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Tristetraprolin Is Required for Full Anti-Inflammatory Response of Murine Macrophages to IL-10

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IL-10 is essential for inhibiting chronic and acute inflammation by decreasing the amounts of proinflammatory cytokines made by activated macrophages. IL-10 controls proinflammatory cytokine and chemokine production indirectly via the transcription factor Stat3. One of the most physiologically significant IL-10 targets is TNF-α, a potent proinflammatory mediator that is the target for multiple anti-TNF-α clinical strategies in Crohn’s disease and rheumatoid arthritis. The anti-inflammatory effects of IL-10 seem to be mediated by several incompletely understood transcriptional and posttranscriptional mechanisms. In this study, we show that in LPS-activated bone marrow-derived murine macrophages, IL-10 reduces the mRNA and protein levels of TNF-α and IL-1-α in part through the RNA destabilizing factor tristetraprolin (TTP). TTP is known for its central role in destabilizing mRNA molecules containing class II AU-rich elements in 3′ untranslated regions. We found that IL-10 initiates a Stat3-dependent increase of TTP expression accompanied by a delayed decrease of p38 MAPK activity. The reduction of p38 MAPK activity releases TTP from the p38 MAPK-mediated inhibition, thereby resulting in diminished mRNA and protein levels of proinflammatory cytokines. These findings establish that TTP is required for full responses of bone marrow-derived murine macrophages to IL-10.


One of the key players in immune homeostasis is IL-10, a cytokine that was discovered 18 years ago as a T cell-secreted factor that inhibited cytokine production by Th1 cells (1). Over the years, it became clear that IL-10 is produced by many cell types including T and B cells, macrophages, dendritic cells, mast cells, keratinocytes, or epithelial cells (2). It is generally considered that the main biological function of IL-10 is to limit or shut down inflammatory responses. This notion is supported by the phenotype of IL-10-deficient mice that develop severe inflammatory bowel disease due to spontaneous chronic inflammation and are susceptible to endotoxin treatment because of acute overproduction of proinflammatory cytokines (3, 4). Many of these phenotypes can be recapitulated by T cell-specific deletion of the IL-10 gene, indicating that T cell-derived IL-10 is primarily responsible for chronic inflammation (5). Under conditions of acute inflammation, the main source of IL-10 are macrophages and dendritic cells (6). On the cellular level, IL-10 inhibits production of proinflammatory cytokines and regulates differentiation and proliferation of various immune cells. These effects depend entirely on the activation of the transcription factor Stat3 by IL-10 (7–11). The events downstream of Stat3 activation that mediate the anti-inflammatory functions of IL-10 remain an area of active research. It is becoming increasingly clear that multiple mechanisms mediate the IL-10 function. On the one hand, IL-10 inhibits transcription of a subset of proinflammatory genes (12, 13). In a mouse model lacking 3′ untranslated regions (UTR)6 in the Tnf gene, the transcriptional mechanism was found to play a major role in IL-10 responses (13). On the other hand, IL-10 was also reported to act posttranscriptionally by increasing the rate of mRNA decay of inflammatory cytokines such as TNF-α or by inhibiting translation (12, 14, 15). The posttranscriptional regulation depends on 3′ UTR containing AU-rich elements (AREs) (16). However, far less is known about the IL-10-regulated effector genes that control the anti-inflammatory response. Several candidates have been described, but so far none have been shown to account for the majority of the anti-inflammatory effects (17). IL-10 was also demonstrated to up-regulate the dual-specificity phosphatase 1 (DUSP1) in LPS-stimulated macrophages, causing a more rapid inactivation of p38 MAPK (18). The reduction of p38 MAPK activity may lead to a decreased stability of ARE-containing mRNAs and/or reduced transcription by transcription factors that depend on p38 MAPK. Recently, the transcriptional repressor ETV3 and

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4 Abbreviations used in this paper: UTR, untranslated region; ARE, AU-rich element; DUSP1, dual-specificity phosphatase 1; BMDM, bone marrow-derived macrophage; TTP, tristetraprolin; WT, wild type; qRT-PCR, quantitative RT-PCR; fwd, forward; rev, reverse; SOCS, suppressor of cytokine signaling; SB, SB203580.
the corepressor SBN02 were characterized as IL-10/Stat3-induced genes that may contribute to the anti-inflammatory IL-10 effects (19). An important aspect of the above-mentioned studies that remains to be resolved is how IL-10 inhibits specifically only a subset of inflammatory genes. These studies indicate that yet unknown IL-10/Stat3 target genes need to be discovered to complement the current knowledge about the anti-inflammatory effects of IL-10.

