The *Listeria monocytogenes* InlC protein interferes with innate immune responses by targeting the IκB kinase subunit IKKα

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Listeria monocytogenes **is an intracellular pathogen responsible for severe foodborne infections. It can replicate in both phagocytic and nonphagocytic mammalian cells. The infectious process at the cellular level has been studied extensively, but how the bacterium overcomes early host innate immune responses remains largely unknown. Here we show that InlC, a member of the internalin family, is secreted intracellularly and directly interacts with IKKα, a subunit of the IκB kinase complex critical for the phosphorylation of IκB and activation of NF-κB, the major regulator of innate immune responses. Infection experiments with WT *Listeria* or the *inlC*-deletion mutant and transfection of cells with InlC reveal that InlC expression impairs phosphorylation and consequently delays IκB degradation normally induced by TNF-α, a classical NF-κB stimulator. Moreover, infection of RAW 264.7 macrophages by the *inlC* mutant leads to increased production of proinflammatory cytokines compared with that obtained with the WT. Finally, in a peritonitis mouse model, we show that infection with the *inlC* mutant induces increased production of chemokines and increased recruitment of neutrophils in the peritoneal cavity compared with infection with WT. Together, these results demonstrate that InlC, by interacting with IKKα, dampens the host innate response induced by *Listeria* during the infection process.**

anti-inflammation | NF-κB | virulence | cytokines | internalin

The Gram-positive bacterium *Listeria monocytogenes* infects human and animal hosts and causes foodborne infections that can lead to bacteremia and meningitis. It mainly affects immunocompromised patients, pregnant women, and newborns. Once inside the host, *L. monocytogenes* can invade both phagocytic and nonphagocytic cell types, replicate intracellularly, and spread directly from cell to cell, thereby escaping the humoral immune response. The successive steps of this intracellular parasitism are dependent on various virulence factors, including the surface proteins InlA and InlB, required for entry into cells; secreted proteins listeriolysin O (LLO) and phospholipases, involved in escape from the primary and secondary vacuoles; and ActA, responsible for actin-based intracellular and intercellular movements. These virulence factors are positively controlled by the transcriptional activator PrfA (1–3).

The complete genome sequence of *L. monocytogenes* strain EGD-e has revealed the presence of 25 genes encoding proteins of the internalin family (4–6). Proteins of this family are characterized by the presence of a leucine-rich repeat (LRR) domain. Most of these are surface proteins attached to the bacterial surface by different anchoring motifs, in particular the LPXTG motif, which mediates covalent binding to the peptidoglycan. Some of these internalin proteins are well-characterized virulence factors, including internalin A (InlA, the prototype of the family) and InlB, which are involved in the crossing of intestinal and placental barriers (7, 8). Only four proteins of the internalin family are predicted to be secreted proteins (5), and among these, only InlC has received attention. This protein, whose gene is present in the pathogenic *L. monocytogenes* and *L. ivanovii* species but absent in nonpathogenic *Listeria* species, has been identified by searching PrfA-regulated genes in strains overexpressing *prfA* (9, 10). *inlC* encodes a small protein of 297 aa that displays a signal peptide of 34 aa, no known anchoring motif, and six LRRs of 22 aa, followed by an Ig-like domain (Fig. 1*A*). This latter domain has been shown in other internalins to stabilize the LRR domain and favor protein–protein interactions. The InlC 3D structure has been determined (11). The LRR domain is similar to that of other internalins. The Ig-like domain displays a high concentration of aromatic residues, suggesting a self-association of InlC molecules or the possibility of InlC association with partner proteins through this domain as well as with the LRR domain.

inlC is transcribed as a monocistronic mRNA from a single promoter displaying a typical consensus PrfA-binding site at position −40 from the transcription start site. The expression of *inlC* has been shown to be highly induced intracellularly at rather late stages of infection (12–14). A recent analysis of the entire *Listeria* transcriptome in various in vitro, ex vivo, and in vivo conditions of growth confirmed stronger *inlC* expression in the intestine and blood than in rich broth media (15).

An *inlC* deletion mutant is significantly attenuated when tested in the mouse model of infection by the i.v. route (10, 16). As reported recently, although the *inlC* deletion does not affect bacterial internalization and intracellular proliferation, it does impair cell-to-cell spread in polarized epithelial cells (17). InlC has been shown to bind the mammalian adaptor protein Tuba, thereby preventing its interaction with N-WASP. Impairment of Tuba–N-WASP interaction by InlC would relieve cortical tension at cell–cell junctions and promote protrusion formation and bacterial spreading.

