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ORIGINAL

TLR2 signals triggered by mycoplasmal lipoprotein/lipopeptide induce K^+ efflux to activate the NLRP3 inflammasome

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ABSTRACT : The proinflammatory cytokine interleukin (IL)-1β plays a crucial role in controlling bacterial infections and is produced after the processing of pro-IL-1β by caspase-1, which is activated by the inflammasome. Mycoplasmal membrane lipoprotein and lipopeptide, which are typical Toll-like receptor 2 (TLR2) ligands, activate the NLRP3 inflammasome to produce IL-1β in macrophages, although the molecular mechanism behind this remains unclear. Here, we found that lipoproteins from *Mycoplasma salivarium* (MsLP) and *M. pneumoniae* (MpLP) and an *M. salivarium*-derived lipopeptide (FSL-1) exhibited IL-1β-inducing activity toward bone marrow-derived macrophages from C57BL/6 mice ($TLR2^{+/+}$ BMMs), whereas the activity toward BMMs from TLR2-deficient mice ($TLR2^{-/-}$ BMMs) was markedly reduced. Microarray analysis suggested that FSL-1 upregulates the potassium voltage-gated channel, subfamily F, member 1 (Kcnf1), which is involved in K^+ efflux as one of the NLRP3 inflammasome activators, in a TLR2-dependent manner. Moreover, we found that a high extracellular concentration of K^+, which blocks K^+ efflux, downregulated the release of IL-1β. Thus, this study is the first to suggest that TLR2-mediated signals triggered by mycoplasmal lipoproteins/lipopeptide upregulate potassium channels to promote K^+ efflux, by which the NLRP3 inflammasome is activated.

Key Words : Toll like receptor 2, interleukin-1β, mycoplasmal lipoprotein, FSL-1, NLRP3 inflammasome

Introduction

Interleukin (IL)-1β plays a crucial role in controlling bacterial infections by amplifying proinflammatory responses[1)]. IL-1β is produced as an inactive precursor (pro-IL-1β) through the activation of nuclear factor-κB by Toll-like receptor (TLR)-mediated signaling and released extracellularly after pro-IL-1β is processed into biologically active IL-1β by activated caspase-1. Caspase-1 activation occurs through the intracellular multiprotein complex known as the inflammasome. The inflammasome is typically composed of a nucleotide-binding oligomerization domain (NOD)-like receptor (NLR), the adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and procaspase-1. Of several types of inflammasome, the NLR family, pyrin domain-containing 3 (NLRP3) inflammasome is the best characterized. This inflammasome is activated upon a diverse spectrum of stimuli including microbial products and damage-associated molecular patterns such as extracellular ATP or K^+ efflux[2–7)].

Mycoplasmas are the smallest self-replicating microbes and lack cell walls[8)]. Although mycoplasmas do not possess cell wall components such as lipopolysaccharide (LPS), lipoteichoic acid, or murein components, they possess membrane-bound lipoproteins, which are recognized by Toll-like receptor 2 (TLR2) and provoke immune responses[9–16)].

We previously found that membrane-bound lipoproteins of *Mycoplasma salivarium* and *M. pneumoniae* (MsLP and MpLP) and the lipopeptide FSL-1 derived from one of MsLP, which are typical TLR2 ligands, activate the NLRP3 inflammasome to produce IL-1β in murine bone marrow-derived macrophages (BMMs)[17)]. However, whether TLR2 signals participate in NLRP3 inflammasome activation remains elusive.

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The present study suggests that TLR2 signals mediated by the diacylated lipopeptide FSL-1 trigger K^+ efflux, by which the NLRP3 inflammasome is activated to release IL-1β in BMMs.

Methods

1) Reagents

FSL-1, a diacylated lipopeptide derived from *M. salivarium*, was synthesized as previously described[18]. Fluorescein isothiocyanate-conjugated FSL-1 (FITC-FSL-1) was purchased from EMC Microcollections GmbH (Tübingen, Germany). Ultrapure *Escherichia coli* LPS was purchased from InvivoGen (San Diego, CA, USA).

