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Rapid reversal of interleukin-6-dependent epithelial invasion in a mouse model of microbially induced colon carcinoma

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Chronic inflammation of mucosal surfaces renders them increasingly susceptible to epithelial cancers both in humans and mice. We have previously shown that anti-inflammatory CD4$^+$ CD45RB^{lo}CD25$^+$ regulatory (Treg or T$_R$) lymphocytes down-regulate inflammation and block development of bacteria-triggered colitis and colorectal cancer (CRC) in 129/SvEv Rag2−/− mice. Interestingly, T$_R$ cells collected from Interleukin (IL)-10-deficient cell donors not only failed to suppress carcinogenesis but instead promoted invasive mucinous colonic carcinoma with a strong gender bias expressing in male mice. We found we show that peritoneal invasion in this model is dependent on pleiotropic cytokine IL-6. Mucinous carcinoma arose rapidly and consistently after treatment with IL10−/− T$_R$ cells, which were found to express Foxp3+ and localize throughout tumor tissue. Carcinogenesis was rapidly reversible with transfer of wild type IL10-competent T$_R$ cells. Likewise, treatment with IL10-Ig fusion protein was sufficient to revert the lesions histologically, and restore inflammatory cytokine and oncogene expression to base line levels. These studies indicate an essential role for IL 6 in this CRC phenotype. Furthermore, immune-competent T$_R$ cells were important not only for preventing pathology but also for constructive remodeling of bowel following tumorigenic microbial insults. These data provide insights into etiopathogenesis of inflammation-associated epithelial invasion and maintenance of epithelial homeostasis.

Introduction

Colorectal cancer (CRC) is among the leading cancer killers in USA and other developed countries (1). Mucinous adenocarcinoma is one type of CRC, that is characterized by abundant pools of mucin surrounded by fibrous stroma and cancerous glands. Mucinous CRC represents ~20% of colonic tumors in humans (2). In human patients, the mucinous lesions are found located most often on the right side of the colon. Molecular characterization studies have revealed that mutations of *K-ras* are more frequent in mucinous carcinomas in contrast to their non-mucinous counterparts (3). In general, mucinous carcinoma is less readily resected by surgery and carries a poorer prognosis compared with other types of CRC in humans (4,5).

We have shown that 129/SvEv recombinase-activating gene 2-deficient (*Rag2*$^{-/-}$) mice, lacking mature lymphocytes, develop several different types of colon cancer associated with colitis, including mucinous CRC, following infection with a widespread enteric bacterial mouse pathogen *Helicobacter hepaticus* (6,7). The inflammatory bowel disease (IBD) and carcinoma that develop in *H.hepaticus*-infected *Rag2*$^{-/-}$ mice are abrogated by treatment with interleukin (IL)-10-competent CD4$^+$CD45RB^{lo}CD25$^+$ regulatory (Treg or T$_R$) cells (7). Other studies using immune-deficient mice have revealed similar protective and therapeutic effects mediated by T$_R$ cells in mice with colitis (8,9).

Interestingly, adoptive transfer of CD4$^+$CD45RB^{lo}CD25$^+$ cells obtained from IL10-deficient donors fail to protect and instead exacerbate a malignant epithelial phenotype, such that 100% of recipient male Rag2−/− mice rapidly develop mucinous tumors in the ascending (right) and transverse colon that invade the peritoneal cavity (7). Mucinous adenocarcinomas in these mice match tumors in human patients according to a National Cancer Institute sponsored consensus report (10). Although it is clear from prior studies that the immune system and T$_R$ cells regulate epithelial cancer progression, the extent to which neoplastic epithelial invasion may be modulated and repaired by down-regulating inflammation has not been characterized.

Here we demonstrate that bacteria-triggered mucinous colonic carcinoma that arises rapidly and consistently in IL10−/− T$_R$ cell-recipient mice is dependent on IL-6 and is completely reversible by wild-type (WT) T$_R$ cells. Likewise, treatment with IL10-immunoglobulin (Ig) fusion protein is sufficient to revert the lesions histologically and restore inflammatory cytokine and oncogene expression levels to baseline. We propose that a signaling pathway comprised, at least in part, of IL-10 and IL-6 is pivotal in maintaining epithelial homeostasis and modulating epithelial invasion during bacterially driven inflammatory diseases.

Abbreviations: IBD, inflammatory bowel disease; Ig, immunoglobulin; IL, interleukin; PCR, polymerase chain reaction; PI, post-infection; Tgf, transforming growth factor; TNF-α, tumor necrosis factor α; T$_R$ or Treg, CD4$^+$CD45RB^{lo}CD25$^+$; wt, wild-type; CRC, colorectal cancer.

Materials and methods

129/SvEv Rag2-deficient mice

All animals, whose health status was as described previously (6), were housed in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facilities in static microisolator cages. 129/SvEv Rag2-deficient mice (originally obtained from Taconic Farms, German town, NY) and transforming growth factor (Tgf)β1-deficient Rag2-deficient mice (obtained from the Mouse Models of Human Cancer Consortium repository, Frederick, MD) were bred in-house to provide animals for these experiments. Experimental mice dosed with *H.hepaticus* were housed separately in a biocontainment area within the same animal facility. Studies used 10–12 mice per group in each experiment, with three or four repetitions (total n = 30–48 mice per group), unless otherwise specified. All experiments using animals were approved by the Institutional Animal Care and Use Committee.

Experimental infection

Helicobacter hepaticus (strain 3B1, American Type Culture Collection #51449) was grown under microaerobic conditions, prepared and confirmed pure as described elsewhere (6,11). Experimental mice received 0.2 ml of fresh inoculum by gastric gavage every other day for a total of three doses. The cecum and colon of mice were collected at necropsy and analysed by polymerase chain reaction (PCR) using *H.hepaticus*-specific primers to confirm *Helicobacter* status (6).

Adoptive transfer with lymphocytes

Rag2$^{-/-}$ recipients of wt or *IL10*-deficient CD4$^+$CD45RB^{lo}CD25$^+$ cells, collected from mice backcrossed at least 10 generations onto a 129/SvEv genetic background, underwent adoptive transfer 48–72 h prior to *H.hepaticus* infection (6,7). All the recipient mice in this study were male, based on the earlier obser-

vation that microbially induced cancer was exacerbated in male mice when compared with female mice of this strain (7). Recipient mice anesthetized with isofluorane were injected intravenously via the retro-orbital sinus with 3×10^5 cells suspended in 0.2 ml of Hank's balanced salt solution. Replicate experiments were conducted with two or three groups of similar size for select experiments.

To obtain viable and highly purified cell populations, single-cell suspensions of $CD4^+CD25^+$ lymphocytes from spleen and mesenteric lymph nodes from *Helicobacter*-free WT or IL10-deficient 129/SvEv donor mice were prepared as described previously (6). Briefly, CD4-positive cells were isolated by using L3T4 Dynabeads (Dynal, Oslo, Norway). Cells were detached from the beads using mouse CD4 DETACHaBEAD (Dynal). $CD45RB^{lo}$ $CD25^+$ cells were further isolated from the $CD4^+$ population by labeling with anti-CD45RB antibodies (PharMingen, San Diego, CA) and anti-CD25 antibodies (PharMingen) and then purified by flow cytometry. For these studies, half of the donor mice were males and half were females. Re-analysis of these cells prior to transfer into mice indicated that they were >96% pure.

