Nucleotide-binding Oligomerization Domain-1 and Epidermal Growth Factor Receptor

CRITICAL REGULATORS OF β-DEFENSINS DURING HELICOBACTER PYLORI INFECTION*

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Parjeet K. Boughan‡, Richard H. Argent§, Mathilde Body-Malapel†, Jong-Hwan Park‡, Katie E. EWings¶, Andrew G. Bowie**, Shao Jin Ong†, Simon J. Cook‡, Ole E. Sorensen§§, Barbara A. Manzo††, Naohiro Inohara‡‡, Nigel J. Klein§§, Gabriel Nuñez¶, John C. Atherton‡‡, and Mona Bajaj-Elliott‡‡‡

From the 1Infectious Diseases and Microbiology Unit, Institute of Child Health, 30 Guilford St, London WC1N 1EH, United Kingdom, the 2Institute of Infection, Immunity, and Inflammation, and the Wolfson Digestive Diseases Centre, Queen’s Medical Centre, University of Nottingham, Nottingham NG7 2UH, United Kingdom, the 3Department of Pathology and Comprehensive Cancer Center, the University of Michigan Medical School, Ann Arbor, Michigan 48109, the 4Department of Biochemistry, Trinity College, Dublin 2, Ireland, the 5Laboratory of Molecular Signaling, The Babraham Institute, Babraham Hall, Cambridge CB2 4AT, United Kingdom, the 6Section for Clinical and Experimental Infectious Medicine, the Department of Clinical Sciences, Lund University, Biomedical Center B14, Tornvågen 10, SE-22184 Lund, Sweden, and the 7Research Centre for Gastroenterology, Institute of Cell and Molecular Sciences, Barts and The London, Queen Mary School of Medicine and Dentistry, London E1 2AD, United Kingdom

Host-pathogen interactions that allow Helicobacter pylori to survive and persist in the stomach of susceptible individuals remain unclear. Human β-defensins (hBDs), epithelial-derived antimicrobial peptides are critical components of host-defense at mucosal surfaces. The role of H. pylori-mediated NF-κB and epidermal growth factor receptor (EGFR) activation on β-defensin expression was investigated. Transient transfection studies utilizing β-defensin promoter constructs were conducted in gastric cells with contribution of individual signaling events evaluated by the addition of specific inhibitors, small interfering RNA (siRNA) including modulation of host innate immunity, proliferation, and apoptosis (13–15). To date studies investigating H. pylori-mediated host innate defense have focused on the regulation of IL-8, a potent neutrophil-chemoattractant, implicated in tissue injury and severity of disease (16–18). Although evidence implicating the cagPAI in disease pathogenesis is unequivocal (11) the exact contribution of the translocated CagA protein in eliciting innate defense remains undefined, as isogenic cagA mutant strains induce IL-8 production to the same extent as the wild-type parent strain (16). More recent studies however, implicate CagA in modulating IL-8 production (19, 20).

Pattern recognition receptors are known to play a crucial role in host innate and adaptive immune responses to microbial pathogens and their products (21–23). Toll-like receptors (TLRs) are evolutionarily conserved, transmembrane receptors that recognize signature motifs on microbes and have the ability to transduce signals leading to the production of cytokines, chemokines, and antimicrobial peptides (24–27). The main TLR implicated in recognition of lipopolysaccharide (LPS) of Gram-negative bacteria is TLR4. Interestingly, evidence to date suggests a minimal role for H. pylori LPS in NF-κB activation and chemotaxis.
mokine gene expression in gastric epithelia (28, 29). The known low biological activity of *H. pylori* LPS combined with variation in signaling components of the TLR4 pathway in different epithelial cell lines leaves the data at present inconclusive. In contrast, evidence for the effect of *H. pylori* LPS in NF-κB activation in monocytic cells is convincing (30). The exact contribution of epithelial TLR2 and TLR5 in *H. pylori* detection remains ambiguous as HEK293 cells show bacterial-mediated IL-8 expression in the absence of TLR2, and *H. pylori* flagellin is known to elicit minimal IL-8 activation (31). NOD1 (nucleotide-binding oligomerization domain) protein, a cytosolic Pattern Recognition receptor has recently been implicated in innate detection of *H. pylori* peptidoglycan moieties. Studies by Vila and colleagues (32) suggest the type IV secretion system acts as a conduit for the intracellular delivery of bacterial muropeptide leading to cellular activation.

