Title: Vanilloid receptor-1 regulates neurogenic inflammation in colon and protects mice from colon cancer.

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Running title: TRPV-1 protects from colitis-associated cancer.

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ABSTRACT

Neuroinflammation driven by the vanilloid-type ion channel receptor TRPV-1 is suspected to play a role in the pathophysiology of inflammatory bowel disease. Since 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 tumors along with elevated expression of IL-6 and IL-11 and activation of the STAT3 and NF-κB signaling pathways. Notably, TPRV-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 pro-inflammatory 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.
INTRODUCTION

The transient receptor potential vanilloid type 1 (TRPV-1), also called vanilloid receptor, is a non-selective cation channel which is predominantly expressed in primary afferent sensory neurons and in the central nervous system (CNS) (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 generation of neurogenic inflammation, which plays a major role in the pathophysiology of inflammatory bowel diseases (IBD) (6, 7). TRPV-1 expression is increased in the colon of IBD patients (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 colonic damage induced by DNBS (13) and DSS (11). In contrast other reports have shown that TRPV-1 activation may exacerbate colon inflammation in different animals models (14-16).

A crosstalk 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 due to its pro-inflammatory nature they are thought to play a significant role in development and pathogenesis of colitis (6). In contrast, other neuropeptides such as VIP and 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 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, tumour development.

Many epidemiological 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 IBD patients are known to increase the risk of colorectal cancer (21). STAT3 and NF-κB signaling pathways play crucial roles in initiation and development of inflammation-induced cancer (20, 22, 23). STAT3 and NF-κB are found constitutively activated in cancer cells and in tumour-associated myeloid cells and regulate in a cooperative manner several pro-inflammatory genes such as IL-6, IL-11, chemokines, growth factors and COX-2, that are crucial for maintaining a pro-carcinogenic inflammatory environment (23, 24). The complex interplay between NF-κB and STAT3 signaling cascades and the role of IL-6 and IL-11 have recently been deciphered in different studies using murine models of CAC (25-27).

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

**MATERIAL AND METHODS**
Animals. C57BL/6J wild type (WT), TRPV-1\(^{-/-}\) (B6.129X1-Trpv1\(^{tm1Jul}\)/J) and APC\(^{Min/+}\) (C57BL/6J-Apc\(^{Min}/J\)) mice (Jackson Laboratory, Bar Harbor, ME, USA) were genotyped using specific primers (Supplemental figure 1) and housed under standard conditions. Compound mutant APC\(^{Min/+}\)TRPV-1\(^{-/-}\) mice were generated by first breeding APC\(^{Min/+}\) males to TRPV-1\(^{-/-}\) females. Males APC\(^{Min/+}\)TRPV-1\(^{+/-}\) where then crossed to TRPV-1\(^{-/-}\) females to generate APC\(^{Min/+}\)TRPV-1\(^{-/-}\) mice. All experiments were performed 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 Cordoba.

Tumour Induction and Analysis. 8–10 week old mice were injected intraperitoneally (i.p.) with 10 mg/kg azoxymethane (AOM) (Sigma-Aldrich, St. Louis, MO). After one week 1% dextran sodium sulphate (DSS, m.w. 36-50 kDa) (MP Biomedicals, Irvine, CA, USA) was given in the drinking water over four 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.

Histological 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 transferred into 70% ethanol, processed and embedded into paraffin. 5 μm sections were cut for haematoxylin and eosin staining and immunohistochemistry. Slides were de-waxed in xylene and rehydrated by passage through graded alcohols to water. Antigen retrieval was performed by microwave (medium power) for 15-20 min in 0.1M sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked using 1.6% H\(_2\)O\(_2\) in methanol and then slides were incubated in 10% normal serum in 1% BSA/PBS for 40 min. Primary incubations were performed with antibodies against Ki67 (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 β-catenin (1:100, Upstate, 05-665) in 1%BSA/PBS for 1 hr at RT. Isotype-matched antibodies were included as negative
controls. Tumour size was determined by image analysis of frozen tissues using imaging software (ImageJ). Size was determined by measuring tumour largest diameter. 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 tumours from each region was measured from at least 7 mice from each genotype. IHC was quantified by counting the number of positive cells visualized per high-power field (HPF; 40x or 20x objective) within each region. The mean positive cells per HPF were calculated (see Supplemental Methods for additional details).

