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J Immunol 2009; 183:8099-8109; doi: 10.4049/jimmunol.0900664
http://www.jimmunol.org/content/183/12/8099

Supplementary Material

http://www.jimmunol.org/content/suppl/2009/12/10/183.12.8099.DC1

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Helicobacter pylori Induces MAPK Phosphorylation and AP-1 Activation via a NOD1-Dependent Mechanism

Cody C. Allison,*† Thomas A. Kufer,‡ Elisabeth Kremmer,§ Maria Kaparakis,*† and Richard L. Ferrero2*†

Helicobacter pylori rapidly activates MAPKs and transcription factors, NF-κB and AP-1, in gastric epithelial cells following host attachment. Activation of these signal transducers is largely dependent on the cag pathogenicity island (cagPAI)-encoded Type IV Secretion System. H. pylori was shown to translocate peptidoglycan through the Type IV Secretion System, which is recognized by the pathogen recognition molecule, NOD1, thus resulting in NF-κB activation. The mechanisms of H. pylori-induced MAPK and AP-1 activation, however, are less well defined and therefore, we assessed the contribution of NOD1 to their activation. For this, we used gastric epithelial cell lines, stably expressing siRNA to either NOD1 or a control gene. In siNOD1-expressing cells stimulated with cagPAI-H. pylori, we observed significant reductions in p38 and ERK phosphorylation (p < 0.05), whereas the levels of Jnk phosphorylation remained unchanged. Consistent with a previous report, however, we were able to demonstrate NOD1-dependent Jnk phosphorylation by the invasive pathogen Shigella flexneri, highlighting pathogen-specific host responses to infection. We also show that NOD1 was essential for H. pylori induction of not only NF-κB, but also AP-1 activation, implying that NOD1 induces robust proinflammatory responses, in an attempt to rapidly control infection. Pharmacological inhibition of p38 and ERK activity significantly reduced IL-8 production in response to H. pylori, further emphasizing the importance of MAPKs in innate immune responses to the pathogen. Thus, for the first time we have shown the important role for NOD1 in MAPK and AP-1 activation in response to cagPAI-H. pylori. The Journal of Immunology, 2009, 183: 8099–8109.

Infection with Helicobacter pylori is associated with chronic gastritis, which is characterized by infiltration of neutrophils, lymphocytes, and other inflammatory mediators into the gastric mucosa (1–3). This immune cell migration is largely regulated by the production of IL-8 and other proinflammatory chemokines by gastric epithelial cells in response to bacterial attachment (1–4). Strains of H. pylori that possess a cag pathogenicity island (cagPAI) are associated with the production of higher levels of IL-8 and more severe disease than cagPAI- strains (5–8). The cagPAI encodes a type IV secretion system (T4SS), through which effectors, such as CagA (9) and peptidoglycan (10), are translocated into the host cell. The secretion of effectors through the T4SS activates a signaling cascade culminating in the migration of the transcription factor NF-κB to the nucleus and transcription of inflammatory mediators, such as IL-8 and MCP-1 (5, 8, 10–14). H. pylori was also reported to rapidly activate another transcription factor, AP-1, in a cagPAI-dependent manner (15, 16). AP-1 is activated by MAPKs and is capable of inducing a strong proinflammatory response, often in concert with NF-κB (11). Indeed, early studies demonstrated that chemokine production could be diminished in H. pylori stimulated cells that had been pretreated with MAPK inhibitors (13, 17–20).

The signaling events leading to rapid MAPK phosphorylation during H. pylori infection are not well understood, although the T4SS is known to be required for complete p38, SAPK/Jnk, and ERK phosphorylation (19, 21). Additional H. pylori factors are believed to be involved in MAPK activation. The vacuolating cytotoxin produced by many isolates, VacA, was found to activate p38 and ERK (22, 23), however, these experiments were performed using purified VacA and not with live bacteria. Subsequent studies identified that H. pylori ΔvacA mutants were able to induce AP-1 transactivation to the same extent as wild-type bacteria (16, 20), implying that VacA is dispensable for AP-1 activity. A second H. pylori factor, CagA, encoded by the cagPAI, induces ERK phosphorylation via the Ras→Raf→Mek→ERK→NF-κB pathway, which can further augment IL-8 production (24). However, the effects of CagA on IL-8 production are not seen until 24 h after infection (24). Also, ERK can still be induced by cagPAI-independent mechanisms (19, 21), though CagA is required for complete AP-1 activation (15, 16), suggesting that CagA plays an additive role in transcription factor activation. Jnk activation during H. pylori infection has also been reported to require a functional T4SS (19, 21).

The cytosolic pathogen recognition molecule, nucleotide binding and oligomerization domain 1 (NOD1), recognizes specific conserved motifs found almost exclusively in the cell wall peptidoglycan of Gram-negative bacteria (25, 26). H. pylori peptidoglycan is delivered to the host cell via the T4SS, where it is recognized by cytosolic NOD1 (10). Upon stimulation with purified...
agonist, NOD1 associates with the receptor-interacting protein serine-threonine kinase 2, (RICK), triggering a proinflammatory response, characterized by NF-κB activation and IL-8 production (27, 28). The involvement of RICK in this signaling cascade, however, has not yet been demonstrated during H. pylori infection.

