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

Varada P. Rao, Theofilos Poutahidis, Zhongming Ge, Prashant R. Nambiar, Chakib Boussahmain, Yan Yan Wang, Bruce H. Horwitz, James G. Fox, and Susan E. Erdman

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 ApcMin/+ mice with an intestinal bacterial pathogen, Helicobacter hepaticus, significantly promotes mammary carcinoma in females and enhances intestinal adenoma multiplicity by a tumor necrosis factor α (TNFα)–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+CD45RBloCD25+ 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 X wild type crosses to provide Apc<sup>Min/+</sup> mice and wild-type littersmates 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 after infection.
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 × 10<sup>5</sup>* IL-10<sup>−/−</sup> *T<sub>R</sub>* cells (*N* = 8), or *1 × 10<sup>5</sup>* wild-type *T<sub>R</sub>* cells (*N* = 32 mice) 24 hours before *H. hepaticus* infection. CD<sup>4</sup><sup>+</sup>CD<sup>45RB</sup><sup>−</sup>CD<sup>25</sup><sup>+</sup> (*T<sub>R</sub>* lymphocytes were isolated from spleen and mesenteric lymph node 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.likewise, *T<sub>R</sub>* cells 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 α 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 α (TNFα) antibody (clone XT-3; BioExpress, West Lebanon, NH) at 200 µg 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 µg 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 chimera gene was cloned into an adenoaviral vector and the infectious virus (AdIL-10g) was generated as described elsewhere (21). AdIL-10g was used to infect *Helicobacter*-free *Rag2<sup>−/−</sup>* B6 mice (*10<sup>11</sup>* 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 ×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 coecolic 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 µg 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). TNFa mRNA levels in mammary tissue were measured using real-time quantitative PCR as previously described (14).

**Statistical analysis.** Total tumor counts were analyzed by one-way ANOVA using Newman-Keuls posttest. Mammary tumor incidence was compared using contingency tables and χ<sup>2</sup> 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*−/− *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* < 0.001) 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*−/− 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* < 0.001) greater multiplicity of intestinal polyps (*μ* = 110 ± 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 ± 0.9 per high-power field) in the sections of intestines (Fig. 2G) when compared with those from *H. hepaticus*–free controls (4.8 ± 0.9 per high-power field), suggesting a role for these cells in pathogenesis. Interestingly, we find a significantly (*P* < 0.005) 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 CD<sup>4</sup><sup>+</sup>CD<sup>45RB</sup><sup>hi</sup> *T<sub>R</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 squamation 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 pathway.
pathway (25). One explanation for the frequency of H. hepaticus–induced tumors in ApcMin/+ may be the recently described immune deficits. Thymic atrophy and lymphopenia (26) may decrease TR cell competency in ApcMin/+ mice, thereby enabling uncontrolled activation of Helicobacter–primed TR 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 TNFα dependent.** To examine whether H. hepaticus infection is accompanied by up-regulation of proinflammatory cytokines, we analyzed gut mucosal expression levels of cytokines including TNFα, IL-12p40, IFNγ, 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 TNFα is a key cytokine implicated in inflammation-associated cancers (28), and treatment with anti-TNFα antibody has been shown to suppress polyp formation in ApcMin/+ mice (15), we asked whether the H. hepaticus–promoted tumorigenesis in Rag2−/−ApcMin/+ is dependent on TNFα. Indeed, neutralization of TNFα 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−/−ApcMin/+ mice. Therapeutic
effects of TNFα neutralizing antibody in these mice suggest that TNFα 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−/− ApcMin/+ mice need further study. Mammary tumors from H. hepaticus–infected Rag2−/− ApcMin/+ mice show an 18-fold increase (P < 0.0001) in TNFα-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 TNFα-mediated innate immune inflammatory response promotes epithelial tumorigenesis locally as well as in other sites such as mammary gland.

CD25+ regulatory T cells inhibit mammary and intestinal tumorigenesis. Prior studies have shown that CD25+ regulatory (Treg) cells are sufficient to prevent H. hepaticus–triggered colitis and colon cancer (20, 27). To determine whether Treg cells can inhibit Helicobacter-promoted tumorigenesis in Rag2−/− ApcMin/+ mice, nine female mice, 2 to 3.5 months of age, were infected with H. hepaticus and adoptively transferred with 3 × 10^5 Treg cells or with anti-TNFα antibody (clone XT-3), 200 μg 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−/− ApcMin/+ 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 μm (A, C, and E); 100 μm (B, D, and F); 25 μm (G and H).
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 uninfected donors was transferred in parallel into recipientsof IL-10-deficient TR cells (N = 8; μ = 100.4 ± 9.91) when compared with untreated control mice (N = 8; μ = 84.13 ± 4.7). These data showing no inhibitory effect on tumorigenesis by IL-10-deficient TR cells, even when the donors were microbiocally 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).

