DOI: 10.1111/hel.12325

ORIGINAL ARTICLE

WILEY Helicobacter

Differential modulation of *Helicobacter pylori* lipopolysaccharide-mediated TLR2 signaling by individual Pellino proteins

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Abstract

Background: Eradication rates for current *H. pylori* therapies have fallen in recent years, in line with the emergence of antibiotic resistant infections. The development of therapeutic alternatives to antibiotics, such as immunomodulatory therapy and vaccines, requires a more lucid understanding of host–pathogen interactions, including the relationships between the organism and the innate immune response. Pellino proteins are emerging as key regulators of immune signaling, including the Toll-like receptor pathways known to be regulated by *H. pylori*. The aim of this study was to characterize the role of Pellino proteins in the innate immune response to *H. pylori* lipopolysaccharide.

Materials and Methods: Gain-of-function and loss-of-function approaches were utilized to elucidate the role of individual Pellino proteins in the Toll-like receptor 2-mediated response to *H. pylori* LPS by monitoring NF-κB activation and the induction of proinflammatory chemokines. Expression of Pellino family members was investigated in gastric epithelial cells and gastric tissue biopsy material.

Results: Pellino1 and Pellino2 positively regulated Toll-like receptor 2-driven responses to *H. pylori* LPS, whereas Pellino3 exerted a negative modulatory role. Expression of Pellino1 was significantly higher than Pellino3 in gastric epithelial cells and gastric tissue. Furthermore, Pellino1 expression was further augmented in gastric epithelial cells in response to infection with *H. pylori* or stimulation with *H. pylori* LPS.

Conclusions: The combination of low Pellino3 levels together with high and inducible Pellino1 expression may be an important determinant of the degree of inflammation triggered upon Toll-like receptor 2 engagement by *H. pylori* and/or its components, contributing to *H. pylori*-associated pathogenesis by directing the incoming signal toward an NF-kB-mediated proinflammatory response.

KEYWORDS

chemokine, *Helicobacter pylori*, lipopolysaccharide, NF-kB, Pellino, toll-like receptor-2

The gastric pathogen *Helicobacter pylori* infects approximately half of the world's population. Infection is causally linked to chronic gastritis, peptic ulcers, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma.[1–3] Disease outcome is influenced by both host factors and strain-specific bacterial components.[4] Eradication rates for current *H. pylori* therapies have fallen in recent years, in line with the emergence of antibiotic resistant infection.[5] The development of therapeutic alternatives to antibiotics, such as immunomodulatory therapy and vaccines, requires a more lucid understanding of host–pathogen interactions. Epithelial cells of the gastric mucosa represent the first line of defense against *H. pylori* infection. Pathogen recognition receptors on gastric epithelial cells, including members of the Toll-like receptor (TLR) family, mediate responses to infection by triggering cell signaling pathways that lead to the induction of host defense genes, including those for inflammatory cytokines, antigen-presenting molecules, and costimulatory molecules.[6–8] Although *H. pylori* infection induces an immune response that contributes to chronic gastric inflammation, the response is frequently not sufficient to eliminate the bacterium.[9,10] The severity of inflammation is highly variable in the host, and the factors determining severity or progression to peptic ulceration or malignancy are incompletely understood. Progression of disease from superficial gastritis to gastric cancer is however linked to the severity of the host inflammatory response.[11–13]

Ten functional TLRs have been identified in humans to date.[7] Upon ligand recognition, all TLRs (apart from TLR3) use the adapter molecule MyD88 to trigger downstream activation of the transcription factor NF-κB. Association of MyD88 with TLRs leads to the recruitment of serine threonine kinases belonging to the IRAK family and subsequent activation of TRAF6, which in turn leads to TAK1-mediated phosphorylation of the IKK (inhibitor of NF-κB (IκB) kinase) complex.[6,7] The phosphorylated IKK complex in turn phosphorylates IκBs, which are subsequently targeted for proteasome-dependent degradation, thus releasing NF-κB to translocate from the cytoplasm to the nucleus to transcriptionally regulate genes with NF-κB-binding elements in their promoters.[6,7] Activated TAK1 also induces the mitogen-activated protein kinase (MAPK) pathway leading to activation of the transcription factor AP-1. The TIR domain-containing adaptor protein inducing IFN-β (TRIF, also known as TICAM1) is involved in the MyD88-independent TLR4 pathway, as well as the TLR3 pathway, mediating both NF-κB signaling and the induction of type I interferon through the activation of the IRF signaling pathway.[6,7]

Several TLRs have been implicated in the innate immune response to *H. pylori*.[11,14–18] In particular, a key role for TLR2 has been described in the response to *Helicobacter* in multiple cell contexts.[11,14,17,19–24] Numerous *H. pylori* components have been suggested to trigger TLR2 signaling including HSP60,[25,26] NapA,[9] the Cag pathogenicity island,[22] and urease.[24] Although there has been substantial investigation into the innate immune response to *H. pylori* lipopolysaccharide (LPS), there have been conflicting findings with regard to the TLR responsible for LPS recognition (recently reviewed[8,18]). Some studies have implicated the classic Gram-negative bacterial LPS receptor TLR4,[11,27–31] while others have suggested a role for TLR2.[12,17,32–34] Our previous studies support a role for TLR2 in the recognition of LPS from both clinical isolates and reference strains of *H. pylori* in epithelial cells.[34] *H. pylori* LPS functioned as a classic TLR2 ligand by signaling through pathways involving MyD88, MAL, IRAK1, IRAK4, TRAF6, IKKβ, and IκBα to activate NF-kB and transcription from the IL-8 promoter and induce expression of the chemokines CXCL1, CXCL2, CXCL3, and CCL20.[34]

Emerging evidence suggests a key role for members of the Pellino family of proteins in modulating TLR signaling.[35] Pellino was first identified as a component of the Toll pathway in *Drosophila melanogaster* as a protein that associates with the serine/threonine kinase Pelle, the *Drosophila* homologue of IRAK.[36] Three mammalian Pellino isoforms (Pellino1, Pellino2, and Pellino3) were subsequently identified.[37–39] Two splice variants of Pellino3 have been described; the longer splice variant designated Pellino3L and the shorter splice variant designated Pellino3S.[39] All three Pellino proteins have been shown to interact with the downstream TLR signaling molecules IRAK1, IRAK4, TRAF6, and TAK1.[39–45] Pellino proteins possess an N-terminal forkhead-associated domain that mediates association with IRAKs[46] and a C-terminal RING-like domain that confers E3 ubiquitin ligase activity.[42,47,48] However, functional differences between the Pellino family members have been described with regard to mediating signaling events in response to specific TLR ligands in a cell context-dependent fashion.[35,49] Thus, the differential expression or activation of Pellino proteins within a distinct cell type or tissue could specifically fine-tune cellular responses and impact on the level and type of pathogenic responses to an organism such as *H. pylori*, which signals through TLR molecules. Given the importance of Pellinos in modulating TLR signaling, this study set out to investigate the role of Pellino proteins in the innate immune response to *H. pylori* LPS.

1 | METHODS

1.1 | Cell culture and reagents

Human embryonic kidney HEK-TLR2 (InvivoGen, Cayla, France) and HEK-Blue-TLR2 cells (Invivogen) were grown in MEM alpha medium (Gibco, Grand Island, NY, USA), supplemented with 10% FCS (Gibco), 2 mmol/L L-glutamine (Sigma-Aldrich, Poole, UK), 100 U/mL penicillin (Sigma-Aldrich), and 100 μg/mL streptomycin (Sigma-Aldrich). The media for HEK-TLR2 and HEK-Blue-TLR2 were further supplemented with 10 μg/mL blasticidin (Invivogen) and 1× HEK-Blue Selection (Invivogen), respectively. MKN45 cells (Health Science Research Resources Bank, Japan) were grown in RPMI 1640 medium (Gibco) supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Pam_2CSK_4 was from Invivogen. Stealth small interfering RNA (siRNA) for Pellino1, Pellino2, and Pellino3 and the nontargeting control were from Invitrogen (Paisley, UK).

1.2 | Patient samples

Ethical permission was granted by the St. James's Hospital Research Ethics Committee, and informed written consent was obtained from all patients. Patients receiving antibiotics, proton-pump inhibitors, steroids, or nonsteroidal anti-inflammatory drugs within 8 weeks of endoscopy were excluded. The *H. pylori* status was determined by the rapid urease test and histopathologic examination of biopsy specimens. Antral biopsies were stored in RNA*later* (Life Technologies, Grand Island, NY, USA) at 4°C overnight to allow the solution to thoroughly penetrate the tissue, and then at −80°C until processed for RNA isolation. The tissue samples were homogenized in TRI reagent (Sigma-Aldrich) and further purified using the RNeasy MinElute cleanup kit (Qiagen, Manchester, UK).