In this study, we demonstrate that IL-10-mediated reduction of TNF-α and IL-1α production by LPS-treated mouse bone marrow-derived macrophages (BMDMs) is less efficient in cells lacking the RNA-destabilizing factor tristetraprolin (TTP). TTP is known to bind and destabilize mRNAs of several proinflammatory cytokines (e.g., TNF-α) containing class II AREs in their 3′ UTRs (20). TTP facilitates degradation of the bound mRNA by initiating the assembly of RNA decay machinery (21, 22). The RNA degradation facilitates degradation of the bound mRNA by initiating the assembly of RNA decay machinery (21, 22). The RNA degradation activity of TTP is negatively regulated by p38 MAPK-dependent phosphorylation of TTP-Flag fragment was cloned into the pGL2-basic (Promega) containing a tetracycline-responsive element (TRE) in the promoter. The resulting plasmid pTRE-TTPfl was transfected into HeLa-Tet-off cells (BD Clontech) using nucleofection (Amaxa). After transfection, the cells were incubated overnight in medium with (no TTP expression) or without (TTP expression) tetracycline (1 μg/ml).

**ELISA**

For ELISA, BMDMs were seeded the day before use at 2 × 10^5 cells/well in a 24-well plate. Supernatants were diluted 1/8 in DEMEM (for TNF-α) or 1/2 (for IL-1α) and cytokines were assayed using ELISA kits (R&D Systems) according to the manufacturer’s instructions.

**Quantitative Western blot**

After treatment, whole cell extracts were prepared and assayed by Western blotting as described elsewhere (29). Detection and quantitation of signals were performed using the infrared imaging system Odyssey (LI-COR Biosciences).

**Quantitation of gene expression by quantitative RT-PCR (qRT-PCR)**

Total RNA was isolated using TRIzol reagent (Invitrogen). Reverse transcription was performed with Moloney murine leukemia virus reverse transcriptase (Fermentas). The following primers were used for HPRT, the housekeeping gene used for normalization, HPRT forward (fwd) 5′-GGATTGGAATTAGTGGTTGCTAT-3′ and HPRT reverse (rev) 5′-ACACCTGCTGTAATTTTCTGCGA-3′; for TTP, TTP fwd 5′-CTCTGGCATCTAGGAGGCT-3′ and TTP rev 5′-GATGGAGTCCCGAGGTATGTC-3′; for TNF-α, TNF-α fwd 5′-CAAAATTAGAGTGAACACCTGCG-3′ and TNF-α rev 5′-GAGATCCATGCCGTTGGC-3′; for suppressor of cytokine signaling (SOCS) 3, SOCS3 fwd 5′-GCTCCGAAAGACGATCTACCAGC-3′ and SOCS3 rev 5′-AGTAGAATCCGCTCTTCGCA-3′; and for TTP primary transcript, TPTt fwd 5′-GACTGGCAAGCTCGTGAACT-3′, TPTt rev 5′-TCTGCTCTACAGCTCGGAAGAGTGTA-3′. For determination of mRNA decay by qRT-PCR, the primers TNF-α fwd 5′-TCTGCTCTACAGCTCGGAAGAGTGTA-3′ and TNF-α rev 5′-ATGTTGAGTCCCGAGGTATGTC-3′ were used. For HPRT, the primer set Q010113505 from Qiagen was used. Amplification of DNA was monitored by SYBR Green (Molecular Probes (30).

**RNA EMSA**

To prepare extracts, cells were washed with cold PBS and lysed in buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 30 mM NaPP3, 50 mM NaF, 2 mM EDTA, 1% Triton X-100, and a protease inhibitor mixture (Roche). Extracts were cleared by centrifugation at 15,000 rpm. Twelve-microliter cell extracts (30 μg of protein) from RAW 264.7 cells or 5 μg of cell extracts (15 μg of protein) from pTRE-TTPfl-transfected HeLa-Tet-off cells were incubated with 0.5 μl of poly(U) RNA (100 μg/ml), 0.5 μl of Cy5.5-labeled TNF-α ARE (1 pmol/μl), 1 μl of Ribonuclease Inhibitor (Fermentas), and 2.5 μl of 5× Gel shift buffer (200 mM KCl, 5 mM MgCl2, 0.5 mM EGTA, 2.5 mM DT, 100 mM HEPES-KOH (pH 7.9), and glycerin 50% (v/v)) for 20 min at room temperature. For supershift assays, 0.5 μl of TTP antisem was added. Samples were then separated on a 6% polyacrylamide gel. The Cy5.5 signal was detected and quantified using the infrared imaging system Odyssey (LI-COR Biosciences). Poly(U) RNA and Cy5.5-labeled TNF-α ARE RNA were purchased from Microsynth. The sequence for Cy5.5-labeled TNF-α ARE was as follows: 5′-AAUUUUUUUUAUUUAUUUUUUUUUUUUUA-3′.

**Statistical analysis**

Data from independent experiments were analyzed using univariate linear regression models and the SPSS program. For qRT-PCR normalized copy numbers and for ELISA pg/ml were log-transformed. Residuals were plotted, visually inspected, and tested for normality. Design matrices were specified such that the coefficients for the relevant comparisons could be calculated, e.g., between the baseline and induced states and between genotypes. The only significant levels are reported.

