TIR-containing Adapter Molecule (TICAM)-2, a Bridging Adapter Recruiting to Toll-like Receptor 4 TICAM-1 That Induces Interferon-β*§

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Lipopolysaccharide (LPS) is an agonist for Toll-like receptor (TLR) 4 and expresses many genes including NF-κB- and interferon regulatory factor (IRF)-3/IFN-inducible genes in macrophages and dendritic cells (DCs). TICAM-1/TRIF was identified as an adapter that facilitates activation of IRF-3 followed by expression of interferon (IFN)-β genes in TLR3 signaling, but TICAM-1 does not directly bind TLR4. Although MyD88 and Mal/TIRAP adapters functions downstream of TLR4, DC maturation and IFN-β induction are independent of MyD88 and Mal/TIRAP. In this investigation, we report the identification of a novel adapter, TICAM-2, that physically bridges TLR4 and TICAM-1 and functionally transmits LPS-TLR4 signaling to TICAM-1, which in turn activates IRF-3. In its structural features, TICAM-2 resembled Mal/TIRAP, an adapter that links TLR2/4 and MyD88. However, TICAM-2 per se exhibited minimal ability to activate NF-κB and the IFN-β promoter. Hence, in LPS signaling TLR4 recruits two types of adapters, TIRAP and TICAM-2, to its cytoplasmic domain that are indirectly connected to two effect adapters, MyD88 and TICAM-1, respectively. We conclude that for LPS-TLR4-mediated activation of IFN-β, the adapter complex of TICAM-2 and TICAM-1 plays a crucial role. This results in the construction of MyD88-dependent and -independent pathways separately downstream of the two distinct adapters.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB091054 and AB100441 for the human and mouse TICAM-2 cDNA sequences, respectively.

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Calmette-Guérin; DBD, DNA-binding domain; DC, dendritic cell; IFN, interferon; IRF, interferon regulatory factor; Mal, MyD88-adaptor-like; MALP-2, macrophage-activating lipoepitope-2; MD-2, myeloid differentiation factor-2; PKR, double strand RNA-dependent protein kinase; siRNA, small interference RNA; TICAM-1, TIR-containing adapter molecule-1; TICAM-2, TIR-containing adapter molecule-2; TIPAR, TIR-containing adapter protein; TLR, Toll-like receptor; mAb, monoclonal antibody; RT, reverse transcriptase; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; IRAK, interleukin-1 receptor-associated kinase; TANK, TRAF family member-associated NF-κB activator.
TLR3- and TLR4-mediated IRF-3 activation and induction of IFN-inducible genes were abrogated in TICAM-1-deficient or -mutated cells (15, 16). This suggested that another factor or mechanism must be involved in the MyD88-independent pathway of TLR4.

Here, we report the identification of a protein that directly binds TLR4. This protein harbored a TIR domain that has significant homology to TICAM-1, and conferred heterophilic dimerization with TICAM-1. We have named this protein TIR-containing adaptor molecule-2, TICAM-2. Based on its functional and physical association analyses, we demonstrated that TICAM-2 helps TLR4 bind TICAM-1 that subsequently induces LPS-mediated IRF-3 activation in the TLR4-mediated MyD88-independent cellular response.

MATERIALS AND METHODS

Cell Culture and Reagents—The human lung fibroblast cell line MRC-5 was obtained from the Riken Cell Bank (Tsukuba, Japan) and the epithelial cell line HeLa was supplied by Tokyo Metropolitan Institute (Tokyo, Japan). Both cell lines were maintained in minimal essential medium supplemented with 5% (HeLa) or 10% (MRC-5) heat-inactivated fetal calf serum (JRH Biosciences) and antibiotics. The mouse macrophage cell line RAW264.7 was maintained in RPMI 1640, 10% fetal calf serum (17). HEK293 cells (obtained from RIKEN Institute, Japan) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. The HEK293 line used expressed TICAM-2 mRNA but no TLR3 mRNA and an extremely low level of TICAM-1 mRNA (data not shown). Poly(I-C) was purchased from Amersham Biosciences. Polyoxymyin B, LPS from Escherichia coli serotype O11:B4, and mouse IgG1 were from Sigma. The mycoplasma lipopeptide MALP-2 was prepared as described (18). These reagents, except for LPS, were treated with polyoxymycin B (10 μg/ml) for 1 h at 37°C before stimulation of cells. MAbs against human TLR3 (TLR3.7) and TLR4 (TLR4.2) were produced in our laboratory as described previously (19, 10). MAb against human TLR4 (20) was a gift from Dr. K. Miyake (University of Tokyo). Anti-IRF-3 antibody was used for determination of IRF-3 activation as noted previously (21). Polyclonal antibodies against human TICAM-1 and TICAM-2 were produced in our laboratory according to the reported method (22).

Identification and Cloning of TICAM-2 cDNA—TICAM-2 expressed sequence tags were identified by BLAST search analysis with the human TICAM-1 protein sequence in the NCBI BLAST server using the blastn program. The full-length TICAM-2 cDNA sequence was predicted by searching for human expressed sequence tags in the DDBJ tblastn program. The full-length TICAM-2 cDNA sequence was predicted by searching for human expressed sequence tags in the DDBJ tblastn program. The putative full sequence of TICAM-2. The BLAST search revealed that the mouse cDNA sequence (accession number AW450623, were found to cover the putative full sequence of TICAM-2. The putative full sequence of TICAM-2 was isolated by RT-PCR using cDNA of NIH3T3 cell lines with the mF1 and mR3 primer sets. The alignment of the adapter molecules was carried out using ClustalW. Expression vectors for TICAM-2 and its mutants were prepared as follows. The cDNA encoding full-length human or mouse TICAM-2 was placed between the Xhol-NotI sites of the pEFBOS plasmid. Regarding human TICAM-2, pEFBOS-TICAM-2 (TIR) was made by inserting the 68–235 amino acid region of TICAM-2 following the cDNA sequence into the Xhol-NotI site of pEFBOS, pEFBOS-TICAM-2-P116H) and -C117H) were made from pEFBOS-TICAM-2 by site-directed mutagenesis changing Cys-117 to His and Pro-116 to His. The plasmids were prepared with an endotoxin-free Plasmid Maxi kit (Qiagen).

