Cot/tpl2-MKK1/2-Erk1/2 controls mTORC1-mediated mRNA translation in Toll-like receptor–activated macrophages

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ABSTRACT **Cot/tpl2 is the only MAP3K that activates MKK1/2-Erk1/2 in Toll-like receptor–activated macrophages. Here we show that Cot/tpl2 regulates RSK, S6 ribosomal protein, and 4E-BP phosphorylation after stimulation of bone marrow–derived macrophages with lipopolysaccharide (LPS), poly I:C, or zymosan. The dissociation of the 4E-BP–eIF4E complex, a key event in the cap-dependent mRNA translation initiation, is dramatically reduced in LPS-stimulated Cot/tpl2-knockout (KO) macrophages versus LPS-stimulated wild-type (Wt) macrophages. Accordingly, after LPS activation, increased cap-dependent translation is observed in Wt macrophages but not in Cot/tpl2 KO macrophages. In agreement with these data, Cot/tpl2 increases the polysomal recruitment of the 5′TOP eEF1α and eEF2 mRNAs, as well as of inflammatory mediator gene–encoding mRNAs, such as tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and KC in LPS-stimulated macrophages. In addition, Cot/tpl2 deficiency also reduces total TNFα, IL-6, and KC mRNA expression in LPS-stimulated macrophages, which is concomitant with a decrease in their mRNA half-lives. Macrophages require rapid fine control of translation to provide an accurate and not self-damaging response to host infection, and our data show that Cot/tpl2 controls inflammatory mediator gene–encoding mRNA translation in Toll-like receptor–activated macrophages.**

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INTRODUCTION

Translational control of gene expression is an important regulatory step in which the amount of protein to be produced of an already transcribed mRNA is modulated. Translation of mRNAs is subject to tight control by cells, providing a rapid and adequate response to external cell stimuli. In addition, cells have also developed mechanisms to control the stability of mRNAs (Chen and Shyu, 1995; Anderson, 2008; Guo *et al.*, 2010). A key limiting step of translation is the initiation step, during which the small ribosome subunit is recruited to the 5′ untranslated region (UTR) of the cap-mRNA and scans toward the start codon (reviewed in Gingras *et al.*, 1999; Sachs and Varani, 2000; Hellen and Sarnow, 2001). In certain transcripts and under defined conditions, the cap is bypassed by use of an internal ribosome entry site (IRES), which is located in the 5′UTR of transcripts (Pestova *et al.*, 2001).

Cap-dependent translation is facilitated by the recognition of the mRNA 5′m^{7}GpppN cap structure by eIF4F, which includes the cap-binding subunit, eIF4E. The assembly of the active eIF4F is blocked by the reversible association of eIF4E with the translation repressors 4E-binding proteins (4E-BPs), in which the release of 4E-BP is the key event in the control of cap-dependent mRNA translation initiation. Dissociation of the eIF4E–4E-BP complex is achieved by 4E-BP phosphorylation, controlled by the mammalian/mechanistic target of rapamycin complex 1 (mTORC1; reviewed in Gingras *et al.*, 1999;

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Abbreviations used: BMDM, bone marrow–derived macrophages; 4E-BP, 4E-binding protein; eEF2k, elongation factor 2 kinase; FBS, fetal bovine serum; HMP, high–molecular weight polysomes; IL-6, interleukin-6; IRES, internal ribosome entry site; KD, kinase dead; KO, knockout; LMP, low–molecular weight polysomes; LPS, lipopolysaccharide; m^{7}GTP, 7-methyl GTP; mTORC, mammalian/mechanistic target of rapamycin complex; NP, nonpolysomal; TLR, toll-like receptor; TNF α, tumor necrosis factor α; 5′TOP, 5′terminal oligopirimidine tract; qRT-PCR, quantitative real-time PCR; UTR, untranslated region; WT, wild type.

Pestova *et al.*, 2001). However, cap-dependent translation of different mRNAs is not equally sensitive to mTORC1 activity, with 5′TOP mRNAs, as well as mRNAs with highly structured 5′UTR, being very sensitive to mTORC1 activity (Topisirovic *et al.*, 2011; Huo *et al.*, 2012; Thoreen *et al.*, 2012). mTORC1 also phosphorylates/activates S6K1 (p70 S6k), which is responsible for the phosphorylation of the ribosomal protein S6. S6K1 also upregulates peptide elongation by phosphorylating and consequently inhibiting elongation factor 2 kinase (eEF2k), which phosphorylates and represses eEF2 (Wang *et al.*, 2001). PI3K signaling connected via Akt, by direct phosphorylation of TSC2, can block the TSC1/TSC2 complex inhibitory action on mTORC1 activity (reviewed in Gingras *et al.*, 1999; Sachs and Varani, 2000; Hellen and Sarnow, 2001). Indeed, the PI3K intracellular pathway controls mRNA translation (Laplante and Sabatini, 2009), including the translation of 5′TOP mRNAs, which contain a 5′-terminal oligopyrimidine tract (5′TOP) and encode components of the translational machinery (Jefferies *et al.*, 1997; Meyuhas, 2000; Tang *et al.*, 2001; Patursky-Polischuk *et al.*, 2009). On the other hand, the activity of mTORC1 and of its downstream effectors is also enhanced by the kinase RSK (Anjum and Blenis, 2008; Pearce *et al.*, 2010). Activation of the RAS-RAF-MKK1/2-Erk1/2 pathway triggers RSK phosphorylation/activation, which subsequently phosphorylates TSC2 (Roux *et al.*, 2004). RSK also phosphorylates the subunit of mTORC1, Raptor (Carriere *et al.*, 2008), and S6 (Roux *et al.*, 2007), as well as eEF2k (Bain *et al.*, 2007). Indeed, both RAF and PI3K pathways stimulate mTORC1 signaling (Anjum and Blenis, 2008; Pearce *et al.*, 2010).

Activation of PI3K occurs by a broad array of different stimuli in a variety of different cell types. In innate immune cells such as macrophages, PI3K activation, among other intracellular signaling pathways, occurs upon activation of different Toll-like receptors (TLRs; reviewed in Fukao and Koyasu, 2003; Chaurasia *et al.*, 2010). Receptors of this family sense infection and are stimulated by different pathogen-associated molecular patterns. Activation of the different intracellular pathways upon TLRs stimulation orchestrates the first line of resistance against infection, triggering the production of cytokines and chemokines critical for host defense (Kawai and Akira, 2011). All TLRs, except TLR3, recruit the adaptor MyD88, which transduces the intracellular signal to activate the kinase TAK1. Consequently, TAK1 activates the p38α and JNK MAP kinase pathways, as well as the canonical IKKs, IKKα and IKKβ. TLR3 and TLR4 recruit the TRIF adaptor, which specifically activates the noncanonical IKKs but also IKKα and IKKβ (reviewed in Akira and Takeda, 2004; O'Neill and Bowie, 2007). Activated IKKβ phosphorylates IκB and p105 NF-κB, targeting IκB to degradation and p105 NF-κB to partial proteolysis. In resting cells, Cot/tpl2 (MAP3K8) forms a stable and inactive complex with p105 NF-κB and ABIN2, protecting Cot/tpl2 from its degradation. The partial proteolysis of p105 NF-κB to p50 NF-κB releases Cot/tpl2 from the complex (reviewed in Gantke *et al.*, 2011; Vougioukalaki *et al.*, 2011). On TLR stimulation, dissociated Cot/tpl2 with an adequate phosphorylation state fully activates MKK1/2 and consequently Erk1/2 (Dumitru *et al.*, 2000; Caivano *et al.*, 2003; Cho and Tsichlis, 2005; Banerjee *et al.*, 2006; Stafford *et al.*, 2006) before being rapidly degraded through the proteasome pathway (Gandara *et al.*, 2003; Waterfield *et al.*, 2003). Cot/tpl2 is the only MAP3K that activates the Erk1/2 pathway under these cell stimulation conditions and fulfils a role in innate immunity and inflammatory hypernociception that cannot be substituted for by any other protein (Cohen, 2009; Soria-Castro *et al.*, 2010; Gantke *et al.*, 2011; Vougioukalaki *et al.*, 2011). Cot/tpl2 participates in the production of interleukin-1β and represses interferon-β transcription (Kaiser *et al.*, 2009; Mielke *et al.*, 2009; Lopez-Pelaez *et al.*, 2011). In addition, Cot/tpl2 has a critical role in the production of tumor necrosis factor α (TNFα) during inflammatory responses; it regulates the processing of the pre-TNFα protein before its secretion (Rousseau *et al.*, 2008), modulates the nuclear export of TNFα mRNA (Dumitru *et al.*, 2000), and also enhances TNFα gene transcription (Ballester *et al.*, 1998).

