**Legionella pneumophila** induces apoptosis via the mitochondrial death pathway

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**INTRODUCTION**

*Legionella pneumophila*, the causative agent of Legionnaires’ disease, has been shown to induce apoptosis within macrophages, monocyte cell lines and alveolar epithelial cells (Muller et al., 1996; Hagele et al., 1998; Gao & Abu-Kwaik, 1999a; Walz et al., 2000). A biphasic model was proposed by which *L. pneumophila* kills the host cell: during the early stages of infection, a low dose of *L. pneumophila* induces apoptosis, which is followed by rapid necrosis upon termination of intracellular bacterial replication due to the presence of a large number of post-exponential bacteria (Gao & Abu-Kwaik, 1999a). The mechanisms of *L. pneumophila*-associated apoptosis are not well understood. It has been demonstrated that induction of apoptosis is not growth-phase-regulated but dose-dependent; it also needs direct adherence of bacteria to host cells, but does not require intracellular bacterial replication or synthesis of new proteins (Gao & Abu-Kwaik, 1999a; Walz et al., 2000). Neither heat-killed bacteria nor bacterial culture supernatants induce apoptosis (Gao & Abu-Kwaik, 1999a). Moreover, the extent of apoptosis induced by a given species belonging to the genus *Legionella* correlates with its human prevalence — species most frequently associated with cases of human legionellosis, such as *L. pneumophila* and *Legionella longbeachae*, are able to induce apoptosis, whereas species less frequently associated with this disease do not induce apoptosis of monocytes (Walz et al., 2000). Muller et al. (1996) showed that neither the Mip protein, a major virulence factor of *L. pneumophila* which contributes to intracellular survival in eukaryotic cells

**Legionella pneumophila** has been shown to induce apoptosis within macrophages, monocyte cell lines and alveolar epithelial cells. The mechanisms and significance of *L. pneumophila*-associated apoptosis are not well understood. It has been speculated that *L. pneumophila* may induce apoptosis through ligation of death receptors by bacterial surface components or by secreted bacterial factors. Translocation of apoptotic factor(s) through the Dot/Icm secretion machinery followed by direct activation of caspases within the cytosol is discussed as another possible mechanism of apoptosis induction by *L. pneumophila*. Here, it is shown that *L. pneumophila* induced the mitochondrial release of cytochrome c in CD95 (Fas/Apo-1)-negative monocyte Mono Mac 6 cells, indicating that *Legionella*-induced apoptosis is mediated via the mitochondrial signalling pathway. In addition, blocking of the death receptor pathway at distinct stages using CD95-, FADD- or caspase-8-deficient Jurkat cells did not affect induction of apoptosis by *L. pneumophila*. Conversely, inhibition of the mitochondrial death pathway by overexpression of the anti-apoptotic protein Bcl-2 potently inhibited the processing of caspases and the induction of apoptosis. Therefore, these findings support a model in which the induction of apoptosis by *L. pneumophila* is mediated by activation of the intrinsic mitochondrial death pathway in the absence of external death receptor signalling.

**Keywords:** Legionnaires’ disease, macrophages, human monocytes

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**Abbreviations:** Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; FADD, Fas-associated death domain; MM6, Mono Mac 6; PARP, poly(ADP-ribose) polymerase; PBMC, peripheral blood mononuclear cell; sg., serogroup; Z-VAD-FMK, N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone.
(Cianciotto et al., 1990; Cianciotto & Fields, 1992), nor the major secretory protein Msp, which is reported to display cytotoxic and proteolytic activities (Quinn et al., 1989), have any influence on monocyte apoptosis. Furthermore, it became evident that Legionella-induced apoptosis does not require the TNF-α-mediated signal transduction pathway (Hagele et al., 1998).

