Helicobacter pylori infection downregulates the DNA glycosylase NEIL2, resulting in increased genome damage and inflammation in gastric epithelial cells

Ibrahim M. Sayed, Ayse Z. Sahan, Tatiana Venkova, Anirban Chakraborty, Dibyabrata Mukhopadhyay, Diane Bimczok, Ellen J. Beswick, Victor E. Reyes, Irina Pinchuk, Debashis Sahoo, Pradipta Ghosh, Tapas K. Hazra, and Soumita Das

From the 1Department of Pathology, University of California San Diego, San Diego, California, USA, 2Department of Internal Medicine, University of Texas Medical Branch, Galveston, Texas, USA, 3Department of Microbiology and Immunology, Montana State University, Bozeman, Montana, USA, 4Department of Medicine, University of Utah, Salt Lake City, Utah, USA, 5Department of Pediatrics, University of Texas Medical Branch, Galveston, Texas, USA, 6College of Medicine, Penn State Health Milton S. Hershey Medical Center, Hershey, Pennsylvania, USA, 7Department of Pediatrics, University of California San Diego, San Diego, California, USA, 8Department of Computer Science and Engineering, Jacob’s School of Engineering, San Diego, California, USA, and 9Department of Medicine and Cellular and Molecular Medicine, John and Rebecca Moore Cancer Center, University of California San Diego, San Diego, California, USA

Infection with the Gram-negative, microaerophilic bacterium Helicobacter pylori induces an inflammatory response and oxidative DNA damage in gastric epithelial cells that can lead to gastric cancer (GC). However, the underlying pathogenic mechanism is largely unclear. Here, we report that the suppression of Nei-like DNA glycosylase 2 (NEIL2), a mammalian DNA glycosylase that specifically removes oxidized bases, is one mechanism through which H. pylori infection may fuel the accumulation of DNA damage leading to GC. Using cultured cell lines, gastric biopsy specimens, primary cells, and human enteroid-derived monolayers from healthy human stomach, we show that H. pylori infection greatly reduces NEIL2 expression. The H. pylori infection-induced downregulation of NEIL2 was specific, as Campylobacter jejuni had no such effect. Using gastric organoids isolated from the murine stomach in coculture experiments with live bacteria mimicking the infected stomach lining, we found that H. pylori infection is associated with the production of various inflammatory cytokines. This response was more pronounced in Neil2 knockout (KO) mouse cells than in WT cells, suggesting that NEIL2 suppresses inflammation under physiological conditions. Notably, the H. pylori-infected Neil2-KO murine stomach exhibited more DNA damage than the WT. Furthermore, H. pylori-infected Neil2-KO mice had greater inflammation and more epithelial cell damage. Computational analysis of gene expression profiles of DNA glycosylases in gastric specimens linked the reduced Neil2 level to GC progression. Our results suggest that NEIL2 downregulation is a plausible mechanism by which H. pylori infection impairs DNA damage repair, amplifies the inflammatory response, and initiates GC.

The World Health Organization and the American Cancer Society (ACS) estimate that gastric cancer (GC) is the fourth most prevalent cancer worldwide and the third leading cause of death in the United States (1). Recognized risk factors for developing GCs include genetic predisposition (variants in DNA repair genes have been reported as key factors in GC susceptibility and survival) (2, 3), dietary factors (for example, high salt intake), obesity, smoking, and Helicobacter pylori infection (4, 5). H. pylori is a Gram-negative, microaerophilic bacterium that infects the gastric mucosa, causing gastritis and often leading to devastating complications, such as peptic ulcers and GC. H. pylori infection increases the risk of developing GCs by 5–6-fold (6). Treatment and eventual eradication of the infection could reduce the risk of developing such complications but cannot completely erase the possible threat. Additionally, an increasing number of studies report antibiotic resistance among H. pylori strains (7, 8). This makes complete eradication of H. pylori infection challenging, and recurrent infections are common without extreme or noticeable symptoms (5, 9, 10).

As for the pathogenesis of GCs, infection with H. pylori of both gastric cell lines and primary gastric epithelial cells has been shown to trigger a unique DNA damage pattern that preferentially targets transcribed regions and regions proximal to telomeres (6, 11). However, how H. pylori infection results in the accumulation of DNA damage remains unknown. Generally, bacterial infection-induced inflammation leads to the generation of reactive oxygen species (ROS) that cause oxidative genome damage in the host cells. Without timely repair, mutagenic DNA lesions can cause several pathologies, including cancer (12–15). Oxidized DNA bases are primarily repaired via the DNA base excision repair (BER) pathway (16, 17). BER is a multistep process that is initiated with recognition and excision of the lesion by a family of repair proteins called DNA glycosylases (DGs). All four DNA bases are subjected to oxidative modifications leading to approximately two dozen lesions; only 5 oxidized base-specific DNA glycosylases with overlapping substrate
specificities have been characterized so far in mammalian cells. Among them, OGG1 and NTH1, which were identified and characterized earlier, primarily remove oxidized purines and pyrimidines, respectively, and were thought to be the major mammalian DNA glycosylases. However, null mice of these two DGs did not show any visible phenotype (16, 18–24). We and several other groups then found that mammalian cells possess a new family of DNA glycosylases and named them E. coli Nei-like (NEIL1–3) proteins. NEILs are capable of removing both purines and pyrimidines, but they are unique with respect to DNA damage recognition. Whereas OGG1 and NTH1 remove the base lesions from duplex DNA, NEILs prefer to initiate excision of lesions that are located in a single-stranded region, a replication fork, or transcription bubble mimic (15, 25, 26). Indeed, it was found that NEIL1 and NEIL3 are mostly involved in repairing the damage in replicating cells, and NEIL2 primarily removes the oxidized DNA bases from transcribed regions (26). Recently, copy number variation analysis of the COSMIC and TCGA databases has clearly shown that, among all DNA repair proteins, NEIL2 is the most affected in various cancers, and its level is generally low (27). A mutant or functional variant of NEIL2 was also reported as a risk factor for lung cancer and squamous cell carcinoma in the oral cavity (28–31), and the loss of NEIL2 is associated with poor survival in patients with estrogen receptor-positive breast cancer (32).

