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Gastric epithelial attachment of *Helicobacter pylori* induces EphA2 and NMHC-IIA receptors for Epstein-Barr virus

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Abstract

Epstein-Barr virus (EBV)-associated gastric cancer belongs to 1 of the 4 subtypes of gastric cancer and accounts for 10% of total gastric cancers. However, most cases of gastric cancer have a history of *Helicobacter pylori* infection. Therefore, we investigated the possibility that *H. pylori* infection promotes the development of EBV-associated gastric cancer. *H. pylori* was exposed to principal EBV receptor, CD21, negative gastric epithelial cells, and then infected with EBV recombinant expressing enhanced green fluorescent protein. Changes in EBV infectivity due to prior *H. pylori* exposure were analyzed using flow cytometry. The treatment of gastric epithelial cells with *H. pylori* increased the efficiency of EBV infection. An increase was also observed when CagA-deficient, VacA-deficient, and FlaA-deficient *H. pylori* strains were used, but not when cag pathogenicity island-deficient *H. pylori* was used. The treatment of epithelial cells with *H. pylori* induced the expression of accessory EBV receptors, EphA2 and NMHC-IIA, and increased the efficiency of EBV infection depending on their expression levels. When gastric epithelial cells were treated with *EPHA2* or *NMHC-IIA* siRNA, EBV infection via *H. pylori* attachment was decreased. The adhesion of *H. pylori* induced the expression of accessory EBV receptors in gastric epithelial cells and increased the efficiency of EBV infection.

Abbreviations: APC, adenomatous polyposis coil; BMRF1, *Bam*HI M fragment rightward open reading frame 1; BRCA, breast cancer susceptibility gene 1; BZLF1, *Bam*HI Z fragment leftward open reading frame 1; CagA, cytotoxin-associated gene A; cagPAI, cag pathogenicity island; EBV, Epstein-Barr virus; EBVaGC, EBV-associated gastric cancer; eGFP, enhanced green fluorescence protein; EphA2, ephrin type-A receptor 2; FlaA, Flagellin A; NMHC-IIA, non-muscle myosin heavy chain IIA; NRP1, Neuropilin-1; OMVs, outer cell membrane vesicles; PBS (−), calcium and magnesium-free phosphate-buffered saline; PC, penicillin; PTEN, phosphatase and tensin homolog deleted from chromosome 10; qPCR, quantitative polymerase chain reaction; SHP1, Src homology region 2 domain-containing phosphatase 1; SM, streptomycin; VacA, vacuolating cytotoxin A.

Sintayehu Fekadu and Yuichi Kanehiro contributed equally to this work.

KEYWORDS
bacterial-viral association, ephrin type-A receptor 2, Epstein-Barr virus, gastric cancer, *Helicobacter pylori*, non-muscle myosin heavy chain IIA

1 | INTRODUCTION

EBV is a ubiquitous gammaherpesvirus that infects B cells and epithelial cells and that sometimes induces cancer.[1] B cell EBV infection is mediated by the high-affinity receptor CD21.[2] However, EBV also infects CD21-negative epithelial cells. In this case, integrins and low-affinity receptors, such as Neuropilin-1 (NRP1), non-muscle myosin heavy chain IIA (NMHC-IIA), and ephrin type-A receptor 2 (EphA2), are used.[1,3-6] The regulation of the expression of these low-affinity receptors is poorly understood. However, if the expression of low-affinity receptors is induced in epithelial cells, EBV infection of epithelial cells is promoted.

EBV was the first human oncogenic virus to be discovered, and its persistent infection promotes the oncogenic transformation of cells. EBVaGC is a representative EBV-positive epithelial cell tumor that accounts for 10% of all gastric cancer cases. EBVaGC tumor cells originate from a single cell clone infected with EBV.[7] In contrast, it has been reported that more than 95% of patients with gastric cancer have a history of *Helicobacter pylori* infection, and *H. pylori* is the major causative agent of gastric cancer.[8-10] Accordingly, it has long been argued that *H. pylori* infection may have some effect on the development of EBVaGC, because it is a subtype of gastric cancer.[11,12]

It has been reported that additional EBV infection assists the oncogenic process of gastric cancer caused by *H. pylori* infection. When gastric epithelial cells are infected with EBV, DNA methylase is induced, and the expression of tumor suppressor genes, such as APC, breast cancer susceptibility gene 1 (*BRCA1*), and phosphatase and tensin homolog deleted from chromosome 10 (*PTEN*), is decreased via promoter methylation.[12] In addition, the expression of Src homology region 2 domain-containing phosphatase 1 (SHP1) is suppressed, resulting in the relative activation of oncogenic SHP2. Therefore, EBV infection promotes the activation of the SHP2-Ras pathway, which is initiated by the phosphorylation of *H. pylori* -derived cytotoxin-associated gene A (CagA).[13]

In contrast, we investigated the possibility that exposure of gastric epithelial cells to *H. pylori* accelerates the tumorigenic steps induced by EBV infection.[14] We observed an increase in EBV copy number in epithelial cells cultured with *H. pylori*. We assumed that 1 of the virulence factors of *H. pylori* assisted the infectivity of EBV. The following pathogenic factors were investigated: CagA, which is encoded by the bacterial cag pathogenicity island (*cagPAI*) gene and promotes the tumorigenicity of gastric epithelial cells,[15] vacuolating cytotoxin A (VacA), which induces cytokines from gastric epithelial cells,[16] and Flagellin A (FlaA), which is a major component of flagella.[17]

We infected gastric epithelial cells with *H. pylori* mutants that are destructive to individual virulence factors, and investigated whether they affected the efficiency of EBV infection. The adhesion of wild-type *H. pylori* to gastric epithelial cells induced the expression of low-affinity EBV receptors. However, the induction was not observed by the *H. pylori* mutant deficient in *cagPAI*. In conclusion, the attachment of *H. pylori* to gastric epithelial cells increases the number of EBV-infected cells, so that people infected with *H. pylori* might be more likely to develop EBVaGC.

2 | MATERIALS AND METHODS

2.1 | Cell lines, bacterial strains, and virus

Human gastric carcinoma-derived AGS cells which were free of parainfluenza virus infection were provided by Lindsey M. Hutt-Fletcher, Louisiana State University Health Sciences Center, LA, USA.[18] Human Burkitt lymphoma Daudi cells were purchased from the American Type Culture Collection. MKN28, which is derived from MKN74, was obtained from the National Institutes of Biomedical Innovation, Health, and Nutrition, JCRB cell bank. GES-1, an immortalized fetal gastric epithelial cell line, was obtained from Beijing Institute for Cancer Research (Beijing, China).[19,20] An EBV-negative Daudi (–) cell line was established via limiting dilution of EBV-positive Daudi cells.[21] AGS cells were infected with recombinant Akata EBV strain, in which the viral thymidine kinase gene was disrupted by enhanced green fluorescence protein (eGFP) and neomycin resistance gene (eGFP-EBV).[22] Cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Thermo Fisher Scientific), 100 units/mL of penicillin (PC), and 100 μg/mL of streptomycin (SM) (Nacalai), at 37°C in a 5% CO_2 incubator. To maintain eGFP-EBV-infected AGS cells [AGS (+)], 500 μg/mL G418 (Promega) was added to the culture medium.

