Nod1 Imprints Inflammatory and Carcinogenic Responses toward the Gastric Pathogen Helicobacter pylori

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Abstract

Helicobacter pylori (H. pylori) is the strongest known risk factor for gastric cancer. The H. pylori cag type IV secretion system is an oncogenic locus that translocates peptidoglycan into host cells, where it is recognized by NOD1, an innate immune receptor. Beyond this, the role of NOD1 in H. pylori–induced cancer remains undefined. To address this knowledge gap, we infected two genetic models of Nod1 deficiency with the H. pylori cag+ strain PMSS1: C57BL/6 mice, which rarely develop cancer, and INS-GAS FVB/N mice, which commonly develop cancer. Infected C57BL/6Nod1−/− and INS-GASM101−/− mice acutely developed more severe gastritis, and INS-GASM101−/− mice developed gastric dysplasia more frequently compared with Nod1+/- mice. Because Nod1 genotype status did not alter microbial phenotypes of in vivo–adapted H. pylori, we investigated host immunologic responses. H. pylori infection of Nod1−/− mice led to significantly increased gastric mucosal levels of Th1, Th17, and Th2 cytokines compared with Nod1 wild-type (WT) mice. To define the role of specific innate immune cells, we quantified cytokine secretion from H. pylori–infected primary gastric organoids generated from WT or Nod1−/− mice that were cocultured with or without WT or Nod1−/− macrophages. Infection increased cytokine production from gastric epithelial cells and macrophages and elevations were significantly increased with Nod1 deficiency. Furthermore, H. pylori infection altered the polarization status of Nod1−/− macrophages compared with Nod1+/- macrophages. Collectively, these studies demonstrate that loss of Nod1 augments inflammatory and injury responses to H. pylori. Nod1 may exert its restrictive role by altering macrophage polarization, leading to immune evasion and microbial persistence.

Significance: These findings suggest that manipulation of NOD1 may represent a novel strategy to prevent or treat pathologic outcomes induced by H. pylori infection.

Introduction

Helicobacter pylori (H. pylori) is the most common bacterial infection worldwide, colonizing more than 4.4 billion people (1). Infection with this pathogen also represents the strongest known risk factor for gastric adenocarcinoma, the third leading cause of cancer-related death (2). However, only a percentage of colonized persons ever develop gastric neoplasia (3). One strain-specific virulence locus that augments cancer risk is the cag pathogenicity island (PAI), which encodes a type IV secretion system (T4SS) that translocates the oncoprotein CagA and microbial DNA into gastric epithelial cells (4–6). Following T4SS-mediated delivery, intracellular CagA undergoes tyrosine phosphorylation (5) and activates a eukaryotic phosphatase (SHP-2), leading to carcinogenic cellular responses.

In addition to CagA and DNA, the cag T4SS delivers peptidoglycan into host cells (7–11). Peptidoglycan is also delivered intracellularly via outer membrane vesicles (12). NOD1, which is expressed by most gastrointestinal epithelial cells, is an innate immune receptor and intracytoplasmic sensor of peptidoglycan components. Most gastrointestinal epithelial cells express NOD1 and activation of NOD1 by the muropeptide γ-D-glutamyl-meso-diaminopimelic acid (β-D-glutamyl-meso-diaminopimelic acid (IDEAP) leads to NF-κB–dependent cytokine production as well as induction of autophagy (13, 14). NOD1 is also expressed and activated within macrophages in vivo (15–17). NOD1 sensing of H. pylori peptidoglycan induces NF-κB activation and expression of type I IFN via IFN-regulatory-Factor 7, MIP-2, and β-defensin (7, 8, 13, 18). In humans, genetic variation in ATG16L1, which encodes a key effector of NOD1-dependent autophagy and inflammation, alters susceptibility to H. pylori infection (19).

However, NOD1 activation is tightly regulated by a negative autocrine feedback system, in which NOD1-dependent effectors such as AP-1 and TRAF3 concomitantly suppress the downstream effects of NOD1 activation (18, 20–22). We previously demonstrated that H. pylori–induced injury can be significantly attenuated in vitro and in outbred Mongolian gerbils by preactivation of NOD1 (22), yet the role of aberrant NOD1 activation by H. pylori...
in gastric carcinogenesis has not been fully investigated. Therefore, we utilized two mouse models of gastric injury and cancer on different genetic backgrounds to precisely define the role of Nod1 in inflammation and inflammation-related cancer that develops in response to *H. pylori*.

