Identification of a Regulatory Acidic Motif as the Determinant of Membrane Localization of TICAM-2

Kenji Funami, Misako Matsumoto, Yoshiaki Enokizono, Noriko Ishii, Megumi Tatematsu, Hiroyuki Oshiumi, Fuyuhiko Inagaki and Tsukasa Seya

*J Immunol* 2015; 195:4456-4465; Prepublished online 25 September 2015; doi: 10.4049/jimmunol.1402628

http://www.jimmunol.org/content/195/9/4456

Supplementary Material: http://www.jimmunol.org/content/suppl/2015/09/25/jimmunol.1402628.DCSupplemental

References: This article cites 43 articles, 18 of which you can access for free at: http://www.jimmunol.org/content/195/9/4456.full#ref-list-1

Subscription: Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions: Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts: Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Copyright © 2015 by The American Association of Immunologists, Inc. All rights reserved.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Identification of a Regulatory Acidic Motif as the Determinant of Membrane Localization of TICAM-2

Kenji Funami,* Misako Matsumoto,* Yoshiaki Enokizono,† Noriko Ishii,* Megumi Tatematsu,* Hiroyuki Oshiumi,*1 Fuyuhiko Inagaki,† and Tsukasa Seya*

TLR4 triggers LPS signaling through the adaptors Toll/IL-1R domain–containing adaptor molecule (TICAM)-2 (also called TRAM) and TICAM-1 (also called TRIF), together with Toll/IL-1R domain–containing adaptor protein (TIRAP) and MyD88. The MyD88 pathway mediates early phase responses to LPS on the plasma membrane, whereas the TICAM pathway mediates late-phase responses, which induce the production of type I IFN and activation of inflammasomes. TICAM-2 bridges TLR4 and TICAM-1 for LPS signaling in the endosome. Recently, we identified an acidic motif, E87/D88/D89 in TICAM-2, that provides the interaction surfaces between TICAM-2 and TICAM-1. In the present study, we found additional D91/E92 in TICAM-2, conserved across species, that is crucial for TICAM-1 activation. The D91A/E92A mutant protein was distributed largely to the cytosol, despite myristoylation, suggesting its importance for assistance of membrane localization of TICAM-2. An ectopically expressed D91A/E92A mutant per se failed to activate TICAM-1, unlike its wild-type counterpart that forms self-aggregation, but it still retained the ability to pass LPS-mediated IFN regulatory factor (IRF)3 activation. In a TICAM-2 knockout human cell line expressing TLR4/MD-2 with or without CD14, overexpression of the D91A/E92A mutant did not activate IRF3, but upon LPS stimulation, it induced sufficient TLR4-mediated IRF3 activation with high coefficient colocalization. Hence, the D91/E92 motif guides TICAM-2 membrane localization and self-activation for signaling. Our results suggest the presence of two distinct steps underlying endosomal LPS signaling on TICAM-2 for TICAM-1 activation: TICAM-2 assembling in TLR4 and/or TICAM-2 self-activation. D91A/E92A of TICAM-2 selectively associates the TLR4-dependent TICAM-2 assembling, but not cytosolic TICAM-2 self-aggregation, to activate TICAM-1. The Journal of Immunology, 2015, 195: 4456–4465.

Innate immunity orchestrates the first line of the host defense by recognizing the pattern molecules of microbes (pathogen-associated molecular pattern [PAMPs]) and host origin (damage-associated molecular patterns) (1). Membrane (2) and cytosolic pattern-recognition receptors (3, 4) have been identified as sensors for PAMPs and damage-associated molecular patterns. LPS of Gram-negative bacteria, a representative PAMP, is recognized by TLR4 on the cell surface to cause inflammatory cytokine production, and it then triggers endosomal trafficking of TLR4 that causes a late wave of inflammatory cytokines production and type I IFN production (5). Notably, LPS forms a complex with LPS-binding protein, and TLR4 sufficiently functions as an LPS receptor in concert with coreceptors CD14 and MD-2 (6).

Alternatively, activation of cytosolic caspase-mediated inflammasome appears to be involved in progressive Gram-negative bacterium septic shock in some cases (7–9). Upon LPS signaling, TLR4 is distributed to both cell surface and endosome and recruits adaptor proteins. Mal/Toll/IL-1R (TIR) domain–containing adaptor protein (TIRAP) and MyD88 facilitate induction of inflammatory cytokine beneath the plasma membrane (5). TIR domain–containing adaptor molecule (TICAM)-1 (also called TRIF) and TICAM-2 (also called TRAM) facilitate induction of both inflammasome response and IFN-inducible genes near the endosomal membrane for signaling (7). As a trigger for LPS stimulation, the IFN-inducing TLR4 adaptor TICAM-1 plays a central role in the detrimental inflammatory response (7, 10). TICAM-2 anchors in the inner membrane where TLR4 is localized nearby and interacts intracellularly with TICAM-1 to bridge the TLR4-mediated LPS signal. TICAM-2 consists of several polarized domains whose functional roles remain undefined.

