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Anti-apoptotic signalling by the Dot/Icm secretion system of *L. pneumophila*

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Summary

The Dot/Icm type IV secretion system of *Legionella pneumophila* triggers robust activation of caspase-3 during early and exponential stages of proliferation within human macrophages, but apoptosis is delayed till late stages of infection, which is novel. As caspase-3 is the executioner of the cell, we tested the hypothesis that *L. pneumophila* triggers anti-apoptotic signalling within the infected human macrophages to halt caspase-3 from dismantling the cells. Here we show that during early and exponential replication, *L. pneumophila*-infected human monocyte-derived macrophages (hMDMs) exhibit a remarkable resistance to induction of apoptosis, in a Dot/Icm-dependent manner. Microarray analyses and real-time PCR reveal that during exponential intracellular replication, *L. pneumophila* triggers upregulation of 12 anti-apoptotic genes that are linked to activation of the nuclear transcription factor kappa-B (NF-κB). Our data show that *L. pneumophila* induces a Dot/Icm-dependent sustained nuclear translocation of the p50 and p65 subunits of NF-κB during exponential intracellular replication. Bacterial entry is essential both for the anti-apoptotic phenotype of infected hMDMs and for nuclear translocation of the p65. Using p65$^{-/-}$ and IKKα$^{-/-}$ β$^{-/-}$ double knockout mouse embryonic fibroblast cell lines, we show that nuclear translocation of NF-κB is required for the resistance of *L. pneumophila*-infected cells to apoptosis-inducing agents. In addition, the *L. pneumophila*-induced nuclear translocation of NF-κB requires the activity of IKKα and/or IKKβ. We conclude that although the Dot/Icm secretion system of *L. pneumophila* elicits an early robust activation of caspase-3 in human macrophages, it triggers a strong anti-apoptotic signalling cascade mediated, at least in part by NF-κB, which renders the cells refractory to external potent apoptotic stimuli.

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Introduction

Upon entering macrophages, *Legionella pneumophila* diverts its phagosome from the 'default' endosomal-lysosomal pathway to form a rough endoplasmic reticulum (ER)-derived replicative niche (Segal *et al*., 1998; Vogel *et al*., 1998; Kagan and Roy, 2002; Roy, 2002). Evasion of the endosomal-lysosomal pathway and biogenesis of the *Legionella*-containing phagosome (LCP) are thought to be mediated by bacterial effectors exported into the host cell through the Dot/Icm secretion system (Nagai *et al*., 2002; Conover *et al*., 2003; Luo and Isberg, 2004). However, activation of human macrophages by IFN-γ results in the maturation of the LCP into phagolysosome, a process that inhibits intracellular replication of *L. pneumophila* (Santic *et al*., 2005). During late stages of intracellular replication, the LCP membrane becomes disrupted and the bacteria escape into the cytoplasm where they continue their terminal rounds of proliferation (Molmeret and Abu Kwaik, 2002; Molmeret *et al*., 2004a,b).

Numerous pathogens modulate the host apoptotic pathways by distinct mechanisms that trigger the extrinsic or intrinsic pathways of apoptosis; both of which converge on the activation of caspase-3, which is the executioner of apoptosis (Gao and Abu Kwaik, 2000a; Wang *et al*., 2005). Under low multiplicity of infection (moi), *L. pneumophila* induces a Dot/Icm-dependent activation of caspase-3 during early stages of infection independent of the classical extrinsic and intrinsic signalling pathways of apoptosis, but high moi triggers the intrinsic pathway (Muller *et al*., 1996; Gao and Abu Kwaik, 1999; Neumeister *et al*., 2002; Zink *et al*., 2002; Molmeret *et al*., 2004b; Abu-Zant *et al*., 2005; Fischer *et al*., 2006). Early activation of caspase-3 by *L. pneumophila* in human macrophages has been shown to be required for evasion of the endosomal-lysosomal pathway (Molmeret *et al*., 2004b). In contrast, in mice macrophages, *L. pneumophila* flagellin triggers caspase-1-mediated immediate pyropoptosis, but caspase-3 is not required for intracellular replication (Molofsky *et al*., 2006; Ren *et al*., 2006; Zamboni *et al*., 2006). Other findings have also shown crucial differences

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in trafficking of *L. pneumophila* in mice versus human quiescent macrophages (Sturgill-Koszycki and Swanson, 2000; Sauer *et al*., 2005). Trafficking of *L. pneumophila* in IFN-γ-activated human and mice macrophages is also very different (Neild *et al*., 2005; Santic *et al*., 2005).

Despite caspase-3 activation by *L. pneumophila* during early and exponential phases of intracellular replication in human macrophages, apoptosis is not triggered till late stages of infection concomitant with termination of intracellular replication (Abu-Zant *et al*., 2005). The delay of apoptosis in *L. pneumophila*-infected macrophages despite caspase-3 activation, suggests that *L. pneumophila* may modulate anti-apoptotic signalling, because induction of apoptosis terminated intracellular replication (Abu-Zant *et al*., 2005).

Apoptosis is regulated by several pro- and anti-apoptotic proteins (Dejean *et al*., 2005; Lucken-Ardjomande and Martinou, 2005). The Bcl-2 family proteins include both pro-apoptotic and anti-apoptotic proteins that regulate the release of cytochrome *c* from the mitochondria (Chao and Korsmeyer, 1998; Dejean *et al*., 2005). The inhibitor of apoptosis proteins (IAPs) regulate apoptosis through the inhibition of both initiator and effector caspases (Salvesen and Duckett, 2002; Shi, 2002). The X-linked inhibitor of apoptosis protein (XIAP) is a potent inhibitor of caspase-3, 7 and 9 (Chai *et al*., 2001; Riedl *et al*., 2001; Shiozaki *et al*., 2003). Both c-IAP1 and c-IAP2 bind and inhibit caspase-3, -7 and -9 (Roy *et al*., 1997; Deveraux *et al*., 1998). The expression of many anti-apoptotic genes including *bcl-2*, *xiap*, *c-iap-1* and *c-iap-2,* is regulated by the nuclear transcription factor kappa-B (NF-κB) (Stehlik *et al*., 1998; Wang *et al*., 1998; Catz and Johnson, 2001).

The transcription factor NF-κB plays crucial role in regulation of apoptosis (Burstein and Duckett, 2003). NF-κB represents a family of homo- and heterodimer transcription factors that are assembled from five structurally related proteins that include p65 (RelA), RelB, c-Rel, NF-κB-1 (p50) and NF-κB-2 (p52). The p65/p50 heterodimer is the most predominant active complex in mammalian cells (Ghosh and Karin, 2002). In resting cells, NF-κB proteins are predominantly sequestered in the cytoplasm by NF-κB inhibitory proteins (IκBs) (Karin and Ben-Neriah, 2000). Crucial to the activation and nuclear translocation of NF-κB, is the IκB kinase (IKK)-mediated phosphorylation, followed by ubiquitination, and proteosomal degradation of IκBs (Karin and Ben-Neriah, 2000; Senftleben and Karin, 2002). The IKK complex consists of the regulatory subunit NF-κB essential modulator (NEMO/IKKγ), and two IKKs, IKKα and IKKβ (Li *et al*., 1999; Coope *et al*., 2002; Dejardin *et al*., 2002; Ghosh and Karin, 2002; Kato *et al*., 2003). Several pathogens have been shown to promote or interfere with apoptosis through the inhibition or activation of NF-κB by targeting NF-κB through type III or type IV secretions systems (Orth *et al*., 1999; Gao and Kwaik, 2000; Tato and Hunter, 2002; Molestina *et al*., 2003; Joshi *et al*., 2004; Schmid *et al*., 2004; Kempf *et al*., 2005).

In this study, we show that despite the documented early robust activation of caspase-3, *L. pneumophila*-infected primary human macrophages are resistant to apoptosis when triggered by potent apoptosis-inducing agents during the exponential phase of intracellular replication. *L. pneumophila*-infected macrophages up-regulate the expression of 12 anti-apoptotic genes. Our data reveal that, *L. pneumophila* induces a sustained Dot/Icm-dependent activation of NF-κB that is, at least in part, required for the exhibition of anti-apoptotic phenotypes in infected cells. Although sustained nuclear translocation of NF-κB by *L. pneumophila* does not require the IKK complex, it requires the functional activity of host cell IKKβ and/or IKKα.

Results

Legionella pneumophila*-infected macrophages are resistant to apoptosis-inducing agents*

Legionella pneumophila induces robust activation of caspase-3 during early and exponential phases of intracellular replication, but the infected human macrophages do not undergo apoptosis until late stages of infection concomitant with termination of intracellular replication (Molmeret *et al*., 2004b; Abu-Zant *et al*., 2005). As caspase-3 is the executioner of the cell, we hypothesized that *L. pneumophila* triggers anti-apoptotic mechanisms to halt human macrophages from committing suicide during exponential intracellular replication. To test this hypothesis, we examined the sensitivity of *L. pneumophila*-infected macrophages to potent apoptosis-inducing agents during the exponential phase of intracellular replication. We utilized the two apoptosis-inducing agents, Staurosporin and TNF-α. Staurosporin activates caspase-3 through both the extrinsic and intrinsic pathways of apoptosis (Bertrand *et al*., 1994). Monolayers of human monocyte-derived macrophages (hMDMs) were infected with either the parental strain AA100 or the *dotA* mutant strain expressing green fluorescent protein (GFP) at a moi of 10 for 1 h, followed by 1 h of gentamicin treatment, which resulted in infection of ~35% of the cells. At 8 h after infection, monolayers were left untreated or treated with 1.0, 0.5 or 0.25 μM Staurosporin for 4 h, followed by TUNEL assays for detection of apoptotic nuclei. Single cell analysis by laser scanning confocal microscopy revealed that in uninfected cells there was a dose-dependent increase of apoptosis where ~83% of the cells became apoptotic in response to 1.0 μM of Staurosporin (Fig. 1A). Similar to uninfected cells,

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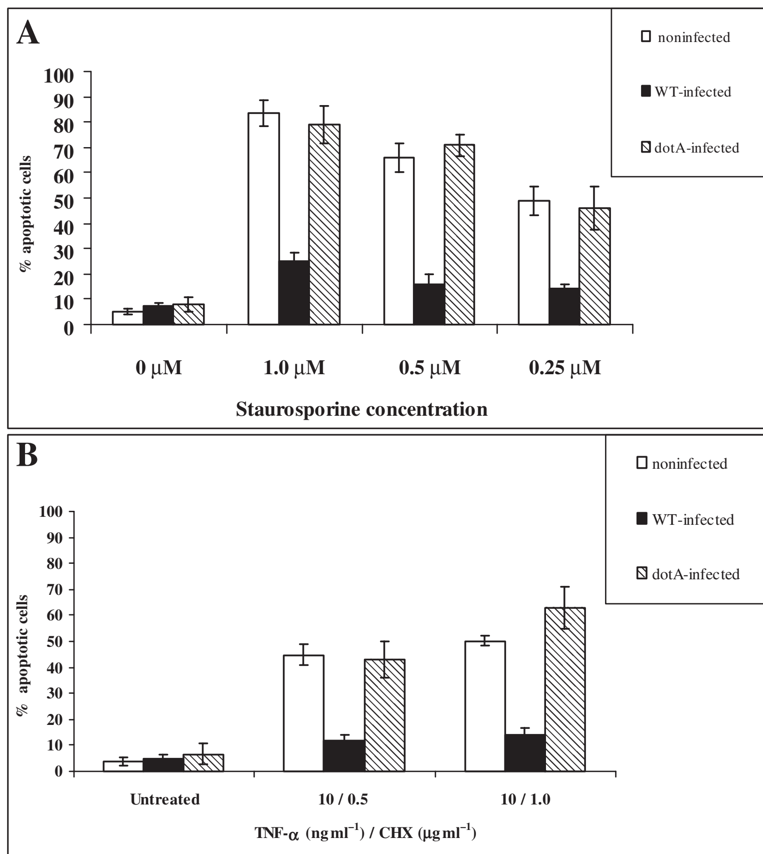

Fig. 1. *L. pneumophila*-infected macrophages are resistant to apoptosis-inducing agents. Monolayers of hMDMs were infected with either the parental strain AA100-GFP or the *dotA*-GFP mutant strain using a moi of 10 for 1 h. At 8 h after infection, monolayers were treated with different concentrations of staurosporine (A) or TNF-α and CHX (B) and labelled by TUNEL. At least 100 cells were scored from different coverslips. The results are representative of two independent experiments and error bars represent standard deviations.

