รายงานวิจัยฉบับสมบูรณ์

โครงการ : การควบคุมการแสดงออกของ Sterile α and HEAT/Armadillo motif containing protein (SARM) ในเซลล์แมคโครฟาจของหนูที่ถูกทรีตด้วย LPS ของเชื้อ *Porphyromonas gingivalis*

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เมษายน 2561

สัญญาเลขที่ MRG5980057

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Abstract

Project Code : MRG5980057

Project Title : Regulation of Sterile α and HEAT/Armadillo motif containing protein (SARM) expression in *Porphyromonas gingivalis* LPS-treated mouse macrophages cell line (RAW264.7)

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At present, it is well established that several negative regulators of TLR signaling pathway have been identified. Among them, SARM (sterile alpha and armadillo motif protein) has been demonstrated as a known negative regulator of TLR signaling for suppression of antimicrobial activity, particularly iNOS expression. The aim of this study is to investigate the involvement of SARM expression in regulation of iNOS expression and NO production in *P. gingivalis* LPS-treated macrophages. In the present study, we demonstrated the low level of nitric oxide and iNOS protein expression were also correlated with the induction of SARM expression in *P. gingivalis* LPS-treated cells. However, in the depletion of SARM, the level of nitric oxide production and iNOS protein expression was not interfere in *P. gingivalis* LPS-treated cells, suggesting that SARM does not involve in iNOS expression. However, we further investigated the regulation of SARM in Pam2CSK4 and Pam3CSK4 (TLR2 ligands). The results demonstrated that the SARM expression was also upregulated at both transcriptional and translational level in time-dependent manner during activation of Pam2CSK4 and Pam3CSK4 in mouse macrophages. Blocking of ligand internalization by cytochalasin D showed interference effect with SARM expression. Moreover, endosomal acidification and TLR9 were required for SARM expression suggesting the essential role of endosomal compartment acidification and TLR9 in regulating SARM expression.

Keywords : *Porphyromonas gingivalis*, RAW264.7, SARM

บทคัดย่อ

รหัสโครงการ: MRG5980057

ชื่อโครงการ: การควบคุมการแสดงออกของ Sterile α and HEAT/Armadillo motif containing protein (SARM) ในเซลล์แมคโครฟาจของหนูที่ถูกทรีตด้วย LPS ของเชื้อ *Porphyromonas gingivalis*

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ในปัจจุบันมีการค้นพบ negative regulators ที่มีผลยับยั้งระบบการส่งสัญญาณผ่านทาง TLR (Toll-like receptor) มากมายและหนึ่งในนั้นคือโมเลกุลที่เรียกว่า SARM (sterile alpha and armadillo motif containing protein) ซึ่งมีบทบาทในการยับยั้ง antimicrobial activity โดยเฉพาะการแสดงออกของ inducible nitric oxide synthase (iNOS) จุดประสงค์ของการศึกษาในครั้งนี้คือการศึกษาบทบาทของการแสดงออกของ SARM ในการควบคุมการแสดงออกของ iNOS และการสร้าง nitric oxide (NO) ในเซลล์แมคโครฟาจของหนูที่ถูกทรีตด้วย *P. gingivalis* LPS จากผลการศึกษาพบว่าปริมาณ nitric oxide และการแสดงออกของ iNOS ที่ลดลงในเซลล์แมคโครฟาจของหนูที่ถูกทรีตด้วย *P. gingivalis* LPS มีความสัมพันธ์กับการแสดงออกของ SARM ที่เพิ่มขึ้น อย่างไรก็ตามเมื่อทำการยับยั้งการแสดงออกของ SARM ในเซลล์แมคโครฟาจของหนูพบว่าปริมาณ nitric oxide และการแสดงออกของ iNOS ไม่ได้มีผลกระทบซึ่งแสดงให้เห็นว่า SARM ไม่ได้มีบทบาทในการควบคุมการแสดงออกของ nitric oxide และ iNOS อย่างไรก็ตามทางผู้วิจัยยังพบว่าลิแกนด์ต่อ TLR2 เช่น Pam2CSK4 และ Pam3CSK4 สามารถที่จะกระตุ้นการแสดงออกของยีนและโปรตีนของ SARM ได้เช่นกัน เมื่อทำการยับยั้งกระบวนการ internalization ของเซลล์แมคโครฟาจด้วย cytochalasin D พบว่ามีผลทำให้การแสดงออกของ SARM ลดลง นอกจากนี้ยังพบว่า endosomal acidification และ TLR9 มีบทบาทสำคัญในการควบคุมการแสดงออกของ SARM