Prior challenge with <i>H. hepaticus</i> 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 × 10<sup>3</sup> CD45RB<sup>+</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 <i>H. hepaticus</i>, or alternatively from donors that remained uninfected, for adoptive transfer into Rag2<sup>−/−</sup>-Apc<sup>Min/+</sup> mice at time of infection with <i>H. hepaticus</i>. We found that T<sub>R</sub> cells isolated from <i>H. hepaticus</i>–exposed donors were significantly (P < 0.001) more effective at suppressing <i>H. hepaticus</i>–induced intestinal tumors when compared with cells from naive donors. It remains to be seen whether CD45RB<sup>+</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>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells from <i>H. hepaticus</i>–exposed mice were efficacious in protecting against <i>H. hepaticus</i>–induced T<sub>R</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 <i>H. hepaticus</i>–induced enhancement in antitumor potency of T<sub>R</sub> cells is limited to <i>H. hepaticus</i>–triggered tumors, the lower dosage of T<sub>R</sub> cells from <i>H. hepaticus</i>–infected as well as uninfected donors was transferred in parallel into Helicobacter–free Rag2<sup>−/−</sup>-Apc<sup>Min/+</sup> recipients. We find that <i>H. hepaticus</i>–experienced T<sub>R</sub> cells are significantly (P < 0.001) more potent compared with cells from naive donors (P < 0.05) at suppressing intestinal adenoma multiplicity in recipients that were not infected with <i>H. hepaticus</i>. 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 <i>H. hepaticus</i>–promoted tumorigenesis.** In murine models, CD4<sup>+</sup>CD25<sup>+</sup> regulatory (TR) 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 microbiocally enhanced antitumor potency, we did adoptive transfer of TR cells from <i>H. hepaticus</i>–infected IL-10-deficient syngeneic donors into uninfected Rag2<sup>−/−</sup>-Apc<sup>Min/+</sup> recipients of IL-10-deficient T<sub>R</sub> cells (N = 8; μ = 100.4 ± 9.91) when compared with untreated control mice (N = 8; μ = 84.13 ± 4.7). These data showing no inhibitory effect on tumorigenesis by IL-10-deficient T<sub>R</sub> cells, even when the donors were microbiocally 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).

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Hh status</th>
<th>No. mice</th>
<th>Small intestine</th>
<th>Large intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apc&lt;sup&gt;Min/+&lt;/sup&gt;</td>
<td>−</td>
<td>9</td>
<td>37.89 ± 3.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>11</td>
<td>67.73 ± 11.36&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.5 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rag&lt;sup&gt;−/−&lt;/sup&gt;-Apc&lt;sup&gt;Min/+&lt;/sup&gt;</td>
<td>−</td>
<td>16</td>
<td>82.69 ± 4.38&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>15</td>
<td>104.7 ± 9.50&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.7 ± 0.6</td>
</tr>
</tbody>
</table>

**A**<sup>n</sup> and **B**<sup>a</sup> are significantly different from each other, P < 0.05; **C**<sup>a</sup>, P < 0.01; **D**<sup>a</sup>, P < 0.001. **E**<sup>a</sup> and **F**<sup>a</sup>, P < 0.001. **G**<sup>a</sup> and **H**<sup>a</sup>, P < 0.001.

**Figure 3. H. hepaticus infection induces up-regulation of proinflammatory cytokines in Rag2<sup>−/−</sup>-Apc<sup>Minn/+</sup> mice.** The intestinal mucosal expression levels of cytokines mRNA from uninfected, H. hepaticus–infected, and H. hepaticus–infected and TR cell (3 × 10<sup>5</sup>)–treated mice are presented: A, IL-12p40; B, TNFα; C, IFNγ; and D, MIP-2. Infection with <i>H. hepaticus</i> significantly increased gene expression of all four cytokines analyzed. Adoptive transfer of wild-type T<sub>R</sub> cells significantly decreased expression of IFNγ (P < 0.05), TNFα, and MIP-2 (both P < 0.001). Cytokine gene expression was analyzed by RQase 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 10-IL-lg 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 10-lg 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-lg 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 TR 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.

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**References**

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