**Materials and Methods**

**Bacteria**

*H. pylori* strains PMSS1 (23) and 7.13 (24), both cag− strains, were maintained on TSA blood agar plates (25). For in vitro and in vivo experiments, *H. pylori* was cultured in Brucella broth (Becton Dickinson) supplemented with 10% heat-inactivated new calf serum (Atlanta Biologicals) overnight at 37°C and 5% CO2.

**Cells**

AGS cells (ATCC CRL-1739) were purchased from ATCC, tested for *Mycoplasma* contamination on July 10, 2018, and determined to be *Mycoplasma* free. Cells passed for <10 passages were grown in RPMI1640 media (Gibco) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals) at 37°C and 5% CO2. L-WRN fibroblasts (ATCC CRL-3276) were obtained from ATCC, tested for *Mycoplasma* contamination on July 10, 2018, and determined to be *Mycoplasma* free. L-WRN cells passed for <10 passages were grown in Advanced DMEM media (Gibco) supplemented with 10% FBS, 500 μg/ml G418 (Gibco), and 500 μg/ml hygromycin (Invitrogen) at 37°C and 5% CO2. Once cells became confluent, antibiotics were removed, and supernatants were collected. Mouse primary gastric epithelial cell monolayers were generated as reported previously (26). Briefly, gastric glands harvested from wild-type (WT) or Nod1+/− mice were embedded into Matrigel (Corning) and cultured in 50% DMEM conditioned media at 37°C and 5% CO2. Once glands formed 3D gastroids, they were trypsinized and plated in collagen-coated plates or transwell filters (Corning) in 5% L-WRN conditioned media at 37°C and 5% CO2 to convert to 2D monolayers. Cell monolayers were then infected with *H. pylori* at a multiplicity of infection (MOI) of 30.

Bone marrow–derived macrophages were obtained from femurs of C57BL/6 WT and Nod1+/− mice. Briefly, marrows were treated with red blood cell lysis buffer (Becton Dickinson) and washed with PBS, and recovered white blood cells were plated in DMEM media (Gibco) supplemented with 10% FBS and 20 ng/ml of M-CSF (Peprotech) for 6 days at 37°C and 5% CO2 for differentiation.

**Animals**

All procedures were approved by the Animal Care Committee of Vanderbilt University (Nashville, TN). All mouse strains were bred and maintained in the same animal facility. C57BL/6 Nod1−/− mice were kindly provided by Dr. Dana Philpott from the University of Toronto (Toronto, Ontario, Canada). FVB/N INS-GAS Nod1−/− mice were generated by crossing FVB/N INS-GAS Nod1−/− mice with C57BL/6 Nod1−/− mice for 12 generations. Nod1−/− and INS-GAS Nod1−/− genotypes were confirmed by PCR and qPCR respectively. Mice were housed in the Animal Care Facility of Vanderbilt University Medical Center in standard plastic cages in a room with a 12-hour light/dark cycle at 21°C to 22°C and fed a standard rodent Chow (SlcD; Purina). Access to food and water was free throughout all experiments. No special pretreatments (acid inhibition, antibiotics) were used prior to orogastric *H. pylori* inoculation or before sacrificing the animals. For C57BL/6 mice, both females and males were used; for FVB/N INS-GAS Nod1+/− and Nod1−/− mice, only males were used in this study (Supplementary Table S1). Mice 6 to 8 weeks of age were challenged with 1 × 10⁷ *H. pylori* at two time points (days 0 and 2) as described previously (27) except for the 2-day time point, where animals were given a single challenge. All mice appeared healthy with no signs of distress noted throughout the infection period up until the time of sacrifice. Serum samples and gastric tissue were harvested. A single pathologist scored indices of inflammation, injury, and cancer as described previously (28).

Specifically, the following variables were graded on a 0 to 3 scale (0, none; 1, mild; 2, moderate; 3, severe) in the gastric antrum and body: acute inflammation (polymorphonuclear cell infiltration) and chronic inflammation (mononuclear cell infiltration independent of lymphoid follicles); thus, a maximum inflammation score of 12 was possible for each animal. Dysplastic mucosa was graded as 0 (absent), 1 (focal), or 2 (extensive) and consisted of irregular, angulated, and occasionally cystically dilated glands with enlarged overlapping hyperchromatic nuclei. Carcinoma was defined as irregular, angulated, and cystically dilated glands with occasional cribriform architecture in the submucosa and muscularis propria, spreading laterally to the surface mucosal component (28). For quantitative *H. pylori* culture, serial dilutions of homogenized tissue were plated on selective antibiotic TSA blood agar plates (27).

**Multiplex bead array**

Gastric linear strips extending from the squamocolumnar junction through the proximal duodenum were lysed in 200 μl of IP lysis buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitors (Roche). Lysates were diluted 1:3 in assay buffer and mixed with magnetic beads according to the manufacturer’s instructions (Millipore; ref. 27). Data were acquired and analyzed using the Millipore software platform.

