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Rafoxanide negatively modulates STAT3 and NF-κB activity and inflammation-associated colon tumorigenesis

Teresa Pacifico[1] | Carmine Stolfi[1] | Lorenzo Tomassini[1] | Anderson Luiz-Ferreira[2] | Eleonora Franzè[1] | Angela Ortenzi[1] | Alfredo Colantoni[1] | Giuseppe S. Sica[3] | Manolo Sambucci[4] | Ivan Monteleone[5] | Giovanni Monteleone[1] | Federica Laudisi[1]

[1]Department of Systems Medicine, University of Rome "Tor Vergata", Rome, Italy

[2]Inflammatory Bowel Disease Research Laboratory, Department of Biological Sciences, Institute of Biotechnology, Federal University of Catalão (UFCAT), Catalão, Brazil

[3]Department of Surgery, University of Rome "Tor Vergata", Rome, Italy

[4]Neuroimmunology Unit, Santa Lucia Foundation IRCCS, Rome, Italy

[5]Department of Biomedicine and Prevention, University of Rome "Tor Vergata", Rome, Italy

Correspondence
Federica Laudisi, Department of Systems Medicine, University of Rome "Tor Vergata", Via Montpellier, 1, 00133 Rome, Italy.
Email: federica.laudisi@uniroma2.it

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Abstract

In the colorectal cancer (CRC) niche, the transcription factors signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB) are hyperactivated in both malignant cells and tumor-infiltrating leukocytes (TILs) and cooperate to maintain cancer cell proliferation/survival and drive protumor inflammation. Through drug repositioning studies, the anthelmintic drug rafoxanide has recently emerged as a potent and selective antitumor molecule for different types of cancer, including CRC. Here, we investigate whether rafoxanide could negatively modulate STAT3/NF-κB and inflammation-associated CRC. The antineoplastic effect of rafoxanide was explored in a murine model of CRC resembling colitis-associated disease. Cell proliferation and/or STAT3/NF-κB activation were evaluated in colon tissues taken from mice with colitis-associated CRC, human CRC cells, and CRC patient-derived explants and organoids after treatment with rafoxanide. The STAT3/NF-κB activation and cytokine production/secretion were assessed in TILs isolated from CRC specimens and treated with rafoxanide. Finally, we investigated the effects of TIL-derived supernatants cultured with or without rafoxanide on CRC cell proliferation and STAT3/NF-κB activation. The results showed that rafoxanide restrains STAT3/NF-κB activation and inflammation-associated colon tumorigenesis in vivo without apparent effects on normal intestinal cells. Rafoxanide markedly reduces STAT3/NF-κB activation in cultured CRC cells, CRC-derived explants/organoids, and TILs. Finally, rafoxanide treatment impairs the ability of TILs to produce protumor cytokines and promote CRC cell proliferation. We report the novel observation that rafoxanide negatively affects STAT3/NF-κB oncogenic activity at multiple levels in the CRC microenvironment. Our data suggest that rafoxanide could potentially be deployed as an anticancer drug in inflammation-associated CRC.

KEYWORDS
anthelmintic drug, colitis-associated colorectal cancer, cytokine, drug repositioning, tumor microenvironment

Teresa Pacifico and Carmine Stolfi contributed equally to this work.

1 | INTRODUCTION

Chronic inflammation is recognized as an important player in the pathogenesis of many types of neoplasia, as it can promote the onset, progression, and invasion of cancer cells.[1] In particular, in patients with ulcerative colitis, one of the main forms of chronic inflammatory bowel disease in humans, there is an increased risk of developing colorectal cancer (CRC),[2] which is related to the duration, extent, and severity of the inflammatory condition.[3,4] However, it is worth mentioning that even sporadic CRCs, which represent the majority of CRC cases, exhibit extensive immune cell infiltrates and prominent inflammatory responses.[5,6]

The transcription factors signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB) lie at the hub of multiple signaling pathways, playing a central role in a multitude of physiological processes, including inflammation, immune cell development, cell proliferation, and survival.[7,8] Typically, STAT3 activation is triggered by binding of cytokines and growth factors to their related receptors. For example, members of the interleukin-6 (IL-6) family of cytokines are potent STAT3 activators, mediating their signal through the pleiotropic gp130 receptor subunit that recruits and triggers the activation of JAK2 by transphosphorylation.[9] JAK2 can then phosphorylate STAT3 on the Y705 residue.[10] Phosphorylated STAT3 can homodimerize, as well as form dimers with STAT1, and then move into the nucleus to exert its functions.[11]

Nuclear factor-κB consists of a family of related transcription factors (i.e., RelA/p65, RelB, c-Rel, p50, and p52) that can hetero- and homodimerize to form at least 12 different identified dimers, and whose interactions, stability, degradation, and transcription activity are controlled by site-specific phosphorylation/s of NF-κB subunits within transactivation domains.[12] In particular, p65 Serine 536 (S536) phosphorylation[13] has been reported to promote its nuclear retention, enhance its association with transcriptional coactivators, inhibit its association with corepressors, and potentiate the activation of NF-κB target genes.[13–17] Dysregulation of STAT3/NF-κB contributes to the development of various autoimmune and inflammatory diseases,[18–20] as well as malignant disorders.[21] In this latter context, accumulating evidence produced in different types of CRC indicates that STAT3 and NF-κB are constitutively activated in both malignant cells and tumor-infiltrating leukocytes (TILs), playing a key role in maintaining cancer cell proliferation/survival, mediating the interaction between cancer cells and immune/inflammatory cells, upregulating the release of inflammatory mediators (i.e., IL-6, tumor necrosis factor-α [TNF-α], and IL-1β cytokines), triggering tumor angiogenesis and invasiveness.[19,21,22] Given the pleiotropic effects of these two transcription factors in the CRC niche, compounds inhibiting STAT3 and NF-κB inappropriate/persistent activation represent promising therapeutic tools and could help combat this neoplasia.

By drug repositioning studies – defined as the investigation of existing drugs for the treatment of pathologies that fall outside the scope of the original medical indication – the halogenated salicylanilide compound rafoxanide, an anthelmintic drug commonly used for the veterinary treatment of fascioliasis and some gastrointestinal roundworms, has recently emerged as a potent antitumor agent for different types of cancer.[23–26] In particular, we reported that rafoxanide exerted selective antimitogenic and proapoptotic effects on CRC cells in vitro and in an experimental model mimicking sporadic CRC.[27] More recently, our follow-up studies in cultured cells and preclinical CRC models identified rafoxanide as a bona fide immunogenic cell death inducer and a selective sensitizer to TNF-related apoptosis-inducing ligand-driven cell death.[28,29]

In this study, we aimed to investigate whether rafoxanide could negatively modulate STAT3/NF-κB activity and inflammation-associated colon tumorigenesis.

2 | MATERIALS AND METHODS

2.1 | Patients

Tissue samples were taken from the tumor area of 12 patients who underwent colon resection for sporadic CRC (all with TNM stages II–III) at the Tor Vergata University Hospital and used for either the generation of organoids or the isolation of TILs. No patients received radiation therapy or chemotherapy prior to surgery. Human studies were approved by the local ethics committee (protocol no. R.S. 131.17) and each patient gave written informed consent. The study methodologies were in accordance with the standards set by the Declaration of Helsinki.

2.2 | Mice

C57BL/6J female mice (6–8 weeks of age) were purchased from Charles River Laboratories Italia Srl. The mice were housed in standard animal cages under specific pathogen-free conditions in the animal facility of the University of Rome "Tor Vergata". Mice were routinely tested (every 3 months) for health status and infections according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). Mice tested negative for all pathogens were included in this protocol. All in vivo experiments were approved by the animal ethics committee according to the Italian legislation on animal experimentation (authorization no. 494/2017-PR) and in accordance with European rules (2010/63/UE).

2.3 | Cell cultures

Unless otherwise noted, reagents were purchased from Merck Life Science. The human CRC cell line HCT-116 was obtained from ATCC and was maintained in McCoy's 5A medium supplemented with 10% FBS and 1% penicillin/streptomycin (P/S). The human CRC cell line DLD1 was obtained from ATCC and was maintained in RPMI-1640 medium supplemented with 10% FBS and 1% P/S. Cell lines were recently authenticated by short tandem repeat

(STR) DNA fingerprinting using the PowerPlex 18D System kit according to the manufacturer's instructions (Promega). The STR profiles of all cell lines matched the known DNA fingerprints. Rafoxanide (PubChem CID: 31475) was purchased from Tokyo Chemical Industry Co., Ltd.

To test whether rafoxanide inhibited STAT3 and/or NF-κB activation in CRC cells, HCT-116 and DLD1 cells were incubated with increasing doses of the drug (1.25–5 μM) for 24 h or with 2.5 μM rafoxanide for 1–6 h.

To shed light on the mechanism/s by which rafoxanide modulates STAT3 Y705 phosphorylation, HCT-116 and DLD1 cells were preincubated or not with the general protein tyrosine phosphatase (PTP) inhibitor Na_3VO_4 (used at 100 μM) for 1 h, and then stimulated with DMSO or 2.5 μM rafoxanide for further 24 h.

To assess the effect of supernatants derived from TILs cultured in the presence of DMSO (TIL SN) or rafoxanide (RFX TIL SN), HCT-116 and DLD1 cells were cultured in the presence of TIL SN, RFX TIL SN, or RPMI-1640 medium (vehicle) (all used at 1:20 final dilution). Cell proliferation was assessed after 24 h using a BrdU assay (Roche Diagnostics) and the expression of phosphorylated (p-)STAT3 Y705 and p-NF-κB/p65 S536 was evaluated after 30 min by western blotting.

