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**Helicobacter pylori Infection Interferes with Epithelial Stat6-Mediated Interleukin-4 Signal Transduction Independent of cagA, cagE, or VacA**

Peter J. M. Ceponis,*† Derek M. McKay,§ Rena J. Menaker,‡ Esther Galindo-Mata,* and Nicola L. Jones²‡*

*Helicobacter pylori* is a bacterial pathogen evolved to chronically colonize the gastric epithelium, evade immune clearance by the host, and cause gastritis, peptic ulcers, and even gastric malignancies in some infected humans. In view of the known ability of this bacterium to manipulate gastric epithelial cell signal transduction cascades, we determined the effects of *H. pylori* infection on epithelial IL-4-Stat6 signal transduction. HEp-2 and MKN45 epithelial cells were infected with *H. pylori* strains LC11 or 8823 (type 1; cagA⁺/cagE⁺/VacA⁺), LC20 (type 2; cagA⁻, cagE⁻, VacA⁻), and cagA, cagE, and vacA isogenic mutants of strain 8823, with some cells receiving subsequent treatment with the Th2 cytokine IL-4, a known Stat6 activator. Immunofluorescence showed a disruption of Stat6-induced nuclear translocation by IL-4 in LC11-infected HEp-2 cells. IL-4-inducible Stat6 DNA binding in HEp-2 and MKN45 cells was abrogated by infection, but MKN45 cell viability was unaffected. A decrease in IL-4-mediated Stat6 tyrosine phosphorylation in nuclear and whole cell lysates was observed following infection with strains LC11 and LC20, while neither strain altered IL-4 receptor chain α or Janus kinase 1 protein expression. Furthermore, parental strain 8823 and its isogenic cagA, cagE, and vacA mutants also suppressed IL-4-induced Stat6 tyrosine phosphorylation to comparable degrees. Thus, *H. pylori* did not directly activate Stat6, but blocked the IL-4-induced activation of epithelial Stat6. This may represent an evolutionarily conserved strategy to disrupt a Th2 response and evade the host immune system, allowing for successful chronic infection.


Infection of the stomach with pathogenic Gram-negative *Helicobacter pylori* is associated with gastritis, peptic ulcer disease, and an increased risk of developing gastric malignancies in humans (1, 2). Current evidence suggests that modulation of host gastric epithelial cell signal transduction responses contributes to pathogenesis of disease (1–3).

Knowledge of *H. pylori* virulence factors, and the epithelial cell signal transduction responses the bacterium modulates, is accumulating but remains incomplete. Some strains of this bacterium harbor the cag pathogenicity island, a horizontally acquired 40-kb segment of DNA that encodes putative virulence factors capable of altering host cell signaling (1). For example, *H. pylori* infection leads to activation of NF-κB and subsequent IL-8 secretion from epithelial cells, illustrating that infection functionally affects host cell transcription factors through direct activation. Bacterial- mediated epithelial IL-8 secretion is dependent on cagE (6, 7), a gene that putatively codes for a component of the *H. pylori* type IV secretion system which is also encoded on the cag pathogenicity island (1). *H. pylori* infection also modulates cytokine signaling.

For example, type IV secretion system-dependent injection of the bacterial protein CagA into the host cell elicits elongation and spreading of gastric epithelial cells through interaction with the cytosolic phosphatase Src homology protein-2 (8). In contrast, *H. pylori* effects can also be independent of genes carried on the cag island, such as degradation of host-derived l-arginine by arginase, the product of the rocF gene, to decrease host production of NO that is capable of killing *H. pylori* (9).

The Janus kinase (Jak) and Stat (Jak/Stat) signal transduction pathway transmits signals from the cell surface to the nucleus to mediate the intracellular effects of many cytokines (10). For example, the Th2-type cytokine IL-4 binds to its cell surface IL-4Rα chain, and either the γ-chain or an IL-13Rα-chain, on most cell types to cause receptor dimerization and subsequent activation of intracellularly associated Jak1 & Jak2 proteins (11). The Jakks then phosphorylate tyrosine residues on the cytoplasmic tail of the receptor, allowing monomeric, cytoplasmic Stat6 proteins to dock via their Src homology 2 domain. Stat6 then becomes tyrosine phosphorylated by the Jakks, dissociates, dimerizes through a reciprocal Src homology 2-domain-phosphotyrosine interaction and translocates to the nucleus to bind DNA and modulate transcription (10, 11). The protective role of Stat6 expression against endotoxemia (12) and parasitic infection (13) highlights its importance in immunity.

