

## Transient Ablation of Regulatory T cells Improves Antitumor Immunity in Colitis-Associated Colon Cancer

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### Abstract

Regulatory T cells (Treg) are supportive to cancer development in most tissues, but their role in colitis-associated colon cancer (CAC) remains unclear. In this study, we investigated the role of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg in a mouse model of CAC and in the peripheral blood of patients with colon cancer. These Treg were increased strongly in number in a mouse model of CAC and in the peripheral blood of patients with colon cancer, exhibiting an activated phenotype as defined by elevated expression of GARP, CD103, CTLA-4, and IL10, along with an increased suppressive effect on the proliferation and Th1 cytokine expression of CD4<sup>+</sup>CD25<sup>-</sup> responder T cells *ex vivo*. Transient ablation of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg during tumor development in the CAC model suppressed tumor outgrowth and distribution, accompanied by an increased number of CD8<sup>+</sup>IFN $\gamma$ /granzyme B-producing effector T cells. Conversely, inactivation of IL10 in Treg did not elevate the antitumor response but instead further boosted tumor development. Our results establish a tumor-promoting function for Treg during CAC formation, but they also suggest that a selective, transient ablation of Treg can evoke antitumor responses, with implications for immunotherapeutic interventions in patients with CAC. *Cancer Res*; 74(16); 4258–69. ©2014 AACR.

### Introduction

A particularly striking example of the link between chronic inflammation and cancer is seen in ulcerative colitis, in which chronic colitis or persistent inflammation of the colon is associated with an elevated risk of colorectal cancer (1). Currently, the pathogenesis of ulcerative colitis is not completely understood, but it is widely accepted that the intestinal pathology is caused by T-cell dysregulation that disturbs the clearance of overreactive and autoreactive cells, in addition to an imbalance of T-cell subsets, including T helper cells (T<sub>h</sub>) and regulatory T cells (Treg; refs. 2, 3).

Treg are crucially involved in the maintenance of gut mucosal homeostasis by suppressing abnormal immune responses against commensal flora or dietary antigens. In particular, they exert their function by producing anti-inflammatory cytokines like IL10 and TGF $\beta$  and by preventing both the activation and the effector function of T cells. Pioneering studies by Powrie and colleagues demonstrated that the pathology in a mouse model of T cell–induced colitis, which mimics human inflammatory bowel disease (IBD), can be prevented by adoptive transfer of Foxp3<sup>+</sup> Treg (4). The number of Treg is lower in the peripheral blood of patients with active IBD than in that of control subjects (5). Conversely, the number of Treg is higher in the intestinal mucosa of patients with IBD, and their function is normal, as demonstrated by their ability to suppress the proliferation of effector cells *in vitro* (6, 7). The promising results obtained from studies using mouse models of intestinal inflammation suggest that Treg may be an auspicious candidate for IBD therapy. The hope is that increasing the number of Treg in the intestine will offer a replacement for the immunosuppressive drugs that are often ineffective.

Nevertheless, although the immunomodulatory effects of Treg may exert a protective or therapeutic effect in intestinal inflammation, the suppression of the immune system can be devastating in cancer immunosurveillance. Indeed, the expression of Foxp3 has been associated with poor prognosis in multiple cancer entities, and Treg have been shown to reduce the host antitumor responses (8, 9). This concept is established in the case of sporadic cancer, but the role of Treg in immunosurveillance of inflammation-associated cancer remains undefined. Studies have highlighted the presence of lymphocyte infiltration and inflammatory mediators in the tumor

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microenvironment, a finding indicating that immune cells play an important role in inflammation-mediated tumor promotion. In addition, the numbers of Foxp3<sup>+</sup> Treg are higher in human colon cancers than in surrounding unaffected mucosa (10). Although some studies of colon cancer have shown a paradoxical and statistically significant association between higher numbers of Treg and a favorable prognosis (11), the function of Treg in inflammation-related colon cancer is poorly understood.

In the current study, we focused on the role of Treg in tumor progression and tumor rejection in a mouse model of colitis-associated colon cancer. We investigated the phenotype and function of Foxp3<sup>+</sup> Treg in the microenvironment of inflammation-induced colon cancer and determined whether Treg ablation during tumor development is a potential therapeutic strategy.

## Materials and Methods

### Mice

All animals used in this study were 8- to 12-week-old female and male mice bred and housed under specific pathogen-free conditions in the Laboratory Animal Facility of the University Hospital Essen. BALB/c mice were obtained from Harlan Winkelmann GmbH. C.B6-Tg(Foxp3-DTR/EGFP)23.2Spar (DEREG) mice, C.Foxp3<sup>tm1(cre)Saka</sup> (FIC) mice, and C.II10<sup>tm1Roer</sup> (IL-10<sup>FL</sup>) mice were established as described (12–14). Crossing FIC mice with IL10<sup>FL</sup> mice resulted in mice specifically deficient in IL-10 in Foxp3<sup>+</sup> T cells (termed IL10<sup>fl/fl</sup>/FIC mice). C.Cg-Foxp3<sup>tm2Tch</sup>/J (termed Foxp3/eGFP) mice (The Jackson Laboratory), DEREG mice, FIC mice, and IL10<sup>fl/fl</sup>/FIC mice were subjected to the azoxymethane/dextran sulfate sodium salt (AOM/DSS) protocol.

### Patient samples

Blood samples were obtained from 15 patients with colon cancer (characteristics are summarized in Supplementary Table S1) before therapy was initiated and from 12 healthy volunteers. The study was approved by the Ethics Committee of the Medical Faculty of the University Duisburg Essen (AZ 05-2882). Patients with colon cancer and healthy volunteers participated after giving informed consent.

### Isolation of peripheral blood mononuclear cells

Blood samples from patients with colon cancer and healthy donors were collected in NH<sub>4</sub>-Heparin Monovette tubes (SARSTEDT). To isolate peripheral blood mononuclear cells (PBMC), we centrifuged blood with Bicol density gradient (Biochrom AG). Isolated cells were washed with PBS containing 2 mmol/L EDTA and 2% fetal calf serum (PAA Laboratories) and either analyzed immediately or stored in cell culture medium containing 10% fetal calf serum and 10% DMSO (Carl Roth GmbH) in liquid nitrogen.

### AOM/DSS protocol

CAC was induced as previously described (15). Mice were injected intraperitoneally with the procarcinogen AOM (12.5 mg/kg of body weight; Sigma-Aldrich). After 1 week, mice received drinking water supplemented with 2.5% DSS (MP

Biomedicals; MW, 36–50 kDa) for 5 to 7 days, followed by 2 weeks of regular water. Because of the enhanced susceptibility of IL10<sup>fl/fl</sup>/FIC mice to DSS treatment, these mice were treated with 2% DSS in the first cycle. The DSS administration was repeated twice with 2% DSS. Mice were sacrificed at week 12. For depletion of Foxp3<sup>+</sup> Treg, DEREG mice received weekly intraperitoneal injections of diphtheria toxin (DT; Merck; 30 ng per g of body weight) either from week 1 to 7 or from week 8 to 12. For additional depletion of CD8<sup>+</sup> T cells, DEREG mice were injected intraperitoneally with 250 µg of anti-mouse CD8 antibodies (Bio X Cell; clone YTS 169.4) twice a week from week 8 to 12.

### Isolation of splenocytes, mesenteric lymph node cells, and lamina propria lymphocytes from the colon

Spleens were rinsed with an erythrocyte lysis buffer, meshed through a 100-µm cell strainer, and washed with PBS containing 2 mmol/L EDTA and 2% fetal calf serum (FCS). Mesenteric lymph node cells (mLN) were meshed through a 100-µm cell strainer and washed with PBS containing 2 mmol/L EDTA and 2% FCS. Lamina propria lymphocytes (LPL) were isolated as described previously (16), with minor modifications. Colons were flushed with PBS to remove feces, and opened longitudinally and cut into 1-cm pieces. Tissue pieces were washed twice in PBS containing 3 mmol/L EDTA for 10 minutes at 37°C with rotation. EDTA was removed by washing colon pieces twice in RPMI-1640 containing 1% FCS, 1 mmol/L EGTA, and 1.5 mmol/L MgCl<sub>2</sub> for 15 minutes at 37°C with rotation. Colon pieces were then vortexed intensely, washed with PBS, and digested in RPMI-1640 containing 20% FCS and 100 U/mL collagenase (Sigma-Aldrich) at 37°C for 90 minutes. Remaining tissue was separated from cells by passing the cell suspension through a 40-µm cell strainer and washing with culture medium.