2.4 | Experimental models of colitis-associated and sporadic CRC

To induce colitis-associated CRC (CAC), cohoused 6–8-week-old female C57BL/6J mice received a first i.p. injection of azoxymethane (AOM; 10 mg/kg) on day 0. After 7 days, mice were given 2% dextran sulfate sodium (DSS) (molecular weight, 9000–20,000; MP Biomedicals LLC) dissolved in the drinking water for 4 consecutive days to induce colitis. Mice received a second i.p. injection of AOM on day 18 (5 mg/kg) and were again given 2% DSS in the drinking water on day 25 for 4 days. The mice were then given regular water until the end of the experiment (day 90). On day 36 mice were randomly divided into two groups and given 7.5 mg/kg rafoxanide (in 10% DMSO in PBS) or 10% DMSO in PBS (control) every other day by i.p. injection until they were killed (day 90). Bodyweight was recorded every week from day 36 to the end of the study. Colonoscopy was carried out on day 88 in a blinded manner for monitoring of tumorigenesis using the Coloview high-resolution mouse endoscopic system (Karl Storz). The lesions observed during endoscopy were counted to obtain the total number of lesions. The size of all lesions in a given mouse was scored using the protocol described by Becker et al.[30] Cohoused 6–7-week-old female *Apc*$^{min/+}$ mice received i.p. injections of 10 mg/kg AOM once a week for 2 weeks to increase colon tumorigenesis as previously reported.[6] Two weeks after the last injection of AOM, mice were randomly divided into two groups and given either 7.5 mg/kg rafoxanide (in 10% DMSO in PBS) or 10% DMSO in PBS (control) every other day by i.p. injections until they were killed (day 90). The dose of rafoxanide was selected according to that currently used in veterinary treatment (i.e., 7.5–10 mg/kg).

2.5 | Western blot analysis

Total proteins were extracted from colon tissue samples isolated from C57BL/6J and *Apc*$^{min/+}$ mice, from CRC cell lines, and TILs using the following lysis buffer: 10 mmol/L HEPES, 1 mmol/L EDTA pH 8.0, 60 mmol/L KCl, 0.2% IGEPAL CA-630 (Nonidet P-40), 1 mmol/L sodium fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L DTT, 1 mmol/L PMSF, and 1 mmol/L sodium orthovanadate, separated on an SDS-PAGE gel and transferred using a Trans-Blot Turbo apparatus (Bio-Rad Laboratories). The samples were then incubated with the following Abs: p-STAT3 Y705 (#9145), p-NF-κB/p65 S536 (#3033), IKKα (#61294), and IKKβ (#8943) (1:1000 final dilution; Cell Signaling Technology), STAT3 (sc-8019), NF-κB/p65 (sc-372), SHP-1 (sc-7289), SHP-2 (sc-7384), JAK2 (sc-390,539), and CDK6 (sc-177) (1:500 final dilution; Santa Cruz Biotechnology, Inc.) and β-actin (A-1978; 1:5000 final dilution) followed by a secondary Ab conjugated to HRP (1:20,000; Dako). Membrane acquisition was carried out in chemiluminescence with the ChemiDoc Imaging System (Bio-Rad Laboratories). Computer-assisted scanning densitometry (Image-Lab 5.2.1; Bio-Rad Laboratories) was used to analyze the intensity of the immunoreactive bands.

2.6 | Enzyme-linked immunosorbent assay

Total proteins extracted from colon tissue samples isolated from C57BL/6J and *Apc*$^{min/+}$ mice were evaluated for the presence of IL-6 and TNF-α by specific ELISA kits (M600OB and MTA00B, respectively; Bio-Techne s.r.l.) according to the manufacturer's instructions. The levels of IL-6 and TNF-α in TIL SN and RFX TIL SN were evaluated using specific ELISA kits (EHL-IL6 and EHL-TNFα, respectively; RayBiotech) according to the manufacturer's instructions.

2.7 | Organ culture

Organ culture experiments were carried out as previously described.[31] Briefly, human CRC explants were placed on Millicell inserts (Min a 6-well plate containing complete RPMI-1640 medium supplemented with 50 μg/mL gentamycin in the presence of DMSO (vehicle) or 2.5 μM rafoxanide for 12 h. Culture was carried out in an organ culture chamber at 37°C in a 5% CO_2/95% O_2 atmosphere.

2.8 | Generation and culture of patient-derived CRC organoids

The resected intestinal tumor tissues were rinsed in HBSS containing antibiotics and cut into 5 mm pieces. These tissue fragments were incubated in Advanced DMEM/F12 medium (Thermo Fisher Scientific) containing 15 mM EDTA and rocked at 4°C for 30 min. The larger tissue fragments were removed and the remaining crypts were centrifugated at 300*g* for 5 min, embedded in Matrigel (CLS356231), and

plated in a warmed 24-well plate. The Matrigel was allowed to solidify for 30 min at 37°C and overlaid with complete medium (#06010 IntestiCult OGM Human; StemCell Technologies) supplemented with 10 μM Y27632, a selective inhibitor of Rho-associated, coiled-coil containing protein kinase (ROCK) (#72302; StemCell Technologies). The entire medium was replaced every 3 days. The established organoids were then cultured with either DMSO or rafoxanide (2.5 μM) for 24 h. The culture medium was then removed and the organoids were washed with PBS and incubated with organoid harvesting solution (3700–100-01; Bio-Techne s.r.l.) for 1 h at 4°C with gentle shaking. The released organoids from the depolymerized Matrigel were then collected and transferred to a cryomold containing optimal cutting temperature compound, frozen, and stored at −80°C.

2.9 | Immunohistochemistry

Cryosections of colon tissue samples isolated from C57BL/6J undergoing AOM/DSS were stained with a primary Ab directed against Ki-67 (clone TEC-3, M7249; Dako), p-eukaryotic translation initiation factor 2α (eIF2α) (Ser51; #3597), and cleaved caspase-3 (Asp175; #9661) (both from Cell Signaling Technology). Cryosections of human CRC explants and CRC patient-derived organoids were stained with a primary Ab against p-STAT3 Y705 (#9145; Cell Signaling Technology), p-NF-κB/p65 S536 (#3033; Cell Signaling Technology), or Ki-67 (clone MIB-1 sc-101,861; Santa Cruz Biotechnology). Positive cells were visualized using the MACH4 Universal HRP Polymer kit with DAB (Biocare Medical) and analyzed using the Leica DMI4000 B microscope using the Leica application suite software (version 4.6.2).

2.10 | Isolation and culture of TILs

Tumor-infiltrating leukocytes were isolated from the lesions of AOM/DSS treated mice by enzymatic digestion with Liberase TM (200 μg/mL, 05401127001; Roche Diagnostics) and DNase I (200 μg/mL, 11284932001; Roche Diagnostics). Cells were filtered through a 70 μm cell strainer, washed in HBSS, and resuspended in complete RPMI-1640 medium.

To isolate human TILs, tumor tissue pieces were dissected from surgical samples within 1 h after resection and rinsed in HBSS containing antibiotics. The samples were then incubated in HBSS containing 1 mM EDTA and antibiotics for 45 min at 37°C to remove epithelial cells. After two washes in HBSS, the samples were minced and incubated with Liberase TM (200 μg/mL, 05401127001; Roche Diagnostics) and DNase I (200 μg/mL, 11284932001; Roche Diagnostics) for 1 h at 37°C. After Liberase digestion, media containing mononuclear cells were collected and washed twice in HBSS. Subsequently, the pellets were resuspended in complete RPMI-1640 and then layered on a Percoll density gradient to isolate the TILs. After two washes in complete RPMI-1640 medium, isolated cells were counted and viability was verified using 0.1% Trypan blue (viability ranged from 90% to 97%). Freshly isolated TILs were resuspended in complete RPMI-1640 medium. An aliquot of cells was seeded and stimulated with DMSO (vehicle) or rafoxanide (2.5 μM) for 16 h, cells were then collected and STAT3/NF-κB activation was assessed by western blotting. An aliquot of cells was cultured in 96-well plates in the presence of DMSO or rafoxanide (2.5 μM) for 16 h. Phorbol 12-myristate 13-acetate (80 pM), ionomycin (1 mg/mL), and monensin (2 μM; eBioscience) were added to the cultures 4 h before the end of stimulation. Cells were then collected and the fraction of IL-6 and/or TNF-α producing TILs were assessed by flow cytometry after staining with specific fluorochrome-conjugated Abs. One million TILs were cultured in 48-well plates in the presence of DMSO or rafoxanide (2.5 μM) to produce 0.5 mL supernatant. Cell-free supernatants were harvested after 16 h.

