CD4^+CD25^+ Regulatory Lymphocytes Require Interleukin 10 to Interrupt Colon Carcinogenesis in Mice

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ABSTRACT

Roles for host immune response in carcinogenesis are not well defined. Recent studies have shown that microbiobially driven inflammation can lead to colon cancer and that prior transfer of regulatory lymphocytes expressing CD4 and CD25 prevents the innate inflammatory events that lead to colon cancer in mice. To further examine the ability of regulatory lymphocytes to inhibit carcinogenesis, 129/SvEv Rag-2-deficient mice were inoculated by gastric gavage with Helicobacter hepaticus, an enteric bacterial pathogen of mice. Mice were then treated at 1, 3, or 12 months after infection with adoptive transfer of CD4^+CD45RB^loCD25^+ regulatory cells. Mice dosed with regulatory cells at 4 or 12 weeks after H. hepaticus infection had reduced severity of inflammatory bowel disease and significantly lower risk of colon cancer during the 8 month observation period, compared with infected mice that had not received cells. This suggested that regulatory cells were able to interrupt the ongoing innate immune events in the stepwise progression to cancer. Transfer of regulatory cells into chronically infected mice with established cancer reduced severity of colitis, epithelial dysplasia, and cancer, but did not eliminate all tumors. Regulatory cells lacking anti-inflammatory cytokine interleukin (IL)-10 were unable to inhibit inflammatory bowel disease, dysplasia, or cancer, showing that IL-10 was required for the protective effects of lymphocytes in this setting. Taken together, the data suggest that IL-10-mediated suppression of host innate inflammatory response was pivotal in interrupting carcinogenesis. Regulatory lymphocytes and cytokines may have implications for novel therapies for colon cancer in humans.

INTRODUCTION

Roles of host immunity in tumorigenesis are poorly understood (1). Epidemiological evidence indicates that humans with IBDs, including CD and UC, have increased risk of developing colon cancer (2). Among many factors contributing to colon neoplasia in humans, host immunity and gut microbial status appear important in progression of lower bowel diseases (3, 4). Mouse models of IBD and colon cancer have facilitated dissection of complex in vivo mechanisms that lead to colorectal cancers in humans (5, 6). Studies using germ-free mice have shown that enteric bacteria are required for colon cancer in some models (7, 8). In addition, infection with Helicobacter hepaticus, an enteric bacterial pathogen in mice, leads to IBD with associated colon cancer in immune-disregulated mice (8, 9). Citrobacter rodentium, another mouse enteric pathogen, also increased intestinal polyps in genetically predisposed mice (10). A bacterial etiology of colon tumorigenesis has not been established in humans; however, gastric carcinoma in humans has been convincingly linked with chronic Helicobacter pylori-induced inflammation (11–13).

Studies using recombinase-activating gene (Rag)-2-deficient mice, which lack functional lymphocytes, convincingly demonstrate that innate immune response to certain pathogenic enteric bacteria are sufficient to initiate colitis and colon carcinogenesis (8, 9, 14–16). In Rag-deficient H. hepaticus-infected mice, IBD was prevented by prior adoptive transfer of CD4^+CD25^+ cells, a subset of T lymphocytes that are important in regulating immune-mediated disease in mice (9, 16, 17). Interestingly, CD4^+CD25^+ cells also blocked H. hepaticus-associated cancer in the colon (9). This indicated a previously unrecognized role for CD4^+ lymphocytes in protecting against primary epithelial tumors. In general, roles for CD4^+CD25^+ cells in carcinogenesis in vivo are not well defined (1, 18).

Data from mouse models have shown that inhibition of colitis by CD4^+ regulatory cells depends primarily upon two key cytokines, IL-10 and TGF-β (16, 19–26). IL-10 has become well accepted as a major regulator of mucosal immune responses in mice based on studies demonstrating that IL-10 directly counteracts IL-12-driven inflammation to maintain homeostasis in the colon (16, 23, 25, 27). Indeed, the balance of IL-10 and IL-12 significantly determines the extent and severity of H. hepaticus-driven colitis in mice (23, 26). However, the possible roles for IL-10 in carcinogenesis are seemingly contradictory (28). Several studies have shown profound inhibition of tumor establishment, growth, and metastasis by IL-10 (29–31). Other studies have suggested that IL-10 may suppress host antitumor responses and promote tumor growth (28).

