Trifluridine/Tipiracil plus Oxaliplatin Improves PD-1 Blockade in Colorectal Cancer by Inducing Immunoegenic Cell Death and Depleting Macrophages

Emeric Limagne1,2,3, Marion Thibaudin1,2,3, Lisa Nuttin1,2,3, Aodrenn Spill1,2,3, Valentin Derangère1,2,3, Jean-David Fumet1,2,3, Nadia Amellal4, Elisa Peranzoni4, Valérie Cattan4, and François Ghiringhelli1,2,3,5,6

Abstract

Trifluridine/tipiracil (FTD/TPI) is a new antimetabolite agent used to treat chemorefractory metastatic colorectal cancer. FTD/TPI induced immunogenic cell death (ICD) in vitro in the microsatellite-stable (MSS) CT26 mouse colon carcinoma cell line, as well as in various human MSS colorectal cancer cell lines (SW620, Caco-2, and Colo-320). The combination of FTD/TPI with oxaliplatin synergized to promote ICD. In vivo, the combination was able to induce ICD, but not the single agents, although all treatment groups showed T-cell dependency. In addition, FTD/TPI and oxaliplatin did not affect regulatory T cells or myeloid-derived suppressor cells but eliminated type-2 tumor-associated macrophages (TAM2), resulting in higher cytotoxic CD8+ T-cell infiltration and activation. This effect was concomitantly associated with PD-L1 expression on tumor cells and PD-1 induction on CD8+ T cells, leading to secondary T-cell exhaustion. Finally, although anti–PD-1 was unable to synergize with FTD/TPI or oxaliplatin monotherapy, concomitant administration of anti–PD-1 to FTD/TPI and oxaliplatin enhanced the antitumor efficacy of the double chemotherapy. Our study showed a novel immunomodulatory role of FTD/TPI and oxaliplatin in depleting TAM2. The combination of oxaliplatin and FTD/TPI induced ICD in vivo, providing a rationale for the use of these drugs to eliminate immunosuppressive cells and boost checkpoint efficacy in patients with metastatic colorectal cancer.

Introduction

Colorectal cancer is the most frequent digestive cancer (1). Chemotherapy, including fluoropyrimidine, oxaliplatin, and irinotecan, is the standard of care for unresectable metastatic colorectal cancer. PD-1/PD-L1 blockade has demonstrated modest clinical responses in a subgroup of patients with metastatic microsatellite instability-high (MSI-H) colorectal cancer. Pembrolizumab and nivolumab, alone or in combination with the anti–CTLA-4 (ipilimumab), are approved by the FDA for the treatment of patients with metastatic MSI-H (up to 4% of patients with metastatic colorectal cancer; refs. 3, 4). However, anti–PD-1 therapies given as monotherapy do not induce clinical responses in patients with microsatellite stability (MSS). Such resistance to immunotherapy can be explained by the reduced expression of neoantigens and the inability of CD8+ T cells to infiltrate the tumor, thus leading to tumor immuno-exclusion (5, 6).

Some cytotoxic drugs have immunogenic properties that can promote activation of the immune system (7, 8). Indeed, 5-fluorouracil (5-FU) selectively depletes myeloid-derived suppressor cells (MDSC) in vivo (9, 10), and oxaliplatin can trigger an immunogenic form of tumor-cell death (immunogenic cell death, ICD) through the cell surface exposure of calreticulin (CRT) and release of high mobility group box 1 protein (HMGB1) and ATP into the extracellular environment (11–13). These processes can contribute to the induction of CD8+ T-cell antitumor immunity and therefore reestablish the cancer-immunity cycle (14, 15). We previously reported that 5-FU combined with oxaliplatin can induce ICD in the murine MSS CT26 colon cancer model and improve anti–PD-1 efficacy, suggesting that the association of...
anti–PD-1 with chemotherapy could be used to reverse the resistance of MSS colon cancer to immunotherapy (16, 17).

Trifluridine/tipiracil (FTD/TPI) is efficacious in advanced and metastatic colorectal and gastric cancer (18, 19). FTD/TPI is an oral combination drug of trifluridine and tipiracil, at a molar ratio of 1:0.5. FTD is a fluorinated thymidine analog (20). Like 5-FU, FTD can inhibit thymidylate synthase (21), but its main effect relies on its incorporation into DNA, resulting in DNA dysfunction (22, 23). TPI enhances the bioavailability of FTD by inhibiting its enzymatic degradation by thymidine phosphorylase, leading to a more durable and sustained response (24). FTD/TPI is used as monotherapy in heavily pretreated patients with metastatic colorectal cancer. In a phase I dose-escalation trial, the combination of FTD/TPI with oxaliplatin (25) had a manageable safety profile combined with some therapeutic efficacy. FTD/TPI and anti–PD-1 treatments can have a synergistic effect in a preclinical colon cancer model; however, the effect on the immune system of this combination therapy was not addressed (26). Here, we tested the immune-modulatory effects of FTD/TPI in the CT26 murine model of KRAS-mutated and MSS colorectal cancer (27), alone and in combination with oxaliplatin, both in vitro and in vivo. Last, we assessed the ability of the combination therapy to improve PD-1 blockade in the intrinsically resistant CT26 model. Overall, our work provides rationale to test the FTD/TPI, oxaliplatin, and anti–PD-1 combination in patients with colorectal MSS cancer.

