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Listeria monocytogenes Activated p38 MAPK and Induced IL-8 Secretion in a Nucleotide-Binding Oligomerization Domain 1-Dependent Manner in Endothelial Cells¹

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Nucleotide-binding oligomerization domain (Nod) proteins serve as intracellular pattern recognition molecules recognizing peptidoglycans. To further examine intracellular immune recognition, we used *Listeria monocytogenes* as an organism particularly amenable for studying innate immunity to intracellular pathogens. In contrast to wild-type *L. monocytogenes*, the nonpathogenic *Listeria innocua*, or *L. monocytogenes* mutants lacking internalin B or listeriolysin O, poorly invaded host cells and escaped into host cell cytoplasm, respectively, and were therefore used as controls. In this study, we show that only the invasive wild-type *L. monocytogenes*, but not the listeriolysin O- or internalin B-negative *L. monocytogenes* mutants or *L. innocua*, substantially induced IL-8 production in HUVEC. RNA interference and Nod1-overexpression experiments demonstrated that Nod1 is critically involved in chemokine secretion and NF- κ B activation initiated by *L. monocytogenes* in human endothelial cells. Moreover, we show for the first time that Nod1 mediated activation of p38 MAPK signaling induced by *L. monocytogenes*. Finally, *L. monocytogenes*- and Nod1-induced IL-8 production was blocked by a specific p38 inhibitor. In conclusion, *L. monocytogenes* induced a Nod1-dependent activation of p38 MAPK signaling and NF- κ B which resulted in IL-8 production in endothelial cells. Thus, Nod1 is an important component of a cytoplasmic surveillance pathway. *The Journal of Immunology*, 2006, 176: 484–490.

The recognition of conserved pathogen-associated molecular patterns by so-called pattern recognition receptors activates the innate immune system (1). The recently found nucleotide-binding oligomerization domain (Nod)³ proteins are in contrast to the transmembranous TLRs localized in the cytoplasm (2). While Nod1 detects peptidoglycan containing *meso*-diaminopimelic acid (3, 4), Nod2 recognizes the muramyl dipeptide MurNAc-L-Ala-D-isoGln which is conserved in basically all kinds of peptidoglycans (5, 6). So far, Nod1 and Nod2 have been shown to activate the NF- κ B, a process in which the receptor-interacting protein 2 (Rip2) as well as the I κ B kinase complex are involved (2). Moreover, we recently indicated that members of the IL-1R/TLR signaling cascade such as IL-1R-associated kinase and

TNFR-associated factor 6 are downstream mediators of Nod proteins (7).

Listeria monocytogenes is a Gram-positive, facultative intracellular bacteria which is particularly amenable to study pathogenesis of infections with cytosolic bacteria, as it enters and grows rapidly in the cytoplasm of a wide variety of tissue culture cells (8, 9). The infectious process of *L. monocytogenes* can be separated into different steps including adhesion, invasion, escape from the phagosomal compartment, intracytosolic replication, and cell-to-cell spread. Although listerial internalin (Inl) A and InlB are crucial for host cell adhesion and invasion, listeriolysin O (LLO) mediates the lysis of bacterium-containing vacuoles and is mandatory for cytosolic entry, intracellular survival, and replication (8, 9). Target cell activation triggered by *L. monocytogenes* is poorly understood, but a cytosolic-triggered cell activation was strongly suggested (10, 11). In contrast to wild-type *L. monocytogenes*, the nonpathogenic *Listeria innocua* very poorly invades human cells (12, 13).

In this study, we demonstrate that Nod1 mediates activation of p38 MAPK and NF- κ B, which contributes to IL-8 production in human endothelial cells infected with invasive *Listeria*.

Materials and Methods

Bacterial strains and growth media

The *L. monocytogenes* serotype 1/2a strain EGD and the *L. innocua* serotype 6b strain ATCC 33090 (INN; American Type Culture Collection) were grown in brain heart infusion broth (Difco). Recombinant strains of *L. monocytogenes* and *L. innocua* were generated as previously described (14, 15). For some experiments, *Listeria* were heat-inactivated (95°C, 30 min).

RNA interference (RNAi) in HUVEC

RNAi experiments in HUVEC were done as described previously (16): control nonsilencing small interference RNA (siRNA) (sense UUCUC CGAACGUGUCACGUt, antisense ACGUGACACGUUCGGAGGAG AAAtt) and different siRNA targeting Nod1 (sequence 1: sense GGGUGAGAC

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³ Abbreviations used in this paper: Nod, nucleotide-binding oligomerization domain; Rip2, receptor-interacting protein 2; Inl, internalin; LLO, listeriolysin O; RNAi, RNA interference; siRNA, small interference RNA; MAPKAPK2, MAPK-activated protein kinase 2; MOI, multiplicity of infection; MALP-2, macrophage-activating lipopeptide; DAPI, 4',6'-diamidino-2-phenylindole; BHI, brain-heart infusion.

CAUCUUCAUctt, antisense GAUGAAGAUGGUCACCCt; sequence 2: sense GGCCAAAGUCUAUGAAGAAtt, antisense AUCUUC AUAGACUUUGGCCtc), Nod2 (sequence 1: sense GGAUUAC CAGUCCCAUUGtt, antisense CAAUGGGACUGGUAUUCCt; sequence 2, sense GGCUGAAAUCAGAAUAUtt, antisense AAUAUUCUGAAUUUCAGCCtc), or TLR2 (sense GCCUUGACCU GUCCAACAAtt, antisense UUGUUGGACAGGUCAAGGctt) were purchased from Ambion. HUVEC were isolated from umbilical cord veins and transfected by using the Amaxa Nucleofector according to the manufacturer's protocol with 2 μ g of siRNA/10⁶ cells (16). Functional studies examining the role of Nod1 in cell activation by *Listeria* were performed 72 h after siRNA transfection.