2.11 | Flow cytometry

Human TILs were stained with the following reagents/mAbs: LIVE/DEAD Fixable Violet Dead Cell Stain kit (L34955; Thermo Fisher Scientific), CD45-eFluor 506 (69-0459-42; eBioscience), CD3-APC-H7 (560176; BD Biosciences), γ-interferon (IFN-γ)-FITC (554700; BD Biosciences), IL-6-PerCP (46-7069-42; eBioscience), and TNF-α-PE (12-7349-82; eBioscience). In parallel, cells were stained with the appropriate control isotype Abs. Mouse TILs were stained with the following reagents/mAbs: LIVE/DEAD Fixable Aqua Dead Cell Stain kit (L34966; Thermo Fisher Scientific), CD45 APC-Cy7 (557659; BD Biosciences), CD3-Pacific Blue (558214; BD Biosciences), CD8-PerCP (45008182; BD Biosciences), DX5-PE (553858; BD Biosciences), IFN-γ FITC (554411; BD Biosciences), Perforin APC (17-9392-80; eBioscience), and granzyme B–Alexa Fluor 700 (372222; BioLegend). In parallel, cells were stained with the appropriate control isotype Abs. Flow cytometry analysis was undertaken using a Gallios flow cytometer (Beckman Coulter).

2.12 | Statistical analysis

Parametric data were analyzed using the two-tailed Student's *t*-test for comparison between two groups or one-way ANOVA followed by Tukey's or Dunnett's post hoc tests for multiple comparisons. Significance was defined as $p < 0.05$.

3 | RESULTS

3.1 | Rafoxanide reduces neoplastic cell proliferation/survival and STAT3/NF-κB activation in a mouse model of CAC

In initial studies, we evaluated the possible antineoplastic effect of rafoxanide in a mouse model mimicking human CAC whereby AOM administration followed by repeated DSS ingestion caused colonic

inflammation and the subsequent development of multiple colonic lesions. To rule out the possibility that rafoxanide could interfere with the ongoing mucosal inflammation, drug treatment was started 1 week after discontinuation of the last treatment with DSS (Figure 1A). At that time (day 36), no visible lesions were detected by endoscopy (not shown). Mice received i.p. injections of rafoxanide (7.5 mg/kg/mouse) or vehicle (control) every other day, until the mice were killed at the end of the study (Figure 1A). Endoscopy on day 88 showed that control mice developed multiple large colonic lesions, whereas the number and size of lesions were reduced in the colons of mice treated

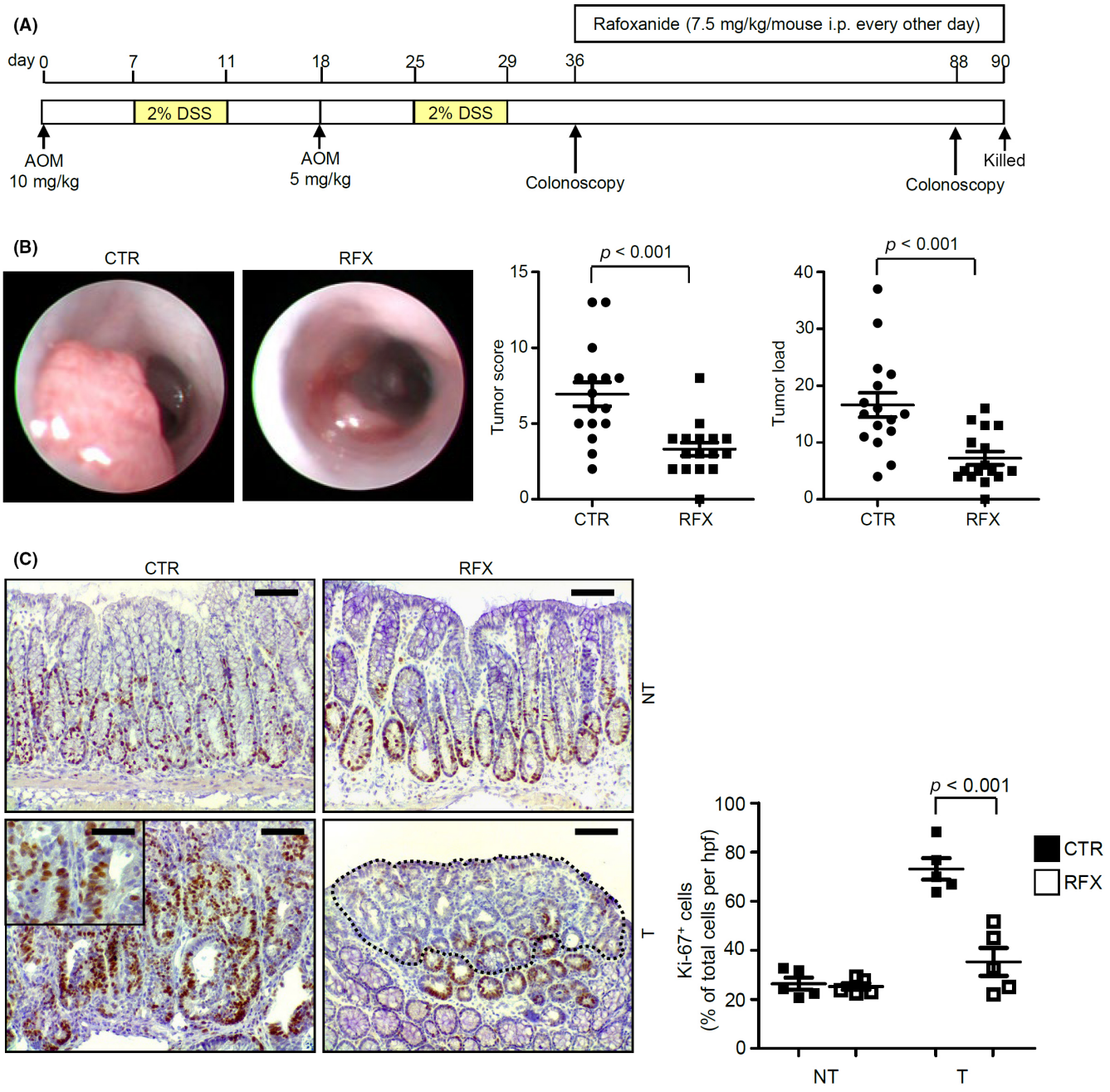


FIGURE 1 Rafoxanide (RFX) limits colonic tumorigenesis in the azoxymethane (AOM)/dextran sulfate sodium (DSS)-driven colitis-associated colorectal cancer (CAC) model. (A) Schematic overview of the protocol used to induce CAC in mice. (B) Left panels show representative endoscopic pictures of colon lesions developed in mice treated with AOM/DSS and receiving vehicle (CTR) or RFX. Graphs show the number of lesions (tumor score) and the endoscopic score (tumor load) of the lesions developed in mice treated as indicated above. Data indicate the mean ± SEM of three independent experiments in which at least four mice per group were considered. (C) Representative images showing Ki-67 immunostaining in colonic sections taken from mice treated as indicated in (B). Scale bars, 20 μm; 10 μm (insets). One of five representative experiments in which similar results were obtained is shown. Right inset, quantification of Ki-67$^+$ epithelial cells in colonic sections taken from mice treated as indicated in (B). Data are presented as mean values of positive cells per high power field ± SEM of two independent experiments in which at least two sections per group were analyzed. NT, nontumor area; T, tumor area.

with rafoxanide (Figure 1B). No significant changes in bodyweight were observed in mice treated with rafoxanide compared to control mice (not shown), in line with rafoxanide treatment experiments performed in healthy mice (pers. obs., 2024). Immunohistochemistry for Ki-67 (Figure 1C) and cleaved caspase-3 (Figure S1A) confirmed the antimitogenic and proapoptotic effects of rafoxanide on neoplastic cells. It should be noted that no significant change in Ki-67 or cleaved caspase-3 staining (Figures 1C and S1A) was detected in the normal colonic mucosa of mice treated with the drug, according to previously reported observations in a mouse model mimicking sporadic CRC.[27] In the AOM/DSS-induced lesions of rafoxanide-treated mice, the increase in the phosphorylation of eIF2α on the S51 residue in transformed colon epithelial cells (Figure S1B), a mandatory event for cells undergoing immunogenic death to emit all the signals required for the initiation of the host immune response,[32] as well as the expanded fraction of T lymphocytes and NK cells producing IFN-γ, a key cytokine in the activation of cellular immunity and, subsequently, stimulation of antitumor immune response,[33] and perforin (Figure S2), are in line with our previous report indicating rafoxanide as a bona fide immunogenic cell death inducer.[28]

Excessive activation of STAT3 and NF-κB transcription factors makes a major contribution to the tumorigenic process in the colon.[21] To determine whether the reduced carcinogenesis observed in mice treated with rafoxanide was associated with a reduced activation of STAT3 and/or NF-κB, we compared the expression of p-STAT3 Y705 and p-NF-κB/p65 S536 in nontumor and tumor tissues derived from colon extracts of control and rafoxanide-treated mice killed on day 90. Robust activation of both STAT3 and NF-κB was observed in the neoplastic areas of control mice compared to those derived from mice treated with rafoxanide (Figure 2A). In the microenvironment characterizing colitis-associated colorectal carcinogenesis, in addition to intrinsic molecular defects in the signaling machinery, aberrant activation of STAT3 and/or NF-κB that occurs in transformed epithelial cells can be increased by the presence of immune cell-derived protumorigenic cytokines.[5] Therefore, next we assessed the expression of IL-6 and TNF-α, among the most potent activators of STAT3 and NF-κB in colonic epithelial cells, respectively,[5,34] in nontumor and tumor colonic tissues taken from mice treated as indicated above. Both IL-6 and TNF-α were reduced in tumors of mice that underwent drug treatment compared to control mice, while such cytokines were barely detectable in nontumor areas of control and rafoxanide-treated mice (Figure 2B). Taken together, our data indicate that rafoxanide reduces the development of neoplastic lesions driven by AOM/DSS colitis and this effect is associated with reduced STAT3/NF-κB activation and production of IL-6 and TNF-α.

