

# Innate Immune Inflammatory Response against Enteric Bacteria *Helicobacter hepaticus* Induces Mammary Adenocarcinoma in Mice

Varada P. Rao,<sup>1</sup> Theofilos Poutahidis,<sup>1,3</sup> Zhongming Ge,<sup>1</sup> Prashant R. Nambiar,<sup>1</sup> Chakib Boussahmain,<sup>1</sup> Yan Yan Wang,<sup>2</sup> Bruce H. Horwitz,<sup>2</sup> James G. Fox,<sup>1</sup> and Susan E. Erdman<sup>1</sup>

<sup>1</sup>Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Massachusetts; <sup>2</sup>Immunology Research Division, Department of Pathology, Brigham and Women's Hospital; Division of Emergency Medicine, Children's Hospital, Boston, Massachusetts; and <sup>3</sup>Laboratory of Pathology, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece

## Abstract

Inflammation associated with bacterial infections is a risk factor for cancers in humans, yet its role in breast cancer remains poorly understood. We have previously shown that innate immune inflammatory response against intestinal bacteria is sufficient to induce colon cancer. Here we report that infecting *Rag2*-deficient C57BL/6 *Apc*<sup>Min/+</sup> mice with an intestinal bacterial pathogen, *Helicobacter hepaticus*, significantly promotes mammary carcinoma in females and enhances intestinal adenoma multiplicity by a tumor necrosis factor  $\alpha$  (TNF $\alpha$ )-dependent mechanism. The mammary and intestinal tumor development as well as the increase in proinflammatory mediators is suppressed by adoptive transfer of interleukin 10-competent CD4<sup>+</sup>CD45RB<sup>lo</sup>CD25<sup>+</sup> regulatory (T<sub>R</sub>) cells. Furthermore, prior exposure of donor mice to *H. hepaticus* significantly enhances antitumor potency of their T<sub>R</sub> cells. Interestingly, these microbially experienced T<sub>R</sub> cells suppress tumorigenesis more effectively in recipient mice irrespective of their tumor etiology. These data suggest that infections with enteric pathogens enhance T<sub>R</sub>-cell potency and protect against epithelial cancers later in life, potentially explaining paradoxical increases in cancer risk in developed countries having more stringent hygiene practices. The possibility that dysregulated gut microbial infections in humans may lead to cancer in anatomically distant organs, such as breast, highlights the need for novel immune-based strategies in cancer prevention and treatment. (Cancer Res 2006; 66(15): 7395-400)

## Introduction

Chronic inflammation promotes carcinogenesis and predisposes susceptible individuals to cancer (1, 2). In humans, infectious inflammation associated with prolonged activation of the host immune system by parasitic, viral, and bacterial agents has been shown to contribute to tumor formation at several sites including bladder (3), liver (4), and stomach (5). Similarly, inflammation of noninfectious nature has been associated with other types of cancer including colorectal cancer (6), lung cancer (7), and cancer

of esophageal/gastric junction (8). Breast cancer, the most frequently diagnosed cancer in North America, nearly thrice its rate in the developing world, has increasing incidence rate at ~4% per annum over the past decade (9). Despite intense efforts to understand etiopathogenesis of breast cancer, no clear explanation for its increasing incidence has been forthcoming.

Cancers of bowel (10) and breast (11) have been associated with mutations in adenomatosis polyposis coli (APC), the gene responsible for multiple intestinal neoplasia in humans and mice (12). Mice heterozygous for *Apc* gene (*Apc*<sup>Min/+</sup>) develop a large number of intestinal polyps by 3 months of age (13) and this process has been shown to be inhibited by adoptive transfer of interleukin 10 (IL-10)-competent T<sub>R</sub> cells (14). Despite their high predilection for intestinal tumors, unmanipulated C57BL/6 *Apc*<sup>Min/+</sup> mice rarely show mammary tumors when housed in our specific pathogen-free animal facilities (15), in contrast to higher tumor incidence reported previously in other animal facilities (13, 16). We reasoned that inflammation induced in the gut by proinflammatory microbial infection could have systemic effects, which would then influence carcinogenic events in other organs including mammary gland. Here, we investigate whether *Helicobacter hepaticus*-triggered inflammatory responses modulate carcinogenesis in *Apc*<sup>Min/+</sup> mice, using a widely applied adoptive T-cell transfer model (17) and recombination-activating gene 2 (*Rag2*)-deficient *Apc*<sup>Min/+</sup> mice, and assess the roles for innate immune inflammatory response in mammary and intestinal tumor development.

## Materials and Methods

**Experimental animals.** All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care–approved facilities and maintained according to protocols approved by the Institutional Animal Care and Use Committee at Massachusetts Institute of Technology. *Apc*<sup>Min/+</sup> mice on a C57BL/6J background were originally obtained from The Jackson Laboratory and bred in house as heterozygous  $\times$  wild type crosses to provide *Apc*<sup>Min/+</sup> mice and wild-type littermates for experimental recipients and donors. Before the study, *Helicobacter*-free status of the mice was confirmed by PCR using *Helicobacter* genus-specific primers as previously described (18).

**Experimental *H. hepaticus* infection.** A total of 71 experimental mice were dosed at 2 to 3 months of age with *H. hepaticus* and housed separately in a bio-containment area within the same animal facility. *H. hepaticus* (strain 3B1, ATCC 51449; ref. 19) was grown under microaerobic conditions, prepared, and confirmed pure as described elsewhere (20). Experimental mice received 0.2 mL of fresh inoculum by gastric gavage every other day for a total of three doses. Cecum and colons were collected 3 to 4 weeks

**Requests for reprints:** Susan E. Erdman, Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA 02139. Phone: 617-252-1804; Fax: 617-258-5708; E-mail: serdman@mit.edu.

©2006 American Association for Cancer Research.  
doi:10.1158/0008-5472.CAN-06-0558

postinfection at necropsy and analyzed by PCR using *H. hepaticus*-specific primers to confirm experimental infection (18).

**Experimental design.** A total of 20 female *Apc*<sup>Min/+</sup> mice and 100 female *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice were included in various treatment regimens or as experimental controls. Some experiments were conducted using separate trials with four to eight mice each. Trials with statistically similar results were then combined for analyses.