~80% of *dotA* mutant-infected macrophages, treated with 1.0 μM of Staurosporin became apoptotic (Fig. 1A). Interestingly, only 25% of wild-type (WT) *L. pneumophila* strain AA100-infected cells treated with 1.0 μM Staurosporin became apoptotic, which was significantly different from *dotA*-infected or uninfected cells ($P < 0.01$, Student's *t*-test) (Fig. 1A). In untreated cells, 5–8% of AA100-infected, *dotA*-infected and uninfected macrophages were apoptotic (Fig. 1A). However, by 16 h post infection, more than 90% of Staurosporin-treated uninfected or AA-100-infected cells became apoptotic, which is consistent with previous observations (data not shown) (Abu-Zant *et al.*, 2005). In contrast, less than 10% of untreated uninfected or *dotA*-infected cells became apoptotic at 16 h post infection (data not shown). To further confirm our observation that *L. pneumophila*-infected human macrophages were resistant to the induction of apoptosis during the exponential phase of intracellular replication, we utilized TNF-α, which activates caspase-3 through the extrinsic pathway of apoptosis (Baud and Karin, 2001). At 8 h after infection, uninfected, AA100-infected and *dotA*-infected hMDMs monolayers were either left untreated or treated with TNF-α (10 ng ml^{-1}) together with either 0.5 or 1 μg ml^{-1} Cycloheximide (CHX). Single cell analysis by laser scanning confocal microscopy revealed that ~55% of treated-uninfected and *dotA*-infected macrophages were apoptotic (Fig. 1B). However, < 10% of TNF-α-treated AA100-infected macrophages were apoptotic, which was significantly different from *dotA*-infected or uninfected cells ($P < 0.01$, Student's *t*-test) (Fig. 1B). In untreated cells, 4–8% of uninfected, AA100-infected and *dotA* mutant-infected cells were apoptotic (Fig. 1B). These data clearly showed that *L. pneumophila*-infected macrophages were resistant to potent apoptosis-inducing agents that trigger apoptosis through the extrinsic and intrinsic pathways of apoptosis. We conclude that *L. pneumophila*-infected macrophages are resistant to the induction of apoptosis during the exponential phase of intracellular replication and this process is Dot/Icm-dependent.

To examine whether bacterial entry was essential for the anti-apoptotic phenotype of human macrophages, we infected hMDMs and treated them with Staurosporin exactly as described above, with the exception that the infection was performed in presence of cytochalasin D to block bacterial entry, as we described previously (Gao *et al.*, 1997). The data showed that bacterial entry was essential for the induction of the anti-apoptotic phenotype of human macrophages, because all cells were equally susceptible to Staurosporin-induced apoptosis (data not shown).

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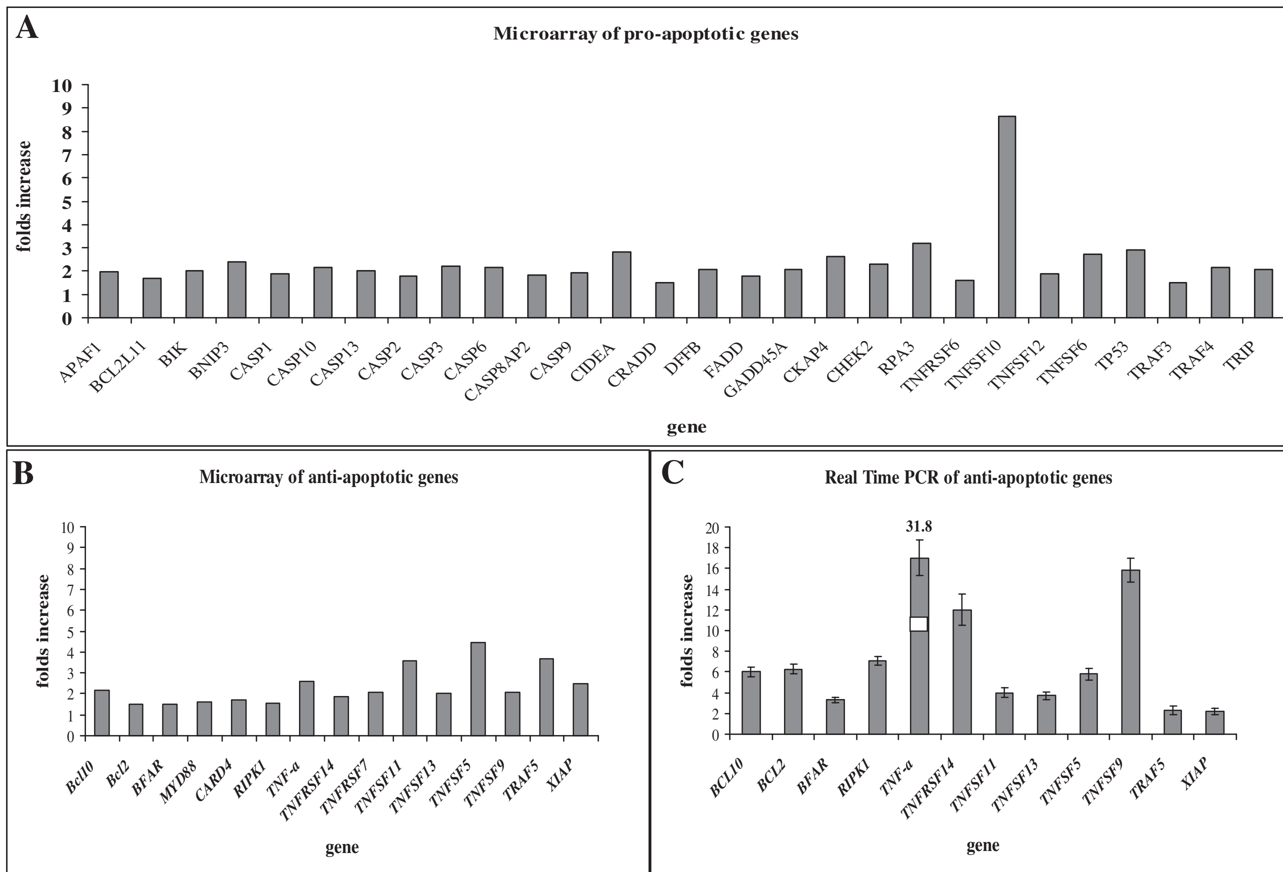

Fig. 2. *L. pneumophila*-infected macrophages upregulate pro-apoptotic and anti-apoptotic genes.
A and B. Monolayers of hMDMs were infected with either the parental strain AA100 or the *dotA* mutant. At 8 h after infection, total RNA was extracted and used to probe GEArray microarrays. The data are representative of two experiments. Expression values are expressed as ratios of infected cells to uninfected cells. A cut-off ≥ 1.5-fold increase was used as an indication for upregulation of gene expression.
C. Upregulation of most of the anti-apoptotic genes in (B) as confirmed by real-time PCR using specific set of primers. The results are representative of three independent experiments and error bars represent standard deviations.

Legionella pneumophila *induces the expression of pro-apoptotic and anti-apoptotic genes*

As *L. pneumophila*-infected macrophages were resistant to the induction of apoptosis, we tested the hypothesis that *L. pneumophila* triggered upregulation of anti-apoptotic mechanisms. We examined the expression profile of 96 pro- and anti-apoptotic genes in *L. pneumophila*-infected hMDMs during the exponential phase of intracellular replication using GEArray microarrays. Monolayers of hMDMs were either left uninfected or infected with the parental strain AA100 or the *dotA* mutant strain, using a moi of 50 for 15 min. There is no detectable toxicity to the cells under these experimental conditions, as determined by Trypan Blue exclusion. Infection of macrophages was followed by 3× washing and 1 h of gentamicin treatment to remove extracellular bacteria. This infection protocol resulted in 30–40% of the cells getting infected. At 2 and 8 h post infection, total hMDMs RNA was extracted and used to synthesize Biotin-labelled cDNA, which was used for hybridization of GEArrays. We decided to use 1.5-fold increases as a cut-off value for upregulation of gene expression in our microarray screen. This cut-off was used to ensure detection of slight changes detected by this screen, which will be validated later. We thought the 1.5 cut-off was essential, because only 30–40% of the cells were infected. The expression levels in AA100-infected or *dotA* mutant-infected hMDMs were expressed as ratios compared with uninfected cells. The data showed that by 2 h after infection, there were no detectable changes in the infected cells (WT or *dotA* mutant) compared with uninfected controls (data not shown). The data showed that by 8 h after infection, hMDMs infected with the parental strain AA100 showed slight upregulation (1.5–2-fold) in the expression of 28 pro-apoptotic), with the exception of TNFSF10, which was upregulated by approximately ninefold (Fig. 2A). There was also strong and significant upregulation of 15 anti-apoptotic genes when compared with uninfected cells (Fig. 2A and B and Table 1). Compared

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Table 1. The anti-apoptotic genes upregulated in *L. pneumophila* AA100-infected hMDMs during exponential phase of intracellular replication.

