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*J Immunol* 2013; 190:6626-6634; Prepublished online 17 May 2013; doi: 10.4049/jimmunol.1203330

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http://www.jimmunol.org/content/suppl/2013/05/17/jimmunol.1203330.DC1

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Helicobacter pylori Infection Inhibits Phagocyte Clearance of Apoptotic Gastric Epithelial Cells

Diane Bimczok,* Lesley E. Smythies,* Ken B. Waites,† Jayleen M. Grams,‡ Richard D. Stahl,† Peter J. Mannon,* Shajan Peter,* C. Mel Wilcox,* Paul R. Harris,§ Soumita Das,§ Peter B. Ernst,¶ and Phillip D. Smith*,#

Increased apoptotic death of gastric epithelial cells is a hallmark of Helicobacter pylori infection, and altered epithelial cell turnover is an important contributor to gastric carcinogenesis. To address the fate of apoptotic gastric epithelial cells and their role in H. pylori mucosal disease, we investigated phagocyte clearance of apoptotic gastric epithelial cells in H. pylori infection. Human gastric mononuclear phagocytes were analyzed for their ability to take up apoptotic epithelial cells (AECs) in vivo using immunofluorescence analysis. We then used primary human gastric epithelial cells induced to undergo apoptosis by exposure to live H. pylori to study apoptotic cell uptake by autologous monocyte-derived macrophages. We show that HLA-DR+ mononuclear phagocytes in human gastric mucosa contain cytokeratin-positive and TUNEL-positive AEC material, indicating that gastric phagocytes are involved in AEC clearance. We further show that H. pylori both increased apoptosis in primary gastric epithelial cells and decreased phagocytosis of the AECs by autologous monocyte-derived macrophages. Reduced macrophage clearance of apoptotic cells was mediated in part by H. pylori–induced macrophage TNF-α, which was expressed at higher levels in H. pylori–infected, compared with uninfected, gastric mucosa. Importantly, we show that H. pylori–infected gastric mucosa contained significantly higher numbers of AECs and higher levels of nonphagocytosed TUNEL-positive apoptotic material, consistent with a defect in apoptotic cell clearance. Thus, as shown in other autoimmune and chronic inflammatory diseases, insufficient phagocyte clearance may contribute to the chronic and self-perpetuating inflammation in human H. pylori infection. The Journal of Immunology, 2013, 190: 6626–6634.

Increased apoptosis of gastric epithelial cells is a hallmark of human Helicobacter pylori gastritis (1). Multiple pathways and bacterial virulence factors that cause H. pylori–induced apoptosis have been identified, including enhanced production of reactive oxygen species (2), upregulation of Fas receptor (3), disruption of mitochondrial membranes by H. pylori VacA (4, 5), and cross-linking of MHC class II molecules by H. pylori urease (6). Enhanced apoptosis during prolonged H. pylori infection provides a persistent stimulus for epithelial cell proliferation, a key process in the cascade of carcinogenic events that promote gastric cancer (1, 7). Microbe-stimulated apoptosis also may cause the induction of Th17 cells (8), crucial cellular contributors to gastric pathology in H. pylori infection (9). However, despite the contribution of H. pylori–induced epithelial cell apoptosis to gastric inflammation and carcinogenesis, the fate of apoptotic epithelial cells (AECs) in inflamed gastric mucosa is not known.

Macrophage and dendritic cell (DC) removal of dying and dead cells prevents the release of proinflammatory signals (10) and is a prerequisite for the maintenance of tissue homeostasis (11). Impaired removal of apoptotic cells allows the dying cell to progress to secondary necrosis, resulting in the loss of membrane integrity and the release of intracellular proinflammatory molecules and autoantigens (12). Defective removal of apoptotic cells has been linked to autoimmune syndromes and chronic inflammatory diseases such as systemic lupus erythematosus (SLE) (13). However, in the gastrointestinal tract, apoptotic cell removal occurs not only by phagocytic uptake (14, 15) but also by luminal extrusion independent of phagocyte activity (16) or a combination of both (17). Also, the mechanisms of AEC elimination differ between species and between individual regions of the gastrointestinal tract (15, 18). Notably, AEC removal in the human gastric mucosa has received little investigative attention.

We previously isolated and characterized HLA-DR+/CD11c− mononuclear phagocytes (MNPs) from human gastric mucosa and showed that these cells take up H. pylori bacteria and promote Th1 responses to H. pylori (19, 20). In this study, we show that normal human gastric MNPs also are involved in the clearance of gastric
epithelial cells that have undergone apoptosis. However, prior exposure of phagocytes to *H. pylori* impairs the cells’ ability, in a TNF-α–dependent manner, to subsequently phagocytose *H. pylori*-treated AECs, resulting in an accumulation of nonphagocytosed apoptotic material in the gastric lamina propria of *H. pylori*-infected individuals. Thus, *H. pylori* upregulates programmed cell death of gastric epithelial cells and downregulates programmed cell removal of AECs by macrophages, features that may provide a potent source of autoimmune stimulatory activity in chronic *H. pylori* infection.

