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Chlamydia pneumoniae harness host NLRP3 inflammasome-mediated caspase-1 activation for optimal intracellular growth in murine macrophages

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ABSTRACT

Chlamydia pneumoniae is an obligate intracellular pathogen that replicates within a vacuole and acquires host cell nutrients. We show that *C. pneumoniae* utilizes host innate immune signaling NLRP3/ASC/caspase-1 inflammasome for intracellular growth. Bone marrow-derived macrophages (BMMs) secreted mature interleukin-1β upon infection with *C. pneumoniae* depending on the NLRP3 inflammasome activation. Intracellular growth of *C. pneumoniae* was severely impaired in BMMs from *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ mice but not wild type or *Nlrc4*$^{-/-}$ mice. Furthermore defective NLRP3 inflammasome components led to accumulation of lipid droplets inside the infected BMMs, suggesting that uptake and/or utilization of lipids is disturbed in the absence of NLRP3 inflammasome activation. These results suggest *C. pneumoniae* has evolved to harness both host innate immune response and NLRP3 inflammasome activation, for the acquisition of essential nutrients necessary for intracellular growth. This unique property of *C. pneumoniae* may shed a new light on how *C. pneumoniae* increase the risk of atherosclerosis and metabolic syndrome.

1. Introduction

Chlamydiae are obligate intracellular pathogens lack the ability to produce sufficient energy and resources for independent growth, therefore can grow only in a parasitophorous vacuole termed 'inclusion' inside host cells. Thus it is quite vital for *Chlamydiae* to acquire essential nutrients containing lipids, nucleotides or amino acids from the host cells [1,2]. *Chlamydiae* acquire host lipids from fragmented golgi [3], lipid droplets (LDs) [4], and sorting vesicles [1,2]. However, the most of molecular mechanisms used by *Chlamydiae* to acquire host lipids are still largely unknown.

Chlamydia pneumoniae causes respiratory tract infections and chronic or recurrent *C. pneumoniae* infections have been associated with development of chronic lung disease such as asthma as well as with development of atherosclerosis [5–7]. It has been shown that the atherogenic effects of *Chlamydia* are dependent on serum cholesterol and specific to *C. pneumoniae* [8]. Foam cell formations containing LDs is the hallmark of early atherosclerosis and it is well established that *C. pneumoniae* induces foam cell formation in toll-like receptor (TLR) 2-, TLR4-, MyD88-, TRIF- and IRF3- dependent manner [9,10]. It is not clear, however, why *C. pneumoniae* but not *Chlamydia trachomatis* posses this unique atherogenic property [11].

Inflammatory cytokines IL-1β and IL-18 are induced by proinflammatory signaling through TLRs and following NF-κB signaling. Premature pro-IL-1β is processed by the cysteine protease caspase-1, followed by secretion from the cell [12]. Activation of caspase-1 requires generation of a protein complex called inflammasome containing procaspase-1 and the crucial adaptor molecules ASC, which bridges procaspase-1 to the Nod-like receptor (NLR) sensor protein [13]. Inflammasomes are classified several types by the unique NLR protein [14]. NLRP3 inflammasome has broad spectrum that detect bacterial toxins, potassium efflux [15], the generation of reactive oxygen species [16], and lysosomal disruption [17]. In contrast NLRC4 (IPAF) senses bacterial flagellin and directly binds to caspase-1 [18,19]. In addition to infectious danger signals, metabolic danger signals such as cholesterol crystal, ceramides and glucose have also been shown to trigger formation of NLRP3

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inflammasome and cause chronic inflammation [20,21]. A number of recent studies have implicated the NLRP3 inflammasome activation in a variety of metabolic disease including obesity, atherosclerosis and type-2 diabetes [22–25]. In this study, we explored the role of NLRP3 inflammasome activation in BMMs during *C. pneumoniae* infection and revealed a role of NLRP3 inflammasome activation on optimal intracellular growth of *C. pneumoniae* via uptake/utilization of LDs into chlamydial inclusion.

