TRAF3-interacting JNK-activating modulator promotes inflammation by stimulating translocation of Toll-like receptor 4 to lipid rafts

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Toll-like receptors (TLRs) are key players of the innate immune system and contribute to inflammation and pathogen clearance. Although TLRs have been extensively studied, it remains unclear how exactly bacterial lipopolysaccharide (LPS)-induced conformational changes of the extracellular domain of the TLRs trigger the dimerization of their intracellular domain across the plasma membrane and thereby stimulate downstream signaling. Here, using LPS-stimulated THP-1-derived macrophages and murine macrophages along with immunoblotting and immunofluorescence and quantitative analyses, we report that in response to inflammatory stimuli, the coiled-coil protein TRAF3-interacting JNK-activating modulator (T3JAM) associates with TLR4, promotes its translocation to lipid rafts, and thereby enhances macrophage-mediated inflammation. T3JAM overexpression increased and T3JAM depletion decreased TLR4 signaling through both the MyD88-dependent pathway and TLR4 endocytosis. Importantly, deletion or mutation of T3JAM to disrupt its coiled-coil-mediated homoassociation abrogated TLR4 recruitment to lipid rafts. Consistently, T3JAM depletion in mice dampened TLR4 signaling and alleviated LPS-induced inflammatory damage. Collectively, our findings reveal an additional molecular mechanism by which TLR4 activity is regulated and suggest that T3JAM may function as a molecular clamp to “tighten up” TLR4 and facilitate its translocation to lipid rafts.

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Toll-like receptors (TLRs)³ are critical for sensing inflammatory stimuli, triggering innate immune responses, and priming adaptive immunity (1–3). TLRs are type I transmembrane proteins with an ectodomain containing leucine-rich repeats for ligand recognition, a transmembrane (TM) domain for subcellular localization, and an intracellular Toll–interleukin 1 (IL-1) receptor (TIR) domain for signal relay (4). The regulatory mechanism of TLR activation has been extensively investigated, ranging from co-receptors, post-translational modifications, cleavage, cellular trafficking, and interactions with negative regulators (5, 6). For example, TLR4 is well known to recognize the lipopolysaccharides (LPS) of the Gram-negative bacteria (7, 8). In a typical scenario, activation of TLR4 is preceded by binding of LPS to CD14 protein anchored in cholesterol- and sphingolipid-rich microdomains of the plasma membrane called lipid rafts. CD14 then transfers LPS to the TLR4/MD-2 complex, which dimerizes and triggers the MyD88-dependent downstream signaling and TLR4 endocytosis, leading to production of pro-inflammatory cytokines and type I interferons (5, 9–11). It is well known that dimerization of TLR4 is required to induce intracellular signaling (12–14), and clustering of TLRs to lipid rafts is important for efficient inflammatory responses (10, 15). In the absence of ligand, the TLR4-inhibitory complex (TIC) associates with TLR4 and maintains the receptor in an inactive conformation. LPS binding to MD2 promotes a conformational change that induces TLR4 ectodomain dimerization, leading to dissociation of TIC from TLR4. In the absence of TIC, the TIR domain of TLR4 spontaneously dimerizes and induces adapter recruitment and downstream signaling (16). It has been shown that homoassociation of TLR4 occurs prior to its translocation and clustering to lipid rafts (17). Currently, it remains partially understood regarding how the TLR signaling is transduced across the plasma membrane and particularly how the translocation of TLR4 to the lipid raft is regulated.
T3JAM (also named TRAF3IP3) was initially identified as a TRAF3-interacting protein abundantly expressed in the immune system (18), and lately it has been characterized as an adaptor protein in the striatin-interacting phosphatase and kinase (STRIPAK) complex (19). Similar to sarcolemmal membrane-associated protein, another STRIPAK adaptor protein, T3JAM, mainly consists of coiled-coil domain followed by a C-terminal transmembrane domain. Recent studies showed a critical role of T3JAM in the development of T and B cells (20, 21). Deficiency of T3JAM in mice impaired positive selection and maturation of thymocytes through disrupting TCR-stimulated MEK/ERK signaling in the Golgi. To date, it is not known whether T3JAM plays a role in macrophage, particularly in the TLR-mediated innate immune response.

