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Involvement of Lysosome Membrane Permeabilization and Reactive Oxygen Species Production in the Necrosis Induced by *Chlamydia muridarum* Infection in L929 Cells

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Chlamydiae, obligate intracellular bacteria, are associated with a variety of human diseases. The chlamydial life cycle undergoes a biphasic development: replicative reticulate bodies (RBs) phase and infectious elementary bodies (EBs) phase. At the end of the chlamydial intracellular life cycle, EBs have to be released to the surrounded cells. Therefore, the interactions between Chlamydiae and cell death pathways could greatly influence the outcomes of *Chlamydia* infection. However, the underlying molecular mechanisms remain elusive. Here, we investigated host cell death after *Chlamydia* infection in vitro, in L929 cells, and showed that *Chlamydia* infection induces cell necrosis, as detected by the propidium iodide (PI)-Annexin V double-staining flow-cytometric assay and Lactate dehydrogenase (LDH) release assay. The production of reactive oxygen species (ROS), an important factor in induction of necrosis, was increased after *Chlamydia* infection, and inhibition of ROS with specific pharmacological inhibitors, diphenylene iodonium (DPI) or butylated hydroxyanisole (BHA), led to significant suppression of necrosis. Interestingly, live-cell imaging revealed that *Chlamydia* infection induced lysosome membrane permeabilization (LMP). When an inhibitor upstream of LMP, CA-074-Me, was added to cells, the production of ROS was reduced with concomitant inhibition of necrosis. Taken together, our results indicate that *Chlamydia* infection elicits the production of ROS, which is dependent on LMP at least partially, followed by induction of host-cell necrosis. To our best knowledge, this is the first live-cell-imaging observation of LMP post *Chlamydia* infection and report on the link of LMP to ROS to necrosis during *Chlamydia* infection.

Keywords: *Chlamydia*, necrosis, lysosome membrane permeabilization, reactive oxygen species production

Introduction

The Chlamydiae are important prokaryotic pathogens that cause infections of the genital tract, eyes, vasculature, respiratory tract, and joints in humans [6]. All *Chlamydia* species are obligate intracellular bacteria characterized by a unique biphasic developmental cycle [21]. *Chlamydia* form of a membrane-enclosed vacuole in the host cells is referred to as an inclusion. Before maturing into replicative, noninfectious, and larger metabolically active reticulate bodies (RB), the initial form of *Chlamydia* infection is metabolically passive and manifested as infectious elementary bodies (EB). During acute infection, the RB can produce up to 1,000 progeny from a single inclusion in the manner of binary

fission. Upon completion of the developmental cycle, the infected cell is lysed, releasing the EBs to infect surrounding cells. It has been shown that interference with the infection stage dominated by RB can prevent completion of the infection cycle [35]. *Chlamydia* replicate exclusively inside the host cells, and their intracellular life cycle concludes upon lysis and death of the host cells. Therefore, the outcome of *Chlamydia* infection relies on the interaction of *Chlamydia* with the cell death pathways [12, 33, 35]. There are three major types of cell death pathways: apoptosis, autophagy, and necrosis. The apoptotic and autophagy pathways in *Chalamydia* infection have been investigated. *Chlamydia*-infected cells are resistant to many experimental apoptotic stimuli, as exhibited with caspase activation inhibition and blockage of cytochrome *c* release from mitochondria [8, 10, 11, 35]. *Chlamydia* lymphogranuloma venereum can lead to autophagy, particulary in the middle stage of the chlamydial developmental cycle (24 h after infection), suggesting that activation of autophagy maybe a result of the metabolic stress caused by chlamydial replication [2, 3, 7, 23, 35]. Necrosis could be detected in Chlamydiae infection [15, 25], but the mechanism still remains to be well characterized.

Recent studies demonstrated tightly intrinsic-cellular-pathway-controlled necrosis, refered to as programmed necrosis, which can be triggered by multiple types of stimuli [13]. During the past 20 years, a lot of molecules and processes related to modulators or effectors of programmed necrosis have been well characterized. These include reactive oxygen species (ROS), a putative key effector of cellular necrosis characterized by cytoplasmic swelling, rounding of the cells, spilling of the intracellular content, and rupture of the plasma membrane [17, 19, 28, 32]. Analyses of tumor necrosis factor (TNF)-induced necrosis, under high-resolution time-lapse microscopy, revealed activation of subcellular events, including oxidative burst, mitochondrial membrane hyperpolarization, plasma membrane permeabilization (PMP), and lysosomal membrane permeabilization (LMP) [29].

