Proinflammatory CD4+CD45RBhi Lymphocytes Promote Mammary and Intestinal Carcinogenesis in ApcMin/+ Mice

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Abstract

Cancers of breast and bowel are increasingly frequent in humans. Chronic inflammation is known to be a risk factor for these malignancies, yet cellular and molecular mechanisms linking inflammation and carcinogenesis remain poorly understood. Here, we apply a widely used T-cell transfer paradigm, involving adoptive transfer of proinflammatory CD4+CD45RBhi (T_E) cells to induce inflammatory bowel disease (IBD) in mice, to investigate roles of inflammation on carcinogenesis in the ApcMin/+ mouse model of intestinal polyposis. We find that transfer of T_E cells significantly increases adenoma multiplicity and features of malignancy in recipient ApcMin/+ mice. Surprisingly, we find that female ApcMin/+ recipients of T_E cells also rapidly develop mammary tumors. Both intestinal polyposis and mammary adenocarcinoma are abolished by cotransfer of anti-inflammatory CD4+CD45RBlo regulatory lymphocytes or by neutralization of key proinflammatory cytokine tumor necrosis factor-α. Lastly, down-regulation of cyclooxygenase-2 and c-Myc expression is observed coincident with tumor regression. These findings define a novel mouse model of inflammation-driven mammary carcinoma and suggest that epithelial carcinogenesis can be mitigated by anti-inflammatory cells and cytokines known to regulate IBD in humans and mice. (Cancer Res 2006; 66(1): 57-61)

Introduction

Colorectal cancer is the leading cause of cancer-related mortality worldwide (1). Breast cancer is the most common nonintegumentary malignancy in women (2). Observations that risk of colorectal cancer (3) and breast cancer (4) are reduced in patients taking aspirin and other nonsteroidal anti-inflammatory drugs indicate that inflammation contributes to intestinal and breast carcinogenesis in humans. However, experimental models of inflammation-driven breast cancer are lacking. ApcMin/+ mice are genetically prone to development of epithelial tumors in intestine and breast (5, 6). Prior studies in our lab (7) as well as from others (8) have raised important questions about roles of inflammation in epithelial tumor development and progression. Yet, no study to date has examined the effect of proinflammation in epithelial tumor development and progression. Several studies using the ApcMin/+ mouse model of intestinal polyposis have raised important questions about roles of inflammation in epithelial tumor development and progression. Several studies using the ApcMin/+ mouse model of intestinal polyposis in epithelial tumor development and progression.

Materials and Methods

ApcMin/+ C57BL/6 mice. All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care–approved facilities and maintained according to protocols approved by the Institutional Animal Care and Use Committee at the Massachusetts Institute of Technology. ApcMin/+ mice on a C57BL/6 background were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in house as (heterozygous × wild type) crosses to provide ApcMin/+ mice and wild-type littermates for experimental recipients and donors.

Experimental design. A total of 102 ApcMin/+ mice were included in various treatment regimens or as experimental controls. Some experiments were conducted using separate trials with four to eight mice each. Trials with statistically similar results were then combined for analyses.

T_E-cell transfer. Sixteen ApcMin/+ mice ages 3.5 to 4 months were dosed with 3 × 10⁶ T_E cells collected from wild-type littermates. For these studies, 10 female and six male recipient mice were used. Mice were euthanized 3 to 4 weeks later and then compared with 14 untreated age-matched ApcMin/+ controls. This experiment was conducted as three separate trials using five or six mice in each trial.

T_E-cell cotransfer. Fourteen ApcMin/+ mice ages 3.5 to 4 months were dosed with both 3 × 10⁶ T_E cells and 3 × 10⁵ T_R cells. For these studies, eight recipient mice were females and six were males. Mice were then euthanized 3 to 4 weeks later and compared with 16 recipients of T_E cells alone as described above. This experiment was conducted as three separate trials using four or five mice in each trial. A second regulatory cell transfer experiment used CD4+CD45RBhiCD25+ regulatory cells collected from wild-type littermates, instead of CD25+ regulatory cells in ApcMin/+ mice, in eight 3.5- to 4-month-old ApcMin/+ recipients of T_E cells.

Tumor necrosis factor-α neutralization. Fourteen ApcMin/+ recipients of T_E cells at age 3.5 to 4 months were treated 3 weeks later with a tumor necrosis factor-α (anti-TNF-α) antibody (clone XT-3) at 200 μg per mouse thrice weekly for 1 week. For these studies, eight recipient mice were females and six were males. Mice were then euthanized (4 weeks after the original T_E-cell transfer) and compared with matched ApcMin/+ recipients of T_E cells that received sham antibody alone (n = 8). This experiment was conducted as two separate trials using seven mice in each trial.