Several reports indicate that H. pylori-induced ROS can cause oxidative DNA damage leading to the accumulation of mutations (33, 34). Hence, it is expected that aberrant expression and/or compromised activities of the DGs will be contributing factors for various pathologies, including carcinogenesis. Surprisingly, despite the accumulation of significantly larger amounts of mutagenic 8-oxoG in Ogg1−/− mice, these animals did not develop tumors when exposed to KBrO3, an oxygen radical-forming agent (35). Moreover, Ogg1-null mice are resistant to H. pylori-induced inflammation. These data suggest that oxidized DNA base lesions alone are not sufficient for developing GCs; some other factor(s) is necessary for developing tumors in the affected tissues.

Based on the link between NEIL2 and various types of cancers, we hypothesized that H. pylori infection triggers the accumulation of mutations via dysregulation of NEIL2. Using the stem cell-based ex vivo technique in which gastric organoids were isolated from the stomach of WT and Neil2 knockout (KO) mice and an in vivo infection model, we report here that the level of NEIL2, but not that of other DGs, is significantly decreased upon H. pylori infection, leading to increased DNA damage accumulation and inflammatory responses. Because ROS, DNA damage, and inflammation are closely intertwined, and because NEIL2 is an anti-inflammatory protein in addition to its role in DNA repair (26), we conclude that the combinatorial effect of the accumulation of oxidized DNA lesions along with chronic inflammation likely plays a critical role in the initiation and progression of H. pylori-associated GCs.

Results

**H. pylori infection is associated with altered expression of NEIL2 in a dose- and time-dependent manner**

To determine the expression of BER proteins in gastric epithelial cells after H. pylori infection, AGS cells were transfected with the C-terminal FLAG-tagged expression plasmids of OGG1, NEIL1, NEIL2 and NTH1, followed by infection with H. pylori. Surprisingly, the level of NEIL2-FLAG (with a 5-fold higher expression than endogenous NEIL2 [Fig. S1]), but not that of the other DNA glycosylases, was significantly decreased in the AGS cell lysate at the protein level (Fig. 1A). To determine whether the stably expressed NEIL2-FLAG is downregulated in transcript level after infection, we specifically detected the NEIL2-FLAG transcript level (Fig. S2A and B). We indeed found its downregulation at 24 h and 48 h postinfection. The empty vector-expressing control cells did not show any amplification of the NEIL2-FLAG transcript, indicating specific amplification in NEIL2-FLAG-expressing cells (Fig. S3A). However, the NEIL2 transcript level was decreased significantly following H. pylori infection in control cells (Fig. S3A and B), indicating the downregulation of the endogenous NEIL2 as well.

To further confirm this, AGS cells were infected with H. pylori, and it was found that the endogenous expression of NEIL2, but not OGG1, was significantly decreased (Fig. 1B). Furthermore, the expression of NEIL2 following H. pylori infection was decreased in a time-dependent manner. NEIL2 protein and its transcript level started to decrease at 6 h postinfection and became undetectable at 24 h and 48 h (Fig. 1C). To test the effect of H. pylori inoculum on NEIL2 expression, AGS cells were infected with H. pylori at a multiplicity of infection (MOI) of 10 and 100, and the expression of NEIL2 was determined by Western blot analysis. The reduction of NEIL2 expression was more pronounced in AGS lysates at higher bacterial load (Fig. 1D), indicating that H. pylori infection is linked to the downregulation of NEIL2.

**H. pylori infection in primary and transformed gastric cells downregulates NEIL2 at the transcript level**

To understand the impact of H. pylori infection on NEIL2 expression in primary and transformed gastric cells (Fig. 2A), we used the epithelial cell lines AGS, NCI-N87 from gastric carcinoma, and normal human fetal gastric/intestinal cells, Hs738 (36). Figure 2B demonstrates the time-dependent changes in the NEIL2 transcript level at 0, 1, 3, 6, and 12 h post-H. pylori infection in AGS cells. The NEIL2 mRNA expression was increased at 1 h postinfection, followed by a significant decrease in the transcript level in a time-dependent manner (Fig. 2B). Similarly, the level of NEIL2 transcription was also downregulated in Hs738, the nontransformed gastric cells from the normal human fetus (Fig. 2C), and in NCI-N87, the transformed epithelial cell line (Fig. 2D), after 12 h of H. pylori infection. Next, we evaluated the physiological relevance of this finding in human gastric biopsy specimens (Fig. 2E, i) that were either H. pylori positive or H. pylori negative. Interestingly, H. pylori-positive specimens had lower NEIL2 expression than H. pylori-negative specimens (Fig. 2E, ii), indicating a link between H. pylori infection and the level of NEIL2 in the stomach. To understand further the effect of H. pylori infection on primary gastric epithelial cells, organoids or enteroids were isolated from healthy human gastric biopsy specimens. The enteroid-derived monolayers (EDMs) showed a significant reduction in the level of NEIL2 following H. pylori infection (Fig. 2E, iii).
The downregulation of NEIL2 is H. pylori-specific and independent of cag pathogenicity island (CagA)