The cytoplasmic TLR4 TIR domain recruits four types of TIR domain–containing adaptor molecules in spatiotemporally different manners (11). MyD88 and TIRAP form a TLR4 plasma membrane complex to induce proinflammatory cytokines via the NF-κB pathway. For MyD88-dependent signaling, TIRAP targets the phosphatidylinositol 4,5-bisphosphate (PIP2)–rich plasma membrane via the polybasic domain (12). In fact, TIRAP anchoring in membrane via this domain is important for the recruitment of both TIRAP and MyD88 to TLR4 upon LPS recognition (12). This molecular complex recruits IL-1R–associated kinase (IRAK)1 and IRAK4 on the cell surface membrane via the...
death domain of MyD88 (designated MyDDosome) (13, 14) to induce proinflammatory responses (15). TIRAP also exists in the endosomal membrane and participates in TLR9/MyD88 signaling (15).

LPS-mediated subcellular trafficking of TLR4 is tightly regulated via the multicellular complex of LPS and intracellular adapter molecules. Upon stimulation with LPS, CD14 delivers TLR4 from the cell surface membrane to the endosome (16, 17), suggesting that CD14 is important for LPS-mediated type I IFN production. Other factors also regulate TLR4 translocation from the plasma membrane to the endosome (18–21). TICAM-1 and TICAM-2 are associated with TLR4 in the endosome, resulting in cytokine shift, including type I IFN signaling (22–25). TICAM-1 also acts as a TLR3 adaptor (22), but the TICAM-2/TICAM-1 complex is required for TLR4-mediated type I IFN induction. In most cases, TICAM-2 knockout or overexpression corroborates the essential role of TICAM-2 in LPS signaling (24, 25), but TICAM-2 overexpression usually leads to self-aggregation, resulting in high baseline activation of TICAM-1–mediated IFN regulatory factor (IRF)3 activation (24). These properties of TICAM-2 have made its functional analysis difficult.

TICAM-2 is reported to contain N-terminal myristoylation and polybasic motifs (10, 26) crucial for its localization to the endosomal membrane where TLR4 is situated. After TLR4 is delivered to the endosome upon LPS stimulation, TICAM-2 interacts with the TLR4 via the TIR domain and the membrane-associated motifs to recruit TICAM-1. TICAM-2 overexpression (i.e., self-aggregation) also leads to activation of TICAM-1, independent of TLR4 stimuli. Once activated, TICAM-1 is soon dissociated from the endosomal membrane to form a signal platform, termed speckle, in the cytosol (27–29). TICAM-1 mediates IRF3 phosphorylation via activation of TBK1 and IκB kinase ε (30, 31). TICAM-2 signaling downstream of TLR4 appears similar to that of TLR3, since the same TICAM-1 supports both pathways. Hence, the TLR4–TICAM-2 and TICAM-2–TICAM-1 interactions are uniquely indispensable for TICAM-1 activation in LPS signaling. Detailed analyses with crystallized proteins have been performed for the identification of functional amino acids critical for TLR4–TICAM-2 interactions (32). We have determined the solution structure of the TIR domains of TICAM-1 and TICAM-2 and showed that an acidic motif in TICAM-2, E87/D88/D89, is important for heterodimerization of TICAM-1 and TICAM-2 (33). However, the precise mechanism ensuring proper localization and self-activation of TICAM-2 is yet to be established.

In this study, we found that TICAM-2 possesses two conserved acidic amino acids, D91 and E92, which regulate TICAM-2 self-activation and signaling. Evidence from D91A/E92A mutant transfection together with human TICAM-2 disrupted cells sheds new insights into the mechanism underlying TICAM-2–mediated signaling.

Materials and Methods

Cells and reagents

HEK293 cells were cultured in the DMEM (Life Technologies) base medium (containing 10% heat-inactivated FCS, penicillin [100 U/ml, Life Technologies], and streptomycin [100 µg/ml, Life Technologies]). HEK293FT cells were cultured in the same DMEM base medium supplemented with MEM nonessential amino acids (Life Technologies) and sodium pyruvate (1 mM, Life Technologies). HeLa cells were cultured in MEM (Nissui) supplemented with 5% heat-inactivated FCS, Ω-glutamine (2 mM, Life Technologies), penicillin, and streptomycin.