**Adoptive transfer of T<sub>R</sub> cells.** A total of 49 *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice, ages 3.5 to 4 months, were dosed with  $3 \times 10^5$  wild-type T<sub>R</sub> cells ( $N = 9$ ),  $3 \times 10^5$  IL-10<sup>-/-</sup> T<sub>R</sub> cells ( $N = 8$ ), or  $1 \times 10^5$  wild-type T<sub>R</sub> cells ( $N = 32$  mice) 24 hours before *H. hepaticus* infection. CD4<sup>+</sup>CD45RB<sup>lo</sup>CD25<sup>+</sup> (T<sub>R</sub>) lymphocytes were isolated from spleen and mesenteric lymph nodes and adoptively transferred as previously described (14). The donor mice for T<sub>R</sub> cells included male and female *H. hepaticus*-infected or *Helicobacter*-free C57BL/6 mice or *H. hepaticus*-infected IL-10-deficient C57BL/6 mice. The T<sub>R</sub>-cell donors were dosed with *H. hepaticus* 8 weeks earlier. The recipients used in this study were all female mice, based on the earlier observation that mammary tumor incidence is greater in female mice (13). Replicate experiments were conducted with two or three groups of similar size for select experiments.

**Tumor necrosis factor  $\alpha$  neutralization.** A total of 11 *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice, ages 3 to 4 months, infected with *H. hepaticus*, were treated 3–4 weeks later with anti-tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) antibody (clone XT-3; BioExpress, West Lebanon, NH) at 200  $\mu$ g per mouse thrice weekly for 1 week as previously described (15). Treated mice ( $N = 11$ ) were compared with age-matched *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice that received sham antibody alone ( $N = 8$ ).

**Treatment with IL-10-Ig fusion protein.** A total of nine *H. hepaticus*-infected *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice, 3 to 4 months of age, were treated with IL-10-Ig fusion protein at 5  $\mu$ g per mouse twice weekly (2–3 days apart) for 1 week. To produce the IL-10-Ig fusion protein, genes for murine IL-10 and immunoglobulin G2a (IgG2a) CH2 were fused and the chimeric gene was cloned into an adenoviral vector and the infectious virus (AdIL-10Ig) was generated as described elsewhere (21). Adv-IL-10Ig was used to infect *Helicobacter*-free *Rag2*-deficient B6 mice ( $10^{11}$  virions per animal). Fusion protein from 10-DPI serum was quantified using an IgG2a-specific ELISA (150 ng/mL of IL-10-Ig = 1 ng/mL of recombinant IL-10 to suppress IL-12 p40 and IP-10 by IL-10-deficient macrophages). Serum containing the required dose of fusion protein was administered by i.p. injection to mice.

**Quantitation of intestinal tumors.** Location of tumors was recorded using a stereomicroscope at  $\times 10$  magnification. Location of tumors in the small intestine was recorded as distance from the pylorus to duodenum, jejunum, and ileum, comprising one third of small intestine each (14).

**Histologic evaluation.** As previously described (15), the formalin-fixed tissues were processed and the H&E-stained tissue sections were evaluated by two veterinary pathologists blinded to sample identity. Macrophages were identified by standard avidin-biotin-complex immunohistochemistry using rat anti-mouse F4/80 and biotinylated goat anti-rat IgG (Serotec, Oxford, United Kingdom).

**Detection of cytokine mRNA expression in colon and mammary tissue.** The RNase protection assay to detect cytokine mucosal mRNA has been described in detail elsewhere (20). Briefly, frozen specimens of cecocolic junction were homogenized into Tri-reagent (MRC, Cincinnati, OH) and RNA was prepared per instructions of the manufacturer. Ribonuclease protection assay analyses were done with 20  $\mu$ g of total RNA using RiboQuant Multi-Probe Template Sets (PharMingen, San Diego, CA). Intensities of the protected fragments were quantitated by phosphorimager analysis and normalized to internal controls as previously described (20). TNF $\alpha$  mRNA levels in mammary tissue were measured using real-time quantitative PCR as previously described (14).