BCL10	Anti-apoptotic, functions as an upstream regulator of NF-κB activation.
BCL2	Anti-apoptotic, an integral outer mitochondrial proteins that blocks apoptosis.
BFAR	Anti-apoptotic, an ER multidomain protein that was originally identified as an inhibitor of Bax-induced apoptosis.
RIPK1	Anti-apoptotic, interacts with TNF-alpha receptor and plays an indispensable role in NF-κB activation.
TNF	Anti-apoptotic, triggers a signalling pathway that activates NF-κB.
TNFRSF14	Anti-apoptotic, activates Fib in response to some viral infection.
TNFSF11	Anti-apoptotic. It is also called receptor activator of nuclear factor kappaB ligand (RANK-L). It activates NF-κB.
TNFSF13	Anti-apoptotic. This protein is a ligand for TNFRSF17/BCMA, a member of the TNF receptor family activates NF-κB to upregulate Bcl-2 and Bcl-x(L), and downregulate Bax.
TNFSF5	Anti-apoptotic, CD40 ligand. Binding to CD40 leads for NF-κB activation.
TNFSF9	Anti-apoptotic, CD137 binding to CD137R. In T cells, it increases the expression of the anti-apoptotic genes bcl-x(L) mediated NF-κB activation.
TRAF5	Anti-apoptotic, TNF receptor-associated factor 5 that is involved in NF-κB activation. NF-kB activation.
XIAP	Anti-apoptotic, inhibits active caspase-3 and 7.

These genes were not triggered by the *dotA* mutant.

with uninfected cells, hMDMs infected with the *dotA* mutant strain, upregulated the expression of only one pro-apoptotic gene, *TNFSF10*, by 2.3-fold. As our goal is to unravel the mechanism by which *L. pneumophila* renders infected macrophages resistant to the induction of apoptosis despite caspase-3 activation, we decided to further confirm upregulation of the anti-apoptotic genes in *L. pneumophila*-infected macrophages by quantitative real-time PCR (qRT-PCR) (see *Experimental procedures*). The qRT-PCR results confirmed that 12 of the anti-apoptotic genes were significantly upregulated by the WT strain, compared with uninfected cells ($P = 0.001$–0.05, Student's *t*-test) (Fig. 2C) and (Table 1). Thus, it is likely that the upregulation of anti-apoptotic genes by *L. pneumophila* during the exponential phase of intracellular replication counteracts and limits, at least in part, the effect of both slight upregulation of pro-apoptotic genes and the activation of caspase-3.

Legionella pneumophila *induces nuclear translocation of NF-κB in infected macrophages*

Interestingly, most of the upregulated anti-apoptotic genes in *L. pneumophila*-infected macrophages are involved in signalling pathways that activate NF-κB (Table 1), which promotes cell survival through the upregulation of several anti-apoptotic genes (Burstein and Duckett, 2003; Kucharczak *et al.*, 2003). In mammalian cells, the most predominant active complex of NF-κB is the p65/p50 heterodimer (Ghosh and Karin, 2002). Thus, we examined whether *L. pneumophila* triggered nuclear translocation of the p65 and p50 subunits of NF-κB in infected macrophages. Monolayers of hMDMs were infected with either the parental strain AA100-GFP or the *dotA*-GFP isogenic mutant strain, at a moi of 10 for 1 h, followed by 3× washing and 1 h of gentamicin treatment. Control hMDMs monolayers were either left untreated or treated with 1 μg ml^{-1} of *Escherichia coli* lipopolysaccharide (LPS) (Faure *et al.*, 2000) as negative and positive controls, respectively, for nuclear translocation of the p65 and p50 subunits. At different time points after infection, monolayers were fluorescence-labelled for both nuclei and the p65 or p50 subunits of NF-κB (see *Experimental procedures*). Single cell analysis by laser scanning confocal microscopy showed that the p65 subunit was translocated to the nuclei of only 6% of uninfected macrophages (Figs 3 and 4A). The p65 subunit was translocated to the nucleus in 87% of *E. coli* LPS-treated macrophages (Figs 3 and 4A). At 2, 4, 8 and 12 h after infection, 92%, 83%, 86% and 76% of the AA100-infected macrophages, respectively, showed nuclear translocation of the p65 subunit (Figs 3 and 4A). At 2 h after infection, 82% of the *dotA* mutant-infected macrophages exhibited nuclear translocation of the p65 subunit (Figs 3 and 4B). However, by 4 h and 8 h after infection, only ~5% of the *dotA* mutant-infected macrophages showed nuclear translocation of the p65 subunit, similar to uninfected cells (Figs 3 and 4B). Very similar results were obtained for nuclear translocation of the p50 subunit (Fig. S1). We conclude that sustained nuclear translocation of the p65 and p50 requires a functional Dot/Icm secretion system.

To examine whether bacterial entry was essential for nuclear translocation of the p65 subunit, we infected human macrophages exactly as described above, with the exception that the infection was performed in presence of cytochalasin D to block bacterial entry, as we described previously (Gao *et al.*, 1997). The data showed that bacterial entry was essential for early (for WT and *dotA* mutant) and sustained nuclear translocation of the p65 (for the WT strain) (Fig. S2). These data are consistent with the above observation that bacterial entry is also essential for the anti-apoptotic phenotype of *L. pneumophila*-infected human macrophages.

Lipopolysaccharide of many Gram-negative bacteria triggers NF-κB activation through Toll-like receptor 4 (TLR4) (Faure *et al.*, 2000; Barton and Medzhitov, 2003).

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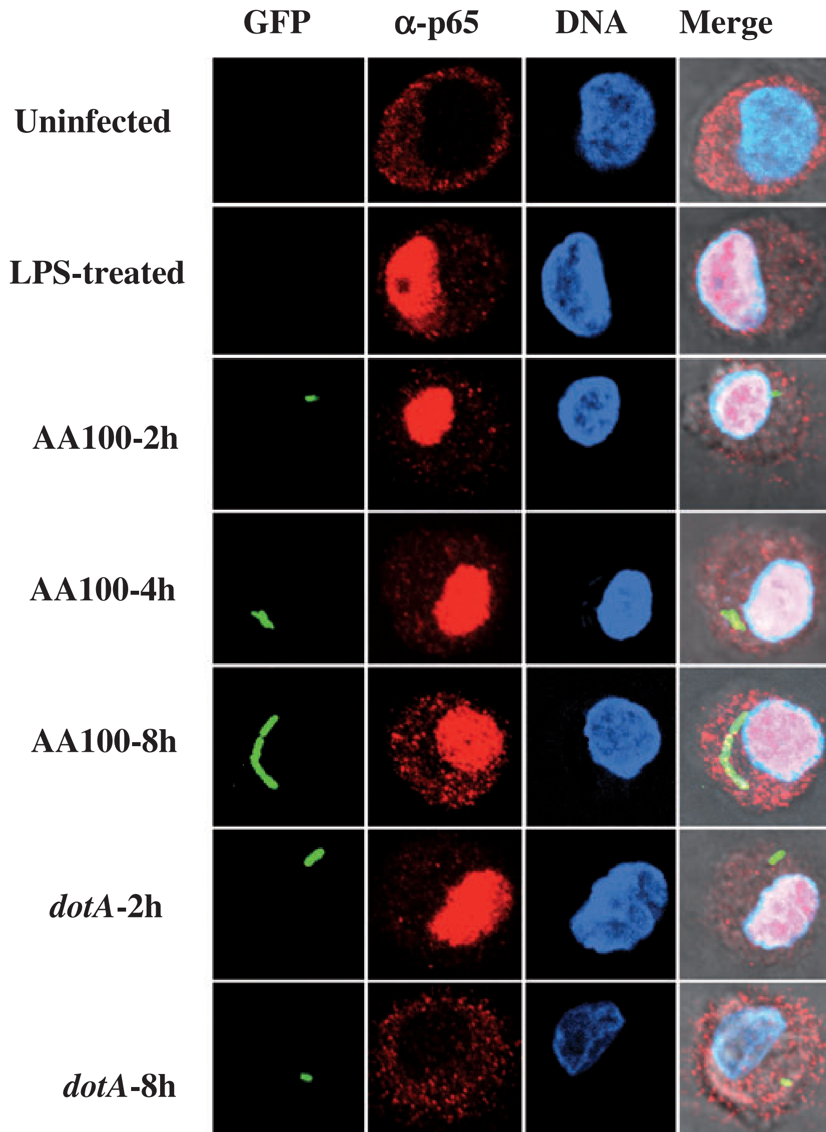

Fig. 3. Representative confocal laser scanning microscopy images for nuclear translocation of the p65 subunit of NF-κB. Monolayers of hMDMs were infected with either the parental strain AA100-GFP or the *dotA*-GFP mutant strain at a moi of 10 for 1 h. The cells were labelled for nuclei using TOTO-3 (blue), and anti-p65 antisera followed by secondary antibodies (red). The images are representative of three independent experiments.

However, the LPS of some Gram-negative bacteria activate NF-κB through TLR2 (Erridge *et al.*, 2004). Similarly, *L. pneumophila* LPS has been shown to trigger the activation of TLR2 rather than TLR4 (Lettinga *et al.*, 2002; Pedron *et al.*, 2003; Akamine *et al.*, 2005). Results from the above experiments indicated that although the *dotA* mutant failed to induce sustained nuclear translocation of the p65 subunit, the *dotA* mutant induced nuclear translocation of the p65 during the first 2 h of infection. To examine whether *L. pneumophila* LPS is involved in *L. pneumophila*-induced nuclear translocation of the p65 subunit of NF-κB, formalin-killed bacteria were used for infection. At 2 and 8 h after infection, nuclear translocation of the p65 subunit was detected in only 6–9% of hMDMs infected with the formalin-killed WT or -*dotA* mutant of *L. pneumophila*, similar to uninfected hMDMs (Fig. 4A and B). These data suggested that the *L. pneumophila* LPS is unlikely to be involved in the early induction of nuclear translocation of p65.