A second experimental study with ApcMin/+ mice of ages 4.5 to 6 months that were treated with anti-TNFα antibody (clone XT-3) at 200 μg per mouse thrice weekly for 1 week and then euthanized immediately afterwards. This experiment was conducted as two separate trials using seven mice in each trial. Treated mice were compared with age-matched ApcMin/+ mice that received sham antibody alone (n = 8).

Adaptive transfer of T cells in ApcMin/+ mice. CD4+ lymphocytes isolated from wild-type littermates (C57BL/6j) using magnetic beads (Dynal Biotech USA, Oslo, Norway) are sorted by hi-speed flow of intestinal polyps. Hence, we follow the cell transfer paradigm of inflammatory bowel disease (IBD) using colitogenic proinflammatory CD4+CD45RBhi (T_E) and colitis-protective anti-inflammatory CD4+CD45RBloCD25+ lymphocyte subsets (9, 10) to test this hypothesis and determine their effect on epithelial carcinogenesis in ApcMin/+ mice.
cytometry (MoFlow) to obtain purified populations of CD4+CD45RBhi or CD4+CD45RBloCD25+ or CD4+CD45RBloCD25− lymphocytes (96% pure) as previously described (7). Anesthetized recipient mice are injected i.v. in the retro-orbital sinus with 3 to 4 x 10^7 T cells as previously described (7).

Quantification 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 (7).

Histologic evaluation. Formalin-fixed tissues were embedded in paraffin, cut at 5 μm, and stained with H&E. Lesions were evaluated by two veterinary pathologists blinded to sample identity. Intramucosal carcinoma, carcinoma in situ, and neoplastic epithelial invasion were assessed based on histopathologic criteria as described elsewhere (11). Quantitative assessment of inflammatory cells was done in standardized areas at the base of adenomas in H&E-stained slides. Multiple representative ×40 high-power fields corresponding to the above mentioned selection criteria were captured using a Nikon eclipse 50i microscope and a Nikon DS-S M-L1 digital camera. Ten images were randomly selected per treatment group. The different inflammatory cells found in each image were counted using the cell count plug-in of the Imagej image processing and analysis program (NIH, Bethesda, MD). Inflammation counts were recorded as the number of granulocytes, lymphocytes, and plasma cells counted per image.

Quantitation of gene expression. Five micrograms of total RNA were prepared (using Trizol, Invitrogen, Carlsbad, CA) from 0.5-cm sections of ileal mucosa harvested at a standardized location 1.0 cm from the base of the cecum to generate cDNA using the High-Capacity Archive kit from Applied Biosystems (Foster City, CA). Levels of c-Myc and cyclooxygenase-2 (Cox-2) transcripts were quantified in the ABI Prism Sequence Detection system 7700 (A/B Applied Biosystems) as described in detail elsewhere (7).

Statistical analyses. The total number of intestinal tumors in mice from different treatment groups and controls was analyzed by unpaired Student’s t test. The prevalence of carcinoma in situ and tumor invasion between groups was compared by the Kruskal-Wallis one-way ANOVA and Dunn’s post-test. Direct comparisons were made by the Mann-Whitney U test. Graphpad Prism 4.0 software was used for all statistical analysis. Statistical significance was set at P < 0.05.

Results and Discussion

Tε cells promote intestinal polyp development and associated malignancy. To study roles for inflammatory cells and cytokines in ApcMin/− mice, we followed an adoptive transfer paradigm widely used to induce IBD in mice (10, 12). Proinflammatory Tε cells isolated from wild-type littermates were adoptively transferred into naive ApcMin/− mice. We find that ApcMin/− mice that receive Tε cells (n = 16) show significantly more frequent (P < 0.001) intestinal tumors (Fig. 1A) and inflammatory cell infiltrates (Table 1) than untreated age-matched ApcMin/− controls (n = 14), when examined 3 to 4 weeks after adoptive transfer. There was a significant (P < 0.05) increase in the number of lymphocytes (Table 1) in polyps of Tε-cell recipient mice matching findings in humans with colorectal cancer (13). Furthermore, polypoid adenomas in recipients of Tε cells show increased frequency of carcinoma in situ and neoplastic epithelial invasion when compared with matched untreated ApcMin/− mice (Table 1). The invasive lesions were characterized by the infiltration of adenocarcinoma glands within the submucosa and muscle layers (Fig. 2A). In general, adenomas from Tε-cell recipients had more frequent dysplastic glands (P < 0.001) showing cellular atypia and pleomorphism (Fig. 2C). These data indicate that proinflammatory Tε cells not only increase multiplicity of intestinal adenomas but also contribute to a malignant phenotype in these mice.

