways involved in LPS-induced TTP expression (Table 1). Dexamethasone (1 μM) had a clear effect and inhibited TTP mRNA expression by 76%. In addition, inhibitors of mitogen-activated protein kinase (MAPK) pathways reduced LPS-induced TTP mRNA expression. Extracellular signal-regulated kinase 1/2 (Erk1/2) inhibitor PD 98059 (10 μM) [20] inhibited TTP mRNA levels by 35% ($P < 0.05$). SP 600125 (10 μM), a novel anthrapyrazolone inhibitor of c-Jun N-terminal kinase (JNK) [21], reduced LPS-induced TTP mRNA by 43% ($P < 0.01$). An inhibitor of p38 MAPK, SB 203580 (1 μM) [22], inhibited TTP mRNA levels by 74% ($P < 0.01$) whereas SB 202474
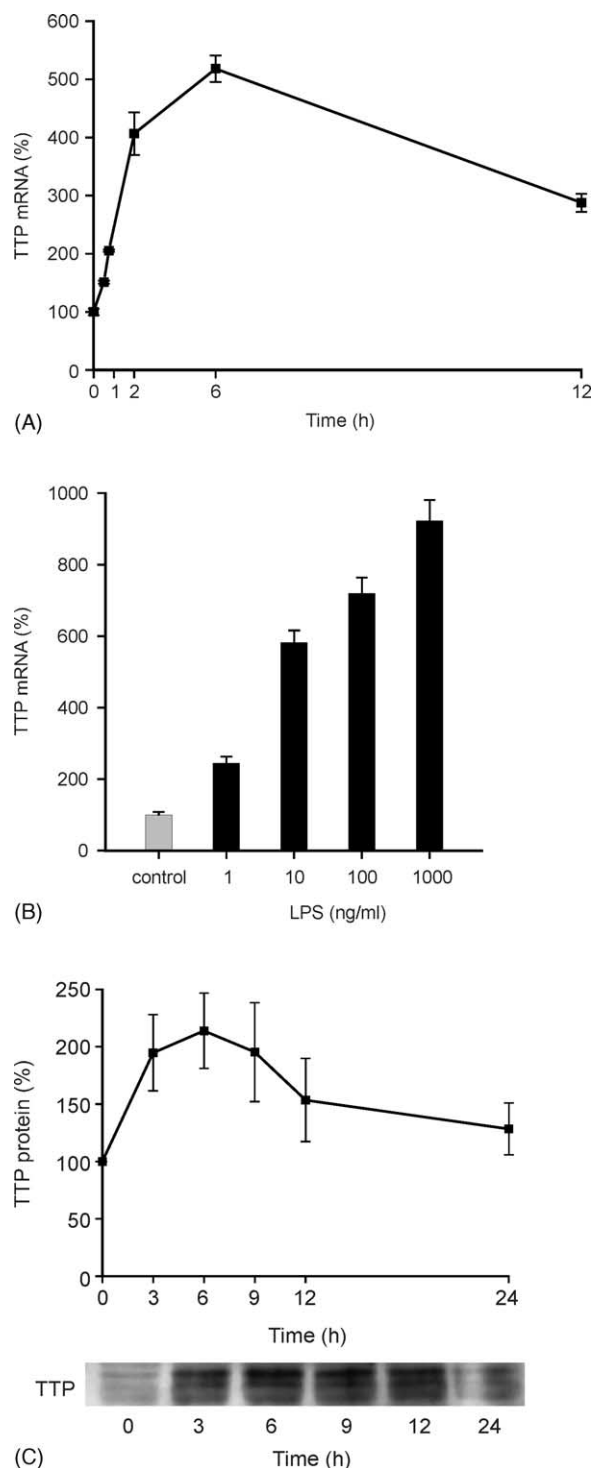


Fig. 1. The effect of LPS on TTP expression in J774 macrophages. (A) LPS (10 ng/ml) was used to stimulate macrophages and total RNA was extracted at the time points indicated. Quantitative PCR was used to measure TTP mRNA and the values were normalized to GAPDH mRNA. Values are mean ± S.E.M. ($n = 3$). (B) The cells were stimulated with the indicated amounts of LPS and total RNA was extracted after 6 h incubation. Quantitative PCR was used to measure TTP mRNA and the values were normalized to GAPDH mRNA. Values are mean ± S.E.M. ($n = 3$). (C) As in A, LPS was used to stimulate macrophages. Proteins were extracted at the time points indicated and TTP was measured by Western blot. Values are mean ± S.E.M. ($n = 5$).

Table 1
The effect of inhibitors of certain signaling pathways on LPS-induced TTP expression

| Treatment | TTP mRNA (% of LPS-induced levels) | |
|---|---|---|
| Untreated | $11 \pm 1$ | |
| LPS (10 ng/ml) | 100 | |
| LPS + dexamethasone (1 μM) | $24 \pm 2$ | $P < 0.01$ |
| LPS + PD 98059 (10 μM) | $65 \pm 7$ | $P = 0.047$ |
| LPS + SP 600125 (10 μM) | $57 \pm 7$ | $P < 0.01$ |
| LPS + SB 203580 (1 μM) | $26 \pm 3$ | $P < 0.01$ |
| LPS + SB 202474 (1 μM) | $96 \pm 12$ | $P > 0.1$ |
| LPS + PDTC (100 μM) | $78 \pm 9$ | $P > 0.1$ |
| LPS + Cyclosporin A (10 μM) | $87 \pm 10$ | $P > 0.1$ |
| LPS + Genistein (100 μM) | $68 \pm 8$ | $P = 0.082$ |
| LPS + AG-490 (10 μM) | $93 \pm 13$ | $P > 0.1$ |

Cells were incubated for 6 h with or without LPS and the inhibitors of interest. Total RNA was extracted and subjected to quantitative PCR. Values are mean $\pm$ S.E.M. ($n = 3$), compared to samples treated with LPS only.

(a structural analog of SB 203580 that does not inhibit p38 [22]) had no effect on TTP mRNA expression.

Pyrrolidine derivative of dithiocarbamate (PDTC), cyclosporin A, genistein and tyrphostin AG-490 were used to evaluate the roles of nuclear factor κB (NF-κB), calcium/calmodulin-dependent phosphatase calcineurin, tyrosine phosphorylation and JAK-2 in TTP mRNA expression respectively. Genistein reduced LPS-induced TTP mRNA expression by $32 \pm 8\%$, ($P = 0.082$) whereas none of the other compounds had any clear effect on TTP mRNA accumulation in LPS-treated J774 macrophages.

### 3.2. Dexamethasone and dissociated steroid RU24858 inhibited LPS-induced TTP expression in J774 macrophages

In subsequent studies, the inhibitory effect of dexamethasone on LPS-induced TTP expression was investigated in more detail. The inhibitory effect of dexamethasone on TTP mRNA accumulation was dose-dependent, and maximal inhibition was reached at drug concentrations of 100–10 000 nM (Fig. 2A). A similar dose-dependent inhibitory effect was also seen in TTP protein expression (Fig. 2B).

Dissociated glucocorticoids such as RU24858 are synthetic glucocorticoid ligands possessing different transactivation and transrepression profiles than those of classical anti-inflammatory steroids. RU24858 has been shown to have transrepression properties similar to those of dexamethasone on AP-1- and NF-κB-dependent gene expression, but it does not induce gene expression through glucocorticoid response element [23,24]. We tested the effect of RU24858 on TTP expression to find out if the TTP-reducing effect of dexamethasone was associated with the transrepression or transactivation mechanisms of glucocorticoids. RU24858 inhibited TTP mRNA expression in a dose-dependent manner, and maximal effect was

reached at 100–1000 nM concentrations (Fig. 2C). Accordingly, an inhibitory effect on TTP protein expression was also found (Fig. 2D). These results suggest that the inhibitory action of glucocorticoids on TTP expression is mediated through their transrepression mechanisms.

In the presence of a glucocorticoid receptor (GR) antagonist, mifepristone (5 μM), dexamethasone or RU24858 did not reduce TTP mRNA expression in LPS-treated macrophages (Fig. 3). These data suggest that the effects of dexamethasone and RU24858 are mediated through the glucocorticoid receptor.

### 3.3. Dexamethasone and dissociated steroid RU24858 did not alter TTP mRNA half-life in LPS-treated J774 macrophages

Dexamethasone reduced LPS-induced TTP mRNA levels when measured at 3 h, 6 h or 9 h after the addition of LPS $\pm$ dexamethasone by 31%, 55% and 38% respectively (Fig. 4A). In the subsequent studies, the effects of dexamethasone and RU24858 on the half-life of TTP mRNA were investigated by actinomycin D assay. Cells were stimulated with LPS (10 ng/ml) in the presence or absence of dexamethasone (100 nM) or RU24858 (100 nM) for 6 h, and thereafter actinomycin D (0.5 μg/ml) was added into the culture to inhibit transcription. Total mRNA was isolated at 1 h intervals after actinomycin D addition, and the TTP mRNA levels were measured.

The half-life on TTP mRNA was ~1 h in LPS-treated cells, and neither dexamethasone nor RU24858 altered the TTP mRNA half-life (Fig. 4B).

### 3.4. Histone deacetylase inhibitors reduced the effects of dexamethasone and RU24858 on TTP expression in LPS-treated J774 macrophages

The suppressive effects of anti-inflammatory steroids on certain genes are mediated through histone deacetylation [25]. We therefore studied the effects of dexamethasone and RU24858 on LPS-induced TTP mRNA expression in the presence and absence of trichostatin A (TSA) and apicidin, which inhibit histone deacetylases leading to histone hyperacetylation, and by that mechanism can abolish the effects of glucocorticoids on histone deacetylation.

In the absence of histone deacetylase inhibitors, dexamethasone (100 nM) and RU24858 (100 nM) inhibited LPS-induced TTP mRNA expression by 53% ($P < 0.05$) and 52% ($P < 0.05$) respectively (Fig. 5A). In the presence of TSA or apicidin the glucocorticoids did not significantly reduce TTP expression (Fig. 5B and C respectively). These results suggest that histone deacetylation and transcriptional silencing may mediate the inhibitory effects of dexamethasone and dissociated steroid RU24858 on TTP expression.
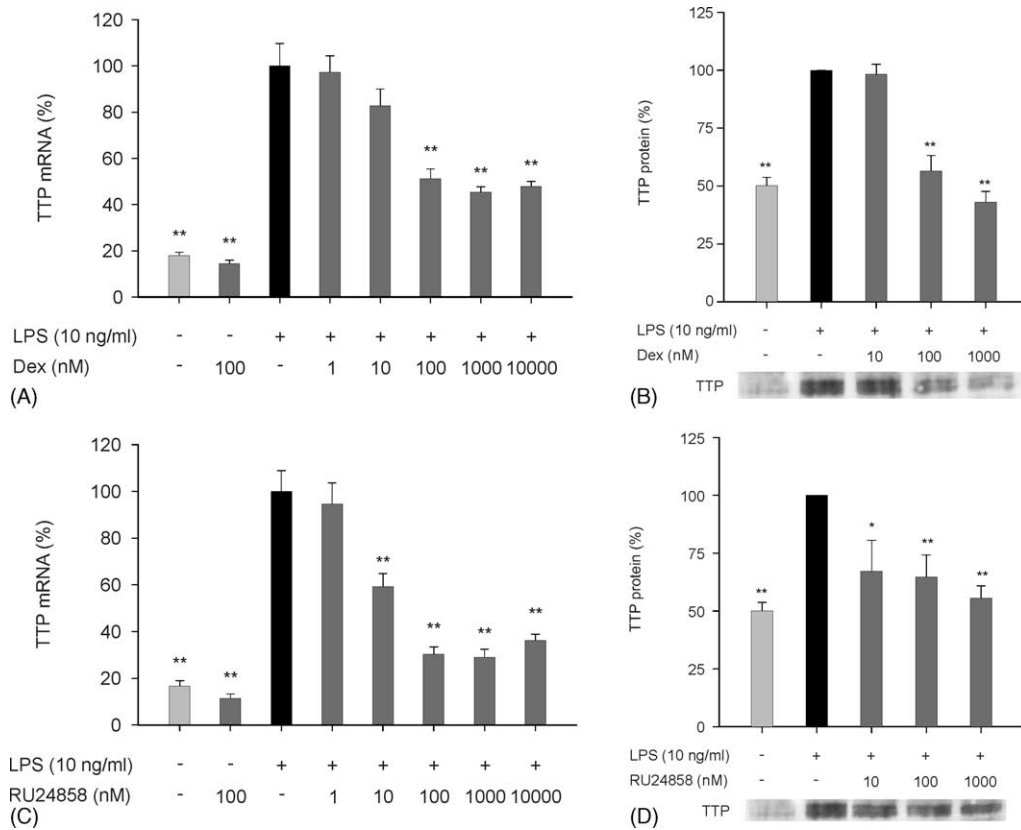
Fig. 2. The effect of dexamethasone (Dex) and RU24858 on LPS-induced TTP expression. Cells were incubated for 6 h with or without LPS and glucocorticoids. (A, C) Quantitative PCR was used to measure TTP mRNA and the values were normalized to GAPDH mRNA. Values are mean ± S.E.M. ($n = 3$–6). (B, D) TTP protein was analysed by Western blot. The gels are representatives of four separate experiments with similar results. Density values are mean ± S.E.M. ($n = 4$). $^{**}P < 0.01$, $^{*}P < 0.05$, when compared to samples treated with LPS only.
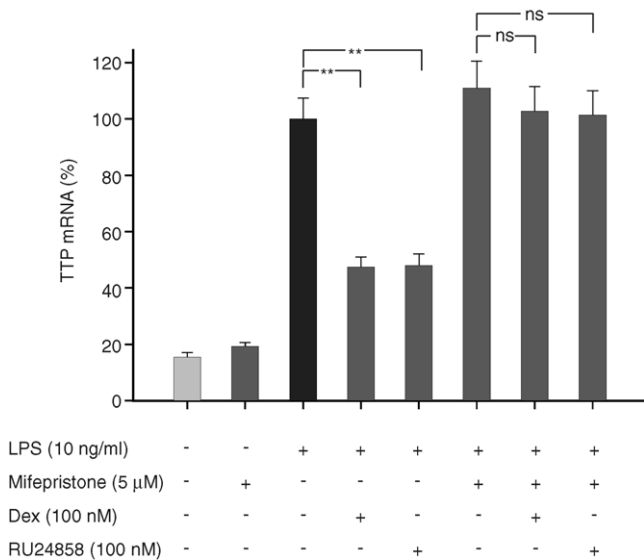


Fig. 3. The effect of glucocorticoid receptor antagonist mifepristone on TTP expression. Cells were cultured with LPS, mifepristone, dexamethasone (Dex) and RU24858 as indicated for 6 h and total RNA was extracted. Quantitative PCR was used to measure TTP mRNA and the values were normalized to GAPDH mRNA. Values are mean ± S.E.M. ($n = 4$). $^{**}P < 0.01$, ns = not significant, when compared to samples treated with LPS only or samples treated with the combination of LPS and mifepristone.

## 4. Discussion

In the present study, we found that dexamethasone and dissociated steroid RU24858 reduced LPS-induced TTP expression in a glucocorticoid receptor-mediated manner. The results suggest that the inhibitory effect of glucocorticoids on TTP expression was mediated through enhanced histone deacetylation and transcriptional silencing. In addition, the inhibitors of p38, JNK and Erk1/2 MAP kinases reduced the amount of TTP mRNA, suggesting a role for these kinases in the regulation of TTP gene expression.

In an inflammatory reaction, TTP is known to regulate the production of pro-inflammatory proteins TNF-α, granulocyte-macrophage colony-stimulating factor, interleukin-3 and cyclooxygenase-2 by destabilizing their mRNAs through A + U-rich elements [13–16]. In the present study we found that TTP is expressed in macrophages in response to bacterial LPS. This result is in line with the earlier findings that certain inflammatory stimuli and growth factors increase TTP expression [1,26], and suggests that TTP is a part of the endogenous machinery regulating the restriction and resolution of the inflammatory process.
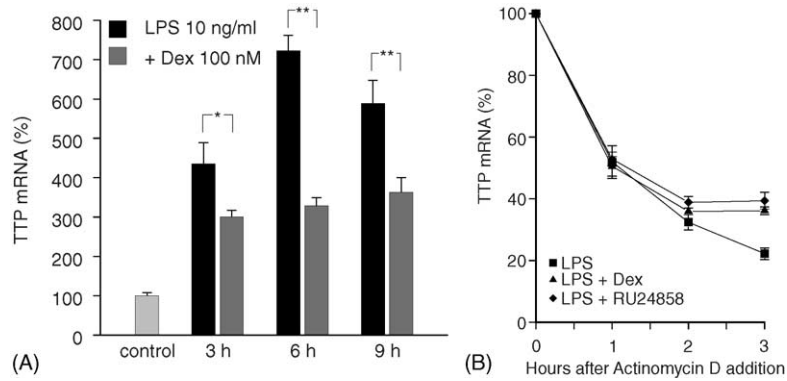
Fig. 4. The kinetics of the effect of dexamethasone on TTP mRNA expression. (A) J774 macrophages were treated with LPS (10 ng/ml) in the presence or absence of dexamethasone (Dex) (100 nM). Total RNA was extracted at the time points indicated. Quantitative PCR was used to measure TTP mRNA and the values were normalized to GAPDH mRNA. Values are mean ± S.E.M. ($n = 3$). $^{**}P < 0.01$, $^{*}P < 0.05$, when compared to samples treated with LPS only. (B) Macrophages were treated with LPS (10 ng/ml) with or without dexamethasone (100 nM) or RU24858 (100 nM) for 6 h, and then actinomycin D (0.5 µg/ml) was added to the cells. Cells were then harvested at indicated time points and total RNA was extracted and subjected to quantitative PCR. The mRNA level of all treatments at actinomycin D addition was set at 100%. Values are mean ± S.E.M. ($n = 3$).

The most interesting finding in the present study was that dexamethasone and dissociated steroid RU24858 inhibit TTP expression in a dose-dependent manner. To our knowledge this effect has not been reported earlier. A core glucocorticoid-like response element has been suggested to reside in TTP promoter [1]. The core glucocorticoid-like response element of TTP resembles more a glucocorticoid response element (GRE) than a negative GRE [27]. Based on these data and on our present results on the effects of dissociated steroid RU24858 it is not likely that the binding of glucocorticoid–GR complex to the core glucocorticoid-like response element is responsible for the inhibition of TTP expression, but rather that the inhibition is mediated through GRE-independent transrepression mechanisms.

Glucocorticoid receptor antagonist mifepristone competes with dexamethasone for the same binding site in glucocorticoid receptor and inhibits the effects of gluco-

corticoids [28,29]. In the present study, dexamethasone and RU24858 had no effect on TTP mRNA expression in the presence of mifepristone. These data suggest that the inhibitory effects of dexamethasone and RU24858 on LPS-induced TTP expression are mediated through GR.

Dexamethasone reduced TTP mRNA expression at early time points after the stimulus, and neither dexamethasone nor RU24858 altered the half-life of TTP mRNA. These data suggest that the effect of glucocorticoids occurs at the transcriptional level. Transcriptional activation is associated with histone acetylation, which facilitates gene transcription [30]. The inhibitory effects of glucocorticoids on the expression of certain genes are mediated through histone deacetylation [25]. In order to study if this is also the case in glucocorticoid-induced suppression of TTP expression, histone deacetylase inhibitors TSA and apicidin were added into the culture. TSA and apicidin reduced
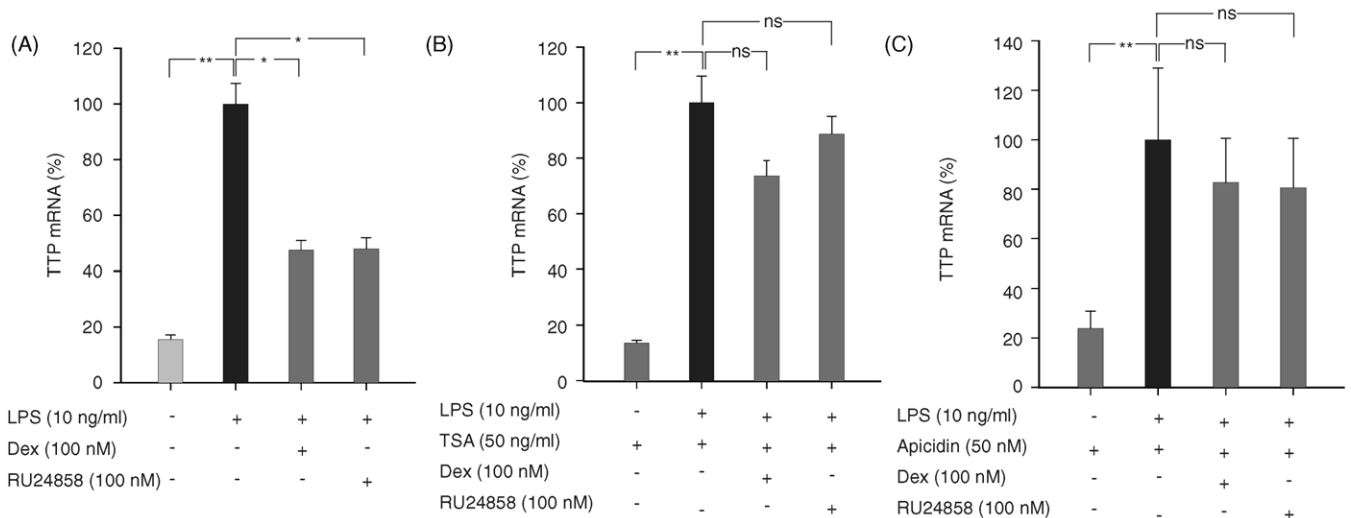


Fig. 5. The effect of histone deacetylase inhibitors TSA and apicidin on the suppressive effect of dexamethasone and RU24858 on LPS-induced TTP expression. Cells were incubated for 6 h with the compounds tested. Quantitative PCR was used to measure TTP mRNA and the values were normalized to GAPDH mRNA. Values are mean ± S.E.M. ($n = 4$). $^{**}P < 0.01$, $^{*}P < 0.05$, ns = not significant, when compared to samples treated with LPS or to samples treated with a combination of LPS and TSA or LPS and apicidin.

the effects of dexamethasone and RU24858 on TTP expression. These results suggest that the effects of dexamethasone and RU24858 on TTP expression are mediated through enhanced histone deacetylation. The mechanisms by which glucocorticoids induce histone deacetylation are not known in detail. It has been reported that GR is a direct inhibitor of CREB-binding protein (CBP)-associated histone acetyltransferase (HAT) activity and it also recruits histone deacetylase 2 to the CBP–HAT complex, and these two mechanisms may enhance histone deacetylation and transcriptional silencing [31]. However, the role of histone deacetylation as a mechanism of anti-inflammatory steroids to regulate the expression of TTP and other genes needs further mechanistic studies.

