

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 adenosquamous carcinoma. 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.

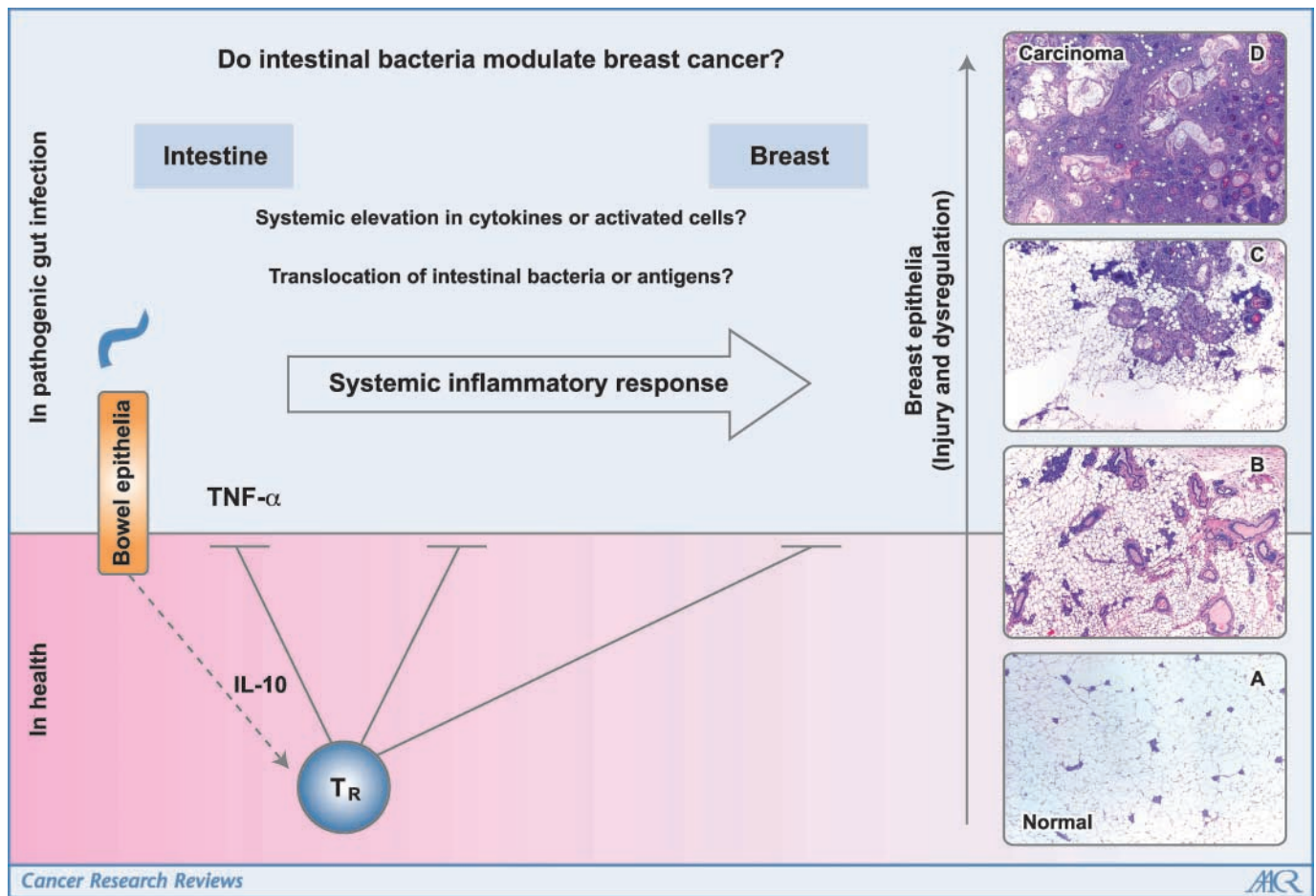


Figure 1. A proposed model of microbially induced breast cancer in women. Microbial flora of mucosal surfaces outnumber total cells in the human body and have essential roles in the development of a competent healthy immune system. However, humans chronically infected with pathogenic gastrointestinal 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.