Gain-of-function mutation of tristetraprolin impairs negative feedback control of macrophages *in vitro*, yet has overwhelmingly anti-inflammatory consequences *in vivo*.

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Running title: Regulation of IL-10 by TTP in vitro and in vivo.

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ABSTRACT

The mRNA destabilizing factor tristetraprolin (TTP) binds in a sequence-specific manner to the 3' untranslated regions of many pro-inflammatory mRNAs and recruits complexes of nucleases to promote rapid mRNA turnover. Mice lacking TTP develop a severe, spontaneous inflammatory syndrome characterized by over-expression of tumor necrosis factor and other inflammatory mediators. However, TTP also employs the same mechanism to inhibit the expression of the potent anti-inflammatory cytokine interleukin 10. Perturbation of TTP function may therefore have mixed effects on inflammatory responses, either increasing or decreasing the expression of pro-inflammatory factors via direct or indirect mechanisms. We recently described a knock-in mouse strain in which the substitution of two amino acids of endogenous TTP protein renders it constitutively active as an mRNA destabilizing factor. Here we investigate the impact on the IL-10-mediated anti-inflammatory response. It is shown that the gain-of-function mutation of TTP impairs IL-10-mediated negative feedback control of macrophage function *in vitro*. However, the *in vivo* effects of TTP mutation are uniformly anti-inflammatory, despite decreased expression of IL-10.

INTRODUCTION

Macrophages are at the forefront of innate immune-mediated defences against infectious pathogens. They are equipped with several pattern recognition receptors (PRRs) with which they detect pathogen-associated molecular patterns (PAMPs) common to different classes of microbes: for example lipopolysaccharide (LPS), a component of the cell wall of gram negative bacteria (1, 2). Engagement of macrophage PRRs initiates very rapid changes of gene expression in order to mobilize antimicrobial effectors, recruit and/or activate other cells of the innate and acquired immune systems, to amplify danger signals and coordinate effective cellular responses. Excessive responses of macrophages to engagement of PRRs is potentially harmful, in the worst case leading to systemic organ failure and death. It is therefore unsurprising that macrophage activation by PAMPs is regulated by complex networks of positive and negative feedback mechanisms that together ensure rapid but proportional and temporally limited pro-inflammatory responses (3-7).

One important feedback mechanism involves the secretion of the anti-inflammatory cytokine interleukin 10 (IL-10). Particularly in tissues like the gut, which are continuously exposed to PAMPs, IL-10 is essential for restraining inflammatory responses to commensal microorganisms (8, 9). One critical function of IL-10 is the suppression of the *Il12b* gene, which codes for the shared p40 subunit of the cytokines IL-12 and IL-23. The constraint of IL-12 and IL-23 expression is required to prevent excessive Th1- and Th17-mediated intestinal inflammation (10-13). Macrophage-specific knock-out of the IL-10 receptor results in severe spontaneous gut inflammation (14, 15). Similarly, polymorphisms in the human IL-10-IL-10R axis are associated with susceptibility to inflammatory bowel disease (16). The anti-inflammatory actions of IL-10 are mediated by the transcription factor STAT3 (signal

transducer and activator of transcription 3), which is phosphorylated and activated by the tyrosine kinases JAK1 and TYK2 upon engagement of the IL-10 receptor. A number of STAT3-regulated target genes have been implicated as mediators of anti-inflammatory functions of IL-10 (6, 17-19). The other side of the coin is that increased expression of IL-10 may contribute to pathogenesis, for example by impairing anti-tumor immunity (20). Several viruses express IL-10 orthologues, apparently as a means of evasion of the innate immune system (21). The expression of endogenous IL-10 is tightly regulated by different mechanisms in the several cell types that can express this anti-inflammatory cytokine (22, 23).

Much is known about how transcription factors regulate the complex programmes of gene expression in activated macrophages (3, 24-27). It is often overlooked that the dynamic regulation of mRNA stability also plays an important role (28, 29). Many inflammatory mediator mRNAs have adenosine/uridine-rich elements (AREs) in their 3' untranslated regions, which act as binding sites for various RNA destabilizing factors, the best characterized of which is tristetraprolin (TTP) (30, 31). TTP promotes rapid turnover of its substrate transcripts by recruiting a number of nuclease complexes; notably the CCR4/NOT complex, which mediates poly-(A) tail shortening, the rate limiting step in the degradation of most mRNA species. In resting macrophages TTP is expressed at very low levels. Its up-regulation in response to pro-inflammatory stimuli constitutes a negative feedback mechanism to limit the duration and strength of inflammatory gene expression (30-32). Myeloid-specific disruption of the TTP gene (formally known as *Zfp36*) causes extreme sensitivity to lethal endotoxic shock, although there is no evident phenotype in the absence of inflammatory challenge (33, 34). In contrast, global disruption of the *Zfp36* gene causes a

spontaneous and severe inflammatory syndrome with some features of rheumatoid arthritis (35). *Tnf* was the first mRNA shown to be post-transcriptionally regulated by TTP, and dysregulation of TNF expression plays an important role in both myeloid-specific and global *Zfp36* knock-out phenotypes (33-36). Nevertheless it is clear that very many additional targets exist, and are dysregulated in cells lacking TTP (30, 37, 38). Puzzlingly, IL-10 was identified as a target of post-transcriptional regulation by TTP, which was over-expressed by *Zfp36-/-* macrophages (38-41). Enhanced IL-10-mediated negative feedback in *Zfp36-/-* cells resulted in paradoxical under-expression of IL-6, IL-12 and IL-23 (39, 41).

The mitogen-activated protein kinase (MAPK) p38 signaling pathway contols the expression of inflammatory mediators via the phosphorylation and inactivation of TTP (42, 43). Hence MAPK p38 inhibitors decrease the expression of several pro-inflammatory mediators, and destabilize the corresponding mRNAs in *Zfp36+/+* but not *Zfp36-/-* macrophages (41, 44). The critical phosphorylations, at serine 52 and serine 178 of murine TTP, are carried out by MAPK-activated protein kinase 2 (MK2), which is downstream of MAPK p38 itself (43, 45-47). They result in the recuitment of 14-3-3 proteins, the disruption of the interaction between TTP and the CCR4/NOT complex, and consequent protection of the target transcript's poly-(A) tail (46, 48-50). The coupling between MAPK p38 signaling and TTP activity contributes to precise timing of gene expression in activated macrophages (33, 38). Prolonged MAPK p38 activation in macrophages lacking the MAPK phosphatase dual specificity phosphatase 1 (DUSP1) promotes over-expression of several inflammatory mediators *in vitro* and *in vivo*, causing exaggerated responses to experimental challenges such as LPS injection (5, 51). To a large extent, this dysregulated inflammatory response is

caused by increased phosphorylation and inactivation of TTP, increased stability of pro-inflammatory mRNAs, and a delay to the off-phase of gene expression (52).

We recently used homologous recombination to generate a knock-in mouse strain in which Ser52 and Ser178 of endogenous TTP protein are substituted by non-phosphorylatable alanine residues (53). The mutated *Zfp36* locus is known as *Zfp36aa*, and its protein product as TTPaa. Since it cannot be inactivated by the MAPK p38 pathway, TTPaa is a constitutive, dominant mRNA destabilizing protein. Its expression protects mice from harmful consequences of systemic LPS injection (53), experimental inflammatory arthritis (54), and inflammatory lung pathologies (43). Differential gene expression was assessed by microarray in *Zfp36aa/aa* macrophages, which express the alanine-substituted mutant form of TTP from the endogenous *Zfp36* locus (52, 53). Several known TTP target mRNAs such as *F3*, *Tnf*, *Cxcl1*, *Cxcl2*, *Ier3* and *Il10* were under-expressed by *Zfp36aa/aa* macrophages, and some putative novel TTP targets were identified.

