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Cytadherence of *Mycoplasma pneumoniae* Induces Inflammatory Responses through Autophagy and Toll-like Receptor 4

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Running title: Cytadherence dependent inflammation by *M. pneumoniae*

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ABSTRACT

Mycoplasma pneumoniae causes pneumonia, tracheobronchitis, pharyngitis, and asthma in humans. The pathogenesis of *M. pneumoniae* infection is attributed to excessive immune responses. We previously demonstrated that *M. pneumoniae* lipoproteins induced inflammatory responses through Toll-like receptor (TLR) 2. In the present study, we demonstrated that *M. pneumoniae* induced strong inflammatory responses in macrophages derived from TLR2 knockout (KO) mice. Cytokine production in TLR2 KO macrophages was increased compared with that in the macrophages of wild-type (WT) mice. Heat-killed, antibiotic-treated, and overgrown *M. pneumoniae* failed to induce inflammatory responses in TLR2 KO macrophages. 3-Methyladenine and chloroquine, inhibitors of autophagy, decreased the induction of inflammatory responses in TLR2 KO macrophages. These inflammatory responses were also inhibited in macrophages treated with the TLR4 inhibitor VIPER and those obtained from TLR2/4 double KO mice. Two mutants that lacked the ability to induce inflammatory responses in TLR2 KO macrophages were obtained by transposon mutagenesis. The transposons were inserted in atpC, an ATP synthase F0F1 subunit ε and hypothetical protein MPN333, respectively. These mutants showed deficiencies in cytadherence. These results suggest that cytadherence of *M. pneumoniae* induces inflammatory responses through TLR4 and

autophagy.

INTRODUCTION

Mycoplasmas are wall-less parasitic bacteria and the smallest organisms capable of self-replication (1). *Mycoplasma pneumoniae* causes pneumonia, tracheobronchitis, pharyngitis, and asthma in humans (2-4). From 2010 to 2012, epidemic of *M. pneumoniae* infection were reported worldwide (e.g., in France, Israel, and Japan) (5). However, pathogenic agents such as endotoxins and exotoxins that cause such diseases have not been identified in *M. pneumoniae*. Cytadherence of invading mycoplasmas to the respiratory epithelium, localized host cell injury, and overaggressive inappropriate immune responses appear to contribute to the pathogenesis of *M. pneumoniae* infection (2, 6).

We previously identified 3 lipoproteins responsible for nuclear factor-kappa B (NF-κB) activation (7). One was MPN602, a subunit b of the F0F1-type ATPase (8). The activation of NF-κB by the subunit b of the F0F1-type ATPase was dependent on TLR1, TLR2, and TLR6, indicating that the subunit b of the F0F1-type ATPase is a diacylated lipoprotein. The others were predicted to be lipoproteins MPN611 and MPN162 and designated as NF-κB-activating lipoprotein (N-ALP) 1 and N-ALP2, respectively. N-ALP1 and N-ALP2 activated TLR signaling through TLR1 and TLR2, indicating that both are triacylated lipoproteins (9). Because mycoplasmas lack cell walls, they do not

contain known pathogen-associated molecular patterns (PAMPS) such as lipopolysaccharide (LPS), peptidoglycan (PGN), or lipoteichoic acid. These findings suggested that lipoproteins are key factors of *M. pneumoniae*-induced inflammatory responses and facilitate the development of pneumonia in humans. However, the existence of lipoproteins in non-pathogenic mycoplasmas suggests the presence of an alternative mechanism by which *M. pneumoniae* induce inflammatory responses.

TLRs are a type of pattern-recognition receptor that play critical roles in early innate recognition and inflammatory responses of the host against invading microbes (10, 11). Among the 10 reported TLR family members, TLR2, TLR4, TLR5, and TLR9 have been implicated in the recognition of different bacterial components. For example, PGN, lipoarabinomannan, zymosan, and lipoproteins from various microorganisms are recognized by TLR2 (12-18), whereas LPS, bacterial flagellin, and bacterial DNA are recognized by TLR4, TLR5, and TLR9, respectively (19-22). These TLR family members have been shown to activate NF-κB via IL-1R-associated signal molecules, including myeloid differentiation protein (MyD88), interleukin-1 receptor-activated kinase (IRAK), tumor necrosis factor receptor-associated factor 6 (TRAF6), and NF-κB-inducing kinase (NIK) (23).

Autophagy is a cellular response that involves sequestration of regions within the

74 cytosol with double membrane compartments. Autophagy has shown to play important
75 roles in response to starvation, cell death, remove of damaged organelles,
76 neurodegenerative diseases (24). Recently, it has been recognized that autophagy is
77 involved in both innate and adaptive immunity against various microorganisms (25).
78 However, the relationship between autophagy and mycoplasma species remains to be
79 elucidated.

80 Cytadherence of *M. pneumoniae* in the respiratory tract is the initial event in infection
81 and is mediated by P1 adhesin and other proteins such as P30 and high-molecular-weight
82 (HMW) proteins (26-29). Although the cytadherence of *M. pneumoniae* is believed to be
83 responsible for its pathogenesis (30, 31), the precise mechanisms by which cytadherence
84 is involved in inflammatory responses remain unknown.

85 In this study, we demonstrated that live *M. pneumoniae* induced pro-inflammatory
86 cytokines through TLR2 independent pathway and investigated the mechanisms of the
87 pathway. The TLR2 independent pathway was inhibited by the autophagy inhibitors and
88 was also decreased in macrophages derived from TLR4 or Myd88 KO mice. Moreover,
89 mutant strains that failed to induce pro-inflammatory cytokines in TLR2 KO
90 macrophages were isolated by transposon mutagenesis. These mutants showed
91 deficiencies in cytadherence. Collectively, these data suggest that the cytadherence

property of *M. pneumoniae* induces inflammatory responses through TLR4 and autophagy.

MATERIALS AND METHODS

***M. pneumoniae* strains.** The *M. pneumoniae* wild-type (WT) strain M129 was cultured in PPLO broth (DIFCO, Franklin Lakes, NJ) containing 10% horse serum, 0.25% glucose, 0.25% yeast extract, and 0.002% phenol red at pH 7.6 till the beginning of a stationary phase (the medium color turned slightly orange). The bacterial concentration was adjusted by optical density (OD_{595}) in PBS. Heat-killed *M. pneumoniae* was obtained by heating at 60°C for 30 min. Sonication of *M. pneumoniae* was carried out for 30 sec at an output 5 using Sonifier cell disruptor 200 (Branson, Danbury, CT). To obtain overgrown *M. pneumoniae*, bacteria in the stationary phase (orange color) were cultured for an additional 48 h. Antibiotic-killed *M. pneumoniae* was prepared by treatment of bacterial cultures with 50 μg/ml of gentamicin for 24 h. In these conditions, heat-killed, sonicated, antibiotics-killed, and overgrown *M. pneumoniae* failed to grow on PPLO agar (DIFCO) containing 10% horse serum, 0.25% glucose, 0.25% yeast extract, and colonies were not observed. To ensure the same amount of *M. pneumoniae* at the same optical density, the DNA amount of *M. pneumoniae* were checked as previously described (32). Briefly, *M. pneumoniae* DNA was purified using the FastPure DNA Kit (Takara, Tokyo, Japan) and quantified using a spectrophotometer at OD_{280}.