Northern Blotting and RT-PCR—Human 12-Lane MTN Blot and Blot III membranes (Clontech) were hybridized under stringent conditions with a 32P-labeled probe of full-length human TICAM-2 cDNA and rehybridized with a labeled β−actin probe. The mRNA signals were detected using a BAS2000 image analyzer (Fuji, Japan) with 4–24 h exposure.

RT-PCR for mRNA identification and semiquantitation were performed with TICAM-2 cDNA for human TICAM-2 or β−actin. The primers used are summarized in Table I.

Yeast Two-hybrid Method—Yeast media were made as described previously (23). Yeast AH109 strain (Clontech) was transformed with the bait and prey plasmids. The transformants were streaked and incubated for 5 to 5 days. BD and AD in figures represent the bait and prey plasmids, respectively. BD-TLR3, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9 were made by inserting the TIR domain of TLRs into the pGBK7 vector (Clontech). The mutant P714H TLR4 was produced according to the previous report (18) and BD-TLR4 P714H (TLR4 PH) was made for this assay. BD-TICAM-2 and AD-TICAM-2 were made by inserting the full-length wild-type TICAM-2 cDNA into the pGBK7 (bait) or pGADT7 (prey). BD-TICAM-1 was made by inserting a TICAM-1 cDNA fragment (from the +1102 to +2142 bp region relative to the start codon). AD-TIRAP and AD-MyD88 were made by inserting the cDNA fragment encoding the full-length protein into the pGADT7 vector. SD-WLH is yeast synthetic dextrorotate medium lacking Trp, Leu, and His amino acids. SD-WLH lacks adenine as well. SD-WLH plates are mild stringency plates and SD-WLHA plates are high stringency plates (24).

Reportor Gene Assay for NF-κB and IFN-β Promoter—HEK293 cells (2 × 105 cells per well) were transiently transfected in 24-well plates using Lipofectamine 2000 reagent (Invitrogen) with pEFBOS for expression of TLR2, TLR3, TLR4 + MD-2 (0.1 μg), TICAM-1, dominant-negative TICAM-1 (TICAM-1 TIR(P434H)), MyD88, TIRAP, TICAM-2, TICAM-2(TIR), TICAM-2(C117H), TICAM-2(P116H) (10, 100, or 200 ng), or vector alone for control, together with a luciferase-linked NF-κB reporter gene (Stratagene, 0.1 μg) or p-125 luc reporter plasmid (0.1 μg). The construct of the dominant negative TICAM-1, TICAM-1 TIR(P434H), was described previously (12). The p-125 luc reporter was provided by Dr. T. Taniguchi (University of Tokyo) and contains the human IFN-β promoter region (−125 to +19) inserted in the Pigeon luciferase reporter plasmid (Toyot Inko) (11). pAP-1-Luc reporter plasmid was purchased from Stratagene. Reporter plasmid containing the ELAM-1 promoter was made in our laboratory (25). The total amount of transfected DNA (0.8–1.0 μg) was adjusted by adding empty vector. Five ng of plasmid pCMVβ (Clontech) was used as an internal control. Where indicated, a dominant-negative expression vector for MyD88 (MD88/TIR)), TIRAP (TIRAP/P125H), TICAM-1 (TICAM-1 TIR(P434H)), or TICAM-2 (TICAM-2(C117H)) was co-transfected into the cells. Twenty-four hours after transfection, cells were stimulated with medium alone, LPS (100 ng/ml), poly(I-C), or polymyxin B-treated poly(I-C) (10 μg/ml) for 6 h. The cells were lysed with lysin buffer (Promega) and both luciferase and β-galactosidase activities were measured according to the manufacturer’s instructions. Values were expressed as mean relative stimulations plus standard deviation (S.D.) for a representative experiment from a minimum of three separate experiments, each performed in duplicate.

Assay for IRF-3 Activation—Two methods were employed to assess IRF-3 activation. Native gel assay was performed according to the report by Fujita et al. (21). Reporter gene assay for IRF-3 was performed as follows (26). HEK293 cells (1 × 105) were transfected with the indicated amounts of the plasmids for TICAM-1 or TICAM-2 together with 5 ng of pCMV-β, 100 ng of p55 UASG-Luc, and/or 25 ng of Gal4 DBD/MRF-5 in Lipofectamine 2000 reagent (Invitrogen). Gal4 BD or DBD only was used as a control. At timed intervals (typically 24 h), cells were harvested and the reporters were determined.

