Molecular Medicine

Absence of TRAM Restricts Toll-Like Receptor 4 Signaling in Vascular Endothelial Cells to the MyD88 Pathway

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Abstract—Mammalian cells respond to bacterial lipopolysaccharide (LPS) through a cognate receptor: Toll-like receptor 4 (TLR4). The signaling pathways, which link TLR4 to the proinflammatory transcription factor nuclear factor κB (NF-κB), occur through the intracellular docking proteins MyD88 and Trif. We hypothesize that unlike antigen-presenting cells, vascular endothelial cells (ECs) lack the Trif protein TRAM and are therefore incapable of eliciting Trif-dependent immune responses to LPS. Stimulation of wild-type mice with LPS leads to the activation of NF-κB in ECs and macrophages in vitro and in vivo. In contrast to macrophages, LPS did not activate endothelial NF-κB or NF-κB–dependent genes in MyD88−/− mice, suggesting the absence of a functional Trif pathway in vascular ECs. Indeed, the Trif-dependent gene cxcl10 was not expressed in ECs after LPS stimulation. This correlated with diminished expression of the Trif accessory TIR protein TRAM in ECs. Overexpression of TRAM cDNA in ECs reconstituted LPS-induced Trif-dependent NF-κB activation and cxcl10 promoter activity. The functional absence of TRAM in vascular ECs restricts TLR4 signaling to MyD88-dependent pathway. This is in contrast to macrophages, which respond to LPS via both Trif- and MyD88-dependent pathways. These findings indicate that vascular ECs do not express the Trif-dependent gene subset. This implies that these genes may be dispensable for the endothelial response to bacterial infection and play no role in the endothelial contribution to the development of atherosclerosis. (Circ Res. 2006;98:1134-1140.)

Key Words: endothelial ■ toll-like ■ Trif ■ TRAM ■ MyD88

The mammalian Toll-like receptors (TLRs) are a family of transmembrane proteins with a critical role in innate immunity through their recognition of pathogen-associated molecular patterns. Binding of microbial antigens to TLR activates the nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling cascades, leading, through gene transcription, to a change in cellular phenotype and ultimately to the elimination of the infecting organism. This signal is transduced through TIR (Toll/interleukin-1 [IL-1] receptor) adaptor proteins to gene transcription pathways, most importantly NF-κB. To date, the TIR adaptor protein family contains six members: MyD88, Mal (TIRAP), Trif (TIR-containing adapter molecule-1, lipopolysaccharide 2 [LPS2]), Trif-related adaptor protein (TRAM), ST2, and SIGIRR. The most widely used of these is MyD88, which is absolutely necessary for the transduction of signal from IL-1 receptor (IL-1R) and also from all TLRs so far described, except TLR3 and TLR4. Although TLR4 (the cognate LPS receptor) uses MyD88 (in conjunction with Mal), this is nonessential because TLR4 can also use Trif to activate MAPK and NF-κB pathways. Trif alone is responsible for mediating the TLR3 signal. MyD88 and Trif are not redundant. In addition to activating NF-κB, Trif activates STAT1, and IRF3 and IRF7 via TBK1.

There is a subset of LPS-responsive genes entirely dependent on TRIF/TBK1/IRF3 (eg, interferon-α [IFN-α] and IFN-β, RANTES, and IFN-γ-inducible protein 10 [IP-10]). The functional ramifications of this bivalent LPS signaling pathway are not yet clear. However, an emerging paradigm suggests that LPS has two distinct functions as an immune mediator. The “innate” function is to induce stromal cells to express molecules that recruit and activate leukocytes to the inflammatory site. The “adaptive” function is to induce the maturation of antigen-presenting cells on exposure to antigen, leading to the effective presentation of antigen, with costimulatory signals, to lymphocytes. It has been suggested that MyD88 mediates the innate function, whereas Trif mediates the adaptive function.