112 **Mice and TLR KO mice.** C57BL mice were purchased from Kyudo (Saga, Japan).
113 TLR2 and TLR4 KO mice originally established by Dr. Akira (Osaka University) (10)
114 were purchased from Oriental Bio Service (Kyoto, Japan). The TLR2 and TLR4
115 (TLR2/4) double KO mouse strain was generated by cross-breeding TLR2 KO mice with
116 TLR4 KO mice. Genotyping was performed with the following primers:
117 5′-GTTTAGTGCCTGTATCCAGTCAGTGCG-3′, specific for the targeted TLR2 gene;
118 5′-TTGGATAAGTCTGATAGCCTTGCCTCC-3′, specific for the TLR2 gene
119 downstream of the targeting construct; 5′-ATCGCCTTCTATCGCCTTCTTGACGAG-3′,
120 specific for the neo resistance gene inserted in TLR2;
121 5′-CGTGTAAACCAGCCAGGTTTTGAAGGC-3′, specific for the targeted TLR4 gene;
122 5′-TGTTGCCCTTCAGTCACAGAGACTCTG-3′, specific for the TLR4 gene upstream
123 of the targeting construct; and 5′-TGTTGGGTCGTTTGTTCGGATCCGTCG-3′,
124 specific for the neo resistance gene inserted in TLR4. All experiments were conducted in
125 compliance with the institutional guidelines and were approved by Yamaguchi University
126 and Kurume University.
127
128 **Isolation of mouse peritoneal macrophages.** Thioglycollate broth (2 ml)
129 (Sigma-Aldrich, St. Louis, MO) was injected into the peritoneal cavities of C57BL,

TLR2 KO, TLR4 KO, and TLR2/4 double KO mice. After 72 h later, peritoneal exudate macrophages were harvested by centrifugation. The cell pellets were suspended in RPMI 1640 medium (Sigma-Aldrich) containing 10% of FCS (Biowest, Nuaillé, France). The cells were allowed to adhere to 48-well culture plates for 2 h at 37°C in an atmosphere of 5% CO_2. Non-adherent cells were removed by washing with PBS and the remaining adherent cells were infected with *M. pneumoniae*.

Cell treatment and infection. Peritoneal macrophages (5×10^5 cells/250 µl) were cultured for 2 h before infection in a 48-well plate and then treated with 100 µM chloroquine (Sigma-Aldrich), 2 µM cytochalasin D, 5 mM 3-Methyladenine (3MA, Sigma-Aldrich), 50 µM VIPER (Imgenex, San Diego, CA), 10 µM OD2088 (Invivogen, San Diego, CA), 10 µM SB203580 (Wako, Osaka, Japan), 10 µM U0126 (Wako), or 50 µM SP600123 (Wako) for 30 min. Next, the cells were infected with 25 µl of *M. pneumoniae* ($OD_{595} = 0.1$) for 1, 3, or 6 h and the culture supernatants were collected, and concentrations of pro-inflammatory cytokines in the supernatants were measured using ELISA. To ensure the same amount of *M. pneumoniae* at the same optical density, the DNA amount of *M. pneumoniae* were checked as previously described (32).

148 **Mouse infection model.** C57BL WT, TLR2 KO and TLR2/4 double KO mice were
149 intranasally infected with 25 µl of *M. pneumoniae* (OD_{595} = 0.1). After 24 h, the mice
150 were again infected with 25 µl of *M. pneumoniae* (OD_{595} = 0.1) and 24 h later,
151 bronchoalveolar lavage fluid (BALF) was obtained by instilling 1 ml of PBS into the
152 lungs and then aspirating the fluid from the trachea of the mice using a tracheal cannula.
153 The cells that infiltrated in BALF were collected by centrifugation (3,000 rpm for 10 min),
154 and the supernatants were stored at −80°C until determination of cytokine concentrations.
155
156 **ELISA.** Concentrations of TNF-α and IL-6 in the supernatants of peritoneal
157 macrophage were measured using Standard ELISA Development Kits (Pepro Tech,
158 Rocky Hill, NJ) according to the manufacturer's instructions. TNF-α concentrations in
159 BALF of WT, TLR2 KO, TLR2/4 double KO mice were measured using the ELISA
160 MAX Standard Kit (Biolegend, San Diego, CA) according to the manufacturer's
161 instructions.
162
163 **Real-time PCR.** Peritoneal macrophages (5×10^5 cells/250 µl) were cultured for 2 h in
164 a 48-well plate and then infected with 25 µl of *M. pneumoniae* (OD_{595} = 0.1) for 1, 2, or 6
165 h. Total RNA was isolated from whole lung tissue by using the NucleoSpin Kit

(Clonetech, Mountain View, CA) and 1 μg of total RNA was used to synthesize cDNA using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). PCR was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo). The following primer sets were purchased from Takara: TNF-α, forward 5′-AAGCCTGTAGCCCACGTCGTA-3′ and reverse 5′-GGCACCACTAGTTGGTTGTCTTTG-3′; IL-6, forward 5′-CCACTTCACAAGTCGGAGGCTTA-3′ and reverse 5′-GCAAGTGCATCATCGTTGTTCATAC-3′; IL-10, forward 5′-GACCAGCTGGACAACATACTGCTAA-3′ and reverse 5′-GATAAGGCTTGGCAACCCAAGTAA-3′; IL-17, forward 5′-CTGATCAGGACGCGCAAAC-3′ and reverse 5′-TCGCTGCTGCCTTCACTGTA-3′; IFN-γ, forward 5′-CGGCACAGTCATTGAAATCCTA-3′ and reverse 5′-GTTGCTGATGGCCTGATTGTC-3′; β-actin, forward 5′-TGACAGGATGCAGAAGGAGA-3′ and reverse 5′-GCTGGAAGGTGGACAGTGAG-3′. All data were normalized to β-actin.

Immunofluorescence microscopy. Peritoneal macrophages (2×10^6 cells/ml) were cultured for 2 h before infection on coverslips in a 12-well plate. Next, the cells were

infected with 100 µl of *M. pneumoniae* M129 or GFP expressing *M. pneumoniae* TK165 (33) (OD_{595} = 0.1) for 6 or 18 h. The samples were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, washed three times with PBS, and incubated successively three times for 5 min in blocking buffer (3% bovine serum albumin in PBS) at room temperature. The samples were permeabilized in 0.1% Triton X-100 and washed three times with PBS, followed by treatment with 2 µg/ml anti-microtubule-associated protein 1A/1B-light chain 3 (LC3) polyclonal goat antibody (Santa Cruz, Dallas, TX) diluted in blocking buffer. After incubation for 1 h at room temperature, the samples were washed three times for 5 min with blocking buffer, stained with fluorescein isothiocyanate (FITC)- or rhodamine-labeled donkey anti-goat IgG (4 µg/ml, Santa Cruz) in blocking buffer, and incubated for 1 h at room temperature. DNA of macrophages and *M. pneumoniae* were stained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescent images were obtained using a LSM 710 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

Transposon mutagenesis. Approximately 10^7 CFU/ml of *M. pneumoniae* were transfected with 5 µg of pISM2062 using Gene Pulser (Bio-Rad, Hercules, CA) at 100 Ω resistance, 25 F capacitance, and 2.5 kV. Transformed *M. pneumoniae* colonies were

selected on PPLO agar containing 50 μg/ml of gentamicin. TLR2 KO peritoneal macrophages (5×10^5 cells/250 μl) were infected with 25 μl of transformed *M. pneumoniae* ($OD_{595} = 0.02$) for 3 h and then the culture supernatants were collected. The strains that showed decreased TNF-α induction were screened by ELISA.

Verification of the transposon-inserted regions. DNA of transposon inserted *M. pneumoniae* was purified using the Fast Pure DNA Kit (Takara, Shiga, Japan). First PCR amplification was performed with the following primers: specific for IS256, 5′-AAGTCCTCCTGGGTATGT-3′; and random primer, 5′-GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC-3′. A second PCR amplification was performed with the following primers: specific for IS256, 5′-CGACTCTAGAGGATCCATTGTACCGTAAAAGGACTG-3′; and specific for random primer, 5′-CGGTACCCGGGGATCGGCCACGCGTCGACTAGTAC-3′. The amplified PCR products were cloned into the pUC19 vector using In-fusion HD Cloning Kit (Clontech), and sequenced using the ABI3130 sequencer (Life Technologies, Carlsbad, CA).

Hemadsorption assay. The hemadsorption assay was performed directly on an agar

plate. In brief, the colonies were overlaid with 15 ml of fresh sheep blood, washed, and resuspended in PBS to a final concentration of 0.5% (v/v). After incubation at 37°C for 30 min, the suspension was carefully discarded and unbound erythrocytes were gently removed by washing 3 times with PBS.

Statistical analysis. All data were compared using one-way analysis of variance and the results are expressed as means and standard deviations. Differences between groups were compared by multiple comparisons using the Bonferroni t-test. Differences were considered significant at *P* values of <0.01 or 0.05.