**Results**

**IL-10 increases TTP expression in LPS-treated macrophages in a Stat3-dependent manner**

TTP expression has been reported to be controlled by the transcription factors Stat1 (in response to IFNs) and Stat6 (in response to IL-4) (27, 31). Affymetrix analysis revealed that LPS-induced TTP expression is further enhanced by IL-10 (data not shown) at
protein levels (Fig. 1B) compared with LPS treatment alone. The IL-10-mediated increase in TTP expression required Stat3 since in LysMcre-Stat3fl/fl cells the TTP levels remained unchanged upon IL-10 treatment. The expression of Stat3 was reduced by 90% in LysMcre-Stat3fl/fl cells (Fig. 1B), thereby confirming the deletion efficiency by LysMcre. Analysis of TTP primary transcripts by PCR (Fig. 1C) and nuclear run-on assays (data not shown) revealed that the IL-10-mediated induction of TTP was caused by increased transcription.

These findings document that IL-10-activated Stat3 increases expression of TTP in LPS-stimulated macrophages. IL-10 alone only weakly increased TTP transcription, and this induction was not sufficient to generate detectable levels of TTP protein. This way of regulation resembles that of IFN-induced TTP expression, which was shown to be activated by IFNs and the IFN-activated Stat1 only if p38 MAPK signaling (e.g., LPS) is stimulated in parallel (27). p38 MAPK is known to increase by yet unclear mechanisms the transcriptional activity of several Stat members (32–34).

**TTP is required for full inhibitory effect of IL-10 on TNF-α and IL-1α production**

To test whether TTP was an effector of the IL-10 anti-inflammatory responses, we measured the amount of secreted TNF-α by ELISA in BMDMs from TTP−/− and control WT littermates that were treated with LPS with or without pretreatment with IL-10. We measured TNF-α production at 6, 8, and 10 h after LPS stimulation. After 10 h, TNF-α was diminished by IL-10 to ~20% in control cells, whereas in TTP−/− cells only a reduction to 60% was achieved (Fig. 2A). Similar results were obtained if cells were treated simultaneously with LPS and IL-10 (Fig. 2B), although the contribution of TTP to the IL-10 response was higher in the pretreatment protocol (Fig. 2A). These data show that TTP contributes to IL-10-mediated inhibition of TNF-α cytokine production.

To investigate whether the incomplete IL-10-mediated inhibition of TNF-α production in TTP−/− BMDMs resulted from differences in mRNA amounts or in mRNA decay, we analyzed the amount of TNF-α mRNA in LPS-stimulated TTP−/− and control BMDMs with or without pretreatment with IL-10. In WT cells, IL-10 caused a reduction of TNF-α mRNA to 40% of the amount present in cells treated with LPS alone, whereas in TTP−/− cells IL-10 caused a reduction to only 80% of the level in cells treated with LPS alone (Fig. 2C). In these experiments, TNF-α mRNA was measured after 2 h of LPS treatment since the amount of TNF-α mRNA peaks at this time point (Ref. 13 and data not shown). To further illustrate the role of TTP in IL-10-mediated decrease of TNF-α mRNA, we analyzed the rate of TNF-α mRNA decay in LPS-stimulated TTP−/− and WT BMDMs with or without IL-10 pretreatment. Transcription was stopped after 3 h of LPS stimulation by addition of actinomycin D, and the degradation of TNF-α mRNA was followed in 15-min intervals for a total of 45 min after imposing the transcriptional stop (Fig. 3A). In LPS-treated control BMDMs, the decay rate of TNF-α mRNA was increased 2.5-fold by IL-10 treatment (half-life 82 min without IL-10 = 32 min; 12, with IL-10 = 13 min). In LPS-treated TTP−/− BMDMs, the residual TNF-α mRNA decayed with a 2.5-fold longer half-life (119 min) compared with WT. This difference is similar to the one reported previously (35). Importantly, IL-10 treatment of LPS-stimulated TTP−/− cells did not increase the decay rate and the remnant mRNA levels were comparable to those in cells treated with LPS alone. LPS-stimulated macrophages produced endogenous IL-10 that is known to mask to some extent the effect of added IL-10 (see Ref. 18). Since TTP was recently
shown to target IL-10 mRNA for degradation (36), we asked whether TTP−/− BMDMs produce more IL-10 after stimulation with LPS. Cytokine measurement revealed that TTP-deficient cells secrete 2- to 3-fold more IL-10 than the WT cells (supplemental Fig. S1A). To assess the contribution of endogenous IL-10 to the IL-10-mediated TNF-α mRNA decay, we compared the TNF-α mRNA levels and decay rates in IL-10−/− and WT cells. IL-10−/− BMDMs expressed 2-fold more TNF-α than WT cells (Fig. 3B). Importantly, the reduction of TNF-α mRNA by exogenous IL-10 was more pronounced in IL-10−/− cells compared with WT cells. The decay rate of TNF-α mRNA was not affected by IL-10 treatment of WT cells stimulated for 1 or 2 h with LPS (Fig. 3C). However, IL-10 accelerated the decay rate in IL-10−/− cells treated for 2 h with LPS, but not for 1 h (Fig. 3D). Therefore, endogenous IL-10 can mask to a certain extent the effect of exogenous IL-10 on the mRNA decay. In addition, the IL-10-mediated increase in mRNA decay rate becomes more apparent at later time points of LPS treatment, consistent with the need for new protein synthesis (e.g., TTP). Note that the duration of LPS treatment in Fig. 3A was 3 h and that the differences in IL-10-imposed inhibition of TNF-α production in TTP−/− vs WT cells increased with time of LPS treatment (Fig. 2, A and B).