Current evidence shows that some microbes disrupt cytokine function by preventing cytokine release, down-regulating cytokine

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Abbreviations used in this paper: Jak, Janus kinase; MOI, multiplicity of infection; EGF, epidermal growth factor.

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receptor expression (14) and disrupting cytokine second messengers (15). Evidence from our laboratory indicates that Stat6 knock-out mice exhibit significantly less gastric colonization following infection with the murine adapted H. pylori strains SSI compared to those with their wild-type littermates (16). Therefore, the aim of the present study was to determine whether H. pylori infection of epithelial cells directly or indirectly modulates Stat6 signaling in vitro.

Materials and Methods

**Epithelial cell culture**

HEP-2 cells have been used as a model epithelium for H. pylori modulation of host cell signal transduction (17, 18) and vacuolating cytotoxin (VacA) treatment (19). The transformed human laryngeal HEP-2 cell line was cultured in MEM supplemented with 15% FBS, 2.5% penicillin-streptomycin, 1.8% sodium bicarbonate, 1.2% Fungizone (all Life Technologies, Grand Island, NY) (20). The gastric adenocarcinoma epithelial cell line MKN45 was cultured in RMPI 1640 supplemented with 10% FBS (21) plus 2% penicillin-streptomycin. For whole cell or nuclear protein extractions, cells were grown to confluency on 6-cm Petri dishes (Falcon; BD Labware, Franklin Lakes, NJ) at 37°C in a 5% CO2 atmosphere. Before bacterial infection, cytokine stimulation, and protein extraction, cells were incubated in antibiotic-free medium containing 1% FBS for 20 h at 37°C. For Stat6 immunofluorescence experiments, HEP-2 cells (~1 × 106) were grown in Labtek four-well chamber slides (Miles Scientific, Naperville, IL) for 6 h in regular medium, then overnight in 1% FBS/antibiotic-free medium.

**Growth of bacteria and conditions of infection**

H. pylori strains used in this study included LC11 (cagA+, cagE+, VacA−), LC20 (cagA+, cagE−, VacA−) (22), the murine adapted strain SSI (cagA+, cagE+, VacA−; Refs. 16 and 17), 8823 (type I: cagA−, cagE−, vacA−), and its isogenic cagA, cagE, and vacA mutants (6). Before infection of epithelial cells, H. pylori strains were cultured on 5% sheep blood-agar plates under microaerophilic conditions at 37°C for 3 days. Bacteria were then transferred to Brucella broth (Difco, Detroit, MI) supplemented with 10% FBS, vancomycin, and trimethoprim, and grown overnight with shaking (120 rpm) under microaerophilic conditions (22). Isogenic mutants of strain 8823 were cultured with supplemental 20 μg/ml kanamycin on plates and in broth (6). One milliliter of bacteria from over-night growth in broth was pelleted, washed, and resuspended in PBS to measure growth spectrophotometrically at 550 nm, where an OD of 1 = 2 × 10^6 bacteria/ml. Before infection, bacteria were washed and resuspended in a total volume of 50 μl of tissue culture medium. Host cells were then infected at a multiplicity of infection (MOI) of 100 bacteria:1 eukaryotic cell, unless otherwise noted. The volume of medium alone served as a vehicle control. Following bacterial infection, cells were rinsed three times with room temperature PBS and the remaining adherent cells were stimulated with either medium alone or medium containing recombinant human IL-4 (50 ng/ml for MKN45 cells, 100 ng/ml for HEP-2 cells; 30 min) (Sigma-Aldrich, Oakville, Ontario, Canada).