### Histology and immunohistochemistry of colon tissues

Tissue sections (4 µm) were prepared from paraffin-embedded tissue blocks, stained with hematoxylin and eosin (H&E), and evaluated histopathologically in a blinded manner. The extent of tumor distribution was estimated as 0 to 100%. Expression of Foxp3 protein was detected on paraffin-embedded colon sections as described previously (17).

### Antibodies and flow cytometry

Splenocytes, mLN, and LPLs were stained with fluorochrome-labeled anti-mouse CD4 (RM4-5), CD8 (53-6.7), CD90.1 (OX-7), CD11b (M1/70), CD11c (HL3), F4/80 (BM8, Invitrogen), CD45R/B220 (RA3-6B2), GARP (YGIC86), CD103 (M290), and CD62L (MEL-14). Intracellular detection of Foxp3, CTLA-4 and granzyme B (Gzmb) was performed using the Foxp3 Staining Kit from eBioscience with anti-Foxp3 (FJK-16s), anti-CTLA-4 (UC10-4F10-11), or anti-Gzmb (GB12; Invitrogen), according to the manufacturer's recommendations. All antibodies used in this study, except for Gzmb and F4/80, were obtained from either BD Biosciences or eBioscience. For analysis of intracellular IFNγ, cells were stimulated for 4 hours with 10 ng/mL PMA and 1 µg/mL ionomycin in the presence of 5 µg/mL Brefeldin A (all Sigma-

Aldrich). After staining with anti-CD8 antibodies, cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% NP-40, and stained for intracellular IFN $\gamma$  with anti-mouse IFN $\gamma$  (XMG1.2). Human PBMCs were stained with fluorochrome-labeled anti-human CD4 (RPA-T4), CD8 (RPA-T8), CD25 (BC96), and intracellular Foxp3 (PCH101), according to the manufacturer's instructions (eBioscience). Cells were analyzed by flow cytometry on a LSR II instrument using DIVA software (both from BD Biosciences).

### Suppression assay

For murine studies, CD4<sup>+</sup>Foxp3<sup>+</sup> (eGFP<sup>+</sup>) Treg were separated from mLNs and colons using a FACSria II cell sorter (BD Biosciences). CD4<sup>+</sup> T cells were purified from spleens of naive Thy1.1 mice with the CD4<sup>+</sup> T Cell Isolation Kit II (Miltenyi Biotec) and labeled with eFluor670 (eBioscience); these cells served as responder T cells. Thy1.1<sup>+</sup>CD4<sup>+</sup> responder T cells ( $1 \times 10^5$ ) were either cultured alone or cocultured with CD4<sup>+</sup>Foxp3<sup>+</sup> (eGFP<sup>+</sup>) Treg ( $1 \times 10^5$ ) for 3 days in the presence of 1  $\mu$ g/mL anti-CD3 (2C11; BD Biosciences). Irradiated splenocytes from naive BALB/c mice served as antigen-presenting cells ( $3 \times 10^5$ ).

For studies of human cells, CD4<sup>+</sup>CD25<sup>high</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells were sorted from PBMCs using a FACSria II cell sorter. CD4<sup>+</sup>CD25<sup>-</sup> responder T cells ( $2 \times 10^4$  to  $8 \times 10^4$ , depending on the blood donor) were labeled with CFSE (Invitrogen) and either cultured alone or cocultured with CD4<sup>+</sup>CD25<sup>high</sup> Treg ( $1-4 \times 10^4$ ) from the same donor at a ratio of 2:1 in the presence of Treg Suppression Inspector (Miltenyi Biotec) for 3 days. All cells were cultured in Iscove's Modified Dulbecco's Medium with GlutaMAX (Invitrogen) supplemented with 10% heat-inactivated FCS (PAA Laboratories), 25 mmol/L HEPES (Biochrom), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (both Sigma-Aldrich). Proliferation of gated Thy1.1<sup>+</sup>CD4<sup>+</sup> murine or CD4<sup>+</sup>CD25<sup>-</sup> human responder T cells was assessed by loss of the fluorescent dye.

### Cytokine detection

IL10 and IFN $\gamma$  protein were quantified by using the Procarta Cytokine Assay Kit (Panomics) according to the manufacturer's recommendations. The assay was run on a Luminex 200 instrument (Luminex Corporation).

### Quantitative RT-PCR

RNA was isolated from sorted cell subsets using the NucleoSpin RNA XS Kit (Macherey-Nagel) according to the manufacturer's instructions. RNA was obtained from colon biopsies using the RNeasy Fibrous Tissue Kit (Qiagen). Following DNase digestion (Qiagen), cDNA was synthesized with M-MLV Reverse Transcriptase (Promega) and Oligo-dT mixed with Random Hexamer primers (Invitrogen). Real-time RT-PCR was performed using the SYBR Green PCR Kit and specific primers for *IL10* and *RPS9* (Supplementary Table S2) on an ABI PRISM cycler (Applied Biosystems, Life Technologies). Relative RNA levels were determined with included standard curves for each individual gene and further normalization to the housekeeping gene *RPS9*.

### Mouse colonoscopy

Numbers and sizes of tumors in the distal part of the colon were determined by microcolonoscopy following a recently described protocol (18). Tumor development was scored by the total number of tumors and the size of tumors. Tumor grade was determined by size: grade 1, very small but detectable tumor; grade 2, tumor covering up to one-eighth of the colonic circumference; grade 3, tumor covering up to one-fourth of the colonic circumference; grade 4, tumor covering up to half of the colonic circumference; and grade 5, tumor covering more than half of the colonic circumference, as recently described (19).

### Statistical analysis

All results were expressed as mean  $\pm$  SEM. Differences were assessed by the Student *t* test or, when means of more than two groups were compared, by 2-way ANOVA followed by Bonferroni multiple comparison test. Kaplan–Meier plots were used to analyze survival. Comparisons of survival curves were made using the log-rank (Mantel–Cox) test. Data analysis was performed with Prism 5.0 software (GraphPad). Statistical significance was set at the level of  $P < 0.05$ .

