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# 5-Fluorouracil Selectively Kills Tumor-Associated Myeloid-Derived Suppressor Cells Resulting in Enhanced T Cell–Dependent Antitumor Immunity

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## Abstract

Myeloid-derived suppressor cells (MDSC) accumulate in the spleen and tumor bed during tumor growth. They contribute to the immune tolerance of cancer notably by inhibiting the function of CD8 (+) T cells. Thus, their elimination may hamper tumor growth by enhancing antitumor T-cell functions. We have previously reported that some anticancer agents relied on T cell–dependent anticancer responses to achieve maximal efficacy. However, the effect of anticancer agents on MDSC has remained largely unexplored. In this study, we observed that gemcitabine and 5-fluorouracil (5FU) were selectively cytotoxic on MDSC. *In vivo*, the treatment of tumor-bearing mice with 5FU led to a major decrease in the number of MDSC in the spleens and tumor beds of animals whereas no significant effect on T cells, natural killer cells, dendritic cells, or B cells was noted. Interestingly, 5FU showed a stronger efficacy over gemcitabine to deplete MDSC and selectively induced MDSC apoptotic cell death *in vitro* and *in vivo*. The elimination of MDSC by 5FU increased IFN-γ production by tumor-specific CD8(+) T cells infiltrating the tumor and promoted T cell–dependent antitumor responses *in vivo*. Altogether, these findings suggest that the antitumor effect of 5FU is mediated, at least in part, by its selective cytotoxic action on MDSC. *Cancer Res; 70(8); 3052–61. ©2010 AACR.*

## Introduction

Myeloid-derived suppressor cells (MDSC) have been identified as a population of immature myeloid cells with the ability to suppress T-cell activation in humans and mice (1–3). These cells accumulate in the blood, lymph nodes, bone marrow, and at tumor sites in many human cancers and animal tumor models, and inhibit both adaptive and innate immunity (4). They notably have the capacity to inhibit $CD8^+$ T cell antigen-specific reactivity by different mechanisms, mainly through their capacities to produce nitric oxide and radical oxygen species (5–7). MDSC are phenotypically characterized in mice by the expression of the cell surface antigens Ly-6C/G (both recognized by the Gr-1 antibody) and CD11b (2). A human counterpart of MDSC was proposed to lie in the CD11b $^+CD33^+CD14^-HLA\text{-}DR^-$ subset (8–11). Elimination of MDSC in mouse tumor models was shown to enhance antitumor responses, resulting in tumor regression. In this regard, strategies aimed at depleting MDSC *in vivo* using agents that target MDSC such as antibodies (12) or cytotoxic agents as gemcitabine have been shown to be the most promising (13–15). Gemcitabine is an antimetabolite chemotherapeutic agent implemented in the clinic since the 1990s and was previously reported to be capable of depleting MDSC in tumor-bearing mice (14).

We and others have previously reported that some anticancer agents, in addition to their direct cytotoxic effects on tumor cells, feature the ability to promote the activation of the immune system of the host, resulting in enhanced antitumor responses (16, 17). To investigate whether conventional anticancer agents were also able to affect the biology of MDSC, we designed an *in vivo* drug screening assay in which we tested the effect of anticancer agents on the proportion of MDSC within tumor-bearing mice. Our results show for the first time that 5FU was able to reduce the number of MDSC in tumor beds by triggering their apoptotic cell death. We also observed that 5FU-mediated MDSC depletion triggered an increase in IFN-γ production by tumor-specific $CD8^+$ T cells infiltrating the tumor bed and promoted a T-dependent antitumor effect. These results suggest that, beyond its direct cytotoxic effect

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on tumor cells, 5FU possesses immunogenic properties that rely on the *in vivo* elimination of MDSC.

## Materials and Methods

***Mice and cell lines.*** EL4, a thymoma cell line syngeneic of C57BL/6, were obtained from the American Type Culture Collection. MSC-1 and MSC-2 are immortalized MDSC cell lines obtained from BALB/c Gr-1$^+$ splenocytes and were given by V. Bronte (University of Padua, Padua, Italy). All cells were cultured in RPMI 1640 (Life Technologies) with 10% fetal bovine serum enriched with 0.4 mmol/L of sodium pyruvate, 4 mmol/L of HEPES, and antibiotics (penicillin, streptomycin, and amphotericin B). BALB/c, C57BL/6, and Nude mice were purchased from the Centre d'Elevage Janvier (Le Genest St. Isle, France), and used at 6 to 10 wk of age. TLR4$^{-/-}$ C57BL/6 mice were provided by Bernhard Ryffel (UMR 6218 CNRS, Orleans, France). Animals were all maintained according to Animal Experimental Ethics Committee Guidelines.

***Tumor model.*** EL4 cells ($2 \times 10^5$/100 μL PBS) were s.c. injected into the flank of mice. Mice were treated when tumor surface was ~100 mm$^2$. EL4 cancer cells were injected s.c. to C57BL/6 mice, *nude* mice, and TLR4$^{-/-}$ C57BL/6 mice. Tumor surfaces were measured thrice a week.

**In vivo *chemotherapeutic treatments.*** For *in vivo* experiments, mice received a unique injection of cyclophosphamide at 80 mg/kg of body weight, paclitaxel (36 mg/kg), raltitrexed (100 mg/kg), gemcitabine (120 mg/kg), doxorubicin (5 mg/kg), 5FU (50 mg/kg), or oxaliplatin (5 mg/kg). All drugs were administrated by i.p. injection, except for doxorubicin, which was injected i.v.

***Isolation of MDSC from spleens and tumors.*** Spleens were mechanically dissociated and individual spleen cells obtained through a 70-μm cellular sieve, then centrifuged, counted and washed once with PBS. To maximize the presence of MDSC, single-cell suspensions prepared from the spleen of tumor-bearing mice were purified by magnetic selection of MDSC, using Gr1-phycoerythrin-cyanine 7 staining followed by anti–phycoerythrin-cyanine 7 magnetic beads (Miltenyi Biotec). Tumors were cut with scalpels in millimetric fragments that were then mechanically dissociated and passed through a 70-μm cellular sieve. Tumor cells were laid down on a leukocyte-separating cushion and centrifuged (1,000 × *g*, 20 min). Floating cells were collected, centrifuged, counted, and washed once with PBS.