Gao & Abu-Kwaik (1999b) have demonstrated that activation of caspase-3, but not caspase-1, is essential for apoptosis induced upon infection with L. pneumophila. However, the molecular mechanism that activates the death proteases remains unknown. Generally, two principal signalling pathways are known that lead to the apoptotic demise of the cell: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. Ligation of death receptors (such as CD95/Fas/Apo-1 or CD120a/TNF receptor-1) with their respective ligand or agonistic antibodies recruits the adapter protein FADD to the active trimeric death receptor. FADD in turn recruits and activates the death protease caspase-8 (Schulze-Osthoff et al., 1998). The mitochondrial death pathway is triggered by a number of apoptotic stimuli, such as anticancer drugs, staurosporine, cycloheximide, UV- and γ-irradiation (Ferrari et al., 1998; Bantel et al., 1999; Belka et al., 2000; Wesselborg et al., 1999). This pathway is initiated at the mitochondrial and is characterized by the mitochondrial release of cytochrome c. In the cytosol, cytochrome c together with dATP binds to the adapter protein Apaf-1, which enables the subsequent binding and activation of caspase-9. The mitochondrial apoptosis pathway can be inhibited by anti-apoptotic members of the Bcl-2 family that block the release of cytochrome c from the mitochondrion (Yang et al., 1997). Activation of both pathways via initiator-caspase-8 or -9 triggers an amplifying cascade of executioner caspases (as caspase-3, -6 and -7) that, after cleavage of vital death substrates, leads to the final demise of the cell.

In the case of apoptosis induction by L. pneumophila, three possible mechanisms for activation of the caspase cascade have been proposed: (i) translocation of apoptotic factor(s) through the Dot/Icm secretion machinery followed by caspase activation within the cytosol; (ii) secretion of bacterial apoptotic factor(s) in close proximity of attachment, which bind to death receptors; or (iii) translocation of bacterial apoptotic factor(s) to the bacterial surface followed by binding to a death receptor (Gao & Abu-Kwaik, 1999b).

In the present study, we show that induction of apoptosis by L. pneumophila does not involve an interaction between the organism and death receptors at the host-cell surface but that it is mediated via the mitochondrial signalling pathway.

METHODS

**Bacteria.** L. pneumophila serogroup (sg.) 1 strain Pontiac was isolated from a patient with severe Legionella pneumonia and was passaged less than three times on BCYE-α agar. The strain was kindly provided by Professor Dr G. Ruckdeschel. L. pneumophila sg. 1 strain Philadelphia-1 ATCC 33152 was isolated from human lung and underwent multiple passages on BCYE-α agar; it was obtained from the American Type Culture Collection (ATCC, Manassas, VA). L. pneumophila sg. 6 strain Heidelberg P10 was isolated from a patient with severe nosocomial Legionella pneumonia and was passaged less than three times on BCYE-α agar. The strain was kindly provided by Dr Christian Lueck (Kohler et al., 1999). Legionella micdadei and Legionella steigerwaltii (a human and an environmental isolate, respectively, passaged less than three times each on BCYE-α agar) were kindly donated by Dr Barry Fields.

All strains were grown on BCYE-α agar (Oxoid) at 37 °C in 5% CO₂ for 5 days.

**Cells**

Mono Mac 6 (MM6) cells. MM6 cells were kindly donated by Professor Dr H. W. L. Ziegler-Heitbrock. They were cultured as replicative non-adherent monocytes under lipopolysaccharide-free conditions in 75 cm² vented culture flasks (Costar) in 25 ml RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (Myocline super plus; Gibco), 2 mM l-glutamine (Gibco), 1% non-essential amino acids (Gibco) and OPI (150 µg oxaloacetate ml⁻¹, 50 µg pyruvate ml⁻¹ and 82 µg bovine insulin ml⁻¹; Sigma) (MM6 medium) at 37 °C in 5% CO₂ as described by Ziegler-Heitbrock et al. (1988) and diluted 1:3 twice a week in fresh medium.

Human peripheral blood mononuclear cells (PBMCs). PBMCs were prepared from buffy coats of citrate/phosphate/dextrose (CPD)-anticoagulated blood donations after informed consent of healthy blood donors and component production of plasma and red blood cell units (Baxter, Heidelberg, Germany) by using the Monocyte Isolation Kit (Miltenyi Biotec) according to the manufacturer’s instructions.