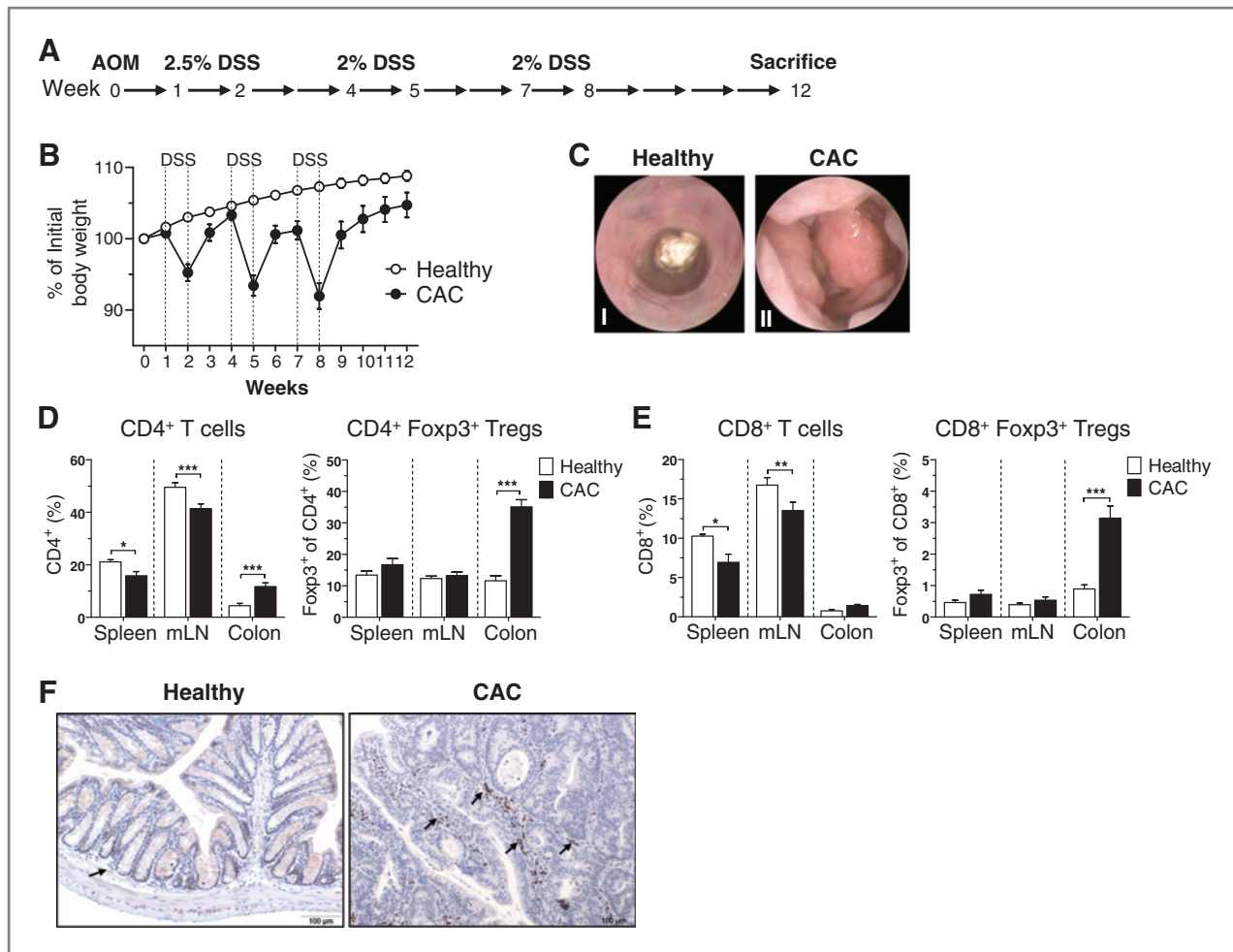
## Results

### Accumulation of Treg in CAC

To determine whether Treg participate in the development of CAC, we subjected Foxp3/eGFP reporter mice to the AOM/DSS regimen (Fig. 1A), a well-established protocol for the induction of CAC (15). The repeated administration of DSS via the drinking water induced a strong inflammatory response in the intestine. Mice lost as much as 10% of their initial body weight during the DSS cycles (Fig. 1B). At week 12 after AOM/DSS treatment, tumor development was documented by endoscopy (Fig. 1C). First, we compared the distribution of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen, mLNs, and the LPLs of colons from healthy mice and CAC mice. We observed a relative reduction of both T cell subsets in the spleens and in the mLNs of CAC mice. Conversely, in tumor-bearing colons, the proportion of CD4<sup>+</sup> T cells was significantly higher but the percentage of CD8<sup>+</sup> T cells remained constant. Most importantly, a strong accumulation of Foxp3<sup>+</sup> regulatory CD4<sup>+</sup> and CD8<sup>+</sup> T cells was detected in the colons but not in the spleens or the mLNs of CAC mice (Fig. 1D and E). Histologic and immunohistochemical analyses revealed that the healthy colonic tissue contained only few Foxp3<sup>+</sup> cells in the lymph follicles of the colon wall. In contrast, the inflammatory cell infiltrate of colonic tumors and their immediate vicinity contained numerous Foxp3<sup>+</sup> cells (Fig. 1F), a finding suggesting an active role of Treg in CAC development.

### Tumor-infiltrating Treg express high levels of CD103, GARP, and CTLA-4 and possess strong suppressive capacity

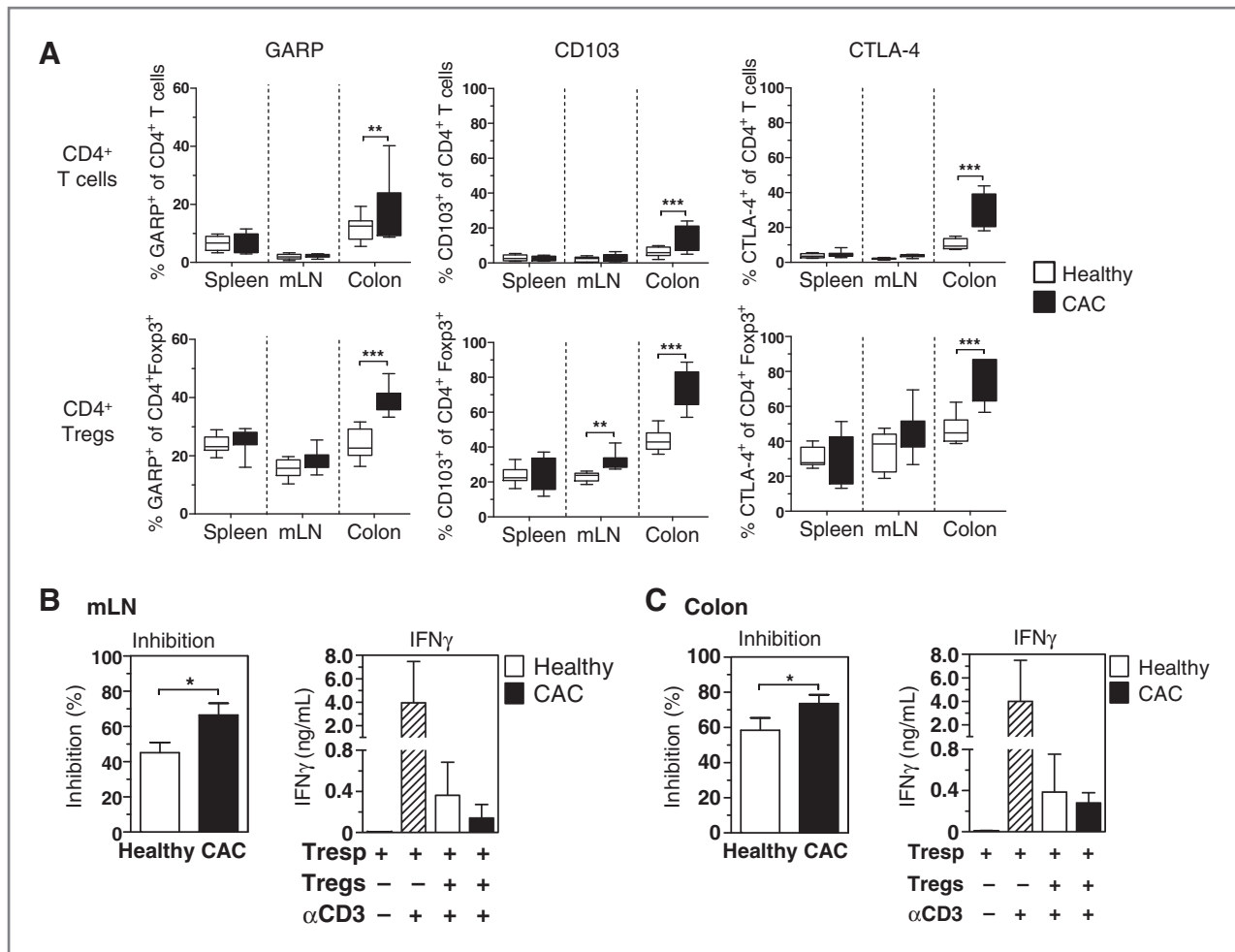
To further explore the role of Treg in CAC, we analyzed their specific phenotype in more detail. Because of the relatively low frequencies of CD8<sup>+</sup>Foxp3<sup>+</sup> Treg in the colons of healthy mice



**Figure 1.** The induction of CAC alters the distribution of T cells and Treg in the colon. **A**, schematic time schedule of the administration of AOM and DSS to induce CAC in Foxp3/eGFP mice. After an intraperitoneal injection of the procarcinogen AOM, three cycles of DSS were given in the drinking water. **B**, weight change of healthy mice (open circles) and AOM/DSS-treated mice (black circles) relative to initial body weight during the course of the experiment. The graph shows data from eight independent experiments (3–4 mice per group). **C**, representative endoscopic images from the distal colon of healthy mice (I) and AOM/DSS-treated mice (II). **D** and **E**, total spleen cells, mLNs, and LPLs from the colons of healthy mice and AOM/DSS-treated mice were isolated and stained with antibodies against CD4 and CD8. The percentages of eGFP<sup>-</sup> (Foxp3<sup>-</sup>) CD4<sup>+</sup> T cells and eGFP<sup>+</sup> (Foxp3<sup>+</sup>) cells among CD4<sup>+</sup> T cells and the percentages of eGFP<sup>-</sup> (Foxp3<sup>-</sup>) CD8<sup>+</sup> T cells and eGFP<sup>+</sup> (Foxp3<sup>+</sup>) cells among CD8<sup>+</sup> T cells were determined by flow cytometry. The data represent the mean  $\pm$  SEM of four independent experiments (2–4 mice per group). Statistical analyses were performed by 2-way ANOVA, followed by the Bonferroni multiple comparison test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . **F**, tissue sections of representative distal colon samples from healthy and CAC mice at week 12 were fixed and stained with H&E to show pathologic changes. Immunohistochemical staining with Foxp3 antibodies shows Treg (black arrows). Images show magnification at  $\times 100$ ; scale bars, 100  $\mu$ m.