***Fluorescence-activated cell sorting analyses of Treg and MDSC.*** Five days after treatment with 5FU, cyclophosphamide, gemcitabine, oxaliplatin, or doxorubicin, tumor-bearing mice were sacrificed. Tumors and spleens were harvested and single cell suspensions were prepared. MDSC were stained with antibodies anti-CD11b, Ly-6C, and Ly-6G for 20 min at 4°C. After one wash with cold PBS, they were analyzed by flow cytometry. Tregs were stained with anti-CD4, anti-CD25, and anti-CD3 monoclonal antibodies for 20 min at 4°C. After one wash with cold PBS, they were permeabilized according to the manufacturer's protocol (Fix/Perm eBioscience), and stained with an anti-Foxp3 antibody (eBiosciences) for 20 min at 4°C. After one wash with cold PBS, cells were analyzed by flow cytometry.

***Flow cytometry analyses.*** Fluorescence-activated cell sorting (FACS) analyses were performed using phycoerythrin (PE) cyanine 7 (Cy7)–conjugated anti-Gr1, allophycocyanin (APC)-conjugated anti-CD11b, fluorescein isothiocyanate (FITC)–conjugated anti-Ly6C, PE-conjugated anti-Ly6G, peridinin-chlorophyll-protein (PerCP)–conjugated anti-CD4, APC-conjugated anti-CD3, PE-Cy7–conjugated anti-CD25, Efluor 450-conjugated anti-Foxp3, FITC-conjugated Annexin V, 7-aminoactinomycin D (7-AAD), APC-conjugated anti-B220, FITC-conjugated anti-CD11c, Alexa 700–conjugated anti-CMH2, PerCP–conjugated anti-CD8, 4′,6-diamidino-2-phenylindole (DAPI), or Alexa 488–conjugated immunized rabbit serum. All antibodies were purchased from BD PharMingen or eBioscience. FITC-Flica caspase-3/7 apoptosis detection kits were purchased from Immunochemistry Technologies. FACS analyses were performed on a LSRII (BD Biosciences) using FACSDiva software (BD Biosciences) and analyzed with FlowJo (TreeStar).

Single cell suspension from tumors and spleens were centrifuged and saturated with 200 μL of PBS containing 2% mouse serum for 15 min at 4°C. After centrifugation, cells were stained for 20 min at 4°C with the following antibodies: CD11b, Ly-6C, and Ly-6G for MDSC; CD3, CD4, and B220 for lymphocytes; and CD11c, CD11b, CMH2, and CD8 for dendritic cells. All cells were stained with 4′,6-diamidino-2-phenylindole or 7-AAD and analyzed by flow cytometry.

***Intracellular stainings.*** For IFNγ intracellular staining, spleens, tumors, and tumor-draining lymph nodes were harvested and dissociated as reported above, 5 d after treatment. When MDSC were reinfused, they were injected i.v. 2 d after treatment. Leukocytes were then cultured *in vitro* in three different conditions: in anti-CD3–coated wells (0.1 μg/well of a 96-well tissue culture plate), in anti-CD3–coated wells with the addition of killed tumor cells (with a ratio of one killed tumor cell per two effectors), or in anti-CD3–coated wells with the addition of soluble anti-CD3 (2 μg/mL), soluble anti-CD28 (2 μg/mL), and interleukin-2 (1,000 UI/mL; Proleukin, Chiron). Stimulation was maintained for 16 h, with brefeldin A (BD GolgiPlug) for the last 4 h. Cells were then harvested and stained for CD4, CD8, and intracellular IFNγ using the manufacturer's recommendations.

***Apoptosis assays.*** Purified MDSC were cultured for 24 h *in vitro* with varying doses of 5FU or gemcitabine. *In vivo*, tumor-bearing mice were treated with 5FU or gemcitabine. Tumors and spleens were harvested and dissociated as described previously and then stained as follows: (*a*) Annexin V/7-AAD: MDSC were washed twice with cold PBS and prepared according to the manufacturer's protocol (BD PharMingen). After staining for 15 min at room temperature with Annexin V and 7-AAD, cells were analyzed by flow cytometry; (*b*) Flica 3/7: cells were manipulated according to the manufacturer's protocol (ImmunoChemistry Technologies). Briefly, cells were cultured for 1 h in the presence of FAM-Flica after pretreatment with PBS or varying doses of 5FU. Cells were then washed and analyzed by flow cytometry in the presence of 7-AAD. In some experiments, cell death was measured using the phenazine methosulfate

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(PMS)/3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega). In PMS/MTS experiments, cells were treated for 1 h with 5FU at varying concentrations in round-bottomed 96-well plates. PMS/MTS was added after 48 h and the plates were read at 540 nm.

***Detection of calreticulin expression on tumor cells.*** EL4 cancer cells were cultured for 24 h in complete culture medium supplemented or not with doxorubicin, gemcitabine, or 5FU. Cells were stained as described elsewhere (18). Briefly, cells were saturated with 200 μL of PBS supplemented with 2% rabbit serum before being stained with a rabbit anti-calreticulin antibody for 20 min at 4°C. Cells were permeabilized or not with BD PharMingen, cells were incubated with anti-calreticulin rabbit antibody (AbCam) for 20 min at 4°C. Cells were then analyzed by flow cytometry.

***Thymidilate synthase expression.*** Total RNA was extracted using Trizol (Invitrogen). One hundred to 300 ng of RNA was reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase, random primers, and recombinant RNasin ribonuclease inhibitor (Promega). cDNA were quantified by real-time PCR using a SYBR Green Real-time PCR kit on a 7500 Fast detection system (Applied Biosystems). Relative mRNA levels were determined using the ΔCt method. Values were expressed relative to mouse cyclophilin A. The same results were obtained when normalized with mouse L32 and mouse hypoxanthine phosphoribosyltransferase (data not shown). The sequences of the oligonucleotides used in this study were as follows: mouse cyclophilin A (forward, 5′-GGCCGATGACGAGCCC-3′; reverse, 5′-TGTCTTTGGAACTTTGTCTGCAA-3′) and mouse thymidylate synthase (TS; forward, 5′-caatggatcccgagattttc-3′; reverse, 5′-gtcatcagggttggttttga-3′).

***Statistical analysis.*** Tests used were Student's *t* test or Mann-Whitney test for parametric and nonparametric means comparison. All tests were performed with GraphPad Prism software.

