CD4+CD25+ Regulatory Lymphocytes Induce Regression of Intestinal Tumors in *Apc*$^{Min/+}$ Mice

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Abstract

Colorectal cancer in humans results from sequential genetic changes in intestinal epithelia commencing with inactivation of the *APC* tumor suppressor gene. Roles for host immunity in epithelial tumorigenesis are poorly understood. It has been previously shown that CD4+CD25+ lymphocytes inhibit colitis-associated epithelial tumors in Rag-deficient mice. Here we show that addition of CD4+CD25+ lymphocytes in *Apc*$^{Min/+}$ mice reduces multiplicity of epithelial adenomas. Interleukin-10 was required in regulatory cells for therapeutic effect. Recipients of regulatory cells showed increased apoptosis and down-regulation of cyclooxygenase-2 within tumors coincident with tumor regression. These data suggest a role for regulatory lymphocytes in epithelial homeostasis in the *Apc*$^{Min/+}$ mouse model of intestinal polyposis. Similarities with cancer of the breast, prostate, lung, and other sites raise the possibility of broader roles for regulatory lymphocytes in prevention and treatment of epithelial cancers in humans. (Cancer Res 2005; 65(10): 3998-4004)

Introduction

Colorectal cancer is a leading cause of morbidity and death in humans (1, 2). A widely used model for human colorectal carcinogenesis is the multiple intestinal neoplasia (Min) mouse, which has a germ-line mutation in the *Apc* tumor suppressor gene (*Apc*^{Min}; ref. 3). Inactivation of *Apc* in this model results in formation of intestinal adenomas and recapitulates early events in human colorectal cancer (4, 5). It has become clear during the past two decades that aspirin and nonsteroidal anti-inflammatory drugs (NSAID) decrease the risk for colon cancer in humans and mice (6–9) at least in part through activities of an apoptotic modulator, cyclooxygenase 2 (COX-2; refs. 6, 10). However, the interplay of immune events in colonic malignancy is not well understood.

Prior studies using adoptive transfer of CD4+CD25+ regulatory lymphocytes in Rag-deficient mice have shown interleukin-10 (IL-10)–dependent suppression of colitis-associated colon cancer (11, 12), suggesting that inhibition of enteric inflammation may be pivotal in intestinal tumorigenesis. Although anti-inflammatories such as NSAIDs have been shown to decrease tumor latency and burden in *Apc*$^{Min/+}$ mice (10, 13), roles for regulatory lymphocytes that inhibit enteric inflammation have not been determined in this model. Thus, we examined whether CD4+CD25+ regulatory cells may modulate development and progression of intestinal tumors using adoptive transfer of purified wild-type CD4+CD25+ regulatory cells into *Apc*$^{Min/+}$ C57BL/6 mice.

Materials and Methods

Apc$^{Min+/-}$ C57BL/6 Mice

All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care–approved facilities in static microisolator cages. Mice had mouse pathogen–free health status as previously described (12). There was no evidence of murine *Helicobacter* spp. by culture or PCR in treated or untreated mice. *Apc*$^{Min/+}$ mice on a C57BL/6J background were originally obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in-house to wild-type (*wt*) C57BL/6J mice to generate *Apc*$^{Min/+}$ and *wt* mice for experimental recipients and donors, as described below.

Experimental Design

Overall, 36 *Apc*$^{Min/+}$ mice served as untreated controls, 44 *Apc*$^{Min/+}$ mice were treated with *wt* regulatory T cells, and 12 *Apc*$^{Min/+}$ mice were treated with IL-10–deficient regulatory T cells. Studies included slightly more males than females in both treatment and control groups. Experiments were conducted using two to three separate trials with six to eight mice each. Initial trials involved two cell transfers timed 3 weeks apart. Subsequent trials involved one adoptive cell transfer only when it was discovered that a single dose of cells was sufficient for preventative or therapeutic effect. Trials with statistically similar results were then combined for analyses.

Experiment 1: Immunotherapy using CD4+ regulatory cells in *Apc*$^{Min/+}$ mice. To determine whether transfer of CD4+CD25+ lymphocytes was able to inhibit intestinal adenomas, a total of 13 *Apc*$^{Min/+}$ mice, 3 to 4 months of age, were dosed with *wt* regulatory cells. Mice were then euthanized 3 weeks later and compared with 13 untreated age-matched *Apc*$^{Min/+}$ controls.

Experiment 2: Role of interleukin-10 in CD4+ regulatory cells. To determine whether IL-10 was necessary in CD4+CD25+ cells for therapeutic effect, 12 *Apc*$^{Min/+}$ mice, 3 to 4 months of age, were treated with regulatory cells derived from C57BL/6 mice lacking IL-10. Findings were compared with 11 age-matched untreated *Apc*$^{Min/+}$ mice and 12 *Apc*$^{Min/+}$ recipients of *wt* regulatory cells. All mice were euthanized at either 3 or 6 weeks after transfer of regulatory cells.

Experiment 3: Immunotherapy using CD4+ regulatory cells in older *Apc*$^{Min/+}$ mice. To determine whether transfer of CD4+CD25+ lymphocytes was able to induce regression of established intestinal tumors, 14 *Apc*$^{Min/+}$ mice, 4.5 to 6 months of age, were treated with *wt* regulatory cells and were euthanized at 3 to 7 weeks after the initial transfer. Treated mice were compared with age-matched untreated *Apc*$^{Min/+}$ mice (n = 18). Data from two trials were similar and were combined in Fig. 2 for these analyses.

