

AD _____

Award Number: W81XWH-05-1-0460

TITLE: Are Anti-inflammatory Lymphocytes Able to Induce Remission of Breast Cancer

PRINCIPLE INVESTIGATOR: Susan E. Erdman, DVM, MPH

CONTRACTING ORGANIZATION: Massachusetts Institute of Technology
Cambridge, MA 02139

REPORT DATE: February 2007

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 01-02-2007		2. REPORT TYPE Final Addendum		3. DATES COVERED 1 Aug 2005 – 31 Jan 2007	
4. TITLE AND SUBTITLE Are Anti-inflammatory Lymphocytes Able to Induce Remission of Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0460	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Susan E. Erdman, DVM, MPH				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Massachusetts Institute of Technology Cambridge, MA 02139				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES Original contains colored plates: ALL DTIC reproductions will be in black and white.					
14. ABSTRACT Recent studies suggest that inflammation is a key contributor to development of breast cancer in women. Increasing scientific and medical data point to immune cells, in particular pro-inflammatory CD4+ effector (TE) cells and anti-inflammatory CD4+regulatory (TR) cells, as pivotal mediators in human health and disease. We have previously demonstrated that anti-inflammatory TR cells prevent colorectal cancer (CRC) in mice by suppressing inflammatory growth factors. We show here that transfer of pro-inflammatory TE cells or infection with pro-inflammatory intestinal bacteria <i>Helicobacter hepaticus</i> rapidly promotes mammary tumor development in the ApcMin/+ mouse model, and that adoptive transfer of TR cells inhibits development of inflammation-associated mammary tumors induced by either pro-inflammatory cells or intestinal bacteria in those mice. Targeting deleterious host inflammatory responses may be more effective and less toxic than traditional chemotherapeutic approaches to neoplasia. Ability to understand and harness the potency of TR cells to suppress inflammatory carcinogenic processes may prevent and ultimately abolish breast malignancies in women.					
15. SUBJECT TERMS breast cancer, lymphocytes, mouse models, inflammation					
16. SECURITY CLASSIFICATION OF:			UU	18. NUMBER OF PAGES 21	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusions.....	5
References.....	6
Appendices.....	6

INTRODUCTION:

Recent studies suggest that inflammation may be a key contributor to development of breast cancer in women (1). Increasing scientific and medical data point to immune cells, in particular the balance between pro-inflammatory CD4⁺ effector (T_E) cells and anti-inflammatory CD4⁺ regulatory (T_R) cells, as pivotal mediators in human health and disease (2). Anti-inflammatory T_R cells inhibit destructive immune responses in both humans and mice; thus, immunotherapy using T_R cells has been proposed to treat diseases such as arthritis and inflammatory bowel disease (IBD) in people. Likewise, T_R cells have been shown to suppress IBD-associated colorectal cancer (CRC) in mice (3) by suppressing tumor necrosis factor (TNF)- α and other inflammatory growth factors required to sustain cancer (4). We recently discovered, during investigations of CRC in Apc^{Min/+} mice, that adoptive transfer of pro-inflammatory T_E cells rapidly promotes mammary tumors in the Apc^{Min/+} mouse model. During subsequent investigations we also discovered that infection a widespread murine pro-inflammatory intestinal bacteria, *Helicobacter hepaticus*, is sufficient to trigger breast carcinogenesis in Apc^{Min/+} mice. These novel inflammation-driven mouse models were used here to determine whether anti-inflammatory T_R cells may ultimately provide an innovative and highly effective approach for preventing and treating breast cancer in women.

BODY:

In order to assess potential of T_R cells to prevent or treat inflammation-associated breast cancer, we performed standard adoptive immune cell transfer techniques (2, 3) using female Apc^{Min/+} mice genetically predisposed to mammary tumorigenesis (5, 6). For the first set of experiments, 8-week-old female C57BL/6J Apc^{Min/+} mice received a single dose of 3X10⁵ highly purified syngeneic pro-inflammatory CD4⁺CD45RB^{hi}CD25⁻ T_E cells by intraperitoneal injection (*ip*), instead of a carcinogen, to induce mammary cancer. This novel model for tumor promotion is attractive because it mimics inflammatory aspects of human disease that are not frequently seen in murine models of breast cancer (6). Half of the mice received a co-transfer of 3X10⁵ highly purified syngeneic anti-inflammatory CD4⁺CD45RB^{lo}CD25⁺ T_R cells *ip*, to assess ability of T_R cells to suppress tumorigenesis. Proof of principle was assessed by comparing mammary tumor frequency, multiplicity and size in T_R cell-treated versus control T_E recipient mice upon euthanasia at age 12-16 weeks. Details of these experiments are provided in an attached manuscript by Rao, *et al* 2006. We demonstrated that adoptive transfer of T_R cells inhibits T_E cell-induced mammary tumorigenesis in Apc^{Min/+} mice.

During studies of bowel homeostasis in mice, we discovered that infection with pro-inflammatory intestinal bacteria *H. hepaticus* also rapidly triggers development of mammary cancer in female Apc^{Min/+} mice. Thus, in a second series of experiments, 8-week-old female C57BL/6J Apc^{Min/+} mice received three doses of *H. hepaticus* bacteria by gastric gavage, instead of a carcinogen, to induce mammary tumors. Half of the mice underwent transfer of 3X10⁵ highly purified syngeneic anti-inflammatory CD4⁺CD45RB^{lo}CD25⁺ T_R cells *ip*, to assess ability to suppress microbially-induced tumorigenesis. Experimental details are provided in a second attached manuscript by Rao, *et al* 2006. We demonstrated that T_R cells inhibit mammary tumors induced by pro-inflammatory intestinal bacteria, and that anti-cancer potency of T_R cells is enhanced with prior microbial challenges.

KEY RESEARCH ACCOMPLISHMENTS:

- demonstration that IL10-dependent functions of CD4⁺ regulatory (T_R) lymphocytes inhibit and suppress inflammation-associated mammary tumorigenesis in mice, and
- discovery that pro-inflammatory intestinal bacteria and balance of intestinal TNF α -mediated inflammatory events modulates mammary cancer progression in mice, and
- discovery that prior challenges with intestinal bacteria enhance anti-cancer capabilities of T_R cells, unveiling novel therapeutic targets involving bowel health and breast cancer progression in women.

REPORTABLE OUTCOMES:

- Two scientific manuscripts published and attached below.
- Publication of an invited review article on this topic in Cancer Research (Feb 1 2007).
- Poster presentation at 2006 AACR meeting.
- Preliminary data for R01 and other grant applications.

CONCLUSIONS:

Anti-inflammatory T_R cells are integral in down-modulating destructive inflammatory responses throughout the body. Targeting deleterious host inflammatory responses may be more effective and less toxic than traditional chemotherapeutic approaches to neoplasia. Research described here revealed novel associations between intestinal bacteria, bowel homeostasis and risk of carcinoma in anatomically-distant extra-intestinal sites such as breast. Ability of T_R cells to down-regulate systemic TNF α -mediated carcinogenic inflammatory events appears to be essential in preventing cancer in this setting. Insights into factors that modulate potency of T_R cells to suppress these carcinogenic inflammatory processes may yield novel therapeutic targets to prevent and ultimately abolish malignancies of the breast in humans.

REFERENCES:

1. Ness RB and Cauley JA. Antibiotics and breast cancer--what's the meaning of this? JAMA 2004; 291: 880-1.
2. Powrie F and Maloy KJ. Immunology: Regulating the regulators. Science 2003; 299: 1030-1.
3. Erdman SE, Poutahidis T, Tomczak M, Rogers AB, Cormier K, Plank B, *et al.* CD4⁺CD25⁺ regulatory T lymphocytes inhibit microbially induced colon cancer in Rag2-deficient mice. Am J Pathol 2003; 162: 691-702.
4. Erdman SE, Sohn JJ, Rao VP, Nambiar PR, Ge Z, Fox JG, Schauer DB. CD4⁺CD25⁺ regulatory lymphocytes induce regression of intestinal tumors in *Apc*^{Min/+} mice. Cancer Res 2005; 65: 3998-4004.
5. Moser AR, Mattes EM, Dove WF, Lindstrom MJ, Haag JD, and 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.
6. Cardiff RD, Anver MR, Gusterson BA, Hennighausen L, Jensen RA, Merino MJ, *et al.* The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting. Oncogene 2000; 19: 968-88.

APPENDICES:

Two manuscripts (resulting from this funding) are attached

Rao VP, Poutahidis T, Ge Z, Nambiar PR, Horwitz BH, Fox JG, **Erdman SE**. Pro-inflammatory CD4⁺CD45RB^{hi} lymphocytes promote mammary and intestinal carcinogenesis in *Apc*^{Min/+} mice. Cancer Res 66 :57-61, 2006.

Rao VP, Poutahidis T, Ge Z, Nambiar PR, Boussahmain C, Horwitz BH, FoxJG, **Erdman SE**. Innate immune inflammatory response against enteric bacterial pathogen *Helicobacter hepaticus* triggers mammary adenocarcinoma in mice. Cancer Res 2006; 66(15): 7395-400.

Proinflammatory CD4⁺CD45RB^{hi} Lymphocytes Promote Mammary and Intestinal Carcinogenesis in *Apc*^{Min/+} Mice

Varada P. Rao,¹ Theofilos Poutahidis,^{1,3} Zhongming Ge,¹ Prashant R. Nambiar,¹ Bruce H. Horwitz,² James G. Fox,¹ and Susan E. Erdman¹

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

Abstract

Cancers of breast and bowel are increasingly frequent in humans. Chronic inflammation is known to be a risk factor for these malignancies, yet cellular and molecular mechanisms linking inflammation and carcinogenesis remain poorly understood. Here, we apply a widely used T-cell transfer paradigm, involving adoptive transfer of proinflammatory CD4⁺CD45RB^{hi} (T_E) cells to induce inflammatory bowel disease (IBD) in mice, to investigate roles of inflammation on carcinogenesis in the *Apc*^{Min/+} mouse model of intestinal polyposis. We find that transfer of T_E cells significantly increases adenoma multiplicity and features of malignancy in recipient *Apc*^{Min/+} mice. Surprisingly, we find that female *Apc*^{Min/+} recipients of T_E cells also rapidly develop mammary tumors. Both intestinal polyposis and mammary adenocarcinoma are abolished by cotransfer of anti-inflammatory CD4⁺CD45RB^{lo} regulatory lymphocytes or by neutralization of key proinflammatory cytokine tumor necrosis factor- α . Lastly, down-regulation of cyclooxygenase-2 and *c-Myc* expression is observed coincident with tumor regression. These findings define a novel mouse model of inflammation-driven mammary carcinoma and suggest that epithelial carcinogenesis can be mitigated by anti-inflammatory cells and cytokines known to regulate IBD in humans and mice. (Cancer Res 2006; 66(1): 57-61)

Introduction

Colorectal cancer is the leading cause of cancer-related mortality worldwide (1). Breast cancer is the most common nonintegumentary malignancy in women (2). Observations that risk of colorectal cancer (3) and breast cancer (4) are reduced in patients taking aspirin and other nonsteroidal anti-inflammatory drugs indicate that inflammation contributes to intestinal and breast carcinogenesis in humans. However, experimental models of inflammation-driven breast cancer are lacking. *Apc*^{Min/+} mice are genetically prone to development of epithelial tumors in intestine and breast (5, 6). Prior studies in our lab (7) as well as from others (8) have raised important questions about roles of inflammation in epithelial tumor development and progression. Yet, no study to date has examined the effect of proinflammatory lymphocytes on polyposis in *Apc*^{Min/+} mice. We hypothesize that addition of proinflammatory cells will increase multiplicity

of intestinal polyps. Hence, we follow the cell transfer paradigm of inflammatory bowel disease (IBD) using colitogenic proinflammatory CD4⁺CD45RB^{hi} (T_E) and colitis-protective anti-inflammatory CD4⁺CD45RB^{lo}CD25⁺ (T_R) lymphocyte subsets (9, 10) to test this hypothesis and determine their effect on epithelial carcinogenesis in *Apc*^{Min/+} mice.