Given that CD4^+CD25^+ regulatory cells are important in immune homeostasis in the lower bowel, we sought to examine whether regulatory cells have broader protective roles in carcinogenesis than described previously. Based upon evidence that IL-10 can block IBD in mice (16, 23, 25–27, 32, 33), we hypothesized that IL-10 may be a key modulator of colon carcinogenesis in this model. Thus, we examined progression of IBD and colon cancer in 129/SvEv Rag-deficient mice using stepwise transfers of regulatory cells, with and without IL-10, after infection with H. hepaticus.

MATERIALS AND METHODS

Rag2 Mice Were Housed in a Helicobacter-free Mouse Facility

All mice were housed in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved facilities in static microisolator cages with health status as previously described (9). Experimental mice dosed with H hepaticus were housed separately in a biocontainment area of the animal facility.

Experimental Infection

H. hepaticus (strain 3B1; ATCC 51449) was grown under microaerobic conditions, prepared, and confirmed pure as described elsewhere (9, 34, 35). Experimental mice received 0.2 ml of fresh inoculum by gastric gavage every other day for a total of three doses.
Experimental Design

To examine whether intervention with regulatory cells could arrest ongoing carcinogenesis, mice were dosed with regulatory cells either early (experiment 1) or late (experiment 2) in the progression of disease. Time points for early intervention and tissue harvest were selected based upon typical progression of IBD and dysplasia as described previously (9).

Cancer type and frequency of colon tumors in mice > 8 months after infection had not been described previously. To determine a suitable time point for late intervention, bowel lesions from a pilot group of 6 _H. hepaticus_-infected aged mice were examined and compared with age-matched uninfected controls. Data from these 6 mice were also included in analyses of aged mice in experiment 2.

For all experiments, helicobacter-free 129SvEv Rag-2-knockout mice, ages 6–8 weeks, were dosed with _H. hepaticus_ suspended in broth. For each adoptive transfer experiment, a cohort of age-matched littermates served as _H. hepaticus_-infected controls. Half of the mice were male and half were female, unless otherwise specified. Replicate experiments were conducted with two or three groups of similar size.

**Experiment 1: Early Intervention.** Forty-eight _H. hepaticus_-infected mice were treated with CD4<sup>+</sup>CD45RB<sup>+</sup>CD25<sup>+</sup>_regulatory cells at either 72 h prior (group 1B; _n_ = 16), 4 weeks after (group 1C; _n_ = 20), or 12 weeks after (group 1D; _n_ = 12) infection. Regulatory cells for this experiment were isolated from helicobacter-free wild-type 129SvEv mice. Twenty-four mice served as _H. hepaticus_-infected controls. Tissues were harvested at 4 or 8 months after infection.

**Experiment 2: Late Intervention.** Eighteen aging infected mice were divided into three groups. One group of 6 mice (2A) was euthanized to determine the frequency and spectrum of colonic adenocarcinoma 8–12 months after infection (as described above). A second group of 6 mice (2B) was treated with regulatory cells at 1 year after infection. The remaining 6 mice (2A) were left untreated for use as infected controls. Six weeks later, tissues from both groups were collected and compared.

**Experiment 3: Role of IL-10.** Sixty-two _H. hepaticus_-infected mice were divided into three groups. One group received IL-10-deficient-regulatory T cells, a second group received wild-type-regulatory cells, and a third group remained untreated. Infected but untreated mice were group 3A. Mice were either pretreated (group 3B; _n_ = 16) at 72 h before infection or posttreated at either 4 weeks (group 3C; _n_ = 12) or 12 weeks (group 3D; _n_ = 12) after infection. Six mice were harvested at 4 and 12 weeks after infection to assess progression of disease upon intervention. Regulatory cells for the third experiment were derived from helicobacter-free 129SvEv/C57BL/6 IL-10-deficient mice that had been backcrossed four generations (N4) onto a 129SvEv background and then intercrossed (F1 or F2) to provide IL-10-deficient or -matched wild-type donors.

**Confirmation of _H. hepaticus_ Infection**

Cecum and colon were collected at necropsy and analyzed by PCR to confirm experimental infection using _H. hepaticus_-specific primers (36). Helicobacter-free status was confirmed in controls using PCR with helicobacter-genus-specific primers (37).

**Quantification of _H. hepaticus_ Infection**

_H. hepaticus_ in the cecum and colon of the infected mice was enumerated by real-time quantitative PCR in the ABI Prism 7700 sequence detection system (PE Biosystems, Foster City, CA) as described previously (38).