We therefore investigated the mechanism of necrosis during *Chlamydia* infection. Our results employing high-resolution time-lapse microscopy demonstrated live-cell-imaging observations of LMP activation during *Chlamydia* infection, followed by necrosis of the infected host cells. The necrosis was associated with a remarkable increase in production of ROS. We further show that inhibition of LMP can reduce the ROS production as well as necrosis. To our best knowledge, this is the first report that provides a link of LMP with ROS and the necrosis process during *Chlamydia* infection. We believe that these findings could contribute to further dissection of the mechanism by which *Chlamydia* species cause the death of infected cells and as such to the understanding on the outcomes of *Chlamydia* infection.

Materials and Methods

Antibodies and Reagents

Chlamydia (*Chlamydia muridarum*, *Chlamydia* mouse strain Nigg II; ATCC, USA); Annexiv-FITC/Propidium Iodide (PI) assay kit (KeyGEN, China); Recombinant mouse TNFα (Invitrogen, USA); ZVAD-FMK (Sigma Aldrich, USA); cycloheximide (Sigma Aldrich, USA); necrostatin-1 (Nec1) (Sigma Aldrich, USA); butylated hydroxyanisole (BHA) (Sigma Aldrich, USA); diphenylene iodonium (DPI) (Sigma Aldrich, USA); SYTOX Green and LysoTracker Red (Molecular Probes – Invitrogen, USA); CM-H2DCFDA (Molecular Probes – Invitrogen, USA); cathepsin inhibitor IV (CA-074-Me, Calbiochem, Germany); CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega, USA).

Cell Culture

L929 cells and BGMK cells were kind gifts from Wenzhou University and University of South China. The cells were grown in RPMI-1640 and Iscove's modified Dulbecco's media supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, respectively. Cells were maintained at a humidified atmosphere at 37ºC with 5% CO_2 and logarithmically growing cells were used for experiments.

Infection of L929 with Live *Chlamydia* EBs

Chlamydia was propagated in BGMK cells and purified by gradient centrifugation. The infectivity titers were determined by titration in BGMK cell monolayers. The inclusion-forming units per milliliter were 1×10^9. L929 cells were seeded at a density of 15×10^4 cells/well into 24-well plates and cultured for 24 h without antibiotics. The cells were then infected with 0.85 Multiplicity of Infection (MOI) live *Chlamydia* EBs. The plates were centrifuged at 1,000 ×*g* for 60 min and then incubated at 37°C for 2 h. Subsequently, the medium was replaced with culture medium with 1 μg/ml cycloheximide.

Analysis of Cell Death

The L929 cells were dispensed in 24-well plates at a density of 1.5×10^5 cells/well. After 24 h incubation, they were treated with or without live *Chlamydia* EBs. Cell apoptosis/necrosis was measured by double-staining with Annexiv-FITC/PI for the indicated time periods. Recombinant mouse TNFα plus ZVAD-FMK treatment was used as the control.

Measurement of Reactive Oxygen Species Production

CM-H2DCFDA, a ROS-sensitive fluorescent probe, was used for estimating the production of intracellular ROS. Cells were incubated

with CM-H2DCFDA for 1 h at 10 μM before flow fluorocytometric analysis and determination of the mean fluorescence intensity of the cell population.

Lactate Dehydrogenase (LDH) Release Assay

The CytoTox96 Non-Radioactive Cytotoxicity Assay kit was used to detect the quantity of LDH released into the culture medium in accordance with the manufacturer's instruction for evaluating cell membrane disruption.

Live-Cell-Imaging Observation with High-Resolution Time-Lapse Microscopy

The imaging setup, acquisition, and analysis of data after acquisition were conducted as previously described [29]. In brief, L929 cells were cultured on cover glass-based eight-well chamber slides (Lab-Tek, Nunc, Belgium). Cells were loaded with fluorescent probes after treatment with live *Chlamydia* EBs or TNF-α plus ZVAD-FMK.

A Leica TCS SP5 II confocal microscope equipped with a 631.40 NA oil-immersion objective lens and an enclosed 37°C chamber for the live cell imaging system (Leica Confocal Microscope, Germany) was used. Time-lapse images were captured with 488 nm and 561 nm laser excitation at the indicated intervals. SYTOX Green was excited with the 488 nm line of the argon laser and the emitted light was filtered through 470 to 525 nm;