Materials and Methods

***Apc*^{Min/+} C57BL/6 mice.** 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 the Massachusetts Institute of Technology. *Apc*^{Min/+} mice on a C57BL/6J background were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in house as (heterozygous $\times$ wild type) crosses to provide *Apc*^{Min/+} mice and wild-type littermates for experimental recipients and donors.

Experimental design. A total of 102 *Apc*^{Min/+} 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.

T_E-cell transfer. Sixteen *Apc*^{Min/+} mice ages 3.5 to 4 months were dosed with 3×10^5 T_E cells collected from wild-type littermates. For these studies, 10 female and six male recipient mice were used. Mice were euthanized 3 to 4 weeks later and then compared with 14 untreated age-matched *Apc*^{Min/+} controls. This experiment was conducted as three separate trials using five or six mice in each trial.

T_R-cell cotransfer. Fourteen *Apc*^{Min/+} mice ages 3.5 to 4 months were dosed with both 3×10^5 T_E cells and 3×10^5 T_R cells. For these studies, eight recipient mice were females and six were males. Mice were then euthanized 3 to 4 weeks later and compared with 16 recipients of T_E cells alone as described above. This experiment was conducted as three separate trials using four or five mice in each trial. A second regulatory cell transfer experiment used CD4⁺CD45RB^{lo}CD25⁺ regulatory cells collected from wild-type littermates, instead of CD25⁺ T_R cells, in eight 3.5- to 4-month-old *Apc*^{Min/+} recipients of T_E cells.

Tumor necrosis factor- α neutralization. Fourteen *Apc*^{Min/+} recipients of T_E cells at age 3.5 to 4 months were treated 3 weeks later with anti-tumor necrosis factor- α (anti-TNF- α) antibody (clone XT-3) at 200 μ g per mouse thrice weekly for 1 week. For these studies, eight recipient mice were females and six were males. Mice were then euthanized (at 4 weeks after the original T_E-cell transfer) and compared with matched *Apc*^{Min/+} recipients of T_E cells that received sham antibody alone ($n = 8$). This experiment was conducted as two separate trials using seven mice in each trial.

A second experiment used 14 *Apc*^{Min/+} mice of ages 4.5 to 6 months that were treated with anti-TNF α antibody (clone XT-3) at 200 μ g per mouse thrice weekly for 1 week and then euthanized immediately afterwards. This experiment was conducted as two separate trials using seven mice in each trial. Treated mice were compared with age-matched *Apc*^{Min/+} mice that received sham antibody alone ($n = 8$).

Adoptive transfer of T cells in *Apc*^{Min/+} mice. CD4⁺ lymphocytes isolated from wild-type littermates (C57BL/6J) using magnetic beads (Dyna Beads, DYNAL, Oslo, Norway) are sorted by hi-speed flow

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-05-3445

cytometry (MoFlow2) to obtain purified populations of CD4⁺CD45RB^{hi} or CD4⁺CD45RB^{lo}CD25⁺ or CD4⁺CD45RB^{lo}CD25⁻ lymphocytes (~96% pure) as previously described (7). Anesthetized recipient mice are injected i.v. in the retro-orbital sinus with 3 to 4 × 10⁵ T cells as previously described (7).

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 and in the colon as distance from ceco-colic junction (7).

Histologic evaluation. Formalin-fixed tissues were embedded in paraffin, cut at 5 μm, and stained with H&E. Lesions were evaluated by two veterinary pathologists blinded to sample identity. Intramucosal carcinoma, carcinoma *in situ*, and neoplastic epithelial invasion were assessed based on histopathologic criteria as described elsewhere (11). Quantitative assessment of inflammatory cells was done in standardized areas at the base of adenomas in H&E-stained slides. Multiple representative ×40 high-power fields corresponding to the above mentioned selection criteria were captured using a Nikon eclipse 50i microscope and a Nikon DS-5 M-L1 digital camera. Ten images were randomly selected per treatment group. The different inflammatory cells found in each image were counted using the cell count plug-in of the ImageJ image processing and analysis program (NIH, Bethesda, MD). Inflammation counts were recorded as the number of granulocytes, lymphocytes, and plasma cells counted per image.

Quantitation of gene expression. Five micrograms of total RNA were prepared (using Trizol, Invitrogen, Carlsbad, CA) from 0.5-cm sections of ileal mucosa harvested at a standardized location 1.0 cm from the base of the cecum to generate cDNA using the High-Capacity Archive kit from Applied Biosystems (Foster City, CA). Levels of *c-Myc* and cyclooxygenase-2 (Cox-2) transcripts were quantified in the ABI Prism Sequence Detection system 7700 (A/B Applied Biosystems) as described in detail elsewhere (7).

Statistical analyses. The total number of intestinal tumors in mice from different treatment groups and controls was analyzed by unpaired Student's *t* test. The prevalence of carcinoma *in situ* and tumor invasion between groups was compared by the Kruskal-Wallis one-way ANOVA and Dunn's post-test. Direct comparisons were made by the Mann-Whitney *U* test. Graphpad Prism 4.0 software was used for all statistical analysis. Statistical significance was set at *P* < 0.05.

Results and Discussion

T_E cells promote intestinal polyp development and associated malignancy. To study roles for inflammatory cells and cytokines in *Apc*^{Min/+} mice, we followed an adoptive transfer paradigm widely used to induce IBD in mice (10, 12). Proinflammatory T_E cells isolated from *wild-type* littermates were adoptively transferred into naive *Apc*^{Min/+} mice. We find that *Apc*^{Min/+} mice that receive T_E cells (*n* = 16) show significantly more frequent (*P* < 0.001) intestinal tumors (Fig. 1A) and inflammatory cell infiltrates (Table 1) than untreated age-matched *Apc*^{Min/+} controls (*n* = 14), when examined 3 to 4 weeks after adoptive transfer. There was a significant (*P* < 0.05) increase in the number of lymphocytes (Table 1) in polyps of T_E-cell recipient mice matching findings in humans with colorectal cancer (13). Furthermore, polypoid adenomas in recipients of T_E cells show increased frequency of carcinoma *in situ* and neoplastic epithelial invasion when compared with matched untreated *Apc*^{Min/+} mice (Table 1). The invasive lesions were characterized by the infiltration of adenocarcinoma glands within the submucosa and muscle layers (Fig. 2A). In general, adenomas from T_E-cell recipients had more frequent dysplastic glands (*P* < 0.001) showing cellular atypia and pleomorphism (Fig. 2C). These data indicate that proinflammatory T_E cells not only increase multiplicity of intestinal adenomas but also contribute to a malignant phenotype in these mice.

T_E cells promote mammary adenocarcinoma in mice. Intriguingly, 70% of female *Apc*^{Min/+} mice (7 of 10 animals) that received T_E cells at age 4 months rapidly developed palpably enlarged mammary glands (Fig. 1B) with histologic features consistent with adenosquamous carcinoma (Fig. 2B) as described previously in *Apc*^{Min/+} mice (6). In contrast, none of the age-matched untreated *Apc*^{Min/+} females (0 of 8 females) had evidence of mammary tumors. Moser et al. (6) also rarely observed mammary tumors in female *Apc*^{Min/+} mice on B6 background when compared with *Apc*^{Min/+} mice on other strain backgrounds. The highly infiltrative neoplastic mammary glands in T_E-cell recipient mice show nonkeratinized and keratinized epithelia arranged in variably sized nests and cords with extensive squamous metaplasia (Fig. 2D). Overall, mammary glands have dense inflammatory cell infiltrates composed primarily of neutrophils and lymphocytes. Macrophages, plasma cells, and mast cells were also readily observed. These findings suggest that addition of proinflammatory T_E cells accelerates development of

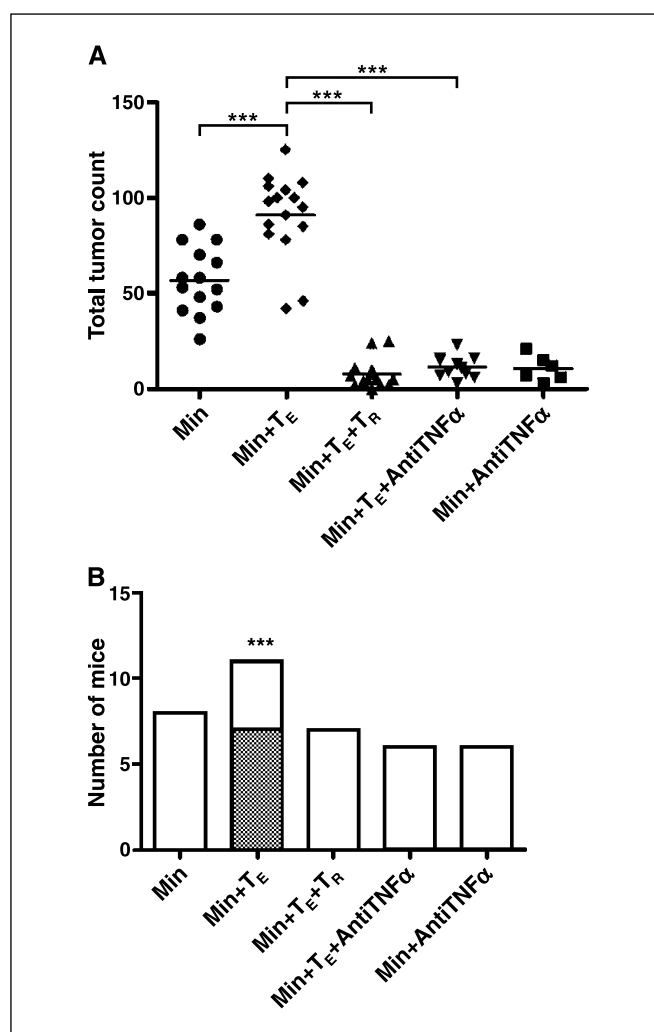


Figure 1. Adoptive transfer of T_E cells increases multiplicity of intestinal polyps (A) and mammary carcinoma incidence (B) in *Apc*^{Min/+} mice. A, points, mean of total intestinal polyps counted in each mouse; bars, SE. Combined from individual experiments with similar data. Tumor counts between groups are significant (*P* < 0.001). B, incidence of mammary tumors within the treatment group (shaded portion). Length of column, number of mice used in each group. ***, *P* < 0.001. ■, with tumor; □, tumor free.

