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BRIEF REPORT

Helicobacter pylori-Induced TLR9 Activation and Injury Are Associated With the Virulence-Associated Adhesin HopQ

Samuel D. R. Dooyema,[1,2] Uma S. Krishna,[3] John T. Loh,[3] Giovanni Suarez,[3] Timothy L. Cover,[1,3,4] and Richard M. Peek Jr[1,3]

[1]Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center, Nashville, Tennessee, USA, [2]Microbe-Host Interactions Training Program, Vanderbilt University, Nashville, Tennessee, USA, [3]Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, USA, and [4]Tennessee Valley Healthcare System, United States Department of Veteran Affairs, Nashville, Tennessee, USA

Helicobacter pylori is the strongest risk factor for gastric adenocarcinoma. The *H. pylori* cancer-associated *cag* pathogenicity island (*cag*-PAI) encodes a type IV secretion system (T4SS), which translocates microbial DNA and activates TLR9; however, most *cag*-PAI$^+$–infected persons do not develop cancer and *cag*-PAI–independent regulators of pathogenesis, including strain-specific adhesins, remain understudied. We defined the relationships between *H. pylori* HopQ adhesin allelic type, gastric injury, and TLR9 activation. Type I *hopQ* alleles were significantly associated with magnitude of injury, *cag*-T4SS function, and TLR9 activation. Genetic deletion of *hopQ* significantly decreased *H. pylori*-induced TLR9 activation, implicating this adhesin in *H. pylori*-mediated disease.

Keywords. *Helicobacter pylori*; gastric cancer; HopQ; TLR9; secretion systems.

Helicobacter pylori incurs the highest known risk for developing gastric cancer [1]; however, only 1%–3% of infected individuals develop gastric adenocarcinoma [2]. One strain-specific *H. pylori* oncogenic determinant is the *cag* pathogenicity island (*cag*-PAI), which encodes a type IV secretion system (T4SS). The *cag*-T4SS translocates effectors, such as CagA, peptidoglycan, heptose-bisphosphate, and DNA into epithelial cells [3]. Translocated DNA subsequently activates Toll-like receptor 9 (TLR9) and *H. pylori* strains that confer a higher risk for gastric cancer are more potent in their ability to activate TLR9 [4]. However, the precise molecular mechanisms regulating *H. pylori*-dependent TLR9 activation remain incompletely defined.

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Correspondence: Richard M. Peek Jr, MD, 2215 Garland Avenue, 1030C Medical Research Building IV, Nashville, TN 37232-0019 (richard.peek@vumc.org).

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Most persons colonized with *cag*-PAI$^+$ strains do not develop gastric cancer, raising the hypothesis that other *H. pylori* constituents may also affect disease risk. The outer membrane protein HopQ, which binds human carcinoembryonic antigen-related cell adhesion molecule (CEACAM) receptors, has been reported to facilitate CagA translocation [5, 6]. *H. pylori hopQ* exhibits a high level of diversity and 2 genetically distinct families of *hopQ* alleles (type I and type II) have been previously described [7]. Type I alleles are present significantly more frequently in *cagA*$^+$ versus *cagA*$^-$ strains [7], suggesting that HopQ may represent a microbial component that can regulate DNA translocation and TLR9 activation and play a role in disease.

METHODS

Clinical Specimens

Gastric antral biopsies were collected for culture and immunohistochemistry [8]. Patients were prospectively enrolled after written informed consent and the study was approved by the institutional review boards of Vanderbilt University and the Nashville Department of Veterans Affairs. Histologic parameters were scored from 0 to 3 as outlined by the Sydney System [8].