Helicobacter pylori strains CPY6271 and CPY3401 were isolated from Japanese gastric cancer patients[23,24] and isogenic *CagA*- and *VacA*-deficient mutants derived from the CPY6271 strain were established via insertion of the kanamycin or chloramphenicol resistance gene following a previously reported method.[25] An isogeneic *FlaA* mutant derived from the CPY3401 strain was also generated via insertion of a kanamycin resistance gene.[26] An isogeneic *cagPAI* mutant derived from the TN2 strain was also generated by inserting a chloramphenicol resistance gene.[27]

Bacteria were inoculated in *Brucella* broth (Becton, Dickinson and Company (BD)) or *Brucella* agar plates supplemented with 5% heat-inactivated horse serum (ThermoFisher Scientific), vancomycin

(10 μg/mL), kanamycin (10 μg/mL), and/or chloramphenicol (8 μg/mL). The culture plate with gas-producing sachets, Anaero-Pack MicroAero (Mitsubishi Gas Chemical Co., Inc) was incubated for 3 d under microaerophilic conditions. Colonies were then transferred to *Brucella* broth and cultured for 18 h under microaerophilic conditions. The bacterial density was determined via optical density measurements at 600 nm (WPA CO 7500 Colorimeter, Biochrom Ltd). The MOI was determined as the number of bacterial colony-forming units divided by the number of epithelial cells in each experimental condition.

Cell-free eGFP-EBV was produced from AGS (+) cells following *Bam*HI Z fragment leftward open reading frame 1 (*BZLF1*) gene transfection using Lipofectamine 2000 (Thermo Fisher Scientific). The culture supernatant was collected, sterilized using a 0.45-μm syringe filter, and concentrated at 16 000 *g* for 90 min at 4°C. The pellet was suspended in fresh cell culture medium, and aliquots were stored at −80°C. The viral infectious titer (IU/mL) was determined by infecting Daudi (−) cells with eGFP-EBV and detecting the eGFP-positive cells using FACS analysis. Similarly, the eGFP-EBV BZLF1 knockout (KO) virus was produced from AGS (+) cells latently infected with BZLF1 KO, following a similar protocol.

2.2 | *Helicobacter pylori*-eGFP-EBV sequential infection of gastric epithelial cells

Gastric epithelial cells (AGS, MKN28, or GES-1) were seeded in cell culture plates and attached to the bottom of the wells for 12 h. *H. pylori* was harvested, washed with PBS (−), and suspended in antibiotic-free cell culture medium. Cells were infected with *H. pylori* at an MOI of 100 and incubated at 37°C and 5% CO_2 for 7 h. To remove *H. pylori*, the cells were washed with PBS (−) and subsequently infected with cell-free eGFP-EBV at an MOI of 125 in the presence of PC/SM. After 48 h of EBV infection, the infection efficiency was assessed using fluorescence microscopy (IX73, Olympus), flow cytometry (BD FACSCalibur, BD), and quantitative polymerase chain reaction (qPCR).

2.3 | Flow cytometry

To determine the efficiency of eGFP-EBV infection, cells were detached using trypsin and suspended in FACS solution (2% FBS in PBS (−)) containing 7-AAD (eBioscience) to distinguish the dead cells. To determine EBV receptor expression, cells were exposed to *H. pylori* (MOI 100) for 7 h under cell culture conditions. Cells were fixed with Mildform 10 N (FUJIFILM Wako Pure Chemical Corp.) for 10 min at 25°C. After washing with PBS (−), the cells were incubated with PBS (−) containing 0.5% saponin (Wako) for 20 min at 25°C. Cells were incubated with PBS (−) containing 0.5% saponin and 5% bovine serum albumin fraction V (Sigma-Aldrich) for 1 h at 25°C. The cells were stained with rabbit anti-human-EphA2 antibody (1C1, Novus Biological) or with mouse anti-human NMHC-IIA (GT218; Genetex) for 30 min on ice. Cells were then washed with FACS solution and stained with goat anti-rabbit IgG conjugated with Alexa Fluor 488 or goat anti-mouse IgG conjugated with Alexa Fluor 488 (Thermo Fisher Scientific) for 30 min on ice. Finally, cells were suspended in FACS solution containing 7-AAD and analyzed using a FACS Calibur flow cytometer and FlowJo software (FlowJo LLC).

2.4 | qPCR

To determine the EBV copy number, genomic DNA was extracted using the GeneElute mammalian genomic DNA Miniprep kit (Sigma-Aldrich), following the manufacturer's instruction. The PCR primers (*Bam*HI W, F: 5′-CCCAACACTCCACCACACC-3′, R: 5′-TCTTAGGAGGCTGTCCGAGG-3′;glyceraldehyde3-phosphatedehydrogenase (*GAPDH)* gene, F: 5′-TGTGCTCCCACTCCTGATTTC-3′, R: 5′-CCTAGTCCCAGGGC TTTGATT-3′; and the double-quenched fluorescent DNA probes (*Bam*HI W: 5′-FAM/CACACACTA/ZEN/CACACACCCACCCGTCTC/IBFQ-3′; *GAPDH*: 5′-FAM/C GGTCACAA/ZEN/TCTCCACGC/IBFQ-3′) were purchased from Integrated DNA Technologies (IDT), Coralville, IA). qPCR was performed using SsoAdvanced Universal Probe Supermix (Bio-Rad Laboratories). A DNA probe-based qPCR assay was used to determine the EBV copy number.[28] GAPDH served as an internal control. Raji cells containing 50 EBV copies per cell were used as positive controls.

2.5 | Reverse transcription (RT)-qPCR

Total RNA was extracted using ISOGEN reagent (Nippon Gene), following the manufacturer's instructions. Subsequently, complementary DNA was synthesized using SuperScript III reverse transcriptase (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. EBV gene expression was determined using the following primers purchased from IDT: *BZLF1* (F: 5′-TCCGACTGGGTCGTGGTT-3′, R: 5′-GCTGCATAAGCTTGAT AAGCATTC-3′); *Bam*HI M fragment rightward open reading frame 1 (*BMRF1*) (F: 5′-GCCGTTGAGGCCCACGTTGT-3′, R: 5′-TGGGAA TGGCAGGCGAGGGT-3′); *ACTB* (F: 5′-TTGCCGACAGGATGCAGAA-3′, R: 5′-GCCGATCCACACGGAGTACT-3′); and *GAPDH* (F: 5′-AAT CCCATCACCATCTTCCA-3′, R: 5′-TGGACTCCACGACGTACTCA-3′). EBV epithelial receptor expression was determined using the following primers: *EPHA2* (F: 5′-TACGTGGACCCCCACACATA-3′, R: 5′-AGCATGCCCTTGTACACCTC-3′); *NMHC-IIA* (F: 5′-CCTCAA GGAGCGTTACTACTCA-3′, R: 5′-CTGTAGGCGGTGTCTGTGAT-3′); *NRP1* (F: 5′-ATAGCCCCTCCTCCTGTTGT-3′, R: 5′-GTGCATTCAA GGCTGTTGGG-3′); integrin-alpha 5 (*ITGAV*) (F: 5′-CTTCTTGGTG GTCCTGGTAGC-3′, R: 5′-TGTGCAGTCCGAGTTG CTAA-3′); integrin-beta 5 (*ITGB5*) (F: 5′-AGTGCCACCTCATGTGAAGA-3′, R: 5′-TCACACCGAGAGGTGATGGA-3′); *ITGB6* (F: 5′-GGAGG TGCAGAAACCTGTGAA-3′, R: 5′-TGGGAGACAGGGTTTTCGATG-3′), and *ITGB8* (F: 5′-TCAAATGCAGC ATCCTGTGC-3′, R: 5′-CTGGACGC