We also studied the involvement of some other signaling mechanisms in LPS-induced TTP expression by pharmacological means. PDTC (NF-κB inhibitor), cyclosporin A (calcineurin inhibitor) and AG-490 (JAK-2 inhibitor) had no effect, suggesting a minor effect of those signaling pathways in TTP expression. Although an NF-κB-like binding site has been described in TTP intron [32] it is not likely that NF-κB is a critical transcription factor for TTP as PDTC had no effect on TTP expression. By contrast, the signaling pathways regulating LPS-induced TTP mRNA expression seem to involve p38, JNK and Erk1/2 kinases. A specific inhibitor of p38, SB 203580 [22] inhibited the expression of TTP mRNA by 74%, whereas SB 202474, a structural analog of SB 203580, that does not inhibit p38 [22], had no effect on TTP expression. These results suggest that p38 positively regulates the expression of TTP. PD 98059 (an inhibitor of the Erk1/2 pathway) reduced the accumulation of TTP mRNA by 35% suggesting that Erk1/2 MAPK also has a role in the regulation of TTP expression. Our results on the involvement of Erk1/2 and p38 in the induction of TTP support the previous findings of Inuzuka et al. [33] and Mahtani et al. [18].

JNK inhibitor SP 600125 [21] reduced TTP mRNA levels by 43% in cells stimulated with LPS. This concurs with the recent findings of Brooks et al. [34] on the inhibitory effect of SP 600125 on TTP expression in THP-1 cells. In addition, cycloheximide (which among its other effects is a strong activator of JNK [35]) has been reported to cause superinduction of TTP [36–39]. These reports together support the role of JNK in the up-regulation of TTP expression.

The mechanisms by which MAP kinases regulate TTP expression are not known in detail. Mitogen- and stress-activated protein kinases-1 and -2 (MSK1, MSK2) have been shown to mediate the effects of p38 and Erk1/2 on the expression of many immediate early genes in inflammation [40] but their role in TTP expression remains to be studied.

Two recent articles evaluated the roles of MAPKs in TTP mRNA stability. Brooks et al. found that in THP-1 cells PD 98059 destabilized TTP mRNA, while SB202190 (an inhibitor of p38) and SP 600125 did not affect LPS-

mediated TTP mRNA stability [34]. In contrast, Tchen et al. reported that another p38 inhibitor SB 203580 destabilized TTP mRNA in RAW264.7 cells [41]. Both reports also describe the involvement of TTP itself in the regulation of TTP mRNA stability [34,41]. In addition to the regulation of TTP expression, p38, JNK and Erk1/2 may affect the activity of TTP, as they have been shown to phosphorylate recombinant TTP in vitro [42].

In the present study, we show that LPS-induced TTP expression in macrophages is down-regulated by anti-inflammatory steroids and inhibitors of p38, JNK and Erk1/2 MAP-kinases. To our knowledge, this is the first report showing that dexamethasone and dissociated steroid RU24858 inhibit TTP expression. Our results suggest that the inhibition of TTP expression by glucocorticoids is mediated through histone deacetylation and transcriptional silencing in a glucocorticoid receptor-dependent manner.

## References

[1] DuBois RN, McLane MW, Ryder K, Lau LF, Nathans D. A growth factor-inducible nuclear protein with a novel cysteine/histidine repetitive sequence. J Biol Chem 1990;265:19185–91.

[2] Heximer SP, Forsdyke DR. A human putative lymphocyte G0/G1 switch gene homologous to a rodent gene encoding a zinc-binding potential transcription factor. DNA Cell Biol 1993;12:73–88.

[3] Lai WS, Stumpo DJ, Blackshear PJ. Rapid insulin-stimulated accumulation of an mRNA encoding a proline-rich protein. J Biol Chem 1990;265:16556–63.

[4] Ma Q, Herschman HR. A corrected sequence for the predicted protein from the mitogen-inducible TIS11 primary response gene. Oncogene 1991;6:1277–8.

[5] Varnum BC, Lim RW, Sukhatme VP, Herschman HR. Nucleotide sequence of a cDNA encoding TIS11, a message induced in Swiss 3T3 cells by the tumor promoter tetradecanoyl phorbol acetate. Oncogene 1989;4:119–20.

[6] Taylor GA, Lai WS, Oakey RJ, Seldin MF, Shows TB, Eddy Jr RL, et al. The human TTP protein: sequence, alignment with related proteins, and chromosomal localization of the mouse and human genes. Nucl Acids Res 1991;19:3454.

[7] Blackshear PJ. Tristetraprolin and other CCCH tandem zinc-finger proteins in the regulation of mRNA turnover. Biochem Soc T 2002;30:945–52.

[8] Lai WS, Carballo E, Thorn JM, Kennington EA, Blackshear PJ. Interactions of CCCH zinc finger proteins with mRNA. Binding of tristetraprolin-related zinc finger proteins to AU-rich elements and destabilization of mRNA. J Biol Chem 2000;275:17827–37.

[9] Lai WS, Kennington EA, Blackshear PJ. Interactions of CCCH zinc finger proteins with mRNA. Non-binding tristetraprolin mutants exert an inhibitory effect on degradation of AU-rich element-containing mRNAs. J Biol Chem 2002;277:9606–13.

[10] Taylor GA, Carballo E, Lee DM, Lai WS, Thompson MJ, Patel DD, et al. A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. Immunity 1996;4:445–54.

[11] Carballo E, Gilkeson GS, Blackshear PJ. Bone marrow transplantation reproduces the tristetraprolin-deficiency syndrome in recombination activating gene-2 (−/−) mice. Evidence that monocyte/macrophage progenitors may be responsible for TNFα overproduction. J Clin Invest 1997;100:986–95.

[12] Carballo E, Lai WS, Blackshear PJ. Feedback inhibition of macrophage tumor necrosis factor-α production by tristetraprolin. Science 1998;281:1001–5.

[13] 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;19:4311–23.

[14] 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;95:1891–9.

[15] Stoecklin G, Ming XF, Looser R, Moroni C. Somatic mRNA turnover mutants implicate tristetraprolin in the interleukin-3 mRNA degradation pathway. Mol Cell Biol 2000;20:3753–63.

[16] Sawaoka H, Dixon DA, Oates JA, Boutaud O. Tristetraprolin binds to the 3'-untranslated region of cyclooxygenase-2 mRNA. A polyadenylation variant in a cancer cell line lacks the binding site. J Biol Chem 2003;278:13928–35.

[17] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.

[18] Mahtani KR, Brook M, Dean JLE, Sully G, Saklatvala J, Clark AR. 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;21:6461–9.

[19] Zhu W, Brauchle MA, Di Padova F, Gram H, New L, Ono K, et al. Gene suppression by tristetraprolin and release by the p38 pathway. Am J Physiol-Lung C 2001;281:L499–508.

[20] Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. J Biol Chem 1995;270:27489–94.

[21] Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, et al. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA 2001;98:13681–6.

[22] Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 1994;372:739–46.

[23] Vayssière BM, Dupont S, Choquart A, Petit F, Garcia T, Marchandeau C, et al. Synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression exhibit antiinflammatory activity *in vivo*. Mol Endocrinol 1997;11:1245–55.

[24] Vanden Berghe W, Francesconi E, De Bosscher K, Resche-Rigon M, Haegeman G. Dissociated glucocorticoids with anti-inflammatory potential repress interleukin-6 gene expression by a nuclear factor-kappaB-dependent mechanism. Mol Pharmacol 1999;56:797–806.

[25] Adcock IM. Glucocorticoids: new mechanisms and future agents. Curr Allergy Asthma Rep 2003;3:249–57.

[26] Beauchamp RD, Papaconstantinou J, Henderson AM, Sheng HM, Townsend CMJ. Thompson JC. Activation of hepatic proliferation-associated transcription factors by lipopolysaccharide. Surgery 1994;116:367–76.

[27] Newton R. Molecular mechanisms of glucocorticoid action: what is important? Thorax 2000;55:603–13.

[28] Gagne D, Pons M, Crastes de Paulet A. Analysis of the relation between receptor binding affinity and antagonist efficacy of antiglucocorticoids. J Steroid Biochem 1986;25:315–22.

[29] Bourgeois S, Pfahl M, Baulieu EE. DNA binding properties of glucocorticosteroid receptors bound to the steroid antagonist RU-486. EMBO J 1984;3:751–5.

[30] Urnov FD, Wolffe AP. Chromatin remodeling and transcriptional activation: the cast (in order of appearance). Oncogene 2001; 20:2991–3006.

[31] Ito K, Barnes PJ, Adcock IM. Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1β-induced histone H4 acetylation on lysines 8 and 12. Mol Cell Biol 2000;20: 6891–903.

[32] Lai WS, Thompson MJ, Blackshear PJ. Characteristics of the intron involvement in the mitogen-induced expression of Zfp-36. J Biol Chem 1998;273:506–17.

[33] Inuzuka H, Nanbu-Wakao R, Masuho Y, Muramatsu M, Tojo H, Wakao H. Differential regulation of immediate early gene expression in preadipocyte cells through multiple signaling pathways. Biochem Biophys Res Commun 1999;265:664–8.

[34] Brooks SA, Connolly JE, Rigby WFC. The role of mRNA turnover in the regulation of tristetraprolin expression: evidence for an extracellular signal-regulated kinase-specific, AU-rich element-dependent, autoregulatory pathway. J Immunol 2004;172:7263–71.

[35] Newton R, Stevens DA, Hart LA, Lindsay M, Adcock IM, Barnes PJ. Superinduction of COX-2 mRNA by cycloheximide and interleukin-1β involves increased transcription and correlates with increased NF-κB and JNK activation. FEBS Lett 1997;418:135–8.

[36] Arenander AT, Lim RW, Varnum BC, Cole R, de Vellis J, Herschman HR. TIS gene expression in cultured rat astrocytes: multiple pathways of induction by mitogens. J Neurosci Res 1989;23:257–65.

[37] Varnum BC, Lim RW, Kujubu DA, Luner SJ, Kaufman SE, Greenberger JS, et al. Granulocyte-macrophage colony-stimulating factor and tetradecanoyl phorbol acetate induce a distinct, restricted subset of primary-response TIS genes in both proliferating and terminally differentiated myeloid cells. Mol Cell Biol 1989;9:3580–3.

[38] Lau LF, Nathans D. Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc. Proc Natl Acad Sci USA 1987;84:1182–6.

[39] Varnum BC, Ma QF, Chi TH, Fletcher B, Herschman HR. The TIS11 primary response gene is a member of a gene family that encodes proteins with a highly conserved sequence containing an unusual Cys-His repeat. Mol Cell Biol 1991;11:1754–8.

[40] Shi Y, Gaestel M. In the cellular garden of forking paths: how p38 MAPKs signal for downstream assistance. Biol Chem 2002; 383:1519–36.

[41] Tchen CR, Brook M, Saklatvala J, Clark AR. The stability of tristetraprolin mRNA is regulated by mitogen-activated protein kinase p38 and by tristetraprolin itself. J Biol Chem 2004; 279:32393–400.

[42] Cao H, Dzineku F, Blackshear PJ. Expression and purification of recombinant tristetraprolin that can bind to tumor necrosis factor-α mRNA and serve as a substrate for mitogen-activated protein kinases. Archiv Biochem Biophys 2003;412:106–20.

# Salbutamol increases tristetraprolin expression in macrophages

Ulla Jalonen, Tiina Leppänen, Hannu Kankaanranta, Eeva Moilanen *

*The Immunopharmacology Research Group, Medical School, University of Tampere, and Research Unit, Tampere University Hospital, 33014 Tampere, Finland*

## Abstract

Tristetraprolin (TTP) is a tandem zinc finger protein that can bind to AU-rich elements (AREs) in the 3′-untranslated regions (3′-UTR) in mRNAs of transiently expressed genes, e.g. tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and granulocyte macrophage colony-stimulating factor (GM-CSF). TTP increases the turnover rate of the target mRNAs, thereby reducing, for example, the expression of TNF-$\alpha$ and GM-CSF. We examined the role of $\beta_2$-agonists, cAMP analogs, and forskolin (an activator of adenylate cyclase) on TTP mRNA and protein expression by quantitative real-time RT-PCR and Western blotting in J774 murine macrophages and THP-1 human macrophages. All of these agents increased TTP expression. A nonspecific inhibitor of phosphodiesterases (PDEs) 3-isobutyl-1-methylxanthine (IBMX) and type IV PDE-inhibitor rolipram further enhanced the increase in TTP expression levels, suggesting a cAMP-mediated effect. A possible mediator of these effects is transcription factor activator protein 2 (AP-2), whereas nuclear factor $\kappa$B (NF-$\kappa$B) seemed not to play any role. This mechanism may, at least in part, explain the anti-inflammatory effects which $\beta_2$-agonists have been reported to have in macrophages.
© 2007 Elsevier Inc. All rights reserved.

*Keywords:* Activator protein 2; Asthma; $\beta_2$-agonist; cAMP; Inflammation; Salbutamol; Tristetraprolin

## Introduction

Tristetraprolin (TTP) (Lai et al., 1990), also known as Nup475 (DuBois et al., 1990), TIS11 (Ma and Herschman, 1991; Varnum et al., 1989), G0S24 (Heximer and Forsdyke, 1993), or Zfp36 (Blackshear, 2002) is an RNA-binding protein that affects the stability of certain mRNAs and its role in the post-transcriptional regulation of its known substrates has become clearer in recent years. Studies on TTP knockout (KO) mice showed a direct connection between TTP and inflammation. The mice developed a systemic inflammatory syndrome with arthritis, autoimmunity, and myeloid hyperplasia (Taylor et al., 1996). The best-studied target of TTP-mediated mRNA instability derived from KO mice is tumor necrosis factor-$\alpha$ (TNF-$\alpha$). TTP can promote the deadenylation and decay of TNF-$\alpha$ mRNA by binding to the conserved AU-rich element (ARE) in the 3′-untranslated region (3′-UTR) of TNF-$\alpha$ mRNA (Lai and Blackshear, 2001; Lai et al., 1999), thereby decreasing the amount of TNF-$\alpha$. Other known targets of TTP include

granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-2 (Carballo et al., 2000; Ogilvie et al., 2005). Recently, cells from TTP-KO mice were subjected to microarray analysis to identify new targets of TTP-mediated mRNA decay, revealing more than 250 transcripts whose mRNA was stabilized in the absence of TTP (Lai et al., 2006). Those containing two or more TTP binding sites were taken for further study. The most dramatic changes in mRNA levels were found in the expression of immediate-early response 3 (Ier3), a regulator of blood pressure. In addition, cyclo-oxygenase-2 (COX-2), IL-3, IL-6 and plasminogen activator inhibitor type 2 have been found to be destabilized by TTP in other cell types using overexpression and gene silencing methods (Sawaoka et al., 2003; Stoecklin et al., 2000, 2001; Yu et al., 2003). In our recent study, IL-12 and macrophage inflammatory proteins 2 (a homologue to human IL-8) and 3$\alpha$ were identified as potential targets of TTP by antibody array method (Jalonen et al., 2006). By destabilizing proinflammatory mRNAs, TTP down-regulates their expression and thereby modulates inflammatory responses towards anti-inflammatory and immunosuppressive direction.

Two of the best-studied targets of TTP, proinflammatory cytokines TNF-$\alpha$ and GM-CSF, also play a major role in

---

Table 1
Primer and probe sequences

| Primer | Sequence |
| --- | --- |
| mTTP forward | 5′-CTCAGAAAGCGGGCGTTGT-3′ |
| mTTP reverse | 5′-GATTGGCTTGGCGAAGTTCA-3′ |
| mTTP probe | 5′-Fam-CCAAGTGCCAGTTTGCTCACGGC-Tamra-3′ |
| mGAPDH forward | 5′-GCATGGCCTTCCGTGTTC-3′ |
| mGAPDH reverse | 5′-GATGTCATCATACTTGGCAGGTTT-3′ |
| mGAPDH probe | 5′-Fam-TCGTGGATCTGACGTGCCGCC-Tamra-3′ |
| hTTP forward | 5′-CCCCAAATACAAGACGGAACTC-3′ |
| hTTP reverse | 5′-GGGCCGCCAGGTCTTC-3′ |
| hTTP probe | 5′-Fam-CCCTACGGCTCTCGCTGCCACTT-Tamra-3′ |
| hGAPDH forward | 5′-AAGGTCGGAGTCAACGGATTT-3′ |
| hGAPDH reverse | 5′-GCAACAATATCCACTTTACCAGAGTTAA-3′ |
| hGAPDH probe | 5′-Fam-CGCCTGGTCACCAGGGCTGC-Tamra-3′ |

asthma. High levels of TNF-α correlate with asthmatic complications and compounds that reduce TNF-α levels may be successful in the treatment of asthma (Mukhopadhyay et al., 2006). The expression levels of GM-CSF in asthmatic patients compared to those in non-asthmatic individuals are much higher in response to different stimuli and GM-CSF may be one of the factors leading to events that provoke airway inflammation and asthma (Ritz et al., 2002). Thus, agents that decrease the amount of TNF-α and GM-CSF may be useful in the treatment of asthma.

β₂-agonists are widely used as bronchodilatators in the treatment of asthma and chronic obstructive pulmonary disease. Activation of β₂-adrenoceptors leads to a series of events that induce airway smooth muscle relaxation and also to some anti-inflammatory effects (Bissonnette and Befus, 1997; Broadley, 2006; Giembycz and Newton, 2006; Johnson, 2002, 2006; Sitkauskiene and Sakalauskas, 2005). β₂-adrenoceptors activate adenylate cyclase that results in increased intracellular cAMP levels and activation of protein kinase A (PKA), which in turn regulates phosphorylation (activation) of target molecules and gene expression.
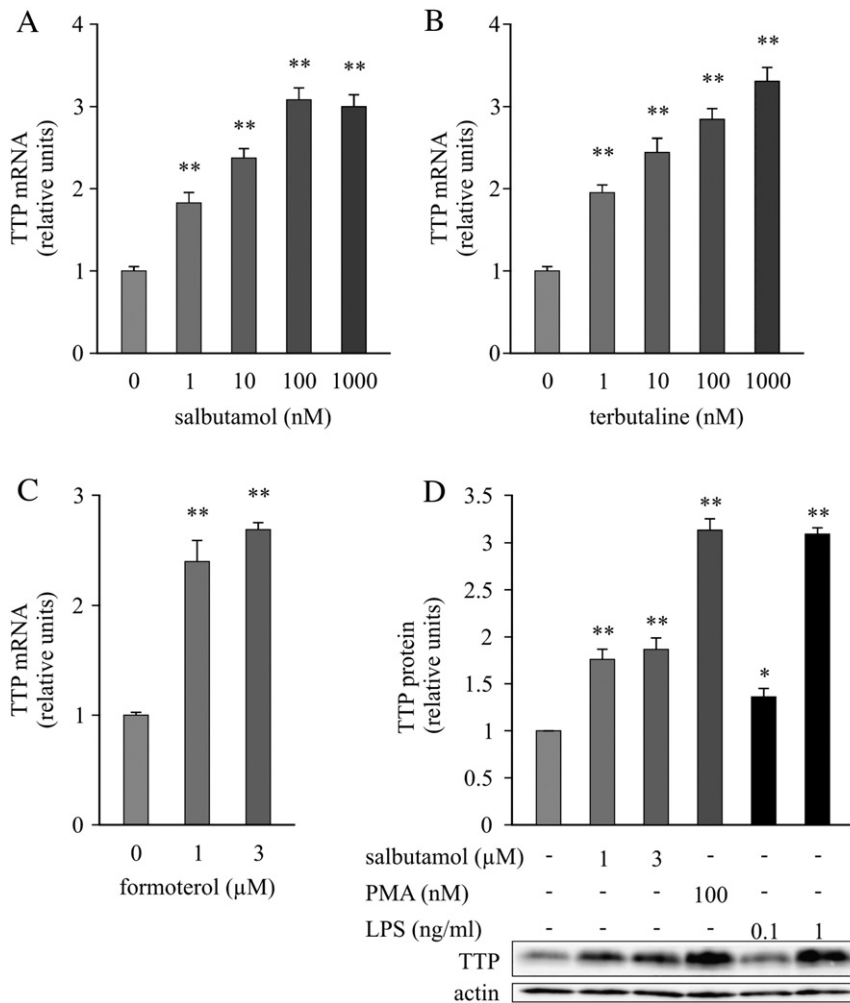


Fig. 1. Effects of β₂-agonists on TTP mRNA and protein expression in J774 macrophages. J774 macrophages were treated with increasing concentrations of (A) salbutamol, (B) terbutaline or (C) formoterol for 1 h, after which total RNA was extracted. Quantitative RT-PCR was used to measure TTP mRNA and the values were normalized to GAPDH mRNA. The TTP mRNA level in the control samples was set at 1 and the other values were related to that. Values are mean±S.E.M. ($n=4$). **$P<0.01$ when compared to the control. (D) The cells were stimulated with salbutamol (1 and 3 μM), PMA (100 nM) or LPS (0.1 and 1 ng/ml) for 4 h. Proteins were extracted and TTP and actin (as a loading control) proteins were measured by Western blot. The TTP protein level in the control samples was set at 1 and the other values were related to that. The blot is a representative of six with similar results. Values are mean±S.E.M. ($n=6$). **$P<0.01$, *$P<0.05$ when compared to the control.

$\beta_2$-agonists have been shown to inhibit TNF-$\alpha$ and GM-CSF release from human mast cells, blood monocytes, macrophages, lyphocytes, and epithelial cells, and demonstrate their anti-inflammatory effects possibly through elevation of cAMP levels (Bissonnette and Befus, 1997; Broadley, 2006; Izeboud et al., 1999; Johnson, 2002; Sitkauskiene and Sakalauskas, 2005). There is tentative data that an activator of adenylate cyclase, forskolin, and an analog of cAMP, dibutyryl-cAMP, may increase TTP mRNA levels (DuBois et al., 1990; Kaneda et al., 1992). Therefore we hypothesized that $\beta_2$-agonists may enhance TTP expression. Here we show that $\beta_2$-agonists and cAMP-elevating agents increase TTP mRNA and protein expression, possibly through the activation of transcription factor activator protein 2 (AP-2). We therefore suggest that $\beta_2$-agonists in part fulfill their anti-inflammatory responses by increasing the expression of TTP.

## Materials and methods

### Materials

Forskolin was purchased from Tocris Cookson Ltd. (Ellisville, MO, USA). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

### Cell culture

The murine J774 macrophages (European Collection of Cell Cultures, Salisbury, UK) were maintained in an atmosphere of 5% carbon dioxide at 37 °C in Dulbecco's modified Eagle medium with UltraGlutamine 1 (Cambrex Bioproducts Europe, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Cambrex Bioproducts Europe), penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml) (Gibco, Paisley, Scotland, UK). Cells were seeded on 6- or 24-well plates and grown to confluence prior to the experiments.

The human THP-1 promonocytes (American Type Culture Collection, Manassas, VA, USA) were cultured at 37 °C in humidified 5% carbon dioxide atmosphere in RPMI 1640 (Cambrex Bioproducts Europe) adjusted to contain 2 mM L-glutamine, 10 mM HEPES (Cambrex Bioproducts Europe), 1 mM sodium pyruvate (Cambrex Bioproducts Europe), 4.5 g/l glucose, and 1.5 g/l bicarbonate (Cambrex Bioproducts Europe) and supplemented with 10% heat-inactivated fetal bovine serum (Cambrex Bioproducts Europe), penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml) (Gibco), and 0.05 mM 2-mercaptoethanol. The cells were differentiated by adding the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (100 nM) 72 h before the experiments at the time of seeding of the cells on 6- or 24-well plates.