Foxp3 characterization

For flow cytometry, after being stained with anti-CD4-APC and anti-CD25-PE (Becton Dickinson Biosciences PharMingen), cells were fixed and permeabilized for intracellular staining with Foxp3- fluorescein isothiocyanate (BD Bioscience PharMingen) or isotype control fluorescein isothiocyanate-labeled antibody. A minimum of 50 000 events was collected for each sample. FACScalibur was used for data analysis. For immunohistochemistry, after deparaffinization, formalin-fixed sections were antigen retrieved with pepsin (Zymed, San Francisco, CA) for 10 min at 37°C and were labeled with rat monoclonal antibody recognizing mouse Foxp3 antigen (# 14-5773 rat anti-mouse; eBioscience, San Diego, CA). Primary antibody binding was detected with species-appropriate biotinylated secondary antibodies (Sigma chemical company), streptavidin peroxidase and 3,3-diaminobenzidieine (Vector Labs, Burlingame, CA). Immunohistochemical assays were performed on an automated immunostainer (i6000; Biogenex, San Ramon, CA).

Carcinoma intervention using adoptive transfer of lymphocytes

Rag2$^{-/-}$ recipients of *IL10*-deficient $CD4^+CD25^+$ cells underwent a second adoptive transfer of *wt* T_R cells at 6–8 weeks after *H.hepaticus* infection, when mice had already developed mucinous carcinoma and localized peritoneal invasion, to determine ability of T_R cells to induce cancer regression.

Subsets of Rag2−/− mice ($n = 8$ mice per group) that received *H.hepaticus* and IL10−/− deficient cells, as above, subsequently underwent adoptive transfer of a lower dose of *wt* T_R cells at 6–8 weeks post-infection (PI) to ascertain and compare therapeutic potency. A dosage of 1.0×10^5 cells was predetermined to be the minimum effective dosage of lymphocytes in suppressing carcinoma.

In vivo ultrasound imaging

To confirm the presence of mucinous colonic masses prior to the onset of treatment, selected mice underwent imaging of the abdominal cavity using high-resolution ultrasound (VisualSonics, Toronto, Canada). Imaging was performed both before (at 8–12 weeks PI) and after (2 weeks) treatment with T_R cells, using a 707B scan head, with isofluorane inhalant anesthesia.

Treatment with IL10-Ig fusion protein

To produce the IL10-Ig fusion protein, murine IL10 was fused to the IgG2a CH2-CH3 regions, mutated at the Fc receptor-binding site, using a PCR cloning strategy and the chimeric gene was cloned into an adenoviral vector and infectious virus generated (Ad-IL-10-Ig) as described previously (12,13). We determined that 150 ng/ml of IL-10-Ig was comparable with 1 ng/ml of recombinant IL10 in its ability to inhibit the production of IL-12 p40 and IP-10 by IL10-deficient macrophages (data not shown). Serum containing 2–5 μg of fusion protein was administered by intraperitoneal injection to mice with established invasive cancer, according to the data from other mice that were euthanized from the same cohort during initial studies, twice weekly for 7–10 days.

Determination of serum IL6 protein by enzyme-linked immunosorbent assay

To determine serum IL6 concentration in treated and untreated mice, a sandwich enzyme-linked immunosorbent assay was performed using Quantikine mouse IL6 kit (R&D Systems, MN) as per the manufacturer's instructions. The IL6 standard curve and sample concentrations were determined by measuring absorbance at 450 nm and after applying correction for plate background (Biotek Instruments, VT).

Treatment with cytokine-neutralizing antibodies

For neutralization of tumor necrosis factor α (TNF-α), *Rag2*$^{-/-}$recipients of *IL10*$^{-/-}$ lymphocytes also infected with *H.hepaticus* were treated for 6–8 weeks PI, with anti-TNF-α antibody (clone XT-3; BioExpress, NH) at a dose of 200 μg per mouse ($n = 8$) intraperitoneally thrice weekly for 7–10 days as described previously (14). To determine whether IL6 is required to sustain colonic cancer, mice with established carcinoma ($n = 8$) were treated with 500 μg of rat anti-mouse IL6 (clone MP5-20F3; eBioscience) by intraperitoneal injection twice weekly for 1 week. Matched control mice ($n = 8$) received the same concentration of rat IgG1 (eBioscience).

Histologic evaluation

Formalin-fixed tissues were embedded in paraffin, cut at 5 μm and stained with hematoxylin and eosin. Lesions were scored by two pathologists blinded to sample identity. Hyperplastic and inflammatory lesions were graded on a scale of 0–4 with ascending severity as described previously (6,15). Intestinal epithelial dysplasia and neoplasia were graded using a scale of 0–4 based on a recently described grading scheme (6,10). Non-parametric data are presented as median score and range (in parentheses) for each group.

Epithelial purification

RNA from colonic epithelium was purified according to Whitehead *et al*. (16) with minor modifications. Briefly, colons were removed and flushed with ice-cold 1× phosphate-buffered saline. Colons were then opened lengthwise and incubated in 3 mM ethylenediaminetetraacetic acid, 0.05 mM dithiothreitol for 60 min on ice at 4°C. Following the 1 h incubation, tissues were rinsed once in cold 1× phosphate-buffered saline. New phosphate-buffered saline was added and the tissue was shaken vigorously to dislodge epithelium. In an independent assay, preparations were found to be >95% pure for intestinal epithelial cells.

Gene expression analysis

The samples for analyses of colonic epithelial genes were centrifuged to pellet the purified epithelia (as above), whereas other gene expression assays utilized snap-frozen 0.5–1.0 cm full-thickness sections of ascending colon. Supernatant was removed and replaced with 1 ml of Trizol (Invitrogen). RNA was isolated according to manufacturer's instructions. After Trizol, the RNA was further purified using Qiagen's RNeasy Kit. Colonic epithelial cDNA was produced from 1 μg of purified RNA using Superscript III reverse transcriptase (Invitrogen). For each treatment group, RNA was obtained from colons of at least three different animals per group for purified colonic epithelia and at least eight animals per group for whole bowel. Gene expression analysis was performed by *Taq*Man analysis (Applied Biosystems, Foster City, CA) on an ABI Prism 7000 Sequence Detection System. All expression assays were designed by ABI (Assays-on-Demand, www.appliedbiosystems.com).

Statistical analyses

Analyses of colonic lesion scores were performed using a Mann–Whitney *U* non-parametric test for ordinal data. Comparisons of frequency of carcinoma between groups were performed using a two-sided Fisher's exact test. Statistical analysis of gene expression data was performed by Wilcoxon Rank Sum test using the Mstat computer program (http://mcardle.oncology.wisc.edu/mstat/).

Results

Infection with H.hepaticus bacteria triggers carcinoma

We demonstrated previously that *H.hepaticus* infection triggers colitis-associated carcinoma in 129/SvEv *Rag2*$^{-/-}$ mice (6,7). In that model, microbially triggered invasive epithelial lesions matched mucinous colonic carcinoma in humans according to a published consensus report on mouse models of colon cancer (10). We discovered that frequency and extent of epithelial invasion in mucinous carcinoma in *Rag2*$^{-/-}$ mice was accelerated and greatly exacerbated by prior adoptive transfer of $CD4^+CD45RB^{lo}CD25^+$ lymphocytes derived from donors lacking IL-10 (7) and that male mice were more susceptible to CRC than females (7). To determine whether carcinogenic effects of *IL10*-deficient lymphocytes require pathogenic bacterial challenge, as in some mouse models of IBD (6,9), we first compared *H.hepaticus*-infected and uninfected *Rag2*$^{-/-}$ recipients of *IL10*$^{-/-}$ T_R cells. Starting as early as 6 weeks PI, colonic carcinoma developed only in animals infected with *H.hepaticus* ($n = 10$, Figure 1C; $n = 12$, Figure 1D). When examined at 6–8 weeks PI, >80% of male mice infected with *H.hepaticus* and receiving *IL10*$^{-/-}$ T_R cells had >0.3 cm diameter locally invasive mucinous tumors in colon. In contrast, only minimal changes in epithelial morphology are found in uninfected recipients of *IL10*$^{-/-}$ T_R cells ($n = 10$, Figure 1A; $n = 10$, Figure 1B). These findings match published data from other murine models (9,17–19) and suggest that risk for intestinal carcinoma and invasive sequellae may increase in susceptible humans after challenge with similar pathogenic bacteria.