Dot/Icm-dependent sustained activation of NF-κB is required for the exhibition of the anti-apoptotic phenotype of infected macrophages

We tested the hypothesis that sustained Dot/Icm-dependent nuclear translocation of NF-κB activation in *L. pneumophila*-infected macrophages is required for the induction of apoptosis resistance in infected macrophages. We utilized caffeic acid phenethyl ester (CAPE), which inhibits NF-κB activation in response to various stimuli, and also induces apoptosis (Natarajan *et al.*, 1996; Watabe *et al.*, 2004). Apoptosis of infected

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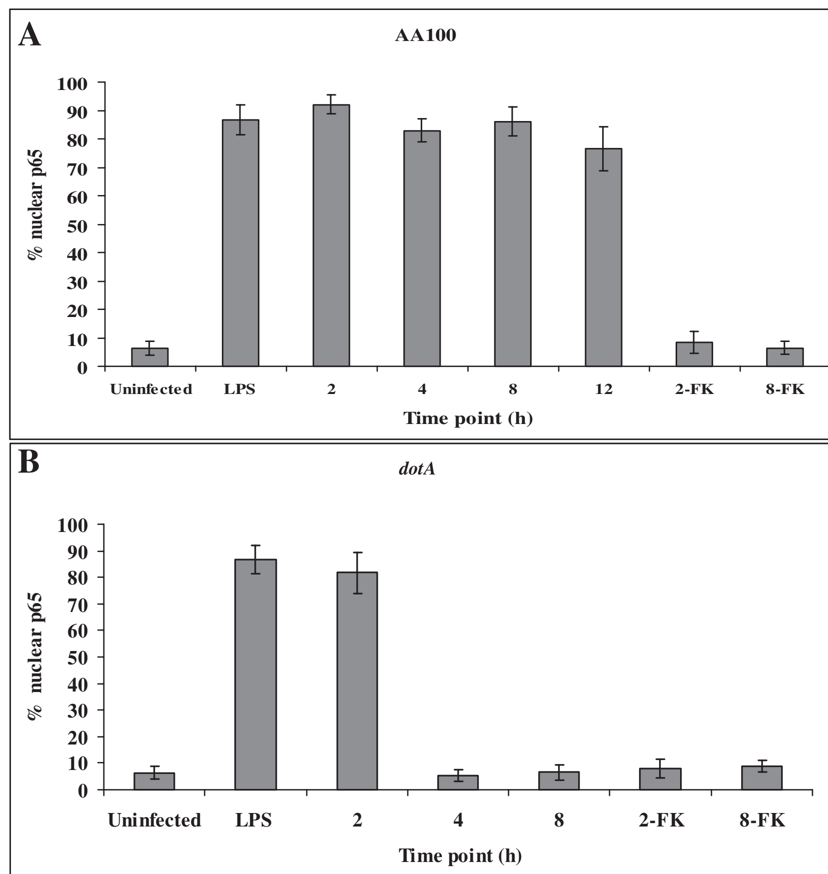

Fig. 4. Quantitative analysis of p65 nuclear translocation in *L. pneumophila*-infected macrophages. Monolayers of hMDMs were infected with live or formalin-killed (FK) AA100-GFP (A) *dotA*-GFP (B) strain at a moi of 10 for 1 h. At several time points after infection, the cells were labelled for the p65 subunit of NF-κB and nuclei. Uninfected monolayers and *E. coli* LPS-treated monolayers were included as negative and positive controls. Approximately 100 infected macrophages were analysed from different coverslips. The results are representative of three independent experiments and error bars represent standard deviations.

macrophages is associated with termination of intracellular proliferation of *L. pneumophila* (Abu-Zant *et al.*, 2005). As CAPE induces apoptosis, we initially examined the effect of different concentrations of CAPE on intracellular replication of *L. pneumophila*. Growth kinetics data revealed that by 24 h after infection, the parental strain AA100 exhibited 1.5 log increase in colony-forming units (cfu) in both untreated cells and cells treated with 20 μg ml^{-1} of CAPE (Fig. 5A). However, in CAPE-treated cells, the parental strain AA100 did not show further increase in cfu after 24 h, in contrast to untreated cells (Fig. 5A). Lower concentrations of CAPE (5, 10 and 15 μg ml^{-1}) had no effect on intracellular replication (data not shown). Our data showed that at 2 h and 8 h after infection, nuclear translocation of the p65 subunit was detected in ~80% of AA100-infected macrophages in the presence or absence of CAPE (Fig. 5B). In contrast, only 26% of CAPE-treated of *dotA*-infected hMDMs showed nuclear translocation of the p65 subunit (Fig. 5B). In the absence of CAPE, nuclear translocation of the p65 subunit was detected at 2 h in 72% of *dotA*-infected hMDMs. As expected, by 8 h after infection, only 5–9% of *dotA*-infected hMDMs that were either untreated or treated with CAPE exhibited nuclear translocation of the p65 subunit (Fig. 5B). Similarly, CAPE blocked *E. coli* LPS-induced nuclear translocation of the p65 subunit (Fig. 5B). Thus, although CAPE inhibited nuclear translocation of the p65 subunit in *E. coli* LPS-treated and *dotA* mutant-infected macrophages, it did not block nuclear translocation of the p65 subunit in AA100-infected macrophages.

TUNEL labelling for apoptotic nuclei revealed that 5–8% of untreated uninfected, *dotA*-infected and AA100-infected cells were apoptotic by 8 h after infection (Fig. 5C). On the other hand, in CAPE-treated cells, 28%, 13% and 33% of uninfected, *dotA*-infected and AA100-infected cells, respectively, were apoptotic (Fig. 5C). We conclude that the mechanism of sustained nuclear translocation of the p65 subunit by the *L. pneumophila* Dot/Icm secretion system is distinct from that induced by *E. coli* LPS and at 2 h post infection by the *dotA* mutant. These observations further support our data that sustained nuclear translocation of the p65 subunit by *L. pneumophila* requires a functional Dot/Icm secretion system.

Expression of TNF-α by infected hMDMs is independent of the Dot/Icm system

In addition to the role of NF-κB in expression of anti-apoptosis genes, it is a primary transcription factor

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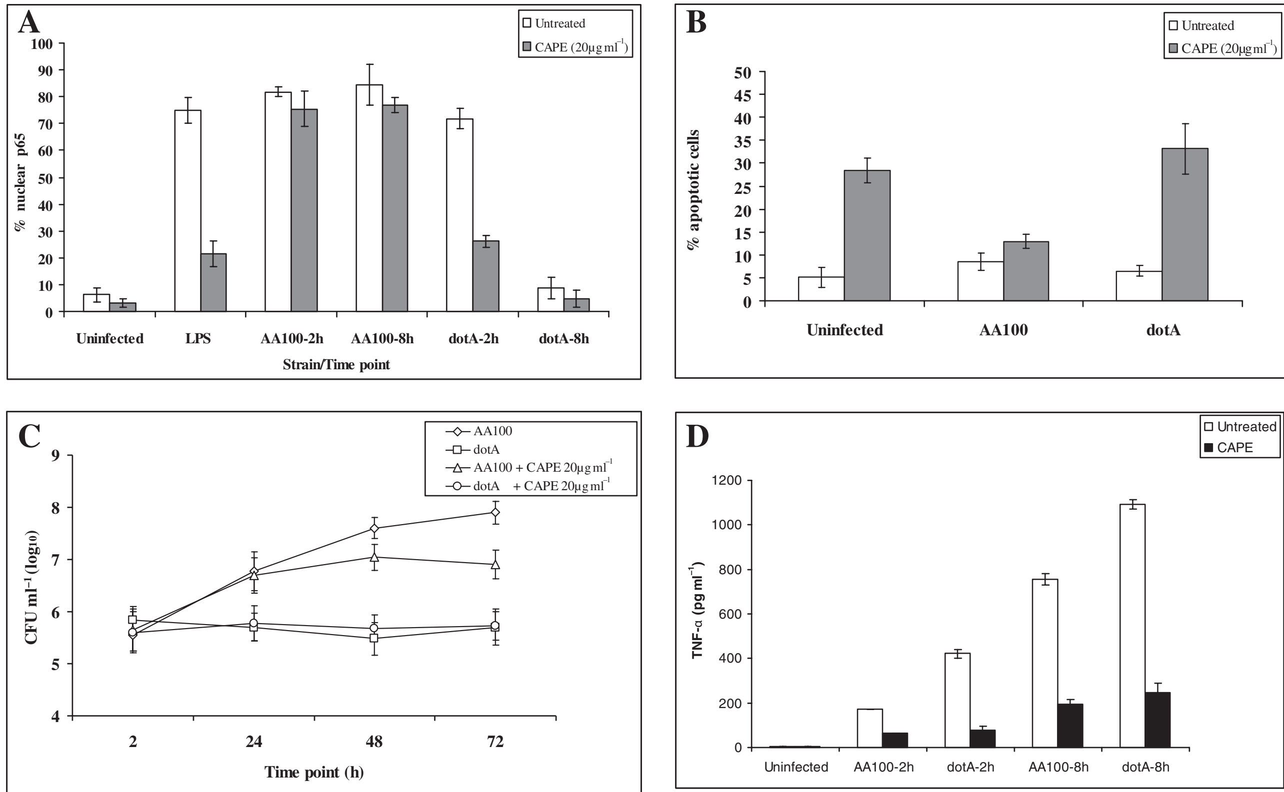

Fig. 5. CAPE does not inhibit *L. pneumophila* Dot/Icm-induced sustained nuclear translocation of the p65 subunit, but inhibits expression of TNF-α.

A. Monolayers of hMDMs were either untreated or treated with 20 μg ml^{-1} of CAPE 30 min prior to infection. Cells were infected with the parental strain AA100 or the *dotA* mutant strain at a moi of 10. At the indicated time points after infection, the cfu in infected monolayers was enumerated.

B. AA100- and *dotA* mutant-infected cells were labelled for p65 and nuclei. Uninfected and *E. coli* LPS-treated (1 μg ml^{-1} for 20 min) monolayers were included as a negative and positive control respectively.

C. At 8 h after infection, cells were labelled for apoptotic nuclei by TUNEL. Approximately 100 macrophages were analysed for (B) and (C) by confocal microscopy from different coverslips.

D. Levels of TNF-α in the supernatant of untreated or CAPE-pretreated hMDMs infected by the WT stain AA100 or its *dotA* isogenic mutant at 2 h or 8 h post infection. All the results in A–C are representative of three independent experiments, while D is one of two representative experiments, and error bars represent standard deviations.

involved in the expression of pro-inflammatory cytokines (Medzhitov, 2001; Barton and Medzhitov, 2003), many of which are triggered by *L. pneumophila*, such as TNF-α (Brieland *et al*., 1995; Friedman *et al*., 2002). We utilized ELISA assays to examine expression of TNF-α in the supernatant of monolayers of hMDMs at 2 and 8 h post infection, and the potential role of NF-κB in this process (Fig. 5D). Our data showed that TNF-α was induced slightly at 2 h and much higher at 8 h post infection by the WT strain and the *dotA* mutant (Fig. 5D). Inhibition of NF-κB by CAPE diminished expression of TNF-α by both the WT strain the *dotA* mutant (Fig. 5D). We conclude that while the anti-apoptotic activity triggered by *L. pneumophila* is Dot/Icm-dependent, expression of TNF-α by *L. pneumophila*-infected macrophages is independent of the Dot/Icm type IV secretion.

