WDFY1 mediates TLR3/4 signaling by recruiting TRIF

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Abstract

Toll-like receptors (TLRs) are pattern recognition receptors that sense a variety of pathogens, initiate innate immune responses, and direct adaptive immunity. All TLRs except TLR3 recruit the adaptor MyD88 to ultimately elicit inflammatory gene expression, whereas TLR3 and internalized TLR4 use TIR-domain-containing adaptor TRIF for the induction of type I interferon and inflammatory cytokines. Here, we identify the WD repeat and FYVE-domain-containing protein WDFY1 as a crucial adaptor protein in the TLR3/4 signaling pathway. Overexpression of WDFY1 potentiates TLR3- and TLR4–mediated activation of NF-κB, interferon regulatory factor 3 (IRF3), and production of type I interferons and inflammatory cytokines. WDFY1 depletion has the opposite effect. WDFY1 interacts with TLR3 and TLR4 and mediates the recruitment of TRIF to these receptors. Our findings suggest a crucial role for WDFY1 in bridging the TLR–TRIF interaction, which is necessary for TLR signaling.

Keywords signal transduction; TLR; TRIF; WDFY1
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Introduction

The vital roles of Toll-like receptors (TLRs) have been demonstrated in both innate and adaptive immune responses [1]. All 13 TLRs identified so far in humans and mice contain an extracellular leucine-rich repeats (LRR) domain, which recognizes a distinct set of pathogen-associated molecular patterns (PAMPs), and an intracellular signaling TIR domain, which is conserved among Toll and interleukin-1 receptors [2,3]. Ligand binding to a TLR leads to the recruitment of TIR-domain-containing adaptor proteins, such as myeloid differentiation primary-response gene 88 (MyD88) and/or TIR-domain-containing adapter-inducing interferon-β (TRIF). The MyD88-dependent or TRIF-dependent signaling cascade activates several transcription factors leading to the induction of proinflammatory cytokines and type I interferons (IFNs) [3–5].

All TLRs besides TLR3 depend, at least in part, upon MyD88 signaling [6]. MyD88 contains an N-terminal death domain, which interacts with IL-1R-associated kinase-4 (IRAK4). IRAK4 phosphorylates IRAK1 and IRAK2 leading to the activation of TNF receptor-associated factor 6 (TRAF6) [7]. TRAF6, as an E3 ubiquitin ligase, activates the inhibitor of kappa B (IkB) kinase (IKK) complex resulting in the activation of transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [8,9]. The interferon regulatory transcription factor (IRF) family is also activated. IRF7 is activated by IRAK1 as IRF1 associates with MyD88 directly, and IRF5 associates with both MyD88 and TRAF6 [10–12].

Engagement of TLR3 by double-stranded RNA (dsRNA) causes dimerization and tyrosine phosphorylation of TLR3, which then recruits TRIF [13]. TRIF contains multiple conserved domains that are responsible for further association with downstream molecules receptor-interacting protein 1 (RIP1), TRAF6, and TANK-binding kinase 1 (TBK1). RIP1 and TRAF6 participate in TRIF-mediated NF-κB activation, whereas TBK1 directly phosphorylates transcription factor IRF3, which subsequently undergoes dimerization and translocates to the nucleus [14–16]. The activation of NF-κB and IRFs induces the production of a series of proinflammatory cytokines and IFNs, the latter of which further induces the transcription of a wide range of downstream antiviral genes and subsequent antiviral responses [17,18].

Engagement of TLR4 by lipopolysaccharide (LPS) triggers both MyD88-dependent and TRIF-dependent signaling. TLR4 recognizes ligands at the cell surface resulting in recruitment and signaling through MyD88. Subsequently, TLR4 is internalized into early endosomes and triggers TRIF-dependent signaling [19,20].