RESULTS

TLR2-independent induction of pro-inflammatory cytokines by *M. pneumoniae.*

We previously demonstrated that purified or synthesized lipoproteins of *M. pneumoniae* induced inflammatory responses through TLR2. To examine whether TLR2 is an important receptor for the induction of inflammatory responses in live *M. pneumoniae* infection, peritoneal macrophages derived from TLR2 KO mice were infected with *M. pneumoniae* and induced TNF-α concentrations were measured using ELISA (Fig. 1A). In WT macrophages, infection by both live and heat-killed *M. pneumoniae* induced TNF-α expression. Contrary to expectations, live *M. pneumoniae* induced TNF-α expression in TLR2 KO macrophages, which was slightly increased compared with that in WT macrophages infected with live *M. pneoumoniae*. These results indicate that live *M. pneumoniae* induces inflammatory responses through a TLR2-independent pathway. Similar to lipoproteins of *M. pneumoniae*, heat-killed *M. pneumoniae* induced TNF-α expression in WT macrophages; however, they failed to do so in TLR2 KO macrophages, suggesting that lipoproteins and TLR2 are important to induce inflammatory responses in the infection of heat killed *M. pneumoniae*. To rule out the possibility that heating of *M. pneumoniae* resulted in conformational changes in the surface components of *M. pneumoniae* leading to a reduction in TNF-α production, TLR2 KO macrophages were

247 infected with sonication-killed, overgrown, and gentamicin-killed *M. pneumoniae* (Fig.
248 1B). All treatments decreased the induction of TNF-α expression in TLR2 KO
249 macrophages. These results suggest that biological activity of *M. pneumoniae* play an
250 important role in the TLR2-independent pathway.

251 To investigate the temporal kinetics of *M. pneumoniae*-induced inflammatory
252 response, peritoneal macrophages from WT and TLR KO mice were infected with live
253 and heat-killed *M. pneumoniae* and TNF-α mRNA expression levels at 0.5, 1, 2, or 6 h
254 post-infection were measured by real-time PCR (Fig. 2A). TNF-α was expressed in WT
255 macrophages infected with both live and heat-killed *M. pneumoniae*. TNF-α expression
256 was observed at 0.5 h post-infection and the same level was sustained till 2 h. However,
257 TNF-α expression was not observed at 6 h post-infection. In TLR2 KO macrophages,
258 TNF-α expression was induced by live *M. pneumoniae*, whereas the expression was
259 reduced in the case of heat-killed *M. pneumoniae* infection. TNF-α expression by live *M.*
260 *pneumoniae* was observed at 1 h post-infection and reached a maximum level at 2 h. The
261 expression level at 2 h post-infection in TLR2 KO macrophages was approximately
262 2.5-fold higher than that in WT macrophages. To confirm whether the TLR2-independent
263 pathway was important for the induction of other pro- and anti-inflammatory cytokines,
264 the expressions of TFN-α, IL-6, IL-17, IFN-γ, and IL-10 mRNA were measured (Fig. 2B).

Although live and heat-killed *M. pneumoniae* induced TNF-α, IL-6 and IL-10 expression in WT macrophages, heat-killed *M. pneumoniae* failed to induce expression of these cytokines in TLR2 KO macrophages. *M. pneumoniae*-induced expression of IFN-γ and IL-17 was not detected in either WT or TLR2 KO macrophages (data not shown).

Autophagy and Endocytosis dependent induction of pro-inflammatory cytokines. To elucidate the mechanism by which live *M. pneumoniae* induces TLR2 independent pro-inflammatory cytokine production, we examined the involvement of autophagy and endocytosis. Peritoneal macrophages were treated with autophagy and endocytosis inhibitors and then infected with live *M. pneumoniae* (Fig. 3A). 3-MA inhibits autophagy by blocking autophagosome formation via the inhibition of type III Phosphatidylinositol 3-kinases (PI-3K) (34). When macrophages derived from WT and TLR2 KO mouse were treated with 3-MA and infected with live *M. pneumoniae*, TNF-α induction was inhibited in comparison with that of control PBS-treated cells. Chloroquine is a lysosomotropic agent that prevents both fusion of autophagosome with lysosome, and lysosomal protein degradation (35). In the presence of chloroquine, TNF-α induction in TLR2 KO macrophages was completely inhibited; however, the induction in WT macrophages was not decreased (Fig. 3A). To further examine whether the autophagy participates in

283 pro-inflammatory cytokines induction, localization of autophagy in *M. pneumoniae*
284 infected macrophages were examined. WT and TLR2 KO macrophages were infected
285 with live *M. pneumoniae* and the localizations of DNA of *M. pneumoniae* and autophagy
286 marker protein LC3 were observed with confocal microscopy. After 6 h of infection, *M.*
287 *pneumoniae* was observed as the small particles of DNA in macrophages (Fig. 3B and C,
288 arrows in upper panels), whereas the small particle of DNA was not observed in
289 macrophages without infection (Fig. 3B and C, lower panels). The small DNA particles
290 were co-localized with LC3 in both WT and TLR2 KO mouse (Fig. 3A and B, video S1
291 and S2), whereas the small particles of DNA was completely removed and co-localization
292 with LC3 was not observed at 18 h post infection (Fig. S1A and B). To confirm that small
293 particle of DNA was *M. pneumoniae*, WT and TLR2 KO macrophages were infected with
294 GFP expressing *M. pneumoniae* TK165, and the co-localization of *M. pneumoniae* and
295 LC3 was examined. The co-localization of GFP-expressing *M. pneumoniae* and LC3 was
296 also observed at 6h post infection (Fig. S2A and B). These results suggest that autophagy
297 is necessary for TLR2-independent induction of inflammatory responses.

298 Cytochalasin D is an inhibitor of phagocytosis and macropinocytosis that disrupts
299 actin filaments (36, 37). When TLR2 KO macrophages were treated with cytochalasin D
300 and infected with live *M. pneumoniae*, TNF-α induction was inhibited in comparison with

301 that of control DMSO-treated cells, whereas the induction was not decreased in WT
302 macrophages (Fig. 3D). These results suggest that phagocytosis is also important for
303 TLR2-independent inflammatory responses.
304
305 **TLR4 dependent induction of pro-inflammatory cytokines.** Some of TLRs such as
306 TLR4 and TLR7 serves as a sensor for autophagy (38-40). In addition that, TLR4, TLR7,
307 TLR8, and TLR9 are reported to be activated in mature endosomes and the acidification
308 of endosomes is necessary to induce the down stream signaling (41-43). Therefore the
309 involvement of TLR4 and TLR9 in pro-inflammatory cytokine induction was examined.
310 Macrophages were treated with the TLR4 and TLR9 antagonists VIPER and OD2088,
311 respectively, and infected with *M. pneumoniae* (Fig. 4A). Although OD2088
312 administration did not affect of TNF-α induction, VIPER decreased the induction
313 markedly, suggesting the involvement of TLR4 in the TLR2-independent pathway.
314 To confirm the involvement of TLR4, peritoneal macrophages derived from TLR4 KO
315 mice and TLR2/4 double KO mice were infected with *M. pneumoniae* (Fig. 4B). In TLR
316 4 KO macrophages infected with live *M. pneumoniae*, TNF-α induction was decreased to
317 approximately 60% compared with that in WT macrophages. On the other hand, TNF-α
318 induction in TLR2/4 double KO macrophages was decreased to 40% compared with that

in TLR2 KO macrophages. In contrast, TNF-α induction by heat-killed *M. pneumoniae* was completely dependent on TLR2. These results indicate the involvement of TLR4 in TLR2-independent induction of inflammatory responses.

To further examine the association of TLRs, the involvement of MyD88, a critical adapter protein of TLRs, was examined. Peritoneal macrophages from MyD88 KO mice were infected with live or heat-killed *M. pneuumoniae* (Fig. 4C). The induction of TFN-α by both live and heat *M. pneumoniae* was completely impaired, suggesting that MyD88 is a critical factor for the induction of TLR2-independent inflammatory responses.