There is increasing evidence that human β-defensins (hBDs), a family of endogenous antimicrobial peptides secreted at epithelial mucosal surfaces are critical components of host defense (33–35). We and others (36–40) have previously shown both in vitro and in vivo increased mRNA and peptide expression of gastric β-defensins during *H. pylori* infection. Importantly, we found hBD2 and -3 to be potent bactericidal agents against *H. pylori* (39, 40). The increased expression and high microbicidal activity of hBDs against the bacterium led us to hypothesize that dynamic modulation of host epithelial antimicrobial responses by bacterial virulence factors may be a critical determinant of bacterial adherence and subsequent pathology in a susceptible individual.

In the present study we explored the role of the *H. pylori* type IV secretion system and the effect of virulence factors CagA and VacA on the hBD2 and -3 gene expression. Further, the contribution of NF-κB and MAPK signaling pathways in β-defensin regulation was investigated. We found hBD2 gene expression required the presence of cagPAI and was NOD1-dependent. In contrast, hBD3 expression was NOD1-independent but relied on epidermal growth factor receptor (EGFR)-mediated ERK activation. The diverse nature of cellular events regulating hBD2 and -3 suggests distinct temporal functions for the two antimicrobials during infection.

**MATERIALS AND METHODS**

**Cell Lines**—AGS gastric cells were maintained in RPMI 1640 medium containing 2 mM L-glutamine, 100 μg/ml penicillin-streptomycin, 1% non-essential amino acids, and 10% fetal bovine serum (Invitrogen). HEK293 cells expressing conditional kinase MEKK3:ER* (38, 39) were described previously (38, 39). NOD1 primers utilized were: sense, 5'-GGCTGATGGCTGGGAGCTTC-3'; and reverse, 5'-GGACTTCTGGTTCACCCACAGGC-3'.

**β-Defensin Promoters and NOD1 siRNA Construct**—Fragments 2.1 and 1.2 kb upstream of hBD2 and hBD3 start codons were amplified with primers (listed) and cloned into pGL3-basic firefly luciferase vector (Promega, Southampton, UK) hBD2 forward: 5'-GGCTGAGGCTCTCGAACAATCAGCACCAA-3' (XhoI); hBD2 reverse: 5'-CGGCATT-GGCTGATGGCTGGGAGCTTC-3' (NcoI); hBD3 forward: 5'-CAGGTGTTGTCTTTTTAGCATTTTTTCCCTCT-3' (KpnI); hBD3 reverse: 5'-CGAGATCGCCCTACCCACAGCCTG-3'.

NOD1 siRNA cloning was performed in two steps prior to insertion in pBS/U6 vector (courtesy of Y. Shi, Harvard Medical School, MA) as detailed (47). 21-Nucleotide NOD1 (AF113925) coding sequences were selected, and the primer sets flanked with appropriate restriction sites were: NOD1 Oligo 1, forward: 5'-GGAGACTTCTGGTACACCATGACGATG-3'; NOD1 Oligo 2, reverse: 5'-AGCTTATGTGAGTGACCAGAAGTTCCC-3'.

**Luciferase Assays**—Transfection of cells with reporter constructs was performed as described previously (47). Transfection efficiency was determined by dual-luciferase assay (Promega). After 18–24 h, cells were exposed to bacterial infection. The relative luciferase activity was measured in a luminometer (Anthos Labtech Instruments, Austria). All experiments were conducted three times and performed in triplicate. Luciferase activities were plotted as fold induction compared with activity measured in unstimulated control cells.
Western Blotting—All primary and secondary antibodies utilized in the present study were from Cell Signaling Technology (Hitchin, UK), Santa Cruz Biotechnology (Autogen Bioclear, Wilts, UK), or Gentaur Molecular Products (Brussels, Belgium). Equipment and reagents were from Amersham Biosciences, St Albans, UK. Bacterially infected cell extracts were routinely subjected to 7–10% SDS-PAGE followed by semi-dry transfer onto nitrocellulose membrane. Nonspecific binding was blocked (5% milk/Tris-buffered saline, pH 7.6) for 2 h prior to overnight incubation at 4°C with primary antibody (1:1,000 or 1:10,000 dilution for phospho-tyrosine). After appropriate washes (Tris-buffered saline/Tween 20) incubation in secondary antibody (1:2,000) was performed for 1 h prior to detection by enhanced chemiluminescence. Blots were stripped for 20 min and re-probed with anti-β-actin, EGFR, or corresponding non-phospho-specific MAPK antibody to confirm equal protein loading. Cell lysates and supernatants were quantified by Bio-Rad protein (Bradford) assay, and a total of 150 μg of total protein was subjected to 16% Tris-Tricine PAGE for the detection of hBD2 and hBD3 as detailed previously (48).

