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Cytoadherence-dependent induction of inflammatory responses by *Mycoplasma pneumoniae*

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Summary

Pathogenesis of *Mycoplasma pneumoniae* infection is considered to be in part attributed to excessive immune responses. *Mycoplasma pneumoniae* shows strong cytoadherence to host cells and this cytoadherence is thought to be involved in the progression of pneumonia. However, the interaction between the cytoadherence and the immune responses is not known in detail. In this study, we demonstrated that the induction of pro-inflammatory cytokines in the human monocyte cell line THP-1 is dependent on the property of cytoadherence of *M. pneumoniae*. A wild-type strain of *M. pneumoniae* with cytoadherence ability induced pro-inflammatory cytokines such as tumour necrosis factor-α and interleukin-1β (IL-1β). Whereas, heat-killed *M. pneumoniae* and cytoadherence-deficient mutants of *M. pneumoniae* caused significantly less production of pro-inflammatory cytokines than the wild-type strain. The wild-type strain induced pro-inflammatory cytokines in an endocytosis-independent manners, but the induction by heat-killed *M. pneumoniae* and cytoadherence-deficient mutants was dependent on endocytosis. Moreover, the wild-type strain induced caspase-1 production and ATP efflux, promoting the maturation of IL-1β and release of the pro-IL-1β precursor, whereas heat-killed *M. pneumoniae* and the cytoadherence-deficient mutants failed to induce them. These data suggest that the cytoadherence ability of *M. pneumoniae* activates immune responses and is involved in the pathogenesis of *M. pneumoniae* infection.

Keywords: cytoadherence; inflammasome; mycoplasma; THP-1; toll-like receptors

Introduction

Mycoplasmas are wall-less parasitic bacteria, and the smallest organisms capable of self-replication.[1] *Mycoplasma pneumoniae* causes primary atypical pneumonia, tracheobronchitis, pharyngitis and asthma in humans.[2–4] However, pathogenic agents such as endotoxins and exotoxins that cause such diseases have not been identified in *M. pneumoniae*. Cytoadherence of invading mycoplasma to the respiratory epithelium, localized host cell injury, and overaggressive inappropriate immune responses seem to contribute to the pathogenesis of *M. pneumoniae* infection.[2,5]

We previously identified three lipoproteins responsible for nuclear factor-κB (NF-κB) activation.[6] One was MPN602, a subunit b of F_0F_1-type ATPase (F_0F_1-ATPase),[7] a diacylated lipoprotein. The activation of NF-κB by F_0F_1-ATPase was dependent on Toll-like receptor 1 (TLR1), TLR2 and TLR6. We identified two other lipoproteins, MPN611 and MPN162, and designated them NF-κB-activating lipoprotein 1 (N-ALP1) and N-ALP2, respectively. N-ALP1 and N-ALP2 activated TLR signalling

Abbreviations: Ac-WEHD-CHO, Ac-Trp-Glu-His-Asp-H; DAMPs, stress associated danger signals; F_0F_1-ATPase, F_0F_1-type ATPase; HMW, high molecular weight; IL, interleukin; NF-κB, nuclear factor-κB; NLR, nucleotide-binding domain, leucinerich repeat-containing; oATP, oxidized ATP; PAMPs, pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cells; TLR, toll-like receptor; TNF, tumor necrosis factor; z-VAD-FMK, z-Val-Ala-Asp(oMe)-CH2F.

through TLR1 and TLR2. Both N-ALP1 and N-ALP2 were assumed to be triacylated lipoproteins based on their functional analysis.[8]

Toll-like receptors with the function of pattern-recognition receptors play critical roles in early innate recognition and inflammatory responses by the host against invading microbes.[9,10] Among 10 TLR family members reported, TLR2, TLR4, TLR5 and TLR9 have been implicated in the recognition of different bacterial components. Peptidoglycan, lipoarabinomannan, zymosan and lipoproteins from various microorganisms are recognized by TLR2.[11–17] On the other hand, lipopolysaccharide, bacterial flagellin and bacterial DNA are recognized by TLR4, TLR5 and TLR9, respectively.[18–21] These TLR family members are shown to activate NF-κB via interleukin-1 receptor (IL-1R) -associated signal molecules, including myeloid differentiation protein, IL-1R-activated kinase, tumour necrosis factor receptor-associated factor 6 and NF-κB-inducing kinase.[22]

Interleukin-1β is an important pro-inflammatory cytokine and mature IL-1β is produced by cleavage of the pro-IL-1β precursor by caspase-1.[23] Caspase-1 is activated within a large protein complex called the inflammasome.[24] The inflammasome is composed of nucleotide-binding domain, leucine-rich repeat-containing (NLR) proteins, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain, and caspase-1. The inflammasome responds to a variety of signals. Intracellular bacterial toxins,[25] pathogen-associated molecular patterns (PAMPs) such as muramyl dipeptide[26] or flagellin,[27] the stress-associated danger signals (DAMPs) such as ATP[28–30] or monosodium urate[28] activate the inflammasome. The ATP activates the inflammasome through binding to the P2X7 receptor followed by potassium efflux[31] and recruitment of pore-forming hemi-channel, pannexin-1.[32] Phagocytosis-dependent production of reactive oxygen species is also reported to stimulate the inflammasome.[33,34]

Cytoadherence by *M. pneumoniae* in the respiratory tract is the initial event of infection. The cytoadherence is mediated by P1 adhesin and other additional proteins such as P30 or high molecular weight (HMW) proteins.[35–38] Although the cytoadherence of *M. pneumoniae* is thought to be responsible for the pathogenesis of *M. pneumoniae* infection,[39,40] it is not known precisely how the cytoadherence is involved in inflammatory responses.

In this study, we examined the interaction between the cytoadherence and inflammatory responses. A wild-type strain of *M. pneumoniae* with cytoadherence ability induced pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and IL-1β, whereas heat-killed *M. pneumoniae* or cytoadherence-deficient mutants induced significantly fewer pro-inflammatory cytokines than the wild-type strain. The wild-type strain of *M. pneumoniae* induced TNF-α and the precursor of IL-1β in an endocytosis-independent manner, and also activated caspase-1 production and ATP efflux, promoting the maturation of IL-1β. These data suggest that the cytoadherence capability of *M. pneumoniae* activates inflammatory responses and is involved in the pathogenesis of *M. pneumoniae* infection.

Materials and methods

Mycoplasma pneumoniae *strains*

Cytoadherence-deficient mutants of *M. pneumoniae*, M5, M6, class I, class II, class III and class IV were obtained from Dr Miyata.[41] These mutants originated from wild-type strain M129.[42–45] The *M. pneumoniae* wild-type strain (M129) and cytoadherence-deficient mutants were cultured in Pleuropneumonia-like organisms (PPLO) broth (Difco, Franklin Lakes, NJ, USA) containing 10% horse serum, 0·25% glucose, 0·25% yeast extract, 0·002% phenol red, at pH 7·6. Each strain was cultured to the beginning of a stationary phase (the colour of medium turned slightly orange). The amount of each strain was adjusted by optical density (OD_{595}) in PBS. The DNA of *M. pneumoniae* was purified using a FastPure DNA kit (Takara, Tokyo, Japan). The amounts of DNA were measured by optical density (OD_{280}).

Cells

Cells of a human monocytic cell line, THP-1 and a human lung squamous cell carcinoma cell line, EBC-1 were cultured in RPMI-1640 medium containing 10% fetal calf serum (Mitsubishi Chemical, Tokyo, Japan) and 2 mM L-glutamine. Cells of a human kidney cell line, 293T were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 2 mM L-glutamine. To exclude the possibility of mycoplasma infections in the cell cultures, these cell suspensions were inoculated onto PPLO agar medium (Difco) once a month. Cells were also stained with 4′,6-diamidino-2-phenylindole and contaminated mycoplasmas were checked once a week. Peripheral blood mononuclear cells (PBMC) were separated using Lymphoprep (Axis-Shield PoC, Oslo, Norway) and cultured in RPMI-1640 medium containing 10% fetal calf serum.

