Microenvironment and Immunology

Adverse Immunoregulatory Effects of 5FU and CPT11 Chemotherapy on Myeloid-Derived Suppressor Cells and Colorectal Cancer Outcomes

Julia Kanterman1, Moshe Sade-Feldman1, Moshe Biton1, Eliran Ish-Shalom1, Audrey Lasry1, Aviya Goldshtein1, Ayala Hubert2, and Michal Baniyash1

Abstract

Colorectal cancer is associated with chronic inflammation and immunosuppression mediated by myeloid-derived suppressor cells (MDSC). Although chemotherapy reduces tumor burden at early stages, it tends to have limited effect on a progressive disease, possibly due to adverse effects on the immune system in dictating disease outcome. Here, we show that patients with advanced colorectal cancer display enhanced MDSC levels and reduced CD247 expression and that some conventional colorectal cancer chemotherapy supports the immunosuppressive tumor microenvironment. A FOLFOX combined therapy reduced immunosuppression, whereas a FOLFIRI combined therapy enhanced immunosuppression. Mechanistic studies in a colorectal cancer mouse model revealed that FOLFIRI-like therapy including the drugs CPT11 and 5-fluorouracil (5FU) damaged host immunocompetence in a manner that limits treatment outcomes. CPT11 blocked MDSC apoptosis and myeloid cell differentiation, increasing MDSC immunosuppressive features and mouse mortality. In contrast, 5FU promoted immune recovery and tumor regression. Thus, CPT11 exhibited detrimental immunoregulatory effects that offset 5FU benefits when administered in combination. Our results highlight the importance of developing therapeutic regimens that can target both the immune system and tumor towards improved personalized treatments for colorectal cancer. Cancer Res; 74(21); 6022–35. ©2014 AACR.

Introduction

Colorectal cancer and certain other tumors are characterized by chronic inflammation–induced immunosuppression mediated by proinflammatory cells and mediators (1–4), which subvert the outcome of anticancer therapy. Myeloid-derived suppressor cells (MDSC) are the main cell population causing immnosuppression in numerous cancers including colorectal cancer (3, 5–8). MDSCs are immature myeloid cells expanded in the course of chronic inflammation, co-expressing Gr1+CD11b+ in mice and CD11b+CD14–CD33+, LIN−HLA-DR−CD33+, or CD14+CD11b+ in humans (6, 9).

Chemotherapeutic drugs commonly used to treat cancer, including colorectal cancer, affect not only the tumor but also the immune system, having a crucial impact on antitumor responses and disease outcome (5, 10). Although chemotherapies combat the tumors and lead to their regression, the effects on the tumor microenvironment and the immune system are not clearly understood. Colorectal cancer is usually treated with multiagent regimens, and in some cases, different drugs that act via diverse mechanisms are combined as they may have superior efficacy and effectiveness when administered jointly (11). The most common protocols for colorectal cancer approved by the FDA are combined chemotherapies FOLFIRI [folinic acid, 5-fluorouracil (5FU), and CPT11] or FOLFOX (folinic acid, 5FU, and oxaliplatin). Studies comparing between these regimens indicated that in some cases FOLFOX is superior as it leads to higher overall survival rates (12, 13). However, other studies have demonstrated equal efficacy for these treatments (14).

Even though chemotherapy for stage IV colorectal cancer leads to tumor regression, in most cases, the survival time is limited. We speculated that various drugs may differently alter the immune status of patients with colorectal cancer, thus affecting their therapeutic effectiveness. Monitoring the immune status of patients with stage IV colorectal cancer, before and following FOLFOX or FOLFIRI treatments, revealed that before therapy the patients displayed a suppressed immune status as indicated by the elevated MDSC levels and downregulated CD247, which is a key molecule that “senses” immune functionality and regulates T-cell and natural killer (NK) cell immune responses (15). During chemotherapeutic treatments, while FOLFOX reduced...
accumulation of circulating MDSCs that was accompanied by upregulated CD247 expression. FOLFIRI displayed opposite effects, enhancing the suppressive environment.

To gain better understanding of 5FU and CPT11 adverse effects on host immunity, we used a mouse colorectal cancer model that mimics the human disease (1). Herein we show that similar to the patients, colorectal cancer mice display an immunosuppressive status. In assessing CPT11 and 5FU monotherapies, we discovered that CPT11, but not 5FU, increases immunosuppression by inducing MDSC insensitivity to apoptosis, arresting their differentiation and retaining their suppressive features. Moreover, 5FU + CPT11 combined treatment displays harmful effects, resulting in a dysfunctional immune response associated with cancer progression and short survival, showing that CPT11 antagonizes the anticancer activity of 5FU by exerting its detrimental immunoregulatory effects. Our data suggest a significant impact of a given chemotherapeutic protocol on both the tumor and its immunosuppressive environment.

Patients and Methods

Patients

Peripheral blood samples were collected from 23 patients with stage IV metastatic colorectal cancer before and every 2 months in the course of chemotherapy treatments. All patients who were diagnosed with metastatic colorectal cancer underwent surgery and were not previously treated with chemotherapy. Twenty healthy donors were used as controls. The samples were taken according to the Helsinki approval and analyzed for the indicated immune biomarkers.

