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Pam2CSK4 and Pam3CSK4 induce iNOS expression via TBK1 and MyD88 molecules in mouse macrophage cell line RAW264.7

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Abstract

Objective The aim of this study was to investigate the involvement of TLR adaptor molecules, such as TRIF, MyD88, and TBK1 in the induction of iNOS and nitric oxide (NO) production in Pam2CSK4 and Pam3CSK4-treated mouse macrophages.

Method Mouse macrophage cell line (RAW264.7) was transfected with *trif*, *myd88,* and *tbk1* siRNAs before stimulated with Pam2CSK4 and Pam3CSK4. The iNOS gene and protein expression were determined by RT-PCR and immunoblotting, respectively. The NO production was determined by Griess reaction assay.

Results The results showed that the induction of iNOS expression and NO production by Pam2CSK4 and Pam3CSK4 were diminished in *tbk1* and *myd88*-depleted mouse macrophages but not *trif*-depleted cells.

Conclusion These results suggested that the TBK1 and MyD88 molecules were essential for the induction of iNOS expression and NO production by both Pam2CSK4 and Pam3CSK4 via TLR2 signaling.

Keywords iNOS · MyD88 · TBK1 · TLR2 ligands · TRIF

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Introduction

TLR activation has been extensively demonstrated as an immune response to a broad spectrum of microbial components, such as lipopolysaccharide (LPS), peptidoglycan (PGN), lipopeptide, and microbial nucleic acid [1]. Upon encountering the cognate ligands, pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), TLR homodimers or heterodimers becomes active and recruits downstream signaling proteins to initiate the signaling cascades [2]. Consequently, TLR activation triggers innate immune response resulting in the activation of transcriptional factors including NF-κB, MAPKs, AP-1, IRF-1, and IRF-3. Subsequently, the production of pro-inflammatory cytokines such as IL-6, TNF-α, IL-1β, type I interferons (IFN α/β), IFN-γ, and antimicrobial activity is responsible for the immune response during pathogen infections [3, 4]. Activation of TLR signaling pathways involves five adaptor proteins known as myeloid differentiation primary response gene 88 (MyD88), MyD88 adaptor-like (MAL), TIR-domain-containing adapter-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo-motif-containing protein (SARM) [5]. MyD88 is considered as the key signaling adaptor for most TLRs (MyD88-dependent pathway) during microbial infections. With some exception, TLR3 and a subset of TLR4 signaling pathways can also utilize another adaptor protein called TRIF (TRIF-dependent pathway) [5].

TRIF and MyD88 are two major adaptor molecules utilized by several TLRs family. Although there is an overlap in the downstream signaling pathways and the specific inflammatory mediators, TRIF and MyD88 have their own independent functions [6]. Since MyD88 and

TRIF are mediated by different TIR domains, different set of pro-inflammatory cytokines are produced downstream of their activation [7]. The previous report demonstrated that MyD88-deficient mice were susceptible to various types of pathogens [8–10]. To stimulate the TRIF-dependent pathway, TRIF is recruited to endosomes leading to activation of TANK-binding kinase 1 (TBK1), the protein kinases that activate the production of type I IFNs [11, 12]. Inducible nitric oxide synthase (iNOS) is known to be one of the outcomes of the TRIF-dependent pathway. It is an important enzyme that synthesizes nitric oxide (NO) to restrict both viral and bacterial replication [13]. The role of iNOS in host immune response during bacterial infection has been reported to empower macrophage ability against intracellular organisms [14, 15]. Failure to induce iNOS expression allows the pathogens to survive and multiply inside the host cells.

TLR2 can recognize essential components of Gram-positive bacteria including PGN, lipoteichoic acid and bacterial lipoproteins, whereas TLR4 recognizes bacterial LPS, a major cell wall component of Gram-negative bacteria [16, 17]. In contrast to other homodimer TLRs, TLR2 has an ability to form heterodimers with TLR1 (TLR2/1) or TLR6 (TLR2/6). TLR2/1 can sense triacyl-lipopeptide derived from Gram-negative bacteria while TLR2/6 are able to detect diacyl-lipopeptides derived from mycoplasma and Gram-positive bacteria [18, 19]. Remarkably, the internalization of TLR2 heterodimers into endolysosome is deliberated as the key feature to induce type I IFN and NO responses during synthetic TLR2 ligand treatment in bone marrow-derived macrophages (BMDMs) [20, 21]. In addition, recent studies confirmed that IFN-β production is mediated by TLR2 using both MyD88 and TRIF molecules in peritoneal macrophages [21, 22]. However, the underlying pathway of iNOS expression in response to TLR2 ligands has not been fully elucidated. The involvement of TLR2 and their downstream signaling molecules as well as *inos* gene expression and NO production are examined in mouse macrophages during specific TLR2 ligand treatment (Pam2CSK4 and Pam3CSK4). We herein show that Pam2CSK4 and Pam3CSK4 are able to induce IFN-inducible genes including *ifn-β, inos* and, NO production via the TRIF-independent and the TBK1-dependent pathway in mouse macrophages. Furthermore, MyD88 adaptor molecule is also required for the signal transduction of iNOS expression via the TLR2 response. Our study provides the evidence that TLR2 activation converges with the downstream signaling molecules to activate iNOS expression and NO production in host innate defense mechanism.

Materials and methods

Cell line and culture condition

Mouse macrophage cell line RAW 264.7 (ATCC) was cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone) and 1% L-glutamine (Gibco Labs, Grand Island, NY, USA) at 37 °C under a 5% CO_2 atmosphere.

Reagents

Pam2CSK4 (Synthetic diacylated lipopeptide) and Pam3CSK4 (Synthetic triacylated lipoprotein) were purchased from Invivogen. Bafilomycin A1 was purchased from Sigma-Aldrich. Anti-mouse CD282 (TLR2) monoclonal antibody and mouse IgG1K isotype control were purchased from eBioscience. *Escherichia coli*-LPS O111:B4 (Sigma-Aldrich) was used for comparisons. Each of these ligands was used at a concentration of 100 ng/ml by adding to the macrophage cultures and incubated together until the indicated time points.

