

Responses of macrophages against *Salmonella* infection compared with phagocytosis

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Abstract To explore the responses of host cell after infection with live *Salmonella* compared with phagocytosis to dead bacteria, the responses of mouse macrophage after infection with *Salmonella* enteritidis C50041 and the fixed C50041 (C50041-d) were analyzed. Results indicated that the cytotoxicity induced by C50041 was stronger than C50041-d. Similar changing trends of mitochondrial membrane potential, intracellular concentration of calcium ions, reactive oxygen species and nitric oxide were found between C50041 and C50041-d infection. But the cell responses against C50041 were earlier and stronger than C50041-d. LC3 expression of macrophage induced by C50041 was lower than C50041-d. C50041 significantly inhibited the production of tumor necrosis factor and interleukin (IL)-6. Whereas intracellular caspase-1 activation and IL-1 β release induced by C50041 were stronger than C50041-d, caspase-1 activation and IL-1 β release are the innate defense responses of macrophage. Therefore, it will be beneficial to explore the use of this pathway in the control of *Salmonella* infection.

Keywords *Salmonella* · Macrophage · Cell function · LC3 · Caspase-1 · Interleukin-1 β

Introduction

Salmonellosis is a zoonotic disease caused by the Gram-negative enteric bacterium *Salmonella*. *Salmonella* infection may present a variety of ways, from asymptomatic colonization to inflammatory diarrhea or typhoid fever depending on serovar- and host-specific factors. *Salmonella* is a facultative intracellular bacteria. After infection, it can secrete effector proteins to regulate cell function depending on type III secretion system (T3SS) for their survival in host cells, such as macrophages. Carriers of the bacteria contribute greatly to the propagation of the disease. Sometimes, it can be transmitted to humans through the consumption of contaminated meat or eggs. Nowadays, salmonellosis is one of the leading causes of food-borne disease throughout the world. So, the resistance to carrier-state should be taken into account by researchers, besides the resistance to general salmonellosis (Clavijo et al. 2006; Calenge et al. 2010).

Phagocytosis by macrophages is a critical host defense step against infection and is a beneficial response of the host to discard the bacteria through the formation of phagosome (McGhie et al. 2009), whereas in the process of *Salmonella* infection, macrophages are the predominant host cells of bacteria (McGhie et al. 2009). Internalization of *Salmonella* into host cells can occur via at least two distinct processes: phagocytic uptake of professional phagocytes and invasion through T3SS of bacteria (Ibarra and Steele-Mortimer 2009). In macrophages, *Salmonella* may regulate *Salmonella*-containing vacuole (SCV) biogenesis to avoid the killing of phagosome by blocking SCV-lysosome fusion (Steele-Mortimer 2008). *Salmonella* selectively delivers effectors to cytoplasm to regulate cell function is a significantly different process from phagocytosis (Lara-Tejero et al. 2011; Schliehe et al. 2011). Understanding

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of the difference will be beneficial for the resistance of *Salmonella* survival in host cells.

During the interaction of bacteria and host cells, live *Salmonella* may induce the formation of SCV and phagosome in macrophages, whereas the dead bacteria are mainly phagocytic uptake to form the phagosome. In order to explore the responses of macrophage against *Salmonella* infection compared with phagocytosis, in this study, the virulent strain of *Salmonella* enteritidis C50041 (live) and the fixed C50041 (dead) were selected to infect mouse macrophages.

Materials and Methods

Mice, plasmid and bacteria. Six-week-old female BALB/c mice were obtained from the Comparative Medicine Center of Yangzhou University (Yangzhou, China). Recombinant plasmid pYA3334-dsRed expressing red fluorescence protein (RFP) without antibiotic resistance gene was constructed by Shizhong Geng (Geng et al. 2009). Wild-type *Salmonella* enteritidis strain C50041 was used in this study. BALB/c mouse abdominal cavity-derived macrophage cell line RAW264.7 (ATCC number: TIB-71) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Promega, Madison, WI) and mouse peritoneal macrophage was cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 medium (Promega), all supplemented with 10% fetal bovine serum (FBS, GIBCO, Carlsbad, CA).

