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Activation of inflammasomes in dendritic cells and macrophages by *Mycoplasma salivarium*

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Running title: Activation of inflammasomes by oral mycoplasma

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Abbreviations: NLR, nucleotide-binding domain leucine-rich repeat-containing receptor; NLRP3, NLR-containing protein 3; interleukin, IL; ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; BMM, bone marrow-derived macrophage

SUMMARY

Interleukin-1β (IL-1β) plays crucial roles in the pathogenesis of periodontal disease. IL-1β is produced after the processing of pro-IL-1β by caspase-1, which is activated by the inflammasome-a multiprotein complex comprising NLR, the adaptor protein ASC, and procaspase-1. *Mycoplasma salivarium* preferentially inhabits the gingival sulcus and the incidence and number of organisms in the oral cavity increase significantly with the progression of periodontal disease. To initially clarify the association of this organism with periodontal diseases, this study determined whether it induces IL-1β production by innate immune cells such as dendritic cells or macrophages by using *Mycoplasma pneumoniae* as a positive control. Both live and heat-killed *M. salivarium* and *M. pneumoniae* cells induced IL-1β production by XS106 murine dendritic cells as well as pyroptosis. The activities were significantly downregulated by silencing of caspase-1. Bone marrow-derived macrophage (BMMs) from wild-type and NLRP3-, ASC-, and caspase-1–deficient mice were examined for IL-1β production in response to these mycoplasmas. Live *M. salivarium* and *M. pneumoniae* cells almost completely lost the ability to induce IL-1β production by BMMs from ASC- and caspase-1–deficient mice. Their activities toward BMMs from NLRP3-deficient mice were significantly but not completely attenuated. These results suggest that live *M. salivarium* and *M. pneumoniae* cells can activate several types of inflammasomes including the NLRP3 inflammasome. *M. salivarium* as well as *M. pneumoniae* cells can activate THP-1 human monocytic cells to induce IL-1β production.

Thus, the present finding that *M. salivarium* induces IL-1β production by dendritic cells and macrophages may suggest the association of this organism with periodontal diseases.

INTRODUCTION

Mycoplasmas, the smallest self-replicating and microorganisms without cell walls, cause various infectious diseases in humans and animals, such as atypical pneumonia, non-gonococcal urethritis, and arthritis (Maniloff, 1992). *Mycoplasma salivarium*, a member of the human oral microbial flora, preferentially inhabits the gingival sulcus (Engel *et al.*, 1970). The incidence and number of organisms in the oral cavity increase significantly with the progression of periodontal disease, ostitis, pericoronitis, and temporomandibular disorders (Engel *et al.*, 1970;Kumagai *et al.*, 1971;Watanabe *et al.*, 1986;Watanabe *et al.*, 1998). Furthermore, significantly greater antibody responses occur in patients with periodontal disease than healthy individuals (Kumagai *et al.*, 1971;Watanabe *et al.*, 1986). However, little is known about roles of *M. salivarium* in oral infectious diseases, especially periodontal disease.

Interleukin-1β (IL-1β), a proinflammatory cytokine, induces the production of inflammatory mediators, osteoclast formation, matrix metalloproteinase expression, and the death of matrix-producing cells in periodontal tissues, resulting in the destruction of alveolar bone and periodontal connective tissue (Graves *et al.*, 2003). Thus, IL-1β plays crucial roles in the onset and progression of periodontal disease. IL-1β is produced after pro-IL-1β is processed by caspase-1, which is activated by an inflammasome (Lamkanfi *et al.*, 2012;Mariathasan *et al.*, 2007;Martinon *et al.*, 2009). An inflammasome is an intracellular multiprotein complex comprising “nucleotide-binding domain leucine-rich repeat-containing receptor” (NLR), the adaptor protein “apoptosis-associated speck-like protein containing a caspase-recruitment domain” (ASC), and procaspase-1. Several types of NLRs are involved in inflammasome activation (Davis *et al.*, 2011). Active caspase-1 is required for the processing and subsequent release of active IL-1β. In

addition, inflammasome activation can lead to host cell death, called pyroptosis, in certain types of cells; this mechanism might be important in restricting the intracellular replication of invasive bacterial pathogens (Mariathasan *et al.*, 2007).

The activities by which mycoplasmas induce IL-1β production by mammalian cells are poorly understood. However, *M. pneumoniae*, a key pathogen of atypical pneumonia in humans (Razin *et al.*, 1998), was recently reported to induce IL-1β production by human monocytes; Nonetheless, the types of NLRs involved in this remain unknown (Shimizu *et al.*, 2011).

Therefore, as a first step to elucidate the etiological roles of *M. salivarium* in periodontal diseases, this study determined whether *M. salivarium* can activate the intracellular sensor inflammasome to induce IL-1β production by innate immune cells such as dendritic cells or macrophages and if so, what kinds of inflammasomes are activated by *M. salivarium* including *M. pneumoniae*.

METHODS

Mycoplasmas and culture conditions

M. salivarium ATCC 23064 and *M. pneumoniae* ATCC15492 were grown in 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* and 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, suspended in phosphate-buffered saline to make aliquots, and then stored at

−80°C. When heat-killed cells were used, the cell suspensions were placed in boiling water for 5 min. The protein concentration was determined according to the method of Dully and Grieve (Dulley *et al.*, 1975).

Mice

Sex-matched 8-week-old C57BL/6 (B6) mice were purchased from CLEA Japan (Tokyo, Japan) and maintained in specific pathogen-free conditions at the animal facility of the Graduate School of Medicine, Hokkaido University. Caspase-1–, NLR-containing protein 3 (NLRP3)-, or ASC-deficient mice (caspase-$1^{-/-}$, $NLRP3^{-/-}$, and $ASC^{-/-}$, respectively) of the same genetic background were maintained in specific pathogen-free conditions at the animal facility of the Graduate School of Medicine, University of the Ryukyus.

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

Cell culture

XS106, a murine dendritic cell (DC) line, kindly provided by Professor Akira Takashima (University of Texas Southwestern, Dallas, TX, USA), is a long-established DC line derived from the epidermis of newborn A/J mice (Mohan *et al.*, 2005). Cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% (vol/vol) fetal bovine serum (FBS) (Invitrogen, Durham, NC, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, 100 units/mL penicillin G, 100 μg/mL streptomycin (Sigma), 50 μM 2-mercaptoethanol (Sigma), 0.5 ng/mL murine recombinant GM-CSF (Pepro Tech, Rocky Hill, NJ, USA) and 5% (vol/vol)

culture supernatant derived from NS47 fibroblast cells (hereafter referred to as XS medium) (Ohtani *et al.*, 2012).

A human acute monocytic leukemia cell line, THP-1, was purchased from Health Science Research Resources Bank (Osaka, Japan). Cells were grown at 37ºC in a humidified atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 10% (vol/vol) FBS, 100 units/mL penicillin G, 100 μg/mL streptomycin.

XS106 cells and IL-1β measurement

XS106 cells were added to a 24-well plate at 3×10^5 cells per well in 500 μL XS medium and incubated at 37°C for 16 h at a 5% CO_2 atmosphere. The cultures were centrifuged at 400 × *g* for 5 min, and the cells were washed with supplement-free RPMI 1640 basal medium. The cells were resuspended in 300 μL RPMI 1640 basal medium and incubated at 37°C for 24 h with intact or heat-killed *M. salivarium* and *M. pneumoniae* cells (0, 36.5, or 365 μg protein). The amounts of IL-1β in cell culture supernatants were measured by an ELISA kit (BD OptEIA™ SET Mouse IL-1β, BD Biosciences, San Jose, CA, USA). The cell culture supernatants were subjected to SDS-PAGE to distinguish mature IL-1β from pro-IL-1β, and proteins were transferred to a nitrocellulose transfer membrane and reacted with the appropriate antibody against IL-1β (R&D Systems, Minneapolis, MN, USA).