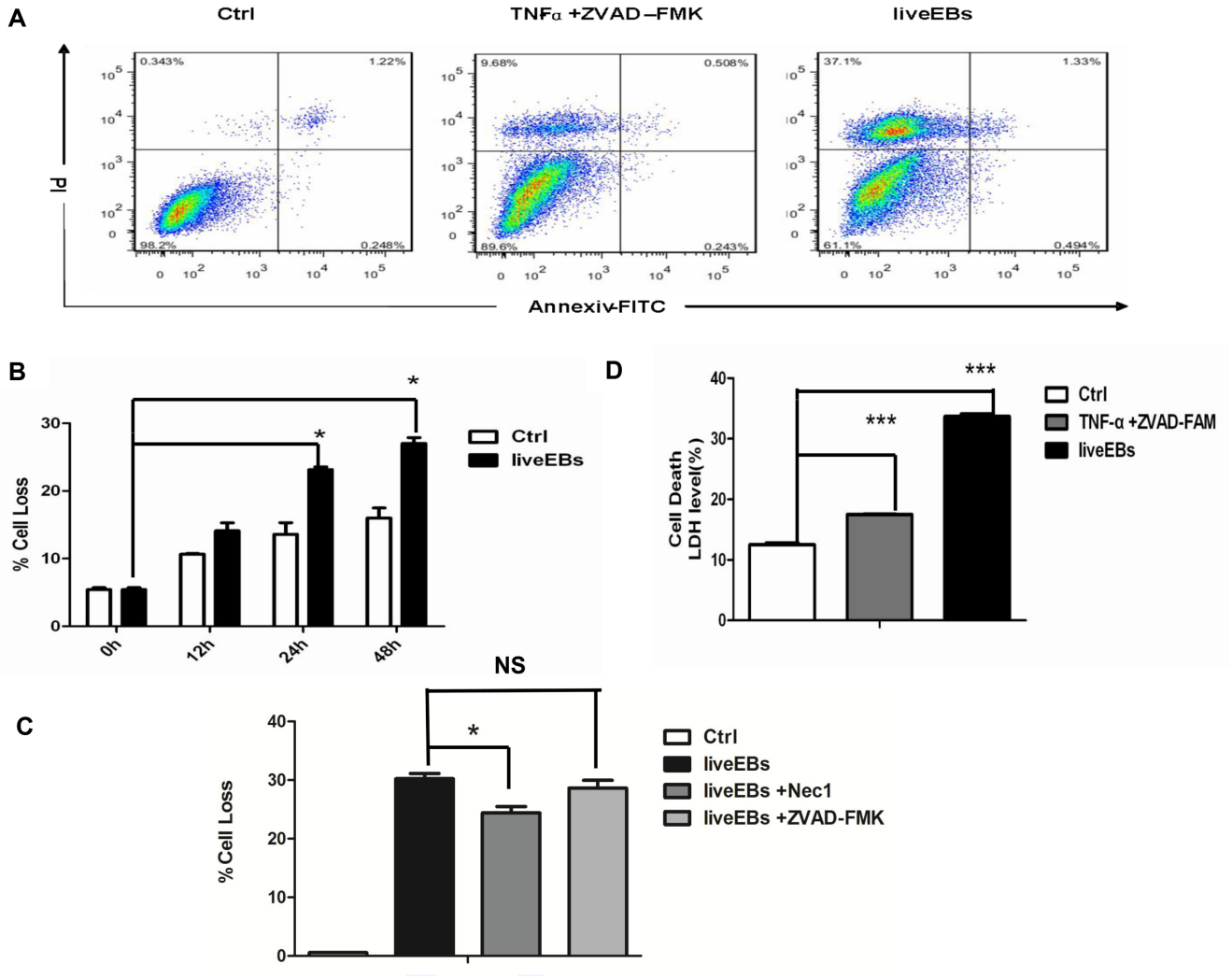

Fig. 1. Cell death assay after *Chlamydia* infection.

L929 cells were infected with live *Chlamydia* EBs (MOI = 0.85). Cell apoptosis/necrosis/autophagy were detected for the indicated time points. (**A**–**C**) Cell apoptosis/necrosis assay; TNF-α (100 ng/ml) plus ZVAD-FMK (25 μM) and PBS treatment as positive/negative control. (**A**) 48 h post infection, cells were double-stained with Annexiv/PI and then assayed by flow cytometry; PI-positive points were shown as necrosis. (**B**) Cell necrosis (% PI-positive cells) was assayed at different time points after infection. (**C**) L929 cells were precultured with Nec1 (30 μM) 3 h/ZVAD-FMK (25 μM) 2 h and then infected with live *Chlamydia* EBs; cell necrosis (% PI-positive cells) was assayed as before. (**D**) Lactate dehydrogenase (LDH) concentration, determined in the culture medium.

LysoTracker Red DND-99 was excited using the 561 nm laser and the fluorescence emissions were detected at the 515 to 590 nm range. All images were single confocal 512 × 512 pixel scans, and time-series images were acquired at 5 min intervals.

To analyze fluorescent signals, cells were monitored at three or four positions (x, y) every time, and more than two independent experiments were run and analyzed for each time. Among each imaging event, the objective was equipped with a Super Z Galvo stage element that allows images to be collected at a step size of 1 μm over a total depth of 10 μm. Images were recorded and processed with LAS-AF-Lite ver. 2.60 software (Leica Microsytems, Nanterre, France).

Data Analysis

All data were presented as the mean ± SD. Statistical analyses for comparison between two groups were performed by Student's t-test using GraphPad Prism software ($n \geq 3$). The value of $p < 0.05$ (*) was considered statistically significant. $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); NS: no statistically significant difference.