To investigate whether the reduced expression of NEIL2 was H. pylori infection specific, the NEIL2 expression level was measured in AGS cells infected with either H. pylori or Campylobacter jejuni, a gastrointestinal pathogen similar to H. pylori (37). We found that NEIL2 expression was downregulated with H. pylori infection but not with C. jejuni infection (Fig. 3A). To investigate if NEIL2 expression depended on specific genes or the virulence factor of H. pylori, we selected two mutant H. pylori strains: 1) alkyl hydroperoxide reductase (AhpC) (an enzyme crucial for oxidative stress resistance and the survival of the bacterium in the host that generates higher amounts of 8-oxo-guanine associated with their DNA [38]), and 2) NapA (proteins are known to regulate oxidative stress and ROS generation [39]). We also tested the effect of H. pylori mutant strains for Cag pathogenicity island or CagA (H. pylori virulence factors) on NEIL2 expression and compared it to the effect of the WT H. pylori 26695 strain. We found that NEIL2 expression was downregulated in all of the mutant strains to a level similar to that of WT H. pylori (Fig. 3B–D). The decrease in NEIL2 expression in epithelial cells was mediated only by bacterial pellets (Fig. 3E, second to last lane), and boiling the pellet abolished the effect (Fig. 3E, last lane), indicating that the bacterial factor responsible for NEIL2 downregulation is heat sensitive.
The inflammatory responses of gastric epithelial cells following H. pylori infection is NEIL2 dependent

To understand the effect of infection on gastric epithelial cells under conditions similar to those in vivo, EDMs were prepared from the stomachs of WT and Neil2 KO mice (Fig. S4) and healthy human subjects (Fig. S6). We established EDMs (Fig. S4B) from gastric spheroids (Fig. S4A) of WT and Neil2 KO mice infected with H. pylori. The EDMs were collected for functional assays as shown in Fig. 4A. To understand the mechanistic basis of NEIL2-mediated inflammation, we conducted a cytokine proteome profiler array on the supernatant collected from the H. pylori-infected EDMs of WT and Neil2 KO mice (Fig. 4B). The cytokines/chemokines at low and high exposures are listed in Table 1. The expression of KC, IL1β, IL-6, TNFα, MCP-1, and IFN-γ was measured in the gastric EDMs of WT and Neil2 KO organoids. Interestingly, the level of proinflammatory cytokines was significantly higher in the KO EDMs after 48 h of H. pylori infection (Fig. 4C). To verify the findings, we also conducted KC, MCP-1, and IL-6 ELISA on the mock-treated and H. pylori-infected EDMs. H. pylori infection showed more inflammatory cytokines that were further upregulated in Neil2 KO EDMs (Fig. 4D–F). A similar result was observed with KO EDMs for TNFα and IL-1β (Fig. S6A and B). We also confirmed that EDMs generated from healthy human...
subjects infected with *H. pylori* had higher levels of the proinflammatory cytokines MCP-1, IL-8, and IL-6 (Fig. S5).

To understand the link between *H. pylori*-mediated inflammatory responses and the role of NEIL2 therein, we transfected AGS cells with vector control or NEIL2-overexpressing plasmids and infected both with *H. pylori* for 12 and 24 h. The multiplex cytokine assay showed IL-8 as the prevalent proinflammatory cytokine after *H. pylori* infection (not shown). Therefore, IL-8 ELISA was performed with control versus NEIL2-overexpressing AGS cells. NEIL2 overexpression resulted in lower levels of the inflammatory cytokine IL-8 (Fig. S6C).

NEIL2 KO mouse stomach showed higher levels of inflammation

To examine the *in vivo* significance of *H. pylori* infection, WT and Neil2 KO mice were infected/inoculated with *H. pylori* via oral gavage (as shown in Fig. 5A). KC (mouse IL-8 analog), TNFα, and IFN-γ mRNA expression levels were measured in the gastric tissue by qRT-PCR (Fig. 5B–D). The level of KC expression was ~12-fold higher, whereas the TNFα and IFN-γ levels were 1.5- and 2-fold higher, respectively, in gastric tissue collected from the infected Neil2 KO mice compared with those of WT-infected mice (Fig. 5B–D). Similarly, the levels of IL-1β and IL-6 expression were higher in KO-infected tissue than in the WT tissue (Fig. 5D–E). To evaluate the role of NEIL2 in preventing gastric lesions following *H. pylori* infection, we performed hematoxylin and eosin (H&E) staining of the gastric specimens. Histology score was measured on the basis of inflammation, as stated in Materials and methods (Fig. 5E). Representative images indicate the differences in the extent of the infiltration of immune cells (Fig. 5F). All the data in Fig. 4 and 5 with KO mouse tissue and EDMs suggest that NEIL2 plays an important role in mediating the inflammatory response against *H. pylori* infection.
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(B) DNA Damage, LA-PCR
RNA: qRT-PCR
Supernatant: ELISA, Proteome Profiler Array

Low Exposure

High Exposure

(C) Relative expression of transcripts

(D) KC (pg/mL)

(E) MCP-1 (pg/mL)

(F) IL-6 (pg/mL)
NEIL2 KO mouse stomach showed increased DNA damage after *H. pylori* infection

To understand the effect of NEIL2 depletion on the accumulation of oxidative DNA base lesions, the supernatants from the coculture of EDMs of WT and Neil2 KO mice with *H. pylori* infection were measured for oxidative DNA adducts. Neil2 KO stomach EDMs accumulated greater oxidative DNA damage at 48 h of *H. pylori* infection than the WT (Fig. 6A). To determine whether ROS-mediated DNA damage can induce NEIL2 expression, we treated the AGS cells with glucose oxidase (GO) for 45 min, followed by recovery of oxidative DNA damage for 3, 6, 12, and 24 h. After GO treatment, we could detect the lower intensity of the long ampiclon for HPRT and Polβ genes (Fig. 6B), indicating the accumulation of oxidative base lesions. Interestingly, after GO treatment, we detected no significant changes in the NEIL2 level (Fig. 6C). Thus, the depletion of NEIL2 is independent of ROS generation, and it is possible that another factor(s) from *H. pylori* can affect the expression of NEIL2. Nonetheless, DNA damage was assessed by long ampiclon quantitative-PCR (LA-qPCR) in representative Polβ and β-globin genes in the genomic DNA extracted from mouse stomach enteroid enteroids upon *H. pylori* infection (Fig. 6D). We observed a significantly larger amount of oxidative damage accumulation in both the Polβ and β-globin genes, particularly after 48 h of *H. pylori* infection in Neil2 KO enteroids compared with that of WT enteroids.