Although much progress has been made in understanding TLR signaling, mechanistic details regarding TLR signaling molecules still need to be elucidated. By screening the possible molecules that are involved in TLR3 signaling, we identified a WD repeat and FYVE-domain-containing 1 protein (WDFY1). It has been previously reported that WDFY1 is localized on the membrane of endosomes by the FYVE domain, but its function is still obscure [21]. In this study, we found WDFY1 potentiates poly(I:C)- or LPS-triggered activation of IRF3 and NF-κB. Knocking down WDFY1 had the opposite
effect. We demonstrate that WDFY1 acts as a critical adaptor protein during TLR3/TLR4 recruitment of TRIF.

Results and Discussion

Identification of WDFY1 as a positive regulator of TLR3 signaling

To identify potential molecules involved in TLR3 signaling, we screened ~10,000 independent human and mouse cDNA expression plasmids by reporter assays in 293-TLR3 cells. These efforts led to the identification of human WDFY1 (clone no. 19G12) (Supplementary Fig S1). WDFY1, containing a single FYVE domain and seven WD-40 repeats, was reported to be localized to early endosomes [21]. Overexpression of WDFY1 markedly potentiated poly(I:C)-induced activation of the IFN-β promoter, ISRE, and NF-κB in a dose-dependent manner in 293-TLR3 cells (Fig 1A). In contrast, WDFY1 had no obvious effects on Sendai virus (SeV)-induced activation of the IFN-β promoter, ISRE, and NF-κB, indicating that WDFY1 selectively functions in virus-sensing TLR3, but not in the RIG-I-like receptor (RLR) pathway (Fig 1B). Furthermore, overexpression of WDFY1 markedly potentiated poly(I:C)-induced transcription of endogenous IFNB1, RANTES, ISG56, TNFA, and CXCL10 genes in 293-TLR3 cells (Fig 1C).

We next examined the role of endogenous WDFY1 in TLR3-mediated signaling. We constructed three RNAi plasmids for WDFY1. Both #2 and #3 RNAi plasmids could efficiently inhibit, to varying degrees, the expression of transfected WDFY1 in 293 cells (Fig 1D). In reporter assays, knockdown of WDFY1 markedly inhibited poly(I:C)-induced, but not SeV-induced, activation of the IFN-β promoter, ISRE, and NF-κB (Fig 1E and Supplementary Fig S2A). Knockdown of WDFY1 inhibited poly(I:C)-induced transcription of endogenous IFNB1, RANTES, TNFA, and IL8 genes in 293-TLR3 cells (Supplementary Fig S2B and C). We also got consistent results in mouse bone marrow-derived macrophages (BMDMs) (Fig 1F). These data suggested that WDFY1 is required for TLR3-mediated antiviral signaling pathways.

Wdfy1 is specifically involved in the Tlr3/4 signaling pathway

Human WDFY1 and its mouse ortholog share about 97% identity at the amino acid level. We next examined the role of endogenous Wdfy1 in other endosome-associated Tlr signaling pathways in mouse RAW264.7 cells. Two RNAi plasmids for mouse Wdfy1 were constructed and verified to be effective (Supplementary Fig S3A and B). Knockdown of Wdfy1 by either #1 or #2 RNAi plasmids inhibited poly(I:C)- and LPS-induced, but not R837- or CpG-induced, transcription of Tnf, Il1b, and Il6 genes (Supplementary Fig S3C-F), suggesting that Wdfy1 participates in Trif-mediated Tlr3 and Tlr4 signaling pathways, but not MyD88-mediated Tlr7, Tlr8, and Tlr9 signaling pathways.