Reporter gene assay

For detection of human TICAM-2–mediated signaling, HEK293FT cells were transfected with TICAM-1 and TICAM-2 expression plasmids based on pEF-BOS vectors, an IFN-β promoter plasmid, and an internal control plasmid encoding Renilla luciferase, using FuGENE HD (Promega). At 24 h after transfection, cells were lysed with reporter lysis buffer (Promega). For LPS stimulation, cells were cotransfected with TLR4, MD-2 (with or without CD14), and various TICAM-2 mutant expression plasmids. Cells were harvested and reseeded onto 96-well plates at 12 h after transfection. After 24 h, LPS stimulation was performed for 6 h, and cells were lysed as above. Luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega). Firefly lucerase activity was normalized to that of Renilla luciferase expressed and folded as solution of the activity of vector-transfected cells. All assays were performed in triplicate.

Immunoprecipitation

TICAM-1–R-HIM mutant (V687A/Q688A/L689A/G690A) (27) and TICAM-2 mutant were cloned into pE-BOS and transfected into HEK293FT cells using FuGENE HD (Promega). At 24 h after transfection, cells were harvested and lysed with lysis buffer (50 mM Tris·HCl [pH 7.5], 150 mM NaCl, 1.0% Nonidet P-40, 5 mM Na3VO4, 30 mM NaF, and 1X protease inhibitor [Roche]). FLAG-tagged TICAM-1 was immunoprecipitated using anti-FLAG mAb (2.5 µg/sample) and protein G-Sepharose (GE Healthcare). For TICAM-2 immunoprecipitation, anti-hemagglutinin (HA) polyclonal Ab (pAb; Sigma-Aldrich, 1.0 µg/sample) was used. Total lysates and immunoprecipitated proteins were resolved with SDS-PAGE, followed by immunoblotting with anti-FLAG pAb (Sigma-Aldrich) and anti-HA mAb (Roche) for the detection of TICAM-1– and TICAM-2, respectively.

Subcellular localization

FLAG-tagged TLR4, MD-2, and various HA- or FLAG-tagged human TICAM-2 mutants were cloned into pE-BOS and transfected into HeLa cells or HEK293 cells or RAW264.7 cells using Lipofectamine 2000 (Life Technologies). At 24 h after transfection, cells were fixed with PBS–4% paraformaldehyde for 30 min and permeabilized with PBS–0.2% Triton X-100 for 15 min. TLR4 was detected with anti-FLAG mAb (Sigma-Aldrich), and TICAM-2 mutants were detected with anti-HA pAb or mAb (Sigma-Aldrich). Anti-calnexin mAb (Abcam), anti-E3A1 Ab (Thermo Scientific), or tetramethylrhodamine isothiocyanate–phallolidin (Invitrogen) was employed for intracellular organelle staining. Alexa Fluor–conjugated goat anti-mouse and rabbit IgG (Invitrogen) were used as secondary Abs. After secondary Ab treatment, ProLong Gold antifade reagent with DAPI (Invitrogen) was used as mounting solution. Cells were visualized at a magnification of ×63 under an LSM 510 META microscope (Zeiss). Colocalization coefficients were determined using an LSM 510 Zeiss LSM image examiner software.

Subcellular fractionation of membranes and cytosol

In cell fractionation, various FLAG-tagged human TICAM-2 mutants were cloned into pE-BOS and transfected into HEK293FT cells using FuGENE HD (Roche). Twenty-four hours after transfection, cells were harvested, washed with PBS, and suspended with hypotonic buffer (20 mM Tris·HCl [pH 7.5], 1X protease inhibitor [Roche]). Cells were homogenized by 20 pestle strokes in a Dounce homogenizer (Wheaton). Cell homogenates were centrifuged at 1000 × g for 10 min. Supernatants were collected and ultracentrifuged at 100,000 × g. Ultracentrifuged pellets were dissolved by cracking buffer (40 mM Tris·HCl [pH 6.8], 5.3 M urea, 5% SDS, 0.1 mM EDTA, 0.04% bromophenol blue) as a membrane fraction. Supernatants were precipitated using trichloroacetic acid. The precipitated pellets were washed with acetone and suspended by cracking buffer as a cytosolic fraction. The ratio of the membrane to the cytosolic fraction was assessed by immunoblotting. Calnexin was stained with anti-calnexin pAb (Enzo) and monitored as a marker for the endoplasmic reticulum (ER) membrane representative of a cytosolic membrane fraction.