1.3 | Growth of *H. pylori* and Preparation of LPS

Bacterial biomass was obtained by growth of the *H. pylori* strain NCTC 11637 on Columbia blood agar under microaerophilic conditions at 37°C, and LPS was isolated and purified as described previously.[34] Before infection of cell cultures, bacteria were inoculated into *Brucella* broth with 10% FCS and grown under microaerophilic conditions at 37°C overnight with shaking. Bacteria were washed in PBS (pH 7.4) and resuspended in antibiotic-free culture medium for the duration of infection. Bacteria were added to cell cultures at a multiplicity of infection of 100:1 for different time points.

1.4 | Plasmids

Myc-tagged Pellino1, Pellino2, Pellino3L, and Pellino3S were expressed from pcDNA3.1/Zeo.[45] The NF-κB luciferase reporter construct contained three κB elements upstream of a minimal conalbumin promoter linked to the firefly luciferase gene.[50] The *Il8* gene promoter reporter construct contained the human *Il8* promoter sequence upstream of an SV40 promoter linked to the luciferase gene. The *Ccl20* gene promoter reporter construct contained the promoter region (from −871 to +58) of the human *Ccl20* gene cloned into pGL2 basic.[51]

1.5 | Transfections and reporter assays

Transfections using plasmid DNA and/or siRNA were performed using Lipofectamine (Gibco) according to the manufacturer's instructions. Forty-eight hours post-transfection, cells were stimulated with either 50 ng/mL of Pam_2CSK_4 or 5 μg/mL *H. pylori* LPS. Cells were harvested 8-hours poststimulation using 1× lysis buffer (Promega, Mannheim, Germany). Luciferase activity was determined from cell extracts by means of the Luciferase Assay System (Promega). Luciferase levels were normalized after determining *Renilla* luciferase activity expressed from a pRL-TK vector (Promega), which was included in all transfections. NF-κB-driven secreted alkaline phosphatase (SEAP) activity in Pam_2CSK_4- or *H. pylori* LPS-treated HEK-Blue-TLR2 cells was monitored by addition of QUANTI-Blue (Invivogen) directly to the cell culture medium according to the manufacturer's instructions, and absorbance was measured at 620 nm.

1.6 | Total RNA extraction, reverse transcription, and PCR

Total RNA was isolated from cell lines using a NucleoSpin RNAII kit (Machery-Nagel GmbH, Düren, Germany), and first-strand cDNA synthesis was performed using a RETROscript kit (Life Technologies) according to the manufacturer's instructions. PCR for *Tlr2* and *Gapdh* was performed using the primers (forward: 5′-TGATGCTGCCATTCTCATTC-3′ and reverse: 5′-CGCAGCTCTCAGATTTACCC-3′) and (forward: 5′-TGA AGGTCGGAGTCAACGGATTTGGT-3′ and reverse: 5′-CATGTGGGCCA TGAGGTCCACCAC-3′), respectively. PCR products (*Tlr2*: 157 bp and *Gapdh*: 983 bp) were analyzed by agarose gel electrophoresis. Quantitative PCR (qPCR) for *Pellino1*, *Pellino2*, *Pellino3*, *Il8*, and *Gapdh* was performed using TaqMan gene expression assays (Life Technologies) and the Applied Biosystems 7900HT real-time PCR system (Applied Biosystems, Cheshire, UK).

1.7 | Immunoblot analysis

Whole cell lysates were prepared using cell lysis buffer (50 mmol/L Hepes pH 7.4, 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L $Na_4O_7P_2$, 50 mmol/L NaF, 50 mmol/L glycerophosphate, 1 mmol/L Na_3VO_4, 1% Triton-X100, 2 mmol/L PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin) and lysed on ice for 30 minutes. Insoluble material was removed by centrifugation at 12000 × g for 5 minutes at 4°C. Protein concentrations were quantified by BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). For immunoblot analysis, equal amounts of protein (20 μg) were separated by SDS-PAGE and transferred to PVDF membrane for probing with antibody. Mouse anti-Myc-tag and mouse anti-β-actin antibodies were from Sigma-Aldrich. Rabbit anti-Pellino3 antibody was generated in-house. Goat anti-mouse HRP and goat anti-rabbit HRP secondary antibodies were from Cell Signaling Technology (Leiden, the Netherlands).

1.8 | Statistical analysis

Mean and standard deviation of triplicates are shown, and data are representative of at least 3 individual experiments. The Student's *t*-test was employed to compare treated vs control samples. The criterion for significance was a P value of $<.05$ for all comparisons.

2 | RESULTS

2.1 | Pellino1 increases TLR2-mediated NF-κB activity and chemokine induction in response to *H. pylori* LPS

To evaluate the role of Pellino proteins during the TLR2-mediated response to *H. pylori* LPS, we first investigated the effect of Pellino1 overexpression on LPS-treated HEK293 cells overexpressing TLR2 (HEK-TLR2 cells). Expression of TLR2 in HEK-TLR2 cells was confirmed by reverse transcription PCR (Fig. 1A). Transfection of HEK-TLR2 cells with increasing quantities of Myc-tagged Pellino1 expression vector resulted in a dose-dependent increase in Pellino1 mRNA (Fig. 1B) and protein (Fig. 1C). Pellino 1 overexpression resulted in a dose-dependent increase in luciferase activity from

