Selective Use of TRAM in Lipopolysaccharide (LPS) and Lipoteichoic Acid (LTA) Induced NF-κB Activation and Cytokine Production in Primary Human Cells: TRAM Is an Adaptor for LPS and LTA Signaling

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*J Immunol* 2007; 178:2148-2154; doi: 10.4049/jimmunol.178.4.2148
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Selective Use of TRAM in Lipopolysaccharide (LPS) and Lipoteichoic Acid (LTA) Induced NF-κB Activation and Cytokine Production in Primary Human Cells: TRAM Is an Adaptor for LPS and LTA Signaling

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TLR signal via Toll-IL-1R (TIR) homology domain-containing adaptor proteins. One of these adaptors, Toll-IL-1R domain-containing adaptor inducing IFN-β-related adaptor molecule (TRAM), has been shown to be essential for TLR4 signaling in TRAM"/" mice and cell lines. Previously, we showed that MyD88 or Mal dominant-negative constructs did not inhibit LPS induction of cytokines in primary human M-CSF-derived macrophages. A possible explanation was redundancy of the adaptors during LPS signaling. TRAM is a suitable candidate to compensate for these adaptors. To investigate a potential role for TRAM in LPS signaling in human M-CSF-derived macrophages, we engineered an adenoviral construct expressing dominant-negative TRAM-C117H (AdTRAMdn). Synovial fibroblasts (SF) and human umbilical endothelial cells (HUVECs) were used as a non-myeloid comparison. AdTRAMdn inhibited LPS-induced signaling in SFs and HUVECs, reducing NF-κB activation and cytokine production, but did not inhibit LPS signaling in M-CSF-derived human macrophages. Further investigation of other TLR ligands showed that AdTRAMdn was also able to inhibit signaling initiated by lipoteichoic acid, a TLR2 ligand, in SFs and HUVECs and lipoteichoic acid and macrophage-activating lipopeptide 2 signaling was also inhibited in TRAM"/" murine embryonic fibroblasts. We conclude that TRAM is an adaptor protein for both TLR4 and TLR2/6 signaling in SFs, HUVECs, and murine embryonic fibroblasts, but cannot demonstrate a role in human macrophages. The Journal of Immunology, 2007, 178: 2148–2154.

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Received for publication February 28, 2006. Accepted for publication November 24, 2006.

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1 This work was supported by The Arthritis Research Council.

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3 Abbreviations used in this paper: LTA, lipoteichoic acid; IRF, IFN regulatory factor; TIR, Toll-IL-1R; Mal, MyD88 adaptor-like; TIRAP, TIR domain-containing adaptor protein; TRIF, TIR domain-containing adaptor-inducing IFN-β; TICAM, TIR-containing adaptor molecule; TRAM, TRIF-related adaptor molecule; SARM, sterile α and HEAT-Armadillo motif; HUVEC, human umbilical endothelial cell; SF, synovial fibroblast; dn, dominant negative; MEF, murine embryonic fibroblast; MOI, multiplicity of infection; Malp, macrophage-activating lipopeptide; IP-10, IFN-inducible protein 10; WT, wild type.

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importance of MyD88 and Mal in primary human myeloid and nonmyeloid cells. It is possible that in this cell system there may be a redundancy between TLR adaptor molecules.

TRAM bears a close structural resemblance to Mal but shares most sequence homology in its TIR domain with TRIF (22). Unlike the other TLR adaptors, TRAM has a cysteine at position 117, where the other adaptors have a conserved proline. This cysteine residue is required for TRAM to form homodimers and to heterodimerize with TRIF (24) (14). TRAM is solely an adaptor for TLR4 signaling, although there is debate whether TRAM functions in IL-1R signaling. Bin et al. (15) were able to immunoprecipitate TRAM with IL-1R and IL-1R accessory protein and show that overexpression of TRAM activated NF-κB but not IFN-β. This was contrary to the finding by Fitzgerald et al. (14) that expression of a TRAMdn C117H inhibited NF-κB activation by LPS, but not IL-1. This finding was confirmed in TRAM knockout cells where responses to dsRNA and IL-1β were normal but LPS signaling was abrogated (22).

In this study, we have assed the involvement of TRAM in LPS signaling in primary human cell systems. We investigated the role of the adaptor molecule TRAM using a C117H dn in M-CSF-derived macrophages, SFs, and HUVECs. We also examined whether TRAM was a specific adaptor for TLR4 or was an adaptor for other TLRs or the IL-1R in primary human cells and TRAM−/− murine embryonic fibroblasts (MEF). The results indicate that there is heterogeneity of the role of TRAM in different cell types, just as there was for MyD88. This emphasizes the concept that signaling pathways may be cell type specific.

