Gasdermin D-independent release of interleukin-1β by living macrophages in response to mycoplasmal lipoproteins and lipopeptides

Short title: Mechanism of lipoprotein-induced IL-1β release

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Abbreviations:

ASC, adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain

BMM, bone marrow-derived macrophage

FBS, fetal bovine serum

GSDMD, gasdermin D

IL-1β, interleukin-1β

LDH, lactate dehydrogenase

LPS;, lipopolysaccharide

MpLP, *Mycoplasma. pneumoniae* lipoproteins

MsLP, *Mycoplasma salivarium* lipoproteins

NLR, nucleotide-binding oligomerization domain-like receptor

NLRP3, nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3

PBS, phosphate-buffered saline

PIP2, phosphatidylinositol-4,5-bisphosphate

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Summary

Interleukin-1β (IL-1β) plays pivotal roles in controlling bacterial infections and is produced after the processing of pro-IL-1β by caspase-1 which is activated by the inflammasome. In addition, caspase-1 cleaves the cytosolic protein, gasdermin-D (GSDMD), whose N-terminal fragment subsequently forms a pore in the plasma membrane, leading to the pyroptic cell death-mediated release of IL-1β. Living cells can also release IL-1β via GSDMD pores or other unconventional secretory pathways. However, the precise mechanisms are poorly defined. Here, we show that lipoproteins from *Mycoplasma salivarium* (MsLP) and *Mycoplasma pneumoniae* (MpLP) and an *M. salivarium*-derived lipopeptide (FSL-1), which are activators of the nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome, induce IL-1β release from mouse bone marrow-derived macrophages (BMMs) without inducing cell death. The levels of IL-1β release induced by MsLP, MpLP and FSL-1 were more than 100 times lower than those induced by the canonical NLRP3 activator nigericin. The IL-1β release-inducing activities of MsLP, MpLP and FSL-1 were not attenuated in BMMs from GSDMD-deficient mice. Furthermore, both active caspase-1 and cleaved GSDMD were detected in response to transfection of FSL-1 into the cytosol of BMMs, but the release of IL-1β was unaffected by GSDMD deficiency. Meanwhile, punicalagin, a membrane-stabilizing agent, drastically downregulated the release of IL-1β in response to FSL-1. These results suggest that mycoplasmal lipoprotein/lipopeptide-induced IL-1β release by living macrophages is not mediated via GSDMD but rather through changes in membrane permeability.

Introduction

Interleukin-1β (IL-1β) plays important roles in controlling bacterial infections by inducing inflammation and recruiting and activating immune cells.[1] Unlike most cytokines, IL-1β lacks a signal peptide and is therefore not secreted through the conventional endoplasmic reticulum–Golgi protein secretion route,[2] although the precise mechanisms by which it is secreted are poorly understood.

IL-1β is produced and released into the extracellular space as a result of the processing of its inactive precursor, pro-IL-1β, by caspase-1, which is activated by the intracellular multiprotein complex known as the inflammasome. Canonical inflammasomes typically consist of a nucleotide-binding oligomerization domain-like receptor (NLR), adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and procaspase-1. Among the several types of inflammasomes, the NLR family pyrin domain containing-3 (NLRP3) inflammasome is the most characterized and is activated in response to a broad spectrum of stimuli including microbial products.[3-8] Moreover, there are non-canonical inflammasomes that are activated by lipopolysaccharide (LPS) from gram-negative bacteria in the cytoplasm, which activate murine caspase-11 (i.e., human caspase-4 and caspase-5).[9, 10]

Caspase-1 and caspase-11/4/5, which are activated by canonical and non-canonical inflammasomes, respectively, trigger a pro-inflammatory form of cell death called pyroptosis, which releases bioactive IL-1β into the extracellular space.[11, 12] These caspases were recently shown to cleave the cytosolic protein, gasdermin D (GSDMD). Upon cleavage, the N-terminal fragment of GSDMD forms a pore in the plasma membrane that alters osmotic pressure, which leads to cell lysis and the rapid release of cellular contents. Therefore, pyroptosis has been redefined as GSDMD-mediated

programmed necrosis.[8, 13-19]

Living cells have also been reported to release IL-1β.[20-30] The cells that release IL-1β in the absence of cell death are considered hyperactive cells.[31] For example, a microbial-hyperactivating stimulus, the *N*-acetyl glucosamine component of bacterial peptidoglycan, induces NLRP3 inflammasome-mediated IL-1β release from living macrophages.[24, 27] Furthermore, IL-1β secretion from living cells can be promoted by GSDMD pores[27, 28] or via mechanisms independent of GSDMD.[29, 32] Furthermore, the secretory mechanism of IL-1β was recently shown to depend on alteration of membrane permeability.[29, 33, 34]

We previously found that membrane-bound lipoproteins from *Mycoplasma salivarium* (MsLP) and *Mycoplasma pneumoniae* (MpLP) as well as FSL-1, a lipopeptide derived from *M. salivarium*, activate the NLRP3 inflammasome to produce IL-1β in murine bone marrow-derived macrophages (BMMs).[35] However, the secretory mechanisms involved remain elusive.

Here, we show that these mycoplasmal lipoproteins/lipopeptides induce IL-1β release by non-pyroptosing living BMMs via mechanisms dependent on plasma membrane permeabilization but not GSDMD.