Results

Cell Death Induced in *Chlamydia*-Infected L929 Cells

Annexiv-FITC/PI double-staining is a classic flow cytometric assay to distinguish apoptosis (Annexiv-positive staining) from necrosis (PI-positive staining). When L929 cells were infected with live *Chlamydia* EBs, the percentage of PI-positive cells was gradually increased, even up to about 30%, over time (Figs. 1A and 1B). This cell death caused by the live EB infection could be inhibited by treatment with Nec1, a common inhibitor of necrosis. In contrast, treatment with ZVAD-FMK did not have obvious affect on cell death. In addition, LDH release was also increased (Fig. 1D).

ROS Production during *Chlamydia*-Induced Necrosis

Flow cytometry analysis of intracellular ROS concentration as analyzed by the fluorescent probe CM-H2DCFDA showed a gradual increase in ROS generation in response to live *Chlamydia* EB infection (Fig. 2). ROS generation and *Chlamydia*-mediated necrosis could be inhibited by DPI and BHA, potent and commonly used inhibitors of ROS (Fig. 3). Taken together, these results suggest that *Chlamydia* infection results in an increase in intracellular ROS concentration with concommitant occurrence of necrosis-like cell death and as such implicate a major role of ROS in *Chlamydia*-induced necrosis.

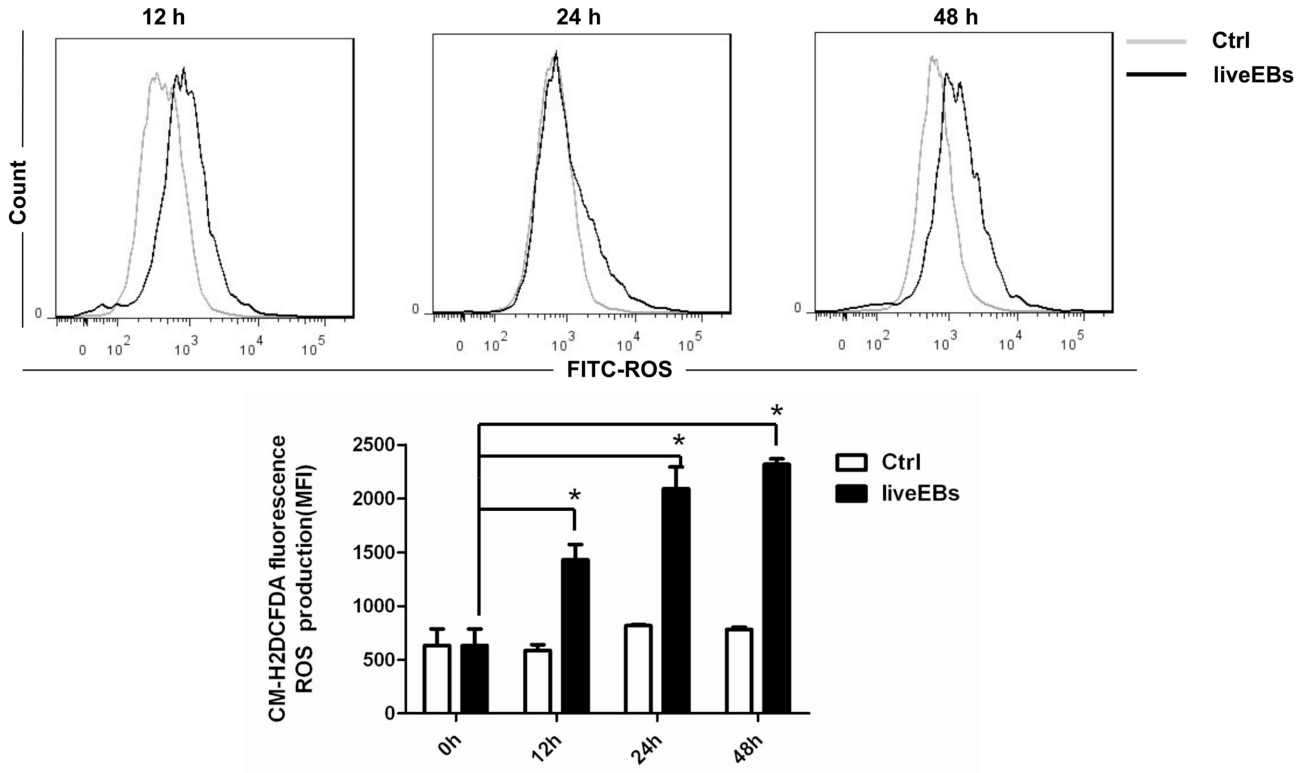

Fig. 2. Production of ROS during *Chlamydia* infection.
L929 cells were infected with live *Chlamydia* EBs and collected at different points. Reactive oxygen species (ROS) production was evaluated by flow cytometry.