In the context of innate immunity, and in addition to the controlled transcriptional activation, the control at the level of translation of cytokine and chemokine mRNAs plays an essential role in the required rapid and accurate response against infection without damage of the host cells (reviewed in Anderson, 2008, 2010; Hao and Baltimore, 2009).

Here we show that Cot/tpl2 controls mTORC1-dependent mRNA translation in TLR-activated macrophages. Cot/tpl2, via Erk1/2, regulates the dissociation of the 4E-BP–eIF4E complex and the mRNA polysomal recruitment of 5′TOP mRNAs. Cot/tpl2 also controls the recruitment onto translating polysomes of mRNAs that encode proteins involved in the innate immune response such as TNFα, IL-6, and KC. In addition, Cot/tpl2 also increases the half-life of these mRNAs, affecting their total mRNA expression levels.

RESULTS

Cot/tpl2 controls, in an Erk1/2-dependent manner, 4E-BP1 phosphorylation in TLR-stimulated macrophages

Cot/tpl2 mediates Erk1/2 activation in the macrophagic cell line RAW, peritoneal macrophages, and bone marrow–derived macrophages (BMDM) upon engagement of lipopolysaccharide (LPS) to TLR4 (Dumitru *et al.*, 2000; Caivano *et al.*, 2003; Figure 1A). Subsequently, activated Cot/tpl2 is rapidly degraded through the proteasome pathway (Gandara *et al.*, 2003; Waterfield *et al.*, 2003). In addition, in LPS-activated BMDM, Cot/tpl2 controls the phosphorylation of Akt on S473 (Lopez-Pelaez *et al.*, 2011; Figure 1A), a residue phosphorylated by mTORC2 (Sarbassov *et al.*, 2005). The phosphorylation of SGK1 on S422, which is also dependent on mTORC2 activity (Garcia-Martinez and Alessi, 2008), was also diminished in LPS-stimulated Cot/tpl2-knockout (KO) BMDM compared with LPS-stimulated wild-type (Wt) BMDM (Figure 1A). However, the loss of Cot/tpl2 expression did not affect the phosphorylation of T308 in the activation loop of Akt by PDK1. These data indicate that Cot/tpl2 deficiency does not affect the activation of PDK1 by PI3K in LPS-treated macrophages but suggest deficient mTORC2 activation in LPS-treated Cot/tpl2 KO BMDM versus their Wt counterparts. Akt needs to be phosphorylated on both residues S473 and T308 to be fully active and to phosphorylate FOXO1 on T24; however, phosphorylation of Akt on T308 is sufficient to phosphorylate TSC2 on S939 (Guertin *et al.*, 2006). Accordingly, T24 FOXO1 phosphorylation was clearly reduced in Cot/tpl2 KO BMDM versus Wt BMDM upon LPS stimulation, whereas S939 TSC2 phosphorylation was only slightly reduced, just by 20% after 90 min of LPS stimulation (Figure 1A).

Compared to their Wt counterparts, LPS-stimulated Cot/tpl2 KO BMDM showed a statistically significant reduction of T573 RSK phosphorylation (Figure 1B), explaining the reduced phosphorylation in the same setting of its substrate TSC2 in residue S1798 and of the downstream effectors S6K1, S6, and eEF2k (Figure 1B). The phosphorylation of 4E-BP by mTORC1 is the key signal that triggers the dissociation of eIF4E from the inactive 4E-BP–eIF4E complex, allowing eIF4E to form the active eIF4F complex. Active eIF4F facilitates the initiation of translation by its binding to the m^7GpppN cap site located at the 5′-terminus of all mRNAs (Gingras *et al.*, 1999; Pestova *et al.*, 2001). Decreased phosphorylation of 4E-BP1 in Cot/tpl2 KO BMDM versus their Wt counterparts was already observed in basal conditions, and upon LPS stimulation, the difference in the 4E-BP1 phosphorylation ratio was further increased by Cot/tpl2

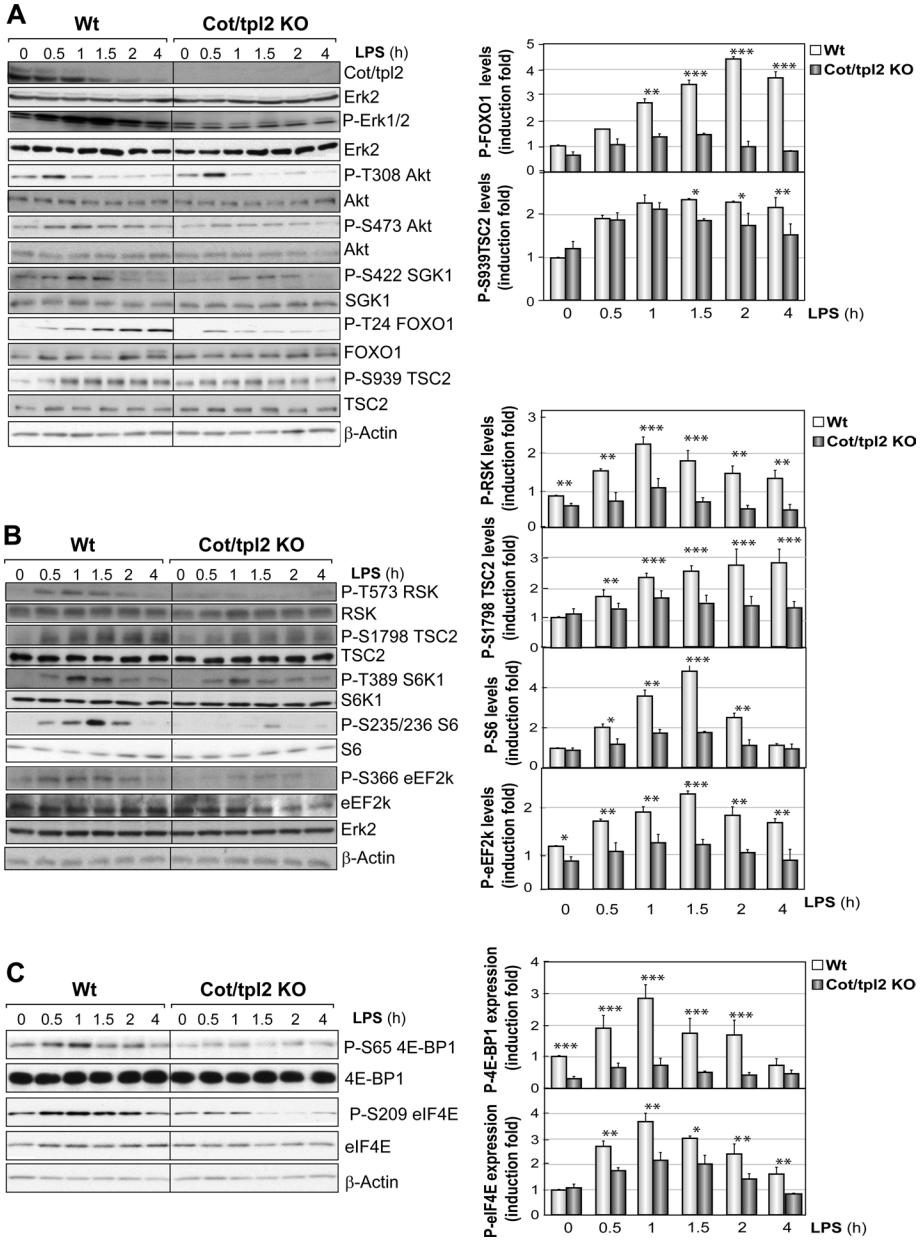

FIGURE 1: Cot/tpl2 governs the phosphorylation state of proteins involved in cap-dependent translation in LPS-stimulated BMDM. (A) Wt and Cot/tpl2 KO BMDM were stimulated with LPS (300 ng/ml), and after the indicated times Cot/tpl2, P-Erk1/2, P-T308 Akt, P-S473 Akt, P-S422 SGK1, P-T24 FOXO1, and P-S939 TSC2 levels were measured by Western blot. Erk2, Akt, SGK1, FOXO1, TSC2, and β-actin levels were determined as a protein loading control. Right, graphs represent the means ± SD from five independent experiments of P-T24 FOXO1 and P-S939 TSC2 fold induction relative to the Wt zero time point, after normalizing values to, respectively, total FOXO1 and total TSC2. (B) Cell extracts obtained as described in A were subjected to Western blot analysis using antibodies against the following phosphoproteins: P-T573 RSK, P-S1798 TSC2, P-T389 S6K1, P-S235/236 S6, and P-S366 eEF2k.The antibodies against total proteins used were RSK, TSC2, S6K1, S6, eEF2k, Erk2, and β-actin. The graphs represent the means ± SD from at least four independent experiments of P-T573 RSK, P-S1798 TSC2, P-S235/236 S6, and P-S366 eEF2k fold induction relative to the Wt zero time point, after normalizing values to, respectively, total RSK, TSC2, S6, and eEF2k. (C) Western blots of cell extracts obtained as described in A were probed against P-S65 4E-BP1, P-S209 eIF4E, 4E-BP1, eIF4E, and β-actin. Right, graphs represent the means ± SD from five independent experiments of P-S65 4E-BP1 and P-S209 eIF4E fold induction relative to the Wt zero time point, after normalizing values to, respectively, total 4E-BP1 and total eIF4E (A, B). Quantification of the induction fold of P-Erk1/2, P-T308 Akt, P-S473 Akt, P-S422 SGK1, and P-T389 S6K1 levels is shown in Supplemental Table S1.