AGCTGGATAGAC-3′). RT-qPCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Targeted genes were amplified and detected using the CFX Connect Real-Time PCR Detection System under the following conditions: 98℃ for 2 min, 95℃ for 10 s, and 60℃ for 30 s for 40 cycles. For the standardization of expression levels of EPHA2, NMHC-IIA, and NRP1, a calibration curve was prepared from the PCR product, and the average molecular weight of 1 bp of the PCR product was calculated as 660 g/mole (Figure S1).[28] *ACTB* or *GAPDH* was used as an internal standard and for normalization of mRNA level in each experiment.

2.6 | Transwell assay

AGS cells were seeded at 2.5×10^4 cells/500 μL on a 24-well plate and the medium was replaced after 12 h. *H. pylori* (CPY3401) strain at an MOI of 100 was placed in the upper layer of a 0.4 μmol/L transwell insert (Corning) and incubated for 7 h. Then, the transwell insert was removed and incubated with EBV at an MOI of 125 for 48 h. After 48 h of EBV infection, eGFP-positive cells were evaluated using FACS analysis.

2.7 | Cell-bacterial contact via centrifugation

AGS cells were seeded at 5×10^3 cells/100 μL in a 96-well plate and the medium was replaced after 12 h. *H. pylori* (CPY3401) strain at an MOI of 100 was added to the medium, and then centrifuged at 300 *g* for 5 min. After centrifugation, the cells were incubated for 7 h and washed with 1× PBS (−). The cells were then infected with EBV at an MOI of 125 in PC/SM-containing medium. After 48 h, eGFP fluorescence was evaluated using FACS. Bacterial cell contact was confirmed using Giemsa staining (Wako).

In total, 300 Giemsa-stained epithelial cells were observed under a microscope, and the number of cells that exhibited a hummingbird appearance was counted.

2.8 | Virus binding assay

For the EBV binding assay, AGS cells were exposed to *H. pylori* (MOI 100) for 7 h, and then infected with EBV at an MOI of 125 in 250 μL of Hank's balanced solution containing 10 mmol/L HEPES buffer (Nacalai) and incubated for 2 h on ice.[5] Cells were washed with 1× PBS (−), and the EBV copy number was quantified using qPCR.

2.9 | Small interfering RNA (siRNA)

AGS cells were seeded at 1×10^4 cells/250 μL on a 48-well plate, and the medium was replaced after 12 h. All siRNAs were obtained from IDT. siRNAs (2 nmol/L) (si*EPHA2-1*: sense, 5′-GCAGCAAGGUGCACGAAUUCCAGAC-3′, antisense, 5′-GUCUGGAAUUCGUGCACCUUGCUGCCG-3′, or si*EPHA2-2*, sense, 5′-CGUAUCUUCAUUGAGCUCAAGUUTA-3′, antisense, 5′-UAAACUUGAGCUCAAUGAAGAUACGCU-3′; si*NMHC-IIA-1*: sense, 5′-GACCGAGAAGAUCAAUCCAUCUUGT-3′, antisense, 5′-UUCUGGCUCUUCUAGUUAGGUAGAACA-3′, or si*NMHC-IIA-2*, sense, 5′-GAUAAGUAUCUCUAUGUGGAUAAAA-3′, antisense, 5′-UUUUAUCCACAUAGAGAUACUUAUCGG-3′) were transfected with RNAiMaX (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. After 24 h, the cells were washed with PBS and infected with *H. pylori* (CPY3401) for 7 h. This was followed by EBV infection at an MOI of 125 in PC/SM-containing medium for 48 h. After 48 h, eGFP fluorescence was evaluated using FACS. The scrambled negative control siRNA was used as a control. The efficiency of the siRNA was confirmed using RT-qPCR.

2.10 | Western blotting

AGS cells exposed or not exposed to *H. pylori* (MOI 100) were lysed by MEM-PER Plus Membrane Protein Extraction Kit (ThermoFisher Scientific)[29] supplemented with protease inhibitor (cOmplete mini, Sigma-Aldrich). Next, 5 μg of membrane fraction were electrophoresed on a 7.5% SDS-page gel and transferred to PVDF membranes (Millipore). The membranes were incubated with antibodies specific to EphA2 (D4A2, Cell Signaling Technology), NMHC-IIA (GT218; Genetex), and Na-K ATPase (EP1845Y, Abcam). After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) or anti-mouse IgG (GE HealthCare). Specific bands were visualized using an Immobilon (Millipore) and detected by X-ray film. Anti-Na-K ATPase antibody was used as an internal control. Luminous intensities were quantified by ImageJ software (https://imagej.nih.gov/ij/).

2.11 | Statistical analyses

Mann-Whitney test was used to analyze and test whether there was a difference between the 2 independent groups. Data are expressed as the mean ± standard deviation (SD). Statistical significance was set at $P < .05$.

3 | RESULTS

3.1 | *H. pylori* pretreatment of gastric epithelial cells enhances EBV infectivity in a *cagPAI*-dependent manner

Gastric epithelial AGS cells[18] were exposed to *H. pylori* at an MOI of 100. After 7 h, AGS cells were infected with eGFP-EBV[22] at an MOI of 125 and incubated for 48 h. Then, eGFP-positive EBV-infected cells were observed using fluorescence microscopy (Figure 1A). We detected eGFP-positive cells more frequently in *H. pylori* -exposed cells than in unexposed cells (Figure 1B, upper panel). FACS analysis

