TRAM Is Required for TLR2 Endosomal Signaling to Type I IFN Induction

Julianne Stack, Sarah L. Doyle, Dympna J. Connolly, Line S. Reinert, Kate M. O'Keeffe, Rachel M. McLoughlin, Søren R. Paludan, and Andrew G. Bowie

Detection of microbes by TLRs on the plasma membrane leads to the induction of proinflammatory cytokines such as TNF-a, via activation of NF-kB. Alternatively, activation of endosomal TLRs leads to the induction of type I IFNs via IFN regulatory factors (IRFs). TLR4 signaling from the plasma membrane to NF-kB via the Toll/IL-1R (TIR) adaptor protein MyD88 requires the TIR sorting adaptor Mal, whereas endosomal TLR4 signaling to IRF3 via the TIR domain–containing adaptor-inducing IFN-b (TRIF) requires the TRIF-related adaptor molecule (TRAM). Similar to TLR4 homodimers, TLR2 heterodimers can also induce both proinflammatory cytokines and type I IFNs. TLR2 plasma membrane signaling to NF-kB is known to require MyD88 and Mal, whereas endosomal IRF activation by TLR2 requires MyD88. However, it was unclear whether TLR2 requires a sorting adaptor for endosomal signaling, like TLR4 does. In this study, we show that TLR2-dependent IRF7 activation at the endosome is both Mal- and TRAM-dependent, and that TRAM is required for the TLR2-dependent movement of MyD88 to endosomes following ligand engagement. TRAM interacted with both TLR2 and MyD88, suggesting that TRAM can act as a bridging adapter between these two molecules. Furthermore, infection of macrophages lacking TRAM with herpes viruses or the bacterium Staphylococcus aureus led to impaired induction of type I IFN, indicating a role for TRAM in TLR2-dependent responses to human pathogens. Our work reveals that TRAM acts as a sorting adaptor not only for TLR4, but also for TLR2, to facilitate signaling to IRF7 at the endosome, which explains how TLR2 is capable of causing type I IFN induction.

The mammalian innate immune system responds to invading pathogens by using pattern recognition receptors such as TLRs to detect conserved pathogen associated molecular patterns. The activation of TLRs initiates signal transduction pathways that determine the type and duration of the host anti-pathogen and inflammatory response (1–3). Upon encountering their cognate PAMP, TLR homodimers or heterodimers become active and recruit downstream signaling proteins.

For example, LPS binding to the TLR4 complex causes recruitment of the MyD88 adaptor–like (Mal) protein and Toll/IL-1R (TIR) domain–containing adaptor-inducing IFN-b (TRIF)–related adaptor molecule (TRAM). Mal and TRAM are bridging and sorting adaptors that recruit and control the localization of the signaling adaptors MyD88 and TRIF, respectively, to TLR4 (4–8). A TLR4/Mal/MyD88 complex is formed at the plasma membrane because of an N-terminal localization domain in Mal that interacts with phosphatidylinositol-4,5 bisphosphate in the plasma membrane (6). This complex mediates MyD88-dependent signaling from the plasma membrane, via IL-1R–associated kinases and TNFR-associated factor (TRAF) 6, leading to activation of MAPKs and of the transcription factors AP-1 and NF-kB. In contrast to Mal, TRAM contains a bipartite N-terminal myristoylation motif and polybasic domain that regulates the intracellular location of TRAM (7). Both domains are required for plasma membrane targeting of TRAM, whereas the myristoylation motif is required for TRAM to localize at endosomes (7, 9). Thus a TLR4/TRAM/TRIF complex is formed at the membrane of endosomal compartments, and this signals via TRAF3 to activate the transcription factor IFN regulatory factor 3 (IRF3) (7). For TLR4 signaling, Mal-dependent NF-kB activation upregulates inflammatory genes such as TNF-a, whereas TRAM-dependent IRF3 activation causes induction of IFN-b.

Apart from TLR4, several other TLRs can signal from endosomes to induce type I IFNs (IFN-a and IFN-b), in response to the detection of viral nucleic acids (10). Thus TLR3

recognizes dsRNA, TLR7 and TLR8 recognize single-stranded RNA, and TLR9 recognizes CpG motifs in DNA (11). For TLR3, type I IFN induction is achieved with TRIF and IRF3, whereas for TLR7, TLR8, and TLR9 the induction pathway involves MyD88- dependent IRF7 activation (10).

Whereas TLR4 responds to LPS from Gram-negative bacteria, recognition of cell surface components of Gram-positive bacteria, such as lipoproteins and lipoteichoic acids, require TLR2 (12). The fatty acid groups of triacylated lipopeptides are the ligand for TLR2/TLR1 heterodimers (13), and the fatty acid groups of diacylated lipopeptides and LTA are ligands for TLR2/TLR6 heterodimers (14, 15). Similar to TLR4 signaling, Mal acts as a bridging adaptor between the TLR2 receptor complex and MyD88, although high TLR2 ligand concentrations can overcome the requirement for Mal in the signaling pathway, whereas some downstream TLR2 signals are entirely Mal-independent (16, 17).

Although TLR2 is best known for its role in recognizing bacterial and fungal cell wall components, it also plays a role in the immune response to viruses. Such responses could be due to direct recognition of viral PAMPs by TLR2 or production of virally induced endogenous TLR2 ligands. Thus, glycoprotein B from human CMV activates TLR2 signaling (18, 19), whereas mouse CMV (20), HSV types 1 and 2 (21, 22), hepatitis C virus (23), lymphocytic choriomeningitis virus (24), measles virus (25), and vaccinia virus (VACV) (26) are also able to elicit TLR2-dependent responses. Activation of TLR2 may benefit the virus; for example, measles virus may have evolved the ability to activate TLR2 as a means of upregulating the viral entry receptor CD150 (25). However, in other instances, TLR2 activation contributes to protection; for example, mice lacking TLR2 are impaired in their ability to mount an innate or adaptive immune response to VACV (26).

It was originally thought that TLR2/TLR1 and TLR2/TLR6 heterodimers elicited proinflammatory, but not type I IFN responses, after ligand engagement (7, 27–30). However, later studies demonstrated that bacterial TLR2 ligands can induce type I IFN responses, whereas live virus-induced type I IFN has been shown to be at least partially TLR2-dependent in the case of VACV, mouse CMV, and murine gammaherpesvirus-68 (MHV68) (31–33). Compared to the mechanism whereby TLR4 and the endosomal TLRs signal to type I IFN induction, much less is known about how TLR2 induces these IFNs. Dietrich et al. (31) showed that, upon stimulation with bacterial TLR2 ligands, the receptor is internalized and transported into endolysosomal compartments from where it induces IFN-b via MyD88 and IRF7. Inhibition of receptor internalization or endosomal acidification could block the induction of IFN-b and IFN-inducible genes, but not proinflammatory cytokines like TNF-a. This finding suggests that TLR2 activation, similar to TLR4, induces proinflammatory and type I IFN responses from distinct subcellular sites: the plasma membrane and endolysosomal compartments, respectively (31). However, apart from the role of MyD88 and IRFs, the signaling pathway of TLR2-dependent production of type I IFNs has yet to be elucidated, and in particular it is unclear whether other TIR adaptor proteins apart from MyD88 are required.

In this article, we show that as well as MyD88, both Mal and TRAM are required for TLR2-stimulated IFN-b induction. TLR2-induced IFN-b but not TNF was sensitive to VIPER (viral inhibitory peptide of TLR4), a viral peptide inhibitor of Mal and TRAM. VIPER is derived from the VACV protein A46 which inhibits TLR4 signaling in the context of a virus infection by disrupting TLR4:Mal and TLR4:TRAM interactions (34, 35). Previously, VIPER was shown to associate with Mal and TRAM, but not MyD88 or TRIF (36), and it is derived from a region of A46 shown to be essential for TLR4 inhibition and to mediate an A46:TRAM interaction (35, 37). In this study, TLR2-induced IFN-b but not TNF-a–required endocytosis. This was impaired in cells lacking Mal or TRAM, whereas TLR2-stimulated IRF7 activation

was blocked by VIPER and required both Mal and TRAM. We demonstrate that stimulation of cells with a TLR2 ligand led to mobilization of MyD88 to intracellular punctate structures in a TRAM-dependent manner. In addition, TRAM interacted with TLR2 and MyD88, suggesting that TRAM acts as a bridging adapter between these two molecules. Furthermore, infection of macrophages lacking TRAM with herpes viruses led to impaired induction of type I IFN, indicating a role for TRAM in this TLR2-dependent pathway, whereas Staphylococcus aureus– stimulated IFN-b was also TRAM-dependent. Thus, TRAM acts as a sorting adaptor not only for TLR4, but also for TLR2, to facilitate signaling to IRF7 at the endosome, which explains how TLR2 is capable of causing type I IFN induction in response to both viral and bacterial pathogens.

Materials and Methods

Cell culture

HEK293T cells were purchased from European Collection of Animal Cell Cultures (Salisbury, U.K.). HEK293 cells stably transfected with TLR4, MD2, and CD14 (HEK293-TLR4) were purchased from InvivoGen (San Diego, CA). HEK293 cells stably transfected with TLR2 (HEK293-TLR2) were a gift from Dr. K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). Immortalized murine wild type (WT), MyD882/2, Mal2/2, TRIF2/2, and TRAM2/2 bone marrow–derived macrophages (iBMDMs) were generated from corresponding knockout mice using J2 recombinant retrovirus carrying v-myc and v raf/mil oncogenes as described previously (17, 38); they were a gift from K. Fitzgerald and D. Golenbock (University of Massachusetts Medical School, Worchester, MA). Cells were maintained in DMEM containing 10% (v/v) FCS, 10 mg/ml Ciproflaxin, and 2 mM L-glutamine. Selection agents were used as follows: HEK293-TLR4 cells, 10 mg/ml Blasticidin

(Sigma), and 50 mg/ml of HygroGold (InvivoGen); HEK293-TLR2 cells, 1 mg/ml G-418 (Sigma).

