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# Macrophage Migration Inhibitory Factor and Interleukin-8 Produced by Gastric Epithelial Cells during *Helicobacter pylori* Exposure Induce Expression and Activation of the Epidermal Growth Factor Receptor▽

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**While a link between *Helicobacter pylori* exposure and gastric cancer has been established, the underlying mechanisms remain unclear. *H. pylori* induces a chronic inflammatory response in infected individuals. A link between chronic inflammation and carcinogenesis has long been suggested but never elucidated. Epidermal growth factor receptor (EGFR) signaling plays an important role in both proinflammatory and procarcinogenic mechanisms and is upregulated on gastric epithelial cells (GECs) during *H. pylori* exposure. The aim of this study was to examine the effects of two important proinflammatory cytokines released during *H. pylori* infection, macrophage migration inhibitory factor (MIF) and interleukin-8 (IL-8), on the expression and transactivation of EGFR and on the proliferation of GECs during *H. pylori* exposure. The expression of EGFR by GECs was increased by exposure to either *H. pylori*, recombinant MIF, or recombinant IL-8. However, *cag* pathogenicity island knockout strains of *H. pylori* had very little effect on expression. MIF and IL-8 also induced phosphorylation of EGFR, signaling events, and proliferation during *H. pylori* exposure, all of which were decreased when they were neutralized by these cytokines or were blocked from their receptors. The overall role of EGFR in these responses to *H. pylori* exposure was assessed by knocking down EGFR expression by small interfering RNA.**

*Helicobacter pylori* infects over 50% of the world's population and is the major cause of gastritis and gastric and duodenal ulcers, as well as gastric carcinoma (3). Infection with *H. pylori* is often chronic, leading to inflammation, mucosal damage, and gastric atrophy. Gastric cancer is the second leading cause of mortality due to cancer in the world, is often undetectable in the early stages, and has an overall survival rate of only 10 to 20% (28). There is now a considerable body of evidence suggesting that *H. pylori* may induce changes in the gastric mucosa of some individuals that are conducive to carcinogenesis (8, 39). During infection, *H. pylori* maintains transient interactions with the epithelial layer. The attachment of *H. pylori* to various epithelial receptors (4, 25, 31, 34) stimulates a multitude of signaling cascades, many of which are important in the regulation of the host inflammatory response (14). There is evidence linking chronic inflammation to gastric cancer, but the mechanisms involved are not well understood.

The signals triggered by *H. pylori* binding to the epithelium lead to various responses, including the production of proinflammatory cytokines and altered cell turnover rates (5). A major virulence factor of *H. pylori*, the cytotoxin-associated pathogenicity island (*cag* PAI) has been shown to be responsible for many signaling pathways associated with the epithelial release of cytokines and chemokines, such as interleukin-8 (IL-8) (9, 22) and macrophage migration inhibitory factor (MIF) (5). Upon *H. pylori* attachment to an epithelial cell, the *cag* PAI, which encodes a type IV secretion system, injects the CagA protein into the cells along with muropeptides that may elicit cell responses (11). Inside the cell, CagA induces various cellular responses, leading to the production of inflammatory mediators, cytoskeleton rearrangement, and cell-to-cell disassociation that may result in disruption of the integrity of the epithelial barrier. Thereby, CagA is the virulence factor most often associated with procarcinogenic host responses. We have previously shown that CagA is responsible for the induction of gastric epithelial cell production of MIF (5). MIF has been shown to mediate both inflammatory and proliferative responses in many cell types by inducing the NF-κB, Erk1/2, and AP-1 signaling pathways (16, 26, 36). Our studies have also shown that MIF binding to CD74 expressed on the surface of gastric epithelial cells (GECs) led to increased cell proliferation (5). IL-8 is another important factor in the inflammatory response to *H. pylori* infection. In addition to being a neutrophil chemotactic factor, IL-8 up-regulates GEC expression of CD74 (6) and has been shown to enhance the cell survival of some cell types (17, 23). One way in which IL-8 may enhance cell survival and proliferation is by aiding in the transactivation of the epidermal growth factor receptor (EGFR) (18).

The EGFR is highly expressed on the surface of cancer cells and, when activated, induces signaling pathways linked to cellular proliferation, such as the Erk1/2 and AKT signaling pathways (19, 20, 24, 29, 30). Transactivation of the EGFR is induced by various stimuli, including G protein-coupled receptors (35) and cytokine receptors (18, 42) that activate metalloproteinases and induce shedding of EGFR ligands. *H. pylori* exposure has been suggested to induce transactivation of the

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EGFR on GECs in a manner that is dependent upon the *cag* PAI (21). Since the *cag* PAI is also involved in the production of MIF and IL-8 by gastric epithelial cells (5, 22), in this study we investigated the direct roles of MIF and IL-8 in the expression and phosphorylation of EGFR during *H. pylori* exposure and the influence of this receptor on gastric epithelial cell proliferation.

## MATERIALS AND METHODS

**Cell lines.** The N87 human gastric carcinoma epithelial cell line was obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics. Hs738.st/int (CRL-7869) nontransformed, human fetal gastric/intestinal cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics.