## Results

***5FU selectively depletes MDSC.*** We tested the capacity of several cytotoxic agents used in conventional cancer chemotherapy to deplete MDSC *in vivo*. To this end, we used cyclophosphamide (an alkylant agent), doxorubicin (a topoisomerase II inhibitor), oxaliplatin (a platinum compound promoting DNA adducts), paclitaxel (a tubulin poison), gemcitabine (a deoxycytidine analogue that inhibits ribonucleotide reductase), and 5FU and raltitrexed [two antimetabolite agents that target thymidilate synthase (TS)]. These drugs were injected once in mice bearing 100 $mm^2$ EL4 thymoma subcutaneous tumors. In our model, MDSC were found to be abundant within the spleens and tumor beds of tumor-bearing mice before chemotherapeutic treatment (Fig. 1A), as previously described (19). Five days after injection of the cytotoxic agent, we observed that only gemcitabine and 5FU were able to significantly decrease the number of MDSC in both the spleen and tumor bed (Fig. 1A; see Supplementary Fig. S1). Interestingly, 5FU had a more drastic effect than gemcitabine in decreasing MDSC frequency in tumor-bearing hosts. It is noteworthy that 5FU did not alter the frequency of the other cell populations within the spleen, except for a non-significant tendency for B cells to increase, compensating the loss of MDSC (Fig. 1B).

MDSC have been described to encompass at least two cell populations: granulocytic cells, $Ly6G^{+}Ly6C^{int}$, and monocytic cells, $Ly6G^{-}Ly6C^{hi}$ (9, 10). We did not find any preferential action from either 5FU or gemcitabine on any MDSC subtype. Indeed, both drugs were similarly effective against granulocytic and monocytic MDSC (Fig. 1C). We also monitored the duration of MDSC depletion induced by 5FU, and found that the nadir was around day 5 posttreatment, then MDSC number increased following tumor growth (Fig. 1D).

Thus, we show that, among the seven cytotoxic agents assayed, only 5FU (and to a lesser extent gemcitabine) was able to lower MDSC number within the tumor bed and spleen of cancer-bearing hosts.

***5FU triggers MDSC apoptosis.*** The disappearance of MDSC from tumors and spleens could possibly be explained by the direct cytotoxic effect of 5FU, which could preferentially trigger their death. We therefore tested whether MDSCs were undergoing apoptosis after 5FU treatment.

Because of spontaneous rapid death of *ex vivo* MDSC, we took advantage of immortalized MDSC cell lines (20). First, we cultured established murine MDSC cell lines in the presence of various concentrations of 5FU and evaluated MDSC cell death using both Annexin V/7-AAD labeling (Fig. 2A, left) and the detection of activated caspases-3 and -7 (Fig. 2A, right). Both methods showed that 5FU triggered MDSC apoptotic cell death in a dose-dependent manner. *In vivo*, we could also note an increase in the presence of activated caspases-3 and -7 in splenic MDSC from 5FU-treated tumor-bearing mice compared with untreated mice, although other immune cells were not killed by 5FU at this dose (data not shown). To our surprise, we also noted that 5FU *in vitro* at low concentrations was more toxic in MSC cell lines than in tumor cells. Indeed, a 20-fold to 50-fold higher concentration of 5FU was required to kill EL4 tumor cells relative to MSC (Fig. 2B). Of note, freshly sorted MDSC underwent spontaneous and rapid death, but surviving cells were also 5FU-sensitive (data not shown).

The cytotoxic action of 5FU was mediated by its metabolites which inhibit TS. Low expression of TS has been shown to be involved in 5FU sensitivity in tumor cells (21–23); therefore, we hypothesized that MDSC's sensitivity to 5FU might be related to lower TS expression. We then performed quantitative PCR on cell extracts to test TS expression (Fig. 2C). We found that MDSC and MSC cell lines expressed fewer TS than splenocytes or tumor cells, thus suggesting that MDSC sensitivity to 5FU may be linked to their low expression of TS. On the whole, these results show that *in vitro* and *in vivo* low concentrations of 5FU selectively induced MDSC apoptotic cell death.

***5FU immunogenic effects are primarily attributable to MDSC depletion.*** Chemotherapeutic agents have been shown to alter the antitumor response (16). Specifically,

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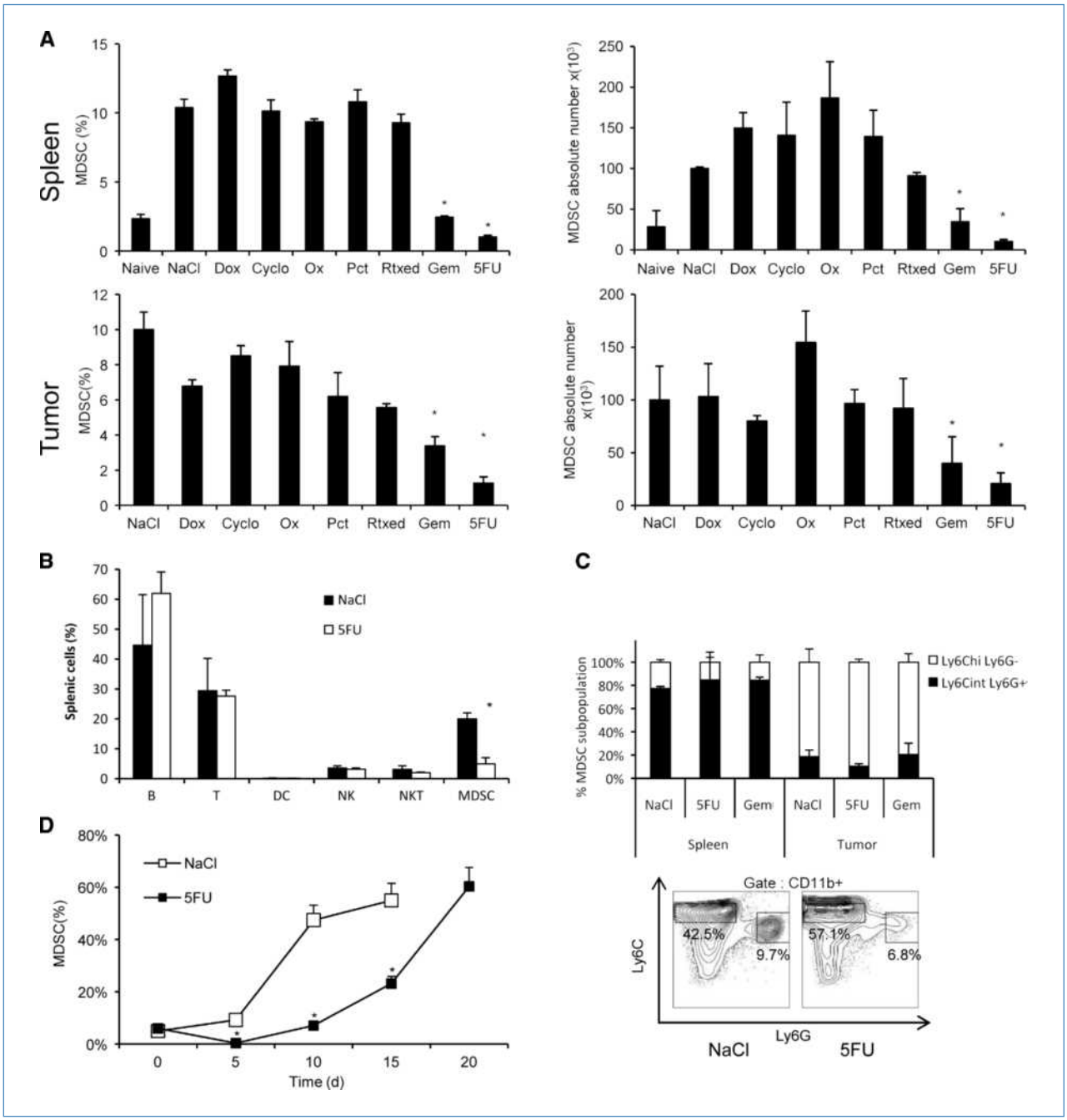