To determine whether the TLR2-independent pathway is involved in the development of pneumonia, inflammatory responses in the lungs of mice were investigated. WT, TLR2 KO, and TLR2/4 double KO mouse were infected with *M. pneumoniae* intranasally and TNF-αinduction in the bronchoalveolar lavage fluid (BALF) was measured using ELISA (Fig. 4D). In TLR2 KO mouse, TNF-αinduction was increased compared with that in WT mice. TNF-α induction in TLR2/4 double KO mice was decreased compared with that in TLR2 KO mice. These results suggest that the TLR2-independent pathway is involved in lung inflammation and TLR4 is an important receptor.

Since Mitogen-activated protein-kinase (MAPK) is thought to be a downstream signaling of TLRs to induce autophagy, the involvement of MAPK was investigated (Fig.

337 4E). When WT macrophages were infected with live *M. pneumoniae*, TNF-α induction
338 was decreased by the c-jun N-terminal kinase (JNK) inhibitor SP600125 and the
339 extracellular signal-regulated kinase (ERK) inhibitor U0126. In contrast, TNF-α
340 induction in TLR2 KO macrophages was inhibited by SP600125, but not by U0126.
341 SB203580, an inhibitor of p38, failed to reduce the TNF-α induction in both WT and
342 TLR2 KO macrophages. These results indicate that TLR2-independent induction of
343 inflammatory responses is MAPK-dependent and JNK is a key factor of this signaling
344 pathway.
345
346 **Cytadherence dependent induction of inflammatory responses.** Next, transposon
347 mutagenesis was conducted to identify bacterial factors that related to the
348 TLR2-independent pathway. *M. pneumoniae* M129 was transformed with pISM2062
349 plasmids containing the IS256 transposon. TLR2 KO macrophages were infected with *M.*
350 *pneumoniae* mutants and those with reduced ability to induce TNF-α expression were
351 selected. Of 2,880 mutants, 2 strains, K2 and K3, were isolated as TNF-α non-inducible
352 mutants in TLR2 KO macrophages. Fig. 6A. To identify the genes responsible for the
353 TLR2-independent induction of inflammatory responses, transposon-inserted regions in
354 the DNA of the K2 and K3 strains were amplified by PCR, cloned into pUC19 plasmids,

and sequenced (Table 1). In the K2 strain, the transposon was inserted in atpC, an ATP synthase F0F1 subunit ε. In the K3 strain, the transposon was inserted within the hypothetical protein, MPN333. The N terminal sequence of MPN 333 had similarity to ATP-binding cassette (ABC)-2 family transporter protein. RNA expression of genes downstream of atpC and MPN333 were not impaired. Although WT *M. pneumoniae* normally bind to the culture flask through sialylated proteins contained in serum (44), these 2 mutants floated in the medium. Therefor, cytadherence properties of these mutants were examined using the hemadsorption assay (Fig. 6B). WT *M. pneumoniae* were able to bind to sheep erythrocytes, but K2 and K3 did not exhibit binding activity, indicating that these mutants lacked cytadherence properties.

365 **DISCUSSION**

366 Mycoplasma species lack cell walls and the cells are surrounded by cell membranes
367 (1). Moreover, mycoplasma cells do not contain TLR ligands such as LPS, PGN, and
368 lipoteichoic acid but contain an abundance of acylated proteins as cell-surface antigens,
369 and many putative lipoprotein-encoding genes have been identified in sequenced
370 mycoplasma genomes (45, 46). These findings suggest that lipoproteins are main
371 components of *M. pneumoniae* that induce inflammatory responses and cause pneumonia
372 in humans. We previously reported that the purified or synthesized lipoproteins of
373 mycoplasma species induce inflammatory responses through TLR2 (7-9). Moreover,
374 lipoproteins derived from various mycoplasmas have been reported to act as PAMPS
375 (47-50). However, the existence of lipoproteins in non-pathogenic mycoplasmas suggests
376 the presence of another mechanism by which *M. pneumoniae* induce inflammatory
377 responses. In this study, we demonstrated that live *M. pneumoniae* was able to induce
378 inflammatory responses even in the lung and macrophage cells of TLR2 KO mice (Fig. 1,
379 2, and 4). Notably, *M. pneumoniae* inactivated by heat, sonication, antibiotics, and
380 overgrowth failed to induce inflammatory responses in TLR2 KO macrophages (Fig. 1B),
381 suggesting that some biological activities of *M. pneumoniae* are necessary to induce
382 TLR2-independent inflammatory responses.

383 To identify the bacterial factor that induces the TLR2-independent inflammation
384 pathway, transposon mutagenesis was conducted. As a result, 2 mutants with decreased
385 abilities to induce TNF-α expression in TLR2 KO macrophages were isolated (Fig. 5A
386 and Table 1). The transposons were inserted in MPN597 and MPN333. MPN597 was
387 atpC, an ATP synthase F0F1 subunit ε. ATP synthase F0F1 subunit ε is a regulatory
388 protein of the F0F1 type ATPase and can inhibit the ATP hydrolysis in the absence of
389 proton motive forces (51). MPN333 is a hypothetical protein with an N-terminal
390 sequence similar to the ABC-2 family transporter protein. ABC-2 family transporter
391 protein is a subfamily of ABC transporters and related to capsular polysaccharide export
392 (52). Notably, these mutants were deficient in cytadherence (Fig. 5B). The cytadherence
393 of *M. pneumoniae* is mediated by attachment organelle, including P1 adhesin and other
394 additional proteins such as P30 or HMW proteins (26-29). These proteins are unique to
395 mycoplasma species, and their homologs have not been identified in any other bacterial
396 species (53). The cytadherence of *M. pneumoniae* is closely linked to the unique
397 movement specific to mycoplasma species, called gliding motility (28). The gliding
398 motility of some mycoplasmas such as *M. mobile* is ATP-dependent (29). In addition, the
399 ABC-2 family transporter protein is reportedly involved in the motility of *Myxococcus*
400 *xanthus* (54). Considering that complementation of mutated genes is impossible in *M.*

401 *pneumoniae*, we could not rule out the possibility that inactivation of downstream genes
402 of transposon-inserted genes resulted in the deficiency in cytadherence. However, the
403 mRNA expressions of down stream genes of atpC and MPN333 (MPN596 and bcrA,
404 respectively) were not impaired (data not shown). Taken together, these results indicate
405 that atpC and MPN333 may be the new virulence factors that are responsible for
406 cytadherence and inflammation in *M. pneumoniae* infections. However, in this study, we
407 failed to screen well-known cytadherence factors such as P1, P30, or HMW. This may
408 suggest that the functions of atpC or MPN333 itself are important for the induction of
409 inflammatory responses. Although further screening of mutants is necessary to clarify the
410 relationship between cytadherence and the induction of inflammatory responses, the
411 relationship between cytadherence and inflammatory responses in host cells are
412 consistent with our previous report that cytadherence of *M. pneumoniae* activates
413 cytokine production in human monocyte cells (32), and earlier reports that a protease
414 treatment decreases the induction of pro-inflammatory cytokines by *M. pneumoniae* (31).
415 Furthermore, *M. pneumoniae* cultured under non-adherent conditions fails to induce IL-4
416 expression in rodent mast cells (30). Moreover, elongated infection time or high
417 concentration of atpC and MPN333 mutants still induced pro-inflammatory cytokines
418 (data not show) in TLR2 KO macrophages. These results suggest that cytadherence is

more likely to be involved in the TLR2 independent induction of inflammatory responses than the functions of atpC and MPN333. However, the mechanism by which *M. pneumoniae* cytadherence activates the induction of pro-inflammatory responses has not been determined. In this study, we observed that the TLR2-independent induction of pro-inflammatory cytokines was dependent on endocytosis, because inhibition of endocytosis with cytochalasin D decreased the TNF-α induction in TLR2 KO macrophages (Fig. 3D). These result indicate that uptake of *M. pneumoniae* by macrophages is necessary to TLR2 independent pathway and suggest that cytadherence of *M. pneumoniae* may enhanced the uptake by macrophages.