**RT2 profiler PCR array**

RNA from primary gastric epithelial cell monolayers was isolated using the RNeasy Kit (Qiagen). Total RNA was treated with RNase-free DNase (Promega) overnight and then used for cDNA synthesis (Applied Biosystems). Real-time PCR master mix, plates, and running protocols were performed following the manufacturer’s instructions for the mouse NF-κB gene target array (Qiagen). Data were analyzed using the online Data Analysis Center provided by Qiagen.

**Real-time RT-PCR**

Total RNA isolated from bone marrow–derived macrophages or AGS gastric epithelial cells (CRL-1739) was subjected to overnight treatment with RNase-free DNase, and then reverse transcribed to cDNA. qPCRs were performed to determine relative differences in expression levels of Nos2, TNFα, IL10, TGFβ, Light, and Ym1, in murine macrophages and CXL8 and CXL2 in AGS cells. Results were then normalized to corresponding levels of GAPDH. For NOD1 inhibition studies, AGS cells were transfected with a mix of shRNAs targeting NOD1 as described previously (22). Colonies were selected using puromycin (10 μg/ml) and tested for NOD1 expression by real-time RT-PCR and Western blot analysis.

**CagA translocation**

AGS cells cocultured with *H. pylori* were lysed in RIPA buffer containing phosphatase and protease inhibitors. Proteins were...
separated using 6% SDS-PAGE mini gels, transferred to PVDF membranes (Thermo Fisher Scientific), and membranes were blocked with 1% BSA (Sigma) overnight. Incubation with primary antibodies (mouse anti-PY99 (Santa Cruz Biotechnology), rabbit anti-CagA (Austral Biologicals), and mouse anti-GAPDH (Millipore) was performed for 1 hour followed by addition of respective HRP-conjugated secondary antibodies (anti-mouse-HRP or anti-rabbit-HRP, Promega). The reaction was developed using ECL (Thermo Fisher Scientific).

**c-Jun immunofluorescence staining**

Monolayers of primary gastric epithelial cells derived from C57BL/6+/+, C57BL/6Nod1fl/fl, FVB/N INS-GASNod1fl/fl, and FVB/N INS-GASNod1−/− mice were infected for 1 hour with *H. pylori* strains 7.13 or PMS11. After infection, monolayers were subjected to c-Jun immunofluorescence staining as previously described (26). Briefly, cells were fixed with 4% paraformaldehyde (Thermo Fisher Scientific) for 1 hour and then blocked with 5% goat serum (Sigma) in PBS for 1 hour. Samples were incubated with an anti-phospho-c-Jun antibody (1:500 dilution; Cell Signaling Technology) overnight at 4°C. Samples were then incubated with Alexa 488-anti-rabbit (1:1,000; Invitrogen), Alexa 568-Phalloidin (1:500; Invitrogen), and Hoechst 33342 (1:1,000; Invitrogen) for 1 hour at room temperature. Slides were mounted using ProLong Glass (Invitrogen), and images were acquired in an Olympus FV-1000 confocal microscopy.

**Statistical analysis**

The Mann–Whitney test was used for two-group comparisons, whereas one-way ANOVA with Newman–Keuls posttest was used for multiple group comparisons. Data were plotted and analyzed using GraphPad Prism 5.6 (GraphPad software Inc). Statistical significance was set at a two-tailed *P* value of <0.05.

**Results**

**Nod1 suppresses the early inflammatory response to *H. pylori* in C57BL/6 mice**

We previously demonstrated that praucivation of NOD1 leads to attenuated cytokine production by *H. pylori in vitro* and reduced inflammation in an outbred model of infection (Mongolian gerbils; ref. 22). We also showed that expression of NOD1 per se and its target genes were significantly decreased in human gastric cancer specimens compared with samples with gastritis alone (22). Therefore, we sought to define mechanisms that regulate this potential anti-inflammatory response within the context of gastric carcinogenesis using genetic models of NOD1 deficiency.

C57BL/6WT and C57BL/6Nod1−/− mice were challenged with the mouse-adapted *H. pylori cag* strain PMS11 or broth alone, and stomachs were harvested and analyzed at 2 (acute), 20 (subacute), and 90 (chronic) days postchallenge. All mice challenged with *H. pylori* were successfully infected. *H. pylori−*infected C57BL/6Nod1−/− mice developed significantly more severe gastric inflammation 20 days postchallenge compared with their infected WT counterparts (*P* < 0.05; Fig. 1A and B). However, by 90 days postchallenge, both groups had similarly elevated inflammation scores (*P* = NS).