Analyses of the transcriptional host responses in cultured human intestinal epithelial cells, murine macrophages, and intestinal tissues infected with *L. monocytogenes* have shown that MAP kinases and NF-κB/Rel pathways are the predominant host

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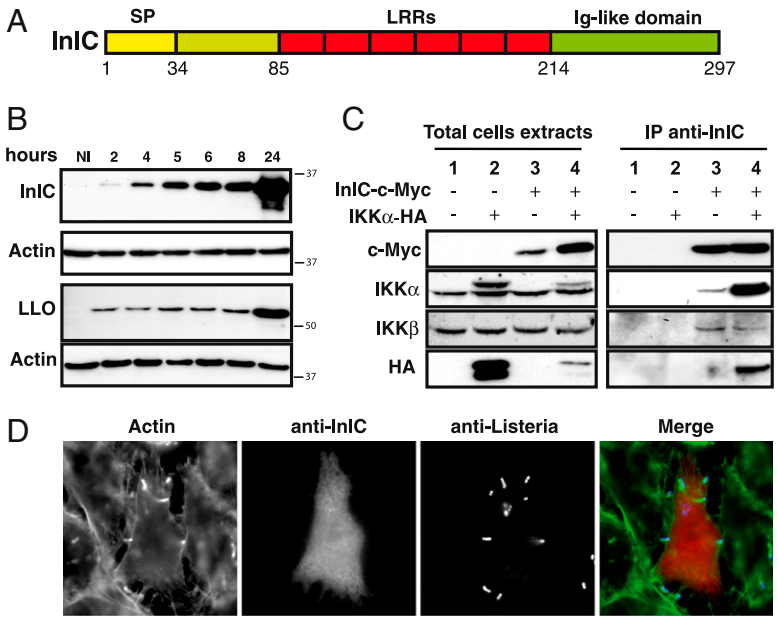

Fig. 1. InlC binds IKKα. (*A*) Schematic representation of InlC, a protein containing a signal peptide (SP), six LRRs, and an Ig-like domain. (*B*) Kinetics of InlC and LLO expression in infected HeLa cells. Total cell extracts at different time points after infection were analyzed by Western-blot using anti-InlC, anti-LLO, and anti-actin antibodies. Actin is used as loading control of protein. (*C*) Interaction of IKKα with InlC in transfected HEK-293 T-REx cells. Whole cell lysates from HEK-293 T-REx cells, nontransfected or transfected with the indicated plasmids (InlC c-Myc, IKKα-HA) were immunoprecipited with anti-InlC antibodies, followed by immunoblotting with anti-c-Myc and anti-HA antibodies. Western blot analysis with anti-IKKα and anti-IKKβ antibodies show the endogenous IKKα and IKKβ. (*D*) Detection of InlC by immunofluorescence in HeLa cells infected for 3 h with the WT strain. Actin was labeled with Alexa Fluor 488–conjugated phalloidin. InlC was detected with anti-InlC antibodies, and bacteria were detected with anti-*Listeria* antibodies.

responses to a *Listeria* infection (13, 18, 19). More specifically, the virulence factor LLO has been shown to induce the NF-κB–mediated transcription of the proinflammatory cytokine IL-8 in endothelial cells (20), whereas InlB induces TNF-α and IL-6 in macrophages (21).

The eukaryotic transcription factor NF-κB consists of a dimeric complex of two subunits, including p65/RelA, c-Rel, RelB, p100/52, or p105/50. In resting cells, NF-κB dimers are sequestered in the cytoplasm and kept inactive through their binding to IκB (22–24). IκB is expressed as different isoforms, of which the alpha isoform (IκBα) is the most abundant and most ubiquitously expressed (24). Certain bacterial infections or other stimuli, such as TNF-α, can activate the IκB kinase complex (IKK), which is composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ/NEMO). Activation of the IKK complex leads to phosphorylation of IκBs, followed by their polyubiquitination and degradation by the 26S proteasome. These events allow translocation of the NF-κB dimers to the nucleus and activation of NF-κB–regulated genes, which are involved mainly in innate immune responses. In the alternative nonclassical pathway, which is involved primarily in the development and maintenance of secondary lymphoid organs, there is no sequestration of NF-κB by IκB proteins. Instead, one of the precursors of NF-κB—the p100 or the p105 subunit before maturation into the p52 or the p50 subunit, respectively—acts as an inhibitor of the complex. In this pathway, the kinase complex comprises only two subunits of IKKα.

