MICROBIAL PATHOGENICITY

Induction of apoptosis of human macrophages *in vitro* by *Legionella longbeachae* through activation of the caspase pathway

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The cytotoxicity of the facultative intracellular bacterium, *Legionella longbeachae*, an important cause of legionellosis, was characterised. Apoptosis was induced in HL-60 cells, a human macrophage-like cell line, during the early stages of infection and induction of apoptosis correlated with cytotoxicity. Apoptosis was confirmed by agarose gel electrophoresis of fragmented DNA, surface exposure of phosphatidylserine and propidium iodide labelling of host cell nuclei. The involvement of macrophage infectivity potentiator (Mip) protein, a known virulence factor of *L. longbeachae*, was also examined. A *mip* mutant of *L. longbeachae* induced apoptosis of HL-60 cells but failed to multiply intracellularly, suggesting that intracellular replication of *L. longbeachae* is not essential for the induction of apoptosis of HL-60 cells. Furthermore, induction of apoptosis of *L. longbeachae*-infected macrophages was mediated by activation of the caspase pathway but might be independent of tumour necrosis factor- α - and Fas-mediated signal transduction pathways.

Introduction

Legionella longbeachae was first described as a new species of Legionellaceae in 1981, following its isolation from a patient with pneumonia who resided in Long Beach, California [1]. Subsequently, *L. longbeachae* serogroup 1 was isolated from a patient in South Australia in 1987 [2] and this was followed by an outbreak of infection in the same Australian state [3, 4]. Recently, c. 50% of all legionellosis in South Australia has been attributed to this species [3, 5]. Epidemiological studies have shown that gardening is a major risk factor in acquiring *L. longbeachae* infection in Australia [6]. Australian potting soil is not sterilised during manufacture and contains a mixture of bacteria and free-living amoebae, which provides a suitable medium for growth of *Legionella* spp. [7]. The first culture-confirmed Japanese case of *L. longbeachae* pneumonia was reported in 1996 by Okazaki *et al.* [8]. The patient was a gardener and potting soil was considered as a possible source of infection. The presence of bacteria in various brands of Japanese potting soil has since been examined: 31 strains of *Legionella* spp. have been isolated from 17 Japanese

potting soils, and 7 potting soils contained *L. longbeachae* [9].

Several investigators have shown that macrophage infectivity potentiator (Mip) protein plays an important role in the intracellular life cycle of *L. longbeachae* [5]. In *L. pneumophila*, the Mip protein is responsible for efficient initiation of intracellular infection of human macrophages, and a *mip* mutant was shown to be less infective for human macrophages, as well as for *Acanthamoeba castellanii* [10, 11]. It was also reported that a mutation in *mip* resulted in attenuated virulence in guinea-pigs [11]. The Mip protein of *L. pneumophila* belongs to the enzyme family of peptidyl-prolyl cis-trans isomerases and is homologous to the FK506 binding protein [12]. A *mip* mutant of *L. longbeachae* serogroup 1 ATCC 33462 could not infect a strain of *Acanthamoeba* sp. Furthermore, the *mip* mutation resulted in reduced virulence in guinea-pigs exposed to aerosol infection [5].

In recent years, several bacterial pathogens have been identified as inducers of apoptosis (programmed cell death). The role of apoptosis differs with each type of bacterial infection. For example, in *Shigella flexneri* [13, 14] and *Salmonella enterica* serovar Typhimurium [15] infections, induction of apoptosis results not only in deletion of host cells but also in initiation of inflammation, by activating interleukin-1 β converting

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enzyme. Several bacteria that produce exotoxins, like *Corynebacterium diphtheriae* [16], *Pseudomonas* spp. [16], *Actinobacillus actinomycetemcomitans* [17], *Bacillus anthracis* [18] and group A streptococci [19] induce apoptosis of macrophages before these cells can ingest and destroy the bacteria. *Mycobacterium tuberculosis* was reported to enhance apoptosis of alveolar macrophages by mechanisms involving tumour necrosis factor (TNF)- α [20]. On the other hand, *M. bovis* and *Brucella suis* prevent apoptosis of human monocytes [21–23] and seem to protect themselves from the host immune system in this way.

