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Cullin 4B Is Recruited to Tristetraproline-Containing Messenger Ribonucleoproteins and Regulates TNF-α mRNA Polysome Loading

Jason R. Pfeiffer* and Seth A. Brooks*,†,‡

TNF-α is a central mediator of inflammation and critical for host response to infection and injury. TNF-α biosynthesis is controlled by transcriptional and posttranscriptional mechanisms allowing for rapid, transient production. Tristetraprolin (TTP) is an AU-rich element binding protein that regulates the stability of the TNF-α mRNA. Using a screen to identify TTP-interacting proteins, we identified Cullin 4B (Cul4B), a scaffolding component of the Cullin ring finger ligase family of ubiquitin E3 ligases. Short hairpin RNA knockdown of Cul4B results in a significant reduction in TNF-α protein and mRNA in LPS-stimulated mouse macrophage RAW264.7 cells as well as a reduction in TTP protein. TNF-α message $t_{1/2}$ was reduced from 69 to 33 min in LPS-stimulated cells. TNF-3′ untranslated region luciferase assays utilizing wild-type and mutant TTP-AA (S52A, S178A) indicate that TTP function is enhanced in Cul4B short hairpin RNA cells. Importantly, the fold induction of TNF-α mRNA polysome loading in response to LPS stimulation is reduced by Cul4B knockdown. Cul4B is present on the polysomes and colocalizes with TTP to exosomes and processing bodies, which are sites of mRNA decay. We conclude that Cul4B licenses the TTP-containing TNF-α messenger ribonucleoprotein for loading onto polysomes, and reduction of Cul4B expression shuts the messenger ribonucleoproteins into the degradative pathway. The Journal of Immunology, 2012, 188: 1828–1839.

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Abbreviations used in this article: ActD, actinomycin D; ARE, AU-rich element; CIP, calf intestine alkaline phosphatase; CRL, Cullin ring finger ligase; Cul4B, Cullin 4B; EDC4, enhancer of decapping protein 4; mRNP, messenger ribonucleoprotein; NT, no tristetraproline; P-body, processing body; qPCR, quantitative PCR; shRNA, short hairpin RNA; TTP, tristetraprolin; UTR, untranslated region; XLMR, X-linked mental retardation syndrome.
ticular interest to our studies, there are specific immunologic phenotypes associated with Cul4B mutations. Leukocytes from mutant Cul4B heterozygotes express only wild-type Cul4B, suggesting that leukocytes expressing mutant Cul4B are strongly selected against in vivo (26). Also, patients with mutant Cul4B exhibit increased monocyte counts and an increase in the percentage of monocytes in total WBCs (26). The precise role of Cul4B in these processes is unclear.

Our data demonstrate that stable knockdown of Cul4B by short hairpin RNA (shRNA) reduces LPS-induced TTP protein levels and blunts LPS-induced TNF-α production in mouse RAW264.7 macrophages. TNF-α mRNA levels are reduced in LPS-stimulated Cul4B shRNA macrophages as is the TNF-α mRNA t1/2 (control shRNA t1/2 = 68.8 min, Cul4B shRNA t1/2 = 33.3 min). An examination of TTP activity by TNF-α 3′ UTR luciferase assays demonstrates that both wild-type TTP and the TTP-AA (Ser52–178– alanine) mutant are significantly more active in Cul4B shRNA macrophages. Neither the combined activation of p38 and ERK nor proteasome inhibition completely blocked TTP activity in the Cul4B shRNA cells. Subcellular fractionation and immunocytochemistry demonstrates the presence of Cul4B on the polysomes and Cul4B colocalization with TTP to both P-bodies and exosome granules. Finally, quantitative PCR (qPCR) demonstrates that less TNF-α is loaded onto the polysomes in Cul4B shRNA macrophages and that the increase in TNF-α mRNA loading in response to LPS stimulation is lower in Cul4B shRNA cells compared with control shRNA cells. We conclude that Cul4B is necessary to effectively load mRNA onto the polysomes in Cul4B shRNA macrophages.

Materials and Methods

Materials

LPS (Escherichia coli 026:B6) was purchased from Sigma-Aldrich. Abs were purchased from the following: Cul4B (2515.00.02; SDIX), tubulin (ab4074; Abcam), p300 (sc-584; Santa Cruz Biotechnology), Rrp45 (ab5703; Abcam), and enhancer of decapping protein 4 (EDC4; sc-137444; Santa Cruz Biotechnology); the anti-TTP Ab was a generous gift of Dr. William Rigby (Dartmouth Hitchcock Medical Center and Dartmouth Medical School).

RNA capture assay for TTP-interacting proteins

The RNA capture assay was previously described (19) with the following variation: 5 × 10⁶ 293 cells were plated in 150-mm tissue culture plates and transfected with 20 μg luciferase reporter construct pGL3-TNF 3′ UTR and 1 μg either pdDNA3.1 HisC or TTP expression vector using Lipofectamine 2000 (Life Technologies). The RNA capture assay was performed on n = 4 biological replicates.

Immunoblotting

Cytoplasmic and nuclear fractions were generated as previously described (5). Cytoplasmic and nuclear lysates were resolved by 8–16% gradient SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted with the indicated Abs according to the manufacturer’s protocol.

Identification of Cul4B

Cul4B knockdown was assayed by Western blot for Cul4B. Promising clones were then successively refined by two additional rounds of limiting dilution seeding and screening by Western blot. Quantification of Cul4B knockdown was performed using ImageJ (National Institutes of Health) as previously published (27). Nontarget control shRNA expressing RAW264.7 cells were maintained as a mixed population selected based on their resistance to 6 μg/ml puromycin.