2) Mycoplasmas and culture conditions

M. salivarium ATCC23064 and *M. pneumoniae* ATCC15492 were grown in pleuropneumonia-like organism (PPLO) broth (Difco Laboratories, Detroit, MI, USA) supplemented with 20 % (vol/vol) horse serum (Gibco, Grand Island, NY, USA), 1 % (wt/vol) yeast extract (Difco), 1 % (wt/vol) L-arginine hydrochloride for *M. salivarium* or 1% (wt/vol) D-glucose for *M. pneumoniae*, and 1,000 units/mL penicillin G.

Cultures were incubated at 37 °C and centrifuged at 15,000 × g for 15 min at late log phase. The cell pellets were washed three times with sterilized phosphate-buffered saline (PBS), suspended in PBS to make aliquots, and then stored at −80 °C. The protein concentration was determined using a DC Protein Assay kit (Bio-Rad, Hercules, CA, USA), in accordance with the manufacturer's instructions.

3) Lipoprotein preparation by Triton X-114 phase separation

M. salivarium and *M. pneumoniae* cells were treated with Triton X-114 to extract lipoproteins, as previously described[19]. The Triton X-114 phase was collected, treated with methanol to precipitate lipoproteins, suspended in sterile PBS, and used for stimulation. The lipoproteins prepared from *M. salivarium* and *M. pneumoniae* were designated MsLP and MpLP, respectively. The protein concentration was determined using a DC Protein Assay kit (Bio-Rad), in accordance with the manufacturer's instructions.

4) Mice

Wild-type C57BL/6 mice ($TLR2^{+/+}$) were purchased from CLEA Japan (Tokyo, Japan). TLR2-deficient mice ($TLR2^{-/-}$) of the same genetic background were kindly provided by Dr. Shizuo Akira, Osaka University. All mice were maintained in specific pathogen-free conditions at the animal facility of Hokkaido University. All experiments were performed in accordance with the regulations of the Animal Care and Use Committee of Hokkaido University.

5) Cell culture of BMMs

Bone marrow cells were prepared from the femurs and tibias of $TLR2^{+/+}$ and $TLR2^{-/-}$ mice. The bone marrow cells were cultured in 10-cm, plastic, non-tissue-culture Petri dishes in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) containing 10 % fetal bovine serum (FBS), 100 U/ml penicillin G, 100 μg/ml streptomycin, and cell-conditioned medium (i.e., culture supernatants derived from L929 fibroblast cells). After 7–9 days of culture, macrophages that loosely adhered to the dishes were harvested using cold PBS and then used as BMMs.

6) IL-1β measurement

BMMs from $TLR2^{+/+}$ and $TLR2^{-/-}$ ($TLR2^{+/+}$ BMMs and $TLR2^{-/-}$ BMMs) were added to a 24-well plate at 4×10^5 cells/well in 500 μl of RPMI 1640 medium containing 10 % (vol/vol) FBS and incubated at 37 °C for 4 h with 10 ng/ml ultrapure *E. coli* LPS. The cells were subsequently resuspended in 300 μl of RPMI 1640 basal medium and incubated at 37 °C with MsLP, MpLP, or FSL-1. Incubation times are indicated in the figures and figure legends. IL-1β in cell culture supernatants was quantified using an ELISA kit for IL-1β (OptEIA™ SET Mouse IL-1β; BD Biosciences, San Jose, CA, USA).

7) Transfection of FSL-1 into the cytosol

$TLR2^{+/+}$ BMMs and $TLR2^{-/-}$ BMMs were added to a poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA)-coated eight-well slide and chamber (Watson, Tokyo, Japan) at 3×10^5 cells per well, in 500 μl of RPMI 1640 medium containing 10 % (vol/vol) FBS and incubated at 37 °C for 4 h with 10 ng/ml ultrapure *E. coli* LPS. The cells were washed with RPMI 1640 basal medium and resuspended in 270 μl of medium. A solution of 30 μl of FITC-FSL-1 (20 μg/ml) dissolved in 20 mM HEPES buffer was mixed with 0.3 μl of PULSin reagent (Polyplus-Tranfection, Illkirch, France) and added to the appropriate wells after 15 min of incubation. After 4 h of incubation at 37 °C, the

cells were washed with PBS and fixed with 4 % paraformaldehyde solution (Nacalai Tesque, Kyoto, Japan); then, the cells were washed with PBS containing 10 mM glycine and sealed in SlowFade™ Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific). Confocal images were taken by a confocal laser scanning microscopy system (Nikon A1 and Ti-E) equipped with a Plan Apo VC 60× objective lens (NA 1.40; Nikon, Tokyo, Japan).