Molecular position of WDFY1 in TLR3 signaling

Upon poly(I:C) stimulation, TLR3 undergoes tyrosine phosphorylation and recruits TRIF, which eventually leads to the activation of transcription factor IRF3 and NF-κB [13]. Overexpression of WDFY1 enhanced poly(I:C)-induced dimerization of IRF3 and phosphorylation of IRF3 and IkBα (Fig 2A and C). WDFY1-knockdown 293-TLR3 cells exhibited a marked reduction in these events (Fig 2B and D). These data further demonstrate that WDFY1 potentiates TLR3 signaling. Moreover, overexpression of WDFY1 enhanced the phosphorylation of TBK1, suggesting that WDFY1 acts upstream of the TBK1 kinase. To determine the molecular position of WDFY1 in TLR3 signaling, we examined the effects of reduced expression of WDFY1 on activation of ISRE or NF-κB by overexpressing various signaling molecules. We selected WDFY1-RNAi-#2 and Wdfy1-RNAi-#1 plasmids for additional experiments described below. The results demonstrated that reduced expression of WDFY1 inhibited TLR3-triggered activation of ISRE and NF-κB, but hardly affected TRIF-, TBK1-, or IRF3-triggered activation of ISRE and TRIF-, TRAF6-, TAK1-, or IKKβ-triggered activation of NF-κB (Fig 2E). Moreover, WDFY1 dose-dependently potentiated TLR3-triggered, but not TRIF-triggered, activation of ISRE and NF-κB (Fig 2F and G). These results indicate that WDFY1 functions at the level of TLR3.

WDFY1 positively regulates TLR3 signaling by mediating the recruitment of TRIF to TLR3

We next investigated whether WDFY1 colocalized and interacted with TLR3. Expression of WDFY1 was localized in early endosomes as reported previously (Fig 3A) [21]. The expression of TLR3 or TRIF partially colocalized with WDFY1, suggesting that these three proteins are spatially related (Fig 3A). Then, we conducted cell fractionation as previously reported [22]. The separated components were further applied to co-immunoprecipitation by anti-TLR3 antibodies. TLR3 and WDFY1 were detected only in the membrane part (P100K), whereas TRIF was found in both cytosol and membrane parts (S100K and P100K). TLR3 had no marked interaction with either WDFY1 or TRIF under physiological condition, but both WDFY1 and TRIF were recruited to TLR3 upon the stimulation of poly(I:C) (Fig 3B and Supplementary Fig S4A). Endogenous poly(I:C)-induced interaction of Wdfy1 and Tlr3 was also confirmed in mouse RAW264.7 cells (Fig 3C). Furthermore, WDFY1 knockdown had no marked effect on the membrane localization of TLR3 in the absence or presence of stimulation (Supplementary Fig S4B).

Since TRIF is indispensable for TLR3 signaling [23], we wondered whether WDFY1 mediated the TLR3–TRIF interaction. Compared to controls, knockdown of WDFY1 reduced the recruitment of TRIF to TLR3 upon the stimulation of poly(I:C) (Fig 3D). Similar results were observed in RAW264.7 cells (Supplementary Fig S4C). Consistently, reduced expression of WDFY1 inhibited the recruitment of downstream RIP1, TRAF6, and TBK1 to TRIF upon poly(I:C) stimulation (Fig 3E). Because tyrosine phosphorylation of TLR3 is important for its activation, we next determined the internal connection between WDFY1–TLR3 interaction and tyrosine phosphorylation of TLR3. The results showed that reduced WDFY1 expression did not affect the tyrosine phosphorylation of TLR3 led by poly(I:C) stimulation (Fig 3F), suggesting that WDFY1–TLR3 interaction occurs after tyrosine phosphorylation of TLR3. We next mutated two tyrosine phosphorylation sites of TLR3, Y759, and Y858, which are necessary for TLR3 signaling [13]. By co-immunoprecipitation, we found that WDFY1 interacted with wild-type TLR3, but not any of the tyrosine mutants (Fig 3G), suggesting that the WDFY1–TLR3 interaction is critically dependent.
Figure 1. WDFY1 regulates TLR3-mediated signaling pathways.

A, B 293-TLR3 cells (A) or 293 cells (B) were transfected with the indicated luciferase reporter plasmids and increasing amounts of WDFY1 plasmid. Twenty hours after transfection, cells were treated with poly(I:C) (50 μg/ml), or SeV, or left untreated, for 8 h before luciferase reporter assays were performed. Graphs show mean ± SD, n = 3.