Materials and Methods
Mouse strains
All animals were bred and maintained according to both the Federation for Laboratory Animal Science Associations (FELASA) and Animal Experimental Ethics Committee Guidelines (N° C 21 464 04 EA, University of Burgundy, France). Female BALB/c and Nude NMRI mice between 7 and 9 weeks of age were purchased from Charles River Laboratories.

Tumor-cell lines and transplantable tumors
CT26 murine colon carcinoma cancer cells were obtained from the ATCC at the beginning of this project. Cells were cultured at 37°C under 5% CO2 in RPMI with 10% (vol/vol) FCS (Dutscher) supplemented with sodium pyruvate (Gibco), penicillin and streptomycin (Gibco), and 10 mmol/L HEPEs (Gibco) and maintained in culture for a maximum of 2 months or 10 passages. Human cell lines were also obtained from the ATCC at the beginning of our study and maintained in culture for a maximum of 2 months or 10 passages. Colo-320 and SW6620 human colon carcinoma cancer cells were cultured at 37°C under 5% CO2 in RPMI with 10% (vol/vol) FCS (Dutscher) supplemented with sodium pyruvate (Gibco), penicillin and streptomycin (Gibco), and 10 mmol/L HEPEs (Gibco). Caco-2 human colon carcinoma cancer cells were cultured at 37°C under 5% CO2 in DMEM with 20% (vol/vol) FCS (Dutscher) supplemented with sodium pyruvate (Gibco), penicillin and streptomycin (Gibco), and 10 mmol/L HEPEs (Gibco). Cells were routinely tested for Mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza). Cell lines were not authenticated in the past year. Tumor formation was induced by subcutaneous injection of 10^6 CT26 cancer cells resuspended in 100 μL of serum-free RPMI into BALB/c or Nude NMRI mice. Mice were randomized and treated as described below for 10 days after tumor-cell implantation.

In vivo chemotherapeutic treatments
For in vivo tumor-growth experiments, oxaliplatin was administered by intraperitoneal injection (100 μL in glucose 5%, Vialo) at a dose of 5 mg/kg/day once at randomization. Anti-mouse PD-1 (BE0146, BioXCell) was injected intraperitoneally (100 μL in Invivopure Dilution Buffer 5%, BioXCell) at a dose of 10 mg/kg/day 3 times per week for 4 weeks. FTD/TPI was prepared by mixing FTP and TPI at a molar ratio of 1:0.5 in 0.5% hydroxypropyl methylcellulose solution (HPMC; Sigma-Aldrich). The dose of FTD/TPI was expressed on the basis of FTD content. FTD/TPI was administered orally (200 μL diluted in HPMC) 5 times per week for 4 weeks at the reported optimal effective dose (150 mg/kg/day; ref. 28). Control groups received 10 mL/kg of vehicle (0.5% HPMC solution) administered orally 5 times per week for 4 weeks, and 0.1 mL of saline was injected intraperitoneally 3 times per week for 4 weeks. In some experiments, macrophage depletion was performed by injecting 150 μL liposome-encapsulated clodronate or liposome control [called Lyophilized Clophosome (F70101C-AL, FormuMax Scientific)] by the intravenous route (100 μL, reconstituted with sterile water) the day before the injection of tumor cells and 3, 6, and 10 days after tumor-cell injection.

IHC
Tumors were collected after 8 days post randomization/treatment, fixed in 4% paraformaldehyde for 24 hours, and embedded in paraffin by our pathology laboratory service. Four-micron-thick slices were cut from formalin-fixed paraffin-embedded tumor samples. Antigen retrieval was carried out by heating slides for 45 minutes at 95°C in citrate buffer (pH 6.1, S1699, Agilent). Several triple stainings were assessed: CD8/Ki67/PD-1; CD8/Mac387/PD-1; CD8/CD45/PD-1; and CD8/TGF-β/PD-1 (antibodies listed below). Hydration of 100 μL of PBS containing 0.1% of BSA (A8806-1G, Sigma-Aldrich) and saturation with 100 μL of PBS containing 5% of goat serum were first carried out. Then, samples were incubated with nonlabeled primary antibody (PD-1, Granzyme B, or PD-L1) diluted in PBS-BSA 0.1% for 1 hour at room temperature and then with secondary antibody (Alexa Fluor 488 goat anti-rabbit for PD-1 and Granzyme B or Alexa Fluor 488 donkey anti-rabbit for PD-L1) for 30 minutes at room temperature. After another saturation step, samples were incubated with Alexa Fluor 555-CD8 antibody and Alexa Fluor 488-Ki67 antibody or Alexa Fluor 555-Ki67 antibody and Alexa Fluor 647-CD45 antibody overnight at 4°C. Finally, nuclei were stained with a DAPI solution (Spectral DAPI, FP1490, PerkinElmer) and the slides mounted in Prolong Diamond Antifade (P36965, Thermo Fisher Scientific) before imaging and analysis. For each slide, three representative areas were imaged using a Mantra Quantitative Pathology Workstation (PerkinElmer). Images were analyzed using inForm Cell Analysis software (PerkinElmer). After nuclear recognition and phenotype learning, phenotyping of CD8-PD1/CD8-Ki67/CD8-Granzyme or PD-L1–positive cells was achieved by the inForm Cell Analysis software. A mean of three areas for each slide was calculated, and one representative image (and associated phenotype map) was chosen for each tumor group.
The following antibodies were used: Alexa Fluor 555-CD8 (0648-R-A55, Bioss antibodies), Alexa Fluor 488-Ki67 (558616, BD Biosciences), Alexa Fluor 555-Ki67 (558616, BD Biosciences), Alexa Fluor 647-CD45 (103124, BioLegend), rabbit anti–PD-1 (D7DSW, Cell Signaling Technology), rabbit anti–Granzyme B (LS-B2783, LSBio), rabbit anti–PD-L1 (MAB90781, R&D Systems), goat-anti rabbit Alexa Fluor 647 (111-605-045, Jackson Immunoresearch), and goat anti-rabbit Alexa Fluor 488 (111-545-045, Jackson Immunoresearch).

