Nitric oxide and TNF-trigger colonic inflammation and carcinogenesis in Helicobacter hepaticus-infected, Rag2-deficient mice

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Nitric oxide and TNF-α 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 Gr1+ neutrophils and elevated tumor necrosis factor-α (TNF-α) expression in colon were required for increased iNOS expression and cancer, whereas interleukin-10 (IL-10) down-regulated TNF-α 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-α expression, and elevated NO production in colon carcinogenesis.

*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 (NO3−) excretion (11). Infected, but not uninfected, mice showed a time-dependent increase (*P* < 0.05) in urinary NO3− 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 those 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]. Induction of iNOS was associated with the presence of 7/4+ polymorphonuclear cells (Table S1) (18, 20, 27), and also correlated with up-regulation of tumor necrosis factor-α (TNF-α), 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


The authors declare no conflict of interest.

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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 74% neutrophils (µ = 0.4 ± 0.32 cells in treated vs. 28.4 ± 3.33 cells in control mice) and significantly decreased macrophages (6.6 ± 1.88 cells in treated vs. 13.8 ± 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. S1 A and C). Depletion also decreased colon mRNA levels of TNF-α (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 ± 4.3 vs. 4.0 ± 1.3 MPO+ cells per 40× 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 κB (NF-κB)-mediated inflammatory signaling is dependent upon TNF-α (32), we postulated that this proinflammatory cytokine would be required for recruitment and activation of Gr-1+ neutrophils. We therefore neutralized TNF-α 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-α 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-α-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 undetectable 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, IL-10 suppressed infection-induced iNOS elevation and tumor formation, whereas IL10-deficient mice were highly susceptible to H. hepaticus-induced 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 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 wt CD4+ cells with low expression of CD45RB that have potent anti-inflammatory properties (16, 18)—would down-regulate TNF-α (18, 27), we performed adoptive i.v. transfer of 3.0 × 105 TREG cells into mice at the time of infection. Mice receiving 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-γ) 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...
previously published findings of others (18). Together, these data indicate that protective properties of IL10-competent lymphocytes reside in the CD45RB<sup>hi</sup> subset of CD4<sup>+</sup> cells. Cotransfer of anti-inflammatory T<sub>REG</sub> cells along with T<sub>EFF</sub> cells in infected mice was sufficient to down-regulate urinary NO<sub>3</sub> (Fig. 1B) and colon pathology (Table 1), indicating that IL10-competent T<sub>REG</sub> cells inhibited TNF-α-mediated inflammatory events leading to cancer.

**Suppression of NO Production and Colon Pathology.** To confirm the role of NO in the induction of colon cancer, mice were administered the iNOS inhibitor NMA in their drinking water continuously, beginning before infection. This treatment resulted in significantly decreased epithelial hyperplasia in colonic (Fig. 3B) and cecal (data not shown) epithelia compared with infected control animals. Decreased epithelial dysplasia was also evident in treated mice at 3-4 months after infection (Fig. 3B), indicative of reduced potential for eventual development of malignancy. Infected animals treated with NMA showed no elevation of NO<sub>3</sub> secretion compared with controls (Fig. 1B), confirming that NMA inhibited NO production. Levels of innate immune inflammatory cells (F4/80<sup>+</sup> macrophages and 7/4<sup>+</sup> neutrophils) remained unchanged in infected mice treated with NMA (Fig. 3A), whereas the number of inflammatory cells expressing iNOS in situ within colonic tissue was reduced by NMA (μ = 4.2 ± 2.86 cells in NMA-treated vs. 7.2 ± 3.35 cells in sham-treated mice). This indicated that inhibition of NO formation by NMA, not a reduction of inflammatory cell counts, was responsible for the observed diminished pathology. Thus, inhibition of NO production in infected mice decreased the probability of colon cancer development.