To investigate the function of InlC in the infectious process, we searched for its potential host partners and performed a large-scale two-hybrid screen in yeast. Among the potential cellular targets, we identified IKKα. In this study, we found that InlC binds to IKKα, impairs the phosphorylation of IκBα, delays the degradation of P-IκBα, prevents NF-κB nuclear translocation, and dampens the NF-κB–associated proinflammatory response both in vitro and in vivo.

Results

InlC Is a Bona Fide Virulence Factor Overexpressed Intracellularly. Because the *inlC* mutant analyzed previously was still expressing a residual polypeptide (10, 16), we generated a complete *inlC*-deletion mutant (Δ*inlC*) in the *L. monocytogenes* strain EGD and reexamined the contribution of the *inlC* gene in the infectious process in vivo. As the original *inlC* mutant, our mutant was growing as well as the WT both in broth medium and intracellularly. It infected cells as efficiently as the WT. Intravenous injections of C57BL/6 female mice with 1×10^5 of the WT bacteria and the Δ*inlC* strain resulted in 100% mortality with the WT bacteria, but only 20% mortality with the *inlC* mutant (Fig. S1). Moreover, the LD_{50} of the Δ*inlC* mutant after i.v. injection in C57BL/6 female mice was 5.6×10^5 CFU, compared with 1.5×10^4 CFU for the WT strain. Together, these results confirm the role of InlC in *Listeria* virulence.

To examine the production of InlC, we generated antibodies against a recombinant InlC protein and examined InlC expression during bacterial infection of HeLa cells. As shown in Fig. 1*B*, in contrast to LLO, which is already expressed at the onset of infection, InlC expression gradually increased inside the cells over time, in perfect agreement with the previously reported transcriptional analysis of the *inlC* gene (12, 13). Moreover, as shown by immunofluorescence analysis, the InlC protein was detected in both the cytosol and the nucleus of infected cells (Fig. 1*D*).

InlC Binds IKKα. To identify partners interacting with InlC in the eukaryotic cell, we used InlC as bait in a large-scale yeast two-hybrid screen. This screen identified IKKα, which had a very high interaction score. To confirm the interaction of InlC with IKKα, InlC tagged with a C-terminal Myc epitope and IKKα tagged with an HA epitope were expressed independently or simultaneously in HEK-293 T-REx cells. Cell extracts were immunoprecipitated with anti-InlC antibodies, and precipitates were analyzed by immunoblotting with anti-Myc, anti-HA, anti-IKKα, and anti-IKKβ antibodies. As shown in Fig. 1*C*, Myc-tagged InlC coimmunoprecipitated with HA-tagged IKKα as revealed by the anti-Myc and anti-HA antibodies. Using an anti-IKKα antibody recognizing both the transfected and the endogenous forms of IKKα, we showed that both forms coimmunoprecipitated with InlC (Fig. 1*C*, lanes 3 and 4). Moreover, by binding IKKα, InlC pulled out IKKβ, in agreement with the known IKKβ–IKKα interaction. Together, these results demonstrate that InlC interacts with the kinase subunit IKKα in mammalian cells.

InlC Impairs the Phosphorylation of IκBα and Delays Its Degradation in Infected and Transfected Cells. To investigate whether InlC, by binding the kinase IKKα, affects IκBα phosphorylation and

degradation, we infected HeLa cells with the WT strain, the Δ*inlC* mutant, and the complemented strain. We then stimulated the cells with TNF-α to induce IκBα phosphorylation via the classical pathway, and compared the kinetics of IκBα phosphorylation and degradation in infected and noninfected cells. As shown in Fig. 2*A*, the phosphorylation of IκBα was detected as early as 3 min after stimulation with TNF-α, with smaller amounts found in WT-infected cells than in noninfected cells. The Δ*inlC*-infected cells showed higher amounts of phospho-IκBα compared with cells infected with WT or the complemented strains. The degradation of the IκBα form (Fig. 2*B*) began concomitantly with the appearance of phospho-IκBα in noninfected cells and in Δ*inlC* mutant–infected cells, but was significantly delayed (by up to 20 min when InlC was present), strongly suggesting that the interaction of InlC and IKKα blocks the phosphorylation of IκBα induced by TNF-α and, consequently, its degradation.