deficiency (Figure 1C). Of note, total 4E-BP1 expression levels were very similar in both Wt and Cot/tpl2 KO BMDM. On the other hand, the phosphorylation of eIF4E on S209 by MNK, a p38α- and Erk1/2-dependent kinase (Marzec *et al.*, 2011), was also diminished by Cot/tpl2 deficiency in LPS-stimulated BMDM.

To examine whether the observed impaired phosphorylation of proteins involved in the initiation of the cap-dependent mRNA translation by Cot/tpl2 deficiency was just due to the lack of Cot/tpl2 activity or to the knockout of Cot/tpl2 protein expression, we used Cot/tpl2 kinase-dead (KD) BMDMs, which express Cot/tpl2 with a point mutation in which the lysine responsible for ATP binding (Lys-167) is replaced by Arg and thereby does not have kinase activity. Indeed, the Cot/tpl2 catalytically inactive protein was not degraded after LPS stimulation, indicating that Cot/tpl2 activity participates in a negative feedback loop ending in its own degradation (Figure 2A). Because S235/236 S6 and S366 eEF2k phosphorylation, as well as the phosphorylation S65 4E-BP1 and S209 eIF4E was also diminished in LPS-stimulated Cot/tpl2 KD BMDM as compared with their Wt counterparts (Figure 2A), we conclude that is just the activity of Cot/tpl2 that is essential in the activation of proteins involved in the cap-dependent mRNA translation. In addition, LPS-stimulated Wt BMDM preincubated with PD 0325901, a specific MKK1/2 inhibitor (Bain *et al.*, 2007), also showed impaired phosphorylation of S6, eEF2k, eIF4E, and 4E-BP1 compared with Wt BMDM after LPS stimulation in the absence of the inhibitor (Figure 2B). Furthermore, pretreatment with UO126, another MKK1/2 inhibitor, or with rapamycin, an inhibitor of mTORC1 activity, also reduced the phosphorylation state of 4E-BP1 in LPS-stimulated Wt BMDM (Figure 2C). Taken together, these data indicate that the capacity of Cot/tpl2 to control Erk1/2 phosphorylation in LPS-activated BMDM is essential for the modulation of the phosphorylation/activation state of proteins involved in the mRNA translation process in LPS-activated macrophages.

LPS signals through both TRIF and MyD88 adaptors after TLR4 activation (Akira and Takeda, 2004; O'Neill and Bowie, 2007). In macrophages with low levels of the receptor dectin-1, zymosan stimulates TLR2/6 and signals through the MyD88 adaptor (Gantner *et al.*, 2003), whereas poly I:C activates TLR3, a receptor that is incapable of recruiting MyD88 and signals through TRIF (O'Neill and Bowie,

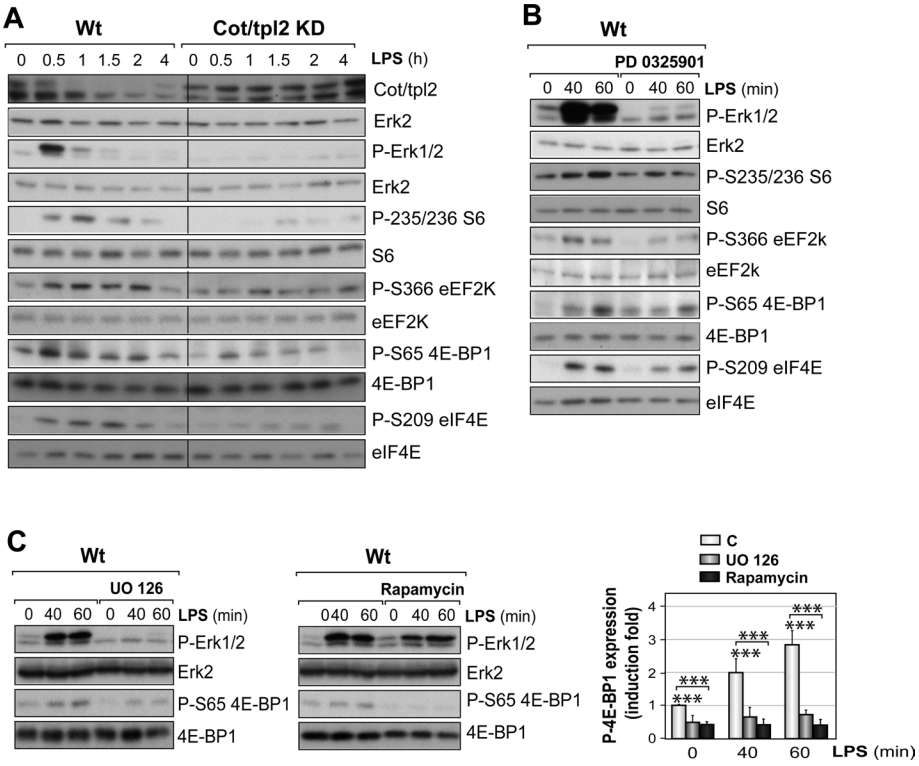

FIGURE 2: Erk1/2-dependent phosphorylation of P-S235/236 S6, P-S366 eEF2k, P-S65 4E-BP1, and P-S209 eIF4E in LPS-stimulated BMDM. (A) Wt and Cot/tpl2 KD BMDM were stimulated for different lengths of time with LPS (300 ng/ml), and the extracts obtained were analyzed by Western blots probed with the phospho antibodies against P-Erk1/2, P-S235/236 S6, P-S366 eEF2k, and P-S65 4E-BP1 and with antibodies against the total protein of Cot/tpl2, Erk2, S6, eEF2k, 4E-BP1, and eIF4E as loading controls. (B) Wt BMDMs were preincubated or not with the MKK1/2 inhibitor PD 0325901 (0.5 μM) for 60 min before stimulation with LPS (300 ng/ml) for the indicated times, after which the levels of P-Erk1/2, P-S235/236 S6, P-S366 eEF2k, P-S65 4E-BP1, and P-S209 eIF4E were determined by Western blot analysis. As a loading control, membranes were also blotted with the following antibodies: Erk2, S6, eEF2k, 4E-BP1, and eIF4E. (C) Wt BMDM were preincubated or not with the MKK1/2 inhibitor UO 126 (10 μM) or rapamycin (20 nM) for 60 min before stimulation with LPS (300 ng/ml) for the indicated times, after which the levels of P-Erk1/2, P-S65 4E-BP1, Erk2, and 4E-BP1 were determined by Western blot analysis. Graph represents the means ± SD from three independent experiments of P-S65 4E-BP1 fold induction relative to the Wt zero time point, after normalizing values to total 4E-BP1. For A–C, one representative experiment of the three independently performed is shown.

2007). Results obtained in Wt and Cot/tpl2 KO BMDM stimulated with poly I:C or with zymosan and analyzed by Western blot indicated that RSK, eIF4E, eEF2k, S6, and S65 4E-BP1 phosphorylation was also controlled by Cot/tpl2 under these cell stimulation conditions (Figure 3). However, Wt and Cot/tpl2 KO BMDM showed similar levels of S65 4E-BP phosphorylation after 15 and 30 min of stimulation with IL-10 (Supplemental Figure S1).