**Immunofluorescence**

HEP-2 cells were washed three times with ice-cold PBS, fixed in 4% paraformaldehyde for 30 min, permeabilized for 4 min with 1% Triton X-100 and blocked for 30 min in 2% BSA/0.1% Triton X-100 (23). Cells were then probed for Stat6 with 1/100 anti-Stat6 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) in 2% BSA/0.1% Triton X-100 overnight at 4°C, washed in PBS, incubated with 1/100 rhodamine-red goat anti-rabbit secondary Ab (The Jackson Laboratory, Bar Harbor, ME) in BSA/Triton X-100 for 1 h at room temperature, followed by washing in PBS. Vectashield (Vector Laboratories, Burlingame, CA) mounting medium for fluorescence was added, slides were sealed with coverslips and then examined under immunofluorescence (Leitz Dialux 22; Leica, Willowdale, Ontario, Canada).

**EMSA**

Nuclear protein extracts were collected by the method of Andrews and Fuller (24), with the following supplements added to the extraction buffers: 15 μg/ml aprotinin, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, and 20 μg/ml PMSF (all obtained from Sigma Aldrich) (25). Extracts were stored at −70°C until the protein concentration of each sample was determined using the Bio-Rad assay (Hercules, CA).

EMSAs were performed as described previously (25). Briefly, 20 μg of nuclear protein extract were mixed with binding buffer (250 μM Tris-Cl (pH 7.5), 40 mM NaCl, 10 mM EDTA (pH 8), 2.5 mM DTT, 10 μM spermidine, 5% autocolaved dH2O and 25% glycerol) and incubated with ~3 × 10^15 cpm of (α-32P)PCTP (NEN Life Sciences, Boston, MA) and labeled double-stranded oligonucleotides bearing a Stat6 binding sequence (25, 26) for 20 min at room temperature. Indicator dye (0.25% w/v bromophenol blue; 5% w/v glycerol) was added to each sample followed by electrophoresis through a 5% polyacrylamide gel (32). Lysates containing 1.25% (v/v) glycerol, 0.7% ammonium persulfate (w/v), 0.05% (v/v) N,N,N’,N’-tetramethylethylenediamine, and 1.25% (v/v) Triton tris-borate-EDTA (10× TBE: 89 mM Tris borate, 2 mM EDTA, pH 8) at 100 V for 3 h at room temperature in 0.25× TBE buffer. Gels were then dried and visualized by autoradiography using Kodak Biomax MR Film (Rochester, NY). Identification of the Stat6 band was accomplished by incubation with a Stat6 Ab (Santa Cruz Biotechnology) and competition with a nonradiolabeled Stat6 DNA probe. Incubation with a Stat1 Ab and a nonradiolabeled Stat1 DNA probe served as negative controls.

**Coomassie-brilliant blue staining**

To verify equal protein loading of nuclear protein on EMSA, 20 μg of nuclear protein extracts was mixed with 2× SDS-PAGE loading buffer and electrophoresed through a 7.5% Tris-HCl SDS-PAGE (111 V for 1.5 h, room temperature). Protein levels in the gel were subsequently visualized by staining with 0.025% Coomassie brilliant blue according to established methods (27).

**Immunoblotting**

For whole cell protein extraction, epithelial cell monolayers were washed three times with ice-cold PBS, scraped with a rubber policeman in 1 ml of PBS and pelleted (12,000 rpm, 10 s). The cell pellet was then resuspended in 100 (for MKN45 cells) or 150 (for HEP-2 cells) μl of RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) supplemented with 50 mM NaF, 150 mM NaCl, 1 mM Na3VO4, 20 μg/ml PMSF, 15 μg/ml aprotinin, 2 μg/ml pepstatin A, and 2 μg/ml leupeptin by vortexing, and left at 4°C for 30 min. Subsequently, the lysate was centrifuged (12,000 rpm) in a Sorvall SS-34 rotor (Mandell Scientific, Guelph, Ontario, Canada), 10 min, 4°C and the supernatant was stored at −70°C as the whole cell protein extract (28).