To assess the release of IL-1β, TLR2$^{+/+}$ BMMs and TLR2$^{-/-}$ BMMs were added to a 24-well plate at 4×10^5 cells per well in 500 μl of RPMI 1640 medium, containing 10 % (vol/vol) FBS, and incubated at 37 °C for 4 h with 10 ng/ml ultrapure *E. coli* LPS. The cells were washed with RPMI 1640 basal medium and resuspended in 270 μl of medium. A solution of 30 μl of 1 μM FSL-1 dissolved in 20 mM HEPES buffer was mixed with 0.3 μl of PULSin reagent and added to the appropriate wells after 15 min of incubation. After the indicated times of incubation at 37 °C, the concentration of IL-1β in the cell culture supernatants was measured using an ELISA kit (BD OptEIA™ Set Mouse IL-1β; BD Biosciences).

8) Statistical analysis

Statistical analysis was performed using Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

1. TLR2-mediated signaling is required for the IL-1β-inducing activity of mycoplasmal lipoprotein/lipopeptide

We previously reported that MsLP, MpLP, and FSL-1, which are typical TLR2 ligands, activate the NLRP3 inflammasome to release IL-1β by BMMs[17]. Therefore, we first investigated whether the IL-1β-inducing activities of MsLP, MpLP, and FSL-1 toward BMMs were mediated by the TLR2 signaling pathway. TLR2$^{+/+}$ and TLR2$^{-/-}$ BMMs were stimulated for 4 h with ultrapure LPS of *E. coli* and then for 24 h with MsLP, MpLP, or FSL-1 (Fig. 1A–C) because LPS priming was previously shown to significantly enhance their IL-1β-inducing activities[20]. Therefore, the following experiments were performed using LPS-primed BMMs. MsLP, MpLP, and FSL-1 exhibited IL-1β-inducing activities toward TLR2$^{+/+}$ BMMs in a dose-dependent manner, whereas the activities of MsLP and MpLP toward TLR2$^{-/-}$ BMMs were significantly attenuated and the activity of FSL-1 completely disappeared. These results suggest that TLR2-mediated signaling is required for expression of the activity. In this context, further experiments were carried out to determine how TLR2 signals participate in this activity.

2. The cytosolic localization of FSL-1 is independent of the expression of TLR2

We previously showed that the localization of FSL-1 in the cytosol is an important step leading to activation of the NLRP3 inflammasome[17]. Therefore, to examine whether the loss of IL-1β release in TLR2$^{-/-}$ BMMs by FSL-1 was due to the attenuated uptake of intracellular FSL-1, TLR2$^{+/+}$ and TLR2$^{-/-}$ BMMs were incubated with FITC-labeled FSL-1 and analyzed for its intracellular localization by confocal microscopy. As shown in Fig. 2A, there was no difference in the level of FSL-1 uptake between TLR2$^{+/+}$ and TLR2$^{-/-}$ BMMs, which was in line with our previous report describing that FSL-1 uptake by murine peritoneal macrophages occurs irrespective of the presence of TLR2[21].

To gain further insights into the role of TLR2 signals in active IL-1β release, we investigated whether the IL-1β release was enhanced by the artificial delivery of FSL-1 into the cytosol of TLR2$^{-/-}$ as well as TLR2$^{+/+}$ BMMs. It was found that the artificial delivery mediated by transfection reagent PULSin drastically enhanced the amount of FSL-1 in the cytosol of both TLR2$^{+/+}$ and TLR2$^{-/-}$ BMMs (Fig. 2A). However, the IL-1β-inducing activity toward TLR2$^{-/-}$ BMMs was significantly lower than that toward TLR2$^{+/+}$ BMMs (Fig. 2B). These results suggest that the cytosolic localization of FSL-1 is independent of TLR2 expression.

