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DOI: 10.4049/jimmunol.1402826

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Citation for published version (Harvard):
Ross, EA, Smallie, T, Ding, Q, O'neil, JD, Cunliffe, HE, Tang, T, Rosner, DR, Klevernic, I, Morrice, NA, Monaco, C, Cunningham, AF, Buckley, CD, Saklatvala, J, Dean, JL & Clark, AR 2015, 'Dominant suppression of inflammation via targeted mutation of the mRNA destabilizing protein tristetraprolin', Journal of Immunology, vol. 195, no. 1, pp. 265-276. https://doi.org/10.4049/jimmunol.1402826

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Dominant Suppression of Inflammation via Targeted Mutation of the mRNA Destabilizing Protein Tristetraprolin

Ewan A. Ross,*1 Tim Smallie,*1 Qize Ding,† John D. O’Neil,* Helen E. Cunliffe,* Tina Tang,* Dalya R. Rosner,* Iva Klevernic,‡ Nicholas A. Morrice,§ Claudia Monaco,¶ Adam F. Cunningham,* Christopher D. Buckley,* Jeremy Saklatvala,*, Jonathan L. Dean,† and Andrew R. Clark*

In myeloid cells, the mRNA-stabilizing protein tristetraprolin (TTP) is induced and extensively phosphorylated in response to LPS. To investigate the role of two specific phosphorylations, at serines 52 and 178, we created a mouse strain in which those residues were replaced by nonphosphorylatable alanine residues. The mutant form of TTP was constitutively degraded by the proteasome and therefore expressed at low levels, yet it functioned as a potent mRNA destabilizing factor and inhibitor of the expression of many inflammatory mediators. Mice expressing only the mutant form of TTP were healthy and fertile, and their systemic inflammatory responses to LPS were strongly attenuated. Adaptive immune responses and protection against infection by Salmonella typhimurium were spared. A single allele encoding the mutant form of TTP was sufficient for enhanced mRNA degradation and underexpression of inflammatory mediators. Therefore, the equilibrium between unphosphorylated and phosphorylated TTP is a critical determinant of the inflammatory response, and manipulation of this equilibrium may be a means of treating inflammatory pathologies. The Journal of Immunology, 2015, 195: 265–276.

The finely orchestrated programs of gene expression in immune cells responding to stimulation are dictated not only by transcriptional regulation but equally by post-transcriptional processes, in particular the control of mRNA stability (1–4). Many cytokines, chemokines, and other immune mediators are encoded by mRNAs that have intrinsically short half-lives, with their rapid turnover being essential for timely termination of immune responses. Furthermore, the modulation of rates of mRNA destruction by pro- and anti-inflammatory agonists is an important means of controlling the duration and quality of those responses. For example, the p38 MAPK signaling pathway is activated by proinflammatory stimuli and mediates transient stabilization of many inflammatory mediator mRNAs (reviewed in Ref. 5). The aberrant expression of proinflammatory mediators in pathological conditions cannot be fully understood without investigating posttranscriptional mechanisms.

Tristetraprolin (TTP) is the founding member of a small family of evolutionarily conserved, sequence-specific RNA binding proteins, which is encoded by the Zfp36 gene in the mouse and recognizes the optimum binding site WUAUUUAUW (where W is adenosine or uridine) (6). TTP binds to this sequence element in the 3′ untranslated region (UTR) of target transcripts, including Tnf and many other inflammatory factors. It then mediates recruitment of the carbon catabolite repression protein 4/carbon catabolite repression protein 4–associated factor 1 deadenylase complex and thus promotes the shortening of the poly(A) tail of the target mRNA (7–11). In most cases, this is rapidly followed by the destruction of the mRNA body (12). Hence, TTP is a critical negative regulator of expression of a large number of proinflammatory genes (6). Zfp36−/− mice lacking TTP protein have a severe, pervasive inflammatory phenotype that includes cachexia, dermatitis, autoimmunity, and inflammatory arthritis. The phenotype is largely (although not exclusively) due to increased stability of Tnf mRNA leading to increased expression of TNF protein in the myeloid compartment (3, 6, 13).

A working model of the posttranscriptional regulation of pro-inflammatory gene expression by the p38 MAPK pathway has been built up using a variety of in vitro assays, transient transfections of reporter construct, and studies of endogenous transcripts in cells derived from knockout mice (reviewed in Refs. 14, 15). According to this model, p38 MAPK activates the downstream kinase MAPK-activated protein kinase 2 (MK2), which phosphorylates TTP at serines 52 and 178 (murine TTP residue numbers), preventing the recruitment of the carbon catabolite repression protein 4/carbon catabolite repression protein 4–associated factor 1 complex and hence inhibiting deadenylation and promoting stabilization of TTP target transcripts. TTP is not expressed by resting macrophages, and for a number of reasons its accumulation depends on the p38 MAPK pathway. First, tran-

*School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, United Kingdom; †Imperial College London, Hammersmith Hospital, London W12 0NN, United Kingdom; ‡Unit of Signal Transduction, Interdisciplinary Cluster for Applied Genoproteomics, University of Liege, University Hospital, 4000 Liege, Belgium; §Beaton Institute for Cancer Research, Bearsden, Glasgow G61 1BD, United Kingdom; and ¶Kennedy Institute of Rheumatology, Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford OX3 7FY, United Kingdom

1E.A.R. and T.S. contributed equally to this work.

Received for publication November 7, 2014. Accepted for publication April 27, 2015.

This work was supported by Medical Research Council UK Project Grant G0800207 and Arthritis Research UK Program Grant 19614.