Cell death assay

XS106 cells were added to a 24-well plate at 3×10^5 cells per well in 500 μL XS medium and incubated at 37°C for 16 h in a 5% CO_2 atmosphere. The cultures were centrifuged at 400 × *g* for 5 min, and the cells were washed with RPMI 1640 basal

medium and incubated at 37°C for 24 h with 365 μg protein of live *M. salivarium* and *M. pneumoniae* cells. The cells were stained by using an Annexin-V-FLUOS Staining Kit (Roche, Branford, CT, USA) and subsequently analyzed by a FACSCalibur flow cytometer (BD Bioscience). Data for 20,000 cells falling within appropriate forward and side light scatter gates were collected from each sample (Ohtani *et al.*, 2012). Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Reactive oxygen species (ROS) production

XS106 cells were added to a 24-well plate at 3×10^5 cells per well in 500 μL XS medium and incubated at 37°C for 16 h in a 5% CO_2 atmosphere. The cultures were centrifuged at 400 × *g* for 5 min, and the cells were washed with RPMI 1640 basal medium. The cells were suspended and incubated at 37°C for 6, 9, or 12 h in RPMI 1640 basal medium containing dihydrorhodamine 123 (Sigma) with live cells (365 μg protein) of *M. salivarium* or *M. pneumoniae* and subsequently analyzed by a FACSCalibur flow cytometer (BD Bioscience). Data for 20,000 cells falling within appropriate forward and side light scatter gates were collected from each sample. Data were analyzed using FlowJo software (Tree Star Inc.).

RNA interference

Caspase-1 was silenced in XS106 cells as follows. siRNAs specific for caspase-1 and non-targeting siRNA as a control (Applied Biosystems, Carlsbad, CA, USA) at final concentrations of 10 μM were transfected into XS106 cells (2×10^6 cells/0.1 mL Opti-MEM) by electroporation using a two-step electroporator (CUY21 Pro-Vitro, NEPA GENE, Tokyo, Japan) at 175 V for 2 ms. The cells were cultured in XS106

medium at 37°C for 40 h to reach 100% confluence in a 10-cm dish. The cells collected from the dish were subsequently inoculated onto a 24-well plate at 3×10^5 cells per well. After an 8-h incubation, the cells were stimulated at 37°C for 24 h with *M. salivarium* or *M. pneumoniae*.

~~NLRP3 was silenced in XS106 cells as follows. The plasmids psiRNA-mNLRP3 and psiRNA-LucGL3 (InvivoGen, San Diego, CA, USA), which express NLRP3-specific and nonspecific siRNA, respectively, were transfected into XS106 cells by electroporation as described above. Transfectants stably expressing NLRP3-specific or nonspecific siRNA were established by selection in the presence of 100 μg/mL Zeocin (Invitrogen) at 37°C for 2 weeks in a 10-cm dish.~~

Total RNA was extracted from the washed cells by using ReliaPrep™ RNA Cell Miniprep System (Promega, Mannheim, Germany), and cDNA was synthesized by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For real-time PCR analysis, PCR amplification was performed in the presence of a TaqMan probe by using a StepOne Real-time PCR system (Applied Biosystems). Specific primers for caspase-1, NLRP3, and GAPDH were purchased from Applied Biosystems. The cycling threshold (C_T) value was defined as the number of PCR cycles in which the fluorescence signal exceeded the detection threshold. The normalized amount of target mRNA (Nt) was calculated from the C_T value obtained for both target and GAPDH mRNAs according to the following equation: $Nt = 2^{Ct(GAPDH) - Ct(target)}$. Relative mRNA expression was obtained by setting Nt to 1 in non-stimulated samples in each experiment.

Bone marrow-derived macrophages (BMMs) and IL-1β measurement

Femurs and tibias prepared from caspase-$1^{-/-}$, NLRP$3^{-/-}$, or ASC$^{-/-}$ mice at the University of the Ryukyus were sent to Hokkaido University. Femurs and tibias from B6 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 containing 10% (vol/vol) FBS (Invitrogen), 100 units/mL penicillin G, and 100 μg/mL streptomycin (Sigma). Cells were suspended by pipetting and washed by centrifugation. The cells were cultured in a non-tissue culture plastic 100-mm petri dish in RPMI 1640 medium containing 10% (vol/vol) FBS (Invitrogen), 100 units/mL penicillin G, 100 μg/mL streptomycin (Sigma), 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 phosphate-buffered saline and used as BMMs (Celada *et al.*, 1984).

BMMs were added to a 24-well plate at 4×10^5 cells per well in 500 μL RPMI 1640 medium (Gibco) containing 10% (vol/vol) FBS and incubated at 37°C for 4 h with 1 μg/mL ultrapure *E. coli* lipopolysaccharide (LPS) (InvivoGen) and then for 24 h with 0, 0.365, or 3.65 μg protein of intact *M. salivarium* and *M. pneumoniae* cells. IL-1β in cell culture supernatants was quantified by using an ELISA kit (OptEIA™ SET Mouse IL-1β, BD Biosciences). To distinguish mature IL-1β from pro-IL-1β, cell culture supernatants were submitted to SDS-PAGE, and the proteins were transferred to a nitrocellulose transfer membrane and reacted with the appropriate antibody against IL-1β (R&D Systems).

THP-1 cells and IL-1β measurement

A 0.5-ml volume of cell suspension (1.2 x 10^6 cells/ml) of THP-1 cells was seeded in each well of a 24-well tissue culture plate. The cells were incubated at 37°C for 12 h in the presence of 20 nM phorbol 12-myristate 13-acetate and then stimulated for 16 h with 0, 36.5, or 365 μg protein of intact *M. salivarium* or *M. pneumoniae* cells. IL-1β in cell culture supernatants was quantified by using an ELISA kit (ELISA MAX™ SET human IL-1β, Biolegend, San Diego, CA) according to the manufacturer's instructions. To distinguish mature IL-1β from pro-IL-1β, cell culture supernatants were submitted to SDS-PAGE, and the proteins were transferred to a nitrocellulose transfer membrane and reacted with the appropriate antibody against human IL-1β (Cell Signaling Technology, Danvers, MA).

RESULTS

IL-1β production by XS106 cells in response to mycoplasma cells

XS106 cells were stimulated for 24 h with various numbers of live or heat-killed *M. salivarium* or *M. pneumoniae* cells, and the amounts of total IL-1β released into the culture supernatant were measured by ELISA. *M. pneumoniae* was used as a positive control, because this species induces IL-1β production by human monocytes/macrophages (Shimizu *et al.*, 2011). Both live and heat-killed *M. salivarium* and *M. pneumoniae* cells induced IL-1β production by XS106 cells (Fig. 1a, b). However, mature IL-1β could not be distinguished from pro-IL-1β by ELISA because of the nature of the anti–IL-1β antibody used in the ELISA kit. Therefore, mature IL-1β (17.5 kDa) production was evaluated according to the molecular weight of the band detected by western blotting, although the possibility that pro-IL-1β is included in the supernatant used as samples for ELISA is very low. The results showed that both live

and heat-killed *M. salivarium* and *M. pneumoniae* cells induced mature IL-1β production by XS106 cells (Fig. 1a, b lower); furthermore, there was no substantial difference in the activity between species (Fig. 1). In addition, the IL-1β–inducing activity of live cells was significantly higher than that of heat-killed cells for both mycoplasma species, suggesting that thermolabile components are included in the active entities of both mycoplasmas.

Pyroptosis induction in XS106 cells by mycoplasma cells

NLRP3 activation triggers an inflammatory caspase-1–dependent death process termed pyroptosis (Miao *et al.*, 2011); pyroptosis is morphologically similar to necrosis, which is characterized by cell expansion and lysis (Lamkanfi *et al.*, 2010). To determine if mycoplasma cells induce pyroptosis in XS106 cells, XS106 cells were stained with PI and Annexin V after stimulation with mycoplasma cells and analyzed by flow cytometry. Live cells of *M. salivarium* and *M. pneumoniae* induced necrosis-like but not apoptosis-like cell death in XS106 cells (Fig. 2); the cell death was considered pyroptosis because it was accompanied by IL-1β production as described above.