Receptor agonists and reagents

Ultrapure LPS from Escherichia coli (99.9% pure in respect to contaminating protein, DNA, and TLR2 agonists) was purchased from Alexis Biochemicals (Plymouth Meeting, PA). N-palmitoyl-S-dipalmitoylglyceryl Cys-Ser-(lys)4 (Pam3CSK4) and macrophage-activating lipopeptide-2 (Malp2) were purchased from InvivoGen (San Diego, CA). Bafilomycin A was purchased from Sigma. Synthetic dsDNA 60-mer derived from nucleotides 144107–144166 of the HSV-1 genome (HSV 60 mer), was obtained from DNA Technology (Aarhus, Denmark). Mouse anti-Flag M2 and anti–b-actin Abs were obtained from Sigma, and rabbit anti-EEA1 was obtained from Abcam (Cambridge, U.K.).

Peptide synthesis and reconstitution

Peptides were synthesized by GenScript (Piscataway, NJ) and were . 95% pure as confirmed by HPLC. Lyophilized peptides were reconstituted aseptically with molecular biology-grade water to a concentration of 10 mM and stored at 280°C.Working stocks of 0.2 or 1 mM were stored at 220°C or kept at 4°C for a maximum of 2 wk.

Plasmids

Sources of expression plasmids were as follows: pCMV-myc empty vector (Clontech, MountainView, CA); phRL-TK vector (Promega, Madison, WI); pFR-luciferase reporter gene (Stratagene/Agilent Technologies, Cork, Ireland); Gal4-IRF3, Gal4-IRF7, Flag-MyD88, and Flag-Mal (K.A. Fitzgerald, The University of Massachusetts Medical School, Worcester, MA); Flag-TRIF (S. Sato, Research Institute for Infectious Diseases, Osaka University,

Japan); Flag-TLR2, CFP-MyD88, GFP-TRAM, YFP-TLR2, YFP-TLR6, glutathione S-transferase (GST), and the GST fusion of TRAM (L.A. O'Neill, Trinity College Dublin, Dublin, Ireland); and the NF-kB reporter gene (described in Ref. 39). Flag-TRAM G2A, which contains a point mutation in the myristoylation motif, was generated from WT Flag-TRAM plasmid using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene).

Quantitative RT-PCR

For mRNA analysis, iBMDMs were seeded at 2 3 105 cell/ml in 24-well plates 24 h prior to treatment. RNA was isolated using High Pure RNA isolation kit from Roche Applied Science (Burgess Hill, U.K.) according to the manufacturer's instructions. RT-PCR was performed using the One-Step RT-PCR Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Quantitative real-time PCR was done using GoTaq qPCR Master Mix (Promega) and the 7500 Fast Real-time PCR System (Applied Biosystems) with the following primers: mTNFa forward, 59-TCCCCAAAGGGATGAGAAGTT-39, and reverse, 59-GTTTGCTACGACGTGGGCTAC- 39; mIFNb forward, 59 ATGGTGGTCCGAGCAGAGAT- 39, and reverse, 59-CCACCACTCATTCTGAGGCA-39. To measure knockdown of Mal or TRAM mRNA by treatment with siRNA (described below), cDNA from HEK293-TLR2 cells was prepared as above and analyzed with qPCR using the following primers: hTRAM forward, 59-TTCCTGCCCTCTTTCTCTCTC-39, and reverse 59-AACATCTCTTCCACGCTCTGA- 3; hTIRAP/Mal forward, 59-CCAGCCTTTCA-CAGGAGAAG-39, and reverse, 59-ATATTCGGGATCTGGGGAAG-39. Relative mRNA expression was calculated using the comparative CT method, normalizing the gene of interest to the housekeeping gene b-actin, and comparing it to an untreated sample as calibrator.

Cytokine analysis

For cytokine production, iBMDMs were seeded at 2 3 105 cell/ml in 96-well plates 24 h prior to treatment. The supernatants were collected and assessed for TNF-a by ELISA (R&D Systems, Minneapolis, MN). IFN-b protein in cell culture supernatants was measured using a custom ELISA originally described elsewhere (40), with a few modifications. In brief, high-binding 96-well polystyrene plates were coated overnight with a 1:1000 dilution of rat anti–mouse IFN-b mAb (Santa Cruz, Heidelberg, Germany) in carbonate buffer (10 mM NaHCO3, 3.4 mM Na2CO3, pH 9.4– 9.6) at 4°C. Plates were washed three times and then blocked with 10% FCS/PBS for 2 h at 37°C. The blocking solution was removed, and 40 ml recombinant IFN-b standard (PBL Biomedical Laboratories, Piscataway, NJ) in triplicates and 1:2 serial dilutions in 10% FCS/PBS starting at 20 U/ml was applied to the plate; 40 ml sample supernatant (undiluted or diluted 1:2 with 10% FCS/PBS) was added to each well and incubated at 4°C overnight. The next day, the plates were washed and incubated overnight with 50 ml/well of rabbit anti-mouse IFN-b pAb (PBL Biomedical Laboratories) diluted 1:2000 in 10% FCS/PBS. After washing, the plates were incubated for 2 h with 50 ml/well of anti-rabbit HRP (Sigma-Aldrich) diluted 1:2000 in 10% FCS/PBS. The plates were washed again and developed with TMB substrate as usual. Experiments were performed three times in triplicate, and data are expressed as mean 6 SD from one representative experiment.

Reporter gene assays

HEK293-TLR4 cells (4 3 104 cells/well) or HEK293-TLR2 cells (2 3 104 cells/well) were seeded into 96-well plates and transfected 24 h later with expression vectors and luciferase reporter genes using GeneJuice (Novagen/Merck, Nottingham, U.K.). For the NF-kB assays, 60 ng kBluciferase reporter gene was used. For the IRF3 and IRF7 assays, IRF3- Gal4 and IRF7-Gal4 fusion vectors (1–3 ng) were used in combination with 60 ng pFR luciferase

reporter as described previously (34). In all cases, 20 ng/well of phRL-TK reporter gene was cotransfected to normalize data for transfection efficiency. The total amount of DNA per transfection was kept constant at 230 ng by the addition of pCMV-Myc. After 24 h, cells were stimulated with the indicated TLR ligands. After an additional 6 h, cells were lysed in Passive Lysis Buffer (Promega), and whole cell lysates were analyzed for luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity, and data are expressed as the mean fold induction, relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Transfection of HEK293-TLR cells with siRNA

HEK293-TLR4 cells (1 3 105 cells/ml) or HEK293-TLR2 (0.5 3 105 cells/ml) cells were seeded into 96-well plates and transfected 24 h later with 50 nM control siRNA or siRNA targeting TRAM or Mal (ONTARGETplus Dharmacon siRNA from Thermo Scientific) using Lipofectamine (Invitrogen). A second transfection was then done 24 h later with relevant reporter assay constructs as described above. NF-kB luciferase activity or IRF7-Gal4 transactivation was measured 24 h later after the relevant stimulations. To measure knockdown of Mal or TRAM mRNA after siRNA treatment, HEK293-TLR2 cells (0.5 3 105 cells/ml) were seeded into 24-well plates and transfected with 50 nM siRNA as above. After 48 h transfection, cDNA was prepared from these cells and Mal or TRAM mRNA measured by qPCR. To measure knockdown of Mal or TRAM protein after siRNA treatment, HEK293-TLR2 cells (0.5 3 105 cells/ml) were seeded into six-well plates and transfected with 50 nM siRNA as above. A second transfection was then done 24 h later with 1 mg of either Flag-Mal or Flag-TRAM. Cell lysates were prepared 24 h later and analyzed with SDS-PAGE and immunoblotting.

Confocal microscopy

HEK293 cells were transfected with plasmids expressing YFP and CFP fusion proteins using GeneJuice (Novagen/Merck, Nottingham, U.K.) according to the manufacturer's protocol. iBMDM cells were transfected with plasmids using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Media was replaced 24 h after transfection and cells were left to recover for an additional 24 h. Cells were treated with TLR agonists as required. For intracellular staining, the cells were fixed with 2% paraformaldehyde in PBS, incubated for 15 min on ice, permeabilized with PEM buffer (80 mM K-Pipes [pH 6.8], 5 mM EGTA, 1 mM MgCl2, 0.05% saponin) for 15 min on ice, quenched of free aldehyde groups in 50 mM NH4Cl with 0.05% saponin for 5 min, and blocked in PBS with 10% FCS and 0.05% saponin for 20 min. The cells were incubated with 5 mg/ml primary Ab in PBS with 0.05% saponin for 60 min at room temperature. Alexa Fluor-labeled secondary Abs (Invitrogen) were added for 30 min at room temperature, and cells were washed three times in PBS with 0.05% saponin. Images were captured using a confocal laser scanning microscopy (Olympus FluoView TM FV1000) at 603 original magnification.

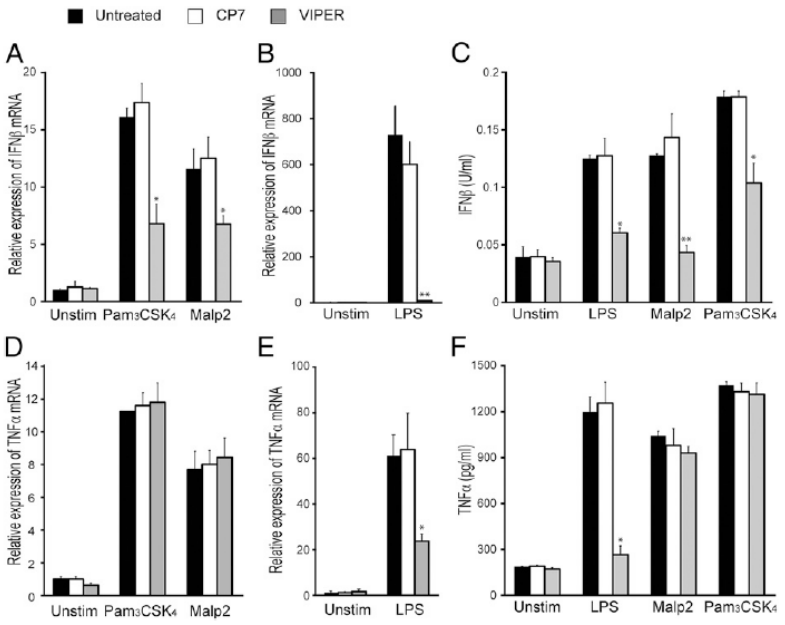

FIGURE 1. VIPER inhibits TLR2-dependent IFN-β induction. (**A**, **B**, **D**, and **E**) WT iBMDMs were treated with 5 μM VIPER or CP7 peptide 1 h prior to being stimulated with 20 nM Malp2, 20 ng/ml Pam_3CSK_4, or 100 ng/ml LPS for 3 h. Induction of IFN-β (A and B) and TNF-α (D and E) mRNA expression was assayed by quantitative RT-PCR, normalized to β-actin, and presented relative to untreated, unstimulated cells. (**C** and **F**) Cells were treated as described above except that TLR stimulations were for 24 h. Production of IFN-β (C) and TNF-α (F) protein was assayed by ELISA. For (A–F) the data are mean ± SD of triplicate samples and are representative of at least three independent experiments. $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$ compared with samples treated with control peptide (CP7).