Elevated NEIL2 gene expression in GCs is correlated to a higher probability of disease-free survival, and NEIL2 is lowered in *H. pylori*-positive atrophic gastritis

NEIL2 mRNA expression levels in GCs were analyzed using publicly available RNA-Seq datasets (41) from the biopsy specimens of the stomach from *H. pylori*-positive and -negative human subjects was analyzed, and the level of NEIL2 was found to be significantly decreased in *H. pylori*-positive gastric atrophy compared with that in the negative specimens. Interestingly, the downregulation of BER proteins in gastric atrophy was prominent only for NEIL2 but not for the other two DNA glycosylases, NEIL1 and OGG1 (Fig. 7F).

Next, we performed computational analysis of gene expression profiles of publicly available datasets (GSE78523) (42) with incomplete metaplasia (IIM-C) and incomplete metaplasia that progressed to GCs (IIM-GC). We chose the incomplete metaplasia group, as it has a higher chance of progression to GCs than the complete metaplasia group (42). The expression data of the BER enzymes showed that, specifically, the level of Neil2 is reduced compared with those of other DNA glycosylases (Neil1, Neil3, NTH1, and Ogg1) in gastric specimens collected from incomplete metaplasia controls (Fig. 8A and 8B). The significant reduction of NEIL2 in incomplete metaplasia controls correlated with the progression of GCs and suggests that the reduced level of Neil2 can accelerate the accumulation of mutations that can initiate cancer progression.

**Discussion**

Chronic infections and infection-induced proinflammatory cytokines can lead to the production of ROS and reactive nitrogen intermediates. These reactive species can induce DNA damage and thereby propel genomic instability during tumor initiation and progression (14, 43). The vicious feed-forward cycle of inflammation and oxidative stress continuously targets cellular macromolecules, including the genomic DNA (gDNA), inducing mostly oxidized base damage. These oxidized DNA base lesions are primarily repaired via the BER pathways (44, 45). Defects in BER will adversely affect cellular function and allow DNA damage to accumulate and may trigger the development of cancers. Many carcinopatogens utilize this paradigm to induce inflammation-induced genomic instability (46, 47) via mechanisms that remain poorly understood. The major

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**Figure 4.** The proinflammatory responses in *H. pylori*-infected EDMs derived from murine gastric tissue depend on NEIL2. A, the schematic diagram shows the plan for functional assays of enteroids isolated from WT or Neil2 KO mice. B, the proteome profiler array was conducted with the supernatant collected from the *H. pylori*-infected WT and Neil2 KO EDMs. Two blots from low exposures and high exposures were analyzed, and the cytokines that appeared in the array are listed in the table. C, the differential expression of selected cytokines was verified by qRT-PCR of RNA samples isolated from EDMs used in panel B. D–F, the supernatants from the EDMs of WT or Neil2 KO mice, either untreated (Un) or infected with *H. pylori* (Hp), were further verified by ELISA for KC, MCP-1, and IL-6 expression. Data were generated from the mean ± S.D. from three independent experiments. *, p < 0.05; **, p < 0.01.

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**Table 1**

| Well no./location | Detail | Cytokine appeared | Cytokine appeared |
|-------------------|--------|-------------------|-------------------|
| A1, A2, A23, A24 | Reference spot | Low exposure | High exposure |
| B1, B2 | CXXL13 | Yes | Yes |
| B3, B4 | CS/CSa | Yes | Yes |
| B7, B8 | GM-CSF | Yes | Yes |
| B15, B16 | IFN-β | Yes | Yes |
| B17, B18 | IL-1α | Yes | Yes |
| B21, B22 | IL-1Rα | Yes | Yes |
| C3, C4 | IL-4 | Yes | Yes |
| C9, C10 | IL-7 | Yes | Yes |
| C17, C18 | IL-16 | Yes | Yes |
| C23, C24 | IL-27 | Yes | Yes |
| D1, D2 | IP-10 | Yes | Yes |
| D3, D4 | I-TAC | Yes | Yes |
| D5, D6 | KC | Yes | Yes |
| D7, D8 | M-CSF | Yes | Yes |
| D9, D10 | CCL2/MCP-1 | Yes | Yes |
| D19, D20 | MIP-2 | Yes | Yes |
| D23, D24 | CXCL12/S.D.F. | Yes | Yes |
| E3, E4 | TIMP-1 | Yes | Yes |
| E5, E6 | TNF-α | Yes | Yes |
discovery we report here is a mechanism by which the carcinogen-pathogen \textit{H. pylori} derails a host defense mechanism that is geared to protect cells from oxidized DNA base lesions by repairing them.