An equal volume of whole cell protein extract was added to 2× SDS-PAGE loading buffer and boiled for 5 min. Alternatively, equal amounts of nuclear protein (20 μg) were adjusted to a 20-μl total volume with 2× buffer, then boiled. Samples were then electrophoresed through a 6% (IL-4Ra) or 7.5% Tris-HCl SDS-PAGE (111 V for 1.5 h, room temperature). Subsequently, proteins were electrophoretically transferred onto a nitrocellulose membrane (BioTrace NT; Pall Corporation, Ann Arbor, MI), at 4°C and 100 V for 1.25 h, and then blocked with 5% low-fat milk, 0.05% Tween 20 in Tris-buffered saline (TBST-M), for 30 min at room temperature. Membranes were probed overnight with either anti-phosphotyrosine Stat6 Ab (936/1); Cell Signaling Technology (New England Biolabs, Pickering, Ontario, Canada); 1/1000), anti-Jak1 (936/4; Santa Cruz Biotechnology; 1/1000), or anti-IL-4Ra (C-20; Santa Cruz Biotechnology; 1/1000), anti-Jak1 (1/400; BioSource International (Camarillo, CA); 1/800), or anti-actin (I-19; Santa Cruz Biotechnology; 1/1000) in TBST-M with shaking at 4°C. Membranes were then washed in TBST, rinsed in dH2O, probed with HRP-conjugated donkey anti-rabbit or goat Ab (Santa Cruz Biotechnology; 1/1000-1/4000) for 1.5 h at room temperature, washed in TBST, and rinsed in dH2O. Bands were visualized by chemiluminescence (Western blotting Luminol Reagent; Santa Cruz Biotechnology) using Kodak Biomax MR Film.

**Determination of epithelial cell viability by fluorescent dye staining**

Acridine orange is a cell-permeable dye that intercalates DNA in viable cells to appear green, whereas ethidium bromide only enters nonviable cells with disrupted membrane integrity to bind RNA and dsDNA and appear orange. Thus, visualization of MKN45 cells stained with an acridine orange-ethidium bromide mixture under UV light allowed for determination of viability based on membrane integrity. In this manner, early and late stages of apoptosis as well as necrosis are determined by the differential uptake and binding of the dyes (22, 28, 29).

MKN45 cells were infected with H. pylori strains LC11, LC20, or 8823 (MOI 100, 1.6 h) and rinsed three times with room temperature PBS. The remaining adherent cells were trypsinized, pelleted by centrifugation, and resuspended in 1 ml of antibiotic-free medium containing 1% FCS. As a positive control to ensure this assay could detect nonviable MKN45 cells, a rubber policeman was used to scrape MKN45 cells from the Petri dish. Next, acridine orange-ethidium bromide in PBS (100 μg/ml) was added to a 0.1-ml cell suspension and a drop of the suspension was applied to a
microscope slide. Viable, necrotic, and apoptotic cells were scored by counting 500 cells at multiple randomly selected fields under a Leitz Dialux 22 microscope (Leica). The percentage of viable cells was then calculated and statistically analyzed by ANOVA.

Results

IL-4-induced nuclear translocation of Stat6 is prevented by H. pylori infection

IL-4 stimulation leads to the nuclear translocation of Stat6, where it binds DNA, in several cell types (10, 11). In this study, immunofluorescence revealed that Stat6 resides mainly in the cytoplasm of unstimulated HEp-2 cells (Fig. 1A). Following IL-4 stimulation (100 ng/ml, 30 min), Stat6 is identified mainly in the nucleus of HEp-2 cells (Fig. 1B). Infection with H. pylori strain LC11 for either 30 min (Fig. 1C) or 4 h (Fig. 1E) did not result in nuclear translocation of Stat6. In contrast, infection for 4 h (Fig. 1F), but not 30 min (Fig. 1D), blocked nuclear translocation of Stat6 when cells were subsequently stimulated with IL-4. These findings indicate that H. pylori infection blocks Stat6 from reaching the nucleus after IL-4 stimulation, in a time-dependent manner.