Bacterial Strains

H. pylori cag-PAI$^+$ strain 26695 (which contains a single type I *hopQ* allele) [9], isogenic mutants, and clinical isolates were maintained on trypticase soy agar plates with 5% sheep blood (Hemostat Laboratories). Allele-specific polymerase chain reactions (PCRs) were used to type *hopQ* [7] and stratify strains into *hopQ* allelic categories based on detection of type I alleles, type II alleles, or both. A kanamycin-resistant 26695 *cagE*$^-$ mutant [4], which lacks functional *cag*-T4SS activity, and a chloramphenicol-resistant 26695 *hopQ*$^-$ mutant (*hopQ*$^-$#1) were previously described [9]. An *H. pylori* 26695 *hopQ* complemented strain was generated by insertion of the *hopQ* gene into the *hp0177/0178* intergenic chromosomal region of *hopQ*$^-$#1. A second independent 26695 *hopQ*$^-$ mutant (*hopQ*$^-$#2) was constructed as previously described by insertional mutagenesis [4]. Additionally, a *hopQ* deletion mutant derivative of strain 7.13 was constructed by inserting chloramphenicol and kanamycin resistance cassettes into the 2 *hopQ* loci [10]. PCR-based typing of clinical isolates was performed for *hopQ* alleles and *cagA* status (Supplementary Table 1) [7, 8]. TLR9 activation, adherence, CagA translocation, and interleukin-8 (IL-8) production assays were performed using the following multiplicities of infection (MOIs): 10:1, 25:1, 50:1, 100:1, and 200:1 (Supplementary Figure 1). Activation with minimal control activation by wild-type *H. pylori* for CagA translocation and IL-8