β-Defensin Expression in a Murine Model of H. pylori Infection—Animal experimentation was performed in accordance with the University of Michigan Committee on Use and Care of Animals guidelines. Wild-type C57BL/6 (Jackson Laboratories) and Nod1 knock-out (KO) mice (6–8 weeks old) (49) on a C57BL/6 background were inoculated intragastrically with cagPAI+ H. pylori strain 26695 (10^8 bacteria in 0.2 ml in phosphate-buffered saline) or with the same volume of phosphate-buffered saline once a day for two consecutive days. Seven days after the first infection, mice were sacrificed, stomachs were harvested, and total RNA was purified using the RNeasy kit (Macherey Nagel, Hoerdt, France) according to the manufacturer’s instructions. Quantitative real-time PCR analyses were performed for mBD2, mBD4, MIP2, and β-actin mRNA expression using sequence-specific PCR primers and SYBR green I (Applied Biosystems, Foster City, CA). The primer sequences were as follows: β-actin forward: 5′-AGAGGGAAATCCTGCGTGAC-3′; β-Actin reverse: 5′-CAATAGTGATGCTGGCCGT-3′; mBD2 forward: 5′-AAAGATTGTAGATCGAGCAGAAGCTTG-3′; mBD2 reverse: 5′-GGAGGAAATCGTGCGTGAGAC-3′; mBD4 forward: 5′-CAATAGTGACCTGGGCTGAC-3′; mBD4 reverse: 5′-CTCGTCTCGTCTCCTCATT-3′; MIP2 forward: 5′-AGTGACCTGGCGTGACATC-3′; and MIP2 reverse: 5′-CCCGCTTGTGAAGTTGCTT-3′.

Quantification of mRNA was performed on an ABI Prism 7000 sequence detector using SYBRgreen (Applied Biosystems, Foster City, CA). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Results were normalized to β-actin expression.

Statistical Analysis—Results are presented as means ± S.E. of three experiments each performed in triplicate. Statistical analyses were performed using GraphPad Instat statistical software, variables were compared using a t test, and a probability value of <0.05 was regarded as significant.

RESULTS

Activation of hBD2 and hBD3 Promoter-Reporter Constructs during Infection with Wild-type and Isogenic Mutant H. pylori Strains—We and others (36–40, 50) have previously shown modulation of both hBD2 and hBD3 mRNA and peptide expression during H. pylori infection, although most studies to date have been confined to the effect of cytotoxic type I strains. To elucidate the role of bacterial virulence factors involved in β-defensin gene regulation, H. pylori isogenic vacA, cagA, cagE mutants of strains 60190 and 84-183, and strain Tx30a that possesses a non-vacuolating s2/m2 VacA and lacks the cag PAI, were employed.

AGS cells were transfected with hBD2 or hBD3 promoter constructs for 24 h prior to bacterial or cytokine stimulation. The results of representative transfection experiments are shown in Fig. 1. A significant (p ≤ 0.02) increase in hBD2 promoter activity was observed during infection with H. pylori strains 60190 and 84-183 (Fig. 1A). Infections in the presence of isogenic cagA and vacA mutant strains showed activation of the hBD2 promoter to a similar extent as that observed for the wild-type strains. In contrast, cagE mutant and strain Tx30a failed to elicit an hBD2 response. Analysis of hBD3 promoter activity in response to a range of bacterial strains revealed a significant increase (p < 0.05) in the presence of H. pylori strains 60190 and 84-183 and the isogenic vacA and cagA mutant strains (Fig. 1B). Interestingly, a modest increase in hBD3 promoter activity over control was observed for cagE mutant and strain Tx30a; however, this increase did not reach statistical significance.

