

Anti-inflammatory Triterpenoid Blocks Immune Suppressive Function of MDSCs and Improves Immune Response in Cancer

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Abstract

Purpose: Myeloid-derived suppressor cells (MDSC) are one of the major factors responsible for immune suppression in cancer. Therefore, it would be important to identify effective therapeutic means to modulate these cells.

Experimental Design: We evaluated the effect of the synthetic triterpenoid C-28 methyl ester of 2-cyano-3,12-dioxooleana-1,9,-dien-28-oic acid (CDDO-Me; bardoxolone methyl) in MC38 colon carcinoma, Lewis lung carcinoma, and EL-4 thymoma mouse tumor models, as well as blood samples from patients with renal cell cancer and soft tissue sarcoma. Samples were also analyzed from patients with pancreatic cancer treated with CDDO-Me in combination with gemcitabine.

Results: CDDO-Me at concentrations of 25 to 100 nmol/L completely abrogated immune suppressive activity of MDSC *in vitro*. CDDO-Me reduced reactive oxygen species in MDSCs but did not affect their viability or the levels of nitric oxide and arginase. Treatment of tumor-bearing mice with CDDO-Me did not affect the proportion of MDSCs in the spleens but eliminated their suppressive activity. This effect was independent of antitumor activity. CDDO-Me treatment decreased tumor growth in mice. Experiments with severe combined immunodeficient–beige mice indicated that this effect was largely mediated by the immune system. CDDO-Me substantially enhanced the antitumor effect of a cancer vaccines. Treatment of pancreatic cancer patients with CDDO-Me did not affect the number of MDSCs in peripheral blood but significantly improved the immune response.

Conclusions: CDDO-Me abrogated the immune suppressive effect of MDSCs and improved immune responses in tumor-bearing mice and cancer patients. It may represent an attractive therapeutic option by enhancing the effect of cancer immunotherapy. *Clin Cancer Res*; 16(6); 1812–23. ©2010 AACR.

In recent years, it has become increasingly clear that tumor-associated immune suppression not only contributes greatly to tumor progression but is also one of the major factors limiting the activity of cancer immunotherapy. Antigen-specific T-cell tolerance is one of the major mechanisms of tumor escape (1–3). The antigen-specific nature of tumor nonresponsiveness explains the fact that tumor-bearing hosts are not capable of maintaining tumor-

specific immune responses while still responding to other immune stimuli (4–6). Recent studies provide evidence that myeloid-derived suppressor cells (MDSC) may represent the major population of antigen presenting cells responsible for the induction of antigen-specific CD8⁺ T-cell tolerance in cancer. These cells have also been implicated in nonspecific immune suppression, as well as in the promotion of tumor vascularization and invasion (reviewed in refs. 7–9).

MDSCs are a group of myeloid cells comprised of hematopoietic progenitor cells and precursors of macrophages, dendritic cells, and granulocytes. These cells are part of normal hematopoiesis but dramatically expand in many types of cancer in mice and men. MDSC are highly active in suppression of T-cell responses. MDSC can phagocytose antigens, migrate to peripheral lymphoid organs, and process and present these antigens to T cells (5, 10, 11). Arginase, nitric oxide (NO), and reactive oxygen species are all implicated in MDSC-mediated T-cell suppression (8).

Realization of the important role of MDSC in suppression of immune responses in cancer prompted attempts to eliminate these cells. Several different approaches have

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Translational Relevance

Myeloid-derived suppressor cells (MDSC) are one of the major factors responsible for immune suppression in cancer and contribute to the limited efficacy of current vaccination strategies. Therefore, it is important to identify therapeutic methods that neutralize these cells. Herein, we report the modulatory effects of the triterpenoid C-28 methyl ester of 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO-Me; bardoxolone methyl) on MDSCs. In different mouse tumor models *in vivo* and blood samples from cancer patients treated with CDDO-Me, we show a potent effect of this compound in neutralizing MDSC function and improving immune responses. These findings have potential for direct clinical relevance because they show that CDDO-Me may represent an attractive therapeutic option by enhancing the effect of cancer immunotherapy.

been tested. They include elimination of MDSC using differentiating agents such as all-*trans* retinoic acid (6, 12), chemotherapy (13, 14), aminobiphosphonates (15), tyrosine kinase inhibitors (sunitinib; refs. 16–18), cyclooxygenase 2 inhibitors (19–21), and inhibition of MDSC function by the phosphodiesterase-5 inhibitors (sildenafil; ref. 22). These compounds have shown promise in pre-clinical testing, and some are currently in clinical trials. However, most of them have pleiotropic effects and can be associated with substantial toxicity.

In search for a specific well-tolerated agent for the therapeutic neutralization of MDSC, we focused on a relatively new class of compounds, synthetic triterpenoids, specifically the methyl ester of 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO-Me; bardoxolone methyl), the most potent representative of this group of molecules. At nanomolar concentrations, CDDO-Me is a potent activator of the nuclear factor-erythroid 2-related factor 2 (NRF2) transcription factor that results in upregulation of several antioxidant genes, including NADPH:quinone oxidoreductase 1 (NQO1), thioredoxin, catalase, superoxide dismutase, and heme oxygenase. This results in reduction of intracellular reactive oxygen species (23). Because upregulation of reactive oxygen species is one of the main mechanisms of MDSC activity, we hypothesized that triterpenoids could be a useful tool in regulating MDSC function in cancer. We tested this hypothesis *in vitro* and *in vivo* in different animal tumor models and in pancreatic cancer patients. This work showed that CDDO-Me was highly effective for the abrogation of immune-suppressive activity of MDSC in tumor-bearing hosts, resulting in improved immune responses.

Materials and Methods

Patients. Experiments *in vitro* were done using samples of peripheral blood collected from nine patients with his-

tologically confirmed, locally advanced or metastatic renal cell carcinoma or soft tissue sarcoma. None of the patients had been treated with chemotherapy or radiation therapy for at least 6 mo before collection of blood.

Peripheral blood mononuclear cells from 19 patients (9 females and 10 males; age, 46–80 y) who were treated in a phase I clinical trial with RTA 402 conducted at Sammons Cancer Center (Dallas, TX) were analyzed (study ID number C-0702). All patients were diagnosed with locally advanced (stage II–III) or metastatic (stage IV) pancreatic adenocarcinoma and were not amenable to resection with curative intent. CDDO-Me (RTA 402) was administered orally daily for 21 d. Nine patients received a dose of 150 mg/d; 2 patients, 200 mg/d; 6 patients, 250 mg/d; and 2 patients, 300 mg/d. All patients were treated with gemcitabine (1,000 mg/m² i.v.) on days 1, 8, and 15 starting the week RTA 402 was initiated. Cycles were repeated every 28 d. Immunologic evaluations were done before the start of treatment and after 2 wk of treatment with CDDO-Me. All patients provided a written informed consent in an Institutional Review Board–approved protocol.

Mice and tumor models. Female C57BL/6 mice with the age of 6 to 8 wk were obtained from the National Cancer Institute (Frederick, MD). Female severe combined immunodeficient (SCID)–beige mice with the age of 6 to 8 wk were purchased from Taconic. Mice were kept in pathogen-free conditions and handled in accordance with the requirements of the Guideline for Animal Experiments. The following s.c. tumor models were used: EL-4 thymoma (obtained from American Type Culture Collection, Lewis lung carcinoma (LLC), and MC38 colon carcinoma (provided by I. Turkova, University of Pittsburgh, Pittsburgh, PA). LLC–IL-1 β cell line was created by transduction of pLXSN/ssIL-1 β plasmid made by R. Apte (Ben Gurion University of Negev, Israel) and provided by S. Ostrand-Rosenberg (University of Maryland, Baltimore, MD) using Nucleofector kit (Amaxa). After transduction, cells were cultured for 48 h, followed by G418 selection (CalBiochem). Clones with highest IL-1 β production were selected.