3.2 | Rafoxanide decreases STAT3/NF-κB activation in colonic neoplastic lesions arising in APC$^{min/-}$ mice and in human CRC explants

In addition to cancers of the digestive tract arising on overt inflammation, sporadic CRC, which represents the majority of CRC cases,

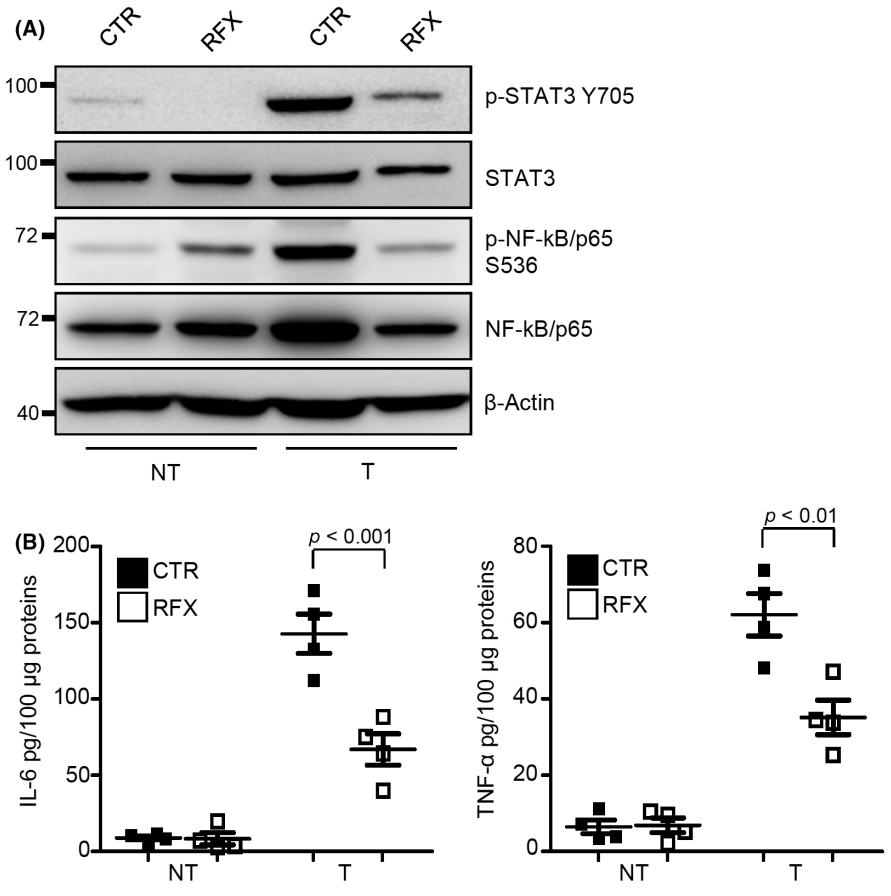


FIGURE 2 Reduced signal transducer and activator of transcription 3 (STAT3)/nuclear factor-κB (NF-κB) activation and interleukin-6 (IL-6)/tumor necrosis factor-α (TNF-α) expression in lesions of mice treated with azoxymethane (AOM)/dextran sulfate sodium (DSS) receiving rafoxanide (RFX). (A) Colonic extracts from mice treated with AOM/DSS receiving vehicle (CTR) or RFX and killed on day 90 were analyzed for phosphorylated (p-)STAT3 Y705, STAT3, p-NF-κB/p65 S536, and NF-κB/p65 expression by western blotting. β-Actin was used as a loading control. One of four representative experiments in which similar results were obtained is shown. (B) Scatter plots showing IL-6 and TNF-α protein expression assessed by ELISA in colonic extracts of mice treated as indicated in (A). Values are mean ± SEM of four experiments. NT, nontumor area; T, tumor area.

shows extensive inflammatory infiltrates with high levels of cytokines that support the growth of CRC cells through the activation of STAT3 and NF-κB.[5] We previously reported the ability of rafoxanide to inhibit colon tumorigenesis in mice carrying a constitutional mutation in the adenomatous polyposis coli (*Apc*) gene (*Apc*$^{min/+}$ mice) and mimicking human sporadic CRC.[27] By comparing the expression of p-STAT3 Y705 and p-NF-κB/p65 S536 in nontumor and tumor tissues derived from colonic extracts of *Apc*$^{min/+}$ mice treated or not with rafoxanide, we showed that the antineoplastic effect of the drug was associated with reduced activation of STAT3 and NF-κB (Figure S3A), as well as with decreased levels of IL-6 and TNF-α (Figure S3B). These results were in line with those obtained in the experimental model of CAC, suggesting that rafoxanide could act on similar pathways to exert its antineoplastic effects in both mouse models of CRC. Next, we sought to determine the effects of rafoxanide on STAT3 and NF-κB activity in the CRC microenvironment. To this end, we compared the expression of p-STAT3 Y705 and p-NF-κB/p65 S536 in patient-derived sporadic CRC explants cultured with rafoxanide or vehicle (control). Immunohistochemistry showed robust activation of STAT3 and NF-κB in control CRC explants compared to those treated with the drug (Figure 3). It is noteworthy that rafoxanide treatment resulted in a reduction in cells positive for p-STAT3 Y705 and p-NF-κB/p65 S536 in transformed epithelial cells and tumor infiltrating cells (Figure 3). Taken together, these results suggest that rafoxanide-driven impairment of STAT3 and NF-κB activation in CRC could rely on a direct effect on the intracellular machinery governing the functionality of these transcription factors in cancer cells, a secondary effect due to inhibition of immune cell-derived STAT3/NF-κB activating factors in the tumor niche, or both.

3.3 | Rafoxanide impairs STAT3 and NF-κB activation in cultured CRC cells and in organoids derived from CRC patients

As a starting point to answer these questions, we initially investigated whether rafoxanide could directly hamper STAT3 and NF-κB activation in CRC cell lines. Treatment of HCT-116 and DLD1 cells with increasing doses (1.25, 2.5, and 5 μM rafoxanide) for 24 h markedly reduced p-STAT3 Y705 and, to a lesser extent, p-NF-κB/p65 S536 levels, in a dose-dependent manner in both cell lines (Figure 4A,B). It should be noted that rafoxanide treatment had no (in DLD1 cells) or minimal (in HCT-116 cells) effects on total STAT3 and p65 total proteins, indicating that the drug could interfere with the molecular machinery controlling the phosphorylation of the transcription factors mentioned above on these specific residues.

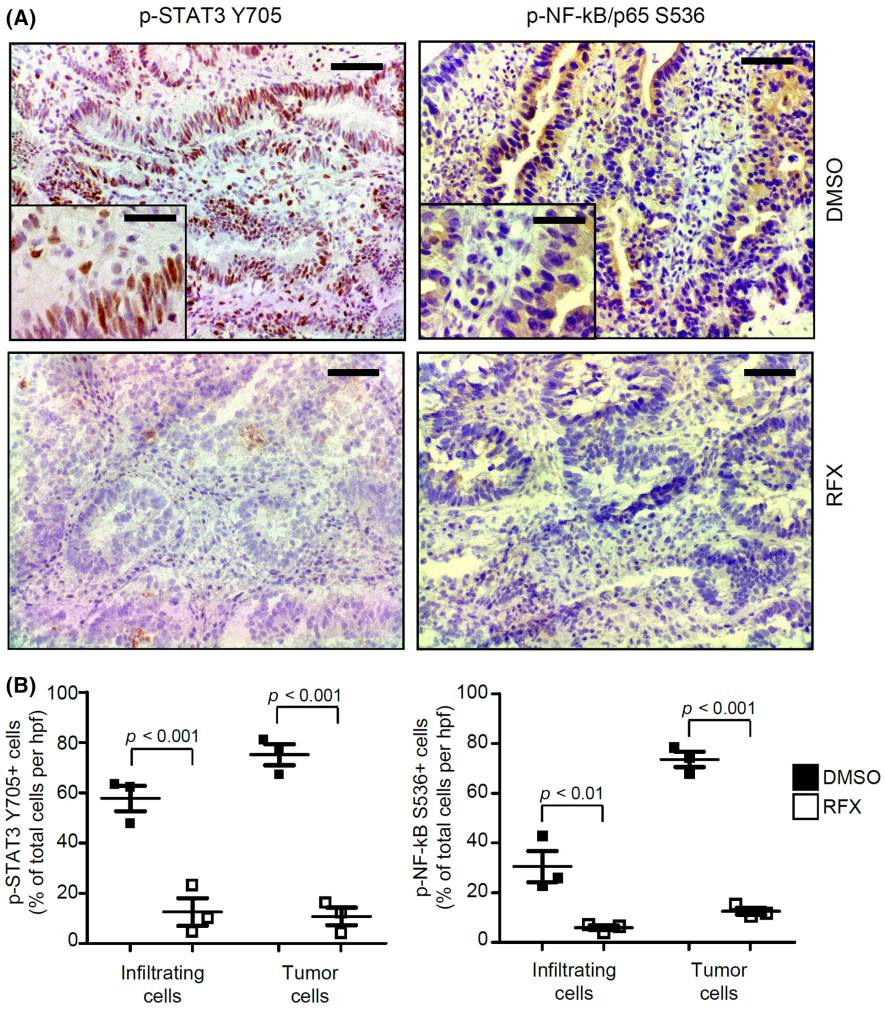


FIGURE 3 Rafoxanide (RFX) treatment reduces signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB) activation in the epithelial and immune compartments of human colorectal cancer (CRC) explants. (A) Representative photographs of phosphorylated (p-)STAT3 Y705- and p-NF-κB/p65 S536-stained sections of freshly obtained human CRC explants treated with DMSO or 2.5 μM RFX for 12 h. Scale bars, 40 μm; 10 μm (insets). (B) Quantification of p-STAT3 Y705- and p-NF-κB/p65 S536-positive infiltrating and tumor cells in human CRC explants treated as indicated in (A). Data are presented as mean values of positive cells per high power field ± SEM of three independent experiments.