and CAC mice, we compared CD4<sup>+</sup>Foxp3<sup>+</sup> Treg with CD4<sup>+</sup> non-Treg cells in healthy and CAC mice. GARP, CD103, and CTLA-4 are known to be induced on activated CD4<sup>+</sup> T cells and to be constitutively expressed by CD4<sup>+</sup>Foxp3<sup>+</sup> Treg (20–22). In the spleens and the mLNs, as many as 10% of the CD4<sup>+</sup> T cells expressed GARP, CD103, and CTLA-4, independent of whether the cells were isolated from healthy or diseased mice. In contrast, a significant increase in the expression of GARP, CD103, and CTLA-4 was detectable in the colons of CAC mice, a finding suggesting an activated phenotype of CD4<sup>+</sup> T cells in the colonic tissue of CAC mice (Fig. 2A and Supplementary Fig. S1). However, the strongest expression of GARP, CTLA-4, and CD103 was detected on CD4<sup>+</sup>Foxp3<sup>+</sup> Treg. In particular, the

expression of GARP, CTLA-4, and CD103 on colonic CD4<sup>+</sup>Foxp3<sup>+</sup> Treg was almost twice as high in CAC mice as in healthy mice. More than 40% of the CD4<sup>+</sup>Foxp3<sup>+</sup> Treg in the colons of CAC mice expressed GARP, whereas approximately 80% expressed CD103 and CTLA-4 (Fig. 2A and Supplementary Fig. S1). To determine whether this activated phenotype was indicative of increased suppressive capacity, we sorted CD4<sup>+</sup>Foxp3<sup>+</sup> Treg from the mLNs and the colons of healthy and CAC mice and cocultured them with CD4<sup>+</sup> responder T cells from naive donor mice. As demonstrated in Fig. 2B and C, we indeed observed that the ability of Treg from the mLNs and the colons of CAC mice to suppress the proliferation and IFN $\gamma$  production of CD4<sup>+</sup> responder T cells was enhanced.

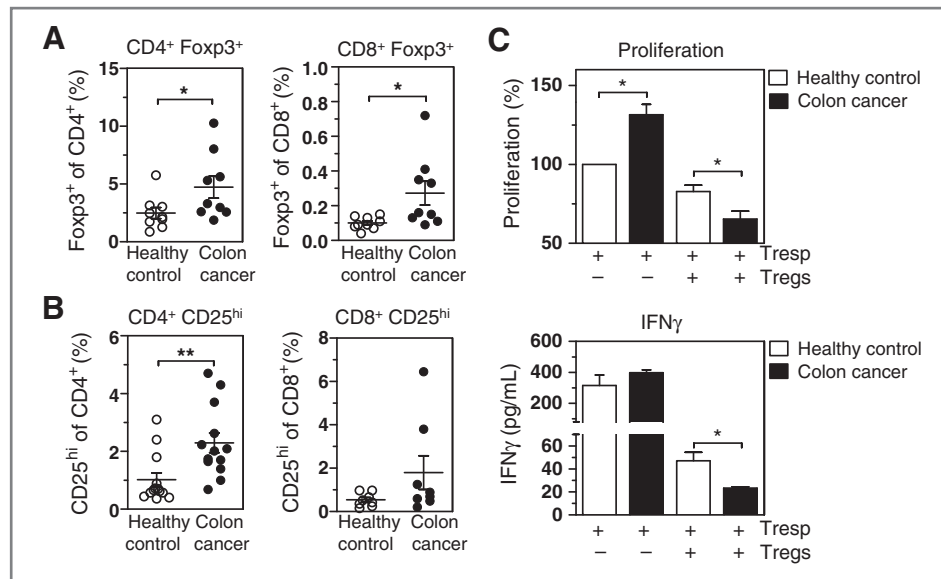


**Figure 2.** Tumor-infiltrating Treg are highly activated and exhibit increased suppressive capability. CAC was induced in Foxp3/eGFP mice. A, at week 12, total spleen cells, mLNs, and LPLs from the colon were isolated and stained with antibodies against CD4, GARP, CD103, and CTLA-4. Cells were analyzed by flow cytometry. Expression of GARP, CD103, and CTLA-4 on gated eGFP<sup>+</sup> (Foxp3<sup>-</sup>) CD4<sup>+</sup> T cells, and on gated CD4<sup>+</sup>eGFP<sup>+</sup> (Foxp3<sup>+</sup>) Treg cells is demonstrated as median (horizontal lines), 25th to 75th percentile (extension of boxes), and range (error bars) of  $n = 2-5$  mice per group. Results of three independent experiments are shown. Statistics were performed by 2-way ANOVA, followed by the Bonferroni multiple comparison test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . To determine the suppressive capability of Treg *in vitro*, we sorted CD4<sup>+</sup>eGFP<sup>+</sup> (Foxp3<sup>+</sup>) T cells (Treg) from mesenteric lymph nodes (B) and colons (C) of AOM/DSS-treated or healthy mice. Treg were cocultured at a ratio of 1:1 with eFluor-labeled CD4<sup>+</sup> responder T cells (Tresp) from naïve Thy1.1 mice and with antigen-presenting cells in the presence or absence of  $\alpha$ -CD3. Proliferation of Tresp was measured by the loss of eFluor dye, and inhibition was calculated. Content of IFN $\gamma$  in the supernatants was determined by Luminex technology. Bar diagrams represent the percentages of inhibition as mean  $\pm$  SEM of at least three independent experiments. Statistical analysis was performed with the Student *t* test. \*,  $P < 0.05$ .

**Increased frequency and suppressive activity of Treg isolated from peripheral blood of patients with colon cancer**

To investigate Treg in human colorectal cancer, we analyzed PBMCs from patients with colorectal cancer and healthy volunteers. We found a significantly higher percentage of Foxp3-expressing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the PBMCs from patients with colorectal cancer as compared with healthy controls (Fig. 3A and Supplementary Fig. S2). Because Foxp3 is not an exclusive marker for human Treg, we also assessed CD25 expression by CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Again, the fraction of CD4<sup>+</sup>CD25<sup>hi</sup> and CD8<sup>+</sup>CD25<sup>hi</sup> T cells was higher in patients with colorectal cancer (Fig. 3B and Supplementary Fig. S2). To

study patient- and healthy donor-derived Treg at a functional level, we purified CD4<sup>+</sup>CD25<sup>hi</sup> cells and cocultured them with autologous CD4<sup>+</sup>CD25<sup>-</sup> responder T cells. CD4<sup>+</sup> responder T cells from patients with colorectal cancer exhibited significantly stronger proliferation and IFN $\gamma$  secretion after stimulation (Fig. 3C). Nevertheless, the suppressive capability of Treg from peripheral blood of patients with colorectal cancer was significantly higher than that of Treg from healthy donors. Moreover, IFN $\gamma$  secretion of CD4<sup>+</sup> responder T cells was decreased when cocultured with Treg from patients with colon cancer (Fig. 3C). In summary, the frequency and the suppressive activity of Treg from patients with colorectal cancer are enhanced and, therefore, may contribute to tumor progression.



**Figure 3.** Increased frequencies and enhanced suppressive capability of Treg in the peripheral blood of patients with colon cancer. PBMCs from healthy donors and from patients with colon cancer were stained for CD4, CD8, CD25, and intracellular Foxp3. A and B, expression of Foxp3 on gated CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells (A) and expression of CD25 on gated CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells (B) was assessed. Scatter dot plots represent the mean  $\pm$  SEM of data from  $n = 8-13$  healthy control donors (open circles) or patients with colon cancer (black circles). C, to measure their ability to suppress CD4<sup>+</sup>CD25<sup>-</sup> responder T cells (Tresp) *in vitro*, CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>hi</sup> Treg were sorted from PBMCs. Tresp were labeled with CFSE and cocultured at a ratio of 2:1 with or without autologous Treg under stimulation with Treg suppression inspector beads. The proliferation of Tresp was measured by the loss of CFSE dye. The proliferation was calculated on the basis of CD4<sup>+</sup>CD25<sup>-</sup> Tresp from healthy donors, which was set to 100%. IFN $\gamma$  in the supernatant was determined by Luminex technology. Bars show the mean  $\pm$  SEM of data from  $n = 4-5$  healthy control donors or patients with colon cancer. Statistical analysis was performed with the Student *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

