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รายงานวิจัยฉบับสมบูรณ์

การศึกษาการควบคุมการสร้าง sterile α-armadillo-motif containing protein (SARM) ในแมคโครฟาจของหนูที่ได้รับเชื้อ *Burkholderia pseudomallei*

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มีนาคม 2554

สัญญาเลขที่ BRG5180003

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

Abstract

Project Code: BRG5180003

Project Title: Regulation of Sterile α Armadillo-Motif (SARM) containing protein in the mouse macrophage cell line infected with *Burkholderia pseudomallei*

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Burkholderiapseudomallei, a causative agent of melioidosis, is a Gram-negative facultative intracellular bacterium that can survive and multiply in macrophages. Previously we demonstrated that *B. pseudomallei* failed to activate gene expression downstream of MyD88-independent pathway, particularly the expression of IFN-β and inducible nitric oxide synthase (iNOS), leading to the inability of macrophages to kill this bacterium. Failure to activate MyD88-independent pathway may due to the fact that *B. pseudomallei* may be able to activate negative regulator of this pathway. Among negative regulator of MyD88-independent pathway Sterile-α and Armadillo Motif (SARM) containing protein has been extensively in many model. In the present report, we extended our study to show that *B. pseudomallei* was able to activate Sterile-α and Armadillo Motif (SARM) containing protein, a known negative regulator of MyD88-independent pathway. Both live and heat-killed *B. pseudomallei* were able to upregulate SARM expression in a time-dependent manner in mouse macrophage cell line RAW 264.7. In addition, the intracellular survival of *B. pseudomallei* was suppressed in SARM-deficient

macrophages. Increased expression of IFN-β, iNOS and degradation of IκBα correlated with enhanced macrophage killing capability. Moreover, the expression of SARM required bacterial internalization as it could be inhibited by cytochalasin D suggested that intracellular receptors may involved in SARM expression. However, *B. pseudomallei*-infected NOD1 and NOD2-depleted macrophages still able to stimulate SARM expression implied that others intracellular receptor but not NOD1 and NOD2 responsible for SARM expression. Altogether, these results demonstrated that *B. pseudomallei* modulated macrophage defense mechanisms by upregulating SARM, thus leading to the suppression of IFN-β and iNOS needed for bacterial elimination.

บทคัดย่อ

รหัสโครงการ : BRG5180003

ชื่อโครงการ : การศึกษาการควบคุมการสร้าง Sterile α- Armadillo-motif containing protein (SARM) ในเซลล์แมคโครฟาจของหนูที่ได้รับเชื้อ *Burkholderia pseudomallei*

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เชื้อเบอร์โคลเดอเรีย สูโดมาลลิไอ (*Burkholderia pseudomallei*) เป็นเชื้อแบคทีเรียชนิดแกรมลบซึ่งเป็นสาเหตุของโรคเมลิออยโดซิส เชื้อ *B. pseudomallei* เป็นแบคทีเรียชนิด facultative intracellular ที่สามารถเจริญเติบโตและเพิ่มจำนวนได้ภายในเซลล์แมคโครฟาจ ก่อนหน้านี้ทางคณะผู้วิจัยได้แสดงให้เห็นว่าเชื้อ *B. pseudomallei* ล้มเหลวในการกระตุ้นการแสดงออกของยีน downstream ของ MyD88-independent pathway รวมถึงการแสดงออกของ IFN-β และ iNOS นำมาซึ่งการที่เซลล์แมคโครฟาจไม่สามารถที่จะทำลายเชื้อแบคทีเรียชนิดนี้ได้ ซึ่งความล้มเหลวในการกระตุ้น MyD88-independent pathway อาจมาจากความจริงที่ว่า เชื้อ *B. pseudomallei* อาจจะกระตุ้น negative regulators ของ MyD88-independent pathway ในบรรดา negative regulators ของ MyD88-independent pathway นั้น SARM เป็นตัวหนึ่งที่ถูกศึกษากันอย่างกว้างขวางในรูปแบบต่างๆ ในรายงานนี้ทางคณะผู้วิจัยได้ทำการศึกษาเพื่อที่จะแสดงให้เห็นว่า เชื้อ *B. pseudomallei* นั้นสามารถที่จะกระตุ้นการแสดงออกของ SARM ได้ทั้งในแบคทีเรียที่มีชีวิตและไม่มีชีวิต อีกทั้งการแสดงออกของ SARM ยังมีการเพิ่มระดับการแสดงออกไปตามเวลาที่เพิ่มขึ้นด้วย นอกจากนี้การดำรงชีวิตของ เชื้อ *B. pseudomallei* นั้นจะลดลงในเซลล์แมคโครฟาจที่ไม่มีการแสดงออกของ SARM และการเพิ่มขึ้นของการแสดงออกของ IFN-β, iNOS และการสลายของ IκB ยังสอดคล้องกับการเพิ่มความสามารถของเซลล์แมคโครฟาจในการทำลาย เชื้อ *B. pseudomallei* อีกด้วย นอกจากที่กล่าวมาแล้วการแสดงออกของ SARM นั้นต้องการการ internalization ของแบคทีเรียให้เข้าไปในเซลล์ซึ่งการแสดงออกของ SARM สามารถถูกยับยั้งด้วยสารยับยั้ง cytochalasin D ซึ่งจากผลการทดลองนี้แสดงให้เห็นว่า intracellular receptors อาจมีบทบาทในการควบคุมการแสดงออกของ SARM อย่างไรก็ตามเมื่อทำการติดเชื้อ *B. pseudomallei* ในเซลล์แมคโครฟาจที่ไม่มีการแสดงออกของ NOD1 และ NOD2 ยังพบว่ามีการแสดงออกของ SARM อยู่ซึ่งอาจจะเป็นไปได้ว่า intracellular receptors ชนิดอื่นมีบทบาทในการควบคุมการแสดงออกของ SARM แต่ถึงอย่างไรก็ตามจากผลการทดลองเหล่านี้แสดงให้เห็นว่าเชื้อ *B. pseudomallei* สามารถที่จะทำการเปลี่ยนแปลงกลไกการป้องกันของเซลล์แมคโครฟาจ โดยการเพิ่มการ

แสดงออกของ SARM ซึ่งนำมาสู่การยับยั้งการแสดงออกของ IFN-β, iNOS ซึ่งมีบทบาทสำคัญในการทำลายเชื้อ *B. pseudomallei*

Executive Summary

Sterile α armadillo-motif (SARM) is one of adaptors molecule which plays a role in TLR signaling. At present, this adaptor is classified as a negative regulator in NF-κB and IRF activation. In resting cells, levels of SARM are low but could be rapidly upregulated when the cells are stimulated with ligands of TLR4 including lipopolysaccharide (LPS) (2). Expression of SARM specifically blocks TRIF-dependent transcription factor activation and gene induction including IFN-β and iNOS. We previously demonstrated that IFN-β, an effector downstream of MyD88-independent pathway, is essential for intracellular killing of *B. pseudomallei*(16). *B. pseudomallei*-infected macrophages fail to activate IFN-β production, leading to a reduced expression of a key enzyme inducible Nitric Oxide Synthase (iNOS) needed for antibacterial activity of the macrophages (16). Exogenous IFN-β can restore the ability of macrophages to activate iNOS expression and promote the killing of intracellular *B. pseudomallei*(16, 17). It should be possible that *B. pseudomallei* may activate SARM leading to the inhibition of signal required for IFN-β and iNOS expression. However, the expression of SARM was inhibited by cytochalasin D suggested that intracellular receptor rather than surface receptor involved in SARM expression.

Besides TLRs, recent studies by several groups of investigators have identified nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) as anadditional innate immune receptor family (6, 8). These intracellular receptors have also been implicated in the pathogenesis of many bacterial infections including *Mycobacterium tuberculosis, Salmonella typhimurium,* and *Shigellaflexneri*, all of which can activate NLRs (6). The best characterized NLRs are NOD1 and NOD2, both of which have been shown to play a critical role inregulating host innate immuneresponse against bacterial infections by sensing the cytosolic presenceof the molecules containing meso-diaminopimelic acid and muramyldipeptide respectively (3, 7, 11). However, *B. pseudomallei*was still able to activate SARM in NOD1 or NOD2-depleted macrophages suggesting that NOD1 and NOD2 did not participate in regulation of SARM expression. Type of intracellular receptor involved in SARM expression remain to be investigated.

Materials and Methods

Cell culture

Mouse macrophage cell line (RAW 264.7) was obtained from American Type Culture Collection (ATCC, Rockville, MD). If not indicated otherwise, the cells were cultured in Dulbeccco's modified Eagles' medium (DMEM) (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (HyClone) at 37°C under a 5% CO_2 atmosphere.