**Bacterial cultures.** *H. pylori* strain 26695 and the mutant strain lacking the entire *cag* PAI (the *cag* PAI mutant) were gifts from Yoshio Yamaoka at Baylor College of Medicine and were constructed as described previously (41). *H. pylori* was grown on blood agar plates (Becton Dickinson, San Jose, CA) at 37°C under microaerophilic conditions, as previously described (30). Bacteria were transferred after 48 h into *Brucella* broth containing 10% FBS for 24 h. After the broth was centrifuged at 2,500 × *g* for 10 min, bacteria were resuspended in sterile phosphate-buffered saline (PBS). The concentration of bacteria was determined by measuring the absorbance (at 530 nm) using a spectrophotometer (model DU-65; Becton Dickinson Instruments, Inc., Fullerton, CA) and comparing the values to those of a standard curve generated by quantifying viable organisms from aliquots of bacteria at various concentrations that were also assessed by absorbance.

**Antibodies and reagents.** The anti-human EGFR-phycoerythrin (PE)-conjugated monoclonal antibodies were obtained from BD Biosciences (San Jose, CA), along with the appropriate isotype controls. Recombinant MIF (rMIF; R&D Systems, Minneapolis, MN) and rIL-8 (Sigma-Aldrich, St. Louis, MO) were run through a Detoxi-Gel column (Pierce Chemical, Rockford, IL) to remove any residual endotoxin. Monoclonal anti-IL-8 neutralizing antibodies were obtained from Sigma and were used at a working concentration of 1 μg/ml, as were anti-MIF neutralizing antibodies, obtained from R&D Systems.

**EGFR staining for flow cytometry.** Gastric epithelial cells ($2 \times 10^5$) were grown in 24-well plates. Cells were incubated with either *H. pylori*, rMIF, or IL-8 for 24 h at 37°C in 7% $CO_2$. Some cultures were treated with 1 μg/ml anti-IL-8- or anti-MIF-neutralizing antibodies before *H. pylori* cells were added. The cells were then washed with PBS and incubated with PE-conjugated anti-EGFR antibodies or with isotype control antibodies for 45 min on ice. Following incubation, the cells were washed and fixed with 2% paraformaldehyde in PBS. Then, 10,000 events per sample were collected for further analysis. The flow cytometric analysis was done on a FACScan cytometer unit (Becton Dickinson, Mountain View, CA), and the data analysis was done using Cell Quest software.

**siRNA nucleofection.** GECs were nucleofected with small interfering RNA (siRNA) for EGFR, using a nucleofector device and a basic nucleofection kit for epithelial cells (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer's instructions, with 1 μg of siRNA, including those with accession numbers NM_00522, NM_201282, NM_201283, and NM_201284 or a negative control siRNA (Ambion, Austin, TX). For N87 cells, program B-023 was used as recommended by Amaxa's Cell Database, while program T-20 was used for HS-738 for optimal cell viability. The knockdown of expression of EGFR was verified by staining cells with anti-EGFR-RPE-conjugated monoclonal antibodies or with an isotype control and analyzed by flow cytometry.

**EGFR ligand enzyme-linked immunosorbent assay (ELISA).** Wells of a 96-well plate were coated with recombinant EGFR (Lab Vision, Fremont, CA) in PBS overnight at 4°C. After cultures were washed, wells were blocked with 10% FBS in PBS for 1 h at room temperature. Three washes were done, and the wells were incubated for 2 h at room temperature with supernatants from treated GECs containing the putative ligands. Wells were washed three times and incubated with 2 ng/ml recombinant transforming growth factor-α (rTGF-α), as a known EGFR ligand, or with fourfold dilutions of TGF-α, starting from 2 ng/ml for a standard curve for 1 h. After cultures received three additional washes, wells were incubated with 5 μg/ml of biotinylated anti-TGF-α for 1 h at room temperature, washed three times, and incubated with streptavidin-horseradish peroxidase for 30 min. After plates received another set of washes, 100 μl of substrate reagent (R&D Systems) was added to each well according to the manufacturer's instructions, and 50 μl of $H_2SO_4$ was added, once the color change was noted, to stop the reaction, and plates were read on a SpectraMax 340pc plate reader device (Molecular Devices, Union City, CA).

**Bio-Plex for phosphorylated EGFR.** Levels of phosphorylated EGFR were measured by phosphoprotein bead array (Bio-Rad, Hercules, CA). Cells exposed to *H. pylori* for 20-min intervals were lysed using a Bio-Plex cell lysis kit according to the manufacturer's instructions. Lysates were normalized to ensure that the same amount of protein was incubated with beads. Beads conjugated with monoclonal antibodies for each were incubated with cell lysates according to the manufacturer's instructions. After samples were washed, they were run on a Bio-Plex system and analyzed using Bio-Plex Manager software (Bio-Rad).

