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Doxorubicin Eliminates Myeloid-Derived Suppressor Cells and Enhances the Efficacy of Adoptive T Cell Transfer in Breast Cancer

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Abstract

Myeloid-derived suppressor cells (MDSC) expand in tumor-bearing hosts and play a central role in cancer immune evasion by inhibiting adaptive and innate immunity. They therefore represent a major obstacle for successful cancer immunotherapy. Different strategies have thus been explored to deplete and/or inactivate MDSC *in vivo*. Using a murine mammary cancer model, we demonstrated that doxorubicin selectively eliminates MDSC in the spleen, blood and tumor beds. Furthermore, residual MDSC from doxorubicin-treated mice exhibited impaired suppressive function. Importantly, the frequency of CD4⁺ and CD8⁺ T lymphocytes and consequently the effector lymphocytes or natural killer (NK) to suppressive MDSC ratios were significantly increased following doxorubicin treatment of tumor-bearing mice. In addition, the proportion of natural killer (NK) and cytotoxic T cell (CTL) expressing perforin and granzyme B and of CTL producing IFN γ was augmented by doxorubicin administration. Of therapeutic relevance, this drug efficiently combined with Th1 or Th17 lymphocytes to suppress tumor development and metastatic disease. MDSC isolated from patients with different types of cancer were also sensitive to doxorubicin-mediated cytotoxicity *in vitro*. These results thus indicate that doxorubicin may be used not only as a direct cytotoxic drug against tumor cells, but also as a potent immunomodulatory agent that selectively impairs MDSC-induced immunosuppression, thereby fostering the efficacy of T cell-based immunotherapy.

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Keywords

Myeloid-Derived Suppressor Cells; Doxorubicin; T helper 1; T helper 17; Reactive oxygen species

Introduction

Myeloid-derived suppressor cells (MDSC) have been described as a heterogeneous population of immature myeloid cells that, in different pathological conditions, are impaired in their ability to terminally differentiate into mature myeloid lineages such as macrophages, dendritic cells or granulocytes (1, 2). MDSC are functionally defined by their capability to inhibit both innate and adaptive immunity. They are potent suppressors of T cell proliferation and activation in humans and mice (1–4). Phenotypically, mouse MDSC express the markers Gr-1 and CD11b. Two main populations have been described in mice based on the relative expression of two epitopes of Gr-1 (Ly6G and/or Ly6C): granulocytic MDSC are CD11b⁺Ly6G⁺Ly6C^{low}, while monocytic MDSC are CD11b⁺Ly6G[−]Ly6C^{high} (5, 6). In human, MDSC are generally identified as CD14[−]CD33⁺CD11b⁺HLA-DR^{neg/low} cells (6). The immunosuppressive function of MDSC depends on multiple mechanisms, including the production of nitric oxide (NO), peroxynitrites, reactive oxygen species (ROS), and the expression of inducible nitric oxide synthase (iNOS), indoleamine 2,3-dioxygenase (IDO) and/or arginase-1 (5, 7, 8).

A substantial expansion of MDSC in the blood, lymph nodes, bone marrow and spleen has been detected in multiple mouse cancer models (5, 9). Similarly, accumulation of MDSC has been reported in the blood, lymph nodes and tumors of patients with various types of cancers, including breast, which correlates with tumor burden and disease stage (10). Tumor-induced MDSC expansion significantly contributes to the mechanisms of cancer-induced immune suppression, and therefore significantly impedes the efficacy of immunotherapeutic approaches (1, 11, 12). Not surprisingly, multiple reports in humans and animal models have indicated that MDSC elimination or inhibition promotes anti-tumor immunity and enhances the response to immunotherapy (11). Different approaches have thus been explored to target these cells, which include the use of specific antibodies (13), all-*trans* retinoic acid (ATRA) (14–16), or chemotherapeutic molecules such as gemcitabine (17, 18), 5-fluorouracil (5-FU) (19) or docetaxel (20).

