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Adverse immunoregulatory effects of 5FU and CPT11 chemotherapy on myeloid-derived suppressor cells and colorectal cancer outcomes

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Running title: Adverse effects of chemotherapies on MDSCs in CRC

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Abstract

Colorectal cancer (CRC) 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 advanced CRC patients display enhanced MDSC levels, reduced CD247 expression and that some conventional CRC chemotherapy supports the immunosuppressive tumor microenvironment. A FOLFOX combined therapy reduced immunosuppression whereas a FOLFIRI combined therapy enhanced immunosuppression. Mechanistic studies in a CRC mouse model revealed that FOLFIRI-like therapy including the drugs CPT11 and 5FU damaged host immune competence 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 in developing improved personalized treatments for CRC.
Introduction

CRC and certain other tumors are characterized by chronic inflammation-induced immunosuppression mediated by pro-inflammatory cells and mediators (1-4), which subvert the outcome of anti-cancer therapy. MDSCs are the main cell population causing immunosuppression in numerous cancers including CRC (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 CRC, affect not only the tumor but also the immune system, having a crucial impact on anti-tumor 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. CRC is usually treated with multi agent 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 CRC approved by the FDA, are combined chemotherapies FOLFIRI (folinic acid, 5FU and CPT11) or FOLFOX (folinic acid, 5FU and oxaliplatin). Studies comparing between these regimens indicated that in some cases FOLFOX is superior since 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 CRC leads to tumor regression, in most cases the survival time is limited. We speculated that various drugs may differently alter the immune status of CRC patients, thus affecting their therapeutic effectiveness. Monitoring the immune status of stage IV CRC patients, prior to and following FOLFOX or FOLFIRI treatments revealed that prior to therapy the patients displayed a suppressed immune status as indicated by the elevated MDSC levels and down-regulated CD247, which is a key molecule that “senses” immune functionality and regulates T- and NK-cell immune responses (15). During chemotherapeutic treatments, while FOLFOX reduced accumulation of circulating MDSCs that was accompanied by up-regulated 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 CRC model that mimics the human disease (1). Herein we show that similar to the patients, CRC-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 anti-cancer 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.

Materials and Methods

Patients

Peripheral blood samples were collected from 23 stage IV metastatic CRC-patients prior to and every 2 months in the course of chemotherapy treatments. All patients that were diagnosed with metastatic CRC, underwent surgery and were not previously treated with chemotherapy. 20 healthy donors were used as controls. The samples were taken according to the Helsinki approval and analyzed for the indicated immune biomarkers in a "blinded test", not knowing the therapy specification. Following analyses completion, the specific treatment regiments and clinical parameters were acquired from medical records of the patients under the care of Dr. Ayala Hubert at the Oncology department, Hadassah University Medical Center Ein Kerem, Jerusalem, and correlation tests were performed.

Mice

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

Reagents

Azoxymethan (AOM) and dextran sulfate sodium (DSS) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and MP biochemicals Inc. (Santa Ana, CA, USA), respectively.

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

Mice were injected intraperitoneally with 10mg/kg body weight of AOM dissolved in physiological saline twice in two weeks intervals. Two weeks later, 2% DSS was given in the drinking water over 7 days, following by 14 days of regular water (21). This cycle was repeated twice. Animals were sacrificed and analyzed three 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 (16).

**Chemotherapeutic drugs**

*In vivo* efficacy of chemotherapeutic FDA-approved drugs on the immune status was determined under: 1) CRC 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 2) 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 50mg/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 (17). 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 >95%. After purification, cells were grown in tissue culture, treated for 24h with 5FU and CPT11 at 1.25μM, 2.5μM and 5μM concentrations for cleaved caspase-3 detection assay and with 5FU, CPT11 and 5FU+CPT11 (2.5μM 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 CRC-mice were administerd intraperitoneally (i.p.) every 3 days with 0.5 mg of anti-Gr1 mAb (RB6-8C5).

**CFSE 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 5μM CFSE (Invitrogen) and subjected to TCR-mediated activation as previously described (16).