Expression plasmids and HEK293 cell overexpression experiments

Expression plasmids for Nod1, Nod2, TLR2, TLR3, and IFN- β luciferase reporter were provided by G. Nuñez (University of Michigan Medical School and Comprehensive Cancer Center, Ann Arbor, MI), C. Kirschning (Technical University of Munich, Munich, Germany), and D. Golenbock (University of Massachusetts Medical School, Worcester, MA), respectively. For reporter gene assays, human embryonic kidney HEK293 cells (American Type Culture Collection) were cultured in 24-well plates with DMEM (Invitrogen Life Technologies) supplemented with 10% FCS. Cells

were cotransfected using the calcium phosphate method (BD Clontech) with 0.05 μ g of the NF- κ B reporter or 0.3 μ g of the IFN- β reporter, 0.05 μ g of the RSV- β -galactosidase reporter, and 0.2 ng of Nod expression vectors. For the experiments in which phosphorylation of p38 MAPK or IL-8 secretion was assessed, HEK293 cells were cultured in 12- or 24-well plates, respectively, and were transfected with 1.2 μ g (12-well plate) or 0.5 μ g (24-well plate) of either an empty control vector or Nod1 expression plasmid by using the Superfect reagent (Qiagen).

Macrophage-activating lipopeptide Infection of HUVEC and HEK293 cells

For experiments studying MAPK or MAPK-activated protein kinase 2 (MAPKAPK2) activation, cells were medium starved for 3 h before stimulation. HUVEC or HEK293 cells were inoculated with *L. monocytogenes* or *L. innocua* at a multiplicity of infection (MOI) of 0.05–5, as indicated, in MCDB131 or DMEM, respectively. Plates were centrifuged at 600 \times g for 30 min to enhance the contact of bacteria with HUVEC. After 60 min, HUVEC were washed with PBS and fresh culture medium containing 100 μ g/ml gentamicin was added to the cells. Cells were then incubated for a further 1 h (MAPK or MAPKAPK2 phosphorylation) or overnight (IL-8 or Nod expression), respectively. For some experiments, cells were incubated