To further substantiate the role of TTP in the anti-inflammatory effects of IL-10, we measured the IL-10-mediated reduction of IL-1α protein and mRNA in TTP−/− and WT cells (Fig. 3, E and F). Efficient LPS-stimulated production of IL-1α, a known IL-10 target (15), depends on a second stimulus, such as ATP, that activates the IL-1-processing function of the inflammasome (37). Although IL-1α is not a direct substrate of caspase 1, production of mature IL-1α has been shown to be inflammasome/caspase 1 dependent (37). BMDMs from WT and TTP−/− animals were stimulated with LPS (10 h) and ATP (1 h before the collection of supernatants) in the presence or absence of IL-10. IL-10 caused a decrease of IL-1α protein to 20% of LPS plus ATP-treated WT cells, whereas in TTP−/− cells the IL-1α production was reduced only to 60% of the LPS plus ATP-treated samples (Fig. 3E). Similar differences between WT and TTP−/− cells were determined also for the IL-10-mediated decrease in IL-1α mRNA (Fig. 3F).

To rule out that the absence of TTP affected activation of Stat3 by IL-10 that might result in reduced IL-10 responsiveness of TTP−/− cells, the IL-10-induced tyrosine phosphorylation of Stat3 was determined in LPS-stimulated TTP−/− and WT BMDMs in the presence or absence of IL-10. The level of tyrosine-phosphorylated Stat3 was under all conditions comparable in both genotypes (supplemental Fig. S2A). In addition, the stimulatory effect of LPS was similar in TTP−/− and WT cells as judged by the activation of p38 MAPK (supplemental Fig. S2B). Thus, a different activation of the critical proinflammatory (p38 MAPK) and anti-inflammatory (Stat3) components in the WT and TTP−/− cells could be excluded as a reason for the observed differences in IL-10 responses.

These data establish that TTP plays an important role in IL-10-mediated down-regulation of two critical inflammatory cytokines (TNF-α and IL-1α).

**TTP function in IL-10 responses depends on IL-10-mediated reduction of p38 MAPK activity at later phase of inflammation**

IL-10 and IL-6 are both known to activate Stat3 yet only IL-10 exhibits anti-inflammatory properties (38–40). To examine whether and to what extent IL-6 was able to stimulate TTP expression, BMDMs were treated with LPS with or without IL-6.

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**FIGURE 2.** TTP is required for full IL-10-mediated inhibition of TNF-α production. A. Reduction of LPS-induced TNF-α cytokine production in cells pretreated with IL-10. BMDMs from WT and TTP−/− mice were treated with LPS or pretreated overnight (o/n) with IL-10 followed by stimulation with LPS (ILon/LPS) for indicated time points. Supernatants were collected and analyzed for TNF-α cytokine levels by ELISA. Reduction of TNF-α cytokine levels by IL-10 pretreatment (IL-10 o/n) relative (in percent) to LPS-alone treatment (100%) is depicted. SDs (n = 4) are indicated. B. Effect of simultaneous treatment with IL-10 and LPS on TNF-α cytokine production performed as described in A. Relative amounts of TNF-α cytokine secreted by LPS-treated WT and TTP−/− BMDMs compared with cells simultaneously treated with IL-10/LPS are shown. SDs (n = 3) are indicated. C. Reduction of LPS-induced TNF-α mRNA in cells pretreated with IL-10. WT-BMDMs and TTP-deficient BMDMs (TTP−/−) were treated 2 h with LPS or pretreated overnight with IL-10 followed by LPS addition (IL-10/L) and analyzed for expression of TNF-α by qRT-PCR, normalized to HPRT mRNA. Shown is the reduction of TNF-α mRNA levels after IL-10/LPS treatment in relation to the sample treated with LPS alone. SDs (n = 3) are indicated.

