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

S. E. Erdman,a1 V. P. Raoa, T. Poutahidis,a,b A. B. Rogers,a C. L. Taylora, E. A. Jacksona, Z. Gea, C. W. Leea, D. B. Schauer,a,c G. N. Woganc,1 S. E. Erdmana,1 V. P. Raa, T. Poutahidis,a,b A. B. Rogers,a C. L. Tailora, E. A. Jacksona, Z. Gea, C. W. Leea, D. B. Schauer,a,c G. N. Woganc,1 S. R. Tannenbaumc, and J. G. Foxa,c

aDivision of Comparative Medicine, bDepartment of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139; and cLaboratory of Pathology, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, 540 06 Thessaloniki, Greece

Contributed by G. N. Wogan, December 4, 2008 (sent for review July 7, 2008)

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-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.

colorectal cancer | IBD | innate immunity

C hronic Helicobacter pylori infection in humans leads to gastritis and has been established as a causative agent for human gastric cancer (1). 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-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.

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.

1To whom correspondence may be addressed. E-mail: serdman@mit.edu or wogan@mit.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0812347106/DCSupplemental.

© 2009 by The National Academy of Sciences of the USA
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<sup>+</sup> cells significantly ($P < 0.01$) decreased NO 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<sup>+</sup> cells eliminated 7/4<sup>+</sup> 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 epithilia 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-$\alpha$ ($P < 0.001$) and IL-6 ($P < 0.05$) (Fig. 2B), suggesting that Gr-1<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells are required for colitis-associated cancer in $H. hepaticus$-infected Rag2<sup>−/−</sup> 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<sup>+</sup> neutrophils. We therefore neutralized TNF-$\alpha$ in Rag2<sup>−/−</sup> 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<sup>−/−</sup>-deficient mice also lacking IL-10 (IL10<sup>−/−</sup>Rag2<sup>−/−</sup>) 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<sup>+</sup> neutrophils, and F4/80<sup>+</sup> 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<sup>−/−</sup>-control mice. Uninfected IL10<sup>−/−</sup>Rag2<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>+</sup> lymphocytes into infected Rag2<sup>−/−</sup> mice. We found that CD4<sup>+</sup> cells collected from wt but not IL10<sup>−/−</sup>-donor mice prevented up-regulation of iNOS (Fig. 2E) and development of cancer (Table 1). To determine whether regulatory T (T<sub>REG</sub>) cells—i.e., the subset of wt CD4<sup>+</sup> 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 $3.0 \times 10^5$ T<sub>REG</sub> cells into mice at the time of infection. Mice receiving wt T<sub>REG</sub> 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<sup>+</sup> cells. Indeed, infected mice that received the CD4<sup>+</sup>CD45RB<sup>−</sup> (T<sub>EFF</sub>) 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). T<sub>EFF</sub> cell recipients also had increased levels of IFN (IFN-?y) and iNOS (Fig. 2E), increased urinary NO<sub>3</sub><sup>−</sup> (Fig. 1B), and a higher frequency of iNOS-bearing F4/80<sup>+</sup> and MPO<sup>+</sup> cells (data not shown), compared with infected controls. In contrast, uninfected recipients showed minimal pathology (Table 1), in agreement with the
in situ within colonic tissue was reduced by NMA (Fig. 2B) and colon pathology (Table 1), indicating that IL10-ergic cell augmentation of anti-inflammatory TREG cells along with TEFF cells in NMA-treated vs. 7.2 inflammatory cytokine production, was responsible for the beneficial effects of blocking NO synthesis beyond IBD-associated colon inflammation-driven mouse models (41), confirmed by studies showing increased inflammatory disease after TEFF cell transfer in Rag2−/− mice (33). Cotransfer of CD4+ TREG and TEFF cells significantly increased NO formation 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 (ApcMin+/− mice) using both inhibitors and mice lacking iNOS (37).