Previous studies have provided evidence for the induction of apoptosis in the HL-60 macrophage-like cell line by *L. pneumophila* [24]. The same organism induces apoptosis of alveolar epithelial cells during the early stages of infection and this plays an important role in its cytotoxicity [25]. However, apoptosis induced by other *Legionella* species has not been investigated. In this study, the ability of *L. longbeachae* to induce apoptosis and cytotoxicity in HL-60 cells was investigated.

Materials and methods

Bacterial strains and growth conditions

The strains used in this study are listed in Table 1. The *mip* mutant B10 of *L. longbeachae* serogroup (SG) 1 ATCC 33462, Australian clinical isolate of *L. longbeachae* SG1 A5H5 and the A5H5 *mip* mutant B8 were kindly provided by Robyn M. Doyle (University of Adelaide, South Australia, Australia) [5]. Strain SG1 96-003 was isolated from the first Japanese case of pneumonia caused by *L. longbeachae* [8]. The other strains (nos 98-066, 98-079, 98-082, 98-083, and 98-084) were isolated from potting soils in Japan. *L. pneumophila* strain SG1 80-045 was isolated from the first Japanese patient identified to have Legionnaires' disease [26]. These strains were kept at -80°C in sterile skimmed milk supplemented with sodium glutamate 1% w/v. Before experiments, the

strains were cultured on buffered charcoal yeast extract- α (BCYE- α) agar plates at 35°C for 48 h, when a thin lawn of bacterial growth was visible [24]. The bacteria were then harvested and suspended in distilled water and their concentration was adjusted to $c. 1 \times 10^9$ cfu/ml by optical density.

HL-60 cell culture

The human leukaemia cell line HL-60 [27] was maintained at 37°C in a medium consisting of RPMI 1640 (pH 7.2; Gibco Laboratories, Grand Island, NY, USA), 10 mM HEPES (Dojin Chemicals, Kumamoto, Japan) and heat-inactivated fetal calf serum (FCS; Whittaker, Walkersville, MD, USA) 10% v/v (RPMI-FCS) in 75-cm² culture flasks (Falcon 3084; Nippon Becton Dickinson, Tokyo, Japan) in humidified air with CO₂ 5%. HL-60 cells were differentiated into macrophage-like cells by incubating them for 2 days with 10^{-8} M phorbol 12-myristate 13-acetate (Sigma) in RPMI-FCS. Adherent cells were washed three times with RPMI-FCS and then incubated in this medium before infection.

Infection of HL-60 cells

Differentiated HL-60 cells, in triplicate, in 96-well microtitration plates containing 1×10^5 cells/well were infected with *L. longbeachae* strains at multiplicities of infection (MOIs; bacteria:macrophage) of 10 and 100 for 2 h. At the end of the infection period (time 0), the monolayers were washed three times to remove unattached extracellular bacteria and maintained at 37°C in the culture medium for various incubation times (0–48 h). The infected cell monolayers and supernates in each well were then harvested in 9.8 ml of sterile distilled water, and the mixture was vortex mixed for 20 s to lyse the cells. These bacterial suspensions were diluted appropriately and 100- μl samples of the dilutions were inoculated on to BCYE- α agar to determine the numbers of viable Legionellae in each well.

Cytotoxicity assay

After infection, washing and incubation of HL-60 cells for various times, as described above, cytotoxicity assays were done. The dye 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 10 μl at a concentration of 5 mg/ml, was added to each well. The microtitration plates were incubated for 4 h at 37°C , under CO₂ 5% in air. The supernates were then removed and 100 μl of isopropyl alcohol containing 0.04 N HCl were added to each well. The plates were read on a microplate reader (model 550, BioRad Laboratories) at a wavelength of 550 nm. The A₅₅₀ values of triplicate wells were averaged to determine the extent of macrophage killing. The percentage cytotoxicity was calculated at each incubation time by the following formula: percentage cytotoxicity

Table 1. Bacterial strains

Strain	Relevant characteristic(s)	Reference or source
<i>L. longbeachae</i> SG 1		
ATCC 33462	Type strain	CDC
96-003	Japanese clinical isolate	8
98-066, 98-079, 98-082, 98-083, 98-084	Japanese environmental isolates	Present study
A5H5	Australian clinical isolate	5
B8	A5H5 with a <i>mip</i> deletion mutation	5
B10	ATCC 33462 with a <i>mip</i> deletion mutation	5
<i>L. pneumophila</i> SG 1		
80-045	Japanese clinical isolate	28

ATCC, American Type Culture Collection, Rockville, MD, USA.
CDC, Centers for Disease Prevention and Control, Atlanta, GA, USA.