Mice

Female C57BL/6 and BALB/c mice (aged 6–8 weeks) were purchased from Harlan and were grown at the Hebrew University specific pathogen-free facility. All experiments were done in accordance with preapproved institutional protocols.

Reagents

Azoxymethane and dextran sulfate sodium (DSS) were purchased from Sigma-Aldrich and MP Biochemicals Inc., respectively.

In vivo mouse models for colorectal cancer and a tumor-free chronic inflammation

Mice were injected intraperitoneally with 10 mg/kg body weight of AOM dissolved in physiological saline twice in 2 weeks’ intervals. Two weeks later, 2% DSS was given in the drinking water over 7 days, following by 14 days of regular water (16). This cycle was repeated twice. Animals were sacrificed and analyzed 3 weeks after the last treatment.

To induce a pathology-free chronic inflammation, we used a previously described protocol subjecting mice to heat-killed Mycobacterium tuberculosis (BCG) treatment (17).

Chemotherapeutic drugs

In vivo efficacy of chemotherapeutic FDA-approved drugs on the immune status was determined under: (i) colorectal cancer conditions: a day after the second DSS administration, chemotherapy treatment was applied intraperitoneally twice a week in a 3-day interval for 3 weeks and (ii) chronic inflammatory tumor-free conditions: a day after the second BCG injection, chemotherapy treatment was applied intraperitoneally twice a week. The chemotherapies were scaled according to FDA-approved dosages: 5FU and CPT11 50 mg/kg each.

The chemotherapeutic drugs’ ex vivo effects were tested on MDSCs isolated from inflamed mice, using a magnetic column separation system (Miltenyi Biotec), as previously described (18). Two cycles of purifications, one with Gr1 antibodies (Biolegend) and a second with CD11b antibodies (Biolegend), were performed. The purity of the cell populations was more than 95%. After purification, cells were grown in tissue culture, treated for 24 hours with 5FU and CPT11 at 1.25, 2.5, and 5 μmol/L concentrations for cleaved caspase-3 detection assay and with 5FU, CPT11, and 5FU + CPT11 (2.5 μmol/L each drug) for nitric oxide (NO−) and reactive oxygen species (ROS) production assay.

In vivo depletion of MDSCs

For the MDSC depletion assay, at the same day of the second DSS administration, CPT11-treated colorectal cancer mice were administered intraperitoneally every 3 days with 0.5 mg of anti-Gr1 mAb (RB6-8C5).

Carboxyfluorescein diacetate succinimidyl ester staining and ex vivo T-cell proliferation assay

Splenocytes or purified T cells isolated by a magnetic column separation system (Miltenyi Biotec) were labeled with 5 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) and subjected to T-cell receptor–mediated activation as previously described (17).

Ex vivo myeloid cell differentiation

MDSCs were isolated from colorectal cancer and control (normal) mice and cultured in the presence or absence of 10 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF; PeproTech) for 3 days. In some samples, 5FU and CPT11 were added to the cells, with or without GM-CSF, followed by phenotyping using flow cytometry.

Flow cytometric analysis

Isolated mouse splenocytes and peripheral blood lymphocytes (PBL) were subjected to cell surface staining as previously described (17), using the following antibodies (Biolegend): fluorescein isothiocyanate (FITC)-labeled anti-Gr1 and anti-CD11c; phycocerythrin (PE)-labeled anti-F4/80, anti-CD3ε, and anti-mNKp46; and biotinylated anti-CD11b detected with streptavidin-Cy5. Intracellular staining for CD247 was performed as previously described (17) by using FITC-labeled...
anti-CD247 or biotinylated anti-CD247 (clone H146), the latter detected with streptavidin-Cy5. Foxxp3 staining was performed according to the manufacturer’s instructions (Miltenyi Biotec). Cleaved caspase-3 staining was performed using primary rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Asp175) and secondary FITC-horse anti-rabbit antibody (Thermo Scientific). For intracellular NO− and ROS detection, dianinofluorescein-2 diacetate (DAF-2DA) reagent (NOS 200-1; Cell Technology) and aminophenyl fluorescein (APF; 4011; Cell Technology) were used, respectively, and determined by flow cytometric analysis.

For human whole blood cell phenotyping, intracellular staining of CD247 cells was performed by first fixing the cells with 1% paraformaldehyde followed by washes and permeabilized with 0.1% saponin. Allophycocyanin-labeled anti-CD11b and anti-CD3, PE-labeled anti-CD33, FITC-labeled anti-HLA-DR, and anti-CD247 were used, all purchased from BD Pharmingen and used according to the manufacturer’s protocol. After surface staining, cells were treated eBioscience-Step Fix/Lyse solution according to the manufacturer’s instructions. All samples were analyzed using FACS Calibur with Cell Quest software (BD).

Cell isolation from the colon

The preparation of single-cell suspensions from colons was performed using a modified version of a previously described protocol (19). Briefly, isolated colons were washed with HBSS 5% FBS (Invitrogen), digested, minced, incubated for 15 minutes at 37°C, and epithelial cell suspension was washed with RPMI. For lamina propria cells, the retained tissue was transferred to collagenase/DNAse (Roche Diagnostic Corporation) solution, incubated for 1 hour at 37°C, filtrated, and washed with RPMI.