Immunoprecipitation—HEK293 cells were transiently transfected in 6-well plates using Lipofectamine 2000 reagent with pCMV/TLR4, FLAG-TLR4, or FLAG-TLR4 P714H (2 μg), pEFBOS/TLR3 or FLAG-
TABLE II

| Target gene          | Sense/antisense | Oligonucleotide sequence[a] |
|----------------------|----------------|---------------------------|
| Mouse TICAM-2 (site A) | Sense          | r(UUCUGAGGAAGCCUCGUUCUGA(TT) |
| Mouse TICAM-2 (site B) | Antisense      | r(CAGGAGACUCCUAAGGCACCUA(TT) |
| Mouse TICAM-1        | Antisense      | r(CCAGGUUGCUUCAAAGAAG(TT)   |
| Control siRNA (human TLR3) | Sense          | r(CCUAGGAGCUCCUGAG(TT)      |
| Control siRNA (Human lamin A/C) | Antisense  | r(GAUCAUGUUGCAUCGAAU(TT)    |
| Mouse TICAM-2        | Antisense      | r(UUGUUCUUCUGGAGUUCAGA(TT)  |

[a] "a" and "d" represent ribo- and deoxyribonucleotide, respectively.

TLR3 (3 μg), pCMV/TLR2 or FLAG-TLR2 (2 μg), and pEFBOS/TICAM-1-HA (0.5 μg) or pEFBOS/MF-2 (1 μg) together with pEFBOS/TICAM-2 or TICAM-2-HA (0.5 μg). The total amount of DNA (4 μg) was kept constant by adding medium vector. Twenty-four hours after transfection, cells were stimulated with medium alone or LPS for 16–20 minutes then lysed in lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 25 mM iodoacetamide, 10 mM EDTA). After centrifugation, the cell lysates were incubated with anti-FLAG M2 mAb (Sigma) or for 2 h at 4 °C. Mouse IgG1 was used for control.

The TLR2, TLR3, and TLR4 complexes were precipitated with protein G-Sepharose (Amersham Biosciences) and washed three times thoroughly with phosphate-buffered saline, 0.02% Triton X-100. Immunoprecipitated proteins were eluted by adding Dulbecco’s phosphate-buffered saline containing 1% SDS, 0.2% Nonidet P-40, and 5% 2-mercaptoethanol, boiled, and resolved on SDS-PAGE followed by immunoblotting using the indicated antibodies. In some experiments, lysates were also directly immunoblotted.

RNAi—The mouse macrophage-like cell line RAW264.7 was employed in this study because no good human cell lines were found for the comparable TIR domains of the reported adapters are shared tandemly arranged basic amino acids and serines with that of TIRAP. Similar to TICAM-1, TICAM-2 lacked the conserved sequences present in other TIR-containing proteins (27), which is essential for the TIR-mediated signaling, was situated in front of the conserved position or replaced with cysteine in TICAM-2 (Fig. 1b). Identities in amino acid composition among the human protein was confirmed by RT-PCR using the primers covering the open reading frame and named TICAM-2 (TIR-containing adapter molecule-2) (Fig. 1a). This protein consisted of 235 amino acids with a short (~70 amino acids) N-terminal Ser/Thr-rich domain, TIR motif, and a C-terminal 20-amino acid stretch. The N- and C-terminal domains of TICAM-2 did not resemble those of TICAM-1. The TIR and C-terminal portion were highly conserved in human and mouse TICAM-2 (Fig. 1a). Although the TIR motif of TICAM-2 had low similarity to the TIR motifs of the reported adapters TIRAP (5, 6) and MyD88 (4), the N-terminal domain of TICAM-2 shared tandemly arranged basic amino acids and serines with those of TIRAP. Similar to TICAM-1, TICAM-2 lacked the conserved sequences present in other TIR-containing proteins (27), which is essential for the TIR-mediated signaling, was situated in front of the conserved position or replaced with cysteine in TICAM-2 (Fig. 1b).

**Specific Binding of TICAM-2 to TLR4 in Yeast**—The associations between TICAM-2 and the reported TIR-containing proteins were examined in yeast. The tails of TLR2, TLR3, TLR5, TLR6, TLR7, TLR8, and TLR9 did not recruit TICAM-2 (Fig. 2a). In a confirmed experiment, TICAM-1 neither bound TLR4 nor its mutant (Fig. 2b).

TICAM-2 homodimerization and TICAM-1-TICAM-2 heterodimerization was observed in yeast, whereas the heterodimerization was weak compared with the homodimerization (Fig. 2c). This weak TICAM-1-TICAM-2 binding was confirmed again by long-term culture (the lower panel of Fig. 2c).

