Functional genomics of silencing TREM-1 on TLR4 signaling in macrophages

M. Ornatowska, A. C. Azim, Wang X, Christman JW, Xiao L, Joo M, Sadikot RT. Functional genomics of silencing TREM-1 on TLR4 signaling in macrophages. Am J Physiol Lung Cell Mol Physiol 293: L1377–L1384, 2007. First published September 28, 2007; doi:10.1152/ajplung.00140.2007.—Triggering receptor expressed on myeloid cells 1 (TREM-1) is a recently discovered molecule that is expressed on the cell surface of monocytes and neutrophils. Engagement of TREM-1 triggers synthesis of proinflammatory cytokines in response to microbes, but the extent and mechanism by which TREM-1 modulates the inflammatory response is poorly defined. In the present study, we investigated the functional effects of blocking TREM-1 on the Toll-like receptor (TLR)4-mediated signaling pathway in macrophages. By transfecting cells with small hairpin interfering RNA molecules to TREM-1 (shRNA), we confirmed that TREM-1 mRNA and protein expression was greatly attenuated in RAW cells in response to treatment with LPS. PCR array for genes related to or activated by the TLR pathway revealed that although the expression of TREM-1 itself was not significantly altered by silencing of TREM-1, expression of several genes, including MyD88, CD14, IkBα, IL-1β, MCP-1, and IL-10 was significantly attenuated in the TREM-1 knockdown cells in response to treatment with LPS. These data indicate that expression of TREM-1 modulates the TLR signaling in macrophages by altering the expression of both adaptor and effector proteins that are critical to the endotoxin response.

TRIGGERING RECEPTOR EXPRESSED ON MYELOID CELLS 1 (TREM-1) is a newly identified member of the immunoglobulin superfamily of receptors present on the macrophage and neutrophils that has also shown to be involved in the triggering of the inflammatory response (4, 5, 7, 8). Although no specific ligands have been identified yet, indirect evidence suggests that TREM-1 can recognize bacterial products, similar to other pattern recognition receptors, or protein ligands that may be secreted in response to microbial infections by the host. TREM-1 has been shown to associate to DAP12, an ITAM-containing adaptor protein, for the initiation of the signaling cascades in monocytes (23). In animal models of severe sepsis, blockade of TREM-1 protects mice from septic shock and death (5, 10, 12, 13, 17, 19, 20). Thus, TREM-1 is a potential target for the development of a novel therapy for septic shock (6, 26, 29). However, the pertinent molecular mechanisms by which these newly identified receptors regulate inflammation have not been characterized.

Toll-like receptors (TLRs) are highly conserved transmembrane proteins that play an important role in the detection and recognition of microbial pathogens. TLRs recognize microbial products termed pathogen-associated molecular patterns in the response to infection (1, 2, 22, 31). In humans, there are at least 10 TLRs that have different pathogen-associated molecular pattern specificities, e.g., TLR4 for LPS, TLR2 for lipoproteins, and TLR3, TLR7, and TLR8 for single- or double-stranded RNA (8, 27). Most TLRs activate nuclear factor-κB (NF-κB; p65) by binding of MyD88 to the cytoplasmic tail of the receptor (the Toll/IL-1 receptor domain) and subsequently triggering IL-1 receptor-associated kinases (IRAKs), TNF receptor-associated factor 6, and TGF-β-activated kinase 1. These events ultimately activate the IκB kinase complex to induce inflammatory genes by initiation of the NF-κB activation pathway (21, 33).

An interaction between TREM-1 and TLRs has been indirectly demonstrated. For example, TREM-1 is significantly upregulated by various ligands for TLRs, including lipoteichoic acid (TLR2), polyinosinic-polycytidylic acid (TLR3), and LPS (TLR4) (3). In addition, binding of agonistic monoclonal antibody to TREM-1 on monocytes in combination with the ligands for TLR2, TLR3, or TLR4 was shown to synergistically amplify the cellular production of proinflammatory cytokines (3, 4, 5). This would amplify the inflammatory response in cells like macrophages and neutrophils that express both TREM-1 and TLRs significantly as opposed to cells that only express the TLRs such as the epithelial cells.