\textit{H. pylori} is a major human pathogen causing chronic, progressive gastric mucosal damage that is linked to atrophic gastritis (AG) \citep{48}. AG is linked to an increased risk for intestinal-type adenocarcinoma and type 1 gastric carcinoid, specifically in the presence of extensive intestinal metaplasia \citep{49}. Studies have shown that eradication of the bacteria can reduce the risk of cancer development \citep{5, 50}. Since \textit{H. pylori} infection generally develops into a chronic infection, the accompanying persistent inflammatory response, ROS production, and accumulation of DNA damage could be a mechanism for the development of GC \citep{51, 52}. The \textit{in silico} data analysis (Fig. 8, \textit{A} and \textit{B}) showed that, among all the BER proteins (NEIL1, NEIL2, NEIL3, OGG1, and NTH1), the level of NEIL2 is significantly reduced in the incomplete metaplasia of gastric specimens that progress to GCs. No study to date has identified the link between NEIL2 or any other BER deficiency with accumulated mutation and their involvement in infection-mediated cancer. Therefore, we studied the BER protein NEIL2 using \textit{Neil2}\textsuperscript{-null} mice that accumulate oxidative genomic damage, mostly in the transcribed regions, and are more responsive than WT mice to inflammatory agents \citep{26}. Interestingly, in gastric epithelial cells, the \textit{H. pylori}-induced accumulation of a unique DNA damage pattern occurs preferentially in transcribed regions \citep{6}.

To establish a link between \textit{H. pylori} infection and the functional role of DNA glycosylases, we assessed the expression level of BER proteins following \textit{H. pylori} infection in human

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\textbf{Figure 5. The proinflammatory responses in gastric tissue following \textit{H. pylori} infection depend on NEIL2.} \textit{A}, schematics show the planning of animal experiments with C57-BL/6 mice to isolate RNA and histopathology. \textit{B}–\textit{D}, the relative mRNA expressions of IL-8 (KC), TNF-\(\alpha\), and IFN-\(\gamma\) were determined by qRT-PCR of gastric tissues from \textit{H. pylori}-infected WT and \textit{Neil2} KO mice. \textit{E}, histology scores of stomach specimens of WT mice and \textit{Neil2} KO mice before and after \textit{H. pylori} infection. *, \(p < 0.05\); ***, \(p < 0.001\). \textit{F}, representative images from the stomach specimens of \textit{H. pylori}-infected WT and \textit{Neil2} KO mouse groups.
gastric epithelial cells (cultured cells as well as in enteroids isolated from gastric biopsies of healthy human subjects). *H. pylori* downregulated NEIL2 expression in a time- and dose-dependent manner but was Cag independent; consequently, DNA damage was accumulated that could lead to GC.

In our study, we showed the link between NEIL2 expression and inflammatory response (IL-8/KC, MCP-1, IL-6, IL-1β, and TNFα); overexpression of NEIL2 led to a lower inflammatory response, whereas *Neil2* KO mice had a greater inflammatory response. Therefore, we hypothesized that NEIL2 expression is linked to both indirect DNA damage caused by the unchecked inflammatory response as well as to a direct build-up of oxidative DNA damage by inhibition of BER. This process implicates the involvement of NEIL2 in gastric carcinogenesis after *H.

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**Figure 6. Evaluation of the role of NEIL2 in *H. pylori* infection-induced accumulation of oxidized DNA bases in stomach epithelial cells.**

A, the stomach epithelial EDM of WT and *Neil2* KO mice were either untreated or infected with *H. pylori* for 48 h. The supernatant at each condition was used to measure the oxidative damage of DNA as mentioned in Materials and methods. B, AGS cells were either untreated or treated with glucose oxidase (GO) for 45 min. The genomic DNA was used for long amplicon (LA) and short amplicon (SA) qPCR for HPRT and POLB. The histogram represents the accumulation of oxidative DNA base lesions after densitometry from 3 independent sets of experiment. One representative gel figure is shown. The band intensity in the control cell was arbitrarily set as 100. The error bars represent the standard deviation of the mean band intensity. ***, p < 0.001.** C, the NEIL2 expression level was measured by Western blotting from nuclear extract of AGS cells following GO treatment for the indicated time. Con, untreated control. HDAC2, histone deacetylase 2, used as a nuclear loading control. D, stomach epithelial enteroids of WT and *Neil2* KO mice were either mock infected or infected with *H. pylori* for 24 h, 48 h, or 72 h and then harvested for genomic DNA isolation. LA-qPCR was performed to evaluate the level of oxidized DNA base lesions after *H. pylori* infection. Representative gels show the amplification of each long fragment (−7–8 kb) (upper) normalized to that of a short fragment (−250 bp) (lower) of the corresponding (Pol β and β-Globin) genes. The histogram represents the accumulation of oxidative DNA base lesions after densitometry from 4 different sets of WT versus *Neil2* KO mice. The band intensity in the WT mice was arbitrarily set as unity (n = 1). The error bars represent the standard deviation from the mean band intensity.
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NEIL2 expression levels in normal versus cancer subjects were analyzed in publicly available RNA-Seq datasets (53) using RRID:SCR_007834. C–E, the Kaplan-Meier plotter [K-M plotter; RRID:SCR_018753] program was used to analyze disease-free survival among 631 patients diagnosed with GCs of various clinical stages (40), stratified into high (red) versus low (black) NEIL2 expression. All stages, C; stage I alone, D; stage II alone, E, F. The levels of NEIL2, NEIL1, and OGG1 were analyzed in the publicly available data set with GDS5338 (41) from the biopsy specimens of stomachs of *H. pylori*-positive and -negative subjects.