Cot/tpl2 regulates cap-dependent translation in LPS-activated macrophages

We next decided to evaluate whether the reduced 4E-BP1 phosphorylation observed by Cot/tpl2 deficiency in TLR-activated BMDM correlated with a diminished dissociation of the 4E-BP1–eIF4E complex. To this end, extracts from Wt and Cot/tpl2 KO BMDM nontreated or treated for 1 or 2 h with LPS were incubated with the m^7GpppN cap affinity resin, and the levels pulled-down of eIF4E and 4E-BP1 proteins were subsequently determined by Western blot analysis (Figure 4A). In basal conditions, Cot/tpl2 KO BMDM already showed increased levels of 4E-BP1 bound to eIF4E compared with Wt BMDM. Furthermore, the amount of 4E-BP1 bound to eIF4E decreased in a statistically significant manner upon LPS stimulation of Wt BMDM, but the absence of Cot/tpl2 expression severely affected the release of 4E-BP1 from the 4E-BP1–eIF4E complex in LPS-stimulated BMDM (Figure 4A). These data prompted us to study the cap-dependent and cap-independent translation rates in LPS-stimulated Wt and Cot/tpl2 KO BMDM. To this end, cells were nucleofected with a biscitronic plasmid in which *Renilla* luciferase cDNA can be only translated by cap-dependent scanning mechanism, whereas translation of Firefly luciferase does not happen unless internal initiation occurs at the IRES element (Figure 4B). Similar levels of cap-independent translation were observed in Wt and Cot/tpl2 KO BMDM stimulated or not with LPS. Nevertheless, cap-dependent translation was increased in Wt BMDM compared with Cot/tpl2 KO BMDM in basal conditions, and, as expected, LPS stimulation of Wt BMDM further increased cap-dependent translation in a statistically significant manner . However, this increase was hardly detected in Cot/tpl2 KO BMDM after LPS stimulation, resulting in a statistically significant increase in the cap-dependent/cap-independent translation ratio in Wt BMDM versus Cot/tpl2 KO BMDM (Figure 4B).

Cot/tpl2 controls the translation of the 5′TOP eEF1α and eEF2 mRNAs

BMDM from Wt and Cot/tpl2 KO mice were treated or not with LPS for 3 h, and sucrose gradient was performed to separate nonpolysomal mRNAs and nontranslating free 40S and 60S ribosomal subunits from moderately and from actively translating mRNA polysomes. The nonpolysomal fraction (NP) contained the pool of mRNAs that were not associated with components of the translation machinery or cosedimented with ribosome subunits (monosomes); thus, they were not considered to be translated. The mRNAs contained in the fraction with small polysomes of low molecular weight (LMP) were considered to be translated at moderate levels. The third mRNA fraction contained mRNAs that were associated with polysomes of high molecular weight (HMP), and they were thus considered to be actively translated (Supplemental Figure S2). On 3 h of LPS stimulation of Wt and Cot/tpl2 KO BMDM, there was an increase in the recruitment of the 5′TOP eEF1α mRNA to HMP in detriment to the levels detected in the inactive pool. However, the increase in the recruitment of eEF1α mRNA on the actively translating polysomes was, in a statistically significant way, lower in Cot/tpl2 KO BMDM versus Wt BMDM, as determined by Northern blot analysis. By contrast, β-actin mRNA, which is known to be constitutively translated, was associated mostly with the HMP fraction in stimulated and nonstimulated cells and irrespective of their genotype (Figure 5A). Similar reduction in the eEF1α mRNA polysomal recruitment was observed in LPS-stimulated Wt BMDM in the presence of UO126 compared with Wt BMDM stimulated with LPS in the absence of this inhibitor (Supplemental Figure S3). According to these

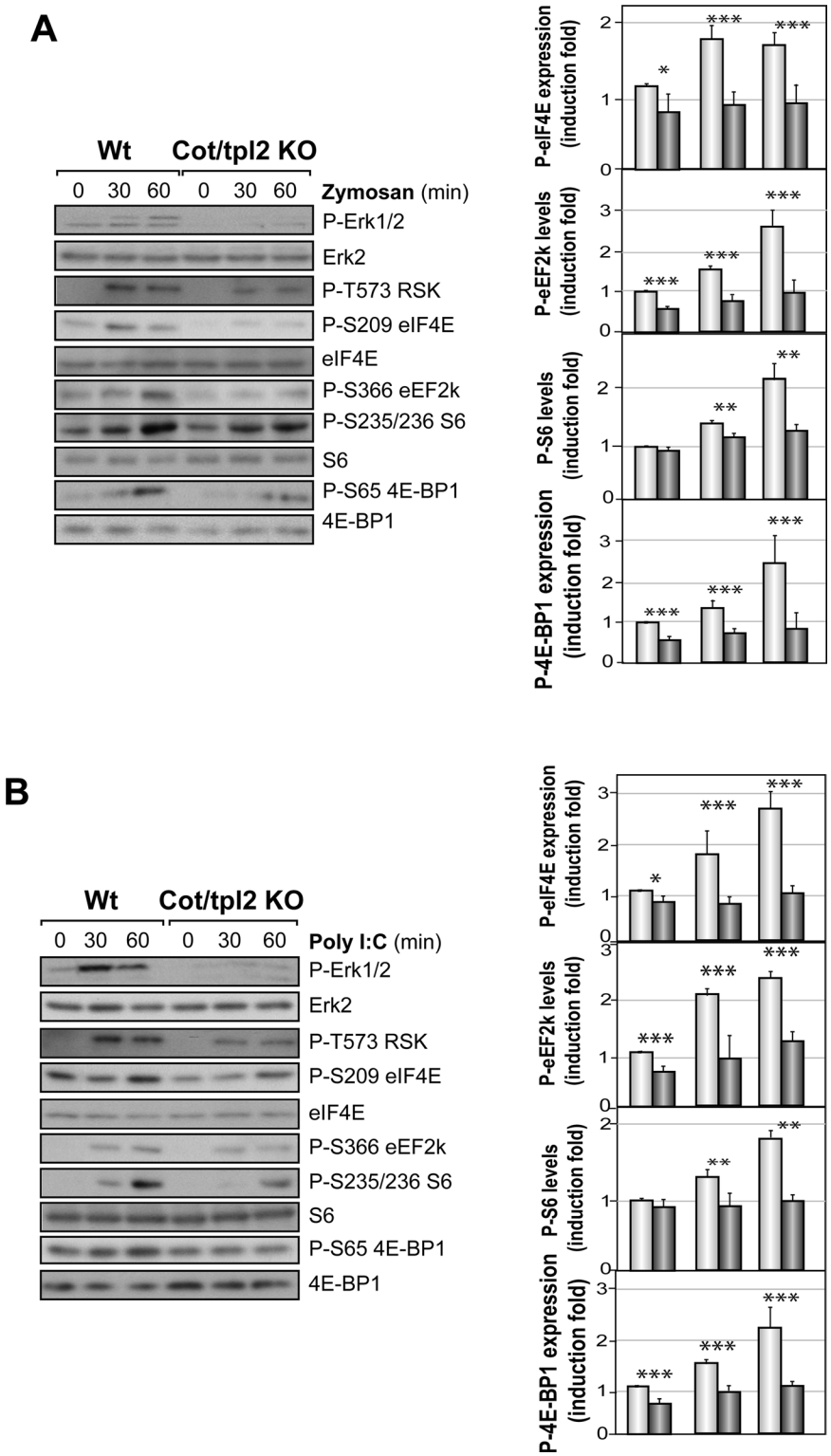

FIGURE 3: P-S65 4E-BP1 phosphorylation in Wt and Cot/tpl2 KO BMDM stimulated with zymosan or poly I:C. Wt and Cot/tpl2 KO BMDM were stimulated with zymosan (10 μg/ml; A) or with poly I:C (50 μg/ml; B) for 30 and 60 min and the expression levels of P-Erk1/2, P-T573 RSK, P-S209 eIF4E, P-S366 eEF2k, P-S235/236 S6, and P-S65 4E-BP1 were determined in Western blots. As a loading control the expression levels of Erk2, eIF4E, S6, and 4E-BP1 were also analyzed. For both A and B, one representative experiment of the three independently performed is shown. Right, graphs represent the means ± SD from five independent experiments of P-S209 eIF4E, eEF2k, P-S235/236 S6, and P-S65 4E-BP1 fold induction relative to the Wt zero time point, after normalizing values to, respectively, total eIF4E, Erk2, S6, and 4E-BP1.

data, Cot/tpl2 deficiency also impaired eEF1α mRNA distribution within polysomal versus nonpolysomal RNA fractions (Supplemental Figure S2) as performed by quantitative real-time PCR (qRT-PCR) analysis (Figure 5B), and again no change in the β-actin distribution was observed in these analysis conditions. To determine whether the effects of Cot/tpl2 were unique for the EF1α mRNA or could be extended to other 5′TOP mRNAs, we also analyzed the recruitment onto polysomes of another 5′TOP mRNA, the eEF2 mRNA. In LPS-activated Wt BMDM this transcript was mainly located in the translated mRNA fraction, but under the same conditions Cot/tpl2 deficiency mainly abolished eEF2 mRNA polysomal recruitment upon LPS stimulation (Figure 5B). Of note, similar levels of total eEF1α, eEF2, or β-actin mRNA were detected in Wt and Cot/tpl2 KO BMDM stimulated or not with LPS (Supplemental Figure S4).