Stimulation of mouse macrophages with TLR ligands

An overnight culture of mouse macrophages (0.5×10^6-cells) in a six-well plate was treated with TLR2 ligands (Pam2CSK4 and Pam3CSK4) and TLR4 ligand (*E. coli*-LPS) at a concentration of 100 ng/ml. The medium was then changed to 1 ml fresh completed DMEM until the experiment was terminated. The pretreated cells were washed twice with 1 ml of PBS and harvested at the indicated time points.

Nitric oxide (NO) production in TLR ligands-treated mouse macrophages

The production of NO was determined by measuring the quantity of nitrite in the supernatant by Griess reaction as described previously [23]. Briefly, RAW264.7 cells were seeded in cell culture plate and incubated overnight. Thereafter, the cells were pretreated with TLR ligands and incubated for 24 h. To measure the nitrite concentration, the culture supernatant (50 μl) was mixed with an equal volume of Griess reagent for 1 min. The nitrite concentration in each culture supernatant was determined by measuring the absorbance at 540 nm (A540) with reference to the standard curve using sodium nitrite.

Knockdown of *trif*, *tbk1*, and *myd88* in mouse macrophages

Mouse macrophages were transfected with *trif*, *tbk1*, and *myd88* small interfering RNAs (siRNAs) (Invitrogen) according to the manufacturer's protocol. In brief, macrophages (2.0×10^5 cells/well) were seeded overnight in a six-well plate. Cells were then transfected with control or *trif*, *tbk1*, and *myd88* siRNAs (60 nM) using Lipofectamine 2000 (Invitrogen). At indicated time points after incubation, the expression of *trif*, *tbk1*, and *myd88* genes and proteins was determined by RT-PCR and immunoblotting, respectively. The siRNA sequences of *trif* are as follows: sense 5′ UCG ACC GUU CUG AAC UGC ACU UUG A 3′ and antisense 5′ UCA AAG UGC AGU UCA GAA CGG UCG A 3′. The siRNA sequences of *tbk1* are as follows: sense 5′ CCA CAA AUU UGA UAA GCA A 3′ and antisense 5′ UUG CUU AUC AAA UUU GUG G 3′. The siRNA sequences of *myd88* are as follows: sense 5′ GCG ACU GAU UCC UAU UAA ATT 3′ and antisense 5′ UUU AAU AGG AAU CAG UCG CTT 3′. As a control, Allstars negativecontrol siRNAs (Qiagen, Hilden, Germany) were used.

Reverse transcriptase PCR

Total RNA was extracted from ligand-treated cells according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany) and used for cDNA synthesis (AMV RT; Promega). PCR was then performed using primer pairs specific for *inos*, *ifn-β*, *trif*, *tbk1*, *myd88*, and *β-actin*. The primer sequences are shown in Table 1. The amplified products were electrophoresed using 1.5% agarose gel and stained with ethidium bromide before visualization under a UV lamp.

Immunoblotting

The treated cells were lysed in lysis buffer containing 20 mM Tris, 100 mM NaCl, and 1% NP-40. The lysates were separated on 10% SDS-PAGE gels. Proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences, Dassel, Germany). The non-specific binding sites on the membrane were blocked with 5% blocking solution (Roche Diagnostics) for 1 h before proteins were allowed to react with specific primary antibodies against iNOS, and Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. A polyclonal rabbit primary antibody against TRIF (Abcam, Cambridge, MA, UK) was used for the detection of TRIF protein expression levels. Polyclonal rabbit primary antibody against TBK1 (Epitomics, Burlingame, CA, USA) was used for the detection of TBK1 protein. Polyclonal rabbit primary antibody against MyD88 (Chemicon International, USA, CA) was used for the detection of MyD88 protein. The membrane was washed three times with 0.1% PBS-Tween 20 (PBST) and incubated with the corresponding horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL, USA) or HRP-conjugated goat anti-mouse IgG (R&D) for 1 h at room temperature. Thereafter, the membrane was washed four times with 0.1% PBST before a chemiluminescence substrate (Roche Diagnostics) was added, and proteins were detected by enhanced chemiluminescence method.

Statistical analysis

Unless indicated otherwise, all experiments in this study were conducted at least three times. Experimental values were expressed as means ± standard errors (SE) of the means. All statistical analysis was performed using SigmaPlot 10.0. Statistical significance of differences between two means was evaluated by Student's *t* test. A *p* value of <0.01 was considered significant.

Results

Pam2CSK4 and Pam3CSK4 activate inducible nitric oxide synthase (iNOS) expression in mouse macrophages

In this study, we demonstrated that TLR2 ligands, Pam2CSK4, and Pam3CSK4, are able to activate *inos* gene

Table 1 Nucleotide primer sequences used for PCR amplification

Primer	Sequences	
	Sense	Antisense
mouse *ifn-β*	TCCAAGAAAGGACGAACATTCG	TGAGGACATCTCCCACGTCAA
mouse *inos*	GCAGAATGTGACCATCATGG	ACAACCTTGGTGTTGAAGGC
mouse *trif*	ATGGATAACCCAGGGCCTTCGC	AGAATGGAGTGGCTGGAAACCA
mouse *tbk1*	CTTCAGGCACTGCTTACCC	CGGCTCGTGACAAAGATAGGA
mouse *myd88*	CCTACCCCCAGAAAAGAAGG	CTGGGGAGAAAACAGCTGAG
mouse *β-actin*	CCAGAGCAAGAGAGGTATCC	CTGTGGTGGTGAAGCTGTAG