FIGURE 1. H. pylori infection prevents the IL-4-induced nuclear translocation of Stat6 in HEp-2 cells in a time-dependent manner. HEp-2 cells were grown on coverslips overnight, serum-starved in antibiotic-free medium, and then infected with H. pylori strain LC11 (MOI 100:1) for either 30 min or 4 h. Bacteria were washed away (three PBS washes, room temperature) and remaining adherent cells received medium alone or medium containing IL-4 (100 ng/ml, 30 min). Cells were fixed, permeabilized, blocked, and subsequently immunostained with rabbit anti-human Stat6 polyclonal Ab. This was followed by rhodamine red goat anti-rabbit Ab staining and visualization by immunofluorescence microscopy to determine Stat6 subcellular localization. A, HEp-2 cells receiving medium only; B, IL-4; C, LC11 (30 min); D, LC11 (30 min) followed by IL-4 stimulation; E, LC11 (4 h); F, LC11 (4 h) followed by IL-4 stimulation (n = 2).
H. pylori infection prevents IL-4-induced Stat6 DNA binding

Nuclear translocation of Stat6 induced by IL-4 is paralleled by its DNA-binding ability. Therefore, we analyzed epithelial nuclear protein extracts by EMSA with a DNA probe specific for Stat6 binding activity in the presence and absence of H. pylori infection and IL-4 stimulation. As shown in Fig. 2A, IL-4 stimulation (50 ng/ml, 30 min) leads to Stat6 DNA binding in gastric epithelial MKN45 cells. The identity of the band as Stat6 was confirmed by the ability of anti-Stat6, but not anti-Stat1, Ab to supershift the band. Furthermore, preincubation of the nuclear extract with a nonradiolabeled Stat6 DNA probe (cold competitor) before addition of the 32P-labeled Stat6 probe competed for the band of interest. In contrast, a Stat1 probe did not compete, indicating binding specificity of the probe for the band of interest. Infection of MKN45 cells with strains LC11 and LC20 (6 h, MOI 100:1) did not activate Stat6 DNA binding (Fig. 2B, upper panel). However, infection with both strains LC11 and LC20 prevented subsequent induction of Stat6 DNA binding by IL-4 (Fig. 2B, upper panel). The lower panel of Fig. 2B shows a Coomassie-stained SDS-PAGE gel of extracts analyzed in the upper panel by EMSA to illustrate that equal protein loading, as determined by protein assay, was achieved on EMSA.

Inhibition of IL-4-induced Stat6 DNA binding after H. pylori infection was confirmed in HEp-2 cells, where Stat6 DNA binding was induced in IL-4-treated, but not unstimulated, cells (Fig. 2C). Four hours of infection with strain LC11 (MOI 100:1) did not activate Stat6 DNA binding. However, infection did abrogate subsequent IL-4 stimulation of Stat6 DNA binding (Fig. 2C). The murine adapted strain SS1 also inhibited IL-4-induced Stat6 DNA binding in HEp-2 cells (data not shown).

H. pylori infection abrogates IL-4-induced tyrosine phosphorylation of Stat6

Tyrosine phosphorylation of Stat proteins is generally required for translocation to the nucleus and DNA binding (10, 11). Fig. 3 shows a lack of Stat6 tyrosine phosphorylation in nuclear extracts obtained from unstimulated HEp-2 cells. In contrast, IL-4 stimulation (100 ng/ml, 30 min) results in activation as demonstrated by tyrosine phosphorylation of Stat6. Infection with H. pylori strain LC11 (4 h, MOI 100:1) alone had no effect on nuclear levels of tyrosine phosphorylated Stat6. However, infection diminished IL-4-stimulated Stat6 tyrosine phosphorylation (Fig. 3). Thus, the decreased DNA-binding ability of Stat6 in nuclear extracts shown in Fig. 2 by EMSA correlated with a decreased level of Stat6 tyrosine phosphorylation.

