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Expression of c-Myc Is Related to Host Cell Death Following *Salmonella typhimurium* Infection in Macrophage

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It has been known that ornithine decarboxylase (ODC) induced by the binding of c-Myc to *odc* gene is closely linked to cell death. Here, we investigated the relationship between their expressions and cell death in macrophage cells following treatment with *Salmonella typhimurium* or lipopolysaccharide (LPS). ODC expression was increased by bacteria or LPS and repressed by inhibitors against mitogen-activated protein kinases (MAPKs) in Toll-like receptor 4 (TLR4) signaling pathway. In contrast, c-Myc protein level was increased after treatment with bacteria, but not by treatment with LPS or heat-killed bacteria although both bacteria and LPS increased the levels of *c-myc* mRNA to a similar extent. c-Myc protein level is dependent upon bacterial invasion because treatment with cytochalasin D (CCD), inhibitors of endocytosis, decreased c-Myc protein level. The cell death induced by bacteria was significantly decreased after treatment of CCD or c-Myc inhibitor, indicating that cell death by *S. typhimurium* infection is related to c-Myc, but not ODC. Consistent with this conclusion, treatment with bacteria mutated to host invasion did not increase c-Myc protein level and cell death rate. Taken together, it is suggested that induction of c-Myc by live bacterial infection is directly related to host cell death.

***Keywords*:** *Salmonella typhimurium*, c-Myc, infection, ornithine decarboxylase, macrophage, cell death

Salmonella is a genus of Gram-negative enteric bacteria that causes enteritis and typhoid fever. *S. enterica* serovar Typhimurium (*S. typhimurium*) can cause a systemic typhoid-like disease in mice, and this animal model of infection is widely used for the study of human typhoid fever caused by *S. typhi* (Pang *et al.*, 1995). Upon enteric infection, *S. typhimurium* induces a lymphocytic response and an innate immune response that eventually leads to cell death (Santos *et al.*, 2001). During bacterial infection, macrophages serve as professional phagocytes, and are key effectors of the innate and adaptive immune responses (Richter-Dahlfors *et al.*, 1997).

Ornithine decarboxylase (ODC) catalyzes the decarboxylation of L-ornithine to form diamine putrescine, a rate-limiting step in the polyamine synthesis pathway (Williams *et al.*, 1992). Previously, it was shown that polyamine synthesis is closely linked to cell death (Davis *et al.*, 1992; Otieno and Kensler, 2000). Elevated expression of ODC increases the concentration of polyamines in cells, including putrescine, spermidine and spermine, and there is evidence that the generation of H_2O_2 during the conversion of spermine to spermidine by spermine oxidase plays an important role in cell death associated with polyamine synthesis (Chaturvedi *et al.*, 2004). The expression of *odc* is activated in macrophages by infection with *S. typhimurium* or treatment with *S. typhimurium*-derived LPS through the activation of TLR4 (Prosser *et al.*, 1984), and the subsequent engagement of downstream effectors, such as the JNK, ERK, and p38 mitogen-activated protein kinases (MAPKs) (Poltorak *et al.*, 1998; Rosenberger *et al.*, 2000).

The oncoprotein c-Myc plays a pivotal role in many cellular processes, including cell proliferation, the suppression of differentiation, and apoptosis (Luscher and Eisenman, 1990; Pelengaris *et al.*, 2002). c-Myc binds to a specific DNA sequence, termed the E-box (Coffino and Chen, 1988; Walhout *et al.*, 1997), that is found all c-Myc-responsive genes. Two E boxes are located in the first intron of *odc*, which suggests that it is a transcriptional target of c-Myc (Bello-Fernandez *et al.*, 1993).

There have been conflicting reports in the literature on the relationship between c-Myc-induced expression of ODC and cell death. The expression of c-Myc was induced in RAW264.7 cells by treatment with *Helicobacter pylori* lysate, resulting in the binding of c-Myc to *odc* and increased ODC levels in infected cells (Cheng *et al.*, 2005). Treatment with c-Myc inhibitor or the expression of a dominant-negative c-Myc mutant decreased the expression of ODC and cell death in cells that were treated with *H. pylori* lysate (Bellmann *et al.*, 2006; Liu *et al.*, 2006). In contrast, while infection of macrophages with *Salmonella* or exposure to *S. typhimurium*-derived LPS induces the expression of both c-Myc and ODC, only bacterial infection induces cell death, presumably because LPS-mediated activation of TLR4 induces the expression of both anti- and pro-apoptotic genes

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(Poltorak *et al.*, 1998; Hsu *et al.*, 2004).