Here, we identify T3JAM functions as a positive regulator of TLR4 signaling activity in macrophage-mediated inflammation. T3JAM associates with TLR4 via its coiled-coil domain, acting as a molecular clamp to facilitate LPS-induced translocation of TLR4 to lipid rafts. This effect depends on coiled-coil-mediated homoassociation of T3JAM. Deficiency of T3JAM impairs LPS-induced recruitment of TLR4 to lipid rafts, which attenuates TLR4 signaling and protects mice from septic shock–induced inflammatory damage.

Results

T3JAM is up-regulated in response to inflammatory stimuli

To evaluate a potential role for T3JAM in innate immunity, we first assessed the correlation of its expression level with infectious diseases by analyzing clinical data from the Gene Expression Omnibus (GEO) database. We observed significant up-regulation of T3JAM mRNA in the peripheral blood samples of patients with sepsis (GEO data sets GSE54514 (p = 0.0007); Fig. 1A), suggesting that T3JAM is involved in innate immune response.

To verify the above observations, we treated THP-1–derived macrophages and mouse peritoneal exudate macrophages (PEMs) with LPS and analyzed the expression of T3JAM. The protein level of T3JAM was elevated gradually within 10 min, corresponding to the progression of TLR4 signaling (Fig. 1B), and peaked at 1 h after LPS stimulation, with IκBα decreased along the time points and then reduced to base levels at 6 h (Fig. 1C). Next we examined in THP-1–derived macrophages and PEMs the kinetics of T3JAM transcription, as well as the expression of genes encoding inflammatory cytokines, such as Tnfa, Il6, and Il1b upon LPS stimulation. Consistent with a previous study (40), Tnfa was rapidly up-regulated, with its mRNA levels peaked at 1 h after LPS challenge. The increase in Tnfa was concomitant with a rapid increase in T3JAM mRNA levels within 10 min of LPS stimulation (Fig. 1D). Subsequently, T3JAM was substantially up-regulated and peaked at 4 h, and then decreased slowly for the next 20 h (Fig. 1E). Taken together, these results showed that the expression of T3JAM dynamically responds to inflammatory stimulation, indicating a possible role of T3JAM in TLR-mediated LPS signaling.

T3JAM positively regulates TLR4 signaling

To investigate the potential role of T3JAM in TLR signaling and inflammation, we generated T3JAM knockout mice using the CRISPR–Cas9 technique (Fig. S2). Depletion of T3JAM dramatically decreased LPS-induced expression of proinflam-
**T3JAM enhances TLR4 signaling**

In contrast, overexpression of T3JAM led to a significant increase in the production of inflammatory cytokines (\textit{Tnfa}, \textit{Il6}, and \textit{Ccl5}) at mRNA levels (Fig. 2A). In contrast, overexpression of T3JAM led to a significant increase in the production of inflammatory cytokines (\textit{Tnfa}, \textit{Il6}, and \textit{Ccl5}) at mRNA levels (Fig. 2A). In contrast, overexpression of T3JAM led to a significant increase in the production of inflammatory cytokines (\textit{Tnfa}, \textit{Il6}, and \textit{Ccl5}) at mRNA levels (Fig. 2A).

Moreover, the survival rate of \textit{T3jam}\textsuperscript{-/-} mice was significantly higher than those of WT mice after LPS challenge (Fig. 2E). In keeping with this observation, the production of proinflammatory cytokines (\textit{Tnfa}, \textit{Il-1\beta}, and \textit{Il-6}) in the lungs of \textit{T3jam}\textsuperscript{-/-} mice was much lower when compared with the control group (Fig. 2F). Taken together, these results indicated that T3JAM positively regulates TLR4 signaling.

**Knockdown of T3JAM retards the late LPS signaling**

\textit{Tnfa}, \textit{Il6}, and \textit{Ccl5} mRNA in PEMs transduced with recombinant lentivirus for the overexpression of T3JAM or empty vector (e.v.) and treated with LPS (1 \mu g/ml) for periods of time (horizontal axes); results were normalized to those of PBS-treated cells transduced with empty vector (e.v.) (Fig. 2B). Immunoblot analysis of phosphorylated (p-) and total IKK, JNK, ERK, and p65, as well as \textit{IxB}a, T3JAM, and \beta-actin; lysates of PEMs were from \textit{WT} and \textit{T3jam}\textsuperscript{-/-} mice. Data are representative of three independent experiments with similar results (Fig. 2C).