**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 Rag<sup>2−/−</sup> 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 cancer is suggested by reduced intestinal adenoma formation in mice that undergo spontaneous loss of heterozygosity of the adenomatous polyposis coli (Apc) gene (*Apc<sup>Min−/−</sup>*) 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<sup>+</sup> granulocytes. Neutrophils also have been shown to be required for infection-induced cancer (38, 39). In our experiments, depletion of Gr-1<sup>+</sup> cells decreased iNOS expression (Fig. 2B), colitis, and carcinoma (Table 1) and reduced iNOS expression within epithelial cells and other inflammatory cells (Fig. S1 B and D). It also decreased TNF-α (*P < 0.001*) and IL-6 (*P < 0.05*) (Fig. 2B), suggesting indirect effects of Gr-1<sup>+</sup> neutrophils, such as escalating host inflammatory response (28) and oxidative stressors (11, 29, 40), that promote cancer.

Supplementation with proinflammatory T<sub>EFF</sub> 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<sub>EFF</sub> cell transfer in *H. hepaticus*-infected Rag<sup>2−/−</sup> 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<sub>EFF</sub> 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<sup>+</sup> T<sub>REG</sub> and T<sub>EFF</sub> cells significantly protected against colon carcinogenesis (41), and NO was linked

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**Table 1. Induction of colon pathology in immune-deficient Rag2<sup>−/−</sup> 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>Inflammation</th>
<th>Hyperplasia</th>
<th>Dysplasia</th>
<th>Carcinoma incidence</th>
<th>Neoplastic invasion</th>
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<tbody>
<tr>
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<td>wt</td>
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<tr>
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<td>3 (2–4)</td>
<td>3 (0–4)&lt;sup&gt;†&lt;/sup&gt;</td>
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<td>2 (2–3)&lt;sup&gt;†&lt;/sup&gt;</td>
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<td>1 (0–3)</td>
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<tr>
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<td>IL10-&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>4 (4–4)</td>
<td>4 (3–4)</td>
<td>3 (3–4)&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
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<td>3 (3–3)</td>
<td>2.5 (2–4)&lt;sup&gt;†&lt;/sup&gt;</td>
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*Median severity score (range) on 0–4 scale.

<sup>†</sup>P < 0.05 vs. control value by Mann–Whitney U test for nonparametric data.
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 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−/− 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−/−* 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 nitrate (54). Decrease of IFN-γ in mice treated with NMA suggests that NMA may prevent or delay the development of malignancy in *H. hepaticus*-infected mice.
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 Tregs 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 129SvEv 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 in this experimental model.

H. hepaticus Infection. Animals were infected with H. hepaticus (strain 381, ATCC S1449) grown and confirmed as pure culture as described previously (16, 54). Mice aged 6–8 weeks received 0.2 mL of fresh inoculum by gavage every other day for a total of 3 doses. Cecum and colon were collected at necropsy and analyzed by PCR using H. hepaticus-specific primers to confirm infection status (16). Subsequent treatments are described below. Time points selected for tissue harvest were based on typical progression of IBD and dysplasia (16). For each adoptive transfer experiment (see below), a cohort of age-matched littermates served as H. hepaticus-infected controls.

Nitrile Excretion. Quantitation of nitrile excretion as a biomarker of endogenous NO production was performed as described previously (55). A total of 160 mice in 3 separate experiments were fed a low-nitrile diet (AYN-76A; Bioserve) for at least 2 weeks before infection to minimize the background rate of nitrile excretion. Mice were housed individually in metabolic cages, and urine was collected weekly in 0.5 M NaOH to inhibit bacterial growth. Total urinary nitrile concentration was determined as described previously (15, 55). One hundred mice served as Helicobacter-free controls. Forty infected mice were used to compare the effects of administration of TREG lymphocytes or neutralization of TNF-α on urinary nitrate concentration. IL-10 and anti-TNF-α fusion protein was administered by i.p. injection to Rag2−/− infected mice or Rag2−/− mice treated by i.p. injection of IL10-lg fusion protein to restore IL-10 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, 2B 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-lg fusion protein to restore IL-10 function. Forty mice served as H. hepaticus-free controls. Mice were euthanized at 3–4 months 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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