Luciferase vectors

The pGL3-Control (Promega) is the backbone vector into which the intron from the 5′ UTR of the pRL-SV40 was amplified by PCR and cloned into the 5′ UTR of pGL3-Control. pGL3-TNF 3′ UTR contains the complete mouse TNF 3′ UTR: nt 865–1619 cloned in to the XbaI site in pGL3-Control containing the intron in its 5′ UTR vector.

Transient transfections and luciferase assays

RAW 264.7 cell transfections were performed in triplicate at least four times (n = 4) using Lipofectamine LTX according to the manufacturer’s protocol (Invitrogen) for RAW264.7 cells. Briefly, 125,000 RAW 264.7 cells per well were plated in 24-well plates and allowed to rest for 24 h. A total of 250 ng luciferase construct and 25 ng mouse TTP expression vector or backbone pcDNA3.1 His C vector were used for each well. For transfections including MEK1 and MEK6 (100 ng each/well), an equal amount of pcDNA3.1 His C was transfected to balance the total amount of DNA. Cells were lysed 24 h after transfection and luciferase values quantified using ONE-Glo luciferase reagent (Promega).

mRNA quantification and stability assays

Total RNA was isolated using the RNAeasy Mini Kit with on-column DNase digestions (Qiagen) and quantified by spectrophotometry. Reverse transcription was performed using Superscript III ( Invitrogen, Carlsbad, CA), 1 μg DNase-treated total RNA, and oligo (d)T according to the manufacturer’s protocol. qPCR for TNF-α and cyclophilin were performed as previously described using the SYBR Green PCR Core Kit (Applied Biosystems, Foster City, CA) and a three-step reaction: 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s (28). The cyclophilin primers have an efficiency of 95.7%, whereas the TNF-α primers have an efficiency of 97.8%; cyclophilin expression changes by <5% over the 4-h LPS stimulation time course (19). Following qPCR, TNF-α expression was normalized to cyclophilin expression. The lower limit of detection for qPCR with these primer sets is 2 aM (19). For RNA stability assays, actinomycin D (ActD) (5 μg/ml) was added to inhibit transcription and RNA isolated at 0, 30, and 60 min post-ActD administration (19). mRNA t1/2 was calculated from the regression fit of the degradation curve generated by plotting the log₂[a] normalized TNF-α as a function of ActD treatment.

Sucrose density fractionation

For continuous sucrose gradient fractionation, cells were washed three times with 1% PBS and resuspended in 500 μl lysis buffer (15 mM Tris-HCl [pH 7.4], 15 mM MgCl₂, 0.3 M NaCl, 1% Triton X-100, and 0.1% [v/v] 2-mercaptoethanol) (Life Technologies). The RNA capture assay was performed on n = 4 biological replicates.

Results

Identification of Cul4B

One of the limitations in the field of posttranscriptional regulation has been the inability to systematically identify proteins that in-
teract with the 3’ UTR of an intact, capped, polyadenylated mRNA transcript that has been exported from the nucleus. Utilizing luciferase vector transfections, we developed an assay to capture luciferase mRNA and its associated proteins using a biotinylated antisense RNA probe (19). To identify proteins that interact with TTP on the TNF-α 3’ UTR, we performed the experiments in HEK 293 cells, which do not express endogenous TTP. HEK 293 cells were transfected with the TNF-3’ UTR luciferase vector and either pcDNA3.1 HisC or a pcDNA3.1 HisC-TTP expression vector, lysed, and used in the RNA capture assay. The captured proteins were then resolved by two-dimensional electrophoresis; proteins present when TTP was cotransfected and absent when pcDNA3.1 HisC was cotransfected were identified using PDQuest software (Fig. 1A). Tandem mass spectrometry of one spot identified a 44% overlap between peptide fragments and the CRL scaffold protein Cul4B (Fig. 1B). Although Cul4A and Cul4B are ~80% homologous, Cul4B contains a distinct N terminus, and it was from this region that sequenced protein spot-matched. Human and mouse Cul4B share 97.1% identity over their 896-aa overlap.

To confirm the presence of Cul4B in the TNF-α mRNA, we performed immunoprecipitations followed by quantitative RT-PCR for TNF-α message. As there is no anti-Cul4B Ab effective for immunoprecipitations, we transfected Cul4B, TTP, or the backbone pcDNA3.1 HisC vector into RAW265.7 cells and immunoprecipitated with an anti-Xpress Ab against the Xpress tag encoded by the vector. Immunoprecipitation-quantitative RT-PCR demonstrates that TNF-α mRNA is present in the TTP (positive control) and Cul4B immunoprecipitates, but absent from the pcDNA 3.1 (negative control) immunoprecipitate (Fig. 1C). Western blotting of mouse macrophage RAW264.7 cells (Fig. 1D) demonstrates that Cul4B localizes in both the cytoplasm and nucleus and that Cul4B expression and cytoplasmic/nuclear distribution are not altered by LPS stimulation.

Cul4B knockdown reduces TNF-α production in RAW264.7 macrophages

To determine if Cul4B influences TNF-α expression, we generated stable Cul4B shRNA RAW264.7 cell lines using two different Cul4B shRNA vectors, each of which targets different portions of the Cul4B 3’ UTR. The use of multiple shRNAs controls for shRNA off-target effects. Fig. 2A shows the level of Cul4B protein knockdown in six Cul4B shRNA clones from the Cul4B shRNA 1, and five clones form the Cul4B shRNA 2. Two clones from each Cul4B shRNA were selected, 1-G8 and 1-E5 from Cul4B shRNA1 and 2-A9 and 2-E27 from Cul4B shRNA 2. Quantitative densitometry reveals a 60–75% reduction in Cul4B protein levels in these clones. We observed no difference in the rate of cell proliferation between any of the clones and the control shRNA.