Bacterial strains

B. pseudomallei parental wild type strain (1026b) was previously described(8). In some experiment, lipopolysaccharide (LPS) mutant (SRM117) that lacks the O-antigenic polysaccharide moiety was used for comparison (4). Bacteria were cultured in Luria-Bertani (LB) at 37°C with agitation at 150 rpm. Overnight cultures were washed twice in phosphate-buffered saline (PBS) and adjusted to a desired concentration by measuring the optical density at 650 nm and the colony-forming unit (CFU) was calculated from the precalibrated standard curve.

Heat-killed bacteria

Non-viable *B. pseudomallei* were prepared by heating a tube of bacteria suspended in phosphate buffered saline (PBS) at 10^8 CFU/ml in a boiling water bath for 15 min. The heat-treated bacteria were washed 3 times with PBS and complete killing was confirmed by inoculating the suspension on tryptic soy agar and observe growth after 48 h.

SARM gene knockdown in macrophage cell line (RAW 264.7)

Mouse macrophage cells (1.5x10^5 cells) were cultured in a 6-well plate overnight. The siRNA (375 ng) was dissolved in DMEM containing a transfection reagent (Qiagen, Hilden, Germany) before adding to the cells, and the cells were incubated at 37˚C and in 5% CO_2 for 24 h. The siRNA sequences are as follows: sense 5' GGA GAU UGU GAC UGC UUU ATT 3' and antisense 5' UAA AGC AGU CAC AAU CUC CTT 3'. Expression of SARM gene and protein was determined by PCR and immunoblotting, respectively.

NOD1 and NOD2 depletion in macrophage cell line RAW 264.7

RAW 264.7 macrophages were transfected with siRNAs against NOD1 and NOD2 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, which was modified as follows. Macrophages (1.5x10^5 cells) were cultured overnight in 6-well plates before the siRNAs were transiently transfected using lipofectamine 2000 (Invitrogen). After 24 h of incubation, the expression of NOD1 and NOD2 were determined by both RT-PCR and immunoblotting. The siRNA sequences used were as follows: NOD1 sense 5' UUG UCC ACC AGA CAC UGA GUG UUC C 3' and antisense 5' GGA ACA CUC AGU GUC UGG UGG ACA A 3' NOD2 sense 5' UAU GGU GUC GGC AUC UCU GUU CAG G 3' and antisense 5' CCU GAA CAG AGA UGC CGA CAC CAU A 3'. As a control, we used AllStars Negative Control siRNA (Qiagen, Hilden, Germany).

Infection of bacteria in mouse macrophage cell line (RAW 264.7)

Mouse macrophages were cultured in a 6-well plate overnight before exposure to bacteria at multiplicity of infection (MOI) of 2 for 1 h. To remove extracellular bacteria, the cells were washed 2 times with 1 ml of PBS before replacing with DMEM containing 250 μg/ml kanamycin (Gibco Labs). At different time intervals, the infected cells were lyzed before subjecting to RT-PCR.

Quantification of intracellular bacteria

To determine intracellular survival and multiplication of the bacteria, a standard antibiotic protection assay was performed as previously described (9). In brief, at the times indicated, the infected cells were washed 3 times with PBS as above, and intracellular bacteria were liberated by lysing the macrophages with 0.1% Triton X-100 and the released bacteria plated on tryptic soy agar. The number of intracellular bacteria expressed as colony forming unit (CFU) was determined by bacterial colony counting.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

In order to determine mRNA expression, mRNA of the infected macrophages was purified as described by manufacture (Roche Diagnostics, Mannheim, Germany). In brief, the infected cells were washed twice with 1 ml PBS and lyzed directly in the culture well with 400 μl of lysis buffer and then transferred to a sterile 1.5 ml microcentrifuge tube. The supernatant was mixed with 350 μl of 70% ethanol and the mixture was gentle mixed before transferring to high pure filter tube and centrifuged at 8,000 rpm for 15 sec. The supernatant was discarded and the pellet was treated with DNase I for 15 min at RT. After incubation, the sample was washed with wash solution I and centrifuged at 8,000 rpm for 15 sec. The supernatant was discarded and wash solution II was added to the tube. After centrifugation, the sample was washed again with wash solution II and centrifuged at maximum speed for 2 min. The filter was transferred to a fresh collection tube before 50 μl of elution buffer and centrifuged at 8,000 rpm for 1 min. Total RNA concentration was determined by using photometrically at 260/280 nm (Multiskan Spectrum, ThermoLabsystems, Helsinki).

The extracted mRNA was subsequently used for cDNA synthesis (Promega, Madison, USA). PCR was then performed using primer pairs specific for *Nod1, Nod2*, *SARM*, *Tnf-α, Il-1β, Il-6* and *β-actin*.

Table 1: Primer specifications for RT-PCR

Gene	Primer	Sequence (5’→3’)
Nod1	Forward	TCTCAGTCTTGCATTCAATGGC
	Reverse	TGTCAGTGTGGTGTTCTGCTT
Nod2	Forward	CTCCTCAGGAAGTTCGTTCG
	Reverse	GTACATGTCCGTGCTGGTTG
SARM	Forward	GGAGGCTCAGTGCATAGGAG
	Reverse	CAGGTCTGGACCTCAGCTTC
Tnf-α	Forward	GTAGCCCACGTCGTAGCAAA
	Reverse	CCCTTCTCCAGCTGGGAGAC
Il-6	Forward	GGTCTCTGGGAAATCGTGGA
	Reverse	GCTGACCCTAGAGCATCCTG
Il-1β	Forward	TCATGGGATGATGATGATAACCTGCT
	Reverse	CCCATACTTTAGGAAGACACGGATT

β–actin	Forward	CCAGAGCAAGAGAGGTATCC
	Reverse	CTGTGGTGGTGAAGCTGTAG

The amplified products were electrophoresed using 1.5% and 2% agarose gel and stained with ethidium bromide before visualization under an ultraviolet lamp.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

1. *Preparation of separating gel*

Electrophoresis was performed on 8x10x0.1 cm gel. A 5 ml of a 8 % acrylamide solution in 3.0 M gel buffer pH 8.8 containing 1% sodium dodecyl sulfate (SDS) (Plusone, Uppsala, Sweden), 0.05% (v/v) N, N, N', N'-tetrametyhlethylenediamine (TEMED) (Plusone) and 0.05% ammonium persulfate (APS) (Plusone) were carefully added and allowed to polymerize at RT for at least 1 h. The comb was gently inserted on the top of the gel to make well for sample application. In either situation, 5 ml of 30% acrylamide in 0.5 M gel buffer pH 6.8 to be used as stacking gel was layered over the separating gel 30 min before used. After polymerization, the comb was carefully removed, samples then electrophoresed immediately.

2. *Preparation of samples*

Cell samples were centrifugation at 10,000 rpm. Supernatant was discharged and the pellet was solubilized in lysis buffer (see appendix) containing 0.0625 M Tris-HCl (USB), pH 6.8, 1% SDS, 10% glycerol (Sigma), 5% 2-mercaptoethanol (2-ME) (BDH) and bromophenol blue. The solution was sonicated on ice for 1 min before heated 5 min in the heat box. The samples were centrifuged at 10,000 rpm, 5 min, 4 °C and the supernatant was subjected to electrophoresis.

3. *Electrophoresis*

The electrophoresis chamber (Hoefer Scientific Instruments, San Francisco, CA) was prepared with cathode at the top and the anode at the bottom chambers. Electrophoresis was carried out at the RT with a constant current of 20 mA per gel in descending direction until the blue dye marker reached the bottom of the gel, approximately 1 h to complete this process.

Immunobloting

After electrophoresis, the gels were electrotransferred to nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, England) at a constant voltage (95volts) for 1 h. The membrane was blocked with 5% blocking solution (Roche Diagnostics, Mannheim, Germany) for 1 h before incubating at 4 °C overnight with 1:1500 rabbit anti-mouse SARM antibody (Santa Cruse, USA) in 5% blocking solution, specific primary antibody against NOD1 (Cell Signaling Technology, Beverly, MA, USA) and NOD2 (eBioscience, CA, USA). The membranes were washed 3 times with 0.1% Tween20 in PBS for 10 min. After that, the blots were then allowed to react with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Pierce, Rockford, USA). Protein bands were detected by enhanced chemiluminescence as recommended by the manufacturer (Roche Diagnostics) which exposed to hyperfilm (Amersham Biosciences).

Statistical Analysis

If not otherwise indicated, all experiments in this study were conducted at least three times. Experimental values were expressed as means ± standard errors of the mean. Statistical significance of differences between two means were evaluated by Student's t test, and a P value < 0.05 was considered significant.