Flow cytometry

To study the infiltration of lymphoid and myeloid cells in the tumor tissue, mice were sacrificed 8 days after randomization/treatment, and tumors were collected. After dissection, tumors were mechanically and enzymatically dissociated using a mouse tumor dissociation kit, according to the manufacturer's recommendations (130-096-730, Miltenyi Biotec). In brief, tumors were cut into small pieces and transferred into the gentleMACS C Tubes containing the enzyme mix. The dissociated tumors were cut into small pieces and transferred into the mouse tumor dissociation kit, according to the manufacturer's instructions. Samples were homogenized before being applied to Heaters and with mouse tumor dissociation 37°C$_\text{m}$TDK$_\text{m}$1. Suspensions were resuspended in RPMI medium at a concentration of 1 x 10^6 cells/mL.

Tumor cell infiltration was analyzed by staining the tumor-cell suspension (10^6 cells) in Flow Cytometry Staining Buffer (FSB, 00-4222-26, eBioscience) with specific antibodies according to the manufacturer's recommendations (antibody details are presented in Supplementary Table S1) for 15 minutes at room temperature in the dark, washed twice in FSB, and analyzed by flow cytometry. Flow cytometry acquisition was performed on a Cytoflex 13C cytometer (Beckman Coulter). Kahuza (Beckman Coulter) was used for the analysis.

Lymphoid cell infiltration was analyzed by staining the tumor-cell suspension with the Foxp3 staining buffer set according to the manufacturer's recommendations (130-093-142, Miltenyi Biotec; antibody details are presented in Supplementary Table S1). For the lymphoid and myeloid cell infiltration assay, viability dye eFluor 780 (65-0865-14, eBiosciences) was used to identify live cells. Flow cytometry acquisition and analysis were performed as described above. For lymphoid and myeloid-cell identification, see the gating strategy presented in Supplementary Figs. S1 and S2.

To study cytokine function of the lymphoid infiltrate, the tumor-cell suspension was cultured on 24-well plates in RPMI medium (Dutscher) containing 10% of FSB and 1% of PSA overnight at 37°C. PMA (phorbol 12-myristate 13-acetate; 20 ng/mL; Sigma-Aldrich), ionomycin (1 µg/mL; Sigma-Aldrich), and brefeldin A (2 µL/mL, eBioscience) were included during the last 4 hours of culture. After staining of surface markers (antibody details in Supplementary Table S1), cells were fixed and permeabilized with the Foxp3 staining buffer set, according to the manufacturer's instructions (130-093-142, Miltenyi Biotec). Whole-cell lysates were prepared, as previously described (29), by lysing the cells in boiling buffer (1% SDS, 1 mmol/L sodium vanadate, 10 mmol/L Tris, pH 7.4) in the presence of complete...
protease inhibitor mixture (11697498001, Sigma-Aldrich). The viscosity of the samples was reduced by sonication. Samples were sonicated for 10 seconds at 30 kHz (Branson 200) at room temperature and then placed immediately on ice. Whole-cell lysates were separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane (GE Healthcare). After incubation for 2 hours at room temperature in 5% nonfat milk in Tris-buffered saline–0.1% Tween-20, membranes were incubated overnight at 4°C with the primary antibody diluted in Tris-buffered saline milk–Twee-20, washed with PBS containing 0.1% of Tween 20 (P1379, Sigma Aldrich), incubated with the secondary antibody for 30 minutes at room temperature, and washed again before analysis using a chemiluminescence detection kit (Amersham). We used the mouse anti-β-actin (A1978) from Sigma, as well as rabbit anti-EF2 (D7/D3) and anti-phospho-EF2 (D9G8) from Cell Signaling Technology. Secondary antibodies horseradish peroxidase–conjugated polyclonal goat anti-mouse and goat anti-rabbit immunoglobulins (Jackson ImmunoResearch) were also used. All antibodies were diluted in PBS containing 5% of BSA (A8806-1G, Sigma-Aldrich).

Real-time qPCR
Total RNA from tumor cells was extracted with TriReagent (Ambion), and RNA content was measured using a NanoDrop 2000 (Thermo Scientific). Three hundred to 500 ng of RNA was reverse transcribed into cDNA using M-MLV Reverse Transcriptase, Random Primers, and RNaseOUT inhibitor (Invitrogen). cDNA was quantified by qRT-PCR using the SYBR Green method (Power SYBR Green PCR Master Mix, 4368700; Thermo Fisher) according to the manufacturer’s instructions, using the QuantStudio 5 Real-time PCR system (Applied Biosystems). Relative mRNA levels were determined using the ΔΔCt method (i.e., 2^(-ΔΔCt)). Expression was normalized to the expression of mouse ACTB. Primers designed to assess gene expression are described in Supplementary Table S2.

Statistical analysis
Statistical analyses were performed using Prism GraphPad software [not significant (ns), P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001]. For in vitro experiments, results are shown as the mean ± SD. Datasets were compared using an unpaired Mann–Whitney–Wilcoxon test. For in vivo experiments, survival data were analyzed using the Kaplan–Meier method and log-rank test. All other analyses were performed using one-way ANOVA or two-way ANOVA followed by the Sidak posttest for multiple comparisons. No statistical corrections were performed.

