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LnaB: a *Legionella pneumophila* activator of NF-κB

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Abstract

Legionella pneumophila possesses a large arsenal of type IV translocated substrates. Over 100 such proteins have been identified, but the functions of most are unknown. Previous studies have demonstrated that *L. pneumophila* activates NF-κB, a master transcriptional regulator of the mammalian innate immune response. Activation of NF-κB is dependent on the *Legionella* Icm/Dot type IV protein translocation system, consistent with the possibility that translocated bacterial proteins contribute to this response. To test this hypothesis, an expression library of 159 known and putative translocated substrates was created to evaluate whether ectopic production of a single *L. pneumophila* protein could activate NF-κB in mammalian cells. Expression of two of these proteins, LnaB (*Legionella* NF-κB Activator B) and LegK1, resulted in ~150-fold induction of NF-κB activity in HEK293T cells, levels similar to the strong induction that occurs with ectopic expression of the known activator Nod1. LnaB is a substrate of the Icm/Dot system, and in the absence of this protein, a partial reduction of NF-κB activation in host cells occurs after challenge by post-exponential phase bacteria. These data indicate that LnaB is an Icm/Dot substrate that contributes to NF-κB activation during *L. pneumophila* infection in host cells.

Introduction

Legionella pneumophila is a Gram negative facultative intracellular bacterial pathogen. Upon inhalation from contaminated water sources *L. pneumophila* can replicate in human alveolar macrophages and epithelial cells leading to a severe pneumonia known as Legionnaires' disease (Chiaraviglio *et al.*, 2008, Fraser, 2005). In the host cell, *L. pneumophila* replicates within a membrane bound vacuole that avoids fusion with late endosomes and lysosomes (Horwitz, 1983b, Horwitz *et al.*, 1984). Instead, the *Legionella*-containing vacuole (LCV) recruits ER-derived secretory vesicles to its surface, altering the morphology of the vacuole to a compartment resembling rough ER (Derre *et al.*, 2004, Horwitz, 1983a, Kagan *et al.*, 2002, Kagan *et al.*, 2004, Tilney *et al.*, 2001). Intracellular growth and evasion of lysosomal fusion depends on Icm/Dot, a chromosomally encoded type IV secretion system (Segal *et al.*, 1998, Vogel *et al.*, 1998). Icm/Dot is made up twenty-six gene products predicted to assemble into a multi-protein membrane spanning complex (Vincent *et al.*, 2006).

Like other bacterial specialized secretion systems, *Legionella's* Icm/Dot system allows the translocation of a large arsenal of proteins known as "Icm/Dot Translocated substrates" (IDTS) (Zusman *et al.*, 2007). Studies over the last six years has led to the identification of approximately 100 proteins that may be delivered by the type IV secretion system into host

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cells (Kubori *et al.*, 2008, Ninio *et al.*, 2007). Of these IDTS, approximately 15 proteins have described activities that include altering vesicle trafficking (Ingmundson *et al.*, 2007, Liu *et al.*, 2007, Machner *et al.*, 2006, Machner *et al.*, 2007, Murata *et al.*, 2006, Pan *et al.*, 2008, Shohdy *et al.*, 2005), promoting macrophage survival (Banga *et al.*, 2007, Laguna *et al.*, 2006), and inhibiting eukaryotic translation (Belyi *et al.*, 2006, Belyi *et al.*, 2008, de Felipe *et al.*, 2005), and mediating host cell release (Chen *et al.*, 2004). However, the majority of the identified IDTS are of unknown function and furthermore, deletion of a single IDTS gene rarely alters intracellular growth (Luo *et al.*, 2004, Machner *et al.*, 2006, Nagai *et al.*, 2002, Ninio *et al.*, 2005, Shohdy *et al.*, 2005). The lack of phenotype is often attributed to the possibility that IDTS are functionally redundant to one another, with multiple proteins targeting a single pathway required for intracellular growth. This is also a common theme with secreted substrates of other bacterial pathogens. The deletion of individual substrates of the type III secretion systems from *Shigella flexneri*, *Salmonella sp*, and Enterohaemorrhagic *Escherichia coli* rarely has strong effects on the pathogenesis of these bacteria. Therefore, additional tools are needed to identify the activities of these translocated proteins and determine how they manipulate cell signaling to promote intracellular growth.

One strategy researchers have used is genetic screens to identify translocated proteins that interfere with essential pathways in *Sacchromyces cerevisiae* (Campodonico *et al.*, 2005, Heidtman *et al.*, 2008, Lesser *et al.*, 2001, Shohdy *et al.*, 2005, Sisko *et al.*, 2006). Although *S. cerevisiae* has served as a useful model system to study bacterial translocated proteins, other expression systems are necessary to evaluate protein activities directed towards pathways that are not conserved in lower eukaryotes. Previous work showed that *L. pneumophila* activates the mammalian transcription factor, NF-κB (Abu-Zant *et al.*, 2007, Bartfeld *et al.*, 2009, Losick *et al.*, 2006, Shin *et al.*, 2008a). Activation could be shown to occur via two pathways: a Toll-like receptor (TLR) dependent path, as well as one that responds to the Icm/Dot translocation system (Abu-Zant *et al.*, 2007, Bartfeld *et al.*, 2009, Losick *et al.*, 2006, Shin *et al.*, 2008a). All previous studies agree that the TLR-dependent response occurs shortly after contact of bacteria with host cells before being down-modulated, whereas the Icm/Dot-dependent activation was persistent and occurred throughout the replication cycle (Abu-Zant *et al.*, 2007, Bartfeld *et al.*, 2009, Losick *et al.*, 2006, Shin *et al.*, 2008a).

NF-κB is one of the master regulators of the mammalian innate immune response leading to activation of pro-inflammatory cytokines, chemokines, and cell survival genes (Karin *et al.*, 2002). Most research has focused on activation of NF-κB through the detection of pathogen associated molecular patterns (PAMPs) that are common to all bacteria, such as lipopolysacchride, peptidoglycan, flagellin, and lipoproteins. The membrane bound Toll-like receptors (TLR) and the cytoplasmic sensors of the Nod-like receptor family (NLR) have been implicated in detecting these PAMPs (Shaw *et al.*, 2008, Uematsu *et al.*, 2008). However, there is emerging evidence that translocated proteins may also play a role in inducing NF-κB activation. For instance, *Helicobacter pylori* delivers the protein CagA via its type IV secretion system into mammalian cells (Backert *et al.*, 2000, Stein *et al.*, 2000). CagA was shown to directly contribute to activation of the innate immune response by activating NF-κB (Brandt *et al.*, 2005). Even ectopic expression of CagA in mammalian cells is sufficient to activate the NF-κB pathway (Brandt *et al.*, 2005). More recently it was shown that LegK1, a *L. pnenumophila* IDTS, activated NF-κB when ectopically expressed in cultured cells, consistent with the idea that Legionella IDTS may be involved in activating this transcription factor (Ge *et al.*, 2009).

L. pneumophila could have multiple proteins contributing to the NF-κB response, so we determined whether the ectopic expression of known or putative *L. pneumophila* IDTS could

be sufficient to activate this pathway. Here we describe the identification of two proteins that can strongly induce an NF-κB response in HEK293T cells.

Results

NF-κB activation is independent of Rip2

Previous studies from our laboratory showed that in bone marrow (BM) macrophages NF-κB activation can occur in the absence of either the TLR signaling adaptor MyD88 or the cytoplasmic peptidoglycan sensor Nod1 (Losick *et al.*, 2006). That signaling can still take place in the absence of TLR signaling is confirmed by the use of macrophages that are lacking the two adaptors MyD88 and Trif, which should eliminate all known TLR signaling (Supplementary Figure S1). Nod1 has been shown to sense the peptidoglycan degradation product iE-DAP released into the host cell cytoplasm (Chamaillard *et al.*, 2003, Girardin *et al.*, 2003a). There is a second peptidoglycan sensor Nod2 (Girardin *et al.*, 2003b, Inohara *et al.*, 2003), and both Nod proteins feed downstream to the protein kinase, Rip2, during peptidoglycan signaling to NF-κB (Kobayashi *et al.*, 2002, Park *et al.*, 2007). To determine whether these cytoplasmic sensors could be functioning redundantly to one another we used BM macrophages from Rip2 knockout mice that retain TLR signaling (Kobayashi *et al.*, 2002).