Jurkat cells. The CD95-negative Jurkat subline was generated by continuous culture of the parental cell line in the presence of an anti-CD95 mAb (1 µg IgG₃, ml⁻¹; BioCheck) for 6 months (kindly provided by Professor Schulze-Osthoff). The caspase-8-deficient and Fas-associated death domain (FADD) protein-negative Jurkat cells were derived from the parental Jurkat cell line by random mutagenesis as described previously and were kindly provided by J. Blenis (Juo et al., 1998, 1999). Stable transfecants of Jurkat cells overexpressing Bcl-2 and their vector-transfected parental cell line were generated as described previously (Belka et al., 2000). All Jurkat cell lines (human leukaemic T-cells) were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 10 mM HEPES (all from Gibco). Cells were grown at 37 °C in a 5% CO₂ atmosphere.

**Reagents.** The broad-range caspase inhibitor N-benzyloxy-carbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK) was purchased from Bachem. The chemotherapeutic drugs etoposide and mitomycin C were obtained from the clinical pharmacy (Medical Clinics, Tübingen, Germany). Mitomycin C was dissolved in methanol and etoposide was dissolved in ethanol; they were both kept as stock solutions at −70 °C. The agonistic anti-CD95 antibody (anti-CD95, IgG₃) was purchased from BioCheck.

**Intracellular multiplication.** Intracellular multiplication within monocytes or Jurkat cells was determined as described for MM6 cells (Walz et al., 2000). Briefly, 2 × 10⁵ cells were...
infected with $2 \times 10^9$ legionellae in 1.5 ml of culture medium without FCS in a well of a six-well tissue-culture plate (Nunc) to provide a bacterium-to-cell ratio of 100:1. After 2 h of co-incubation, non-phagocytized bacteria were killed by the addition of 100 µg gentamicin ml$^{-1}$ for 1 h at 37 °C in 5% CO$_2$. After three washes, the cells were distributed in 1 ml
Fig. 2. For legend see facing page.
The leakage of fragmented DNA from apoptotic nuclei was measured by the method of Nicoletti et al. (1991). Briefly, apoptotic nuclei were prepared by lysing cells in a hypotonic lysis buffer (1% sodium citrate, 0.1% Triton X-100, 50 µg propidium iodide ml⁻¹) and subsequently analysed by flow cytometry. Nuclei to the left of the 2N peak containing hypodiploid DNA were considered as apoptotic. Flow cytometric analyses were performed on a FACScan (Becton Dickinson) using CELLQUEST analysis software.

Alternatively, the Cell Death Detection ELISA system (Roche Applied Science) was used for the quantitative in vitro determination of cytoplasmic histone-associated DNA fragments, as described in the manufacturer’s instructions. The specific enrichment of mono- and oligo-nucleosomes released into the cytoplasm of MM6 or Jurkat cells indicating apoptotic cell death was calculated using the following formula, where mU is the absorbance value at 405 nm.

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\text{mU of infected or ActD-treated cells} - \text{mU of uninfected control cells} = \text{enrichment factor (EF)}
\]

An EF above 1 was indicative of apoptosis.

Fluorometric assay of caspase activity. Cytosolic cell extracts of monocytes or Jurkat cells were prepared by lysing cells in a buffer containing 0.5% Nonidet P-40 (NP-40), 20 mM HEPES (pH 7.4), 84 mM KCl, 10 mM MgCl₂, 0.2 mM EDTA, 0.2 mM ethylene glycol bis (2-aminoethyl)-tetraacetic acid (EGTA), 1 mM DTT, 5 µg aprotinin ml⁻¹, 1 µg leupeptin ml⁻¹, 1 µg pepstatin ml⁻¹ and 1 mM PMSF. Caspase activities were determined by incubation of cell lysates with 50 µM of the fluorogenic substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC; Bachem) in 200 µl buffer containing 50 mM HEPES (pH 7.3), 100 mM NaCl, 10% sucrose, 0.1% CHAPS and 10 mM DTT. The release of aminomethylcoumarin was measured by fluorometry using an excitation wavelength of 360 nm and an emission wavelength of 475 nm. Caspase activity was determined as the slope of the resulting linear regressions and expressed in arbitrary fluorescence units min⁻¹.

