Terminal uridyltransferase 7 regulates TLR4-triggered inflammation by controlling Regnase-1 mRNA uridylation and degradation

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Different levels of regulatory mechanisms, including posttranscriptional regulation, are needed to elaborate regulate inflammatory responses to prevent harmful effects. Terminal uridyltransferase 7 (TUT7) controls RNA stability by adding uridines to its 3′ ends, but its function in innate immune response remains obscure. Here we reveal that TLR4 activation induces TUT7, which in turn selectively regulates the production of a subset of cytokines, including Interleukin 6 (IL-6). TUT7 regulates IL-6 expression by controlling ribonuclease Regnase-1 mRNA (encoded by Zc3h12a gene) stability. Mechanistically, TLR4 activation causes TUT7 to bind directly to the stem-loop structure on Zc3h12a 3′-UTR, thereby promotes Zc3h12a uridylation and degradation. Zc3h12a from LPS-treated TUT7-sufficient macrophages possesses increased oligo-uridylated ends with shorter poly(A) tails, whereas oligo-uridylated Zc3h12a is significantly reduced in Tut7−/− cells after TLR4 activation. Together, our findings reveal the functional role of TUT7 in sculpting TLR4-driven responses by modulating mRNA stability of a selected set of inflammatory mediators.

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Inflammatory cytokines and chemokines are mediators of inflammation in the innate arm of immunity. Innate cells produce inflammatory cytokines and chemokines rapidly upon infection or tissue injury through an array of pattern-recognition receptors (PRRs)\(^1\). Inappropriate expression of inflammatory cytokines dysregulates inflammation, causing numerous disorders, including inflammatory and autoimmune diseases. Therefore, orchestrating the expression of inflammatory cytokines for an effective inflammatory response without harmful immunopathology is an important issue.

Recognition of conserved pathogen-associated molecular patterns (PAMPs) on microorganisms and damage-associated molecular patterns (DAMPs) released from stressed or injured cells by PRRs initiates pro-inflammatory cytokine responses and coordinates adaptive immune responses\(^2\). Amongst all PRR families, Toll-like receptors (TLRs) are the most well-characterized and they play an important role in innate immune responses\(^3\). TLR4 recognizes lipopolysaccharides (LPS) on Gram-negative bacteria\(^4\). Upon engagement with its ligand, TLR4 recruits Myd88, MyD88-related adaptor protein (MyD88) and Toll/IL-1 receptor domain-containing adapter-inducing IFNβ (TRIF) to inhibit activator of transcription factors NF-κB kinase (IKK)/NF-κB and TANK-binding kinase 1 (TBK1)/interferon regulatory factor 3 (IRF3) for the production of pro-inflammatory cytokines/chemokines and type I interferons, respectively\(^5,6\). Inflammatory responses induced by TLRs or other PRRs are controlled by layers of regulatory mechanisms including modulation of signaling transduction, posttranscriptional regulation (PTR), and posttranslational modification (PTM)\(^6,7\). PTR which includes RNA splicing, editing, and decay, is a quick and effective way to control the quantity of mRNA\(^8-10\). The role of PTR in regulation of PRR-triggered inflammatory response has come to light as of late.

Many PRR-triggered cytokine mRNAs have short half-lives which allows rapid control of cytokine production\(^6\). Cytokine mRNAs are subject to various regulatory cis-elements in their 3′-UTRs, such as microRNA (miRNA) target sites, AU-rich elements (AREs), and stem-loop structures. Many RNA-binding proteins (RBPs), including Regnase-1\(^1\), Arid5a\(^12\), Tristetraprolin (TTP)\(^13\), and AU-rich element RNA-binding protein 1 (AUF-1)\(^14\), specifically bind regulatory RNA cis-elements to control the stability of cytokine mRNAs. TTP and AUF1 recognize AREs in the 3′-UTR of numerous cytokines, including tumor necrosis factor (TNF) and IL-6, to initiate their decay\(^13,14\). The ribonuclease (RNase) Regnase-1 (also called Mcpip1 encoded by the Zc3h12a gene) modulates IL-6 mRNA stability when macrophages are activated by LPS or IL-1β\(^15\). Mechanistic studies revealed that Regnase-1 targets the stem-loop structure on Il6 3′-UTR and cleaves Il6 mRNA through cooperation with RNA helicase protein, UPF1\(^11,16\). On the other hand, stimulation with LPS and cytokines also enhances the expression of Arid5a. The presence of Arid5a impedes Regnase-1 binding to Il6 3′-UTR thereby stabilizes Il6 mRNA\(^12\). Therefore, it appears that regulation of spatiotemporal expression of RBPs can fine tune the quantity of cytokine mRNAs during inflammation.

Recent studies show that modification of mRNAs on their 3′ ends by non-templated nucleotide addition is an evolutionary conserved mechanism for the control of RNA stability and its fate\(^17\). Among the known template-independent RNA 3′ terminal modifications, polyadenylation and uridylation are the two most studied mechanisms\(^18\). Uridylation is catalyzed by terminal uridytransferases (TUTs). TUTs belong to a family of non-canonical poly(A) polymerases. Their function in RNA processing is evolutionally conserved from Schizosaccharomyces pombe to human\(^19\). TUT4 (also known as ZCCHC11) and TUT7 (also named ZCCHC6) are primarily responsible for cytoplasmic 3′ uridylation\(^20,21\) of various RNAs, including precursor microRNAs (pre-miRNAs)\(^22,23\), mature miRNAs\(^24\), histone mRNAs\(^25,26\), cellular mRNAs\(^26\), noncoding RNAs\(^27\), and viral RNAs\(^28\) in mammalian cells. Catalytic activities of TUT4/T7 can mono-uridylate or oligo-uridylate pre-miRNA resulting in biogenesis or degradation of miRNAs, respectively\(^21,23\). TUT4/7 are also known to trigger oligo-uridylation of poly(A)-tail-lacking histone mRNA at the end of S phase of a cell cycle to enhance its degradation\(^25,29,29\). Recently, TAIL-seq analysis revealed that TUT4 and TUT7 uridylate mRNAs with short poly(A) tails, leading to 5′-to-3′ or 3′-to-5′ mRNA degradation by recruiting deadenylases, decapping enzymes, and exonucleases\(^20,30,31\). These studies indicate that oligo-uridylation is associated with RNA degradation, but how TUT4/7 uridylate RNAs remains unknown.