**Knockdown of T3JAM retards the late LPS signaling**

Knockdown of T3JAM by specific siRNA substantially blocked the co-localization of TLR4 with the endosome markers (Fig. 3A). To further assess the regulatory effect of T3JAM in the late LPS signaling, we investigated whether T3JAM functions in the endocytosis of TLR4. LPS stimulation induced strong signals for co-localization of TLR4 with the endosome marker EEA1 at 30 min and with the late endosome marker Rab7 at 120 min after LPS challenge in both MEFs and PEMs (Fig. 3A). Knockdown of T3JAM by specific siRNA substantially blocked the co-localization of TLR4 with the endosome markers (Fig. 3A). Further flow cytometry assay combined with fluorophore-conjugated staining showed a rapid process of LPS-induced TLR4 endocytosis, with 50% of TLR4 internalized within 30 min of LPS treatment in PEMs, and TLR4 re-appears at the plasma membrane at late time points (120 min). However, knockdown of T3JAM substantially inhibited TLR4 endocytosis (Fig. 3B). A similar result was obtained in BMDMs (Fig. S3).

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significantly inhibited LPS-triggered TBK1–IRF3 signaling (Fig. 3C). Taken together, these results indicated that T3JAM indeed regulates the endocytosis of TLR4 and the late LPS signaling.

**T3JAM is required for TLR4–MyD88 signalosome assembly**

Because T3JAM is a membrane protein, we speculated that T3JAM could associate with and regulate the assembly of the TLR4–MyD88 signalosome, which is required for the signal

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**Figure 3. Knockdown of T3JAM retards the late LPS signaling.** A, co-localization of TLR4 with EEA1 and Rab7 in MEFs and PEMs transfected with negative control (n.c.) or siRNA of T3JAM (72 h) followed by the indicated time point LPS (1 μg/ml) challenge. Data are representative of three independent experiments with similar results, and each data set was based on n > 30 individual cells. B, PEMs were transfected with negative control or siRNA of T3JAM (72 h) followed by treatment with LPS (1 μg/ml) or PBS for the times indicated. Surface level of TLR4 was measured by flow cytometry. The MFIs of TLR4 from unstimulated or stimulated cells were recorded. The summary of endocytosis efficiency is shown. Data shown as means ± S.D. were collected from three independent experiments, and p values were calculated by unpaired Student’s t test. ****, p < 0.0001; n.s., no significance. C, immunoblot analysis of phosphorylated (p-) and total IRF3, TBK1, IKKe, as well as T3JAM in lysates from THP-1–derived macrophages transfected with n.c. or siRNA of T3JAM (72 h) and then treated with LPS (1 μg/ml) or PBS for the times indicated. Data are representative of three independent experiments with the similar results.
T3JAM enhances TLR4 signaling

To test this possibility, we performed endogenous immunoprecipitation (IP) and immunoblotting assays in THP-1–derived macrophages using an antibody against T3JAM. Both TLR4 and MyD88 were found to be associated with T3JAM, suggesting that T3JAM could be a component of the TLR4–MyD88 signalosome (Fig. 4A). To further dissect the interaction between T3JAM and TLR4, we carried out a super-resolution microscopy analysis, and the result suggested that T3JAM might directly interact with TLR4 (Fig. 4B).

The co-IP assay in PEMs revealed a physical association between endogenous T3JAM and TLR4, which was gradually strengthened within 10 min, and peaked 1 h after LPS challenge (Fig. 4C). Consistent with this observation, a semiquantitative immunofluorescence assay in MEFs also recorded the dynamic interaction between T3JAM and TLR4 during LPS challenge (Fig. 4D).

As expected, we found that anti-TLR4 antibody, but not control IgG, immunoprecipitated endogenous TLR4 and pulled down multiple components of the signalosome, including MyD88, IRAK2, and TRAF6. However, knockdown of T3JAM significantly decreased the association of TLR4 with MyD88 and IRAK2 (Fig. 4E). Moreover, T3JAM was indeed associated with TLR4, and such association was also enhanced by LPS stimulation, hinting at a positive role of T3JAM in the assembly of the TLR4–MyD88 signalosome (Fig. 4E). Taken together, these results indicated that upon LPS stimulation, T3JAM binds to and facilitates the assembly of the TLR4–MyD88 signalosome.