To define mechanisms underpinning these temporal differences in inflammation, we initially investigated whether microbial factors contributed to these phenotypes. No significant differences in levels of *H. pylori* colonization were present between WT and Nod1−/− mice at any time point (Fig. 1A). We next determined whether selective pressure exerted by the genetic loss of Nod1 affected *H. pylori* virulence phenotypes in vivo. *H. pylori* strains were recovered from infected WT or Nod1−/− mice and assessed for the ability to translocate CagA in *vitro* as a measure of cag T4SS functionality. AGS gastric epithelial cells were infected with *in vivo*–adapted *H. pylori* strains and the ratio of phosphor-ylated CagA (intracellular) to total CagA was quantified by Western blot analysis. The vast majority of strains harvested at 20 days postinfection harbored a functional cag T4SS. However, as previously reported (25, 29), loss of cag T4SS function was present in many of the *in vivo*–adapted isolates harvested at 90 days postchallenge (Fig. 1C). Because activation of NOD1 is cag-dependent (7), this may partially explain the lack of difference in severity of inflammation between infected WT or Nod1−/− mice at the 90-day time point. Importantly, host Nod1 status had no effect on T4SS function at either time point (Fig. 1C). Thus, on a genetically defined background, loss of Nod1 resulted in more severe subacute inflammation and injury within the context of *H. pylori* infection and in conjunction with strains that harbored a functional cag T4SS; however, Nod1 deficiency did not alter *H. pylori* cag T4SS phenotypes.

**Loss of Nod1 alters cytokine production within *H. pylori*–infected gastric mucosa**

Having shown that *H. pylori* increases inflammation in Nod1−/− mice despite similar cag T4SS function (Fig. 1), we next performed an unbiased survey to assess the role of Nod1-mediated immunomodulatory effectors that may contribute to differences in inflammation within uninfected or infected gastric tissue. Infection with *H. pylori* induced an increase in the levels of numerous chemokines, as well as Th1, Th2, and Th17 cytokines in gastric mucosa harvested from both WT and Nod1-deficient mice when compared with uninfected controls. For ease of presentation, we have included cytokines and chemokines in Fig. 2 that (i) were statistically different in terms of levels of expression between Nod1−/− and Nod1+/+ mice and (ii) could be classified as either chemokines or Th1, Th2, or Th17 cytokines. The complete sets of data are contained in Supplementary Tables S2–S4. Expression levels of archetypal Th1-secreted cytokines such as IL12, Th1-associated proinflammatory cytokines such as IL1, the Th17 cytokines IL17 and IL23, and the Th2 cytokine IL9 were further increased in *H. pylori–*infected C57BL/6Nod1−/− compared with infected C57BL/6WT mice at each time point (2, 20, and 90 days postchallenge; Fig. 2A–C, Supplementary Tables S2–S4). In contrast to universal elevation of these cytokines and chemokines throughout the duration of infection, there were also more selected differences between *H. pylori–*infected WT versus Nod1-deficient mice that varied by time point (Fig. 2A–C, Supplementary Tables S2–S4). Specifically, at 20 days postchallenge when inflammation was significantly increased in Nod1-deficient mice (Fig. 2B), levels of G-CSF, IL7, IP10, IFNα, IFNβ, and IL4 were exclusively increased in infected C57BL/6Nod1−/− compared with C57BL/6WT mice. In addition, the overall number of cytokines and chemokines that were elevated in infected WT mice increased from 2 to 90 days postchallenge (Fig. 2A–C). Thus, in addition to altering the level of inflammation in a time-dependent manner, loss of Nod1 also temporally modified the portfolio of chemokine and cytokine expression within *H. pylori–*infected gastric mucosa.
H. pylori differentially alters cytokine production in primary gastric organoid systems in a Nod1-dependent manner