$= 100 \times (1 - A_{550} \text{ infected cells} / A_{550} \text{ uninfected cells})$.

DNA fragmentation

Differentiated HL-60 cells in 24-well plates containing 1×10^6 cells/well were infected with *L. longbeachae* strains at a MOI of 10 and 100 for 2 h. At the end of the infection period, the monolayers were washed three times to remove unattached extracellular bacteria and maintained at 37°C in the culture medium. As a positive control for apoptosis or DNA fragmentation, cells were incubated in RPMI-FCS containing actinomycin D (ActD; Sigma) 1 µg/ml. As a negative control, uninfected cells in RPMI-FCS underwent the same washing treatment. To assess whether the bacteria have to be viable to induce apoptosis, heat-inactivated bacteria were used for infection. A bacterial suspension was heated at 95°C for 30 min, and complete killing was verified by plating on BCYE- α . After incubation of HL-60 cells, the cells were lysed by addition of 100 µl of lysis buffer (Triton X-100 0.5%, 10 mM Tris, pH 7.4, 10 mM EDTA, pH 8.0) per well. After incubation for 10 min at 4°C, samples were centrifuged for 20 min at 16 000 rpm. The resulting supernates were digested with 2 µl of RNAase (20 mg/ml) for 1 h at 37°C, and then incubated for a further 1 h at 37°C with 2 µl of proteinase K (20 mg/ml). The lysates were further incubated with 20 µl of 5 M NaCl and 120 µl of isopropanol for 24 h at -20°C to precipitate the DNA. After centrifugation the precipitates were dried to remove isopropanol and then solubilised in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Electrophoresis was performed in an agarose 2% gel, which was then stained with ethidium bromide.

Inhibition of DNA fragmentation in differentiated HL-60 cells by the broad-specificity caspase inhibitor Z-VAD-FMK (Enzyme System Products) [28] was investigated. Cells were pretreated with the inhibitor (100 µM) for 90 min before they were infected with *L. longbeachae* strain ATCC 33462 at a MOI of 100 for 2 h in the presence of the inhibitor, followed by washing of unattached extracellular bacteria and incubation for 3 h in the presence of the inhibitor.

Quantification of apoptosis by flow cytometry

Apoptotic cells were quantified by an assay based on detection of phosphatidylserine (PS) exposed on the outer leaflet of apoptotic cells in the early phase of apoptosis [29]. Cells were stained with fluorescein-conjugated annexin V, which has a high affinity for membrane-exposed PS according to the data provided by the manufacturer (Annexin V FITC Kit, Coulter Immunotech, Tokyo, Japan). Propidium iodide (PI) allowed the discrimination of apoptotic from necrotic cells, as necrotic cells are characterised by loss of cell membrane integrity and staining by PI and simul-

taneous binding of annexin V. Analysis was performed by flow cytometry (Coulter Immunotech).

Measurement of TNF- α

Differentiated HL-60 cells were infected with *L. longbeachae* as described above. After various incubation times, the concentrations of TNF- α were determined by enzyme-linked immunosorbent assay (ELISA) with a commercial kit according to the manufacturer's instructions (Biosource International, Camarillo, USA). In selected experiments, anti-human TNF- α (IgG fraction) (Sigma; 10 ng/ml) was added 1 h before infection.

Detection of Fas expression

Fas expression on the surface of differentiated HL-60 cells was measured by flow cytometry. Uninfected and infected cells (5×10^5) were stained with 20 µl of FITC-conjugated anti-Fas monoclonal antibody (MAb; Medical & Biological Laboratories, Nagoya, Japan) in PBS containing FCS 1% v/v for 30 min at 4°C. As a negative control, cells were stained with mouse IgG1-FITC (Coulter Immunotech). Cells were then analysed by flow cytometry.

Results

Intracellular growth and cytotoxicity of *L. longbeachae* in macrophage-like HL-60 cells

Macrophage-like HL-60 cells were infected with *L. longbeachae* wild strain ATCC 33462 at MOIs of 10 and 100. No detectable intracellular replication of the bacterium was observed by 6 h after infection at either MOI. After incubation for 12 h, intracellular replication of *L. longbeachae* was observed (Fig. 1a). The cytotoxicity of *L. longbeachae* ATCC 33462 was tested by the MTT viability assay. The bacterium killed the HL-60 cells and the cytotoxic effect was dose-dependent, with the maximal toxicity ($74.3\% \pm 1.3\%$) noted at a MOI of 100 at 48 h after infection (Fig. 1b).