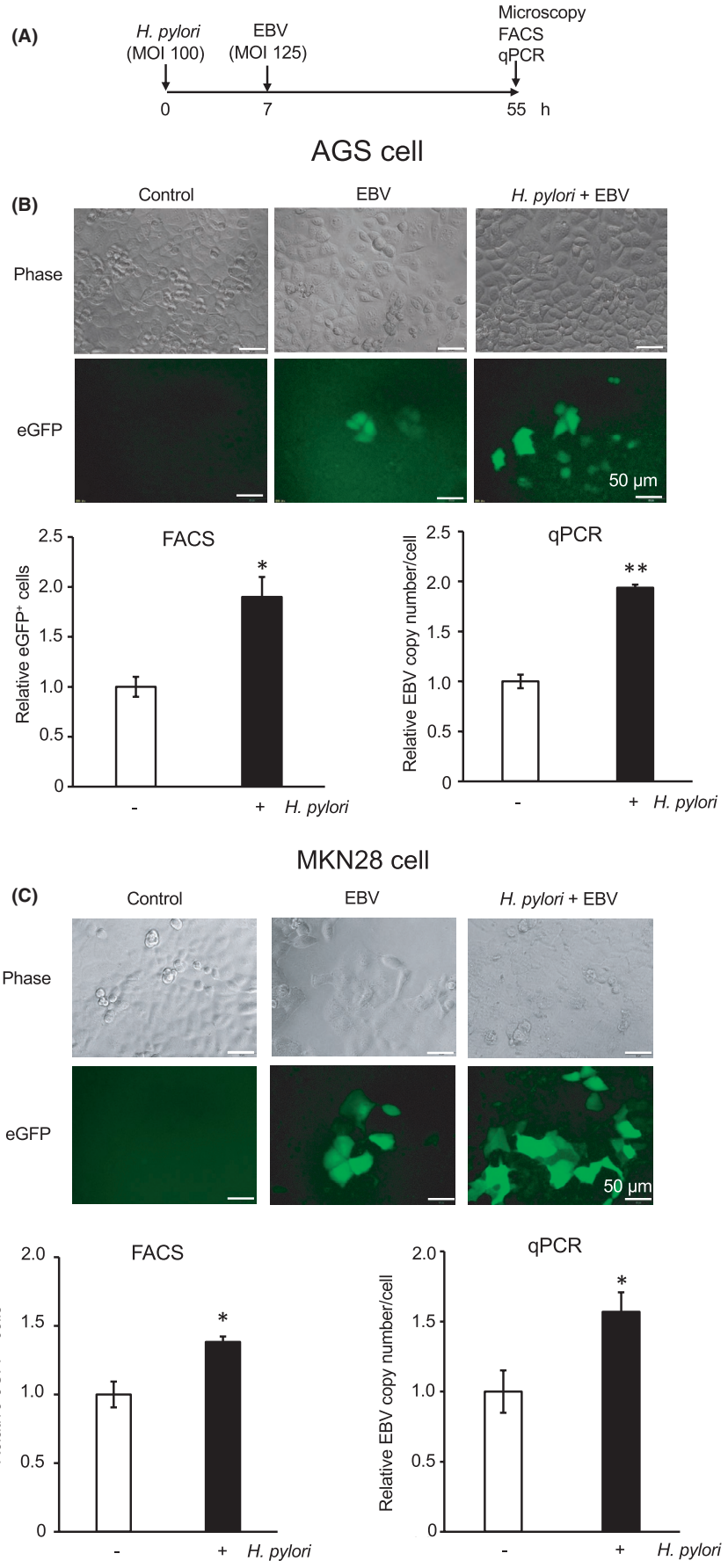


FIGURE 1 (Continued)

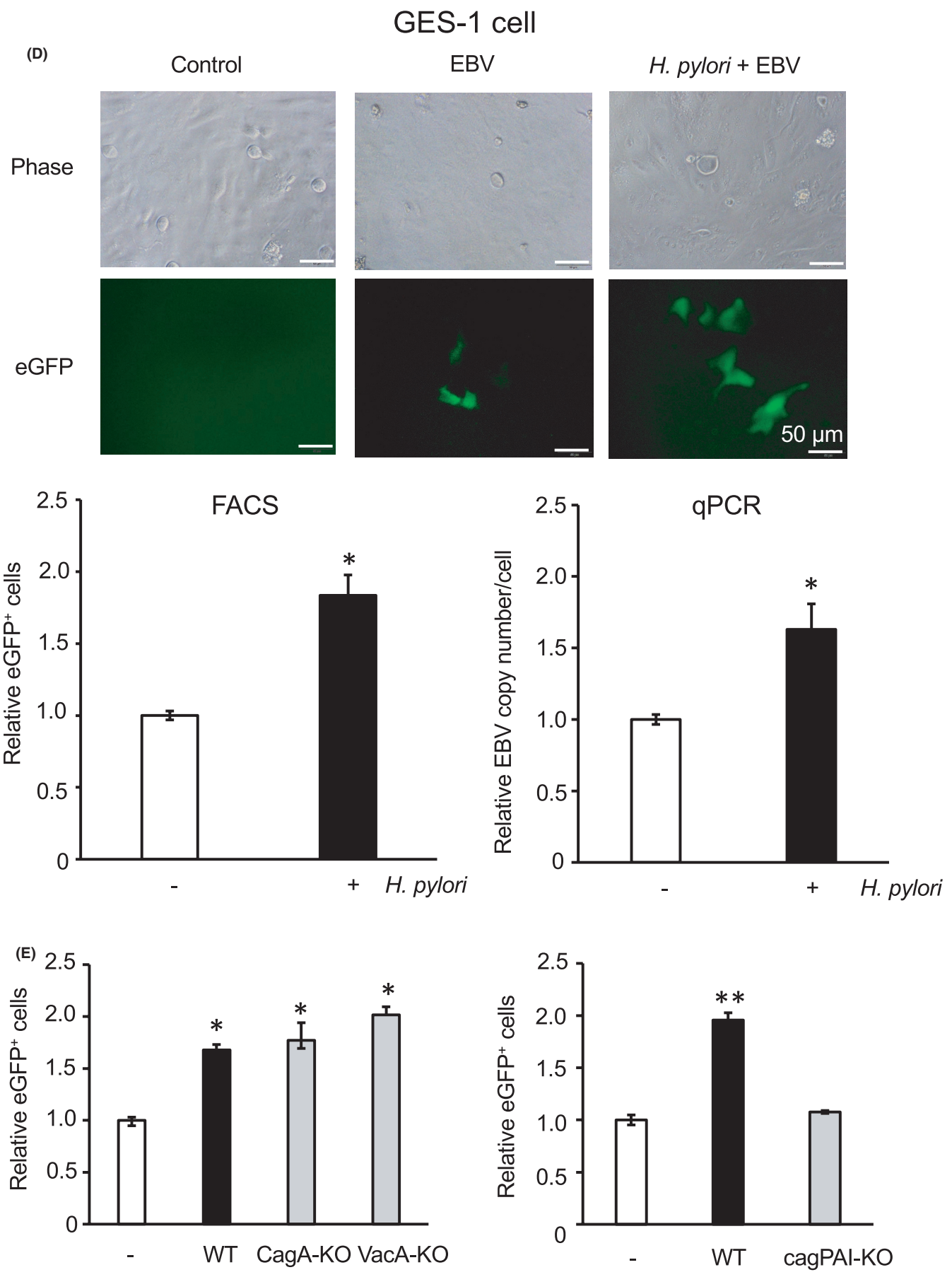

GES-1 cell
(D)
Control
EBV
H. pylori + EBV
Phase
eGFP
50 μm
FACS
Relative eGFP+ cells
2.5
2.0
1.5
1.0
0.5
0
*
-
+ H. pylori
qPCR
Relative EBV copy number/cell
2.5
2.0
1.5
1.0
0.5
0
*
-
+ H. pylori
(E)
Relative eGFP+ cells
2.5
2.0
1.5
1.0
0.5
0
*
*
*
-
WT
CagA-KO
VacA-KO
Relative eGFP+ cells
2.5
2.0
1.5
1.0
0.5
0
**
-
WT
cagPAI-KO

