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Toll IL-1 Receptors Differ in Their Ability to Promote the Stabilization of Adenosine and Uridine-Rich Elements Containing mRNA

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Several ligands for Toll IL-1R (TIR) family are known to promote stabilization of a subset of short-lived mRNAs containing AU-rich elements (AREs) in their 3’ untranslated regions. It is now evident however, that members of the TIR family may use distinct intracellular signaling pathways to achieve a spectrum of biological end points. Using human embryonic kidney 293 cells transfected to express different TIRs we now report that signals initiated through IL-1R1 or TLR4 but not TLR3 can promote the stabilization of unstable chemokine mRNAs. Similar results were obtained when signaling from endogenous receptors was examined using a mouse endothelial cell line (H5V). The ability of TIR family members to stabilize ARE-containing mRNAs results from their differential use of signaling adaptors MyD88, MyD88 adaptor-like protein, Toll receptor IFN-inducing factor (Trif), and Trif-related adaptor molecule. Overexpression of MyD88 or MyD88 adaptor-like protein was able to promote enhanced stability of ARE-containing mRNA, whereas Trif and Trif-related adaptor molecule exhibited markedly reduced capacity. Hence the ability of TIRs to signal stabilization of mRNA appears to be linked to the MyD88-dependent signaling pathway.

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3 Abbreviations used in this paper: TIR, Toll IL-1R; ARE, adenosine and uridine-rich element; UTR, untranslated region; Mal, MyD88 adaptor-like protein; Trif, Toll receptor IFN-inducing factor; Tram, Trif-related adaptor molecule; AciD, actinomycin D; Dox, doxycycline; IRAK, IL-1R-associated kinase; HA, hemagglutinin; TRE, tetracycline responsive sequence element.

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FBS was purchased from BioWhittaker (Walkersville, MD). Formamidase was obtained from International Biotechnologies (New Haven, CT). Magna nylon transfer membrane was obtained from Micron Separations (Westboro, MA). Recombinant human IL-1α and the KC ELISA kit were purchased from R&D Systems (Minneapolis, MN), and poly(I:C), LPS, gentamicin sulfate (G418), and actinomycin D (ActD) were purchased from Sigma-Aldrich (St. Louis, MO). Doxycycline (Dox) was obtained from Clontech Laboratories (Palo Alto, CA). Superfect Transfection Reagent was obtained from Qiagen (Valencia, CA) and Tri-Reagent was purchased from Molecular Research Center (Cincinnati, OH). DuPont-NEN (Boston, MA) was the source of [α-32P]dCTP. The Luciferase Assay System was purchased from Promega (Madison, WI). Protein assay reagents were purchased from Bio-Rad (Richmond, CA).

Cell culture

Human embryonic kidney (HEK) 293 C6 cells expressing elevated IL-1R1 were prepared as previously described (29) and maintained in DMEM supplemented with 10% FBS, penicillin, and streptomycin in humidified 5% CO2. The 293 tet-off cells were prepared by stable transfection of the parental HEK 293 C6 line with a plasmid encoding the bacterial tet-repressor (tetR) protein fused with the VP-16 transactivation domain obtained from Clontech Laboratories and were maintained in G418. The HEK 293 C6 cell line was stably transfected with expression constructs (described below) encoding TLR3 or TLR4 and MD2 and selected on the basis of antibiotic resistance. An immortalized mouse endothelial cell line (H5V) was maintained as previously described (30).

Plasmids

Plasmids encoding KC, IL-8, GRO3, and GAPDH were as previously described (31, 32). A CDNA clone encoding ISG56 was obtained from Dr. G. Sen (Cleveland Clinic Foundation) (33). pTRE2 and pTRE2-luciferase vectors were purchased from Clontech Laboratories. pTRE2-KCdcDNA was constructed as previously described (29). Luciferase reporter plasmids containing the promoter from the ISG56 gene (ISG56-luciferase) or five copies of the kB site from the mouse IFN-γ-inducible protein 10 promoter were as previously described (33, 34). Plasmids encoding TLR4, TLR3, MD2, MyD88, Mal, Trif, and Tram were prepared by amplification of full-length coding sequences by RT-PCR using total RNA prepared from HEK 293 C6 cells or HeLa cells and inserted in the EcoRI site of the expression plasmid pcDNA3.1. Expression constructs were designed to include epitope tags (FLAG or hemagglutinin (HA)) at the carboxyl terminus of the proteins. The cloned cDNAs were verified by sequencing in the Molecular Biotechnology Core facility of the Cleveland Clinic Foundation.

