Nitric oxide and TNF- trigger colonic inflammation and carcinogenesis in Helicobacter hepaticus-infected, Rag2-deficient mice
Nitric oxide and TNF-\(\alpha\) trigger colonic inflammation and carcinogenesis in *Helicobacter hepaticus*-infected, *Rag2*-deficient mice

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Recombinase-activating gene-2-deficient (*Rag2\(^{−/−}\)*) mice lacking functional lymphocytes provide a useful model of chronic inflammatory bowel disease-emulating events in human colon cancer. Infection of *Rag2\(^{−/−}\)* mice with *Helicobacter hepaticus* led to accumulation of macrophages and neutrophils in the colon, a process temporally related to up-regulation of tissue inducible nitric oxide synthase (iNOS) expression at the site of infection and increased nitric oxide (NO) production, as evidenced by urinary excretion of nitrate. Progressive development of increasingly severe inflammation, hyperplasia, dysplasia, and cancer accompanied these changes. Concurrent administration of an iNOS inhibitor prevented NO production and abrogated epithelial pathology and inhibited the onset of cancer. The presence of Gr-1\(^+\) neutrophils and elevated tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) expression in colon were required for increased iNOS expression and cancer, whereas interleukin-10 (IL-10) down-regulated TNF-\(\alpha\) and iNOS expression and suppressed cancer. Anti-inflammatory CD4\(^+\) regulatory lymphocytes also down-regulated iNOS and reduced cancer formation. Collectively, these results confirm essential roles for inflammation, increased TNF-\(\alpha\) expression, and elevated NO production in colon carcinogenesis.

colon cancer | IBD | innate immunity

Chronic *Helicobacter pylori* infection in humans leads to gastritis and has been established as a causative agent for human gastric cancer (1). Inflammatory bowel diseases (IBDs), such as Crohn’s disease and ulcerative colitis, also increase risk for colon cancer (2, 3). Generation of nitric oxide (NO) by inducible NO synthase (iNOS) is a central feature of chronic inflammatory diseases in the gastrointestinal tract (4–9), but precise mechanistic roles for NO in colon cancer development remain undefined.

Colon cancer patients exhibit evidence of nitrosative and oxidative stresses that increase cancer risk (10), resulting from mutagenic reactive oxygen and nitrogen species derived from NO generated by immune cells (6, 11–15). Roles for chronic bacterial infection in IBD and colon cancer have been identified recently in recombinase-activating gene-2-deficient mice (*Rag2\(^{−/−}\)*), which completely lack functional lymphocytes (16–18). We have exploited this mouse model of chronic IBD-associated cancer for studies of the role of NO and its products because it emulates naturally occurring inflammatory events in humans (16, 19, 20).

*Rag2\(^{−/−}\)* mice have been used to assess functions of lymphocytes by adoptive transfer. Populations of CD4\(^+\) T cells with low or high expression of CD45RB (17, 21, 22) or CD25 (16, 18, 23, 24) prevent or accelerate colitis in these animals. In wild-type (wt) mice, protection against inflammatory pathology induced by bacterial infection has been attributed to interleukin-10 (IL-10) and IL-10-dependent functions of CD4\(^+\) cells (18, 20, 25, 26). Collectively, this evidence forms the rationale for the hypothesis that NO overproduction comprises a linkage between *Helicobacter hepaticus*-induced inflammation and induction of colon cancer (16), which we further test in this study by assessing protective effects of N-methyl-arginine (NMA), an iNOS inhibitor, administered in the drinking water of infected animals. All experiments were carried out in *Rag2\(^{−/−}\)* mice unless otherwise stated.

Results

Infection and NO Production. To assess the contribution of NO production to colon cancer development, *Rag2\(^{−/−}\)* and wt mice were infected with *H. hepaticus* (16, 18). Total NO production was measured by urinary nitrate (NO\(_3^-\)) excretion (11). Infected, but not uninfected, mice showed a time-dependent increase (\(P < 0.05\)) in urinary NO\(_3^-\) excretion beginning 2 weeks after infection (Fig. 1A), which was temporally associated with development of IBD and carcinoma (Table 1). In contrast, infected wt mice, showed no elevation of urinary nitrate and did not develop cancer (Fig. 1B) (16), indicating that infection alone was not sufficient to induce cancer.