The inductions of pro-inflammatory cytokines were inhibited by the inhibitors of autophagy, and *M. pneumoniae* was co-localized with autophagy marker protein LC3 in WT and TLR2 KO macrophages (Fig. 3). These results indicate that autophagy play an important role in induction of pro-inflammatory cytokines by *M. pneumoniae*. An autophagy inhibitor, chloroquine inhibits the fusion of lysosomes and autophagosomes. In this study, chloroquine treatment completely decreased the TLR2 independent induction of pro-inflammatory cytokines, suggesting that degradation of *M. pneumoniae* in autophagosomes is required for the induction. The TLR2-independent induction of pro-inflammatory cytokine was also dependent on TLR4, Myd88 (Fig. 4). It was reported

437 that some TLRs such as TLR4 and TLR7 serves as a sensor for autophagy in Myd88
438 dependent manner, and MAPK is important downstream signal of this pathway (38-40,
439 55). These results suggest that recognition of *M. pneumoniae* with TLR4 induces
440 autophagy, followed by induction of pro-inflammatory cytokines. The TLR2 independent
441 pathway was also dependent on JNK MAPK in this study (Fig. 4E). This result was
442 consistent with the report that JNK MAPK was involved in induction of autophagy and
443 cell death (56).

444 Generally, bacteria that are retained in, or escape from phagosome can be targeted by
445 autophagy. Recently, LC3-associated phagocytosis (LAP) was shown to uptake and
446 degrade the bacteria without the ability to retain in or escape form phagosome (57).
447 Similar to autophagy, LAP is consistent of autophagy-related protein including LC3,
448 autophagy-related gene (ATG) 5, and ATG7. However, unlike autophagy, double
449 membrane structures are not formed around the LPA (58). Since it is still controversial
450 whether *M. pneumoniae* can escape from phagosome and grow intracellular (59), we
451 could not rule out the possibility that the TLR2 independent induction of
452 pro-inflammatory cytokines were dependent on LPA but not on autophagy.

453 In this study, TLR4 seems key receptor to induce autophagy and following
454 inflammatory responses (fig. 4). TLR4 is essential for the recognition of LPS, which is

455 composed of lipid A, a core oligosaccharide, and an O-antigen. TLR4 recognizes lipid A
456 of LPS. Because mycoplasma species lack cell walls and dos not contain LPS, the ligands
457 for TLR4 in mycoplasmas remain unclear. Other than LPS, TLR4 also recognizes fungal
458 mannan and glucuronoxylomannan (60), protozoan glycoinositolphospholipids (61, 62),
459 and viral proteins (63, 64). Mycoplasma species also express unique glycolipids,
460 phosphoglycolipids (65), and polysaccharides (66, 67). Although further studies are
461 needed to determine the exact ligands of *M. pneumoniae* for TLR4, these molecules may
462 be potential TLR4 ligands. TNF-α induction was only partially decreased in
463 VIPER-treated TLR2 KO macrophages and TLR2/4 double KO macrophages (Fig. 3 and
464 4A). In contrast, TNF-α induction was completely inhibited in MyD88 KO macrophages
465 (Fig. 4B). Because MyD88 is an essential adaptor protein in TLR signaling, these results
466 suggest that TLRs are necessary to induce inflammatory responses in TLR2 KO
467 macrophages, and that other TLRs are associated with this induction in concert with
468 TLR2 and TLR4.

469 In conclusion, our results suggest that *M. pneumoniae* induced inflammatory
470 responses in TLR4 and autophagy dependent manner, and the cytadherence property of *M.*
471 *pneumoniae* is a key factor. Hence, the proteins involved in cytadherence including atpC
472 and MPN333, or TLR4 ligands present potential targets for the development of

473 alternative strategies to prevent and treat *M. pneumoniae* infection.

474 Table 1. Transposon-inserted genes in *M. pneumoniae* mutants

Strains	Locus tag	Gene name	Inserted position	Function
K2	MPN597	atpC	31	ATP synthase F0F1 subunit ε
K3	MPN333	F10_orf750	768	ABC-2 family transporter protein[a]

475 ABC-2 family transporter protein[a], The N terminal sequence of MPN333 is similar to

476 ABC-2 family transporter protein.

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679 **FIGURE LEGENDS**

680 Figure 1. TNF-α induction in TLR2 KO macrophages. (A) Peritoneal macrophages
681 derived from WT and TLR2 KO mice were infected with live or heat-killed *M.*
682 *pneumoniae*. After 6 h of incubation, TNF-α concentrations in the culture medium were
683 measured using ELISA. (B) Peritoneal macrophages derived from TLR2 KO mice were
684 infected with heat-, sonication-, overgrown-, or antibiotic-killed *M. pneumoniae*. After 6
685 h of incubation, TNF-α concentrations in the culture medium were measured using
686 ELISA. All values are represented as the means and SD of 3 assays. *: $P < 0.01$ compared
687 with PBS by multiple comparison.

688

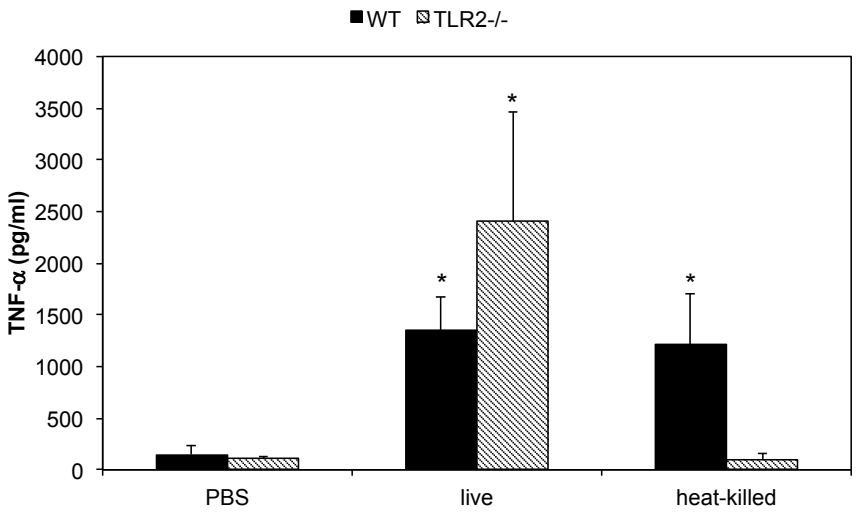
689 Figure 2. mRNA expression of pro-inflammatory cytokines by *M. pneumoniae* infection.
690 (A) Peritoneal macrophages derived from WT and TLR2 KO mice were infected with
691 live or heat-killed *M. pneumoniae*. After 1, 2, 3, and 6 h of incubation, total RNA was
692 isolated and TNF-α mRNA expression levels were measured using real-time PCR. All
693 values are represented as the means and SD of 3 assays. *: $P < 0.01$ compared with PBS
694 by multiple comparison. (B) Peritoneal macrophages derived from WT and TLR2 KO
695 mice were infected with live and heat-killed *M. pneumoniae*. After 6 h of incubation, total
696 RNA was isolated and the expression level of TNF-α, IL-6, and IL-10 mRNA were

697 measured using real-time PCR. All values are presented as the means and SD of 3 assays.
698 *: $P < 0.01$ compared with WT macrophages by multiple comparison.
699
700 Figure 3. Autophagy dependent induction of TNF-α. (A) Peritoneal macrophages derived
701 from TLR2 KO mice were treated with 100 μM chloroquine or 5 mM 3MA for 30 min.
702 The treated cells were infected with live *M. pneumoniae*. After 6 h of incubation, TNF-α
703 concentration in the culture medium were measured using ELISA. (B, C) Peritoneal
704 macrophages were infected with live *M. pneumoniae* for 6 h. LC3 was stained with
705 anti-LC3 antibody and FITC-labeled secondary antibody (green). DNA of macrophages
706 and *M. pneumoniae* were stained with DAPI (blue). Small DNA particles derived from *M.*
707 *pneumoniae* were shown with arrows. Scale bar = 20 μm. (D) Peritoneal macrophages
708 derived from TLR2 KO mice were treated with 2 μM cytochalasin D for 30 min and then
709 infected with *M. pneumoniae*. After 6 h of incubation, TNF-α concentrations in the
710 culture medium were measured using ELISA. All values are presented as the means and
711 SD of 3 assays. *: $P < 0.01$ compared with PBS or DMSO treatment by multiple
712 comparison. **: $P < 0.05$ compared with PBS or DMSO treatment by multiple
713 comparison.
714