Cell transfection and RNA and luciferase analysis

Pools of 293 tet-off cells were transiently transfected using Superfect Transfection Reagent according to the manufacturer’s protocol. Three hours after transfection, the cultures were subdivided into 60-mm dishes and rested for 24 h before individual treatments. Total RNA was prepared using Tri-Reagent following the manufacturer’s protocol. Three pools of 293 tet-off cells were transiently transfected using Superfect Transfection Reagent according to the manufacturer’s instructions. The levels of RNA were analyzed and quantified by Northern blot hybridization as previously described (20, 32). Transfected 293 tet-off cells were used to prepare cell extracts and assess luciferase activity according to the manufacturer’s protocol.

KC protein analysis to estimate mRNA decay

Pools of 293 tet-off cells were transiently transfected as previously described (29). Twenty-four hours after transfection, the supernatants were removed and replaced with fresh medium. After 3 h, the supernatant was harvested and used to determine the amount of KC protein expression during 3 h before selective termination of reporter gene transcription. The plates were washed and fresh medium containing Dox with or without IL-1α was added and the cultures incubated for 3 h to allow mRNA decay to occur in the absence of transcription. The supernatants were discarded and cultures were washed extensively before addition of fresh medium containing Dox with or without IL-1α as indicated. After a final 3 h incubation, KC protein levels were determined in the supernatant by ELISA, reflecting the KC protein produced from mRNA remaining after 3 h of decay. The data are presented as the percentage of KC protein produced after Dox-induced transcription termination relative to that produced by the same culture before addition of Dox.

Western blot analysis

Western blot analysis was done essentially as previously described using 10% denaturing PAGE of total cytosolic extracts and ECL detection technology (35).

Results

The IL-1R1, TLR3, and TLR4 members of the TIR family represent the prototypes for use of different TIR adaptor molecules; IL-1R requires only MyD88, TLR3 requires only Trif, and TLR4 requires MyD88, Mal, Trif, and Tram (22, 23, 27, 28, 36). Using the 293 cell line as parent, we prepared a series of cell lines stably expressing IL-1R1 along with either TLR3 or TLR4 to determine the relative capacity of each receptor to couple with processes promoting stabilization of ARE-containing mRNAs. The parental line 293 C6 has been stably transfected to express high levels of the IL-1R1 protein (29). These cells show good induction of the chemokine gene IL-8 in response to stimulation with human IL-1α but are unresponsive to either LPS (for TLR4) or poly(I:C) (for TLR3) (Fig. 1). When cells expressing TLR4/MD2 were stimulated with LPS, IL-8 mRNA was readily induced although these cells remained unresponsive to poly(I:C). HEK 293 C6 cells transfected with TLR3 were unresponsive to LPS but were highly sensitive to the action of poly(I:C) as indicated by the expression of the human ISG56 mRNA, a product known to be sensitive to dsRNA and both type I and type II IFNs (33). Interestingly, poly(I:C) treatment of 293 cells expressing TLR3 resulted in only modest chemokine mRNA expression. This finding suggested that signaling from TLR3 might be unable to promote stabilization of these chemokine mRNAs.

To test this possibility, the three cell lines (293, TLR4/MD2, and TLR3) were stimulated with their corresponding ligands for 2 h and ActD was added to inhibit further mRNA synthesis. Total RNA was prepared immediately or following additional incubation for 2 or 4 h with ActD and the levels of IL-8 mRNA were again determined (Fig. 2). In response to IL-1α or LPS, IL-8 mRNA was stable over the entire incubation period. In contrast, the modest levels of IL-8 mRNA induced in response to poly(I:C) disappeared rapidly indicating that poly(I:C) acting through TLR3 was unable to promote the stability of this chemokine mRNA.