BM macrophages from C57Bl/6J (B6) or the Rip2 knock out (Rip2KO) were infected with *L. pneumophila ΔflaA* strains, which are permissive for intracellular growth within this mouse strain background (Machner *et al.*, 2006, Molofsky *et al.*, 2006, Ren *et al.*, 2006). NF-κB activation was measured at 7 hours post infection (hpi) by staining for the subcellular localization of NF-κB in *L. pneumophila* infected macrophages within the monolayer (Losick *et al.*, 2006). Using this assay we observed that *L. pneumophila* was still able to cause efficient nuclear translocation of NF-κB independently of Rip2, since ~80% of the macrophages harboring wild-type (Lp02 *ΔflaA*) bacteria had strong nuclear staining of NF-κB (Fig. 1, data not shown). As was observed previously, the Icm/Dot mutant (*dotA ΔflaA*) had a reduction in the percent of cells with NF-κB in the nucleus, indicating that NF-κB translocation is dependent on *L. pneumophila* Icm/Dot system in both B6 and Rip2KO macrophages (Fig. 1; *T*-test p-value ≤ 0.01). This is consistent with recent findings in Rip2KO macrophages in which it was observed that IκBα, the negative regulator of NF-κB, was degraded in Icm/Dot dependent manner (Shin *et al.*, 2008a). Therefore, we predicted that Icm/Dot translocated substrates (IDTS) were likely contributing to NF-κB activation, since peptidoglycan signaling through Rip2 was not required for this response in BM macrophages.

Construction of a *L. pneumophila* Icm/Dot translocated substrate library to assay for NF-κB activity

There are approximately 85 proteins identified to have translocation signals recognized by the Icm/Dot system. Studies from our laboratory indicate that an additional 100 or more proteins could be delivered into the host cell via the Icm/Dot system (L. Huang, and R. Isberg, unpublished). We hypothesized that some of these proteins could be contributing to NF-κB activation. To do so, a library of 159 known and candidate *L. pneumophila* IDTS was created to evaluate whether the ectopic expression of IDTS would be sufficient to activate NF-κB. Eighty of the 159 proteins in the library have been demonstrated to be secreted by at least one of the following approaches: direct immmunofluorescence (Conover *et al.*, 2003, Laguna *et al.*, 2006, Machner *et al.*, 2006), Cre/*loxP* interbacterial translocation assay (Luo *et al.*, 2004), CyaA reporter system (Chen *et al.*, 2004), β-lactamase translocation assay (de Felipe *et al.*, 2008), and/or a protein fusion translocation assay (Huang, unpublished, Luo *et al.*, 2004, VanRheenen *et al.*, 2006) (Table S1). Other *Legionella*

proteins included were either paralogs of known substrates, proteins with eukaryotic-like domains (de Felipe *et al.*, 2005), proteins regulated by the response regulator, PmrA (Zusman *et al.*, 2007), or hypothetical proteins unique to *L. pneumophila* (Table S1). *L. pneumophila* genes were cloned using the Gateway® system and expressed in mammalian cells as fusion proteins to the green fluorescent protein (GFP) (Supplementary Figure S2). The expression of most of these GFP bacterial fusions could be detected by Western blot with anti-GFP antibodies. Of the 159 GFP fusions, full-length proteins were detected for 120 (~75% of total number; data not shown).

NF-κB activation in HEK293T cells is dependent on *L. pneumophila's* Icm/Dot translocation system and is independent of flagella recognition

A transfectable mammalian system was required to test whether the ectopic production of the cloned IDTS could stimulate NF-κB. Human embryonic kidney (HEK293T) cells and the NF-κB luciferase reporter were chosen, since HEK293T cells are easily transfectable and the NF-κB luciferase reporter assay is a sensitive detection method. To evaluate this reporter system we first tested the NF-κB response to *L. pneumophila* in the HEK293T cell-type, to verify that the pattern of NF-κB activation is similar to our previously characterized human and mouse macrophage systems (Losick *et al.*, 2006). As we observed previously in a human macrophage cell line (U937 cells), NF-κB activation in HEK293T increased over a time course of infection (Fig. 2A). By 4 hours post infection (hpi) there was a 5-fold increase in NF-κB activity, and by 10 hpi there was 25-fold increase in activity compared to uninfected cells (Fig. 2A). In agreement with our previous studies in macrophages, NF-κB activation in HEK293T was dependent on the Icm/Dot translocation system. The *dotA* strain did not significantly stimulate NF-κB activity (Fig. 2A). This was in contrast to previous studies that showed an early response in the absence of Icm/Dot, using much higher MOI than used in this figure (Abu-Zant *et al.*, 2007, Bartfeld *et al.*, 2009).

Unlike mouse bone marrow derived macrophages, HEK293T cells are known to express Toll-like receptor 5, which senses bacterial flagellin (Gewirtz *et al.*, 2001). Flagellin is also known to be important for promoting uptake of *L. pneumophila* (Molofsky *et al.*, 2005), and high MOI infections of an immortalized lung epithelial line have been associated with a flagellin-dependent NFκ-B response (Bartfeld *et al.*, 2009, Molofsky *et al.*, 2005). To determine whether flagellin sensing could be contributing to the NF-κB response in HEK293T cells, we challenged cells with an *L. pneumophila* flagellin deficient strain, *ΔflaA* (Ren *et al.*, 2006). At 8 hpi, the *ΔflaA* strain could induce NF-κB activity, but the induction was dependent on the multiplicity of infection (MOI) (Fig. 2B). Only by infecting cells with *ΔflaA* strain at MOI=100 was there an equivalent induction of NF-κB activity when compared to the wild-type (Lp02) strain at MOI=1 (Fig. 2B). The lower levels of NF-κB activation in the Δ*flaA* strain appeared to be due to a defect in cell association. There was a 38-fold defect in the uptake of bacteria at 2 hpi (MOI 1) for the *ΔflaA* strain, as measured by plating for colony forming units (CFU) (Fig. 2C). When the dose of the *ΔflaA* strain was increased to MOI = 100, the dose at which NF-κB activation by this strain was identical to Lp02, the uptake efficiencies of the Lp02 and *ΔflaA* were identical (Fig. 2C). In contrast, there was low NF-κB activation by the Icm/Dot-defective mutant *dotA*, but increasing the MOI could not increase NF-κB activation levels to that observed in the Lp02 strain (Fig. 2B). When cells were challenged with the *dotA* strain at MOI=10, there was no uptake defect of the mutant (Fig. 2C). Therefore when these defects are taken into account, the Icm/Dot translocation system, but not flagella, significantly contributes to NF-κB activation consistent with our previous finding in human and mouse macrophages. The HEK293T cell type and the NF-κB luciferase reporter together are a valid system to test whether *L. pneumophila* IDTS can activate NF-κB.

L. pneumophila IDTS can activate NF-κB

HEK293T cells were co-transfected with plasmids encoding the NFκB-luciferase reporter and GFP fusion proteins (Fig. 3A), then assayed for luminescence (Experimental Procedures). As a positive control, the luciferase reporter construct was co-transfected with a vector encoding GFP-Nod1, which strongly stimulates NF-κB (Inohara *et al.*, 1999,Bertin *et al.*, 1999). Of the 159 proteins tested, the expression of 13 *L. pneumophila* proteins each caused more than 3-fold increase in NF-κB activity relative to empty vector. These were placed in three groups based on expression levels observed in HEK293T cells (Fig. 3A). Those causing milder effects included known translocated substrates LidA, SidM, SidE, SidA, SdbA, VpdA, SidH, LegA5 and LegA12, as well as candidate substrates, SdeB (a paralog of SidE) and SdhB (a paralog of SidH) (Figs. 3B and 3C). Of the ones causing mild effects, the most striking IDTS was VpdA, which showed 14-fold induction of the reporter despite being poorly expressed (Fig. 3A and 3B).

Of the 159 *L. pneumophila* genes screened, expression of two candidate *L. pneumophila* IDTS LegK1 and Lpg2527 caused ~150-fold induction of NF-κB activity (Figs. 3A and 3C). As was true of VpdA, LegK1 was poorly expressed, but it generated 10-fold higher induction relative to VpdA, consistent with previous observations (Ge *et al.*, 2009). The gene for the candidate IDTS Lpg2527, which was more efficiently expressed than LegK1, was named *lnaB* for *Legionella* NF-κB activator B, and encodes a predicted protein that has no sequence similarity to any known proteins. LegK1, on the other hand, is predicted to be a serine/threonine kinase (de Felipe *et al.*, 2005), with a conserved catalytic asparatic acid residue at amino acid 223. As previously demonstrated, mutation of this residue to alanine in the GFP-LegK1 construct (D223A) eliminated induction of the reporter encoding LegK1 in HEK293T cells (Fig. 4).