คำหลัก : *Porphyromonas gingivalis*, RAW264.7, SARM

Executive summary

Periodontitis is a chronic inflammatory disease caused by gram-negative anaerobic bacteria, *Porphyromonas gingivalis*. The pathogenesis of this bacterium can lead to tissue destruction of the attachment apparatus of the teeth. Periodontitis has also been implicated in systemic conditions such as atherosclerosis and stroke [1]. *P. gingivalis* possesses a variety of virulence factors include lipopolysaccharide (LPS), fimbriae, outer membrane vesicles, hemolysins, capsules, and gingipains [2]. These factors allow the bacteria to colonize and disrupt host-microbe homeostasis. In addition, *P. gingivalis* is capable of invading cells and tissues, thus avoiding the host immune responses by applying different escape mechanisms to survive and persist resistance in the periodontal pocket [3]. Interaction of microbial components and host-expressed TLRs is a key control elements of the innate immune response to microbial infection [1]. Several TLRs are expressed on multiple cell types located at the site of infection and their activation lead to the induction of inflammatory cytokines. Innate TLR-mediated host immune responses to microbial challenge are crucial for host protection against these pathogens. It is well established that a number of gram-negative bacteria have evolved strategies to modify the component of bacterial LPS, particularly lipid A portions for evasion of host recognition by TLR4. In contrast to LPS of other gram-negative bacteria recognized by TLR4, LPS of *P. gingivalis* is predominantly a TLR2 agonists [4]. For example, Darveau *et al.* also demonstrate the inability of *P. gingivalis* LPS to activate host innate immune responses by blocking E-selectin expression and inhibition of neutrophil adhesion [5]. Additionally, production of antagonistic lipid A structure of *P. gingivalis* displays attenuated production of proinflammatory mediators and evasion of inflammasome activation, thus facilitating bacterial survival in macrophages [6].

The family of TLRs can be subdivided into two groups according to their localization. TLR1, 2, 4, 5, 6 and 11 are expressed on the cell surface whereas TLR3, 7, 8 and 9 are located in the endosomal compartments [7]. Upon stimulation, engagement of TLRs results in the activation of several signaling cascades leading to the induction of innate immune response genes. The TLR/ligand binding can further recruit an intracellular Toll-interleukin (IL)-1 receptor (TIR) domain-containing adaptor molecules, including myeloid differentiation factor 88 (MyD88), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF), TIR-associated protein (TIRAP), and TRIF-related adaptor molecule (TRAM). MyD88 adaptor molecule is considered as the main activator of all TLRs, except TLR3. In contrast to other TLRs, TLR4 recruits both MyD88 and TRIF adaptor molecules to sense the signaling cascades [8]. The main signal of MyD88 is to activate NF-κB family members and mitogen-activated protein kinase resulting in the induction of pro-inflammatory cytokines such as IL-6 and IL-12. On the other hand, TRIF adaptor molecule mainly activates IRF family members and tends to stimulate the induction of type I interferons (IFNs), an anti-viral response. Type I IFN is also activated through endosomal TLR such as TLR9 by recruitment of the key adaptor molecule MyD88 leading to IRF7 activation. Responsiveness of TLR signaling results in secretion of pro-inflammatory cytokines and type I IFNs that are crucial for host defensive mechanisms against invading pathogens.

However, the overstimulation of TLR signaling may lead to the pathogenesis of autoimmune, chronic inflammatory and infectious diseases [9]. In order to avoid harmful effect of TLR response, several negative regulators are often induced to fine-tune the activation of TLR signaling pathway. Among negative regulators, sterile α- and armadillo-motif-containing protein or SARM is known to inhibit TRIF-dependent pathway of TLR4 [10]. The significance of SARM has also been demonstrated in microbial infection [11]. Invading pathogens could

develop strategies to evade host immune responses by taking the advantage of the negative regulators to suppress the TLR signaling [9]. For example, subversion of TRIF-dependent pathway by overexpression of SARM in bacterial infection such as *Burkholderia pseudomallei* could suppress antimicrobial defense mechanism, particularly iNOS, leading to an increase in the intracellular bacterial survival in *B. pseudomallei*-infected mouse macrophages [11]. These results suggested that a negative regulator of TLR signaling pathway is not only important for preventing overstimulation in non-infectious diseases but is also involved in the pathogenesis of some infectious microbes. The aim of this study is to investigate the involvement of SARM expression in regulation of iNOS expression and NO production in *P. gingivalis* LPS-treated macrophages. However, we further extended our study to investigate the involvement of TLRs in the regulation of SARM expression during activation of Pam2CSK4 and Pam3CSK4.

Materials and Methods

1. Cell line and culture condition

Mouse macrophage cell line (RAW 264.7) was obtained from American Type of Culture Collection (ATCC). If not indicated otherwise, the cells were cultured in Dulbecco's modified Eagles' medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone) and 1% L-glutamine (Gibco Labs, Grand Island, NY, USA) at 37 oC under a 5% CO2 atmosphere.

2. Reagents

Porphyromonas gingivalis LPS, *Escherichia coli* LPS, Pam2CSK4, Pam3CSK4 and ODN1826 were purchased from invivogen. Monoclonal TLR2 antibody (T2.5 antibody) and IgG1

isotype control were purchased from eBioscience (eBioscience, San Diego, CA, USA). The inhibitor of endosomal acidification, Bafilomycin A1 and Chloroquine, were purchased from Sigma-Aldrich (Sigma-Aldrich, Darmstadt, Germany).

3. Incubation of TLR ligands in mouse macrophages

Mouse macrophages ($5x10^5$ cells/well) were seeded in a 6-well plate for overnight. Then the cells were stimulated with several TLR ligands at the different concentrations. At indicated time points, the supernatant was collected for NO assay and the treated cells were lyzed in lysis buffer for immunoblotting.

4. Depletion of SARM in mouse macrophages

Mouse macrophages (1.5×10^5 cells/well) were seeded overnight in a 6-well plate. The cells were then transfected with 60 nM each of negative control siRNA and SARM siRNA using Lipofectamine 2000 (Invitrogen). After 24 h of incubation, the expression of SARM was determined by immunoblotting. Viability of the siRNA treated macrophages was determined by trypan blue staining method.

5. Depletion of TLR9 in mouse macrophages

Mouse macrophages (1.5×10^5 cells/well) were seeded overnight in a 6-well plate. The cells were then transfected with 60 nM each of negative control siRNA and TLR9 siRNA using Lipofectamine 2000 (Invitrogen). After 24 h of incubation, the expression of SARM was determined by immunoblotting. Viability of the siRNA treated macrophages was determined by trypan blue staining method.