**Figure 1.** 5FU and gemcitabine eliminate MDSC *in vivo*. A, mice were tumor-free (Naïve) or inoculated s.c. with EL4 tumor cells. Five days after the indicated chemotherapy (NaCl, control mice; Dox, doxorubicin; CTX, cyclophosphamide; Ox, oxaliplatin; Pct, paclitaxel; Rtxed, raltitrexed; Gem, gemcitabine; 5FU, 5-fluorouracil), spleens and tumors were harvested and MDSC infiltration was determined by FACS analysis. Graphics show the percentage of splenic and tumor infiltration MDSC and the absolute number of MDSC in spleen and tumors. B, effect of 5FU on other splenic cell populations. C, the percentages of monocytic ($Ly6C^{hi}Ly6G^{-}$) and granulocytic ($Ly6C^{int}Ly6G^{+}$) subpopulation of MDSC after 5FU treatment was determined by FACS analysis. Bottom, representative FACS. D, tumor-bearing mice were treated or not with 5FU, and spleens were harvested at various posttreatment times. Graphic shows the percentage of splenic MDSC. Columns, mean; bars, SEM. The experiments were performed twice with similar results, $n = 4$ per group (*, $P < 0.05$; **, $P < 0.01$).

some chemotherapeutic agents could promote Treg cell killing (24–26) or affect the biology of dendritic cells (27, 28). We therefore tested if, in addition to its action on MDSC, 5FU exerted some of these reported effects on the immune system. To this end, we first compared 5FU and cyclophosphamide for their ability to trigger regulatory T-cell depletion *in vivo*. Although cyclophosphamide administration led to a reduction in Treg numbers in the tumor bed and in the

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spleen, 5FU had no such effect in the same setting (Fig. 3A). Similarly, we verified that 5FU (in contrast to mafosphamide) exerts no toxic effect on regulatory T cells *in vitro* (data not shown).

Cancer chemotherapy may also enhance the anticancer immune response by promoting the maturation of antigen-presenting cells. We thus tested the effect of a single systemic injection of 5FU, gemcitabine, or Gram-negative bacteria lipopolysaccharide on the maturation pattern of splenic dendritic cells. In this setting, although lipopolysaccharide induced a massive upregulation of CD86 and CD40, 5FU administration did not significantly alter the expression of those markers, ruling out a putative involvement of 5FU in inducing dendritic cell maturation (Fig. 3B).

We have previously shown that the *in vivo* efficacy of doxorubicin relied on its ability to trigger an "immunogenic" cell death of cancer cells. Treatment of tumor cells by doxorubicin leads to the cell surface exposure of calreticulin, which is responsible for tumor cell phagocytosis, and to the release of HMGB1, which is critical for the Toll-like receptor 4 (TLR4)–mediated cross-presentation of tumor antigens from dendritic cells to T cells (29). To test the ability of 5FU to trigger an immunogenic form of cell death *in vivo*, we first monitored the expression of cell surface calreticulin on EL4 cells treated