In addition to activation of the classical NF-κB pathway, NOD1 was reported to be essential for p38 phosphorylation in endothelial cells during Listeria monocytogenes infection (30). This NOD1-dependent p38 activation was also found to induce IL-8 production (30). Furthermore, purified NOD1 agonist rapidly induced activation of p38, ERK, and JNK in murine macrophages (31). Despite the knowledge that NOD1 is clearly capable of activating MAPKs, however, the role of NOD1 in MAPK-dependent inflammatory responses during H. pylori infection is unknown.

The mechanisms of H. pylori-induced MAPK activation are unclear and though many factors are thought to play a role, most are expendable. To better understand the mechanisms of H. pylori-dependent MAPK activation, we examined the requirement for NOD1 in MAPK and AP-1 activation during H. pylori infection. We observed that NOD1 was necessary for complete MAPK activation in the early stages of infection and that NOD1 was essential for the activation of both NF-κB and AP-1, as well as the release of proinflammatory cytokines in response to H. pylori. Thus, NOD1-dependent MAPK activation may represent an additional signaling pathway through which this pathogen controls the transcription of a subset of novel downstream target genes during infection.

Materials and Methods

Cell culture and reagents
Human adenocarcinoma gastric epithelial cells (AGS) as well as AGS cells stably expressing siRNA to either the caspase-activation and recruitment domain (CARD) of NOD1 (AGS siNOD1) or an irrelevant gene, enhanced GFP, EGFP (AGS siEGFP), were cultured in RPMI 1640 (Life Technologies) containing 10% FBS (Thermo Electron). The detailed characterization of this cell line will be reported elsewhere (A. Grunbaum, M. Kaparakis, J. Viaala, C. Allison, L. Badea, A. Karrar, I. Boneca, L. Le Bourhis, S. Restuccia, I. Smith, et al., manuscript in preparation). The human embryonic kidney cell line, HEK293, was cultured in DMEM (Life Technologies) containing 10% (v/v) FBS. All cell lines were supplemented with 1% (v/v) penicillin-streptomycin (Life Technologies) and 1% (v/v) Glutamax (Life Technologies) and grown at 37°C, with 5% CO₂. Additionally, AGS siNOD1 and siEGFP were supplemented with 400 μg/ml Geneticin (Life Technologies).

For MAPK inhibition experiments, cells were pretreated with pharmacological MAPK inhibitors, SB203580 (p38 inhibitor) and U0126 (ERK inhibitor) (10 μM) (all from Calbiochem) for 1 h before the addition of bacteria. As a positive control, TNF-α (10 ng/ml) (Chemicon) was added to cells for indicated periods of time.

The NOD1-specific mAb was prepared as described previously (32). In brief, Abs were raised in rats immunized with the GST-tagged CARD of NOD1 (29, 33) or AP-1 luciferase (34) and 250 ng/well of dTK renilla plasmid vector (Promega). The total amount of DNA to be transfected was standardized to 1150 ng/well by the addition of pCDNA3 (10). AGS and HEK293 cells were seeded in triplicate in 24-well plates at a concentration of 1 × 10⁵ cells/ml and incubated for 18–24 h. Transfection was achieved using 4 μg of polyethyleneimine (Polysciences) to 1 μg of total DNA. Cells were cultured in a final volume of 1 ml of complete culture medium per well and incubated for 16–24 h at 37°C in 5% CO₂. Following stimulation, cells were lysed by adding 100 μl of Reporter Lysis Buffer (Promega). Sample aliquots (20 μl) were incubated with either coelenterazine renilla (50 μl) (Synchem) or Luciferin substrate (30 μl) (Promega) solutions. Luminescence was measured using a TECAN infinite M200 luminometer (TECAN) and Magellan version 6.0 software.

Vector constructs expressing RICK (29), NOD1 (35), and NOD1 ΔCARD (35) have been described previously. In brief, AGS cells were seeded in 2-well tissue culture plates at a concentration of 1 × 10⁵ cells/ml and incubated for 18–24 h. Cells were transfected with 100 ng/well of NOD1, NOD1 ΔCARD, or RICK. Cells were incubated for 16 h before stimulation with bacteria.

Bacterial strains and isogenic mutants

H. pylori strains 251 (36) and G27 (37) are clinical isolates and isogenic 251 ΔagpPAI was constructed by natural transformation (38, 90) and G27 ΔcagA and ΔcagM mutants were constructed as described previously (10, 39). Bacteria were routinely cultured on blood agar medium, supplemented or not with 10 μg/ml kanamycin, under microaerophilic conditions (40). Liquid broth cultures were incubated overnight at 37°C with shaking at 125 rpm in 25-cm² tissue culture flask (Iwaki) containing 10 ml of brain heart infusion broth (Oxoid) with 10% (v/v) newborn calf serum (Life Technologies).