**Physical Association of TICAM-2 with TLR4**—Physical binding of TICAM-2 to the TIR domain of TLR4 was confirmed by immunoprecipitation in HEK293 cells expressing TLR2, TLR3, or TLR4 + MD-2, and TICAM-2 with HA-tag (Fig. 3a).
FIG. 1. Molecular cloning and tissue distribution of TICAM-2. a, sequence alignment of human and mouse TICAM-2. Overlined, TICAM-2 TIR-like motifs. Asterisks, identical residues; double dots, conserved substitutions; single dots, semiconserved substitutions; hu, human; mu, murine. b, alignment of the TIR domains of TICAM-2, TICAM-1, TIRAP, MyD88, and the TIR of TLR2. The TIR domains of human TICAM-2 (amino acids 75–213), murine TICAM-2-(72–210), human TICAM-1-(394–532), murine TICAM-1-(396–534), human MyD88-(160–296) (accession number U70451), human TIRAP-(85–214) (accession number AF406652), and the TIR of human TLR2-(640–784) (accession number U88878) were aligned. Symbols are defined as in a. For details about Boxes 1, 2, and 3 see Ref. 13. c, alignment of amino acids for identities among the TIR domains of the adapters. d, schematic representation of the four human adapter molecules. The TIR domains are shaded. e, Northern blot of TICAM-2 mRNAs from specified tissues. Northern blots for human tissues (Clontech) were probed with the TICAM-2 or control/H9252-actin cDNA. Notice the two band profiles of 3.6 and 3.3 kb in tissues such as lymph node, prostate, stomach, thyroid, muscles, and lung. An additional 1.8-kb band was specific only for placenta. f, TICAM-2 mRNA in various human cells and cell lines. Total RNA was isolated from the indicated types of human cells/cell lines, and RT-PCR was performed using primers for TICAM-2 (35 PCR cycles) or control/H9252-actin (20 cycles). iDC, immature DC.
molecular complex containing both TLR4 and TICAM-2 was detected by immunoprecipitation using anti-FLAG antibody and immunoblotting using anti-HA antibody (Fig. 3a, lane 6). Association of TICAM-2 with TLR2 or TLR3 was barely detectable under these conditions (Fig. 3a, lanes 1–4). Even when co-expressed with MD-2, TLR2 failed to co-precipitate TICAM-2 (Fig. 3a, lane 3 and 4). These results were confirmed with specific mAbs against TLR2, TLR3, and TLR4 (data not shown). Stimulation of TLR4-expressing cells with LPS had no affect on this molecular association (Fig. 3b). The binding of TICAM-2 to TICAM-1 and TLR4 was tested next using TICAM-1 (HA-tag), TICAM-2 (with HA tag or FLAG tag), and TLR4 (FLAG tag). Results show that TLR4 recruited TICAM-1 only in the presence of TICAM-2 (Fig. 3c, lane 6). Under the same conditions, TLR3 (12) but not TLR4 (data not shown) directly recruited TICAM-1 into a complex. TICAM-2 formed a dimer because TICAM-2-HA and TICAM-2-FLAG were co-precipitated (Fig. 3c, lane 1). TICAM-2-TICAM-1 complex was only marginally detected in the absence of TLR4 (Fig. 3c, lane 4). The two TICAM-2 mutants (P116H and C117H) (see Fig. 4b) lost the ability to dimerize with TICAM-1 and TICAM-2 (data not shown) as shown in yeast. Thus, the TICAM-2 mutants, although still bind the tail of TLR4, cannot be coupled with intact TICAM-1, which may reflect its dominant-negative properties. These results are essentially in accord with those observed in yeast (Fig. 2).
The P714H mutant of TLR4 did not recruit TICAM-2, thereby disassembling TICAM-1 (Fig. 3d). A similar TLR4–TICAM-1 relationship was confirmed with the specific mAb against TLR4 (data not shown). Thus, simultaneous expression of normal TLR4 is crucial for assembling TICAM-2 and TICAM-1.

Functional Analysis of TICAM-2—The HEK293 cell line was used throughout the functional study because this human cell line express no TLR4, a minute amount of TICAM-1, and detectable TICAM-2 by immunoblotting (Supplemental Materials Annex 1). Overexpression of the TICAM-2 protein in HEK293 cells led to mild induction of an NF-κB-dependent luciferase reporter gene and the Luc-IFN-β promoter, and virtually no activation of AP-1 (Fig. 4a). To characterize the overexpressed TICAM-2 function, we generated a series of TICAM-2 mutants: TICAM-2(TIR(P434H) and TICAM-2(C117H) that contained a histidine substitution at amino acids 116 and 117, respectively (Fig. 4b). Finally, TICAM-1 TIR(P434H) and TICAM-2(C117H) were used as dominant-negatives for TICAM-1 and TICAM-2, respectively (see Supplemental Materials Annex 2 and Fig. 4, e and f, for their detailed properties).

The activation of the IFN-β promoter and NF-κB induced by TICAM-2 overexpression was largely suppressed by TICAM-1 TIR(P434H) (Fig. 4c). This TICAM-1 TIR(P434H) effect was specific to TICAM-2 function blocking, because the dominant-negative versions of MyD88 and TIRAP did not block the TICAM-2-mediated activation of the IFN-β promoter or NF-κB (Fig. 4c). Hence, TICAM-2-mediated activation of NF-κB and IFN-β promoter largely depends on intrinsic TICAM-1. Furthermore, we noticed that TICAM-2(C117H) did not suppress TICAM-1-mediated IFN-β or NF-κB activation if TICAM-1 was overexpressed (Fig. 4d), reinforcing the idea that TICAM-1 recruited to TICAM-2 rather than TICAM-2 per se is critical for the effector function. Actually, TICAM-2(C117H) and -(P116H) (not shown) acted as dominant-negatives for TICAM-1 and TICAM-2, respectively.

Dominant-negative TICAM-1 TIR(P434H) was used instead of TICAM-2(C117H) and activation of the IFN-β promoter and NF-κB was analyzed using HEK293 cells expressing TLR3 or TLR4 + MD-2 (Fig. 4f). LPS-TLR4-mediated IFN-β induction was inhibited by the expression of TICAM-1 TIR(P434H), although the efficacy of inhibition appeared less than that observed for poly(I:C)-TLR3-mediated IFN-β induction (left panel of Fig. 4f). In contrast, NF-κB activation was not inhibited by the addition of the TICAM-1 TIR(P434H) vector in cells expressing TLR4 + MD-2 + CD14 (right panel of Fig. 4f). TICAM-2 overexpression-mediated NF-κB activation was inhibited by TICAM-1 TIR(P434H) (Fig. 4c), which was not reflected in the LPS-TLR4-mediated NF-κB activation (Fig. 4f), suggesting the presence of another critical adapter, presumably MyD88. Under the same conditions, the TICAM-1 TIR(P434H) dominant-negative construct inhibited poly(I:C)-dependent NF-κB activation in cells expressing TLR3 (right panel of Fig. 4f). Thus, at least TICAM-1 and TICAM-2 are involved in LPS-triggered TLR4-mediated IFN-β induction.