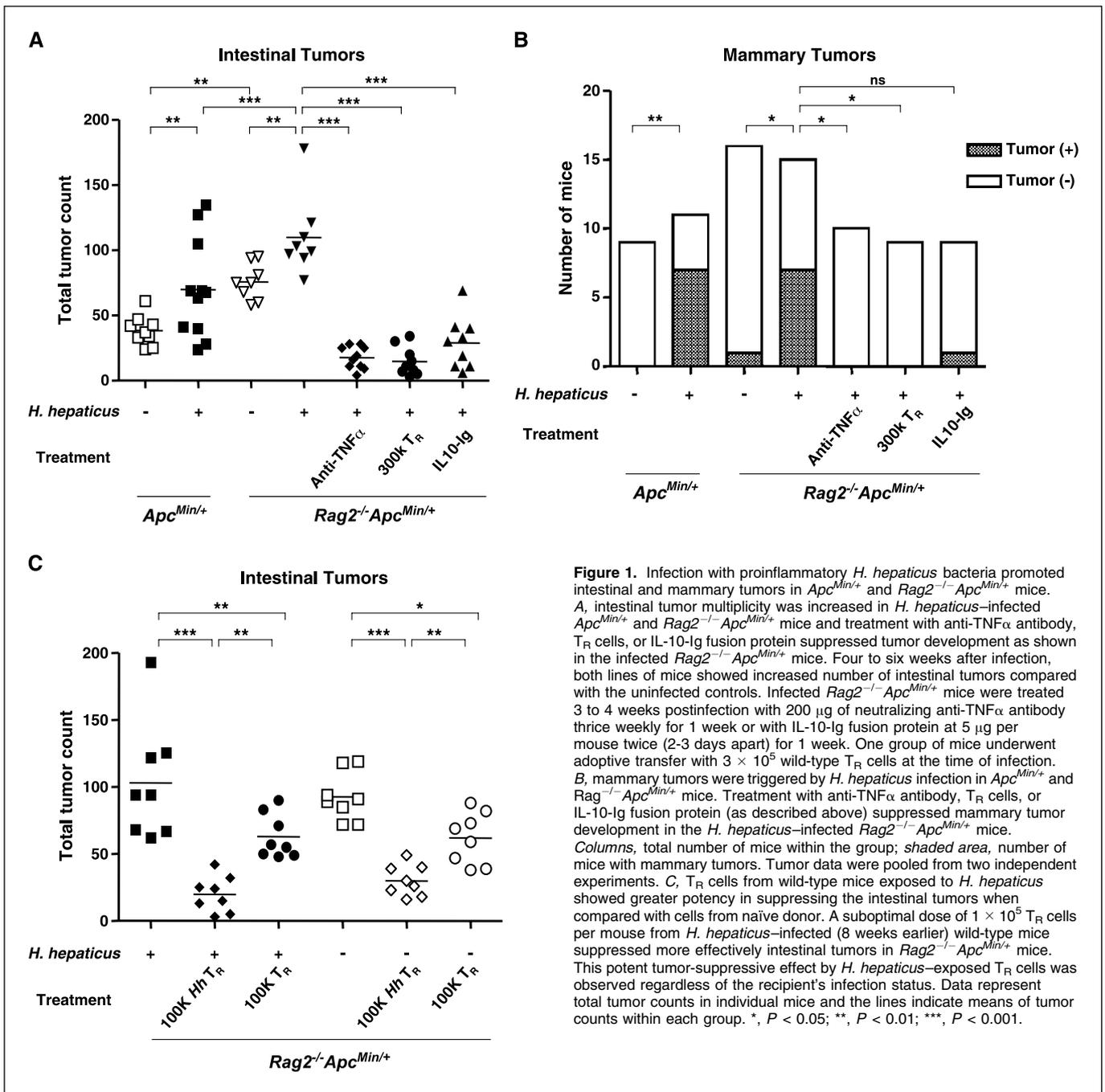
**Statistical analyses.** Total tumor counts were analyzed by one-way ANOVA using Newman-Keuls posttest. Mammary tumor incidence was compared using contingency tables and  $\chi^2$  analysis. Macrophage counts were compared by unpaired two-tailed *t* test. Small and large intestine tumor multiplicities were compared by unpaired *t* test with Welch's correction. For all statistical analyses, GraphPad Prism version 4.0 for windows (GraphPad Software, San Diego, CA) was used.

## Results and Discussion

**Innate immunity is sufficient for mammary and intestinal tumor development.** Previously, we showed that the innate immune inflammatory response was sufficient to promote colorectal carcinoma in *Rag2*-deficient 129/SvEv mice (20, 22). In the present study, we first sought to determine whether lymphocytes are essential for mammary and intestinal tumorigenesis in *Apc*<sup>Min/+</sup> mice. We find that unmanipulated *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice between 4 and 4.5 months of age develop significantly ( $P < 0.01$ ) more frequent adenomas in the small bowel (Fig. 1A) when compared with age-matched *Apc*<sup>Min/+</sup> controls housed under the same health status conditions. In addition, one *Helicobacter*-free *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> female mouse (1 of 16; 6%) developed a palpable mammary tumor. The findings of intestinal tumors and mammary tumors in *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice indicated that adaptive immunity is not required for tumorigenesis in the *Apc*<sup>Min/+</sup> mouse model. Further, the development of significantly higher intestinal tumor multiplicity in *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice over their *Apc*<sup>Min/+</sup> counterparts suggests that tumorigenesis is enhanced in the absence of lymphocytes.

***H. hepaticus* infection promotes intestinal and mammary tumorigenesis.** Because *H. hepaticus* infection has been shown to induce colonic cancer in 129 strain *Rag2*<sup>-/-</sup> mice devoid of lymphocytes (20), we asked whether infecting C57BL/6 *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice with *H. hepaticus* promotes intestinal and mammary tumor development. Four to six weeks after *H. hepaticus* infection, female *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice ( $N = 8$ ) developed significantly ( $P < 0.01$ ) greater multiplicity of intestinal polyps ( $\mu = 110 \pm 10.7$ ; Figs. 1A and 2C) when compared with age-matched uninfected female control mice. Likewise, *H. hepaticus*-infected mice had significantly ( $P < 0.001$ ) more frequent F4/80<sup>+</sup> macrophages ( $12.6 \pm 0.9$  per high-power field) in the sections of intestines (Fig. 2G) when compared with those from *H. hepaticus*-free controls ( $4.8 \pm 0.9$  per high-power field), suggesting a role for these cells in pathogenesis. Interestingly, we find a significantly ( $P < 0.05$ ) higher frequency of mammary tumors in *H. hepaticus*-infected *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice (7 of 15 mice; 43%; Figs. 1B and 2D) when compared with age-matched uninfected control female *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice. Adenosquamous mammary carcinoma in *Helicobacter*-infected *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice showed minimal squamous metaplasia (Fig. 2D) when compared with mammary tumors in *Apc*<sup>Min/+</sup> mice promoted by proinflammatory CD4<sup>+</sup>CD45RB<sup>hi</sup> T<sub>E</sub> lymphocytes (15). In addition, mammary tumors of *H. hepaticus*-infected *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice had dense inflammatory infiltrates of F4/80<sup>+</sup> macrophages (Fig. 2H), consistent with inflammation-associated breast cancer in humans (23, 24).