Figure 7. NEIL2 level is specifically downregulated in *H. pylori*-positive atrophic gastritis, and low levels of expression in early GCs lead to a poor prognosis. A–B, NEIL2 mRNA expression is elevated in both the intestinal (A) and diffuse (B) subtypes of GCs. Expression levels of NEIL2 mRNA in normal versus cancer subjects were analyzed in publicly available RNA-Seq datasets (53) using RRID:SCR_007834. C–E, the Kaplan-Meier plotter [K-M plotter; RRID:SCR_018753] program was used to analyze disease-free survival among 631 patients diagnosed with GCs of various clinical stages (40), stratified into high (red) versus low (black) NEIL2 expression. All stages, C; stage I alone, D; stage II alone, E, F. The levels of NEIL2, NEIL1, and OGG1 were analyzed in the publicly available data set with GDS5338 (41) from the biopsy specimens of stomachs of *H. pylori*-positive and -negative subjects.

*pylori* infection and could represent a potential biomarker for GC progression and prevention. From a database search, we found that NEIL2 is expressed at higher levels in various gastric adenocarcinomas than in normal gastric tissue. However, higher NEIL2 expression within each stage of cancer led to a higher probability of survival than that of patients with lower NEIL2 expression (53). In addition to being a potential biomarker for risk assessment and the diagnosis of carcinogenesis,
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NEIL2 expression may act as a prognostic biomarker to aid in determining the course of the disease. In future studies, it will be important to determine the NEIL2 expression after *H. pylori* infection and correlate expression levels to gastric carcinogenesis to identify a more significant and clinically relevant link between *H. pylori* infection, NEIL2 expression, and gastric carcinogenesis.

Besides NEIL2 downregulation, we found that *H. pylori* infection increased the inflammatory response (IL-8 production) *in vitro* (AGS cells, Hs738, and enteroid-derived monolayers) and
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In vivo (human biopsy samples and mice). Since inflammation is a risk factor for cancer development (47, 54), we hypothesized that *H. pylori* could promote cancer progression by increasing inflammatory signaling. To link IL-8 expression with the function of NEIL2, Neil2 KO mice and WT mice were tested for several inflammatory cytokines (IL-8/KC, MCP-1, IL-6, IL-1β, and TNFα) upon infection with *H. pylori*. We found that Neil2 KO mice had a greater inflammatory response than the WT. We confirmed our findings by infecting EDM prepared from WT and Neil2 KO mice with *H. pylori*. IL-8 levels decreased in the Neil2 KO overexpressed cells. All the inflammatory cytokines are increased in Neil2 KO mice or enteroid-derived cells, further confirming the regulatory role of NEIL2 on the resolution of inflammation. This reveals an additional immune-homeostatic role of NEIL2 apart from its DNA glycosylase activity, and this is in conjunction with recent findings that show that OGG1 (another dedicated DNA glycosylase) facilitates NF-κB’s binding to its consensus sequence and promotes inflammatory responses (24, 55–57).

Inflammatory cytokines can induce DNA damage that, in turn, promotes senescence (58, 59). This is further established as shown in Fig. S8, where an increase in IL-8 (KC) and IL-6 levels in KO EDMs started at 24 h and increased further at 48 h. However, the DNA damage is significantly increased in Neil2 KO EDMs only after 48 h of infection (Fig. 6D), suggesting that the inflammatory cytokines induce ROS generation, followed by DNA damage. We provide a perspective on DNA repair inhibition and inflammatory response and propose that both phenomena are regulated by NEIL2 and exacerbate another, leading to the accumulation of tumorigenic DNA damage (Fig. 8C). Moreover, we found that the regulation of NEIL2 expression is independent of the presence of ROS and that the expression of NEIL2 is *H. pylori* dose and time dependent. It was demonstrated earlier that the bacterial effector EspF from enteropathogenic *Escherichia coli* (EPEC) abrogates host cell mismatch repair (MMR) (60). Thus, we postulate that such bacterial effectors are also involved in downregulating NEIL2 expression in *H. pylori*-mediated infection. Hence, how the factors from *H. pylori* can downregulate NEIL2 expression will be an exciting area of future research. The knowledge gained from such studies should help in understanding the mechanistic link between infection-mediated inflammation, DNA damage, and GC, which will ultimately have a great translational impact to benefit human health.

**Materials and methods**

**Spheroid isolation and maintenance**

Gastric stem cells were isolated from WT and Neil2 KO mice, as described previously (61, 62). The pylorus section of the stomach was removed, washed with PBS, and then incubated with collagenase type I solution containing gentamicin (50 μg/mL) at 37 °C for 30 min with mixing at 10-min intervals. The cells were filtered through a 70-μm cell strainer, and the density and viability of the gastric stem cells were checked using trypan blue and a hemocytometer. The cells were suspended in Matrigel, and 50% conditioned medium (CM) was added to each well (61). Spheroid cultures were passaged every 3 to 4 days using 0.25% trypsin in PBS-EDTA. EDM was prepared as previously described (22, 63). The spheroids were briefly trypsinized, and the isolated cells were filtered, counted, suspended in 5% CM, and pelleted with Matrigel (1:30 dilution) in the apical part of the transwell at a density of 5 × 10^5 cells/well. The 5% CM was added to the base of the transwell. A similar isolation and culture protocol was used to generate spheroids from human stomach tissue, as previously described (64, 65).

**Cell line maintenance**

AGS cells were cultured in ATCC-formulated F-12K medium (catalog no. 30-2004) containing 10% fetal bovine serum (FBS), penicillin (1 units/ml), and streptomycin (100 μg/mL) and then kept in an incubator at 37 °C and 5% CO_2_.

**H. pylori infection**

*H. pylori* strain ATCC-26695 was incubated with Brucella broth supplemented with 10% FBS 1 day before infection and left on a shaker at 37 °C in 10% CO_2_. EDM and gastric epithelial cell lines were infected with the bacteria at a multiplicity of infection (MOI) of 100:1 for 24 h and 48 h. The Cag and CagA mutants were described in our previous study (66). The AhpC and NapA mutants were grown as published previously (38, 39). For the supernatant and bacterial pellet, the bacterial culture was centrifuged down at 10,000 rpm and the supernatant was separated from the bacterial pellet. The same number of bacteria was used for preparing the whole bacteria, pellet, and supernatant.