Cot/tpl2 modulates polysomal recruitment of TNFα, IL-6, and KC mRNA in LPS-activated BMDM

We previously showed that in LPS-activated macrophages Cot/tpl2 deficiency triggered a fivefold increase in the IRF1 mRNA levels but only approximately twofold in the IRF1 protein levels respect to Wt BMDM in the same cell conditions (Lopez-Pelaez *et al.*, 2011). The polysomal mRNA distribution of this transcription factor, involved in macrophage activation (reviewed in Tamura *et al.*, 2008), indicated that Cot/tpl2 expression is required to increase the recruitment of IRF1 mRNA to polysomes upon LPS stimulation of BMDM (Supplemental Figure S5). Furthermore, the recovery of IκBα protein levels after TNFα stimulation of mouse embryonic fibroblasts (Das *et al.*, 2005) or upon LPS stimulation of BMDM (Lopez-Pelaez *et al.*, 2011) is diminished in the absence of Cot/tpl2. The similar expression levels of IκBα mRNA after LPS stimulation of Wt and Cot/tpl2 KO BMDM (Supplemental Figure S5) could not explain the previously reported impaired IκBα protein expression; however, Cot/tpl2 deficiency reduced IκBα mRNA levels in the polysomes fraction upon 3 h of LPS stimulation (Supplemental Figure S5), arguing that Cot/tpl2 stimulates the translation of IκBα mRNA. Of interest, IκBβ showed similar protein expression levels in Wt and Cot/tpl2 KO BMDM after LPS stimulation (unpublished data), and Cot/tpl2 did not appear to regulate IκBβ mRNA recruitment to polysomes (Supplemental Figure S5).

Cot/tpl2 controls TNFα production in LPS-activated macrophages (Dumitru *et al.*, 2000; Rousseau *et al.*, 2008; Figure 6A). In addition, an analysis of the TNFα mRNA distribution within the polysomal and nonpolysomal RNA fractions upon LPS stimulation of Wt and Cot/tpl2 KO BMDM indicated that Cot/tpl2 increased TNFα mRNA polysomal recruitment (Figure 6B).

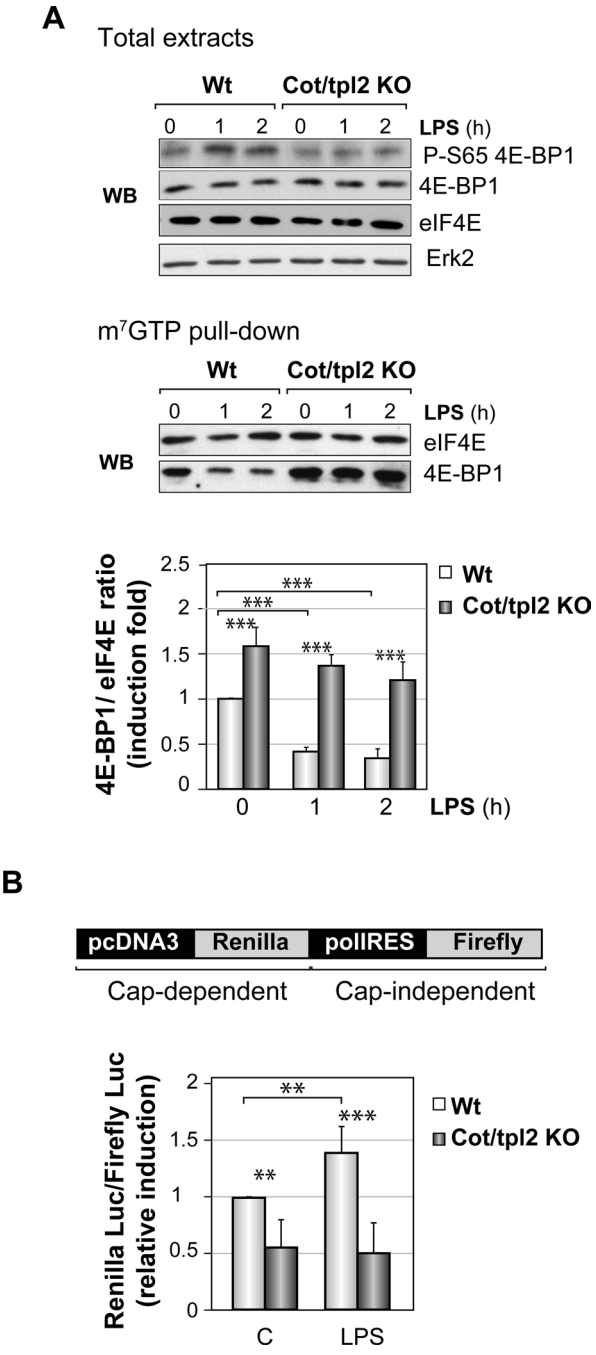

FIGURE 4: Cot/tpl2 promotes dissociation of the 4E-BP1–eIF4E complex and cap-dependent translation. (A) Cell extracts from Wt and Cot/tpl2 KO BMDM stimulated or not for 1 or 2 h with LPS (300 ng/ml) were subjected to m^{7}GTP–Sepharose bead pull-down assays, and the amount of 4E-BP1 bound to eIF4E was analyzed by Western blot. Expression levels of P-S65 4E-BP1, 4E-BP1, eIF4E, and Erk2 in total cell extracts are also shown. Representative experiments of the three independently performed are shown. Graph represents the means ± SD from three independent experiments of the 4E-BP1/eIF4E values, giving the value of 1 to the one obtained in Wt BMDM at zero point time. (B) Wt and Cot/tpl2 KO BMDM were nucleofected as indicated in *Materials and Methods*, with the biscitronic plasmid specified in the figure. Cells were harvested, and *Renilla* and Firefly luciferase luminescence was quantified using a luminometer. Graph represents the means ± SD from three independent experiments of *Renilla*/firefly luciferase values, giving the value of 1 to the one obtained in Wt BMDM at zero time point.

Furthermore, Cot/tpl2 deficiency diminished the levels of other two inflammatory mediators, IL-6 and KC, in the incubation media of BMDM upon LPS stimulation (Figure 6A), and LPS-stimulated Cot/tpl2 KO BMDM showed a severely diminished recruitment of IL-6 and KC mRNAs to polysomes compared with their Wt counterparts (Figure 6B).

Cot/tpl2 increases TNFα, IL-6, and KC mRNA stability in LPS-activated BMDM

In addition to the reduced recruitment of TNFα, KC, and IL-6 mRNA to polysomes, Cot/tpl2 deficiency also reduced total mRNA levels of TNFα, KC, and IL-6 ~3-, 2.5-, and 6.5-fold, respectively, in 3 h LPS-stimulated BMDM (Figure 7A). To determine whether Cot/tpl2 enhances the stability of these mRNAs, Wt and Cot/tpl2 KO BMDM were first treated with LPS for 3 h to induce their expression, and subsequently, to block transcription, actinomycin D was added to the cell culture media and the decay in the expression levels of TNFα, KC, and IL-6 mRNA was examined. In LPS-stimulated BMDM, Cot/tpl2 stabilizes TNFα, KC, and IL-6 mRNA, with an increase in the half-life of ~2.5-fold for TNFα and IL-6 mRNA and ~5-fold for the KC transcript (Figure 7B), indicating that the increase in the total mRNA levels of these cytokines and chemokine by Cot/tpl2 after LPS stimulation could be mainly due to a stabilization of their mRNAs. Of note, the half-life of β-actin mRNA was not affected by Cot/tpl2 deficiency. Taken together, these data indicate that Cot/tpl2 not only controls the translation of inflammatory mediator gene–encoding mRNAs but also controls the mRNA stability of cytokine and chemokine transcripts in the context of the innate immune response generated upon LPS stimulation of macrophages.