**Ex vivo myeloid-cell differentiation**

MDSCs were isolated from CRC and control (normal) mice and cultured in the presence or absence of 10ng/ml 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 cytometry analysis

Isolated mouse splenocytes and peripheral blood lymphocytes (PBLs) were subjected to cell surface staining as previously described (16), using the following antibodies (Biolegend): FITC-labeled anti-Gr1, and anti-CD11c; 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 (16) by using FITC-labeled anti-CD247 or biotinylated anti-CD247 (Ione H146), the latter detected with streptavidin-Cy5. Foxp3 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, diaminofluorescein-2 diacetate (DAF-2DA) reagent (NOS 200-1; Cell Technology) and aminophenyl fluorescein (APF) (4011; Cell Technology) were used respectively and determined by flow cytometry analysis.

For human whole blood cell phenotyping, intracellular staining of CD247 cells was performed by first fixing the cells with paraformaldehyde 1% followed by washes and permeabilized with 0.1% saponin. APC-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 (18). Briefly, isolated colons were washed with HBSS 5%FBS (Invitrogen), digested, minced, incubated for 15 min 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 h 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 (16). The sequences of the oligonucleotides used are listed in Supplementary Table 1.
Western blot analysis

Cells isolated from the spleen or colon were analyzed by Western blotting for the expression of various proteins as previously described (16). The antibodies used for immunoblotting were: anti-S100A9, anti-S100A8, and anti-αTubulin. Specific antibodies were detected by anti-rabbit or anti-goat antibodies conjugated to horseradish peroxidase (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 CRC, CRC-5FU or CRC-CPT11 treated and control-untreated mice and stained with hemotoxylin 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 immuno-peroxidase polymer for mouse tissues (414311F; Histofine) was used, based on a horseradish peroxidase (HRP)-labeled polymer conjugated to anti-Rat. After incubation for 30 min, slide staining was completed by 3-5 min 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 ± s.e.m. When comparing two groups, statistical significance was determined using two-tailed Student's t-test. When more than two groups were investigated, an analysis of variance (ANOVA) was performed. Survival analyses were assessed using Fisher's 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 CRC groups were investigated by ANOVA.
Results

FOLFOX and FOLFIRI therapies of CRC-patients display opposite effects on CD11b⁺CD33⁺HLA-DR⁻ myeloid cells and immune status

Progression of CRC 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 stage IV CRC patients prior to 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%, \( p < 0.01 \)) as compared to healthy donors (5.35%±1.05%) (Fig. 1A). Moreover we found a significant increased production of both NO⁻ and ROS in MDSCs, as compared to healthy donors (Fig. 1B), showing their immunosuppressive features in the blood of CRC patients. We also found an inverse correlation between the percentage of circulating MDSCs and CD247 expression in the CRC-patients (Fig. 1C), suggesting an impaired immune status associated with the disease. To examine the impact of FOLFOX and FOLFIRI on immune parameters of stage IV CRC patients, 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 six month. PBL analysis revealed decreased levels of circulating MDSCs following FOLFOX treatments (Fig. 1D, left panel), which were associated with a tendency of up-regulated CD247 expression (Fig. 1D, right panel). By contrast, during the course of FOLFIRI treatment of CRC patients the MDSC percentage continuously increased, correlating with CD247 down-regulation (Fig. 1E). These results suggest an adverse impact of the different chemotherapies on the CRC-patients’ immune status.

CPT11 but not 5FU treatment increases MDSC accumulation at the tumor site and supports CRC growth