The microarray data presented in this article have been submitted to the Gene Expression Omnibus at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) under accession number GSE68449.

Address correspondence and reprint requests to Prof. Andrew R. Clark, School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, U.K. E-mail address: a.r.clark@bham.ac.uk

The online version of this article contains supplemental material.

Abbreviations used in this article: BMM, bone marrow–derived macrophage; MK2, MAPK-activated protein kinase 2; TTP, tristetraprolin; UTR, untranslated region.

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phorylated TTP. The experiments described in this study were intended to test in vivo consequences of perturbing the MAPK p38-mediated hypothesis. The experiments provide circumstantial evidence in support of this.

When p38 MAPK activity subsequently falls, TTP is activated via dephosphorylation, likely by PP2A (19), and drives the off-phase of the inflammatory response by promoting the rapid degradation by the proteasome (17, 18).

Materials and Methods

Reagents

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 when the endotoxin concentration was >0.1 U/ml.

Generation of a cell line stably expressing flag-TTP

RAW-MB01, a RAW264.7 clone expressing a tetracycline-responsive (Tet-OFF) transcription factor, was generated by stable transfection and selection using blasticidin (2 μg/ml). LPS-induced expression of Tet mRNA and TNF protein in this clone was indistinguishable from that in the parental RAW264.7 line (data not shown). Murine TTP cDNA with an N-terminal flag tag was subcloned into the tetracycline-responsive expression vector pTRE2Hyg (TaKaRa Bio). The vector was transfected into RAW-MB01, and several clones resistant to hygromycin (100 μg/ml) were isolated. Clone RAW-MB01-WT3.1 was used in the present study. Expression of flag-TTP was found to be strongly inducible by LPS but only weakly responsive to tetracycline, because of leakiness of the tetracycline-regulated promoter in RAW264.7 cells (data not shown).

Phosphoproteomics

RAW-MB01-WT3.1 cells were stimulated for 2 h with 10 ng/ml LPS and lysed in buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1% Triton X-100, 5% (w/v) Nonidet P-40, 1 mM DTT, 1 μg/ml pepstatin, 1 mM PMSF, 10 mM MgCl2). All purification steps were at 4°C. Lysates were precleared overnight using protein G–agarose beads and then applied to M2 anti-flag agarose beads for 4 h. Beads were washed six times with LB plus 0.15 M NaCl and three times with LB plus 0.15 M NaCl, and then flag-TTP was eluted in EB (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1 mM EGTA) containing 0.25 mg/ml 3X flag peptide. The eluate was subjected to SDS-PAGE, bands were visualized using colloidal Coomassie blue, excised, digested with trypsin, and analyzed by liquid chromatography–mass spectrometry on an LTQ Orbitrap–XL essentially as described previously. Peptide mixtures were separated on a 150 × 0.075 mm PepMap C18 column fitted to a Proxeon Easy-LC and liquid chromatography–tandem mass spectrometry was performed using multistage activation in the LTQ. Raw data files were searched against Swiss-Prot database (160613) using Mascot 2.4 (Matrix Science) and processed using Proteome Discoverer 1.3 software (Thermo Scientific). Search criteria were: precursor mass tolerance, 20 ppm; tandem mass spectrometry mass tolerance, 0.6 Da; enzyme, trypsin (KP/RP bonds cleaved, two missed cleavages permitted); variable modifications, phosphorylation (STY) and oxidation (M); minimum peptide ion score, 20. Extracted ion chromatography was performed using Proteome Discoverer 1.3 and Xcalibur software (Thermo Scientific).

Generation and testing of phospho-specific antisera

An antisera specific for phosphorylated Ser52 was generated by immunization of rabbits with the peptide LRGSpSpGSLPSR (Division of Signal Transduction Therapy, University of Dundee). The serum was precleared using the equivalent nonphosphorylated peptide and then affinity purified using the immunizing peptide. Codons 52 and 178 in pGEX-TTP (20) were mutated from serine to alanine codons using QuikChange (Agilent Technologies). Wild-type GST-TTP and GST-TTP-S52A/I78A were purified as described (20). In vitro phosphorylations were performed at 30°C for 30 min with 3 μg recombinant TTP substrate and 100 μM ATP in the absence or presence of 0.01 μM purified MK2 (Millipore). Aliquots containing 100 ng TTP were transferred to nitrocellulose and blotted with either phospho-specific antisera or antisera raised against total TTP (20).

Generation of Zfp36aa/aa strain

Targeting of the Zfp36 allele was performed with the assistance of Genoway (see schematic in Fig. 2A). Homology arms were generated by PCR from 129Sv genomic DNA and sequenced in their entirety. The targeting construct was created by sequential ligation of the two homology arms, a neomycin cassette flanked by Cre recombinase recognition sites and a synthetic DNA fragment (Top Gene Technologies) corresponding to part of the Zfp36 intron and part of the second exons of the second exon, incorporating serine to alanine substitutions at codons 52 and 178. The construct encodaspases a 9.5-kb region orthologous to Chr7:28,372,130-28,381,646 of the C57BL/6J genome (build 38). The position of insertion of the neomycin cassette corresponds to Chr7:28,378,880. The targeting construct was transfected into 129Sv embryonic stem cells. Correct recombination of the Zfp36 locus was confirmed by Southern blotting, PCR, and sequencing of genomic DNA for two independent neomycin-resistant embryonic stem cell clones. Recombinant embryonic stem cells were injected into C57BL/6J blastocysts, which were then reimplanted into pseudo-pregnant C57BL/6J females. Three males with >50% coat color chimerism were selected for breeding with C57BL/6J female mice constitutively expressing Cre recombinase (Cre deleter strain). Twenty-three of 23 pups had Agouti coat color, suggesting germline transmission of the modified Zfp36 allele. In three of those pups, correct excision of the neomycin cassette was confirmed by Southern blotting, genomic DNA sequencing, and PCR of genomic DNA with primers flanking the site of the neomycin insert: CLA1 forward (5'-CACACCTGCTGATGCCTCCTGCT-3') and CLA1 reverse (5'-CCCCAATCAAGACACAGCTGTCGTC-3'). Two Zfp36+/aa males were used as founders of a breeding colony, with genotyping using the above oligonucleotides. Initial experiments used littermate Zfp36+/aa and Zfp36aa/aa mice. The strain has now been back-crossed against C57BL/6J for 10 generations. Parallel, true breeding Zfp36+/aa versus Zfp36+/aa matings, and were used in all experiments described in the present study. Differences between Zfp36+/aa and Zfp36aa/aa mice were consistent whether littermates or true-breeding Zfp36+/aa and Zfp36aa/aa lines were used.

