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Activation of NLRP3 inflammasome in dendritic cells and macrophages by *Mycoplasma salivarium*  

*(Mycoplasma salivarium による樹状細胞ならびにマクロファージにおける NLRP3 インフラマソーム活性化)*

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Abstract

The proinflammatory cytokine IL-1β plays crucial roles in the pathogenesis of periodontal disease. IL-1β is produced after processing of pro-IL-1β by caspase-1, which is activated by the multiprotein complex consisting of NLR, an adaptor protein and procaspase-1, called an the inflammasome. Mycoplasma salivarium, human oral microbial flora, preferentially inhabits the gingival sulcus and is considered to play an etiological role in periodontal diseases. As a first step to clarify the etiological role of the organism in periodontal diseases, this study was designed to clarify whether it induces production of IL-1β by innate immune cells such as dendritic cells or macrophages by using M. pneumoniae, which is known to induce production of IL-1β by human macrophages, as a positive control.

It was found that both live and heat-killed cells of M. salivarium induced production of IL-1β by the murine dendritic cell line XS106 cells and also necrotic cell death called pyroptosis in the cells. The activity was significantly and partially downregulated by silencing of caspase-1 and NLRP3, respectively. BMMs from wild-type and NLRP3-, ASC- and caspase-1-deficient mice were examined for IL-1β production in response to the mycoplasma. Live cells of M. salivarium almost completely lost the activity to induce IL-1β production by macrophages derived from ASC- and caspase-1-deficient mice. In addition, the activity toward BMMs from NLRP3-deficient mice was significantly but not completely attenuated. These results suggest that live cells of M. salivarium are able to activate several types of inflammasome, including the NLRP3 inflammasome, to produce IL-1β.

Taken together, the results of this study suggests that M. salivarium plays an etiological role in periodontal diseases through induction of inflammatory IL-1β.
production by dendritic cells and macrophages that have infiltrated inflamed gingival connective tissue.

**Introduction**

Mycoplasmas, the smallest self-replicating and cell wall-less microorganisms, cause various infectious diseases in humans and animals such as atypical pneumonia, non-gonococcal urethritis, and arthritis (1). *Mycoplasma salivarium*, a member of the human oral microbial flora, preferentially inhabits the gingival sulcus (2). The incidence and number of organisms in oral cavities increase significantly with the advancement of periodontal disease, osteitis, pericoronitis and temporomandibular disorders (2-5). Furthermore, significantly higher antibody responses occur in patients with periodontal disease than in healthy individuals (3, 4). Thus, *M. salivarium* is considered to play some etiological role in oral infectious diseases, especially periodontal disease.

Interleukin-1β (IL-1β), a proinflammatory cytokine, induces production of inflammatory mediators, osteoclast formation, matrix metalloproteinase expression and death of matrix-producing cells in periodontal tissues, resulting in destruction of alveolar bone and periodontal connective tissue (6). Thus, IL-1β plays crucial roles in the onset and progression of periodontal disease. IL-1β is produced after processing of pro-IL-1β by caspase-1 activated by an inflammasome (7-9). An inflammasome is an intracellular multiprotein complex consisting of NLRs (nucleotide-binding domain leucine-rich repeat-containing receptor), the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase-recruitment domain) and procaspase-1. 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,
certain cell type, which might be important in restricting the intracellular replication of invasive bacterial pathogens (9).

Therefore, as a first step to address etiological roles of the organism in periodontal diseases, the aim of this study was to determine whether M. salivarium induces IL-1β production by innate immune cells such as dendritic cells or macrophages through activation of the intracellular sensor inflammasome.

**Materials and methods**

**Mycoplasmas and culture conditions**

M. salivarium ATCC 23064 and M. pneumoniae ATCC15492 were grown in PPLO broth (Difco Laboratories, Detroit, Mich.) supplemented with 20% (v/v) horse serum (Gibco, Grand Island, NY), 1% (w/v) yeast extract (Difco), 1% (w/v) L-arginine hydrochloride for M. salivarium or 1% (w/v) D-glucose for M. pneumoniae and 1000 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. When heat-killed cells were used, the cell suspensions were treated for 5 min in boiling water. The protein concentration was determined by the method of Dully and Grieve (10).