Similarly, we also find that *Apc*<sup>Min/+</sup> mice ( $N = 11$ ) infected with *H. hepaticus* showed an increase in the adenoma multiplicity in the small and large intestine (Fig. 1; Table 1) when compared with *Helicobacter*-free age-matched controls. In addition, 63% (7 of 11;  $P < 0.001$ ) of female *Apc*<sup>Min/+</sup> mice that received *H. hepaticus* 4 to 6 weeks earlier had palpably enlarged mammary glands (Fig. 1B) with histologic features of adenosquamous mammary carcinoma (Fig. 2B) when examined at 3 to 4 months of age. In contrast, no mammary tumors were found in *Helicobacter*-free *Apc*<sup>Min/+</sup> females (0 of 8 animals) during the course of the study. Microscopically, the tumors in *Apc*<sup>Min/+</sup> mice had more locally invasive borders and had increased squamification when compared with neoplastic mammary glands of *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice (compare Fig. 2B and D). Differences in histologic appearance of tumors may reflect adaptive immune-mediated alterations in the Wnt signaling

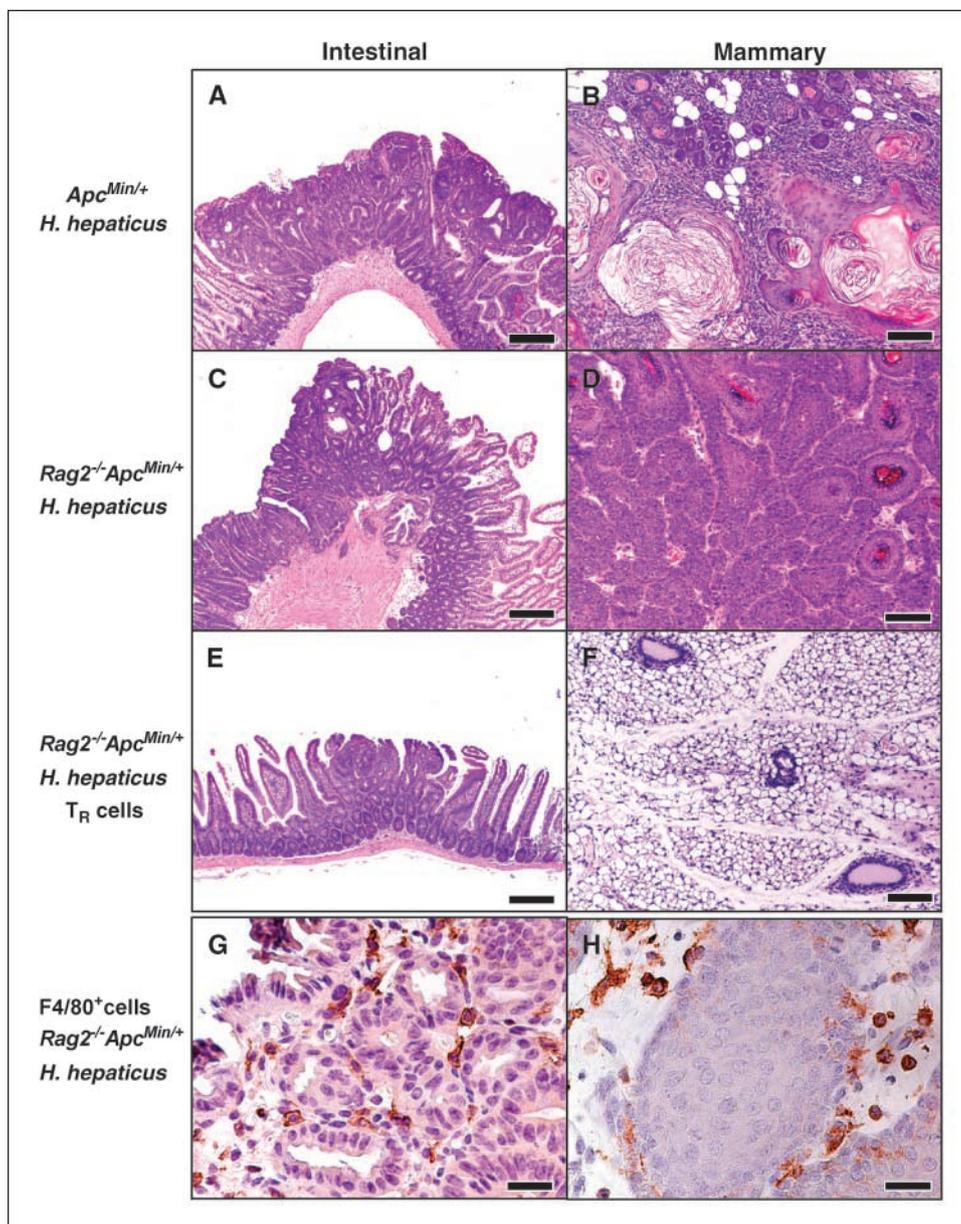


**Figure 1.** Infection with proinflammatory *H. hepaticus* bacteria promoted intestinal and mammary tumors in *Apc<sup>Min/+</sup>* and *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice. **A**, intestinal tumor multiplicity was increased in *H. hepaticus*-infected *Apc<sup>Min/+</sup>* and *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice and treatment with anti-TNF $\alpha$  antibody, T<sub>R</sub> cells, or IL-10-Ig fusion protein suppressed tumor development as shown in the infected *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice. Four to six weeks after infection, both lines of mice showed increased number of intestinal tumors compared with the uninfected controls. Infected *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice were treated 3 to 4 weeks postinfection with 200  $\mu$ g of neutralizing anti-TNF $\alpha$  antibody thrice weekly for 1 week or with IL-10-Ig fusion protein at 5  $\mu$ g per mouse twice (2-3 days apart) for 1 week. One group of mice underwent adoptive transfer with  $3 \times 10^5$  wild-type T<sub>R</sub> cells at the time of infection. **B**, mammary tumors were triggered by *H. hepaticus* infection in *Apc<sup>Min/+</sup>* and *Rag<sup>-/-</sup>Apc<sup>Min/+</sup>* mice. Treatment with anti-TNF $\alpha$  antibody, T<sub>R</sub> cells, or IL-10-Ig fusion protein (as described above) suppressed mammary tumor development in the *H. hepaticus*-infected *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice. **Columns**, total number of mice within the group; **shaded area**, number of mice with mammary tumors. Tumor data were pooled from two independent experiments. **C**, T<sub>R</sub> cells from wild-type mice exposed to *H. hepaticus* showed greater potency in suppressing the intestinal tumors when compared with cells from naïve donor. A suboptimal dose of  $1 \times 10^5$  T<sub>R</sub> cells per mouse from *H. hepaticus*-infected (8 weeks earlier) wild-type mice suppressed more effectively intestinal tumors in *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice. This potent tumor-suppressive effect by *H. hepaticus*-exposed T<sub>R</sub> cells was observed regardless of the recipient's infection status. Data represent total tumor counts in individual mice and the lines indicate means of tumor counts within each group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

pathway (25). One explanation for the frequency of *H. hepaticus*-induced tumors in *Apc<sup>Min/+</sup>* may be the recently described immune deficits. Thymic atrophy and lymphopenia (26) may decrease T<sub>R</sub> cell competency in *Apc<sup>Min/+</sup>* mice, thereby enabling uncontrolled activation of *Helicobacter*-primed T<sub>E</sub> cells (27) or other cells of adaptive immunity, which may promote mammary and intestinal carcinogenesis. Nonetheless, the observation that *H. hepaticus* infection promotes mammary tumors in both lines of mice raises the likelihood that proinflammatory intestinal bacterial infections contribute to breast tumorigenesis in other mouse models as well as in women.