Results

Expression of SARM mRNA in macrophages infected with *B. pseudomallei*

To investigate the expression of *SARM* mRNA, we performed RT-PCR to determine the *SARM* expression in mouse macrophages infected with *B. pseudomallei* and *B. pseudomallei* LPS mutant (*S. typhi* used as a comparison). As shown in Fig. 1, the infected cells with all types of bacteria were able to induce the expression of *SARM* mRNA. In contrast to *B. pseudomallei* (1026b), *B. pseudomallei* LPS mutant and *S. typhi* activated the low level of *SARM* mRNA. These results demonstrated that only *B. pseudomallei* but not LPS mutant or *S. typhi* induce SARM expression.

Expression of SARM mRNA in macrophages infected with heat-killed *B. pseudomallei*

To elucidate whether the expression of *SARM* mRNA depends on the viability of bacteria, the gene expression from the macrophages exposed to heat-killed *B. pseudomallei* was analyzed. The macrophages were treated with heat-killed bacteria at concentration similar to MOI of 1:1, 10:1 and 100:1. After eight hours of infection, heat killed bacteria at concentration similar to MOI of 10 was able to activate the high level of *SARM* mRNA as shown in Fig. 2.

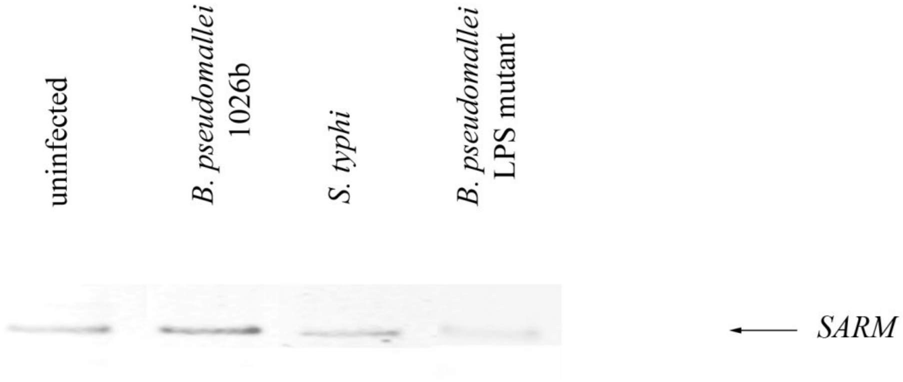


Figure 1. Expression of *SARM* mRNA in *B. pseudomallei*, *B. pseudomallei* LPS and *S. typhi*-infected cells. The mouse macrophages cell line (1x10^6 cells/well) was infected with *B. pseudomallei*, *B. pseudomallei* LPS and *S. typhi* at MOI of 2:1. After 4 hours of infection, the cells were lysed and the *SARM* mRNA expression was determined by RT-PCR. Data are representative of three independent experiments.

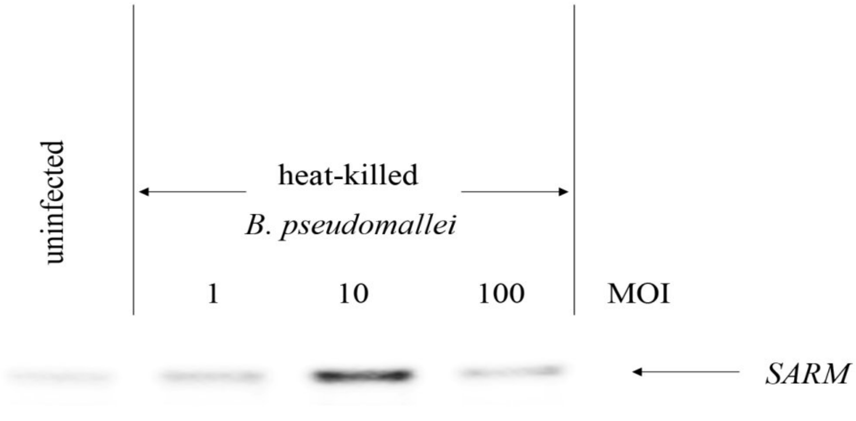


Figure 2. Expression of *SARM* mRNA in heat-killed *B. pseudomallei*-treated cells. The mouse macrophages cell line (1x10^6 cells/well) was treated with heat-killed *B. pseudomallei* at MOI of 1:1, 10:1 and 100:1. After eight hours of activation, the expression of *SARM* mRNA was determined by RT-PCR. Data are representative of three independent experiments.

Kinetics of SARM expression in *B. pseudomallei*-infected mouse macrophage cell line (RAW 264.7)

To investigate SARM expression in *B. pseudomallei*-infected cells, mouse macrophages were co-cultured with the bacteria at MOI of 2. After 15, 30, 60 and 120 min of infection, the cells were harvested and the levels of SARM were analyzed by RT-PCR. Expression of SARM protein was determined by immunoblotting at 15, 30, 60, 120 and 360 of infection. The results showed that *B. pseudomallei* was able to induce SARM expression within 30 min after infection and gradually increased with time (Fig. 3). Previously we demonstrated that *B. pseudomallei* lacking the O-antigenic polysaccharide moiety (LPS mutant) was more susceptible to macrophage killing during the early phase of infection, therefore this LPS mutant strain was used for comparison (1). In contrast to the wild type *B. pseudomallei*, the LPS mutant failed to upregulate SARM expression (Fig. 3).

To determine if the expression of SARM by *B. pseudomallei*-infected macrophages requires viable bacteria, heat-killed bacilli were co-cultured with macrophages at MOI of 10. After different time intervals, the infected cells were harvested and the levels of SARM mRNA and protein were analyzed by RT-PCR and immunoblotting respectively. The results (Fig. 4) showed that, similar to live bacteria, only heat-killed *B. pseudomallei* but not LPS mutant was able to induce SARM expression in a time dependent manner.

In order to rule out the possibility that SARM expression induced by *B. pseudomallei* was mediated through other mediators including cytokines, similar experiment was performed in the presence of a protein synthesis inhibitor. The cells

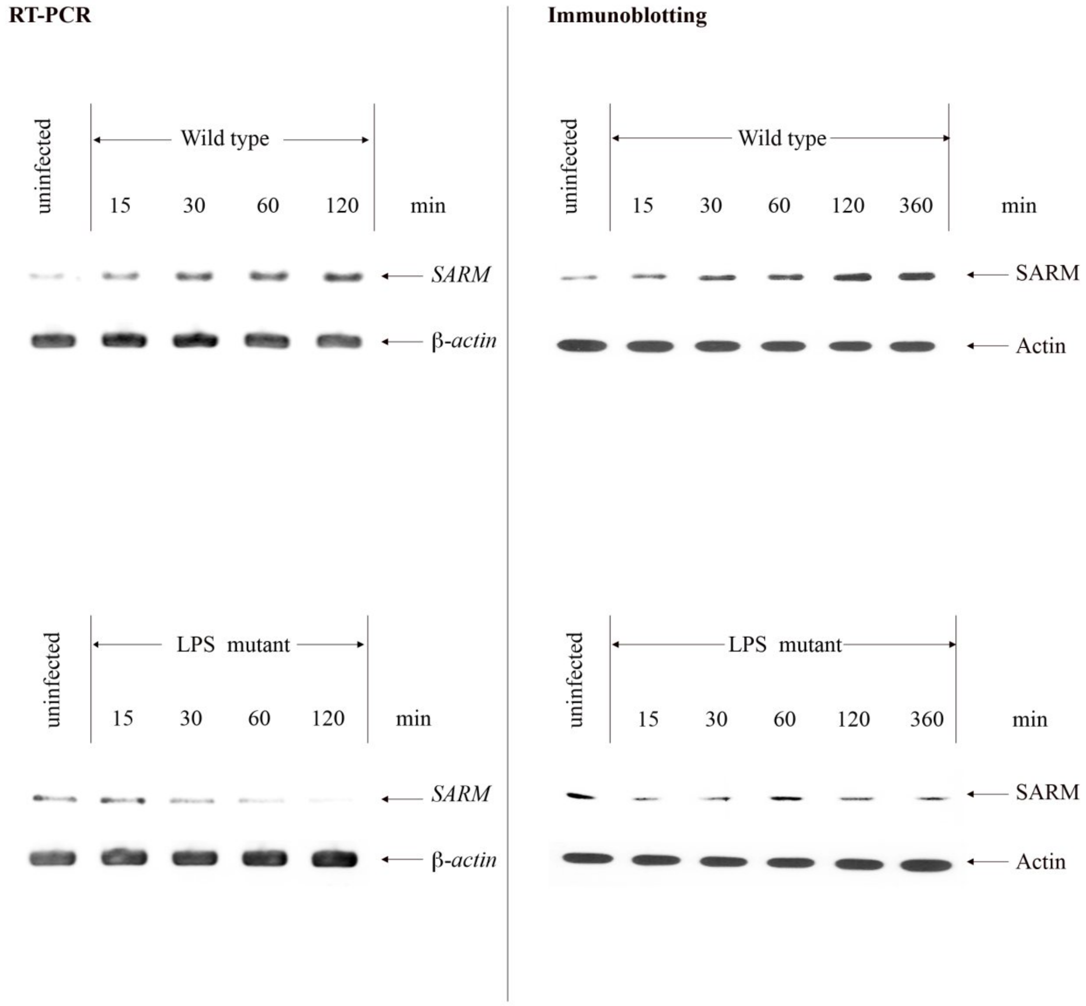


Figure 3. *B. pseudomallei*-induces SARM expression in mouse macrophage cell line (RAW 264.7). Macrophages were infected with either wild type or LPS mutant bacteria at MOI of 2. At different time intervals, the infected cells were lyzed and the kinetics of SARM mRNA and protein levels were determined by RT-PCR and immunoblotting respectively. Data are representative of three independent experiments.