Having established Cul4B knockdown, we performed ELISAs to examine the levels of TNF-α released into the media of resting and LPS-stimulated control and Cul4B shRNA macrophages. TNF-α protein expression and Cul4B protein expression from control shRNA cells are nearly identical to that of wild-type RAW264.7 cells (Supplemental Fig. 1). The Cul4B shRNA clones produce significantly less TNF-α at 1, 2, and 4 h following LPS stimulation (p < 0.01) (Fig. 2B, 2C). TNF-α protein levels in resting cells exposed to fresh media for 4 h is also significantly reduced by Cul4B shRNA (p < 0.01) (Fig. 2D).

To definitively establish that Cul4B knockdown results in reduced TNF-α production, we performed rescue experiments by transfecting a Cul4B expression vector into one clone from each of the two Cul4B shRNA cell lines (Fig. 2E, 2F). Please note, both Cul4B shRNAs target distinct sequences in the Cul4B 3’ UTR, and only the Cul4B coding region was cloned into the expression

**FIGURE 1.** RNA capture assay. A. Top panel, Schematic of the captured TNF-α 3’ UTR luciferase mRNA with and without TTP cotransfection. Bottom panel, Overlay of a representative TNF-3’ UTR (green) and TNF-3’ UTR + TTP (red) capture gels. The capture experiment was performed on n = 4 biological replicates. The white square indicates Cul4B. B, Tandem mass spectrometry of a TTP-interacting protein identified by two-dimensional gel electrophoresis resulted in a 44% overlap with the amino portion of Cul4B; overlap indicated in boldface. C, RAW264.7 cells were transfected with Cul4B, TTP, or pcDNA 3.1 HisC (backbone vector) treated + LPS for 90 min and lysed. Immunoprecipitation with anti-Xpress followed by qPCR demonstrates the presence of TNF-α mRNA in the Cul4B and TTP immunoprecipitates and absence of TNF-α in the pcDNA3.1 immunoprecipitate. The lower limit of detection in this assay is 2 aM. D, Western blot examining the cytoplasmic and nuclear distribution of Cul4B in resting and LPS-stimulated RAW264.7 macrophages. Cul4B is a doublet in RAW264.7 cells, with the smaller band migrating around the predicted size of 110 kDa. Nuclear matrix protein p300 serves as a nuclear loading control and α-tubulin serves as a cytoplasmic loading control (n = 4).
vector. Transfecting 500 ng of Cul4B expression vector into either clone 1-E5 (Fig. 2E) or 2-A9 (Fig. 2F) results in an increase in secreted TNF-α protein in both the 1-E5 and 2-A9 Cul4B shRNA clones compared with the same clones transfected with an equal amount of pcDNA 3.1. Given the transfection efficiency of RAW264.7 cells with Lipofectamine LTX (60–70%; Invitrogen), complete recovery of TNF-α expression is unlikely. There was no effect of Cul4B transfection on TNF-α production in control shRNA cells.

*Cul4B is necessary for TNF-α mRNA stabilization*

Having established that Cul4B knockdown reduces TNF-α protein levels, we examined the effect of Cul4B knockdown (clone 1-G8) on TNF-α mRNA levels (Fig. 3). LPS stimulation of the control shRNA cells results in a dramatic increase in TNF-α mRNA levels, peaking at ~2 h and then beginning to decline (Fig. 3A), consistent with our previous findings (19, 30). In contrast, TNF-α mRNA levels are significantly reduced in the Cul4B shRNA cells (p < 0.01 at 1, 2, and 4 h LPS). Thus, loss of Cul4B reduces both TNF-α mRNA and protein in RAW264.7 macrophages following LPS stimulation. Interestingly, there was no difference between control and Cul4B shRNA cells with respect to TNF-α mRNA levels in the unstimulated cells (Fig. 3B), even though TNF-α secretion is reduced by knockdown of Cul4B under these conditions (Fig. 2D).

Next, we examined if Cul4B knockdown alters TNF-α mRNA stability (Fig. 3C, 3D). mRNA stability assays were performed by adding ActD (5 μg/ml) to cultures to inhibit transcription, following which RNA isolated at 0, 30, and 60 min after ActD treatment. In resting cells, there is no difference in the TNF-α mRNA t1/2 between control and Cul4B (t1/2 = 27.7 min.) and Cul4B (t1/2 = 27.7) shRNA cells (Fig. 3C). Following 90 min of LPS stimulation (Fig. 3D), the TNF-α mRNA t1/2 increases to 68.8 min in control shRNA cells, consistent with our previous findings in RAW24.7 cells (30). In contrast, the TNF-α mRNA t1/2 in the Cul4B shRNA cells is 33.3 min. The remaining RNA is significantly reduced at both 30 and 60 min in Cul4B shRNA cells compared with control shRNA cells (p < 0.01) (Fig. 3D). Thus, reduced Cul4B expression impacts TNF-α mRNA stability in activated, but not resting, macrophages.