Results
FTD/TPI synergized with oxaliplatin to induce ICD in vitro
We tested the cytotoxic effect of FTD/TPI or oxaliplatin alone or in combination in vitro on murine colon carcinoma CT26 cells (MSS and Kras-mutant cell line; ref. 27). Both viability (crystal violet staining) and cell death (Annexin-V/DAPI labeling) assays showed a synergistic effect for the FTD/TPI and oxaliplatin combination after 24 hours of treatment. We selected a dose of 5 and 50 μmol/L of each drug, the latter inducing approximately 50% of cell death (Supplementary Fig. S4A and S4B). ICD is a molecular process, induced by some anticancer agents, which triggers a CD8-dependent immune response. ICD requires cell surface CRT exposure, induction of EF2α-dependent reticulum stress, HMGB1 and ATP release, and expression of type 1 IFNs (Ifna1 and Ifnb1) and chemokines (Cxcl9 and Cxcl10; refs. 7, 8, 11–13). We validated the capacity of oxaliplatin and FTD/TPI or combination to induce ICD of CT26 cells in vitro (Fig. 1A) by assessing CRT exposure (Fig. 1B), EF2α activation (Fig. 1C), HMGB1 release (Fig. 1D), the extracellular release of ATP (Fig. 1E), and mRNA expression of type 1 IFNs (Ifna1 and Ifnb1; Fig. 1F and G) and chemokines (Cxcl9 and Cxcl10; Fig. 1H and I). Indeed, treatment with FTD/TPI alone induced expression of all ICD markers in CT26 cells at 5 or 50 μmol/L, except for Ifna1 and Cxcl9 mRNA expression (Fig. 1F). In contrast, oxaliplatin alone induced ICD only at 50 μmol/L (Fig. 1F). The combination of the drugs was synergistic and significantly enhanced ICD induction as compared with control or monotherapies (Fig. 1). We also performed similar experiments on a panel of three human MSS colorectal cancer cell lines: KRAS wild-type Caco-2 and Colo-320 and KRAS-mutant SW620 (ref. 30; for genetic characteristics, see: Supplementary Fig. 5A). We evaluated cell viability and ICD, and we compared effect of the monotherapies and combination therapy with the control and the monotherapies to the combination therapy. Caco-2 cells were intrinsically resistant to FTD/TPI and oxaliplatin-induced cell death (Supplementary Fig. SS and SSC) and consequently did not exhibit ICD markers. Only a high dose of oxaliplatin induced ER stress and ATP release in Caco-2 cells (Fig. 2A–D). Colo-320 cells were very sensitive to oxaliplatin but weakly sensitive to FTD/TPI (Supplementary Fig. SSC). Both drugs were required to trigger markers of ICD, including EF2α activation, CRT exposure, and ATP release (Fig. 2E–H). In contrast, SW620 cells were sensitive to oxaliplatin and FTD/TPI, displaying higher apoptosis compared with control (Supplementary Fig. SDD). Both drugs were able to induce all markers of ICD alone and in combination as compared with chemotherapies alone (Fig. 2I–L). Overall, these data demonstrate that FTD/TPI induces ICD in vitro in cell lines that are sensitive to FTD/TPI in both mouse and human cancer models. In some cases, oxaliplatin further increased the effect of FTD/TPI.

FTD/TPI and oxaliplatin triggered ICD in vivo
We addressed the in vivo capacity of the drugs to induce ICD and thus act on tumor growth by this mechanism by treating CT26 tumor-bearing mice with FTD/TPI or oxaliplatin alone or the combination of the two drugs. Upon chemotherapeutic treatment, HMGB1 has been observed to translocate from the nucleus to the cytoplasm and is then released into the extracellular compartment (11). Only the combination of FTD/TPI and oxaliplatin was able to induce HMGB1 cytoplasmic relocalization, similar to that observed after treatment with doxorubicin, used as a positive control (Fig. 3A and B). Similarly, only the FTD/TPI plus oxaliplatin combination induced the phosphorylation of the reticulum stress marker EF2α in vivo (Fig. 3C and D). A comparison of tumor growth in immunodeficient and immunocompetent mice showed that the anti-tumor effect only occurred in immunocompetent mice with higher activity of the combination therapy. Despite the absence of HMGB1 cytoplasmic relocalization and EF2α activation, FTD/TPI or oxaliplatin monotherapy had an anti-tumor effect in immunocompetent mice. These observations suggest an ICD-independent immune effect, such as depletion of an immunosuppressive population by FTD/TPI or oxaliplatin (Fig. 3E and F).
CT26 cells treated for 24 hours as indicated in
not significant; *** P<0.001; **** P<0.0001. Data sets were compared using an unpaired Mann–Whitney–Wilcoxon test. DXR, doxorubicin; OxPt, oxaliplatin.

