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DIPLOMARBEIT

The search for novel mRNAs targeted by the RNA-destabilizing protein tristetraprolin for degradation

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

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Wien, am 28. April 2010

Index

1. Summary

1.1 Summary

Gene expression is regulated at the level of transcription, mRNA stability and translation. mRNA stability plays a particularly important role in the expression of inflammatory genes. The stability of mRNA is in most cases regulated by cis-acting regulatory elements located in the 3´ untranslated regions. The best characterized elements are the so called adenine-uridine-rich sequences (AREs). These AREs are recognized by RNA-binding proteins that may have stabilizing or destabilizing effects. One of these proteins is tristetraprolin (TTP), encoded by the gene *zfp36,* that is known to suppress inflammation by accelerating the degradation of cytokine mRNAs. Binding of TTP to the ARE leads to decapping, deadenylation and subsequent nuclease digestion. Several mRNAs have been found to be destabilized by the activity of TTP. Among the mRNA targets of TTP is GM-CSF and tumor necrosis factor alpha (TNFα), a major factor in the regulation of the inflammatory response. By promoting mRNA decay, TTP significantly contributes to cytokine homeostasis. In this diploma thesis three new TTP targets were characterized. This study shows that TTP can bind and destabilize the mRNA of IL1α, IL6 and Cxcl2 through assays performed with both primary macrophages and through rabbit β-globin reporter assays. Additionally, the presence of TTP significantly reduces the expression of a luciferase gene fused to the 3´ UTR of IL1α, IL6, Cxcl2 and IL12β in reporter assay experiments.

mRNA stability is an important gene expression-regulating mechanism that is critically involved in immune responses and tumorigenesis. Some of TTPs targets, such as VEGF, COX-2 and IL3, have been shown to play a fundamental role in the formation and progression of various cancers, including colon cancer and mast-cell carcinoma. In this thesis, an involvement of TTP in *v-abl* induced B-cell lymphoma formation is described. Cells isolated from TTP-deficient mice show an increased ability to form transformed cell lines after transduction with the *v-abl* oncogene. Subcutaneous injection of v-abl-transformed wild-type and $TTP^{-/-}$ B-cells into recipient mice show an increase in tumor size, further solidifying the hypothesis that TTP acts as a tumor suppressor.

1.2 Zusammenfassung

Genexpression ist auf den Ebenen der Transkription, mRNA Stabilität und Translation reguliert. Bei der Regulation von inflammatorischen Genen ist die mRNA Stabilität von besonderer Bedeutung. Die Stabilität der mRNA wird in den meisten Fällen von regulatorischen Elementen, welche cis-Aktivität besitzen und sich in der 3´ untranslatierten Region der mRNA befinden, reguliert. Die am besten charakterisierten Elemente sind die sogenannten Adenin-Uridin-reichen Elemente (ARE). AREs werden von RNA-bindenden Proteinen erkannt, welche stabilisierende oder destabilisierende Effekte bewirken können. Eines dieser Proteine ist Tristetraprolin (TTP). TTP wird von dem Gen *zfp36* codiert und ist dafür bekannt, Entzündungsreaktionen durch die Beschleunigung von Cytokin mRNA Abbau zu unterdrücken. Die Bindung von TTP an eine Ziel mRNA führt zur Entfernung der 5´ Cap-Struktur, Deadenylierung und darauffolgendem Abbau der mRNA. Die bereits identifizierten Ziel-mRNAs von TTP schließen GM-CSF, IL10, INFγ und TNFα ein. TNFα, das bedeutendste Ziel von TTP, ist ein wichtiger Faktor in der Regulation des Entzündungsprozesses. Die Aktivität TTP bewirkt eine Beschleunigung des mRNA Abbaus, was bedeutende Auswirkungen auf das Cytokin Gleichgewicht in der Zelle hat. In dieser Arbeit beschreiben wir drei neue mRNAs welche von TTP reguliert werden. Durch Untersuchung von primären Makrophagen und Reporter Systemen zeigen wir, dass TTP die mRNAs von IL1α, IL6 und Cxcl2 binden und destabilisieren kann. Zusätzlich zeigen wir, dass die Expression eines Luziferase-Reporters in Anwesenheit von TTP deutlich reduziert wird wenn die Luziferase mRNA an die 3´ UTR von IL1α, IL6, IL12β und Cxcl2 gekoppelt ist.

Die Rolle von mRNA Stabilität ist ein wichtiger Mechanismus in der Regulation von Entzündungsreaktionen und Tumorentwicklung. Einige der identifizierten Ziele von TTP, wie VEGF, COX-2 und IL3, sind essentiell an der Entstehung und dem Fortschreiten von Tumoren beteiligt. In dieser Arbeit beschreiben wir die Rolle von TTP in der Entwicklung von *v-abl* bedingtem B-Zell Lymphom. Zellen, welche von $TTP^{-/-}$ Mäusen isoliert wurden, zeigen eine erhöhte Tendenz stabile Kolonien zu bilden nachdem das *v-abl* Onkogen eingebracht wurde. In Versuchen an lebenden Mäusen, welchen transformierte Zellen von Wildtyp und $TTP^{-/-}$ Mäusen injiziert wurden, bilden $TTP^{-/-}$ Zellen signifikant größere Tumore aus, was die Rolle von TTP als Tumor-Unterdrücker weiter untermauert.

2. General Introduction

2.1 The post-transcriptional regulation of gene expression by mRNA stability

mRNA stability is, besides translational control mechanisms and transcriptional control, the most determining mechanism for the regulation of final protein levels. Once in the cytoplasm mRNAs undergo progressive deadenylation at rates that are specific for each mRNA species. This provides a timing mechanism that confers a defined lifetime, rather than a stochastic half-life, on each mRNA species. The mRNA therefore contains information that defines its own life span. The best characterized stabilizing/destabilizing elements are the adenine-uridine-rich elements (AU-rich elements), which are bound by a number of proteins to either extend or shorten mRNA life span, often in response to extracellular signals [1]. The major steps in mRNA degradation are deadenylation, decapping, 3´-5′- and 5´-3´-exonuclease digestion. AU-rich elements (AREs) can be found in the 3´ UTR of many mRNAs. The 3´ UTR of an mRNA contains several motifs controlling polyadenylization, stability, localization and the efficiency of translation.

AREs were identified to mediate the stability of messenger RNAs through the binding of ARE binding proteins more than 20 years ago [2]. It is estimated that up to 8 % of mRNAs contain ARE sequences [3]. 3 classes of these elements are characterized [4]. The class I ARE contains a sequence of AUUUA and is mainly found in the mRNA of transcription factors and cell cycle regulatory proteins like c-fos [5], c-myc or cyclin A. The class II AREs is made up by at least 2 overlapping UUAUUUA(A/U)(A/U) sequences and can be found in many cytokine mRNAs. This class of ARE has been subdivided into several subclasses depending on the number of consecutive AUUUA repeats found in the 3´ UTR. Class III AREs are less well defined and describe a stretch of nucleotides which is U-rich but does not contain the AUUUA motif. Like the class I AREs, this class is also found in the mRNA of transcription factors and cell cycle regulatory factors. The impact on the stability of the mRNAs is conferred by the binding of ARE-binding proteins [6]. This binding can either expand the half-life of the bound mRNA or mediate its decay [7]. The fact that, depending on the ARE, 15-20 different ARE-binding proteins can bind to the mRNA highlights the heterogenous effects these sequences can have on the stability of an mRNA.

The importance of AREs has been highlighted through the analysis of TNFα mutant mouse models lacking the ARE in the 3´ UTR [8-9]. These experiments showed a drastic increase in the stability of the TNFα mRNA resulting in increased expression of the gene. This overexpression resulted in a perpetual inflammation of the mice.

AREs have also been shown to play an important role in the orchestration of the immune response. In a recent publication [10], the temporal order of a cellular response to TNFα-treatment was addressed. Genes, which were expressed rapidly during the inflammatory response, were highly unstable. A comparison of the half-life and the 3´ UTR sequences resulted in a correlation between stability and the amount of AREs in the 3´ UTR. A finding that not only shows that these mRNAs contain a method of their temporal regulation but also shows the importance of AREs and mRNA stability in the inflammatory response.

2.2 AU-binding proteins

2.2.1 ELAV family

The ELAV (embryonic lethal abnormal vision) protein family, first described in Drosophila, comprises a group of RNA-binding proteins. In Drosophila, the *elav* locus is essential for the development and maintenance of the nervous system. Unlike other members of the ELAV family of proteins (HuB, HuC and HuD) who are only expressed in the brain, HuR (HuA/ELAVl1) is also expressed in thymus, intestine, spleen and reproductive organs [11]. HuR's human homologue is composed of 326 amino acids and is localized to the human chromosome 19 [12]. It is predominantly nuclear, but upon cellular activation, it translocates to the cytoplasm, binds selected mRNAs and causes their stabilization. The protein possesses three RNA-recognition motifs that are used to bind ARE-containing mRNAs with high affinity. This binding increases the stability and translation of the target mRNAs. HuR has been described to influence the stability of a number of ARE-containing mRNAs of proteins involved in cancer and inflammation, for example TNFα, GM-CSF, COX-2, VEGF, IL8, cycline A,B,D and p21 [12].

Genetic ablation of HuR revealed that HuR is essential for the survival of midgestational embryos [13]. The absence of HuR led to defects in placental architecture resulting in the death of the embryos. With the use of conditional genetic ablation methods using Tie1 Cre

recombinase (deletion of HuR in the embryo while HuR expression in the placenta is not affected), the mice displayed distortions in skeletal segments leading to a reduced size of the skeleton. This phenotype was triggered by altered BMP-4 expression pattern in the HuR-deficient embryos causing delays in skeletal ossification. Additionally, HuR was found to greatly influence the patterning and outgrowth of limbs and the development of the spleen.

In experiments using tetracycline-regulated transgenic HuR cells, resulting in a 2.5-to 3-fold overexpression of HuR, the increase in HuR led to significant stabilization of TNFα and COX-2 mRNAs while the stability of TGFβ1 (transforming growth factor-β1; does not contain AREs but is controlled by HuR on a transcriptional level) mRNA remained unaffected [14]. In these experiments, the transgenic cells were stimulated with lipopolysaccarides (LPS) and subsequently, transcriptional termination was induced through actinomycin-D-treatment. Polysomal distribution analysis revealed that HuR-overexpression resulted in a reduction of TNFα, COX-2 and TGFβ1 mRNA-translation. *In-vivo* experiments using the HuR-overexpressing mice, induction of transgenic HuR by doxycycline-treatment led to significantly decreased production of TNFα, IL1β and TGFβ1. This overexpression was not sufficient to alter the animal's sensitivity to LPS-induced shock. HuR-overexpression reduced both *ex-vivo* and *in-vivo* inflammatory responses. These studies show the role of HuR as an mRNA-stabilizing factor while expanding on the function of HuR as a translational inhibitor.

2.2.2 AUF1

AUF1 (ARE/poly-(U)-binding/degradation factor 1), also called hnRNPD (heterogeneous nuclear ribonucleoprotein D), was originally found to promote mRNA decay [15], as determined from studies using cultured cells expressing different levels of AUF1, as well as using AUF1-deficient mice. In some cases, AUF1 has also shown to enhance the stability of target mRNAs and to promote their translation. However the most pronounced effect of AUF1 is mRNA destabilization. It displays high affinity for poly-(U) RNA. There are 4 different isoforms of AUF1 (p37, p40, p42 and p45), which arise as a result from differential splicing of the mRNA. All of the AUF1 isoforms contain two RNA recognition motifs through which they bind to a select group of mRNAs.

AUF1 has been shown to bind the AREs of mRNAs that encode stress response and proliferative proteins including TNFα, GM-CSF, FOS, Cyclin D and COX-2. Interestingly, the effect of AUF1-binding can vary from mRNA stabilization to the promotion of decay

depending on cell type [16-17]. AUF1 has been shown to influence the steady-state mRNA levels both in untreated conditions and in response to treatment with damaging or growth-regulatory stimuli. Through its actions on these and other mRNAs, AUF1 was shown to play a central role in differentiation, carcinogenesis and in the cellular response to mitogenic factors and immune factors [18].

2.2.3 TIA1 and TIAR

T cell-restricted intracellular antigen-1 (TIA1) and TIA-related protein (TIAR) are able to bind U-rich elements [19]. The two proteins were shown to bind to the ARE in the TNFα mRNA 3´ UTR in macrophages. While the TAI1 knock-out does not lead to increased levels of TNFα, LPS-treatment of the knock-out mice causes a 2-fold increase of TNFα expression compared to the wild-type [20]. As the deficiency of TIA1 was shown to result in increased association of the RNA with polysomes, TIA was suggested to function as a translational silencer.

2.2.4 KSRP

KSRP (KH-type splicing regulatory protein), also known as FBP1, a member of the FBP (far upstream sequence element-binding protein), family of proteins has originally been described as single-stranded DNA-binding protein activating a far upstream element of c-myc [21]. KSRP has been described to function in a multiprotein-complex where it mediates splicing [22]. KSRP has later been found to be an ARE-binding protein that recruits the exosome to a subset of mRNA targets thereby promoting their degradation. The central part of the protein is organized into four domains capable of binding single-stranded nucleic acids, the so-called K-homology domains [23]. Studies have shown that binding by more than one domain is necessary to attain high binding affinity. Protein complexes containing FBP1 have been shown to bind to AREs of both COX-2 and TNFα. FBP2, a member of the same protein family, has been shown to bind to the AREs of IL2 and c-Fos.

2.3 Tristetraprolin (TTP)

The 33.6 kDa TTP, also known under the name Tis11 and Nup475 contains two CCCH zinc-fingers and three tetraproline (PPPPG) motives and is encoded by the immediate-early mitogen induced gene *zfp-36* in mice and *ZFP36* in humans. The gene was initially discovered in a screen for genes that were rapidly turned on by exposure of cultured fibroblasts to insulin, serum or tumor promoting phorbol esters [24-27]. This induction, combined with the presence of the two nearly identical putative zinc fingers [25] and nuclear localisation, led to the conclusion that TTP was likely to be a transcription factor. Upon further investigation, TTP was found to shuttle from the nucleus to the cytosol [28] and that TTP was phosphorylated by the same agents that stimulated the transcription of the *zfp-36* gene. There are two other family members of TTP known, TIS11b (*zfp36L1*) and TIS11d (*zfp36L2*), containing the same tandem zinc finger motif [29].

TTP has been shown to bind to class II AREs and upon binding of TTP to those elements, the mRNA is destabilized by several mechanisms (see chapter 2.3.2). The human TTP protein is 87 % identical to the murine TTP and the zinc finger domain is conserved between human TTP, murine TTP and the rat variant cMG1 [30]. The human gene encoding TTP is localized to chromosome 19 and the mouse homologue is located in chromosome 7 [30].

When induced with LPS, TTP from macrophages visualized on western blots stained with a TTP antibody takes the form of multiple bands which collapse to higher mobility bands following phosphatase-treatment [31], indicating phasphorylation of the protein.

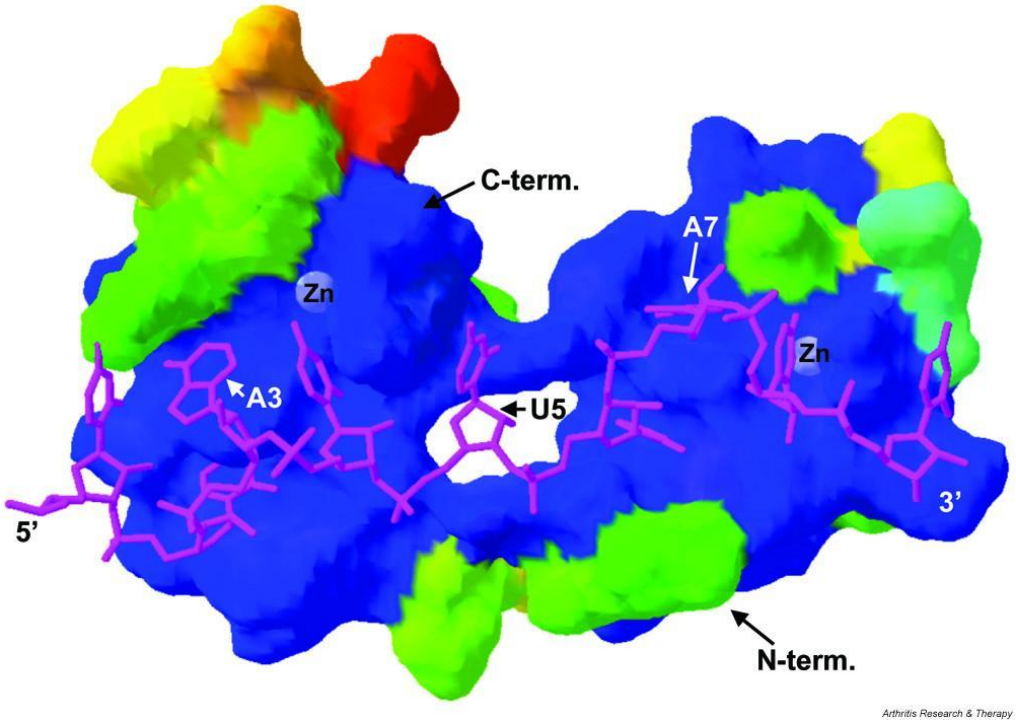


Fig 1: Structure of TTP including bound RNA oligonucleotide [32]

2.3.1 Phenotype of the TTP knock-out mouse

The biological function of TTP was discovered with the help of a TTP knock-out mouse [32]. These animals, although they appear normal at birth [33], soon develop a systemic inflammatory phenotype that includes erosive polyarticular arthritis, cachexia, dermatitis, myeloid hyperplasia and the mice exhibit the 'kangaroo' hunched posture that is characteristic of many inflammatory syndromes in mice. The severity of the phenotype can vary drastically among the individual mice and the mice are unable to breed homozygously. The phenotype is accompanied by the loss of secondary adipose tissue in all depots. Additional phenotypes of the TTP knock-out mouse include granulocyte infiltration of the skin and foci of granulocytes in the liver, spleen, lymph nodes and other extramedullary sites. The mice also display significant splenomegaly and lymph-adenopathy. In the blood, a two-fold increase in white blood cells due to a 4-fold increase in GR-1^+ granulocytes, a 3-fold increase in F4/80^+ macrophages and an increased population of PK135^+ natural-killer (NK) cells can be observed [32]. The mice also displayed serological differences to the wild-type. Autoimmunity in the form of high titres of antinuclear antibodies as well as antibodies against both double-stranded and single-stranded DNA was found in the TTP-deficient mice. TTP was found to be

expressed in spleen, thymus, lung, large intestine, and liver and weakly expressed in brain and pancreas but is not expressed at all in the testis and uterus [34].

The phenotype of the TTP-deficient animals, particularly cachexia, suggested that the mutant mice suffered from an excessive production of tumor necrosis factor alpha (TNFα, also known as cachectin). Another indication for the involvement of TNFα was the apparent symmetrical arthritis, manifested in reddened, swollen paw joints, a phenotype often associated with TNFα excess syndromes. These aspects implicated TNFα as the main contributor to the phenotype. To address the causal role of TNFa in the phenotype, the TTP-deficient mice were treated with anti-mouse TNFα monoclonal antibodies, a treatment that remarkably prevented the development of most of the symptoms associated with the TTP deficiency [33]. Later studies using double knock-outs of TTP and both types of TNFα receptors confirmed the hypothesis that chronically elevated levels of TNFα were the cause of the phenotype. The knock-out mouse also exhibits upregulation of expression of various other genes, most notably GM-CSF [35].

Macrophages, isolated from fetal liver, adult bone marrow or the peritoneal cavity of TTP knock-out mice, produce considerably more TNFα than macrophages of their respective littermates [36]. This increase was then thought to be caused by an increase in the quantities of TNFα mRNA in the cell by either increased gene transcription or decreased turnover rate of the mRNA. The role of macrophages in the formation of the $TTP^{-/-}$ phenotype was further addressed through bone marrow transplantation experiments [36]. Analysis of LPS treated primary macrophages then revealed that TTP-deficient cells displayed a decreased TNFα mRNA turnover that was likely to be responsible for the inflammatory phenotype of the TTP-deficient mice [36]. Consistently, RNA immunoprecipitations and gel-shift assays showed that TTP could bind to the ARE located in the 3´ UTR of TNFα mRNA [36].

2.3.2 The effects of TTP binding to target mRNA

The binding of TTP to the ARE in the 3´ UTR of its target mRNA leads to decapping and cleavage of the poly-(A) tail which is followed by rapid 3´-5’ and 5´-3´ exonuclease activity [37-38]. TTP exerts its function by interacting with components of the basic mRNA decay machinery. Whether these interactions are direct, indirect or linked through the RNA-binding has not yet been determined as most of its interaction partners are subunits of larger protein complexes. TTP interacts with several components of processing bodies (P-bodies) [39],

small cytoplasmic foci that contain enzymes required for mRNA decay. MicroRNA-silenced mRNAs and mRNAs targeted for decay are transported to the P-bodies. In these foci, TTP can interact with Ccr4, a component of the Ccr4-Caf1-Not mRNA deadenylation complex [40] and components of the decapping complex including Dcp2, Dcp1a, Edc3 and Hedls [40-41]. Additionally, TTP interacts with Xrn1, a 5´-3´ exonuclease. TTP may also play a role in miRNA-based silencing through its interaction with Ago2 and Ago4, central components in the RNA-induced silencing process [42].

Additionally to the interaction partners in the P-bodies TTP has been shown to bind to the two exosomal subunits PM-Scl75 and Rrp4 [40, 43]. The components of the exosome include at least 10 exoribonucleases and RNA-binding proteins required for 3´-5´ digestion of RNA. TTP also activates the poly(A)-specific ribonuclease (PARN) [44] and it can bind to another ARE-binding protein KSRP [45]. Figure 2 shows a summary of the function of TTP and its interplay with other cellular factors to promote mRNA decay of bound targets.

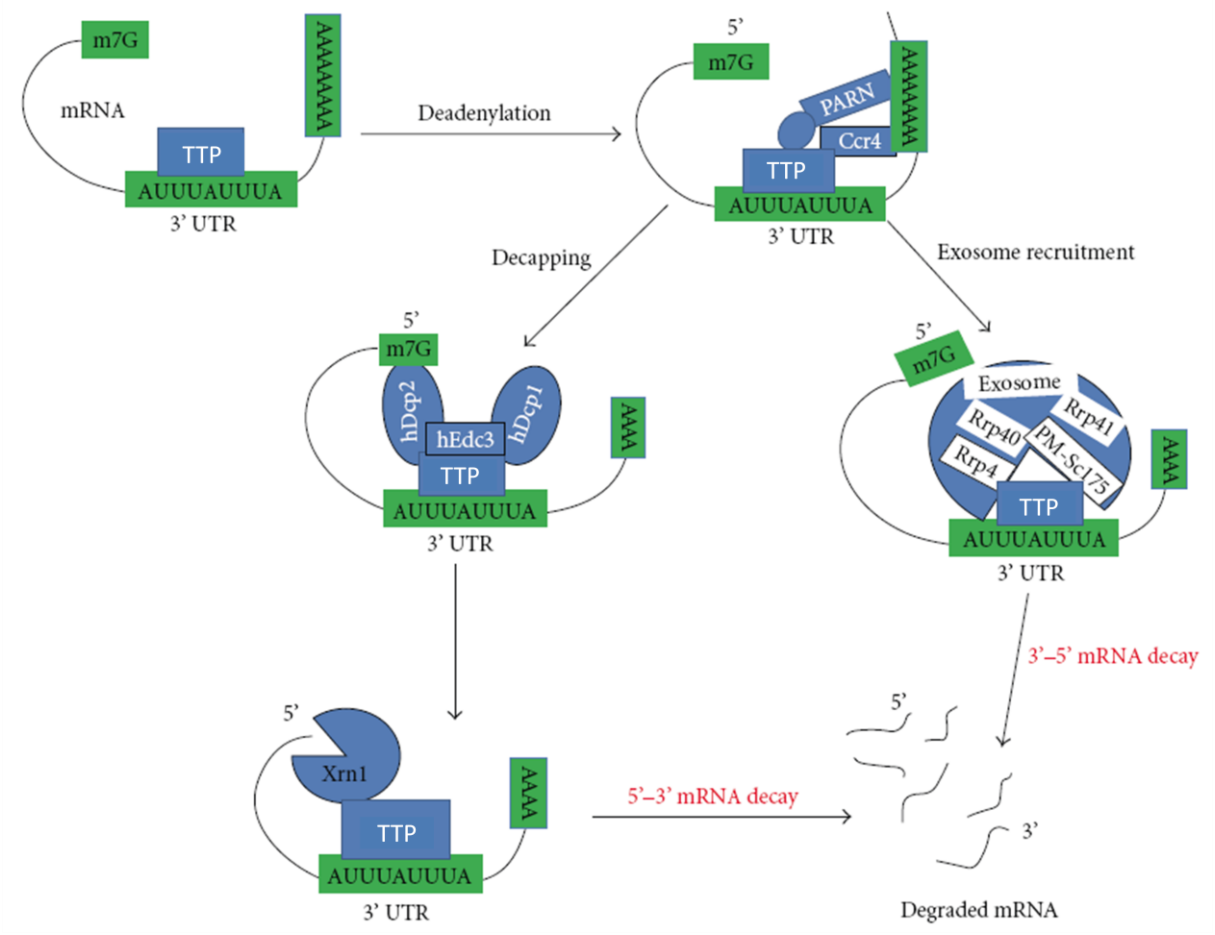


Fig 2: Sequential effects of TTP-binding to a target mRNA [38]

2.3.3 Regulation of TTP activity by expression and protein modification

TTP activity is regulated by the activity of several signaling cascades including ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase) [46], PI3K (phosphoinositide 3-kinase) [47] and p38 MAPK [48].

TTP expression has been shown to be induced by IL4 [49], TGFβ [50] and TNFα [36, 51]. The expression of the TTP protein is also regulated by the p38 MAPK-MK2 pathway [52]. TTP upregulation has been shown in LPS-stimulated murine macrophages. It was also shown that addition of IFN-γ to LPS treated primary mouse bone marrow macrophages further increased induction of TTP mRNA in comparison with LPS treated cells [53].

TTP expression is also regulated at the post-transcriptional level. The 3´ UTR of TTP contains AREs which can be bound by the TTP protein resulting in a negative feedback-loop in which TTP regulates its own mRNA stability [54].