6. Immunoblotting

The treated cells were lyzed in lysis buffer containing 20 mM Tris, 100 mM NaCl and 1% NP40. The lysates then were separated in 8% SDS-PAGE. 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 at 4°C overnight. The membranes were washed 3 times with 0.1% PBST and was incubated with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford) for 1 h at room temperature. Thereafter, the membranes were washed 4 times with 0.1% PBST before a chemiluminescence substrate (Roche Diagnostics) was added and protein band was detected by enhanced chemiluminescence.

7. NO assay

To elucidate the production of NO in treated cells, the supernatant was determined by measuring the quantity of nitrite in the supernatant by Griess reaction, as described. Briefly, the 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.

8. Reverse transcriptase PCR

Total RNAs were extracted from ligand-stimulated cells according to the manufacturer's instruction (GE Healthcare, Buckinghamshire, UK) and used for cDNA synthesis (AMV RT, Promega). PCR was then performed using primer pairs specific for sarm and β-actin. The

sequences were: for *sarm*, sense 5'-GGA GCTCAGTGCATAGGAG-3' and antisense 5'- CAG GTC TGG ACC TCA GCT TC-3' ; for *inos,* sense 5'-GCA GAA TGT GAC CAT CAT GG-3' and antisense 5'-ACA ACC TTG GTG TTG AAG GC-3' ; for *β-actin*, sense 5'-CCA GAG CAA GAG AGG TAT CC-3' and antisense 5'-CTG TGG TGG TGA AGC TGT AG-3'. For *tlr9,* sense 5'-GCA CAG GAG CGG TGA AGG T-3' and antisense 5'-GCA GGG GTG CTC AGT GGA G-3'. The amplified products were electrophoresed using 1.5% and 2% agarose gel and stained with ethidium bromide before visualization under an ultraviolet lamp.

Results

1. *P. gingivalis* LPS induces SARM expression in mouse macrophage cell line (RAW264.7)

It has been demonstrated that SARM interacts TRIF adaptor molecule and suppresses the gene expression downstream of TRIF-dependent pathway, particularly iNOS expression. In order to investigate the expression of SARM and iNOS protein expression, mouse macrophages were treated with either *P. gingivalis* LPS or *E. coli* LPS at different concentration. At indicated time, the treated cells were lyzed with lysis buffer and the protein expression was determined by immunoblotting. As shown in Fig. 1, the upregulation of SARM expression was observed in *P. gingivalis* LPS-treated cells but not in *E. coli LPS*-treated cells. The induction of iNOS protein expression was also correlated with the expression of SARM as shown in Fig. 1. These results indicated that SARM expression may involve in the expression of iNOS in *P. gingivalis*-treated mouse macrophages.

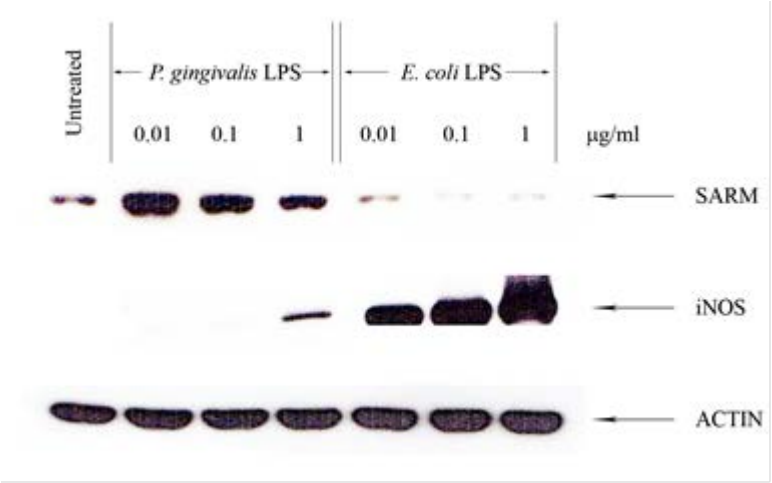


Figure 1. Expression of SARM and iNOS in *P. gingivalis* LPS and *E. coli* LPS-treated mouse macrophages. Mouse macrophages (RAW264.7) were treated with either *P. gingivalis* LPS or *E. coli* LPS at a different concentrations for 24 h. *E. coli* LPS was used as a control. At indicated time, the treated cells were lyzed. The SARM and iNOS protein expression were determined by immunoblotting. Actin protein expression was used as a loading control.

2. Induction of nitric oxide production in *P. gingivalis* LPS and *E.coli* LPS –treated mouse macrophages

In order to demonstrate the nitric oxide production, mouse macrophages were treated with the different concentrations of these two bacterial LPS (10, 100, 1000 ng/ml) for 24 h. As shown in Fig. 2, the level of nitric oxide production by *P. gingivalis* LPS and *E. coli* LPS-treated mouse macrophages was increased in a concentration dependent. These results were also consistent with the iNOS protein expression.