The aim of this study was to determine the functional effects of silencing TREM-1 on the genes induced by the TLR pathway in the inflammatory response to LPS in macrophages. To define the effects of TREM-1 on TLR signaling, we generated a TREM-1 knockdown cell line using small interfering RNA molecules. Silencing of TREM-1 resulted in attenuation of adaptor and TLR interacting proteins and downstream target genes such as pro- and anti-inflammatory cytokines, induced by the TLR pathway in response to LPS, thus implying a cooperation between the two receptors in generation of inflammatory response.

MATERIALS AND METHODS

Cell culture. A murine macrophage cell line RAW 264.7 (ATCC, Rockville, MD) was maintained in DMEM (Cellgro) containing 10% FBS (Hyclone), penicillin (100 U/ml)/streptomycin (100 μg/ml) (Invitrogen), and 2 mM glutamine (Sigma).

Cell lines with small hairpin interfering RNA TREM-1: TREM-1 RNA interference constructs. Small hairpin interfering RNA (shRNA) TREM-1 were prepared according to Invitrogen design software.
L1378  SILENCING OF TREM-1 GENE

(BLOCK-iT). Constructs that contain the DNA sequence from 101–121 bp (construct named 101) and 399–420 bp (construct named 399) of the TREM-1 coding sequence, and exhibit homology lower than 17 bp to the other coding sequence from NCBI data base, were used. Each PCR reaction was performed with the primers named shRNA-101 and shRNA. The primers used for annealing and ligation are as follows: shRNA-101-5'-CAC CGC CAG ACT TGG ACA GTG AAG TCA TCT CAG TGT CAA AGT CTG-3', shRNA-101-bottom 5'-AAA AGC CAG ACT TGG ACA GTG AAG TTA CGA CTT CAG TCT CAG TCA AAG TCT CCT TAC CCT TCC CGG TCT CAG ATG TCT CTC CCT AGG GTT CTT CAG ATG TCG AAA CAT CAG AAG CCT CTT GC-3', shRNA-399-top 5'-CAC CGA CCA AGG GTT CCT CAA CTT CAG ATG TCG AAA CAT CAG AAG CCT CTT TG-3', shRNA-399-5'-AAA AGA CCA AGG GTT CCT CAG ATG TCT CCA CAT GAC AAC CCT TGG TG-3'. The ligation products containing the sequence in a sense orientation (21 bp) followed by loop sequence /H11032

The cDNA was synthesized from 1 l of total RNA using 200 units of MMLV reverse transcriptase (Fermentas), and 20 units of Ribonuclease inhibitor (Fermentas). Following a denaturation step of 5 min at 70°C, RNA was reverse transcribed to single-stranded cDNA using oligo(dT) primers (Fermentas). The reverse transcription reaction was performed in a total volume of 20 μl containing 0.2 mM of each dNTP (Fermentas), 40 units of MMLV reverse transcriptase (Fermentas), and 20 units of Ribonuclease inhibitor (Fermentas) at 37°C for 60 min and 70°C for 10 min.