We and others have discussed the possibility of therapeutic targeting of TTP in order to exert anti-inflammatory effects (31, 43). Proof of principle was generated by treatment of experimental arthritis using two compounds that promote PP2A-mediated dephosphorylation and activation of TTP (54). A significant concern is that altering TTP function could give rise to unwanted pro-inflammatory effects by disrupting endogenous negative feedback mechanisms, for example those involving IL-10 (37, 39-41). Here we investigated *in vitro* and *in vivo* consequences of the impaired expression of IL-10 caused by a gain-of-function mutation of TTP.

MATERIALS AND METHODS.

Materials.

LPS (*Escherichia coli* serotype EH100) was purchased from Enzo Life Sciences. Other biochemicals were purchased from Sigma-Aldrich unless otherwise stated. All media and sera were routinely tested for endotoxin using the Limulus amebocyte lysate test (Lonza) and were rejected if the endotoxin concentration exceeded 0.1 U/ml.

Generation of mouse strains.

Generation of a *Zfp36aa/aa* mouse strain on C57BL/6 background was described previously (49). The *Dusp1-/-* strain was a generous gift from Bristol-Myers Squibb, and was back-crossed to C57BL/6 for ten generations prior to experiments described here. The double-targeted *Dusp1-/-* : *Zfp36aa/aa* line was generated by three generations of crossing, with genotyping performed by PCR of genomic DNA, and thereafter was maintained as a pure-breeding line. Bone marrow from *Il10-/-* mice was generously provided by Prof. Fiona Powrie (Oxford).

In vivo experiments and cell isolation.

All animal experiments were approved by local ethical committees and performed under UK Home Office Project Licences. C57BL/6 mice were purchased from Harlan Laboratories. All mice used were between 6 and 12 weeks of age. To assess the systemic response to LPS, mice were injected i.p. with 5 mg/kg purified LPS in 200 µl sterile PBS. Mice were humanely culled 3h or 12 h after challenge and peripheral blood was collected by cardiac puncture for serum isolation. Spleens were excised and snap frozen in liquid nitrogen for later isolation of RNA.

Bone marrow was isolated from humanely culled mice, and BMMs (bone marrow derived macrophages) obtained by differention *in vitro* with either 100 ng/ml macrophage-colony stimulating factor (M-CSF) (PeproTech), or 50 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), in RPMI 1640 containing 10% heat-inactivated FCS and penicillin/streptomycin for 7 d. BMMs were plated at a density of 1 x 10^6/ml in the appropriate cell culture plate at least 1 d prior to stimulation.

Neutralization of endogenous secreted IL-10 was achieved by in vitro incubation with an IL-10 neutralising antibody (BioLegend, catalogue number 501407) at 10 µg/mL for the duration of the assays. Controls were treated with an equal concentration of isotype-matched antibody.

Measurements of mRNA.

RNA was extracted from BMMs using QIAshredder columns and RNeasy Mini kit (Qiagen). cDNA was generated using the iScript cDNA Synthesis Kit (Biorad). Gene expression was quantified by qRT-PCR on a Lightcycler 480 II (Roche) using Superscript III platinum RT-PCR kit and custom-synthesised oligonucleotide primers (Eurofins MWG) with SYBR Premix Ex Taq (Lonza). Relative gene expression was calculated using the ΔΔCt method with *Gapdh* or *B2m* mRNA for normalization of RNA levels. Primary transcript PCR was performed using primer pairs that crossed exon-intron boundaries, with an additional DNaseI step to remove contaminating genomic DNA from RNA samples (Qiagen). Control PCR reactions were carried out in the absence of RT in order to monitor genomic DNA contamination, which in all cases was negligible. Sequences of oligonucleotides designed to detect primary or mature transcripts are available on request from the authors.

The half-life of mRNA was estimated using the transcription inhibitor actinomycin D (Sigma) at 5 µg/mL, followed by measurement of mRNA by quantitative real time PCR.

Microarray analyses were performed using SurePrint G3 Mouse GE 8x60K slides (Agilent) and Partek Genomics Suite version 6.6, build 6.13.0315 (Partek) as previously described (49). For generation of volcano plots, transcripts were first filtered for significant upregulation in response to LPS (> 2-fold increase, adjusted p-value < 0.05) and weakly expresed transcripts were removed by application of an arbitrary filter of 200 RMA. Plots (log_2 fold difference of expression vs -log_{10} ANOVA p-value) were constructed using ggplot2s in the statistical package "R", with subset cut-offs at p-value < 0.05 and fold difference of expression > 1.5.

Assessment of protein expression.

Secreted factors in tissue culture supernatants and sera were quantified by ELISA according to manufacturer's instructions (eBioscience), or by using Bio-Plex bead capture assays and a Bio-Plex 200 analyzer (BioRad).

Statistical analysis.

GraphPad Prism software (Version 5.03) was used for statistical analysis. Mann Whitney U test was used for comparison of two groups. For analysis of multiple groups, ANOVA was used with Bonferroni correction for multiple comparisons. The following marks are used throughout: *,p<0.05; **,p<0.01;***,p<0.005; n.s., not statistically significant.

Accession number.

Microarray data discussed in this paper were deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE68449.

RESULTS

Anomalous expression of certain TTP targets by *Zfp36aa/aa* macrophages.

Microarray analysis was performed to identify transcripts differentially expressed by *Zfp36aa/aa* M-CSF-differentiated bone marrow-derived macrophages (M-BMMs) after stimulation with LPS. The microarray data have been deposited at the Gene Expression Omnibus site (GSE68449), and some aspects of their analysis previously reported (52, 53). Figure 1 illustrates analysis of LPS-induced transcripts (> 2-fold increase of expression in response to LPS, $p < 0.05$). The data are presented in the form of volcano plots, in which transcripts with a greater than 1.5-fold difference of expression beween *Zfp36+/+* and *Zfp36aa/aa* M-BMMs and a corrected p value less than 0.05 are shaded black. Differential expression data are summarized in Supplemental Table 1. As expected for a dominant negative regulator of gene expression, many transcripts were under-expressed by *Zfp36aa/aa* M-BMMs. Several of the changes of expression at 1h were previously reported, including the under-expression of *Tnf*, *Il10* and *Cxcl1* mRNAs (53). By 4h after addition of LPS, both *Tnf* and *Cxcl1* mRNAs had returned almost to basal levels, and differences between *Zfp36+/+* and *Zfp36aa/aa* M-BMMs were no longer statistically significant (also see Fig 4A). *Il10* mRNA was significantly underexpressed by *Zfp36aa/aa* M-BMMs at both 1h and 4h time points. Contrary to expectation, *Il6*, which was previously characterized as a TTP target (38, 41, 55, 56), was not significantly under-expressed by *Zfp36aa/aa* M-BMMs (Fig. 1). Several other transcripts were significantly over-expressed by *Zfp36aa/aa* M-BMMs. One example, *Il12b*, is highlighted in Fig. 1.