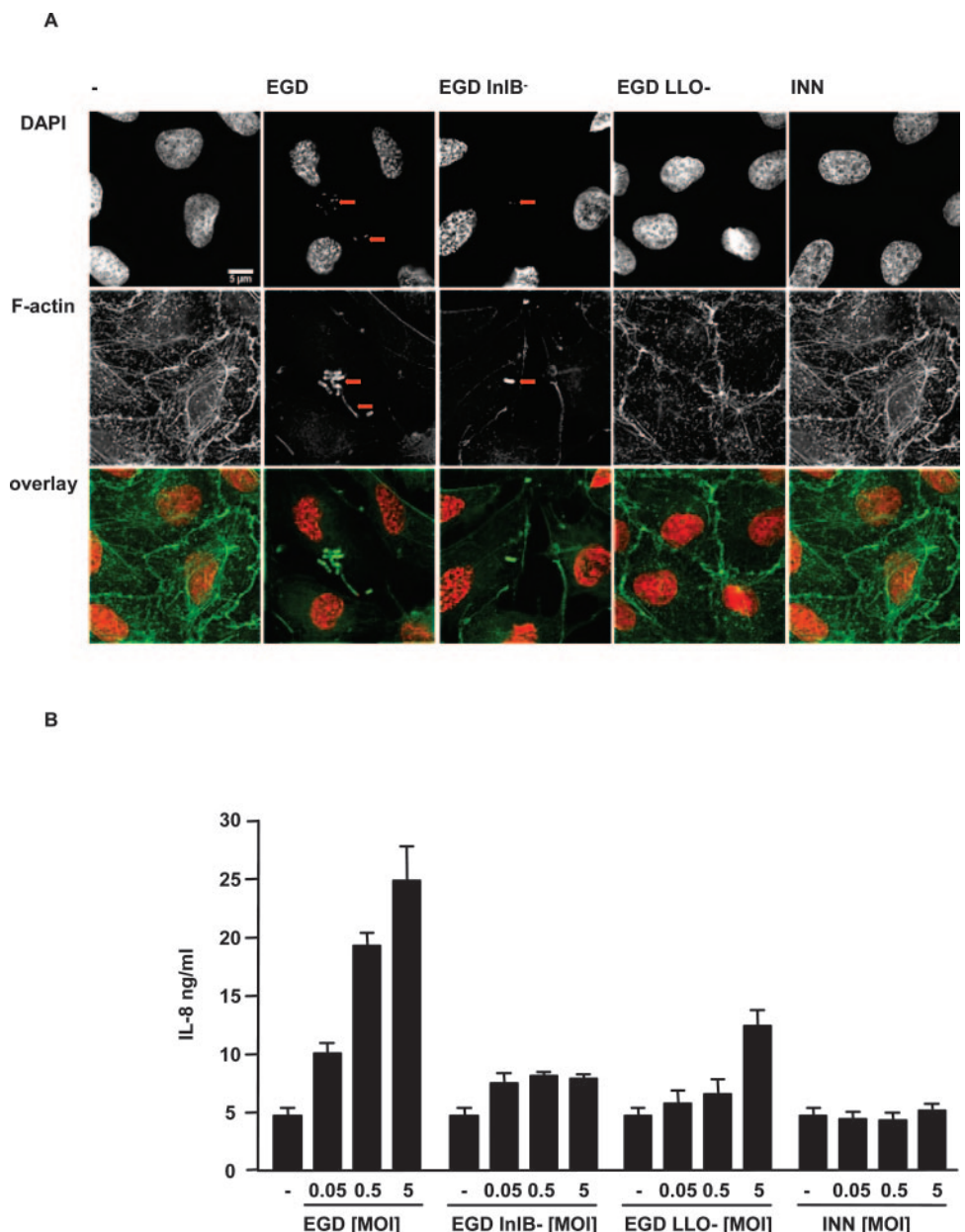


FIGURE 1. Invasiveness and capability to induce IL-8 of different *Listeria*. *A*, HUVEC were infected with *L. monocytogenes* (EGD), *L. monocytogenes* lacking internalin B (EGD InIB⁻), or listeriolysin (EGD LLO⁻), or *L. innocua* (INN). Intracellular bacteria were detected by confocal laser scanning microscopy as described in *Materials and Methods*. *B*, HUVEC were infected with bacteria as indicated, and supernatants were analyzed for IL-8 secretion by ELISA. Data presented are mean \pm SE of three different experiments performed in duplicate.

with the specific p38 MAPK inhibitor SB202190 (Calbiochem) or an inactive control peptide SB202474 (Calbiochem), in concentrations as indicated. TNF- α (10–30 ng/ml), LPS (1 μ g/ml), poly(I:C) (25 μ g/ml), or macrophage-activating lipopeptide 2 (MALP-2) (100 ng/ml) were used as controls. To investigate NF- κ B activation in HEK293 cells by heat-inactivated *Listeria*, heat-inactivated bacteria were added to the cells in the presence of calcium phosphate (during cDNA transfection) for 5 h to allow their entry into the host cells as reported (4, 6).

Confocal laser scanning microscopy

HUVEC monolayers were infected with *Listeria*, subsequently fixed with 3% paraformaldehyde (w/v; Sigma-Aldrich) for 20 min, and 4',6'-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) staining for cellular and bacterial DNA was performed. For visualization of intracellular *L. monocytogenes*, *Listeria* mutants or *L. innocua*, additional F-actin staining was conducted with Phalloidin Alexa488 (Molecular Probes). Fluorescent images were acquired using an Axioskop 2 mot (objective: PlanNeoFluar \times 100, NA 1.4) equipped with an AxioCam MRM cooled greyscale camera (Zeiss). Deconvolution and digital image processing was executed by ImageProPlus 5.0 software (Media Cybernetics). To reach higher contrast for bacterial DNA and F-actin cladding in overlay images, red color was given for the DAPI channel.

Luciferase assay

Relative luciferase activities were obtained as described previously (7, 16).

IL-8 ELISA

IL-8 concentrations in HUVEC or HEK293 cell supernatants were assessed as described previously (16).