NF-κB is required for exhibition of the anti-apoptotic phenotype of L. pneumophila*-infected cells*

To examine the role of NF-κB in apoptosis resistance of infected cells directly, we utilized p65 knockout mouse embryonic fibroblast (MEF p65$^{-/-}$). These cells have been recently utilized in apoptotic studies on *Legionella* (Fischer *et al*., 2006). Infection efficiency of the MEF was ~25% using infection conditions similar to hMDMs. Similar to hMDMs, at 8 h after infection, ~7% of *dotA*-infected wild-type MEF (MEF-WT) exhibited nuclear translocation of the p65 subunit (Fig. 6A). In contrast, 73% of AA100-infected MEF-WT, exhibited nuclear translocation of the p65 subunit (Fig. 6A). Nuclear translocation of the p65 subunit was detected in 5% and 54% of uninfected and LPS-treated control cells respectively. As expected, no

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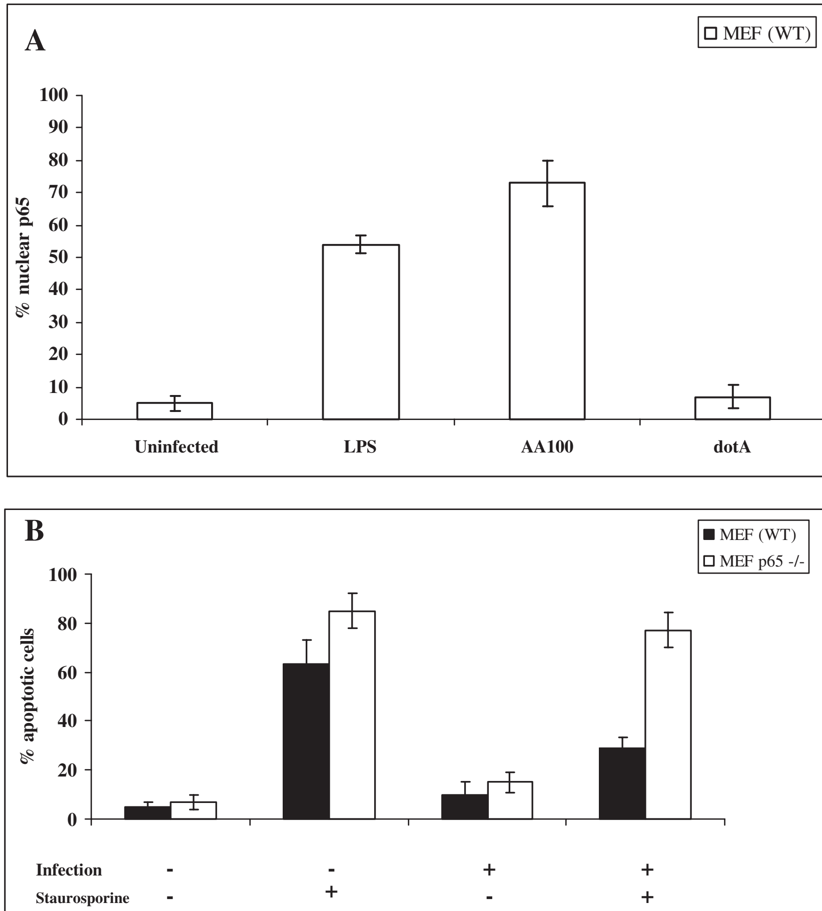

Fig. 6. The p65 subunit is required for induction of the anti-apoptotic phenotype in *L. pneumophila*-infected MEF:
A. MEF-WT cells were infected with the parental strain AA100-GFP or the *dotA*-GFP mutant strain at a moi of 10. At 8 h after infection, the cells were labelled for p65 and nuclei. Uninfected monolayers and LPS-treated (1 μg ml^{-1} for 20 min) monolayers were included as a negative and positive control respectively.
B. MEF-WT and MEF $p65^{-/-}$ cells were infected for 1 h with the parental strain AA100-GFP at a moi of 10. At 8 h after infection, infected and uninfected cells were either left untreated or treated with 0.3 μM Staurosporin for 4 h. Then the cells were fixed, permeabilized and labelled for apoptotic nuclei by TUNEL. Approximately 100 infected macrophages were analysed from different coverslips. The results are representative of three independent experiments and error bars represent standard deviations.

fluorescence labelling for the p65 subunit was detected in the p65 deficient MEF (data not shown).

To examine the role of NF-κB in the anti-apoptotic phenotype of infected cells, monolayers of MEF-WT and MEF $p65^{-/-}$ were either left uninfected or infected with the parental strain AA100 at a moi of 10 for 1 h. At 8 h after infection, monolayers were either left untreated or treated with 0.3 μM Staurosporin for 4 h, followed by TUNEL labelling. In untreated cells, 5–7% of uninfected MEF-WT and MEF $p65^{-/-}$ were apoptotic (Fig. 6B). In infected cells, there was no difference in apoptosis between MEF-WT and MEF $p65^{-/-}$ where only 10–15% of the infected cells were apoptotic (Fig. 6B). However, the WT strain AA100 of *L. pneumophila* failed to protect MEF $p65^{-/-}$ from Staurosporin-induced apoptosis, while MEF-WT was protected. Staurosporin induced apoptosis in only 29% of AA100-infected MEF-WT. In contrast, 77% of AA100-infected MEF $p65^{-/-}$ were apoptotic (Fig. 6B). In uninfected cells, ~63–85% of Staurosporin-treated MEF-WT or MEF $p65^{-/-}$ cells were apoptotic (Fig. 6B). We conclude that *L. pneumophila* protected MEF-WT but not MEF $p65^{-/-}$ from Staurosporin-induced apoptosis, by a process that requires, at least in part, NF-κB.

The IKKγ is dispensable for L. pneumophila*-induced nuclear translocation of p65*

The NF-κB essential modulator (NEMO/IKKγ)-binding domain (NBD) peptide has been shown to be a potent specific inhibitor of NF-κB activation (May *et al.*, 2000; Tas *et al.*, 2005). The NBD peptide inhibitor (DRQIKIWFQNRRMKWKK-TALDWSWLQTE) consists of NBD (underlined) of IKKα and IKKβ, fused with an amino acid sequence that mediates membrane translocation (May *et al.*, 2000). The NBD domain mediates the association of IKKγ with IKKα and IKKβ for the formation of the active IKK kinase complex. The NBD peptide inhibitor prevents the formation of active IKK complex by interfering with the association of IKKγ with the IKKα and IKKβ subunits, and thus blocks NF-κB activation (May *et al.*, 2000). Our data showed that pretreatment of hMDMs with 50 μM NBD peptide inhibitor for 2 h prior to infection had no effect on *L. pneumophila* intracellular replication (Fig. 7A). We examined the effect of the NBD peptide on both nuclear translocation of the p65 subunit of NF-κB and apoptosis. The NBD negative control (NBD-NC) peptide (DRQIKIWFQNRRMKWKK-TALDASALQTE),

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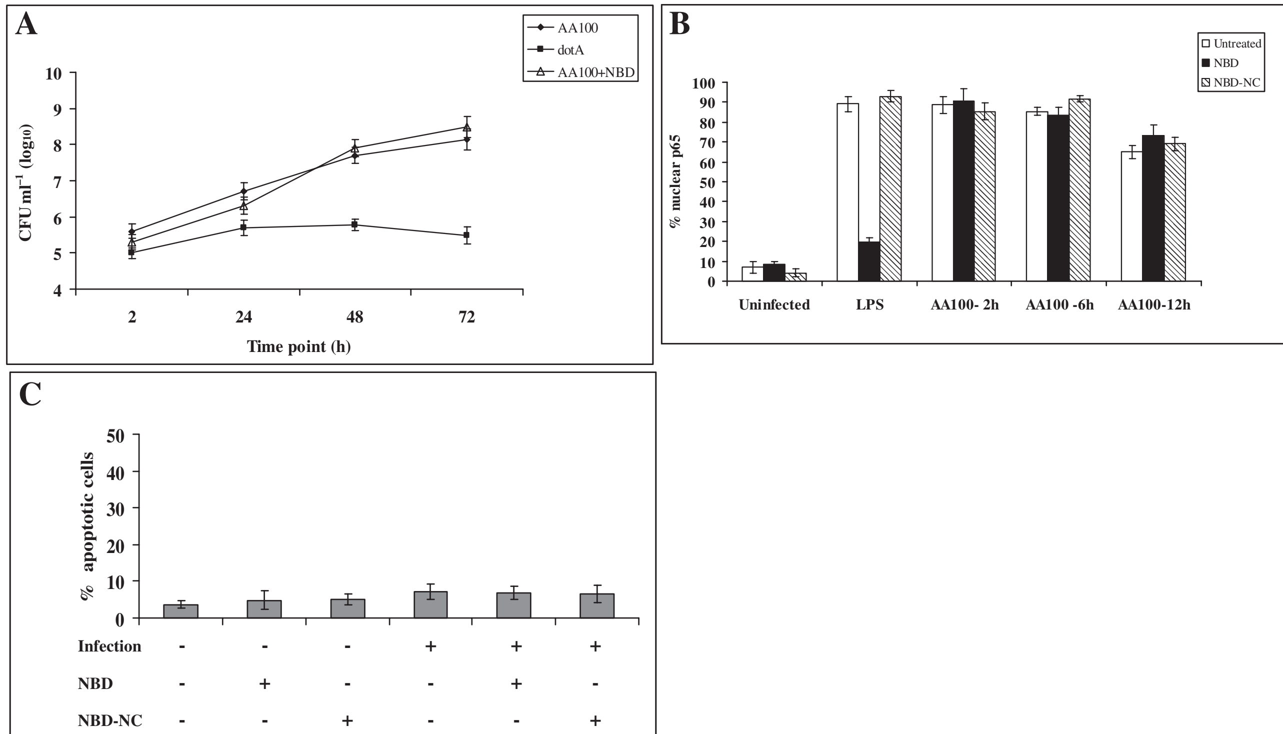

Fig. 7. Inhibition of the IKK kinase complex by NBD does not block *L. pneumophila*-induced nuclear translocation of the p65 subunit and does not affect intracellular replication.
A. Untreated and NBD-treated monolayers were infected with the parental strain AA100 at a moi of 1 for 1 h. At the indicated time points after infection, the cfu was enumerated.
B. Monolayers of hMDMs were either untreated or treated with 50 μM NBD or NBD-NC peptide for 2 h prior to infection. The cells were infected with the parental strain AA100-GFP strain at a moi of 10 for 1 h, and labelled for p65 and nuclei at several time points after infection. Untreated and LPS-treated monolayers (1 μg ml^{-1} for 20 min) served a positive and negative control for nuclear translocation of the p65 subunit.
C. Monolayers of hMDMs untreated or treated with NBD or NBD-NC peptide were infected with the parental strain AA100-GFP strain at a moi of 10 for 1 h. At 12 h after infection, the cells were labelled for apoptotic nuclei by TUNEL. For (B) and (C), approximately 100 infected macrophages were analysed by confocal microscopy from different coverslips. The results are representative of three independent experiments and error bars represent standard deviations.

with tryptophan to alanine substitution, which results in abrogation of the inhibitory activity of the NBD peptide, was used as a control (May *et al.*, 2000). Nuclear translocation of the p65 subunit was detected in ~90% of *E. coli* LPS-treated control hMDMs in presence or absence of the NBD-NC (Fig. 7B). However, LPS-induced nuclear translocation of the p65 subunit was inhibited in NBD-treated cells (Fig. 7B). At 2, 8 and 12 h after infection, neither the NBD nor the NBD-NC peptide had any detectable effect on WT *L. pneumophila*-induced p65 nuclear translocation (Fig. 7B). In addition, neither the NBD nor the NBD-NC peptide had any detectable effect on resistance of *L. pneumophila*-infected macrophages to apoptosis (Fig. 7C). Thus, although the NBD peptide inhibited nuclear translocation of the p65 subunit in LPS-treated cells, it had no effect on p65 nuclear translocation in WT *L. pneumophila*-infected macrophages. In contrast, at 2 h after infection, NBD but not NBD-NC blocked p65 nuclear translocation in *dotA* mutant-infected cells, which was similar to LPS-treated cells (data not shown). We conclude that WT *L. pneumophila* induces sustained nuclear translocation of the p65, which is likely to be independent of IKKγ.