The key stromal cell type involved in the innate immune response to LPS is the endothelial cell (EC). Kubes et al reported recently that endothelial TLR4 expression is required for LPS-induced neutrophil sequestration in mouse lung, whereas leukocyte TLR4 is dispensable. As well as being critical in the innate immune response against mi-
crobes, EC activation by proinflammatory mediators has been strongly implicated in the pathogenesis of atherosclerosis. Both TLR4 and MyD88 deficiency reduced atherosclerosis in apolipoprotein E−/− mice and reduced neointimal formation in two murine arterial injury models. Administration of LPS to cholesterol-fed rabbits increased atherogenesis, and in the human, there is a correlation between circulating LPS and the development of carotid atherosclerosis. Furthermore, there is a suggestion that human tlr4 polymorphisms are associated with protection from atherosclerosis, although further studies are required.

We hypothesize that the proatherogenic effect of LPS is mediated through the activation of vascular ECs. The precise signaling events that occur in ECs downstream of TLR4 activation have not been studied in detail. It has long been understood that, like other cell types, ECs respond to LPS by activation of NF-κB. This, in turn, initiates transcription of a number of proinflammatory genes implicated in the development of atherosclerosis, such as IL-6, IL-8, MCP-1, intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin. More recent studies have shown that this effect is mediated through TLR4. It is not clear whether the bivalent MyD88/Trif pathways described in monocytes macrophages are present in ECs. It has been suggested that ECs and other stromal cells differ from leukocytes in this regard, with an essential role for MyD88. However, the role of Trif in LPS signal transduction in ECs is not known. Characterization of these pathways would have significant implications for the understanding of the EC response to LPS in the context of bacterial infection and in atherogenesis.

In this study, we undertook to describe LPS signaling in ECs in detail. We confirmed that, unlike in the macrophage, LPS activation of NF-κB in ECs is entirely dependent on MyD88, with no Trif-mediated NF-κB activation. Furthermore, we found that LPS does not induce the expression of the Trif-dependent gene IP-10 in ECs. Absence of a functional Trif pathway was attributable to the lack of the expression of the accessory TIR protein TRAM (TIR-containing adapter molecule-2).

**Materials and Methods**

For full details of the Materials and Methods, please refer to the online supplement, available at http://circres.ahajournals.org. EC activation in vivo was assessed by immunofluorescent staining for endothelial adhesion molecules on snap-frozen mouse tissues. Peritoneal macrophages, dendritic cells (DCs), monocytes, and ECs were obtained and cultured for in vitro studies. Adhesion molecule and chemokine expression of cultured cells was quantified by ELISA. Monocyte adhesion to EC monolayers was determined under laminar flow conditions using videomicroscopy. NF-κB activity in cultured cells was determined either by p65 immunolocalization or by electrophoretic mobility shift assay (EMSA) on nuclear extracts. Specific mRNAs were detected by RT-PCR, qRT-PCR, and Northern blotting, and expression of TRAM protein by Western blotting. Human IP-10 (cxcl10) promoter activity was assayed using dual luciferase reporter transfection in bovine aortic ECs (BAECs). TRAM overexpression in mouse ECs was achieved using a lentivirus vector.

**Results**

**No Endothelial Activation to LPS Without MyD88**

To evaluate the role of the MyD88-independent pathway in ECs in vivo, we administered LPS (1 mg/kg IP) systemically to wild-type and MyD88−/− mice (n = 3) for 4 hours before preparing heart and kidney for quantification of EC adhesion molecule expression by immunofluorescence. In wild-type controls, LPS induced the expression of E-selectin (and VCAM-1; data not shown) in the myocardial vascular endothelium and renal glomerular endothelium when compared with mice treated with vehicle alone (Figure 1). No such increase was observed in MyD88−/− mice. We confirmed this observation in vitro by the isolation and culture of mouse heart microvascular ECs (MHECs) from wild-type and MyD88−/− mice, and the quantification of E-selectin and VCAM-1 after LPS by cell-based ELISA (Figure 2). We also used IL-1α stimulation because both IL-1α and IL-1β are dependent on MyD88 for signal transduction downstream of IL-1R. Neither IL-1α nor LPS increased E-selectin or VCAM-1 in MyD88−/− ECs, whereas both caused induction with a characteristic time course in wild-type cells. These data are consistent with the published macrophage studies and show the requirement for MyD88 in LPS and IL-1-induced transcription of proinflammatory genes. Furthermore, they appear to suggest that the requirement for MyD88 in LPS-induced EC activation is absolute. To confirm this, we tested whether LPS could increase monocyte–EC interaction under flow in the absence of MyD88 (Figure 3). We found that on wild-type MHECs, LPS stimulation over 4 or 24 hours increased U937-LAM monocyte accumulation two- to threefold. This was equivalent to the increase in monocyte accumulation seen with tumor necrosis factor-α (TNF-α) stimulation. However, when MyD88−/− MHECs were used, there was no increase in U937-LAM accumulation over baseline after LPS stimulation. These results were consistent at 0.5 and 1.0 dyne/mm² of shear stress. In contrast, the accumulation of monocytes on MyD88−/− MHECs reached similar levels to wild-type when the stimulus was TNF-α.
No NF-κB Activation to LPS in ECs Without MyD88