2. Materials and methods

2.1. Mice and reagents

C57BL/6J mice were purchased from Japan SLC. C57BL/6 background *Asc*$^{-/-}$, *Casp1*$^{-/-}$, *Nlrc4*$^{-/-}$ and *Nlrp3*$^{-/-}$ knockout mice were housed in pathogen-free facility of University of the Ryukyus. All experiments were performed in accordance with institutional guidelines of Fukuoka University and University of the Ryukyus. Ac-YVAD-CHO was purchased from Merck. Recombinant mouse IL-1β and IL-18 were from R&D systems.

2.2. Bacterial cultures and infections

C. pneumoniae (strain:AR-39, ATCC 53592), *C. trachomatis* serovar D (strain:UW-3/Cx, ATCC VR-885), *C. trachomatis* serovar L2 (strain:434, ATCC VR-902B), and *Chlamydia muridarum* (mouse pneumonitis strain Nigg II, ATCC VR-123) were propagated in HEp-2 or HeLa cells growing in DMEM (WAKO) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) at 37 °C or 35 °C (*C. pneumoniae*) in a 5% CO_2 environment. Chlamydial EBs were purified using urografin (Bayer) density gradient centrifugation and re-suspended in sucrose-phosphate-glutamate buffer and stored at −80 °C until use. All *Chlamydia* stocks were confirmed negative for *Mycoplasma* contamination. Cells were infected with *Chlamydiae* under centrifugation at 900×*g*, 25 °C for 1 h. All specific inhibitors were separately added to growth medium after centrifugation at indicated concentrations.

2.3. Preparation of bone marrow-derived macrophages (BMMs)

Bone marrow cells were flushed out from femurs and tibiae of 8 week-old mice with RPMI 1640 medium (WAKO). Cells were suspended in growth medium (RPMI 1640 with 10% heat-inactivated FBS, 10 mM HEPES, 1 mM sodium pyruvate and 100 μg/ml streptomycin sulfate) supplemented with 20 ng/ml of recombinant mouse M-CSF (R&D systems) at 1×10^6 cells/ml concentration for 7 days. The adherent cells were harvested, plated out in growth media at 1×10^6 cells/ml and incubated at least 12 h for resting.

2.4. Cytokine ELISA and LDH assay

ELISA assays for IL-1β and IL-6 were performed using DuoSet ELISA development system (R&D systems) according to manufacturer's instructions. ELISA plates were read via Model 680 microplate reader (BIO-RAD). Released lactate dehydrogenase (LDH) from dead cell was measured using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega).

2.5. Real-time quantitative reverse transcription-PCR

Total RNA was isolated using TRIzol® Reagent (Life Technologies) and cDNA synthesis was performed via SuperScript® III First Strand Synthesis System (Life Technologies). Each gene expression was measured with the 7500 Real-Time PCR system (Applied Biosystems) using the SYBR Premix DimerEraser (TaKaRa Bio). The relative quantification of target mRNA levels was compared to the level in control sample. Primer sequences used in this study were described as follows: MmIL-1βFw (5′-AAGGAGAACCAAGC AACGACAAAA-3′), MmIL-1βRv (5′-TGGGGAACTCTGCAGACTCAAA CT-3′), Mm18SrRNAFw (5′-TCAAGAACGAAAGTCGGAGGTT-3′), Mm18SrRNARv (5′-GGACATCTAAGGGCATCACAG-3′), Cpn16SrR NAFw (5′-GATTGCCAGTATAGATGCTTGTGAG-3′), Cpn16SrRNARv (5′-CTATGTCACTACTAACCCTTCCGCCACT-3′).

2.6. Immunocytochemistry and immunofluorescence

Cells on cover slips were fixed with ice-cold methanol or 4% paraformaldehyde for 15 min at RT. Cells were permeabilized with 0.1% Triton X-100 in PBS containing 2% FBS for 10 min before incubating with biotin-conjugated goat anti-*Chlamydia* polyclonal antibody (GeneTex). Alexa 488-conjugated streptavidin (Life Technologies) were used. For detection of cytoplasmic LDs, cells were fixed and treated with 100 ng/ml Nile red (SIGMA) in PBS for 20 min. Immunofluorescent imaging was obtained by BIOREVO BZ-9000 fluorescence microscope (KEYENCE).

