Activation of NLRP3 inflammasome in macrophages by mycoplasmal lipoproteins and lipopeptides

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SUMMARY

The NLRP3 inflammasome, an intracellular sensor consisting of the NOD-like receptor family, pyrin domain containing 3 (NLRP3), the apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and procaspase-1, plays critical roles in host defense against microbial pathogens by inducing production of interleukin (IL)-1β and IL-18. *Mycoplasma salivarium* and *Mycoplasma pneumoniae* cells activated murine bone marrow-derived macrophages (BMMs) to induce production of IL-1α, IL-1β, and IL-18. The IL-1β production-inducing activities of these mycoplasmas toward BMMs from Toll-like receptor 2 (TLR2)-deficient mice were significantly attenuated, compared with those from C57BL/6 (B6BMMs). This result suggests the possibility that their lipoproteins as TLR2 agonists are involved in the activity. Lipoproteins of *M. salivarium* and *M. pneumoniae* (MsLP and MpLP), and the *M. salivarium*-derived lipopeptide FSL-1 induced IL-1β production by B6BMMs, but not by BMMs from caspase-1-, NLRP3- or ASC-deficient mice. The activities of MsLP and MpLP were not downregulated by the proteinase K treatment, suggesting that the active sites are their N-terminal lipopeptide moieties. B6BMMs internalized the mycoplasmal N-terminal lipopeptide FSL-1 at least 30 min after incubation, FSL-1-containing endosomes started to fuse with the lysosomes around 2 h, and then FSL-1 translocated into the cytosol from LAMP-1^{+} endosomes. FSL-1 as well as the representative NLRP3 inflammasome activator nigericin induced the NLRP3/ASC speck in the same way as, but FSL-1 located in the compartment different from the NLRP3/ASC specks. The artificial delivery of FSL-1 into the cytosol of B6BMMs drastically enhanced the IL-1β production-inducing activity.

INTRODUCTION

Inflammasomes are intracellular multiprotein complexes that play critical roles in host defense against microbial pathogens and in the development of inflammatory diseases by inducing the production of proinflammatory cytokines.[1-5] Inflammasomes typically consist 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. They mediate the activation of caspase-1 in response to pathogen- and danger-associated molecular patterns (PAMPs and DAMPs, respectively). Active caspase-1 promotes the processing and subsequent release of active interleukin (IL)-1β and IL-18. Several types of NLRs are involved in inflammasome activation.[1-5]

Mycoplasmas are characterized by a wall-less envelope.[6] They do not possess bacterial modulins such as LPS, lipoteichoic acid, or murein components, but do possess membrane-bound lipoproteins, which are recognized by Toll-like receptor 2 (TLR2) and elicit immune responses.[7-14] Mycoplasmas are generally commensal in humans, but some species are pathogens capable of causing a wide variety of diseases.[6]

Mycoplasma salivarium is a member of the human oral microbial flora which preferentially inhabits the gingival sulcus[15] and is suspected to play some etiological role in periodontal diseases,[15-17] although its etiological roles remain unknown. IL-1β plays a pivotal role in the pathogenesis of periodontal diseases, by inducing the production of inflammatory mediators, osteoclast formation, matrix metalloproteinase expression, and the death of matrix-producing cells.[18] In contrast, *Mycoplasma pneumoniae* is a key pathogen of atypical pneumonia in humans.[19] Respiratory infection with *M. pneumoniae* triggers secretion of several proinflammatory cytokines, including

IL-1β.[20] In addition, it has been recently reported that the NLR family, pyrin domain containing-3 (NLRP3) inflammasome activation, leading to IL-1β secretion plays an important role in recruiting and activating the innate immune cells in the lung that are critical for *M. pneumoniae* clearance.[21] Thus, IL-1β production through inflammasome activation is a key regulator of the host response to pathogens and to disease outcomes.

Recently, several studies have reported that mycoplasmas activate inflammasomes to induce IL-1β production.[21-26] We have also shown that *M. salivarium* and *M. pneumoniae* cells activate the NLRP3 inflammasome to produce IL-1β in murine dendritic cells and macrophages.[27] However, the detailed mechanism of inflammasome activation triggered by mycoplasmas is not yet fully clarified. In this study, therefore, attempts were made to determine what kinds of mycoplasmal components activate the NLRP3 inflammasome to induce IL-1β production and how they (or it) activate(s) the inflammasome.

METHODS

Chemicals

The diacylated lipopeptide FSL-1 derived from *M. salivarium* was synthesized as described previously.[28] Fluorescein isothiocyanate-conjugated FSL-1 (FITC-FSL-1) was purchased from EMC Microcollections (GmbH, Germany). Ultrapure *Escherichia coli* lipopolysaccharide (LPS) was purchased from InvivoGen (San Diego, CA, USA).

Mycoplasmas and culture conditions

M. salivarium ATCC 23064 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 a 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 by using a DC Protein Assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Preparation of lipoproteins by Triton X-114 phase separation

M. salivarium and *M. pneumoniae* cells were treated with Triton X-114 to extract lipoproteins according to the method described previously.[29] The Triton X-114 phase was collected and treated with methanol to precipitate lipoproteins, then used for stimulation after being suspended in sterile PBS. The lipoproteins prepared from *M. salivarium* and *M. pneumoniae* were named MsLP and MpLP, respectively. Protein concentration was determined by using a DC Protein Assay kit (Bio-Rad) according to the manufacturer's instructions.

Mice

Sex-matched 8-week-old C57BL/6 (B6) mice 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 the Hokkaido University. Caspase-1-, NLRP3-, or ASC-deficient mice (caspase-1$^{-/-}$, NLRP3$^{-/-}$, or ASC$^{-/-}$,

respectively) of the same genetic background were maintained in specific pathogen-free conditions at the animal facility of the Tokyo Medical and Dental University.

All experiments were performed in accordance with the regulations of the Animal Care and Use Committees of both universities.

Cell culture

Femurs and tibias prepared from caspase-1$^{-/-}$, NLRP3$^{-/-}$, or ASC$^{-/-}$ mice at the Tokyo Medical and Dental University were sent to Hokkaido University. Femurs and tibias from B6 or TLR2$^{-/-}$ mice were prepared at Hokkaido University.

Bone marrow cells were obtained from femurs and tibias as follows. The ends of the bones were cut off, and marrow tissue was eluted by irrigation with RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) containing 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Cells were suspended by pipetting and washed by centrifugation. The cells were cultured in a non-tissue-culture plastic 10 cm Petri dish in RPMI 1640 medium containing 10% 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 loosely adhered to the dishes were harvested by using cold PBS and then used as bone marrow derived macrophages (BMMs).[30]

Cytokines

BMMs from B6, TLR2$^{-/-}$, caspase-1$^{-/-}$, NLRP3$^{-/-}$, or ASC$^{-/-}$ mice were added to a 24-well plate at 4×10^5 cells per well in 500 μl 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 resuspended in 300 μl RPMI 1640 basal medium and incubated at 37 °C for 24 h with intact *M. salivarium* or *M. pneumoniae* cells (0, 36.5, 365 μg/ml protein), MsLP or MpLP (0, 0.4, 4 μg/ml protein), or FSL-1(0, 10, 100 nM).

IL-1β, IL-1α, and IL-18 in cell culture supernatants were quantified by using ELISA kits for IL-1β (OptEIA™ SET Mouse IL-1β, BD Biosciences, San Jose, CA, USA), IL-1α (DuoSet mouse IL-1α, R&D systems, Minneapolis, MN, USA), and IL-18 (Mouse IL-18 ELISA Kit, MBL, Nagoya, Japan).