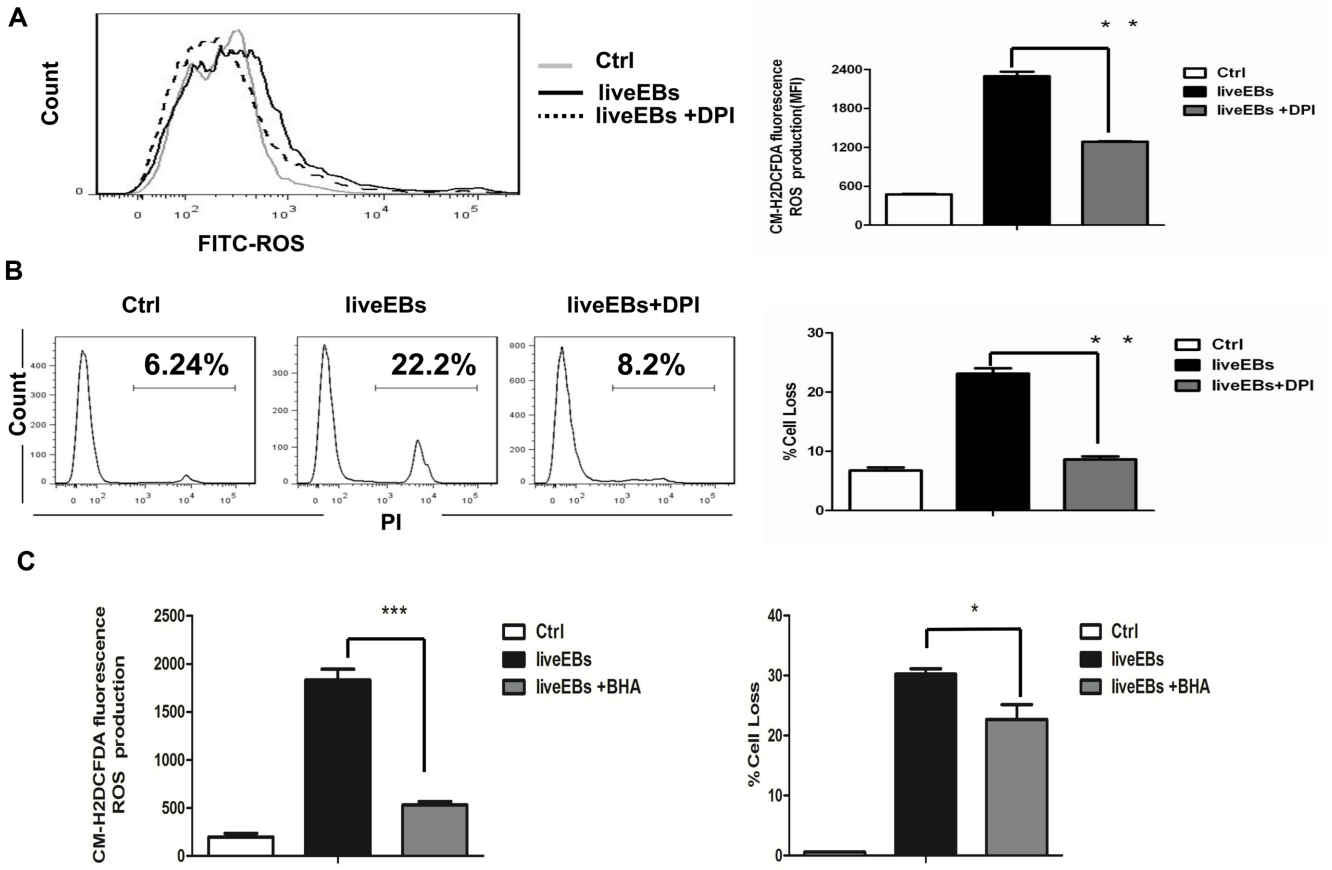

Fig. 3. *Chlamydia*-induced necrosis depends on ROS production.
(**A**, **B**) L929 cells were infected with or without live *Chlamydia* EBs for 18 h, and then were incubated in the presence or absence of 1 nmol/l DPI for another 9 h, followed by assay of ROS production and cell necrosis assay. (**C**) L929 cells were pretreated with BHA (100 μM) for 1 h and then infected with or without live *Chlamydia* EBs for 48 h followed by assay as before.