***H. hepaticus*-triggered intestinal and mammary carcinoma is TNF $\alpha$  dependent.** To examine whether *H. hepaticus* infection is

accompanied by up-regulation of proinflammatory cytokines, we analyzed gut mucosal expression levels of cytokines including TNF $\alpha$ , IL-12p40, IFN $\gamma$ , and macrophage inflammatory protein 2 (MIP-2). We find increased expression of all four cytokines (Fig. 3) consistent with our prior findings in colitis and colon cancer (20). Because TNF $\alpha$  is a key cytokine implicated in inflammation-associated cancers (28), and treatment with anti-TNF $\alpha$  antibody has been shown to suppress polyp formation in *Apc<sup>Min/+</sup>* mice (15), we asked whether the *H. hepaticus*-promoted tumorigenesis in *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* is dependent on TNF $\alpha$ . Indeed, neutralization of TNF $\alpha$  by antibody significantly suppressed both intestinal ( $P < 0.001$ ) and mammary tumors ( $P < 0.05$ ; Fig. 1A and B) in the *H. hepaticus*-infected *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice. Therapeutic



**Figure 2.** *H. hepaticus* infection promotes intestinal and mammary tumorigenesis in *Apc<sup>Min/+</sup>* and *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice. **A**, typical adenomatous polyp seen in infected *Apc<sup>Min/+</sup>* mice showing high-grade dysplasia and carcinoma *in situ*. **B**, mammary gland adenosquamous carcinoma seen in infected *Apc<sup>Min/+</sup>* mice showing increased squamous component when compared with *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice. The enlarged keratinized neoplastic glands have invasive borders and lamellated keratin in the center. Small nonkeratinized and keratinized glands and the dense inflammatory cell infiltrate can be seen. **C**, adenomatous intestinal polyp with early invasion of neoplastic glands into the muscular layers often seen in *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice. **D**, mammary adenosquamous carcinoma with neoplastic glands showing variable degrees of squamous differentiation. In contrast to high squamous component of tumors in *Apc<sup>Min/+</sup>* mice (as illustrated above in **B**), nonkeratinized neoplastic glandular structures predominate in *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice. **E**, minute polyp with remnant dysplastic glands close to surface epithelium. This typical regressive intestinal cancer morphology is seen throughout the intestine in mice treated once with  $3 \times 10^5$  T<sub>R</sub> cells or with anti-TNF $\alpha$  antibody (clone XT-3), 200  $\mu$ g per mouse thrice weekly for 1 week. **F**, normal mammary gland tissue and mammary fat. Intestinal (**G**) and mammary (**H**) tumors of *H. hepaticus*-infected *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice showing high number of macrophages as identified by avidin-biotin-complex immunohistochemistry using rat anti-mouse F4/80 antibody and biotinylated goat anti-rat IgG. **A** to **F**, H&E; **G** and **H**, 3,3-diaminobenzidine, hematoxylin counterstain. Bars, 250  $\mu$ m (**A**, **C**, and **E**); 100  $\mu$ m (**B**, **D**, and **F**); 25  $\mu$ m (**G** and **H**).

effects of TNF $\alpha$  neutralizing antibody in these mice suggest that TNF $\alpha$  or its downstream signaling mediators are required to sustain intestinal and mammary tumors in this setting.

The underlying cellular and molecular mechanisms of mammary tumor promotion by *H. hepaticus* in *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice need further study. Mammary tumors from *H. hepaticus*-infected *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>* mice show an 18-fold increase ( $P < 0.0001$ ) in TNF $\alpha$ -gene expression when compared with mammary tissues of uninfected mice, indicating a localized inflammatory response. Tumorigenesis may be initiated by systemic increases in proinflammatory factors and/or by the trafficking of activated innate immune cells to target tissues. Another possibility is translocation of *Helicobacter* organisms or their antigens to mammary tissue in infected mice with attendant localized proinflammatory host response, which subsequently promotes development of mammary cancer. Taken together, these data indicate that *H. hepaticus*-triggered TNF $\alpha$ -mediated innate immune inflammatory response

promotes epithelial tumorigenesis locally as well as in other sites such as mammary gland.

**CD25<sup>+</sup> regulatory T cells inhibit mammary and intestinal tumorigenesis.** Prior studies have shown that CD25<sup>+</sup> regulatory (T<sub>R</sub>) cells are sufficient to prevent *H. hepaticus*-triggered colitis and colon cancer (20, 27). To determine whether T<sub>R</sub> cells can inhibit *Helicobacter*-promoted tumorigenesis in *Rag2<sup>-/-</sup>Apc<sup>Min/+</sup>*, nine female mice, 2 to 3.5 months of age, were infected with *H. hepaticus* and adoptively transferred with  $3 \times 10^5$  cells per recipient of T<sub>R</sub> cells collected from *Helicobacter*-free wild-type donors. When examined 3 to 4 weeks later, we found a significant ( $P < 0.001$ ) reduction in intestinal adenoma multiplicity ( $\mu = 14.8 \pm 3.69$ ; Fig. 1A) and observed no mammary tumors ( $P < 0.05$ ) in these infected T<sub>R</sub> cell-recipient mice. Additionally, treatment of *H. hepaticus*-infected mice with T<sub>R</sub> cells resulted in a significant ( $P < 0.001$ ) decrease in the levels of intestinal mucosal proinflammatory cytokines including TNF $\alpha$  and MIP-2 (Fig. 3). These