Figure 1. FTD/TPI synergized with oxaliplatin to induce ICD in vitro. A, CT26 cells were treated, or not, in vitro with 5 or 50 μmol/L oxaliplatin, 5 or 50 μmol/L FTD/TPI, or a combination of 5 μmol/L doxorubicin. CT26 cells were recovered after 24 hours of treatment, and ICD was analyzed. B, CRT exposure was detected by flow cytometry of DAPI−/CRT+ cells treated as indicated in A for 24 hours. A plot showing the percentage of DAPI−/CRT+ cells is presented on the left, and representative histograms are shown on the right. C, Western blot analysis of phosphorylated and total EIF2α and β-actin in CT26 cells treated for 24 hours as indicated in A. The heatmap indicates the phospho-EIF2α/CRT− ratio calculated by a densitometry analysis. D and E, HMGB1 was measured in supernatants of CT26 cells treated for 24 hours as indicated in A by ELISA (D) and ATP levels determined by luminescence assay (E). F–I, The relative expression of Ifna1 (F), Ifnb1 (G), Cxcl9 (H), and Cxcl10 (I) mRNA was determined by qRT–PCR of CT26 cells treated for 24 hours as indicated in A. Representative data from three independent experiments are shown (mean and SD), except in C, for which representative data from one of three independent experiments are presented. ns, not significant; *, P<0.05; **, P<0.01; *** P<0.001; and ****, P<0.0001.
Chemotherapy Improves Anti-PD-1 in MSS Colorectal Cancer

Figure 2.
FTD/TPI synergized with oxaliplatin to induce ICD in vitro. Caco-2, Colo-320, and SW620 cells were treated, or not, in vitro with 5 or 50 μmol/L oxaliplatin, 5 or 50 μmol/L FTD/TPI, or a combination or 5 μmol/L doxorubicin. Cancer cells were recovered after 48 hours of treatment and ICD analyzed. A, CRT exposure was detected by flow cytometry on DAPI−CRT− cells, or a combination of 5 or 50 ng/mL FTD/TPI, or a combination or 5 or 50 μmol/L oxaliplatin. Western blot analysis of phosphorylated total Eif2α and β-actin in Caco-2 cells treated as indicated above. The percentage of DAPI− CRT+ cells is presented. B, Western blot analysis of phosphorylated and total Eif2α and β-actin in Caco-2 cells treated as indicated above. The heatmap indicates the phospho-Eif2α/total Eif2α ratio calculated by a densitometry analysis. C and D, HMGB1 was measured in supernatants of Caco-2 cells treated for 48 hours, as indicated above, by ELISA (C) and ATP levels determined by luminescence assay (D). E and F, Same as in A, but for Colo-320 (E) and SW620 (F) cells. G and H, Same as in B, but for Colo-320 (G) and SW620 (H) cells. I and J, Same as in D, but for Colo-320 (I) and SW620 (J) cells. Representative data from three independent experiments are shown (mean and SD), except in B, F, and J, for which representative data from one of three independent experiments are presented. ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001. Data sets were compared using an unpaired Mann–Whitney–Wilcoxon test. DXR, doxorubicin; OxPt, oxaliplatin.
Figure 3.
FTD/TPI and oxaliplatin triggered ICD in vivo. A and B, CT26 tumor–bearing mice (n = 5/group) were treated with 0.5% HPMC solution (control) or oxaliplatin (5 mg/kg), FTD/TPI (150 mg/kg/day), or a combination of oxaliplatin and FTD/TPI or doxorubicin (5 mg/kg), and the tumors were harvested 3 days after treatment. A, Representative images of an HMGB1 localization study performed by IHC and a representative phenotyping map obtained after analysis for each tumor group with Inform software. B, Graph showing the percentage of cytoplasmic HMGB1 in each tumor group. C and D, CT26 tumor–bearing mice (n = 3–4/group) were treated with 0.5% HPMC solution (control), oxaliplatin (5 mg/kg), FTD/TPI (150 mg/kg/day), or a combination of oxaliplatin and FTD/TPI or doxorubicin (5 mg/kg), and the tumors were harvested 3 days after treatment. C, Western blot analysis of phosphorylated and total EIF2α and β-actin in each control or treated tumor. D, Graph of the densitometry analysis of the effect of each treatment on EIF2α phosphorylation using total EIF2α as reference. E and F, BALB/c (E) and Nude NMRI (F) mice under control (0.5% HPMC solution), oxaliplatin (5 mg/kg), FTD/TPI (150 mg/kg/day), or a combination of oxaliplatin and FTD/TPI treatments were inoculated with CT26 cells, and tumor growth was monitored over 7 weeks (n = 10/groups). Survival curves are presented on the left. Monitoring of tumor growth is shown on the right. Each line represents an individual mouse. Experiment was performed once. ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001. Data sets were compared using an unpaired Mann–Whitney–Wilcoxon test, and survival data were analyzed using the Kaplan–Meier method and log-rank test. DXR, doxorubicin; OxPt, oxaliplatin.
The FTD/TPI and oxaliplatin combination induced a CD8+ T-cell immune response

An efficient antitumor immune response is promoted by the capacity of the chemotherapies to affect immunosuppressive populations and/or induce ICD, which result in subsequent T-cell recruitment (7,8). Thus, we studied the effect of each drug on immunosuppression and effector immune response. First, we analyzed CD8+ and CD4+ T-cell infiltration in the tumor microenvironment. As compared with untreated mice, only the FTD/TPI and oxaliplatin combination therapy induced an increase in the CD8+ T-cell compartment among immune cells, whereas the CD4+ T-cell fraction did not change (Fig. 4A). Importantly, the combination therapy promoted CD8+ T-cell infiltration not only of the tumor periphery but also of the tumor core, as observed by immunofluorescence on tumor samples (Fig. 4B, Supplementary Figs. S6 and S7). We then analyzed the impact of these therapies on the four main tumoral immunosuppressive cell populations: regulatory T cells (Treg), neutrophilic-MDSC, monocytic-MDSC, and tumor-associated macrophages (TAM; ref. 8). Tregs were not affected by the treatment. MDSCs, particularly polymorphonuclear-MDSCs and unlike 5-FU/oxaliplatin treatment, were not depleted by FTD/TPI or FTD/TPI plus oxaliplatin. FTD/TPI, oxaliplatin, or the combination induced TAM depletion, particularly of TAM2, resulting in a change in the TAM1/TAM2 ratio (Fig. 4C–F, Supplementary Fig. S8). We assessed the capacity of cytotoxic T cells by spectral microscopy and flow cytometry. As compared with untreated mice, only the combination therapy could enhance granzyme B, IFNγ, and TNFα production in intratumoral CD8+ T cells, whereas the monotherapies induced the production of only one or two cytokines (Fig. 4G and H).