Time course studies confirmed the inhibitory effect of the drug on the expression of p-STAT3 Y705 and p-NF-κB/p65 S536 (Figure 4C). In particular, the decrease in p-STAT3 Y705 expression was already evident from 1 h of incubation with rafoxanide and preceded the inhibition of p-NF-κB/p65 S536 in DLD1 and, to a lesser degree, HCT-116 cells (Figure 4C).

To figure out how rafoxanide negatively affected STAT3 Y705 phosphorylation, we first investigated whether rafoxanide treatment could hinder the expression of JAK2, a key STAT3 Y705 targeting kinase.[10] However, we were unable to see any change in JAK2 expression after rafoxanide treatment in either HCT-116 or DLD1 cells (Figure S4). Further work indicated that the rafoxanide-mediated inhibitory effect on STAT3 Y705 phosphorylation was reverted by the addition of the protein tyrosine phosphatase inhibitor Na_3VO_4 (Figure 5A), suggesting a role for rafoxanide in modulating STAT3 dephosphorylation. The precise mechanism/s underlying the rafoxanide-driven p-STAT3 Y705 downregulation remains to be determined as our first results did not show any effect of rafoxanide in modulating the expression of SHP-1 or SHP-2, two key STAT3 tyrosine phosphatases (Figure 5B).[35]

Concerning the mechanism/s by which rafoxanide negatively regulates p-NF-κB activity, it is worth mentioning that multiple, distinct protein kinases that phosphorylate the transcriptionally most active NF-κB subunit p65 at S536 exist, such as IKKα, IKKβ, and CDK6,[36] with the latter requiring association with cyclins D1–D3 for full activation.[37] Rafoxanide treatment downregulated CDK6, but not IKKα or IKKβ, protein expression in HCT-116 and DLD1 cells (Figure 6). Furthermore, rafoxanide was recently proposed (through molecular docking studies) as a dual CDK4/CDK6 inhibitor,[38] and we have previously shown that rafoxanide treatment markedly reduced cyclin D1 expression in CRC cells.[27] These pieces of evidence suggest that the impairment of CDK6/cyclin D1 expression/activity might be the mechanism, or one of the mechanisms, by which the drug inhibits phosphorylation of NF-κB/p65 on S536 in CRC. To investigate whether rafoxanide was able to affect the proliferation and STAT3/NF-κB activation of human primary CRC cells, we took advantage of the generation and culture of intestinal organoids derived from CRC patients, which are able to faithfully recapitulate the molecular steps of disease evolution and the anatomical and functional hallmarks of the real organ.[39] Rafoxanide was added to organoids derived from CRC patients and the expression of Ki-67, p-STAT3 Y705, and p-NF-κB/p65 S536 was evaluated after 24 h by immunohistochemistry. Rafoxanide inhibited cell proliferation and, consistent with the results obtained in cultured CRC cells, STAT3/NF-κB activation in this experimental setting (Figure 7). Together, our data clearly show the ability of rafoxanide to directly inhibit STAT3 and NF-κB in CRC cells.

3.4 | Rafoxanide negatively affects STAT3/NF-κB activation and the production of protumorigenic cytokines in TILs

Beyond their direct effects in sustaining CRC cell proliferation and survival, STAT3 and NF-κB play an important role in shaping the immune microenvironment of CRC.[40,41] For example, STAT3 negatively regulates the T helper 1 (Th1)-type antitumor immune response and promotes Th17 cell differentiation,[42] while activation of NF-κB in immune cells contributes to the increased production of proinflammatory cytokines (such as TNFα, IL-1, and IL-6).[43] Thus, in subsequent studies, we investigated whether rafoxanide could modulate immune cell-related STAT3/NF-κB activity and protumorigenic signals in the CRC niche. Specifically, TILs isolated from explants derived from sporadic CRC patients were cultured in the presence of rafoxanide or DMSO (vehicle) for 16 h and the expression of p-STAT3 Y705 and p-NF-κB/p65 S536 was evaluated by immunoblotting. In parallel experiments, an aliquot of cells was used to evaluate changes in cell viability and cytokine production (that is, IFN-ɣ, IL-6, and TNF-α) by flow cytometry. Treatment with rafoxanide inhibited the activation of STAT3 and NF-κB in TILs (Figure 8A). This effect was associated with an increased frequency of CD3+ IFN-ɣ-producing cells, and with a decreased frequency of CD45+ IL-6- and TNF-α-producing cells (Figure 8B).

3.5 | Rafoxanide reduces TIL-derived culture supernatant-mediated cell proliferation and STAT3/NF-κB activation in CRC cells

Cytokine-producing immune/inflammatory cells contribute to the progression of CRC in part through the activation of STAT3/NF-κB signaling in transformed epithelial cells.[5] Finally, to address the biological relevance of the results observed in TILs after rafoxanide exposure, we investigated the effects of cell culture supernatants derived from DMSO- and rafoxanide-treated TILs on CRC cell proliferation and STAT3/NF-κB activation. Specifically, HCT-116 and DLD1 cells were cultured with cell-free supernatants derived from TILs treated with rafoxanide or DMSO (vehicle) for 16 h. As expected, TIL-derived supernatants robustly increased the expression of p-STAT3 Y705 and p-NF-κB/p65 S536, as well as cell proliferation in both HCT-116 and DLD1 cells (Figure 9). In particular, such responses were markedly hampered in cells cultured in the presence of cell-free supernatants derived from TILs treated with rafoxanide (Figure 9), which presented a reduced amount of IL-6 and TNF-α (Figure S5).

4 | DISCUSSION

In this study, we present data indicating that the anthelmintic drug rafoxanide is a powerful inhibitor of STAT3 and NF-κB activation in the CRC microenvironment.

We first reported that systemic administration of rafoxanide reduced the multiplicity and size of lesions in a mouse model that mimicked human CAC. In line with data from our previous report assessing the antineoplastic effects of rafoxanide in mice carrying a constitutional mutation in the *Apc* gene ($Apc^{min/+}$ mice) and mimicking sporadic CRC,[27] the drug exerted antimitogenic effects in transformed but not in normal colonic epithelial cells, further sustaining