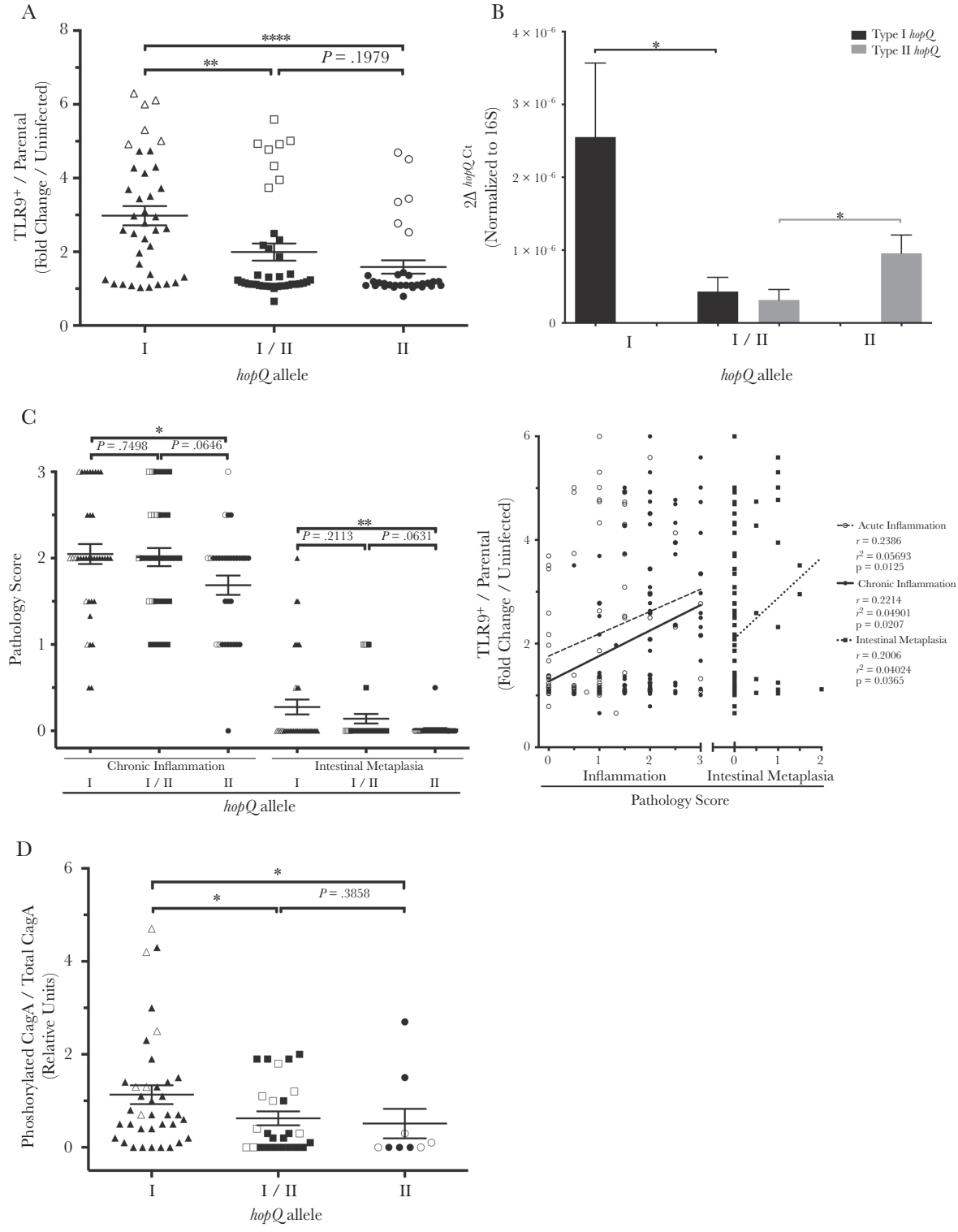

Figure 1. Clinical Helicobacter *pylori* type I *hopQ* strains enhance Toll-like receptor 9 (TLR9) activation and are more virulent than type I/II or type II strains. The highest *H. pylori* responder strains for each *hopQ* allelic category (type I, n = 6; type I/II, n = 8; type II, n = 6) are indicated by open symbols (A); data originating from gastric tissue harvested from patients infected with these same strains (C) and levels of CagA translocation by these same strains (D) are also indicated by open symbols. *A*, TLR9-reporter or parental cells were challenged with clinical *H. pylori* strains. Data are fold change in infected TLR9$^+$ cells/parental cells over uninfected controls. Each strain was tested in duplicate at least 3 times (type I, n = 38; type I/II, n = 39; type II, n = 32). *B*, Expression of either type I *hopQ* or type II *hopQ* was assessed by real-time PCR (RT-PCR) on a random selection of clinical strains (type I, n = 7; type I/II, n = 8; type II, n = 9). $2\Delta^{hopQ\,Ct}$ is the expression level of *hopQ* normalized to the reference gene 16S rRNA. *C*, Inflammation and intestinal metaplasia scores from patients infected with either *H. pylori* type I, type I/II, or type II *hopQ* strains. Each data point represents the score from an individual patient (type I, n = 38; type I/II, n = 39; type II, n = 32). TLR9 activation levels induced by the corresponding infecting *H. pylori* strains (n = 109) are shown as fold change in infected TLR9$^+$ cells/parental cells over uninfected controls. Spearman correlation was performed to determine linear correlation. *D*, AGS cells were co-cultured

induction occurred at an MOI of 50:1 and for adherence and TLR9 activation at an MOI of 100:1.

Real-Time PCR

RNA was extracted using the RNAeasy Mini Kit (Qiagen) from log-phase *H. pylori* cultures. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher) and quantitative real-time PCR was performed using Power SYBR Green Master Mix (ThermoFisher) with gene-specific primers (Supplementary Table 1).

TLR9 and NOD1 Activation Assays

HEK-Blue-hTLR9 cells (TLR9$^+$), HEK-Blue-hNOD1 cells (NOD1$^+$), and HEK-Blue-Null1 (parental) cells were seeded in 96-well plates (Corning) in Dulbecco's Modified Eagle's Medium without antibiotics and challenged with agonist, *H. pylori,* or sterile phosphate-buffered saline for 24 hours. Supernatants were added to QUANTI-Blue solution (Invivogen) and analyzed by spectrophotometer (Bitoek) at 650 nm.

Statistical Analysis

All experiments were repeated at least 3 times. The Mann-Whitney test or student *t* test was used for 2 group comparisons, while 1-way analysis of variance (ANOVA) with Bonferroni correction was used for multiple group comparisons. Statistical significance was set at a *P* value of $< .05$.