Isolation of cells and functional assays. MDSCs were immune-magnetically isolated from spleens of tumor-bearing mice using biotinylated anti-Gr1 antibody (BD Pharmingen) and streptavidin beads (Miltenyi). The purity of Gr-1⁺CD11b⁺ cell populations was >95%. Splenocytes from OT-1 mice were used in functional tests. CD8⁺ T cells from these mice have a T cell receptor (TCR) that recognize ovalbumin-derived peptide SIINFEKL. The number of IFN- γ -producing cells in response to stimulation with 10 μ g/mL specific or control (RAHYNIVTF) peptide was evaluated in an Enzyme-Linked ImmunoSpot (ELISPOT) assay as described earlier (24). The spots were counted in triplicate and calculated using an automatic ELISPOT counter (Cellular Technology, Ltd.). Peptides were purchased from American Peptide Company. Cell proliferation induced by antigen-specific or CD3/CD28 antibody stimulation (0.5 and 5 μ g/mL, respectively) was evaluated using [³H]thymidine incorporation as described previously (24).

For some assays, 2 million splenocytes were incubated with the relevant antibodies, and then, flow cytometry data were acquired using a FACSCalibur flow cytometer (BD Biosciences) and were analyzed using FlowJo software (Tree Star).

Dendritic cell culture and isolation of MDSC and T cells. Dendritic cells were generated from Mononuclear cells (MNC) obtained from healthy donor leukocyte-enriched buffy coat (Florida Blood Services) by 5-d culture with 50 ng/mL recombinant human granulocyte macrophage colony-stimulating factor (PeproTech, Inc.) and 6 ng/mL IL-4 (PeproTech, Inc.). T cells were purified from another unrelated healthy donor buffy coat by enrichment from peripheral blood mononuclear cells using human T-cell enrichment columns (R&D Systems, Inc.) according to the manufacturer instructions.

For isolation of MDSC, MNC isolated from 30 mL of patient's peripheral blood were cultured overnight in complete media at a concentration of 4×10^6 /mL. For the evaluation of phenotype, cells were labeled with appropriate panels of antibodies, and viable cells (4',6-diamidino-2-phenylindole negative) were sorted into Lin⁻HLA-DR⁻CD33⁺ or CD14⁻CD33⁺CD11b^{high} cells using a FACSARIA cell sorter (Becton Dickinson). Lineage (Lin) markers included CD3, CD14, CD19, and CD56. All monoclonal antibodies used were from BD Pharmingen. The phenotype of the cells and postsort purity was evaluated by multicolor flow cytometry using a FACSARIA cytometer (BD Biosciences) using FlowJo software.

Qualitative and quantitative identification of T cells secreting IFN- γ (ELISPOT assay). The IFN- γ ELISPOT assay was done in 96-well MultiScreen-HA plates (Millipore) coated overnight at 4°C with 1.4 μ g/mL purified anti-human IFN- γ monoclonal antibody (clone 1-D1K; Mabtech, Inc.) in Dulbecco's Phosphate Buffered Saline (DPBS) (Thermo Scientific). T cells (2×10^5) and autologous dendritic cells were placed into each well at a 50:1 ratio in triplicates with or without MDSC at different ratios (1:2, 1:4, and 1:8). CDDO-Me was added at a concentration of 200 or 300 nmol/L. Triplicates for positive controls (T cells + 5 μ g/mL phytohemagglutinin; Sigma-Aldrich) and negative controls (T cells alone) were also added to the assay. Following 48-h incubation at 37°C in a humidified 5% CO₂ incubator, plates were washed in DPBS and incubated for 2 h with 1 μ g/mL biotinylated anti-human IFN- γ monoclonal antibody (clone 7-B6-1; Mabtech, Inc.), followed by 1 h incubation with 1 μ g/mL streptavidin-horseradish peroxidase at room temperature. Spots were visualized with 50 μ L per well 3,3',5,5'-tetramethylbenzidine-hydrogen (TMB-H) peroxidase substrate (Moss, Inc.). The spots were counted with an ImmunoSpot System (Cellular Technology, Ltd.).

Reactive oxygen species production. The oxidation-sensitive dye DCFDA (Molecular Probes/Invitrogen) was used for the measurement of reactive oxygen species production by MDSC. Cells were incubated at room temperature in serum-free RPMI media in the presence of 3 μ mol/L DCFDA with or without 300 nmol/L phorbol 12-myristate 13-acetate

for 30 min, washed with PBS, and then labeled with anti-CD11b-PE-Cy7 and anti-Gr-1-APC antibodies. After incubation on ice for 20 min, cells were washed with PBS and analyzed using flow cytometry.

Arginase activity. Arginase activity was measured in cell lysates as previously described (25).

NO production. Equal volumes of culture supernatants (100 μ L) were mixed with Greiss reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in double-distilled water). After 10 min incubation at room temperature, the absorbance at 550 nm was measured using a microplate plate reader (Bio-Rad). Nitrite concentrations were determined by comparing the absorbance values for the test samples to a standard curve generated by serial dilution of 0.25 mmol/L sodium nitrite.

Quantitative real-time PCR. Total RNA was extracted from cells with Trizol (Invitrogen). Traces of DNA were removed by treatment with DNase I. The cDNA was synthesized from 1 μ g of total RNA using random hexamers and Superscript II reverse transcription (Invitrogen) according to the manufacturer's protocol. PCR was done with 2.5 μ L cDNA, Taqman Universal PCR Master Mix (Applied Biosystems), and target gene assay mix containing sequence-specific primers for NQO1 and 6-carboxyfluorescein dye-labeled Taqman minor groove binder probe (assay ID Mm00500821_m1; Applied Biosystems). Amplification with 18S endogenous control assay mix was used for controls. Data quantitation was done using the comparative Δ - Δ Ct method. Expression levels of the genes were normalized by 18S rRNA.

Dendritic cell vaccine. Bone marrow cells were obtained from the femurs and tibias of mice and were cultured in complete RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 ng/mL granulocyte macrophage colony-stimulating factor, 10 ng/mL IL-4, and 50 mmol/L 2-mercaptoethanol. On day 5, cells were collected, and dendritic cells were enriched by centrifugation over a Nycoprep gradient (Accurate Chemical and Scientific Corporation). Dendritic cells (2×10^6) were washed and infected with adenoviruses encoding full-length survivin gene (5,000 viral particles per cell). The details of this construct were described elsewhere (26). Dendritic cells were activated with lipopolysaccharide (100 ng/mL) for 18 h and washed in PBS, and 5×10^5 cells were injected s.c. into mice.

Statistical analysis. For *in vitro* experiments, statistical analysis was done using two-tailed Mann-Whitney or Wilcoxon rank sum test with significance level of 0.05. To compare two related groups, Wilcoxon signed rank test was used. For tumor measurements, the Anderson-Darling statistics and normal curves were examined to assess normality, and if tumor measurements were normally distributed, then ANOVA test was used. If the normality assumption was violated but the distributions were symmetric, Kruskal-Wallis test was used to determine whether there was significant difference in tumor sizes measured across treatment groups at each time. Tukey's method was applied for all pairwise group comparison. All statistical analyses were done using SAS (version 9.1; SAS Institute).