**Mice**

Sex-matched 8-week-old C57BL/6 (B6) mice were purchased from Japan Clea (Tokyo, Japan) and maintained in specific pathogen-free conditions in our animal facility at the Graduate School of Medicine, Hokkaido University. Caspase-1-, NLRP3-
or ASC-deficient mice (caspase-1<sup>−/−</sup>, NLRP3<sup>−/−</sup> or ASC<sup>−/−</sup>) on the same genetic background were maintained in specific pathogen-free conditions in our animal facility at the Graduate School of Medicine, University of the Ryukyus.

All experiments were carried out in accordance with the regulations of the Animal Care and Use Committee of both universities.

**Cell culture**

XS106, a murine DC cell line, kindly provided by Professor Akira Takashima (University of Texas South-Western, TX), is a long-established DC line derived from the epidermis of newborn A/J mice (11). This cell line was cultured in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO) containing 10% (v/v) FBS (Invitrogen Corp., Durham, NC), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, 100 μg/ml each of penicillin G and streptomycin (Sigma), 50 μM 2-mercaptoethanol (Sigma), 0.5 ng/ml murine recombinant GM-CSF (Pepro Tech. Rocky Hill, NJ) and 5%(v/v) culture supernatant derived from the NS47 fibroblast cell line (hereafter referred to as XS medium) (12).

**Measurements of IL-1β**

XS106 cells were added to a 24-well plate at a concentration of 3×10<sup>5</sup> cells per well in 500 μl of XS medium and incubated 37 °C for 16 h at an atmosphere of 5% CO<sub>2</sub>. The cultures were centrifuged at 400 g for 5 min and the cells were washed with RPMI1640 base medium, which does not contain any supplements. The cells were resuspended in 300 μl of RPMI1640 base medium and incubated at 37 °C for 24 h with 0, 36.5 and 365 μg of proteins of intact cells or heat-killed cells of *M. salivarium* and *M. pneumoniae*. 
The amounts of IL-1β in cell culture supernatants were measured by an ELISA kit (OptEIA™ SET Mouse IL-1β, BD Biosciences, San Jose, CA). To distinguish mature IL-1β from pro-IL-1β, SDS-PAGE of cell culture supernatants was performed, and proteins were transferred to a Nitrocellulose Transfer Membrane and reacted with the appropriate antibody against IL-1β (R&D Systems, Minneapolis, MN).

**Cell death assay**

XS106 cells were added to a 24-well plate at a concentration of $3 \times 10^5$ cells per well in 500 μl of XS medium and incubated 37 °C for 16 h at an atmosphere of 5% CO₂. The cultures were centrifuged at 400 g for 5 min and the cells were washed with RPMI1640 base medium and incubated at 37 °C for 24 h with 365 μg of proteins of live cells of *M. salivarium* and *M. pneumoniae*. The cells were stained by using an Annexin-V-FLUOS Staining Kit (Roche, Branford, CT) and then analyzed by a FACS Calibur flow cytometer (BD Bioscience). Data for 20000 cells falling within appropriate forward and side light scatter gates were collected from each sample (12). Data were analyzed using CellQuest software (BD Bioscience).

**Reactive oxygen species (ROS) production**

XS106 cells were added to a 24-well plate at a concentration of $3 \times 10^5$ cells per well in 500 μl of XS medium and incubated 37 °C for 16 h at an atmosphere of 5% CO₂. The cultures were centrifuged at 400 g for 5 min and the cells were washed with RPMI1640 base medium. The cells were suspended and incubated at 37 °C for 6, 9 and 12 h in RPMI1640 base medium containing Dihydrorhodamine123 (Sigma) with 365 μg of proteins of live cells of *M. salivarium* and *M. pneumoniae* and then analyzed by a FACS
Calibur flow cytometer (BD Bioscience). Data for 20000 cells falling within appropriate forward and side light scatter gates were collected from each sample. Data were analyzed using CellQuest software (BD Bioscience).

**RNA interference**

Silencing of caspase-1 in XS106 cells was performed as follows. siRNA specific for caspase-1 (Applied Biosystems) or non-targeting siRNA as a control, final concentrations of which were 10 μM, was transfected into XS106 cells (2×10^6 cells/0.1 ml of Opti-MEM) by electroporation using a two-step electroporator (CUY21 Pro-Vitro, NEPA GENE, Tokyo, Japan) at 175 V and 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 then inoculated to a 24-well plate at a concentration of 3×10^5 cells per well. After 8-h incubation, the cells were stimulated at 37°C for 24 h with *M. salivarium* or *M. pneumoniae*.