NF-κB Is Involved in the Regulation of hBD2 but Not hBD3 Gene Expression—Gastric epithelial cells respond to H. pylori infection by activating multiple signal transduction cascades. Activation of NF-κB transcription factor by H. pylori is well established, especially in the regulation of the innate immune gene, IL-8 (30, 51). One study to date has implicated NF-κB in H. pylori-mediated hBD2 gene regulation (50); however, upstream signaling events leading to NF-κB-mediated hBD2 induction and, further, any potential effect of this transcription factor on hBD3 expression are unknown. Prior to investigating the potential role of NF-κB in β-defensin gene regulation, we first confirmed the activation of NF-κB in our co-culture infection model by following the degradation of its inhibitor IκBα in the presence of two cytotoxic H. pylori strains. Detectable levels of IκBα were present in control unstimulated AGS cells (Fig. 2A). Exposure to H. pylori strain 60190 resulted in rapid (15–30 min) IκBα degradation with protein levels returning to that of control by 60 min. Similar results were obtained using H. pylori strain 84-183 (data not shown). To our knowledge this is the first report investigating kinetics of IκBα degradation in AGS cells infected with H. pylori strains 60190 and 84-183. Although most studies to date have concentrated on the role of IκBα in H. pylori-mediated NF-κB activation, nearly half of the NF-κB in cells is sequestered in the cytoplasm by another major inhibitor isoform, IκBβ (52). We followed IκBβ degradation during infection with H. pylori strain 60190 and observed a minimal effect on IκBβ protein levels5 up to 4 h post-infection. This suggests that during H. pylori infection NF-κB is activated by specific removal of IκBα and any potential IκBα/NF-κB complexes remain sequestered in the cytoplasm. Another level of regulation in this pathway is the potential modification of NF-κB-p65 subunit via phosphorylation (53). At present the p65 phosphorylation status, and its effect on β-defensin gene expression, during H. pylori infection are unknown. We observed a modest increase in the levels of phosphorylated p65 2–3 h post infection when compared with unstimulated control AGS cells (Fig. 2B, upper panel). In contrast, IL-1β caused a more potent, transient increase in phosphorylated p65, which was noted as early as 30-min post stimulation (Fig. 2B, lower panel). These kinetic studies suggest differential regulation of NF-κB activation in response to cytokine and bacterial stimuli.

To evaluate any potential contribution of NF-κB to hBD2 and hBD3 gene regulation, infections in the presence of MG132, a specific proteasome inhibitor were conducted. We observed a dramatically significant
reduction in hBD2 promoter activity \((p < 0.02, \text{Fig. 2C})\), which is likely to be related to its ability in preventing proteasomal degradation of IκB. In contrast to its effect on hBD2 promoter function, MG132 had no significant effect on hBD3 activity. This further supports the critical role of NF-κB in mediating hBD2 but not hBD3 expression (50, 54).

**β-Defensin Induction by H. pylori Is Not Mediated by IL-1β—**Studies investigating innate defense in skin suggest that direct microbial stimuli results in a modest induction in hBD2 expression; this increase however, is dramatically enhanced in the presence of other innate immune cells and their secretory products (46, 55). For example, it has been shown that LPS-stimulated macrophages secrete IL-1β, which further enhances hBD2 induction in a paracrine fashion (46). To elucidate if *H. pylori*-mediated NF-κB activation and subsequent hBD2 gene expression was a result of direct microbial assault or an indirect, autocrine effect of cytokine release during infection, we followed several approaches. Firstly, we measured any potential induction of IL-1β

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**FIGURE 1.** hBD2 and hBD3 promoter activity in response to infection. AGS cells were transiently transfected with hBD2 (A) or hBD3 (B) promoter-luciferase constructs. Cells were exposed to wild-type and isogenic mutant *H. pylori* strains for 8 h. Promoter luciferase activity was assayed by normalization to Renilla luciferase. Data are expressed as n-fold increases in luciferase activity when compared with uninfected, control cells. Error bars indicate ± S.E. of values representative of three experiments conducted in triplicates \((*, p < 0.05)\).
mRNA in our co-culture experiments and found no expression of the cytokine during the 24-h period of infection. Secondly, the effect of exogenous IL-1 receptor antagonist (IL-1RA) was evaluated by conducting cytokine and bacterial stimulation of AGS cells in the presence and absence of IL-1RA (Fig. 3). In the presence of IL-1β or H. pylori an increase in both hBD2 and hBD3 was noted compared with unstimulated control cells, though the hBD2 response to IL-1β was the greatest (Fig. 3A). Inclusion of IL-1RA caused a dramatic inhibition of hBD2 gene expression in IL-1β-stimulated cells whereas the H. pylori-mediated hBD2 expression remained unchanged. IL-1β caused a small increase in hBD3 expression, which was abolished by IL-1RA; in comparison, IL-1RA had no effect on H. pylori-mediated hBD3 expression.