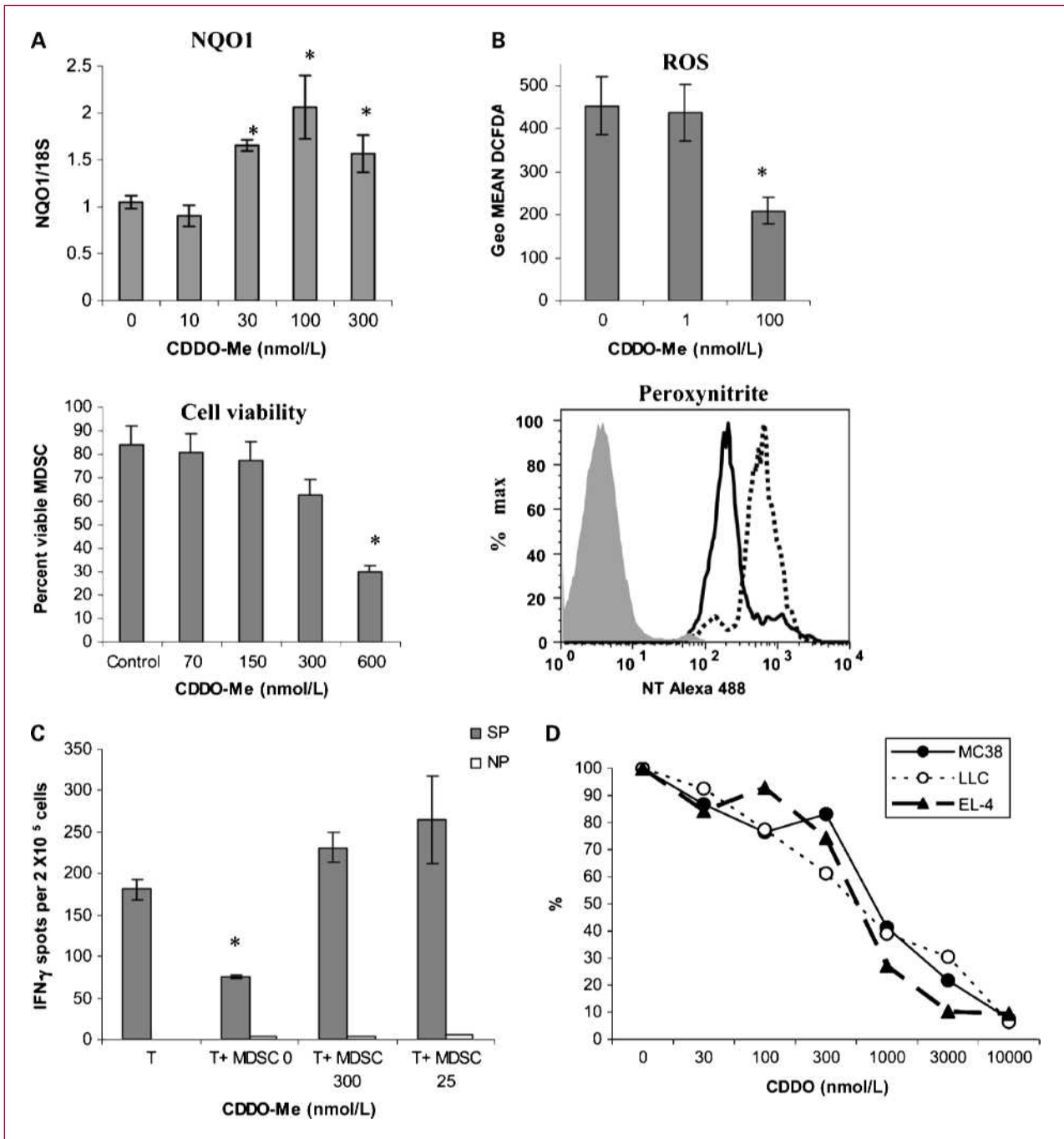


Fig. 1. Effect of CDDO-Me on MDSC *in vitro*. A and B, MDSC were isolated from spleens of EL-4 tumor-bearing mice and treated with different concentrations (10–300 nmol/L) of CDDO-Me for 24 hr. *, statistically significant difference from control ($P < 0.05$). A, RNA was extracted from isolated MDSC, and expression of NQO1 was measured in triplicates by quantitative real-time PCR. CDDO-Me-treated MDSC were stained with propidium iodide, anti-CD11b-FITC, and anti-Gr1-APC antibodies and analyzed in triplicates by flow cytometry. Bars, percent of viable CD11b⁺Gr1⁺ cells. B, the level of reactive oxygen species in cells was measured using DCFDA staining and flow cytometry. ROS, reactive oxygen species. MDSC were stained with antibodies against NT-Alexa 488, CD11b-PE, and Gr1-APC, and NT staining was analyzed using flow cytometry. Representative histogram of CDDO-Me-treated MDSC (CD11b⁺ Gr1⁺ gated NT⁺ cells): shaded area, isotype control; solid line, nontreated MDSC; dashed line, 100 nmol/L CDDO-Me-treated MDSC. C, splenocytes from EL-4 tumor-bearing mice were treated with 300 and 25 nmol/L CDDO-Me for 24 h. MDSC were isolated and cultured with 2×10^5 OT-1 transgenic T cells at a 1:3 ratio and stimulated with specific peptide, and the number of IFN- γ -producing cells was evaluated in quadruplicates by an ELISPOT assay. D, tumor cells (EL-4, MC38, and LLC) were seeded in triplicates in 96-well plates at a concentration of 2×10^5 /mL. The number of viable tumor cells was assessed after 24-h incubation with indicated concentrations of CDDO-Me in a standard MTT assay. Percent sign, percentage of viable cells to untreated control. The statistically significant decrease in number of cells was detected for all cell lines at 1 μ mol/L concentration of CDDO-Me ($P < 0.05$ in Mann-Whitney test).