Quantitative real-time polymerase chain reactions. Real-time PCR was conducted using Applied Biosystems (ABI Prism 7900HT) according to the manufacturer’s instructions. The mRNA expression levels of TREM-1, and an endogenous housekeeping gene encoding for GAPDH as a reference, were quantified using real-time PCR analysis (SYBR green I dye) on an ABI Prism 7900HT. Amplification of specific PCR product was performed using the SYBR Green PCR Master Mix (Applied Biosystems). All primers employed were cDNA specific and were purchased from Sigma Genosys (Sigma). TREM-1 (NH2 and COOH terminus) and GAPDH designed primers were: 1) TREM-1 forward primer, 5'‐TAT GCC AAC AGG CAG AGC GC-3'; TREM-1 reverse primer, 5'‐GTC TCC CTT CCC GTC TCG TA-3'; 2) TREM-1c forward primer, 5'-CTA CAA CCC GAT CCC TAC CCA-3'; TREM-1c reverse primer, 5'-GAC CAG GAG AAA CCA CCG-3'; 3) GAPDH forward primer 5'-CAT CTT GTC CCT TCC TCC G-3'; 4) GAPDH reverse primer, 5'-TCT ACC CCC ATC ACA AAC ACG-3'. The qRT-PCR was performed in triplicate in a total reaction volume of 25 μl containing 12.5 μl of SYBR Green PCR Master Mix, 300 nM forward and reverse primers, 11 μl of distilled H2O, and 1 μl of cDNA from each sample. Samples were heated for 10 min at 95°C and amplified for 40 cycles of 15 s at 95°C and of 60 s at 60°C. Blank and positive controls (calibrators) were run in parallel to determine amplification efficiency within each experiment. During the extension step, the ABI Prism 7900HT Sequence Detection System monitored PCR amplification in real time by quantitative analysis of the emitted fluorescence. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimmer. Quantification was performed using the ∆ΔCt method. The target amount of each mRNA sample was subsequently divided by the control gene amount (which was assigned a value of 1 arbitrary unit) to obtain a normalized target value. Each primer efficiency was tested using sequential annealing temperatures and Mg2+ concentrations (2–7 mM). Primer efficiency was calculated using the standard curve method (E = 10 – 1/slope, where E represents the primer efficiency). Each standard curve was determined with multiple dilution steps and replicates and stored as a coefficient file used for the analysis.

RT-PCR. RT-PCR was conducted using Peltier Thermal Cycler (MJ Research) according to the manufacturer’s instructions. Reactions were performed in a 25-μl volume with 0.2 mM dNTPs, 2.5 μl of reaction buffer, 0.1 μM primers, 2.5 mM MgCl2, 1.25 units of Taq polymerase (all reagents were from Fermentas), and 4 μl of cDNA. Actin was chosen as housekeeping gene for relative quantification to normalize target gene expression. The following primers were used for RT-PCR amplification: TREM-1 forward (5'-CGG AAT TCG AGC TTG AAG GAT GAA GGC-3'), TREM-1 reverse (5'-AAT CCA GAG TCT GTC AGT AAG TCA GTC-3'), CD4 forward (5'-CTGATCTCAACCCCTGTC-3'), CD4 reverse (5'-CGTGAGCTGAGTTTTCTTTG-3'), MyD88 forward (5'-CGT GTG CTG CAT CTG CAT-3'), MyD88 reverse (5'-AGTACACGGTTTCCCCACTTG-3'). The ligation products containing the DNA sequence from 101–399 of the TREM-1 gene were used in a pENTRY/U6/ vector (Invitrogen) via gateway technology and subsequently used for a stable shRNA expression in RAW 264.7 cell line. The integrity of the sequences was confirmed by DNA sequencing. We subcloned two knockdown lines of RAW cells, which are referred to as 399 and 101, and compared them with the wild-type RAW cells. The 101 line of RAW cells targets the NH2 terminus of the TREM-1 gene, whereas the 399 line targets the COOH-terminal region of the gene.

shLacZ construct prepared in a similar manner was used for control shRNA. LacZ DNA oligo primers, top strand 5'-cagcgtccaaaacatcattcaggtcgaagatgtagtcg-3', bottom strand 5'-aaactacataacatcattcaggtcgaagatgtagtcg-3', were used in a pENTRY/U6/ vector for the transient expression of shLacZ in RAW cells or subcloned into pDEST vector (BLOCK-iT Lentiviral vector) for the stable expression in RAW 264.7 cell line.

RNA extractions. Total cellular RNA was extracted using the Qiagen RNAeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. The RNA samples were treated with DNase I (Qiagen) and stored at –80°C until used. The RNA quality was examined using gel electrophoresis and using A260/A280 ratio (SMARTspec, Bio-Rad). The cDNA was synthesized from 1 μg of total RNA by using SuperScript first-strand synthesis system (Fermentas). Following a denaturation step of 5 min at 70°C, RNA was reverse transcribed to single-stranded cDNA using oligo(dT) primers (Fermentas). The reverse transcription reaction was performed in a total volume of 20 μl containing 0.2 mM of each dNTP (Fermentas), 40 units of MMuLV reverse transcriptase (Fermentas), and 20 units of RiboLock Ribonuclease inhibitor (Fermentas) at 37°C for 60 min and 70°C for 10 min.