**Proliferation assay.** Cell proliferation was measured by CellTiter 96 nonradioactive cell proliferation kit (Promega, Madison, WI). Cells were cultured in a 96-well plate overnight in serum-free medium at $1 \times 10^4$ cells per well. A standard curve was established by adding known amounts of cells to wells. Cells nucleofected with EGFR or negative control siRNA were treated with 1:1, 10:1, or 100:1 bacterium-to-cell ratios of *H. pylori* for 24 h or 0.1, 1, or 10 ng/ml of MIF or IL-8. Cells were then washed with medium to remove bacteria, and the dye solution was added according to the manufacturer's instructions for 1 h at 37°C. After the stop solution was added, absorbance was read at 490 nm using an ELISA plate reader device. Cell numbers were calculated using a standard curve of known cell numbers.

**Statistical analysis.** Data were analyzed using one-way analysis of variance. A $P$ value of $<0.05$ was considered significant.

## RESULTS

**EGFR expression is increased on GECs during *H. pylori* exposure.** Since several studies have suggested that EGFR may be important in GEC responses during *H. pylori* exposure, the expression of this receptor on GECs was investigated after cells were exposed to *H. pylori*. N87 gastric epithelial cells and HS-738 nontransformed fetal gastric cells were exposed to an *H. pylori*-to-cell ratio of 100:1 for 24 h to allow for transcription, translation, and transport of new receptor to the cell surface. The surface expression of EGFR was analyzed by flow cytometry. EGFR expression by both N87 and HS-738 cells was increased after *H. pylori* exposure (Fig. 1A and B). When EGFR expression on GECs was assayed after cells were exposed to the *H. pylori* 26695 *cag* PAI knockout strain, expression was found to have minimally increased for both cells examined (Fig. 1A and B).

**MIF and IL-8 up-regulate EGFR expression on GECs during *H. pylori* exposure.** During *H. pylori* infection, the *cag* PAI genes have been shown to play an important role in the proinflammatory responses of the gastric epithelium. We have also shown that MIF is produced by the epithelium in a CagA-dependent manner after exposure to *H. pylori* (5). Therefore, the role of proinflammatory cytokines in EGFR expression by GECs during exposure with *H. pylori* was examined. To investigate this, anti-MIF or anti-IL-8 neutralizing antibodies were added to GEC cultures before *H. pylori* was added. As seen in Fig. 2A and B, GEC cultures incubated with anti-MIF antibodies prevented the level of EGFR expression induced during *H. pylori* exposure, whereas the control antibody had no effect. Similar observations were made with cells incubated with anti-IL-8 neutralizing antibodies (Fig. 2C and D). Interestingly, neither antibody treatment alone reduced expression to that of basal levels. In order to further examine the role of these cytokines in EGFR expression, rMIF and IL-8 were added to GEC cultures in amounts within a range relevant to that produced after 24 h of *H. pylori* exposure (5, 6). Figures 2E and F show that rMIF increases EGFR expression to levels similar to