Materials and Methods

Reagents

Cell culture reagents used were penicillin-streptomycin, RPMI 1640, and DMEM obtained from Cambrex, indomethacin obtained from Sigma-Aldrich, and FBS obtained from PAA. Human recombinant M-CSF was a gift of Genetics Institute. The TLR ligands used were chloroform-extracted *Escherichia coli* LPS and *PamCyS*-Ser,Lys₄ (Pam3C) obtained from Alexius. LTA obtained from *Bacillus subtilis*, resiquimod (R-848) obtained from InvivoGen, and macrophage-activating lipopeptide (Malp-2) and flagellin (purified) obtained from Alexis. Western blotting was performed with a TRAM Ab designed in our laboratory and raised against an N-terminal peptide corresponding to residues 11–22 (INSQPLSLSWGK RHS) (25). The Ab to IkBα was purchased from Santa Cruz Biotechnology and anti-mouse RHS) (25). The Ab to IkBα was purchased from Santa Cruz Biotechnology and anti-mouse HRP was obtained from DakoCytomation.

Cell culture

Primary human SFs, human umbilical vein endothelial cells (HUVECs) and peripheral blood monocytes were isolated and cultured as previously described (26–29). Macrophages were derived from monocytes after differentiation for 4 days with 100 ng/ml M-CSF as previously described (27). All cell types used in this study showed equivalent levels of infection. Western blotting and EMSA were used to examine TRAM protein expression, equal numbers were lysed directly into SDS-PAGE lysis buffer and proteins were separated on a 10% (w/v) polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane for Western blotting for IkBα. Nuclear extracts (5–10 μg) were examined for NF-κB DNA-binding activity by EMSA as previously described (35).

ELISA

SF, HUVECs, and macrophages were plated and left uninfected or infected with adenovirus for 1 h in serum-free medium, after which the virus was removed and replaced with complete medium. Twenty-four hours after infection, cells were cultured for 18 h. SF and HUVECs were cultured with 20 μg/ml LTA or 10–100 ng/ml LPS in medium. M-CSF macrophages were stimulated with 10 ng/ml Pam3Cys, 1 ng/ml chloroform-extracted *E. coli* LPS, 10 ng/ml flagellin, 3 ng/ml Malp-2, 20 μg/ml LTA, or 1 μg/ml R-848 in complete medium. MEFs were stimulated with 10 ng/ml Pam3C, 100 ng/ml Malp-2, 20 μg/ml LTA, 100 ng/ml LPS, or 20 ng/ml IL-1 for 24 h. All reagents were tested for LPS using the Limulus amebocyte lysate assay from Cambrex (36), all reagents were found to have undetectable levels apart from one of four batches of LTA used in preliminary studies that had <10 pg/ml LPS at the working concentration, which is below the threshold for stimulation of the cells used in this study.

Sandwich ELISAs were used to measure TNF, IL-6, IL-8 (BD Pharmingen), IFN-inducible protein 10 (IP-10) and murine IL-6 (R&D Systems). Absorbance was read on a spectrophotometric ELISA plate reader (Labsystems Multiscan Biochromatic) and analyzed using Ascent software V2.6 (Thermo Labsystems). All results are expressed as the mean cytokine concentration ± SD obtained from triplicate cultures per condition. Cell viability was not significantly affected over this time period when examined

bination with AdEasy1 in BJS138 recombination-competent *E. coli* (30). Recombinant genomes were transfected into the HEK 293 packaging cell line to yield mature recombinant adenoviral particles using Lipofectamine (Invitrogen Life Technologies), according to the manufacturer’s instructions. Adenoviral propagation to high titers was as previously described (31). Two groups have previously used this mutated version of TRAM as a dn construct in TLR signaling (14, 24). Other viruses used were a GFP adenovirus (AdGFP) containing no insert as a control, dn MyD88 (AdMyD88dn), and dn MAL (AddMALdn), generated as previously described (32) and a NF-κB luciferase reporter adenovirus (33).

Adenoviral infection of human primary cells

Macrophages and HUVECs were infected in serum-free medium with adenoviruses at a multiplicity of infection (MOI) of 100. For passaged SF, an MOI of 500 was used, as these cells are more resistant to adenoviral infection. These MOIs have been used in the past by our group and other laboratories resulting in >90% of cells expressing the transgene of interest (27). All cell types used in this study showed equivalent levels of infection.