All cells are not infected during infection of tissue cultured cells, precluding a precise evaluation of events occurring in each cell—in our case, the effect of InlC on the phosphorylation of IκBα. Therefore, to definitively demonstrate the effect of InlC on the phosphorylation of IκBα, we analyzed the fate of phospho-IκBα in human HEK-293 T-Rex cells cotransfected with plasmids expressing InlC and mouse IκBα, taking advantage of the fact that mouse IκBα and human IκBα migrate differently in acrylamide SDS/PAGE gels (25, 26). In this scenario, all cells expressing InlC also express the mouse IκBα. After stimulation by TNF-α, (Fig. S2*A*), lower amounts of mouse phospho-IκBα were detected in cells cotransfected with the plasmids expressing inlC or IκBα compared with cells cotransfected with IκBα and the control plasmid. Moreover, the phosphorylation of mouse IκBα was delayed, appearing only after 6 min. Similarly, IκBα's degradation also was delayed, and it was still detectable 20 min after stimulation in cells expressing mouse IκBα cotransfected with the plasmid expressing InlC compared with the control plasmid (Fig. S2*B*). These findings, in full agreement with the results obtained with the *Listeria*-infected cells, reinforce the data showing that InlC prevents the phosphorylation and degradation of IκBα.

Taken together, these results establish that InlC, by binding IKKα, prevents the phosphorylation of IκBα induced by TNF-α and delays the degradation of phospho-IκBα.

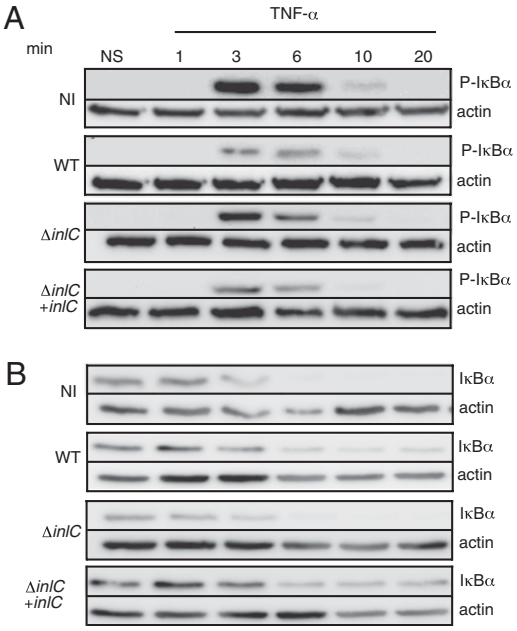

Fig. 2. InlC delays the degradation of IκBα in infected cells. HeLa cells noninfected (NI) or infected by *L. monocytogenes* WT or by the Δ*inlC* mutant (Δ*inlC*) and the complemented-Δ*inlC* mutant (Δ*inlC* + *inlC*), and stimulated by TNF-α (10 ng/mL) were lysed at the indicated times points after stimulation. Extracts were submitted to immunoblot analysis with antibodies recognizing phosphorylated-IκBα (*A*) and IκBα (*B*). Membranes were reprobed using antibodies to actin for loading control. NS, nonstimulated.

InlC Prevents Translocation of NF-κB to the Nucleus in Infected Cells. To assess whether InlC could block the translocation of NF-κB to the nucleus after stimulation by TNF-α, we performed immunofluorescence assays in noninfected cells (NI) and in cells infected with WT *Listeria*, the Δ*inlC* mutant, and the complemented strain (Δ*inlC* + *inlC*). Cells were fixed and stained with anti-p65 antibodies to label NF-κB, with anti-InlC antibodies to identify the cells where InlC was secreted, and with DAPI to visualize nuclei and bacteria. As shown in Fig. 3 *A* and *B*, at 30 min after stimulation by TNF-α, all of the noninfected cells were positive for p65 in the nucleus, confirming that TNF-α had activated the classical NF-κB pathway and that the p65 subunit of NF-κB had translocated to the nucleus. In cells infected with WT *Listeria*, the NF-κB complex was present only in the cytoplasm and was not detected in the nucleus. In Δ*inlC*-infected cells, activated NF-κB was detectable in the nucleus. In Δ*inlC* + *inlC*–infected cells, the NF-κB complex was not detectable in the nucleus. Together, these immunofluorescence findings unambiguously demonstrate that InlC expression prevents NF-κB translocation to the nucleus.