**Adoptive Transfer of T Cells in Rag-2-knockout Mice**

To examine ability of regulatory cells to modulate carcinogenesis in _H hepaticus_-infected Rag2 mice, we transferred purified CD4<sup>+</sup> T lymphocytes from helicobacter-free wild-type 129SvEv donors into Rag2-deficient mice. Half of the donor mice were males, and half of them were females. Anesthetized mice were injected i.v. in the retro-orbital sinus with 3 × 10<sup>8</sup> T cells suspended in 0.2 ml of HBSS. Mice were injected with regulatory T cells 72 h before or 1, 3, or 12 months after infection with _H. hepaticus_, as described above.

**Purification of T Cells for Adoptive Transfer**

To obtain viable and highly purified populations of T cells for adoptive transfer, single cell suspensions from spleen and mesenteric lymph nodes from helicobacter-free 129SvEv donor mice were prepared as described previously (9). The purified cells were suspended in HBSS before injection as described previously. Reanalysis of these cells before transfer into mice indicated that purity was >95%.

**Histological Evaluation**

Formalin-fixed tissues were embedded in paraffin, cut at 5 μm, and stained with H&E. Lesions were scored by a pathologist blinded to sample identity. Pathology was not evaluated in a 0.5-mm zone surrounding the anus to minimize concerns about interpretation of dysplasia involving rectal prolapse. The cecal and colonic hyperplastic and inflammatory lesions were graded on a scale of 0 to 4 with ascending severity (0 = none, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe) modified from Berg et al. (9, 32). Epithelial dysplasia and neoplasia were graded using a scale of 0–4 based on a recently described scheme (9, 39–42). Grade 0 = normal, 1 = mild dysplastic changes, 2 = low-grade gastrointestinal intraepithelial neoplasia, 3 = high-grade gastrointestinal intraepithelial neoplasia (carcinoma _in situ_ or intramucosal carcinoma), and 4 = invasive carcinoma (9). Nonparametric data are presented as median score and range (in parentheses) for each group.

**Immunohistochemical Assessment of Colon Lesions**

Normal and neoplastic epithelium was demonstrated by pancytokeratin (AE1/AE3; Dako, Carpinteria, CA) using standard immunohistochemistry as described elsewhere (9).

**Statistical Analyses**

Analyses of cecal and colonic lesion scores were performed using a Mann-Whitney _U_ nonparametric test for ordinal data. Comparisons of frequency of carcinoma between groups were performed using a two-sided Fisher’s exact test. _Helicobacter_ quantification data were analyzed using a two-tailed _t_ test.

**RESULTS**

**Early Intervention with CD4<sup>+</sup>CD25<sup>+</sup>_regulatory T Cells Abrogated Inflammation and Inhibited Onset of Cancer.** It has been previously shown that infection with _H. hepaticus_ triggered IBD (9, 16) and carcinoma (9) in Rag-deficient 129SvEv mice. Pretreatment with CD4<sup>+</sup>regulatory T cells inhibited onset of cancer in this model (9). To examine whether regulatory cells may also interrupt the events leading to cancer, we performed adoptive transfer experiments after infection. Experiment 1 involved early intervention at either 4 (for moderate-severe inflammation) or 12 (for moderate-severe dysplasia) weeks after infection with _H. hepaticus_. Twelve weeks of postinfection were selected for intervention because of the high frequency of epithelial dysplasia and early invasive cancer lesions characteristic of this interval postinfection (9).

Mice that received regulatory cells at 4 weeks after infection (group 1C; _n_ = 20) had reduced IBD and dysplasia during the following 7 months (Table 1). Suppression of dysplasia was highly significant (_P_ < 0.005) in both cecum [0 (0–1)] and descending colon [0 (0–0)] at 8 months after infection. In general, mice that received regulatory cells had fewer neutrophils in mucosal erosions compared with infected Rag<sup>−/−</sup> controls. Eosinophils and neutrophils were the predominant inflammatory cell type at time of intervention (at 4 weeks after infection) in matched infected control Rag<sup>−/−</sup> mice.

A similar pattern of inhibition was observed in mice that received regulatory cells at 12 weeks after infection (group 1D; Table 1). In this group, some dysplasia remained at 4 months after infection but not at 8 months after infection. Because high-grade dysplasia was a feature at the time of intervention with regulatory cells (at 12 weeks after infection), 1 month (between treatment and harvest) may have been
insufficient time to suppress pathology in some mice. In general, inflammation and dysplasia in *H. hepaticus*-infected untreated Rag−/− control mice were more severe in the colon than in the colon at 4 months after infection but of similar severity in cecum and colon at 8 months after infection (Table 1). Epithelial dysplasia was clearly associated with inflammatory foci in both cecum and colon.