Western blotting and EMSA

To examine TRAM protein expression, equal cell numbers were lysed directly into SDS-PAGE lysis buffer and proteins were separated on a 10% Tris-HEPES gel from Pierce. For EMSAs, cells were stimulated with either 20 ng/ml IL-1α, 20 μg/ml LTA, or 10–100 ng/ml LPS for 1–2 h 24 h after infection, and cytosolic and nuclear extracts were prepared as described (34). Cytosolic proteins were subsequently separated by SDS-PAGE on a 10% (w/v) polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane for Western blotting for IkBα. Nuclear extracts (5–10 μg) were examined for NF-κB DNA-binding activity by EMSA as previously described (35).
by the MTT assay (Sigma-Aldrich) (37). Experiments were repeated in cells from three separate donors.

**Luciferase assay**

Cells cultured in a 96-well plate were infected with recombinant adenovirus containing a NF-κB luciferase reporter gene at a MOI of 500:1 for SFs or 100:1 for macrophages and HUVECs. The cells were allowed to rest for at least 4 h before an additional infection with replication deficient AdTRAMdn or AdGFP. After stimulation for 6 h, the cells were washed once in PBS and lysed with 100 μl of chloramphenicol acetyltransferase lysis buffer (0.65% (v/v) of Nonidet P-40, 10 mM Tris-HCl (pH 8), 0.1 mM EDTA (pH 8), 150 mM NaCl). Fifty microliters of cell lysate were mixed with 120 μl of luciferase assay buffer (25 mM Tris-phosphate (pH 7.8), 8 mM MgCl2, 1 mM EDTA, 1% (v/v) Triton X-100, 1% (v/v) glycerol, 1 mM DTT, 0.5 mM ATP) in the well of a luminometer cuvette strip. Luciferase activity was measured with a Luminometer (Thermo Labsystems) by adding 30 μl of luciferin (Bright-Glo Luciferase Assay System; Promega) per assay point.

**Statistical methods**

Mean, SD, and statistical significance were calculated using GraphPad version 3 (GraphPad Software). For statistical analysis, a one-tailed Student’s t test of paired data was used with a 95% confidence interval.

**Results**

**Expression and delivery of TRAM**

Endogenous expression of TRAM was detected by RT-PCR (Fig. 1A) in M-CSF macrophages, SFs, and HUVECs. Adenoviral gene transfer of a dn form of TRAM C117H (AdTRAMdn) at an MOI of 100:1 for macrophages and HUVECs and 500:1 for SF, overexpressed the protein in comparison to endogenous levels of TRAM in M-CSF-derived macrophages, SFs, and HUVECs (Fig. 1B).
TRAMdn does not inhibit cytokine production or NF-κB activation in human M-CSF macrophages stimulated with TLR ligands

Previously we showed that AdMyD88dn and AdMALdn did not inhibit LPS signaling in M-CSF-derived macrophages. We hypothesized that this may be due to redundancy between the adaptor proteins in human macrophages. A candidate for this role in LPS signaling is TRAM. An initial investigation of NF-κB activation by EMSA and a Western blot of IkBα showed no inhibition of LPS, LTA, or IL-1 signaling (Fig. 2A). Further investigation with a NF-κB luciferase reporter gene assay in cells stimulated with a range of TLR ligands, Pam3Cys (TLR 1/2), LPS (TLR4), flagellin (TLR5), Malp-2 (TLR2/6), LTA (TLR2), or R-848 (TLR7/8) did not show any inhibition of NF-κB activation (Fig. 2B) or the production of TNF (Fig. 2C), IL-6, or IL-8 (data not shown) in cells expressing TRAMdn.

TRAM is an adapter protein for both TLR2 and TLR4 signaling in synovial fibroblasts

As we had previously observed MyD88 and Mal were required for LPS signaling in human primary SFs (23), we investigated the role of TRAM in TLR2 (LTA) and TLR4 (LPS) signaling in these cells. In contrast to the effect in macrophages, TRAMdn inhibited IL-6 (Fig. 3A) and IL-8 (Fig. 3B) production in response to LPS stimulation. Unexpectedly, we also found that AdTRAMdn inhibited IL-6 (Fig. 3C) and IL-8 production (Fig. 3D) in response to LTA stimulation via TLR2. TRAMdn had its greatest effect on LPS signaling significantly inhibiting IL-6 (p = 0.0069) and IL-8 (p = 0.0103) production but gave a partial yet significant inhibition of LTA stimulation of IL-6 (p = 0.0261) and IL-8 (p = 0.0294) production. As a control to check for nonspecific effects of TRAMdn, we examined flagellin signaling via TLR5 that only uses MyD88 as its adaptor. TRAMdn did not inhibit IL-6 production induced by flagellin (Fig. 3E). Specificity was further confirmed by IL-1 activation of NF-κB in the presence of TRAMdn (see below).