Materials and methods

Reagents

FSL-1, a diacylated lipopeptide derived from *M. salivarium*, was synthesized as previously described.[36] 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). Nigericin and punicalagin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycine was purchased from Promega (Madison, WI, USA).

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 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. Protein concentration was determined by using a DC Protein Assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

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.[37] 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. Protein concentration was determined by using a DC Protein Assay kit (Bio-Rad) according to the manufacturer's instructions.

Mice

Wild-type C57BL/6 (WT) mice were purchased from CLEA Japan (Tokyo, Japan) and maintained in specific pathogen-free conditions at the Animal Facility of Hokkaido University. GSDMD-deficient ($GSDMD^{-/-}$) mice of the same genetic background[38] were kindly provided by Dr. Toshihiko Shiroishi (RIKEN BioResource Research Center) and maintained in specific pathogen-free conditions at the animal facility of Kanazawa University. All experiments were performed in accordance with the regulations of the Animal Care and Use Committees of Hokkaido University (14-0066, 19-0059) and Kanazawa University (AP-173853).

Cell culture of BMMs

Bone marrow cells were prepared from the femurs and tibias of $GSDMD^{-/-}$ mice at Kanazawa University and sent to Hokkaido University for analysis. Bone marrow cells from the femurs and tibias of WT mice were prepared at Hokkaido University.

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 by using cold PBS and then used as BMMs.

IL-1β measurement

BMMs from WT or $GSDMD^{-/-}$ mice were added to a 24-well plate at 4×10^5 cells/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 subsequently resuspended in 300 μL RPMI 1640 basal medium and incubated at 37°C with MsLP or MpLP (0, 0.4, or 4 μg/mL protein), FSL-1(0, 10, or 100 nM), or nigericin (5 μM; a representative pyroptotic NLRP3 inflammasome stimulator used as a positive control). Incubation times are indicated in figures and figure legends. IL-1β in cell culture supernatants was quantified by using ELISA kits for IL-1β (OptEIA™ SET Mouse IL-1β, BD Biosciences, San Jose, CA, USA).

LDH release assay

The LDH released from cells into culture supernatants was measured using a CytoTox 96 kit (Promega) according to the manufacturer's instructions. Cytotoxicity was calculated against the maximum release of LDH, which was obtained by cell lysis with 0.09% Triton X-100. The percentage of cytotoxicity was calculated as LDH released in [tested sample (A490)/maximum LDH release (A490)] × 100.

FSL-1 transfection, immunoblot analysis, and ELISA

BMMs from WT mice were added to a 6-well plate at 1.6×10^6 cells/well in 2 mL RPMI 1640 medium containing 10% 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 900 μL medium. Next, 100 μL FSL-1 solution (1 μM) dissolved in 20 mM HEPES buffer was mixed with 1 μL PULSin reagent (Polyplus-Tranfection, Illkirch, France) and added to the appropriate wells after 15 min of incubation. After 5 h of incubation at 37°C, the cells were lysed in cell lysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% [w/v] IGEPAL® CA-630 [Sigma-Aldrich], and complete

protease inhibitors [Roche, Mannheim, Germany]) and combined with the supernatant precipitated with 6% TCA.

BMMs from WT mice were added to a 6-well plate at 1.6×10^6 cells per well in 2 mL RPMI 1640 medium containing 10% FBS and incubated at 37°C for 4 h with 10 ng/mL ultrapure *E. coli* LPS. The cells were resuspended in 1 mL RPMI 1640 basal medium and incubated with 5 μM nigericin at 37°C for 1 h. The cells were then lysed in cell lysis buffer and combined with the supernatant precipitated with 6% TCA. The samples were treated with SDS sample buffer and submitted to SDS-PAGE analysis. Proteins were transferred to a PVDF membrane (Bio-Rad) and reacted with rabbit anti-mouse caspase-1 antibody (sc-514, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-mouse GSDMD antibody (ab209845, Abcam, Cambridge, UK), goat anti-mouse IL-1β antibody (AF-401, R&D Systems, Minneapolis, MN, USA), and anti–β-actin (AC-15, Sigma-Aldrich).

To assess the release of IL-1β, BMMs from WT or $GSDMD^{-/-}$ mice were added to a 24-well plate at 4×10^5 cells/well in 500 μL RPMI 1640 medium containing 10% (vol/vol) FBS and incubated with 10 ng/mL ultrapure *E. coli* LPS at 37°C for 4 h. The cells were washed with RPMI 1640 basal medium and resuspended in 270 μL medium. Then, 30 μL FSL-1 solution (1 μM) dissolved in 20 mM HEPES buffer was mixed with 0.3 μL PULSin reagent and added to the appropriate wells after 15 min 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).

Localization of FSL-1 in the cytosol

BMMs from WT mice were added to a 6-well plate at 8×10^5 cells/well in 2 mL RPMI 1640 medium containing 10% FBS and incubated at 37°C for 4 h with 10 ng/mL ultrapure *E. coli* LPS. The cells were resuspended in 1 mL RPMI-1640 basal medium and incubated at 37°C for 2 h with 2 μg/mL of FITC-FSL-1 in the absence or the presence of punicalagin (25 μM). After being washed 3 times with PBS, the cells were fixed for 15 min with 4% paraformaldehyde solution (Nacalai Tesque, Kyoto, Japan), and then washed 3 times 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).