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5 The online version of this article contains supplemental material.
Interestingly, IL-6 was able to increase TTP expression in LPS-treated macrophages almost to the same extent as IL-10 (Fig. 4A). Yet, consistent with the known properties of IL-6, IL-6 was not able to inhibit TNF-α/H9251 (Fig. 4B) and IL-1α/H9251 (Fig. 4C) in either WT or TTP−/− mice. These data also implicate that the up-regulation of TTP expression by IL-10 cannot solely explain the role of TTP in the anti-inflammatory effects of this cytokine. We speculated that the role of TTP in IL-10 responses might be explained by an IL-10-dependent increase in TTP activity. TTP function is known to be negatively regulated by p38 MAPK and its downstream kinase MK2 (41). IL-10 has been reported to modestly inhibit p38 MAPK in the later phases of LPS treatment (18). The reduction of p38 MAPK activity is caused by the IL-10-mediated up-regulation of the dual-specificity phosphatase DUSP1 (Ref. 18 and Fig. 4F). We reasoned that such a reduction in p38 MAPK could relieve the p38 MAPK-dependent inhibition of TTP, thereby increasing the ability of TTP to down-regulate its target mRNAs. To investigate whether IL-6 was also able to reduce p38 MAPK activity, BMDMs were treated with LPS for 2 h with LPS in the presence or absence of IL-10. The amount of TNF-α mRNA in these cells was determined by qRT-PCR. Remnant TNF-α mRNA in percentage of the amount at the time point 0 of actinomycin D treatment is depicted. SDs (n = 3) are indicated. B, Effects of endogenous IL-10 on TNF-α mRNA induction in LPS- or LPS/IL-10-treated BMDMs. BMDMs derived from WT or IL-10−/− animals were treated for 1 or 2 h with LPS in the presence or absence of IL-10. The amount of TNF-α mRNA was determined at the indicated times by qRT-PCR. Remnant TNF-α mRNA in percentage of the amount at the time point 0 of actinomycin D treatment is depicted. E, Reduction of IL-1α cytokine by IL-10. BMDMs from WT and TTP−/− mice were treated with LPS for 10 h in the presence or absence of IL-10. ATP was added 1 h before the collection of supernatants. Supernatants were collected and analyzed for IL-1α cytokine levels by ELISA. Reduction of IL-1α cytokine levels by IL-10 pretreatment relative (in percent) to LPS alone treatment (100%) is depicted. SDs are indicated, n = 3. F, Reduction of IL-1α mRNA by IL-10. BMDMs from WT and TTP−/− mice were treated with LPS for 2 h in the presence or absence of IL-10. IL-1α expression was analyzed by qRT-PCR. Reduction of IL-1α mRNA by IL-10 treatment relative (in percent) to LPS alone treatment (100%) is depicted.
p38 MAPK activity was not reduced by treatment with IL-6 at any time point examined (Fig. 4, D and E, and supplemental Fig. S3A). These findings are in agreement with the induction of mRNA for the MAPK phosphatase DUSP1 by IL-10 but not IL-6 in LPS-treated macrophages (18). To further support the role of DUSP1 in the IL-10-mediated effect on p38 MAPK activity, we examined p38 MAPK phosphorylation in DUSP1−/− BMDMs treated with LPS alone or along with either IL-10 or IL-6 for 4 h. In DUSP1−/− cells, IL-10 was no longer able to reduce p38 MAPK phosphorylation at treatment (Fig. 4F). Consistent with the IL-10-mediated decrease in p38 MAPK phosphorylation, at the 4 h-time point, the induction of DUSP1 by IL-10 plus LPS compared with LPS alone was more apparent than after a shorter treatment (1 h; supplemental Fig. S3B). The different effect of IL-10 and IL-6 on p38 MAPK is in agreement with the anti-inflammatory properties that are exhibited by IL-10 but not IL-6. The difference between IL-10 and IL-6 with regard to their anti-inflammatory properties has been attributed to SOCS3 that, as part of the negative feedback loop, binds to the gp130 subunit of the IL-6 receptor but not to the IL-10 receptor (38–40). Thus, SOCS3, a Stat3 target gene, is able to inhibit signaling elicited by IL-6 but not by IL-10. Consequently, IL-10 induces a prolonged Stat3 activation, whereas IL-6-mediated Stat3 activation is rapidly shut down. Consistently, in LPS-treated BMDMs IL-10 and IL-6 caused a comparable Stat3 activation after 30 min of cytokine treatment, whereas after 2 h Stat3 remained active only in cells stimulated with IL-10 but not IL-6 (supplemental Fig. 3C). These data suggest that a sustained Stat3 activation is needed for inhibition of p38 MAPK.