Doxorubicin, is an anti-neoplastic drug broadly used in the treatment of hematological malignancies, soft tissue sarcomas and several types of carcinomas including breast cancer (21). Extensive evidence has been provided that besides its direct tumoricidal activity, doxorubicin also promotes anti-tumor immunity (22–26). This drug has indeed been shown to induce an “immunogenic type” of tumor cell death leading to the stimulation of dendritic cell (DC) antigen presenting function (23). Doxorubicin administration has also been reported to enhance the proliferation of tumor-specific CD8⁺ T cells (27), and to increase the permeability of tumor cells to granzyme B produced by cytotoxic T lymphocytes (CTL) (28). However, whether this drug may impact tumor-induced immunosuppression, specifically by negatively targeting MDSC, remains unclear. In the current study, we investigated the effects of doxorubicin on MDSC in the murine breast cancer model 4T1 and explored the possibility of combining this chemotherapeutic drug with immunotherapy. We demonstrated for the first time that doxorubicin eliminated MDSC by triggering apoptosis of these cells. ROS may contribute to doxorubicin-mediated elimination of MDSC. In addition, residual MDSC from doxorubicin-treated mice were impaired in their suppressive function. Of importance, doxorubicin administration led to improved T and NK cell function. The proportion of T and NK cells expressing perforin and granzyme B was indeed significantly

augmented following treatment of tumor-bearing mice with doxorubicin. In addition, doxorubicin increased the proliferation status of T lymphocytes and NK cells. Substantiating these observations, doxorubicin exhibited selective cytotoxic effects on MDSC isolated from cancer patients. Furthermore, the combination of doxorubicin with T helper (Th) 1 or with recently identified Th17 lymphocytes impaired tumor development and metastatic spreading. These findings therefore highlight a novel property of doxorubicin as a potent selective MDSC-targeting agent, which may be used to enhance the efficacy of immunotherapeutic regimens.

Materials and Methods

Patients

A total of n=10 patients with confirmed solid cancers (two patients with lung, one patient with ovarian, one patient with prostate, one patient with bladder, one patient with colon, one patient with stomach, one patient with kidney, one patient with pancreatic and one patient with breast cancer) were enrolled in the study before treatment after giving written informed consent in accordance with the Declaration of Helsinki. The study was approved by the Ethic Committee of the University Hospital of Dijon. None of the patients had received chemotherapy or any other immunosuppressive treatment during the previous 3 months.

Animals and tumor cell lines

Six to eight week-old Balb/c and C57BL/6 mice were purchased from the National Cancer Institute (NCI). Six to eight week-old gp91^{phox}^{-/-} (C57BL6-Cybb^{tm1Din}) were purchased from Jackson Laboratory. The mice were housed and cared for according to the University of Arizona Institutional Animal and Care Guidelines and Use Committee (IACUC). The 4T1 and EMT6 murine mammary tumor cell lines (Balb/c origin) and the EL4 thymoma cell line (C57BL/6 origin) were obtained from the American Type Culture Collection (ATCC). 4T1-luc was generated using luciferase reporter plasmid (PGL4-51) (InvivoGen). Briefly, the transfection was performed using Fugene6 reagent (Promega) according to the manufacturer's protocol. Stably transfected cells were clonally selected in presence of G418 (800 µg/ml) and luciferase expression was confirmed using the luciferase reporter assay system and a luminometer (Femtomaster FB12, Berthold Detection System), according to the manufacturer's recommendation (Promega).

Bioluminescence imaging

Tumor-bearing animals were injected (i.p.) with D-luciferin (4.29 mg/mouse; Xenogen). Mice were anesthetized using isoflurane (1.5 L/min oxygen, 4% isoflurane) and kept in an induction chamber. Images were captured with an AMI1000 imager (Spectral Instruments Imaging). Light emission was measured over an integration time of 1 minute, 10 minutes after injection of luciferin. Luciferase activity was analyzed using the AMI1000 Software (Spectral Instruments Imaging) to quantify tumor region flux (photons per second) and to assess tumor growth.