### RNA extraction and quantitative real-time reverse transcriptase (RT)-PCR

The protocol for RNA extraction and quantitative real-time RT-PCR has been described in (Jalonen et al., 2005). Primers and probes (Table 1) for human and mouse TTP (hTTP and mTTP respectively) and also human and mouse glyceraldehyde-3-phosphate dehydrogenase (hGAPDH and mGAPDH
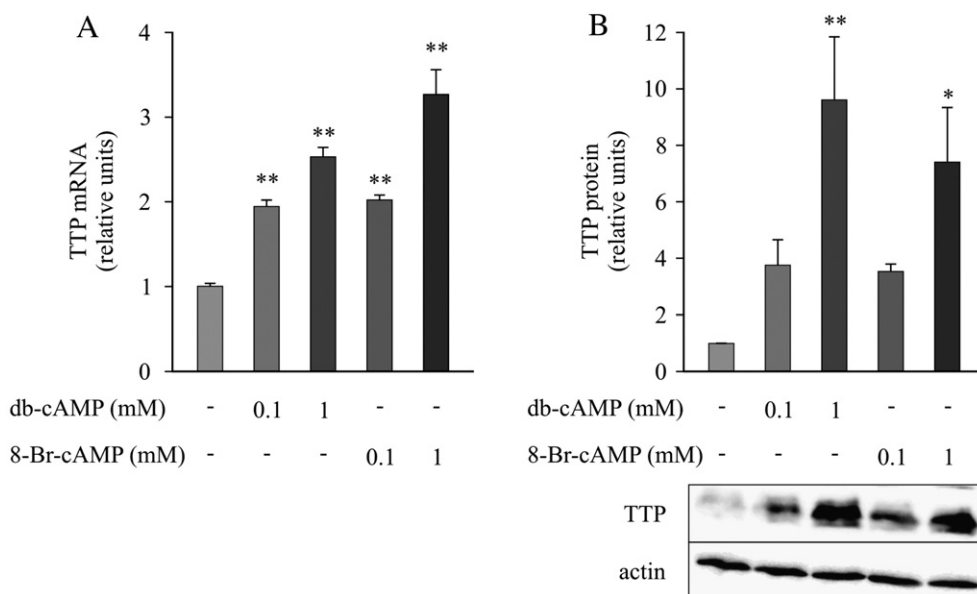


Fig. 2. Effects of cAMP analogs on TTP mRNA and protein expression in J774 macrophages. (A) Db-cAMP and 8-Br-cAMP (0.1 and 1 mM) were used to stimulate macrophages for 1 h, after which total RNA was extracted. Quantitative RT-PCR was used to measure TTP mRNA and the values were normalized to GAPDH mRNA. The TTP mRNA level in the control samples was set at 1 and the other values were related to that. Values are mean±S.E.M. ($n=3$). **$P<0.01$ when compared to the control. (B) The cells were stimulated with db-cAMP and 8-Br-cAMP (0.1 and 1 mM) for 6 h. Proteins were extracted and TTP and actin proteins were measured by Western blot. The TTP protein level in the control samples was set at 1 and the other values were related to that. The blot is a representative of three with similar results. Values are mean±S.E.M. ($n=3$). **$P<0.01$, *$P<0.05$ when compared to the control.

respectively) were designed using Primer Express® Software (Applied Biosystems, Foster City, CA, USA) and supplied by Metabion (Martinsried, Germany).

### Western blotting

The protocol for Western blotting has been described in (Jalonen et al., 2005). The gels were loaded with 35 or 20 µg of protein for TTP or transcription factor Western blots respectively. Actin, AP-2, and lamin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), nuclear factor κB (NF-κB) antibody from Cell Signaling Technology Inc. (Danvers, MA, USA), and the mouse TTP antibody (Cao et al., 2004) was a kind gift from Dr. Perry Blackshear (NIEHS, Research Triangle Park, NC, USA). The bound antibodies were detected using SuperSignal® West Pico (for actin and lamin) or Dura (for AP-2, NF-κB, and TTP) chemiluminescent substrate for HRP detection (Pierce, Cheshire, U.K.), and FluorChem™ 8800 imaging system (Alpha Innotech, San Leandro, CA, USA). The chemiluminescent signals were measured with FluorChem™ software v. 3.1.

### Preparation of nuclear extracts for AP-2 and NF-κB Western blotting

At indicated time points, the cells were rapidly washed with ice-cold PBS and solubilized in hypotonic buffer A (10 mM HEPES–KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1 mM NaF and 0.1 mM EGTA). After incubation for 10 min on ice, the cells were vortexed for 30 s and the nuclei were separated by centrifugation at 4 °C, 21 000×g for 10 s. The nuclei were resuspended in buffer C (20 mM HEPES–KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1 mM NaF and 0.1 mM EGTA) and incubated for 20 min on ice. The nuclei were vortexed for 30 s and nuclear extracts were obtained by centrifugation at 4 °C, 21 000×g for 2 min. The protein contents of the nuclear extracts were measured by the Coomassie blue method (Bradford, 1976).

### Statistics

Results are expressed as the mean±standard error of mean (SEM). The significance of differences was calculated by analysis of variance supported by Dunnett's adjusted significance levels. A difference between treatment groups was considered significant when $P < 0.05$.

## Results

### β2-agonists, cAMP analogs, and forskolin-induced TTP expression in murine J774 macrophages

Resting J774 macrophages expressed low levels of TTP mRNA. When the cells were treated with increasing concentra-

tions of salbutamol or terbutaline (1–1000 nM) for 1 h, a maximum of 3 to 3.5-fold increase in TTP mRNA levels was found (Fig. 1A and B). Maximal effect was achieved at 100 nM salbutamol. Similarly, formoterol 1 and 3 µM increased TTP mRNA levels approximately 2.5-fold (Fig. 1C). A clear increase was observed also in TTP protein levels (as detected by Western blot) when J774 macrophages were treated with salbutamol (1 and 3 µM) for 4 h (Fig. 1D). Phorbol 12-myristate 13-acetate (PMA, 100 nM) and lipopolysaccharide (LPS, 0.1 and 1 ng/ml) were used as control compounds to evaluate the level of TTP protein expression. PMA (100 nM) and LPS (1 ng/ml) increased TTP protein levels, and their effect was somewhat stronger than that of salbutamol.

Since β2-agonists are known to elevate cAMP levels, we were curious to know whether cAMP analogs and other cAMP-elevating agents were able to promote TTP expression. The
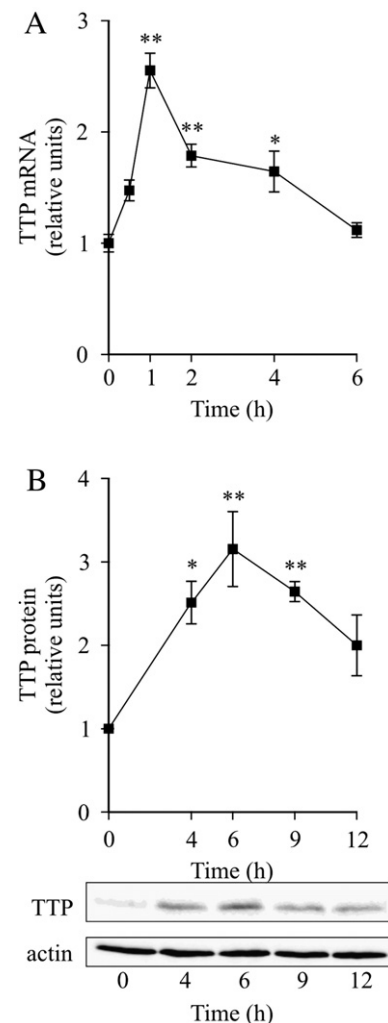


Fig. 3. Forskolin-induced TTP mRNA and protein expression in J774 macrophages. The cells were incubated with forskolin (100 µM). (A) Total mRNA and (B) proteins were extracted at indicated time points and quantitative RT-PCR and Western blot were used to measure TTP and GAPDH mRNA, and TTP and actin protein levels respectively. The TTP mRNA or protein level in the control samples was set at 1 and the other values were related to that. The blot is a representative of three blots with similar results. Values are mean±S.E.M. ($n=3$). **$P < 0.01$, *$P < 0.05$ when compared to the control.

cells were treated with cAMP analogs $N^6,2'$-$O$-dibutyryladenosine $3',5'$-cyclic monophosphate sodium salt (db-cAMP) and 8-bromoadenosine $3',5'$-cyclic monophosphate sodium salt (8-Br-cAMP) at concentrations of 100 μM and 1 mM for 1 h and mRNA levels were quantitated. A 2 to 3-fold increase in TTP mRNA levels was detected with the concentrations used (Fig. 2A). Db-cAMP and 8-Br-cAMP also increased TTP protein levels when measured by Western blot after 6 h incubation (Fig. 2B).

An activator of adenylate cyclase forskolin (100 μM) also induced TTP mRNA and protein expression in J774 macrophages. TTP mRNA expression peaked at 1 h and declined to basal level after 6 h incubation (Fig. 3A). TTP protein expression followed mRNA expression and peaked at 6 h showing a 3-fold increase in that time point (Fig. 3B).

The induction of TTP mRNA expression by $β_2$-agonists, cAMP analogs, and forskolin was clear and statistically significant but weaker than that induced by LPS (1 ng/ml). The ΔCt value between control and LPS-treatment was around 3, while with the $β_2$-agonists, cAMP analogs, and forskolin the ΔCt value between control and treatment was around 2.

*Phosphodiesterase (PDE) inhibitors increased salbutamol and forskolin-induced TTP expression in murine J774 macrophages*

The J774 macrophages were incubated with a nonspecific inhibitor of PDEs, 3-isobutyl-1-methylxanthine (IBMX, 100 μM), and type IV PDE inhibitor, rolipram (20 μM), for 30 min. Thereafter salbutamol (1 μM) or forskolin (100 μM)
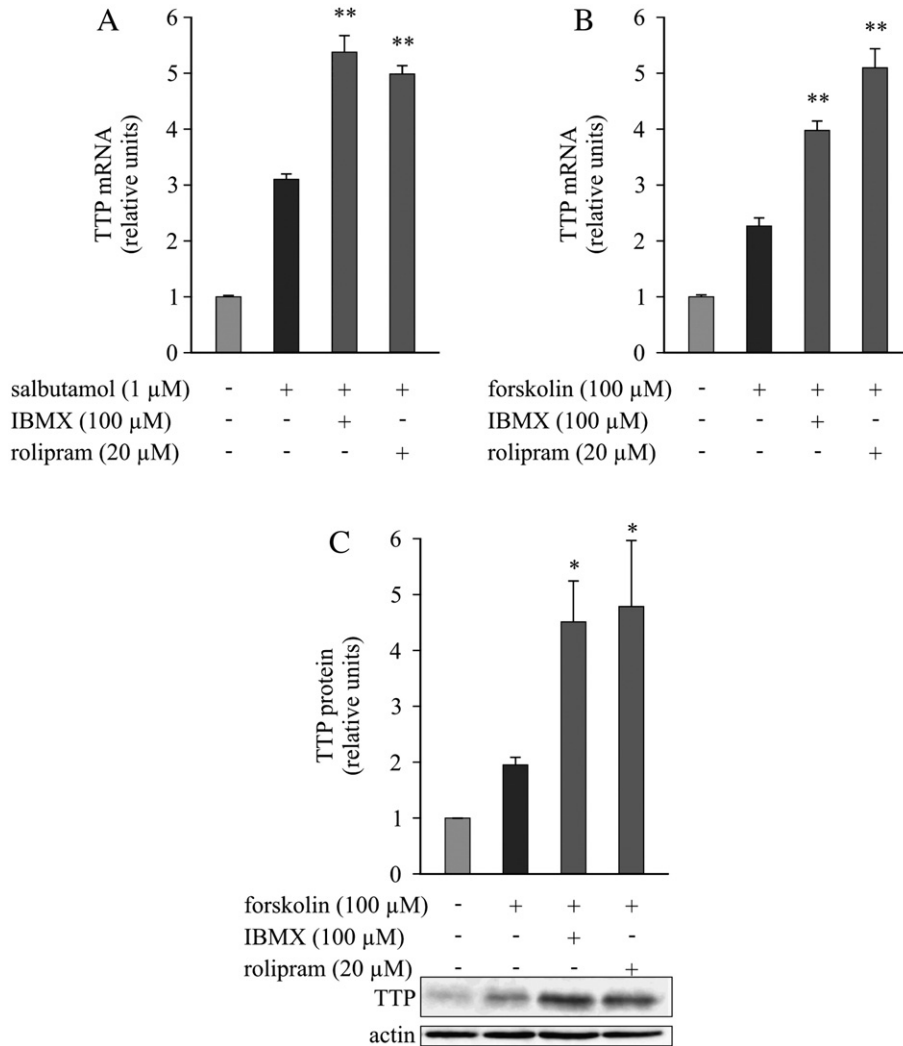


Fig. 4. PDE-inhibitors enhanced salbutamol and forskolin-induced TTP mRNA and protein expression in J774 macrophages. The cells were incubated with IBMX (100 μM) and rolipram (20 μM) for 30 min prior to the addition of (A) salbutamol (1 μM) or (B) forskolin (100 μM). Total RNA was extracted after 1 h incubation and used in quantitative RT-PCR to detect TTP and GAPDH mRNA. The TTP mRNA level in the control samples was set at 1 and the other values were related to that. Values are mean ± S.E.M. ($n=4$ in A, $n=3$ in B). **$P<0.01$ when compared to samples treated with salbutamol (A) or forskolin (B) alone. (C) The cells were incubated with IBMX (100 μM) and rolipram (20 μM) for 30 min prior to the addition of forskolin (100 μM) and proteins were extracted after 6 h incubation. Proteins were analyzed by Western blot to detect TTP and actin protein. The TTP protein level in the control samples was set at 1 and the other values were related to that. A representative blot of three with similar results is shown. Values are mean ± S.E.M. ($n=3$). *$P<0.05$ when compared to samples treated with forskolin (100 μM) alone.

Table 2
Effects of salbutamol and terbutaline on TTP mRNA expression in human THP-1 cells

| Treatment | TTP mRNA (relative units) |
|---|---|
| control | $100 \pm 9.4$ |
| Salbutamol (1 μM) | $139.4 \pm 20.1$ |
| Salbutamol (3 μM) | $153.8 \pm 12.1$* |
| Terbutaline (1 μM) | $169.3 \pm 17.3$** |
| Terbutaline (3 μM) | $171.4 \pm 12.8$** |

The cells were cultured with $\beta_2$-agonists for 1 h. Total RNA was extracted and used to detect TTP and GAPDH mRNA by quantitative RT-PCR. The TTP mRNA level in the control samples was set at 100 and the other values were related to that. Values are mean $\pm$ S.E.M. ($n=4$). **$P<0.01$, *$P<0.05$ when compared to the control.

was added to induce TTP expression for 1 h. In the presence of these compounds salbutamol-induced TTP mRNA levels were further increased (Fig. 4A), as was also forskolin-induced TTP mRNA expression (Fig. 4B). PDE inhibitors also augmented forskolin-induced TTP protein expression levels (Fig. 4C).

### $\beta_2$-agonists, cAMP analogs, and forskolin-induced TTP expression in human THP-1 macrophages

We were interested whether the same phenomena could be seen in human cells, and used human THP-1 macrophages in the subsequent experiments. Resting THP-1 macrophages expressed low levels of TTP mRNA. A 39–71% increase in TTP mRNA levels was seen when the cells were incubated with $\beta_2$-agonists salbutamol and terbutaline (1 and 3 μM) for 1 h (Table 2). Forskolin (100 μM) and db-cAMP (1 mM) increased TTP mRNA levels by 126% and 151% respectively (Table 3). In addition, PDE-inhibitor IBMX (100 μM) resulted in a 48% increase in salbutamol-induced TTP mRNA in THP-1 cells suggesting a cAMP-mediated response (Data not shown).

### Forskolin-induced nuclear translocation of AP-2 but not NF-κB in murine J774 macrophages

The TTP promoter region has been shown to include AP-2 binding site (Lai et al., 1995) and the single intron to contain NF-κB-like binding motif (Lai et al., 1998), which are assumed to be important regulators of TTP expression. Therefore we

tested the effect of forskolin on the activation of AP-2 and NF-κB, which was assessed by measuring their nuclear translocation by Western blot. The results showed that AP-2 relocated to the nucleus when cells were treated with forskolin (100 μM) or LPS (1 ng/ml), which was used as a control compound (Fig. 5A). This implies that AP-2 may be involved in forskolin-induced TTP expression. On the other hand, forskolin treatment (100 μM) for 30 min was not able to relocate NF-κB to the nucleus (Fig. 5B). LPS was used as a control compound and induced nuclear translocation of NF-κB, which peaked at 30 min.



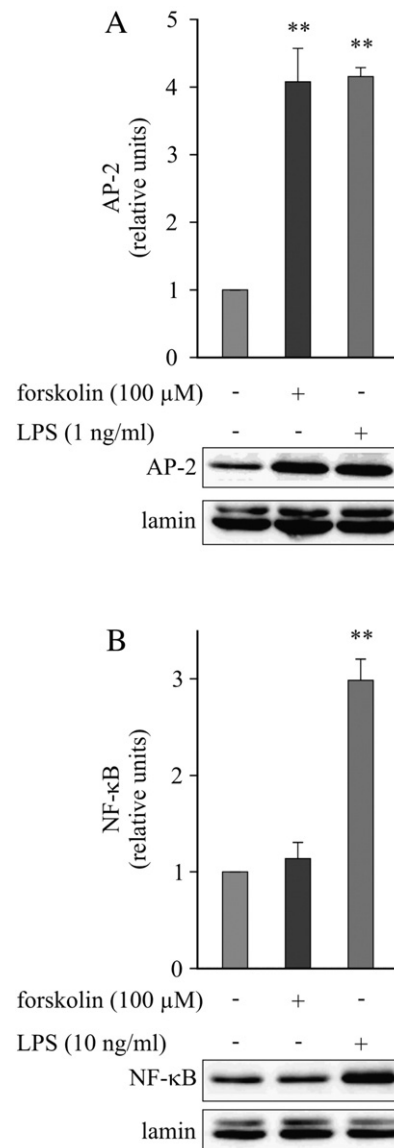Fig. 5. Nuclear translocation of AP-2 and NF-κB in response to forskolin treatment in J774 macrophages. The cells were stimulated with forskolin (100 μM), or LPS (1 or 10 ng/ml), which was used as a control compound, for 30 min. Nuclear extracts were prepared as described in materials and methods. AP-2 (A), NF-κB (B) and lamin protein were detected in nuclear extracts by Western blot. The AP-2 (A) and NF-κB (B) level in control samples was set at 1 and the other values were related to that. Values are mean $\pm$ S.E.M. ($n=3$). **$P<0.01$ when compared to the control.

Table 3
Effects of forskolin and db-cAMP on TTP mRNA expression in human THP-1 cells

| Treatment | TTP mRNA (relative units) |
|---|---|
| Control | $100 \pm 1.8$ |
| Forskolin (100 μM) | $225.7 \pm 15.0$** |
| db-cAMP (1 mM) | $251.1 \pm 16.5$** |

The cells were stimulated with forskolin or db-cAMP for 3 h, after which total RNA was extracted and used to detect TTP and GAPDH mRNA by quantitative RT-PCR. The TTP mRNA level in the control samples was set at 100 and the other values were related to that. Values are mean $\pm$ S.E.M. ($n=4$). **$P<0.01$ when compared to the control.

## Discussion

In the present study, $\beta_2$-agonists, cAMP analogs, and forskolin (an activator of adenylate cyclase) were shown to increase TTP expression in both mouse and human macrophages. PDE-inhibitors further enhanced the response. A possible mediator for the effect is transcription factor AP-2, whereas NF-$\kappa$B seemed to play no role in forskolin-induced TTP expression.

The significant role of TTP in inflammation was discovered when the TTP-KO mice were generated and investigated (Taylor et al., 1996). TTP-KO mice produce proinflammatory cytokines TNF-$\alpha$ and GM-CSF in excess due to increased mRNA stability of the two factors (Carballo et al., 1998, 2000), and this leads to inflammatory syndrome including severe arthritis, autoimmunity, and myeloid hyperplasia (Phillips et al., 2004; Taylor et al., 1996). The symptoms could be alleviated by injections of TNF-$\alpha$ antibody (Taylor et al., 1996). TTP expression has been shown in various tissues and the high expression of TTP in mouse and rat lung tissues is of interest (Cao et al., 2004; Lai et al., 1990; Smoak and Cidlowski, 2006). Only little is known about the pharmacological regulation of TTP expression. In addition to the $\beta_2$-agonists used in the present study, dexamethasone has been shown to increase TTP mRNA and protein levels in resting human A549 lung epithelial cells and in rat lung tissue (Smoak and Cidlowski, 2006). On the other hand, dexamethasone reduced LPS-induced TTP expression in J774 macrophages (Jalonen et al., 2005). Interferons have been reported to up-regulate TTP expression, which in turn results in down-regulation of several proinflammatory genes (Sauer et al., 2006). Cinnamon extract and cinnamon polyphenols have been shown to increase both the protein and mRNA levels of TTP in mouse adipocytes (Cao et al., 2007b). In addition, green tea, which is also associated with anti-inflammatory effects, increased the levels of TTP mRNA in rat liver and skeletal muscle (Cao et al., 2007a).

TNF-$\alpha$ and GM-CSF, whose expression is regulated by TTP at post-transcriptional level, play a major role in asthma. Here we show that $\beta_2$-agonists, which are used as bronchodilatators by asthmatic patients, and also cAMP analogs and forskolin, increase the expression of TTP in mouse and human macrophages. The results are supported by earlier findings that forskolin and db-cAMP increase TTP mRNA levels in mouse fibroblasts and rat pheochromocytoma cells (DuBois et al., 1990; Kaneda et al., 1992). A nonspecific PDE-inhibitor IBMX and type IV PDE-inhibitor rolipram further increased the level of TTP mRNA suggesting a cAMP-mediated effect.

Little is known about transcription factors that bind to TTP promoter and enhance TTP mRNA transcription. Computer analyses of sequence elements in TTP promoter of mouse, human, and rat origin have given some indications of such factors (DuBois et al., 1990; Heximer and Forsdyke, 1993; Kaneda et al., 2000; Lai et al., 1995; Smoak and Cidlowski, 2006). The involvement of the single intron of TTP gene has also been characterized (Lai et al., 1998). NF-$\kappa$B-like binding site in TTP intron has been reported to be involved in TNF-$\alpha$ induced TTP expression (Lai et al., 1998), but according to the present results it seems not to be involved in cAMP-mediated TTP expression.