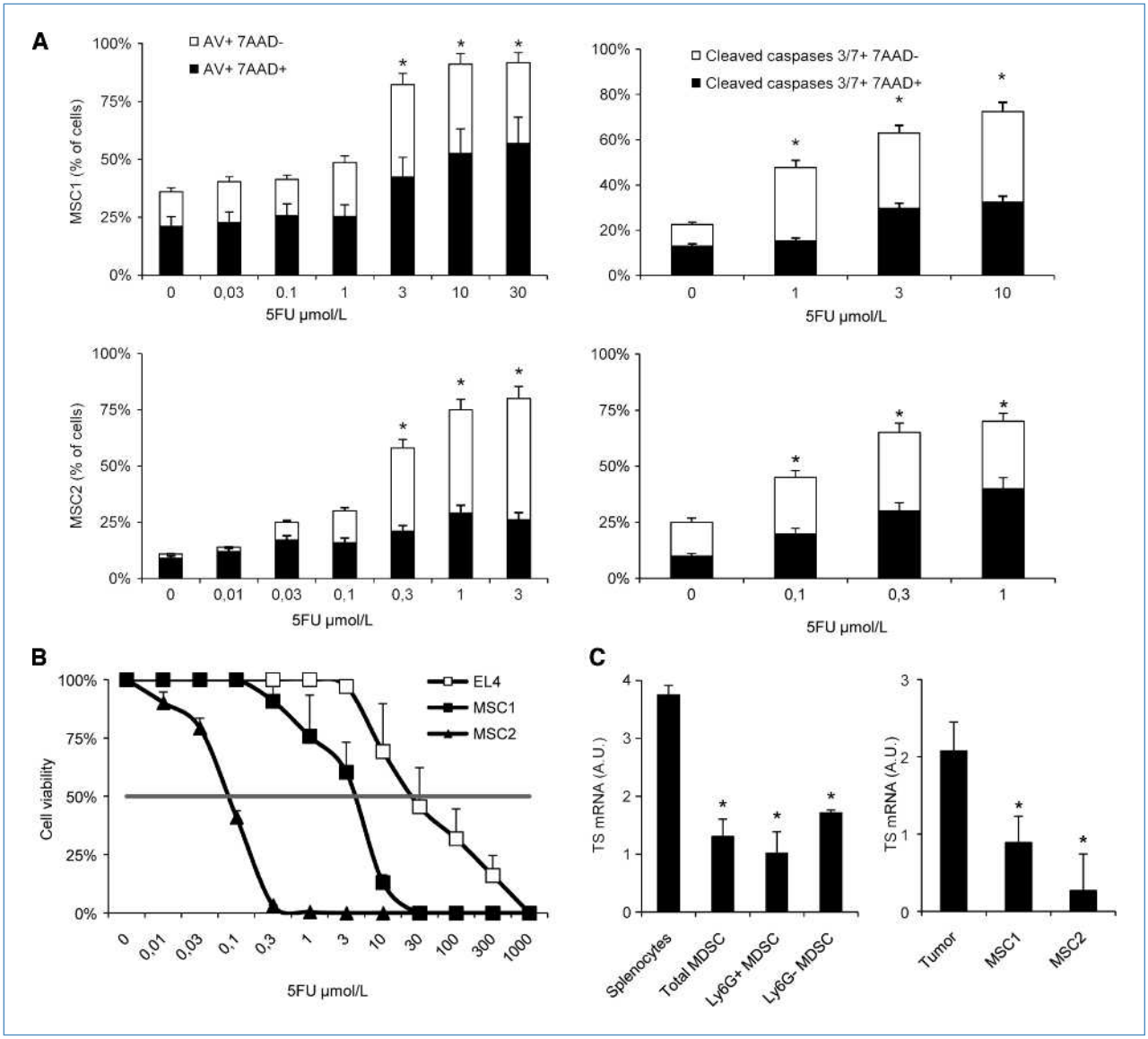


Figure 2. 5FU induces MDSC apoptosis. A, the effect of 5FU on MSC1 and MSC2 cell lines was tested *in vitro* for apoptosis induction. FACS analysis of Annexin V/7-AAD staining (left) or activated caspase-3/7-AAD (right) staining results, respectively, in MSC1 (top) or MSC2 (bottom). B, compared dose-effect relation of 5FU-induced cell death on MSC cell lines and EL4 tumor cell lines. The experiments were performed thrice with identical results. C, quantitative RT-PCR for TS was performed on extracts from the indicated cellular preparation. Total MDSC were prepared as usual, granulocytic ($Ly6G^+$) or monocytic ($Ly6G^-$) cells were sorted by FACS from spleens of tumor-bearing mice. The experiments were performed twice with similar results (*, $P < 0.05$).

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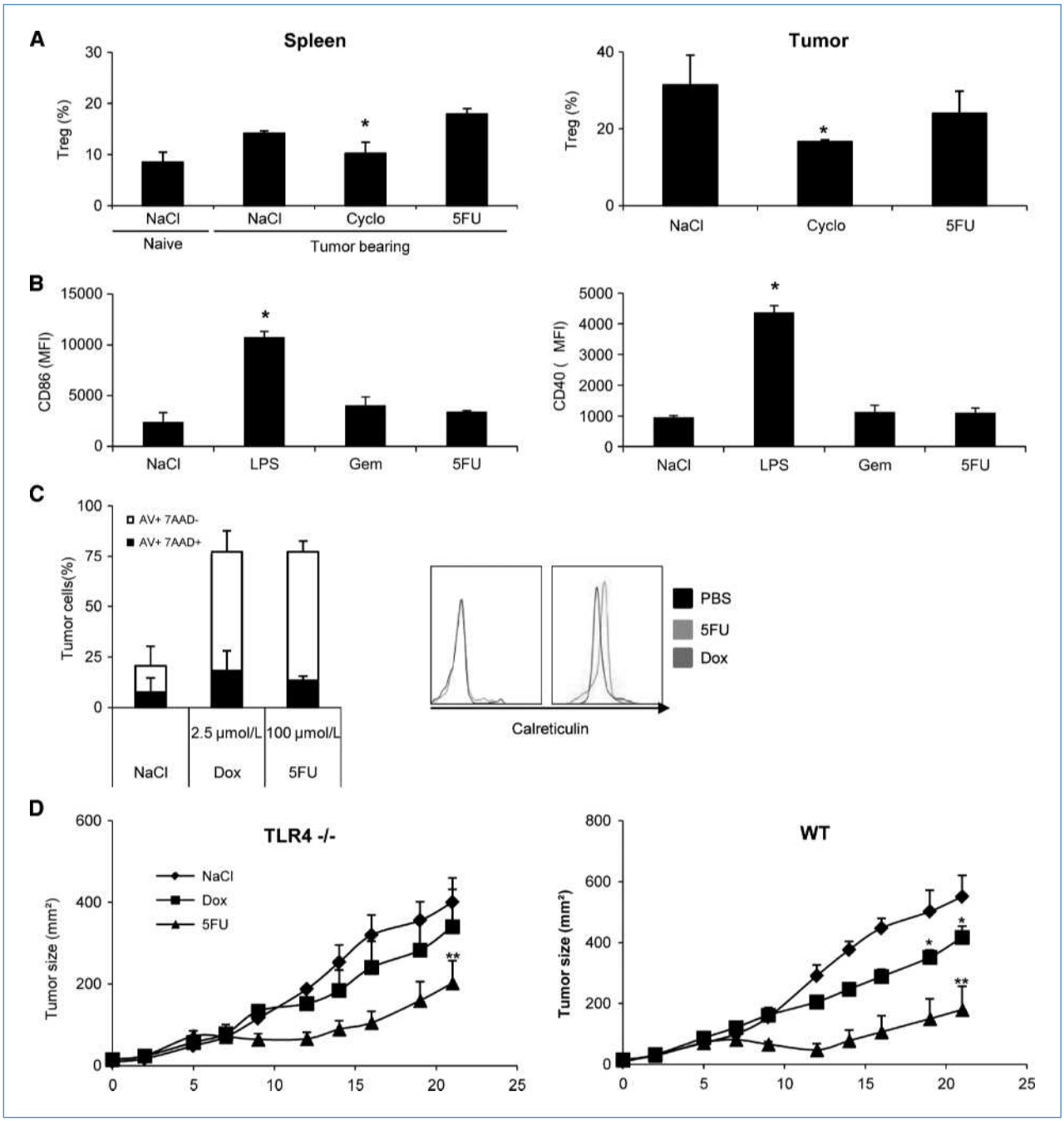