715 Figure 4. TLR4- and MyD88-dependent TNF-α induction. (A) Peritoneal macrophages
716 derived from TLR2 KO mice were treated with 10 μM OD2088 or 50 μM VIPER for 30
717 min and then infected with *M. pneumoniae*. After 6 h of incubation, TNF-α
718 concentrations in the culture medium were measured using ELISA. All values are
719 presented as the means and SD of 3 assays. *: $P < 0.01$ compared with PBS treatment by
720 multiple comparison. (B) Peritoneal macrophages derived from TLR2 KO, TLR4 KO,
721 and TLR2/4 double KO mice were infected with *M. pneumoniae*. After 6 h of incubation,
722 TNF-α concentration in the culture medium were measured using ELISA. All values are
723 presented as the means and SD of 3 assays. *: $P < 0.01$ compared with WT macrophages
724 by multiple comparison. (C) Peritoneal macrophagesderived from TLR2 KO, TLR4 KO,
725 and MyD88 KO mice were infected with *M. pneumoniae* ($OD_{595} = 0.1$). After 6 h of
726 incubation, TNF-α concentrations in the culture medium were measured using ELISA.
727 All values represent the means and SD of three assays. All values are presented as the
728 means and SD of 3 assays. *: $P < 0.01$ compared with WT macrophages by multiple
729 comparison. (D) WT, TLR2 KO, and TLR2/4 double KO mice were intranasally infected
730 with *M. pneumoniae*. After 24h, the mice were infected with same amount of *M.*
731 *pneumoniae* again for an additional 24h. TNF-α concentrations in the BALF were
732 measured using ELISA. All values represent the means and SD of three assays. All values

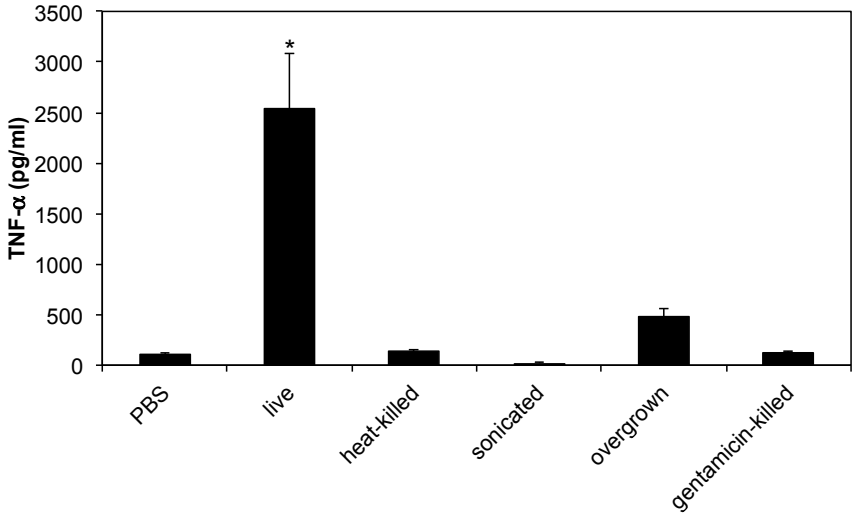
733 are presented as the means and SD of 3 assays. **: $P < 0.05$ compared with WT mice by
734 multiple comparison. (E) Peritoneal macrophagesderived from TLR2 KO mice were
735 treated with 10 μM SB203580, 10 μM U0126, or 50 μM SP600123 for 30 min. The
736 treated cells were infected with *M. pneumoniae*. After 6 h of incubation, TNF-α
737 concentration in the culture medium were measured using ELISA. All values are
738 presented as the means and SD of 3 assays. *: $P < 0.01$ compared with DMSO treatment
739 by multiple comparison. **: $P < 0.05$ compared with DMSO treatment by multiple
740 comparison.
741
742 Figure 5. Cytadherence-dependence TNF-α induction. (A) Transposon mutagenesis of *M.*
743 *pneumoniae* was performed as described in the Materials and Methods section. Peritoneal
744 macrophages derived from TLR2 KO mice were infected with transformed *M.*
745 *pneumoniae* for 3h. TNF-α concentrations in the culture medium were measured usig
746 ELISA. All values are presented as the means and SD of 3 assays. *: $P < 0.01$ compared
747 with M129 by multiple comparison. (B) PPLO agar plates were overlaid with 15 ml of
748 fresh sheep blood, washed, and resuspended in PBS to a final concentration of 0.5% (v/v).
749 After incubation at 37°C for 30 min, the plates were washed with PBS. Bar = 1mm.

750 Fig. 1A



751

752 Fig. 1B



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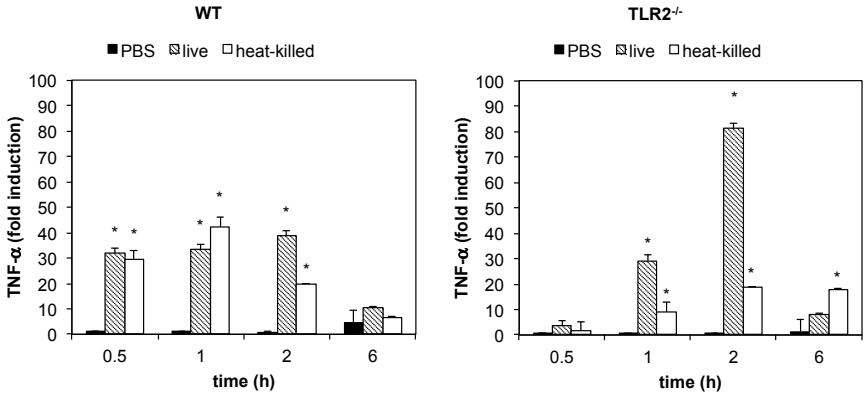
754

755 Fig. 2A



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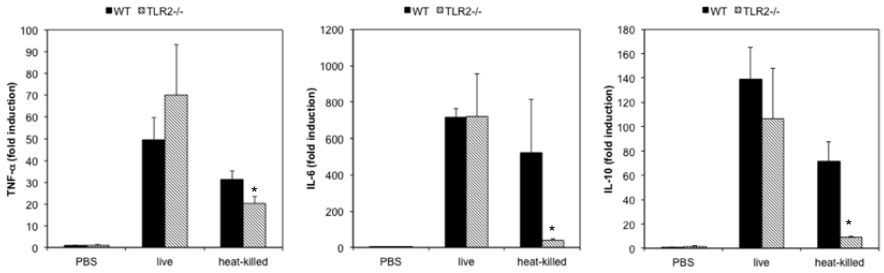
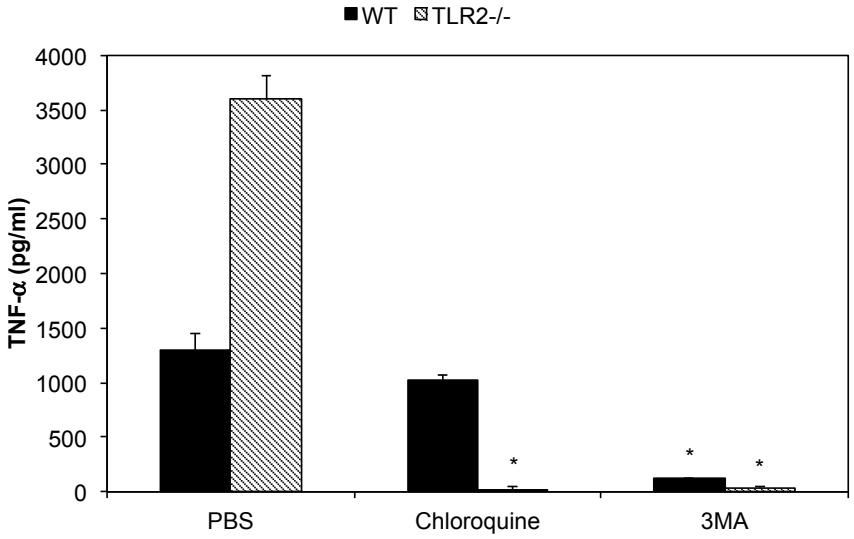
758 Fig. 2B