T3JAM regulates TLR4 signaling through interaction with TLR4

Next, we performed a pulldown assay using in vitro translated proteins of T3JAM, TLR4, and MyD88. Indeed, the results showed that T3JAM could interact with TLR4 but not MyD88 (Fig. 5A). Subsequent domain mapping by co-IP assays revealed that the TM (amino acids 527–544) of T3JAM is important for its interaction with TLR4, although the TIR domain of TLR4 (amino acids 633–778) is required for binding T3JAM (Fig. 5B).
Following the pulldown assay, we showed that T3JAM (amino acids 265–544), including its coiled-coil (CC) domain and TM domain, could interact with TLR4 (Fig. 5C).

To corroborate these observations, we transfected HEK-Blue™-hTLR4 cells (stably expressing human TLR4, MD-2, and CD14 co-receptor) with plasmids of an NF-κB luciferase reporter T3JAM and its truncations. Consistent with the binding analysis, wildtype (WT) T3JAM strongly promoted the activation of NF-κB, but T3JAM and its truncations’ lack of the coiled-coil domain (DelCC) and the transmembrane domain (DelTM) failed to induce NF-κB activation (Fig. 5D). Taken together, these results indicated that the CC and TM domains of T3JAM mediate the association of T3JAM with TLR4.

Figure 5. T3JAM regulates TLR4 signaling through interaction with TLR4. A, FLAG pulldown assay for analyzing the interaction between FLAG-tagged T3JAM and HA-tagged TLR4 (left) and the interaction between FLAG-tagged MyD88 and HA-tagged T3JAM (right). Fused proteins were all expressed from the cell-free system. Data are representative of three independent experiments with similar results. B, top, schematic illustration of the domain organization of T3JAM and TLR4. Bottom, IP mapping of specific domains responsible for T3JAM interaction with TLR4. Data are representative of three independent experiments with similar results. Of note: T3JAM (DelCC), delete amino acids 265–512; T3JAM (DelTM), delete amino acids 527–544; TLR4 (DelTIR), delete amino acids 633–778. C, HA pulldown assay for detecting the interaction between TLR4 and WT T3JAM and its truncation (CC+/TM). Data are representative of three independent experiments with similar results. Of note: T3JAM (CC+/TM): amino acids 265–544. D, luciferase activity of HEK-Blue hTLR4 cells transfected with NF-κB luciferase reporter and indicated plasmids. Data shown as means ± S.D. were collected from three independent experiments, and p values were calculated by unpaired Student’s t test. ***, p < 0.001; n.s., no significance.
T3JAM facilitates interaction between FLAG-tagged TLR4 and HA-tagged TLR4

Because T3JAM is a coiled-coil transmembrane protein that co-localized with TLR4 upon LPS stimulation, we speculated that T3JAM might directly regulate TLR4 activity through coiled-coil-mediated homoassociation. To test this hypothesis, we first transfected 293FT cells with FLAG-tagged TLR4 and HA-tagged TLR4, as well as empty vector and increasing doses of T3JAM. Our IP analysis showed that FLAG-tagged TLR4 could interact with HA-tagged TLR4 to a certain extent, whereas overexpression of T3JAM apparently increased such interaction (Fig. 6A). Consistent with this observation, confocal microscopic analysis also indicated a stabilizing effect of T3JAM toward the interaction between FLAG-tagged TLR4 and HA-tagged TLR4 in a dose-dependent manner (Fig. 6B).