**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 cancer 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).

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>
<th>Carcinoma incidence</th>
<th>Neoplastic invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>wt</td>
<td>−</td>
<td>0</td>
<td>0/8</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>wt</td>
<td>−</td>
<td>1 (0–2)</td>
<td>0 (0–1)</td>
<td>0/8</td>
</tr>
<tr>
<td>+</td>
<td>wt</td>
<td>−</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
<td>0/8</td>
</tr>
<tr>
<td>−</td>
<td>Rag2−/−</td>
<td>−</td>
<td>0</td>
<td>0/8</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>Rag2−/−</td>
<td>−</td>
<td>4 (2–4)</td>
<td>4 (3–4)</td>
<td>3 (0–4)†</td>
</tr>
<tr>
<td>+</td>
<td>Rag2−/−</td>
<td>wt TEFF</td>
<td>1 (0–2)</td>
<td>0.5 (0–1)</td>
<td>0/8</td>
</tr>
<tr>
<td>+</td>
<td>Rag2−/−</td>
<td>wt TREG</td>
<td>0 (0–4)</td>
<td>0 (0–3)</td>
<td>0/8</td>
</tr>
<tr>
<td>+</td>
<td>Rag2−/−</td>
<td>wt TEFF</td>
<td>0</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>+</td>
<td>Rag2−/−</td>
<td>IL10/−/−</td>
<td>1.5 (1–3)</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>−</td>
<td>Rag2−/−</td>
<td>IL10−/−/−</td>
<td>4 (4–4)</td>
<td>4 (4–4)</td>
<td>3 (3–4)†</td>
</tr>
<tr>
<td>+</td>
<td>Rag2−/−</td>
<td>IL10−/−/−</td>
<td>1</td>
<td>0 (0–2)</td>
<td>0.5 (0–1)</td>
</tr>
<tr>
<td>−</td>
<td>IL10−/−/−Rag2−/−</td>
<td>−</td>
<td>4 (4–4)</td>
<td>4 (4–4)</td>
<td>3 (3–4)†</td>
</tr>
<tr>
<td>+</td>
<td>IL10−/−/−Rag2−/−</td>
<td>−</td>
<td>3 (3–3)</td>
<td>3 (3–3)</td>
<td>2.5 (2–4)†</td>
</tr>
</tbody>
</table>

*Median severity score (range) on 0–4 scale.

†P < 0.05 vs. control value by Mann-Whitney U test for nonparametric data.

previously published findings of others (18). Together, these data indicate that protective properties of IL10-compotent lymphocytes reside in the CD45RB+B subset of CD4+ cells. Cotransfer of anti-inflammatory TREG cells along with TEFF cells in infected mice was sufficient to down-regulate urinary NO3− excretion 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 NO3− excretion compared with controls (Fig. 1B), confirming that NMA inhibited NO production. Levels of innate immune inflammatory cells (F4/80+ macrophages and 7/4+ 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 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 cancer 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+ 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+ 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 TEFF 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 pericolonic region (33). Cotransfer of CD4+ T REG and TEFF cells significantly protected against colon carcinogenesis (41), and NO was linked
with increased IL-10-mediated recruitment of protective anti-inflammatory T\(_{REG}\) cells (43). Prior challenge with *H. hepaticus* induced IL-10-dependent T\(_{REG}\) cells that protected against IBD in mice (26). T\(_{REG}\) 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 T\(_{REG}\) subsets are most effective in the regulation of TNF-\(\alpha\)/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 NO\(_2\) (31). NO\(_2\) is a strong one-electron oxidant capable of both oxidation and nitration of nucleic acids, proteins, and lipids (45–49). Products generated by NO\(_2\) 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/\(11002\)/\(11002\)/ 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*\(11002\)/\(11002\)/ 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 reduced by NMA treatment.

**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-\(\alpha\) (\(C\)) decreased iNOS expression. (\(D\)) Infected double-knockout mice showed significant increases in iNOS and IFN-\(\gamma\) gene expression. (\(E\)) T\(_{EFF}\) cells increased levels of IFN (IFN-\(\gamma\)) 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.