3. Depletion of SARM expression fails to enhance nitric oxide and iNOS protein expression in *P. gingivalis* LPS-treated macrophages

In order to elucidate the involvement of SARM in regulation of the nitric oxide and iNOS protein expression in *P. gingivalis* LPS-treated mouse macrophages. Mouse macrophages were transfected with SARM siRNA prior to incubation with *P. gingivalis* LPS for 24 h. At the indicated time intervals, the production of nitric oxide and iNOS protein expression was determined by Griess assay and immunoblotting, respectively. As shown in Fig. 3, the nitric oxide production in *P. gingivalis* LPS-treated SARM-depleted mouse macrophages was not significantly different from control siRNA. This result was also consistent with iNOS protein expression in SARM-depleted macrophages (Fig. 4). These results suggested that SARM expression was not involved the production of nitric oxide and iNOS expression in *P. gingivalis* LPS-treated mouse macrophages.

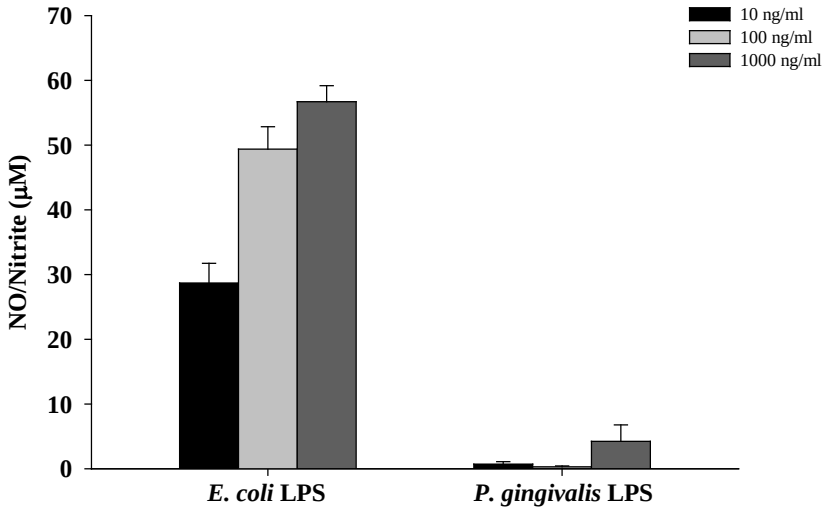


Figure 2. Nitric oxide production in *P. gingivalis* LPS and *E.coli* LPS-treated mouse macrophages. Mouse macrophages (RAW264.7) were treated with *P. gingivalis* LPS and *E.coli* LPS (used for comparison) at the different concentrations for 24 h. At the indicated time, supernatant was collected and NO production was determined by Griess assay. Data represent the mean and standard error (SE) of three independent experiments.

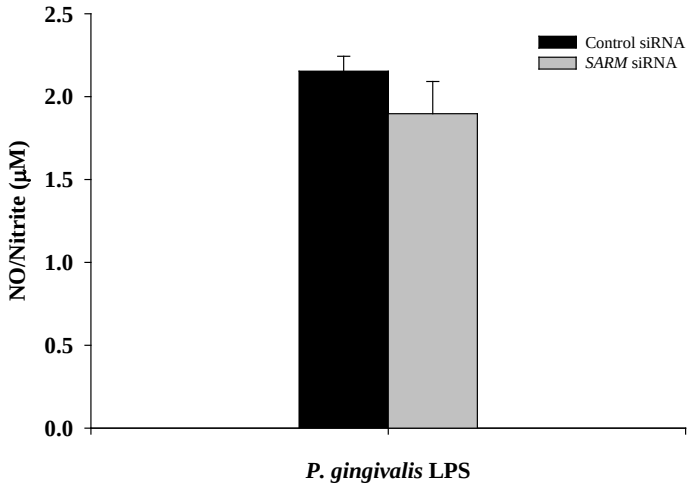


Figure 3. Nitric oxide production in *P. gingivalis* LPS-treated *SARM*-depleted mouse macrophages. Mouse macrophages (RAW264.7) were transfected with *SARM* siRNA (60 nM) for 24 h. After incubation, the transfected cells were treated with *P. gingivalis* LPS at a concentration of 1 μg/ml for 24 h. At 24 h of stimulation, supernatant was collected and NO production was determined by Griess assay. Data represent the mean and standard error (SE) of three independent experiments.

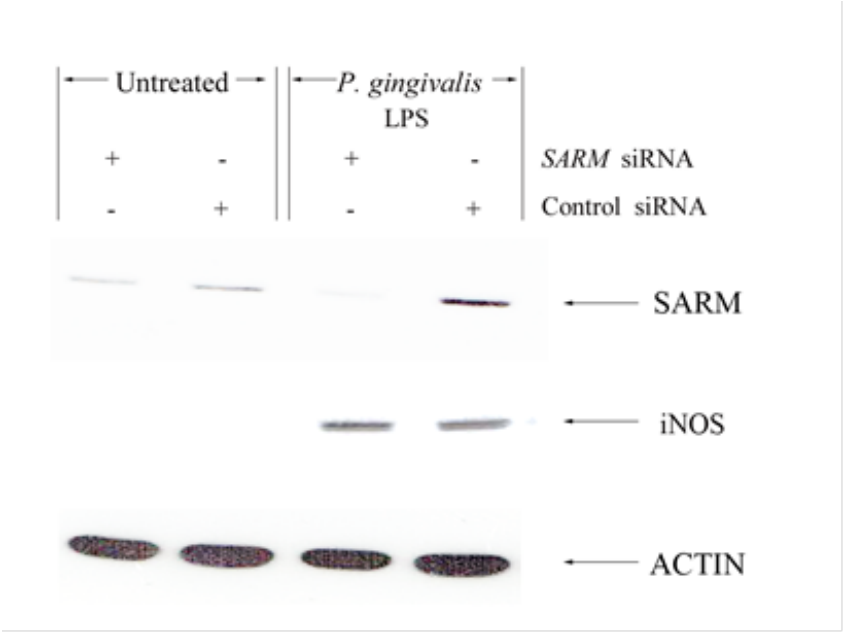


Figure 4. *P. gingivalis* LPS induced iNOS expression did not alter in *SARM*-depleted macrophages. Mouse macrophages (RAW264.7) were transfected with *SARM* siRNA (60 nM) for 24 h. After incubation, the transfected cells were treated with *P. gingivalis* LPS at a concentration of 1 μg/ml for 24 h. At 24 h of stimulation, the treated cells were lyzed and the protein expression was determined by immunoblotting. Data represent the mean and standard error (SE) of three independent experiments.