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T3JAM enhances TLR4 signaling

Interactions of LPS with the MD-2/TLR4 complex induce structural rearrangements that trigger the homoassociation of TLR4 and its migration and clustering to lipid rafts, facilitating signal transduction through the TLR4 cytoplasmic domain (17). To further evaluate the regulatory effect of T3JAM on TLR4, we performed a fluorescence resonance energy transfer (FRET) experiment. To this end, we constructed two TLR4 constructs, C-terminally tagged with GFP and mCherry, respectively. The FRET efficiency between GFP-tagged TLR4 and mCherry-tagged TLR4 was then measured in HEK-Blue™ hTLR4 cells transfected with WT or a mutant of T3JAM. After photobleaching mCherry-tagged TLR4, the energy was transferred to GFP-tagged TLR4 if the mCherry-tagged TLR4 and GFP-tagged TLR4 were physically adjacent to each other. Apparently, overexpression of WT T3JAM significantly increased the FRET efficiency, again suggesting a promoting effect of T3JAM on the homoassociation of TLR4 (Fig. 7A). However, T3JAM mutants...
with deletion of the transmembrane domain or the coiled-coil domain did not have the FRET efficiency as did the WT T3JAM to promote the FRET signal (Fig. 7A).

Given that homoassociation of TLR4 is essential for its translocation to lipid rafts (17, 26, 27), we reasoned that T3JAM could be important for the translocation of TLR4 to lipid rafts. To verify this hypothesis, we examined the enrichment of TLR4 in lipid rafts by extraction of membrane lipid microdomains and sucrose density gradient flotation centrifugation (28, 29). As indicated by Western blotting of TLR4 and Flotillin-1, LPS stimulation of THP-1-derived macrophages significantly increased the abundance of TLR4 protein in the fractions (fractions 4–5) corresponding to lipid rafts; knockdown of T3JAM abrogated the LPS-induced

Figure 7. T3JAM is essential for LPS-induced TLR4 translocation to lipid rafts. A, FRET assay of TLR4-mediated homoassociation, assessed with constructs corresponding to empty vector (e.v.) (far left) and T3JAM (WT, DelTM, and DelCC) with PBS or LPS (1 μg/ml) treatment 30 min before and after bleaching. Bottom right, relative FRET efficiency shown as means ± S.D. were normalized to initial FRET values. Data are representative of three independent experiments with similar results, and each data set was based on n ≥ 30 individual cells. p values were calculated by unpaired Student’s t test. ***, p < 0.001; n.s., no significance. B, membrane lipid microdomains extracted by nondetergent (Na2CO3) solution and fractionated with a three-step sucrose gradient. Flotillin-1 and TLR4 were detected by Western blotting in THP-1-derived macrophages with PBS or LPS (1 μg/ml) treatment for 30 min. Data are representative of three independent experiments with similar results. C, lipid rafts extracted in MEFs similar to B.
enrichment of TLR4 in the lipid rafts (Fig. 7B). Similar results were obtained in MEF cells (Fig. 7C). Taken together, these results indicated that T3JAM is required for LPS-induced translocation of TLR4 to lipid rafts.

Discussion

Despite the well-known function of TLR4 as an inflammatory player, it is not fully understood how TLR4, especially its intracellular TIR domain, forms a homo-oligomer upon LPS binding to the extracellular domain (17, 30). It has been reported that TLR4 could exist as preformed dimers in the absence of ligand and that complete activation of TLR4 needs not only ligand binding but also apposition of the TIR domains (31). It has been long thought that additional factors proximal to the plasma membrane may facilitate the homoassociation of TLR4 during its activation. This study identifies T3JAM functions as a positive regulator of TLR4, which associates with TLR4 to promote its translocation to lipid rafts, a process essential for its activation and downstream signaling. T3JAM is a single-pass α-helical membrane protein with its N-terminal domain targeted to the cytosol. T3JAM could be localized to the plasma membrane, as well as an intracellular membrane system such as Golgi (20). Our study suggested that T3JAM might bind to preformed dimers of TLR4, and such binding is enhanced by LPS stimulation, leading to increased translocation of TLR4 to lipid rafts for full activation (Fig. 8). Multiple regulators of TLR4 have been identified to act toward the extracellular part of the receptor. For example, upon LPS detection, CD14 transfers LPS to the TLR4/MD-2 complex, leading to their heterodimerization (32–34). We propose that T3JAM represents a new type of TLR4 regulator targeting the intracellular TIR domain.

Of note, LPS-induced TLR4 association with CD14 has been shown to take place in the lipid rafts (35–39). Moreover, homoassociation of TLR4 occurs prior to its migration to the lipid rafts (17). In our study, knockdown of T3JAM abolished TLR4 translocation to lipid rafts, supporting the role of T3JAM as a molecular clamp during activation of TLR4.