The coiled-coil domain in LnaB is required for NF-κB activation

LnaB is a 558 amino acid protein with a small putative coiled-coil domain located between 361-401 amino acids. Truncation and deletion constructs of LnaB were generated to determine whether NF-κB activity was dependent on the coiled-coil domain. LnaB was divided into regions that included or excluded the coiled-coil domain, and an in-frame deletion of the coiled domain (ΔCC). All constructs were designed to be in-frame with EGFP at the N-terminus (Fig. 5A). Co-transfection of HEK293T cells with the NF-κB reporter and EGFP-LnaB fusions revealed that the N-terminal region of LnaB including the coiled-coil domain (amino acids 1-401) was sufficient to induce NF-κB activity (Fig. 5C). However, the level of NF-κB activity in the presence of this N-terminal fragment was reduced to one third of the activity observed for full-length protein: the full-length EGFP-LnaB fusion resulted in ~120 fold induction, whereas the EGFP-$LnaB_{1\text{-}401}$ construction showed only a ~40 fold increase in luminescence (Fig. 5C). The coiled-coil domain was required for activation, because expression of the ΔCC construct, or one containing the amino terminus truncated at the amino terminus of this domain (amino acid residues 1-361) showed almost no NF-κB induction (Fig. 5C). Each of these fusions showed very similar amounts of protein expression, arguing that the differences in phenotypes were due to the loss of specific amino acid residues (Fig. 5B). Similarly, shorter fusions also failed to show induction (Fig. 5C), and these showed even more robust expression than the wild type fusion (Fig. 5B).

Unlike GFP-LegK1, which localized diffusely in the cytoplasm of HEK293T cells (data not shown), GFP-LnaB localized to the nuclear envelope and to a perinuclear region reminiscent of an ER compartment in all mammalian cell lines tested including HEK293T and Hela cells (Fig. 5D; data not shown). To determine whether NF-κB activity correlated with ER localization, Hela cells were co-transfected with the EGFP-LnaB fusions and the ER

membrane protein Sec61 fused to the fluorescent protein mCherry. By fluorescence microscopy it appeared that the 401-558 amino acid C-terminal region was sufficient for ER localization (Fig. 5D, EGFP panel). In contrast, the residue 1-401 fragment that has NF-κB activating activity was diffuse within the cell and did not co-localize with Sec61 (Fig. 5D, EGFP panel). Therefore, the region of the protein that promotes NF-κB activation can be separated from the region that allows ER localization, indicating that ER localization of LnaB is not required for NF-κB activation.

LnaB is a substrate of *L. pneumophila's* Icm/Dot translocation system

LnaB was fused to the catalytic domain of *Bordetella pertussis* adenylate cyclase protein, CyaA, to determine whether this protein is a substrate of *L. pneumophila's* Icm/Dot translocation system. The cytoplasmic activation of CyaA by calmodulin leads to an increase in cAMP in the host cell (Sory *et al.*, 1994). The cAMP level can then be quantified by ELISA, thereby acting as an indicator of the translocation of the CyaA fused protein. The human U937 cell-line was incubated with *L. pneumophila* expressing CyaA (negative control), CyaA-RalF (positive control), or CyaA-LnaB, and assayed for cAMP levels after 1 hour. The Icm/Dot deficient strain, *dotA*, was used as negative control. Both Lp02 CyaA-RalF and Lp02 CyaA-LnaB resulted in a ~30-fold increase in cAMP as compared to uninfected or Lp02 CyaA infected U937 cells (Fig. 6A). The cAMP induction by CyaA-RalF and CyaA-LnaB was dependent on the Icm/Dot system, since there was no significant increase in cAMP with the *dotA* strains (Fig. 6A). CyaA fusions were expressed at high levels in all bacterial strains before infection (Fig. 6B). These data demonstrate that LnaB has an Icm/Dot translocation signal. A recent study also presented evidence that LnaB (designated lpg2527) is a Icm/Dot substrate (Kubori *et al.*, 2008), supporting our independent observations.

LnaB and *legK1* are dispensable for intracellular growth, but *lnaB* contributes to NF-κB activity

As LnaB and LegK1 can activate NF-κB when ectopically expressed, we next determined if knockout mutations in *ΔlnaB* or *ΔlegK1* affect *L. pneumophila* intracellular growth and/or NF-κB activation. The in-frame deletions *ΔlnaB* or *ΔlegK1* were generated in the wild-type Lp02 strain and assayed for the ability to grow intracellularly. Mouse macrophages were challenged with Lp02, *ΔlnaB*, or *ΔlegK1* mutants to assay for their efficiencies of intracellular growth. *L. pneumophila* growth was monitored over a three-day growth curve by lysing macrophages and enumerating CFUs. As is true of many strains harboring single IDTS mutation (Luo *et al.*, 2004, Machner *et al.*, 2006, Nagai *et al.*, 2002, Ninio *et al.*, 2005, Shohdy *et al.*, 2005, VanRheenen *et al.*, 2006), the absence of *ΔlnaB* or *ΔlegK1* did not appear to influence *L. pneumophila* intracellular growth. The growth of both deletion strains was equivalent to wild-type Lp02 (Fig. 7A, B).

To investigate whether strains lacking *lnaB* and *legK1* are defective for initiating intracellular replication, bacterial replication at the single cell level was followed after low MOI challenge at time points in which NF-κB activation was maximal (Fig. 7C,D). At 9 h post-infection, the wild type strain (Lp02), mutants lacking either *lnaB* or *legK1*, as well as a double mutant lacking both genes, were equally efficient at initiating replication in bone marrow derived macrophages. For each strain, almost the entire population of infected macrophages showed multiple intracellular bacteria, with intracellular loads nearly equivalent for each of the strains (Fig. 7C,D). Consistent with this observation, the lack of these two translocated substrates had little effect on the survival of macrophages harboring bacteria. For each of the single mutants, as well as the double *ΔlnaBΔlegK1* mutant, greater than 60% of the infected macrophages showed normal nuclei after 9 hours incubation with bacteria, with similar kinetics of cell death after challenge with each strain (Fig. 7E).

Similarly, there was little effect of the mutations on avoiding targeting to a late endosomal compartment (Fig. 7F), based on colocalization of the late endosomal marker LAMP-1. For each of the mutants, there were similar levels of co-localization of the replication vacuole with LAMP-1, although strains harboring the*ΔlnaBΔlegK1* mutations showed a small increase relative to wild type (Fig. 7F).

To assay for NF-κB activation, HEK293T cells transfected with the NF-κB reporter were challenged with *L. pneumophila* strains for 7 hours and luciferase activity was determined and normalized to CFU to ensure that the readout was not affected by bacterial load (Experimental Procedures). The deletion of *ΔlegK1* did not affect NF-κB activity when assayed in this system (Fig. 8A). In contrast, the absence of *ΔlnaB* modestly reduced NF-κB activation, and this phenotype could be complemented by re-integration of $lnaB^+$ on the chromosome (Fig. 8B; *T*-test p-value < 0.05). The double mutant lacking both *lnaB* and *legK1* showed no further reduction in NF-κB activation (data not shown). Western blots of extracts probed with anti-LnaB serum demonstrated that the deletion mutant expressed no detectable LnaB, whereas the strain having a chromosomally encoded $lnaB^+$ showed levels of expression equivalent to the wild-type Lp02 (Fig. 8C). The strains were grown to post-exponential phase prior to infection when LnaB is most highly expressed (Fig. 8D). Therefore the delivery of LnaB during *L. pneumophila* infection contributes to NF-κB activation when *L. pneumophila* is grown to post-exponential phase.

Discussion

In this study we identified *Legionella pneumophila* Icm/Dot translocated substrates (IDTS) that have an impact on host cell signaling when expressed within mammalian cells. A large swath of known and potential IDTS was chosen, based on either bioinformatics or the presence of an experimentally identified translocation signal, and plasmids encoding these genes were introduced into a screening system that allowed rapid identification of a phenotype. The NF-κB-luciferase screening procedure described here was developed because previous studies demonstrated that NF-κB activation was dependent on the presence of the Icm/Dot translocation system (Abu-Zant *et al.*, 2007, Losick *et al.*, 2006, Shin *et al.*, 2008a). Here we demonstrate that several known IDTS modestly activated an NF-κB-luciferase reporter, whereas two candidate IDTS expressed in cultured cells, LnaB and LegK1, resulted in robust activation. Full activation of this promoter after challenge of cells with post-exponential phase *L. pneumophila* required the presence of LnaB.