TUTs are recently shown to modulate inflammatory responses in mammalian cells. TUT4 uridylates and degrades IL-6-targeting miR-26a/b, leading to control of the quantity of Il6 mRNA in A549 cells following TNF treatment\(^24\). TUT7 is also known to modulate miR-26b uridylation and stability in IL-1β-stimulated chondrocytes through which positively regulates IL-6 expression\(^32\). A recent report revealed that TUT7 impedes the expression of a specific set of pro-inflammatory cytokines (including IL-6) in mice after challenge with Streptococcus pneumoniae\(^33\). It is thus evident from in vitro and in vivo studies that TUT7 modulates inflammatory response. However, it remains an interesting question whether TUT7 can modulate inflammation by direct targeting mRNAs.

In this study, we reveal that TLR4 activation in macrophages upregulates TUT7 expression and TUT7 selectively regulates the expression of a subset of inflammatory cytokines. Both in vitro and in vivo studies show that TUT7 is critical for IL-6 and IL-12p40 upon LPS challenge and it is dependent on its nucleotidytransferase activity. We further demonstrate that TUT7 directly controls Zc3h12a mRNA decay by uridyling its 3′ end, which subsequently prevents Regnase-1 from degrading Il6. Interestingly, Zc3h12a isolated from LPS-treated TUT7-deficient bone marrow-derived macrophages (BMDMs) contains increased oligo-uridines (≥2 U) with short poly(A) tails at its 3′ end, whereas reduced oligo-uridylation is found in Zc3h12a transcripts from TUT7-deficient cells after the same treatment. Together, our results demonstrate that TUT7 functions as a regulator in TLR4-driven inflammatory responses by mediating uridylation of and thus destabilizing the mRNAs of inflammatory mediators including Zc3h12a.

**Results**

**TUT7 regulates cytokine production in macrophage response to LPS.** TUTs possess the ability to uridylate small RNAs and/or mRNAs, thereby regulates their stability\(^17,21,29\). Transcriptome analysis of GEO Datasets (GDS5623) obtained from the National Center for Biotechnology Information shows that TUT7 is upregulated in both BMDMs and RAW 264.7 macrophages upon LPS stimulation (https://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS5623). We discovered that LPS treatment dramatically increased TUT7 expression in BMDMs (Fig. 1a). LPS-induced TUT7 expression required the activities of IKK and p38 Mitogen-activated protein kinase (MAPK), whereas suppression of JNK and ERK MAPKs had no effect on TUT7 expression (Supplementary Fig. 1a). In addition, other TLR agonists, including Pam3CSK4 (TLR1/2), poly(I:C) (TLR3), R848 (TLR7/8) and CpG-1826 (TLR9) induced moderate levels of TUT7 expression (Supplementary Fig. 1b). IKK, but not JNK, activation was required for Pam3CSK4-driven inflammatory responses induced by TLR4 (Supplementary Fig. 1c).

To assess the role of TUT7 in TLR4-driven inflammatory response, we knocked down its expression in murine RAW 264.7...
macrophages by lentivirus-mediated shRNA transduction. Silencing Tut7 in RAW 264.7 macrophages significantly decreased Il6, Il12b, and Il1b mRNA, and cytosolic pro-IL-1β, and IL-6 and IL-12p40, but not Tnf and Nfkbia mRNA or TNF protein expression upon LPS challenge (Supplementary Fig. 1d–h). Nevertheless, LPS-induced IkBa degradation, activation of MAPKs JNK and p38, and nuclear translocation of IRF3 were comparable in control and TUT7-silenced cells (Supplementary Fig. 1i, j). These
results suggest a regulatory role for TUT7 in TLR4-mediated immune response.

We generated Tut7−/− mice using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system (Supplementary Fig. 2 and Supplementary Table 1). Results in Fig. 1b showed that TUT7 deficiency decreased the levels of Il6, Il12b, and Il1b mRNA and proteins (Fig. 1b, c, e) but not Tnf and Nfkbia mRNA or TNF protein in BMDMs response to LPS (Fig. 1d). Neither did TUT7 deficiency affect LPS-induced activation of IKK and MAPKs (Fig. 1). RNA Sequencing (RNA-Seq) was applied to further assess the effect of TUT7 in global gene expression during TLR4 activation. Surprisingly, TUT7-modulated 352 out of a total 7637 of LPS-induced genes. Among the 352 genes, 111 genes involved in the innate immune response were downregulated in Tut7−/− cells. These include pro-inflammatory cytokines (IL-6, IL-12p40 (Il12b), and IL-23p19 (Il23a)), cluster of differentiation (CD) markers CD24a (Cd24a) and CD55 (Cd55), and chemokine CCL17 (Cd17) (Fig. 1g and Supplementary Table 2). By contrast, 68 innate immune-related genes were upregulated in LPS-treated Tut7−/− BMDMs (Fig. 1g and Supplementary Table 3).