GST pull-down assays

Empty pGEX.4T2 or pGEX.4T2 plasmid containing TRAM were transformed into Escherichia coli Rosetta-Gami B Host Strains (Novagen/Merck, Nottingham, U.K.) and grown in Luria–Bertani broth. Protein expression was induced with 0.5 mM IPTG at 30°C. Bacterial cells were harvested by centrifugation after 6 h induction and were lysed using BugBuster (Merck/ Millipore, Nottingham, U.K.). Insoluble fractions were removed by centrifugation. The remaining soluble fractions were cleared by glutathione Sepharose 4B affinity chromatography (Amersham Biosciences) and levels of protein expression confirmed by SDS-PAGE and Coomassie staining of the gel. HEK293T cells were seeded into 15-cm dishes (3 3 106 cells) 24 h before transfection with GeneJuice. Cells were transfected with the relevant signaling molecule or pCMV-myc plasmid. Cells were harvested after 48 h in 850 ml lysis buffer (50 mM HEPES, pH 7.5, 250 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40 containing 0.01% aprotinin, 1 mM sodium orthovanadate, and 1 mM PMSF) for 30 min on ice. Whole cell lysates were clarified by centrifugation. Fifty microliters of cleared lysate was retained for analysis of protein expression (i.e., input lysate), and the remainder was divided in two and added to either purified GST or purified GST-fusion protein coupled to glutathione-sepharose and incubated for 2 h at 4°C. The immune complexes were precipitated and washed four times in lysis buffer. Pull-downs were analyzed with SDS-PAGE and immunoblotting.

IFN-a/b bioassay of virally infected bone marrow-derived macrophages

The viruses used were HSV-1 (F+ strain), HSV-2 (333 strain), MHV68, and Sendai virus (Cantell strain). HSV-2 and HSV-1 were amplified in Vero cells, whereas MHV68 was amplified in BHK-21 cells. iBMDMs were seeded in 48-well plates at a density of 4 3 105 cells/well and were infected with viruses or transfected with Lipofectamine alone or with

dsDNA as indicated. IFN-a/b bioactivity was measured with an L929 cell-based bioassay. L929 cells (2 3 104 cells/well in 100 ml) in MEM with 5% FCS were incubated overnight at 37°C in successive 2-fold dilutions of samples or murine IFN-a/b as standard. Subsequently, vesicular stomatitis virus (VSV/V10) was added to the wells, and the cells were incubated for 2–3 d. The dilution mediating 50% protection was defined as 1 U/ml IFN-a/b (41).

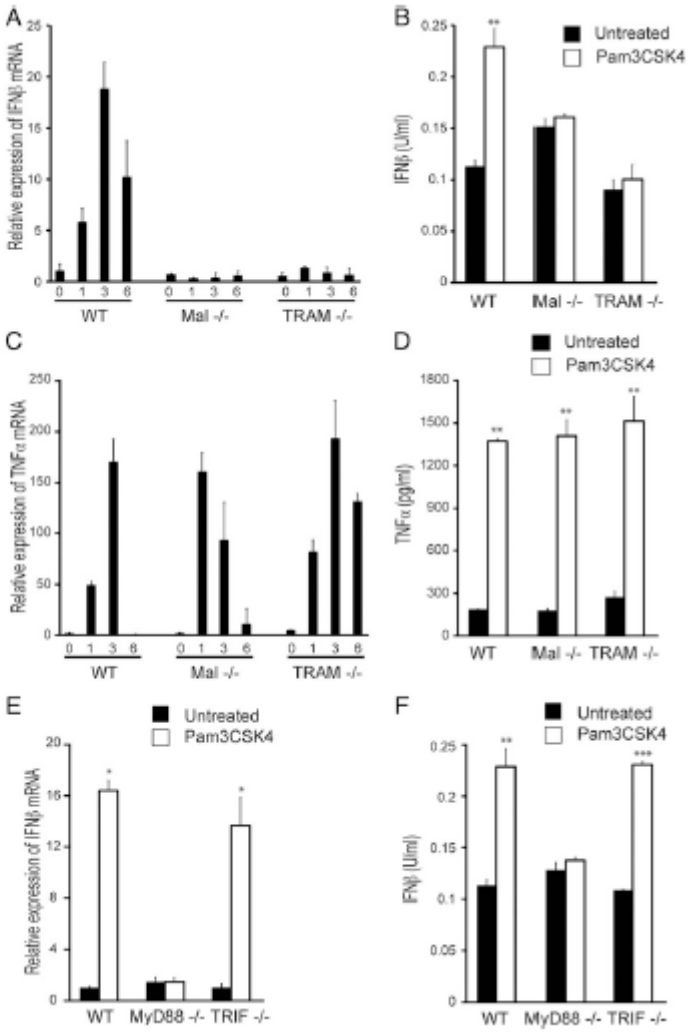

FIGURE 2. Mal and TRAM are required for TLR2-dependent IFN-β induction. (**A** and **C**) WT, $Mal^{-/-}$ and $TRAM^{-/-}$ iBMDMs were stimulated with 20 ng/ml Pam_3CSK_4 for 1, 3, and 6 h. Induction of IFN-β (A) and TNF-α mRNA (C) expression was assayed with quantitative RT-PCR, normalized to β-actin, and presented relative to untreated, unstimulated cells. (**B**, **D** and **F**) WT, $Mal^{-/-}$, $TRAM^{-/-}$, $MyD88^{-/-}$, and $TRIF^{-/-}$ iBMDMs were stimulated with 20 ng/ml Pam_3CSK_4 for 24 h. Production of IFN-β (B and F) and TNF-α (D) protein was measured by ELISA. (**E**) WT, $MyD88^{-/-}$, and $TRIF^{-/-}$ iBMDMs were stimulated with 20 ng/ml Pam_3CSK_4 for 3 h. Induction of IFN-β mRNA expression was assayed by quantitative RT-PCR, normalized to β-actin and presented relative to untreated, unstimulated cells. The data are mean ± SD of triplicate samples and are representative of at least three independent experiments. $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$ compared with untreated samples.

Infection of BMDMs with S. aureus

S. aureus strain SH1000 has been described previously (42, 43). Bacteria were cultivated from frozen stocks for 24 h at 37°C on agar plates. Bacterial suspensions were then prepared in PBS, and the concentrations were estimated by measuring the absorbance of the

suspension at 600 nm. iBMDM cells were infected with live S. aureus at multiplicities of infection (MOIs) of 10 and 100 for the indicated times as previously described (43). Supernatants were then collected and assayed for IFN-b by ELISA.

Statistical analysis

Statistical analysis was performed using paired Student t test.

Results

VIPER inhibits TLR2-dependent IFN-b production

We previously showed that the peptide VIPER derived from the VACV protein A46 could block TLR4-dependent gene induction by antagonizing TRAM and Mal, but not MyD88 nor TRIF (35–37). Consistent with this result, VIPER did not inhibit TNF-a production mediated by TLRs 2, 3, and 9 (36). Because stimulation of TLR2/1 and TLR2/6 heterodimers with Pam3CSK4 or Malp2, respectively, leads to production of type I IFNs via a signaling pathway that has yet to be fully elucidated, we tested the effect of VIPER on this pathway. Stimulation of iBMDM cells with Pam3CSK4 or Malp2 led to induction of IFN-b mRNA, which was significantly inhibited by pretreatment of cells with VIPER, but not with CP7 control peptide (Fig. 1A). As expected from previously published work (36), VIPER, but not CP7, could also impair induction of LPS-induced IFN-b mRNA (Fig. 1B). Although LPS stimulation induces a much higher level of IFN-b mRNA in WT iBMDMs than either Pam3CSK4 or Malp2, a similar level of IFN-b protein was produced by these cells in response to all three ligands, and VIPER significantly inhibited production of IFN-b for each of these ligands (Fig. 1C). In contrast, only LPS-induced TNF-a mRNA and protein (Fig. 1E, 1F), and not Pam3CSK4- or Malp2-induced TNF-a mRNA and protein (Fig. 1D, 1F), were

inhibited by VIPER treatment of cells. These data demonstrate that VIPER can impair TLR2-dependent production of IFN-b but not TNF-a, which was suggestive of a role for Mal, TRAM, or both in TLR2-induced type I IFN.