One of the transcription factors emerging in most of the analyses and is required for full serum inducibility of mouse TTP (Lai et al., 1995) is AP-2. cAMP pathway is one of the signaling pathways that activates AP-2 (Imagawa et al., 1987). There is evidence that shuttling between cytoplasm and nucleus regulates the activity of AP-2 (Mazina et al., 2001). In the present study, forskolin-induced nuclear translocation of AP-2. This effect may well explain the increases in TTP expression detected.

In addition to bronchodilatation, salbutamol and other $\beta_2$-agonists have been shown to have some anti-inflammatory effects (Bissonnette and Befus, 1997; Broadley, 2006; Johnson, 2002; Sitkauskiene and Sakalauskas, 2005). Inflammatory cells including macrophages express $\beta_2$-adrenoceptors on their cell surface. Activation of these receptors on inflammatory cells results in the inhibition of the release of modulators such as leukotrienes and histamine and suppression of the secretion of cytokines such as TNF-$\alpha$, GM-CSF and interleukins 2, 3 and 6 (Bissonnette and Befus, 1997; Sitkauskiene and Sakalauskas, 2005). TNF-$\alpha$, GM-CSF, IL-2, IL-3 and IL-6 have all been reported to be targets of TTP (Carballo et al., 1998, 2000; Ogilvie et al., 2005; Stoecklin et al., 2000, 2001). Therefore it is tempting to speculate that TTP mediates, at least to some extent, the anti-inflammatory effects of $\beta_2$-agonists in macrophages.

In the present study, salbutamol and other $\beta_2$-agonists were found to elevate TTP expression in human and murine macrophages, possibly through increased intracellular cAMP levels and activation of transcription factor AP-2. cAMP analogs and forskolin had very similar effects. The TTP-enhancing effect described here may be one of the mechanisms by which $\beta_2$-agonists regulate cytokine expression in macrophages and display anti-inflammatory properties in inflammation.

## Acknowledgements

## References

Bissonnette, E.Y., Befus, A.D., 1997. Anti-inflammatory effect of $\beta_2$-agonists: inhibition of TNF-$\alpha$ release from human mast cells. The Journal of Allergy and Clinical Immunology 100 (6 Pt 1), 825–831.

Blackshear, P.J., 2002. Tristetraprolin and other CCCH tandem zinc-finger proteins in the regulation of mRNA turnover. Biochemical Society Transactions 30 (Pt 6), 945–952.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Broadley, K.J., 2006. $\beta$-Adrenoceptor responses of the airways: for better or worse? European Journal of Pharmacology 533 (1–3), 15–27.

Cao, H., Tuttle, J.S., Blackshear, P.J., 2004. Immunological characterization of tristetraprolin as a low abundance, inducible, stable cytosolic protein. Journal of Biological Chemistry 279 (20), 21489–21499.

Cao, H., Kelly, M.A., Kari, F., Dawson, H.D., Urban Jr., J.F., Coves, S., Roussel, A.M., Anderson, R.A., 2007a. Green tea increases anti-inflammatory tristetraprolin and decreases pro-inflammatory tumor necrosis factor mRNA levels in rats. Journal of Inflammation (London, England) 4 (1) 12 pages.

Cao, H., Polansky, M.M., Anderson, R.A., 2007b. Cinnamon extract and polyphenols affect the expression of tristetraprolin, insulin receptor, and glucose transporter 4 in mouse 3T3-L1 adipocytes. Archives of Biochemistry and Biophysics 459 (2), 214–222.

Carballo, E., Lai, W.S., Blackshear, P.J., 1998. Feedback inhibition of macrophage tumor necrosis factor-α production by tristetraprolin. Science 281 (5379), 1001–1005.

Carballo, E., Lai, W.S., Blackshear, P.J., 2000. Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability. Blood 95 (6), 1891–1899.

DuBois, R.N., McLane, M.W., Ryder, K., Lau, L.F., Nathans, D., 1990. A growth factor-inducible nuclear protein with a novel cysteine/histidine repetitive sequence. Journal of Biological Chemistry 265 (31), 19185–19191.

Giembycz, M.A., Newton, R., 2006. Beyond the dogma: novel $\beta_2$-adrenoceptor signalling in the airways. The European Respiratory Journal 27 (6), 1286–1306.

Heximer, S.P., Forsdyke, D.R., 1993. A human putative lymphocyte $G_0/G_1$ switch gene homologous to a rodent gene encoding a zinc-binding potential transcription factor. DNA and Cell Biology 12 (1), 73–88.

Imagawa, M., Chiu, R., Karin, M., 1987. Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell 51 (2), 251–260.

Izeboud, C.A., Monshouwer, M., van Miert, A.S.J.P.A.M., Witkamp, R.F., 1999. The β-adrenoceptor agonist clenbuterol is a potent inhibitor of the LPS-induced production of TNF-α and IL-6 in vitro and in vivo. Inflammation Research 48 (9), 497–502.

Jalonen, U., Lahti, A., Korhonen, R., Kankaanranta, H., Moilanen, E., 2005. Inhibition of tristetraprolin expression by dexamethasone in activated macrophages. Biochemical Pharmacology 69 (5), 733–740.

Jalonen, U., Nieminen, R., Vuolteenaho, K., Kankaanranta, H., Moilanen, E., 2006. Down-regulation of tristetraprolin expression results in enhanced IL-12 and MIP-2 production and reduced MIP-3α synthesis in activated macrophages. Mediators of Inflammation 2006 (Article ID 40691), 8 pages.

Johnson, M., 2002. Effects of $\beta_2$-agonists on resident and infiltrating inflammatory cells. The Journal of Allergy and Clinical Immunology 110 (6 Suppl), S282–S290.

Johnson, M., 2006. Molecular mechanisms of $\beta_2$-adrenergic receptor function, response, and regulation. The Journal of Allergy and Clinical Immunology 117 (1), 18–24.

Kaneda, N., Oshima, M., Chung, S.Y., Guroff, G., 1992. Sequence of a rat *TIS11* cDNA, an immediate early gene induced by growth factors and phorbol esters. Gene 118 (2), 289–291.

Kaneda, N., Murata, T., Niimi, Y., Minamiyama, M., 2000. Cloning and characterization of the rat TIS11 gene. Molecular and Cellular Biochemistry 213 (1–2), 119–126.

Lai, W.S., Blackshear, P.J., 2001. Interactions of CCCH zinc finger proteins with mRNA: tristetraprolin-mediated AU-rich element-dependent mRNA degradation can occur in the absence of a poly(A) tail. Journal of Biological Chemistry 276 (25), 23144–23154.

Lai, W.S., Stumpo, D.J., Blackshear, P.J., 1990. Rapid insulin-stimulated accumulation of an mRNA encoding a proline-rich protein. Journal of Biological Chemistry 265 (27), 16556–16563.

Lai, W.S., Thompson, M.J., Taylor, G.A., Liu, Y., Blackshear, P.J., 1995. Promoter analysis of *Zfp-36*, the mitogen-inducible gene encoding the zinc finger protein tristetraprolin. Journal of Biological Chemistry 270 (42), 25266–25272.

Lai, W.S., Thompson, M.J., Blackshear, P.J., 1998. Characteristics of the intron involvement in the mitogen-induced expression of *Zfp-36*. Journal of Biological Chemistry 273 (1), 506–517.

Lai, W.S., Carballo, E., Strum, J.R., Kennington, E.A., Phillips, R.S., Blackshear, P.J., 1999. Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. Molecular and Cellular Biology 19 (6), 4311–4323.

Lai, W.S., Parker, J.S., Grissom, S.F., Stumpo, D.J., Blackshear, P.J., 2006. Novel mRNA targets for tristetraprolin (TTP) identified by global analysis of stabilized transcripts in TTP-deficient fibroblasts. Molecular and Cellular Biology 26 (24), 9196–9208.

Ma, Q., Herschman, H.R., 1991. A corrected sequence for the predicted protein from the mitogen-inducible *TIS11* primary response gene. Oncogene 6 (7), 1277–1278.

Mazina, O.M., Phillips, M.A., Williams, T., Vines, C.A., Cherr, G.N., Rice, R.H., 2001. Redistribution of transcription factor AP-2α in differentiating cultured human epidermal cells. The Journal of Investigative Dermatology 117 (4), 864–870.

Mukhopadhyay, S., Hoidal, J.R., Mukherjee, T.K., 2006. Role of TNFα in pulmonary pathophysiology. Respiratory Research 7 (125) 9 pages.

Ogilvie, R.L., Abelson, M., Hau, H.H., Vlasova, I., Blackshear, P.J., Bohjanen, P.R., 2005. Tristetraprolin down-regulates *IL-2* gene expression through AU-rich element-mediated mRNA decay. Journal of Immunology 174 (2), 953–961.

Phillips, K., Kedersha, N., Shen, L., Blackshear, P.J., Anderson, P., 2004. Arthritis suppressor genes TIA-1 and TTP dampen the expression of tumor necrosis factor α, cyclooxygenase 2, and inflammatory arthritis. Proceedings of the National Academy of Sciences of the United States of America 101 (7), 2011–2016.

Ritz, S.A., Stämpfli, M.R., Davies, D.E., Holgate, S.T., Jordana, M., 2002. On the generation of allergic airway diseases: from GM-CSF to Kyoto. Trends in Immunology 23 (8), 396–402.

Sauer, I., Schaljo, B., Vogl, C., Gattermeier, I., Kolbe, T., Müller, M., Blackshear, P.J., Kovarik, P., 2006. Interferons limit inflammatory responses by induction of tristetraprolin. Blood 107 (12), 4790–4797.

Sawaoka, H., Dixon, D.A., Oates, J.A., Boutaud, O., 2003. Tristetraprolin binds to the 3′-untranslated region of cyclooxygenase-2 mRNA. A polyadenylation variant in a cancer cell line lacks the binding site. Journal of Biological Chemistry 278 (16), 13928–13935.

Sitkauskiene, B., Sakalauskas, R., 2005. The role of $\beta_2$-adrenergic receptors in inflammation and allergy. Current Drug Targets — Inflammation and Allergy 4 (2), 157–162.

Smoak, K., Cidlowski, J.A., 2006. Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling. Molecular and Cellular Biology 26 (23), 9126–9135.

Stoecklin, G., Ming, X.-F., Looser, R., Moroni, C., 2000. Somatic mRNA turnover mutants implicate tristetraprolin in the interleukin-3 mRNA degradation pathway. Molecular & Cellular Biology 20 (11), 3753–3763.

Stoecklin, G., Stoeckle, P., Lu, M., Muehlemann, O., Moroni, C., 2001. Cellular mutants define a common mRNA degradation pathway targeting cytokine AU-rich elements. RNA 7 (11), 1578–1588.

Taylor, G.A., Carballo, E., Lee, D.M., Lai, W.S., Thompson, M.J., Patel, D.D., Schenkman, D.I., Gilkeson, G.S., Broxmeyer, H.E., Haynes, B.F., Blackshear, P.J., 1996. A pathogenetic role for TNFα in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. Immunity 4 (5), 445–454.

Varnum, B.C., Lim, R.W., Sukhatme, V.P., Herschman, H.R., 1989. Nucleotide sequence of a cDNA encoding TIS11, a message induced in Swiss 3T3 cells by the tumor promoter tetradecanoyl phorbol acetate. Oncogene 4 (1), 119–120.

Yu, H., Stasinopoulos, S., Leedman, P., Medcalf, R.L., 2003. Inherent instability of plasminogen activator inhibitor type 2 mRNA is regulated by tristetraprolin. Journal of Biological Chemistry 278 (16), 13912–13918.

# Compounds That Increase or Mimic Cyclic Adenosine Monophosphate Enhance Tristetraprolin Degradation in Lipopolysaccharide-Treated Murine J774 Macrophages

Ulla Jalonen, Erja-Leena Paukkeri, and Eeva Moilanen

The Immunopharmacology Research Group, Medical School, University of Tampere and Research Unit, Tampere University Hospital, Tampere, Finland

## ABSTRACT

Tristetraprolin (TTP) is a *trans*-acting factor that can regulate mRNA stability by binding to the *cis*-acting AU-rich element (ARE) in the 3′-untranslated region in mRNAs of certain transiently expressed genes. The best-studied target of TTP is tumor necrosis factor (TNF)-$\alpha$. By binding to ARE, TTP increases the degradation of TNF-$\alpha$ mRNA, thereby reducing the expression of TNF-$\alpha$. We examined the effects of cAMP analogs and the cAMP-elevating agents forskolin and $\beta_2$-agonists on lipopolysaccharide (LPS)-induced TTP mRNA and protein expression by quantitative real-time reverse transcriptase-polymerase chain reaction and Western blotting in activated macrophages. All of these agents caused a slight increase in LPS- induced expression of TTP mRNA. However, TTP protein levels were significantly reduced when the cells were treated with the combination of LPS and cAMP-elevating agent compared with LPS alone. Proteasome inhibitors MG132 (*N*-[(phenylmethoxy)-carbonyl]-L-leucyl-*N*-[(1*S*)-1-formyl-3-methylbutyl]-L-leucinamide) and lactacystin increased TTP protein levels and abolished the effects of cAMP-enhancing compounds on TTP protein levels. The results suggest that mediators and drugs that enhance intracellular cAMP reduce TTP expression in macrophages exposed to inflammatory stimuli by increasing TTP degradation through the proteasome pathway.

Post-transcriptional regulation of gene expression by *cis*-acting elements and *trans*-acting factors has been an object of growing interest in recent years. One of the *trans*-acting factors regulating the stability of transiently expressed mRNAs is tristetraprolin (TTP), also known as Nup475, TIS11, G0S24, or Zfp36 (Blackshear, 2002). The *cis*-acting element to which TTP binds resides in the AU-rich element (ARE) in the 3′-untranslated region of mRNAs of many inflammatory and other transiently expressed genes. The stability of an increasing number of transcripts has been shown to be regulated by TTP in studies with overexpression of TTP or by knockout (KO) or knockdown of TTP expression. Most

studies have focused on the post-transcriptional regulation of tumor necrosis factor (TNF)-$\alpha$ mRNA. TTP can promote the deadenylation and decay of TNF-$\alpha$ mRNA by binding to the conserved ARE in the 3′-untranslated region of TNF-$\alpha$ mRNA (Lai et al., 1999; Lai and Blackshear, 2001), thereby decreasing the amount of proinflammatory cytokine TNF-$\alpha$. TTP KO mice developed a complex inflammatory syndrome characterized by arthritis, autoimmunity, cachexia, and myeloid hyperplasia due to increased levels of TNF-$\alpha$ (Taylor et al., 1996; Carballo et al., 1998).

Other suggested and/or partially confirmed targets of TTP include inflammatory modulators such as granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-2, IL-3, IL-6, IL-12, macrophage inflammatory protein-2, macrophage inflammatory protein-3$\alpha$, inducible nitric oxide synthase, and cyclooxygenase-2 (Carballo et al., 2000; Stoecklin et al., 2000, 2001; Sawaoka et al., 2003; Linker et al., 2005; Ogilvie et al., 2005; Jalonen et al., 2006). The most recent members in the group of mRNAs possibly regulated post-

**ABBREVIATIONS:** TTP, tristetraprolin; ARE, AU-rich element; KO, knockout; TNF, tumor necrosis factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; SOCS3, suppressor of cytokine signaling 3; MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide; MG132, *N*-[(phenylmethoxy)carbonyl]-L-leucyl-*N*-[(1*S*)-1-formyl-3-methylbutyl]-L-leucinamide; SB203580, 4-(4-fluorophenyl)-2-(4-methyl-sulphinylphenyl)-5-(4-pyridyl)-1*H*-imidazole; PCR, polymerase chain reaction; RT, reverse transcriptase; TAMRA, 6-carboxytetramethylrhodamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; db-cAMP, $N^6$,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate sodium salt.

transcriptionally by TTP include suppressor of cytokine signaling 3 (SOCS3), transforming growth factor β1, and transcription factor E47 (Chang et al., 2007; Ehlting et al., 2007; Frasca et al., 2007). However, studies using cells derived from TTP KO mice have not confirmed TTP as a sole regulator of mRNA stability of these genes. For instance, in the case of SOCS3, the results showed that in NIH 3T3 cells, down-regulation of TTP by small interfering RNA and TTP overexpression affected SOCS3 mRNA stability, but SOCS3 mRNA half-life was similar in cells from wild-type and TTP KO mice (Ehlting et al., 2007).

In addition, there is increasing evidence that the p38 mitogen-activated protein kinase (MAPK) pathway is a crucial regulator of the expression, stability, and function of TTP (Mahtani et al., 2001; Brook et al., 2006; Hitti et al., 2006). Phosphorylation of particular serine residues of TTP increases binding of 14-3-3 proteins, thereby excluding TTP from stress granules and inactivating TTP and increasing the stability of target mRNAs (Johnson et al., 2002; Chrestensen et al., 2004; Stoecklin et al., 2004). In addition to stress granules, other sites where degradation of ARE-containing mRNAs has been shown to occur are the processing body (Franks and Lykke-Andersen, 2007; Stoecklin and Anderson, 2007), the exosome (Chen et al., 2001), and the proteasome (Laroia et al., 1999). Micro-RNAs have also been reported to have a role in TTP-mediated mRNA degradation (Jing et al., 2005). Whatever the site of mRNA degradation is, TTP protein has been proposed to be degraded by the proteasome (Brook et al., 2006; Deleault et al., 2008).

We have recently shown that β2-agonists, cAMP analogs, and forskolin increase TTP mRNA and protein expression in resting cells, possibly by increasing the activation of transcription factor activator protein 2 (Jalonen et al., 2007). In the present study, we found that TTP protein expression was differently regulated by cAMP-increasing compounds in cells exposed to LPS (which mimics the inflammatory situation) than in our earlier study in resting cells. Even though TTP mRNA amounts were increased, the TTP protein levels decreased when J774 macrophages were treated with a combination of LPS and cAMP-elevating agents. A possible mechanism of these effects is the increased degradation of TTP protein through the proteasome pathway because the inhibition of proteasome activity by proteasome inhibitors MG132 and lactacystin inhibited the decrease in TTP protein levels found with the combination of LPS and cAMP-elevating agents compared with treatment with LPS alone.

## Materials and Methods

**Materials.** Reagents were purchased as follows: forskolin, MG132, and SB203580 from Tocris Cookson Inc. (Ellisville, MO), and ubiquitin aldehyde was from Boston Biochem (Cambridge, MA). All other reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

**Cell Culture.** The murine J774 macrophages (European Collection of Cell Cultures, Porton Down, Wiltshire, UK) were maintained in an atmosphere of 5% carbon dioxide at 37°C in Dulbecco's modified Eagle's medium with UltraGlutamine 1 (Lonza Verviers SPRL, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Lonza Verviers SPRL), penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml) (Invitrogen, Paisley, UK). Cells were seeded on six- or 24-well plates and grown to confluence before the experiments. Tested compounds were added to the cell culture at the same time as LPS, unless otherwise stated.

**Cell Viability Test.** Cell viability was tested using the Cell Proliferation Kit II (Roche Diagnostics, Mannheim, Germany). Cells were incubated with LPS (1 ng/ml) or LPS (1 ng/ml) and forskolin (100 μM) for 12 h before the addition of the sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate labeling reagent (final concentration, 0.3 mg/ml) and N-methyl dibenzopyrazine methyl sulfate (2.5 μg/ml). Then, cells were incubated for 3 h, and the amount of formazan accumulated in the growth medium was assessed spectrophotometrically. No difference in cell viability between treated and untreated cells was detected. Triton X-treated cells were used as a positive control resulting in 97% reduction in cell viability.

**RNA Extraction and Quantitative Real-Time Reverse Transcriptase-PCR.** The protocol for RNA extraction and quantitative real-time reverse transcriptase (RT)-PCR has been described by Jalonen et al. (2005). Primers and TaqMan 6-carboxyfluorescein (Fam)-TAMRA probes (Table 1) for TTP, TNF-α, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer Express Software (Applied Biosystems, Foster City, CA) and supplied by Metabion (Martinsried, Germany).

**Western Blotting.** The protocol for Western blotting has been described by Jalonen et al. (2005). When preparing cell lysates for ubiquitin Western blotting, lysis buffer also contained ubiquitin aldehyde (20 μg/ml) and MG132 (25 μM) to prevent deubiquitinylation of the sample. The gels were loaded with 35 μg of protein for TTP and actin Western blots and 240 μg for ubiquitin Western blotting. Actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), the mouse TTP antibody (Cao et al., 2004) was kindly provided by Dr. Perry Blackshear (National Institute of Environmental Health Sciences, Research Triangle Park, NC), ubiquitin antibody was from Zymed Laboratories (South San Francisco, CA), p38 mitogen-activated protein kinase and phospho-p38 mitogen-activated protein kinase (Thr180/Tyr182) antibodies were from Cell Signaling Technology Inc. (Danvers, MA). The bound antibodies were detected using SuperSignal West Dura or Femto chemiluminescent substrate for horseradish peroxidase detection (Pierce Chemical, Cramlington, Northumberland, UK) and the FluorChem 8800 imaging system (Alpha Innotech, San Leandro, CA). Actin was used as a loading control, and it was detected from the same membrane as TTP after stripping the membrane. The chemiluminescent signals were measured with FluorChem software, version 3.1.

TABLE 1
Primer and probe sequences

| Primer | Sequence |
| --- | --- |
| mTTP forward | 5′-CTCAGAAAGCGGGCGTTGT-3′ |
| mTTP reverse | 5′-GATTGGCTTGGCGAAGTTCA-3′ |
| mTTP probe | 5′-Fam-CCAAGTGCCAGTTTGCTCACGGC-TAMRA-3′ |
| mTNF-α forward | 5′-AATGGCCTCCCTCTCATCAGTT-3′ |
| mTNF-α reverse | 5′-TCCTCCACTTGGTGGTTTGC-3′ |
| mTNF-α probe | 5′-Fam-CTCAAAATTCGAGTGACAAGCCTGTAGCCC-TAMRA-3′ |
| mGAPDH forward | 5′-GCATGGCCTTCCGTGTTC-3′ |
| mGAPDH reverse | 5′-GATGTCATCATACTTGGCAGGTTT-3′ |
| mGAPDH probe | 5′-Fam-TCGTGGATCTGACGTGCCGCC-TAMRA-3′ |

**Statistics.** Results are expressed as the mean ± S.E.M. The significance of differences was calculated by analysis of variance supported by Dunnett's adjusted significance levels. A difference between treatment groups was considered significant when $p < 0.05$.

## Results

**cAMP Analogs, Forskolin, and $\beta_2$-Agonists Decreased LPS-Induced TTP Protein Expression in J774 Macrophages.** In resting cells, low levels of TTP protein were detected. LPS (1 ng/ml) induced transient expression of TTP protein, which enhanced rapidly, remained relatively constant for 4 to 9 h, and declined thereafter (data not shown). After incubation with LPS (1 ng/ml) for 9 h, TTP protein amount was increased 5-fold in J774 macrophages compared with resting cells (Fig. 1A). cAMP analogs $N^6,2'$-$O$-dibutyry-ladenosine 3′,5′-cyclic monophosphate sodium salt (db-cAMP; 1 mM) and 8-bromoadenosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cAMP; 1 mM) decreased LPS-induced TTP protein expression by 40% (Fig. 1A). At lower concentrations (0.1 mM), db-cAMP was less efficient than 8-Br-cAMP in decreasing TTP protein levels. Likewise, forskolin (an activator of adenylate cyclase; 100 µM) decreased LPS-induced TTP protein levels by 45% when measured after 9 h of incubation (Fig. 1B). In addition to the cAMP analogs and forskolin, $\beta_2$-agonists salbutamol and terbutaline (0.3 and 1 µM) decreased LPS-induced TTP protein levels by 41 to 50% when measured after 9-h incubation (Fig. 1C).