Silencing of NLRP3 in XS106 cells was performed as follows. The plasmids psiRNA-mNLRP3 and psiRNA-LucGL3 (InvivoGen, San Diego, CA), which express NLRP3-specific and nonspecific siRNA, respectively, were transfected into XS106 cells by electroporation as described above. Each transfectant that stably expresses NLRP3-specific or nonspecific siRNA was established by being selected 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 the ReliaPrep™RNA Cell Miniprep System (Promega, Mannheim, Germany), and cDNA was synthesized with the High Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). 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\textsubscript{T}) value is defined as the number of PCR cycles in which the fluorescence signal exceeds the detection threshold value. The normalized amount of target mRNA (Nt) was calculated from the C\textsubscript{T} value obtained for both target and GAPDH mRNA with the equation \( N_t = 2^{C_t(GAPDH)-C_t(target)} \). Relative mRNA expression was obtained by setting Nt in non-stimulated samples to 1 in each experiment.

**Bone marrow-derived macrophages (BMMs) and measurements of IL-1\(\beta\)**

Femurs and tibias prepared from caspase-1\(^{-/-}\), NLRP3\(^{-/-}\) or ASC\(^{-/-}\) mice at 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% FBS (Invitrogen), 100 \(\mu\)g/ml each of penicillin G and streptomycin (Sigma). Cells were suspended by pipetting and washed by centrifugation. The cells were cultured in a nontissue culture plastic 100 mm petri dish in RPMI-1640 medium containing 10% FBS (Invitrogen), 100 \(\mu\)g/ml each of penicillin G and streptomycin (Sigma), and cell conditioned medium (culture supernatant derived from the L929 fibroblast cell line). After 7-9 days of culture, macrophages that were loosely adherent to the dishes were harvested with cold PBS and used as BMMs (13).

BMMs were added to a 24-well plate at a concentration of 4\(\times\)10\(^5\) cells per well in 500 \(\mu\)l of RPMI1640 medium (GIBCO) containing 10% (v/v) FBS and incubated 37 \(^\circ\)C
for 4 h with 1 μg/ml ultrapure *E. coli* LPS (InvivoGen) and then for 24 h with 0, 0.365 and 3.65 μg of proteins of intact cells of *M. salivarium*. The amounts of IL-1β (IL-1β) in cell culture supernatants were measured by an ELISA kit (OptEIA™ SET Mouse IL-1β, BD Biosciences). To distinguish mature IL-1β from pro-IL-1β, SDS-PAGE of cell culture supernatants was performed, and the proteins were transferred to a Nitrocellulose Transfer Membrane and reacted with the appropriate antibody against IL-1β (R&D Systems).

**Results**

**Production of IL-1β by XS106 cells in response to mycoplasma cells**

XS106 cells were stimulated for 24 h with various amounts of live or heat-killed cells of *M. salivarium* or *M. pneumoniae*, 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 the mycoplasma has been shown to induce IL-1β production by human monocytes/macrophages (14). Both live and heat-killed cells of *M. salivarium* induced production of IL-1β by XS106 cells (Fig. 1a) as did *M. pneumoniae* cells (Fig. 1b). However, mature IL-1β is not discriminated from pro-IL-1β by ELISA because of the nature of the anti-IL-1β antibody used in the ELISA kit. Therefore, production of mature IL-1β (17.5 kDa) was evaluated by the molecular weight of the band detected by Western blotting. As a result, it was found that both live and heat-killed cells of *M. salivarium* or *M. pneumoniae* induced production of mature IL-1β by XS106 cells (Fig. 1a and b lower) and there was no great difference in the activity between *M. salivarium* and *M. pneumoniae* (Fig. 1). In addition, the IL-1β-inducing activity of live cells was significantly higher than that of heat-killed
Involvement of caspase-1 in IL-1β-inducing activity