To estimate levels of NO synthesis at the site of infection (20), iNOS expression was examined by quantitative RT-PCR on sections of ascending colon. Tissue from infected mice contained 200-fold higher (\(P < 0.001\)) levels of iNOS mRNA than were present in uninfected *Rag2\(^{−/−}\)* or wt controls (Fig. 2A). Expression of iNOS protein in situ in ascending colon was localized by FIHC to epithelial cells and macrophages, and less frequently to granulocytes [Fig. 1 C and D and supporting information (SI) Fig. S1].

Infection of iNOS-deficient mice with the presence of 7/4\(^+\) polymorphonuclear cells (Table S1) (18, 20, 27), and also correlated with up-regulation of tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), IL-6, and MIP-2 (Fig. 2A) (28). The iNOS-dependent activities of bone marrow-derived 7/4\(^+\) neutrophils have been shown previously to be required for induction of IBD in mice (29).

Immune Cell Modulation of Infection-Induced NO Production and Colon Pathology. Myeloid differentiation antigen Gr-1 (or Ly-6G) is expressed on bone marrow precursors and peripheral neutrophils and is considered a good marker for these populations. Treatment with anti-Gr-1 antibody has been shown previously to deplete mature neutrophils and abrogate *Helicobacter*-induced gastritis in mice (30). For further assessment of the role of neutrophils in colon carcinogenesis in infected *Rag2\(^{−/−}\)* mice, we...
performed depletion experiments by intraperitoneal injection of anti-Gr-1 antibody thrice weekly for 10 days beginning at 6 weeks after infection. Depletion of Gr-1+ cells significantly \( (P < 0.01) \) decreased iNOS gene expression levels (Fig. 2B) and the severity of colon pathology (Table S1) in infected mice compared with infected, sham IgG-treated animals. Interestingly, depletion of Gr1+ cells eliminated 7/4+ neutrophils (\( \mu = 0.4 \pm 0.32 \) cells in treated vs. 28.4 \( \pm 3.33 \) cells in control mice) and significantly decreased macrophages (6.6 \( \pm 1.88 \) cells in treated vs. 13.8 \( \pm 1.14 \) cells in control mice). iNOS expression within epithelia and other inflammatory cells in the colon also was suppressed (Fig. S1 B and D) compared with sham-treated infected mice (Fig. S1A and C). Depletion also decreased colon mRNA levels of TNF-\( \alpha \) \( (P < 0.001) \) and IL-6 \( (P < 0.05) \) (Fig. 2B), suggesting that Gr-1+ cells may induce iNOS expression through cytokine-mediated processes. Similarly, their depletion decreased the frequency of myeloperoxidase (MPO)-positive cells (21.3 \( \pm 4.3 \) vs. 4.0 \( \pm 1.3 \) MPO+ cells per 40\( \times \) field in sham and anti-Gr-1 mice, respectively; \( P < 0.01 \)) in the colon (Fig. S1). Taken together, these results demonstrate that Gr-1+ cells are required for colitis-associated cancer in \( H. \) hepaticus-infected Rag2/+ mice, and suggest a functional relationship between iNOS and MPO in the carcinogenesis process (31).

Because nuclear factor \( \kappa B \) (NF-\( \kappa B \))-mediated inflammatory signaling is dependent upon TNF-\( \alpha \) (32), we postulated that this proinflammatory cytokine would be required for recruitment and activation of Gr-1+ neutrophils. We therefore neutralized TNF-\( \alpha \) in Rag2/+ mice by injection of anti-TNF antibody, which significantly \( (P < 0.05) \) decreased expression of IL-6, MIP-2, and iNOS (Fig. 2C) and reduced bowel pathology (Table S1) compared with uninfected controls. These results suggest a sequence of events in which bacterial infection triggers increased TNF-\( \alpha \) expression, leading to up-regulation of iNOS and contributing to development of colon cancer in infected mice.