In the current study, we examined the expression of ODC and c-Myc in RAW264.7 and mouse peritoneal macrophage cells infected with *S. typhimurium* or treated with *S. typhimurium*-derived LPS. We show that LPS induces the expression of ODC, but not c-Myc, and that the induction of c-Myc by bacterial infection is directly related to host cell death.

Materials and Methods

Bacteria, cells, and culture conditions

Wild-type *S. typhimurium* strain SCH2005 and its mutants, invF$^-$, sipB$^-$, and ssrAB$^-$ (lab stock), were cultured aerobically at 37°C in LB broth. To prepare heat-killed *S. typhimurium*, bacteria (equivalent in number to the live bacteria used for infection) were pelleted, washed with phosphate-buffered saline (PBS), resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), and then boiled for 10 min.

RAW264.7 cells or mouse peritoneal macrophages (3×10^6 cells/well) were cultured in a 6-well plate in DMEM medium. For infection, bacteria were added to cultured RAW264.7 and peritoneal macrophage cells at a multiplicity of infection (MOI) of 100 and 10, respectively, for 30 min. Cells were washed twice with PBS and then incubated for the indicated periods of time in culture medium containing 10 μg/ml gentamicin (Sigma, USA) without other antibiotics. For LPS treatment, cells were cultured as described above for infection, with the addition of 5 μg/ml *S. typhimurium*-derived LPS to the medium (Sigma). Where indicated, cells were cultured in the presence of 10 μM SB202190 (Calbiochem, USA), 25 μM SP600125 (Calbiochem) or 25 μM PD98059 (Calbiochem) after bacterial infection for 30 min. For the inhibition of bacterial endocytosis or c-Myc activity, cells were cultured in the presence of 0.5 μg/ml *Zygosporium mansonii* cytochalasin D (CCD) (Sigma) or 0.5 μM Int-H1-S6A, F8A c-Myc inhibitor (Biomol, USA) after bacterial infection for 30 min.

Peritoneal macrophages were prepared as previously described (Largen and Tannenbaum, 1986).

Western blot analysis

Cells in culture were treated with a solution of trypsin/EDTA (Invitrogen, USA), washed once with PBS, and then lysed in 0.2 ml lysis buffer [10 mM Tris-HCl; pH 7.5, 150 mM NaCl, 1 mM EDTA; pH 8.0, 1% NP-40, and one tablet of protease inhibitor cocktail (Roche, Germany)] on ice for 30 min. Following centrifugation at 10,000×g, the supernatants subjected to 12% SDS-PAGE, and then proteins were transferred to a nitrocellulose membrane (Bio-Rad, USA). Protein levels were measured with rabbit polyclonal anti-c-Myc (Santa Cruz Biotechnology, USA) or monoclonal anti-ODC antibodies (Sigma) (1:400 dilution). As a quantitative control for protein levels, the membrane was stripped and re-probed using anti-actin antibodies.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analysis

The levels of *odc* mRNA were measured by Northern blot analysis, as follows. An *odc*-specific oligonucleotide probe was generated by PCR using cDNA prepared from RAW264.7 cells as a template, and the following *odc*-specific primers: 5'-TAAAAGCTCTTCCCCGCGTC and 5'-CCTGCTGGTTTT GAGTGTGG. The amplified fragment was radiolabeled with [α-^{32}P]dCTP using a random primer labeling kit (GE Healthcare, UK). Total RNA from RAW264.7 cells was isolated using the TRIzol reagent (Invitrogen). RNA (20 μg) was separated by 1.2% agarose gel electrophoresis in the presence of formaldehyde, and then transferred to a Hybond^{TM}-N membrane (Amersham Bioscience, UK) for 16 h. The membrane was exposed twice to 1200 J of UV light, dried in a 64°C oven, and then incubated with the probe.