**Materials and Methods**

**Tissue specimens**

Gastric tissue specimens for cell isolation and histological analyses were obtained with Institutional Review Board approval and informed consent from adult subjects at the University of Alabama at Birmingham undergoing elective gastric bypass for obesity or diagnostic esophagogastroduodenoscopy. Abnormal gastric biopsies or 1 g gastric mucosa obtained from gastric bypass donors were minced with a scalpel blade and digested for 1 h at 37°C, 200 rpm, with a digestion solution containing RPMI 1640, collagenase (0.5 FALGPA units/ml; Sigma-Aldrich, St. Louis, MO), dispase (1.25 U/ml; Roche, Mannheim, Germany), DNAse (0.2 mg/ml; Sigma-Aldrich), and BSA (0.3%; Fisher, Fair Lawn, NJ). Recovered cells were suspended in F12K medium containing 10% FBS, amphotericin (125 ng/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml) and plated on collagen-I–coated plates (Biocoat; BD Biosciences, San Jose, CA). Nonadherent cells were removed after 18 h of culture. Phenotypic analysis of gastric epithelial cells and then digested using collagenase solution (0.5 FALGPA units/ml; Sigma-Aldrich) and digested for 1 h at 37°C, 200 rpm, with a digestion protocol described by Smoot et al. (21). Briefly, 10–20 gastric biopsies or 1 g gastric mucosa obtained from gastric bypass donors were minced with a scalpel blade and digested for 1 h at 37°C, 200 rpm, with a digestion solution containing RPMI 1640, collagenase (0.5 FALGPA units/ml; Sigma-Aldrich, St. Louis, MO), dispase (1.25 U/ml; Roche, Mannheim, Germany), DNAse (0.2 mg/ml; Sigma-Aldrich), and BSA (0.3%; Fisher, Fair Lawn, NJ). Recovered cells were suspended in F12K medium containing 10% FBS, amphotericin (125 ng/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml) and plated on collagen-I–coated plates (Biocoat; BD Biosciences, San Jose, CA). Nonadherent cells were removed after 18 h of culture. Phenotypic analysis of gastric epithelial cells was performed using anti–ZO-1 (clone 1), anti-cytokeratin (CAM5.2, speciﬁc for mucosal epithelial cell associated Moll’s peptides #7 and #8), anti-human CD104 (439-9B), anti-human CD90 (5E10), anti-human CD45 (CD45.2; BD Biosciences). Titration experiments with *H. pylori* were performed with fresh *H. pylori* (2 × 10^6^ bacteria/ml) or camptothecin (CPT; 5 μM; Sigma-Aldrich). Apoptosis was determined by Annexin-V FITC binding (Southern Biotechnologies, Birmingham, AL), and necrosis was determined by propidium iodiode (PI) uptake and LysotrackerRed labeling (both from Invitrogen). Cells were analyzed using an LSRII flow cytometer (BD Biosciences) and FlowJo 7.5.5. software (Tree Star, Ashland, OR).

**Apoptotic cell uptake by primary gastric MNPs**

MNPs in cytosplasms of gastric lamina propria cells or cryosections prepared from healthy gastric tissue were labeled with anti–HLA-DR–PE or –Cy3. Apoptotic DNA was detected by the TUNEL using the Fluorescein In situ Cell Death Detection kit (Roche, Mannheim, Germany), and epithelial material was detected with an anti–cytokeratin-FITC Ab (CAM5.2; BD Biosciences). For immunofluorescence labeling, slides were fixed in ice cold acetone, washed in PBS/G0.05% Tween-20, blocked (Dako Protein Block; DakoCytomation) and then incubated with the appropriate Abs. For

![Image](http://www.jimmunol.org/DownloadedFrom/6627/)
quantitative analysis of apoptotic cell uptake in gastric mucosa, formalin-fixed, paraffin-embedded tissue from nine H. pylori–infected and seven healthy adults organized on a single slide as a tissue microarray (22) was treated with proteinase K (20 μg/ml; Millipore, Billerica, MA) for 20 min at room temperature, subjected to Ag retrieval with Dako Target Retrieval Solution (DakoCytomation) at 98°C for 20 min, labeled for apoptotic material using the ApopTag Plus Kit (Millipore) together with an anti-Digoxigenin-FITC Fab fragments (Roche), and then labeled for MNPs using an anti–HLA-DR (LN-3; Abcam, Cambridge, MA) and a goat antimouse-Cy3 secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). In all experiments, cell nuclei were stained with DAPI. Samples were analyzed on a Nikon Eclipse T2000-U fluorescent microscope (Nikon) equipped with a CoolSnap ES digital camera (Roper Scientific, Martinstried, Germany) and NIS Elements BR2.30 (Nikon). HLA-DR* cells, TUNEL* particles, and DAPI* cell nuclei were counted using the taxonomy feature of the software, with the investigator blinded to the identity of the samples.