Although p38 MAPK is needed for TFP expression and protein stability (24, 25, 42), the kinase negatively regulates TFP activity at least at two levels. First, phosphorylation of TFP at Ser212 and Ser178 by MAPK-activated protein kinase kinase 2 (MK2), a kinase downstream of p38 MAPK, provides binding sites for 14-3-3 proteins that reduce the destabilizing activity of TFP (23, 43). Second, phosphorylation of TFP by MK2 was reported to negatively regulate its binding to AREs (25). To show that the IL-10-mediated decrease in p38 MAPK activity was able to change TFP properties in terms of its phosphorylation and binding to AREs, we analyzed the mobility of TFP in SDS-PAGE and binding of TFP to AREs in EMSA experiments. TFP appears in SDS-PAGE in the form of multiple bands that reflect various degree of predominantly p38 MAPK-dependent phosphorylation (24). To demonstrate p38 MAPK effects on TFP phosphorylation and binding to AREs, we first used an inducible expression of TFP in HeLa-Tet-off cells. This system allows manipulation of p38 MAPK activity without affecting the transcription of the TFP gene that is known to require p38 MAPK activity (27, 42). Stimulation of HeLa-Tet-off cells expressing TFP (i.e., without tetracycline) with anisomycin (a p38 MAPK agonist (44)) resulted in a more pronounced appearance of a slower migrating hyperphosphorylated TFP band, whereas the p38 MAPK inhibitor SB reduced the amount of the hyperphosphorylated TFP and also the total TFP level due to TFP protein destabilization (Fig. 5A).
the same extracts in a RNA EMSA experiment revealed that anisomycin reduced binding of TTP to the TNF-α ARE by 50% (Fig. 5B), whereas inhibition of p38 MAPK resulted in a similar amount of ARE-bound TTP as in untreated cells (Fig. 5B) despite a reduced TTP protein level present in that sample (compare SB-labeled lanes in Fig. 5A and 5B). Consistent with the IL-10-mediated p38 MAPK inhibition, the treatment of BMDMs with LPS plus IL-10 or IL-6 were analyzed for TTP expression and SDS-PAGE mobility by Western blotting using Ab to TTP. Equal protein loading was controlled by reprobing with an anti-ERK Ab. The position of hyperphosphorylated (hyper-p.) and hypophosphorylated (hypo-p.) TTP is marked. D, Whole cell extracts of RAW 264.7 cells treated for 6 h with LPS alone or cotreated with IL-10 or IL-6 were analyzed as in C. E, Whole cell extracts of RAW 264.7 cells treated for 4 or 6 h with LPS alone or cotreated with IL-10 or IL-6 were assayed for in vitro binding of TTP to TNF-α ARE using RNA EMSA. The relative intensity of the TTP-ARE complexes (as indicated by the numbers 1, 2, 1.3, 1.3, 1.8, and 1.3) was quantitated using the LI-COR Odyssey software (supplementary Fig. 4B). To control the position of the TTP-ARE complexes extracts from HeLa-Tet-off cells expressing TTP (−Tet) or without TTP expression (+Tet) were used. The data are representative of three independent experiments.
mRNAs in the presence of the p38 MAPK inhibitor SB in TTP−/− and control WT BMDMs. Two hours after LPS stimulation, SB was added in combination with actinomycin D. p38 MAPK inhibition caused a 4-fold decrease of mRNA stability of TNF-α (TNF-α without SB: t1/2 = 31 min; TNF-α with SB: t1/2 = 8 min) and an ~8- to 10-fold decrease in IL-1α mRNA stability (IL-1α without SB: t1/2 > 2 h; IL-1α with SB: t1/2 = 14 min; Fig. 6). In TTP−/− cells, the inhibition of p38 MAPK caused only a modest (2-fold) decrease in mRNA stability of TNF-α mRNA (TNF-α without SB: t1/2 = 80 min; TNF-α with SB: t1/2 = 37 min with SB) and no detectable decrease of IL-1α mRNA stability (IL-1α without SB: t1/2 = >2 h; IL-1α with SB: t1/2 = >2 h). These data indicate that the augmentation of mRNA decay by inhibition of p38 MAPK is to a large part TTP dependent. The contribution of TTP to the effects of p38 MAPK inhibition depends on the nature of the target mRNA.

We conclude that the IL-10-mediated reduction of p38 MAPK activity increases TTP-dependent mRNA decay. The combined effect of IL-10 on p38 MAPK activity and TTP expression contributes to the anti-inflammatory properties of IL-10.

Discussion
This study provides evidence that the mRNA-desaturizing factor TTP plays an important role in anti-inflammatory effects of IL-10. BMDMs deficient in TTP show a strongly reduced anti-inflammatory response to IL-10. Two cytokines, TNF-α and IL-1α, were here demonstrated to be inhibited by IL-10 in a TTP-dependent manner. IL-10 appears to regulate TTP function in two ways (Fig. 7). First, IL-10 increases TTP expression in LPS-treated BMDMs. This augmented expression depends on Stat3, the key immediate effector of IL-10 effects. Second, IL-10 reduces at later time points the activity of p38 MAPK from the p38 MAPK-mediated inhibition. Since the increased expression of TTP is not sufficient to initiate an anti-inflammatory response (as demonstrated by IL-6-mediated up-regulation of TTP), we conclude that the TTP-dependent IL-10 effects can be best explained by the combination of higher TTP expression and reduction of p38 MAPK activity.