Secretion of mature IL-1β was induced after processing of pro-IL-1β by caspase-1, which is activated by the inflammasome (7). Therefore, the next experiment was designed to clarify the involvement of caspase-1 in the IL-1β-inducing activities of these mycoplasmas by silencing of caspase-1 mRNA with transfection of caspase-1-specific siRNA. The transfection induced approximately 75% downregulation of the mRNA (Fig. 2a) and downregulated by more than 90% the IL-1β-inducing activities of *M. salivarium* and *M. pneumoniae* (Fig. 2b), strongly suggesting that caspase-1 was 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 (7). The inflammasome is a complex consisting of pro-caspase-1, ASC and cytosolic NLR (7). NLRP3 is a representative cytosolic NLR that recognizes various bacterial pathogens (15) (16). Therefore, we hypothesized that these 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, we established transfectants, as described above, that express NLRP3-specific or non-specific siRNA. These transfectants were examined for production IL-1β after being stimulated with live cells of *M. salivarium* and *M. pneumoniae*. Although the expression of NLRP3 mRNA was downregulated by
approximately 20% in the transfectant expressing NLRP3-specific siRNA when compared with that in the transfectant expressing non-specific siRNA (Fig. 3a), the IL-1β-inducing activities of both mycoplasmas toward the transfectant expressing NLRP3-specific siRNA were significantly or not significantly lower than those toward the transfectant expressing non-specific siRNA (Fig. 3b)

**Production of ROS by XS106 cells in response to mycoplasma cells**

ROS are known to trigger activation of NLRP3 inflammasome (15). In addition, we have reported that mycoplasma cells induce ROS production through recognition by Toll-like receptor 2 (17). Therefore, the next experiment was carried out to determine whether mycoplasma cells induce ROS production by XS106 cells. ATP was used as a positive control, because it is known that ATP induces ROS production (18). We found that both mycoplasmas accelerated ROS production by XS106 cells, although the cells produce ROS spontaneously without any stimulators (Fig. 4a, b).

The results suggest that the NLRP3 inflammasome activator ROS are involved in the IL-1β-inducing activities of these mycoplasma cells. In order to further confirm this, we examined the effects of NAc (N-acetylcysteine, an ROS inhibitor) on the activities. It was found that NAc downregulated the activities in a dose-dependent manner (Fig. 4c). These results also suggest that these mycoplasma cells induce activation of the NLRP3 inflammasome in murine dendritic cells to produce IL-1β.

**IL-1β-inducing activity toward BMMs from B6, caspase-1<sup>−/−</sup>, NLRP3<sup>−/−</sup> or ASC<sup>−/−</sup> mice**

As stated above, live cells of *M. salivarium* and *M. pneumoniae* are able to activate
the NLRP3 inflammasome to produce IL-1β in murine dendritic cells. The NLRP3 inflammasome is a protein complex consisting of NLRP3, ASC and procaspase-1 (9, 15). In order to further confirm whether live cells of \textit{M. salivarium} activate the NLRP3 inflammasome, BMMs from B6 and NLRP3-, ASC- and caspase-1-deficient mice were examined for IL-1β production in response to live cells of the mycoplasma. BMMs were stimulated for 4 h with LPS and then for 24 h with mycoplasma cells, because LPS priming significantly enhanced the IL-1β-inducing activities of these mycoplasma cells (Fig. 5). LPS itself had activity to induce production of IL-1β by B6-derived BMMs (Fig. 5), which had already been reported (19, 20). However, live cells of \textit{M. salivarium} almost completely lost the activity to induce IL-1β production by BMMs derived from caspase-1\textsuperscript{-/} and ASC\textsuperscript{-/} mice (Fig. 6a and 6c). In addition, the activity toward BMMs from NLRP3\textsuperscript{-/} mice was significantly but not completely attenuated (Fig. 6b). These results suggest that live cells of \textit{M. salivarium} are able to activate some types of inflammasome including the NLRP3 inflammasome.

**Induction of pyroptosis of XS106 cells by mycoplasma cells**

NLRP3 activation triggers an inflammatory caspase-1-dependent death process termed pyroptosis (21). Pyroptosis is similar morphologically to necrosis, which is characterized by cell expansion, explosion and lysis (22). To determine whether mycoplasma cells induce pyroptosis in XS106 cells, XS106 cells were stained with PI and Annexin V after stimulation with these mycoplasma cells and analyzed by flow cytometry. Both live cells of \textit{M. salivarium} and \textit{M. pneumoniae} induced necrosis-like cell death in XS106 cells (Fig.7). This cell death was considered to be pyroptosis because it was accompanied by NLRP3 inflammasome-dependent IL-1β production as
described above.