Table 1. Quantitative assessment of intestinal tumor pathology and composition of cellular infiltrate in *Apc*^{Min/+} mice

Treatment group	Tumors (mean ± SE)	Percent tumors with		Intratumor infiltrate (mean ± SE)		
		Carcinoma <i>in situ</i>	Neoplastic invasion	Plasma cells	Lymphocytes	Granulocytes
Min	56.7 ± 4.6 ^{a, b, c}	38 (26/75) ^a	2 (1/75) ^a	16.7 ± 1.9 ^d	7.7 ± 0.9 ^{d, g}	3.1 ± 1.0
Min + T _E	90.9 ± 5.5 ^{a, b, c}	70 (52/74) ^{a, b, c, d}	7 (6/74) ^g	33.1 ± 2.7 ^{a, d, g, h}	14.8 ± 3.2 ^{e, g, h}	6.8 ± 1.5
Min + T _E +T _R	7.8 ± 2.0 ^a	25 (7/30) ^b	0 (0/30) ^{a, d, e, g}	23.4 ± 2.2 ^{g, j}	14.5 ± 1.4 ^{d, f, i}	5.7 ± 2.4
Min + T _E + anti-TNF-α	11.5 ± 7 ^b	42 (20/50) ^d	4 (2/50) ^d	22.0 ± 3.6 ^{h, i}	7.0 ± 1.0 ^{h, i}	5.6 ± 1.6
Min + anti-TNF-α	10.7 ± 2.7 ^c	19 (5/25) ^c	0 (0/25)	10.7 ± 4.0 ^{a, i, j}	5.9 ± 0.6 ^{e, f}	7.8 ± 1.9

NOTE: Data within a column that share a superscript letter are significantly different from other groups in that column. ^{a, b, or c}, $P < 0.001$; ^{d, e, or f}, $P < 0.01$; ^{g, h, i, or j}, $P < 0.05$. Parenthesis include fields with lesions/total fields.

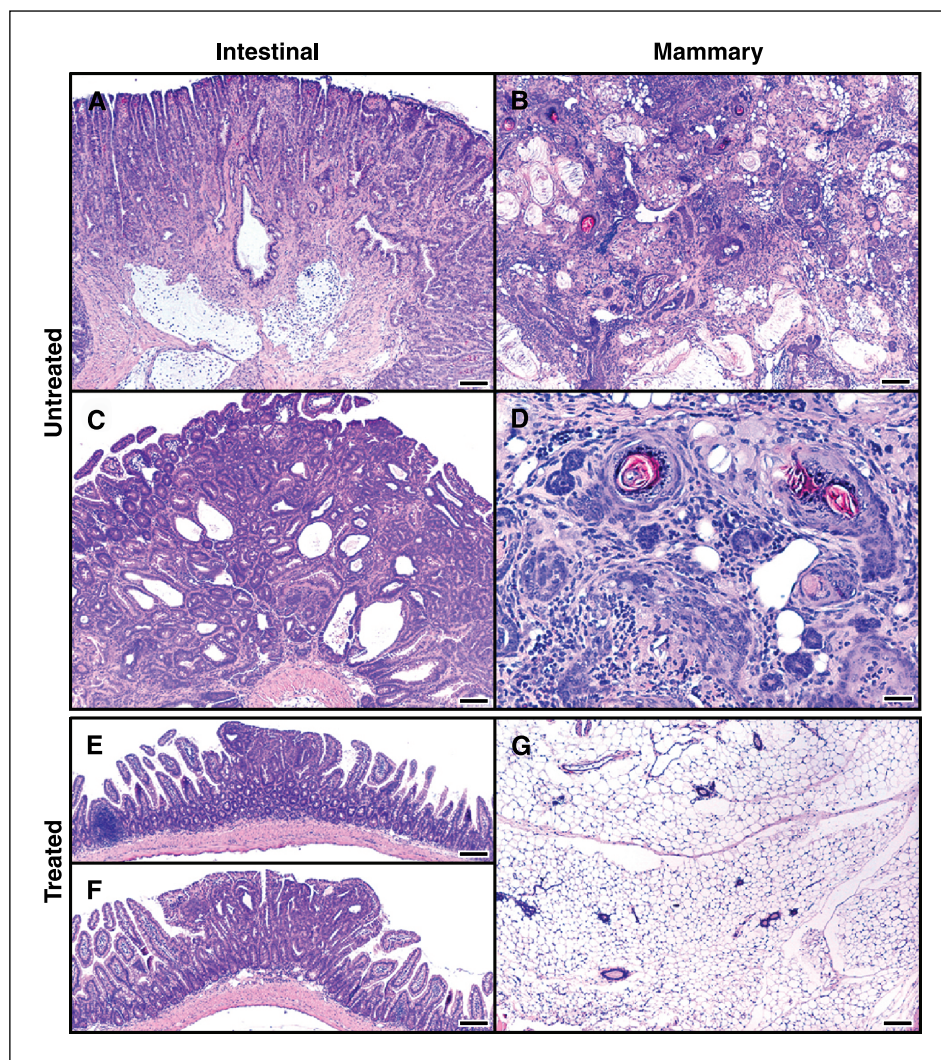


Figure 2. Left, representative histopathology of small intestine in left column (A, C, E, and F); right, representative histopathology of mammary gland (B, D, and G) from T_E-cell recipient *Apc*^{Min/+} mice. *Apc*^{Min/+} mice that received purified T_E cells but no further treatment showed high frequency of invasive adenocarcinoma in the intestine and mammary gland. A, ileum. Well-differentiated glands invading through the muscularis propria. The advancing edge of the neoplastic lesion shows typical mucinous adenocarcinoma morphology. B, mammary gland. Highly infiltrative adenosquamous carcinoma contains glandular structures with or without squamous differentiation. Note the dense inflammatory cell infiltrate and desmoplastic reaction. Higher magnification in (D) clearly illustrates the typical morphology of adenosquamous carcinoma with admixed nonkeratinized and keratinized (keratin pearls) neoplastic glands. Recipients of T_E cells also showed increased frequency of adenomatous polyps in ileum (C). Adenomatous polyps were enlarged and had increased frequency of abnormal glandular architecture with epithelial dysplasia and carcinoma *in situ*. Min recipients of cotransfer of T_E and T_R cells (E and G) or anti-TNF-α antibody (F) showed regression of intestinal tumors in ileum (E and F). Remaining minute polyps showing minimal evidence of remaining dysplasia on surface epithelium. Normal mammary gland tissue and mammary fat (G) in recipients of T_R cells. H&E.

cancer in breast tissue in these genetically susceptible mice and thus reveal a model of inflammation-driven breast cancer in humans. Prior studies in mice with IBD (9, 10, 14) led us to hypothesize that cotransfer of anti-inflammatory T_R cells will inhibit inflammatory factors that may drive mammary and intestinal carcinoma in *Apc*^{Min/+} mice.

T_R cells inhibit T_E cell-induced epithelial carcinogenesis. To determine whether T_E cell-mediated carcinogenic events of gut and breast can be inhibited by anti-inflammatory CD4⁺CD45RB^{lo}CD25⁺ (T_R) regulatory cells, *Apc*^{Min/+} mice that received T_E cells simultaneously underwent adoptive transfer with T_R cells (cotransfer group). We find that cotransfer recipients (*n* = 14) show significantly (*P* < 0.001) fewer intestinal tumors (Fig. 1A) and do not develop mammary adenocarcinoma (Fig. 1B and Fig. 2G). Tissues from these mice have decreased frequency of epithelial dysplasia (Table 1) and are similar in appearance to those of wild-type C57BL/6 mice (Fig. 2E). Interestingly, however, sections of intestines from the cotransfer recipients did not differ significantly when scored for number of inflammatory cells (Table 1) from those of T_E-cell recipients despite complete disappearance of invasive adenocarcinoma and restoration to normal epithelial homeostasis (Fig. 2E). These data match earlier findings showing that T_R cells suppress tumors in *Apc*^{Min/+} mice (7) and also inhibit IBD in cell transfer models using immunodeficient mice (10, 11, 15).

In addition to the CD25⁺ population, Kullberg et al. have shown that CD25⁺ cells of CD4⁺CD45RB^{lo} phenotype also act as potent inhibitors of IBD in mice (16). To test antineoplastic efficacy of CD25⁺ cells in this setting, we transferred CD25⁺ cells from wild-type littermates into *Apc*^{Min/+} mice. We find that *Apc*^{Min/+} recipients of CD4⁺CD45RB^{lo}CD25⁺ cells (*n* = 8) also show significantly (*P* < 0.001) fewer intestinal adenomas (mean = 6.2 ± 2.3) when compared with untreated mice (mean = 56.7 ± 4.6). Furthermore, female *Apc*^{Min/+} cotransfer recipients of CD4⁺CD45RB^{lo}CD25⁺ cells (*n* = 6) also had complete lack of mammary adenocarcinoma. These data show that CD4⁺CD45RB^{lo} cells, in general, have antineoplastic functions in mice with enteric flora matched with their donors. Studies are in progress to investigate whether specific enteric antigens modulate anti-inflammatory and antineoplastic potency of CD4⁺ regulatory cells in this model.

Neutralization of proinflammatory cytokine TNF-α inhibits intestinal and mammary carcinogenesis. TNF-α is a potent effector cytokine in the pathogenesis of IBD in humans (17) and in mice (18, 19) and has been associated with poor prognosis in several human cancers, including mammary carcinoma (20). To determine whether TNF-α is critical for intestinal and mammary carcinoma seen in our model, we treated *Apc*^{Min/+} mice that received T_E cells (*n* = 14) with anti-TNF-α neutralizing antibody (21). We find that mice that receive 200 μg/mouse of anti-TNF-α antibody thrice weekly for 1 week had significantly (*P* < 0.001) fewer intestinal adenomas when compared with *Apc*^{Min/+} mice that receive sham antibody alone (*n* = 8; Fig. 1A). Intestinal tumors had less frequent epithelial dysplasia and neoplastic invasion than tumors of untreated *Apc*^{Min/+} counterparts (Table 1; Fig. 2F). Furthermore, mammary gland neoplasia was not observed in any of T_E-cell recipient female mice (*n* = 8) following 1 week of treatment with anti-TNF-α antibody (Fig. 1B). These findings

indicate that proinflammatory cytokine TNF-α is required to sustain tumors in breast and bowel, revealing a key cytokine mediator of carcinogenesis in animals predisposed to epithelial tumors. Preliminary studies in *Apc*^{Min/+} mice on a C57BL/6 Rag^{-/-} background reveal that TNF-α from cells of innate immunity is sufficient to trigger both intestinal and mammary tumors in this model.⁴ Tumor regression and restoration of epithelial homeostasis at two anatomically distinct sites (i.e., intestines and mammary gland) after treatment with anti-inflammatory T_R cells or after anti-TNF-α antibody suggest that these less toxic approaches should be considered for future cancer treatment in humans.