In vivo experiments and cell isolation

All animal experiments were approved by local ethical committees and performed under UK Home Office project licenses. C57BL/6 mice were purchased from Harlan Laboratories UK. All mice used were between 6 and 12 wk of age. To assess the response to LPS-induced systemic shock, mice were injected i.p. with 5 mg/kg purified LPS (Serotype EH100, Enzo Life Sciences) in 200 μl sterile PBS. Mice were culled or 12 h after challenge, and hindleg tissues were collected for histology. Serum markers of tissue damage were measured by the Clinical Pathology Service Laboratory at MRC Harwell (Didcot, U.K.). The response to bacterial infection was assessed by challenging mice with 5 × 105 attenuated Salmonella enterica serovar typhimurium as previously described (21).

Bone marrow–derived macrophages (BMMs) were generated from hindlimbs of humanly and chemically killed animals and differentiated in vitro with 100 ng/ml M-CSF (PeproTech) in 10% heat-inactivated FCS RPMI 1640 containing penicillin/streptomycin for 7 d. BMMs were plated at a density of 1 × 106/ml in the appropriate cell culture plate at least 1 d prior to...
stimulation. Primary peritoneal macrophages were harvested by lavaging the peritoneal cavity with 5 ml PBS containing 2 mM EDTA. Lavage fluid was collected and cells were resuspended at 2 × 10^6/ml in DMEM supplemented with 10% heat-inactivated FCS and penicillin/streptomycin. Cells were plated and allowed to adhere for 1 h at 37°C, before being washed twice with media. The remaining adherent cells were >90% F4/80+ macrophages as assessed by flow cytometry. Cells were rested overnight before stimulation. Mesenteric and inguinal lymph node T cells were negatively selected using Pan T Cell Isolation Kit II (Miltenyi Biotec) and stimulated for 72 h with plate-bound anti-CD3e and anti-CD28 (Bio-scientific). Spleen cells were harvested as described (21) and stimulated for 6 h with plate-bound anti-CD3e and anti-CD28, the last 3 h in the presence of brefeldin A (3 μg/ml). Intracellular IFN-γ was measured as described (22), using a Cyan ADF flow cytometer (Dako) and FlowJo software (Tree Star).

**Measurement of mRNA**

RNA was extracted from BMMs using QIAshredder columns and RNeasy Mini kits (Qiagen). cDNA was generated using iScript cDNA synthesis kit (Bio-Rad). Gene expression was quantified by real-time PCR on a LightCycler 480 II (Roche) using SuperScript III platinum RT-PCR kit and custom-synthesized oligonucleotide primers (Eurofins MWG) with SYBR Premix Ex Taq (Tosho). Relative gene expression was calculated using the ∆∆Ct method with Gapdh mRNA for normalization of RNA levels. Sequences of oligonucleotides are available upon request from the authors.

For microarray analysis, RNA was extracted as described and purified using RNA Clean and Concentrator kits (Cambridge Bioscience). Hybridization and analysis were performed by Oxford Gene Technology. Total RNA or control RNA were converted to labeled cRNA with Cy3 or Cy5, respectively, using two-color low input Quick Amp labeling kit (Agilent Technologies). Samples were hybridized onto SurePrint G3 mouse GE 60k slides and read using a G2505C scanner (Agilent Technologies). The scanned images were analyzed with Agilent Feature Extraction software (7.3.1.3) using default parameters. Processed signal intensities were background subtracted and spatially detrended.

**RNA immunoprecipitation**

BMMs (~2 × 10^7) were left untreated or stimulated with 10 ng/ml LPS for 1 h, harvested, and washed twice with ice-cold PBS, then lysed in 1 ml ice-cold polyethylene glycol lysis buffer (100 mM KCl, 10 mM HEPES [pH 7.0], 5 mM MgCl2, 0.5% Nonidet P-40, 1 mM EDTA, 100 U/ml RNase inhibitor, protease inhibitor mixture [Roche]). Lysates were frozen at −80°C, thawed, and cleared by centrifugation in a chilled benchtop centrifuge at 13,000 rpm for 5 min. Protein A beads were washed three times in NT2 buffer (150 mM NaCl, 50 mM HEPES [pH 7.4], 1 mM MgCl2, 5% BSA) and then incubated overnight with TFP-specific anti-serum or rabbit IgG. Beads were washed four times with buffer NT2 minus BSA, plus 1 mM DTT, 15 mM EDTA, 100 U/ml RNase inhibitor, and protease inhibitors. Beads were resuspended in the same modified NT2 buffer, added to macrophage lysates, and incubated overnight. Beads were washed four times in buffer NT2, and RNA was extracted using TRIzol reagent according to the manufacturer’s instructions. An equal volume of isopropanol and 20 μg glycogen were added, and RNA was precipitated by incubation at −20°C for 1 h and centrifugation at 13,000 rpm for 5 min at 4°C. The pellet was washed with 70% ethanol, resuspended in distilled water, and used as a template for RT-PCR as described above.