Immunoblotting

The cell culture supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) to distinguish mature IL-1β from pro-IL-1β, and proteins were transferred to a PVDF membrane (Bio-Rad). These proteins were reacted with the appropriate polyclonal antibody (Ab) against IL-1β (R&D Systems).

Digestion of lipoproteins by proteinase K

MsLP or MpLP suspensions (400 μg/ml in PBS) were pretreated at 37 °C for 1 h with various concentrations (0, 0.8, 4 or 40 μg /ml) of proteinase K (Promega, Madison, WI, USA), and then boiled for 10 min to inactivate the enzyme. SDS-PAGE of the MsLP or MpLP digested by proteinase K was performed in 10% polyacrylamide gels and stained using a Silver Stain kit (Bio-Rad) in accordance with the manufacturer's instruction. Precision Plus Protein™ All Blue Standards (Bio-Rad) were used to estimate molecular weights.

Transfection of FSL-1 into the cytosol

B6BMMs were added to a poly-l-lysine (Sigma-Aldrich, St. Louis, MO, USA)-coated 8 well Slide and Chamber (WATSON, Tokyo, Japan) at 3×10^5 cells per well, in 500 μl 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 medium. A 30-μl FITC-FSL-1 solution (20 μg/ml) suspended 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 incubation. After a 4 h incubation at 37 °C, the cells were washed with PBS and fixed with 4% paraformaldehyde solution (Nacalai Tesque, Kyoto, Japan), and then the cells were washed with PBS containing 10 mM glycine and sealed in VECTASHIELD Antifade Mounting Medium (Vector Laboratories, Burlingame, Ca, USA). 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, B6BMMs were added to a 24-well plate at 4×10^5 cells per well in 500 μl 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 medium. A 30-μl 1 μM FSL-1 solution dissolved in 20 mM HEPES buffer was mixed with 0.3 μl of PULSin reagent and added to the appropriate wells after 15 min incubation. After a 24 h incubation at 37 °C, the concentration of IL-1β in the cell culture supernatants were measured using an ELISA kit (BD OptEIA™ Set Mouse IL-1β, BD Biosciences).

Localization of FSL-1 in the cytosol

B6BMMs were added to a poly-l-lysine (Sigma-Aldrich)-coated 8 well Slide and Chamber (WATSON) at 3×10^5 cells per well in 500 μl 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 resuspended in 300 μl RPMI 1640 basal medium and incubated at 37 °C for various periods of time (30 min, 1, 2, 5 h) with 7 μg/ml of FITC-FSL-1. After being washed three times with PBS, the cells were fixed for 15 min with 4% paraformaldehyde in PBS, and then washed three times with PBS containing 10 mM glycine, and permeabilized for 5 min with 0.1% Triton X-100 in PBS, and blocked for 30 min at room temperature with 3% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS. The cells were then stained for 90 min at 37 °C with eFluor660-conjugated anti-CD107a (Lamp-1) (2 μg/ml) (eBio1D4B; Thermo Fisher Scientific) in 3% BSA in PBS. The cells were washed five times with PBS and sealed in SlowFade™ Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific).

For double immunofluorescent staining for NLRP3 and ASC, fixed and permeabilized cells were blocked for 1 h at room temperature with 1% BSA in PBS. The cells were incubated for 1 h at room temperature with anti-NLRP3 mouse monoclonal Ab (1/300) (Cryo-2; AdipoGen) and anti-ASC rabbit polyclonal Ab (1/200) (AL177; AdipoGen) in 1% BSA in PBS. The cells were then stained with Alexa 647-conjugated anti-mouse IgG Ab and Alexa 594-conjugated anti-rabbit IgG Ab (A21236 and A11012; Thermo Fisher Scientific) for 1 h at room temperature. Confocal images were taken by a confocal laser scanning microscopy system (Nikon A1 and Ti-E, Tokyo, Japan) equipped with a Plan Apo VC 60× objective lens (NA 1.40, Nikon).

Statistical analysis

218 The statistical analyses were performed using the Student's t test. Differences were
219 considered significant at a P value of < 0.05.

RESULTS

IL-1β production by BMMs in response to mycoplasmal lipoprotein/lipopeptide

We have previously shown that *M. salivarium* and *M. pneumoniae* cells induce the IL-1β production by BMMs derived from B6 mice.[27] It is well known that these mycoplasmas are recognized by TLR2.[11-14] Therefore, we first investigated whether the IL-1β-inducing activities of *M. salivarium* and *M. pneumoniae* cells toward BMM were mediated by the TLR2 signaling pathway. BMMs from B6 or TLR2$^{-/-}$ mice (B6BMMs or TLR2$^{-/-}$BMMs) were stimulated for 4 h with ultrapure LPS of *E. coli* and then for 24 h with these mycoplasma cells, because LPS priming significantly enhanced the IL-1β-inducing activities of these mycoplasma cells as shown previously.[27] In this study, we measured IL-1α and IL-18 as well as IL-1β in the culture supernatant. We found that mycoplasma cells induced secretion of IL-1α (Fig. 1C, D), IL-18 (Fig. 1E, F), and IL-1β (Fig. 1A, B) in a dose-dependent manner by B6BMMs. However, the levels of secretion of these cytokines were significantly attenuated in TLR2$^{-/-}$ BMMs (Fig. 1A–F), suggesting that the mycoplasmal membrane-bound lipoproteins were involved, because they are a representative TLR2 ligand in mycoplasmas.[7-14] Therefore, we prepared membrane-bound lipoproteins from *M. salivarium* and *M. pneumoniae* (MsLP and MpLP, respectively) by Triton X-114 phase separation. BMMs from B6 mice were stimulated with LPS for 4 h and then for 24 h with MsLP or MpLP. The diacylated lipopeptide FSL-1, synthesized based on the N-terminal structure of LP44 from *M. salivarium*[28], was also used as a stimulator, because FSL-1 is directly recognized by TLR2.[11,12,14] In the following studies, only IL-1β was measured, because the amount of IL-1β secreted was higher than IL-1α and IL-18 secretion (Fig. 1). It was demonstrated that MsLP, MpLP, and FSL-1 induced IL-1β secretion by B6BMM (Fig. 2A, B and C).

IL-1β production by BMMs from B6, caspase-1$^{-/-}$, NLRP3$^{-/-}$, or ASC$^{-/-}$ mice in response to mycoplasmal lipoprotein/lipopeptide

We have previously shown that *M. salivarium* and *M. pneumoniae* cells activate the NLRP3 inflammasome to produce IL-1β in murine dendritic cells and macrophages.[27] Therefore, the next experiment was carried out to investigate whether MsLP, MpLP. and FSL-1 activate the NLRP3 inflammasome to produce IL-1β in BMMs. It was found that MsLP, MpLP, and FSL-1 induced IL-1β production by B6BMMs, but not by BMMs from caspase-1-, NLRP3-, or ASC-deficient mice (caspase-1$^{-/-}$, NLRP3$^{-/-}$, or ASC$^{-/-}$ BMMs) (Fig. 3A, B and C). These results suggest that one of the mycoplasmal active entities that activate the NLRP3 inflammasome to induce IL-1β production are lipoproteins/lipopeptides.