RT-PCR

Immunoblotting
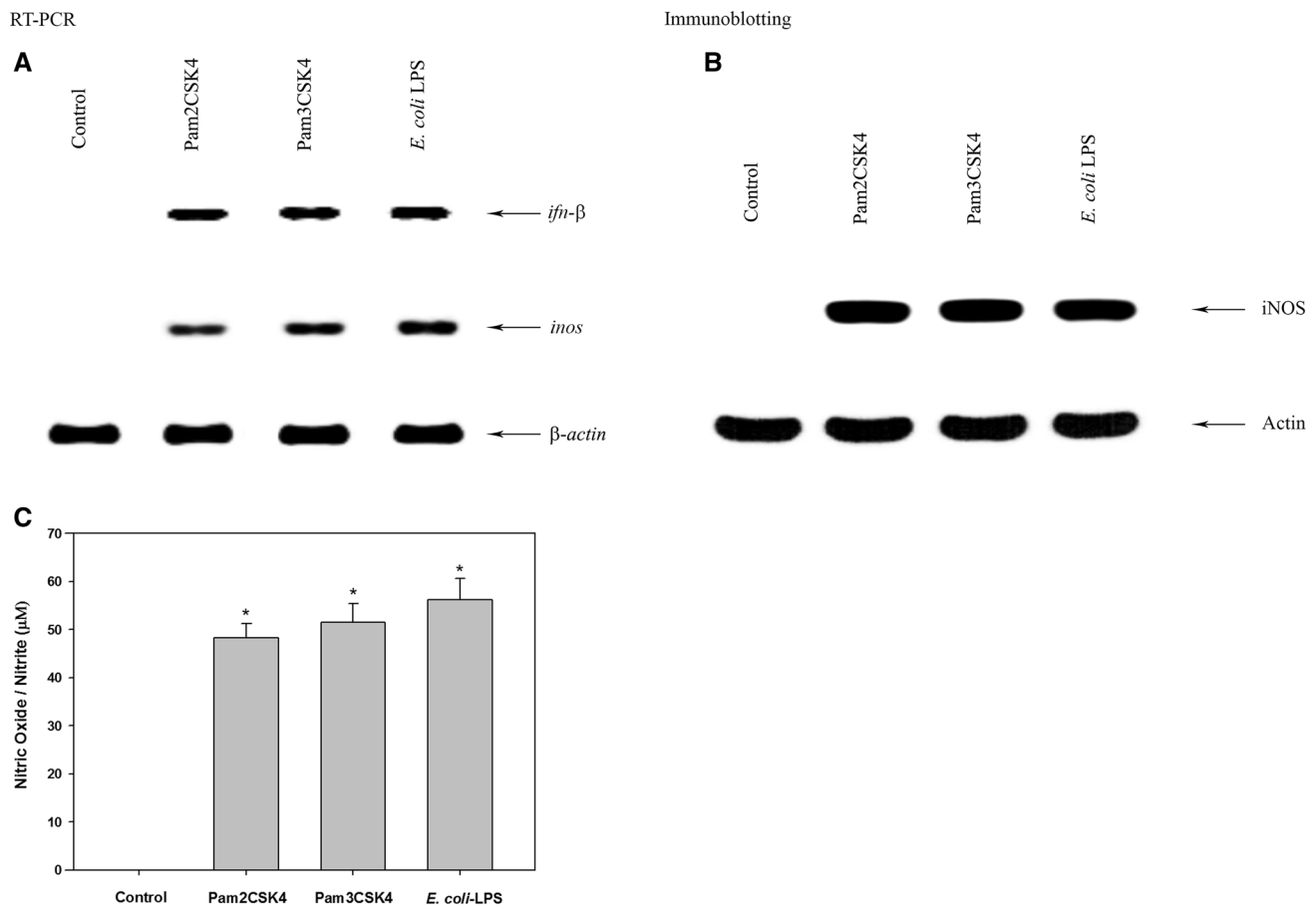
Fig. 1 Activation of TLRs by TLR2 and TLR4 ligands in mouse macrophages. An overnight culture of mouse macrophages (0.5×10^6 cells) was treated with Pam2CSK4, Pam3CSK4, and *E. coli*-LPS at a concentration of 100 ng/ml. 6 hours after stimulation, the treated cells were lysed, and gene expression was determined by RT-PCR (**a**). 10 hours of post-stimulation, the treated cells were lysed and the levels of iNOS proteins in the cell lysates were determined by immunoblotting (**b**). The supernatant of treated cell culture was also harvested at 24 h and NO production was determined by Griess assay (**c**). The results of NO production represent mean μM ± SE of three independent experiments and the data were analyzed by Student's *t* test (*p < 0.01)

(Fig. 1a) and protein (Fig. 1b) expression as determined by RT-PCR and immunoblotting, respectively. Consistent with gene and protein expression, Pam2CSK4 and Pam3CSK4 were able to activate NO production (Fig. 1c). These results suggested that TLR2 ligands (Pam2CSK4 and Pam3CSK4) could activate iNOS expression similar to TLR4 ligand (*E. coli*-LPS).

Neutralizing antibody against TLR2 suppresses the induction of iNOS expression

To determine whether iNOS expression by Pam2CSK4 and Pam3CSK4 are a consequence of the TLR2 ligands binding, mouse macrophage cell line was pretreated with TLR2 neutralizing monoclonal antibody for 2 h before exposing to Pam2CSK4 and Pam3CSK4 at a concentration of 100 ng/ml. The expression of *inos* gene and protein was determined at 6 and 10 h post-stimulation by RT-PCR and

Fig. 2 Neutralizing antibody against TLR2 suppresses iNOS expression and NO production during TLR2 ligands treatment. An overnight culture of mouse macrophages (0.5×10^6 cells) was pretreated with 1 μg/ml TLR2 neutralizing monoclonal antibody (TLR2-mAb) or isotype control for 2 h before stimulating with Pam2CSK4, Pam3CSK4, and *E. coli*-LPS at a concentration of 100 ng/ml. The levels of *inos* gene expression (**a**), as well as iNOS protein expression (**b**), were analyzed after 6 and 10 h of post-stimulation by RT-PCR and immunoblotting, respectively. The supernatant of treated cell culture was also harvested at 24 h and NO production was determined by Griess assay (**c**). The results of NO production represent mean μM ± SE of three independent experiments and the data were analyzed by Student's *t* test (*p < 0.01)

immunoblotting, respectively. As shown in Fig. 2, TLR2 neutralizing antibody could inhibit iNOS expression and NO production in Pam2CSK4 and Pam3CSK4-activated macrophages. In contrast to TLR2 ligands, co-treatment with TLR2 neutralizing antibody was unable to diminish iNOS expression and NO production in *E. coli*-LPS-treated