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Tε \text{ cells promote mammary adenocarcinoma in mice. Intriguingly, 70% of female } Apc^{Min/−} \text{ mice (7 of 10 animals) that received } Tε \text{ cells at age 4 months rapidly developed palpably enlarged mammary glands (Fig. 1B) with histologic features consistent with adenosquamous carcinoma (Fig. 2B) as described previously in } Apc^{Min/−} \text{ mice (6). In contrast, none of the age-matched untreated } Apc^{Min/−} \text{ females (0 of 8 females) had evidence of mammary tumors. Moser et al. (6) also rarely observed mammary tumors in female } Apc^{Min/−} \text{ mice on B6 background when compared with } Apc^{Min/−} \text{ mice on other strain backgrounds. The highly infiltrative neoplastic mammary glands in } Tε-\text{cell recipient mice show nonkeratinized and keratinized epithelia arranged in variably sized nests and cords with extensive squamous metaplasia (Fig. 2D). Overall, mammary glands have dense inflammatory cell infiltrates composed primarily of neutrophils and lymphocytes. Macrophages, plasma cells, and mast cells were also readily observed. These findings suggest that addition of proinflammatory } Tε \text{ cells accelerates development of }\]

![Graph](image-url)
Table 1. Quantitative assessment of intestinal tumor pathology and composition of cellular infiltrate in Apc<sup>Min/+</sup> mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Tumors (mean ± SE)</th>
<th>Percent tumors with</th>
<th>Intratumor infiltrate (mean ± SE)</th>
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<tr>
<td></td>
<td>Carcinoma &lt;i&gt;in situ&lt;/i&gt;</td>
<td>Neoplastic invasion</td>
<td>Plasma cells</td>
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<tr>
<td>Min</td>
<td>56.7 ± 4.6&lt;sup&gt;a, b, c&lt;/sup&gt;</td>
<td>38 (26/75)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 (1/75)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Min + T&lt;sub&gt;E&lt;/sub&gt;</td>
<td>90.9 ± 5.5&lt;sup&gt;a, b, c&lt;/sup&gt;</td>
<td>70 (52/74)&lt;sup&gt;b, c, d&lt;/sup&gt;</td>
<td>7 (6/74)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Min + T&lt;sub&gt;E&lt;/sub&gt; + T&lt;sub&gt;R&lt;/sub&gt;</td>
<td>7.8 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25 (7/30)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0 (0/30)&lt;sup&gt;a, d, e, g&lt;/sup&gt;</td>
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<tr>
<td>Min + T&lt;sub&gt;E&lt;/sub&gt; + anti-TNF-α</td>
<td>11.5 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42 (20/50)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4 (2/50)&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>Min + anti-TNF-α</td>
<td>10.7 ± 2.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19 (5/25)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0 (0/25)</td>
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NOTE: Data within a column that share a superscript letter are significantly different from other groups in that column. <sup>a, b, or c</sup>, P < 0.001; <sup>d, e, or f</sup>, P < 0.01; <sup>g, h, or i</sup>, P < 0.05. Parenthesis include fields with lesions/total fields.

Figure 2. Left, representative histopathology of small intestine in left column (A, C, E, and F): right, representative histopathology of mammary gland (B, D, and G) from T<sub>E</sub>-cell recipient Apc<sup>MV/+</sup> mice. Apc<sup>MV/+</sup> mice that received purified T<sub>E</sub> cells but no further treatment showed high frequency of invasive adenocarcinoma in the intestine and mammary gland. A, ileum. Well-differentiated glands invading through the muscularis propria. The advancing edge of the neoplastic lesion shows typical mucinous adenocarcinoma morphology. B, mammary gland. Highly infiltrative adenosquamous carcinoma contains glandular structures with or without squamous differentiation. Note the dense inflammatory cell infiltrate and desmoplastic reaction. Higher magnification in (D) clearly illustrates the typical morphology of adenosquamous carcinoma with admixed nonkeratinized and keratinized (keratin pearls) neoplastic glands. Recipients of T<sub>E</sub> cells also showed increased frequency of adenomatous polyps in ileum (C). Adenomatous polyps were enlarged and had increased frequency of abnormal glandular architecture with epithelial dysplasia and carcinoma <i>in situ</i>. Min recipients of cotransfer of T<sub>E</sub> and T<sub>R</sub> cells (E and G) or anti-TNF-α antibody (F) showed regression of intestinal tumors in ileum (E and F). Remaining minute polyps showing minimal evidence of remaining dysplasia on surface epithelium. Normal mammary gland tissue and mammary fat (G) in recipients of T<sub>R</sub> cells. H&E.
cancer in breast tissue in these genetically susceptible mice and thus reveal a model of inflammation-driven breast cancer in humans. Prior studies in mice with IBD (9, 10, 14) led us to hypothesize that cotransfer of anti-inflammatory T<sub>R</sub> cells will inhibit inflammatory factors that may drive mammary and intestinal carcinoma in Apc<sup>Min</sup>/<sup>+</sup> mice.