Bacteria infection. Plasmid pYA3334-dsRed was electroporated into C50041 and the recombinant bacteria, C50041-(Red), were cultured in Luria–Bertani (LB) agar plates overnight at 37°C. The RFP-positive colony was subcultured for many times by rotation culturing in LB medium without antibiotics to assay the stable expression of RFP. Partial C50041(Red) was fixed with 70% alcohol overnight and the fixed bacteria, C50041(Red)-d, were cultured in LB agar plates for their viability assay. The RFP expression of bacteria was analyzed by flow cytometry after washing with phosphate buffer saline (PBS).

RAW264.7 cells were plated in 24-well plates at a density of 2×10^5 cell/well with DMEM medium containing 10% FBS (without antibiotics). The infection of bacteria was performed as previously described (Hoffmann et al. 2010). Briefly, RAW264.7 cells were allowed to adhere for 2 h (37°C, 5% CO₂) and washed with fresh medium to remove unattached cells. C50041(Red) and C50041(Red)-d were diluted in the medium and then added to the cells to the multiplicity of infection of 150, respectively. Cell plates were centrifuged (500×g, 10 min) to enhance contact of bacteria with cells. Cells were incubated at 37°C for 30 min. The uninfected bacteria were removed by washing with medium. Then, the medium containing 5% penicillin–streptomycin was added

(this point was determined as 0 h) and continued culturing for 20 h. Cell growth was observed under a TS100-F microscope (Nikon, Japan). Then the RFP expression of cells was analyzed by flow cytometry.

The same methods were used in the following infection experiments. The fixed C50041 were named as C50041-d.

Lactate dehydrogenase release and intracellular MMP, Ca²⁺, ROS, NO, and pH assay. After infection, cell plates were centrifuged (250×g, 5 min) and the supernatants were collected for measuring lactate dehydrogenase (LDH) release using the cytotoxicity detection kit (Roche, Switzerland). The relative amount of released LDH was calculated as follows: % released LDH (sample)=(sample–medium background)/(-total LDH–medium background)×100% (Lightfield et al. 2011). Cells were collected for the mitochondrial membrane potential (MMP) assay stained with rhodamine 123 (Rh123, Beyotime Institute of Biotechnology, China), the intracellular concentration of calcium ions ([Ca²⁺]_i) assay stained with Fluo-3AM (Beyotime), the intracellular level of reactive oxygen species (ROS) assay stained with DCFH-DA (Beyotime) (Itoh et al. 2010), the production of nitric oxide (NO) assay stained with DAF-FM DA (Beyotime) and the intracellular level of pH value ([pH]_i) assay stained with BCECF AM (Beyotime) (Chow and Hedley 1997). All protocols were performed according to the manufacturer's instructions.

Expression of Microtubule-associated protein light chain 3 (LC3). After infection, cell plates were washed with PBS and the cells were lysed with lysing solution containing 1 mM phenylmethanesulfonyl fluoride (Westang, Shanghai) according to the manufacturer's instructions. The lysing products were centrifuged (5,000×g, 5 min) and the supernatants were collected for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting assay. Briefly, protein samples separated by SDS-PAGE were transferred onto a poly(vinylidene fluoride) membrane. After incubation with 5% non-fat dry milk in tris-buffered saline containing 0.1% Tween-20 for 1 h, the membranes were then incubated with rabbit anti-LC3 antibodies (1:1,500) and rabbit anti-β-actin antibodies (1:15,000, Sigma-Aldrich, St. Louis, MO) at 4°C overnight. Following incubation with peroxidase-conjugated goat anti-rabbit IgG (1:15000, Sigma-Aldrich), the immunoreactive bands were visualized with an enhanced chemiluminescence (Millipore, Billerica, MA) detection kit. The detection of β-actin was used as an internal control.