These findings indicated that regulatory cells suppressed innate immune IBD and dysplasia leading to cancer and facilitated homeostasis. Next, we were interested to know whether regulatory cells might also inhibit the variety of established cancers typical in aged *H. hepaticus*-infected mice.

**Helicobacter hepaticus** Induced a High Frequency of Colon Adenocarcinoma in Aged Rag-deficient Mice. Early progression of *H. hepaticus*-induced inflammation (9, 16) and cancer (9) has been well-characterized. This previously documented (9) progression of inflammation and dysplasia was used to select time points for early intervention. However, the spectrum and frequency of colon adenocarcinoma in this model at >8 months after infection had not been described previously. Thus, we examined colon cancer in a pilot group of 6 Rag−/− mice that had been chronically infected with *H. hepaticus* to define a target for late intervention (after the development of adenocarcinoma) with regulatory cells.

At 8–12 months after infection, Rag−/− mice (n = 6) had inflammation [4 (2–4)], hyperplasia [4 (2–4)], dysplasia [4 (1–4)], and adenocarcinoma in the lower bowel. Dysplasia and carcinoma were associated with foci of inflammation. In contrast, minimal bowel disease was observed in uninfected control mice (Fig. 1A). *Helicobacter*-free Rag−/− littermates, examined at 15 months of age (n = 10), had minimal inflammation in the cecum [0 (0–1)] or colon [0 (0–1)], minimal epithelial hyperplasia in the cecum [0 (0–1)] or colon [0 (0–1)], and no dysplasia or cancer in the cecum [0 (0–0)] or colon [0 (0–0)]. This reaffirmed that *H. hepaticus* infection was required for IBD and cancer in this model (9, 16).

All 6 mice had at least two types of cancer, including carcinoma in
Table 2. Histologic findings in H. hepaticus-infected Rag-deficient mice with or without transfer of regulatory cells at 1 year after infection

<table>
<thead>
<tr>
<th>Group</th>
<th>H. hepaticus</th>
<th>Cell transfer</th>
<th>Cecum</th>
<th>Ascending colon</th>
<th>Transverse colon</th>
<th>Descending colon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inflammation</td>
<td>Dysplasia</td>
<td>Inflammation</td>
<td>Dysplasia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2A</td>
<td>Yes</td>
<td>Rag (none)</td>
<td>0 (0–1)</td>
<td>0 (0–0)</td>
<td>0 (0–1)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>2B</td>
<td>Yes</td>
<td>Post 1-yr Treg</td>
<td>3.5 (1–4)</td>
<td>2.1 (1–3)</td>
<td>2 (1–4)</td>
<td>0 (0–3)</td>
</tr>
</tbody>
</table>

* Mann Whitney U test, comparison between bowel dysplasia in H. hepaticus-infected mice after dosing with regulatory cells at 1 year after infection. P < 0.05.

Table 3. Frequency of adenocarcinoma in H. hepaticus-infected Rag-deficient mice with or without treatment with wild-type regulatory cells at 1 year after infection

<table>
<thead>
<tr>
<th>Types of carcinomas</th>
<th>Untreated</th>
<th>Treated</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All carcinomas</td>
<td>11/12 (94%)</td>
<td>2/6 (33%)</td>
<td>P = 0.022</td>
</tr>
<tr>
<td>Invasive carcinomas</td>
<td>6/12 (50%)</td>
<td>0/6 (0%)</td>
<td>P = 0.054</td>
</tr>
<tr>
<td>Mucinous carcinoma</td>
<td>3/12 (25%)</td>
<td>0/6 (0%)</td>
<td>P = 0.515</td>
</tr>
<tr>
<td>Intramucosal carcinoma</td>
<td>4/12 (33%)</td>
<td>2/6 (33%)</td>
<td>P = 1.000</td>
</tr>
<tr>
<td>Poorly differentiated (intramucosal)</td>
<td>3/12 (25%)</td>
<td>2/6 (33%)</td>
<td>P = 1.000</td>
</tr>
</tbody>
</table>

REGULATORY CELLS INTERRUPT COLON CARCINOGENESIS

Carcinoma in situ (6 of 6; 100%) and various types of intramucosal carcinomas (6 of 6; 100%). Carcinoma in situ was most common in the cecolic junction. Three (50%) of the Rag-deficient mice that were dosed with H. hepaticus had invasive cancer. Muscle invasion was most common in the transverse and descending colon. In general, the spectrum of dysplasia and cancer resembled lesions already described in detail elsewhere (9). Additional details are described below.