FIGURE 1. IL-8 expression induced by distinct TLR/ligand pairs. Parental HEK 293 C6 cells expressing high levels of IL-1R1 were stably transfected with expression plasmids encoding either TLR4/MD2 or TLR3. The resulting cell lines (293, 293/TLR3, and 293/TLR4/MD2) were untreated or stimulated with IL-1α (10 ng/ml), LPS (100 ng/ml), or poly(I:C) (25 μg/ml) for 2 h before preparation of total RNA and analysis of IL-8, ISG56, and GAPDH mRNA levels by Northern hybridization and autoradiography. Similar results were obtained in three separate experiments.
To determine whether the behavior of TLR signaling in 293 cells reflects the activity of these receptors when expressed from endogenous genes, we examined the response of a mouse endothelial cell line (HSV) to stimulation with IL-1α, LPS, or poly(IC) for 2 h before addition of ActD. Individual cultures were harvested at the indicated times and used to determine expression levels for CXCL1 or KC mRNA (homologue of human GROα) by Northern hybridization (Fig. 3A). Although both IL-1α and LPS could induce KC mRNA expression with prolonged expression, poly(IC) was a weak inducer, and in the presence of ActD, the message decayed rapidly. To examine the effects of each stimulus on KC mRNA stability independently of their role in transcription, we exploited the prior finding that TNF-α treatment can induce KC mRNA expression but cannot stabilize the message (31). HSV cells were stimulated with TNF-α for 2 h followed by the addition of ActD alone or in the presence of either IL-1α, LPS, or poly(IC) for additional times (Fig. 3B). As reported previously, TNF-α could stimulate KC mRNA expression but in the presence of ActD, it decayed rapidly, whereas IL-1α treatment prolonged the half-life considerably. Consistent with the findings previously presented, poly(IC) did not stabilize KC mRNA. Interestingly, although LPS was capable of stabilizing KC when also used as the inducer (Fig. 3A), it was relatively inactive when added in the presence of ActD (Fig. 3B).

These studies suggest that IL-1α and LPS, acting through their corresponding TIRs, can modulate the stability of ARE-containing mRNAs. However, because these stimuli are also necessary to promote the transcriptional induction of IL-8 or KC mRNAs, we cannot assess message decay in their absence. To more directly test the ability of these receptors to modulate mRNA stability, we used an experimental system in which transcription of an ARE-containing reporter mRNA is regulated by a tetracycline responsive promoter. The 293 cells stably expressing the tetR-VP-16 fusion protein (293 tet-off) were transiently transfected with a plasmid containing the full-length mouse CXCL1 (KC) cDNA downstream of a TRE. The KC gene is a mouse chemokine gene closely related to IL-8 that also exhibits both instability and stabilization in response to IL-1α and LPS (20, 31). Following addition of the tetracycline analog Dox this unstable ARE-containing chemokine mRNA decays with a half-life of between 60 and 90 min (Fig. 4, A and B). Treatment of the cells with IL-1α at the same time as Dox results in a significant increase in the half-life of KC mRNA (to 150 to 180 min), mRNA stability in this system can also be measured by comparing the amount of KC protein secreted from transfected cells before and after the addition of Dox (Fig. 4C). IL-1α stimulation resulted in a 2-fold increase in the amount of KC protein secreted from cells after termination of transcription by the addition of Dox.