A

RT-PCR

B

Immunoblotting

C
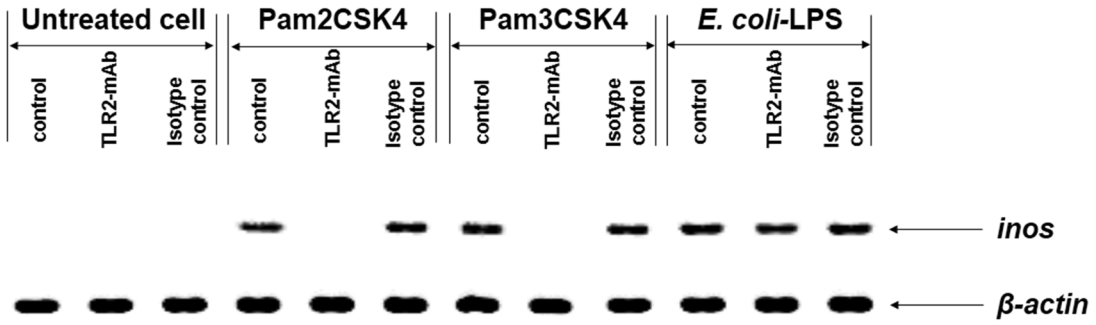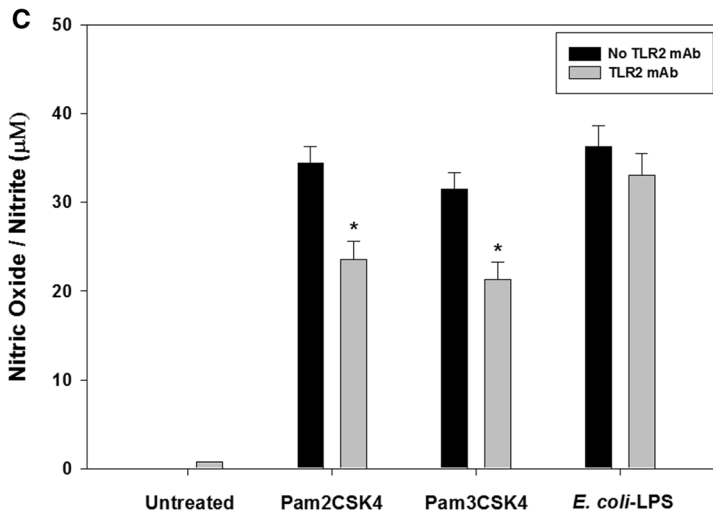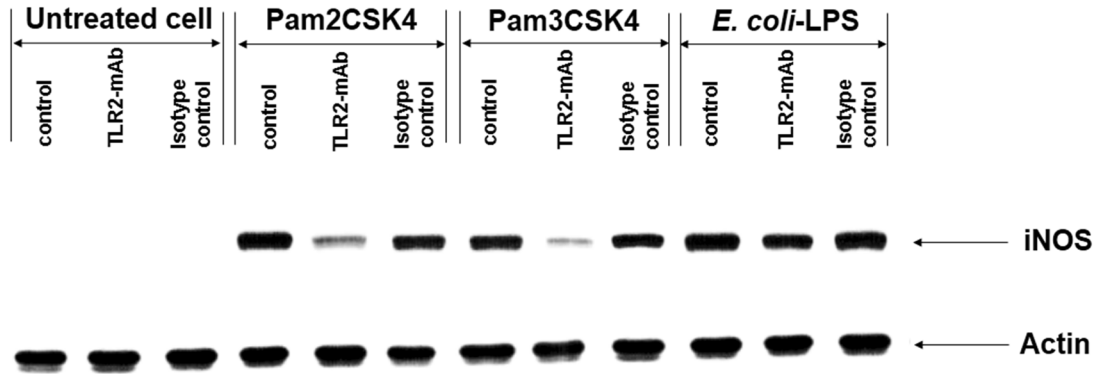
cells, suggesting that the induction of iNOS expression and NO production by Pam2CSK4 and Pam3CSK4 utilized TLR2 as a receptor in mouse macrophages.

Pam2CSK4 and Pam3CSK4 activate iNOS and NO via the TRIF-independent pathway

TRIF adaptor molecule is known to play an essential role in induction of IFN-inducible genes, particularly *inos* through TLR4 signaling [12]. To investigate the involvement of TRIF in Pam2CSK4 and Pam3CSK4-induced iNOS expression and NO production, *trif*-depleted mouse macrophages were performed using siRNAs. The levels of iNOS expression and NO production were determined by RT-PCR, immunoblotting, and Griess reaction assay. In contrast to the *E. coli*-LPS treatment, iNOS expression and NO production by Pam2CSK4 and Pam3CSK4 were not impaired in *trif*-depleted mouse macrophages (Fig. 3a–c). These results indicated that the activation of iNOS expression and NO production by Pam2CSK4 and Pam3CSK4 in mouse macrophages do not require TRIF adaptor molecule.

Induction of iNOS and NO response by Pam2CSK4 and Pam3CSK4 is impaired in *tbk1*-depleted mouse macrophages

TBK1 is known as an essential downstream molecule of the TRIF signaling pathway. Even though Pam2CSK4 and Pam3CSK4 do not utilize TRIF molecule, the involvement of other downstream signaling molecules in iNOS activation and NO production should be investigated. To address the role of TBK1 in this pathway, iNOS expression and NO production in *tbk1*-depleted mouse macrophages stimulated with Pam2CK4 and Pam3CSK4 were analyzed. The results showed that iNOS expression and NO production in *tbk1*-depleted cells treated with both the TLR2 ligands (Pam2CSK4 and Pam3CSK4) and also the TLR4 ligand (*E. coli*-LPS) were significantly impaired when compared to that of the negative control (Fig. 4a–c). These results indicated that TBK1 molecule is required for the induction of iNOS expression and NO production by both TLR2 and TLR4 ligands in mouse macrophages.