Involvement of caspase-1 in IL-1β–inducing activity

Mature IL-1β secretion was induced after pro-IL-1β processing by caspase-1, which is activated by the inflammasome (Martinon *et al.*, 2009). Therefore, in order to clarify the involvement of caspase-1 in the IL-1β–inducing activities of the mycoplasmas, caspase-1 mRNA was silenced by transfection of caspase-1–specific siRNA. Transfection downregulated caspase-1 mRNA by approximately 75% (Fig. 3a) and downregulated the IL-1β–inducing activities of *M. salivarium* and *M. pneumoniae* by

more than 90% (Fig. 3b). These findings strongly suggest that caspase-1 is involved in these IL-1β–inducing activities.

Involvement of NLRP3 in IL-1β–inducing activity

Caspase-1 is the central effector protein of the inflammasome, which acts as a molecular scaffold for caspase-1 activation (Martinon *et al.*, 2009). NLRP3 is a representative cytosolic NLR that recognizes various bacterial pathogens (Sutterwala *et al.*, 2014;Vladimer *et al.*, 2013). Therefore, we hypothesized that the mycoplasma cells also activate the NLRP3 inflammasome to induce IL-1β production~~. In order to determine the involvement of NLRP3 in IL-1β–inducing activity, transfectants that express NLRP3-specific or non-specific siRNA were generated; these transfectants were examined for IL-1β production after stimulation with live cells of *M. salivarium* and *M. pneumoniae*. Although NLRP3 mRNA expression was downregulated by approximately 20% in the transfectant expressing NLRP3-specific siRNA compared to that in the transfectant expressing non-specific siRNA (Fig. 4a), the IL-1β–inducing activities of both mycoplasmas toward the transfectant expressing NLRP3-specific siRNA were significantly lower or tended to be lower than those toward the transfectant expressing non-specific siRNA (Fig. 4b). Therefore, we~~ and determined if XS106 cells produced ROS in response to these mycoplasma cells, because ROS are known to trigger the activation of NLRP3 inflammasomes (Sutterwala *et al.*, 2014). In addition, we previously found that mycoplasma cells induce ROS production through recognition by Toll-like receptor 2 (Into *et al.*, 2005). Therefore, we determined if mycoplasma cells induce ROS production by XS106 cells. ATP was used as a positive control because it induces ROS production (Hussen *et al.*, 2012). Both mycoplasmas accelerated ROS

production by XS106 cells, although the cells produced ROS spontaneously without any stimulators (Fig.4a, b). The results suggest that NLRP3 inflammasome-activating ROS are involved in the IL-1β-inducing activities of these mycoplasma species. To confirm this, we examined the effects of *N*-acetylcysteine (NAc), an ROS inhibitor, on the IL-1β-inducing activities. NAc downregulated the activities in a dose-dependent manner (Fig. 4c). These results also suggest that the mycoplasma cells induce the activation of the NLRP3 inflammasome in murine dendritic cells to produce IL-1β.

IL-1β–inducing activity toward BMMs from B6, caspase-1$^{-/-}$, NLRP3$^{-/-}$, and ASC$^{-/-}$ mice

As mentioned above, the NLRP3 inflammasome appears to be involved in the IL-1β–inducing activities of live *M. salivarium* and *M. pneumoniae* cells toward XS106 murine dendritic cells. The NLRP3 inflammasome is a protein complex comprising NLRP3, ASC, and procaspase-1 (Mariathasan *et al.*, 2007;Sutterwala *et al.*, 2014). In order to confirm that live cells of both mycoplasmas activate the NLRP3 inflammasome, the IL-1β production of BMMs from B6 and NLRP3-, ASC-, and caspase-1–deficient mice in response to live cells of these mycoplasmas were examined. BMMs were stimulated for 4 h with LPS followed by 24 h with mycoplasma cells, because LPS priming significantly enhanced the IL-1β–inducing activities of these mycoplasma cells (Fig. 5). LPS itself induced IL-1β production by B6-derived BMMs (Fig. 5) as reported previously (Bauernfeind *et al.*, 2009;Hiscott *et al.*, 1993). However, live *M. salivarium* and *M. pneumoniae* cells almost completely lost their activity to induce IL-1β production by BMMs derived from caspase-1$^{-/-}$ and ASC$^{-/-}$ mice (Fig. 6a, c, 7a, c). In addition, the activities of these mycoplasmas toward BMMs from NLRP3$^{-/-}$ mice were

significantly but not completely attenuated (Fig. 6b and 7b). These results suggest that live *M. salivarium* and *M. pneumoniae* cells can activate some types of inflammasomes including the NLRP3 inflammasome.

IL-1β–inducing activity toward THP-1 cells

Live *M. salivarium* and *M. pneumoniae* cells can activate inflammasomes to induce IL-1β production by murine dendritic cells and macrophages. Therefore, next experiments were designed to determine whether they can induce IL-1β production by THP-1 human monocytic cells, although *M. pneumoniae* cells have already known to activate the cells to induce IL-1β production (Shimizu *et al.*, 2011). It was found that *M. salivarium* as well as *M. pneumoniae* cells can activate THP-1 cells to induce IL-1β production in a dose-dependent manner (Fig. 8).

DISCUSSION

Various bacterial pathogens induce caspase-1–dependent IL-1β production through the activation of the NLRP3 inflammasome (Franchi *et al.*, 2012;Koizumi *et al.*, 2012;Vladimer *et al.*, 2013). This study demonstrates that caspase-1 is involved in the IL-1β–inducing activities of *M. salivarium* and *M. pneumoniae* toward the murine dendritic cell line XS106 by silencing caspase-1 mRNA (Fig. 3a, b). However, the involvement of NLRP3 in IL-1β–inducing activity of these mycoplasmas, which was detected by experiments with the XS106 transfectant expressing NLRP3-specific or nonspecific siRNA, was ambiguous. Therefore, in order to confirm the involvement of NLRP3 in the IL-1β–inducing activity of these mycoplasmas, we demonstrated that ROS, which activate NLRP3 (Sutterwala *et al.*, 2014), were produced in XS106 cells in

response to these mycoplasmas and that NAc inhibited this activity (Fig.4). In addition, BMMs derived from B6-, NLRP3-, ASC-, and caspase-1–deficient mice were examined for IL-1β production in response to *M. salivarium* and *M. pneumoniae* cells after LPS priming. As a result, *M. salivarium* and *M. pneumoniae* completely lost their activity to induce IL-1β production by BMMs derived from caspase-$1^{-/-}$ and $ASC^{-/-}$ mice, whereas their activities toward BMMs derived from $NLRP3^{-/-}$ mice were significantly but not completely downregulated (Fig. 6b, 7b). These results suggest that live *M. salivarium* and *M. pneumoniae* cells can activate the NLRP3 inflammasome as well as other types of inflammasomes that require ASC and caspase-1, such as NLRP1, NLRC4, and AIM2. This is corroborated by previous findings that whole *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Mycobacterium tuberculosis* cells can induce IL-1β production via some types of inflammasomes (Franchi *et al.*, 2012;Mishra *et al.*, 2010;Saiga *et al.*, 2012;Vladimer *et al.*, 2013). *M. salivarium* and *M. pneumoniae* very likely activate the AIM2 inflammasome in addition to the NLRP3 inflammasome, because the former is activated by bacterial DNA (Sutterwala *et al.*, 2014) and mycoplasma cells are easily disrupted because of their lack of a cell wall.