Recently, T3JAM has been reported to regulate T cell development through Golgi-specific ERK signaling (20). Thus, it appears that T3JAM is involved in both innate and adaptive immunity. Meanwhile, it is worth noting that T3JAM is also a component of the STRIPAK complexes containing both kinases and phosphatase. Interestingly, we have previously reported that MST4, a major kinase component of STRIPAK, plays a key role in macrophages through direct regulation of TLR4–TRAF6 signaling (40). At this stage, it remains to be investigated whether the regulatory function of T3JAM on TLR4 depends on the STRIPAK complex.

Experimental procedures

Cell culture

The human monocyte cell line THP-1 and 293FT cells were purchased from the Cell Resource Center (Shanghai Institutes
for Biological Sciences, Chinese Academy of Sciences). HEK-Blue™-hTLR4 cells were obtained by co-transfection of the human TLR4, MD-2, and CD14 co-receptor genes, and an inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene into HEK293 cells (InvivoGen, hkb-hTLR4, and 16116-MM). THP-1–derived macrophages were maintained in RPMI 1640 medium, whereas 293FT and MEF cells were maintained in Dulbecco’s modified Eagle’s medium. For MEFs, mice were given intraperitoneal injection of thioglycollate broth to elicit peritoneal macrophages. BMDMs were generated after culture for a week with complete RPMI 1640 medium adding G-MSF. All cell cultures were supplemented with 10% fetal bovine serum and penicillin, streptomycin, or Normocin™.

**Antibodies and reagents**

LPS (Escherichia coli, serotype 055:B5), anti-FLAG (F3165), and anti-β-actin (A2228) were from Sigma. Antibody to p-IKKβ (2694) and IKKβ (2678), p-JNK (4376), IκBα (4812), HA (3724), pNF-κB p65-Ser-536 (3033), and NF-κB p65 (4764), and antibodies specific for p-IRF3-Ser-396 (29047), IRF3 (11904), p-TBK1/NAK-p65-Ser-536 (3033), and NF-κB (4764), and antibodies specific for p-IRF3-Ser-396 (29047), IRF3 (11904), p-TBK1/NAK-ser-172, TBK1/NAK (3504), and p-IRKα-ser-172 (8766), and IKKe (2905) were from Cell Signaling. Anti-ERK (sc-292838), anti-T3JAM (sc-366384), anti-TLR4 (sc-293072), anti-MYD88 (sc-11356), anti-TRAF6 (sc-8409), anti-IRAK2 (sc-367746), anti-caveolin-1 (sc-894), and anti-Flotillin-2 (sc-25506) were from Santa Cruz Biotechnology. Antibody for TLR4 (immuno-fluorescence staining, ab22048), EEA1 (ab196186), and Rab7 (ab198337) were from Abcam. PE anti-mouse TLR4 (145404) and PE anti-human TLR4 (312806) were from Biolegend. FITC anti-mouse CD14 (11-0141), FITC anti-human CD14 (11-0149-42) was from ebioscience. Pierce goat anti-mouse IGG (31430) and Pierce goat anti-rabbit IGG (31460) were from ThermoFisher Scientific.

**Plasmids**

Mammalian expression vectors for FLAG-MyD88, FLAG-IRAK1, FLAG-TRAF6, and HA-p65 have been described previously (25, 40, 41). T3JAM and its variants were subcloned into pCDA-3.1 with a sequence encoding a FLAG, HA, GFP, or mCherry tag. All lentiviral plasmids were constructed in a modified pCdh vector.

**siRNAs**

Duplexes of siRNA targeting T3JAM and negative control were synthesized by Gene pharma (Shanghai, China). The siRNA sequences are as follows: human-T3JAM-950, 5′-GAC-CACAUAUAAGAAGATT-3′ (F) and 5′-UCGUUCUUA-UUGUUGGCTTT-3′ (R); human-T3JAM-1133, 5′-GGAA-GGGACAGCUUAUGATT-3′ (F) and 5′-UCAUUAAGCU-GUCCCUUCCCT-3′ (R); human-T3JAM-1611, 5′-CCAGGA-CCUACAGAUCAATT-3′ (F) and 5′-UUGAUCCUUAG-GUGCCUGGT-3′ (R); mouse-T3JAM-922, 5′-GAAGAGGC-AUUGCCAAACTT-3′ (F) and 5′-UUGUGCCAAUGCCUUCCU-CTT-3′ (R); mouse-T3JAM-996, 5′-CAUCAUCAACACUCUGGAATTT-3′ (F) and 5′-UUCGGAGAGUUGAAGUAG-3′ (R); and mouse-T3JAM-1115, 5′-GGAAGGGACAG-CUUAAUGATT-3′ (F) and 5′-UCAUUAAGCUGUCCCUUCCTT-3′ (R).