FIGURE 1 *Helicobacter pylori* exposure increased the frequency of EBV infection of gastric epithelial cells. A, Experimental EBV infection to *H. pylori*-exposed epithelial cells is performed in accordance with the illustrated time schedule. B-D, AGS cells (B), MKN28 cells (C), and GES-1 cells (D) were exposed to *H. pylori*, then infected with eGFP-EBV. The efficiency of infection was determined by detecting eGFP using a fluorescence microscope (top) or flow cytometry (left bottom). The efficiency of infection was also determined by EBV copy number analysis (right bottom). E, Neither CagA nor VacA was involved in the enhancement of EBV infection through *H. pylori* exposure, but cagPAI was involved. Before incubation with EBV, AGS cells were exposed to wild-type, CagA knockout (KO), VacA-KO, and cagPAI-KO *H. pylori* strains, respectively. The frequency of EBV infection was confirmed using FACS analysis. $^{*}P < .05$, $^{**}P < .01$. White column: unexposed cells, black column: wild-type *H. pylori*-exposed cells, gray column: CagA, VacA, or cagPAI-KO *H. pylori*-exposed cells. WT: wild-type *H. pylori*. All experiments were performed 3 times and the average value and SD were described

revealed that *H. pylori* treatment increased the number of eGFP-positive cells by a factor of 1.9, compared with unexposed cells (1.9 ± 0.2, $^{*}P < .05$) (Figure 1B, lower left, Figure S2A). qPCR analysis also showed that exposure to *H. pylori* increased EBV copy numbers per infected cell (1.9 ± 0.07, $^{**}P < .001$) (Figure 1B, lower right). Moreover, *H. pylori* contact significantly increased the numbers of both EBV-infected cells and EBV genome copies in another gastric cancer cell line, MKN28 cells (Figures 1C and S2B), as well as in non-malignant fetal gastric epithelial GES-1 cells (Figures 1D and S2C).

To clarify the bacterial factors that enhanced EBV infection, AGS cells were exposed to CagA-KO or VacA-KO *H. pylori* prior to EBV infection. However, we could not detect any difference in the efficiency of EBV infection after treatment with either wild-type, CagA-KO, or VacA-KO *H. pylori* strains (Figure 1E left). Conversely, enhanced EBV infection of gastric epithelial cells was not observed by exposure to *cagPAI*-KO *H. pylori* strain (Figure 1E right).

3.2 | Enhancement of EBV infection in *H. pylori*-exposed gastric epithelial cells is mediated by direct contact with bacteria

Helicobacter pylori migrates using flagella and attaches to gastric epithelial cells.[17] AGS cells were exposed to wild-type or FlaA-KO *H. pylori*. The number of eGFP-positive cells was lower in AGS cells exposed to FlaA-KO *H. pylori* than in cells exposed to wild-type *H. pylori* (Figure 2A). To clarify whether the increase in EBV infection caused by *H. pylori* exposure is affected by bacterial motility, a transwell insert was used to separate AGS cells from *H. pylori*. The transwell culture showed a similar efficiency of EBV infection between cells exposed to wild-type and FlaA-KO *H. pylori* (Figure 2B). The direct contact of cells with *H. pylori* increased the efficiency of EBV infection.

The attachment of *H. pylori* to gastric epithelial cells is known to involve the injection of CagA via a type IV secretion system and induce a hummingbird phenotype.[30] Giemsa staining showed the morphological changes in the epithelial cells exposed to bacteria. Staining was also visible for bacteria that had adhered to the epithelial cells. FlaA-KO *H. pylori* adhered less efficiently to cells than wild-type *H. pylori*. Moreover, although the epithelial cells exposed to wild-type *H. pylori* showed a hummingbird phenotype, the cells exposed to FlaA-KO *H. pylori* did not (Figure 2C upper). However, bacterial attachment to AGS cells was promoted to a similar extent in wild-type and FlaA-KO *H. pylori* using centrifugation. Epithelial cells exposed to either wild-type or FlaA-KO *H. pylori* showed a hummingbird phenotype (Figure 2C lower).

In the uncentrifuged conditions, the hummingbird phenotype was less often detected in cells exposed to FlaA-KO *H. pylori* than in cells exposed to wild-type *H. pylori* (WT 12.7% ± 2.2%, FlaA-KO 5.2% ± 2.5%) (Figure 2D, dotted). In contrast, in the centrifuged conditions, the hummingbird phenotype was similarly detected between cells exposed to wild-type and FlaA-KO *H. pylori* (WT 16.2% ± 0.8%, FlaA-KO 16.2% ± 0.8%) (Figure 2D, hatched). The centrifugation of bacteria strengthened the bacterial cell contact to a similar extent between wild-type and FlaA-KO *H. pylori*. The number of eGFP-positive EBV-infected cells was measured after bacterial attachment to AGS cells. EBV infection efficiency was similar between the wild-type and FlaA-KO *H. pylori* (Figure 2E).

3.3 | Increase in EBV infection by *H. pylori* exposure is not due to the induction of viral lytic infection, but to an increase in the viral attachment to cells

To clarify whether the enhancement of EBV infection by *H. pylori* exposure is due to the entry of the virus into cells or due to an enhancement of viral replication, the expression levels of the immediate early *BZLF1* gene and early *BMRF1* gene were measured using RT-qPCR. When the expression level of the viral genes was normalized based on the EBV copy number, the levels of *BZLF1* and *BMRF1* genes were not significantly increased in *H. pylori*-exposed cells (Figure 3A).

To confirm that the enhancement of EBV infection by *H. pylori* exposure was not due to the induction of viral replication, *H. pylori*-exposed cells were infected with BZLF1-KO EBV. As expected, *H. pylori* exposure resulted in a 1.4-fold increase in the number of eGFP-positive cells (Figure 3B). Based on these results, we speculated that the increase in the efficiency of EBV infection by *H. pylori* might be receptor dependent.

Next, gastric epithelial cells were exposed to *H. pylori* for 7 h, and then infected with eGFP-EBV for 2 h on ice. After washing the cells with cold phosphate buffer to remove the unattached EBV, cell-bound EBV DNA was extracted together with the host DNA. EBV copy numbers were calculated using qPCR by using the *GAPDH* gene to standardize the number of cells. The results showed that *H. pylori* exposure enhanced the binding of EBV to cells (Figure 3C).