DISCUSSION

Cot/tpl2 has emerged as an attractive target to develop new and improved anti-inflammatory drugs (Cohen, 2009; Gaestel *et al.*, 2009) since it plays a role after TLR activation of macrophages that affects the innate immune response (reviewed in Gantke *et al.*, 2011). Here we show a novel function for Cot/tpl2 in controlling the activation state of proteins involved in cap-dependent translation initiation. The PI3K-Akt-TSC2-mTORC1 pathway plays, through their downstream effectors, a key role in the maintenance of translation (Laplante and Sabatini, 2009). However, the fact that in TLR-activated macrophages Cot/tpl2 deficiency does not alter the phosphorylation of Akt on T308 but only on S473 indicates that Cot/tpl2 does not modulate 4E-BP phosphorylation through Akt, since the partial activation of Akt, P-T308 Akt, by PI3K-PDK1 upon TLR stimulation is sufficient to transduce the activating signal to TSC2 (Guertin *et al.*, 2006). On the other hand, the RAS-RAF-MKK1/2-Erk1/2 pathway, by activating the Erk1/2-dependent kinase RSK, also triggers mTORC1 activation (reviewed in Anjum and Blenis, 2008; Pearce *et al.*, 2010). Stimulation of the receptors of the TLR/IL-1R superfamily does not activate any of the RAF proteins, and the activation of the MKK1/2-Erk1/2 pathway is entirely mediated by Cot/tpl2 (Caivano *et al.*, 2003; Cho and Tsichlis, 2005; Banerjee *et al.*, 2006; Rodriguez *et al.*, 2006; Gantke *et al.*, 2011). On the basis of the data shown here, and taking into account the mechanism by which Cot/tpl2 is activated (Rodriguez *et al.*, 2006; Gantke *et al.*, 2011; Vougioukalaki *et al.*, 2011), we can conclude that after TLR activation the TAK1-IKKβ-Cot/tpl2-MKK1/2-Erk1/2 pathway also has RSK as a downstream effector. In addition, Cot/tpl2-dependent activation of RSK links Cot/tpl2, via Erk1/2, with the capacity to control cap-dependent mRNA translation initiation, the key rate-limiting step in the translation process. Nevertheless, we cannot rule out the possibility that Cot/tpl2-MKK1/2-Erk1/2 also inhibits the TSC1-TSC2 complex and consequently increases

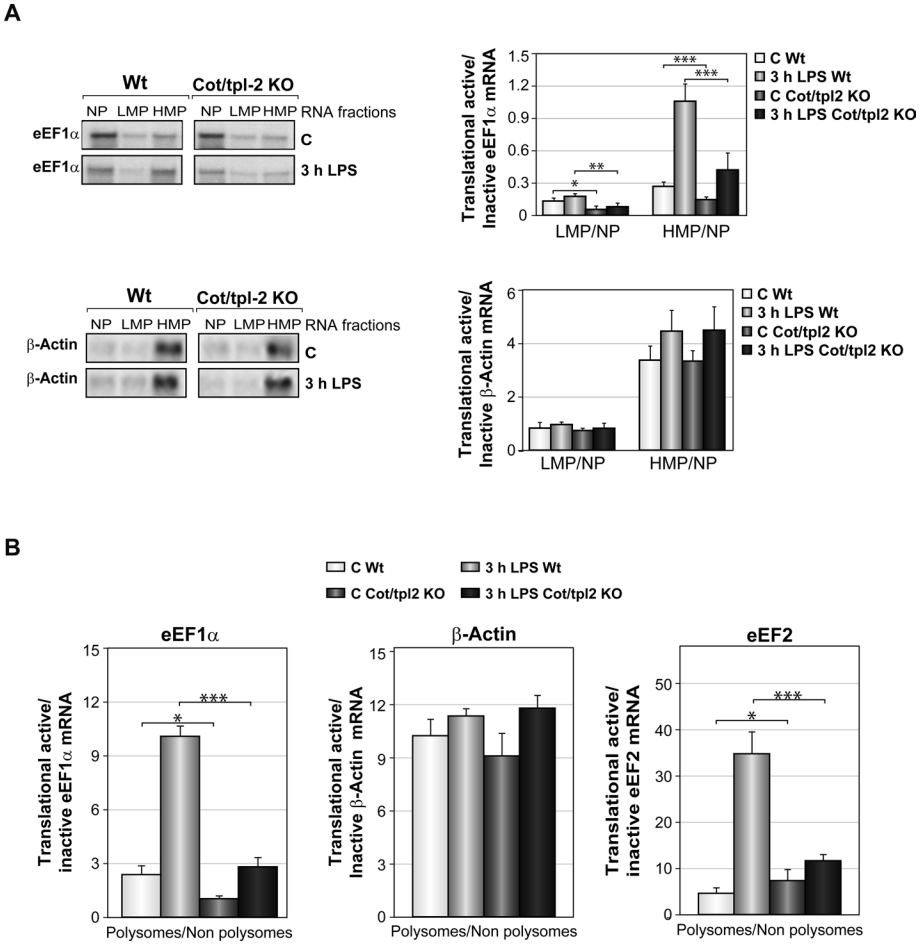

FIGURE 5: Cot/tpl2 regulates polysomal recruitment of eEF1α and eEF2 mRNA in LPS-stimulated BMDM. Cell lysates of Wt and Cot/tpl2 BMDM stimulated for 3 h or not with LPS (300 ng/ml) were subjected to sucrose gradient, and different RNA fractions were pooled. The RNA isolated from the different RNA fractions was subjected to Northern blot or qRT-PCR analysis. (A) Three mRNA fractions from the sucrose gradient were isolated: nontranslated mRNA (nonpolysomal components, consisting of nonpolysomal mRNAs and nontranslating free 40S and 60S ribosomal; NP), moderately translated mRNA (LMP), and actively translated mRNA (HMP). These mRNA fractions were subjected to Northern blot analysis. The eEF1α and β-actin mRNA expression in the NP, LMP, and HMP fractions of nonstimulated and 3-h LPS-stimulated Wt and Cot/tpl2 KO BMDM is shown. Right, graphs represent the means ± SD from five independent experiments of the quantification of the LMP/NP and HMP/NP eEF1α and β-actin mRNA ratios in the different cell conditions. (B) eEF1α, β-actin, and eEF2 mRNA expression in the nonpolysomal and polysomal fractions analyzed by qRT-PCR. Graphs represent the means ± SD from five independent experiments of the quantification of the polysome/nonpolysome mRNA ratio in the different cell conditions.

mTORC1 activity by Erk1/2 direct phosphorylation of TSC2 on S664 (Ma *et al.*, 2005). These data indicate a novel role of Cot/tpl2 in modulating the now evident important role that mTORC1 substrates have in innate immunity development (reviewed in Thomson *et al.*, 2009; Weichhart and Saemann, 2009).

4E-BP phosphorylation by mTORC1 is the key event in the regulation of cap-dependent translation. Here we show that Cot/tpl2-MKK1/2-Erk1/2 triggers 4E-PB1 phosphorylation by activating mTORC1, since not only MKK1/2 inhibitors, but also rapamycin, without inhibiting Erk1/2 activation, inhibits 4E-BP1 phosphorylation in LPS-stimulated Wt BMDM. Dissociation of phosphorylated 4E-BP from the eIF4E–4E-BP complex allows the formation of the active eIF4F that facilitates the initiation of translation by its binding to the m^7GpppN located in the 5′ region of most mRNA sequences (Gingras *et al.*, 1999; Pestova *et al.*, 2001). Accordingly, with the deficient 4E-BP1 phosphorylation in LPS-stimulated Cot/tpl2 KO BMDM versus Wt BMDM, Cot/tpl2 deficiency also prevents in a statistically significant manner the dissociation of the eIF4E–4E-BP complex and diminishes translation of the cap-dependent *Renilla* luciferase. Taken together, these data indicate that Cot/tpl2 controls the activation of the cap–dependent translational machinery in TLR-activated macrophages. Accordingly, mRNA recruitment to polysomes of transcripts encoding proteins that are involved in the innate immune response, such as TNFα, IL-6, and KC, is severely diminished by Cot/tpl2 deficiency.

This newly described capacity of Cot/tpl2 to control the activation state of proteins involved in the mRNA translation process occurs independent of the type of adaptor, MyD88 or TRIF, used by TLRs to transduce the activating signals since it is observed upon both poly I:C and zymosan stimulation of macrophages. Nevertheless, this capacity of Cot/tpl2 is so far a selective and specific event triggered by TLR activation, since Cot/tpl2 has no effect on 4E-BP phosphorylation in BMDM stimulated with IL-10, which uses a receptor distinct from the TLR family (Kotenko *et al.*, 1997), and does not activates Cot/tpl2 (Lopez-Pelaez *et al.*, 2011). In this context, it was recently reported that Cot/tpl2 is also the only MAP3K that activates Erk1/2 in signals initiated by the thrombin-activated, G protein–coupled, receptor protease–activated receptor-1 (Hatziapostolou *et al.*, 2011); thus the possibility that Cot/tpl2 could control cap-dependent translation initiation in other biological processes cannot be excluded.