Fig. 1 TUT7 deficiency alters gene expression profile in BMDM response to LPS. a-f BMDMs from wild-type (Tut7+/+) and Tut7−/− mice were incubated with 100 ng/ml LPS for indicated times. TUT7 protein expression was analyzed by immunoblotting (a). Il6, Il12b, and Il1b mRNA expression was determined by RT-qPCR (b, d). The levels of indicated inflammatory cytokines in cultural media were analyzed by ELISA (c, d). The levels of pro-IL-1β, IL-8, and phosphorylation of MAP kinases were analyzed by immunoblotting (e). g Volcano plot of the changes of innate immune-related gene expressions in Tut7−/− BMDMs after 4 h treatment with 100 ng/ml LPS against wild-type BMDMs. The transcriptome of BMDMs from wild-type and Tut7−/− was compared to baseline. The x-axis indicates the logarithm of p values to the base 2 of the fold-change (FC) and the y-axis reveals the negative logarithm of that to the base 10. Black horizontal dash line indicates significance at 5% criteria (p value = 0.05). log2FC > +0.2 and log2FC < −0.2 indicate increase of transcript levels by >20% and decreased by >20%, respectively. Green and red dots denote transcripts related to innate immune responses that were significantly lower (n = 111) and higher (n = 68) in response to LPS, respectively. The innate immune-related genes that are significantly differentially expressed are listed in Supplementary Tables 2, 3. h 8-week-old mice were injected intraperitoneally with LPS (50 μg/kg). Whole blood was collected 2 h after LPS challenge, and the concentrations of IL-6, IL-12p40, and TNF in sera were determined by ELISA. Data (except g) presented are representative of three independent experiments with triplicates in each experiment (error bars, mean ± S.D.). The p values were obtained from two-tailed Student’s t test and are shown in the figure if p < 0.05. Source data are provided as a Source Data file.
Il6 is responsible for TUT7-mediated effects, we generated luciferase reporter constructs with either altered or similar stem-loop structures as the wild-type (Fig. 2g). Deletion of the internal loop structure (Δloop) abolished the suppressive effect of TUT7 (Fig. 2g, h). In addition, m1 and m2 mutants (with altered stem structures) both dampened TUT7-modulated inhibition of the luciferase activity; whereas m(1+2) mutant with an intact stem structure that was restored by combining both mutations.
Fig. 2 Regulation of Il6 mRNA stability by TUT7 is dependent on its 3′-UTR_{66-104}. a) TUT7-silenced RAW 264.7 macrophages were reconstituted with either flag-tagged wild-type TUT7 (hTUT7) or activity-dead TUT7 (hTUT7(DADA)) and challenged with 100 ng/ml LPS for 4 h. The expression of Il-6, Il-12p40, and TNF mRNA and TUT7 protein were analyzed by RT-qPCR. b) RAW 264.7 macrophages expressing shControl and shTut7 were treated with 100 ng/ml LPS for 4 h followed by incubation with 5 μg/ml actinomycin D for the indicated times. Total RNAs were extracted and analyzed by RT-qPCR. c) Schematic diagram of the firefly luciferase reporter constructs containing full-length (FL) and truncated forms of mouse (red line) or human (blue line) Il6 3′-UTR. d-f) Control and TUT7-depleted RAW 264.7 cells were co-transfected with the indicated firefly luciferase reporter plasmids containing various mouse or human Il6 3′-UTR (mIl6 3′-UTR or hIl6 3′-UTR) or Tnf 3′-UTR and TK-renilla control reporter plasmid. At 48 h post-transfection, cells were treated with 100 ng/ml LPS for 8 h and the luciferase activities were determined. g) Schematic diagram of the predicted stem-loop structure in human and mouse Il6 3′-UTR, and mutants whose stem-loop structure (m1 and m2) or loop structure (∆loop) were disrupted or deleted. The stem-loop structure of mIl6 3′-UTR or hIl6 3′-UTR was predicted by ref. 14. Mutant (m1 + 2) was generated to swap sequences on the 5′ and 3′ sides of the stems without disrupting the stem-loop structure in mIl6 3′-UTR. h) Control and shTut7-expressing RAW 264.7 cells were co-transfected with the indicated luciferase reporter plasmids and TK-renilla control reporter plasmid for 48 h. Cells were stimulated with 100 ng/ml LPS for 8 h and harvested for the analysis of luciferase activity. Data presented are representative of three independent experiments with triplicates in each experiment (error bars, mean ± S.D.). The p values were obtained from two-tailed Student’s t test and are shown in the figure if p < 0.05. Source data are provided as a Source Data file.

TUT7 controls Il6 mRNA stability through Regnase-1. Previous studies showed that TUT7 uridylates target RNAs to modulate their stability20,25,26. However, our RNA-immunoprecipitation (RNA-IP) data demonstrated that TUT7 did not associate with Il6 transcripts (Fig. 3a). Regnase-1 is reported to bind and control Il6 stability via the conserved stem-loop structure in Il6 3′-UTR_{66-104}. Therefore, we hypothesized that TUT7 controls Il6 mRNA destabilization through regulating Regnase-1 expression. Results showed that the levels of mRNA and protein of Regnase-1 increased in LPS-stimulated TUT7-depleted RAW 264.7 cells (Fig. 3b, c) and BMDMs (Fig. 3d, e) compared with that in control cells. Regnase-1 belongs to the Zc3h12 family that is characterized by its conserved CCCH-type zinc-finger15. The mRNA level of another family member Zc3h12c was not altered in LPS-treated cells, regardless of the presence or absence of TUT7 (Supplementary Fig. 6), suggesting a specific regulatory role of TUT7 in Zc3h12a expression. The half-life of Zc3h12a was longer (30 vs. 80 min) in TUT7-deficient RAW 264.7 macrophages than control cells upon LPS challenge (Fig. 3f). In addition, LPS-induced Zc3h12a expression was reduced in TUT7-depleted cells reconstituted with wild-type TUT7, but not with hTUT7(DADA) mutant (Fig. 3g), indicating that TUT7-mediated Zc3h12a expression after LPS stimulation depends on its nucleotidyltransferase activity. To further confirm TUT7-mediated TLR4-induced cytokine production is through Regnase-1, we depleted Regnase-1 in TUT7+/− BMDMs by lentivirus-mediated shRNA and challenged these cells with LPS. Deletion of Regnase-1 in TUT7+/− BMDMs significantly increased Il6 and Il12b expression in response to LPS (Fig. 3h). However, LPS-induced Tnf and Nfkbia were similar in TUT7+/− BMDMs expressing control and Zc3h12a shRNA (Fig. 3h). These data together demonstrate that TUT7 regulates TLR4-induced Il6 and Il12b expression by suppressing Regnase-1 expression.