Caspase-1 activation and cytokines release. Primary peritoneal exudate cells (PECs) of BALB/c mice were separated (Hu et al. 2012) and seeded in a 96-well plate with RPMI medium containing 10% FBS (without antibiotics) at 37°C overnight. The non-adherent cells were discarded and the adherent cells were ensured to be 2×10^4 cells/well. The induction of caspase-

l activation and interleukin (IL)-1 β release were determined as previously described (Pelegrin et al. 2008; Hoffmann et al. 2010). Briefly, the lipopolysaccharide of *E. coli* (1 μ g/ml) were added (100 μ l/well) and the cells were incubated for 3 h. The infection of bacteria C50041 and C50041-d were performed as above. After incubation for 3.5 h following the killing of

uninfected bacteria by 5% antibiotics, the supernatants were collected for the analysis of cytokine release, including IL-1 β , IL-6, MCP-1, and tumor necrosis factor (TNF), using cytometric bead array system kit (BDIS) according to the manufacturer's instructions. The adherent cells were collected, washed with PBS, and the caspase-1 activation were determined by FLICA™ Caspase 1 Detection Kit (Immunochemistry Technologies Inc., Bloomington, MN).

Flow cytometry and statistical analysis. Flow cytometry and statistical analysis were determined as previously described (Hu et al. 2012). Briefly, Cells were harvested and labeled with dye and then analyzed with flow cytometry using a FACSaria with FACSDiva software (Becton-Dickinson Immunocytometry Systems, BDIS, San Jose, CA). Cellular debris was eliminated from analyses by gating using forward and side scatters. At least 10,000 cells were analyzed in each sample. Every experiment was repeated at least three times. All statistical analyses were performed by Student's *t* test using SPSS software (Version 13.0 for windows, Chicago, IL). A value of $p \leq 0.05$ was considered to be significant.

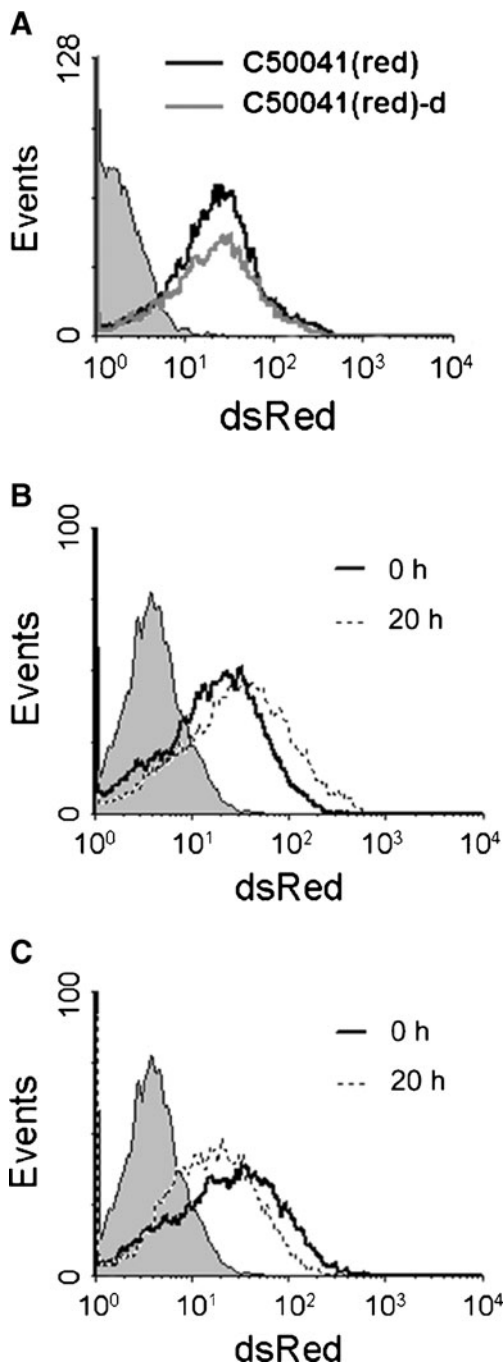


Figure 1. Dynamics assay of bacteria infection by flow cytometry. The red fluorescence protein expression of C50041(Red) and fixed C50041(Red)-d (A), C50041(Red)-infected cells (B), and C50041(Red)-d-infected cells (C).