Cell treatment

A total of 2×10^5 cells/500 μl THP-1 or EBC-1 were cultured for 24 hr before infection in a 24-well plate. Cells were infected with 50 μl *M. pneumoniae* ($OD_{595} = 0{\cdot}1$) for 6 hr and the cells were harvested by centrifugation for 10 min at 300 ***g***. A total of 5×10^5 cells/500 μl PBMC were cultured for 3 hr before infection in a 24-well plate. Cells were infected with 50 μl *M. pneumoniae*

(OD_{595} = 0·01) for 3 hr and the cells were harvested by centrifugation for 10 min at 300 ***g***. Concentrations of pro-inflammatory cytokines in supernatants were measured by ELISA. The TLR2 was blocked by 10 μg/ml mouse anti-human TLR2 monoclonal antibody, IMG-416 (Imgenex, San Diego, CA, USA). Endocytosis was examined in the presence of 1·0 μM cytochalasin D or 100 nM bafilomycin A1 (Wako, Osaka, Japan). P2X7 receptor was blocked using 300 μM oxidized ATP (Sigma, St Louis, MO, USA). Inhibition of caspase-1 was performed with 100 μM z-Val-Ala-Asp(oMe)-CH2F (z-VAD-FMK) or Ac-Trp-Glu-His-Asp-H (Ac-WEHD-CHO) (Bachem, Bubendorf, Switzerland).

ELISA

Amounts of TNF-α in the supernatants of cell culture were measured using ELISA development kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Amounts of IL-1*β* in the supernatants of cell culture were measured using ELISA development kits (R&D Systems). This kit detects both precursor and mature forms of IL-1*β*. Amounts of mature caspase-1 were measured using a Quantikine ELISA kit (R&D Systems).

ATP concentration

THP-1 cells were cultured in the presence of 200 μM ecto-ATPase inhibitor ARL 1 hr before infection. The cells were infected with *M. pneumoniae* for 6 hr and the levels of ATP in the supernatant were determined using ENLITEN luciferase reagent (Promega, Madison, WI, USA).

Western blot analysis

A total of 2×10^5 cells/500 μl THP-1 cells were cultured for 24 hr before infection in a 24-well plate. The cells were infected with 50 μl *M. pneumoniae* (OD_{595} = 0·1) for 6 hr. Cells and supernatants were collected by centrifugation for 10 min at 3000 ***g***, and then mixed with lithium dodecyl sulphate sample buffer (Invitrogen, Carlsbad, CA, USA) and heated at 70° for 10 min. The proteins were resolved by SDS–PAGE and transferred to PVDF membranes. The membranes were probed with goat anti-human IL-1*β* (R&D Systems) and horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein bands were visualized using ECL Advance (GE Healthcare, Amersham, UK).

Lipopeptides

A synthetic lipopeptide containing the N-terminal 20 amino acids of F_0F_1-ATPase derived from *M. pneumoniae* (FAM20) was synthesized by Bio Synthesis (Lewisville, TX, USA).[7]

The (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-*N*-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH, 3HCl (P3CSK4)[11] was purchased from Calbiochem (Darmstadt, Germany).

Expression vectors

To prepare TLR2 expression vectors (pFLAG-TLR2), the coding regions of TLR2 minus the respective N-terminal signal sequences were amplified by PCR from a cDNA of THP-1 and cloned into the expression vector pFLAG-CMV1 (Sigma), in which a preprotrypsin leader precedes an N-terminal FLAG epitope. An NF-κB Cis-Reporting System containing pNF-kB-luc was purchased from Stratagene (La Jolla, CA, USA).

Transfection and luciferase assay

Transient transfection was performed using FuGENE6 (Roche, Basel, Switzerland) according to the manufacturer's instructions. A total of 1×10^5 293T cells were transfected with 0·1 μg pFLAG-TLR2, 0·1 μg pNF-kB-luc and 0·01 μg pRL-TK internal control plasmid (Promega) in 24-well plates. After 48 hr, the transfected cells were infected with *M. pneumonia*e or stimulated with lipopeptides. After a further 6 hr of incubation, the cells were lysed and assayed for luciferase activity using a Dual-Luciferase Reporter Assay System (Promega). Both firefly and *Renilla* luciferase activity were monitored with a Lumat LB9507 luminometer (Berthold, Wildbad, Germany). Normalized reporter activity is expressed as the firefly luciferase value divided by the *Renilla* luciferase value. Relative fold induction is calculated as the normalized reporter activity of the test samples divided by the unstimulated samples.

Statistical analysis

Results expressed as means and standard deviations were compared using one-way analysis of variance. The differences between each group were compared by multiple comparisons (Bonferroni *t*-test). Differences were considered significant at $P < 0{\cdot}01$.

Results

Pro-inflammatory cytokine induction by *M. pneumoniae* strains and heat-killed *M. pneumoniae*

To examine the interaction between cytoadherence and inflammatory responses, wild-type strain (M129), cytoadherence-deficient mutants (M5 and M6) originating from M129[42–45] or heat-killed *M. pneumoniae* were infected on THP-1 cells and the levels of pro-inflammatory cytokines induced by infection were measured by ELISA. As

M. pneumoniae strongly adheres to itself, it is difficult to determine a precise colony-forming unit count. Therefore, the amounts of mycoplasma cells were adjusted using the OD_{595} in PBS. Table 1 shows the DNA amounts of wild-type (M129) and cytoadherence-deficient mutants (M5 and M6) of *M. pneumoniae* in 100 μl PBS at 0·1 OD_{595}. M129 showed the same DNA amount as M5 and M6 at the same optical density.

When THP-1 cells were infected with M129, TNF-α and IL-1β were strikingly induced (Fig. 1a,b). In contrast, heat-killed M129 induced the production of TNF-α at lower levels, but the induction of IL-1β was completely decreased to the control level. Next, THP-1 cells were infected with M129 and cytoadherence-deficient mutants (M5 and M6) at graded concentrations (Fig. 2a,b). M129 induced TNF-α and IL-1β in a dose-dependent manner. Although M5 at high doses (OD_{595} = 0·2) induced TNF-α as much as M129, M5 at low doses (OD_{595} = 0·1 and 0·05) and M6 at all doses induced lower levels of TNF-α than M129. In contrast, M5 and M6 at all doses induced lower levels of IL-1β than M129. We also tested four other cytoadherence-deficient mutants, class I, class II, class III and class IV (data not shown). Similar to M5 and M6, the induction levels of TNF-α and IL-1β by these mutants were also decreased compared with M129. To examine the time kinetics of pro-inflammatory cytokines induced by *M. pneumoniae*, THP-1 cells were infected with M129, M5 and M6. The induced TNF-α and IL-1β were measured by ELISA at 0, 1, 3, 6, 12 and 24 hr post-infection (Fig. 2c,d). The induction levels of TNF-α were maximal at 3 and 6 hr whereas the maximal induction levels of IL-1β were observed at 6 and 12 hr. When THP-1 cells were infected with M5 and M6, the induction levels of TNF-α and IL-1β were strikingly low compared with M129 at all time-points. To confirm the importance of cytoadherence in cytokine production of different types of cells, human PBMC and a human lung squamous cell carcinoma cell line, EBC-1 cells were infected with M129, M5 and M6. $CD14^+$ cells in PBMC were 12·8% by flow cytometry analysis. When PBMC were infected with M129, high levels of TNF-α and IL-1β were induced. In contrast, M5 and M6 induced low levels of these cytokines (Supplementary materials, Fig. S1a,b). Likewise, in EBC-1 cells, M129 induced high levels of IL-8, but M5 and M6 failed to induce IL-8 (Fig. S1c). These results indicate that the adherent *M. pneumoniae* possesses the ability to induce potent inflammatory responses compared with the cytoadherence-deficient mutants.