Finally, we employed two well characterized Vaccinia virus-encoding proteins, A46R and A52R, which are known to inhibit both IL-1β- and TLR-mediated NF-κB activation by targeting shared downstream signaling molecules (56, 57). Plasmids encoding A46R and A52R were co-transfected with the hBD2 promoter-construct prior to stimulation with H. pylori or IL-1β. A significant reduction of hBD2-luciferase activity in the presence of A46R was observed in IL-1β-stimulated cells, but there was no significant effect on H. pylori-mediated hBD2 promoter function (Fig. 3B). Similarly, the presence of A52R showed no effect on H. pylori-induced hBD2 regulation. These observations suggest that H. pylori-mediated hBD2 signaling does not occur via the IL-1β or membrane-associated TLR signaling pathways.

**NOD1 and EGFR Regulate H. pylori-mediated hBD Expression**

**FIGURE 2. Role of NF-κB activation in H. pylori-mediated hBD2 gene regulation.** AGS cells were co-cultured with wild-type H. pylori strain 60190, and NF-κB activation was followed by degradation of IκBα (A). Phosphorylation status of NF-κB p65 subunit during infection (B, upper panel) and IL-1β stimulation (B, lower panel) was evaluated. Promoter activity of hBD2 and hBD3 was investigated in the presence of 25 μM MG132, a proteasome inhibitor (C).
gene expression was verified in AGS cells. NOD1 mRNA was constitutively expressed in control uninfected cells, and expression remained throughout infection with all the strains (wild-type and isogenic mutants) studied.5 Secondly, we conducted experiments utilizing a NOD1 siRNA encoding plasmid and commercially available NOD1 siRNA (detailed under “Materials and Methods”). Cells were transfected with NOD1 siRNA reagents for 24–48 h prior to transfection of hBD2 or hBD3 promoter constructs. Down-regulation of NOD1 mRNA and protein was verified by RT-PCR and Western blotting (Fig. 4, A and B). The presence of NOD1 siRNA in IL-1β/H9252 stimulated cells did not cause reduction in hBD2 promoter activity, suggesting no role for NOD1 in IL-1β-mediated hBD2 expression (Fig. 4C). In contrast hBD2 promoter activity was dramatically (p < 0.05) reduced during H. pylori infection, highlighting a critical role for NOD1 in H. pylori-induced hBD2 gene expression. When similar experiments were conducted in the presence of the hBD3 promoter construct we found no effect of NOD1 siRNA on IL-1β- or bacterial-mediated hBD3 expression (Fig. 4D).

To confirm a role for NOD1 in vivo we performed H. pylori infections in wild-type and Nod1 KO mice and assessed the expression of β-defensin 4 (mBD4) a mouse orthologue of hBD2 (58, 59), in the stomach of infected mice. Seven days after infection, the expression of mBD4 was clearly induced in the stomach of wild-type mice when compared with uninfected mice (Fig. 5). In contrast, the expression of mBD4 was impaired in the stomach of Nod1 KO after H. pylori infection (Fig. 5). In control experiments, the expression of mBD2 (another member of the β-defensin family) was very low or undetectable in mouse stomach before and after H. pylori in both wild-type and Nod1 KO mice when compared with ileal tissue, clearly indicating differential regulation of members of the β-defensin family in response to H. pylori infection (Fig. 5). Furthermore, expression of macrophage inflammatory protein-2, a functional analogue of the human interleukin 8, was higher in Nod1 KO mice than wild-type mice after H. pylori (Fig. 5). The latter results might be explained by increased H. pylori colonization in the stomach of Nod1 KO mice 7 days after infection (32). Thus, both in vitro and in vivo studies described identify NOD1 as a major signal transduction player involved in differential β-defensin gene regulation during H. pylori infection.