*Cul4B knockdown inhibits TNF-α 3′ UTR luciferase expression*

Given that we identified Cul4B as present on TNF-α 3′ UTR-containing mRNPs only in the context of TTP protein, we examined the effect of Cul4B knockdown on TTP function using transient transfections. These studies employ pGL3-Control and pGL3-TNF-α UTR luciferase reporters, which differ only in the presence of the TNF-α 3′ UTR, as well as several TTP expression constructs (5, 19, 30–33). Our hypothesis for these experiments was that TNF-α luciferase expression would be lower in the Cul4B shRNA cells compared with the control shRNA cells. However, initial experiments reveal that luciferase expression from the pGL3-Control vector in the Cul4B shRNA cells is ~60% higher (p < 0.01) compared with the control cells (Fig. 4A). The mechanism mediating the increase in luciferase expression is unclear. LPS stimulation (100 ng/ml, 90 min) did not alter pGL3-Control luciferase expression in either of the cell lines, consistent with previous findings with pGL3-Control luciferase expression (19, 30). Luciferase expression from the pGL3-TNF-3′ UTR vector is significantly lower than expression from the pGL3-Control vector, due to the presence of the TNF-α 3′ UTR, consistent with our previous findings (30, 32). Importantly, TNF-3′ UTR luciferase expression is only 5% higher in the Cul4B shRNA cells compared with the control shRNA cells (Fig. 4B), in contrast to the 60% difference in pGL3-Control luciferase expression, even though all of the sequences in the pGL3-Control vector are present in the pGL3-TNF-3′ UTR vector. Although the mechanism mediating the increase in pGL3-Control luciferase expression in Cul4B shRNA cells is unknown, the loss of this effect with the inclusion of the TNF-3′ UTR is consistent with the decrease in TNF-α mRNA and protein in Figs. 2 and 3. To better illustrate the reduced TNF-3′ UTR luciferase expression in the Cul4B shRNA cells, we examined the data as the ratio of TNF-3′ UTR luciferase expression to control luciferase expression in the two cell types (Fig. 4C). Consistent with our previous observations (S. A. Brooks, unpublished observations), pGL3-TNF-3′ UTR luciferase expression in resting control shRNA cells is ~15% of the pGL3-Control vector due to destabilizing and translationally repressing cis-elements in the TNF-α 3′ UTR. In contrast, TNF-3′ UTR luciferase expression is only ~10% of control luciferase expression in the Cul4B shRNA cells, which is significantly lower than the ratio in control shRNA cells (p < 0.001).
Knockdown of Cul4B has no effect on TNF-α mRNA levels in Cul4B clone 1-G8 shRNA cells (Fig. 3). In resting cells (0 LPS), TNF-α mRNA levels are comparable in control (46.7 aM) and Cul4B shRNA clone 1-G8 (45.4 aMol) shRNA cells (n = 4). C, Knockdown of Cul4B has no effect on TNF-α mRNA stability in resting cells compared with control cells: t1/2 = 27.6 min in control shRNA and t1/2 = 27.7 min in Cul4B clone 1-G8 (n = 4). D, LPS stimulation for 90 min results in a dramatic increase in TNF-α mRNA stability in control shRNA cells (t1/2 = 68.8 min) but not in Cul4B clone 1-G8 shRNA cells (t1/2 = 33.3 min), with the percent remaining mRNA significantly different at both 30 and 60 min post-ActD addition (n = 4). **p < 0.01.

Ninety minutes of LPS stimulation results in an 80% increase in TNF-α 3′ UTR luciferase expression from the control shRNA cell line and a 60% increase in TNF-α 3′ UTR luciferase expression from the Cul4B shRNA cell line. Both increases are significant compared with resting TNF-α 3′ UTR luciferase expression (p < 0.01) and of a magnitude consistent with our previous findings (19, 30). Induction of TNF-α 3′ UTR luciferase expression by LPS is lower in the Cul4B shRNA cells compared with the control cells. Although the magnitude of the effect is smaller, these data are consistent with the endogenous TNF-α mRNA and protein findings in Figs. 2 and 3.

Cul4B knockdown enhances TTP function

Next, we performed the same experiments as in Fig. 4 with wild-type and mutant TTP. To clearly illustrate changes in TTP function, the no TTP (NT) transfection condition is set to 1 in both control and Cul4B shRNA cell lines (Fig. 5). The NT condition is transfected with an equal amount of pcDNA 3.1 HisC, the backbone vector for the TTP expression construct. Consistent with previous results (19, 30–32), transfection of TTP into control shRNA cells results in an ~25% decrease in TNF-3′ UTR luciferase expression in resting cells (p < 0.01) (Fig. 5A) (30–32). Use of the TTP-AA mutant (S52A, S178A) (18), which cannot be inhibited by p38/MK2 phosphorylation, results in a further reduction in TNF-3′ UTR luciferase expression (51% decrease) that is significantly different from wild-type TTP (p < 0.01, consistent with previous reports (32). Use of a TTP zinc finger mutant (TTP-M1.2) (34), which cannot bind RNA due to mutations in both zinc finger domains, abrogates the effect of TTP transfection.

Performing the same experiment in Cul4B shRNA cells demonstrates that cotransfected wild-type TTP mediates an ~49% decrease in TNF-α 3′ UTR expression (p < 0.01). Use of the TTP-AA mutant again significantly enhances TTP function (p < 0.01 WT-TTP versus TTP-AA), resulting in a 62% decrease in TNF-3′ UTR luciferase expression. Importantly, both wild-type TTP and TTP-AA are significantly more active in the Cul4B shRNA cells compared with the control shRNA cells (p < 0.01). As above, the TTP M1.2 mutant has no effect on TNF-3′ UTR luciferase expression.

The experiment in Fig. 5A was then performed in the context of LPS stimulation (Fig. 5B). As in Fig. 4B, 90 min of LPS stimulation results in a significant increase in TNF-α 3′ UTR luciferase expression in both control and Cul4B shRNA cells; the data are graphed as in Fig. 5A to allow a direct comparison. Consistent with our previous work (30–32), LPS stimulation does not overcome the effect of TTP transfection in either cell type. The results of wild-type TTP transfection, TTP-AA transfection, and TTP-M1.2 transfection are all comparable to those seen in resting cells. In both cell lines, TTP and TTP-AA significantly reduce TNF-3′ UTR luciferase expression relative to NT transfection (p < 0.01), and TTP-AA is significantly more active than wild-type TTP in control (p < 0.01) and Cul4B (p < 0.05) shRNA cells. Wild-type TTP is significantly more active in the Cul4B shRNA cells compared with control shRNA cells (p < 0.01), as is TTP-AA (p < 0.05).