Quantitative PCR analysis

Total RNA was recovered from colon cells, splenocytes, or isolated MDSCs and subjected to real-time PCR analysis as previously described (17). The sequences of the oligonucleotides used are listed in Supplementary Table S1.

Western blot analysis

Cells isolated from the spleen or colon were analyzed by Western blotting for the expression of various proteins as previously described (17). The antibodies used for immunoblotting were: anti-S100A9, anti-S100A8, and anti-α-tubulin. Specific antibodies were detected by anti-rabbit or anti-goat antibodies conjugated to horseradish peroxidase (HRP; Jackson ImmunoResearch), followed by enhanced chemiluminescence and exposure at blotting reader (Bio-Rad software).

Histopathology and immunohistochemistry

Paraffin-embedded colon tissue sections were prepared from colorectal cancer, colorectal cancer–5FU, or colorectal cancer–CPT11–treated and control untreated mice and stained with hematoxylin and eosin solution. For immunohistochemistry, after antigen retrieval, sections were incubated at 4°C with primary antibodies: anti-β-catenin (BD) and anti-Gr-1 (Biolegend). For immunohistochemical staining, universal immunoperoxidase polymer for mouse tissues (414311F; Histofine) was used, based on an HRP-labeled polymer conjugated to anti-rat. After incubation for 30 minutes, slide staining was completed by 3- to 5-minute incubation with DAB + Chromogen (Lab Vision), followed by counterstaining with hematoxylin. As a control, samples were stained with each antibody and reagent individually.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.04. Averaged values are presented as the mean ± SEM. When comparing two groups, statistical significance was determined using 2-tailed Student t test. When more than two groups were investigated, an ANOVA was performed. Survival analyses were assessed using the Fisher exact test.

For the human experiments, paired t test was used to compare samples from the same patients before and after FOLFOX or FOLFIRI treatment. Control and colorectal cancer groups were investigated by ANOVA.

Results

FOLFOX and FOLFIRI therapies of patients with colorectal cancer display opposite effects on CD11b+/CD33+/HLA-DR+ myeloid cells and immune status

Progression of colorectal cancer involves the development of a chronic inflammatory and immunosuppressive milieu. We therefore proposed that some chemotherapies may have limited beneficial effects due to their harmful impact on the patients’ immune status. To verify this possibility, we first assessed the immune status of 23 patients with stage IV colorectal cancer before a given chemotherapy in comparison to 20 healthy donors. The percentage of CD11b+/CD33+/HLA-DR+ MDSCs in the patients’ peripheral blood was significantly higher (12.65% ± 1.35%, P < 0.01) than in healthy donors (5.35% ± 1.05%; Fig. 1A). Moreover, we found a significant increased production of both NO− and ROS in MDSCs, as compared with healthy donors (Fig. 1B), showing their immunosuppressive features in the blood of patients with colorectal cancer. We also found an inverse correlation between the percentage of circulating MDSCs and CD247 expression in the patients with colorectal cancer (Fig. 1C), suggesting an impaired immune status associated with the disease. To examine the impact of FOLFOX and FOLFIRI on immune parameters of patients with stage IV colorectal cancer, we followed the effects of these drugs on the kinetics of MDSC levels and the association with CD247 expression. All patients were treated with chemotherapy for at least 6 months. PBL analysis revealed decreased levels of circulating MDSCs following FOLFOX treatments (Fig. 1D, left), which were associated with a tendency of upregulated CD247 expression (Fig. 1D, right). In contrast, during the course of FOLFIRI treatment of patients with colorectal cancer, the MDSC percentage continuously increased, correlating with CD247 downregulation (Fig. 1E). These results suggest an adverse impact of the different chemotherapies on the immune status of patients with colorectal cancer.
CPT11 but not 5FU treatment increases MDSC accumulation at the tumor site and supports colorectal cancer growth

The harmful effects of FOLFIRI treatment on the immune status of patients with colorectal cancer and the reported beneficial effects of 5FU, which induces MDSC apoptosis and tumor regression in mice (20), suggest that CPT11 might antagonize the anticancer activity of 5FU by exerting its detrimental immunoregulatory effects. To further investigate whether indeed 5FU and CPT11 display an adverse effect on the immune system, we used a mouse-inducible colorectal cancer model based on AOM/DSS treatments (Fig. 2A). In this model, developing colorectal cancer is accompanied by chronic inflammation and immunosuppression, reminiscent of
spontaneous human colorectal cancer (21). Kinetic analysis of MDSC during colorectal cancer development revealed their gradual accumulation in the blood along with disease progression (Fig. 2B). When MDSC level became stable and adenomas were evident, mice were treated with either CPT11 or 5FU for 3 weeks. CPT11 monotherapy did not prevent tumorigenesis as no apparent differences in tumor loads within the colon were observed when compared with untreated colorectal cancer mice (Fig. 2C). This stood in sharp contrast to the dramatic effect of 5FU toward a decreased tumor load and recovery of colon architecture (Fig. 2C). Moreover, immunohistochemical analysis showed a massive β-catenin accumulation in the nuclei of tumor cells both in colons from untreated and CPT11-treated colorectal cancer mice (Supplementary Fig. S1), suggesting