**Table 1.** Comparison of intestinal tumor frequency between *Apc*<sup>Min/+</sup> and *Rag*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice

Group	<i>Hh</i> status	No. mice	Tumor multiplicity (mean ± SE)	
			Small intestine	Large intestine
<i>Apc</i> <sup>Min/+</sup>	-	9	37.89 ± 3.57 <sup>a, e</sup>	0.5 ± 0.3 <sup>d</sup>
	+	11	67.73 ± 11.36 <sup>a, c</sup>	2.5 ± 0.2 <sup>d</sup>
<i>Rag</i> <sup>-/-</sup> <i>Apc</i> <sup>Min/+</sup>	-	16	82.69 ± 4.38 <sup>b, e</sup>	1.4 ± 0.3
	+	15	104.7 ± 9.50 <sup>b, c</sup>	2.7 ± 0.6

NOTE: Data sharing a superscript letter are significantly different from each other. <sup>a, b, and c</sup>,  $P < 0.05$ ; <sup>d</sup>,  $P < 0.01$ ; <sup>e</sup>,  $P < 0.001$ . Mice were either uninfected or dosed with *H. hepaticus* (*Hh*) and tumor multiplicity in small and large intestine was quantitated as described in Materials and Methods. Total tumor numbers in small and large intestine were separately analyzed between groups by using unpaired *t* test with Welch's correction using Prism 4.0 software as described in Materials and Methods.

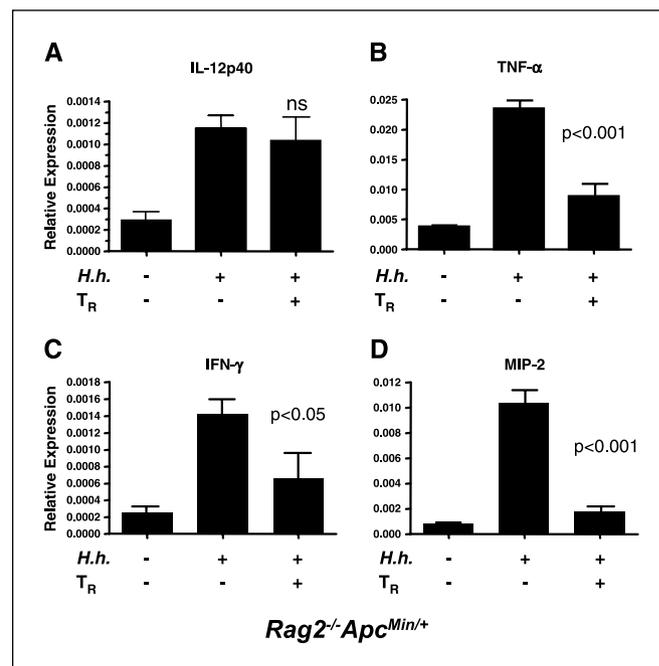
data indicate that T<sub>R</sub> cells are sufficient to inhibit mammary and intestinal tumorigenesis in *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice. These findings match earlier data from our laboratory (14, 15) as well as by others (29) showing that T<sub>R</sub> cells are not only capable of regulating other T cells but are also capable of suppressing inflammation resulting from chronic activation of the innate immune system.

**Prior challenge with *H. hepaticus* enhances antitumor potency of T<sub>R</sub> cells.** Microbes or microbial products enhance survival, proliferation, and cytokine production by T<sub>R</sub> cells (30). To test whether protective antitumor effects of T<sub>R</sub> cells can be enhanced by prior microbial challenge, we first determined a suboptimal dosage of  $1 \times 10^5$  CD45RB<sup>lo</sup>CD25<sup>+</sup> wild-type T<sub>R</sub> cells per recipient (31). We then used this lower dose of T<sub>R</sub> cells derived from donors that were infected at least 8 weeks earlier with *H. hepaticus*, or alternatively from donors that remained uninfected, for adoptive transfer into *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice at time of infection with *H. hepaticus*. We found that T<sub>R</sub> cells isolated from *H. hepaticus*-exposed donors were significantly ( $P < 0.001$ ) more effective at suppressing *H. hepaticus*-induced intestinal tumors when compared with cells from naïve donors. It remains to be seen whether CD45RB<sup>lo</sup>CD25<sup>-</sup> T<sub>R</sub> cells, or other cell subsets with regulatory functions, act similarly as potent promoters of epithelial homeostasis after microbial challenges. Kullberg et al. (31) have previously shown that CD45RB<sup>lo</sup>CD25<sup>-</sup> T<sub>R</sub> cells from *H. hepaticus*-exposed mice were efficacious in protecting against *H. hepaticus*-induced T<sub>E</sub> cell-mediated colitis. Studies in progress may reveal dynamics of immune tolerance involving both innate immunity and host T<sub>R</sub> cell competency.

To test whether the *H. hepaticus*-induced enhancement in antitumor potency of T<sub>R</sub> cells is limited to *H. hepaticus*-triggered tumors, the lower dosage of T<sub>R</sub> cells from *H. hepaticus*-infected as well as uninfected donors was transferred in parallel into *Helicobacter*-free *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> recipients. We find that *H. hepaticus*-experienced T<sub>R</sub> cells are significantly ( $P < 0.001$ ) more potent compared with cells from naïve donors ( $P < 0.05$ ) at suppressing intestinal adenoma multiplicity in recipients that were not infected with *H. hepaticus*. The finding that tumor multiplicity

is significantly inhibited by *Helicobacter*-challenged donor T<sub>R</sub> cells in all recipients irrespective of their *Helicobacter* status suggests that prior proinflammatory challenges broadly enhance antitumor potency of T<sub>R</sub> cells, even against tumors of unknown etiology. In light of recent studies showing that probiotic intestinal bacteria (32) and parasite antigens (33) enhance IL-10 and the protective functions of T<sub>R</sub> cells, it will be interesting to examine whether these agents will affect antitumor potency of T<sub>R</sub> cells in our model.