2.7. Statistical analyses

PRISM Graphpad software was used for statistical analysis. Differences between experimental groups within each experiment were analyzed using the unpaired Student's *t*-test and were considered significant if $p < 0.05$.

3. Results

3.1. C. pneumoniae more easily activates inflammasome in murine BMMs

We initially examined whether there are any differences in cytokine productions of infected BMMs with different chlamydial strains. At low multiplicity of infection (MOI = 1), both *C. pneumoniae* and *C. trachomatis* D specifically mediates rapid induction of IL-1β mRNA expression in infected BMMs (Fig. 1A) and intracellular pro-IL-1β protein was highly produced at 6 h post infection (Fig. 1B). However, only *C. pneumoniae* infected-BMMs secreted mature IL-1β into culture supernatant (Fig. 1C). When high dose (MOI = 10), all tested *Chlamydiae* species/serovar induced the secretion of IL-1β with highest secretion from *C. pneumoniae*-infected BMMs (Fig. 1D). Next we investigated the role of inflammasome on IL-1β secretion. Addition of active caspase-1 inhibitor ac-YVAD-CHO abrogated IL-1β secretion (Fig. 1E) but not IL-6 (data not shown) from *C. pneumoniae*-infected BMMs. Furthermore, IL-1β secretion from *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ or *Casp1*$^{-/-}$ BMMs was nearly completely abolished compared with WT or *Nlrc4*$^{-/-}$ BMMs (Fig. 1G), whereas the expression of IL-1β mRNA were equally induced (Fig. 1F). These results clearly indicated that *C. pneumoniae* more easily induce NLRP3 inflammasome activation than other *Chlamydiae*.

3.2. NLPR3 inflammasome activation is required for the optimal intracellular replication of C. pneumoniae in BMMs

We next investigated the possible role of NLRP3 inflammasome on chlamydial growth. We found that remarkable effects on the chlamydial inclusions; smaller, fragmented inclusions were observed in RAW264.7 cell treated with ASC-, caspase-1- and NLRP3-shRNA compared to those of WT or NLRC4-shRNA cells (Fig. 2A). In addition, dramatic decrease of *C. pneumoniae* growth were observed in BMMs derived from *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *Casp1*$^{-/-}$ mice but not *Nlrc4*$^{-/-}$ mice or WT mice (Fig. 2B). We confirmed that the chlamydial replication is restricted in *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ and *Casp1*$^{-/-}$ BMMs by measuring the expressions of chlamydial