Effect of proteinase K on the IL-1β-inducing activity of mycoplasmal lipoproteins

We have previously shown that the production of IL-6 and tumor necrosis factor (TNF)-α by normal human gingival fibroblasts and the human monocytic cell line THP-1 in response to MsLP depends on the N-terminal lipid moiety, but not the proteinous moiety.[28] In addition, the recognition site of mycoplasmal lipoproteins by TLR2/6 is the N-terminal lipopeptide.[12] Therefore, the IL-1β-inducing activities of MsLP and MpLP digested by proteinase K were examined. MsLP and MpLP were clearly digested by proteinase K (Fig. 4A). However, the activities of MsLP and MpLP were not downregulated, but rather upregulated by the proteinase K treatment (Fig 4B), suggesting that the active entities of these lipoproteins were proteinase K-resistant, possibly their N-terminal lipopeptide moieties.

Transfection of mycoplasmal lipopeptide into the cytosol

It has been reported that cytosolic entry of bacteria or bacterial ligands is required for activation of inflammasomes upon infection with various bacteria.[5] Therefore, we investigated whether the mycoplasmal lipopeptide FSL-1 exists in the cytosol of B6BMM by using FITC-labeled FSL-1, and found that FSL-1 does indeed exists within the cytosol (Fig. 5A). In addition, we next tried to investigate whether IL-1β production was enhanced by the artificial delivery of FSL-1 into the cytosol of BMMs with the protein transfection reagent PULSin. It was found that the transfection drastically enhanced the amount of FSL-1 in the cytosol (Fig. 5A) and also enhanced the IL-1β-inducing activity (Fig. 5B). These results suggest that the localization of FSL-1 in the cytosol is an important process leading to the activation of the NLRP3 inflammasome.

The subcellular distribution of internalized lipopeptide

We confirmed that FSL-1 localizes in the cytosol of B6BMM at 4 h after incubation with FSL-1 (Fig. 5A). Previously, we have found that FSL-1 is internalized into a murine macrophage cell line, RAW 264.7 cells in a clathrin-dependent endocytic pathway.[31] Therefore, we wanted to know how FSL-1 translocates into the cytosol of B6BMMs. Firstly, we investigated the time course of the subcellular distribution of internalized FSL-1. B6BMMs were incubated with FITC-FSL-1 and counterstained with the lysosome marker LAMP-1 at several time points (Fig. 6). After 30 min or 1 h of incubation, most of the FSL-1 was found in endosome-like compartments of the cytosol, but did not colocalize with LAMP-1^{+}. However, after 2 h of incubation, some

FSL-1-containing endosomes colocalized with the LAMP-1^+ compartments and some FSL-1 has already localized in the cytosol. The majority of FSL-1 existed in the cytosol after 5 h incubation, although small part of FSL-1 still existed in both the LAMP-1^+ or LAMP-1^- compartments. These results suggest that BMMs internalize FSL-1 at least 30 min after incubation and FSL-1-containing endosomes start to fuse with the lysosomes around 2 h, then FSL-1 moves to cytosol from LAMP-1^+ endosome by unknown mechanisms.

Formation of the NLRP3/ASC speck in response to mycoplasmal lipopeptide

Upon activation by nigericin or ATP as the NLRP3 stimulators, NLRP3 forms a inflammasome complex with the adaptor molecule ASC and the complex is visualized by the appearance of large aggregates called 'speck' or 'pyroptosome' [32-35]. Therefore, the next experiment was carried out to investigate whether FSL-1 induces and is colocalized with the NLRP3/ASC speck. It was found that FSL-1 as well as the representative NLRP3 inflammasome stimulator nigericin induced the NLRP3/ASC speck, but FSL-1 was not colocalized with the speck (Fig. 7).

DISCUSSION

This study demonstrated that one of the active entities of *M. salivarium* and *M. pneumoniae* capable of stimulating the NLRP3 inflammasome to produce IL-1β in murine BMMs are their membrane-bound lipoproteins (Fig 2, 3). Consistent with our results, the ability of mycoplasmal lipoproteins and lipopeptides to induce the secretion of proinflammatory cytokines including IL-1β, TNF-α, and IL-6 has been reported.[36] We also showed that the active entities which stimulate the NLRP3 inflammasome were

316 proteinase K-resistant, and were possibly their N-terminal lipopeptide moieties (Fig 4A,
317 B). Several lines of evidence indicate that TLR2 recognizes the N-terminal lipopeptide
318 moiety of a variety of microbial lipoproteins.[8-10,12,13,28,37-41] Taken together, these
319 findings indicate that lipoproteins have immunostimulating activities, through the
320 activation of NLRP3 inflammasome, as well as TLR2, and that these activities are
321 attributed to their N-terminal lipopeptide moieties.
322 The activation of inflammasomes by microbial lipoproteins and lipopeptides has
323 been reported previously, yet the precise mechanisms have remained unclear. In
324 particular, a recent report by Khare *et al.*[23] demonstrated that synthetic microbial
325 lipopeptides, diacylated lipopeptides such as FSL-1 and MALP-2 derived from
326 mycoplasmas, Pam_2CSK_4, and triacylated Pam_3CSK_4, induce IL-1β and IL-18 through
327 the NLRP7 inflammasome in human macrophages. It is known that there are differences
328 in the repertoire of human and mouse NLRs, and that NLRP7 is not present in the
329 mouse genome.[2] Another report by Kanneganti *et al.*[42] showed that P2X7-mediated
330 large pore formation by pannexin-1 allows extracellular PAMPs including Pam_3CSK_4 to
331 access the cytosol in ATP-pulsed murine BMMs, which in turn activates the NLRP3
332 inflammasome. In addition, Muñoz-Planillo *et al.*[43] showed that lipoproteins released
333 from *Staphylococcus aureus* activate the NLRP3 inflammasome in combination with
334 hemolysins also secreted by the bacterium, in murine BMMs. They found that the
335 activity did not depend on the ATP-P2X7 receptor axis, but on K^+ efflux caused by
336 hemolysins. That is, they considered that cell membrane permeation by hemolysins may
337 allow the cytosolic delivery of lipoproteins. In our study, the addition of FSL-1 to
338 BMMs resulted in internalization of FSL-1 into the cytosol and secretion of IL-1β (Fig
339 5). In addition, we also showed that the artificial delivery of FSL-1 into the cytosol of

BMM with the protein transfection reagent PULSin drastically enhanced the IL-1β-inducing activity (Fig 5B), suggesting that translocation of FSL-1 into the cytosol is an important process leading to the activation of NLRP3 inflammasome. Consistent with these results, Shimizu *et al.*[22] showed that the artificial delivery of lipopeptides, such as FAM20 derived from *M. pneumoniae*, and Pam_3CSK_4, into the cytosol of a human monocytic cell line, THP-1, by lipofection significantly augmented the release levels of IL-1β. It is thus conceivable that the cytosolic localization of microbial lipoproteins and lipopeptides is a key event sensed by inflammasomes.

We have previously found that FSL-1 is internalized into a murine macrophage cell line, RAW 264.7, via a clathrin-dependent endocytic pathway.[31] Consistent with our result, Motoi *et al.*[44] also have shown that FSL-1 and Pam_3CSK_4 are uptaken by dynamin, a key component of clathrin-coated pits[45], dependent endocytosis in murine BMM and bone marrow-derived conventional dendritic cells. In addition, these two reports have shown that the endosome containing lipopeptides then fuses with lysosomes. However, it remains unclear how microbial lipoproteins and lipopeptides translocate into the cytosol across the endosomal membrane. To address this question, first of all, we investigated the time course of subcellular distribution of internalized FSL-1. We found that BMM internalized FSL-1 at least 30 min after incubation and FSL-1-containing endosomes started to fuse with the lysosomes around 2 h, then FSL-1 translocated into the cytosol from $LAMP\text{-}1^+$ endosomes by unknown mechanisms (Fig 6).