The levels of *c-myc* mRNA were measured by semiquantitative RT-PCR. cDNA was synthesized from total RNA (5 μg) using the PrimeScript^{TM} kit (TaKaRa, Japan). PCR was carried using cDNA derived from RAW264.7 cells as a template, and the following *c-myc* primers: 5'-GCCCAGTG AGGAATATCTGGA and 5'-ATCGCAGATGAAGCTCTGGT. The expected product size was 226 bp. As a quantitative control for mRNA levels, *β-actin* mRNA was also amplified using the same conditions.

Measurement of cell death

Cell death was measured by lactate dehydrogenase (LDH) activity released in the media after indicated time using CytoTox96 nonradioactive assay (Promega, USA) and quantified by measuring wavelength absorbance at 490 nm. Data are normalized to the amount of LDH released from lysis buffer treated cells and are corrected for baseline LDH released from cells exposed to culture media only.

Results

ODC expression is induced by *S. typhimurium* infection and LPS treatment in RAW 264.7 cells

We assessed the protein and mRNA levels of ODC in RAW264.7 cells after the infection with *S. typhimurium*, or treatment with *S. typhimurium*-derived LPS (Fig. 1). ODC protein levels were first detected at 4 h, and remained elevated for up to 8 h following exposure to bacteria or LPS (Fig. 1A). We also detected a steady increase in *odc* mRNA levels under both conditions for up to 8 h (Fig. 1B). These results indicated that *Salmonella* infection or treatment with LPS activates *odc* expression, presumably through TLR4-mediated signaling (Royle *et al.*, 2003), and that ODC protein levels are tightly regulated. To determine whether *odc* expression was mediated by TLR4-associated signaling pathways, cells were infected with bacteria for 30 min, then cultured in the presence of MAPK inhibitors that block the phosphorylation of p38, JNK, and ERK (SB202190, SP600125, and PD98059, respectively) (Nemoto *et al.*, 1998), or cultured in the presence of both LPS and inhibitors, for 4 h (Fig. 1C and D). Protein and mRNA levels were analyzed by Western and Northern blot, respectively. All of the MAPK inhibitors effectively blocked the induction of ODC protein (Fig. 1C) and mRNA (Fig. 1D) levels by bacterial infection and LPS treatment. These results suggested that *odc* expression is induced by TLR4-mediated signaling through the activation of p38, JNK, and ERK.

