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Unifying roles for regulatory T cells and inflammation in cancer

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Abstract

Activities of $CD4^+$ regulatory (T_{REG}) cells restore immune homeostasis during chronic inflammatory disorders. Roles for T_{REG} cells in inflammation-associated cancers, however, are paradoxical. It is widely believed that T_{REG} function in cancer mainly to suppress protective anticancer responses. However, we demonstrate here that T_{REG} cells also function to reduce cancer risk throughout the body by efficiently downregulating inflammation arising from the gastrointestinal (GI) tract. Building on a "hygiene hypothesis" model in which GI infections lead to changes in T_{REG} that reduce immune-mediated diseases, here we show that gut bacteria-triggered T_{REG} may function to inhibit cancer even in extraintestinal sites. Ability of bacteria-stimulated T_{REG} to suppress cancer depends on interleukin (IL)-10, which serves to maintain immune homeostasis within bowel and support a protective antiinflammatory T_{REG} phenotype. However, under proinflammatory conditions, T_{REG} may fail to provide antiinflammatory protection and instead contribute to a T helper (Th)-17-driven procarcinogenic process; a cancer state that is reversible by downregulation of inflammation. Consequently, hygienic individuals with a weakened IL-10 and T_{REG}-mediated inhibitory loop are highly susceptible to the carcinogenic consequences of elevated IL-6 and IL-17 and show more frequent inflammation-associated cancers. Taken together, these data unify seemingly divergent disease processes such as autoimmunity and cancer and help explain the paradox of T_{REG} and inflammation in cancer. Enhancing protective T_{REG} functions may promote healthful longevity and significantly reduce risk of cancer.

Keywords

gut bacteria; regulatory T cells; IL-6; IL-17; colon cancer; breast cancer

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Although improvements in cleanliness have reduced the incidence of many serious infections, with increased hygiene there has also been a concomitant increase in the incidence of several life-threatening ailments including allergies, autoimmune diseases and some types of cancer.[1-3] The "hygiene hypothesis" suggests beneficial effects of infections. This is supported by the observations that early childhood infections are associated with reduced frequency of aberrant immune reactions such as allergies and asthma later in life.[1] The observation that childhood infections suppress diseases associated with inflammation raises the possibility that intestinal bacterial infections may also protect against inflammation-associated cancers.

Risk of developing inflammation-associated cancer, such as colorectal cancer (CRC), is increased in societies with rigorous hygiene practices.[2] In the context of cancer, inflammation is widely believed to represent the body's fight against the tumor cells.[4,5] Recent data, however, suggest just the opposite; inflammation may be a cause of cancer and is a powerful stimulus for tumor growth and invasion.[6-8] It would follow logically that measures aimed at decreasing inflammation would be beneficial for cancer. Indeed, systemic nonsteroidal antiinflammatory drug (NSAID) use has been linked with a significant decrease in both sporadic and familial colon cancer[9-11] and a trend toward decreased cancer of the breast.[12] These opposing observations are not easily reconciled, and this is the basis for the paradox of inflammation and cancer.

Prior work in our laboratory[13-15] and others[16,17] supports a model in which enteric infections suppress inflammatory bowel disease (IBD) and cancer by modulating regulatory T cell (T_{REG}) responses, consistent with the observations of Belkaid and Rouse.[3] Specifically, we showed that the beneficial cancer-suppressing effects of microbial infections are dependent on Interleukin (IL)-10,[13,15,18,19] a cytokine that also provides suppressive and feedback inhibitory effects on allergies and autoimmune responses.[20] Early life exposures to microbial products have been well studied regarding the etiologies of allergies and asthma. It follows that reduced or delayed exposures to microbiota or their products in childhood might hinder normal immune functions in adult life. Although the hygiene hypothesis has been considered in depth for etiology of autoimmune diseases, few studies other than our own[13-15] have addressed these concepts as they may relate to cancer in bowel or extraintestinal sites such as the breast.

Recent studies by Kuchroo and coworkers[21,22] show that ability of T_{REG} cells to inhibit autoimmune diseases depends on levels of inflammation and IL-6. In this paradigm, elevated levels of T helper type (Th)-17 cytokines contribute to autoimmune diseases. Taken together, these observations link the immune system, gastrointestinal infections and seemingly divergent downstream phenotypes: allergies, autoimmune disease and cancer. These observations suggest that T_{REG} may do more to modulate cancer than block constructive anticancer responses.[4,5] Using a model in which childhood infections protect from inflammatory diseases later in life, here we test whether T_{REG} cell biology may help explain unanswered questions of cancer risk and modern lifestyle.

Material and Methods

Experimental animals

All animals were housed in AAALAC accredited facilities and maintained according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Massachusetts Institute of Technology. The 129 strain wild type and *Rag2*-deficient mice were obtained from Taconic Farms and then bred in-house to provide cell donors and experimental animals. IL-10-deficient mice on a C57BL/6 background were originally purchased from Jackson Labs, then backcrossed for 12 generations onto a 129 strain background and subsequently bred in-house for experiments. $Apc^{Min/+}$ mice on a C57BL/6J background were originally obtained from Jackson Labs and bred in-house as (heterozygous × wt) crosses to provide $Apc^{Min/+}$ mice and wt littermates for experimental mice, recipients and donors. FVB strain MMTV-*HER2/neu* mice were purchased from Jackson Labs. FVB wt mice were purchased from Jackson Labs and then bred in-house to produce experimental mice and cell donors.

Mice used in these experiments were specific pathogen free for pathogenic gut bacteria including *Salmonella* species, *Citrobacter rodentium* and *Helicobacter sp*., and also confirmed to be negative for murine viral pathogens and ecto- and endoparasites. Importantly, mice were *Helicobacter hepaticus*-free unless specifically experimentally infected with *H. hepaticus*.

Experimental design

A total of 298 mice were used for these experiments. One hundred twenty-four 129-strain mice were included in various treatment regimens or as experimental controls or as cell donors. The 129 strain mice were infected with *H. hepaticus* and then euthanized at 8–12 weeks postinfection (14–16 weeks of age). Eighty-eight C57BL/6 *wt* or $Apc^{Min/+}$ mice were included in various treatment regimens or as experimental controls as outlined in Table 1. For the survival curve (Fig. 2*d*), mice were humanely euthanized according to institutional criteria (*i.e*., poor body condition score and large tumor size) or when exhibiting other signs of distress. An additional cohort of Treg-treated $Apc^{Min/+}$ mice ($N = 6$) and wt ($N = 6$) counterparts were permitted to age normally for this survival curve comparison. Finally, 86 FVB strain *wt* or MMTV-*HER2/neu* mice were included in various treatment regimens as recipients of cells or anti-TNF or bacterial sonicates or used as experimental controls. For cell transfer experiments, FVB strain wt donor mice were infected with *H. hepaticus* ($N = 8$ mice per group) at 4–6 weeks of age and then underwent tissue collections for cell transfer 8 weeks later. Experiments were conducted using 5–8 mice per group as noted throughout the text.

Experimental Helicobacter hepaticus infection

A total of 142 experimental mice were dosed at 2–3 months of age with *H. hepaticus* and housed separately in a biocontainment area within the same animal facility. *H. hepaticus* (strain 3B1, ATCC no. 51449) was grown under microaerobic conditions, prepared and confirmed pure as described elsewhere.[13] Experimental mice received 0.2 ml of fresh inoculum by gastric gavage every other day for a total of 3 doses. Cecae and coli were

collected 3–4 weeks postinfection at necropsy and analyzed by PCR using *H. hepaticus*-specific primers to confirm experimental infection.[13]

Adoptive transfer of T cells into recipient mice

$CD4^+$ lymphocytes isolated from wt or IL-10-deficient mice using magnetic beads (Dynal) are sorted by hi-speed flow cytometry (MoFlow2) to obtain purified populations of $CD4^+CD45RB^{hi}$ (T_{EFF}) or $CD4^+CD45RB^{lo}$ $CD25^+$ (T_{REG}) lymphocytes and determined to be ~96% pure as previously described elsewhere.[13] Anesthetized recipient mice are injected intravenously in the retro-orbital sinus with 3×10^5 T cells as previously described.