**RNA analysis.** Transcript levels were quantified by RT-PCR with specific primers for each gene, and Quantitative Real-time PCR reaction samples were prepared as a mixture with the qPCR SYBR Green PCR Kit (Promega, Madison, WI, USA). Amplifications were performed using the iCycler PCR Detection system (Bio-Rad, Hercules, CA, USA). The primer sequences used and PCR conditions are shown in supplemental methods. The expression profile of 84 key genes involved in the inflammatory response (Supplemental figure 2) or in cancer pathways was studied by real-time PCR using RT² Profiler PCR Arrays (PAMM-011A and PAMM-033A; SABiosciences, Frederick, MD, USA). 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 ºC until processed for protein extraction. Comparable amounts of tissue from distal or medial colon were homogenized in lysis buffer and western blots were performed under standard conditions. The antibodies used and conditions are shown in supplemental methods.

**Isolation and culture of mouse dendritic cells.** Bone marrow-derived dendritic cells (DCs) were isolated as described in supplemental methods. DCs were seeded in 6-well plates (5-6 × 10⁶ cells per well) in a final volume of 2 ml. Cells were stimulated with LPS
(1 μg/ml) from *Escherichia coli* 0111:B4, (Sigma-Aldrich), in the presence or absence of VIP or PACAP38 (EMD Biosciences, Inc., Darmstadt, Germany) at 10^−6 M for 12 hours. Cell-free supernatants were harvested at designated time points and stored at -20 °C for measurement of cytokines by ELISA.

**Analysis of CD11b* and CD11c* cell populations.** Colon lamina propria mononuclear cells (LPMCs) were isolated as described in supplemental methods. LPMCs preparations were stained with FITC-conjugated anti-CD11c (Miltenyi Biotech, Auburn, CA, USA) and APC-conjugated anti-CD11b (clone M1/70, BD Biosciences), and analyzed by FACS.

**Cytokine and neuropeptide quantitation.** Quantitative measurements of mouse IL-6 and IL-11 in cell culture supernatants and tissues were performed by ELISA following manufacturer’s instructions. VIP and PACAP levels were quantified by ELISA as described in supplemental methods. For the measurement of mouse IL-11 the supernatants were previously concentrated using Amicon Ultra-4 Centrifugal Filter Units (Millipore, Tremecula, CA, USA) following manufacturer’s instructions.

**Statistical Analysis.** Data are expressed as mean ± SEM. Differences were analyzed by Generalized estimating equations (GEE), Fisher’s exact test or Student’s t test. *P < .05 was considered significant (*), P < .01 very significant (**), and P < .001 extremely significant (***)
RESULTS

Genetic ablation of TRPV-1 increases CAC tumourigenesis.

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

Flat, nodular, or polypoid-like tumours developed were mainly located in the middle and/or distal colon (Supplemental Figure 3A). Interestingly, significant differences in tumours distribution were found. While WT mice developed mainly flat tumours in the medial colon, neoplasm observed in TRPV-1$^{-/-}$ mice were predominantly polypoid-like tumours with prevalence in the rectum and distal colon (Figure 1B). Only in one third (32%) of WT mice distal tumours were observed, whereas ablation of TRPV-1 resulted in enhanced incidence (77.4%) (Figure 1E). The number and size of tumours that arose in the distal colon were also very significantly increased in TRPV-1$^{-/-}$ mice (Figure 1F and Supplemental Figure 3B), showing therefore a higher frequency of larger adenomas than WT mice (Supplemental Figure 3C). A higher expression of TRPV-1 in the distal colon from WT animals compared to other regions was confirmed by qRT-PCR and western blot (data not shown). No differences in PGP9.5 distribution in TRPV-1$^{-/-}$ compared with WT mice point out that the observed effects are not due to developmental abnormalities in the enteric nervous system (Supplemental Figure 3D).
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 tumourigenesis we evaluated cell proliferation by analyzing the number of Ki-67 expressing cells. We found increased percentage of Ki-67+ cells in the colon of AOM/DSS-treated TRPV-1−/− animals compared to WT. The increased cell proliferation was evident both in medial and distal colon, being highly significant in the latter localization (Figures 2, A-C), and correlated with the levels of IL-6 protein in tissues (Supplemental Figure 4A). Changes in Ki67 staining did not correlate with the expression of stem cell marker Lgr5 studied by qPCR (Supplemental Figure 4B). Accumulation of β-catenin in the nucleus of tumour cells is a key feature in colon cancer (28). Therefore, the expression of β-catenin was analyzed by immunostaining in the representative tumours that appeared in TRPV-1−/− and in WT mice. Accumulation of β-catenin was detected in the nucleus of tumour cells in WT mice, but a higher number of stained nuclei were observed in tumours from TRPV-1−/− mice sacrificed at week 12 (Figure 2D).