**RNA isolation and RT-PCR**

RNA was isolated from uninfected and *H. pylori*-infected cells using the RNeasy Micro kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. cDNA was synthesized using the superscript kit (Invitrogen). qPCR was carried out using the SYBR Green master mix for target genes and normalized to the housekeeping gene encoding GAPDH or β-actin using the ΔΔCT method (30). Primers were designed using the Roche Universal Probe Library Assay Design software (Table 2).

Figure 8. The heat map and the schematic summarize the role of NEIL2 in the initiation of GC. A, a heatmap was generated after analyzing the expression profile of base excision repair genes in the microarray of the publicly available data set (GSE78523) (42). The microarray was performed with the RNA extracted from FFPE cuts of gastric biopsies from a total of 45 human subjects from different disease groups: healthy controls (HC), incomplete metaplasia (IM-C), and complete metaplasia (IM-GC). Incomplete metaplasia had a higher progression rate to GC than complete metaplasia. The level of NEIL2 was significantly reduced compared with those of all the genes analyzed in the heatmap. B, NEIL1, NEIL2, NEIL3, OGG1, and NTH1 expression were detected in this cohort. C, *H. pylori* infection has serious negative effects on the gastric mucosa because of chronic mucosal inflammation leading to DNA damage and down-regulation of the base-excision repair protein NEIL2. NEIL2, which actively repairs the damage that occurs during transcription, has impaired repair activity following *H. pylori* infection and, therefore, accumulates mutations that can lead to GC. D, the schematic diagram summarizes the findings of the current study.
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Table 2
List of primers used in the study

| Gene       | Species | Forward primer (5′→3′)                      | Reverse primer (5′→3′) |
|------------|---------|---------------------------------------------|------------------------|
| NEIL2      | Mouse   | CTCCGCGCTTTGAGGTCTCT                     | TCTGGATGAAACCGAAGGA   |
| IL-8/KC    | Human   | GGGGGCGCAAGGCTGACCTTC                    | GGAATATTTCACCTCAATGAGG |
| IFN-γ      | Human   | GCCACTGTATGGTCACTTGGCTC                | AGAGGCTGACCACTGAGGA   |
| MCP-1      | Mouse   | AGGTTGAACTGCGACATGGAG            | AGGAGGCTGAGGACACAC    |
| TNF-α      | Mouse   | CCACACACCTGGCTGGTGGTCT                | AGAGGCTGAGGACACAC    |
| IL-1β      | Mouse   | GAAATGCCACCTTTGAGAGT           | CTGAGATCTGATCTCAGGACA |
| B-actin    | Mouse   | GAGGAGCCATGGTCAATCGAT           | ACATGCTGAGGAAGGGGAC   |
| NEIL2 FLAG | Mouse   | AGAGGCTGAGGACACAC            | AGGAGGCTGAGGACACAC    |
| Endogenous NEIL-2 | Human | ATGCCGAAGGGGCGCGTG | GGAGAAGCTGACAGCTGCTG |

Western blotting

Cell lysates, collected from uninfected and H. pylori-infected monolayers and cell lines, were tested for NEIL2 protein expression using a purified anti-NEIL-2 antibody (dilution, 1:500) as described previously (67).

Quantification of NEIL2 transcript level

AGS cells stably expressing NEIL2-FLAG or empty FLAG vector, as a control, were infected with H. pylori at different time points (0 h, 1 h, 6 h, 12 h, 24 h, and 48 h), total RNA was isolated, and the levels of both NEIL2-FLAG and endogenous NEIL2 mRNA were determined by RT-PCR using the primers listed in Table 2.

ELISA

Supernatants collected from apical and basolateral parts of uninfection and H. pylori-infected monolayers on transwells were tested for IL-8 using the mouse CXCL1/KC DuoSet ELISA kit (R&D Systems) according to the manufacturer’s instructions. The level of IL-8 was compared in H. pylori-infected monolayers on transwells versus the uninfected monolayers with the same cell line.

Infection of Neil2-KO mice with H. pylori

Mice used in the study were WT and Neil2 KO C57BL/6 mice and were generated and characterized as previously described (26). The maintenance, breeding, and infection study protocols were approved by the Animal Care and Ethics Committee of UTMB, Galveston, TX. Mice were inoculated with H. pylori strain SS1 (10^8 bacterial counts/dose) via oral gavage for 3 days/week as described previously (68). Animals were sacrificed after 3 months, and stomach specimens were collected for RNA and DNA isolation.

Histopathology of Neil2 KO mice following H. pylori infection

Gastric tissues from H. pylori-infected WT and Neil2 KO mice were stained with H&E. The slides were scored using the criteria used in the published literature (69, 70). The scoring system was graded as 1, mild multifocal; 2, mild widespread or moderate multifocal; 3, mild widespread and moderate multifocal; 4, moderate multifocal; 5, moderate widespread and severe multifocal; and 6, severe widespread.

Induction of ROS by GO for NEIL2 expression

Human gastric epithelial (AGS) cells with GO (final concentration, 200 ng/ml) were treated for 1 h, a period known to generate ROS, and the cells were allowed to recover for 24 h following induction of ROS to measure the NEIL2 level.

Induction of ROS by GO to determine its effect on NEIL2 expression

AGS cells were treated with GO (final concentration, 200 ng/ml; 45-min incubation in reduced serum medium), known to generate ROS, and cells were allowed to recover in complete medium for 24 h following induction of ROS. Cells were collected at various time points for isolation of nuclear extract (NE) to assess the NEIL2 level by Western blotting.