**AEC uptake in vitro**

Gastric epithelial cells treated with H. pylori or CMP to induce apoptosis, as described above, were labeled with CellTracker Green CMFDA (2 μg/ml; Invitrogen, Eugene, OR) for 1 h at 37°C and harvested using trypsin-EDTA. Simultaneously, macrophages were labeled with CellTracker Red CMTPX (2 μg/ml; Invitrogen) for 1 h, washed, and then harvested using a cell scraper. Macrophages and epithelial cells (1 × 10⁴ each) then were cocultured for 2.5 h at 37°C. Control cultures were maintained at 4°C. To inhibit macrophage phagocytosis, macrophages were pretreated with cytochalasin D (1 μM) for 45 min prior to coculture, with continuing exposure of the cells to cytochalasin D during coculture. To block binding of apoptotic cells to macrophages, epithelial cells were pretreated with recombinant RGD-thrombospondin repeat (TSR) region of the brain angiogenesis inhibitor 1 (BAI1) ectodomain (residues 202–585), prepared, and purified as described previously (23, 24) for 15 min prior to coculture, with continuing exposure of the cells to BAI1 during coculture. Macrophage phagocytic activity also was analyzed by feeding the macrophages fluorescein-labeled latex beads (Fluoresbrite YM Microspheres; Polysciences, Warrington, PA) or GFP-labeled H. pylori (MOI 50). After culture, cells were washed, fixed in Cytofix (BD Biosciences), and analyzed on an LSRII flow cytometer (BD Biosciences). For confocal analysis, cells were imaged on an LSM 710 Laser Confocal Scanning Microscope equipped with Zen 2008 4.2.3 software (Zeiss, Thornwood, NY).

**Macrophage treatment**

To determine the influence of H. pylori on the ability of macrophages to engulf apoptotic cells, macrophages were treated with H. pylori 60190 at an MOI of 10 for 6 h prior to the phagocytosis experiment. Macrophages also were treated with cell-free supernatants from cultures of H. pylori–treated macrophages or rhTNF-α (10 ng/ml; R&D Systems). In addition, macrophages were treated with H. pylori as above plus neutralizing anti-TNF-α Ab (0.02–2 μg/ml; R&D Systems) or an isotype control Ab. The amount of TNF-α in the culture supernatants was determined by ELISA (R&D Systems).

**Quantitative RT-PCR**

RNA was isolated from snap-frozen gastric biopsy samples using the RNeasy Minikit (Qiagen, Valencia, CA), and cDNA was generated using iScript Reverse Transcriptase (Bio-Rad, Hercules, CA). Target genes were amplified in 25 μl reactions containing TaqMan Universal PCR Master Mix and primer probe sets for TNF-α (FAM/MGB, Hs00174129_m1, ref. seq. NM_000594.2), GAPDH (VIC/TAMRA, ref. seq. X03205.1), and 18s rRNA (VIC/TAMRA, ref. seq. NM_002046.3), all from Applied Biosystems (Foster City, CA). Real-time PCR reactions were run for 40 cycles (15 s, 95°C; 60 s, 60°C) on a Chromo4 PCR system (Bio-Rad) and analyzed with Opticon Monitor software, version 3.1. Relative expression rates were calculated using the Pfaffl method (25) with gastric tissue 18s rRNA as the reference gene. Results are presented as the geometric mean of both reactions.

**Study data and statistical analysis**

Data were analyzed using Microsoft Excel 2003 and Analyze-it for Excel, version 1.73 (Microsoft). Results are presented as mean ± SEM. Differences between values were analyzed for statistical significance by the two-tailed Student t test, unless stated otherwise. Differences were considered significant at p < 0.05.

**Results**

**MNPs in human gastric mucosa engulf apoptotic gastric epithelial cells**

The mechanism by which AECs are cleared in human gastric mucosa is largely unknown. In this study, we analyzed whether HLA-DR* MNPs are involved in the removal of apoptotic gastric epithelial cells. Cytosplasm of gastric lamina propria mononuclear cells obtained from H. pylori–negative donors were stained for HLA-DR and, using the TUNEL method, for apoptotic DNA. Microscopic image analysis (Fig. 1A, 1B) showed that 10–15% of the gastric MNPs contained TUNEL* inclusions in their cytoplasm, indicating the cells had taken up apoptotic material.