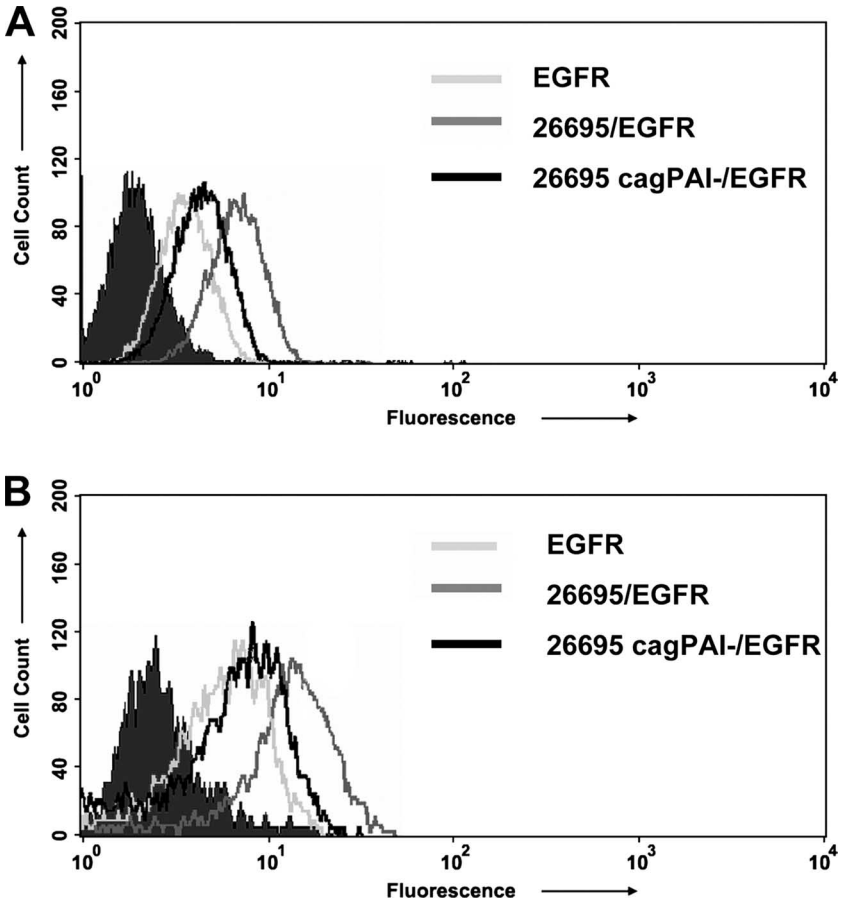


FIG. 1. EGFR expression is increased on GECs during *H. pylori* exposure in a *cag* PAI-dependent manner. EGFR expression was examined by staining cells with anti-EGFR-PE-conjugated antibodies for flow cytometry after a 24-h incubation of (A) N87 with *H. pylori* strain 26695 or 26695 *cag* PAI knockout and (B) HS-738 with *H. pylori* strain 26695 or 26695 *cag* PAI knockout. Isotype controls are represented by solid peaks. Representative figures are shown for four flow cytometry experiments.

those noted during cell exposure to *H. pylori*, while Fig. 2G and H show that IL-8 also increases EGFR expression.

**MIF and IL-8 induce the phosphorylation of EGFR on GECs during *H. pylori* exposure.** Recent studies have suggested that EGFR can undergo transactivation by pathways involving proinflammatory factors, such as IL-8, which accelerate the processing of EGF ligands (18). Therefore, we sought to determine if proinflammatory cytokines play a role in the activation of EGFR during *H. pylori* exposure. To do this, phosphorylated levels of EGFR were determined by Bio-Plex bead array at 20-min intervals during *H. pylori* exposure with the wild-type and the *cag* PAI knockout bacteria. Wild-type bacteria induced phosphorylation starting at 40 min, while the *cag* PAI knockout strain induced significantly less phosphorylation (Fig. 3A). Since MIF and IL-8 are induced by *cag* PAI, the induction of EGFR phosphorylation in GECs by these cytokines was also assessed. rMIF and rIL-8 induced phosphorylation starting at 20 min (Fig. 3B), since they were added directly and there was no time lapse while the cells released them as they did during *H. pylori* exposure. Since MIF and *H. pylori* can both utilize CD74 as a receptor (4, 5) and MIF and IL-8 may both utilize CXCR2 as a receptor (2), the role of these receptors in the induction of EGFR transactivation was examined. Furthermore, CXCR4 expression was also examined because MIF was recently shown to bind to this receptor on T cells, and we confirmed its expression on GECs by flow cytometry (data not shown). Figure 3C demonstrates that CD74, CXCR1, CXCR2, and CXCR4 all play roles in the induction of EGFR phosphorylation by GECs during *H. pylori* infection, which was assessed at 80 min, based on the preceding data showing that phosphorylated EGFR is seen at this time. When CD74 or CXCR4 was blocked with monoclonal antibodies before *H. pylori* exposure, the amount of EGFR phosphorylation induced by *H. pylori* was reduced to as much as 50% less than that of cells incubated with the isotype control antibodies before *H. pylori* exposure. Blocking CD74 decreased *H. pylori* attachment, which may reduce the cytokines released or it may reduce MIF binding so it may play multiple roles in this system. Decreases in EGFR phosphorylation were also seen when CXCR1 or CXCR2 was blocked, and blocking of all of these receptors resulted in 70% decreased phosphorylation of EGFR. These results indicate that the binding of MIF and IL-8 to their receptors plays a crucial role not only in the expression of EGFR but also in the initiation of its activation.