Generation of Th1 and Th17

Naive CD4⁺CD25⁻CD62L⁺ T lymphocytes were isolated from the spleen of 6 to 8 week-old Balb/c mice according to the manufacturer's instructions (Miltenyi Biotec). Cells were subsequently cultured at a concentration of 10⁶ cells/ml in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 0.5 × MEM nonessential amino acids, and 1 mM sodium pyruvate. Cells were stimulated with anti-CD3 and anti-CD28-coated beads (Invitrogen). Th1 cells were generated in the presence of IL-12 (10 ng/ml), IL-2 (10 ng/ml), IL-7 (20 ng/ml) and blocking anti-IL-4

antibodies (5 µg/ml) (3 day culture). For the generation of Th17 cells, naive T cells were cultured with IL-6 (40 ng/ml), TGFβ (0.5 ng/ml), blocking anti-IFNγ (5 µg/ml) and anti-IL-4 (5 µg/ml) antibodies for 3 days. Th17 cultures were then prolonged in fresh medium containing IL-23 (40 ng/ml) for an additional 3 days. All cytokines were purchased from R&D Systems or Peprotech.

Chemotherapy and chemoimmunotherapy

4T1 or 4T1-Luc cells were injected (1×10^6 cells) orthotopically (mammary fat pad). In some experiments, seven and twelve days after tumor injection, mice were administered with doxorubicin (2.5 or 5 mg/kg; intravenously), cyclophosphamide (50 mg/kg; intraperitoneally), fludarabine (50 mg/kg; intraperitoneally), melphalan (5 mg/kg; intraperitoneally), vincristine (1 mg/kg; intravenously), etoposide (5 mg/kg; intraperitoneally) or control PBS. Spleens, blood and tumors were collected 14, 17 and 23 days after tumor cell injection. For evaluating the effects of doxorubicin plus T helper lymphocyte combination therapy, mice were injected with doxorubicin (5 mg/kg) on days 7 and 12 and with Th1 or Th17 cells (1×10^6 intravenously; 2×10^6 intratumorally) on days 9 and 14 after tumor cell injection. Mice were euthanized for ethical reasons when they exhibited severe morbidity signs due to overwhelming metastatic spreading (endpoint at 4–4.5 weeks) in compliance with IACUC regulations.

MDSC isolation

Spleens were harvested, dissociated and red blood cells were lysed in lysis buffer (BD biosciences). MDSC were purified using a mouse MDSC isolation kit according to the manufacturer's instructions (Miltenyi Biotec). The purified cells were used in other experiments.

Flow cytometry analysis

Cell suspensions from blood, spleens or tumors were filtered and red blood cells were lysed. For extracellular staining cells were incubated with the indicated combinations of antibodies (CD11b, Ly6C, Ly6G (Gr-1), CD8, CD49b, CD4, CD25, F4/80, CD11c, CCR7 and CD206) or isotype controls (1 hr, 4°C). For intracellular staining, cells were fixed and permeabilized immediately after cell surface staining according to the manufacturer's description (Affymetrix eBioscience). For IFNγ staining, cells were incubated with a leukocyte stimulating kit (BD biosciences) for 4 hours. For perforin or granzyme B staining, cells were incubated with anti-CD3 and anti-CD28-coated beads (Invitrogen) and IL-2 (4 ng/ml) for 24 hours. The proliferation status of the cells was assessed by staining with Ki67 according to the manufacturer's protocol (Ki67 detection protocol; BD biosciences). All antibodies and isotype controls were purchased from BD Biosciences or Affymetrix eBioscience. For the detection of apoptosis, spleen and blood samples from tumor-bearing mice, untreated or treated with doxorubicin, were dissociated and processed as described above. Cells were then stained with Annexin V and propidium iodide (PI) according to the manufacturer's protocol (Apoptosis detection kit; Affymetrix eBioscience). Fluorescence data were collected on a FACSVerse or LSRII Fortessa (BD biosciences). The data was analyzed using FlowJo software (Tree star Inc.).

Suppression assays

Spleens from naïve mice were collected, dissociated and cells were incubating on nylon wool columns (37°C, 45 minutes) after red blood cell lysis. More than 90% of the eluted cells were T lymphocytes based on TCRαβ expression. These T cells were labeled with 5mM CellTrace™ Violet according to the manufacturer's instructions (Invitrogen) and were plated in 96-well plates with anti-CD3 and anti-CD28-coated activation beads (Invitrogen).