Immunoblotting. Cleavage of poly(ADP-ribose) polymerase (PARP) and caspases was detected by immunoblotting. Jurkat cells (2 × 10⁶) were washed in cold PBS and lysed in 1% Triton X-100, 50 mM Tris (pH 7.6) and 150 mM NaCl containing 3 µg aprotinin ml⁻¹, 3 µg leupeptin ml⁻¹, 3 µg pepstatin A ml⁻¹ and 2 mM PMSF. Subsequently, proteins were separated under reducing conditions by 8–15% gradient SDS-PAGE and electroblotted onto a PVDF membrane (Amersham Pharmacia Biotech). The loading and transfer of equal amounts of protein were confirmed by staining the membrane with Ponceau S. Membranes were blocked for 1 h with 5% non-fat dry milk powder in Tris-buffered saline (TBS) and then immunoblotted for 1 h with a rabbit anti-PARP polyclonal antibody (1:2000; Roche Applied Science) and caspase-9 (kindly provided by Y. A. Lazebnik) (Fearnhead et al. 1998), and mouse mAbs directed against caspase-8 (BioCheck) and caspase-3 (Transduction Laboratory, Heidelberg, Germany). Membranes were washed four times with TBS/0.02% Triton X-100 and incubated with the respective peroxidase-conjugated affinity-purified secondary antibody for 1 h. Following extensive washing, the reaction was developed by enhanced chemiluminescent staining using ECL reagents (Amersham Pharmacia Biotech).

Measurement of cytochrome c release. For analysis of cytochrome c release, 2 × 10⁶ MM6 or Jurkat cells infected with L. pneumophila were collected by centrifugation, washed with ice-cold PBS and resuspended in 5 vols of buffer A containing 250 mM sucrose, 20 mM HEPES (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 10 µg leupeptin ml⁻¹ and 10 µg aprotinin ml⁻¹. The cells were homogenized with 12 strokes in a douncer and the homogenates were centrifuged at 1000 g for 10 min at 4 °C to remove cell nuclei. The supernatants were transferred to a fresh tube and centrifuged at 10000 g for 10 min at 4 °C to deplete mitochondria. The resulting supernatants, designated as the cytosolic S10 fractions, from each sample were loaded onto a 15% SDS-polyacrylamide gel. Cytochrome c release was analysed by immunoblotting with the mouse mAb 7H8.2C12 (PharMingen).

Fig. 2. Inhibition of CD95 signalling does not affect Legionella-induced apoptosis and caspase activation. (a, b) Induction of apoptosis by different Legionella species in Jurkat cells. Parental or CD95-negative Jurkat cells were stimulated with different Legionella species, mitomycin C, etoposide or anti-CD95 as described in Fig. 1(a, b). Data are expressed as means ± SD of three independent experiments. Light-grey bars, parental; dark-grey bars, CD95-negative. (c) Processing of caspase-3, caspase-8, caspase-9 and the caspase-specific substrate PARP in immunoblot analysis. Parental and CD95-negative Jurkat cells were infected with L. pneumophila or treated with etoposide (25 µg ml⁻¹), mitomycin C (25 µg ml⁻¹) or anti-CD95 (1 µg ml⁻¹) for the indicated amount of time. Where indicated, cells were pre-incubated with the broad-spectrum caspase inhibitor Z-VAD-FMK (100 µM). Cellular proteins were resolved by SDS-PAGE and caspase activity was detected by cleavage of PARP, caspase-3, caspase-8 and caspase-9 using immunoblot analysis. Solid arrowheads indicate the uncleaved form and open arrowheads indicate the cleaved form of the respective protein. A representative of three independent experiments is shown. L1P, L. pneumophila sg. 1 strain Pontiac. (d) Caspase activity in parental (grey bars) and CD95-negative (solid bars) Jurkat cells was measured fluorometrically as described in Fig. 1(c). Mean values of duplicate experiments are shown. (e) The release of cytochrome c by parental Jurkat cells was determined as described in Fig. 1(d). A representative experiment is shown.