**Assessment of protein expression**

Secreted factors in tissue culture supernatants and sera were quantified by ELISA according to the manufacturer’s instructions (E Bioscience), or by using multiplex bead capture assays and a Bio-Plex 200 analyzer (Bio-Rad). Cell lysates were resolved on SDS-PAGE gels, probed with primary Abs, and immunoreactive proteins were visualized with HRP-coupled secondary Abs and chemiluminescence reagents (Bio-Rad, Pierce, or Cell Signaling Technology). Blots were visualized using the ChemiDoc MP imaging system and in some cases were quantified using Image Lab software (Bio-Rad).

**Adenoviral reporters**

The construction of adenoviral reporter constructs containing the human TNF 5’ region, the TNF 5’ region, and 3’ UTR, or five tandem copies of an NFκB consensus sequence has previously described, as was the method of macrophage infection (23). Levels of viral infection were quantified by flow cytometric assessment of GFP fluorescence. Luciferase assays were performed using the Bright-Glo luciferase assay system (Promega). Luminescence was measured with a Centro LB 960 luminometer (Berthold Technologies).

**Histology and confocal microscopy**

Spleens were snap frozen in liquid nitrogen immediately after excision and stored at −80°C. Tissues were subsequently embedded in OCT compound (Sakura Finetek) and 6-μm sections were cut and fixed in acetone at 4°C for 20 min. Sections were rehydrated in TBS and blocked in 10% horse serum in TBS for 1 h at room temperature. Primary Abs (rabbit anti-mouse TFP, H-120, Santa Cruz Biotechnology; sheep anti-mouse IgG, Abcam; rat anti-mouse F4/80, AbD Serotec) or appropriate species isotype controls were incubated overnight at 4°C in blocking buffer. Sections were washed three times in TBS and incubated for 1 h at room temperature with secondary Abs (donkey anti-rabbit IgG–Alexa Fluor 555, donkey anti-sheep IgG–Alexa Fluor 488, donkey anti-rat IgG–Alexa Fluor 647; all Jackson Immunoresearch Laboratories). Sections were washed three times and mounted in DABCO (2.5% [w/v] diabicyclo-octane in 90% glycerol/10% PBS [pH 8.6]). Images were captured by confocal microscopy using a Zeiss Axiosvert UV microscope with a ×40 objective and processed using an LSM image browser (Zeiss). Paraffin sections were dewaxed with xylene and rehydrated to water through graded alcohol steps. H&E staining was performed using standard methodologies.

**Statistical analysis**

GraphPad Prism (version 5.03) was used for statistical analysis. An unpaired, two-tailed Student t test was applied for comparison of two groups. For analysis of multiple groups, ANOVA was used with Bonferroni correction for multiple comparisons. A p value <0.05 was considered significant.

Probe intensity values for the microarray were analyzed with Partek Genomics Suite version 6.6, build 6.13.0315. A two-way mixed model ANOVA was performed on the entire dataset (55,681 probes) to calculate pairwise contrasts consisting of a corrected step-up p value (false discovery rate by the Benjamini–Hochberg step-up method, integrated into the Partek software) and a fold change or ratio for difference in gene expression. The ANOVA was calculated using the following factors: genotype, Zfp36+/+ versus Zfp36a−/−; condition, unstimulated versus 1 h LPS. Weakly expressed genes were filtered out when their sample intensity values (inverse log2) did not exceed an arbitrary value of 100 in at least two of three replicate samples from LPS-treated BMMs of either genotype. Probes were also filtered out that contained aberrant outliers (SD of three replicates exceeding average of three replicates). The microarray data described in this study have been submitted to the Gene Expression Omnibus at National Center for Biotechnology Information (GSE68449). (http://www.ncbi.nlm.nih.gov/).

**Results**

**Gene targeting to disrupt MK2-mediated phosphorylation of TFP**

Previous studies described extensive phosphoproteomic analysis of exogenous human TFP in HEK293 cells, which express little or no endogenous TFP protein and were not stimulated (24, 25). To identify phosphorylations occurring in a myeloid cell responding to a proinflammatory stimulus, epitope-tagged TFP was immunoprecipitated from a stably transfected and LPS-stimulated RAW264.7 cell line, which expressed exogenous TFP at a level only 2- to 3-fold higher than that of endogenous TFP. Phosphoproteomic analysis unequivocally identified 14 sites of phosphorylation (11 serines, 2 threonines, and 1 tyrosine). Among these were serines 52 and 178 (Fig. 1A). One tryptic peptide, PPGEPSPSPTSPTATPTTSSR, was phosphorylated at up to three of the underlined residues. A second large peptide corresponding to residues 188–234 of murine TTP contained up to four discrete phosphorylations that could not be accurately assigned. Broadly, the pattern of phosphorylation was similar to that of human TFP in HEK293 cells (24, 25), although the previous studies did not report phosphorylation of Ser52 (which corresponds to Ser52 of the murine protein). It was confirmed by in vitro phosphorylation of recombinant wild-type or mutated TFP mass spectrometry,
and two dimensional phosphopeptide mapping that MK2 efficiently phosphorylates both Ser\(^52\) and Ser\(^178\) (data not shown).