Anti-inflammatory activities of IL-10 have been shown to inhibit TNF-\( \alpha \)-dependent responses to pathogenic bacteria in several mouse models (18, 20, 25). We therefore used Rag2/−/− deficient mice also lacking IL-10 (IL10/−/−Rag2/−/−) to determine whether IL-10 was capable of preventing iNOS up-regulation triggered by infection. Infected double-knockout mice showed significant \( (P < 0.01) \) increases in iNOS gene expression (Fig. 2D), 7/4+ neutrophils, and F4/80+ macrophages (Table S2), as well as inflammatory pathology (Fig. S2 C–E) and colon tumors \( (P < 0.05; \) Table 1), when compared with infected Rag2/+ control mice. Uninfected IL10/−/−Rag2/−/− mice had undetectably low levels of inflammatory cytokines (Fig. 2D) and colon pathology (Table 1). Replacement of IL-10 by injection of IL10-Ig fusion protein restored iNOS and inflammatory cytokine expression to baseline levels (Fig. 2D). Likewise, infected Rag2/−/− mice treated with IL10-Ig fusion protein had significantly \( (P < 0.05) \) decreased inflammation and tumor development when compared with sham-treated animals (Table 1). Thus, IL10 suppressed infection-induced iNOS elevation and tumor formation, whereas IL10-deficient mice were highly susceptible to \( H. \) hepaticus-infected bowel disease (Table 1 and Table S2).

To study further the role of IL-10 in modulating infection-induced responses, we performed adoptive transfer of purified IL10-producing CD4+ lymphocytes into infected Rag2/−/− mice. We found that CD4+ cells collected from \( \text{wt} \) but not IL10/−/− donor mice prevented up-regulation of iNOS (Fig. 2E) and development of cancer (Table 1). To determine whether regulatory T (TReg) cells—i.e., the subset of \( \text{wt} \) CD4+ cells with low expression of CD45RB that have potent anti-inflammatory properties (16, 18)—would down-regulate TNF-\( \alpha \) (18, 27), we performed adoptive i.v. transfer of 3.0 \( \times 10^5 \) TReg cells into mice at the time of infection. Mice receiving \( \text{wt} \) TReg cells had reduced \( (P < 0.005) \) epithelial dysplasia in the cecum (Table S3) and colon, as well as decreased inflammatory gene expression (Fig. 2E) 3–4 months after infection. However, this inhibitory effect was not a property of all CD4+ cells. Indeed, infected mice that received the CD4+CD45RBhi (TEFF) subset of cells developed more severe IBD and mucinous carcinoma (Fig. S2 and Table 1), a variant of colorectal cancer in humans that arises in about 20% of patients with IBD (33). TEFF cell recipients also had increased levels of IFN (IFN-\( \gamma \)) and iNOS (Fig. 2E), increased urinary NO3− (Fig. 1B), and a higher frequency of iNOS-bearing F4/80+ and MPO+ cells (data not shown), compared with infected controls. In contrast, uninfected recipients showed minimal pathology (Table 1), in agreement with the

Fig. 1. Urinary nitrate excretion after infection with \( H. \) hepaticus. (A) Temporal increase in urinary nitrate excretion followed infection with \( H. \) hepaticus. (B) Nitrate excretion was elevated in infected Rag2/+ mice with cancer but not \( \text{wt} \) mice without cancer. Treatment with NMA or TReg cells decreased urinary nitrate levels to that of uninfected Rag2/+ mice and prevented cancer development in infected Rag2/+ mice. (C) IFHC showed colocalization of F4/80 and iNOS proteins in the colon of an \( H. \) hepaticus-infected mouse. (Original magnification, 100\( \times \)) (D) Higher magnification (1,000\( \times \)) of C reveals iNOS expression in inflammatory cells.

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in situ within colonic tissue was reduced by NMA (Fig. 3), significantly decreased epithelial hyperplasia in colonic (Fig. 3), and dysplasia without having an impact on inflammatory cell levels in tissues (Fig. 3), in agreement with previous studies (35, 36). Lack of change in cellular inflammatory cell infiltrates and inflammation in NMA-treated mice (Fig. 3A) points to dysregulation of NO production rather than the presence of inflammatory cells per se in the pathogenesis of colon cancer in this system. The host benefit of blocking NO synthesis beyond IBD-associated colon inflammation (37) is suggested by reduced intestinal adenoma formation in mice that undergo spontaneous loss of heterozygosity of the adenomatous polyposis coli (Apc) gene (ApcMin/+ mice) using both inhibitors and mice lacking iNOS (37).

The proinflammatory cytokine TNF-α and bacteria-triggered TNF-α-dependent increases in inflammatory cell infiltrates and NO production have been shown to be required for development of colon cancer in mice (14, 15, 29). Beck et al. (29) revealed the main source of NO to be bone marrow-derived inflammatory cells, in particular iNOS-bearing 7/4+ granulocytes. Neutrophils also have been shown to be required for infection-induced cancer (38, 39). In our experiments, depletion of Gr-1 neutrophils, such as escalating host inflammation-driven mouse models (41), confirmed by studies showing indirect effects of Gr-1 neutrophils, such as escalating host inflammatory response (28) and oxidative stressors (11, 29, 40), that promote cancer.