Mal and TRAM are required for TLR2-dependent IFN-b production

To determine whether TRAM or Mal are required for TLR2- mediated IFN-b production, iBMDMs deficient in Mal or TRAM were examined for their ability to produce IFN-b mRNA and protein in response to Pam3CSK4. Time course analysis of mRNA induction showed that Pam3CSK4 induced IFN-b transcription in WT iBMDMs with a rapid kinetic, peaking at 3 h (Fig. 2A), as shown previously (31). However, Mal2/2 or TRAM2/2 iBMDMs were incapable of IFN-b mRNA induction in response to Pam3CSK4 (Fig. 2A), indicating that both Mal and TRAM are required for TLR2-dependent IFN-b induction. In agreement with these data, 24 h of stimulation with Pam3CSK4 led to IFN-b protein production in WT, but not Mal2/2 or TRAM2/2, iBMDMs (Fig. 2B). Both Mal2/2 and TRAM2/2 iBMDMs were responsive to Pam3CSK4, as evidenced by the induction of TNF-a mRNA and protein in these cells, which was comparable to WT iBMDMs (Fig. 2C, 2D). It has been shown previously that MyD88 is required for the production of TLR2-dependent type I IFN (31, 32, 44), and our results agree with those findings; compared with WT cells, stimulation of MyD882/2 iBMDMs with Pam3CSK4 did not lead to induction of IFN-b mRNA (Fig. 2E) or protein (Fig. 2F). It has been shown previously that TRIF is not involved in this pathway (32), and this was also confirmed because the Pam3CSK4–IFNb response was not impaired in TRIF2/2 iBMDMs (Fig. 2E, 2F). These data confirm that TLR2-dependent induction of IFN-b requires MyD88 and not TRIF, and they reveal a role for Mal and TRAM in this pathway.

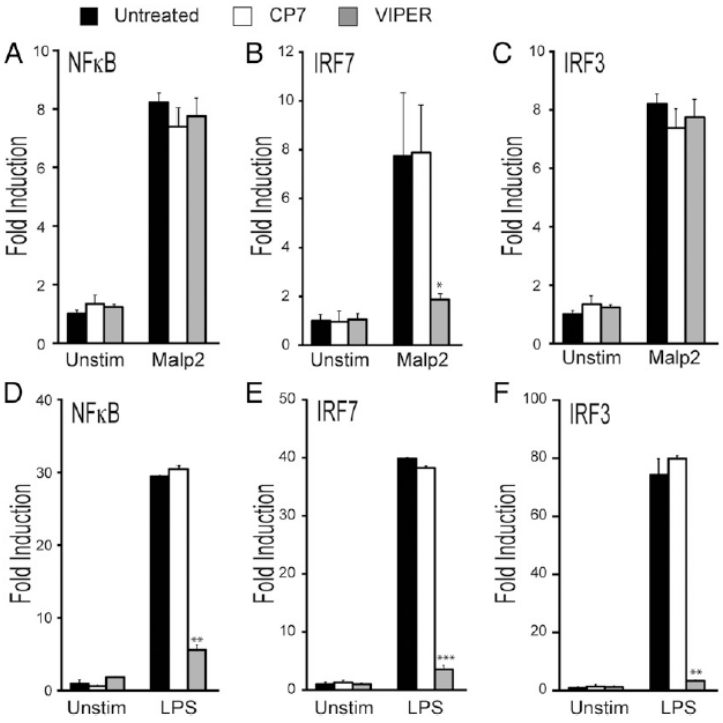

FIGURE 3. VIPER inhibits TLR2-mediated IRF7 transactivation. HEK293-TLR2 cells (**A**–**C**) or HEK293-TLR4 cells (**D**–**F**) were transfected for 24 h with the NF-κB luciferase reporter gene (A and D), or the pFR luciferase reporter gene along with plasmid expressing either IRF7-Gal4 (B and E) or IRF3-Gal4 (C and F). Cells were pretreated with 5 μM VIPER or CP7 peptide 1 h prior to being stimulated with 20 nM Malp2 (A–C) or 10 ng/ml LPS (D–F) for 6 h, and luciferase reporter gene activity was measured. The data are mean ± SD of triplicate samples and are representative of at least three independent experiments. $*p < 0.05$, $**p < 0.005$, or $***p < 0.0005$ compared with stimulated samples treated with control peptide (CP7).

TLR2 activates IRF7 via Mal and TRAM

IFN-b mRNA induction by TLRs is regulated by IRF3, IRF7, and NF-kB (45). To assess the involvement of these transcription factors in the TLR2–IFN-b signal transduction pathway, HEK293 cells stably transfected with TLR2 were used (HEK293 cells naturally express the TLR2 coreceptors TLR1 and TLR6) (46). The ability of Malp2 to activate IRF3, IRF7, and NF-kB and the effect of VIPER on transcription factor activation were measured with reporter gene assay (34, 47). Fig. 3A–C shows that Malp2 treatment stimulated activation of all three transcription factors; however, VIPER only inhibited IRF7 activation (Fig. 3B). The lack of an effect of VIPER on TLR2-stimulated NF-kB activation was consistent with the inability of VIPER to inhibit TNF mRNA induction by TLR2 (Fig. 1D), which is strongly NF-kB dependent. Furthermore, the effect of VIPER on TLR2-stimulated IRF7 activation and not on IRF3 implicated IRF7 in the VIPER-sensitive TLR2-mediated IFN-b mRNA induction (Fig. 1A). In agreement with a previous study showing that VIPER inhibits all

signals downstream of TLR4 (36), pretreatment of HEK293-TLR4 cells with VIPER potently blocked LPS-dependent activation of NF-kB, IRF7, and IRF3 (Fig. 3D–F).

Because TLR2-stimulated IRF7 was sensitive to VIPER, we next determined whether TRAM, Mal, or both were involved in this pathway using siRNA. We used a combination of four anti-Mal or anti-TRAM siRNAs, provided as single reagents, and a combination of four nontargeting siRNAs as a control, and we assessed the role of Mal and TRAM in IRF7 activation, compared with NF-kB activation. This comparison demonstrated a role for Mal in both NF-kB and IRF7 activation by TLR2 (Fig. 4A, 4B), whereas TRAM was required for TLR2-stimulated IRF7 but not NF-kB activation. Similar results were obtained for TLR4-stimulated transcription factor activation, whereby both Mal and TRAM were required for NF-kB activation (Fig. 4C) and TRAM but not Mal was required for IRF7 activation (Fig. 4D). We confirmed the efficacy of the siRNAs by showing reduced mRNA induction of the targets (Fig. 4E, 4F) and reduced protein expression of overexpressed Mal (Fig. 4G) and TRAM (Fig. 4H), because commercially available anti-Mal and anti-TRAM Abs were not successful in our hands for detecting endogenous Mal or TRAM in HEK 293 cells. These data show that both Mal and TRAM are required for TLR2 signaling to IRF7.

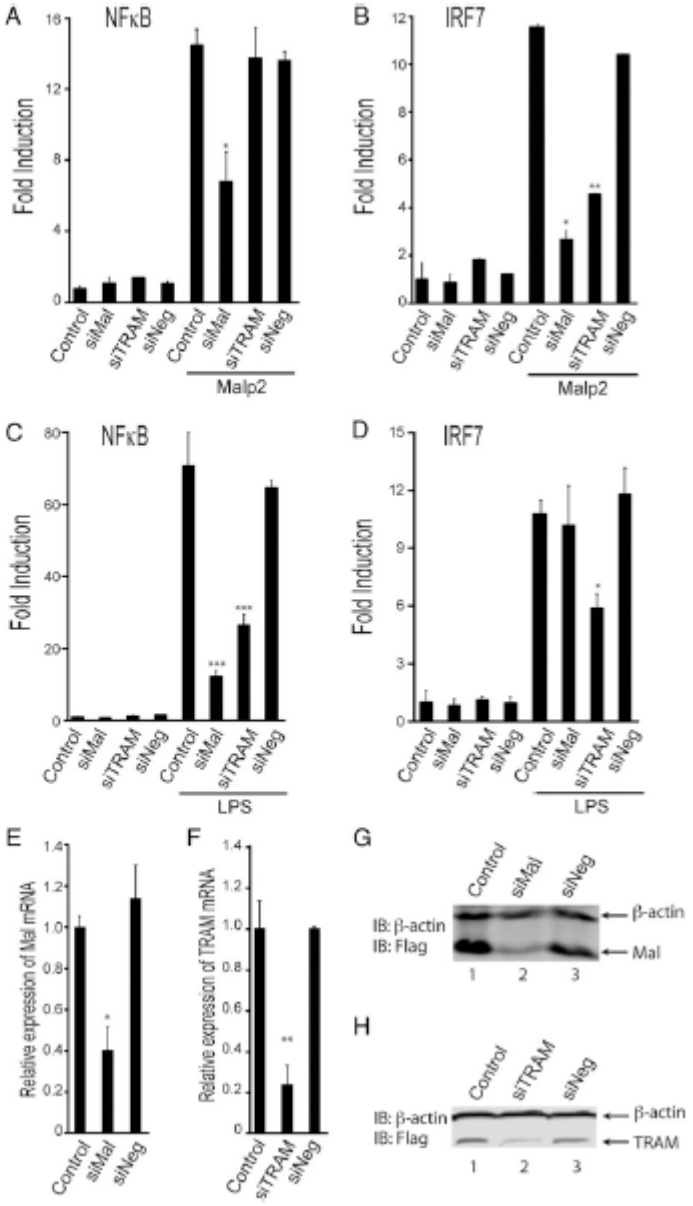

FIGURE 4. TLR2 activates IRF7 via Mal and TRAM. (**A**–**D**) HEK293-TLR2 cells (A and B) or HEK293-TLR4 cells (C and D) were transfected for 24 h with siRNA targeting Mal (siMal), TRAM (siTRAM), or control siRNA (siNeg). Twenty-four hours later, cells were transfected with the NF-κB luciferase reporter gene (A and C), or the pFR luciferase reporter gene along with plasmid expressing IRF7-Gal4 (B and D). Cells were stimulated with 20 nM Malp2 (A and B) or 10 ng/ml LPS (C and D) for 6 h and luciferase reporter gene activity was measured. (**E** and **F**) HEK-293 TLR2 cells were transfected for 48 h with siNeg and siMal (E) or siTRAM (F) for 48 h. Mal and TRAM mRNA expression was assayed with quantitative RT-PCR, normalized to β-actin, and presented relative to untreated (Control) cells. (**G** and **H**) HEK-293 TLR2 cells were transfected with siRNAs and 24 h later transfected with 1 μg plasmid expressing Flag-Mal (G) or Flag-TRAM (H). Cell lysates were prepared 24 h later and analyzed with SDS-PAGE and immunoblotting. The data are mean ± SD of triplicate samples and are representative of at least three independent experiments. $*p < 0.05$, $**p < 0.005$ compared with stimulated samples treated with control siRNA (siNeg).