**Discussion**

It has been reported that various bacterial pathogens induce caspase-1-dependent IL-1β production though activation of the NLRP3 inflammasome (16, 23). In this study, we also demonstrated that caspase-1 is involved in the IL-1β-inducing activities of *M. salivarium* and *M. pneumoniae* by silencing of caspase-1 mRNA (Fig. 2a, b). The IL-1β-inducing activities of both mycoplasmas toward the XS106 transfectant expressing NLRP3-specific siRNA were significantly or not significantly downregulated compared with those toward the transfectant expressing non-specific siRNA (Fig. 3b). Thus, the involvement of NLRP3 in IL-1β-inducing activity of these mycoplasmas was ambiguous. Therefore, next experiment was designed to determine whether XS106 cells produce ROS in response to these mycoplasmas, because ROS are known to trigger activation of the NLRP3 inflammasome (15) and we have reported that mycoplasma cells induce ROS production by monocytes through the recognition by Toll-like receptor 2 (17). We found that mycoplasma cells induce ROS production by XS106 cells. These results suggest that *M. salivarium* and *M. pneumoniae* activate the NLRP3 inflammasome, possibly through ROS production, in XS106 cells to induce IL-1β production. In order to further confirm this, BMMs derived from B6, NLRP3-, ASC- and caspase-1-deficient mice were examined for IL-1β production in response to *M. salivarium* cells after LPS priming. As a result, it was found that *M. salivarium* completely lost the activity to induce IL-1β production by BMMs derived from caspase-1<sup>+</sup> and ASC<sup>+</sup> mice, whereas the activity toward BMMs derived from NLRP3<sup>−/−</sup> mice was significantly but not completely downregulated (Fig. 6a). These results
suggest that live cells of *M. salivarium* are able to activate the NLRP3 inflammasome and also some types of inflammasome, such as NLRP1, NLRC4 and AIM2, that require ASC and caspase-1. At present, we think it very likely that *M. salivarium* activates the AIM2 inflammasome, because it is activated by bacterial DNA (15) and mycoplasma cells are easily disrupted due to the lack of cell wall. In addition, it has been reported that whole cells of one bacterial strain are able to activate plural types of inflammasome to produce IL-1β. For example, *Listeria monocytogenes, Pseudomonas aeruginosa, Candida albicans* and *Mycobacterium tuberculosis* induce IL-1β production through some types of inflammasome (23) (16) (24) (25). Therefore, studies are in progress to investigate involvement of the AIM2 inflammasome in IL-1β-inducing activity of *M. salivarium*.

As described earlier, *M. salivarium*, human oral microbial flora, preferentially inhabits the gingival sulcus and plays some etiological roles in periodontal disease (2-5). IL-1β plays pathological roles in the disease by inducing production of inflammatory mediators, osteoclast formation, matrix metalloproteinase expression and death of matrix-producing cells in the periodontal tissues (6). It has been reported that the level of IL-1β is increased in periodontitis patients compared with that in healthy individual (26). The representative periodontopathogenic bacterium *Porphyromonas gingivalis* has been reported to induce IL-1β secretion and pyroptosis through activation of NLRP3 and AIM2 inflammasomes (26). In addition, *Aggregatibacter actinomycetemcomitans*, a representative pathogen in aggressive periodontitis, has also been reported to induce IL-1β secretion by macrophages, although the mechanism remains unknown (27).

Taken together, the present finding that *M. salivarium* has activity for inducing IL-1β production and pyroptosis in dendritic cells and macrophages through activation
of an inflammasome strongly suggests that *M. salivarium* plays some etiological role in the onset and progression of periodontal disease as described previously (2-4).

**References**


Figure legends

Fig. 1. Production of IL-1β by XS106 cells in response to mycoplasma cells.
XS106 cells were stimulated at 37 °C for 24 h with various amounts (0, 36.5, 365 μg/ml of proteins) of live or heat-killed cells of *M. salivarium* (Ms) (a) or *M. pneumonia* (Mp) (b). The amounts of total IL-1β released into the culture supernatant were measured by ELISA. Results are expressed as mean ± SD of values from three separate experiments. 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. 2. Involvement of caspase-1 in 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. Results are expressed as mean ± SD of values from four separate experiments (a). The transfected cells were stimulated at 37 °C for 24 h with various amounts (0, 36.5, 365 μg of protein/ml of proteins) of live cells of *M. salivarium* (Ms) or *M. pneumonia* (Mp). The amounts of total IL-1β released into the culture supernatant were measured by ELISA. Results are expressed as mean ± SD of values from three separate experiments (b).

Student’s t test (*, 0.01< P < 0.05, **, 0.01< P < 0.001, ***, P < 0.001).