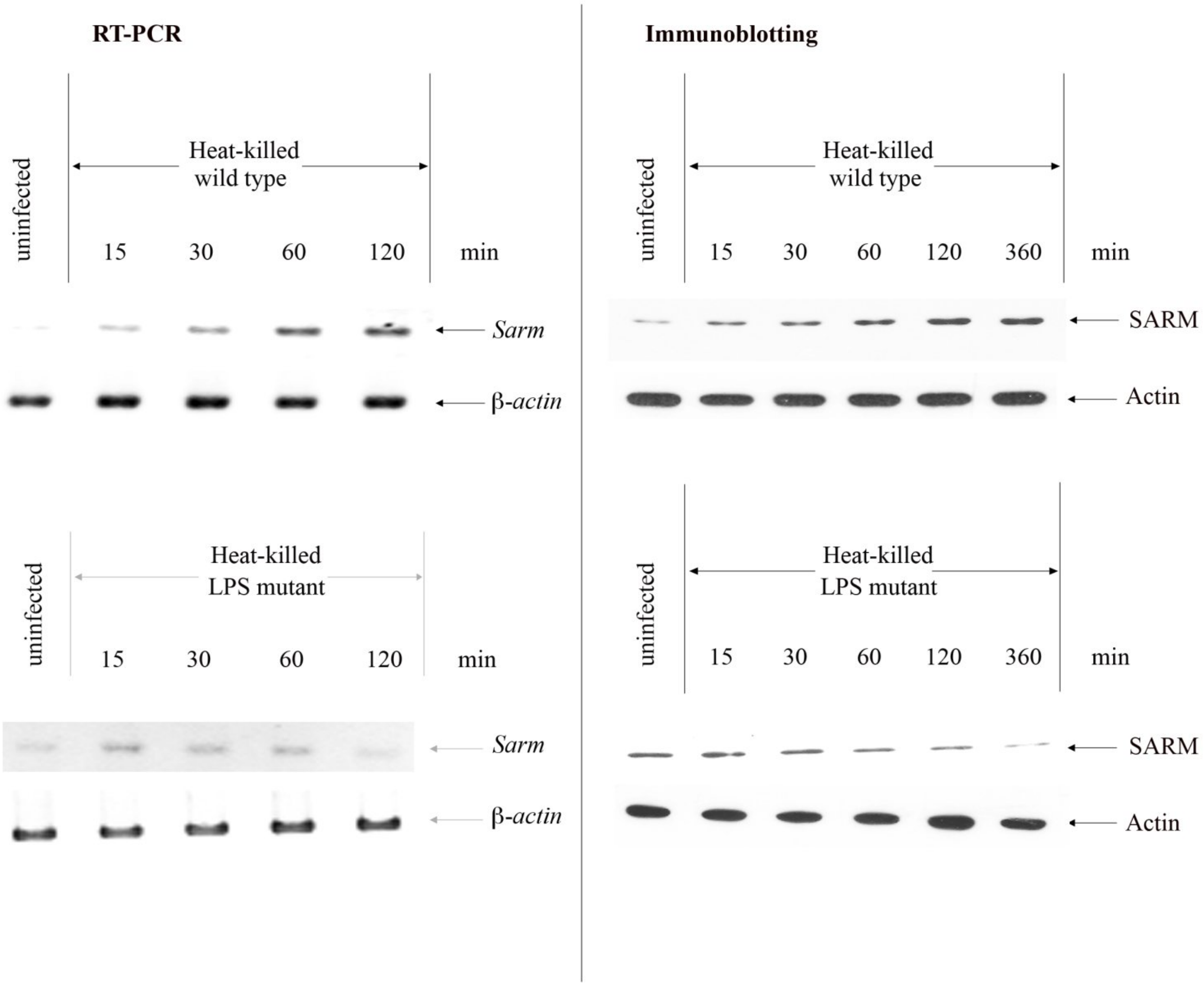


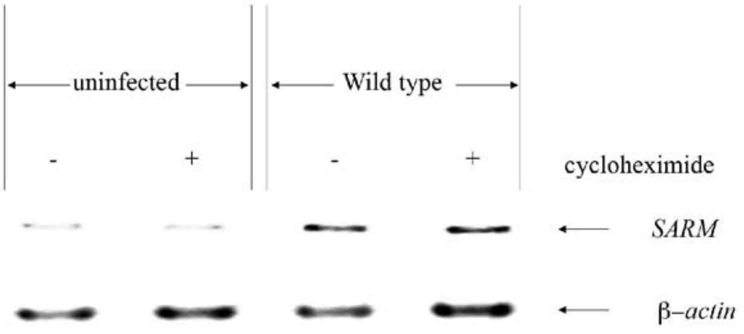
Figure 4. Heat-killed bacteria were co-cultured with macrophages at ratio equivalent to MOI of 10. The expression of SARM mRNA and protein were determined as described in Figure 3. β-actin mRNA and protein were used as internal loading control. Data are representative of three independent experiments.

were pretreated with cyclohexamide (5 μg/ml) for 1 h before infection. As shown in Fig. 5, the levels of SARM mRNA expression were not affected by cyclohexamide. In contrast, as to be expected, the SARM protein level was markedly reduced in the presence of this inhibitor. It should be mentioned that cyclohexamide at the concentration used was not interfere with cell viability (data not shown). These results suggested that the expression of SARM was directly induced by *B. pseudomallei* rather than mediated through cytokine.

Cytochalasin D inhibits SARM expression in *B. pseudomallei*-infected macrophages

To examine a possible relationship between bacterial internalization and SARM expression, the macrophages were pretreated with cytochalasin D (2 μg/ml) for 1 h immediately before exposure to *B. pseudomallei*. This condition of cytochalasin D treatment was selected as it was previously shown to significantly reduce the number of intracellular *B. pseudomallei* in mouse macrophages without interfering with cell viability (5). The level of SARM was markedly reduced in cytochalasin D-treated macrophages, as judged by a decrease in the band intensity (Fig. 6). Moreover, the result presented in Fig. 6 showed that cytochalasin D markedly enhanced iNOS expression. These results implied that upregulation of SARM might require intracellular receptors.

A) RT-PCR



B) Immunoblotting

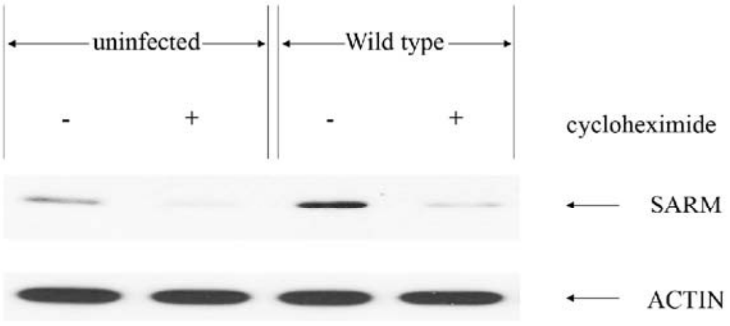
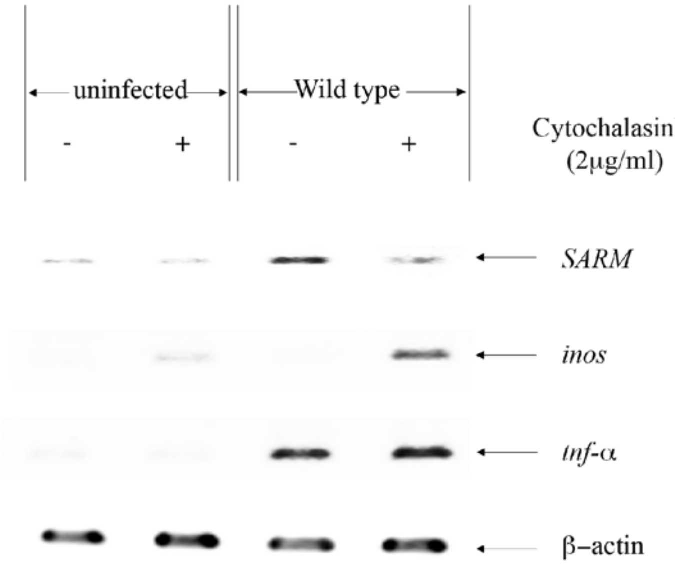


Figure 5. *B. pseudomallei*-induced SARM expression in the presence of cyclohexamide. Macrophages were pretreated with cyclohexamide (5 mg/ml) for 1 h prior to infection. The pretreated cells were infected with *B. pseudomallei* at MOI of 2. The levels of SARM mRNA and protein expression in the infected macrophages were determined at 4 h after infection by RT-PCR and immunoblotting respectively. Data are representative of three independent experiments.