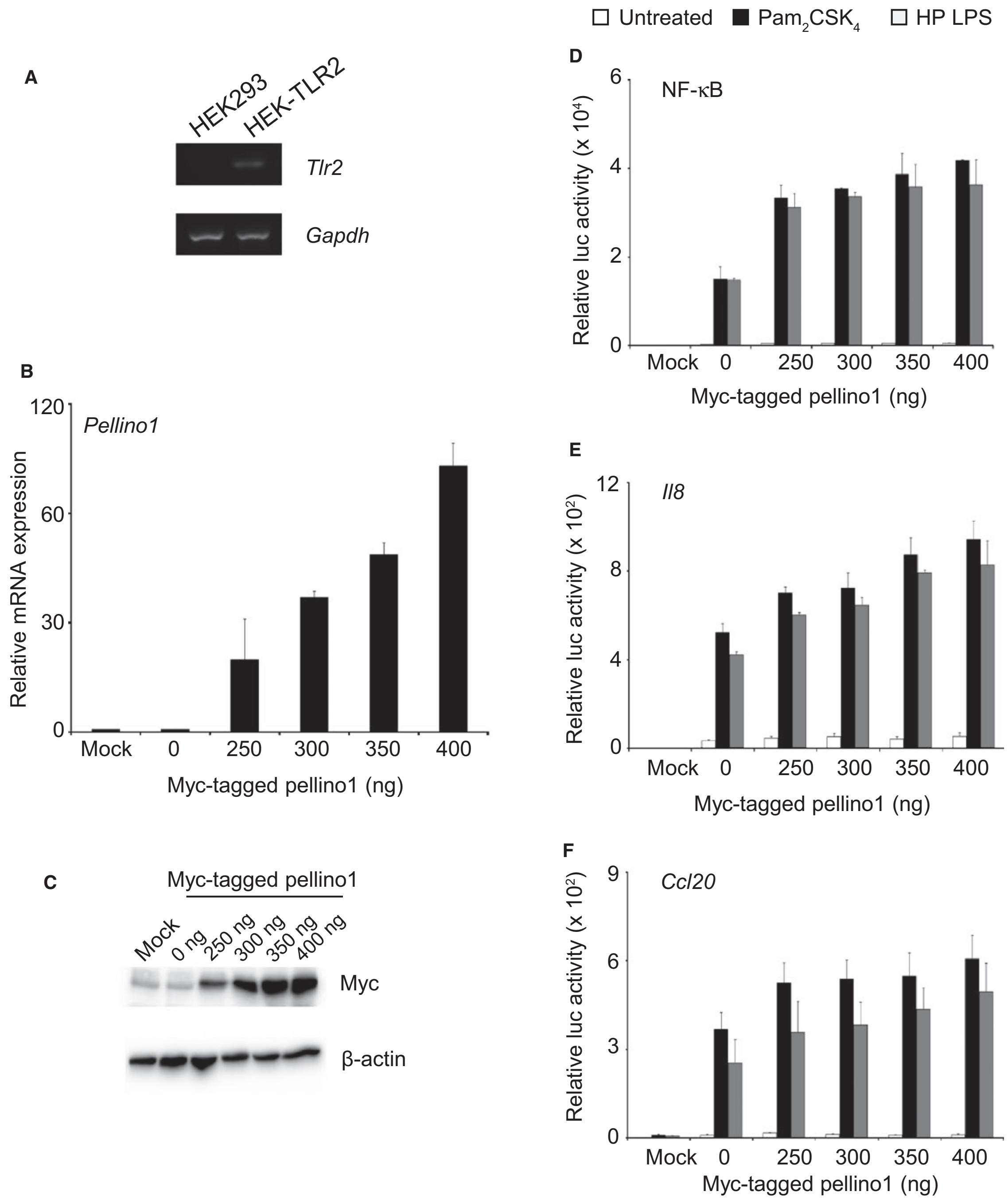

FIGURE 1 Pellino1 overexpression enhances *H. pylori* LPS-mediated activation of NF-κB and the *Il8* and *Ccl20* promoters. (*A*) Agarose gel electrophoresis images of reverse transcription PCR products for *Tlr2* and *Gapdh* in HEK293 and HEK-TLR2 cells. (*B*) qPCR analysis of *Pellino1* mRNA expression in HEK-TLR2 cells transfected for 48 hours with increasing quantities of a Myc-tagged Pellino1 expression vector. Total plasmid DNA concentration was constant across transfections through addition of the empty vector pcDNA3.1/Zeo. Results are normalized to *Gapdh* mRNA expression and presented relative to untransfected cells (mock). (*C*) Immunoblot analysis of Myc-tagged Pellino1 and β-actin expression in HEK-TLR2 cells transfected for 48 hours with increasing quantities of a Myc-tagged Pellino1 expression vector. Total plasmid DNA concentration was constant across transfections through addition of the empty vector pcDNA3.1/Zeo. Luciferase activity in lysates of HEK-TLR2 cells cotransfected for 48 hours with increasing quantities of a Pellino1 expression vector together with an NF-κB responsive reporter construct (*D*), an *Il8* promoter reporter construct (*E*), or a *Ccl20* promoter reporter construct (*F*), and stimulated with either 50 ng/mL Pam_2CSK_4 or 5 μg/mL *H. pylori* LPS for 8 hours. Total plasmid DNA concentration was constant across transfections through addition of the empty vector pcDNA3.1/Zeo. Results are normalized to values for renilla luciferase and are presented relative to those of unstimulated untransfected cells (mock)

an NF-κB-dependent reporter construct in response to both *H. pylori* LPS and the TLR2 ligand Pam_2CSK_4 (Fig. 1D). Similarly, Pellino1 overexpression augmented Pam_2CSK_4- and *H. pylori* LPS-mediated activation of an *Il8* promoter reporter construct (Fig. 1E) and a *Ccl20* promoter reporter construct (Fig. 1F). Using a loss-of-function approach, endogenous Pellino1 expression was inhibited using siRNA,

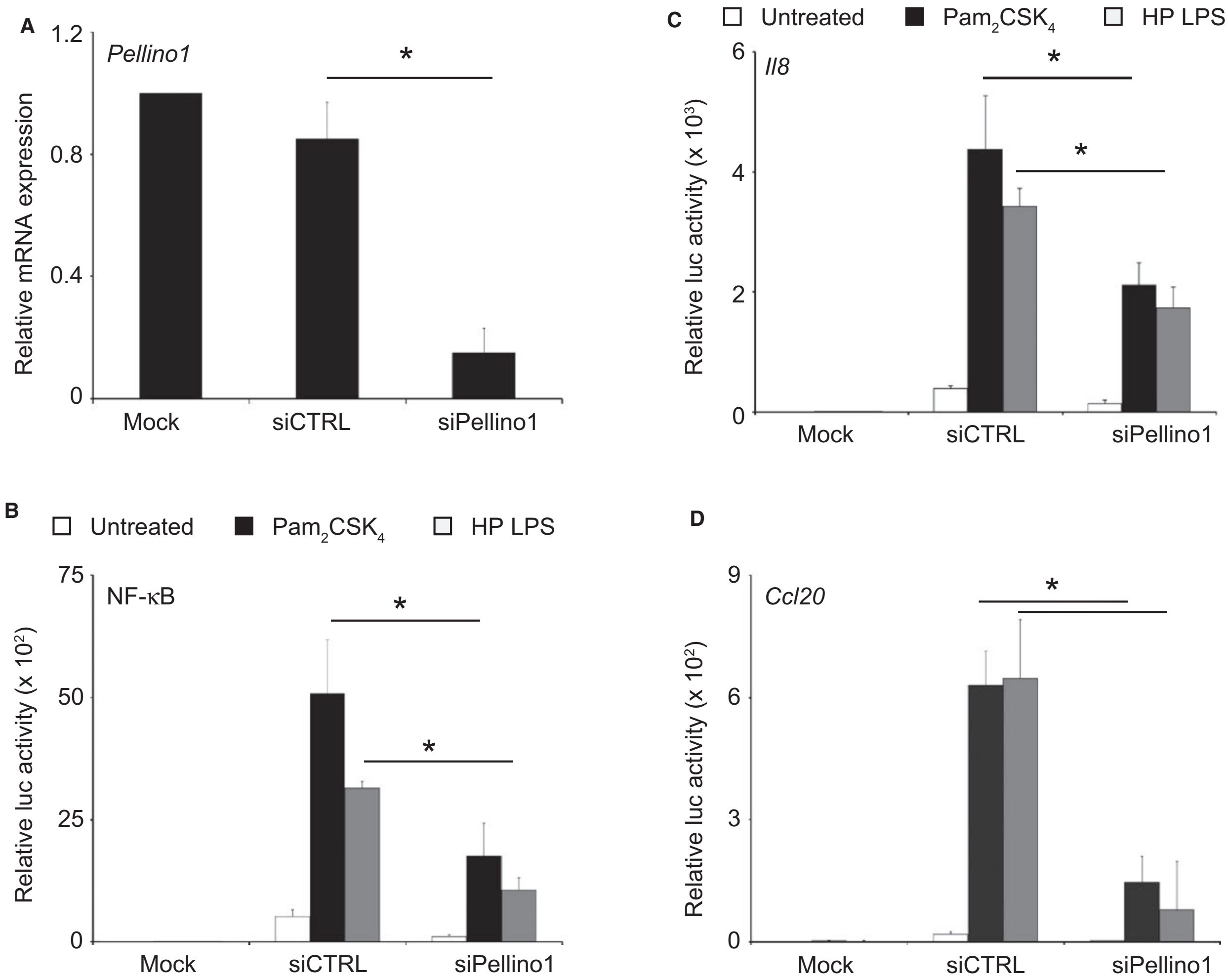

FIGURE 2 Pellino1 knockdown inhibits *H. pylori* LPS-mediated activation of NF-κB and the *Il8* and *Ccl20* promoters. (*A*) Quantitative PCR analysis of *Pellino1* mRNA expression in HEK-TLR2 cells following transfection with siRNA for *Pellino1* (siPellino1) or a scrambled siRNA negative control (siCTRL). Results are normalized to *Gapdh* mRNA expression and presented relative to untransfected cells (mock). (*B*, *C*, *D*) Luciferase activity in lysates of HEK-TLR2 cells cotransfected for 48 hours with either siPellino1 or siCTRL together with an NF-κB responsive reporter construct (*B*), an *Il8* promoter reporter construct (*C*), or a *Ccl20* promoter reporter construct (*D*), and stimulated with either 50 ng/mL Pam_2CSK_4 or 5 μg/mL *H. pylori* LPS for 8 hours. Results are normalized to values for renilla luciferase and are presented relative to those of unstimulated untransfected cells (mock). *$P<.05$

resulting in approximately 85% decrease in *Pellino1* mRNA expression (Fig. 2A). Inhibition of Pellino1 expression led to a decrease in Pam_2CSK_4- and *H. pylori* LPS-mediated activation of NF-κB (Fig 2B), and transcription driven by the *Il8* (Fig. 2C) and *Ccl20* (Fig. 2D) gene promoters. Taken together, these data support a positive regulatory role for Pellino1 during the TLR2-mediated response to *H. pylori* LPS.