Induction of iNOS expression and NO production by Pam2CSK4 and Pam3CSK4 are suppressed in *myd88*-depleted mouse macrophages

According to the results shown in Fig. 3, the iNOS expression and NO production by Pam2CSK4 and Pam3CSK4 occured through the TRIF-independent pathway. This implied that MyD88 molecule may be required in the induction of iNOS via the TLR2 signaling. To
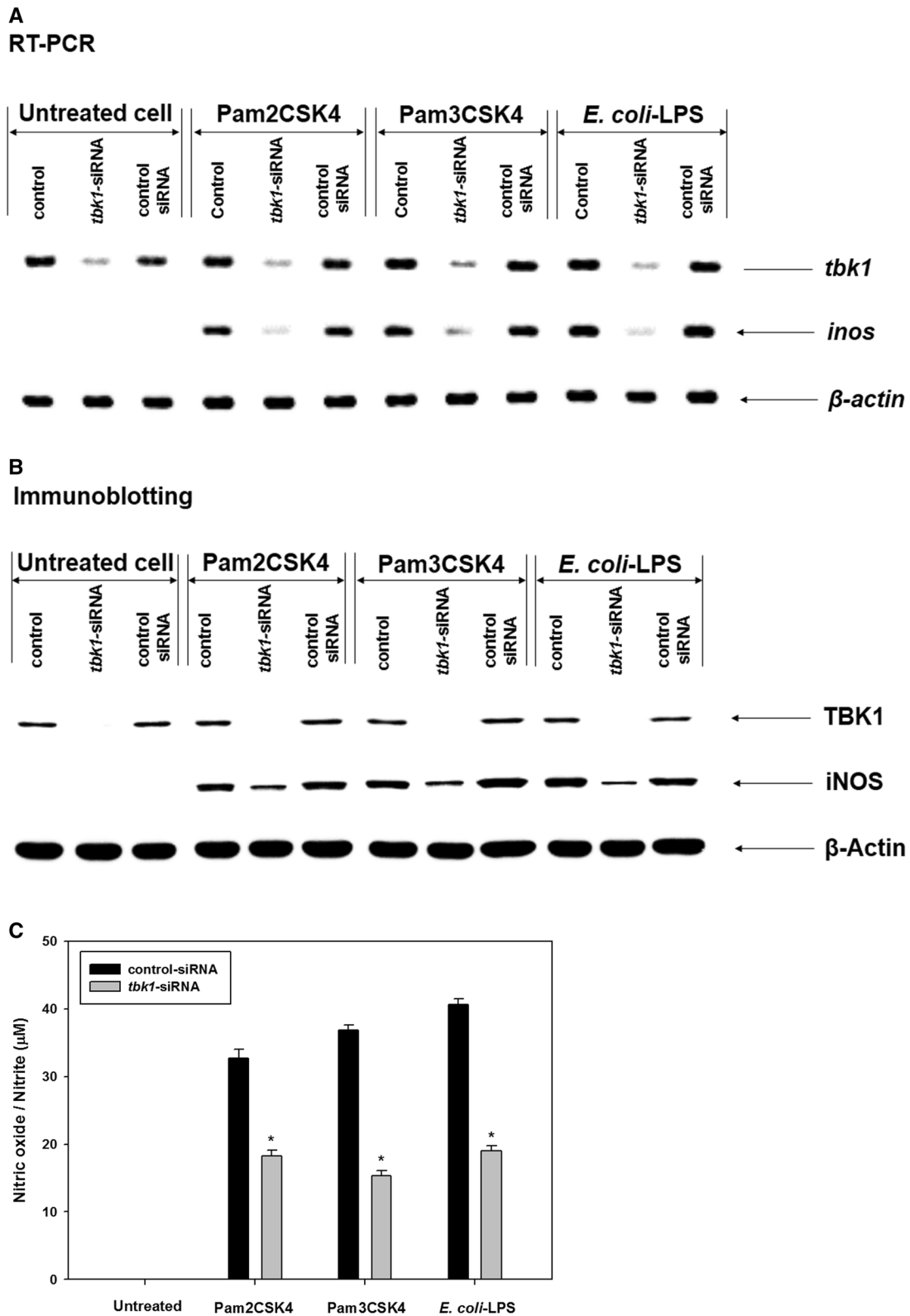
Fig. 3 Pam2CSK4 and Pam3CSK4 induce iNOS expression and NO ▶ production via the TRIF-independent pathway in mouse macrophages. Mouse macrophages (2.0×10^5 cells/well) were transfected with siRNAs against *trif* prior to stimulation with either Pam2CSK4 or Pam3CSK4. *E. coli*-LPS was used as a positive control. The levels of *trif* and *inos* genes (**a**), protein (**b**), as well as NO production (**c**) were analyzed after 6, 10, and 24 h of post-stimulation by RT-PCR, immunoblotting, and Griess reaction, respectively. Data are representative of three independent experiments and were analyzed by Student's *t* test (*$p < 0.01$). The *bars* and *lines* represent mean and SE, respectively

investigate the involvement of MyD88 in this process, the iNOS expression and NO production in *myd88*-depleted macrophages stimulated with Pam2CSK4 and Pam3CSK4 were determined. The results demonstrated that Pam2CSK4 and Pam3CSK4 are able to utilize MyD88-dependent pathway in the induction of iNOS expression in mouse macrophages (Fig. 5). Stimulation of *myd88*-depleted macrophages with Pam2CSK4 and Pam3CSK4, however, led to impair in the induction of iNOS expression and NO production. These results indicated that MyD88 molecule plays an essential role in the iNOS expression and NO production though the TLR2-dependent response in mouse macrophages.

Discussion

It is well documented that the activation of TLR2 by Gram-positive bacterial components, such as PGN and lipopeptide utilizes the MyD88-dependent signaling pathway. The activation of NF-κB-dependent pathway and the production of inflammatory cytokines are the consequence of the TLR2 response against infections [24]. In contrast to TLR2, TLR4 requires both MyD88 and TRIF adaptor molecules to trigger the activation of inflammatory cytokines and type I IFNs, respectively. The previous report demonstrated that TLR2 agonists poorly induced IFN-β and also IFN inducible gene, such as MCP-5, IP-10, and iNOS compared to the TLR4 agonist in murine macrophage [25]. However, recently several reports demonstrated the unconventional role of TLR2 for the TLR2-dependent induction of IFN-β in response to virus and synthetic TLR2 ligands [22, 26, 27]. For example, the synthetic TLR2 agonists, Pam2CSK4 and Pam3CSK4, are able to activate the expression of type I IFN-inducible genes in BMDMs [22]. A similar result was also observed in the production of type I IFN in vaccinia virus-infected bone marrow cells [26]. In this study, we demonstrated that antibody against TLR2 was able to inhibit iNOS expression in Pam2CSK4- and Pam3CSK4-treated cells. The result indicated that these two ligands could bind and activate cells via TLR2. However, unlike *E. coli*-LPS, TRIF molecule is not essential for iNOS expression and NO