Wild-type Shigella flexneri (M90T serotype 5A) and its noninvasive derivative (BS176) cured of the 220K virulence plasmid pWR100, have been described previously (29, 41, 42). S. flexneri strains were routinely cultured on Trypticase Soy Agar (BD Biosciences), supplemented with 0.01% (v/v) Congo Red (British Drug Houses), to differentiate between colonies with and without a virulence plasmid. Liquid broth cultures were prepared by inoculating 8 ml of trypticase soy broth with a single colony. Cultures were grown shaking at 160 rpm overnight at 37°C.

Infection of AGS and HEK293 cells with bacteria

Overnight H. pylori broths were washed twice in PBS and pelleted at 1250 × g for 10 min. Bacterial pellets were resuspended in RPMI 1640 or DMEM and added to AGS or HEK293 cells, respectively, at a multiplicity of infection of 1:10. H. pylori was centrifuged onto cells at 200 × g for 5 min before incubation for the appropriate times. For IL-8 assays, bacteria were washed off in PBS after 1 h of stimulation and the wells replaced with fresh tissue culture medium followed by further incubation.

For S. flexneri coculture experiments, overnight cultures were used to inoculate trypticase soy broth at a 1/100 dilution. Bacteria were grown at 37°C to mid-exponential phase, washed twice in PBS, and then pelleted at 3220 × g for 10 min. Pellets were resuspended in RPMI 1640 and used to infect AGS cells at a multiplicity of infection of 1:50. S. flexneri was centrifuged onto cells at 200 × g for 5 min. Following a 20 min incubation, cells were washed three times with PBS and fresh medium containing gentamicin (50 μg/ml) (Pfizer) was added to wells. This was considered to be time 0 h.

Preparation of H. pylori outer membrane vesicles (OMVs)

OMVs were prepared from H. pylori 251 ΔagpPAI (90). In brief, bacteria were grown in brain heart infusion broth, supplemented with 1.8% (w/v) β-cyclodextrin (Sigma-Aldrich), with shaking at 125 rpm for 16 h. Bacteria were pelleted for 10 min at 3220 × g and the culture supernatants vacuum filtered through a 0.22 μm pore stericup filter (Millipore). OMVs were collected by ultra-centrifugation at 100,000 × g for 2 h at 4°C and quantified by Bradford protein assay (Bio-Rad).

Detection of MAPK phosphorylation

AGS siNOD1 and siEGFP cells were incubated with H. pylori or S. flexneri for the appropriate times. Cells were lysed by the addition of 100 μl boiling Laemmli buffer and subjected to SDS-PAGE. Proteins were transferred to 0.4-μm Transblot nitrocellulose membrane (Bio-Rad), followed by membrane blocking using 5% (w/v) skim milk in TBS. Immunodetection of phosphorylated or total MAPKs was performed by incubating membranes with respective anti-p38,-ERK or -Jnk primary Abs (Cell Signaling Technology) at a dilution of 1/1000 in 5% BSA (Roche), prepared in TBST. Secondary goat anti-rabbit (Chemicon) Abs was used at a dilution of 1/1000 in 5% skim milk in TBST. Detection of actin was performed using primary actin and secondary goat anti-rabbit (Chemicon) Abs at a dilution of 1/1000 in 5% skim milk in TBST. Western blots were developed using ECL detection reagent (GE Healthcare) and exposed to Super RX film (Fuji).
**MAPK densitometry**

Densitometry was performed on Western blots to quantify MAPK activation. Blots from three independent biological replicates were analyzed using ImageQuant software (v7.0). Bands representing either phosphorylated p38, Jnk, or ERK MAPKs were standardized to their corresponding actin bands. Samples were ranked and statistical significance determined using a Mann-Whitney U test.

**Preparation of nuclear extracts and EMSA**

AGS siNOD1 and siEGFP cells were grown in six-well tissue culture plates and stimulated with H. pylori. Cells were washed twice in PBS and resuspended in ice-cold PBS containing protease (Roche) and phosphatase (Sigma-Aldrich) mixture inhibitors. Cells were lysed in 1× hypotonic buffer (20 mM HEPES (pH 7.9), 0.1 mM EDTA, 10 mM KCl, and 1 mM DTT), supplemented with 1% Nonidet P-40. Nuclear pellets were washed in 1× low-salt buffer (10 mM HEPES (pH 7.9), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 25% (v/v) glycerol), then resuspended in 1× high-salt buffer (10 mM HEPES (pH 7.9), 400 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 25% (v/v) glycerol), supplemented with 4.5 μl of 1 M NaCl. Nuclear extracts were collected by centrifugation at 15,000 × g and protein concentrations determined using the Bradford protein assay. Oligonucleotides to NF-κB (5′-AGTTAGGGGACTTTCCCAGGC-3′) and AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) consensus sequences (Promega) were used in EMSA. Nuclear extracts (5 μg) were incubated with 5× binding buffer (2.5 mM HEPES (pH 7.8), 50 mM NaCl, 0.5 mM EDTA, 25% (v/v) glycerol), 1 mg/ml poly dI-dC (Sigma-Aldrich) and labeled oligo for 30 min at room temperature. For supershift assays, Abs specific for NF-κB p50 and p65 subunits and to c-Jun, c-Fos, and ribosomal-S6 (all from Cell Signaling Technology) were incubated with nuclear protein extracts on ice for 20 min, then incubated with labeled probes for 30 min at room temperature. The DNA-protein complexes were resolved by electrophoresis using 6% nondenaturing polyacrylamide gels, which were vacuum dried and exposed to x-ray film.