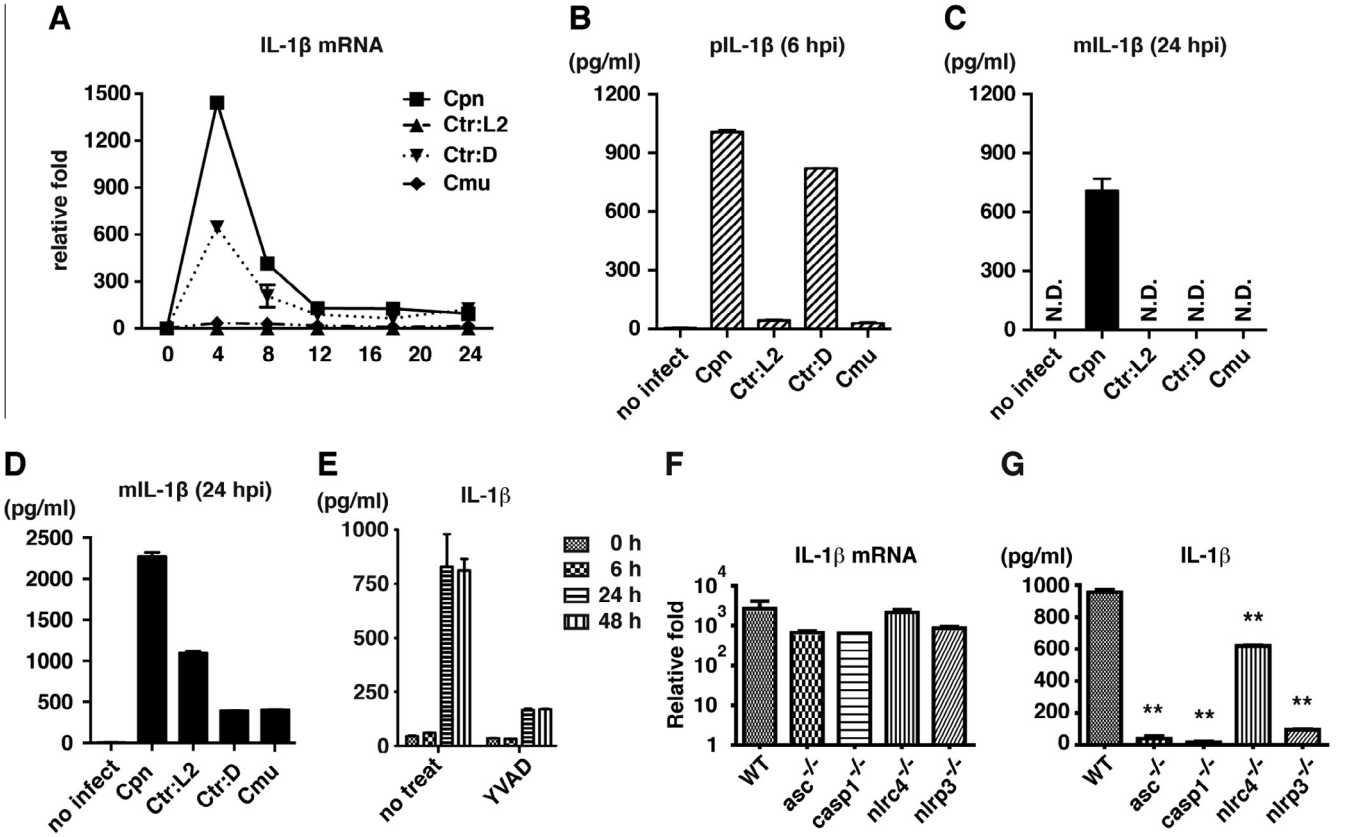

Fig. 1. *C. pneumoniae* infection specifically evokes caspase-1 activation and subsequent secretion of IL-1β in BMMs. (A) Murine BMMs were infected with *C. pneumoniae* AR39 (Cpn), *C. trachomatis* serovar L2, D (Ctr: L2 and D) and *C. muridarum* (Cmu) at MOI = 1 and cells were harvested at indicated time-points. Total RNA were separately extracted and real-time RT-PCR analysis was performed for mouse IL-1β mRNA. The result was normalized to the value for the mouse 18S rRNA. (B) Whole cell lysates were collected at 6 h post infection (hpi) and intracellular pro-IL-1β was measured using ELISA. (C) Culture supernatants were collected at 24 hpi and each secreted mature IL-1β was measured. N.D., not detected. (D) BMMs were separately infected with *Chlamydiae* at MOI = 10 and secreted mIL-1β was verified as in (C). (E) BMMs were infected with *C. pneumoniae* at MOI = 1 and cultured with or without 50 μM ac-YVAD-CHO, then supernatants were collected at indicated times. Secreted IL-1β was measured. (F) The level of IL-1β mRNA in WT or knockout BMMs were verified at 4 hpi. (G) Secreted IL-1β protein was measured at 48 hpi. $^{**}P < 0.001$, compared to WT BMMs by the Student's *t*-test. Every experiment had been done for three times, and experiment shown is a representative experiment. Data are presented as mean ± S.D. of triplicate samples.