Myristoylation assay

HEK293FT cells were transfected with FLAG-tagged TICAM-2 or its mutant constructs and subsequently treated with Click-IT myristic acid azide (Invitrogen) for 6 h. After labeling, cells were harvested and immunoprecipitation was performed as above. Immunoprecipitated proteins were lysed with Tris·HCl (pH 8.0)–1% SDS. Myristoylated proteins were labeled using biotin alkyne based on the manufacturer’s protocol (Invitrogen). Biotin-labeled proteins were resolved with SDS-PAGE, followed by immunoblotting using HRP-streptavidin (Invitrogen).

Generation of TICAM-2 knockout cells

TICAM-2 knockout HEK293 cells were generated using the CRISPR/Cas9 system. Cas9 and target plasmids were obtained from Addgene. Plasmid
FIGURE 1. TICAM-2 D91A/E92A displays reduced downstream signaling function. (A) Comparison of TICAM-2 primary structures in mammals. Putative secondary structures, myristoylation, and polybasic domains in the amino terminal regions are indicated as white rectangles above the sequence. Residues mutated in TICAM-2 are marked with black squares. (B and C) Reporter assays using IFN-β promoter vector (B) or ELAM promoter vector (C) and internal control vector (phRL-TK). TICAM-1–HA and various TICAM-2 mutant expression plasmids were transfected into HEK293FT cells. After 24 h, cells were harvested and promoter activity was analyzed. Data are representative of three independent experiments. (D) Physical interaction between TICAM-1 and TICAM-2 mutants. TLR4 (200 ng), MD-2 (200 ng), TICAM-1-HA (200 ng), and various TICAM-2 mutant (300 ng) expression plasmids were transfected into HEK293FT cells. At 24 h after transfection, cells were harvested and lysed, and TICAM-2 was immunoprecipitated using anti-FLAG mAb (2.5 μg/sample). A representative of more than three experiments is shown.
Acidic motifs in TICAM-2 are important for the TICAM-2–mediated signaling pathway

Initially, we analyzed downstream signaling activity mediated by TICAM-2 mutants. Ectopically expressed TICAM-2 mediated IFN-β promoter activation via TICAM-1 interactions without participation of TLR4. TICAM-2–S156A normally mediated IFN-β promoter activation (Supplemental Fig. 1), similar to wild-type TICAM-2 (33). However, signaling activity was significantly reduced with TICAM-2–E87A/D88A/D89A and D91A/E92A, compared with wild-type TICAM-2 (Fig. 1B). Coexpression of TLR4 and MD-2 did not affect TICAM-2 signaling (Supplemental Fig. 2A). Similar results were obtained for TICAM-2–mediated NF-κB activation (Fig. 1C, Supplemental Fig. 2B). These results suggest that both E87/D88/D89 and D91/E92 acidic motifs are indispensable for TICAM-2 downstream signaling.

**D91/E92 in TICAM-2 is dispensable for binding to TICAM-1**

To further investigate the involvement of E87/D88/D89 and D91/E92 motifs in regulation of TICAM-2 downstream signaling, we examined the binding activity between TICAM-1 and TICAM-2 mutants in HEK293FT cells. Binding of TICAM-2–E87A/D88A/D89A to TICAM-1 significantly diminished, compared with that of wild-type TICAM-2 (Fig. 1D), consistent with the reduced signaling activity of the TICAM-2 mutant (33). In contrast, TICAM-2–D91A/E92A interacted with TICAM-1 to a similar extent as did wild-type TICAM-2 (Fig. 1D). Our findings suggest that the D91A/E92A mutant of TICAM-2 lacks TICAM-2–mediated signaling without affecting the binding between TICAM-1 and TICAM-2.

**TICAM-2–D91A/E92A interacts normally with TLR4**

TICAM-2–D91A/E92A binds normally to TICAM-1 but displays reduced IFN-inducing signaling activity. We next examined whether the D91A/E92A mutation affects TLR4-mediated signaling. HEK293 cells were used for analysis, because LPS-mediated signaling could not be reproduced in HEK293FT cells (data not shown). As reported previously (24), TICAM-2–C117H significantly inhibited TLR4-mediated IFN-β promoter activation upon LPS stimulation. In contrast, TICAM-2–D91A/E92A had marginal detrimental effect on LPS-derived downstream signaling.
Wild-type TICAM-2 with higher input increased the baseline control with no LPS, which suggests that self-assembly of TICAM-2 activates TICAM-1 as previously reported (24). Notably, this baseline increase was not observed in the D91A/E92A mutant as in C117H TICAM-2, which has a defect in self-aggregation (24). Furthermore, TICAM-2–D91A/E92A dis-
played similar binding activity to TLR4 as did wild-type TICAM-2 (Fig. 2B). We conclude that the reduced signaling activity of TICAM-2–D91A/E92A is not caused by defective interactions with TLR4 or TICAM-1, based on the expression analysis.