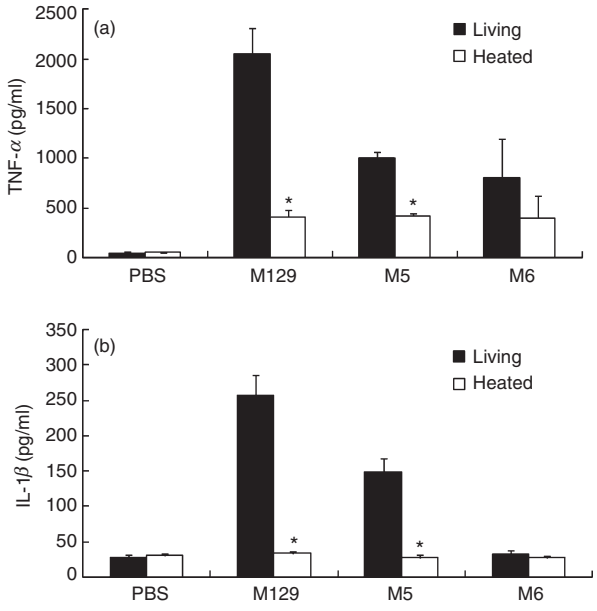

Figure 1. Induction of tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) by heat-killed *Mycoplasma pneumoniae*. The *M. pneumoniae* was killed by heating at 60° for 30 min. THP-1 cells (2×10^5 cells/500 μl) were treated with 50 μl living or heat-killed *M. pneumoniae* (OD_{595} = 0.1). After 6 hr incubation, amounts of TNF-α (a) and IL-1β (b) in culture medium were measured by ELISA. All values represent the means and SD of three assays. An asterisk indicates that the *P*-value is < 0.01 for a comparison with living *M. pneumoniae* by multiple comparison.

Table 1. Strains of *Mycoplasma pneumoniae* used in this study

Strain	Missing proteins	Mutated gene	DNA[1] (μg)	Reference
M129	–	–	4·57 ± 0·67	–
M5	P90, P40	NR[2]	4·39 ± 0·62	[42]
M6	HMW1, P30	*hmw1*, *p30*	4·42 ± 0·71	[43–45]

[1]DNA, the amounts of *M. pneumoniae* in 100 μl PBS were adjusted to OD_{595} = 0·1 in 96-well plates. DNA was purified as described in the Materials and methods. The amounts of DNA were measured by optical density at 280 nm. All values represent the means and SD of three assays.
[2]NR, not reported.

Induction of TLR2 signalling

We previously demonstrated that lipoproteins of *M. pneumoniae* induced inflammatory responses through TLR2.[7,8] To examine whether M129 augments TLR2 signalling, the levels of NF-κB induction through TLR2 were measured using NF-κB reporter assay system. A human kidney cell line, 293T cells, transfected with TLR2 expression vector (pFLAG-TLR2) and NF-κB reporter vector (pNF-kB-luc) was infected with *M. pneumoniae* and the induced luciferase activity was measured (Fig. 3a). The *M. pneumoniae* failed to induce NF-κB activation in 293T cells transfected with pNF-kB-luc alone, indicating that TLR2 is necessary for *M. pneumoniae* to induce the NF-κB activation. Surprisingly, M5 and M6 as well as M129 induced comparable levels of NF-κB activation

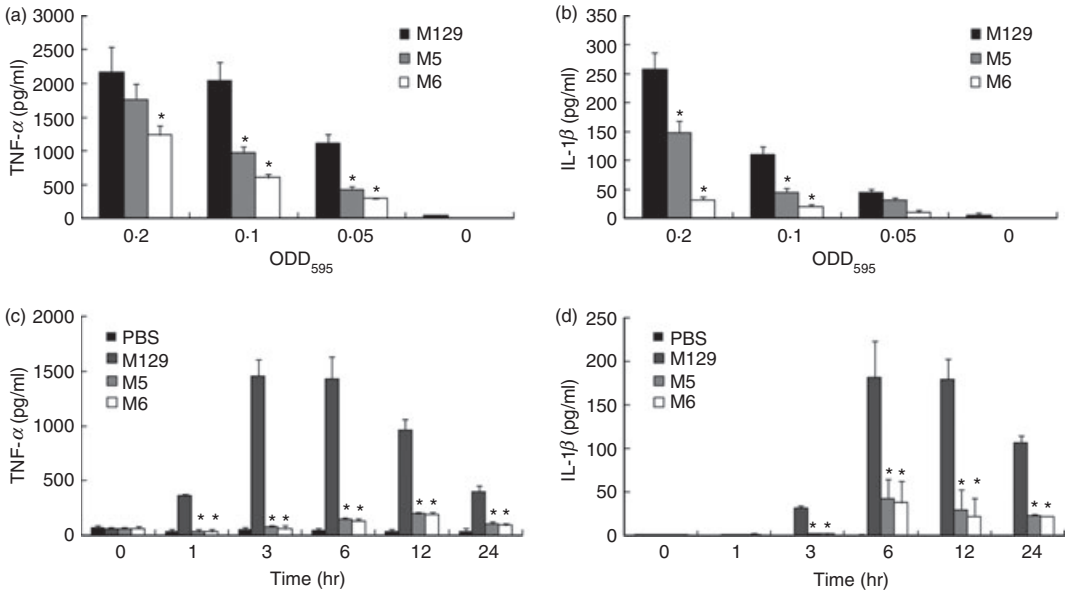

Figure 2. Induction of tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) by cytoadherence-deficient mutants of *Mycoplasma pneumoniae*. THP-1 cells (2×10^5 cells/500 μl) were infected with 50 μl of the indicated amounts of wild-type or mutants of *M. pneumoniae*. After 6 hr incubation, amounts of TNF-α (a) and IL-1β (b) in culture medium were measured by ELISA. THP-1 cells (2×10^5 cells/500 ml) were infected with 50 ml of *M. pneumoniae* (OD_{595} = 0.1). Amounts of TNF-α (c) and IL-1β (d) in culture medium were measured by ELISA at the indicated time post-infection. All values represent the means and SD of three assays. An asterisk indicates that the *P*-value is < 0.01 for a comparison with wild-type of *M. pneumoniae* by multiple comparison.