<table>
<thead>
<tr>
<th>Hh Treatment</th>
<th>Neutrophils</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ NMA</td>
<td>42.9 ± 4.62</td>
<td>19.5 ± 2.24</td>
</tr>
<tr>
<td>+ Sham (CH(_3)COONH(_4))</td>
<td>42.9 ± 4.76</td>
<td>19.9 ± 1.67</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Colonic Inflammation</th>
<th>Colonic Hyperplasia</th>
<th>Colonic Dysplasia/Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hh</td>
<td>Hh+HMA</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>*</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>**</td>
<td>***</td>
<td>****</td>
</tr>
<tr>
<td>***</td>
<td>****</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>****</td>
<td>**</td>
<td>****</td>
</tr>
<tr>
<td>(p&lt;0.001)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 nitrite. Progressive development of increasingly severe inflammation, hyperplasia, dysplasia, and cancer accompanied these changes. Concurrent administration of an iNOS inhibitor prevented NO production and abrogated the epithelial...
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 microisolator cages and were approved by the institutional Animal Care and Use Committee. Each experiment included 6–10 mice per group, except when indicated otherwise. All mice were between 12–22 weeks of age, unless otherwise specified. Treatment groups included equal numbers of males and females unless otherwise specified.

H. hepaticus Infection. Animals were infected with H. hepaticus (strain 3B1, 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 to achieve 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.

Nitrate Excretion. Quantitation of nitrate 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-nitrate diet (AYN-76A; Bioserve) for at least 2 weeks before infection to minimize the background rate of nitrate excretion. Mice were housed individually in metabolic cages, and urine was collected weekly in 0.5 M NaOH to inhibit bacterial growth. Total urinary nitrate concentration was determined as described previously (15, 55). One hundred mice served as Helicobacter-free controls. Forty infected mice were used to generate baseline nitrate excretion data. At 24 h before infection, 30 mM NMA (EMD Chemicals) replaced drinking water for 10 mice (2 replications; total n = 20) and equimolar ammonium acetate (Sigma Chemical) for 10 mice (2 replications; total n = 20) to serve as sham controls. Mice were euthanized at 3–4 months after infection.

Lymphocyte Transfer. For adoptive transfer of CD4^+ lymphocytes, 160 mice (treatment groups of 10 recipient mice, each with 2 replications) underwent the procedure as described previously (16, 41). Single-cell suspensions of CD4^+ lymphocytes were prepared as described previously (56, 57) from spleen and mesenteric lymph nodes from Helicobacter-free wild-type or IL10^-/- donor mice backcrossed at least 10 generations onto the 129/SvEv genetic background. Reanalysis confirmed purity (>-95%) and phenotype of the cells before transfer. Rag2^-/- recipients of wt or IL10^-/- lymphocytes underwent adoptive transfer 48 h before infection (16). For replacement of IL-10, murine IL-10 was fused to IgG2a by using a PCR cloning strategy and cloned into an adenoviral vector as described previously (27, 57). Serum containing 1 μg of fusion protein was administered by i.p. injection to H. hepaticus-infected IL10^-/-Rag2^-/- or Rag2^-/- mice twice weekly for 6 weeks.

Neutrophil or TNF-α Depletion. For experiments involving depletion of Gr-1^- cells or neutralization of TNF-α, 40 infected mice were subdivided into groups of 10 each, and each was dosed with 0.2 mL of anti-Gr-1 or anti-TNF antibody, or sham isotype IgG control, as described elsewhere (41). Anti-Gr-1 antibody is widely used to label mature neutrophils. Neutrophils were depleted in 10 infected Rag2^-/- mice at 6 weeks after infection by i.p. injection of 500 μg/mouse Ly-6G antibody (BioExpress) thrice weekly for 10 weeks. Ten mice infected with H. hepaticus 6 weeks earlier were injected for 10 days with anti-TNF-α antibody (clone XT-3; BioExpress) at 200 mg per mouse thrice weekly as previously described (44). Mice were euthanized at 3–4 months after infection, and tissues were evaluated as described below.

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 IL-10 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 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.).

ACKNOWLEDGMENTS. We thank the following: S. Xu, N. S. Taylor, J. Cline, E. B. Armon, K. Hardy, G. A. Paradis, M. J. Jennings, K. Cormier, J. Bajko, E. Stefanovich, K. Rydstrom, and C. Boussahmain for technical assistance; and M. Hudelson, E. Robbins, and L. J. Trudel for manuscript preparation. Financial support was provided by National Institutes of Health Grants P01 26736 (to J.G.F., R01CA108854 (to S.E.E.), R01CA67529 (to J.G.F.), R01A151404 (to J.G.F.), R01AI052267 (to S.E.E.), P30 ES02109 (to J.G.F.), and T32RV0736 (to J.G.F.), and from European Union and Greek Ministry of National Education and Religious Affairs Pythagoras II Grant 80860 (to T.P.).