Phospho-specific Abs were raised against Ser\(^52\) and Ser\(^178\), and their specificity was established using wild-type or mutant GST-TTP phosphorylated in vitro by MK2 (Fig. 1B). The ability of the Abs to recognize recombinant TTP was dependent on MK2 and intact phospho-modified proteins. The phospho-specific Abs both recognized endogenous TTP immunoprecipitated from LPS-treated RAW264.7 cells (Fig. 1C), providing further evidence that these sites are phosphorylated in the context of the response to LPS. Incubation of RAW264.7 cells with a p38 inhibitor decreased both Ser\(^52\) and Ser\(^178\) phospho-TTP signals (data not shown). As previously reported (17), inhibition of p38 MAPK also decreased levels of total TTP. Therefore, at this stage it cannot be formally concluded that in vivo phosphorylation of S52 and S178 is dependent on p38 MAPK. Evidence in support of this hypothesis is presented below.

Functional consequences of TTP phosphorylation have so far been studied in vitro or in transfected cells but not in vivo. Therefore, homologous recombination was used to replace both serine 52 and 178 codons with alanine codons in the endogenous murine Zfp36 locus (Fig. 2A). We refer to the modified locus as Zfp36aa/aa pups were underexpressed by 8% (Table I). The proportion of LPS-induced transcripts that were underexpressed by Zfp36aa/aa BMMs significantly underexpressed 165 genes when the stimulus was applied for 4 h rather than 1 h (Table I, third panel). Selected impairment of responses to LPS in Zfp36aa/aa macrophages

Microarray analysis was carried out to identify genome-wide effects of Zfp36 gene targeting on the LPS responses of macrophages. Of 2130 transcripts significantly induced by 1 h treatment with LPS, Zfp36aa/aa BMMs significantly underexpressed 165 (8%) (Table I). The proportion of LPS-induced transcripts that were underexpressed by Zfp36aa/aa BMMs remained similar when a 3-, 5-, or 10-fold cut-off for LPS response was applied, or when the stimulus was applied for 4 h rather than 1 h (Table I, Supplemental Table I, GSE68449). Clearly, mutation of the Zfp36 gene did not globally impair macrophage responses to LPS. Instead, expression of a distinct subset of genes was altered. Fig. 3A

**FIGURE 1.** Identification of sites of phosphorylation of TTP in LPS-stimulated myeloid cells. (A) Schematic of phosphorylation sites identified in RAW264.7 cells expressing flag-TTP. Serines 52 and 178 are boxed. Pale gray bars indicate tetraprolin (PPPP) motifs. The dark gray bar indicates a large tryptic peptide that contained up to four phosphorylations, only one of which (S188) could be assigned with confidence. Dotted lines indicate positions of C\(_3\)H zinc finger motifs. (B) Validation of phospho-specific antiserum. Phospho-specific antisera were raised against the phosphopeptides LTGRSTpSLVEGR (S52) and LRQSIpSFSGLPSGR (S178). GST-TTP and GST-TTP-S52A/S178A (-TTPaa) were mock treated (−) or phosphorylated in vitro using MK2 (+) as described in Materials and Methods, transferred to nitrocellulose, and dot blotted using either a previously described anti-TTP antiserum (20) or the phospho-specific antiserum. (C) LPS-induced phosphorylation of endogenous murine TTP at serines 52 and 178. RAW264.7 cells were stimulated with 10 ng/ml LPS for the indicated times, lysates were prepared, and endogenous TTP was immunoprecipitated using an antiserum raised against the N terminus of murine TTP (20). Immunoprecipitates were Western blotted using either phospho-specific antiserum or an anti-TTP antiserum, with HRP-conjugated protein G for detection. The experiment was performed twice for each phospho-specific antiserum.

TTPaa is weakly expressed

Zfp36aa mRNA was expressed at lower levels than Zfp36 mRNA in BMMs (Fig. 2B), and this was accompanied by a decrease in mRNA stability (Fig. 2C). TTP is thought to autoregulate its expression via binding to adenosine/uridine-rich elements in the Zfp36 3′ UTR and destabilization of Zfp36 mRNA (3, 16, 26). The simplest interpretation of our findings is that the mutant form of TTP has greater mRNA destabilizing activity and enhances the degradation of its own (i.e., Zfp36aa) mRNA.

Relative to its wild-type counterpart, TTPaa was weakly expressed in LPS-treated BMMs (Fig. 2D). On the basis of scanning densitometry in four independent experiments, we estimate an 80% decrease in levels. Because this was greater than the decrease in mRNA expression, cycloheximide chases were performed to investigate protein stability (Fig. 2E). Wild-type TTP was relatively stable (top panel, lanes 2–4), but was rapidly degraded following addition of a p38 MAPK inhibitor (top panel, lanes 5 and 6). In contrast, TTPaa was rapidly degraded whether the p38 MAPK inhibitor was absent or present (third panel, lanes 2–6). A proteasome inhibitor protected TTPaa from degradation (Fig. 2F, last two lanes). Taken together, these observations confirm that p38 MAPK-dependent phosphorylation of Ser\(^52\) and Ser\(^178\) protects TTP protein from degradation by the proteasome (17, 18). Therefore, the diminished expression of TTPaa appears to result from (at least) two distinct phenomena: 1) an increased rate of degradation of Zfp36aa mRNA, and 2) an increased rate of degradation of TTPaa protein by the proteasome.