2.2 | Pellino2 increases TLR2-mediated chemokine induction in response to *H. pylori* LPS

Next, we assessed the role of Pellino2 during *H. pylori* LPS-driven cell signaling events. Transfection of HEK-TLR2 cells with increasing quantities of Myc-tagged Pellino2 expression vector resulted in a dose-dependent increase in Pellino2 mRNA (Fig. 3A) and protein (Fig. 3B). In contrast to Pellino1, increased Pellino2 expression in HEK-TLR2 cells did not enhance NF-κB-dependent luciferase activity in response to either *H. pylori* LPS or Pam_2CSK_4 (Fig. 3C). However, similar to the findings following Pellino1 overexpression, increased Pellino2 expression led to enhanced TLR2-mediated activation of the *Il8* promoter (Fig. 3D) and the *Ccl20* promoter (Fig. 3E). Knockdown of endogenous Pellino2 expression by RNA interference (RNAi) resulted in an 80% decrease in Pellino2 mRNA expression (Fig. 4A). This decrease in Pellino2 expression in HEK-TLR2 cells was not accompanied by any significant effect on NF-κB activity in response to either *H. pylori* LPS or Pam_2CSK_4 (Fig 4B). Using an alternative approach, Pellino2 inhibition did not significantly alter LPS- or Pam_2CSK_4-mediated NF-κB-driven SEAP activity in HEK-TLR2-Blue cells (Fig. 4C). However, Pellino2 knockdown led to an inhibition in Pam_2CSK_4- and LPS-mediated *Il8* (Fig. 4D) and

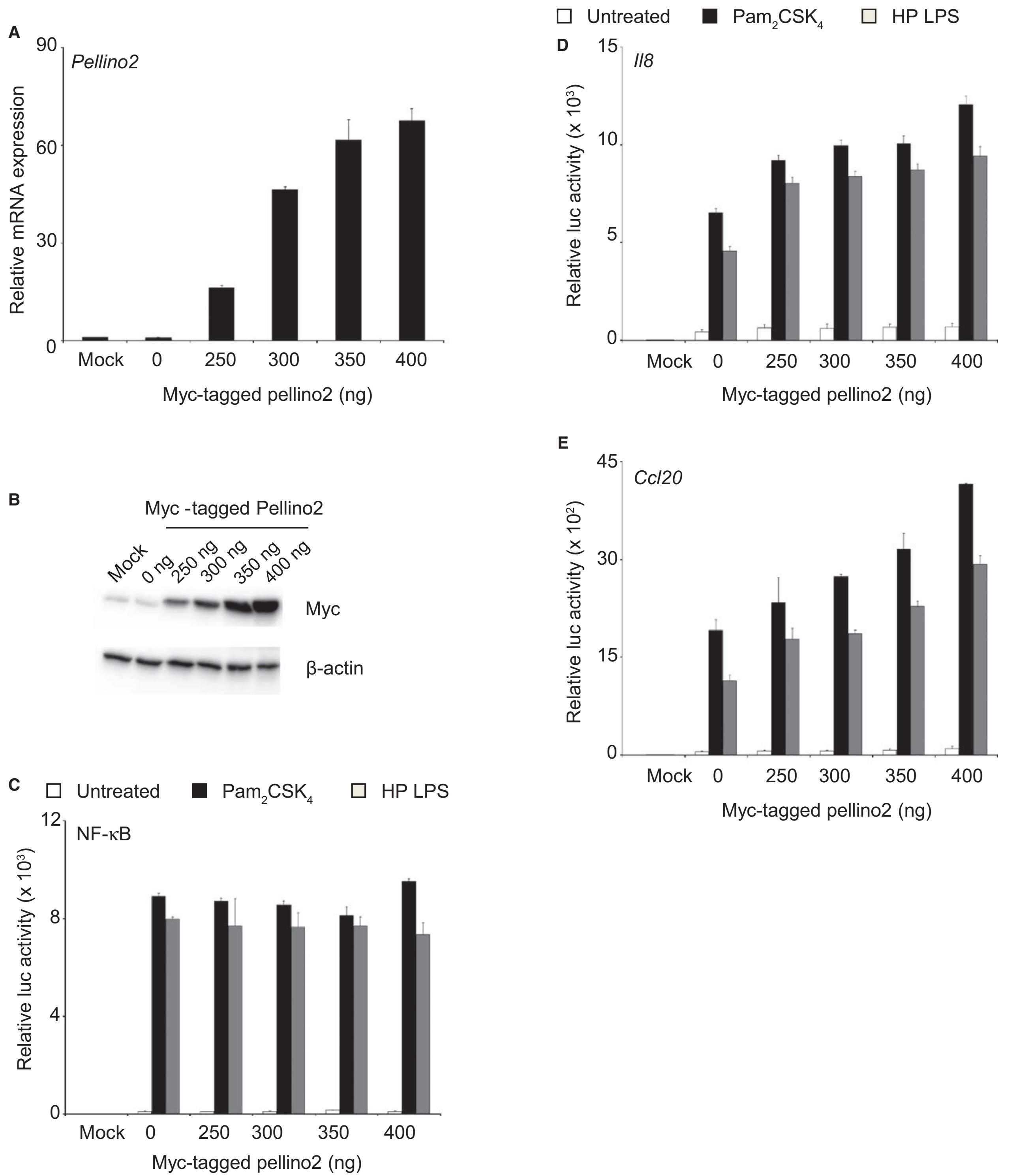

FIGURE 3 Pellino2 overexpression enhances *H. pylori* LPS-mediated activation of the *Il8* and *Ccl20* promoters. (*A*) Quantitative PCR analysis of *Pellino2* mRNA expression in HEK-TLR2 cells transfected for 48 hours with increasing quantities of a Myc-tagged Pellino2 expression vector. Total plasmid DNA concentration was constant across transfections through addition of the empty vector pcDNA3.1/Zeo. Results are normalized to *Gapdh* mRNA expression and presented relative to untransfected cells (mock). (*B*) Immunoblot analysis of Myc-tagged Pellino2 and β-actin expression in HEK-TLR2 cells transfected for 48 hours with increasing quantities of a Myc-tagged Pellino2 expression vector. Total plasmid DNA concentration was constant across transfections through addition of the empty vector pcDNA3.1/Zeo. Luciferase activity in lysates of HEK-TLR2 cells cotransfected for 48 hours with increasing quantities of a Myc-tagged Pellino2 expression vector together with an NF-κB responsive reporter construct (*C*), an *Il8* promoter reporter construct (*D*), or a *Ccl20* promoter reporter construct (*E*), and stimulated with either 50 ng/mL Pam_2CSK_4 or 5 μg/mL *H. pylori* LPS for 8 hours. Total plasmid DNA concentration was constant across transfections through addition of the empty vector pcDNA3.1/Zeo. Results are normalized to values for renilla luciferase and are presented relative to those of unstimulated untransfected cells (mock)