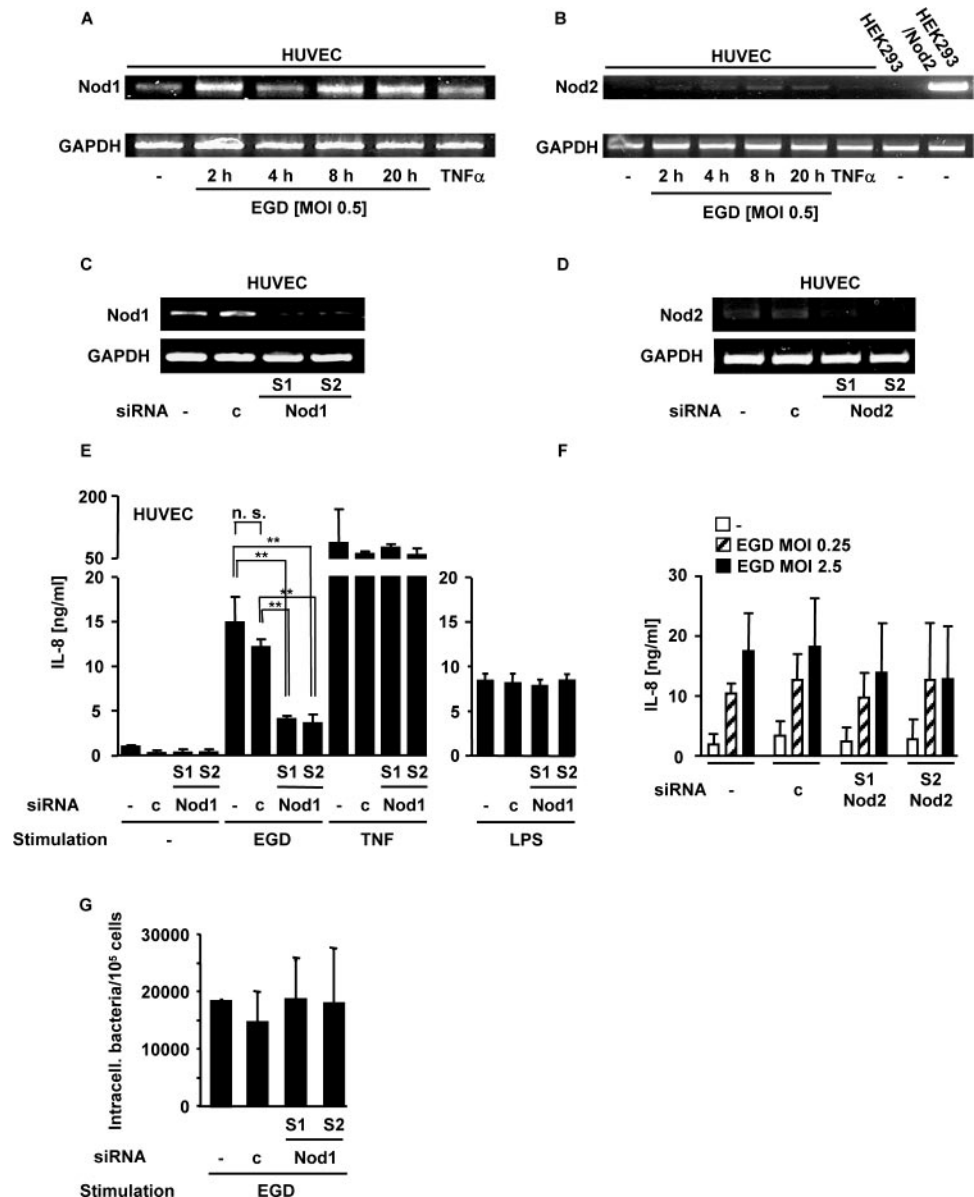
Antibiotic protection assay

HUVEC were transfected with siRNAs as described above, and cultured in 48-well plates. Listerial invasion of HUVEC and potential influence of Nod1-siRNAs hereon was assessed by antibiotic protection assay. HUVEC were infected with *L. monocytogenes* at a MOI of 0.5, and centrifuged at $600 \times g$ for 30 min to enhance the contact of bacteria with HUVEC. After 60 min, cells were washed twice with PBS and fresh culture medium containing 100 μ g/ml gentamicin (to kill extracellular bacteria) was added to the cells for 1 h. Cells were again washed twice with PBS, and incubated overnight with culture medium containing no antibiotics. Subsequently, HUVEC were lysed with 0.2% Triton X-100 for 20 min, and plated on brain-heart infusion (BHI) agar to assess numbers of intracellular *Listeria*. Control experiments demonstrated sufficient killing of extracellular *Listeria* (data not shown).

RT-PCR analysis

Total RNA from HUVEC or HEK293 cells was isolated with the RNeasy Mini kit (Qiagen) and reverse-transcribed using AMV reverse transcriptase (Promega). The generated cDNA was amplified by PCR using specific primers (Nod1: sense, 5'-AAGCGAAGAGCTGACCAAAT-3', antisense,

FIGURE 2. *L. monocytogenes*-induced IL-8 production in HUVEC was inhibited by siRNAs targeting Nod1. Nod1 (A) and Nod2 (B) mRNA levels during listerial infection in HUVEC were analyzed by RT-PCR (Nod1, 31 cycles; Nod2, 34 cycles). HEK293 cells overexpressing Nod2 were used as a positive control for Nod2 RT-PCR. HUVEC were left unaffected or were transfected with Nod1 (C) or Nod2 (D) siRNAs (S1–2, sequences 1–2) or control nonsilencing siRNA (C). After 72 h, gene-silencing abilities of the RNA duplexes used were assessed by RT-PCR. E and F, HUVEC were transfected with the siRNAs as indicated. Seventy-two hours later, *L. monocytogenes* (MOI 0.5 in E), TNF- α (10 ng/ml), or LPS (1 μ g/ml) were added to HUVEC. The next day, IL-8 concentrations in the supernatants were analyzed by ELISA. Data presented in E represent mean \pm SE of two independent experiments of four experiments with similar results, or mean \pm SE of four independent experiments. G, HUVEC were transfected with siRNAs as indicated, infected with *Listeria* after 72 h, and an antibiotic protection assay was performed. The next morning, numbers of internalized bacteria were determined by assessing CFU surviving antibiotic treatment. Data represent mean \pm SE of two independent experiments performed in duplicate.



5'-TTCATAGACTTTGGCCTCCTC-3'; Nod2: sense, 5'-AGCCATTGT CAGGAGGCTC-3', antisense, 5'-CGTCTCTGCTCCATCATAGG-3'). Primers were purchased from TIB MOLBIOL. After 31 (Nod1) or 34 (Nod2) amplification cycles, the PCR products were analyzed on 1.5% agarose gels, stained with ethidium bromide, and subsequently visualized. To confirm equal amounts of RNA in each experiment, all samples were checked for GAPDH mRNA expression.

Immunoblotting

Cytoplasmic cell extracts were separated by SDS-PAGE and blotted. Membranes were exposed to Abs specific to phosphospecific p38 MAPK (Cell Signaling Technology), phosphospecific MAPKAPK2 Thr²²² (BIOMOL) or p42 MAPK (Santa Cruz Biotechnology), respectively. Subsequently, membranes were simultaneously incubated with secondary Abs (IRDye 800-labeled anti-rabbit and Cy5.5-labeled anti-mouse (Rockland Immunochemicals), respectively). Simultaneous detection of target proteins and a loading control requires primary Abs of different species, therefore, we used Abs against p42 (mouse) to check equal loading in the pp38 or pMAPKAPK2 (rabbit Ab) immunoblots. Proteins were detected by using an Odyssey infrared imaging system (LI-COR).

Statistics

Stimulatory effects of *Listeria* as well as inhibitory effects of siRNA or inhibitors used were statistically evaluated using the Student *t* test. Throughout the figures, *p* values of <0.05 are indicated by one asterisk, *p* values of <0.01 by double asterisks.