759

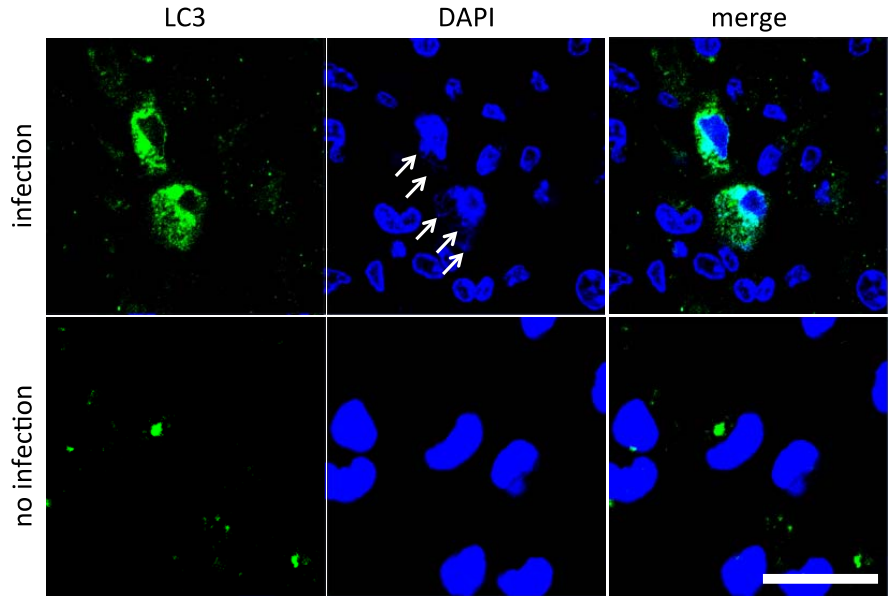
760

761 Fig. 3A



762

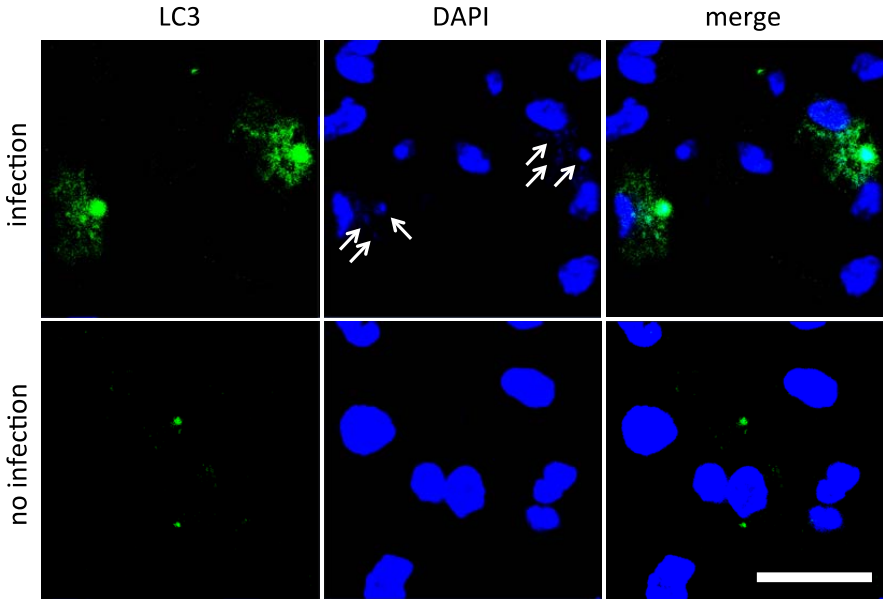
763 Fig. 3B



764

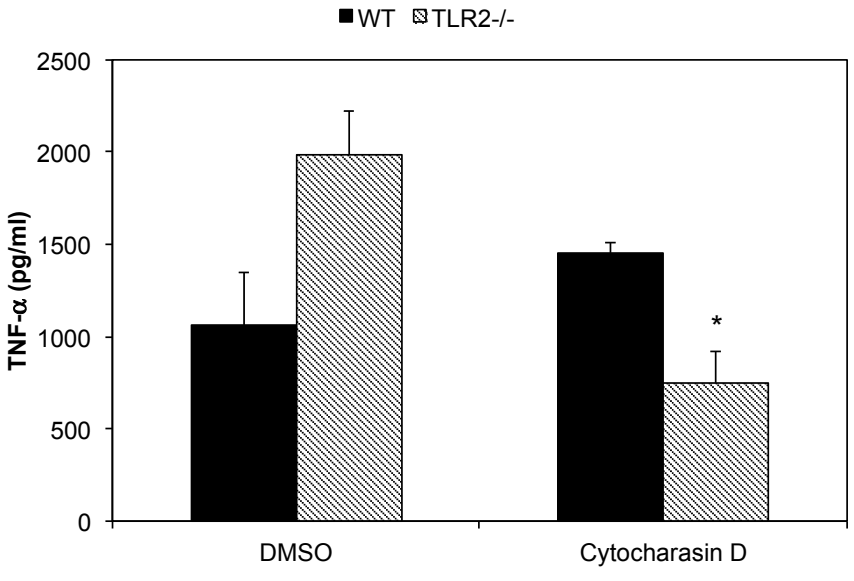
765

766 Fig. 3C



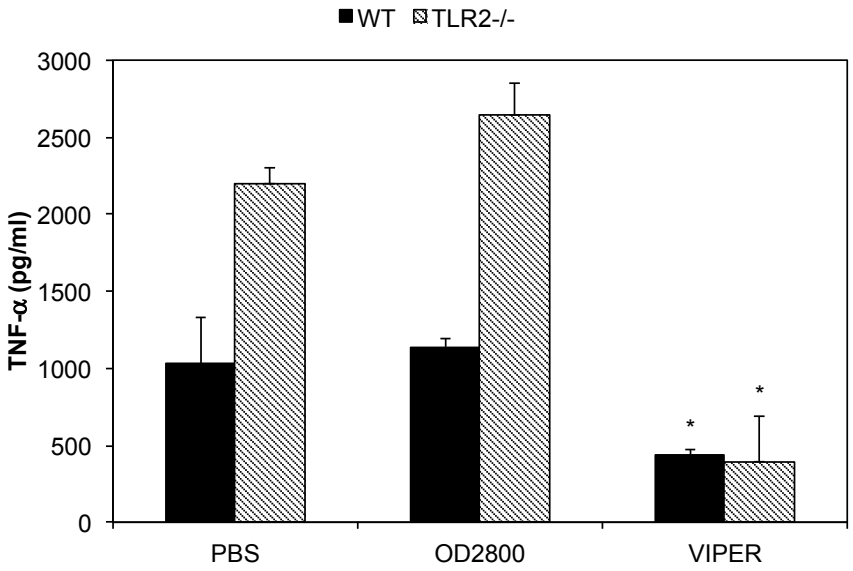
767

768 Fig. 3D



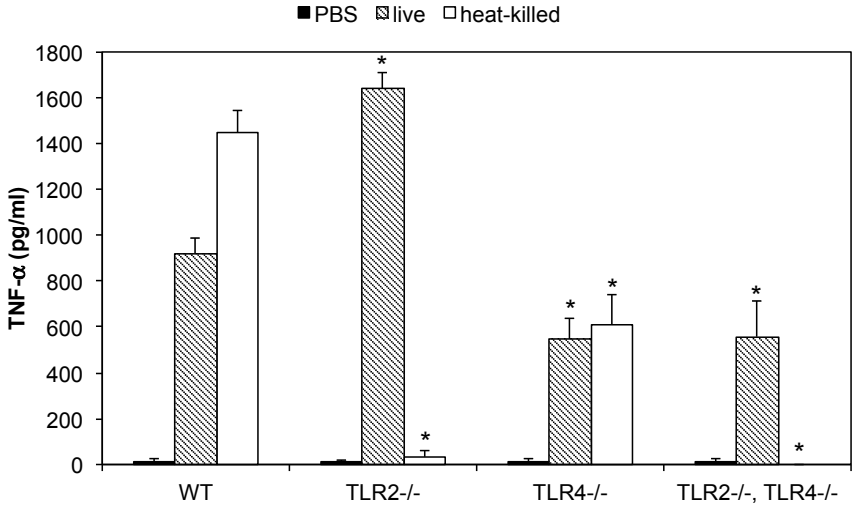
769

770 Fig. 4A



771

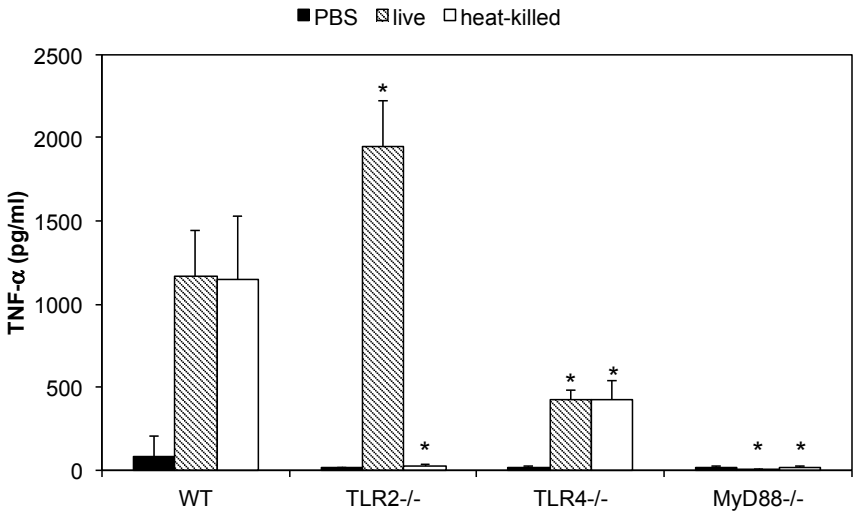
