

## Vanilloid Receptor-1 Regulates Neurogenic Inflammation in Colon and Protects Mice from Colon Cancer

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### Abstract

Neuroinflammation driven by the vanilloid-type ion channel receptor transient receptor potential vanilloid type 1 (TRPV-1) is suspected to play a role in the pathophysiology of inflammatory bowel disease. Because inflammatory bowel disease is known to elevate the risk of colon cancer, we examined postulated roles for TRPV-1–driven neuroinflammation in promoting colitis-associated and spontaneous colon cancer development. Using a well-established model of colitis-associated cancer (CAC), we found that mice genetically deficient in TRPV-1 showed a higher incidence and number of tumors in the distal colon. In like manner, genetic deficiency of TRPV-1 in the APC<sup>Min/+</sup> model of spontaneous colon cancer accentuated the number of colonic adenomas formed. Mechanistic analyses in the CAC model revealed an increased infiltration of inflammatory cells into the tumors along with elevated expression of interleukin (IL)-6 and IL-11 and activation of the STAT3 and NF- $\kappa$ B signaling pathways. Notably, TRPV-1–deficient mice exhibited a defect in expression of the anti-inflammatory neuropeptides, vasoactive intestinal peptide (VIP), and pituitary adenylate cyclase-activating peptide (PACAP) which contributed to the generation of a local proinflammatory environment. Together, our findings argue that by limiting neuroinflammatory processes, TRPV-1 exerts a protective role that restricts the initiation and progression of colon cancer. *Cancer Res*; 72(7): 1–12. ©2012 AACR.

### Introduction

The transient receptor potential vanilloid type 1 (TRPV-1), also called vanilloid receptor, is a nonselective cation channel which is predominantly expressed in primary afferent sensory neurons and in the central nervous system (CNS; refs. 1, 2). In the gastrointestinal tract, TRPV-1 is preferentially expressed in the distal colon (3). TRPV-1 is a molecular integrator of multiple noxious stimuli and a regulator of the body temperature (4). This receptor is activated by a plethora of stimuli including noxious heat, tissue acidosis, inflammatory mediators, and plant-derived vanilloids such as capsaicin with depolarization leading to burning pain (5).

Neurogenic inflammation is produced by overstimulation of peripheral nociceptor terminals in inflamed tissues. Notably, capsaicin-sensitive sensory neurons participate in the gener-

ation of neurogenic inflammation, which plays a major role in the pathophysiology of inflammatory bowel diseases (IBD; refs. 6, 7). TRPV-1 expression is increased in the colon of patients with IBD (8), and this overexpression is thought to contribute to the ongoing pain and visceral hypersensitivity in these patients. Therefore, enormous efforts are being made to identify novel TRPV-1 antagonists with a potential therapeutic profile. However, recent evidence suggests a possible protective role for TRPV-1 in inflammatory states and particularly in the gastrointestinal tract (9). Several approaches have been made to elucidate whether TRPV-1 exerts beneficial effects against colitis. Genetic ablation of the TRPV-1 receptor or desensitization of capsaicin-sensitive afferent neurons in rodents results in a much more pronounced development of colitis (10–12). Furthermore, activation of TRPV-1 receptor by capsaicin reduces the colonic damage induced by 2,4-dinitrobenzene sulfonic acid (DNBS; ref. 13) and dextran sodium sulfate (DSS; ref. 11). In contrast, other reports have shown that TRPV-1 activation may exacerbate colon inflammation in different animal models (14–16).

A cross-talk between the enteric nervous system and the immune system seems to be a crucial factor in the pathophysiology of intestinal inflammation (6). Thus, the release of neuropeptides by the enteric nervous system can amplify or modulate the inflammatory response. Among them, substance P and calcitonin gene-related peptide (CGRP) are the best characterized, and because of its proinflammatory nature, they are thought to play a significant role in the development and pathogenesis of colitis (6). In contrast, other neuropeptides such as vasoactive intestinal peptide (VIP) and pituitary

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adenylate cyclase-activating peptide (PACAP) are endowed with anti-inflammatory properties and they have been suggested to protect colon from inflammation (17–19). Thus, activation of enteric sensory neurons might lead to the secretion of both pro- and anti-inflammatory neuropeptides, being the balance between both types of neuropeptides what determines the degree of inflammation and in last term, tumor development.

Many epidemiologic and experimental studies have highlighted the relevance of inflammation as a predisposing cause of cancer (20). Chronic inflammatory conditions such as those occurring in the gastrointestinal tract of patients with IBD are known to increase the risk of colorectal cancer (21). STAT3 and NF- $\kappa$ B signaling pathways play crucial roles in the initiation and development of inflammation-induced cancer (20, 22, 23). STAT3 and NF- $\kappa$ B are found to be constitutively activated in cancer cells and in tumor-associated myeloid cells and regulate in a cooperative manner several proinflammatory genes such as interleukin (IL)-6, IL-11, chemokines, growth factors, and COX-2 that are crucial for maintaining a procarcinogenic inflammatory environment (23, 24). The complex interplay between NF- $\kappa$ B and STAT3 signaling cascades and the role of IL-6 and IL-11 have recently been deciphered in different studies using murine models of colitis-associated cancer (CAC; refs. 25–27).

In this study, we sought to determine the role of TRPV-1 in 2 different models of colon cancer and we found that TRPV-1 has a protective role against colon cancer development.

## Materials and Methods

### Animals

C57BL/6J wild-type (WT), TRPV-1<sup>-/-</sup> (B6.129 × 1-*Trpv1*<sup>tm1Jul/J</sup>), and APC<sup>Min/+</sup> (C57BL/6J-Apc<sup>Min/J</sup>) mice (Jackson Laboratory) were genotyped using specific primers (Supplementary Fig. S1) and housed under standard conditions. Compound mutant APC<sup>Min/+</sup>TRPV-1<sup>-/-</sup> mice were generated by first breeding APC<sup>Min/+</sup> males to TRPV-1<sup>-/-</sup> females. Males APC<sup>Min/+</sup>TRPV-1<sup>+/-</sup> were then crossed to TRPV-1<sup>-/-</sup> females to generate APC<sup>Min/+</sup>TRPV-1<sup>-/-</sup> mice. All experiments were carried out according to the Institutional Guidelines for the Care and Use of Laboratory Animals in Research and the approval of the local ethic committee from the University of Córdoba, Córdoba, Spain.

### Tumor induction and analysis

Eight- to ten-week-old mice were injected intraperitoneally (i.p.) with 10 mg/kg azoxymethane (AOM; Sigma-Aldrich). After 1 week, 1% DSS (m.w. 36–50 kDa; MP Biomedicals) was given in the drinking water for more than 4 days, followed by 17 days of regular water. This cycle was repeated once more and mice were sacrificed 12 weeks after the AOM injection. Body weight and presence of blood in stool were measured every week.

### Histologic analysis

Intestines were removed and processed by standard procedures. Colons were fixed as "Swiss-rolls" overnight in 10% neutral-buffered formalin, briefly washed with PBS and trans-

ferred into 70% ethanol, processed and embedded into paraffin. Five-micrometer sections were cut for hematoxylin and eosin staining and immunohistochemistry. Slides were dewaxed in xylene and rehydrated by passage through graded alcohols to water. Antigen retrieval was conducted by microwave (medium power) for 15 to 20 minutes in 0.1 mol/L sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked using 1.6% H<sub>2</sub>O<sub>2</sub> in methanol and then slides were incubated in 10% normal serum in 1% bovine serum albumin (BSA)/PBS for 40 minutes. Primary incubations were conducted with antibodies against Ki-67 (Clone TEC-3, 1:125, Dako, M7249), CD3 (1:150, Dako, A0452), F4/80 (1:50, Serotec, MCAP497), CD45R/B220 (1:200, BD Pharmingen, 553086), and  $\beta$ -catenin (1:100, Upstate, 05–665) in 1% BSA/PBS for 1 hour at room temperature. Isotype-matched antibodies were included as negative controls. Tumor size was determined by image analysis of the frozen tissues using imaging software (ImageJ). Size was determined by measuring the largest diameter of the tumor. Images were taken with a scale bar and lengths were measured in pixels and correlated to the known distance in scale bars. A minimum of 15 tumors from each region were measured from at least 7 mice from each genotype. Immunohistochemistry was quantified by counting the number of positive cells visualized per high-power field (HPF; 40 $\times$  or 20 $\times$  objective) within each region. The mean positive cells per HPF were calculated (see Supplementary Methods for additional details).