InlC Inhibits the NF-κB–Regulated Promoter Response on TNF-α Activation. To investigate whether InlC through its interaction with IKKα could modify the expression of NF-κB–regulated genes, we transfected cells with a plasmid encoding InlC and the reporter plasmid Igκ3-luciferase in which the luciferase gene is under the control of a NF-κB–regulated promoter. Luciferase activity was assayed at 6 h after TNF-α stimulation (Fig. 4) and found to be 35-fold greater in cells stimulated by TNF-α compared with unstimulated cells. Transfection of the InlC-encoding plasmid significantly decreased the total luciferase activity, with decreases ranging from 60% to 85% depending on the amount of plasmid used for transfection. We found no effect of InlC on luciferase activity when the cells were not stimulated by TNF-α. Moreover, neither the basal luciferase activity of nonstimulated cells nor that of TNF-α–stimulated cells was affected by the expression of LacZ used as a control. Together, these results indicate that InlC blocks TNF-α–induced activation of NF-κB and NF-κB–regulated downstream events.

InlC Decreases the Proinflammatory Cytokine Response to Infection. Because NF-κB plays a role in regulating various genes involved in inflammation (24), we next addressed whether InlC could control the expression of NF-κB–dependent proinflammatory cytokines during infection. We used ELISA to quantify the expression of TNF-α and IL-6 in supernatants of RAW 264.7 macrophages infected by *Listeria* WT, Δ*inlC*, or complemented strains. As shown in Fig. 5, IL-6 and TNF-α production was increased in cells infected by the *inlC* mutant compared with those infected by the WT. Complementation of Δ*inlC* restored the capacity of bacteria to down-regulate IL-6 and TNF-α production. Together, these results indicate that inactivation of *inlC* leads to the induction of a stronger proinflammatory response on infection in vitro.

InlC Counteracts the Inflammatory Process Induced by *Listeria* and LPS in Vivo. To further investigate the function of InlC in vivo, we used a mouse model of peritonitis in which WT *Listeria*, the Δ*inlC* mutant, and the complemented Δ*inlC* strains were injec-

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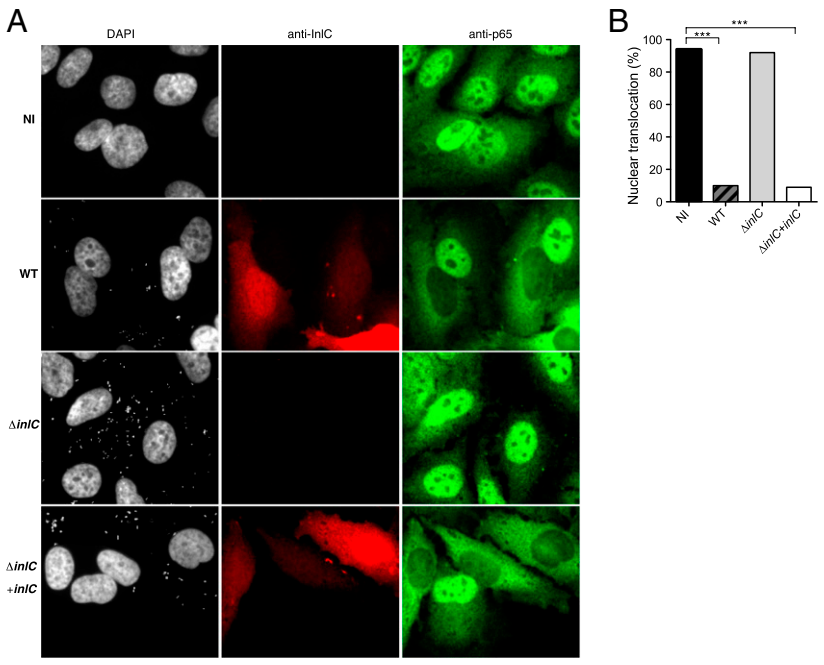

Fig. 3. InlC prevents the translocation of NF-κB-p65 to the nucleus in infected cells. (*A*) HeLa cells noninfected (NI) or infected by *Listeria monocytogenes* WT, the Δ*inlC* mutant (Δ*inlC*), or the complemented-Δ*inlC* mutant (Δ*inlC* + *inlC*) were stimulated with TNF-α (50 ng/mL) for 30 min, fixed, and labeled by immunofluorescence with DAPI for the nucleus and bacteria, anti-InlC antibodies to detect the secretion of InlC in the cytoplasm of cells, and anti-p65 to recognize the NF-κB subunit. (*B*) To quantify the results shown in *A*, the percentage of nuclear translocation of NF-κB (P65) was determined by counting at least 100 noninfected or WT, Δ*inlC*, or Δ*inlC*+*inlC* infected cells using fluorescence microscopy. The experiment was performed twice, and typical results are shown. ***$P <$ 0.0005, χ^2 test.