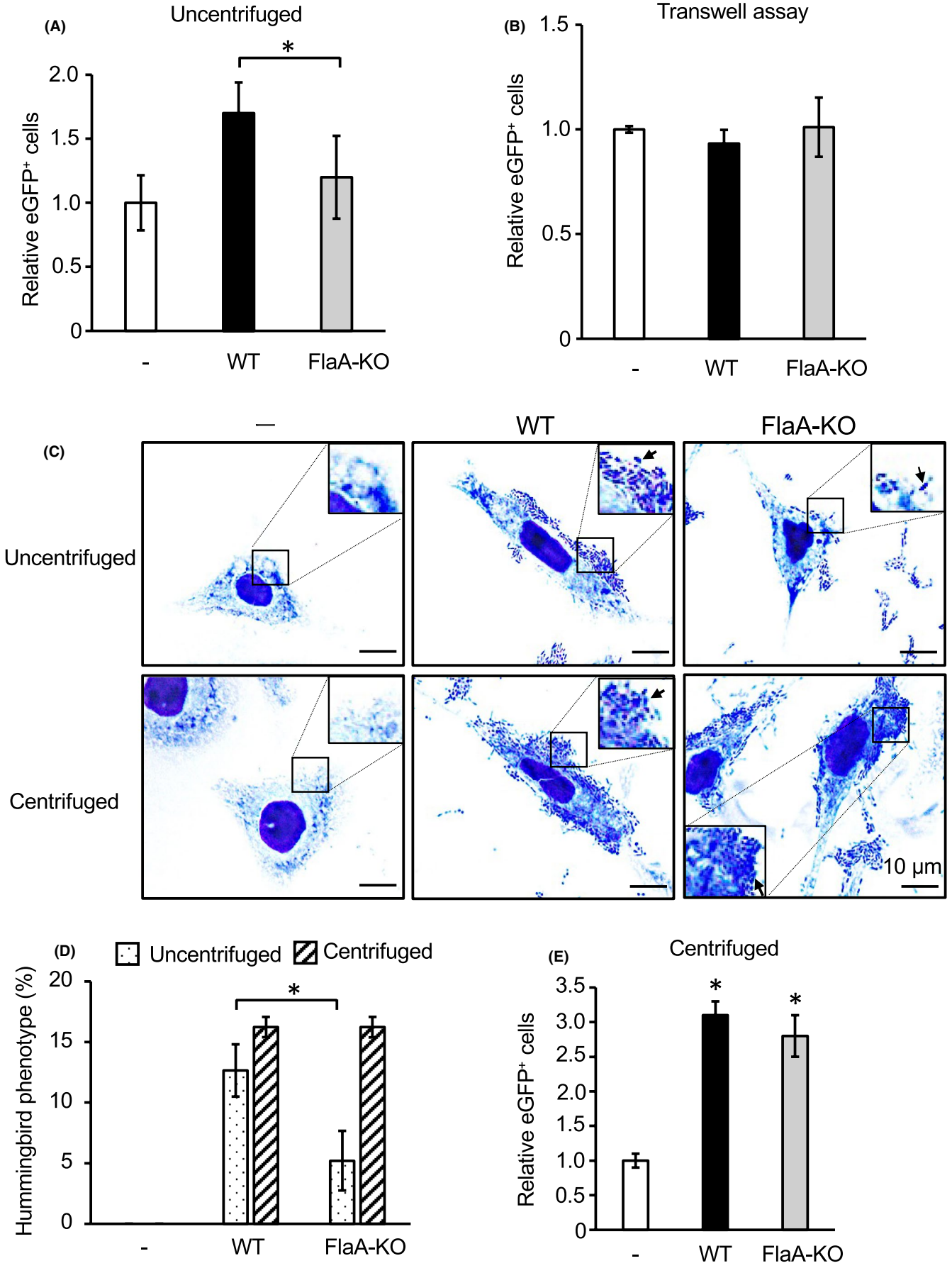
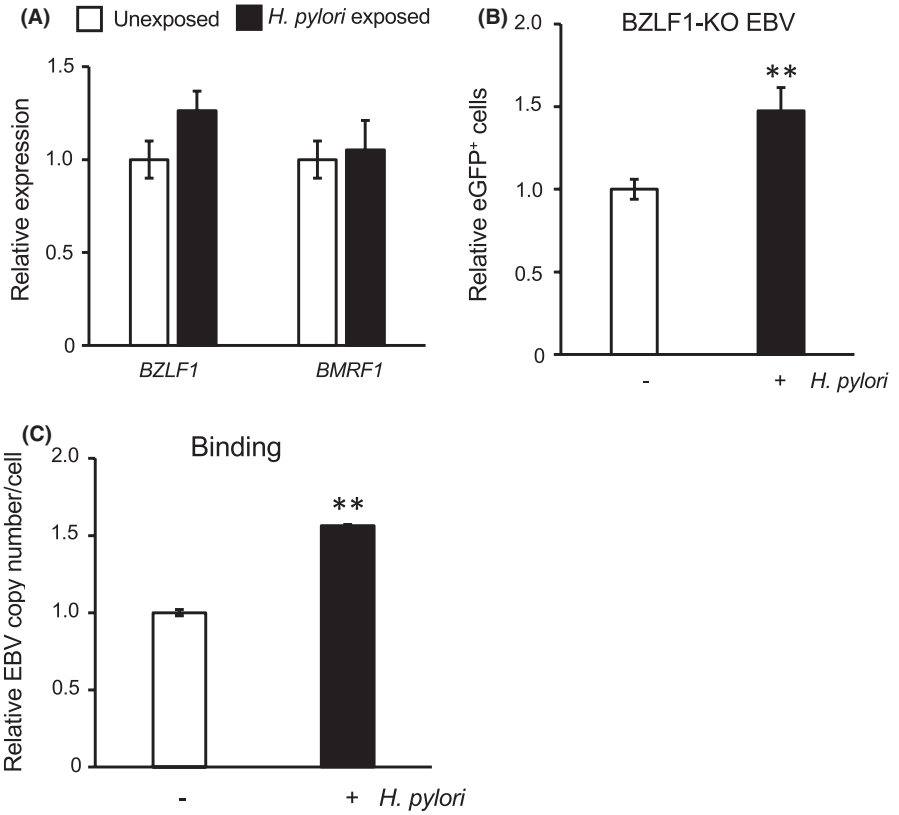

(A)
Uncentrifuged
Relative eGFP+ cells
2.0
1.5
1.0
0.5
0
*
-
WT
FlaA-KO
(B)
Transwell assay
Relative eGFP+ cells
1.5
1.0
0.5
0
-
WT
FlaA-KO
(C)
—
WT
FlaA-KO
Uncentrifuged
Centrifuged
10 μm
(D)
Uncentrifuged
Centrifuged
Hummingbird phenotype (%)
20
15
10
5
0
*
-
WT
FlaA-KO
(E)
Centrifuged
Relative eGFP+ cells
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
-
WT
FlaA-KO

FIGURE 2 *Helicobacter pylori*-mediated promotion of EBV infection is initiated by direct contact between bacteria and cells. A, Expression of eGFP in epithelial cells exposed to wild-type or FlaA-KO *H. pylori*. B, Expression of eGFP in epithelial cells through blocking bacterial contact with cells using a transwell insert. C, Giemsa staining of epithelial cells showing a hummingbird phenotype and bacteria attached to epithelial cells under uncentrifuged (upper) and centrifuged (lower) conditions. Part of each figure is enlarged and shown in a small frame in the corner so that *H. pylori* adhesion to cells can be observed, where arrows indicate stained *H. pylori* cells. D, Detection of the hummingbird phenotype under noncentrifuged (dotted area) and centrifuged (hatched area) conditions. E, Frequency of eGFP-positive EBV-infected cells measured by FACS analysis. Both wild-type and FlaA-KO bacteria were attached to epithelial cells via centrifugation. AGS cells were used in all experiments. $^{*}P < .05$. White column: unexposed cells, black column: *H. pylori*-exposed cells, WT: wild-type *H. pylori*, gray column: FlaA-KO *H. pylori*-exposed cells. All experiments were performed 3 times and the average value and SD were described
[Correction added on 28 Sept 2021, after first online publication: Figure 2D has been corrected.]