**IL-10 is critical to suppress *H. hepaticus*-promoted tumorigenesis.** In murine models, CD4<sup>+</sup>CD25<sup>+</sup> regulatory (T<sub>R</sub>) cells require anti-inflammatory cytokine IL-10 to inhibit inflammatory bowel disease (27, 31), colon cancer (20, 22), and intestinal polyposis (14). To determine whether IL-10 is dispensable in T<sub>R</sub> cells endowed with microbially enhanced antitumor potency, we did adoptive transfer of T<sub>R</sub> cells from *H. hepaticus*-infected IL-10-deficient syngeneic donors into uninfected *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice. Clearly, no reduction in tumor burden was seen in *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> recipients of IL-10-deficient T<sub>R</sub> cells ( $N = 8$ ;  $\mu = 100.4 \pm 9.91$ ) when compared with untreated control mice ( $N = 8$ ;  $\mu = 84.13 \pm 4.7$ ). These data showing no inhibitory effect on tumorigenesis by IL-10-deficient T<sub>R</sub> cells, even when the donors were microbially challenged, are consistent with our recent observations in this model (14) and parallel our earlier studies in 129/SvEv *Rag2*<sup>-/-</sup> mice showing no protection from colitis and colon cancer when T<sub>R</sub> cells donors lack IL-10 (20, 22).



**Figure 3.** *H. hepaticus* infection induces up-regulation of proinflammatory cytokines in *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice. The intestinal mucosal expression levels of cytokines mRNA from uninfected, *H. hepaticus*-infected, and *H. hepaticus*-infected and T<sub>R</sub> cell ( $3 \times 10^5$ )-treated mice are presented: A, IL-12p40; B, TNF $\alpha$ ; C, IFN $\gamma$ ; and D, MIP-2. Infection with *H. hepaticus* significantly increased gene expression of all four cytokines analyzed. Adoptive transfer of wild-type T<sub>R</sub> cells significantly decreased expression of IFN $\gamma$  ( $P < 0.05$ ), TNF $\alpha$ , and MIP-2 (both  $P < 0.001$ ). Cytokine gene expression was analyzed by RNase protection and the intensity of the protected fragments was quantified after normalization to glyceraldehyde-3-phosphate dehydrogenase, which is used as internal control. Columns, mean relative mRNA expression for each cytokine from a group of six to eight mice; bars, SE. Data were analyzed and compared as described in Materials and Methods for statistical significance.

To determine whether exogenously administered IL-10 will inhibit mammary and intestinal tumors, we treated *H. hepaticus*-infected *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice (*N* = 9) with IL-10-Ig fusion protein for 1 week. In all nine mice, we observed significantly (*P* < 0.001) fewer intestinal adenomas when compared with untreated infected control mice (Fig. 1A). Likewise, when compared with the untreated group, only one of nine animals in the IL-10-Ig treated group had a mammary tumor (Fig. 1B). These data support that IL-10 is sufficient to suppress tumors in the absence of lymphocytes. Although the cellular and molecular mechanism(s) through which IL-10 inhibits carcinogenesis are not well understood (34), whether through suppression of inflammatory cytokines, promotion of epithelial homeostasis, or induction of tolerogenic dendritic cells, the *in vivo* inhibitory effect(s) of IL-10-Ig on epithelial cancers in mice lacking lymphocytes is promising and may facilitate new studies in this area.

In summary, the study shows for the first time that an enteric microbial infection promotes cancer in the mammary gland. It is plausible that other proinflammatory bacteria including *H. pylori* also exert extraintestinal carcinogenic effects. Mammary tumor suppression by anti-inflammatory regimens in the present and prior study (15) matches the clinical and epidemiologic data on the protective effects of anti-inflammatory therapies in women with breast cancer. That the wild-type donor T<sub>R</sub> cells inhibit intestinal and mammary tumors in *Apc*<sup>Min/+</sup> and *Rag2*<sup>-/-</sup>*Apc*<sup>Min/+</sup> mice

highlights the prophylactic potential of T<sub>R</sub> cells in inflammation-associated cancers. The observation that T<sub>R</sub> cells from bacterially challenged mice possess greater antitumor potency suggests that microbial challenges in early life may augment protection against the inflammation-associated maladies, including cancer, later in life. It is tempting to speculate that stringent hygiene practices may decrease competency in T<sub>R</sub> cells, which, when coupled with other risk factors, could contribute to increases in breast and other epithelial cancers in Western countries. Nevertheless, due to their anti-inflammatory functions and pivotal roles in epithelial homeostasis, future studies with T<sub>R</sub> cells may offer important clues to the design of more effective treatment and prevention strategies for inflammation-associated cancers in humans.

## Acknowledgments

Received 2/20/2006; revised 4/12/2006; accepted 5/25/2006.

**Grant support:** DOD contract W81XWH-05-01-0460 (S.E. Erdman), R01CA108854 (S.E. Erdman and J.G. Fox), R01 AI5226701 (B.H. Horwitz and S.E. Erdman), NIH R01CA67529 (J.G. Fox), P01 CA26731 (J.G. Fox and S.E. Erdman), P30 ES02109 (J.G. Fox), R01 AI50952 (J.G. Fox), T32RR07036 (J.G. Fox), EU and GMNERA, Pythagoras II 80860 (T. Poutahidis), and R01 DK52413 (J.G. Fox).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Kathy Cormier and Erinn Stefanich for help with histology and immunohistochemistry, Glenn Paradis and Michele Perry for assistance with cell-sorting, and Shilu Xu and Nancy Taylor for preparing bacteria to infect mice. Finally, we thank Brian D. Morrison, Chung-Wei Lee and Elaine Robbins for help with figures.