In an approach that was similar to the one used here, LegK1 was shown to induce expression of an NF-κB reporter in cultured cells (Ge *et al.*, 2009). Interestingly LegK1 was shown to phosphorylate IκB in mammalians cells, although it is unclear whether this activity occurs during a natural infection, particularly in the natural host amoebae, which have no known NF-κB signaling systems. When *legK1* was deleted, bacteria lacking this protein showed normal levels of NF-κB activation, suggesting that LegK1 may be a minor player in inducing NF-κB *in vivo*. Therefore, the exact role of this protein during *Legionella* infection is not known.

The activation of NF-κB in response to low doses of *L. pneumophila* has been hypothesized to positively contribute to intracellular growth by upregulating genes encoding antagonists of apoptosis (Abu-Zant *et al.*, 2007, Losick *et al.*, 2006, Bartfeld et al., 2009, Shin et al. 2008a). In addition, a conflicting property of this level of control is that NF-κB activates an anti-microbial response. The best characterized routes of NF-κB activation in response to bacterial infection occurs through the stimulation of surface localized Toll-like receptors (TLR) and/or the cytoplasmic peptidoglycan sensors Nod1 and Nod2 via common molecules liberated by both pathogenic and nonpathogenic microbes (Shaw *et al.*, 2008, Uematsu *et*

al., 2008). *L. pneumophila* activation of NF-κB can occur independently of TLR signaling, as macrophages lacking the TLR adaptor MyD88 (Losick *et al.*, 2006), or double knockouts of both required TLR adaptors MyD88 and Trif (Supplementary Figure S1) still support NF-κB nuclear localization in response to *L. pneumophila*.

The role of Nod proteins in Icm/Dot-dependent signaling to NF-κB is not clearcut. Rip2 knock-out macrophages, missing the downstream kinase necessary for Nod1 and Nod2 signaling to NF-κB (Inohara *et al.*, 2000, Kim *et al.*, 2008, Ogura *et al.*, 2001) supported Icm/Dot-dependent NF-κB nuclear translocation (Fig. 1), paralleling our previous results with Nod1 knock out macrophages (Losick *et al.*, 2006). Therefore, there exists a peptidoglycan-independent, Icm/Dot-dependent signal to NF-κB in bone marrow macrophages. However, recent results from Shin et al., (2008a) suggest that the Rip2-independent, Icm/Dot-dependent response appears to require the TLR system in bone marrow macrophages. We tested NF-κB activation in HEK293T cells knocked down for Nod1 and found that NF-κB activation was reduced in response to *L. pneumophila* infection (Supplementary Figure S3). HEK293T cells do not express a number of TLRs present in bone marrow macrophages suggesting there may indeed be cross talk between TLR and Nod signaling to activate NF-κB in response to *L. pneumophila*. So perhaps the Rip2-independent activation in bone marrow macrophages results from cross talk between an IDTS and TLR-signaling, or due to an IDTS preventing downregulation of TLR signaling. This emphasizes the hypothesis that multiple IDTS could affect NF-κB activation via mechanistically distinct, but redundant, strategies.

The novel strong NF-κB activator we identified was LnaB (Lpg2527). We and Kubori *et al.*, 2008 have demonstrated that Lpg2527 is translocated into host cells via the *L. pneumophila* Icm/Dot translocation system. In addition, eight of the eleven modest NF-κB activators are previously characterized IDTS (LidA, SidM, SidA, SidE, SidH, VpdA, LegA12, and LegA5) (Cambronne *et al.*, 2007, Luo *et al.*, 2004, Machner *et al.*, 2006, Murata *et al.*, 2006, VanRheenen *et al.*, 2006). Therefore multiple *L. pneumophila* translocated proteins could contribute to NF-κB activation when introduced into the host cell during infection, indicating that there may be more than one route that leads to manipulation of the NF-κB signaling pathway. In line with this argument is the behavior of an *L. pneumophila ΔlnaB* mutant. Using the HEK293T NF-κB reporter assay there was a partial defect in the activation of this transcription factor. There must be other microbial molecules that contribute to activation, because LnaB is only expressed in post-exponential phase (Fig. 8D).

There is precedence for NF-κB activation by translocated bacterial proteins. The *Helicobacter pylori* type IV secretion system delivers the CagA protein which, when ectopically expressed in mammalian cells, strongly induces NF-κB activation (Brandt *et al.*, 2005, Hatakeyama, 2008). CagA has been shown to influence a diverse set of host cell signaling pathways including, cell motility and cell proliferation. Any of these CagA stimulated pathways could feed into NF-κB signaling. Therefore it is possible that like CagA, LnaB acts indirectly to stimulate the NF-κB pathway in mammalian cells. Alternatively, the protein may be “sensed” by a host cell receptor to stimulate this pathway. It is important to point out that the peak of Icm/Dot-dependent induction of NF-κB occurs between 6–8 hr after challenge with *L. pneumophila*, and this timing is independent of the host cell type challenged by the bacteria (Losick *et al.*, 2006, Bartfeld *et al.*, 2009). This result is supported by other work showing waves of NF-κB induction in response to *L. pneumophila* (Bartfeld *et al.*, 2009). This delay could also be explained by accumulation of translocated substrates or peptidoglycan fragments (Shin *et al.*, 2008a) within the cell, or due to paracrine signaling from outside the cell, as inflammatory mediators accumulate in the culture medium dependent on Icm/Dot activity.

In plants there are Nod-like receptor (NLR) proteins known as R-proteins that either interact directly with injected proteins from bacterial pathogens or recognize the activities of these proteins to trigger a defense response (Grant *et al.*, 2006). Plants may have more than a hundred R-proteins that can recognize the activities of these diverse bacterial translocated proteins. In mammalian cells, with a more limited number of NLRs, sensing appears to be limited to common microbe-associated molecules presented into the host cell cytoplasm (Jones *et al.*, 2006, Proell *et al.*, 2008). However, alterations to the actin cytoskeleton (Kustermans *et al.*, 2005, Legrand-Poels *et al.*, 2007) perturbations of the plasma membrane and disruption of ER homeostasis have been shown to activate NF-κB (Schroder, 2008). Many bacterial secreted proteins do just that, inhibiting the actin cytoskeleton for the purpose of blocking phagocytosis (Trosky *et al.*, 2008), disrupting the plasma membrane or altering membrane trafficking events (Ramsden *et al.*, 2007, Shin *et al.*, 2008b). Thus, in response to microbial attack, it is conceivable that the disruption or manipulation of these cellular pathways by bacterial translocated proteins serve as triggers for activation of mammalian innate immune responses as well. Furthermore, the response of host cells to common microbial molecules, such as peptidoglycan fragments, could be amplified as a result of misregulation of cellular processes by translocated substrates. In support of the possibility that IDTS could either cause or potentiate an existing NF-κB response, we observed that LidA and SidM, two IDTS that modulate the host cell vesicle trafficking GTPase Rab1 (Machner *et al.*, 2006, Murata *et al.*, 2006), can also induce NF-κB activity when ectopically expressed.

LnaB associates with the ER when ectopically expressed, however the protein contains no predicted transmembrane domains by a Kyle Doolittle hydropathy plot (data not shown). We found that the C-terminal region between amino acids 401-558 was sufficient for ER localization, but not NF-κB activation. Therefore ER localization is not sufficient to induce NF-κB activity. In addition, more than thirty other GFP-fusions in our library co-localized to an ER compartment but did not cause activation of the NF-κB sensitive promoter when ectopically expressed (Losick V.P., et al., unpublished), further indicating that ER localization is not sufficient to induce this response. In contrast, the coiled-coil domain of LnaB is required for NF-κB activation and the N-terminal region containing the coiled-coil domain (from amino acids 1-401) is sufficient to induce this activity despite being localized to the cytoplasm. We propose that LnaB is likely to have a binding partner that targets the coiled-coil domain of this protein. Interaction with this region either is important for an activity that results in the NF-κB response, or the protein directly interacts with a component of a pathway upstream of this transcriptional regulator.

In conclusion, we have identified several *L. pneumophila* Icm/Dot translocated proteins that can stimulate NF-κB activation when ectopically produced in a mammalian cell-line. In particular the IDTS LnaB strongly activates NF-κB and a deletion of Δ*lnaB* is sufficient to cause reduction of transcription of this pathway when post-exponential bacteria challenge mammalian cells. The mechanism by which LnaB induces NF-κB activity still remains to be elucidated, but our study lends further support to the role of translocated proteins in contributing to activation of the innate immune response in mammalian cells.