Isolated MDSC from 4T1 tumor-bearing mice, treated or not with doxorubicin, were then added to the culture (MDSC to T cell ratio= 1:2). After 4 days, cells were harvested, stained with anti-CD4, anti-CD8 and anti-CD25 (Affymetrix eBioscience). Cell proliferation was determined by measuring the dilution of cell trace violet by flow cytometry after gating on the CD4⁺ or CD8⁺ cell populations. The proliferation index (pi) was determined using the Modfit software (Verity Software House) and percent proliferation was calculated as follows: proliferation (%) = $(T^+ - T) - (S - T) / (T^+ - T) \times 100$, with T⁺: pi of the control stimulated T cells without MDSC; T: pi of control non-stimulated T cells without MDSC; S: pi of stimulated T cells in the presence of MDSC).

Western Blot analysis

Cells were lysed in RIPA buffer and sonicated. Lysates were cleared by centrifugation at 14,000 rpm and protein concentration was determined with the Thermo Scientific BCA protein assay using BSA as standard. Equal amounts of proteins (30 µg) were separated on 10% or 16% SDS-PAGE gels, transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore) and probed with primary Abs specific for P-STAT3, STAT3, Arginase-1, IDO, cleaved caspase-3 (Cell Signaling Company), S100A9, S100A8 (R&D Systems) and actin (Sigma-Aldrich) followed by secondary antibody (Jackson ImmunoResearch). Reactive bands were visualized by exposure to film using Super Signal Chemiluminescent Substrate (Thermo Scientific).

ROS detection

Splenocytes from untreated or doxorubicin-treated mice were incubated for 30 min with the oxidation-sensitive dye Dichlorodihydrofluorescein diacetate (DCFDA, 5 µM, Molecular Probes/Invitrogen). Samples were then labeled with anti-Gr-1 and anti-CD11b antibodies. The level of ROS was detected using flow cytometry as described (29).

Real-time PCR

Cells were collected and total RNA was isolated using Trizol (Invitrogen). PCR reactions were set up in 96-well plates containing 10 µl 2x IQ Supermix (Bio-Rad), 1 µL TaqMan[®] primer/probe set (ABI, Foster City, CA), 2 µL of the cDNA synthesis reaction (10% of RT reaction) and 7 µL of nuclease-free water. Reactions were run and analyzed on a Bio-Rad iCycler iQ real-time PCR detection system. Primers for IFN γ and IL-17 were obtained from Applied Biosystems (Invitrogen).

ELISA

IL-17 and IFN γ concentration was detected in Th1 and Th17 cell culture supernatant using ELISA according to the manufacturer's instructions (Affymetrix eBioscience).

Immunofluorescent staining

Tumors and spleens were harvested and frozen. Serial sections (5 µm) were performed from each tissue and mounted. Frozen slides were first fixed in 100% cold methanol for 10 min. After blocking for 1 hour at room temperature (RT) (10% normal chicken serum in Tris-Buffered Saline and 0.1% Tween 20 (TBS-T)/1% BSA) slides were incubated (overnight, 4°C) with anti-Gr-1 (Affymetrix eBioscience) (0.02 mg/mL in TBS-T). Slides were washed with TBS-T and incubated with a secondary chicken anti-rat antibody conjugated with Alexa Fluor 647 for 45 min at RT (1:200 in TBST, Invitrogen). Slides were washed and incubated (2 hours, RT) with a rat anti-mouse CD11b antibody conjugated with FITC (Affymetrix eBioscience) (0.02 mg/mL in TBS-T). Nuclear counterstaining was performed using Sytox orange according to the manufacturer's instructions (Invitrogen). Slides were

mounted with fluorescence mounting medium (Dako North America) and visualized using a confocal microscope (Zeiss LSM 510-META NLO).

Statistical Analysis

Experiments were performed with 4–8 mice per groups as indicated. Mice were individually processed and analyzed separately unless specified otherwise. All analyses were carried out using GraphPad Prism software (GraphPad Software). Analyses were performed by one-way ANOVA or two-way ANOVA with a Bonferroni or Dunnett posttest, or a paired *t* test where appropriate. Statistically significant *p* values were labeled as follows: *****P* <0.001; ***P* <0.01 and **P* <0.05.