The harmful effects of FOLFIRI treatment on the immune status of CRC patients and the reported beneficial effects of 5FU, which induces MDSC apoptosis and tumor regression in mice (19), suggest that CPT11 might antagonize the anti-cancer 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 CRC model based on AOM-DSS treatments (Fig. 2A). In this model, developing CRC is accompanied by chronic inflammation and immunosuppression, reminiscent of spontaneous human CRC (20).
Kinetic analysis of MDSC during CRC 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 three weeks. CPT11 monotherapy did not prevent tumorigenesis as no apparent differences in tumor loads within the colon were observed when compared to untreated CRC-mice (Fig. 2C). This stood in sharp contrast to the dramatic effect of 5FU towards 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 CRC-mice (Supplementary Fig. S1), suggesting tumor progression (21). Histological analyses of colons from CPT11 treated CRC-mice demonstrated not only a loss of entire crypts 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 CRC-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 (LP) and epithelium, depicted increased MDSC numbers in the CPT11 treated as compared to untreated CRC-mice (Fig. 2E). A significantly reduced MDSC infiltration was observed in both the LP and epithelium following 5FU treatment (Fig. 2E and Supplementary Fig. S2A). Furthermore, a significant reduction of NO− production was found in LP and epithelial MDSCs from 5FU treated CRC-mice (Fig. 2F), whereas after CPT11 treatment the NO− levels remained elevated as compared to the CRC untreated mice. These results strengthen the CPT11 harmful effects 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 CRC-mice display stronger inflammatory response as indicated by the enlarged spleen size as compared to 5FU treated CRC- or control-mice (Fig. 3A), and by the significantly decreased MDSC numbers in spleens of 5FU treated CRC-mice as compared to those of the untreated CRC-mice or those treated with CPT11 or 5FU/CPT11 (Fig. 3B). Interestingly, none of the monotherapies altered the high percentage of regulatory T-cells (CD4+Foxp3+Tregs) observed in CRC-mice (Supplementary Fig. S3), showing that mainly MDSCs are affected by 5FU and CPT11.
Moreover, we found that while MDSCs from 5FU treated CRC-mice displayed a significantly reduced NO\textsuperscript{−} and ROS production, MDSCs from CPT11 or 5FU/CPT11 treated CRC-mice displayed elevated levels, as compared to untreated CRC-mice (Fig. 3C). Ex\_vivo studies showed that 5FU administration does not alter NO\textsuperscript{−} 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\textsuperscript{+} Ly6C\textsuperscript{high} Ly6G\textsuperscript{−}) and granulocyte (CD11b\textsuperscript{+} Ly6C\textsuperscript{low} Ly6G\textsuperscript{+}) cell populations showed increased NO\textsuperscript{−} (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\textsuperscript{−} production, while granulocytic population showed more ROS production (Supplementary Fig. S4A and B). 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\textsuperscript{+} T-cells (Supplementary Fig. S4C) from all experimental groups along with the expression levels of CD247 in CD3\textsuperscript{+} (Fig. 3E) and in CD8\textsuperscript{+} 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 CRC-mice did not affect T-cell proliferation, as compared to untreated CRC-mice. Moreover, T-cell function was correlated with CD247 expression levels; low CD247 levels were obtained in T-cells from CRC-mice untreated and treated with CPT11 or a 5FU/CPT11 combination, while elevated levels were obtained upon 5FU treatment. Similar results were found in T-cells isolated from the LP 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 CRC-mice (Fig. 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 anti-tumor effect. Indeed, in\_vivo MDSC depletion in CPT11 treated CRC-mice (Supplementary Fig. S5) as indicated by the negligible MDSC levels as compared to CPT11 and 5FU/CPT11 treated CRC-mice (Fig. 4A), led to an almost complete regression of the tumors (Fig. 4B).
Moreover, histopathologic analysis revealed a differentiated adenocarcinoma in the colons of CRC-mice, CPT11 and 5FU/CPT11 treated CRC-mice, while only few aberrant crypt foci and tumors were detected in colons of 5FU treated CRC-mice and CPT11 treated MDSC depleted CRC-mice (Fig. 4C). This pattern indicates a beneficial effect of 5FU that attenuates CRC 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 CRC-mice treated with CPT11 or 5FU/CPT11, as compared to the 5FU, or CPT11 treated MDSC depleted CRC-mice, or even to untreated CRC-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 (19). Indeed, a significant increased cleaved caspase-3 expression, an indicator for apoptosis, was observed within splenic MDSCs from 5FU treated CRC-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 CRC-mice (Fig. 5A). Moreover, ex vivo studies revealed that 5FU but not CPT11 leads to cleaved caspase-3 up-regulation in purified cultured MDSCs in a dose-dependent manner (Fig. 5B and C). Interestingly, 5FU-induced apoptosis was evident only in non-differentiated MDSCs (Fig. 5D) while DCs (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 pro-inflammatory 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, 16, 22). While 5FU treatment of CRC-mice induced a significant decrease in S100A8/9 mRNA and protein levels in the spleen as compared to 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 immature suppressive stage towards differentiated non-suppressive myeloid phenotype (16). Indeed, 5FU treatment of CRC-mice resulted in a significant shift towards differentiated DCs and macrophages (Fig. 6C) and to matured antigen presenting cells (APCs), 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 CRC-derived MDSC differentiation, we detected that, similar to the in vivo effects, CPT11 prevented cell differentiation after both 48h (data not shown) and 72h (Fig. 6E) as compared to MDSCs incubated with GM-CSF only. In contrast, 5FU enabled MDSC differentiation to DCs and macrophages (Fig. 6F). Interestingly, ex vivo CPT11-mediated MDSC differentiation blockade was associated with increased mRNA levels of the pro-inflammatory mediators TNFα (Supplementary Fig. S7B) S100A9 (Supplementary Fig. S7C), while the 5FU-induced MDSC differentiation correlated with decreased levels of these factors. Thus, 5FU directly affect the differentiation pathway of MDSCs whereas CPT11 exhibits 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 method section and (Fig. 7A). The 5FU beneficial and CPT11 harmful effects were similar to those observed in the CRC model. 5FU significantly reduced MDSC levels (Fig. 7B, 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 to 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 (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 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 D). Similar effects were also detected when analyzing NK-cells; down-regulated CD247 expression detected in NK-cells from chronically inflamed mice was almost completely recovered upon 5FU but not CPT11 treatment (Supplementary Fig. S8E). Since NK-cell activity is mediated via natural cytotoxicity receptors (NCRs), 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- and NK-cell responsiveness under chronic inflammatory conditions.