Nuclear translocation of the p65 subunit in L. pneumophila*-infected cells requires the IKK activity*

As the NBD-peptide did not inhibit *L. pneumophila*-induced nuclear translocation of the p65 subunit of NF-κB, we decided to examine whether the *L. pneumophila*-induced nuclear translocation of the p65 subunit bypasses the requirement of IKKα and IKKβ. We examined the ability of *L. pneumophila* to induce nuclear translocation of the p65 subunit in MEF IKKαβ double knockout (MEF $IKK\alpha^{-/-}$ $IKK\ \beta^{-/-}$). The data showed that nuclear translocation of the p65 subunit was detected in only 2–4% of uninfected, LPS-treated and AA100-infected MEF $IKK\alpha^{-/-} IKK\beta^{-/-}$ (Fig. 8). The failure of *L. pneumophila*

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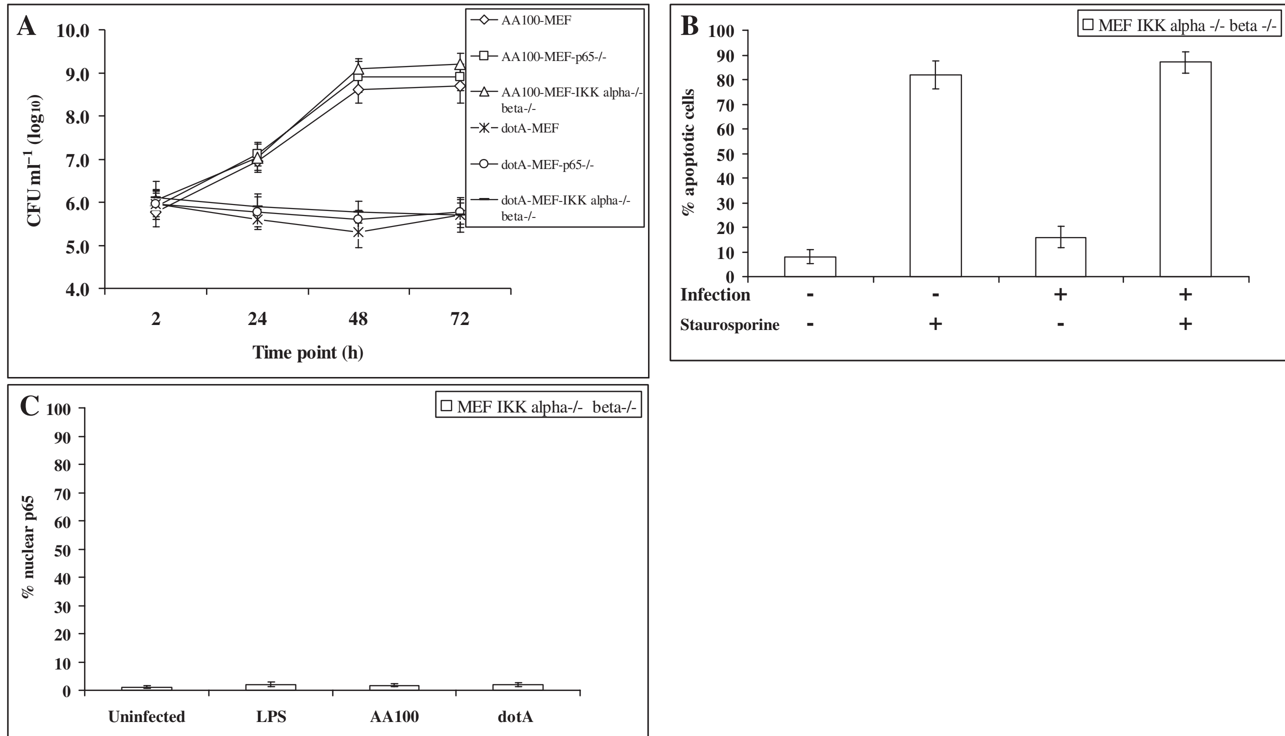

Fig. 8. The IKK is required for nuclear translocation of the p65 subunit, and apoptosis resistance but not for intracellular proliferation in MEF.
A. Growth kinetics of *L. pneumophila* in MEF-WT, MEF p65$^{-/-}$ and MEF IKKα$^{-/-}$ IKKβ$^{-/-}$ cells. Monolayers were infected with either the parental strain AA100 or the *dotA* mutant strain at a moi of 1 for 1 h. At the indicated time points after infection, the cfu in infected monolayers was enumerated.
B. MEF IKKα$^{-/-}$ IKKβ$^{-/-}$ double knockout cells were infected for 1 h with the parental strain AA100-GFP at a moi of 10. At 8 h after infection, cells were either left untreated or treated with 0.3 μM staurosporine for 4 h and labelled for apoptotic nuclei by TUNEL. For (A) and (B) approximately 100 infected macrophages from different coverslips were analysed by confocal microscopy.
C. MEF IKKα$^{-/-}$ IKKβ$^{-/-}$ double knockout cells were infected with the parental strain AA100-GFP or the *dotA*-GFP mutant strains at a moi of 10 for 1 h. At 8 h after infection, the cells were permeabilized and fluorescence-labelled for the p65 of NF-κB and nuclei. Uninfected monolayers and LPS-treated (1 μg ml^{-1} for 20 min) monolayers were included as a negative and positive control respectively. The results are representative of three independent experiments and error bars represent standard deviations.

to induce nuclear translocation of the p65 subunit in MEF IKKα$^{-/-}$ IKKβ$^{-/-}$ prompted us to examine the sensitivity of *L. pneumophila*-infected MEF IKKα$^{-/-}$ IKKβ$^{-/-}$ to Staurosporin-induced apoptosis. The data showed that both uninfected and infected cells were similarly sensitive to Staurosporin-induced apoptosis where 82–87% of the cells were apoptotic (Fig. 8B). In untreated monolayers, only 8% and 17% of uninfected and *L. pneumophila*-infected cells, respectively, were apoptotic (Fig. 8B). We conclude that the functional activity of IKKβ and/or IKKα is required for *L. pneumophila*-induced nuclear translocation of the p65 subunit in MEF, and for the anti-apoptotic phenotype of *L. pneumophila*-infected cells.

We examined intracellular replication of *L. pneumophila* in MEF-WT, MEF p65$^{-/-}$ and MEF IKKα$^{-/-}$ IKKβ$^{-/-}$. The data showed that the parental strain AA100 replicated to the same level in all cell lines, but the *dotA* mutant control failed to replicate (Fig. 8). These results were expected, because there were no significant differences among all infected MEF cells in apoptosis upon infection by *L. pneumophila*. We conclude that the p65 subunit of NF-κB and the IKK complex are dispensable for intracellular replication of *L. pneumophila* in MEF, but they are required for the resistance of infected cells to apoptosis-inducing agents.

Discussion

Activation of caspase-3 by *L. pneumophila* in human macrophages is mediated by the Dot/Icm type IV secretion system and is independent of the classical pathways of apoptosis (Zink *et al.*, 2002; Molmeret *et al.*, 2004b). Despite caspase-3 activation during early and exponential intracellular replication of human macrophages, host cell apoptosis is delayed until late stages of infection (~18 h) concomitant with termination of intracellular replication (Abu-Zant *et al.*, 2005). Our data show that *L. pneumophila*-infected macrophages upregulate the expression of 12 anti-apoptotic genes during exponential intracellular replication. Most of the upregulated anti-

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apoptotic genes in *L. pneumophila*-infected macrophages are involved in signalling pathways that activate NF-κB (Table 1) or their expression is regulated by NF-κB, such as *bcl2* and *xiap* (Stehlik *et al*., 1998; Catz and Johnson, 2001). Activation of NF-κB promotes cell survival through upregulating the expression of anti-apoptotic genes (Burstein and Duckett, 2003; Kucharczak *et al*., 2003). Bcl2 inhibits the intrinsic pathway of apoptosis by inhibiting the release of cytochrome *c* from the mitochondria (Chao and Korsmeyer, 1998), while XIAP inhibits caspase-3 and caspase-7 through direct binding to the catalytic site (Scott *et al*., 2005).

Several intracellular pathogens, such as *Toxoplasma gondii* and *Rickettsia rickettsii,* inhibit apoptosis of their host cells by a mechanism that involves NF-κB activation and the consequent upregulation of anti-apoptotic genes (Clifton *et al*., 1998; Molestina *et al*., 2003). Our data show that *L. pneumophila* triggers a sustained nuclear translocation of the p65 and p50 subunits during early and exponential intracellular replication, which correlates with the induction of apoptosis resistance and with upregulation of anti-apoptotic genes in infected macrophages. In contrast to the resistance of *L. pneumophila*-infected MEF-WT to apoptosis-inducing agents, *L. pneumophila*-infected MEF p65$^{-/-}$ and MEF IKKα$^{-/-}$ IKKβ$^{-/-}$ are susceptible to these agents. Thus, NF-κB is essential, at least in part, for the anti-apoptotic phenotype of *Legionella.* However, the *L. pneumophila*-infected MEF p65$^{-/-}$ and MEF IKKα$^{-/-}$ IKKβ$^{-/-}$ do not undergo apoptosis upon infection by *L. pneumophila*. This indicates that NF-κB-independent anti-apoptotic processes are also triggered by *L. pneumophila*. This may not be surprising because this pathogen has been shown to exhibit tremendous redundancy in the Dot/Icm effectors as well as the cellular pathways that are manipulated to ensure intracellular proliferation (Chen *et al*., 2004; Luo and Isberg, 2004; Dorer *et al*., 2006; VanRheenen *et al*., 2006).

In addition to its role in anti-apoptotic signalling, NF-κB is involved in expression of pro-inflammatory cytokines (Medzhitov, 2001; Barton and Medzhitov, 2003), which are triggered by *L. pneumophila* in infected macrophages *in vitro* as well as in the lungs of experimental animals (Brieland *et al*., 1995; Friedman *et al*., 2002). Our data show that expression of TNF-α is triggered by *L. pneumophila* independent of the Dot/Icm system, which is distinct from the Dot/Icm-dependent anti-apoptotic signalling. Furthermore, CAPE inhibits expression of TNF-α by *L. pneumophila*-infected macrophages but does not inhibit the Dot/Icm-mediated anti-apoptotic signalling. These data suggest that activation of NF-κB by *L. pneumophila* is mediated by multiple mechanisms, some of which are Dot/Icm-dependent and some are not. The data also suggest that while the Dot/Icm-independent activation of NF-κB is mediated by a classical pathway sensitive to inhibition by CAPE, the Dot/Icm-dependent activation of NF-κB is mediated by a process not inhibited by CAPE. It is likely that the multiple mechanisms involved in activation of NF-κB by *L. pneumophila* play dual roles in triggering anti-apoptotic signalling as well as expression of pro-inflammatory cytokines. Although anti-apoptotic events are predicted to benefit the pathogen by maintaining viability of the replicative niche, pro-inflammation has been shown to limit and control the infection by *L. pneumophila* (Brieland *et al*., 1995; Friedman *et al*., 2002). The balance between the two events during the disease is expected to be detrimental to the outcomes of the infection, and is most likely to involve multiple signalling and overlapping pathways.