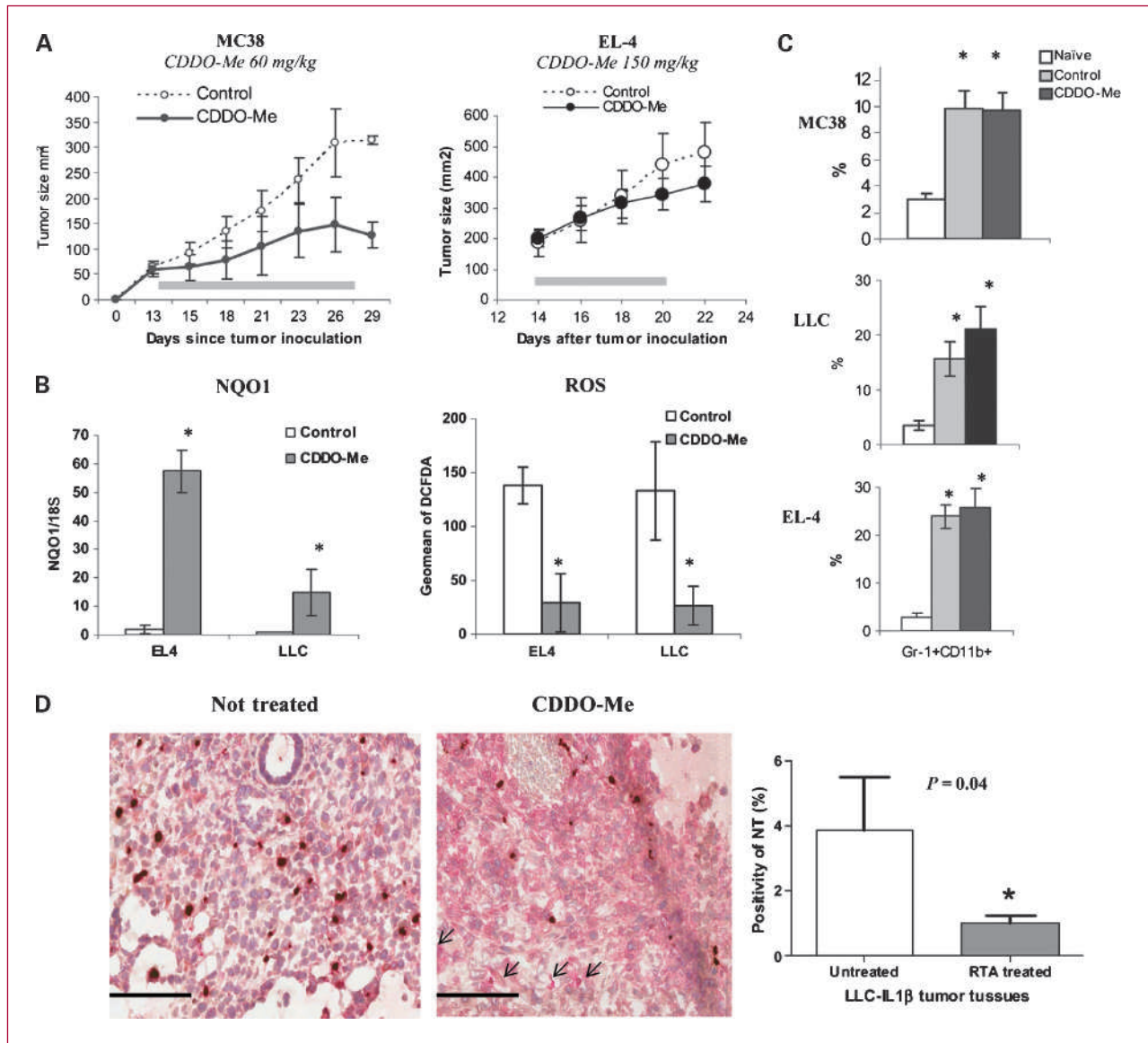


Fig. 2. Effect of CDDO-Me on MDSC *in vivo*. A, tumors were established s.c. in C57BL/6 mice. Two weeks after tumor inoculation, mice were treated with either control or CDDO-Me (60-150 mg/kg chow) food for the indicated periods (gray line). Tumor size (mean and SD) was monitored every 2 to 3 d. Each group includes five mice. B, RNA was extracted from MDSC, and the expression of NQO1 was measured by quantitative real-time PCR. The level of reactive oxygen species in MDSC in CDDO-Me-treated tumor-bearing mice was measured using DCFDA staining and flow cytometry. C, splenocytes from MC38, EL-4, or LLC tumor-bearing mice treated with CDDO-Me for 7 d were stained with anti-CD11b and anti-Gr-1 antibodies, and the percentage of Gr-1⁺CD11b⁺ cells was analyzed using flow cytometry. Each group included three mice. *, statistically significant differences from control ($P < 0.05$). D, LLC-IL-1 β tumors were established in C57BL/6 mice. When tumor reached 1 cm in diameter, mice were treated with 150 mg/kg chow CDDO-Me for 7 d. After that time, tumor tissues were collected and stained with rabbit anti-NT antibody (Millipore-Upstate) and rat anti-Gr-1 antibody (BD Pharmingen). The corresponding secondary antibody were labeled with either peroxidase or alkaline phosphatase, and colors were developed using VECTASTAIN ABC kit (Vector). Dark brown, NT staining; red, Gr-1. Images were taken by digital slide scanner Scanscope (Aperio) and analyzed by Aperio software. Arrows point on Gr-1⁺ NT⁻ cells. Bottom, percentage (mean \pm SD) of NT⁺ cells per field. Ten randomly selected fields ($4 \times 10^5 \mu\text{m}^2$) were counted. Bar, 100 μm .

Results

Regulation of MDSC function by CDDO-Me in vitro. To assess the possible effect of triterpenoid on MDSC, Gr-1⁺CD11b⁺ cells were isolated from spleens of EL-4 tumor-bearing mice and then cultured for 24 hours in the pres-

ence of 10 ng/mL granulocyte macrophage colony-stimulating factor and different concentrations of CDDO-Me. First, we evaluated whether CDDO-Me-induced upregulation of a specific target gene in MDSC. Previous studies have shown that CDDO-Me selectively upregulated expression of NQO1 (27). Therefore, we used NQO1 as an

indicator of triterpenoid activity in these cells. CDDO-Me significantly increased NQO1 expression at concentrations starting from 30 nmol/L (Fig. 1A). At these concentrations, CDDO-Me did not affect MDSC viability. Significant cell death was observed only at a concentration of 600 nmol/L (Fig. 1A). Several major factors are implicated in MDSC-mediated immune suppression. These include arginase,

NO, and reactive oxygen species (8). We investigated the effect of CDDO-Me on these factors. At all tested concentrations, CDDO-Me did not affect arginase activity or NO level in MDSC (Supplementary Fig. S1). In contrast, CDDO-Me significantly reduced the level of reactive oxygen species in MDSC (Fig. 1B). Increased production of peroxynitrite, which is a result of interaction between