Quantitative real-time polymerase chain reactions. Real-time PCR was conducted using Applied Biosystems (ABI Prism 7900HT) according to the manufacturer’s instructions. The mRNA expression levels of TLR-1, and an endogenous housekeeping gene encoding for GAPDH as a reference, were quantified using real-time PCR analysis (SYBR green I dye) on an ABI Prism 7900HT. Amplification of specific PCR product was performed using the SYBR Green PCR Master Mix (Applied Biosystems). All primers employed were cDNA specific and were purchased from Sigma Genosys (Sigma). TLR-1 (NH2 and COOH terminus) and GAPDH designed primers were: 1) TLR-1 forward primer, 5'-TAT GCC AAC AGG CAG AGC GC-3'; TLR-1 reverse primer, 5'-GTC TCC CTT CCC GTC TCG TA-3'; 2) TLR-1c forward primer, 5'-CTA CAA CCC GAT CCC TAC CCA-3'; TLR-1c reverse primer, 5'-GAC CAG GAG AAA CCA CCG-3'; 3) GAPDH forward primer 5'-CAT CTT GTC CCT TCC TCC G-3'; 4) GAPDH reverse primer, 5'-TCT ACC CCC ATC ACA AAC ACG-3'. The qRT-PCR was performed in triplicate in a total reaction volume of 25 μl containing 12.5 μl of SYBR Green PCR Master Mix, 300 nM forward and reverse primers, 11 μl of distilled H2O, and 1 μl of cDNA from each sample. Samples were heated for 10 min at 95°C and amplified for 40 cycles of 15 s at 95°C and of 60 s at 60°C. Blank and positive controls (calibrators) were run in parallel to determine amplification efficiency within each experiment. During the extension step, the ABI Prism 7900HT Sequence Detection System monitored PCR amplification in real time by quantitative analysis of the emitted fluorescence. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimmer. Quantification was performed using the ∆ΔCt method. The target amount of each mRNA sample was subsequently divided by the control gene amount (which was assigned a value of 1 arbitrary unit) to obtain a normalized target value. Each primer efficiency was tested using sequential annealing temperatures and Mg2+ concentrations (2–7 mM). Primer efficiency was calculated using the standard curve method (E = 10 − 1/slope, where E represents the primer efficiency). Each standard curve was determined with multiple dilution steps and replicates and stored as a coefficient file used for the analysis.

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compared using the ΔΔC(t) method to determine the fold change [fold change = \[2^{\Delta \Delta CT}\]]. The significance value for the fold change in each gene fold change was calculated as the difference in gene expression between test sample (shRNA for TREM-1) and control sample (shRNA for control). A positive value indicates gene upregulation, and a negative value indicates gene downregulation. All data are expressed as means ± SE. P < 0.05 was considered significant. All statistical analyses were performed using Microsoft Excel software.

RESULTS

TREM-1 message and protein induction was inhibited in response to LPS in cells with shRNA TREM-1. To define the functional consequences of silencing TREM-1 in the RAW macrophage cell lines, we made cells that express the TREM-1 shRNA. Although the specific ligand for TREM-1 has not been identified, it has been shown that TREM-1 is induced in response to LPS. We first confirmed that the TREM-1 mRNA
and protein induction of TREM-1, in response to LPS treatment, is inhibited in cells with shRNA of TREM-1. Wild-type RAW cells expressing a lacZ shRNA vector before treatment with either PBS or LPS (100 ng/ml) for 24 h were used as controls. We have shown that the induction of TREM-1 in response to LPS occurs by 4 h, and the expression of TREM-1 protein lasts until 24 h (35). There was no basal expression of TREM-1 mRNA in the lacZ shRNA RAW cells, but upon treatment with LPS, TREM-1 mRNA and protein production was induced in the lacZ shRNA RAW cells. These data were compared with the expression of TREM-1 by cells that expressed two different TREM-1 shRNA constructs. The 101 TREM-1 shRNA lines of RAW cells targets the NH2 terminus of the TREM-1 gene, whereas the 399 line of TREM-1 shRNA RAW cells targets the COOH-terminal region of the gene. Compared with the LPS-treated lacZ shRNA RAW cells, the 101 TREM-1 shRNA RAW cells consistently showed at least 80% reduction in the expression of TREM-1 mRNA and TREM-1 protein levels (Fig. 1, A and B), thus confirming that these cells were true TREM-1 knockdown cells. The 399 TREM-1 shRNA RAW cells were intermediate in the reduction of TREM-1 gene expression between the lacZ shRNA RAW cells and the 101 TREM shRNA RAW cells. Densitometric analysis showed a statistically significant difference in the expression of TREM-1. The attenuation of TREM-1 in these cells was also confirmed by FACS analysis (Fig. 1C).