**IL-8 ELISA**

IL-8 concentrations in cell culture supernatants were quantified by ELISA (BD Biosciences), according to the manufacturer’s instructions. The minimum detection limit of the assay was 3.125 pg/ml.

**Statistical analysis**

The Student t test was used for numerical data, whereas the Mann-Whitney U test was used for categorical data as appropriate. p values of <0.05 were considered statistically significant.

**Results**

**NOD1 is required for rapid MAPK activation during H. pylori infection**

Previous studies have reported rapid MAPK activation within epithelial cells in response to H. pylori stimulation (19, 21). To determine the kinetics of activation, a time course of MAPK phosphorylation was performed at 15, 30, 45, and 60 min following stimulation with wild-type H. pylori and ΔcagA and ΔcagM mutants on the G27 strain background (37). Experiments were performed using AGS cells that stably express siRNA to either NOD1 (siNOD1) or an irrelevant gene, EGFP (siEGFP), p38, ERK, and Jnk MAPKs were rapidly phosphorylated in AGS siEGFP cells within 30 min of stimulation with H. pylori, and activation was sustained after 60 min (Fig. 1, A–F). Activation of p38, Jnk, and ERK was dependent on a functional type IV secretion system, as stimulation with an H. pylori G27 ΔcagM mutant, which has a nonfunctional secretion apparatus, induced significantly lower levels of MAPK phosphorylation than wild-type bacteria at the appropriate times (i.e., 30, 45, and 45 min, respectively; p < 0.05) (Fig. 1, A–F). From experiments with H. pylori ΔcagA mutant bacteria, it was shown that CagA was required for maximal p38 and ERK phosphorylation in AGS siEGFP cells at 30 and 45 min poststimulation, respectively (Fig. 1, A–F; supplemental Fig. 1).4

4 The online version of this article contains supplemental material.

In contrast, CagA translocation was dispensable for Jnk phosphorylation in these cells (Fig. 1, C and D).

NOD1 was previously shown to be essential for Jnk activation in response to S. flexneri in HEK293 epithelial cells and for p38 activation by L. monocytogenes in HUVEC endothelial cells, respectively (29, 30). To assess whether NOD1 was involved in H. pylori-induced MAPK activation, we determined p38, ERK, and Jnk phosphorylation in AGS siNOD1 cells. In all experiments outlined here and below, IL-8 production was measured to indirectly verify a reduction in NOD1 expression (data not shown). In addition, Western blot analysis was performed using whole cell extracts to confirm that NOD1 synthesis had been “knocked down” in these cells (Fig. 1G). Wild-type H. pylori G27 bacteria induced maximal p38 activation within 30 min in siEGFP cells, whereas parallel stimulation of siNOD1 cells resulted in significantly reduced p38 phosphorylation (p < 0.05) (Fig. 1, A and B; supplemental Fig. 1). After 45 min, activation in the siNOD1 cell line was restored to levels seen in the siEGFP cells. Similar findings were observed in cells stimulated with H. pylori 251 wild-type and mutant strains, thereby excluding possible strain-specific NOD1-dependent p38 activation (supplemental Fig. 2). In the case of ERK, NOD1 appeared to be important for phosphorylation of this MAPK at 45 min poststimulation, but not earlier (Fig. 1, E and F). Interestingly, H. pylori was found to induce Jnk phosphorylation in a NOD1-independent, but T4SS-dependent manner (Fig. 1, C and D).

NOD1 is required for Jnk phosphorylation in response to S. flexneri, but not H. pylori stimulation

Previous reports demonstrated that NOD1 is required for rapid Jnk activation following S. flexneri infection of HeLa cells (29). As we found that this did not appear to be the case during H. pylori stimulation (Fig. 1, C and D), AGS siNOD1 cells were cocultured with virulent S. flexneri M90T to exclude cell-specific effects. As reported previously in HeLa cells (29), stimulation with S. flexneri M90T resulted in rapid Jnk phosphorylation within 15 min in the siEGFP cells, however, phosphorylation was substantially diminished in the siNOD1 cells at both time points examined (Fig. 1H). When stimulated with avirulent Shigella BS176, Jnk was not activated in either cell line at 15 or 30 min (Fig. 1H). As above (Fig. 1, C and D), wild-type H. pylori induced strong Jnk phosphorylation after 30 min, irrespective of whether NOD1 had been knocked down or not (Fig. 1H). Jnk phosphorylation in S. flexneri M90T-infected siEGFP cells was substantially reduced at 30 min, which is consistent with previous findings that Shigella actively inhibits host signaling via the action of a phosphothreonine lyase, OsF, which dephosphorylates MAPKs (43). The differences in NOD1 signaling between H. pylori and S. flexneri may reflect the different mechanisms (bacterial secretion systems vs invasion) used by these bacteria to activate the NOD1 pathway.