Anti-inflammatory treatment regimens down-regulate c-Myc expression. Up-regulation of oncogene *c-Myc* has been well documented in cancers of the breast (22) and bowel (23) in humans and in *Apc*^{Min/+} mice (24). To determine whether anti-inflammatory treatments modulate *c-Myc* levels, we measured oncogene expression levels using quantitative reverse transcription-PCR (Taqman) in intestinal mucosa samples from mice undergoing treatments as described above. We find that *c-Myc* levels were decreased by 10- to 20-fold in intestinal mucosal samples of mice from T_R and anti-TNF-α treatment groups (Fig. 3). Likewise, we observed a significant decrease in Cox-2 expression levels in these samples correlating with down-regulation of inflammation (Fig. 3), matching earlier findings in *Apc*^{Min/+} mice (7) and humans with intestinal polyposis (3). The disappearance of carcinoma and associated malignant lesions as well as restoration of epithelial

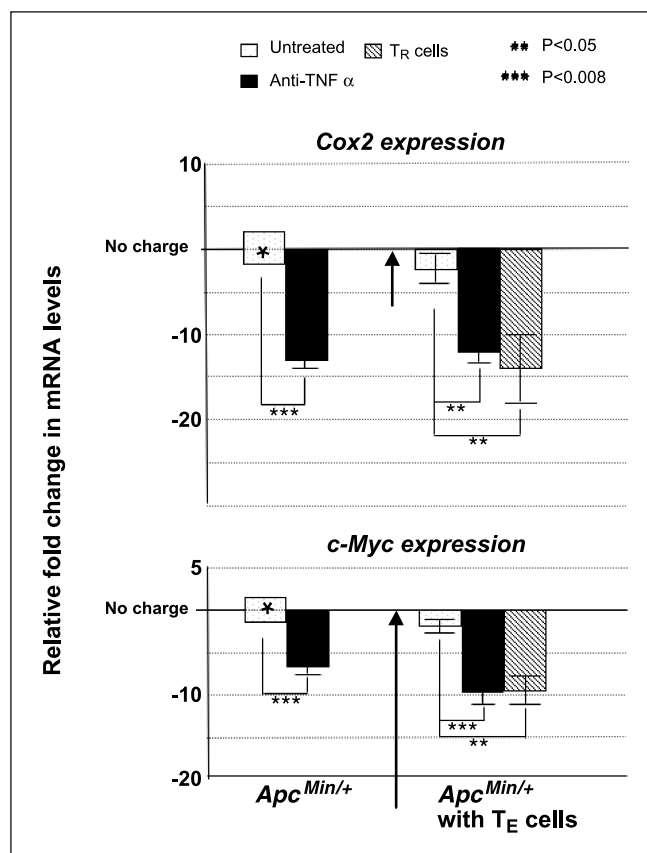


Figure 3. Relative levels of *Cox-2* and *c-Myc* mRNA. In each sample, *Cox-2* or *C-myc* mRNA was normalized to that of the "housekeeping" gene *Gapdh*. Columns, mean fold change of *Cox-2* or *c-Myc* mRNA levels in reference to untreated *Apc*^{Min/+} group; bars, SE. ref, no change (open column with *).

⁴ Unpublished data.

homeostasis brought about by anti-inflammatory T_R cells or anti-TNF- α antibody coincide with reversal to basal expression levels of *c-Myc*, suggesting its potential role in inflammation-driven carcinogenesis. Thus, carcinogenesis in *Apc*^{Min/+} mice seems to be reversibly linked with *c-Myc* expression, which is regulated by the balance of proinflammatory and anti-inflammatory mediators.

That chronic inflammation predisposes humans and animals to cancer is becoming increasingly clear (25). However, the interplay of events stemming from chronic inflammation leading to malignancy still remains poorly understood. Here, in *Apc*^{Min/+} mice, we show that development of breast cancer and intestinal carcinoma can be triggered by adoptive transfer of proinflammatory T_E lymphocytes. Additional studies are required to examine mechanisms by which proinflammatory CD45RB^{hi} cells promote mammary and intestinal carcinoma in these mice. As excessive production of inflammatory mediators, including TNF- α , during chronic inflammation has been implicated in oncogenesis (26), it may be that similar mechanisms involving COX-2 and *c-MYC* are relevant in T_E-cell recipient *Apc*^{Min/+} mice. Tumor regression and restoration of epithelial homeostasis in intestines and mammary

gland seen after treatment with T_R cells or anti-TNF- α antibody in this model support the clinical observations showing reduction in the risk of colorectal cancer (3) and breast cancer (4) in patients receiving anti-inflammatory drugs. These findings allude to broader applicability of these therapies in cancers of prostate (27) and other sites responsive to anti-inflammatory therapies in humans (28, 29). Ultimately, efforts to harness the potency of cells and cytokines with anti-inflammatory function will help develop less toxic cancer immunotherapies in humans.

Acknowledgments

Received 9/26/2005; revised 10/28/2005; accepted 11/16/2005.

Grant support: Department of Defense contract W81XWH-05-01-0460 (S.E. Erdman) and NIH grants R01CA108854 (S.E. Erdman and J.G. Fox), R01CA67529 (J.G. Fox), R01 AI50952 (J.G. Fox), T32RR07036 (J.G. Fox), R01 DK52413 (J.G. Fox), P01CA26731 (J.G. Fox and S.E. Erdman); and P30ES02109 (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 Chakib Boussahmain, Kathy Cormier, and Erinn Stefanich for histology and immunohistochemistry; Glenn A. Paradis and Michele Perry for their expert assistance with cell sorting; and Brian D. Morrison, Chung-Wei Lee, and Elaine Robbins for assistance with figures in this article.

References

- Pitari GM, Zingman LV, Hodgson DM, et al. Bacterial enterotoxins are associated with resistance to colon cancer. *Proc Natl Acad Sci U S A* 2003;100:2695–9.
- American Cancer Society. Estimated new cancer cases and deaths by sex for all sites. In: US cancer facts figures 2004. Atlanta (GA): American Cancer Society. p. 4.
- Gupta RA, DuBois RN, Wallace MC. New avenues for the prevention of colorectal cancer: targeting cyclooxygenase-2 activity. *Best Pract Res Clin Gastroenterol* 2002;16:945–56.
- Arun B, Goss P. The role of COX-2 inhibition in breast cancer treatment and prevention. *Semin Oncol* 2004;31:22–9.
- Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 1990;247:322–4.
- Moser AR, Mattes EM, Dove WF, et al. *Apc*^{Min}, 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.
- Erdman SE, Sohn JJ, Rao VP, et al. CD4⁺CD25⁺ regulatory lymphocytes induce regression of intestinal tumors in *Apc*^{Min/+} mice. *Cancer Res* 2005;65:3998–4004.
- Kettunen HL, Kettunen AS, Rautonen NE. Intestinal immune responses in wild-type and *Apc*^{Min/+} mouse, a model for colon cancer. *Cancer Res* 2003;63:5136–42.
- Powrie F, Correa-Oliveira R, Mauze S, et al. Regulatory interactions between CD45RB^{high} and CD45RB^{low} CD4⁺ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J Exp Med* 1994;179:589–600.
- Maloy K, Salaun L, Cahill R, et al. CD4⁺CD25⁺ T_R cells suppress innate immune pathology through cytokine-dependent mechanisms. *J Exp Med* 2003;197:111–9.
- 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.
- Powrie F, Leach MW, Mauze S, et al. Inhibition of Th1 responses prevents inflammatory bowel disease in SCID mice reconstituted with CD45RB^{hi} CD4⁺ T cells. *Immunity* 1994;1:553–62.
- Svennevig JL, Lunde OC, Holter J. *In situ* analysis of the inflammatory cell infiltrates in colon carcinomas and in the normal colon wall. *Acta Pathol Microbiol Immunol Scand [A]* 1982;90:131–7.
- Asseman C, Fowler S, Powrie F. Control of experimental inflammatory bowel disease by regulatory T cells. *Am J Respir Crit Care Med* 2000;162:S185–9.
- Singh B, Read S, Asseman C, et al. Control of intestinal inflammation by regulatory T cells. *Immunol Rev* 2001;182:190–200.
- 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.
- Wang J, Fu YX. Tumor necrosis factor family members and inflammatory bowel disease. *Immunol Rev* 2005;204:144–55.
- Hibi T, Ogata H, Sakuraba A. Animal models of inflammatory bowel disease. *J Gastroenterol* 2002;37:409–17.
- Mueller C. Tumour necrosis factor in mouse models of chronic intestinal inflammation. *Immunology* 2002;105:1–8.
- Robinson SC, Coussens LM. Soluble mediators of inflammation during tumor development. *Adv Cancer Res* 2005;93:159–87.
- Menezes-de-Lima-Junior O, Werneck-Barroso E, Cordeiro RS, Henriques MG. Effects of inhibitors of inflammatory mediators and cytokines on eosinophil and neutrophil accumulation induced by *Mycobacterium bovis* bacillus Calmette-Guerin in mouse pleurisy. *J Leukoc Biol* 1997;62:778–85.
- Hatsell S, Rowlands T, Hiremath M, et al. Beta-catenin and Tcfs in mammary development and cancer. *J Mammary Gland Biol Neoplasia* 2003;8:145–58.
- Tanaka K, Nagaoka S, Takemura T, et al. Incidence of apoptosis increases with age in colorectal cancer. *Exp Gerontol* 2002;37:1469–79.
- Colnot S, Niwa-Kawakita M, Hamard G, et al. Colorectal cancers in a new mouse model of familial adenomatous polyposis: influence of genetic and environmental modifiers. *Lab Invest* 2004;84:1619–30.
- Balkwill F, Coussens LM. Cancer: an inflammatory link. *Nature* 2004;431:405–6.
- Wilson J, Balkwill F. The role of cytokines in the epithelial cancer microenvironment. *Semin Cancer Biol* 2002;12:113–20.
- Sabichi AL, Lippman SM. COX-2 inhibitors and other nonsteroidal anti-inflammatory drugs in genitourinary cancer. *Semin Oncol* 2004;31:36–44.
- Rao CV, Reddy BS. NSAIDs and chemoprevention. *Curr Cancer Drug Targets* 2004;4:29–42.
- Harris RE, Beebe-Donk J, Doss H, et al. Aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs in cancer prevention: a critical review of non-selective COX-2 blockade (review). *Oncol Rep* 2005;13:559–83.

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

Varada P. Rao,¹ Theofilos Poutahidis,^{1,3} Zhongming Ge,¹ Prashant R. Nambiar,¹ Chakib Boussahmain,¹ Yan Yan Wang,² Bruce H. Horwitz,² James G. Fox,¹ and Susan E. Erdman¹

¹Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Massachusetts; ²Immunology Research Division, Department of Pathology, Brigham and Women's Hospital; Division of Emergency Medicine, Children's Hospital, Boston, Massachusetts; and ³Laboratory 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*^{Min/+} 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 (TNF α)-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⁺CD45RB^{lo}CD25⁺ regulatory (T_R) cells. Furthermore, prior exposure of donor mice to *H. hepaticus* significantly enhances antitumor potency of their T_R cells. Interestingly, these microbially experienced T_R cells suppress tumorigenesis more effectively in recipient mice irrespective of their tumor etiology. These data suggest that infections with enteric pathogens enhance T_R-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*^{Min/+}) 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_R cells (14). Despite their high predilection for intestinal tumors, unmanipulated C57BL/6 *Apc*^{Min/+} 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*^{Min/+} mice, using a widely applied adoptive T-cell transfer model (17) and recombination-activating gene 2 (*Rag2*)-deficient *Apc*^{Min/+} 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*^{Min/+} 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*^{Min/+} 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*^{Min/+} mice and 100 female *Rag2*^{-/-}*Apc*^{Min/+} 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_R cells. A total of 49 *Rag2*^{-/-}*Apc*^{Min/+} mice, ages 3.5 to 4 months, were dosed with 3×10^5 wild-type T_R cells ($N = 9$), 3×10^5 IL-10^{-/-} T_R cells ($N = 8$), or 1×10^5 wild-type T_R cells ($N = 32$ mice) 24 hours before *H. hepaticus* infection. CD4⁺CD45RB^{lo}CD25⁺ (T_R) lymphocytes were isolated from spleen and mesenteric lymph nodes and adoptively transferred as previously described (14). The donor mice for T_R 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_R-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 α neutralization. A total of 11 *Rag2*^{-/-}*Apc*^{Min/+} mice, ages 3 to 4 months, infected with *H. hepaticus*, were treated 3–4 weeks later with anti-tumor necrosis factor α (TNF α) antibody (clone XT-3; BioExpress, West Lebanon, NH) at 200 μ g per mouse thrice weekly for 1 week as previously described (15). Treated mice ($N = 11$) were compared with age-matched *Rag2*^{-/-}*Apc*^{Min/+} mice that received sham antibody alone ($N = 8$).