As described earlier, *M. salivarium* is member of the human oral microbial flora and preferentially inhabits the gingival sulcus; thus, it plays etiological roles in periodontal disease (Engel *et al.*, 1970;Kumagai *et al.*, 1971;Watanabe *et al.*, 1986;Watanabe *et al.*, 1998). IL-1β plays pathological roles by inducing the production of inflammatory mediators, osteoclast formation, matrix metalloproteinase expression, and the death of matrix-producing cells in periodontal tissues (Graves *et al.*, 2003). IL-1β levels are reported to be higher in periodontitis patients than healthy individuals (Park *et al.*, 2014). The representative periodontopathogenic bacterium *Porphyromonas gingivalis* is

reported to induce IL-1β secretion and pyroptosis through the activation of the NLRP3 and AIM2 inflammasomes (Park *et al.*, 2014). In addition, *Aggregatibacter actinomycetemcomitans*, a representative pathogen in aggressive periodontitis, is also reported to induce IL-1β secretion by macrophages, although the underlying mechanism remains unknown (Kelk *et al.*, 2008). *M. salivarium* can induce IL-1β production and pyroptosis in dendritic cells and macrophages through the activation of several types of inflammasomes, although LPS priming is required in the case of macrophages. Judging from the fact that many Gram-negative bacteria, which contain their own LPS on the outer membrane, inhabit the gingival sulcus and most periodontopathogenic bacteria are Gram-negative, LPS priming definitely occurs in the gingival pockets.

In conclusion, this study suggests that *M. salivarium* in cooperation with Gram-negative periodontopathogenic bacteria may be associated with the onset and progression of periodontal diseases as described previously (Engel *et al.*, 1970;Kumagai *et al.*, 1971;Watanabe *et al.*, 1986).

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Figure legends

Fig. 1. IL-1β production by XS106 cells in response to mycoplasma cells

XS106 cells were stimulated with various amounts (i.e., 0, 36.5, or 365 μg/mL protein) of live or heat-killed cells of *M. salivarium* (Ms) (a) or *M. pneumoniae* (Mp) (b) at 37°C 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. All of the experiments were repeated at least twice and similar results were obtained. Mature IL-1β was evaluated according to molecular weight (mature IL-1β: 17.5 kDa, pro-IL-1β: 31 kDa) of the bands detected by western blotting.

Student's t test; *, $0.01 < P < 0.05$, **, $0.01 < P < 0.001$, ***, $P < 0.001$.

Fig. 2. Pyroptosis induction of XS106 cells by mycoplasma cells

XS106 cells were stimulated with live cells (i.e., 365 μg/mL protein) of *M. salivarium* (Ms) or *M. pneumoniae* (Mp) at 37°C for 24 h. XS106 cells were stained with PI and Annexin V after stimulation and analyzed by flow cytometry. Percentages are the sum of Q1 and Q2, which indicate the percentages of cells that died from necrosis.

Fig. 3. Involvement of caspase-1 in the IL-1β–inducing activity of mycoplasmas

XS106 cells were transfected with caspase-1–specific siRNA or non-targeting siRNA. Relative mRNA expression was determined by real-time PCR analysis. The results are expressed as the mean ± SD of triplicate assays of a representative experiment (a). All of the experiments were repeated at least twice and similar results

were obtained. The transfected cells were stimulated with various amounts (i.e., 0, 36.5, or 365 μg protein/mL protein) of live cells of *M. salivarium* (Ms) or *M. pneumoniae* (Mp) at 37°C 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; **, $0.01 < P < 0.001$, ***, $P < 0.001$.

~~Fig. 4. Involvement of NLRP3 in the IL-1β-inducing activity of mycoplasmas~~

~~XS106 cells were transfected with psiRNA-mNLRP3 or psiRNA-LucGL3, which express NLRP3-specific and non-specific siRNA, respectively. Relative mRNA expression was determined by real-time PCR analysis. The results are expressed as the mean ± SD of triplicate wells from three separate experiments (a). The transfected cells were stimulated with various amounts (i.e., 0, 36.5, or 365 μg protein/mL protein) of live cells of *M. salivarium* (Ms) or *M. pneumoniae* (Mp) at 37°C 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; *, $0.01 < P < 0.05$, **, $0.01 < P < 0.001$, ***, $P < 0.001$.~~

Fig. 4. ROS production by XS106 cells in response to mycoplasma cells

XS106 cells were stimulated with live cells of *M. salivarium* (Ms) or *M. pneumonia* (Mp) (i.e., 365 μg protein) or ATP (5 mM) in RPMI 1640 basal medium containing

dihydrorhodamine 123 at 37°C for 6, 9, or 12 h; ROS production was subsequently measured by flow cytometry (a, b). XS106 cells were pretreated with the ROS inhibitor NAc (5, 10 μM) for 1 h before stimulation with mycoplasmas (c). XS106 cells were stimulated at with live cells of Ms or Mp (365 μg protein) at 37°C 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. 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. 5. Effect of LPS priming on the IL-1β–inducing activity of mycoplasmas toward BMMs from B6 mice

BMMs were stimulated with or without LPS at 37°C for 4 h and then for 24 h with live cells (i.e., 0, 0.365, or 3.65 μg protein) of *M. salivarium* (Ms) or *M. pneumonia* (Mp). 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; ***, $P < 0.001$).

Fig. 6. IL-1β production by BMMs from B6, caspase-$1^{-/-}$, NLRP$3^{-/-}$, or ASC$^{-/-}$ mice in response to *M. salivarium* cells

BMMs were stimulated with LPS at 37°C for 4 h and then for 24 h with live cells (i.e., 0, 0.365, or 3.65 μg protein) of *M. salivarium*. 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. Mature IL-1β was evaluated by molecular weight (mature IL-1β: 17.5 kDa, pro-IL-1β: 31 kDa) of the band detected by western blotting.

Student's t test; *, $0.01 < P < 0.05$, **, $0.01 < P < 0.001$, ***, $P < 0.001$.

Fig. 7. IL-1β production by BMMs from B6, caspase-$1^{-/-}$, NLRP$3^{-/-}$, or ASC$^{-/-}$ mice in response to *M. pneumoniae* cells

BMMs were stimulated with LPS at 37°C for 4 h and then for 24 h with live cells (i.e., 0, 0.365, or 3.65 μg protein) of *M. pneumoniae*. 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. Mature IL-1β was evaluated by molecular weight (mature IL-1β: 17.5 kDa, pro-IL-1β: 31 kDa) of the bands detected by western blotting.

Student's t test; *, $0.01 < P < 0.05$, **, $0.01 < P < 0.001$, ***, $P < 0.001$.

Fig. 8. IL-1β production by THP-1 cells in response to M. *salivarium* and *M. pneumoniae* cells

A 0.5-ml volume of cell suspension (1.2×10^6 cells/ml) of THP-1 cells was seeded in each well of a 24-well tissue culture plate. The cells were incubated at 37°C for 12 h in the presence of 20 nM phorbol 12-myristate 13-acetate and then stimulated for 16 h with 0, 36.5, or 365 μg protein of intact *M. salivarium* or *M. pneumoniae* cells. The

amounts of total IL-1β released into the culture supernatant were measured by ELISA. The results are expressed as the mean ± SD of three separate experiments, each of which were performed in duplicate. Mature IL-1β was evaluated by molecular weight (mature IL-1β: 17 kDa) of the bands detected by western blotting.

Student's t test: ***, $P < 0.001$).