TUT7 controls Zc3h12a mRNA stability through its 3′-UTR. We then determined whether TUT7 controls Zc3h12a mRNA stability via its 3′-UTR using luciferase reporter assay. We generated a set of luciferase reporter constructs containing full-length or different truncation of the Zc3h12a 3′-UTR and analyzed their luciferase activities in control and TUT7-depleted RAW 264.7 cells (Fig. 4a). The luciferase activity of full-length Zc3h12a 3′-UTR was upregulated in LPS-stimulated TUT7-depleted RAW 264.7 cells compared to that in control cells (Supplementary Fig. 7). To further assess whether TUT7 directly regulates Zc3h12a expression via its 3′-UTR, luciferase reporter constructs together with either wild-type or enzymatic inactive TUT7 were co-expressed in human embryonic kidney 293 (HEK 293) cells. Like most mRNA 3′-UTRs, full-length mouse Zc3h12a 3′-UTR decreased luciferase activity compared to luciferase reporter control (Fig. 4b). This repressive effect was further enhanced by co-expression of wild-type TUT7, but not the hTUT7(DADA) mutant, suggesting that TUT7 directly regulates Zc3h12a in a nucleotidyltransferase activity-dependent manner (Fig. 4b). In addition, while TUT7 overexpression reduced Zc3h12a 3′-UTR_{100-250} luciferase activity, it failed to reduce the luciferase activities of Zc3h12a 3′-UTR_{533-698} and Zc3h12a 3′-UTR_{660-865} (Fig. 4b). We further shortened Zc3h12a 3′-UTR_{100-250} and found that Zc3h12a 3′-UTR_{100-250} rendered TUT7-mediated suppressive effect similar to full-length Zc3h12a 3′-UTR and Zc3h12a 3′-UTR_{100-250} (Fig. 4c). Zc3h12a 3′-UTR_{100-250} was previously predicted to be able to form a stem-loop structure11. We then investigated the role of this stem-loop structure in TUT7-modulated Zc3h12a mRNA stability by constructing luciferase reporters containing Zc3h12a 3′-UTR_{100-250} mutants whose stem-loop structures were eliminated (Fig. 4d)11. Surprisingly, disruption of the stem-loop structure (St1m and St2m) lost TUT7-mediated inhibition of the luciferase activity, whereas restoring stem-loop structure (St1 + 2m) regained the effect of TUT7 (Fig. 4e), suggesting a requirement for the stem-loop structure of Zc3h12a 3′-UTR in TUT7-mediated suppression. Taken together, TUT7 modulates Zc3h12a mRNA stability by recognition of the stem-loop structure in its 3′-UTR_{100-250}.

TUT7 binds and uridylates Zc3h12a. Our results in Fig. 3g revealed that TUT7 directly modulated Zc3h12a mRNA stability through its 3′-UTR in an uridytransferase activity-dependent manner. TUT7 possesses the ability to uridylate both mRNA and miRNA and to facilitate their degradation20,23. We thus hypothesized that TUT7 uridylates and degrades Zc3h12a after TLR4 engagement. To test this possibility, we first did RNA-IP to assess the association of TUT7 and Zc3h12a. Results in Fig. 5a, b showed that TUT7 was associated with Zc3h12a by 2 h after LPS treatment.

To further investigate whether TUT7 directly binds to Zc3h12a stem-loop structure, we performed RNA electrophoresis mobility shift assay (EMSA) using synthesized RNA probes. In line with the results of RNA-IP, TUT7 bound to murine Zc3h12a stem-loop, but not that of Il6 (Fig. 5c). A previous study showed that the length of poly(A) tails negatively correlates with the frequency of uridylation20. Interestingly, TUT7 bound to Zc3h12a stem-loop regardless of the length of poly(A) tails (Supplementary
Fig. 8c), suggesting that TUT7 associating with Zc3h12a is dependent on its stem-loop structure rather than the length of its poly(A) tails. Furthermore, the association of TUT7 and Zc3h12a was dependent on its stem-loop structure rather than its sequence as the addition of RNA oligomers containing wild-type Zc3h12a or St(1 + 2)m, but not St1m nor St2m, stem-loop mutant abolished their binding (Fig. 5d). To assess whether TUT7 is able to uridylate Zc3h12a, we set up in vitro uridylation assay
using immunopurified TUT7 from HEK293T cells overexpressing Flag-tagged TUT7. RNA substrates were in vitro transcribed Zc3h12a or Tnf containing 3′-UTR (Fig. 5e). Interestingly, regardless of the coding sequences (CDSs) of the substrates, TUT7 was only capable of uridylating transcripts containing Zc3h12a 3′-UTR, but not those without 3′-UTR or those with Tnf 3′-UTR (Fig. 5f). Consistently, TUT7 did not uridylate transcripts containing Ile6 3′-UTR albeit with the CDS of Zc3h12a (Fig. 5g). Furthermore, TUT7 preferentially uridylated transcripts containing Zc3h12a stem-loop rather than that containing Ile6 stem-loop (Fig. 5h). Pull-down and immunoblotting experiments showed that Regnase-1 was not co-immunoprecipitated with TUT7 (Supplementary Fig. 8a, b), which excludes the possibility of the involvement of Regnase-1 in TUT-mediated uridylation. These data together support the notion that upon LPS stimulation TUT7 directly binds and uridylates Zc3h12a 3′-UTR.