**AEC uptake in vitro**

Gastric epithelial cells treated with H. pylori or CMP to induce apoptosis, as described above, were labeled with CellTracker Green CMFDA (2 μg/ml; Invitrogen, Eugene, OR) for 1 h at 37°C and harvested using trypsin-EDTA. Simultaneously, macrophages were labeled with CellTracker Red CMTPX (2 μg/ml; Invitrogen) for 1 h, washed, and then harvested using a cell scraper. Macrophages and epithelial cells (1 × 10⁴ each) then were cocultured for 2.5 h at 37°C. Control cultures were maintained at 4°C. To inhibit macrophage phagocytosis, macrophages were pretreated with cytochalasin D (1 μM; Sigma-Aldrich) for 45 min prior to coculture, with continuing exposure of the cells to cytochalasin D during coculture. To block binding of apoptotic cells to macrophages, epithelial cells were pretreated with recombinant RGD-thrombospondin repeat (TSR) region of the brain angiogenesis inhibitor 1 (BAI1) ectodomain (residues 202–585), prepared, and purified as described previously (23, 24) for 15 min prior to coculture, with continuing exposure of the cells to BAI1 during coculture. Macrophage phagocytic activity also was analyzed by feeding the macrophages fluorescein-labeled latex beads (Fluoresbrite YM Microspheres; Polysciences, Warrington, PA) or GFP-labeled H. pylori (MOI 50). After culture, cells were washed, fixed in Cytofix (BD Biosciences), and analyzed on an LSRII flow cytometer (BD Biosciences). For confocal analysis, cells were imaged on an LSM 710 Laser Confocal Scanning Microscope equipped with Zen 2008 4.2.3 software (Zeiss, Thornwood, NY).

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similar proportion of gastric MNPs contained epithelial cell-specific cytokeratin inclusions consistent with the epithelial origin of the apoptotic material. The TUNEL and cytokeratin inclusions were discrete and varied in size, suggesting that fragments of different sizes were ingested. Importantly, TUNEL and cytokeratin inclusions also were detected in HLA-DR MNPs in gastric tissue sections (Fig. 1C), confirming that AEC phagocytosis occurs in situ and is not an artifact associated with cell isolation. Thus, under steady-state conditions, apoptotic gastric epithelial cells are cleared at least in part by MNPs in human gastric mucosa.

H. pylori interaction with primary human gastric epithelial cells triggers epithelial cell apoptosis and subsequent clearance by MNPs

Human gastric H. pylori infection is associated with increased epithelial cell apoptosis (1), and direct induction of apoptotic cell death by H. pylori bacteria has been shown in various gastric epithelial cell cultures. This study investigated the role of MNPs in the clearance of apoptotic gastric epithelial cells induced by H. pylori.

**FIGURE 3.** Apoptotic gastric epithelial cells are phagocytosed by monocyte-derived macrophages through a phosphatidylserine-dependent pathway. (A and B) Gastric epithelial cell cultures were treated with H. pylori (Hp), CMP (5 μM), or with medium alone for 6–8 h to induce apoptosis and then labeled with CMFDA (green). Monocyte-derived macrophages (MΦ) were stained with CMTPX (red), and equal numbers of stained MΦ and AECs were cocultured at 4˚C or 37˚C for 2.5 h. (A) MΦ were analyzed by confocal microscopy (top right panels) or by flow cytometry (bottom right panels). Scale bar, 10 μm. Representative data; left panels show gating strategy. (B) Cumulative data (mean ± SEM) from 3 (untreated) or 10 (Hp, CMP) experiments. (C) To block uptake, MΦ were pretreated with cytochalasin D (CytD; 1 μg/ml) for 45 min prior to coculture with H. pylori–treated AECs (cytochalasin D also present during coculture). Representative (left panel) and cumulative data (right panel); n = 3. (D and E) Gastric epithelial cells with apoptosis induced by CMP (CMP-AEC) or H. pylori (Hp-AEC) were treated for 15 min with control GST or recombinant BAI1 RGD-TSR, which neutralizes surface phosphatidylserine, and then cultured with MΦ. (D) Representative (left panel) and cumulative data (right panel) from four experiments with 10 μg/ml of BAI1 RGD-TSR or control GST. Phagocytosis is expressed as percent MΦ that contained BAI1-treated AECs relative to GST-treated AECs (100%). (E) GST and BAI1 used at the indicated concentration, mean ± SEM of two (CMP) or three (H. pylori) experiments. SSC, Side scatter.
epithelial cell lines (2–6). In this study, we investigated whether primary human gastric epithelial cells undergo cell death post-infection with live H. pylori and are phagocytosed by autologous macrophages in vitro. The cultured gastric epithelial cells expressed epithelial cell–specific markers, including cytokeratin and tight junction protein ZO-1 (Supplemental Fig. 1). As shown in Fig. 2A and 2B, incubation of epithelial monolayers for 6–8 h with H. pylori (2 × 10^7 bacteria/ml; MOI ≈25), compared with monolayers incubated in media alone, induced a marked increase in the proportion of apoptotic (Annexin-V+/PI−) epithelial cells (56.0 ± 4.9 versus 14.7 ± 2.0%; n = 9; p < 0.001) but only a small increase in necrotic (Annexin-V+/PI+) epithelial cells (16.7 ± 3.2 versus 12.5 ± 2.1%; n = 9; p = 0.7). In addition, lysosomal membrane permeabilization, an early event of necrosis, was only detected after 24 and 48 h, but not after 6 and 12 h, of epithelial cell exposure to H. pylori (Supplemental Fig. 2A). Thus, the majority of the epithelial cells exposed to H. pylori for 6 h underwent apoptosis rather than necrotic cell death. Notably, the relatively high background in the PI channel in Fig. 2A was due to binding of bacteria to the cell surface instead of nuclear staining of membrane-permeable necrotic cells (Supplemental Fig. 2C), whereas ethanol-treated necrotic cells were present exclusively in the PI-high gate (Supplemental Fig 2D). In agreement with earlier reports (26), H. pylori induction of apoptosis in primary gastric epithelial cells was associated with expression of VacA, as VacA-deficient mutants of the 60190 strain did not induce a significant level of apoptosis (Supplemental Fig. 2D, 2E). In contrast, CagA– and urease-deficient mutants were each as capable of inducing apoptosis as the wild-type strain. Interestingly, primary gastric epithelial cells were significantly more susceptible to H. pylori–induced apoptosis than cells of the AGS gastric cell line (Supplemental Fig. 2A, 2B), and, surprisingly, CMP was less efficient than H. pylori at inducing cell death in primary gastric epithelial cells within 6 h (Fig. 2A, 2B). At 24 h, the epithelial monolayers incubated with both H. pylori and CMP, but not the control monolayer, showed prominent architectural distortion and disrupted intercellular ZO-1, consistent with epithelial cell death (Fig. 2C).