16S rRNA via real-time RT-PCR (Fig. 2C). In addition we performed lactate dehydrogenase (LDH) release assay to measure viability of BMMs. There were not much increases of dead cells in all infected BMMs (Fig. 2D). These results suggest that the NLRP3 inflammasome activation promotes the optimal intracellular growth of *C. pneumoniae* in BMMs.

To gain insights into the role of NLRP3 inflammasome activation on *C. pneumoniae* growth, we examined the effects of exogenously added IL-1β and IL-18 on the growth of *C. pneumoniae*. Neither exogenously added IL-1β nor IL-18 affected *C. pneumoniae* replication in both WT and *Casp1*$^{-/-}$ BMMs (Fig. 3A), indicating IL-1 signaling was not related for the growth of *C. pneumoniae* in BMMs. On the contrary, exogenous ATP treatment, the trigger for NLRP3 inflammasome activation significantly increased *C. pneumoniae* replication in BMMs (Fig. 3B, *left*), while rather decreased *C. muridarum* growth (Fig. 3B, *right*). In line with this, intracellular replication of *C. muridarum* was augmented in *Casp1*$^{-/-}$ BMMs compared to wild type (Fig. 3C). Taken together these results strongly indicate that only *C. pneumoniae* harness NLRP3 inflammasome activation for the optimal intracellular replication in macrophages.

3.3. Defective caspase-1 activation leads accumulation of lipid droplets inside the infected macrophages

C. pneumoniae should acquire essential nutrients including lipids from host cells. It is also reported that blocking the proliferation of LDs with triacsin C (fatty acyl-CoA synthetase inhibitor) impairs chlamydial replication, suggesting that the pathogen takes advantage of these organelles [26]. This information prompted us to seek whether the activation of NLRP3 inflammasome by *C. pneumoniae* is linked to formation of LDs and/or the uptake of LDs into chlamydial inclusions. We examined foam cell formation and *C. pneumoniae* intracellular growth in NLRP3 inflammasome-deficient BMMs. The number of lipid laden foamy BMMs (more than 10-LDs per cell) were determined by Nile-red staining. The number of LD positive infected-BMMs from *Asc*$^{-/-}$, *Casp1*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice were surprisingly further increased compared with those of infected BMMs of WT or *Nlrc4*$^{-/-}$ mice (Fig. 4A and B), This result suggested that defective NLRP3 inflammasome activation leads to accumulation of LDs inside the infected BMMs. In contrast, *C. muridarum* infection did not cause any significant induction of LDs in both WT and *Casp1*$^{-/-}$ BMMs (Fig. 4C). These results strongly suggest that NLRP3 inflammasome activation is closely associated with uptakes or utilization of LDs into chlamydial inclusions in *C. pneumoniae*-infected BMMs. Although the precise mechanism remains largely unknown, these results strongly suggest that *C. pneumoniae* harness host NLRP3 inflammasome activation for intracellular replication via increased uptake or utilization of lipids into chlamydial inclusions.

4. Discussion

It is commonly believed that *C. pneumoniae* promotes both foam cell formation and atherosclerosis, and several animal experiments supported this opinion [27–29]. This is the unique feature of *C.*