Our findings in gastric mucosa provided important insights into mechanisms through which Nod1 may restrain early proinflammatory responses to H. pylori. However, gastric tissue contains a myriad of cell types, including epithelial cells and macrophages among others. Gastroids are polarized, replenishable epithelial culture systems that can be readily generated from nontransformed gastric epithelium (30). We have previously developed and optimized gastroid models of H. pylori infection originating from murine tissue (31); therefore, we capitalized on this reductionist ex vivo model to begin to dissect the role of specific cell types in regulating phenotypes linked to Nod1 deficiency (Fig. 3). Polarized 2-dimensional gastroid monolayers were generated from uninfected C57BL/6 WT and C57BL/6 Nod1-/- mice, infected ex vivo with H. pylori strains PMSS1 or 7.13 for 6 or 24 hours, and then coculture total mRNA was subjected to real-time RT-PCR to quantify expression levels of NF-κB...
loss of Nod1 alters cytokine production in gastric mucosa of C57BL/6 mice infected with H. pylori -Fig. 3E and F, we cocultured gastroids isolated from Nod1+/− or Nod1−/− mice with H. pylori and examined c-Jun activation by immunofluorescence. In contrast to the pattern observed with NF-κB, Nod1 deficiency suppressed c-Jun activation in response to H. pylori infection (Fig. 3E and F). Thus, Nod1 can exert differing effects on signaling pathways following infection with H. pylori. Collectively, these results indicate that gastric epithelial cells likely represent an important source of cytokine production that is under Nod1-dependent control during H. pylori infection.

NOD1 regulates cytokine production in multiple innate immune cells following H. pylori infection

Gastritis is an initiating event for the development of most gastric adenocarcinomas, and macrophages are required for gastritis to develop in response to H. pylori (34). Therefore, we enriched our gastroid monolayer system by adding macrophages derived from WT or Nod1-deficient mice to epithelial cells derived from the same mice.

Gastroid epithelial monolayers from uninfected WT or Nod1-deficient mice were seeded in the upper chamber of a transwell system and macrophages from the same mice were placed in the lower chamber in different combinations. H. pylori strain PMSS1 was then added to epithelial monolayers for 24 hours and cytokine production was quantified in supernatants. Loss of Nod1 in H. pylori–infected epithelial cells cocultured with WT macrophages resulted in significantly enhanced levels of chemokines and cytokines, including KC, MIP-2, IL6, MCP-1, and IL1β (Fig. 4A) when compared with infected WT epithelial cells cocultured with WT macrophages. However, loss of Nod1 in both epithelial cells and macrophages resulted in a markedly enhanced cytokine response to H. pylori (Fig. 4B). These results indicate that loss of Nod1 in multiple innate immune cells likely contributes to enhanced inflammation observed in H. pylori–infected Nod1-deficient mice.
We also capitalized on this system to assess cytokine expression in macrophages per se. RNA was isolated from macrophages subjected to uninfected and infected transwell cocultures and expression of prototype M1 (classically activated), M2 (alternatively activated), and Mreg (regulatory) macrophage genes was determined by real-time RT-PCR. Uninfected WT macrophages harbored a predominant M2 phenotype, which shifted dramatically to an M1 phenotype after \( H. pylori \) infection. In contrast, uninfected Nod1-deficient macrophages displayed a more profound M2 phenotype than uninfected WT macrophages; following \( H. pylori \) infection, the profile of Nod1-deficient macrophages shifted to a hybrid M1/M2 phenotype. There were no significant differences in Mreg profiles between macrophages from WT or Nod1-deficient mice (Fig. 4C). Thus, loss of Nod1 alters cytokine profiles in both epithelial cells and macrophages during coinubcation experiments in response to \( H. pylori \).

Loss of Nod1 accelerates gastric carcinogenesis in a mouse model of stomach cancer

Our studies described above using a mouse model of \( H. pylori \)-induced gastritis demonstrated that C57BL/6\(^{\text{Nod1}^{-/-}}\) mice develop more severe inflammation in response to \( H. pylori \) infection than C57BL/6\(^{\text{WT}}\) mice (Fig. 1A and B). Because specific genetic backgrounds can influence disease outcome in different mouse models (35), we next determined whether the increased inflammatory phenotype induced by loss of Nod1 in C57BL/6 mice could be recapitulated in \( H. pylori \)-infected mice on a different genetic background that are also susceptible to gastric cancer.

INS-GAS\(^{\text{Nod1}^{+/+}}\) and INS-GAS\(^{\text{Nod1}^{-/-}}\) mice on a FVB/N background were challenged with \( H. pylori \) strain PMSS1 or broth alone, and stomachs were harvested and analyzed 2, 20, 40, and 90 days postchallenge. The 40-day time point was added due to accelerated inflammation and damage previously seen in the mouse model.
observed in *H. pylori*-infected INS-GAS mice at this time point (36). All mice challenged with *H. pylori* were successfully infected. *H. pylori*-infected INS-GAS<sup>Nod1<sup>−/−</sup></sup> mice developed significantly more severe acute and chronic inflammation compared with their infected WT Nod1 counterparts at 20 and 40 days postchallenge (Fig. 5A and B). Of interest, loss of Nod1 per se in uninfected INS-GAS mice led to increased levels of inflammation and injury at 40 and 90 days postchallenge compared with uninfected INS-GAS mice with a WT Nod1 genotype (Fig. 5A and B). Thus, on two genetically distinct backgrounds, loss of Nod1 resulted in a time-dependent pattern of more severe inflammation and injury within the context of *H. pylori* infection.