Figure 2. Differential effects of 5FU and CPT11 monotherapies on the tumor and colon microenvironments in colorectal cancer mice. A, schematic representation of the mouse model for colorectal cancer. B, kinetic study of Gr1+CD11b+ (MDSC) accumulation. Blood samples were collected from mice during colorectal cancer development and progression at the indicated time points and tested for MDSC accumulation by flow cytometry. C, histopathology of colonic late neoplasms developed in AOM/DSS-treated mice was determined by hematoxylin and eosin staining. Original magnifications, ×200 and insets ×40. D, immunostaining for MDSCs in colons (top) and tumors (bottom). Original magnifications, ×200. E, MDSC accumulation within the colons was evaluated by flow cytometric analysis. Graphs represent the absolute number of MDSCs within the lamina propria (left) and the epithelium (right). F, the lamina propria (left) and epithelium (right) fractions isolated from the colons of colorectal cancer mice were analyzed for NO production by flow cytometric analysis gating on the MDSC population. Graphs represent production levels, as shown by mean fluorescence intensity (MFI). All in vivo experiments involved six mice per group and were repeated three times, yielding similar results. Graphs (means of triplicates ± SEM, n = 6) are representative of a typical experiment of three performed. *, P < 0.05; ***, P < 0.001; ns, nonsignificant.
tumor progression (16). Histologic analyses of colons from CPT11-treated colorectal cancer mice demonstrated not only a loss of the crypt and surface epithelial layer but also a massive leukocyte infiltration into the mucosa (Fig. 2C). Importantly, immunohistochemical probing of MDSCs within the colon revealed elevated levels in untreated and in CPT11-treated but not in 5FU-treated colorectal cancer mice (Fig. 2D, top). The same correlation between MDSC accumulation and the given treatment was also observed when testing tumors in colons (Fig. 2D, bottom). Analysis of cells generated from the colon lamina propria and epithelium depicted increased MDSC numbers in the CPT11 treated as compared with untreated colorectal cancer mice (Fig. 2E). A significantly reduced MDSC infiltration was observed in both the lamina propria and epithelium following 5FU treatment (Fig. 2E and Supplementary Fig. S2A). Furthermore, a significant reduction of NO production was found in lamina propria and epithelial MDSCs from 5FU-treated colorectal cancer mice (Fig. 2F), whereas after CPT11 treatment, the NO levels remained elevated as compared with the colorectal cancer untreated mice. These results strengthen the harmful effects of CPT11 supporting MDSC accumulation and suppressive features at the tumor site.

CPT11 treatment increases systemic immunosuppression and counteracts 5FU beneficial effects

We next investigated whether CPT11 and 5FU also differently affect the systemic immunosuppressive state. Untreated, CPT11-, and 5FU + CPT11-treated colorectal cancer mice display stronger inflammatory response as indicated by the enlarged spleen size as compared with 5FU-treated colorectal cancer or control mice (Fig. 3A) and by the significantly decreased MDSC numbers in spleens of 5FU-treated colorectal cancer mice as compared with those of the untreated colorectal cancer mice or those treated with CPT11 or 5FU + CPT11 (Fig. 3B). Interestingly, none of the monotherapies altered the high percentage of regulatory T cells (CD45+Foxp3+ Tregs) observed in colorectal cancer mice (Supplementary Fig. S3), showing that mainly MDSCs are affected by 5FU and CPT11.

Moreover, we found that while MDSCs from 5FU-treated colorectal cancer mice displayed a significantly reduced NO− and ROS production, MDSCs from CPT11- or 5FU + CPT11-treated colorectal cancer mice displayed elevated levels, as compared with untreated colorectal cancer mice (Fig. 3C). Ex vivo studies showed that 5FU administration does not alter NO− or ROS production in purified cultured MDSCs, but the addition of CPT11 or 5FU + CPT11 to the medium resulted in their elevation (Supplementary Fig. S4A), suggesting a direct effect of the drugs on MDSC suppressive features. Both monocytic (CD11b+Ly6C+Ly6G−) and granulocytic (CD11b+Ly6C−Ly6G+) cell populations showed increased NO− (Supplementary Fig. S4B, left) and ROS (Supplementary Fig. S4B, right) production upon CPT11 or 5FU + CPT11 treatment. However, the monocytic population displayed a more pronounced NO− production, whereas granulocytic population showed more ROS production (Supplementary Fig. S4A and S4B). Thus, CPT11 supports the immunosuppressive environment when applied alone or in a combination with 5FU, affecting the whole MDSC population.

We next evaluated the effect of the given chemotherapies on the immune status and tested the function of the whole T-cell population (Fig. 3D) and CD8+ T cells (Supplementary Fig. S4C) from all experimental groups along with the expression levels of CD247 in CD3+ (Fig. 3E) and in CD8+ T cells (Supplementary Fig. S4D). The results revealed a decreased T-cell proliferative ability following both CPT11 and 5FU + CPT11 treatments. In contrast, 5FU treatment of colorectal cancer mice did not affect T-cell proliferation, as compared with untreated colorectal cancer mice. Moreover, T-cell function was correlated with CD247 expression levels; low CD247 levels were obtained in T cells from colorectal cancer mice untreated and treated with CPT11 or a 5FU + CPT11 combination, whereas elevated levels were obtained upon 5FU treatment. Similar results were found in T cells isolated from the lamina propria and epithelium, inversely correlating with the local generated immunosuppressive environment (Supplementary Fig. S2B). Thus, CPT11 and 5FU adversely affect not only the tumor but also the immune system, pointing at the dominating harmful effects of CPT11 when combined with 5FU.