A) RT-PCR



B) Immunoblotting

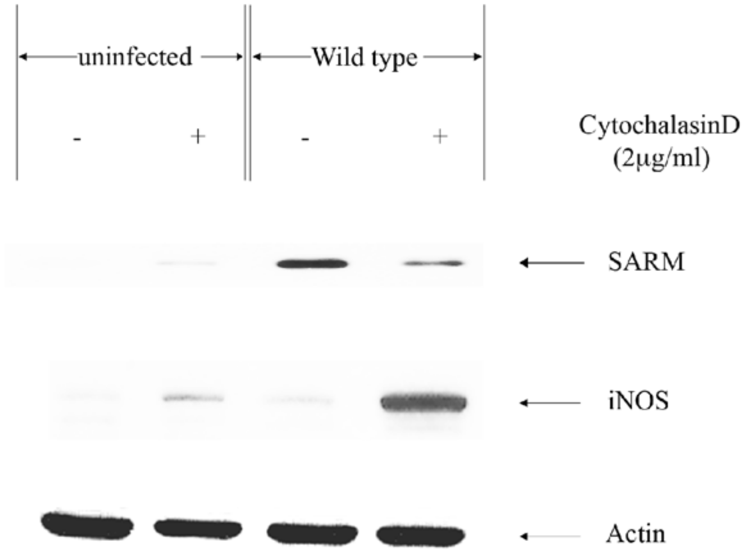


Figure 6. Cytochalasin D inhibits SARM expression. Cytochalasin D (2mg/ml) was added to macrophages for 1 h prior to infection. Infected cells were lyzed after 4 h and 6 h of infection and SARM mRNA (A) and protein (B) were analyzed. Data are representative of three independent experiments.

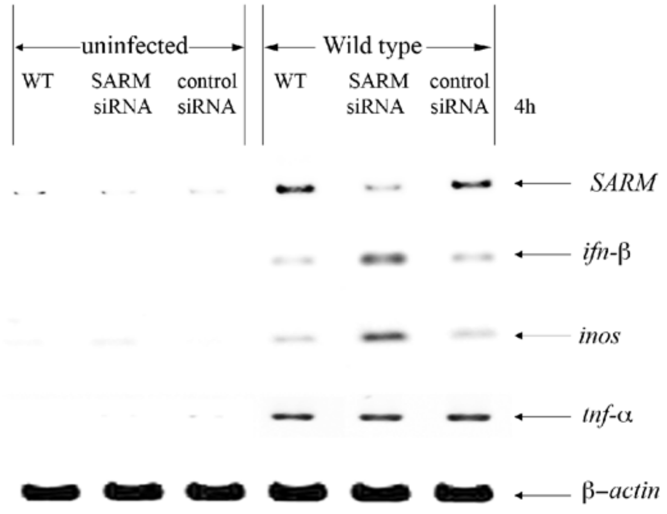
SARM depletion enhances *ifn-β* and *inos* expression in *B. pseudomallei*-infected macrophages

SARM is known to interfere with gene expression downstream of TRIF-dependent pathway including that of *ifn-β* (2). To elucidate the function of SARM in *B. pseudomallei*-infected macrophages, the cells were first transfected with *SARM* siRNA 24 h before infection. The levels of SARM mRNA and protein in SARM-depleted macrophages were markedly decreased compared with those of wild type macrophages (Fig. 7A and B), respectively. To determine the inhibition of gene expression downstream of TRIF, the level of *ifn-β* and *inos* mRNA was analyzed 4 h after infection. As shown in Fig. 7A, the *ifn-β* and *inos* mRNA expression was markedly increased in SARM-depleted macrophages compared to that of the wild type or control siRNA transfected cells. Because it was previously demonstrated that IFN-β was required for iNOS induction (1, 16), we assessed iNOS expression in SARM depleted cells. As shown in Fig. 7A and B, iNOS was highly upregulated in infected SARM-deficient macrophages. These results suggested that SARM upregulation interfered with IFN-β and iNOS expression in *B. pseudomallei*-infected macrophages.

Depletion of SARM increases the rate of IκBα degradation

In order to determine the effect of SARM on IκBα degradation, SARM-deficient or wild type macrophages were infected with bacteria at MOI of 2. At 15, 30 and 60 min after infection, the levels of IκBα expression were determined by immunoblotting. The rate of IκBα degradation in *B. pseudomallei*-infected macrophages was lower than that

A) RT-PCR



B) Immunoblotting

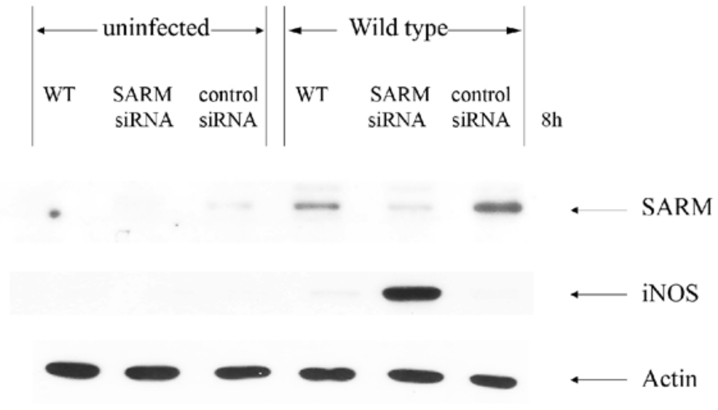


Figure 7. Alteration of mRNA and protein expression profiles in SARM-deficient mouse macrophages. Mouse macrophages ($1.5x10^5$ cells) were transfected with siRNAs against *SARM* prior to infection with *B. pseudomallei* at an MOI of 2. The infected cells were lyzed after 4 h and 8 h of infection for mRNA (A) and protein (B) analyses respectively. Data are representative of three independent experiments.

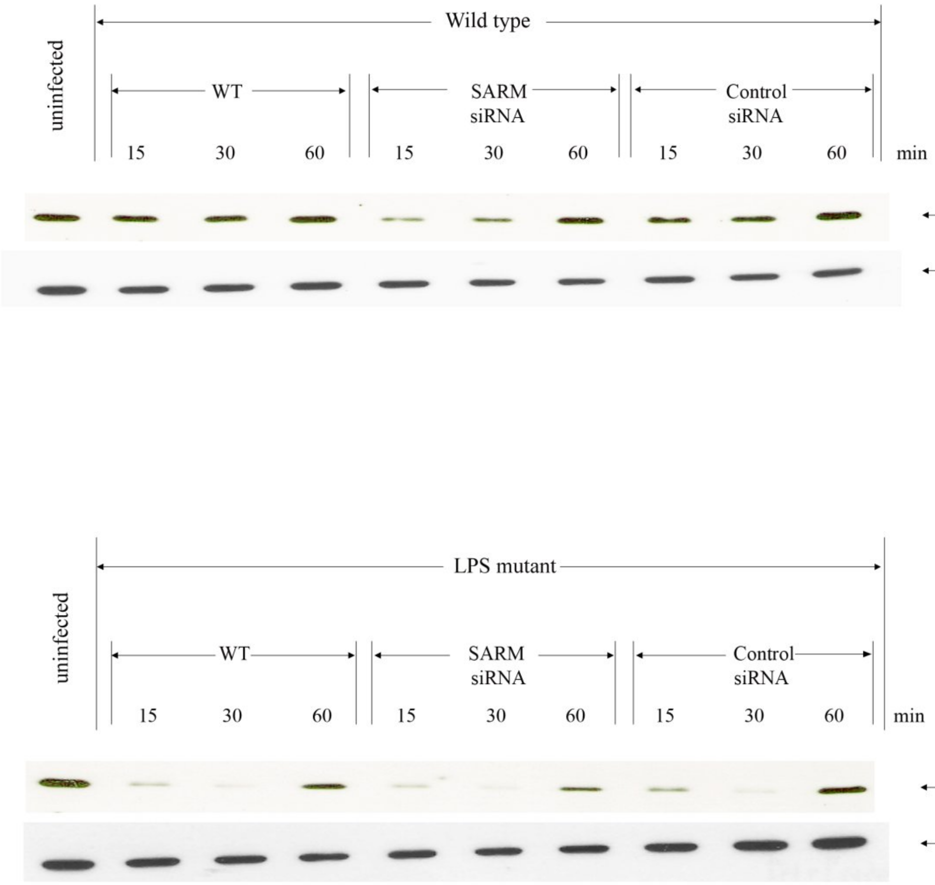


Figure 8. Increased IκBα degradation in *B. pseudomallei*-infected SARM-deficient mouse macrophages. Mouse macrophages ($1.5x10^5$ cells) were transfected with siRNAs against *SARM* prior to infection with either wild type or LPS mutantat an MOI of 2. The infected cells were harvested at 15, 30 and 60 min of infection and IκBα levels were analyzed by immunoblotting. Data are representative of three independent experiments.

of the LPS mutant infected cells (Fig. 8). However, increased IκBα degradation was observed in *B. pseudomallei*-infected SARM-depleted macrophages compared to that of control cells. These results suggested that SARM decreased the rate of IκBα degradation in *B. pseudomallei*-infected macrophages.