**cAMP Analogs, Forskolin, and Salbutamol Increased LPS-Induced TTP mRNA Levels in J774 Macrophages.** Low levels of TTP mRNA were found in unstimulated cells, and that was significantly increased when LPS (1 ng/ml) was added into the culture. J774 macrophages were incubated with LPS (1 ng/ml) and 8-Br-cAMP or db-cAMP (0.1 or 1 mM) for 1 h (Fig. 2A). 8-Br-cAMP (0.1 and 1 mM) increased TTP mRNA levels by 49 and 79%, respectively, compared with cells treated with LPS only. db-cAMP (0.1 and 1 mM) had a similar effect and enhanced TTP mRNA levels by 37 and 93% compared with LPS-treated cells. When the cells were incubated with LPS (1 ng/ml) in combination with forskolin (100 µM), TTP mRNA levels were higher than when treated with LPS (1 ng/ml) alone (Fig. 2B). The difference between the treatments was statistically significant after 1- and 2-h incubation. A slight increase in TTP mRNA levels was also observed when the cells were treated with LPS (1 ng/ml) and increasing concentrations (0.1–3 µM) of salbutamol for 1 h
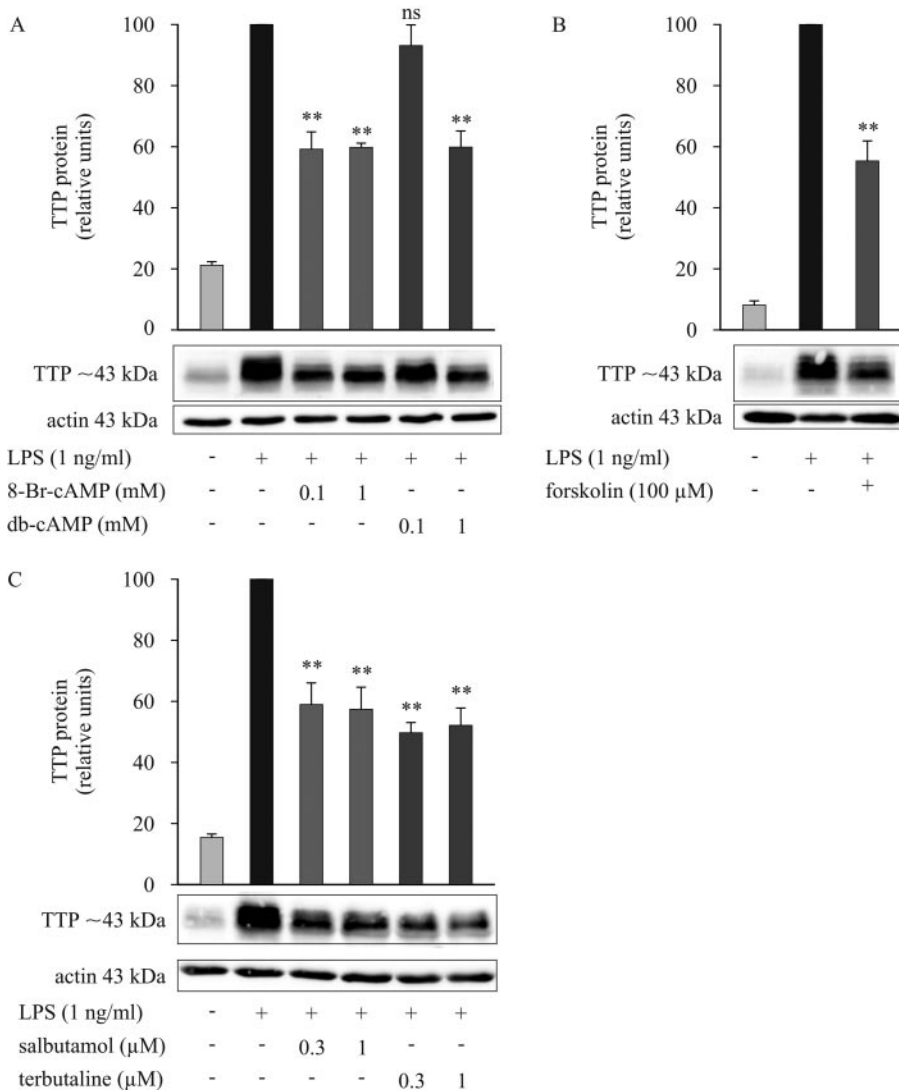


**Fig. 1.** Effects of cAMP analogs, forskolin, and $\beta_2$-agonists on LPS-induced TTP protein expression in J774 macrophages. The cells were treated with LPS (1 ng/ml) and indicated concentrations of 8-Br-cAMP or db-cAMP (A), forskolin (B), or salbutamol or terbutaline (C) for 9 h. Proteins were extracted, and TTP and actin proteins were measured by Western blot. The TTP protein level in the LPS-treated samples was set at 100, and the other values were related to that. The blot is a representative of six (in A and C) or three (in B) with similar results. Values are mean ± S.E.M. ($n = 6$ in A and C, $n = 3$ in B). ∗∗, $p < 0.01$ when compared with the LPS-treated samples.
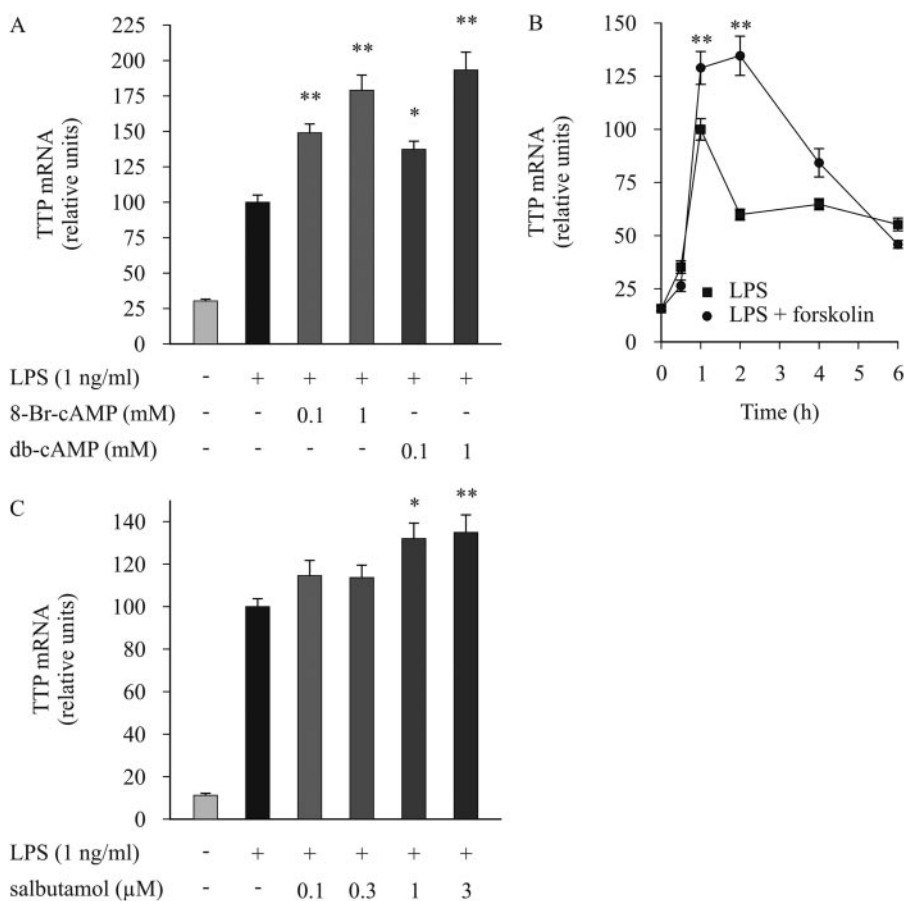
Fig. 2. Effects of cAMP analogs, forskolin, and salbutamol on LPS-induced TTP mRNA expression in J774 macrophages. A, LPS (1 ng/ml) and indicated concentrations of 8-Br-cAMP or db-cAMP were used to stimulate macrophages for 1 h, after which total RNA was extracted. Quantitative RT-PCR was used to measure TTP mRNA, and the values were normalized to GAPDH mRNA. The mean of TTP mRNA levels in the LPS-treated samples was set at 100, and the other values were related to that. Values are mean $\pm$ S.E.M. ($n = 3$). ∗∗, $p < 0.01$ and ∗, $p < 0.05$ when compared with the LPS-treated samples. B, cells were stimulated with LPS (1 ng/ml) in combination with forskolin (100 $\mu$M). Total RNA was extracted at the time points indicated. Quantitative RT-PCR was used to measure TTP mRNA, and the values were normalized to GAPDH mRNA. The mean of TTP mRNA levels in the LPS-treated samples at the 1-h time point was set at 100, and the other values were related to that. Values are mean $\pm$ S.E.M. ($n = 3$). ∗∗, $p < 0.01$ when compared with the LPS-treated samples in each time point. C, LPS (1 ng/ml) and indicated concentrations of salbutamol were used to stimulate macrophages for 1 h, after which total RNA was extracted. Quantitative RT-PCR was used to measure TTP mRNA, and the values were normalized to GAPDH mRNA. The TTP mRNA level in the LPS-treated samples was set at 100, and the other values were related to that. Values are mean $\pm$ S.E.M. ($n = 3$). ∗∗, $p < 0.01$ and ∗, $p < 0.05$ when compared with the LPS-treated samples.

(Fig. 2C). Salbutamol (3 $\mu$M) increased LPS-induced TTP mRNA levels by 35%.

**Forskolin Did Not Affect TTP mRNA Half-Life in LPS-Treated J774 Macrophages.** To find out the reasons for the differences between mRNA and protein expression, we first examined the degradation rate of TTP mRNA by actinomycin D assay (Fig. 3). Cells were treated with LPS (1 ng/ml) in the presence or absence of forskolin (100 $\mu$M) for 1 h; thereafter, actinomycin D (0.5 $\mu$g/ml) was added to the
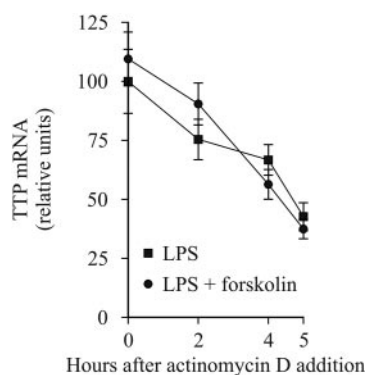


Fig. 3. Effects of forskolin on TTP mRNA decay in LPS-treated J774 macrophages. The cells were treated with LPS (1 ng/ml) with or without forskolin (100 $\mu$M) for 1 h before actinomycin D (0.5 $\mu$g/ml) was added to the culture. Total RNA was extracted 2, 4, and 5 h after actinomycin D addition, and quantitative PCR was used to detect TTP mRNA. GAPDH mRNA was measured for normalization. The mean of TTP mRNA levels in the LPS-treated samples at the time of actinomycin D addition was set at 100, and the other values were related to that. Values are mean $\pm$ S.E.M. ($n = 3$).

culture to inhibit transcription. Total RNA was extracted 2, 4, and 5 h after addition of actinomycin D, and the TTP mRNA levels were measured. Forskolin did not alter the TTP mRNA degradation rate, and the half-life of TTP mRNA with both treatments was approximately 4.5 h.

**Forskolin, cAMP Analogs, and $\beta_2$-Agonists Decreased LPS-Induced TTP Protein Expression in a Time-Dependent Manner.** Because forskolin had no effect on TTP mRNA half-life, we examined its effects on TTP protein expression after different incubation times (Fig. 4A). Macrophages were incubated with LPS (1 ng/ml) with or without forskolin (100 $\mu$M), and proteins were extracted after 4, 6, 9, and 12 h of culture. With both treatments, TTP protein levels were almost equal when measured after 4-h incubation. After 6-h incubation, TTP protein levels were 10% lower in forskolin-treated cells. After 9- and 12-h incubations, LPS-induced TTP protein levels were 51 and 57% lower in forskolin-treated cells, respectively. In addition, TTP mRNA levels were measured at the same time points (Fig. 4B). As expected, after 4-h incubation, TTP mRNA levels were higher in forskolin-treated cells. In contrast, after 6-, 9-, and 12-h incubation, the TTP mRNA levels in LPS + forskolin-treated cells were similar to those in cells treated with LPS only.

Thereafter, we investigated the effects of cAMP analogs and the $\beta_2$-agonist on TTP protein levels (Fig. 5). The cells were treated with LPS (1 ng/ml) with or without the cAMP analogs or $\beta_2$-agonist. Proteins were extracted after 4- and 9-h incubation, and TTP protein was detected by Western blot. After 4-h incubation, LPS-induced TTP protein levels were approximately similar in control cells and in cells
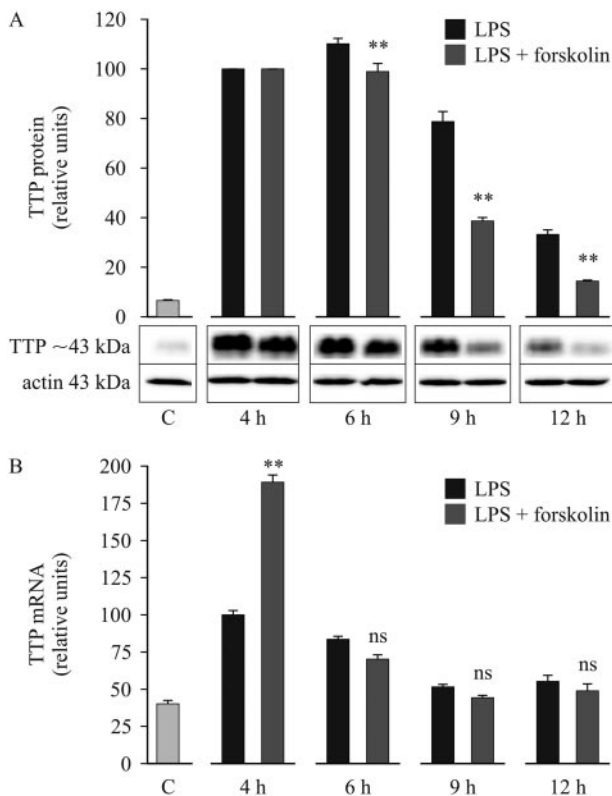
**Fig. 4.** Effects of forskolin on TTP protein and mRNA expression in LPS-treated J774 macrophages. The cells were incubated with LPS (1 ng/ml) with or without forskolin (100 $\mu$M) for 4, 6, 9, or 12 h, after which proteins (A) or total RNA (B) were extracted. A, TTP and actin proteins were measured by Western blot. The TTP protein levels in the LPS- and LPS + forskolin-treated samples at 4 h were set at 100, and the other values gained with the same treatment were related to those. The blot is a representative of three with similar results. Values are mean $\pm$ S.E.M. ($n$ = 3). C, control. **, $p < 0.01$ when compared between the LPS- and LPS + forskolin-treated samples. B, quantitative PCR was used to detect TTP mRNA, and GAPDH mRNA was measured for normalization. The mean of TTP mRNA levels in the LPS-treated samples at the 4-h time point was set at 100, and the other values were related to that. Values are mean $\pm$ S.E.M. ($n$ = 4). **, $p < 0.01$ and N.S. (ns) when compared between the LPS- and LPS + forskolin-treated samples.

treated with 8-Br-cAMP, db-cAMP, or salbutamol. After 9-h incubation, all of the three compounds significantly decreased the level of TTP protein expression. The cAMP analogs 8-Br-cAMP and db-cAMP decreased TTP protein levels after 9-h incubation by 52 and 40%, respectively. Likewise, salbutamol decreased the amount of TTP protein by 41%. In conclusion, the results suggest that cAMP analogs and cAMP-elevating agents increase the rate of TTP protein degradation.

**Proteasome Inhibitors Abolished Differences between TTP Protein Levels Extracted from LPS- and LPS + Forskolin-Treated J774 Macrophages.** We used two inhibitors of proteasome, lactacystin and MG132, to investigate whether the proteasome-mediated degradation of TTP protein is increased with forskolin in LPS-treated cells. First, we wanted to confirm that lactacystin, an inhibitor of 20S and 26S proteasomes, has a functional effect as a proteasome inhibitor in the cell culture conditions used. When protein degradation through the proteasome is inhibited, ubiquitinated proteins accumulate in the cells. Therefore, we examined the effects of lactacystin on the levels of ubiquitinated cellular proteins (Fig. 6A). The cells were first treated

with or without LPS (10 ng/ml) for 8 h, and then lactacystin (10 $\mu$M) was added to inhibit the activity of proteasome. Total proteins were extracted as described under *Materials and Methods* after 16-h incubation with lactacystin. Ubiquitinated proteins were detected with ubiquitin antibody. Addition of lactacystin to untreated cells increased the amount of ubiquitinated proteins as can be seen in Fig. 6A, lanes 1 and 2. An even greater increase of ubiquitinated proteins in cells by lactacystin could be seen in samples extracted from LPS-treated cells (see Fig. 6A, lanes 3 and 4).

J774 macrophages were incubated for 5 h with LPS (1 ng/ml) in the presence or absence of forskolin (100 $\mu$M) before lactacystin (20 $\mu$M) was added into the culture (Fig. 6B). Incubations were continued for 4 h, after which proteins were extracted and detected by Western blot. Addition of lactacystin increased LPS-induced TTP protein levels by 68%, suggesting that the degradation of TTP protein was decreased during inhibition of the proteasome. In the absence of lactacystin, forskolin reduced the amount of LPS-induced TTP protein by 45%. When lactacystin was added to the culture, forskolin had no effect on TTP protein levels.

Another inhibitor of proteasome, MG132, was used to assess the effect of forskolin on TTP protein degradation in a similar experiment (Fig. 6C). The cells were first incubated with LPS (1 ng/ml) with or without forskolin (100 $\mu$M) for 6 h before MG132 (10 $\mu$M) was added. Incubation was continued for another 6 h. After protein extraction, TTP and actin proteins were detected by Western blot. MG132 increased TTP protein levels of LPS-treated samples more than 10-fold, indicating that without the inhibitor of proteasome large amounts of TTP protein are degraded by the proteasome. In the absence of MG132, forskolin decreased LPS-induced protein levels by 55%. In the presence of MG132, forskolin did not alter TTP protein levels.

**LPS and LPS + Forskolin Activated MAPK p38 at 30 min but Not at 9 h.** Activation of p38 by phosphorylation was studied by Western blot (Fig. 7A). J774 macrophages were treated with LPS (1 ng/ml) with or without forskolin (100 $\mu$M) for 30 min or 9 h, after which proteins were extracted, and phosphorylated and total p38 were detected by Western blot. Thirty-minute incubation with LPS or LPS + forskolin increased the amount of phosphorylated p38 3 or 4-fold, respectively, compared with control. After 9-h incubation, phospho-p38 levels were very low, and there were no differences between the treatments.

The p38 pathway is one of the regulators of TTP transcription. To find out whether the inhibition of p38 activation affects the mRNA levels of TTP, we treated the J774 macrophages with different combinations of LPS (1 ng/ml), forskolin (100 $\mu$M), and p38 inhibitor SB203580 (1 $\mu$M) (Fig. 7B). Total RNA was extracted after 1-h incubation, and TTP mRNA was measured by quantitative PCR. Both LPS-induced and LPS + forskolin-induced TTP mRNA levels were inhibited by the p38 inhibitor SB203580 by 51 to 53%, indicating that p38 regulates TTP mRNA expression both in LPS and LPS + forskolin-treated cells.

**Forskolin Decreased TNF-$\alpha$ mRNA Decay in LPS-Treated J774 Macrophages.** Because TNF-$\alpha$ mRNA is a well known target of TTP, and its decay is increased by TTP, we measured TNF-$\alpha$ mRNA decay by actinomycin D assay at a time point when the forskolin-induced reduction in TTP protein amount was most significant (Fig. 8). Macrophages
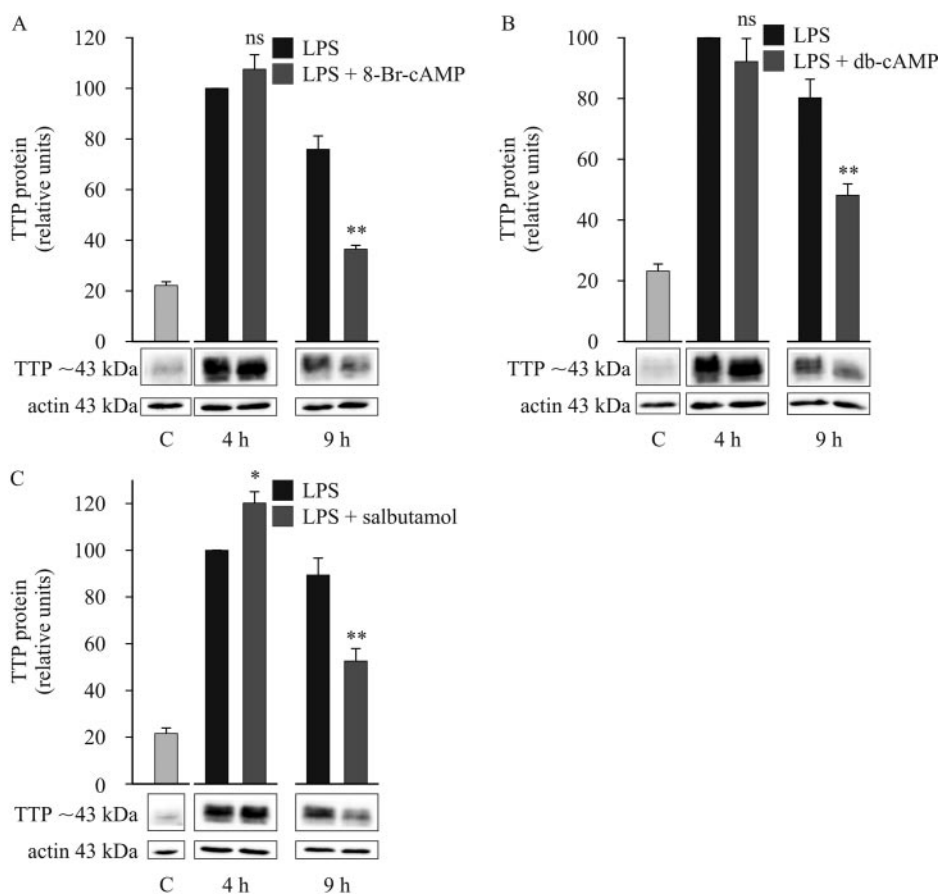
**Fig. 5.** The effects of cAMP analogs and $\beta_2$-agonist on TTP protein expression in LPS-treated J774 macrophages. The cells were incubated with LPS (1 ng/ml) with or without 8-Br-cAMP (1 mM) (A), db-cAMP (1 mM) (B), or salbutamol (1 $\mu$M) (C) for 4 or 9 h, after which proteins were extracted. TTP and actin proteins were measured by Western blot. The TTP protein levels in the LPS-treated samples at the 4-h time point were set at 100, and the other values were related to those. C, control. The blot is a representative of six with similar results. Values are mean $\pm$ S.E.M. ($n = 6$). **, $p < 0.01$ and *, $p < 0.05$ when compared between the LPS and LPS + cAMP analog or LPS + $\beta_2$-agonist-treated samples at each time point.

were treated with LPS (1 ng/ml) in the presence or absence of forskolin (100 $\mu$M) for 9 h; thereafter, actinomycin D was added. Total RNA was extracted at 60 and 90 min after addition of actinomycin D, and TTP mRNA levels were measured. As seen in Fig. 8, TNF-$\alpha$ mRNA decay was slower in LPS + forskolin-treated cells that in cells exposed to LPS only.