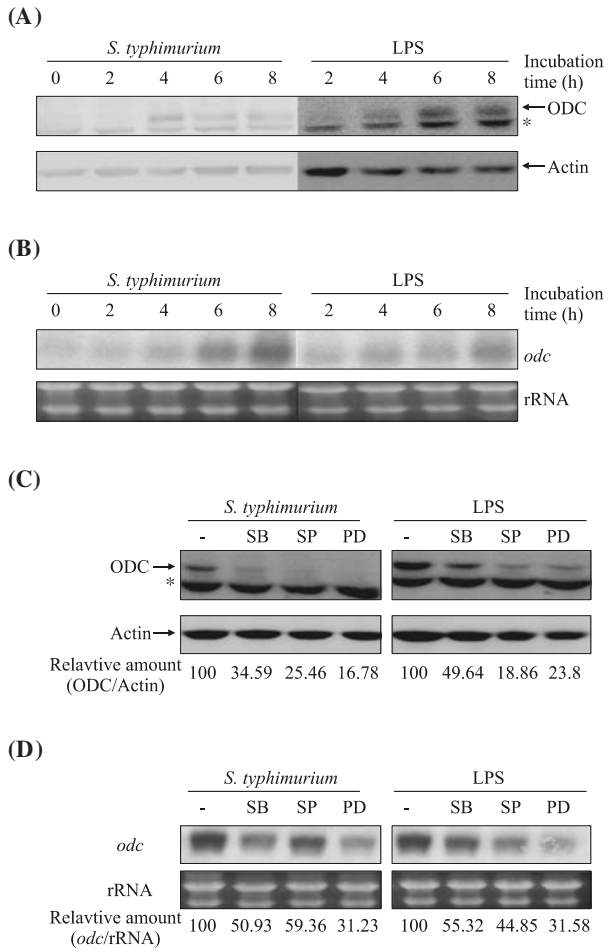

Fig. 1. ODC expression in RAW264.7 cells treated with *S. typhimurium* or LPS. (A) Western blot analysis of ODC protein levels. Proteins were probed with anti-ODC (upper panel) or anti-actin (lower panel) antibodies. An asterisk (*) indicates non-specific antibody binding. (B) Northern blot analysis of *odc* mRNA levels. Total RNA was separated on an agarose gel containing formaldehyde (lower panel). *odc* mRNA levels were detected using a radiolabeled probe (upper panel). (C) The effect of MAPK inhibitors on ODC expression. The treated cells were cultured for 4 h in the presence of the indicated MAPK inhibitors. SB, SB202190; SP, SP600125; PD, PD98059. The relative amount of ODC to actin in each sample was measured by densitometry. Minus (-) indicates cells cultured in the absence of inhibitor. Western analysis was performed, as described for Fig. 1A. (D) Northern blot analysis of *odc* mRNA levels. Total RNA was isolated from the same cells in Fig. 1C. The relative amount of *odc* mRNA to rRNA in each sample was measured by densitometer.

c-Myc protein is induced by *S. typhimurium* infection, but not LPS treatment

It has been reported that *odc* contains c-Myc binding sites and that its expression is activated by c-Myc (Bello-Fernandez *et al.*, 1993). We next investigated whether *S. typhimurium* infection or LPS treatment affected c-Myc expression in RAW264.7 cells (Fig. 2). c-Myc protein was detected 1 h after bacterial infection, and remained elevated for 8 h (Fig. 2A). In contrast, LPS treatment did not affect the levels of c-Myc protein. Note, however, that *c-myc* mRNA was detected by semiquantitative RT-PCR following LPS stimulation as well as bacterial infection (Fig. 2B). These results suggested that c-Myc mRNA is not efficiently translated or that the protein is rapidly degraded in LPS-treated cells. To confirm these results, RAW264.7 and mouse peritoneal macrophage cells were treated for 4 h with heat-killed bac-