The activation of TTP and the post-transcriptional regulation of cytokines by TTP have been mainly studied in lipopolysaccaride (LPS)-stimulated macrophages. In this system, the p38 MAPK-MK2 pathway has been shown to represent a major factor in the regulation of TTP expression and activity [37]. Murine MK2-deficient macrophages have shown severely reduced levels in TNFα, IL1, IL6 and INF-γ [55-56].

MK2 has been shown to directly phosphorylate TTP at the residues Ser^{52} and Ser^{178} [31, 57], allowing for 14-3-3 adaptor molecules to bind and inhibit the destabilizing activity of TTP [57]. In experiments using a non-phosphorylatable TTP mutant, 14-3-3 adaptor proteins were unable to bind resulting in constitutively active TTP which prevented TNFα upregulation during LPS stimulation in murine macrophages [48].

The TTP deactivating phosphorylation by MK2 is counteracted by a directly competing binding of the phosphatase PP2A with the 14-3-3- adaptor [48]. PP2A is then able to dephosphorylate TTP at serine residue Ser^{178} and possibly Ser^{52} resulting in the activation of mRNA decay by TTP.

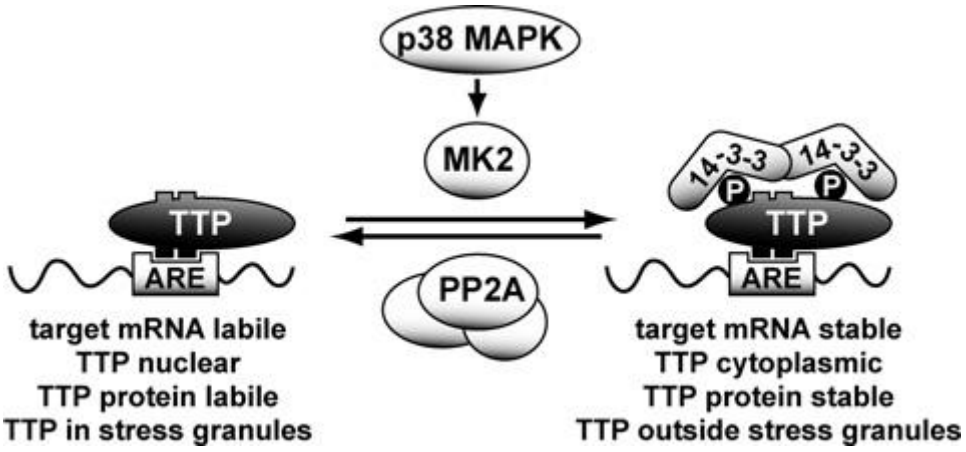


Fig 3: Regulation of TTP activity via p38 MAPK-MK2 [37]

Additionally to the expression and activation, the localization and stability of TTP are important criteria for the effect of TTP on the stability of its target mRNAs. These aspects are also regulated via the phosphorylations at the residues Ser^{52} and Ser^{178} [31]. These phosphorylation events performed by MK2 directly prevent decay of the protein by the proteasome thereby increasing its stability [31]. Additionally, the binding of the 14-3-3 adaptor promotes relocalization of TTP from the nucleus to the cytoplasm. TTP is also directly phosphorylated by p38 MAPK (α- and β-isoform) [58]. These phosphorylation events were analyzed *in-vitro* and showed the multiple bands comparable to the bands displayed by activated TTP after LPS stimulation when visualized via western blot.

2.3.4 TTP homologues

As mentioned above, the *Zfp36* family of genes consists of more members, Tis11b (*Zfp36l1*), Tis11d (*Zfp36l2*) and *Zfp36l3* [38, 59] (only described in rodents and not detected in human tissues). Just as TTP, these proteins contain tandemly repeated zinc finger motifs through which they bind to adenine-uridine-rich elements in mRNA and mediate ARE-mediated mRNA decay. Tis11b and Tis11d have been shown to exhibit an mRNA destabilizing effect on transcripts with the ARE of TNFα inserted into the 3´ UTR. Just like TTP, Tis11b and Tis11d have been shown to destabilize TNFα, IL3 and GM-CSF mRNA. Tis11d also has an effect on VEGF levels but unlike TTP, Tis11d does not influence the mRNA stability of VEGF but influences the translation of the mRNA [60].

While TTP-deficient mice appear normal at birth and only later they develop the inflammatory phenotype, Tis11b knock-out mice die *in-utero* (between days E8 and E13) [60-61]. Mice with a truncated form of Tis11d appeared normal at birth but displayed female infertility [62].

The homologues of TTP also display differences in their tissue expression. TIS11b is highly expressed in kidneys, liver, lung, pancreas, and heart, and weakly in skeletal muscle, colon, thymus, spleen, small intestine, brain, and peripheral blood leukocytes [63]. TIS11d is highly expressed in lung, liver, skeletal muscle, kidneys, pancreas, placenta, and less strongly in heart and brain [64].

2.3.5 Established targets for TTP and its homologues

TTP		Tis11b	Tis11d
TNFα [36]	IL12 [65]	TNFα	TNFα
GM-CSF [35]	c-myc	GM-CSF	GM-CSF
IL3 [66]	cyclin D1	IL3	IL3
IL6 [67]	IL10	VEGF	
COX-2	Ier3	c-IAP2	
PAI type 2	MIP-2	STAR	
Pitx2	p21		
TTP [54]	E47		
IL2	VEGF		
Cxcl2 [65]	polo-like kinase 3		
1,4galactosyltransferase			

Table 1: Established mRNA targets for destabilization by TTP and its homologues Tis11b and Tis11d.

2.3.6 Role of stress-regulated kinases in TTP function

As previously described, stress kinases, especially p38 MAPK, play an important role in the regulation of TTP expression as well as in processes such as activation, localization and stability of TTP.

2.3.6.1 p38 MAPK

Mitogen-activated protein kinases (MAPKs) participate in signaling induced by a wide variety of extracellular signals. Normally, MAPKs are activated by a series of phosphorylation events

in which one kinase phosphorylates another, resulting in a "MAPK cascade". This cascade results in the dual phosphorylation of MAPKs on a conserved Thr-X-Tyr motif. The p38 MAPK family has four members, α, β, γ and δ [68]. The established role of the p38 family is as a transducer of responses to environmental stress including hyperosmolarity, heat shock, UV irradiation, and inhibition of protein synthesis, as well as to receptor occupancy by pro-inflammatory molecules such as LPS, TNFα, and IL1. This signaling is involved in processes ranging from the induction of cytokine genes, apoptosis, cell survival, differentiation and the activation of effector functions.

The canonical activation of p38 is performed through the dual phosphorylation of a Thr-Gly-Tyr motif by its upstream MAPK kinases (in the case of TTP activation, this role is performed by MKK6). The function of p38 MAPK can be pharmacologically inhibited by the administration if the high-specificity inhibitor SB203580 [69].

TTP has been shown to be a substrate of the p38α and p38β isoforms [58]. The phosphorylation of TTP at the residues Ser^{52} and Ser^{178}, responsible for the increased stability and decreased activity, are performed by p38's downstream kinase MK-2.

2.3.6.2 ERK

The extracellular signal-regulated kinases (ERKs) belong to the family of MAPKs. ERKs are involved in the regulation of mitosis, meiosis and are also responsible for postmitotic functions in differentiated cells. The ERK pathway in activated by various stimuli including growth factors, cytokines and G-protein coupled receptor ligands.

The involvement of the ERK signaling pathway was first indicated by the increase in mobility of TTP after pharmacological inhibition of the ERK/MEK pathway [31]. This increase in electrophoretic mobility was due to a decrease in phosphorylation events. While the inhibition on ERK alone had no impact on the expression of TTP, combined inhibition of ERK and p38 MAPK completely shuts down LPS-induced TTP expression although this effect is caused primarily by the inhibition of p38 MAPK.

ERK also has an effect on the localization of TTP in the cell [31]. In this case, ERK acts synergistically with p38 MAPK. Pharmacological inhibition of p38 MAPK and ERK leads to an increased shift in the localization of TTP compared to an exclusive p38 inhibition. The combined inhibition also affects the stability of the TTP protein synergistically.

2.3.6.3 JNK

The c-Jun N-terminal kninases (JNKs) belong to the family of MAPKs and are involved in inflammatory responses, cytokine production (mediated by activating proteins such as IL8 and GM-CSF) but also cell differentiation, proliferation and apoptosis.

The involvement of JNK in the induction of TTP [70] was shown through the TNFα-induced expression of TTP. In this experiment, the induction of TTP after TNFα stimulation was shown in human TM4 cells (testicular cell line) in which this induction could be blocked by pharmacological inhibition of JNK through the inhibitor SP600125.

2.3.7 Role of TTP in IL10 responses

The biological function of IL10 is generally considered to limit or shut down inflammatory responses. On the cellular level, IL-10 inhibits production of proinflammatory cytokines and regulates differentiation and proliferation of various immune cells. One of the effects of IL10 is the destabilization of several inflammatory mRNAs. It has recently been discovered that this effect is caused by a TTP-dependent mechanism and that TTP was needed for the full anti-inflammatory function of IL10 [71].

IL10-mediated inactivation of p38 MAPK constitutes a major factor in later stages of the inflammatory response. In macrophages, LPS initially strongly induces expression of TTP that, however, displays a low mRNA-destabilizing activity since at this phase the p38 MAPK activity is high. Only at the later phase of the inflammatory response, when p38 MAPK is diminished due to the endogenous IL10 production, TTP acquires its full mRNA-destabilizing function. This inactivation of p38 MAPK is an essential part in the resolution phase of the inflammation. For experimental procedures, the p38 MAPK inhibitor SB203580 can be used to mimic the IL10-mediated inhibition of the p38 MAPK activity and, consequently, to increase the mRNA-destabilizing activity of TTP.

2.3.8 Aims and rationales

Our understanding of the role that TTP plays in the organism is highly dependent on the identification of TTPs target mRNAs. As the characterization of the TTP deficiency phenotype is far from completed, a detailed list of all target mRNAs of TTP is highly

important. Even though the role of TTP in the regulation of TNFα-expression has been researched in detail, this function of TTP is not sufficient to fully explain the phenotype of the TTP-deficient mice. During the course of this thesis, we aimed to establish a reliable method to identify yet unknown target mRNAs of TTP. This was achieved through the use of several established methods like RNA-immunoprecipitation, quantitative RT-PCR and reporter assays.

2.4 TTP in hematopoiesis

The TTP knock out mouse displays several characteristics that suggest a role of TTP in hematopoiesis. The TTP-deficient mouse shows an almost twofold increase in white blood cells. This increase can be attributed to a fourfold increase in Gr-1^+ granulocytes, a threefold increase in F4/80^+ macrophages and increases in PK135^+ NK cells. Additionally, there were decreases in the B and T lymphocyte populations [32] (populations in the bone marrow were unaffected).

Additionally, TTP has been described to have an impact on the stability of IL3 [66], leading to an enhanced expression of IL3 in TTP-deficient mice. This increase in IL3 causes an enhanced differentiation of multipotent hematopoietic stem cells into myeloid progenitor cells. This also enhances the proliferation of myeloid linage cells such as granulocytes, monocytes and dendritic cells. Allthough the TTP-deficient mouse displayed differences in B and T cell populations [32], no effect on IL7, the interleukin responsible for stimulating multipotent hematopoietic stem cells to differentiate into lymphoid progenitor cells, is known to date.

2.4.1 TTP involvement in tumor formation

During tumor formation, a normally unstable mRNA sometimes becomes abnormally stable leading to an increased expression of the gene [72]. This change may happen because of mutations of the mRNA's ARE or through changes in the activity and expression of ARE-binding mRNA stabilizing or destabilizing proteins.

The involvement of TTP in tumor formation has been shown using a mast cell tumor model. In this system, the IL3 [73] mRNA undergoes an increase in stabilization in the *v-H-ras*

transformed PB-3c mast cell line. This transformation is not sufficient for malignant transformation but leads to the development of autocrine, IL3-secreting tumors after a long latency in *in-vivo* studies. The role of TTP was highlighted in this study as the cell line was transfected with a TTP expression vector (additional to the endogenous TTP of the cell line) which led to an increased latency of the TTP expressing cell line during *in-vivo* tumor formation assays. Alternatively, addition of a TTP expression vector into an established tumor line led to a decrease in IL3 secretion. This decrease was lost after seven weeks and the additional TTP expression was not present anymore, a result that was not visible in cells transfected with an inactive TTP C139R mutant (leading to a disruption of the zinc-finger domain), in which IL3 secretion was not affected. Tumor growth during *in-vivo* experiments was also highly affected by the addition of a TTP expression vector. This result suggested a possible role of TTP as a tumor suppressor.

The role of TTP as a tumor suppressor was also supported by the destabilization of VEGF mRNA by TTP. The expression of VEGF, the most potent mitogen for vascular endothelial cells, which has emerged as a very important factor in angiogenesis, correlates strongly with tumor progression in many human cancers. The expression of VEGF [74] is regulated on multiple levels including mRNA stability by means of ARE-binding proteins. The AU-binding protein HuR is known to bind and enhance the stability of VEGF mRNA while TTP mediates VEGF mRNA decay and its homologue Tis11b (BRF1) decreases the expression of VEGF at the translational level.

In an analysis of primary human colorectal adenocarcinoma and adenomas, a distinct down-regulation of TTP could be observed in the colorectal tumors with intermediary decreases in TTP expression in the adenomas compared to normal colonic mucosa [75]. As VEGF expression is closely linked to tumorigenesis in the colon, an inverse pattern of expression between VEGF and TTP could be found in the analyzed tissues. This phenomenon was also found in tumor cell lines. TTP overexpression in colon cancer cell lines showed a decrease in VEGF expression due to enhanced instability of the VEGF mRNA while siRNA-mediated knock-down of TTP led to an increased VEGF expression. The destabilizing function of TTP was further evaluated through a luciferase vector system showing that the luciferase reporter expression including the VEGF ARE was inhibited.

Additionally to the expression of VEGF leading to angiogenesis in the tumorigenic tissue, it was also shown that an overexpression of TTP in colorectal cell lines led to decreased proliferation in vitro [75]. This effect was however not linked to the established TTP target

mRNAs c-myc or Cyclin-D1 but was instead caused by a decrease in secreted VEGF in the TTP overexpressing cells and the effect could be remedied by the addition of VEGF protein to the medium.

In addition of the TTP-mediated upregulation of VEGF, overexpression of the ARE-containing- and TTP-target gene COX-2 coincided with elevated HuR and loss of TTP expression in colon carcinoma cells [76]. Cyclooxygenases (COX) are key enzymes in the production of prostaglandins, and overexpression of the inducible isoform COX-2 has been shown to occur at multiple stages of colon carcinogenesis allowing for elevated prostaglandin synthesis to occur in the tumor microenvironment [77]. Furthermore, the presence of TTP in normal colon epithelium suggests that it serves in a protective capacity by controlling inflammatory mediator expression levels.

2.4.2 Aims and rationales

The role of TTP as a tumor suppressor has been introduced in several publications [73, 75-76] although none of them showed tumor studies involving TTP knock-out cells. Thanks to collaboration with the lab of Veronika Sexl, we had the opportunity to employ the method of *v-abl*-mediated transformation [78] of B-cells obtained from mice of wild-type C57Bl/6 and C57Bl/6 $TTP^{-/-}$ background.

One necessary step in this experiment is to compare possible differences in the development of B-cells between wild-type and $TTP^{-/-}$ mice. The acquired cell lines are then used for subcutaneous injection in *in-vivo* tumor studies in wild-type C57Bl/6 mice to visualize a possible impact of the absence of TTP on tumor progression.

The aim of this project is to find out whether TTP deficiency has an impact on B-cell lymphoma formation and progression. A further goal was to unravel the mechanism behind the possible TTP-dependent effects in the tumors.

3. MATERIALS AND METHODS

3.1 Western blotting

3.1.1 Buffer

6x Sample buffer preparation:

- 0.5M Tris-HCl pH 6.8 5 ml
- Glycerol 2.5 ml
- 10 % SDS 4 ml
- Beta-mercaptoethanol 2 ml
- 0.05 % bromphenol blue (w/v) 0.2 ml
- dH_2O 9.5 ml

1x Running buffer

- 20 mM Tris base
- 192 mM glycine
- 0,1 % SDS

4x Separation gel buffer:

- 1.5 M Tris-HCl pH 8.8
- autoclave

4x Stacking gel buffer:

- 0.5 M Tris-HCl pH 6.8
- autoclave

Frackelton buffer:

- 10 mM Tris
- 50 mM NaCl
- 30 mM$NaPP_i$
- 50 mM NaF

- 1 % Triton X-100
- Adjust to pH 7-7.5
- Store at 4°C

20 % (w/v) APS

- Ammoniumpersulfate 1 g
- dH_2O to 5 ml
- store at -20°C

Anode buffer I:

- 0.3 M Tris
- 20 % Methanol

Anode buffer II:

- 2.5 mM Tris
- 20 % Methanol

Cathode buffer:

- 0.04 M amino-caprionic acid
- 20 % Methanol
- 0.01 % SDS

1x TBST:

- 10 mM Tris pH 8.0
- 150 mM NaCl
- 0.05 % Tween 20

Stripping buffer:

- 200 mM Glycin
- 150 mM NaCl
- 0.5 % (v/v) Tween 20
- Adjust to pH 2.5
- Autoclave

Ponceau staining solution:

- 0,1 % Ponceau
- 3 % Trichloracetacid

Pille:

- 1 protease inhibitor cocktail tablet (Roche Diagnostics)
- Dissolve in 1 ml Frackelton buffer

Lysis buffer:

- Frackelton buffer 1 ml
- Pille 50 µl
- 1 M dithiothreitol (DTT) 1 µl
- Pervanadate 10 µl

3.1.2 SDS PAGE (polyacrylamide gel electrophoresis)

Sample preparation

6 cm cell culture dishes are used

- Prepare lysis buffer
- Wash cells 1x with 1x PBS
- Add 120 µl lysis buffer to the cell culture dishes and distribute equally
- Scrape off cells using a plastic scraper
- Transfer lysed cell solution to an Eppendorf tube
- Centrifuge 5’ at 13.200 rpm at 4°C to remove cellular debris
- Transfer supernatant to a new tube
- Add 24µl 6x SDS sample buffer
- Incubate samples 10’ at 95°C
- Cool samples to room temperature and collect sample through centrifugation
- Use samples for SDS PAGE or store at -20°C

SDS PAGE

- Prepare minigel apparatus (0.75 cm spacers)
- Mix separation gel (values for one 10 % gel)
    - dH_2O 2,2 ml
    - Tris 1,5 M pH: 8.8 1 ml
    - 50 % AcAm 0,8 ml
    - 10 % SDS 40 µl
    - 20 % APS 12 µl
    - Temed 8 µl
- Cast gel into the apparatus leaving ~1.5 cm empty
- Overlay with isopropanol
- Wait until the gel is polymerized
- Mix separation gel
    - dH_2O 1,25 ml
    - Tris 0,5 M pH: 6,8 0,5 ml
    - 30 % AcAm 0,25 ml
    - 10 % APS 6 µl
    - Temed 4 µl
- Remove isopropanol, cast stacking gel and insert comb
- Wait until the gel is polymerized
- Place gel into electrophoresis tank
- Remove the comb
- Fill apparatus with 1x running buffer
- Load protein extracts (10-15 µl)
- Run gel with 200 V- 20 mA/gel
- Run gel until the sample buffer dye reaches the bottom

3.1.3 Western Transfer

Semi-dry blotting

- Prepare membrane (nitrocellulose) and whatman paper in appropriate size
- Prepare blot:

  -pole

  5 whatman papers (Cathode buffer)

  SDS page gel (cathode buffer)

  Nitrocellulose membrane (Anode buffer II)

  3 whatman papers (Anode buffer II)

  5 whatman papers (Anode buffer I)

  +pole
- Blot 2 h 25 V- 60 mA/gel

Ponceau staining

The proteins are detected through staining. To determine if the blotting procedure was successful and to determine where to cut the membrane without destroying protein bands, the membrane is stained with Ponceau S.

- Wash membrane with dH_2O
- Incubate membrane 1' with 2 ml Ponceau S solution
- Remove dye and wash membrane with dH_2O until the bands are clearly visible
- Completely remove the dye through washing with 1xTBST prior to blocking

Blocking

- Incubate membrane for 1.5 h with 1xTBST 15 % fat-free milk powder on shaker
- Wash membrane 3x 10' with 1xTBST

3.1.4 Antibody incubation

Primary antibody

- Incubate membrane in ~10 ml primary antibody solution (appropriate quantity of antibody diluted with 1xTBST, 1 % BSA, 0.05NaN_3) on shaker, 4°C, o/n
- Retrieve antibody solution and store at 4°C

- Wash membrane 3x 10’ with 1xTBST

Secondary antibody

- Prepare the dilution (1:20.000 in 1xTBST) of the fluorescence-labeled secondary antibody
- Incubate membrane 20’ on shaker at RT
- Wash membrane 3x 5’ with 1xTBST
- Scan membrane using the Odyssey Infrared Imaging System

Stripping

- Add stripping buffer (enough to cover the membrane)
- Incubate membrane 5’ on shaker on RT
- Wash membrane 3x 10’ with 1xTBST
- Use membrane for blocking

3.2.5 Antibodys used for western blotting

Antibody	origin	used dilution	producer
panERK	mouse	1:2000	BD
phosphoERK	rabbit	1:1000	Santa Cruz
phospho p38	rabbit	1:1000	Cell Signalling
TTP	rabbit	1:1000	Lab Kovarik
p38	rabbit	1:2000	Santa Cruz
anti-rabbit green	goat	1:2000	Rockland
anti-rabbit-red	goat	1:2000	Rockland
anti-mouse-green	goat	1:2000	Rockland
anti-mouse-red	goat	1:2000	Invitrogen

3.2 Cell culture

3.2.1 Cultivation

3.2.1.1 Medium preparation

DMEM:

- Dulbecco's Modified Eagle Medium (Dulbecco)
- 10 % fetal calf serum
- Penicillin and Streptomycin (1:1000)

DMEM +L-conditioned (L cell-derived CSF-1):

L-conditioned medium is necessary to supply the cells with macrophage colony stimulating factor. (preparation described in chapter 3.2.7)

- Dulbecco's Modified Eagle Medium
- 10 % fetal calf serum
- Penicillin and Streptomycin (1:1000)
- 15 % L-conditioned medium (production described in [79])

RPMI complete:

- RPMI medium (+glutamine) (PAA)
- 2 % fetal calf serum
- Penicillin and Streptomycin (1:1000)
- β-mercapto ethanol (1:1000 of a 50 mM solution)

Phosphate Buffered Saline (PBS)

- 80 g/l NaCl
- 2 g/l KCl
- 16,5 g/l Na_2HPO_4
- 2 g/l KH_2PO_4

1000x Penicillin/Streptomycin

- 0,6 g Penicillin
- 1 g Streptomycin
- To 1 ml ddH_2O
- Sterilize using a filter
- Store at -20°C

3.2.1.2 Growth conditions

- 37°C
- 5 % CO_2
- 95 % humidity

3.2.1.3 Cell lines

Primary bone marrow-derived macrophages (BMDM) are grown in DMEM +L-conditioned and transformed B-cells are grown in RPMI complete medium. HeLa cells are grown in DMEM in separate incubators as primary cells.

3.2.2.1 Passaging by trypsinization

- Remove medium completely
- Wash 1x with 1xPBS
- Remove residual PBS
- Add 1 ml 1xTrypsin-EDTA
- Incubate 3’ in the incubator
- Resuspend cells in appropriate volume of medium

3.2.2.2 Passaging by scraping (for macrophages)

- Remove medium leaving 1-2 ml
- Flame the scraper and completely scrape down the cells

- Resuspend the cells using a pipette and split in the appropriate ratio (measure cell density with a cell counter)
- Keep the scraper in 70 % ethanol

3.2.3 Preparation of macrophages from mouse bone marrow

- Isolate the femur and tibia (keep in cold 1xPBS on ice)
- Transfer the specimen to a cell culture bench
- Abscise the tips of the bone and flush the bone marrow with DMEM +L-conditioned medium using a syringe with a G27 needle (transfer the bone marrow of the individual animal into a single tube)
- Centrifuge the solution 5' 1.200 rpm
- Resuspend pellet in 40 ml L-conditioned medium
- Fill 4 100 cm^2 petri dishes with 5 ml DMEM +L-conditioned medium and add 10 ml cell suspension
- Incubate in the incubator
- Feed the cells approximately 3 days after isolation through addition of 5 ml DMEM +L-conditioned medium, which promotes differentiation of the precursor cells to form macrophages
- The cells can be split after 3 additional days or if enough cells have settled down.

3.2.4 Freezing bone marrow

- Isolate bone marrow as described
- Centrifuge 5' 1.400 rpm
- Resuspend pellet in 1 ml FCS, 10 % DMSO
- Transfer to cryo-tube
- Freeze and store at -80°C

3.2.5 Preparation of mouse embryonic fibroblasts (MEFs)

- Use approx. 13 days pregnant mouse
- Isolate uterus

- Isolate embryos and store them in DMEM
- Fill embryos into a syringe and separate cells by forcing the tissue through a G21 needle
- Seed cells on 10 cm dishes
- Change medium after 2 days
- Split cells 1:3 if plates are full (every 2 days at the start) for up to 2 months
- Monitor cell morphology continuously

3.2.6 LPS induction of cells

Isolated LPS from *Escherichia coli* 055:B5 used were bought from Sigma Aldrich.