Mice aged 3 months were injected with 3×10^5 T_{EFF} cells ($N = 40$ mice), 3×10^5 T_{REG} cells ($N = 22$ mice) or 1×10^5 T_{REG} cells ($N = 34$ mice). Twenty-six of these mice received cotransfer of T_{EFF} cells and T_{REG} cells, as noted. For T_{REG} cell assays, the donor mice for T_{EFF} or T_{REG} cells included both male and female syngeneic mice. Infected cell donors were dosed with *H. hepaticus* at least 8 weeks earlier.

T_{REG} cell titration assay

To test whether antitumor potency of T_{REG} cells can be enhanced by prior microbial challenge, we first established a suboptimal dose of $CD45RB^{lo}CD25^+$ *wt* T_{REG} cells (*i.e.*, 1×10^5 cells/recipient) that reduces tumor burden approximately to 50% levels of the untreated controls within 4 weeks.[13] Later, we used this lower dose regimen with either uninfected or infected cell donors as shown in Table 1.

Flow cytometry

Cells from the mesenteric lymph node or spleen were harvested after euthanasia and stimulated with PMA and Ionomycin for 4 hr. Golgiplug was added for the last 2 hr. Cells were fixed and permeabilized and then stained with anti-CD4, anti-Foxp3, anti-IL-10 and anti-IL-17 as described elsewhere.

TNF-α neutralization

Six MMTV-*HER2/neu* mice at age 6 months were treated with anti-TNFα antibody (clone XT-3; BioExpress, Lebanon, NH) at 100 μg per mouse 3 times weekly for 10 days. Mice were then euthanized and compared with age-matched mice that received sham IgG antibody alone ($N = 6$) or untreated controls ($N = 8$).

Feeding of *H* hepaticus sonicate

To investigate *in vivo* whether sterile preparations of *H. hepaticus* antigens alone are sufficient to generate protective host responses that prevent cancer, we treated 10 HER2/neu mice with sonicate of *H hepaticus* daily in drinking water and then examined mammary tumor frequency and burden. Bacterial sonicate was confirmed to be sterile by aerobic and anaerobic cultures and again on necropsy of mice. To achieve daily dosing, the equivalent of 10^9 live organisms that underwent sonication was suspended in drinking water.

Detection of systemic cytokine protein expression

Serum cytokine levels of 6 animals per group were analyzed using the Bioplex assay system (BioRad, Hercules, CA) according to the manufacturers protocol. Samples were analyzed in duplicate on a Bio-Plex 200 system (BioRad, Hercules, CA). Statistical analysis was performed using 2-tailed Student's *t* test; a p value < 0.05 was considered statistically significant.

Histologic evaluation and immunohistochemistry

As described previously,[13,19,23] the formalin-fixed tissues were processed, and tissue-sections were stained by H&E, Toluidine-Blue or IHC- evaluated by a veterinary pathologist blinded to sample identity. The frequency of mice with lesions was recorded for the various experimental groups.

Primary antibodies used for IHC included rat anti-Foxp3 (eBioscience, Inc., San Diego, CA), rabbit anti-IL-17 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), goat anti-IL6 (Santa Cruz Biotechnology) and rabbit-anti CD3 (Cell Marque, Rocklin, CA). Heat-induced antigen retrieval was performed with EDTA buffer pH8 for Foxp3 and IL-17 or with CC1 epitope retrieval solution (Ventana Medical Systems, Inc., Tucson, AZ) for IL-6 and CD3 detection. Anti-IL-17 and CD3 antibody binding was detected with goat anti-rabbit IgG Poly-HRP (Chemicon International, Temecula, CA). Anti-foxp3 and anti-IL-6 antibody binding was detected with species-appropriate biotinylated secondary antibodies (Serotec, Oxford, UK) and streptavidin peroxidase (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). Color was developed with DAB substrate-chromogen system (DakoCytomation, Glostrup, Denmark) and tissues were counterstained with hematoxylin.

Foxp3+ or IL-17+ cells were quantitatively assessed as previously described.[15] Multiple representative 40× high-power fields 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. Cell counts were recorded as the number of positive cells counted per image.

Mammary tumor counts were based on grossly evident tumor nodules in mammary tissue, and then compared between groups by unpaired Student's *t* test. Mammary tumor volumes (cm^3) were estimated based on dimensions of solid tumor tissue (excluding fluid-filled cysts) on necropsy, and then were compared between groups using Mann–Whitney *U* analyses.

Detection of cytokine mRNA expression

Total RNA from mammary or bowel tissue was prepared using Trizol reagent according to the supplier's instruction (Invitrogen, Carlsbad, CA). Five micrograms of the respective RNA were used to generate cDNA using the High Capacity Archive Kit from A/B Applied Biosystems according to the supplier's procedure. Levels of *IL-6* and *IL-17* transcripts in the cDNA samples were quantified with the corresponding commercial primers and probes in the ABI Prism Sequence Detection system 7700 (A/B Applied Biosystems). The levels of these cytokines among the samples were normalized by the level of the *GAPDH* transcripts

and compared between the T_{REG} cells-treated and untreated mice by the DDCt method described by A/B Applied Biosystems (User Bulletin no. 2).

Statistical analyses

Total lesion counts were analyzed by unpaired Student's *t* test. Cell counts between groups were compared using Mann–Whitney *U* analyses. Mammary tumor volume (cm^3) was estimated based on dimensions of solid tumor tissue. For all statistical analyses Graphpad Prism version 4.0 for windows, GraphPad software, San Diego, CA, was used.

Results

Bacteria, IL-10 and T_{REG} cells in IBD-associated colon cancer

To test whether the paradigm of T_{REG}, IL-6 and Th-17 described in autoimmunity may help explain protective relationships between gut bacteria and increased cancer risks in developed countries, we first used mice lacking antiinflammatory cytokine Interleukin (IL)-10. An essential role for IL-10 in immune homeostasis has been amply demonstrated in animals devoid of IL-10 (*IL10−/−*), which are highly susceptible to bacteria-triggered inflammatory bowel disease (IBD).[16,24] After infection with *H. hepaticus*, 129 strain mice without IL-10 (*N* = 8 mice per group) developed IBD and mucinous carcinoma resembling CRC seen in humans with colitis[25]; however, their wt counterparts infected with *H. hepaticus* had only minimal bowel pathology (*N* = 8 mice) (Table 1). Thus, mice required IL-10, a cytokine that inhibits allergies and autoimmune responses,[20] to reduce likelihood of IBD and cancer.

On the basis of the earlier data,[13,18,26,27] we postulated that protection from cancer resided in activities of IL-10 competent lymphocytes. We found that adoptive transfer of CD4+ lymphocytes from *wt* donor mice inhibited *H. hepaticus*-induced IBD and cancer in *Rag2−/−* mice; in contrast, IL-10-deficient CD4+ lymphocytes were not at all protective from IBD and CRC.[27] Subdivision of *wt* CD4+ subsets revealed that purified CD4+CD45RB^{hi} (T_{EFF}) cells from *wt* donor mice did not protect from *H. hepaticus* gut bacteria-triggered cancer, whereas CD4+CD45RB^{lo} (T_{REG}) cells completely eliminated IBD and carcinoma in recipient mice (Table 1). This showed that protection from cancer resided in the CD4+CD45RB^{lo} T_{REG} subset. Indeed, T_{REG} cells are widely recognized as pivotal in immune homeostasis and overall health due, at least in part, to coordinated antiinflammatory activities of cytokines IL-10 and transforming growth factor (TGF)-β.[28]

Intruigingly, transfer of T_{REG} from *IL-10−/−* donor mice into 129 *Rag2−/−* mice not only failed to inhibit pathology but also significantly ($p < 0.05$) increased risk of bacteria-triggered neoplastic invasion (*N* = 8; Table 1). Absence of IL-10 in T_{REG} also led to bacteria-triggered overexpression of IL-6 and IL-17 (Fig. 1*a*) that was not detected in matched recipients of *wt* T_{REG} cells. Overexpression of IL-17 was evident at the site of *H. hepaticus* infection in lower bowel but most prominent within enlarged lymph nodes throughout the body (Fig. 1*b*). This demonstrated that lack of antiinflammatory IL-10 raised risk for a gut bacteria-triggered inflammatory host response, and this effect extended beyond the bowel.[27,29] These findings supported a simplistic model with a GI bacteria-triggered

systemic immune response that shifted CD4+ cells toward a Th-17 phenotype in the absence of IL-10.