Similar results were obtained by infection of a clinical isolate of *L. pneumophila*, strain 80-045, and other Japanese isolates of *L. longbeachae* under the same conditions (data not shown). The cytotoxic activities of the Australian clinical isolate A5H5 of *L. longbeachae* and its *mip* mutant were also compared (Fig. 2). The *mip* mutant strain B8 exhibited a similar level of cytotoxicity compared to the wild-type strain in the later stages of infection (24 and 48 h after infection). However, at the earlier stage of infection (6 h after infection), strain A5H5 was more cytotoxic than strain B8 (15.5% and -7.2%, respectively).

Apoptosis of *L. longbeachae*-infected HL-60 cells

DNA fragmentation is a unique event of apoptosis.

Fig. 1. (a) Intracellular growth kinetics of *L. longbeachae* ATCC 33462 in macrophage-like HL-60 cells. Cells were infected with the bacteria at MOIs of 10 ($\diamond$) and 100 ($\blacksquare$) for 2 h; the numbers of intracellular bacteria were determined at intervals thereafter. (b) Cytotoxic effect of *L. longbeachae* ATCC 33462 on macrophage-like HL-60 cells. Cytotoxicity was determined by the MTT assay at different times after infection at MOIs of 10 ($\square$) and 100 ($\blacksquare$). Data are the means and SD of triplicate wells and are representative of three experiments.

Apoptosis of *L. pneumophila*-infected HL-60 cells has been observed at 24–48 h after infection at MOIs of 10 and 50 [24]. Apoptosis of U937 macrophages by *L. pneumophila* was also reported during the early stages of infection at MOIs of 0.5, 5, and 50 when various methods were used to identify apoptosis [25]. Based on these early studies, apoptosis of *L. longbeachae*-infected macrophage-like HL-60 cells was examined by agarose gel electrophoresis to detect DNA fragmentation. The DNA from macrophage-like HL-60 cells that had been infected with live *L. longbeachae* for 24–48 h showed the typical nucleosome pattern of DNA ladder formation (Fig. 3). The

Fig. 2. Comparison of the cytotoxicity of *L. longbeachae* strain A5H5 ($\blacksquare$) and strain B8 ($\square$), a *mip* mutant derivative of A5H5. HL-60 cells were infected at a MOI of 100. Data are the means and SD of triplicate wells and are representative of three experiments.

pattern of the DNA ladder was similar to that in actinomycin-D-treated cells (positive control), whereas the DNA pattern of cells treated with heat-inactivated bacteria was similar to that of uninfected cells (negative control), which exhibited mainly intact DNA (Fig. 3). The DNA from HL-60 cells infected with *L. pneumophila* 80-045 and other Japanese isolates of *L. longbeachae* also showed ladder formation (data not shown).

To examine whether the potential virulence factor Mip protein is involved in the induction of apoptosis, *mip* mutant strains B8 and B10 were used for infection. The ladder pattern of DNA fragmentation in HL-60 cells induced by the *mip* mutant strains were similar to these induced by the parental strains (Fig. 4b). These results suggest that Mip protein of *L. longbeachae* is not involved in the induction of apoptosis of HL-60 cells.

Apoptosis of the macrophage-like HL-60 cells was also assessed by flow cytometry after staining with fluorescein-conjugated annexin V and PI. In normal cells, PS is located in the inner leaflet of the cell membrane bilayer. The surface expression of PS is an early feature of apoptosis and occurs before the loss of membrane integrity. Cells in the early apoptotic process bind annexin V which has high affinity for PS, but exclude PI, while those in the late process or necrotic cells stain simultaneously with annexin V and PI. Macrophage-like HL-60 cells were infected with *L. longbeachae* ATCC 33462 at MOIs of 10 and 100. At several time points after infection, cells were labelled with fluorescein-conjugated annexin V and PI and the quantity of label was determined by random