- Prepare the correct LPS concentration
    - Dissolve 5 mg LPS in 5 ml 1xPBS (→1mg/ml)
    - Further dilute LPS in DMEM for later use
- Add appropriate amount of LPS to the medium of the culture dishes
- Incubate for appropriate time

3.2.7 Preparation of L cell-derived CSF-1

- Day 0:
    - Thaw L929 cells
    - Add 10 ml DMEM immediately
    - Collect cells 5' at 1.200 rpm
    - Discard supernatant and resuspend in DMEM
    - Seed cells on 10 cm cell culture dishes
    - Incubate in the incubator
- Day 2:
    - Monitor cell growth using the microscope
    - Split cells if they have grown to confluency (through trypsin)
- Day 9:
    - Split 10 confluent 10 cm cell culture dishes (ratio: 1:2)
    - Transfer 1 ml cell suspension into a 175 cm^2 tissue culture flask (in 50 ml DMEM)

- Day 10:
    - If cells are 80 % confluent remove medium
    - Add 100 ml starvation medium (DMEM without FCS or Penicillin/Streptomycin)
- Day 21:
    - Passage supernatant through a 0,22 µm vacuum-driven bottle top filter to sterilize the solution
    - Store sterile L cell-derived CSF-1 solution at -20°C

3.3 Transfection protocols

3.3.1 Nucleofection using the Amaxa kit

- Grow cells to 60-90 % conflucency
- Harvest the cells using trypsin
- Measure cell density with a cell counter
- Transfer $5x10^6$ cells into a 15 ml falcon tube
- Collect the cells through centrifugation for 5’ at 1.200 rpm
- Remove the medium
- Resuspend cells in 100 µl Amaxa reaction solution (VCA-1001 for HeLa cells)
- Add plasmid(s) in appropriate concentration (up to 5 µg) and mix carefully
- Transfer to an electroporation vessel
- Electroporate (setting T-20 for HeLa cells)
- Immediately add ~500 µl 37°C DMEM and resuspend
- Transfer cells to cell culture dish

3.3.2 Transformation of cells using the Exgen-500 reagent

- Grow cells to ~50 % confluency
- Mix appropriate amount of pasmid with 450 µl sterile DMEM
- Add 10 µl Exgen-500 reagent and mix carefully
- Incubate for 30’ at room temperature

- Add DNA-reagent mixture to the cell culture dish
- Incubate over night

3.4 Preparation of RNA

3.4.1 Preparation of RNA using TRIZOL

- Grow on 10 cm dishes and treat cells according to the experiment
- Remove medium
- Add 750 µl TRIZOL reagent
- Harvest cells using a rubber policeman
- Transfer cell solution into an endotoxin-free Eppendorf tube
- Freeze sample at -80°C until all other samples are prepared
- Thaw and add 200 µl of $CHCl_3$
- Vortex extensively and incubate 15' at RT
- Centrifuge for 15' at 12.000 rpm at 4°C
- Transfer upper phase into a new tube
- Add 500 µl isopropanol and mix gently to precipitate the nucleic acids
- Incubate 10' at RT
- Centrifuge 10' at 12.000 rpm at 4°C
- Discard supernatant and wash with 1 ml 75 % EtOH
- Vortex and centrifuge 5' at 12.000 rpm
- Remove supernatant and dry pellet 10-15'
- Resuspend pellet in 40 µl RNase free dH_2O
- Check RNA quantity with a Nano-drop apparatus or load 2 µl in 10 µl RNA sample buffer (10' 65°C) on a RNA-agarose gel

3.4.2 RNA immunoprecipitation

3.4.2.1 Buffer preparation

Gradient buffer:

- 0,2 M KCl
- 20 mM $MgCl_2$
- 20 mM Tris pH 7,4

Lysis buffer:

- 0,2 M KCl
- 20 mM $MgCl_2$
- 20 mM Tris pH 7,4
- 0,5 % NP-40
- 1 mM PMSF
- 200 u/ml Ribo-lock
- Pille (see chapter 3.1.1)

TES buffer:

- 10 mM Tris
- 0,5 mM EDTA
- 0,5 % SDS pH 8,0

3.4.1.2 RNA-IP

- Grow cells (HeLa or BMDM were used) on 15 cm dishes
- Wash cells with 1xPBS
- Add 1 ml 1xTrypsin-EDTA and incubate 3’ in the incubator
- Resuspend cells and transfer to a fresh tube
- Wash cells 3x with cold 1xPBS (3’ 4000 rpm)
- Resuspend cells in 300 µl lysis buffer
- Lyse cells by rotating 5’ at 4°C
- Centrifuge 10’ 13.200 rpm at 4°C

- Transfer 50 µl aliquot to -70°C (use for RNA preparation as input)
- Add 25 µl resuspended Protein-A Sepharose beads coated with BSA and tRNA to the remaining 250 µl lysate
- Rotate for 40’ at 4°C
- Collect beads 20’’ at maximum speed
- Transfer supernatant to a fresh tube
- Add lysis buffer up to 500 µl and add 0,8 µl antibody (prepare one duplicate per sample as mock using unspecific antibody)
- Rotate 30’ at 4°C
- Add 35 µl beads
- Rotate 45’ at 4°C
- Collect beads 20’’ at maximum speed and discard supernatant
- Wash beads 2x with lysis buffer; 1x with gradient buffer
- Resuspend beads in 200 µl TES buffer
- Incubate 15’ at 65°C
- Collect beads 20’’ at maximum speed
- Transfer supernatant to a new tube and use for RNA preparation

3.5 Quantitative RT-PCR (qRT-PCR)

3.5.1 cDNA preparation using RevertAid Reverse Transcriptase

- Mix:
    - 1 µl Oligo dT (100 pmol/µl)
    - 3-10 µg RNA (depends on concentration)
    - dH_2O to a volume of 11 µl
- Incubate 5’ at 70°C
- Add per sample:
    - 4 µl 5x reaction mix
    - 2 µl 10 mM dNTP
    - 2 µl dH_2O
- Incubate 5’ at 37°C
- Add 200 u (1 µl) RevertAid reverse transcriptase (Fermentas)

- Incubate 60’ at 42°C
- Incubate 10’ at 70°C to stop the reaction
- Store cDNA at -20°C

3.5.2 qRT-PCR using SYBR-green

- Prepare a 96 well PCR plate
- Prepare the appropriate dilution of the cDNA samples (1/10)
- Dilute one sample of cDNA for a standard curve (1/1, ½, ¼, 1/8)
- Distribute the template cDNA in the appropriate volume and concentration into the labeled wells
- Prepare the wells in duplicate or triplicate and duplicate the layout for the HPRT standard
- Prepare a master mix (n+1): final concentration
    - 5 µl Template
    - 2.5 µl 10x buffer 1x
    - 2.5 µl 25 mM $MgCl_2$ 1.5 mM
    - 0.5 µl 10 mM dNTP 200 µM
    - 0.075 µl 100 µM primer1 300 nM
    - 0.075 µl 100 µM primer2 300 nM
    - 1 µl SYBR Green (1:1000)
    - 0.2 µl Ampli Taq 1 u add at the very last moment
    - dH_2O to final volume of 25 µl

  Prepare another master mix for the housekeeping gene HPRT as an internal standard using the appropriate primers

- Create the PCR program and heat the lid
- Add the master mix
- Close the PCR plate using a sealing tape
- Transfer the plate into the qRT-PCR cycler
- Run the program
- Discard the PCR plate

3.5.2.1 qRT-PCR program

- 95°C 5:00
- 95°C 0:15
- 60°C 0:15 } 40x
- 72°C 0:20
- 95°C 0:15
- 60°C 0:15
- 60-95°C 20:00 (melting curve)
- 95°C 0:15

3.5.2.2 Primers for RT PCR

Name	Sequence	Tm [°C]
Luc RT FW	AGCTTACTGGGACGAAGACGAACA	59.9
Luc RT RV	TTGACTGGCGACGTAATCCACGAT	60.3
RbGl-RT-FW	TCCTAAGGTGAAGGCTCATGGCAA	60.3
RbGl-RT-RV	GTGGTATTTGTGAGCCAGGGCATT	59.9
Hprt Hs rt FW	TGTGTGCTCAAGGGGGGC	61
Hprt Hs rt RV	CGTGGGGTCCTTTTCACC	56.1

3.6 Luciferase Assay (Promega dual luciferase detection kit)

During this procedure, cells transfected with plasmids expressing firefly luciferase, renilla luciferase and a third plasmid depending on the experiment are used. In the luminometer, the activity of the firefly luciferase is measured first (LAR1 buffer + PLB cell extract). The firefly luciferase activity is then inhibited by the addition of the Stop and Go buffer, which also provides substate for the renilla luciferase which is measured for normalization of the firefly luciferase data (to account for differences in the transfection efficiency). In the procedure used in this thesis, the buffers are added manually and the luciferase activity is measured individually for renilla and firefly luciferase.

- Seed $5x10^6$ Amaxa transfected cells on 6 well dishes
- Incubate 24 h
- Treat cells according to the experiment

- Add 500 µl 1x Promega Passive Lysis buffer (PLB)
- Incubate 15’ on shaker
- Transfer lysate to a fresh tube
- Dilute sample with 1x PLB if necessary
- Transfer 20 µl lysate into an luminometer tube
- Adjust the luminometer (LUMAT LB 950I; measuring time 20’’)
- Add 100 µl LAR1 buffer and mix
- Measure Firefly luciferase activity
- Add 100 µl Stop and Go buffer and mix
- Measure renilla luciferase activity
- Discard sample

3.7 Transformation of B-cells

3.7.1 *v-abl* transformation of B-cells

- Prepare Bone Marrow as described in M&M 3.2.3
- Prepare virus
    - 1 ml *v-abl* viral supernatant (A-MuLV (Abelson murine leukemia virus) containing the *v-abl* oncogene obtained from 54C12 producer cells)
    - 1:1000 2-Mercaptoethanol (stock 50 mM)
    - 1:1000 IL7 (stock 10 µg/ml)
    - 1:1000 Polybrene (hexadimethrine bromide; stock 10 mg/ml)
- Prepare Mock (RPMI complete instead of viral supernatant)
- Collect cells 5’ 1.000 rpm
- Resuspend one aliquot in 1 ml virus and the other in 1 ml Mock
- Incubate 1 h at 37°C, vortex cells every 10’ for 10’’
- Collect cells 5’ 1.000 rpm
- Resuspend in 3 ml RPMI complete +1:1000 IL7
- Incubate in 6 well dishes for 12 days

3.7.2 Colony formation Assay

- Use *v-abl* transformed cells (16 h after infection)
- Resuspend cells
- Take 750 µl aliquot
- Collect cells 5' 1.000 rpm
- Resuspend cells in 500 µl RPMI
- Count cells using counting chamber
- Transfer $1x10^6$ cells into a sterile FACS tube with 800 µl RPMI complete
- Add 3,8 ml methylcellulose medium (highly viscous; no antibiotics added)
- Carefully pipette up and down approx. 20 times without causing any bubbles
- Pipette 2 ml cell suspension into 4cm dishes
- Transfer dishes into one 15 cm dish with one uncovered 1xPBS filled dish in the middle
- Incubate dishes in the incubator until colonies are visible (approximately after 12 days)
- Count colonies

3.8 *In-vivo* experiments

3.8.1 Preparation of cells

- Use stable *v-abl* transformed B-cells
- Grow cells in 10 cm dishes in RPMI complete
- Resuspend and transfer cells to a 50 ml Falcon tube
- Collect cells 10' 800 rpm
- Resuspend cells in 10 ml RPMI complete medium
- Count cells using a cell counter (diluted 1:10) and measure live cell percentage
- Transfer appropriate amount into 15 ml Falcon tube
- Collect cells 5' 1.000 rpm (is cell viability >90 %) or 10' 800 rpm
- Resuspend in appropriate volume of sterile, physiological NaCl solution
- Count cells (diluted 1:20 to minimize loss)
- Calculate volume to be injected to achieve desired cell count

3.8.2 Subcutaneous injections

- Shave necessary part of the mouse (tibia or abdomen)
- Prepare cells in a syringe with a G27 needle ($5x10^5$ to $5x10^6$ depending on experiment)
- Wash the affected area with 70 % EtOH
- Carefully insert the needle just puncturing the skin, lift the needle and move the needle just under the skin
- Slowly inject the desired volume
- Slowly pull out the needle to minimize the volume of exiting cell suspension
- Incubate mice for 7-14 days in sealed cages

3.9 FACS

3.9.1 Preparation of cells for analysis

Blood:

- Bleed mice through the eye (approx 250 µl blood)
- Collect blood in EDTA coated tubes (MiniCollect 0,25 ml K3E K3EEDTA) and vortex
- Store blood samples on room temperature for short periods
- Transfer 50 µl blood to a new tube
- Add 3 ml sterile filtered erythrocyte lysis buffer
- Incubate 5’ at RT
- Add 1 ml RPMI complete
- Collect cells 5’ 1.000 rpm
- Resuspend in 500 µl 1xPBS
- Use 50 µl for FACS

Lymph nodes:

- Isolate lymph nodes from the knee cavity

- Disrupt tissue by grinding it through a 70 µm filter using a plunger
- Add 500 µl RPMI complete and transfer cell suspension to a new tube
- Collect cells 5’ 1.000 rpm
- Resuspend cells in 500 µl 1xPBS
- Use 50 µl for FACS

Spleen:

- Isolate spleen
- Disrupt tissue by grinding it through a 70 µm filter using a plunger
- Add 500 µl RPMI complete and transfer cell suspension to a new tube
- Collect cells 5’ 1.000 rpm
- Resuspend cells in 1 ml sterile filtered erythrocyte lysis buffer
- Incubate 5’ at RT
- Collect cells 5’ 1.000 rpm
- Resuspend cells in 1 ml PBS
- Use 50 µl for FACS

Bone Marrow:

- Isolate the femur and tibia (keep in cold 1xPBS on ice)
- Remove the tips of the bone and move the bone marrow into RPMI complete medium using a medium-filled syringe with a G27 needle
- Centrifuge the solution 5’ 1.000 rpm
- Resuspend pellet in 1 ml RPMI complete
- Transfer a 50 µl aliquot into a fresh tube for further FACS procedure
- Collect 5’ 1.000 rpm
- Resuspend cells in 1 ml sterile filtered erythrocyte lysis buffer
- Incubate 5’ at RT
- Collect cells 5’ 1.000 rpm
- Resuspend cells in 500 µl 1xPBS
- Use 50 µl for FACS

The erythrocyte lysis step can introduce discrepancies between independent experiments as slight differences in incubation time either led to too many remaining erythrocytes influencing the measurements or led to lysis of other cells.

3.9.2 Preparation of staining master mixes for B-cell differentiation analysis

Staining of the samples was performed using the following antibodies in a total volume of 50 µl in 1xPBS. Staining is performed in 2 separate master mixes as the number of fluorescence-dyes is limited. (Cell populations are further explained in Results 4.4.2)

Staining for cell populations A-C:

Antibody	**Fluorescent dye**	**volume [µl]**
CD19	FITC	0,5
B220	PerCP-Cy5.5	0,7
CD43	PE	0,5
BP-1	APC-Cy7	0,5
Fc-Block	-	0,5

Staining for cell populations D-F:

Antibody	**Fluorescent dye**	**volume [µ]**
B220	PerCP-Cy5.5	0,7
CD43	PE	0,5
IgD	APC-Cy7	0,5
IgM	FITC	0,5
Fc-Block	-	0,5

3.9.3 Preparation of staining master mixes for cell linage analysis

Staining of the samples was performed using the following antibodies in a total volume of 50 µl in 1xPBS.

Staining for granulocytes and macrophages:

Antibody	**Fluorescent dye**	**volume [µ]**
CD19	FITC	0,5
CD3	PerCP-Cy5.5	0,7
Mac-1	PE	0,5
GR-1	APC	0,5
Fc-block	-	0,5

Staining for T-cells and B-cells:

Antibody	Fluorescent dye	volume [µ]
CD3	PerCP-Cy5.5	0,5
CD4	PE	0,5
CD8	APC	0,5
Fc-block	-	0,5

3.9.3.1 FACS antibodies: (ordered from Beckton Dickinson)

Antibody	Dilution	Source
Fc-block	1:100	BD 553142
CD19-FITC	1:100	BD 553785
CD43-PE	1:100	BD 553271
BP-1-biotin	1:100	BD 553159
SAV-APC	1:100	BD 554067
B220-PerCP	1:65	BD 552771
IgD-biotin	1:100	BD 553509
IgM-FITC	1:100	BD 553408

3.9.4 Preparation of FACS samples

- Transfer 50 µl cell suspension in 1xPBS into FACS tubes (for all samples)
- Prepare a mixture of the spleen and bone marrow cells of multiple samples for single staining (total of 50 µl per staining)
- Wash samples with 2 ml 1xPBS
- Collect cells 5’ 1.000 rpm
- Discard supernatant (approx. 50 µl supernatant remaining)
- Prepare staining solution master mix according to experiment
- Add 50 µl master mix to the samples (perform single staining)
- Vortex samples
- Incubate 20’ at 4°C without light
- Collect cells 5’ 1.000 rpm
- Resuspend in approx. 300 µl 1xPBS
- Vortex and measure samples with FACScan and FACScanto (Beckton-Dickinson) and evaluate the data using CellQuestPro and FACS Diva software

3.10 Cloning methods

3.10.1.1 Media and Plates

Lysogeny broth (LB)-medium:

- 10 g/l Peptone
- 10 g/l NaCl
- 5 g/l Yeast extract
- Adjust to pH 7 (important for correct function of certain antibiotics)

LB-Agar:

The medium is prepared, autoclaved and cooled down to approx. 50°C. Ampicillin is added to a concentration of either 50 or 100 µg/ml. The medium is poured into 10 cm Petri-dishes and allowed to harden. The dishes can be stored at 4°C for up to 6 weeks.

- 10 g/l Peptone
- 10 g/l NaCl
- 5 g/l Yeast extract
- 15 g/l Agar
- Adjust to pH 7

Ampicillin (stock):

- 100 mg/ml Ampicillin (Merck) in dH_2O
- Aliquots are stored at -20°C

Tetracycline (stock):

- 1 mg/ml Tetracycline (Serva) in dH_2O
- Aliquots are stored at -20°C

3.10.1.2 Buffer preparation

TE buffer:

- 10 mM Tris-HCl pH 7,5
- 1 mM EDTA

TENS buffer:

- Prepare fresh directly before use
- 28,95 ml TE buffer
- 300 µl 10 N NaOH
- 750 µl 20 % SDS

3M NaAc pH 5.5

- 24,6 g NaAc
- 100 ml dH_2O
- Adjust to pH 5,2 with CH_3COOH

TAE buffer (50x):

- 57,1 ml CH_3COOH
- 242 g Tris base
- 100 ml EDTA 0,5 M
- To 1 l dH_2O

3.10.2 PCR for cloning

- Thaw plasmid, which is stored at -20°C
- Prepare reaction mix for Pwo Polymerase (Roche)
    - Reaction mix I
        - Template cDNA 5 µl
        - dH_2O 6.4 µl
        - primer 100 pM 0.3 µl each
        - dNTPs 10 mM 0,5 µl
    - Reaction mix II
        - Pwo polymerase buffer 2.5 µl

- $MgSO_4$ 2.5 µl
- dH_2O 7.2 µl
- Pwo Polymerase 0.3 µl

- Mix the final reaction solution
- Use the specific annealing temperature for each primer pair
- Run the reaction:
  - 95°C 3:00
  - 95°C 0:45
  - X°C 0:45
  - 72°C 5:00

  } 35x
  - 72°C 7:00
- Add 6x Sample buffer and load on a 1 % agarose gel
- Run at 100 V
- Cut out the DNA fragments and store at -20°C

3.10.2.1 Primers for cloning

Underlined sequences contain the specific restriction site used for the cloning.

Name	Sequence	Tm [°C]
IL1a3UTRPflMIFW2	TTTTTTCCAACCTATGGGCAGCCTTATTTCGGGAGTCTA	56.6
IL1a3UTRStyIRV2	TTTTGTCCTTGGGTTGATAGTTACATGACACTGTGG	53.5
IL63UTRPflMIfw	TTCCAACCTATGGGCGTTATGCCTAAGCATATCAG	53.7
IL63UTRStyIrv	AACCTTGGTTTGTTTGAAGACAGTCTAAACAT	51.0
IL12b3UTRPflMIfw	TTCCAACCTATGGGCAACGTTGGAAAGGAAAGAAAAG	55.4
IL12b3UTRPflMIrv	TTCCATAGGTTGGGAGACAGAATTTCTGTGTGGCAC	56.2
Cxcl23UTRPflMIFW	TTTTTTCCAACCTATGGGAAAGGAGGAGCCTGGGCTG	60.4
Cxcl23UTRPflMIRV	TTTTTTCCATAGGTTGGCATGAATAAATAAATGTGTCCACTTC	51.0
TNFa3UTRPflMlFW2	TTTTTTCCAACCTATGGAGGGAATGGGTGTTCATCCAT	56.3
TNFa3UTRStylRV2	TTTTGTCCTTGGATTTCTCTCAATGACCCGTAG	51.9
Hprt3´UTRPflM1FW	TTTTTTCCAACCTATGGTGAGCGCAAGTTGAATCTGCA	57.6
Hprt3´UTRSty1RV	TTTTGTCCTTGGATTTAAAAGGAACTGTTGACAACG	52.3
bGl3´UTRPflM1FW	TTTTTTCCAACCTATGGGCCCCTTTTCTGCTATTGTCT	55.2
bGl3´UTRSty1RV	TTTTGTCCTTGGGCTAGATGCCCAAAGGTCTTC	55.7
RbGl-IL1 FW	TTTTTTGGATCCGCAGCCTTATTTCGGGAGTCTA	56.6
RbGl-IL1 RV	TTTTTTAGATCTGTTGATAGTTACATGACACTGTGG	53.5
RbGl-IL6 FW	TTTTTTGGATCCGCGTTATGCCTAAGCATATCAG	53.7
RbGl-IL6 RV	TTTTTTAGATCTTTTGTTTGAAGACAGTCTAAACAT	51.0
RbGl-Cxcl2 FW	TTTTTTGGATCCGAAAGGAGGAGCCTGGGCTG	60.4

RbGl-Cxcl2 RV	TTTTTTAGATCTCATGAATAAATAAATGTGTCCACTTC	51.0
RbGl-HPRT FW	TTTTTTGGATCCTGAGCGCAAGTTGAATCTGCA	57.6
RbGl-HPRT RV	TTTTTTAGATCTATTTAAAAGGAACTGTTGACAACG	52.3

3.10.3 DNA agarose gel electrophoresis

- Dilute 1 g agarose in 100 ml TAE buffer (1 % gel for 1-8 kb size)
- Melt and completely dissolve the agarose in a microwave oven
- Cool solution to ~50°C
- Prepare container
- Add 6 µl ethidium bromide (5 mg/ml)
- Cast gel in the container and add the comb
- Wait until the gel is completely solidified

3.10.4 Elution of DNA from an agarose gel using the Fermentas kit

- The DNA is eluted from the gel using the Fermentas kit according to the manufacturer's protocol.

3.10.5 DNA precipitation

DNA precipitation is used to remove buffers or to achieve higher concentrations of DNA for subsequent reactions.

- Adjust DNA solution to a salt concentration of 0,3 M using 3M NaAc pH5,5
- Add 3 volumes of 2-propanol (in minimal volume of 500 µl)
- Mix and incubate 2 h at -20°C (longer incubation periods reduce loss of DNA)
- Centrifuge 10' 13.200 rpm at 4°C
- Wash pellet 1x with 1 ml 96 % EtOH
- Centrifuge 2' 13.200 rpm at 4°C
- Remove supernatant completely
- Let the pellet dry for 10-15' with open cap
- Resuspend pellet in appropriate volume dH_2O

3.10.6 Digestion of plasmids

- Prepare reaction mix:
    - 40 µl plasmid (5 µg)
    - 5 µl buffer
    - 4 µl enzyme
    - 1 µl dH_2O
- Incubate o/n at 37°C
- Load digestions onto a 1 % agarose gel to remove buffer
- Cut out bands and elute
- Use digested plasmid for 2nd digestion using adjusted volumes, enzyme and buffer
- Store DNA at -20°C

3.10.7 Dephosphorylation of DNA

Vector DNA dephosphorylation is used to prevent vector recircularization during the ligation step when either blunt-end ligation is necessary or both insert and vector are digested with the same enzyme.

- Prepare reaction mix:
    - 40 µl plasmid (5 µg)
    - 5 µl CIAP buffer (or Fermentas buffer R+)
    - 1 µl Calf Intestinal Alkaline Phosphatase (CIAP)
    - 4 µl dH_2O
- Incubate 1 h at 37°C (longer incubation reduces ligation yield significantly)

3.10.8 Ligation of DNA fragments with a linearized vector

- Concentration of digested insert and vector DNA is measured through a Nano-drop (Thermo Scientific) apparatus
- Prepare reaction mix:
    - 50 ng vector DNA
    - 150 ng insert DNA

    } Adjust ratio to 1:9 if necessary
    - 2 µl buffer containing ATP

    - 1 µl viral T4 ligase
    - Add dH_2O to 20 µl
- Prepare controls
    - Without ligase and fragment
    - Without fragment
- Incubate o/n at 16°C

3.10.8.1 Primers used directly for ligation

Underlined sequences highlight the sticky ends used for the ligation reaction. The primers were annealed and diluted in dH_2O prior to their use.

Name	Sequence
TNFa core ARE FW	ATGGTTATTTATTTATTATTTATTTATTTC
TNFa core ARE RV	CAAGGAAATAAATAAATAATAAATAAATAACCATAGG
Random Oligo FW	ATGGCCGCCCGCCCGCCGCCCGCCCGCCCC
Random Oligo RV	CAAGGGGGCGGGCGGGCGGCGGGCGGGCGGCCATAGG

3.10.9. Heat shock transformation

3.10.9.1 Preparation of competent E-coli

- Inoculate colonies from XL-1 blue E-coli in 50 ml LB medium without antibiotics
- Incubate over night
- Inoculate 500 ml LB medium with 10 ml overnight culture
- Incubate at 37°C and measure optical density frequently against fresh LB medium
- Grow culture to an OD of 0,2
- Centrifuge 10’ 5.500 rpm at 4°C
- Resuspend pellet in 250 ml ice cold, sterile 0,1 M $CaCl_2$
- Incubate 30’ at 4°C
- Centrifuge 10’ 5.500 rpm at 4°C
- Remove supernatant completely
- Resuspend pellet in 5 ml ice cold, sterile 0,1 M $CaCl_2$/20 % Glycerol
- Use competent cells fresh for highest yield

- Prepare aliquots in pre-cooled tubes and immediately freeze using liquid nitrogen
- Use competent cells for control transfections and negative controls
- Store competent cells at -70°C

3.10.9.2 Heat-shock transformation of competent E-coli

- Thaw competent cells on ice
- Mix 10 µl ligation mix (1 µl if a purified plasmid is used) with 100 µl competent cells
- Incubate on ice for 30'
- Heat shock 2' on 42°C
- Incubate on ice for 5'
- Add 1 ml LB medium
- Incubate 50' at 37°C on a shaker
- Plate 200 µl on LB plates with the selective antibiotic
- Concentrate through centrifugation (6.000 rpm for 3'- partially remove supernatant)
- Resuspend pellet in the remaining ~150 µl medium
- Plate medium on LB plates with the selective antibiotic
- Incubate o/n at 37°C

3.10.10 Plasmid preparations

3.10.10.1 Plasmid Mini-Prep using the 5-prime Mini-Prep kit

Buffers and columns are provided by the PerfectPrep^{tm} Spin Mini Kit

- Pick colonies from a LB plate
- Inoculate 8-50 ml LB medium with the selective antibiotic
- Incubate o/n on a shaker at 37°C
- Add 500 µl buffer BL to a CB3 spin column to equilibrate
- Spin down 6 ml bacterial solution (3x in 2 ml Eppendorf tube) 4' at 3.000 rpm
- Discard supernatant
- Resuspend in 250 µl buffer PR1
- Add 250 µl PL2 (lysis) buffer –invert 6x

- Add 350 µl PN3 (neutralization) buffer –invert 6x
- Centrifuge 10’ at 12.000 rpm
- Transfer supernatant to a filter spin column
- Centrifuge 2’ at 12.000 rpm
- Transfer filtered solution to a CB3 Spin column
- Centrifuge 1’ at 12.000 rpm
- Add 500 µl buffer PD (desalting solution)
- Centrifuge 1’ at 12.000 rpm
- Add 700 µl buffer PW (washing solution)
- Centrifuge 1’at 12.000 rpm
- Add 500 µl buffer PW
- Centrifuge 1’at 12.000 rpm
- Centrifuge 1’ at 12.000 rpm to remove residual buffer
- Let columns dry for 2’ at RT
- Incubate columns with 50 µl dH_2O for 2’
- Centrifuge 2’ 12.000 rpm
- Measure concentration using the Nano-drop apparatus
- Store plasmids at -20°C

3.10.10.2 Quick gram negative plasmid preparation

This protocol was used for quick and inexpensive preparation of plasmid DNA from a large quantity of clones. The resulting quality of the plasmid DNA is sufficient for analytic digestions and transformation of bacteria but not for sequencing.