RESULTS

We first PCR-genotyped a cohort of clinical strains for *hopQ* allelic type and analyzed their ability to activate TLR9. Type I strains induced significantly higher levels of TLR9 activation compared to type I/II or type II strains (Figure 1A and Supplementary Figure 2). To determine whether gene expression may be associated with these differences, we analyzed expression of *hopQ* alleles in a subset of clinical strains by real-time PCR. Expression of type I *hopQ* was significantly greater in type I strains compared to type I/II strains while expression of type II *hopQ* was significantly higher in type II strains compared to type I/II strains (Figure 1B). To study potential downstream ramifications of these findings in vivo, severity of inflammation and premalignant lesions (eg, intestinal metaplasia) in biopsies obtained from the source patients was stratified by *hopQ* genotypes of the infecting *H. pylori* isolates. Severity of chronic inflammation and intestinal metaplasia were significantly increased in patients infected with type I strains compared to patients infected with type II strains (Figure 1C), while levels of acute inflammation were no different (Supplementary Figure 3). We also directly compared levels of *H. pylori*-induced TLR9 activation in vitro to the severity of gastric inflammation and injury induced by the same strains in vivo. Levels of TLR9 activation were significantly associated, albeit of weaker magnitude, with the intensity of chronic inflammation, intestinal metaplasia, as well as acute inflammation (Figure 1C), suggesting that the capacity of *H. pylori* strains to induce higher levels of TLR9 activation in vitro is related in part to the extent of damage induced by these same strains in vivo. *H. pylori cagA*$^+$ type I *hopQ* strains also translocated significantly higher amounts of CagA per level of total CagA/strain compared to strains containing type I/II alleles or a type II allele; however, there were no significant associations between type I *hopQ* expression levels and pathologic outcomes or levels of CagA translocation (Figure 1D, Supplementary Figure 4, and Supplementary Figure 5). Collectively, these results indicate that *H. pylori* type I strains induce more intense TLR9 activation in vitro and enhanced chronic inflammation and damage in vivo. This may reflect enhanced translocation of CagA, which has been shown to induce inflammation and promote the development of intestinal metaplasia [11].

We next more definitively defined the role of HopQ in TLR9 activation by genetically inactivating *hopQ*. *H. pylori* wild-type strain 26695 robustly induced TLR9 activation when compared to uninfected cells, while a *cagE*$^-$ mutant minimally activated TLR9. TLR9 activation was significantly diminished following co-culture with the *hopQ*$^-$ mutant compared to *H. pylori* wild-type 26695-infected cells, and complementation fully restored levels of TLR9 activation to levels induced by wild-type strain 26695 (Figure 2A). To more strongly implicate *hopQ* allele status in TLR9 activation, we generated a second independent isogenic *hopQ*$^-$ mutant in the *H. pylori* 26695 strain background as well as a double *hopQ* mutant in *H. pylori* strain 7.13, which contains 2 identical copies of type I *hopQ*. Levels of TLR9 activation induced by 26695 *hopQ*$^-$#2 and the 7.13 *hopQ*$^-$ mutant were significantly reduced compared to levels induced by the wild-type strains (Figure 2A). Of note, levels of activation in parental cells infected by either of the wild-type strains were higher than in uninfected parental cells although this was not statistically significant, which may represent residual *cag*-T4SS-dependent but HopQ-independent activation of nuclear factor-κB (NF-κB). Downstream signaling effectors activated by TLR9 include type I interferons (IFN-α and IFN-β). To investigate consequences of TLR9 activation, we co-cultured wild-type *H. pylori* strain 26695 and the 26695 isogenic *hopQ*$^-$ mutant with AGS cells and quantified IFN-α and IFN-β production. Similar to TLR9, wild-type *H. pylori* strain 26695 induced significantly higher levels of type I interferons compared to the 26695 *hopQ*$^-$ mutant (Figure 2B).

with *cagA*$^+$ clinical *H. pylori* strains (n = 71) at multiplicities of infection 50:1 for 4 hours. Levels of translocated CagA were quantified in cell lysates by Western blotting for phosphorylated CagA and total CagA. Data are represented as phosphorylated CagA over total CagA (type I, n = 37; type I/II, n = 25; type II n = 9). Mean ± SEM are shown for all groups. Mann-Whitney tests or student *t* test were used to determine statistical significance between groups $^*P < .05$, $^{**}P < .01$, $^{****}P < .0001$.