Discussion

CRC 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 progress in detection and treatment, CRC 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, 16, 26, 27), poses a serious obstacle in cancer chemotherapy, attenuating the capacity of conventional drugs to evoke a robust anti-tumor 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 stage IV CRC patients show that prior to treatment the patients displayed an immunosuppressive status indicated by elevated MDSC levels and the down-regulated CD247 expression, which is critical for T- and NK-cell activities. Moreover, we demonstrate that while FOLFOX treatment of CRC patients led to a decrease in MDSC levels and a gradual up-regulation of CD247 expression, FOLFIRI had opposite and harmful effects. Such adverse effects between drugs could have a significant impact on the overall anti-tumor 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 (19). Hence, the harmful effects of FOLFIRI on the immune status of CRC patients, suggested that CPT11 might have detrimental immunoregulatory effects that offset the anti-cancerous impact of 5FU when given together. This was confirmed by testing the effects of 5FU and CPT11 on a CRC mouse model, showing that CRC-mice display immunosuppressive features, similar to CRC patients manifested by elevated MDSC levels, impaired T-cell function associated with down-regulated 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 to the beneficial effects of 5FU alone. These results suggest that CPT11 reinforces the immunosuppressive environment in CRC-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 CRC-mice, 5FU reduced MDSC levels, both by inducing their apoptotic death and by enforcing myeloid cell differentiation to mature macrophages and DCs. The former drug effect is associated with elevated levels of cleaved caspase-3 and the latter involves a decreased expression of the pro-inflammatory S100A8/9 proteins, known to induce MDSC differentiation arrest (16, 22, 28). In contrast, treatment with CPT11 had opposite effects on CRC associated MDSCs. CPT11 also directly affects MDSCs by boosting secretion of pro-inflammatory cytokines such as TNFα, which is a key MDSC regulator, inducing their differentiation arrest via S100A8/9 and increasing their suppressive activity (16). Thus, in untreated and CPT11 treated CRC-mice, but not in 5FU treated CRC-mice, multiple changes occur in the tumor micro- and macro-environments that directly enhance tumor growth and support indirectly its progression by inhibiting anti-tumor immunity.

Immunosuppressive MDSCs that accumulate during CRC 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 carboxylatedglycans expressed on CRC cells, thereby promoting activation of MAPK and NF-κB signaling pathways. These, in turn, up-regulate a cohort of proteins that promote leukocyte recruitment, angiogenesis, tumor migration and establishment of pre-metastatic niches in
distal organs (28). Moreover, the generated pro-inflammatory environment can affect Wnt/β-catenin signaling as reflected herein by nuclear accumulation of β-catenin in tumors of untreated and CPT11 treated CRC-mice. The attenuated immunosuppression during 5FU treatment improves the therapeutic outcome since it enables the generation of anti-cancer 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 IL-1β, production of IL-17 and tumor growth. NLRP3 activation in the tumor microenvironment could diminish anti-tumor 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 IL-1β 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 CRC-mice, upon 5FU treatment MDSCs and their suppressive activity are significantly decreased. These effects were sufficient to improve T- and NK-cell anti-tumor 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, since 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 CRC patients at earlier stages when monitored prior to 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 anti-tumor chemotherapy or to the currently developing anti-CRC 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 anti-tumor combined therapy should lead to an improved design of future cancer personalized treatments.

