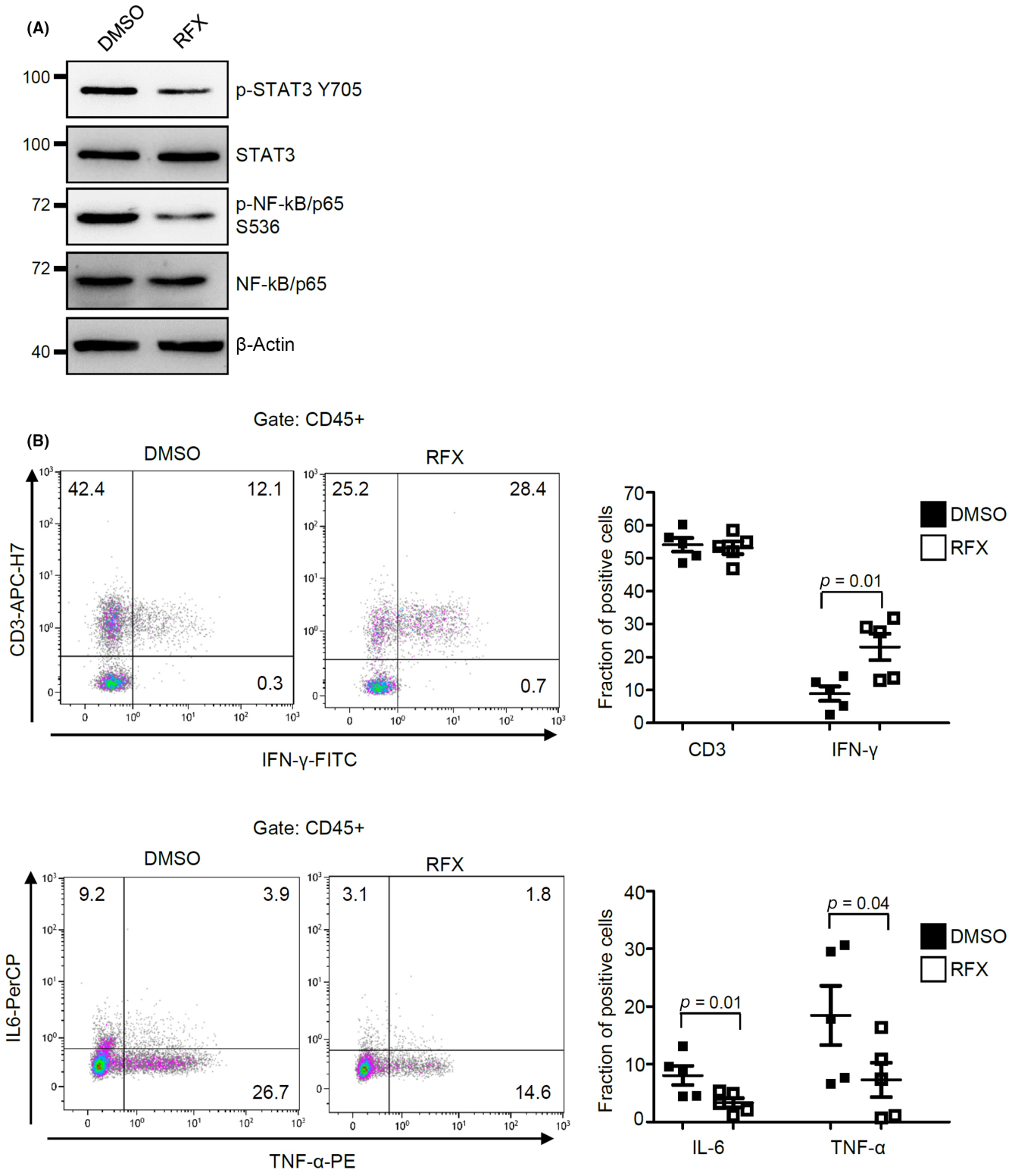

FIGURE 8 Rafoxanide (RFX) hampers signal transducer and activator of transcription 3 (STAT3)/nuclear factor-κB (NF-κB) activation and the production of protumorigenic cytokines in tumor infiltrating leukocytes (TILs). (A) Expression of phosphorylated (p-)STAT3 Y705, STAT3, p-NF-κB S536, and NF-κB/p65 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.