D91/E92 mutation in TICAM-2 alters its subcellular localization

TICAM-2 contains the N-terminal myristoylation motif (10). Myristoylated TICAM-2 forms hydrophobic interactions with the plasma membrane and intracellular vesicles, such as endosomes. This membrane anchoring is critical for TICAM-2–mediated signaling. Based on these findings, we examined the subcellular localization of TICAM-2–D91A/E92A. Wild-type TICAM-2 was localized to the plasma membrane and intracellular vesicles, as expected (Fig. 3A, 3B). Notably, TICAM-2–D91A/E92A was predominantly localized to the cytosolic region distant from the plasma membrane (Fig. 3A, 3B), although the dominant-negative mutant, TICAM-2–C117H, localized to plasma membrane (Fig. 3B). Data from the localization assay supported the finding that the TICAM-2–D91A/E92A mutant did not compete with endogenous TICAM-2 for membrane situation (Fig. 2). These results were adaptable to the immune cells: we showed an example of a macrophage-like cell line, that is, RAW264.7 cells transfected with TICAM-2 and its mutant. The TICAM-2–D91A/E92A mutant also localized to cytosol in RAW264.7 cells on confocal microscopy (Fig. 3C).

The cytosolic region contains cytoplasm and intracellular vesicles. To further confirm the localization of the TICAM-2 mutants, we made cell fractionation experiments (Fig. 3D, where the protocol is shown). TICAM-2–transfected cells were homogenized and separated into the membrane and cytosolic fractions (Fig. 3D). In our experimental conditions, calnexin, an ER membrane protein, was highly concentrated on the membrane fraction in both TICAM-2– and mutant-expressing cells. In this setting, TICAM-2 was distributed almost equally to the membrane and cytosolic fractions, whereas TICAM-2–D91A/E92A was enriched in the cytosolic fraction (Fig. 3D). A similar analysis was performed with the G2A mutant (with no myristoylated tail), and the distribution profile of the G2A mutant was contrasted to that of wild-type TICAM-2, reflecting the cytosolic situation of the G2A mutant (Fig. 3D). Thus, the D91A/E92A mutant largely remains in the cytosol, with minimal distribution to the membrane.

A metabolic labeling assay showed that the myristoylation-defective mutant, TICAM-2–G2A, was not myristoylated (Fig. 4). Alternatively, TICAM-2–D91A/E92A was myristoylated as much as wild-type TICAM-2 (Fig. 4). These results clearly suggest that the D91A/E92A mutant undergoes N-terminal myristoylation but this modification is only insufficient for TICAM-2 to be fixed to the endosomal membrane, where TICAM-1 is recruited for activation; at least, defective membrane localization does not cause functional deficiency of TICAM-2–D91A/E92A.

Colocalization between TICAM-2–D91A/E92A and TLR4 is increased by LPS stimuli

Endogenous wild-type TICAM-2 in cells used for assay may have influenced the function of transfected TICAM-2 mutants. To overcome this problem, we generated TICAM-2 knockout HEK293 cell sublines using Cas9/CRISPR technology, with a view to determining the specific functions of the conserved residues of TICAM-2. Genomic PCR data revealed that junk DNAs were inserted into the TICAM-2 gene in TICAM-2–null cell lines (Supplemental Fig. 3A). No TICAM-2 mRNA was detected in these cells with quantitative PCR (Supplemental Fig. 3B). In TICAM-2–null cells, IRF3 activation induced by TLR4/MD-2 and LPS was significantly diminished (Fig. 5A). Using the TICAM-2–targeted cell line, a reconstitution assay was performed by overexpression of TICAM-2 constructs. In TICAM-2–null cells expressing intact TICAM-2, IRF3 was constitutively activated as observed as an increase of control baseline (i.e., spontaneous self-activation), and we set the conditions to see the effect of LPS-mediated activation of IRF3 over the baseline values (Fig. 5B). LPS stimulation clearly upregulated IRF3 activation in cells with a low TICAM-2 input (Fig. 5B). Alternatively, in TICAM-2–null cells expressing TICAM-2–D91A/E92A, the baseline IRF3 acti-
FIGURE 5. TICAM-2 D91A/E92A mutant sustains the ability to support TICAM-1 signaling. (A) Establishment of TICAM-2 knockout cell clones. The Ticam2-null state of these sublines is shown in Supplemental Fig. 3. TLR4 and MD-2 expression plasmids (150 ng/well) were cotransfected into TICAM-2 knockout HEK293 cells with GAL4-IRF3 (160 ng/well), UAS promoter vector (40 ng/well), and internal control vector.