- Use 6 ml overnight cultures
- Collect 4 ml medium (2x2 ml Eppendorf tube) 4’ at 3.000 rpm
- Resuspend in 50 µl residual LB medium
- Add 300 µl TENS buffer and vortex
- Add 150 µl 3 M NaAc pH 5,2 and vortex
- Centrifuge 2’ 13.200 rpm
- Transfer supernatant to a fresh tube
- Add 900 µl 96 % EtOH (cooled to -20°C) mix by inverting the tube
- Centrifuge 2’ 13.200 rpm

- Remove supernatant
- Wash pellet 2x with 1 ml 70 % EtOH
- Dry pellet (incubate 2’ 65°C with open cap)
- Solve in 30 µl RNase-containing dH_2O (10 µg/ml)
- Use plasmid DNA for analytic digestion

3.10.10.3 Plasmid Maxi-Prep using the 5-prime Maxi-Prep Kit

This method was used when large quantities of plasmid were required (e.g. to amplify a finished vector). Plasmids were prepared according to the Protocol provided by the 5-prime Maxi-Prep Kit.

3.10.11 Sequencing of plasmid DNA

The isolated plasmids with confirmed restriction digestion pattern were sent to Eurofins / MWG operon for sequencing using specific primers:

Name	Sequence	Tm [°C]
SeqpGL2bas_1 FW	GTGGTGTAAATAGCAAAGCAAGC	55.0
SeqpGL2bas_2 RV	ACCTTACTTCTGTGGTGTGAC	54.4
SeqIL1_1 RV	AAGCACACTCAATGCATTTGG	54.4
SeqIL1_2 RV	ATGGAACATCCTTAAATCCTCTGA	53.7
SeqIL12 RV	TTGAATATTTCATGTGCTCGTGGC	56.2
SeqCXCL2 RV	TTGGGAACTAGCTACATCCCA	55.4
SeqTNFa RV	TGCCTCTGTCTCAGAATGAGG	56.3
SeqRbGl FW	CCTGGGCAACGTGCTGGTTATTGT	62.0
SeqRbGl RV	CCCATATGTCCTTCCGAGTGAGAG	58.4

3.11 Genotyping

3.11.1 Preparation of DNA from mouse tails

- Tails are supplied by the technician
- Add 200 µl Direct PCR Lysis Reagent (Tail) containing 10 µl Proteinase K (20 mg/ml)
- Incubate 3 h-over night 55°C on shaker
- Incubate 45’ at 85°C
- Centrifuge 10’’
- Store at -20°C

3.11.2 Preparation of DNA from mouse hair

- Hair is supplied by the technician
- Add 500 µl Hom-buffer +2 µl Proteinase K (20 mg/ml)
- Incubate 2 h at 60°C
- Vortex, spin down
- Transfer supernatant to an Eppendorf tube
- Add 200 µl $NaCl_2$ +700 µl CIA (Chloroform: Isoamylalcohol 24:1)
- Incubate 10’ on shaker
- Centrifuge 10’ at 14.000 rpm
- Transfer 500 µl of the upper-phase to another tube
- Add 1vol. Isopropanol to precipitate DNA
- Incubate 10’ at RT
- Centrifuge 10’ at 14.000 rpm
- Wash pellet with 70 % ETOH
- Dry completely and resuspend in 25 µl dH_2O
- Store at -20°C

3.12 Immunofluorescence

- Prepare 6-well cell culture dishes with sterile cover slips inside
- Seed cells in cover slips, treat and grow cells according to the experiment
- Add 1 % Formaldehyde (55 µl of 36,5 % stock in 2 ml medium) to fix the cells
- Incubate cells 5’ on shaker
- Discard medium
- Permeabilize cells by adding 2 ml 1xTBST +1 % Triton X-100
- Incubate 5’ on shaker
- Wash 3x 5’ with 1xTBST
- Place cover slips upside down on the inverted lid of the 6-well dish onto a 30 µl drop of primary antibody solution
- Incubate 1 h at RT in wet storage
- Place the cover slip back into the 6-well dish
- Wash 3x 5’ with 1xTBST
- Put cover slip upside down on 30 µl of secondary antibody
- Incubate 30’ at RT in the dark
- Wash the cover slip 3x 5’ with 1xTBST in the 6-well dish in the dark
- Put the slip upside down onto a drop of moviol on a microscope slide

3.12.1 Antibodies for immunofluorescence

Antibody	origin	used dilution	Source
TTP	rabbit	1:2000	Lab Kovarik
anti-Flag	mouse	1:2000	Sigma
anti-mouse	goat	1:2000	Invitrogen
anti rabbit	goat	1:2000	Molecular probes

3.13 Plasmid list

Plasmid	Resistance	Source
pGL2-basic	Ampicillin	Promega
pTRE-tight	Ampicillin	Lab Beug
pGL2-TRE	Ampicillin	This study
pGL2-TRE-IL1a	Ampicillin	This study
pGL2-TRE-IL6	Ampicillin	This study
pGL2-TRE-IL12	Ampicillin	This study
pGL2-TRE-Cxcl2	Ampicillin	This study
pGL2-TRE-TNFa	Ampicillin	This study
pGL2-TRE-bGlo	Ampicillin	This study
pGL2-TRE-HPRT	Ampicillin	This study
pGL2-TRE-5´ARE	Ampicillin	This study
pRNL-NULL	Ampicillin	Vet. Med.
pRNL-CMV	Ampicillin	Vet. Med.
pCMV-TTP-Flag	Neomycin	Lab Kovarik
pTet-BBB	Ampicillin	Lab Bohjanen
pTet-BBB-IL2	Ampicillin	Lab Bohjanen
pRbGl-IL1	Ampicillin	This study
pRbGl-IL6	Ampicillin	This study
pRbGl-Cxcl2	Ampicillin	This study
pRbGl-TNFa	Ampicillin	This study
pCMV-Cre	Ampicillin	Vet. Med.

4. RESULTS

4.1 Analysis of the target candidates

In the search for novel mRNA targets for mediation of decay by TTP, several target candidates were discovered by means of RNA immunoprecipitation (Kratochvill et. al. 2010 manuscript submitted). This RNA-IP showed the ability of TTP to bind to the mRNA of IL1α (Interleukin 1 alpha), IL6, IL12β and Cxcl2 (chemokine (C-X-C motif) ligand2). The candidates were further evaluated by measuring their mRNA decay in murine bone marrow-derived macrophages (BMDM) of WT or TTP knock-out origin stimulated with LPS. Decay was measured by termination of transcription via actinomycin-D, pharmacological inhibition of p38 MAPK using SB203580 and subsequent preparation of RNA for qRT-PCR after various time points.

The p38 MAPK inhibitor SB203580 was used to relieve the inhibitory effect of p38 MAPK on TTP (see Introduction 2.3.3). This step was made necessary as the constant stimulation with LPS led to phosphorylated (inactive) TTP. In this experiment, the target candidates showed significant TTP-mediated decay indicating that they are indeed target mRNAs of TTP.

As a first step in the process of target verification, we looked for the minimal ARE, the AUUUA pentamer, in the 3´ UTR of the target candidates which we obtained from the Ensembl database (http://www.ensembl.org/). Additionally, we looked for the heptamer UAUUUAU showing significantly higher binding affinity to TTP, and the (A/U)UAUUUAU(A/U) nonamer that represents the ideal TTP binding site [80].

GCAGCCTTATTTCGGGAGTCTATTCACTTGGGAAGTGCTGACAGTCTGTATGT
ACCATGTACAGGAACCTTCCTCACCCTGAGTCACTTGCACAGCATGTGCTGA
GTCTCTGTAATTCTAAATGAATGTTTACCCTCTTTGTAAGAGAAGAGCAAACC
CTAGTGGAGCCACCCCGACATATGATACTATCTGTTATTTTAAAGAGTACCCTA
TAGTTTGCTCAGTACTAATCATTTTAATTACTATTCTGCATGGCATTCTTAGGA
GGATCAAAAAGACTCTACACATATTACAGATGGGTTAACAAAGGGATAAAAC
AACTGAAAAGCACACTCAATGCATTTGGAATATAAATTCACAGACCAATCTC
ACTGTGCACCTTCGGCTTCAAAATGCCAGTTGAGTAGGATAAAGGTATAAGA
ACTTAATGCTGTCATTTTCAAAAGGAAGGGGACAATAGCTACATCTTTCCTAC
CTCAGTGGGTTTTACTCCAGTGAGATCATTTGGATGAAATCCTCCTGTAACAG
ACCTCAAGAAGGAGACAGACTGTTGAATGTTATTTTTAAGTTATTTTATCTAT
GT**ATTTA**TAAATAT**ATTTA**TGATAATTATATT**ATTTA**TGGAACATCCTTAAATCC
TCTGAGCTTGACGGCACCCTCGCAGCAGGGTTTTCTAGGTGGTCAGTTAGAT
GTAGTCTCCTCTAGAGCTCCATGCTACAGACTTTTACACTTTTTCCACAGCCA
CGAAGCTCTCCGTACATTCCTGCACTTGGGAGCCCTTTCATCATGATCTTAAT
CTGTGCTGTTTACTTTGTGCATCTAAAATGATAATTGAGTCAGTCTTTCTCCCT
CCCGTCCTTAAAGCTGTCTGGGTATTCTTACATCATTCAGTCTCACCTGTAACT
AACACCAACCATCTAAAGATGGAAAGAGCTTAACTGTGACAACCACATCACT
GATACCTGAAGTTTCTTTTCTAGAATGTAATCAGTGTTTCCCCTGGATTCCAAT
TTTTTTTTCAAACCACAGTGTCATGTAACTATCAAC

Fig 4: 3´ UTR of IL1α. Three UAUUUAU heptamers (underlined) can be found in the 3´ UTR

TGCGTTATGCCTAAGCATATCAGTTTGTGGACATTCCTCACTGTGGTCAGAAA
ATATATCCTGTTGTCAGGTATCTGACTTATGTTGTTCTCTACGAAGAACTGACA
ATATGAATGTTGGGACACTATTTTAATTATTTTTA**ATTTA**TTGATA**ATTTA**AATA
AGTAAACTTTAAGTTA**ATTTA**TGATTGATT**ATTTA**TTATTTTTATGAAGTGTCAC
TTGAAATGTTATATGTTATAGTTTTGAAATGATAACCTAAAAATCTATTTGATAT
AAATATTCTGTTACCTAGCCAGATGGTTTCTTGGAATGTATAAGTTTACCTCAA
TGAATTGCTA**ATTTA**AATATGTTTTTAAAGAAATCTTTGTGATGTATTTTTATAA
TGTTTAGACTGTCTTCAAACAAATAAATTATATTATATTT

Fig 5: The 3´ UTR of IL6 contains 4 AUUUA pentamers (bold) and one AUAUUUAUU nonamer (waving underline)

GATGCAACGTTGGAAAGGAAAGAAAAGTGGAAGACATTAAGGAAGAAAA**A**
TTTAAACTCAGGATGGAAGAGTCCCCCAAAAGCTGTCTTCTGCTTGGTTGGC
TTTTTCCAGTTTTCCTAAGTTCATCATGACACCTTTGCTGATTTCTACATGTAA
ATGTTAAATGCCCGCAGAGCCAGGGAGCTAATGTATGCATAGATATTCTAGCA
TTCCACTTGGCCTTATGCTGTTGAAAT**ATTTA**AGTA**ATTTA**TGT**ATTTA**TTA**AT**
TTATTTCTGCATTTCACATTTGTATACCAAGATGTATTGAATATTTCATGTGCTC
GTGGCCTGATCCACTGGGACCAGGCCCTATTATGCAAATTGTGAGCTTGTTAT
CTTCTTCAACAGCTCTTCAATCAGGGCTGCGTAGGTACATTAGCTTTTGTGAC
AACCAATAAGAACATAATATTCTGACACAAGCAGTGTTACATATTTGTGACCA
GTAAAGACATAGGTGGTATTTGGAGACATGAAGAAGCTGTAAAGTTGACTCT
GAAGAGTTTAGCACTAGTTTCAACACCAAGAAAGACTTTTTAGAAGTGATAT
TGATAAGAAACCAGGGCCTTCTTTAGAAGGGTACCTAA**ATTTA**AAAGAATTT
TGAAAGGCTGGGTATCGGTGGTATATGCTTTTAATTCCAGCACTCAGGAGACC
AAGGCAGGCAGATCTCTGTGAGTTTGAGGACAGCCTGGTGTACAGAGGGAG
TTCCAGCACAGCCAGTGCCACACAGAAATTCTGTCTC

Fig 6: The 3´ UTR of IL12β contains 5 AUUUA pentamers (minimal AREs; bold) and one UAUUUAU heptamer (underlined).

GAAAGGAGGAGCCTGGGCTGCTGTCCCTCAACGGAAGAACCAAAGAGAAA
GAAAAAAACAAACAGCACCCGGGAAGCCTGGATCGTACCTGATGTGCCTCG
CTGTCTGAGAGTTCACTT**ATTTA**TTTATCTATGT**ATTTATTTATTTA**TTAATTC
CATTGCCCAGATGTTGTTATGTTTATTTTGAT**ATTTA**AAGATATGCATTCGCTAA
TTCACTGTAATATCTTAAAAGGTCATTTTAATATGTTAAAGTTTATTTTAATAAT
GTTTAATGTGTTCAATTAAAGTT**ATTTA**ACTTATATAGTTGGAAGGTGATAAAT
TTTTAAACCT**ATTTA**TTCATTAGTTTCTGGGGAGAGGGTGAGTTGGGAACTA
GCTACATCCCACCCACACAGTGAAAGAGACTGGGGATAAGGGGTGGGGGTG
GGGACAAATAGATGCAGTCGGATGGCTTTCATGGAAGGAGTGTGCATGTTCA
CATCATTTTTTTGTAAGCACCGAGGAGAGTAGAACAGCTGTT**ATTTA**GGTTTC
AGTGTTTGTAAACTGTATGTACAACATTTTTGATGCTGGATTTCAATGTAATGT
TGTGAGTAACCCTTGGACATTTTATGTCTTCCTCGTAAGGCACAGTGCCTTGC
TTAGCAATTGTTTTGTCATGCCTTTTCGTGTCTTGAAGTGGACAC**ATTTA**TTTA
TTCATG

Fig 7: The 3´ UTR of Cxcl2 contains 3 AUUUA pentamers (bold) one UAUUUAU heptamer (underlined), one independent UUAUUUAUU nonamer (waving underline) and one overlapping class II ARE containing three AUUUA entamers. Among the target candidates, the 3´ UTR of Cxcl2 shows the highest potential to bind to TTP.

AGGGAATGGGTGTTCATCCATTCTCTACCCAGCCCCCACTCTGACCCCTTTAC
TCTACCCCTTTATTGTCTACTCCTCAGAGCCCCCAGTCTGTATCCTTCTAACTT
AGAAAGGGATTATGGCTCAGGGTCCAACTCTGTGCTCAGAGCTTTCAACAAC
TACTCAGAAACACAAGATGCTGGGACAGTGACCTGGACTGTGGGCCTCTCAT
GCACCACCATCAAGGACTCAAATGGGCTTTCCGAATTCACTGGAGCCTCGAA
TGTCCATTCCTGAGTTCTGCAAAGGGAGAGTGGTCAGGTTGCCTCTGTCTCA
GAATGAGGCTGGATAAGATCTCAGGCCTTCCTACCTTCAGACCTTTCCAGATT
CTTCCCTGAGGTGCAATGCACAGCCTTCCTCACAGAGCCAGCCCCCCTCT**ATTTA**TATTTGCACTTATT**ATTTATTTATTTATTTATTTATTTATTTATTTA**TTTGCTTATGA
ATGT**ATTTA**TTTGGAAGGCCGGGGTGTCCTGGAGGACCCAGTGTGGGAAGC
TGTCTTCAGACAGACATGTTTTCTGTGAAAACGGAGCTGAGCTGTCCCCACC
TGGCCTCTCTACCTTGTTGCCTCCTCTTTTGCTTATGTTTAAAACAAAAT**ATTTA**TCTAACCCAATTGTCTTAATAACGCTGATTTGGTGACCAGGCTGTCGCTACA
TCACTGAACCTCTGCTCCCCACGGGAGCCGTGACTGTAATCGCCCTACGGGT
CATTGAGAGAAAT

Fig 8: 3´ UTR of TNFα. The 3´ UTR of TTPs major target TNFα contains 3 UAUUUAU heptamers (underlined) and one extended, overlapping class II ARE (waving underline) containing 5 AUUUA pentamers.

The analysis of the 3´ UTRs of the target candidates showed that while all target candidates contained at least one class 1 ARE, only Cxcl2 displayed a class 2 ARE similar to the pronounced overlapping ARE of the established TTP target mRNA TNFα.

Although the RNA IPs and mRNA decay studies provided a good indication that mRNAs of IL6, Cxcl2 and IL1α were TTP targets for the following reasons, further experiments were needed to confirm the data: 1) RNA IP may give positive results even in case of an indirect interaction of TTP with RNA, 2) mRNA decay assays employing transcriptional stop by act-D introduce a very artificial situation of a complete transcriptional blockage in the cells, and 3) these assays do not address the role of 3´ UTR. To clarify these issues, we prepared a reporter system to show the TTP-mediated decay of the target candidates. We chose a tetracycline-dependent transcriptional regulation of the reporter as this system allows for selective transcriptional termination compared to the total termination of transcription of the actinomycin-D-treatment. In this system, the reporter gene, either firefly luciferase or rabbit β-globin, is regulated by a tetracycline responsive promoter. The reporter gene is fused to the complete 3´ UTR of the suspected TTP targets. The vector is transfected in a HeLa Tet-Off cell line (Clontech) which expresses a tetracycline-dependent transcriptional activator that can only bind the tetracycline responsive promoter in the absence of tetracycline. In this system, the effect of TTP was studied without possible secondary effects of actinomycin-D as the

transcription of the reporter could be terminated at will without affecting the overall transcription in the cell.

Additionally, the stimulation of the cells with LPS was not necessary as TTP expression was achieved through co-transfection of the HeLa Tet-Off cells with the TTP expression plasmid pCMV-TTP-Flag.

4.2.1 Cloning of luciferase reporters

In order to verify the TTP-dependent decay of the target mRNA candidates, we prepared a tetracycline-regulated reporter system. The reporter vector pGL2-basic constitutes a 5598 base pair (bp) long, high copy plasmid and allowed selection for ampicillin for amplification in E-coli cells.

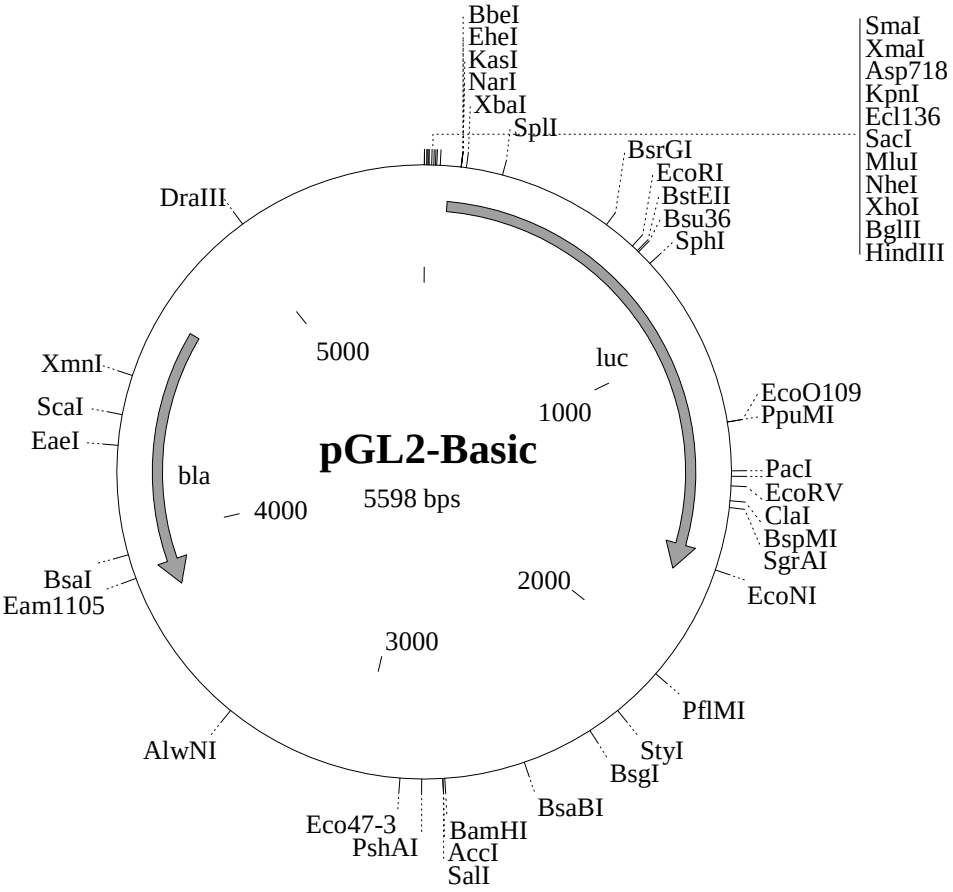


Fig 9: Vector map of pGL2-basic. Abbreviations: bla: β-lactamase (for ampicillin resistance); luc: luciferase reporter gene.

To put the expression of the luciferase reporter under the control of a tetracycline-dependent promoter, the tetracycline-regulated element (TRE) of the vector pTRE-tight was inserted into the multi cloning site (MCS) of pGL2-Basic. Therefore, both vectors were digested with XhoI and HindIII. The linearized pGL2-basic vector and the 387 bp fragment of pTRE-tight were joined using viral T4 ligase (Fermentas) and used for transformation of competent XL1-blue E-coli cells.

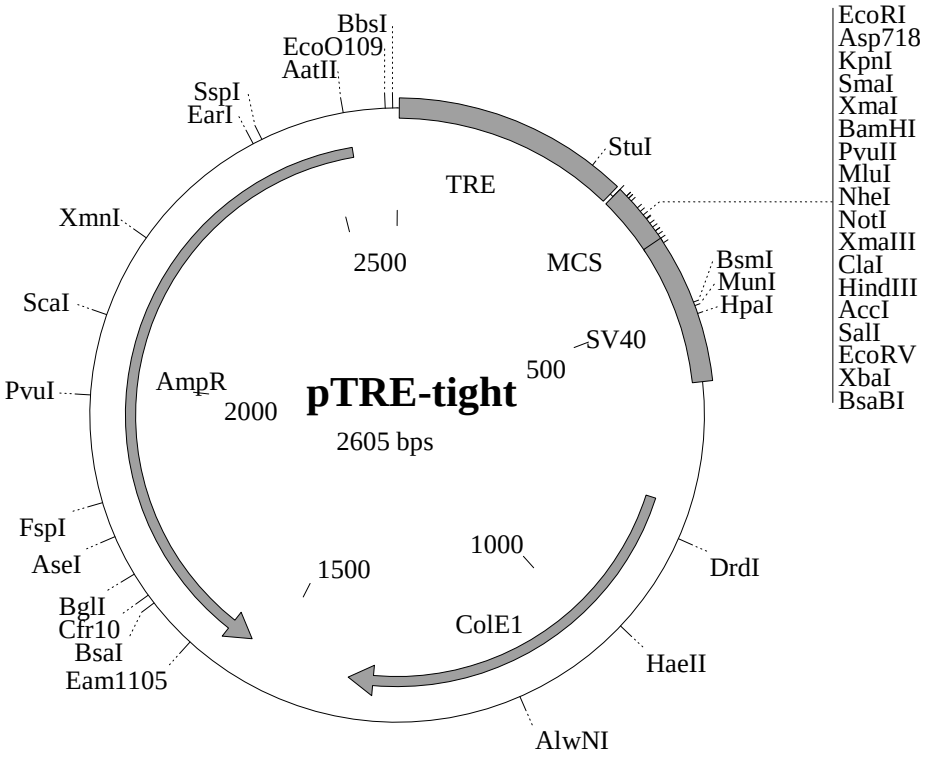


Fig 10: Vector map pTRE-tight. Abbreviations: AmpR: ampicillin resistance cassette; TRE: tetracycline responsive element.

Several candidates were analyzed and cloning was confirmed with analytical digestions using HindIII/XhoI or EcoRI resulting in the plasmid pGL2-TRE (Figure 11).