In analysis of the effect of TICAM-2 on limiting amounts of TICAM-1 in HEK293 cells, a marked additive activation of the IFN-β promoter was observed by overexpression of TICAM-2 (100 ng of plasmid) in TICAM-1 (0.5–10 ng of plasmid)-expressing cells (Fig. 4g). This may reflect TICAM-2–TICAM-1 dimerization. Endogenous TICAM-1 was a neck in the pathway from TICAM-2 to the IFN-β promoter in HEK293 cells. These functional results, together with biochemical molecular associateFig. 2. Association between TICAM-2 and TLRs or adapters. a, interactions between TICAM-2 and the TIRs of TLR2–9 in the yeast two-hybrid system. Strong association was observed between TICAM-2 and TLR4 (SD-WLHA plate), whereas weaker TICAM-2-TLR3 association was observed in SD-WLH plates. b, interaction between TICAM-2 and the tail of TLR4 or its P714H mutant. Strong association was observed between TICAM-2 and the normal TIR of TLR4. The association was not detected between TICAM-2 and the mutant TIR of TLR4 (TIR(P434H)). TICAM-1 was used as a control and no association was observed between TICAM-1 and two TLR4 TIRs, whereas TICAM-1 strongly bound TLR3. c, interactions between the TICAM-2 or TICAM-2 mutant (TICAM-2 C117H, TICAM-2CH) and other adapter molecules in yeast. TICAM-2 homodimerization and weak TICAM-1-TICAM-2 heterodimerization were observed. TICAM-2CH failed to couple with other intact TICAM-2 or TICAM-1. The TICAM-1-TICAM-2 heterodimerization was confirmed in the WLHA plate (lower panel). The yeast growth was usually observed <3 days, whereas in this case 6 days were required to confirm the growth. d, interactions between TICAM-2 and the other adapter molecules in yeast. No association was observed between TICAM-2 and MyD88 or Mal/TIRAP under the conditions where TICAM-2 strongly homodimerized. AD-Mal/TIRAP and AD-MyD88 constructs were functional, because we observed the interaction among these prey and some TLR bait plasmids (data not shown). SD-WLH, reflecting weak interaction; SD-WLHA, reflecting strong interaction. BD and AD represent bait and prey plasmids, respectively.
**TICAM-1 and TICAM-2 Cooperatively Activate IRF-3**—

Based on the biochemical analysis, we tested whether TICAM-2 together with endogenous TICAM-1 activates IRF-3 that in turn mediates IFN-β induction in the TLR4-induced MyD88-independent response (4). HEK293 cells were transfected with variable amounts of the TICAM-2 plasmid and the levels of IRF-3 activation were measured by IRF-3 reporter assay. In parallel with IFN-β promoter activation (Fig. 4a), IRF-3 was activated by simple transfection with >50 ng of TICAM-2 (Fig. 5a). Potentiating TICAM-1-mediated IRF-3 activation was observed when TICAM-2 (50–100 ng) was simultaneously expressed with 0–10 ng of TICAM-1 (Fig. 5b). Next, HEK293 cells were transfected with TICAM-1 or TICAM-2 and IRF-3 activation was assessed by native gel migration (Fig. 5c). TICAM-1 (0.3 μg) or TICAM-2 (0.5 μg or more) could activate IRF-3. Under similar conditions, neither HEK293 cells expressing TLR3 (Fig. 5c) nor MyD88 or TIRAP in HEK293 cells as well as HeLa cells (data not shown) induced IRF-3 dimerization in response to poly(I:C). Thus, TICAM-1 and TICAM-2 are largely responsible for IRF-3 activation followed by IFN-β induction. The participation of an IRF-3 bypass factor (for example, MAP kinase-mediated activation of PKR (28)) in induction of IFN-β, however, could not be ruled out.

**TICAM-2 Is Involved in TLR4-mediated IFN-β Production**—To clarify the involvement of TICAM-1/TICAM-2-mediated IFN-β production, TICAM-2-knockdown cells were generated with mouse RAW264.7 macrophage-like cells by siRNA transfection and tested for IFN-β induction in response to LPS (Fig. 6). RAW cells were transfected twice with buffer, nonspecific siRNAs, or two siRNAs for mouse TICAM-2 site A or B (Fig. 6a). The transfected cells were stimulated with LPS and total RNAs were extracted before or 6 h after stimulation, and IFN-β levels induced by LPS were assessed by quantitative PCR. Results show that the mRNA levels of TICAM-2 were suppressed by ~60 or 26% by the site A or site B RNAi target, respectively (Fig. 6b). TICAM-2 knockdown was accompanied by specific inhibition of IFN-β induction by ~60 and 35% by site A or site B siRNA, respectively (Fig. 6c). Functional uncoupling of TICAM-2 with TLR3 was again confirmed in TICAM-2-knockdown cells (Supplemental Materials Annex 3).