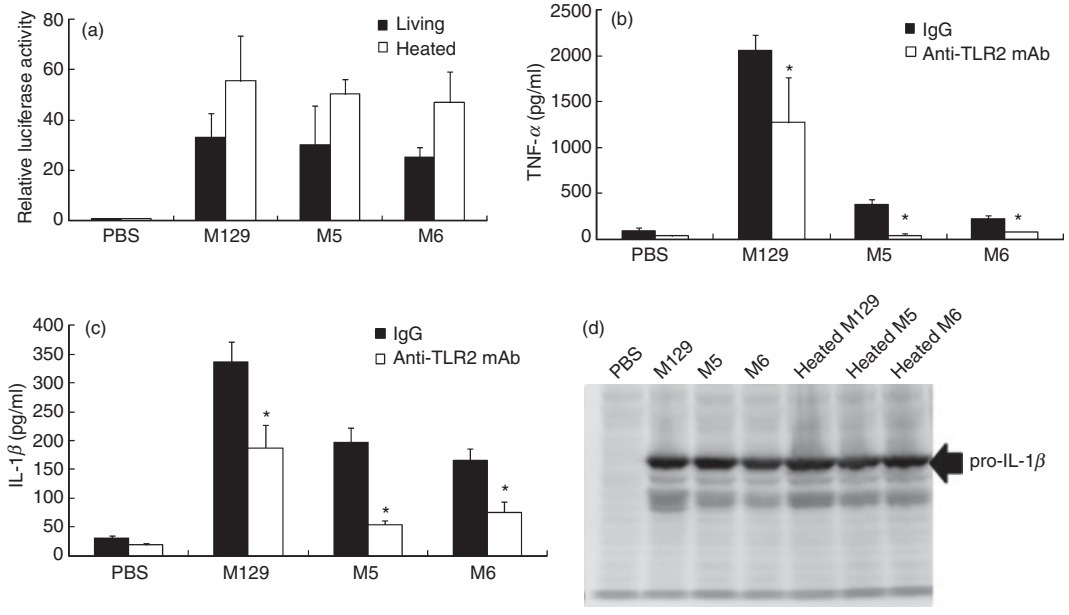

Figure 3. Induction of Toll-like receptor 2 (TLR2) signalling. (a) 293T cells (2×10^5 cells/500 μl) were transfected with the 0.1 μg/ml pFLAG-TLR2, 0.01 μg/ml pNF-kB-luc and 0.01 μg/ml pRL-TK. After 24 hr incubation, the cells were infected with 50 μl wild-type or mutants of *Mycoplasma pneumoniae* (OD_{595} = 0.1). After 6 hr incubation, the activity of luciferase was measured as described in the Materials and methods. All values represent the means and SD of three assays. (b, c) THP-1 cells (2×10^5 cells/500 μl) were treated with 10 μg/ml anti-TLR2 monoclonal antibody and infected with 50 μl wild-type or mutants of *M. pneumoniae* (OD_{595} = 0.1). After 6 hr incubation, amounts of tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in culture medium were measured by ELISA. All values represent the means and SD of three assays. An asterisk indicates that the *P*-value is < 0.01 for a comparison with control IgG by multiple comparison (d) THP-1 cells (2×10^5 cells/500 μl) were treated with 50 μl living or heat-killed *M. pneumoniae* (OD_{595} = 0.1). After 6 hr incubation, THP-1 cells were harvested and the induction of pro-IL-1β precursor was investigated by Western blotting.

through TLR2. Heat-killed *M. pneumoniae* seemed to slightly increase the induction levels of NF-κB compared with living mycoplasmas. Anti-TLR2 monoclonal antibody decreased the levels of TNF-α and IL-1β induction by M5 and M6 down to the control levels. In contrast, the induction levels of TNF-α and IL-1β by M129 were partially blocked by the antibody (Fig. 3b,c).

The wild-type strain of *M. pneumoniae* strongly induced IL-1β production compared with cytoadherence-deficient mutants (Figs 1b and 2b). The induction of pro-IL-1β precursor in THP-1 cells was therefore examined by Western blotting (Fig. 3d). Similar to the results of TLR 2 signalling, the pro-IL-1β precursor was induced at the same levels by treatment with M129, M5, M6 and heat-killed mycoplasmas. These results suggest that the enhancement of inflammatory responses by cytoadherent mycoplasma is mostly TLR2-independent.

Endocytosis-independent induction of pro-inflammatory cytokines

To elucidate the mechanism by which cytoadherent mycoplasma enhances pro-inflammatory cytokine induction, we examined the involvement of endocytosis. THP-1 cells were treated with cytochalasin D, which disrupts actin filaments and inhibits the initiation of endocytosis.[46] Cytochalasin D completely inhibited TNF-α production by M5, M6 and heat-killed mycoplasma strains, whereas TNF-α production by M129 was not affected (Fig. 4a). Interleukin-1β production by M129 was slightly decreased by cytochalasin D, but the production by M5, M6 and heat-killed mycoplasma strains was diminished (Fig. 4b). Subsequently, induction of pro-IL-1β precursor was examined by Western blotting. The induction levels of M5, M6 and heat-killed mycoplasma strains were lowered in the presence of cytochalasin D, but the induction by M129 was unaffected (Fig. 4c and data not shown). These results suggest that cytoadherent mycoplasma activates TNF-α and pro-IL-1β precursor production in an endocytosis-independent manner, whereas endocytosis is important for cytoadherence-deficient mycoplasma strains to induce TNF-α and IL-1β.

To further examine whether the maturation of endosome participates in the induction of pro-inflammatory cytokines, THP-1 cells were treated with bafilomycin A1, which inhibits the maturation of early endosome via inhibition of the vacuolar H^+ ATPase system.[47] In the presence of bafilomycin A1, the induction of TNF-α by mycoplasma infection was slightly enhanced (Fig. 4a). Interleukin-1β induction was slightly increased by bafilomycin A1 when THP-1 cells were treated with M129 and heat-killed mycoplasmas, but the induction was unaffected in the case of M5 and M6 infection (Fig. 4b). Taken together, these results indicate that cytoadherent *M. pneumoniae* induced pro-inflammatory cytokines in an endocytosis-independent manner, and that the early endosome was important for the induction of pro-inflammatory cytokines by heat-killed and cytoadherence-deficient *M. pneumoniae*.

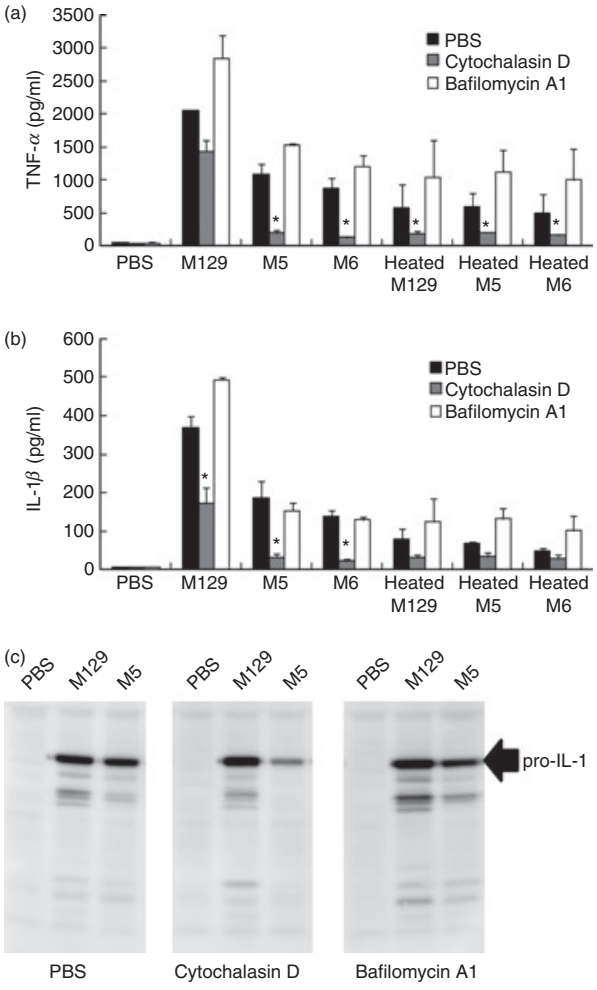

Figure 4. Endocytosis-independent induction of pro-inflammatory cytokines. THP-1 cells (2×10^5 cells/500 μl) were cultured in the presence of 1.0 μM cytochalasin D or 100 nM bafilomycin A1 for 1 hr, and treated with 50 μl of living or heat-killed *Mycoplasma pneumoniae* ($OD_{595} = 0.1$). After 6 hr incubation, amounts of tumour necrosis factor-α (TNF-α) (a) and interleukin-1β (IL-1β) (b) in culture medium were measured by ELISA. Cells were harvested and the induction levels of pro-IL-1β precursor were determined by Western blotting (c). All values represent the means and SD of three assays. An asterisk indicates that the *P*-value is < 0.01 for a comparison with PBS treatment by multiple comparison.