To determine whether H. pylori infection decreased overall levels of IL-4-induced Stat6 tyrosine phosphorylation or just prevented the IL-4-induced nuclear translocation of Stat6, whole cell protein lysates from noninfected and infected epithelial cells ± IL-4 stimulation were analyzed. Fig. 4A shows that the gastric epithelial cell line MKN45 displayed inducible Stat6 tyrosine phosphorylation with IL-4 stimulation (50 ng/ml, 30 min). Neither strain LC11 nor LC20 infection alone for 6 h (MOI 100:1) induced Stat6 tyrosine phosphorylation. However, infection with strain LC11 abrogated IL-4-induced Stat6 tyrosine phosphorylation to a greater extent than LC20. The effects of strain LC11 on Stat6 activation were confirmed in Fig. 4B. HEp-2 cell Stat6 tyrosine phosphorylation was not constitutive, but was inducible following IL-4 stimulation (100 ng/ml, 30 min). Infection with strain LC11 (MOI 100:1, 4 h) had no effect on Stat6 in unstimulated cells, but did...
H. pylori infection abrogates IL-4-induced Stat6 tyrosine phosphorylation. Collectively, these results suggest that H. pylori infection prevents nuclear translocation of Stat6, potentially by down-regulating IL-4-induced Stat6 tyrosine phosphorylation throughout the cell.

Inhibition of IL-4-Stat6 signaling is not due to H. pylori-induced MKN45 cell death

To determine whether decreased epithelial viability was responsible for the decrease in IL-4-induced Stat6 activation in MKN45 cells, cell death was assayed by fluorescent staining with acridine orange/ethidium bromide. These results are displayed in Table I, showing that MKN45 cells in medium alone were 95.5% viable. As a positive control to ensure this assay could detect nonviable MKN45 cells, a rubber policeman was used to scrape MKN45 cells from the Petri dish, which induced a statistically significant amount of cell death (24.8% viable; p < 0.0001 by ANOVA). However, cells infected with H. pylori strains LC11, LC20, or 8823 (MOI 100:1, 6 h) were as viable as cells receiving medium only (Table I). Thus, at the same time point when inhibition of Stat6 signaling was observed, H. pylori infection had no effect on the viability of MKN45 cells.

LC11 inhibition of IL-4-induced Stat6 tyrosine phosphorylation is dose-dependent

Next, we determined the dose-dependent kinetics behind the H. pylori strain LC11 abrogation of IL-4-induced Stat6 tyrosine phosphorylation. Infection with strain LC11 for 6 h prevented the IL-4-induced tyrosine phosphorylation of Stat6 at MOIs of 250:1 and 100:1 (Fig. 5). An MOI of 20:1 with strain LC11 minimally reduced IL-4-mediated Stat6 activation (Fig. 5).

Table I. H. pylori infection does not decrease MKN45 cell viability after 6 h of infection

<table>
<thead>
<tr>
<th>MKN45 Cell Treatment</th>
<th>% Viable Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>95.5 ± 0.9</td>
</tr>
<tr>
<td>Scraping</td>
<td>24.8 ± 7.6*</td>
</tr>
<tr>
<td>LC11</td>
<td>96.2 ± 1.7</td>
</tr>
<tr>
<td>LC20</td>
<td>97.8 ± 1</td>
</tr>
<tr>
<td>8823</td>
<td>96.8 ± 1.1</td>
</tr>
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</table>

*Confluent MKN45 cells in 6-cm Petri dishes were serum-starved overnight, infected with H. pylori strains LC11, LC20, or 8823 (MOI 100:1, 6 h), and then rinsed in PBS. Adherent cells were then trypsinized, pelleted by centrifugation, and scored for viable, necrotic, and apoptotic cells by the acridine orange/ethidium bromide fluorescent dye staining technique; n = 2–3.

H. pylori strain 8823 disrupts IL-4-induced Stat6 tyrosine phosphorylation independent of cagA, cagE, and vacA

We observed a difference between the ability of the type I strain LC11 and type II strain LC20 to disrupt IL-4-induced Stat6 tyrosine phosphorylation. Therefore, to determine the potential bacterial factor responsible for these findings, whole cell lysates from MKN45 cells infected with the parental type I strain 8823 and its isogenic cagA, cagE, and vacA mutants ± IL-4 stimulation (50 ng/ml, 30 min) were analyzed by immunoblotting for Stat6 tyrosine phosphorylation. Fig. 6 shows that infection with the parental strain 8823 (MOI 100:1, 6 h) inhibited IL-4-induced Stat6 phosphorylation. Furthermore, the cagA, cagE, and vacA mutants also inhibited Stat6 activation. These results indicate that type I H. pylori suppresses IL-4-Stat6 signaling independent of the presence of cagA, cagE, and vacA.