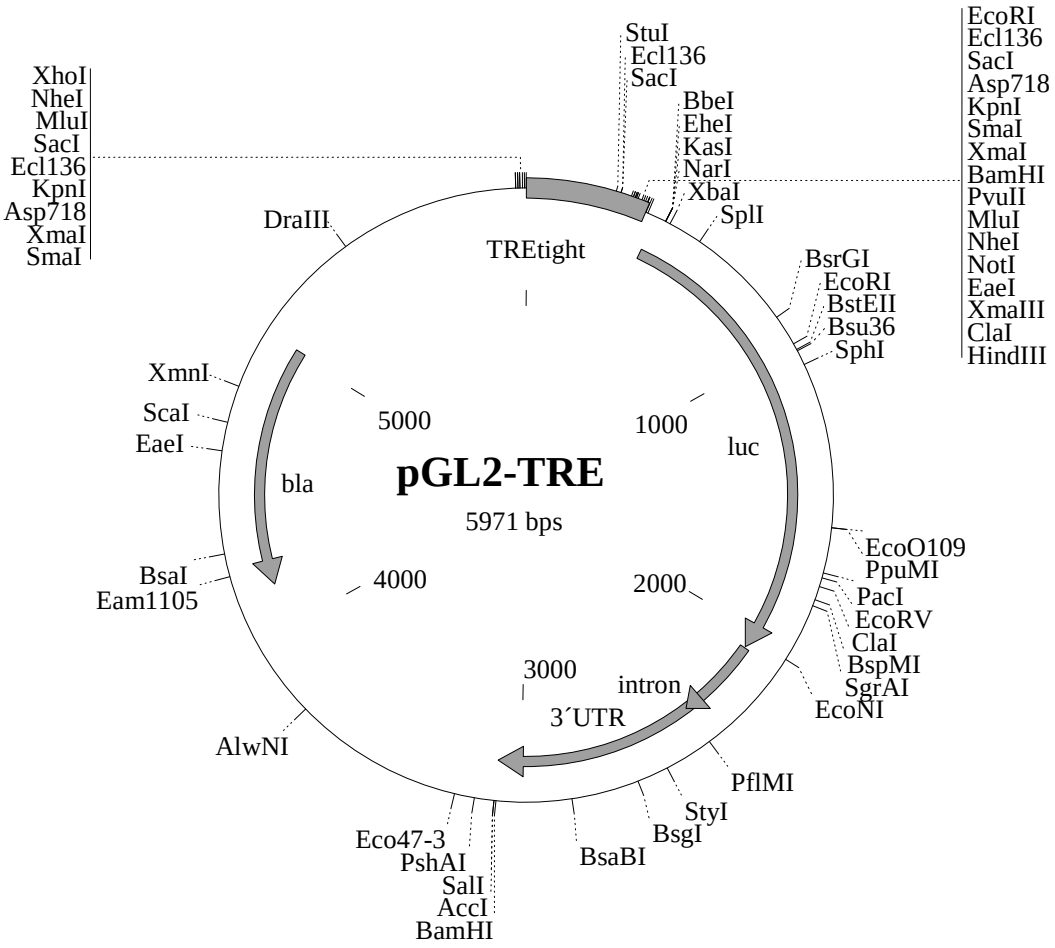


Fig 11: Vector map of pGL2-TRE. Abbreviations: TREtight: inserted tetracycline responsive element of pTRE-tight; luc: luciferase reporter gene; 3´ UTR: 3´ untranslated region of the luciferase reporter gene; intron: 65 bp intron in the 3´ UTR; bla: β-lactamase (ampicillin resistance);

The pGL2-TRE vector was tested regarding the function of the tetracycline-regulated promoter. Therefore, HeLa Tet-Off cells were transfected with the vector using the Exgen 500 reagent. The transfected cells were treated with either 1 or 0.5 µg/ml tetracycline and luciferase assays were performed.

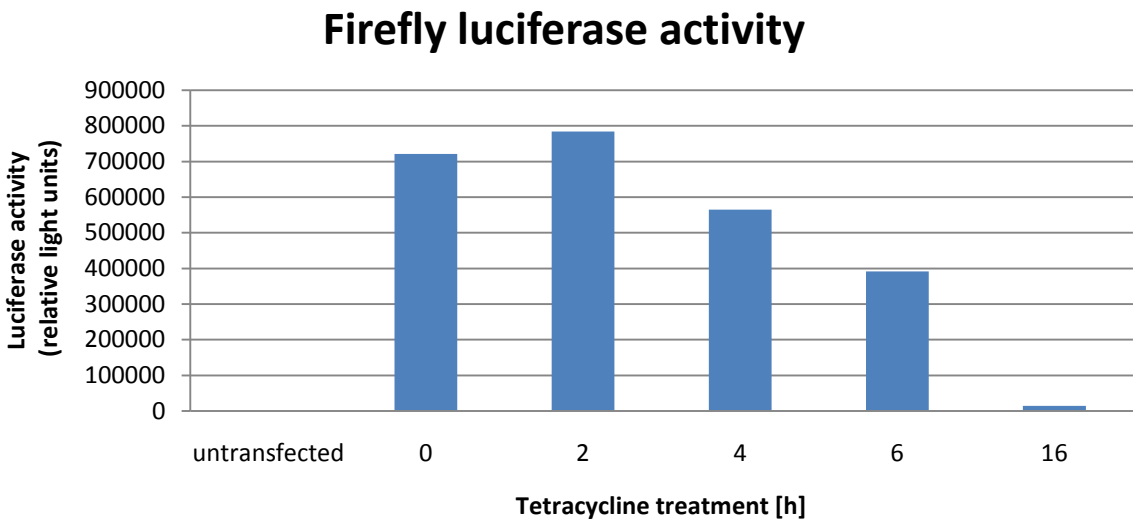


Fig 12: Luciferase assay for determination of the function of pGL2-TRE's inserted tetracycline responsive element. HeLa Tet-off cells were transfected with 20 µl Exgen 500, 10 µg pGL2-TRE plasmid DNA. Treatment with 1 µg/ml tetracycline led to complete termination of the expression or the reporter.

The pGL2-TRE plasmid was consequently used for further cloning to fuse the luciferase reporter mRNA with the 3´ UTRs of the putative TTP target mRNAs IL1α, IL6, IL12β and Cxcl2 as well as the controls TNFα, HPRT and rabbit-β-globin.

Target 3´ UTRs were PCR amplified using the primers depicted in M&M 3.10.2.1. The primers for IL1α, IL6, HPRT, TNFα, and rabbit-β-globin were modified by addition of an upstream PflMI and a downstream StyI restriction site. For the other targets, IL12 and Cxcl2, the restriction site PflMI was added upstream and downstream. The target cDNAs were obtained by reverse transcription of murine RNA, prepared after 6 hours of LPS-treatment of mouse primary macrophages. PCRs were performed for the 3´ UTRs of IL1α (IL1a3UTRPflMIFW2, IL1a3UTRStyIRV2), IL6 (IL63UTRPflMIfw, IL63UTRStyIrv), IL12β (IL12b3UTRPflMIfw, IL12b3UTRPflMIrv), Cxcl2 (Cxcl23UTRPflMIFW, Cxcl23UTRPflMIRV), TNFα (TNFa3UTRPflMlFW2, TNFa3UTRStylRV2), HPRT (Hprt3´UTRPflM1FW, Hprt3´UTRSty1RV) and rabbit-β-globin (bGl3´UTRPflM1FW, bGl3´UTRSty1RV) using high fidelity Pwo DNA Polymerase (Roche) to ensure accurate DNA synthesis.

The PCR fragments of IL1α, IL6, HPRT, TNFα, rabbit β-globin and the pGL2-TRE vector were digested with PflMI and StyI, purified and ligation was performed using viral T4 ligase. This digestion removed a 148 bp fragment of the 3´UTR of the luciferase reporter gene. The fragments of IL6 and Cxcl2 were digested with PflMI alone and inserted into a PflMI digested

and CIAP dephosporylated pGL2-TRE vector. Due to complications regarding the stability of the vector, the incubation temperature of the transformed E-coli had to be reduced to 28°C with an ampicillin concentration of 50 µg/ml.

Fig 13: insertion of target 3´ UTRs into the pGL2-TRE vector

In addition to these constructs, plasmids containing either the core-ARE of TNFα or the inverted (T-C/A-G) sequence of the TNFα core ARE were cloned using primers (see M&M 3.10.8.1) already containing the sticky ends necessary for ligation. These sequences were annealed and directly used for ligation and subsequent transformation of competent E-Coli cells.

Candidate vectors were screened by quick gram negative plasmid preparation followed by control digestions verified by sequencing of the insert using primers flanking the insert (M&M 3.10.11). For inserts exceeding 500 bp, additional primers were used to achieve higher precision of the sequencing process. For this cause, primers were designed for the individual target 3´ UTRs (see Materials and Methods).

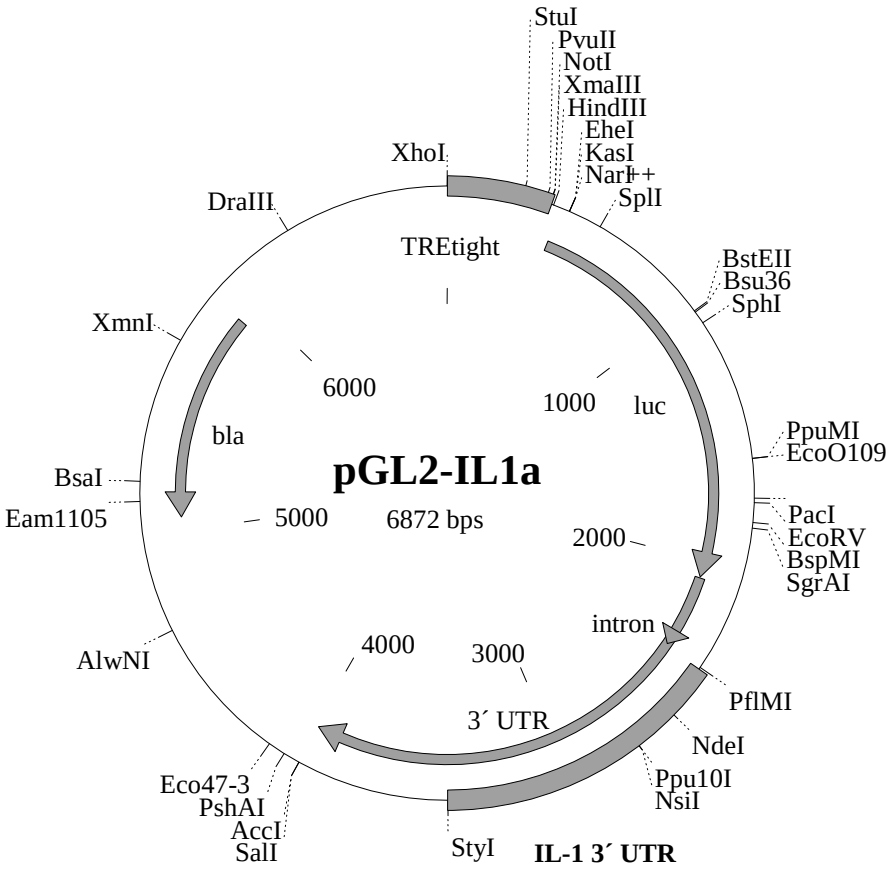


Fig 14: Vector map of pGL2-IL1a, representative for the vectors pGL2-IL1a, pGL2-IL6, pGL2-IL12b, pGL2-Cxcl2, pGL2-TNFa, pGL2-HPRT, pGL2-RbGl, pGL2-TNFcore, pGL2-random (maps added in supplementary data). The 3´ UTR of IL1α is inserted between the PflMI and StyI restriction site.

4.2.2 Luciferase Assay

In order to measure the effect of TTP on the luciferase protein expression, we put the luciferase gene under the control of each TTP target candidate 3´ UTR. We co-transfected HeLa Tet-off cells with the individual luciferase reporter vectors and the TTP expression vector pCMV-TTP-Flag. Luciferase assay samples were generated by nucleofection (Amaxa kit) of $5x10^6$ HeLa Tet-off cells with 2.5 µg pGL2-target vector, 1.5 µg pRL-NULL and 0.75 µg pCMV-TTP-Flag.

The renilla luciferase expression vector pRL-NULL, containing a minimal promoter, was used to account for discrepancies in transfection efficiency which could arise during the nucleofection procedure using multiple plasmids. The expression of TTP was controlled by

the addition of the vector pCMV-TTP-Flag, therefore avoiding the necessity to stimulate the cells with LPS.

The transfected cells were incubated for 16 hours before samples were prepared. For the luciferase assay, samples were diluted if necessary to achieve a signal of 80.000 to 800.000 (relative light units) of the firefly luciferase activity. The values of luciferase reporter activity were normalized to the activity of the renilla luciferase, encoded by the pRL-NULL vector.

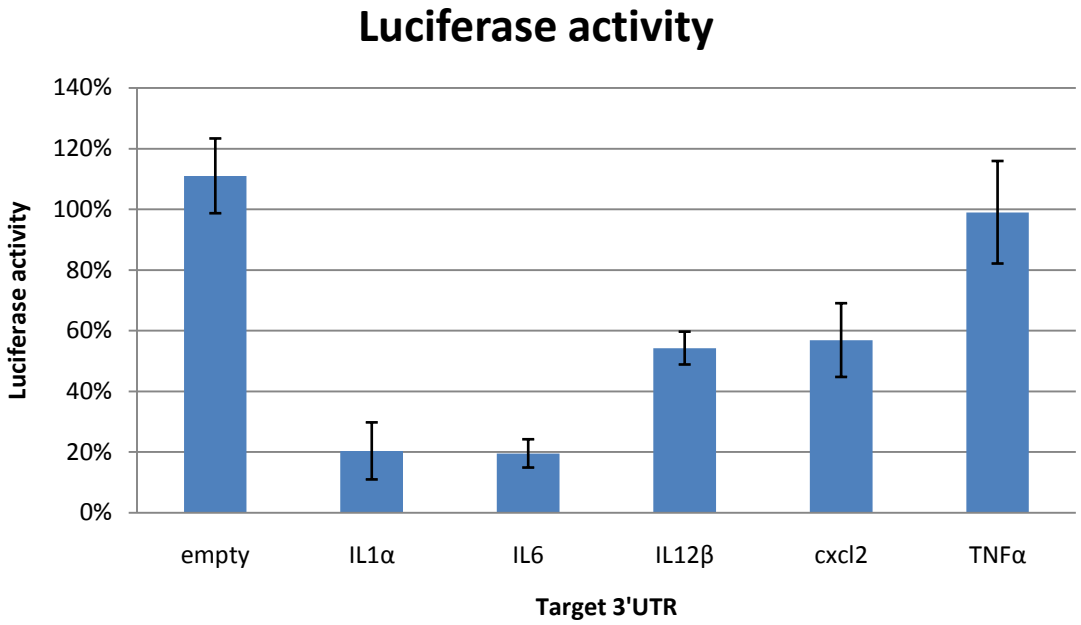


Fig 15: Luciferase assay of reporters containing the 3´ UTRs of IL1α, IL6, IL12β, Cxcl2 and TNFα. Luciferase activity values were calculated by normalizing firefly luciferase to renilla luciferase activity; values represent the luciferase activity of TTP expressing cells relative to cells without TTP expression. The assay was performed 3 times using independent cell extracts.

In the luciferase assay, we found that in cells, containing both the reporter and the TTP expression vector, we could measure a significant reduction of reporter activity when compared to the cells lacking TTP expression. While the signal of the negative control pGl2-TRE displayed virtually no change in magnitude, the signal of IL1α and IL6 controlled luciferase was reduced to 20 % compared to the non-TTP-containing cells. The signal of IL12β and Cxcl2 controlled reporter was reduced to 50 %. Although the positive control TNFα didn't show the anticipated reaction to the presence of TTP, we could see that the overall signal of the luciferase reporter was up to 30 times weaker than the signal of the negative control and the targets even without TTP suggesting that the pGL2-TNFa vector was

extremely unstable. The Cxcl2 reporter showed a similar reduction but only to about 5-10 times weaker than the other targets. Conclusively, the luciferase assays revealed a TTP-dependent reduction of luciferase protein expression. The magnitude of this effect was dependent on each target 3´ UTR.

As the luciferase assay using the pGL2-TNFa construct containing the complete 3´ UTR of TNFα did not yield a strong signal, possibly due to the extreme instability of the mRNA, we wanted to show the effect of the isolated TNFα class 2 ARE. We expected that this core ARE-containing vector would display less instability than the complete TNFα and performed additional luciferase assays using the vector pGL2-TNFcore and as control the vector pGL2-random. Luciferase samples were prepared and luciferase activity was measured as previously described.

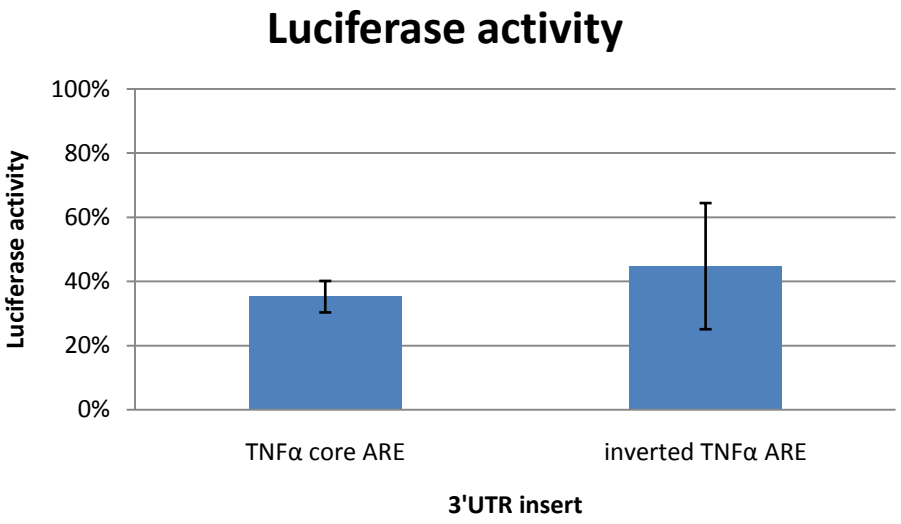


Fig 16: Luciferase reporter assay using the TNFα core ARE (pGL2-TNFc) and inverted ARE (pGL2-random) controlled plasmids. Mean value of 3 independent experiments is shown. Luciferase activity values were calculated as described in Figure 15.

In this assay we could see a reduction of luciferase signal to 35 % with TTP expression. Unfortunately, the negative control pGL2-random also displayed a reduction to 44 % although the signal prior to normalization was, as desctibed before for pGl2-TNFα, vastly reduced in the case of pGL2-TNFcore.

4.2.3 Tetracycline timecourse of luciferase vectors

As the luciferase assay showed a TTP-dependent decrease in target gene expression, we wanted to gain insight in the TTP-dependent effects on stability of the luciferase reporter. We transfected $5x10^6$ HeLa Tet-off cells with 2.5 µg pGL2-target vector, 1.5 µg pRL-NULL and 0.75 µg pCMV-TTP-Flag. The transfected cells were thoroughly resuspended and seeded in 4 aliquots on 6 well dishes and incubated for 16 h. Luciferase samples were prepared after the termination of transcription through either16, 6, 3 or 0 h 1 µg/ml tetracycline-treatment. Unfortunately, decay could not be measured due to the enormous differences in luciferase activity of the samples with and without TTP expression vectors.

The second attempt was made using qRT-PCR as a detection method for the luciferase mRNA thereby preventing any possible interference on the translational level. $5x10^6$ HeLa Tet-off cells were transfected with 2.5 µg pGL2-target vector and 0.75 µg pCMV-TTP-Flag and distributed onto 3 10 cm cell culture dishes. RNA was prepared after 0, 45 and 90 minutes of tetracycline-treatment and was reverse-transcribed. qRT-PCR using the primers Luc RT FW and Luc RT RV was performed while primers for human HPRT were used for normalization. In this attempt, the incomplete removal of vector DNA contamination from the isolated RNA samples was the reason that we were unable to quantify changes in mRNA stability.

Conclusively, the impact of TTP on the stability of the reporter, both the mRNA (RT-PCR) and protein (luciferase assay) could not be studied with this system due to methodical problems with the luciferase reporter vectors.

4.2.4 RNA immunoprecipitation of luciferase reporter vectors

In order to confirm the validity reporter assays, we wanted to show that TTP can bind to the 3´ UTRs inserted in the pGL2-target vectors. $5x10^6$ HeLa cells were transfected with 2.5 µg pGL2-target vector, 1.5 µg pRL-NULL and 0.75 µg pCMV-TTP-Flag and subsequently seeded on 15 cm cell culture dishes. In this experiment, the renilla luciferase expression vector pRL-NULL was not necessary, but the vector was included for consistency with the previous luciferase protein activity assays. 2 days after transfection, a RNA-IP was performed using anti-TTP antibodies (see M&M 3.4.2). After RNA preparation and reverse transcription,

the obtained cDNA was used for quantitative PCR using Taq DNA Polymerase and the primers Luc RT FW and Luc RT RV screening for luciferase mRNA.

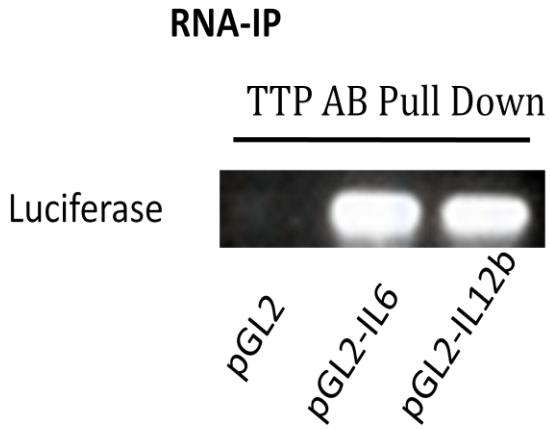


Fig 17: RNA-IP using the vectors pGL2-TRE, pGL2-IL6 and pGL2-IL12β. PCR was stopped after 27 cycles and loaded onto a 1 % agarose gel.

The RNA-IP was successfully used on the reporter vectors for IL6, IL12β and the empty vector. Unfortunately, further analysis of all target vectors was not possible as the removal of plasmid DNA during the process of reverse transcription was not sufficient and the remaining plasmid generated a strong signal in the quantitative PCR which exceeded the signal obtained by the cDNA. Even when the RNA samples were pretreated with DNase, the signal from the plasmid DNA was too intense.

4.3.1 Cloning of the rabbit β-globin vectors

The cloning of these new vectors was necessary due to the fact that the impact of TTP on the stability of the target 3´ UTRs could not be measured in the luciferase vector system. Tetracycline-regulated rabbit β-globin reporter vectors were prepared to measure the impact of TTP on the stability of the target mRNA candidates. The vector pTET-BBB was provided by the group of PR Bohjanen [81]. The pTET-BBB vector is a high copy, 5003 bp plasmid, containing an ampicillin resistance cassette for selection in E-coli (Figure 18). It expresses the stable rabbit β-globin mRNA that contains a 572 bp intron, which made the use of qRT-PCR possible.

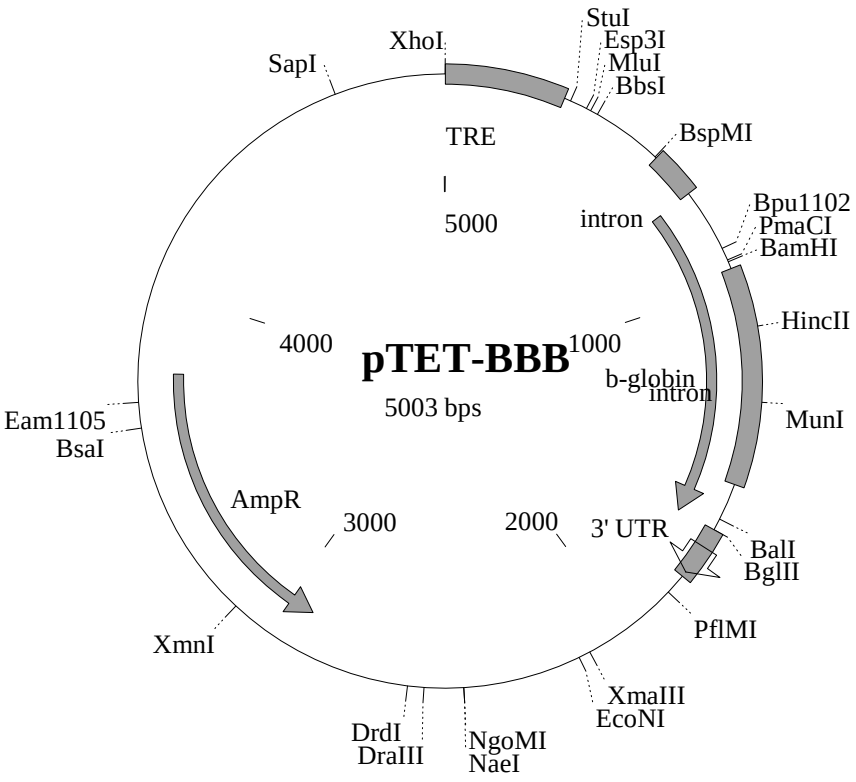


Fig 18: Vector map of pTET-BBB.

Primers, containing the asymmetrical restriction site BglII, were designed for the murine target candidates IL1α, IL6, Cxcl2 and the negative control HPRT. The PCR fragments were amplified from the pGL2-TRE plasmids containing each target 3´ UTR using the high fidelity Pwo DNA polymerase and purified by DNA precipitation. The pTET-BBB vector and the fragments were digested using BglII, purified by agarose electrophoresis and vector DNA was dephosphorylated using CIAP.

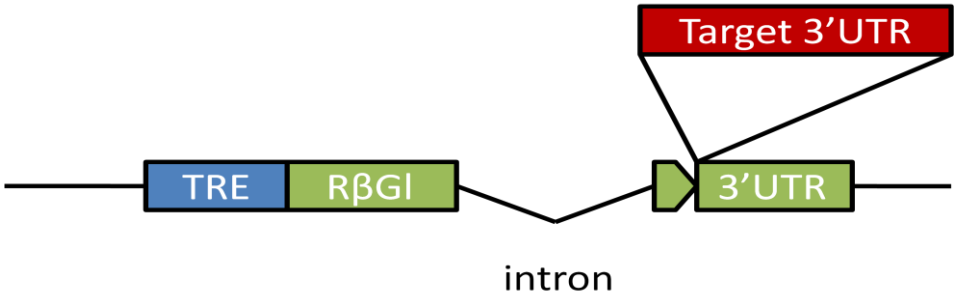


Fig 19: insertion of the target candidate 3´ UTR at the BglII restriction site at the start of the rabbit β-globin (RβGl) 3´ UTR. The reporter gene contains a 572 bp intron. The rabbit β-globin gene is inder the control of a tetracycline responsive element (TRE) able to terminate transcription through tetracycline-treatment.