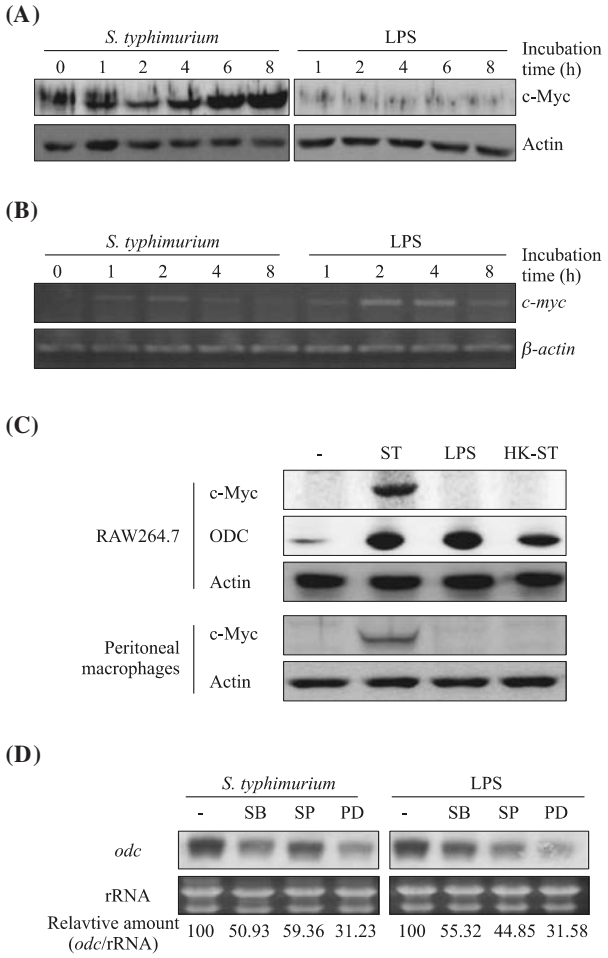

Fig. 2. The induction of c-Myc expression in macrophage cells treated with *S. typhimurium* or LPS. RAW264.7 cells were treated with *S. typhimurium* or LPS as described for Fig. 1. (A) Western blot analysis of c-Myc protein levels. Proteins were probed with anti-c-Myc (upper panel) or anti-actin (lower panel) antibodies. (B) Semiquantitative RT-PCR analysis of *c-myc* mRNA levels. PCR was carried out using *c-myc* (upper panel) or *β-actin* specific primers (lower panel). (C) c-Myc expression in macrophages treated with heat-killed *S. typhimurium*. RAW264.7 or mouse peritoneal macrophage cells were treated with heat-killed *S. typhimurium*. After 4 h, protein levels were analyzed by Western blot using anti-c-Myc and anti-ODC antibodies, and anti-actin antibodies as a control, as indicated. Minus (-), non-treated cells; ST, cells infected with live bacteria; LPS, cells treated with LPS; HK-ST, cells treated with heat-killed bacteria. (D) The effect of MAPK inhibitors on the induction of c-Myc protein by *S. typhimurium* infection. RAW264.7 cells were infected with *S. typhimurium*, and then cultured for 4 h in the presence of the MAPK inhibitors SB202190 (SB), SP600125 (SP), and PD98059 (PD). Protein levels were detected by Western blot using anti-c-Myc (upper panel) or anti-actin antibodies (lower panel).

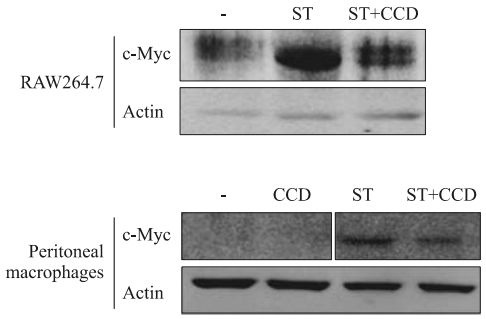

Fig. 3. The effect of an endocytosis inhibitor on the induction of c-Myc expression by *S. typhimurium* infection. Cells were cultured in the absence or presence of CCD prior to *Salmonella* infection. c-Myc and actin were measured by Western blot using anti-c-Myc and anti-actin antibodies, respectively.

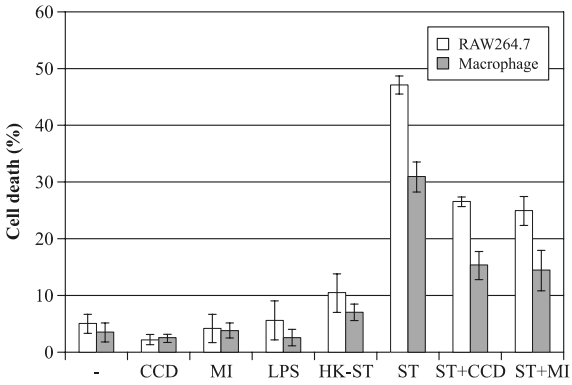

Fig. 4. The effect of inhibitors of endocytosis and c-Myc on *S. typhimurium*-induced cell death. RAW264.7 (open bars) and mouse peritoneal macrophage cells (closed bars) were cultured in the indicated condition for 8 and 4 h, respectively, and cell death percentage was measured. Minus (-), un-treated cells; CCD, cells treated with CCD; MI, cells treated with a c-Myc inhibitor; LPS, the cells treated with LPS; HK-ST, cells treated with heat-killed bacteria; ST, cells infected with live bacteria; ST+CCD, cells infected with live bacteria in the presence of CCD; ST+MI, cells infected with live bacteria in the presence of c-Myc inhibitor.

terial lysate (Fig. 2C), since LPS activity is heat-stable (Konadu *et al.*, 1994). Neither heat-killed lysate nor LPS increased c-Myc protein levels, as shown by Western blot analysis. In contrast, the level of ODC induction by heat-killed lysate was comparable to treatment with intact (live) bacteria or LPS. These results indicated that the induction of c-Myc protein by *S. typhimurium* is due to a bacterial component other than LPS.

We next examined whether the increase in c-Myc protein levels by bacterial infection was mediated by TLR4. RAW 264.7 cells were treated with MAPK inhibitors for 4 h after *Salmonella* infection for 30 min (Fig. 2D), and the levels of c-Myc were analyzed by Western blot. The induction of c-Myc protein in the presence of MAPK inhibitors was similar to control cells that were not treated with inhibitors, which indicated that the induction of c-Myc protein in response to bacterial infection is not mediated by such MAPK pathway.