To determine the presence or absence of an MyD88-independent pathway from TLR4 to NF-κB in ECs, we stimulated wild-type and MyD88−/− MHECs with LPS and identified NF-κB activation initially by immunolocalization of the p65 (RelA) subunit. In unstimulated cells, p65 is cytoplasmic but translocates to the nucleus on TLR stimulation. As a positive control for the effect of MyD88 deficiency, we also stimulated the ECs with lipoteichoic acid, a TLR2 agonist, and flagellin, a TLR5 agonist. NF-κB activation to these agonists is known to be solely MyD88 dependent. As expected (Figure 4), nuclear translocation of p65 did not occur after TLR2 and TLR5 ligation except in the presence of MyD88. Furthermore, we detected no p65 translocation after TLR4 stimulation in the absence of MyD88. This result suggested the absence of an MyD88-independent pathway to NF-κB in ECs, in contrast to reported findings in macrophages.

To confirm this observation, we quantified NF-κB activation by an alternative method: p65 and p50 DNA binding using EMSA (Figure 5). For this experiment, we used thioglycollate-elicited peritoneal macrophages as a positive control for the detection of the MyD88-independent pathway. As expected, both wild-type and MyD88−/− macrophages (Figure 5C) underwent NF-κB activation after exposure to 100 ng/mL LPS for 1 hour (this upregulation has a slower time course in MyD88−/− compared with wild-type macrophages, but by 1 hour, there is no difference). In contrast, whereas wild-type MHECs evinced NF-κB activation to LPS over 8 hours with a peak at 1 hour, in MyD88−/− MHECs, there was no NF-κB activation over the entire time period. To confirm that this was not attributable to a general defect in NF-κB activation in these cells, we stimulated them with TNF-α and showed that NF-κB activation to this ligand was unimpaired.
Initially, we looked for these gene products in unstimulated macrophages, MHECs, and MAECs by super-shift as indicated in A and C. These data are consistent with absence of TRAM in ECs, whereas IFN-γ induces IP-10 in macrophages and ECs in an MyD88-independent manner.

Trif-Dependent LPS Gene Transcription Is Absent in ECs

There is a clearly defined subset of LPS-induced genes in macrophages that do not require the presence of MyD88 and instead are dependent on Trif. The implication of the data presented above is that LPS would not be expected to induce these genes in ECs. We tested this with a classically described Trif-dependent gene, cxcl10, encoding the chemokine IP-10. Both macrophages and ECs isolated from mouse aorta (MAECs) were obtained from wild-type and MyD88−/− mice (as indicated) and elicited macrophages from WT and MyD88−/− mice (C) were stimulated with LPS 100 ng/mL (A, C, and D) or TNF-α 10 ng/mL (B) for the indicated times. Nuclear protein was extracted and subjected to EMSA for NF-κB binding. LPS led to NF-κB activation in wild-type ECs and WT and MyD88−/− macrophages but not MyD88−/− ECs. NF-κB activation to TNF-α was observed in MyD88−/− ECs. NF-κB-bound nuclear proteins were confirmed as p65 (RelA) and p50 by super-shift as indicated in A and C.