Treatments that inhibit TTP in control shRNA cells only partially inhibit TTP in Cul4B shRNA cells

We have previously demonstrated that although p38 activation is necessary to inhibit TTP function, it is not sufficient; rather, both p38 and ERK activation are required to inhibit wild-type TTP in wild-type RAW264.7 cells (30). In light of the enhanced effect of TTP and TTP-AA in Cul4B cells, we examined the effect of Cul4B knockdown on wild-type TTP and TTP-AA in the context of ERK/p38 activation (Fig. 6A). Given that TTP-M1.2 behaved as expected and was not different between control and Cul4B shRNA cells, it was dropped from further analysis. Transfection of constitutively active MEK1 and MEK6, upstream activators of ERK and p38, results in ~3.6 times more TNF-3′ UTR luciferase expression in control shRNA cells, consistent with our previous findings with these vectors (30), and ~3.3 times more TNF-3′ UTR luciferase expression in Cul4B shRNA cell lines (not shown). As above, to facilitate comparison with Fig. 5, the data for the NT transfection are normalized to 1. In control shRNA cells, transfected TTP is inhibited by the activation of ERK and p38, consistent with previous findings, whereas the TTP-AA mutant remains active (p < 0.001 versus either NT or TTP), as it cannot be phosphorylated by the activated p38 pathway. In contrast, in the Cul4B shRNA cells, both wild-type TTP and TTP-AA remain active in the context of ERK and p38 activation. However, the activity of both wild-type TTP and the TTP-AA mutant is comparable to that found in resting or LPS-activated control shRNA cells rather than Cul4B cells, indicating a partial loss of TTP function following MEK1/6 activation in the Cul4B cells.
shRNA cells. TTP-AA remains significantly more active than wild-type TTP ($p < 0.05$) in the Cul4B shRNA cells. Thus, ERK/p38 activation only partially inhibits TTP activity in the Cul4B shRNA cells.

Next, we examined the effect of inhibiting the proteasome (10 μM MG-132, 120 min) on TTP function in the control and Cul4B cell lines (Fig. 6B). The proteasome treatment conditions employed in this study are consistent with our previous work and that of others (30, 35). Proteasome inhibition increased TNF-α 3’ UTR luciferase expression by ∼10% in both control and Cul4B shRNA cell lines (not shown) over DMSO-treated cells. We previously reported that MG-132 treatment inhibits TTP function in RAW264.7 cells (30). Given that TTP appears to be more rapidly turned over in the Cul4B cells, we hypothesized that TTP function would be inhibited in both control and Cul4B shRNA cells. Proteasome inhibition abrogates wild-type TTP function in the control shRNA cells and reduces, but does not completely inhibit the activity of the TTP-AA mutant. In the Cul4B shRNA cells, proteasome inhibition only partially inhibits the activity wild-type TTP and TTP-AA, as seen with ERK/p38 activation. However, following proteasome inhibition, the TTP-AA mutant is no longer more active than wild-type TTP. Similar results were obtained with MG-132 treatment followed by LPS stimulation (10 μM MG-132, 30 min pretreatment, followed by addition of LPS for 90 min) (data not shown). In total, treatment conditions that inhibit wild-type TTP in control shRNA cells only partially inhibit TTP function in Cul4B shRNA cells.

Cul4B knockdown reduces TTP protein levels

TTP protein turnover is thought to be linked with TNF-α mRNA turnover (30, 35, 36); we thus examined the effect of Cul4B knockdown on TTP protein expression. Following LPS stimulation, TTP protein levels are reduced in Cul4B shRNA cells compared with control shRNA cells (Fig. 7A). Importantly, TTP levels increase in both cell types in response to LPS and TTP protein size increases in both cell types, consistent with previous findings (30, 35). Although loss of TTP protein expression, as occurs with TTP knockout cells (6), results in increased TNF-α mRNA stability and increased TNF-α protein production, increased TTP protein turnover, as seen MK2/MK3 knockout cells, results in failure to stabilize TNF-α mRNA (36).

Given that Cul4B is an established component of CRL4B E3 ligases, we tested the hypothesis that Cul4B activity is essential for TTP protein stability either by directly ubiquitinating TTP or indirectly by altering the levels or activity of a protein that regulates TTP protein turnover. To this end, we examined the effect of proteasome inhibition on TTP protein levels in control and Cul4B shRNA cells. Fig. 7B demonstrates the effect of treating cells with either DMSO or MG-132 (10 μM MG-132, 30 min pretreatment, followed by LPS) and treating the resulting cytoplasmic lysates with or without calf intestine alkaline phosphatase (CIP) to dephosphorylate TTP protein. Dephosphorylation of lysates from