Involvement of the inflammasome

Despite the same induction levels of pro-IL-1β precursor, cytoadherent *M. pneumoniae* induced a higher level of IL-1β than cytoadherence-deficient mutants (Figs 1b and 3b). Mature IL-1β is produced by cleavage of the pro-IL-1β precursor by caspase-1.[23] Caspase-1 is activated within

a large protein complex, called the inflammasome.[24] The inflammasome plays a central role in sensing various intracellular bacterial components. To examine the involvement of the inflammasome, we transfected diacylated lipopeptide derived from *M. pneumoniae* (FAM20) and triacylated lipopeptide (P3CSK4) into the cytoplasm of THP-1 cells by lipofection. The induction levels of TNF-α and IL-1β remained low when THP-1 cells were treated with either FAM20 or P3CSK4 alone, but the induction levels were augmented when the lipopeptides were transfected using lipofectamin (Fig. 5b). These results suggest that PAMPs like mycoplasmal lipopeptides are recognized by an intracellular receptor such as the inflammasome, leading to the release of IL-1β.

Next, we examined the activation of caspase-1. Active caspase-1 is composed of 20 000 and 10 000 molecular

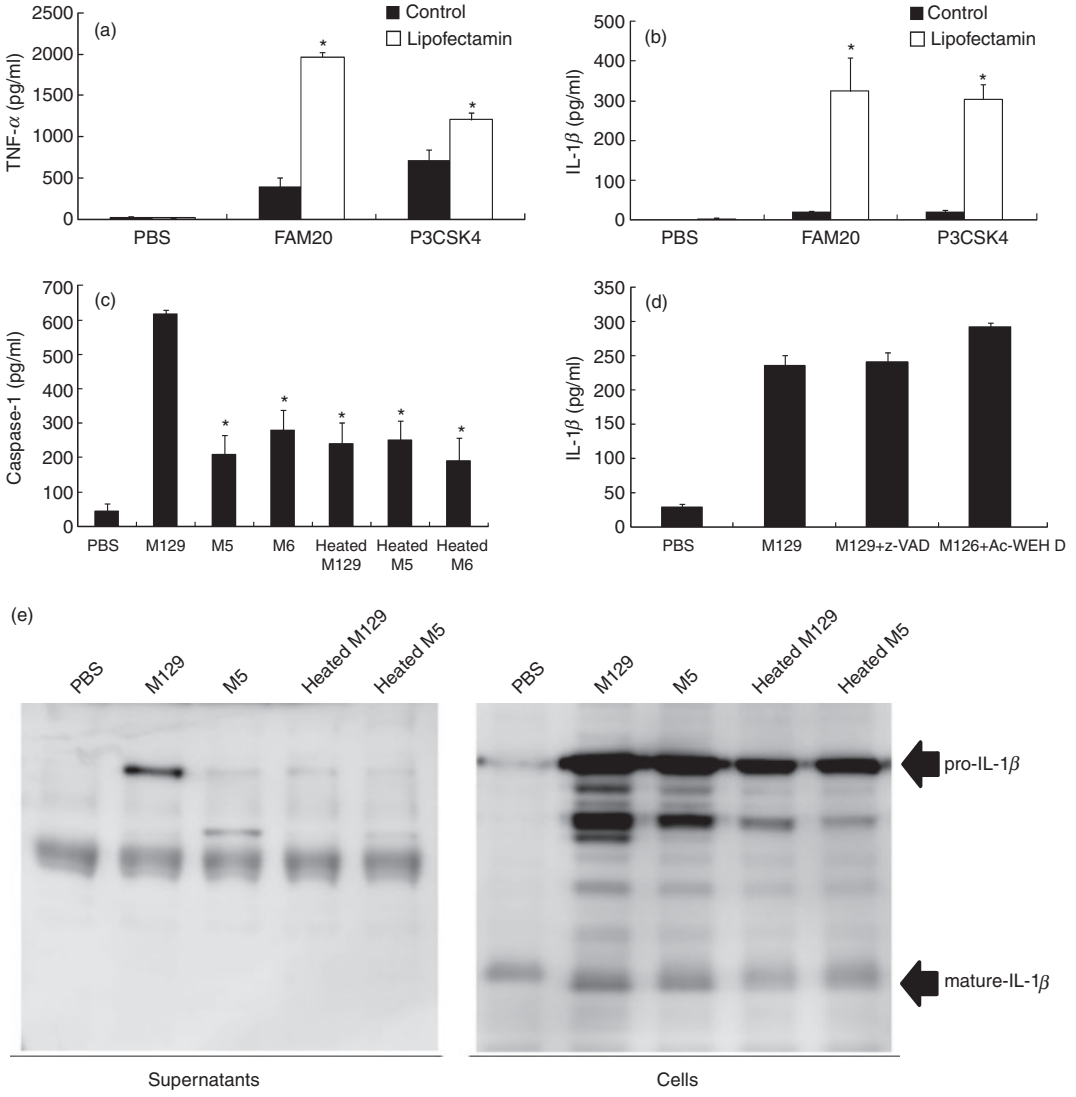

Figure 5. Involvement of the inflammasome. (a, b) 1000 ng/ml of FAM20 was incubated with 1% of lipofectamin. THP-1 cells (2×10^5 cells/500 µl) were transfected with 100 ng/ml FAM20. After 6 hr incubation, the amount of tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in the culture medium was measured by ELISA. All values represent the means and SD of three assays. An asterisk indicates that the *P*-value is < 0.01 for a comparison with control by multiple comparison. (c) THP-1 cells (2×10^5 cells/500 µl) were infected with 50 µl of indicated *Mycoplasma pneumoniae* ($OD_{595} = 0.1$). After 6 hr incubation, levels of caspase-1 in culture medium were measured by ELISA. All values represent the means and SD of three assays. An asterisk indicates that the *P*-value is < 0.01 for a comparison with M129 by multiple comparison. (d) THP-1 cells (2×10^5 cells/500 µl) were cultured in the presence of 100 µM z-Val-Ala-Asp(oMe)-CH_2F (z-VAD-FMK) or Ac-Trp-Glu-His-Asp-H (Ac-WEHD-CHO) for 1 hr, and infected with 50 µl M129 ($OD_{595} = 0.1$). After 6 hr incubation, amounts of IL-1β in culture medium were measured using ELISA. All values represent the means and SD of three assays. (e) THP-1 cells (2×10^5 cells/500 µl) were infected with 50 µl of the indicated *M. pneumoniae* ($OD_{595} = 0.1$). After 6 hr incubation, the IL-1β levels in the supernatants or infected cells were determined by Western blotting.

weight polypeptides (p20 and p10) derived from the processing of a cytosolic 45 000 MW precursor protein (p45).[48] After caspase-1 is activated within the inflammasome, both p20 and p10 are released from cytoplasm to outside the cells.[49] We measured the amount of caspase-1 p20 in the culture medium of THP-1 cells infected with *M. pneumoniae* (Fig. 5c). Infection of M129 released high concentrations of active caspase-1 p20, but the release of the p20 was depressed when M5, M6 and heat-killed *M. pneumoniae* were used for infection or treatment. To further confirm the involvement of caspase-1, we used several caspase inhibitors in the assay. Interestingly, the release of IL-1β was unaffected when THP-1 cells were treated with a pan-caspase inhibitor, z-VAD or a caspase-1 inhibitor, ac-WEHD (Fig. 5d). To check whether caspase-1 cleaves the pro-IL-1β precursor, the levels of IL-1β precursor in the cells and supernatants of THP-1 cells were examined by Western blotting (Fig. 5e). Surprisingly, pro-IL-1β precursor was cleaved and the mature form of IL-1β was detected in the cells, but only pro-IL-1β precursor was released into the supernatants when the cells were infected with M129. When the cells were treated with M6 or heat-killed *M. pneumoniae*, lower amounts of mature IL-1β were induced and pro-IL-1β precursor was not released into the supernatant. These results indicate that cytoadherent *M. pneumoniae* induces caspase-1 activation and maturation of IL-1β, but only pro-IL-1β precursor was released from the cell.