Silencing of TREM-1 modulates the expression of selective pro- and anti-inflammatory cytokine genes and receptors. After confirming that TREM-1 expression is inhibited in the 101 TREM-1 shRNA RAW cells, we performed a TLR4 microarray to elucidate the response to LPS in wild-type cells and cells with TREM-1 knockdown. This array included 84 TLR-dependent genes including the TLRs, adaptor proteins, downstream transcription factors such as NF-kB, JNK/p38 pathway, NF-IL-6, and IRF pathways and specific cytokines and chemokines that are regulated by this pathway.

LacZ shRNA RAW cells and 101 TREM-1 shRNA RAW cells were treated with PBS or LPS (100 ng/ml), and cells were harvested at 4 h. Total RNA was extracted from the cells, and PCR microarray was performed by using the RT Profiler PCR array for murine TLR pathway. There was very little basal induction of these cytokines in untreated cells. Upon treatment with LPS, the gene expression of proinflammatory cytokines and cytokine receptors MCP-1, CXCL10, IL-10, IL1r1, IL-1β, IL-2, GM-CSF, and IL-6r were significantly decreased in the 101 TREM-1 shRNA RAW cells compared with the lacZ shRNA RAW cells that were treated with LPS (100 ng/ml) (Table 1 and Fig. 2A). There were no significant difference in the expression of G-CSF, INF-γ, IL-6, IL-1α, or TNF-α gene between the lacZ shRNA RAW cells and the 101 TREM-1 shRNA RAW cells (Table 1). Verification of the microarray data in selected downregulated genes was performed by RT-PCR or Western blotting. As shown in Fig. 2A, there was a large reduction in the expression of IL-1β mRNA and IL-10 mRNA by RT-PCR (Fig. 2, B and C, respectively), and there were differences in IL-10 and MCP-1 protein expression by Western blot analysis that are shown in Fig. 2D. These data suggest that the activation of TREM-1 amplifies the expression of selective cytokine genes (e.g., MCP-1, IL-1β, and IL-10) that are regulated by the TLR4 pathway in macrophages.

The induction of CD14 and MyD88 is significantly attenuated in TREM-1 knockdown cells. We also evaluated the gene expression of the adaptor and TLR interacting proteins that are critical to the TLR4 response to LPS in cells with the TREM-1 knockdown cells. The expression of MyD88, Cd14, and Pgytrp1 mRNA was significantly decreased in the 101 TREM-1 shRNA RAW cells compared with the lacZ shRNA RAW cells that were treated with LPS (100 ng/ml) (Table 2 and Fig. 3A). This difference in expression was confirmed by RT-PCR for MyD88 mRNA (Fig. 3B) and by Western blot analysis for CD14 (Fig. 3B). Induction of other adaptor proteins, such as MD2, RIP2, Ticom1, Ticom2, Tirap, CD80, or CD86, was not altered in the TREM-1 knockdown cell lines (Table 2). Similarly, there was no significant difference in the expression of effector proteins such as Irak1, Irak2, and Tradd in the 101 TREM-1 shRNA RAW cells compared with control. Expression of TLR4 itself was also not altered in the TREM-1 knockdown cells.