Unlike C57BL/6 mice, which do not develop cancer prior to 15 months after *H. pylori* infection (37), INS-GAS mice rapidly develop premalignant lesions as early as 6 to 12 weeks following *H. pylori* challenge (36). At 40 days postchallenge, none of the uninfected or *H. pylori*-infected INS-GAS<sup>Nod1<sup>−/−</sup></sup> developed gastric dysplasia. However, loss of Nod1 led to the development of dysplasia in 50% of uninfected mice, and the frequency of dysplasia rose significantly in conjunction with *H. pylori* infection (Fig. 5C). At 90 days postchallenge, these effects were magnified.
Infection of INS-GAS\(Nod1^{+/+}\) with \(H. pylori\) significantly increased the prevalence of dysplasia compared with uninfected WT controls, which did not demonstrate any evidence of premalignant lesions. In uninfected INS-GAS\(Nod1^{-/-}\) mice, the prevalence of gastric dysplasia was significantly higher than in uninfected INS-GAS\(Nod1^{+/+}\) mice; however, infection with \(H. pylori\) similarly augmented this effect as 95% of infected INS-GAS\(Nod1^{-/-}\) mice developed gastric dysplasia by this time point.

To assess the levels of microbial colonization in each group, we quantified colony-forming units (CFU) from each infected animal. There were no differences in colonization between WT and \(Nod1^{-/-}\) INS-GAS mice at any time point (Fig. 5A). Collectively, these data indicate that the increased injury phenotype that develops in \(H. pylori\)-infected \(Nod1\)-deficient mice is not an artifact of a single host genetic background.

**Nod1 deficiency increases gastric mucosal cytokine production in \(H. pylori\)-infected INS-GAS mice**

We next sought to elucidate the mechanisms through which loss of \(Nod1\) resulted in increased inflammation and premalignant lesions among \(H. pylori\)-infected INS-GAS mice by quantifying \(Nod1\)-mediated immune effectors within uninfected or infected gastric tissue. Similar to our results in C57BL/6 mice (Fig. 2A–C; Supplementary Tables S2–S4), infection with \(H. pylori\) induced an increase in the levels of chemokines, as well as Th1, Th2, and Th17 cytokines in both WT and \(Nod1\)-deficient mice when compared with uninfected controls and these changes were augmented within the context of \(Nod1\) deficiency (Fig. 6A–D; Supplementary Tables S10–S13). For ease of presentation, we have included cytokines and chemokines in Fig. 6 that (i) were statistically different in terms of levels of expression between \(Nod1^{+/+}\) and \(Nod1^{-/-}\) mice and (ii) could be classified as either chemokines or Th1, Th2, or Th17 cytokines. The complete sets of data are contained in Supplementary Tables S10–S13. Because inflammation was more severe in \(Nod1\) deficient INS-GAS mice at 20 and 40 days postinfection (Fig. 5A and B), we focused on selected differences between \(H. pylori\)-infected WT versus \(Nod1\)-deficient mice at these time points (Fig. 6B and C). Specifically, at 20 days postchallenge, levels of IL12p40 and TNF\(\alpha\) were exclusively increased in infected INS-GAS\(Nod1^{-/-}\) compared with INS-GAS\(WT\) mice and at 20 and 40 days postinfection, levels of IL6 were exclusively increased in the same pattern. There was also a shift toward more chemokines and cytokines being increased in INS-GAS\(Nod1^{+/+}\) mice compared with their \(Nod1\)-deficient
Figure 6.

Nod1 deficiency increases gastric mucosal chemokine/cytokine production in H. pylori–infected INS-GAS mice and human gastric epithelial cells. Expression of chemokines and cytokines in gastric mucosa of INS-GAS Nod1+/+ and INS-GAS Nod1−/− mice infected with H. pylori strain PMSS1 for 2 (A), 20 (B), 40 (C), and 90 (D) days. Bars, mean ± SEM. Light shaded bars, upregulated in INS-GAS Nod1−/− mice; dark shaded bars, upregulated in INS-GAS Nod1+/+ mice. *, P < 0.05; **, P < 0.01. E, AGS cells stably transfected with either nontargeting shRNA or shRNA specific for NOD1 were cocultured with strains 7.13 or PMSS1 at MOI 30 for 6 and 24 hours. Expression levels of CXCL8 (left axis) and CXCL2 (right axis) were determined by real-time RT-PCR. Primer sequences are shown in Supplementary Table S14. Data represent mean ± SEM from at least two experiments. *, P < 0.05; **, P < 0.01.
counterparts as the duration of infection progressed (Figs. 6A–D). Collectively, these results and results from C57BL/6 mice demonstrate that loss of Nod1 alters the severity of mucosal damage as well as the portfolio of chemokine and cytokine expression within H. pylori–infected gastric mucosa in two different murine models of gastric injury. However, the effects of Nod1 deficiency wane over time, suggesting that Nod1 exerts its effects predominantly on innate immune responses, which is consistent with our epithelial/macrophage guttoid coulture results.