**Purified H. pylori OMVs activate MAPKs in a NOD1-dependent manner**

Recent work in our laboratory has shown that H. pylori can also induce NOD1 signaling via a casPAl-independent mechanism involving OMVs, which are released during normal bacterial growth (44). Purified H. pylori OMVs contain peptidoglycan and enter nonphagocytic cells to induce NOD1-dependent, but TLR-independent, host cell responses in vitro and in vivo (90). Given that H. pylori OMVs appear to selectively induce NOD1 signaling in host cells, we therefore wished to determine whether these structures could induce NOD1-dependent MAPK phosphorylation, in a manner analogous to casPAl+ H. pylori bacteria. Stimulation of AGS...
NOD1 is required for rapid MAPK activation during *H. pylori* infection. AGS siEGFP or siNOD1 cells were stimulated with wild-type *H. pylori* G27 or the isogenic mutants ΔcagA and ΔcagM for 15, 30, 45, or 60 min. Cells were lysed in boiling Laemmli buffer and samples analyzed by immunoblot (A, C, and E) and densitometry (B, D, and F) to assess phosphorylation of: p38, Jnk, and ERK. G, NOD1 expression was assessed by immunoblot in AGS siEGFP and siNOD1 cells to confirm NOD1 knock down. H, AGS siEGFP or siNOD1 cells were stimulated with wild-type *H. pylori* 251, *S. flexneri* M90T or *S. flexneri* BS176 for 15 and 30 min. Cells were lysed in boiling Laemmli buffer and samples analyzed by immunoblot to assess phosphorylation of Jnk MAPK. All membranes were reprobed with anti-actin Abs to ensure equal loadings. Blots are representative of three independent experiments. Statistical analysis of densitometry was performed by normalizing band intensity across replicate experiments. Statistical significance was determined using the Mann-whitney test. *p < 0.05.
NOD1 is required for activation of the transcription factors NF-κB and AP-1

Previous studies demonstrated NOD1-dependent activation of NF-κB using reporter assays (10, 45); however, the nuclear translocation of this transcription factor was not investigated. Additionally, the role of NOD1 in *H. pylori*-induced AP-1 activation has not been investigated before. Thus, EMSA were performed using nuclear extracts isolated from infected cells to confirm the effect of NOD1 on downstream transcription factor activation. Strong NF-κB and AP-1 binding activity was observed in AGS siEGFP cells at 2 h poststimulation with wild-type *H. pylori* (Fig. 3, A–C). This activity was not observed in nonstimulated cells and was reduced in those stimulated with *H. pylori* ΔcapPAI mutant bacteria. Moreover, siRNA inhibition of NOD1 expression resulted in reduced levels of NF-κB nuclear translocation and completely abrogated AP-1 translocation in response to capPAI⁺ *H. pylori*. This finding confirms that NOD1-dependent recognition of *H. pylori* directs signaling via the downstream activation and nuclear translocation of both transcription factors.

The transcriptional activity of NF-κB and AP-1 can vary substantially depending on their subunit composition (46–48). To assess the composition of these transcription factors in response to *H. pylori*, supershift assays were performed using nuclear extracts from infected cells. Previous work has demonstrated that capPAI⁺ *H. pylori* strains induce NF-κB complexes composed of p50/p65 subunits (14, 49). The addition to nuclear extracts of Abs to p50 caused a shift in the NF-κB complexes induced in response to NOD1 signaling (Fig. 3B). p65 Abs did not shift the complex, though a substantial decrease in the corresponding NF-κB band can be observed, which is evident of a specific interaction between the Ab and NF-κB complex. The failure to result in a supershift suggests that the binding of the Ab prevented the labeled oligonucleotide from binding to the complex, which has been reported previously for EMSAs (49). We next determined the role of NOD1 in *H. pylori*-induced AP-1 activation. As for *H. pylori*-mediated NF-κB activation, this pathogen was shown to induce the nuclear translocation of AP-1 complexes by a NOD1-/capPAI⁻-dependent mechanism. Supershift assays performed using Abs directed against c-Jun and c-Fos, two known components of the AP-1 complex, revealed that Abs to c-Jun/c-Fos proteins.