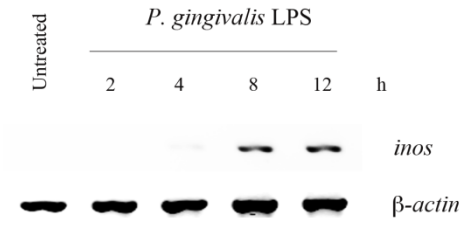
4. Kinetics of iNOS expression in *P. gingivalis* LPS-treated mouse macrophages

We further investigated the regulation of iNOS expression in TLR activation. To address the kinetics of iNOS expression in *P. gingivalis*-treated cells, mouse macrophages were treated with *P. gingivalis* LPS at a concentration of 10 μg/ml. At different time intervals, the iNOS gene and protein expression were determined by RT-PCR and immunoblotting, respectively. As shown in Figure 5, the iNOS gene (A) and protein (B) expression were stimulated at 4 hours and peaked at 8 hours after incubation.

5. *P. gingivalis* LPS induced iNOS expression requires TLR9

Recently, it has been demonstrated that TLR9 activation contributes to stimulation of inflammatory pathway in *P. gingivalis* infection [12]. Activation of TLR9 resulted in macrophage activation as judged by the increase in nitric oxide production [13]. In order to investigate the involvement of TLR9 in regulation of iNOS expression, depletion of TLR9 in mouse macrophages was also performed by using siRNA. As shown in Fig. 6, iNOS mRNA expression was markedly decreased in *P. gingivalis* LPS-treated TLR9-depleted cells when compared to that of the control siRNA transfected cells. In this study, ODN 1826 (TLR9 ligand) was used as a positive control and *E. coli* LPS (TLR4 ligand) was used as a negative control. This result indicated that the upregulation of iNOS expression by *P. gingivalis* LPS requires TLR9.

A) RT-PCR



B) Immunoblotting

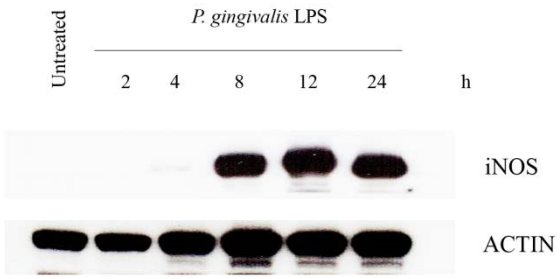


Figure 5. *P. gingivalis* LPS induce iNOS expression in mouse macrophage cell line (RAW 264.7). Mouse macrophages were treated with *P. gingivalis* LPS at a concentration of 10 μg/ml. At different time intervals, the activated cells were lyzed and the kinetics of iNOS mRNA expression (A) and protein expression (B) were determined by RT-PCR and immunoblotting, respectively. Actin mRNA and protein were used as internal loading control. Data are representative of three independent experiments.

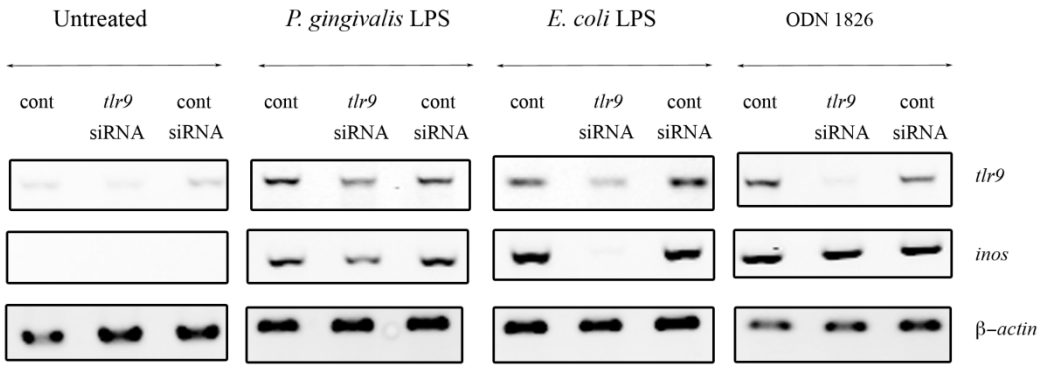


Figure 6. TLR9 regulates the expression of iNOS in *P. gingivalis* LPS-treated mouse macrophages. Depletion of TLR9 was performed in mouse macrophages prior to activation with *P. gingivalis* LPS at a concentration of 10 μg/ml for 6 hours. The treated cells were lyzed and the iNOS gene expression was determined by RT-PCR. Data are representative of three independent experiments.