### FIGURE 4. TNF-α 3’ UTR luciferase expression is reduced in Cul4B shRNA cells compared with control shRNA cells. A, Luciferase expression from the pGL3-Control vector in resting and LPS-stimulated (100 ng/ml, 90 min) control and Cul4B shRNA RAW264.7 macrophages. Luciferase expression is ∼60% higher in the Cul4B shRNA cells compared with the control shRNA cells. There was no effect of LPS stimulation on luciferase expression from the pGL3-Control luciferase vector ($n = 4$). B, Luciferase expression from the pGL3-TNF-3’ UTR vector in resting and LPS-stimulated (100 ng/ml, 90 min) control and Cul4B shRNA RAW264.7 macrophages. Luciferase expression in the context of the TNF-α 3’ UTR is significantly lower than control luciferase expression. TNF-3’ UTR luciferase expression is ∼5% higher in the Cul4B shRNA cells compared with the control shRNA cells. LPS stimulation results in a significant increase in TNF-3’ UTR luciferase expression in both control and Cul4B shRNA cells ($n = 4$). C. Ratio of unstimulated TNF-3’ UTR luciferase expression to control luciferase expression, expressed as a percentage. The ratio from the control shRNA cell is significantly larger than the Cul4B shRNA cells, indicating that the presence of the TNF 3’ UTR results in significantly less luciferase expression in Cul4B shRNA cells compared with control shRNA cells. *$p < 0.01$.
FIGURE 5. TTP activity is significantly increased in Cul4B shRNA cells. Control and Cul4B shRNA cells were transfected with either the pGL3-Control or pGL3–TNF-3’-UTR luciferase vectors and pcDNA3.1, wild-type mouse TTP, mouse TTP-AA mutant (S52A, S178A), or mouse TTP zinc-finger mutant (TTP-M1,2). The data are presented to illustrate the net effect of TTP transfection on TNF-3’-UTR luciferase expression by setting the NT transfection (pcDNA) to 1. A. Effect of wild-type and mutant TTP cotransfection in resting control and Cul4B shRNA cells (n = 4) on TNF-α 3’-UTR relative luciferase expression. B. Effect of wild-type and mutant TTP cotransfection in stimulated (LPS, 100 ng/ml, 90 min) control and Cul4B shRNA cells (n = 4). *p < 0.05, **p < 0.01.

DMSO-treated cells collapses the broad diffuse TTP band into a tighter band, consistent with previous findings (35), with no difference in the effect of CIP between control and Cul4B shRNA cell lines. The increased intensity of the tighter TTP band in both cell lines is presumably a function of the concentration of the TTP protein to a more narrow size range. Importantly, an 8–10 kDa larger TTP band remains after CIP treatment, indicated by the < symbol on the right of the image (Fig. 7), which could represent a monoubiquitinated TTP. This larger TTP is also phosphorylated as the band, although not as broad as the lower m.w. TTP band, decreases in size with CIP treatment. As previously reported (30), inhibiting the proteasome with MG-132 treatment for 30 min prior to LPS stimulation partially inhibits the increase in TTP protein in response to LPS stimulation by blocking NF-κB activation, and this effect is consistent across both cell lines. Dephosphorylating MG-132–treated cell lysates with CIP results in a consolidation and collapse of the TTP bands as seen in the non–MG-132–treated cells. There is perhaps more of the higher m.w. TTP in the MG-132–treated cells, but the effect is not very robust. Most importantly, although there is less TTP in the Cul4B shRNA cells, it does not appear that TTP posttranslational modifications are altered by Cul4B knockdown, nor do TTP protein levels increase in the Cul4B shRNA cells following proteasome inhibition. These findings indicate that Cul4B is likely not targeting TTP protein, either directly or indirectly, for stabilization or degradation.

The zero LPS time point in Fig. 7B is difficult to interpret due to the low signal; to better examine the effects of the treatments and differences between the control and Cul4B shRNA cell lines, we loaded 450 μg of the zero LPS time point lysate per well and examined TTP protein levels (Fig. 7C). The results of the CIP treatment are consistent with those seen in Fig. 7B. In resting cells, proteasome inhibition increases TTP protein levels, consistent with our previous findings (30). What does become clear from this examination, however, is that the levels of TTP protein in the resting control and Cul4B shRNA cells are comparable. This finding would explain the lack of a difference in TNF-α mRNA and mRNA stability in the resting cells (Fig. 3B).

Subcellular localization of Cul4B

Given that we isolated Cul4B as a protein present in a TTP-containing mRNP, we sought to determine the subcellular distribution of the protein. We have previously established that TTP localizes to polysome and nonpolysome cellular fractions (32). To establish the distribution of Cul4B, we performed sucrose density fractionation (5–45%) of resting and LPS-activated RAW264.7 cell cytoplasm to determine the localization of Cul4B within the gradient and whether localization is altered by LPS stimulation (Fig. 8). These data demonstrate that Cul4B is present in all fractions of the gradient, and the distribution of Cul4B in the gradient does not change in response to LPS stimulation. Quantification of the RNA concentration in each fraction was previously reported (19). We also plotted the gradients for TTP (Fig. 8). TTP is present through the entire gradient, and TTP expression dramatically increases in response to LPS stimulation, consistent with previous data (32). Fractionation of the cytoplasm in this way reveals a much broader spectrum of TTP bands than is visible in total cytoplasmic lysate.

Cul4B shRNA cells have reduced polysome-associated TNF-α mRNA

Having established that Cul4B expression affects TNF-α mRNA stability and that Cul4B is associated with translating mRNA, we examined if TNF-α mRNA polysome loading was altered in the Cul4B shRNA cells. Polysomes were isolated from resting and LPS-stimulated (90 min) control and Cul4B shRNA cells, RNA isolated, and TNF-α and cyclophilin levels quantified (Fig. 9). TNF-α mRNA levels on the polysomes are significantly reduced (p < 0.05) in resting Cul4B shRNA cells compared with control shRNA cells, even though total TNF-α message levels are comparable between the unstimulated cell lines (Fig. 3B). LPS stimulation (90 min) results in an order of magnitude increase in TNF-α polysome loading in both control and Cul4B shRNA cells, but...
there remains significantly more polysome-loaded TNF-α mRNA in the control shRNA cells compared with the Cul4B shRNA cells ($p < 0.05$). For comparison, we also examined polysome cyclophilin levels in control and Cul4B shRNA cells (Fig. 9B). Cyclophilin in control and Cul4B shRNA cells is comparable in both stimulated and unstimulated cells; LPS stimulation results in a significant increase in cyclophilin polysome-associated mRNA, but not nearly to the levels seen with TNF-α. Given these data, we examined if the difference in polysome TNF-α mRNA levels following LPS stimulation is simply a function of less TNF-α mRNA in the Cul4B shRNA cells or if there is a difference in the fold induction of TNF-α mRNA polysome loading between Cul4B shRNA cells and control shRNA in response to LPS stimulation (Fig. 9C). For cyclophilin, there is no difference in the polysome loading between the two cell lines. For TNF-α, the fold induction is significantly reduced ($p < 0.05$), from 12.1 in control shRNA cells to 9.5 in Cul4B shRNA cells.