Recently, several mechanisms for the entry of microbial ligands to the host cytosol have been reported.[46] For example, Nakamura *et al.*[47] demonstrated that the NOD2 ligand muramyl dipeptide (MDP) is transported from endosomes into the cytosol

through endosomal membrane peptide transporters, solute carrier (SLC)15A3, and SLC15A4 in dendritic cells and macrophages. In addition, recent studies reported that LPS is released from gram-negative bacterium-containing vacuoles into the cytosol by guanylate-binding protein-mediated lysis of the vacuoles and then directly binds to pro-caspase-11, leading to non-canonical inflammasome activation in macrophages.[48-50] The cytosolic entry mechanisms of microbial components, such as MDP and LPS, might be involved in those of lipoproteins and lipopeptides. Therefore, studies are in progress to address this possibility.

It has been reported that the NLRP3 inflammasome activators such as nigericin or ATP induce the formation of a inflammasome complex with the adaptor molecule ASC in cytosol of mammalian cells, which is visualized by the appearance of large aggregates called ‘speck’ or ‘pyroptosome’ [32-35]. Therefore, we also investigated whether FSL-1 induces and is colocalized with the NLRP3/ASC speck and found that FSL-1 as well as the representative NLRP3 inflammasome stimulator nigericin induced the NLRP3/ASC speck (Fig. 7). However, FSL-1 was not colocalized with the speck (Fig. 7) as most of NLRP3 agonist. Multiple indirect mechanisms have been proposed for the activation of NLRP3 inflammasome, including K^+ efflux due to pore formation by bacterial toxins, ATP release, lysosomal destabilization, changes in intracellular calcium levels, the release of oxidized mitochondrial DNA, generation of reactive oxygen species, or mitochondrial dysfunction.[2-5] Recently, in addition to these indirect mechanisms, Bose *et al.* have shown that community-acquired respiratory distress syndrome toxin, which is an ADP-ribosylating and vacuolating toxin produced by *M. pneumoniae*, activates the NLRP3 inflammasome by ADP-ribosylation.[25]

Considering these reports and our results, it is likely that after clathrin-mediated

endocytosis, the endosomes containing lipoproteins and lipopeptides fuse with lysosomes, and then lipoproteins and lipopeptides translocate into the cytosol by unknown mechanisms, which leads to the NLRP3 inflammasome activation. Further studies are needed to elucidate the mechanisms by which lipoproteins and lipopeptides enter the cytosol and activate the NLRP3 inflammasome.

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539 **Figure legends**

540 **Fig. 1** IL-1α, IL-1β and IL-18 production by B6BMM or TLR2$^{-/-}$BMM in response to
541 mycoplasma cells.
542 BMMs from B6 or TLR2$^{-/-}$ mice were stimulated with LPS (10 ng/ml) for 4 h and then
543 for 24 h with live cells of *Mycoplasma salivarium* (Ms) (A, C, E) or *Mycoplasma*
544 *pneumoniae* (Mp) (B, D, F) (i.e. 0, 36.5, 365 μg/ml protein). The amounts of total IL-1β
545 (A, B), IL-1α (C, D), or IL-18 (E, F) released into the culture supernatant were
546 measured by ELISA. The results are expressed as the mean ±SD of triplicate assays of a
547 representative experiment. All of the experiments were repeated at least twice, and
548 similar results were obtained. Mature IL-1β (mIL-1β) was evaluated by molecular
549 weight (mIL-1β: 17.5 kDa, pro-IL-1β: 31 kDa) of the band detected by Western blotting
550 (A, B). Student's t-test; *$0.01 < P < 0.05$, **$0.01 < P < 0.001$, ***$P < 0.001$.
551
552 **Fig. 2** IL-1β production by B6BMMs in response to MsLP, MpLP, and FSL-1.
553 B6BMMs were stimulated with LPS (10 ng/ml) for 4 h and then for 24 h with MsLP (A)
554 or MpLP (B) (i.e. 0, 0.4, 4 μg/ml protein) or FSL-1 (i.e. 0, 10, 100 nM) (C). The
555 amounts of total IL-1β released into the culture supernatant were measured by ELISA.
556 The results are expressed as the mean ±SD of triplicate assays of a representative
557 experiment. All of the experiments were repeated at least twice, and similar results were
558 obtained. Mature IL-1β (mIL-1β) was evaluated by molecular weight (mIL-1β: 17.5
559 kDa, pro-IL-1β: 31 kDa) of the band detected by Western blotting. Student's t-test;
560 *$0.01 < P < 0.05$, **$0.01 < P < 0.001$.
561
562 **Fig. 3** IL-1β production by BMMs from B6, caspase-1$^{-/-}$, NLRP3$^{-/-}$, or ASC$^{-/-}$ mice in

response to MsLP, MpLP, and FSL-1.

BMMs from B6, caspase-$1^{-/-}$, $NLRP3^{-/-}$, or $ASC^{-/-}$ mice were stimulated with LPS (10 ng/ml) for 4 h and then for 24 h with MsLP (i.e. 0, 0.4, 4 μg/ml protein) (A) or MpLP (i.e. 0, 0.4, 4 μg/ml protein) (B) or FSL-1 (i.e. 0, 10, 100 nM) (C) The amounts of total 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; *0.01 < P < 0.05, **0.01 < P < 0.001, ***P < 0.001.

Fig. 4 Effect of proteinase K on the IL-1β-inducing activity of MsLP and MpLP.

MsLP or MpLP suspension (400 μg/ml of PBS) was pretreated at 37 °C for 1 h with proteinase K (0, 0.8, 4, 40 μg /ml), and then boiled for 10 min to inactivate the enzyme. MsLP or MpLP (i.e. 4 μg protein) digested by proteinase K was run in 10% gel and stained using a Silver Stain kit (A). Precision Plus Protein™ All Blue Standards (M lane) were used to estimate molecular weights. B6BMMs were stimulated with LPS (10 ng/ml) for 4 h and then for 24 h with MsLP or MpLP (i.e. 0, 4 μg/ml protein) digested by proteinase K (B). The amounts of total 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.

Fig. 5 Induction of IL-1β by cytosolic FSL-1 in B6BMM.

B6BMMs were stimulated with LPS (10 ng/ml) for 4 h and then cultured with or without (None) 2 μg/ml of FITC-FSL-1 in the absence (-) or the presence (+) of PULSin.

After incubation for 4 h, the cells were fixed, and observed by using a confocal microscope. FITC-FSL-1 (green) and differential interference contrast (DIC) were shown separately. The merged images with FITC-FSL-1 and DIC were also shown. Scale bar indicates 10 μm (A). B6BMMs were stimulated with LPS (10 ng/ml) for 4 h and then cultured with (+) or without (-) FITC-FSL-1 (i.e. 0, 100 nM) in the absence (-) or the presence (+) of PULSin. After incubation for 24 h, the amounts of total 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; *$0.01 < P < 0.05$.

Fig. 6 The subcellular distribution of internalized FSL-1 in B6BMM

B6BMMs were stimulated with LPS (10 ng/ml) for 4 h and then for various periods of time (30 min, 1, 2, 5 h) with 7 μg/ml of FITC-FSL-1 (green). The cells were immunostained with eFluor660-conjugated anti-LAMP-1 antibody (red). Cell nuclei were stained with DAPI (blue). Samples were observed by using a confocal microscope. The merged images with eFluor660 Lamp-1, FITC-FSL-1, and DAPI are shown. Differential interference contrast (DIC) are also shown. White arrows indicate colocalization of FSL-1-containing endosomes with the LAMP-1^+ compartments. Scale bar indicates 10 μm. The experiments were repeated at least twice, and similar results were obtained.