Results

Doxorubicin selectively eliminates tumor-induced MDSC in mice bearing established 4T1 mammary tumors

The immunomodulatory effects of doxorubicin have been extensively studied (23, 26). However, the possible impact of this chemotherapeutic agent on suppressive MDSC remains elusive. To address this question, Balb/c mice were injected with 4T1 breast cancer cells and treated with doxorubicin (2.5 and 5 mg/kg; non-curative doses), 7 and 12 days after tumor implantation (Figure 1A). We first confirmed that 4T1 tumor growth is associated with substantial MDSC expansion and determined the basal proportion of MDSC in the absence of doxorubicin therapy at different time points following injection of tumor cells (Supplemental Figure S1) (30, 31). Doxorubicin significantly reduced the proportion and absolute number of 4T1 tumor-induced MDSC in the spleen (Figure 1B and 1C) and blood (Figure 1D) of treated animals. It is noteworthy that while doxorubicin-mediated elimination of MDSC was prominent on day 14 and 17 post-tumor cell injection, these cells were reconstituted by day 23. These results were further confirmed by confocal microscopy analysis of spleen sections (Figure 1E). Furthermore, MDSC were also depleted within the tumor beds in mice treated with doxorubicin (Supplemental Figure S2A–C). 4T1 tumor development is primarily associated with the expansion of granulocytic MDSC (30, 32). Our results indicate that doxorubicin did not significantly affect monocytic MDSC, which were low in frequency even in untreated animals, but significantly depleted granulocytic MDSC (Supplemental Figure S2D). These results thus demonstrate that doxorubicin induced elimination of tumor-induced MDSC.

Doxorubicin minimally affects effector immune cell populations

To optimally promote anti-tumor immunity, immunomodulatory drugs should negatively target immunosuppressive cells while sparing immune effectors. We therefore sought to investigate whether doxorubicin may affect other immune cells, specifically anti-tumoral effector T lymphocytes and NK cells. Interestingly, the proportion of CD4⁺ and CD8⁺ T lymphocytes was significantly increased in the spleen and blood of doxorubicin-treated mice (Figure 2A and 2B). NK frequency increased in the blood and was not altered in the spleen of the treated animals (Figure 2C). Consistent with these results, the proliferation status of CD4⁺ and CD8⁺ T cells was augmented in doxorubicin-treated animals (Figure 2D). This preferential targeting of MDSC resulted in a significant increase in the ratios of effector CD8⁺ T, CD4⁺ T or NK cells to suppressive MDSC (Supplemental Figure S3A). Importantly, the proportion of NK cells and CD8⁺ T lymphocytes expressing perforin and granzyme B was significantly increased in doxorubicin-treated 4T1 tumor-bearing mice (Figure 2E and Supplemental Figure S3B). In line with these results, doxorubicin administration resulted in augmented frequency of IFN γ -producing CD8⁺ T lymphocytes (Figure 2F). However, no change in the proportion of IFN γ -producing CD4⁺ T cells was observed (Figure 2F). It is noteworthy that doxorubicin did not change immunosuppressive

Treg frequency (Supplemental Figure S3C). Together these results indicate that doxorubicin-mediated MDSC depletion was associated with augmented effector immune cell proliferation and function.

Importantly, we further determined that doxorubicin was significantly more potent at depleting MDSC than other chemotherapeutic agents such as cyclophosphamide, fludarabine, melphalan (Supplemental Figure S4A), vincristine or etoposide (Supplemental figure S4B) which exhibited limited effects on these cells. Of note, these drugs promoted a decrease in tumor volume similar to that induced by doxorubicin. Doxorubicin was also unique at substantially increasing the effector lymphocytes (or NK) to suppressor MDSC ratios, indicating that it is endowed with a higher degree of selectivity compared to these other drugs (Supplemental Figure S4A, B). Furthermore, even in combination with cyclophosphamide, doxorubicin significantly triggered MDSC elimination and increased T lymphocyte frequency (supplemental Figure S4C).