A

RT-PCR

B

Immunoblotting

C

A
RT-PCR

B
Immunoblotting

C
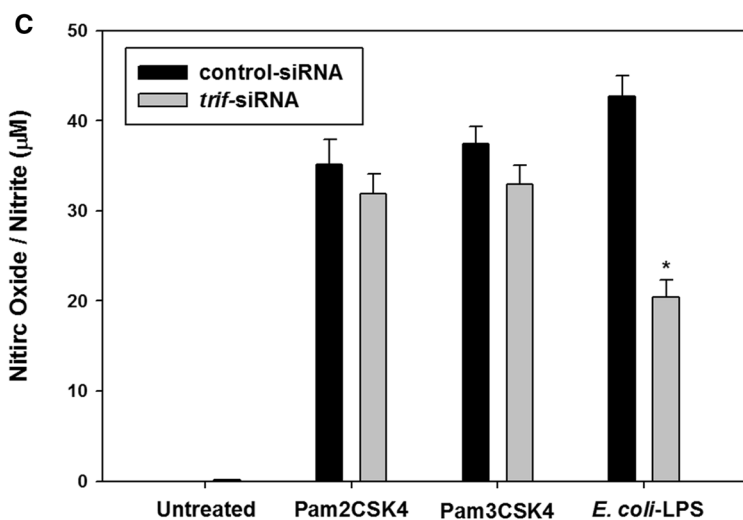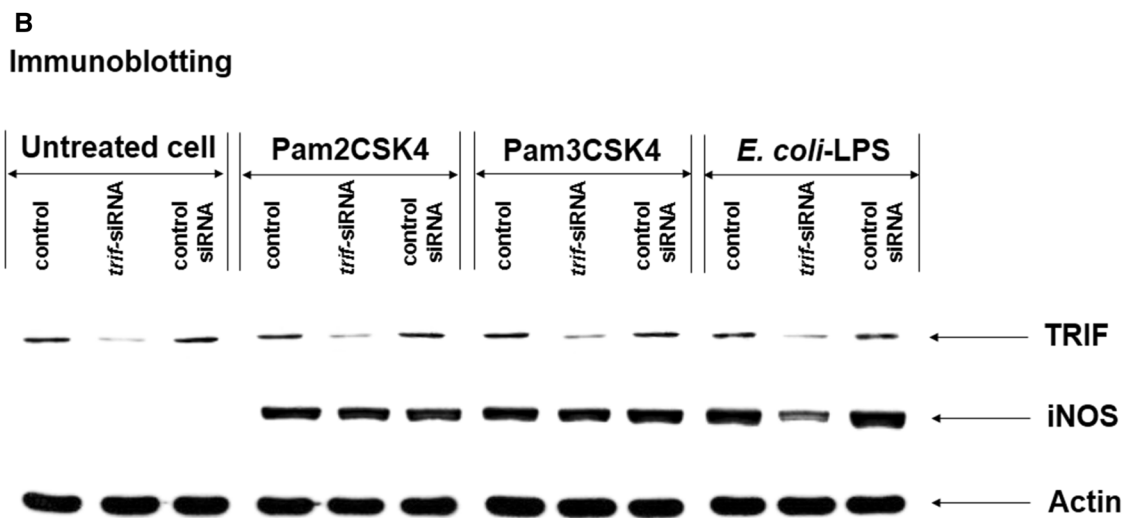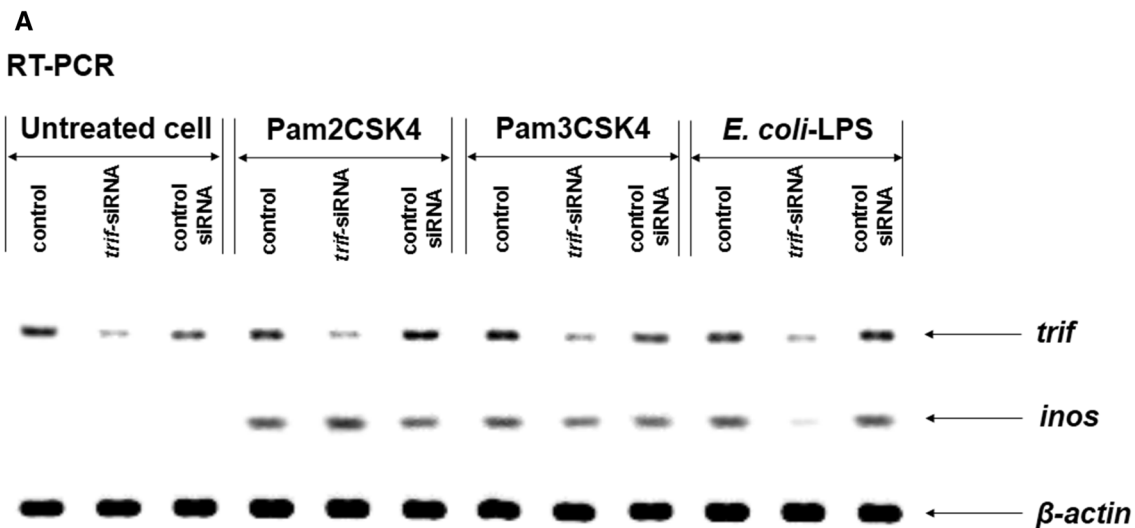
Fig. 4 TLR2 mediated NO response is impaired in *tbk1*-depleted mouse macrophages. Mouse macrophages (2.0×10^5 cells/well) were transfected with siRNAs against *tbk1* prior to stimulation with either Pam2CSK4 or Pam3CSK4. *E. coli*-LPS was used as a positive control. The levels of *tbk1* and *inos* genes (**a**), protein (**b**) as well as NO production (**c**) were analyzed after 6, 10, and 24 h of post-stimulation by RT-PCR, immunoblotting, and Griess reaction, respectively. Data are representative of three independent experiments and were analyzed by Student's *t* test (*$p < 0.01$). The *bars* and *lines* represent mean and SE, respectively