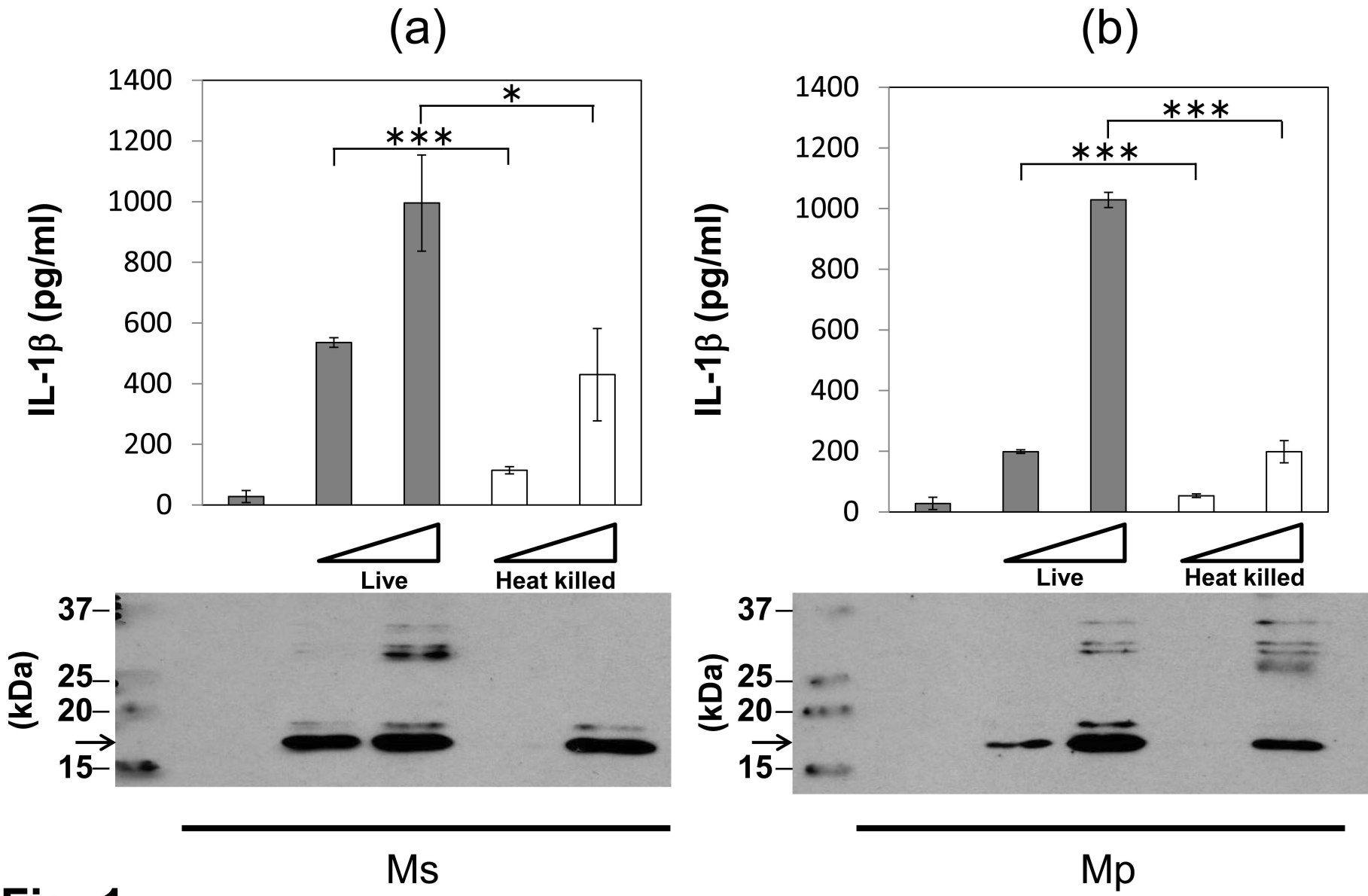
(a)
(b)
IL-1β (pg/ml)
1400
1200
1000
800
600
400
200
0
***
*
Live
Heat killed
(kDa)
37
25
20
15
Ms
Mp

Fig. 1

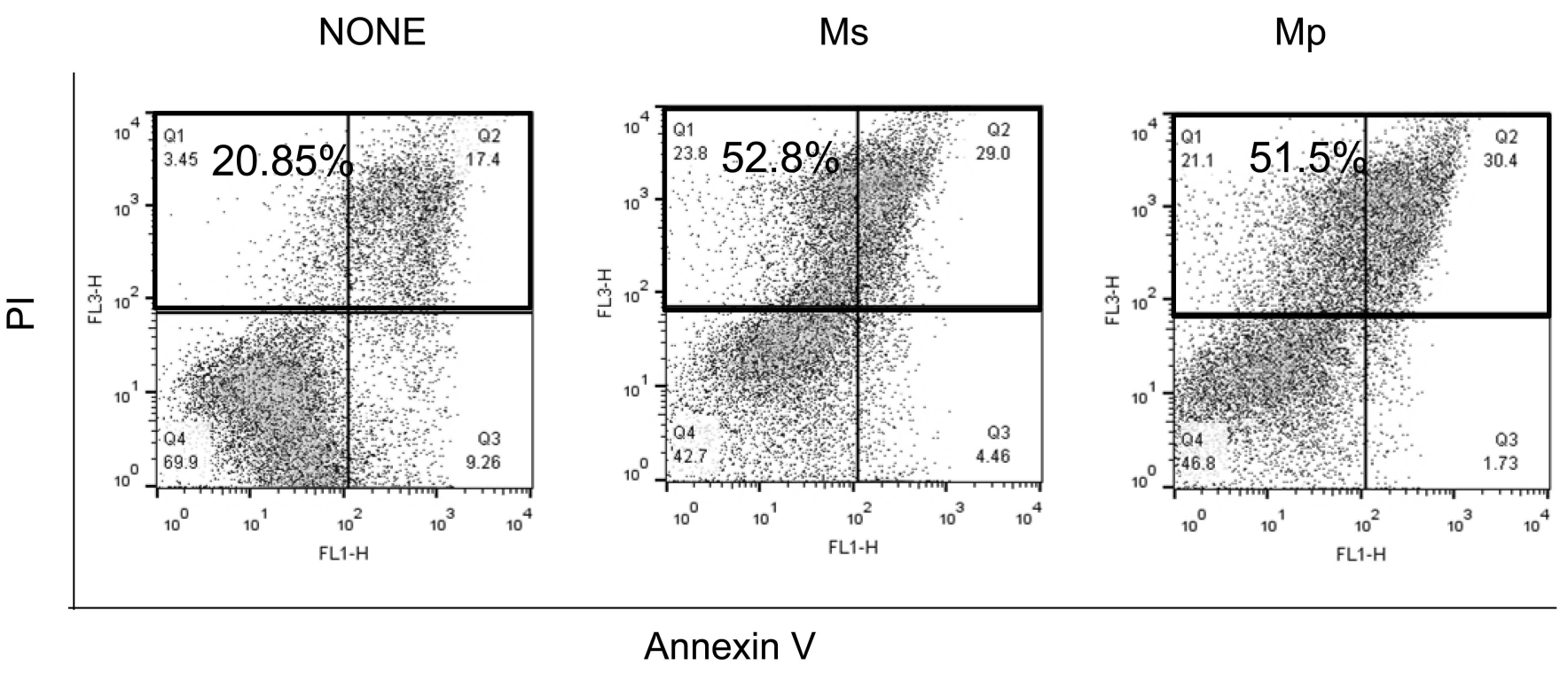
NONE
Ms
Mp
PI
Annexin V
FL3-H
FL1-H
Q1
3.45
20.85%
Q2
17.4
Q4
69.9
Q3
9.26
Q1
23.8
52.8%
Q2
29.0
Q4
42.7
Q3
4.46
Q1
21.1
51.5%
Q2
30.4
Q4
46.8
Q3
1.73