We next generated monocyte-derived macrophages and labeled the macrophages with a red CellTracker dye (CMTPX). Epithelial cells obtained from matched donors were cultured with H. pylori (2 × 10^7 bacteria/ml) or CMP (5 μM) for 6–8 h to induce apo-

**FIGURE 4.** H. pylori stimulation inhibits macrophage phagocytic activity for apoptotic gastric epithelial cells. (A and B) Monocyte-derived macrophages (MoΦ) were treated with H. pylori (Hp; MOI 10) for 6–8 h or left untreated and then stained with CMTPX (red). Gastric epithelial cells were cultured for 3 d on collagen-coated plates, treated with H. pylori (2 × 10^5/ml) or CMP (5 μM) for 6–8 h, and labeled with CMFDA (green). MoΦ and AECs then were harvested and incubated at a ratio of 1:1 at 37°C for 2.5 h to allow MoΦ engulfment of epithelial cells. Representative FACS plots (A) and individual values (diamonds) and means (bars) (B); n = 10. (C) Phagocytosis of AECs after MoΦ pretreatment with different concentrations of live H. pylori; n = 3, mean ± SEM, one-way ANOVA with Tukey’s post hoc test. *p ≤ 0.05. (D and E) Soluble mediators released by H. pylori–treated MoΦ inhibit AEC phagocytosis. Cell-free supernatants (SN) of MoΦ cultured with or without H. pylori for 6–8 h were added to untreated MoΦ for 6 h prior to the phagocytosis experiment. Representative FACS plots (D) and individual values (diamonds) and means (bars) (E); n = 6. (F and G) H. pylori–induced inhibition of MoΦ phagocytosis is not specific to the uptake of AECs. MoΦ were pretreated with either H. pylori, culture SN from H. pylori–treated MoΦ, or media and then assayed for phagocytosis of Hp-AEC, CMP-AEC, GFP-labeled H. pylori (MOI 50), or YG fluorescent latex beads (40 beads/cell). (F) The relative efficiency of H. pylori–treated MoΦ was determined by comparing the phagocytosis of treated MoΦ to that of untreated MoΦ for the different targets; mean ± SEM, n = 4. (G) Representative FACS plots for the data shown in (F).
ptosis or with media alone and then labeled with a green CellTracker dye (CMFDA). After equivalent numbers of red-labeled monocyte-derived macrophages and green-labeled \textit{H. pylori}–treated AECs had been cultured for 2.5 h at 37°C, 32.5 ± 3.2% of the macrophages had acquired green fluorescence, indicating apoptotic cell phagocytosis (Fig. 3A, 3B). In contrast, flow cytometric analysis of control cultures incubated at 4°C showed only a small population of green fluorescent macrophages (7.3 ± 1.3%; n = 10; p < 0.001 for 37°C versus 4°C) after coculture, likely representing macrophages that had bound epithelial cells on their surface. A slightly lower proportion of macrophages phagocytosed CMP-treated apoptotic gastric epithelial cells (23.7 ± 5.5% at 37°C versus 6.3 ± 1.2% at 4°C; n = 10; p = 0.01), consistent with the lower rate of apoptotic cell death after this treatment (Fig. 3A, 3B). The presence of green particles inside red macrophages, indicating phagocytosed apoptotic gastric epithelial cells, was confirmed by confocal microscopy (Fig. 3A, top panels).