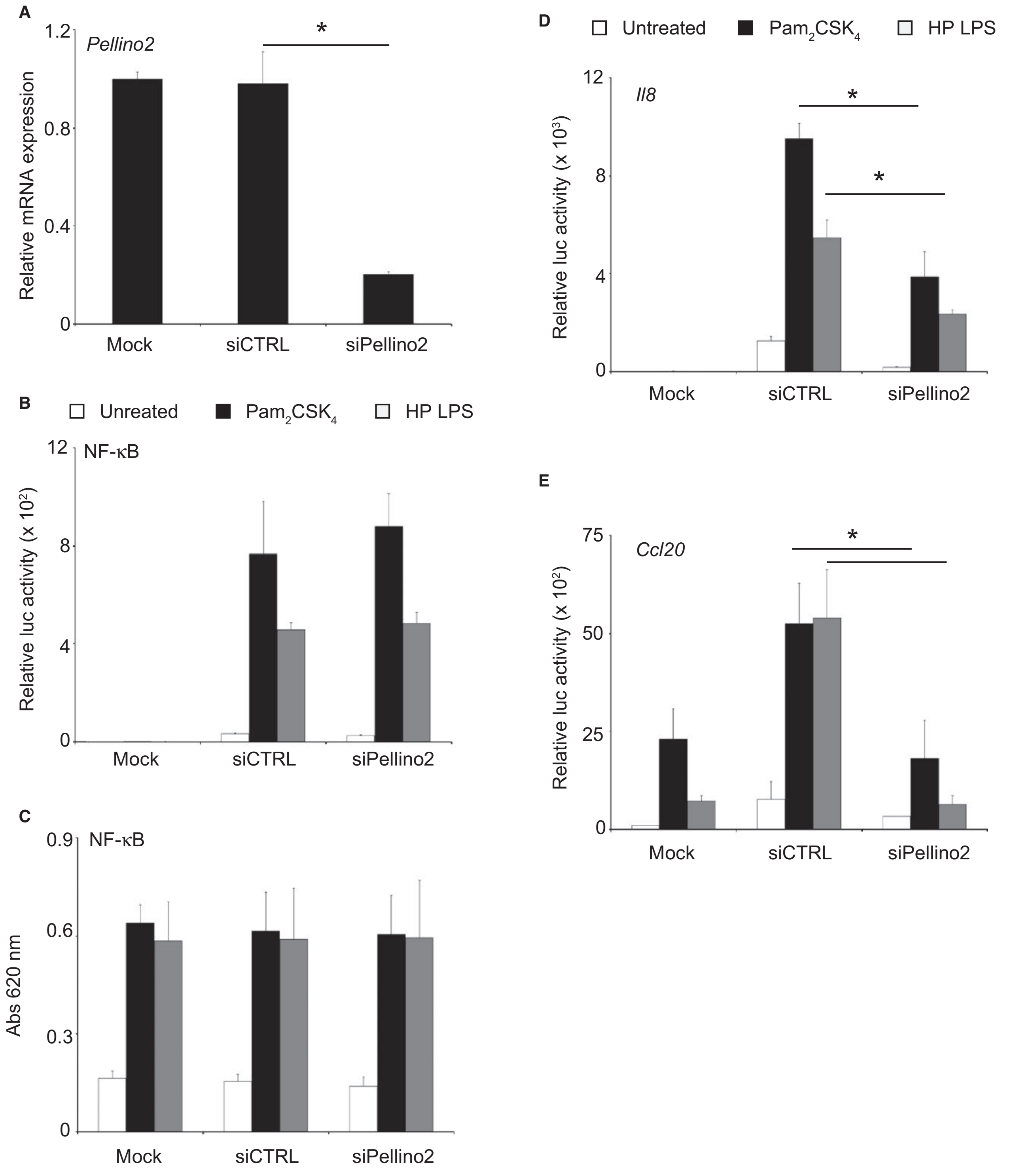

FIGURE 4 Pellino2 knockdown inhibits *H. pylori* LPS-mediated activation of the *Il8* and *Ccl20* promoters. (*A*) Quantitative PCR analysis of *Pellino2* mRNA expression in HEK-TLR2 cells following transfection with siRNA for *Pellino2* (siPellino2) or siCTRL. Results are normalized to *Gapdh* mRNA expression and presented relative to untransfected cells (mock). (*B*) Luciferase activity in lysates of HEK-TLR2 cells cotransfected for 48 hours with siPellino2 or siCTRL together with an NF-κB responsive reporter construct and stimulated with 50 ng/mL Pam_2CSK_4 or 5 μg/mL *H. pylori* LPS for 8 hours. (*C*) SEAP activity in lysates of HEK-Blue-TLR2 cells transfected for 48 hours with siPellino2 or siCTRL and stimulated with 50 ng/mL Pam_2CSK_4 or 5 μg/mL *H. pylori* LPS for 8 hours. (*D, E*) Luciferase activity in lysates of HEK-TLR2 cells cotransfected for 48 hours with siPellino2 or siCTRL together an *Il8* promoter reporter construct (*D*), or a *Ccl20* promoter reporter construct (*E*), and stimulated with 50 ng/mL Pam_2CSK_4 or 5 μg/mL *H. pylori* LPS for 8 hours. Results are normalized to values for renilla luciferase and are presented relative to those of unstimulated untransfected cells (mock). *P<.05

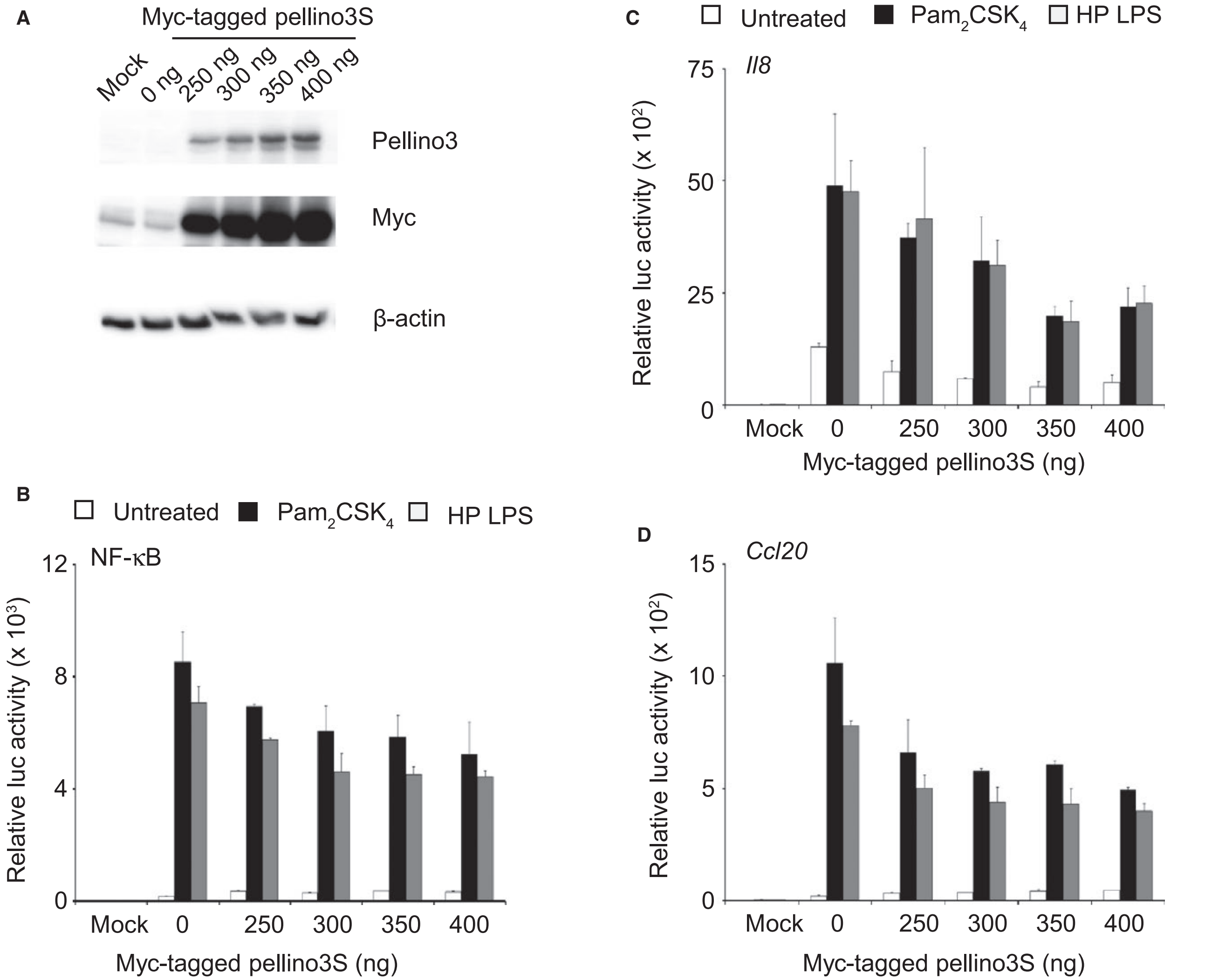

FIGURE 5 Pellino3S overexpression inhibits *H. pylori* LPS-mediated activation of NF-κB and the *Il8* and *Ccl20* promoters. (*A*) Immunoblot analysis of Pellino3, Myc-tagged Pellino3, and β-actin expression in HEK-TLR2 cells transfected for 48 hours with increasing quantities of a Myc-tagged Pellino3S expression vector. Total plasmid DNA concentration was constant across transfections through addition of the empty vector pcDNA3.1/Zeo. Luciferase activity in lysates of HEK-TLR2 cells cotransfected for 48 hours with increasing quantities of a Myc-tagged Pellino3S expression vector together with an NF-κB responsive reporter construct (*B*), an *Il8* promoter reporter construct (*C*), or a *Ccl20* promoter reporter construct (*D*), and stimulated with either 50 ng/mL Pam_2CSK_4 or 5 μg/mL *H. pylori* LPS for 8 hours. Total plasmid DNA concentration was constant across transfections through addition of the empty vector pcDNA3.1/Zeo. Results are normalized to values for renilla luciferase and are presented relative to those of unstimulated untransfected cells (mock)

Ccl20 (Fig. 4E) promoter activity. These findings suggest that although Pellino2 does not play a role in *H. pylori* LPS-mediated NF-κB activation, it positively modulates *Il8* and *Ccl20* gene promoter activity.