Results

Only cytosolic *L. monocytogenes*, but not *Listeria* deficient in adhesion and cytosol invasion significantly induced IL-8 production in human endothelial cells

To characterize host cell activation by intracellular vs extracellular bacteria, we incubated HUVEC with wild-type *L. monocytogenes* (EGD), InlB-negative, or LLO-negative mutants of *L. monocytogenes*, or nonpathogenic *L. innocua* (INN). Wild-type *L. monocytogenes* could be seen in the cytoplasm of human endothelial cells examined and recruited host cell actin (Fig. 1A). *L. monocytogenes* deficient in InlB hardly entered, and *L. innocua* failed to enter, into the host cell cytoplasm. Moreover, the LLO-negative *L. monocytogenes* could not be seen neither in actin or in DAPI staining, demonstrating an insufficient entry of the bacteria into the cytoplasm, and suggesting a deficient propagation of the bacteria and/or sufficient killing of these vacuole-trapped bacteria (Fig. 1A). Interestingly, the induction of IL-8 in endothelial cells strongly correlated with the invasiveness: only wild-type *L. monocytogenes*, but not the nonvirulent strain *L. innocua* or the invasion-deficient *L. monocytogenes* mutants, induced IL-8 in HUVEC (Fig. 1B). Moreover, overexpression of LLO in *L. innocua* resulted only in very little restoration of the capability to activate HUVEC with respect to IL-8 secretion (data not shown). For *Listeria*, the ability to reside within the cytoplasm of HUVEC seemed therefore to be essential for cell activation, raising the question of whether intracellular immune receptors might be involved.

L. monocytogenes-induced IL-8 production was inhibited by siRNA targeting *Nod1*

In the next step, we tested the hypothesis that cytoplasmic Nod proteins mediate proinflammatory activation of endothelial cells by intracellular *Listeria*. We detected Nod1-mRNA in HUVEC by RT-PCR experiments (Fig. 2A), whereas only very little Nod2-mRNA could be found in HUVEC after listerial infection (Fig. 2B). In contrast, Nod2 was clearly seen in control cells overexpressing Nod2, indicating the reliability of our assay. To address the role of Nod1 and Nod2 in *L. monocytogenes*-induced IL-8 secretion in endothelial cells, we performed RNAi experiments to inhibit the expression of endogenous Nod1 and Nod2. First, we evaluated different siRNAs for their ability to reduce Nod mRNA expression. Only the Nod1- or Nod2-specific oligonucleotides, re-

spectively, inhibited Nod1 or Nod2 mRNA levels (Fig. 2, C and D). Both siRNAs targeting Nod1 (but not the nonsilencing siRNA or Nod2 siRNAs) significantly blocked the IL-8 production induced by *L. monocytogenes* in HUVEC (Fig. 2E, left panel, and F). In contrast, the TNF- α -related cytokine production was not reduced by any siRNA used. Moreover, while *Listeria*-induced IL-8 secretion was clearly reduced by Nod1-siRNA, IL-8 production stimulated by LPS (TLR-dependent) was not affected by the siRNAs (Fig. 2E, right panel). Nod2 has been indicated as an antibacterial factor constraining bacterial survival (17, 18). Thus, we examined whether Nod1 siRNAs affect *Listeria*-induced IL-8 production indirectly by altering numbers of intracellular bacteria. In our antibiotic protection assay, we could not see a significant influence of any siRNAs used on the numbers of intracellular *Listeria* 20 h postinfection (Fig. 2G). Overall, our observations demonstrate that Nod1 is important for *L. monocytogenes*-induced, but not TNF- α - or LPS-induced, signaling leading to IL-8 production in HUVEC.

TLR2 mediates *Listeria*-induced NF- κ B activation in HEK293 cells, but is not crucial for *Listeria*-induced IL-8 secretion in HUVEC

TLR2 has been implicated in *Listeria*-induced cell activation (Chinese hamster ovary fibroblast cell lines) and host responses against *Listeria* in mice (19, 20). As can be seen in Fig. 3A, HEK293 cells overexpressing TLR2 responded to *L. monocytogenes* and *L. innocua*, indicating that TLR2 mediated HEK293 cell activation by