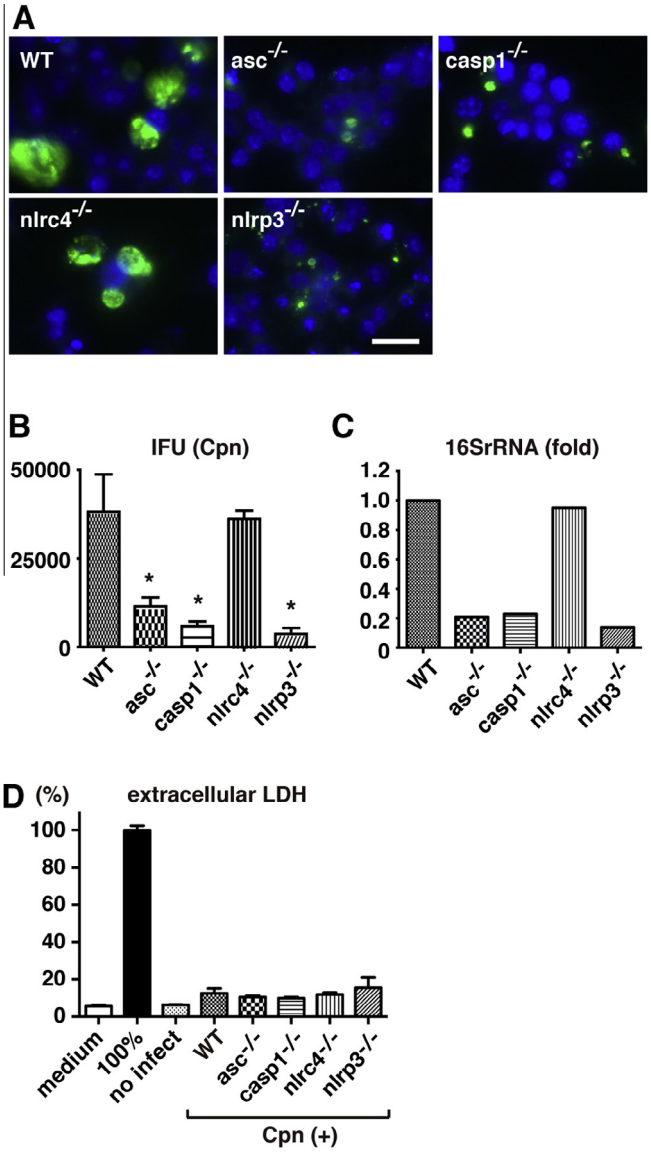

Fig. 2. NLPR3 inflammasome mediated caspase-1 activation is required for the optimal intracellular replication of *C. pneumoniae* in macrophages. (A) RAW264.7 cells were infected with *C. pneumoniae* AR39 (MOI = 1) respectively, then fixed and chlamydial inclusions were stained at 48 hpi. Blue staining; Hoechst 33342 (cellular nuclei). Scale bar: 25 μm. (B) Infected BMMs harvested at 48 hpi were examined for intracellular growth of *C. pneumoniae* in BMMs by IFU assay. $^{*}P < 0.05$ compared with control group by the Student's *t*-test. (C) Total RNA was isolated from infected BMM at 24 hpi respectively and each chlamydial 16S rRNA was measured by quantitative RT-PCR. Mouse 18S rRNA from host cell was used as normalization. (D) Culture supernatants were collected at 48 hpi and released LDH was measured. 0.1% Triton X-100 added sample was used as 100% control.

pneumoniae and other *Chlamydia* species does not accelerate atherosclerosis even respiratory tract infection [11]. Why only *C. pneumoniae* promotes atherosclerotic disease is almost unknown, hence it is very intriguing. We anticipated that there is a special response of infected macrophages with *C. pneumoniae*, and used the *in vitro* BMM infection system, then we acquired two novel findings.

First, only *C. pneumoniae* more easily promotes both NLRP3 inflammasome activation and production of various cytokines at low dose infection than other *Chlamydia*. We tried to examine at low-dose infection in most experiments and acquired some different results between *C. pneumoniae* and others. We believe that the experiment with the lowest threshold value to which an inflammation response is occurred is very effective to comparing pathogenic differences in two or more bacteria species and immune responses to them. Our current findings are of particular interests when we think of the possible relationship between *C. pneumoniae* infection and atherosclerosis. Duewell et al. [23] reported NLRP3 inflammasome is required for atherogenesis and activated by cholesterol crystals that form early in disease. We think this unique characteristic or ability to stimulate NLRP3 inflammasome by *C. pneumoniae* but not other *Chlamydia* species may be fundamentally involved in the acceleration of atherosclerosis by *C. pneumoniae* infection.