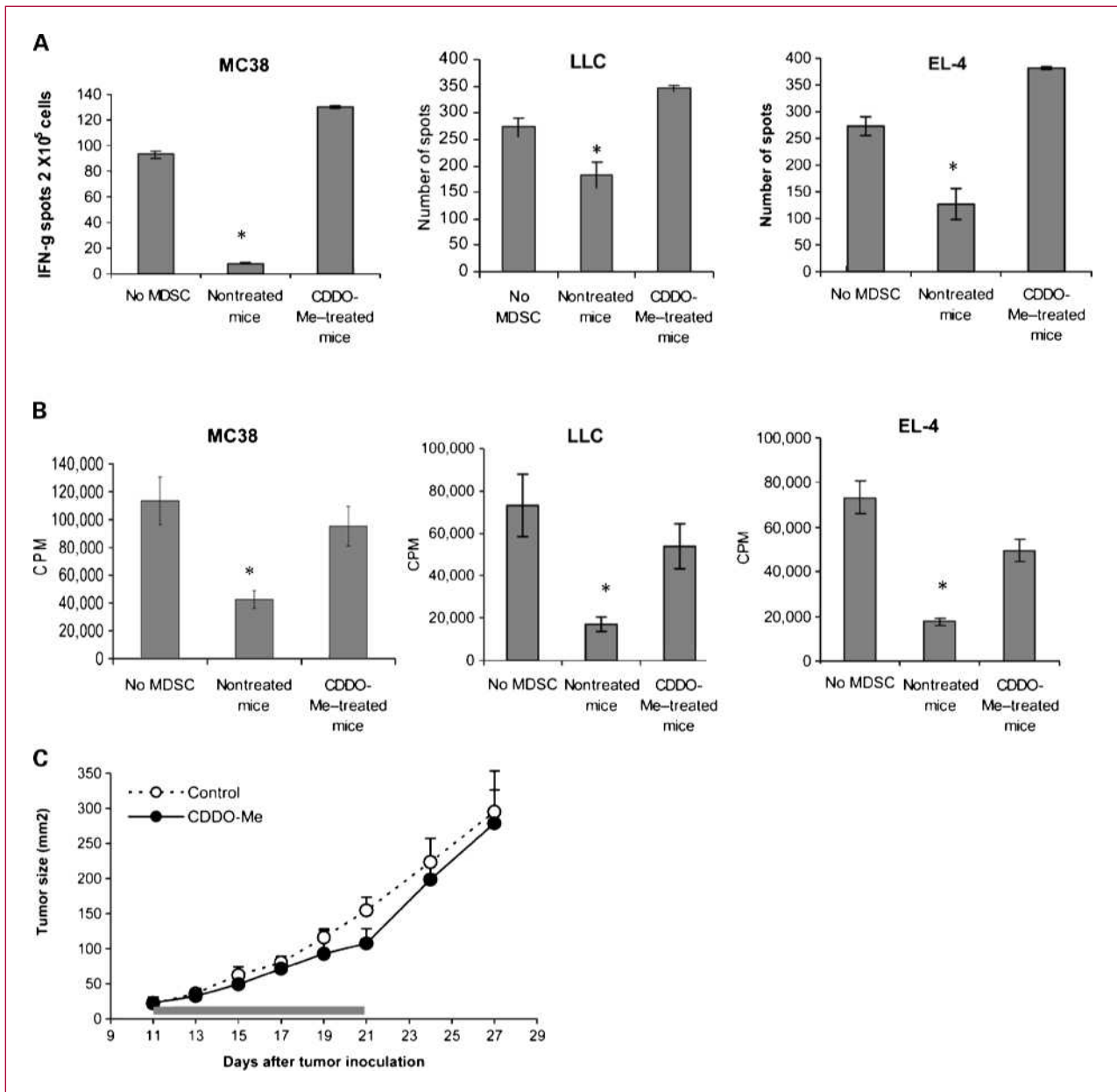


Fig. 3. Effect of synthetic triterpenoid on antigen-specific T-cell responses. A, splenocytes from OT-1 transgenic mice were cultured with MDSC isolated from spleens of MC38, LLC, or EL-4 tumor-bearing mice at a 3:1 ratio. A, an IFN- γ ELISPOT assay was done in quadruplicates with two mice per group. The experimental values obtained in the presence of control peptide were subtracted from the values obtained in the presence of specific peptide. B, cell proliferation was measured in triplicate cultures using [³H]thymidine assay with two mice in each group. The experimental values obtained in the presence of control peptide were subtracted from the values obtained in the presence of specific peptide. CPM, counts per minute. *, statistically significant differences from control ($P < 0.05$). C, MC38 tumors were established s.c. in SCID-beige mice. Treatment with CDDO-Me (150 mg/kg chow) was started on day 11 and continued for 10 d (gray line). Tumor size (mean and SD) was monitored every 2 to 3 d ($n = 4$ per group).

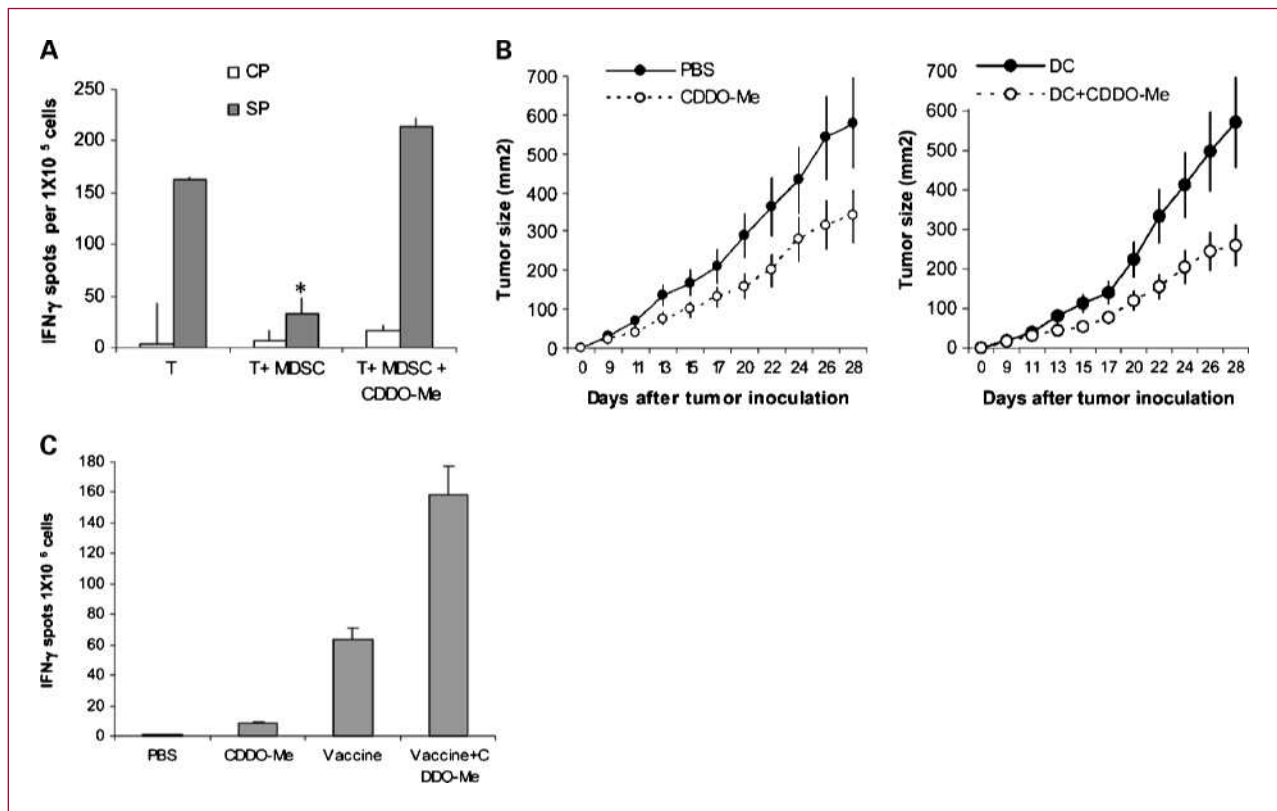


Fig. 4. Combination of synthetic triterpenoid treatment and dendritic cell vaccine. OT-1 T cells ($4\text{--}5 \times 10^6$) were transferred i.v. into naïve C57BL/6 mice. Two days later, mice were transferred i.v. with 3 to 4×10^6 MDSC isolated from spleens of nontreated or CDDO-Me-treated tumor-bearing mice. Recipient mice were pretreated with CDDO-Me for 5 d. Eight days after the transfer, antigen-specific CD8⁺ T cells were evaluated by an IFN- γ ELISPOT assay. A, results of IFN- γ ELISPOT assay done in quadruplicate. Two mice in each group were used. CP, cells stimulated with control peptide; SP, cells stimulated with specific peptide. B, EL-4 tumor cells were inoculated s.c. into C57BL/6 mice. Three days after tumor inoculation, mice were treated thrice with either PBS or dendritic cells infected with adenoviruses encoding full-length survivin gene, with 7-d interval. Mice were treated with CDDO-Me (150 mg/kg chow) in three short cycles (4 d each) on days 3 to 7, 10 to 14, and 17 to 21 after tumor inoculation. Tumor size (mean and SD) was monitored twice to thrice a week. Each group included six mice. DC, dendritic cells. C, T cells were isolated from spleens of tumor-bearing mice on day 28, mixed with naïve splenocytes, and incubated in triplicates with 10 $\mu\text{g}/\text{mL}$ of survivin-specific (CPTENEPDL) or control (SIINFEKL) peptides for 48 h, and IFN- γ production was evaluated by an ELISPOT assay. The number of IFN- γ -producing cells was calculated per 10^6 cells. The values of IFN- γ production in the presence of control peptide were subtracted from the values obtained in the presence of specific peptide. Each group included three mice.

reactive oxygen species (superoxide) and NO, is one of the hallmarks of MDSC functional activity (5, 8, 28, 29). CDDO-Me dramatically reduced the level of nitrotyrosine (NT) in MDSC, which reflects peroxynitrite activity (Fig. 1B).

Inhibition of antigen-specific CD8⁺ T cells is the main characteristic of MDSC. To assess the effect of CDDO-Me on the suppression by MDSCs of T-cell responses, Gr-1⁺ CD11b⁺ cells were isolated from spleens of EL-4 tumor-bearing mice and cultured with OT-1 T cells in the presence of a specific peptide (SIINFEKL). MDSC significantly reduced T-cell responses to the specific peptide. CDDO-Me completely abrogated this suppressive activity (Fig. 1C).

We evaluated direct antitumor effect of CDDO-Me *in vitro*. In all three tested mouse tumor lines, CDDO-Me showed significant antitumor activity. However, the effect was observed only at concentrations $>1 \mu\text{mol}/\text{L}$ (Fig. 1D). Thus, CDDO-Me at low to midrange nanomolar concentrations did not affect MDSC viability. However, it blocked reactive oxygen species production, decreased levels of per-

oxynitrite, and abrogated MDSC suppressive activity against antigen-specific CD8⁺ T cells.