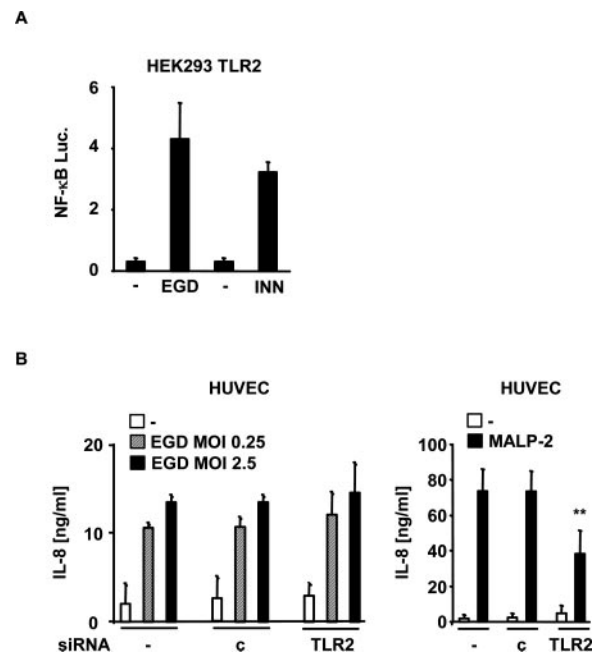


FIGURE 3. TLR2 mediates *Listeria*-induced NF- κ B activation in HEK293 cells, but is not crucial for *Listeria*-induced IL-8 secretion in HUVEC. **A**, HEK293 cells were cotransfected with TLR2, as well as an NF- κ B reporter and a β -galactosidase reporter plasmid. The next day, cells were infected with *L. monocytogenes* or *L. innocua*; relative luciferase activities were obtained and shown as means \pm SE expressed as fold inductions for one representative experiment of three independent experiments, each transfection performed in duplicate. **B**, HUVEC were transfected with siRNA targeting TLR2, and after 72 h, were infected with *L. monocytogenes*, or MALP-2 (100 ng/ml) were added to HUVEC. The next day, IL-8 concentrations in the supernatants were analyzed by ELISA. Data represents mean \pm SE of three independent experiments. In contrast to the *Listeria*-induced IL-8 secretion, the MALP-2-induced IL-8 production (73.85 \pm 12.28 ng/ml) was significantly inhibited by TLR2 siRNA (38.53 \pm 12.88 ng/ml).

invasive and noninvasive bacteria. The behavior of TLR2/HEK293 cells is in contrast to our results obtained in HUVEC, showing that only intracellular *Listeria* but not the noninvasive bacteria induced IL-8 secretion in these cells, thus suggesting a minor role of TLR2 in HUVEC activation by *Listeria*. To further exclude an involvement of TLR2 in *Listeria*-induced HUVEC activation, we performed RNAi experiments: siRNA targeting TLR2 did not abrogate IL-8 production induced by *L. monocytogenes* in HUVEC, but did significantly inhibited the HUVEC activation by the TLR2 agonist MALP-2 (Fig. 3B). Overall, our data indicate that TLR2 is not crucial for *Listeria*-induced IL-8 production in HUVEC.

Nod1 and Nod2 mediated NF- κ B activation by L. monocytogenes in HEK293 cells

To further confirm the role of Nod proteins in target cell activation by *Listeria*, we performed overexpression assays. Therefore, HEK293 cells were transfected with an NF- κ B reporter, and with an empty control vector, Nod1 or Nod2 expression vectors. Intracellular stimulation with heat-inactivated bacteria was done as it has been performed earlier (4, 6), and as described in *Materials and Methods*. In this experiment, bacteria activated the NF- κ B reporter only when Nod1 or Nod2 were overexpressed (Fig. 4A). In contrast to the NF- κ B reporter, an IFN- β promoter-dependent reporter was only very slightly activated by *Listeria* via Nod1 or Nod2 but cells responded with strong IFN- β promoter activation after stimulation with poly(I:C) through TLR3 (Fig. 4B). Overall, data obtained indicate that Nod proteins mediated *L. monocytogenes*-induced NF- κ B activation but are not likely involved in IFN- β induction.

siRNAs targeting Nod1 reduced p38 MAPK phosphorylation induced by L. monocytogenes in HUVEC

Besides NF- κ B signaling, activation of p38 MAPK has been shown to be fundamental for inflammatory processes (21). To ad-

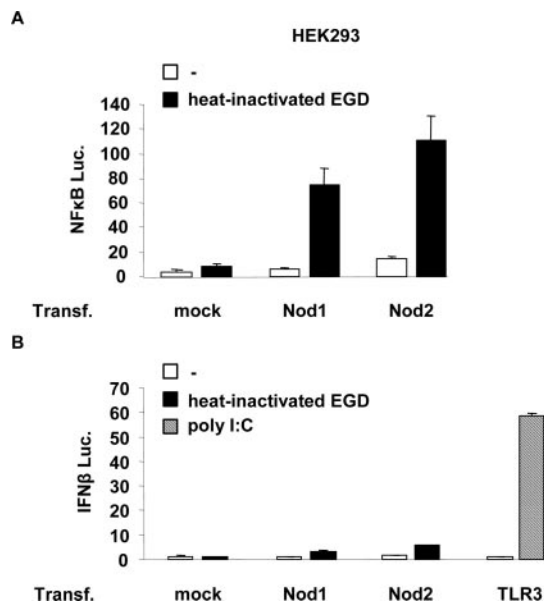


FIGURE 4. *L. monocytogenes* induced NF- κ B activation. HEK293 cells were cotransfected with a control vector (mock), Nod1, Nod2, or TLR3 expression plasmids, as well as an NF- κ B-reporter (A) or IFN- β -reporter construct (B). Moreover, the heat-inactivated bacteria were added to the cells during transfection to allow internalization of bacterial components. Poly(I:C) stimulation (TLR3 agonist) was performed the next day. Relative luciferase activities were obtained and shown as means \pm SE expressed as fold inductions for one representative experiment of three independent experiments, each transfection performed in duplicate.

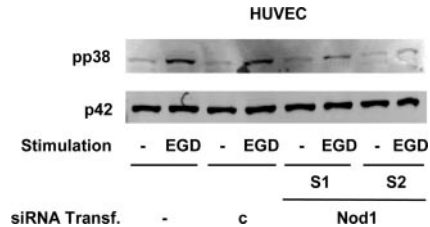


FIGURE 5. Nod1 is essential for *L. monocytogenes*-induced p38 MAPK phosphorylation in HUVEC. HUVEC were transfected with siRNAs as indicated. Seventy-two hours after transfection, cells were infected with *L. monocytogenes* (EGD) at a MOI of 1. Total cell lysates were then fractionated by SDS-PAGE and analyzed by immunoblotting using an Ab detecting the phosphorylated form of p38 MAPK (pp38). Abs against p42 were used to check equal loading. Data represent one of four independent experiments.

dress the question of whether Nod1 activates p38 MAPK, we used HUVEC, which were infected with *L. monocytogenes*. Indeed, *L. monocytogenes* induced phosphorylation of p38 MAPK in HUVEC (Fig. 5). The *Listeria*-induced activation of p38 examined was reduced by siRNAs targeting Nod1, but not by a nonspecific control siRNA (Fig. 5). Thus, our results strongly suggest an involvement of Nod1 in *L. monocytogenes*-induced activation of p38.