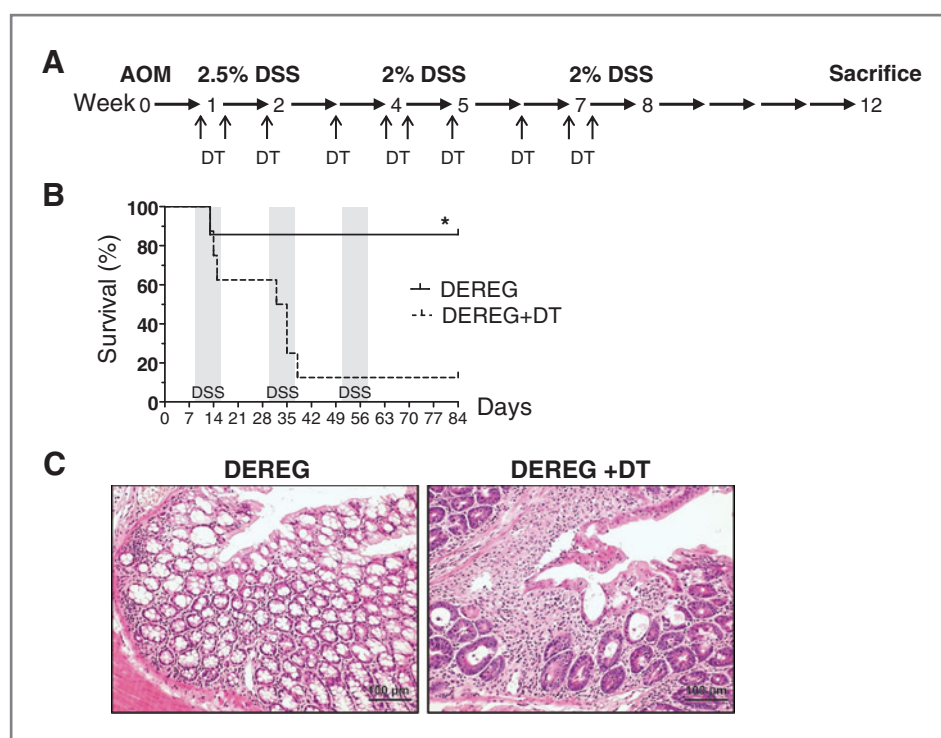
### Treg are essential to control inflammation during CAC induction

Treg may suppress intestinal inflammation and thereby limit inflammation-mediated tumor development. On the other hand, suppression of immune surveillance by Treg might facilitate tumor development in the colon. To tackle this conundrum in a murine model of CAC, we made use of DERE mice, which express a DT receptor (DTR)-enhanced GFP fusion protein under the control of the *foxp3* locus and allow conditional ablation of Foxp3<sup>+</sup> Treg by the injection of DT (12). First, we evaluated the impact of Treg ablation during the acute inflammation phase. Therefore, DERE mice were subjected to the AOM/DSS treatment and DT was applied during the DSS cycles from week 1 to week 7 (Fig. 4A). Importantly, Treg depletion during acute inflammation in AOM/DSS-treated DERE mice, resulted in significantly reduced survival with severe weight loss (more than 20% of initial body weight) early after DSS treatments (Fig. 4B). Histologic analysis of the colons confirmed that mice died as a result of intestinal inflammation shown by increased necrosis of the epithelial layer, crypt damage, crypt abscesses and infiltration of leucocytes in the lamina propria of DT-treated DERE mice (Fig. 4C), demonstrating that Treg are indispensable for the control of the inflammatory process.

### Treg ablation in the late phase of CAC attenuates tumor growth by expanded CD8<sup>+</sup> T-cell effector cells

To ensure the ablation of Treg during the process of tumor formation, we injected DT on a weekly basis starting directly

after the last DSS cycle (Fig. 5A). At week 12, the percentages of Foxp3<sup>+</sup>CD4<sup>+</sup> in the spleens and the mLNs of DERE mice with CAC were up to 70% lower than in untreated DERE mice, whereas the percentages of Foxp3<sup>+</sup>CD8<sup>+</sup> Treg were reduced by approximately 30%. This effect could also be observed in the colon, where the induction of CAC led to the highest accumulation of Foxp3<sup>+</sup>CD4<sup>+</sup> and CD8<sup>+</sup> Treg (Fig. 5B and Supplementary Fig. S3). Interestingly, the absolute number of Foxp3<sup>+</sup>CD4<sup>+</sup> Treg was also decreased but the absolute number of CD8<sup>+</sup>Foxp3<sup>+</sup> Treg remained constant (Supplementary Fig. S4). Cytotoxic CD8<sup>+</sup> T cells play a central role in the antitumor immune response, because they can eliminate dysplastic target cells (23, 24). Remarkably, the ablation of Treg during intestinal tumor formation led to an increase of CD8<sup>+</sup> effector T cells in the colon (Fig. 5C). Increased frequencies of colonic CD8<sup>+</sup>CD62L<sup>low</sup> T cells in AOM/DSS/DT-treated mice were accompanied by enhanced cytotoxic activity, as demonstrated by elevated secretion of IFN $\gamma$  and Gzmb (Fig. 5D and Supplementary Fig. S3). To assess the impact on tumor progression, we performed histologic analyses and colonic endoscopy. The ablation of Treg led to a significant reduction of tumor distribution in the colons (Fig. 5E). Furthermore, colonoscopy revealed reduced numbers and tumor sizes in the distal part of the colon when Treg were depleted (Fig. 5F and G). Thus, Treg play an important role in the control of tumor growth in this murine model of CAC. Ablation of Treg during the late phase of inflammation or the early phase of tumor development effectively reduces the formation and growth of colon tumors. To assess whether



**Figure 4.** Ablation of Treg during the induction of CAC leads to severe inflammation in the colon. **A**, schematic time schedule of administration of AOM and DSS to induce CAC in DEREg mice. To deplete Foxp3<sup>+</sup> Treg during the inflammatory phase, we administered DT twice weekly by intraperitoneal injection from week 1 (before the first DSS treatment) to week 7. **B**, Kaplan–Meier survival curve comparing DEREg mice (black solid line,  $n = 7$ ) and DEREg mice treated with DT (dashed line,  $n = 8$ ). There was a significant difference in survival between DEREg mice and DEREg mice receiving DT (log-rank test,  $^*P = 0.012$ ). Data are pooled from two independent experiments. **C**, at day 14, after the first DSS cycle, representative tissue sections of colon samples from DEREg mice and DEREg mice treated with DT were fixed and stained with H&E to show pathologic changes. Images show magnification at  $\times 100$ ; scale bars, 100  $\mu\text{m}$ .

the expansion of CD8<sup>+</sup> effector T cells during Treg ablation is responsible for the reduced tumor growth, we depleted Treg in the presence of a CD8-depleting antibody (Fig. 6A–C). The depletion of CD8<sup>+</sup> T cells during tumor growth in DEREg mice already slightly increased the numbers and the sizes of tumors but strikingly, the concomitant depletion of Treg and CD8<sup>+</sup> T cells completely attenuated the Treg ablation–mediated antitumor effect in DEREg mice (Fig. 6D and E). Together, these results indicate that Treg control the function of CD8<sup>+</sup> effector T cells during tumor growth in CAC.