**Cul4B localizes to P-bodies and exosomes**

Finally, we performed immunocytochemistry to determine if Cul4B colocalizes with TTP, P-bodies, and exosomes. P-bodies are cytoplasmic foci containing translationally repressed mRNA; messages within P-bodies can be degraded in a 5’ to 3’ manner or stored for a return to translation (37). Exosomes and exosome granules are sites of 3’ to 5’ mRNA degradation. To determine if Cul4B localizes to P-bodies, we employed Abs to the P-body marker EDC4 (29) as well as TTP. These studies demonstrate that Cul4B strongly colocalizes with TTP and, to a lesser degree, EDC4 in LPS-stimulated cells (Fig. 10A); a subset of the granules contains all three proteins. We did not examine the colocalization in resting cells due to the very low levels of TTP protein present.

Next, we examined the localization of Cul4B with exosome granules. Fig. 10B shows that Cul4B colocalizes with the structural exosome component Rrp45 and that, as with P-bodies, at least a subset of exosome granules colocalizes TTP and Cul4B. Fig. 11 presents a model of Cul4B regulated TNF-α polysome loading.

**Discussion**

We identified Cul4B as a component of the TNF-α mRNA complex in the context of TTP protein expression (Fig. 1) (19). The Cul4B gene was recently identified by a chromosome-wide whole-exon sequencing study as one of the more commonly mutated genes in patients with XLMR. It has been proposed that Cul4B-mediated...
ubiquitination results in proteasome targeting of the histone methylase H3K4, and the loss of Cul4B activity in patients with Cul4B mutations results in aberrant histone methylase by H3K4, altering gene expression (23). This hypothesis is consistent with a growing list of epigenetic regulators implicated in XLMR, including: the SMCX (KDM5C/JARID1C) (38, 39), neuron-restrictive silencing factor NRSF/REST (40), and plant homeodomain finger 8 (38, 41). Our data demonstrating that Cul4B can serve as a translational regulator links it with another XLMR protein, fragile-X mental retardation protein, mutations of which result in the most common single gene inherited mental retardation. The contribution of each of these gene expression regulatory mechanisms to the diverse phenotype associated with Cul4B mutations remains to be determined.

The data support a posttranscriptional role for Cul4B in TNF-α production. If Cul4B were acting as a transcriptional regulator of TTP protein, such that reduced Cul4B levels result in reduced TTP protein expression, then we would expect an increase in TNF-α mRNA stability as occurs in the TTP knockout mice (6). Instead, TNF-α mRNA levels (Fig. 3B) and stability (Fig. 3C) are the same in resting control and Cul4B shRNA cells, as are TTP protein levels (Fig. 7C). These data indicate that reduced Cul4B is not impacting basal TTP activity. Following LPS stimulation, Cul4B shRNA cells do not fully stabilize the TNF-α mRNA (Cul4B shRNA t1/2 = 33.3 min versus control shRNA t1/2 = 68.8 min) (Fig. 3D), and these cells exhibit a reduction in TTP protein levels (Fig. 7A). Together, these data are consistent with increased TTP activity or a failure to fully inhibit TTP activity in LPS-stimulated Cul4B shRNA cells.

Transient transfection experiments confirm enhanced TTP activity in the Cul4B shRNA cells (Figs. 5, 6). These data demonstrate a stepwise increase in TTP activity with wild-type TTP transfected into control shRNA cells, the least active, and the TTP-AA mutant transfected into the Cul4B shRNA cells, the most active. In between are TTP-AA in control shRNA cells and wild-type TTP in Cul4B shRNA cells, each of which exhibits about the same level of activity. The enhanced function of the TTP-AA mutant in the Cul4B shRNA cells implies that the impact of Cul4B on TTP function is independent of TTP phosphorylation at Ser⁵² and Ser¹⁷⁸. If dephosphorylation of TTP at Ser⁵² and Ser¹⁷⁸ were all that was required to drive TTP activity, then we would predict that the TTP-AA mutant would have equal activity in the control and Cul4B shRNA cells. This interpretation is further supported by the finding that constitutive activation of p38 and ERK, which completely inhibits wild-type TTP function in control shRNA cells but not a complete loss of TTP activity (Fig. 6A). Thus, it appears that Cul4B is necessary to fully inhibit TTP function and that Cul4B is operating independent of the established mechanism regulating TTP activity.

We previously reported that proteasome antagonists inhibit TTP function, and thus, proteasome-mediated turnover of TTP is likely a step involved in TNF-α mRNA decay (30). Data from the non-p38 phosphorylatable TTP-AA mutant and the Cul4B shRNA cells demonstrate that although proteasome blockade inhibits wild-type TTP function in control shRNA cells, results in a stepwise decrease in TTP activity in the Cul4B shRNA cells but not a complete loss of TTP activity (Fig. 6A). Thus, it appears that Cul4B is operating independent of the established mechanism regulating TTP activity.