**DSS-induced inflammation is enhanced in the distal colon of TRPV-1−/− mice compared to TRPV-1+/+ mice**

We next examined the histological 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 compared to WT mice, which showed large regions with preserved epithelial structures (Figure 3A). Inflammatory infiltrates were mainly composed of T cells (CD3+) and myeloid cells (F4/80+) but also some aggregates of B cells (B220+) 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−/− mice compared to WT animals (Figure 3B, 3C). TRPV-1−/− 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−/− mice compared to WT (Figure 3D, 3E). No differences were found when comparing untreated WT and TRPV-1−/− mice (data not shown). However, FACS analysis of CD11b+ and CD11c+ populations from LPMC showed no significant changes in percentage of positive cells in TRPV-1−/− animals compared to WT (Figure 3F). Interestingly, the myeloid cells infiltrating the colon of TRPV-1−/− mice were significantly increased at week 12 from AOM injection even in the absence of the DSS-induced inflammation (Supplemental Figure 5).

Next, we explored the mRNA expression of pro-inflammatory genes in the colon of TRPV-1−/− and WT mice. We found that upon AOM/DSS treatment, the expression of several genes such as COX-2, TNFα, IL-1β and IL-6 was upregulated in both WT and TRPV-1−/− animals (Figure 4, A-D). Clearly, in animals lacking TRPV-1, COX-2 and IL-6 were greatly increased in the distal colon compared to WT mice (Figure 4, A and D). We next studied a larger number of genes involved in inflammation by quantitative RT-PCR array (Supplemental figure 2). Strikingly, we found a dramatic increase in the expression of IL-11 in the distal colon of AOM/DSS-treated TRPV-1−/− mice (Figure 4E). IL-11 was also induced by DSS alone but to a lesser extent than in animals treated with AOM/DSS (Figure 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−/− mice when compared to 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−/− and WT animals treated with AOM/DSS. TRPV-1−/− mice exhibited increased expression of several genes involved in angiogenesis, cell cycle control, adhesion or metastasis
(Supplemental Figure 6A). Of all these genes, Matrix metalloproteinase 9 (Mmp9) was specially increased at the mRNA level. In addition, we also found by zymography a clear increased expression of MMP-9 dimers and MMP-2 in TRPV-1-/- mice (Supplemental Figure 6B). Altogether, these results suggest that in TRPV-1-/- mice the tumoural process is more invasive and aggressive than in WT mice.

**TRPV-1 protects from spontaneous tumour 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 APCMin/+ 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 APCMin/+ background, compound-mutant heterozygous (APCMin/+TRPV-1+/-) or knock out for TRPV-1 (APCMin/+TRPV-1-/-) animals (Supplemental Figure 7A) were sacrificed at 4 months of age and the development of colonic tumours analyzed (Supplemental Figure 7B). In the absence of just one or the two alleles of TRPV-1, 100% of mice presented tumours in colon (Figure 5A). APCMin/+ mice developed mainly medial tumours, and only a small number of animals had a single tumour in the distal part of the large bowel (15.79%). Noteworthy, in compound-mutant mice, a significant increase in the number of mice with distal tumours was observed, with the lack of one allele (APCMin/+TRPV-1+/-: 45.45%) or the two alleles (APCMin/+TRPV-1-/-: 92.86%) (Figure 5B), highlighting the possible protective role of TRPV-1 in this part of the gastrointestinal tract. Quantification of macroscopic tumours revealed significant differences between APCMin/+ and APCMin/+TRPV-1-/- mice, where the total number of tumours developed in colon was nearly 2.5 fold increased in APCMin/+TRPV-1-/- mice compared to APCMin/+ (Figure 5C). Moreover, an increased multiplicity in the distal colon of APCMin/+ /TRPV-1-/- was also evident (Figure 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 to APC\(^{Min/+}\) mice, the distal colon of APC\(^{Min/+}\)TRPV-1\(^{-/-}\) 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) (Figure 5E), which have been shown to have an important role in immune surveillance and tumour suppression (30). In addition, up-regulated expression of some genes was also observed. As shown in Figure 5E, Spp1 (Osteopontin), Ccr6 and its ligand Ccl20, were increased in the distal colon of TRPV-1\(^{-/-}\) mice. Accordingly, Osteopontin is overexpressed in a variety of cancers including colorectal cancer (31), and Ccr6 has been shown to be associated to development of Crohn\(^-\)s disease (32).