TLR2-mediated IRF7 activation requires endocytosis

TLRs appear to signal from the endosome to stimulate IRF activation. TLR3, TLR7, TLR8, and TLR9 are naturally located at endosomal compartments, whereas for TLR4 stimulation of IRF activity, it is necessary for the receptor to move to endosomal compartments after ligand engagement (7). For TLR2, Dietrich et al. (31) showed that upon activation, TLR2 is internalized and transported into endolysosomal compartments from where it induces IFN-b, because Malp2 or Pam3Csk4-stimulated IFN-b (but not TNF-a) induction was blocked by

bafilomycin A, an inhibitor of the endosomal proton pump. Bafilomycin A inhibited Pam3Csk4-stimulated IRF7 but not NF-kB activation (Fig. 5A, 5B), and as expected LPS-stimulated IRF7 and not NF-kB (Fig. 5C, 5D). Therefore, similar to TLR4, the TLR2 signaling pathway to IRF7 requires both TRAM and endocytosis. Consistent with this finding, when the myristoylation motif of TRAM, which is required for TRAM to localize at endosomes (7, 9), was mutated at a single amino acid residue, the ability of TRAM to activate IRF7 was impaired. Fig. 5E shows that expression of TRAM but not TRAM G2A led to activation of IRF7.

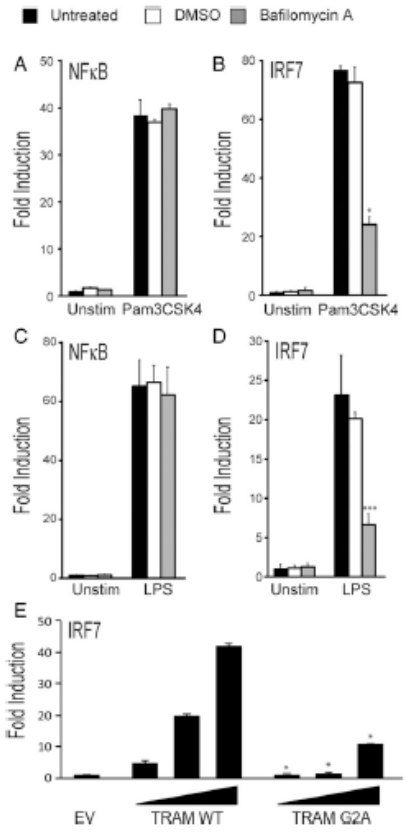

FIGURE 5. TLR2-mediated IRF7 activation requires endocytosis. HEK293-TLR2 cells (**A** and **B**) or HEK293-TLR4 cells (**C** and **D**) were transfected for 24 h with the NF-κB luciferase reporter gene (A and C) or the pFR luciferase reporter gene along with plasmid expressing IRF7-Gal4 (B and D). Cells were pretreated with 100 nM bafilomycin A or DMSO (vehicle) 1 h before stimulation with either 20 ng/ml Pam3CSK4 (A and B) or 10 ng/ml LPS (C and D). Luciferase reporter gene activity was measured 6 h later. (**E**) HEK293-TLR2 cells were transfected with empty vector (EV) or 10, 60, or 120 ng (wedge) of plasmid encoding TRAM WT or TRAM G2A, and the pFR luciferase reporter gene along with plasmid expressing IRF7-Gal4. Luciferase reporter gene activity was measured 30 h later. The data are mean ± SD of triplicate samples and are representative of at least three independent experiments. $*p < 0.05$, $***p < 0.0005$ compared with stimulated samples treated with DMSO (B and D) or compared with TRAM WT (E).

TLR2-stimulated intracellular mobilization of MyD88 is TRAM-dependent

The data so far have demonstrated that TLR2 signaling to IFN induction requires MyD88, TRAM, endocytosis, and IRF7 activation, and that TLR2 stimulation causes TRAM to relocate to endosomes. This result suggested that, similar to the case for TLR4, TRAM could be required for TLR2 as a sorting adaptor to mobilize a signaling adaptor to an endosome, which is TRIF in the case of TLR4 (7). If TRAM is a sorting adaptor for the TLR2-IRF7 pathway, then TLR2 ligand stimulation should cause relocalization of MyD88 within the cell in a TRAM-dependent manner. To test this prediction, HEK293 cells were transfected with TLR2- YFP, TLR6-YFP, and MyD88-CFP and then treated with Malp2. In untreated cells, TLR2/6 was observed at the periphery of the cells, whereas MyD88 was spread diffusely throughout the cell (Fig. 6A, upper panel). After 20 min of Malp2 treatment, TLR2/6 was observed in punctate structures throughout the cell, and MyD88 and TLR2/6 were observed to colocalize in large intracellular structures (Fig. 6A, lower panel, arrows). After 20 min of Malp2 treatment, an average of 61% of transfected cells contained MyD88 in large intracellular structures compared with 2% of unstimulated cells (Fig. 6B), which is strongly suggestive of TLR2-stimulated relocalization of MyD88 to intracellular compartments. (Fig. 6C, top panels). After 20 min of Malp2 treatment, MyD88 could be observed in endosome-like structures, reminiscent of those observed in the HEK293 cells. Compellingly, this mobilization of MyD88 could be observed only in the WT and not in the TRAM2/2 iBMDMs (Fig. 6C, lower panels). Furthermore, after 20 min of Malp2 treatment, an average 81% of WT iBMDMs contained MyD88 in punctate structures compared with 5% of TRAM2/2 iBMDMs (Fig. 6D). Thus, intracellular movement of MyD88 after TLR2 stimulation is TRAM-dependent.

Under basal conditions TRAM is known to localize to the plasma membrane and to endosomal compartments. In response to LPS, TRAM is initially mobilized to EEA1-positive

early endosomes, and it can be found in Rab11-positive sorting and recycling endosomes (48, 49). Thus, we next investigated whether the compartments to which MyD88 localized after Malp2 stimulation were early endosomes. As seen in Fig. 6E (left panel), the MyD88-positive, TLR2/6-positive compartments (white arrows) did not correlate with the EEA1-positive early endosomes. This observation was paralleled in WT BMDMs (Fig. 6E, right panel, arrow). To ensure that the cells were responding normally, TRAMGFP was transfected into WT iBMDMs, and cells were stained for EEA1. As expected, TRAM localized to the membrane (Fig. 6F, first panel, green arrow) and to endosomal structures (Fig. 6F, first panel, white arrow) in untreated cells. After 20 min of LPS treatment, TRAM was found to colocalize with EEA1-positive early endosomes (Fig. 6F, second panel, arrows). Interestingly, after 20 min of Malp2 treatment, TRAM localization was distinctly different from that observed after LPS treatment, as TRAM was observed in large EEA1-negative endosome-like structures reminiscent of the Malp2-inducible MyD88-TLR2/6-positive compartments (Fig. 6F, third panel, arrows). As a control, we showed that ligand stimulation of TLR7 with CLO75, while increasing the number of EEA1-positive endosomes, did not induce observable TRAM mobilization, with the vast majority of TRAM localized in a similar pattern to the untreated cells (Fig. 6F, fourth panel). Thus, TLR2 causes relocalization of both MyD88 and TRAM to large intracellular compartments that are distinct from the EEA1-positive endosomes that TLR4 stimulates TRAM to locate to. Together, these data indicate that MyD88 and TRAM likely localize together at the same TLR2/6-positive compartment in response to Malp2, a structure that is distinct from the early endosomes involved downstream of LPS signaling, and that the TLR2 stimulation of MyD88 movement to these compartments requires the sorting adaptor TRAM.

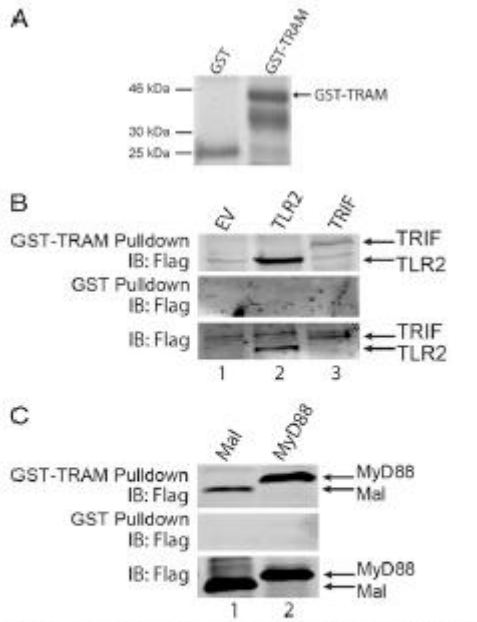

FIGURE 7. TRAM interacts with TLR2 and MyD88. (**A**) GST and GST-TRAM were analyzed with SDS-PAGE and Coomassie staining to demonstrate equal inputs for GST pulldown assays in (**B**) and (**C**). (B and C) HEK293T cells were transfected with 8 μg empty vector (EV), Flag-TLR2, or Flag-TRIF (B) or with 4 μg Flag-Mal or Flag-MyD88 (C). After 48 h, lysates were incubated with GST-TRAM or GST alone as indicated, and together with input lysates, were analyzed with SDS-PAGE and immunoblotting. Data are representative of at least three independent experiments. *Nonspecific band.

TRAM interacts with TLR2 and MyD88

To gain further evidence that TRAM acts as a sorting adaptor for TLR2 and controls MyD88 location after TLR2 stimulation, we tested whether TRAM interacted with TLR2 and MyD88, as is the case for TLR4 and TRIF. For this test, a GST pull-down assay was used, whereby the ability of the TIR proteins expressed in cells to interact with a GST fusion of TRAM was examined. Cell lysates from TLR2- or TIR adaptor–expressing cells were incubated with equal amounts of either GST or GST-TRAM (Fig. 7A). Immunoblot of GST pull-downs demonstrated that TLR2 interacted with GST-TRAM (Fig. 7B, top panel, lane 2), but not with GST alone (Fig. 7B, middle panel). The known TRAM-interaction partner in the TLR4 receptor complex, TRIF, also associated with GST-TRAM in this assay (Fig. 7B, top panel, lane 3). Consistent with the notion that TRAM would provide a link between TLR2 and MyD88, GST-TRAM also interacted with MyD88 (Fig. 7C, top panel, lane 3). In agreement with published data (47), GST-TRAM also interacted with Mal in this assay (Fig. 7C, top

panel, lane 2). Thus, TRAM can interact with all the known components of the TLR2 complex, namely Mal, MyD88, and TLR2 itself.