Two (2 of 6; 33%) of the Rag-deficient mice had large mucinous colon tumors (Fig. 1, B and C). Mucinous carcinoma had moderately to well-differentiated atypical epithelium infiltrating the submucosa and muscle. Tumors obliterated the normal architecture, as described in Boivin et al. (39). Extracellular mucin was a prominent feature. Variably shaped large mucin pools were partially lined by neoplastic epithelium or bounded by host stroma. There was a moderate to severe desmoplastic reaction.

Two (2 of 6; 33%) of the infected Rag−/− mice had a variant of intramucosal carcinoma consisting of multiple large mucosal tumors in the descending colon and rectum (Fig. 1, D–F). These nodules appeared to expand within the mucosa, compressing and displacing adjacent structures. The surface epithelium overlying the nodules was either intact or effaced by extensive areas of coagulative necrosis. The tumors were composed of a relatively uniform population of large polygonal cells with abundant slightly eosinophilic cytoplasm and round to oval nuclei with finely dispersed chromatin and conspicuous nucleoli. Mitotic figures were rare. The tumor cells were arranged in trabeculae or nests separated by a fine fibrous stroma. Nodules were heavily infiltrated by neutrophils. Nests of abnormal glands were found associated with some tumor nodules, most probably representing a more typical, moderately differentiated, intramucosal adenocarcinoma component. The tumor cells were pancytokeratin positive (Fig. 1D). Based upon histology, these medullary tumors were classified as poorly differentiated. Several of these tumors had morphological features of a type of medullary adenocarcinoma referred to as hepatoid adenocarcinoma in humans (43). There were no detectable metastases to lymph nodes or liver.

Based upon the high frequency (100%) and wide variety (two or more types of cancer in most mice) of colon neoplasia at this time, we selected 1 year after infection as the time point for late intervention to assess inhibition of cancer (see experiment 2 below).

Late Intervention Reduced Overall Frequency of Carcinoma but Did Not Suppress all Types of Cancer. In the first experiment, early intervention with regulatory cells inhibited IBD and cancer. To determine whether regulatory cells were also able to inhibit a wide spectrum of well-established adenocarcinomas, aged H. hepaticus-infected Rag-deficient mice received i.v. injections of regulatory cells at 12 months after infection. At this time point after infection, all 6 mice in the pilot group had cancer, and half had carcinoma with muscle invasion. Data from the 6 mice used in the pilot study, above, were then combined with another 6 infected untreated controls for a total group of 12 controls. H. hepaticus-infected mice that were untreated with regulatory cells, in experiment 2.

Among the 12 untreated H. hepaticus-infected Rag−/− mice examined, 11 (92%) had carcinoma, including carcinoma in situ (11 of 12; 92%), intramucosal carcinoma (8 of 12; 75%), and high-grade villus adenoma (2 of 12; 16%) as described previously (9). Six (50%) mice had invasive bowel cancer. Three (3 of 12; 25%) mice had poorly differentiated mucosal carcinoma. All 8 (100%) of the males in this experiment developed carcinoma. Three (3 of 4; 75%) females had cancer, and all mice had IBD.

Transfer of regulatory cells (n = 6) at 1 year after infection inhibited IBD and dysplasia in the colon (Table 2). There was significantly less (P = 0.022) lower bowel cancer in late-treated (2 of 6; 33%) versus untreated (11 of 12; 94%) mice (Table 3). There were also fewer invasive lesions in treated (0 of 6) than untreated (6 of 12; 50%) mice, although this difference did not achieve statistical significance (P = 0.054). Likewise, there was no (0 of 6) mucinous cancer seen in mice treated with regulatory cells in contrast to 3 (3 of 12; 25%) mice with mucinous colon tumors (P = 0.515) in the untreated group. The small sample size of six mice treated with regulatory T cells made it difficult to interpret these findings or achieve statistical significance.

Late treatment with regulatory cells did not entirely eliminate adenocarcinoma in 2 (33%) of the mice. Both mice had large poorly differentiated mucosal tumors in the descending colon (Fig. 2B). Carcinoma in situ was also present in both of the regulatory cell-treated mice that had cancer (33%). The frequency of the large poorly differentiated medullary tumors was similar in the treated (33%) versus untreated (25%) groups. These findings (summarized in Table 3) suggested a trend that regulatory cells reduced invasive cancers, but not all types of mucosal carcinoma, in these mice, although larger numbers of mice are needed for statistical significance.