CPT11 harmful effects on the host’s immune function are mediated via MDSCs

The observed MDSC elevation and increased tumor load in CPT11-treated colorectal cancer mice (Figs. 2–4) suggest an impact of CPT11 on MDSC-induced cancer progression. We hypothesized that MDSC depletion could reduce CPT11 harmful effect on the immune status, thus enhancing the antitumor effect. Indeed, in vivo MDSC depletion in CPT11-treated colorectal cancer mice (Supplementary Fig. S5) as indicated by the negligible MDSC levels as compared with CPT11 and 5FU + CPT11-treated colorectal cancer mice (Fig. 4A) led to an almost complete regression of the tumors (Fig. 4B). Moreover, histopathologic analysis revealed a differentiated adenocarcinoma in the colon of colorectal cancer mice, CPT11-, and 5FU + CPT11–treated colorectal cancer mice, whereas only few aberrant crypt foci and tumors were detected in colons of 5FU-treated colorectal cancer mice and CPT11-treated MDSC-depleted colorectal cancer mice (Fig. 4C). This pattern indicates a beneficial effect of 5FU that attenuates colorectal cancer progression with a harmful contribution of CPT11, supporting immunosuppression and tumor progression via its effects on MDSCs. The daily recorded vitality and survival of the mice show a rapid deterioration, with increased death rates in colorectal cancer mice treated with CPT11 or 5FU + CPT11, as compared with the 5FU, or CPT11-treated MDSC-depleted colorectal cancer mice, or even to untreated colorectal cancer mice (Fig. 4D).

MDSCs are insensitive to apoptosis under CPT11 treatment but become susceptible after 5FU treatment

We next aimed to explore the mechanisms responsible for the opposite effects of 5FU and CPT11 on MDSC accumulation. These drugs could differently affect MDSC levels by changing their sensitivity to apoptosis, as previously reported for 5FU.
(20). Indeed, a significant increased cleaved caspase-3 expression, an indicator for apoptosis, was observed within splenic MDSCs from 5FU-treated colorectal cancer mice, similar to that detected in MDSCs from control mice (Fig. 5A). In contrast, following CPT11 or 5FU + CPT11 treatment, MDSCs displayed decreased cleaved caspase-3 levels, as in splenic MDSCs of untreated colorectal cancer mice (Fig. 5A). Moreover, ex vivo studies revealed that 5FU but not CPT11 leads to cleaved caspase-3 upregulation in purified cultured MDSCs in a dose-dependent manner (Fig. 5B and C). Interestingly, 5FU-induced apoptosis was evident only in nondifferentiated MDSCs (Fig. 5D) whereas dendritic cells (Fig. 5E) and macrophages (Fig. 5F) were insensitive, suggesting an exclusive effect of 5FU on the immature myeloid cell population. We also found that 5FU controls both monocytic and granulocytic purified cultured MDSC populations, with the monocytic population being more sensitive, as indicated by the enhanced cleaved caspase-3 expression upon 5FU addition or when combined with CPT11 (Supplementary Fig. S6A). We also show that the drugs did not affect the apoptotic state of other immune cells as T (CD3+ and B (B220+) lymphocytes (Supplementary Fig. S6B). These results underscore the direct apoptotic effects of 5FU on immature MDSCs as opposed to the apoptosis insensitivity of MDSCs to the CPT11 or CPT11 + 5FU treatments.

**5FU and CPT11 directly affect myeloid cell maturation and suppressive activity.**

To investigated whether 5FU and CPT11 also affect MDSC maturation, we first assessed expression of S100A8/9...
Proinflammatory proteins, which are induced in the course of tumorigenesis and chronic inflammation and playing a role in controlling MDSC accumulation and retention in their immature suppressive state (7, 17, 22). While 5FU treatment of colorectal cancer mice induced a significant decrease in S100A8/9 mRNA and protein levels in the spleen as compared with control mice, increased S100A8/9 levels were observed following CPT11 treatment (Fig. 6A and B). Similar results were obtained when assessing the colon (Supplementary Fig. S7A), suggesting that 5FU supports MDSC transition from an

Figure 4. CPT11 harmful effects supporting tumor progression are mediated via MDSCs. Colorectal cancer mice were subjected to 5FU, CPT11, or a 5FU + CPT11 combination starting at week 8 or left untreated. At the same day of the second DSS administration, CPT11-treated colorectal cancer mice were randomly separated into two groups; one group continued with CPT11 treatment, whereas the second group was treated with anti-Gr1 mAb for MDSC depletion in addition to CPT11 treatment. Three weeks after the second DSS treatment, mice were sacrificed and PBLs (A) and colons (B and C) were analyzed. A, MDSC accumulation was measured in PBLs by flow cytometric analysis. Graph represents the percentage of MDSCs in each experimental group. Representative colon structures are presented in B, and histopathology analyses of colons in colorectal cancer mice were determined by hematoxylin and eosin staining in C. Original presented magnification, ×100. D, Kaplan–Meier curve (n = 20) of colorectal cancer, 5FU-treated, CPT11-treated, 5FU + CPT11–treated, or MDSC-depleted CPT11–treated colorectal cancer mice. All in vivo experiments involved six mice per group and were repeated three times, yielding similar results. Graphs (means of triplicates ± SEM, n = 6) are representative of a typical experiment of three performed. **, P < 0.01; ***, P < 0.001.
immature suppressive stage toward differentiated nonsuppressive myeloid phenotype (17). Indeed, 5FU treatment of colorectal cancer mice resulted in a significant shift toward differentiated dendritic cells and macrophages (Fig. 6C) and to matured antigen-presenting cells, shown by the induced CD80 and MHCII expression (Fig. 6D). In contrast, CPT11 treatment blocked myeloid cell differentiation in vivo (Fig. 6C and D).