In addition, we performed a correlation analysis between CD8+ cytokine production and the infiltration of the immunosuppressive populations (Supplementary Fig. S9). As expected, the percentage of TAM2 was associated with lower CD8+ cytokine secretion, whereas Tregs and other myeloid populations were not (Supplementary Fig. S9). We confirmed that macrophage depletion was important for the CD8+ T-cell response using clodronate liposomes, known to deplete TAM2 (31). Clodronate liposome-induced TAM2 depletion and reduced tumor growth, similarly to FTD/TPI and oxaliplatin (Supplementary Fig. S10A and S10B). This depletion did not modulate CD8+ T-cell recruitment but enhanced IFNγ or TNFα production by CD8+ T cells (Supplementary Fig. S10C and S10D). Overall, these data demonstrated the capacity of the FTD/TPI and oxaliplatin combination to promote the CD8+ T-cell cytotoxic immune response by both modulating immunosuppression in the tumor microenvironment and inducing T-cell effector recruitment via ICD.

FTD/TPI and oxaliplatin improved anti–PD-1 efficacy

We have previously shown that the 5-FU and oxaliplatin combination can induce adaptive immune tolerance, which includes both T-cell exhaustion and the induction of IFNγ-dependent PD-L1 expression (16). We therefore studied PD-L1 expression by flow cytometry and observed that FTD/TPI oxaliplatin combination therapy increased the proportion of PD-L1+ on tumor cells but not tumor-infiltrating leukocytes, for which PD-L1 expression was lower compared with control (Fig. 5A). Among tumor-infiltrating immune cells, PD-L1 was mainly expressed by TAM, particularly TAM2 (Fig. 5B). These data suggest that PD-L1 expression on CD45+ was decreased by chemotherapy due to TAM depletion (Figs. 4E and 5B). We observed that FTD/TPI and oxaliplatin combination therapy reduced Ki67 expression and enhanced PD-1+ expression on CD8+ T cells by spectral microscopy, thus suggesting that intratumoral T cells may undergo exhaustion (Fig. 5C–E). Moreover, FTD/TPI plus oxaliplatin affected the proportion of exhausted CD8+ T-cell subsets. We observed that combitherapy increased the ratio of progenitor exhausted (PE: CD44+ PD-1– Tim-3–) to terminally exhausted CD8+ T cells (TE: CD44+ PD-1+ Tim-3–; Fig. 5F). We thus hypothesized that blocking PD-1 could enhance the efficacy of chemotherapies, as FTD/TPI and oxaliplatin combination therapy not only induced ICD, activated CD8+ T cells, and reduced TAM2, but also upregulated the expression of PD-L1 and PD-1. Anti–PD-1 did not have a significant additive effect on FTD/TPI treatment in CT26 tumor-bearing mice but enhanced the antitumor efficacy of the combination therapy and improved mice survival as compared to all other groups (Fig. 5G). We also observed a lack of toxicity of this chemoinmunotherapy combination (Supplementary Fig. S11). Overall, these data suggested that immunogenic FTD/TPI/oxaliplatin chemotherapies could be used in combination with anti–PD-1 blockade in the clinic to improve antitumor efficacy.

Discussion

Here, we investigated the immunoregulatory effect of FTD/TPI and oxaliplatin in a mouse model of MSS Kras-mutated cancer and in human MSS cell lines of colorectal cancer. The combination therapy induced ICD in both human and mouse cell lines. FTD/TPI or oxaliplatin alone or their combination depleted both TAM1 and TAM2, with a stronger effect on TAM2, thus promoting CD8+ T-cell activation. Together, ICD induction and macrophage depletion resulted in better CD8+ activation and recruitment but also led to adaptive immune tolerance. Finally, the combination of FTD/TPI and oxaliplatin improved anti–PD-1 efficacy in vivo in the CT26 model.

FTD/TPI induced ICD in vitro in the murine CT26 cell line. Moreover, FTD/TPI improved the capacity of oxaliplatin to induce ICD. However, not all human cell lines were sensitive to drug-mediated cell death. We observed similar effects on SW620 or Colo-320 human cell lines, in which FTD/TPI plus oxaliplatin combination induced a significant amounts of ICD. It was not the case for the Caco-2 cell line, which was intrinsically resistant to FTD/TPI plus oxaliplatin-mediated cell death. Chemotherapies that induce ICD have been reported to synergize with immunotherapy in a CD8+ T-cell–dependent manner in either lung or colorectal cancer models (16,32). The capacity of FTD/TPI to trigger ICD has not been reported and in vitro FTD/TPI induced CRT exposure and ATP and HMGB1 release. Few studies have demonstrated whether chemotherapy also triggers ICD in vivo. In most studies, the presence of ICD was deduced by comparing the antitumoral effect of chemotherapy between immunocompetent and immunodeficient mice (33,34). In our study, we observed that neither oxaliplatin nor FTD/TPI alone could induce ICD stigmas in vivo, whereas the combination therapy did. This case for the Caco-2 cell line, which was intrinsically resistant to chemotherapy in vivo, which could not be reached in vivo.