2.3 | Pellino3 decreases TLR2-mediated NF-κB activity and chemokine induction in response to *H. pylori* LPS

Using similar gain-of-function and loss-of-function approaches to those described above, the role of both isoforms of Pellino3 in *H. pylori* LPS-triggered signaling was evaluated. Increased expression of either Myc-tagged Pellino3S or Myc-tagged Pellino3L led to increased expression levels of these proteins as detected by a Pellino3-specific antibody and Myc-tagged antibody (Figs 5A and 6A). Pellino3S overexpression led to a decrease in Pam_2CSK_4- and *H. pylori* LPS-mediated activation of NF-kB (Fig. 5B) and the *Il8* (Fig. 5C) and *Ccl20* gene promoters (Fig. 5D) in HEK-TLR2 cells. Similar results were obtained upon overexpression of Pellino3L (Fig. 6B–D), indicating that increased Pellino3 expression inhibits TLR2-driven responses to *H. pylori* LPS. Endogenous Pellino3 expression was subsequently inhibited in HEK-TLR2 cells using an siRNA molecule that targeted both Pellino3 isoforms resulting in a 77% inhibition in *Pellino3* mRNA expression (Fig. 7A). Knockdown of Pellino3 expression enhanced the ability of Pam_2CSK_4 and *H. pylori* LPS to activate NF-kB (Fig. 7B) and the *Il8* (Fig. 7C) and *Ccl20*

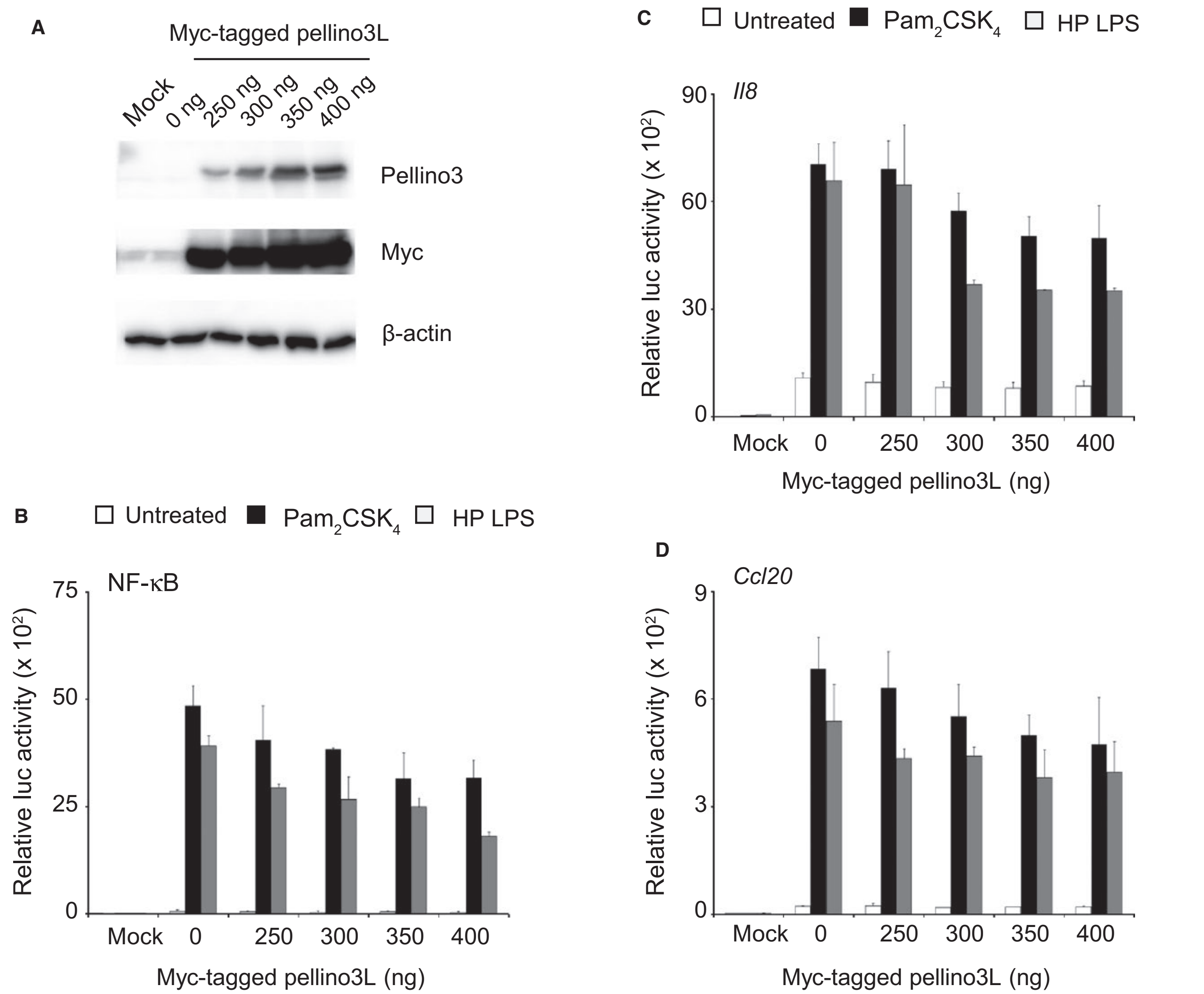

FIGURE 6 Pellino3L overexpression inhibits *H. pylori* LPS-mediated activation of NF-κB and the *Il8* and *Ccl20* promoters. (*A*) Immunoblot analysis of Pellino3, Myc-tagged Pellino3, and β-actin expression in HEK-TLR2 cells transfected for 48 hours with increasing quantities of a Myc-tagged Pellino3L expression vector. Total plasmid DNA concentration was constant across transfections through addition of the empty vector pcDNA3.1/Zeo. Luciferase activity in lysates of HEK-TLR2 cells cotransfected for 48 hours with increasing quantities of a Myc-tagged Pellino3L expression vector together with an NF-κB responsive reporter construct (*B*), an *Il8* promoter reporter construct (*C*), or a *Ccl20* promoter reporter construct (*D*), and stimulated with either 50 ng/mL Pam_2CSK_4 or 5 µg/mL *H. pylori* LPS for 8 hours. Total plasmid DNA concentration was constant across transfections through addition of the empty vector pcDNA3.1/Zeo. Results are normalized to values for renilla luciferase and are presented relative to those of unstimulated untransfected cells (mock)

(Fig. 7D) promoters. Taken together, these findings suggest that Pellino3 is a negative regulator of the activating properties of *H. pylori* LPS.

2.4 | Differential expression of Pellinos in Gastric epithelial cells and gastric tissue biopsies

Having characterized the role of individual Pellino family members during the TLR2-mediated response to *H. pylori* LPS using HEK-TLR2 cells, expression of Pellinos in the gastric epithelial cell line MKN45 and in gastric biopsy samples was investigated next. *Pellino1* was the most abundantly expressed of the Pellinos in MKN45 cells (Fig. 8A,B; time 0 hour). *Pellino2* and *Pellino3* were also detected but expressed at a significantly lower level relative to *Pellino1* (82% and 75%, respectively, Fig. 8A,B; time 0 hour). Stimulation of MNK45 cells with *H. pylori* LPS (Fig. 8A) or infection with *H. pylori* (Fig. 8) resulted in a transient increase in *Pellino1* mRNA expression. Neither *Pellino2* nor *Pellino3* expression was significantly altered in response to *H. pylori* LPS (Fig. 8A) or infection (Fig. 8B). In gastric biopsy tissue samples, expression of Pellino1 was the highest among the Pellinos, with an expression level of just 10% for Pellino3 relative to Pellino1 (Fig. 8C). There was no significant difference in expression of Pellino proteins in biopsies isolated from *H. pylori*-negative individuals compared to those isolated from *H. pylori*-infected patients with chronic gastritis (data not shown). To confirm the functional roles of Pellino family members in response