Treatment with IL-10-Ig fusion protein. A total of nine *H. hepaticus*-infected *Rag2*^{-/-}*Apc*^{Min/+} mice, 3 to 4 months of age, were treated with IL-10-Ig fusion protein at 5 μ 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 μ 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 α 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 χ^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*^{Min/+} mice. We find that unmanipulated *Rag2*^{-/-}*Apc*^{Min/+} 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*^{Min/+} controls housed under the same health status conditions. In addition, one *Helicobacter*-free *Rag2*^{-/-}*Apc*^{Min/+} female mouse (1 of 16; 6%) developed a palpable mammary tumor. The findings of intestinal tumors and mammary tumors in *Rag2*^{-/-}*Apc*^{Min/+} mice indicated that adaptive immunity is not required for tumorigenesis in the *Apc*^{Min/+} mouse model. Further, the development of significantly higher intestinal tumor multiplicity in *Rag2*^{-/-}*Apc*^{Min/+} mice over their *Apc*^{Min/+} 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*^{-/-}*Apc*^{Min/+} mice with *H. hepaticus* promotes intestinal and mammary tumor development. Four to six weeks after *H. hepaticus* infection, female *Rag2*^{-/-}*Apc*^{Min/+} 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⁺ 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 ($P < 0.05$) higher frequency of mammary tumors in *H. hepaticus*-infected *Rag2*^{-/-}*Apc*^{Min/+} mice (7 of 15 mice; 43%; Figs. 1B and 2D) when compared with age-matched uninfected control female *Rag2*^{-/-}*Apc*^{Min/+} mice. Adenosquamous mammary carcinoma in *Helicobacter*-infected *Rag2*^{-/-}*Apc*^{Min/+} mice showed minimal squamous metaplasia (Fig. 2D) when compared with mammary tumors in *Apc*^{Min/+} mice promoted by proinflammatory CD4⁺CD45RB^{hi} T_E lymphocytes (15). In addition, mammary tumors of *H. hepaticus*-infected *Rag2*^{-/-}*Apc*^{Min/+} mice had dense inflammatory infiltrates of F4/80⁺ macrophages (Fig. 2H), consistent with inflammation-associated breast cancer in humans (23, 24).

Similarly, we also find that *Apc*^{Min/+} 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*^{Min/+} 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*^{Min/+} females (0 of 8 animals) during the course of the study. Microscopically, the tumors in *Apc*^{Min/+} mice had more locally invasive borders and had increased squamification when compared with neoplastic mammary glands of *Rag2*^{-/-}*Apc*^{Min/+} mice (compare Fig. 2B and D). Differences in histologic appearance of tumors may reflect adaptive immune-mediated alterations in the Wnt signaling

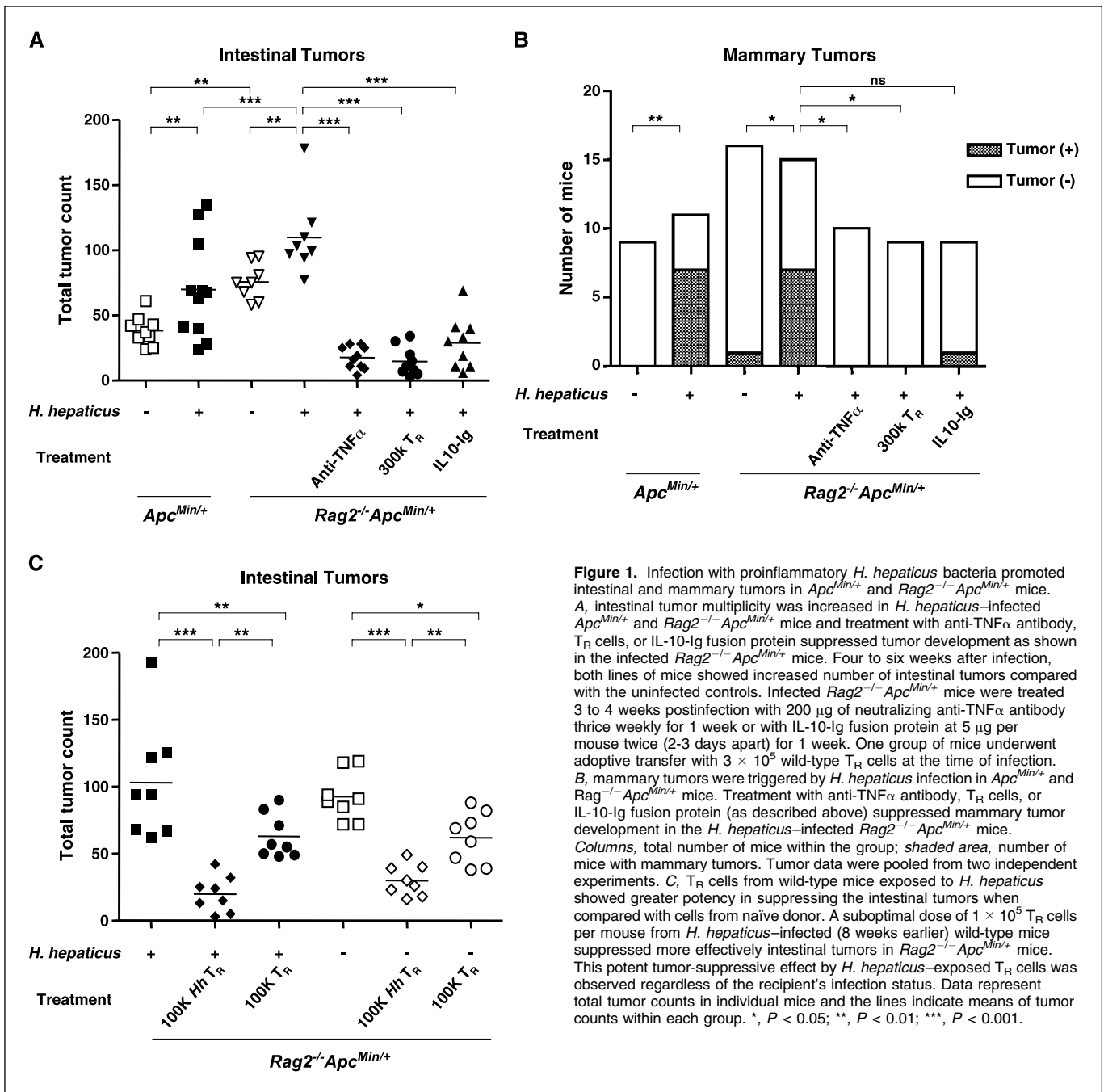


Figure 1. Infection with proinflammatory *H. hepaticus* bacteria promoted intestinal and mammary tumors in *Apc^{Min/+}* and *Rag2^{-/-}Apc^{Min/+}* mice. **A**, intestinal tumor multiplicity was increased in *H. hepaticus*-infected *Apc^{Min/+}* and *Rag2^{-/-}Apc^{Min/+}* mice and treatment with anti-TNF α antibody, T_R cells, or IL-10-Ig fusion protein suppressed tumor development as shown in the infected *Rag2^{-/-}Apc^{Min/+}* mice. Four to six weeks after infection, both lines of mice showed increased number of intestinal tumors compared with the uninfected controls. Infected *Rag2^{-/-}Apc^{Min/+}* mice were treated 3 to 4 weeks postinfection with 200 μ g of neutralizing anti-TNF α antibody thrice weekly for 1 week or with IL-10-Ig fusion protein at 5 μ g per mouse twice (2-3 days apart) for 1 week. One group of mice underwent adoptive transfer with 3×10^5 wild-type T_R cells at the time of infection. **B**, mammary tumors were triggered by *H. hepaticus* infection in *Apc^{Min/+}* and *Rag2^{-/-}Apc^{Min/+}* mice. Treatment with anti-TNF α antibody, T_R cells, or IL-10-Ig fusion protein (as described above) suppressed mammary tumor development in the *H. hepaticus*-infected *Rag2^{-/-}Apc^{Min/+}* mice. **C**, mammary tumors were triggered by *H. hepaticus* infection in *Apc^{Min/+}* and *Rag2^{-/-}Apc^{Min/+}* 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_R 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 1×10^5 T_R cells per mouse from *H. hepaticus*-infected (8 weeks earlier) wild-type mice suppressed more effectively intestinal tumors in *Rag2^{-/-}Apc^{Min/+}* mice. This potent tumor-suppressive effect by *H. hepaticus*-exposed T_R 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^{Min/+}* may be the recently described immune deficits. Thymic atrophy and lymphopenia (26) may decrease T_R cell competency in *Apc^{Min/+}* mice, thereby enabling uncontrolled activation of *Helicobacter*-primed T_E 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 α 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 α , IL-12p40, IFN γ , 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 α is a key cytokine implicated in inflammation-associated cancers (28), and treatment with anti-TNF α antibody has been shown to suppress polyp formation in *Apc^{Min/+}* mice (15), we asked whether the *H. hepaticus*-promoted tumorigenesis in *Rag2^{-/-}Apc^{Min/+}* is dependent on TNF α . Indeed, neutralization of TNF α 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^{-/-}Apc^{Min/+}* mice. Therapeutic