3. K^+ efflux is required for the IL-1β-inducing activities of FSL-1

To further assess how TLR2 signals triggered by FSL-1 participate in NLRP3 inflammasome activation, we examined the differences in transcripts triggered by FSL-1 between TLR2$^{+/+}$ and TLR2$^{-/-}$ BMMs by microarray analysis using Agilent SurePrint G3 Mouse GE Ver. 2.0 8x60K. In the inflammasome-related microarray data, we noticed that the expression of potassium voltage-gated channel, subfamily F, member 1 (Kcnf1), was significantly downregulated in TLR2$^{-/-}$ BMMs (data not shown). K^+ efflux through the potassium channels is known to trigger activation of the NLRP3 inflammasome[5–7, 22, 23]. Therefore, we hypothesized that the TLR2 signals triggered by FSL-1 were required for K^+ efflux through

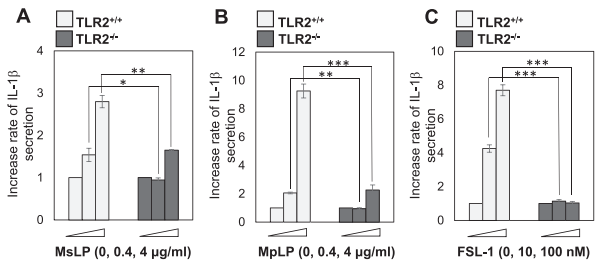

Fig.1 IL-1β release by $TLR2^{+/+}$ or $TLR2^{-/-}$ BMMs in response to MsLP, MpLP, and FSL-1

$TLR2^{+/+}$ or $TLR2^{-/-}$ BMMs were stimulated with LPS (10 ng/ml) for 4 h and then for 24 h with MsLP (0, 0.4, and 4 μg/ml protein) (A), MpLP (0, 0.4, and 4 μg/ml protein) (B), or FSL-1 (0, 10, and 100 nM) (C). The total amounts of IL-1β released into the culture supernatant were measured by ELISA. Increase rate of IL-1β secretion was calculated as [(IL-1β secretion in the presence of MSLP, MpLP or FSL-1) / (IL-1β secretion in the absence of MSLP, MpLP or FSL-1)]. The results are expressed as the mean ± SD of triplicate assays of a representative experiment. All of the experiments were repeated at least twice and similar results were obtained. Student's t-test: *P < 0.05, **P < 0.01, ***P < 0.001.

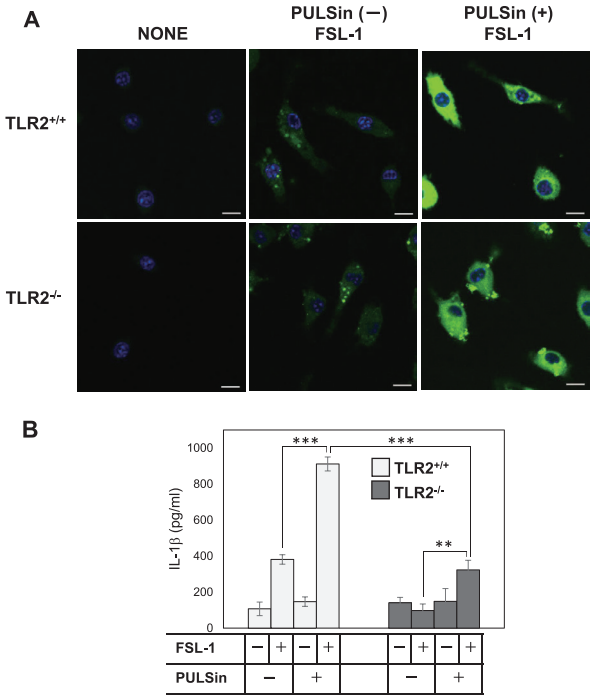

Fig. 2 Induction of IL-1β by cytosolic FSL-1 in $TLR2^{+/+}$ or $TLR2^{-/-}$ BMMs