## Discussion

The principal finding in the present study was that cAMP analogs, forskolin, and $\beta_2$-agonists decreased LPS-induced TTP protein expression by increasing the rate of TTP degradation via proteasome. The role of p38 in the regulation of TTP expression was also studied, indicating a role for p38 in the regulation of TTP mRNA expression at early time points. The results suggest a novel mechanism by which mediators or drugs that increase intracellular cAMP concentrations may participate in the up-regulation of the expression of inflammatory genes.

Experiments on TTP KO mice and cells derived from them have shown that TTP is a physiological regulator to reduce mRNA stability and, hence, the expression of two important proinflammatory cytokines, TNF-$\alpha$ and GM-CSF (Taylor et al., 1996; Carballo et al., 1998, 2000). Due to increased levels of TNF-$\alpha$ and GM-CSF, the TTP KO mice suffer from arthritis-like symptoms, autoimmunity, and myeloid hyperplasia (Taylor et al., 1996; Phillips et al., 2004). In this study, we showed that forskolin reduced LPS-induced TTP protein levels significantly after 9-h incubation (i.e., by approximately 50% based on the Western blot results). Forskolin also reduced TNF-$\alpha$ mRNA decay, suggesting that the forskolin-induced changes in TTP levels may be functionally signifi-

cant. In our earlier study, down-regulation of TTP expression by small interfering RNA by approximately 50% resulted in increased release of TNF-$\alpha$ and some other cytokines from activated macrophages (Jalonen et al., 2006). That result further supports that down-regulation of TTP expression by the degree found in the present study may have significant effects in macrophages.

In addition to arthritis, recent studies suggest that TTP has a potential role in cancer and in obesity-related metabolic complications (Bouchard et al., 2007; Carrick and Blackshear, 2007). The possibility to regulate TTP expression to treat these conditions with future drugs requires intensive study of the regulation TTP expression and the effects of TTP over- and underexpression. Only little is known about the pharmacological regulation of TTP expression. Dexamethasone has been shown to increase TTP mRNA and protein levels in resting human A549 lung epithelial cells and in rat lung tissue (Smoak and Cidlowski, 2006). On the other hand, dexamethasone reduced LPS-induced TTP expression in J774 macrophages (Jalonen et al., 2005). Interferons have been reported to up-regulate TTP expression, which in turn results in down-regulation of several proinflammatory genes (Sauer et al., 2006). Cinnamon extract and cinnamon polyphenols have been shown to increase both the protein and mRNA levels of TTP in mouse adipocytes (Cao et al., 2007b). In addition, green tea, which is also associated with anti-inflammatory effects, increased the levels of TTP mRNA in rat liver and skeletal muscle (Cao et al., 2007a).

We recently reported that the expression of TTP mRNA and protein could be induced by $\beta_2$-agonists, cAMP analogs,
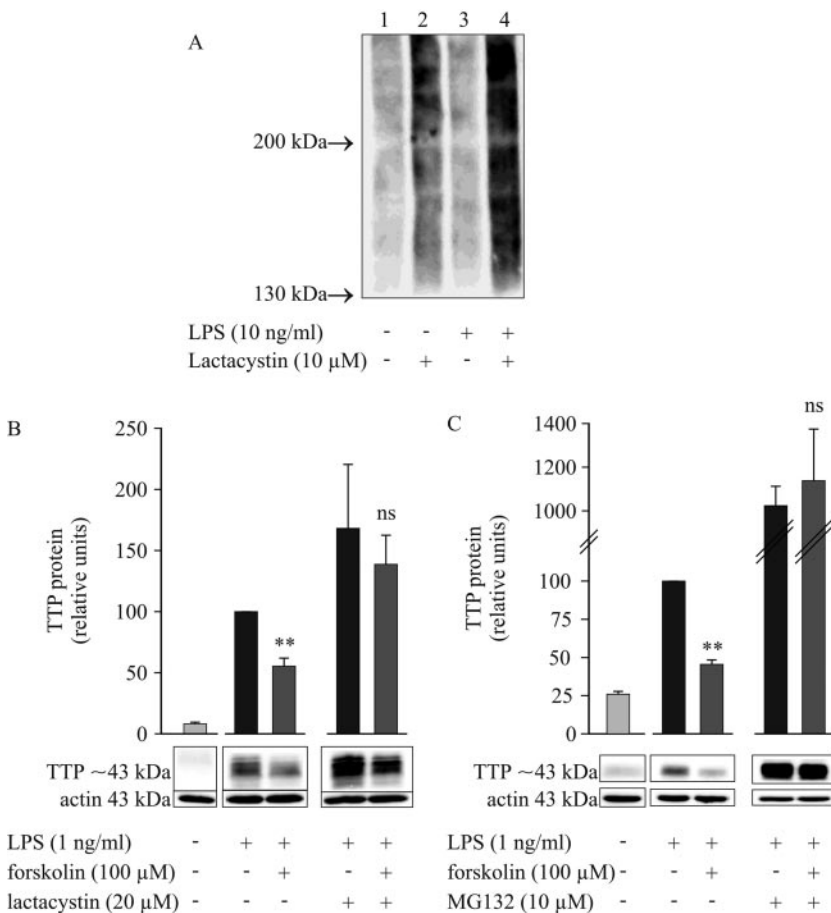
**Fig. 6.** Effects of proteasome inhibitors on TTP protein expression in J774 macrophages. A, cells were incubated with or without LPS (10 ng/ml) for 8 h before the addition of lactacystin (10 μM). Proteins were extracted 16 h after the addition of lactacystin, and protein ubiquitination was analyzed by Western blot. The blot is a representative of three with similar results. B, J774 macrophages were treated with LPS (1 ng/ml) with or without forskolin (100 μM) for 5 h, and then lactacystin (20 μM) was added. Incubation was continued for 4 h, after which proteins were extracted. TTP and actin proteins were measured by Western blot. The TTP protein level in the LPS-treated samples was set at 100, and the other values were related to that. The blot is a representative of three with similar results. Values are mean ± S.E.M. ($n = 3$). **, $p < 0.01$ and N.S. (ns) when compared with the LPS- and LPS + lactacystin-treated samples. C, J774 macrophages were incubated with LPS (1 ng/ml) with or without forskolin (100 μM) for 6 h before MG132 (10 μM) was added, and incubation was continued for another 6 h. Proteins were extracted, and TTP and actin protein were detected by Western blot. The TTP protein level in the LPS-treated samples was set at 100, and the other values were related to that. The blot is a representative of three with similar results. Values are mean ± S.E.M. ($n = 3$). **, $p < 0.01$ and N.S. (ns) when compared with the LPS- and LPS + MG132-treated samples.

and forskolin in resting macrophages, and that was probably mediated by increased activator protein 2 activation (Jalonen et al., 2007). Here, we have shown that when these compounds were given in combination with LPS, which mimics the inflammatory situation, TTP protein levels were significantly decreased. The decrease in TTP protein amounts could be prevented by treating the cells with proteasome inhibitors MG132 and lactacystin. The observed decrease in TTP protein levels in cells treated with LPS in combination with cAMP-elevating agents compared with treatment with LPS alone may be caused by enhanced activity of the proteasome activated by cAMP. This assumption is supported by earlier findings that phosphorylation of an ATPase in the 19S cap of the mammalian proteasome, Rpt6, by cAMP-dependent protein kinase A enhances proteasome activity (Zhang et al., 2007). cAMP seems to regulate the expression of TTP differently in resting cells and in cells exposed to inflammatory stimulus. It is interesting to note that very similar results have been reported with glucocorticoid dexamethasone. Dexamethasone, in combination with LPS, decreased TTP expression in mouse macrophages (Jalonen et al., 2005), but in resting human A549 lung epithelial cells and in rat lung tissues (in the absence of inflammatory stimuli), dexamethasone increased TTP mRNA and protein levels (Smoak and Cidlowski, 2006).

In contrast to TTP mRNA, which is transiently expressed, TTP protein has been regarded to be fairly stable, and the stability is regulated by p38 MAPK and its downstream target, MAPK-activated protein kinase 2, and extracellular signal-regulated kinase (Cao et al., 2004; Brook et al., 2006;

Hitti et al., 2006; Deleault et al., 2008). It has been suggested that dephosphorylation of TTP directs TTP to the proteasome by an unknown mechanism to be rapidly degraded (Brook et al., 2006; Deleault et al., 2008). TTP protein sequence contains three PEST domains (proline, glutamic acid, serine, and threonine) that target proteins for degradation by proteasome, but studies with mutated domains have not yet shown the functional activity of these domains (Rigby et al., 2005). Here, we provide further evidence that TTP protein expression is down-regulated by degradation through proteasome by showing that two proteasome inhibitors (lactacystin and MG132) enhanced TTP protein levels. Phosphorylation of p38 was also examined at 30 min and 9 h to gain mechanistic information on the regulation of TTP expression. The results suggest that at early time points, when p38 is activated by phosphorylation, it activates TTP transcription. At later time points, when p38 has been inactivated by phosphatases, the phosphorylation status of TTP also may decrease, enabling the degradation of TTP protein. We suggest that the degradation through the proteasome is an inducible mechanism to withdraw TTP from the cells and serves as a regulatory mechanism that enhances the inflammatory reaction by limiting the duration of TTP expression. Although the expression of TTP is regulated in a similar manner in resting mouse and human macrophages by cAMP-elevating agents (Jalonen et al., 2007), the results described in the present study require confirmation in human macrophages. Many factors increase intracellular cAMP levels through G-protein-coupled receptors in cell membrane, indicating that several
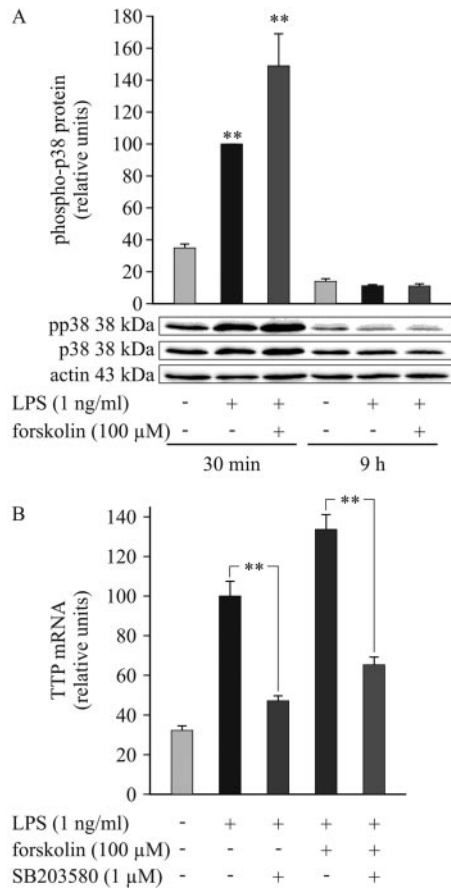
**Fig. 7.** Effects of LPS and forskolin on p38 activation and the suppressive effect of p38 inhibitor SB203580 on TTP mRNA expression in J774 macrophages. A, cells were treated with LPS (1 ng/ml) and forskolin (100 μM) for 30 min or 9 h. Proteins were extracted, and phospho-p38 (pp38), total p38, and actin proteins were measured by Western blot. The phospho-p38 protein level in the LPS-treated samples was set at 100, and the other values were related to that. The blot is a representative of four with similar results. Values are mean ± S.E.M. ($n = 4$). ∗∗, $p < 0.01$ when compared with the control samples at the 30-min time point. B, cells were incubated with LPS (1 ng/ml), forskolin (100 μM), and p38 inhibitor SB203580 (1 μM) as indicated for 1 h, after which total RNA was extracted. Quantitative RT-PCR was used to measure TTP mRNA, and the values were normalized to GAPDH mRNA. The mean of TTP mRNA levels in the LPS-treated samples was set at 100, and the other values were related to that. Values are mean ± S.E.M. ($n = 4$). ∗∗, $p < 0.01$ when compared with the LPS-treated or LPS + forskolin-treated sample.

other factors than those tested in the present study may also increase TTP protein degradation in a similar manner.

The data in the present study suggest that activation of cAMP-mediated mechanisms by cAMP analogs, forskolin, and β₂-agonists decreases LPS-induced TTP protein expression in macrophages possibly through the activation of proteasome. The results described here provide a mechanism by which the expression of TTP can be turned off at sites of inflammation. β₂-Agonists have been reported to have anti-inflammatory effects in various in vitro conditions but these actions do not clearly translate into the in vivo situation, e.g., in inflamed lung tissue in patients with asthma (Sitkauskiene and Sakalauskas, 2005). The mechanism described in the present study and in our earlier study (Jalonen et al., 2007) could, at least partly, explain the discrepancy between these in vitro and in vivo findings. In vivo, in situations with an ongoing inflammation, the expression of TTP can be down-regulated by β₂-agonists. In the absence of an
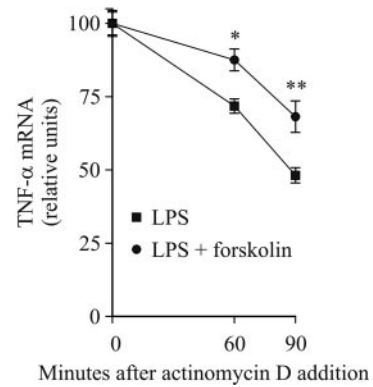


**Fig. 8.** Effects of forskolin on TNF-α mRNA decay in LPS-treated J774 macrophages. The cells were treated with LPS (1 ng/ml) with or without forskolin (100 μM) for 9 h before actinomycin D (0.5 μg/ml) was added to the culture. Total RNA was extracted 60 and 90 min after actinomycin D addition, and quantitative PCR was used to detect TNF-α mRNA. GAPDH mRNA was measured for normalization. The mean of TNF-α mRNA levels at the time of actinomycin D addition was set at 100, and the other values were related to that. Values are mean ± S.E.M. ($n = 4$). ∗∗, $p < 0.01$ and ∗, $p < 0.05$ when compared with the LPS-treated samples.

LPS-like inflammatory stimulus, these agents could increase the amount of TTP. Actually, the increased TTP degradation may be involved in the enhancement of asthmatic inflammation sometimes seen in asthma patients treated with β₂-agonists only without concomitant inhaled anti-inflammatory steroids. Our results suggest that compounds that increase or mimic cAMP decrease LPS-induced TTP protein expression, possibly by directing TTP for degradation through the proteasome, which is likely to enhance the expression of factors regulated by TTP.

### Acknowledgments

### References

Blackshear PJ (2002) Tristetraprolin and other CCCH tandem zinc-finger proteins in the regulation of mRNA turnover. *Biochem Soc Trans* **30:**945–952.

Bouchard L, Tchernof A, Deshaies Y, Marceau S, Lescelleur O, Biron S, and Vohl MC (2007) *ZFP36*: a promising candidate gene for obesity-related metabolic complications identified by converging genomics. *Obes Surg* **17:**372–382.

Brook M, Tchen CR, Santalucia T, McIlrath J, Arthur JSC, Saklatvala J, and Clark AR (2006) Posttranslational regulation of tristetraprolin subcellular localization and protein stability by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways. *Mol Cell Biol* **26:**2408–2418.

Cao H, Kelly MA, Kari F, Dawson HD, Urban JF Jr, Coves S, Roussel AM, and Anderson RA (2007a) Green tea increases anti-inflammatory tristetraprolin and decreases pro-inflammatory tumor necrosis factor mRNA levels in rats. *J Inflamm* **4:**1.

Cao H, Polansky MM, and Anderson RA (2007b) Cinnamon extract and polyphenols affect the expression of tristetraprolin, insulin receptor, and glucose transporter 4 in mouse 3T3–L1 adipocytes. *Arch Biochem Biophys* **459:**214–222.

Cao H, Tuttle JS, and Blackshear PJ (2004) Immunological characterization of tristetraprolin as a low abundance, inducible, stable cytosolic protein. *J Biol Chem* **279:**21489–21499.

Carballo E, Lai WS, and Blackshear PJ (2000) Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability. *Blood* **95:**1891–1899.

Carballo E, Lai WS, and Blackshear PJ (1998) Feedback inhibition of macrophage tumor necrosis factor-α production by tristetraprolin. *Science* **281:**1001–1005.

Carrick DM and Blackshear PJ (2007) Comparative expression of tristetraprolin (TTP) family member transcripts in normal human tissues and cancer cell lines. *Arch Biochem Biophys* **462:**278–285.

Chang X, Fan Y, Karyala S, Schwemberger S, Tomlinson CR, Sartor MA, and Puga A (2007) Ligand-independent regulation of transforming growth factor β1 expression and cell cycle progression by the aryl hydrocarbon receptor. *Mol Cell Biol* **27:**6127–6139.

Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJM, Stoecklin G, Moroni C, Mann M, and Karin M (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell* **107:**451–464.

Chrestensen CA, Schroeder MJ, Shabanowitz J, Hunt DF, Pelo JW, Worthington MT, and Sturgill TW (2004) MAPKAP kinase 2 phosphorylates tristetraprolin on *in vivo* sites including Ser[178], a site required for 14-3-3 binding. *J Biol Chem* **279:**10176–10184.

Deleault KM, Skinner SJ, and Brooks SA (2008) Tristetraprolin regulates TNF-α mRNA stability via a proteasome dependent mechanism involving the combined action of the ERK and p38 pathways. *Mol Immunol* **45:**13–24.

Ehlting C, Lai WS, Schaper F, Brenndörfer ED, Matthes RJ, Heinrich PC, Ludwig S, Blackshear PJ, Gaestel M, Häussinger D, et al. (2007) Regulation of suppressor of cytokine signaling 3 (SOCS3) mRNA stability by TNF-α involves activation of the MKK6/p38[MAPK]/MK2 cascade. *J Immunol* **178:**2813–2826.

Franks TM and Lykke-Andersen J (2007) TTP and BRF proteins nucleate processing body formation to silence mRNAs with AU-rich elements. *Genes Dev* **21:**719–735.

Frasca D, Landin AM, Alvarez JP, Blackshear PJ, Riley RL, and Blomberg BB (2007) Tristetraprolin, a negative regulator of mRNA stability, is increased in old B cells and is involved in the degradation of E47 mRNA. *J Immunol* **179:**918–927.

Hitti E, Iakovleva T, Brook M, Deppenmeier S, Gruber AD, Radzioch D, Clark AR, Blackshear PJ, Kotlyarov A, and Gaestel M (2006) 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* **26:**2399–2407.

Jalonen U, Lahti A, Korhonen R, Kankaanranta H, and Moilanen E (2005) Inhibition of tristetraprolin expression by dexamethasone in activated macrophages. *Biochem Pharmacol* **69:**733–740.

Jalonen U, Leppänen T, Kankaanranta H, and Moilanen E (2007) Salbutamol increases tristetraprolin expression in macrophages. *Life Sci* **81:**1651–1658.

Jalonen U, Nieminen R, Vuolteenaho K, Kankaanranta H, and Moilanen E (2006) Down-regulation of tristetraprolin expression results in enhanced IL-12 and MIP-2 production and reduced MIP-3α synthesis in activated macrophages. *Mediators Inflamm* **2006:**40691.

Jing Q, Huang S, Guth S, Zarubin T, Motoyama A, Chen J, Di Padova F, Lin SC, Gram H, and Han J (2005) Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell* **120:**623–634.

Johnson BA, Stehn JR, Yaffe MB, and Blackwell TK (2002) Cytoplasmic localization of tristetraprolin involves 14-3-3-dependent and -independent mechanisms. *J Biol Chem* **277:**18029–18036.

Lai WS and Blackshear PJ (2001) Interactions of CCCH zinc finger proteins with mRNA: tristetraprolin-mediated AU-rich element-dependent mRNA degradation can occur in the absence of a poly(A) tail. *J Biol Chem* **276:**23144–23154.

Lai WS, Carballo E, Strum JR, Kennington EA, Phillips RS, and Blackshear PJ (1999) Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. *Mol Cell Biol* **19:**4311–4323.

Laroia G, Cuesta R, Brewer G, and Schneider RJ (1999) Control of mRNA decay by heat shock-ubiquitin-proteasome pathway. *Science* **284:**499–502.

Linker K, Pautz A, Fechir M, Hubrich T, Greeve J, and Kleinert H (2005) Involvement of KSRP in the post-transcriptional regulation of human iNOS expression-complex interplay of KSRP with TTP and HUR. *Nucleic Acids Res* **33:**4813–4827.

Mahtani KR, Brook M, Dean JL, Sully G, Saklatvala J, and Clark AR (2001) 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* **21:**6461–6469.

Ogilvie RL, Abelson M, Hau HH, Vlasova I, Blackshear PJ, and Bohjanen PR (2005) Tristetraprolin down-regulates *IL-2* gene expression through AU-rich element-mediated mRNA decay. *J Immunol* **174:**953–961.

Phillips K, Kedersha N, Shen L, Blackshear PJ, and Anderson P (2004) Arthritis suppressor genes TIA-1 and TTP dampen the expression of tumor necrosis factor α, cyclooxygenase 2, and inflammatory arthritis. *Proc Natl Acad Sci U S A* **101:**2011–2016.

Rigby WF, Roy K, Collins J, Rigby S, Connolly JE, Bloch DB, and Brooks SA (2005) Structure/function analysis of tristetraprolin (TTP): p38 stress-activated protein kinase and lipopolysaccharide stimulation do not alter TTP function. *J Immunol* **174:**7883–7893.

Sauer I, Schaljo B, Vogl C, Gattermeier I, Kolbe T, Müller M, Blackshear PJ, and Kovarik P (2006) Interferons limit inflammatory responses by induction of tristetraprolin. *Blood* **107:**4790–4797.

Sawaoka H, Dixon DA, Oates JA, and Boutaud O (2003) Tristetraprolin binds to the 3′-untranslated region of cyclooxygenase-2 mRNA. A polyadenylation variant in a cancer cell line lacks the binding site. *J Biol Chem* **278:**13928–13935.

Sitkauskiene B and Sakalauskas R (2005) The role of β₂-adrenergic receptors in inflammation and allergy. *Curr Drug Targets Inflamm Allergy* **4:**157–162.