**T<sub>R</sub> cells inhibit T<sub>E</sub> cell–induced epithelial carcinogenesis.** To determine whether T<sub>R</sub> cell–mediated carcinogenic events of gut and breast can be inhibited by anti-inflammatory CD4<sup>+</sup>CD45RB<sup>lo</sup>CD25<sup>+</sup> (T<sub>R</sub>) regulatory cells, Apc<sup>Min</sup>/<sup>+</sup> mice that received T<sub>R</sub> cells simultaneously underwent adoptive transfer with T<sub>E</sub> cells (cotransfer group). We find that cotransfer recipients (n = 14) show significantly (P < 0.001) fewer intestinal tumors (Fig. 1A) and do not develop mammary adenocarcinoma (Fig. 1B and Fig. 2G). Tissues from these mice have decreased frequency of epithelial dysplasia (Table 1) and are similar in appearance to those of wild-type C57BL/6 mice (Fig. 2E). Interestingly, however, sections of intestines from the cotransfer recipients did not differ significantly when scored for number of inflammatory cells (Table 1) from those of T<sub>E</sub>-cell recipients despite complete disappearance of invasive adenocarcinoma and restoration to normal epithelial homeostasis (Fig. 2F). These data match earlier findings showing that T<sub>R</sub> cells suppress tumors in Apc<sup>Min</sup>/<sup>+</sup> mice (7) and also inhibit IBD in cell transfer models using immunodeficient mice (10, 11, 15).

In addition to the CD25<sup>+</sup> population, Kullberg et al. have shown that CD25<sup>-</sup> cells of CD4<sup>+</sup>CD45RB<sup>lo</sup> phenotype also act as potent inhibitors of IBD in mice (16). To test antineoplastic efficacy of CD25<sup>-</sup> cells in this setting, we transferred CD25<sup>-</sup> cells from wild-type littermates into Apc<sup>Min</sup>/<sup>+</sup> mice. We find that Apc<sup>Min</sup>/<sup>+</sup> recipients of CD4<sup>+</sup>CD45RB<sup>lo</sup>CD25<sup>-</sup> cells (n = 8) also show significantly (P < 0.001) fewer intestinal adenomas (mean = 6.2 ± 2.3) when compared with untreated mice (mean = 56.7 ± 4.6). Furthermore, female Apc<sup>Min</sup>/<sup>+</sup> cotransfer recipients of CD4<sup>+</sup>CD45RB<sup>lo</sup>CD25<sup>-</sup> cells (n = 6) also had complete lack of mammary adenocarcinoma. These data show that CD4<sup>+</sup>CD45RB<sup>lo</sup> cells, in general, have antineoplastic functions in mice with enteric flora matched with their donors. Studies are in progress to investigate whether specific enteric antigens modulate anti-inflammatory and antineoplastic potency of CD4<sup>+</sup> regulatory cells in this model.