Effect of synthetic triterpenoid on MDSC function in tumor-bearing mice *in vivo*. To assess the effect of CDDO-Me on MDSC *in vivo*, three experimental models were used: EL-4 thymoma, LLC lung carcinoma, and MC38 colon carcinoma. All tumors were established s.c. Treatment with CDDO-Me was started on day 14 after tumor inoculation when tumor reached 7 to 8 mm in diameter. Mice received CDDO-Me dissolved in ethanol and Noebee oil before being mixed into powdered chow, and the concentration was calculated per chow weight. We tested several doses of CDDO-Me. Toxicity was evaluated based on animal weight and gross signs of distress. The doses of 60 to 100 mg/kg chow did not cause toxicity during at least 14 days of continuous administration unlike 150 mg/kg, which was only tolerated for 7 to 10 days.

Initially, we treated mice bearing MC38 tumors at 60 mg/kg dose for 14 days and observed significant ($P < 0.05$)

decrease in tumor growth (Fig. 2A). However, when we treated EL-4-bearing mice with the same dose of CDDO-Me, no effect on tumor growth was observed (data not shown). Increasing the dose of CDDO-Me to 100 mg/kg resulted in only a slight delay in tumor growth (Supplementary Fig. S2), and further escalation of the dose to 150 mg/kg (given for 7 days) did not enhance the effect of the compound in this tumor model (Fig. 2A). Similar results were obtained in LLC tumor-bearing mice (Supplementary Fig. S2). Analysis of the MDSC population in mice with different tumor sizes would not be very useful because tumor burden would have a direct effect on MDSC generation and function. Therefore, we evaluated MDSC after 1 week of treatment with 150 mg/kg CDDO-Me (3 weeks after tumor inoculation) when differences in tumor sizes were not statistically significant. Triterpenoid caused a dramatic upregulation of the target gene *NQO1* and a significant decrease in the level of reactive oxygen species in MDSC (Fig. 2B) without affecting arginase activity in MDSC (data not shown). All three tumor models caused significant increase in the presence of MDSC in spleens. In all these models, treatment with CDDO-Me did not affect the proportion (Fig. 2C) or absolute number (data not shown) of MDSC.

To assess the effect of CDDO-Me on the presence of NT⁺ MDSC *in vivo*, we injected mice with LLC cells that overexpress IL-1 β . LLC-IL-1 β tumor-bearing mice had dramatic expansion of MDSC in spleens (data not shown) and large presence of Gr-1⁺ cells in tumor tissues (Fig. 2D). Practically all these cells in tumor tissues were NT⁺ indicating production of peroxynitrite. Seven-day treatment of mice with CDDO-Me resulted in significant decrease in the presence of NT⁺ cells. In contrast to nontreated mice tumors from CDDO-Me treated mice contained Gr-1⁺ NT⁻ cells (Fig. 2D).

Suppression of antigen-specific CD8⁺ T cells was evaluated using IFN- γ ELISPOT and proliferation assays. MDSC from all three tumor models inhibited CD8⁺ antigen-specific T-cell responses, and treatment with CDDO-Me completely abrogated this suppression (Fig. 3A and B). Because the synthetic triterpenoid had significant antitumor effect in MC 38 tumor-bearing mice, we asked whether it was mediated by an immune system response. To address this question, MC38 tumors was established in SCID-beige mice. Mice were treated with 150 mg/kg CDDO-Me for 10 days. The antitumor effect of CDDO-Me in these mice was not statistically significant (Fig. 3C).

It was still possible that triterpenoid affected MDSC function in tumor-bearing mice indirectly by manipulating the tumor microenvironment. To evaluate the effect of triterpenoid on MDSC *in vivo* in the tumor-free host, OT-1 T cells were transferred *i.v.* into naïve tumor-free C57BL/6 mice. MDSC were isolated from the spleens of EL-4 tumor-bearing mice and transferred into the tumor-free recipients described above 2 days later, followed by immunization with the specific peptide SIINFEKL. Recipient mice were pretreated with CDDO-Me for 5 days before transfer of MDSC, followed by three additional days of treatment after MDSC transfer (Supplementary Fig. S3).

Eight days later, lymph nodes were collected and stimulated with specific peptide. Response was evaluated by an IFN- γ ELISPOT assay. In the absence of MDSC transfer, the T cells in recipient mice showed a strong antigen-specific response. Adoptive transfer of MDSC dramatically reduced this response. Treatment of mice with CDDO-Me completely abrogated this suppression (Fig. 4A).

Next, we asked whether triterpenoid might affect the immune response in tumor-bearing mice vaccinated with a tumor-associated antigen. EL-4 tumor-bearing mice were vaccinated with dendritic cells transduced with full-length survivin (26) on days 3, 10, and 17 after tumor inoculation. To maximize the effect of CDDO-Me we used several short cycles of CDDO-Me at a dose of 150 mg/kg chow. Mice were treated on days 3 to 7, 10 to 14, and 17 to 21 after tumor inoculation. Treatment of mice with several cycles of CDDO-Me substantially reduced tumor growth ($P = 0.02$; Fig. 4B). Vaccine by itself had only a moderate effect ($P > 0.1$). Tumor growth slowed down during the first 2 weeks of treatment, and then, it resumed at the previous rate. However, addition of CDDO-Me to the cancer vaccine substantially delayed tumor progression ($P = 0.004$; Fig. 4B). To assess survivin-specific immune responses, T cells were isolated from mice at the end of the treatment and restimulated with a survivin-derived peptide (26). Nontreated or CDDO-Me alone-treated mice showed no specific response to the peptide (Fig. 4E). Moderate specific response was observed in mice vaccinated with dendritic cells-survivin vaccine. In contrast, mice treated with the combination of the vaccine and CDDO-Me showed significantly ($P < 0.05$) higher antigen-specific response (Fig. 4C).

Effect of synthetic triterpenoid on MDSC in cancer patients. The data described above showed that triterpenoid abrogated the suppressive activity of MDSC in mice. We asked whether a similar effect could be observed in cancer patients. We have developed an experimental system that allows for a direct evaluation of immune suppressive activity of MDSC in cancer patients. This system uses the ability of MDSC to present antigens in an allogeneic mixed leukocyte reaction. T cells isolated from healthy donors were used as responders. These cells were stimulated with dendritic cells generated from unrelated healthy donors. At a dendritic cell-T cell ratio 1:50, this allogeneic system generated robust T-cell responses. MDSC were isolated from peripheral blood of cancer patients and added at different ratios to this cell mix, and IFN- γ production was evaluated by ELISPOT assays. We used two previously suggested (6, 30, 31) combinations of markers to isolate these cells: Lin⁻HLA-DR⁻CD33⁺ and CD14⁻CD11b⁺CD33⁺ cells (Supplementary Fig. S4). MDSC were sorted from the blood of patients with renal cell carcinoma or soft tissue sarcoma and incubated with the dendritic cell-T cell mix (described above) at 1:2 to 1:8 (MDSC-T cell) ratio. For each combination of markers, blood from three different patients was tested. CD14⁻CD11b⁺CD33⁺ MDSC caused profound inhibition of T-cell responses to allogeneic dendritic cells at a 1:2 ratio in all three tested patients. At a 1:4 ratio, this effect was reduced but still remained statistically