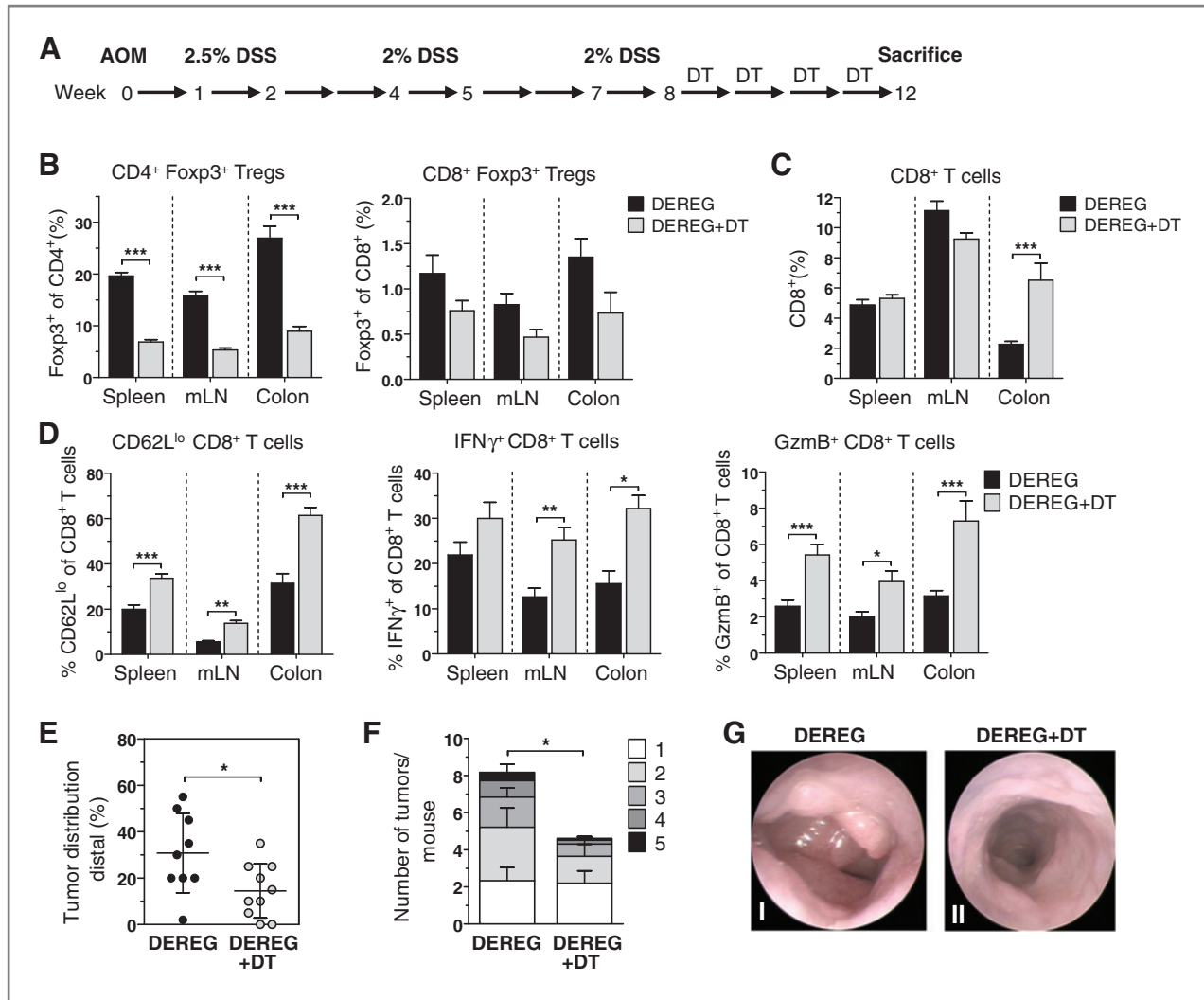
#### Production of IL10 in the colon is increased in mice with CAC

IL10 is particularly important for the function of Treg at environmental interfaces at which tolerance induction is the hallmark of intestinal homeostasis. To assess how activated Treg exert their inhibitory mechanism during CAC, we determined the production of IL10 in biopsy specimens from the colons of healthy mice and CAC mice. *IL10* mRNA and IL10 protein was significantly increased in tumor tissues from CAC mice compared with colonic tissues from healthy control mice (Fig. 7A). Further analysis revealed that the elevated expression of *IL10* during CAC depends on CD45<sup>+</sup> lamina propria mononuclear cells. In particular, Treg, macrophages, and dendritic cells expressed higher levels of *IL10* (Fig. 7A).

#### Deletion of IL10 from Treg is associated with increased tumor progression

To determine whether increased IL10 production of colonic CD4<sup>+</sup>Foxp3<sup>+</sup> Treg in CAC mice is a key factor in

the suppression of efficient antitumor immune responses, we induced CAC in mice with IL10-deficient Treg (IL10<sup>fl/fl</sup>/FIC). When using the standard protocol of CAC induction by the administration of 2.5% DSS in the drinking water, almost all mice with IL10-deficient Treg died from intestinal inflammation (data not shown). Thus, we modified the AOM/DSS protocol to administer 2.0% DSS in the first cycle of DSS treatment. Even then, we could detect enhanced inflammation in IL10<sup>fl/fl</sup>/FIC mice, characterized by pronounced loss of body weight (Fig. 7B). However, all AOM/DSS-treated IL10<sup>fl/fl</sup>/FIC transgenic mice survived until the end of the experiment. To further characterize the role of Treg, we measured the percentage of Foxp3<sup>+</sup>CD4<sup>+</sup> and CD8<sup>+</sup> Treg in spleens, mLNs, and colons 12 weeks after CAC induction. Interestingly, the inactivation of IL10 in Treg led to an increase in the percentage of Foxp3<sup>+</sup>CD4<sup>+</sup> and CD8<sup>+</sup> Treg in mLNs, and even more prominently in the colons of AOM/DSS-treated mice (Fig. 7C). Simultaneously, a slight reduction in the percentage of CD8<sup>+</sup> T cells in the mLN of IL10<sup>fl/fl</sup>/FIC mice compared with FIC control mice was observed, and the frequency of CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> effector T cells from IL10<sup>fl/fl</sup>/FIC mice was decreased, with the highest prevalence in the mLN and the colon (Fig. 7D). Furthermore, we detected an increased tumor burden in the distal part of the colons from IL10<sup>fl/fl</sup>/FIC mice (Fig. 7E and F). Whereas FIC mice developed only a few small tumors (grades 1 and 2), endoscopy revealed enhanced tumor dissemination and larger tumors in the colons of IL10<sup>fl/fl</sup>/FIC mice (Fig. 7F and G). Taken together, these findings indicate that the deletion of IL10 from Treg during inflammation-mediated colon cancer provokes a more severe inflammation, resulting in enhanced tumor formation and growth.



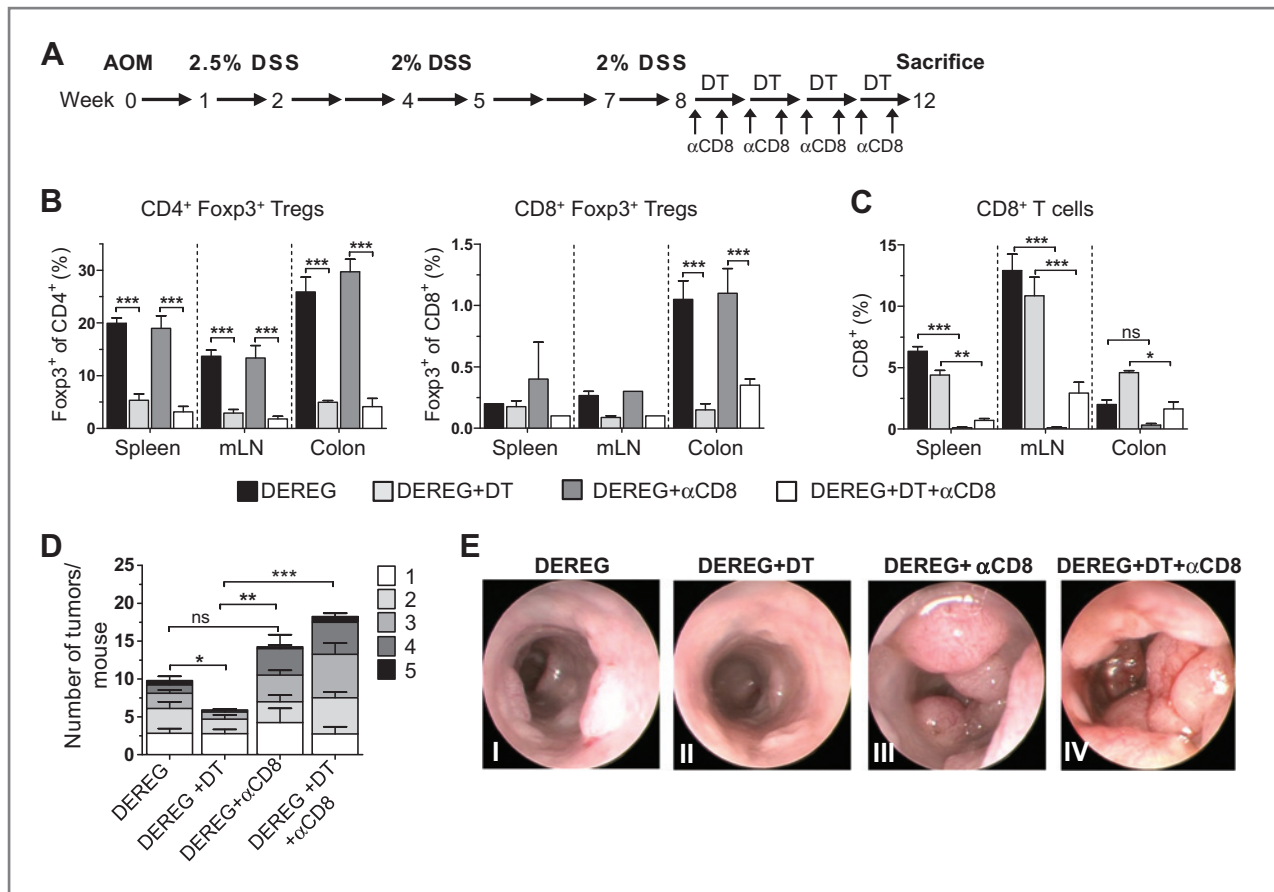
**Figure 5.** Impact of Treg ablation on carcinogenesis and adaptive immunity. **A**, schematic time schedule of administration of AOM and DSS to induce CAC in Dereg mice. To deplete Foxp3<sup>+</sup> Treg during the recovery period after the last DSS treatment, we administered DT weekly by intraperitoneal injection. At week 12, total spleen cells, mLN, and LPLs from the colons of Dereg mice and Dereg mice treated with DT were isolated and stained for the expression of CD4, CD8, CD62L, intracellular IFN $\gamma$ , and GzmB. **B**, the expression of eGFP (Foxp3) on CD4<sup>+</sup> and CD8<sup>+</sup> T cells was determined by flow cytometry. **C**, CD8<sup>+</sup> T cells were gated and analyzed for the expression of IFN $\gamma$ , CD62L, and GzmB (**D**). Bars represent the mean  $\pm$  SEM of data from at least three independent experiments (with 3–4 mice per group). Statistical analyses were performed by 2-way ANOVA, followed by the Bonferroni multiple comparison test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . **E**, to determine the distribution of tumors in the distal colon at week 12, we scored histopathology in a blinded fashion. The scatter dot plot comprises the results of three independent experiments (with 3–4 mice per group), and the statistical analysis was performed with the Student  $t$  test. \*,  $P < 0.05$ . **F**, the average number and size of tumors in the distal part of the colon of each mouse was determined by murine endoscopy. Bars represent the mean  $\pm$  SEM of data from three experiments (with 3–4 mice per group). Statistical analyses were performed by the Student  $t$  test. \*,  $P < 0.05$ . **G**, representative endoscopic images from the distal colon of Dereg mice (I) and Dereg mice treated with DT (II).