When testing the ex vivo direct effects of the drugs on GM-CSF–mediated colorectal cancer–derived MDSC differentiation, we detected that, similar to the in vivo effects, CPT11 prevented cell differentiation after both 48 hours (data not shown) and 72 hours (Fig. 6E) as compared with MDSCs incubated with GM-CSF only. In contrast, 5FU enabled MDSC differentiation to dendritic cells and macrophages, shown by the induced CD80 and MHCII expression (Fig. 6D). Interestingly, ex vivo CPT11-mediated MDSC differentiation blockade was associated with increased mRNA levels of the proinflammatory mediators TNFα (Supplementary Fig. S7B) S100A9 (Supplementary Fig. S7C), whereas the 5FU-induced MDSC differentiation correlated with decreased levels of these factors. Thus, 5FU directly affects the differentiation pathway of MDSCs, whereas CPT11 exerts a differentiation blockade capacity when added to GM-CSF–treated MDSCs.

5FU and CPT11 opposing effects on MDSCs are tumor-independent

To examine whether the immunoregulatory effects of 5FU and CPT11 are tumor-dependent, we used a mouse model for chronic inflammation and associated immunosuppression (15), described in the Materials and Methods and Fig. 7A. The 5FU beneficial and CPT11 harmful effects were similar to those observed in the colorectal cancer model. 5FU significantly reduced MDSC levels (Fig. 7B and C and Supplementary Fig. S8A) and NO− and ROS production (Fig. 7D). Treatment with 5FU also elevated cleaved caspase-3 levels (Fig. 7E), as compared with inflamed-untreated mice. In contrast, CPT11 or 5FU + CPT11 treatments induced opposite effects (Fig. 7B–E).

No changes in CD4+ Foxp3+ Tregs percentage were detected (Supplementary Fig. S8B), confirming that these chemotherapies specifically affect MDSCs.

We next assessed whether the 5FU and CPT11 opposite effects on MDSCs have different impacts on the host’s immune competence. Assessment of the drugs’ effects on total T-cell

Figure 5. 5FU and CPT11 directly and differently affect MDSC sensitivity to apoptosis. A, splenic MDSCs from each group were analyzed for the expression of activated (cleaved) caspase-3 by flow cytometric analysis, gating on MDSCs. To assess the direct effect of 5FU (B) and CPT11 (C) on cleaved caspase-3 expression, primary MDSCs isolated from spleens of colorectal cancer mice (n = 6) were ex vivo incubated with various doses of the drugs for 3 days and subjected to flow cytometric analysis. To assess which cells are affected by the chemotherapeutic drugs, MDSCs isolated from spleens of colorectal cancer mice were cultured ex vivo with 10 ng/mL of GM-CSF in the absence or presence of scaled doses (0, 1.25, 2.5, and 5 µmol/L) of 5FU or CPT11 for 3 days. Cleaved caspase-3 levels were then evaluated on the primary MDSCs (D), differentiated CD11c+CD11b+ dendritic cells (E), and F4/80+CD11b+ macrophages (F). All experiments involved six mice per group and were repeated three times, yielding similar results. Graphs (means of triplicates ± SEM, n = 6) are representative of a typical experiment of three performed. *, P < 0.05; **, P < 0.001; ns, nonsignificant.
Figure 6. 5FU and CPT11 directly affect myeloid cell differentiation, maturation, and suppressive activity. S100A8/9 mRNA (A) and protein (B) levels were evaluated in MDSCs isolated from the spleen of colorectal cancer mice or colorectal cancer mice treated with 5FU or CPT11 (n = 4). a-Tubulin levels served as a control. C and D, the differentiation and maturation of myeloid cells within the spleens of the different experimental groups were evaluated by testing the levels of CD11c+CD11b+ dendritic cells and F4/80+CD11b+ macrophages (C), and the CD80 and MHCII expression (D), respectively. E and F, MDSCs isolated from spleens of colorectal cancer mice were ex vivo cultured with 10 ng/mL GM-CSF in the absence or presence of scaled doses (0, 1.25, 2.5, 5, and 10 μmol/L) of CPT11 (E) or 5FU (F) for 3 days. The phenotype of differentiated dendritic cells (left) and macrophages (right) was then evaluated. All in vivo experiments involved six mice per group and were repeated three times, yielding similar results. Graphs (means of triplicates ± SEM, n = 6) are representative of a typical experiment of three performed. Ex vivo experiments involved four mice per group and were repeated three times, yielding similar results. Graphs (means of triplicates ± SEM, n = 4) are representative of a typical experiment of three performed. Data shown are mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (2-way ANOVA). ns, nonsignificant.
Figure 7. 5FU and CPT11 opposite effects on the chronic inflammatory environment are tumor independent. A, mouse model for chronic inflammation was established by three repeated injections of heat-killed BCG bacteria. A day after the second BCG injection, mice were treated twice a week with 5FU, CPT11, or a 5FU + CPT11 combination. PBls (B) and spleens (C) from normal, inflamed, inflamed 5FU-treated, CPT11-treated, or 5FU + CPT11–treated mice were analyzed for MDSC accumulation by flow cytometric analysis. Representative dot plots of MDSCs (C, left) and the percentage within the spleen (C, right) and PBls (B) are shown. D, splenocytes were analyzed for NO (left) and ROS (right) production by flow cytometric analysis, gating on the MDSC population. Graphs represent production levels as shown by mean fluorescence intensity (MFI). E, the expression of cleaved caspase-3 was analyzed by flow cytometry, gating on MDSC populations. F, splenocytes were labeled with CFSE and activated with anti-CD3 and anti-CD28 antibodies or left nonactivated. The proliferative response was assessed by monitoring cell divisions of gated CFSE-labeled Thy1.2+ (CD90+) T cells. Representative histograms of proliferative activity are shown (left), and the percentage of proliferating cells was calculated and compared with steady-state levels of nonactivated cells in each group (right). G, NK cell–mediated clearance of CFSE-labeled allogeneic (CFSElow) and syngeneic (CFSEhigh) splenocytes was evaluated by monitoring the ratio between CFSElow/CFSEhigh in the spleen (top) and PBls (bottom) in each experimental group. Graphs (means of triplicates ± SEM, n = 5) are representative of a typical experiment of three independent performed. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, nonsignificant.