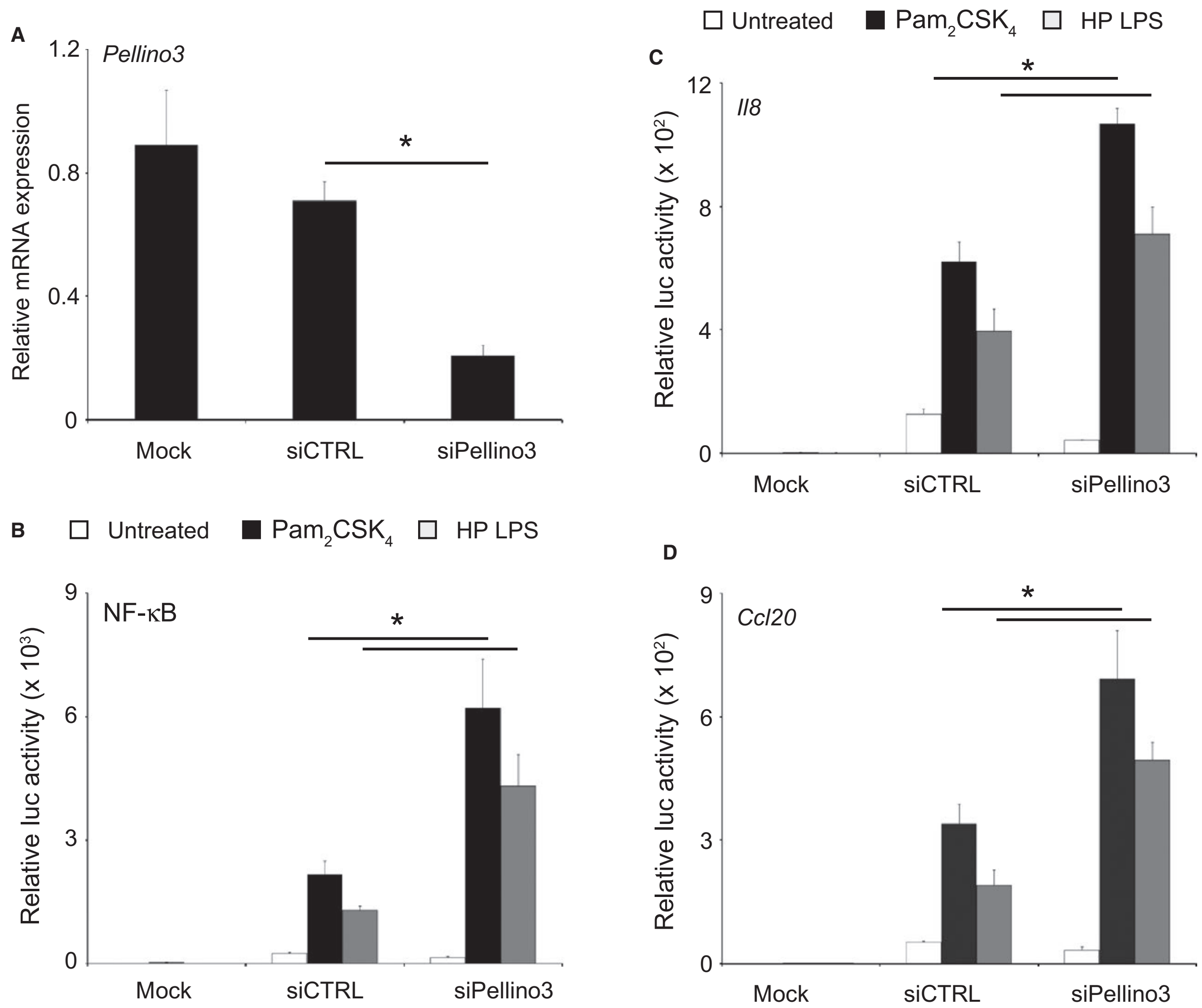

FIGURE 7 Pellino3 knockdown increases *H. pylori* LPS-mediated activation of NF-κB and the *Il8* and *Ccl20* promoters. (*A*) Quantitative PCR analysis of *Pellino3* mRNA expression in HEK-TLR2 cells following transfection with siRNA for *Pellino3* (siPellino3) or siCTRL. Results are normalized to *Gapdh* mRNA expression and presented relative to untransfected cells (mock). (*B, C, D*) Luciferase activity in lysates of HEK-TLR2 cells cotransfected for 48 hours with either siPellino3 or siCTRL together with an NF-κB responsive reporter construct (*B*), an *Il8* promoter reporter construct (*C*), or a *Ccl20* promoter reporter construct (*D*), and stimulated with either 50 ng/mL Pam_2CSK_4 or 5 µg/mL *H. pylori* LPS for 8 hours. Results are normalized to values for renilla luciferase and are presented relative to those of unstimulated untransfected cells (mock). $^*P<.05$

to *H. pylori* LPS in gastric MKN45 cells, the effect of their inhibition on *Il8* mRNA expression was investigated. Firstly, qPCR for *Pellino1*, *Pellino2*, and *Pellino3* expression following knockdown of the individual Pellino proteins demonstrated that the siRNAs for each Pellino protein did not result in off-target inhibition of the other Pellino family members (Fig 8D). Knockdown of either *Pellino1* or *Pellino2* expression led to decreased *Il8* mRNA induction in response to *H. pylori* LPS (Fig. 8E). In contrast, LPS-mediated *Il8* expression was augmented in cells where *Pellino3* was inhibited (Fig. 8E). Taken together, these data indicated that the positive regulator of *H. pylori* LPS-mediated signaling, Pellino1, is highly expressed in gastric epithelial cells and gastric tissue relative to Pellino2 and in particular to the negative regulator Pellino3. In addition, both *H. pylori* infection and LPS have the ability to modulate Pellino1 expression by increasing its expression in gastric epithelial cells.

3 | DISCUSSION

Pellino proteins are emerging as key regulators of immune signaling pathways and mediators of infection, inflammation, and cancer (reviewed in[35,49]). Indeed, a recent study has highlighted the therapeutic potential for specifically targeting Pellino1 in experimental models of sepsis.[52] A number of functional roles for Pellino1 have been reported in terms of TLR signaling. Initially, using a variety of cell types from Pellino1-deficient mice, Pellino1 was shown to positively regulate TLR3- and TLR4-mediated NF-κB activation and

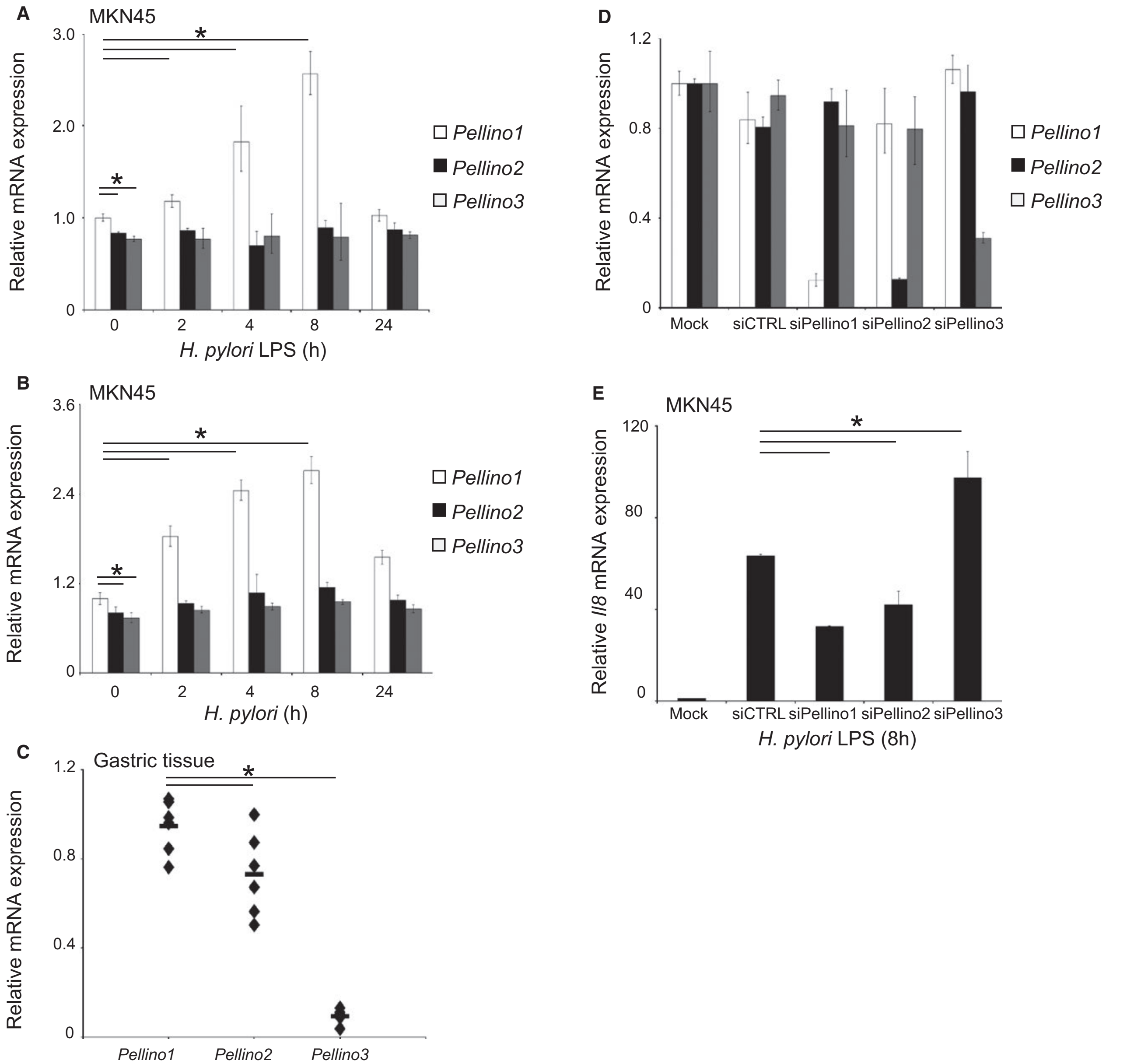

FIGURE 8 Differential expression of Pellino mRNAs in gastric epithelial cell lines and gastric tissue. Quantitative PCR analysis of *Pellino1*, *Pellino2*, and *Pellino3* mRNA expression in MKN45 gastric epithelial cells stimulated with *H. pylori* LPS (*A*) or intact *H. pylori* (*B*) over time. Results are normalized to *Gapdh* mRNA expression and presented relative to *Pellino1* mRNA levels in unstimulated cells (time 0 hour). Quantitative PCR analysis of *Pellino1*, *Pellino2*, and *Pellino3* mRNA expression in biopsy tissue samples from *H. pylori*-negative patients (*C*; N=6). Results are normalized to *Gapdh* mRNA expression and presented relative to *Pellino1* mRNA levels. (*D*) Quantitative PCR analysis of *Pellino1*, *Pellino2*, *and Pellino3* mRNA expression in MKN45 cells following transfection with siCTRL, siPellino1, siPellino2, or siPellino3 for 48 hours. Results are normalized to *Gapdh* mRNA expression expressed relative to untransfected cells (mock). (*E*) Quantitative PCR analysis of *Il8* mRNA expression in MKN45 cells following transfection with siCTRL, siPellino1, siPellino2, or siPellino3 for 48 hours, followed by stimulation with 5 µg/mL *H. pylori* LPS. Results are normalized to *Gapdh* mRNA expression expressed relative to untransfected unstimulated cells (mock). **P*<.05