Role of MAPK Pathways in H. pylori-mediated β-Defensin Gene Regulation—Activation of transcription factor c-fos/c-jun (AP-1 complex) via the MAPK pathways during H. pylori infection has been well studied (15, 60–62); however, the role of these cellular events in β-defensin expression during infection is limited. In this series of experiments we first confirmed the activation of all three MAPK pathways in our co-culture system (Fig. 6A). Secondly, pharmacological inhibitors selective for individual pathways were utilized to determine their contribution in hBD2 and -3 gene regulation during infection. RT-PCR and transient transfections allowed the assessment of gene expression. The effect of each inhibitor is represented as a percentage of total hBD2 and
FIGURE 4. NOD1 plays a major role in hBD2 but not hBD3 expression. AGS cells were transiently transfected with a plasmid encoding NOD1 siRNA or a commercially available NOD1 siRNA 24–48 h prior to the introduction of β-defensin promoter constructs and 24 h later, cells were stimulated with IL-1β or H. pylori for 8 h. Down-regulation of NOD1 mRNA (A) and protein was confirmed (B). Luciferase activities for hBD2 (C) and hBD3 (D) were evaluated, and error bars indicate ± S.E. of values obtained from three independent experiments conducted in triplicates. Statistical significance was determined by t test; *, p < 0.02.
FIGURE 5. Impaired murine β-defensin 4 (mBD4) expression in Nod1-deficient mice after *H. pylori* infection. Mice were inoculated intragastrically with *H. pylori* or phosphate-buffered saline (uninfected) and sacrificed 7 days post-infection. Results denote expression levels of indicated genes in the stomach tissue after normalization to β-actin as determined by quantitative real-time PCR. 1 and 2 represent experiment numbers; each was derived from different mice and performed independently. Ileum denotes expression analysis in ileal tissue from a mouse.

FIGURE 6. Potential role of MAPK pathways in *H. pylori*-mediated β-defensin regulation. Time-dependent activation of ERK, JNK, and p38 MAPKs in the presence of cytotoxic 60190 strain was followed by Western blotting with phospho-specific antibodies (A). The data are representative of three individual experiments. β-Defensin promoter transfected AGS cells were pre-treated with specific inhibitors (p38, SB203580 25 μM; JNK, SP100625 50 μM; and ERK, U0126 25 μM) and promoter activity quantified 8 h post-infection. Results are presented as percentage inhibition of hBD2 (B) and hBD3 (C) promoter activity compared with bacterial infection alone. Values are expressed as mean ± S.E. from a representative of three experiments conducted in triplicates.
NOD1 and EGFR Regulate H. pylori-mediated hBD Expression

In the present study we identified NOD1 and EGFR as central regulators of hBD2 and hBD3 expression during H. pylori infection. This is the first report suggesting differential regulation of two epithelial antibacterial peptides in response to a bacterial infection.

We confirmed hBD2 gene expression to be exclusively dependent on the presence of an intact bacterial secretion system (Fig. 1A). The effect of bacterial virulence factors CagA and VacA on defensins has not been studied to date. Our data suggest a minimal role for CagA and VacA in β-defensin gene regulation (Fig. 1). Crabtree and colleagues (16) have reported similar findings for IL-8 production. Collectively, these studies suggest that signaling events involved in cytoskeletal changes in the presence of CagA and VacA (humming bird phenotype and vacuolation, respectively) do not influence early epithelial innate immune responses. However, due to the constraints of our in vitro model system, long-term effects of CagA and VacA on epithelial antimicrobial barrier function cannot be ruled out.

Although the transcription factor NF-κB has been implicated in both IL-1β and H. pylori-mediated hBD2 gene expression, molecular interactions leading to NF-κB activation and identity of upstream signaling events remain unclear. We found no evidence of IkBβ degradation with minimal increase in p65 phosphorylation during H. pylori infection. In
contrast, IL-1β stimulation led to a more rapid and potent increase in p65 phosphorylation (Fig. 2B, lower panel), highlighting differential NF-κB activation in the presence of the two stimuli. We propose that p65 phosphorylation may contribute to the marked hBD2 induction observed in the presence of IL-1β, compared with the more modest increase during infection (Fig. 3A). To delineate signaling events upstream of NF-κB-mediated hBD2 gene expression, we explored the potential role of secondary IL-1β stimulation and TLR-mediated NF-κB activation. For this purpose we utilized Vaccinia virus-encoded proteins A46R and A52R. These viral proteins target the Toll/interleukin-1/receptor (TIR) domain of adapter proteins, including myeloid differentiation factor 88 protein, myeloid differentiation factor 88 adapter-like protein, and TIR domain-containing adaptor inducing interferon β protein, leading to inhibition of downstream signaling events responsible for NF-κB activation (56, 57). Studies performed in the presence of A46R showed dramatic inhibition of IL-1β-induced hBD2 expression. However, the presence of A46R had no effect on bacterial-mediated hBD2 expression (Fig. 3B). This approach, along with our observations that IL-1β was not produced in our co-culture system and that IL-1RA did not affect hBD2 expression, provided further evidence to exclude IL-1β signaling in mediating H. pylori-induced hBD2 expression. However, it is likely that IL-1β paracrine effects following release from inflammatory cells would be an additional stimulus to epithelial cell hBD2 expression in vivo in the gastric mucosa.
NOD1 and EGFR Regulate H. pylori-mediated hBD Expression