Experimental Procedures

Bacterial strains, media, and plasmids

L. pneumophila Philadelphia-1 strains Lp02 (*thyA*) and Lp03 (*dotA3* point mutant; referred to as *dotA)* are derivatives of Lp01 (*hsdR rpsL*) (Berger and Isberg, 1993). Lp02 *ΔflaA* was generously provided by Drs. Tao Ren and William Dietrich (Ren *et al.*, 2006). *L. pneumophila* strains were maintained on buffered charcoal yeast extract (BCYE) solid medium and ACES-buffered yeast extract (AYE) broth culture media (Feeley *et al.*, 1979,

Gabay *et al.*, 1985, Swanson *et al.*, 1995). For infections, *Legionella* was patched from a single colony onto BCYE containing 100 μg/ml of thymidine and/or antibiotics: 5 μg/ml of chlroamphenicol, 20 μg/ml of kanamycin, if required. After 2 days at 37°C, patches were used to inoculate AYE broth culture. Cultures were grown overnight in AYE broth with appropriate additives and grown to post-exponential phase (A_{600} ~ 4.0 – 4.5) prior to infection. The in-frame deletion of *ΔlnaB* (VPL2) and *ΔlegK1* (VPL1) in Lp02 were constructed as previously described (Luo *et al.*, 2004, Roy *et al.*, 1997). The VPL11 (Lp02::SR47s) and VPL12 (*ΔlnaB*::SR47s) were constructed by selecting for Kan^R colonies that integrated the SR47s suicide vector into the *lnaB* chromosomal locus. The complementing strain VPL13 (*ΔlnaB*::SR47s-*LnaB*$^+$) was constructed by selecting for Kan^R colonies that integrated the suicide vector containing the full-length *lnaB* under its endogenous promoter onto the chromosome.

pJB-CyaA and CyaA translocation assay plasmids and related procedures were generously provided by Dr. Joseph Vogel (Bardill *et al.*, 2005). All *L. pneumophila* plasmids were constructed using PCR fragments amplified from Lp02 genomic DNA isolated using DNeasy kit, Qiagen. The *cyaA* plasmids p*cyaA-ralF*, p*cyaA-lnaB*, and p*cyaA-legK1* were cloned in-frame with *cyaA* at *Bam*HI and *Sal*I sites. The mammalian expression plasmid pEGFP-C1 (Clontech) has a constitutive CMV promoter. *LnaB* was cloned in-frame with EGFP using *Eco*RI and *Bam*HI sites. Competent DH5α and DH5αλpir were prepared by the Tufts GRASP center. See Table S2, S3, and S4 for more information and details on primers, plasmids, and strains used in this study.

Cell culture

HEK293T and HeLa cells were maintained in DMEM plus 10% fetal bovine serum (FBS). U937 cells (ATCC) were grown in RPMI 1640 (Invitrogen) supplemented with 10% FBS and 1 mM glutamine (Invitrogen). 5×10^7 U937 cells were differentiated using 10 ng/ml 12-tetradecanoyl phorbol 13-acetate (TPA) for 48 hr, after which cells were washed, replated with fresh media in the absence of TPA, and used for infections with *L. pneumophila* ~16 hours later. Bone marrow (BM) macrophages were flushed from the femurs of 6- week to 3-month old mice and differentiated in bone marrow macrophage media, BMM (RPMI, 1mM glutamine, 10% FBS, 30% L-cell supernatant) (Swanson *et al.*, 1995). BM macrophages were differentiated for 7–8 days, collected, and frozen for use in multiple experiments in medium containing 20% serum, 10% DMS0. BM macrophages were replated in BMM media containing 200 μg/ml of thymidine.

L. pneumophila growth curves in BM macrophages

BM macrophages from A/J mice were plated at a density of 4×10^5 cells per well of a 24 well plate and allowed to settle overnight. *L. pneumophila* strains were grown to post-exponential phase, as determined by the presence of highly motile bacteria, and incubated with macrophages at MOI = 0.05. After infection, plates were spun at 1,000 rpm for 5 minutes at room temperature to promote contact of bacteria with the macrophages. Incubations proceeded at 37°C, 5% $C0_2$ for 2 hours, after which the monolayers were washed three times with pre-warmed BMM medium plus 200 μg/ml of thymidine. After 2, 24, 48, and 72 hours, three independent wells were lysed with 0.2% saponin, and then wells were washed with 1ml of sterile water. CFUs were enumerated on BCYE plates and colonies were counted 4 days post-plating.

NF-κB nuclear translocation assay

NF-κB nuclear translocation in bone marrow-derived macrophages was performed as previously described (Losick *et al.*, 2006). Cells were considered positive if there was intense staining for NF-κB in the nucleus at 7 hours post infection (hpi).

NF-κB luciferase reporter assay

HEK293T cells were replated at a density of 3×10^4 cells per well of 96-well white Corning clear-bottomed plates and left overnight to adhere. Cells were co-transfected with 50 ng of the indicated plasmid and 200 ng of ultra pure NFκB-luciferase (Stratagene) reporter plasmid per well using 0.5 μl of Lipofectamine 2000 according to manufacturer's instructions (Invitrogen) for ~24 hours. For *L. pneumophila* infections after 16 hours of tranfection with the reporter plasmid, HEK293T cells were incubated with *Legionella pneumophila* grown to post-exponential phase at MOI indicated. Plates were spun at 1,000 rpm for 5 minutes at room temperature. After 2 hours post infection, cells were gently washed twice with warm DMEM plus 10% FBS and 200 μg/ml thymidine. Incubations were allowed to proceed for times indicated, after which the medium was aspirated and replaced by 50 μl of sterile PBS plus 50 μl of Steadylite HTS (Perkin-Elmer) luminescent reagent. The plates were read immediately in a 96-well plate reader. For each well, the relative light units (RLU) reporter was the mean data from nine points within the well, scanned for 1 second each. The fold NF-κB activity is the mean RLU of the indicated infection versus uninfected cells. Standard error is based on triplicate wells from a representative experiment.

If indicated, the NF-κB activity was normalized to mean bacterial uptake from three independent wells to ensure that activity was not affected by subtle differences in bacterial load. HEK293T cells were replated and infected as described above, except at 2 hpi three independent wells were lysed with 0.2% saponin and washed with equal volume of sterile water. CFUs were enumerated on BCYE plates and colonies were counted after 4 days at 37°C.

Quick-change mutagenesis of LegK1 D223A

The PAGE purified primer, 5′GCATTTGATTCATCGAG**C**TATAAAACCAGG-3′ (IDT), was used to mutate the putative catalytic asparatic acid residue (D223) of the LegK1 Ser/Thr kinase domain to an alanine according to the Quick Change protocol described by Stratagene. Underlined is the nucleotide change used for this mutation. Plasmids were screened by digest and sequenced to confirm the mutation.

CyaA translocation assay

TPA treated U937 cells were plated at density of $1 – 2 \times 10^6$ cells per well of 24-well plate. *L. pneumophila* pCyaA strains were grown overnight in AYE plus 100 μg/ml thymidine and 5 μg/ml chloramphenicol to A_{600} ~2.5. CyaA protein expression was induced by adding 100 μM IPTG and strains were allowed to grow for additional 2 to 3 hours until the bacteria were in post-exponential phase and motile. The equivalent of 1 OD unit of cells was pelleted, resuspended and boiled in 150 ul 1 × SDS sample buffer. $1/20^{th}$ of the sample was used for Western blot analysis of CyaA fusions with anti-CyaA. The CyaA expressing *L. pneumophila* strains were used to infect U937 cells at MOI = 1. After addition of the bacteria, 24 well plates were spun at 1,000 rpm for 5 minutes and incubated for 1 hour at 37°C, 5% CO_2. Cells were washed 3 times with PBS and cells were lysed by adding 200 μl of lysis buffer (50mM HCl, 0.1% Triton X-100) and incubated on ice for 10 minutes. Lysates were collected and boiled immediately for 5 minutes, then neutralized by addition of 12 μl of 0.5 M NaOH. The cAMP present in the extracts was precipitated by adding 400 μl of cold 95% EtOH (65% final) and incubated on ice for 5 minutes. Insoluble material was removed by spinning samples in a microfuge for 5 minutes at 4°C at 13,000 rpm. Supernatants containing the cAMP were dried under vacuum and resuspended in 200 μl dH_20. The cAMP concentration was measured using Amersham Biotrak cAMP ELISA Kit according to manufactures instructions.