Fig. 3. Induction of internucleosomal DNA cleavage by infection of HL-60 cells with *L. longbeachae* ATCC 33462. Cellular DNA was isolated 24 or 48 h after infection from macrophage-like HL-60 cells infected with *L. longbeachae* ATCC 33462 at MOIs of 10 or 100. Lanes 1 and 12, molecular size marker (123 bp); 2 (24 h) and 7 (48 h), cellular DNA from uninfected HL-60 cells; 4 (24 h) and 9 (48 h), cellular DNA from HL-60 cells infected with heat-inactivated bacteria; 3 (24 h) and 8 (48 h), cellular DNA from actinomycin D-treated (1 μ g/ml) HL-60 cells. 5 (24 h) and 10 (48 h), cellular DNA from HL-60 cells infected at a MOI of 10; 6 (24 h) and 11 (48 h), cellular DNA from HL-60 cells infected at a MOI of 100.

Fig. 4. (a) Intracellular growth kinetics of macrophage-like HL-60 cells infected with *L. longbeachae* strain A5H5 (▲) and the *mip* mutant strains B8 (■) and B10 (◆). Cells were infected at a MOI of 100 and incubated for 48 h. Data are means and SD of triplicate samples. (b) DNA fragmentation in HL-60 cells. Lane 1, 123-bp molecular size marker; 2, uninfected cells; 3, DNA from HL-60 cells infected with strain A5H5; 4, DNA from HL-60 cells infected with strain B8; 5, DNA from HL-60 cells infected with strain B10.

counting of 5×10^3 cells. As shown in Fig. 5, the maximal percentage of early apoptotic cells was noted at 6 h after infection at different MOIs (MOI 10: 21.5%; MOI 100: 28.5%). The results of confocal microscopic examination (not shown) were similar to those of flow cytometric analysis. In the late stage of infection (48 h after infection) the percentage of late apoptotic cells and necrotic cells had increased, especially with the higher MOI (MOI 10: from 0.6 to 20.8%; MOI 100: from 2.5 to 39.1%). Based on these results, *L. longbeachae*-induced apoptosis was dose-dependent. DNA fragmentation demonstrated by agarose gel electrophoresis also showed that induction of apoptosis was dose-dependent (Fig. 3).

Apoptosis of infected cells was induced during the early stage of infection, before intracellular bacterial replication. Intracellular replication of the *mip* mutants in infected cells was not observed even at 48 h after infection, whereas DNA fragmentation of infected cells

Fig. 5. Quantification of apoptosis in *L. longbeachae*-infected macrophage-like HL-60 cells by flow cytometry. Cell monolayers were infected at MOIs of 10 and 100 and labelled simultaneously with fluorescein-conjugated annexin V and PI; 5×10^3 cells were counted at random and data are expressed as percentage means and SD of triplicate samples. Similar results were obtained in two independent experiments. The numbers of annexin-positive cells (□) and annexin and PI-positive cells (■) are shown.

was observed by agarose gel electrophoresis (Fig. 4). These results suggested that intracellular bacterial replication is not required for induction of apoptosis of HL-60 cells.

Mediation of L. longbeachae-induced apoptosis by activation of the caspase cascade

To determine the involvement of caspases in *L. longbeachae*-induced apoptosis, HL-60 macrophages were treated with a broad-spectrum, cell-permeable, caspase inhibitor (Z-VAD-FMK) for 90 min, at a concentration of $100 \mu\text{M}$. Monolayer cells were infected with strain ATCC 33462 at a MOI of 100 for 2 h in the presence of the inhibitor, washed three times to remove unattached extracellular bacteria and incubated for a further 6 h in the presence of the inhibitor. DNA was isolated at the end of the 6-h incubation period and examined by agarose gel electrophoresis for detection of DNA fragmentation. DNA fragmentation in *L. longbeachae*-infected HL-60 cells was completely blocked by Z-VAD-FMK (Fig. 6).