(Figure legend continues)
vation diminished, whereas the response to LPS remained high (Fig. 5B). D91A/E92A mutants were functionally comparable to wild-type TICAM-2 in cells expressing TLR4 and MD-2 without CD14 upon LPS stimulation, whereas the baseline IRF3 activation due to the overexpression was significantly reduced in the mutant compared with wild-type TICAM-2 (Fig. 5B, lower panel). Although the D91A/E92A mutant lacks self-activation by TICAM-2–TICAM-2 assembling in the cytosol, it might be compatible with wild-type TICAM-2 if LPS and TLR4 are supplied to facilitate the formation of the TLR4–TICAM-2 complex underneath the endosomal membrane. In either membrane- or cytosol-dominant localization, the D91A/E92A mutant maintains a capacity to assemble in the TLR4–TICAM-1 complex to deliver TICAM-1 signaling.

After transfection of TLR4 and MD-2 expression plasmids, TICAM-2–null cells were stimulated with LPS and the localization of TICAM-2 or D91A/E92A mutant was examined in comparison with that of TLR4 (Fig. 5C). The merging profile and colocalization ratio between TLR4 and the D91A/E92A mutant were increased more than those between TLR4 and wild-type TICAM-2 in response to LPS stimuli (Fig. 5C, 5D), suggesting that LPS stimulation induces the ability to assemble TLR4 and the D91A/E92A mutant as well as TICAM-2. The results of the *Ticam2<sup>−/−</sup>* cells with wild-type TICAM-2 are essentially consistent with those of the cells having endogenous TICAM-2 (Figs. 2A, 3A). The results were comparable with cells additionally expressing CD14 (Fig. 5B). That is, the cytoplasmic mutant still retains the ability to bridge TLR4 and TICAM-1 upon LPS stimulation. LPS stimulation is indispensable for the functional correction of the mislocalization mutant TICAM-2.

**Discussion**

In the present study, we identified a novel functional motif in human TICAM-2 participating in TICAM-1 signal activation in response to LPS. Only the overexpression of TICAM-2 without LPS stimulation allows the cells to enhance IFN-β promoter activation (24), but overexpression of the D91A/E92A mutant does not (Fig. 1B, 1C). D91A/E92A of TICAM-2 appears dispensable for TLR4–TICAM-2 or TICAM-1–TICAM-2 binding (Figs. 1D, 2B), but it is important for subcellular localization (Fig. 3). The molecular assembly of TICAM-2 near the membrane (TLR4) diminishes in the D91A/E92A mutant in nonstimulated cells (Fig. 3), and TICAM-2 self-activation by overexpression is reduced in cells with the D91A/E92A mutant (Fig. 5B). In contrast, TLR4–TICAM-2 interaction can be detected upon LPS stimulation even in the D91A/E92A mutant. Less aggregation tendency of the mutant was observed in not only HeLa cells but also other types of cells (Fig. 3B, 3C). Upon LPS stimulation, however, the D91A/E92A mutant exhibits sufficient IRF3-activating response in cells expressing TLR4/MD-2. The presence of CD14 appears to minimally affect the functional efficacy of TICAM-2 or the mutant upon LPS stimulation (Fig. 5B). The experiments in both wild-type and *Ticam2* mutant cells reflect a tendency of the D91A/E92A mutant to act as a signal transducer only in the presence of LPS stimuli. Our results provide insight into the activation mechanism of the IFN-β promoter by TICAM-2, which consists of LPS/TLR4-dependent and TICAM-2 self-assembling modes for IRF3 activation via TICAM-1.

On the cell surface, TLR4 induces production of proinflammatory cytokines, such as TNF-α and IL-6, in response to LPS, and TIRAP acts as a bridging adaptor for the MyD88-mediated cellular events. TIRAP assembles in the plasma membrane with its PI3P-binding domain. After activation of the NK-kB pathway through TIRAP-MyD88, TIRAP is removed from the plasma membrane and degraded by a protease, calpain (21). In this context, ARF6, a regulator of PI(4)P5K, and PI2P2 production are required for appropriate TIRAP distribution and MyD88-dependent TLR4 signaling (20). In another report, p110<sub>β</sub>, an isoform of PI3K, participates in endosomal translocation of TLR4 by accelerating PI<sub>3</sub>P2–phosphatidylinositol 3,4,5-trisphosphate conversion that dissociates TIRAP from TLR4 (21). Alternatively, in endosome, the TLR4 TIR interacts with TICAM-2 in the endosomal membrane, which is crucial for LPS-derived type I IFN production (24). TICAM-2 is myristoylated in its N terminus that enables TICAM-2 to anchor the plasma membrane (10), but in the present study we show that additional motifs in TICAM-2 may assist the membrane localization. Our sequential studies clarified the regions in TICAM-2, which are essentially involved in the molecular interactions between TICAM-2 and TLR4, TICAM-2, or TICAM-1 in LPS signaling.