Regulatory cells were more effective at reducing IBD in the colon (P = 0.0016) than in the cecum (P = 0.072) in this experiment (Table 2). Among the 6 mice treated with regulatory cells, 3 (50%) had moderate inflammation in the cecum, whereas these same mice had little or no inflammation in the descending colon. Only 1 (16%) mouse among the regulatory cell recipients in group 2B had minimal inflammation (score ≤ 1) and dysplasia (score = 0) in both cecum and colon.

These findings indicated that late intervention with regulatory cells reduced overall frequency of cancer but did not suppress selected poorly differentiated mucosal cancers. Next, we examined whether.
the key mucosal regulatory cytokine IL-10 was needed for the protective effects of regulatory cells.

**IL-10 Was Required to Interrupt the Stepwise Progression of IBD and Colon Cancer.** Anti-inflammatory cytokine IL-10 is pivotal in preventing IBD in several widely used mouse models (27, 32, 33). To examine the role of IL-10 in carcinogenesis in this Rag-deficient mouse model, we performed adoptive transfer of regulatory cells collected from helicobacter-free IL-10-deficient or matched wild-type mice.

Transfer of CD4⁺CD25⁻ regulatory cells lacking IL-10 either before (n = 8) or 4 (n = 6) or 12 weeks (n = 6) after infection failed to protect against IBD and cancer compared with recipients of wild-type-regulatory cells at each time point (Table 4). Using the scoring system originally adopted from Berg et al. (9, 32), there were no significant differences between inflammation in ascending colon, transverse colon or descending colon in mice that had received IL-10⁻/⁻ regulatory cells versus those that received no cells (Table 4). As in experiment 1, there were more neutrophils present in mucosa of *H. hepaticus*-infected Rag⁻⁻ mice and IL-10-deficient cell recipients compared with recipients of wild-type-regulatory cells (Fig. 3). There were few differences in the character of dysplasia among untreated Rag⁻⁻ mice and IL-10⁻⁻ recipients, with the exception of increased invasiveness (denoted with a dysplasia score = 4) with mucinous lesions in the ascending and transverse colon of recipients of IL-10⁻⁻ cells as described below. As in experiment 1, wild-type-regulatory cells significantly suppressed both inflammation and dysplasia when compared with *H. hepaticus*-infected Rag⁻⁻ mice (Table 4). This indicated that IL-10 was required for the protective effects of regulatory cells in IBD and cancer.

Interestingly, 5 (5 of 8) mice that received IL-10-deficient cells before *H. hepaticus* infection developed large mucinous carcinoma masses in the colon that were not observed in recipients of wild-type cells (0 of 8; P = 0.026) or untreated (0 of 10; P = 0.007) infected control mice at 4 months after infection (Fig. 3). Four (4 of 5; 80%) of the mice with locally invasive tumors were males, and 1 (1 of 3; 33%) of the affected mice was female. There were no significant differences between inflammation scores in the cecum or colon of males versus females in experiment 3, but there was significantly more dysplasia in the ascending colon (P = 0.04) and the transverse colon (P = 0.04) of males compared with females. The spectrum of dysplasia and carcinoma in recipients of IL-10-deficient cells resembled the adenomas and carcinomas, described above, in aged *H. hepaticus*-infected Rag-deficient mice. However, the lesions were more frequent and severe and appeared more rapidly after infection than in Rag-deficient mice that did not receive cells. Mucinous carcinoma was the predominant cancer in mice treated with IL-10-deficient cells (Fig. 3C). In the most severe cases, the tumors invaded the peritoneal cavity and compressed adjacent viscera at 4 months after infection.

Using quantitative PCR, the counts of *H. hepaticus* organisms/μg mouse bowel DNA in infected mice were 10⁶–10⁷ for cecum and 10³–10⁵ for colon. There were no statistically significant differences among the groups of mice, which indicated that treatment with regulatory cells, either with or without IL-10, had minimal influence on the colonization efficiency of *H. hepaticus* in cecum or colon. The lack of correlation between helicobacter counts and IBD or cancer indicated that host inflammatory response to *H. hepaticus* rather than the quantity of *H. hepaticus* lead to cancer in this model.

Results from all three experiments are summarized in Fig. 4. In experiment 1, early intervention with regulatory cells blocked IBD and cancer. Late intervention in experiment 2 inhibited some cancers but not poorly differentiated mucosal tumors. Regulatory cells lacking IL-10 did not inhibit IBD or cancer in this model, in experiment 3, suggesting an IL-10-dependent mechanism.

### Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell transfer</th>
<th>Cecum</th>
<th>Ascending Colon</th>
<th>Transverse Colon</th>
<th>Descending Colon</th>
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<td>Rag (none)</td>
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<td>Pre IL-10 Treg</td>
<td>4 (4–4)</td>
<td>3.5 (3–4)</td>
<td>4 (1–4)</td>
<td>3 (0–4)</td>
</tr>
<tr>
<td>3C</td>
<td>Pre WT Treg</td>
<td>2 (1–4)</td>
<td>1 (0–3)</td>
<td>1.5 (1–4)</td>
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<tr>
<td>3D</td>
<td>Post 4-wk IL-10 Treg</td>
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a Mann-Whitney U test, comparison between inflammation or dysplasia in *H. hepaticus*-infected Rag2-deficient mice with or without IL-10-deficient-regulatory cells at 4 months after infection. P < 0.05.

b Mann-Whitney U test, comparison between inflammation or dysplasia in *H. hepaticus*-infected Rag2-deficient mice with or without WT-regulatory cells at 4 months after infection. P < 0.05.
DISCUSSION

These results indicated that CD4+CD25+ regulatory cells play a broader role in inhibiting microbially induced cancer in the bowel than appreciated previously. Although prior studies have focused primarily upon preventing onset of inflammation (9, 16), the present study showed that stepwise intervention of colitis and dysplasia with regulatory cells arrested ongoing inflammation and significantly reduced risk of associated colon cancer. Early intervention with regulatory cells blocked IBD and reduced dysplasia and cancer. Treatment of established IBD and cancer with regulatory T cells in aged infected mice reduced colitis and cancer but did not eliminate some types of colon tumors. Regulatory T cells lacking IL-10 were unable to prevent colitis or cancer in this model, suggesting that protection from IBD and cancer was dependent upon IL-10.

The observation that early intervention with regulatory cells prevented later development of carcinoma suggested that onset of inflammation alone was insufficient and that sustained inflammation is required for cancer progression. There is growing evidence in other models, including H. pylori-induced gastritis and gastric adenocarcinoma, that bacterially induced activation of immune cells increases cancer risk by causing oxidative damage that is directly or indirectly mutagenic (12). Perhaps 12 weeks of innate immune activation was too brief to initiate carcinogenesis in this Rag-deficient model. Alternatively, regulatory cells may have uncharacterized abilities to arrest or reverse epithelial changes that would ultimately lead to cancer. Studies in SCID and nude mouse tumor models have shown that IL-10, a key regulatory cell cytokine, inhibited tumor growth in mice by a T-cell-independent mechanism (28). Examination of markers of early molecular changes in bowel epithelia would additionally define the spectrum of regulatory cell- and IL-10-mediated activities early in carcinogenesis.

Given the compelling evidence that IL-10 prevents H. hepaticus-induced IBD (16, 23–27) and that IL-10-deficient mice may develop colon cancer in some settings (32, 44), it is not surprising that IL-10 was required to block tumorigenesis in this model. It is well established that regulatory cell-mediated IL-10 prevents onset of colitis by blocking IL-12-mediated events (16, 23, 25, 26). It has been previously shown in the 129SvEv Rag-deficient model that IL-12 is pivotal in driving IBD during the initial weeks after experimental infection with H. hepaticus (16). The ability of regulatory cells to inhibit inflammation when introduced at longer intervals after infection suggests that additional innate immune-mediated mechanisms of suppression may also be involved. Suppression of the other potent proinflammatory cytokines tumor necrosis factor α, IL-1β, and IL-6 are likely possibilities (16, 28). Beyond these pivotal anti-inflammatory bioactivities, roles of regulatory cells and IL-10 in carcinogenesis are less well defined (1, 28). A comprehensive pathway linking DNA damage, tumorigenesis, and IL-10 has not been elucidated (28). Gene therapy studies have demonstrated IL-10-mediated inhibition of tumor growth and metastases (29–31), but cancer outcome is variable and appears to depend upon host immune competency and levels of IL-10 expression (28). The complex interplay between host immune cells, cytokines, and epithelia makes it challenging to evaluate specific contributions to mucosal integrity by selectively adding, blocking, or deleting cytokines in vivo.