Western blotting of GFP fusions and other proteins

To analyze expression in cultured cells of the GFP fusions expressed from pDEST53, HEK293T cells were plated at a density of 5×10^4 cells per well in 96-well plates and 200 ng plasmid was transfected using 0.5 μl Lipofectamine 2000 for 24 – 48 hours. Samples were analyzed by Western blotting using rabbit anti-GFP (Invitrogen) (1:5000). Detection of full-length GFP-fusions were marked as positive (+) (Table S1). The other primary antibodies used in this study included rabbit anti-Myc (Santa Cruz) (1:3000); mouse anti-Tubulin (Sigma) (1:10,000); rabbit anti-CyaA (Santa Cruz) (1:10,000); rabbit anti-LnaB affinity purified (1:2000); rabbit anti-ICDH (1:10,000) was kind gift Dr. Abraham Sonenshein at Tufts University.

To generate anti-LnaB antibodies, *lnaB* (lpg2527) was introduced into pDEST17 to generate an N-terminal His fusion under the control of arabinose according to manufacturer's instructions (Invitrogen). The His-LnaB fusion protein expressed in *E. coli* strain BL21-A1 was insoluble, so the recombinant protein was isolated from the pellet. One liter of BL21-A1 expressing His-LnaB was pelleted and resuspended in 50 ml of 6 M guandine hydrochloride in 100 mM NaH_2PO4, 10 mM Tris-HCl, pH 7.5. His-LnaB was bound to Ni-NTA beads (Qiagen) by incubating at 4°C overnight. His-LnaB was eluted with 6 M guandine hydrochloride buffer containing 500 mM imidazole at pH 7.5. The eluant was dialyzed overnight into 6 M urea buffer containing 20 mM Tris-HCl pH 7.4, 20% glycerol, 500 mM NaCl, then again in a two-fold stepwise dialysis to remove the urea. The fusion protein, which was now soluble in the absence of denaturant, was used to immunize rabbits by the Quick draw protocol from Pocono Rabbit Farm (Canadensis PA).

Subcellular localization

1×10^5 Hela cells were co-transfected with 0.5 μg of EGFP fusion plasmids and 0.2 μg of pmCherry-Sec61 with 1 μl of lipofectamine 2000. After 24 hours, the transfected cells were fixed for 15 minutes at room temperature in 4% paraformaldhyde in PBS. Representative images were taken using a 63X lens on a Zeiss IM200 fluorescent microscope. For all images the contrast and brightness of individual channels were adjusted linearly in Adobe Photoshop.

LAMP-1 targeting assay

L. pneumophila strains were tested for avoidance of a LAMP-1-containing compartment as targeting described, challenging bone marrow-derived macrophages from A/J mice (Conover *et al.*, 2003). The number of LAMP-1-positive phagosomes per bacterial strain was quantified by counting 100 bacteria-associated macrophages on each of three coverslips. The mean and standard error were calculated a typical experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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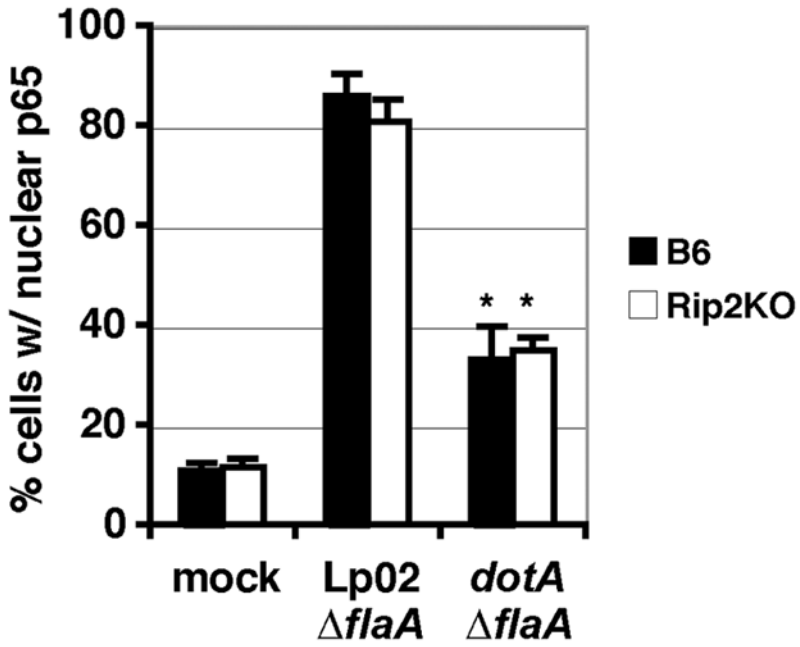

Figure 1. *L. pneumophila* induced NF-κB activation is not dependent on Rip2

BM macrophages from C57Bl/6J (B6) and Rip2 knockout (Rip2KO) mice were challenged with *L. pneumophila* strain Lp02 *ΔflaA* and *dot ΔflaA* at MOI 1 for 7 hours. Fixed cells were stained for NF-κB, probed with anti- *L. pneumophila* antibody and the percent of cells harboring bacteria with detectable nuclear NF-κB staining were scored. Data represents the mean ± SE from three independent experiments, with 300 cells scored. * *T*-test *p*-value ≤ 0.01.

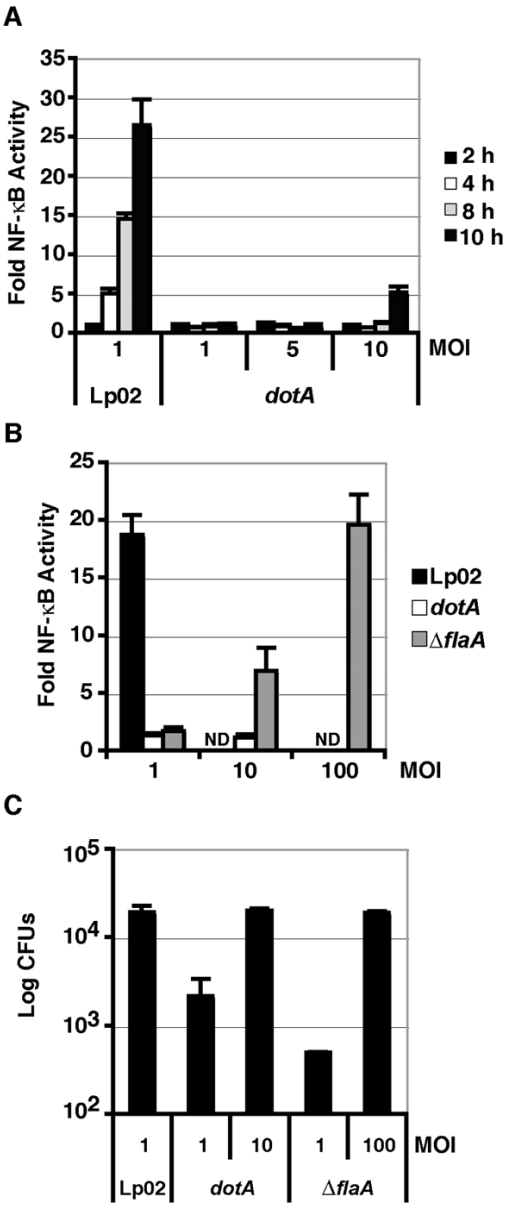

Figure 2. NF-κB activation by *L. pneumophila* in HEK293T cells is dependent on the Icm/Dot translocation system and independent of flagellin

(A) Time course of NF-κB activation at 2 (black bars), 4 (white bars), 6 (gray bars), or 10 (dark gray bars) hours post infection (hpi) with wild-type *L. pneumophila* (Lp02) or a Icm/Dot mutant (*dotA*) at the MOI indicated. (B) Dose dependent activation of NF-κB in response to Lp02, *dotA*, or a flagellin mutant (*ΔflaA*). MOI is indicated on the *x*-axis. Luminescence was assayed after 8 hours post infection (hpi). ND: not determined. HEK293T cells were transfected with the NFκB-luciferase reporter plasmid, and then challenged with the *L. pneumophila* strains indicated. Fold NF-κB activity is expressed as the light units of samples relative to transfected cells incubated in absence of bacteria. (C) High efficiency *L. pneumophila* uptake into HEK293T cells requires flagellin. Uptake was measured at 2 hpi by lysing cells and enumerating CFUs. Data represent the mean and SE of triplicate samples from a representative experiment. Experiments were repeated in duplicate.