In addition, to establish the kinetics of tumor regression, 11 *Apc*$^{Min/+}$ mice, 5 to 6 months of age, were treated with regulatory cells and then euthanized 2 to 4 days later. Data from three trials were similar and were combined for these analyses. Four age-matched untreated *Apc*$^{Min/+}$ control mice were statistically similar to other age-matched untreated *Apc*$^{Min/+}$ controls, and were combined for these analyses.

Note: S.E. Erdman and J.J. Sohn contributed equally to this work.

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Adoptive Transfer of T Cells in *Apc*$^{Min/+}$ Mice

To determine the ability of T lymphocytes to modulate polyp formation, we transferred purified CD4$^+$CD45RB^{lo}CD25$^+$ T lymphocytes from *wt* C57BL/6 or IL-10–deficient C57BL/6 donors into *Apc*$^{Min/+}$ mice. Half of the donor mice were male and half were female for each cell transfer experiment, thus, all mice received both male and female lymphocytes. To obtain viable and highly purified populations of lymphocytes, single-cell suspensions from spleen and mesenteric lymph nodes from donor mice were prepared as described previously (12). Reanalysis of these cells before transfer into mice indicated that they were >96% pure. Anesthetized mice were injected i.v. in the retro-orbital sinus with 3×10^5 to 4×10^5 T cells suspended in 0.2 mL of HBSS.

Quantitation of Intestinal Tumors

Location of tumors was recorded using a stereomicroscope at 10× magnification. Location of tumors in the small intestine was recorded as distance from the pylorus, and in the colon as distance from ceco-colic junction.

Histologic Evaluation

Formalin-fixed tissues were processed, embedded in paraffin, sectioned at 5 μm, and stained with H&E. Lesions were evaluated by a board-certified veterinary pathologist blinded to sample identity. Inflammation within intestinal tissue sections was graded on a scale of 0 to 4 with ascending severity as previously described (12, 14). Categorical lesion scores are presented as median score and range (in parentheses) for each group.

Immunohistochemistry

Immunohistochemical analyses of formalin-fixed paraffin-embedded intestinal sections from *Apc*$^{Min/+}$ mice were carried out to assess apoptosis and proliferation *in situ* using anti–caspase-3 rabbit polyclonal antibody (Cell Signaling Technologies, Inc., Beverly, MA) according to the recommendations of the manufacturer. Anti-Ki67 antibody (BD Biosciences, San Jose, CA) was used as a proliferation marker. Briefly, 1:50 dilution of the Ki67 antibody was used with the ARK kit (DAKO Cytomation, Carpenteria, CA) according to the recommendations of the manufacturer. Antigen-antibody binding for both caspase-3 and Ki67 was visualized using diaminobenzidine as substrate and all sections were counterstained with hematoxylin. High power fields (×400) of the tumors in the tissue section from each animal were acquired using a Nikon DXM 1200 digital camera and an Olympus BX50 microscope. The resolution (number of pixels) was held constant for each image. Using Adobe Photoshop (Version 6.0) color range tool, the total number of pixels within the caspase-3 positive nuclei (brown) with associated morphology of apoptotic cells was calculated from each field. The average of the total number of pixels that comprised the apoptotic bodies was subsequently calculated.

Quantitation of Gene Expression

For these assays, tumors were removed at the base and snap-frozen in liquid nitrogen. Total RNA from ileal tumors of *Apc*$^{Min/+}$ mice was prepared using Trizol reagent according to the recommendations of the manufacturer (Invitrogen, Carlsbad, CA). Five micrograms of total RNA were used to generate cDNA using the High Capacity Achieve Kit from Applied Biosystems (Foster City, CA) according to the recommendations of the manufacturer. Levels of COX-2, IFN-γ, IL-12p40, and tumor necrosis factor (TNF)-α transcripts were quantified with Applied Biosystems predesigned primers and probes (TaqMan Gene Expression Assays) in an ABI Prism Sequence Detection System 7700 (Applied Biosystems). Transcript levels were normalized to the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and expressed as fold change compared with untreated control tumors using the Comparative C_T method (Applied Biosystems User Bulletin no. 2).

Statistical Analyses

Distribution of data was determined by the Kolmogorov-Smirnov test. Similarity of SDs between groups was determined by the method of Bartlett. Tumor multiplicity between multiple groups, with normally distributed data and similar SDs, was analyzed using ANOVA and Dunn's posttest. Multiple comparisons between normally distributed groups with dissimilar SDs ($P > 0.05$) were done using Kruskal-Wallis test, followed by Dunnett's comparison test. For tumor multiplicity data that were not normally distributed, a Kruskal-Wallis test, followed by a Dunn's posttest, was used. Comparisons between individual groups were done with a two-tailed t test for normally distributed data, a two-tailed t test with Welch's correction for normally distributed data with differing SDs, or a Mann-Whitney U test for data that were not normally distributed. Regions of the gastrointestinal tract (stomach, duodenum, jejunum, ileum, cecum, and colon) were compared for differences in tumor multiplicity. The duodenum, jejunum, and ileum were defined as the proximal, middle, and distal thirds of the small intestine, respectively. Aggregate tumor multiplicity for the entire gastrointestinal tract was also compared. Statistical analyses of categorical inflammation scores were carried out with a nonparametric Kruskall-Wallis test and a *post hoc* Dunn's multiple comparison test. For proliferation, apoptosis, and quantitative reverse transcription-PCR (RT-PCR) for transcript abundance, a nonparametric Mann-Whitney U test was performed.