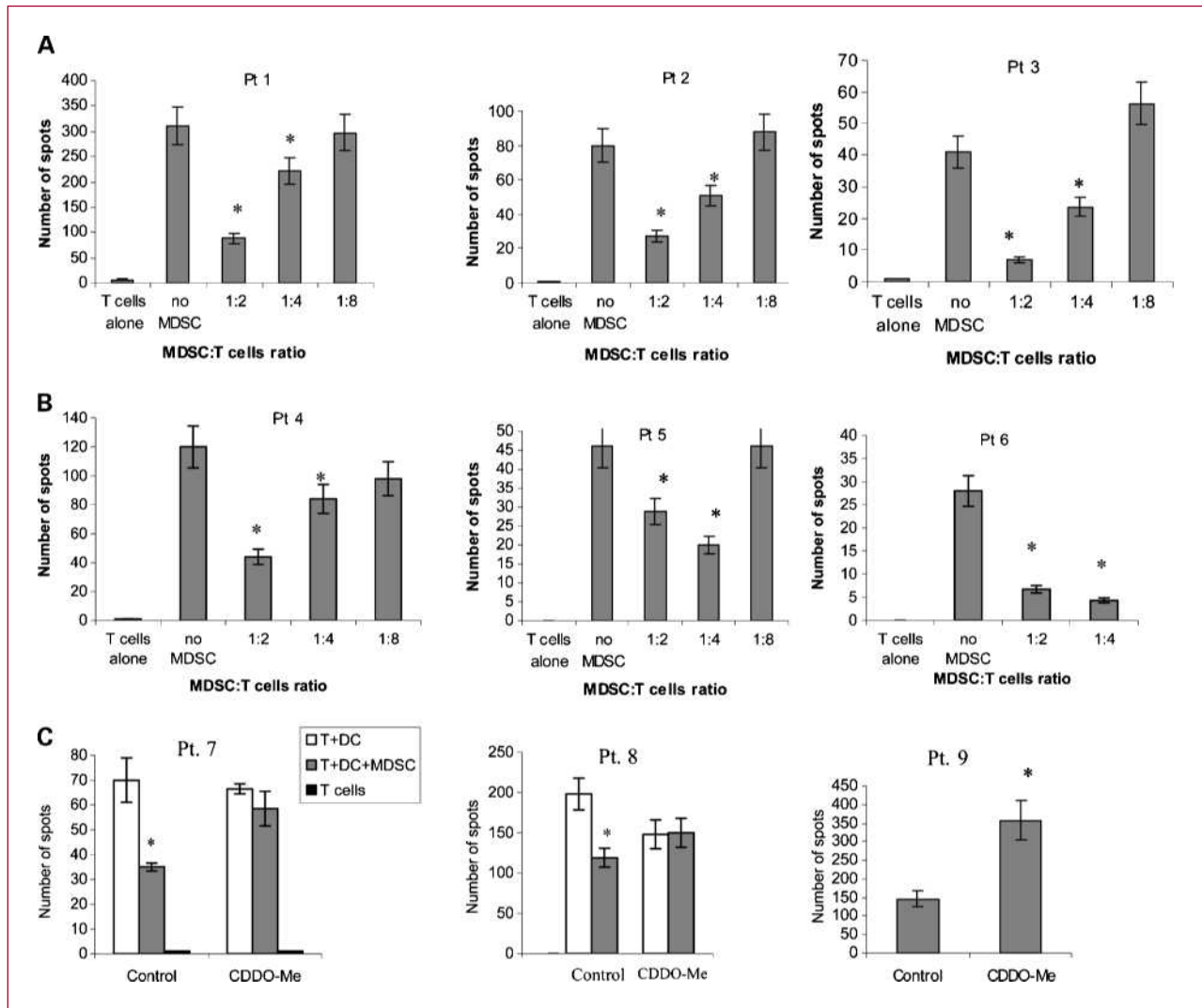


Fig. 5. Abrogation of MDSC suppressive activity by synthetic triterpenoid in cancer patients. A, MDSCs were sorted from peripheral blood of cancer patients using the antibody panel: CD3,CD14,CD19,CD56⁻ (Lin⁻), HLA-DR⁻CD33⁺ cells (patients 1, 2, and 3). Pt, patient. B, CD14⁻CD33⁺CD11b^{high} (patients 4, 5, and 6). The sorted MDSC were cultured at different ratios with T cells from a healthy donor and tested for the production of IFN- γ in an ELISPOT assay. C, sorted CD14⁻CD33⁺CD11b^{high} MDSC cultured with T cells in the presence of allogeneic dendritic cells were either untreated or treated with 200 nmol/L (patient 7) or 300 nmol/L (patients 8 and 9) CDDO-Me and tested for their ability to produce IFN- γ in an ELISPOT assay. *, statistically significant differences compared with controls ($P < 0.05$).

significant. At a 1:8 ratio, the inhibitory effect was no longer observed (Fig. 5A). Lin⁻HLA-DR⁻CD33⁺ MDSC also induced profound inhibition of T-cell responses at a 1:2 ratio in all three tested patients. In two of three patients, this effect was not diminished at a 1:4 ratio and, in one patient, even at a 1:8 ratio (Fig. 5B). Thus, both combinations of markers were suitable for isolating human MDSC. We used the former combination to evaluate the effect of CDDO-Me *in vitro*. MDSC isolated from three patients with renal cell carcinoma were incubated with a mix of allogeneic dendritic cells and T cells in the presence of 200 to 300 nmol/L of CDDO-Me. Triterpenoid completely abrogated the inhibitory effect of MDSC (Fig. 5C).

To evaluate the effect of CDDO-Me on MDSC and immune responses *in vivo*, we analyzed samples from 19 patients with pancreatic adenocarcinoma that were treated in a phase I clinical trial RTA 402-C-0702. Patients were treated with gemcitabine (1,000 mg/m²) i.v. weekly on days 1, 8, and 15. CDDO-Me (RTA 402; bardoxolone methyl) was administered orally once daily for 21 days. Although treatment consisted of several 28-day cycles, immunologic evaluations were done only before the start and after 2 weeks of treatment. Such a relatively short period of evaluation was selected to minimize the possible effect of gemcitabine on MDSC and the immune system.

No toxicity attributed to CDDO-Me was observed. Treatment with RTA 402 and gemcitabine did not significantly affect the proportion of MDSC (Fig. 6A). No differences were also observed in the proportion of Lin⁻HLA-DR⁺ dendritic cells (Fig. 6A). However, 2-week treatment with CDDO-Me and gemcitabine resulted in a significant increase in the patients' T-cell responses to tetanus toxoid and phytohemagglutinin (Fig. 6B).

Discussion

Our study has shown that the synthetic triterpenoid CDDO-Me was able to neutralize MDSC activity in tumor-bearing mice and cancer patients. The important role of MDSC in tumor-associated immune suppression is now well established (8, 32). MDSC exert their suppressive effect through several mechanisms. They include upregulation of arginase and inducible nitric oxide synthase (iNOS), release of transforming growth factor β , downregulation of L-selectin on T cells, etc.; refs. 7, 32, 33). We and others have previously shown that increased production of reactive oxygen species, especially peroxynitrite, plays a critical role in the function of these cells (5, 11, 29, 34). Therefore, we investigated means to block reactive oxygen species in these cells. The synthetic oleanane triterpenoid CDDO-

Me was shown to have a potent antioxidant activity in different experimental systems (27). Therefore, we tested the hypothesis that it might be useful in neutralizing the activity of these cells. Our experiments *in vitro* showed that CDDO-Me activated the target gene *NQO1* and blocked reactive oxygen species in MDSC at 100 nmol/L, which was consistent with previously reported data (23). Interestingly, CDDO-Me did not affect the level of NO in these cells. The absence of the effect was probably due to the low basal level of NO in splenic MDSC. We have previously reported that, in contrast to the cells in tumor sites, MDSC from peripheral lymphoid organs did not have substantial upregulation of iNOS and NO (24, 34). Blockade of reactive oxygen species was sufficient to completely abrogate the immunosuppressive function of MDSC, which was consistent with previous data, underscoring the critical role of reactive oxygen species in MDSC-mediated immune suppression. Inhibition of reactive oxygen species in MDSC is consistent with the recently reported observation that CDDO-Me increases the number of mature dendritic cells in tumor-bearing mice (35). Reactive oxygen species was shown to be an important factor for preventing dendritic cell differentiation in tumor-bearing mice, and the blockade of reactive oxygen species in MDSC promoted their differentiation into dendritic cells (36).