Bacterial endocytosis induces c-Myc expression in macrophages

As the induction of c-Myc protein by *S. typhimurium* was not involved with bacterial LPS in above results, we accessed the induction of c-Myc in bacterial invasion into host cells. To determine whether the induction of c-Myc expression was dependent on bacterial endocytosis by macrophages, RAW264.7 and mouse peritoneal macrophage cells were infected with bacteria in the presence of the endocytosis inhibitor CCD for 4 h (Fig. 3). CCD treatment significantly decreased c-Myc protein levels in both cell types, indicating that bacterial endocytosis is the primary mechanism of induction of c-Myc protein levels in macrophage cells.

S. typhimurium induces severe cell death in macrophage cells

Previously, it was shown that increased expression of ODC or c-Myc leads to cell death by apoptosis (Gobert *et al.*, 2002). We were interested in whether the induction of ODC and c-Myc expression by bacterial infection and LPS treatment also induced cell death in macrophages. Cell death was determined by LDH assay. This method is known to measure apoptosis and pyroptosis (Rupper and Cardelli, 2008). RAW264.7 cells and mouse peritoneal macrophages were treated with live or heat-killed *S. typhimurium* or with LPS (Fig. 4), and the rate of cell death in treated and untreated cells was examined 4 h after treatment. Whereas cell death in LPS-treated cells was similar to non-treated control cells, it was significantly increased in both types of macrophage cells following infection with live *S. typhimurium.* These results indicated that the expression of c-Myc, but not ODC, results in cell death in host macrophages. Consistent with the role of c-Myc in bacteria-induced cell death, the rate of cell death induced by heat-killed bacteria was significantly less than live bacteria.

To confirm that c-Myc expression induces cell death in macrophages, cells were cultured for 4 h in the presence of CCD or the c-Myc inhibitor Int-H1-S6A, F8A, prior to bacterial infection. The rate of bacteria-induced cell death in the presence of either inhibitor was significantly less than in the absence of inhibitor. These results indicated that the induction of c-Myc expression in RAW264.7 cells by *S. typhimurium* is dependent on bacterial endocytosis, and results in cell death.

The c-Myc level and cell death in bacterial infection are dependent upon bacterial invasion, but not upon subsequent proliferation

S. typhimurium genome has two pathogenicity islands (SPI) with genes encoding type III secretion systems for virulence proteins (Galan, 2001). SPI1 is required for bacterial invasion into host cells and SPI2 is important for the subsequent proliferation of bacteria (Ochman *et al.*, 1996). To assess which stage of bacterial infection affects c-Myc protein level and cell death, we treated RAW264.7 cells with mutant bacteria which were defective of invasion or intracellular proliferation (Fig. 5). c-Myc proteins were not detected significantly in 8 h after the treatment of SPI1 mutants (invF$^-$ or

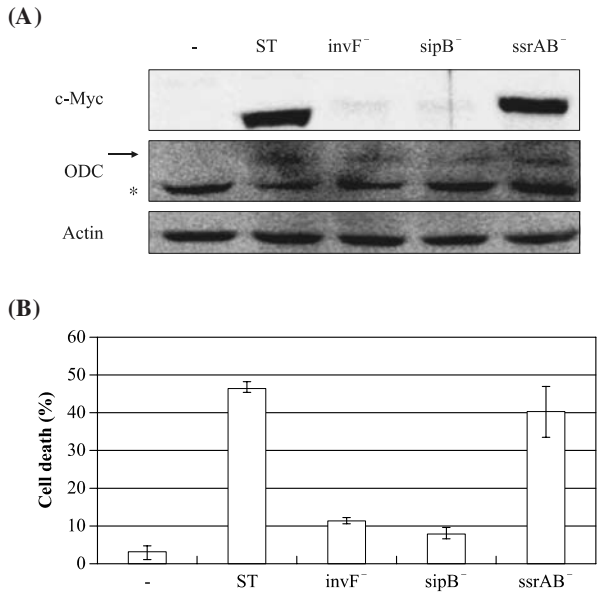

Fig. 5. c-Myc level and cell death are dependent upon bacterial invasion stage. AW 264.7 cells were cultured with SPI1 (invF$^-$ or sipB$^-$) or SPI2 (ssrAB$^-$) *S. typhimurium* mutants for 8 h. (A) Western blot analysis against c-Myc and ODC proteins. The protein levels were analyzed by Western blotting using anti-c-Myc and anti-ODC antibodies and anti-actin antibodies were used as a control. (B) Cell death percentage was measured 8 h after infection. Minus (-), untreated cells; ST, cells infected with wild-type bacteria; invF$^-$, cells infected with *invF*-deficient mutant; sipB$^-$, cells infected with *sipB*-deficient mutant; ssrAB$^-$, cells infected with *ssrAB*-deficient mutant.

sipB$^-$) (Fig. 5A). In contrast, c-Myc proteins in SPI2 mutant (ssrAB$^-$) were at the similar level in those in wild-type bacteria. Consistent with this observation, the wild-type bacteria and SPI2 mutant increased cell death in 8 h, whereas SPI1 mutant induced low level of cell death (Fig. 5B).