Fig. 2

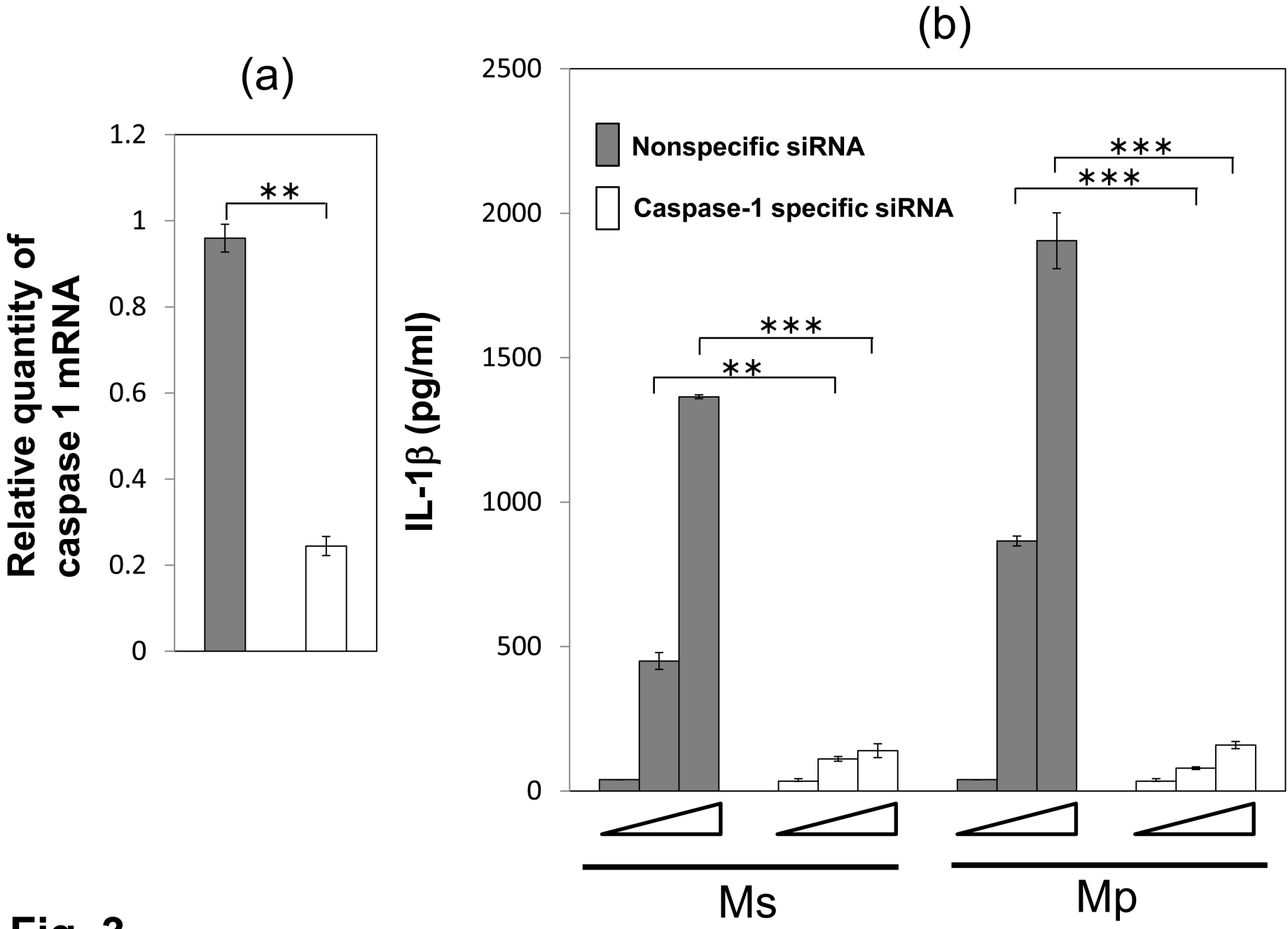
(a)
Relative quantity of caspase 1 mRNA
1.2
1
0.8
0.6
0.4
0.2
0
**
(b)
IL-1β (pg/ml)
2500
2000
1500
1000
500
0
Nonspecific siRNA
Caspase-1 specific siRNA
***
**
***
***
Ms
Mp

Fig. 3

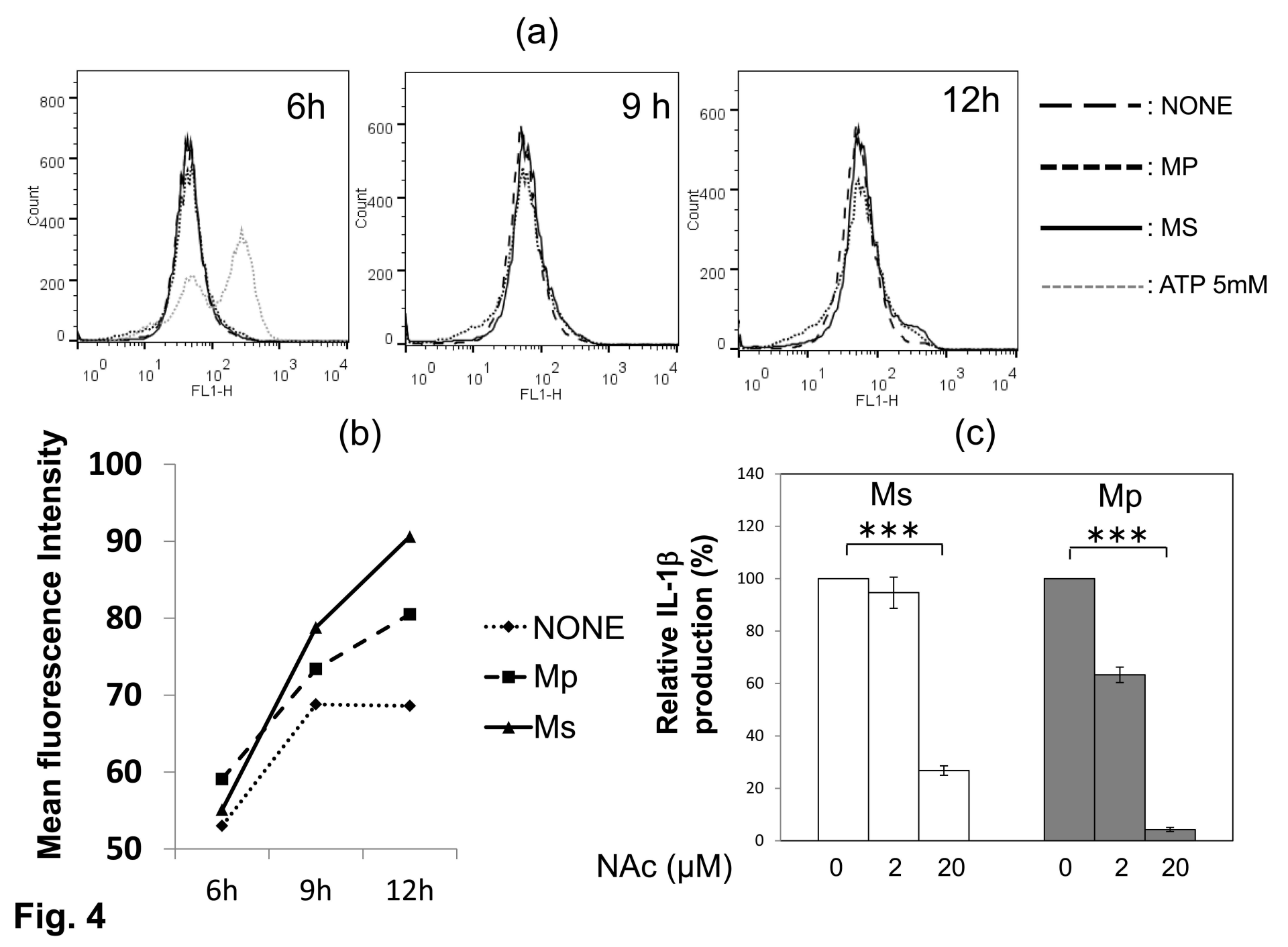
(a)
6h
9 h
12h
Count
FL1-H
: NONE
: MP
: MS
: ATP 5mM
(b)
Mean fluorescence Intensity
100
90
80
70
60
50
6h
9h
12h
NONE
Mp
Ms
(c)
Relative IL-1β production (%)
140
120
100
80
60
40
20
0
Ms
Mp
***
***
NAc (μM)
0
2
20
0
2
20