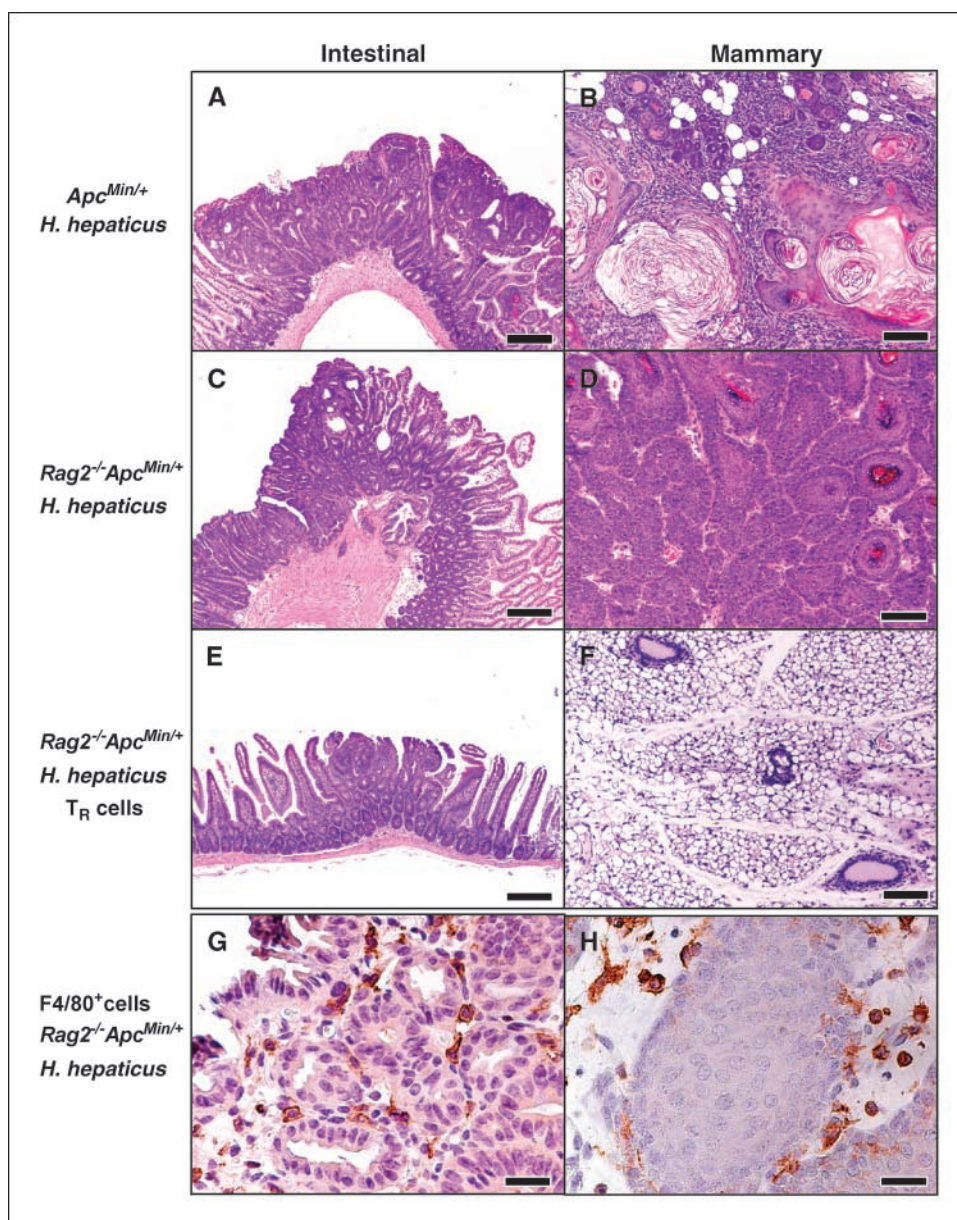


Figure 2. *H. hepaticus* infection promotes intestinal and mammary tumorigenesis in *Apc*^{Min/+} and *Rag2*^{-/-}*Apc*^{Min/+} mice. **A**, typical adenomatous polyp seen in infected *Apc*^{Min/+} mice showing high-grade dysplasia and carcinoma *in situ*. **B**, mammary gland adenocarcinoma seen in infected *Apc*^{Min/+} mice showing increased squamous component when compared with *Rag2*^{-/-}*Apc*^{Min/+} 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*^{-/-}*Apc*^{Min/+} mice. **D**, mammary adenocarcinoma with neoplastic glands showing variable degrees of squamous differentiation. In contrast to high squamous component of tumors in *Apc*^{Min/+} mice (as illustrated above in **B**), nonkeratinized neoplastic glandular structures predominate in *Rag2*^{-/-}*Apc*^{Min/+} 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 3×10^5 T_R cells or with anti-TNF α antibody (clone XT-3), 200 μ 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*^{-/-}*Apc*^{Min/+} 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 μ m (**A**, **C**, and **E**); 100 μ m (**B**, **D**, and **F**); 25 μ m (**G** and **H**).

effects of TNF α neutralizing antibody in these mice suggest that TNF α 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*^{-/-}*Apc*^{Min/+} mice need further study. Mammary tumors from *H. hepaticus*-infected *Rag2*^{-/-}*Apc*^{Min/+} mice show an 18-fold increase ($P < 0.0001$) in TNF α -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 α -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 (T_R) cells are sufficient to prevent *H. hepaticus*-triggered colitis and colon cancer (20, 27). To determine whether T_R cells can inhibit *Helicobacter*-promoted tumorigenesis in *Rag2*^{-/-}*Apc*^{Min/+}, nine female mice, 2 to 3.5 months of age, were infected with *H. hepaticus* and adoptively transferred with 3×10^5 cells per recipient of T_R 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_R cell-recipient mice. Additionally, treatment of *H. hepaticus*-infected mice with T_R cells resulted in a significant ($P < 0.001$) decrease in the levels of intestinal mucosal proinflammatory cytokines including TNF α and MIP-2 (Fig. 3). These

Table 1. Comparison of intestinal tumor frequency between *Apc*^{Min/+} and *Rag*^{-/-}*Apc*^{Min/+} mice

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

NOTE: Data sharing a superscript letter are significantly different from each other. ^a, ^b, and ^c, $P < 0.05$; ^d, $P < 0.01$; ^e, $P < 0.001$. 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_R cells are sufficient to inhibit mammary and intestinal tumorigenesis in *Rag2*^{-/-}*Apc*^{Min/+} mice. These findings match earlier data from our laboratory (14, 15) as well as by others (29) showing that T_R 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_R cells. Microbes or microbial products enhance survival, proliferation, and cytokine production by T_R cells (30). To test whether protective antitumor effects of T_R cells can be enhanced by prior microbial challenge, we first determined a suboptimal dosage of 1×10^5 CD45RB^{lo}CD25⁺ wild-type T_R cells per recipient (31). We then used this lower dose of T_R 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*^{-/-}*Apc*^{Min/+} mice at time of infection with *H. hepaticus*. We found that T_R 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^{lo}CD25⁺ T_R 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^{lo}CD25⁺ T_R cells from *H. hepaticus*-exposed mice were efficacious in protecting against *H. hepaticus*-induced T_E cell-mediated colitis. Studies in progress may reveal dynamics of immune tolerance involving both innate immunity and host T_R cell competency.

To test whether the *H. hepaticus*-induced enhancement in antitumor potency of T_R cells is limited to *H. hepaticus*-triggered tumors, the lower dosage of T_R cells from *H. hepaticus*-infected as well as uninfected donors was transferred in parallel into *Helicobacter*-free *Rag2*^{-/-}*Apc*^{Min/+} recipients. We find that *H. hepaticus*-experienced T_R 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_R cells in all recipients irrespective of their *Helicobacter* status suggests that prior proinflammatory challenges broadly enhance antitumor potency of T_R 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_R cells, it will be interesting to examine whether these agents will affect antitumor potency of T_R cells in our model.

IL-10 is critical to suppress *H. hepaticus*-promoted tumorigenesis. In murine models, CD4⁺CD25⁺ regulatory (T_R) 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_R cells endowed with microbially enhanced antitumor potency, we did adoptive transfer of T_R cells from *H. hepaticus*-infected IL-10-deficient syngeneic donors into uninfected *Rag2*^{-/-}*Apc*^{Min/+} mice. Clearly, no reduction in tumor burden was seen in *Rag2*^{-/-}*Apc*^{Min/+} recipients of IL-10-deficient T_R 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_R 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*^{-/-} mice showing no protection from colitis and colon cancer when T_R cells donors lack IL-10 (20, 22).

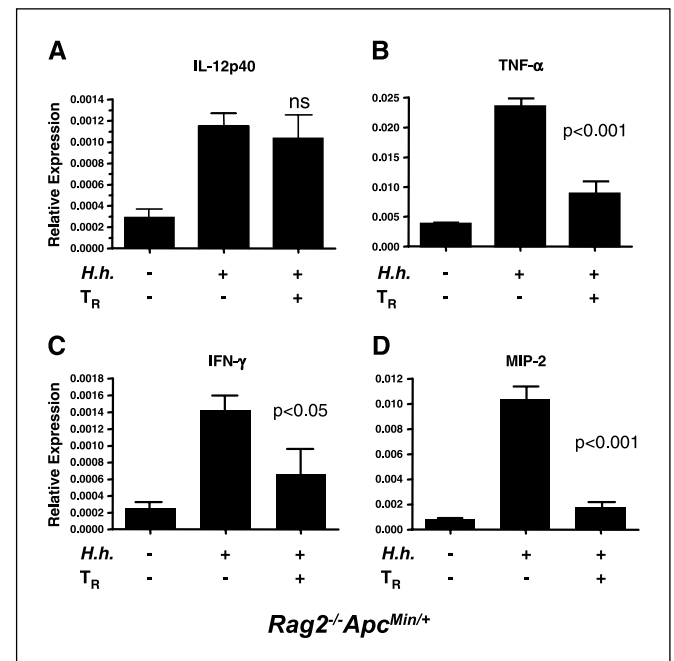


Figure 3. *H. hepaticus* infection induces up-regulation of proinflammatory cytokines in *Rag2*^{-/-}*Apc*^{Min/+} mice. The intestinal mucosal expression levels of cytokines mRNA from uninfected, *H. hepaticus*-infected, and *H. hepaticus*-infected and T_R cell (3×10^5)-treated mice are presented: A, IL-12p40; B, TNF-α; C, IFN-γ; and D, MIP-2. Infection with *H. hepaticus* significantly increased gene expression of all four cytokines analyzed. Adoptive transfer of wild-type T_R cells significantly decreased expression of IFN-γ ($P < 0.05$), TNF-α, 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*^{-/-}*Apc*^{Min/+} 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_R cells inhibit intestinal and mammary tumors in *Apc*^{Min/+} and *Rag2*^{-/-}*Apc*^{Min/+} mice

highlights the prophylactic potential of T_R cells in inflammation-associated cancers. The observation that T_R 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_R 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_R 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, McNeely 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 CD45RB^{low}CD4⁺ 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. Cytotoxic 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.

Breast Cancer: Should Gastrointestinal Bacteria Be on Our Radar Screen?

Varada P. Rao,¹ Theofilos Poutahidis,^{1,2} James G. Fox,¹ and Susan E. Erdman¹

¹Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Massachusetts and ²Laboratory of Pathology, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece

Abstract

Anti-inflammatory drugs and antibiotics alter the risk of breast cancer in women, but roles for bacteria and inflammation in breast malignancies are poorly understood. A recent study in mice suggests that intestinal bacteria can trigger mammary carcinoma. The mechanisms involved in this effect suggest that dysregulated host immune responses to enteric bacteria can influence the development of extraintestinal cancers, highlighting the opportunities for prevention and treatment aimed at promoting intestinal homeostasis. [Cancer Res 2007;67(3):847–50]

Introduction

Breast cancer is the most common malignancy, accounting for 30% of all cancers diagnosed in women each year (1). The risk of developing breast cancer is increasing at a rate of 4% annually (2). Although it is widely accepted that breast cancer risk is based on genetic predisposition, only 5% to 10% of the total breast cancer incidence has been attributed directly to heritable risk factors. Multiple factors are likely to contribute to breast cancer incidence, but all recognized risk factors together account for only 40% of the variability in incidence (3), leaving the majority of risk factors to be determined.