In this 129 mouse model, however, not only lymphocytes but also antigen presenting cells (APCs) were a major source of IL-17 after transfer of IL-10−/− CD4+ lymphocytes into *Rag2*−/− mice (Fig. 1*b*). Adoptive transfer of IL-10-deficient T_{REG} was also associated with increased frequency of IL-6- and Tgfβ-1-bearing neutrophils within the developing carcinoma (Fig. S1). Thus, although *wt* T_{REG} completely inhibited cancer and maintained bowel homeostasis, transfer of IL-10−/− CD4+ cells led to just the opposite: increased innate immune inflammatory cell infiltrates, IL-6, IL-17 and cancer.

We have previously shown that supplementation with exogenous IL-10-Ig fusion protein was sufficient to normalize IL-6 and Tgfβ signaling within bowel and induce regression of colonic carcinoma and neoplastic invasion.[15] Taken together, these data led us to postulate that individuals with insufficient IL-10 are more susceptible to uncontrollable elevations in IL-6 and IL-17 and may show more frequent inflammation-associated cancers in response to proinflammatory challenges such as with pathogenic enteric bacteria (Fig. 1*c*).

Gut bacteria trigger protective IL-10-dependent T_{REG} cells

To test directly in IL-10-competent mice whether infections earlier in life - such as childhood exposure to bacterial products in humans - may protect from immune-mediated diseases later in life, we used a T_{REG} cell titration assay as in Rao *et al*.[13] Various concentrations of 129 strain *wt* donor cells, starting at 300K and progressing incrementally downward to 100K per mouse, were injected into *H. hepaticus*-infected *Rag2*−/− recipient animals. Importantly, Rag-deficient mice otherwise lack functional lymphocytes,[26,30] providing a model animal to display isolated activities of purified lymphocytes. We found, when examining recipients of the lowest dosages (1×10^5 cells) of cells, that potency to protect from *H hepaticus*-induced IBD-associated CRC was increased when T_{REG} came from *wt* donor mice that were previously exposed to *H. hepaticus* (Table 1). Importantly, T_{REG} from *H. hepaticus*-infected donors required IL-10 to protect from IBD or cancer, even when added at the highest dosages (Table 1). These data matched earlier findings of Kullberg *et al*.[16] showing *in vivo* and *in vitro* that prior *H. hepaticus* infection in T_{REG} cell donors conveyed disease protection—not achievable with naïve T_{REG}—and specifically attributable to production of IL-10 by T_{REG} following challenge.

Interestingly, the lowest dosages (1×10^5 cells) of T_{REG} cells from uninfected donor mice (*e.g*., “hygienic” donors) did not inhibit cancer and instead actually led to increased neoplastic epithelial invasion, similar to the outcome observed in *Rag2*−/− recipients of IL10−/− T_{REG} cells (Table 1). We found that adoptive transfer of “hygienic” T_{REG} into *Rag2*−/− led to increased frequency of IL-6-bearing neutrophils and macrophages within inflamed mucosa, a feature of inflammation that is likely to contribute to cancer and dysregulated T_{REG} functions.[21,31] Although T_{REG} cells derived under these varying health status conditions performed differently, these differences were not the result of differential expansion or recruitment of cells within spleen or lymph nodes (Fig. 1*d*), as the percentage of Foxp3+ CD4+ cells in lymph node (25% in protected mice and 31% in mice with carcinoma) were similar or slightly increased in animals at greatest risk for carcinoma.

Taken together, cancer risk was lowered in these animals by protective roles of bacteria-triggered T_{REG}. Exposures to bacteria earlier in life served to increase efficiency of IL-10-dependent T_{REG}-mediated resolution of inflammatory challenges arising later in life.

T_{REG} cells, IL-6 and IL-17 in polyposis

To test whether roles for T_{REG} in sporadic CRC also conform to the aforementioned paradigm of IL-6 and IL-17 in autoimmunity, we examined C57BL/6 mice heterozygous for a mutation in the *Apc* gene ($Apc^{Min/+}$), which are genetically prone to intestinal polyps that mimic early stages of sporadic CRC in humans.[32,33] Although risk for sporadic CRC is reduced by NSAID usage in humans, intestinal polyps are less clearly associated with inflammation than is IBD-CRC. We find, that despite a lack of overt inflammation, there were higher systemic levels of TNF-α, IL-6 and IL-17 in $Apc^{Min/+}$ mice with intestinal polyposis (Fig. 2*a*). Importantly, this matches findings in colon cancer in humans.[34]

To test whether rigorous hygiene practices may also impact cancer risk less clearly linked with inflammation, as in this $Apc^{Min/+}$ model, we again applied adoptive transfer of lymphocytes. We used mice that were maintained in a *helicobacter*-free facility. To exacerbate carcinogenesis without use of *H hepaticus* infection, we instead applied adoptive transfer of proinflammatory CD4+CD45RB^{hi} (T_{EFF}) cells, a mouse model used elsewhere to emulate autoimmune pathology in humans.[28] In this model, $Apc^{Min/+}$ mice developed CRC without IBD,[23] and *H. hepaticus* infection was not required for CRC. Interestingly, female $Apc^{Min/+}$ recipients of T_{EFF} also have increased risk for mammary carcinoma.[23] These mice developed thyroiditis and splenic extramedullary hematopoeisis (data not shown) as in women with autoimmune disorders.[35] We found that expression levels of IL-6 and IL-17 were upregulated in bowel tissue (Fig. 2*b*) after T_{EFF} cells, when compared with untreated $Apc^{Min/+}$, and this was even in the absence of *H. hepaticus* infection.

In humans, the risk of developing CRC is lower in countries that have less stringent hygiene practices.[2,36] To test this concept of whether T_{REG} cells may fail to protect from pathology under more “hygienic” conditions in the adenoma-prone $Apc^{Min/+}$ model, we again used a T_{REG} cell titration assay. T_{REG} were isolated from *H. hepaticus*-exposed or uninfected *wt* C57BL/6 littermate mice and then injected at different dosages into uninfected “hygienic” $Apc^{Min/+}$ recipient animals. We found that at lower dosages (1×10^5 cells per mouse), T_{REG} cells collected from the uninfected “hygienic” donor mice failed to provide significant protection against intestinal tumor development (Table 1). However, T_{REG} cells from *H. hepaticus*-infected donors consistently provided complete protection, even at lowest dosages, when transferred into helicobacter-free “hygienic” $Apc^{Min/+}$ recipient mice (Table 1). Furthermore, we found that T_{REG} cells collected from *H. hepaticus*-exposed donor animals were more effective at suppressing both IL-6 and IL-17 levels in tumor-prone tissues when compared with suppressive ability of uninfected “hygienic” T_{REG} cells (Fig. 2*b*). Therefore, bacteria-primed T_{REG} cells did indeed suppress inflammation under hygienic recipient host conditions, and their ability to do so was more dependent on the prior conditions of the donor T_{REG}, rather than that of the recipient animals.

"Hygienic" T_{REG} cells led to increased mammary tumor burden

Surprisingly, *Apc*$^{Min/+}$ recipients of T_{REG} from the "hygienic" uninfected donor animals had a significantly ($p < 0.05$) greater mammary tumor burden (Table 2; $N = 8$ per group; 4.65 ± 1.15 cm^3 in hygienic T_{REG} cell corecipients of T_{EFF} cells compared with 1.63 ± 0.47 cm^3) than in matched T_{EFF} cell recipients. In contrast, T_{REG} cells collected from previously *H. hepaticus*-infected *wt* donors were completely protective against mammary tumor development (no tumors observed, Table 1). Importantly, expression of IL-17 was elevated in the mammary tissue from animals that received the T_{REG} of uninfected "hygienic" donors (Fig. 2*b*), when compared with recipients of T_{REG} from infected donor animals. The observation that adoptive transfer of T_{REG} from "hygienic" donors actually increased rather than suppressed carcinoma was coincident with overexpression of IL-17 and matched our findings with T_{REG} from "hygienic" donors in IBD-CRC in 129 strain *Rag2*−/− mice.

Using IHC, tissues surrounding the mammary tumors of *Apc*$^{Min/+}$ recipients were infiltrated by large numbers of cells expressing Foxp3 (Fig 2*c*), which is a widely accepted marker of T_{REG} cells.[21] Accumulation of Foxp3+ cells was detected only in recipients of T_{REG} from "hygienic" donors at high risk for mammary tumors and not in recipients of T_{REG} from "infected" donors. Importantly, T_{REG} collected from donor mice infected with *H. hepaticus* provided complete protection from mammary tumors and did not demonstrate accumulations of Foxp3+ cells in mammary tissue. This bacteria-endowed T_{REG}-mediated protection from mammary tumors emulated bacteria-endowed benefit of T_{REG} in IBD.[15-17] All these findings conformed with the "hygiene hypothesis" model in humans.