The observations that doxorubicin reduces MDSC frequency and absolute number and increases effector lymphocyte proportion, resulting in an increased effector T cells to suppressor MDSC ratios, were further confirmed in the EL4 (thymoma) and EMT6 (breast cancer) mouse models (Supplemental Figure S5A, B). Additionally, residual MDSC isolated from EL4 or EMT6 tumor-bearing mice treated with doxorubicin exhibited reduced suppressive function (data not shown).

Doxorubicin preferentially triggers MDSC apoptotic program

We next investigated the mechanisms underlying doxorubicin-mediated elimination of MDSC and specifically explore whether this drug may trigger the MDSC apoptotic program. Flow cytometry analysis after staining of spleen cells with anti-Gr-1, anti-CD11b antibodies, Annexin V and PI, and gating on the Gr-1⁺CD11b⁺ population indicated that doxorubicin increased the proportion of apoptotic (Annexin V⁺, PI⁻) and secondary necrotic (Annexin V⁺, PI⁺) MDSC (Figure 3A). Consistent with these data, caspase-3 cleavage was detected in MDSC isolated from 4T1 tumor-bearing doxorubicin-treated animals (Figure 3B). Importantly, in line with the results depicted in Figure 2A and 2B, the number of dead (apoptotic and necrotic) T lymphocytes (CD4⁺ or CD8⁺) was not significantly modified by doxorubicin (Figure 3C). These data thus demonstrate that doxorubicin preferentially induced apoptosis of MDSC with no detectable toxic effect on effector T lymphocytes.

MDSC isolated from untreated tumor-bearing mice were more sensitive to doxorubicin *in vitro* than the MDSC-depleted cell population or than 4T1 cells (Figure 3D and data not shown). Treatment of these purified MDSC with *N*-acetylcysteine (NAC, a ROS scavenger) prevented their killing by doxorubicin, suggesting that ROS may play a role in this process (Figure 3D). Consistent with this result, the levels of ROS were increased in the immediate hours following doxorubicin treatment of MDSC *in vitro* (Figure 3E), and MDSC isolated from EL4 tumor-bearing gp91^{-/-} mice (lacking the gp91phox glycosylated subunit of the NADPH oxidase flavocytochrome b558, responsible for the production of superoxide ion O₂^{·-}) were less sensitive to doxorubicin-mediated cytotoxicity *in vitro* (Figure 3F). Interestingly, *in vivo*, the effects of doxorubicin administration on MDSC were partially impaired in EL4 tumor-bearing gp91^{-/-} mice compared to wild-type mice (data not shown).

Doxorubicin impedes the suppressive activity of residual MDSC isolated from tumor bearing mice

A cardinal characteristic of MDSC is their ability to suppress the activation and proliferation of T cells. MDSC depletion by doxorubicin was not complete as ~15% of residual MDSC could still be detected 17 days post doxorubicin administration. Therefore, it was important

to determine whether the suppressive function of these remaining MDSC was affected by doxorubicin. As expected and previously reported (32), MDSC isolated from non-treated tumor-bearing mice significantly inhibited T lymphocyte (CD4⁺ and CD8⁺) proliferation (Figure 4A and Supplemental Figure S6) and activation (Figure 4B). The suppressive function of residual MDSC isolated from doxorubicin-treated tumor-bearing animals was significantly impaired (Figure 4A, B and Supplemental Figure S6). Exposure of MDSC isolated from untreated 4T1 tumor-bearing mice to non-cytotoxic concentration of doxorubicin *in vitro* impaired their immunosuppressive function (Figure 4C).