FIGURE 3 *Helicobacter pylori* exposure enhanced EBV infection by increasing virus attachment. A, *H. pylori* exposure does not induce viral lytic gene expression. Relative EBV mRNA expression was calculated based on the EBV copy number. B, *H. pylori* exposure enhanced the infection of the BZLF1 KO EBV strain to gastric epithelial cells. C, EBV binding assay after treatment of cells with *H. pylori*. AGS cells were used in all experiments. $^{**}P < .01$. White column: unexposed cells, black column: *H. pylori*-exposed cells. All experiments were performed 3 times and the average value and SD were described



3.4 | *Helicobacter pylori* enhances EBV infection of epithelial cells through induction of viral receptors

Total RNAs were extracted from both AGS cells and MKN28 cells after treatment with *H. pylori* for 7 h. *H. pylori* infection did not enhance the mRNA expression levels of *NRP1*, *ITGAV*, *ITGB5*, *ITGB6*, and *ITGB8*, but significantly increased the mRNA expression levels of *EphA2* and *NMHC-IIA* in AGS cells and MKN28 cells (Figures S3A,B, and 4A). FACS analysis also showed that *H. pylori* exposure increased the expression of EphA2 and NMHC-IIA proteins (Figure 4B). Moreover, immunoblotting of fractionated cell membrane proteins showed that expression of EphA2 and NMHC-IIA protein was upregulated in wild-type *H. pylori*-exposed cells, but not in *cagPAI*-KO *H. pylori*-exposed cells (Figure 4C). Moreover, *H. pylori* treatment increased EphA2 protein expression by 2.8-fold (2.8 ± 0.2) (Figure S4A). To examine whether EphA2 and/or NMHC-IIA enhanced EBV infection by *H. pylori* exposure, we used siRNA against either *EPHA2* or *NMHC-IIA*. siRNA treatment significantly reduced the expression of *EphA2* and *NMHC-IIA* (Figure S4B). We used 2 sets of si*EPHA2* or si*NMHC-IIA*. In accordance with the reduction in *EPHA2* or *NMHC-IIA* mRNA, we observed reduction of EBV infection to *H. pylori*-exposed cells (Figures 4D and S4C,D). Therefore, both EphA2 and NMHC-IIA assisted EBV entry into epithelial cells.

4 | DISCUSSION

EBVaGC is a gastric cancer characterized by high methylation in host DNA and a high expression of PD-L1, and most patients have a history of *H. pylori* infection.[7,31] We reported that EBVaGC is frequently found at the atrophic border of chronic atrophic gastritis lesions caused by *H. pylori* infection, which is associated with strong lymphocyte infiltration.[11] In response to this, molecular biological analysis of EBV and *H. pylori* co-infection showed that EBV promoted the development of gastric cancer induced by *H. pylori* infection.[13] In the study, EBV infection induced promoter methylation in gastric

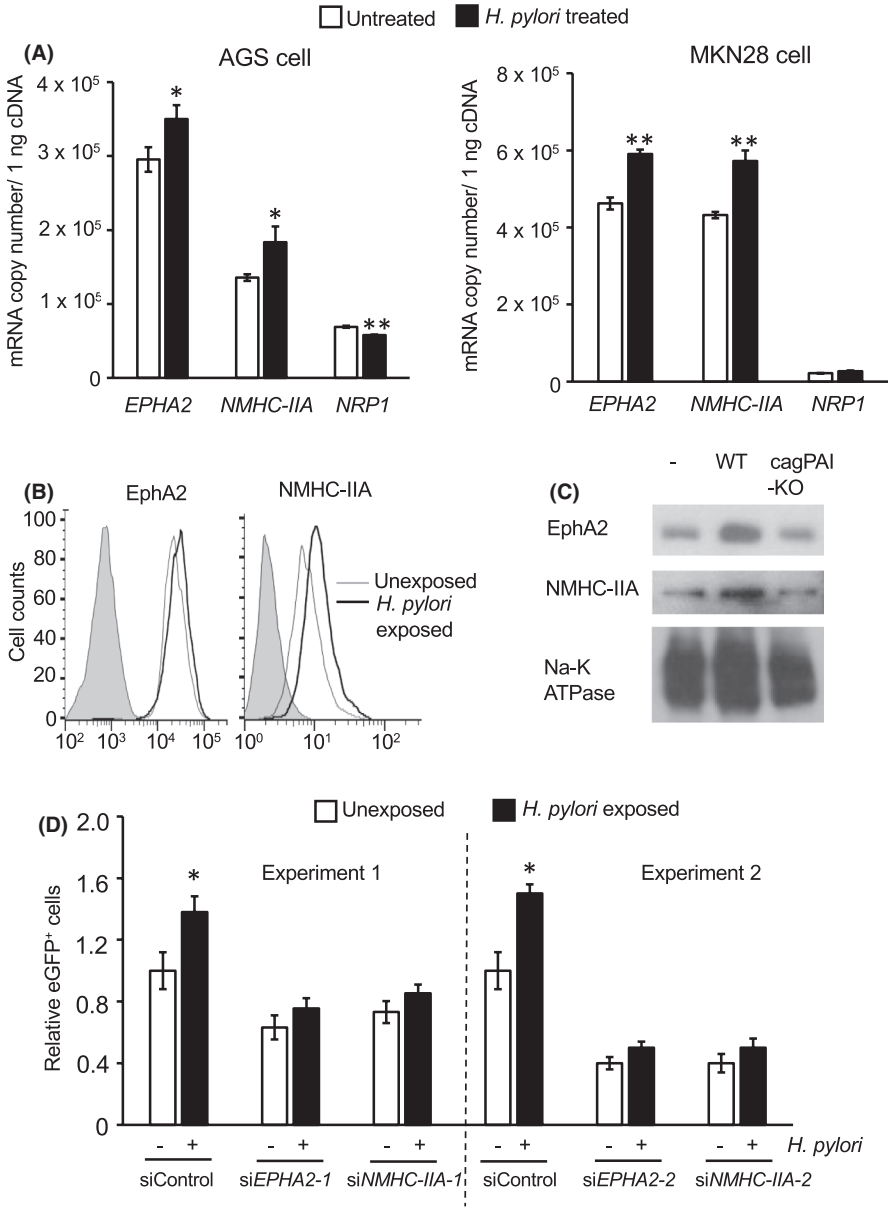


FIGURE 4 *Helicobacter pylori* exposure increased the efficiency of EBV infection through induction of viral accessory receptors. A, Expression of low-affinity EBV receptors after *H. pylori* exposure to gastric epithelial AGS cells (left) and MKN28 cells (right). B, Cell surface expression of EphA2 and NMHC-IIA on *H. pylori*-exposed AGS cells was confirmed using flow cytometry. C, EphA2 and NMHC-IIA expression in wild-type or *cagPAI*-KO *H. pylori*-exposed AGS cells. Na-K ATPase, a protein constitutively expressed on the cell membrane, is used as a control. D, Efficiency of EBV infection of AGS cells treated with siRNA against *EPHA2* or *NMHC-IIA*, followed by *H. pylori* exposure. Two different set of siRNAs were used. The eGFP-positive cells were counted using FACS analysis. $^{*}P < .05$, $^{**}P < .01$. All experiments were performed 3 times and the average value and SD were described

epithelial cells and reduced SHP1 expression, making SHP2 relatively susceptible to phosphorylated CagA, and to the stimulation of the CagA-SHP2-Ras cascade. In contrast, we wondered whether *H. pylori* infection promotes or suppresses EBV infection in gastric epithelial cells.