We further examined Zc3h12a 3′-terminal uridylation in Tut7+/+ and Tut7−/− BMDMs using gene specificTAIL-Seq31 (Fig. 6a). As expected, LPS significantly increased the frequency of uridylation on Zc3h12a, especially that of oligo-uridylation (≥2 U) (Fig. 6b). Deleting Tut7 significantly decreased uridylation on Zc3h12a (12% mono-uridine and 14% oligo-uridines among 1714 reads) after LPS stimulation compared to that of control (14% mono-uridine and 24% oligo-uridines among 1067 reads) (Fig. 6b). However, there is no observable difference in uridylation on Ile6 mRNA between Tut7-deficient macrophages (9% mono-uridine and 5% oligo-uridine among 1730 reads) and control (9% mono-uridine and 6% oligo-uridine among 1756 reads) in response to LPS (Supplementary Fig. 9). Consistent with published studies, the frequency of oligo-uridine (≥2 U) addition to Zc3h12a 3′ end negatively correlated with the length of poly(A) tails in wild-type cells but not in Tut7-deficient cells upon LPS challenge (Fig. 6c). Furthermore, Tut7 deficiency did not affect non-templated addition of cytosine and guanine to the 3′ end of Zc3h12a (Fig. 6d). Together, our results demonstrate that TUT7 binds Zc3h12a in macrophage stimulated by LPS and regulates Zc3h12a expression by uridylylating its 3′ tail.

TUT4 and TUT7 regulate Zc3h12a expression through different mechanisms. TUT7 and TUT4 are highly homologous among members of the TUTase family. They have been reported to share various similar biological functions. We thus examined whether TUT4 like TUT7 possesses the modular function of TLR4-driven effect through regulating Zc3h12a expression. We first assessed TUT4 expression in Tut7−/−deficient cells and found that the levels of TUT4 mRNA and protein were similar in TUT7-silenced and control cells (Supplementary Fig. 10a, b). However, unlike TUT7, depletion of TUT4 only marginally reduced Regnase-1 mRNA and protein expression after LPS stimulation (Supplementary Fig. 10c, d), indicating that TUT4 and TUT7 participate in TLR4-triggered inflammatory responses through different mechanisms.

Discussion

Inflammatory cytokines are quickly induced upon infection in order to eliminate pathogens and repair damaged tissues. To prevent their detrimental effect on the host, their production must be tightly regulated. PTR is a quick and effective mechanism to modulate the expression of inflammatory cytokines. The involvement of PTR in regulation of cytokine production during innate immune response is recently emerged. As shown in Fig. 7, we reveal that terminal uridyltransferase TUT7 expression is induced by TLR4 ligand LPS, and, in turn participates in the regulation of the expression of a subset of cytokines, including IL-6 and IL-12. TUT7 positively regulates Ile6 mRNA stability through controlling Zc3h12a expression by its terminal nucleotidyltransferase activity after TLR4 activation. We further demonstrate that upon TLR4 engagement TUT7 directly binds and uridylates Zc3h12a to downregulate its expression, resulting in stabilization of Ile6 mRNAs. Our results together demonstrate that TUT7 is a modulator of TLR4-triggered inflammatory response (Fig. 7). TUT7 fine-tunes the expression of IL-6, and we speculate other inflammatory cytokines as well, through directly uridylylating Zc3h12a transcript.

Accumulating data have revealed that a number of TLRs-inducible proteins play a role in modulation of inflammatory responses. In this study, we extend this list to include TUT7. Our data indicate that TLR4-induced TUT7 expression requires IKK activity, and p38, but not JNK nor ERK, MAPK also contributes to its expression. These results suggest that transcription factors downstream of IKK and p38 MAPK may be required for TLR4-induced TUT7 expression. We and others have previously reported that p38 MAPK modulates the expression of certain LPS-induced genes via several transcription factors, including CCAAT/Enhancer binding protein β (C/EBPβ) and cAMP response element-binding protein (CREB). Interestingly, online prediction tool PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) identified the conserved binding motifs for C/EBPβ and NF-κB on both human and mouse TUT7 promoters although it remains to be determined whether p38 MAPK regulates TLR4-triggered TUT7 expression through C/EBPβ.

Regnase-1 is an RNAse. By controlling the degradation of target mRNAs, such as Ile6 and Il12b, Regnase-1 is crucial for restraining inflammatory response during TLRs and IL-1 receptor (IL-1R) activation. Therefore, the dynamics of Regnase-1 expression following TLRs and IL-1β stimulation ensures timely regulation of cytokine production. Accumulating data revealed that both PTR and PTM contribute to the regulation of Regnase-1 expression upon TLRs- and IL-1R activation. Regnase-1 recognizes the conserved stem-loop structure in its 3′-UTR to...
degrade its own mRNA at the early phase of an inflammatory response. Interestingly, Regnase-1 protein is quickly phosphorylated and degraded through IKK complex upon TLRs and IL-1R activation. It may thereby unable to effectively eliminate its own mRNA, impeding the subsequent cytokine production.

Our findings demonstrate that TUT7 induces Zc3h12a degradation through uridylation on its 3' end in the early phase of TLR4-triggered inflammatory response. Our data together with a previous study indicate that at least two distinct PTR mechanisms triggered by TLR4 destabilize Zc3h12a to promote the production of inflammatory response.
of cytokines and inflammatory mediators in the early phase of innate immune responses.