TTP is known for its mRNA-desaturizing activity most notably, but not exclusively, toward ARE-containing mRNAs. The activity of DUSP1 can be depicted by question marks. The other effector is TTP that requires the three effector genes. The still unidentified repressors of transcription are depicted by question marks. The other effector is TTP that requires the activity of a third effector, the DUSP1 phosphatase, that reduces the activity of p38 MAPK, thereby increasing the TTP-desaturizing activity toward specific ARE-containing mRNAs. The activity of DUSP1 can be mimicked by, for example, the p38 MAPK inhibitor SB.
TTP-deficient cells are known to produce 2- to 3-fold more TNF-α (both mRNA and cytokine) than WT cells (26, 48). At the same time, IL-10 mRNA was shown to be targeted by TTP for degradation (36) and we found that the TTP-deficient BMDMs produce more IL-10 cytokine (supplemental Fig. S1). Thus, TTP cells produce more of both the proinflammatory TNF-α and the anti-inflammatory IL-10. Yet, the TTP cells still respond to IL-10 treatment (by e.g., Stat3 activation) and LPS treatment (by e.g., p38 MAPK activation) similarly as the WT cells (Fig. 4). We conclude that despite the 2- to 3-fold higher production of pro- and anti-inflammatory cytokines the TTP-deficient cells display comparable immediate-early response to pro- (LPS) or anti-inflammatory (IL-10) stimuli as WT cells.

We were not able to detect any differences in the TTP expression or p38 MAPK activity, the two key factors influencing the TTP function, when we compared the two protocols for IL-10 treatment. Thus, the higher absolute contribution of TTP to IL-10 responses in the pretreatment protocol suggests that IL-10 induces a cofactor of TTP during the pretreatment period. TTP serves as an adaptor protein linking mRNA to the mRNA processing and degradation machinery located in stress granules and processing bodies (21, 22). Some of the components of these complexes may be up-regulated by IL-10 during the pretreatment phase to enhance TTP function. Alternatively, IL-10-activated Stat3 may increase transcription of micro-RNAs such as micro-R16 that was shown to assist TTP in degradation of ARE-containing mRNAs (49). The influence of the experimental protocol on the IL-10 effects may also partially explain the discrepancy between our study and the known properties of IL-10. For example, the efficient and dominant inhibition of the proinflammatory p38 MAPK by IL-10 at the later time points regardless of applied protocol for IL-10 treatment (supplemental Fig. S2, A and B). These data are in agreement with several other studies showing no effect of IL-10 on p38 MAPK activity at early time points (2, 12, 50, 51). In a kinetics analysis of p38 MAPK activity, IL-10 was found to exhibit a modest inhibitory effect on p38 MAPK activity at later time points (after 3 h of LPS plus IL-10 treatment) (18). The IL-10-mediated inhibition of p38 MAPK correlated with the induction of the dual-specificity phosphatase DUSP1, a MAPK phosphatase (18). We observed a similar reduction in p38 MAPK phosphorylation in terms of both, the magnitude and kinetics, and found that DUSP1 was required for this effect. The inhibition of p38 MAPK was caused specifically by IL-10 but not by IL-6 and correlated with the ability of IL-10 (but not IL-6) to induce sustained Stat3 activation. Consistently, IL-10 but not IL-6 was reported to increase expression of DUSP1 in LPS-treated macrophages (18). DUSP1 functions as a critical p38 MAPK-inactivating enzyme provides the most likely explanation for the susceptibility of DUSP1−/− mice to LPS-mediated toxic shock (52, 53). We speculate that the inhibition of the proinflammatory p38 MAPK by IL-10 at the later stages of macrophage stimulation is a key factor in immune homeostasis since p38 MAPK influences inflammation-related transcription, RNA stability, translation, as well as secretion. To mimic the inhibition of p38 MAPK by IL-10 at the late phase of stimulation, we used the p38 MAPK inhibitor SB and examined the effect of p38 MAPK on the TTP-dependent decrease of target mRNA molecules. Interestingly, whereas in the case of TNF-α a different decay rate (i.e., different decay rate in TTP+/+ and TTP−/− cells) was observed also without p38 MAPK inhibition, for IL-1α mRNA the TTP-dependent detectable only if p38 MAPK was inhibited (Fig. 5). A similar requirement for p38 MAPK inhibition has been recently described also for the TTP target CXCL1 (KC) mRNA (54). These findings indicate that the p38 MAPK-mediated control of TTP activity has a differential impact on target mRNAs. Although the TTP-dependent decay of some mRNAs (e.g., TNF-α) proceeds in the presence of p38 MAPK activity, the degradation of other mRNAs (e.g., IL-1α and CXCL1) is strongly dependent on the kinase inhibition. This mechanism may play an important role in the specificity of TTP-mediated RNA decay: depending on the activation status of p38 MAPK or other kinases implicated in regulation of TTP activity (e.g., ERK of MK2), TTP would discriminate between various targets. The critical role that p38 MAPK plays in TTP-mediated RNA decay also suggests that the time point that is taken to determine RNA stability has a decisive effect on the outcome of the assay. The activation/inactivation profile of p38 MAPK is likely to vary between different experimental settings (e.g., amount and quality of LPS, the use of primary or immortalized cells, the origin of primary cells such as bone marrow or peritoneum) so that this aspect may also contribute to the variability of published data. For example, we did not observe an IL-10-mediated increase in TNF-α mRNA decay in peritoneal-derived macrophages.