The fragments were then ligated with the linearized pTET-BBB vector, a process that left only one BglII restriction site at the 3´ ends of each inserted fragment. The ligated constructs were then used for transformations of competent XL-1 blue E-coli cells. The plasmids were screened by gram negative plasmid preparation and correct insertion was controlled by restriction digestions. Correct clones were further verified by sequencing of the inserts.

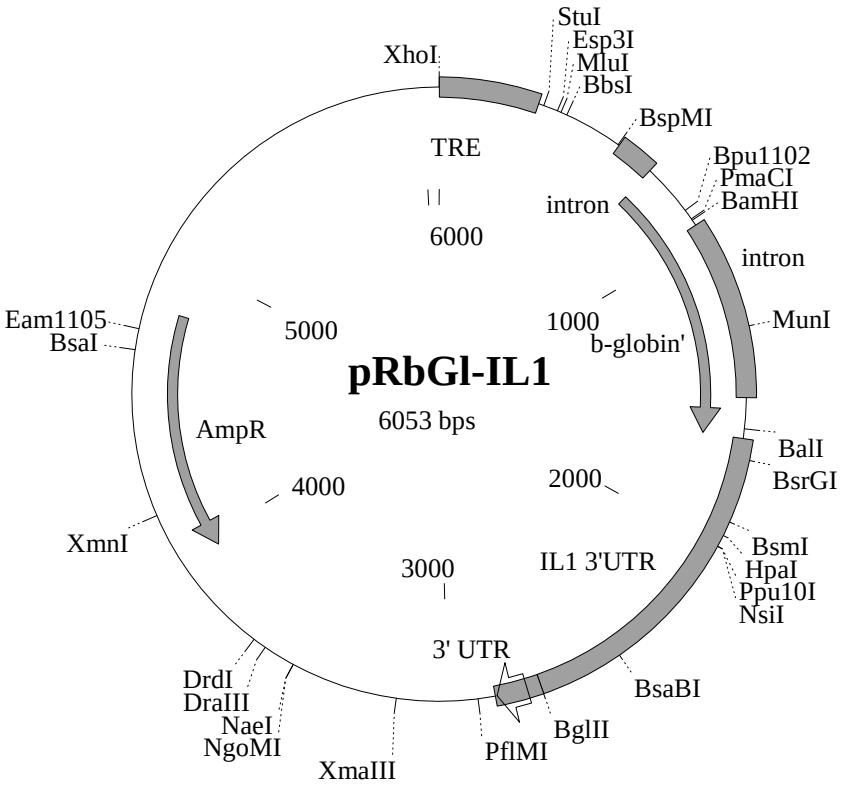


Fig 20: Vector map of pRbGl-IL1, representative for the vectors pRbGl-IL1, pRbGl-IL6, pRbGl-Cxcl2, pRbGl-HPRT (maps added in supplementary figures). The 3´ UTR of IL1α was inserted at the BglII restriction site at the start of the rabbit β-globin 3´ UTR.

4.3.2 Decay of rabbit β-globin constructs after tetracycline-induced transcriptional termination

To analyze the impact of TTP on the decay of the reporter constructs containing the 3´ UTRs of IL1α, IL6 and Cxcl2, we measured mRNA stability of the rabbit β-globin reporters using qRT-PCR. We transfected $5x10^6$ HeLa Tet-off cells with 4 µg of pRbGl-target and 1 µg pCMV-TTP-Flag expression vector or an empty CMV vector used for control. The transfected cells were incubated for one day before transcription of rabbit β-globin was terminated through addition of 1 µg/ml tetracycline. RNA was prepared after 0, 10, 20 and 30

minutes after treatment and qRT-PCR screening for rabbit β-globin (RbGl-RT-FW/RbGl-RT-RV) and the HPRT internal standard (HPRT HS rt FW/HPRT HS rt RV) was performed.

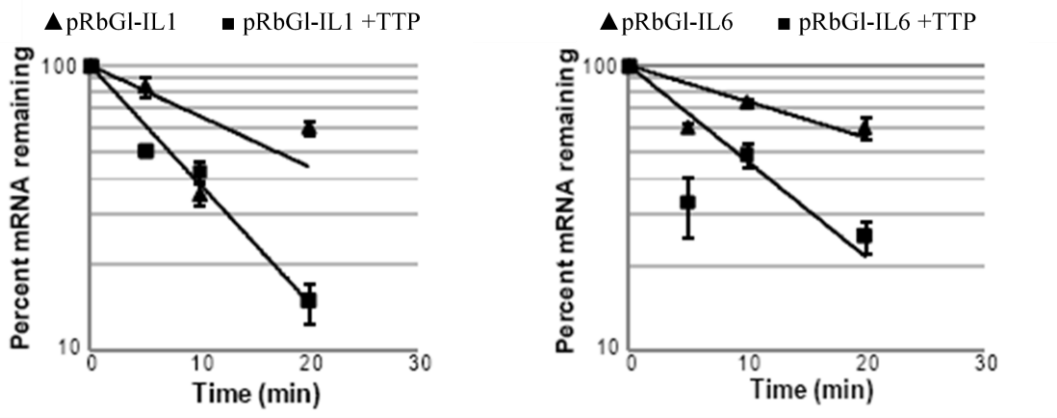


Fig 21: qRT-PCR of the rabbit β-globin decay assay using the reporter constructs for IL1α (pRbGl-IL1) and IL6 (pRbGl-IL6). mRNA levels were measured after tetracycline-induced transcriptional termination. In both targets, the addition of TTP led to a significant destabilization of the rabbit β-globin mRNA compared to the reporter vector without additional TTP.

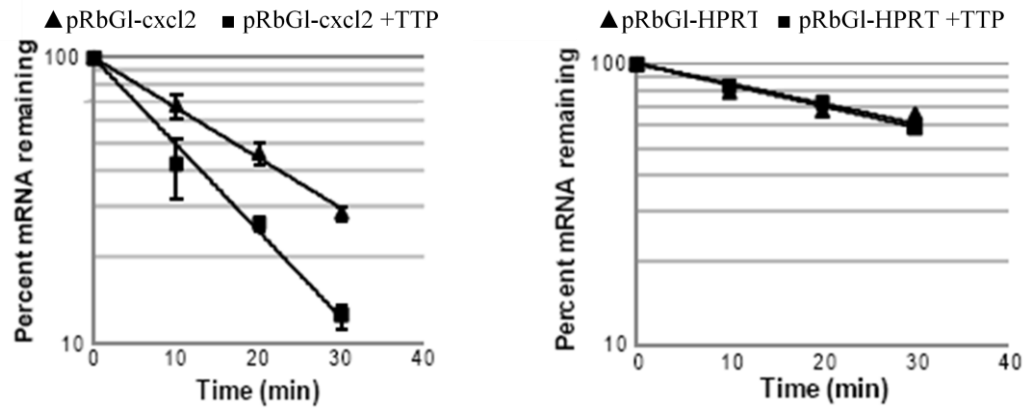


Fig 22: qRT-PCR of the rabbit β-globin decay assay using the reporter constructs for Cxcl2 (pRbGl-Cxcl2) and the negative control HPRT (pRbGl-HPRT). mRNA levels were measured after tetracycline-induced transcriptional termination. In the case of the Cxcl2 3´ UTR-containing vector, the addition of TTP led to enhanced destabilization of the reporter mRNA while the HPRT 3´ UTR-containing rabbit β-globin mRNA showed no TTP-dependent change in stability.

The qRT-PCR analysis of the TTP-dependent destabilization of rabbit β-globin constructs under the control of TTP target candidate 3´ UTRs showed that all mRNAs of all three target candidate reporter constructs, IL1α, IL6 and Cxcl2, were destabilized by the addition of TTP. The mRNA stability of the reporter constructs showed a significant decrease when the cells

co-expressed the exogenous TTP originating from the vector pCMV-TTP-Flag. The 3´ UTR of the housekeeping gene HPRT had absolutely no impact on the rabbit β-globin mRNA.

4.4 The role of TTP in hematopoiesis

4.4.1 Lineage analysis of wild-type and $TTP^{-/-}$ macrophages

We conducted a FACS analysis using antibodies for granulocyte and macrophage markers to check whether the enhancement of the population of GR-1+ cells (a cellular marker expressed on neutrophils, dendritic cells and monocyte cell subpopulations) in TTP-deficient mice of mixed background described by P. Blackshear [32] were also present in the C57Bl/6 background $TTP^{-/-}$ mice used in our experiments. The Mac-1 marker was used for the identification of macrophages (Mac-1 is a macrophage cell surface receptor binding the complement component C3b). The double-positive cells constitute a population of immature myeloid cells that develop under high cytokine levels and can yet differentiate to macrophages, granulocytes and dendritic cells. The experiments were performed using 2 mice per genotype. One knock-out, mouse 472, was genotyped again after the experiment and could possibly be $TTP^{+/-}$ explaining the intermediate cell percentages between wild-type and the one $TTP^{-/-}$ mouse. In the analysis, the mouse was counted as a TTP knock-out. If the suspected heterozygous mouse was not included in this study, the TTP-dependent effect would be more pronounced.

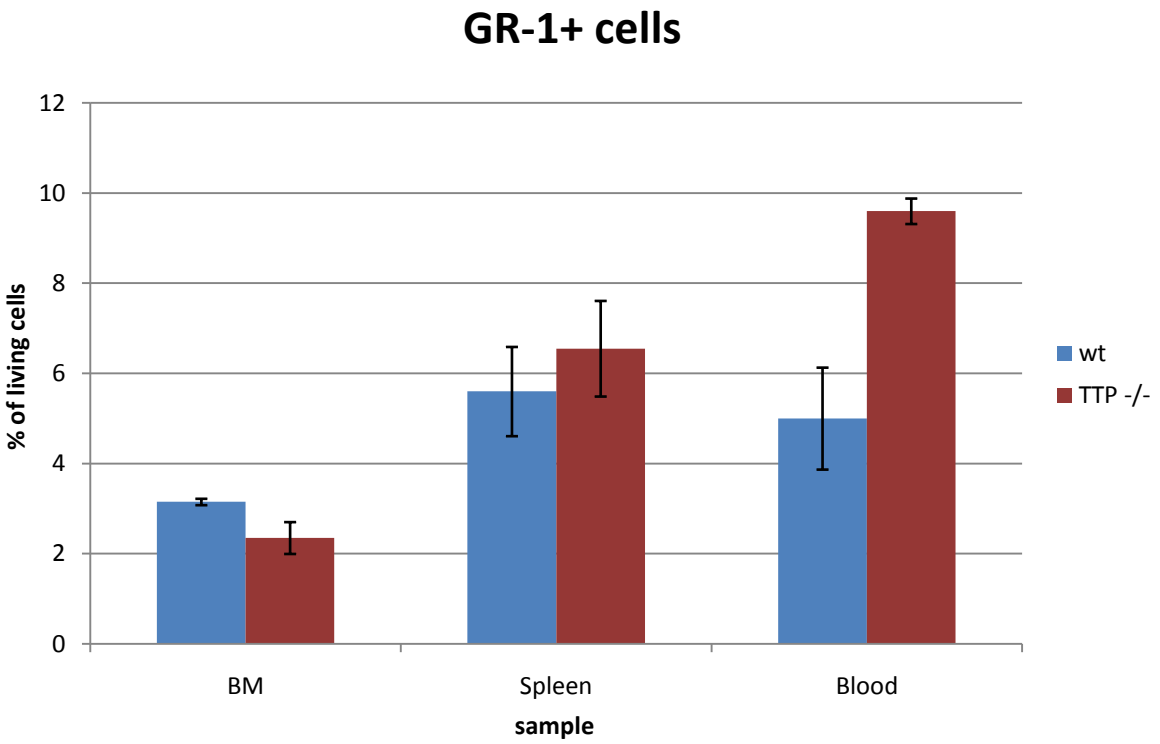


Fig 23: FACS analysis of GR-1^{+} granulocyte populations in WT and TTP$^{-/-}$ mice. In the peripheral blood, the TTP-deficient mouse possessed nearly double the GR-1^{+} granulocytes than the wild-type.

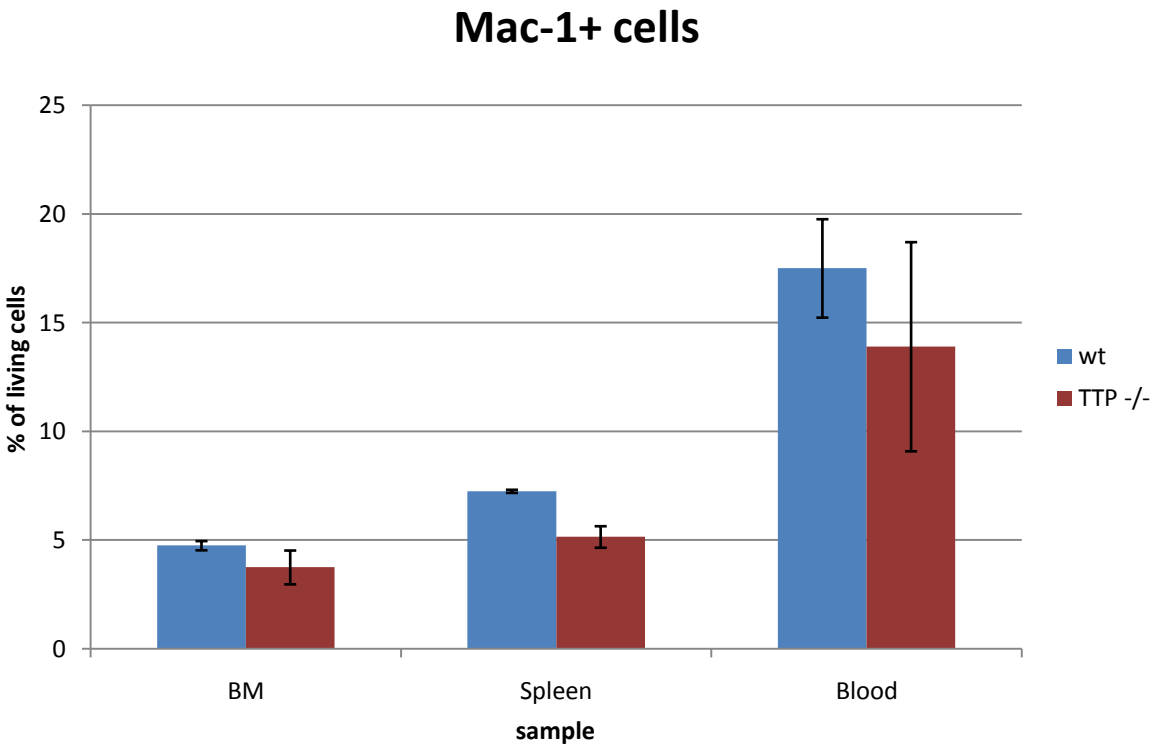


Fig 24: The FACS analysis showed a slight decrease in the population of Mac-1^{+} macrophages in the TTP-deficient mouse.

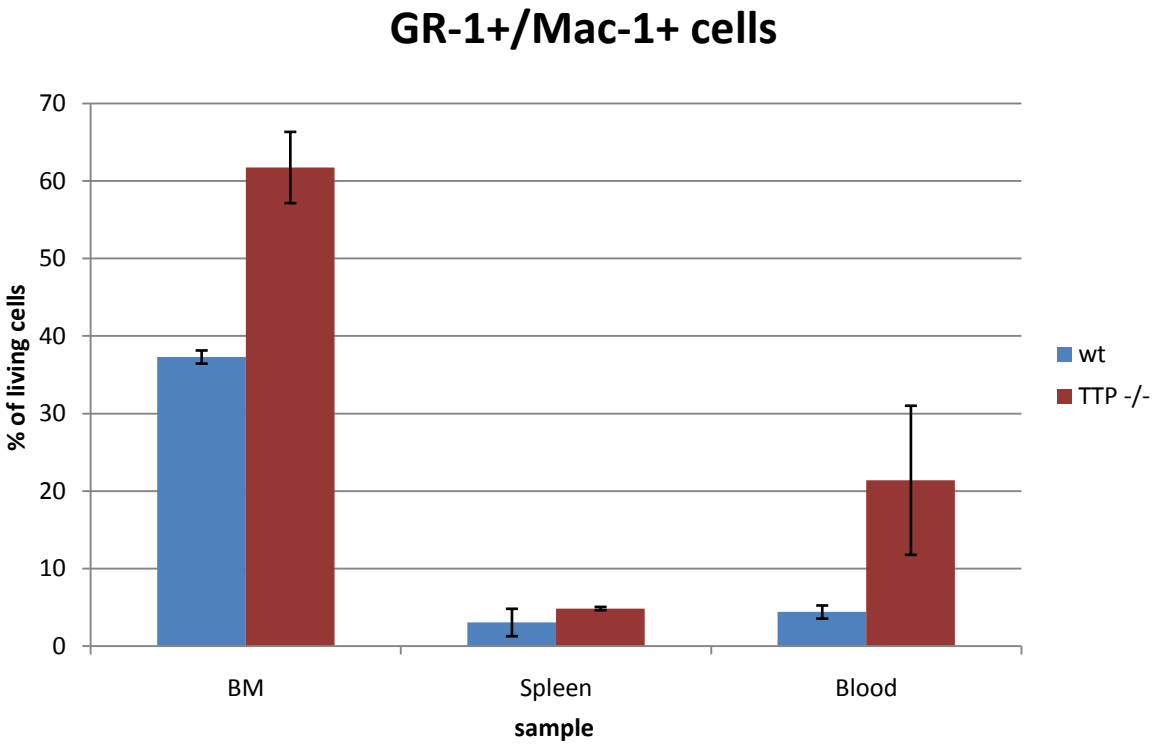
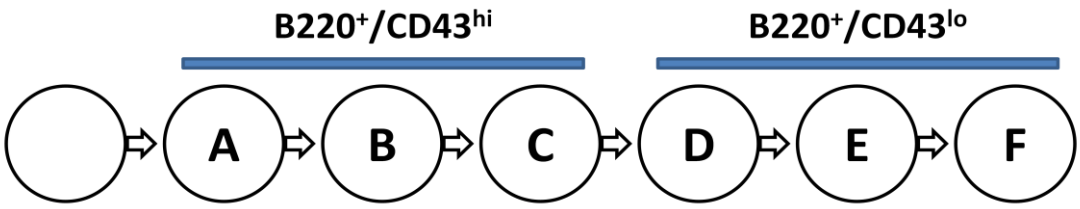


Fig 25: FACS analysis of GR-1/Mac-1 double positive cells. This cell population was markedly increased in TTP-deficient cells, especially so in the peripheral blood.

The cell linage FACS showed a significant increase in GR-1^{+} granulocytes in the TTP-deficient mice although the effect was not as pronounced as previously described [32]. The difference may have originated from the enormous population difference in GR-1^{+}/Mac-1^{+} cell population which was not mentioned in this publication.

4.4.2 B-cell development in TTP$^{-/-}$ mice

We analyzed differences in the maturation of B-cells between wild-type C57Bl/6 mice and TTP$^{-/-}$ mice of the same genetic background. For this, we prepared FACS samples of the peripheral blood, lymph nodes, spleen and bone marrow (according to M&M 3.9.1). Values represent the average of 3 individual experiments with at least 2 mice per phenotype each. A mean value of each experiment, normalized to the wild-type percentage of B-cell precursor (B220^{+} CD43^{hi}) cells for cell populations (developmental stadiums) A-C and B220^{+} CD43^{lo} for populations D-F, was calculated before combining the experiments to minimize sample preparation related differences.



		pre-pro B-cell	early pre B-cell	late pre B-cell	Pro B-cell	immature B-cell	mature B-cell
B220	-	+	+	+	+	+	+
CD43	hi	hi	hi	hi	lo/-	lo/-	lo/-
CD19	-	-	+	+	+	+	+
BP-1	-	-	-	+	n.d.	n.d.	n.d.
IgM	-	-	-	-	-	+	+
IgD	-	-	-	-	-	-	+

Fig 26: Chart of B-cell development steps. The maturation stadium of the cells is determined by the presence of surface markers. The cell populations (developmental stages) A-C and D-F in the figures 27-32 were measured independently as the number of different fluorescent dyes should be kept small to reduce interference.

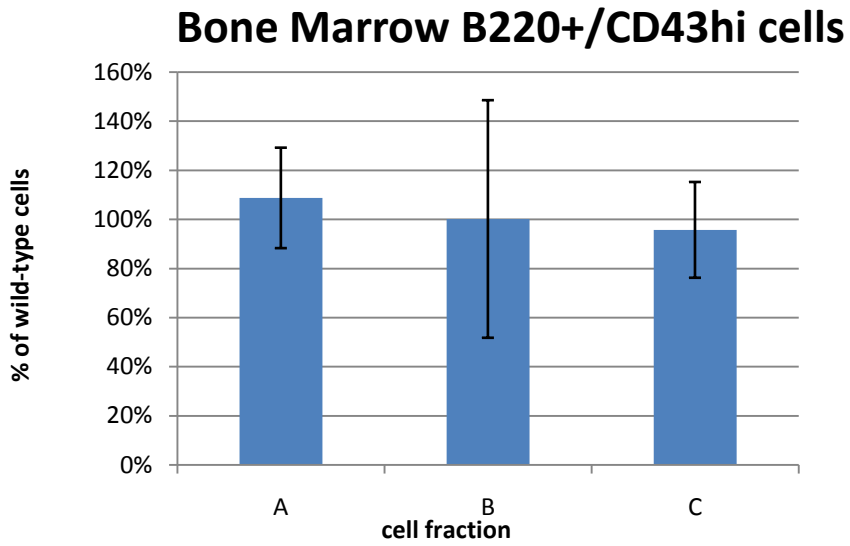


Fig 27: Analysis of B-cell development by FACS of bone-marrow samples of wild-type and $TTP^{-/-}$ mice in early B-cell populations. TTP does not seem to have an impact the development of early B-cell fractions in bone marrow samples. Values represent the percentage of $TTP^{-/-}$ B-cell progenitor cells containing the markers $B220^{+}/CD43^{hi}$ relative to the cell count in the wild-type mice. Cell populations are explained in Figure 26.

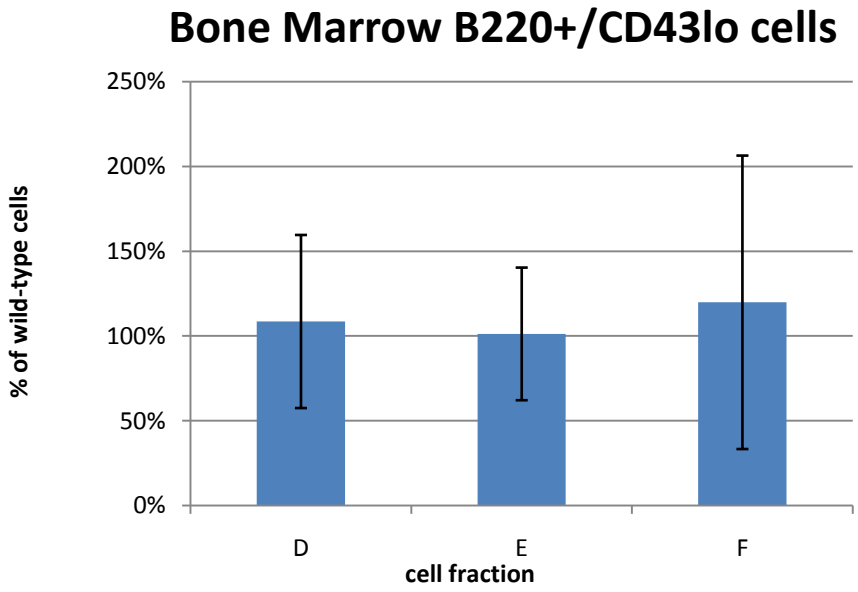


Fig 28: Analysis of B-cell development by FACS of bone-marrow samples of wild-type and $TTP^{-/-}$ mice in later stages of B-cell development. Later maturation of B-cells was not significantly affected by absence of TTP in bone marrow samples. Values represent the percentage of $TTP^{-/-}$ B-cell progenitor cells containing the markers $B220^{+}/CD43^{lo}$ relative to the cell count in the wild-type mice.

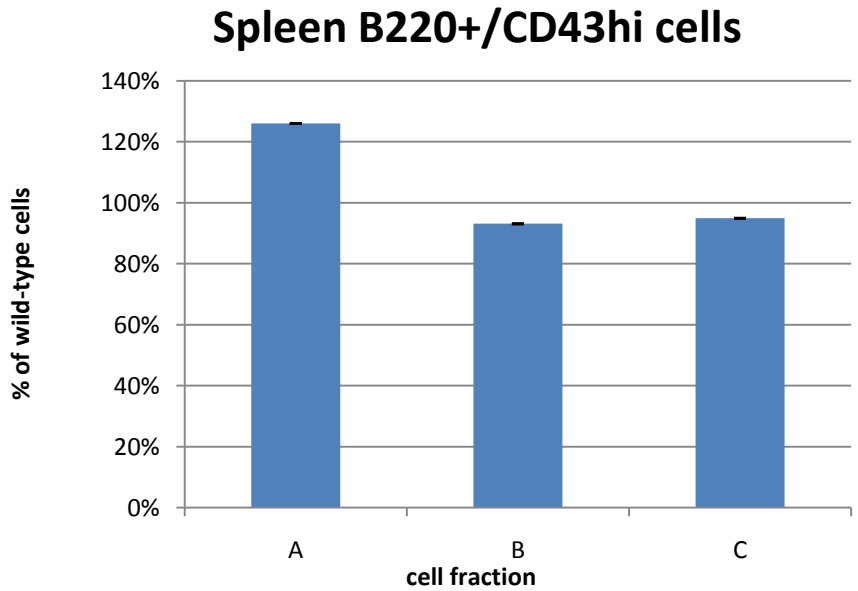


Fig 29: Analysis of B-cell development by FACS of spleen samples of wild-type and $TTP^{-/-}$ mice in early stages of B-cell development. This experiment was only carried out once with 3 mice per genotype (for consistency with the other experiments, no error bars are possible). TTP deficiency did not have an impact on the early B-cell population in the spleen. Values represent the percentage of $TTP^{-/-}$ B-cell progenitor cells containing the markers $B220^{+}/CD43^{hi}$ relative to the cell count in the wild-type mice.