Among the members of the TUTase family, TUT7 shares structural and functional homology with TUT4. Both TUT7 and TUT4 function to uridylate noncoding RNAs, i.e., miRNAs, leading to biogenesis or degradation of miRNA22,23,27,42. Recent reports indicate that TUT7 and TUT4 act redundantly in NIH3T3 and HeLa cells to control the stability of global mRNAs
Fig. 5 TUT7 binds and uridylates Zc3h12a. a Raw 264.7 cells were treated with 100 ng/ml LPS for 2 h and cell lysates were incubated with control IgG or protein A-agarose beads-conjugated anti-TUT7 antibody at 4 °C for 4 h. TUT7-interacting RNAs were extracted and analyzed by RT-PCR (a) and RT-qPCR (b). c Synthetic RNAs containing Il6 or Zc3h12a wild-type stem-loop or mutant variants were incubated with immunopurified Flag-tagged wild-type TUT7 at 37 °C for 30 min. d Cy3-labeled RNA containing Zc3h12a stem-loop was incubated with immunopurified TUT7 in the presence or absence of 10 or 5× fold excess of unlabeled RNA containing Zc3h12a wild-type or the indicated stem-loop mutant at 37 °C for 30 min. All RNA samples were resolved on a 10% native PAGE and visualized by SYBR Green II staining (c) and Cy3 fluorescent signal (d). e Schematic diagram of different constructs used for in vitro transcription. Flowchart on the right shows the procedure for in vitro uridylation. RNA transcripts were prepared by in vitro transcription. Transcripts were incubated with Flag-tagged wild-type (WT) or enzyme-dead (DADA) TUT7 mutant prepared by immunoprecipitation of HEK293T cells transfected with Flag-tagged TUT7- or DADA mutant-expressing plasmids in the presence of α-32P-UTP at 37 °C for 1 h. f-h Immunoprecipitation of flag-tagged wild-type and DADA mutant TUT7 was confirmed by immunoblotting (the upper panel of f). RNA samples were separated on a 1% formaldehyde-agarose gel and visualized by SYBR Green II staining and autoradiography (the bottom panel of f, g, h). Data presented are representative of three independent experiments. Source data are provided as a Source Data file. Asterisk (*) represents the nonspecific binding.

Fig. 6 TUT7 mediates oligo-uridylation of Zc3h12a on 3′ end in response to LPS. a Flowchart showing the experimental procedure of specific gene TAIL-Seq to analyze 3′ tails of Zc3h12a mRNA transcripts. b The frequency of Zc3h12a uridylation assessed by specific gene TAIL-Seq. Uridylation frequency is the percentage of uridylated Zc3h12a reads out of the total number of Zc3h12a reads. c The correlation between the mono-uridylation (upper panel) and oligo-uridylation (lower panel) of Zc3h12a and their poly(A) tail lengths. Uridylation frequency presents the percentage of uridylated reads among the total Zc3h12a reads. d The frequency of non-templated 3′ end modifications on Zc3h12a tails. The Y-axis presents the percentage of uridine, cytosine, and guanine additions among total Zc3h12a reads (for cytosine and guanine additions, 75 and 22 among 466 reads, 144 and 40 among 1067 reads, 65 and 12 among 311 reads, and 174 and 60 among 1714 reads in Tut7+/-, Tut7+/- (+LPS), Tut7−/-, and Tut7−/- (+LPS) cells, respectively. The value of uridylation is from b). The p value in (c) was assessed by Pearson correlation analysis. Source data are provided as a Source Data file.

through uridylation, especially oligo-uridylation (≥2 U) as oligo-uridylation is crucial for mRNA decay20,31. Both TUTs also function redundantly to eliminate both maternal mRNA during early embryogenesis and viral RNAs in mammalian cells28,43,44. Our results show that upon TLR4 activation, TUT7, but not TUT4, is upregulated and TUT7 subsequently associates with and oligouridylates Zc3h12a to regulate cytokine production. On contrast, TUT4 positively regulates Zc3h12a expression response to LPS through a yet-to-be investigated mechanism. TUT7 and TUT4 were reported to regulate Il6 expression through translation of miR-26, yet miR-26 uridylation is pervasive24,32. Our study demonstrates that mRNA uridylation can be specific and
inducible and that TUT7 is functionally distinct from TUT4 in innate immune responses. However, it is worth noting that LPS-induced Zc3h12a oligo-U-tails is not completely diminished in Tut7-deficient cells, indicating the involvement of additional TUT in Zc3h12a uridylation response to LPS.

It is still unclear how TUT4 and TUT7 discriminate and mediate uridylation on the 3′ end of their target mRNAs. Generally, it is proposed that when the poly(A) tails of the target mRNAs become shorter than 20 nts, poly(A)-binding proteins are dissociated from the poly(A) tails, thereby allowing the binding of TUTs to mRNAs for subsequent uridylation. This model is based on the assumption that uridylation by TUTs is an activity downstream of deadenylated but it excludes the possibility that TUTs may participate in deadenylated. In addition, it remains to be determined whether any cis-element on 3′-UTR is required for TUT-mediated uridylation. Our data reveal that TUT7 directly binds to Zc3h12a through the conserved stem-loop structure in its 3′-UTR and catalyzes uridylation, which probably marks the mRNA for degradation.

Regnase-1 is also known to target the same conserved stem-loop structure as TUT7 does to facilitate its own mRNA decay. It is unclear why Regnase-1 can recognize the stem-loop structures appearing in both Zc3h12a and Il6 3′-UTRs, whereas TUT7 only interacts with Zc3h12a, but not Il6, stem-loop structure. Structural and functional studies suggest that Regnase-1 prefers binding to the stem-loop structure with a UAU loop and 5 to 9 base pairs in the stems. Interestingly, the stem-loop structures in Il6 and Zc3h12a 3′-UTR are distinct: Il6 stem-loop structure harbors a typical Y-R-Y loop and 9 base pairs in the stem, whereas the one in Zc3h12a 3′-UTR is composed of a 5-nucleotide loop and 5 base pairs in the stem. A recent study employing Hamiltonian scores to predict how TUT7 distinguishes its target pre-let-7a from other unrelated RNAs suggests a critical role of the loop, but not the stem, structure for TUT7 binding. These data together suggest that TUT7 may prefer to interact with the loop in Zc3h12a rather than Il6 3′-UTR, but this notion needs confirmation. In addition, Lin28 is shown to recognize a GGAG loop and subsequently recruit TUT4 to pre-let-7. UPF1 helicase is also reported to cooperate with Regnase-1 to control Zc3h12a destabilization. Therefore, we cannot exclude the possibility that an unknown RNA-binding molecule associates with the stem-loop structure of Zc3h12a and enslists TUT7.