772 Fig. 4B



773

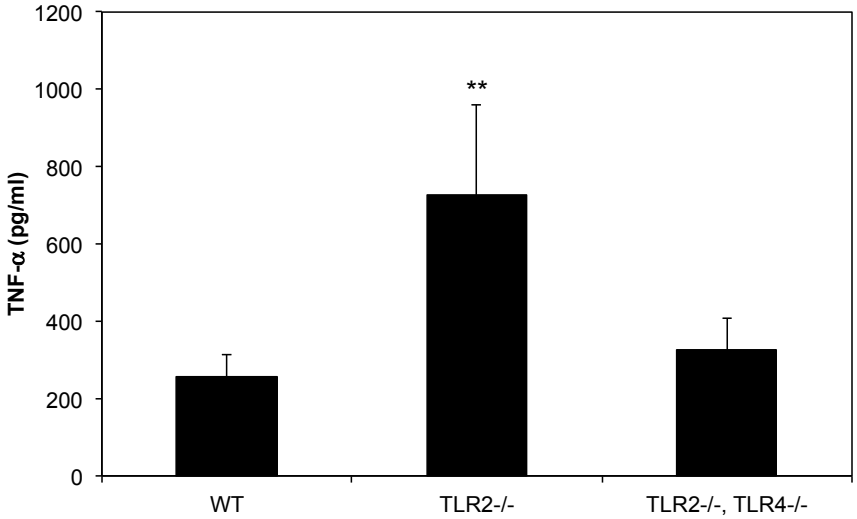
774

775 Fig. 4C



776

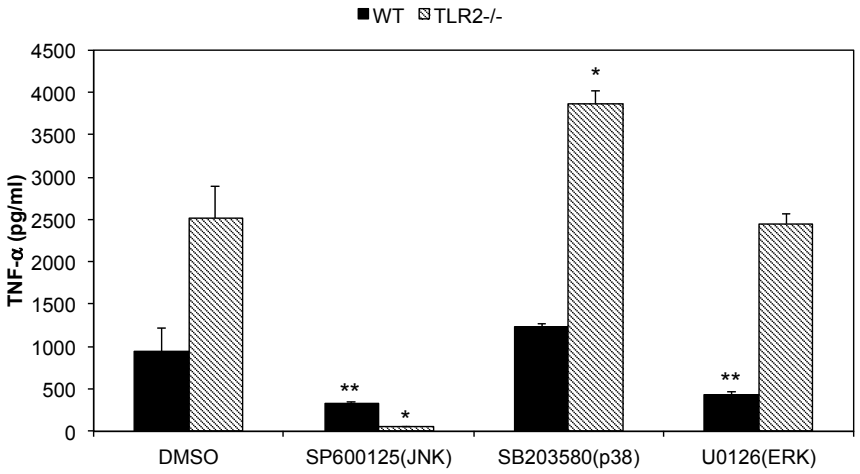
777 Fig. 4D



778

779

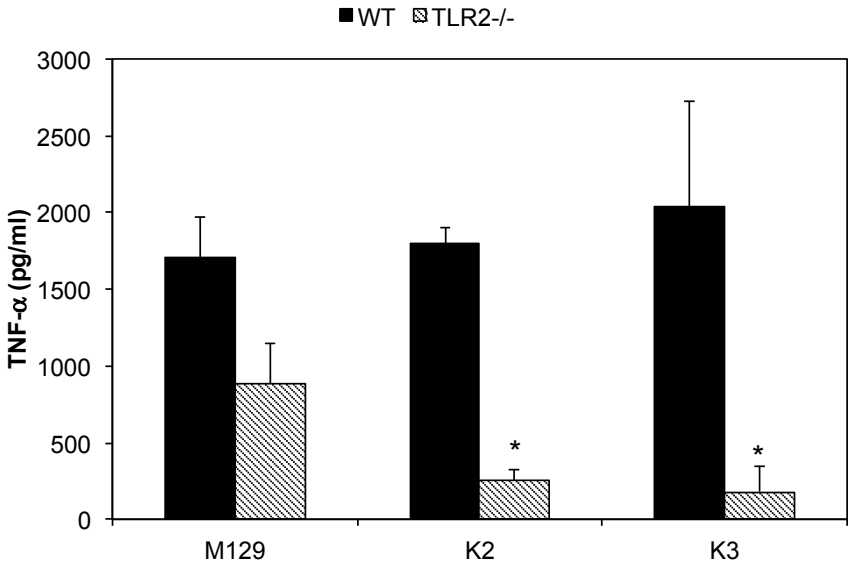
780 Fig. 4E



781

782

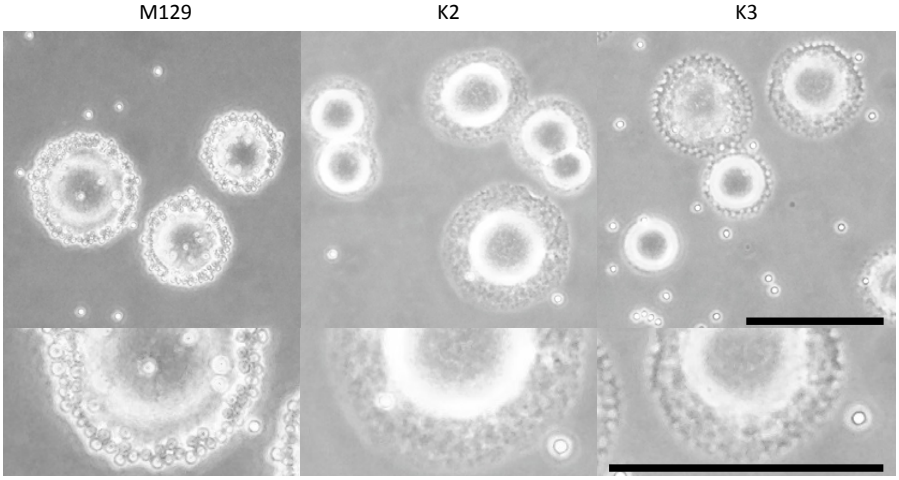
783 Fig. 5A



784

785

786 Fig. 5B



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