Gene ontology (GO) analysis was performed on transcripts differentially-expressed by *Zfp36aa/aa* M-BMMs (Supplemental Table 2). At both 1h and 4h, under-expressed transcripts were highly enriched in several GO terms relating to response to stimulus, defense response or inflammatory response. Enrichment of these terms was a consequence of the differential expression of transcripts including *Tnf*, *Cxcl1*, *Cxcl2*, *Clcf1*, *Ier3*, *S100a8*, *Zfp36*, *Mefv*, *Ltf* and *Il10*, most of which have been previously described as TTP targets (30). These results are arguably trivial, given that LPS-induced transcripts were selected prior to the analysis of differential gene expression. GO analysis on the over-expressed transcripts was more informative. At 1h, several GO terms related to cell migration were very significantly enriched ($p < 10^{-10}$). All of these enrichments arose from the increased expression of four transcripts, namely *Cited2*, *Cxcl10*, *Sdc4* and *Trib1*. At 4h, the most significantly enriched GO terms were related to phosphorylation of the transcription factor STAT5 (signal transducer and activator of transcription 5) or the regulation of the extrinsic apoptosis pathway (Supplemental Table 2). The corresponding lists of differentially expressed transcripts were dominated by *Il12b*, *Csf2*, *Cx3cl1*, *Kdr* and *Rasip1*. These findings suggest that the genes over-expressed by *Zfp36aa/aa* M-BMMs are functionally distinct.

TTP can negatively regulate gene expression by inhibiting translation rather than destabilizing mRNA (37, 57, 58). LPS-induced expression of TNF, IL-6, IL-10 and IL-12p40 proteins was therefore compared between *Zfp36+/+* and *Zfp36aa/aa* M-BMMs. Multiple experiments were performed using different numbers and densities of M-BMMs, depending whether the primary aim was to harvest RNA for microarrays, RNA for qPCR, secreted protein for ELISA and multiplex, or intracellular protein for western blotting. Regardless of

experimental conditions, expression of both TNF and IL-10 proteins was consistently diminished in *Zfp36aa/aa* M-BMMs (by approximately 5-fold and 3-fold, respectively; Fig. 2A and 2B). In contrast, the pattern of expression of IL-6 was inconsistent, with unchanged expression by *Zfp36aa/aa* M-BMMs in most cases, but both over-expression and under-expression in some experiments. Overall there was no statistically significant difference in expression of IL-6 between *Zfp36+/+* and *Zfp36aa/aa* M-BMMs. (Fig. 2C). Expression of IL-12p40 protein was invariably higher in *Zfp36aa/aa* than in matched *Zfp36+/+* M-BMMs, although the extent of over-expression was quite variable (Fig. 2D).

Anomalous expression of certain TTP targets by *Dusp1-/-* macrophages.

In *Dusp1-/-* macrophages, enhanced and prolonged MAPK p38 activation in response to LPS promotes over-expression of several cytokines and chemokines via increased phosphorylation and inactivation of TTP (52). The defining characteristic of genes that are regulated by the DUSP1-TTP axis is that the over-expression caused by disruption of the *Dusp1* gene can be prevented by combining this with the *Zfp36aa/aa* genotype. Dysregulated MAPK p38 signaling fails to promote over-expression of target genes if TTP cannot be phosphorylated and inactivated. At protein and mRNA level, the expression of both TNF and the anti-inflammatory cytokine IL-10 conformed to the pattern described, being elevated in *Dusp1-/-* but not *Dusp1-/- : Zfp36aa/aa* M-BMMs (Fig. 3, first two lines). *Il6* mRNA levels were decreased in M-BMMs of all three genetically modified genotypes, but there were no significant differences in protein expression (Fig. 3, third line). At 4h the expression of *Il12b* mRNA was actually decreased rather than increased in *Dusp1-/-* M-BMMs, and increased rather than decreased in *Zfp36aa/aa* M-BMMs, whereas in *Dusp1-/-* : *Zfp36aa/aa* M-BMMs the expression of *Il12b* mRNA was not significantly different from that

in wild type controls (Fig. 3, fourth line). The pattern of expression of IL-12p40 protein was similar.

Targeted mutation of TTP paradoxically increases transcription of *Il6* and *Il12b* genes. *Tnf*, *Il6, Il10* and *Il12b* mRNAs can all be physically recognized by TTP (38), yet show very different responses to mutations that influence TTP phosphorylation and activity. To make sense of these disparate patterns we monitored gene expression at the levels of steady state mRNA, primary transcript (as a surrogate for transcription rate), mRNA stability and protein secretion in LPS-treated *Zfp36+/+* and *Zfp36aa/aa* M-BMMs (Fig. 4).

Abundance of both *Tnf* and *Il10* steady state mRNAs was decreased in *Zfp36aa/aa* M-BMMs (Fig. 4A). Expression of the corresponding proteins was diminished (Fig. 4D) as previously shown (53, Fig. 2). There were no significant differences of *Tnf* or *Il10* primary transcript levels (Fig. 4B), but the stabilities of *Tnf* and *Il10* mRNAs were decreased in *Zfp36aa/aa* M-BMMs (Fig. 4C), as previously reported (53). The behavior of both *Tnf* and *Il10* genes is therefore consistent with direct regulation by TTP at the level of mRNA stability. Since *Zfp36* gene mutation had a greater effect on TNF and IL-10 protein than on *Tnf* and *Il10* mRNA, the phosphorylation of TTP may also have some impact at the translational level, as has been previously suggested (37, 57, 58).

Only a minor difference of steady state *Il6* mRNA abundance was detected at 4 h (Fig 4A), and IL-6 secretion did not differ between *Zfp36+/+* and *Zfp36aa/aa* M-BMMs (Fig 4D). There appeared to be an almost perfect balance between an increase in the rate of *Il6* transcription (Fig. 4B) and an increase in the rate of *Il6* mRNA degradation (Fig. 4C). *Il12b*

mRNA was highly stable in both *Zfp36+/+* and *Zfp36aa/aa* M-BMMs (Fig. 4C). Increased expression of *Il12b* mRNA (Fig 4A) and IL-12p40 protein (Fig 4D) by *Zfp36aa/aa* M-BMMs appeared to be driven by an enhanced transcriptional response to LPS (Fig 4B).

Disruption of IL-10-mediated negative feedback contributes to the anomalous expression of IL-6 and IL-12p40 by *Zfp36aa/aa* macrophages.

IL-10 is a well characterized target of TTP (38-41), which is under-expressed by *Zfp36aa/aa* M-BMMs (Fig 5), and known to negatively regulate the expression of TNF, IL-12p40 and IL-6 (7, 19). Noticeably, the expression of IL-12p40 in macrophages of differing genetic backgrounds was an almost perfect mirror image of IL-10 expression (Fig. 3). It was therefore speculated that disruption of IL-10-mediated autocrine negative feedback mechanisms contributed to differences of IL-6 and IL-12p40 expression *in vitro*.

It is not known whether pro-inflammatory genes display the same dose response to inhibition by IL-10. Differential sensitivity to IL-10 could influence the outcomes of dysregulated IL-10 biosynthesis in macrophages. Therefore, *Il10-/-* M-BMMs were used to investigate the relative sensitivity of TNF, IL-12p40 and IL-6 expression to exogenous IL-10, in the absence of interference from endogenous IL-10 (Fig. 5). The three pro-inflammatory cytokines displayed quite distinct dose-responses. IL-12p40 was the most sensitive in terms of both EC_{50} (estimated 0.15 ng/ml) and maximal inhibition (93%). TNF was inhibited by a maximum of 76%, and with an EC_{50} of approximately 0.29 ng/ml. IL-6 biosynthesis was insensitive to low concentrations of IL-10. Maximal inhibition was 81%, and estimated EC_{50} was 1.52 ng/ml.