Statistical analysis

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

Results

IL-1β release by BMMs in response to mycoplasmal lipoproteins/lipopeptides is independent of cell death

We previously showed that MsLP, MpLP and FSL-1 activate the NLRP3 inflammasome to produce IL-1β in BMMs.[35] Inflammasome-dependent cell death, i.e., pyroptosis, is a mechanism that involves IL-1β release into the extracellular space.[11, 12] Therefore, to determine if MsLP, MpLP or FSL-1 induces pyroptosis in BMMs, the LDH released into the culture supernatants was quantified. Nigericin, a representative

pyroptotic NLRP3 inflammasome stimulator, was used as a positive control. Concordant with previous studies,[27, 29] 1 h of stimulation with LPS-primed BMMs with nigericin resulted in the release of extremely large quantities of IL-1β as well as cell death (Fig. 1a). The cell death was not essential for the release of IL-1β, because the cytoprotective agent glycine downregulated the LDH release in a dose-dependent manner but not the IL-1β release. However, FSL-1 induced less release of IL-1β, but the quantities increased significantly in a linear fashion up to 24 h after stimulation without inducing cell death (Fig. 1b). Like FSL-1, MsLP and MpLP also induced the release of similar quantities of IL-1β without inducing cell death (Fig. 1c). Therefore, in the subsequent experiments, the supernatants of MsLP, MpLP and FSL-1–treated BMMs after LPS priming were examined at 24 h unless otherwise noted.

These results suggest that stimulation with nigericin rapidly induces substantial IL-1β release by BMMs with the induction of pyroptosis, whereas stimulation with MsLP, MpLP or FSL-1 slowly induces IL-1β release without inducing cell death. Therefore, the next experiment was performed to determine how MsLP, MpLP and FSL-1 induce IL-1β release by living macrophages.

GSDMD is not required for the IL-1β release by BMMs in response to mycoplasmal lipoproteins/lipopeptides

In addition to cleaving IL-1 family cytokines, active caspase-1 cleaves the cytosolic protein GSDMD. Upon cleavage, the N-terminal fragment of GSDMD forms a pore in the plasma membrane, which leads to induction of pyroptosis.[13-18] Furthermore, recent studies demonstrate that under certain conditions, GSDMD pores do not induce

pyroptosis but rather promote IL-1β secretion from living cells.[27, 28] Therefore, to investigate whether the release of IL-1β by living BMMs in response to MsLP, MpLP or FSL-1 is mediated via GSDMD pores, IL-1β release was examined in BMMs from WT and GSDMD$^{-/-}$ mice. Nigericin was used as a control because it triggers the GSDMD-dependent release of IL-1β from BMMs.[27, 28] Similar to previous findings, in GSDMD$^{-/-}$ BMMs, nigericin did not induce IL-1β or LDH release (Fig. 2a). However, IL-1β release induced by MsLP, MpLP or FSL-1 was not attenuated in GSDMD$^{-/-}$ BMMs (Fig. 2b-d). Furthermore, MsLP, MpLP and FSL-1 did not induce LDH release from WT or GSDMD$^{-/-}$ BMMs (Fig. 2b-d). These results collectively suggest that GSDMD is not required for the release of IL-1β by BMMs in response to mycoplasmal lipoproteins/lipopeptides.

FSL-1 transfection by PULSin cleaves GSDMD

GSDMD is a substrate of both caspase-1 and caspase-11/4/5, which are activated by the inflammasome.[13, 14] Because the processing of pro-IL-1β in response to MsLP, MpLP or FSL-1 is dependent on caspase-1 after the activation of NLRP3 inflammasome,[35] whether GSDMD is cleaved by caspase-1 activated by MsLP, MpLP or FSL-1 was determined. Caspase-1 activation and GSDMD cleavage in cell lysates and culture supernatants were examined by western blotting using anti–caspase-1 and anti-GSDMD antibodies, respectively. Consistent with previous reports,[14, 30, 34, 39, 40] nigericin activated caspase-1 (10 kDa) and cleaved GSDMD (32 kDa) (Fig. 3a). However, the band of active caspase-1 was not detected in FSL-1–treated BMMs under the assay conditions used (data not shown). This was likely attributable to the level of

the IL-1β–inducing activity and the detection sensitivity of western blotting. We previously found that the artificial delivery of FSL-1 into the cytosol of BMMs using the PULSin protein transfection reagent enhances the IL-1β–inducing activity.[35] Accordingly, under this assay condition, both active caspase-1 and cleaved GSDMD as well as IL-1β (17.5 kDa) were detected (Fig. 3a). Therefore, to clarify the involvement of GSDMD cleavage in the induction of IL-1β release by FSL-1, the IL-1β and LDH released into the culture supernatants in both WT and GSDMD$^{-/-}$ BMMs after transfection with FSL-1 at several points in time were quantified. GSDMD deficiency did not affect IL-1β release in response to FSL-1 transfection (Fig. 3b). Moreover, LDH was released from neither WT nor GSDMD$^{-/-}$ BMMs (Fig. 3c). Taken together, these results indicate that GSDMD-independent mechanisms contribute to the IL-1β release from BMMs in response to mycoplasmal lipoproteins/lipopeptides, although they can activate caspase-1 and cleave GSDMD.