Oligo-uridylation on mRNAs in mammalian cells is considered to be a tag for RNA degradation. Addition of uridine to the 3′ end of RNAs by TUT7 and TUT4 induces 5′-to-3′ and 3′-to-5′ degradation by the XRN family of exoribonucleases and the exosome complex, respectively. The 3′-to-5′ exoribonuclease Tris3L2 has been shown to form complex with TUT4/7 to degrade cytosolic noncoding RNAs, independent of the exosome. However, recent studies indicate that Dis3L2 only plays a modest role in human cells to degrade global mRNAs with uridylated tails. Rather it is a predominant exoribonuclease to eliminate uridylated mRNA during apoptosis.

Here we reveal that LPS-induced TUT7 uridylates Zc3h12a and accelerates its mRNA degradation thereby promotes the production of its target cytokines. It appears that this regulatory mechanism to suppress Regnase-1 expression is at work in the early phase of inflammation when mRNAs of cytokines, such as IL-6 and IL-12β, are being actively translated. Inflammation is a highly dynamic and intricate process. Multiple factors collaborate to spatially and temporally control the production of inflammatory mediators at the levels of transcription, PTR, or PTM. A recent study demonstrated that Regnase-1 functions to degrade translationally active cytokine mRNAs during inflammation. In addition, our transcriptome analysis revealed that 111 and 68 innate-related genes were up- and down-regulated, respectively, in Tut7−/− cells upon TLR4 activation. TUT7 therefore may act in concert with other molecules to finely tune the expression of different sets of cytokine mRNAs. How different molecules and mechanisms work together to elaborately control inflammatory response is worth further investigation.

In summary, our study uncovers TUT7-mediated Zc3h12a uridylation as a posttranscriptional mechanism in regulation of TLR4-driven inflammatory cytokine response. This molecular mechanism involving TUT7-restricted Regnase-1 expression may...
contribute to the generation of a specific set of inflammatory cytokines to shape a beneficial inflammation. Given the prominent role of IL-6 in inflammation, the TUT7/Regnase-1 axis pathway may also contribute to the pathogenesis of IL-6 mediated inflammatory diseases.

### Methods

#### Plasmids

The coding regions (CDS) of mouse TUT7, TNF, and Regnase-1 were amplified by PCR using cDNA library and cloned into pcDNA3. Post-267.4 macrophages as templates and cloned into pcDNA3 (Invitrogen, Carlsbad, CA) containing an N-terminal Flag tag. The 3′-UTR of mouse Zc3h12a, Il6, and Tnf were also PCR amplified from RAW 264.7 DNA libary and subcloned to the immediate downstream of the CDS of TNF, IL-6 or Regnase-1 in pcDNA to obtain pcDNA-Zc3h12a-UTR, pcDNA-Zc3h12a-ΔUTR, pcDNA-Zc3h12a-3′-UTR, pcDNA-Zc3h12a-5′UTR, pcDNA-Zc3h12a-Δ5′UTR, pcDNA-Zc3h12a-Δ3′UTR, pcDNA-Zc3h12a-Δ3′UTR-Δ5′UTR, pcDNA-Zc3h12a-Δ3′UTR-Δ5′UTR-stem-loop, and pcDNA-Zc3h12a-Δ3′UTR-Δ5′UTR-stem-loop plasmids used for in vitro transcription. Human TUT7 and TUT4 CDSs were amplified by PCR using pCK-FLAG-TUT7 and pCK-FLAG-TUT4 plasmids.

Provided by Dr. V. Nary Kim (National University of Seoul, Korea) as templates and cloned into the pLKO.AS2.neo vector (National RNAi Core Facility, Academia Sinica, Taiwan). Luciferase reporter plasmids pcGL3 and pRL-TK were obtained from Promega (Madison, WI). The pcGL-3′-UTR full-length (1–1403), truncated constructs (1–70, 56–173, and 172–264) were obtained with standard protocols.

**Methods.**

#### Immunoblotting and immunoprecipitation

Cells were harvested after centrifugation of cell mixtures, and cell lysates were harvested after centrifugation of cell mixtures, and the supernatants were subjected to the addition of 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin. HEK293T and HEK 293T cells were cultured in DMEM containing 10% FBS and 100 μM penicillin/streptomycin. All cells were maintained at 37 °C in a humidified 5% CO2 atmosphere. BMDMs were prepared as previous description. Briefly, femurs and tibia bones were collected from 6 to 8-week-old mice and bone marrow was flushed out with DMEM medium using a 25-gauge syringe. The bone marrow progenitor cells were harvested, and differentiated in high-glucose DMEM medium containing 20% L929 cell-conditioned medium for 7 days. Adherent BMDMs were collected and cultured in DMEM medium containing 10 ng/ml M-CSF overnight for further experiments.

### RNA extraction and Real-time quantitative RT-PCR

Total cellular RNA was isolated using NucleoZol (MACHEREY-NAGEL, Düren, Germany). One μg of total RNA was used to synthesize cDNA with the ReverTra Aid H Minus First-Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL) following the manufacturer’s instructions. The amount of CDNA was determined by Real-time quantitative PCR (RT-qPCR) using Maxima® SYBR Green/Fluorescein qPCR Master Mix (#4376659; Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions. All RT-qPCR values of interesting genes were normalized to cyclophilin A transcript as an internal control. All data were presented as fold-change relative to the unstimulated sample. The primer sequences are listed in Supplementary Table S5.