Gut bacteria-triggered T_{REG} cells not only inhibited cancer but also increased longevity in recipient mice

Examination of additional *Apc*$^{Min/+}$ animals, which were not euthanized at age 6 months but instead allowed to age normally, revealed that longevity was increased in recipients of T_{REG} when compared with untreated *Apc*$^{Min/+}$ animals. It was not determined whether increased survival (Fig. 2*d*) was directly attributable to lowered tumor burden alone or also the result of reduced systemic sequelae as related with IL-6 in humans.[37] Only recipients of T_{REG} cells from *H. hepaticus*-infected donors had benefit of increased longevity. Longevity-promoting effects of bacterial infection in *Apc*$^{Min/+}$ mice were isolated to functions of bacteria-triggered T_{REG} cells, rather than chronic infection with *H. hepaticus, per se*, which instead decreased lifespan of *Apc*$^{Min/+}$ mice (Fig. 2*d*). This raised the possibility that microbial or synthetic antigen-triggered CD4+ cells may ultimately be applied to protect against a wide variety of chronic inflammatory disorders to promote healthful longevity in humans living under modernized "hygienic" conditions.

Mammary tumor growth requires sustained inflammation

We previously showed that mammary tumorigenesis in *Apc*$^{Min/+}$ mice required TNF-α-dependent inflammation.[13,23] In those earlier studies, intraperitoneal injection with anti-TNF-α antibody abrogated mammary tumor development mediated by CD4+ lymphocytes in *Apc*$^{Min/+}$ mice.[23] We postulated that initial failure to control bowel and systemic levels of TNF-α and inflammation may result in sustained subversion of T_{REG} antiinflammatory functions leading to a downward immunological spiral and culminating in cancer growth.

This possibility was best tested directly in immunologically intact animals without overt intestinal diseases such as IBD or intestinal polyposis. For this reason, we used female FVB strain MMTV-*HER-2/neu* (*ErbB2*) transgenic mice with a genetic predilection to mammary tumors resembling those seen in 30% of humans with breast cancer.[12,38-41]

To directly test the requirement for inflammation and T_{REG} in mammary cancer, we used anti-TNF-α treatment in *HER2/neu* mice. We selected 6-month-old *HER2/neu* mice with an established tumor burden. Anti-TNF-α was delivered by intraperitoneal injection 3× weekly for 10 days, and then tumors were evaluated upon necropsy. We found that neutralization of inflammation with anti-TNF-α led to a significantly ($p < 0.05$) reduced mammary tumor burden (anti-TNF-α = 0.047 ± 0.03 cm^3/mouse *vs*. sham = 4.04 ± 2.11 *vs*. untreated 3.61 ± 1.04)(Fig. 3*a*). In addition, a trend emerged with lower mammary tumor multiplicity ($p = 0.07$) in mice treated with anti-TNF-α (μ = 1.0 ± 0.25 tumors/mouse) when compared with control animals (sham IgG μ = 2.7 ± 0.91 tumors/mouse and untreated μ = 3.1 ± 1.2) after only 10 days of antiinflammatory treatment.

Anti-TNF-α therapy also significantly ($p < 0.001$) increased frequency of intratumoral Foxp3+ cells coincident with decreased ($p < 0.05$) expression of IL-17 within tumors (Table 3 and Fig. 3*b*). In untreated mice, however, we found that intratumoral Foxp3+ cells were entirely absent from the mammary tumors (untreated or sham IgG-treated *HER2/neu* mice in Table 3; Fig. 3*b*). However, during cancer growth, Foxp3+ cells—while absent from within tumors—did accumulate in large numbers within lymph nodes and the desmoplastic tissue surrounding the mammary cancer in untreated and sham-treated mice (Fig. 3*b*), as they had in $Apc^{Min/+}$ recipients of "hygienic" T_{REG} (Fig. 2*b*). Underlying commonalities in these diverse mouse models are summarized in Figure 4. Taken together, these data helped explain how uncontrolled inflammation in the mammary tumor microenvironment may disable T_{REG}, and then lead to increased IL17 and increased tumor growth. Neutralization of inflammation rapidly restored homeostasis and normalized T_{REG} cell distribution.

Feeding of sterile gut bacteria protects from Erb2-associated mammary tumors

We hypothesized that optimally functioning T_{REG} routinely inhibit inflammation arising from bowel to maintain systemic immune homeostasis and reduce tumor risk. Indeed, we found that adoptive transfer of purified T_{REG} from *H. hepaticus*-infected wt FVB donor mice was sufficient to reduce risk of mammary cancer ($p < 0.05$; compared with untreated MMTV-*HER-2/neu* mice; Table 1), even when *HER2/neu* recipients were not themselves infected with *H. hepaticus*. Thus, anticancer protection imparted by gut bacteria *via* T_{REG} was crossprotective and broad spectrum, involving more than protection from allergies or asthma, and extending to inflammation-associated cancers arising in distant tissues later in life.

To test practically whether exposure to gut bacterial antigens alone may protect from cancers in distant sites, *HER2/neu* mice were treated for 4 weeks with sterile (sonicated) equivalent of 10^9 *H. hepaticus* organisms added to drinking water. Treatment was initiated at age 20 weeks to precede overt tumor development. We found that *HER-2/neu* mice had a significantly ($p < 0.05$) reduced tumor burden when examined 4-6 weeks later upon euthanasia at 24-26 weeks (Table 2). Strikingly, Foxp3+ cells were significantly ($p < 0.001$;

Table 3) reduced in the desmoplastic region surrounding the tumors after only 4 weeks of feeding of sonicates ($N = 10$; 5.4 ± 1.18 cells/field) when compared with untreated HER2/neu mice ($N = 6$; 17.8 ± 3.45 cells). Similarly, Foxp3+ cells that accumulated in large numbers within lymph nodes of untreated mice (68.3 ± 6.8 cells) were significantly ($p < 0.01$) reduced after feeding of sterile *H. hepaticus* sonicates (39.5 ± 3.51 cells).

Taken together, these data show that although pathogenic bacterial infections may trigger chronic inflammation leading to cancer, sterile enteric bacterial products may be utilized to stabilize immunity and offer protection from cancer. This raises the possibility that feeding of sterile bacterial products or crossreactive glycoproteins may be developed to provide protection from a wide variety of inflammatory disorders including cancers in humans.

Discussion

Data presented here, using mouse models that mimic cancer in humans, were consistent with the "hygiene hypothesis" and unified seemingly divergent disease phenotypes including autoimmunity and cancer. Findings matched the paradigm presented by Kuchroo and coworkers[21,22] involving elevated levels of TNF-α and IL-6 that disabled antiinflammatory T_{REG} and upregulated a T helper type (Th)-17 host response. Ability of bacteria-triggered T_{REG} to protect from inflammation-associated cancer matched findings of Kullberg *et al.*, Powrie and Maloy, and others[28,42] showing IL-10-dependent ability of CD4+ cells to efficiently and constructively modulate host inflammatory responses and health. Under normal physiological conditions T_{REG} responses are beneficial to the host to ignore or reinforce protective acute inflammation. Afterwards, T_{REG} regain protection from pathologic sequellae of chronic inflammation.[14] These normal protective processes that efficiently restore homeostasis seem to be disabled during chronic inflammatory diseases. Taken together, these observations connect the immune system, gastrointestinal infections and diverse immune-mediated diseases including allergies, autoimmune disease and cancer.