ted i.p. in BALB/c mice. We first analyzed the cytokine response in the peritoneal cavity after 12 h of infection. Despite the reduced number of viable mutant bacteria (30% less than WT bacteria), the *inlC* mutant induced a stronger cytokine response than the WT, particularly for the chemokines KC (the murine IL8 homolog) and MIP1α (Fig. 6*A*). This enhanced cytokine response was abolished in mice infected with the complemented strain, in which CFU recovery was similar to that in mice infected with the WT bacteria. We next investigated the recruitment of leukocytes in the peritoneal cavity after infection. We found significantly increased recruitment of neutrophils in response to infection with the *inlC* mutant compared with infection with the WT or the complemented strain (Fig. 6*B*). These findings indicate that InlC down-regulates the inflammation induced in response to *Listeria* infection.

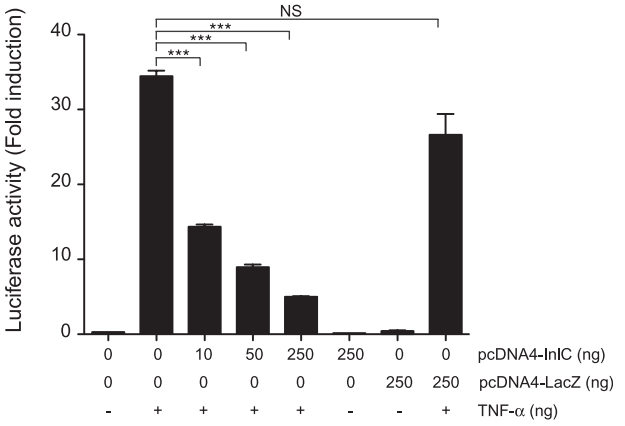

Fig. 4. InlC inhibits activation of an NF-κB–regulated promoter. HEK-293 T-REx cells were cotransfected with different plasmids, an NF-κB–dependent luciferase reporter, pRL-Tkluc, and pCDNA4-*inlC* or pCDNA4-*lacZ* at the indicated amount per well. The cells were then stimulated with TNF-α (10 ng/mL). Firefly luciferase activity was normalized against Renilla luciferase activity. Bars indicate fold induction compared with unstimulated cells. Values are mean ± SD of six wells. Each experiment was repeated three times. ***$P <$ 0.0005, Welch's *t* test. NS, not significant.

Discussion

To overcome host defenses, bacterial pathogens or viruses often interfere with the NF-κB pathway, the major pathway involved in the regulation of innate host responses (27). In this pathway, stimulation with an agonist, such as TNF-α, activates the trimeric IKK complex (i.e., IKKα, IKKβ, and NEMO). Various other pathways, including TLR and NOD pathways, also lead to activation of the IKK complex. This complex then phosphorylates IκB inhibitor proteins, which normally sequester the NF-κB proteins in the cytosol. Phosphorylated IκBs then become targets for ubiquitination and degradation, allowing the translocation of free NF-κB to the nucleus and consequent activation of downtream genes. Strikingly, several viral and bacterial pathogens interfere with the NF-κB pathway; however, only a few interfere with the degradation of IκB to inhibit the activation of NF-κB (28). Among these, cowpox virus, racoon pox virus, and certain strains of vaccinia virus can prevent degradation of phosphorylated IκBα (29). It has been proposed that this inhibition may result from dephosphorylation of IκB or interference with its degradation after phosphorylation. Similarly, HIV uses various strategies to manipulate NF-κB activation. The HIV type I Vpu protein competitively inhibits the β-TrCP/ubiquitin ligase–dependent degradation of IκB, thereby keeping NF-κB in the cytosol and resulting in inhibition of NF-κB activity in T cells (30, 31). The *Salmonella* SseL effector protein deubiquitinates IκBα and prevents its degradation, thereby impairing NF-κB activation (32, 33); another *Salmonella* effector, AvrA, also has an IκBα deubiquitinase activity (34). Similarly, ChlaDub1 of *Chlamydia* deubiquitinates IκB (35). These three bacterial effector proteins are secreted by a type III secretion system (T3SS) and translocated through the *Salmonella* or *Chlamydia* vacuolar membrane into the cytosol. Their mode of action differs from that of YopP/YopJ protein of *Yersinia*, which acetylates IKKα and IKKβ, thereby preventing phosphorylation of IκB (36, 37). Enteropathogenic *Escherichia coli* (EPEC) also has been shown to prevent the phosphorylation of IκB through the use of two T3SS effectors, NleE and NleB, which interact with the TAK1-IKK