Depletion of SARM suppresses *B. pseudomallei* intracellular survival

Our previous findings are consistent with the prediction that *B. pseudomallei* escapes macrophage killing by interfering with IFN-β production, resulting in suppression of iNOS expression (1, 17). In the present study, we showed that SARM depletion led to an increase in iNOS expression (Fig. 7). Because this process might affect the intracellular fate of *B. pseudomallei*, additional experiments were performed. In order to demonstrate this relationship, SARM-depleted macrophages were infected with *B. pseudomallei* at MOI of 2 and after 2 and 6 h of infection, the infected cells were lyzed and the number of intracellular bacteria was analyzed. As shown in Fig. 9A, at 2 h after infection, the number of intracellular bacteria was similar between the three groups. These results suggested that SARM did not interfere with *B. pseudomallei* internalization. However, depletion of SARM resulted in an enhancement of the macrophage ability to kill *B. pseudomallei* (Fig. 9B) as judged by a significant reduction in the number of viable intracellular bacteria, thus compatible with the prediction that SARM expression suppresses antibactericidal activity of macrophages.

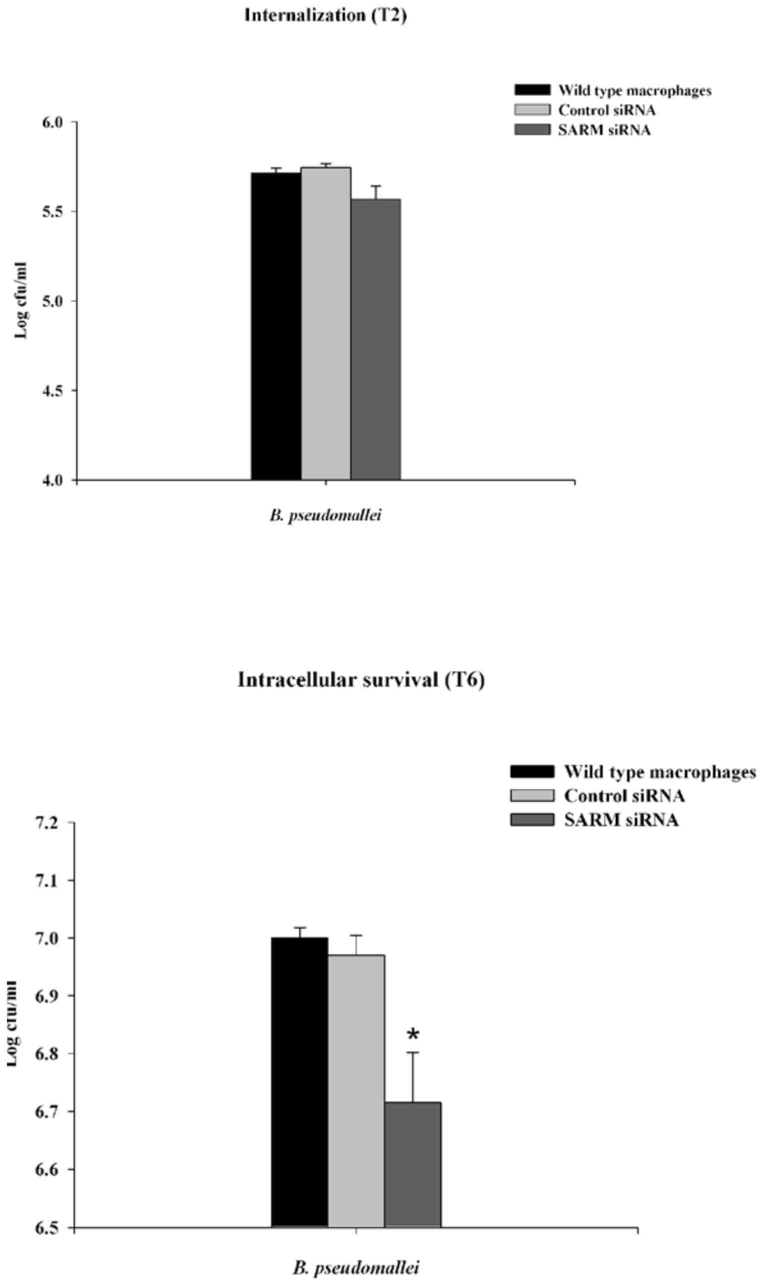


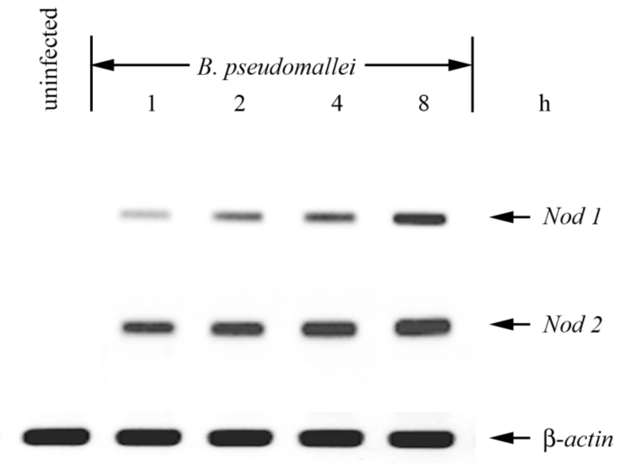
Figure 9. Intracellular fate of *B. pseudomallei* in SARM-deficient macrophages. SARM-deficient macrophages were infected with *B. pseudomallei* at MOI of 2. At 2 h (A) and 6 h (B) after infection, the number of bacterial internalization and intracellular replication were determined by standard antibiotic protection assay as described in Materials and Methods. The data indicate the means and standard deviations of three separate experiments, each carried out in duplicate. *, $P < 0.05$.

Role of NOD1 and NOD2 in SARM expression

As shown in Fig. 6, expression of SARM was inhibited in the presence of cytochalasin D. These results suggested that intracellular receptors may responsible for SARM expression. Among them NOD1 and NOD2 have been extensively study in bacterial infection. It is possible that these two intracellular receptors may involve in regulation in SARM expression.

To investigate the role of NOD1 and NOD2 expression, mouse macrophages were infected with *B. pseudomallei*at a MOI of 2. At specific times after infection, the levels of NOD1 and NOD2 mRNAs were determined by RT-PCR. With the condition used, NOD1 and NOD2 mRNAs could not be detected in the uninfected macrophages (Fig. 10A). In contrast, *B. pseudomallei* was able to activate the transcription of both receptorswithin 1 h of infection and the levels gradually increase with time of incubation. Trace quantity of NOD1 and NOD2 proteins could be detected as early as 4 and 2 h after the infection respectively (Fig. 10B). In order to determine if the expression of NOD1 or NOD2 by the macrophages requires viability of the bacteria, the cells were exposed to heat-killed *B. pseudomallei* at concentration equivalent to MOI of 10. At 1, 2, 4 and 8 h of infection, the infected cells were harvested and mRNA and protein of NOD1 or NOD2 were assessed as described above. The results from Fig. 11 indicated that exposure of macrophages to nonviable *B. pseudomallei* yielded NOD1 and NOD2 expression patterns that were similar to those obtained using viable bacteria (Fig. 10), thus suggesting that the expression of both receptors does not require active invasion of the macrophages by live *B. pseudomallei*.