Results

CD4$^+$CD25$^+$ regulatory cells inhibit development of adenomas in *Apc*$^{Min/+}$ mice. We have previously shown that CD4$^+$CD25$^+$ regulatory cells are able to prevent and treat colitis-associated colon cancer in Rag-deficient mice (11, 12). To determine whether CD4$^+$CD25$^+$ regulatory cells similarly disrupt intestinal polyp development in the *Apc*$^{Min/+}$ mouse model of human colorectal cancer, adoptive transfer of highly purified *wt* syngeneic CD4$^+$CD45RB^{lo}CD25$^+$ cells was done in *Apc*$^{Min/+}$ C57BL/6 mice at 3 to 4 months of age. In a series of two trials, *Apc*$^{Min/+}$ mice that received CD4$^+$ regulatory cells (n = 13) had significantly fewer (4.38 ± 2.9) intestinal adenomas than untreated age-matched *Apc*$^{Min/+}$ controls (n = 13; 45.9 ± 20.4; $P < 0.01$) when examined 3 weeks after adoptive transfer (Fig. 1*A*). These findings show that adoptive immunotherapy using competent CD4$^+$CD25$^+$ regulatory cells was sufficient to inhibit the development of ~90% of the intestinal tumors in *Apc*$^{Min/+}$ mice.

Interleukin-10 is required in CD4$^+$ regulatory lymphocytes to prevent adenoma development in *Apc*$^{Min/+}$ mice. We have previously shown that CD4$^+$CD25$^+$ cells require IL-10 to disrupt colitis-associated cancer in Rag-deficient mice (11). To determine whether IL-10 is necessary for CD4$^+$CD25$^+$ cells to prevent intestinal adenoma development, *Apc*$^{Min/+}$ mice received regulatory cells from IL-10–deficient C57BL/6 donors (n = 12) or from syngeneic *wt* mice (n = 19). In a series of experiments, there were no significant differences in tumor multiplicity between mice receiving IL-10–deficient CD4$^+$CD25$^+$ lymphocytes (60.4 ± 16.6) and age-matched untreated control *Apc*$^{Min/+}$ mice (n = 18; 50.3 ± 20.7; 20.7; $P > 0.05$; Fig. 1*B*). In contrast, *Apc*$^{Min/+}$ recipients of *wt* CD4$^+$CD25$^+$ cells (n = 19) had significantly fewer aggregate intestinal tumors (8.54 ± 6.32) than age-matched control untreated *Apc*$^{Min/+}$ mice ($P < 0.01$; Fig. 1*B*). In comparing the individual trials, there was lower multiplicity of tumors in *Apc*$^{Min/+}$ mice or *Apc*$^{Min/+}$ mice treated with *wt* regulatory cells at 3 weeks after treatment (4.38 ± 2.9) than at 6 weeks after treatment (13.3 ± 5.75; P = 0.0113). There was no difference in tumor multiplicity between trials in untreated control *Apc*$^{Min/+}$ mice treated with IL-10–deficient cells at 3 and 6 weeks after treatment (P = 0.6373), thus all 3- to 4-month-old mice are shown together in Fig. 1*B*. Taken together, these data show that IL-10 is necessary for CD4$^+$CD25$^+$ cells to prevent adenoma development in *Apc*$^{Min/+}$ mice.

CD4$^+$CD25$^+$ cells induce regression of established adenomas in older *Apc*$^{Min/+}$ mice. To examine whether CD4$^+$CD25$^+$ regulatory cells are able to treat established intestinal tumors in