### RNA analysis

Transcript levels were quantified by real-time PCR (RT-PCR) with specific primers for each gene, and quantitative RT-PCR (qRT-PCR) reaction samples were prepared as a mixture with the qPCR SYBR Green PCR Kit (Promega). Amplifications were conducted by the iCycler PCR Detection System (Bio-Rad). The primer sequences used and PCR conditions are shown in Supplementary Methods. The expression profile of 84 key genes involved in the inflammatory response (Supplementary Fig. S2) or in cancer pathways was studied by RT-PCR using RT<sup>2</sup> Profiler PCR Arrays (PAMM-011A and PAMM-033A; SABiosciences). A pool of total RNA from distal colon of 3 mice (equal amounts) was used for each array.

### Western blot analysis

The intestines were opened out and snap-frozen at  $-80^{\circ}\text{C}$  until processed for protein extraction. Comparable amounts of tissue from distal or medial colon were homogenized in lysis buffer and Western blotting was done under standard conditions. The antibodies used and conditions are shown in Supplementary Methods.

### Isolation and culture of mouse dendritic cells

Bone marrow-derived dendritic cells (DCs) were isolated as described in Supplementary Methods. DCs were seeded in 6-well plates ( $5 \times 10^6$  to  $6 \times 10^6$  cells per well) in a final volume of 2 mL. Cells were stimulated with lipopolysaccharide (LPS; 1  $\mu\text{g}/\text{mL}$ ) from *Escherichia coli* (0111:B4; Sigma-Aldrich), in the presence or absence of VIP or PACAP38 (EMD Biosciences) at  $10^{-6}$  mol/L for 12 hours. Cell-free supernatants were

harvested at designated time points and stored at  $-20^{\circ}\text{C}$  for measurement of cytokines by ELISA.

#### Analysis of CD11b<sup>+</sup> and CD11c<sup>+</sup> cell populations

Colon lamina propria mononuclear cells (LPMC) were isolated as described in Supplementary Methods. LPMCs preparations were stained with fluorescein isothiocyanate-conjugated anti-CD11c (Miltenyi Biotech) and allophycocyanin (APC)-conjugated anti-CD11b (clone M1/70; BD Biosciences) and analyzed by fluorescence-activated cell sorting (FACS).

#### Cytokine and neuropeptide quantitation

Quantitative measurements of mouse IL-6 and IL-11 in cell culture supernatants and tissues were conducted by ELISA following manufacturer's instructions. VIP and PACAP levels were quantified by ELISA as described in Supplementary Methods. For the measurement of mouse IL-11, the supernatants were previously concentrated with Amicon Ultra-4 Centrifugal Filter Units (Millipore) following manufacturer's instructions.

#### Statistical analysis

Data are expressed as mean  $\pm$  SEM. Differences were analyzed by generalized estimating equations (GEE), Fisher

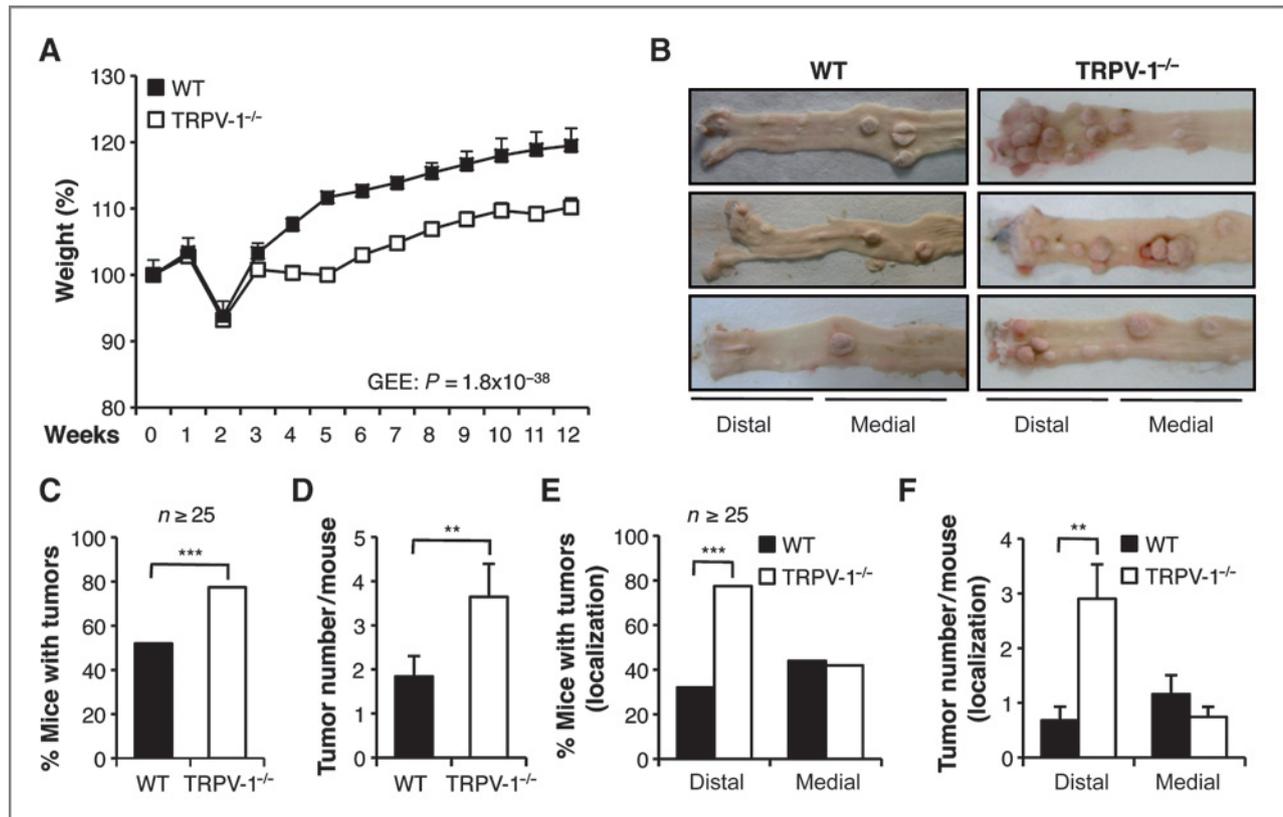
exact test, or Student *t* test.  $P < 0.05$  was considered significant (\*),  $P < 0.01$  very significant (\*\*), and  $P < 0.001$  extremely significant (\*\*\*)