Lysosomal Membrane Permeabilization during *Chlamydia*-Induced Necrosis

It has been reported that LMP plays a crucial role in apoptosis and necrosis processes [15]. We therefore used LysoTracker Red DND-99 and SYTOX Green to analyze the lysosomal and plasmal integrity following live *Chlamydia* EB infection. In response to the live *Chlamydia* EB-induced necrosis, LMP increased at the initial times following infection showing the typical oncosis morphology afterwards, (Fig. 4A) and this was reminiscent of TNF-α plus ZVAD-FMK treatment (Fig. 4B). Unstimulated cells did not exhibit LMP. The live-cell imaging data therefore suggest that LMP may have an initiating role in *Chlamydia*-induced necrosis. Furthermore, we hypothesize that the disruption of local lysosomal membranes in turn facilitates *Chlamydia*-induced necrosis. We also analyzed the effect of CA-074-Me, and the results showed that it could significantly block *Chlamydia*-induced necrosis (Fig. 5A).

ROS Production Decreased after CA-074-Me Treatment in *Chlamydia*-Infected Cells

We next directly investigated whether LMP is required for ROS production in response to infection. For this, DCF fluorescence was monitored 48 h post infection in L929 cells treated with CA-074-Me and infected with *Chlamydia*. CA-074-Me treatment attenuated ROS production upon *Chlamydia* infection (Fig. 5B). These results suggest that LMP is required for *Chlamydia*-induced ROS production.

Discussion

Chlamydiae belong to intracellular bacterial pathogens, which are able to cause a wide range of diseases in humans and animals [35]. However, so far, the relationship between infection and host cell death is still not fully delineated [21]. In this study, we observed cell death in *Chlamydia*-infected L929 cells. First, to discriminate apoptosis from

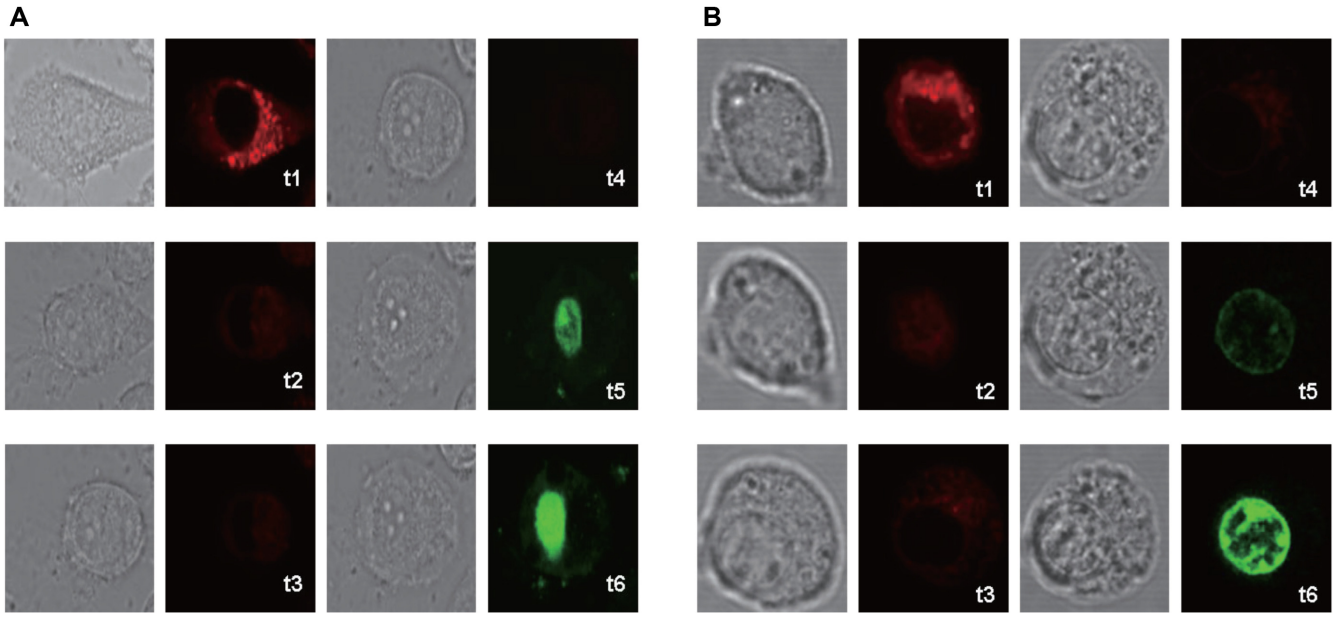

Fig. 4. Live-cell imaging reveals distinct lysosomal leakage in *Chlamydia* or TNFα plus ZVAD-FMK-induced necrosis.
Light fluorescence microscopic analysis of lysosomal leakage in L929 cells at different time points after 24 h of live *Chlamydia* EBs infection (**A**: t1: 24 h; t2: 33 h; t3: 34 h; t4: 36 h; t5: 36 h 25 min; t6: 36 h 55 min) or TNF-α plus ZVAD-FMK treatment (**B**: t1: 0 min; t2: 60 min; t3: 120 min; t4: 180 min; t5: 189 min; t6: 237 min) and subsequent PMP. Cells were loaded with LysoTracker Red DND-99 (1 μM, red, inversely proportional to lysosomal leakage) and SYTOX Green (10 nM, green, indicating PMP). Graphic representations of the mean intensities of LysoTracker Red DND-99 and SYTOX Green during necrosis of a representative single cell in L929 cells.