To clarify the above question, we conducted an experiment in which *H. pylori* was contacted with gastric epithelial cells and then infected with EBV. In this experiment, we used AGS cells, MKN28 cells, and GES-1 cells. AGS cells and MKN28 cells are gastric cancer cell lines with different cell differentiation and gene mutation patterns.[32,33] Conversely, GES-1 cells are non-malignant fetal gastric epithelial cells.[19,20] Exposure to *H. pylori* increased the number of EBV-infected cells and the number of viral genomes in all 3 cells (Figure 1B-D). We assume that this phenomenon is common to gastric epithelial cells. Our experimental results showed that the number of EBV-infected cells increased by *c*. 1.5-2 times after only 7 h of exposure to *H. pylori*. Because the infection of *H. pylori* into the stomach extends from childhood to several decades,[34] a continuous increase in the number of EBV-infected cells will occur during the life-long gastric inflammation caused by *H. pylori*. Moreover, activation of cell signaling pathways associated with inflammation often induces expression of viral transcription factor BZLF1, which is a molecular switch for EBV from latent infection to lytic replication that causes dissemination of newly produced virus.[14] Furthermore, de novo EBV infection of epithelial cells progresses to lytic proliferative infection rather than to latent stable infection.[35] Taken together, it can be speculated that the life-long *H. pylori* infection in the stomach increases the number of EBV-infected cells and promotes EBVaGC.

Subsequently, we conducted experiments to detail the molecular mechanism of the increase in the number of EBV-infected cells and the number of viral genomes due to *H. pylori* exposure.

We showed that the enhancement of EBV infection by *H. pylori* exposure did not depend on CagA or VacA, but on FlaA (Figures 1E and 2A). FlaA is a major component of flagellin, and the FlaA-KO *H. pylori* strain has reduced motility due to flagellar hypoplasia.[17] FlaA acts as a ligand for Toll-like receptor 5 (TLR5), but *H. pylori*'s FlaA induces much less inflammation than the flagellar component FliC.[36] When cells were incubated with FlaA-KO *H. pylori*, we did not observe any increase in the efficiency of EBV infection. However, when the FlaA-KO *H. pylori* strain was adhered to the cells via centrifugation, EBV infection was enhanced to the same extent as that observed with wild-type *H. pylori* (Figure 2C). These results suggested that FlaA-dependent promotion of EBV infection was not due to the stimulation of TLR5 signaling by FlaA, but to an increase in *H. pylori* attachment to cells. However, a previous report mentioned that *H. pylori* enhances EBV infection in gastric epithelial cells in a CagA-dependent manner.[12] In the report, *H. pylori* and epithelial cells were co-cultured for a longer period than in our study. We speculated that a large amount of the collapsed bacterial components possibly leaked into the culture medium, which might have stimulated gastric epithelial cells and enhanced EBV infection.

Helicobacter pylori enhanced EBV infection through cell contact rather than via CagA or VacA activity (Figures 1 and 2). In addition to CagA, *H. pylori* also injects ADP-Hepatose and bacterial DNA into cells using a type IV secretion system.[37-39] We showed that cells exposed to *cagPAI*-KO *H. pylori* did not enhance EBV infection (Figure 1E). This result suggests that bacterial type IV secretion system is, to some extent, involved in the enhancement of EBV infection by *H. pylori* exposure. It is also reported that *H. pylori* stimulates gastric epithelial cells by adhering bacterial OMVs to the epithelial cell membrane.[40] Therefore, *H. pylori* may enhance viral infection through multiple factors.

Helicobacter pylori treatment induced the hummingbird phenotype in 10%-15% of AGS cells (Figure 2D). Increase in bacterial cell contact also increased the hummingbird phenotype and EBV infection efficiency (Figure 2D,E). As the MOI of *H. pylori* infection was 100, it is expected that all cells were exposed to *H. pylori*. Nevertheless, only some cells showed the hummingbird phenotype. The reason may be that the hummingbird phenotype is frequently expressed in isolated cells and less likely in cells that are strongly attached to the surroundings. In addition, in our assay, the direct relationship between the hummingbird phenotype and EBV infection is unknown. This is because cells were exposed to *H. pylori*, washed with PBS containing antibiotics, and infected with EBV. The cells showing the hummingbird phenotype had disappeared when the efficiency of EBV infection was calculated 48 h after EBV infection.

In epithelial cells that do not express the high-affinity receptor CD21, EBV adheres to cells via gHgL and causes infection.[41] Infection of epithelial cells of EBV without high-affinity receptors is carried out via low-affinity receptors, such as EphA2 and NMHC-IIA.[4-6] Upon contact with gastric epithelial cells, *H. pylori* increased EphA2 and NMHC-IIA expression and enhanced receptor-dependent EBV infection (Figures 3 and 4A,B). Conversely, the suppression of EphA2 and NMHC-IIA expression reduced EBV infection during *H. pylori* treatment (Figure 4D). It is possible that the interaction between EphA2 and NMHC-IIA may facilitate epithelial cell infection of EBV, because in Kaposi sarcoma-associated herpesvirus, which is the other human gammaherpesvirus, the binding of EphA2 to phosphorylated NMHC-IIA initiates infection.[42-44] *H. pylori* attachment to cells will increase the expression of EphA2 and NMHC-IIA and promote assembly of the 2 proteins, resulting in enhancement of EBV infection.

It has been shown that cell surface localization of EphA2 is important for EBV infection.[45] We have shown that cells exposed to *H. pylori* increased EphA2 expression up to 2.8-fold at the protein level, while 1.3-fold at the mRNA level (Figure 4A and Figure S4A). The increase in EphA2 protein in the membrane fraction is more pronounced than the increase in *EPHA2* mRNA extracted from whole cells, suggesting that *H. pylori* exposure may facilitate the intracellular relocalization of low-affinity EBV receptors.

Our results showed that the treatment of cells with si*EPHA2* and si*NMHC-IIA* canceled *H. pylori*-exposure-mediated enhancement of EBV infection. Based on these results, EphA2 and NMHC-IIA may also interact with EBV infection, and further investigation is necessary.

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DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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