Extracellular ATP serves as a danger signal that alerts the immune system by binding to the receptor P2X7, thereby activating the inflammasome and caspase-1.[28–31] Binding of ATP to P2X7 receptor also augments the release of pro-IL-1β precursor.[50] It was reported that *M. fermentans* and *M. salivarium* induced the extracellular ATP and regulated cell death through the P2X7 receptor in lymphocytes and monocytes.[51] Then, we investigated the involvement of extracellular ATP and the P2X7 receptor. Initially, THP-1 cells were infected with *M. pneumoniae*, and the amounts of extracellular ATP were measured. The extracellular ATP concentration reflects the balance between ATP release and ATP hydrolysis by ectonucleotidase.[52] Therefore, to determine the levels of ATP release, THP-1 cells were pre-treated with the ecto-ATPase inhibitor ARL and in turn infected with *M. pneumoniae*. The levels of ATP release by M129 were increased about threefold compared with M5, M6 and heat-killed *M. pneumoniae* strains (Fig. 6a). To further examine the role of the P2X7 receptor, THP-1 cells were treated with an inhibitor of the P2X7 receptor, oxidized ATP (oATP). Although the induction of pro-IL-1β precursor by M129 was unaffected by oATP (Fig. 6b), the release of IL-1β was strikingly inhibited by oATP (Fig. 6c). Taken together, these results suggest that cytoadherent *M. pneumoniae* activates the inflammasome via 'an ATP autocrine system,' leading to the release of IL-1β.

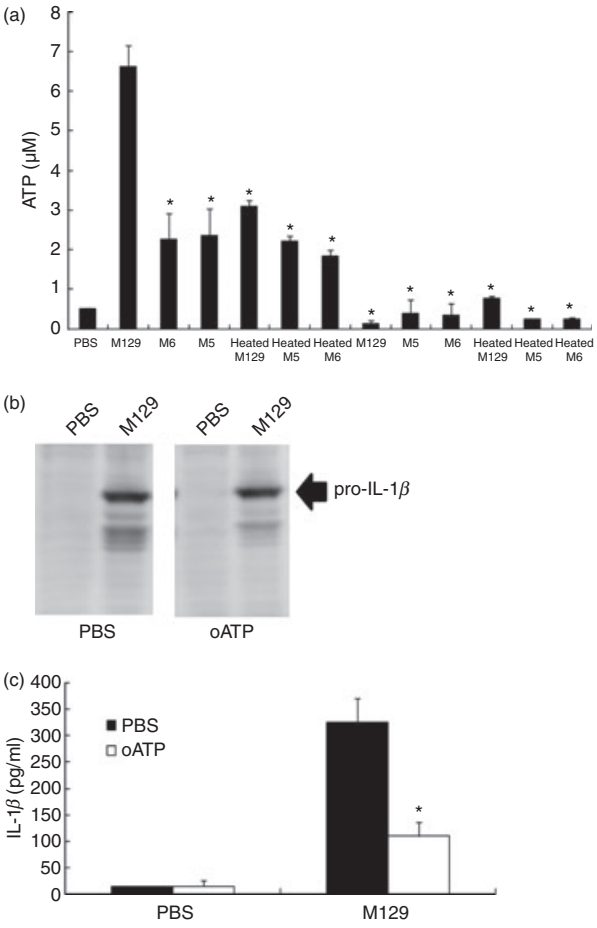

Figure 6. Involvement of ATP in the interleukin-1β (IL-1β)induction. (a) THP-1 cells (2×10^5 cells/500 μl) were cultured in the presence of 200 μM ecto-ATPase inhibitor, ARL for 1 hr, and infected with 50 μl of the indicated *Mycoplasma pneumoniae* $sOD_{595} = 0.1$). After 6 hr incubation, concentrations of ATP were measured as described in the Materials and methods. All values represent the means and SD of three assays. An asterisk indicates that the *P*-value is < 0.05 for a comparison with M129 by multiple comparison. (b) THP-1 cells (2×10^5 cells/500 μl) were cultured in the presence of 300 μM oxidized ATP for 1 hr and infected with 50 μl M129 ($OD_{595} = 0.1$). After 6 hr incubation, THP-1 cells were harvested and the induction levels of pro-IL-1β precursor were determined by Western blotting. (c) THP-1 cells were treated with oxidized ATP and infected with M129 as described above. Release levels of IL-1β were measured by ELISA. All values represent the means and SD of three assays. An asterisk indicates that the *P*-value is < 0.01 for a comparison with PBS treatment by multiple comparison.

Discussion

The cytoadherence of *M. pneumoniae* is mediated by attachment organelle, including P1 adhesin and other additional proteins such as P30 or HMW proteins.[35–38] These proteins are unique in mycoplasma species, and their homologues are not identified in any other bacteria.[53] In this study, we found that cytoadherent

M. pneumoniae enhanced the induction of pro-inflammatory cytokines TNF-α and IL-1β compared with cytoadherence-deficient mutants and heat-killed *M. pneumoniae* (Figs 1 and 2). These results indicate that the property of cytoadherence is critical for the progression of diseases caused by *M. pneumoniae*. These results are consistent with the earlier reports that a protease treatment decreases the induction of pro-inflammatory cytokines by *M. pneumoniae*,[40] and that *M. pneumoniae* cultured in non-adherent condition fails to induce IL-4 from rodent mast cells.[39] Although the cytoadherence of *M. pneumoniae* is suggested to be involved in the induction of pro-inflammatory cytokines, the mechanism whereby cytoadherence of *M. pneumoniae* activates the induction of pro-inflammatory responses has not been clarified.

We previously reported that *M. pneumoniae* induced pro-inflammatory cytokines through TLR2.[6] Interestingly, cytoadherence-deficient *M. pneumoniae* as well as cytoadherent *M. pneumoniae* induced TLR2 signalling and pro-IL-1β precursor (Fig. 3a,d). Anti-TLR 2 antibody inhibited the TNF-α and IL-1β production by cytoadherence-deficient mutants, but only partially inhibited the production by cytoadherent *M. pneumoniae* (Fig. 3b). In addition, wild-type *M. pneumoniae* had TNF-α inducing activity in macrophages of TLR 2 knockout mice (data not shown). These findings suggest that augmentation of pro-inflammatory cytokine induction by cytoadherent *M. pneumoniae* is independent of TLR 2 signalling. The NLR proteins have been reported as recognizing intracellular components of microbes and endogenous molecules associated with inflammation, known as PAMPs and DAMPs, respectively.[54] Some of the NLR proteins such as NOD1, NOD2, NLRX1 and NLRP12 induce NF-κB and mitogen-activated protein kinase signalling leading to TNF-α induction.[55] In this study, we found that lipofection of lipopeptides derived from *M. pneumoniae* enhances TNF-α production (Fig. 5a). Recognition of intracellular components of *M. pneumoniae* by intracellular receptors, such as NLR proteins, may explain TLR2-independent pro-inflammatory cytokine induction by cytoadherent *M. pneumoniae*. The mechanisms of the injection of mycoplasmal components into the cytoplasm of host cells remain unclear. The secretion system in mycoplasma species has not been found. Although it is believed that mycoplasmas remain attached to the surface of epithelial cells, some mycoplasmas including *M. pneumoniae* can reside within non-phagocytic cells under certain circumstances.[56] Moreover, several reports suggest that mycoplasma species are wall-less bacteria and the cell membranes of mycoplasma fuse with the membranes of host cells.[57,58] Invasion and fusion may result in the direct delivery of mycoplasmal components into host cells. Alternatively, a pore-forming hemi-channel, pannexin-1 induced by ATP may participate in the process. Pannexin-1-mediated infiltration of bacterial components was reported to activate the production of pro-inflammatory cytokines.[32]