Selective impairment of responses to LPS in Zfp36aa/aa macrophages

Microarray analysis was carried out to identify genome-wide effects of Zfp36 gene targeting on the LPS responses of macrophages. Of 2130 transcripts significantly induced by 1 h treatment with LPS, Zfp36aa/aa BMMs significantly underexpressed 165 (8%) (Table I). The proportion of LPS-induced transcripts that were underexpressed by Zfp36aa/aa BMMs remained similar when a 3-, 5-, or 10-fold cut-off for LPS response was applied, or when the stimulus was applied for 4 h rather than 1 h (Table I, Supplemental Table I, GSE68449). Clearly, mutation of the Zfp36 gene did not globally impair macrophage responses to LPS. Instead, expression of a distinct subset of genes was altered. Fig. 3A
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FIGURE 2. Generation and initial characterization of the Zfp36aa/aa strain. (A) Schematic of gene targeting strategy. Exons are shown as boxes, Flox sites as arrowheads. The first downward arrow represents homologous recombination of the targeting fragment into the Zfp36 locus, and the second represents Cre-mediated excision of the neomycin cassette. Left and right arrows represent the primers used in genotyping, that is, CLA1-For and CLA1-Rev. A representative genotyping gel is shown for three litters of mice born from Zfp36+/+×aa crosses. The wild-type locus yields a 234-bp PCR product, the modified locus a 328-bp product. (B) Zfp36+/+ and Zfp36aa/aa BMMs were treated with 10 ng/ml LPS for 1, 2, 4, and 8 h, and Zfp36 mRNA was measured by quantitative PCR with normalization first against Gapdh then against expression level in Zfp36+/+ cells treated with LPS for 1 h. Means ± SEM of four independent experiments are shown. **p < 0.005. (C) Zfp36+/+ and Zfp36aa/aa BMMs were treated with 10 ng/ml LPS for 4 h, actinomycin D (AMD; 5 μg/ml) and DRB (50 μM) were added, and the subsequent decay of Zfp36 mRNA was monitored by quantitative PCR. Means ± SEM of four independent experiments are shown. (D) Zfp36+/+ and Zfp36aa/aa BMMs were treated as in (B) and levels of TTP protein were assessed by Western blotting. (E) As indicated in the schematic, Zfp36+/+ and Zfp36aa/aa BMMs were treated with LPS for 2 h, then 5 μg/ml cycloheximide (CHX) was added in the presence of 5 μM SB202190 (SB) or vehicle control (0.01% DMSO). Cells were harvested at 0, 2, 3, or 6 h as indicated, and TTP was detected by Western blotting. (F) As indicated in the schematic, Zfp36+/+ and Zfp36aa/aa BMMs were treated with LPS for 4 h, then cycloheximide (CHX) was added in the presence of 5 μg/ml MG132 or vehicle control (0.01% DMSO). Cells were harvested at 0, 4, 5, or 6 h as indicated, and TTP was detected by Western blotting. Western blots in (D)–(F) are representative of at least three independent experiments.

illuminates in heat map form 24 transcripts that were induced at least 3-fold by LPS and significantly underexpressed by Zfp36aa/aa BMMs. So far, 12 of these differentially expressed transcripts have been validated by quantitative RT-PCR (Figs. 2B, 3B and data not shown; also see Fig. 5B), with good agreement between microarray-based and quantitative RT-PCR–based measurements (Fig. 3B). The list of underexpressed transcripts at 1 h included Tnf, Cxcl1, Cxcl2, and Il10, all of which are well-established targets of negative regulation by TTP in myeloid cells (6). Zfp36 itself was also significantly underexpressed, consistent with the suggestion that TTP autoregulates its own expression. Two alternative F3 transcripts were underexpressed by Zfp36aa/aa BMMs. F3 encodes tissue factor, an initiator of coagulation that has recently been identified as a target of TTP in macrophages (27). Ier3, Pim1, and Lif were previously identified as TTP targets in cell types other than primary macrophages (28–30). To our knowledge, Areg and Clcf1 have not been identified as targets of TTP; however, their 3’ UTRs have highly conserved adenosine/uridine-rich elements with matches to the consensus TTP binding sequence UAUAUAU (Fig. 3C). As expected, Tnf and Cxcl1 mRNAs were detected in TTP immunoprecipitates from LPS-activated wild-type BMMs (Fig. 3D). F3 and Clcf1 mRNAs were also enriched in TTP immunoprecipitates, whereas Bcl3 mRNA, which lacks a consensus TTP binding sequence, was strongly induced by LPS but did not interact with TTP. As an additional negative control, Gapdh mRNA was not enriched in TTP immunoprecipitates. These observations confirm that tissue factor expression is regulated by TTP, and they suggest that Clcf1 may also be an authentic novel target of TTP. Genome-wide alterations of expression may therefore be a useful starting point for the identification of transcripts regulated by the p38 MAPK/MK2/TTP axis in macrophages or other cell types.