#### shRNA-based gene silencing and lentiviral infection

Lentiviral short hairpin RNA (shRNA) constructs expressing shRNAs against mouse TUT7, TUT4, and Regnase-1 were produced by BioVector (Fermentas, Schwerte, Germany) according to the manufacturer’s recommendations. Cultural medium containing the lentivirus was harvested 48 and 72 h after transfection. RAW 264.7 macrophages were infected overnight with lentiviruses in the presence of 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO) to promote lentivirus attachment to cells followed by culturing in fresh medium for another 24 h. The infected cells were selected in 5 μg/ml puromycin-containing medium until the uninfected cells were completely eliminated. The stable colonies were pooled for further experiments. TUT7+/− and TUT7−/− macrophage progenitors were infected with lentiviruses expressing Zc3h12a shRNA on days 1 and 2 of differentiation into macrophages as mentioned previously. After 4 days, cell medium was changed to fresh L929-conditioned medium and the cells were differentiated for 3 more days.

#### mRNA stability assay

RAW 264.7 macrophages were challenged with 100 ng/ml LPS for 4 h, and then incubated with actinomycin D (5 μg/ml) for the indicated times. The cells were collected and total cellular RNAs were prepared for mRNA quantitation by RT-qPCR as described above.

#### Immunoblotting and immunoprecipitation

Cells were collected and lysed in cold All Purpose Buffer (APB, 0.05 M Tris, pH 7.5, 0.25 M NaCl, 3 mM EDTA, 3 mM EGTA, 0.5 M NaCl, 0.02% Triton X-100, 0.05% SDS, 0.5% glycerol, 20 mM EGTA, 1% Triton X-100, 0.5% NP-40, 40 mM β-glycerophosphate, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM p-nitrophenyl phosphate (p-NPP), 1 mM sodium orthovanadate, and protease inhibitor cocktail including 2 μg/ml aprotinin, 1 μg/ml benzamidine hydrochloride hydrate, 1 μg/ml leupeptins, and 2 μg/ml leupeptins) followed by incubation on ice for 15 min. The cell lysates were harvested after centrifugation of cell mixture, and protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Cellular extracts in

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#### Supplementary information

**Supplementary information**

- **Supplementary Table S1:** List of primers used in this study.
- **Supplementary Table S2:** List of antibodies used in this study.
- **Supplementary Table S3:** List of shRNA constructs used in this study.
- **Supplementary Table S4:** List of lentiviral constructs used in this study.
- **Supplementary Table S5:** Primer sequences used in this study.

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400 µl APB buffer was incubated with the appropriate antibody and Protein A-conjugated agarose beads overnight at 4 °C. The immunocomplexes were pelleted by centrifugation, washed three times with lysis buffer, and resuspended in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample-loading buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 1.25% β-mercaptoethanol, and 0.1% bromophenol blue). Protein samples were separated by SDS-PAGE, and transferred to the PVDF membranes. The PVDF membranes were incubated with the indicated primary antibody overnight at 4 °C, followed by incubation of HRP-conjugated secondary antibody for 1 h at 25 °C. The immunoreactive signals were detected using Western Lighting™ Plus-ECL (PerkinElmer, Waltham, MA) based on the manufacturer’s instructions.

**Dual-luciferase reporter and enzyme-linked immunosorbent assays.** Cells were co-transfected with the indicated dual-luciferase reporter plasmid, pRL-TK-renilla luciferase plasmid, and TUT7 or empty control plasmid, and cultured for 48 h. Cells were harvested and lysed, and firefly and renilla luciliaferase activities were determined using the Dual-Luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer’s instructions. The levels of cytokines in culture supernatants were measured by DuoSet ELISA systems (R&D Systems, Minneapolis, MN) following the manufacturer’s instructions.

**In vitro transfection and in vitro uridylation.** In vitro transfection was carried out by T7 RNA polymerase (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, 5 µl of linearized DNA template was incubated with 40 units T7 RNA polymerase in 100 µl reaction mixtures containing 40 mM Tris, pH 7.9, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 2.5 mM NTP mix, 100 units RNase inhibitor, for 2 h at 37 °C. RNA transcripts were then purified by NucleoZol, precipitated with 100% isopropanol (Sigma-Aldrich, St. Louis, MO), and resolved in 10 µl RNase-free water. For in vitro uridylation assay, 1 µg in vitro transcribed RNA was incubated with wild-type or enzyme-dead TUT7 immuno-nucleoprecipitated from HEK293T cells transfected with TUT7 expression plasmid and α-<sup>32</sup>P-UTP for 1 h at 37 °C. RNA samples were separated on a 1% formaldehyde-agarose gel electrophoresis and visualized by SYBR Green II staining and radiography.

**RNA immunoprecipitation.** Cells were harvested and lysed in APB lysis buffer containing protease inhibitors and RNase inhibitors. 5% of cell lysates were kept as inputs for the immunoprecipitation. The remaining beads and the inputs were resuspended in 200 µl nuclear fractionation buffer B (5 mM HEPES, 1.5 mM KCl, 10 mM MgCl₂, 300 mM NaCl, 0.05% NP-40, 0.5% Nonidet P-40, 0.5% Triton-X 100, 0.1% SDS, 20% glycerol, 0.05% bromophenol blue) and separated on a 6% native PAGE, stained with bromophenol blue, and scanned (GE Healthcare, Little Chalfont, UK). The exact values are shown in the figures if p < 0.05, which is considered statistically significant.

**Data availability** RNA-Seq and Tail-Seq data have been deposited into NCBI GEO under the accession number GSE136161 and GSE146259, respectively. The datasets of LPS-induced BMDMs and RAW 264.7 cells were obtained from GEO with the accession number GDS5623. The data that support the findings of this study are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions
C.-C.L., Y.-R.S., and L.-C.H., designed the research. C.-C.L. and Y.-R.S. performed the experiments. I.-S.Y. generated the Tut7−/− mice. C.-C.L., Y.-R.S., L.-C.H., and H.-Y.T. analyzed the data. S.-Y.G., C.-C.C., Y.-Y.Y., T.-Y.L., and C.-Y.L. assisted with some experiments. C.-H.C. generated TUT7 antibody. C.-C.L. and L.-C.H. wrote the paper. All authors discussed the results and approved the paper.

Competing interests
The authors declare no competing interests.

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