Mycoplasmal lipopeptide-induced IL-1β release is dependent on plasma membrane permeabilization

Changes in plasma membrane permeability were recently shown to be required for IL-1β release by both pyroptotic and living cells.[29, 33, 34] Punicalagin is reported to stabilize plasma membrane lipids and consequently inhibits changes in membrane permeability, leading to the blockade of IL-1β release.[33] Herein, punicalagin downregulated nigericin-induced IL-1β and LDH release from BMMs in a dose-dependent manner (Fig. 4a) as previously reported.[29, 33, 34] Furthermore, punicalagin downregulated IL-1β release in response to FSL-1 in a dose-dependent manner (Fig.

4b). To rule out the possibility that the downregulated IL-1β release was due to the reduction of FSL-1 uptake into the cytosol of BMMs by punicalagin, which is an important process leading to the activation of the NLRP3 inflammasome,[35] experiments were carried out to determine whether punicalagin downregulated internalization of FSL-1. BMMs were incubated with FITC-labeled FSL-1 in the absence or presence of punicalagin and it was found that punicalagin had no effects on FSL-1 uptake by BMMs (Fig. 4c). These results suggest that changes in membrane permeability play important roles in IL-1β release from living BMMs.

Discussion

Although various bacterial stimuli can activate the NLRP3 inflammasome to produce IL-1β, cell-fate outcomes are complicated, and there might not be a unified pathway that causes IL-1β release. Some bacterial stimuli, such as nigericin, induce IL-1β release via pyroptotic cell death, whereas others, such as peptidoglycan, induce a hyperactivated state in living cells whereby IL-1β is released through GSDMD pores.[27] The present study shows that mycoplasmal lipoproteins (i.e., MsLP and MpLP) and a lipopeptide (i.e., FSL-1) induce the release of IL-1β by murine BMMs without inducing cell death (Fig. 1b, c). Therefore, we thought the possibility that they are one of hyperactivating stimuli to murine BMMs. However, these mycoplasmal lipoproteins/lipopeptides induced IL-1β release by living murine BMMs independent of GSDMD pores (Fig. 2b-d).

As such, the mechanisms that mediate IL-1β release independent of GSDMD remain poorly understood. Nevertheless, Monteleone et al. recently showed that slow

IL-1β release does not require GSDMD whereas rapid IL-1β release indeed requires GSDMD.[29] They demonstrated that pro-IL-1β cleavage by caspase-1 removes the negatively charged pro-piece, allowing positively charged mature IL-1β to relocate from the cytosol to negatively charged phosphatidylinositol-4,5-bisphosphate (PIP2)-enriched plasma membrane projections and surface ruffles via electrostatic interaction, which leads to the slow release of IL-1β. In the present study, MsLP, MpLP and FSL-1 slowly induced IL-1β release independent of GSDMD over a long period of time (Fig. 1b, 1c, 2b–d). Therefore, the IL-1β release induced by MsLP, MpLP or FSL-1 is likely due to the slow release mechanism described above. Indeed, the membrane-stabilizing agent punicalagin drastically downregulated the IL-1β release in response to FSL-1 (Fig. 4b).

Recent studies have also shown that gasdermin-E (GSDME) is proteolytically activated by caspase-3 during apoptosis, and its N-terminal fragment forms a pore in the plasma membrane, which causes secondary necrosis/pyroptosis and the release of IL-1β .[41, 42] In fact, we have previously reported that mycoplasmal lipoproteins induce caspase-3 activation, which was assessed by cleavage of poly (ADP-ribose) polymerase (PARP), a substrate of caspase-3 in lymphocytic and monocytic cell lines.[43-45] Therefore, it is likely that the IL-1β release induced by MsLP, MpLP or FSL-1 might be mediated through GSDME pores. However, at present, it is unknown whether GSDME pores facilitate IL-1β release from living cells in the hyperactivated state. Further studies are in progress in our laboratories to determine whether MsLP, MpLP or FSL-1 induce GSDME pores in living BMMs.

Thus, a more modest abundance and more continuous release of IL-1β from living macrophages in response to mycoplasmal lipoproteins/lipopeptides, as compared to

pyroptotic cells, might facilitate chronic and persistent inflammation in mycoplasma-infected tissue.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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Figure legends

Fig. 1 Mycoplasmal lipoproteins/lipopeptides induce IL-1β release by BMMs independent of cell death

(a) BMMs from WT mice were stimulated with LPS (10 ng/mL) for 4 h, followed by glycine (0, 0.5, 5, or 20 mM) with nigericin (5 μM) for 1 h. Quantification of total IL-1β and LDH released into the culture supernatant. (b) BMMs from WT mice were stimulated with LPS (10 ng/mL) for 4 h, followed by FSL-1 (100 nM) for the indicated times. Quantification of total IL-1β and LDH released into the culture supernatant. (c) BMMs from WT mice were stimulated with LPS (10 ng/mL) for 4 h, followed by *M. salivarium* or *M. pneumoniae* lipoproteins (MsLP and MpLP, respectively; 0, 0.4, or 4 μg/mL protein) for 24 h. Quantification of total IL-1β and LDH released into the culture supernatant. The results are expressed as the mean ± SD of duplicate assays of a representative experiment. All experiments were repeated at least twice, and similar results were obtained. Student's *t*-test. n.s., not significant; $*P < 0.05$.