To confirm the findings for AP-1 activation by EMSA (Fig. 3C), we performed luciferase reporter assays in HEK293 cells. Both *H. pylori* wild-type and ΔcapA mutant bacteria induced similar levels of AP-1 reporter activity after 4 h infection (Fig. 3D). The *H. pylori* ΔcapM mutant, however, was unable to induce AP-1 activation (p < 0.01), thus confirming the requirement of a functional T4SS for AP-1 responses to *H. pylori* infection. In agreement with these data, as well as those obtained by EMSA (Fig. 3C), *H. pylori*-induced AP-1 reporter activity in AGS cells occurred in a T4SS-/NOD1-dependent manner (Fig. 3E). Additionally, siNOD1 cells stimulated with OMVs produced significantly less AP-1 reporter activity than OMV-stimulated siEGFP cells (p < 0.001). These results indicate that the relatively subtle and transient influence of NOD1 on MAPK activation has significant effects on the ability of MAPKs to mediate downstream AP-1 activation.

**FIGURE 2.** Purified *H. pylori* OMVs activate MAPKs in a NOD1-dependent manner. AGS siEGFP or siNOD1 cells were stimulated with *H. pylori* 251 ΔcapPAI OMVs for 15, 30, 45, or 60 min. Cells were lysed in boiling Laemmli buffer and samples were analyzed by immunoblot to assess phosphorylation of p38 (A), ERK (B), and Jnk (C) MAPKs. Membranes were re-probed with anti-actin Abs to ensure equal loadings. Blots are representative of two independent experiments.
MAPK inhibitors significantly impair IL-8 responses to 
H. pylori infection

To assess the role of MAPKs in IL-8 production, AGS cells were treated with inhibitors of either p38, ERK, or both, before stimulation with H. pylori or, as a control, TNF-α. Cells pretreated with the p38 MAPK inhibitor, SB203580, produced significantly less IL-8 in response to H. pylori (p < 0.05) and TNF-α (p < 0.01), as did cells pretreated with the ERK inhibitor, U0126 followed by stimulation with H. pylori (p < 0.01) or TNF-α (p < 0.01) (Fig. 4B). Pretreatment with both inhibitors, resulted in a further reduction in the levels of IL-8 produced in response to H. pylori stimulation (p < 0.001). Treatment with SB203580, however, did not noticeably reduce the levels of p38 phosphorylation (Fig. 4A and D). It was previously reported that the effects of this inhibition may be exerted downstream on the ability of p38 to phosphorylate signaling molecules (50). SB203580 treatment also slightly augmented ERK and Jnk phosphorylation (Fig. 4B), which can occur due to antagonistic effects between the MAPKs (51, 52). Likewise, U0126 treatment augmented p38 phosphorylation (Fig. 4A) and caused a modest decrease in ERK phosphorylation. This compensatory MAPK activation may explain why IL-8 production is not completely abrogated following pretreatment with inhibitors. The impairment of IL-8 production following MAPK inhibition, confirms the importance of MAPKs in H. pylori-induced IL-8 production, which was not fully appreciated in previous studies that instead focused on the role of NF-κB.

The effects of NOD1 over-expression on MAPK activation in response to H. pylori

To evaluate the role of the NOD1 signaling pathway on MAPK activation, AGS cells were transfected to express either wild-type H. pylori 251, 251 ΔcagPAI, or 251 ΔcagPAI OMVs for 4 h. Mean ± SEM are shown. All the data presented here are representative of three independent experiments. Statistical significance was determined using the Mann-Whitney test. **p < 0.01; ***p < 0.001.
of NOD1 (ΔCARD) (35). Over-expression of NOD1 in AGS cells did not increase IL-8 production (Fig. 5A), nor did it enhance MAPK phosphorylation in response to wild-type \( H. pylori \) (Fig. 5B). Interestingly, however, NOD1 over-expression significantly increased IL-8 production in cells stimulated with a \( H. pylori /H9004 \) cag PAI mutant (Fig. 5A), in addition to modest increases in p38 and Jnk phosphorylation (Fig. 5B). This may suggest that an abundance of NOD1 enhances the ability of the cell to respond to the peptidoglycan of \( cag \) PAI \( H. pylori \) bacteria, which are otherwise unable to inject bacterial components into the cell via a T4SS.

Over-expression of RICK, a cytosolic signaling molecule known to interact with NOD1 during \( Listeria monocytogenes \) (55, 57) and \( Chlamydia trachomatis \) (58) infections, significantly augmented IL-8 production above the limit of detection in response to wild-type \( H. pylori \). This is the first direct evidence that RICK may also be important in NOD1 signaling to \( H. pylori \). Nevertheless, similar to the results for NOD1 over-expression, RICK over-expression enhanced IL-8 production but did not increase the levels of phosphorylation in any of the studied MAPKs (Fig. 5B). This may suggest that although we have demonstrated the importance of NOD1 in p38 and ERK phosphorylation, endogenous levels of NOD1 and RICK are sufficient to induce MAPK phosphorylation, or that the effects of over-expression are exerted downstream on NF-κB and AP-1 activation. Of interest is the fact that over-expression of any construct had no effect on Jnk phosphorylation in response to wild-type \( H. pylori \) or the \( H. pylori /251 \) cagPAI mutant (Fig. 5B), however, IL-8 production varied substantially (Fig. 5A). This further supports our data that NOD1-dependent proinflammatory responses are occurring independently of Jnk,
which as previously shown, is activated in a T4SS-dependent, but NOD1-independent manner (39).