It has been well established in humans and in mice that chronic inflammation increases the risk of CRC.[13,15,23,26,27,34,43,44] It is paradoxical, then, that the risk for developing CRC is actually lower in countries that have less stringent hygiene practices.[2,36] This paradox was explained here using titration assays of CD4+ cells in adoptive transfer experiments. Increased likelihood of cancer due to infectious agents was offset by enhanced antiinflammatory protection from bacteria-triggered T_{REG}. Thus, exposure to bacteria earlier in life serve to increase efficiency of T_{REG}-mediated resolution of future inflammatory challenges. Titration assays revealed that "hygienic" T_{REG} may under some circumstances serve to promote carcinogenesis and increase cancer risk. This may be due to inability of "hygienic" T_{REG} to suppress uncommitted CD4+ cells recruited to Th-17, or this may also be due to Foxp3+ "hygienic" T_{REG} redirected to Th-17 phenotype. In either event, we postulate this carcinogenic effect arises from dysregulation of protective inflammatory responses. In an aging or genetically susceptible host, elevated levels of inflammation, namely cytokines TNF-α and IL-6, serve to disable antiinflammatory T_{REG} functions, which then leads to aberrant wound healing and contributes to cancer growth. CD4+ cell functions Foxp3+ redirected to Th-17 identity under these sustained are proinflammatory conditions, as described previously in humans and in mice.[45,46] In contrast, T_{REG} from a host with prior

bacterial challenges (*i.e.*, "infected" T_{REG}) effectively suppressed inflammatory challenges, rapidly restored immune homeostasis and prevented cancer growth. T_{REG} are widely recognized as pivotal in immune homeostasis and overall health.[3,28] Requirement for IL-10 has been amply demonstrated in murine models at high risk for IBD.[16,24,47,48] Extrapolating these results to humans, individuals with a naïve immune system and weakened IL-10- and T_{REG}-mediated inhibitory loop would be more susceptible to uncontrollable inflammation and more frequent inflammation-associated cancers throughout the body later in life.

Ability of gut bacteria to inhibit mammary cancer was isolated to functions of CD4+ T_{REG} cells. We hypothesize that benefit of T_{REG} is a manifestation of CD4+ cell-mediated oral tolerance to gut bacterial antigens. Following this line of reasoning, host ability to regulate inflammation is critical. A robust TNF-α-triggered inflammatory response benefits the host to eliminate a pathogen.[21,22,49-53] However, ability of T_{REG} to regain homeostasis is disrupted when the bowel is chronically inflamed. This results in a downward spiral of sustained systemic inflammation with further disabling effects on T_{REG} protective functions. Faubion *et al*.[54] and others have demonstrated that TNF-α dependent inflammation arising from bowel leads to impaired thymic and peripheral T_{REG} functions. This downward spiral is preventable with microbial challenges earlier in life that may serve to recruit IL-10-dependent CD4+ cells that protect from uncontrolled GI inflammation (shown in Fig. 5). Kullberg *et al*.[16] showed that prior exposures to gut bacteria increase efficiency of IL-10-dependent T_{REG} to downregulate inflammation and subsequently tip the systemic balance toward antiinflammatory activities and homeostasis. That these events rely on systemic interactions was displayed in an autoimmune condition such as multiple sclerosis (MS). Korn *et al*.[49] showed that during MS the T_{REG} relied on normalized levels of TNF-α and educated T_{REG} to efficiently restore homeostasis. In support of this, immune homeostasis was restored in the present study using anti-TNF-α which yielded tumor remission in our mice.

Ability of properly functioning T_{REG} to downregulate TNF-α and IL-6 and suppress cancer transcended gender differences in the animals examined here. In humans, men are up to 70% more likely to suffer dysregulated levels of IL-6 and develop colon cancer than are women.[55,56] Any anti-cancer protective effect of being female is lost after ovary removal or menopause, implicating steroidal hormones such as estrogen in protecting against cancer. A pivotal role for steroidal hormones in cancer is also supported by epidemiological studies in women showing that[57] hormone replacement therapy (HRT) with estrogen reduces risk of CRC.[58-60] One possible explanation is that estrogen drives expansion of IL-10-dependent CD4+CD25+ T_{REG} cells,[61-64] a feature of ovulation and pregnancy that minimizes maternal rejection.[65] This tolerance-based effect toward gut bacteria may serve to downregulate carcinogenic inflammation in the bowel in female animal; levels of cytokine IL-6 are generally lower than in males.[34,66,67] It was surprising that gut bacteria-primed T_{REG} were sufficient to inhibit mammary tumors. Perhaps, overexpression of IL-6 and IL-17 as shown here contribute to malignancy in breast and ovarian tissue.[4] Important pathogenic roles for inflammatory cytokines TNF-α and IL-6 have also emerged in social stress, bereavement and aging in humans and animal models,[68-71] perhaps providing clues as to why individuals living under these conditions may be at increased risk for cancer.

Foxp3 is a widely accepted marker of T_{REG} cells.[21] Interestingly, in this study large numbers of Foxp3+ cells accumulated in tumor periphery and lymph nodes during development of cancer. However, Foxp3+ cells were seen intratumorally only during restorative treatments with 1) anti-TNF-α or 2) gut bacteria-educated "infected" T_{REG} or 3) sterile gut bacterial sonicates. Within CRC lesions in bowel, intratumoral Foxp3+ cells were recently correlated with a favorable clinical outcome in human patients.[72] This dearth of Foxp3+ cells within the tumor parenchyma during malignancy was most clearly demonstrated in Figure 3*b* using HER2/neu transgenic mice that have well-circumscribed mammary tumor tissue. These findings contrasted with those of Ambrosino *et al*.[73] One possible explanation for this polarized distribution of Foxp3+ cells is that T_{REG} may accumulate in the tumor vicinity and then may differentiate into IL-17-producing cells[31,45,46] within the inflammatory milieu of the tumor. In any event, these effects are reversible using anti-TNF-α. Neutralization of systemic inflammation by treatment with anti-TNF-α resulted in fewer tumors, decreased Foxp3+ cell accumulations in surrounding tissues and also led to increased Foxp3+ cell counts within the tumors (Fig. 3*b*).

We postulate that host ability to rapidly restore immune homeostasis explains the "hygiene hypothesis" paradox and provide a basis for healthful longevity. Recent works of Mazmanian *et al*.[17,42] involving protective cross-reactivity of gut bacterial antigens in IBD support this concept, along with our earlier findings in mouse models.[13-15] In this study, exposure to gut bacterial antigens - either in the form of *H. hepaticus* sonicates fed to a host or *H. hepaticus*-educated T_{REG} transferred into a host, served to protect from pathology whether or not cancer was induced by *H. hepaticus*. Immediate benefits of this type could be achieved in humans using *in vitro* stimulation with bacterial antigens to educate and tolerize CD4+ cells[74] that may afterward be returned to a host. Similarly, feeding of bacterial antigens may lower cancer risk with population-based prophylactic and therapeutic potential. This approach would address the underlying "cause" of susceptibility to cancer, rather than targeting a "symptom" of accumulation of T_{REG} during antitumor host responses.[4,5] Many unanswered questions remain to optimize this process, especially regarding T_{REG} identity and T_{REG} recruitment during younger years rather than in later life.

Taken together, these data help explain the paradox of gut bacteria, inflammation and T_{REG} cells in cancer. We discovered here that T_{REG} triggered by gut bacterial antigens promote healthful longevity. T_{REG} arising under hygienic conditions seem to be captive to the paradigm of IL-6 and IL-17, as in autoimmunity elucidated by Kuchroo and co-workers.[21,22,49-53] Proinflammatory stimuli arising in a "hygienic" host may induce uncommitted CD4+ cells and possibly also Foxp3+ T_{REG} themselves to fuel carcinogenic events by favoring a Th-17 host response. Thus, insufficient microbial exposures in modern societies may undermine protective immunity arising from bowel. This results in lower thresholds for future carcinogenic events in distant tissues such as prostate, breast or lung. Two faces of T_{REG} have emerged here that highlight the importance of better understanding T_{REG} biology. The apparent dichotomy of T_{REG} in promoting healthful longevity, or, alternatively, exacerbating carcinogenesis, highlights the importance of exploiting T_{REG} biology as a powerful tool in cancer therapy.