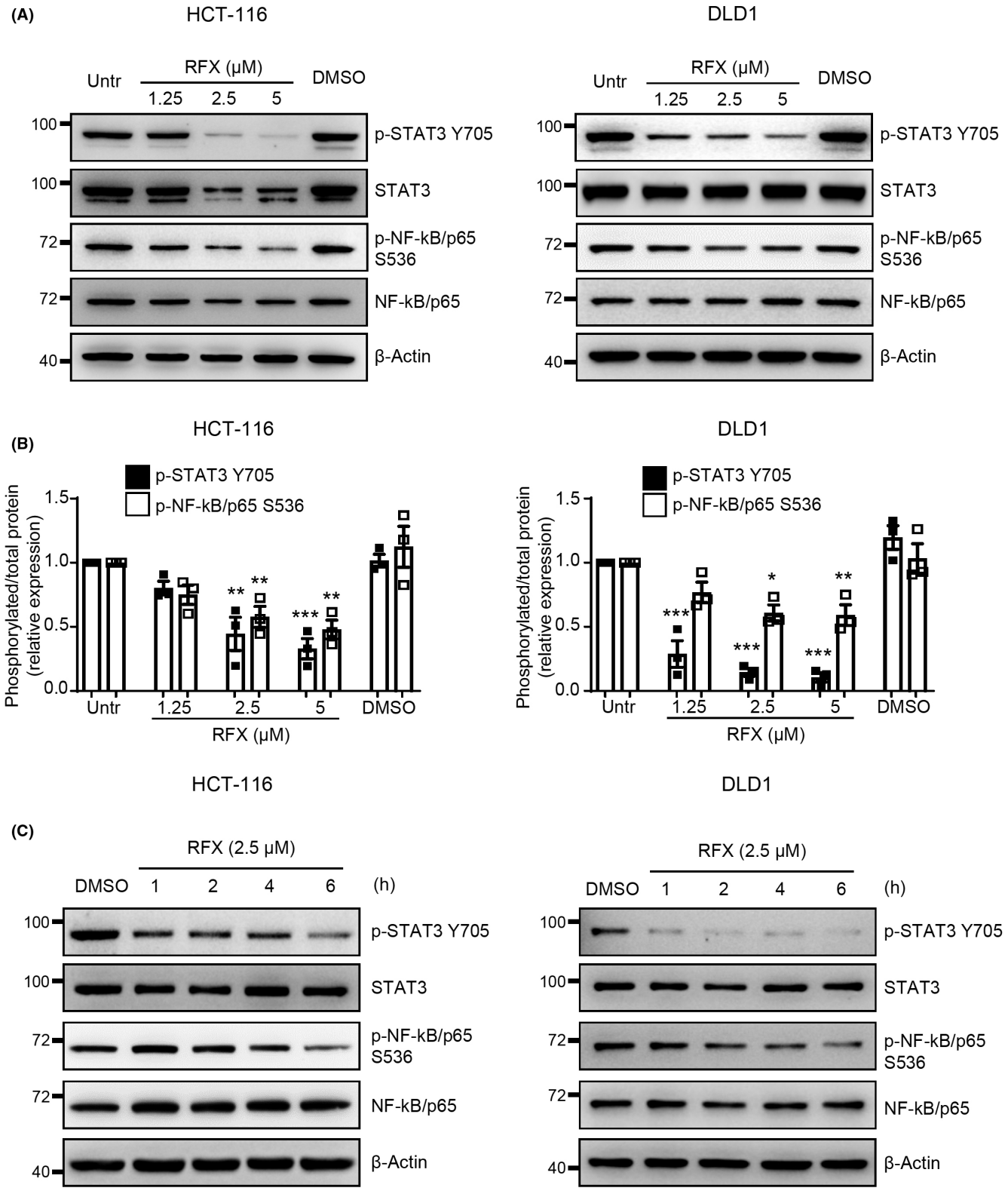


FIGURE 4 Treatment with rafoxanide (RFX) reduces the activation of signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB) in cultured colorectal cancer cells. (A) HCT-116 and DLD1 cells were left untreated (Untr) or treated with the indicated doses of RFX or DMSO for 24 h. Protein extracts were evaluated for phosphorylated (p-)STAT3 Y705, STAT3, p-NF-κB/p65 S536, and NF-κB/p65 by immunoblotting. β-Actin was used as a loading control. One of three independent experiments in which similar results were obtained is shown. (B) Quantitative analysis of the p-STAT3 Y705/STAT3 protein ratio and p-NF-κB/p65 S536/NF-κB/p65 protein ratio in total extracts of HCT-116 and DLD1 cells stimulated as indicated in (A). Values are the mean ± SEM of three independent experiments (DMSO- vs. RFX-treated cells). (C) HCT-116 and DLD1 cells were stimulated with DMSO or 2.5 μM RFX for the indicated time points. Protein extracts were evaluated for p-STAT3 Y705, STAT3, p-NF-κB/p65 S536 and NF-κB/p65 by immunoblotting. β-Actin was used as a loading control. One of three independent experiments in which similar results were obtained is shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

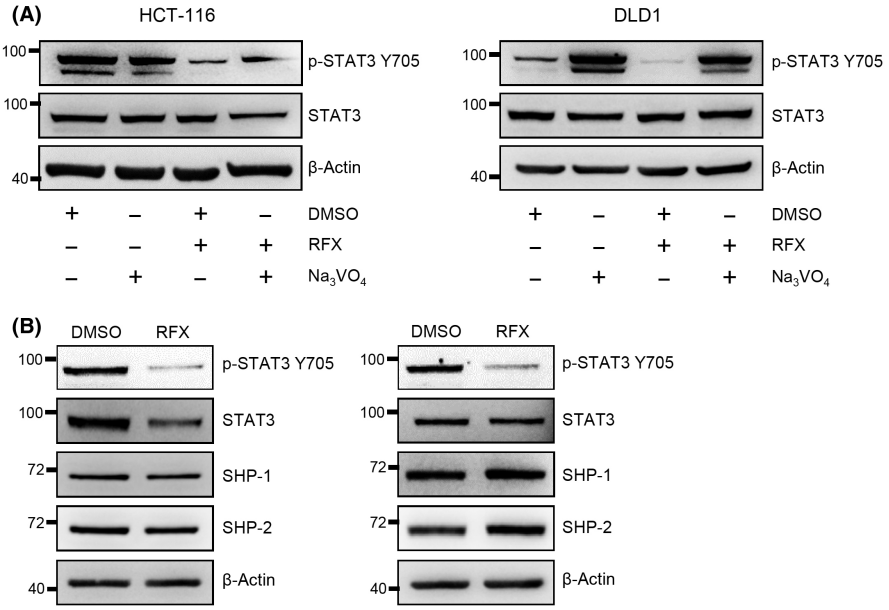
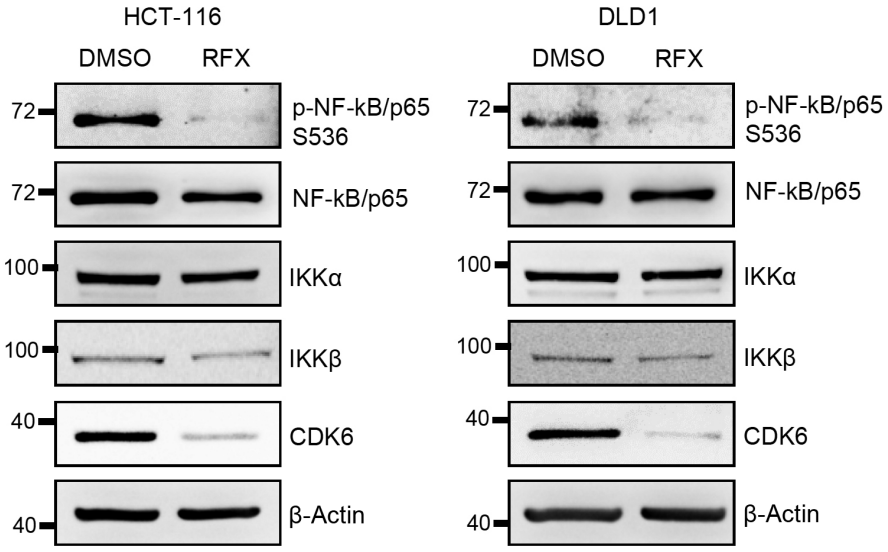


FIGURE 5 Role of protein tyrosine phosphatases (PTP) on rafoxanide-driven phosphorylated signal transducer and activator of transcription 3 (p-STAT3) Y705 downregulation. (A) Addition of the general PTP inhibitor Na_3VO_4 to HCT-116 and DLD1 cell cultures reduces the STAT3 Y705 dephosphorylation induced by rafoxanide (RFX). Cells were preincubated with Na_3VO_4 (used at 400 μM) for 1 h and then stimulated with DMSO or 2.5 μM RFX for 24 h. At the end, total extracts were prepared and the expression of p-STAT3 Y705 and STAT3 assessed by western blotting. β-Actin was used as loading control. One of three representative experiments in which similar results were obtained is shown. (B) RFX does not affect the expression of the key STAT3 targeting PTP SHP-1 and SHP-2. Total proteins extracted from HCT-116 and DLD1 cells treated with DMSO or RFX for 24 h were evaluated for p-STAT3 Y705, STAT3, SHP-1, and SHP-2 expression by western blotting. β-Actin was used as a loading control. One of three representative experiments in which similar results were obtained is shown.

FIGURE 6 Effect of rafoxanide (RFX) on phosphorylated nuclear factor-κB (p-NF-κB)/p65 S536 targeting kinases. Total proteins extracted from HCT-116 and DLD1 cells treated with DMSO or 2.5 μM RFX for 24 h were evaluated for p-NF-κB/p65 S536, NF-κB/p65, IKKα, IKKβ, and CDK6 expression by western blotting. β-Actin was used as a loading control. One of three representative experiments in which similar results were obtained is shown.



the evidence that rafoxanide preferentially interferes with biological pathways that support malignant cell growth in vivo. Consistent with our previous observations in *Apc*$^{min/+}$ mice, in our mouse model mimicking inflammation-associated colon tumorigenesis, rafoxanide would seem to be well tolerated, as no significant changes in bodyweight were observed in mice treated with the drug as compared to control mice. The apparent in vivo safety of rafoxanide is further supported by the evidence reported by Xiao et al. in a multiple myeloma xenograft model, showing no significant side-effects of the drug in mice receiving i.p. injections of rafoxanide at 15 mg/kg every other day for 14 days,[26] and, more recently, by Hu et al. in a non-small-cell lung cancer xenograft model, describing no obvious pathological damage in the liver or kidney as well as no significant changes in blood biochemical parameters related to liver or kidney function in mice treated with daily i.p. injections of rafoxanide at 15 mg/kg for 2 weeks.[24] However, further experimentation of the

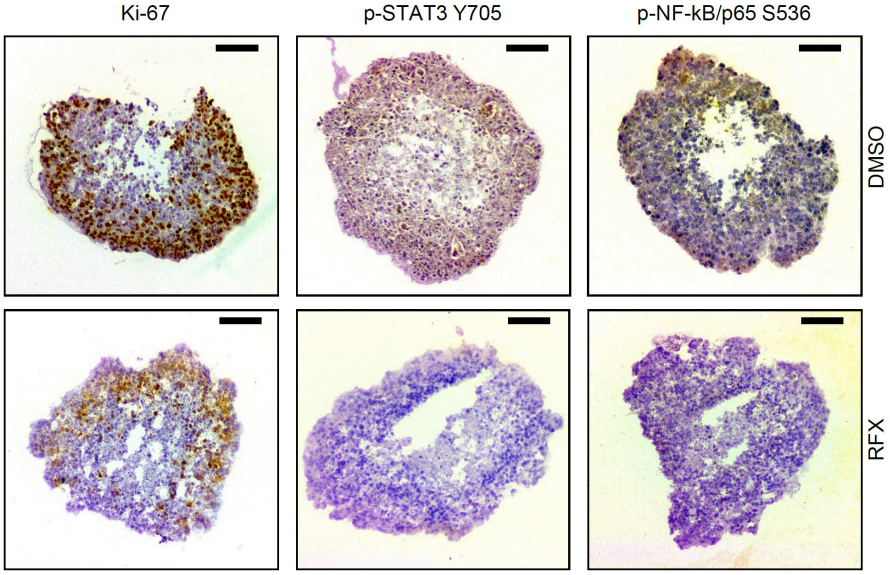


FIGURE 7 Treatment with rafoxanide (RFX) inhibits cell proliferation and signal transducer and activator of transcription 3 (STAT3)/nuclear factor-κB (NF-κB) activation in patient-derived colorectal cancer (CRC) organoids. Representative pictures of Ki-67, phosphorylated (p-) STAT3 Y705, and p-NF-κB/p65 S536-stained sections of CRC explant-derived organoids treated with either DMSO or 2.5 μM RFX for 24 h. One of three independent experiments in which similar results were obtained is shown. Scale bar, 200 μm.