## Results

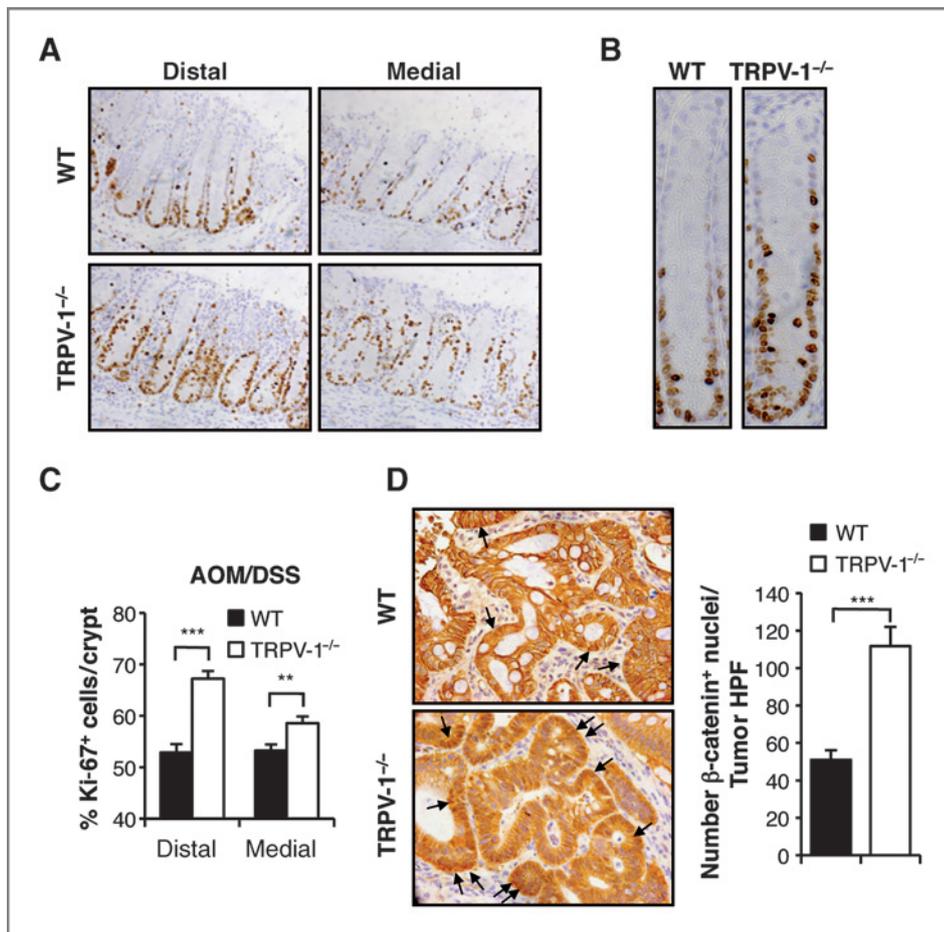
### Genetic ablation of TRPV-1 increases CAC tumorigenesis

To investigate the role of TRPV-1 in inflammation and colon carcinogenesis, we used a model of CAC in TRPV-1<sup>-/-</sup> and WT mice. Upon AOM/DSS treatment, TRPV-1<sup>-/-</sup> mice exhibited profound body weight loss compared with WT (Fig. 1A). Macroscopic colonic neoplasms developed in either WT or TRPV-1<sup>-/-</sup> animals showed a different incidence and multiplicity (Fig. 1B). Only half of the WT mice treated with AOM/DSS (52%) developed tumors in colon and this incidence was significantly higher in the mice lacking TRPV-1 (77.4%; Fig. 1C). None of the mice (WT or TRPV-1<sup>-/-</sup>) given either AOM or DSS alone had any macroscopic colonic tumors (data not shown). The multiplicity of colonic neoplasms (number of tumors/mouse) was also significantly increased in animals lacking TRPV-1 (Fig. 1D).

Flat, nodular, or polypoid-like tumors developed were mainly located in the middle and/or distal colon (Supplementary Fig. S3A). Interestingly, significant differences in the



**Figure 1.** TRPV-1<sup>-/-</sup> mice exhibit increased AOM/DSS-induced tumor formation. A, changes in body weight of WT and TRPV-1<sup>-/-</sup> mice during CAC. Data represent mean  $\pm$  SEM.  $P = 1.8 \times 10^{-38}$  by GEE. B, macroscopic view of the large bowel showing the location of tumors. C, percentage of mice with tumors (incidence). \*\*\*,  $P < 0.001$  by Fisher's exact test. D, histogram showing average tumor number per mouse. \*\*,  $P < 0.01$  by Student *t* test. E, percentage of mice with tumors in the distal and medial colon from WT and TRPV-1<sup>-/-</sup> mice. \*\*\*,  $P < 0.001$  by Fisher's exact test. F, number of tumors per mouse in the distal and medial part of the colon. Data represent average tumor number  $\pm$  SEM. \*\*,  $P < 0.01$  by Student *t* test ( $n \geq 25$  per group).



**Figure 2.** Lack of TRPV-1 expression results in increased proliferation in distal colon and accumulation of  $\beta$ -catenin in colon tumors. A, the extent of intestinal epithelial cell proliferation in colons of AOM/DSS-treated mice was determined by Ki-67 immunohistochemistry (40 $\times$ ). B, Ki-67 expression on representative crypts of WT and TRPV-1<sup>-/-</sup> distal colons of AOM/DSS-treated animals. C, quantification of Ki-67<sup>+</sup> cells in WT and TRPV-1<sup>-/-</sup> crypts from distal and medial part of colon after 12 weeks of AOM/DSS. Quantification was conducted from crypts in tumor-free areas. Results are averages  $\pm$  SEM (\*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  by Student  $t$  test). D, immunohistochemistry for  $\beta$ -catenin from paraffin-embedded sections of tumor-containing colons of WT and TRPV-1<sup>-/-</sup> mice (40 $\times$ ). Black arrows indicate typical nuclear  $\beta$ -catenin accumulation. Histogram showing quantification of nuclear  $\beta$ -catenin per HPF (40 $\times$  objective) from tumors of WT and TRPV-1<sup>-/-</sup> mice. Results are averages  $\pm$  SEM. (\*\*\*,  $P < 0.001$  by Student  $t$  test).

distribution of tumors were found. While WT mice developed mainly flat tumors in the medial colon, neoplasm observed in TRPV-1<sup>-/-</sup> mice were predominantly polypoid-like tumors with prevalence in the rectum and distal colon (Fig. 1B). Only in one third (32%) of WT mice, distal tumors were observed, whereas ablation of TRPV-1 resulted in enhanced incidence (77.4%; Fig. 1E). The number and size of tumors that arose in the distal colon were also very significantly increased in TRPV-1<sup>-/-</sup> mice (Fig. 1F and Supplementary Fig. S3B), therefore showing a higher frequency of larger adenomas than WT mice (Supplementary Fig. S3C). A higher expression of TRPV-1 in the distal colon from WT animals than in other regions was confirmed by qRT-PCR and Western blotting (data not shown). No differences in PGP9.5 distribution in TRPV-1<sup>-/-</sup> compared with WT mice point out that the observed effects are not due to the developmental abnormalities in the enteric nervous system (Supplementary Fig. S3D). Taken together, these data suggest that TRPV-1 may play an important role in distal colon cancer development.