These data suggest that modulation of the inflammatory response by TREM-1 through TLR pathway may be a result of altered expression of important adaptor molecules such as MyD88 and CD14. These molecules are known to associate with TLR4 to activate signaling cascades to generate an inflammatory response to LPS. Thus the inhibition of these molecules may be a mechanism by which TREM-1 attenuates the inflammatory response induced by the TLR4 signaling pathway in response to endotoxin.

Expression of selective genes regulating the NF-κB pathway was downregulated in TREM-1 knockdown cells. Of the genes encoding the activation of NF-κB pathway, the expression of p100 (the precursor to p50), IkBa, and IkBβ was significantly downregulated in the 101 TREM-1 shRNA RAW cells that were treated with LPS compared with the lacZ shRNA RAW cells (Fig. 4). The expression of CEBP-β was also downregulated in the 101 TREM-1 shRNA RAW cells. However, there was no significant difference in the expression of the MAP kinases (MEK3, MEK4, or MEK1) or JNK or Fos expression in the TREM-1 shRNA RAW cells (Table 3). Thus silencing of
TREM-1 modulates the activity of selective proteins that are involved in the transcriptional regulation of downstream effector proteins. These data suggest that the interaction of TREM-1 with the TLR4 signaling pathway occurs at multiple levels.

**DISCUSSION**

In this study, we have elucidated the contribution of TREM-1 expression to the genes regulated by the TLR4 pathway in response to LPS by employing PCR microarray. Verification of the transcriptional profiling was obtained for selected genes by reverse transcription PCR or by Western blotting. Our data indicate that silencing of TREM-1 gene in macrophages modulates the expression of certain selective genes including adaptor and TLR interacting molecules, genes related to the NF-κB signaling pathway, and pro- and anti-inflammatory molecules that define the TLR response to endotoxin. In particular, the expression of CD14, MyD88, IL-10, IL-1β, MCP-1, and IL-6 was significantly attenuated in TREM-1 knockdown cells compared with the control cells. This selective modulation of genes regulated by the TLR pathway by activation of TREM-1 is a potential mechanism by which the inflammatory response may be accentuated in cells that express both TREM-1 and TLRs such as macrophage and neutrophils.

TREM-1 is a newly identified receptor present on the macrophage and neutrophils that has also shown to be involved in the triggering of the inflammatory response (4, 7, 10). Although no specific ligands have been identified yet, it has been shown that TREM-1 is induced in response to bacterial products, similar to other pattern recognition receptors, or protein ligands that may be secreted in response to microbial infections by the host. In animal models of severe bacterial infection,
blockade of signaling via TREM-1 was able to protect mice against septic shock and death (13). Levels of soluble TREM-1 in bronchoalveolar lavage fluid and serum of patients with pneumonia have shown to be an indicator and prognostic factor in sepsis and acute respiratory distress syndrome induced by microbial infections (11, 14, 18, 22, 28). Thus, TREM-1 is an attractive therapeutic target and a diagnostic marker for infectious inflammatory diseases. The molecular mechanisms by which TREM-1 modulates the inflammatory response have not been well defined.

Inflammation constitutes the initial and essential response of the host against infection and injury. In the presence of infection, inflammation is triggered through the recognition of microorganisms, subsequently initiating signaling pathways important in the generation of cytokines, chemokines, antimicrobial peptides, and upregulation of adhesion and costimulatory molecules involved in innate and acquired immune responses (32, 34). To date, 10 human and 11 murine TLRs have been identified, and these receptors signal principally by using the adaptor myeloid differentiation factor 88 (MyD88) and IRAKs (9, 33). However, the consequences of TREM-1 activation on TLR signaling have not been characterized.

TREM-1 has been shown to amplify the TLR-initiated responses to invading pathogens, allowing the secretion of proinflammatory chemokines and cytokines (5, 24). TREM-1 has been shown to be underexpressed in macrophages in the human intestines, which are constantly exposed to the gut bacteria, thus implying their capacity to potentiate the TLR-induced responses (30). Bouchon et al. (5) activated neutrophils and macrophages using specific nonblocking agonistic monoclonal antibodies to TREM-1. They showed that neutrophils activated by the TREM-1 antibody via TREM-1 release IL-8 and myeloperoxidase. Monocytes/macrophages activated by TREM-1 release a variety of inflammatory mediators that include MCP-1, MIP-1α, IL-8, TNF-α, and GM-CSF (1). In a mouse model of fecal peritonitis, inhibition of TREM-1 resulted in altered production of cytokines and chemokines, in particular IL-1β, IL-6, TNF-α, and IL-10 (19). In our study, we