Fig. 2 Mycoplasmal lipoproteins/lipopeptide induce IL-1β release by BMMs independent of GSDMD

(a) BMMs from WT or GSDMD$^{-/-}$ mice were stimulated with LPS (10 ng/mL) for 4 h, followed by nigericin (5 μM) for 1 h. Quantification of total IL-1β and LDH released

into the culture supernatant. (b-d) BMMs from WT or GSDMD$^{-/-}$ mice were stimulated with LPS (10 ng/mL) for 4 h, followed by MsLP or MpLP (0, 0.4, or 4 μg/mL protein) or FSL-1 (an *M. salivarium*-derived lipopeptide; 0, 10, or 100 nM). Quantification of total IL-1β and LDH released into the culture supernatant. The results are expressed as the mean ± SD of (a) duplicate or (b–d) triplicate assays of a representative experiment. All experiments were repeated at least three times, and similar results were obtained. Student's *t*-test. n.s., not significant; *$P < 0.05$.

Fig. 3 FSL-1 transfection by PULSin cleaves GSDMD

BMMs were stimulated with LPS (10 ng/mL) for 4 h before transfection with FSL-1 or stimulation with nigericin. BMMs from WT mice were transfected with FSL-1 (100 nM) by PULSin or stimulated with nigericin (Nig) (5 μM) and then incubated for the indicated times. (a) Combined supernatants and cell lysates were analyzed for activated caspase-1, GSDMD, IL-1β, and β-actin by immunoblot analysis. (b, c) BMMs from WT or GSDMD$^{-/-}$ mice were transfected with FSL-1 (100 nM) by PULSin and then incubated for the indicated times. Quantification of total (b) IL-1β and (c) LDH released into the culture supernatant. The results are expressed as the mean ± SD of triplicate assays of a representative experiment. All experiments were repeated at least twice, and similar results were obtained.

Fig. 4 Mycoplasmal lipopeptide-induced IL-1β release is dependent on plasma membrane permeabilization

(a) BMMs from WT mice were stimulated with LPS (10 ng/mL) for 4 h, followed by punicalagin (0, 2.5, or 25 μM) with nigericin (5 μM) for 1 h. Quantification of total IL-

1β and LDH released into the culture supernatant. (b) BMMs from WT mice were stimulated with LPS (10 ng/mL) for 4 h, followed by punicalagin (0, 2.5, or 25 μM) with FSL-1 (100 nM) for 24 h. Quantification of total IL-1β and LDH released into the culture supernatant. (c) BMMs from WT mice 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 punicalagin (25 μM). After incubation for 2 h, the cells were fixed. Cell nuclei were stained with DAPI (blue). Samples were 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, DAPI and DIC were also shown. Scale bar indicates 10 μm. The results are expressed as the mean ± SD of (a) duplicate or (b) triplicate assays of a representative experiment. All experiments were repeated at least twice, and similar results were obtained. Student's *t*-test. n.s., not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

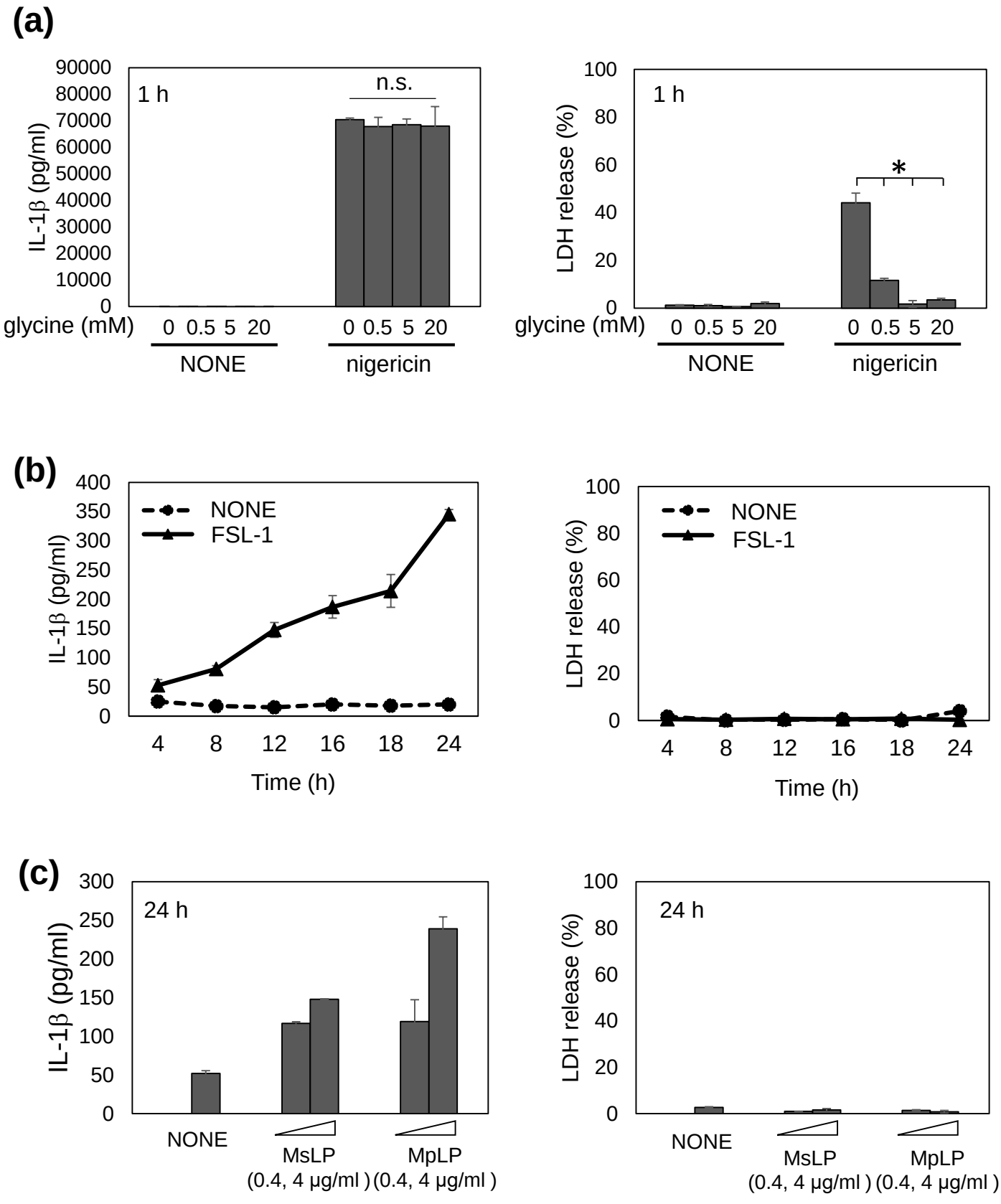
(a)
1 h
n.s.
IL-1β (pg/ml)
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
glycine (mM)
0 0.5 5 20
0 0.5 5 20
NONE
nigericin
1 h
LDH release (%)
100
80
60
40
20
0
*
glycine (mM)
0 0.5 5 20
0 0.5 5 20
NONE
nigericin
(b)
NONE
FSL-1
IL-1β (pg/ml)
400
350
300
250
200
150
100
50
0
4
8
12
16
18
24
Time (h)
NONE
FSL-1
LDH release (%)
100
80
60
40
20
0
4
8
12
16
18
24
Time (h)
(c)
24 h
IL-1β (pg/ml)
300
250
200
150
100
50
0
NONE
MsLP
(0.4, 4 μg/ml)
MpLP
(0.4, 4 μg/ml)
24 h
LDH release (%)
100
80
60
40
20
0
NONE
MsLP
(0.4, 4 μg/ml)
MpLP
(0.4, 4 μg/ml)