Mucinous adenocarcinomas seen in one-third of aged helicobacter-bacterium-infected Rag-deficient mice were pervasive in recipients of regulatory cells lacking IL-10. Lesions in both the Rag-deficient mice and IL-10−/− cell recipients were morphologically similar to carcinoma previously described in mice lacking TGF-β1 and SMAD3 (19, 39) and also resembled colorectal cancer seen in humans with IBD. In humans, these cancers have been characterized by microsatellite in-
stability (45), although this has not been clearly demonstrated in mice (19, 39). IL-10 has been previously shown to inhibit tumor growth and invasion in other models (28), but the increased frequency of mucinous tumors in recipients of IL-10-deficient regulatory cells seen in this study was unexpected. One possible explanation is enhancement of proinflammatory activity after transfer of regulatory cells that lack IL-10. Mucinous carcinoma is a feature of IBD after adoptive transfer of proinflammatory CD4⁺/CD45RB⁺ hi cells alone (unpublished finding). The lack of significant increases in inflammation among recipients of IL-10−/− cells, when compared with Rag−/− mice, does not support this hypothesis; however, inflammation scores were near maximal in mice that did not receive cells, thus detection of increases using this scoring method would be difficult. Another interesting possibility is dysregulation of key regulatory cytokine, TGF-β, in the absence of IL-10 in this model. Indeed, TGF-β has diverse activities and may stimulate tumor progression as well as suppression (46). Recently, Engle et al. (8) showed that TGF-β-deficient mice required enteric microbiota, including H. hepaticus, to develop the characteristic mucinous tumor phenotype. Taken together, the similarities between these mutant models suggest that perturbation of IL10 or TGFβ during a proinflammatory microbial insult may lead to invasive tumors, perhaps through dysregulation of mucosal repair. It remains unclear whether the activities of CD4⁺CD25⁺ cells were predominantly anti-inflammatory in nature or involved other mechanisms as well.

Transfer of regulatory cells did not reduce frequency of medullary mucosal carcinoma in the colon of these mice. These poorly differentiated tumors had morphological features of adenocarcinomas previously described in the stomach (43) and also in UC (39, 47) of humans. The prognosis for survival with some of these cancers is very poor in humans with or without chemotherapy, possibly because the tumors are aggressive and readily metastasize to other sites (43). To our knowledge, this is the first study of any type of medullary colon cancer in mice (39). Because medullary tumors in humans have a high frequency of microsatellite instability (45), additional molecular characterization of this and the other microbially induced colon tumors in this model are needed to assess the spectrum of mutations and malignancy. The inability of CD4⁺CD25⁺ cells to inhibit some types of colon tumors in this model begins to define limits of homeostatic potential of regulatory cells.

A male predominant gender effect for cancer was most pronounced in recipients of IL-10-deficient-regulatory cells in this study, although carcinoma was also more frequent in helicobacter-infected males than females that had not received cells. A predilection for cancer in males paralleling syndromes in humans has been previously described in H. pylori-infected mice with gastric cancer (48) and H. hepaticus-infected A/J mice with hepatocellular carcinoma (34, 49, 50).

Neutrophils were a frequent feature of lower bowel inflammation and dysplasia in this model, but the precise cause-and-effect relationship between these innate immune cells and carcinogenesis is unclear. The finding may reflect robust host response to a mucosal microbial insult; however, granulocyte-mediated oxidative damage may also contribute to carcinogenesis (12). The observation that recipients of wild-type-regulatory cells had fewer granulocytes associated with mucosal erosions than untreated controls supported earlier evidence that regulatory T cells may inhibit neutrophil activation in this model (16). In situ phenotypic markers will be needed to more fully characterize the etiopathogenesis of this disease.
Helicobacter infection was required for IBD and cancer in these mice as previously shown (9, 16). However, helicobacter counts were not directly correlated with cancer. This additionally supported the concept that host inflammatory response to these bacteria rather than a direct quantitative effect of H. hepaticus organisms leads to cancer in this model. An inverse correlation between severity of pathology and H. hepaticus counts was previously seen in mice and attributed to a robust T-helper 1-mediated host response (33). In the present Rag-deficient model, inflammation was more severe in the cecum, the primary site of H. hepaticus colonization (16) rather than in the colon during early infection. Interestingly, prominent tumors, including mucinous and poorly differentiated mucosal adenocarcinomas, were more frequent in the transverse and descending colon and not in the cecum of these aged mice. This suggested that factors beyond chronic inflammation alone, perhaps underlying physiological differences between cecal and colonic mucosa or other enteric microbiota, also contributed to carcinogenesis in this model.

Given that humans with IBD have an increased risk of colon cancer (2) and that colonic dysplasia is a detectable premalignant condition, it will be important to identify targets and strategies for intervention in early and late stages of bowel disease. Therapeutic potential of transforming growth factor β will be important to identify targets and strategies for intervention in early and late stages of bowel disease. Therapeutic potential of transforming growth factor β1-deficient mice. Cancer Res., 59: 3379–3386, 1999.


CD4+CD25+ Regulatory Lymphocytes Require Interleukin 10 to Interrupt Colon Carcinogenesis in Mice