To further investigate the mechanism of macrophage uptake of AECs, we examined the effect of cytochalasin D, an inhibitor of actin polymerization and thus engulfment, on macrophage phagocytosis of apoptotic gastric epithelial cells. Macrophages treated with cytochalasin D (1 μg/ml) before and during the incubation with \textit{H. pylori}–treated gastric epithelial cells were significantly less capable of phagocytosing the epithelial cells (n = 3; p < 0.001) (Fig. 3C). Because the expression of phosphatidylserine on the outer leaflet of the plasma membrane is an essential macrophage engulfment signal (12), we assessed macrophage phagocytosis of apoptotic gastric epithelial cells after blockade of surface phosphatidylserine using the recombinant soluble fragment RGD-TSR of BAII1. BAII1 is a phospholipid receptor with a phosphatidylserine binding domain that consists of five TSR motifs (23, 24). Treatment of apoptotic gastric epithelial cells with the recombinant RGD-TSR region of BAII1 prior to coculture with the macrophages reduced, but did not completely abrogate, macrophage phagocytosis of the epithelial cells in a dose-dependent manner (Fig. 3D, 3E). A similar level of suppression was reported in our earlier study on BAII1-mediated bacterial uptake (23). Notably, higher doses of the RGD-TSR caused significant macrophage cell death, likely due to the glycerol stabilizer present in the peptide preparation. These data support previous reports of phosphatidylserine exposure as an engulfment signal molecule that mediates macrophage phagocytosis of apoptotic gastric epithelial cells.

\textit{H. pylori} interaction with MNPs inhibits engulfment of apoptotic gastric epithelial cells through a TNF-α–dependent mechanism

In the \textit{H. pylori}–infected gastric mucosa, phagocytes likely encounter both \textit{H. pylori} bacteria and AECs. \textit{H. pylori} have been identified in close contact with lamina propria macrophages (27), and we have shown that human gastric macrophages contain \textit{H. pylori} surface proteins (28). \textit{H. pylori} inhibits its own uptake by macrophages (29), raising the possibility that macrophage interaction with \textit{H. pylori} in the gastric mucosa might interfere with the uptake of AECs. Therefore, we investigated the effect of \textit{H. pylori} on MNP uptake of apoptotic gastric epithelial cells in vitro. Monocyte-derived macrophages were pretreated with live \textit{H. pylori} for 6 h, incubated with \textit{H. pylori}–treated apoptotic gastric epithelial cells for 2.5 h, and then analyzed for engulfed epithelial cells. Exposure of the macrophages to live \textit{H. pylori} caused a dose-dependent reduction in subsequent engulfment of the AECs with a 38% reduction in phagocytosis at an MOI of 10 (35.6 ± 3.5 to 22.1 ± 2.2%; n = 10; p = 0.006) (Fig. 4A–C).

\textbf{FIGURE 5.} TNF-α mediates reduced apoptotic cell clearance by \textit{H. pylori}–treated macrophages (MΦ). (A) TNF-α concentrations in culture supernatants of MΦ treated with live \textit{H. pylori} (MOI = 10) or medium for 6 h were determined by ELISA; n = 4. (B) Human \textit{H. pylori} infection increases gastric expression of TNF-α. Gastric biopsies obtained from noninfected or \textit{H. pylori}–infected human subjects were analyzed for TNF-α gene expression by quantitative real-time PCR; n = 8. (C) MΦ pretreatment with rhTNF-α (10 ng/ml) inhibits phagocytosis of AECs. Representative FACS plots (left panels) and cumulative data from four experiments; mean ± SEM (right panel). (D) Neutralization of TNF-α in \textit{H. pylori}–treated MΦ cultures reverses the suppressive effect of \textit{H. pylori} on MΦ clearance of AECs. MΦ were pretreated for 6–8 h with both \textit{H. pylori} and anti–TNF-α Abs (or isotype control) prior to coculture with Hp–AECs. Representative FACS plots (top panels) and cumulative data from three experiments; mean ± SEM (bottom panel).