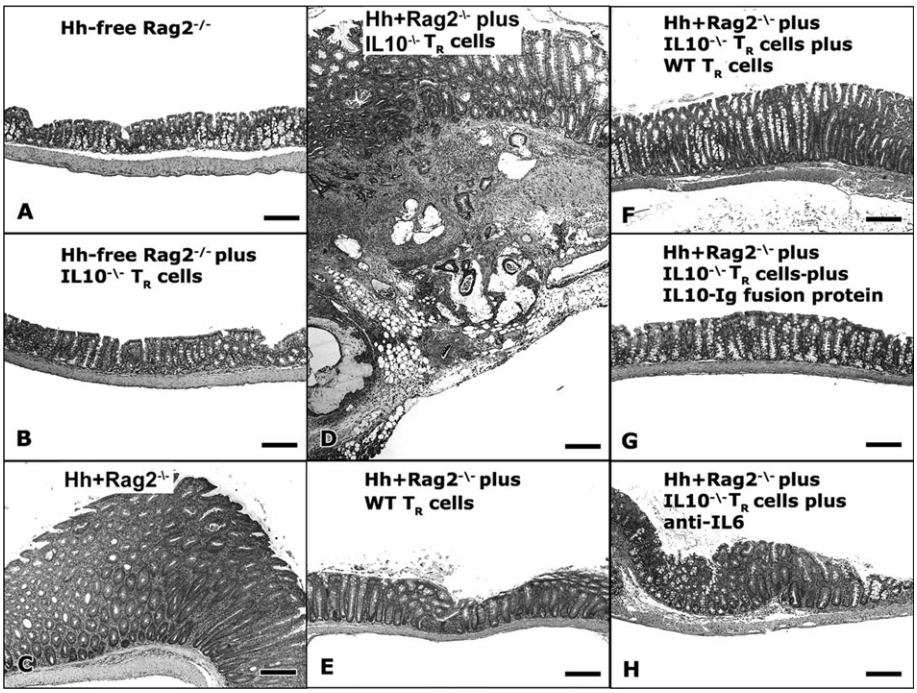


Fig. 1. Histopathology of bowel. Photomicrographs of ascending and transverse colon of (**A**) uninfected *Rag2*$^{-/-}$ mice ($n = 10$); (**B**) uninfected *Rag2*$^{-/-}$ mice + IL10-deficient CD4^{+}CD25^{+} regulatory cells ($n = 10$); (**C**) *Rag2*$^{-/-}$ mice infected with *Helicobacter hepaticus* ($n = 10$); (**D**) *Rag2*$^{-/-}$ mice + IL10-deficient CD4+CD25+ cells, then infected with *H.hepaticus,* then examined at 6–8 weeks PI ($n = 12$); (**E**) *Rag2*$^{-/-}$ mice infected with *H.hepaticus* given *wt* IL10-competent regulatory cells ($n = 10$); (**F**) *Rag2*$^{-/-}$ mice given IL10-deficient CD4+CD25+ cells and infected with *H.hepaticus,* then given IL10-competent regulatory cells ($n = 10$); (**G**) *Rag2*$^{-/-}$ + IL10-deficient CD4+CD25+ cells infected with *H.hepaticus,* then given IL10-Ig ($n = 10$); (**H**) *Rag2*$^{-/-}$ mice given IL10-deficient CD4+CD25+ cells and infected with *H.hepaticus,* then treated with anti-IL6 antibody. There were significant increases in colitis and carcinoma in *Rag2*$^{-/-}$ mice treated with IL10-deficient CD4+CD25+ cells, but only after infection with *H.hepaticus*. When IL6 was neutralized or when IL10 was introduced, either through adoptive transfer of lymphocytes or treatment with fusion protein at 6 weeks PI, there were significant decreases in colonic inflammation and dysplasia, as well as evidence of regression of invasive neoplastic epithelia. 4X, H&E; bar = 250 μm

Colonic carcinoma in these mice demonstrated a highly invasive mucinous phenotype (Figure 1D) that appeared to arise from areas of severe colitis. Neutrophils were the predominant inflammatory cell type associated with both ulcerative and invasive epithelial lesions in these mice. The rapid (at 6–8 weeks PI) and uniform (>80% penetrance) phenotype of macroscopically evident carcinoma in male mice led us to utilize this mouse model to determine whether immune cells and cytokines may modulate epithelial invasion and provide insight into development and regression of certain types of tumors.

IL-10-deficient CD4+CD25+ cells express Foxp3 and localize in colonic tumors

We described previously that adoptive transfer with *IL10*-deficient regulatory (T_R) cells rapidly induces colonic carcinoma, resembling that seen in *H.hepaticus*-infected *Rag2*$^{-/-}$ mice receiving pro-inflammatory CD4^{+}CD45^{RBhi} effector lymphocytes (7). However, from those studies, it was unclear whether CD4^{+}CD45RB^{lo}CD25^{+} cells collected from IL10-deficient donors have a regulatory cell identity, as defined by the expression of the forkhead/winged-helix family member Foxp3 (20,21). In order to determine this, Foxp3 status of purified lymphocytes was examined in spleen tissue derived from *IL10*-deficient and *wt* mice, and found to be comparable (9.34 and 9.43% of CD4+ cells, respectively) and approximating the anticipated 10% frequency (21) in both genotypes of mice (Figure 2A and B). Interestingly, immunohistochemistry of colonic tissue revealed that recipients of *IL10*−/− T_R cells had numerous Foxp3+ cells scattered throughout the mucinous carcinoma masses (Figure 2C), matching findings of Foxp3+ cells in IBD patients (22,23). In contrast, *wt* IL10-competent Foxp3+ cells, which effectively suppress IBD and carcinoma, were relatively infrequent and localized only within the lymphoid follicles (Figure 2D) of quiescent colonic mucosa. These data indicate that expression of Foxp3 in T_R cells does not necessarily correlate with a normally functioning immune-suppressive phenotype.

IL-10-competent T_R cells abrogate colitis and reverse epithelial invasion

We demonstrated previously that adoptive transfer with IL10-competent wild type (*wt*) regulatory (T_R) cells inhibits development of carcinoma in *H.hepaticus*-infected *Rag2*$^{-/-}$ mice (7). However, from those studies, it was unclear whether competent T_R cells are able to restore epithelial homeostasis in recipients with advanced carcinoma with localized invasion in the peritoneal cavity. In order to determine this, mice underwent subsequent adoptive transfer with 3×10^5 *wt* (IL10 competent) T_R cells per recipient at 6–8 weeks after initial transfer of *IL10*$^{-/-}$ cells and infection with *H.hepaticus*. The timing of 6 weeks PI corresponds to mucinous carcinoma in >80% of mice receiving IL10$^{-/-}$ cells and *H.hepaticus* during four repetitions with at least 10 mice per group. When examined at 1 week after treatment with *wt* T_R cells, mice with invasive tumors that received T_R cells ($n = 10$) had significantly less colitis ($P < 0.01$) and cancer ($P < 0.001$) than untreated controls ($n = 12$) (Figure 1 and Table I).