**Transfection and luciferase assay**

Transient transfection of cells was performed using Lipo-fectamine 2000 or RNAiMax from Invitrogen, according to the manufacturer’s instructions. Luciferase activities were determined using the Dual-Luciferase assay system (Promega), and 293FT cells were transfected with plasmid encoding the NF-κB luciferase reporter.

**Real-time PCR**

Real-time PCR was performed on an Applied Biosystems step two real-time PCR system (Applied Biosystems) using the comparative C_{T} quantitation method. Real-time PCR master mix (Toyobo) was used to detect and quantify the expression level of the target gene. GAPDH was used as an internal control.

The primers used were as follows: mT3jam, 5′-AGGCCCTA-CATAGGGAGCTGA-3′ (F) and 5′-AGAGCTGTGTTTCTA-GGGATCTT-3′ (R); mll6, 5′-TCTATACCCCTCAAGTG-CGGA-3′ (F) and 5′-GAATTGGCATTCACAACCTTCTTT-3′ (R); mTnfa, 5′-CCTGTAGGCCACCGTCTAG-3′ (F) and 5′-GGAGTGAGCAAGGTGAC-3′ (R); mCcl5, 5′-GCT-GTCTTGGCCTACTCTCC-3′ (F) and 5′-TCGAGTGAACA-ACAGCTGC-3′ (R); mGapdh, 5′-TGAAGCAGCGATCT-GAGGG-3′ (F) and 5′-CAAGGAGTGGAAAGTGAGG-3′ (R).

**ELISA**

Serum and cell culture supernatants were collected and assayed for cytokines. Cytokine production was measured by ELISA of TNFα (STA00C for human; SMTA00B for mouse), IL-1β (SLB50 for human; SMLB00C for mouse), and IL-6 (S6050 for human; SM600B for mouse) according to the protocol of the manufacturer (R&D Systems).

**Immunoprecipitation and protein expression**

For immunoprecipitation experiments, whole-cell extracts were prepared after transfection or stimulation and were incubated overnight with the appropriate antibodies (identified above), together with protein A/G beads (Santa Cruz Biotechnology). Beads were then washed three times with lysis buffer, and immunoprecipitates were eluted with SDS loading buffer and resolved by PAGE. The proteins were transferred to a polyvinylidene difluoride membrane and further incubated with the appropriate antibodies. For protein expression, we use the TnT® Quik-coupled Transcription/Translation Systems (Promega) for in vitro translation according to the manufacturer’s instructions.

**Immunofluorescence**

MEF, 293FT cells, or THP-1-derived macrophages were plated on 35-mm glass bottom dishes and grown for 24 h before the indicated treatment. Cells were washed once with PBS and fixed in 4% formaldehyde in PBS for 15 min. After permeabilization with Triton X-100 (0.05%) in PBS for 5 min, cells were blocked with PBS containing BSA (5%) for 1 h and then incu-
bated with primary antibodies for 1 h. After three separate washes, cells were incubated with a secondary antibody for another hour and then stained with 4',6-diamidino-2-phenylindole for 2 min. Images were captured using an Olympus FV1200 confocal microscope or using SIM equipped with Nikon Apo TIRF 100× NA 1.49 oil immersion objective.