Because IL-1β and TLR signaling was found not to be crucial in modulating β-defensin expression, we hypothesized that NOD1 may play a role in bacterial-mediated β-defensin gene regulation. We observed dramatic reduction of hBD2 promoter activity in NOD1 siRNA-transfected cells when exposed to *H. pylori*, implicating NOD1 engagement in NF-κB-mediated hBD2 expression (Fig. 4C). Interestingly, no effect of NOD1 siRNA was noted on hBD3 expression (Fig. 4D).

We were further able to confirm a major role for NOD1 in modulating host anti-microbial function in vivo. The expression of mBD4, an orthologue of hBD2, was found to be profoundly impaired in Nod1 KO mice when compared with control wild-type mice 7 days post-infection (Fig. 5). A previous study showed that Nod1 KO mice exhibit increased *H. pylori* colonization when compared with wild-type mice, which was dependent on the presence of cagPAI (32). Our results suggest that the role of Nod1 in controlling *H. pylori* colonization might be explained, at least in part, through the regulation of mBD4 as impairment in mBD4 expression and function is likely to result in reduced bacterial killing and increased colonization. The absolute requirement for cagPAI for NOD1 activity (32), combined with the present study showing dependence of hBD2 expression on cagPAI and NOD1, highlights NOD1 not only as a crucial regulator of bacterial sensing but also as a major player in the induction of a subset of innate defense genes, including IL-8 and hBD2.

Activator protein (AP)-1, a family of transcription factors, is also known to play a role in regulation of host defense genes, for example IL-8 and β-defensin promoters encode potential AP-1 sites (54, 60, 66). Activation of the AP-1 family is dependent on upstream MAPK signaling pathways (67, 68). In the present study we confirmed the activation of all three during *H. pylori* infection and usage of specific inhibitors implicated all three pathways to varying degrees in both hBD2 and hBD3 gene regulation (Fig. 6, B and C). Due to the complexity of cross-talk between cellular events during infection, it was not surprising that the inhibitor studies did not yield greater molecular detail. To untangle the cross-talk and gain better understanding of the role of individual MAPK pathways in β-defensin gene regulation we employed HEK293 cell lines stably transfected with conditional kinase mutants. This novel strategy allowed us to selectively activate individual MAPK pathways in the absence of any other signaling event. The most contrasting data between the inhibitor studies and the conditional mutants was obtained for hBD2 gene expression, because the use of U0126 suggested ERK activation to be involved in both hBD2 and -3 gene expression (Fig. 6); however, ERK activation alone was unable to induce hBD2 expression (Fig. 7B). This important finding suggests that ERK pathway must synergize or cross-talk with other pathways to modulate hBD2 expression during *H. pylori* infection. In contrast, ERK pathway alone was sufficient to induce hBD3 expression. Addition of specific inhibitors in HM3 cell system revealed a crucial role for the JNK pathway in defense expression, whereas p38 was found to be dispensable. A similar dependence for JNK but not the p38 or ERK pathway in hBD2 expression has also been observed during *Escherichia coli Nissle* 1917-mediated infection of intestinal epithelial cells (54).