C 293-TLR3 cells were transfected and treated with poly(I:C) (50 μg/ml) for 3 h before real-time PCR was performed. Graphs show mean ± SD, n = 3. *P < 0.05; **P < 0.01.

D 293 cells were transfected with Flag-tagged WDFY1, MPP5, and WDFY1-RNAi plasmids. The expression levels of WDFY1 were detected by immunoblot analysis using the indicated antibodies.

E 293-TLR3 cells were transfected with the indicated luciferase reporter and RNAi plasmids. Twenty hours after transfection, cells were treated with poly(I:C) (50 μg/ml), or left untreated, for 8 h before luciferase assays were performed. Graphs show mean ± SD, n = 3. *P < 0.05; **P < 0.01.

F Mouse BMDMs were transfected with siRNAs to Wdfy1 or control siRNA. Cells were treated with poly(I:C) (50 μg/ml), or left untreated, for 3 h before real-time PCR was performed. Graphs show mean ± SD, n = 3. **P < 0.01.

Source data are available online for this figure.
on tyrosine phosphorylation of TLR3. The above data suggested that WDFY1 is associated with tyrosine-phosphorylated TLR3 in a ligand binding-dependent manner and mediates the recruitment of TRIF to TLR3.

**FYVE domain of WDFY1 is required for TLR3 signaling**

To examine the minimal regions of TLR3 and WDFY1 responsible for their interaction, we constructed a series of truncation plasmids...
Figure 3. WDFY1 interacts with TLR3 in a ligand-dependent manner.

A 293 cells were transiently transfected with the indicated plasmids and observed by confocal microscopy.

B 293-TLR3 cells were treated with poly(I:C) (50 µg/ml) for the indicated times and then applied to cell fractionation. Separated cell fractions were applied to co-immunoprecipitation and then analyzed by immunoblotting with the indicated antibodies. The experiments were repeated three times with similar results.

C RAW264.7 cells were treated with poly(I:C) (50 µg/ml) for the indicated times. The cell lysates were immunoprecipitated with anti-Wdfy1 and analyzed by immunoblotting. The experiments were repeated three times with similar results.

D–F WDFY1-knockdown or control 293-TLR3 cells were treated with poly(I:C) (50 µg/ml). The cell lysates were immunoprecipitated with anti-TLR3 (D, F) or anti-TRIF (E) and analyzed by immunoblotting. The experiments were repeated three times with similar results.

G 293 cells were transiently transfected with HA-WDFY2, Flag-TLR3, and TLR3 mutants. The cell lysates were immunoprecipitated with anti-Flag and analyzed by immunoblotting. The experiments were repeated three times with similar results.

Source data are available online for this figure.
of TLR3 and WDFY1 to perform co-immunoprecipitation assays. The results showed that WDFY1 was associated with the cytosolic TIR domain of TLR3 and the second WD repeat domain of WDFY1 was required for its binding to TLR3 (Fig 4A and B and Supplementary Fig S4D and E).

The FYVE domain is responsible for WDFY1 localization to early endosomes [21]. We next determined whether the FYVE domain is required for the function of WDFY1 in TLR3 signaling. Overexpression of the ΔFYVE mutant construct, which is a FYVE domain-deleted WDFY1 mutant, had no marked effect on poly(I:C)-induced activation of the IFN-β promoter, ISRE, and NF-κB, compared to its wild-type counterpart (Fig 4C). Consistently, ΔFYVE had minimal effects on TLR3-mediated transcription of endogenous IFNBI, ISG15, and IL8 genes (Fig 4D). Moreover, an RNAi off-target WDFY1 mutant (WDFY1-M), with three nucleotide nonsense mutations in the target sequence of the #2 WDFY1-RNAi plasmid, rescued the #2 WDFY1-RNAi-mediated inhibition of poly(I:C)-induced phosphorylation of IRF3 and IκBα, but the RNAi off-target ΔFYVE mutant (ΔFYVE-M) had no marked effects (Fig 4E). Furthermore, WDFY1-M, but not ΔFYVE-M, could restore the inhibition of TLR3–TRIF interaction caused by WDFY1 knockdown (Fig 4F). These data strongly suggest that the FYVE domain of WDFY1 is required for TLR3 signaling.