Fig. 7 Formation of NLRP3 /ASC specks in response to FSL-1 in B6BMM

B6BMMs were stimulated with LPS (10 ng/ml) for 4 h and then for24 h with 7 μg/ml of

611 FITC-FSL-1 (green). The immunofluorescent staining for NLRP3 (blue) and ASC (red)
612 was carried out. Samples were observed by using a confocal microscope. The merged
613 images with NLRP3, ASC, and FITC-FSL-1 are shown. Differential interference
614 contrast (DIC) are also shown. White arrows indicate colocalization of NLRP3 with
615 ASC (specks). Scale bar indicates 10 μm. The experiments were repeated at least twice,
616 and similar results were obtained.
617

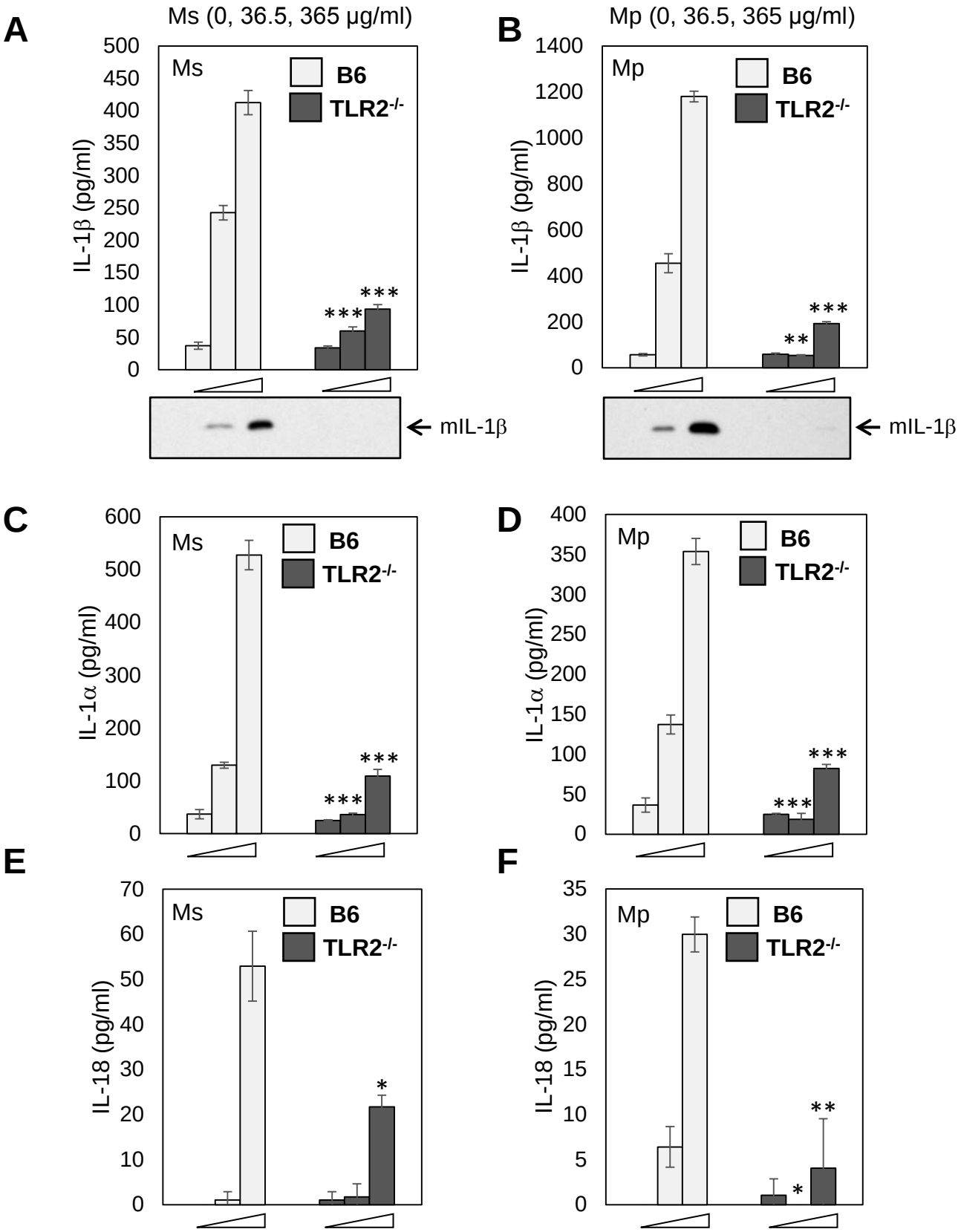

A
Ms (0, 36.5, 365 μg/ml)
Ms
B6
TLR2-/-
IL-1β (pg/ml)
mIL-1β
B
Mp (0, 36.5, 365 μg/ml)
Mp
C
IL-1α (pg/ml)
D
E
IL-18 (pg/ml)
F

Fig. 1

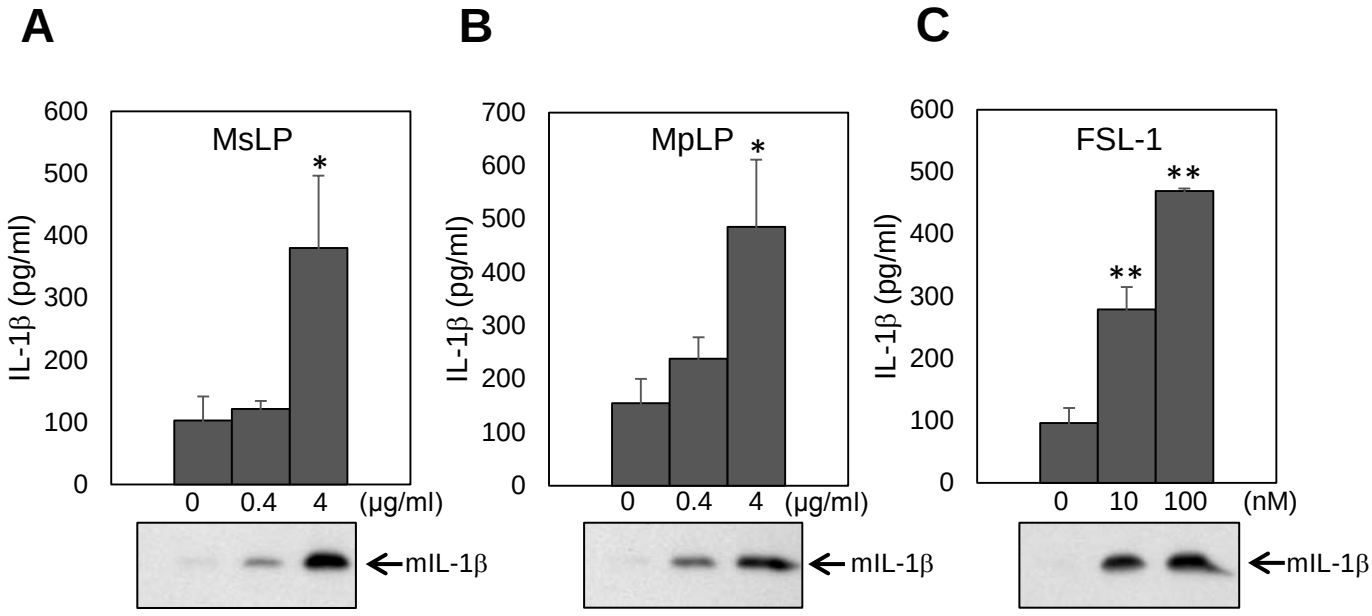
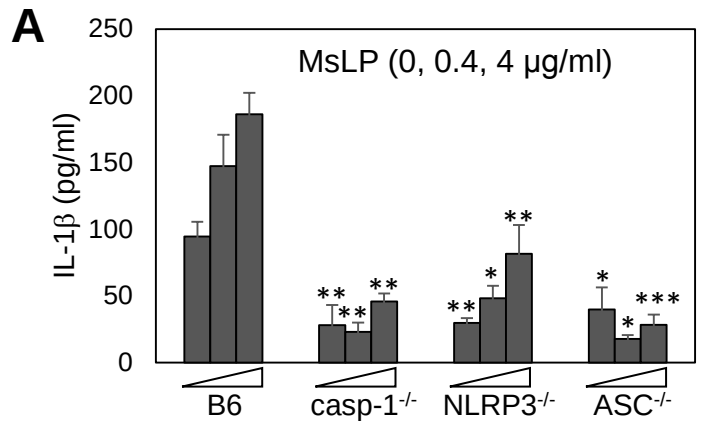