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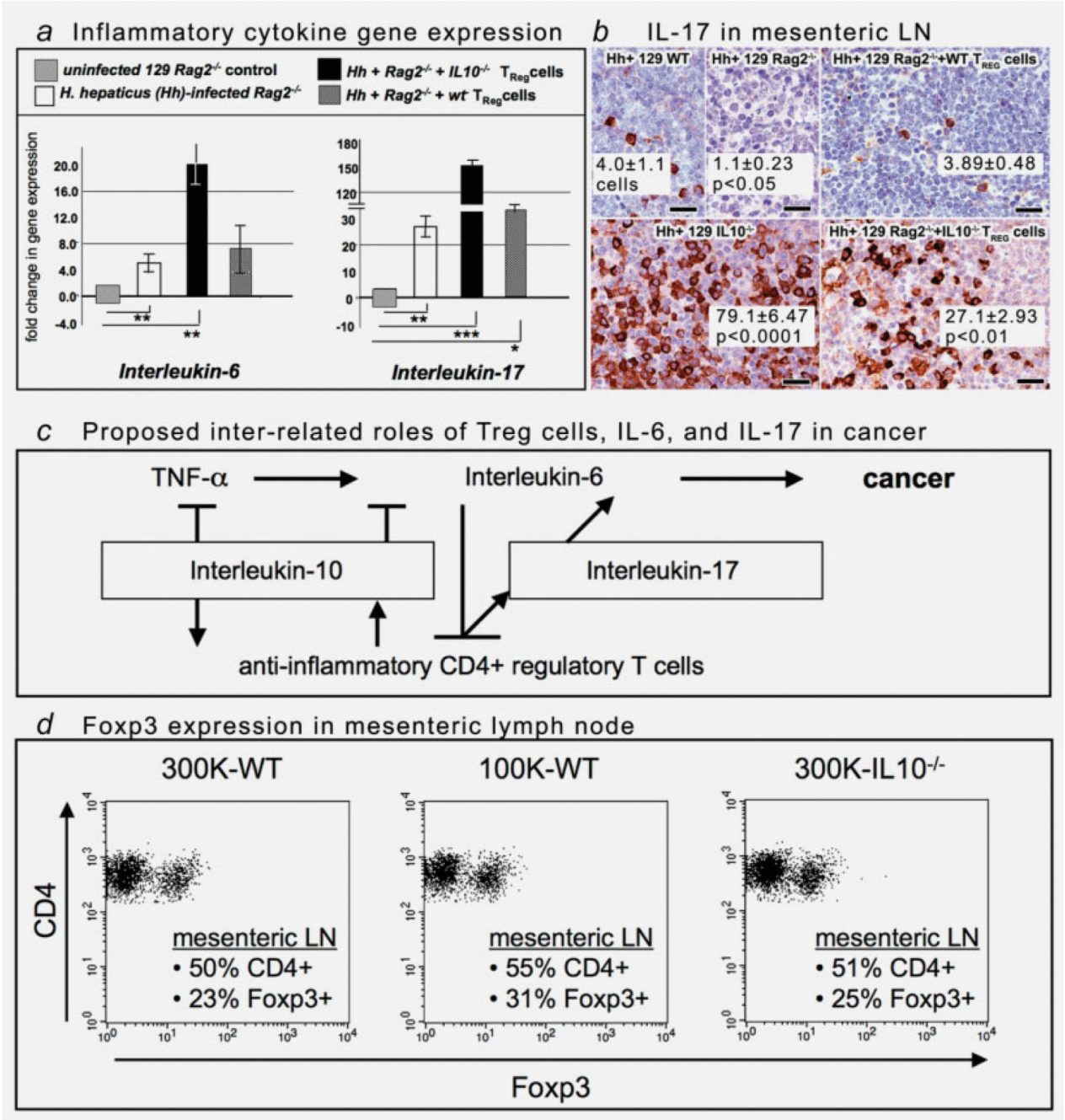

Figure 1.

Interleukin-10-dependent functions of regulatory T cells restore immune homeostasis in 129 strain Rag2-deficient mice. (*a*) Gene expression of IL-6 and IL-17 were elevated ($p < 0.05$) within colonic tissues of infected recipients of *IL10*-deficient Treg cells. For comparison of mRNA levels, the target mRNA was normalized to that of the housekeeping gene *GAPDH*. Numbers on the *y*-axis represent mean fold change of target mRNA levels in reference to the control levels (*B6 wt*, defined as 0, standard deviation represented by solid bars). mos = age in months on necropsy. **p* values were compared with the control. (*b*)

Immunohistochemical staining illustrates overexpression of IL-17 in the mesenteric lymph nodes of mice lacking IL-10. The aberrant immunostaining pattern observed in IL-10−/− animals after infection with *H. hepaticus* can be appreciated by comparison with tissues of wt mice (top row). The carcinogenic effect was isolated to IL-10-deficient lymphocytes and was correlated with overexpression of IL-17. 3,3-Diaminobenzidine, hematoxylin counterstain. Bars: 25 μm. (*c*) Overview of interrelated pathways of inflammation involving Treg cells, IL-6 and IL-17 contributing to cancer development and growth. (*d*) Purified T_{REG} cells of differing dosages or genotypes functioned differently, but these differences are not the result of differential expansion or recruitment of cells within lymph nodes. Each plot indicates % of MLN cells in the live cell gate that express CD4. The second number represents the % of CD4+ cells that expressed Foxp3.

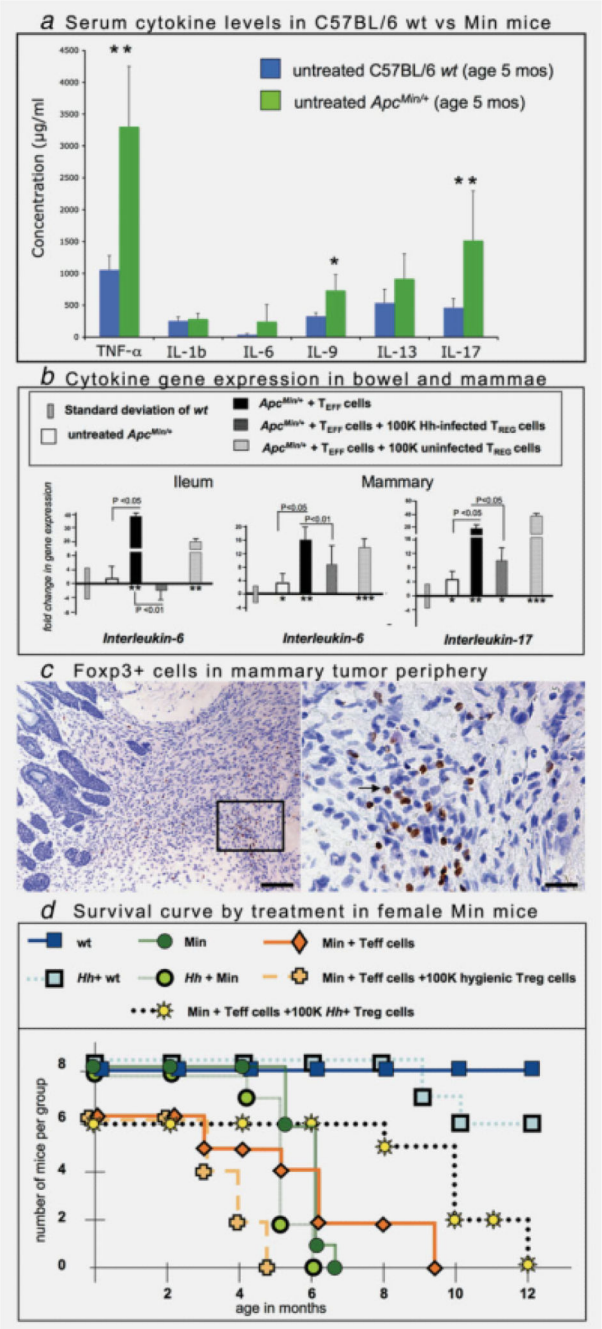

Figure 2.

Gut bacteria-triggered T_{REG} cells protect against intestinal and mammary neoplasia and increase survival. (*a*) Levels of TNF-α and IL-17 were significantly ($p < 0.01$) elevated in female *Min* mice. Serum cytokine levels of 5 months old C57Bl/6 *wt* (blue bars) and $APC^{Min/+}$ mice (green bars) were measured using the Bioplex assay system. Statistically significant higher levels of TNF-α, IL-9 and IL-17 were detected in $APC^{Min/+}$ mice. *$p < 0.05$; **$p < 0.01$. (b) T_{REG} cells from "hygienic" Treg cell donors failed to reduce gene expression of *IL-6* and *IL-17*. Levels of *IL-6* and *IL-17* were elevated ($p < 0.05$) within ileal

and mammary tissues of recipients of T_{EFF} cells. (*c*) Adoptive transfer of purified T_{EFF} cells cause highly infiltrative mammary adenosquamous carcinoma in female $APC^{Min/+}$ mice. Foxp3+ cells (right panel arrow) locate within tumor-associated inflammatory cell foci at the margins of tumors. The right panel provides a higher magnification of the same field. 3,3-Diaminobenzidine, hematoxylin counterstain. Bars: left panel: 100 μm; right panel: 25 μm. (*d*) Survival curve illustrating increased lifespan in $APC^{Min/+}$ mouse recipients of T_{REG} cells from *H. hepaticus*-infected syngeneic *wt* donor mice. For the survival curve, mice were humanely euthanized using institutional criteria (*i.e.*, poor body condition score, large tumor size) or when exhibiting other signs of distress.