The two acidic regions around the hinge (aa 87–92) of the TICAM-2 molecule (Supplemental Fig. 1) participate in activation of TICAM-1. One motif, E87/E88/D89, regulates direct binding of TICAM-2 to TICAM-1 (33). In the present study, we characterized the other region, D91/E92, which plays a critical role in IFN-β promoter activation in TICAM–2-overexpressing cells. Whereas the D91/E92 motif was dispensable for TICAM-2's direct interactions with TICAM-1 or TLR4, self-aggregation–mediated IRF3 activation was largely abrogated, resulting in low baseline IFN-β promoter activation in cells with TICAM-2–D91A/E92A (Figs. 2A, 5B). These results suggest that physical interactions between TICAM-2 and TICAM-1 are not the sole factor determining TICAM-2 downstream signaling. Because the two acidic regions are located nearby, this hinge region appears crucial for the quaternary structure of the TLR4/TICAM-1/2 complex.

TICAM-2 localization to the endosome was recently found to be critical for downstream signaling (26). Myristoylation of this mutant is similar to that of wild-type TICAM-2, suggesting that

(60 ng/well). Twenty-four hours after transfection, cells were stimulated with LPS (final 1 μg/ml) for 6 h. Cells were harvested, lysed with passive lysis buffer (Promega), and promoter activity was analyzed using a Dual-Luciferase reporter assay system (Promega). (B) *Ticam2<sup>−/−</sup>* cells recover IRF3 activation activity by transfection with wild-type TICAM-2, but less with D91A/E92A mutant. TLR4, MD-2, CD14 (150 ng/well), and various TICAM-2 construct expression plasmids (3–30 ng/well) were transfected into TICAM-2 knockout HEK293 cells with pGAL4-IRF3 vector (160 ng/well), UAS promoter vector (40 ng/well), and internal control vector (60 ng/ml). Twenty-four hours after transfection, cells were stimulated with LPS (final 300 ng/ml) for 6 h. Promoter assay was performed as for (A). Data are representative of three independent experiments. (C) Confocal analysis with TLR4 and D91A/E92A mutant in LPS stimulation. TLR4, MD-2, and TICAM-2 mutant expression plasmids were transfected into TICAM-2 knockout cells. Twenty-four hours after transfection, cells were stimulated with 300 ng/ml LPS for 0–60 min. After stimulation, cells were fixed, permeabilized, and stained with anti-FLAG mAb for TLR4 and anti-HA pAb for TICAM-2. Scale bars, 10 μm. (D) Colocalization coefficients of TICAM-2–D91A/E92A with TLR4. Colocalization coefficients of TICAM-2–D91A/E92A or wild-type TICAM-2 with TLR4 were calculated using an LSM 510 Zeiss image examiner software. Single-plane data were used and at least 60 cells per condition were measured for quantification of colocalization between TICAM-2 and TLR4. 1, colocalization coefficients means relative number of colocalizing pixels as compared with total number of TICAM-2<sup>−/−</sup> pixels; 2, weighted colocalization coefficients means sum of intensities of colocalizing pixels as compared with the overall sum of TICAM-2<sup>−/−</sup> pixel intensities.
this modification itself is insufficient for proper localization of TICAM-2. It is proposed that a second signal is required to establish a firm localization of myristoyl proteins to the membrane (34). Several types of second signals for myristoylation have been determined to date. In the case of TICAM-2, a polybasic cluster at the N terminus acts as the second signal for interactions with the head groups of acidic phospholipids (26). D91/E92 may therefore act as a stabilizer of this second signal or another motif by interacting with other membrane-targeting molecules. Our findings suggest that the myristoylation of TICAM-2 does not fully support membrane localization and requires additional motifs of TICAM-2 for maintaining localization and tight interaction with other molecules. The D91/E92 motif is an example of a supporter for membrane anchoring of myristoyl proteins.