## References

- Shacter E, Weitzman SA. Chronic inflammation and cancer. *Oncology* (Williston Park) 2002;16:217–26, 29; discussion 30–2.
- O'Byrne KJ, Dalgleish AG. Chronic immune activation and inflammation as the cause of malignancy. *Br J Cancer* 2001;85:473–83.
- Badawi AF, Mostafa MH, Probert A, O'Connor PJ. Role of schistosomiasis in human bladder cancer: evidence of association, aetiological factors, and basic mechanisms of carcinogenesis. *Eur J Cancer Prev* 1995;4:45–59.
- Imperial JC. Natural history of chronic hepatitis B and C. *J Gastroenterol Hepatol* 1999;14 Suppl:S1–5.
- Blaser MJ. Linking *Helicobacter pylori* to gastric cancer. *Nat Med* 2000;6:376–7.
- Rhodes JM, Campbell BJ. Inflammation and colorectal cancer: IBD-associated and sporadic cancer compared. *Trends Mol Med* 2002;8:10–6.
- Malkinson AM, Bauer A, Meyer A, et al. Experimental evidence from an animal model of adenocarcinoma that chronic inflammation enhances lung cancer risk. *Chest* 2000;117:228S.
- McCann J. Esophageal cancers: changing character, increasing incidence. *J Natl Cancer Inst* 1999;91:497–8.
- ACS cancer facts and figures 2004. US Cancer Fact and Figures; 2004.
- Fodde R, Kuipers J, Rosenberg C, et al. Mutations in the APC tumour suppressor gene cause chromosomal instability. *Nat Cell Biol* 2001;3:433–8.
- Furuuchi K, Tada M, Yamada H, et al. Somatic mutations of the APC gene in primary breast cancers. *Am J Pathol* 2000;156:1997–2005.
- van Es JH, Giles RH, Clevers HC. The many faces of the tumor suppressor gene APC. *Exp Cell Res* 2001;264:126–34.
- Moser AR, Luongo C, Gould KA, McNeley MK, Shoemaker AR, Dove WF. ApcMin: a mouse model for intestinal and mammary tumorigenesis. *Eur J Cancer* 1995;31A:1061–4.
- Erdman SE, Sohn JJ, Rao VP, et al. CD4+CD25+ regulatory lymphocytes induce regression of intestinal tumors in ApcMin/+ mice. *Cancer Res* 2005;65:3998–4004.
- Rao VP, Poutahidis T, Ge Z, et al. Proinflammatory CD4+ CD45RB(hi) lymphocytes promote mammary and intestinal carcinogenesis in Apc(Min/+) mice. *Cancer Res* 2006;66:57–61.
- Moser AR, Mattes EM, Dove WF, Lindstrom MJ, Haag JD, Gould MN. ApcMin, a mutation in the murine Apc gene, predisposes to mammary carcinomas and focal alveolar hyperplasias. *Proc Natl Acad Sci U S A* 1993;90:8977–81.
- Powrie F, Correa-Oliveira R, Mauze S, Coffman RL. Regulatory interactions between CD45RBhigh and CD45RBlowCD4+ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J Exp Med* 1994;179:589–600.
- Ge Z, Feng Y, Whary MT, et al. Cytolethal distending toxin is essential for *Helicobacter hepaticus* colonization in outbred Swiss Webster mice. *Infect Immun* 2005;73:3559–67.
- Fox JG, Dewhirst FE, Tully JG, et al. *Helicobacter hepaticus* sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. *J Clin Microbiol* 1994;32:1238–45.
- Erdman SE, Poutahidis T, Tomczak M, et al. CD4+CD25+ regulatory T lymphocytes inhibit microbially induced colon cancer in Rag2-deficient mice. *Am J Pathol* 2003;162:691–702.
- Mihara M, Tan I, Chuzhin Y, et al. CTLA4Ig inhibits T cell-dependent B-cell maturation in murine systemic lupus erythematosus. *J Clin Invest* 2000;106:91–101.
- Erdman SE, Rao VP, Poutahidis T, et al. CD4(+)CD25(+) regulatory lymphocytes require interleukin 10 to interrupt colon carcinogenesis in mice. *Cancer Res* 2003;63:6042–50.
- Lewis CE, Leek R, Harris A, McGee JO. Cytokine regulation of angiogenesis in breast cancer: the role of tumor-associated macrophages. *J Leukoc Biol* 1995;57:747–51.
- Leek RD, Harris AL. Tumor-associated macrophages in breast cancer. *J Mammary Gland Biol Neoplasia* 2002;7:177–89.
- Miyoshi K, Rosner A, Nozawa M, et al. Activation of different Wnt/β-catenin signaling components in mammary epithelium induces transdifferentiation and the formation of pilar tumors. *Oncogene* 2002;21:5548–56.
- Coletta PL, Muller AM, Jones EA, et al. Lymphodepletion in the ApcMin/+ mouse model of intestinal tumorigenesis. *Blood* 2004;103:1050–8.
- Maloy KJ, Salaun L, Cahill R, Dougan G, Saunders NJ, Powrie F. CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. *J Exp Med* 2003;197:111–9.
- Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002;420:860–7.
- Coombes JL, Robinson NJ, Maloy KJ, Uhlig HH, Powrie F. Regulatory T cells and intestinal homeostasis. *Immunol Rev* 2005;204:184–94.
- Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol* 2005;6:353–60.
- Kullberg MC, Jankovic D, Gorelick PL, et al. Bacteria-triggered CD4(+) T regulatory cells suppress *Helicobacter hepaticus*-induced colitis. *J Exp Med* 2002;196:505–15.
- Di Giacinto C, Marinaro M, Sanchez M, Strober W, Boirivant M. Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-β-bearing regulatory cells. *J Immunol* 2005;174:3237–46.
- Wilson MS, Taylor MD, Balic A, Finney CA, Lamb JR, Maizels RM. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J Exp Med* 2005;202:1199–212.
- Mocellin S, Marincola FM, Young HA. Interleukin-10 and the immune response against cancer: a counterpoint. *J Leukoc Biol* 2005;78:1043–51.

# Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

## Innate Immune Inflammatory Response against Enteric Bacteria *Helicobacter hepaticus* Induces Mammary Adenocarcinoma in Mice

Varada P. Rao, Theofilos Poutahidis, Zhongming Ge, et al.

*Cancer Res* 2006;66:7395-7400.

**Updated version** Access the most recent version of this article at:  
<http://cancerres.aacrjournals.org/content/66/15/7395>

**Cited articles** This article cites 30 articles, 12 of which you can access for free at:  
<http://cancerres.aacrjournals.org/content/66/15/7395.full#ref-list-1>

**Citing articles** This article has been cited by 16 HighWire-hosted articles. Access the articles at:  
<http://cancerres.aacrjournals.org/content/66/15/7395.full#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://cancerres.aacrjournals.org/content/66/15/7395>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.