Pathogen-induced type I IFN is TRAM-dependent

Since TRAM-dependent TLR2-stimulated type I IFN would likely be relevant for antiviral innate immune sensing, we tested whether herpes virus–induced type I IFN was TRAM-dependent in mouse macrophages. HSV, an alpha herpes virus, is detected by TLR2 and TLR9 through recognition of an unidentified viral surface component and viral genomic DNA, respectively (22, 50–52), whereas MHV68 induces type I IFN via TLR2 in MEFs and in vivo (33). Infection of WT iBMDMs with HSV-1, HSV-2, or MHV68 led to induction of type I IFN as measured with bioassay (Fig. 8A). This herpes virus–stimulated type I IFN induction was strongly impaired in TRAM2/2 cells (Fig. 8A). In contrast, type I IFN produced by Sendai virus infection, or dsDNA transfection, of BMDMs was identical in WT or TRAM2/2 BMDMs (Fig. 8A). These data suggest a role for TRAM in the TLR2-dependent type I IFN response to HSV and MHV68. We also tested the ability of S. aureus, a gram-positive bacterium for which TLR2 is a major PRR in macrophages and in vivo (53–58), to stimulate IFN-b production BMDMs. Fig. 8B shows that infection of WT BMDMs with S. aureus strain SH1000 at an MOI of 10 produced a comparable amount of IFN-b production as the pure TLR2 ligands Pam3CSK4 and Malp2, and even higher IFN-b production than from the pure TLR2 ligands at an MOI of 100. Similar to the case for Pam3CSK4 and Malp2, live S. aureus–induced IFN-b was significantly impaired in TRAM2/2 BMDMs compared with WT cells, for all MOIs and time points tested (Fig. 8B), indicating that in addition to pure TLR2 ligands, S. aureus–induced IFN-b in macrophages is TRAM-dependent.

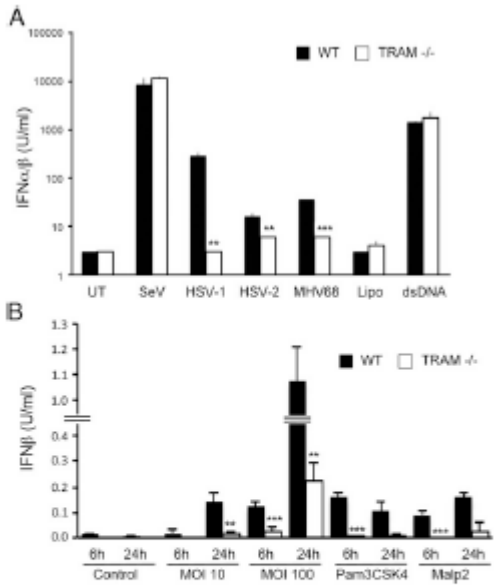

FIGURE 8. TRAM-dependent pathogen-induced type I IFN. (**A**) WT or TRAM-deficient iBMDMs were left untreated (UT) or infected with herpes viruses HSV-1 (MOI of 3), HSV-2 (MOI of 3), MHV68 (MOI of 50), or with Sendai virus (SeV; MOI of 0.001), or transfected with lipofectamine (Lipo) alone or with 1 μg of dsDNA, all for 10 h. Supernatants were harvested, and the amount of type I IFN produced was determined with a bioassay. (**B**) WT or TRAM-deficient iBMDMs were left untreated (Control), stimulated with Pam3CSK4 or Malp2, or infected with *S. aureus* strain SH1000 at MOIs of 10 and 100 for the indicated times. Supernatants were harvested, and IFN-β production was measured with ELISA. The data are mean ± SD of triplicate samples and are representative of two independent experiments. $^{**}p < 0.005$, $^{***}p < 0.0005$ compared with samples from WT iBMDMs.

Discussion

In recent years, there has been a growing appreciation that upon encountering either viral or bacterial ligands, TLR2 heterodimers signal induction of not only proinflammatory cytokines, but also type I IFN. Thus, similar to TLR4 and to the well-characterized endosomal TLRs such as TLR3 and TLR7, TLR2 signaling would activate IRFs. Indeed, Dietrich et al. (31) showed that induction of IFN-b by bacterial ligands of TLR2 was IRF7-dependent in BMDMs. They also showed that similar to other TLRs, TLR2 signaling to IRFs occurred at endosomes, and not at the plasma membrane. However, apart from the requirement of MyD88 for TLR2-stimulated IRF7 activation, it was unclear how TLR2 signaling at the endosome would be enabled, and in particular whether other TIR adaptor proteins apart from MyD88 are required. For TLR4, TRAM is known to be the key sorting adaptor that facilitates endosomal-dependent signaling, in that case via TRIF.

In this study, we show that TLR2-dependent IRF activation at the endosome requires both Mal and TRAM, and that TRAM is required for the TLR2-stimulated movement of MyD88 to endosomes after ligand engagement. This pathway operates for both TLR2/1 and TLR2/6 receptor complexes because we always obtained almost identical results for Pam3CSK4 and Malp2. Furthermore, we demonstrate that TRAM interacts with both TLR2 and MyD88, and that both herpes virus– and S. aureus–stimulated type I IFN induction in BMDMs is TRAM-dependent. Thus, our work reveals a novel and broader role for TRAM than was previously appreciated, in that TRAM acts as a sorting adaptor not only for TLR4, but also for TLR2, to facilitate signaling to IRF7 at the endosome. Our data do not exclude the possibility that other IRFs apart from IRF7 are also involved in TRAM-dependent induction of type I IFNs by TLR2. Indeed, Dietrich et al. (31) showed that in BMDMs lacking IRF7 or IRF1 (but not IRF3), TLR2-dependent IFN-b production was impaired, whereas Liljeroos et al.

(44) showed that in RAW264.7 macrophages both IRF1 and IRF2 had a role in TLR2-dependent IFN-a production. Whether other IRFs such as IRF5 might also be involved in TLR2 response pathways remains to be determined, as does the exact mechanism whereby TRAM would regulate a specific IRF downstream of TLR2.

Interestingly, Sacre et al. (59) previously hinted at a role for TRAM in TLR2 signaling using dominant-negative TRAM. They used an adenoviral construct expressing TRAM mutated in the key signaling BB loop domain (TRAM-C117H) and observed that this mutant inhibited LPS- and LTA- (i.e., TLR2-) induced NF-kB activation and cytokine production in human synovial fibroblasts, human umbilical endothelial cells, and MEFs, but not in human macrophages. They concluded that TRAM is an adaptor protein for both TLR4 and TLR2/6 signaling in specific cell types (59). We used three different approaches to examine the role of TRAM in TLR2 signaling, namely the VIPER peptide inhibitor of TRAM, TRAM siRNA, and TRAM2/2 iBMDMs. These approaches revealed that TRAM was required for IRF7

activation and IFN-b induction, but not for TLR2-dependent NF-kB activation or proinflammatory cytokine production. Other studies using TRAM deficient murine peritoneal macrophages (60) or TRAM dominantnegative HEKTLR2 cells (61) concur with our conclusion of no role for TRAM in proinflammatory signaling initiated by TLR2. However, the exact role of TRAM in TLR2 responses may vary in different cell types.

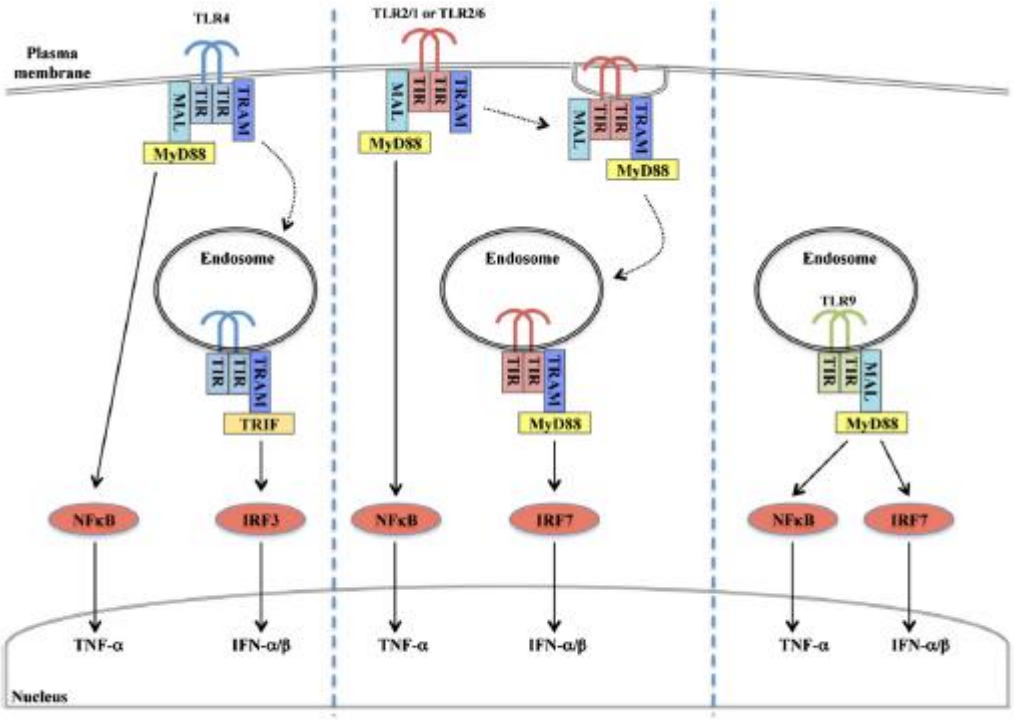

FIGURE 9. Role of TRAM in endosomal TLR2-stimulated IRF activation. Model for TRAM-dependent endosomal signaling to IRF7 for TLR2 (center), compared with endosomal TLR4 (*left*) and TLR9 (*right*) signaling.