**Flow cytometry**

PEMs and BMDMs were treated as indicated at 37˚C and then washed with 1 ml of cold PBS and stained for appropriate antibodies on ice in the cold room for 20–30 min. 2% mouse serum or rat serum were used as the blocking reagent to reduce nonspecific binding of the antibodies. The stained cells were then washed with 1 ml of cold PBS and resuspended in 200 ml of PBS. Staining of the surface receptors was analyzed with BD LSR II (BD Biosciences). The mean fluorescence intensity (MFI) of TLR4 from unstimulated or stimulated cells was recorded. The percentage of surface receptor staining at the indicated time points, which is the ratio of the MFI values measured from the stimulated cells to those measured from the unstimulated cells, was plotted to reflect the efficiency of receptor endocytosis. Flow cytometry graphs shown under “Results” were representative data from at least three independent experiments.

**FRET**

The plasmids GFP-tagged TLR4 and mCherry-tagged TLR4 were transfected into HEK-Blue™-hTLR4 cells at a ratio of 1:1. To calculate the apparent efficiency of FRET, we used the following two spectra obtained during the process of generating the FRET-emission spectrum. The GFP-emission spectrum was obtained once before and once after photobleaching mCherry. Each data set was based on n >30 individual cells. FRET efficiency was calculated with the following formula: FRET(%) = (GFP after bleaching – GFP before bleaching)/GFP after bleaching) × 100. Statistical significance was determined with an unpaired t test. Images were captured using a Leica TCS SP5 microscope.

**Extraction of membrane lipid microdomains and sucrose density gradient flotation centrifugation**

MEF and THP-1–derived macrophage cells were sorted with or without LPS treatment. Nondetergent sodium carbonate extraction mixture was placed at the bottom of an ultracentrifuge. The gradient was formed in a Beckman centrifuge tube and centrifuged at 180,000 × g for 20 h in a SW41 rotor (Beckman) at 4˚C. Twelve fractions were collected from the top of the sucrose gradient.

**Generation of T3jam KO mice using CRISPR–Cas9 genome editing**

Cas9 knockout mice (C57BL/6J) background were kindly provided by Prof. Dawang Zhou from Xiameng University. T3JAM sgRNA sequences are as follows: sgT3jam-1, 5'-GGGCACAAAAAGGAAAGA-3'; sgT3jam-2, 5'-CTGGAAAGGACAGGTTTAA-3'; sgT3jam-3, 5'-GGACCTCAAGGTCAACTA-3'; sgT3jam-4, 5'-GCCAACAATAGAAGATGAT-3'; sgT3jam-5, 5'-CCTGGAAAGGAAGAGAAGAAA-3'; and sgT3jam-6, 5'-CTGGCATCTATCGTGAGTA-3'.

A control sgRNA sequence was designed to target the lacZ gene from E. coli. The pH-responsive diblock copolymers and the resulting nanoparticles were synthesized and characterized following protocols previously described. Briefly, reversible addition fragmentation chain transfer polymerization was used to polymerize dimethylaminoethyl methacrylate (DMAEMA) blocks. The polymerization was conducted in a nitrogen atmosphere in N,N-dimethylformamide (DMF) at 30˚C for 12 h. To add the second block of the diblock copolymer, pDMAEMA macroCTA was isolated and added to DMAEMA, propyl acrylic acid, and butyl methacrylate in DMF at 1:1:2 molar ratios. Diblock copolymers were solubilized in highly concentrated stock solutions (~1 g/ml) in ethanol and diluted to 2 mg/ml in PBS. This solution was then used to form complexes with sgRNA and injected into mice (i.v.). The size of complexes (or polymer alone) was 50–60 nm. The mice were randomly divided into two groups and received either control sgRNA nanoparticles or sgT3jam nanoparticles.

**Experimental sepsis model**

For LPS challenge, LPS (20 mg/kg) was administered intraperitoneally (i.p.). All animals were randomly assigned to treatment groups in all experiments, and experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute of Biochemistry and Cell Biology. The approval ID for the use of animals was No. 081 issued by the Animal Core Facility of SIBCB.

**Statistical analysis**

Both cellular and animal studies tended to be underpowered. Estimated sample size for a planned comparison of two independent means using a two-tailed test was undertaken using an on-line calculator and the SAS statistical software package (version 9.1.3). Data are expressed as means ± S.D. for continuous variables and as frequencies and proportions for categorical variables. Continuous data were compared using Student’s t test. For correlation, the Spearman rank correlation was used. Survival curves were calculated according to the Kaplan-Meier method; survival analysis was performed using the log rank test. p < 0.05 was considered to indicate a significant difference.

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