Differentiation of BMMs in the presence of GM-CSF rather than M-CSF is reported to generate a cell population (GM-BMMs) that expresses relatively little IL-10, but higher amounts of TNF, IL-6 and IL-12p40 in response to LPS (59, 60). It was confirmed that wild-type GM-BMMs expressed 5-fold less IL-10 than M-BMMs generated in parallel from the same donor mice (Fig. 6). Expression of the pro-inflammatory cytokines TNF, IL-6 and IL-12p40 was elevated 2.5-fold, 5.4-fold and 56.3-fold in GM-BMMs compared to M-BMMs generated in parallel. *Zfp36+/+* and *Zfp36aa/aa* GM-BMMs did not differ in their expression of IL-10 after stimulation with LPS. Therefore, in GM-BMMs, the targeted mutation of TTP cannot indirectly influence gene expression by perturbing IL-10-mediated autocrine/paracrine feedback mechanisms. Targeted mutation of the *Zfp36* locus decreased the expression of TNF in both M-BMMs and GM-BMMs. *Zfp36* mutation did not affect IL-6 expression in M-BMMs, whereas in GM-BMMs the mutation significantly decreased the expression of IL-6. Expression of IL-12p40 was elevated as a consequence of *Zfp36* mutation in M-BMMs. Contrastingly, in GM-BMMs the *Zfp36* mutation had no significant effect on IL-12p40 expression.

The observations described above support the hypothesis that changes in the expression of IL-10 influence the phenotype caused by substitution of two TTP phosphorylation sites. To test the hypothesis more rigorously, *Zfp36+/+* and *Zfp36aa/aa* M-BMMs were stimulated with LPS in the presence of an IL-10 neutralizing antibody or isotype control (Fig. 7). In the presence of the neutralizing antibody, IL-6 was under-expressed by *Zfp36aa/aa* M-BMMs. IL-10 neutralization increased the expression of IL-12p40 in *Zfp36+/+* M-BMMs, but had relatively little effect in *Zfp36aa/aa* M-BMMs. As a consequence, the degree of overexpression of IL-12p40 by *Zfp36aa/aa* M-BMMs was diminished. Together, these data

341 indicate that the aberrant patterns of expression of IL-6 (unaltered in *Zfp36aa/aa* M-BMMs)
342 and IL-12p40 (elevated in *Zfp36aa/aa* M-BMMs) are at least partly due to alterations in IL-
343 10-mediated negative feedback. The expression of TNF appears minimally sensitive to this
344 phenomenon.
345
346 **Targeted mutation of TTP has overwhelmingly anti-inflammatory consequences *in vivo*.**
347 Next, *Zfp36+/+* and *Zfp36aa/aa* mice were injected intraperitoneally with LPS, and serum
348 cytokines and chemokines were measured after 3 and 12 h (Fig.8). Decreased expression of
349 TNF and IL-10 by *Zfp36aa/aa* mice was previously reported (53) (indicated by † in Fig. 8).
350 CCL3, CCL4, CCL11, CSF2, IFN-γ, IL-1α, IL-5 and IL-13 were also significantly under-expressed
351 by *Zfp36aa/aa* mice. Most notably, expression of IL-6 was decreased by almost four-fold at
352 3h and by more than 200-fold at 12 h in *Zfp36aa/aa* mice. The elevated expression of IL-
353 12p40 in LPS-treated *Zfp36aa/aa* M-BMMs was not recapitulated *in vivo*. Serum
354 concentrations of IL-12p40 were not significantly different between LPS-treated *Zfp36+/+*
355 and *Zfp36aa/aa* mice at 3h, but signficantly lower in *Zfp36aa/aa* mice at 12h.
356
357 Finally, to test whether cytokine expression *in vivo* was regulated by DUSP1 via alterations in
358 TTP phosphorylation, wild type, *Dusp1-/-*, *Zfp36aa/aa* and *Dusp1-/-* : *Zfp36aa/aa* mice were
359 injected intraperitoneally with LPS, culled after 3 h, and the expression of *Tnf*, *Il10*, *Il6* and
360 *IL12b* mRNAs was measured in spleens (Fig. 9). *Tnf* and *Il10* mRNAs were elevated in spleens
361 of LPS-treated *Dusp1-/-* mice and decreased in those of *Zfp36aa/aa* mice. When *Dusp1-/-*
362 and *Zfp36aa/aa* genetic modifications were combined, the *Zfp36aa/aa* phenotype was
363 dominant, and the expression of both mRNAs remained low. These patterns of gene
364 expression are very similar to those previously described *in vitro* (Fig. 3a), and in serum of

LPS-treated mice (52), consistent with DUSP1 regulating gene expression via modulation of the phosphorylation state of TTP in both contexts. Strikingly, the pattern of expression of *Il6* mRNA was the same: increased in *Dusp1-/-* spleens but decreased in both *Zfp36aa/aa* and *Dusp1-/-* : *Zfp36aa/aa* spleens, indicating a similar mechanism of regulation. IL-6 protein was also very strongly elevated in serum of LPS treated Dusp1-/- mice, but decreased in serum of both *Zfp36aa/aa* and *Dusp1-/-* : *Zfp36aa/aa* mice (data not shown). Differences in expression of *Il12b* mRNA between the four genotypes of mice were not significant. The dysregulation of *Il12b* gene expression in isolated *Zfp36aa/aa* M-BMMs was therefore not recapitulated *in vivo* in *Zfp36aa/aa* mice (also see Fig. 8).

DISCUSSION

The phosphorylation and dephosphorylation of TTP constitutes a molecular switch for turning gene expression on and off during responses to inflammatory stimuli (43). In *Dusp1-/-* cells the dysregulation of MAPK p38 signaling pushes the switch in the "on" direction, promoting the phosphorylation and inactivation of TTP, enhancing and sustaining the expression of TTP target genes by increasing mRNA stability. In *Zfp36aa/aa* cells the switch is locked in the "off" position, and the expression of TTP target genes is diminished as a consequence of enhanced mRNA decay. This locked switch prevents over-expression of TTP target genes in cells in which the two genetic modifications are combined (43, 52). Here and in previous publications (52, 53), the expression of both TNF and IL-10 conformed exactly to the pattern described above, whether investigated *in vitro* or *in vivo*. The behavior of these genes can be simply understood in terms of our working model of TTP function. However, it remains puzzling at first sight, that the same molecular mechanism is used to regulate the expression of a powerful pro-inflammatory and a powerful anti-inflammatory cytokine. It is

likely that this shared regulation contributes to the resolution of inflammation. Any stimulus that provokes strong expression of TNF via the activation of MAPK p38 and the phosphorylation of TTP will also provoke strong expression of IL-10, with a built in delay owing to the slower transcriptional activation of the latter. IL-10 then acts in as an autocrine or paracrine negative feedback regulator of the inflammatory response.