Authors' Contributions

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References

Figure legends

Figure 1

FOLFOX and FOLFIRI therapies display opposite effects on CD11b⁺CD33⁺HLA-DR⁻ myeloid cells and CD247 expression in metastatic CRC-patients. (A) CD33⁺HLA-DR⁻ MDSCs were detected in PBLs of healthy donors and CRC-patients, gating on CD11b⁺ cells by flow cytometry analysis. Representative dot plots of MDSC levels in healthy donors and CRC-patients is presented (left), as well as the MDSC percentages of 20 healthy donors and 23 patients (right). (B) NO⁻ (left) and ROS (right) levels produced by MDSCs from healthy donors and CRC-patients are presented as MFI, gating on CD11b⁺CD33⁺HLA-DR⁻ cells. (C) PBLs from the healthy donors and CRC-patients described in (A and B) were analyzed by flow cytometry for CD247 expression presented as MFI, gating on CD3⁺ cells. (D and E) The percentage of circulating MDSCs (left) and the expression of CD247 in T-cells (right) from CRC-patients before (control) and after FOLFOX (patients 1-6) (D) or FOLFIRI (patients 7-10) (E) treatments. Dashed lines represent the normal mean values of %MDSCs and CD247 levels that were measured in gated CD3⁺ cells and are presented as the expression in the experimental group relative to the mean of expression in healthy donors (as 100%).**, P<0.01; ***, P<0.001.

Figure 2

Differential effects of 5FU and CPT11 monotherapies on the tumor and colon microenvironments in CRC-mice. (A) A schematic representation of the mouse model for CRC. (B) Kinetic study of Gr1⁺CD11b⁺ (MDSC) accumulation. Blood samples were collected from mice during CRC 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-eosin stain, original magnifications, x200 and inserts x40. (D) Immunostaining for MDSCs in colons (top) and tumors (bottom), original magnifications, x200. (E) MDSC accumulation within the colons was evaluated by flow cytometry 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 CRC-mice were analyzed for NO⁻ production by flow cytometry analysis gating on the MDSC population. Graphs represent production levels, as shown by MFI. All in vivo experiments involved 6 mice per group and were repeated three times yielding similar results. Graphs (means of triplicates ± s.e.m., n=6) are representative of a typical experiment out of three performed. *, P<0.05; ***, P<0.001; ns = non-significant.

Figure 3

A combined 5FU/CPT11 therapy abrogates recovery from immunosuppression during CRC progression. CRC-mice were either subjected to 5FU, CPT11 or a 5FU/CPT11 combination starting at week eight or left untreated. Three weeks after the second DSS treatment mice were sacrificed and spleens were analyzed. (A) Representative spleens of the different experimental groups are presented. (B) MDSCs accumulation was measured in the spleen by flow cytometry analysis and theo percentage and absolute numbers are presented. (C) Splenocytes isolated from normal, CRC and 5FU-, CPT11- and 5FU/CPT11-tretaed CRC-mice, were analyzed for
NO− and ROS production by flow cytometry analysis, gating on MDSCs. Graphs represent mean fluorescence intensity (MFI). (D) T-cell proliferative response was assessed by monitoring cell divisions of gated CFSE-labeled Thy1.2+ (CD90+) T-cells upon TCR-CD28 mediated activation. Representative histograms of proliferative activity are shown (left), and the percent of proliferating cells was calculated and compared to steady-state levels of non-activated cells in each group (right). (E) Splenocytes from the experimental groups were analyzed for CD247 expression levels indicated by MFI, gating on CD3+ cells and. All in vivo experiments involved 6 mice per group and were repeated three times yielding similar results. Graphs (means of triplicates ± s.e.m., n=6) are representative of a typical experiment out of three performed. *, P<0.05; **, P<0.01; ***, P<0.001; ns = non-significant.