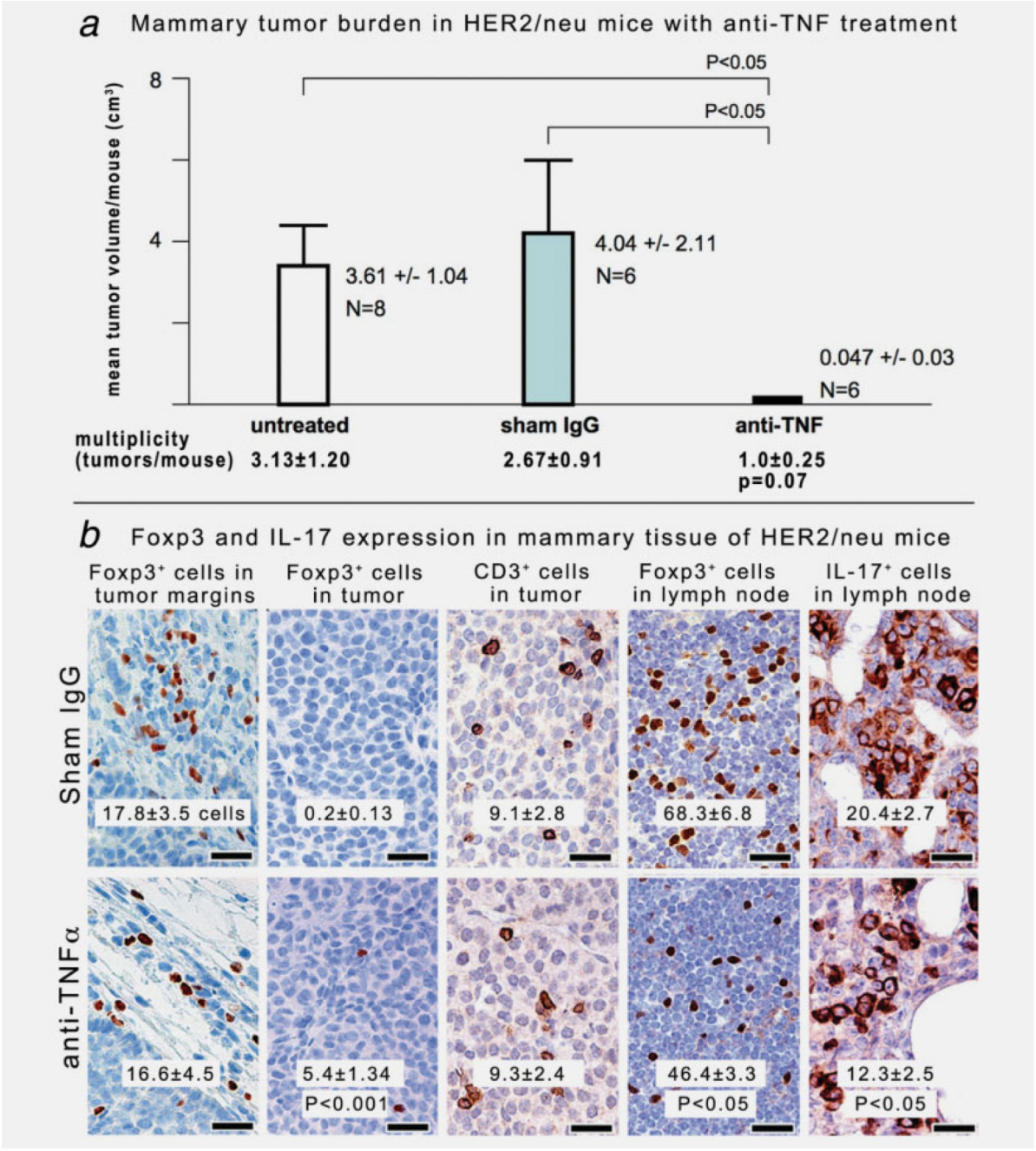

Figure 3.

Neutralization of TNF-α restores epithelial and immune homeostasis in FVB strain HER2/neu mice. To directly test requirements for inflammation in mammary cancer, we used anti-TNF-α treatment in HER2/neu mice. We selected 6-month-old HER2/neu mice with a small but established tumor burden. Anti-TNF-α was delivered by intraperitoneal injection ×3 weekly for 10 days and then underwent necropsy immediately afterward. Mammary tumor counts were based on grossly evident tumor nodules in mammary tissue on necropsy and then compared between groups by unpaired Student's *t* test. Mammary tumor

volumes (cm^3) were estimated based on dimensions of solid tumor tissue (excluding fluid-filled cysts) on necropsy and then were compared between groups using Mann–Whitney *U* analyses. (*a*) Blockade of TNF-α led to decreased tumor burden with significantly ($p < 0.05$) reduced volume and a trend ($p = 0.07$) toward fewer tumors in 6-month-old female HER2/neu mice. (*b*) Foxp3+ cells in untreated or sham mice were located within lymph nodes and inflammatory foci adjacent to, but not inside, mammary tumors. Treatment with anti-TNF-α significantly increased frequency of Foxp3+ cells within tumors. Similarly, anti-TNF-α treatment reduced ($p < 0.05$) the numbers of IL-17+ cells in mammary tumors (shown in right panels). Cell counts were performed as described in Material and Methods section. 3,3-Diaminobenzidine, hematoxylin counterstain. Bars: 25 μm.

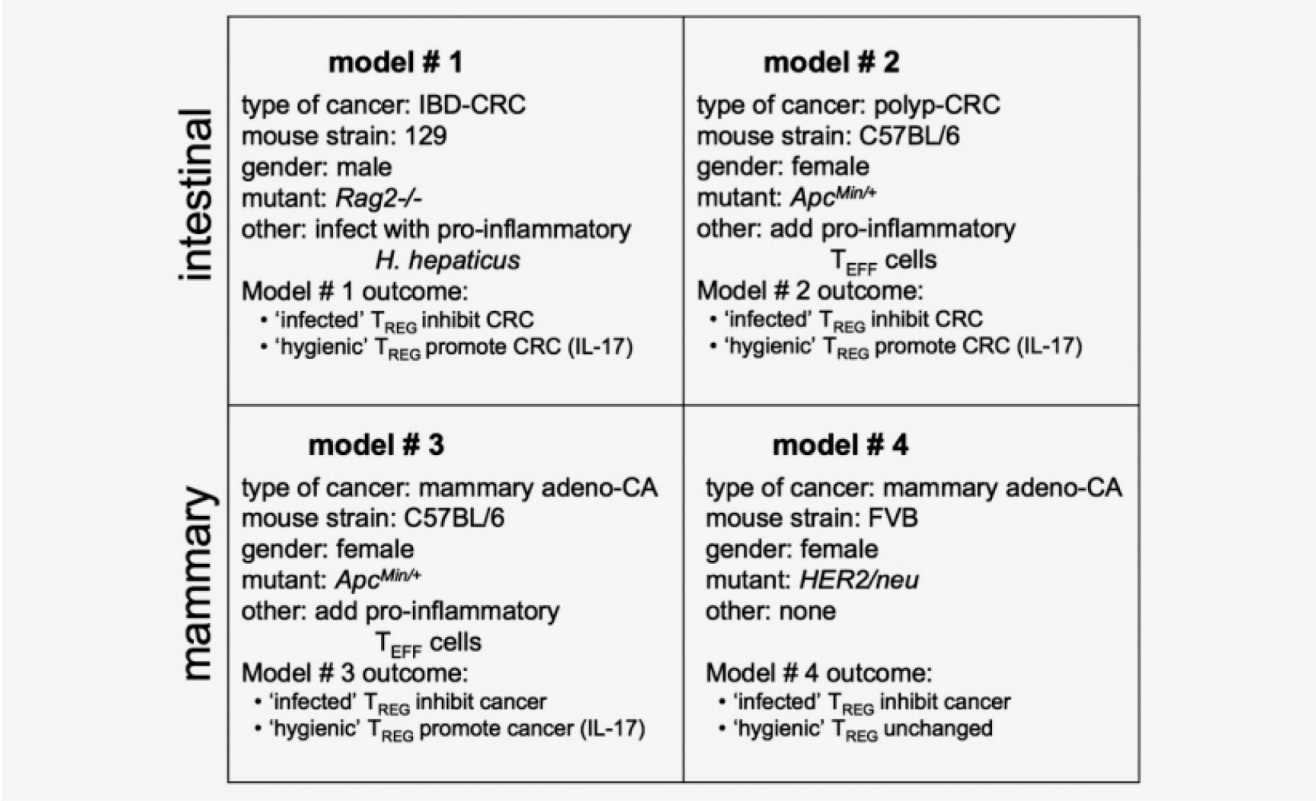

Figure 4.