A second cohort of *H.hepaticus*-infected recipients of *IL10*−/− T_R cells was examined at a later interval of 12 weeks PI when frequency of mucinous peritoneal invasion was 100% (10/10). Use of high-resolution ultrasound imaging allowed *in vivo* visualization of intestinal tumors (Figure 3A) before (Figure 3B) and 1 week after (Figure 3F) treatment with *wt* T_R cells. Postmortem examination confirmed normal bowel morphology and epithelial histology (Figure 3C–E) at 14 days after transfer of *wt* T_R cells. The individual animal shown in Figure 3 had restored appetite and activity and gained 9 g (from 18.5 to 27.8 g) of body weight during the 14 days after transfer of *wt* T_R cells. Antitumor-suppressive effects of *wt* T_R cells were prolonged such that other mice examined up to 18 months after *wt* T_R cell transfer

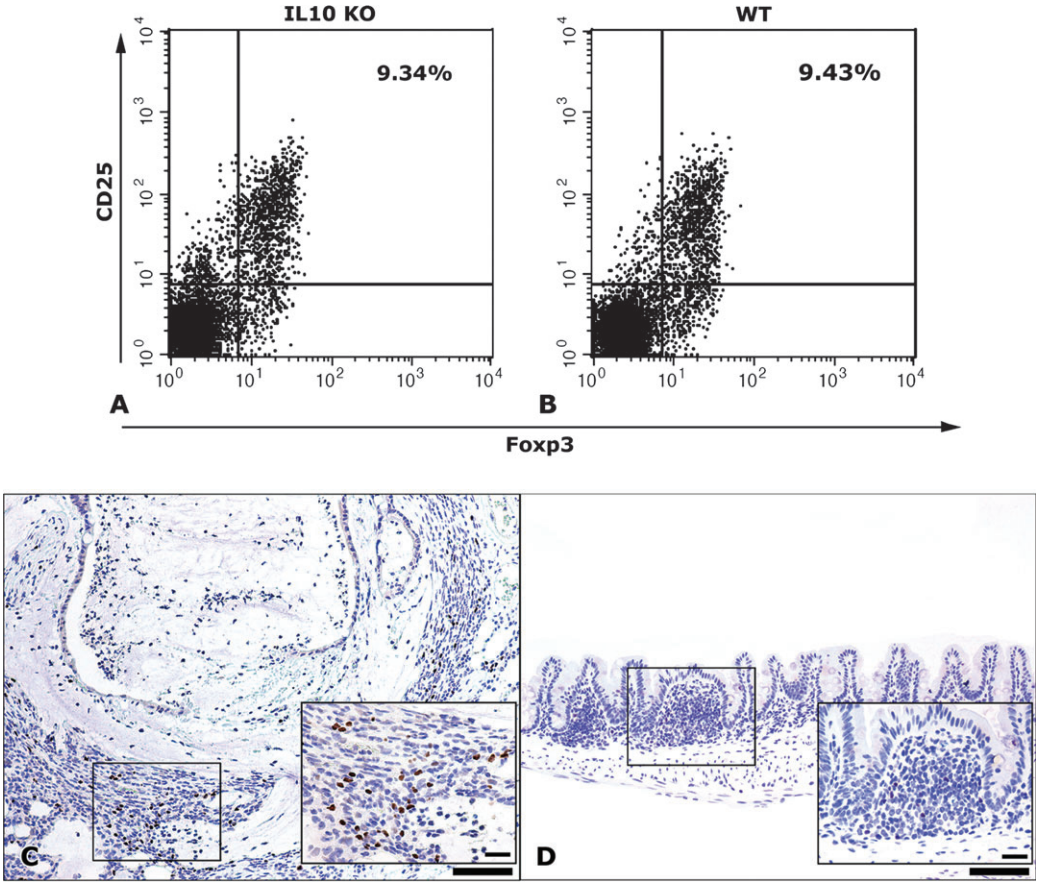


Fig. 2. IL10-deficient CD4+CD25+ T_R cells are Foxp3+ and localize in tumors. Flow cytometry analysis plots of splenocytes from mice used as lymphocyte donors. Total splenocytes were harvested from groups of IL-10-deficient (**A**) and WT (**B**) mice ($n = 4$) and stained with CD4, CD25 and Foxp3 as described in Materials and methods and analysed by flow cytometry. The percent of CD4+ cells that express Foxp3 is shown in the upper right corner. Plots depict results from individual animals representative of each group ($n = 4$). (**C** and **D**) Foxp3-specific immunohistochemistry of colon sections from *Helicobacter hepaticus*-infected $Rag2^{-/-}$ mice that received $CD4^+$ $CD25^+$ cells derived from lymphocyte populations matching those shown in plot A (C) or plot B (D). The inflammatory infiltrate at the invasive front of the mucinous tumor shown in (C) includes a large number of $IL10^{-/-}$ cells showing strong Foxp3 immunoreactivity. Note, intense nuclear brown-colored signal in (C) inset. In contrast, transfer of IL10-competent WT $CD4^+$ $CD25^+$ cells prevent and suppress *hepaticus*-induced colitis and cancer (D) resulting in normalized colon with small numbers of Foxp3-positive cells. Those cells localized mainly in lymphoid follicles (D inset). 3,3-diaminobenzidine, hematoxylin counterstain. Bars, 100 μm (C and D); 25 μm (C and D insets).

Table I. Histopathology scores in colon according to treatment

Infection status	*Helicobacter hepaticus* free		*Helicobacter hepaticus* infected			
Genotype treatment	Rag 2−/−	Rag 2−/− + IL10−/− cells	Rag2−/− + IL10−/− cells	Rag 2−/− + IL10−/− cells plus competent Treg cells	Rag 2−/− + IL10−/− cells plus IL10-Ig	Rag2−/− + IL10−/− cells plus anti-IL6
Sample size	$n = 10$	$n = 10$	$n = 12$	$n = 10$	$n = 10$	$n = 8$
Inflammation	0 (0–1)	1 (0–2)	4 (4–4)	2 (0–4)[a]	2 (0–4)[a]	2 (0–4)[a]
Hyperplasia	0 (0–1)	0 (0–1)	4 (3–4)	2 (0–4)	1 (0–4)	2 (0–4)
Dysplasia	0 (0–0)	0 (0–0)	4 (3–4)	1 (0–4)[b]	0 (0–3)[b]	0 (0–3)[b]
Carcinoma (%)	0/10 (0)	0/10 (0)	11/12 (92)	1/10 (10)	0/10 (0)	0/8 (0)

Histology scores presented as median score (range). Inflammation and dysplasia were evaluated histologically and scored 0–4 as described in the text. Mice underwent adoptive transfer of IL10-deficient lymphocytes and infection with *H.hepaticus* at 6–8 weeks of age. Mice then underwent 1 week of treatment with lymphocytes, IL-10 fusion protein or anti-IL6 antibody starting 6–8 weeks after infection with *H.hepaticus*. Mice were harvested at 1 week after onset of treatment and compared with matched controls. Data were subjected to Mann–Whitney *U*-test by comparison of each criterion of disease in the colon. Data are presented as median score and range. There were significant differences in inflammation ($P < 0.01$) and dysplasia ($P < 0.001$) when IL10 was introduced, either through adoptive transfer with lymphocytes or treatment with fusion protein, or when cytokine IL6 was neutralized with blocking antibody.

[a]Mann–Whitney *U*-test, comparison between colon inflammation in *H.hepaticus*-infected recipients of IL10-deficient regulatory cells, untreated versus post-treated with IL10-competent regulatory cells or IL10-Ig.

[b]Mann–Whitney *U*-test, comparison between colon dysplasia in *H.hepaticus*-infected recipients of IL10-deficient regulatory cells, untreated versus post-treated with IL10-competent cells or IL10-Ig.