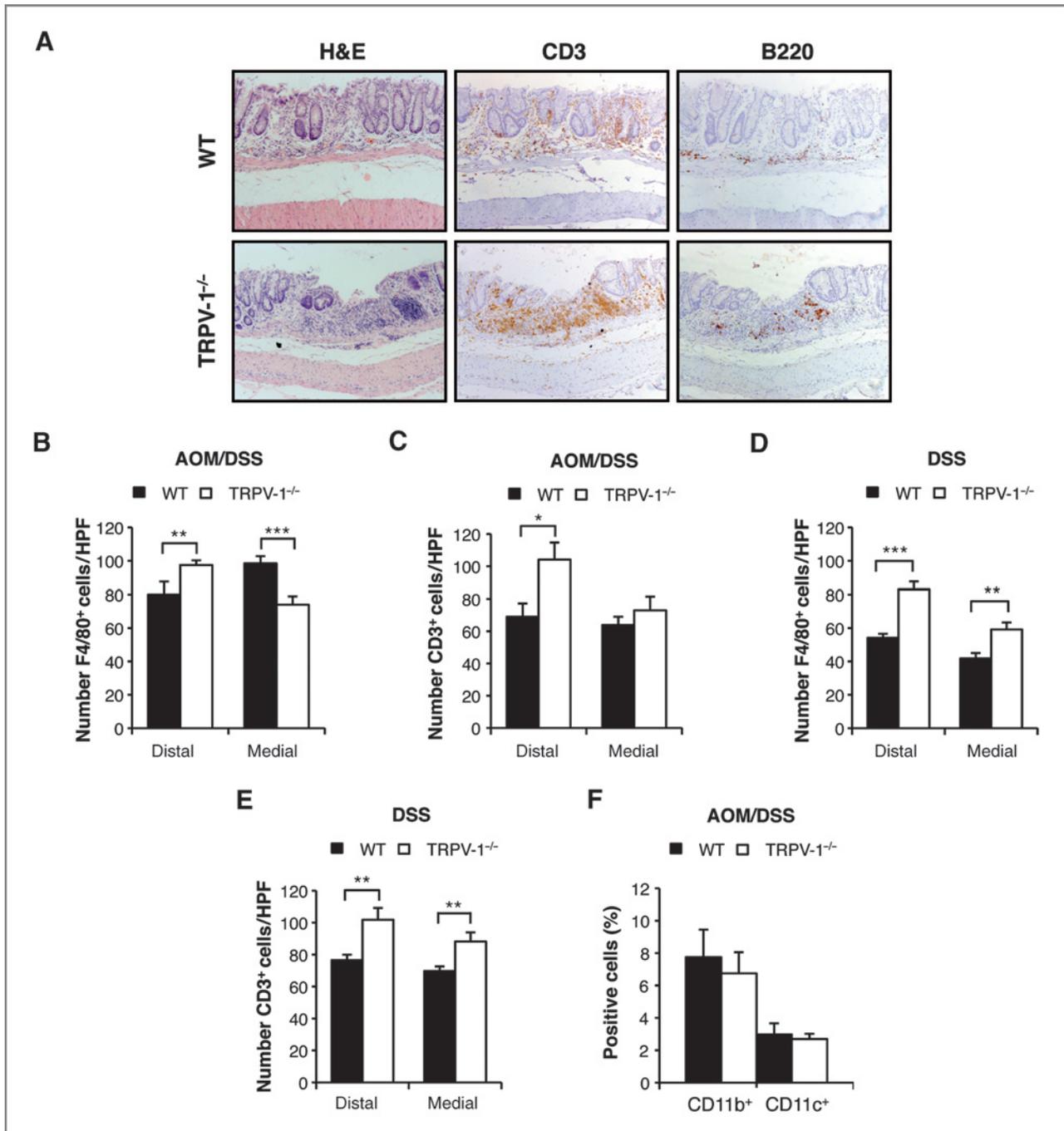
#### TRPV-1 deficiency increases colonic epithelial cell proliferation in AOM/DSS-treated mice

To further investigate the role of TRPV-1 on tumorigenesis, we evaluated cell proliferation by analyzing the number of Ki-67-expressing cells. We found increased percentage of Ki-67<sup>+</sup>

cells in the colon of AOM/DSS-treated TRPV-1<sup>-/-</sup> animals compared with WT. The increased cell proliferation was evident both in medial and distal colon, being highly significant in the latter localization (Fig. 2A-C), and correlated with the levels of IL-6 protein in tissues (Supplementary Fig. S4A). Changes in Ki-67 staining did not correlate with the expression of stem cell marker *Lgr5* studied by qPCR (Supplementary Fig. S4B). Accumulation of  $\beta$ -catenin in the nucleus of tumor cells is a key feature in colon cancer (28). Therefore, the expression of  $\beta$ -catenin was analyzed by immunostaining in the representative tumors that appeared in TRPV-1<sup>-/-</sup> and WT mice. Accumulation of  $\beta$ -catenin was detected in the nucleus of tumor cells in WT mice, but a higher number of stained nuclei were observed in tumors from TRPV-1<sup>-/-</sup> mice sacrificed at week 12 (Fig. 2D).

#### DSS-induced inflammation is enhanced in the distal colon of TRPV-1<sup>-/-</sup> mice compared with TRPV-1<sup>+/+</sup> mice

We next examined the histologic damage and inflammatory cell infiltrate in the distal colon of AOM/DSS-treated mice. We found more ulcerative lesions in the distal colon of mice lacking TRPV-1 than in WT mice, which showed large regions with preserved epithelial structures (Fig. 3A). Inflammatory infiltrates were mainly composed of T cells (CD3<sup>+</sup>) and myeloid cells (F4/80<sup>+</sup>) and also some aggregates of B cells (B220<sup>+</sup>)



**Figure 3.** Inflammatory parameters in TRPV-1<sup>-/-</sup> and WT mice after AOM/DSS challenge. A, representative hematoxylin and eosin (H&E)-stained sections and immunohistochemical analysis of CD3 and B220 of distal colons from WT and TRPV-1<sup>-/-</sup> mice after CAC induction (20 $\times$ ). B, quantification of F4/80<sup>+</sup> cells per HPF in distal and medial colon from WT and TRPV-1<sup>-/-</sup> mice after CAC induction. Data represent mean  $\pm$  SEM. \*,  $P = 0.0283$ ; \*\*\*,  $P = 0.0004$  by Student  $t$  test. C, quantification of CD3<sup>+</sup> cells per HPF in colons of WT and TRPV-1<sup>-/-</sup> mice at the end of AOM/DSS challenge (12 weeks). D, quantification of F4/80<sup>+</sup> cells per HPF in distal and medial regions of colons from WT and TRPV-1<sup>-/-</sup> mice treated with 2 cycles of DSS and sacrificed at week 12. Data represent mean  $\pm$  SEM. \*\*,  $P = 0.0016$ ; \*\*\*,  $P = 0.0001$  by Student's  $t$  test. E, quantification of CD3<sup>+</sup> cells per HPF in colons of WT and TRPV-1<sup>-/-</sup> mice at the end of DSS challenge (12 weeks). F, percentage of CD11b<sup>+</sup> and CD11c<sup>+</sup> cells determined by FACS in LPMCs isolated from WT and TRPV-1<sup>-/-</sup> mice colons after AOM/DSS treatment (12 weeks). Results are mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  by Student  $t$  test.

were detected. Next, we carried out microscopic quantifications of the immunostained cells and we found a significant increase of myeloid and T cells infiltrating the distal colon in TRPV-1<sup>-/-</sup> mice compared with WT animals (Fig. 3B and C).

TRPV-1<sup>-/-</sup> mice also exhibited an increased infiltration of inflammatory cells when chronic inflammation was chemically induced by repeated administration of DSS in the absence of AOM. In this case, immunostaining of CD3 and F4/80 revealed

that the number of lymphocytes and myeloid cells were markedly increased in both distal and medial colon of TRPV-1<sup>-/-</sup> mice compared with WT (Fig. 3D and E). No differences were found when comparing untreated WT and TRPV-1<sup>-/-</sup> mice (data not shown). However, FACS analysis of CD11b<sup>+</sup> and CD11c<sup>+</sup> populations from LPMCs showed no significant changes in percentage of positive cells in TRPV-1<sup>-/-</sup> animals compared with WT (Fig. 3F). Interestingly, the myeloid cells infiltrating the colon of TRPV-1<sup>-/-</sup> mice were significantly increased at week 12 from AOM injection even in the absence of the DSS-induced inflammation (Supplementary Fig. S5).

Next, we explored the mRNA expression of proinflammatory genes in the colon of TRPV-1<sup>-/-</sup> and WT mice. We found that upon AOM/DSS treatment, the expression of several genes such as *COX-2*, *TNF- $\alpha$* , *IL-1 $\beta$* , and *IL-6* were upregulated in both WT and TRPV-1<sup>-/-</sup> animals (Fig. 4A–D). Clearly, in animals lacking *TRPV-1*, *COX-2* and *IL-6* were greatly increased in the distal colon compared with WT mice (Fig. 4A and D). We next studied a larger number of genes involved in inflammation by qRT-PCR array (Supplementary Fig. S2). Strikingly, we found a dramatic increase in the expression of *IL-11* in the distal colon of AOM/DSS-treated TRPV-1<sup>-/-</sup> mice (Fig. 4E). *IL-11* was also induced by DSS alone but to a lesser extent than in animals treated with AOM/DSS (Fig. 4F). In addition to *COX-2*, *IL-6*, and *IL-11*, other genes playing distinct roles in inflammation (e.g., *Cxcl1*, *Spp1*, *IL1F8*, *Ccl2*) were also differentially regulated in TRPV-1<sup>-/-</sup> mice when compared with their WT counterparts.