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RESULTS

Multiplication of Legionella species and induction of apoptosis in human monocytic cells

Infection of MM6 cells and human PBMCs with different Legionella species resulted in varying degrees of apoptosis, as documented by DNA fragmentation. In general, the fresh clinical isolate L. pneumophila sg. 1 Pontiac, which was highly replicative (3–4 log within 72 h; data not shown), was most potent in inducing apoptosis (Fig. 1a, b). The two other strains of L. pneumophila tested as well as L. micdadei showed intracellular growth of about 1 log (data not shown) and were also able to induce programmed cell death, although to a lesser extent (Fig. 1a, b). L. steigerwaltii was not able to replicate within MM6 cells or PBMCs and did not cause apoptosis (Fig. 1a, b). In addition, L. pneumophila sg. 1 Pontiac was able to cleave the fluorogenic caspase substrate Ac-DEVD-AMC (Fig. 1c) and to induce the release of cytochrome c from MM6 cells (Fig. 1d). It should be noted that, in contrast to PBMCs, MM6 cells did not show apoptosis (Fig. 1a, b) or release of cytochrome c (Fig. 1d) after stimulation with anti-CD95, due to the lack of CD95 expression (B. Neumeister et al., unpublished observation), while the extent of apoptosis induced by bacteria and anticancer drugs did not differ between the PBMCs and the MM6 cells (Fig. 1a, b).

Multiplication of Legionella species and induction of apoptosis in Jurkat T-cells

In contrast to their strong multiplication within human monocytes, the three strains of L. pneumophila showed a weak intracellular growth of less than 1 log within all of the Jurkat cell lines tested (data not shown). The two non-L. pneumophila species tested (i.e. L. micdadei and L. steigerwaltii) did not possess intracellular replication (data not shown). Nevertheless, infection of the human leukaemic T-cell line Jurkat resulted in levels of apoptosis comparable to those of monocytic cells (Figs 2–4). This indicates that Legionella-induced apoptosis is not restricted to cells of monocytic origin but can also be triggered in human T lymphocytes independent of the extent of intracellular replication.

Ligation of CD95 (Fas/Apo-1) is not necessary for apoptosis induced by Legionella

The induction of mitochondrial release of cytochrome c in CD95-negative MM6 cells by L. pneumophila (Fig. 1e) indicates that death receptor signalling might not be involved in Legionella-induced apoptosis. To further determine whether apoptosis induced by L. pneumophila involves ligation of CD95, we compared the levels of apoptosis induced by several strains and species of the genus Legionella in parental Jurkat cells with the levels of apoptosis induced in CD95-negative Jurkat cells. Infection of CD95-negative Jurkat cells resulted in levels of host-cell apoptosis similar to those of the parental cell line as revealed by both enrichment of histone-associated DNA fragments (Fig. 2a) and assessment of hypodiploid apoptotic nuclei (Fig. 2b). Since L. pneumophila sg. 1 Pontiac was the most potent inducer of apoptosis in both cell lines, we used this strain to further investigate the involvement of caspases in Legionella-associated apoptosis by measuring the cleavage of known caspase substrates in immunoblot analysis. As shown in Fig. 2(c), the caspase-specific substrate PARP was cleaved into the characteristic 85 kDa fragments in both parental and CD95-negative Jurkat cells after infection with L. pneumophila within the same time range. In addition, the initiator caspases, caspase-8 and -9, as well as the executioner caspase-3 were all processed in parental and CD95-negative Jurkat cells after infection with L. pneumophila to the same extent (Fig. 2c). Since the analysis by Western blotting allowed us to examine the processing of caspases but did not indicate whether the cleavage products were enzymically active, caspase activity was determined by cleavage of the fluorogenic substrate Ac-DEVD-AMC in a fluorometric assay. Again, no difference in caspase activity between parental and CD95-negative Jurkat cells could be detected (Fig. 2d). Since L. pneumophila induces similar amounts of apoptosis and caspase activation in CD95-resistant and CD95-sensitive host cells, ligation of CD95 seems not to be involved in the induction of programmed cell death by Legionella infection. This hypothesis is further supported by the fact that L. pneumophila was able to induce cytochrome c release in CD95-negative MM6 cells (Fig. 1d) as well as in Jurkat parental cells (Fig. 2e).