Fig. 4

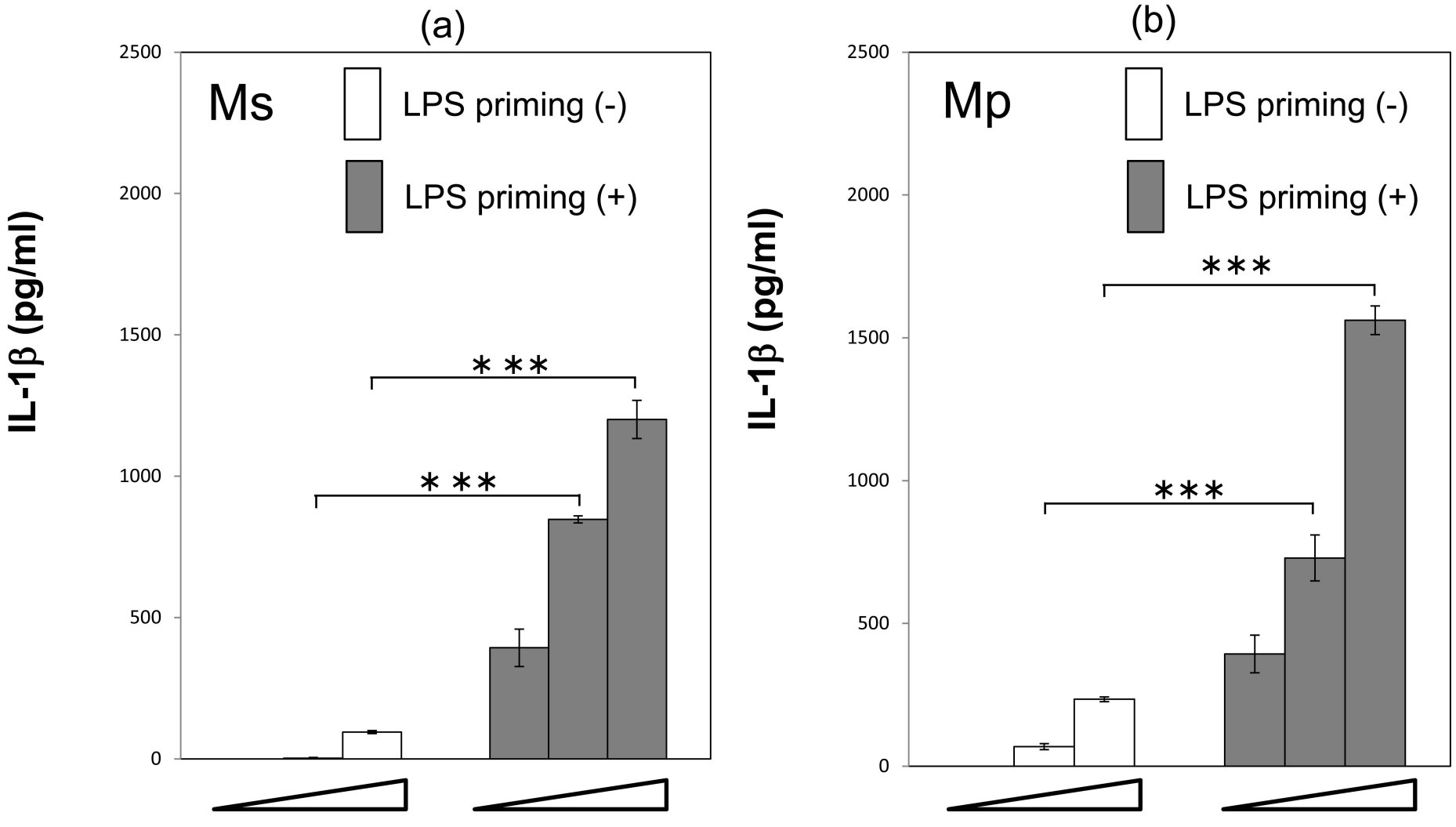
(a)
Ms
LPS priming (-)
LPS priming (+)
IL-1β (pg/ml)
2500
2000
1500
1000
500
0
***
***
(b)
Mp
LPS priming (-)
LPS priming (+)
IL-1β (pg/ml)
2500
2000
1500
1000
500
0
***
***

Fig. 5

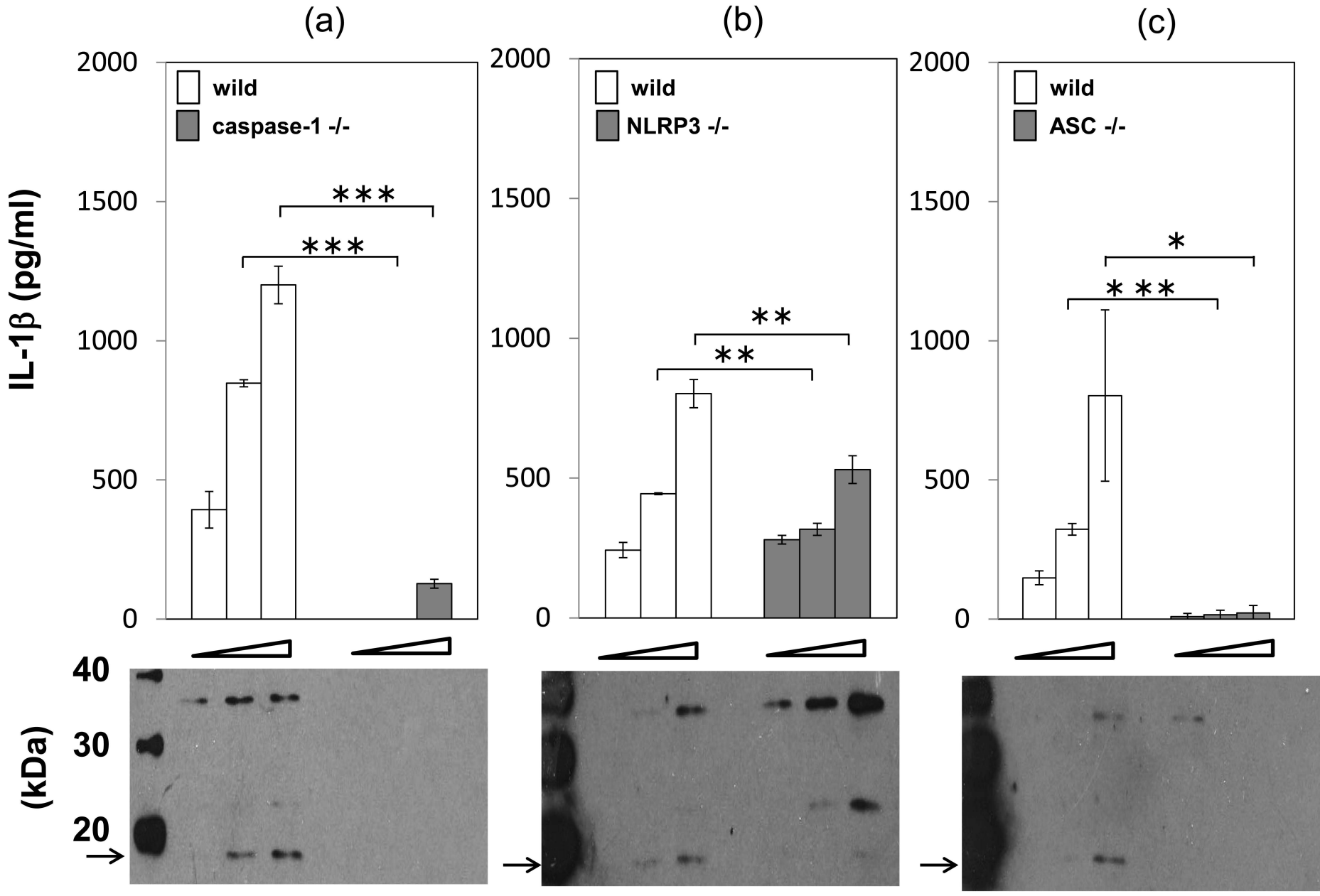
(a)
(b)
(c)
IL-1β (pg/ml)
2000
1500
1000
500
0
wild
caspase-1 -/-
NLRP3 -/-
ASC -/-
***
***
**
**
*
***
(kDa)
40
30
20