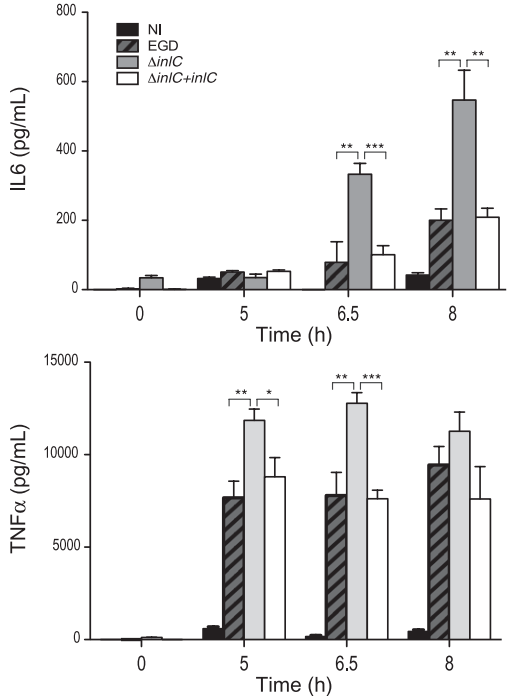

Fig. 5. InlC impairs the proinflammatory response in vitro. RAW264.7 cells were infected with *Listeria monocytogenes* WT, the Δ*inlC* mutant, or the complemented Δ*inlC* mutant (Δ*inlC* + *inlC*) (MOI_{10}), and levels of IL-6 and TNF-α in the supernatant were measured by ELISA at the indicated times points. Cytokines released from RAW264.7 exposed to LPS (50 ng/mL) were used as positive controls (data not shown). Values are mean ± SEM; $n = 6$. *$P < 0.01$; **$P < 0.005$; ***$P < 0.0005$, Student *t* test.

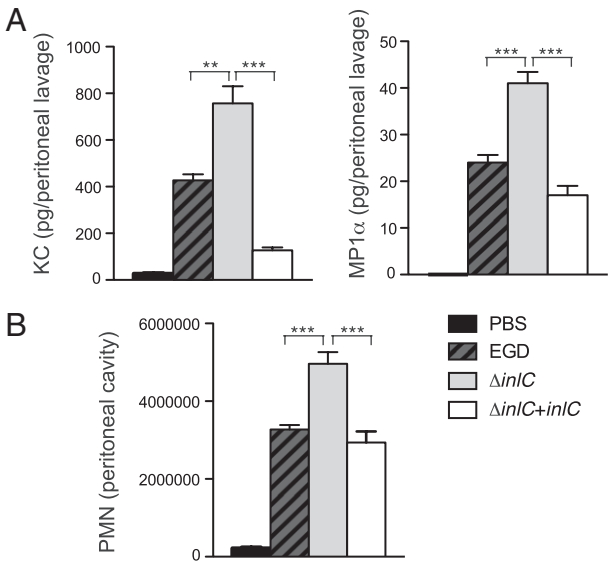

Fig. 6. InlC modulates the inflammatory process in vivo. (*A*) Mice were inoculated i.p. with PBS or with *L. monocytogenes* WT (EGD), the Δ*inlC* mutant, or the complemented Δ*inlC* mutant (Δ*inlC* + *inlC*), and levels of KC and MIP1α in the peritoneal lavage fluid were measured by ELISA at 12 h post-infection. (*B*) Leukocytes present in the peritoneal lavage fluid were pelleted and processed by flow cytometry, and the number of neutrophils (PMN) was determined. Values are mean ± SEM; $n = 6$. *$P < 0.01$; **$P < 0.005$; ***$P < 0.0005$, Student *t* test.

pathway, albeit in an unknown fashion (38). Finally, *Shigella* has at least two effectors, OspG and IpaH9.8, that interfere with the NF-κB pathway (25, 39). IpaH9.8 is an ubiquitin ligase that interacts and interferes with NEMO, thereby impairing IκB phosphorylation. In contrast, OspG acts directly on IκBα. This T3SS is a kinase that binds various ubiquitin-conjugating enzymes, including UbcH5, which promotes ubiquitination of IκBα. Thus, OspG prevents the degradation of IκBα and negatively controls the infammatory response (25).