Our studies so far implicated NOD1-dependent NF-κB activation and the JNK pathway in hBD2 gene regulation. In contrast to hBD2, we found that hBD3 expression was NOD1-independent but ERK- and JNK pathway-dependent. As the ERK pathway can be activated by cag-PAI-negative strains (15), we suggest a potential role for this pathway in the modest hBD3 expression observed in the presence of cagE and Tc30a strains (Fig. 1B). We further hypothesized that the EGF receptor is the most likely candidate upstream of the ERK pathway for several reasons. Firstly, gastric expression levels of EGFR ligands (EGF and HB-EGF) and EGFR itself are elevated during *H. pylori* infection (64, 65), and secondly, EGFR transactivation has been documented in the presence of *Cag*+ and *Cag*− strains (64). Keates and colleagues (69) have shown unequivocally that EGFR-mediated Ras activation is an upstream signaling event leading to ERK activation in *H. pylori*-infected AGS cells. Finally, a recent study has implicated EGFR transactivation in hBD3 gene expression in a skin model of inflammation (55). In the present study EGFR transactivation was observed as early as 1 h post-infection (Fig. 8A). The molecular events leading to EGFR transactivation during infection were not investigated in the present study, although metalloprotease cleavage of HB-EGF may play a role (64). Critical to our hypothesis, we found that specific inhibition of EGFR transactivation by inhibitors or in the presence of a neutralizing EGFR antibody resulted in significant reduction in hBD3 mRNA and peptide expression (Fig. 8, B and C), thus providing evidence for EGFR-mediated hBD3 regulation in our model of infection. Importantly, inhibition of this pathway had no effect on hBD2 gene or peptide expression. Based on the present evidence we suggest that, like NOD-1, EGFR may also function as a critical regulator of a subset of innate response genes with hBD3 a prominent member.

Activation of the EGFR with its array of ligands is known to play a critical role in mucosal repair and wound healing processes during infection and inflammation (70). The intimate linkage of tissue repair with induction of a potent antimicrobial barrier, i.e. increased hBD3 expression, should aid in limiting potential infection and microbial invasion at a site undergoing extensive tissue remodeling. It would be of great interest in the future to observe the addition of other critical innate genes involved in tissue repair to this subset. All studies to date show a dramatic increase in hBD2 expression during inflammation, suggesting its antimicrobial and chemotactic properties provide a first line of defense, however, during the repair phase of tissue homeostasis, inflammation recedes with declining hBD2 levels, and we propose that at this stage the antimicrobial effects of hBD3 come into play.

In conclusion, we found *H. pylori* to activate a spectrum of signaling events that play unique and overlapping roles in modulation of epithelial innate defense. Whether these cellular events have similar functions in modulating host immunity in other innate immune cells is a matter of future research.

REFERENCES

1. Blaser, M. J., and Atherton, J. C. (2004) *J. Clin. Invest.* 113, 321–333
2. Atherton, J. C., Tao, P., Peek, R. M., Tummuru, M. K., Blaser, M. J., and Cover, T. L. (1995) *J. Biol. Chem.* 270, 17771–17777
3. Blaser, M. J. (1996) *Sci. Am.* 274, 104–107
4. Israil, D. A., and Peek, R. M. (2001) *Aliment. Pharmacol. Ther.* 15, 1271–1280
5. Blaser, M. J., and Berg, D. E. (2001) *J. Clin. Invest.* 107, 767–773
6. Atherton, J. C. (1997) *Gut* 40, 701–703
7. Levenstein, S. (1999) *Can. J. Gastroenterol.* 13, 753–759
8. El Omar, E. M., Carrington, M., Chow, W. H., McColl, K. E., Brew, J. H., Young, H. A., Herrera, J., Lissowska, J., Yuan, C. C., Rothman, N., Larygon, M., Martin, M., Fraumeni, J. F., Jr., and Rabin, C. S. (2000) *Nature* 404, 398–402
9. El Omar, E. M., Rabin, C. S., Gammion, M. D., Vaughan, T. L., Risch, H. A., Schoenberg, J. B., Stanford, J. L., Mayne, S. T., Goedert, J., Blot, W. J., Fraumeni, J. F., Jr., and Chow, W. H. (2003) *Gastroenterology* 124, 1193–1201
10. Covacci, A., Censini, S., Bugnoli, M., Petracca, R., Burroni, D., Macchia, G., Massone, A., Papini, E., Xiang, Z., and Figura, N. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 5791–5795
11. Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Rappuoli, R., and Covacci, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 9369–9374
12. Naumann, M., and Crabtree, J. E. (2004) *Trends Microbiol.* 12, 29–36
13. Crabtree, J. E., Xiang, Z., Lindley, J. I., Tompkins, D. S., Rappuoli, R., and Covacci, A. (1993) *J. Clin. Pathol.* 48, 967–969
14. Crabtree, J. E., Kersulyte, D., Li, S. D., Lindley, J. I., and Berg, D. E. (1999) *J. Clin. Pathol.* 52, 653–657