Preparation of Nuclear Extract NE

NEs were prepared as described previously (26). Briefly, cells were lysed in buffer A (10 mM Tris-HCl [pH 7.9], 0.54 M sucrose, 3 mM CaCl_2, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40 [NP-40], and 1× protease inhibitor mixture [Roche]) and centrifuged at 3,500 × g for 15 min. Nuclear pellets were washed with buffer A without NP-40 and then lysed in buffer B (20 mM HEPES [pH 7.9], 3 mM EDTA, 10% glycerol, 150 mM potassium acetate, 1.5 mM MgCl_2, 1 mM DTT, 0.1% NP-40, 1 mM sodium orthovanadate [vanadate], and 1× protease inhibitors) by homogenization. Supernatants were collected after centrifugation at 15,000 × g for 30 min, and DNA/RNA in the suspension was digested with 0.15 units/μl Benzonase (Novagen) at 37 °C for 1 h. The samples were centrifuged at 20,000 × g for 30 min and the supernatants collected as NEs.

Biopsy samples

Gastric biopsy samples were collected from gastro-esophageal-duodenoscopy patients according to Institutional Review Board-approved human subject protocols. All human studies abide by the Declaration of Helsinki principles. Histopathology...
and rapid urease testing were used to detect the *H. pylori* infection. NEIL2 transcript expression was determined using qRT-PCR.

**DNA damage analysis**

WT and Neil2 KO mice were infected with *H. pylori*, and gDNA was isolated from stomach epithelial enteroids of uninfected control and *H. pylori*-infected mice using a genomic-tip 20/G kit (Qiagen, catalog no. 10223, with corresponding buffer sets) per the manufacturer’s protocol. This kit has the advantage of minimizing DNA oxidation during the isolation steps; thus, it can be used reliably for isolation of high-molecular-weight DNA with excellent template integrity for detecting endogenous DNA damage using LA-qPCR. The extracted gDNA was quantified by Pico Green (Molecular Probes) in a 96-well black-bottomed plate, and the presence of *H. pylori* was confirmed by PCR. To induce strand breaks at the sites of unrepaired oxidized DNA base lesions, 150 ng of gDNA was treated with 4 units of *E. coli* enzyme Fpg (New England Biolabs) for 1 h at 37 °C. The enzyme was inactivated by incubating the reaction mixture at 65 °C for 15 min. The gene-specific DNA damage was measured by LA-qPCR using 15 ng of Fpg-treated gDNA per PCR reaction by following the protocol reported earlier by Chakraborty et al. (20). LA-qPCR was carried out to amplify a 6.5-kb region of polβ and the 8.7-kb region of globin genes, as done previously (26). To maintain linearity, the final PCR condition was optimized at 94 °C for 30 s (94 °C for 30 s, 55–60°C for 30 s depending on the oligo annealing temperature, 65 °C for 10 min) for 25 cycles and 65 °C for 10 min. Since amplification of a small region would be independent of DNA damage, a small DNA fragment for each gene was also amplified to normalize the amplification of large fragments, as done before (20). The amplified products then were visualized on gels and quantified with an ImageJ automated digitizing system (National Institutes of Health) based on two independent replicate PCRs. The extent of damage was calculated in terms of the relative band intensity with the WT mice considered as one. A similar method was followed to measure the oxidative DNA damage following GO treatment of AGS cells.

**Measurement of DNA/RNA oxidative damage**

The level of DNA damage in WT stomach EDM and NEIL2 KO EDM was challenged with *H. pylori* infection and quantified with the DNA/RNA oxidative damage ELISA kit (Cayman Chemical, USA) according to the manufacturer’s instructions and previous papers (71, 72). This immunoassay detects oxidized guanine species: 8-hydroxy-2′−deoxaguanosine from DNA and 8-hydroxyguanine from either DNA or RNA. Briefly, this assay is based on competition between oxidatively damaged guanosine species and 8-hydroxy deoxaguanosine acetylcholinesterase conjugate (DNA/RNA oxidative damage tracer) for a limited amount of DNA/RNA oxidative damage mAb. The amount of tracer, which is held constant, that will bind to the mAb is inversely proportional to the concentration of oxidatively damaged guanine species in the well. The secondary antibody (goat polyclonal anti-mouse IgG) has been previously attached to the well and will react with antibody oxidatively damaged guanine complex. The reaction is developed by the addition of Ellman’s reagent that has substrate to acetylcholinesterase. The intensity of the color is determined spectrophotometrically at a wavelength of 405–420 nm and is proportional to the amount of oxidative damage tracer bound to the well, which is inversely proportional to the amount of free 8-hydroxy guanosine present in the well. According to the manufacturer’s instructions, standard curves were developed using standards of 3000 pg/ml to 10.3 pg/ml and were prepared using the same culture media of the infection experiment to ensure that the matrix of standards is comparable to the samples.

**Statistics**

Results are expressed as mean ± S.D. and were compared by using a two-tailed Student *t* test. Differences were considered significant if *p* values were lower than 0.05.

**Data availability**

All data are contained within the manuscript.

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*Conflict of interest*—All authors declare that they have no conflict of interest.

*Abbreviations*—The abbreviations used are: GC, gastric cancer; EDMs, enteroid-derived monolayers; MOI, multiplicity of infection; BER, base excision repair; DGs, DNA glycosylases; LA-qPCR, long amplicon quantitative PCR; AG, gastric atrophy; ; ROS, reactive oxygen species; qRT-PCR, quantitative reverse transcription-PCR; GO, glucose oxidase; CM, conditioned medium; NE, nuclear extract; gDNA, genomic DNA.
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