Certain cytokines of the TNF ligand family and their receptors, including TNF- α receptor and Fas (also known as Apo-1 or CD95), are potent triggers of apoptosis in many cells [30]. TNF- α and Fas ligands induce apoptosis by binding to their respective death domain-containing receptors, TNF-RI and Fas [31]. TNF- α is secreted in the lung during *L. pneumophila*

Fig. 6. Inhibition of DNA fragmentation in *L. longbeachae*-infected HL-60 macrophages by caspase inhibitor Z-VAD-FMK. Cell monolayers were treated with $100 \mu\text{M}$ Z-VAD-FMK for 90 min then infected with ATCC 33462 at MOIs of 10 and 100 for 2 h in the presence of Z-VAD-FMK. Cells were washed to remove extracellular bacteria and incubated for a further 6 h in the presence of Z-VAD-FMK. Lane 1, 123-bp molecular size marker; 2, Z-VAD-FMK-treated cells at a MOI of 100; 3, Z-VAD-FMK-treated cells at a MOI of 10; 4, Z-VAD-FMK-untreated cells at MOI of 100; 5, Z-VAD-FMK-untreated cells at a MOI of 10; 6, uninfected cells.

infection and inhibits growth of *L. pneumophila* *in vivo* in the lung and *in vitro* [32]. As shown in Fig. 7a, TNF- α production was significantly increased in *L. longbeachae*-infected HL-60 macrophages. To determine whether TNF- α is involved in triggering the death signal in *L. longbeachae*-infected HL-60 macrophages, the cells were treated with anti-human TNF- α for 1 h before infection. TNF- α activity in the supernate of infected cells was inhibited by 95% up to 48 h after infection (Fig. 7a) and it was lower than in the supernate of uninfected cells. The addition of anti-human TNF- α did not inhibit apoptosis or necrosis of infected cells (Fig. 7b). The expression of Fas in infected cells was also examined by flow cytometry. A

slight increase of Fas was noted on the surface of infected cells only at a late stage of apoptosis (8.23% at 48 h after infection with a MOI of 100) (Fig. 7c). These results indicated that apoptosis of *L. longbeachae*-infected HL-60 macrophages does not seem to be dependent on TNF- α - and Fas-mediated pathways.

Discussion

Few studies have examined the virulence of *L. longbeachae*, an important causative organism of legionellosis [33]. *L. longbeachae* replicates within and is cytotoxic to macrophages [9, 34]. Several recent

Fig. 7. Induction of apoptosis in *L. longbeachae*-infected HL-60 macrophages is not mediated by TNF- α and FAS. (a) TNF- α concentration in cell culture supernates of uninfected cells ($\diamond$), infected cells ($\blacksquare$), and infected cells pretreated with anti-human TNF- α antibody ($\blacktriangle$). (b) Percentage of apoptotic cells. (c) Expression of Fas-positive cells. In (b) and (c), uninfected cells are represented by $\square$, cells infected at MOI of 100 by $\blacksquare$, and infected cells pretreated with anti-human TNF- α antibody by $\blacksquare$. Data are expressed as percentage means and SD of triplicate samples. Similar results were obtained in two independent experiments.

studies have investigated the mechanisms of cell death induced by *L. pneumophila* [24, 25, 35, 36]. Müller *et al.* [24] showed that *L. pneumophila* could induce apoptosis of HL-60 macrophages at 24 h and 48 h after infection. Furthermore, Gao *et al.* [25] reported that *L. pneumophila* induced apoptosis of macrophages and epithelial cells during the early stage of infection at MOIs of 0.5, 5 and 50. They concluded that induction of apoptosis by *L. pneumophila* correlated with cytotoxicity. However, the cytotoxicity and the mechanisms of host cell death due to infection by other *Legionella* spp. has not been well examined. The present study investigated whether *L. longbeachae* could multiply within human macrophages and could be cytotoxic, and whether *L. longbeachae* infection could induce apoptosis of macrophage-like HL-60 cells. Various criteria of apoptosis were used, including DNA fragmentation, flow cytometric analysis, and terminal deoxynucleotidyl transferase-mediated triphosphate (dUTP)-biotin nick end labelling (TUNEL; data not shown) to confirm for the first time that *L. longbeachae* induced apoptosis of these cells. *L. longbeachae* induced apoptosis after a few hours of infection at MOIs of 10 and 100. Viability of bacteria is also an essential feature of *L. longbeachae*-induced apoptosis of host cells. *Mip* mutant strains of *L. longbeachae* could induce apoptosis of macrophages, despite their defective intracellular replication. Recent studies have shown that *Mip* protein plays an important role in the intracellular life cycle of *L. longbeachae* and is required for full virulence [5]. However, the data from the present study suggested that *Mip* protein was not essential for the induction of apoptosis of macrophages. Gao *et al.* [25] examined 46 *pmi* (protozoan and macrophage infectivity loci) mutants of *L. pneumophila* that were defective, to various degrees, in intracellular replication within host cells. Among them, *dotA* and *icmWXYZ* mutants were completely defective in induction of apoptosis and cytotoxicity, suggesting that *dot* and *icm* express possible apoptosis-inducing factors [25]. Thus, further studies of the *dot* and *icm* homologues in *L. longbeachae* are warranted.