Lack of TRAM in ECs Explains the Absence of an MyD88-Independent LPS Pathway

Our findings suggest that TRAM is required for TLR4 to be able to interact with Trif and the downstream elements of the pathway. To confirm this, we reconstituted TRAM expression in ECs and demonstrated that this restores the Trif pathway. We did this initially by testing for NF-κB activation (using p65 immunolocalization) in MyD88−/− MAECs. We overexpressed a TRAM–GFP fusion protein using a lentiviral vector (Figure 8). In MyD88−/− MAECs transduced with the control lentivirus (overexpressing GFP), LPS did not induce copious amounts of IP-10 from both ECs and macrophages. In contrast, LPS was able to induce IP-10 from macrophages but not ECs, and this was independent of the presence of MyD88. These data are consistent with absence of a functional Trif pathway in ECs.
activate NF-κB. However, in the TRAM–GFP-transfected cells, NF-κB activation by LPS was restored, as evinced by p65 translocation to the nucleus. To confirm this finding, we determined whether TRAM overexpression could restore cxcl10 (IP-10) transcription, which is Trif pathway dependent and therefore absent in wild-type ECs (Figure 6). As a control, we overexpressed TLR4, which, according to our model, would not be able to drive IP-10 induction in the absence of TRAM. This experiment was performed using transient transfection because the TLR4 construct was too big to package into the lentivirus vector. Because the mouse primary ECs were found to be refractory to transfection, this experiment was performed in BAECs. We found that, like mouse ECs, BAECs do not express TRAM, first by probing BAEC RNA with the full-length murine TRAM probe (data not shown) and second by Western blot in BAECs lysate using anti-TRAM antibody (Figure 7D). BAECs were co-transfected with the IP-10 luciferase reporter plasmid Renilla luciferase transfection control plasmid and empty, TRAM or TLR4 overexpression plasmid. TRAM overexpression caused a significant increase in IP-10 promoter activity (P<0.05; Student t test; data shown representative of three experiments), whereas TLR4 overexpression did not.

Discussion
We have shown that proinflammatory responses to LPS in vascular ECs, namely the upregulation of adhesion molecules and the recruitment of leukocytes, are entirely dependent on MyD88 in vivo and in vitro. This finding is in contrast to monocyte/macrophages in which MyD88 is not essential for NF-κB activation because of the presence of the Trif path-
way. Indeed, our data showed that unlike macrophages, ECs do not undergo NF-κB activation in response to LPS in the absence of MyD88. The implication of this is that the Trif pathway is not functional in ECs and predicts that genes specifically dependent on this pathway would not be transcribed in LPS-stimulated ECs. Such is the case with the gene cxc10, which encodes for the CXC chemokine IP-10.

We further hypothesized that the expression of candidate genes involved in the Trif pathway may be lacking in ECs, which could explain the absence of this pathway. Abundance of all interrogated mRNAs was similar to that found in macrophages, except for one, that of the accessory TIR domain protein TRAM, which was markedly diminished in ECs. TRAM protein was at low or undetectable levels in mouse, bovine, and human ECs. To confirm that this alone explains lack of MyD88-independent signal in ECs, we overexpressed TRAM cDNA in ECs and found that this successfully reconstituted MyD88-independent NF-κB activation in response to LPS and resulted in the activation of the IP-10 promoter.

Proinflammatory signaling through both TLR4 and MyD88 is directly implicated in the pathogenesis of atherosclerosis. It is not clear whether this effect is exerted on the leukocyte population or on the stromal cells (ie, ECs) of the vascular wall, but the key target tissue for the canonical TLR4 ligand LPS appears to be the vascular endothelium rather than the leukocytes. Our study identifies MyD88 as the essential signaling intermediate for TLR4 signaling in ECs, as it is for all other TLRs thus far described except for TLR3. As such, MyD88 is critical for the EC response to most pathogen-derived stimuli and also to putative endogenous TLR ligands, including hyaluronan fragments, minimally modified low-density lipoprotein, and human hsp60. This observation suggests that the endothelial contribution to the development of atherosclerosis may be dependent on the MyD88-inducible gene subset and independent of Trif-dependent genes.