**MIF and IL-8 induce EGFR ligand shedding by GECs during *H. pylori* exposure.** To examine the mechanism with which

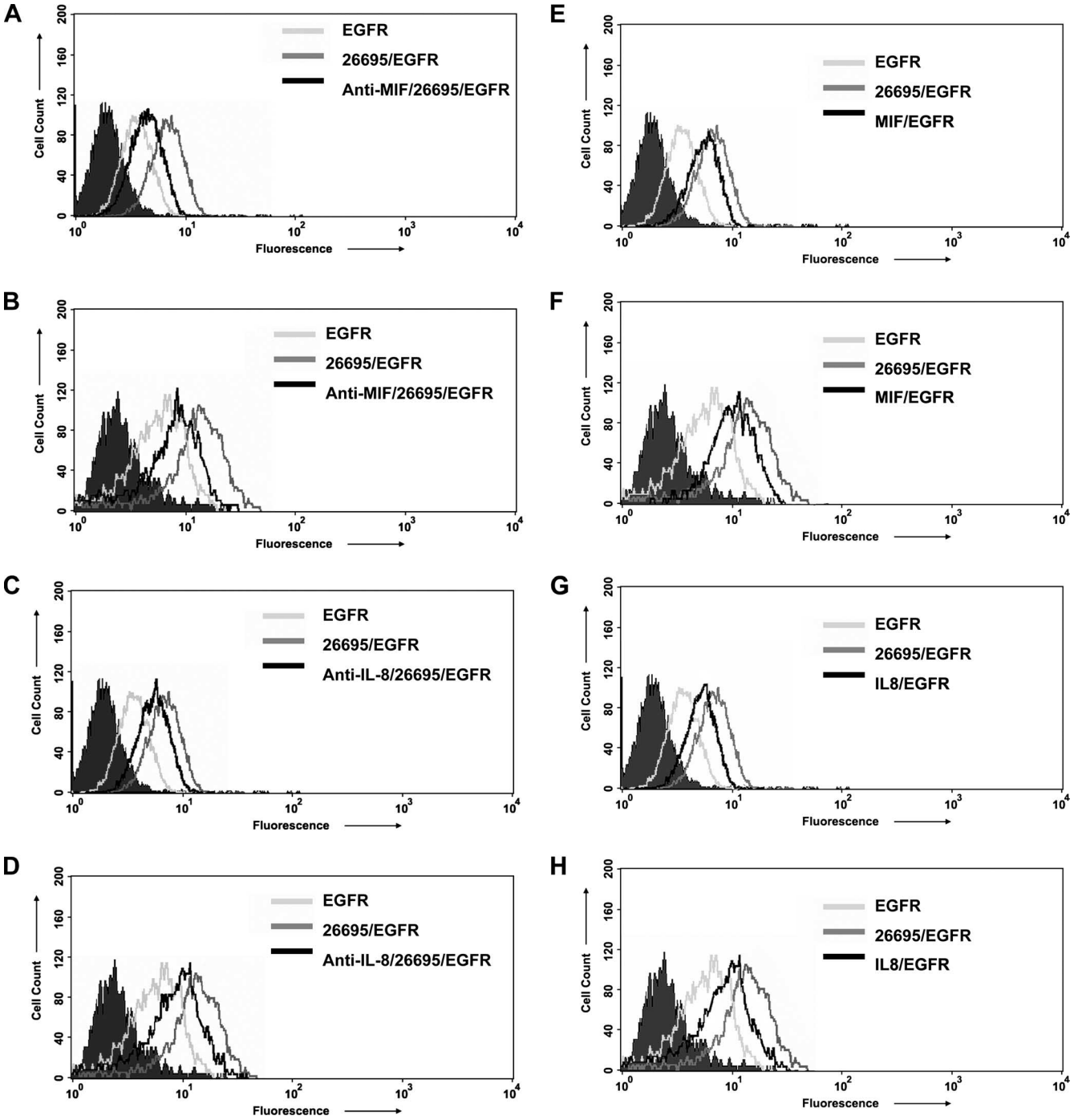


FIG. 2. MIF and IL-8 increase EGFR expression in GECs during *H. pylori* exposure. EGFR expression was examined by staining with anti-EGFR-PE-conjugated antibodies for flow cytometry after a 24-h incubation of (A) N87 with anti-MIF neutralizing antibodies or isotype control antibodies and 26695; (B) HS-738 with anti-MIF neutralizing antibodies or isotype control antibodies and 26695; (C) N87 with anti-IL-8 neutralizing antibodies or isotype control antibodies and 26695; (D) HS-738 with anti-IL-8 neutralizing antibodies or isotype control antibodies and 26695; (E) N87 with rMIF; (F) HS-738 with rMIF; (G) N87 with rIL-8; and (H) HS-738 with rIL-8. Isotype controls are represented by solid peaks. Representative figures are shown for four experiments.

MIF and IL-8 induce EGFR phosphorylation, the ability of these cytokines to induce GECs to shed EGFR ligands was investigated by ELISA. Since EGFR has multiple known ligands that epithelial cells may release, an assay was developed that could detect the binding of any of them. Wells were coated with recombinant EGFR (rEGFR) and incubated with cell culture supernatants from GECs exposed to *H. pylori*, the *cag* PAI knockout, or rMIF or rIL-8. Any EGFR ligands secreted by GECs into the supernatant would bind to the rEGFR coated onto each well. In order to check for the binding of