Although extracellular TLRs trigger NF-κB upon binding to conserved microbial ligands such as LPS, flagellin or peptidoglycan (Medzhitov, 2001; Barton and Medzhitov, 2003), it is unlikely they play a role in the *L. pneumophila*-mediated activation of NF-κB (Lettinga *et al*., 2002; Akamine *et al*., 2005). Although TLR2 has been shown to be triggered by the *L. pneumophila* LPS (Lettinga *et al*., 2002; Akamine *et al*., 2005), and the downstream signalling cascade leads to NF-κB activation (Medzhitov, 2001; Barton and Medzhitov, 2003), it is unlikely that this is the mechanism by which *L. pneumophila* trigger NF-κB activation. This is supported by our data, which show that formalin-killed bacteria as well as live extracellular *L. pneumophila* do not trigger NF-κB activation. It is possible that intracellular sensing molecules belonging to the NOD family of proteins are involved in sensing *L. pneumophila* molecules that activates NF-κB, which triggers expression of both anti-apoptotic proteins and pro-inflammatory cytokines (Fortier *et al*., 2005).

Apoptosis resistance of *Bartonella henselae*-infected cells depends on functional integrity of the type IV secretion system (Schmid *et al*., 2004). In addition, Type IV secretion systems have been shown to be required for NF-κB activation by *Helicobacter pylori* and *B. henselae* (Forsyst-Ludwig and Naumann, 2000; Schmid *et al*., 2004; Brandt *et al*., 2005). The effector protein CagA of *H. pylori* type IV secretion system has been shown to trigger an IKK signalling pathway that leads to NF-κB activation (Brandt *et al*., 2005). *L. pneumophila* has been shown to translocate several effector proteins into the host cell through the Dot/Icm type IV secretion system (Nagai *et al*., 2002; Conover *et al*., 2003; Chen *et al*., 2004; Luo and Isberg, 2004). Thus, the *L. pneumophila* Dot/Icm system plays a key role in triggering a sustained nuclear translocation of the p65 and p50 subunits of NF-κB, and induction of high resistance apoptosis in infected macrophages. Bacterial entry is essential for the anti-apoptotic phenotype of the WT-infected cells in addition to the nuclear translocation of the p65. We conclude that the Dot/Icm secretion system is essential for nuclear translo-

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cation of NF-κB and for strong anti-apoptotic signalling by intracellular *L. pneumophila*.

The ability of the *dotA* mutant to trigger nuclear translocation of the p65 and p50 subunits only at very early stages of infection (2 h) indicates that a Dot/Icm-independent mechanism is likely to be involved in early induction of nuclear translocation of the p65 and p50 subunits. The inability of formalin-killed *L. pneumophila* to induce nuclear translocation of the p65 subunit suggests that *L. pneumophila* LPS may not be involved in early nuclear translocation of the p65 subunit in *L. pneumophila*-infected cells. In addition, nuclear translocation of the p65 subunit is blocked by CAPE and NBD in *E. coli* LPS-treated and *dotA* mutant-infected cells, but not in WT-infected cells at 2 h after infection. Therefore, early nuclear translocation of the p65 subunit in *L. pneumophila*-infected macrophages is likely to be mediated by both Dot/Icm-dependent and -independent process.

Caffeic acid phenethyl ester induces apoptosis in both uninfected and *dotA*-infected cells, but not in AA100-infected cells. It is possible that resistance of AA100-infected cells to CAPE-induced apoptosis is due to sustained nuclear translocation of NF-κB. On the other hand, the absence of sustained nuclear translocation of NF-κB in uninfected and *dotA*-infected cells renders them susceptible to CAPE-induced apoptosis. Partial inhibition of intracellular replication of *L. pneumophila* in CAPE-treated macrophages is likely due to induction of apoptosis among uninfected cells but not among infected cells within the same monolayers, and thus decreasing the number of cells susceptible for intracellular replication during a second round of invasion.

Several stimuli, including LPS, activate NF-κB through the classical pathway, which requires the functional activity of the IKK complex (IKKα, IKKβ and IKKγ) (Ghosh and Karin, 2002). As the NBD peptide and CAPE, which are potent inhibitors of NF-κB activation, do not block nuclear translocation of the p65 subunit in WT *L. pneumophila*-infected macrophages, the Dot/Icm-induced sustained nuclear translocation of p65 is independent of the classical pathway of NF-κB activation. However, failure of *L. pneumophila* to induce nuclear translocation of the p65 subunit in MEF IKKα$^{-/-}$ IKKβ$^{-/-}$ indicates that the functional activity of IKKα and/or IKKβ is essential for the *L. pneumophila* Dot/Icm-induced p65 nuclear translocation. However, we cannot exclude the possibility that different anti-apoptotic processes involved in signalling through NF-κB are triggered by *L. pneumophila* in MEFs and hMDMs. Interestingly, although IKKβ and IKKα are involved in transient NF-κB activation in *Rickettsia rickettsii*-infected cells during early stages of infection, IKKα may play a major role in the induction of sustained NF-κB activation during later stages of infection (Clifton *et al*., 2005).

Although *L. pneumophila* triggers nuclear translocation of the p65 subunit in MEF and that the p65 subunit is required for resistance to staurosporine-induced apoptosis in *L. pneumophila*-infected cells, the p65 subunit and the IKK complex are dispensable for intracellular replication in MEF. Apoptosis resistance of *Chlamydia pneumoniae*-infected macrophages depends on NF-κB activation (Wahl *et al*., 2001; 2003). However, inhibition of apoptosis in *Chlamydia pneumoniae*-infected HeLa cells is independent of NF-κB activation (Fischer *et al*., 2001). A recent study has shown that signalling pathways that inhibit pathogen-induced macrophage apoptosis involves, in addition to NF-κB activation, activation of the cAMP responsive transcription factor CREB (Park *et al*., 2005). However, CREB activation in other cells types, such as neurones and β-cells, induces expression of anti-apoptotic genes such as Bcl-2 and IRS2 (Lonze *et al*., 2002; Mantamadiotis *et al*., 2002). In addition, CREB activation is involved in apoptosis resistance of human T-cell leukaemia virus-infected murine fibroblasts (Trevisan *et al*., 2004). Accordingly, it is possible that *L. pneumophila* induces, in addition to NF-κB, the activation of other transcription factor, such as CREB that promotes anti-apoptotic phenotype in MEF. However, protection against apoptosis in *L. pneumophila*-infected cells upon exposure to Staurosporin requires NF-κB, because *L. pneumophila*-infected MEF p65$^{-/-}$ and MEF IKKα$^{-/-}$ IKKβ$^{-/-}$ are sensitive to Staurosporin-induced apoptosis. These data clearly show that *L. pneumophila*-infected cells are remarkably resistant to strong apoptotic stimuli that trigger both the intrinsic and extrinsic pathways of apoptosis. This may be very crucial for the disease process, because apoptosis of the infected cells results in termination of intracellular proliferation of *L. pneumophila* (Abu-Zant *et al*., 2005). On the other hand, one may argue that *L. pneumophila* may not need to trigger anti-apoptosis during Legionnaires' disease. Although this is a possibility that cannot be excluded, it is more likely that the anti-apoptotic events play a role during the disease process. Our data clearly show that the Dot/Icm-mediated activation of NF-κB plays a role in protecting the infected cells from external apoptotic stimuli to maintain viability of the infected cells to ensure bacterial proliferation till termination of the intracellular infection.

Modulation of apoptosis within human macrophages by the *L. pneumophila* Dot/Icm type IV secretion system seems to be novel. At early stages of infection, the *L. pneumophila* Dot/Icm secretion system induces robust caspase-3 activation, but also triggers strong anti-apoptotic mechanism that is associated with upregulation of anti-apoptotic genes and sustained NF-κB activation. The strong anti-apoptotic signalling cascades may counteract the effect of caspase-3 activation during early stages of intracellular replication and, thus, delaying the

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induction of host cell apoptosis till late stages of infection. As *L. pneumophila* does not trigger apoptosis in its protozoan environmental hosts (Gao and Abu Kwaik, 2000b), it seems that interaction of *L. pneumophila* with the pro- and anti-apoptotic pathways is mammalian host-specific. The level of sophistication of interaction of *Legionella* with the pro- and anti-apoptotic machineries of human macrophages may argue against the idea that simple availability of bacterial transmission by droplets was sufficient for *Legionella* to make the jump from a protozoan parasite into a human pathogen, which has been proposed by several investigators (Cianciotto and Fields, 1992; Shuman *et al*., 1998; Harb *et al*., 2000).

Experimental procedures

Bacterial strain and eukaryotic cells

The virulent clinical isolate of *L. pneumophila* strain AA100 and its isogenic *dotA* mutant, and their cultivations have been described previously (Abu Kwaik and Engleberg, 1994; Zink *et al*., 2002). *L. pneumophila* AA100-GFP and *dotA*-GFP strains were created by transforming AA100 and *dotA* strains with pMIP-GFP plasmid vector that constitutively expresses GFP (Kohler *et al*., 2000). Human U937 and mouse J774A.1 macrophage cell lines were maintained in RPMI-1640 tissue culture medium (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine (Invetrogen, Carlsbad, CA) and were grown at 37°C in the presence of 5% CO_2. Before infection, U937 cells were differentiated for 48 h using phorbol 12-myristate 13-acetate (PMA) as described previously (Zink *et al*., 2002). Isolation and preparation of the hMDMs was carried out as described previously (Welsh *et al*., 2004). 3T3 MEF cell lines, MEF-WT, p65 knockout (MEF $p65^{-/-}$) and MEF $IKK\alpha^{-/-}$ $IKK\beta^{-/-}$ (Welsh *et al*., 2004) were obtained from Anthony Sinai (University of Kentucky) maintained in minimum essential medium (Hyclone, Logan, UT) supplemented with 7% FBS. For intracellular growth kinetic experiments, the cells were cultured in 96 well plates at a concentration of 1×10^5 cell per well. For confocal microscopy experiments, the cells were seeded onto glass coverslips in 24 well plates at a concentration of 5×10^5 cell per well. For microarray experiment, hMDMs were cultured in 6 well plates at a concentration of 1.5×10^6 cell per well.

Intracellular growth kinetics experiments

Unless otherwise indicated, cells were infected with either the parental strain AA100 or the mutant strain *dotA* at a moi of 10 (moi-1) for 1 h, followed by 3× washing with the cell culture medium and 1 h of gentamicin treatment (50 μg ml^{-1}) to remove extracellular bacteria. The numbers of cfu in infected monolayers were enumerated at the indicated time points after infection (Gao *et al*., 1998). For studying the effect of the NF-κB inhibitors, NBD peptide (Calbiochem, La Jolla, CA) and CAPE (Sigma, St Louis, MO), on intracellular growth kinetics of *L. pneumophila*, monolayers of macrophages were either left untreated, or treated with different concentrations of CAPE for 30 min prior to infection or with 50 μM NBD peptide 2 h prior to infection.