Figure 4. The FYVE domain of WDFY1 is required for TLR3 signaling.

A, B Mapping of the minimal interaction domains between WDFY1 and TLR3.
C, D 293-TLR3 cells were transfected with the indicated plasmids prior to treatment with poly(I:C) (50 μg/ml). Luciferase assays (C) or real-time PCR (D) was then performed. Graphs show mean ± SD, n = 3. *P < 0.05; **P < 0.01.
E WDFY1-knockdown 293-TLR3 cells were transfected with RNAi-resistant mutant WDFY1-M or ΔFYVE-M prior to treatment with poly(I:C) (50 μg/ml). Cell lysates were applied to immunoblot analysis using the indicated antibodies. The experiments were repeated three times with similar results.
F WDFY1-knockdown 293-TLR3 cells were transfected with the indicated plasmids. The cell lysates were immunoprecipitated with anti-Flag and analyzed by immunoblotting. The experiments were repeated three times with similar results.

Source data are available online for this figure.
WDFY1 is required for TLR4 signaling

Because reduced expression of Wdfy1 by RNAi also inhibited LPS-induced expression of downstream genes (Supplementary Fig S3D), we wondered whether WDFY1 functions in TLR4 signaling by the same mechanism as in TLR3 signaling. As shown in Fig 5A, overexpression of mouse Wdfy1 markedly potentiated the LPS-induced activation of the IFN-β promoter, ISRE, and NF-κB in a dose-dependent manner in RAW264.7 cells. Similarly, Wdfy1 had no marked interaction with Tlr4 unless the cells were stimulated by A

Figure 5. WDFY1 regulates the TLR4-mediated signaling pathway.
A RAW264.7 cells were transfected with the indicated plasmids and treated with LPS (0.5 µg/ml). Luciferase reporter assays were then performed. Graphs show mean ± SD, n = 3.
B LPS-treated RAW264.7 cells were immunoprecipitated with anti-Tlr4 and analyzed by immunoblotting. The experiments were repeated for three times with similar results.
C WDFY1-knockdown RAW264.7 cells were treated with LPS (0.5 µg/ml). Cell lysates were immunoprecipitated with anti-Tlr4 and analyzed by immunoblotting. The experiments were repeated three times with similar results.
D, E 293 cells were transient transfected with the indicated plasmids. Cell lysates were immunoprecipitated with anti-Flag and analyzed by immunoblotting. The experiments were repeated three times with similar results.

Source data are available online for this figure.
LPS (Fig 5B). Knockdown of Wdfy1 by RNAi largely inhibited the recruitment of Trif to Tlr4 upon LPS stimulation (Fig 5C). These results suggest that WDFY1 is also required for the recruitment of TRIF to TLR4 upon LPS stimulation. It is known that TRIF-related adaptor molecule (TRAM) is needed for TLR4–TRIF interaction. To investigate whether the function of WDFY1 is overlaid or complementary with that of TRAM, we conducted co-immunoprecipitation assays. The results showed that WDFY1 knockdown had no marked effect on TLR4–TRAM or TRAM–TRIF interaction, but significantly reduced the recruitment of TRIF to TLR4 (Fig 5D and E), suggesting that WDFY1 is a new adaptor protein mediating the recruitment of TRIF to TLR4 other than TRAM.

In this study, we provide strong evidence that demonstrates the essential role of WDFY1 for TRIF-mediated TLR3/4 signaling. Firstly, overexpression and RNAi assays consistently demonstrate that WDFY1 promotes TLR3/4 ligand-induced activation of transcription factors IRF3 and NF-κB, as well as the production of IFN-β and inflammatory cytokines in both human 293-TLR cells and mouse macrophage lines. WDFY1 is not required for either dsRNA-recognition RLR signaling or endosome-associated TLR7-9 signaling, which mechanistically do not require TRIF to signal. Secondly, WDFY1 knockdown reduces overexpressed TLR3-triggered, but not TRIF-, TBK1-, and TRAF6-triggered, activation of IRF3 or NF-κB. Thirdly, WDFY1 knockdown markedly affects the recruitment of TRIF to activated TLR3 or TLR4. Lastly, restoring the expression of WDFY1 in WDFY1-knockdown cells could rescue the TLR3–TRIF interaction.