Finally, because H. pylori is a human gastric pathogen, we ascertained the effects of infection on Nod1–dependent signaling in human gastric epithelial cells. For these studies, stably transduced human AGS gastric epithelial cells harboring either NOD1-targeting or control shRNA were used as described previously (22). Inhibition of NOD1 prior to infection with the H. pylori cag+ strains PMSS1 or 7.13 increased production of CXCL8 and CXCL2, confirming our data in mouse gastroids that suppression of Nod1 can augment inflammatory responses to this pathogen (Fig. 6E).

Discussion

The innate immune system is exquisitely poised to detect and respond to bacteria residing at mucosal surfaces (38, 39). However, chronic mucosal pathogens have developed multiple strategies to subvert this facet of the immune response (40). H. pylori has coevolved with its cognate human host for over 100,000 years and is uniquely adapted to survive for decades within the harsh environment of the stomach (41). This has necessitated the development of mechanisms to induce inflammation as well as strategies to evade detection and downregulate the host immune response.

H. pylori harbors multiple pathogen-associated mucosal patterns that interact differently with innate immune effectors than the respective counterparts in other acute mucosal pathogens. For example, H. pylori LPSA is a noninflammatory molecule in terms of its ability to activate TLR5 (42). H. pylori LPS contains an anergic lipid A core that induces an attenuated TLR4-mediated response (43, 44). The cag T4SS delivers peptidoglycan into host cells, where it is recognized by NOD1 (7–11), and although NF-κB activation is a prototypical response to NOD1 activation, we and others have shown that preactivation of NOD1 suppresses subsequent H. pylori–induced NF-κB signaling via activation of a negative feedback loop, and that deacetylation of peptidoglycan allows H. pylori to evade host clearance (22, 45–47). More recent work has also indicated that NOD1 may suppress gastric inflammation in response to H. pylori. Tran and colleagues used a mouse model of gastritis to demonstrate that H. pylori promotes the activation of IL33, a mediator of Th2 immune responses, via Nod1 signaling (48). Importantly, H. pylori cag+ strains specifically activated Nod1 in mouse gastric epithelial cells, leading to enhanced levels of IL33 in gastric mucosa and spleenocytes, which was linked with reduced IFNγ responses (48). Our current results are consistent with these data, as loss of Nod1 in two independent models of H. pylori–induced inflammation and injury augmented damage within the gastric niche. However, there are additional pathways that can regulate NF-κB activation following H. pylori infection that are independent of NOD1. Gall and colleagues demonstrated that NF-κB activation can be induced by TNF receptor–associated factor (TRAF)–interacting protein with forkhead-associated domain (TIFA), and that this occurs independently from NOD1–mediated NF-κB activation (49). Furthermore, NF-κB activation following H. pylori infection occurs in a temporally regulated manner, with TIFA induction occurring early, which is subsequently followed by NOD1–dependent NF-κB activation (49). Selective activation of these additional pathways under different experimental conditions may account for differences in our current results when compared with previous results published by Viala and colleagues (7). Therefore, future studies should focus on assessing both NOD1 and TIFA innate immune signaling pathways in primary organoid systems as well as animal models of infection, utilizing both WT H. pylori cag+ strains and mutant strains with defective cag secretion systems, to precisely elucidate the combinatorial contributions of these constituents to H. pylori pathogenesis.

In this study, Nod1–/– genotypes did not alter the microbial phenotype of H. pylori output derivatives in terms of T4SS function. However, Nod1–deficient mice developed more severe inflammation compared with WT mice when colonized with H. pylori strains harboring a functional cag T4SS. Using a multiplex cytokine array to examine distinct host immune effectors that may contribute to this increase in inflammation, we observed that, as expected, H. pylori infection broadly increased levels of Th1, Th2, and Th17 cytokines, and these changes were augmented in the presence of Nod1 deficiency. However, differences stratified on the basis of Nod1 genotype waned over time. To dissect this further, we utilized an innovative reductionist system in which the effects of H. pylori on WT or Nod1–/– epithelial cells alone or epithelial cells cocultured with macrophages of varying Nod1 genotype could be ascertained. We worked for the first time that loss of Nod1 in both epithelial cells and macrophages augmented the production of proinflammatory cytokine production in response to H. pylori infection, further supporting the premise that NOD1 primarily alters innate immune responses to this pathogen. To more fully define the respective contributions of each of these cellular constituents, however, would require eliminating the macrophage and the gastric epithelial cell component of Nod1–dependent signaling, both individually and in combination. This could be done by crossing Nod1–/– mice and Fox3-Cre mice (which directs expression of Cre recombinase to gastric epithelium; ref. 50) in conjunction with selective depletion of macrophages using agents such as clodronate liposomes and our current results have provided an important framework for these future studies.