A
RT-PCR

B
Immunoblotting

C
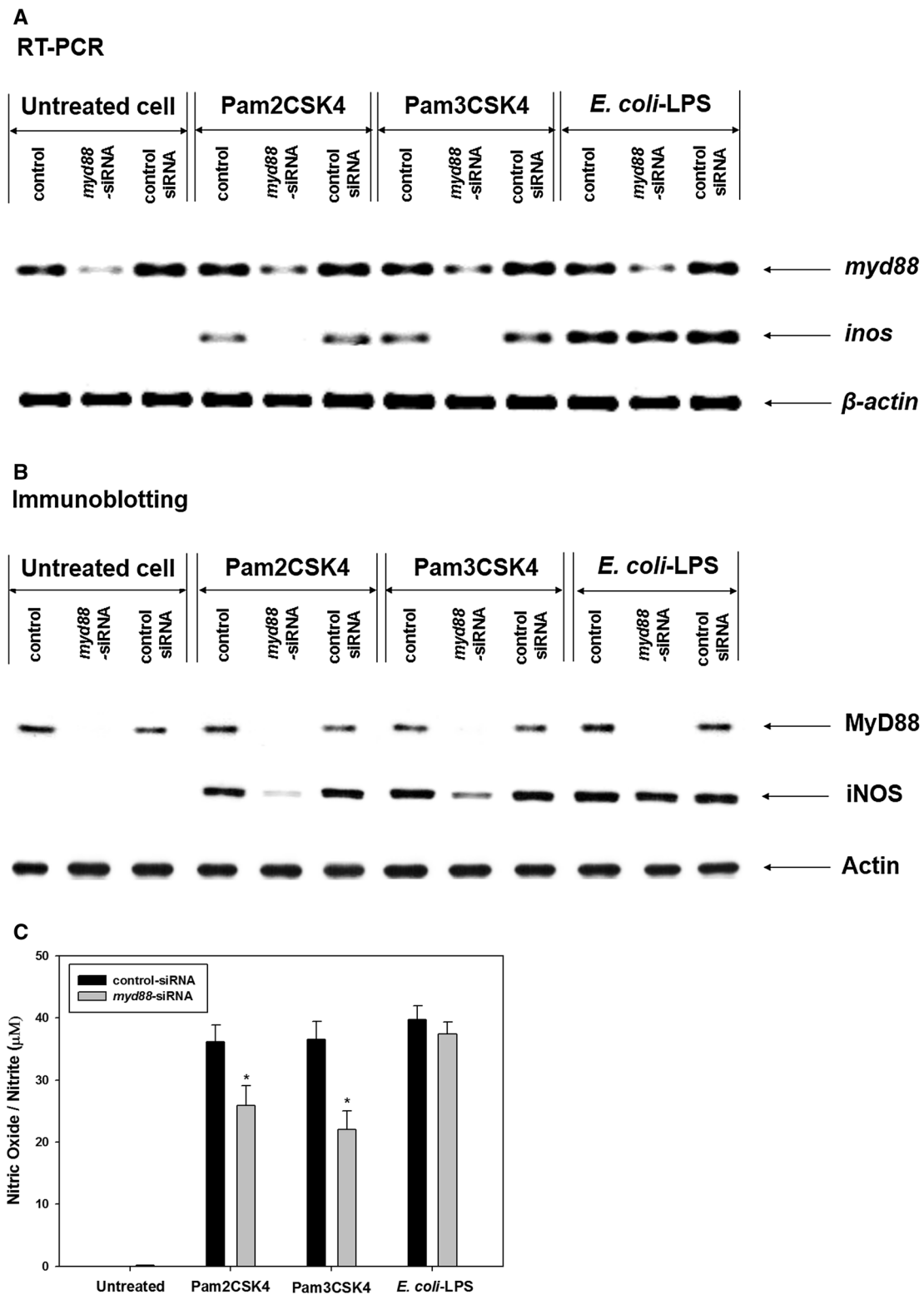
Fig. 5 Pam2CSK4 and Pam3CSK4 induce iNOS expression and NO production via the MyD88-dependent pathway in mouse macrophages. Mouse macrophages (2.0×10^5 cells/well) were transfected with siRNAs against *myd88* prior to stimulation with either Pam2CSK4 or Pam3CSK4. *E. coli*-LPS was used as a positive control. The expression levels of *myd88* and *inos* genes (**a**), protein (**b**), as well as NO production (**c**) were analyzed after 6, 10 and 24 h of post-stimulation by RT-PCR, immunoblotting, and Griess reaction, respectively. Data are representative of three independent experiments and were analyzed by Student's *t* test (*$p < 0.01$). The *bars* and *lines* represent mean and SE, respectively

production in Pam2CSK4- and Pam3CSK4-treated mouse macrophages.

Since LPS is a major heat-stable component on the outer surface of Gram-negative bacteria, including that of *E. coli*, a typical LPS can activate the TRIF signaling pathway and induce type I IFN responses via TLR4 [17]. In contrast to TLR4, the lack of TRIF molecule in the TLR2 signaling pathway could drive type I IFN production which requires the internalization of the TLR2 receptor into the endosomal compartments via the MyD88-dependent pathway [22, 26]. Endocytotic internalization of TLR2 can recruit MyD88 molecule via the TRAM adaptor molecule [20]. The formation of TLR2 signaling components in endolysosome activates the MyD88-dependent pathway resulting in IFN-β production [20]. Blocking the endocytic maturation using Bafilomycin A1, an inhibitor of endosome acidification, severely inhibited the TLR2-mediated IFN-β and type I IFN-dependent response in Pam2CSK4- and Pam3CSK4-treated BMDM cells [22]. Consistent with our results, Pam2CSK4 and Pam3CSK4 failed to activate iNOS expression in mouse macrophages treated with Bafilomycin A1 (data not shown). Moreover, iNOS expression and NO production were also inhibited in *myd88*-depleted macrophages activated with these two ligands. These data suggested that induction of iNOS expression by Pam2CSK4 and Pam3CSK4 requires both the endosomal acidification and MyD88 adaptor molecule.