A) RT-PCR



B) Immunoblotting

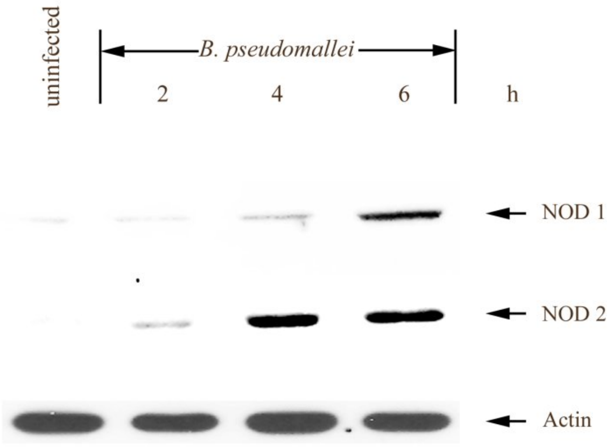
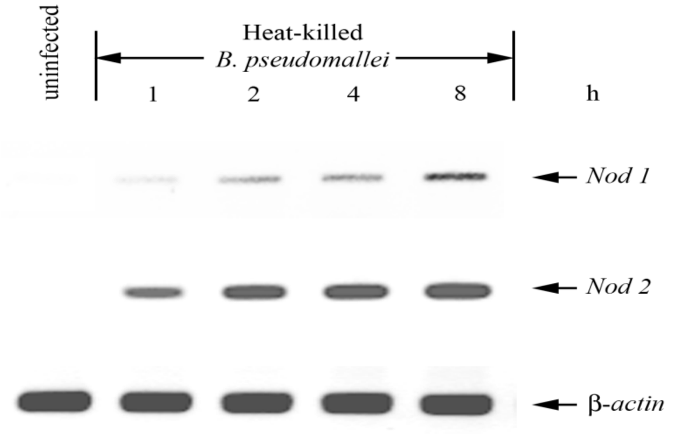


Figure 10. Upregulation of NOD1 and NOD2 expression in *B. pseudomallei*-infected mouse macrophages. The macrophages were infected with the bacteria at MOI of 2. At different time intervals, the infected cells were lysed and the kinetics of NOD1 and NOD2 mRNA (A) and protein (B)were determined by RT-PCR and immunoblotting respectively. β-actin mRNA was served as an internal control. Data are representative of three independent experiments.

A) RT-PCR



B) Immunoblotting

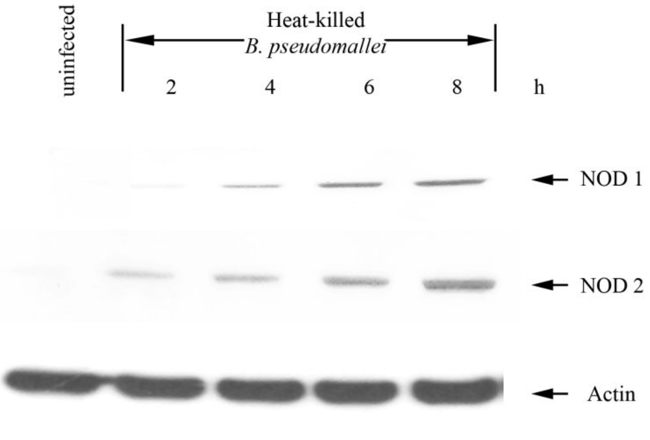


Figure 11. Kinetics of NOD1 or NOD2 expression in mouse macrophages treated with heat-killed *B. pseudomallei*. The macrophages were exposed to heat-killed *B. pseudomallei* at concentration equivalent to MOI of 10. Expressions of mRNA and protein were determined as described in Fig. 10. Data are representative of three independent experiments.

SARM expression is not regulated by NOD1 or NOD 2

In order to determine the possible involvement of NOD1 and/or NOD2 in SARM expression, the macrophages were first treated with NOD1 or NOD2 siRNAs before infecting with *B. pseudomallei*. The mRNA level and protein expression of NOD1 or NOD2 of uninfected and *B. pseudomallei*-infected macrophages were assayed 24 h after transfection. Results presented in Fig. 12Aand B showed that when the cells were treated with siRNAs, both mRNA and protein of NOD1 and NOD2 were diminished. However, the expression of SARM mRNA and protein did not alter in *B. pseudomallei*-infected either NOD1 or NOD2 knockdown macrophages (Fig. 12A and B). In addition, the mRNA expression of TNF-α, IL-1β and IL-6 was not also affected in both *B. pseudomallei*-infected NOD1 and NOD2 knockdown macrophages (Fig. 12B). These results demonstrated that the other intracellular receptors may involve in SARM expression.

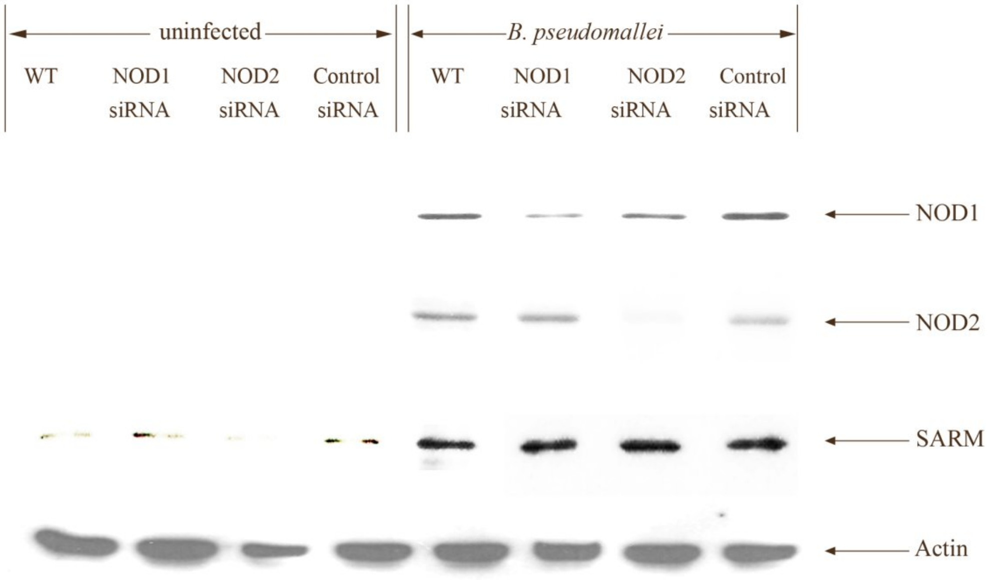


Figure 12A. Alteration of protein expression profiles in NOD1 and NOD2 knockdown mouse macrophages infected with *B. pseudomallei*. The wild type and NOD knockdown macrophages were infected with *B. pseudomallei* as described in Fig. 10. The infected cells were lyzed after 6 h of infection for protein analysis. The expression of uninfected cells serves as control. Data are representative of three independent experiments.

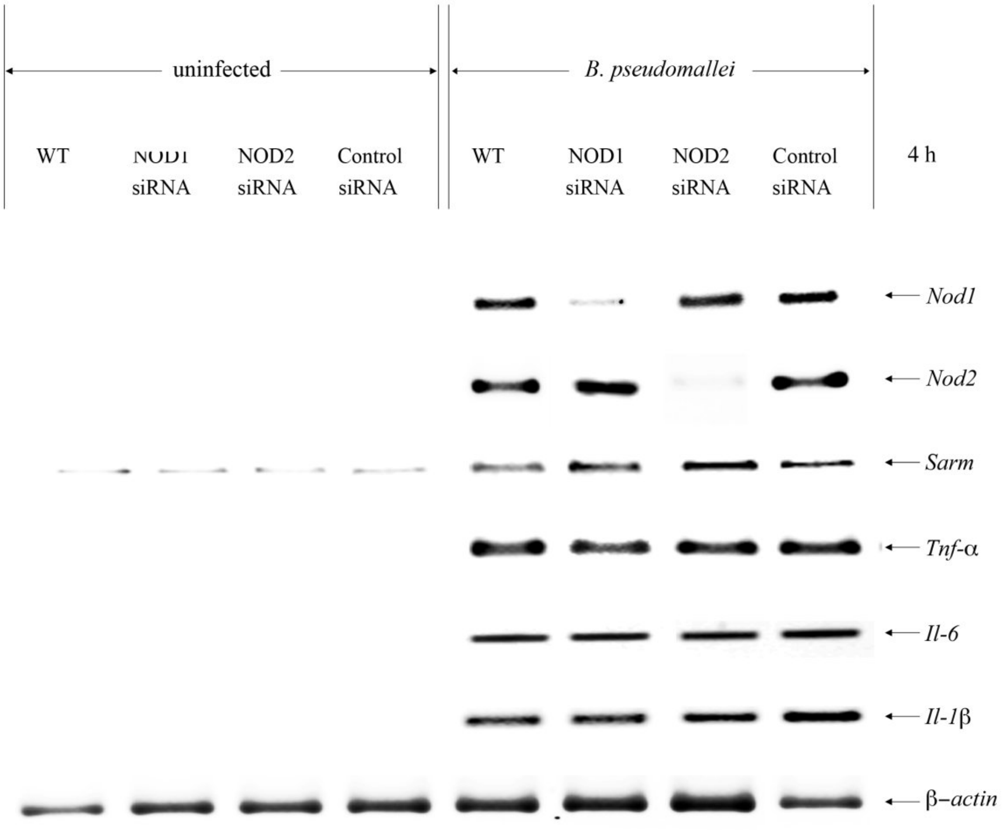
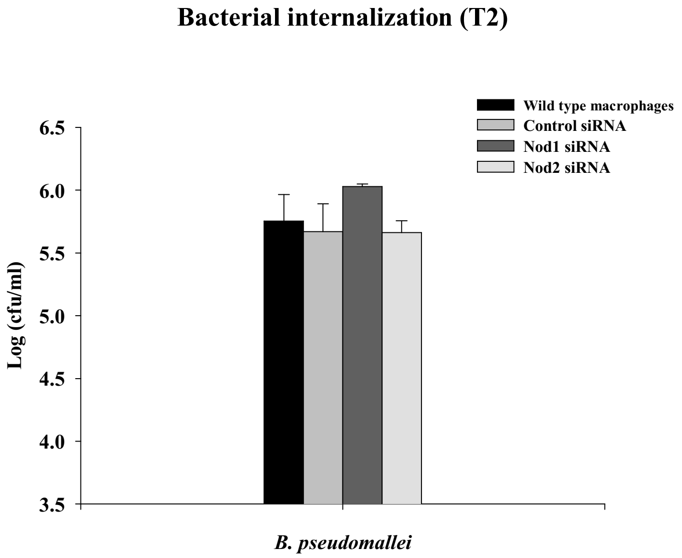