TRAMdn inhibits LPS and LTA induced NF-κB activation in synovial fibroblasts

The cytokines IL-6 and IL-8 are regulated by NF-κB. To establish whether the effect of TRAMdn on cytokine production in cells stimulated by LPS and LTA (Fig. 3) was due to inhibition of this effect.
transcription factor, the degradation of IκBα and the nuclear binding of NF-κB were examined by Western blot and EMSA, respectively. TRAMdn inhibited IκBα degradation in cells stimulated with LPS and LTA but had no effect on IL-1-stimulated cells which were used as a control (Fig. 4A). These results were also confirmed using a NF-κB luciferase assay where we observed significant inhibition of LPS (p = 0.0013) and LTA (p = 0.0033) luciferase activity, while there was no inhibition in cells stimulated by IL-1 (Fig. 4B).

TRAMdn inhibits LPS and LTA induced NF-κB activation in HUVECs

Inhibition of LPS and LTA signaling by TRAMdn was not restricted to SFs. HUVECs, nonmyeloid primary cells similar to SFs require MyD88 and Mal as adaptor proteins in LPS signaling (23). TRAMdn significantly inhibited production of IL-6 (p = 0.001) (Fig. 5A) and IL-8 (p = 0.0047) production in response to LPS stimulation (Fig. 5B). LTA stimulation of IL-6 (p = 0.0079) (Fig. 5C) and IL-8 (p = 0.0012) production was also significantly inhibited (Fig. 5D). A NF-κB luciferase assay was performed on cells stimulated by LPS, LTA, and IL-1 (Fig. 5E). TRAMdn significantly inhibited NF-κB activation in the cells stimulated with LPS (p = 0.0039) and LTA (p = 0.015) but not IL-1. An EMSA was not performed due to the number of cells required.

TLR4 and TLR2/6 but not TLR2/1 signaling is inhibited in TRAM−/− MEFs

TRAM has previously been demonstrated to be an adaptor for TLR4 but not IL-1 (14). To confirm our results from human non-myeloid cells, indicating a role for TRAM in TLR2 signaling, we investigated the role of TRAM in MEFs from gene-deficient mice. Cells were stimulated with IL-1 and LPS as controls. The TRAM−/− cells showed impaired LPS induced IL-6 (p = 0.0013) compared with the wild-type (WT) cell but there was no significant difference in IL-1-stimulated WT and TRAM−/− cells. The TLR2/1 ligand Pam3C also showed no significant inhibition but IL-6 production. However, IL-6 production was inhibited in TRAM−/− cells stimulation with the TLR2/6 ligands LTA (p = 0.0077) and Malp-2 (p = 0.0011) (Fig. 6).

Discussion

This is the first study in primary human cells to investigate the role of TRAM in endogenous TLR signaling. Previously, we observed that whereas MyD88 and Mal are necessary for LPS signaling in SFs and HUVECs, this was not the case for M-CSF macrophages (23). Compensation by TRIF or TRAM may explain this finding in macrophages. On investigation TRAM was found to also be required for LPS signaling in SFs and HUVECs, but not in macrophages. Macrophages infected with constructs of AdTRAMdn, AdMyD88dn, and AdMaldn still produced cytokines in response to LPS. Inhibition of TRIF or other signaling molecules may also be necessary to effectively block LPS signaling in macrophages but has not been investigated here, as the first reported dn of TRIF (the TIR domain of TRIF), was found to be nonspecific (12). However, AdTRAMdn inhibited both LPS and LTA induced cytokine production and NF-κB activation in SFs, HUVECs, and MEFs, reinforcing the heterogeneity of signaling in different cell lineages.

TRAM is able to activate both IRFs and NF-κB (14, 16, 22). To assess the role of TRAM in signaling to IRFs, we measured IP-10, but observed that LPS induction of IP-10 was not inhibited by TRAMdn in macrophages. IP-10 is poorly induced if at all by TLR2 stimulation in macrophages (38), while SFs and HUVECs did not produce detectable levels of IP-10 in response to LPS, in agreement with previous publications (39). Thus, we were unable to show a role for TRAM in the MyD88-independent pathway. The arrangement of the TLR signalosome in macrophages may differ from SFs and HUVECs and might necessitate a more potent inhibition to successfully suppress signaling. AdMyD88dn is able to inhibit IL-1R signaling in macrophages (23) and AdMyD88dn or AdMaldn can inhibit TLR signaling in macrophages when stimulated with other TLR ligands, for example LTA, indicating that the dn can indeed inhibit signaling in macrophages.