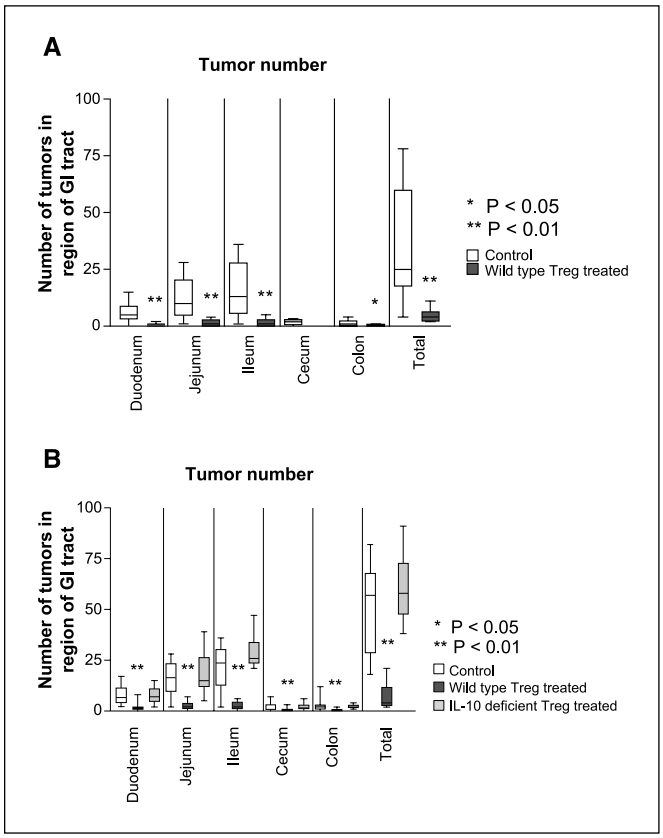

Figure 1. Adoptive immunotherapy using $CD4^+CD25^+$ regulatory cells prevents development of intestinal tumors in 3-month-old $Apc^{Min/+}$ mice in an IL-10–dependent manner. Tumor multiplicity is represented for each region of the intestinal tract and in aggregate (total) for untreated control (*unshaded*), *wt* regulatory cell–treated (*darkly shaded*), and IL-10–deficient regulatory cell–treated (*lightly shaded*) $Apc^{Min/+}$ mice. *Columns,* mean and middle quartiles; *bars,* SE. *A,* 3- to 4-month-old $Apc^{Min/+}$ mice treated with *wt* regulatory cells (n = 13) had fewer tumors in every region of the intestine and throughout the entire intestinal tract than untreated $Apc^{Min/+}$ mice (n = 13) at 3 weeks after treatment. Treated $Apc^{Min/+}$ mice did not develop any tumors in the cecum. *B,* treatment of 3- to 4-month-old $Apc^{Min/+}$ mice with IL-10–deficient regulatory cells (n = 12) did not prevent the development of intestinal tumors in any region of the intestine or throughout the intestinal tract compared with untreated control $Apc^{Min/+}$ mice (n = 18). In contrast, 3- to 4-month-old $Apc^{Min+/-}$ mice treated with *wt* regulatory cells (n = 19) had fewer tumors in every region of the intestine and throughout the entire intestinal tract than untreated $Apc^{Min/+}$ mice (n = 18) and recipients of IL-10–deficient regulatory cells (n = 12). Results have been combined for 3- to 4-month-old mice at 3 and 6 weeks posttreatment. Tumor multiplicity was not significantly different between these two time points among untreated control $Apc^{Min/+}$ mice and $Apc^{Min/+}$ mice treated with IL-10–deficient regulatory cells, but $Apc^{Min/+}$ mice treated with *wt* regulatory cells had fewer tumors at 3 weeks than at 6 weeks after treatment (see Results for details).

older mice, 14 $Apc^{Min/+}$ C57BL/6 mice at 4.5 to 6 months of age (mean age = 5.6 months) were given *wt* $CD4^+CD25^+$ cells. $Apc^{Min/+}$ mice that received *wt* $CD4^+$ regulatory cells had significantly fewer (14.8 $\pm$ 10.0) intestinal adenomas than age-matched $Apc^{Min/+}$ untreated controls (n = 18; 63.2 $\pm$ 15.2; P < 0.01; Fig. 2). Tumor multiplicity was significantly reduced in $CD4^+CD25^+$ cell–treated $Apc^{Min/+}$ mice in all regions of the gastrointestinal tract except the cecum, where the low multiplicity of tumors in treated (1.71 $\pm$ 1.54) and control (2.22 $\pm$ 1.63) mice probably accounts for this finding.

In addition, we found that older $Apc^{Min/+}$ recipients of $CD4^+$ regulatory cells were alert and active when euthanized at the end of the experiment, up to 7.5 months of age, whereas all 18 untreated $Apc^{Min/+}$ mice had to be euthanized due to morbidity before 6 months of age. The remaining tumors in the intestine of regulatory cell–treated mice appeared smaller (Fig. 3*B*) or showed an umbilicated center characterized by central necrosis and ulceration with underlying granulation tissue (Fig. 3*E*). Two tumors within one of the treated animals showed stromal vascular thrombosis (Fig. 3*C*). There was no evidence of neoplastic invasion in regulatory cell–treated mice, whereas invasive neoplastic epithelia were seen in adenomas of two age-matched untreated $Apc^{Min/+}$ mice (Fig. 3*D*).

Remarkably, a reduction in tumor multiplicity was evident as early as 2 to 4 days after adoptive immunotherapy with regulatory cells (n = 11; 28.7 $\pm$ 16.5; P < 0.01; Fig. 2). In treated $Apc^{Min/+}$ mice, remaining tumors appeared grossly to be flattened with a depressed center, suggestive of increased cell death, in contrast to polypoid nodular tumors in untreated control $Apc^{Min/+}$ mice.

$CD4^+CD25^+$ regulatory cells induce apoptosis in intestinal adenomas in $Apc^{Min/+}$ mice. To determine whether treatment with $CD4^+CD25^+$ cells induced regression of tumors through increased cell death, apoptosis within intestinal tissues was quantitated *in situ* in both age-matched untreated control $Apc^{Min/+}$ mice and *wt* regulatory cell recipients. We found a significant increase (P = 0.011) in apoptosis within tumors after treatment with regulatory cells (n = 7; 6,710 $\pm$ 6,330 pixels) compared with untreated control mice (n = 5; 4,816 $\pm$ 6,471 pixels; Fig. 4*A*). Increased apoptosis was most evident within adenomas of mice receiving regulatory cells 2 to 4 days after treatment (P = 0.002; Fig. 4*C*) relative to untreated mice (Fig. 4*B*). There were no differences in epithelial proliferation determined *in situ* using Ki67 (data not shown) between treated and untreated tumors at either interval posttreatment. These data suggest that adoptive immunotherapy with $CD4^+CD25^+$ lymphocytes decreased tumor multiplicity through rapid induction of apoptosis in intestinal tumors in $Apc^{Min/+}$ mice.