To further investigate the effect of blocking NOD1 signaling on MAPK activation, we over-expressed the NOD1 ΔCARD mutant, which has a deleted CARD domain (35), thus preventing interaction with downstream signaling partners. As reported previously (10), significantly decreased IL-8 production was observed in response to wild-type *H. pylori* (*p* < 0.001) (Fig. 5A). Additionally, this decrease in IL-8 production coincided with decreased levels of p38 MAPK phosphorylation (Fig. 5B) and a modest reduction in the phosphorylation of ERK, but not Jnk in response to wild-type bacteria (Fig. 5B). These data are consistent with the findings reported above for the role of p38 and ERK in NOD1-dependent induction of IL-8 production, demonstrating that in addition to NF-κB activation, NOD1-dependent recognition of cagPAI+

**Discussion**

*H. pylori* rapidly activates MAPKs upon contact with gastric epithelial cells (19, 21). A number of bacterial factors have been implicated in MAPK activation, including VacA (22, 23) and CagA (19, 21). However, it is clear that a T4SS is crucial for complete phosphorylation of p38, ERK, and Jnk MAPKs (19, 21).

Despite the knowledge that a functional T4SS is required, the mechanism(s) of T4SS-dependent MAPK activation are not well understood. Previous studies reported that CagA is capable of activating ERK MAPK (24, 60–62), though ERK can also be activated via CagA-independent mechanisms (19, 21, 63). Consistent with these data and with the reported time-dependent effects of CagA on ERK phosphorylation (63), we found that a *H. pylori ΔcagA* mutant with a functional T4SS was still capable of inducing wild-type levels of ERK phosphorylation in siEGFP cells at 30 min poststimulation (Fig. 1E), but not at 45 min (Fig. 1, E and F).

Of more interest, however, we found that following stimulation with the *H. pylori ΔcagA* mutant, the levels of p38 activation in siEGFP cells were significantly reduced at 30 min poststimulation (Fig. 1, A and B). Moreover, this effect was more pronounced when NOD1 was knocked down (Fig. 1, A and B). To our knowledge, this is the first report to describe CagA-dependent p38 phosphorylation during *H. pylori* infection.

Opitz et al. (30) reported NOD1-dependent p38 activation in endothelial cells following stimulation with *L. monocytogenes*. Likewise, we found that the levels of *H. pylori*-induced p38 and, to a lesser extent, ERK phosphorylation were diminished in AGS cells stably expressing siRNA to *NOD1* (Fig. 1, A, B, E, and F). This inhibition in siNOD1 cells was transient, however, as phosphorylation was restored to levels seen in the siEGFP cells, 60 min after stimulation (Fig. 1, A, B, E, and F). Interestingly, p38 phosphorylation was dependent only on a functional T4SS after 60 min (Fig. 1A), indicating the involvement of an additional NOD1-independent pathway. We found that Jnk phosphorylation was unaffected at any time point in the AGS siNOD1 cells, though its activation was still dependent on a functional TFFS (Fig. 1, C and D). These findings are in contrast to those from in vitro kinase assays, showing that *S. flexneri* activated Jnk in a NOD1-dependent manner in HeLa cells (29). However, when AGS siNOD1 cells were stimulated with the same virulent *S. flexneri* M90T strain used by those authors (29), we confirmed the NOD1-dependent Jnk activation they reported (Fig. 1H), indicating that the differences in the findings could not be attributed to the different cell culture models used. This indicates that the NOD1-independent Jnk phosphorylation during *H. pylori* infection is specific to this pathway. In confirmation of our findings, Snider et al. (59) reported that *H. pylori*-induced Jnk phosphorylation occurred independently of NOD1 and other known upstream kinases such as, Cdc42, Rac1, P3K, MKK4, and MKK7. Instead, Jnk was found to be activated via Src family kinases (59), which are activated when the TFSS-associated CagL protein binds to α5β1 integrin on host cells (64). Thus, Src kinase activation of p38 during *H. pylori* infection may be responsible for the T4SS-dependent/NOD1-independent phosphorylation seen 60 min poststimulation (Fig. 1, A and B).

In confirmation of previous reports (10), we found that *H. pylori* induces NF-κB reporter activity in a NOD1-dependent manner, however, this is the first report to demonstrate that the nuclear translocation of the transactivating NF-κB complex is also dependent on NOD1 (Fig. 3, A and B). In addition, cagPAI+ *H. pylori* induce higher levels of NF-κB translocation, however, ΔcagPAI mutants can still induce NF-κB activation, though to a lesser extent (Fig. 3B). This has been described previously (65, 66), with non-cagPAI-encoded proteins, such as OipA and VacA, known to play a role in NF-κB-dependent proinflammatory responses (22, 67).