Results

Bacteria could maintain in host cells for a long time. In order to assay the dynamics of C50041 infection, the plasmid pYA3334-dsRed was transformed into C50041 and the recombinant bacteria, C50041(Red), were screened based on the RFP expression (Fig. 1A). The expression of RFP was stable in the subculturing of bacteria without antibiotics. The C50041(Red) were fixed by 70% alcohol to maintain the whole body of bacteria. After fixation, all bacteria were dead and the fluorescent intensity of bacteria retained (Fig. 1A). Flow cytometric assay indicated that the percentage of RFP-positive cells were about 87% after incubation with C50041(Red) for 30 min and no significant difference were found compared with C50041(Red)-d infection. Twenty hours after infection, the bacteria, C50041(Red) and C50041(Red)-d, still maintained in the host cells. But, the mean fluorescence intensity of C50041(Red)-infected cells was increased, whereas that of C50041(Red)-d-infected cells was decreased (Fig. 1B, C). In the following experiments, cell responses within several hours after infection were analyzed.

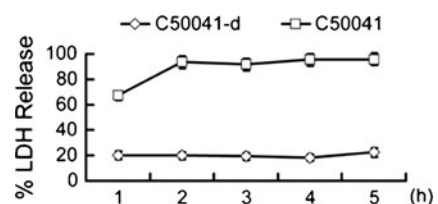


Figure 2. Cytotoxicity assay by LDH release after infection with C50041 and C50041-d, respectively.

Cytotoxicity induced by C50041 infection. Twenty hours later, no obvious suspension cells were found in both infection group compared with uninfected group. Compared with C50041-d infection, partial dead cells were found 5 h after C50041 infection. These dead cells were still adherent on the plate with destroyed cell membrane and damaged cell shape.

With the elongation of time, the dead cells increased. LDH release indicated the cytotoxicity of C50041 infection was higher than for C50041-d. One hour later, the relative amount of released LDH of C50041 infection was about 66%. Two hours later, it was increased to about 93%, and was retained at the level of 18–22% for C50041-d infection (Fig. 2). Two