6. Kinetics of SARM expression in Pam2CSK4- and Pam3CSK4-treated mouse macrophages

Besides *P. gingivalis* LPS, we extended our study to elucidate the SARM expression in other TLR2 ligands, Pam2CSK4 and Pam3CSK4. To address the kinetics of SARM expression in TLR2 ligand-treated cells, mouse macrophages were treated with Pam2CSK4 and Pam3CSK4. At indicated time point, the protein expression was determined by immunoblotting. As shown in Figure 7, SARM protein was rapidly upregulated within 1 hour after stimulation. In contrast, *E. coli* LPS-treated mouse macrophages (TLR 4 ligand) failed to upregulate SARM expression within this time frame. These results demonstrated that TLR2 but not TLR4 ligand can promptly upregulate both gene and protein expression of SARM in mouse macrophage cell line (RAW264.7).

7. Neutralizing antibody against TLR2 interferes with SARM expression in Pam2CSK4- and Pam3CSK4-treated mouse macrophages

In order to confirm that the increase in SARM expression observed was a result of binding of the ligand to TLR2 not other contaminated components, mouse macrophages were pretreated with TLR2 neutralizing monoclonal antibody for 2 hours before the activation with either Pam2CSK4 or Pam3CSK4. As shown in Figure 8, neutralizing antibody against TLR2 was able to suppress SARM expression in both TLR2 ligand treatment, indicating the significance of TLR2 in the regulation of SARM expression in Pam2CSK4- and Pam3CSK4-treated mouse macrophages.

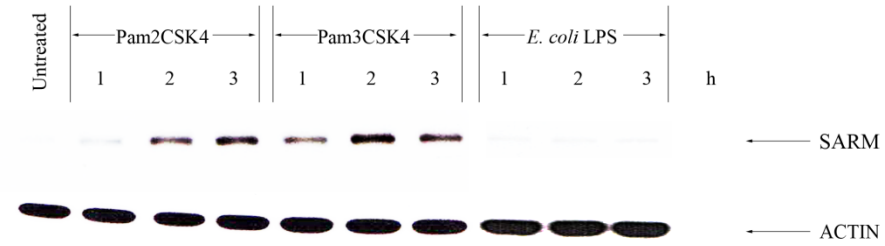


Figure 7. PAM2CSK4 and PAM3CSK4 induce SARM expression in mouse macrophage cell line (RAW 264.7). Mouse macrophages were treated with Pam2CSK4 and Pam3CSK4 at a concentration of 100 ng/ml or *E. coli* LPS at a concentration of 10 ng/ml. At different time intervals, the activated cells were lyzed and the kinetics of SARM protein expression were determined by immunoblotting. Actin protein was used as internal loading control. Data are representative of three independent experiments.

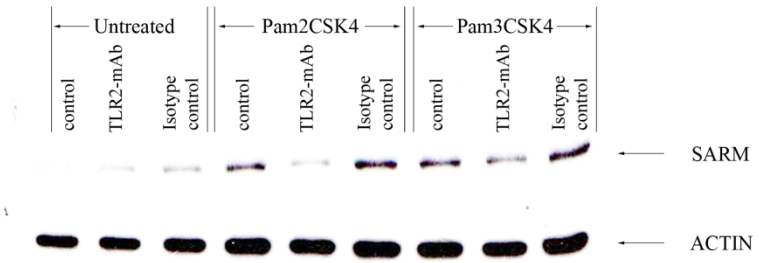


Figure 8. TLR2 neutralizing antibody suppresses SARM expression in Pam2CSK4- and Pam3CSK4-treated mouse macrophages. Mouse macrophages were pretreated with 1 μg/ml of TLR2 neutralizing monoclonal antibody or isotype control for 2 hours before activation with Pam2CSK4 and Pam3CSK4 at a concentration of 100 ng/ml for 3 hours. The treated cells were lyzed and the SARM expression was determined by immunoblotting. Data are representative of three independent experiments.

8. Activation of SARM by Pam2CSK4 and Pam3CSK4 requires internalization

To investigate the requirement of TLR2 ligands internalization for SARM upregulation, mouse macrophages were pretreated with an inhibitor of actin polymerization, cytochalasin D, at a concentration of 2 μg/ml for 2 hours prior to the stimulation with Pam2CSK4 and Pam3CSK4. As shown in Figure 9, suppression of SARM protein expression was observed in the presence of cytochalasin D. These results suggested that the internalization of TLR2 and its ligands is essential for the regulation of SARM expression.

9. Endosomal acidification is required for SARM expression in Pam2CSK4- and Pam3CSK4-treated mouse macrophages

Endosomes are known to play an essential role in innate immune signaling pathway. Since SARM expression requires TLR2 ligand internalization (Figure 8), we extended our finding to demonstrate whether endosomal maturation involves in this process. In order to elucidate this hypothesis, mouse macrophages were pretreated with endosomal maturation/acidification inhibitors, bafilomycin A1 or chloroquine, for 2 hours prior to the stimulation with Pam2CSK4 and Pam3CSK4 ligands. In the presence of these inhibitors, expression of SARM was markedly decreased as shown in Figure 10A and 10B. These results indicated that acidification of endosomal compartments may also be involved in the induction of SARM in Pam2CSK4- and Pam3CSK4-treated mouse macrophages.