We next investigated whether the reduced macrophage phagocytosis caused by \textit{H. pylori} was a direct effect of the bacteria or...
due to a soluble mediator released from the macrophages in response to *H. pylori*. Monocyte-derived macrophages were cultured with *H. pylori* for 6 h, the cell- and bacteria-free supernatants were harvested and added to fresh macrophages for 6 h, and the cells’ phagocytic activity for apoptotic gastric epithelial cells was analyzed as above. As shown in Fig. 4D and 4E, culture supernatants from *H. pylori*-treated macrophages (compared with supernatants from macrophages incubated in media alone) significantly suppressed the mean level of macrophage engulfment of the epithelial cells by 35% (from 45.0 ± 5.4 to 28.3 ± 3.0%; n = 6; p = 0.03). Culture supernatants from the same concentrations of live *H. pylori* alone did not impair macrophage phagocytosis (data not shown). These findings implicate a soluble mediator released by the *H. pylori*-treated macrophages in the reduced phagocytosis of apoptotic gastric epithelial cells. Both *H. pylori* and culture supernatants from *H. pylori*-treated macrophages also suppressed macrophage phagocytosis of CMP-treated apoptotic gastric epithelial cells, GFP-labeled *H. pylori*, and YG-labeled latex beads (Fig 4F, 4G). Thus, both *H. pylori* bacteria and an *H. pylori*-inducible mediator reduced macrophage clearance of AECs, as well as clearance of bacteria and foreign material, suggesting a global reduction in macrophage phagocytic activity that could contribute to the accumulation of proinflammatory material in the mucosa in *H. pylori* gastritis.

Because TNF-α has been shown to inhibit macrophage clearance of apoptotic cells (30), we measured TNF-α secretion of monocyte-derived macrophages incubated with *H. pylori* (MOI = 10) for 6 h and tested whether the released TNF-α could inhibit macrophage phagocytosis of apoptotic gastric epithelial cells. Indeed, *H. pylori*-stimulated macrophages released significant amounts of TNF-α (>6000 pg/ml; n = 4; P = 0.01) (Fig. 5A). The in vivo relevance of this finding is reflected in the >4-fold increase in TNF-α mRNA expression in the gastric tissue of *H. pylori*-infected subjects compared with that of uninfected subjects (Fig. 5B; n = 8; P = 0.013), confirming our previous observation (31). Moreover, pretreatment of macrophages with rhTNF-α (10 ng/ml) caused a significant reduction in macrophage engulfment of AECs (26.8 ± 5.2%; n = 4; p = 0.004) (Fig. 5C), and, conversely, incubation of *H. pylori*-treated macrophages with a neutralizing anti–TNF-α, but not an isotype control, Ab completely restored the ability of the macrophages to phagocytose AECs (Fig. 5D). In summary, these results implicate TNF-α release by macrophages in response to *H. pylori* as a potent inhibitor of the cells’ capacity to phagocytose apoptotic gastric epithelial cells.

**Gastric mucosa from *H. pylori*-infected subjects** contains increased amounts of nonphagocytosed apoptotic material

To determine whether phagocytosis of apoptotic cells is impaired in human *H. pylori* gastritis, we analyzed gastric mucosa from *H. pylori*-infected (n = 9) and noninfected (n = 7) subjects for the presence of HLA-DR+ MNPs and TUNEL+ material, with an average of 954 ± 157 cells analyzed per sample (Fig. 6A–D). As anticipated, *H. pylori*-infected gastric mucosa contained significantly higher numbers of TUNEL+ cells in the gastric epithelial layer, consistent with *H. pylori* induction of epithelial cell apoptosis (Fig. 6B). Surprisingly, in contrast to our published digital image analysis study (19), we did not find more HLA-DR+ MNPs in the gastric mucosa of *H. pylori*-infected versus uninfected subjects (data not shown). This discrepancy could be due to the exclusion of tertiary lymphoid follicle areas, which are rich in DCs but do not allow accurate cell counts, from the current study. The percentage of MNPs containing apoptotic material did not differ significantly between noninfected and *H. pylori*-infected mucosa, with 2.6 ± 1.4 and 4.7 ± 1.5% of positive cells, respectively (Fig. 6C). Notably, the percentage of MNPs containing TUNEL inclusions in gastric tissue was lower than that seen in isolated cells on cytospins (Fig. 1B), possibly reflecting additional apoptotic cell uptake by the MNPs during the collagenase digestion procedure. Importantly, the amount of TUNEL+ apoptotic material not associated with HLA-DR+ MNPs was significantly increased in *H. pylori*-infected compared with noninfected tissue (Fig. 6D). Thus, results of the HLA-DR/TUNEL analysis of gastric tissue support the hypothesis that chronic *H. pylori* gastritis...
is associated with insufficient apoptotic cell clearance in the human gastric mucosa.

**Discussion**

Chronic *H. pylori* gastritis is associated with self-perpetuating inflammation that contributes to the development of autoimmune atrophic gastritis and gastric adenocarcinoma and may persist even after clearance of the bacteria in advanced disease (32, 33). The mechanism for this chronic and persistent inflammation is not fully understood. In this study, we present findings that support inefficient clearance of AECs by gastric MNPs as a pathogenic mechanism in chronic *H. pylori* gastritis.