Summary of tumor outcomes in different mouse models of cancer. Mouse models that mimic cancer in humans were consistent with the "hygiene hypothesis" and connected seemingly divergent disease phenotypes including autoimmunity and cancer. Bacteria-triggered IL-10-dependent functions of T_{REG} cells protect from inflammation-associated cancer.

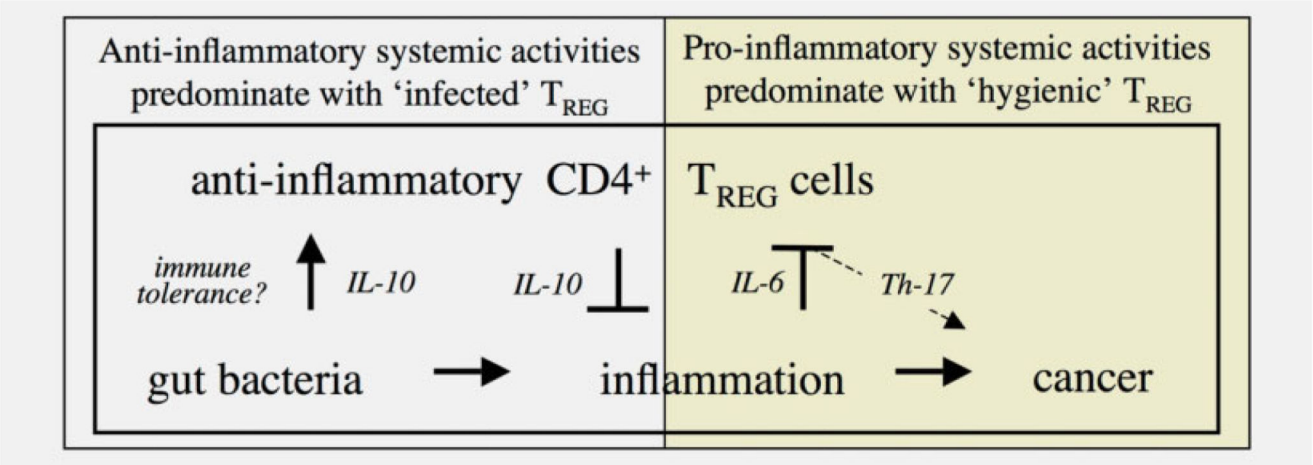

Figure 5.

Conceptual overview of immune factors contributing to cancer development and growth under hygienic host conditions. Under normal physiological conditions, T_{REG} responses are beneficial to the host by reinforcing protective acute inflammation and then afterward regain protection from pathologic sequellae of chronic inflammation. During a proinflammatory microbial challenge, elevated levels of IL-6 upregulate a T helper type (Th)-17 host response that contributes to cancer growth and invasion. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 1

Frequency of bowel and mammary cancer by treatment group

Strain	Murine model	Sex	Infection (recipient)	Bowel cancer (No. of mice with carcinoma/total)	Mammary AdenoCA (No. of mice with adenoCA/total mice)
129/SvEv	WT	M	-	0/8	0/8
	IL10−/−	M	-	0/8	0/8
	Rag2−/−	M	-	0/8	0/8
	WT	M	*Hh*	0/8	0/8
	IL10−/−	M	*Hh*	0/8	0/8
	Rag2−/−	M	*Hh*	1/8	0/8
	IL10−/−Rag2−/−	M	*Hh*	1/8	0/8
	Rag2−/− + 300K Teff	M	*Hh*	2/8	0/8
	Rag2−/− + 300K Teff + 300K Treg	M	*Hh*	0/8	0/8
	Rag2−/− + 300K Treg	M	*Hh*	0/8	0/8
	Rag2−/− + 300K IL10−/−Treg	M	*Hh*	8/8[1]	0/8
	Rag2−/− + 300K Hh+ IL10−/−Treg	M	*Hh*	8/8	0/8
	Rag2−/− + 100K Hh+ Treg	M	*Hh*	1/8[1]	0/8
	Rag2−/− + 100K hygienic Treg	M	*Hh*	7/8	0/8
C57BL/6	WT	F	-	0/8	0/8
	Min	F	-	0/8	0/8
	WT	F	*Hh*	0/8	0/8
	Min	F	*Hh*	4/8	5/8
	Min + Teff	F	-	3/6	4/6
	Min + Teff + 300KTreg	F	-	0/6	0/6
	Min + Teff + 100K Hh+ Treg	F	-	0/6	0/6
	Min + Teff + 100K hygienic Treg	F	-	4/6	6/6
FVB	WT	F	-	0/8	0/8
	HER2/neu	F	-	0/8	8/8
	WT+ *Hh* sterile sonicate	F	-	0/8	0/8
	HER2/neu+*Hh* (sterile) sonicate	F	-	0/10	4/10[1]
	HER2/neu + 100K Hh+ Treg	F	-	0/6	2/6 [1]
	HER2/neu + 100K hygienic Treg	F	-	0/6	4/6

[1] $p < 0.05$.

[2] $p < 0.01$.

Table 2

Mammary tumor burden in mice

Mouse model	Treatment	Cell donor health status	Recipient health status	Sample size (*n*)	Tumor volume (mammary)
B6 strain Min	-	-	Hh-free	8 mice	None found
Min+T_{EFF}	+T_{EFF}	Hh-free	Hh-free	8	1. 63 ± 0.47 cm^3
Min+T_{EFF}+naive T_{REG}	+T_{EFF}+ naiveT_{REG}	Hh-free	Hh-free	8	4.65 ± 1.15 cm^3[1]
Min+T_{EFF}+HhT_{REG}	+T_{EFF}+Hh infected T_{REG}	Hh -infected	Hh-free	8	None found
FVB strain HER2/neu	-	-	Hh-free	8	3.61 ± 1.04 cm^3
FVB strain HER2/neu	sham IgG	-	Hh-free	6	4.04 ± 2.11 cm^3
HER2/neu+antiTNF	Anti-TNF	-	Hh-free	6	0.047 ± 0.03 cm^3[1]
HER2/neu+HhT_{REG}	Hh infectedT_{REG}	Hh -infected	Hh-free	6	*1*
HER2/neu+Hh sonicate	Hh sonicate	-	Hh-free	10	0.31 ± 0.18 cm^3[1]

1 $p < 0.05$.

Table 3

Localization of Foxp3+ cells (mammary)

Model	Treatment	Sample size (*n*)	Health status	Foxp3+ cells in lymph node	Foxp3+ cells in tumor periphery	Foxp3+ cells within tumor
FVB HER2/neu	-	8	Hh-free	68.3 ± 6.8 cells	17.8 ± 3.45 cells	0.2 ± 0.13 cells
HER2/neu sham	Sham IgG	6	Hh-free	nd	16.5 ± 2.75	0
HER2/neu +anti-TNF	Anti-TNF	6	Hh-free	46.4 ± 3.27	16.6 ± 4.47	5.4 ± 1.34
HER2/neu + sonicate	Hh sonicate (sterile)	10	Hh-free	39.5 ± 3.51	5.4 ± 1.18	1.10 ± 0.48

Cell counts between groups were compared using Mann–Whitney *U* analyses.

Foxp3+ lymph node (LN): $p < 0.01$; untreated (68.3 ± 6.8 cells) *vs*. anti-TNF-α (46.4 ± 3.27).

Foxp3+ in LN: $p < 0.001$; untreated (68.3 ± 6.8) *vs*. sterile *Hh* sonicate (39.5 ± 3.51).

Foxp3+ in tumor periphery: $p < 0.001$; untreated (17.8 ± 3.45) *vs*. *Hh* sonicate (5.4 ± 1.18).

Foxp3+ within tumor: $p < 0.001$; sham IgG (0 cells) *vs*. anti-TNF (5.4 ± 1.34).