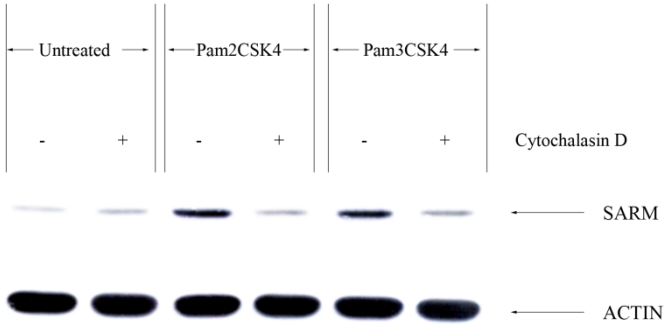


Figure 9. Cytochalasin D suppresses SARM expression. Cytochalasin D (2μg/ml) was added to macrophages for 2 hours prior to stimulation with TLR2 agonists, Pam2CSK4 (100 ng/ml) and Pam3CSK4 (100 ng/ml) for 3 hours. The activated cells were lyzed and the SARM expression was determined by immunoblotting. Data are representative of three independent experiments

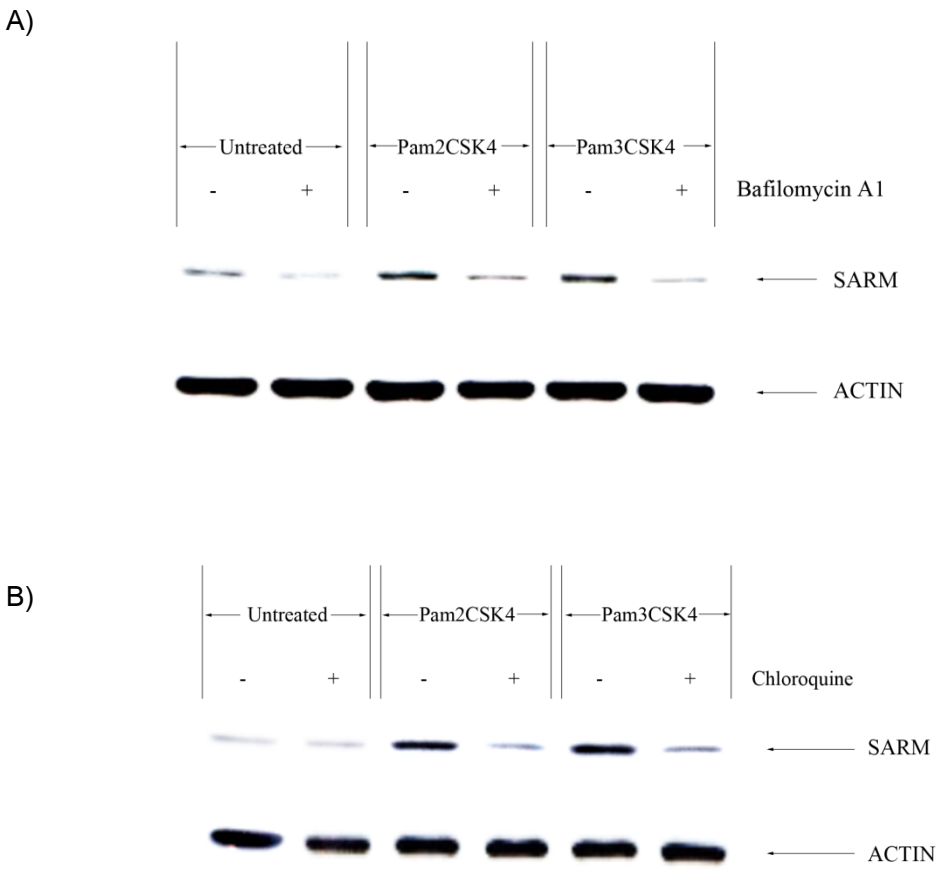


Figure 10. Bafilomycin A1 and chloroquine suppress SARM expression. Mouse macrophages were pretreated with inhibitor of endosomal acidification, bafilomycin A1 (200 nM) (A) or chloroquine (2.5 μg/ml) (B) for 2 hours. After incubation, the cells were treated with Pam2CSK4 and Pam3CSK4 at a concentration of 100 ng/ml for 3 hours. The activated cells were lyzed and the SARM expression was determined by immunoblotting. Data are representative of three independent experiments.

10. TLR9 is essential for the increased SARM expression by Pam2CSK4 and Pam3CSK4

Endosomal acidification has been demonstrated to play an essential role in endosomal TLRs function [13]. Among endosomal TLRs, TLR9 involve in bacterial infection by sensing CpG motif of bacterial DNA. In this study, we further investigated the role of TLR9 in the regulation of SARM expression. As shown in Figure 11, Pam2CSK4 and Pam3CSK4 failed to stimulate SARM expression in TLR9-depleted cells when compared to that of the control siRNA transfected cells. This result indicated that the upregulation of SARM expression by Pam2CSK4 and Pam3CSK4 requires TLR9.

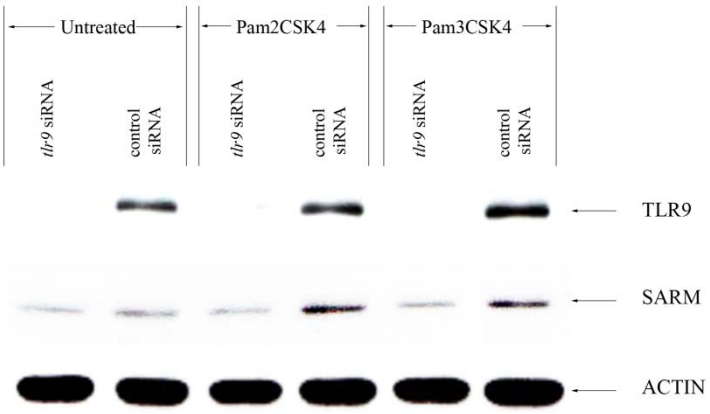


Figure 11. TLR9 regulates the expression of SARM in Pam2CSK4- and Pam3CSK4-treated mouse macrophages. Depletion of TLR9 was performed in mouse macrophages prior to activation with Pam2CSK4 and Pam3CSK4 at a concentration of 100 ng/ml for 3 hours. The treated cells were lyzed and the SARM expression was determined by immunoblotting. Data are representative of three independent experiments.