Upon LPS stimulation of TICAM-2-null cells expressing TICAM-2–D91A/E92A, colocalization between TLR4 and TICAM-2–D91A/E92A was increased (Fig. 5C). The increased coefficient ratios based on TICAM-2 signals were more prominent in the D91A/E92A mutant than in TICAM-2 (Fig. 5D), which is consistent with an increased merging profile of TLR4 and D91A/E92A compared with TLR4 and TICAM-2 (Fig. 5C). Under these conditions, the overexpressed mutant mediated IRF3 activation upon LPS stimulation. Although we could not give sufficient explanation for different dynamics of TICAM-2 and this mutant in colocalization coefficient with TLR4 upon LPS stimuli, the D91A/E92A region is eligible for temporally interacting with TLR4 in response to LPS (Fig. 5C). CD14 minimally affects the action of the D91/E92 mutant. Without LPS stimuli, TICAM-2–D91A/E92A did not mediate IRF3 activation in TICAM-2-null cells. When overexpressed, only a part of wild-type TICAM-2, but not the mutant, is stably localized to endosome, unlike the case of LPS stimulation (Fig. 3A), suggesting that LPS stimulation promotes interactions of TLR4 with the D91A/E92A mutant, which induces less self-aggregation than does TICAM-2, but evokes downstream IFN signaling through TICAM-1. The mutant appears more mobile than wild-type TICAM-2 in response to LPS stimulation, because the mutant is mainly localized to the cytosol (Fig. 4D). Why the three-dimensional move required by the mutant sufficiently supports LPS signaling, however, remains unanswered. Further study on how this behavior happens in the D91A/E92A mutant is needed.

TLR4 signaling plays a pivotal role in protection against microbial invasion. Loss-of-function studies suggest that LPS signaling profoundly involves the TLR4 pathway, including CD14, TIRAP, MyD88, and IRAK proteins. Alternatively, inflammatory responses, particularly causing serious manifestations such as sepsis, are not always attributable solely to TLR4–MyD88, but to cytoplasmic events (7–9). Gram-negative bacteria appear to be phagocytosed, and their constituents, including polysaccharides (7–9) and RNAs (15), stimulate NALP3 inflammasome formation. Recently, several reports suggested that inflammatory caspases–4/11 acted as cytoplasmic messengers for TICAM-1–dependent LPS signaling (8, 9, 35), and that in collaboration with the TLR4 pathway, the complex manifestations of LPS-mediated sepsis was completed. TICAM-1 activation, either TLR4-dependent or -independent, is a key for inflammasome activation in LPS lethality in mice (7–9). When TLR4 triggers the upregulation of the pro-forms of these caspases and IRF3 activation as in bacterial infection (7), TICAM-2 confers the master function of TLR4/TICAM-1 activation in LPS signaling and toxicity (7, 24, 36). TICAM-2 functions in TLR4 signaling as a bridging adaptor for activation of the TICAM-1 pathway may be extrapolated to the TLR2 and TLR5 signaling, particularly in macrophages (37, 38). To generalize the TICAM-2 functions, the TICAM-2 mutants we have provided will be good tools to further consolidate the TICAM signaling in other TLR pathways.

Several studies have focused on modulation of TLR4 signaling (39, 40), and TICAM–1–specific TLR4 agonists were recently generated (39, 41). Although switching the molecular relationship between TLR4 and TIRAP or TICAM-2 is an attractive strategy for control of bacterial pathogenesis (39–41), it is difficult to achieve specific regulation discriminating between the MyD88–TIRAP and TICAM–1–TICAM-2 pathways without cell damage in vivo by the currently available methods. TICAM-1 harbors potential to activate multifarious intracellular signaling pathways that evoke live and death events in cells with TLR3 stimulation (27, 42, 43). Our present findings support the importance of the hinge region of TICAM-2 for induction of the TLR4-mediated IFN response (10, 26, 41), and they identify the specific portion of D91/E92 that participates in control of self-activation of TICAM-2. Although biological significance of the hinge region remains unknown, chemical biology based on structural analysis for designing TICAM-2 antagonists, particularly targeting the hinge region, would provide an efficient approach for therapeutic manipulation of LPS signaling without affecting TICAM-1 and other cytotoxic pathways.

Acknowledgments

We thank all laboratory members in our department for valuable discussions. We are grateful to Haruko Sato for secretarial help. Consilhiarl Menda (Hokkaido University) kindly checked English usage in the final version of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Solution structure of the TICAM-2-TIR domain.

The amino acids identified are indicated.
Supplemental Figure 2. TICAM-2 D91A/E92A displays reduced downstream signaling functions in the presence of TLR4 and MD2

IFN-β promoter vector (A) or ELAM promoter vector (B), internal control vector (phRL-TK), TLR4, MD-2, TICAM-1 and various TICAM-2 mutant expression plasmids were transfected into HEK293FT cells. After 24 h, cells were harvested and promoter activity was analyzed. Data are representative of three independent experiments.
Supplemental Figure 3. Generation of TICAM-2 knockout cells

(A) A junk DNA sequence was inserted in a TICAM-2 knockout stable cell line (#2-6-5). The target sequence is depicted in red, the start codon in blue, and stop codons in inserted junk DNA are depicted in green. (B) TICAM-2 mRNA was quantified in selected TICAM-2 knockout cell lines.