Nod1 mediated phosphorylation of p38 induced by Listeria in HEK293 cells

To further clarify the association of Nod1 with MAPK activation, we performed overexpression studies in HEK293 cells. Cells were transfected with either a control vector or with Nod1, and infected with *L. monocytogenes*. The *L. monocytogenes*-induced phosphorylation of p38 MAPK in HEK293 cells was increased following overexpression of Nod1 (Fig. 6), confirming the critical role of Nod1 for p38 MAPK activation induced by intracellular bacteria. The somewhat moderate effects of Nod1 overexpression could be explained by the fact that in our transient overexpression assays, only 50% of the cells expressed the transfected gene of interest (data not shown).

siRNAs targeting Nod1 reduced pMAPKAPK2 phosphorylation induced by L. monocytogenes in HUVEC

p38 MAPK signaling leads to phosphorylation and activation of downstream effector proteins such as the MAPKAPK2 (22). To address the question of whether Nod1 also mediates MAPKAPK2 activation by *Listeria*, we used HUVEC that were infected with *L. monocytogenes*. Indeed, Nod1 siRNAs inhibited the *L. monocytogenes*-induced phosphorylation of MAPKAPK2 in HUVEC (Fig. 7). Thus, our results indicated that in *Listeria* infection of HUVEC, Nod1 is important for signaling events downstream of p38.

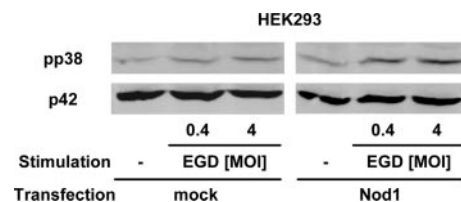


FIGURE 6. Phosphorylation of p38 MAPK by *L. monocytogenes* in HEK293 cells is dependent on Nod1. HEK293 cells were either transfected with an empty control vector (mock) or Nod1 expression plasmid using Superfect reagent. The next day, cells were infected with *L. monocytogenes* (EGD). Cells were harvested and subjected to SDS-PAGE. Phosphospecific p38 (pp38) and p42 MAPK were simultaneously detected by an infrared imaging system. Data represent one of three independent experiments.

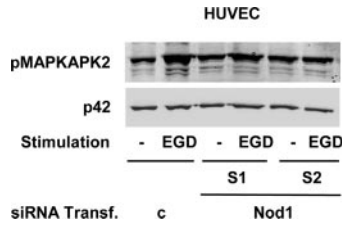


FIGURE 7. Nod1 is essential for *L. monocytogenes*-induced MAPKAP2 phosphorylation in HUVEC. HUVEC were transfected with siRNAs as indicated. Seventy-two hours after transfection, cells were infected with *L. monocytogenes* (EGD) at a MOI of 1. Total cell lysates were then fractionated by SDS-PAGE and analyzed by immunoblotting using an Ab detecting the phosphorylated form of MAPKAPK2 (pMAPKAPK2). Abs against p42 were used to check equal loading. Data represent one of three independent experiments.

Importance of p38 MAPK for L. monocytogenes- and Nod1-induced IL-8 production

Following our observation that *L. monocytogenes* induced p38 MAPK activation as well as IL-8 secretion via Nod1 in HUVEC, we sought to examine the role of p38 MAPK in IL-8 production induced by *L. monocytogenes* and Nod1. Therefore, cells were pretreated with a specific p38 inhibitor (SB202190) or with an inactive control peptide (SB202474). *L. monocytogenes*-induced

IL-8 production in HUVEC was reduced in a dose-dependent fashion by the p38 MAPK inhibitor (Fig. 8A). In contrast, the control peptide hardly showed any inhibitory effect with respect to *L. monocytogenes*-related IL-8 secretion. Moreover, TNF- α -induced IL-8 secretion was only slightly affected by any inhibitor used.

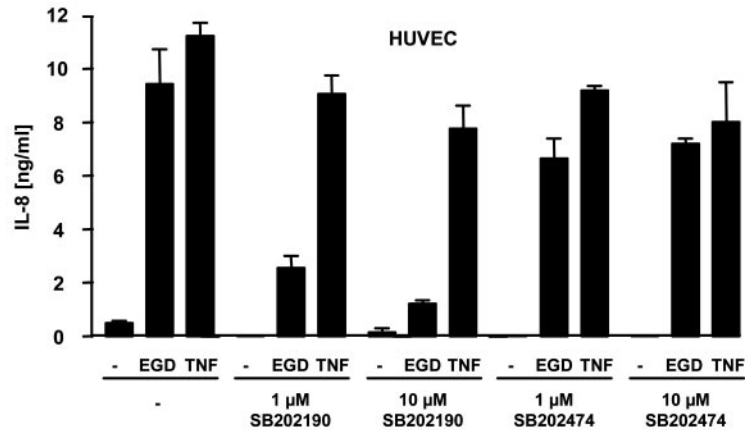
Furthermore, Nod1 overexpression induced IL-8 production in HEK293 cells (Fig. 8B). This Nod1-dependent chemokine secretion was reduced by the specific p38 MAPK inhibitor but not by the control peptide (Fig. 8B and data not shown). Overall, data obtained demonstrate that IL-8 production by *L. monocytogenes* and Nod1 is dependent on p38 MAPK activation.