To further investigate the influence of TRPV-1 in carcinogenesis, we studied the expression of several cancer-related genes in the distal colon of both TRPV-1<sup>-/-</sup> and WT animals treated with AOM/DSS. TRPV-1<sup>-/-</sup> mice exhibited increased expression of several genes involved in angiogenesis, cell-cycle control, adhesion, or metastasis (Supplementary Fig. S6A). Of all these genes, Matrix metalloproteinase 9 (*MMP-9*) was specially increased at the mRNA level. In addition, we also found by zymography a clear increased expression of MMP-9 dimmers and MMP-2 in TRPV-1<sup>-/-</sup> mice (Supplementary Fig. S6B). Altogether, these results suggest that in TRPV-1<sup>-/-</sup> mice, the tumoral process is more invasive and aggressive than in WT mice.

### TRPV-1 protects from spontaneous tumor formation triggered by *Apc* mutation

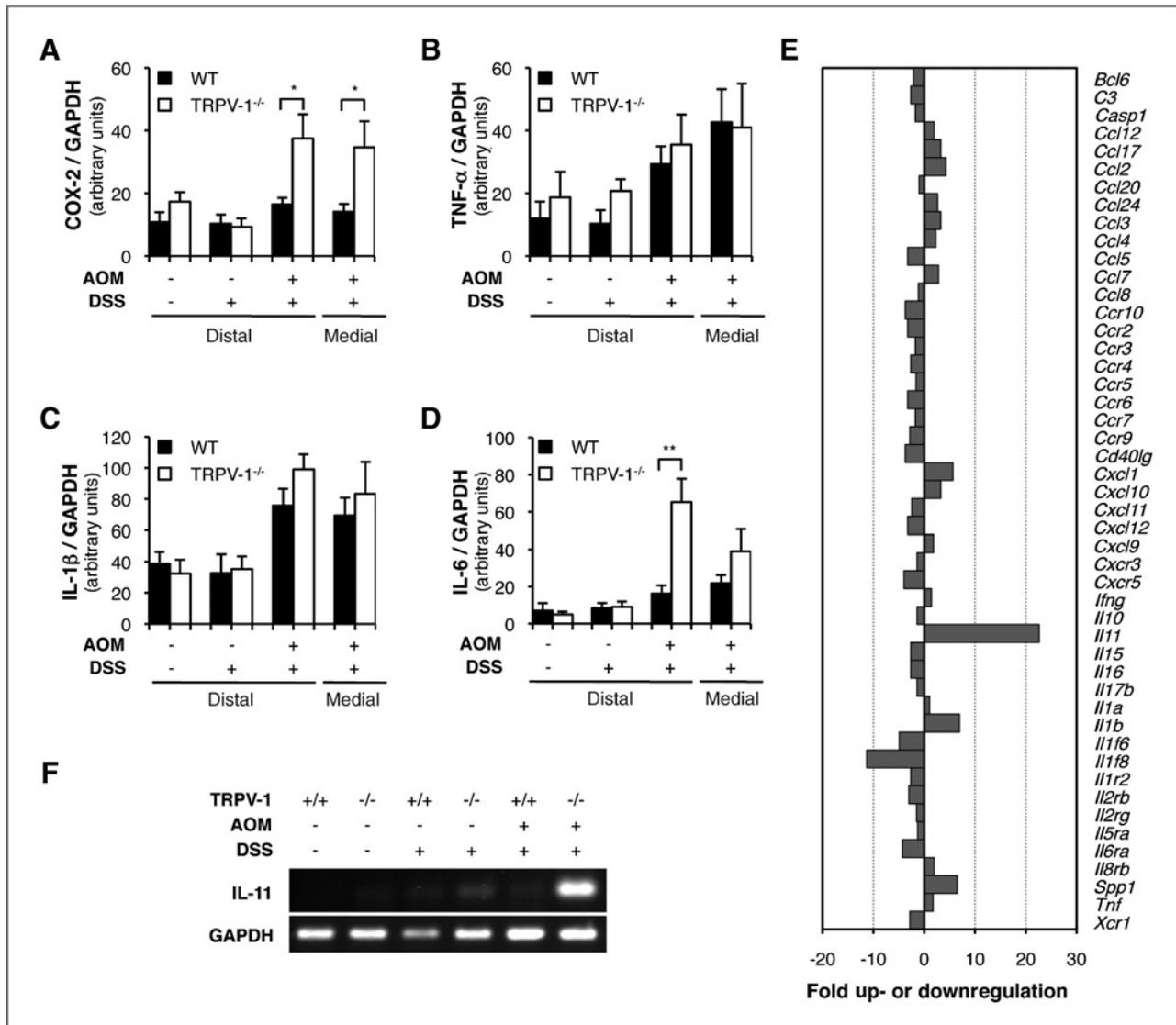
To investigate the role of TRPV-1 in the carcinogenesis process of genetically predisposed animals, we investigated the effect of TRPV-1 deletion in APC<sup>Min/+</sup> mice. *Apc* mutation triggers spontaneous development of adenomas mainly throughout the small intestine but also in the large bowel (29). To examine the effect of the loss of TRPV-1 in the APC<sup>Min/+</sup> background, compound-mutant heterozygous (APC<sup>Min/+</sup>TRPV-1<sup>+/-</sup>) or knockout for TRPV-1 (APC<sup>Min/+</sup>TRPV-1<sup>-/-</sup>) animals (Supplementary Fig. S7A) were sacrificed at 4 months of age and the development of colonic tumors analyzed (Supplementary Fig. S7B). In the absence of just 1 or 2 alleles of TRPV-1, 100% of mice presented tumors

in colon (Fig. 5A). APC<sup>Min/+</sup> mice developed mainly medial tumors, and only a small number of animals had a single tumor in the distal part of the large bowel (15% and 79%). Noteworthy, in compound-mutant mice, a significant increase in the number of mice with distal tumors was observed, with the lack of 1 allele (APC<sup>Min/+</sup>TRPV-1<sup>+/-</sup>; 45% and 45%) or 2 alleles (APC<sup>Min/+</sup>TRPV-1<sup>-/-</sup>; 92% and 86%; Fig. 5B), highlighting the possible protective role of TRPV-1 in this part of the gastrointestinal tract. Quantification of macroscopic tumors revealed significant differences between APC<sup>Min/+</sup> and APC<sup>Min/+</sup>TRPV-1<sup>-/-</sup> mice, where the total number of tumors developed in colon was nearly 2.5-fold increased in APC<sup>Min/+</sup>TRPV-1<sup>-/-</sup> mice compared with APC<sup>Min/+</sup> (Fig. 5C). Moreover, an increased multiplicity in the distal colon of APC<sup>Min/+</sup>TRPV-1<sup>-/-</sup> was also evident (Fig. 5D).

We analyzed the mRNA expression of inflammation-related genes to determine the inflammatory pathways that contribute to the development of tumors spontaneously triggered by *Apc* mutation. We found that in comparison with APC<sup>Min/+</sup> mice, the distal colon of APC<sup>Min/+</sup>TRPV-1<sup>-/-</sup> showed a markedly decreased expression in IFN- $\gamma$ , as well as several chemokine ligands (*Cxcl9*, *Cxcl10*, *Cxcl11*, *Ccl4*, *Ccl5*) and receptors (*Cxcr3*, *Ccr3*, *Ccr4*, *Ccr5*; Fig. 5E), which have been shown to have an important role in immune surveillance and tumor suppression (30). In addition, upregulated expression of some genes was also observed. As shown in Fig. 5E, the expression of *Spp1* (osteopontin), *Ccr6*, and its ligand *Ccl20* was increased in the distal colon of TRPV-1<sup>-/-</sup> mice. Accordingly, osteopontin is overexpressed in a variety of cancers including colorectal cancer (31), and *Ccr6* has been shown to be associated with the development of Crohn's disease (32).