Discussion

In the current study, we have shown that the infection of macrophages by *S. typhimurium* and the treatment of cells with *S. typhimurium*-derived LPS induces the expression of ODC to a similar extent. Thus, under both conditions, polyamine synthesis would also be similarly elevated. However, whereas bacterial infection resulted in macrophage cell death, LPS treatment did not. LPS-mediated activation of TLR4 results in the induction of both anti- and pro-apoptotic genes in macrophages, which might explain the differential effects of bacterial infection and LPS treatment (Hsu *et al.*, 2004). In particular, p38 MAPK has been shown to activate anti-apoptotic genes, thus, LPS-induced cell death may require the inhibition of p38 activation (Park *et al.*, 2002). It is also possible that other genes are induced in response to LPS treatment that mitigates the effects of increased expression of ODC.

The level of c-Myc protein was increased by infection with *S. typhimurium*, but not by exposure to LPS, and this increase in c-Myc correlated with increased cell death in RAW264.7 and peritoneal macrophages. Since LPS induced the expression of ODC, similar to live bacteria, these results indicate that the activation of *odc* by c-Myc is not critical for cell death, and suggest that unidentified gene(s) involved in cell death are induced by c-Myc in response to bacterial infection. This conclusion were supported that pre-treatment of alpha-difluoromethylornithin (DFMO), a specific ODC inhibitor, was no effect on the inhibition of caspase activity by LPS and TNF (Tantini *et al.*, 2002). Consistent with the role of c-Myc in bacteria-induced cell death, treatment of cells with an inhibitor of c-Myc significantly decreased cell death following infection by *S. typhimurium*. This wasn't limited to macrophages. We observed that the induced c-Myc increased cell death in mouse breast cancer cell line, 4T-1 (data not shown).

While the levels of *c-myc* mRNA were increased by both bacterial infection and LPS treatment, c-Myc protein levels were increased only following bacterial infection. Increased levels of c-Myc have a severe effect on normal cell functions, including proliferation, differentiation, and apoptosis (Luscher and Eisenman, 1990), (Pelengaris *et al.*, 2002). Thus, it is not surprising that the expression and function of c-Myc are regulated at multiple levels. Our results suggest that the regulation of c-Myc in response to bacterial infection is most likely at the level of posttranslational modification (Vervoorts *et al.*, 2006). There are several post-translational modifications that affect the stability of c-Myc, including phosphorylation, ubiquitinylation, and acetylation. Our results raise the possibility that LPS treatment and bacterial infection differentially regulate the signaling pathways involved in c-Myc stability and degradation. Additional studies are required to clarify this issue.

Host cell death is a common outcome of bacterial invasion (Monack *et al.*, 1996). The ability of a pathogen to survive and even replicate within phagocytic cells is a potent method of evading the defense mechanisms of the host. A number of pathogens, including *Salmonella*, survive within macrophages after phagocytosis, and this is a major factor in their virulence (Richter-Dahlfors *et al.*, 1997). The invasion of host cells by *Salmonella* requires actin polymerization and endocytosis (Tilney and Portnoy, 1989). Our results demonstrated that CCD, an inhibitor of actin polymerization, also decreases c-Myc protein levels in cells infected with bacteria. These results indicate that bacterial endocytosis is required for bacteria-induced cell death, through c-Myc. We also showed that SPI1, but not SPI2 was related with c-Myc protein level and cell death. This conclusion is consistent with the report that apoptosis in macrophages in response to *S. typhimurium* infection is rapid, specific, and depend on the type III secretion system encoded within SPI1 (Cook *et al.*, 2007). Here, we reported that *Salmonella* infection increased c-Myc protein level in host cells during bacterial endocytosis and sequentially induced host cell death.

Acknowledgements

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