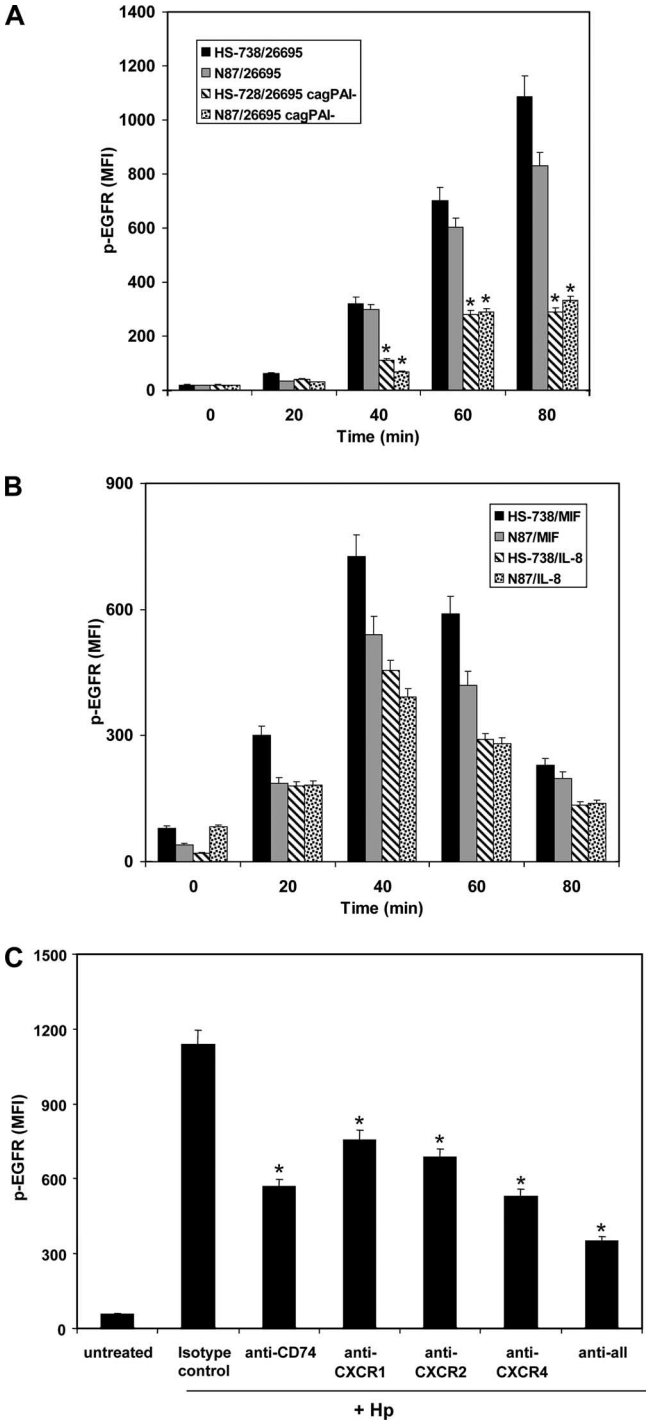


FIG. 3. MIF and IL-8 induce phosphorylation of EGFR during *H. pylori* (Hp) exposure. Phosphorylation of GEC EGFR was assessed by Bio-Plex phosphoprotein bead assay after incubation with (A) *H. pylori* strain 26695 and 26695 *cag* PAI knockout, (B) 10 ng/ml rMIF or rIL-8, and (C) 26695 at 80 min when blocking MIF and IL-8 receptors. Results are shown as the means of four experiments ($n = 8$). *, $P < 0.05$.

these ligands, wells were then incubated with rTGF-α, a known EGFR ligand that would bind to unoccupied EGFR. The presence of TGF-α binding was detected with specific antibodies, and its level of binding was inversely proportional to the level

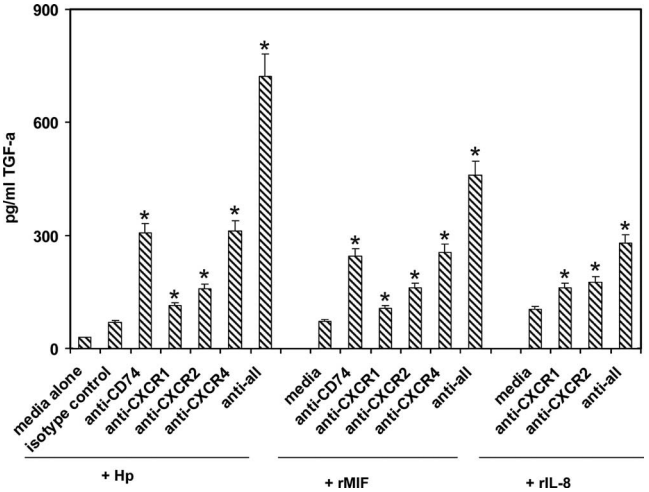


FIG. 4. MIF and IL-8 induce EGFR ligand shedding during *H. pylori* (Hp) exposure. EGFR ligand shedding was measured by ELISA, where wells were coated with rEGFR, incubated with medium from GECs exposed to *H. pylori*, then incubated with rTGF-α, and finally detected with biotinylated anti-TGF-α and developed. Since TGF-α binds to EGFR, the more that binds, the less EGFR ligand is present. The means of four experiments ($n = 8$) are shown here. *, $P < 0.05$.

of EGFR ligands captured in the wells. If rTGF-α bound to free rEGFR, this indicated that EGFR ligands were minimally present in the cell culture supernatant, while less TGF-α binding indicated that EGFR ligands were present in the cell culture supernatant after treatment. Amounts of rTGF-α were calculated by a comparison of values to those of a standard curve. Figure 4 shows that as CD74, CXCR1, CXCR2, and CXCR4 were blocked with monoclonal antibodies on GECs before exposure to *H. pylori*, rMIF, or rIL-8, increased binding of rTGF-α was seen, indicating that when these receptors were blocked, fewer EGFR ligands were produced by cells. Blocking CD74 may decrease MIF binding, and it may decrease *H. pylori* attachment and subsequent IL-8 production, as we have previously shown (4), both of which would effect EGFR ligand shedding. These results demonstrate that the MIF and IL-8 produced during *H. pylori* exposure lead to GEC production of EGFR ligands, which then go on to induce EGFR phosphorylation as seen in Fig. 3A, B, and C.