Recent studies on the effects of anti-inflammatory drugs suggest that inflammation is a key contributor to development of breast cancer in humans (4). Preclinical and clinical data suggest that overexpression of cyclooxygenase (COX)-2 and increased production of prostaglandins contribute to cancer development at many sites including breast (5). Clinical trials with COX-2 inhibitors and nonsteroidal anti-inflammatory drugs have shown a reduction in both incidence and invasive pathology of breast cancer. Prolonged immune activation due to pathogenic bacterial infections, such as *Helicobacter pylori*, in people clearly contributes to elevated COX-2 production and cancer in susceptible individuals (6). Although inflammatory mediators produced during chronic bacterial infections are likely to affect breast cancer incidence and pathology, no study has directly examined this issue until recently. And precisely how mediators of chronic inflammation enhance the risk of developing breast cancer in women remains to be elucidated.

Malignancies induced in mice after exposure to external agents such as bacteria provide valuable model systems to interrogate cancer pathogenesis otherwise not feasible in humans. For example, murine models have greatly facilitated our current

understanding of *H. pylori* infection-associated gastric cancer in humans (7). Explicit requirements for bacteria in carcinogenesis have been shown in highly cancer-prone mutant mice that fail to develop cancer under germ-free housing conditions. Use of immunodeficient mice has revealed pivotal roles for cells of innate immunity in colorectal cancer due to chronic bacterial infection with *Helicobacter hepaticus* (8)—an enteric pathogen of mice closely related to *H. pylori*. Anti-inflammatory CD4⁺ regulatory lymphocytes, crucial players in the induction of peripheral tolerance to self and foreign antigens, abrogate *Helicobacter*-triggered inflammatory bowel disease (IBD; refs. 8, 9) and IBD-associated colorectal cancer (8) by down-regulating host inflammatory responses triggered by intestinal bacteria. Over the years, the data accumulated from mouse models have led to proposals to augment regulatory T cells to treat inflammatory disorders such as IBD in people (10). Although inflammation clearly contributes to breast malignancy in women (4), murine models of inflammation-associated breast neoplasia have been lacking (11).

Pathogenic Gut Bacteria: Can They Trigger Breast Cancer?

During investigations of colorectal cancer, we serendipitously discovered that orogastric infection of C57BL/6 *Apc*^{Min/+} (*Min*) mice with *H. hepaticus* bacteria rapidly promotes extraintestinal tumors in mammary tissue (12). As a result of a mutation in the *Apc* gene, *Min* mice are genetically predisposed to epithelial tumors of intestine (13) and are highly responsive to COX-2 and prostaglandin inhibitor therapy (14). Although genetically predisposed to mammary carcinoma, *Min* mice on a C57BL/6 strain background rarely develop mammary tumors under standard housing conditions (15). However, within 4 to 6 weeks of infection with *H. hepaticus*, more than two thirds of 12-week-old female *Min* mice develop mammary adenocarcinoma. Studies using *Rag2*-deficient *Min* mice lacking lymphocytes have determined that breast cancer arises from intestinal bacteria-triggered innate immune events requiring proinflammatory cytokine tumor necrosis factor (TNF)- α (12, 16). TNF- α has established roles in cancer progression in bowel, liver, breast, and other sites in mice (17, 18). The finding that *H. hepaticus* induces TNF- α expression (12) even in IBD-resistant mouse strains (8, 9) raises the possibility that overt IBD may not necessarily accompany breast cancer in susceptible women. Importantly, our data linking pathogenic *Helicobacter* bacterial infection and increased TNF- α with mammary tumors in mice match prior findings in women with elevated TNF- α levels and poor breast cancer outcome, and also correlate with the lower risk of breast cancer seen in women treated with anti-inflammatory drugs (4). Our preliminary data showing that *Campylobacter jejuni*, a common enteric pathogen in humans, has similar oncogenic potential in mice warrants further study.

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.

©2007 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-06-3468

Adenosquamous carcinoma in C57BL/6 *Min* and *Rag2*-deficient *Min* mice seems to arise in areas of inflammation from hyperplastic epithelia of the mammary ducts and ductules (see Fig. 1), matching features of breast malignancies in women involving the terminal ductal lobular unit (11). Orogastric infection with *H. hepaticus* induces low-grade and high-grade hyperplasias of mammary epithelia and these changes are accompanied by periductal accumulation of small numbers of mast cells, neutrophils, and macrophages in infected *Rag2*-deficient *Min* mice, suggesting that these cells may have roles in the initiation of tumorigenesis. A spectrum of precancerous mammary lesions analogous to those observed in women is evident in female *Rag2*-deficient *Min* and *Min* mice; however, the progression of hyperplasia to mammary intraepithelial neoplasia in *Min* mice has concurrent squamous metaplasia with or without formation of keratin. The squamous metaplasia observed may be a secondary species-related phenomenon because hyperplastic repair processes in response to inflammation are often accompanied by squamous metaplasia in

the mammary epithelia in mice (11). Squamous metaplasia is less frequent in *H. hepaticus*-induced mammary tumors in *Rag2*-deficient *Min* mice (12), when compared with *Min* mice receiving proinflammatory CD4⁺ effector T lymphocytes (16), perhaps providing important clues about adaptive immunity in breast carcinogenesis in women.

Precisely how gastrointestinal bacteria may trigger carcinogenesis in the mammae of *Min* mice is not yet clear. Mammary lymphadenopathy is evident within days of gastric gavage with *H. hepaticus* in C57BL/6 *Rag2*-deficient *Min* mice, matching earlier data in 129 *Rag2*-deficient showing systemic immune activation and splenomegaly after infection with *H. hepaticus* (9, 19), which indicates that enteric bacterial infection triggers a systemic innate immune inflammatory response. Studies are under way to determine whether additional events, such as translocation of intestinal bacteria to mammary tissue, may explain the rapid onset of mammary cancer in these genetically susceptible mice.

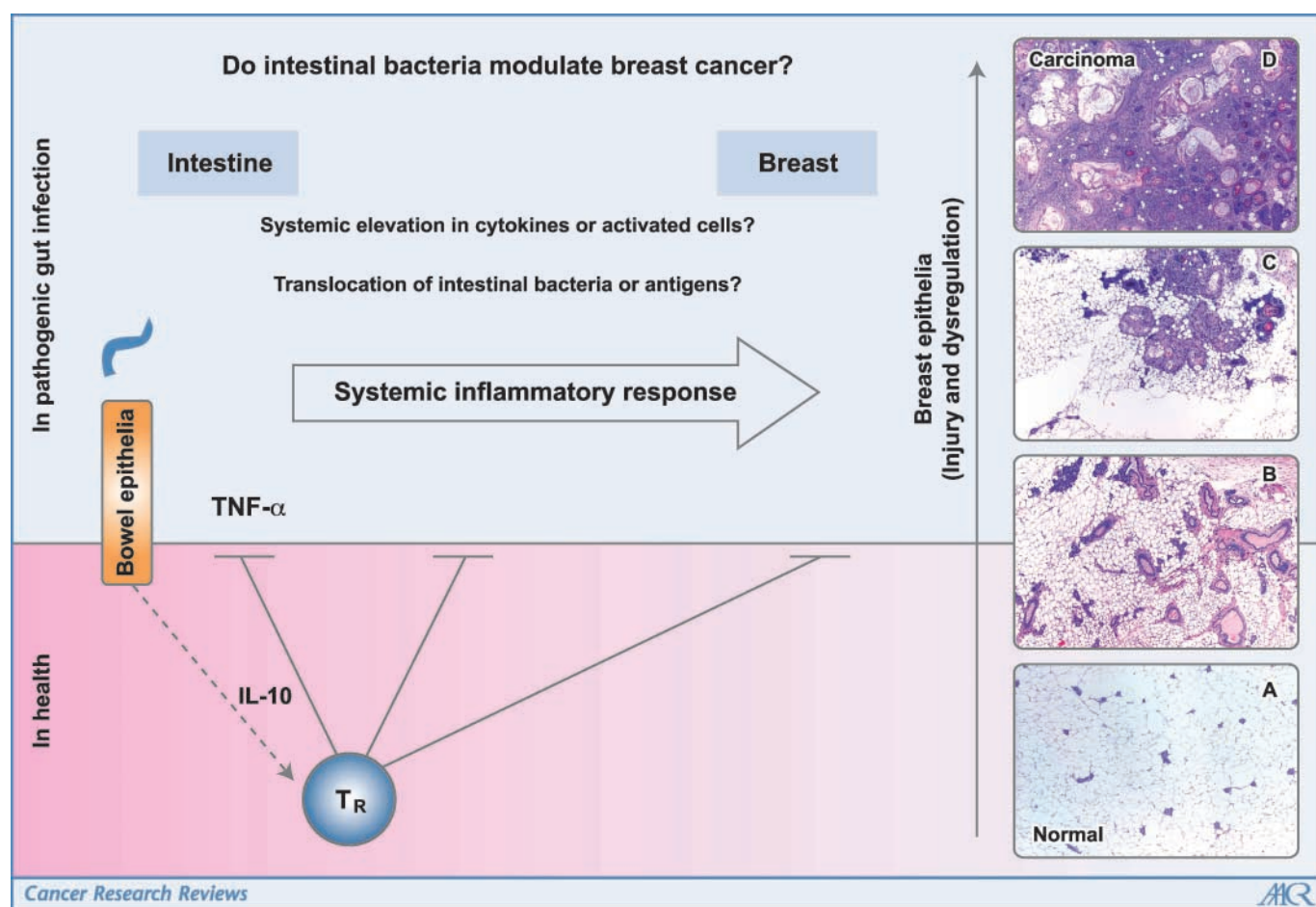


Figure 1. A proposed model of microbially induced breast cancer in women. Microbial flora of mucosal surfaces outnumber total cells in the human body and have essential roles in the development of a competent healthy immune system. However, humans chronically infected with pathogenic gastrointestinal bacteria often develop inflammation and cancer. The compromised intestinal epithelial barrier in individuals with chronic gut infections leads to submucosal translocation of bacteria thereby triggering persistent activation of cells of immune inflammatory response including dendritic cells, macrophages, granulocytes, and lymphocytes, culminating in a systemic inflammatory response that may lead to cancer at extraintestinal sites such as the breast. In immunocompetent hosts, regulatory T cells expand in response to the microbial challenge and down-regulate inflammatory events to help restore gut epithelial homeostasis. In hosts with immune dysregulation, inflammatory cytokines such as TNF- α are produced in excessive quantities and the downstream activities are poorly regulated favoring tumorigenesis. Mammary gland carcinoma arises in mice infected with *H. hepaticus* bacteria from the foci of inflammation and hyperplastic epithelia in mammary ducts and ductules. The spectrum of morphologic intermediates from normal mammary gland (A) to preneoplastic (B and C) and neoplastic (D) states are shown: ductal proliferation (B) with focal alveolar hyperplasia (B, inset), early adenosquamous metaplasia (C) with ductal carcinoma *in situ* (mammary intraepithelial neoplasia) and apocrine cytoplasmic differentiation (C, inset), and finally adenocarcinoma (D). H&E staining. Magnification, 40 $\times$ (A–D); 400 $\times$ (insets).

Anti-inflammatory Lymphocytes: Can They Prevent Breast Cancer?