Discussion

Toll-like receptors (TLRs) play a front-line role in the initiation of innate immune responses against invading pathogens [14]. Signaling transduction by TLRs involves five adaptor molecules known as MyD88, Mal, TRIF, TRAM and SARM [15]. On the contrary to other adaptor molecules, SARM has been identified as a negative regulator of TLR signaling pathway by directly binding to TRIF molecule resulting in suppression of signaling pathway downstream of TRIF-dependent pathway [10]. Previously, we demonstrated that *B. pseudomallei*, a gram-negative intracellular bacterium, upregulated SARM expression in mouse macrophage cell line (RAW264.7) [11]. Overexpression of this negative regulator molecule lead to the inhibition of iNOS expression. However, the decrease of *B. pseudomallei* intracellular survival was observed in SARM-depleted mouse macrophages suggesting that SARM expression is important for intracellular killing of infected macrophages [11]. In the present study, we demonstrated that *P. gingivalis* LPS stimulated the low level of nitric oxide production at the concentration as high as 1000 ng/ml when compared to *E. coli* LPS (Fig. 1). As expected, the low level of nitric oxide and iNOS protein expression were also correlated with the induction of SARM expression (Fig. 2). These results implied that SARM may involve in the activation of iNOS expression and nitric oxide production in *P. gingivalis* LPS-treated mouse macrophages. Therefore, in order to study the involvement of SARM in *P. gingivalis* LPS induced iNOS expression, mouse macrophages were knockdown with *SARM* siRNA. However, as shown in Fig.3 and Fig. 4, the level of nitric oxide production and iNOS protein expression were not increased in SARM-depleted macrophages. These results suggested that SARM expression is not involved in the production of nitric oxide and iNOS expression in *P. gingivalis* LPS-treated mouse macrophages. However, we further investigated the SARM expression by

using Pam2CSK4, Pam3CSK4 (TLR2 ligands) and *E.coli* LPS (TLR4 ligand) as activators. These results demonstrated, only Pam2CSK4 and Pam3CSK4 could upregulate both mRNA and protein of SARM in a time dependent manner (Fig. 7). These results indicated that SARM expression requires TLR2 as demonstrated in Fig. 8. Furthermore, in the presence of cytochalasin D, an inhibitor that blocks actin polymerization, the expression of SARM was markedly decreased (Fig. 9). Our results were also correlated with the reduction of SARM expression in *B. pseudomallei*-infected mouse macrophages in the presence of cytochalasin D [11], suggesting that internalization of surface receptor and ligand is important for SARM expression.

Endosomal acidification facilitates TLR conformational change which requires for initiating the signal transduction after receptor internalization. Blocking of this process could also interfere with the TLR signaling resulting in suppression of cytokine production. For example, bafilomycin A1 severely inhibited the TLR2-driven IFN-β and type I IFN-dependent responses [16]. This result also consistent with another report suggesting that regulation of type I IFN production by TLR2 also requires endosomal acidification [17]. In the present study, we hypothesized that endosomal TLR might be involved in the regulation of SARM. The inhibitory activity of bafilomycin A1 and chloroquine on endosomal TLRs was performed. Interestingly, we observed that Pam2CSK4- and Pam3CSK4-induced SARM expression was also suppressed in the presence of these inhibitors (Fig. 10). These results indicated that besides the signal from TLR2, the signal released from endosomal TLRs may also contribute to the initiation of SARM upregulation in Pam2CSK4 and Pam3CSK4-treated mouse macrophages.

Toll-like receptor 9 (TLR9) is the member of pattern recognition receptors (PRRs) that senses microbial nucleic acids, particularly the bacterial DNA (CpG DNA). TLR9 triggers the innate immune responses and plays an essential role in initiation of adaptive immunity [18]. Inhibition of endosomal maturation/acidification by chloroquine resulted in the suppression of nitric oxide (NO) production and iNOS expression from CpG ODN-treated mouse macrophages, suggesting that maturation/acidification of endosome is involved in TLR9 activation/signaling pathway [13]. In the present study, we further indicated that TLR9 participates in the regulation of SARM expression. Depletion of TLR9 in mouse macrophages showed the markedly suppression of Pam2CSK4- and Pam3CSK4-induced SARM expression, indicating the TLR9 involvement of the SARM regulation in Pam2CSK4- and Pam3CSK4-treated mouse macrophages (Fig. 11). Furthermore, our results also demonstrated the induction of iNOS expression required TLR9 in *P. gingivalis* LPS-treated cells (Fig. 6). However, the underlying mechanism of this TLR in *P. gingivalis* LPS-treated mouse macrophages is under investigated.

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1. International Journal Publication

1.1 **Pudla M,** Kulsantiwong P, Srisaowakarn C, Utaisincharoen P. Regulation of sterile α- and armadillo motif (SARM) containing protein expression in Pam2CSK4- and Pam3CSK4-activated mouse macrophage cell line (RAW264.7) requires TLR9. Inflamm Res. 2017.66(12):1099-1105.

1.2 Induction of inducible nitric oxide synthase (iNOS) in *P. gingivalis* LPS-treated mouse macrophage cell line (RAW264.7) requires TLR9. (preparation)