Figure 4

CPT11 harmful effects supporting tumor progression are mediated via MDSCs

CRC-mice were either subjected to 5FU, CPT11 or a 5FU/CPT11 combination starting at week eight or left untreated. At the same day of the second DSS administration, CPT11-treated CRC-mice were randomly separated into two groups; one group continued with CPT11 treatment while 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) MDSCs accumulation was measured in PBLs by flow cytometry analysis. Graph represents the percent of MDSCs in each experimental group. Representative colon structures are presented (B) and histopathology analyses of colons in CRC-mice determined by hematoxylin-eosin staining (C). Original presented magnification, x100. (D) Kaplan-Meyer curve (n = 20) of CRC, 5FU-treated, CPT11-treated, 5FU/CPT11-treated or MDSC-depleted CPT11-treated CRC-mice. All in vivo experiments involved 6 mice per group and were repeated three times yielding similar results. Graphs (means of triplicates ± s.e.m., n=6) are representative of a typical experiment out of three performed. **, P<0.01; ***, P<0.001.

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 cytometry 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 CRC mice (n=6) were ex-vivo incubated with various doses of the drugs for 3 days and subjected to flow cytometry analysis. To assess which cells are affected by the chemotherapeutic drugs, MDSCs isolated from spleens of CRC mice were cultured ex-vivo with 10ng/ml of GM-CSF in the absence or presence of scaled-doses (0, 1.25, 2.5, and 5µmol/L) of 5FU or CPT11 for 3 days. Cleaved caspase-3 levels were then evaluated on the primary MDSCs (D), differentiated CD11c+CD11b+DCs (E) and F4/80+CD11b+ macrophages (F). All experiments involved 6 mice per group and were repeated three times yielding similar results. Graphs (means of triplicates ± s.e.m., n=6) are representative of a typical experiment out of three performed. *, P<0.05; **, P<0.001; ns = non-significant.
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 CRC-mice, or CRC-mice treated with 5FU or CPT11 (n=4), α-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+DCs and F4/80+CD11b+ macrophages (C), and the CD80 and MHCII expression (D), respectively. (E and F) MDSCs isolated from spleens of CRC mice were ex vivo cultured with 10ng/ml GM-CSF in the absence or presence of scaled-doses (0, 1.25, 2.5, 5 and 10µmol/L) of CPT11 (E) or 5FU (F) for 3 days. The phenotype of differentiated DCs (left) and macrophages (right) was then evaluated. All in vivo experiments involved 6 mice per group and were repeated three times yielding similar results. Graphs (means of triplicates ± s.e.m., n=6) are representative of a typical experiment out of three independent performed. Ex vivo experiment involved 4 mice per group and were repeated three times yielding similar results. Graphs (means of triplicates ± s.e.m., n=4) are representative of a typical experiment out of three performed. Data shown are the mean ± s.e.m. *, P<0.05; **, P<0.01; ***, P<0.001. (2-way ANOVA).

Figure 7

**5FU and CPT11 opposite effects on the chronic inflammatory environment are tumor independent.** (A) 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 cytometry 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 cytometry analysis gating on the MDSC population. Graphs represent production levels, as shown by 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 non-activated. 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 percent of proliferating cells was calculated and compared to steady-state levels of non-activated 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 CFSEflow/CFSEhigh in the spleen (top) and PBLs (bottom) in each experimental group. Graphs (means of triplicates ± s.e.m., n=5) are representative of a typical experiment out of three independent performed. *, P<0.05; **, P<0.01; ***, P<0.001; non-significant.
Figure 1

A
Healthy donors | CRC patients
- CD11b+CD33+HLA-DR- cells (%)
  - Healthy donors: 3.65%, 3.84%, 7.06%
  - CRC patients: 25.07%, 19.57%, 28.60%

B
Healthy donors | CRC patients
- NO production (MFI)
  - Healthy donors: 30
  - CRC patients: 20

C
Healthy donors | CRC patients
- ROS production (MFI)
  - Healthy donors: 20
  - CRC patients: 15

D
5FU/FOLFOX treatment
- CD11b+CD33+HLA-DR- cells (%)
  - Patient 1: 10, 20, 30
  - Patient 2: 20, 30, 40
  - Patient 3: 30, 40, 50
  - Patient 4: 40, 50, 60
  - Patient 5: 50, 60, 70
  - Patient 6: 60, 70, 80

E
FOLFIRI treatment
- CD11b+CD33+HLA-DR- cells (%)
  - Patient 7: 10, 20, 30
  - Patient 8: 20, 30, 40
  - Patient 9: 30, 40, 50
  - Patient 10: 40, 50, 60