**The activation of NF-\(\kappa\)B and STAT3 signaling pathways is enhanced in DSS-treated TRPV-1\(^{-/-}\) mice.**

IL-6 and IL-11 are members of the same family of pro-inflammatory 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\(^{-/-}\) 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\(^{-/-}\) 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\(^{-/-}\) and WT animals. IL-6 mRNA expression was clearly increased in TRPV-1\(^{-/-}\) compared to WT mice (Figure 6A), with no differences in IL-11 expression. Next, we analyzed the NF-\(\kappa\)B, MAPKs 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 IκBα and STAT3 in the distal colon, which was clearly enhanced in TRPV-1−/− mice (Figure 6B). These results fit well with the increased expression of IL-6 (Figures 6A and 6C), 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-κB 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. In order to address this hypothesis we examined the distal colon of TRPV-1−/− 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 (Figure 6D). Next, we measured the levels of VIP and PACAP peptides in colons of TRPV-1−/− and WT mice. We found that both VIP and PACAP levels were higher in WT compared with TRPV-1−/− animals and also the levels of both peptides were higher in distal colon compared with 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 (Figures 6E and 6F).
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−/− and WT mice were equally competent to induce IL-6 mRNA after LPS stimulation, indicating that TRPV-1−/− DCs are fully functional to produce cytokines (Supplemental Figure 8A). 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 (Figure 7A, 7B and Supplemental Figure 8B). Moreover, VIP and PACAP were able to inhibit LPS-induced release of the pro-inflammatory cytokines IL-6 (Figure 7C) and IL-11 (Figure 7D). We found that PACAP was more effective than VIP in the 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−/− animals during CAC.
DISCUSSION

Proliferation and survival of tumour cells can be deeply affected by interactions with the surrounding inflammatory microenvironment. During the initial stages, immune and stromal cells provide signals that favour malignant-cell growth, whereas in advanced stages, they can promote tumour-cells invasion and metastasis (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 demonstrate that TRPV-1 contributes to protection of tumour 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 crosstalk with TRPV1/VIP 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 isolated from TRPV-1−/− 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−/− 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−/− 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 pro-inflammatory response (17, 18). Moreover, induction of a mild colitis in mice deficient in PACAP has been shown to trigger rapid development of colorectal tumours without the use of a carcinogen. Absence of PACAP led to increased tumour incidence and severity as a consequence of the enhanced inflammatory response to DSS (19). As in the TRPV-1−/− mice subjected to CAC challenge, clinical symptoms, inflammatory changes, and pro-inflammatory cytokine responses were significantly more severe in PACAP KO 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 tumour cells (42). We hypothesized that under pro-inflammatory 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-κB activation and carcinogenesis in epithelial cells (Supplemental figure 9).

We found that the mechanisms underlying the increased carcinogenesis in TRPV-1−/− mice were tightly related to inflammation. Therefore, the expression profile of inflammation and cancer-related genes of TRPV-1−/− 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 like IL-6, IL-1 and TNF-α promote expression of MMP9, via NF-κB 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-κB and STAT3 up-regulated pathways, which orchestrate an increased inflammatory milieu (Supplemental figure 9).

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 pathological condition. For instance, a pharmacological 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 favors colon carcinogenesis in patients with previous inflammatory diseases and/or genetically predisposed.
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FIGURE LEGENDS

Figure 1. TRPV-1−/− mice exhibit increased AOM/DSS-induced tumour formation. (A) Changes in body weight of WT and TRPV-1−/− mice during CAC. Data represent mean ± SEM. \( P = 1.8\text{e-}38 \) by Generalised Estimating Equation. (B) Macroscopic view of the large bowel showing the location of tumours. (C) Percentage of mice with tumours (incidence). ***\( P < .001 \) by Fisher’s exact test. (D) Histogram showing average tumour number per mouse. **\( P < .01 \) by Student’s \( t \) test. (E) Percentage of mice with tumours in the distal and medial colon from WT and TRPV-1−/− mice. ***\( P < .001 \) by Fisher’s exact test. (F) Number of tumours per mouse in the distal and medial part of the colon. Data represent average tumour number ± SEM. **\( P < .01 \) by Student’s \( t \) test (\( n \geq 25 \) per group).

Figure 2. Lack of TRPV-1 expression results in increased proliferation in distal colon and accumulation of β-catenin in colon tumours. (A) The extent of intestinal epithelial cell proliferation in colons of AOM/DSS treated mice was determined by Ki-67 immunohistochemistry (40x). (B) Ki-67 expression on representative crypts of WT and TRPV-1−/− distal colons of AOM/DSS treated animals. (C) Quantification of Ki-67+ cells in WT and TRPV-1−/− crypts from distal and medial part of colon after 12 weeks of AOM/DSS. Quantification was performed from crypts in tumour-free areas. Results are averages ± SEM. (**\( P < .01 \), ***\( P < .001 \) by Student’s \( t \) test). (D) Immunohistochemistry for β-catenin from paraffin-embedded sections of tumour-containing colons of WT and TRPV-1−/− mice (40x). Black arrows indicate typical nuclear β-catenin accumulation. Histogram showing quantification of nuclear β-catenin per HPF (40x objective) from tumours of WT and TRPV-1−/− mice. Results are averages ± SEM. (***\( P < .001 \) by Student’s \( t \) test).