Complete immunomonitoring showed that both drugs induced TAM2 depletion and, consequently, the activation of CD8+ T cells. TAM2 are frequently associated with tumor-induced tolerance, notably by their capacity to produce immunosuppressive cytokines or metabolites (34). Murine CT26 tumors, similar
Figure 4.
The FTD/TPI and oxaliplatin combination induced a CD8\(^+\) T-cell immune response. CT26 tumor-bearing mice (n = 5/group) were treated with 0.5% HPMC solution (control), oxaliplatin (5 mg/kg), FTD/TPI (150 mg/kg/day), or a combination of oxaliplatin and FTD/TPI, and tumors were harvested 8 days after treatment. Experiment was performed two independent times. A, Frequency of CD8\(^+\) and CD4\(^+\) tumor-infiltrating lymphocytes (TIL) among CD45\(^+\) cells measured by flow cytometry in each control or treated group of mice (for the gating strategy, see Supplementary Fig. S1). B, Frequency of CD8\(^+\) cells analyzed by IHC at the invasive margin and the tumor core for each tumor group. Representative images of CD8 staining are presented on the left for the control and FTD/TPI + oxaliplatin-treated groups of mice (to see all images, see Supplementary Fig. S6). C–E, Frequency of Treg (C), PMN-MDSC and Mo-MDSC (D), and total TAM, TAM1, and TAM2 (E) among CD45\(^+\) TILs measured by flow cytometry in each control or treated group of mice. F, Analysis of the TAM1/TAM2 ratio for each control and treated group of mice (for the gating strategy, see Supplementary Figs. S1 and S2). G, Frequency of Granzyme B\(^+\) CD8\(^+\) cells analyzed by IHC for each tumor group. Representative composite images and the corresponding phenotype map obtained after Inform analysis of CD8/Granzyme B staining are presented at the top for each group of mice. At the bottom, the histograms indicate the percentage of Granzyme B\(^+\) cells among CD8 cells. GrzB, Granzyme B. H, Frequency of IFN\(\gamma\)\(^+\) CD8 and TNF\(\alpha\)\(^+\) CD8 TILs among CD45\(^+\) cells measured by flow cytometry in each control and treated group of mice (for the gating strategy, see Supplementary Fig. S3). GranzB, Granzyme B. ns, not significant; *, P < 0.05; **, P < 0.01; and ***, P < 0.001. Data sets were compared using an unpaired Mann–Whitney–Wilcoxon test. Mo-MDSC, monocytic-MDSC; OxPt, oxaliplatin; PMN-MDSC, polymorphonuclear-MDSC.
Chemotherapy Improves Anti–PD-1 in MSS Colorectal Cancer

Figure 5.
FTD/TPI and oxaliplatin improved anti–PD-1 efficacy. CT26 tumor-bearing mice (n = 5/group) were treated with 0.5% HPMC solution (control), oxaliplatin (5 mg/kg), FTD/TPI (500 mg/kg/day), or a combination of oxaliplatin and FTD/TPI, and tumors were harvested 8 days after treatment. This experiment was performed once. A, Frequency of PD-L1 on tumor cells (left) and leukocytes (right) measured by flow cytometry in each control and treated group of mice (for the gating strategy, see Supplementary Fig. S2). B, Frequency of PD-L1 on PMN-MDSC, Mo-MDSC, TAM, TAM1, and TAM2 populations measured by flow cytometry in each control and treated group of mice. C-E, The frequency of PD-1–/Ki67– (left) and PD-1+Ki67+ (right) CD8+ tumor-infiltrating lymphocytes (TIL) measured by IHC in each control and treated group of mice is presented in D and E. Frequency of Ki67– CD8+ cells (D) and PD-1– CD8+ cells (E) analyzed by IHC in each tumor group. Representative images of each staining for each group of mice (above) and representative phenotype maps obtained after Inform analysis for each tumor group (below) are shown. F, Ratio of progenitor to terminally exhausted CD8+ TILs (PE/TE) was calculated in different conditions of treatment after analysis by flow cytometry. Representative dot plots of PD-1 and Tim-3 expression of CD8+ CD44+ cells in control and FTD/TPI + oxaliplatin groups (PE = PD-1– Tim-3– and TE = PD-1+ Tim-3+) are shown. G, CT26 tumor growth was monitored over 10 weeks in BALB/c mice under control (0.5% HPMC solution), oxaliplatin (5 mg/kg) + FTD/TPI (500 mg/kg/day), FTD/TPI + anti–PD-1 (30 mg/kg/day), or oxaliplatin + FTD/TPI + anti–PD-1 treatment. The monitoring of tumor growth is shown, and each line represents an individual mouse. The survival curves are also presented. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001. Survival data were analyzed using the Kaplan–Meier method and log-rank test. Mo-MDSC, monocytic-MDSC; OxPt, oxaliplatin; PMN-MDSC, polymorphonuclear MDSC.
to human colorectal cancer, are massively invaded by TAM2, which are associated with a poor prognosis (35–37). We observed a strong inverse correlation between the frequency of TAM2 and that of activated T cells, suggesting that the depletion of TAM could favor CD8+ T-cell reactivation. Liposome clodronate is a classical treatment to deplete macrophages, especially TAM2 (38). In our model, we observed that liposome clodronate treatment restored CD8 function similarly to FTD/TPI or oxaliplatin. These data strongly support the hypothesis that FTD/TPI and oxaliplatin mainly exert their antitumor immune effect by the direct elimination of TAM2. This observation demonstrated a novel immune effect of FTD/TPI and oxaliplatin. We thus hypothesized that monotherapy with FTD/TPI or oxaliplatin can promote immune activation via the depletion of TAM2, whereas the combination therapy was able to induce either TAM2 depletion or ICD. These data show that chemotherapies could have pleiotropic effects on the immune response and that exhaustive immunomonitoring is required to describe any immune effect of chemotherapy. This article will help to better define clinical trials that combine chemotherapy and immunotherapy to treat cancer.