Confocal microscopy

In all confocal microscopy experiments, the cells were infected with bacteria at a moi of 10 for 1 h, followed by 3× washing and 1 h of gentamicin treatment (50 μg ml^{-1}) to remove extracellular bacteria. All experiments were done in triplicate and a minimum of 100 cells per sample were examined from different coverslips. On average, 8–15 0.2 μm serial *z* sections of each image were captured and stored for further analyses, using Adobe Photoshop 6.0 (Adobe Photoshop).

Apoptosis resistance and expression of TNF-α by L. pneumophila*-infected cells*

To examine the sensitivity of *L. pneumophila*-infected hMDMs to TNF-α- and Staurosporin-induced apoptosis, hMDMs infected with AA100-GFP or the mutant strain *dotA*-GFP were either left untreated or treated TNF-α (10 ng ml^{-1} $)^{-1}$ (BD Pharmingen, San Jone, CA) or different concentrations of Staurosporin (0.25, 0.5 or 1.0 μM) (Sigma, St Louis, MO). Treatment with TNF-α was carried out for 6 h starting at 7 h after infection in the presence of either 0.5 or 1.0 μg ml^{-1} Cycloheximide (Sigma, St Louis, MO). Treatment with staurosporine was carried out for 4 h starting at 8 h after infection. Control uninfected monolayers were either left untreated or treated with the above mentioned concentrations of TNF-α/CHX or staurosporine for the same time periods. Then, the cells were fixed at room temperature with 4% paraformaldehyde in 1× phosphate-buffered saline (PBS) (pH 7.2) for 30 min, followed with 3× washing with 1× PBS. The fixed cells were permeabilized on ice with 0.1% Triton X100 and 0.01% sodium citrate in PBS for 15 min, followed by 3× washing with 1× PBS. Labelling for apoptotic nuclei was carried out by TUNEL using In Situ Cell Death Detection kit, TMR red as recommended by the manufacturer (Roche Diagnostics, IN). Infected cells were examined for apoptotic nuclei by laser scanning confocal microscopy. To examine the sensitivity of *L. pneumophila*-infected hMDMs cells to CAPE-induced apoptosis, hMDMs were treated with 20 μg ml^{-1} CAPE for 30 min prior to infection. Then, the cells were infected with the parental strain AA100-GFP. At 8 h after infection, the cells were labelled and examined for apoptotic nuclei. To examine the sensitivity of *L. pneumophila*-infected MEF to staurosporine-induced apoptosis, monolayers of MEF-WT, MEF $p65^{-/-}$, and MEF $IKK\alpha^{-/-}$ $IKK\beta^{-/-}$ cell were infected with the parental strain AA100-GFP. At 8 h after infection, uninfected control cells and infected cells were either left untreated or treated with 0.3 μM staurosporine for 4 h. Then, the cells were labelled and examined for apoptotic nuclei. To study the effect of NBD peptide on apoptosis induction in *L. pneumophila*-infected macrophages, hMDMs were either left untreated or treated for 2 h with 50 μM of either the NBD peptide or NBD-NC peptide (Calbiochem, La Jolla, CA). Then, the cells were either left uninfected or infected with AA100-GFP. At 12 h after infection, the cells were labelled and examined for apoptotic nuclei.

To examine expression of TNF-α- by *L. pneumophila*-infected hMDMs, the infection and treatments of the cells was performed as described above. The supernatant of monolayers was obtained at 2 h or 8 h post infection of macrophages. The levels of TNF-α was determined by ELISA assays using OptiEIA sets, as described by the manufacturer (BD Pharmingen, San Jone, CA). Measurements were performed using an E-max Precision plate reader (Molecular Devices, Sunnyvale, CA). The levels of

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TNF-α were derived from a standard curve of known quantities of TNF-α subjected to same measurements.

Nuclear translocation of the p65 and p50 subunits of NF-κB in L. pneumophila*-infected cells*

In all experiments, uninfected untreated cells and *E. coli* LPS-treated cells (1 μg ml^{-1} for 20 min) (Sigma, St Louis, MO) were used as a negative and positive controls, respectively, for nuclear translocation of the p65 and p50 subunits of NF-κB. To examine nuclear translocation of the p65 or p50 subunit in *L. pneumophila*-infected cells, monolayers of hMDMs MEF-WT, MEF $p65^{-/-}$, and MEF $IKK\alpha^{-/-}$ $IKK\beta^{-/-}$ infected with either AA100-GFP or *dotA*-GFP. At the indicated time points after infection (see *Results*), the cells were fixed and permeabilized as mentioned above. Then, the cells were blocked with 3% bovine serum albumin in RPMI with 10% FBS for 1 h at 37°C. Immunofluorescence labelling for the p65 subunit of NF-κB was carried out in blocking buffer using Rabbit polyclonal anti-p65 or anti-p50 antibodies primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), followed $Alexafluor^{555}$ donkey anti-Rabbit IgG secondary antibodies (1:4000 fold dilution) (Molecular Probes, Eugene, OR). Fluorescence labelling for nuclei was carried out using TOTO-3 iodide (Molecular Probes, Eugene, OR) at 1:4000 fold dilution, which was used together with the secondary antibodies. To examine the role of *L. pneumophila* LPS in the induction of nuclear translocation of the p65 subunit, hMDMs were infected with either formalin-killed AA100-GFP or formalin-killed *dotA*-GFP. Nuclear translocation of the p65 subunit was examined at 2 and 8 h after infection. For studying the effect of NBD peptide on LPS- and *L. pneumophila*-induced nuclear translocation of the p65 subunit, hMDMs were either left untreated or treated with 50 μM of either the NBD peptide or NBD-NC peptide for 2 h prior to infection or LPS treatment. Untreated and treated cells were infected with the AA100-GFP. Nuclear translocation of the p65 subunit in infected cells was examined at 2 and 8 h after infection. NBD peptide- and NBD-NC peptide-treated cells were exposed to *E. coli* LPS at 1 μg ml^{-1} for 20 min. To examine the effect of CAPE on LPS- and *L. pneumophila*-induced nuclear translocation of the p65 subunit, hMDMs were treated with 20 μg ml^{-1} CAPE for 30 min prior to infection or LPS treatment. Untreated and treated cells were infected with AA100-GFP or *dotA*-GFP. Nuclear translocation of the p65 subunit was examined in infected cells at 2 and 8 h after infection. To examine the effect of CAPE on LPS-induced nuclear translocation p65, CAPE-treated cells were exposed for 20 min to 1 μg ml^{-1}*E. coli* LPS.

Microarray and real-time PCR

Expression level of apoptosis-related genes in *L. pneumophila*-infected hMDMs was determined using GEArray HS-002 kit (SuperArray Bioscience, Frederick, MD). Monolayers of hMDMs (~1.5×10^6 cell) were infected with either the parental strain AA100 or *dotA* mutant strain at a moi of 50 for 15 min, followed by 3× washing with the cell culture medium and 1 h of gentamicin treatment (50 μg ml^{-1}) to remove extracellular bacteria. At 8 h after infection, total RNA was extracted from both uninfected and infected hMDMs using RNeasy Mini Kit (Qiagen, Valencia, CA) as recommended by the manufacturer. RNA integrity was assessed by visualizing ethidium promide-stained 1% agarose gel. Approximately, 5 μg of total RNA was used for cDNA probe synthesis. Synthesis and Biotin-16-dUTP (Roche Diagnostics, Indianapolis, IN) labelling of the cDNA probes was carried out using AmpLabelling-LPR Kit (SuperArray Bioscience, Frederick, MD) as recommended by the manufacturer. GEArray nylon membranes pre-hybridization, hybridization with the Biotin-16-dUTP labelled probes, and washing steps was carried out according to GEArray™ Q and S series kits user manual instructions (SuperArray Bioscience, Frederick, MD). Chemiluminescence detection was carried out using Chemiluminescence Detection Kit (SuperArray Bioscience, Frederick, MD) as recommended by the manufacturer. Image data acquisition and gene expression analysis were carried out using *ScanAlyze* and *GEAArray* Analyzer softwares respectively (http://www.GEASuite.superarray.com). The increase in the expression level of each of the apoptosis related genes in AA100- or *dotA*-infected cells was determined using a cut-off value of 1.5-fold.

Real-time quantitative PCR (qPCR) was used to confirm the increase the expression level of anti-apoptotic genes in AA100-infected hMDMs. Approximately, 5 μg of total RNA isolated from both uninfected and AA100-infected was treated with DNase I (Ambion, Austin, TX) at 37°C for 30 min and converted to first-strand cDNA by random priming using Superscript™ III Reverse Transcriptase kit as recommended by the manufacturer (Invetrogen, Carlsbad, CA). The generated cDNA was fivefold diluted with RNase free water. Real-time qPCR was done using the DNA Engine Opticon System (MJ Research). Real-time qPCR was carried out in triplicate using the DyNAmo SYRB Green qPCR Kit in 20 μl reaction volume, as recommended by the manufacturer (New England Biolabs, Ipswich, MA). Each of the indicated anti-apoptotic genes was amplified by a specific set of primers (SuperArray Bioscience, Frederick, MD), using 2 μl of the fivefold diluted cDNA/qPCR reaction as a template. PCR conditions were 5 min at 94°C, 15 s at 96°C and 15 s at 72°C for 30 cycles. The concentration of each gene was determined by the comparative C_T method (threshold cycle number at the cross-point between amplification plot and threshold) and normalized values to the internal *β-actin* gene control.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Representative confocal laser scanning microscopy images and quantification of nuclear translocation of the p50

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subunit of NF-κB. Monolayers of hMDMs were infected with either the parental strain AA100-GFP or the *dotA*-GFP mutant strain at a moi of 10 for 1 h. The cells were labelled for nuclei using TOTO-3 (Blue), and anti-p50 antisera followed by secondary antibodies (Red). The images are representative of three independent experiments. Quantitative analyses on the right are results representative of the mean of three independent experiments ± standard deviations.

Fig. S2. Extracellular *L. pneumophila* does not trigger nuclear translocation of the p65 subunit of NF-κB. Quantitative analyses of p65 nuclear translocation in hMDMs infected by *L. pneumophila* in presence of cytochalasin D to block bacterial entry. The infection and labelling are identical to Fig. 4 with the exception that during infection cytochalasin D was used to block bacterial entry. Monolayers of hMDMs were infected with live or formalin-killed (FK) AA100-GFP (A) *dotA*-GFP (B) strain at a moi of 10 for 1 h. At several time points after infection, the cells were labelled for the p65 subunit of NF-κB and nuclei. Uninfected monolayers and *E. coli* LPS-treated monolayers were included as negative and positive controls. Approximately 100 infected macrophages were analysed from different coverslips.

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