LPS-stimulated induction of IFN-β in TICAM-1-depleted RAW cells was examined by a similar RNAi method. The level of TICAM-1 mRNA was suppressed by ~70% by the mouse TICAM-1-specific siRNA in RAW cells (data not shown). Such TICAM-1 knockdown suppressed LPS-mediated IFN-β induction irrespective of the level of TLR3 (Fig. 6d). TICAM-2 site B knockdown also reduced the IFN-β mRNA level by ~60% (Fig. 6d). Thus, both TICAM-1 and TICAM-2 are required for full induction of LPS-mediated IFN-β in macrophage-like cells. LPS-mediated activation of IRF-3 was severely impaired in TICAM-1- or TICAM-2-knockdown cells paralleling suppression of IFN-β induction (Fig. 6e). In TICAM-2 knockdown cells, transfection of TICAM-1 resulted in recovery of IRF-3 activation that is presumably its dimer formation, but in TICAM-1

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**Fig. 3. Association of TICAM-2 with TLR4.** In all panels, immunoprecipitation was carried out with anti-FLAG antibody and blots were probed with anti-HA antibody (upper panel) and reprobed with anti-FLAG antibody (lower panel). a, TLR4, but not TLR2 and TLR3, coprecipitates with TICAM-2. HEK293 cells were transfected with corresponding vectors for expression of the indicated proteins. Lysates from the cells transfected with the indicated vectors were immunoprecipitated (IP) with anti-FLAG antibody or mouse IgG1 (data not shown), and the samples were resolved on SDS-PAGE and immunoblotting (lanes 1–6). The superscript F represents transfection of vectors for expression of FLAG-tagged relevant TLRs. Results with lysates immunoprecipitated (IP) with mouse IgG1 are not shown, because no specific band was observed in samples with FLAG-tagged and untagged TLRs. The trace band of ~50 kDa is nonspecific as it was seen in all lanes. Upper panel, HA-tagged TICAM-2 (arrow); lower panel, FLAG-tagged TLR proteins (parentheses). b, lack of effect of LPS on TLR4-TICAM-2 interaction. HEK293 cells were transfected with vectors for expression of the indicated proteins. Cells were stimulated with LPS in samples of lanes 2 and 4. TICAM-2 levels co-precipitated with TLR4-FLAG were compared between lanes 1 and 2, and lanes 3 and 4. Arrow indicates the TICAM-2 band. The 50-kDa band is nonspecific as seen in panel a. The reprobed blot (stained with anti-FLAG antibody) is shown in the lower panels. Closed arrowhead shows TLR4. c, TICAM-2 co-precipitates with TICAM-1, TICAM-2, and TLR4. HEK293 cells were transfected with vectors for TICAM-1-HA, TICAM-2, TICAM-2-HA, TICAM-2-FLAG, TLR4-Flag + MD-2, and empty vectors. Cells were either stimulated (not shown) or not stimulated with LPS (lanes 1–6). Cell lysates were immunoprecipitated with anti-FLAG mAb (lanes 1–6). TICAM-2 formed homodimers, because TICAM-2-FLAG and TICAM-2-HA were co-precipitated (lane 1). The background TICAM-2-HA level (substituted TICAM-2-FLAG with unlabeled TICAM-2) was minimal under the same conditions (lane 2). TICAM-2 and TLR4 were associated (lane 3). TICAM-2 barely bound TICAM-1 in the absence of TLR4 (lane 4). No association between TICAM-1 and TLR4 was observed (lane 5). TICAM-1-TLR4 interaction was visible in the presence of TICAM-2 (lane 6). The positions of TICAM-1 and TICAM-2 are indicated by arrows. No difference was observed before and after LPS challenge between TICAM-1-TICAM-2 association (data not shown). Reprobed blot stained with anti-FLAG is shown in the lower panel. Position of TLR4 is indicated by an open arrow. d, failure of the P714H TLR4 mutant to recruit TICAM-2 and TICAM-1. The experiment was performed principally as in panel c. HEK293 cells were transfected with the indicated vectors and 24 h later, cells were lysed and immunoprecipitated with anti-FLAG mAb (lanes 1–4). The blot was probed with the anti-HA antibody (upper panel). Positions of TICAM-1 (upper arrow) and TICAM-2 (lower arrow) are indicated. The blot was reprobed with anti-FLAG antibody (lower panel). Positions of TLR4 and its mutant are indicated by an open arrow.
knockdown cells, transfection of TICAM-2 virtually did not (Fig. 6f). These results reinforce our interpretation of the TICAM-1/2 dominant-negative overexpression analyses (Fig. 4): the TICAM-2 is required for the LPS-TLR4-mediated MyD88-independent pathway and TICAM-1 is the critical factor for IRF-3 activation in this pathway. Of note, the tumor

![Image of TICAM-2 overexpression](image1)