The differences between these gene subsets may reflect a different physiologic role for ECs compared with monocytes–macrophages. MyD88-dependent genes are generally proinflammatory, whereas Trif-dependent genes facilitate effective antigen presentation for adaptive immune responses. These Trif-dependent responses include antigen-presenting cell proliferation (cyclin D2, ISG20), microbial processing (inducible NO synthase), T-cell recruitment (IP-10, RANTES, MIP-1β, I-TAC), T-cell activation (IL-15, IL-18, CD40), and DC maturation (type I interferons). Because of these differences, we suggest that specific downregulation of the Trif/TRAM pathway could provide therapy for autoimmune diseases (for instance, systemic lupus erythematosus) without affecting EC responsiveness to LPS required for the elimination of bacterial infections.

The findings of this study raise a number of intriguing questions. For example, it is not known whether the activation of p42/p44, p38, and JNK MAPK pathways by LPS also depends entirely on MyD88 in ECs. In macrophages, these pathways behave like NF-κB and are activated by both MyD88 and Trif. Because we found no evidence of gene transcription downstream of Trif in ECs, we suspect that in the absence of MyD88, LPS does not activate these pathways in ECs. Nevertheless, although IP-10 is a useful marker for the Trif pathway, there are a large number of other Trif-dependent genes that need to be considered. Thus, a gene array analysis in MyD88- and Trif-deficient ECs is planned to determine the full extent of genes dependent on each particular pathway, similar to those performed in macrophages.

With regard to the Trif pathway, we did not specifically determine whether the MyD88-independent transcription pathways activated by Trif remained unresponsive to LPS in ECs. For example, IRF3, IRF7, and STAT1 are activated in parallel with NF-κB in macrophages. However, because we found no evidence of downstream transcription involving Trif, it is likely that these factors are not activated in ECs. It should be noted that we found that TRAM deficiency extends across species, being evident in mouse, human, and bovine ECs. In contrast to the TLR4–Trif pathway, the TLR3–Trif pathway is present in ECs as TLR3 leads to the induction of type I interferon expression in human ECs. This is consistent with our model that the Trif pathway defect in ECs is caused by absence of TRAM because TLR3–Trif signaling has been shown to be entirely independent of TRAM.

It has been proposed that Trif cannot interact directly with TLR4 but requires the interposition of TRAM for signal transduction. In this model, TRAM would interact with TLR4 and provide a docking site for a bridging interaction with Trif. We attempted to address this using protein–protein interaction studies but could not arrive at a conclusion because of complete degradation of TLR4 after LPS stimulation (data not shown). As reported, this is likely attributable to specific ubiquitination of TLR4 by TRADD, which targets it for subsequent proteasomal degradation. Because of the instability of the TLR4 signaling complex, we could not definitively show a TLR4–Trif interaction in the nonoverexpression system of primary ECs and macrophage culture.

In summary, we propose that TRAM is a critical Trif-associated TLR4 signal transducer, the absence of which in normal vascular ECs renders the Trif pathway nonfunctional in that cell type. It remains to be determined whether the lack of TRAM prevents ECs from participating in adaptive immune responses.

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Supplemental Materials and Methods

Materials

MyD88−/− mice were kindly provided by Shizuo Akira (Osaka University). Wild-type controls were littermates. Human TNFα, mouse IL-1α, IFNγ and GM-CSF were from Peprotech, IFNα was from PBL. LPS from E. coli 0111:B4, purified by ion exchange, and lipoteichoic acid (LTA) from S. aureus were from Sigma, flagellin from Invivogen. Antibodies to p65 and p50 were from Santa Cruz, to E-selectin from PharMingen, to VCAM-1 from SouthernBiotech, to TRAM from Fabgennix, to α-tubulin from Sigma, to CD11c form eBioscience. Lentivirus constructs and packaging vectors were generously provided by Carlos Lois 1. Full-length TRAM was amplified by RT-PCR from mouse macrophage RNA and cloned into the lentiviral construct pFUGW downstream of the ubiquitin C promoter to express TRAM with GFP fused at the C-terminal by a 4-glycine linker. Lentiviruses were packaged in 293T cells and purified as described. TRAM-GFP was subcloned into expression vector pcDNA3.1 (Invitrogen) according to standard methods. Full-length TLR4 was amplified by RT-PCR from mouse macrophage RNA, and cloned into pcDNA3.1 with a 5’ FLAG sequence.