Increased gastric epithelial cell apoptosis is well established in *H. pylori* infection and is thought to contribute to gastric carcinogenesis (1). In this regard, our results show that primary human gastric epithelial cells cultured in vitro are highly susceptible to *H. pylori*-induced cell death. Epithelial cell apoptosis was significantly associated with *H. pylori* expression of the virulence factor VacA, which causes loss of mitochondrial membrane potential through formation of an anion channel in the inner mitochondrial membrane, thereby inducing apoptosis through the intrinsic pathway (26).

The increased epithelial cell death in *H. pylori*-infected gastric mucosa results in a higher demand for AEC removal. In addition, chronic active *H. pylori* gastritis also involves infiltration of the gastric mucosa with short-lived neutrophils and lymphocytes (32), further increasing the need for apoptotic cell clearance. In this study, we show that AEC-derived material was present in infected mucosa to clear apoptotic cells. Importantly, the amount is consistent with a reduced capacity by MNPs in chronic *H. pylori* gastritis.

Reduced clearance of AECs in the *H. pylori*-infected human gastric mucosa could exacerbate chronic gastritis by three putative mechanisms. First, nonphagocytosed apoptotic cells that undergo secondary necrosis in the gastric mucosa could release proinflammatory intracellular molecules including nucleic acids, which promote innate immune cell activation (39). This likely results in additional release of proinflammatory cytokines, including TNF-α, by MNPs, which may further inhibit apoptotic cell clearance.

Second, epithelial cell autoantigens released from necrotic cells may induce gastric autoimmune responses. In this connection, gastric autoimmunity has been closely associated with *H. pylori* infection, and ~50% of *H. pylori*-infected subjects possess autoantibodies to gastric epithelial cell Ags (40). Conversely, most persons diagnosed with autoimmune gastritis show signs of active or past *H. pylori* infection (41). A third potential consequence of reduced phagocytosis of AECs in *H. pylori* infection is decreased tolerization due to reduced APC engulfment of apoptotic cells that contain *H. pylori* Ags, leading to enhanced inflammatory responses.

Our results indicate that *H. pylori*-induced macrophage release of TNF-α suppressed apoptotic gastric epithelial cell clearance in an auto- and paracrine manner. Thus, our results extend previous reports that TNF-α inhibits macrophage clearance of apoptotic neutrophils (30, 42). TNF-α levels in gastric tissue of *H. pylori*-infected subjects were increased compared with noninfected subjects, confirming our previous finding (31), and macrophages treated with *H. pylori* released significant amounts of TNF-α. A number of different pathways for the induction of TNF-α by *H. pylori* have been described to date. *H. pylori* TNF-α–inducing protein (43) and protein HP986 (44), both of which signal through NF-kB, have been shown to induce TNF-α secretion directly. In addition, bacterial danger signals that trigger MyD88 signaling through activation of TLRs are involved in the induction of TNF-α and other proinflammatory cytokines in response to *H. pylori* stimulation (45). Notably, phagocytosis of *H. pylori* is required for maximal stimulation of TNF-α secretion (46). Although *H. pylori* bacteria have been identified in the gastric lamina propria, often in close contact with MNPs (27), direct contact between these cells and *H. pylori*, resulting in autocrine TNF-α signals, is likely infrequent. However, the majority of gastric MNPs in the *H. pylori*-infected gastric mucosa will be exposed to paracrine TNF-α signals, which may derive from both MNPs and *H. pylori*-reactive Th1 cells (47). Notably, the suppressed phagocytosis by *H. pylori*-treated macrophages was not specific to the uptake of AECs, because macrophage uptake of latex beads and of *H. pylori* bacteria also was reduced, corroborating previous observations that *H. pylori* can inhibit its own uptake by macrophages (29).

In conclusion, we propose that an increased apoptotic cell load in the *H. pylori*-infected human gastric mucosa exacerbates the persistent inflammatory state that leads to gastric atrophy and adenocarcinoma through a mechanism in which *H. pylori*-induced MNP secretion of TNF-α suppresses apoptotic cell clearance. The observed insufficient apoptotic cell clearance in the gastric mucosa may then result in a release of inflammatory components and autoantigens from the dying cells, which further enhances gastric inflammation, independent of *H. pylori* bacteria.

**Acknowledgments**

We thank Donna Crabb and Amy Ratliff for preparation of *H. pylori* cultures. We also thank the Analytic and Preparative Cytometry Facility, the Digestive Diseases Research Development Center Human Cell/Tissue Core Human Cell/Tissue Core, and the University of Alabama at Birmingham High Resolution Imaging Facility for supporting this study.
Disclosures
The authors have no financial conflicts of interest.

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