Figure 3. 5FU immunomodulatory effects are restrained to MDSC depletion. A, effect of 5FU or cyclophosphamide on Tregs. Mice bearing EL4 tumors either received no treatment (PBS) or one s.c. administration of cyclophosphamide (Cyclo) or 5FU. Splenic (top) or tumor-infiltrating (bottom) Tregs, respectively, 5 d after drug administration. B, effect of 5FU or gemcitabine on splenic dendritic cell maturation. Tumor-free mice received an injection of lipopolysaccharide (LPS), gemcitabine (Gem), or 5FU. Twenty-four hours after treatment, dendritic cells were isolated from the spleen and examined by flow cytometry after labeling with antibodies to CD86 (left) or CD40 (right). MFI, mean fluorescence index. C, tumor cells were treated with PBS, doxorubicin (Dox), or 5FU then mortality was evaluated with Annexin V/7-AAD labeling (left), membrane calreticulin presence was assayed by flow cytometry (right). D, effect of 5FU and doxorubicin on tumor growth in WT (right) or TLR4$^{-/-}$ (left) mice ($n$ = 5 per group). The experiments were performed twice with identical results (*, $P < 0.05$; **, $P < 0.01$).

with 5FU or doxorubicin as a positive control. Although doxorubicin treatment could trigger cell surface expression of calreticulin, we failed to show any significant upregulation of calreticulin by treating EL4 cells with 5FU (Fig. 3C). We then compared the effect of 5FU on EL4 tumor growth in wild-type (WT) and TLR4-deficient mice. Although the efficacy of doxorubicin was largely impaired in TLR4-deficient hosts, we observed that 5FU had a TLR4-independent

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antitumor activity (Fig. 3D). These results suggest that the antitumor effects of 5FU cannot be explained by its ability to trigger an immunogenic form of tumor cell death. Altogether, these data show that the 5FU immune-mediated effects specifically rely on the elimination of MDSC.

***5FU-induced specific activation of CD8+ T cells.*** As MDSC are known to inhibit antigen-dependent CD8+ T cell proliferation and Tc1 differentiation (3), we tested whether 5FU could affect Th1- or Tc1-specific polarization in tumor-bearing animals. To this end, leukocytes from the spleen, tumor-draining lymph nodes, and tumor bed from EL4 tumor-bearing mice treated or not with 5FU were stimulated either with dead EL4 tumor cells or nonspecifically with anti-CD3 plus anti-CD28 antibodies. We then performed intracellular staining for IFN-γ production. We did not detect any significant IFN-γ production by either $CD4^+$ or $CD8^+$ T cells obtained from spleen or draining lymph node of tumor-bearing animals, even after specific T-cell restimulation (data not shown). In contrast, antigen-specific restimulation of tumor-infiltrating $CD8^+$ T cells (but not $CD4^+$ T cells) produced detectable levels of IFN-γ, which was enhanced after 5FU treatment of EL4 tumor-bearing mice (Fig. 4A and B). Moreover, adoptive transfer of MDSC from tumor-bearing mice drastically impeded IFN-γ production by $CD8^+$ T cells (Fig. 4A and B). These data collectively suggest that the selective cytotoxic activity of 5FU on MDSC could locally enhance the Tc1 polarization of $CD8^+$ T cells.

***5FU exerts an MDSC-dependent antitumor effect and acts synergistically with the depletion of regulatory T cells.*** We first treated WT or nude mice bearing a large EL4 tumor with gemcitabine or 5FU (Fig. 5A and B). These two treatments efficiently slowed down tumor growth in WT mice but exerted only a minor effect on tumor growth in nude mice (Fig. 5A and B). These data show that these chemotherapies depend on T cells to mediate their activity. In addition, we observed in WT mice bearing tumors that an adoptive transfer of MDSC from tumor-bearing mice 1 day after 5FU injection blunted the *in vivo* antitumor effect of 5FU (Fig. 5C). Altogether, these data suggest that 5FU exerts its activities through the elimination of MDSC and permits the restoration of T cell–dependent antitumor responses.

Tregs represent another population of immunosuppressive cells that exerts a major negative effect on antitumor immune response (25, 26, 30). We showed previously that a low dose of cyclophosphamide could deplete this population and restore latent antitumor immunity (25, 26). Cyclophosphamide had a T-dependent antitumor effect that was annihilated by an adoptive transfer of Treg cells (25, 30). These