Second, *C. pneumoniae* may uses NLRP3 inflammasome activation for optimal replication in macrophages, which is the notable finding in this study. Our results that *C. pneumoniae* promotes NLRP3 inflammasome activation (Fig. 1), that is good consistent with several previous reports [30–32]. However, it is surprising that the absence of NLRP3 inflammasome activation leads to the suppression of intracellular growth in BMMs (Fig. 2). Decreased *C. pneumoniae* IFU in NLRP3 inflammasome defective BMMs were apparently associated with increased accumulation of LDs in BMMs (Figs. 3 and 4), suggesting that NLRP3 inflammasome activation is not required for the step of LDs formation, but the uptake/utilization of LDs. It has been reported that *Chlamydia* acquires host lipids from various organelles. Heuer et al. [3] showed that *C. trachomatis* uptake lipids from fragmented golgi in infected cells and they found that both caspases and calpains are involved in the successive cleavage of golgin-84. *C. trachomatis* uptakes LDs into chlamydial inclusion and chlamydial effector protein Lda3 is related to this step [4]. Activated caspase-1 may be related to lipid metabolic pathway or membrane biogenesis through inducing activation of the sterol regulatory element binding proteins (SREBPs) [33], it will be of interest to determine the precise requirements or mechanisms of these innate immune signaling for lipogenesis and lipolysis in *C. pneumoniae* infection.

Shimada et al. [32] reported that both caspase-1 and IL-1β productions are critical for bacterial clearance and host immune defense in a mouse model. In contrast, our *in vitro* BMMs infection model revealed intracellular proliferation of *C. pneumoniae* has diminished in the absence of NLRP3 inflammasome activation. He et al. [31] reported that defect of IL-1 signaling is unrelated to the bacterial burden following *C. pneumoniae* infection, in good agreement with our result (Fig. 3). Such discrepancy may be explained that different type cells such as epithelial or T cells are present *in vivo* and form a delicate cross-talk, which leads to different results against *in vitro* systems.

There are increasing numbers of reports that pathogens escape from host immune responses such as TLR/NLR activations but few reports that pathogens use these host immune responses for optimal growth. It has been reported that TLR signaling is required for *Salmonella typhimurium* virulence [34]. *S. typhimurium* uses signals downstream of TLRs to broadly coordinate expression of virulence genes required for intracellular growth. This is conceptually quite unique that *S. typhimurium* has evolved to require host-resistance signals for proper expression of virulence genes, distinct from those previously well described antagonistic strategy such as escape from host innate immune system. In line with this, our current results also imply fascinating *C. pneumoniae*'s strategy to cope with host innate immune responses such as NLR recognition and subsequent inflammatory responses. Rather than simply escaping or avoiding host immune responses, *C. pneumoniae*, highly co-evolved in human, has chosen to harness host innate immune responses for the purpose to promote intracellular growth in BMMs.

In conclusion, our study may provide a novel and unique strategy of *C. pneumoniae* that utilizes host immune response in order to efficient nutrition acquirement. Elucidating the mechanisms of how *C. pneumoniae* harness the host NLRP3