In summary, this study identifies WDFY1 as a novel critical mediator in TLR3 and TLR4 signaling pathways. WDFY1 bridges the interaction of TLR3/4 with TRIF, which provides new insight into the complicated molecular mechanisms of TLR signaling.

Materials and Methods

More detailed information can be found in Supplementary Materials and Methods.

Constructs

NF-κB, ISRE, and the IFN-β promoter luciferase reporter plasmids, mammalian expression plasmids for HA- or Flag-tagged TLR3, TLR4, TRAM, TRIF, TRAF6, TAK1, TAB 1, IKKβ, IRF3, and TBK1 were previously described [24–28]. Mammalian expression plasmids for Flag-, HA-, GFP-, or Cherry-tagged WDFY1 and its mutants, Cherry-tagged TLR3 and its truncations or point mutants, and Cherry-tagged TRIF were constructed by standard molecular biology techniques. pSuper.retro RNAi plasmid was purchased from Oligoengine.

Expression cloning

The cDNA expression clones encoding 10,000 independent human and mouse cDNAs were obtained from Origene. The clones were transfected into 293-TLR3 cells together with an IFN-β promoter reporter plasmid for 20 h, then treated with or without poly(I:C) for 8 h before luciferase assays were performed.

RNAi experiments

Double-stranded oligonucleotides for RNA interference corresponding to the target sequences were inserted into pSuper.retro RNAi plasmid (Oligoengine) according to protocols recommended by the manufacturer. The target sequences for human WDFY1 cDNA were as follows: #1: 5′-GGGCTGTGATGGAATTTCA-3′; #2: 5′-GACAGATCGTGTTGGC-3′. The target sequences for mouse Wdfy1 cDNA were as follows: #1: 5′-CATCCGAGTATGGCTGAAA-3′; #2: 5′-GGGCTGTGATGGAATTTCA-3′.

RNAi-transduced stable cells

293-TLR3 cells stably expressing WDFY1-RNAi and RAW264.7 cells stably expressing Wdfy1-RNAi were constructed as previously described [29–31].

Transfection and reporter assays

293 or 293-TLR3 cells were transfected by standard calcium phosphate precipitation or retroviral transduction. RAW264.7 cells were transfected by retroviral transduction. Mouse bone marrow-derived macrophages (BMDMs) were transfected by Lipofectamine 2000 (Invitrogen). Luciferase reporter assays were performed with a dual-specific luciferase assay kit (Promega). To normalize the transfection efficiency, 0.01 µg pRL-TK (Renilla luciferase) reporter plasmid was added to each transfection. All reporter assays were repeated at least three times. Data shown were mean values ± SD from one representative experiment.

Subcellular fractionation experiments

293-TLR3 cells were stimulated with poly(I:C), then lysed in a hypotonic buffer (50 mM Hepes, 7.3, 10 mM potassium chloride, 2 mM magnesium chloride, 1 mM dithiothreitol, plus a protease inhibitor cocktail) by subsequent homogenization in a dounce homogenizer. Cell extracts were cleared by centrifugation at 1,000 g to remove the nuclei and intact cells. The supernatant fractions were subjected to subcellular fractionation at 100,000 g to separate the membrane pellets from the cytosol. The different fractions were then lysed and subjected to co-immunoprecipitation and immunoblot analysis.

Statistical analysis

Student’s t-test was performed for all experiments. The level of significance was shown in each figure (*P < 0.05; **P < 0.01).

Supplementary information for this article is available online: http://embor.embopress.org

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Conclusion
The authors declare that they have no conflict of interest.

Reference