necrosis, we used a classic method of dual staining with fluorescent Annexin V and PI [24]. Our results showed that the percentage of PI-positive cells gradually increased, even up to about 30% after 48 h post infection, but with no effect on Annexin-V-positive cells. When Nec1, an inhibitor of necrosis, was added, the cell death decreased, whereas ZVAD-FMK, a pan caspase inhibitor, did not show obvious inhibitory effects. In addition, the LDH, which was used to evaluate plasma membrane integrity, showed release of cellular content into the culture medium after *Chlamydia* infection. Together, these results suggest that *Chlamydia* infection can induce cell necrosis to a great extent in L929 cells.

In contrast to extensive studies on apoptosis and autophagy during *Chlamydia* infection, the role of necrosis has remained very elusive. This paper thus focused on the mechanism of necrosis during *Chlamydia* infection. Necrosis has been characterized as a passive type or an accidental cell death caused by non-physiological stress [34]. However, increasing evidence has clearly demonstrated that different types of intrinsic cellular programs closely control necrosis. Therefore, we wondered if *Chlamydia*-induced necrosis is regulated by a distinct signal transduction pathway.

As an important regulator of programmed necrosis, generation of ROS can be induced by many triggers such as tumor necrosis factor. Moreover, during *Chlamydia trachomatis* infection, basal levels of ROS are generated by NADPH oxidase [1, 30]. Here, flow cytometry analysis of the intracellular ROS concentration by use of the fluorescent probe CM-H2DCFDA showed that the ROS generation gradually increased after infection. Both DPI (an inhibitor of NADPH) and BHA (a ROS scavenger) were able to cause significant reduction of *Chlamydia*-induced ROS production, as shown in Fig. 3 [1, 31]. We then tested whether the attenuation of ROS production can affect *Chlamydia*-induced necrosis by measuring the percentage of PI-positive cells. Our results showed that indeed, treatment of *Chlamydia*-infected L929 cells with DPI and BHA can inhibit necrosis post-infection compared with controls. These results suggest that ROS production during *Chlamydia* infection is essential for *Chlamydia*-induced necrosis.

Lysosome has shown ambiguous roles in *Chlamydia* infections. Beatty [4] reported that lysosome-mediated repair, by a mechanism of lysosome-plasma membrane fusion, enabled host cell survival and chlamydial persistence following *Chlamydia trachomatis* serovar L2 infection in HEp-2 cells. Ouellette SP *et al.* [22] also confirmed that blocking lysosomal acidification and functions impaired