$CD4^+CD25^+$ cells down-regulate cyclooxygenase-2 expression in intestinal adenomas in $Apc^{Min/+}$ mice. To determine whether $CD4^+CD25^+$ cells down-regulate COX-2 expression within

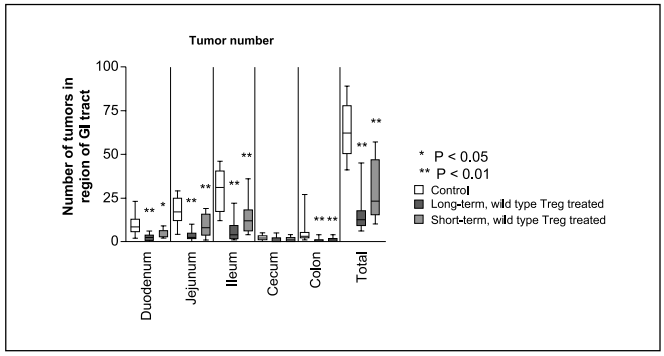

Figure 2. Adoptive immunotherapy using $CD4^+CD25^+$ regulatory cells induces rapid regression of intestinal tumors in $Apc^{Min/+}$ mice. Tumor multiplicity is represented for each region of the intestinal tract and in aggregate (total) for untreated control $Apc^{Min/+}$ mice (*unshaded*), *wt* regulatory cell–treated mice at >3 weeks after treatment (*darkly shaded*), and *wt* regulatory cell–treated mice at 2 to 4 days after treatment (*lightly shaded*). *Columns,* mean and middle quartiles; *bars,* SE. Compared with untreated control $Apc^{Min/+}$ mice (n = 18), $Apc^{Min/+}$ mice treated with *wt* regulatory cells had fewer tumors in every region of the intestine and throughout the entire intestinal tract at 3 or more weeks after treatment (n = 14). Tumor regression was evident as early as 2 to 4 days after treatment (n = 11). Low multiplicity of tumors in the cecum is likely to account for the lack of significant differences in this region of the intestine.