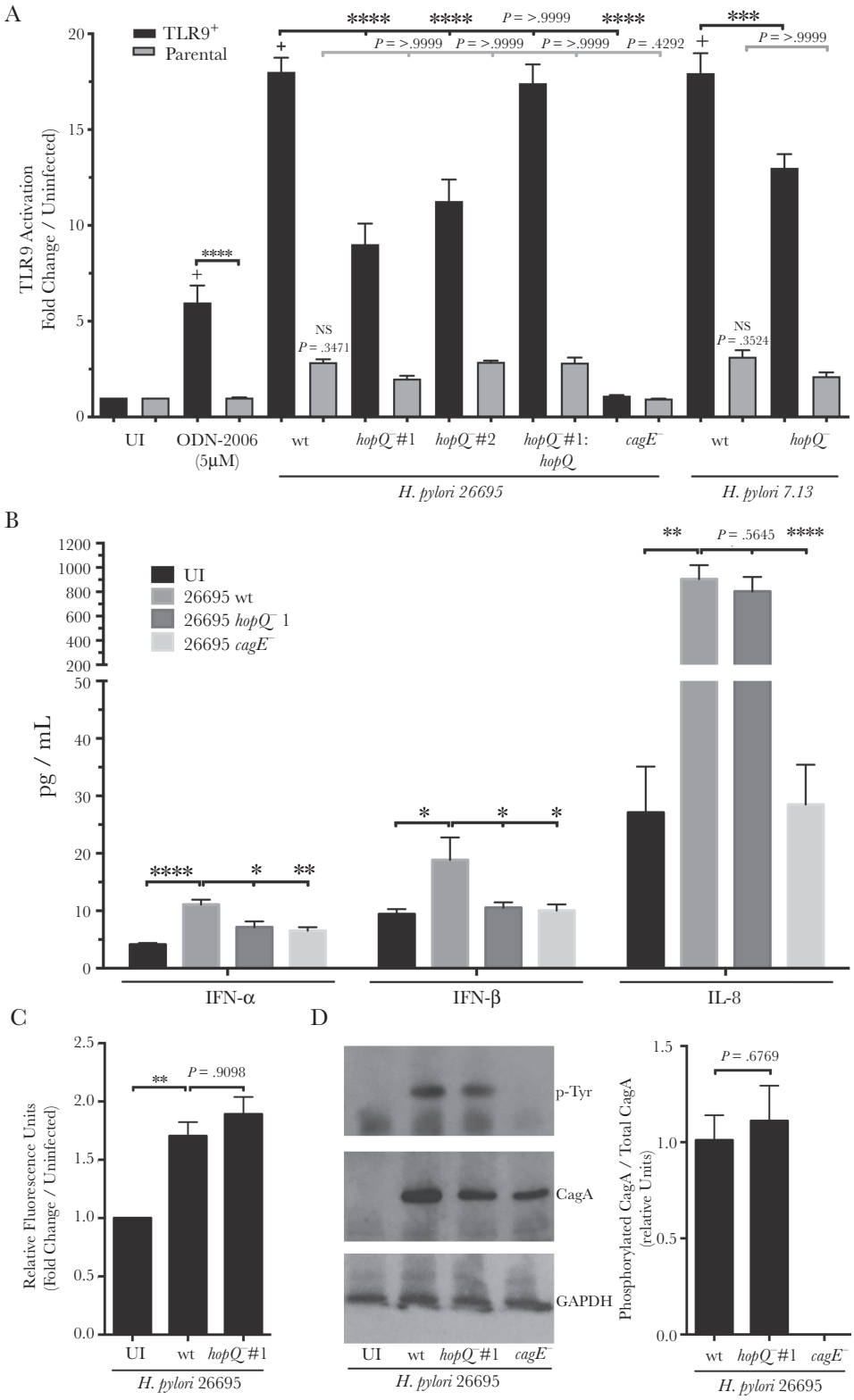

Figure 2. Deletion of *hopQ* significantly decreases TLR9 activation independent of cellular adhesion and *cag*-T4SS function. *A*, TLR9-reporter or parental cells were challenged with TLR9 agonist ODN-2006, Helicobacter *pylori* wild-type *cag*-PAI$^+$ strain 26695, wild-type *cag*-PAI$^+$ strain 7.13, respective *hopQ*$^-$ or *cagE*$^-$ isogenic mutant strains, or a complemented 26695 *hopQ* mutant. Samples were tested in duplicate at least 3 times and data are fold change in infected over uninfected controls. *B*, Levels of IFN-α, IFN-β, and IL-8 were determined via ELISA in *H. pylori*:AGS cell supernatants. In each experiment, strains were tested at least 3 times and mean ± SEM are shown. *C*, Fluorescently labeled *H. pylori* wild-type strain 26695 or a 26695 *hopQ*$^-$ isogenic mutant were co-cultured with AGS cells for 4 hours and analyzed for fluorescence. Strains were tested in duplicate and data are fold change of infected over uninfected control. *D*, CagA translocation was determined by quantifying levels of phospho-CagA in AGS cell lysates during *H. pylori* co-culture by Western blotting. Representative Western blots and densitometric analysis normalizing levels of phosphorylated CagA to total