Several molecules such as TNF receptor-associated factor (TRAF3), TRAF family member-associated NF-κB activator (TANK), TANK-binding kinase 1 (TBK1), and Inhibitor-κB kinase ε (IKKε) have been known to participate downstream of the TRIF signaling pathway [28, 29]. Among them, TBK1 is an essential molecule to regulate IFN-inducible gene expression. Consistent with our study, we also demonstrated the important role of TBK1 in the expression of iNOS and NO production as observed in Pam2CSK4- and Pam3CSK4-treated *tbk1*-depleted mouse macrophages. This observation suggested that TBK1 is also an essential molecule in the regulation of iNOS expression.

In conclusion, our findings provide a novel insight into the TLR2 signaling pathway. We demonstrated that by binding to TLR2, TLR2 ligands could activate iNOS expression. Moreover, TBK1, which is known to be an essential molecule downstream of TRIF pathway, is also associated with MyD88 in the induction of iNOS expression and NO production. However, the mechanism by which MyD88 interact with TBK1 is still under investigation.

Compliance with ethical standards

Conflict of interest There are no conflict of interest.

References

1. Mifsud EJ, Tan AC, Jackson DC. TLR agonists as modulators of the innate immune response and their potential as agents against infectious disease. Front Immunol. 2014;5:79.
2. Gay NJ, Symmons MF, Gangloff M, Bryant CE. Assembly and localization of Toll-like receptor signalling complexes. Nat Rev Immunol. 2014;14:546–58.
3. Medzhitov R, Janeway C Jr. Innate immune recognition: mechanisms and pathways. Immunol Rev. 2000;173:89–97.
4. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124:783–801.
5. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol. 2007;7:353–64.
6. Akira S. Toll receptor families: structure and function. Semin Immunol. 2004;16:1–2.
7. Akira S, Takeda K. Functions of toll-like receptors: lessons from KO mice. C R Biol. 2004;327:581–9.
8. Albiger B, Sandgren A, Katsuragi H, Meyer-Hoffert U, Beiter K, Wartha F, et al. Myeloid differentiation factor 88-dependent signalling controls bacterial growth during colonization and systemic pneumococcal disease in mice. Cell Microbiol. 2005;7:1603–15.
9. Wiersinga WJ, Wieland CW, Roelofs JJ, van der Poll T. MyD88 dependent signaling contributes to protective host defense against *Burkholderia pseudomallei*. PLoS ONE. 2008;3:e3494.
10. Russo BC, Brown MJ, Nau GJ. MyD88-dependent signaling prolongs survival and reduces bacterial burden during pulmonary infection with virulent *Francisella tularensis*. Am J Pathol. 2013;183:1223–32.
11. Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nat Immunol. 2008;9:361–8.
12. Nilsen NJ, Vladimer GI, Stenvik J, Oming MP, Zeid-Kilani MV, Bugge M, et al. A role for the adaptor proteins TRAM and TRIF in toll-like receptor 2 signaling. J Biol Chem. 2015;290:3209–22.
13. Ullah MO, Sweet MJ, Mansell A, Kellie S, Kobe B. TRIF-dependent TLR signaling, its functions in host defense and inflammation, and its potential as a therapeutic target. J Leukoc Biol. 2016;100:27–45.
14. Utaisincharoen P, Anuntagool N, Arjcharoen S, Limposuwan K, Chaisuriya P, Sirisinha S. Induction of iNOS expression and antimicrobial activity by interferon (IFN)-beta is distinct from IFN-gamma in *Burkholderia pseudomallei*-infected mouse macrophages. Clin Exp Immunol. 2004;136:277–83.
15. Brennan RE, Russell K, Zhang G, Samuel JE. Both inducible nitric oxide synthase and NADPH oxidase contribute to the control of virulent phase I *Coxiella burnetii* infections. Infect Immun. 2004;72:6666–75.
16. Pietrocola G, Arciola CR, Rindi S, Di Poto A, Missineo A, Montanaro L, et al. Toll-like receptors (TLRs) in innate immune defense against *Staphylococcus aureus*. Int J Artif Organs. 2011;34:799–810.
17. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine. 2008;42:145–51.

18. Buwitt-Beckmann U, Heine H, Wiesmuller KH, Jung G, Brock R, Akira S, et al. TLR1- and TLR6-independent recognition of bacterial lipopeptides. J Biol Chem. 2006;281:9049–57.

19. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol. 2002;169:10–4.

20. Stack J, Doyle SL, Connolly DJ, Reinert LS, O'Keeffe KM, McLoughlin RM, et al. TRAM is required for TLR2 endosomal signaling to type I IFN induction. J Immunol. 2014;193:6090–102.

21. Aubry C, Corr SC, Wienerroither S, Goulard C, Jones R, Jamieson AM, et al. Both TLR2 and TRIF contribute to interferon-beta production during *Listeria* infection. PLoS ONE. 2012;7:e33299.

22. Dietrich N, Lienenklaus S, Weiss S, Gekara NO. Murine toll-like receptor 2 activation induces type I interferon responses from endolysosomal compartments. PLoS ONE. 2010;5:e10250.

23. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem. 1982;126:131–8.

24. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol. 2010;11:373–84.

25. Toshchakov V, Jones BW, Lentschat A, Silva A, Perera PY, Thomas K, et al. TLR2 and TLR4 agonists stimulate unique repertoires of host resistance genes in murine macrophages: interferon-beta-dependent signaling in TLR4-mediated responses. J Endotoxin Res. 2003;9:169–75.

26. Barbalat R, Lau L, Locksley RM, Barton GM. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. Nat Immunol. 2009;10:1200–7.

27. Li J, Lee DS, Madrenas J. Evolving bacterial envelopes and plasticity of TLR2-dependent responses: basic research and translational opportunities. Front Immunol. 2013;4:347.

28. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Front Immunol. 2014;5:461.

29. Yu T, Yi YS, Yang Y, Oh J, Jeong D, Cho JY. The pivotal role of TBK1 in inflammatory responses mediated by macrophages. Mediators Inflamm. 2012;2012:979105.