A number of cytokine- and chemokine-encoding transcripts were found among those underexpressed by Zfp36aa/aa BMMs. To

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\begin{align*}
\text{Table I. Genome-wide dysregulation of responses to LPS in Zfp36aa/aa BMMs} \\
\hline
\text{LPS-Induced Transcripts} & \text{1 h} & \text{4 h} \\
\text{↓ in Zfp36aa/aa} & \text{↑ in Zfp36aa/aa} & \text{↓ in Zfp36aa/aa} & \text{↑ in Zfp36aa/aa} \\
\hline
\text{All} & 2130 & 165 (8%) & 60 (3%) & 4933 & 291 (6%) & 151 (3%) \\
\text{>3-fold} & 434 & 24 (6%) & 14 (3%) & 1441 & 103 (7%) & 47 (3%) \\
\text{>5-fold} & 250 & 17 (7%) & 10 (4%) & 897 & 70 (8%) & 29 (3%) \\
\text{>10-fold} & 142 & 14 (10%) & 6 (4%) & 476 & 37 (8%) & 13 (3%) \\
\hline
\end{align*}
\]
extend this observation a preliminary experiment was carried out using an Ab array to survey secretion of inflammatory mediator proteins by LPS-treated Zfp36aa/aa BMMs (Supplemental Fig. 1). Expression of TNF, CCL3, CXCL1, CXCL2, CXCL10, IL-1B, CSF2, and other cytokines or chemokines was lower in Zfp36aa/aa BMMs (Supplemental Fig. 1). Although targeted mutation of Zfp36+/+ mice display decreased systemic responses to LPS

Tissue-specific deletion of the Zfp36 gene in myeloid cells rendered mice extremely sensitive to LPS-induced endotoxemia (3, 13). It was hypothesized that a gain of function mutation of TTP would have the opposite effect. Systemic challenge of Zfp36+/+ mice by i.p. injection of LPS increased the expression of TTP protein in spleen (Fig. 6A). TTP staining was predominantly cytoplasmic as expected, weak in the B and T cells of the white pulp,
Acquired immune responses to S. typhimurium are not strongly impaired in Zfp36aa/aa mice

The mutation of two serine codons effectively increased TTP activity by preventing its inactivation, impaired the expression of several inflammatory mediators, and conferred significant protection in an experimental model of endotoxemia. However, these codons are subject to strong positive selective pressure, apparently being conserved throughout vertebrate evolution (25). It was hypothesized that the loss of phosphorylation-mediated dynamic control of TTP function may impair adaptive immune responses, causing increased susceptibility to pathogens. By way of precedent, disruption of the Mk2 gene (encoding the kinase that phosphorylates serines 52 and 178 of TTP) increased susceptibility to the intracellular pathogen Listeria monocytogenes (35). To test the hypothesis, Zfp36+/+ and Zfp36aa/aa mice were inoculated with an attenuated strain of S. typhimurium. In this well-established model of infectious disease (22, 36), bacteria typically peak in numbers in the liver and spleen ~7 d after inoculation, with most being cleared by day 55. Clearance of this strain of Salmonella is mediated by a Th1 adaptive immune response and is highly dependent on IFN-γ, but not TNF (22, 37).

Bacterial loads were significantly higher in spleens and livers of Zfp36aa/aa mice 21 d after inoculation, but they were comparable to those in wild-type mice by day 55 (Fig. 7A). Pathogen-induced expansion and subsequent contraction of the spleen followed identical time courses in Zfp36+/+ and Zfp36aa/aa mice (Fig. 7B). Zfp36+/+ and Zfp36aa/aa mice were vaccinated twice with PBS or purified S. typhimurium porin proteins prior to challenge with live S. typhimurium. The immunization gave similarly strong (>97.5%) protection against the establishment of bacterial infection in both strains of mice (Fig. 7C).

IFN-γ is reported to be a target of negative regulation by TTP (38). The ability of Zfp36aa/aa mice to clear an S. typhimurium infection was therefore surprising and prompted us to investigate the effect of TTP mutation on the expression of IFN-γ by T cells. Stimulation of Zfp36+/+ and Zfp36aa/aa splenocytes via CD3 and CD28 induced similar numbers of IFN-γ-expressing cells and similar secretion of IFN-γ protein (Fig. 7D, 7E). In summary, we have not yet found evidence of impaired adaptive immune responses in the Zfp36aa/aa mouse, consistent with its ability to both clear an S. typhimurium infection and mount an effective response to vaccination against this pathogen.

TTPaa functions as a dominant inhibitor of inflammatory gene expression

Substitution of two phospho-acceptor sites of TTP by non-phosphorylatable residues appeared to cause a gain of function, increasing the rate of degradation of target mRNAs and impairing the expression of inflammatory mediators. To test whether the mutant allele exerts a dominant phenotype in the presence of a wild-type allele, Zfp36+/+ and Zfp36aa/aa mice were bred to generate heterozygotes. All of these mice are derived from the same breeding program and have as near as possible the same genetic background with the exception of the Zfp36 locus. As previously shown, the expression of TTP protein was high in Zfp36+/+ BMMs and comparatively low in Zfp36aa/aa BMMs (Fig. 8A). In heterozygous BMMs, the levels of TTP were intermediate, with an estimated 10–20% of this being the mutant form (Fig. 8B). CXCL1, CXCL2, IL-10, IL-12p70, and TNF were all significantly underexpressed by both Zfp36+/+ and Zfp36aa/aa BMMs, with no significant difference between these
Genotypes (Fig. 8C). The LPS-induced expression of CCL2 did not differ significantly between Zfp36+/+, Zfp36+/aa, and Zfp36aa/aa BMMs, confirming that the targeted mutation of TTP phosphoacceptor sites does not globally impair the response to engagement of TLR4. After i.p. injection of LPS, serum levels of CXCL1, CXCL2, IL-10, and IL-12p70 were significantly lower in both Zfp36aa/aa and Zfp36+/+ than in Zfp36+/+ control mice (Fig. 8D). In the cases of CXCL1 and IL-10, there were small but statistically significant differences in expression between Zfp36aa/aa and Zfp36+/+ mice. There was a small but statistically significant reduction in the levels of CCL2 in serum of LPS-treated Zfp36aa/aa and Zfp36+/+ mice. TTPaa therefore appears to function as a dominant mRNA destabilizing factor and inhibitor of inflammatory gene expression, even when in competition with a large excess of wild-type protein.