Figure 12B. Alteration of mRNA expression profiles in NOD1 and NOD2 knockdown mouse macrophages infected with *B. pseudomallei*. The wild type and NOD knockdown macrophages were infected with *B. pseudomallei* as described in Fig. 10. The infected cells were lyzed after 4 h of infection for mRNA analysis. The expression of uninfected cells serves as control. Data are representative of three independent experiments.

NOD1 and NOD2-depleted macrophages do not interfere intracellular replication of *B.pseudomallei*

In order to determine the involvement of NOD1 and NOD2 in bacterial intracellular replication, the number of intracellular replication of *B. pseudomallei* was determined at 2 and 8 h after infection. As shown in Fig. 13A and B, at 2 h and 8 h after infection, the number of intracellular bacteria was similar between the three groups. These results consistent with Fig. 12 suggested that the regulation of SARM expression did not require NOD1 and NOD2.



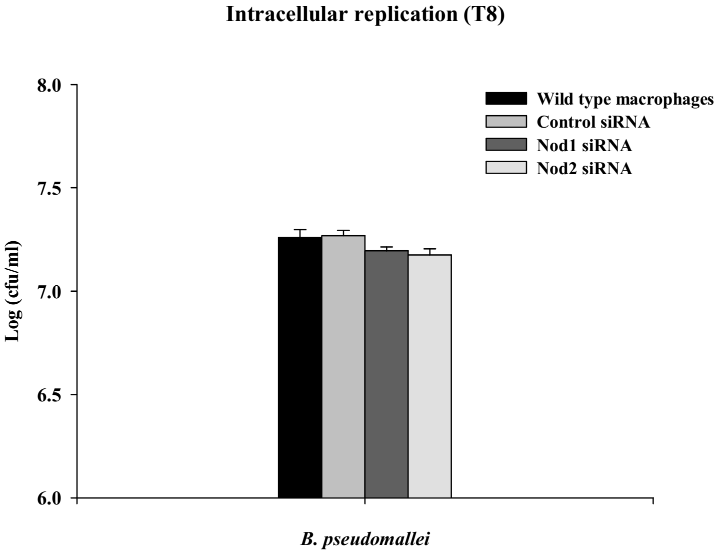


Figure 13. Intracellular fate of *B. pseudomallei* in NOD1 and NOD2-deficient macrophages. NOD1 and NOD2-deficient macrophages were infected with *B. pseudomallei* at MOI of 2. At 2 h (A) and 8 h (B) after infection, the number of bacterial internalization and intracellular replication were determined by standard antibiotic protection assay as described in Materials and Methods. The data indicate the means and standard deviations of three separate experiments, each carried out in duplicate. *, $P < 0.05$.

Discussion

In our present study, we designed additional experiments to demonstrate the role of SARM in *B. pseudomallei* infection. This negative regulator was upregulated in a time-dependent manner (Fig. 3). In contrast LPS mutant failed to activate SARM expression (Fig. 1 and 3). Moreover, heat-killed *B. pseudomallei* is able to upregulate SARM expression suggesting that the activation of SARM does not require active invasion of the macrophages (Fig. 4). In addition, the expression of SARM can be rapidly induced by either live or heat-killed *B. pseudomallei* (within 30 min) and cyclohexamide did not interfere with SARM mRNA (Fig. 5). These results imply that SARM upregulation is initiated directly by the physical contact of bacteria rather than indirectly by mediators released from the host. Furthermore, inhibition of bacterial internalization by cytochalsin D decreases SARM expression in *B. pseudomallei*-infected cells (Fig. 6). These data suggested that the signal generated by the interaction of *B. pseudomallei* with appropriate receptor(s) at the surface of macrophages is sufficient to activate TNF-α expression, but insufficient for SARM upregulation. It is possible that intracellular pattern recognition receptors may be involved in this process. In addition, this adaptor molecule has been shown to inhibit signaling pathways downstream of MyD88-independent pathway including that of *ifn-β* and *inos* (2). Our conclusion was reached by the fact that increased *ifn-β* and *inos* mRNA expressions were observed in the *B. pseudomallei*-infected SARM knockdown macrophages (Fig. 7). These results are also consistent with the finding that both living and heat-killed LPS mutant does not activate SARM expression (Fig. 1, 3 and 4) resulting in the upregulation of *ifn-β* and *inos* expression levels previously observed (1, 14).

It is well documented that IκBα regulates NF-κB activation by forming a complex with NF-κB, thereby preventing it from entering into the nucleus (19). In response to stimuli, IκBα is degraded by the proteasome resulting in NF-κB nuclear translocation. Previously we demonstrated that the level of IκBα degradation in human lung epithelial A549 cells infected with *B. pseudomallei*is lower than that in cells infected with other Gram-negative bacteria including *Salmonella typhi* (18). The lower level of IκBα degradation was also observed in *B. pseudomallei*-infected macrophages when compared to that observed in LPS mutant (Fig. 8). However, IκBα degradation level was noticeably increased in *B. pseudomallei*-infected SARM-deficient macrophages (Fig. 8) suggesting that SARM could influence IκBα processing. These results are also in accord with a recent report which demonstrated a correlation between SARM expression and reduced IκBα degradation (13). The mechanism by which SARM reduces IκBα degradation may relate to its ability to inhibit the MAPK pathway such as by suppressing p38 phosphorylation(12, 13). Although depletion of SARM does not interfere with internalization of *B. pseudomallei*, it influences the bacterial intracellular survival (Fig. 9) which correlates with increased iNOS expression (Fig. 7). However, intracellular suppression of *B. pseudomallei* observed in SARM depleted macrophage was not as efficient as IFN-γ treatment as previously reported by our group (15, 19). It is possible that regulation of SARM by *B. pseudomallei*may only partially involve in inhibition of genes expression downstream of MyD88. Recent evidence demonstrates that SARM directly interact with TRIF (2) therefore it is possible that other negative regulators of TLR signaling downstream of TRIF such as MAP kinase phosphatase (MKP)-1 or signal regulatory proteinα (SIRPα) which negatively regulate phosphorylation of p38 and TBK-1 respectively (10, 20), may also participate in *B. pseudomallei* infection. The involvement of these molecules is under investigation by our group.

As shown in Fig. 6, level of SARM expression was suppressed in the presence of cytochalasin D. Therefore, we extended the study to investigate the involvement of intracellular pattern recognition receptors in SARM expression. The most well characterized cytosolic receptors are NOD1 and NOD2. NOD1 senses peptidoglycan-derived meso-diaminopimelic acid (DAP)-containing muramypeptides which are a structural component of cell wall of gram-negative bacteria, whereas NOD2 senses muramyldipeptide (MDP) present in both gram-positive and gram-negative bacteria (3, 7, 11). As shown in Fig. 10, the activation processes of NOD1 and NOD2 are associated with *B. pseudomallei* components as shown by the upregulation of both NOD1 and NOD2 expression in the infected macrophages. The expression of these cytosolic receptors could also be induced by heat-killed *B. pseudomallei*, suggesting the involvement of heat stable components most likely present on the surface of *B. pseudomallei* (Fig. 11). Moreover, we attempted to identify if these cytosolic receptor(s) involved in SARM expression. However, the results presented in Fig. 12 showed both two intracellular receptors did not involve in SARM expression. Then results also consistent with the number of intracellular replication did not alter in NOD1 and NOD2-depleted macrophages (Fig. 13). However, the type of intracellular receptor that involved in SARM expression remain to be investigated.

Altogether, the results presented here suggest that *B. pseudomallei* interferes with MyD88-independent signaling pathway at least in some part by inducing SARM expression which results in the inhibition of MyD88 independent pathway therefore the bacteria can survival inside macrophages.

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