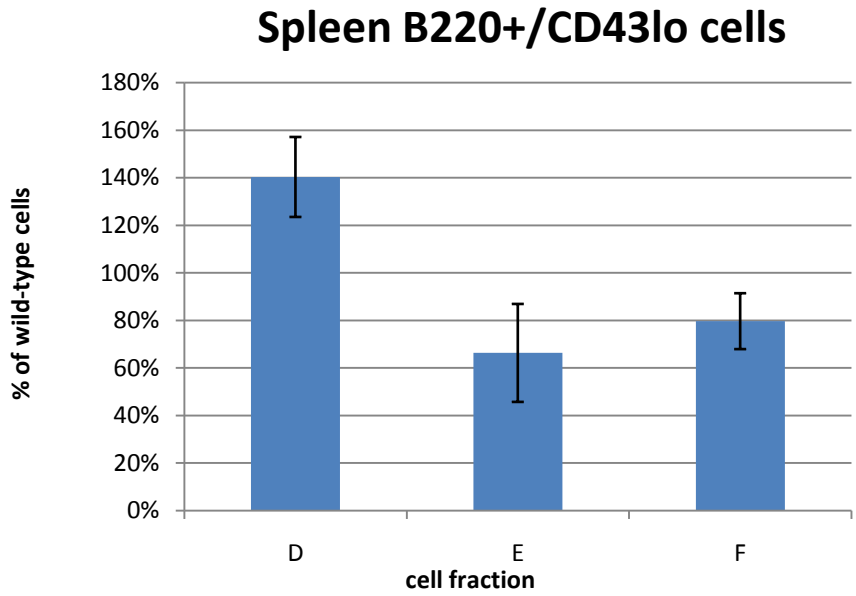


Fig 30: Analysis of B-cell development by FACS of spleen samples of wild-type and $TTP^{-/-}$ mice in later stages of B-cell development. Late B-cell populations were slightly decreased by TTP deficiency. Values represent the percentage of $TTP^{-/-}$ B-cell progenitor cells containing the markers $B220^{+}/CD43^{lo}$ relative to the cell count in the wild-type mice.

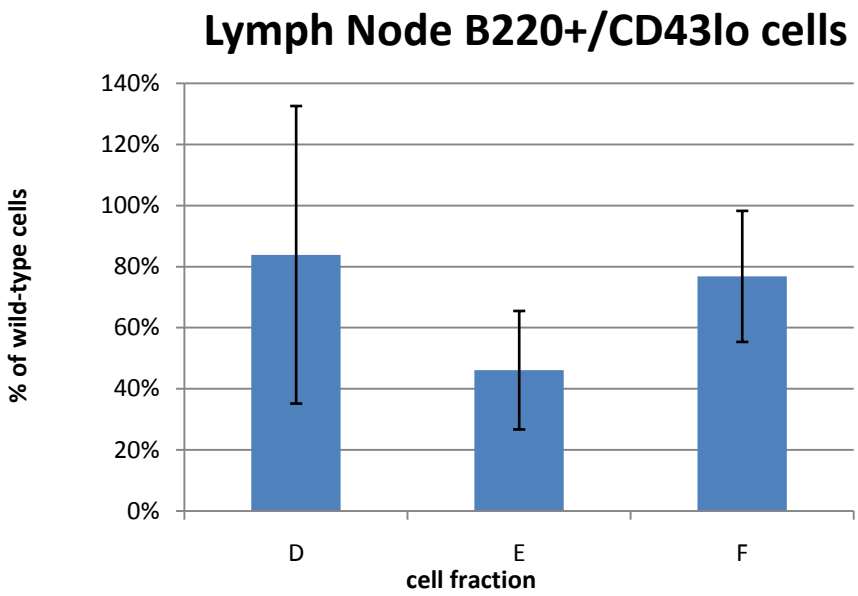


Fig 31: Analysis of B-cell development by FACS of lymph node samples of wild-type and $TTP^{-/-}$ mice in later stages of B-cell development. Late B-cell populations were decreased by TTP deficiency. Values represent the percentage of $TTP^{-/-}$ B-cell progenitor cells containing the markers $B220^{+}/CD43^{lo}$ relative to the cell count in the wild-type mice.

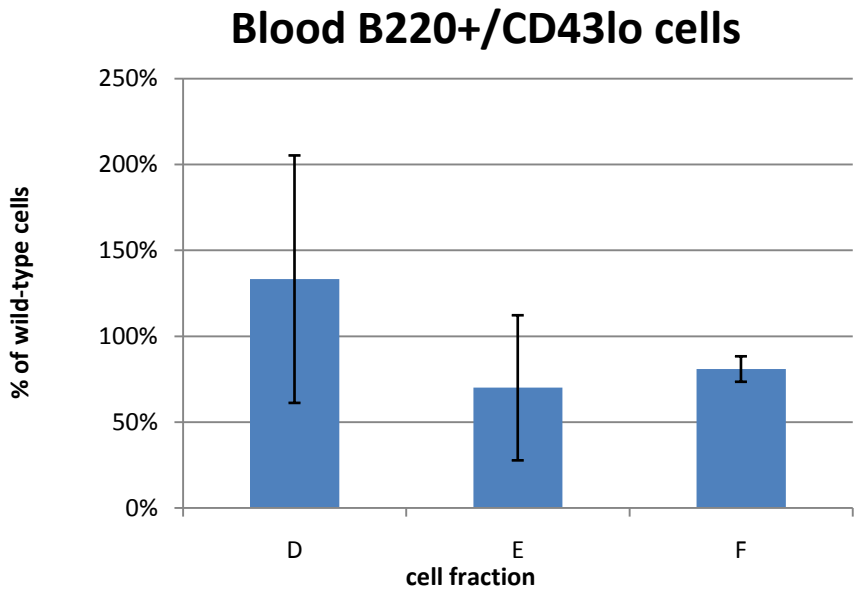


Fig 32: Analysis of B-cell development by FACS of blood samples of wild-type and $TTP^{-/-}$ mice in later stages of B-cell development. Late B-cell populations were slightly decreased by TTP deficiency. Analysis of blood samples resulted in high discrepancies between the different samples and experiments as the erythrocyte lysis (procedure explained in M&M 3.9.1) was a very important step in this analysis. Values represent the percentage of $TTP^{-/-}$ B-cell progenitor cells containing the markers $B220^{+}/CD43^{lo}$ relative to the cell count in the wild-type mice.

The B-cell development FACS showed that according to the already published data, the TTP knock-out mice displayed no change in B-cell differentiation in the bone marrow while they displayed a minor decrease in late differentiation stages in spleen, lymph node and peripheral blood.

4.4.3 *v-abl* transformation of B-cell progenitor cells

The results of the FACS analysis of the B-cell development showed that there was no major difference in B-cell precursor quantities in the bone marrow of wild-type and $TTP^{-/-}$ mice. This result suggested that B-cells from wild-type and $TTP^{-/-}$ mice were comparable at the differentiation stage examined. To acquire transformed B-cells, bone marrow of 8 week old C57Bl/6 wild-type and $TTP^{-/-}$ mice was isolated and used for infection with viral supernatant. The oncogenic properties of *v-abl* are based on a fusion of the retroviral *gag* gene with a C-terminal portion of the *c-abl* gene [82]. The resulting fusion product codes for a constitutively active tyrosine kinase located in the cytoplasm. The cells were transformed using a replication-deficient virus encoding *v-abl* that transforms B-lymphoid precursors. The

transformation was conducted in 2 batches, the first one included the WT mice 432 and 433 (genotyped wild-type B6 mice of the $TTP^{-/-}$ breeding stock) and the TTP KO mice 434 and 435. The second batch of animals contained of 3 C57Bl/6 mice ordered from Charles River and the $TTP^{-/-}$ mice 463, 465 and 469. The infection with the *v-abl*-containing virus (supernatant obtained from 54C12 producer cells containing A-MuLV particles) was carried out according to protocol.

4.4.4 Colony formation assay

We used a colony formation assay to determine if B-cell progenitor cells of $TTP^{-/-}$ or wild-type mice displayed differences in the potential to be transformed by *v-abl*. For this, we seeded the infected cells in a highly viscous methylcellulose medium (see procedure in M&M 3.7.2). After 2 weeks, the colonies were counted and normalized to the $B220^{+}$/ $CD34^{hi}$ cell percentage determined by FACS analysis of bone marrow for each individual mouse.

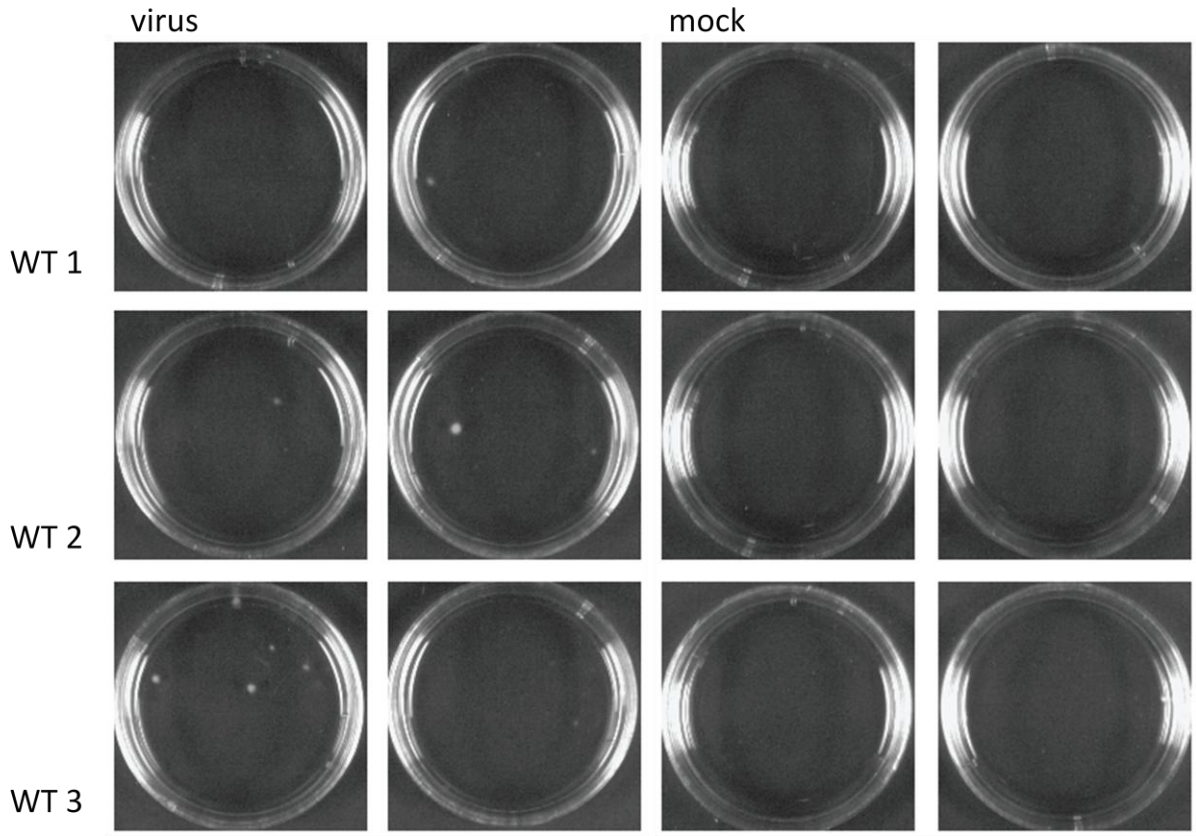


Fig 33: Methylcellulose dishes used for the colony formation assay for C57Bl/6 wild-type cells in duplicates for both *v-abl* transformed and mock treated cells. Mean colony count of *v-abl* transformed cells per wild-type dish: 5.25. Colonies were counted via microscope without additional staining.

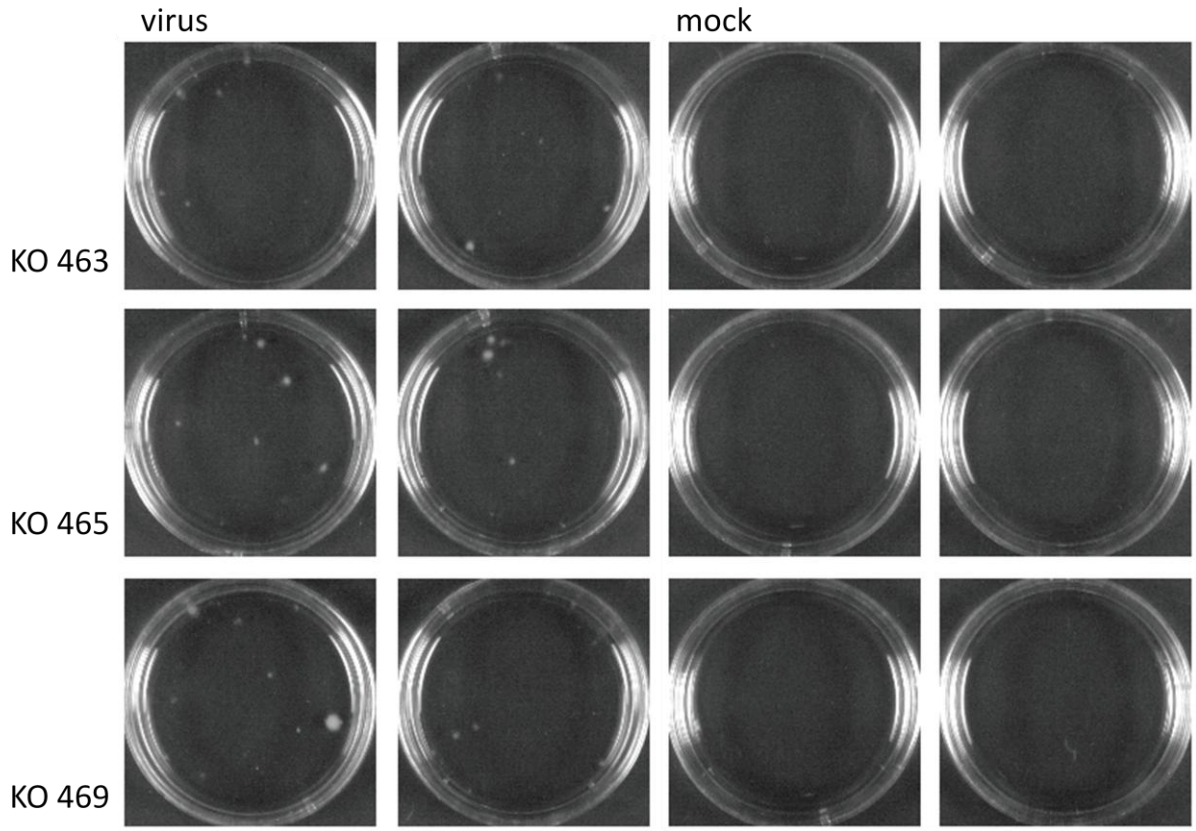


Fig 34: Methylcellulose dishes used for the colony formation assay for C57Bl/6 $TTP^{-/-}$ cells in duplicates for both *v-abl* transformed and mock treated cells. Mean colony count of *v-abl* transformed cells per knock-out dish: 10.08.

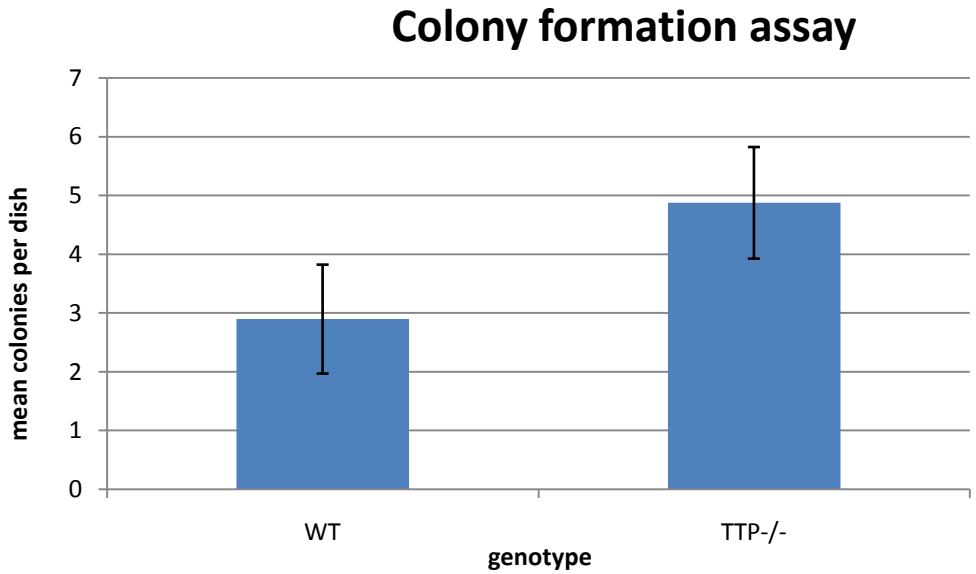


Fig 35: Colony formation assay; mean values for WT and $TTP^{-/-}$ cells normalized to the percentage of $B220^{+}/CD34^{hi}$ cells analyzed via FACS. TTP knock-out cells displayed an enhanced ability to form colonies after *v-abl* transformation.

The colony formation assay showed that TTP-deficient cells display a 70 % increased tendency to undergo the necessary steps for growth factor idependence compared to wild-type cells.

4.4.5 B-cell lymphoma formation

In order to analyze the potential impact of TTP on tumor formation, we injected *v-abl* transformed B-cells of C57Bl/6 wild-type and $TTP^{-/-}$ mice subcutaneously into the thigh of C57Bl/6 mice. For this procedure, the affected area had to be shaved to allow for more precise injection of the cells. As only a few transformed cell lines were at our disposal in this first experiment, only two wild-type, 432 and 433, and one knock-out, 435, cell lines were used. The cells were injected in 8 week old wild-type mice from the TTP knock-out breeding colony, 2 mice for each the wild-type clone and 4 mice for the knock out cells. The mice were injected with $1x10^6$ cells, measured using a cell counter, and monitored for 8 days.

During the incubation period, the growth of the subcutaneous tumor could be monitored at the shaved area of the thigh. After 8 days, the mice were killed and the tumor was removed surgically. In some cases, the tumor infiltrated the skin making isolation of the tissue difficult.

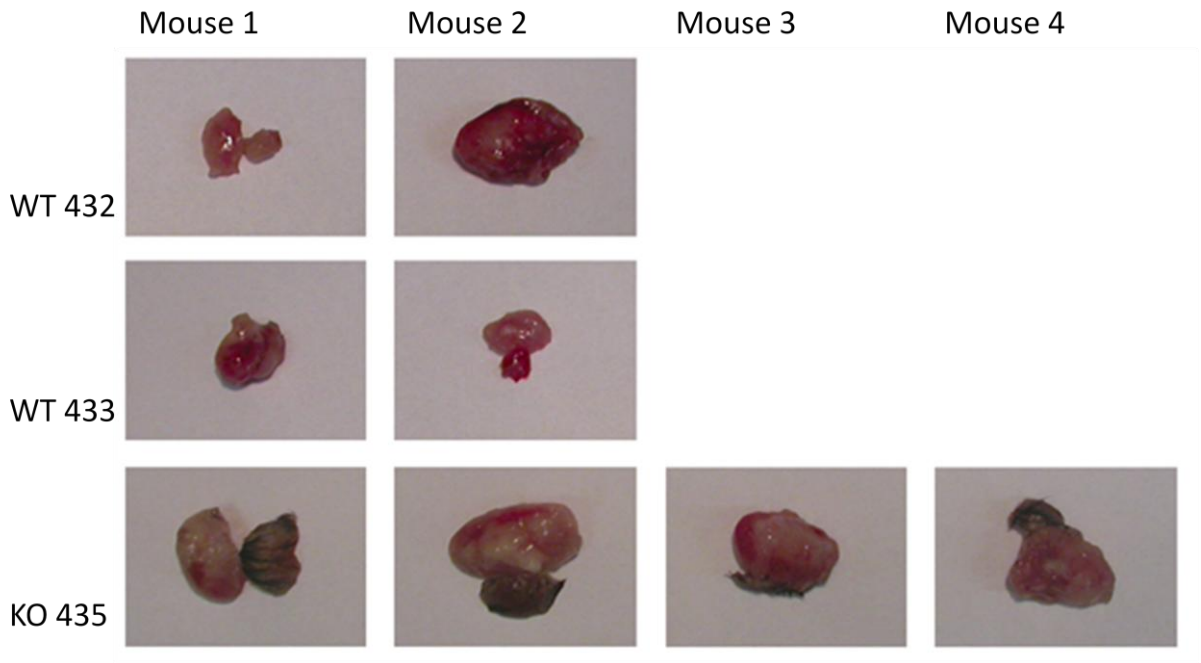


Fig36: B-cell tumors 8 days after injection with $1x10^6$ cells. Tumors originating from B-cell line KO 435 were significantly larger than the tumors resulting from injection with the two wild-type cell lines. (The first tumor on

the right side still contained some surrounding tissue and was therefore not included in the analysis of the experiment; tumors of cell line KO 435 contained some skin tissue due to infiltration of the tumor)

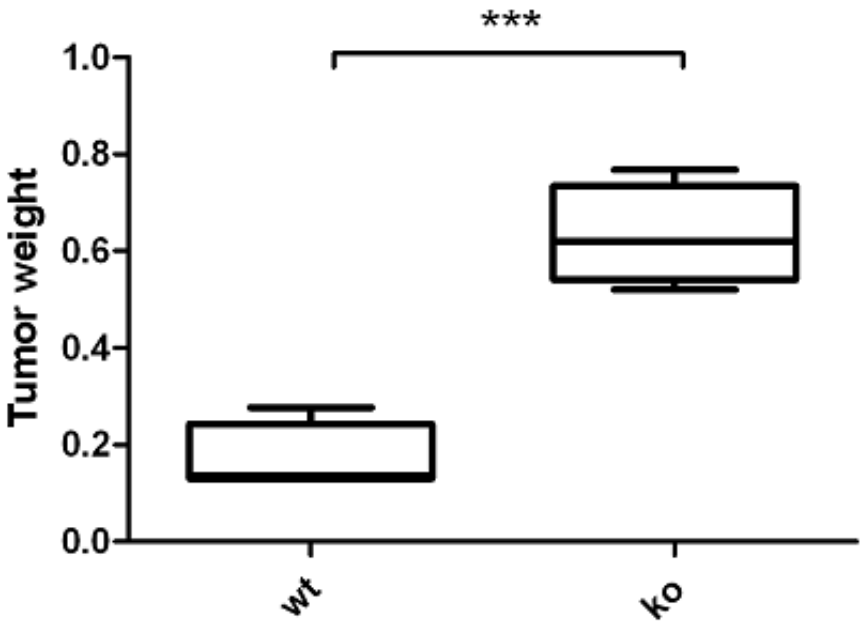


Fig 37: B-cell tumor weight [g] 8 days after B-cell injection. Value for wild-type (wt) is the mean value of clones WT432 (mouse1) and WT433 (mouse1 and mouse2), TTP knock-out (ko) value includes all 4 mice of clone KO435.

The B-cell lymphoma formation showed a distinctly enhanced tumor growth for the TTP-deficient cells compared to the wild-type cells. As only one $TTP^{-/-}$ clone could be used for this experiment, the effect may be caused by the ability of this particular cell line to proliferate rapidly. To further evaluate the effect of TTP on tumor growth in this B-cell lymphoma model, further studies using more a larger number of cell lines are necessary.

5. Discussion

5.1 Analysis of TTP target candidates

During the course of this thesis, new TTP targets have been characterized. The selective analysis of several TTP target mRNAs through RNA-immunoprecipitation showed the ability of TTP to bind to the mRNAs of IL1α, IL6, IL12β and Cxcl2. This finding indicates that TTP can directly or indirectly bind to the selected mRNAs. Additionally, an electrophoretic mobility shift assay (EMSA) was performed (Kratochvill et. al. 2010 manuscript submitted) which showed the ability of selected target AREs to compete with the established TTP binding motif of TNFα for binding to TTP. The EMSA verified the hypothesis that TTP can bind to the 3´ UTR of the target candidates although other criteria are still needed to confirm the targets.

The analysis of the 3´ UTR sequences of the putative TTP targets showed that even though all mRNAs analyzed contained AREs to some extent, only the mRNA of Cxcl2 displayed the established TTP binding motif of overlapping class II AREs. This analysis was supported by the findings of the RNA-immunoprecipitation in which the mRNA of Cxcl2 was the most reliable target. The putative TTP target mRNA IL1α contained only three UAUUUAU heptamers although these motifs were located in an AU-rich region of the 3´ UTR. Even though TTP-binding is not exclusive for the (A/U)UAUUUAU(A/U) nonamer, the heptamer and the pentamer (described in Results 4.1) display significantly reduced binding affinities to TTP [80]. This suggests that mRNAs which do not contain the overlapping nonamers ideal for TTP-binding require a higher amount of active TTP in the cell to be destabilized than the TNFα mRNA.

Due to the binding of the selected mRNAs to TTP *in-vivo* and *in-vitro* these mRNAs were classified as putative TTP targets. As such, we expected that these mRNAs would be more abundant in the TTP-deficient cells because of reduced mRNA decay. The analysis of the mRNA levels revealed that only the mRNA of Cxcl2 showed an increase in the TTP knock-out cells while the mRNAs of IL1α, IL6 and IL12β even displayed reduces levels. This finding can be explained by secondary effects resulting from the overtly disregulated cytokine production in TTP-deficient cells. For example, the increased TNFα in the supernatants of

$TTP^{-/-}$ cells might cause hyperactivation of the TNFα signaling pathway that in turn could activate or inhibit various pathways involved in the expression of other cytokine genes. Although this finding further emphasized the role of Cxcl2 as a target for TTP, additional experiments were needed for target confirmation.

As TTP activity causes accelerated decay of its target mRNAs, we looked at the effect of TTP on the mRNA stability of our putative targets (Kratochvill et. al. 2010 manuscript submitted). In this assay, we stimulated murine wild-type and TTP-deficient macrophages with LPS and terminated all cellular transcription through actinomycin-D-treatment. The mRNA decay was then measured in a 45 minute timeframe through qRT-PCR analysis. This assay showed no change in mRNA decay of the putative TTP targets in the TTP-deficient cells. The increased TTP-dependent decay described for TNFα mRNA [71, 83] could not be observed in the target candidates. The fact that the target mRNA remained equally stable in wild-type and $TTP^{-/-}$ cells could be explained by the longer half-life of the target candidates compared to TNFα. Although a longer time frame of the decay assay (i.e. several hours instead of 90 min) might have revealed decay differences between wild-type and $TTP^{-/-}$ cells such an assay is not advisable due to the adverse effects of prolonged treatment of cells with actinomycin-D.