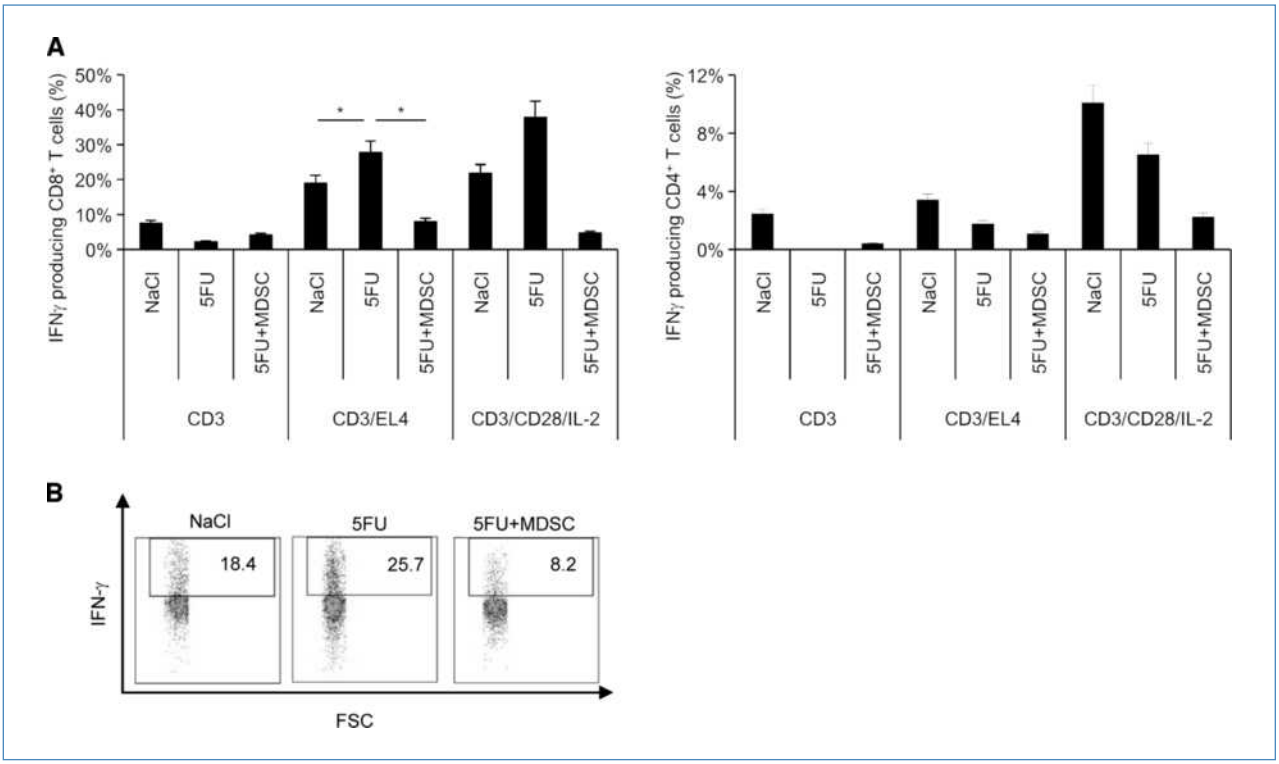


Figure 4. 5FU-induced specific activation of CD8+ T cells. A, EL4 tumor-bearing mice were treated with 5FU (or NaCl for controls; 50 mg/kg body weight) and injected (when indicated) with $5 \times 10^6$ MDSC isolated from the spleen of tumor-bearing mice. Tumor-infiltrating leukocytes isolated 5 d after 5FU treatment were stimulated with immobilized anti-CD3 alone, anti-CD3 plus killed EL4 cells, or anti-CD3 plus anti-CD28 for 12 h. IFN-γ production in tumor-infiltrating T CD8 (left) or T CD4 (right) lymphocytes, respectively. B, representative FACS showing the effect of 5FU treatment on IFN-γ production by $CD8^+$ T lymphocytes from EL4 tumor infiltrate and its modulation by passive transfer of MDSC. The experiments were performed thrice with identical results (*, $P < 0.05$).

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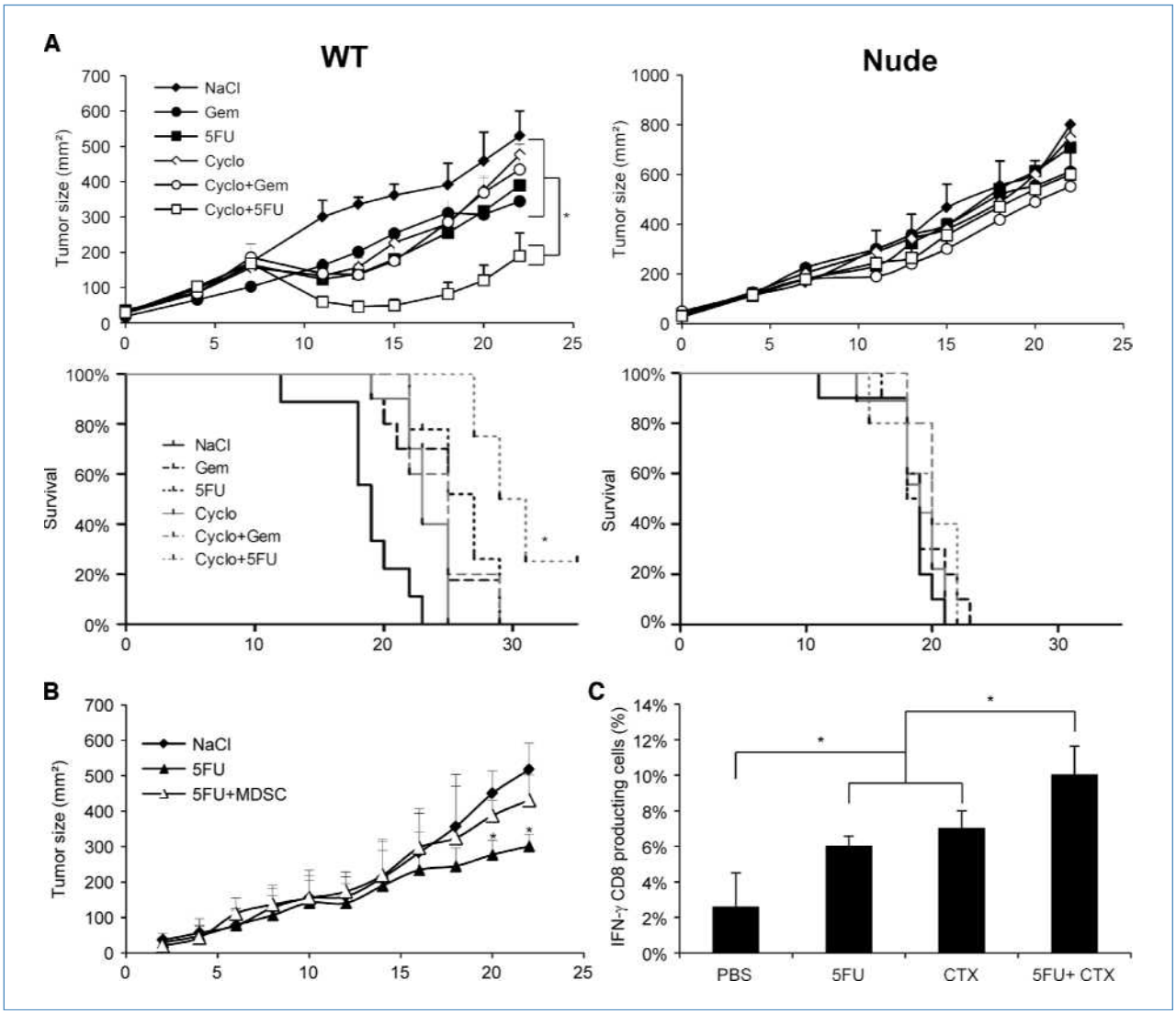