Fig. 3. Involvement of NLRP3 in IL-1β-inducing activity of mycoplasmas.
XS106 cells were transfected with psiRNA-mNLRP3 or psiRNA-LucGL3 which express NLRP3-specific and non-specific siRNA. Relative mRNA expression was
determined by real-time PCR analysis. Results are expressed as mean ± SD of values from four separate experiments (a). The transfected cells were stimulated at 37 °C for 24 h with various amounts (0, 36.5, 365 μg of protein/ml of proteins) of live cells of *M. salivarium* (Ms) or *M. pneumonia* (Mp). The amounts of total IL-1β released into the culture supernatant were measured by ELISA. Results are expressed as mean ± SD of values from three separate experiments (b).

Student’s t test (*, 0.01< P < 0.05, **, 0.01< P < 0.001, ***, P < 0.001).

Fig. 4. Production of ROS by XS106 cells in response to mycoplasma cells.

XS106 cells were stimulated at 37 °C for 6, 9 and 12 h with live cells of *M. salivarium* (Ms) or *M. pneumonia* (Mp) (365 μg of protein) or ATP (5 mM) in RPMI1640 base medium containing Dihydrorhodamine123, and then ROS production was 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 37 °C for 24 h with live cells of Ms or Mp (365 μg of protein). The amounts of total IL-1β released into the culture supernatant were measured by ELISA. Results are expressed as mean ± SD of values from three separate experiments.

Student’s t test (*, 0.01< P < 0.05, **, 0.01< P < 0.001, ***, P < 0.001).

Fig. 5. Effect of LPS priming on IL-1β–inducing activity of mycoplasmas toward BMMs from B6 mice.

BMMs were stimulated at 37 °C for 4 h with or without LPS and then for 24 h with live cells (0, 0.365, 3.65 μg of protein) of *M. salivarium* (Ms) or *M. pneumonia* (Mp). The amounts of total IL-1β released into the culture supernatant were measured by
ELISA.

Results are expressed as mean ± SD of values from three separate experiments.

Student’s t test (*, 0.01< P < 0.05, **, 0.01< P < 0.001, ***, P < 0.001).

Fig. 6. Production of IL-1β by BMMs from B6, caspase-1<sup>−/−</sup>, NLRP3<sup>−/−</sup> or ASC<sup>−/−</sup> mice in response to mycoplasma cells.

BMMs were stimulated at 37 °C for 4 h with LPS and then for 24 h with live cells (0, 0.365, 3.65 μg of protein) of <i>M. salivarium</i> (Ms). The amounts of total IL-1β released into the culture supernatant were measured by ELISA. Results are expressed as mean ± SD of values from three separate experiments. 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. Induction of pyroptosis of XS106 cells by mycoplasma cells.

XS106 cells were stimulated at 37 °C for 24 h with live cells (365 ug/ml of proteins) of <i>M. salivarium</i> (Ms) or <i>M. pneumonia</i> (Mp). XS106 cells were stained with PI and Annexin V after stimulation and analyzed by flow cytometry. The number of percentage in the square surrounded by bold line is the sum of Q1 and Q2, which shows percentages of cells died by necrosis.
Fig. 1

IL-1β (pg/ml)

(a) Live vs. Heat killed

(b) Live vs. Heat killed

Ms

Mp

15 20 25 37 (kDa)

15 20 25 37 (kDa)
Relative quantity of caspase 1 mRNA

IL-1β (pg/ml)

Nonspecific siRNA
Caspase 1 specific siRNA

Fig. 2
Fig. 3

(a) Relative quantity of NLRP3 mRNA

(b) IL-1β (pg/ml)

Nonspecific siRNA
NLRP3 specific siRNA

Ms
Mp

* n.s
**
Fig. 4

(a) Mean fluorescence intensity over time for different treatments:
- NONE
- MP
- MS
- ATP 5mM

(b) Relative IL-1β production (%)

(c) Graph showing relative IL-1β production with NAc (μM) levels:
- Ms
- Mp

* Significant difference
Fig. 5

**Ms**
- LPS priming (-)
- LPS priming (+)

**Mp**
- LPS priming (-)
- LPS priming (+)

IL-1β (pg/ml)

LPS priming (+) compared to LPS priming (-) shows a significant increase in IL-1β levels. The graphs indicate a statistically significant difference denoted by asterisks: **p < 0.01** and ***p < 0.001***.
Fig. 6

(a) 

![Chart](chart-a.png)

(b) 

![Chart](chart-b.png)

(c) 

![Chart](chart-c.png)
Fig. 7