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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 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

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 (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 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. 1C 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

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The authors declare no conflict of interest.

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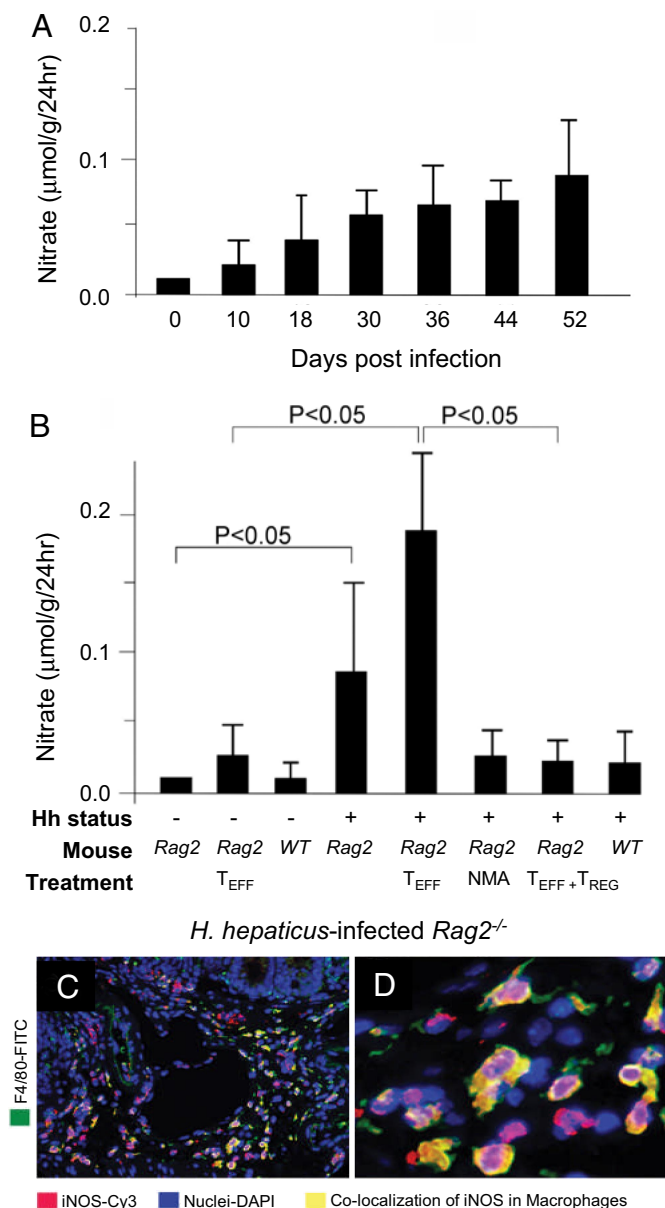


Fig. 1. Urinary nitrate excretion after infection with *H. hepaticus*. (A) Temporal increase in urinary nitrate excretion followed infection with *H. hepaticus*. (B) Nitrate excretion was elevated in infected *Rag2*^{-/-} mice with cancer but not WT mice without cancer. Treatment with NMA or T_{REG}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

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 Gr-1⁺ cells eliminated 7/4⁺ neutrophils ($\mu = 0.4 \pm 0.22$ 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 κ B (NF- κ B)-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*^{-/-} 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, 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 (T_{REG}) 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 3.0×10^5 T_{REG} cells into mice at the time of infection. Mice receiving *wt* T_{REG} 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⁺CD45RB^{hi} (T_{EFF}) 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_{EFF} cell recipients also had increased levels of IFN ($\text{IFN-}\gamma$) 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

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⁺ T_{REG} 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 and was repeated twice (total $n = 12$ –20 mice per group) 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 51449) 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.

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 in the evaluation of NMA effects. 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 μ g 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 μ g/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 μ m, 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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