CD4⁺CD25⁺ regulatory cells constitute a naturally occurring potent lymphocyte subset of thymic origin with critical roles in induction of peripheral tolerance to self and foreign antigens (20). These cells are potent suppressors of the activation and proliferation of other CD4⁺ and CD8⁺ T cells, as well as cells of innate immunity, and contribute to immune homeostasis in subjects with autoimmune diseases, chronic inflammatory disease, and cancer (10). The molecular basis of their suppressive activity has not been fully elucidated (20). Although there is general consensus that regulatory cells are essential in maintaining immune homeostasis through down-regulation of physiologic and pathologic immune responses, their roles in the progression of inflammation-associated cancers are less clear.

Murine models of IBD have convincingly shown that anti-inflammatory CD4⁺ regulatory lymphocyte subsets suppress destructive host immune responses during pathogenic intestinal bacterial infections, such as with *H. hepaticus* in mice (8, 9). We have shown, using a widely applied adoptive cell transfer paradigm in mice (10), that CD4⁺CD45RB^{lo}CD25⁺ regulatory T cells also suppress *H. hepaticus*-induced IBD-associated colorectal cancer in 129 *Rag2*-deficient mice (8). Likewise, adoptive transfer of regulatory T cells inhibits mammary tumorigenesis attributable to *H. hepaticus* infection in female *Min* mice (12). However, the potent antineoplastic efficacy of regulatory T cells is not restricted to *H. hepaticus*-triggered tumors alone because these cells equally suppressed the spontaneous development of intestinal polyps in C57BL/6 *Min* mice (21) and mammary carcinogenesis induced by proinflammatory CD4⁺ effector T-lymphocyte transfer in female *Min* mice (16). Supplementation with regulatory T cells in *Min* mice suppresses expression of COX-2 (21), previously linked with several cancers in humans (5), and also normalizes downstream expression of the *c-myc* oncogene (16), previously linked with breast cancer in women (11). It is probable that these antineoplastic effects of regulatory T cells, as previously shown in 129 *Rag2*-deficient mice (9, 22), are achieved through down-regulation of inflammatory cytokines, rather than by decreasing pathogenic bacterial counts within the bowel. Regulatory T cells may also function by inhibiting systemic trafficking of inflammatory cells (9) or perhaps bone marrow-derived mesenchymal stem cells (23), as the latter have been recently shown to contribute to carcinoma (23). These possibilities remain to be explored in our future studies.

Modern Hygiene Practices: A Double-Edged Sword

From the above discussion, it is clear that pathogenic gut bacteria may pose a trigger for breast cancer. However, this seems to be only half the story. It does not explain why breast cancer risk is increasing in developed countries with more rigorous hygiene practices, or answer how chronic use of prescribed antibiotics enhances the risk for breast cancer in women (4). The "hygiene hypothesis" is based on the observation that early childhood infections reduce the incidence of allergies (24). A later counter-regulatory model of the hygiene hypothesis, forwarded by Wills-Karp et al. (24), postulates that microbial infections have a beneficial role in the developing immune system and that the anti-inflammatory cytokine interleukin 10 (IL-10), produced by cells of both innate and adaptive immune systems during bacterial infections, has suppressive and feedback inhibitory effects on autoimmunity and allergy and is central to immune homeostasis (24). Following this reasoning, we hypothesize

that the reduced infectious burden due to stringent hygiene practices and excessive antibiotic use in developed countries may lead to weakening of this interleukin (IL)-10 feedback inhibitory loop and, thus, predispose susceptible individuals to develop more frequently chronic inflammatory diseases and inflammation-associated cancers. Therefore, in this context, bacterial infections are not necessarily entirely adversarial and may impart some long-term health benefits by reducing risk for chronic debilitating diseases, such as autoimmunity and cancer, later in life.

Recent experimental evidence (25) suggests that microbial infections in mice up-regulate the function of regulatory T cells and their ability to produce IL-10. Lymphocyte titration experiments in our laboratory (12) also show an IL-10-dependent increase in the antineoplastic potency of regulatory T cells from mice with prior exposure to *H. hepaticus* bacteria. Importantly, the enhanced antineoplastic potency extends protection against tumors arising in *Helicobacter*-free *Min* mice (12) and also suggests that prior exposures to intestinal bacteria may reduce risk for carcinoma in humans arising from other inflammation-associated disorders in later life. Whether the increased protection against cancer involves only regulatory T cells of thymic origin (19) or also peripherally recruited IL-10-dependent regulatory subsets is not well understood. We speculate that immune competency may be suboptimal in individuals with more stringent hygiene practices, and when combined with other known risk factors of Western lifestyle this contributes to the paradoxical increase in inflammation-associated cancers seen in developed countries. Likewise, antibiotics may deplete intestinal bacteria directly or indirectly essential for enteric homeostasis, thereby leading to increased risk of breast cancer in women undergoing chronic antimicrobial therapy (4). Interestingly, it seems that the long-term health benefits imparted by intestinal bacterial infections early in life may also be achieved in other ways. Recently, probiotic bacteria were shown to reduce IBD in mice through an IL-10-dependent regulatory lymphocyte-mediated mechanism (26), and clinical trials in humans using IL-10-expressing probiotic organisms are under way. Taken together, these data suggest that elimination of bacteria from the environment may have detrimental effects on the ability of the immune system to constructively regulate subsequent systemic inflammatory responses. The use of probiotic bacteria may provide the needed immune stimulatory input while minimizing interaction with pathogenic organisms.

The extraintestinal outcome of the interplay between proinflammatory and anti-inflammatory immune mediators in the bowel is readily shown in female *Min* mice that develop mammary tumors after infection with *H. hepaticus* bacteria. Although *Min* mice have both B and T lymphocytes, they show accelerated thymic involution (27) and develop lymphopenia at 3 months of age, including loss of regulatory lymphocytes (28). As a result, the persistent and unopposed activation of the remaining lymphocytes by *H. hepaticus* infection culminates in a state of chronic inflammation that exerts carcinogenic effects on mammary glandular epithelium, which may not have been evident in mice with a competent immune system. Similarly, a single injection of 3×10^3 proinflammatory effector T cells triggers development of mammary tumors predominantly adenocarcinoma in nature, whereas cotransfer with anti-inflammatory regulatory T cells from syngeneic wild-type cell donors completely prevents tumorigenesis (16). Unpublished data showing that antibody-mediated depletion of CD25⁺ cells significantly hastens the onset of mammary tumors in 2-month-old female *Min* mice corroborate

our prior work and suggest that regulatory T cells may possess previously unrecognized roles in the prevention of breast cancer. In addition, preliminary studies reveal that a single dose of 1.0×10^3 regulatory T cells can enhance the longevity of *Min* mice from their average life span of 4 months to up to 1 year of age. In general, however, the roles for regulatory T cells in cancer progression are far from clear. Data from many other mouse models suggest that regulatory T cells may dampen the protective CD4⁺ and CD8⁺ T-cell responses important in the elimination of neoplastic cells. Despite strong experimental data accumulated from mouse models in this regard, attempts of cancer immunotherapy and cancer vaccines in humans have thus far been disappointing (29).

Treading a Fine Line between Protection and Pathology

The possibility that dysregulated host immune responses to enteric bacteria lead to cancer in extraintestinal organs highlights the need to better understand factors that regulate immune and epithelial homeostasis in bowel and breast. It is clear that regulatory lymphocytes have evolved to play a sophisticated

balancing act of ignoring the host protective acute inflammatory response during infections and later regaining suppressive roles that limit deleterious pathologic sequelae of chronic inflammation. These inherent homeostatic properties of a competent immune system can be targeted for optimization in population-based approaches for cancer prevention using probiotics or vaccines. Selective enhancement of host beneficial anti-inflammatory activities may offer potent yet less toxic means when compared with traditional cancer treatment approaches such as radiation and chemotherapy. Further studies are warranted to gain better understanding of the interplay between microbes and cells of innate and adaptive immunity, which may help us to selectively expand or deplete relevant cells and their biological activities. The challenge now is to integrate findings from basic science with those from clinical studies, so we can sufficiently understand the cross-talk between microbial and immune homeostatic mechanisms that govern our health in the bowel to achieve optimal protection from cancer in the breast and other sites.

Acknowledgments

Received 9/22/2006; revised 11/6/2006; accepted 11/21/2006.

References

- Bange J, Zwick E, Ullrich A. Molecular targets for breast cancer therapy and prevention. *Nat Med* 2001;7: 548–52.
- ACS cancer facts and figures 2004. US cancer fact and figures 2004.
- Madigan MP, Ziegler RG, Benichou J, Byrne C, Hoover RN. Proportion of breast cancer cases in the United States explained by well-established risk factors. *J Natl Cancer Inst* 1995;87:1681–5.
- Ness RB, Cauley JA. Antibiotics and breast cancer—what's the meaning of this? *JAMA* 2004;291:880–1.
- Wang D, Dubois RN. Cyclooxygenase-2: a potential target in breast cancer. *Semin Oncol* 2004;31:64–73.
- Moss SF, Blaser MJ. Mechanisms of disease: inflammation and the origins of cancer. *Nat Clin Pract Oncol* 2005;2:90–7; quiz 1 p following 113.
- Fox JG, Wang TC. Inflammation, atrophy and gastric cancer. *J Clin Invest* 2007;117:60–9.
- 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.
- 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.
- Powrie F, Maloy KJ. Immunology. Regulating the regulators. *Science* 2003;299:1030–1.
- Cardiff RD, Anver MR, Gusterson BA, et al. The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting. *Oncogene* 2000;19:968–88.
- Rao VP, Poutahidis T, Ge Z, et al. Innate immune inflammatory response against enteric bacteria *Helicobacter hepaticus* induces mammary adenocarcinoma in mice. *Cancer Res* 2006;66:7395–400.
- Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 1990;247:322–4.
- Corpet DE, Pierre F. Point: from animal models to prevention of colon cancer. Systematic review of chemoprevention in min mice and choice of the model system. *Cancer Epidemiol Biomarkers Prev* 2003;12: 391–400.
- 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.
- 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.
- Karin M, Lawrence T, Nizet V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell* 2006;124:823–35.
- Balkwill F, Coussens LM. Cancer: an inflammatory link. *Nature* 2004;431:405–6.
- Maloy KJ, Antonelli LR, Lefevre M, Powrie F. Cure of innate intestinal immune pathology by CD4⁺CD25⁺ regulatory T cells. *Immunol Lett* 2005;97:189–92.
- Sakaguchi S, Ono M, Setoguchi R, et al. Foxp3CD25CD4 natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 2006; 212:8–27.
- 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.
- 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.
- Houghton J, Stoicov C, Nomura S, et al. Gastric cancer originating from bone marrow-derived cells. *Science* 2004;306:1568–71.
- Wills-Karp M, Santeliz J, Karp CL. The germless theory of allergic disease: revisiting the hygiene hypothesis. *Nat Rev Immunol* 2001;1:69–75.
- Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol* 2005;6:353–60.
- 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.
- Coletta PL, Muller AM, Jones EA, et al. Lymphodepletion in the ApcMin/+ mouse model of intestinal tumorigenesis. *Blood* 2004;103:1050–8.
- Gounari F, Chang R, Cowan J, et al. Loss of adenomatous polyposis coli gene function disrupts thymic development. *Nat Immunol* 2005;6:800–9.
- Pure E, Allison JP, Schreiber RD. Breaking down the barriers to cancer immunotherapy. *Nat Immunol* 2005;6: 1207–10.