Our data differs from studies in TRAM-deficient murine peritoneal macrophages (22) and studies using a TRAMdn in 293 HEK/TLR2 cells (14) which do not show a role for TRAM in TLR2 signaling initiated by peptidoglycan or Malp-2. To confirm a role for TRAM in TLR2 signaling, we repeated these experiments in WT and TRAM−/− MEFs. The results confirmed that TRAM was required for LPS as has been previously demonstrated but also showed a selective requirement for TLR2/6 signaling but not TLR2/1 signaling. The disparity between this study and other published data on TRAM use may be due to the use of different cell systems, Fitzgerald et al. (14) did not observe a role for TRAM in TLR2-transfected HEK293 cells stimulated with Malp-2, however, this could be due to a requirement for TLR6 and/or CD36 to be present for recruitment of TRAM. TRM6 has been shown to be required for MEF stimulation by Malp-2 (40) and cotransfection of CD36 with TLR2/6 has been reported to amplify LTA-induced TNF production in HEK293 cells (41). All of our work was performed in primary cells for closer relevance to the clinical situation.

Previous observations have revealed differences in the requirement of adaptors between cell types (23) and other groups have demonstrated that different ligands for the same TLR can activate different combinations of adaptors depending on the presence of cell surface cofactors. Functional CD14 was essential for LTA signaling but a mutated version of CD14 only partially inhibited Malp-2 and had no effect on Pam3C-induced production of TNF (42). This different requirement by TLR2 ligands for CD14 may be translated to their use of the adaptor proteins. Interestingly, TLR2 mediated induction of secretary leukocyte protease inhibitor stimulated by Mycobacterium tuberculosis has been shown to be MyD88, Mal, and TRIF independent (43), suggesting the possibility of TRAM or an as yet unidentified molecule being the adaptor. Another indication that TRAM could signal from TR2 comes from the observation that TRAM interacts with Mal or a Mal (P125H) mutant in transfected 293T cells as shown by immunoprecipitation (14). Although yeast two-hybrid experiments did not show association between Mal and TRAM (24), a direct interaction may go undetected by yeast two-hybrid screening if either adaptor requires phosphorylation for the interaction to occur.
IL-1 was used as a control in EMSAs, Ixβo degradation and NF-κB luciferase assays in SFs and in stimulation of cytokine production from MEFs. AdTRAMdn inhibited TLR signaling but did not inhibit IL-1 stimulation. TRAM does not appear to be an adaptor for the IL-1R. This result along with the finding that TRAMdn did not inhibit flagellin (TLR5) in SFs suggests that TRAMdn is not inhibiting TLR2 due to a nonspecific interaction with other TIR-containing molecules. To allay any concerns about LPS contamination or LTA signaling through TLR4, batches of LTA were tested for LPS using the Limulus amebocyte lysate assay and IP-10 was measured in macrophages stimulated with LPS or LTA. Only cells stimulated with LPS produced IP-10 (data not shown), which suggests that LTA does not stimulate TLR4 or have LPS contamination. Preliminary data has shown that LPS but not LTA induced TNF from TLR2−/− bone marrow-derived macrophages when compared with WT cells (data not shown). We also discovered that AdMyD88dn and AdMaldn were able to inhibit LTA induction of TNF in macrophages, whereas we have previously shown that AdMyD88dn and AdMaldn do not inhibit LPS induction of TNF in macrophages (23) also suggesting LTA does not signal through TLR4. LTA from B. subtilis has previously been reported as a TLR2 ligand that does not signal through TLR4 (41, 44).

In summary, this study confirms the role of TRAM in LPS signaling and shows for the first time a role in TLR2/6 signaling in SFs, HUVECs, and MEFs. The TLR adaptor protein(s) required for LPS signaling in human macrophages remains unclear, but the data suggests that a more complex LPS-signaling mechanism may exist in these cells. TRIF is still a candidate that requires investigation, but it is important to consider the role of other signaling molecules, e.g., kinases. Identifying the adaptors involved in TLR4 signaling in macrophages is now crucial as these cells have an important role in infectious and inflammatory diseases.

Acknowledgments

We thank Profs. Douglas Golenbock and Dr. Kate Fitzgerald from the University of Massachusetts Medical School (Worcester, MA) for providing the TRAM construct used in this study and their helpful comments. TRAM−/− MEFs were also donated by Dr. K. Fitzgerald (University of Massachusetts) from mice generated by Prof. S. Akira (Osaka University, Japan). We also express gratitude to Dr. John Simms from Amgen for providing the original plasmid for the dn of MAL.

Disclosures

The authors have no financial conflict of interest.

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