The role of TRAM in TLR2-stimulated IRF activation revealed in this study is reminiscent of a similar requirement of TLR4 for TRAM. TLR4 initially signals at the plasma membrane (via Mal and MyD88) and is then internalized into early endosomes, where signaling switches to TRAM and TRIF (Fig. 9) (7, 62). Thereafter, TLR4 moves to late endosomes where TRIF-dependent signaling is suppressed by TRAM adaptor with GOLD domain, a splice variant of TRAM (48). Endosomal location and IRF signaling for TLR4 are both dependent on TRAM, and they are compromised when the myristoylation motif of TRAM is mutated. In this study, we showed TLR2-stimulated relocalization of MyD88 to intracellular

compartments was TRAM-dependent in the same way that TLR4-stimulated relocalization of TRIF to endosomes is TRAM-dependent. Furthermore, TRAM interacted with both TLR2 and MyD88, consistent with the notion that it acts as a bridging adaptor to link TLR2 and MyD88, as well as a sorting adaptor to facilitate TLR2 and MyD88 locating at the endosome.

That TRAM and MyD88 interact was also recently demonstrated by Ohnishi et al. (63), who also showed that TRAM was required for IL-18 signaling and thus suggested a role for TRAM as a bridging adapter between MyD88 and the IL-18R. How exactly TRAM engages with MyD88 remains to be determined, although those authors showed that the binding site for TRAM on MyD88 overlaps with the binding site used by MyD88 to engage Mal (63). Therefore, during TLR2 signaling, it is conceivable that MyD88 may be transferred directly from Mal to TRAM as the TLR2 complex moves from the plasma membrane (Fig. 9). Alternatively, the TLR2 signaling complex may engage MyD88 via TRAM from the outset of initiation of signaling. Discriminating between these possibilities will require further study.

TLR2 has been shown to be internalized after ligand engagement (64). In that study, the authors concluded that TLR2 internalization was not actually required for signaling; however, they examined only NF-kB activation, which we showed here does not require TRAM or endocytosis. Apart from TRAM, we also showed that Mal, which is required for some but not all TLR2-dependent signaling events (16, 17), is essential for TLR2-dependent IRF7 activation and IFNb induction. It has been hypothesized that the reason TLRs signal to IRFs from the endosome and not the plasma membrane is that TRAF3 can engage with TLR signaling only at the former location (7). Whether TRAF3 is required for the endosomal TLR2-TRAM-IRF pathway remains to be confirmed. Which downstream kinases activate IRFs after TLR2-TRAM signaling at the endosome also remain to be determined.

The data lead to the following model for TRAM involvement in TLR2 signaling to IRFs for type I IFN induction (Fig. 9): Upon ligand engagement at the plasma membrane, TLR2 recruits MyD88 via Mal, giving rise to NF-kB activation. This part of the signaling process for TLR2 is TRAM-independent (Fig. 4A). Thereafter, a TLR2-TRAM-MyD88 complex would be internalized endocytotically. This internalization leads to formation of a TLR2-signaling competent endosome to affect IRF7 activation. Within this part of the signaling process, Mal might directly transfer MyD88 to TRAM to license the complex for endosomal localization and subsequent signaling. Alternatively, Mal itself may also be retained in the endosomal TLR2 signaling complex. Because our data show that both Mal and TRAM were required for IRF7 activation, further work will be required to distinguish these two possibilities. Interestingly, it was shown recently that for natural ligands, TLR9 also needs a sorting adaptor for endosomal signaling, apart from the signaling adaptor MyD88, which in that case was shown to be Mal (Fig. 9) (65). It will be of interest to re-examine whether other endosomal TLRs (TLR3, TLR7, TLR8) have any requirement for TRAM or Mal for signaling, which might be the case in certain cell types or for particular ligands.

Acknowledgments

We thank Drs. K. Fitzgerald, D. Golenbock, S. Sato, L. O'Neill, and J. McCarthy for the kind gift of expression plasmids and cell lines.

Disclosures

A.G.B. holds a patent on VIPER as an inhibitor of TLR4.

References

1. Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. 2: 675–680.

2. Kawai, T., and S. Akira. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. 11: 373–384.

3. Underhill, D. M., and A. Ozinsky. 2002. Toll-like receptors: key mediators of microbe detection. Curr. Opin. Immunol. 14: 103–110.

4. Gay, N. J., M. Gangloff, and L. A. O'Neill. 2011. What the Myddosome structure tells us about the initiation of innate immunity. Trends Immunol. 32: 104–109.

5. Jenkins, K. A., and A. Mansell. 2010. TIR-containing adaptors in Toll-like receptor signalling. Cytokine 49: 237–244.

6. Kagan, J. C., and R. Medzhitov. 2006. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell 125: 943–955.

7. Kagan, J. C., T. Su, T. Horng, A. Chow, S. Akira, and R. Medzhitov. 2008. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferonbeta. Nat. Immunol. 9: 361–368.

8. O'Neill, L. A., and A. G. Bowie. 2007. The family of five: TIR-domaincontaining adaptors in Toll-like receptor signalling. Nat. Rev. Immunol. 7: 353–364.

9. Rowe, D. C., A. F. McGettrick, E. Latz, B. G. Monks, N. J. Gay, M. Yamamoto, S. Akira, L. A. O'Neill, K. A. Fitzgerald, and D. T. Golenbock. 2006. The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction. Proc. Natl. Acad. Sci. USA 103: 6299–6304.

10. G€urtler, C., and A. G. Bowie. 2013. Innate immune detection of microbial nucleic acids. Trends Microbiol. 21: 413–420.

11. Kawai, T., and S. Akira. 2006. Innate immune recognition of viral infection. Nat. Immunol. 7: 131–137.

12. Kirschning, C. J., and R. R. Schumann. 2002. TLR2: cellular sensor for microbial and endogenous molecular patterns. Curr. Top. Microbiol. Immunol. 270: 121–144.

13. Jin, M. S., S. E. Kim, J. Y. Heo, M. E. Lee, H. M. Kim, S. G. Paik, H. Lee, and J. O. Lee. 2007. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell 130: 1071–1082.

14. Morath, S., A. Stadelmaier, A. Geyer, R. R. Schmidt, and T. Hartung. 2002. Synthetic lipoteichoic acid from Staphylococcus aureus is a potent stimulus of cytokine release. J. Exp. Med. 195: 1635–1640.

15. Deininger, S., A. Stadelmaier, S. von Aulock, S. Morath, R. R. Schmidt, and T. Hartung. 2003. Definition of structural prerequisites for lipoteichoic acidinducible cytokine induction by synthetic derivatives. J. Immunol. 170: 4134–4138.

16. Santos-Sierra, S., S. D. Deshmukh, J. Kalnitski, P. K€uenzi, M. P. Wymann, D. T. Golenbock, and P. Henneke. 2009. Mal connects TLR2 to PI3Kinase activation and phagocyte polarization. EMBO J. 28: 2018–2027.

17. Kenny, E. F., S. Talbot, M. Gong, D. T. Golenbock, C. E. Bryant, and L. A. O'Neill. 2009. MyD88 adaptor-like is not essential for TLR2 signaling and inhibits signaling by TLR3. J. Immunol. 183: 3642–3651.

18. Boehme, K. W., M. Guerrero, and T. Compton. 2006. Human cytomegalovirus envelope glycoproteins B and H are necessary for TLR2 activation in permissive cells. J. Immunol. 177: 7094–7102.

19. Compton, T., E. A. Kurt-Jones, K. W. Boehme, J. Belko, E. Latz, D. T. Golenbock, and R. W. Finberg. 2003. Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. J. Virol. 77: 4588–4596.

20. Szomolanyi-Tsuda, E., X. Liang, R. M. Welsh, E. A. Kurt-Jones, and R. W. Finberg. 2006. Role for TLR2 in NK cell-mediated control of murine cytomegalovirus in vivo. J. Virol. 80: 4286–4291.

21. Sato, A., M. M. Linehan, and A. Iwasaki. 2006. Dual recognition of herpes simplex viruses by TLR2 and TLR9 in dendritic cells. Proc. Natl. Acad. Sci. USA 103: 17343–17348.

22. Kurt-Jones, E. A., M. Chan, S. Zhou, J. Wang, G. Reed, R. Bronson, M. M. Arnold, D. M. Knipe, and R. W. Finberg. 2004. Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. Proc. Natl. Acad. Sci. USA 101: 1315–1320.

23. Chang, S., A. Dolganiuc, and G. Szabo. 2007. Toll-like receptors 1 and 6 are involved in TLR2-mediated macrophage activation by hepatitis C virus core and NS3 proteins. J. Leukoc. Biol. 82: 479–487.

24. Zhou, S., E. A. Kurt-Jones, L. Mandell, A. Cerny, M. Chan, D. T. Golenbock, and R. W. Finberg. 2005. MyD88 is critical for the development of innate and adaptive immunity during acute lymphocytic choriomeningitis virus infection. Eur. J. Immunol. 35: 822–830.

25. Bieback, K., E. Lien, I. M. Klagge, E. Avota, J. Schneider-Schaulies, W. P. Duprex, H. Wagner, C. J. Kirschning, V. Ter Meulen, and S. Schneider- Schaulies. 2002. Hemagglutinin protein of wild-type measles virus activates toll like receptor 2 signaling. J. Virol. 76: 8729–8736.

26. Zhu, J., J. Martinez, X. Huang, and Y. Yang. 2007. Innate immunity against vaccinia virus is mediated by TLR2 and requires TLR-independent production of IFN-beta. Blood 109: 619–625.

27. Doyle, S., S. Vaidya, R. O'Connell, H. Dadgostar, P. Dempsey, T. Wu, G. Rao, R. Sun, M. Haberland, R. Modlin, and G. Cheng. 2002. IRF3 mediates a TLR3/ TLR4-specific antiviral gene program. Immunity 17: 251–263.

28. Hoshino, K., T. Kaisho, T. Iwabe, O. Takeuchi, and S. Akira. 2002. Differential involvement of IFN-beta in Toll-like receptor-stimulated dendritic cell activation. Int. Immunol. 14: 1225–1231.