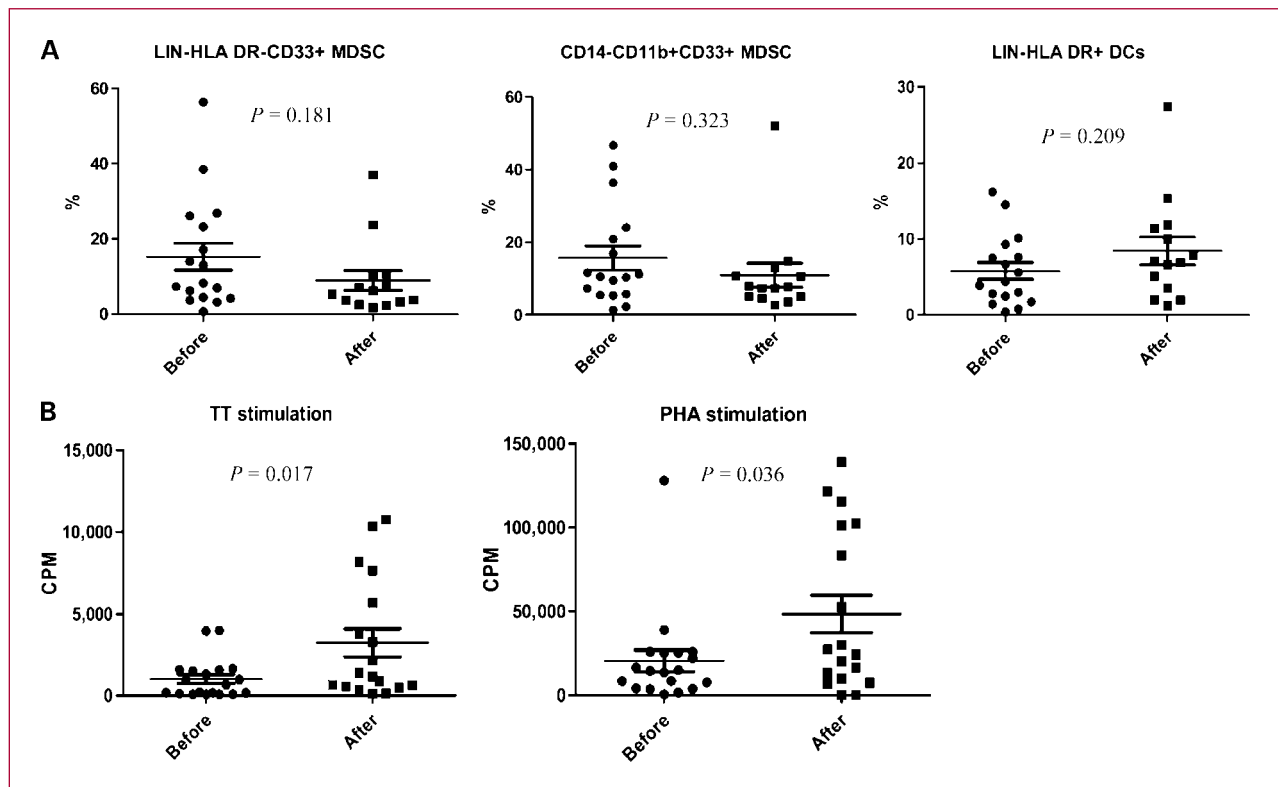


Fig. 6. Effect of synthetic triterpenoid on MDSC in cancer patients. A, effect of CDDO-Me treatment on the proportion of MDSC and dendritic cells. B, MNC (2×10^5 /well) were incubated in triplicates with 0.1 μ g/mL tetanus toxoid or 5 μ g/mL phytohemagglutinin for 4 d. TT, tetanus toxoid; PHA, phytohemagglutinin. [3 H]thymidine (1 μ Ci) was added to each well 18 h before cell harvesting, and proliferation was evaluated in a standard 3 H assay. Values are for each tested patient.

A number of studies have shown that CDDO-Me inhibits signal transducers and activators of transcription 3 (STAT3) activity in tumor cells and that results in tumor cell apoptosis (37–39). STAT3 is a critical factor responsible for the expansion of MDSC. Blockade of STAT3 dramatically reduces the presence of MDSC in tumor-bearing mice (40, 41). Therefore, we expected that CDDO-Me would affect the viability of MDSC *in vitro* and halt their expansion *in vivo*. However, this was not the case. CDDO-Me neutralized MDSC activity at concentrations substantially lower than that reported necessary to block STAT3 activity (100 nmol/L versus 1–5 μ mol/L). Apparently, the concentrations of CDDO-Me achieved *in vivo* were also not sufficient to block STAT3 activity in MDSC because no effect on MDSC expansion was observed.

The synthetic triterpenoid had an antitumor effect *in vivo* with all three tested experimental systems. It is known that the tumor burden directly affects the expansion of MDSC. Therefore, we evaluated the effect of this compound on MDSC after 1 week of treatment before the tumor size in control and treatment groups became significantly different. Our data, together with the results of experiments involving adoptive transfer of MDSC to tumor-free mice, indicate that the effect of CDDO-Me on MDSC *in vivo* was not mediated through its possible effect on the tumor microenvironment. Moreover, experiments with SCID-beige mice suggested that the antitumor effect of this compound was mediated to a large degree by its effect on the immune system but not on tumor cells. SCID-beige mice lack functional T, B, and natural killer cells, major components of the immune system, and cannot develop or mount immune responses. In these mice, CDDO-Me did not affect tumor growth. These data suggest, at least in these models, that the effect of CDDO-Me is mediated through improvement of antitumor immunity, probably by blocking the immune-suppressive effect of MDSC. This conclusion was also based on the results of the experiment involving combination of survivin vaccine and CDDO-Me treatment. We observed significantly higher levels of survivin-specific immune responses in mice treated with CDDO-Me with the vaccine compared with vaccine alone.

The effect of triterpenoid on MDSC was confirmed in experiments with cells isolated from cancer patients. Direct detection of the immune suppressive effect of human MDSC represented a substantial challenge due to the complex nature of the phenotype of these cells in humans and of their sensitivity to isolation procedures. Most of the previous studies have used negative depletion of these cells to evaluate their immune suppressive activity. We have developed methods allowing for a direct evaluation of human

MDSC using cell sorting and allogeneic mixed leukocyte reaction using two unrelated donors. In this experimental system, MDSC exerted a potent immune suppressive effect, but CDDO-Me eliminated that suppressive activity.

We also evaluated the possible effects of CDDO-Me on MDSC in cancer patients *in vivo* by obtaining samples of blood from patients treated in a phase I clinical trial of CDDO-Me (RTA 402) with gemcitabine. This trial design was not primarily to assess the effect of the compound on the immune system. One of the major limitations from these data set is the fact that patients were treated with gemcitabine, which is known to affect the immune system. However, we believe our results still provided useful and important information even in the settings of this design limitation. Gemcitabine is known to cause profound immune suppression (42). On the other hand, it was reported that, in tumor-bearing mice, gemcitabine could eliminate MDSC (13, 43). Therefore, we limited our analysis to only 2 weeks after the start of the treatment. Our data showed that, during that time, gemcitabine in combination with CDDO-Me did not affect the proportion of MDSC in patient's blood, which was consistent with the effect of CDDO-Me in tumor-bearing mice *in vivo*, suggesting that, during the initial 2-week treatment, gemcitabine did not affect MDSC. However, 2-week treatment was enough to observe a significant improvement of the immune responses in these patients. This suggested that the improvement was the result of the CDDO-Me effect. However, these preliminary findings need to be confirmed in a trial specifically designed to address this question.

In summary, our data in tumor-bearing mice and preliminary findings in cancer patients strongly suggest that CDDO-Me neutralizes the activity of MDSC and improves antitumor immune responses. These findings warrant further testing of this compound in clinical trials.

Disclosure of Potential Conflicts of Interest

C. Meyer is employed by and is an advisory board member of Reata Pharmaceuticals, Inc. No potential conflicts of interest were disclosed by the other authors.

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