**Depletion of EGFR expression with siRNA decreases *H. pylori*-induced GEC proliferation.** Since the most well known role of EGFR is induction of tumor cell proliferation, and since we have recently shown that *H. pylori*-induced GEC proliferation is dependent upon the *cag* PAI (5), the role of EGFR in *H. pylori*-induced GEC proliferation was examined by knocking down its expression by using validated siRNAs. After cells were transfected with siRNAs, cell viability was examined by the soluble tetrazolium salt (MTS) assay and compared to that of control transfected cells and nontransfected cells. The viabilities of cells transfected with control or EGFR siRNAs were similar and decreased by approximately 10% compared to those of nontransfected cells at 24 h (data not shown). Proliferation rates of control cells and EGFR knockdown cells were investigated by a nonradioactive cell proliferation assay during *H. pylori* exposure, utilizing MTS. Figure 5A demonstrates that siRNA specific for EGFR prevented its expression

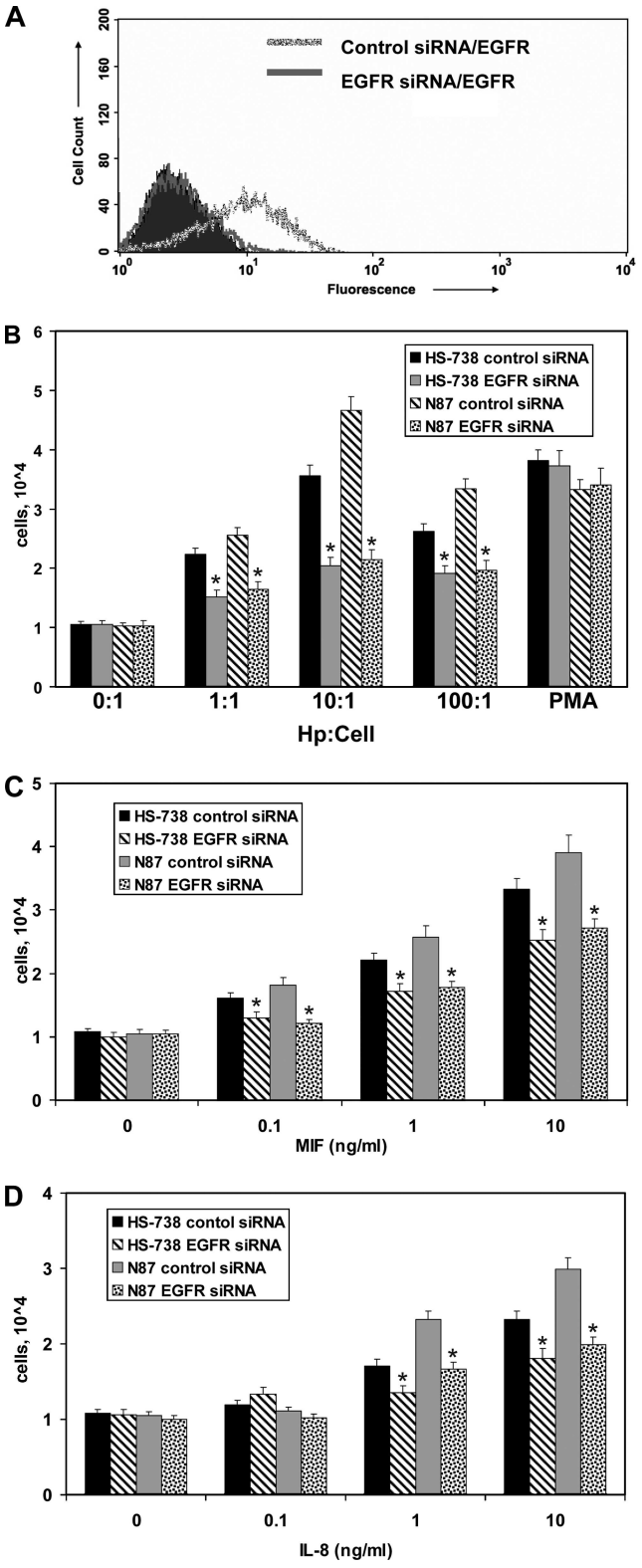


FIG. 5. EGFR activation induces the proliferation of GECs during *H. pylori* (Hp) exposure. (A) EGFR expression was assessed by flow cytometry after siRNA knockdown and GEC proliferation was measured by CellTiter 96 nonradioactive cell proliferation assay and compared to a standard curve of known amounts of cells incubated with (B) *H. pylori* strain 26695 and 26695 *cag* PAI knockout, (C) rMIF, and (D) rIL-8. The means are shown as the results of duplicates in four experiments ($n = 8$). *, $P < 0.05$.