In addition to differences in cytokine levels between Nod1–/– and Nod1+/– mice, we also found differences in the levels of certain cytokines when we compared C57BL/6 with INS-GAS FVB/N mice. These results likely reflect differences in the genetic backgrounds of the mice under study as well as the presence of hypergastrinemia, which is inherent to INS-GAS mice bearing the human gastrin transgene (51). Gastrin exerts growth factor–like effects on gastric epithelial cells, which may induce the production of certain chemokines and cytokines (51). Furthermore, INS-GAS mice are on a FVB/N genetic background that has previously been shown to augment the risk for carcinogenesis when compared with C57BL/6 mice. Specifically, this may be due to a polymorphism within the Pchtl gene, which encodes an inhibitory receptor for ligands of the Hedgehog gene family (52). Within the context of our results, it was notable that, among others, expression levels of IL9 were downregulated in INS-GAS Nod1–/– mice when compared with C57BL/6 Nod1–/– mice. IL9 is a cytokine produced by Th9 cells, a subpopulation of CD4+ T cells, and several studies have demonstrated that IL9 suppresses tumor
growth (53, 54). Thus, decreased levels of IL9 in H. pylori–infected INS-GAS Nod1−/− mice may contribute to the enhanced carcinogenic phenotype seen in these mice following infection with this pathogen.

Alterations in the composition of functional macrophage phenotypes within a specific inflamed niche can significantly alter the risk for carcinogenesis (55). M1 (classically activated) macrophages clear pathogens via intracellular microbialid activity and by secreting inflammatory mediators that promote a Th1-type response, while simultaneously dampening Th2-type responses (55). M2 (alternatively activated) macrophages promote wound healing by secreting components of the extra-cellular matrix and anti-inflammatory effectors that promote Th2-directed responses while dampening Th1 responses (5). Mregs (regulatory macrophages) are also anti-inflammatory, but fail to deposit extracellular matrix (55). Our results utilizing epithelial/gastrointestinal macrophage cocultures with or without H. pylori revealed that NOD1 likely plays a role in macrophage polarization as Nod1 WT macrophages cocultured with epithelial cells developed a profound M1 phenotype following exposure to H. pylori compared with a mixed M1/M2 phenotype exhibited by infected Nod1−/− macrophages. Such a hybrid phenotype may render Nod1-deficient macrophages ineffective in dampening respective Th1- or Th2-directed responses, thereby leading to a more severe pattern of global inflammation; our current results provide an important framework for defining such mechanisms in future work. Thus, the capacity of NOD1 to regulate macrophage phenotypes may also contribute to the ability of H. pylori to evade host immune clearance.

In conclusion, this study demonstrates that loss of NOD1 augments inflammatory responses to H. pylori within the context of gastric carcinogenesis. NOD1 may exert its restrictive role by altering macrophage polarization, thereby leading to immune evasion and microbial persistence. These studies lay the foundation for further exploration into the role of NOD1–H. pylori interactions in human hosts and suggest that manipulation of NOD1 may represent a novel strategy to prevent or treat pathologic outcomes induced by H. pylori infection.

Disclosure of Potential Conflicts of Interest

R.M. Peek Jr is the editor of Gastroenterology at AGA. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: G. Suarez, R.M. Peek Jr Development of methodology: G. Suarez, R.M. Peek Jr Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Suarez, J. Romero-Gallo, M.B. Piazuelo, J.C. Sierra, A.G. Delgado, M.K. Washington Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Suarez, K.T. Wilson, R.M. Peek Jr Writing and review, and/or revision of the manuscript: G. Suarez, M.K. Washington, S.C. Shah, K.T. Wilson, R.M. Peek Jr Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Suarez, J. Romero-Gallo, R.M. Peek Jr Study supervision: G. Suarez, R.M. Peek Jr

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Supplementary Material

Supplementary Fig. 1. NOD1 expression in human macrophages. doi:10.1158/0008-5472.CAN-18-2651.

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