A
B
C
MsLP
MpLP
FSL-1
IL-1β (pg/ml)
600
500
400
300
200
100
0
700
*
**
0
0.4
4
(μg/ml)
10
100
(nM)
mIL-1β

Fig. 2


A
MsLP (0, 0.4, 4 μg/ml)
IL-1β (pg/ml)
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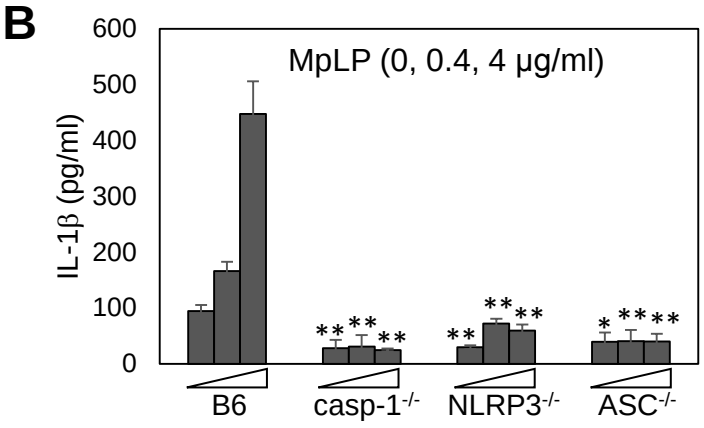
200
150
100
50
0
B6
casp-1-/-
NLRP3-/-
ASC-/-


B
MpLP (0, 0.4, 4 μg/ml)
IL-1β (pg/ml)
600
500
400
300
200
100
0
B6
casp-1-/-
NLRP3-/-
ASC-/-

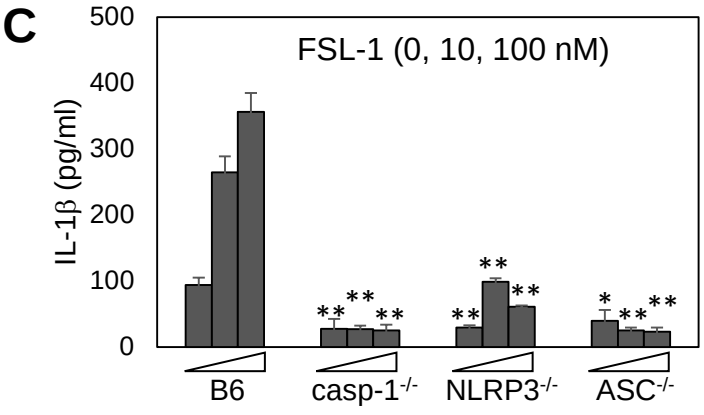
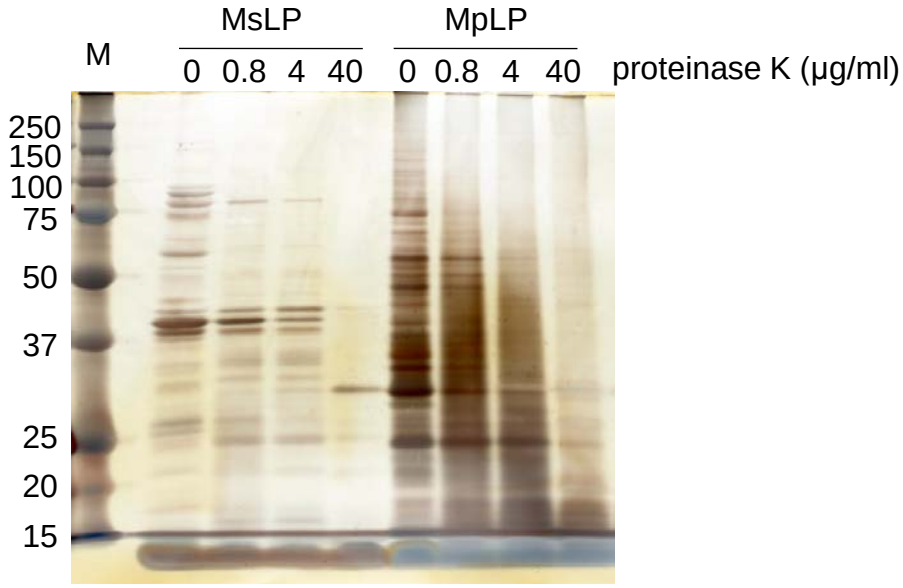

C
FSL-1 (0, 10, 100 nM)
IL-1β (pg/ml)
500
400
300
200
100
0
B6
casp-1-/-
NLRP3-/-
ASC-/-

Fig. 3

A


MsLP
MpLP
M
0 0.8 4 40
0 0.8 4 40
proteinase K (μg/ml)
250
150
100
75
50
37
25
20
15