**Fig. 4. TICAM-2 overexpression activates NF-κB and IFN-β promoter.** The doses of each vector are indicated below the x-axis. a, HEK293 cells were transfected with empty vector or increasing amounts of expression vector for TICAM-2 with luciferase-linked NF-κB, p-125 luc reporter (IFN-β), and AP-1 reporter plasmids. Twenty-four hours after transfection, luciferase reporter gene activity was assayed and expressed as relative stimulation. Notice the overexpressed TICAM-2, together with endogenous minute TICAM-1, activated NF-κB and the IFN-β promoter, but not AP-1 in HEK293 cells. b, the structures of mutant TICAM-2. The black boxes indicate the TIR domain. TICAM-2-TIR contains the 68–235 amino acid region. Pro-116 and Cys-117 were substituted by His (open circle). c, cells were transfected with TICAM-2 together with NF-κB or IFN-β reporter and dominant-negative forms of TICAM-1, MyD88, or TIRAP where indicated. TICAM-1 inhibited the signal for IFN-β and NF-κB induced by TICAM-2 expression. d, the effects of dominant-negative TICAM-2 on TICAM-1 overexpression. TICAM-2(C117H) did not inhibit the TICAM-1 activity. e, transfection of HEK293 cells with TLR4 plus MD-2 or TLR3 together with NF-κB or IFN-β promoter reporter and with wild-type or mutant TICAM-2, wherever indicated. Cells were stimulated with 100 ng/ml LPS or 50 μg of poly(I-C) for 6 h and luciferase activities were measured. TICAM-2(C117H) (10 and 100 ng) inhibited the induction of transcription from the IFN-β promoter by TLR4 stimulation. The dominant-negative TICAM-2 inhibited activation of both the IFN-β promoter and NF-κB in response to TLR4, but not in response to TLR3 stimulation. f, TICAM-1 TIR(P434H) inhibited activation of NF-κB by TLR3 stimulation and activation of the IFN-β promoter by TLR3 or TLR4 stimulation. TICAM-1 TIR(P434H) did not inhibit activation of NF-κB by function of TLR4, supporting the crucial role of the MyD88-dependent pathway in NF-κB activation. g, overexpression of TICAM-2 and limiting amounts of TICAM-1 to assess whether their effects were synergistic. Doses of TICAM-1 input were in parallel with the levels of IFN-β promoter activation in a range of low doses in HEK293 cells (left bars). The synergy enhanced IFN-β promoter activation in the presence of high doses of TICAM-2 (right bars), suggesting that TICAM-1 is a neck in this pathway.
necrosis factor-α levels were reduced at most by 15% in TICAM-2-knockdown cells (not shown), whereas >60% were reduced in TIRAP- or MyD88-knockdown cells made by the method previously reported (Ref. 12, data not shown). These results are consistent with those obtained with MyD88-deficient and TIRAP-deficient mouse cells (8, 9). Absolute requirement of TICAM-1 for the MyD88-independent pathway was later confirmed by TRIF (TICAM-1)-deficient or -mutant mice (15, 16). Our results revealed the missing link between the essential TICAM-1 for LPS-TLR4-mediated signaling (15, 16) and no physical link of TICAM-1 to TLR4 (12). This, together with the biochemical results (Fig. 3), implies that TICAM-2 is an adapter that physically bridges the TIR domain of TLR4 and TICAM-1 for LPS-mediated activation of IRF-3 followed by induction of IFN-β as part of the MyD88-independent pathway.

**DISCUSSION**

We have identified a novel adapter TICAM-2 that participates in LPS-TLR4-mediated IFN-β promoter activation. TICAM-2 is not ubiquitously expressed and TICAM-2 per se
even overexpressed barely activates AP-1 and only weakly activates NF-κB and the IFN-β promoter. TICAM-2 binds tightly to the TIR domains of TLR4 and TICAM-1, but has weak or no binding affinity for other TLRs and adapters. Requirement of both TICAM-2 and TICAM-1 to complete the LPS-TLR4-triggered IFN-β production was finally confirmed by the RNAi-knockdown analysis (Fig. 6). The point that TICAM-1 is the crucial effector in TLR3 and TLR4 was confirmed after the submission of this study by two groups using gene disruption and forward genetics (15, 16).
functional analyses including the IRF-3 assay, suggest that the TICAM-2–TICAM-1 complex is the adapter that mediates TLR4-triggered activation of IRF-3. Collectively, the most marked properties of TICAM-2 are to physically bridge TLR4 and TICAM-1 and transmit TLR4 signaling to the downstream TICAM-1. TICAM-1 is the effector that exerts potent IFN-β promoter activation in TLR4, as well as TLR3 (12, 14). If at all, the induction of IFN-β is largely supported by TICAM-1-mediated IRF-3 activation in these TLRs. The IFN-β promoter appears to be more potently activated by transfection with TICAM-2 and TICAM-1 than the IRF-3-responsive element (Fig. 4g versus Fig. 5b). It has been reported that in the TLR3 pathway NF-κB and MAP kinases are activated through an IRAK-independent pathway that contains PKR (28). Thus, other factors than IRF-3 may be involved in the TICAM-1-de- pendent IFN-β induction. At least, TIRAP and MyD88 are not responsible for relevant IFN-β induction (8, 9), although they induce robust NF-κB activation with virtually no IRF-3 activation.

Direct binding of TICAM-1 to the tail of TLR3 has been demonstrated in a previous report (12, 14). We have not observed any involvement of TICAM-2 in poly(I-C)-TLR3-mediated IRF-3 activation (Fig. 5c, and Supplemental Materials Annex 3). Thus, at least two modes, direct and indirect binding, sustain the molecular association between TLRs and TICAM-1 (29). Currently, TLR3, TLR4, TLR7, and TLR9 have been reported to induce type 1 IFN. TLR3 provides a typical instance of the direct binding mode and TLR4 represents the indirect binding mode. TICAM-1 is phosphorylated during TLR3 stimulation (12) and we noticed that TICAM-2 is also phosphorylated during TLR4 stimulation. Signal transduction may be retarded and vulnerable for modulation at multiple checkpoints in the indirect mode (30, 31). The modulation of LPS signaling would be related to an unknown part of LPS toler- ance (4, 32). Requirement for TLR signaling in the indirect mode remains to be further investigated.