on transfected cells. When *H. pylori* was added to cultures in bacterium-to-cell ratios of 1:1, 10:1, and 100:1, the most effective ratio for proliferation was 10:1 (Fig. 5B). Control cells had almost double the proliferation rates as cells with siRNA knockdown of EGFR expression. These results suggest a significant role for EGFR in *H. pylori*-induced GEC proliferation. Since we previously showed that rMIF could induce GEC proliferation (5), and since others have shown that IL-8 can also induce GEC proliferation (18), the role of EGFR in MIF- and IL-8-induced proliferation was investigated. Figure 5C and D verify that rMIF and rIL-8, in concentrations similar to those we have previously shown to be produced during *H. pylori* infection (4, 5), induce proliferation of both N87 and HS-738 cells, and proliferation increases as concentrations of rMIF and rIL-8 increase. However, the proliferation levels of cells nucleofected with EGFR siRNA induced significantly less proliferation than cells incubated with control siRNA. These results suggest that EGFR is a crucial player in the proliferative response seen during *H. pylori* exposure.

## DISCUSSION

The EGFR is overexpressed in various cancers and is involved in the proinflammatory responses and procarcinogenic events including cell proliferation, migration, and invasion. Expression of this receptor is well documented in many cancers, including gastric cancer (10), and the identification of it as an oncogene has prompted the development of anticancer drugs that target EGFR. One study suggests a correlation between EGFR expression on tumor cells, proliferation, and prognosis in gastric cancer (19), and another study showed that the treatment of EGFR with antisense RNA inhibited gastric tumor growth in a mouse model of gastric cancer (15). In addition to its roles in cell proliferation and carcinogenesis, EGFR has also been suggested to play a role in proinflammatory signaling and responses in a variety of diseases, such as airway inflammation in asthma, respiratory syncytial virus infection (13, 27), and inflammatory bowel disease (37). Although EGFR expression is increased with many inflammatory diseases and with cancers where inflammation is prevalent, there are few data on how this receptor expression is increased. In this study, we investigated the expression of the EGFR on the N87 gastric carcinoma cell line, which was chosen because its phenotype is most similar to those of normal GECs (1) and HS-738 nontransformed fetal gastric cells. We found that EGFR expression was increased on GECs by *H. pylori* exposure and that this was dependent on the *cag* PAI. Furthermore, we found that the production of both MIF and IL-8 could increase the expression of EGFR during *H. pylori* expression. The induced expression of EGFR is decreased with the *cag* PAI knockout strain compared to that induced by the wild type; however, there remains a small increase in expression of EGFR over that of the basal levels. This may be due to IL-8 produced by GECs independent of the *cag* PAI, which has been shown in several studies (7, 40), or to other cytokines that may be produced by GECs. While one study mentions a possible correlation between IL-8 production and EGFR expression (13), the current study has shown a direct link between the proinflammatory cytokines MIF and IL-8 and the expression of

EGFR, which may be important in the general correlation between chronic inflammation and cancer.

The findings in this study are in agreement with those of other studies that indicate EGFR phosphorylation is partially dependent upon the *cag* PAI of *H. pylori* (21). Further studies have shown that IL-8, which can be induced by the *cag* PAI (9), can induce phosphorylation of the EGFR (18, 38). The full pathogenicity island knockout was utilized and not the *cagA* knockout, since studies have shown that along with CagA, other *cag* PAI proteins may induce IL-8, along with muropeptides also injected through the type IV secretion system (11). Our results indicate that the intracellular stores of proinflammatory cytokines MIF and IL-8 are rapidly released from cells upon exposure to *H. pylori*. Both cytokines induce phosphorylation of the EGFR, and that raises the possibility that multiple proinflammatory cytokines are capable of this mechanism. IL-8 may induce phosphorylation of the EGFR, since the IL-8 receptors CXCR1 and CXCR2 are G protein-coupled receptors that, upon activation, induce the ADAM proteins to shed EGFR ligands (18, 32). MIF induction of EGFR phosphorylation appears to act through multiple receptors, e.g., CD74, CXCR2, and CXCR4, but since MIF can induce IL-8 production, that is perhaps why MIF induces higher responses than IL-8. There are studies showing that MIF can upregulate the expression of metalloproteinases (33), which is similar to the IL-8 mechanism, and upon activation could lead to EGFR ligand shedding. The results shown here suggest that both MIF and IL-8 induce some EGFR ligand shedding by GECs during *H. pylori* infection.

Since EGFR plays a crucial role in cell proliferation and carcinogenesis, we investigated its role in GEC proliferation. Proliferation was increased during low levels of *H. pylori* exposure, such as a bacterium-to-cell ratio of 10:1, while these levels began to decrease at 100:1. Perhaps apoptotic signaling is increased at higher bacterial ratios, which is similar to what we found in a previous study (5). This proliferation was considerably reduced when EGFR was knocked down. Similarly, both rMIF and rIL-8 could induce GEC proliferation, and it was also reduced when EGFR expression was decreased. The cell proliferation induced by the activation of EGFR during *H. pylori* exposure may be an important early step in carcinogenesis, and the role MIF and IL-8 play in this mechanism represents an important step in the link between chronic inflammation and carcinogenesis.

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