Fig. 1

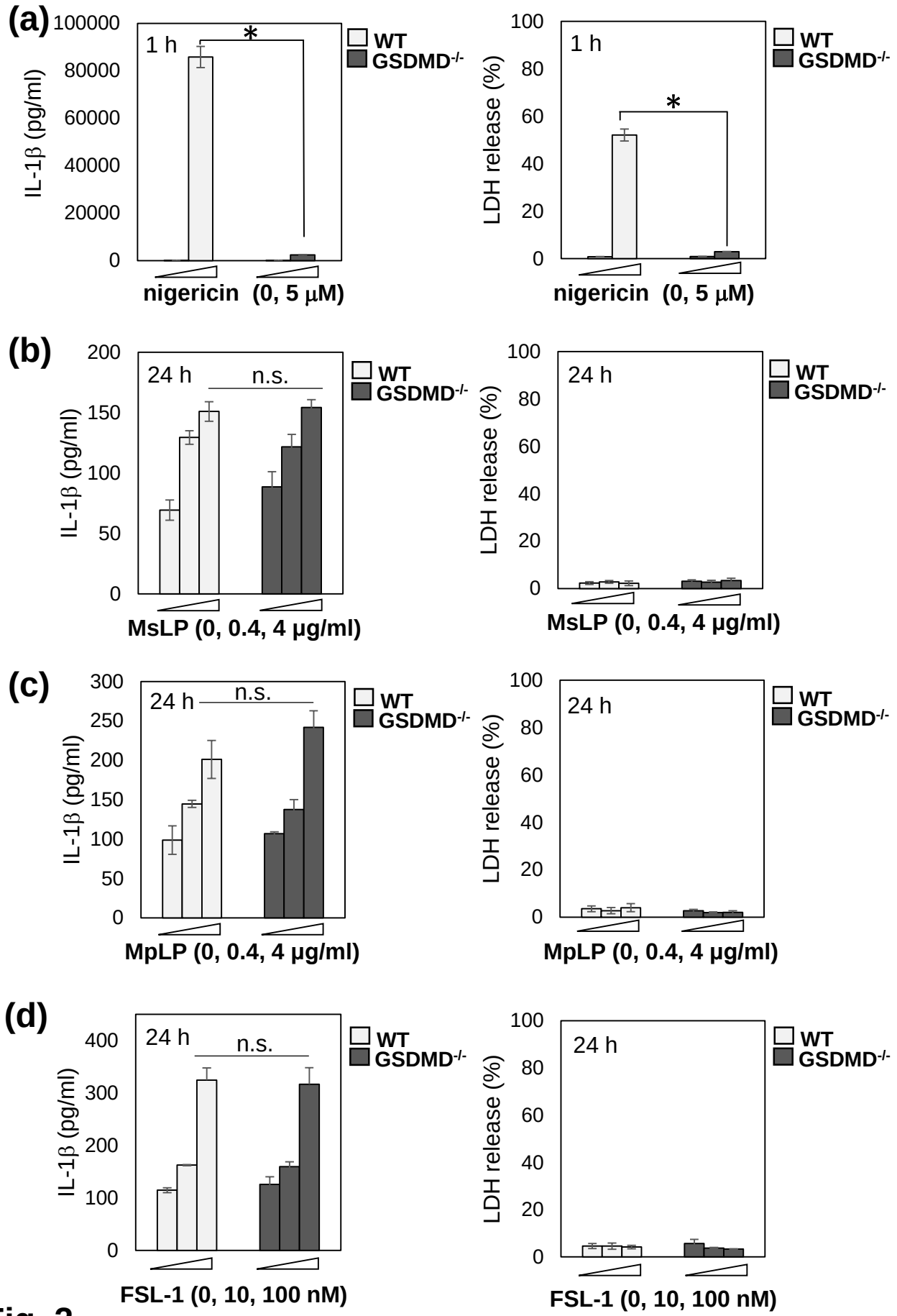
(a)
1 h
IL-1β (pg/ml)
100000
80000
60000
40000
20000
0
*
WT
GSDMD-/-
nigericin (0, 5 μM)
LDH release (%)
100
80
60
40
20
0
(b)
24 h
n.s.
200
150
100
50
MsLP (0, 0.4, 4 μg/ml)
(c)
300
250
200
150
MpLP (0, 0.4, 4 μg/ml)
(d)
400
300
FSL-1 (0, 10, 100 nM)