## Discussion

IBD, including ulcerative colitis and Crohn disease, is prototypic chronic inflammatory disease of the gastrointestinal tract, which is associated with an increased risk of colorectal cancer development (1). The hallmarks of inflammation-related cancer include the presence of inflammatory cells and inflammatory cytokines or chemokines in tumor tissues. However, medical and scientific data from human and mouse studies indicate that it is the balance between proinflamma-

tory and anti-inflammatory activities that strongly influences clinical outcome (25). In this study, we showed that the induction of CAC augments the frequency of highly activated Treg in colonic tumor tissues. This augmentation may facilitate tumorigenic processes, because transient ablation of Treg dampens tumor progression and results in an improved CD8<sup>+</sup> T-cell response. The clinical relevance is supported by our finding of increased frequencies and suppressive capability of Treg in the peripheral blood of patients with colorectal cancers.

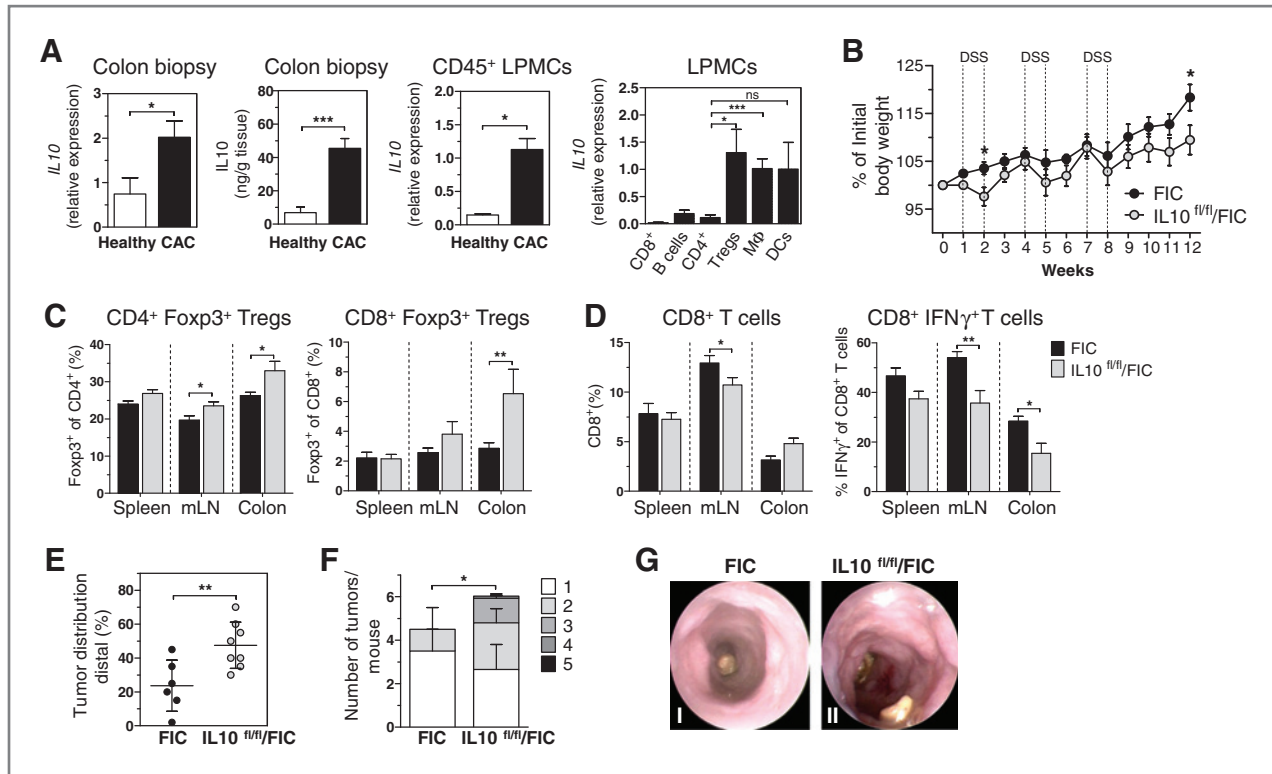




**Figure 6.** Impact of Treg and CD8<sup>+</sup> T-cell ablation on carcinogenesis. A, schematic time schedule of administration of AOM and DSS to induce CAC in DEREg mice. To deplete Foxp3<sup>+</sup> Treg and CD8<sup>+</sup> T cells during the recovery period after the last DSS treatment, we administered DT and/or  $\alpha$ CD8 antibodies weekly by intraperitoneal injection. At week 12, total spleen cells, mLN, and LPLs from the colons of DEREg mice, DEREg mice treated with  $\alpha$ CD8, DEREg mice treated with DT, and DEREg mice treated with DT and  $\alpha$ CD8 were isolated and stained for the expression of CD4 and CD8. B and C, the expression of eGFP (Foxp3) on CD4<sup>+</sup> and CD8<sup>+</sup> T cells (B) and the percentages of CD8<sup>+</sup> T cells (C) were determined by flow cytometry. Bars represent the mean  $\pm$  SEM of data from one experiment (with 3–4 mice per group). Statistical analyses were performed by 2-way ANOVA, followed by the Bonferroni multiple comparison test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . D, to determine the distribution and progression of tumors, the average number and size of tumors in the distal part of the colon of each mouse was determined by murine endoscopy at week 12. Bars represent the mean  $\pm$  SEM of data from one experiment (with 3–4 mice per group). Statistical analyses were performed by the Student *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . E, representative endoscopic images from the distal colon of DEREg mice (I), DEREg mice treated with DT (II), DEREg mice treated with  $\alpha$ CD8 (III), and DEREg mice treated with DT and  $\alpha$ CD8 (IV).

Treg are crucially involved in the maintenance of gut mucosal homeostasis by suppressing abnormal immune responses against commensal flora, dietary antigens, or autoantigens (26, 27). Adoptive transfer of Treg effectively alleviates or prevents symptoms of colitis (28, 29), and in consequence reduces the incidence of inflammation-induced colon cancer (30). Interestingly, we detected significantly elevated numbers of Treg within tumors from CAC mice. In various solid tumors, including ovarian and pancreatic cancer, Treg accumulation in tumors and peripheral blood has been generally linked to an unfavorable disease outcome (31, 32). Conversely, no significant association between the absolute number of tumor-infiltrating Treg and prognosis was established in several studies involving patients with colorectal cancer. Some studies indicate that a high frequency in tumor-infiltrating Treg is associated with a favorable rather than a dismal prognosis. It is hypothesized that Treg suppress bacteria-driven inflammation that promotes

carcinogenesis and, thus, benefits the host (33, 34). However, evidence suggests that Treg may have different effects depending upon the extent of inflammation and tumor development or progression (35). Here, we demonstrated that the ablation of Treg during acute colitis tremendously exacerbates the inflammatory response, resulting in significant less survival, but depletion of Treg in the late phase of inflammation abrogates the tumor growth accompanied with an increased frequency of CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup>GzmB<sup>+</sup> cytotoxic T cells, which are the main effectors of an adaptive antitumor response. This is associated with reduced tumor numbers and sizes. Our finding is well in line with a recent study showing that a high density of CD8<sup>+</sup> T cells with a low density of Foxp3<sup>+</sup> cells in the colon is associated with a favorable outcome in patients with colorectal cancer (36). Therefore, our findings suggest that CD8<sup>+</sup> effector T cells and Foxp3<sup>+</sup> Treg may interact to regulate the antitumor immune response during CAC.