Fig. 6

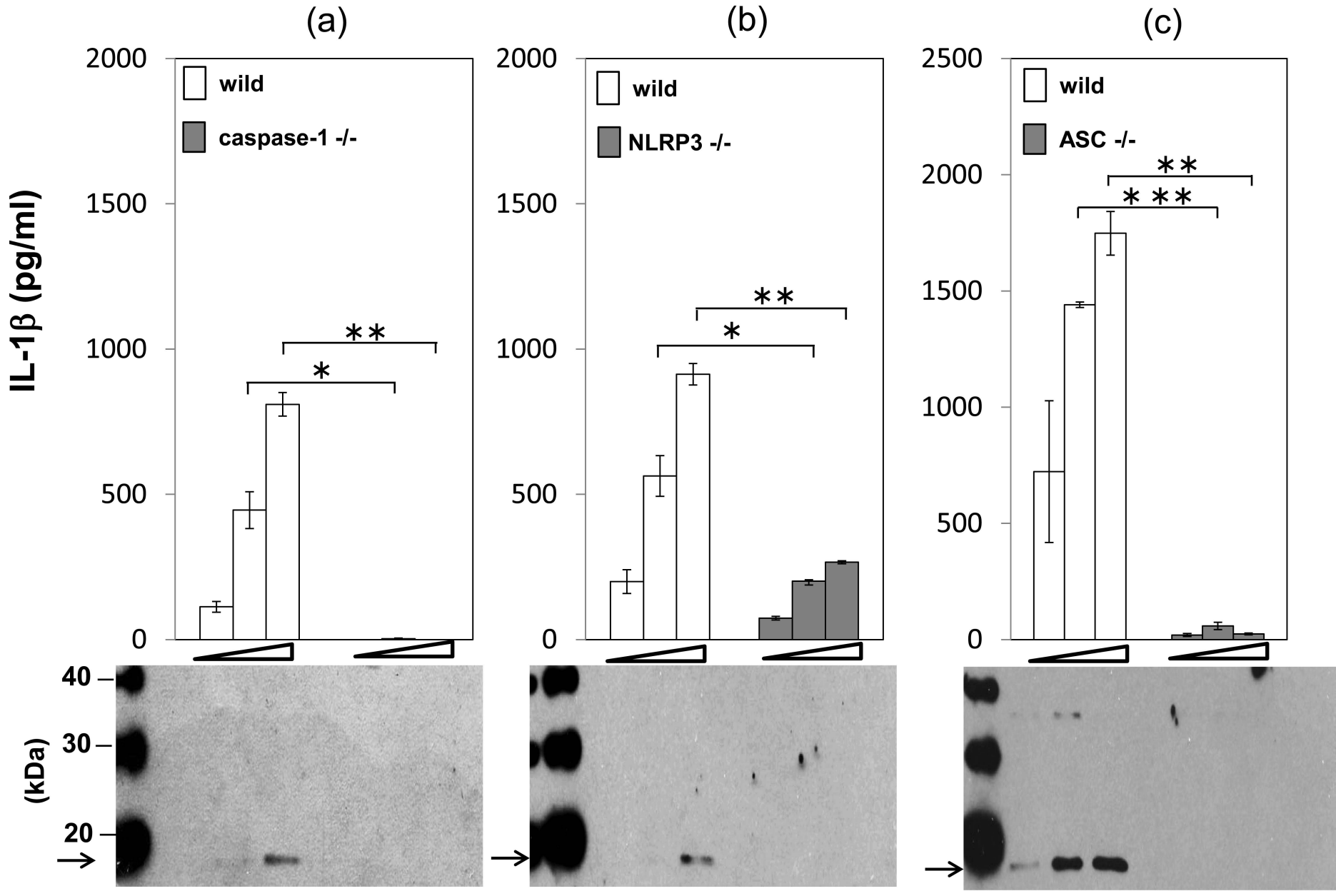
(a)
(b)
(c)
IL-1β (pg/ml)
2000
1500
1000
500
0
2500
wild
caspase-1 -/-
NLRP3 -/-
ASC -/-
*
**
***
(kDa)
40
30
20

Fig. 7

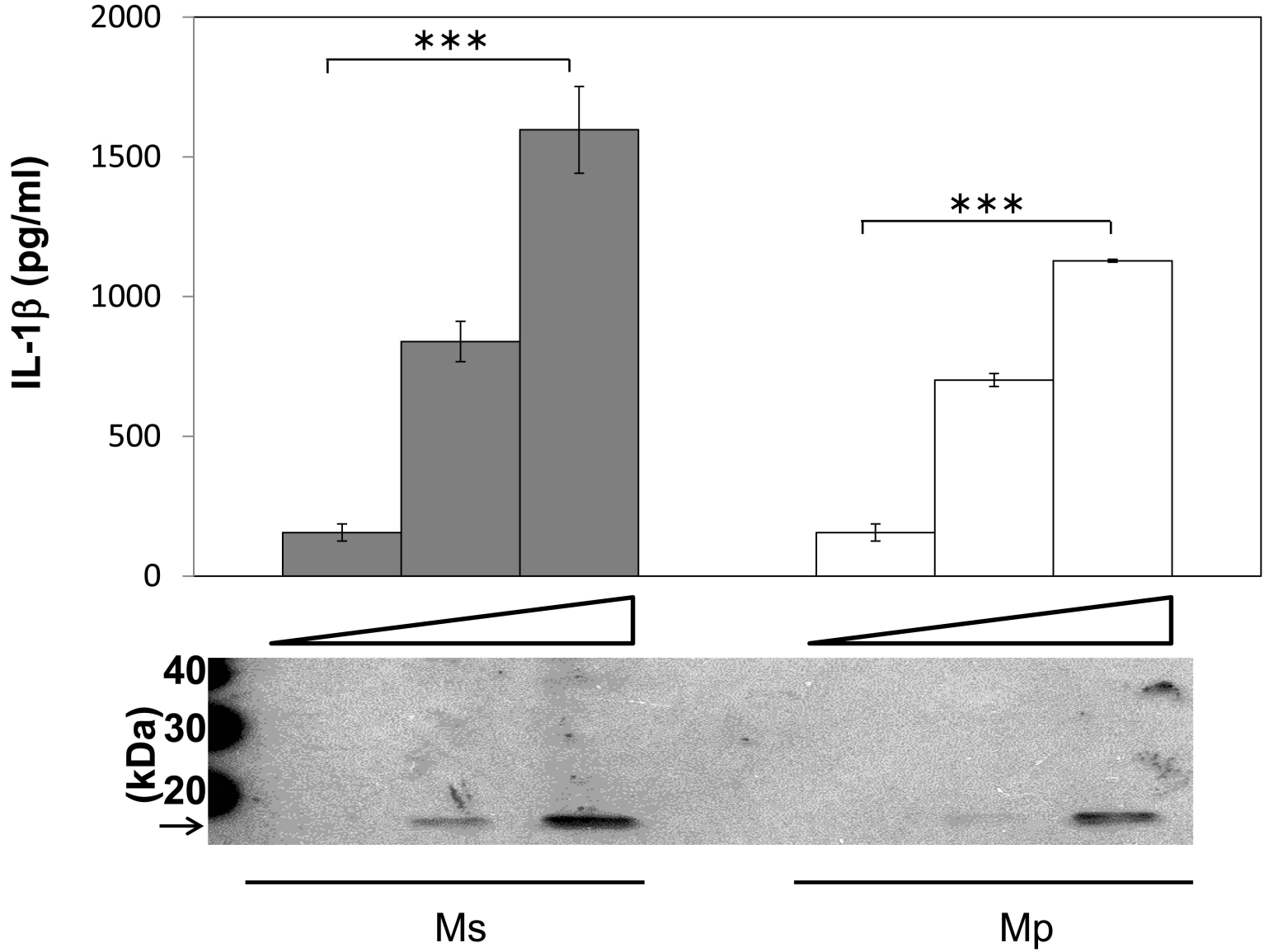

Fig. 8