Another possible reason for the magnitude in which the target mRNA stabilities were affected is the specificity of TTP-binding to the respective AREs. The 3´ UTR of the TNFα mRNA contains the ideal TTP binding site which is characterized as multiple overlapping AUUUA pentamers. In contrast, the candidate target mRNAs contain a smaller number of the pentamer AREs so that the amount of active and/or available TTP may be a more limiting factor in the destabilization of these mRNAs.

As mentioned above, one possible method of analyzing the decay of long lived TTP target mRNAs would be by the expansion of the timeframe of the decay experiment. However, due to the complete transcription blockage by actinomycin-D the cells become gradually depleted of unstable proteins. These proteins may comprise phosphatases or nucleases. The lack of phosphatases would increase activity of kinase-regulated pathways whereas the depletion of nucleases would result in an increased stability of nucleic acids, e.g. RNA. For these reasons, a prolonged treatment with actinomycin-D should be avoided to reduce the side effects of the act D treatment to a minimum.

To analyze the influence of TTP on the decay of the putative TTP targets, we chose to pharmacologically inhibit the activity of p38 MAPK (see introduction 2.3.7). For this

purpose, the inhibitor SB203580 was added at the point of transcription termination resulting in a rapid activation (dephosphorylation) of TTP. This method gave us the opportunity to induce TTP activity leading to an experimental procedure in which TTP mediated destabilization of mRNA can be monitored regardless of differences in mRNA half-life. The modified experiment including SB203580 revealed a TTP-dependent decrease in mRNA stability of the target candidate mRNAs.

The results obtained from the mRNA stability assay demonstrated that the target candidate mRNAs are destabilized by TTP. However, the use of actinomycin-D as a transcriptional terminator and the inhibition of p38 MAPK were artificial factors in the experiment. To further substantiate the decay data, we needed other, independent methods of showing the TTP-dependent effect on the stability of the putative TTP target mRNAs.

5.1.1 TTP-dependent reduction in reporter luciferase activity

We analyzed TTP-dependent effects on the expression of a firefly luciferase gene under the control of the complete 3´ UTRs of IL1α, IL6, IL12β, Cxcl2 and the established TTP target TNFα. The reporter constructs were cloned and the binding of TTP to the mRNA was confirmed using RNA immunoprecipitation for the target vectors IL6 and IL12β (Figure 17). In this system, pharmacological inhibition of p38 MAPK was not necessary as active TTP was introduced through an expression plasmid.

The analysis of the reporter gene expression using firefly luciferase activity revealed a significant impact of TTP. The relative luciferase activity was reduced to 20 % for the targets IL6 and IL1α if the TTP expression vector was co-transfected (Figure 15). The other target candidates also displayed a 50 % reduction in luciferase activity while the luciferase vector lacking a target 3´ UTR did not display any TTP-dependent change. These results indicate a TTP-dependent reduction in the expression of the reporter protein when it is under the control of the 3´ UTR of the target candidates.

Although the luciferase reporter assay showed a TTP-dependent effect on the luciferase activity, it doesn't reveal a possible mechanism. As only the luciferase activity is measured, there is no possibility to directly obtain information whether the TTP-dependent activity decrease is caused by a decrease in mRNA stability. More importantly, the possibility that the

observed effect is caused by a TTP-dependent reduction of mRNA translation cannot be excluded.

As the luciferase system was not suitable for the analysis of RNA stability, a rabbit β-globin reporter [81, 84] system was generated.

5.1.2 TTP-dependent destabilization of 3´ UTR-containing reporter constructs

The stability assay through the use of the rabbit β-globin reporter had the advantage that we were independent of actinomycin-D transcriptional termination and pharmacological p38 MAPK-inhibition. Instead of the total termination of all cellular transcription through actinomycin-D the tetracycline-inducible regulation gave us the opportunity to selectively terminate the transcription of the reporter gene. In addition, no further manipulation of cells was needed for the induction of TTP expression (e.g. stimulation by LPS or treatment with the p38 MAPK inhibitor) since TTP was introduced through the co-transfection of a TTP expression plasmid. These characteristics of the reporter assay provided us with a method which is independent of the mRNA stability assays performed using primary macrophages.

Using the reporter assay, we have shown a TTP dependence in the mRNA decay of the rabbit β-globin reporter vectors under the control of target candidate 3´ UTRs (Figures 21 and 22). The qRT-PCR showed that TTP expression markedly destabilizes the reporter mRNAs under the control of IL1α, IL6 and Cxcl2 3´ UTRs while having no effect on the negative control.

Although the reporter assay generated data which is consistent with other experiments of this project, we have to be aware of the possible problems resulting from reporter systems. The fact that we fused the complete 3´ UTR sequences to the rabbit β-globin gene could result in a changed secondary structure of the mRNA leading to different binding properties compared to the endogenous target mRNA. Additional problems could occur as the reporter assay depends on the transfection of two plasmids into the HeLa cells. During this process, a possible difference in the plasmid's ability to be taken up in the cell can occur due to the different lengths of the target constructs (there is a significant difference between IL1α and IL6 3´ UTR length).

In this project we have performed two separate assays using different methods to show the influence of TTP on the stability of its target mRNAs. Because of the completely different circumstances of the two experiments we now have separate results, both leading to the

conclusion that the candidates are indeed post-transcriptionally-regulated by the mRNA decay mediating factor TTP. We have now shown that IL1α, IL6 and Cxcl2 mRNA can be bound and destabilized by TTP.

5.1.2.1 Interleukin 1-alpha (IL1α)

The IL1 family consists of 3 proteins IL1α, IL1β and IL receptor antagonist [85]. IL1α is generally retained within the cell or expressed on the cell surface and consequently it is believed to function as an autocrine messenger. Binding of IL1α to specific receptors on the surface of target cells leads to intracellular signal transduction resulting in gene expression. IL1α can bind to two receptors. While IL1R1-binding leads to changes in cellular gene expression, IL1R2 serves as a decoy receptor. IL1α is produced in monocytes and macrophages and is known to be involved in hematopoiesis, the immune response and inflammatory processes.

In the course of this project we have shown that TTP has a significant influence on IL1α mRNA stability. Although IL1α has the fewest AUUUA pentamers in its 3´ UTR and does not possess a class 2 ARE, our experiments have shown a TTP dependence in its mRNA stability. The fact that TTP has been shown to bind to IL1α mRNA and mediate its decay in both primary macrophages and in the rabbit β-globin reporter assay, shows that IL1α mRNA is a target for destabilization by TTP.

5.1.2.2 Interleukin 6 (IL6)

IL6 is a secreted protein [86] that has anti-inflammatory and pro-inflammatory functions. It is secreted by T-cells and macrophages and stimulates energy mobilization in fatty tissue increasing body temperature.

IL6 mRNA was already reported to be a target for TTP [67]. In this publication, a similar experiment as the reporter assay described in this thesis was performed, with the exception that transcriptional termination was induced through actinomycin-D-treatment instead of a reporter-specific method. Additionally, we could show the mRNA decay of IL6 using the more sensitive method of qRT-PCR instead of a quantification of a Northern-blot. The combination of the various methods performed over the course of this thesis confirms the

hypothesis that the mRNA of IL6 is a target of TTP. We have shown that IL6 mRNA can be bound and destabilized by TTP. IL6 mRNA displays 4 AUUUA pentamers and one nonamer but like IL1α is has no overlapping class 2 ARE in its 3´ UTR.

5.1.2.3 Chemokine (C-X-C motif) ligand 1 (Cxcl2)

The candidate Cxcl2 displayed the strongest destabilization by TTP. The Cxcl2 3´ UTR contains 8 AUUUA pentamers including one class 2 ARE (class 2D [38]). In the luciferase activity assays, Cxcl2 appears to only be weakly affected by the presence of TTP, but as described in 4.2.2 Cxcl2-containing mRNA was very unstable even without TTP. During immunoprecipitations using primary murine macrophages and anti TTP antibodies (Kratochvill et. al. 2010 manuscript submitted), Cxcl2 provided the most intense and reliable signal of all target candidates. Additionally to the stability assays using p38 MAPK inhibition and reporter systems, Cxcl2 mRNA stability has also shown to be affected in primary macrophages without p38 inhibition if the correct time frame was used. Taken together, all results regarding Cxcl2 point to the mRNA of Cxcl2 being a target for degradation by TTP.

5.1.2.4 Interleukin 12-beta (IL12β)

IL12β is the only target candidate for which no rabbit β-globin mRNA stability assay was performed. Previous experiments show a TTP-dependent decrease in reporter luciferase activity and the ability of TTP to bind to IL12β mRNA. mRNA stability assays in primary macrophages using p38 inhibition show an influence of TTP an mRNA stability and the IL12β ARE has been shown to successfully compete with the TNFα ARE for TTP-binding in EMSA experiments. IL12β has 6 AUUUA pentamers in its 3´ UTR, 3 of them in U rich regions.

Conclusively, we can state that we have found novel mRNA targets for destabilization by TTP. In addition, a reliable approach for further target characterizations has been established. As some of our new targets do not contain the specific class II ARE that was described as a prequisite for TTP-binding, further analysis of the possible effect of flanking nucleotide sequences are an interesting future prospect of the research on TTP.

5.2 TTP in hematopoiesis

We have shown through FACS analysis, that TTP-deficient mice of C57Bl/6 background have an elevated level of GR-1^{+} granulocytes in the peripheral blood (Figure 23). This increase has already been published for $TTP^{-/-}$ mice of mixed background [32]. Furthermore we analyzed the effect of TTP deficiency on the development of B-cells (Figures 27-32). We could detect small decreases in late B-cell stadiums in spleen, lymph nodes and peripheral blood but the bone marrow was unaffected in all B-cell populations. As the B-cell development was primarily analyzed to exclude effects on the *v-abl*-induced transformation and the affected tissue showed no significant change in B-cell populations we were ready to proceed to the transformation of the B-cells.

5.2.1 TTP has an effect on B-cell transformation by *v-abl*

We have shown that bone marrow from TTP-deficient mice displays an increased tendency to form colonies through *v-abl*-induced transformation (Figures 33-35). The reason for this increase was not yet addressed but the role of TTP as a tumor suppressor suggests a possible role of the stabilization one or more TTP target mRNAs [72]. As transformation of the B-cells was achieved through the *v-abl* oncogene, we can exclude a role of the TTP target IL3, as the transformation procedure renders the cells independent of IL3.

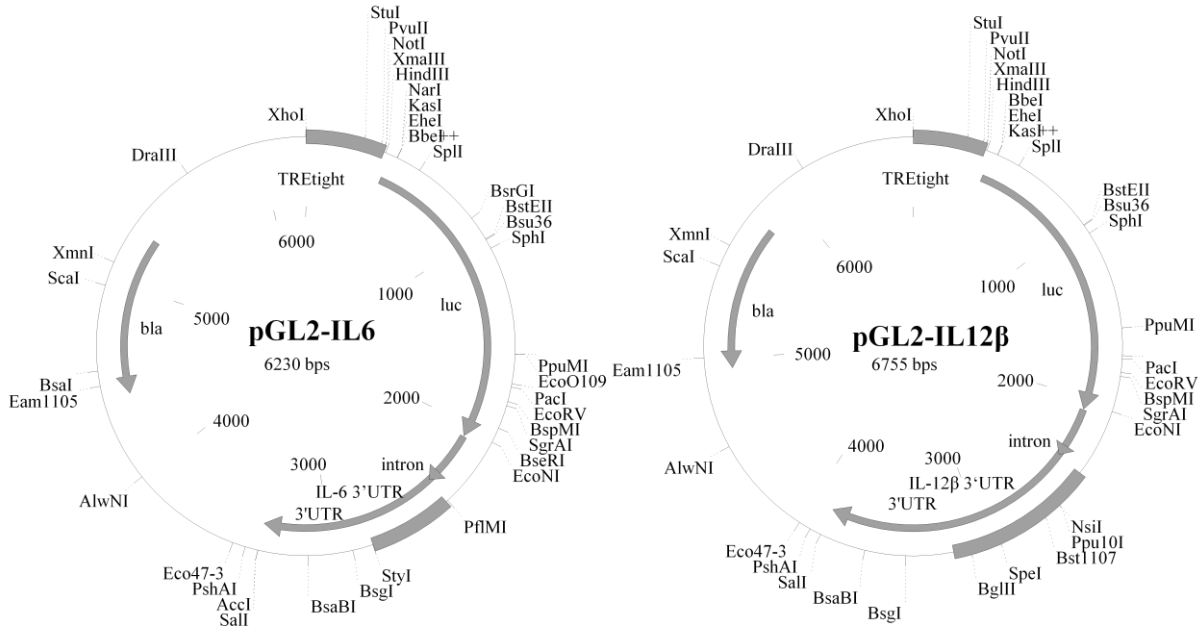
5.2.2 TTP-dependent effects on B-cell lymphoma formation

Through subcutaneous *in-vivo* injections of transformed B-cells, originating from wild-type C57Bl/6 and TTP-deficient C57Bl/6 mice, we have shown a TTP dependence in tumor growth. As several important experiments have not been completed yet, the exact reason for this increase in tumor growth of TTP-deficient B-cells, we can only speculate by which mechanism TTP interacts with tumor growth. Several publications describe TTP as a tumor suppressor [72, 74-75]. Because of the method with which the B-cells were transformed, we can exclude an effect of interleukin 3 and a possible connection of IL7, a hematopoietic growth factor that stimulates the proliferation of cells of a lymphoid linage, to TTP has not been shown. One possible route for TTP to influence tumor growth is the stabilization of VEGF resulting from TTP deficiency. Further analysis of the tumors is necessary to confirm or rule out VEGF as the effector of TTP in this tumor model, preferably through histology of

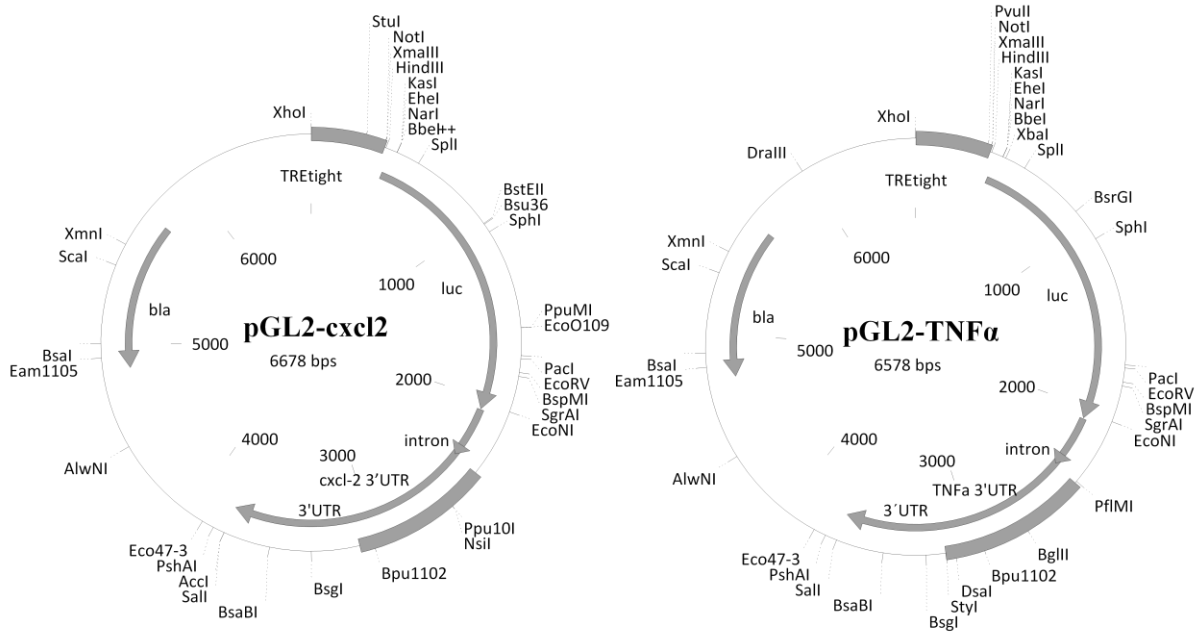
the tumors highlighting vascularization. One cannot exclude a possible effect of TTP on cell proliferation, but these assays have not been carried out yet. Additionally, the *in-vivo* tumor formation assay was only carried out using 1 TTP-deficient B-cell line so cell line specific differences regarding cell proliferation have to be taken into consideration when interpreting this result.

Conclusively, the tumor formation assay supports the idea that TTP functions as a tumor suppressor but additional work has to be done to understand the mechanism of how TTP influences the formation of B-cell lymphomas.

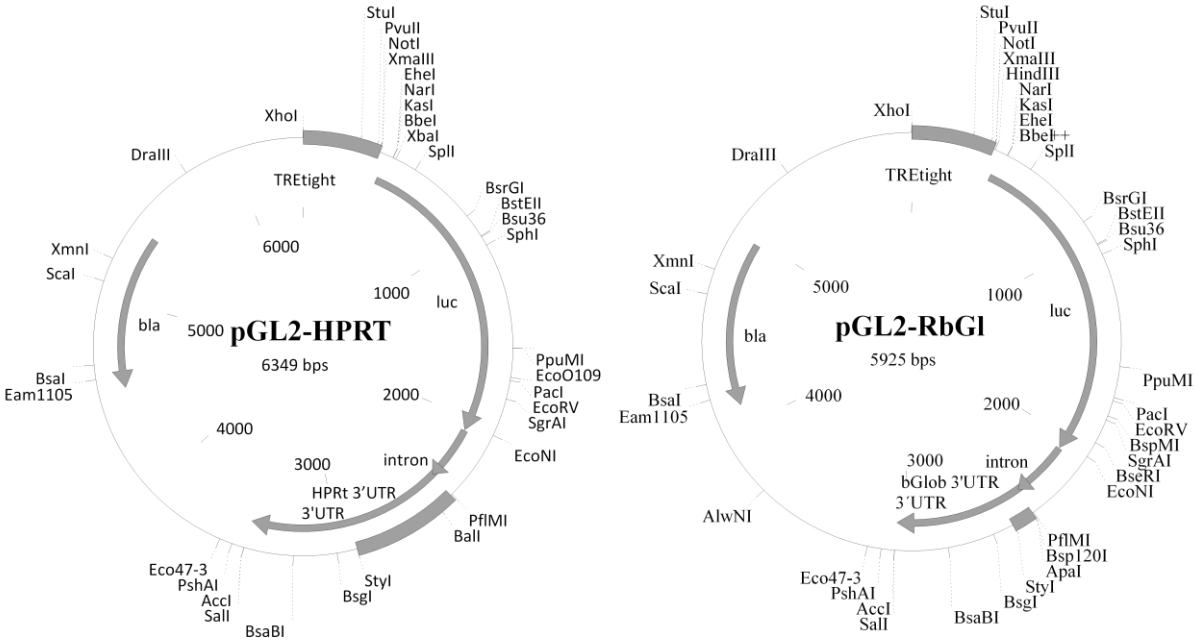
6. Supplementary figures



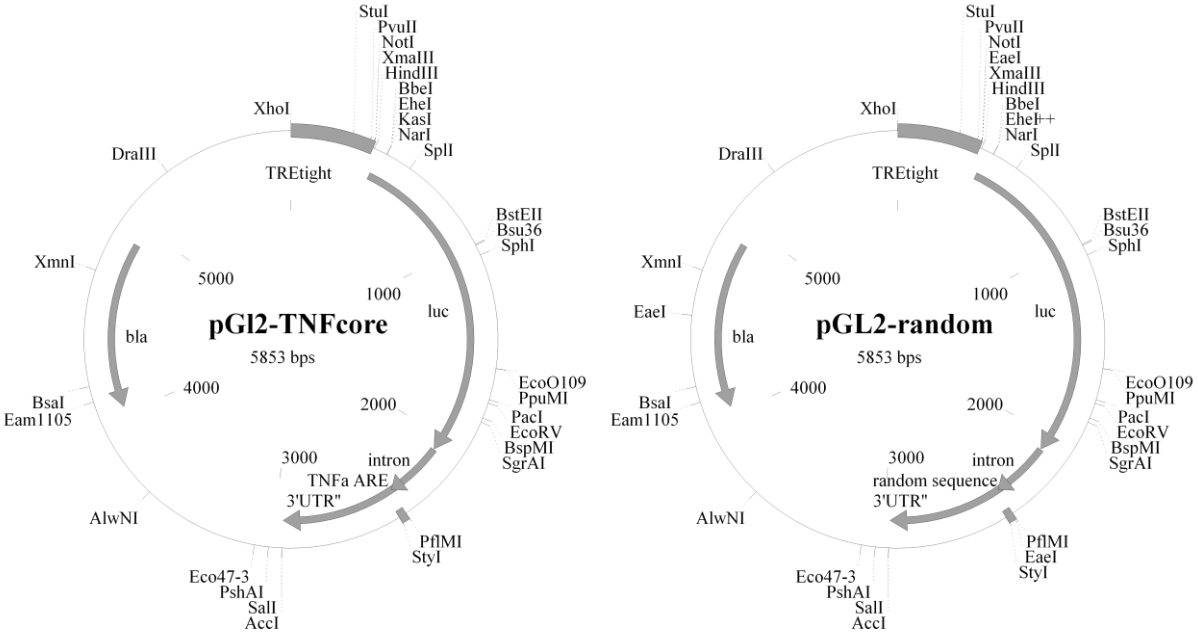
Supp. Fig. 1: pGl2-TRE vectors with IL6 and IL12β 3´ UTR insertions



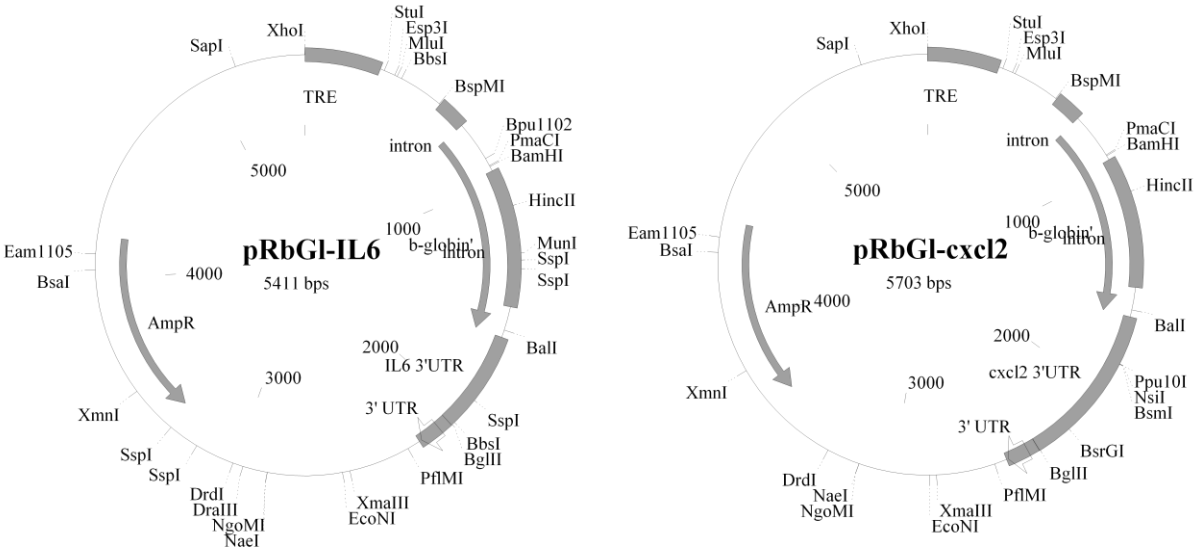
Supp. Fig. 2: pGl2-TRE vectors with Cxcl2 and TNFα 3´ UTR insertions



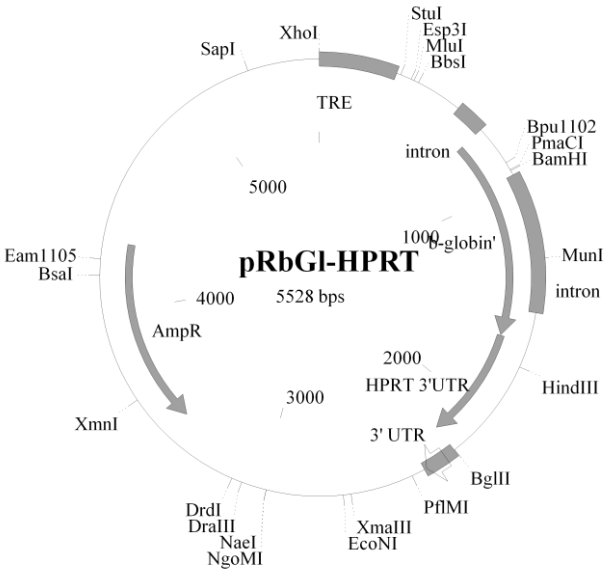
Supp. Fig. 3: pGl2-TRE vectors with HPRT and rabbit β-globin 3´ UTR insertions



Supp. Fig. 4: pGl2-TRE vectors with TNFα core ARE insertion and a pGL2-TRE vector with an inverted (A/T to C/G) TNFα ARE



Supp. Fig. 5: pTET-BBB vectors with IL6 and Cxcl2 complete 3´ UTR insertions at the BglII restriction site



Supp. Fig. 6: pTET-BBB vector with the HPRT complete 3´ UTR inserted at the BglII restriction site

7. References

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8. Appendix

8.1 *Curriculum Vitae*

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8.2 Acknowledgements

I owe a great deal to my colleagues, who have continually supported me through sharing their own research experience and provided valuable advice during the course of my diploma thesis. I would like to extend thanks to our group leader, Professor Pavel Kovarik, PhD, for giving me the possibility to work on this project and for assembling a team which provided a positive atmosphere at the working place.

I am especially thankful to my supervising tutor, Franz Kratochvill, PhD, who helped me overcome obstacles, introduced me to the working methods and provided me with countless pieces of good advice. I would like to thank my colleagues Mag. Nina Gratz, Barbara Schaljo, PhD, Mag. Joanna Bancerek, Mag. Ivana Mikulitz, Harald Hartweger and Stefan Badelt for their help and wish everyone the best for their scientific career. Additionally, I would like to thank Professor Thomas Decker, PhD, Professor Manuela Baccarini, PhD, Professor Emmanuelle Charpentier, PhD, and their groups for assistance and for providing input and inspiration. I would also like to thank Professor Veronika Sexl, PhD, and her group for valuable input regarding the tumor-formation project and for granting me access to their facilities. Eva-Maria Putz and Nina Neugebauer provided me with all the help I could ask for and I owe them a great deal for their assistance.

I am deeply thankful for the continued support of my family and friends, who helped me tremendously during the course of this thesis.