**Figure 7.** Induction of CAC leads to the increased secretion of IL10 in the colon and inactivation of IL10 in Treg favors tumor growth. **A**, CAC was induced in Foxp3/eGFP mice. At week 12, total RNA was isolated from biopsy specimens of the colons of healthy and CAC mice, and relative expression of IL10 was determined by quantitative RT-PCR. Bars show mean  $\pm$  SEM of data from  $n = 6-8$  individual mice. A small biopsy from the colon of healthy and CAC mice was incubated for 6 hours in culture medium. IL10 concentrations in the supernatants were determined by Luminex technology. Bars show the mean of the amount of IL10 per gram of tissue  $\pm$  SEM from  $n = 5-6$  individual mice. CD45<sup>+</sup> lymphocytes were sorted from the colons of healthy and CAC mice, RNA was isolated, and the relative expression of IL10 was measured by quantitative RT-PCR. Bars show mean  $\pm$  SEM of data from  $n = 2$  mice. Lymphocytes were isolated from the colons of CAC mice and CD8<sup>+</sup>CD11c<sup>-</sup>eGFP<sup>-</sup> (Foxp3<sup>-</sup>) T cells (CD8<sup>+</sup>), B220<sup>+</sup>CD11c<sup>-</sup>eGFP<sup>-</sup> (Foxp3<sup>-</sup>) B cells, CD4<sup>+</sup>eGFP<sup>-</sup> (Foxp3<sup>-</sup>) T cells (CD4<sup>+</sup>), CD4<sup>+</sup>eGFP<sup>+</sup> (Foxp3<sup>+</sup>) Treg, CD11b<sup>+</sup>F4/80<sup>+</sup>CD11c<sup>int</sup> macrophages (Mφ), and CD11b<sup>+</sup>CD11c<sup>+</sup>F4/80<sup>-</sup> dendritic cells (DC) were sorted, RNA was isolated, and the relative expression of IL10 was measured by quantitative RT-PCR. Bars show mean  $\pm$  SEM of data from  $n = 3-6$  CAC mice. Statistical analysis was performed with the Student *t* test. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ . **B**, CAC was induced in FIC mice and in mice specifically deficient in IL10 in CD4<sup>+</sup>Foxp3<sup>+</sup> T cells, termed IL10<sup>fl/fl</sup>/FIC mice. Weight change of AOM/DSS-treated FIC mice (black circles) and IL10<sup>fl/fl</sup>/FIC mice (gray circles) relative to initial body weight. The graph shows data from three independent experiments (4–5 mice per group). Statistical analysis was performed with the Student *t* test. \*,  $P < 0.05$ . **C** and **D**, at week 12, total spleen cells, mLN, and LPLs from the colons of FIC and IL10<sup>fl/fl</sup>/FIC mice were stained for the expression of CD4, CD8, intracellular Foxp3, and IFN $\gamma$ . The expression of Foxp3 on gated CD4<sup>+</sup> or CD8<sup>+</sup> T cells (**C**) and the percentage of CD8<sup>+</sup> T cells (**D**) were assessed. Gated CD8<sup>+</sup> T cells were analyzed for the expression of IFN $\gamma$ . Bars represent the mean  $\pm$  SEM of data from one of three independent experiments (with 4–5 mice per group). Data were analyzed by 2-way ANOVA, followed by the Bonferroni multiple comparison test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . **E** and **F**, the distribution of tumors at week 12 was scored by histopathology in a blinded fashion. The average number and size of tumors in the distal part of the colon of each mouse were determined by endoscopy. Data of two independent experiments (with  $n = 2-4$  mice) are shown and the statistics was determined with the Student *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . **G**, representative endoscopic images from the distal colon of FIC mice (I) and IL10<sup>fl/fl</sup>/FIC mice (II).

Given the detrimental role of Treg on tumor progression, we attempted to identify target molecules that could bring about selective ablation of these cells. We detected strong expression of GARP, CTLA-4, and CD103 by Treg in the colons of CAC mice. GARP has recently been reported to be a highly specific molecule for activated Foxp3<sup>+</sup> Treg with a strong suppressive function (22, 37). The frequency of GARP<sup>+</sup>Foxp3<sup>+</sup> Treg was found to be highly elevated in advanced hepatocellular carcinoma, and the depletion of GARP<sup>+</sup> T cells in combination with other markers restored CD8<sup>+</sup> T cell-dependent GzmB production (38). This finding is well in line with our results, because Treg isolated from colonic tumors exhibited a higher suppressive capacity than Treg isolated from healthy colons. CTLA-4 is a coinhibitory molecule expressed by activated T

cells and by a large proportion of Foxp3<sup>+</sup> Treg. Ipilimumab, an antibody against CTLA-4 molecule was shown to inhibit Treg-induced suppression *in vivo* and has been approved for treatment of metastatic malignant melanoma (39, 40). Because of the increased expression of GARP and CTLA-4 by Treg in colonic tumors, further exploration of therapeutic ablation of GARP<sup>+</sup> T cells and blocking CTLA-4 in CAC is warranted.

In this study, we demonstrated that most tumor-associated Treg in CAC mice express CD103, a cell-surface protein of the integrin family. It has been previously shown that CD103 expression defines a subset of Treg with specific properties: these Treg exhibit enhanced migration into inflamed tissues (41), a finding explaining the accumulation of these cells in the tumors of CAC mice. Another important property of these cells

is their improved suppressive function. Tumor-derived CD103<sup>+</sup> Treg suppress CD8<sup>+</sup> T-cell responses more strongly than CD103<sup>-</sup> Treg (42, 43). The observed prevalence of CD103<sup>+</sup> Treg in our model of CAC may explain this enhanced immunosuppression.

IL10 is an immunomodulatory cytokine that mainly limits and terminates inflammatory responses. Experimental findings indicate that IL10 may play a role in the pathogenesis of IBD and CAC (44, 45). Here, we found increased levels of IL10 in colonic tissue of CAC mice. Further, we identified Treg together with macrophages and dendritic cells as the main source of IL10 in the colon. These results imply an IL10-dependent control of the antitumor response. However, we observed that specific inactivation of IL10 in Treg during CAC development led to an increased tumor burden rather than a stronger antitumor response. The reduced activity of IL10-deficient Treg was seemingly compensated for by an increased percentage of Treg in the colons of IL10<sup>fl/fl</sup>/FIC mice with CAC. Thus, under conditions of low IL10, there is poor regulation of inflammation, which then may further contribute to cancer growth. Whether the specific ablation of IL10 during tumor progression rather than during the inflammation phase would be a beneficial option remains unclear.

Most of the analyses performed in this study are based on CD4<sup>+</sup>Foxp3<sup>+</sup> Treg. Interestingly, we also detected an increase in the percentage of CD8<sup>+</sup>Foxp3<sup>+</sup> Treg in colonic tumors of CAC mice and in the peripheral blood of patients with colon cancer, but the frequency of CD8<sup>+</sup>Foxp3<sup>+</sup> Treg in the colonic tumors was 100-fold less than the frequency of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg. CD8<sup>+</sup>Foxp3<sup>+</sup> T cells were recently found in prostate cancer specimen (46). In addition, Chaput and colleagues identified a CD8<sup>+</sup>Foxp3<sup>+</sup> T-cell population in human colorectal tumors with a similar phenotype to CD4<sup>+</sup>Foxp3<sup>+</sup> Treg. These CD8<sup>+</sup> Treg showed an activated phenotype with upregulation of CTLA-4. Moreover, they were able to suppress CD4<sup>+</sup> T-cell proliferation and T<sub>H</sub>1 cytokine production *ex vivo* (47), suggesting that CD8<sup>+</sup>Foxp3<sup>+</sup> Treg may contribute to tumoral immune escape. Nevertheless, CD8<sup>+</sup>Foxp3<sup>+</sup> Treg represent only a small fraction of CD8<sup>+</sup> T cells *in vivo*, and their clinical relevance remains to be determined.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** A. Frede, M. Schuler

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