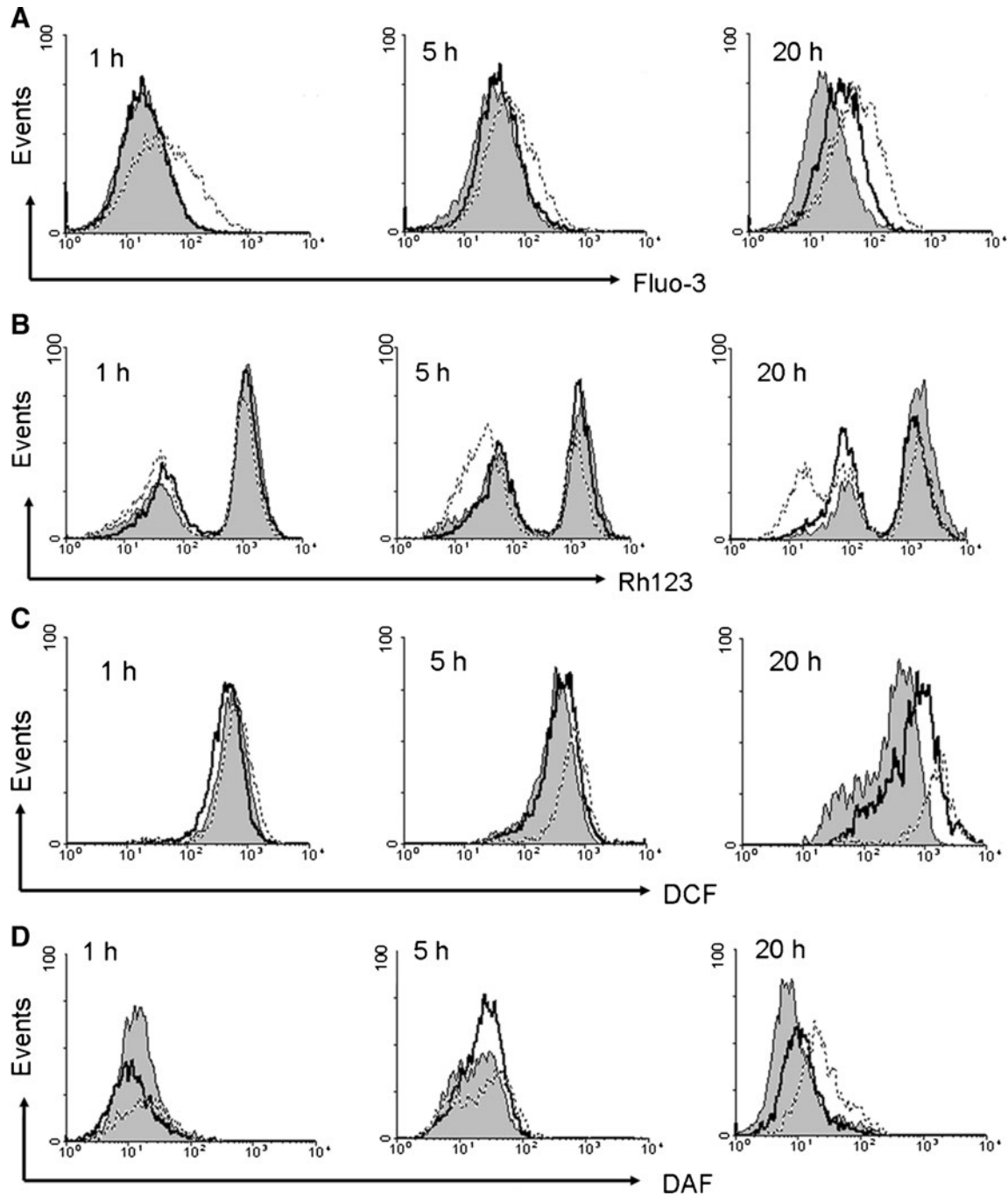


Figure 3. Cell function assay. (A) The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) stained with Fluo-3AM; (B) The mitochondrial membrane potential (MMP) stained with rhodamine 123 (Rh123); (C) The reactive oxygen species (ROS) stained with DCFH-DA; (D) The nitric oxide

(NO) stained with DAF-FM DA. Shaded areas represent the uninfected control, solid lines represent C50041-d infection, dashed lines represent C50041 infection.

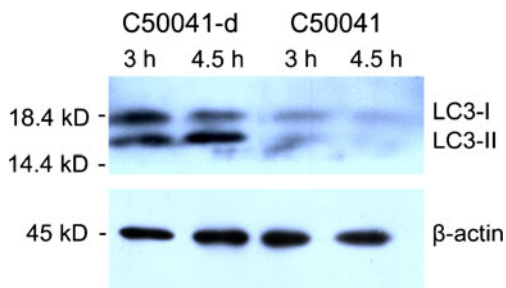


Figure 4. Western blotting of LC3 level expressed by bacteria-infected cells.

days later, only few viable cells were found after C50041 infection compared with C50041-d infection.

Similar functional cellular responses induced by C50041 and C50041-d. RAW264.7 cells were analyzed using fluorescent dye after 1, 5, 20 h culturing. The ratio of fluorescence intensity (520 nm/640 nm) of BCECF-staining cells was used for the assay of $[pH]_i$. The ratio of C50041 infection (about 2.47) was slightly lower than that for C50041-d (about 2.6) and control group (about 2.56). This indicated that it is more acid in C50041-infected cells. The levels of $[Ca^{2+}]_i$, MMP, ROS, and NO were proportional to the intensity of intracellular Fluo-3, Rh123, DCF, and DAF. Flow cytometric assay indicated that C50041- and C50041-d-infected cells were shown the similar change, including the increase of $[Ca^{2+}]_i$, ROS, and NO and the decrease of MMP, with the elongation of culturing time. But, the responses of C50041-infected cells were earlier and stronger than C50041-d-infected cells (Fig. 3).

Lower LC3 expression in macrophages induced by C50041 than C50041-d. Western blotting assay indicated that LC3-I and LC3-II were all found in both infection groups after 3 and 4.5 h culturing. But the contents of C50041-infected group were lower than C50041-d infection (Fig. 4).