Here we also describe that Cot/tpl2 regulates the translation of the 5′TOP mRNAs eEF1α and eEF2. The 5′TOP mRNAs contain a 5′-terminal oligopyrimidine tract and encode ribosomal proteins involved in the processing of mRNA, and their translation is very sensitive to mTORC1 activity (Jefferies *et al.*, 1997; Meyuhas, 2000; Tang *et al.*, 2001; Patursky-Polischuk *et al.*, 2009; Thoreen *et al.*, 2012). In this context, it has been shown that sensitivity of mRNA translation to mTORC1 activation varies depending on the mRNA species (Topisirovic *et al.*, 2011; Huo *et al.*, 2012; Thoreen *et al.*, 2012). Indeed, β-actin mRNA translation is barely dependent on mTORC1 activation and 4E-BP1 phosphorylation (Colina *et al.*, 2008; Huo *et al.*, 2012; Thoreen *et al.*, 2012), and our data here show that Cot/tpl2 modulates the downstream effectors of mTORC1 but not polysomal recruitment of β-actin.

Not only does Cot/tpl2 modulate TNFα, KC, and IL-6 mRNA polysomal recruitment in activated macrophages, but it also participates in the expression of their total mRNA levels upon LPS stimulation of macrophages. Total mRNA levels are the result of both the

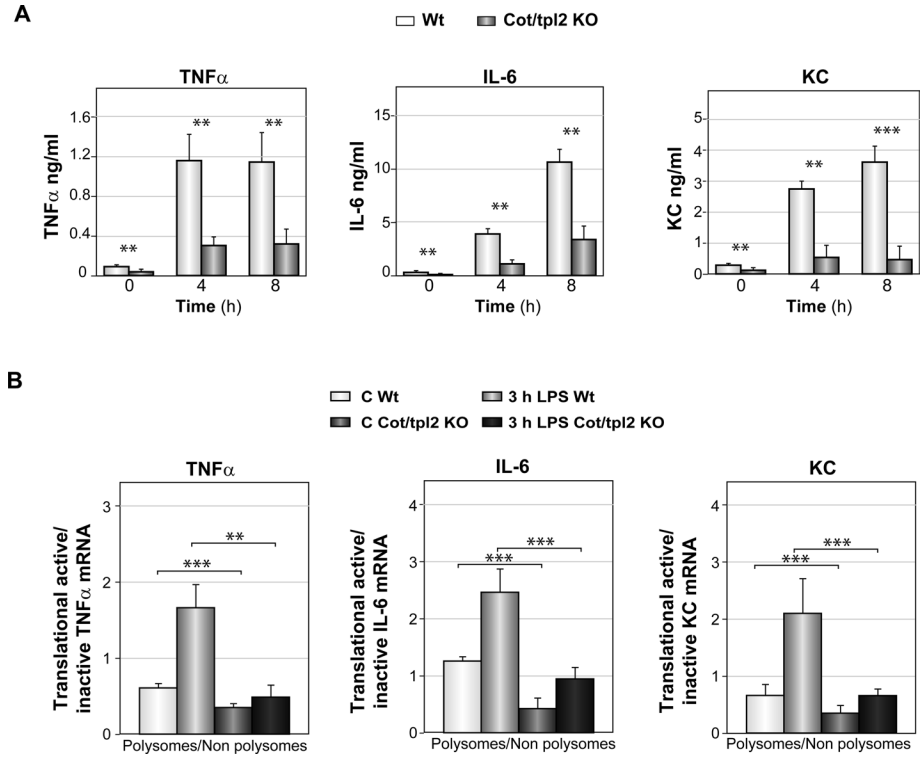

FIGURE 6: Cot/tpl2 controls TNFα, KC, and IL-6 production and polysomal recruitment of their mRNAs in LPS-stimulated BMDM. (A) The concentration of TNFα, IL-6, and KC in the supernatant of Wt and Cot/tpl2 KO BMDM stimulated or not for 3 or 8 h with LPS (300 ng/ml) was determined using a Luminex 100 system (Upstate, Millipore, Billerica, MA), according to the manufacturer's instructions. Graphs show the means ± SD from three independent experiments. (B) TNFα, KC, and IL-6 mRNA expression in the nonpolysomal and polysomal RNA fractions described in Figure 5B was analyzed by qRT-PCR. Graphs represent the means ± SD from five independent experiments of the quantification of the polysome/nonpolysome mRNA ratio in the different cell conditions.

rate of gene transcription and the stability of the mRNA itself. Our data here demonstrate that the increase in the total mRNA levels by Cot/tpl2 after LPS stimulation correlates with the induction in their mRNA stability induced by Cot/tpl2. All three TNFα, IL-6, and KC mRNAs contain AU-rich elements in the 3′ UTR, which are determinant for modulating their half-life in the context of inflammation (reviewed in Anderson, 2008; Mazumder *et al.*, 2010). In this context it was reported that Cot/tpl2 modulates cytoplasmic TNFα mRNA expression by a mechanism that targets the AU-rich element in the 3′ UTR of the TNF-α mRNA upon LPS stimulation (Dumitru *et al.*, 2000). Indeed, MAPKAP-2, as well as MNK1 and 2, downstream kinases of Erk1/2 (Roux and Blenis, 2004), phosphorylate and inhibit hnRP40 activity an AU-rich sequence-specific RNA-binding protein that destabilizes TNFα mRNA (Rousseau *et al.*, 2002; Buxade *et al.*, 2005).

Translational control offers a strategic advantage because the use of the preexisting mRNAs bypasses lengthy nuclear control mechanisms (e.g., transcription, splicing, and transport). At the same time, it provides reversibility through modifications of regulatory intermediates, mainly via reversible phosphorylation, and provides macrophages with required rapid and accurate response capacity. Innate immune system cells are the first line of defense against pathogen infection, and fine control of the translation levels of inflammatory mediators by these cells is a crucial requirement for the proper development of innate immune response. Indeed, the host response against infection is a double-edged sword, as an uncontrolled response can exacerbate tissue damage, and the control of inflammatory mediators at the level of translation has emerged as a key step in adjusting the magnitude of the response (reviewed in Anderson, 2008; Thomson *et al.*, 2009; Mazumder *et al.*, 2010).

Cot/tpl2 has emerged as an interesting new anti-inflammatory target by its capacity to activate Erk1/2, without involving the RAF proteins, during the innate immune response. Here we show that Cot/tpl2 controls mRNA stability and cap-dependent mRNA translation in the context of the innate immune response generated upon the stimulation of macrophages by LPS, modulating at the posttranscriptional level the production of inflammatory mediators involved in the host response.

MATERIALS AND METHODS

Mice, BMDM preparation, and stimulation

C57BL/6J wild type and C57BL/6J Cot/tpl2 KO animals were produced from heterozygous mice (Cot/tpl2 $KO^{+/-}$ × Cot/tpl2 $KO^{+/-}$), and C57BL/6J Cot/tpl2 KD animals were produced from heterozygous C57BL/6J Cot/tpl2 $KD^{+/-}$ mice. All animals received care according to methods approved under institutional guidelines for the care and use of laboratory animals in research. BMDM were obtained as previously described (Lopez-Pelaez *et al.*, 2011), and cells were then stimulated with LPS (*Salmonella typhimurium*; L726; Sigma-Aldrich, St. Louis, MO), poly I:C (Invitrogen, Carlsbad, CA), zymosan (Invitrogen), or IL-10 (PeproTech, Rocky Hill, NJ). Actinomycin D was purchased from Sigma-Aldrich and UO 126 from Tocris Bioscience (Ellisville, MO). PD 0325901 and rapamycin were gifts from, respectively, Philip Cohen (Dundee, Scotland) and Victor Calvo (Madrid, Spain).

Western blot analysis

Cell extracts were examined by Western blot as previously described (Rodriguez *et al.*, 2008), using primary antibodies raised against the following proteins: from Santa Cruz Biotechnology (Santa Cruz, CA), Cot/tpl2, Erk2, P-T389 S6K1, RSK, S6K1, and SGK1; from Cell Signaling Beverly, MA), P-T202/Y204 Erk1/2, P-S473 Akt, P-T308 Akt, Akt, P-T24 FOXO1, FOXO1, P-S939 TSC2, TSC2, β-actin, P-T573 RSK, P-S235/236 S6, P-S366 eEF2k, eEF2k, P-S65 4E-BP1, 4E-BP1, P-S209 eIF4E, and eIF4E; and from Pierce Thermo Fisher Scientific (Rockford, IL), P-S1798 TSC2. Western blot analysis using P-S422 SGK1 (sc-16745R; Santa Cruz Biotechnology) was performed as described previously (Garcia-Martinez and Alessi, 2008). The secondary antibodies used were raised against rabbit (Cell Signaling), goat (Dako, Glostrup, Denmark), and mouse (Amersham, GE Healthcare Bio-Sciences, Piscataway, NJ).