Supplementation with proinflammatory T eff cells increased IFN-γ-dependent and TNF-α-dependent malignancy in inflammation-driven mouse models (41), confirmed by studies showing increased inflammatory disease after T eff cell transfer in H. hepaticus-infected Rag2−/− mice (18, 22, 42). Increased IFN-γ secretion and NO (11, 15) have been found in humans with gastrointestinal carcinoma as well (1). Targeting inflammation and NO is clinically relevant, because mucinous colonic carcinoma seen in T eff cell recipient mice most often affects young humans and has a poor prognosis associated with invasion of adjacent viscera and lymph nodes beyond the pericolic region (33). Cotransfer of CD4+ T reg and T eff cells significantly protected against colon carcinogenesis (41), and NO was linked previously published findings of others (18). Together, these data indicate that protective properties of IL10-competent lymphocytes reside in the CD45RBlo subset of CD4+ cells. Cotransfer of anti-inflammatory T reg cells along with T eff cells in infected mice was sufficient to down-regulate urinary NOx (Fig. 1B) and colon pathology (Table 1), indicating that IL10-competent T reg cells inhibited TNF-α-mediated inflammatory events leading to cancer.

**Table 1. Induction of colon pathology in immune-deficient Rag2−/− mice by H. hepaticus (Hh) infection and modulating effects of immune cell augmentation**

<table>
<thead>
<tr>
<th>Hh status</th>
<th>Genotype</th>
<th>Treatment</th>
<th>Severity of colon pathology*</th>
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<tbody>
<tr>
<td>−</td>
<td>wt</td>
<td>−</td>
<td>Inflammation</td>
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<tr>
<td>+</td>
<td>wt</td>
<td>−</td>
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<td>+</td>
<td>wt</td>
<td>−</td>
<td>1 (0–2)</td>
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<tr>
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<td>−</td>
<td>0 (0–1)</td>
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<tr>
<td>+</td>
<td>Rag2−/−</td>
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<td>+</td>
<td>Rag2−/−</td>
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*Median severity score (range) on 0–4 scale.

**Discussion**

Observations of Virchow more than a century ago (32) are supported by many epidemiological studies illustrating that chronic inflammation increases cancer risks in humans. The features of colon carcinoma induced in Rag2−/− mice by H. hepaticus infection closely resemble those of colon cancer in humans (34). Epithelial dysplasia and carcinoma arise in situ from inflammatory cell foci and epithelial ulceration, then progress to intramucosal carcinoma, and highly invasive mucinous carcinoma (16, 20, 27). Although a bacterial etiology has not been confirmed for IBD in humans, H. pylori infection has been shown to lead to gastritis-associated gastric cancer (1). Treatment of infected mice with NMA reduced epithelial dysplasia without having an impact on inflammatory cell levels in tissues (Fig. 3), in agreement with previous studies (35, 36). Lack of change in cellular inflammatory cell infiltrates and inflammation in NMA-treated mice (Fig. 3A) points to dysregulation of NO production rather than the presence of inflammatory cells per se in the pathogenesis of colon cancer in this system. The host benefit of blocking NO synthesis beyond IBD-associated colon inflammation (37) is suggested by reduced intestinal adenoma formation in mice that undergo spontaneous loss of heterozygosity of the adenomatous polyposis coli (Apc) gene (ApcMin/+ mice) using both inhibitors and mice lacking iNOS (37).
with increased IL-10-mediated recruitment of protective anti-inflammatory TREG cells (43). Prior challenge with H. hepaticus induced IL-10-dependent TREG cells that protected against IBD in mice (26). TREG cells collected from H. hepaticus-infected donors had greater potency to protect against intestinal and mammary cancer than those from uninfected donors (44). Identifying which TREG subsets are most effective in the regulation of TNF-α/H9251 and NO to prevent or treat infection-associated colon cancer is a future goal.

Our findings suggest a connection between NO and MPO, an enzyme present in granulocytes, in which NO is converted to nitrite, and nitrite is oxidized by MPO to NO2 (31). NO2 is a strong one-electron oxidant capable of both oxidation and nitration of nucleic acids, proteins, and lipids (45–49). Products generated by NO2 oxidation of DNA are toxic and highly mutagenic (50, 51), and lipid peroxidation products yield reactive carbonyls that also modify DNA to form promutagenic bases (52). Studies in MPO/H11002/H11002 mice showed that formation of nitrotyrosine, a product of NO oxidation to peroxynitrite, required MPO following Klebsiella pneumoniae infection that induced primarily neutrophil infiltration (53). Only when both nitrite and MPO were present did nitrotyrosine form.