B

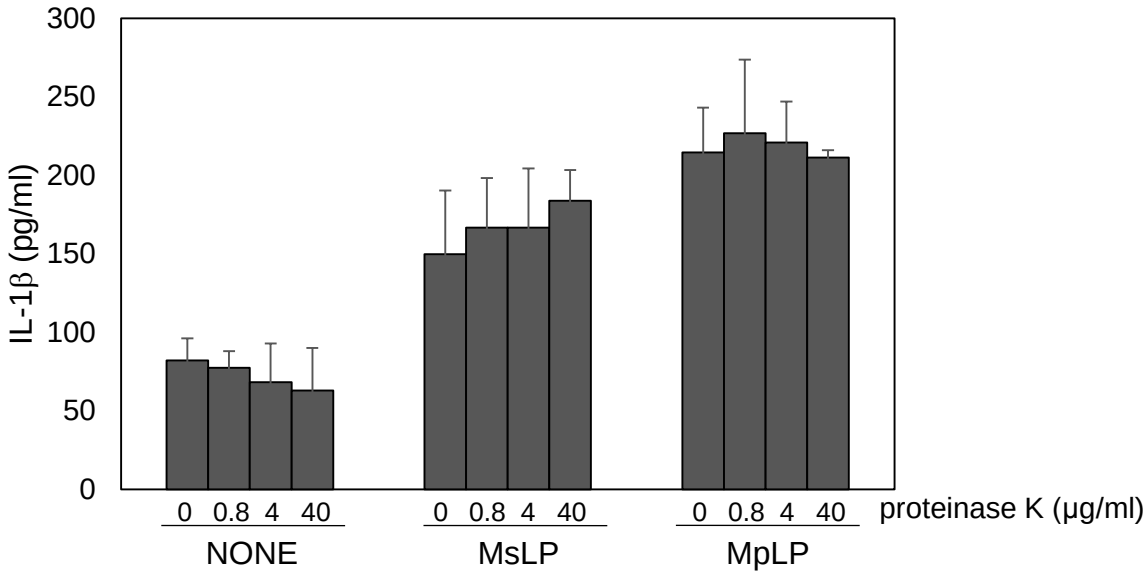
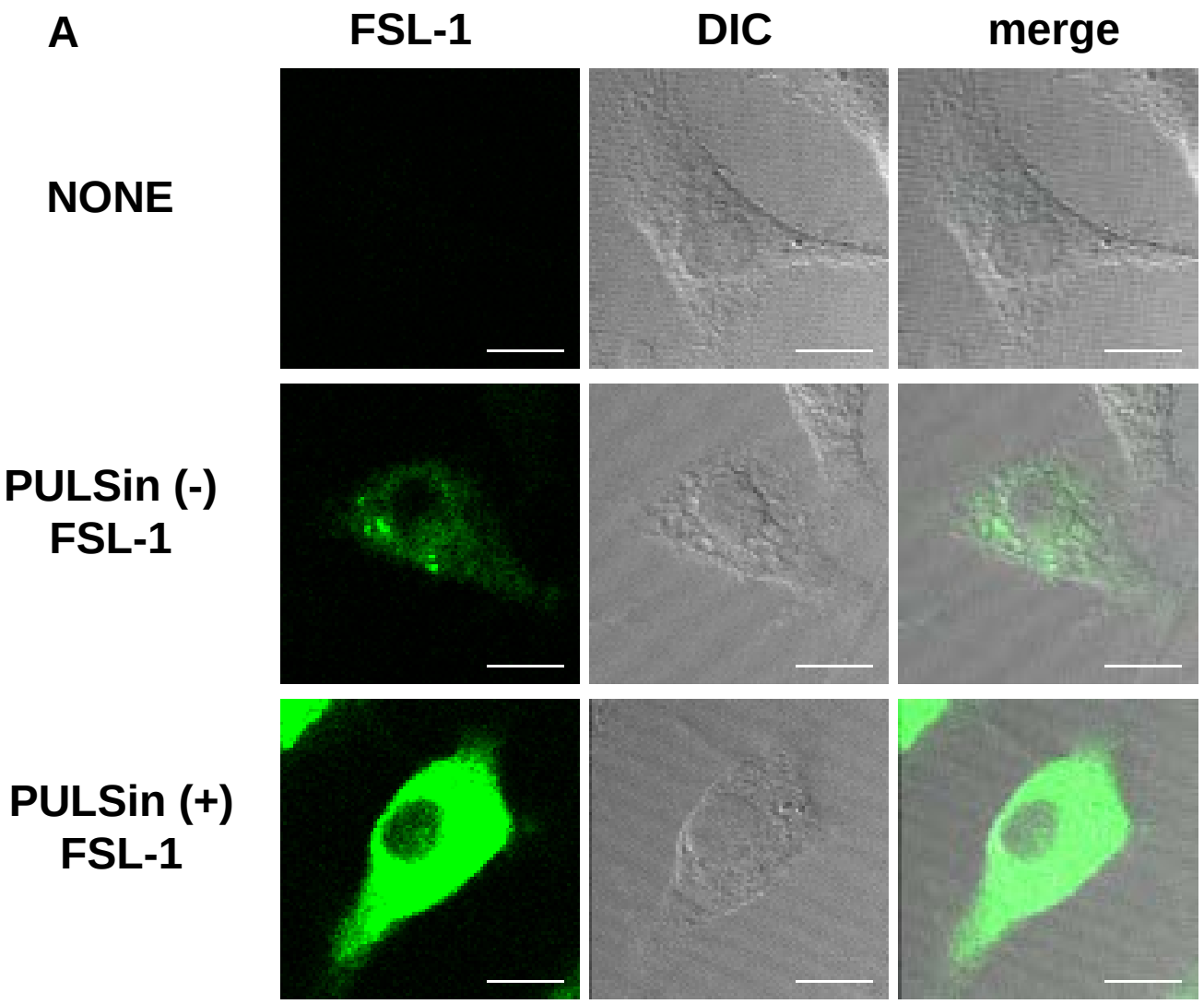

IL-1β (pg/ml)
300
250
200
150
100
50
0
0 0.8 4 40
0 0.8 4 40
0 0.8 4 40
proteinase K (μg/ml)
NONE
MsLP
MpLP

Fig. 4


A
FSL-1
DIC
merge
NONE
PULSin (-)
FSL-1
PULSin (+)
FSL-1

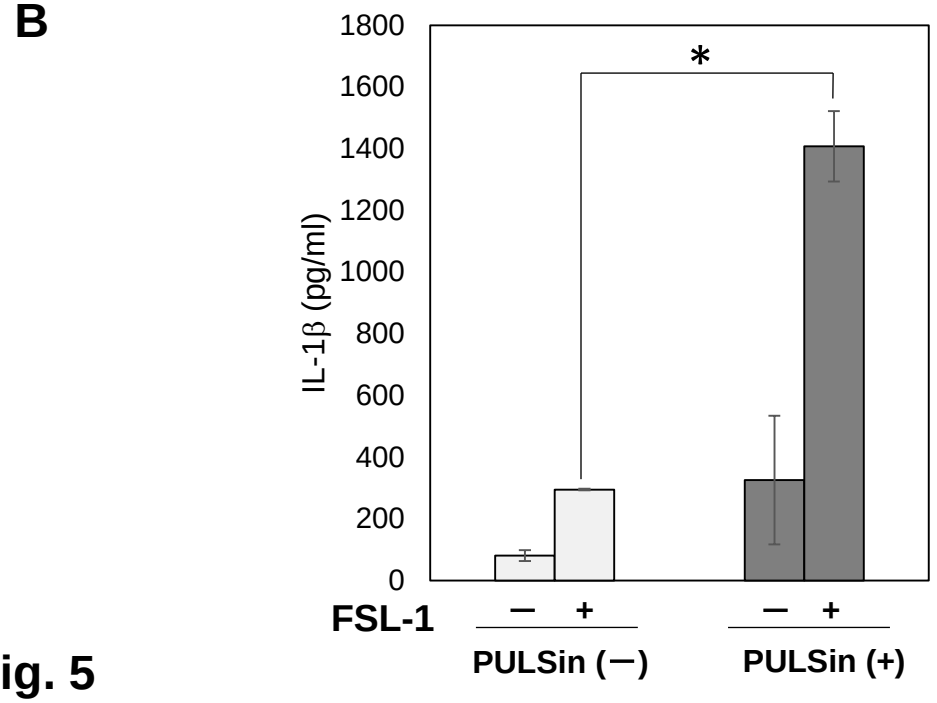

B
1800
1600
1400
1200
1000
800
600
400
200
0
IL-1β (pg/ml)
*
FSL-1
−
+
−
+
PULSin (−)
PULSin (+)