Suppression of inflammatory responses by IL-10 is one of the key features in immune homeostasis. Despite many years of research, the question of how a single cytokine can specifically inhibit various inflammatory reactions with such a high efficiency remains unresolved. Recent studies suggest that known as well as yet unknown IL-10 effectors interfere on different levels and by different mechanisms with the intracellular inflammatory networks. This multitasking system employed by IL-10 is likely to be essential for the efficiency and specificity of the anti-inflammatory properties of IL-10. For example, the efficient and dominant inhibition of Tnf gene transcription by IL-10 still requires the remaining TNF-α mRNA to be removed from the system, i.e., by TTP. Although the primary IL-10-elicited signaling events that involve the IL-10 receptor, Jak1 and Tyk2 kinases, as well as the transcription factor Stat3, are to a large part common to all cell types, the more complex effects downstream of Stat3 may be cell type dependent. Thus, the function and activity of the Stat3-induced IL-10 effectors may be regulated by the environment within the particular cell type, hereby helping to explain the still incoherent and sometimes contradictory studies of the anti-inflammatory effects of IL-10.

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Disclosures
The authors have no financial conflict of interest.

References


Supplemental Figure S1. TTP-deficient BMDMs produced more IL-10 cytokine after stimulation with LPS. WT and TTP-/- BMDMs were stimulated for 6, 8 or 10 h with LPS. Amounts of IL-10 in supernatants were determined by ELISA.
**Supplemental Figure S2.** p38MAPK and Stat3 activation by LPS or IL-10, respectively, are not affected by inactivation of the TTP gene. 

**A.** Activation of Stat3 and p38MAPK in LPS-treated TTP^{+/+} and TTP^{{-/-}} BMDMs. Cells treated for 30 min with IL-10 (IL), LPS (L), or IL-10 + IL-10 (IL/L) were examined for activation of p38MAPK by Western blotting using phospho-p38MAPK antibody (pp38). Membranes were reprobed with p38 antibody for loading control, and phospho-Stat3 antibody (pY-S3) to reveal activation of Stat3. 

**B.** Activation of p38MAPK in LPS-treated TTP^{+/+} and TTP^{{-/-}} BMDMs. Whole cell extracts of TTP^{+/+} and TTP^{{-/-}} BMDMs treated for 30 min, 2 h or 4 h with LPS (L) or left untreated (-) were analyzed for activation of p38MAPK by Western blotting using phospho-p38MAPK antibody (pp38). Membrane was reprobed with p38MAPK antibody (p38) for loading control.
**Supplemental Figure S3.** IL-10 causes a decrease in p38MAPK phosphorylation, increase in DUSP1 expression, and a prolonged activation of Stat3. 

**A.** Whole cell extracts of BMDMs treated for 30 min, 2 h, 4 h and 6 h with LPS+IL-6 or LPS+IL-10 were analyzed for activation of p38MAPK by Western blotting using antibody to activated p38MAPK (pp38). Antibody to total p38MAPK (p38) was used for loading control. 

**B.** Whole cell extracts of DUSP1-/- and IL10-/- BMDMs treated for 1 h and 4 h with LPS alone or LPS+IL-10 were analyzed for DUSP1 expression by Western blotting. Antibody to tubulin was used for loading control. IL-10-/- cells were used instead of WT BMDMs in order to increase the sensitivity to treatment with exogenous IL-10. 

**C.** BMDMs were treated for 30 min and 2 h with LPS alone or in combination with either IL-10 or IL-6. Stat3 activation was determined by Western blotting of whole cell extracts with antibody directed to tyrosine-phosphorylated Stat3 (pY-S3). Antibody to total Stat3 (S3) was used for loading control.
Supplemental Figure S4. Quantitative analysis of TTP-ARE complexes. The fluorescence signal of the Cy5.5-labeled TNFa ARE within the equally-sized rectangles positioned at the TTP-ARE complexes was measured and quantified (median background setting) using the LI-COR Odyssey system. The signal intensities are depicted. A, EMSA from the Fig. 5B. Equal amounts of the same extracts used for this EMSA were also used for the TTP Western blot in Fig. 5A, thus allowing for TTP loading control. B, EMSA from the Fig. 5E. The signal of TTP-ARE complexes (lower rectangles) were first normalized to the unspecific signals (upper rectangles) in the corresponding lanes before the relative intensities were calculated for the Fig. 5E.