### The activation of NF- $\kappa$ B and STAT3 signaling pathways is enhanced in DSS-treated TRPV-1<sup>-/-</sup> mice

IL-6 and IL-11 are members of the same family of proinflammatory cytokines, which are connected with the STAT3 and NF- $\kappa$ B signaling pathways in CAC (26, 27). We have previously found that IL-6 and IL-11 were greatly enhanced in the distal colon of TRPV-1<sup>-/-</sup> mice in the final steps of the AOM/DSS challenge. To analyze whether TRPV-1 was contributing to the control of inflammation from the initial steps, we treated TRPV-1<sup>-/-</sup> and WT mice with a short AOM/DSS protocol where the animals were injected with AOM and 5 days later challenged with DSS at 3.5% in drinking water during 5 days. After a recovery phase of 5 days, proteins and mRNA from distal colon were isolated. IL-6 and IL-11 mRNA expression was found upregulated in both TRPV-1<sup>-/-</sup> and WT animals. IL-6 mRNA expression was clearly increased in TRPV-1<sup>-/-</sup> compared with WT mice (Fig. 6A), with no differences in IL-11 expression. Next, we analyzed the NF- $\kappa$ B, mitogen-activated protein kinase (MAPK), and STAT3 activation pathways by measuring the phosphorylation status of I $\kappa$ B $\alpha$ , JNK1+2, ERK1+2, and STAT3 proteins, respectively. Treatment with AOM/DSS induced the phosphorylation of I $\kappa$ B $\alpha$  and STAT3 in the distal colon, which was clearly enhanced in TRPV-1<sup>-/-</sup> mice (Fig. 6B). These results fit well



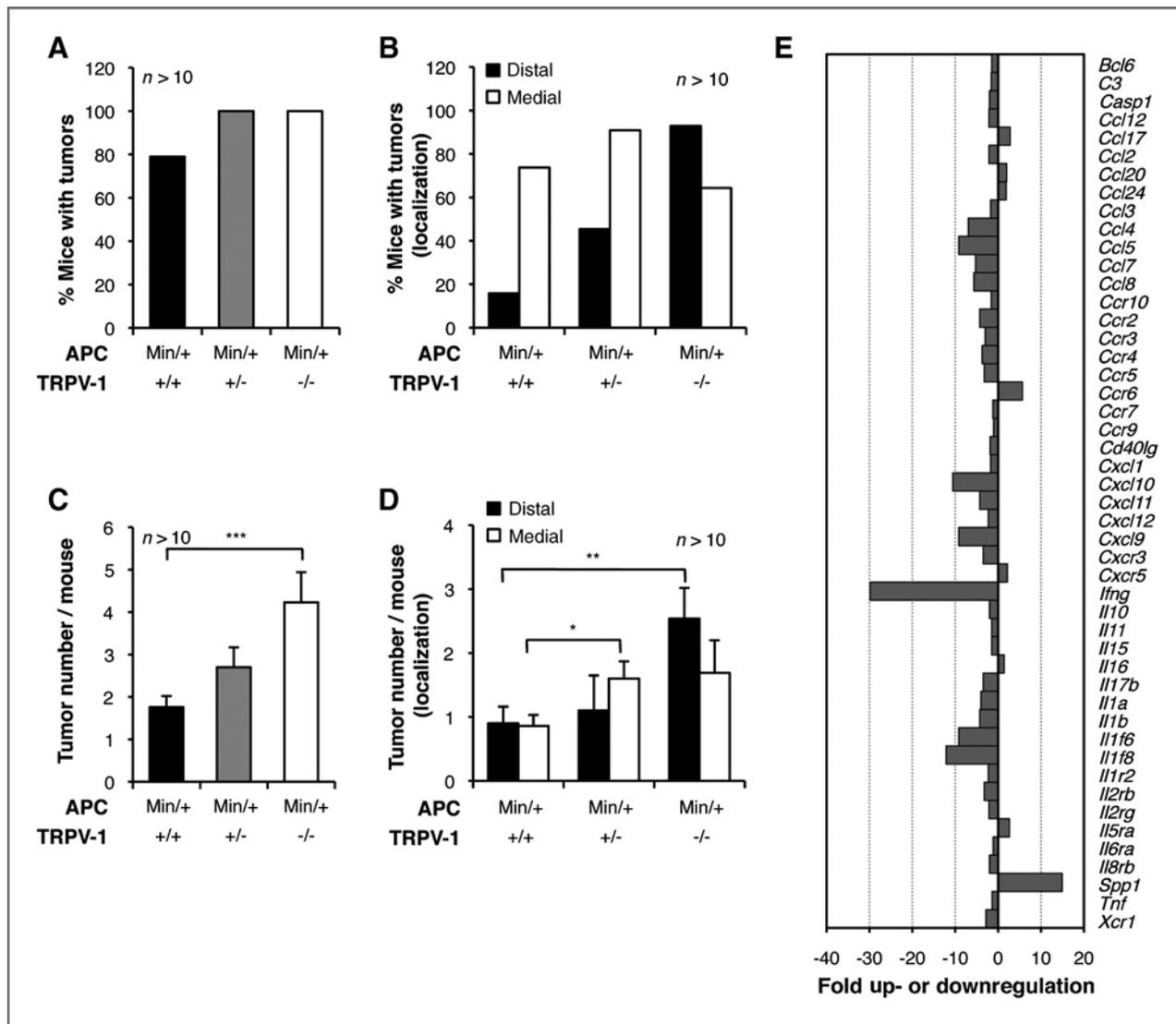
**Figure 4.** Ablation of TRPV-1 increases expression of genes involved in inflammation during CAC induction. A–D, histograms showing relative expression of proinflammatory cytokines obtained by RT-PCR from RNA isolated from whole colonic mucosa from a minimum of 3 WT or TRPV-1<sup>-/-</sup> mice receiving no treatment, DSS alone, or AOM/DSS and sacrificed at week 12 ( $n \geq 3$ ). The data are normalized to GAPDH and represent mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  by Student's *t* test. E, inflammatory gene expression. Data show representative changes in expression of genes from TRPV-1<sup>-/-</sup> mice compared with WT after CAC challenge (see Supplementary Fig. S2 for complete list of genes). F, expression of IL-11 was analyzed by RT-PCR from distal colon of WT and TRPV-1<sup>-/-</sup> mice receiving no treatment, DSS, or AOM/DSS at week 12. One representative experiment is shown ( $n \geq 3$ ). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

with the increased expression of IL-6 (Fig. 6A and C), suggesting that this cytokine, in cooperation with IL-11, is a major player in the enhanced inflammatory process that occurs in the absence of TRPV-1.

#### TRPV-1 is required for VIP and PACAP neuropeptides mRNA expression in the distal colon of DSS-treated mice

Sensory neurons can release anti-inflammatory neuropeptides such as VIP and PACAP in response to specific stimuli. These neuropeptides are able to inhibit NF- $\kappa$ B activation (33) and VIP has been shown to reduce the expression of STAT3 and phospho-STAT3 (34). Thus, we reasoned that the

enhanced inflammation observed in the gastrointestinal tract in absence of TRPV-1 could reflect the lack of anti-inflammatory peptides. To address this hypothesis, we examined the distal colon of TRPV-1<sup>-/-</sup> and WT animals in the recovery phase of DSS-induced acute colitis. Strong induction of VIP and PACAP mRNA expression was observed in WT mice. In contrast, the mRNA for these neuropeptides was not detected in the distal colon of DSS-treated mice lacking TRPV-1 (Fig. 6D). Next, we measured the levels of VIP and PACAP peptides in colons of TRPV-1<sup>-/-</sup> and WT mice. We found that both VIP and PACAP levels were higher in WT than in TRPV-1<sup>-/-</sup> animals and also the levels of both