Smoak K and Cidlowski JA (2006) Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling. *Mol Cell Biol* **26:**9126–9135.

Stoecklin G and Anderson P (2007) In a tight spot: ARE-mRNAs at processing bodies. *Genes Dev* **21:**627–631.

Stoecklin G, Ming XF, Looser R, and Moroni C (2000) Somatic mRNA turnover mutants implicate tristetraprolin in the interleukin-3 mRNA degradation pathway. *Mol Cell Biol* **20:**3753–3763.

Stoecklin G, Stoeckle P, Lu M, Muehlemann O, and Moroni C (2001) Cellular mutants define a common mRNA degradation pathway targeting cytokine AU-rich elements. *RNA* **7:**1578–1588.

Stoecklin G, Stubbs T, Kedersha N, Wax S, Rigby WFC, Blackwell TK, and Anderson P (2004) MK2-induced tristetraprolin: 14-3-3 complexes prevent stress granule association and ARE-mRNA decay. *EMBO J* **23:**1313–1324.

Taylor GA, Carballo E, Lee DM, Lai WS, Thompson MJ, Patel DD, Schenkman DI, Gilkeson GS, Broxmeyer HE, Haynes BF, et al. (1996) A pathogenetic role for TNFα in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity* **4:**445–454.

Zhang F, Hu Y, Huang P, Toleman CA, Paterson AJ, and Kudlow JE (2007) Proteasome function is regulated by cyclic AMP-dependent protein kinase through phosphorylation of Rpt6. *J Biol Chem* **282:**22460–22471.

**Address correspondence to:** Dr. Eeva Moilanen, Medical School, Immunopharmacology, FIN-33014 University of Tampere, Finland. E-mail: eeva.moilanen@uta.fi

*Research Communication*

# Down-Regulation of Tristetraprolin Expression Results in Enhanced IL-12 and MIP-2 Production and Reduced MIP-3$\alpha$ Synthesis in Activated Macrophages

**Ulla Jalonen, Riina Nieminen, Katriina Vuolteenaho, Hannu Kankaanranta, and Eeva Moilanen**

*The Immunopharmacology Research Group, Medical School, University of Tampere, and Tampere University Hospital, Research Unit, 33014 Tampere, Finland*

In inflammation, the post-transcriptional regulation of transiently expressed genes provides a potential therapeutic target. Tristetraprolin (TTP) is of the factors regulating decay of cytokine mRNAs. The aim of the present study was to identify cytokines whose expression is regulated by TTP. We established a TTP knock-down cell line by expressing shRNA against TTP (shTTP cell line). A cytokine antibody array was used to measure cytokine production in macrophages exposed to lipopolysaccharide (LPS). Cytokines IL-6, IL-12, TNF-$\alpha$, and MIP-2 (a homologue to human IL-8) were expressed at higher levels whereas MIP-3$\alpha$ was produced at lower levels in LPS-treated shTTP cells than in control cells suggesting that the expression of these cytokines is regulated by TTP. The present data provide IL-12, MIP-2, and MIP-3$\alpha$ as novel inflammatory cytokine targets for TTP-mediated mRNA decay and stress the role of TTP in the regulation of the inflammatory process.

## INTRODUCTION

In inflammation, the post-transcriptional regulation of transiently expressed genes provides a potential therapeutic target. The regulation of mRNA stability through AU-rich element (ARE)-containing areas in the 3′-untranslated region has been found an important means to regulate cytokine production. Tristetraprolin (TTP) is one of the factors known to regulate mRNA stability and expression of proinflammatory cytokines especially tumor necrosis factor (TNF)-$\alpha$. TTP (synonyms: Nup475, TIS11, G0S24, and Zfp36) expression is induced by inflammatory and related stimuli including bacterial products, growth factors, 12-$O$-tetradecanoylphorbol-13-acetate ester and serum [1–6]. TTP is a member of a CCCH tandem zinc finger protein family that also contains Zfp36-like 1, Zfp36-like 2 and the recently found Zfp36-like 3 [7, 8]. TTP has been reported to bind the ARE of certain mRNAs, which leads to mRNA deadenylation and degradation [9, 10]. Recent articles suggest that TTP is a component of both stress granules and processing bodies [11] and that the zinc finger domain is needed to localize TTP into the stress granules, which are sites of stalled translational preinitiation complexes [12]. TTP also recruits and activates enzymes needed in ARE-containing mRNA decay [13], and TTP seems to have a multifunctional role in mRNA degradation.

Inflammatory tissues such as spleen, lymph nodes, and thymus express TTP mRNA [2, 3]. The significant role of TTP in inflammation was first discovered in TTP knock-out mice, which developed a set of severe inflammatory symptoms due to high levels of TNF-$\alpha$ [14]. In TTP deficient animals, the levels of TNF-$\alpha$ were elevated because of increased TNF-$\alpha$ mRNA stability [9, 15]. The mRNAs of granulocyte macrophage colony stimulating factor (GM-CSF), interleukin (IL)-2, IL-3, IL-6, cyclooxygenase-2 (COX-2), and plasminogen activator inhibitor type 2 have also been reported to be destabilized by TTP [16–21]. In contrast, TTP has been shown to inhibit human inducible nitric oxide synthase (iNOS) mRNA degradation. TTP did not bind to the iNOS mRNA but its effect was mediated through interaction with the KH-type splicing regulatory protein (KSRP) [22].

In the present study, we established a cell line expressing shRNA against TTP resulting in reduced TTP expression in response to inflammatory stimulus. In the further studies, we used a cytokine antibody array to measure the effects of TTP down-regulation on cytokine production in macrophages exposed to LPS.

## MATERIALS AND METHODS

### Cell culture

J774 murine macrophages (American Type Culture Collection, Rockville, Md, USA) were cultured at 37°C in humidified 5% carbon dioxide atmosphere in Dulbecco's modified Eagle medium with Ultraglutamine 1 (DMEM/U1, Cambrex Bioproducts Europe, Verviers, Belgium) supplemented with 10% heat-inactivated FBS (EuroClone, Wetherby, UK), penicillin (100 units/mL), streptomycin (100 $\mu$g/mL) and amphotericin B (250 ng/mL) (Gibco, Paisley, Scotland, UK).

### Cell lines expressing short hairpin RNAs (shRNA) against TTP or a negative control sequence

The nucleotides (Table 1) (Metabion, Planegg-Martinsried, Germany) were annealed and ligated into the p*Silencer* neo vector (Ambion Inc, Austin, Tex, USA) with T4 DNA ligase (Fermentas Inc, Burlington, Ontario, Canada). One Shot TOP10 Competent Cells (Invitrogen, Paisley, UK) were chemically transformed according to the manufacturer's instructions. Plasmids were isolated with Plasmid Mini kit (QIAGEN Inc, Santa Clarita, Calif, USA) and transfected with FuGENE 6 Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, Ind, USA) into J774 macrophages. G418 disulfide salt (Sigma Chemical Co, St Louis, Mo, USA) was used to select and maintain the J774 cell lines expressing shRNA against TTP (shTTP) and negative control shRNA (shNEG).

### Stimulation of shTTP and shNEG cell lines

For the cytokine protein array, shTTP and shNEG cells were plated on 6 well plates 24 h prior to the experiment. Cells were first incubated in DMEM/U1 + FBS with or without LPS (100 ng/mL) (Sigma, St Louis, Mo, USA). After 1 h of incubation medium without FBS was changed to the wells and incubation was continued for 48 h. Thereafter, cell culture mediums were collected and stored at −20°C until assayed.

For Western blot shTTP and shNEG cells were plated on 6 well plates and grown to confluence. Cells were treated with or without LPS (100 ng/mL) for 6 h and proteins were extracted as described [23].

### Western blotting

The protocol for Western blotting was described in [23]. The gels were loaded with 50 $\mu$g of protein. Actin antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, Calif, USA and the mouse TTP antibody was a kind gift from Dr Perry Blackshear (NIEHS, Research Triangle Park, NC, USA). The bound antibodies were detected using Super Signal West Pico (for actin) or Dura (for TTP) chemiluminescent substrate for HRP detection (Pierce, Cheshire, UK) and FluorChem 8800 imaging system (Alpha Innotech, San Leandro, Calif, USA). The chemiluminescent signals were measured with FluorChem software v. 3.1.

### TNF-$\alpha$ enzyme-linked immunosorbent assay (ELISA)

TNF-$\alpha$ concentrations in culture media were determined by mouse TNF-$\alpha$ DuoSet ELISA kit (R&D Systems, Inc, Minneapolis, Minn, USA) according to the manufacturer's instructions.

### Cytokine antibody array

Cytokines were detected in cell culture media with Mouse Cytokine Antibody Array III (RayBiotech, Inc, Norcross, Ga, USA), which measures 62 cytokines and other inflammatory mediators. The array membranes were blocked with 2 mL of 1X blocking buffer for 30 min and then incubated with the sample (1 mL) for 2 h at room temperature. The membranes were washed three times with 2 mL of 1X wash buffer I and twice with 2 mL of 1X wash buffer II at room temperature. The membranes were then incubated in diluted primary antibodies over night at +4°C. The membranes were washed as described earlier and incubated with diluted HRP-conjugated streptavidin for 2 h at room temperature and washed. Detection buffer C and detection buffer D were combined and applied on the membranes for 2 min. Each membrane was exposed for 1 min and images were taken with FluorChem 8800 imaging system (Alpha Innotech Corp, San Leandro, Calif, USA) and chemiluminescent signals for each spot were measured with FluorChem software v. 3.1. The average chemiluminescence of each cytokine and control was calculated for all the treatments separately. The average of positive controls of each treatment was set to 100 and all cytokines of the same treatment were compared to that.

### Statistics

Results are expressed as the mean ± standard error of mean (SEM). The significance of differences was calculated by analysis of variance supported by Dunnett's adjusted significance levels. A difference between treatment groups was considered significant when $P < .05$.

## RESULTS

### Down-regulation of TTP expression in macrophages transfected with shTTP

J774 macrophages were transfected with shTTP expression vector (shTTP cell line) or with shNEG negative control vector (shNEG cell line) and maintained under G418 disulfide salt selection. Neither of the cell lines expressed detectable amounts of TTP protein when cultured in the absence of lipopolysaccharide (LPS). When LPS (100 ng/mL) was added into the culture, TTP was clearly expressed in shNEG cell line whereas TTP protein expression was markedly lower in shTTP cells (Figure 1(a)).

TTP knock-out mice have been shown to have increased levels of circulating TNF-$\alpha$ due to increased TNF-$\alpha$ mRNA stability in the absence of TTP [15, 24]. LPS-induced TNF-$\alpha$ levels produced by shNEG and shTTP cell lines were

TABLE 1: Target sequences and primers of shTTP and shNEG.

shTTP target sequence 5′-AACAUAAACUCGGACUCCAUC-3′
shTTP sense 5′-GATCCGCATAAACTCGGACTCCATCTTCAAGAGAGATGGAGTCCGAGTTTATGTTTTTTGGAAA-3′
shTTP antisense 5′-AGCTTTTCCAAAAAACATAAACTCGGACTCCATCTCTCTTGAAGATGGAGTCCGAGTTTATGCG-3′

shNEG target sequence 5′-AAACUACCGUUGUUAUAGGUG-3′
shNEG sense 5′-GATCCACTACCGTTGTTATAGGTGTTCAAGAGACACCTATAACAACGGTAGTTTTTTTGGAAA-3′
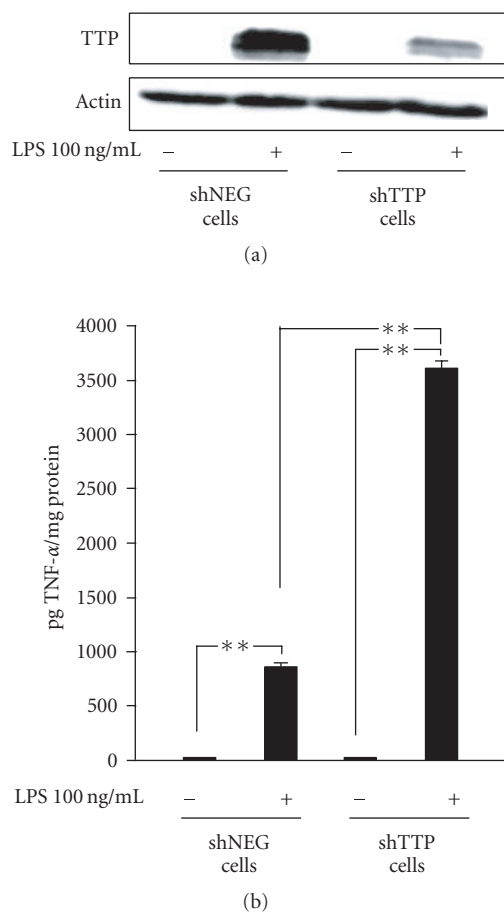shNEG antisense 5′-AGCTTTTCCAAAAAAACTACCGTTGTTATAGGTGTCTCTTGAACACCTATAACAACGGTAGTG-3′



(a)



(b)

FIGURE 1: Down-regulation of TTP expression and enhancement of TNF-$\alpha$ production in shTTP cells. (a) shNEG and shTTP cells were stimulated with LPS (100 ng/mL) and proteins were extracted after 6 h of incubation. TTP and actin were detected by Western blot. The blot is a representative of three blots with similar results. (b) shNEG and shTTP cells were stimulated with LPS (100 ng/mL) for 1 h. Thereafter the medium was changed and the cells were incubated for another 48 h. TNF-$\alpha$ concentrations in the culture media were measured by ELISA. Values are mean ± SEM ($n = 3$). ** = $P < .01$.

determined by ELISA. The results show that the TNF-$\alpha$ levels were more than three fold higher in LPS-treated shTTP cells than in shNEG cells (Figure 1(b)) confirming the functional consequences of TTP knock-down in shTTP cells.

### Cytokine production in shNEG and shTTP cell lines

Cytokine production in shNEG and shTTP cell lines was measured by using an antibody array that detects 62 cytokines and other inflammatory mediators (Figure 2(a)). shNEG and shTTP cell lines were incubated with or without LPS (100 ng/mL) and cytokines produced into the culture medium were measured after 48 h incubation. An example membrane of each treatment is shown in Figures 2(b)–2(f).

When analyzing the results of the cytokine antibody array the positive control was set as 100 and the cytokine results were related to the positive control. The average of negative controls and blanks obtain values < 2.5. The immunoreactivity of different cytokines in the cell culture medium was < 5 when compared to the positive controls.

In the absence of LPS, shNEG, and shTTP cell lines produced five out of the 62 measured cytokines into the culture medium, that is, cutaneous T-cell attracting chemokine (CTACK), CXCL16, MIP-1$\alpha$, MIP-1$\gamma$ and thymus and activation-regulated chemokine (TARC). Between the shNEG and shTTP cell lines, CXCL16 was expressed at higher levels in shNEG cell line whereas only minor differences between shNEG and shTTP cell lines were found in the other cytokines. The results are shown in Table 2.

### LPS induced changes in cytokine production in shNEG and shTTP cell lines

In shNEG cell line the expression of 11 out of the 62 measured cytokines was changed following LPS treatment (Table 3). With LPS treatment the expression of 9 cytokines [GCSF, IL-6, LPS induced C-X-C chemokine (LIX), MCP-1, MCP-5, MIP-2, MIP-3$\alpha$, RANTES, and sTNF RII] and the IL-12 p40 subunit increased. IL-12 p40 detects p40 subunit in the active IL-12 p70 as well as all otherwise engaged p40 subunits. On the contrary, the expression of CXCL16 in LPS-treated shNEG cells decreased as compared to untreated shNEG cells.

In shTTP cell line, the production of 10 cytokines (GCSF, IL-6, IL-12, LIX, MCP-1, MCP-5, MIP-2, RANTES, sTNF RII, and TNF-$\alpha$) and IL-12 p40 subunit were increased following LPS treatment (Table 4).

### Differences in LPS-induced cytokine expression in shNEG and shTTP cell lines

The LPS-induced production of six cytokines (IL-6, IL-12, IL-12 p40 subunit, MIP-2, MIP-3$\alpha$, and TNF-$\alpha$) was altered

|   | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | POS | POS | POS | POS | Blank | Ax1 | BLC | CD30 L | CD30 T | CD40 | CRG-2 | CTACK | CXCL16 | Eotaxin |
| 2 | NEG | NEG | NEG | NEG | Blank | Ax1 | BLC | CD30 L | CD30 T | CD40 | CRG-2 | CTACK | CXCL16 | Eotaxin |
| 3 | Eotaxin-2 | Fas Ligand | Frac-talkine | GCSF | GM-CSF | IFN-$\gamma$ | IGFBP-3 | IGFBP-5 | IGFBP-6 | IL-1$\alpha$ | IL-1$\beta$ | IL-2 | IL-3 | IL-3 R$\beta$ |
| 4 | Eotaxin-2 | Fas Ligand | Frac-talkine | GCSF | GM-CSF | IFN-$\gamma$ | IGFBP-3 | IGFBP-5 | IGFBP-6 | IL-1$\alpha$ | IL-1$\beta$ | IL-2 | IL-3 | IL-3 R$\beta$ |
| 5 | IL-4 | IL-5 | IL-6 | IL-9 | IL-10 | IL-12 p40 | IL-12 | IL-13 | IL-17 | KC | Leptin R | Leptin | LIX | L-Selectin |
| 6 | IL-4 | IL-5 | IL-6 | IL-9 | IL-10 | IL-12 p40 | IL-12 | IL-13 | IL-17 | KC | Leptin R | Leptin | LIX | L-Selectin |
| 7 | Lympho-tactin | MCP-1 | MCP-5 | M-CSF | MIG | MIP-1$\alpha$ | MIP-1$\gamma$ | MIP-2 | MIP-3$\beta$ | MIP-3$\alpha$ | PF-4 | P-Selectin | RANTES | SCF |
| 8 | Lympho-tactin | MCP-1 | MCP-5 | M-CSF | MIG | MIP-1$\alpha$ | MIP-1$\gamma$ | MIP-2 | MIP-3$\beta$ | MIP-3$\alpha$ | PF-4 | P-Selectin | RANTES | SCF |
| 9 | SDF-1$\alpha$ | TARC | TCA-3 | TECK | TIMP-1 | TNF-$\alpha$ | sTNF RI | sTNF RII | TPO | VCAM-1 | VEGF | Blank | Blank | Blank |
| 10 | SDF-1$\alpha$ | TARC | TCA-3 | TECK | TIMP-1 | TNF-$\alpha$ | sTNF RI | sTNF RII | TPO | VCAM-1 | VEGF | Blank | POS | POS |

(a)



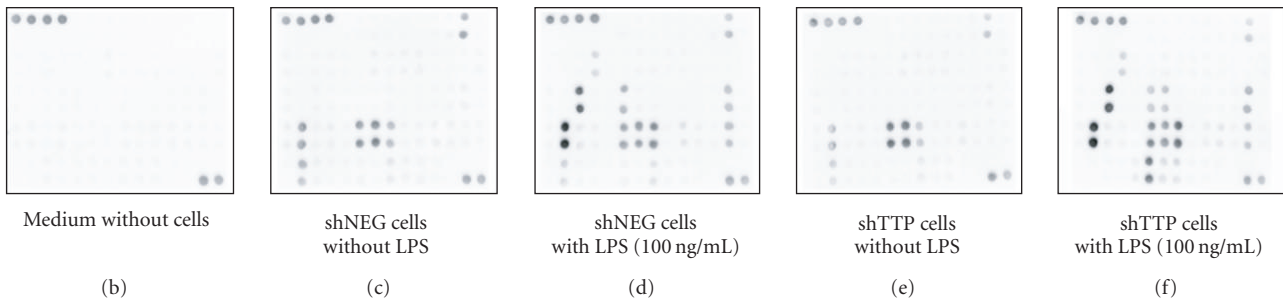| Medium without cells | shNEG cells without LPS | shNEG cells with LPS (100 ng/mL) | shTTP cells without LPS | shTTP cells with LPS (100 ng/mL) |
|---|---|---|---|---|
| (b) | (c) | (d) | (e) | (f) |

FIGURE 2: Cytokine antibody array. (a) A schematic diagram of the Mouse Cytokine Antibody Array III shows the locations of controls and the duplicate spots of cytokines. (b)–(f) Images of the membranes treated with cell culture media from the following experiments: (b) culture medium without cells, (c) shNEG cells, 49 h incubation, (d) shNEG cells, stimulated for 1 h with LPS (100 ng/mL) and incubated thereafter for 48 h, (e) shTTP cells, 49 h incubation, (f) shTTP cells, stimulated for 1 h with LPS (100 ng/mL) and incubated thereafter for 48 h. Representative membranes of three with similar results are shown. POS = positive control, NEG = negative control.

TABLE 2: Cytokines secreted spontaneously by shNEG and shTTP cell lines. Arbitrary units compared to positive controls (100) are presented. Mean $\pm$ SEM ($n = 3$).

| Cytokine | Medium without cells | shNEG cell line | shTTP cell line |
|---|---|---|---|
| CTACK | 1.9 | 5.8 | 6.3* |
| CXCL16 | 1.1 | 72.3** | 35.7* |
| MIP-1$\alpha$ | 2.9 | 82.3* | 117.5** |
| MIP-1$\gamma$ | 1.8 | 118.4** | 128.8** |
| TARC | 2.5 | 41.3** | 28.1* |

** = $P < .01$, * = $P < .05$ as compared to the medium without cells.

in shTTP cells as compared to shNEG cells suggesting that the expression of these six cytokines is regulated by TTP. Cytokines IL-6, MIP-2, IL-12, and TNF-$\alpha$ were expressed at higher levels in shTTP cell line than in shNEG cell line (Figure 3). In contrast, MIP-3$\alpha$ and IL-12 p40 subunit were expressed at lower levels in LPS-treated shTTP than in LPS-treated shNEG cell lines (Figure 4).

## DISCUSSION

The role of mRNA turnover in the regulation of inflammatory gene expression has been recognized pathophysiologically and therapeutically important. TTP is one of the factors regulating mRNA decay. Here we report that TTP downregulation resulted in increased expression of TNF-$\alpha$, MIP-2, IL-6, and IL-12 in macrophages exposed to bacterial LPS. On the other hand, the levels of MIP-3$\alpha$ were reduced in LPS-treated TTP knock-down cells. The possible new targets for TTP discovered in the present study are IL-12, MIP-2 (a homologue to human IL-8) and MIP-3$\alpha$.