Because HopQ is a bacterial adhesin, we determined whether reductions in TLR9 activation induced by the *hopQ*$^{-}$ mutant were dependent upon decreased adherence. No differences in binding to AGS cells were identified between the wild-type strain 26695 and the *hopQ*$^{-}$ isogenic mutant (Figure 2C). Soft agar motility assays demonstrated that *H. pylori* parental strain 26695 exhibited similar motility to other *H. pylori* strains included in this study (data not shown). Type I *hopQ* alleles are in linkage disequilibrium with the *cag*-T4SS [7] and specific HopQ-CEACAM interactions have been reported to be required for translocation of CagA into epithelial cells [5, 6]. Thus, to discern whether reductions in TLR9 activation were due to inactivation of *hopQ* per se and not due to concomitant loss of *cag*-T4SS function, we analyzed *cag*-T4SS-associated phenotypes in the *hopQ*$^{-}$ mutant. Both wild-type strains 26695 and 7.13 and their respective *hopQ*$^{-}$ or complemented *hopQ* mutants were similar in their ability to translocate CagA, as determined by levels of phosphorylated CagA per level of total CagA/strain, while, as expected, the *cagE*$^{-}$ mutant failed to translocate CagA (Figure 2D and Supplementary Figure 6). Furthermore, no significant differences in levels of IL-8 production were observed between 26695 and 7.13 wild type, *hopQ*$^{-}$, and *hopQ* complemented mutant-infected samples (Figure 2B and Supplementary Figure 6). There were also no differences between wild-type *H. pylori* strain 26695 and the *hopQ*$^{-}$ mutant in the ability to activate an independent *cag*-T4SS–dependent effector, NOD1 (Supplementary Figure 7).

DISCUSSION

We identified a strain-variable *cag*-PAI–independent *H. pylori* component, HopQ, that is associated with TLR9 activation and is linked to carcinogenic potential. There are several potential mechanisms that may underpin these observations. Structural analyses comparing type I HopQ to type II HopQ proteins have revealed a differential ability to bind specific CEACAMs [12]. Type I HopQ harbors a higher affinity for human CEACAM1 versus CEACAM6, raising the possibility that HopQ-CEACAM1 interactions are necessary for translocation of microbial DNA and TLR9 activation. Of interest, *H. pylori cagA*$^{+}$ strains induce higher levels of CEACAM expression than *cagA*$^{-}$ strains, and TLR9-regulated transcription factors such as NF-κB and activator protein *1* (AP-1) are linked to *H. pylori* infection and CEACAM regulation [5]. Although further studies linking discrete cell signaling cascades to specific HopQ alleles and CEACAMs will be required, *H. pylori* has likely evolved to harbor different alleles of *hopQ* that may confer selective binding and molecular signaling capacities.

Our current results also indicate that *cag*-T4SS function in *H. pylori* strains 26695 and 7.13 is not dependent on HopQ. Some, but not all, previous studies have demonstrated a more direct role for HopQ in *cag*-PAI functions [5, 6]. However, these studies utilized independent *H. pylori* strains in different cell models under different conditions compared to our current study, which may account for the varying results.

We recognize that not all of the in vitro and in vivo data are fully aligned and speculate that the lack of absolute concordance represents fundamental differences that exist between the reductionist in vitro TLR9 activation assay and the in vivo milieu colonized by *H. pylori*. For example, varying expression levels of CEACAM proteins may be present within different patient samples, which may alter HopQ function. Further, other microbial constituents and host signaling pathways, such as peptidoglycan and NOD1 as well as heptose-bisphosphate and NF-κB, have been shown to affect inflammation in vivo [3]. The complexity of such interactions is heightened when comparing results from human tissue to rodent tissue. In humans, genetic polymorphisms within TLR9 have been linked to *H. pylori* persistence [13]. We previously demonstrated that *H. pylori* strains harvested from persons at increased risk for gastric cancer activated TLR9 more robustly than strains isolated from patients residing in a low-risk cancer region [4]. These data are consistent with the current results and with data from Qin et al demonstrating that *H. pylori* and *H. pylori* DNA induce TLR9-dependent proliferation, migration, and invasion of human gastric epithelial cells [14]. However, our group also reported enhanced inflammation in *H. pylori*-infected *tlr9*$^{-/-}$ mice when compared to *H. pylori*-infected wild-type mice [15]. We speculate that this discordance may be related to the duration of infection (years in humans, weeks in mice) as well as inherent differences in host responses to microbial pathogens across species, which for TLR9 carries increased complexity because activation of TLR9 can lead to either pro- or anti-inflammatory responses in vivo depending on cellular context.