Cell Isolation and Culture

Peritoneal macrophages were elicited with 1 ml 4% Brewer’s thioglycollate medium (Difco) i.p. and harvested 4 days later by lavage with 10% FCS in RPMI-1640 (Invitrogen). They were seeded in 10% FCS in high glucose DMEM and allowed to adhere for 1 hour before washing and stimulation. EC were isolated and cultured from
mouse heart or thoracic aorta according to published methods. Briefly, harvested tissues were digested with collagenase (Worthington) and EC isolated with two rounds of magnetic bead purification (Dynal), the first with anti-PECAM-1 and the second with anti-ICAM-2 (PharMingen). EC purity was confirmed by staining Isolectin IB4 (Molecular Probes) and for PECAM-1 and smooth muscle actin (Sigma). Bovine aortic EC and human EC from aorta, umbilical vein and saphenous vein, were harvested using type II collagenase (Worthington) and cultured (bovine: DMEM with 10% FCS, penicillin and streptomycin, human: medium 199, 20 mM HEPES, 50 µg/ml endothelial cell growth serum, 100 µg/ml heparin sulfate, 5 mM L-glutamine, 10% FCS, penicillin, streptomycin and Fungizone). EC purity was confirmed with Factor VIII immunostaining. Only early passage EC (four or less) were used in experiments. THP-1 monocyte cell line was obtained from the American Type Culture Collection and grown in standard media. U937 monocytes stably expressing human L-selectin (U937-LAM) were prepared as previously described. Mouse dendritic cells (DC) were differentiated \textit{ex vivo} from harvested bone marrow, resuspended in RPMI 1640 with 10% FCS, and seeded at 250,000 cells/ml. Mouse GM-CSF was added at a concentration of 20 ng/ml, halved to 10 ng/ml 3 days later, and again to 5 ng/ml, 3 days later. After a further 2 days, identity of \textit{ex vivo} differentiated DC was confirmed by FACS analysis for CD11c.

\textbf{Immunofluorescence and ELISA}\textbf{}

Tissues were harvested after perfusion fixation with 4% paraformaldehyde, then snap frozen in OCT (TissueTec) over liquid nitrogen. 5 micron sections were cut for staining. Cells were grown on chamber slides and fix-permeabilized in acetone for 2 minutes at –
20°C. Tissue sections or cells were blocked with 3% normal goat serum (Dako) and incubated with primary antibody, then with AlexaFluor-conjugated secondary antibody (Molecular Probes). For cell-based ELISA, cells were fixed with paraformaldehyde-lysine-periodate and blocked with glycine-BSA. After primary antibody incubation in 10% normal rabbit serum, biotinylated secondary then Streptavidin ABC (Dako) were applied in succession, followed by developing with OPD (Sigma) in citrate-phosphate buffer. Reaction was stopped with 2M sulfuric acid and absorbance read at 490 nm. Chemokine ELISA kits were purchased from R&D Systems.

Leukocyte adhesion under defined laminar flow conditions.