We also found that an inhibitor of initiation of endocytosis, cytochalasin D inhibited the induction of TNF-α and IL-1β by cytoadherence-deficient mutants, demonstrating that the induction of TNF-α and IL-1β by cytoadherence-deficient *M. pneumoniae* was completely dependent on endocytosis. In contrast, cytochalasin D partially inhibited the induction by cytoadherent *M. pneumoniae*, indicating that cytoadherent *M. pneumoniae* induces pro-inflammatory cytokines in an endocytosis-independent as well as a dependent manner (Fig. 4). In our experimental system, an inhibitor of maturation of early endosome, bafilomycin A1, failed to affect the induction levels of TNF-α and IL-1β. The results indicate that *M. pneumoniae* induces the production of pro-inflammatory cytokines through the early endosome rather than the late endosome. This result is inconsistent with the recent report that late endosome inducing reactive oxygen species are necessary for IL-1β induction by influenza virus or silica.[33,34]

Mature IL-1β is produced by cleavage of the pro-IL-1β precursor by caspase-1.[23] Caspase-1 is activated within a large protein complex, called the inflammasome.[24] Activation of the inflammasome is also mediated by ATP through the P2X7 receptor.[28–31] Our results suggest that the inflammasome is involved in the release of IL-1β by *M. pneumoniae*. Lipofection with the lipopeptide FAM20 derived from *M. pneumoniae* augmented the release levels of IL-1β (Fig. 5b). Cytoadherent *M. pneumoniae* strongly induced caspase-1 compared with cytoadherence-deficient mutants (Fig. 5c). Cytoadherent *M. pneumoniae* efficiently induced efflux of ATP from THP-1 cells (Fig. 6a,b). The oATP, an inhibitor of the P2X7 receptor, decreased the release levels of IL-1β by *M. pneumoniae* (Fig. 6c). In the present study, however, caspase inhibitors such as z-VAD and ac-WEHD failed to affect the release of IL-1β (Fig. 5d). Pro-IL-1β was cleaved and mature IL-1β was produced in the cells infected with cytoadherent *M. pneumoniae*, but only pro-IL-1β was released into the supernatants of infected cells (Fig. 5e). The findings suggest that cytoadherent *M. pneumoniae* induced caspase-1 activation followed by maturation of IL-1β, but only pro-IL-1β precursor was released in an ATP- and P2X7 receptor-dependent manner. These results are consistent with the earlier report that binding of ATP to P2X7 receptor augments the release of pro-IL-1β precursor.[50] Although a recent report demonstrated that ATP is released by monocytes stimulated with pathogen-sensing receptor ligands followed by induction of IL-1β,[59] the mechanisms by which such ligands release ATP from host cells are still unknown. To our knowledge, this is the first report that the cytoadherence of bacteria enhances the release of ATP from the host cells. Released pro-IL-1β precursor was reported to be cleaved by neutrophil- and macrophage-

derived serine proteases such as protease3, elastase and cathepsin-G.[60,61] In particular, the crucial role of neutrophil-dependent activation of pro-IL-1β precursor has been validated by the experiments using neutrophil-derived protease inhibitors.[62] In *M. pneumoniae* infection, a large amount of neutrophils are present in human broncho-alveolar lavage fluid,[2] and lipoproteins derived from *M. pneumoniae* induce many neutrophils in mouse broncho-alveolar lavage fluid.[6] These evidences indicate that such released pro-IL-1β precursor would be cleaved into mature IL-1β in the extracellular milieu.

In conclusion, our results suggest that cytoadherence of *M. pneumoniae* is one of the key factors to induce inflammatory responses leading to pneumonia by *M. pneumoniae*. Hence, the proteins such as P1 and the HMW proteins involved in cytoadherence might be molecules for use in the development of alternative strategies for the prevention and treatment of *M. pneumoniae* infection.

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Disclosures

The authors declare no financial conflicts of interest.

References

1 Razin S. Peculiar properties of mycoplasmas: the smallest self-replicating prokaryotes. *FEMS Microbiol Lett* 1992; **79**:423–31.
2 Waites KB, Talkington DF. *Mycoplasma pneumoniae* and its role as a human pathogen. *Clin Microbiol Rev* 2004; **17**:697–728, table of contents.
3 Gil JC, Cedillo RL, Mayagoitia BG, Paz MD. Isolation of *Mycoplasma pneumoniae* from asthmatic patients. *Ann Allergy* 1993; **70**:23–5.
4 Kraft M, Cassell GH, Henson JE, Watson H, Williamson J, Marmion BP, Gaydos CA, Martin RJ. Detection of *Mycoplasma pneumoniae* in the airways of adults with chronic asthma. *Am J Respir Crit Care Med* 1998; **158**:998–1001.
5 Tryon VV, Baseman JB. Pathgenic determinant and mechanisms. In: Maniloff J, McElhaney RN, Finch LR, Baseman JB, eds. *Mycoplasmas – Molecular Biology and Pathogenesis*. Washington, D.C: American Society for Microbiology, 1992:457–89.
6 Shimizu T, Kida Y, Kuwano K. *Mycoplasma pneumoniae*-derived lipopeptides induce acute inflammatory responses in the lungs of mice. *Infect Immun* 2008; **76**:270–7.
7 Shimizu T, Kida Y, Kuwano K. A dipalmitoylated lipoprotein from *Mycoplasma pneumoniae* activates NF-kappa B through TLR1, TLR2, and TLR6. *J Immunol* 2005; **175**:4641–6.
8 Shimizu T, Kida Y, Kuwano K. Triacylated lipoproteins derived from *Mycoplasma pneumoniae* activate nuclear factor-kappaB through toll-like receptors 1 and 2. *Immunology* 2007; **121**:473–83.
9 Akira S, Takeda K. Toll-like receptor signaling. *Nat Rev Immunol* 2004; **4**:499–511.
10 Kopp EB, Medzhitov R. The toll-receptor family and control of innate immunity. *Curr Opin Immunol* 1999; **11**:13–8.
11 Aliprantis AO, Yang RB, Mark MR *et al.* Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science* 1999; **285**:736–9.
12 Brightbill HD, Libraty DH, Krutzik SR *et al.* Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science* 1999; **285**:732–6.
13 Lien E, Sellati TJ, Yoshimura A *et al.* Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J Biol Chem* 1999; **274**:33419–25.
14 Means TK, Lien E, Yoshimura A, Wang S, Golenbock DT, Fenton MJ. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for toll-like receptors. *J Immunol* 1999; **163**:6748–55.
15 Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 1999; **11**:443–51.
16 Takeuchi O, Kaufmann A, Grote K, Kawai T, Hoshino K, Morr M, Muhlradt PF, Akira S. Preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. *J Immunol* 2000; **164**:554–7.
17 Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, Bassetti M, Aderem A. The toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature* 1999; **401**:811–5.
18 Hayashi F, Smith KD, Ozinsky A *et al.* The innate immune response to bacterial flagellin is mediated by toll-like receptor 5. *Nature* 2001; **410**:1099–103.
19 Hemmi H, Takeuchi O, Kawai T *et al.* A toll-like receptor recognizes bacterial DNA. *Nature* 2000; **408**:740–5.
20 Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 1999; **162**:3749–52.
21 Poltorak A, He X, Smirnova I *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998; **282**:2085–8.
22 Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, Janeway CA Jr. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell* 1998; **2**:253–8.
23 Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 2002; **10**:417–26.
24 Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. *Annu Rev Immunol* 2009; **27**:229–65.
25 Boyden ED, Dietrich WF. Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genet* 2006; **38**:240–4.
26 Martinon F, Agostini L, Meylan E, Tschopp J. Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome. *Curr Biol* 2004; **14**:1929–34.
27 Miao EA, Andersen-Nissen E, Warren SE, Aderem A. TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system. *Semin Immunopathol* 2007; **29**:275–88.
28 Mariathasan S, Weiss DS, Newton K *et al.* Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 2006; **440**:228–32.
29 Sutterwala FS, Ogura Y, Szczepanik M *et al.* Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* 2006; **24**:317–27.
30 Kanneganti TD, Ozoren N, Body-Malapel M *et al.* Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* 2006; **440**:233–6.
31 Franchi L, Kanneganti TD, Dubyak GR, Nunez G. Differential requirement of P2X7 receptor and intracellular K^+ for caspase-1 activation induced by intracellular and extracellular bacteria. *J Biol Chem* 2007; **282**:18810–8.
32 Kanneganti TD, Lamkanfi M, Kim YG, Chen G, Park JH, Franchi L, Vandenabeele P, Nunez G. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of toll-like receptor signaling. *Immunity* 2007; **26**:433–43.
33 Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 2008; **320**:674–7.
34 Allen IC, Scull MA, Moore CB *et al.* The NLRP3 inflammasome mediates *in vivo* innate immunity to influenza A virus through recognition of viral RNA. *Immunity* 2009; **30**:556–65.
35 Krause DC, Balish MF. Structure, function, and assembly of the terminal organelle of *Mycoplasma pneumoniae*. *FEMS Microbiol Lett* 2001; **198**:1–7.
36 Balish MF, Krause DC. Cytoadherence and the cytoskeleton. In: Razin S, Herrmann R, eds. *Molecular Biology and Pathogenicity of Mycoplasmas*. New York: Kluwer Academic/Plenum Publishers, 2002:491–518.
37 Miyata M. Centipede and inchworm models to explain Mycoplasma gliding. *Trends Microbiol* 2008; **16**:6–12.
38 Miyata M. Molecular mechanism of mycoplasma gliding – a novel cell motility system. In: Lenz P, ed. *Cell Motility*. New York: Springer Science, 2008:137–75.
39 Hoek KL, Duffy LB, Cassell GH, Dai Y, Atkinson TP. A role for the *Mycoplasma pneumoniae* adhesin P1 in interleukin (IL)-4 synthesis and release from rodent mast cells. *Microb Pathog* 2005; **39**:149–58.
40 Yang J, Hooper WC, Phillips DJ, Talkington DF. Regulation of proinflammatory cytokines in human lung epithelial cells infected with *Mycoplasma pneumoniae*. *Infect Immun* 2002; **70**:3649–55.