L. pneumophila does not induce apoptosis by stimulation of other death receptors

The preceding experiments have shown that the ligation of CD95 is not a prerequisite for apoptosis induced by L. pneumophila. Consequently, we investigated the involvement of other death receptors in apoptosis. Since all known death receptors mediate apoptosis via the adapter protein FADD, we used a FADD-deficient Jurkat cell line to investigate whether death receptors other than CD95 are involved in Legionella-associated apoptosis. In addition, caspase-8-deficient Jurkat cells were used to investigate whether the initiator caspase of the death-receptor-dependent activation pathway is involved in Legionella-associated apoptosis. As previously shown, the absence of FADD and caspase-8 protected Jurkat cells from anti-CD95-induced apoptosis but not from anticancer-drug-mediated apoptosis (Engels et al., 2000; Juo et al., 1998, 1999), whereas infection of host cells with different Legionella species induced comparable levels of apoptosis in parental and FADD-deficient host cells (Fig. 3a). Western-blot analysis revealed that processing of caspases after infection with L. pneumophila did not differ between parental and FADD-deficient cells (Fig. 3b). In addition, caspase-8 was processed in FADD-deficient cells after infection with L. pneumophila. As expected, caspase-8 was not processed in caspase-8-deficient cells due to the genetic deletion of this enzyme. Although caspase-8 was absent...
from these cells, PARP and caspase-3 were processed, indicating an alternative pathway of apoptosis induction by *L. pneumophila*. Caspase activity, as determined by cleavage of Ac-DEVD-AMC, did not reveal differences between parental and FADD-deficient cells after infection with *L. pneumophila* (Fig. 3c). The reduced caspase activity in caspase-8-deficient Jurkat cells indicates that caspase-8 might contribute to *Legionella*-induced apoptosis in a death-receptor-independent way. In this context, we have previously demonstrated that in the mitochondrial death pathway, caspase-8 is cleaved downstream of caspase-9 and might serve as an amplification loop (Engels et al., 2000).

### Induction of apoptosis by *L. pneumophila* can be inhibited by the overexpression of Bcl-2 in Jurkat host cells

The preceding experiments have shown that apoptosis induced by *L. pneumophila* is independent of death receptor signalling. To address the question as to whether *L. pneumophila* induces apoptosis via the mitochondrial pathway, we infected Jurkat cells stably overexpressing Bcl-2 or the vector alone with several strains and species of the genus *Legionella*. In vector control cells, mainly *L. pneumophila* was able to induce apoptosis (Fig. 4a, b). The level of apoptosis was substantially reduced in Jurkat cells overexpressing Bcl-2 (Fig. 4a, b). Induction of apoptosis by *L. pneumophila* in the vector control cells was paralleled by activation of caspases, as demonstrated by proteolytic processing of caspase-3, caspase-8, caspase-9 and PARP (Fig. 4d) as well as fluorometric measurements of the cleavage of Ac-DEVD-AMC (Fig. 4e). Conversely, blocking of the mitochondrial pathway by Bcl-2 completely abrogated the activation of caspases, as demonstrated by immunoblot analysis and fluorometry (Fig. 4d, e). As a control, cells were stimulated via the death receptor pathway with anti-CD95 or via the mitochondrial pathway using etoposide and mitomycin C (Fig. 4c). As demonstrated previously, overexpression of Bcl-2 only affects activation of the mitochondrial cytochrome c/Apaf-1 pathway by anticancer drugs but has no or little effect on death receptor signalling (Engels et al., 2000). Accordingly, induction of apoptosis and activation of caspases by mitomycin C and etoposide was completely blocked by the overexpression of Bcl-2, whereas CD95 signalling was unaffected or only partially reduced by overexpression of Bcl-2 (Fig. 4a–c).

Taken together, the results from this study showed that despite differences in intracellular bacterial replication, the levels of apoptosis, the degrees of caspase activity and the degrees of cytochrome c release induced by legionellae were similar in monocytes and lymphocytic host cells. In addition, no differences could be observed between CD95-, FADD- and caspase-8-deficient cells when compared to their parental Jurkat cells. Conversely, overexpression of Bcl-2 inhibited the induction of apoptosis and processing of caspases. We therefore conclude that legionellae induce apoptosis via the
Fig. 4. For legend see facing page.
mitochondrial death pathway rather than via ligation to death receptors.