7-Methyl-GTP pull-down assays

BMDM were washed twice in ice-cold phosphate-buffered saline, lysed in lysis/binding buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 0.2% NP-40, 1 U/ml

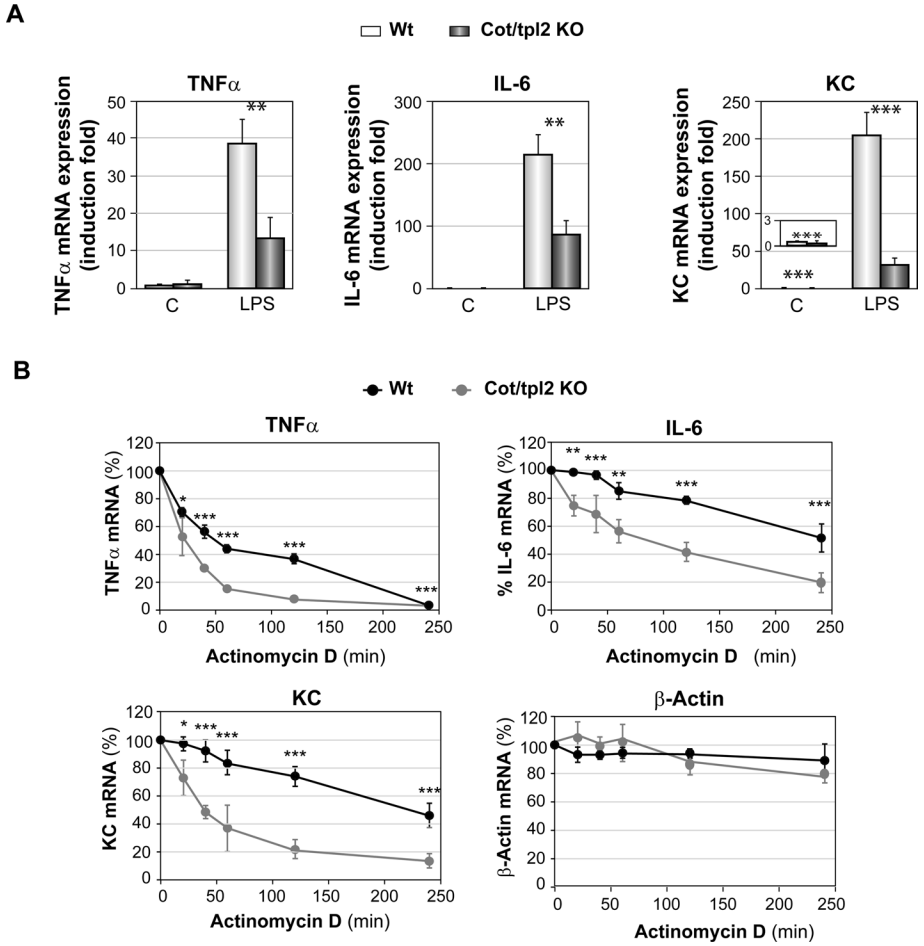

FIGURE 7: Cot/tpl2 controls TNFα, KC, and IL-6 mRNA stability in LPS-stimulated BMDM. (A) Total RNA from Wt and Cot/tpl2 KO BMDM stimulated or not for 3 h with LPS (300 ng/ml) was extracted, and TNFα, KC, and IL-6 mRNA expression was determined by qRT-PCR analysis. TNFα, KC, and IL-6 expression levels were normalized to levels of 18Sr mRNA expression in each assay. Graphs show the mean ± SD from five independent experiments, giving the value of 1 to the one obtained in control Wt BMDM. (B) The stability of TNFα, KC, IL-6, and β-actin mRNA in 3-h LPS-stimulated Wt and Cot/tpl2 KO BMDM was determined. Wt and Cot/tpl2 KO BMDM were stimulated for 3 h with LPS (300 ng/ml), and then actinomycin D (5 μg/ml) was added. At the indicated times cells were lysed, and subsequently the TNFα, IL-6, KC, and β-actin mRNA levels were analyzed by qRT-PCR and normalized by the expression of 18Sr mRNA. Graphs shown the means ± SD from three independent experiments, giving the value of 100% to the normalized mRNA levels obtained for Wt BMDM 3 h after the LPS stimulation.

RNase OUT [Invitrogen], 1 mM phenylmethylsulfonyl fluoride, plus 1 tablet/50 ml of Roche [Indianapolis, IN] inhibitors) and centrifuged at 1400 rpm for 15 min at 4°C. One milligram of supernatant protein was added to 50 μl of 7-methyl GTP (m^7GTP) Sepharose (Amersham), previously precleared with washing buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 1.5 mM $MgCl_2$). Samples were tumbled at 4°C overnight and, after five washes in 1 ml of washing buffer plus 1 mM DTT, were boiled in Laemmli sample buffer and separated on 7–15% SDS–PAGE gels. Western blot analysis was performed to determine coprecipitated 4E-BP1 and eIF4E proteins.

BMDM nucleofection

BMDM were nucleofected with the biscitronic pcDNA3rLuc-polI-RESfLuc plasmid (1 μg/1 × 10^6 cells), generously provided by Alexey Benyumov (University of Minnesota) using the Ingenio Electroporation Kit (Mirus Bio, Madison, WI) and the Nucleofector System (Amaxa Biosystems, Lonza, Cologne, Germany) and performed according to the manufacturer's instructions. Four hours after transfection, cells were washed with RPMI plus 0.5% fetal bovine serum (FBS) and incubated overnight in RPMI, 0.5% FBS, and gentamicin (80 μg/ml). Subsequently, cells were stimulated with LPS (300 ng/ml) for 3 h, and the *Renilla* and Firefly luciferase activities were measured in the cell extracts according to the manufacturer's instructions (Promega, Madison, WI).

Isolation of polyribosomal RNA and Northern blot analysis

BMDMs (5 × 10^6 cells) stimulated or not for 3 h with LPS (300 ng/ml) were washed twice on ice with hypotonic buffer (5 mM Tris-HCl, pH 7.5, 1.5 mM KCl, 2.5 mM $MgCl_2$), lysed in lysis buffer (hypotonic buffer plus 0.5% Triton X-100, 0.5% Na deoxycholate, 120 U/ml of RNase inhibitor, and 3 mM DTT), and centrifuged at 6000 rpm for 8 min at 4°C. After measurement of protein concentration, heparin (1 mg/ml) was added to the extracts. Extract were loaded on a linear sucrose gradient (0.5 and 1.5 M sucrose in 20 mM Tris-HCl, pH 8, 80 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT) and centrifuged in a Beckman SW41 rotor at 36,000 rpm for 2 h at 4°C. Gradients were fractionated, following the RNA absorbance profile at 254 nm, in different fractions. Once collected, fractions were incubated with proteinase K (5 μg/ml) in proteinase K buffer (100 mM Tris-HCl, pH 7.5, 50 mM EDTA, 5% SDS) for 30 min at 37°C. RNA for Northern blot analysis was isolated using phenol/chloroform, and hybridization of Northern blots with the eEF1α and β-actin probes was performed as described previously (Shima *et al.*, 1998).

qRT-PCR

Total RNA was isolated from BMDMs (5 × 10^6 cells) stimulated or not for 3 h with LPS (300 ng/ml), using the miRNeasy Mini Kit and according to the manufacturer's instructions (Qiagen, Valencia, CA). Recruited mRNA to polysomes was isolated using phenol/chloroform and further purified as described previously (del Prete *et al.*, 2007). Briefly, RNA was subjected to a precipitation with 2 M LiCl at –20°C overnight. After centrifugation (12,000 × *g*, 15 min at 4°C), pellets were washed twice with 70% ETOH (prestored at –20°C), air-dried, and resuspended in RNase-free water. qRT-PCR was performed as described previously (Rodriguez *et al.*, 2008). The specific TAQMAN primers (Applied Biosystems, Foster City, CA) TNFα, IL-6, KC, β-actin, and 18Sr were used.

Statistical analysis

Experiments shown were performed at least three times. The graphical data presented, as the means ± SD, were analyzed with Student's *t* test. Values were taken to be statistically significant at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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