Collectively, the current results aid in delineating the route by which microbial DNA is delivered to host cells, and also may reveal the impact that DNA translocation has on carcinogenesis in vivo.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the

CagA from 3 replicates are shown. GAPDH served as a loading control. ANOVA with Bonferroni correction or student *t* test was used to determine statistical significance between groups. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$; + $P < .00001$ compared to uninfected TLR9^{+} cells; NS, nonsignificant compared to uninfected parental cells. Abbreviations: ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IL-8, interleukin-8; TLR9, Toll-like receptor 9; UI, uninfected; wt, wild type.

authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Amieva M, Peek RM Jr. Pathobiology of *Helicobacter pylori*-induced gastric cancer. Gastroenterology **2016**; 150:64–78.
2. Wroblewski LE, Peek RM Jr, Wilson KT. *Helicobacter pylori* and gastric cancer: factors that modulate disease risk. Clin Microbiol Rev **2010**; 23:713–39.
3. Backert S, Haas R, Gerhard M, Naumann M. The *Helicobacter pylori* type IV secretion system encoded by the *cag* pathogenicity island: architecture, function, and signaling. Curr Top Microbiol Immunol **2017**; 413:187–220.
4. Varga MG, Shaffer CL, Sierra JC, et al. Pathogenic *Helicobacter pylori* strains translocate DNA and activate TLR9 via the cancer-associated cag type IV secretion system. Oncogene **2016**; 35:6262–9.
5. Javaheri A, Kruse T, Moonens K, et al. *Helicobacter pylori* adhesin HopQ engages in a virulence-enhancing interaction with human CEACAMs. Nat Microbiol **2016**; 2:16189.
6. Zhao Q, Busch B, Jiménez-Soto LF, et al. Integrin but not CEACAM receptors are dispensable for *Helicobacter pylori* CagA translocation. PLoS Pathog **2018**; 14:e1007359.
7. Cao P, Cover TL. Two different families of hopQ alleles in *Helicobacter pylori*. J Clin Microbiol **2002**; 40:4504–11.
8. Peek RM Jr, van Doorn LJ, Donahue JP, et al. Quantitative detection of *Helicobacter pylori* gene expression in vivo and relationship to gastric pathology. Infect Immun **2000**; 68:5488–95.
9. Loh JT, Torres VJ, Algood HM, McClain MS, Cover TL. *Helicobacter pylori* HopQ outer membrane protein attenuates bacterial adherence to gastric epithelial cells. FEMS Microbiol Lett **2008**; 289:53–8.
10. Loh JT, Beckett AC, Scholz MB, Cover TL. High-salt conditions alter transcription of *Helicobacter pylori* genes encoding outer membrane proteins. Infect Immun **2018**; 86:e00626-17.
11. Yokoyama K, Higashi H, Ishikawa S, et al. Functional antagonism between *Helicobacter pylori* CagA and vacuolating toxin VacA in control of the NFAT signaling pathway in gastric epithelial cells. Proc Natl Acad Sci U S A **2005**; 102:9661–6.
12. Moonens K, Hamway Y, Neddermann M, et al. *Helicobacter pylori* adhesin HopQ disrupts trans dimerization in human CEACAMs. EMBO J **2018**; 37:e98665.
13. Loganathan R, Nazeer M, Goda V, et al. Genetic variants of TLR4 and TLR9 are risk factors for chronic *Helicobacter pylori* infection in South Indian Tamils. Hum Immunol **2017**; 78:216–20.
14. Qin XR, Wu J, Yao XY, Huang J, Wang XY. *Helicobacter pylori* DNA promotes cellular proliferation, migration, and invasion of gastric cancer by activating toll-like receptor 9. Saudi J Gastroenterol **2019**; 25:181–7.
15. Varga MG, Piazuelo MB, Romero-Gallo J, et al. TLR9 activation suppresses inflammation in response to *Helicobacter pylori* infection. Am J Physiol Gastrointest Liver Physiol **2016**; 311:G852–8.