**DISCUSSION**

Modulation of apoptosis is a widely used strategy among extra- and intra-cellular bacteria but the exact mechanisms by which bacteria are able to affect the endogenous suicide programme still remain largely unresolved. The pro-apoptotic arsenal of bacteria comprises direct activation of caspase-1 by bacterial toxins such as IpAB of *Shigella flexneri* or SipB of *Salmonella enterica* serovar Typhimurium (Hilbi et al., 1998; Hersh et al., 1999), and the action of pore-forming factors such as the α-toxin of *Staphylococcus aureus* (Jonas et al., 1994), PorB of *Neisseria gonorrhoeae* (Muller et al., 1999), the lipopeptide haemolysin of *Burkholderia cepacia* (Hutchison et al., 1998) and the leukotoxin of *Pasteurella haemolytica* (Stevens & Czuprynski, 1996), which induce apoptosis by a still unknown mechanism. Other known mechanisms involved in apoptosis induction by bacteria are inhibition of the NF-κB-mediated pathways by YopP/J of *Yersinia* spp. (Palmer et al., 1998; Schemer et al., 1998), upregulation of CD95 at the surface of host cells by *Pseudomonas aeruginosa* (Jendrossek et al., 2001) and *Helicobacter pylori* (Wang et al., 2000), release of CD95L after phagocytosis of *Staphylococcus aureus* (Baran et al., 2001) and down-regulation of anti-apoptotic proteins, such as Bcl-2, by mycobacteria (Klingler et al., 1997). However, some bacterial pathogens, such as low numbers of *Mycobacterium tuberculosis* (Durrbaum-Landmann et al., 1996), *Brucella suis* (Gross et al., 2000), the human granulocytic ehrlichiosis agent (Yoshiie et al., 2000) and *Chlamydia pneumoniae* (Rajalingam et al., 2001), are able to prevent spontaneously occurring or induced apoptosis of host cells.

We have previously described that members of the genus *Legionella* induce apoptosis in the human monocytic cell line MM6 (Walz et al., 2000). We observed that the level of apoptosis was dose- and virulence-dependent and that apoptosis was triggered by direct adherence of bacteria to the host cell rather than by intracellular multiplication. Gao & Abu-Kwaik (1999b) demonstrated that activation of caspase-3, but not caspase-1, is essential for apoptosis induced upon infection by *L. pneumophila*, but the initial events of caspase induction remained unclear. Here, we have shown that members of the genus *Legionella* induce a comparable extent of apoptosis in CD95-positive (PBMC) and CD95-negative (MM6) human mononuclear cells. The apoptotic death of MM6 cells induced by *L. pneumophila* was accompanied by caspase activation and mitochondrial cytochrome c release. This suggested that apoptosis of human mononuclear cells induced by legionellae is independent of the interaction between bacteria or bacterial products with the death receptor CD95.