**Figure 5.** The antitumor effect of 5FU relies on MDSC. A, WT (left) or nude (right) mice were injected with $2 \times 10^5$ EL4 cells and treated when tumor reached 100 $mm^2$ with one s.c. injection of the indicated chemotherapy. WT (left) or nude (right) mice, respectively. Tumor growth was measured every 2 d (top) and survival was monitored (bottom; $n = 10$). B, WT mice were treated when tumor reached 200 $mm^2$ with one s.c. injection of 5FU (50 mg/kg) or 5FU plus an adoptive transfer of $5 \times 10^6$ splenic MDSC cells from tumor-bearing mice ($n = 10$). Top, tumor growth in each group; bottom, mice survival using Kaplan-Meier curves. The experiments were performed thrice with similar results. C, WT mice were injected with $2 \times 10^5$ EL4 cells and treated when tumor reached 100 $mm^2$ with the combination of 5FU (50 mg/kg) plus cyclophosphamide (CTX, 80 mg/kg), or 5FU alone or were left untreated. Cells isolated from tumor-infiltrating lymphocytes were stimulated with anti-CD3 plus dead EL4 cells. Histogram represents the percentage of IFN-γ–producing CD8 T cells in tumor-infiltrating CD8 T lymphocyte ($n = 4$). The experiments were performed twice with similar results (*, $P < 0.05$).

data provided the impetus to combine Treg and MDSC depletion to enhance T-cell functions and antitumor responses. The two combinations (cyclophosphamide plus gemcitabine and cyclophosphamide plus 5FU) showed a synergic effect in WT mice compared with monotherapies but had little or no effect on tumors growing in nude mice (Fig. 5A and B). Interestingly, the cyclophosphamide + 5FU combination showed a significantly superior antitumor effect compared with the cyclophosphamide + gemcitabine combination in immunocompetent mice, and mice survival was improved in the cyclophosphamide + 5FU group ($P = 0.04$, log rank test). In line with the *in vivo* results, we verified *ex vivo* that the combination of 5FU and cyclophosphamide enhanced the number of tumor-specific IFN-γ producing specific intratumoral CD8 T cells compared with monotherapies (Fig. 5D).

Altogether, these data show that cyclophosphamide and 5FU could exert a T-dependent synergistic antitumor effect and could lead to the cure of some animals bearing large tumors.

## Discussion

The recognition that immune suppression is crucial to promote tumor progression, which might explain the failure of some cancer vaccines, has resulted in a paradigm shift

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regarding approaches to cancer immunotherapy. It indeed becomes clear that successful cancer immunotherapy will only be achieved when associated with the elimination of suppressive cells (31–33). Two major immunosuppressive cell types are mainly involved in tumor-induced immunosuppression: Tregs and MDSC. The elimination of Treg cells using a low dosage of cyclophosphamide as a metronomic regimen proved its efficacy in many rodent models as well as in humans (25, 26, 30, 34–37). Many strategies have been tested to dampen the immunosuppressive actions of MDSC, including treatments designed to favor their differentiation, or to inhibit their expansion or their inhibitory function (13, 38). However, the most promising results have been obtained with the selective depletion of these cells. Some groups tested the *in vivo* administration of monoclonal antibodies against Gr-1, aimed at depleting Gr-1$^+$ MDSC. This treatment gave interesting results in restoring T cell antitumor activity, both in terms of general reduction of tumor progression and in terms of prevention of relapse (39, 40). Unfortunately Gr-1 is not a specific marker of MDSC as it is also expressed in granulocytes, implying the possibility that tumor-bearing hosts treated with such depleting antibodies might undergo opportunistic infections. This consideration emphasizes the interest of screening drugs aimed at selectively depleting MDSC. It was recently shown that several cytotoxic agents used for cancer chemotherapy not only featured a direct cytotoxic effect on tumor cells, but also featured an interesting side effect by eliminating leukocyte subpopulations involved in the suppression of antitumor immunity (35). However, up to now, only gemcitabine has been shown to be able to eliminate MDSC, one of the major cell populations involved in tumor tolerance (14, 15).

Here, we show for the first time that 5FU was also able to reduce the number of MDSCs not only in the spleen but also in the tumor bed. The effect of 5FU on MDSC was obtained at low doses and seemed to be selective as we did not observe any *in vivo* drop in T cells, B cells, or dendritic cell numbers. This effect was mediated by inducing the apoptotic death of MDSC with activation of caspase-3 and caspase-7 both *in vitro* and *in vivo*. The effect of 5FU lasted for ~10 days *in vivo*, and could be related to a lower TS expression in this cell type. Moreover, the effect of 5FU on MDSC seemed more pronounced than the effect of gemcitabine, thus suggesting that this agent could be more efficient in enhancing antitumor immunity.

MDSC are well known inhibitors of CD8$^+$ T cell activation (3). We showed here for the first time that 5FU induces MDSC depletion and could enhance the intratumoral CD8$^+$ T cell antigen-specific capacity to produce IFN-γ. This effect was reversed by adoptive transfer of MDSC thus providing the evidence that reinfused MDSC could migrate to the tumor and blunt T cell reactivity *in situ*. When monitoring tumor growth, we observed that its inhibition by 5FU was strictly dependent on T cells and was also completely hindered by MDSC transfer. Based on these results, we propose that most of the antitumor effect of 5FU could be explained by its capacity to eliminate MDSC and restore CD8$^+$ cell capacity to produce IFN-γ. Finally, we have shown that the association of Treg and MDSC depletion obtained with cyclophosphamide and 5FU, respectively, has a synergistic effect on the suppression of tumor growth. This data is of importance because it shows that therapies acting on tumor immunosuppression may be sufficient to restore T-cell function and obtain therapeutic effects. The antitumor activity obtained by the 5FU-cyclophosphamide association in our experimental tumor model strongly suggests that controlling tumor-induced tolerance is at least of equal importance as activating tumor immunity with a tumor vaccine for the purpose of obtaining immune eradication of cancers.

5FU, an analogue of uracil, the metabolites of which misincorporate in RNA and DNA and inhibit TS, is a cytotoxic agent widely used in the treatment of colorectal and breast cancers, as well as cancers of the aerodigestive tract (21). Its efficacy in cancer chemotherapy is currently considered to result directly from its toxic effect on tumor cells. Here, we report that 5FU could also hinder tumor growth by selectively destroying the tumor-associated MDSC, thus inhibiting their suppressive effect on the T cell–mediated control of the tumor. Even if this effect was briefly discussed in a very recent review (33), our report is the first one to actually bring up experimental evidence on the cytotoxic effect of 5FU on MDSC. Interestingly, in our experimental tumor model, the effect of 5FU on MDSC was predominant over its direct effect on tumor cells, thus explaining why the antitumor effect of 5FU is hampered by MDSC adoptive transfer. In summary, the results of our study would support the addition of 5FU to the expanding list of chemotherapeutic agents whose antitumor effects depend, at least in part, on their capability to enhance the anticancer immune response (16).

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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