When gene expression was initially studied in *Zfp36aa/aa* M-BMMs, we were puzzled that IL-6 did not behave as expected. IL-6 has been well characterized as a target of TTP (30, 55, 56), yet its expression was not consistently diminished in *Zfp36aa/aa* M-BMMs, and in many experiments was actually increased (Fig. 2). Microarray analysis also revealed surprising over-expression of several transcripts by *Zfp36aa/aa* M-BMMs (Fig. 1), amongst which *Il12b* was prominent. We hypothesized that impairment of IL-10-mediated negative feedback control contributed to these anomalies. Being a secondary response gene, the peak of expression of *Il6* occurred at a time when endogenous IL-10 protein had already accumulated in tissue culture supernatants (Fig.4). IL-6 expression was sensitive to inhibition by IL-10, with a very steep dose respose between 1 and 3 ng/ml (Fig. 5), similar to the range of IL-10 concentrations produced by *Zfp36+/+* and *Zfp36aa/aa* M-BMMs (Fig. 4). As predicted, IL-6 was under-expressed by *Zfp36aa/aa* GM-BMMs (Fig. 6) and by *Zfp36aa/aa* peritoneal macrophages (data not shown), both of which produce very low levels of IL-10. Neutralization of IL-10 also restored the expected gene expression pattern, in which IL-6 was under-expressed by *Zfp36aa/aa* M-BMMs (Fig. 7). It therefore appears that differences in the levels of IL-10 in tissue culture have a profound effect on expression of the *Il6* gene, resulting in variable under- or over-expression by *Zfp36aa/aa* M-BMMs. Although *Tnf* gene expression is also negatively regulated by IL-10 (Fig. 5) (19, 61), its immediate early pattern

of expression (61, 62) (Fig. 4) presumably allows it to escape the influence of such fluctuations in the efficacy of negative feedback control. By the time that levels of endogenous IL-10 accumulate significantly, the transcriptional activity of the *Tnf* gene is already declining.

The production of IL-12p40 protein was profoundly sensitive to IL-10-mediated inhibition (Fig. 5). The paradoxical over-expression of IL-12p40 by *Zfp36aa/aa* M-BMMs was caused by an increase in transcription (Fig. 4). Like others (63, 64), we could find no evidence that TTP regulated *Il12b* mRNA stability (Fig. 4). This increase of expression was not observed in *Zfp36aa/aa* GM-BMMs, which express little IL-10 (Fig. 6). IL-10 is reported to inhibit *Il12b* transcription via induction of the transcriptional repressor NFIL3 (nuclear factor, IL-3 regulated) (13). 4h after addition of LPS, the expression of *Nfil3* mRNA was significantly lower in *Zfp36aa/aa* than in *Zfp36+/+* M-BMMs (2135±35 vs 4940±497 RMA, $p < 0.005$). It is therefore possible that impaired negative feedback via NFIL3 contributes to the enhanced transcriptional induction of the *Il12b* gene in *Zfp36aa/aa* M-BMMs (Fig. 4). However, neutralization of IL-10 only partially reversed the over-expression of IL-12p40 by *Zfp36aa/aa* M-BMMs, suggesting that additional mechanisms may be involved. TTP has been reported to inhibit *Il12b* gene expression by modulating the function of the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (65). However, we were unable to detect any differences of NF-κB function in *Zfp36aa/aa* M-BMMs (53). Additionally, it is difficult to explain increases in expression of only a few genes by increased activity of a transcription factor that regulates much of the macrophage response to LPS. Therefore, the aberrant expression of IL-12p40 by *Zfp36aa/aa* M-BMMs has been only partially accounted for.

437

438 Previous reports have identified IL-10 as a target of TTP (39, 41), describing increased
439 expression of IL-10 and consequent under-expression of both IL-6 and IL-12 by *Zfp36-/-*
440 macrophages. The phenomenon described here is a mirror image, in which gain of function
441 of TTP impairs IL-10-mediated negative feedback control *in vitro*, and promotes increased
442 expression of these two cytokines. As we and others are interested in the concept of
443 therapeutic targeting of TTP (31, 43, 53), the impairment of negative feedback control was a
444 source of concern, prompting us to test the *in vivo* effects of the gain of function mutation.
445 Despite the fact that LPS-induced IL-10 expression was diminished in *Zfp36aa/aa* mice, we
446 could detect no corresponding increase in the expression of either IL-6 or IL-12p40 (Fig. 8,
447 9). In fact, IL-6 was consistently expressed at very low levels by *Zfp36aa/aa* mice following
448 systemic LPS challenge (Fig. 8), injection of zymosan into dorsal airpouches, or induction of
449 experimental arthritis (53). Increased circulating levels of either IL-12 or IL-23, which share
450 the common IL-12p40 subunit, would be expected to promote Th1 and Th17 responses,
451 characterized by elevated expression of IFN-γ and IL-17. In fact, both of these effector
452 cytokines were present at lower levels in serum of LPS-injected *Zfp36aa/aa* mice (49, Fig.
453 8).These findings suggest that agonists which activate TTP may exert anti-inflammatory
454 effects without incurring immune activation via increased expression of IL-6, IL-12 or IL-23.
455 It will be important to test this further in experimental settings such as inflammatory bowel
456 disease, where impaired expression of IL-10 may have profound consequences (8, 12).

457

458 A remaining question is why the behavior of *Il6* and *Il12b* genes is so different when studied
459 *in vitro* or *in vivo*. Although *in vitro* experiments are invaluable for gaining insight into
460 molecular mechanisms of gene regulation, it is easy to forget how poorly they model

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inflammatory responses *in vivo*. In many senses the *in vitro* assay system is artificial. The population of cells is homogeneous, their exposure to stimulus is synchronous, and they remain thereafter in a static milieu, in which local concentrations of cytokines and other effectors may become high, and autocrine or paracrine effects may be amplified. *In vivo* the population of cells responding to LPS is heterogeneous, their time of exposure to the agonist will vary somewhat, and the local accumulation of cytokines and other immune effectors will be limited by circulation. Whatever the explanation of the divergent behaviors of IL-6 and IL-12p40 *in vitro* and *in vivo*, our principal conclusion remains the same. Gain of function of TTP causes dysregulation of IL-6 and IL-12p40, at least in part indirectly, via changes in IL-10 expression. However, aberrant over-expression of IL-6 and IL-12p40 does not occur in *Zfp36aa/aa* mice.

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FIGURE LEGENDS

Figure 1. Differentially expressed transcripts in *Zfp36aa/aa* M-BMMs. M-BMMs were generated from 3 *Zfp36+/+* and 3 *Zfp36aa/aa* mice, and treated with 10 ng/ml LPS for 1 h (A) or 4 h (B). RNA was isolated and transcript abundance analyzed using Agilent microarrays and Partek Genomics Suite. Transcripts expressed above an arbitrary threshold of 200 RMA in at least two replicates and demonstrating upregulation by LPS (> 2X, $p < 0.05$) were selected for display. Data are illustrated in the form of volcano plots, in which transcripts with a greater than 1.5-fold difference of expression between *Zfp36+/+* and *Zfp36aa/aa* M-BMMs and a corrected p value less than 0.05 are shaded black. Under-expressed transcripts are to the left of the origin. Several transcripts are highlighted.