We previously reported that 5-FU selectively eliminates MDSCs (9, 10). This effect is dependent of its effect on thymidylate synthase. In contrast to 5-FU, FTD/TPI could not induce MDSC depletion but depleted type-2 macrophages. FTD/TPI mainly acts by DNA incorporation, which leads to DNA dysfunction during DNA duplication (23, 38). Such a molecular mechanism may explain the difference between the immune effect of FTD/TPI and that of 5-FU. Oxaliplatin also induces single- and double-strand breaks, which are involved in cell death during DNA replication, corroborating our previous study on the association of 5-FU and oxaliplatin (39). Such common molecular mechanisms of DNA damage may explain the similar effect on type-2 macrophages. Targeting only immunosuppressive cells by depleting them through 5-FU treatment is not sufficient, as 5-FU does not enhance the efficacy of anti–PD-1 monoclonal antibody (mAb; ref. 16). In contrast, the association of an MDSC-depleting agent and oxaliplatin acts in synergy with anti–PD-1 (16). This report supports the idea that FTD/TPI could have the same effect by depleting TAM2 in the tumor. Further studies are warranted to test whether the addition of 5-FU to the FTD/TPI-oxaliplatin combination can improve antitumor efficacy and enhance the effect of the anti–PD-1 mAb.

Immunogenic chemotherapies can lead to adaptive tolerance due to their capacity to reactivate IFNγ production by CD8+ T cells (31). Indeed, we observed a decrease in T-cell proliferation and upregulation of PD-1 and PD-L1 following chemotherapeutic treatment. These observations are in accordance with the concept of adaptive immune resistance, which has been defined as a dominant mechanism of tumor escape in the tumor microenvironment (16, 40). Our data confirm previous reports from the group of Pittet and our own and provide a rationale for the association of anti–PD-1 treatment with immunogenic chemotherapies, despite the mechanism underlying their immunogenicity (16, 32). A previous report suggests that CD44++ PD-1+ Tim-3+ cells are enriched in progenitor exhausted, whereas CD44++ PD-1+ Tim-3- cells are enriched in terminally exhausted CD8+ T cells (41). In that report, the authors suggest that exhausted progenitors are more responsive to anti–PD-1 check-point inhibitors. We observed that FTD/TPI plus oxaliplatin increased the ratio of progenitor to terminal exhausted CD8+ T cells. These data support that FTD/TPI plus oxaliplatin treatment could be used to modify T-cell phenotype to improve PD-1 blockade efficacy.

In conclusion, we reported the capacity of FTD/TPI and oxaliplatin to eliminate TAM2 and restore CD8 function. We also demonstrated that FTD/TPI can induce ICD in vitro but required the addition of oxaliplatin to induce ICD in vivo. Blockade of the PD-1/PD-L1 pathway counteracts the emergence of the immune adaptive resistance induced by immunogenic chemotherapies (32). Previous data suggest that FOLFOX plus anti–PD-1 combination has an acceptable toxicity profile and displayed clinical efficacy in patients with mismatch repair (MMR)-proficient colorectal cancer (42). Thus, we anticipate a similar profile of tolerance to FTP/TPI plus oxaliplatin plus anti–PD-1. Clinical trial of FTP/TPI plus oxaliplatin plus anti–PD-1 in patients is currently ongoing (NCT02848443). FTP/TPI plus oxaliplatin is safe and seemingly efficacious in early human clinical trials (25).

Our study showed that therapies such as FTD/TPI and oxaliplatin in combination with immune-checkpoint inhibitors may constitute a new option for the treatment of patients with metastatic MSS colorectal cancer.

Disclosure of Potential Conflicts of Interest
N. Amellal is a project director for Servier. E. Peranzoni is Research Scientist at Institut de Recherche Servier. V. Cattan is an employee of Institut de Recherches Internationales Servier. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: E. Limagne, V. Derangère, N. Amellal, V. Cattan, F. Ghiringhelli
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Thibaudin, L. Nuttin, A. Spill, J.-D. Fumet
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Limagne, M. Thibaudin, L. Nuttin, F. Ghiringhelli
Writing, review, and/or revision of the manuscript: E. Limagne, M. Thibaudin, J.-D. Fumet, N. Amellal, E. Peranzoni, V. Cattan, F. Ghiringhelli
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Thibaudin, L. Nuttin, A. Spill, V. Derangère
Study supervision: E. Limagne, V. Cattan, F. Ghiringhelli
Other (part of the whole project under my responsibility): N. Amellal

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References
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Trifluridine/Tipiracil plus Oxaliplatin Improves PD-1 Blockade in Colorectal Cancer by Inducing Immunogenic Cell Death and Depleting Macrophages

Emeric Limagne, Marion Thibaudin, Lisa Nuttin, et al.


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