cytokine induction.[53] Pellino1 deficiency did not significantly affect cytokine induction in response to other TLR ligands, suggesting that Pellino1 was required for proinflammatory gene induction mainly in response to TLR3 and TLR4 stimulation.[53] While TLR3- and TLR4-mediated TRIF-dependent activation of NF-κB was inhibited in Pellino1-deficient cells, no effect on IRF activation or IFN-β expression was observed, implying specificity for Pellino1 in positively regulating the NF-κB axis of TRIF-dependent TLR3 and TLR4 signaling.[53] By contrast, studies using bone marrow-derived macrophages and dendritic cells from a knockin mouse expressing an inactive form of Pellino1 showed normal levels of TLR3- and TLR4-induced NF-κB activation and induction of proinflammatory cytokines, but reduced IFN-β expression.[54] In support of Pellino1 selectively impacting the NF-κB signaling axis in response to TLR3 stimulation, studies using primary human bronchial epithelial cells demonstrated that Pellino1 was required for TLR3 ligand- or rhinovirus-mediated induction of IL-6 and CXCL8, but not IFN-related genes.[55]

Although Pellino1 deficiency did not impact TLR2-or TLR4-mediated cytokine gene induction (*Tnf*, *Il12p40*, and *Il6*) in bone marrow-derived dendritic cells from the Pellino1-deficient mice discussed above,[53] a role for Pellino1 in TLR2- and TLR4-mediated signaling has recently been described in human cell lines. Using overexpression and gene inhibition approaches, Pellino1 was shown to be a positive regulator of NF-κB activation and *Il8* gene induction in transfected HEK-TLR2 and HEK-TLR4 cells in response to Pam_3CSK_4 and *E. coli* LPS, respectively.[56] Furthermore, Pellino1 positively influenced TLR2- and TLR4-mediated induction of both MyD88- and TRIF-dependent cytokine genes in differentiated human THP1 cells. The studies presented herein confirm the positive regulatory role for Pellino1 in terms of NF-κB activation and *Il8* gene induction in HEK-TLR2 cells in response to the synthetic TLR2 ligand Pam_2CSK_4. Additionally, our studies extend this role by providing evidence that Pellino1 enhances NF-kB activation and induction of the *Il8* and *Ccl20* gene promoters in response to *H. pylori* LPS. Pellino1 was also necessary for optimal *Il8* mRNA expression in human MKN45 gastric epithelial cells.

In terms of TLR signaling, the role of Pellino2 has been less well studied and a Pellino2-deficient mouse has not to date been generated. Ectopic expression of an antisense Pellino2 construct inhibited TLR4-mediated activation of *Il8* promoter activity in mouse embryonic fibroblasts.[40] Inhibition of Pellino2 expression in the mouse macrophage cell line Raw 264.7 using siRNA led to decreased TLR4-mediated activation of an NF-kB luciferase reporter construct.[57] In the current study, we did not observe a role for Pellino2 in NF-κB activation with regard to TLR2-mediated signaling in response to either Pam_2CSK_4 or *H. pylori* LPS using two separate NF-κB reporter assays. We did, however, observe a positive regulatory role for Pellino2 in TLR2-driven *Il8* and *Ccl20* reporter gene activity in HEK-TLR2 cells and *Il8* mRNA expression in MKN45 cells. Several elements are involved in the regulation of the *Il8* and *Ccl20* promoters, including NF-κB, AP-1, and C/EBPβ.[58–60] Moreover, it has been shown that activation of both AP-1 and NF-κB is involved in the regulation of *Il8* gene expression in MKN45 cells in response to *H. pylori* infection.[61] Additionally, overexpression of Pellino2 has been demonstrated to activate the ERK and JNK MAPK pathways,[44] and inhibition of Pellino2 expression attenuates TLR4-mediated activation of these kinases and the induction of proinflammatory cytokine genes.[62] As such, it is possible that Pellino2 regulates chemokine expression through pathways involving the MAPK axis of the TLR2-mediated response.

Pellino3 has been reported to modulate both TLR3 and TLR4 signaling. Studies involving Pellino3-deficient mice have elucidated a negative regulatory role for Pellino3 with respect to type I IFN regulation. While cells from the Pellino3-deficient mice were shown to respond normally to a range of TLR ligands with respect to the induction of proinflammatory genes and type I IFNs, Pellino3 deficiency led to enhanced TLR3-induced expression of IFN-β.[63] With regard to TLR4 signaling, a negative regulatory function for Pellino3 has been described whereby low-density lipoprotein inhibits TLR4-mediated IFN-β expression by activating Pellino3.[64] More recently, Pellino3 has been shown to negatively regulate TLR2- and TLR4-mediated cell signaling events in macrophages.[65] Here, we show a negative regulatory role for both isoforms of Pellino3 in terms of NF-κB activation and proinflammatory gene induction during the TLR2-mediated response to *H. pylori* LPS in epithelial cells.

Taken together, our findings indicate that both Pellino1 and Pellino2 positively influence the induction of proinflammatory chemokines in response to *H. pylori* LPS, while Pellino3 has a negative modulatory role. In the context of the growing list of diverse roles for Pellino proteins that is currently emerging, these findings highlight a lack of functional redundancy among the Pellino family members, despite their sequence homology and their common RING-like and FHA structural domains. Although Pellino proteins have many common binding partners, specific protein interactions have also been reported for individual Pellinos, for example, Pellino1 interacts with SMAD6,[52] providing insight into a possible basis for the observed functional diversity. In the context of *H. pylori* infection, the observed differences in regulatory roles are noteworthy, given the differential expression of the Pellino family members in gastric epithelial cells and gastric biopsy tissue. We have shown that Pellino1 is the most abundantly expressed member of the Pellino family, with expression levels tenfold greater than those of Pellino3 in gastric tissue. Although we observed that Pellino1 expression was induced in MKN45 cells in response to *H. pylori* infection and *H. pylori* LPS, we did not detect any increase in Pellino1 in gastric biopsy samples from *H. pylori*-infected patients with chronic gastritis compared to uninfected controls. Using microarray analysis to compare gene expression profiles in patients with premalignant gastric mucosa and gastric cancer, others have shown increased Pellino1 expression in patients with intestinal metaplasia and intestinal gastric cancer compared to those with chronic gastritis.[66] Therefore, it is likely that the degree of Pellino1 induction in infected patients is linked to the virulence of the infecting *H. pylori* strain and host genetic factors, influencing the degree of inflammation and disease progression.

In summary, the combination of low Pellino3 levels together with high and inducible Pellino1 expression may be an important determinant of the level of inflammation triggered upon TLR2 engagement by *H. pylori* and/or its components by directing the incoming signal toward an NF-kB-mediated proinflammatory response. Chronic induction of NF-kB could also contribute to the pathogenesis of *H. pylori*-associated malignancy. Further studies are required to determine whether differential expression and activation of Pellinos could impact on disease severity and outcomes in the pathogenesis of *H pylori*-related disease.

ACKNOWLEDGEMENTS

This work was supported by a Science Foundation Ireland Strategic Cluster award to DPK and PNM. We thank Prof Anthony Moran for providing *H. pylori* LPS, Ms Martina Regan and Ms Aoife Kilkenny for technical assistance, Dr Salah Ahmed, Dr Abdelhafeez Mohamed, and Dr David Prichard for assistance with biopsy collection, Prof Aideen Long for reagents and Dr Aisling Dunne for cell lines.

DISCLOSURES OF INTERESTS

The authors have no conflict of interests to declare.

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