Here we have shown that InlC, a protein of the *L. monocytogenes* internalin family whose gene is regulated by the global virulence gene activator PrfA, is overexpressed and secreted in the cytosol by *Listeria* once the bacterium has escaped from the internalization vacuole. InlC interacts with IKKα. Interestingly, the InlC–IKKα interaction decreases phosphorylation of the inhibitory component IκB and delays degradation of its phosphorylated form, thereby impairing NF-κB translocation to the nucleus and subsequent activation of NF-κB–regulated genes, particularly genes encoding proinflammatory cytokines. Like all bacteria, *Listeria* expresses a number of components that stimulate nonspecific innate immune responses via the NF-κB pathway. As in many other Gram-positive bacteria, lipoteichoic acids in the *Listeria* cell wall stimulate the NF-κB pathway and the production of proinflammatory cytokines (40). The virulence factors LLO and InlB also have been reported to activate the NF-κB pathway (20, 21).

Nevertheless, similar to other bacterial pathogens [e.g., *Shigella* (41)], *Listeria* has evolved several mechanisms to counteract proinflammatory processes. We previously reported that deacetylation of the peptidoglycan by the deacetylase PgdA is a major anti-inflammatory mechanism that down-regulates the production of proinflammatory cytokines (42). We also have shown that the dephosphorylation of histone H3 leads to down-regulation of a subset of genes including genes involved in immune responses (43), and that LLO plays a critical role in this process. As reported herein, InlC also contributes to down-regulate the inflammatory response both in vitro and in vivo. Similar to several other bacterial factors, InlC dampens the inflammatory response by acting on one of the most appropriate pathway, the NF-κB pathway. InlC is highly induced inside eukaryotic cells and interacts with a key component of the NF-κB pathway. It is highly likely that IKKα interacts with InlC via the InlC LRR domain, given that InlC is a small protein composed almost entirely of LRRs. It will be interesting to examine whether other bacterial LRR-containing factors behave like InlC. It also will be interesting to investigate whether InlC, which was shown here to interfere with the classical NF-κB pathway, also modulates other pathways regulated by IKKα (e.g., histone phosphorylation), especially in vivo. Interestingly, InlC was recently shown to also interact with the cytoskeleton-associated protein Tuba. This interaction also occurs late in infection and affects the efficiency of the cell-to-cell spread of *Listeria* (17). The finding that InlC has several functions is not unexpected for a virulence factor. Indeed, it is well known that bacterial pathogen effectors can be multifunctional, be modular, or display cooperative activities. InlC is another example of such a bacterial effector that exerts several activities during infection. However, its effect on virulence is subtler than that of bona fide virulence factors, such as LLO and ActA (3, 44). Thus, InlC might be more appropriately considered a "virulence modulator" that acts at different stages of the infectious process. In conclusion, this study reinforces the importance of the internalin family in listerial pathogenesis, highlighting that this family is involved not only in the entry of *Listeria* into cells, but also in other critical events in the infectious process.

Materials and Methods

Bacterial Strains, Reagents, Antibodies, and Immunofluorescence Microscopy. The bacterial strains and growth conditions, reagents, antibodies, and mi-

croscopy procedures used in this study are described in *SI Materials and Methods* and Table S1.

Cell Culture and Infections, Luciferase Reporter Assay, and ELISA. The cell lines and protocols for this study are described in *SI Materials and Methods*.

Plasmids and Oligonucleotides. The plasmids and oligonucleotides used in this study are listed in Tables S2 and S3. The construction of plasmids pcDNA4-*inlC*, pGEX-4T-*inlC*, and pAD-*inlC* is described in *SI Materials and Methods*.

Mutant Construction. The Δ*inlC* isogenic deletion mutant was constructed as described in *SI Materials and Methods*.

Identification of InlC Interactor by Yeast Two-Hybrid Screening. The Y2H screen was performed on a whole human genome cDNA placental library as described in *SI Materials and Methods*.

Immunoprecipitation and Immunoblotting. HEK-293 T-REx or HeLa cells were transfected or infected, and cells lysates were prepared. Details of immunoprecipitation and Western blot analysis are provided in *SI Materials and Methods*.

Murine Infection Experiments and Cell Counting. Infection procedures (i.v. and i.p.) and leukocyte counting in peritoneal lavage fluid are described in *SI Materials and Methods*.

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