41 Seto S, Miyata M. Attachment organelle formation represented by localization of cytoadherence proteins and formation of the electron-dense core in wild-type and mutant strains of *Mycoplasma pneumoniae*. *J Bacteriol* 2003; **185**:1082–91.
42 Layh-Schmitt G, Harkenthal M. The 40- and 90-kDa membrane proteins (ORF6 gene product) of *Mycoplasma pneumoniae* are responsible for the tip structure formation and P1 (adhesin) association with the Triton shell. *FEMS Microbiol Lett* 1999; **174**:143–9.
43 Layh-Schmitt G, Hilbert H, Pirkl E. A spontaneous hemadsorption-negative mutant of *Mycoplasma pneumoniae* exhibits a truncated adhesin-related 30-kilodalton protein and lacks the cytadherence-accessory protein HMW1. *J Bacteriol* 1995; **177**:843–6.
44 Romero-Arroyo CE, Jordan J, Peacock SJ, Willby MJ, Farmer MA, Krause DC. *Mycoplasma pneumoniae* protein P30 is required for cytadherence and associated with proper cell development. *J Bacteriol* 1999; **181**:1079–87.
45 Jordan JL, Berry KM, Balish MF, Krause DC. Stability and subcellular localization of cytadherence-associated protein P65 in *Mycoplasma pneumoniae*. *J Bacteriol* 2001; **183**:7387–91.
46 Mimura N, Asano A. Synergistic effect of colchicine and cytochalasin D on phagocytosis by peritoneal macrophages. *Nature* 1976; **261**:319–21.
47 Bowman EJ, Siebers A, Altendorf K. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc Natl Acad Sci USA* 1988; **85**:7972–6.
48 Yamin TT, Ayala JM, Miller DK. Activation of the native 45-kDa precursor form of interleukin-1-converting enzyme. *J Biol Chem* 1996; **271**:13273–82.
49 Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, Panther E, Di Virgilio F. The P2X7 receptor: a key player in IL-1 processing and release. *J Immunol* 2006; **176**:3877–83.
50 Mehta VB, Hart J, Wewers MD. ATP-stimulated release of interleukin (IL)-1beta and IL-18 requires priming by lipopolysaccharide and is independent of caspase-1 cleavage. *J Biol Chem* 2001; **276**:3820–6.
51 Into T, Okada K, Inoue N, Yasuda M, Shibata K. Extracellular ATP regulates cell death of lymphocytes and monocytes induced by membrane-bound lipoproteins of *Mycoplasma fermentans* and *Mycoplasma salivarium*. *Microbiol Immunol* 2002; **46**:667–75.
52 Joseph SM, Buchakjian MR, Dubyak GR. Colocalization of ATP release sites and ecto-ATPase activity at the extracellular surface of human astrocytes. *J Biol Chem* 2003; **278**:23331–42.
53 Himmelreich R, Hilbert H, Plagens H, Pirkl E, Li BC, Herrmann R. Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. *Nucleic Acids Res* 1996; **24**:4420–49.
54 Bryant C, Fitzgerald KA. Molecular mechanisms involved in inflammasome activation. *Trends Cell Biol* 2009; **19**:455–64.
55 Ting JP, Duncan JA, Lei Y. How the noninflammasome NLRs function in the innate immune system. *Science* 2010; **327**:286–90.
56 Baseman JB, Lange M, Criscimagna NL, Giron JA, Thomas CA. Interplay between mycoplasmas and host target cells. *Microb Pathog* 1995; **19**:105–16.
57 Dimitrov DS, Franzoso G, Salman M, Blumenthal R, Tarshis M, Barile MF, Rottem S. *Mycoplasma fermentans* (incognitus strain) cells are able to fuse with T lymphocytes. *Clin Infect Dis* 1993; **17**(Suppl. 1):S305–8.
58 Franzoso G, Dimitrov DS, Blumenthal R, Barile MF, Rottem S. Fusion of *Mycoplasma fermentans* strain incognitus with T-lymphocytes. *FEBS Lett* 1992; **3**:251–4.
59 Piccini A, Carta S, Tassi S, Lasiglie D, Fossati G, Rubartelli A. ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1beta and IL-18 secretion in an autocrine way. *Proc Natl Acad Sci USA* 2008; **105**:8067–72.
60 Dinarello CA. Biologic basis for interleukin-1 in disease. *Blood* 1996; **87**:2095–147.
61 Coeshott C, Ohnemus C, Pilyavskaya A, Ross S, Wieczorek M, Kroona H, Leimer AH, Cheronis J. Converting enzyme-independent release of tumor necrosis factor alpha and IL-1beta from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3. *Proc Natl Acad Sci USA* 1999; **96**:6261–6.
62 Greten FR, Arkan MC, Bollrath J *et al.* NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta. *Cell* 2007; **130**:918–31.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Induction of cytokines in peripheral blood mononuclear cells and EBC-1 cells by cytoadherence-deficient mutants of *Mycoplasma pneumoniae*.

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