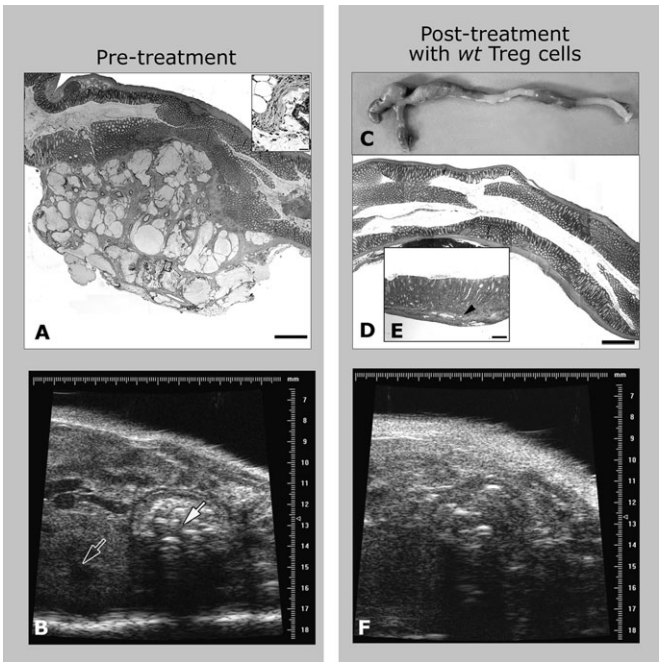


Fig. 3. Regression of mucinous adenocarcinoma in colon after treatment with *wt* regulatory T cells. Images depicting restoration of normal intestinal morphology at 2 weeks after transfer of 3×10^5 IL10-competent T_R cells. (**A**) Transverse colon of Rag2−/− mice receiving IL10-deficient T_R cells and infected with *Helicobacter hepaticus*, shown here at 12 weeks PI ($n = 10$) corresponding to pre-treatment status of bowel. Mucinous colonic carcinoma invading peritoneal cavity was present in 100% (10/10) of male mice at 12 weeks PI. The area in the box is shown in the higher magnification inset. Note, neoplastic epithelium within mucin pool and juxtaposed remnants of peritoneal fat. (**B**) *In vivo* imaging of abdominal cavity using high-resolution ultrasound of a Rag2−/− mouse with IL10−/− T_R cells and also infected with *H.hepaticus* (as in A), examined prior to treatment at 12 weeks PI. Note right kidney (open arrow) and colonic mucinous mass (white arrow). (**C**) Entire bowel (cecum and colon) of the same mouse as in (B) upon necropsy at 2 weeks after treatment with *wild-type* T_R cells. (**D** and **E**) Photomicrographs from different areas of the transverse part of the colon shown in (C). The overview of the largest part of transverse colon is highly suggestive for the absence of neoplastic lesions (D). Multiple serial sectioning of the paraffin block containing the whole bowel shown in (C) revealed only one area in the entire large bowel showing minute neoplastic epithelium and mucin pools within submucosa (arrow head in E). Those structures most probably represent remnants after regression of the tumor shown in (B). (F) *In vivo* high-resolution ultrasound image of the abdominal cavity of the same mouse as in (B) at 1 week after adoptive transfer of *wild-type* T regulatory cells. (A, D and E): hematoxylin and eosin; Bars, (A and D) = 1000 μm; (A) inset = 25 μm and (E) = 250 μm.

had no evidence of carcinoma ($n = 4$; data not shown). Taken together, these data indicate that IL10-competent T_R cells not only prevent carcinoma but also suppress epithelial invasion and promote re-epithelialization following microbially induced mucosal injury.

Although T_R cells in this study were derived from naive helicobacter-free donors, epithelial ulceration was not seen in any (0/40) recipients of 3.0×10^5 *wt* T_R cells. Prior knowledge that microbes or microbial products enhance survival and proliferation of T_R cells prompted us to question whether prior microbial challenges in donor mice may further enhance anti-neoplastic protection of transferred T_R cells in this model. To test this possibility, we adapted a titration assay (14,19) utilizing a suboptimal lower dose of 1×10^5 cells per recipient of $CD4^+CD45RB^{lo}CD25^+$ *wt* T_R cells. This lower dose regimen of T_R cells was transferred from donors either uninfected or previously infected with *H.hepaticus* 8 weeks earlier separately into *Rag2*$^{-/-}$ recipients of *IL10*$^{-/-}$ T_R cells ($n = 8$) at the time of infection with *H.hepaticus*. We found that T_R cells prepared from *H.hepaticus*-infected donors (supplementary figure is available at *Carcinogenesis* Online) were significantly ($P < 0.05$) more effective at inhibiting inflammation-associated bowel pathology than those derived from naive donors. While mucinous differentiation was typically absent from recipients of the lower dosage of cells from either infected or naive cell donors, an important contrast between treatment groups was the complete lack of epithelial ulceration among mice receiving T_R cells from *H.hepaticus*-exposed cell donors. There was extensive ulceration remaining only in bowel of mice that received the lower dose of naive T_R cells, which were collected from mice not previously exposed to *Helicobacter* sp bacteria. Taken together, this suggests that IL-10-competent hosts benefit from prior enteric bacterial challenges through enhanced ability of T_R cells to prevent epithelial ulceration and neoplastic sequellae.

IL-10 reverses epithelial invasion and restores epithelial homeostasis

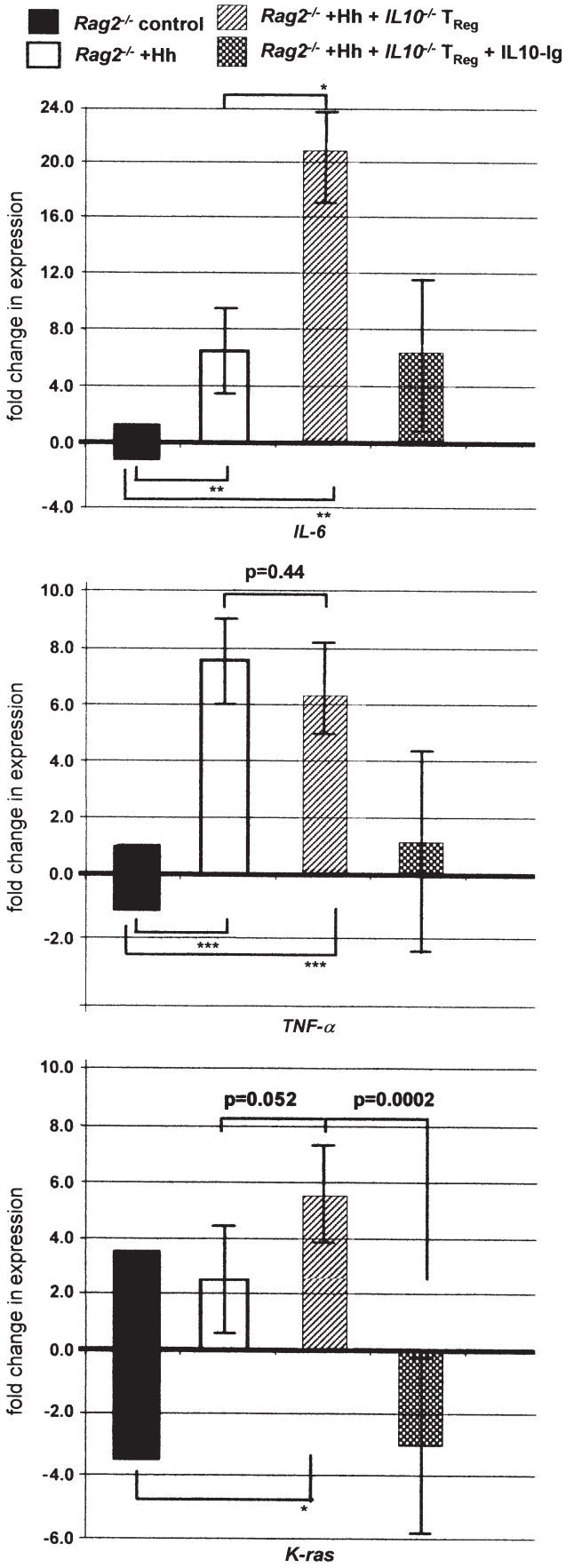
We next examined whether exogenous IL10 was sufficient to reverse malignancy and restore epithelial homeostasis in recipients of *IL10*$^{-/-}$ cells. Mice with established mucinous colonic carcinoma at 6–8 weeks PI were dosed twice weekly intraperitoneally with recombinant IL10-IgG2a fusion protein (IL10-Ig). A similar fusion protein was shown previously to exhibit IL10-like activity *in vitro* and *in vivo* (24). Colonic tissues were then examined at 1 week after onset of treatment. Recipients of IL10-Ig fusion protein ($n = 10$) had minimal colitis ($P < 0.01$) and no invasive cancer (Figure 1G and Table I), when compared with untreated mice or recipients of isogenic sham antibody alone ($n = 8$; data not shown). Assessment of oncogene expression using qRT–PCR (*Taq*Man) revealed that *K-ras* was up-regulated in *H.hepaticus*-infected mice with carcinoma (Figure 4), matching data from humans with mucinous CRC (3), and that *K-ras* returned to baseline after treatment with IL10-Ig fusion protein (Figure 4). These data suggest that IL10 restores epithelial homeostasis in mice through down-regulation of bacteria-triggered carcinogenic pro-inflammatory cytokines.