H. pylori infection does not alter expression of either IL-4R-α or Jak1 protein

Expression of both the IL-4R-α chain and its associated Jak1 protein are necessary for IL-4-induced activation of Stat6 (10, 11). Fig. 7 shows that infection with H. pylori strains LC11 and LC20 (MOI 100:1, 6 h) did not alter the expression levels of either the IL-4R-α chain or Jak1 compared with uninfected controls in MKN45 whole cell protein lysates. Actin levels demonstrate approximately equivalent protein loading. These results suggest that infection with either a type I or type II H. pylori strain does not disrupt IL-4 activation of Stat6 at the proximal aspect of the pathway.
and primary human bronchial epithelial cells (32) are known to indicating that epithelial Stat proteins play a central role in epi-crucial to elicit airway hyperreactivity and mucus production (31), that IL-13-induced Stat6 activation in airway epithelial cells was on B cells (30). It was recently shown in a murine model of asthma expression of immune molecules such as MHC class II and CD23 involved in the development of a Th2 phenotype in T cells and expression of cellular machinery involved in negative regulation of H. pylori

**FIGURE 7.** *H. pylori* infection does not alter expression of either IL-4Rα or Jak1 proteins. Confluent MKN45 cells grown on 6-cm Petri dishes were infected with the type I *H. pylori* strain LC11 or type II strain LC20 (MOI 100:1, 6 h), rinsed with cold PBS, and whole cell protein was extracted from the adherent cells for immunoblot analysis of IL-4Rα and Jak1. Actin levels were also monitored to demonstrate equivalent protein loading among samples (n = 3).

**Discussion**

As transcription factors, the DNA-binding ability of Stat proteins is crucial to their function. Our results demonstrate for the first time that IL-4 stimulation of MKN45 gastric epithelial cells acti-vates Stat6 as assessed by immunoblotting and EMSA, and con-firm this in a second epithelial cell line, HEP-2. Furthermore, we show that *H. pylori* did not affect MKN45 epithelial cell viability, but did prevent the IL-4 induction of Stat6 DNA binding after 6 h of infection. Decreased DNA binding correlated with decreased tyrosine phosphorylation of Stat6 in nuclear and whole cell protein extracts, indicating that overall Stat6 phosphorylation was affected and that the decreased DNA binding was not due strictly to defec-tive nuclear translocation. The effect on Stat6 tyrosine phos-phorylation was more prominent following infection with the type 1-*H. pylori* strain LC11, but was independent of the expression of cagA, cagE, and vacA. Neither IL-4Rα nor Jak1 protein expression were affected by infection, suggesting that *H. pylori* may induce expression of cellular machinery involved in negative regulation of cytokine signal transduction.

IL-4 stimulation elicits Stat6 tyrosine phosphorylation and sub-sequent DNA binding in several cell types, and is functionally involved in the development of a Th2 phenotype in T cells and expression of immune molecules such as MHC class II and CD23 on B cells (30). It was recently shown in a murine model of asthma that IL-13-induced Stat6 activation in airway epithelial cells was crucial to elicit airway hyperreactivity and mucus production (31), indicating that epithelial Stat proteins play a central role in ep-thelial function during disease. T84 colonic epithelial cells (25) and primary human bronchial epithelial cells (32) are known to respond to IL-4 stimulation by activating Stat6, and these are known to respond to IL-4 stimulation by activating Stat6, and that this activation can be blocked by *H. pylori* infection. Humans infected with *H. pylori* generally mount a Th1 response, typified by high levels of IFN-γ production from gastric mucosal T cells (33). However, IL-4 secretion in the inf-ected stomach has been noted (34) and exhibits protective effects following *H. pylori* in some animal models of infection (35) by, for example, reducing bacterial colonization load in the host (reviewed in Ref. 36). Thus, inhibition of signal transduction induced by IL-4 may be of relevance to *H. pylori*-mediated disease. Given the lim-ited knowledge of Stat6 responsive genes in epithelial cells, this area of research deserves future investigation, as abrogation of IL-4-Stat6 signaling may be a mechanism by which *H. pylori* sup-presses a host immune response capable of decreasing bacterial colonization or clearing infection.