dosage and long-term toxicity is needed and is currently ongoing to confirm the therapeutic potential and clinical benefit of rafoxanide. A reduced activation of STAT3/NF-κB was observed in tumor tissues from AOM/DSS treated mice that received rafoxanide compared to control mice, as well as a decrease in the expression of the immune cell-derived protumorigenic cytokines IL-6 and TNF-α, among the most potent activators of STAT3 and NF-κB in colonic epithelial cells, respectively,[5] and playing a key role in the development of CRC.[34] Taking advantage of the lysates derived from our previous study assessing the *Apc*$^{min/+}$ mice, we showed that the antineoplastic effect of the drug was associated with a reduced activation of STAT3/NF-κB and with decreased levels of IL-6 and TNF-α also in this experimental setting, raising the possibility that rafoxanide could affect the interplay between immune/inflammatory and malignant cells in the CRC niche independently of the presence of overt inflammation.

Our data produced in the AOM/DSS model are in line with the knowledge that blocking IL-6 or the downstream transcription factor STAT3 causes a significant suppression of CAC development in mouse models, indicating that STAT3 is indispensable for inflammation-associated colonic tumorigenesis.[44,45] However, our results in *Apc*$^{min/+}$ mice appear to contradict the paper by Oshima et al., which states that epithelial STAT3 is not necessary for intestinal tumor development in a Wnt activation-driven model of sporadic tumorigenesis.[46] This apparent discrepancy could be due to the fact that, different to the genetic ablation of STAT3, which was limited to the mouse intestinal epithelium,[46] the rafoxanide-driven STAT3 inhibition is not limited to the colon transformed epithelial cells but it likely extends to the immune cell compartment resulting in the modulation of both pro- and antitumor cytokines/factors. We previously reported that rafoxanide reduced transformed epithelial cell proliferation in sporadic CRC explants without substantially affecting the growth of normal colonic epithelial cells.[27] In the same context, to extend our in vivo observations to human CRC, we showed that rafoxanide negatively affected STAT3 and NF-κB activation. Notably, this effect was evident not only in transformed epithelial cells but also in tumor infiltrating cells, raising the possibility that rafoxanide could negatively impact STAT3 and NF-κB activity through a direct effect on cancer cells and/or by exerting a secondary inhibitory effect on immune cell-derived STAT3/NF-κB activating factors in the tumor microenvironment. Treatment of cultured CRC cells with rafoxanide revealed that the drug could directly affect STAT3/NF-κB phosphorylation/activation in cancer cells, in agreement with other studies reporting inhibitory properties of several anthelmintic drugs on STAT3 (i.e., pyrvinium, pyrvinium pamoate, niclosamide, albendazole, and flubendazole)[47–52] and NF-κB (e.g., niclosamide and flubendazole)[53,54] in different types of cancers (i.e., lung cancer, gastric cancer, CRC, glioblastoma, triple-negative breast cancer, and acute myelogenous leukemia). Although our initial results point to the involvement of STAT3 phosphatases in the downregulation of p-STAT3 Y705 driven by rafoxanide, more in-depth experimentation is needed to determine whether rafoxanide treatment might somehow modulate the activity of SHP-1 and/or SHP-2 and/or the expression/activity of other STAT3 Y705 targeting phosphatases (e.g., PTPRD, PTPRT, PTPRK).[35] To extend our observations to primary human cells, we generated patient-derived CRC organoids. Given their ability to recapitulate the anatomical and functional hallmarks of the real organ, organoids represent an additional step between 2D in vitro laboratory research and preclinical models and optimal systems for the screening of new drugs.[39] Of note, we showed that the culture of CRC organoids in the presence of rafoxanide resulted in a striking reduction in STAT3 and NF-κB activation as compared to the control.

The potential antitumor effects of various anthelmintic drugs are not limited to cytostatic and proapoptotic properties, as several studies highlighted their ability to modulate host immunity.[55] In particular, the activity of different subsets of immune cells can be influenced by some of these compounds (e.g., niclosamide and

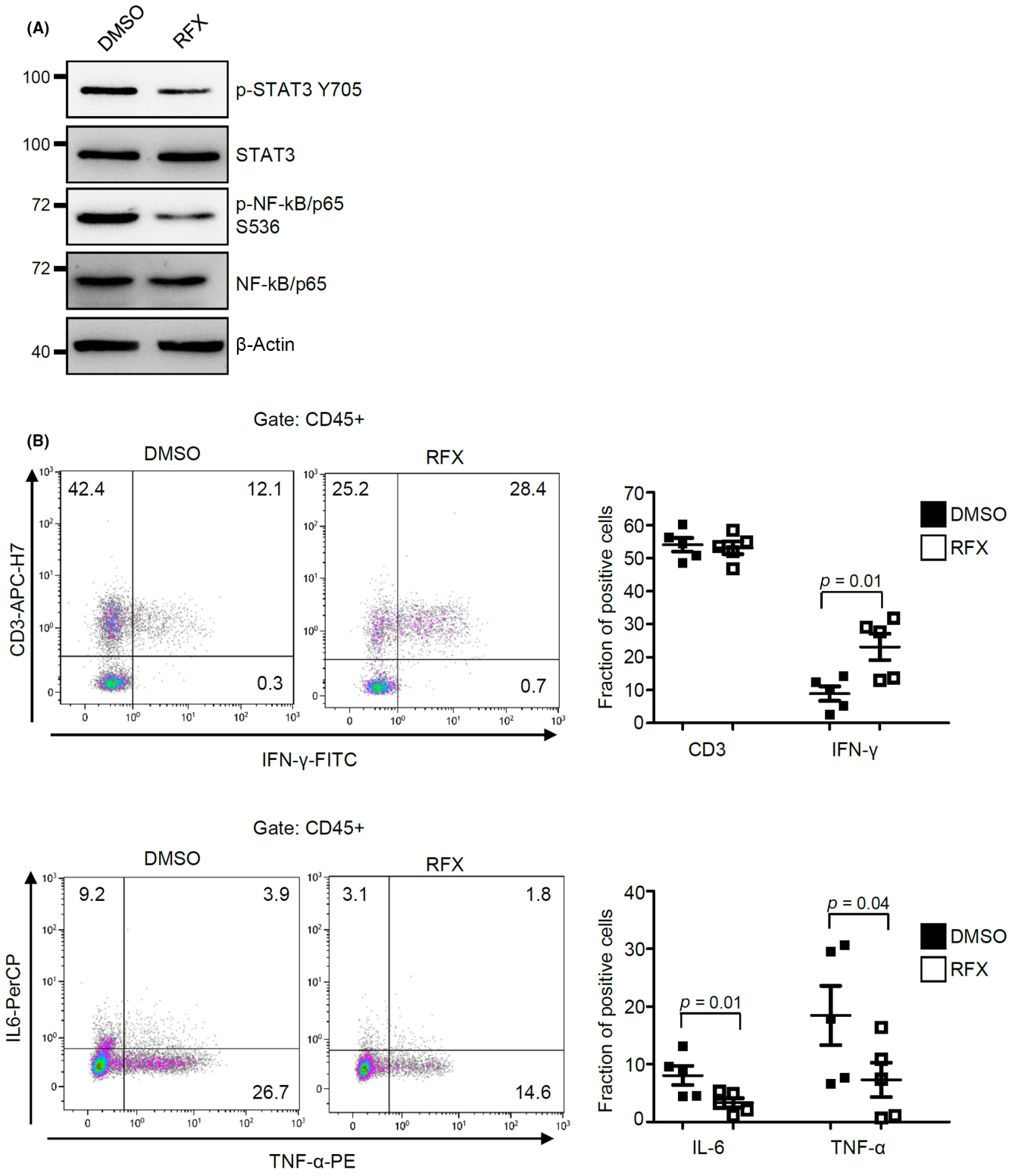


FIGURE 8 Rafoxanide (RFX) hampers signal transducer and activator of transcription 3 (STAT3)/nuclear factor-κB (NF-κB) activation and the production of protumorigenic cytokines in tumor infiltrating leukocytes (TILs). (A) Expression of phosphorylated (p-)STAT3 Y705, STAT3, p-NF-κB S536, and NF-κB/p65 was evaluated by immunoblotting in TILs isolated from colorectal cancer (CRC) explants stimulated with DMSO or 2.5 μM RFX for 16 h. β-Actin was used as a loading control. One of three independent experiments in which similar results were obtained is shown. (B) Representative dot plots from flow cytometry analysis showing the frequencies of viable γ-interferon (IFN-γ)-producing cells (upper panels) and viable interleukin-6 (IL-6)- and/or tumor necrosis factor-α (TNF-α)-producing cells in TILs isolated from CRC explants stimulated with DMSO or RFX (2.5 μM) for 16 h. Numbers indicate the percentage of cells in the designated quadrants. Right insets, scatter plots showing the fraction of IL-6- and/or TNF-α-producing cells in TILs isolated and cultured as indicated in (B). Values are mean ± SEM of five independent experiments in which TILs isolated from five independent CRC samples were used.