IL-6 is associated with a reversible neoplastic epithelial phenotype

We hypothesized that uncontrolled up-regulation of pro-inflammatory cytokines (14,25–27), such as TNF-α and IL-6, contribute to inflammation-associated carcinogenesis. Indeed, treatment with TNF-α-neutralizing antibody is sufficient to significantly ($P < 0.05$) reduce epithelial invasion and restore epithelial homeostasis in *H.hepaticus*-infected *Rag2*$^{-/-}$ recipients of *IL10*−/− cells ($n = 8$), when compared with sham antibody-injected matched control mice ($n = 8$), as shown previously in other immune-deficient mouse models (14). However, a downstream pleiotropic cytokine, IL6, has also clearly been linked with neoplastic epithelial invasion in colon (28,29) and other sites in humans and mice. To further investigate roles for bacteria-triggered inflammatory cytokines, we examined pro-inflammatory cytokine gene expression using qRT–PCR in *H.hepaticus*-infected mice at 6–8 weeks PI. Significant elevations in *IL6* (Figure 4) but not *TNF-α* gene expression were observed among groups of mice with rapidly invading epithelia. Adoptive transfer with *wt* T_R cells suppressed pathology and pro-inflammatory cytokine expression; in contrast, supplementation with regulatory cells lacking IL-10 up-regulated expression of IL-6 (Figure 4) and increased frequency of neoplastic invasion (Figure 1). In addition, IL6 protein was elevated ($n = 4$; $\mu = 61.4$ pg/ml) in sera of *H.hepaticus*-infected mice with colitis and cancer, but not in *Helicobacter*-free counterparts ($n = 4$; $\mu = 4.28$ pg/ml). Taken together, these data indicate that localized and systemic up-regulation of cytokine IL-6 was linked with an invasive epithelial phenotype following intestinal microbial infection.

Epithelial oncogene expression is associated with a reversible neoplastic phenotype

Disruption of Tgfβ signaling (30) is a common feature of CRC in humans (2). Given that the mucinous colonic phenotype of our mice is similar to that reported previously in *H.hepaticus*-infected Tgfβ1-knockout mice (31,32), we sought to determine whether Tgfβ



signaling was disrupted after pathogenic bacterial infection in our model. We analysed the expression of Tgfβ pathway members (30) in *H.hepaticus*-infected *Rag2*$^{-/-}$ recipients of IL10$^{-/-}$ cells using qRT–PCR on purified colonic epithelia. We found that *Tgfβ1* was significantly over-expressed in mice 4–6 weeks after infection with *H.hepaticus* (Figure 5). Treatment with IL10-Ig led to normalized expression of *Tgfβ1*. We also analysed the expression of *TgfβRI*, *TgfβRII* and *Smad4* in purified colonic epithelia. The expression of *TgfβRI* was not significantly affected by infection with *H.hepaticus*. In contrast, expressions of *TgfβRII* and *Smad4* were significantly increased in mice infected with *H.hepaticus*. The over-expression of *Tgfβ1*, *TgfβRII* and *Smad4* may be compensatory for a defect further downstream in the signaling pathway. This hypothesis is consistent with the finding that *TgfβRII* and *Smad4* are also over-expressed in *H.hepaticus*-infected Tgfβ1-knockout mice that develop mucinous colonic carcinoma (Figure 5).

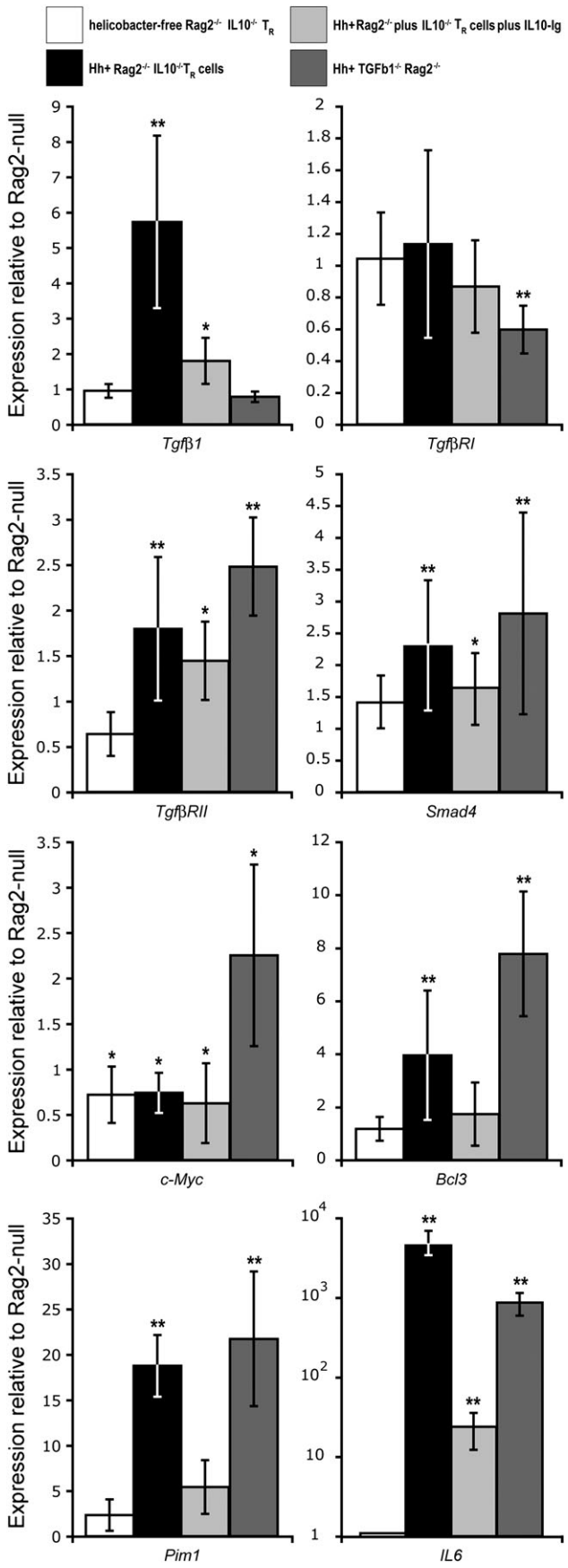
We postulated that other epithelial oncogenes downstream of IL-10 and Tgfβ may contribute to the neoplastic phenotype in *H.hepaticus*-infected mice. When examined at 6–8 weeks PI, purified colonic epithelia from *H.hepaticus*-infected mice had significant increases in the expression of *Pim1*, but not *Bcl3 or c-Myc* (Figure 5). The expression of *Bcl3* is only slightly elevated in infected mice when compared with *Pim1*, which demonstrated a 20-fold increase in expression level after infection. Interestingly, changes in *Pim1* gene expression were evident throughout the entire colonic epithelium and not restricted to the invasive carcinomas. It is noteworthy that *IL6* and *Pim1* are similarly dysregulated in *Rag2*$^{-/-}$ mice entirely lacking *Tgfβ1* (Figure 5) that are also highly susceptible to *H.hepaticus*-induced mucinous carcinoma (31). The over-expression of *IL6* and oncogene *Pim1* is especially intriguing given the proposed roles for these factors in prostate cancer in men (33), and our prior finding that male mice are predisposed to *H.hepaticus*-induced CRC.