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activity and specifically CD8+ T cells revealed a significant recovery of CD247 expression in the spleen and PBLs as well as T-cell proliferation following 5FU but not CPT11 or 5FU + CPT11 treatments (Fig. 7F and Supplementary Fig. S8C and S8D). Similar effects were also detected when analyzing NK cells; downregulated CD247 expression detected in NK cells from chronically inflamed mice was almost completely recovered upon 5FU but not CPT11 treatment (Supplementary Fig. S8E). Because NK cell activity is mediated via natural cytotoxicity receptors (NCR), which associate with and depend on CD247, we assessed NK cell in vivo function by monitoring the clearance of adoptively transferred allogeneic cells. A complete recovery of NK cell activity both in the spleen and PBLs was detected following 5FU treatment (Fig. 7G), along with a significant decreased clearance of allogeneic cells after CPT11 or 5FU + CPT11 treatments. These results indicate that 5FU and CPT11 opposite effects on MDSCs are tumor-independent, displaying a significant impact on effector T-cell and NK cell responsiveness under chronic inflammatory conditions.

Discussion

Colorectal cancer appears in most cases as adenocarcinoma that develops from the lining of the large intestine (colon) and rectum and is supported and progressed by chronic intestinal inflammation as in patients with inflammatory bowel disease (23–25). Despite the clinical progression in detection and treatment, colorectal cancer remains one of the major causes of cancer-related death. In inflammation-driven tumors, an immunosuppressive microenvironment, which is characterized by MDSC accumulation within the tumor and periphery (1, 8, 17, 26, 27), poses a serious obstacle in cancer chemotherapy, attenuating the capacity of conventional drugs to evoke a robust antitumor immunity.

In the present study, we highlight novel mechanisms underlying the action of the commonly used 5FU and CPT11 chemotherapies, showing their effect not only on the tumor but also on its immunosuppressive environment. Our initial studies with patients with stage IV colorectal cancer show that before treatment the patients displayed an immunosuppressive status indicated by elevated MDSC levels and the downregulated CD247 expression, which is critical for T-cell and NK cell activities. Moreover, we demonstrate that while FOLFOX treatment of patients with colorectal cancer led to a decrease in MDSC levels and a gradual upregulation of CD247 expression, FOLFIRI had opposite and harmful effects. Such adverse effects between drugs could have a significant impact on the overall antitumor response and disease outcome. Our results were obtained using a limited number of patients as an initial proof of concept. Randomized clinical studies using larger patient cohort should be performed comparing these therapies to validate our initial observations.

Recent data demonstrated that 5FU treatment leads to a selective MDSC apoptosis and tumor regression in mice (20). Hence, the harmful effects of FOLFIRI on the immune status of patients with colorectal cancer suggested that CPT11 might have detrimental immunoregulatory effects that offset the anticancerous impact of 5FU when given together. This was confirmed by testing the effects of 5FU and CPT11 on a colorectal cancer mouse model, showing that colorectal cancer mice display immunosuppressive features, similar to patients with colorectal cancer manifested by elevated MDSC levels, impaired T-cell function associated with downregulated CD247 expression. A comparison between the effects of 5FU and CPT11 mono or combined 5FU + CPT11 therapies revealed that the overall effect of the CPT11 in both therapies was harmful; yielding a strong immunosuppression mediated via MDSCs and associated with a rapid disease progression and decreased survival as compared with the beneficial effects of 5FU alone. These results suggest that CPT11 reinforces the immunosuppressive environment in colorectal cancer mice and patients and dominates the beneficial effects of 5FU in the FOLFIRI regimen, thus leading to an overall harmful effect.