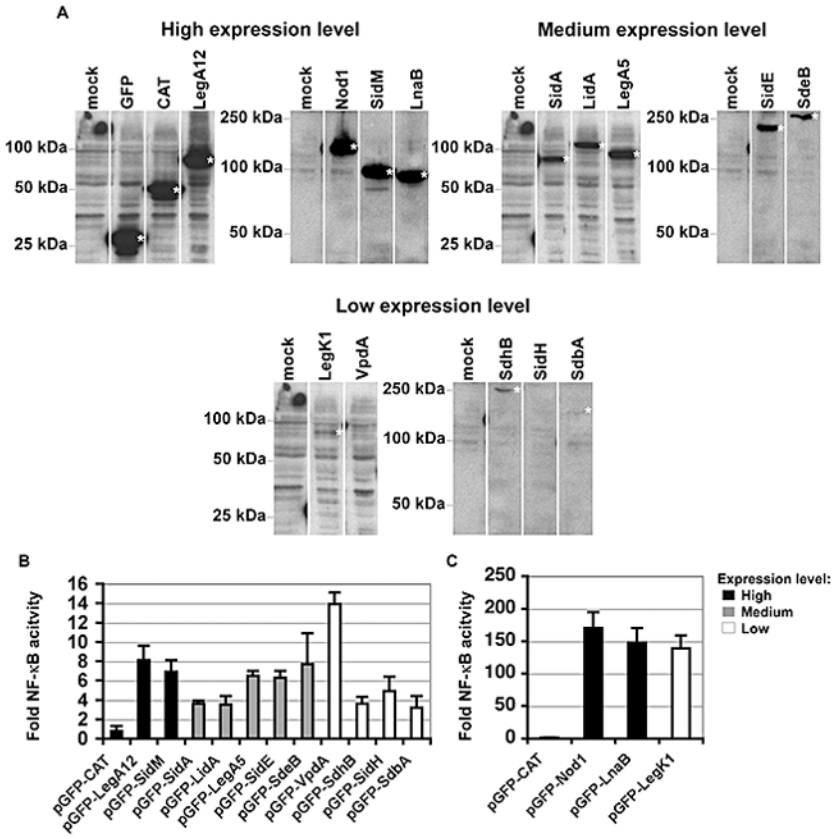

Figure 3. Candidate IDTS can activate NF-κB

(A). Varied expression levels of *L. pneumophila* IDTS expressed in 293T cells. Protein expression levels of the indicated GFP fusions that had enhanced NF-κB activation are displayed on immunoblots, probing with anti-GFP. To allow clearer comparisons of the relative increases in NFκB activity, IDTS are grouped according to their expression levels in HEK293T cells. (B) Low level activation of NF-κB by *L. pneumophila* IDTS. *Legionella* NF-κB activators fused to GFP are marked on the x-axis. Shown are those IDTS that show statistically significant activation of NF-κB (*T*-test *p*-value ≤ 0.05). Bars representing those IDTS expressed at high levels are marked in black. Medium and low expression levels are shown in grey and white, respectively. (C) High level NF-κB activation by two *Legionella* IDTS. HEK293T cells were co-transfected with 200 ng of pNFκB-luciferase reporter and either 50 ng of the control plasmid (pGFP) or the indicated pGFP fusions and luminescence was assayed at 24 hours. pGFP-CAT (chloramphenicol aminotransferase) served as a negative control and pGFP-Nod1 was used as a positive control for NF-κB activation. Fold NF-κB activity is the relative light units of pGFP fusion compared to the pGFP control. Data represents the mean ± SE from three independent experiments preformed in triplicate.

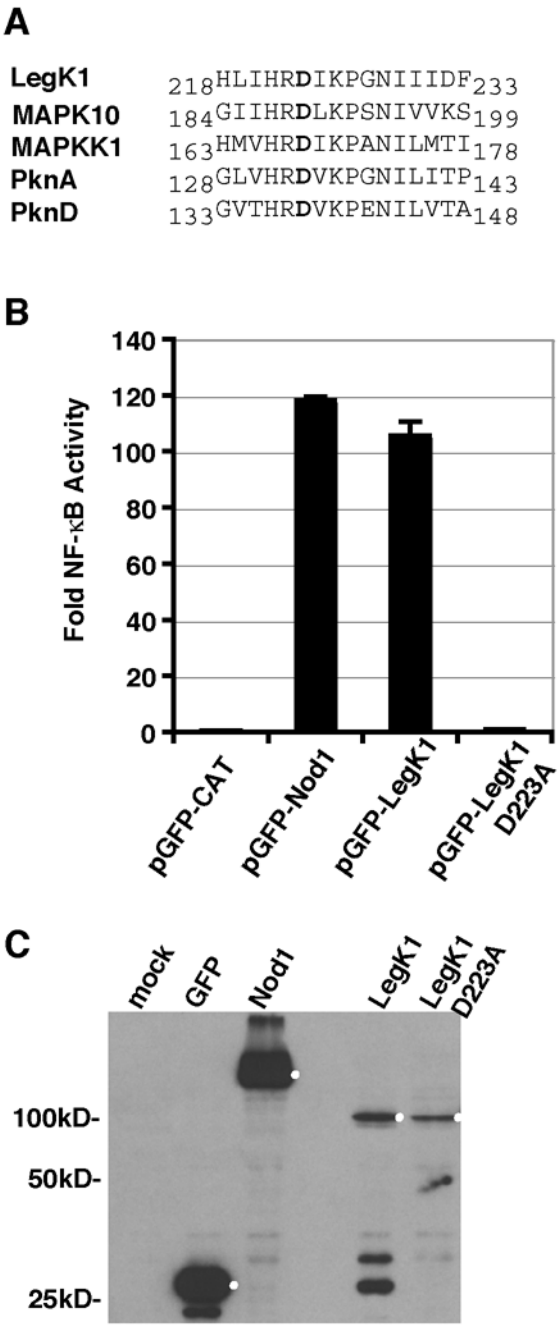

Figure 4. The ser/thr kinase activity of LegK1 is required for NF-κB activation

(A) Alignment of the predicted catalytic domain of *L. pneumophila* LegK1 with other ser/thr kinase domain containing proteins: Human MAPK10 (NP_002744), *Chlamydomonas reinhardtii* MAPKK (XP_001696437), *Mycobacterium tuberculosis* PknA (NP_214529) and PknB (NP_214528). The catalytic aspartate (shown in bold) is preceded by an arginine in all the ser/thr kinases. (B) Mutation of the putative LegK1 catalytic aspartate to alanine eliminates NF-κB activation. HEK293T cells were co-transfected with 200 ng per well of pNFκB-luciferase and either 50ng of pGFP fused to CAT (negative control), Nod1 (positive control), LegK1, or catalytically inactive mutant LegK1 D223A. Data represent the mean ± SE from triplicate samples of a representative experiment. The experiment was preformed in

triplicate. (C) Western blot of the protein expression levels of GFP, GFP-Nod1, GFP-LegK1, and GFP-LegK1-D223A probed with anti-GFP antibodies. HEK293T cells were transfected for 48 hours with 200 ng of the indicated plasmids; the expected protein based on molecular weight are marked by a white dot.