TTP is a regulator of the stability of some transiently expressed ARE-containing cytokine mRNAs. TTP has been reported to down-regulate the expression of TNF-$\alpha$, GM-CSF, IL-2, IL-3, and IL-6 by destabilizing their mRNAs [9, 15–19]. In the present study, we established a cell line where TTP was knocked down by expressing an shRNA against TTP, and evaluated the levels of 62 cytokines secreted by the cells into the culture medium by cytokine antibody array. Protein antibody array has proved to be a useful tool to measure the expression of multiple proteins in cells and tissues at the

TABLE 3: Cytokines secreted by untreated and LPS- (100 ng/mL) treated shNEG cells during 48 h incubation. Arbitrary units compared to positive controls (100) are presented. Mean ± SEM ($n = 3$).

| Cytokine | Medium without cells | shNEG cells | |
|---|---|---|---|
| | | Untreated | LPS-treated |
| CXCL16 | 1.1 | 72.3 | 43.0* |
| GCSF | 2.3 | 3.3 | 21.4** |
| IL-6 | 2.4 | 3.8 | 172.3** |
| IL-12 p40 | 1.8 | 2.7 | 68.0** |
| LIX | 4.0 | 6.1 | 62.5** |
| MCP-1 | 4.4 | 81.2 | 293.6** |
| MCP-5 | 3.3 | 4.3 | 13.5** |
| MIP-2 | 4.2 | 51.0 | 128.1** |
| MIP-3$\alpha$ | 3.9 | 5.3 | 18.1** |
| RANTES | 3.0 | 4.3 | 53.8** |
| sTNF RII | 3.9 | 11.4 | 20.6* |

** $= P < .01$, * $= P < .05$ for the difference between untreated and LPS-treated shNEG cells.

TABLE 4: Cytokines secreted by untreated and LPS- (100 ng/mL) treated shTTP cells during 48 h incubation. Arbitrary units compared to positive controls (100) are presented. Mean ± SEM ($n = 3$).

| Cytokine | Medium without cells | shTTP cells | |
|---|---|---|---|
| | | Untreated | LPS-treated |
| GCSF | 2.3 | 3.6 | 32.0** |
| IL-6 | 2.4 | 3.5 | 258.0** |
| IL-12 p40 | 1.8 | 2.9 | 41.6** |
| IL-12 | 1.7 | 3.6 | 44.3** |
| LIX | 4.0 | 6.2 | 85.2** |
| MCP-1 | 4.4 | 46.3 | 349.1** |
| MCP-5 | 3.3 | 3.5 | 11.0** |
| MIP-2 | 4.2 | 33.6 | 182.3** |
| RANTES | 3.0 | 4.6 | 57.5** |
| sTNF RII | 3.9 | 12.1 | 27.3** |
| TNF-$\alpha$ | 3.7 | 7.2 | 149.0** |

** $P < .01$ for the difference between untreated and LPS-treated shTTP cells.

same time. The results obtained are more significant than when using cDNA microarrays as mRNAs are subjected to post-transcriptional and post-translational processes and the amount of mRNA may not correlate with protein expression [25]. The advantage of protein array as compared to ELISA is the simultaneous measurement of multiple proteins, but it is regarded as a semiquantitative method. In the present study, when TTP expression was induced by LPS, the TTP knock-down cell line showed clearly reduced TTP protein levels as compared to control cells. The reduction was functionally significant as it resulted in a clear increase in TNF-$\alpha$ production that is in line with the previous literature on the effects of TTP on TNF-$\alpha$ production [14, 24].

In experiments with TTP knock-out mice and cells derived from them, TTP has been shown to mediate the

degradation of TNF-$\alpha$ and GM-CSF mRNAs [15, 16]. Both transcripts contain AREs [26] and TTP binds to the nonamer UUAUUUAUU in these structures with its zinc finger domains [9, 27–30]. ARE-binding proteins such as TTP and KSRP seem to accelerate the degradation of ARE-mRNAs via the exosome pathway by recruiting the exosome to the mRNA [31]. It has also been shown that TTP can promote mRNA deadenylation by stimulating poly(A) ribonuclease [32]. The N-terminal domain of TTP also associates with mRNA decay enzymes involved in decapping, deadenylation, and exonucleolytic decay [13]. In addition, TTP has been proposed to direct certain mRNAs through stress granules to processing bodies to be degraded there [11]. However, the detailed mechanisms involved in the TTP-mediated mRNA decay are not known.

IL-6 is another proinflammatory cytokine produced by macrophages that is known to be regulated by TTP. It was shown that IL-6 mRNA degradation was impaired in a HT1080-derived mutant cell line. When the cells were then stably transfected with TTP, the IL-6 mRNA decay restored to the wild-type levels [19]. Here, we show that down-regulation of TTP expression in activated macrophages resulted in increased IL-6 production. These data suggest that in wild-type cells IL-6 mRNA is destabilized by TTP.

In addition to TNF-$\alpha$ and IL-6 [9, 15, 19], TTP has been reported to down-regulate the expression of GM-CSF, IL-2, and IL-3 by destabilizing their mRNAs [16–18]. In the present experiments, we were not able to detect these cytokines in our macrophage cultures and could not draw any conclusions on the effects of TTP deficiency on those cytokines.

IL-12 is a proinflammatory cytokine involved, for example, in the pathogenesis of autoimmune diseases [33, 34]. IL-12 is a heterodimer comprised of p35 and p40 subunits and designated as IL-12 p70 [34]. The subunits are encoded by two distinct genes and they need to be expressed simultaneously by the same cell to generate the biologically active heterodimer p70. Neither of the subunits is active on its own. The p40 subunit can also form homodimers (IL-12 p80), which act as endogenous IL-12 antagonists by binding to the IL-12 receptor without inducing a cellular response. The p40 subunit is also a part of IL-23. Our results showed that IL-12 was produced at higher levels in TTP-knock-down cells than in control cells in response to LPS. The result suggests a similar pattern of regulation of one or both of the subunits of IL-12 as with TNF-$\alpha$ and IL-6 where TTP destabilizes the mRNA and promotes its rapid degradation. To our knowledge, this is the first time to report that the expression of IL-12 is regulated by TTP.

In addition, we found a decrease in the expression of IL-12 p40 subunit in LPS-treated shTTP cells as compared to shNEG cells. In macrophages, the p40 subunit is secreted in several-fold excess as compared to the p35–p40 heterodimer, and the formation of active IL-12 (p35 and p40 heterodimer) is regulated by the synthesis of p35 subunit [35]. The mRNA of p35 (GenBank accession number NM_008351) contains one copy of UUAUUUAUU nonamer which is the preferred TTP binding site [30], where as none of those are found
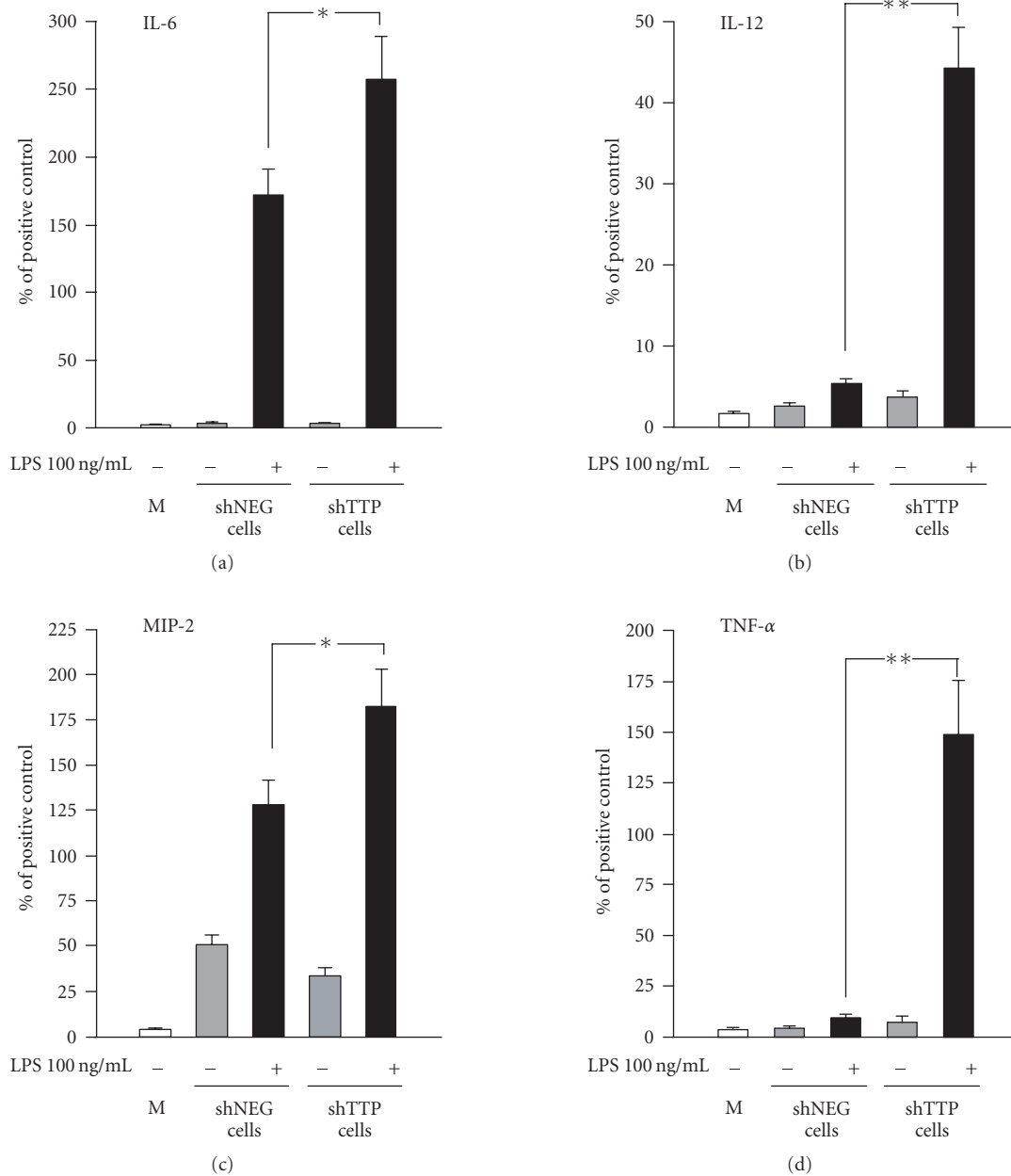
(a)

(b)

(c)

(d)

FIGURE 3: TTP down-regulation (shTTP cell line) enhanced the production of IL-6, IL-12, MIP-2, and TNF-$\alpha$ in response to LPS. Cytokine antibody array membranes were incubated in medium control (M) or in media from cultures of TTP knock-down cells (shTTP cell line) or control cells (shNEG cell line) after treatment with or without LPS (100 ng/mL). Values are arbitrary units compared to positive controls (100) and are presented as mean ± SEM ($n = 3$). ** = $P < .01$, * = $P < .05$.

in p40 mRNA (GenBank accession number NM_008352). Therefore we suggest that the target for TTP might be the p35 subunit, which could be expressed at lower levels in the LPS-treated shNEG cells than in shTTP cells. Further studies are needed to understand the mechanisms how TTP regulates IL-12 production.

A member of the CXC chemokine superfamily, MIP-2 (a homologue to human IL-8), is a potent chemoattractant for neutrophils [36]. In the present study, we found that MIP-2 production in response to LPS was higher in TTP knock-down cells than in control cells. We are not aware of earlier reports on the regulation of MIP-2 by TTP, and the results suggest a role for TTP as a destabilizer of MIP-2 mRNA. The sequence of MIP-2 mRNA (GenBank accession number NM_009140) contains three copies of the preferred nonamer binding site of TTP (UUAUUUAUU), which further supports MIP-2 mRNA as a target of the destabilizing effect of TTP.
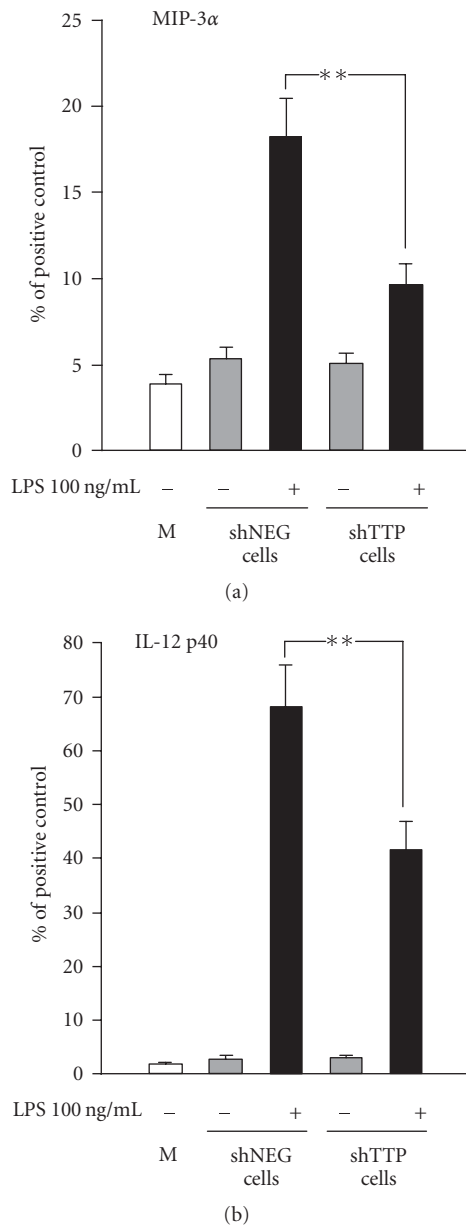
(a)



(b)

FIGURE 4: TTP down-regulation (shTTP cell line) reduced the production of MIP-3α and IL-12 p40 subunit in response to LPS. Cytokine antibody array membranes were incubated in medium control (M) or in media from cell cultures of TTP knock-down cells (shTTP cell line) or control cells (shNEG cell line) after treatment with or without LPS (100 ng/mL). Values are arbitrary units compared to positive controls (100) and are presented as mean ± SEM ($n = 3$). ** = $P < .01$.

MIP-3α (synonyms: CC chemokine ligand 20, CCL20; liver and activation-regulated chemokine, LARC; and Exodus-1) is a chemokine which has a potential role in rheumatoid arthritis [37]. In the present study, we found that MIP-3α production in response to LPS was lower in cells with reduced TTP expression than in control cells. That is interesting, because TTP has been recognized as a factor that

down-regulates the expression of certain inflammatory genes by destabilizing their mRNAs. MIP-3α seems to be an exception to that rule. Our result is supported by the fact that MIP-3α mRNA (GenBank accession number NM_016960) contains no nonamer binding sites for TTP (UUAUUUAUU), and therefore it is unlikely a direct target of TTP. Recently, Fechir and coworkers reported that TTP was able to enhance human iNOS expression by stabilizing its mRNA in cytokine-treated bronchial epithelial cell line [22]. TTP did not bind to human iNOS mRNA but was shown to enhance the half-life of iNOS mRNA by an indirect mechanism. TTP was found to interact with KSRP and it was proposed to inhibit the degradation of iNOS mRNA and enhance iNOS expression by capturing KSRP [22]. In the present study, we found that down-regulation of TTP resulted in reduction of MIP-3α production. To our knowledge, this is the first report to show that TTP regulates the production of MIP-3α. It remains to be studied if TTP regulates MIP-3α expression in a similar mechanism as has been reported for human iNOS.

The results presented here provide IL-12, MIP-2, and MIP-3α as novel inflammatory cytokine targets for TTP-regulated mRNA decay. The data are implicated in the understanding of basic mechanisms of the inflammatory process and in the development of novel anti-inflammatory drugs.

## ACKNOWLEDGMENTS

## REFERENCES

[1] Varnum BC, Lim RW, Sukhatme VP, Herschman HR. Nucleotide sequence of a cDNA encoding TIS11, a message induced in Swiss 3T3 cells by the tumor promoter tetradecanoyl phorbol acetate. *Oncogene.* 1989;4(1):119–120.

[2] Lai WS, Stumpo DJ, Blackshear PJ. Rapid insulin-stimulated accumulation of an mRNA encoding a proline-rich protein. *Journal of Biological Chemistry.* 1990;265(27):16556–16563.

[3] DuBois RN, McLane MW, Ryder K, Lau LF, Nathans D. A growth factor-inducible nuclear protein with a novel cysteine/histidine repetitive sequence. *Journal of Biological Chemistry.* 1990;265(31):19185–19191.

[4] Taylor GA, Lai WS, Oakey RJ, et al. The human TTP protein: sequence, alignment with related proteins, and chromosomal localization of the mouse and human genes. *Nucleic Acids Research.* 1991;19(12):3454.

[5] Ma Q, Herschman HR. A corrected sequence for the predicted protein from the mitogen-inducible TIS11 primary response gene. *Oncogene.* 1991;6(7):1277–1278.

[6] Heximer SP, Forsdyke DR. A human putative lymphocyte G0/G1 switch gene homologous to a rodent gene encoding a zinc-binding potential transcription factor. *DNA and Cell Biology.* 1993;12(1):73–88.

[7] Blackshear PJ. Tristetraprolin and other CCCH tandem zinc-finger proteins in the regulation of mRNA turnover. *Biochemical Society Transactions*. 2002;30(6):945–952.

[8] Blackshear PJ, Phillips RS, Ghosh S, Ramos SVB, Richfield EK, Lai WS. *Zfp36l3*, a rodent X chromosome gene encoding a placenta-specific member of the tristetraprolin family of CCCH tandem zinc finger proteins. *Biology of Reproduction*. 2005;73(2):297–307.

[9] 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. *Molecular and Cellular Biology*. 1999;19(6):4311–4323.

[10] Lai WS, Blackshear PJ. Interactions of CCCH zinc finger proteins with mRNA. Tristetraprolin-mediated AU-rich element-dependent mRNA degradation can occur in the absence of a poly(A) tail. *Journal of Biological Chemistry*. 2001;276(25):23144–23154.

[11] Kedersha N, Stoecklin G, Ayodele M, et al. Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *Journal of Cell Biology*. 2005;169(6):871–884.

[12] Murata T, Morita N, Hikita K, Kiuchi K, Kiuchi K, Kaneda N. Recruitment of mRNA-destabilizing protein TIS11 to stress granules is mediated by its zinc finger domain. *Experimental Cell Research*. 2005;303(2):287–299.

[13] 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 and Development*. 2005;19(3):351–361.

[14] Taylor GA, Carballo E, Lee DM, et al. A pathogenetic role for TNFα in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity*. 1996;4(5):445–454.

[15] Carballo E, Lai WS, Blackshear PJ. Feedback inhibition of macrophage tumor necrosis factor-α production by tristetraprolin. *Science*. 1998;281(5379):1001–1005.

[16] 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(6):1891–1899.

[17] Ogilvie RL, Abelson M, Hau HH, Vlasova I, Blackshear PJ, Bohjanen PR. Tristetraprolin down-regulates IL-2 gene expression through AU-rich element-mediated mRNA decay. *Journal of Immunology*. 2005;174(2):953–961.

[18] Stoecklin G, Ming X-F, Looser R, Moroni C. Somatic mRNA turnover mutants implicate tristetraprolin in the interleukin-3 mRNA degradation pathway. *Molecular and Cellular Biology*. 2000;20(11):3753–3763.

[19] Stoecklin G, Stoeckle P, Lu M, Muehlemann O, Moroni C. Cellular mutants define a common mRNA degradation pathway targeting cytokine AU-rich elements. *RNA*. 2001;7(11):1578–1588.

[20] Sawaoka H, Dixon DA, Oates JA, Boutaud O. Tristetraprolin binds to the 3′-untranslated region of cyclooxygenase-2 mRNA: a polyadenylation variant in a cancer cell line lacks the binding site. *Journal of Biological Chemistry*. 2003;278(16):13928–13935.

[21] Yu H, Stasinopoulos S, Leedman P, Medcalf RL. Inherent instability of plasminogen activator inhibitor type 2 mRNA is regulated by tristetraprolin. *Journal of Biological Chemistry*. 2003;278(16):13912–13918.

[22] Fechir M, Linker K, Pautz A, et al. Tristetraprolin regulates the expression of the human inducible nitric-oxide synthase gene. *Molecular Pharmacology*. 2005;67(6):2148–2161.

[23] Jalonen U, Lahti A, Korhonen R, Kankaanranta H, Moilanen E. Inhibition of tristetraprolin expression by dexamethasone in activated macrophages. *Biochemical Pharmacology*. 2005;69(5):733–740.

[24] Carballo E, Gilkeson GS, Blackshear PJ. Bone marrow transplantation reproduces the tristetraprolin-deficiency syndrome in recombination activating gene-2 (-/-) mice. Evidence that monocyte/macrophage progenitors may be responsible for TNFα overproduction. *Journal of Clinical Investigation*. 1997;100(5):986–995.

[25] Templin MF, Stoll D, Schrenk M, Traub PC, Vöhringer CF, Joos TO. Protein microarray technology. *Drug Discovery Today*. 2002;7(15):815–822.

[26] Chen C-YA, Shyu A-B. AU-rich elements: characterization and importance in mRNA degradation. *Trends in Biochemical Sciences*. 1995;20(11):465–470.

[27] Worthington MT, Pelo JW, Sachedina MA, Applegate JL, Arseneau KO, Pizarro TT. RNA binding properties of the AU-rich element-binding recombinant Nup475/TIS11/tristetraprolin protein. *Journal of Biological Chemistry*. 2002;277(50):48558–48564.

[28] Blackshear PJ, Lai WS, Kennington EA, et al. Characteristics of the interaction of a synthetic human tristetraprolin tandem zinc finger peptide with AU-rich element-containing RNA substrates. *Journal of Biological Chemistry*. 2003;278(22):19947–19955.

[29] Brewer BY, Malicka J, Blackshear PJ, Wilson GM. RNA sequence elements required for high affinity binding by the zinc finger domain of tristetraprolin: conformational changes coupled to the bipartite nature of AU-rich mRNA-destabilizing motifs. *Journal of Biological Chemistry*. 2004;279(27):27870–27877.

[30] Lai WS, Carrick DM, Blackshear PJ. Influence of nonameric AU-rich tristetraprolin-binding sites on mRNA deadenylation and turnover. *Journal of Biological Chemistry*. 2005;280(40):34365–34377.

[31] Chen C-Y, Gherzi R, Ong S-E, et al. AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell*. 2001;107(4):451–464.

[32] Lai WS, Kennington EA, Blackshear PJ. Tristetraprolin and its family members can promote the cell-free deadenylation of AU-rich element-containing mRNAs by poly(A) ribonuclease. *Molecular and Cellular Biology*. 2003;23(11):3798–3812.

[33] McInnes IB, Gracie JA. Targeting cytokines beyond tumor necrosis factor-α and interleukin-1 in rheumatoid arthritis. *Current Rheumatology Reports*. 2004;6(5):336–342.

[34] Becker C, Wirtz S, Neurath MF. Stepwise regulation of $T_H1$ responses in autoimmunity: IL-12-related cytokines and their receptors. *Inflammatory Bowel Diseases*. 2005;11(8):755–764.

[35] Snijders A, Hilkens CMU, van der Pouw Kraan TCTM, Engel M, Aarden LA, Kapsenberg ML. Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. *Journal of Immunology*. 1996;156(3):1207–1212.

[36] Driscoll KE. TNFα and MIP-2: role in particle-induced inflammation and regulation by oxidative stress. *Toxicology Letters*. 2000;112-113:177–183.

[37] Schutyser E, Struyf S, Van Damme J. The CC chemokine CCL20 and its receptor CCR6. *Cytokine and Growth Factor Reviews*. 2003;14(5):409–426.