Figure 3. Inflammatory parameters in TRPV-1−/− and WT mice after AOM/DSS challenge. (A) Representative H&E-stained sections and immunohistochemical
analysis of CD3 and B220 of distal colons from WT and TRPV-1−/− mice after CAC induction (20x). (B) Quantification of F4/80+ cells per HPF in distal and medial colon from WT and TRPV-1−/− mice after CAC induction. Data represent mean ± SEM. *P = .0283, ***P = .0004 by Student’s t test. (C) Quantification of CD3+ cells per HPF in colons of WT and TRPV-1−/− mice at the end of AOM/DSS challenge (12 weeks). (D) Quantification of F4/80+ cells per HPF in distal and medial regions of colons from WT and TRPV-1−/− mice treated with 2 cycles of DSS and sacrificed at week 12. Data represent mean ± SEM. **P = .0016, ***P = .0001 by Student’s t test. (E) Quantification of CD3+ cells per HPF in colons of WT and TRPV-1−/− mice at the end of DSS challenge (12 weeks). (F) Percentage of CD11b+ and CD11c+ positive cells determined by FACS in lamina propria mononuclear cells isolated from WT and TRPV-1−/− mice colons after AOM/DSS treatment (12 weeks). Results are mean ± SEM. *P < .05, **P < .01 by Student’s t test.

**Figure 4.** Ablation of TRPV-1 increases expression of genes involved in inflammation during CAC induction. (A-D) Histograms showing relative expression of pro-inflammatory cytokines obtained by RT-PCR from RNA isolated from whole colonic mucosa from a minimum of 3 WT or TRPV-1−/− mice receiving no treatment, DSS alone, or AOM/DSS and sacrificed at week 12 (n ≥ 3). The data are normalized to GAPDH and represent mean ± SEM. *P < .05, **P < .01 by Student’s t test. (E) Inflammatory gene expression. Data show representative changes in expression of genes from TRPV-1−/− mice compared to WT after CAC challenge (see supplementary figure 2 for a complete list of genes). (F) Expression of IL-11 was analyzed by RT-PCR from distal colon of WT and TRPV-1−/− mice receiving no treatment, DSS, or AOM/DSS at week 12. One representative experiment is shown (n ≥ 3).

**Figure 5.** Absence of TRPV-1 alleles increases tumour formation in APCMin/+ mice. (A) Percentage of mice with tumours (incidence) in the colon and (B) in distal and
medial regions. (C) Average tumour number per mouse. Data represent mean ± SEM. ***p = 0.0006 by t test. (D) Histogram showing number of tumours per mouse in the distal and medial part of the colon. Data represent mean ± SEM. *p = 0.023, **p = 0.0025 by t test. (A, B, C and D) For each genotype, APCMin/+ , APCMin/+ TRPV1−/− or APCMin/+ TRPV-1−/− the number of animals analysed (n values) are 19, 11 and 14 respectively. (E) Expression of genes involved in inflammation was analyzed by quantitative RT-PCR superarrays. Data show representative changes in expression of genes from APCMin/+ TRPV-1−/− mice compared to APCMin/+ sacrificed at 4 months of age. A pool of total RNA from distal colon of 3 mice was used for each array (see supplementary figure 2 for a complete list of genes).

Figure 6. TRPV-1−/− 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−/− distal colons. (B) Western blot analysis of protein lysates from WT and TRPV-1−/− distal colons. (C) IL-6 levels detected by ELISA in protein lysates from WT and TRPV-1−/− distal colon. (D) mRNA expression of VIP and PACAP in WT and TRPV-1−/− distal colons (E and F) ELISA analysis of (F) VIP and (G) PACAP peptide levels in protein lysates extracted from WT and TRPV-1−/− distal and medial parts of colon after short AOM/DSS challenge. Data represent mean ± SEM. *P < .05, **P < .01, ***P < .001 by Student’s t test.

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 DCs culture supernatants was analyzed by ELISA. Data represent mean ± SEM. *P < .05, **P < .01, ***P < .001 by Student’s t test.
Vanilloid receptor-1 regulates neurogenic inflammation in colon and protects mice from colon cancer.

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