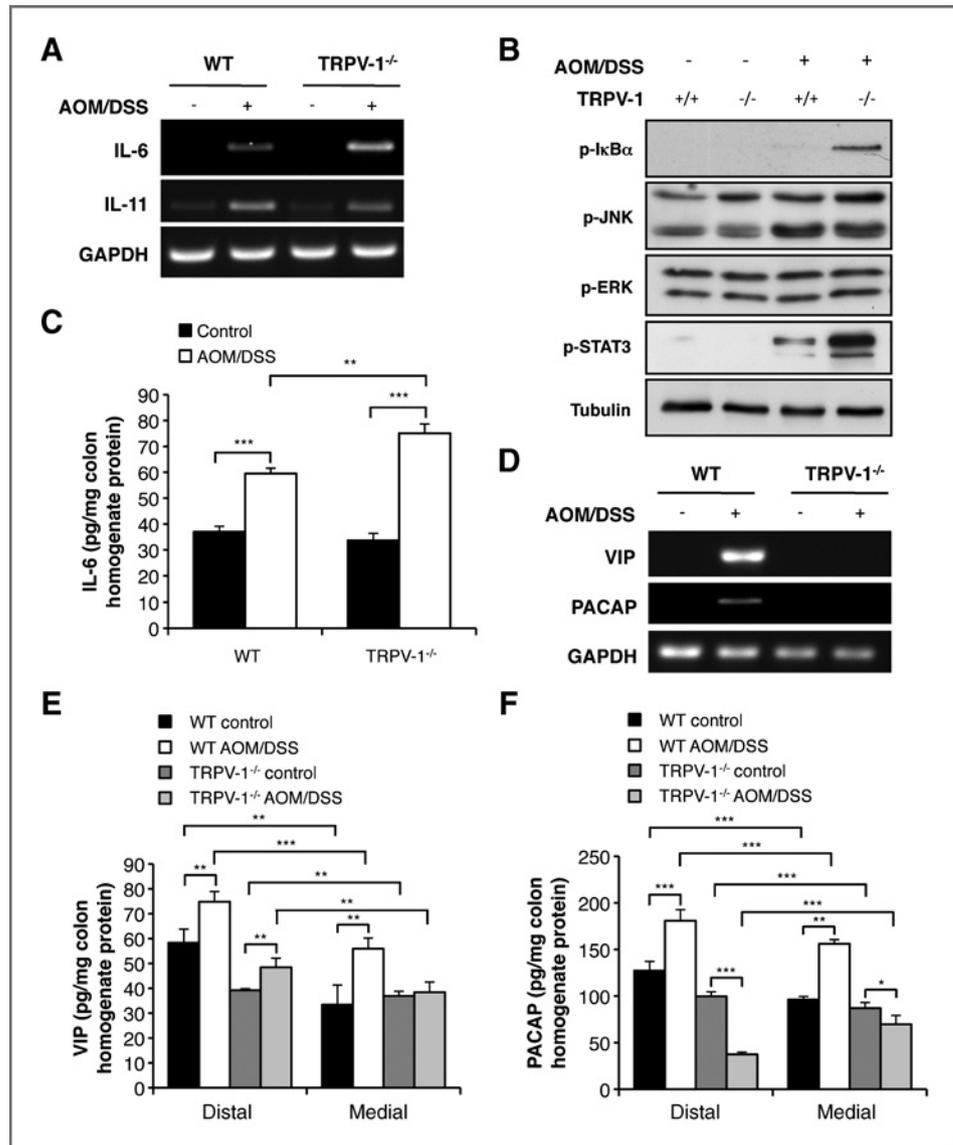


**Figure 5.** Absence of TRPV-1 alleles increases tumor formation in  $APC^{Min/+}$  mice. A, percentage of mice with tumors (incidence) in the colon and (B) in distal and medial regions. C, average tumor number per mouse. Data represent mean  $\pm$  SEM.  $***, P = 0.0006$  by Student  $t$  test. D, histogram showing number of tumors per mouse in the distal and medial part of the colon. Data represent mean  $\pm$  SEM.  $*, P = 0.023$ ;  $**, P = 0.0025$  by Student  $t$  test. A–D, for each genotype,  $APC^{Min/+}$ ,  $APC^{Min/+} TRPV-1^{+/-}$ , or  $APC^{Min/+} TRPV-1^{-/-}$ , the number of animals analyzed ( $n$  values) are 19, 11, and 14, respectively. E, expression of genes involved in inflammation was analyzed by qRT-PCR superarrays. Data show representative changes in expression of genes from  $APC^{Min/+} TRPV-1^{-/-}$  mice compared with  $APC^{Min/+}$  sacrificed at 4 months of age. A pool of total RNA from distal colon of 3 mice was used for each array (see Supplementary Fig. S2 for a complete list of genes).

peptides were higher in distal colon than in medial colon. AOM/DSS treatment resulted in a significant increase in both VIP and PACAP peptides in both distal and medial colons in WT animals. However, the induction of VIP was attenuated in the distal colon of  $TRPV-1^{-/-}$  mice and completely prevented in the medial colon of these animals. Interestingly, AOM/DSS treatment greatly reduced the levels of PACAP in the distal colon of  $TRPV-1^{-/-}$  mice (Fig. 6E and F).

Previous studies have found that myeloid cells, and in particular DCs, are the major contributors of IL-6 production during the initial stages of colitis (25, 35) and in developed CAC (27). Therefore, we isolated DCs from  $TRPV-1^{-/-}$  and WT mice

to test their ability to induce mRNA IL-6 expression. We found that DCs isolated from either  $TRPV-1^{-/-}$  or WT mice were equally competent to induce IL-6 mRNA after LPS stimulation, indicating that  $TRPV-1^{-/-}$  DCs are fully functional to produce cytokines (Supplementary Fig. S8A). Next, we investigated whether the neuropeptides VIP and PACAP were able to influence the expression of IL-6 and IL-11 in DCs. Incubation of the cells in the presence of VIP or PACAP significantly decreased the LPS-induced mRNA expression of both IL-6 and IL-11 (Fig. 7A and B and Supplementary Fig. S8B). Moreover, VIP and PACAP were able to inhibit LPS-induced release of the proinflammatory cytokines IL-6 (Fig. 7C) and IL-11 (Fig. 7D). We found that PACAP was more effective than VIP in the



**Figure 6.** TRPV-1<sup>-/-</sup> mice show increased expression of IL-6 and IL-11 and decreased expression of neuropeptides VIP and PACAP in the distal colon. **A**, IL-6 and IL-11 mRNA expression in WT and TRPV-1<sup>-/-</sup> distal colons. **B**, Western blot analysis of protein lysates from WT and TRPV-1<sup>-/-</sup> distal colons. ERK, extracellular signal-regulated kinase. **C**, IL-6 levels detected by ELISA in protein lysates from WT and TRPV-1<sup>-/-</sup> distal colon. **D**, mRNA expression of VIP and PACAP in WT and TRPV-1<sup>-/-</sup> distal colons. **E** and **F**, ELISA analysis of (E) VIP and (F) PACAP peptide levels in protein lysates extracted from WT and TRPV-1<sup>-/-</sup> distal and medial parts of colon after short AOM/DSS challenge. Data represent mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  by Student *t* test.