Transient over-expression of TLR4 along with MD2 is known to mimic the effects of LPS stimulation resulting in the activation of NFκB and other signaling events (37). When 293 tet-off cells were cotransfected with the TRE regulated KC cDNA and plasmids encoding the genes for TLR4 and MD2, KC mRNA stability was strongly enhanced as indicated by increased KC mRNA half-life and protein secretion (Fig. 4, A–C). Consistent with the findings presented in Fig. 2, over-expression of TLR3 was not able to enhance mRNA stability. This did not reflect an inability of overexpressed TLR3 to stimulate cellular response as demonstrated by induction of luciferase activity controlled by the ISG56 promoter (Fig. 4D). Although both IL-1α stimulation and over-expression of TLR4/MD2 could activate strong NFκB-driven luciferase expression, over-expression of TLR3 was able to promote only modest NFκB activation (Fig. 4E). The ability of IL-1α or over-expression of TLR4/MD2 to promote enhanced stability of KC mRNA was dependent upon the 3′UTR of KC mRNA because constructs in which the 3′UTR of the rabbit β-globin gene was substituted for the KC 3′UTR were highly stable and exhibited no sensitivity to stimulation (data not shown).
FIGURE 4. IL-1α and TLR4/MD2 stabilize KC mRNA in 293 tet-off cells. A. A pool of 293 cells stably expressing the tetR-VP-16 fusion protein (293 tet-off cells) were transiently transfected with pTRE2/KCcDNA and empty vector (pcDNA3) or pcDNA encoding TLR3 or TLR4/MD2 (total plasmid concentration was 2 μg/10⁶ cells). After 3 h each pool was subdivided into four separate culture dishes. Following a 24 h rest, individual cultures were treated or not with Dox (100 ng/ml) with or without IL-1α (10 ng/ml) as indicated. Following the indicated incubation times, total RNA was prepared and used to determine levels of KC and GAPDH mRNAs by Northern hybridization. B. Supernatants were used to measure residual KC protein production following addition of Dox as outlined in Materials and Methods. C. The 293 tet-off cells were transfected as described in A and supernatants were used to measure residual KC protein production following addition of Dox as outlined in Materials and Methods. D. Treatment with the MyD88 expression construct could only activate Trif shown strong luciferase expression driven from both NFκB and ISG56 promoter constructs whereas IL-1α treatment or transfection with the MyD88 expression construct could only activate NFκB-dependent transcription (Fig. 5D). Furthermore, Western blot analysis using Abs to epitope tags (FLAG for MyD88, Mal, and Tram and HA for Trif) showed comparable expression for all four constructs (Fig. 5E).

Discussion

TIR family receptors promote the initiation and amplification of inflammatory responses at least in part through induced expression of multiple proinflammatory gene products. Control of these responses has been demonstrated at multiple mechanistic steps including transcription, mRNA decay, and mRNA translation (7–12). Though different members of the TIR family share many features, they appear to use at least two interacting but distinct pathways distinguished by their dependence on the downstream signaling TIR domain containing adaptor protein MyD88 (3, 38). Although the IL-1R1 depends only on MyD88, TLR3-initiated responses are fully MyD88-independent. TLR4-initiated responses exhibit both MyD88-dependent and -independent components. The objectives of the present study were to determine whether these three prototypic TLRs possess comparable capacity to promote the stabilization of ARE-containing mRNAs encoding inflammatory chemokines and further, whether mRNA stabilization could be achieved by MyD88-dependent or -independent signaling pathways. The results clearly establish that the signaling pathways used by these receptors are differentially coupled to this endpoint.
Although stimulation through both IL-1R1 and TLR4/MD2 can promote stabilization in several different experimental settings, TLR3 appears to be entirely inactive. The differential capacity of each receptor reflects their selective use of the MyD88-dependent signaling pathway. Both MyD88 and Mal, used by IL-1R and TLR4, can when over-expressed, effectively promote enhanced stability of the ARE-containing KC mRNA whereas Trif and Tram, associated with the MyD88-independent pathway exhibit much lower efficacy.

TLR3 provides recognition of dsRNA and host defense against viruses that use dsRNA structures in their replication cycle (39, 40). Signaling from TLR3 leads to stimulation of type I IFN expression through the activation of IFN regulatory factor 3, a distinguishing hallmark of the MyD88-independent pathway (24, 26, 41). TLR3 activation is also capable of activating NFκB, though the magnitude and temporal pattern of this response are diminished and delayed, respectively, as compared with NFκB activation through either IL-1R1 or TLR4 (27, 28). This modest activation of NFκB via TLR3 appears to be insufficient to promote the transcription of cytokine and chemokine genes as evidenced by the relatively poor induction of IL-8 or KC mRNA expression demonstrated in the experiments shown in Figs. 1–3. Furthermore, the inability to stabilize these mRNAs will contribute to their very limited accumulation as well. It is noteworthy, however, that poly(IC) is known to be a potent stimulus of cytokine gene expression in multiple cell types (39). This may reflect, in part, the existence of non-TLR3-dependent responses to poly(IC), as evidenced by residual dsRNA sensitivity in mice deficient for the TLR3 gene (40). Little is known, however, regarding the structure and function of such events.