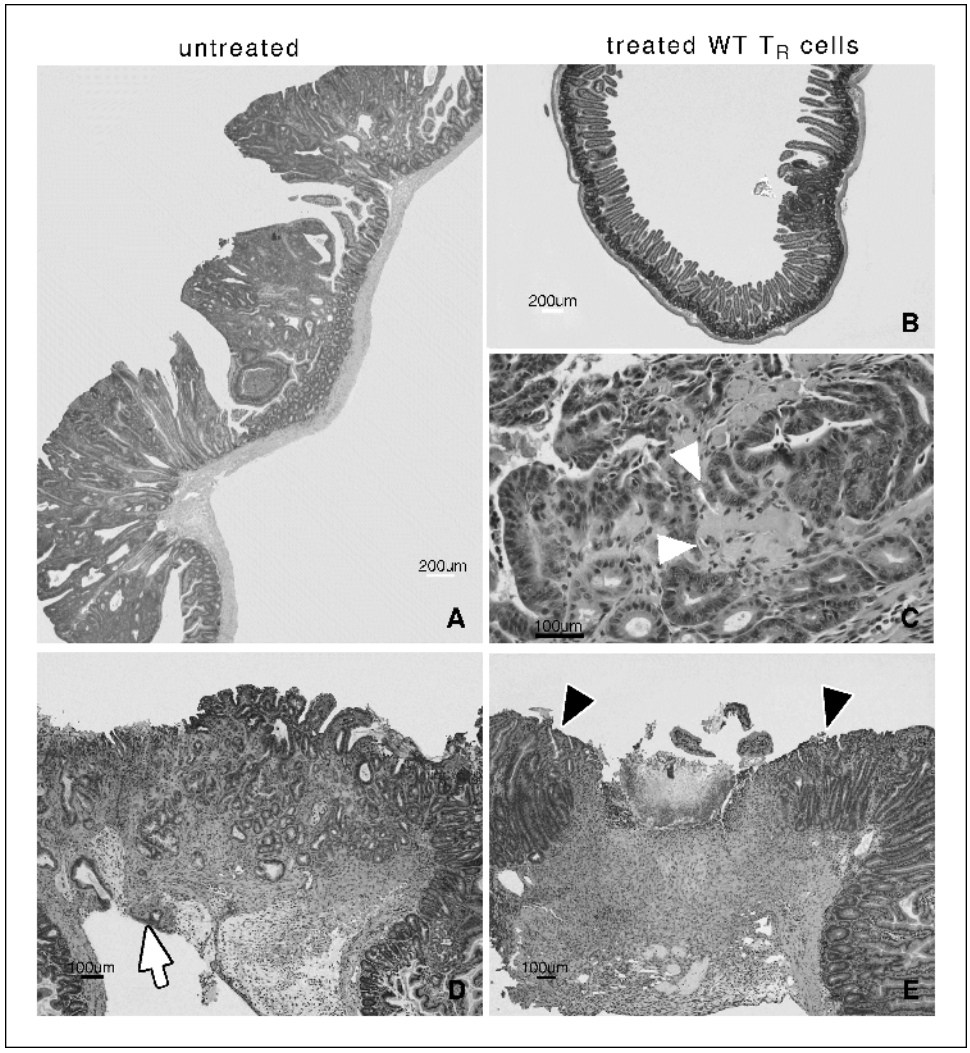

Figure 3. Histomorphology of the small intestinal tumors in $Apc^{Min/+}$ mice. *A,* small intestine (ileum) from a 6-month-old untreated $Apc^{Min/+}$ mouse. Note the polypoid adenomas protruding into the intestinal lumen, which occupy most of the mucosa, compared with ileum from a 6-month-old $Apc^{Min/+}$ mouse (*B*) treated with $CD4^+CD25^+$ regulatory lymphocytes showing the regressing adenomatous focus (*arrowhead*) in the mucosa. *C,* higher magnification of another tumor in the mouse from *B*. Note that multiple blood vessels within the stroma of the tumor have intravascular thrombi (*arrows*). *D,* ileum from a 6-month-old untreated $Apc^{Min/+}$ mouse. The neoplastic crypts have infiltrated the muscular wall and occasionally are present within the serosa (*arrow*). *E,* ileum from a 6-month-old $Apc^{Min/+}$ mouse treated with $CD4^+CD25^+$ regulatory lymphocytes. The tumor is umbilicated with a central area of necrosis, ulceration, and subadjacent granulation tissue. Note the remnant adenomatous crypts at the periphery.

adenomas, as previously described in mice undergoing NSAID treatment (9), message from intestinal tumors was analyzed for COX-2 gene expression using quantitative RT-PCR (TaqMan). COX-2 expression was reduced 2.23-fold in *wt* regulatory cell–treated $Apc^{Min/+}$ mice compared with untreated control $Apc^{Min/+}$ mice ($P < 0.05$) at 4 to 7 weeks after treatment; however, decreases in COX-2 expression were not significant at 2 to 4 days after treatment (Fig. 5).

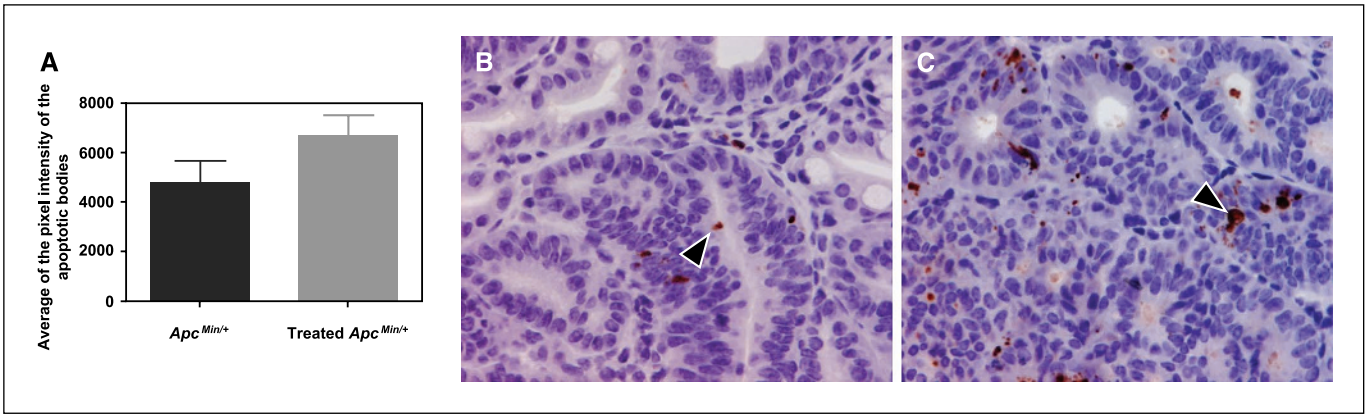

Figure 4. Immunohistochemical analysis of intestinal tumors for the apoptosis marker caspase-3 in untreated $Apc^{Min/+}$ mice and $Apc^{Min/+}$ mice treated with $CD4^+CD25^+$ cells. *A,* average of the total number of pixels of positively stained apoptotic bodies within the tumors from both untreated and treated $Apc^{Min/+}$ mice. *B* and *C,* apoptotic bodies (*arrowheads*) are noted within the intestinal tumors of the untreated (*B*) and treated $Apc^{Min/+}$ mice (*C*), showing increased apoptotic bodies in epithelia of treated mice (*C*). Caspase-3 antibody was used as a marker for apoptosis and the antigen-antibody reaction was visualized by using diaminobenzidine as a substrate. Hematoxylin stain.