Fig. 2

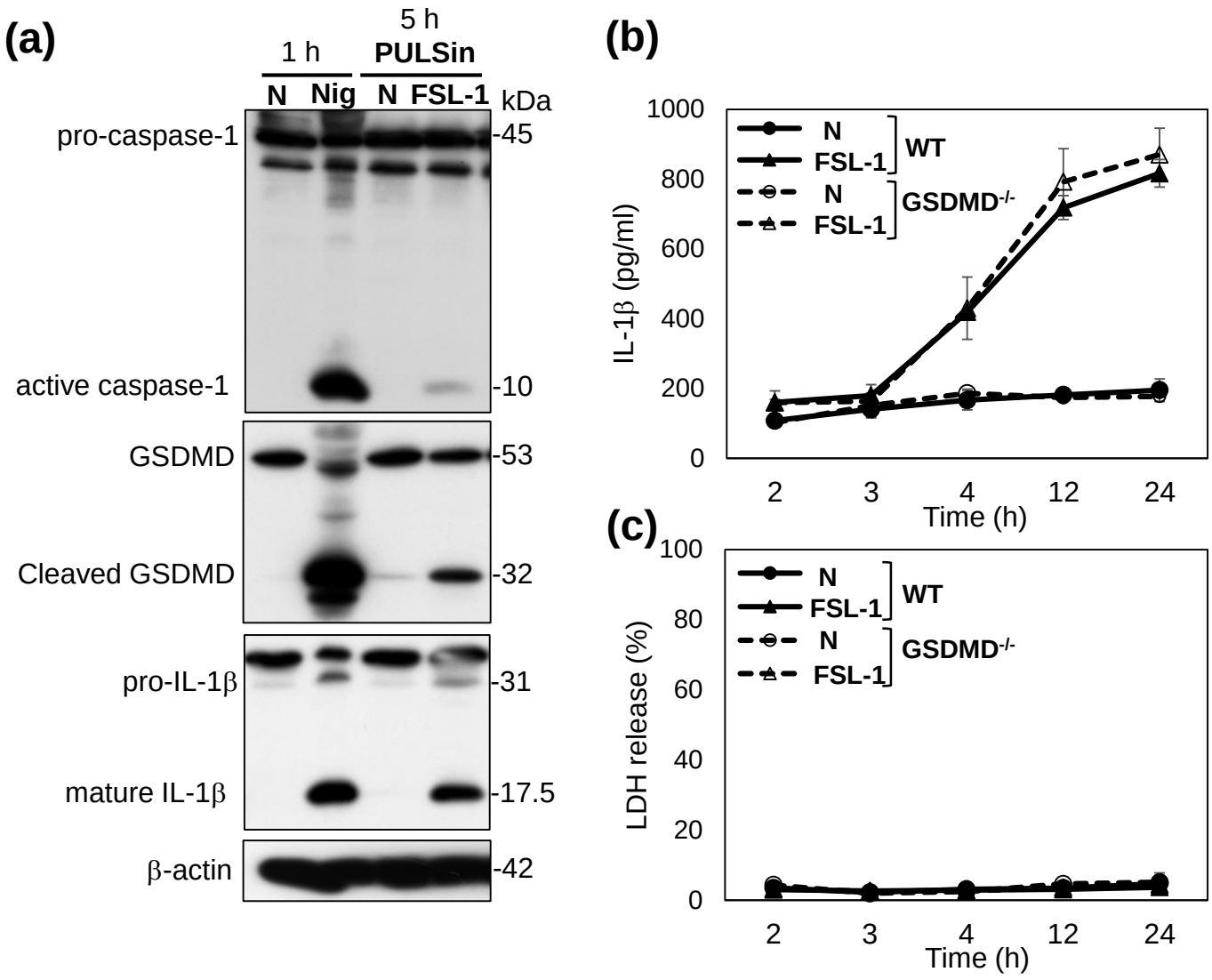
(a)
1 h
5 h
PULSin
N
Nig
N
FSL-1
kDa
pro-caspase-1
-45
active caspase-1
-10
GSDMD
-53
Cleaved GSDMD
-32
pro-IL-1β
-31
mature IL-1β
-17.5
β-actin
-42
(b)
IL-1β (pg/ml)
1000
800
600
400
200
0
N
FSL-1
WT
N
FSL-1
GSDMD-/-
2
3
4
12
24
Time (h)
(c)
LDH release (%)
100
80
60
40
20
0
N
FSL-1
WT
N
FSL-1
GSDMD-/-
2
3
4
12
24
Time (h)