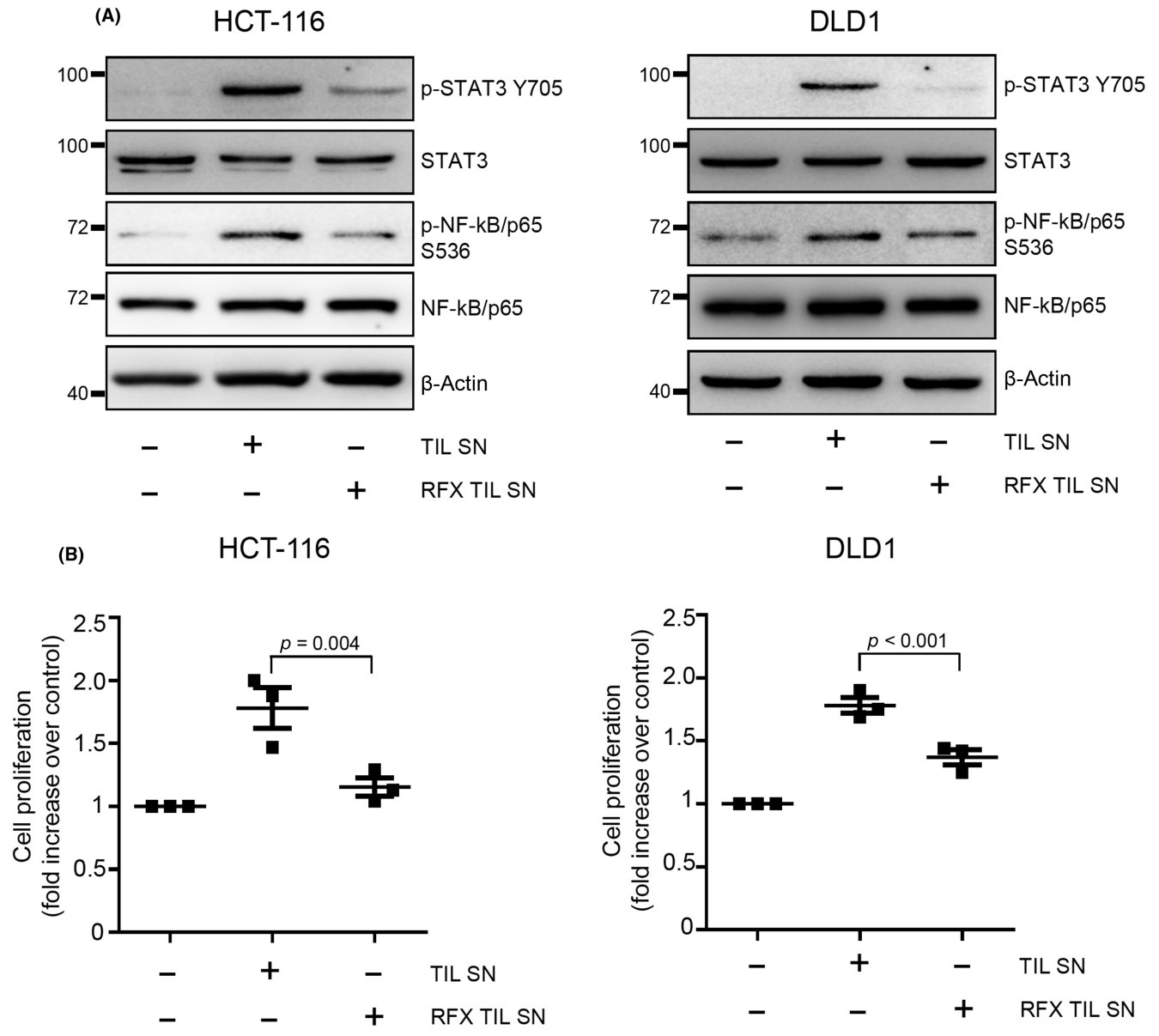

FIGURE 9 Effect of supernatants derived from tumor infiltrating leukocytes (TILs) cultured in the presence of rafoxanide (RFX TIL SN) or DMSO (TIL SN) on colorectal cancer (CRC) cell proliferation and signal transducer and activator of transcription 3 (STAT3)/nuclear factor-κB (NF-κB) activation. (A) 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).

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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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