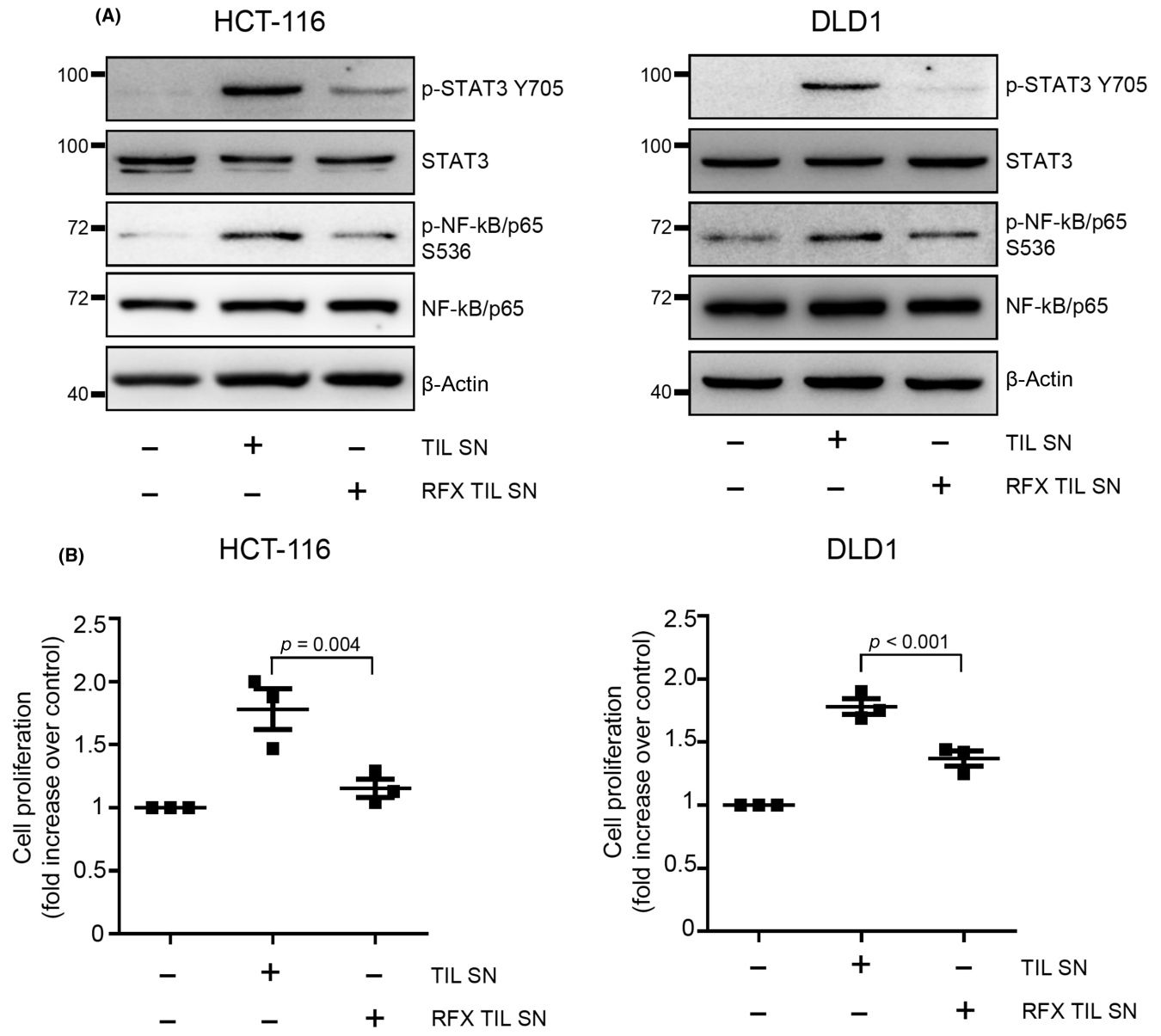


FIGURE 9 Effect of supernatants derived from tumor infiltrating leukocytes (TILs) cultured in the presence of rafoxanide (RFX TIL SN) or DMSO (TIL SN) on colorectal cancer (CRC) cell proliferation and signal transducer and activator of transcription 3 (STAT3)/nuclear factor-κB (NF-κB) activation. (A) HCT-116 and DLD1 cells were cultured in the presence of TIL SN, RFX TIL SN, or RPMI-1640 medium (vehicle) (all used at a final dilution of 1:20) for 30 min. Protein extracts for phosphorylated (p-)STAT3 Y705, STAT3, p-NF-κB/p65 S536, and NF-κB/p65 were evaluated by immunoblotting. β-Actin was used as a loading control One of three independent experiments in which similar results were obtained is shown. Supernatants derived from TILs isolated from three independent CRC specimens were used. (B) HCT-116 and DLD1 cells were cultured in the presence of TIL SN, RFX TIL SN, or RPMI-1640 medium (vehicle) (all used at a final dilution of 1:20) for 24 h. Cell proliferation was evaluated by a BrdU assay. Values are the mean ± SEM of three independent experiments in which supernatants derived from TILs isolated from three independent CRC samples were used.

mebendazole) through modulation of specific transcription factors, including STAT3 and NF-κB.[56–58] Consistent with these observations, we report that rafoxanide impaired STAT3/NF-κB activation in TILs, and this effect was associated with an increased frequency of $CD3^+$ IFN-γ-producing cells, and a decreased frequency of protumorigenic $CD45^+$ IL-6- and TNF-α-producing cells. We cannot exclude the possibility that the reduction of TNF-α outside the tumor after rafoxanide treatment could eventually increase susceptibility to infections. However, our data showing that rafoxanide does not affect TNF-α levels in the nontumor area of AOM/DSS-treated mice, and the fact that treatment of human and mouse TILs with the drug results in a change in the inflammatory profile, as documented by the increase of the fraction of immune cells producing IFN-γ, a cytokine released to provide protection against bacteria, parasites, and fungi,[59] rather than in a generic suppression of inflammatory cytokines/factors, would seem to rule out

this drawback. However, further experimental efforts in specific preclinical models are needed to comprehensively address this potential issue.

As immune/inflammatory cells that produce cytokines contribute to the progression of CRC in part through the activation of STAT3/NF-κB signaling in transformed epithelial cells,[5] finally, to corroborate the biological relevance of the results observed in TILs treated with rafoxanide, we showed that rafoxanide reduced TIL-derived culture supernatant-mediated cell proliferation and STAT3/NF-κB activation in CRC cells. Future studies will aim to characterize in depth which immune cells are preferentially targeted by rafoxanide and the effect of the drug on the production of other cytokines that are supposed to be protumorigenic in CRC (e.g., IL-17A, IL-21, and IL-22) and cytotoxic factors (e.g., perforin and granzyme B).

As STAT3 and NF-κB aberrant activation significantly contributes to tumor invasion and metastasis in advanced stages of the disease,[60,61] future efforts will focus on assessing the effects of rafoxanide in experimental models of metastasis. In this context, preliminary data showed the ability of rafoxanide to impair the invasiveness of the human CRC cell line HCT-116 in a Matrigel-based cell invasion assay, as well as the RNA transcripts of STAT3-related MMPs, namely MMP-1 and MMP-3 (pers. obs., 2021).

In conclusion, we report the novel observation that rafoxanide negatively affects NF-κB and STAT3 protumorigenic activity in the CRC microenvironment at multiple levels, further strengthening the anticancer properties of the drug.

AUTHOR CONTRIBUTIONS

Teresa Pacifico: Data curation; formal analysis; investigation; visualization; writing – original draft. **Carmine Stolfi:** Data curation; formal analysis; investigation; visualization; writing – original draft. **Lorenzo Tomassini:** Data curation; formal analysis; investigation. **Anderson Luiz-Ferreira:** Data curation; formal analysis; investigation. **Eleonora Franzè:** Data curation; formal analysis; investigation. **Angela Ortenzi:** Investigation. **Alfredo Colantoni:** Investigation. **Giuseppe S. Sica:** Investigation. **Manolo Sambucci:** Data curation. **Ivan Monteleone:** Formal analysis; writing – review and editing. **Giovanni Monteleone:** Writing – review and editing. **Federica Laudisi:** Conceptualization; supervision; writing – original draft.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

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None declared.

ETHICS STATEMENT

Approval of the research protocol by an institutional review board: Human studies were approved by the local ethics committee (protocol no. R.S. 131.17).
Informed consent: Informed consent was obtained from the patients.
Registry and the registration no. of the study/trial: N/A.
Animal studies: All in vivo experiments were approved by the animal ethics committee according to the Italian legislation on animal experimentation (authorization no. 494/2017-PR) and in accordance with European rules (2010/63/UE).

ORCID

Federica Laudisi https://orcid.org/0000-0001-7380-0269

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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