Fig. 3

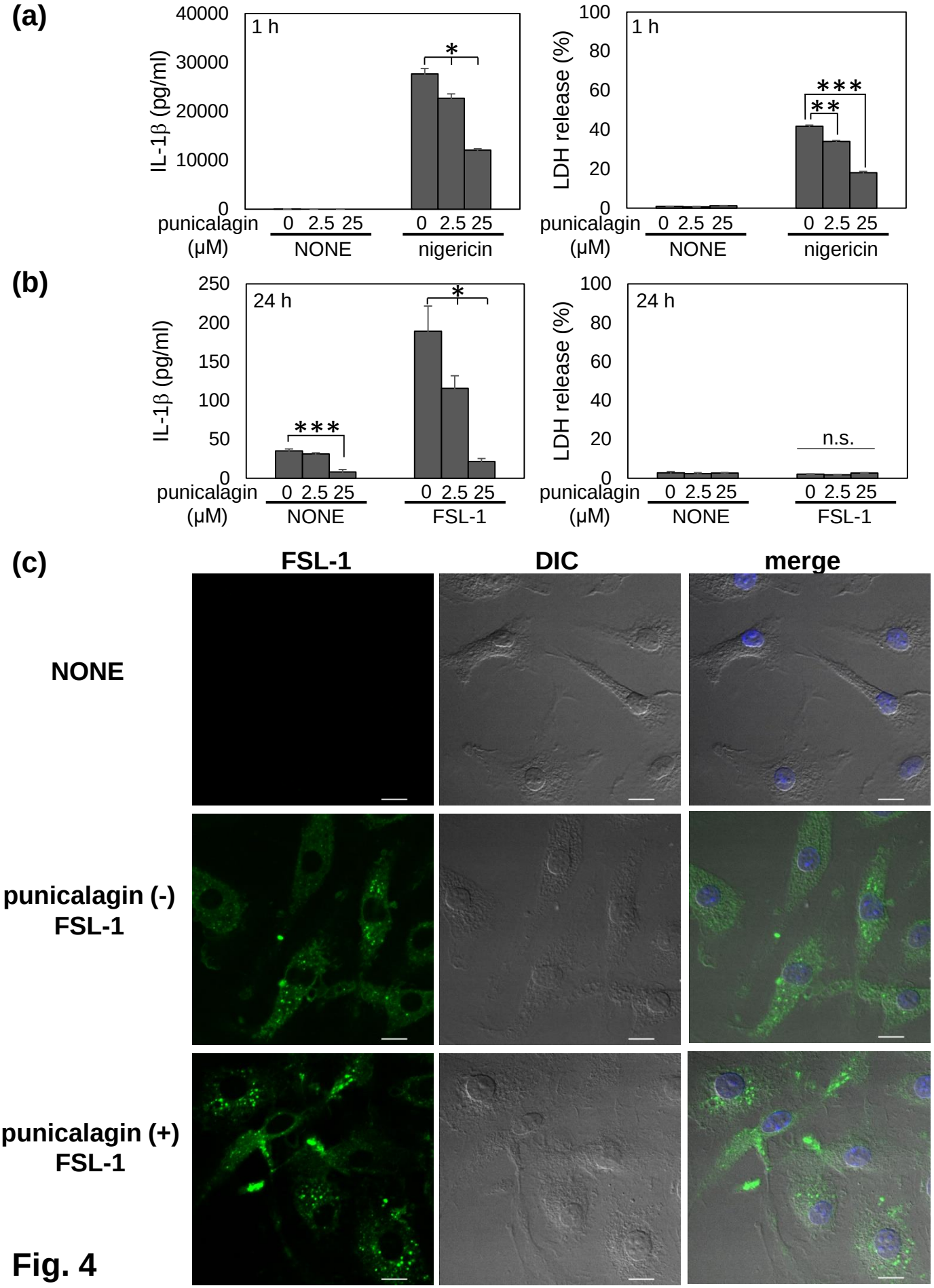
(a)
1 h
IL-1β (pg/ml)
40000
30000
20000
10000
0
*
punicalagin (μM)
0 2.5 25
NONE
0 2.5 25
nigericin
1 h
LDH release (%)
100
80
60
40
20
0
***
**
punicalagin (μM)
0 2.5 25
NONE
0 2.5 25
nigericin
(b)
24 h
IL-1β (pg/ml)
250
200
150
100
50
0
*
***
punicalagin (μM)
0 2.5 25
NONE
0 2.5 25
FSL-1
24 h
LDH release (%)
100
80
60
40
20
0
n.s.
punicalagin (μM)
0 2.5 25
NONE
0 2.5 25
FSL-1
(c)
FSL-1
DIC
merge
NONE
punicalagin (-)
FSL-1
punicalagin (+)
FSL-1

Fig. 4