Disruption of host cytokine signaling has been proposed as a mechanism of bacterial immune evasion and survival (14). For example, IFN-γ induction of Stat1 DNA binding is disrupted in macrophages by the pathogenic bacteria *Listeria monocytogenes* (15) and *Ehrlichia chaffeensis* (37). Enteroheamorrhagic Esche-richia coli O157:H7 inhibits both IFN-γ induction of Stat1 activa-tion (38) and TNF-α activation of NF-κB (39) in epithelial cells. Also, IFN-γ-induced Stat1, but not IL-4-induced Stat6, activation was prevented in primary human tracheobronchial epithelia by ade-novirus infection (40). Thus, our results identify *H. pylori* as the first human pathogen to abrogate Stat6 activation in epithelial cells. Whether this suppression of cytokine signaling by *H. pylori* also oc-curs with different cytokines and Stats, and in other cell types impor-tant to the host immune response, deserves further investigation.

With respect to the host cell, activation of Stat6 by IL-4 is presum-ably contingent upon a viable cell with an intact IL-4 signaling pathway. Although *H. pylori* infection can induce epithelial cell death at later time points (e.g., 72 h) (22), this was not the case at the time point when we documented inhibition of IL-4-induced Stat6 activation (e.g., 6 h in MKN45 cells), in agreement with an earlier viability study in MKN45 cells (41). Taken into account with *H. pylori* induction of epidermal growth factor (EGF) recep-tor phosphorylation after a 4-h infection of AGS gastric epithelial cells (6), and activation of NF-κB to elicit continuously increasing IL-8 secretion from MKN45 cells over a 16-h time period (7), this indicates that not all epithelial signal transduction pathways are negatively regulated by infection. Together, these results suggest that inhibition of IL-4 signal transduction observed in this study is a specific effect on the host cell. Furthermore, *Mycobacterium avium* infection of macrophages down-regulates IFN-γ receptor 1 and 2 protein expression (42), and *Porphyromonas gingivalis* outer membrane vesicles decrease the expression and activity levels of Jak1 and Jak2 in endothelial cells (43). In contrast, in this study we found that *H. pylori* infection did not decrease the protein expres-sion levels of IL-4Rα or Jak1, similar to its suppression of EGF-related peptide signaling in MKN28 gastric mucosal cells without affecting EGF receptor expression (44). Because suppression of IL-4-induced Stat6 activation was time-dependent, it may suggest that induction of epithelial cell signal transduction and gene tran-scription are important here. For example, *L. monocytogenes* in-fection of macrophages leads to transcription of suppressor of cy-tokine signaling 3 in a p38 mitogen-activated protein kinase-depen-dent manner (15). Moreover, activation of the mitogen-activated protein kinases, whose activation interferes with cytokine signal transduction (15, 45) and which are activated by *H. pylori* infection of epithelial cells (46, 47), may also be involved.

The type I strain LC11 inhibited IL-4-induced Stat6 tyrosine phosphorylation to a greater degree than the type II strain LC20, suggesting that the cag pathogenicity island or VacA may be in-volved. We tested the type I parental strain 8823 and its isogenic cagA, cagE, and vacA mutants and found them to equally inhibit IL-4-induced Stat6 tyrosine phosphorylation, indicating a different bacterial factor is involved. Indeed, *H. pylori* expresses other factors that may be important to disease. For example, further genes on the cag pathogenicity island remain incompletely characterized, such as cagF, which encodes an immunogenic outer membrane protein (48). Also, the chromosomal oipA gene encodes an outer membrane protein that modulates epithelial cell signal transduction and can be expressed by both cag-positive and cag-negative strains (49). Defining the *H. pylori* factor responsible for suppressing IL-4 induction of Stat6 tyrosine phosphorylation is the focus of ongoing work.

In conclusion, these data identify IL-4-induced activation of Stat6 in gastric epithelial cells that is disrupted by *H. pylori* in-fection. This provides insight into the immunobiology of *H. pylori*...
infection, and may represent an important virulence strategy for successful chronic colonization of the host and evasion of its immune responses.

Acknowledgments

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References