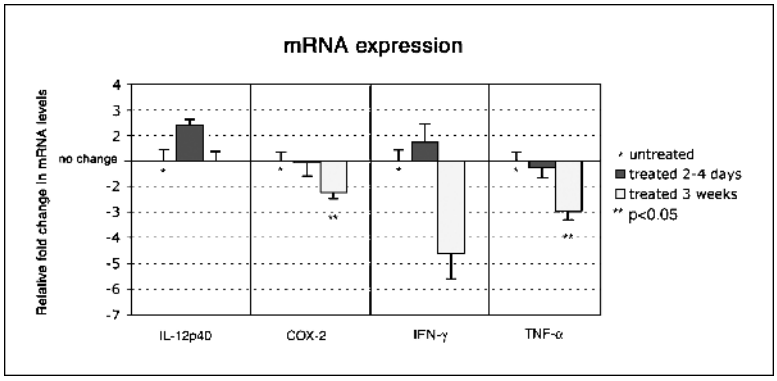

Figure 5. COX-2 and proinflammatory gene expression is reduced in tumors from $CD4^+CD25^+$ regulatory cell–treated $Apc^{Min/+}$ mice versus untreated $Apc^{Min/+}$ mice. Quantitative RT-PCR was performed on mRNA isolated from individual tumors collected from untreated control $Apc^{Min/+}$ mice ($n = 6$) and regulatory cell–treated $Apc^{Min/+}$ mice at 2 to 4 days ($n = 6$) and 4 to 7 weeks ($n = 4$) after treatment. Expression was normalized to the endogenous control gene *GAPDH*, and is shown as mean ± SE fold changes relative to tumors from untreated control $Apc^{Min/+}$ mice. COX-2, TNF-α, and IFN-γ are reduced 2.23-fold, 2.98-fold, and 4.59-fold, respectively, in regulatory cell–treated tumors at 4 to 7 weeks after treatment, although changes were negligible at 2 to 4 days after treatment. IL-12p40 was increased 2.4-fold at 2 to 4 days after treatment, but was not different from untreated control tumors at 4 to 7 weeks after treatment.

$CD4^+CD25^+$ regulatory cells decrease proinflammatory cytokine gene expression in intestinal adenomas in $Apc^{Min/+}$ mice. Whereas severity and extent of inflammation within tumors of regulatory cell–treated [2 (0-3.5); $n = 6$] versus untreated [2.5 (1.5-2.75); $n = 6$] $Apc^{Min/+}$ mice were not significantly different ($P = 0.07$), there were decreases in expression of TNF-α (2.98-fold; $P < 0.05$) and IFN-γ (4.59-fold) within tumors after treatment with $CD4^+CD25^+$ cells compared with tumors in untreated control $Apc^{Min/+}$ mice (Fig. 5). These findings show an association between adoptive immunotherapy and down-regulation of proinflammatory cytokine gene expression in the intestinal tumors of $Apc^{Min/+}$ mice.

Discussion

Here we show that adoptive immunotherapy using $CD4^+CD25^+$ regulatory cells prevents the development of intestinal adenomas in the $Apc^{Min/+}$ mouse model of intestinal cancer. IL-10 is required in $CD4^+CD25^+$ cells for therapeutic effect. We also show that $CD4^+CD25^+$ regulatory cells induce regression of established adenomas in 4.5- to 6-month-old $Apc^{Min/+}$ mice. Tumor burden is significantly decreased throughout all regions of the small and large bowel after adoptive cell transfer. Adoptive immunotherapy using $CD4^+CD25^+$ cells induced apoptosis in tumor epithelia, coincident with tumor regression as early as 2 to 4 days after lymphocyte transfer. Treatment with competent regulatory cells also induced down-regulation of COX-2 and proinflammatory cytokines within intestinal polyps.

Intervention with NSAIDs has previously been shown to decrease frequency of some intestinal cancers in humans (6–9), at least in part through inhibition of COX-2, which is expressed at high levels in intestinal adenomas of humans and in $Apc^{Min/+}$ mice (10). A key role for COX-2 in intestinal tumorigenesis is further supported by studies in $Apc^{Min/+}$ mice lacking COX-2 that show fewer intestinal tumors than $Apc^{Min/+}$ mice that do express COX-2 (15). Our finding that COX-2 expression was decreased over 2-fold in tumors by 3 weeks after treatment with $CD4^+CD25^+$ lymphocytes is consistent with the hypothesis that regulatory cells directly or indirectly suppress COX-2 expression in intestinal tumors, resulting in decreased prostaglandin production, increased cell death, and a reduction in tumor multiplicity (9). Compared with untreated control $Apc^{Min/+}$ mice, tumor multiplicity in mice at ages 4.5 to 6 months was reduced by ~50% after 2 to 4 days of treatment and by ~75% at 4 to 7 weeks after treatment. Although COX-2 inhibition is clearly linked with chemoprevention and apoptosis of epithelial tumors (6), the precise role of COX-2 in adoptive immunotherapy of $Apc^{Min/+}$ mice will require additional studies.

Whereas IL-10 is most widely known as a potent anti-inflammatory cytokine (16), it has also been shown to modulate apoptosis and suppress angiogenesis during tumor regression (16, 17). Natural killer (NK) cells have been linked with at least some of these activities (17). Whether the requirement for IL-10 in preventing tumor development in $Apc^{Min/+}$ mice by regulatory cells is associated with a loss-of-function or a gain-of-function effect remains to be determined. While IL-10 may simply be a critical secreted molecule by which regulatory cells exert their effect, it is also possible that absence of IL-10 leads to a dysregulated effector phenotype in $CD4^+$ lymphocytes. This is plausible given that IL-10 is pivotal in regulatory cell differentiation and function (18). Indeed, transfer of IL-10–deficient cells actually enhanced intestinal tumorigenesis in $Rag2^{-/-}$ mice (11). A similar trend was seen in $Apc^{Min/+}$ mice, although it did not achieve statistical significance. Insufficiency in IL-10 may also inhibit $CD4^+CD25^+$ regulatory cell–mediated recruitment of other $CD4^+$ subsets or NK cells during inhibition of inflammation (18–20). It was previously shown that glioma-specific $CD4^+$ Tr1-like cells similarly required IL-10 for glioma rejection capabilities (21). In that model, IL-10–mediated IFN-γ activity was enhanced rather than suppressed, suggesting a complex interplay of IL-10–mediated activities in tumorigenesis. Studies are underway in our laboratory using exogenous IL-10 to elucidate the role of this critical cytokine in adoptive immunotherapy.