**Neutralization of proinflammatory cytokine TNF-α inhibits intestinal and mammary carcinogenesis.** TNF-α is a potent effector cytokine in the pathogenesis of IBD in humans (17) and in mice (18, 19) and has been associated with poor prognosis in several human cancers, including mammary carcinoma (20). To determine whether TNF-α is critical for intestinal and mammary carcinoma seen in our model, we treated Apc<sup>Min</sup>/<sup>+</sup> mice that received T<sub>E</sub> cells (n = 14) with anti-TNF-α neutralizing antibody (21). We find that mice that receive 200 μg/mouse of anti-TNF-α antibody thrice weekly for 1 week had significantly (P < 0.001) fewer intestinal adenomas when compared with Apc<sup>Min</sup>/<sup>+</sup> mice that receive sham antibody alone (n = 8; Fig. 1A). Intestinal tumors had less frequent epithelial dysplasia and neoplastic invasion than tumors of untreated Apc<sup>Min</sup>/<sup>+</sup> counterparts (Table 1; Fig. 2F). Furthermore, mammary gland neoplasia was not observed in any of T<sub>E</sub>-cell recipient female mice (n = 8) following 1 week of treatment with anti-TNF-α antibody (Fig. 1B). These findings indicate that proinflammatory cytokine TNF-α is required to sustain tumors in breast and bowel, revealing a key cytokine mediator of carcinogenesis in animals predisposed to epithelial tumors. Preliminary studies in Apc<sup>Min</sup>/<sup>+</sup> mice on a C57BL/6 Rag<sup>-/-</sup> background reveal that TNF-α from cells of innate immunity is sufficient to trigger both intestinal and mammary tumors in this model. Tumor regression and restoration of epithelial homeostasis at two anatomically distinct sites (i.e., intestines and mammary gland) after treatment with anti-inflammatory T<sub>R</sub> cells or after anti-TNF-α antibody suggest that these less toxic approaches should be considered for future cancer treatment in humans.

**Anti-inflammatory treatment regimens down-regulate c-Myc expression.** Up-regulation of oncogene c-Myc has been well documented in cancers of the breast (22) and bowel (23) in humans and in Apc<sup>Min</sup>/<sup>+</sup> mice (24). To determine whether anti-inflammatory treatments modulate c-Myc levels, we measured oncogene expression levels using quantitative reverse transcription-PCR (Taqman) in intestinal mucosal samples from mice undergoing treatments as described above. We find that c-Myc levels were decreased by 10- to 20-fold in intestinal mucosal samples of mice from T<sub>R</sub> and anti-TNF-α treatment groups (Fig. 3). Likewise, we observed a significant decrease in Cox-2 expression levels in these samples correlating with down-regulation of inflammation (Fig. 3), matching earlier findings in Apc<sup>Min</sup>/<sup>+</sup> mice (7) and humans with intestinal polyposis (3). The disappearance of carcinoma and associated malignant lesions as well as restoration of epithelial homeostasis of two anatomically distinct sites (i.e., intestines and mammary gland) after treatment with anti-inflammatory T<sub>R</sub> cells or after anti-TNF-α antibody suggest that these less toxic approaches should be considered for future cancer treatment in humans.

**Figure 3.** Relative levels of Cox-2 and c-Myc mRNA. In each sample, Cox-2 or c-Myc mRNA was normalized to that of the “housekeeping” gene Gapdh. Columns, mean fold change of Cox-2 or c-Myc mRNA levels in reference to untreated Apc<sup>Min</sup>/<sup>+</sup> group; bars, SE. ref, no change (open column with *).

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4 Unpublished data.
homeostasis brought about by anti-inflammatory Th2 cells or anti-TNF-α antibody coincide with reversal to basal expression levels of c-Myc, suggesting its potential role in inflammation-driven carcinogenesis. Thus, carcinogenesis in Apc<sup>Min/−</sup> mice seems to be reversibly linked with c-Myc expression, which is regulated by the balance of proinflammatory and anti-inflammatory mediators.

That chronic inflammation predisposes humans and animals to cancer is becoming increasingly clear (25). However, the interplay regulated by the balance of proinflammatory and anti-inflammatory c-Myc seems to be reversibly linked with driven carcinogenesis. Thus, carcinogenesis in ApcMin/+ mice, we show that development of breast cancer and intestinal carcinoma can be triggered by adoptive transfer of proinflammatory Th2 lymphocytes. Additional studies are required to examine mechanisms by which proinflammatory CD45RB<sup>hi</sup> cells promote mammary and intestinal carcinoma in these mice. As excessive production of inflammatory mediators, including TNF-α, during chronic inflammation has been implicated in oncogenesis (26), it may be that similar mechanisms involving COX-2 and c-MYC are relevant in Th2-cell recipient Apc<sup>Min/−</sup> mice. Tumor regression and restoration of epithelial homeostasis in intestines and mammary gland seen after treatment with Th<sub>2</sub> cells or anti-TNF-α antibody in this model support the clinical observations showing reduction in the risk of colorectal cancer (3) and breast cancer (4) in patients receiving anti-inflammatory drugs. These findings allude to broader applicability of these therapies in cancers of prostate (27) and other sites responsive to anti-inflammatory therapies in humans (28, 29). Ultimately, efforts to harness the potency of cells and cytokines with anti-inflammatory function will help develop less toxic cancer immunotherapies in humans.

References

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