Figure 2. Expression of TNF, IL-10, IL-6 and IL-12p40 proteins by *Zfp36+/+* and *Zfp36aa/aa* M-BMMs. Matched *Zfp36+/+* and *Zfp36aa/aa* M-BMMs were cultured in 24-, 12- or 6-well dishes and stimulated with 10 ng/ml LPS for 4 h. ELISA or multiplex bead assay were used to measure TNF (A), IL-10 (B), IL-6 (C) and IL-12p40 (D). In the cases of TNF and IL-6, 5 of 14 experiments employed matched *Zfp36+/+* and *Zfp36aa/aa* littermates. All other experiments employed at least three mice of each genotype, mean cytokine concentrations being plotted. Cytokine quantities expressed by matched *Zfp36+/+* and *Zfp36aa/aa* M-BMMs or sets of M-BMMs are connected by lines. The graphs on the right of each panel show mean cytokine expression in *Zfp36aa/aa* M-BMMs relative to that in matched *Zfp36+/+* controls. n.s., not statistically significant; ***, $p < 0.005$; **, $p < 0.01$ (Wilcoxon matched pairs test).

Figure 3. Regulation of inflammatory mediators by DUSP1 and TTP. Wild type, *Dusp1-/-*, *Zfp36aa/aa* and *Dusp1-/-* : *Zfp36aa/aa* M-BMMs were stimulated with 10 ng/ml for 1 h or 4 h. A) Expression of selected mRNAs was determined by microarray, and B) corresponding proteins were measured by ELISA or multiplex bead assay. Graphs represent mean ± SEM of 3 independent M-BMM cultures . n.s., not statistically significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (one way ANOVA). The microarray experiment used to generate panel A was previously described (52)

Figure 4. TTP mutation affects gene expression at different levels. *Zfp36+/+* and *Zfp36aa/aa* M-BMMs were treated with LPS for the times indicated, supernatants were collected and RNA isolated. A) Steady state mRNA abundance was measured by quantitative PCR. B) Primary transcripts were measured by quantitative PCR. C) Actinomycin D chase experiments were performed at peak of gene expression (1 h in the case of *Tnf*, 4 h in the cases of *Il10*, *Il6* and *Il12b*). D) Secreted proteins were measured by ELISA or multiplex bead assay. Graphs represent mean ± SEM of three independent M-BMM cultures in each case. Pairwise comparisons that showed no statistical significance are not indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (Mann Whitney).

Figure 5. Dose dependent inhibition of pro-inflammatory gene expression by IL-10. *Il10-/-* M-BMMs were stimulated with 10 ng/ml LPS for 4 h in the presence of different concentrations of recombinant IL-10. TNF, IL-6 and IL-12p40 were quantified by ELISA. Cytokine levels were normalized against those in the absence of IL-10. Graphs show mean ± SEM of at least 8 independent M-BMM cultures.

529 **Figure 6.** Differential gene expression in *Zfp36+/+* and *Zfp36aa/aa* M-BMMs and GM-BMMs.
530 Macrophages were differentiated from bone marrow of *Zfp36+/+* and *Zfp36aa/aa* mice in
531 the presence of either M-CSF of GM-CSF, and treated with 10 ng/ml LPS for 4 h. Cytokines
532 were measured by ELISA or multiplex bead assay. Graphs represent mean ± SEM from 4
533 (TNF, IL-10 and IL-12p40) or 7 (IL-6) independent cultures. n.s., not statistically significant; *,
534 $p < 0.05$ (Mann Whitney).
535
536 **Figure 7.** Endogenous IL-10 differentially affects expression of pro-inflammatory cytokines.
537 *Zfp36+/+* and *Zfp36aa/aa* M-BMMs were treated with 10 ng/ml LPS for 4 h (TNF and IL-6) or
538 8 h (IL-12p40) in the presence of 10 µg/ml IL-10 neutralizing antibody or isotype control.
539 Cytokines were measured by multiplex bead assay or ELISA. Graphs represent mean ± SEM
540 from 4 independent M-BMM cultures. n.s., not statistically significant; ***, $p < 0.005$ (Mann
541 Whitney).
542
543 **Figure 8.** *In vivo* responses to LPS are broadly impaired in *Zfp36aa/aa* mice. *Zfp36+/+* and
544 *Zfp36aa/aa* mice were injected intraperitoneally with 5 mg/kg LPS and humanely sacrificed
545 after 3 or 12 h. Serum cytokines were measured by multiplex bead assay or ELISA. Graphs
546 represent mean ± SEM from 5 untreated mice and 10 LPS-injected mice at each time point. *, $p <$
547 0.05; **, $p < 0.01$; ***, $p < 0.005$ (Mann Whitney). The IL-10 and TNF measurements (indicated by †)
548 were previously reported in (53), and are reproduced here with permission.
549
550 **Figure 9. Regulation of cytokine expression by the DUSP1-TTP axis *in vivo*.** *Dusp1+/+* : *Zfp36+/+*
551 (open bars), *Dusp1-/-* : *Zfp36+/+* (pale grey bars), *Dusp1+/+* : *Zfp36aa/aa* (black bars) or *Dusp1-/-* :

552 *Zfp36aa/aa* mice (dark grey bars) were injected intraperitoneally with 5 mg/kg LPS and humanely
553 sacrificed after 3 h. Spleens were excised, RNA was prepared and the indicated transcripts were
554 quantified by qPCR, with normalization against *B2m* mRNA and then against the untreated wild type
555 (*Dusp1+/+* : *Zfp36+/+*) control. Graphs represent mean ± SEM from 2 untreated and 4 LPS treated
556 mice of each genotype. n.s., not statistically significant; **, $p < 0.01$; ***, $p < 0.005$ (ANOVA).

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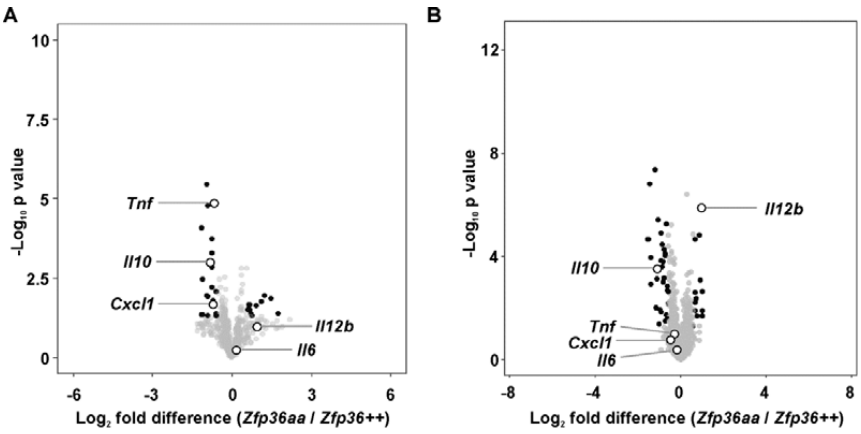

Figure 1. Differentially expressed transcripts in *Zfp36aa/aa* M-BMMs. M-BMMs were generated from 3 *Zfp36+/+* and 3 *Zfp36aa/aa* mice, and treated with 10 ng/ml LPS for 1 h (A) or 4 h (B). RNA was isolated and transcript abundance analyzed using Agilent microarrays and Partek Genomics Suite. Transcripts expressed above an arbitrary threshold of 200 RMA in at least two replicates and demonstrating upregulation by LPS (> 2X, $p < 0.05$) were selected for display. Data are illustrated in the form of volcano plots, in which transcripts with a greater than 1.5-fold difference of expression between *Zfp36+/+* and *Zfp36aa/aa* M-BMMs and a corrected p value less than 0.05 are shaded black. Under-expressed transcripts are to the left of the origin. Several transcripts are highlighted.