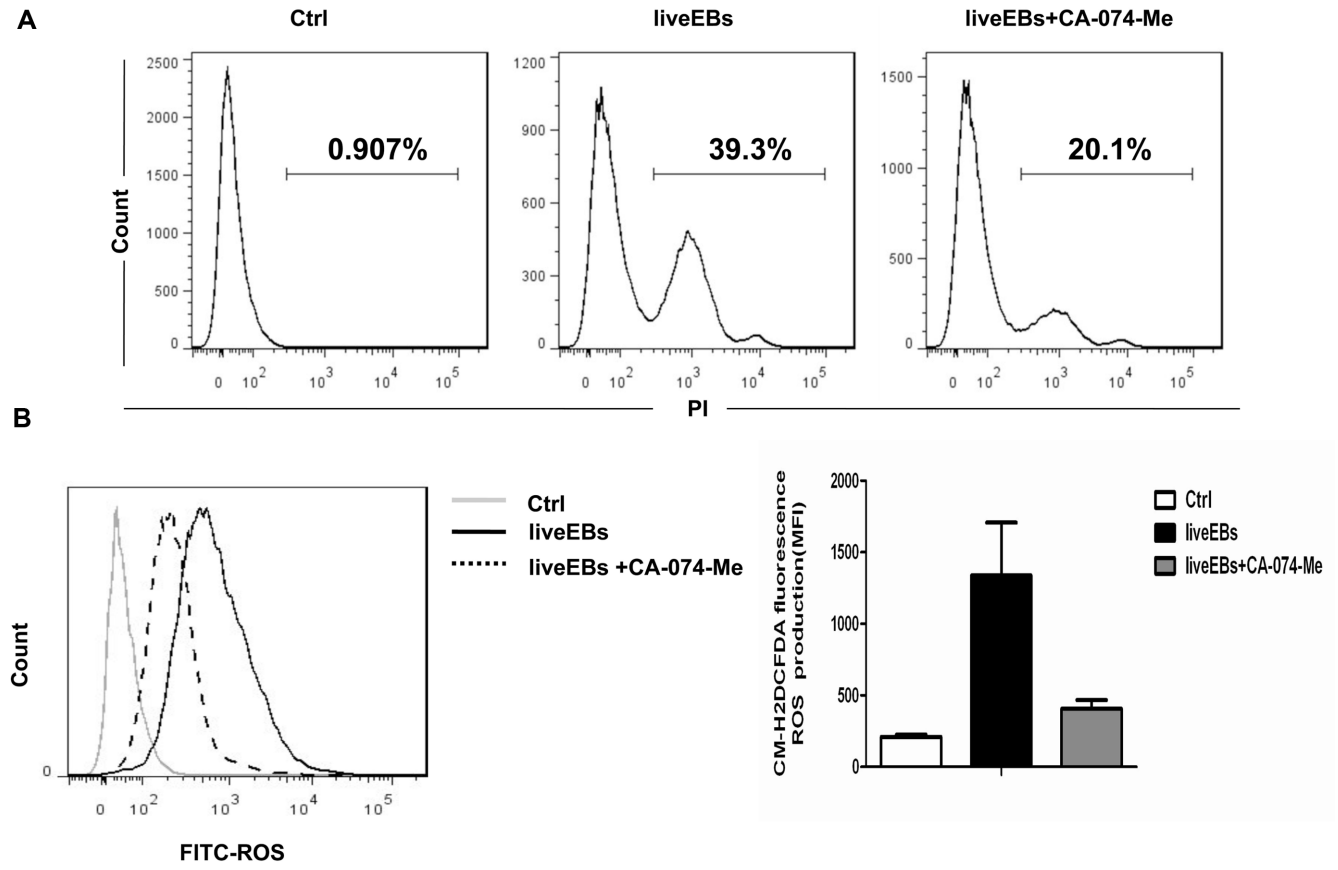

Fig. 5. CA-074-Me inhibits *Chlamydia*-induced necrosis and ROS production.
L929 cells were infected with live *Chlamydia* EBs and cultured for 42 h, and 100 μM CA-074-Me was then added or not, and the cells were cultured for an additional 6 h. Cell necrosis (**A**) and ROS production (**B**), as assessed by flow cytometry assay.

the growth of *C. trachomatis* and *C. pneumoniae* in the infected HEp-2 or MEFs cells, since the lysosome could serve an obligate role for Chlamydiae as a source of amino acids.

However, Sun *et al*. [26] showed that *C. trachomatis* growth could be suppressed by macrophages through rapidly targeting the bacteria to lysosomes, with activation of autophagy at later stages of *Chlamydia* infection, which might contribute to chlamydial antigen presentation [27]. At the present time, however, the role of lysosome compartment in *Chlamydia* infection-induced cell deaths remains elusive, although some reports showed LMP to be involved in cell death [2, 14].

Intensive studies have been performed to unravel the mechanisms of apoptosis-associated signaling induced by LMP. More specifically, when lysosome membranes rupture, the hydrolytic enzymes can be released from inside lysosomes into the cytosol and this can in turn trigger the apoptotic signaling pathway. However, sometimes a massive exodus of lysosomal enzymes occurs inside the cytosol after lysosomal membrane rapture, resulting in indiscriminate digesting of cellular components. This can further cause deleterious cytoplasmic acidification and ultimately induce necrosis instead of apoptosis [9, 16].

By high-resolution live-cell imaging observation, our study analyzed the changes in LMP during *Chlamydia*-mediated necrosis in L929 cells. Fig. 4 present that cells show LMP at the beginning and manifest the typical oncosis morphology afterwards. Finally, cells showed PMP and died. To test the hypothesis that the disruption of local lysosomal membranes facilitates *Chlamydia*-induced necrosis, CA-074-Me, an inhibitor of upstream to LMP events [5, 18], was employed. Treatment of cells with CA-074-Me reduced *Chlamydia*-induced necrosis, demonstrating that lysosomal disruption is a significant event in this process.

As both ROS and LMP are indispensable for *Chlamydia*-induced necrosis, we wondered if they are associated in this type of necrosis. In addition, it has been shown that NADH-driven lysosomal respiration is a permanent source

of cellular ROS release [20]. In this study, with the addition of CA-074-Me, *Chlamydia*-induced ROS production was dramatically attenuated, whereas it had no obvious effect on the control group.

In conclusion, the production of ROS during *Chlamydia* infection resulted in host-cell necrosis. We show for the first time that LMP is associated with late induction of oxidative stress of ROS, culminating in necrosis during *Chlamydia* infection. Our findings might provide new insights into *Chlamydia*-host interplays and deepen our understandings of the relationship between mechanisms of cell death and *Chlamydia* infection, and as such provide novel clues for the development of effective prophylactic and therapeutic approaches to treat *Chlamydia* infection.

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