Although NSAIDs and other anti-inflammatory drugs decrease tumor frequency in humans and mice, overt mucosal inflammation is not a prominent feature of the bowel during sporadic colon cancer in humans or in the $Apc^{Min/+}$ model. Indeed, there was minimal histologic evidence of intestinal inflammation in mice in our study. However, earlier studies have shown that bacterial infections in the intestine, with concomitant mucosal inflammation, facilitate the development of intestinal adenomas in $Apc^{Min/+}$ mice (22). Regulatory lymphocytes have well-documented abilities to suppress bacterially triggered inflammatory responses in the bowel of mice (23–27). Perhaps the ability of $CD4^+CD25^+$ regulatory cells to traffic and suppress inflammation throughout the host (28) explains the therapeutic efficacy in both the small and large bowel in the present study. An intriguing possibility is that inflammatory mediators, even in the absence of overt inflammatory cell infiltration, drive tumor development. If so, then regulatory cells, NSAIDs, and other anti-inflammatory drugs are all likely to exert their effect by modulating the levels of these molecules. Decreased expression of these putative inflammatory mediators could result in decreased mitogenic stimuli, increased apoptosis, or both. Indeed, the almost 3-fold and 5-fold decrease in TNF-α and IFN-γ,

respectively, in remaining adenomas after regulatory cell treatment support this hypothesis. Such effects are not necessarily limited to neoplastic cells. Indeed, TNF-α and other proinflammatory cytokines produced by stromal cells have been linked with angiogenesis and apoptosis in neoplastic cells (29). In those studies, inhibition of TNF-α induced programmed cell death of transformed hepatocytes and reduced frequency of liver tumors in mice (30). Perhaps IL-10–mediated down-regulation of TNF-α induces regression of intestinal polyps in $Apc^{Min/+}$ mice by a similar mechanism. The significance of the transient increase in IFN-γ and IL-12p40 expression in $Apc^{Min/+}$ tumors at 2 to 4 days after regulatory cell treatment is not clear and will require further study.

The recent observation of thymic depletion (31) coincident with increased tumorigenesis in $Apc^{Min/+}$ C57BL/6 mice at age 3 to 4 months supports a pivotal role for lymphocytes in the progression of adenomas in this model. It has been shown elsewhere that $CD4^+CD25^+$ regulatory cells are derived from the thymus of mice (28), and that these thymus-derived cells subsequently recruit peripheral $CD4^+CD25^+$ regulatory cells (18). Further, functions of regulatory cells may be compromised during thymic atrophy (32). Whether regulatory cell quantity or function is compromised in aging $Apc^{Min/+}$ mice is not known. We show here that addition of competent regulatory cells at age 3 to 4 months prevents the development of ~90% of intestinal polyps in $Apc^{Min/+}$ mice. On the other hand, $CD4^+CD25^+$ regulatory lymphocytes have also been shown to promote epithelial cancer by inhibiting beneficial host antitumor responses in other models (28, 33, 34). It remains to be proven whether regulatory cell deficiency contributes to the development of intestinal tumors in this model.

In humans, intestinal adenomas with mutations in *APC* become malignant and metastasize through a series of additional genetic changes (4, 5). Although $Apc^{Min/+}$ mice on a C57BL/6 background generally have fewer invasive neoplastic foci than $Apc^{Min/+}$ mice of other strain backgrounds (35), several 4.5- to 6-month-old untreated $Apc^{Min/+}$ mice had localized neoplastic epithelial invasion in our study. Whether or not antineoplastic activities of $CD4^+CD25^+$ lymphocytes extend beyond adenomatous polyps and *Apc* alone is unknown. Our data indicate that these immunomodulatory lymphocytes do have a potent antineoplastic role in epithelial carcinogenesis in $Apc^{Min/+}$ mice.

In summary, we have shown that adoptive immunotherapy using $CD4^+CD25^+$ regulatory cells prevents the development of tumors and rapidly induces regression of established tumors in the $Apc^{Min/+}$ mouse model of human intestinal cancer. Whereas regulatory cells can thwart anticancer surveillance activities (28, 33, 34), it is also clear that suppression of active inflammation by regulatory cells can prevent and treat inflammation-associated cancer (11, 12). The role of regulatory cells in enhancing or preventing sporadic colon cancer in patients requires further investigation. $CD4^+$ regulatory cells in immunotherapeutic strategies for colon cancer should not be discounted. A recent long-term trial using sulindac in familial adenomatous polyposis patients has been discouraging (36), suggesting that early regression of polyps may not necessarily reduce the long-term risk of colon cancer. Perhaps adoptive immunotherapy holds promise for chemoprevention of greater efficacy and duration. Down-regulation of COX-2, previously linked with cancer of the breast, prostate, lung, and urinary bladder (10), also suggests that adoptive immunotherapy using $CD4^+$ regulatory lymphocytes may prove beneficial in the prevention and treatment of a wide range of epithelial cancers in humans.

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