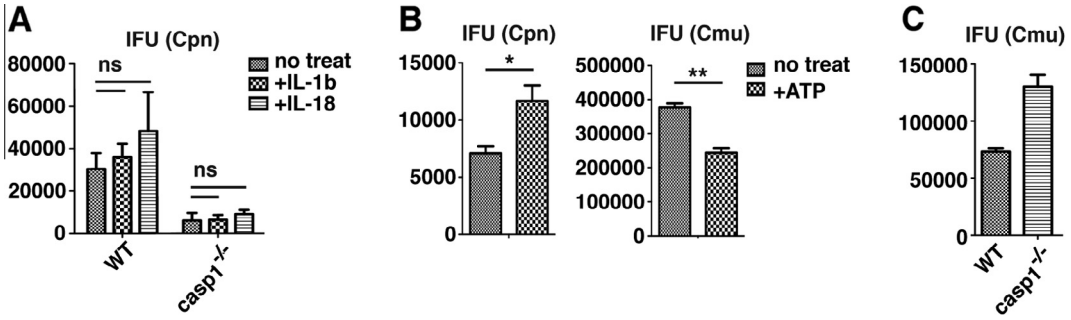

Fig. 3. NLPR3 inflammasome activation regulates *C. muridarum* growth in BMMs. (A) *C. pneumoniae*-infected BMMs incubated for 48 h with or without 100 ng/ml recombinant IL-1β or IL-18, respectively, were examined for intracellular *C. pneumoniae* growth. (B) ATP-pretreated (5 mM, 5 min) BMMs were infected with *C. pneumoniae* (Cpn) or *C. muridarum* (Cmu) and chlamydial IFU was examined at 48 h after infection. (C) Wild-type or *Casp1*$^{-/-}$ BMMs were separately infected with *C. muridarum* and incubated for 48 h and chlamydial IFU score is measured. ns; not significant, $^{*}P < 0.05$, $^{**}P < 0.001$ compared with control group by the Student's *t*-test. Every experiment had been done for 3 times, and experiment shown is a representative experiment. Data are presented as mean ± S.D. of triplicate samples.

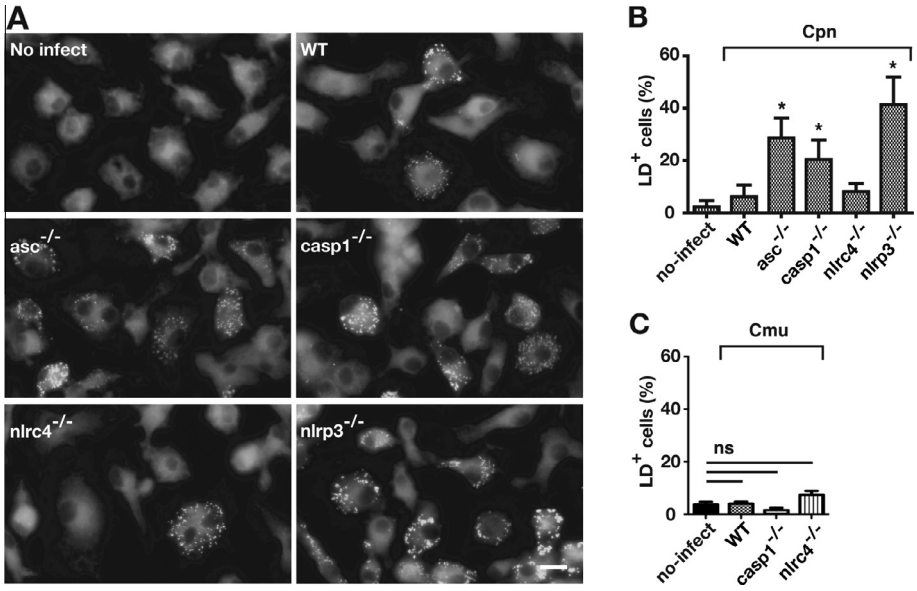

Fig. 4. Defective NLRP3 inflammasome mediated caspase-1 activation causes the accumulation of lipid droplets in *C. pneumoniae* infected-BMMs. (A) BMMs were infected with *C. pneumoniae* and incubated for 48 h, and cellular lipid droplets were stained with 100 nM Nile-red. Scale bar: 10 μm (B and C) Stained BMMs were identically taken 10 frames for each samples, and then LD positive cells (⩾10 LDs per cell) were represented by percentage for all cells. ns, not significant. Data are presented as mean ± S.D. of triplicate samples. ns, not significant, $^{*}P < 0.05$ compared with control group by the Student's *t*-test. Every experiment had been done for 3 times, and experiment shown is a representative experiment.

inflammasome activation to acquire host lipids will be important not only for chlamydial biology but also for targeting new immune intervention against *C. pneumoniae* infection. Now we are investigating how activated caspase-1 acts on the process of uptake/utilization of LDs by *C. pneumoniae*.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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