An adapter protein TIRP (33) was reported after completing this study. TIRP is structurally identical to our reported protein TICAM-2. TIRP has a TRAF6-binding motif PXEXX and actually interacts with TRAF6 (33). Overexpression and dominant-negative analysis in HEK293 cells suggested that TIRP is associated with interleukin-1 receptors and also interacts with kinase-inactive mutants of IRAK-4, IRAK-2, and IRAK-M. The authors reasoned that TIRP functions upstream of IRAK and TRAF6 in the interleukin-1-induced NF-κB activation pathway (33). If this is the case, TIRP/TICAM-2 acts encom- passing the interleukin-1-NF-κB pathway and the TLR-IFN-β pathway. Their results opposing those of our TICAM-2 are that TIRP could not activate the IFN-β promoter (data not shown) and their dominant-negative TIRP (TIRP-(78–171)) did not inhibit TLR4-mediated NF-κB activation. Perhaps, the point mutation at Pro-116 or Cys-117 is essential for the relevant dominant-negative function (Supplemental Materials Annex 2). However, we have no idea in terms of the discrepancy on the TIRP/TICAM-2 function of IFN-β promoter activation. TIRP/ TICAM-2 may be a regulatory adapter that bridges TIR do- mains and other molecules. Gene disruption studies will fur- ther clarify the unique bimodal function of TIRP/TICAM-2.

Thus far, Gram-negative bacterial LPS, lipid A, the anti- tumor agent Taxol, and the respiratory syncytial virus (respi- ratory syncytial virus) F protein are agonists for TLR4 (34, 35). The cell wall skeleton of bacillus Calmette-Guérin (BCG-cell wall skeleton) (36) and listeriolysin O (37) activates TLR2 and TLR4. It is of interest whether the TLR4 ligands other than LPS (including the F protein of respiratory syncytial virus) can induce the genes regulated by IRF-3 and IFN-β (38). Some TLR4 agonists such as BCG-cell wall skeleton do not up-regu- late the IFN-inducible genes mediated via TICAM-1 but acti- vate NF-κB via the MyD88-dependent pathway in macro- phages and DCs (7, 39). Hence, the properties of agonists may affect the selection of the downstream signaling in TLR4. If this is the case, LPS rather than TLR4 itself is fastidious in IFN-β signaling.

The intermolecular complex formed by TLRs, TICAM-2, and TICAM-1 appears analogous to the intermolecular association among TLRs, TIRAP, and MyD88 (29). TIRAP –/– cells show a loss-of-function phenotype similar to MyD88 –/– cells (8, 9). TIRAP probably serves as a linker between TLR2/4 and MyD88, and MyD88 acts as an effector for activation of down- stream signaling. Structural findings also support this idea. MyD88 possesses the death domain (40) that adheres to the death domain of IRAK4 in the IRAK-TRAF6 complex leading to the activation of NF-κB and p38 MAPK/AP-1 (41). On the other hand, TIRAP resembles TICAM-2 in its overall features and has no particular domains in its N and C termini (8, 9), endorsing their common properties as bridging adapters. Unlike TICAM-2, TICAM-1 has unique proline-rich motifs in its N and C termini, and novel molecules bind these stretches. Based on the topology of TIRAP and MyD88, we can delineate the mole- cular association between TICAM-2 and TICAM-1 and thus propose the two important pathways downstream of TLR4 (29). TLR4 recruits two bridging adapters, making it possible to affect NF-κB/MAPK (AP-1) and IRF-3 activation. This is why LPS induces a larger variety of cellular responses than other agonists of TLRs.

LPS is highly toxic to mammals. The first Toll discovered in humans was TLR4 (42). Identification of an LPS-resistant gene as a mutated TLR4 by positional cloning (43) is an important landmark for its physiological importance. In fact, the P714H mutant of TLR4 failed to recruit both TICAM-2/TICAM-1 (Fig. 3d) and TIRAP/MyD88 (3, 44). Molecular complexing involving TLR4 is the most complicated compared with other TLRs. A stable TLR4 dimer is present in conjunction with MD-2 (45) and signals the presence of LPS complexed with lipopolysac- charide-binding protein or bactericidal/permeability increasing protein (46). CD14 on the same cell membrane binds the LPS complex and in turn transfers LPS to TLR4 (47). MD-2 intrin- sically confers species specificity on the recognition of lipid A (48), the active principle of LPS. To our knowledge on the TLR4 scenario, LPS responsiveness is highly sophisticated, which is sustained by a receptor complex encompassing intra- and extracellular regions. At least, the fish lacks TLR4 (49) and TICAM-2 and LPS sensitivity is incomplete in reptiles, am- phibians, and fish (50). The indirect mode of TLR4 signaling probably emerged as a result of evolution of the TLR system.

Many molecules other than those in the TICAM-1-related pathway are implicated in IRF-3 activation. Proteins with RNA-binding motifs, such as PKR (21) and PACT (51), can sense poly(I-C) to induce IFN-β inside cells and are activated secondarily to sensing viral replication. Virus-associated ki- nases (52) have not yet been identified but are present as activators of IRF-3. At present it is not possible to fully depict the IRF-3 activation pathway for IFN-β induction at the mol- ecular level (53). However, TICAM-1-related pathways are being elucidated through recent topics: two reports implicated in the TICAM-1/2-related pathways were recently published (39, 54). The first report is that two TANK-binding kinases, TBK-1 and iκB kinase ε, are involved in TICAM-1-mediated

2 M. Sasai, H. Oshima, M. Matsumoto, and T. Seya, unpublished data.

3 H. Oshima, A. Matsuo, M. Sasai, M. Matsumoto, and T. Seya, unpublished data.
activation of IRF-3 and NF-κB (54). The TANK-binding kinase complex act upstream of IRF-3 (54) and second report (55) suggested that it is a component of virus-associated kinase. A tantalizing point is whether TICAM-1 can directly bind and activate TLR1 and TLR2. IκB kinase ε. Although TRIF (14), i.e. TICAM-1, reportedly coprecipitated with IRF-3, we believe that some other molecules are positioned between TICAM-1 and IRF-3. Some of the TICAM-1-binding proteins we collected would be candidates to satisfy the missing link between TICAM-1 and the TANK-binding kinases.

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