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Figure 2

A

Screen 1

Screen 2

Screen 3

Screen 4

Screen 5

Harvest

B

Gr1+CD11b+ cells (%)

C

Normal  CRC  CRC+5FU  CRC+CPT11

D

Normal  CRC  CRC+5FU  CRC+CPT11

E

Gr1+CD11b+ cells (x10^6)

F

NO production (MEF)
Figure 3

A

Normal CRC  CRC+  CRC+  CRC+  5FU  CPT11  5FU+  CPT11

1cm

B

Gr1+CD11b+ cells (%)

Normal CRC  CRC+  CRC+  CRC+  5FU  CPT11  5FU+  CPT11

** NS

C

NO production (MFI)

Normal CRC  CRC+  CRC+  CRC+  5FU  CPT11  5FU+  CPT11

* NS

D

T-cell proliferation (%)

Normal CRC  CRC+  CRC+  CRC+  5FU  CPT11  5FU+  CPT11

* NS

E

CD247 expression (MFI)

Normal CRC  CRC+  CRC+  CRC+  5FU  CPT11  5FU+  CPT11

** NS
Figure 4

A

Gr1+ CD11b+ cells (%)

Normal  CRC  CRC+  CRC+  CRC+  CRC+

5FU    CPT11 5FU+ CPT11+ CPT11 anti-Gr1

B

Normal  CRC  CRC+  CRC+  CRC+  CRC+

5FU    CPT11 5FU+ CPT11+ CPT11 anti-Gr1

C

Normal  CRC  CRC+  CRC+  CRC+  CRC+

5FU    CPT11 5FU+ CPT11+ CPT11 anti-Gr1

D

Survival (%)

Days

Normal  CRC  CRC+  CRC+  CRC+  CRC+

5FU    CPT11 5FU+ CPT11+ CPT11 anti-Gr1
Figure 5

A. Normal CRC, CRC+, CRC+ 5FU, CRC+ CPT11, CRC+ 5FU+CPT11

B. Cleaved caspase-3 (MFI) at 0, 1.25, 2.5, and 5 µmol/L 5FU.

C. Cleaved caspase-3 (MFI) at 0, 1.25, 2.5, and 5 µmol/L CPT11.

D. Comparison of 5FU and CPT11 at 0, 1.25, 2.5, and 5 µmol/L.

E. Cleaved caspase-3 (MFI) for 5FU and CPT11 at 0, 1.25, 2.5, and 5 µmol/L.

F. Comparison of 5FU and CPT11 at 0, 1.25, 2.5, and 5 µmol/L.
Figure 6

A

mRNA (RQ)

S100A8

S100A9

CRC

CRC+5FU

CRC+CPT11

**

* NS

B

CRC

CRC+5FU

CRC+CPT11

S100A8

S100A9

αTub

C

CD11b+CD11c+ cells (%)

Normal

CRC

CRC+5FU

CRC+CPT11

NS

**

D

CD11b+F4/80+ cells (%)

Normal

CRC

CRC+5FU

CRC+CPT11

NS

**

E

CD11b+CD11c+ cells (%)

F4/80+MHCIICD80+ cells (%)

Normal

CRC

CRC+5FU

CRC+CPT11

NS

***

F

CD11b+CD11c+ cells (%)

Control

GM-CSF

CRC

CRC+5FU

CRC+CPT11

NS

5FU µmol/L
Figure 7

A

Days

BCG injection
1st
Chemotherapy treatment
2nd
3rd
Harvest

B

Gr1+ CD11b+ cells (%)

Inflamed
5FU
CPT11

NS

C

Normal
Inflamed
Inflamed+
5FU
Inflamed+
CPT11
Inflamed+
5FU+CPT11

Gr1+ CD11b+ cells (%)

D

NO production (MFI)

Inflamed
5FU
CPT11

NS

ROS production (MFI)

Inflamed
5FU
CPT11

NS

Cleaved caspase-3 (MFI)

Inflamed
5FU
CPT11

NS

E

Inflamed+
5FU+
CPT11
18.02%
18.36%
41.89%
19.41%
63.02%

T-cell proliferation (%)

Inflamed
5FU
CPT11

NS

F

Specific allogeneic cell clearance (%)

Inflamed
5FU
CPT11

NS