Interestingly, the *H. pylori* 251 strain used in many of the experiments herein expresses an “on” form of the oipA gene, required for OipA functionality (data not shown). Nevertheless, the composition of the NF-κB complexes, composed primarily of proinflammatory p50/p65 heterodimers, remained unchanged after NOD1 knock down. Additionally, we have shown that NOD1 is required for AP-1 activation, as siRNA to NOD1 abrogated nuclear translocation of AP-1 during *H. pylori* stimulation (Fig. 3C). This finding was of particular interest, as although NOD1 was initially required for complete MAPK activation, it was not required for phosphorylation 60 min poststimulation (Fig. 1, A–F). This suggests that multiple signaling events activated during *H. pylori* infection are collectively required to initiate a global transcriptional response to infection. The AP-1 complex activated during *H. pylori* infection was primarily found to be composed of c-Jun/c-Fos heterodimers (Fig. 3C), which is in agreement with previous findings for *H. pylori* T4SS-dependent AP-1 activation (11, 15, 16).

Many proinflammatory cytokines are dually regulated by NF-κB and AP-1, which can act in concert to amplify the transcriptional response. Examples of this include IL-8 (68, 69), for which maximal transcription in response to *H. pylori* was also shown to require the binding of both NF-κB and AP-1 to upstream promoters (11, 13, 17, 19, 20). Likewise, in this study, we have shown that pretreatment of AGS cells with p38 and ERK inhibitors significantly inhibited IL-8 production in response to *H. pylori* stimulation (Fig. 4A). These data reinforce our findings that the dual activation of both NF-κB and AP-1 by *H. pylori* rapidly induces a robust proinflammatory response upon recognition of *H. pylori*.

NOD1 over-expression was previously found to affect modest increases in NF-κB activation (26, 54). Interestingly, we found that IL-8 production was not significantly elevated in AGS cells transfected with NOD1 before wild-type *H. pylori* stimulation (Fig. 5A), though the reasons for this are unclear. Alternatively, the lack of amplified IL-8 production or MAPK activation following NOD1 and/or RICK over-expression may suggest the involvement of additional signaling molecules, which are required for initial host responses to *H. pylori*. Indeed, Fukazawa et al. (70), recently found that the guanine nucleotide exchange factor was critical for the NOD1-dependent signaling responses to *S. flexneri*. It may be that molecules such as guanine nucleotide exchange factor are also required for the initiation of NOD1-mediated proinflammatory signal cascades during *H. pylori* infection.

Interestingly, over-expression of RICK substantially increased IL-8 production in cells stimulated with wild-type bacteria (Fig. 5A), suggesting that RICK over-expression may be amplifying
NF-κB-dependent responses to \(H. pylori\). Although previous reports have demonstrated that over-expression of RICK in mouse embryonic fibroblasts and HEK293 cells led to amplified NF-κB responses (54, 71, 72), this is the first study to show a potential link between RICK and NOD1 responses to caspase-1 \(H. pylori\). The association of NOD1 with RICK has been reported to activate the TGF-β associated kinase, TAK1, resulting in NF-κB activation (27). TAK1 is also known to activate p38 and Jnk (73, 74) and this pathway may represent a mechanism through which NOD1 induces MAPK activation during \(H. pylori\) infection.

In contrast to the data for over-expression of NOD1 and RICK, expression of the dominant-negative NOD1 ACARD construct not only affected IL-8 production in cells, but also resulted in diminished levels of p38 and to a lesser extent, ERK phosphorylation in response to \(H. pylori\). No effect, however, was observed for Jnk phosphorylation. These results are in agreement with those of the NOD1 siRNA studies (Figs. 1 and 2), showing the importance of p38 and ERK in NOD1-dependent IL-8 responses to \(H. pylori\).

NOD1 has been shown to be an important regulator of innate immune responses to bacterial pathogens (10, 25, 26, 29–32, 55–58, 70, 75–84), however, research has mainly focused on the role of NOD1 in activating NF-κB. In this study, we show that NOD1 is critical, not only for NF-κB activation, but also for the activation of MAPKs and AP-1 during \(H. pylori\) infection. This in turn may induce the transcription of a novel subset of proinflammatory genes that are exclusively activated in an AP-1-dependent manner during \(H. pylori\) infection. One example of this is the decay-accelerating factor (DAF), a \(H. pylori\) cellular receptor previously shown to be up-regulated following contact with \(H. pylori\) (85). Although the DAF promoter contains a κB response element, it was found that DAF expression in response to \(H. pylori\) was dependent on p38 phosphorylation and totally independent of NF-κB (86–89). Further studies are therefore warranted to investigate the role of NOD1 in \(H. pylori\)-induced DAF expression, as well as in other MAPK regulated host factors.

Acknowledgments
We thank Drs. D. Philpott and L. Le Bourhis (University of Toronto, Canada) for providing Shigella strains for our coculture experiments. Additionally, we thank Dr. J. Magalhaes for his advice regarding the culture of Shigella.

Disclosures
The authors have no financial conflict of interest.

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