Our principal findings can be summarized as follows. Infection of Rag2/H11002/H11002 mice with H. hepaticus led to infiltration of macrophages and neutrophils into the colon, which was temporally related to up-regulation of iNOS expression at the site of infection and increased NO production, evidenced by urinary excretion of nitrite. Progressive development of increasingly severe inflammation, hyperplasia, dysplasia, and cancer were the consequences of reduced iNOS and IFN-γ gene expression. (E) T EFF cells increased levels of IFN (IFN-γ) and iNOS expression.

Fig. 2. Modulation of expression of iNOS and inflammatory cytokines in colon tissue. (A) Expressions of proinflammatory cytokines and iNOS were significantly increased after infection with H. hepaticus (Hh). Depletion of Gr-1+ cells (B) and TNF-α (C) decreased iNOS expression. (D) Infected double-knockout mice showed significant increases in iNOS and IFN-γ gene expression. (E) T EFF cells increased levels of IFN (IFN-γ) and iNOS expression.

Fig. 3. NO synthase inhibitor NMA inhibits development of malignancy in mice. (A) Numbers of 7/4+ neutrophils and F4/80+ macrophages were unchanged by NMA treatment; therefore, protective effects of NMA were not attributable to reduced numbers of inflammatory cells. (B) Histopathologic indices of dysplasia and cancer were reduced by NMA treatment.
pathology and inhibited the onset of cancer. The presence of Gr-1− cells and elevated TNF-α expression in colon were required for increased iNOS expression and cancer, whereas IL-10 down-regulated TNF-α and iNOS expression and suppressed cancer. Anti-inflammatory CD4+ Treg lymphocytes down-regulated iNOS and reduced cancer formation. Collectively, these results confirm essential roles for elevated NO production and TNF-α expression in the carcinogenesis process in this experimental model.

Materials and Methods

Animals. Experiments were conducted in 129/SvEv Rag2−/− and wt mice (16) housed in Association for Assessment and Accreditation of Laboratory Animal Care-approved facilities in static microisolation cages and were approved by the institutional Animal Care and Use Committee. Each experiment included 6–10 mice per group and was repeated twice (total 10 with 2 trials; total n = 20) as described elsewhere. Additionally, 8 IL10−/− Rag2−/− mice were treated by i.p. injection of IL10-Ig fusion protein to restore IL10 function. Forty mice served as H. hepaticus-free controls. Mice were euthanized at 6–8 weeks after infection.

IL-10 Modulation. In experiments to determine effects of IL-10 on iNOS expression, 28 IL10−/− Rag2−/− mice were infected and compared with Rag2−/− mice (n = 10 with 2 trials; total n = 20) as described elsewhere. Additionally, 8 IL10−/− Rag2−/− mice were treated by i.p. injection of IL10-Ig fusion protein to restore IL10 function. Forty mice served as H. hepaticus-free controls. Mice were euthanized at 6–8 weeks after infection.

Histopathology. For histologic evaluation, formalin-fixed tissues were embedded in paraffin, cut at 5 μm, and stained with hematoxylin and eosin. Lesions were scored by 2 pathologists blinded to sample identity. Hyperplastic and inflammatory lesions were graded on a scale of 0 to 4 with ascending severity, as described previously (16, 34). Macrophages and neutrophils were identified in intestinal tissue sections with standard avidin-biotin complex immunohistochemistry and were quantified as described previously (41). Immunofluorescence was performed as described elsewhere (58).

Gene Expression. Gene expression analysis was conducted on snap-frozen 0.5- to 1.0-cm full-thickness sections of ascending colon by TaqMan analysis (Applied Biosystems) using expression assays designed by Applied Biosystems (Assays-on-Demand: www.appliedbiosystems.com).

Statistical Analysis. Statistical analyses of colonic lesion scores were performed by using a Mann–Whitney U nonparametric test for ordinal data. Comparisons of frequency of carcinoma between groups were performed by using a 2-sided Fisher exact test. Macrophage and neutrophil counts, urinary nitrates, and cytokine gene expression were compared by using Student’s t test. Statistical analyses used Graphpad Prism 4.0 (GraphPad Software, Inc.).

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