Stronger caspase-1 activation and IL-1 β release induced by C50041 than C50041-d. Caspase-1 activation and IL-1 β release are innate responses of macrophages. RAW264.7 cells

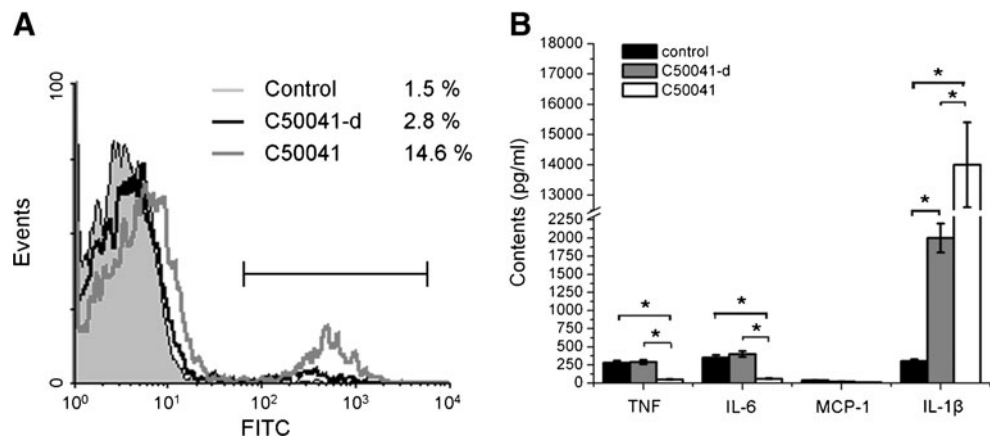
do not express apoptosis-associated speck-like protein containing a C-terminal caspase-activating recruiting domain and limit the activation of caspase-1 (Pelegriin et al. 2008). In the study, the PECs of BALB/c mice were selected for this assay. Four hours after infection, the levels of caspase-1 activation in PECs induced by C50041 were higher than C50041-d infection ($p < 0.05$). The contents of TNF and IL-6 of C50041-d group in supernatants were similar as uninfected control and higher than that of C50041 group ($p < 0.05$). This indicated that C50041 inhibited the secretion of TNF and IL-6 in macrophages. Interestingly, C50041 and C50041-d all could induce the secretion of IL-1 β , but C50041 could induce a much higher level of IL-1 β secretion than C50041-d ($p < 0.05$, Fig. 5).

Discussion

Phagocytosis is normally believed to be a protective process of host. After englobement, the phagosome are fused with lysosome and the non-self particles are then cleared. As the major host cells of *Salmonella*, phagocytes promote the invasion of *Salmonella*. In order to avoid the killing of phagosome, *Salmonella* can limit the maturation of phagosome and promote the formation of SCV (Steele-Mortimer 2008; Lahiri et al. 2010). So, the response of phagocytes after *Salmonella* infection is different from phagocytosis. In the present study, the cell responses after englobement of fixed bacteria were used as control for the exploration of cell responses against live *Salmonella*. The expression of RFP was stable in the subculturing of bacteria and could be used as a marker for the dynamics assay of C50041 infection. Both bacteria could retain in cells 20 h after infection. The changes of the fluorescence intensity of C50041(Red)- and C50041(Red)-d-infected cells 20 h after infection may be due to the replication of live bacteria in host cells and the clearance of dead bacteria by macrophages. So, the responses of host cells within several hours after infection were selected for the synchronic comparison.

In the study, the cell morphology and LDH release indicated that the cytotoxicity induced by C50041 were much higher

Figure 5. Caspase-1 activation and IL-1 β release after bacteria infection for 4 h. (A) Activation of intracellular caspase-1 in primary peritoneal exudate cells (PECs) using FLICA staining. Results represent one of four independent experiments. (B) Cytokines release of bacteria-infected PECs using cytometric bead array system kit.



than phagocytosis to C50041-d. The changing trends of MMP, $[Ca^{2+}]_i$, ROS, and NO in host cells induced by C50041 were similar to C50041-d infection. But, the responses of C50041-infected cells were earlier and stronger than C50041-d infection. The intracellular ROS and NO production are essential to kill the bacteria (Rinaldi et al. 2008; Mendez-Samperio et al. 2009, Paul et al. 2011). In lower pH condition, the physiological function of *Salmonella* decrease (Dunkley et al. 2008). So, the decrease of $[pH]_i$ and the increase of ROS and NO in this study were the protective responses of host cells. The decrease of MMP reflects the damage of mitochondrial membrane (Soto et al. 2009; Itoh et al. 2010). $[Ca^{2+}]_i$ is also a regulating response of cells to external stimuli (Guthrie et al. 2011). This indicated that the cells were damaged in the interaction process between cells and bacteria.