The balance between IL-6, IL-10 and Tgfβ predicts epithelial phenotype

We hypothesized that bacteria-triggered up-regulation of IL-6 contributes to a neoplastic colonic epithelial phenotype in helicobacter-infected male animals. To determine whether IL10 restores epithelial homeostasis through down-regulation of IL6, we examined mice that received IL10-Ig fusion protein. Recipients of IL10-Ig fusion protein demonstrated recovery of normalized *IL6* expression in colonic tissue (Figure 4), within days of the onset of treatment (data not shown), and coincident with reversion to normal epithelial morphology. Likewise, elevated serum IL6 protein levels returned to near baseline following 1 week of treatment with IL10-Ig ($n = 4$; $\mu = 11$ pg/ml). This matches prior data showing that IL 10 down-regulates IL 6 in colitis in humans and mice (13,34–36) and led us to propose a mechanistic overview as shown in Figure 6.

It was next tested whether treatment with IL6-neutralizing antibodies restores epithelial homeostasis. Treatment with anti-IL6 antibody alone induced significant ($P < 0.05$) regression of mucinous carcinoma (Figure 1H and Table I) in colonic epithelia of *H.hepaticus*-infected mice. Treatment with IL 6-neutralizing antibody significantly ($P < 0.01$) down-regulated epithelial *K-ras* and *Pim 1* gene expression (data not shown), which suggests that epithelial oncogene expression in mice is readily modulated by bacterially induced

Fig. 4. Relative mRNA levels of *IL-6*, *TNF-α* and *K-ras*. In each sample, the target mRNA was normalized to that of the 'house-keeping' gene *Gapdh*. Numbers represent mean fold change of the individual mRNA levels in reference to the control group (defined as 0 for no change) with _ representing the standard deviation. Expression of *IL6*, but not TNF-α, was significantly correlated with neoplastic epithelial invasion in this mouse model when examined at 4–6 weeks PI. Expression of *K-ras* was also correlated with neoplastic epithelial invasion, and treatment with IL10-Ig fusion protein returned oncogene expression to baseline in this mouse model. Bars, standard deviations. *P* values using the unpaired Student's *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$.



pro-inflammatory gene expression, and that oncogene expression is readily reversible through activities of anti-inflammatory cells or cytokines.

Discussion

We show here that subserosal epithelial invasion resembling mucinous colonic adenocarcinoma in humans arises from bacterially triggered inflammation and depends upon effects of IL-6. Supplementation with IL10-competent regulatory T_R cells inhibited ulceration and invasion and restored epithelial homeostasis through interrelated activities of IL 10 and Tgfβ signaling. The rapid restoration of epithelial integrity after neoplastic invasion indicated that T_R cell-mediated remodeling and ability to restore and maintain epithelial homeostasis (6,37,38) may be more extensive than previously thought. In human patients, similar mucinous tumors carry a poorer prognosis than other types of CRC (4,5). Thus, harnessing constructive endogenous host immune-modulatory capabilities may provide adjunct therapies for IBD-associated CRC and similar neoplastic processes.

Induction of colonic tumors in these mice required pathogenic intestinal bacteria and was greatly exacerbated by the addition of IL10-deficient $CD4^+CD45RB^{lo}CD25^+$ T_R cells. In this situation, IL-6 was significantly up-regulated by addition of Foxp3+ IL10−/− lymphocytes rather than suppressed as with the *wt* IL10-competent T_R cell counterparts. The finding that T_R cells collected from IL10−/− donors demonstrated Foxp3 (21) reveals that Foxp3 expression does not uniformly equate with immune-suppressive function in lymphocytes. The observation that IL10-deficient Foxp3+ T_R cells accumulated in tumors matches the data in patients with intestinal and other cancers (39). In contrast, *wt* IL10-competent Foxp3+ cells were identified only in lymphoid follicles of quiescent colonic mucosa (because prevention of colitis and CRC after *wt* T_R cells approached 100%). However, unpublished data from our laboratory indicate that normally functioning *wt* T_R cells may also accumulate in *H.hepaticus*-induced colonic tumors in $Rag2^{-/-}$ mice. For example, Foxp3+ *wt* T_R cells accumulate at early time points during the tumor regression process (data not shown). Importantly, not all physiologically relevant IL10-secreting regulatory cells express Foxp3 (40). Indeed, peripherally recruited IL10-dependent $CD45RB^{lo}CD25^-$Treg subsets may not

Fig. 5. Gene expression analysis. The relative expression of genes in purified colonic epithelium was measured using qRT–PCR (*Taq*Man) analyses. The expression level of each gene was normalized to that of Tbp and then compared with its expression in uninfected $Rag2^{-/-}$ mice. Treatment groups are as follows: uninfected $Rag2^{-/-}$ mice + IL10-deficient regulatory cells (white); $Rag2^{-/-}$ mice + IL10-deficient regulatory cells infected with *Helicobacter hepaticus* (black); $Rag2^{-/-}$ mice + IL10-deficient regulatory cells infected with *H.hepaticus* then given IL10-Ig fusion protein (light gray) and $Tgf\beta1^{-/-}$ $Rag2^{-/-}$ infected with *H.hepaticus* (dark gray). There is a 5.8-fold increase in the expression of *Tgfβ1* in animals infected with *H.hepaticus* relative to uninfected mice. The expression of *TgfβRII* and *Smad4* was also slightly higher in infected animals, whereas *TgfβRI* did not change. The expression of *c-Myc* was significantly higher in $Tgf\beta1^{-/-}$; $Rag2^{-/-}$ mice infected with *H.hepaticus*, but was unchanged in mice with WT *Tgfβ*1. In contrast, the expression of *Bcl3* was significantly higher in animals infected with *H.hepaticus*. The oncogene whose expression was most affected by *H.hepaticus* infection was *Pim1*, with a 20-fold increase in expression level. This increase in *Pim1* expression is coincident with a very large increase in *IL6* expression. Asterisks denote statistical significance when compared with uninfected $Rag2^{-/-}$ mice ($^*P < 0.05$ and $^{**}P < 0.01$). There is a 5.8-fold increase in the expression of *Tgfβ1* in animals infected with *H.hepaticus* relative to uninfected mice. The expression of *TgfβRII* and *Smad4* was also slightly higher in infected animals, whereas *TgfβRI* did not change. The expression of *c-Myc* was significantly higher in *Tgfβ1−/−; Rag2−/−* mice infected with *H.hepaticus*, but was unchanged in mice with WT *Tgfβ*1. In contrast, the expression of *Bcl3* was significantly higher in animals infected with *H.hepaticus*. The oncogene whose expression was most affected by *H.hepaticus* infection was *Pim1*, with a 20-fold increase in expression level. This increase in *Pim1* expression is coincident with a very large increase in *IL6* expression. Asterisks denote statistical significance when compared with uninfected *Rag2−/−* mice ($^*P < 0.05$ and $^{**}P < 0.01$).

express Foxp3 but are shown to be potent down-regulators of *H.hepaticus*-induced IBD (19). Future studies will examine whether regulatory cells with bacterial antigen specificity (19) may provide enhanced antitumor protection. These cells may act synergistically with CD25+ cells to optimize epithelial wound repair (40). CD4+CD25+ cells utilized for the present experiments are derived from spleen and mesenteric lymph nodes of adult mice and are likely to include diverse regulatory subsets.