![Fig. 3. A: the expression of adaptor proteins MyD88, CD14, and PGRP mRNA was significantly reduced in 101 TREM-1 shRNA RAW cells compared with lacZ shRNA RAW cells treated with LPS (100 ng/ml) at 24 h as determined by PCR microarray. B: RT-PCR shows the reduction in MyD88 expression in 101 TREM-1 shRNA RAW cells compared with lacZ shRNA RAW cells. C: Western blot analysis confirms attenuation of CD14 expression in 101 TREM-1 shRNA RAW cells compared with lacZ shRNA RAW cells in response to treatment with LPS.]

![Table 3. Effect of TREM-1 deficiency on gene expression of transcription factors and related kinases]

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Fold difference in gene downregulation of transcription factor-related proteins between control cells expressing lacZ shRNA and TREM-1 shRNA after treatment with LPS (100 ng/ml) in RAW cells at 24 h. Average results of 3 individual experiments are shown, n = 3. *P < 0.001.
did not see a significant difference in TNF-α or IL-6. This may be related to the fact that in vivo neutrophils contribute to the inflammatory response, and the effects may depend on the cell type involved with the response. Besides, Gibot et al. (19) used a model of peritonitis where the induction of TNF-α may dominate the inflammatory response. Mechanisms for the synergistic effect of TREM-1 and TLRs are not defined but can be postulated. One of the potential links may be activation of transcription factors such as NF-κB and AP-1. It is well established that NF-κB is activated by the TLR signaling pathway. TREM-1-mediated tyrosine phosphorylation, activation of MAPK, and mobilization of Ca²⁺ might also lead to the activation of transcription complexes, which could have a synergistic effect with NF-κB in promoting the expression of proinflammatory genes (8, 23). Thus, activation of TREM-1 may synergize with various TLRs in promoting the production of proinflammatory cytokines through activation of NF-κB and MAPK, tyrosine phosphorylation, and mobilization of Ca²⁺. We have shown that expression of TREM-1 is transcriptionally regulated by NF-κB (35). Thus the regulation of TREM-1 is NF-κB dependent, and at the same time, the induction of NF-κB in response to TLR ligands appears to be at least partially dependent on activation of TREM-1.

In the present study, we have shown that the gene expression of adaptor proteins and transcription factors related to the TLR signaling pathway is modulated in macrophages. LPS, which is the cell wall component of gram-negative bacteria, binds to TLR4 and recruits adaptor proteins of which CD14 and MD2 are critical for generating the inflammatory response. The downstream effects are then signaled in an MyD88-dependent and -independent manner. The importance of both these proteins in the TLR signaling pathway has been demonstrated in murine and human studies. It has been shown that LPS fails to elicit a response in CD14 knockout mice (25). In human studies, an association between susceptibility to septic shock and genetic polymorphisms in the CD14 locus has been reported (15). Our data show that in TREM-1 knockout cell lines, the gene expression of both CD14 and MyD88 was attenuated, thus implying that TREM-1 plays a role in the induction of the genes that are proximal in the TLR signaling pathway and may be a mechanism by which the expression of effector cytokines and chemokines such as IL-1β, IL-10, and MCP-1 may be influenced. However, the specific mechanisms by which TREM-1 modulates the gene expression of the proximal proteins involved in the TLR signaling need further study.

In conclusion, we have shown that activation of TREM-1 modulates the TLR signaling response by activating genes such as MyD88, CD14, IL-1β, MCP-1, IκBα, and IL-10. Thus activation of these genes in cells such as the macrophage may amplify the TLR response compared with cells that do not express TREM-1, such as the epithelial or endothelial cells.

GRANTS

This work was supported by the U. S. Department of Veterans Affairs and National Heart, Lung, and Blood Institute Grants HL-075557 and HL-66196.

REFERENCES