The maturation process of autophagosome is similar as phagosome and is believed to be a protective step to maintain the homeostasis of cells (Yi et al. 2012). Autophagy can limit the early localization of *Salmonella* in cytoplasm due to the coexistence of LC3 and bacteria in the cytoplasm in the early stage of infection (Campoy and Colombo 2009), whereas, facultative intracellular bacteria, *Listeria monocytogenes* and *Tuberculosis mycobacteria*, can inhibit the autophagy process for their survival in host cells. LC3 is the marker molecule of autophagosome. Toll-like receptor (TLR) signaling can promote the recruitment of LC3 to phagosome and then activate autophagy process (Shi and Kehrl 2008; Liu et al. 2011), which is named as TLR–autophagy–phagosome–lysosome innate immunity pathway (Sanjuan et al. 2007). The contents of β -actin expressed in different cells are relatively constant and can be used as internal control for the sample. In the study, the low level of LC3 expression in C50041-infected cells in the study might be due to the regulation of *Salmonella* to host cells and the inhibition of autophagosome maturation.

Intestinal phagocytes (iMPs) are important in maintaining the stability of intestinal tract. Colonic iMPs show hyporesponsive against microbes infection due to the production of antiinflammatory molecules. After pathogenic *Salmonella* infection, iMPs do not produce TNF and IL-6 (Franchi et al. 2012). The low-level expression of TNF and IL-6 induced by C50041 infection proved this point. In macrophages, NOD-like receptors (NLR) recognize intracellular components of microbe and then trigger the form of inflammasome. After enrichment to inflammasome, the enzyme precursor procaspase-1 is split to activated caspase-1. The precursor pro-IL-1 β produced by activated macrophages is then hydrolyzed to IL-1 β by caspase-1 (Broz et al. 2010). iMPs can induce the production of IL-1 β depending on the NLRC4 (NLR family, CARD domain containing 4) inflammasome pathway after pathogenic *Salmonella* infection. This represents a specific response to discriminate pathogenic from commensal bacteria and promote the host defense in intestinal tract (Franchi et al. 2012; Franklin and Latz 2012). In the study, the activation of

caspase-1 and the IL-1 β release of macrophages after C50041 infection were higher than that for C50041-d ($p < 0.05$). The difference of cytokines secretion may be due to the regulation of live *Salmonella* to host cells.

Strong cellular immunologic response is needed for the immunity against intracellular microbe infection. Live *L. monocytogenes* can induce cellular immunologic response, whereas the dead bacteria cannot induce protective immunity (Witte et al. 2012). In the study, we further found that the levels of caspase-1 activation and IL-1 β release of host cells induced by the live *Salmonella* were significantly higher than that for the dead bacteria. The activation of caspase-1 and the release of IL-1 β reflect the activation of inflammasome in macrophages. The inflammasome is first named by Tschopp's group in 2002 (Martinon et al. 2002). Many proteins have been reported to activate this pathway. Flagellin and PrgJ proteins are the major components of flagellar filament and the rod of the *Salmonella* pathogenicity island (SPI)-1 T3SS, respectively. After *Salmonella* infection, they can be secreted to cytoplasm and trigger NLRC4-dependent caspase-1 activation, induce the pyroptosis of host cells and the release of IL-1 β and IL-18, and promote cellular immunologic response and autophagy function (Miao et al. 2010; Ashida et al. 2011; Miao and Rajan 2011; Puri et al. 2012). In the evolution process, *Salmonella* have developed some evasion strategies to prevent NLRC4 detection during intracellular replication in macrophages and the evasion of NLRC4 is essential for *Salmonella* virulence. Flagellin is repressed in the intracellular environment and the rod protein is different from PrgJ when SPI-2 T3SS is active (Miao and Rajan 2011). Therefore, the activation of NLRC4 inflammasome is one of the key points of host cells to inhibit the survival of *Salmonella* in host. It will be beneficial to explore the utilization of this pathway in the control of *Salmonella* infection.

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