Leukocyte interactions with MHEC from either WT or MyD88−/− mice were examined under conditions of fluid shear stress in a parallel plate flow chamber as detailed previously 5. Briefly, confluent MHEC were grown on glass coverslips (25-mm dia, Carolina Biological Supply) coated with 5 μg/mL fibronectin (Sigma), were stimulated with TNFα (10 ng/mL) or LPS (100 ng/ml) for 4 or 24 hours and inserted into the flow chamber. U937-LAM cells were suspended in flow buffer (Dulbecco phosphate-buffered saline, 0.75 mM Ca^{2+} and Mg^{2+}, containing 0.1% human serum albumin) and drawn through the flow chamber at an estimated shear stress of 0.5 or 1.0 dynes/cm². U937-LAM accumulation was measured after 3 min of flow by counting stably adherent U937-LAM cells in four different fields. U937-LAM interactions with MHEC of WT or MyD88−/− mice were viewed videomicroscopy (20x phase contrast objective) and digitally recorded to a PC using VideoLab software.
Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were isolated by incubating cells in hypotonic solution (10 mM HEPES [pH 7.8], 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA supplemented with a protease inhibitor cocktail [EDTA-free mini, Roche]) followed by addition of 1% Igepal CA-630 (Sigma). After brief centrifugation, the nuclear pellet was resuspended in extraction buffer (50 mM HEPES [pH 7.8], 50 mM KCl, 350 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol). After 30 minutes on ice and centrifugation at 14000 rpm for 10 minutes the supernatant was recovered and protein quantified with MicroBCA assay (Pierce). Binding reactions were performed with 5 µg of nuclear extract in 20 µL containing 0.1 mg/ml poly-dIdC (Roche), 0.1 mg/ml bovine serum albumin, 2 mM dithiothreitol, 4% Ficoll and the ³²P-labeled NF-κB consensus binding sequence (Santa Cruz) for 30 minutes at 30°C. 2 µg of supershift antibody was included in the binding mixture 10 minutes before addition of the probe. Bound complexes were separated from the unbound probe by non-denaturing 5% PAGE. Gels were dried for 1 hour at 80°C and then exposed to film in auto-radiograph cassette at -80°C overnight.

RNA and protein detection

RNA was obtained using standard methods with RNA-Bee (Isotex). RT reactions were performed with Superscript II (Invitrogen) and PCR with the following primers: β-tubulin ATC GGT GCT AAG TTC TGG GA forward, AGG GAC ATA CTT GCC ACC TG reverse, Trif AAC ACC TTC AAG ACA CAG AAG C forward, TAT GGA CAT AGG CTG TGT AGG C reverse, TRAM ACT CTG TCA TGG GTG TTG GG forward, CAC TGC TCT GCT CCA CAA AA reverse, IKKi GTG TCA CCG CAA CCT ATG G...
forward, TGT AAC CGT TCG ATG AGT CG reverse, TBK1 GCA GTC TTC AGG ACA TCA GC forward, TCT GTG ATG CAG TTC AAC AGG reverse, IRF3 CTT CGT GGC AGA TCT GAT TG forward, CAA GTC CAC GGT TTT CAG TG reverse, Tie2 TTG GAT TGT CAC GAG GTC AA forward, GGG GTG CCT CCT AAG CTA AC reverse. qRT-PCR was performed with the Quantitect SYBR Green kit (Qiagen) on a Roche LightCycler, using gene specific primers above, cDNA standards, and water as negative control. TRAM abundance was normalized to β-tubulin. For northern blotting, equal amounts of total RNA (10 µg/lane) were separated by 1% formaldehyde-agarose gel electrophoresis, transferred overnight onto nylon membranes, UV crosslinked prior to prehybridization in PerfectHyb plus (Sigma). Radiolabeling of TRAM cDNA probe was performed using Random Primer Labeling kit (Takara). The membranes were hybridized overnight at 68°C and washed (0.1X SSC, 0.1% SDS at 60°C) prior to autoradiography with intensifying screen at -80°C for 48 hours. For western blotting, whole cell protein lysates were extracted using RIPA buffer with PMSF 1 mM and complete mini EDTA-free protease inhibitor cocktail (Roche), separated by denaturing PAGE, and electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked (5% skim milk in TBS/0.1% Tween-20), and incubated with anti-TRAM (FabGennix) or anti-α-tubulin (Sigma), then HRP-coupled anti-rabbit secondary antibody, sequentially, with three TBS/0.1% Tween-20 washes after each step. Immunodetection was accomplished using ECL chemiluminescence (Amersham). TRAM protein was identified as a 27 kD band (Uni-ProtKB/TrEMBL database accession number Q86XR7).
Absence of TRAM in endothelial cells

**IP-10 reporter assay**

Human IP-10 (cxcl10) promoter luciferase reporter construct was kindly provided by Dr Andrew Luster (Massachusetts General Hospital)\(^6\). Early passage BAEC were seeded in 24-well plates and transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. Reporter assay was carried out using Dual-Luciferase with pRL-CMV as transfection control (Promega) and a Biotek KC4 luminometer.

**References**