Several recent studies have demonstrated that apoptosis plays an important role in local immune responses against micro-organisms. Several pathogens, including *Sh. flexneri* [13], *Yersinia enterocolitica* [37], *Bordetella pertussis* [38], *A. actinomycetemcomitans* [17], *Listeria monocytogenes* [39], *M. tuberculosis* [20, 40] and *S. Typhimurium* [15], promote the destruction of monocytic phagocytes by apoptosis, to destroy the host immune system and promote the development of infection. On the other hand, some intracellular bacteria such as *M. bovis* [22], Chlamydiae [41], *Rickettsia rickettsii* and *Br. suis* [23], inhibit apoptosis to protect themselves from the host immune system, and to multiply in host cells. Gross *et al.* [23] suggested that intracellular bacteria use two strategies, the first is inhibition of apoptosis which enhances their survival

and replication within phagocytes, as well as their continued presence at the site of infection. Another group of bacteria including *L. pneumophila*, promotes apoptosis of phagocytes and finally reaches epithelial cells [25]. In this study, apoptosis induced by *L. longbeachae* did not inhibit bacterial multiplication. Further studies are necessary to examine bacterial growth in macrophages treated with various inhibitors of apoptosis.

Following confirmation of *L. longbeachae*-induced apoptosis, the mechanisms of this process, including the role of the caspase cascade were investigated. The caspase family of cysteine proteases is known to play important roles in regulation of apoptosis and the inflammatory response [42]. *Sh. flexneri*-induced apoptosis of macrophages not only permits bacterial survival but also triggers inflammation [43]. Invasion plasmid antigen B (Ipa B), which is secreted from *Sh. flexneri*, binds to caspase-1 (interleukin (IL)-1 β converting enzyme) and activates the enzyme. The activated enzyme converts pro IL-1 β to IL-1 β , and also allows magnification of the inflammation cascade [44]. *Y. enterocolitica*-induced apoptosis is independent of caspase-1, but a broad-spectrum caspase inhibitor still blocks the completion of the *Yersinia*-induced apoptotic programme [37]. However, *Chlamydia psittaci*-induced apoptosis is independent of either pro-apoptotic caspase-1 or caspase-3 [44]. In the present study, the broad-spectrum caspase inhibitor, Z-VAD-FMK, completely blocked induction of apoptosis of *L. longbeachae*-infected HL-60 macrophages. This result is similar to the findings in *L. pneumophila*-induced apoptosis in macrophages [25]. In this study, pro-inflammatory cytokines, such as TNF- α (Fig. 7a) and IL-1 β (data not shown), were released by *L. longbeachae*-infected macrophages. However, the role of these released cytokines in *L. longbeachae* infection remains to be determined. TNF- α is known to activate mononuclear phagocytes to inhibit intracellular parasites [45] and to potentiate the antimicrobial action of interferon (IFN)- γ [46]. Furthermore, *L. pneumophila* induces TNF- α secretion *in vitro* and *in vivo* [32], suggesting a potential role for this cytokine in host defence in legionellosis [47].

TNF- α and Fas are well known as common death signals, but other death signalling pathways have been reported [48, 49]. Apoptosis of *L. pneumophila*-infected monocytes is independent of the TNF- α -mediated pathway [36]. Host cell death by other organisms such as *C. psittaci* [44] and *Entamoeba histolytica* [50] does not involve Fas and TNF- α pathways. The results of the present study also suggested that *L. longbeachae*-induced apoptosis is independent of both TNF- α - and Fas-mediated pathways.

In conclusion, *L. longbeachae*-infected HL-60 cells undergo apoptosis during the early stages of infection

and induction of apoptosis plays an important role in cytotoxicity. Furthermore, induction of apoptosis of *L. longbeachae*-infected macrophages was mediated by activation of the caspase pathway but might be independent of TNF- α - and Fas-mediated signal transduction pathways. Further studies are necessary to determine the bacterial factor(s) that induce apoptosis, which is potentially involved in *L. longbeachae* pathogenesis.

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