Fig. 5

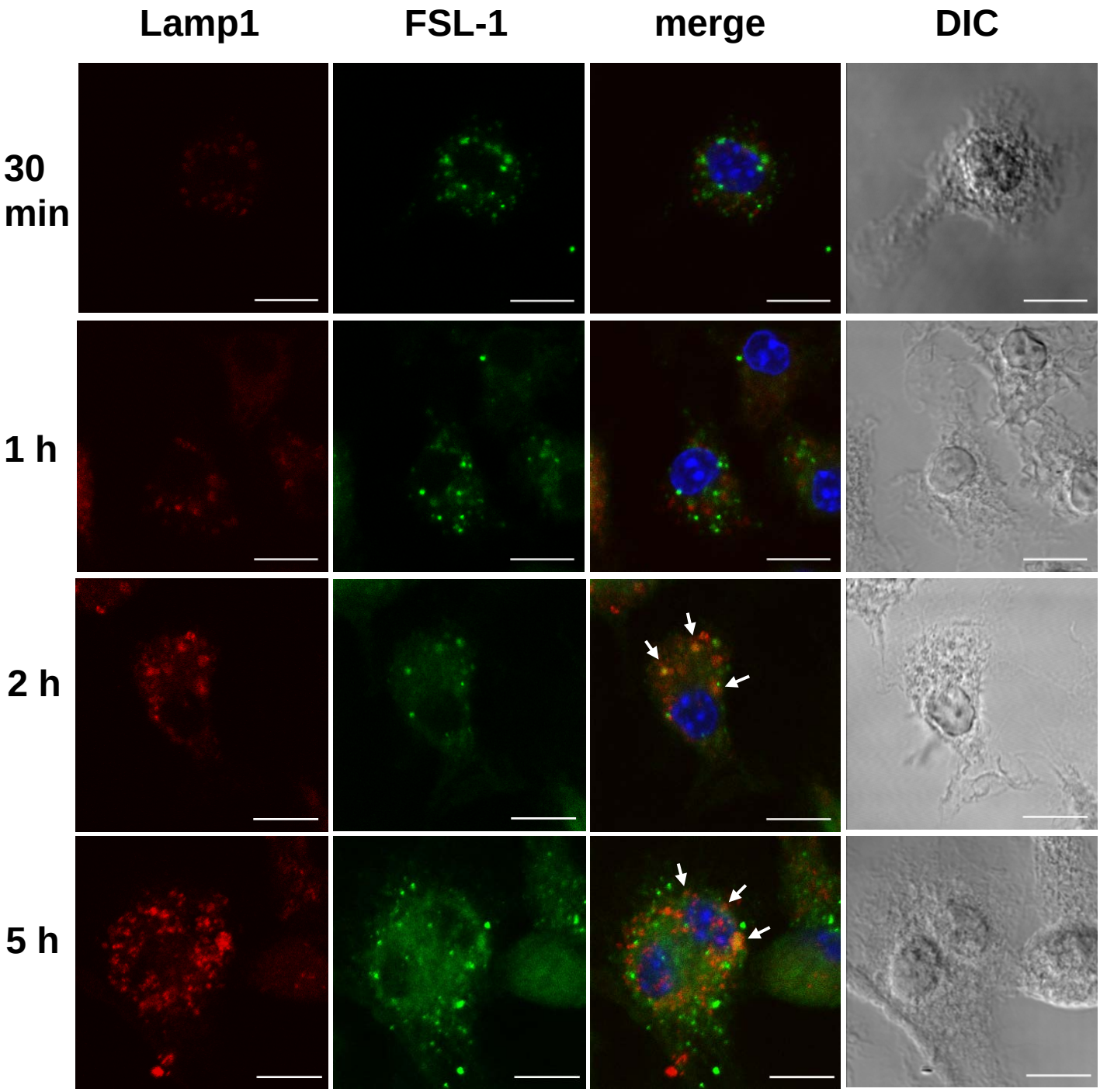


Fig. 6

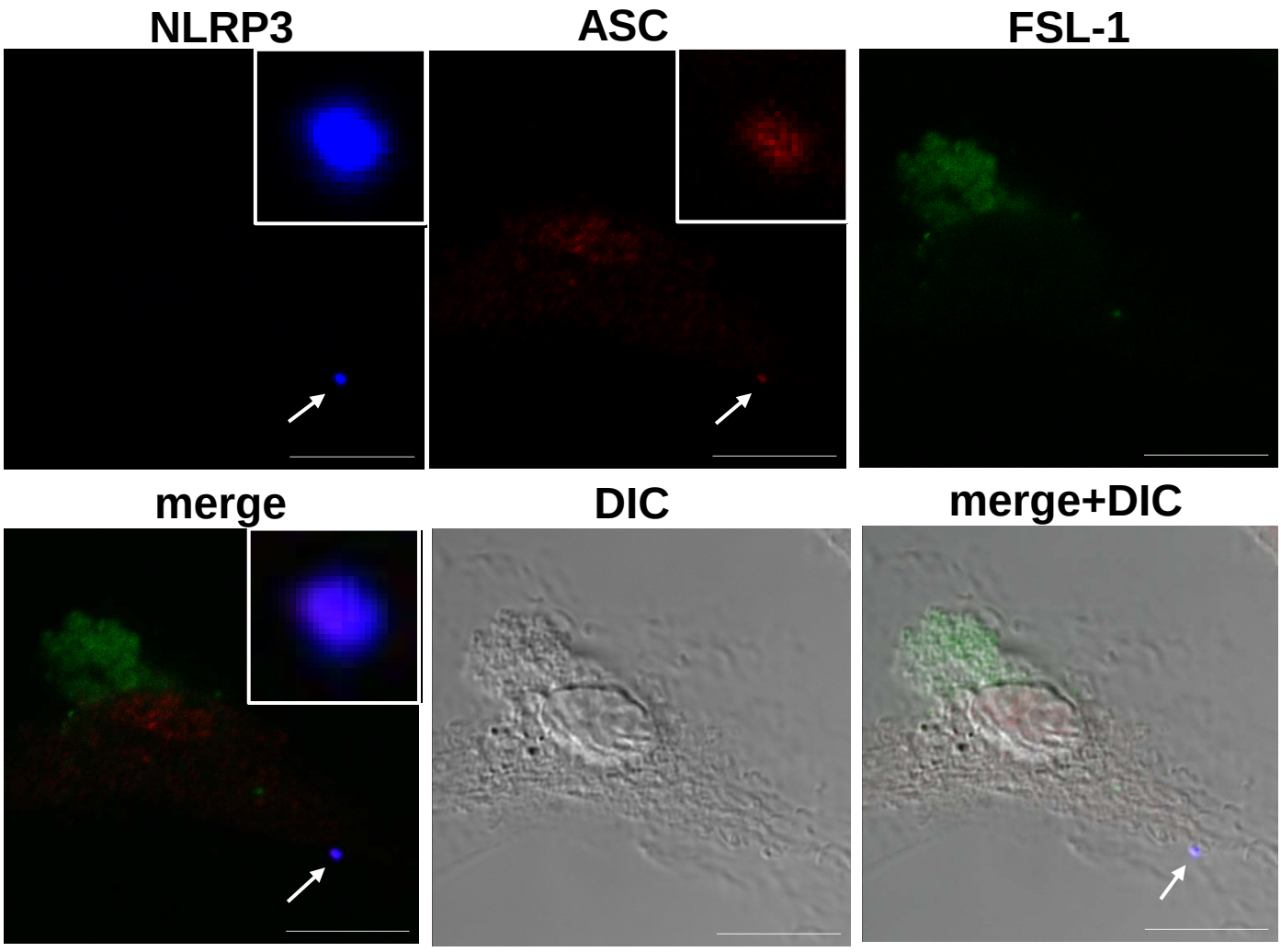


Fig. 7