Since appropriate mononuclear cells are not available, we used Jurkat cell lines deficient in the expression of CD95, the adaptor molecule FADD or caspase-8 and stable transfectants overexpressing the death antagonist Bcl-2 to investigate whether *Legionella* induces apoptosis via the death-receptor-dependent pathway or the mitochondrial apoptosis pathway. Although the *Legionella* species under investigation have shown only modest intracellular multiplication in Jurkat cells in comparison to their behaviour in monocytes, they were able to induce apoptosis within these T-cell lines. This again supports the previous results that attachment rather than intracellular replication within host cells is necessary for apoptosis induction by *Legionella* (Walz et al., 2000). As we have already demonstrated for monocytes (Walz et al., 2000), a fresh isolate of *L. pneumophila* sg. 1 also induced the highest levels of apoptosis in Jurkat cells. Since we could not observe differences in the level of apoptosis and caspase activation between parental cells and CD95-negative, FADD-deficient or caspase-8-deficient Jurkat cells, ligation of death receptors by bacterial apoptotic factor(s) can be excluded. Conversely, blocking of the mitochondrial death pathway by overexpression of the anti-apoptotic protein Bcl-2 abrogated the activation of caspases and induction of apoptosis by legionellae. Therefore, we conclude that apoptosis induced by *Legionella* is mediated via the intrinsic mitochondrial death pathway and does not rely on external death receptor signalling. Although we could not completely rule out that death receptors might be involved in the induction of apoptosis, we could demonstrate that death receptors are not a prerequisite for *Legionella*-induced apoptosis. Interestingly, caspase-8, the major initiator caspase of the death receptor pathway, was also activated by legionellae in CD95- and FADD-deficient Jurkat cells. In this context, we have previously demonstrated that anticancer drugs induce the processing of caspase-8 via the mitochondrial death pathway in the absence of death receptor signalling (Ferrari et al., 1998; Wesselsborg et al., 1999). In addition, we and others have shown that caspase-8 is not a prerequisite for anticancer-drug-induced apoptosis and...
that in the mitochondrial death pathway, caspase-8 is processed downstream of caspase-9 and caspase-3 (Chinnaiyan et al., 1997; Slee et al., 1999; Engels et al., 2000). However, activation of caspase-8 might constitute an amplification loop via the BID-mediated activation of the mitochondrial death pathway (Engels et al., 2000; Slee et al., 2000). Thus, the reduced caspase-3-like activity in caspase-8-deficient Jurkat cells (Fig. 3b, c) might be explained by the absence of this mitochondrial amplification loop. Since overexpression of Bcl-2 prevented the processing of caspase-8 (as well as that of caspase-3 and -9) (Fig. 4d), we presume that caspase-8 does not play an essential role in Legionella-induced apoptosis. However, since the Legionella-mediated caspase activity was decreased in caspase-8-deficient Jurkat cells in contrast to FADD-deficient or parental cells (Fig. 3c), mitochondrially processed caspase-8 might contribute to the extent of Legionella-induced apoptosis.

The bacterial factor(s) of L. pneumophila that are responsible for the activation of the mitochondrial pathway have yet to be determined. In contrast to the situation in Chlamydia trachomatis (Jendro et al., 2000), cell-free supernatant of macrophages or Jurkat cells infected with L. pneumophila sg. 1 Pontiac could not induce apoptosis in Jurkat cells (data not shown). This supports the hypothesis that contact-mediated export of a bacterial factor after attachment of bacteria to host cells may play a role in apoptosis induction (Gao & Abu-Kwaik, 1999a). It has been shown that expression and/or export of the apoptosis-inducing factor(s) of L. pneumophila is regulated by the icm/dot virulence system, since icm/dot mutants are defective in apoptosis induction (Gao & Abu-Kwaik, 1999a, b; Zink et al., 2002). Prospective studies have to clarify which of the gene products are associated with apoptosis induction. In N. gonorrhoeae, a porin (PorB) is transported to the mitochondria of infected cells and the subsequent apoptosis can be blocked by overexpression of Bcl-2 (Muller et al., 2000). Since L. pneumophila is able to insert pores of less than 3 nm in diameter into the plasma membrane of host cells using the dot/icm transport machinery, the dot/icm complex may not only play a role in altering endocytic trafficking events leading to the establishment of a replicative vacuole (Kirby et al., 1998) but may also be associated with Legionella-induced apoptosis. Whether induction of apoptosis is a prerequisite for the alteration of the specialized Legionella phagosome remains to be determined.

The biological plausibility of apoptosis of the host cells induced by legionellae is unclear. Modulation of apoptosis is often a prerequisite to the establishment of a host–pathogen relationship. Apoptotic bodies have been shown to have an immunosuppressive effect by enhancing the production of the anti-inflammatory cytokine IL-10 and reducing the secretion of the pro-inflammatory cytokines TNF-α, IL-1β and IL-12 (Voll et al., 1997). Bacterial induction of apoptosis offers an advantage for attachment of enterohaemorrhagic Escherichia coli by augmenting outer leaflet levels of the phosphatidylethanolamine receptor (Barnett-Foster et al., 2000). In vivo, apoptosis of macrophages induced by YopJ facilitates the establishment of a systemic infection in mice (Monack et al., 1998). However, apoptosis may also augment the antimicrobial immune response. Internalization of apoptotic macrophages infected with Salmonella typhimurium results in the presentation of bacterial antigenic epitopes after internalization by dendritic cells (Yrlid & Wick, 2000). Thus, further experiments are necessary to characterize the physiological function of apoptosis during Legionella infection.

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