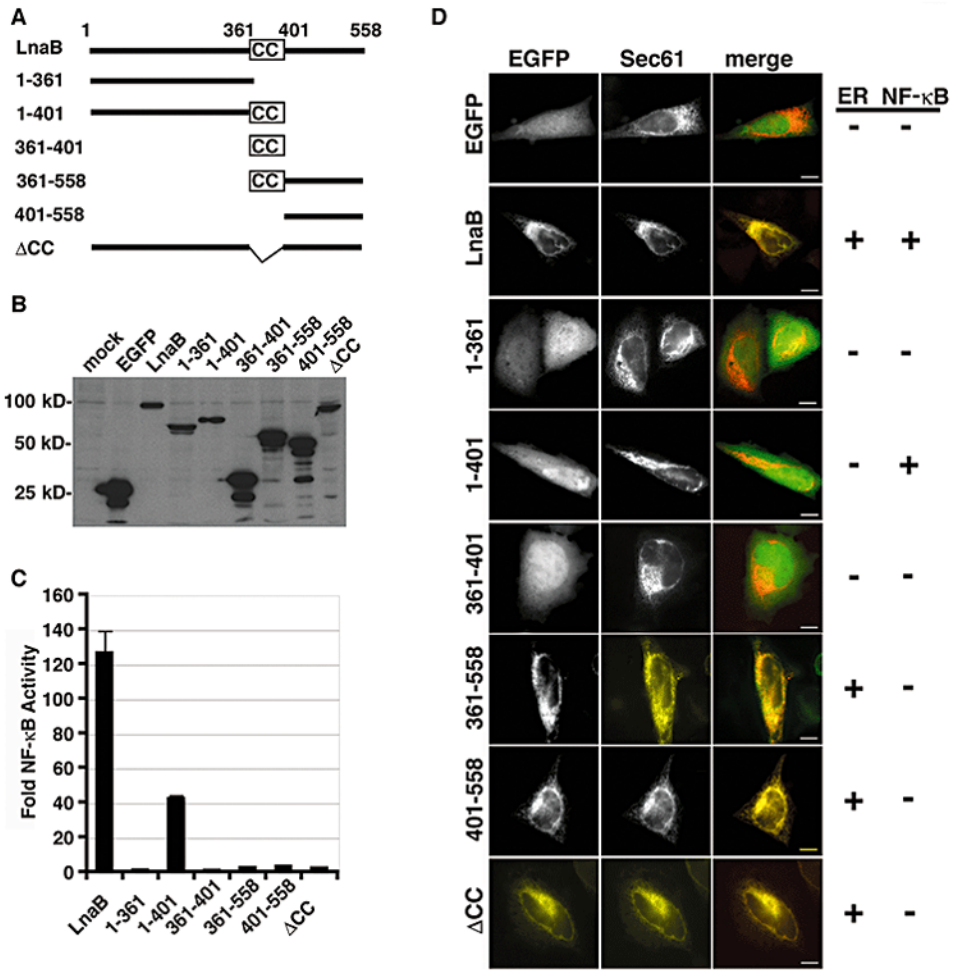

Figure 5. The coiled-coil domain of LnaB is required for NF-κB activity

(A) Schematic of EGFP-LnaB fusions. Full-length LnaB is 558 amino acids and contains a putative coiled-coil domain (CC) between amino acids 361-401. (B) Expression of EGFP fusions, 24 hours after transfection of HEK293T cells with the corresponding plasmids as detected by Western blot with anti-GFP. (C) HEK293T cells were co-transfected with 200 ng of pNFκB-luciferase and 50 ng of indicated EGFP fusion and luminescence was assayed after 24 hours. Fold NF-κB activity is the relative light units of pEGFP fusions compared to the pEGFP control. Data represents the mean ± SE from triplicate samples of a representative experiment. The experiment was preformed in triplicate. (D) Subcellular localization of indicated EGFP-LnaB fusions in HeLa cells expressing the ER marker Sec61 (red) and EGFP (green) in the merged image. Constructs that co-localized with Sec61 or activated NF-κB are marked with a +. Scale bar = 2μm.

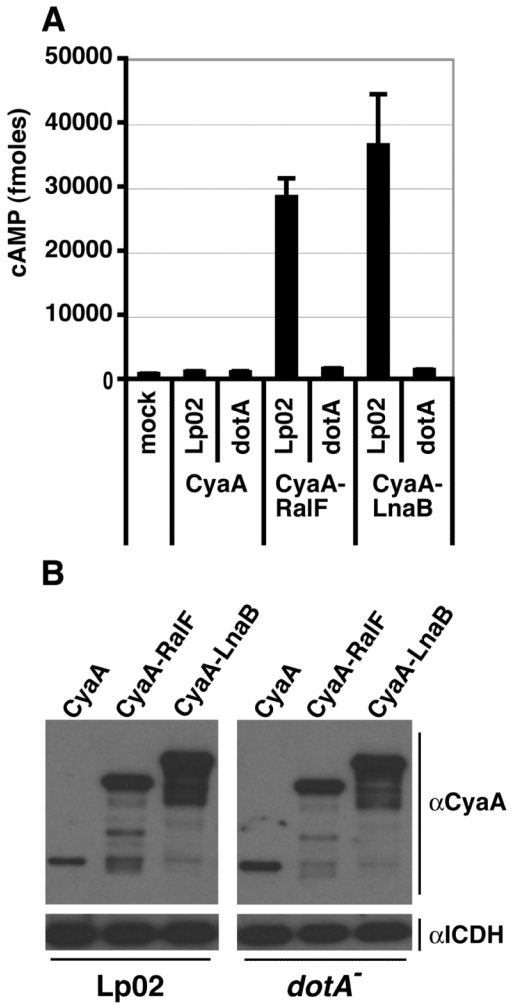

Figure 6. LnaB is a substrate of the Icm/Dot translocation system

(A) U937 cells were challenged with the indicated *L. pneumophila* strains expressing CyaA (negative control), CyaA-RalF (positive control), CyaA-LnaB. After a 1 hour incubation, cAMP was extracted and relative cAMP levels were measured. Data represent the mean ± SE of triplicate samples from a representative experiment performed in triplicate. (B) Protein expression of CyaA, CyaA-RalF, CyaA-LnaB in the corresponding *L. pneumophila* strains prior to challenge of U937 cells. Isocitrate dehydrogenase (ICDH) was used as a loading control.

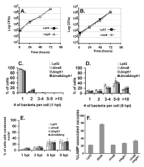

Figure 7. LnaB is dispensable for intracellular growth and protection from cell death

(A and B). Intracellular growth of *L. pneumophila* within A/J mouse BM macrophages. Displayed is the wild-type (Lp02) strain compared to *ΔlegK1* (A) or *ΔlnaB* (B). At 2, 24, 48, and 72 hpi macrophages were lysed and colony forming units (CFUs) were enumerated (Experimental Procedures). Data represents the mean ± SE from three independent infections of a representative experiment preformed in duplicate. (C,D) Bacterial replication at 1 hpi and 9 hpi. The percentage of A/J mouse BM macrophages harboring the denoted number of bacteria is shown. The wild type (Lp02) was compared to *ΔlnaB*, *ΔlegK1* and *ΔlnaBΔlegK1* after infection of MOI=1. (E) Host cell survival. At the denoted time points post infection with wild type (Lp02), *ΔlnaB*, *ΔlegK1* and *ΔlnaBΔlegK1*, condensed nuclei (cell death) were observed by Hoechst DNA staining and immunofluorescence microscopy. Average and SE of three cover slips of the respective experiments are displayed. (F). Intracellular targeting of the *L. pneumophila* replication vacuole in mutants defective for NF-κB activators. Bone marrow-derived macrophages from A/J mice were challenged with the noted *L. pneumophila* strains at MOI = 1.0 for one hour (Experimental Procedures). Cells were fixed, probed as described for *L. pneumophila* and late endocytic marker LAMP-1 (Experimental Procedures) and visualized by indirect immunofluorescence microscopy. Data are the mean ± SD of nine coverslips from three experiments for samples except *ΔlegK1* which was the mean ± SD of 6 coverslips from two experiments.

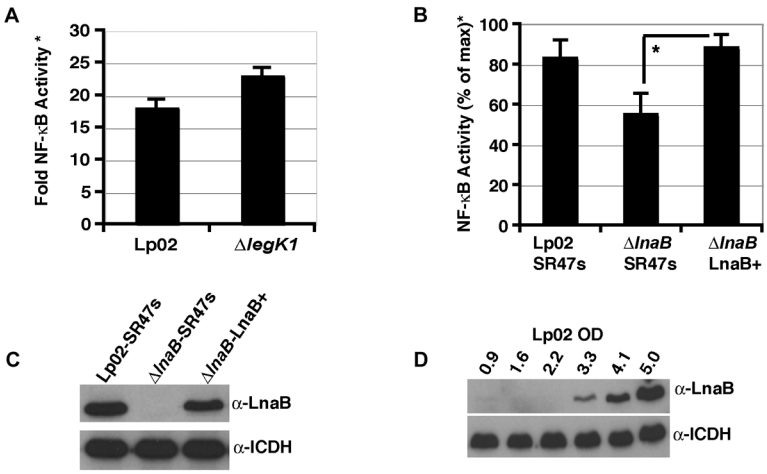

Figure 8. LnaB is required for full activation of NF-κB

(A and B) HEK293T cells were transfected with the NF-κB-luciferase reporter plasmid and then challenged with the wild-type *L. pneumophila* (Lp02), the *ΔlegK1* (A) or the *ΔlnaB* (B) strains at MOI = 1 for 7 hours. Fold NF-κB activity* is the relative light units of cells incubated with bacteria compared to cells incubated without bacteria. Samples were normalized to bacterial uptake at 2 hpi (Experimental Procedures). Data represents the mean ± SE of triplicate samples from a representative experiment performed in duplicate (A) or the mean ± SE from 4 independent experiments performed in triplicate (B) in which the maximum NF-κB activity was normalized to 100%. **T-test p*-value < 0.05. (C) LnaB production in wild-type (Lp02::SR47s), Δ*lnaB*::SR47s, and Δ*lnaB*-LnaB^{+} complement strains. (D) LnaB expression is induced in post-exponential phase of growth. Lp02 was grown overnight to indicated A_{600} and immunoblotted with anti-LnaB or anti-ICDH as loading control.