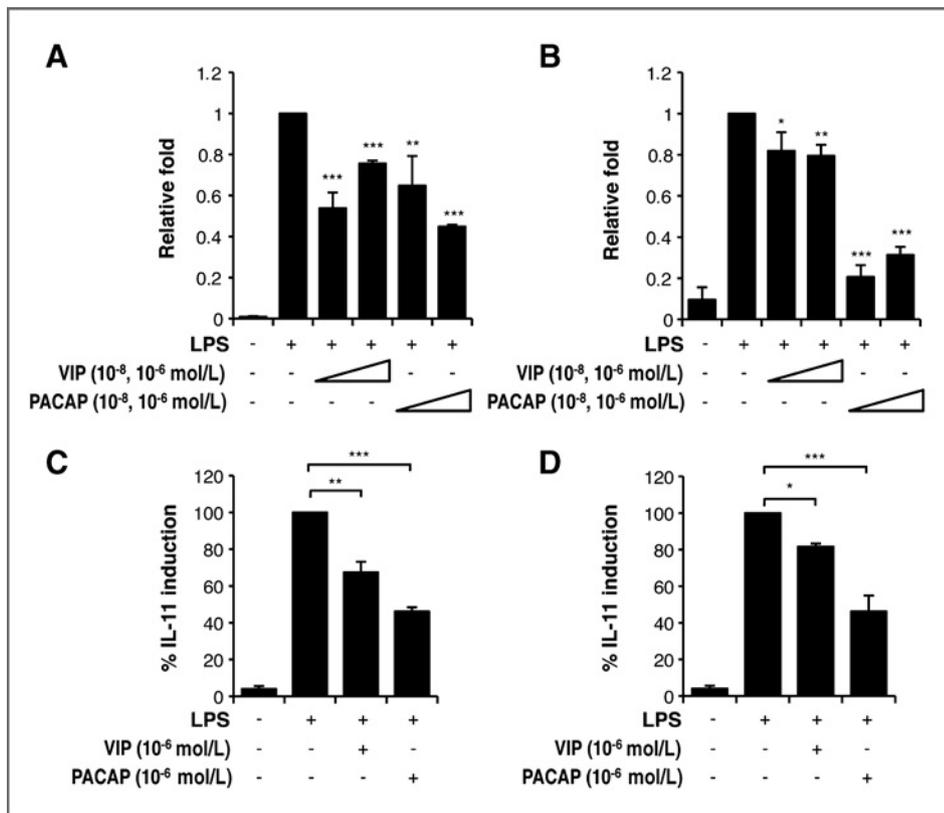
inhibition of IL-6 and IL-11 at the concentrations tested. These findings suggest that the reduced expression of the neuropeptides VIP and PACAP can be involved in the exacerbated expression of IL-6 and IL-11, found in the distal colon of TRPV-1<sup>-/-</sup> animals during CAC.

## Discussion

Proliferation and survival of tumor cells can be deeply affected by interactions with the surrounding inflammatory microenvironment. During the initial stages, immune and stromal cells provide signals that favor malignant cell growth, whereas in advanced stages, they can promote the invasion and metastasis of tumor cells (36). The expression of TRPV-1 on sensory nerves is essentially associated with pain and neurogenic inflammation (37). However, the role of TRPV-1 in colon cancer was not investigated. Our findings show that TRPV-1 contributes to protection of tumor development in the lower

gastrointestinal tract of mice subjected to chronic inflammation and in mice genetically predisposed to develop colon adenomas, which implies that TRPV-1 may be also relevant to modulate the homeostasis of the colonic immune system. It has been recently shown that TRPV-1-immunoreactive nerve fibers present in the rectum colocalize with CGRP, confirming the neuropeptidergic nature of TRPV-1 expressing sensory fibers (38). However, it has been reported that colon neurons expressing markers for A- and C-fibers express VIP but not TRPV-1, suggesting the existence of separate populations of neurons projecting from colon to CNS (39). Thus, it is possible that TRPV-1-expressing neurons exert some type of cross-talk with TRPV-1<sup>-</sup>/VIP<sup>+</sup> neurons to release anti-inflammatory peptides in the colon.

TRPV-1 expression has been also detected in DCs (40). However, other studies were unable to detect expression of functional TRPV-1 on these cells (41). We found that DCs



**Figure 7.** Effects of VIP and PACAP on cytokines expression in LPS-stimulated DCs. Relative mRNA expression (qPCR) of IL-6 (A) and IL-11 (B) in isolated DCs stimulated with LPS in the presence or absence of different doses of neuropeptides. Release of IL-6 (C) and IL-11 (D) proteins in DC culture supernatants was analyzed by ELISA. Data represent mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  by Student *t* test.

isolated from TRPV-1<sup>-/-</sup> and WT mice are fully functional in response to LPS, and therefore it is unlikely that the inflammatory effects found in mice lacking TRPV-1 are due to deregulated DCs (i.e., gain of function). We did not find an increase in the percentage of DCs in the colon of TRPV-1<sup>-/-</sup> mice, suggesting that immunosuppressive factors such as VIP and PACAP may control the release of IL-6 and IL-11 in the inflammatory focus. Accordingly, the neuropeptides VIP and PACAP inhibited LPS-induced secretion of IL-6 and IL-11 in isolated DCs. In support of our hypothesis, we found decreased expression of VIP and PACAP and enhanced expression of IL-6 and IL-11 in the distal colon of DSS-treated TRPV-1<sup>-/-</sup> mice. VIP and PACAP have been shown to exert important beneficial actions in the treatment of murine models of Crohn's disease by downregulating the proinflammatory response (17, 18). Moreover, induction of a mild colitis in mice deficient in PACAP has been shown to trigger rapid development of colorectal tumors without the use of a carcinogen. Absence of PACAP led to increased tumor incidence and severity as a consequence of the enhanced inflammatory response to DSS (19). As in the TRPV-1<sup>-/-</sup> mice subjected to CAC challenge, clinical symptoms, inflammatory changes, and proinflammatory cytokine responses were significantly more severe in PACAP knockout versus WT controls. VIP and PACAP have multiple actions and in addition to their anti-inflammatory activity, these neuropeptides can also control the growth and survival of tumor cells (42). We hypothesized that under proinflammatory conditions, TRPV-1 is activated not only to transmit pain sensation but also to release anti-inflammatory

neuropeptides that control cytokine release by myeloid cells, which in turn can enhance STAT3 and NF- $\kappa$ B activation and carcinogenesis in epithelial cells (Supplementary Fig. S9).

We found that the mechanisms underlying the increased carcinogenesis in TRPV-1<sup>-/-</sup> mice were tightly related to inflammation. Therefore, the expression profile of inflammation and cancer-related genes of TRPV-1<sup>-/-</sup> mice suggested a more aggressive and severe process, which could be a direct consequence of the exacerbated colon inflammation. Invasiveness and metastasis of cancer cells requires proteolysis of the extracellular matrix at the invasive front. Interestingly, inflammatory cells are an important source of proteases, and cytokines such as IL-6, IL-1, and TNF- $\alpha$  promote expression of MMP9, via NF- $\kappa$ B and STAT3 pathways (43). Both pathways mediate the increased expression of a wide variety of anti-apoptotic proteins (Bcl2l1), proangiogenic factors, inflammatory cytokines and chemokines, (CXCL1, CCL2, CCL7), and metastasis and invasion factors (MMP9, osteopontin, Twist1, Plau). Thus, results obtained in mice genetically modified to lack TRPV-1 could be a consequence of NF- $\kappa$ B and STAT3 upregulated pathways, which orchestrate an increased inflammatory milieu (Supplementary Fig. S9).

The gastrointestinal protection produced by capsaicin-induced stimulation of sensory neurons is associated with a marked increase of mucosal blood flow and mucus secretion (44). Therefore, both activation and inhibition of TRPV-1 could represent a therapeutic option depending on the target disease or pathologic condition. For instance, a pharmacologic TRPV-1 blockade without secondary effects at the CNS could be

beneficial for the treatment of acute abdominal pain and IBD active phases. However, a long-term use of TRPV-1 inhibitors could favor colon carcinogenesis in patients with previous inflammatory diseases and/or genetic predisposition.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

**Conception and design:** A.G. Vinuesa, C. García-Limones, E. Muñoz

**Development of methodology:** A.G. Vinuesa, M.A. Calzado

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** R. Sancho, C. García-Limones, A. Behrens, P. ten Dijke, M.A. Calzado

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** A.G. Vinuesa, R. Sancho, C. García-Limones, M.A. Calzado

**Writing, review, and/or revision of the manuscript:** A.G. Vinuesa, R. Sancho, C. García-Limones, P. ten Dijke, M.A. Calzado, E. Muñoz

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** M.A. Calzado  
**Study supervision:** E. Muñoz

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## Vanilloid Receptor-1 Regulates Neurogenic Inflammation in Colon and Protects Mice from Colon Cancer

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