**FIGURE 5.** The adaptor proteins MyD88, Mal, Trif, and Tram differentially couple to mRNA stabilization. A, The 293 tet-off cells were transiently cotransfected with pTRE2/KCcDNA and pcDNA3 or pcDNA3 containing cDNAs for MyD88, Mal, Trif, or Tram as indicated. After 3 h each pool of cells was subdivided into separate culture dishes and allowed to rest for 24 h. Individual cultures were treated with Dox for the indicated times before preparation of total RNA and determination of KC and GAPDH mRNA levels by Northern hybridization. B, The autoradiograph (A) was quantified using the NIH Image software package and plotted as a percentage of remaining RNA vs time after Dox treatment. C, C6 293 tet-off cells were transiently transfected with pTRE2/KCcDNA and the indicated plasmid concentrations of pcDNA3 containing MyD88, Mal, Trif, or Tram. Total plasmid DNA was maintained at a level of 4 μg/10^6 cells with empty vector. Residual KC protein production following addition of Dox was measured as described in Fig. 4. D, The 293 tet-off cells were transiently cotransfected with pcDNA3 or pcDNA3 encoding MyD88 or Trif and either the 5 copies xβ-luciferase or ISG56 promoter-luciferase reporter. The pcDNA3 transfected cells were treated with IL-1α for 6 h. Cell extracts were prepared and used to assess luciferase activity. E, C6 293 tet-off cells were transfected with 2.5 μg of plasmids encoding MyD88, Mal, Trif, or Tram as previously described. After 24 h, cell lysates were prepared and used to measure protein levels by Western blot with Ab to FLAG (MyD88, Mal, and Tram) or to HA (Trif). Similar results were obtained in three separate experiments.
are both potent stimuli of enhanced RNA stability whereas Trif and Tram do not efficiently modulate mRNA decay. Though Trif and Tram also exhibit some capacity to stimulate this pathway when expressed at high levels, the quantitative difference between MyD88 or Mal and Trif or Tram is between 10- and 100-fold (2.5 μg of Trif plasmid is required to promote stabilization comparable to that seen with 0.025 μg of MyD88 plasmid). Thus although Trif and Tram exhibit potential to couple with the process of mRNA stabilization, their activation through ligand/receptor stimulation is apparently unable to achieve the required level under physiologic circumstances.

The signaling events downstream from the IL-1R1 leading to activation of NFkB are understood in some detail (29, 42–48). MyD88 assembles a complex composed of IL-1R-associated kinase (IRAK), IRAK4, and TRAF6 at the receptor. Following dissociation from the receptor, this complex relocates to the plasma membrane and recruits TAB1, TAB2 and TAK1. This complex then dissociates from the membrane and TAK1 is activated in the cytosol via a process that appears to involve ubiquitin ligase activation and phosphorylation of TAK1. Activated TAK1 is believed to be responsible for the activation of the 1xkB kinase complex and the subsequent phosphorylation, ubiquitination, and degradation of 1xBs with the attendant release and nuclear translocation of free NFkB. TAK1 is also linked with activation of downstream stress-activated kinases including p38 via intermediate activation of MAPK kinases MKK6 or MKK3 and these have been reported to couple with the stabilization of chemokine and cytokine mRNAs (49, 50). The linkage of the TIR family receptors to mRNA stability appears to involve this MyD88-dependent pathway. TLR3, acting through Trif and TRAF6 also results in the activation of TAK1 via assembly of a cytoplasmic complex that does not depend upon MyD88 or IRAK (51) (X. Li, unpublished observation). This complex can activate NFkB but results in very modest cytokine expression and this may reflect, at least in part, its relative inefficiency to couple with the mRNA stabilization pathway. In this regard, although multiple ligand receptor pairs are known to activate one or more of the MAPK modules including p38, not all couple effectively with the stabilization of ARE-containing mRNAs. For example, TNF- 

References

TLR3 DIFFERENTIALLY STABILIZE CHEMOKINE mRNA