In the context of cancer as 'a wound that does not heal' (41), the present data indicate that constructive interplay between T_R cells and epithelia are essential for epithelial cancer prevention. This is not surprising given the earlier findings (6,38) showing that T_R cells are important for intestinal homeostatis. In this capacity, T_R cells function, at least in part, through coordinated activities of cytokines IL 10 and Tgfβ1 (34) that are otherwise linked with epithelial wound repair (30,42,43). There is considerable prior evidence identifying complex and interrelated roles for IL 10 and Tgfβ among lymphocytes (44) and in epithelia (45) in mice with colitis. Within gut epithelial cells, IL 10 was shown previously to be required for normal Tgfβ signaling and SMAD-complex translocation following intestinal bacterial infection with colitogenic *Enterococcus fecalis* (45). Thus, *H.hepaticus* infection may trigger a similar Tgfβ signaling blockade within enteric epithelia of *Rag2*-deficient mice mimicking the neoplastic phenotype in *Tgfβ1*-deficient (31) and *SMAD3*-deficient (46,47) mice. An interesting possibility is that IL 10 expression in lymphocytes is required for proper signaling and elaboration of Tgfβ in our model. The precise relationships between IL 10 and Tgfβ within the tumor microenvironment remain unclear and are the subject of future investigations.

It is interesting that murine mutants with genetic disruptions in the *Tgfβ* signaling pathway, i.e. deficiency in *Tgfβ1* (31) or *SMAD3* (47), are also highly susceptible to *H.hepaticus*-induced colitis and neoplastic invasion. It is probable that the carcinoma phenotype in these models also arises from insufficiently down-regulated inflammatory response to bacterial challenge, i.e. cytokines such as IL-6, and downstream oncogenes such as *K-ras* rather than bacteria burden *per se*. Indeed, earlier data showed that *H.hepaticus* colonization levels were not significantly different between untreated *Rag2*$^{-/-}$ mice and recipients of competent T_R cells or their *IL10*-deficient counterparts (7,9). The present finding of elevated *TβRII* gene expression matches the molecular signature of tumors within pancreas, uterus and other sites in humans (48), raising the possibility of a similar etiopathogenesis.

Neutralization of TNF-α activity alone is sufficient to suppress carcinogenesis in this and other murine models (14,25), indicating that TNF-α over-expression is necessary to sustain carcinoma. In chronic inflammatory diseases such as IBD and arthritis, it is proposed that prolonged elevations in TNF-α arising from bowel induce thymic and peripheral T_R cell insufficiency—leading to further deterioration of health due to unmitigated systemic elevations in inflammatory factors (49). Therapeutic interventions in this setting would then focus upon strategies that promote or restore immune homeostasis in the bowel. Population-based cancer prevention may focus upon enhancing anti-inflammatory functions and potency of relevant regulatory cell subsets. Considering the relatively short half-life for IL-10 fusion protein (13), exogenous IL-10 supplementation is perhaps better suited for selective interventions to stabilize a dysregulated bowel.

The present data support a model that subscribes to the activation of downstream cytokines, such as IL-6, rather than TNF-α *per se*, that are directly linked with the invasive phenotype. Along these lines, one potential therapeutic target is the STAT3 signaling molecule, which appears pivotal in IL-6-mediated progression of malignancy (36,50,51). Carcinogenic effects of IL-6 may also be modulated by oxidative stress and other effects of neutrophils, which are prevalent in invasive epithelial foci (7) and shown previously to be highly responsive to up-regulation of IL 6 (52). Not discounting the risk of neutropenia, therapeutic blockade of IL 6 may be less disruptive to host-protective immunity than neutralization of TNF-α or supplementation with IL10-Ig for treatment of arthritis and other systemic immune disorders. Gut bacteria-triggered elevations in circulating IL 6 levels, as documented in the present mice, may destructively impact systemic immune status and carcinogenic processes in distant organs. IL 6 may contribute to generalized immune impairment through inhibition of anti-inflammatory functions of T_R cells (29,34,36,50). Beyond IL-6-mediated disruption of Tgfβ expression in T lymphocytes (50), IL-6 may drive T_R cells toward a pathogenic T helper (Th)-17 phenotype (36).

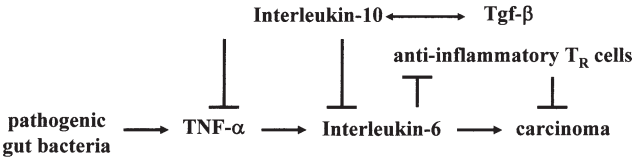


Fig. 6. Proposed mechanistic overview. Pathogenic gut bacteria trigger pro-inflammatory cytokines including TNF-α and IL-6. IL-10 down-regulates bacteria-triggered IL-6 and prevents development of carcinoma. Whether *wt* T_R cells simply secrete IL-10 or require IL-10 for recruitment and anti-inflammatory functions is undetermined.

IL-10, whether integrated with activities of regulatory cells or not, appears to be fundamental in directly down-regulating TNF-α and IL-6 (see Figure 6), in addition to stabilizing Tgfβ signaling (45), during constructive wound repair and prevention of cancer. Whether T_R cells simply secrete IL10 in this setting or also require IL10 for proper development and recruitment of cells with anti-inflammatory phenotype is unclear. It has been shown elsewhere that IL-10 facilitates recruitment of peripheral CD4+ cells to a regulatory phenotype that suppresses ongoing inflammatory processes (40,53). In spite of overwhelming evidence that IL 10 down-regulates pathogenic sequellae of gut microbial infections (35), roles for IL-10 in cancer development and progression are far from clear. For example, IL-10 is also a well-documented suppressor of antitumor immunity (36).

The finding that T_R cells induce regression of carcinoma in this setting also conflicts with other data showing that regulatory T cells inhibit beneficial anti-cancer inflammatory responses (39,54). Whether outcomes differ due to varying etiopathogeneses, diverse regulatory T cell subsets, divergent tumor sites or host immune competency—as present studies utilize *Rag2*-deficient mice otherwise lacking lymphocytes—is unclear. It will be interesting to test whether supplementation with T_R cells is able to rescue a mucinous colon carcinoma phenotype in other mouse models such as *H.hepaticus*-infected SMAD3-deficient mice that have a full repertoire of lymphocytes (47), as has been shown with T_R cells in *Apc*$^{Min/+}$ mice (37). In immune-competent humans, it is probable that dietary, hormonal and stress factors all contribute—along with bacterial triggers and ensuing immune dysregulation—to a multi-factorial process leading to cancer.

The over-expression of oncogene *Pim1* in colonic carcinoma is intriguing, given its proposed role in prostate cancer in men (33), and the increased susceptibility of male mice to *H.hepaticus*-induced CRC in this model (7). In human male patients, IL 6 up-regulation is linked with prostatitis and prostate cancer and IL 6 is known to regulate *Pim1* (33). This is the first report connecting expression of *Pim1* with colitis-associated colon cancer. Reversible up-regulation of epithelial oncogenes correlates with the histologic status of the colonic epithelium in our model and suggests that sustained over-expression of oncogenes may be required for cancer. Chin *et al*. (55) previously demonstrated that sustained over-expression of oncogenic H-*ras* is required to maintain melanoma growth in transgenic mice (55). In the present model, expression of *K-ras* was dependent upon bacteria-triggered cytokine IL 6. Targeted deletion of candidate oncogenes such as *Kras* and *Pim1* is needed to determine whether oncogene expression is coincident or required for tumor maintenance in inflammation-associated carcinoma.

In summary, we have demonstrated that invasive colonic carcinoma is rapidly reversible through IL-10-mediated restoration of epithelial homeostasis. The ability of competent T_R cells to normalize epithelial signaling and restore epithelial homeostasis substantiates links between host immunity, epithelial homeostasis and malignancy. Adoptive transfer of IL10-competent regulatory cells also improved overall body condition and activity level of recipient mice, perhaps providing

insights into links with systemic health. The finding that microbially triggered colitis induces universal up-regulation of IL-6 highlights possible roles for IL-6-mediated inflammatory responses throughout the body. Because dysregulation of IL-6 is a frequent feature of invasive malignancies, it will be important to consider and target immune-mediated effects among the steps initiating or modulating cancer and associated neoplastic invasion in humans.

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Supplementary material

Supplementary material can be found at http://carcin.oxfordjournals.org/

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