Discussion

The study presented demonstrates that Nod1 is crucially involved in endothelial cell activation induced by the cytosolic pathogen *L. monocytogenes*. Data show that intracellular *L. monocytogenes* induces p38 MAPK and MAPKAPK2 activation, NF- κ B signaling, and subsequent IL-8 production via Nod1. The identification of p38 MAPK as a downstream kinase of Nod1 expands the potential biological effector systems affected by Nod1-dependent cell stimulation.

Our results are consistent with recently published studies indicating a cytosol-specific surveillance mechanism involved in *L. monocytogenes*-induced target cell activation (10, 11). It was shown that cytosol-, but not the vacuole-, localized bacteria induced

A



B

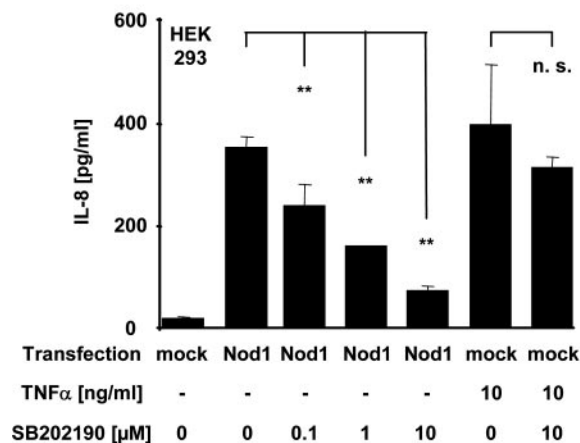


FIGURE 8. Role of p38 MAPK in *L. monocytogenes*- and Nod1-induced IL-8 production. **A**, HUVEC were pretreated with either a specific p38 inhibitor (SB202190) or with a control peptide (SB202474) for 45 min, and then stimulated with *L. monocytogenes* (EGD, MOI 0.5) or TNF- α (10 ng/ml). The next day, supernatants were subjected to IL-8 ELISA. Data presented are mean \pm SE of one representative experiment of four independent experiments. **B**, HEK293 cells transfected with a control vector (mock) or Nod1. Twenty-four hours later, cells were incubated with SB202190 in different concentrations and with TNF- α where indicated for a further 12 h, and supernatants were analyzed by ELISA. Data are shown as means \pm SE for three experiments with transfection performed in triplicate.

NF- κ B and p38 phosphorylation (11). Moreover, two recently published studies demonstrated that IFN- β induction by *Listeria* was independent on the TLRs, and was also independent on Rip2 and Nod2 (23, 24). In this study, we demonstrate that Nod1 is also unlikely to be crucial for IFN- β induction. A possible explanation would be that a so far unidentified receptor might be involved in the IFN- β induction induced by *Listeria*. Taken in consideration that in the TLR family all members mediate NF- κ B activation but only some members are involved in type I IFN induction (25), we propose a model in which Nod1 (and Nod2 in Nod2-expressing cells (Fig. 4A and Ref. 26) mediates NF- κ B and MAPK activation by *Listeria*, whereas other innate immune receptors mediate activation of IFN regulatory factors leading to IFN- β induction.

One of the initial reports about Nod protein functions demonstrated that JNK activation induced by *Shigella flexneri* was inhibited by dominant-negative Nod1 in epithelial cells (27). Our results obtained by RNAi experiments add p38 MAPK signaling to the events triggered by intracellular bacteria via Nod1. Our data furthermore suggest that p38 MAPK are involved in Nod1-induced signaling and subsequent gene expression. Hence, Nod1-related p38 MAPK activation may contribute significantly to *L. monocytogenes*-induced epigenetic modifications paving the way to forced expression of inflammatory genes (28).

Our results demonstrating a fundamental role of Nod1 in *L. monocytogenes*-triggered cell activation are in line with the fact that Nod1 recognizes peptidoglycan containing *meso*-diaminopimelic acid (3, 4), found primarily in Gram-negative bacteria but also in Gram-positive *L. monocytogenes* (29). Nod2 seemed to not be critically involved in endothelial cell activation by *Listeria*, because RT-PCR analysis show very little Nod2 expression in HUVEC, and siRNA targeting Nod2 did not significantly inhibit the HUVEC activation by *L. monocytogenes*. However, Nod2 is very likely to be involved in *Listeria* recognition in other cell types (Fig. 4A and Ref. 26).

A critical involvement of TLRs in *L. monocytogenes*-induced endothelial cell activation is unlikely. TLR2 would be the candidate for sensing bacteria examined (19, 20), but TLR2 siRNA did not abrogate *Listeria*-induced HUVEC activation (Fig. 3B). Moreover, both *L. monocytogenes* and *L. innocua* are equally capable of activating TLR2-expressing HEK293, but only the cytosol-localized *L. monocytogenes* induced IL-8 secretion in HUVEC (Figs. 1B and 3A).

In summary, we have identified Nod1 as a fundamental mediator of endothelial cell innate immune responses. Our results suggest a signaling pathway triggered by *L. monocytogenes* in HUVEC, involving Nod1, p38, as well as NF- κ B, leading to gene expression. Thus, our data expand the knowledge about the recently described cytosolic surveillance system in eukaryotic cells.

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Disclosures

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