29. Kawai, T., O. Takeuchi, T. Fujita, J. Inoue, P. F. M€uhlradt, S. Sato, K. Hoshino, and S. Akira. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J. Immunol. 167: 5887– 5894.

30. Toshchakov, V., B. W. Jones, P. Y. Perera, K. Thomas, M. J. Cody, S. Zhang, B. R. Williams, J. Major, T. A. Hamilton, M. J. Fenton, and S. N. Vogel. 2002. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. Nat. Immunol. 3: 392–398.

31. Dietrich, N., S. Lienenklaus, S. Weiss, and N. O. Gekara. 2010. Murine toll-like receptor 2 activation induces type I interferon responses from endolysosomal compartments. PLoS ONE 5: e10250.

32. Barbalat, R., L. Lau, R. M. Locksley, and G. M. Barton. 2009. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. Nat. Immunol. 10: 1200–1207.

33. Michaud, F., F. Coulombe, E. Gaudreault, J. Kriz, and J. Gosselin. 2010. Involvement of TLR2 in recognition of acute gammaherpesvirus-68 infection. PLoS ONE 5: e13742.

34. Stack, J., I. R. Haga, M. Schro¨der, N. W. Bartlett, G. Maloney, P. C. Reading, K. A. Fitzgerald, G. L. Smith, and A. G. Bowie. 2005. Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. J. Exp. Med. 201: 1007–1018.

35. Stack, J., and A. G. Bowie. 2012. Poxviral protein A46 antagonizes Toll-like receptor 4 signaling by targeting BB loop motifs in Toll-IL-1 receptor adaptor proteins to disrupt receptor:adaptor interactions. J. Biol. Chem. 287: 22672– 22682.

36. Lysakova-Devine, T., B. Keogh, B. Harrington, K. Nagpal, A. Halle, D. T. Golenbock, T. Monie, and A. G. Bowie. 2010. Viral inhibitory peptide of TLR4, a peptide derived from vaccinia protein A46, specifically inhibits TLR4 by directly targeting MyD88 adaptor-like and TRIF-related adaptor molecule. J. Immunol. 185: 4261–4271.

37. Fedosyuk, S., I. Grishkovskaya, E. de Almeida Ribeiro, Jr., and T. Skern. 2014. Characterization and structure of the vaccinia virus NF-kB antagonist A46. J. Biol. Chem. 289: 3749–3762.

38. Verstak, B., K. Nagpal, S. P. Bottomley, D. T. Golenbock, P. J. Hertzog, and A. Mansell. 2009. MyD88 adapter-like (Mal)/TIRAP interaction with TRAF6 is critical for TLR2- and TLR4-mediated NF-kappaB proinflammatory responses. J. Biol. Chem. 284: 24192–24203.

39. Bowie, A., E. Kiss-Toth, J. A. Symons, G. L. Smith, S. K. Dower, and L. A. O'Neill. 2000. A46R and A52R from vaccinia virus are antagonists of host IL-1 and toll-like receptor signaling. Proc. Natl. Acad. Sci. USA 97: 10162–10167.

40. Roberts, Z. J., N. Goutagny, P. Y. Perera, H. Kato, H. Kumar, T. Kawai, S. Akira, R. Savan, D. van Echo, K. A. Fitzgerald, et al. 2007. The chemotherapeutic agent DMXAA potently and specifically activates the TBK1-IRF-3 signaling axis. J. Exp. Med. 204: 1559–1569.

41. Sørensen, L. N., L. S. Reinert, L. Malmgaard, C. Bartholdy, A. R. Thomsen, and S. R. Paludan. 2008. TLR2 and TLR9 synergistically control herpes simplex virus infection in the brain. J. Immunol. 181: 8604–8612.

42. Horsburgh, M. J., J. L. Aish, I. J. White, L. Shaw, J. K. Lithgow, and S. J. Foster. 2002. sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from Staphylococcus aureus 8325-4. J. Bacteriol. 184: 5457–5467.

43. Maher, B. M., M. E. Mulcahy, A. G. Murphy, M. Wilk, K. M. O'Keeffe, J. A. Geoghegan, E. C. Lavelle, and R. M. McLoughlin. 2013. Nlrp-3-driven interleukin 17 production by gdT cells controls infection outcomes during Staphylococcus aureus surgical site infection. Infect. Immun. 81: 4478–4489.

44. Liljeroos, M., R. Vuolteenaho, S. Rounioja, B. Henriques-Normark, M. Hallman, and M. Ojaniemi. 2008. Bacterial ligand of TLR2 signals Stat activation via induction of IRF1/2 and interferon-alpha production. Cell. Signal. 20: 1873–1881.

45. Honda, K., and T. Taniguchi. 2006. IRFs: master regulators of signalling by Tolllike receptors and cytosolic pattern-recognition receptors. Nat. Rev. Immunol. 6: 644–658.

46. Hise, A. G., K. Daehnel, I. Gillette-Ferguson, E. Cho, H. F. McGarry, M. J. Taylor, D. T. Golenbock, K. A. Fitzgerald, J. W. Kazura, and E. Pearlman. 2007. Innate immune responses to endosymbiotic Wolbachia bacteria in Brugia malayi and Onchocerca volvulus are dependent on TLR2, TLR6, MyD88, and Mal, but not TLR4, TRIF, or TRAM. J. Immunol. 178: 1068–1076.

47. Fitzgerald, K. A., D. C. Rowe, B. J. Barnes, D. R. Caffrey, A. Visintin, E. Latz, B. Monks, P. M. Pitha, and D. T. Golenbock. 2003. LPS-TLR4 signaling to IRF- 3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J. Exp. Med. 198: 1043–1055.

48. Palsson-McDermott, E. M., S. L. Doyle, A. F. McGettrick, M. Hardy, H. Husebye, K. Banahan, M. Gong, D. Golenbock, T. Espevik, and L. A. O'Neill. 2009. TAG, a splice variant of the adaptor TRAM, negatively regulates the adaptor MyD88- independent TLR4 pathway. Nat. Immunol. 10: 579–586.

49. Husebye, H., M. H. Aune, J. Stenvik, E. Samstad, F. Skjeldal, O. Halaas, N. J. Nilsen, H. Stenmark, E. Latz, E. Lien, et al. 2010. The Rab11a GTPase controls Toll-like receptor 4-induced activation of interferon regulatory factor-3 on phagosomes. Immunity 33: 583–596.

50. Aravalli, R. N., S. Hu, T. N. Rowen, J. M. Palmquist, and J. R. Lokensgard. 2005. Cutting edge: TLR2-mediated proinflammatory cytokine and chemokine production by microglial cells in response to herpes simplex virus. J. Immunol. 175: 4189–4193.

51. Krug, A., G. D. Luker, W. Barchet, D. A. Leib, S. Akira, and M. Colonna. 2004. Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9. Blood 103: 1433–1437.

52. Lund, J., A. Sato, S. Akira, R. Medzhitov, and A. Iwasaki. 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. J. Exp. Med. 198: 513–520.

53. Watanabe, I., M. Ichiki, A. Shiratsuchi, and Y. Nakanishi. 2007. TLR2-mediated survival of Staphylococcus aureus in macrophages: a novel bacterial strategy against host innate immunity. J. Immunol. 178: 4917–4925.

54. Schmaler, M., N. J. Jann, F. Ferracin, L. Z. Landolt, L. Biswas, F. Go¨tz, and R. Landmann. 2009. Lipoproteins in Staphylococcus aureus mediate inflammation by TLR2 and iron-dependent growth in vivo. J. Immunol. 182: 7110–7118.

55. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of gramnegative and gram-positive bacterial cell wall components. Immunity 11: 443–451.

56. Underhill, D. M., A. Ozinsky, A. M. Hajjar, A. Stevens, C. B. Wilson, M. Bassetti, and A. Aderem. 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 401: 811–815.

57. Takeuchi, O., K. Hoshino, and S. Akira. 2000. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. J. Immunol. 165: 5392–5396.

58. Hoebe, K., P. Georgel, S. Rutschmann, X. Du, S. Mudd, K. Crozat, S. Sovath, L. Shamel, T. Hartung, U. Za¨hringer, and B. Beutler. 2005. CD36 is a sensor of diacylglycerides. Nature 433: 523–527.

59. Sacre, S. M., A. M. Lundberg, E. Andreakos, C. Taylor, M. Feldmann, and B. M. Foxwell. 2007. Selective use of TRAM in lipopolysaccharide (LPS) and lipoteichoic acid (LTA) induced NF-kappaB activation and cytokine production in primary human cells: TRAM is an adaptor for LPS and LTA signaling. J. Immunol. 178: 2148–2154.

60. Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K. Takeda, and S. Akira. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat. Immunol. 4: 1144–1150.

61. Ding, A., H. Yu, J. Yang, S. Shi, and S. Ehrt. 2005. Induction of macrophagederived SLPI by Mycobacterium tuberculosis depends on TLR2 but not MyD88. Immunology 116: 381–389.

62. Blasius, A. L., and B. Beutler. 2010. Intracellular toll-like receptors. Immunity 32: 305–315.

63. Ohnishi, H., H. Tochio, Z. Kato, N. Kawamoto, T. Kimura, K. Kubota, T. Yamamoto, T. Funasaka, H. Nakano, R. W. Wong, et al. 2012. TRAM is involved in IL-18 signaling and functions as a sorting adaptor for MyD88. PLoS ONE 7: e38423.

64. Nilsen, N. J., S. Deininger, U. Nonstad, F. Skjeldal, H. Husebye, D. Rodionov, S. von Aulock, T. Hartung, E. Lien, O. Bakke, and T. Espevik. 2008. Cellular trafficking of lipoteichoic acid and Toll-like receptor 2 in relation to signaling: role of CD14 and CD36. J. Leukoc. Biol. 84: 280–291.

65. Bonham, K. S., M. H. Orzalli, K. Hayashi, A. I. Wolf, C. Glanemann, W. Weninger, A. Iwasaki, D. M. Knipe, and J. C. Kagan. 2014. A promiscuous lipid-binding protein diversifies the subcellular sites of toll-like receptor signal transduction. Cell 156: 705–716.