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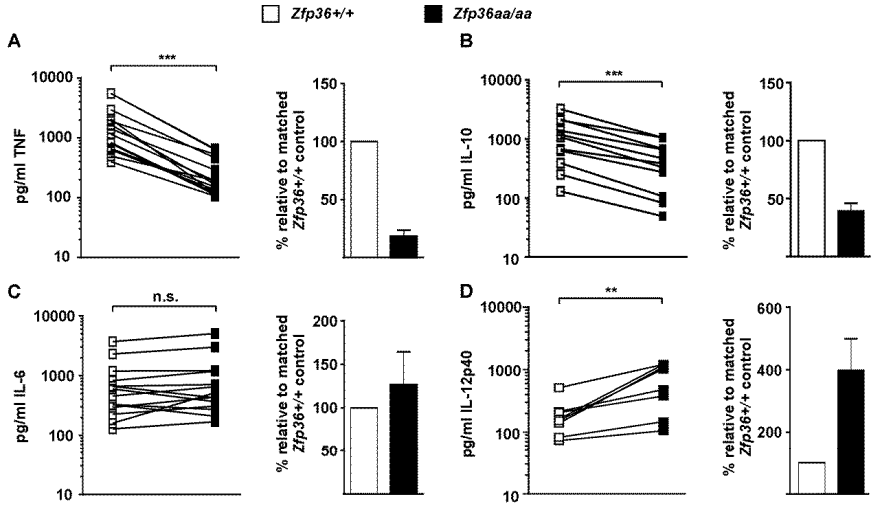

Figure 2. Expression of TNF, IL-10, IL-6 and IL-12p40 proteins by *Zfp36+/+* and *Zfp36aa/aa* M-BMMs. Matched *Zfp36+/+* and *Zfp36aa/aa* M-BMMs were cultured in 24-, 12- or 6-well dishes and stimulated with 10 ng/ml LPS for 4 h. ELISA or multiplex bead assay were used to measure TNF (A), IL-10 (B), IL-6 (C) and IL-12p40 (D). In the cases of TNF and IL-6, 5 of 14 experiments employed matched *Zfp36+/+* and *Zfp36aa/aa* littermates. All other experiments employed at least three mice of each genotype, mean cytokine concentrations being plotted. Cytokine quantities expressed by matched *Zfp36+/+* and *Zfp36aa/aa* M-BMMs or sets of M-BMMs are connected by lines. The graphs on the right of each panel show mean cytokine expression in *Zfp36aa/aa* M-BMMs relative to that in matched *Zfp36+/+* controls. n.s., not statistically significant; ***, $p < 0.005$; **, $p < 0.01$ (Wilcoxon matched pairs test).

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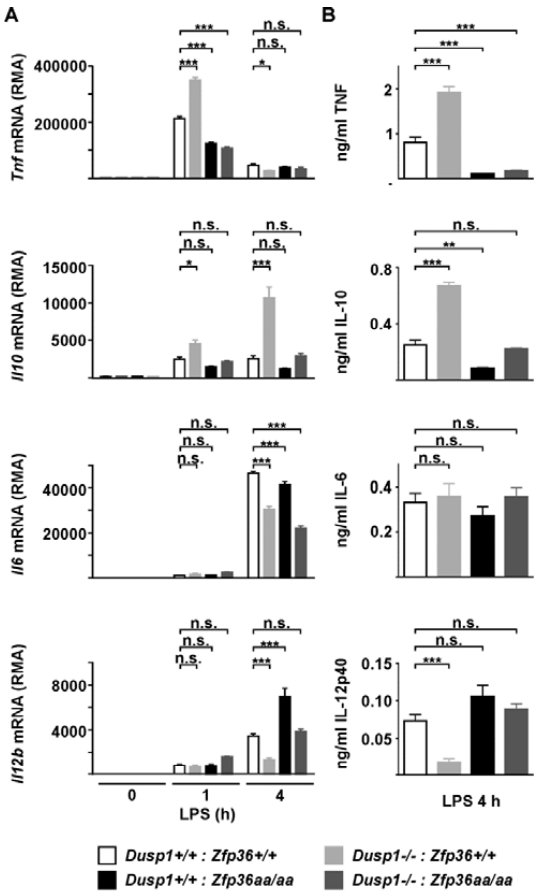

Figure 3. Regulation of inflammatory mediators by DUSP1 and TTP. Wild type, *Dusp1-/-*, *Zfp36aa/aa* and *Dusp1-/-* : *Zfp36aa/aa* M-BMMs were stimulated with 10 ng/ml for 1 h or 4 h. A) Expression of selected mRNAs was determined by microarray, and B) corresponding proteins were measured by ELISA or multiplex bead assay. Graphs represent mean ± SEM of 3 independent M-BMM cultures . n.s., not statistically significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (one way ANOVA).

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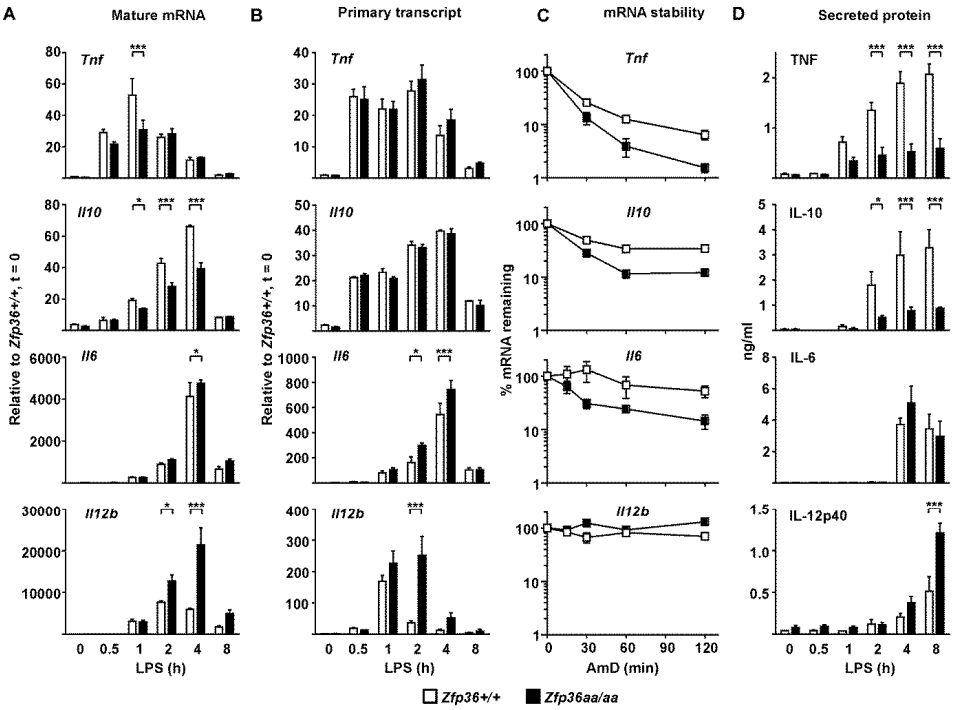

Figure 4. TTP mutation affects gene expression at different levels. *Zfp36+/+* and *Zfp36aa/aa* M-BMMs were treated with LPS for the times indicated, supernatants were collected and RNA isolated. A) Steady state mRNA abundance was measured by quantitative PCR. B) Primary transcripts were measured by quantitative PCR. C) Actinomycin D chase experiments were performed at peak of gene expression (1 h in the case of *Tnf*, 4 h in the cases of *Il10*, *Il6* and *Il12b*). D) Secreted proteins were measured by ELISA or multiplex bead assay. Graphs represent mean ± SEM of three independent M-BMM cultures in each case. Pairwise comparisons that showed no statistical significance are not indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (Mann Whitney).