Detailed analysis of the mechanisms underlying CPT11 and 5FU adverse effects and the affected pathways revealed that in colorectal cancer mice, 5FU reduced MDSC levels, both by inducing their apoptotic death and by enforcing myeloid cell differentiation to mature macrophages and dendritic cells. The former drug effect is associated with elevated levels of cleaved caspase-3 and the latter involves a decreased expression of the proinflammatory S100A8/9 proteins, known to induce MDSC differentiation arrest (17, 22, 28). In contrast, treatment with CPT11 had opposite effects on colorectal cancer–associated MDSCs. CPT11 also directly affects MDSCs by boosting secretion of proinflammatory cytokines such as TNFα, which is a key MDSC regulator, inducing their differentiation arrest via S100A8/9 and increasing their suppressive activity (17). Thus, in untreated and CPT11-treated colorectal cancer mice, but not in 5FU-treated colorectal cancer mice, multiple changes occur in the tumor micro- and macroenvironments that directly enhance tumor growth and support indirectly its progression by inhibiting antitumor immunity.

Immunosuppressive MDSCs that accumulate during colorectal cancer and upon treatment with CPT11 or a 5FU + CPT11 combination produce elevated levels of NO− and ROS that could induce DNA damage and formation of colonic adenomas (29). In addition, S100A8/9 produced upon CPT11 treatment could interact with receptors for advanced-glycation end products (RAGE) and carbonylated glycans expressed on colorectal cancer cells, thereby promoting activation of MAPK and NF-κB signaling pathways. These, in turn, upregulate a cohort of proteins that promote leukocyte recruitment, angiogenesis, tumor migration, and establishment of premetastatic niches in distal organs (28). Moreover, the generated proinflammatory environment can affect Wnt/β-catenin signaling as reflected herein by nuclear accumulation of β-catenin in tumors of untreated and CPT11-treated colorectal cancer mice. The attenuated immunosuppression during 5FU treatment improves the therapeutic outcome as it enables the generation of antitumor immunity and superior tumor regression.

Our findings regarding the immunosuppression relief by 5FU are corroborated by a recent report demonstrating that...
5FU does not induce ROS activation in MDSCs. In this study, however, the authors also demonstrated that 5FU induces a direct activation of the NLRP3 inflammasome through the release of cathepsin B from lysosomes, leading to secretion of IL1β, production of IL17, and tumor growth. NLRP3 activation in the tumor microenvironment could diminish antigen immunity by facilitating migration of MDSCs to the tumor site (30). Hence, multiple regulatory pathways of MDSC function are targeted by 5FU and the drug susceptibility of both the tumor and its environment will dictate the therapy outcome; 5FU could accelerate IL1β secretion and inflammasome activation (5) as well as attenuate Treg activity and promote autoreactive T-cell expansion (31). Herein, we show that even though Tregs are elevated in both inflamed and colorectal cancer mice, upon 5FU treatment, MDSCs and their suppressive activity are significantly decreased. These effects were sufficient to improve T-cell and NK cell antitumor activities, indicating that in the absence of MDSCs, other factors do not necessarily worsen host immune function.

Taken together, we demonstrate that conventional measurements of chemotherapeutic effects on the tumor are insufficient to evaluate their curative effectiveness. Rather, it is mandatory to assess the drug effects on the immune function as well, as the combined impact on both the tumor and immune system will dictate the disease outcome. We wish to stress the significance of MDSC-mediated immunosuppressive environment and its sensitivity to chemotherapy in determining the most appropriate therapeutic regimen. While our study alludes to patients with advanced disease, it may bear even greater relevance to patients with colorectal cancer at earlier stages when monitored before and following treatment. We also suggest that by monitoring the host’s immune function using unique biomarkers as CD247 and MDSCs, the efficacy of a given treatment could be evaluated and modified accordingly if required. Once MDSC-mediated immunosuppression is detected, modalities leading to MDSC elimination, differentiation, or neutralization should be considered as auxiliary therapy (32) in addition to the antitumor chemotherapy or to the currently developing anti-colorectal cancer immune-based treatments as specific tumor-infiltrating lymphocytes or anti-CTLA4 and/or anti-PD1 antibodies (33, 34) that will necessitate a functional immune system to gain efficient treatment efficacies. Thus, a selection of appropriate antitumor combined therapy should lead to an improved design of future cancer personalized treatments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J. Kanterman, M. Sade-Feldman, M. Biton, E. Ish-Shalom, M. Baniyash
Development of methodology: J. Kanterman, M. Sade-Feldman
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Kanterman, M. Sade-Feldman, A. Hubert
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): J. Kanterman, M. Sade-Feldman, M. Biton, E. Ish-Shalom, A. Goldstein, A. Laery
Writing, review, and/or revision of the manuscript: J. Kanterman, M. Sade-Feldman, M. Biton, M. Baniyash

Study supervision: M. Baniyash

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References

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Julia Kanterman, Moshe Sade-Feldman, Moshe Biton, et al.


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