Discussion

The substitution of only two codons of the endogenous murine locus encoding TTP gave rise to a strong and dominant hypoinflammatory phenotype that is (as far as we are aware) unprecedented for such a minimal genetic modification. The expression of several inflammatory mediators was impaired in LPS-treated peritoneal or bone marrow–derived Zfp36aa/aa macrophages. Organ damage and systemic expression of inflammatory mediators in response to LPS were likewise diminished in Zfp36aa/aa mice. In some cases (e.g., CXCL2, IL-17), serum levels were >10-fold lower in Zfp36aa/aa mice than in wild-type controls. We also found that the genetically modified mice were protected in other models of inflammatory pathology (E.A. Ross, T. Smallie, C.D. Buckley, J.L. Dean, and A.R. Clark, manuscripts in preparation). Note that TTP has been identified as a putative tumor suppressor, which limits the expression of many regulators of cell cycle progression and survival. Loss of expression or phosphorylation-mediated inactivation of TTP have been linked to poor prognosis in several types of cancer (39–41). This raises the intriguing prospect, now under investigation, that the Zfp36aa/aa mouse may have a degree of protection in some experimental models of cancer.

It has been suggested that TTP decreases expression of inflammatory mediators in part by impairing the activation of NF-κB (31, 42, 43). Although we did not directly test this hypothesis, our observations suggest that the enhanced anti-inflammatory function of TTPaa is related to the canonical mechanism of mRNA destabilization rather than involving inhibition of NF-κB signaling. Accordingly, several known mRNA targets of TTP were degraded at increased rates in Zfp36aa/aa cells, whereas there was no evidence of impaired NF-κB function or decreased transcription of Tnf, a well-characterized NF-κB–dependent gene. The phenotype reflects the inability of TTPaa to be inactivated via p38 MAPK–MK2 signaling. TTP has also been reported to impair translation of target transcripts, and phosphorylation of Ser52 and Ser178 to alleviate this effect (13, 15). In our hands, decreases in expression of cytokines and chemokines by Zfp36aa/aa BMMs were generally accompanied by decreases in the stability and the steady-state levels of the corresponding mRNAs. However, it cannot be ruled out that decreased mRNA translation also contributes to the underexpression of inflammatory mediators by Zfp36aa/aa cells or animals. If this proves to be correct, experiments such as microarrays, based on quantification of steady-state mRNA, may underestimate the impact of the Zfp36 mutation.
Expression of TTPaa was ∼5-fold lower than that of the wild-type protein. At least two distinct phenomena contributed to this underexpression. First, the stability of Zfp36 mRNA was decreased in cells expressing the mutated form of TTP, supporting previous suggestions that TTP autoregulates its expression via destabilization of its own mRNA (3, 16, 26). Second, TTPaa was consti-
tutively unstable, whereas wild-type TTP protein was stabilized in a p38 MAPK-dependent manner. This confirms that p38 MAPK-dependent phosphorylation of TTP at Ser52 and Ser178 protects it from proteasomal degradation (17, 18). Despite this decrease of expression, the phenotype represents a gain of function, as demonstrated most clearly by the behavior of heterozygous (Zfp36+/aa) mice or macrophages derived from them. Their expression of inflammatory mediators was as low, or almost as low, as in Zfp36aa/aa mice or macrophages. Similar half-lives of target mRNAs and levels of expression of inflammatory mediators in Zfp36+/aa and Zfp36aa/aa macrophages imply that TTPaa effectively competes with an excess of wild-type TTP to promote more rapid mRNA degradation. One possible explanation is that the phosphorylation of Ser52 and Ser178 decreases the affinity of TTP for RNA, so that the nonphosphorylatable mutant has a competitive advantage in binding to target sequences. The difference in expression of both TTPaa protein and its mRNA targets makes it extremely difficult to test this hypothesis directly in Zfp36aa/aa macrophages. However, other researchers have used biochemical methods to demonstrate MK2-mediated decreases in mRNA binding affinity of TTP (44, 45).

The results described in the present study emphasize that the equilibrium between phosphorylated and unphosphorylated TTP (with respect to serines 52 and 178) plays a crucial role in determining the inflammatory output of macrophages. The heterozygous phenotype suggests that quite a small shift in favor of the dephosphorylated form could have strong anti-inflammatory consequences. It follows that endogenous factors or therapeutic reagents could exert anti-inflammatory effects by influencing the phosphorylation/dephosphorylation equilibrium. As an example of the former, an accompanying paper shows that dual specificity phosphatase 1 limits inflammatory responses in vitro and in vivo partly by modulating the phosphorylation status of TTP (34).

There are at least four possible methods for therapeutic targeting of the equilibrium: 1) Inhibitors of p38 MAPK destabilize inflammatory mediator mRNAs by activating TTP (46, 47). However, despite clear therapeutic effects in experimental models of acute and chronic inflammation, these compounds do not have sustained clinical benefits in humans (48). A possible explanation for their transient anti-inflammatory action is that in a chronic setting they prevent the accumulation of TTP protein and therefore have little or no effect on target mRNA stability (46, 49). 2) Disruption of the gene encoding MK2 impairs inflammatory responses of macrophages, identifying MK2 as a candidate target for novel anti-inflammatory drugs (15). However, there are significant technical challenges to the development of MK2 inhibitors (50). 3) It may be possible to increase TTP activity and downregulate expression of inflammatory mediators via agonism of PP2A. Increasing PP2A activity is already under investigation as a therapeutic approach in cancer and neurodegenerative disease.
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