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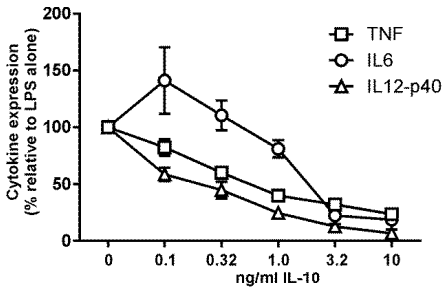

Figure 5. Dose dependent inhibition of pro-inflammatory gene expression by IL-10. *Il10-/-* M-BMMs were stimulated with 10 ng/ml LPS for 4 h in the presence of different concentrations of recombinant IL-10. TNF, IL-6 and IL-12p40 were quantified by ELISA. Cytokine levels were normalized against those in the absence of IL-10. Graphs show mean ± SEM of at least 8 independent M-BMM cultures.

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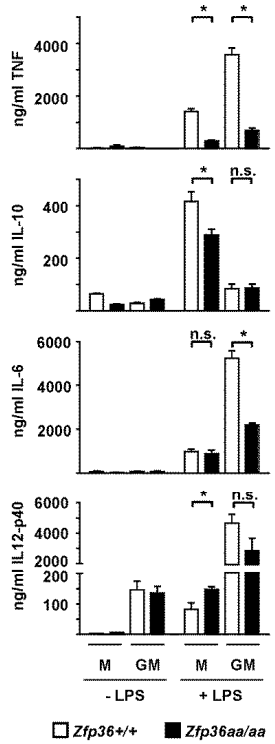

Figure 6. Differential gene expression in *Zfp36+/+* and *Zfp36aa/aa* M-BMMs and GM-BMMs. Macrophages were differentiated from bone marrow of *Zfp36+/+* and *Zfp36aa/aa* mice in the presence of either M-CSF of GM-CSF, and treated with 10 ng/ml LPS for 4 h. Cytokines were measured by ELISA or Luminex. Graphs represent mean ± SEM from 4 (TNF, IL-10 and IL-12p40) or 7 (IL-6) independent cultures. n.s., not statistically significant; *, $p < 0.05$ (Mann Whitney).

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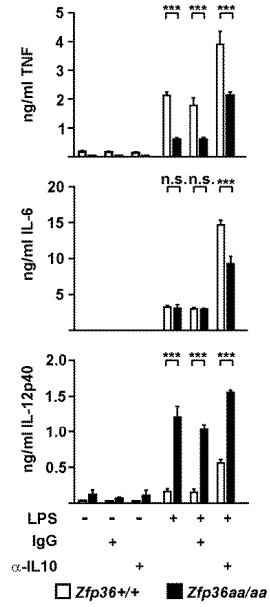

Figure 7. Endogenous IL-10 differentially affects expression of pro-inflammatory cytokines. *Zfp36+/+* and *Zfp36aa/aa* M-BMMs were treated with 10 ng/ml LPS for 4 h (TNF and IL-6) or 8 h (IL-12p40) in the presence of 10 μg/ml IL-10 neutralizing antibody or isotype control. Cytokines were measured by Luminex or ELISA. Graphs represent mean ± SEM from 4 independent M-BMM cultures. n.s., not statistically significant; ***, $p < 0.005$ (Mann Whitney).

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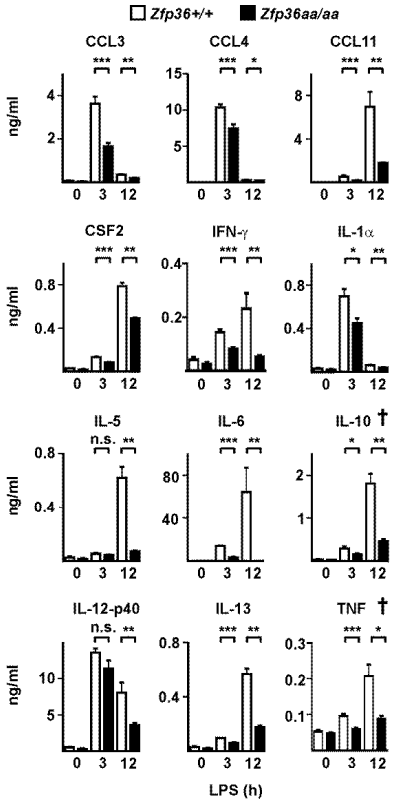

Figure 8. *In vivo* responses to LPS are broadly impaired in *Zfp36aa/aa* mice. *Zfp36+/+* and *Zfp36aa/aa* mice were injected intraperitoneally with 5 mg/kg LPS and humanely sacrificed after 3 or 12 h. Serum cytokines were measured by ELISA. Graphs represent mean ± SEM from 5 untreated mice and 10 LPS-injected mice at each time point. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (Mann Whitney). The IL-10 and TNF measurements (indicated by †) were previously reported in (49), and are reproduced here with permission.

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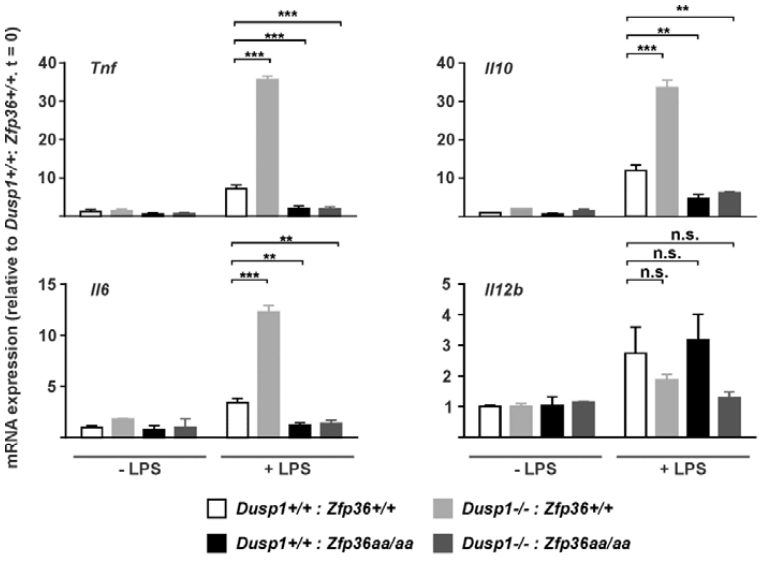

Figure 9. Regulation of cytokine expression by the DUSP1-TTP axis *in vivo*. *Dusp1+/+* : *Zfp36+/+* (open bars), *Dusp1-/-* : *Zfp36+/+* (pale grey bars), *Dusp1+/+* : *Zfp36aa/aa* (black bars) or *Dusp1-/-* : *Zfp36aa/aa* mice (dark grey bars) were injected intraperitoneally with 5 mg/kg LPS and humanely sacrificed after 3 h. Spleens were excised, RNA was prepared and the indicated transcripts were quantified by qPCR, with normalization against *B2m* mRNA and then against the untreated wild type (*Dusp1+/+* : *Zfp36+/+*) control. Graphs represent mean ± SEM from 2 untreated and 4 LPS treated mice of each genotype. n.s., not statistically significant; **, $p < 0.01$; ***, $p < 0.005$ (ANOVA).

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