$TLR2^{+/+}$ or $TLR2^{-/-}$ BMMs were stimulated with LPS (10 ng/ml) for 4 h and then cultured with or without (NONE) 2 μg/ml FITC-FSL-1 in the absence (–) or presence (+) of PULSin. After incubation for 4 h, the cells were fixed and observed by confocal microscopy. Cell nuclei were stained with DAPI (blue). Samples were observed using a confocal microscope. Merged images with FITC-FSL-1 (green) and DAPI are shown. Scale bar indicates 10 μm (A). $TLR2^{+/+}$ or $TLR2^{-/-}$ BMMs were stimulated with LPS (10 ng/ml) for 4 h and then cultured with (+) or without (–) FSL-1 (0 and 100 nM) in the absence (–) or presence (+) of PULSin. After incubation for 24 h, the total amounts of IL-1β released into the culture supernatant were measured by ELISA. The results are expressed as the mean ± SD of triplicate assays of a representative experiment (B). All of the experiments were repeated at least twice and similar results were obtained. Student's t-test: **P < 0.01, ***P < 0.001.

the potassium channels to activate the NLRP3 inflammasome. To confirm this, we examined the IL-1β-inducing activity in the presence of a high extracellular concentration of K^+, which blocks K^+ efflux. We found that KCl downregulated the IL-1β release in a dose-dependent manner (Fig. 3), suggesting that TLR2-mediated signals by FSL-1 trigger K^+ efflux.

Discussion

Two signals are known to be required for the release of IL-1β in macrophages. The first signal induces the expression of pro-IL-1β and inflammasome components, which is mediated by pattern recognition receptors, such as TLRs. The second signal triggers assembly of the inflammasome complex and initiates its activation, which induces the processing of pro-IL-1β by caspase-1 and the release of biologically active IL-1β. Several types of inflammasome are activated by different stimuli. Of these, the NLRP3 inflammasome is the best characterized[2–7]. We previously showed that the typical TLR2 ligands, MsLP, MpLP, and FSL-1, activate the NLRP3 inflammasome to produce IL-1β in BMMs[17]. The present study demonstrated that the IL-1β-inducing activities of these mycoplasmal lipoproteins and lipopeptide toward $TLR2^{-/-}$ BMMs were markedly reduced compared with those toward $TLR2^{+/+}$ BMMs, suggesting that the TLR2 signaling pathway plays important roles in such activity (Fig. 1). Therefore, we examined the differences in transcripts triggered by FSL-1 between $TLR2^{+/+}$ and $TLR2^{-/-}$ BMMs by microarray analysis and found that FSL-1 upregulates the potassium voltage-gated channel, subfamily F, member 1 (Kcnf1) (data not shown), which is involved in K^+ efflux as one of the NLRP3 inflammasome activators[5–7, 22, 23]. We found that a high extracellular concentration of K^+, which blocks K^+ efflux, downregulated the release of IL-1β (Fig. 3). These results suggest that TLR2-mediated signals by FSL-1 trigger K^+ efflux and then induce NLRP3 inflammasome activation.

Thus, the present study is the first to suggest that

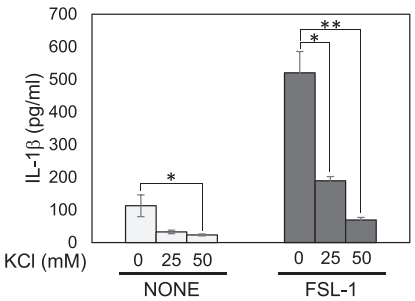

Fig. 3 K^+ efflux is required for the IL-1β-inducing activities of FSL-1

$TLR2^{+/+}$ BMMs were stimulated with LPS (10 ng/ml) for 4 h and then transfected with FSL-1 (100 nM) by PULSin in the absence or presence of KCl (0, 25, and 50 mM). After incubation for 4 h, the total amounts of IL-1β released into the culture supernatant were measured by ELISA. The results are expressed as the mean ± SD of triplicate assays of a representative experiment. All of the experiments were repeated at least twice and similar results were obtained. Student's t-test: $^*P < 0.05$, $^{**}P < 0.01$.

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