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TTP protein levels are reduced in Cul4B shRNA cells following LPS stimulation (Fig. 7A). Examining TTP protein levels in control and Cul4B shRNA cells following proteasome inhibition and CIP treatment demonstrates that TTP protein levels in MG-132–treated Cul4B cells do not increase to levels comparable to the levels in the control cells (Fig. 7B, 7C). It thus does not appear that the TTP protein itself is the direct or indirect target of Cul4B ubiquitination and proteasome-mediated degradation. The finding

![Figure 10](image-url)

**FIGURE 10.** Cul4B colocalizes with TTP in P-bodies and exosome. RAW264.7 cells were stimulated with LPS (100 ng/ml) for 90 min and processed for immunocytochemistry. The white box in the immunocytochemistry image (1 μM) is enlarged on the right to show specific colocalization. A, Immunocytochemistry for Cul4B (red), TTP (green), and the P-body marker EDC4 (blue). B, Immunocytochemistry for Cul4B (red), TTP (green), and the exosome protein Rrp45 (blue).

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![Figure 11](image-url)

**FIGURE 11.** Model of Cul4B activity. Cul4B is present only if TTP protein is present on the TNF-α mRNP. TTP is not the target of Cul4B-containing E3 ligase (CRL4B). Cul4B shRNA cells exhibit reduced TNF-α mRNA polysome loading and an increase in TNF-α mRNA decay. Importantly, mRNA decay occurs even in the context of treatment conditions (ERK/p38 activation, proteasome inhibition) that normally inhibit TTP function. This schematic is not intended to indicate all proteins present on the TNF-α mRNP or specific binding relationships among TTP, Cul4B, and the CRL4B target.
that TTP levels are comparable in resting control and Cul4B cells is consistent with the comparable TNF-α mRNA levels and stability in resting cells (Fig. 3). We believe the reduction in TNF-α protein production in resting cells (Fig. 2), which appears to conflict with the TNF-α mRNA data from resting cells, is a function of a small percentage of activated cells present in macrophage cultures. The small number of activated cells releases a disproportionate amount of TNF-α protein into the culture media, as the resting cells produce very little TNF-α protein, whereas their mRNA contribution is averaged out by the mRNA contribution by the total cell population.

The current data support the conclusion that Cul4B, which is present on the polysomes (Fig. 8), acts to regulate loading of TTP bound TNF-α mRNA onto the polysomes (Fig. 9), and we present a model of this activity in Fig. 11. In response to LPS stimulation, Cul4B acts to license loading of the TTP-bound TNF-α mRNA onto the polysomes. In the absence of licensing, some of the TNF-α mRNPs fail to load, shunting them back to the mRNA decay pathway. This accounts for: 1) the stepwise drop in TNF-α 3′ UTR luciferase expression with TTP cotransfection between the control and Cul4B shRNA cells; 2) the failure to fully stabilize the TNF-α message in response to LPS stimulation; and 3) the failure to fully inhibit wild-type TTP in the MEK1/6-activated and proteasome-inhibited Cul4B shRNA cells. Indeed, these data are consistent with TTP involvement in TNF-α mRNA translation.

Previous work has demonstrated that lipoxygenase mRNA is translationally silenced in erythroid precursor cells by hnRNPs K and E1, which, following binding to the 3′ UTR differentiation control element, act to inhibit joining of the 60S large ribosomal subunit to the 43S preinitiation complex (42). It is possible that a similar mechanism is operating in TNF-α mRNA translation with Cul4B specifically targeting a blocking protein for destruction. However, it is unclear if the continued presence of the Cul4B target would result in the complete or near complete blockade of TNF-α translation, as occurs with lipoxygenase, or if the mechanism operates more as an enhancer, similar to the enhanced TNF-α export that occurs following LPS stimulation (33, 43). Given that the loss of TTP results in TNF-α overexpression, either the protein repressing translation is also recruited by TTP or translational blockade is incomplete.

We assume that Cul4B is operating as part of CRL4 ubiquitin E3 ligase that specifically ubiquitinates a component of the mRNP either to cause the destruction of the protein or modification of its conformation of activity. It is tempting to speculate that the Cul4B/CRL4 target protein is phosphorylated by p38, resulting in its ubiquitination by CRL4 and removal from the mRNP, enhancing TNF-α mRNA polysome loading.

In conclusion, TTP was identified as promoting TNF-mRNA decay (6, 44). Initially, it was hypothesized that TTP binding to the ARE was the event regulating TTP function and that TTP binding was altered by p38 phosphorylation (45). Our previous data demonstrating TTP association with translating messages prompted us to hypothesize that TTP remained associated with its cognate mRNA during translation (32). Subsequent work (12, 46) has demonstrated that phosphorylation of TTP does not alter TTP binding to reporter mRNA containing the TNF-α ARE, confirming our original hypothesis. The current model of TTP activity involves TTP binding to Not1 and recruiting the CAFI1 deadenylase to the mRNA, resulting in degradation of the message (12, 13, 46). Activation of macrophages results in the phosphorylation of TTP at Ser25 and Ser178 (Ser60 and Ser160 in humans) by MK2, inhibiting the interaction of TTP with Not1 and thus preventing recruitment of CAF1 and degradation of the message (12, 13, 46). The p38–MK2 signaling axis also promotes TNF-α translation via an un-defined mechanism. Our finding that TTP recruits Cul4B to the mRNP and that the presence of Cul4B promotes TNF-α translation indicates a more complex role for TTP. The data are consistent with the hypothesis that TTP is part of the complex promoting TNF-α mRNA translation. In this model, TTP operates as a switch integrating the activation status of the cell, resulting in mRNA decay in resting macrophages and promoting TNF-α message translation in activated cells. How other TNF-α ARE binding proteins integrate into this model remains to be determined.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1.  

Supplemental Figure 1. TNF-α protein production from Wild-Type and Control shRNA RAW264.7 cells. Inset: Western blot demonstrates comparable Cul4B protein levels in Wild-Type and Control shRNA RAW264.7 cells. Tubulin serves as a loading control.