Various mechanisms have been implicated in MDSC suppressive function including the production of reactive oxygen species or the expression of arginase-1 or indoleamine 2, 3-dioxygenase (IDO) involved in the catabolism of arginine or tryptophan, respectively (1). Depletion of these amino acids from the microenvironment results in the inhibition of T cell proliferation, notably through the down-regulation of the ζ -chain of the T cell receptor (TCR) complex (1, 11). Similarly, ROS exhibit suppressive effects on T lymphocytes (1, 33). Five days after doxorubicin treatment of 4T1 tumor-bearing mice, the production of ROS (Figure 5A) and the expression of arginase-1 (Figure 5B) and IDO (Figure 5C) by residual MDSC was impaired. Additional molecules reported for their role in MDSC development and/or immunosuppressive function such as CD73, CD39, S100A8/9, or STAT-3 were not significantly altered by doxorubicin treatment (Supplemental Figure S7A and S7B).

Previous reports have indicated that some chemotherapeutic drugs such as docetaxel can promote MDSC differentiation into macrophages (M1) (20). To evaluate this possibility, MDSC from doxorubicin-treated mice were analyzed for the expression of markers expressed by M1 (CCR7) or M2 (CD206; Mannose Receptor) macrophages. The expression of these two cell surface markers was not detected on MDSC following doxorubicin treatment, indicating that this drug did not promote MDSC differentiation into macrophages (Supplemental Figure S7C). Finally, further investigation indicated that the proliferation status (Ki67 expression) of residual 4T1 tumor-induced MDSC was reduced by doxorubicin therapy (not shown).

Altogether, these results indicate that 5 days after doxorubicin administration, MDSC that have not been eliminated by the drug exhibited reduced expression of arginase-1 and IDO, decreased ROS level and curtailed immunosuppressive activity.

Doxorubicin acts synergistically with Th1 or Th17 cell therapy

Doxorubicin depleted tumor-induced MDSC and curtailed the suppressive function of residual MDSC, thereby averting one major mechanism of cancer-mediated immunosuppression. Moreover, the proliferation status and activation of responder effector lymphocytes and NK were restored in doxorubicin-treated mice. We therefore reasoned that this drug may create a favorable environment that may allow for successful combinatory immunotherapy. To address this hypothesis, we evaluated a chemo-immunotherapy regimen consisting of doxorubicin followed by infusion of T helper lymphocytes.

Th17 lymphocytes represent a recently described subset of T helper cells with controversial effects on tumor development. Recent reports have demonstrated that Th17 generated *in vitro* have the potential to promote the development of CD8⁺ T lymphocyte-dependent immune response and to impair tumor growth (34). Th1 can also promote antitumor immunity (35). In our current study, mice bearing established 4T1 tumors were treated with doxorubicin and received either Th1 or Th17 lymphocytes generated *in vitro* from naïve CD4⁺ T cells (Supplemental Figure S8A, B) as depicted in Figure 6A. The chemotherapeutic regimen significantly reduced the number of 4T1 metastatic

nodules in the lungs and impaired tumor growth when compared to the monotherapies (Figure 6B and supplemental Figure S8C). Importantly, MDSC depletion persisted in mice treated with doxorubicin plus Th1 or Th17 cells, while these cells eventually re-expanded post-treatment in mice receiving doxorubicin alone (Figure 6C and 6D). Consistent with these results, the frequency of CD8⁺ and CD4⁺ T lymphocytes was significantly increased in doxorubicin plus Th1 or Th17 -treated animals (Figure 6D), but NK proportion remained unchanged (data not shown). These data thus indicate that doxorubicin administration resulted in the promotion of a favorable environment fostering the anti-tumoral efficacy of Th1 and Th17 lymphocytes.

Doxorubicin induces apoptosis of MDSC isolated from cancer patients

To determine whether similar effects of doxorubicin can be observed on human MDSC, CD33⁺ cells were isolated from the blood of patients with different types of cancer (n=10). CD33⁺ cells exhibited a phenotype consistent with that reported for human MDSC (36) (Figure 7A) and importantly were endowed with significant suppressive capabilities (Figure 7B). Our results indicate that these immunosuppressive cells were sensitive to doxorubicin-induced cell death (Figure 7C, E). Importantly, CD33⁻ depleted cells (CD33⁻) from the same patients were significantly less sensitive to doxorubicin (Figure 7D, E), indicating that this chemotherapeutic molecule preferentially targets MDSC. CD3⁺ T cells were also minimally affected by doxorubicin (not shown). These results thus suggest that doxorubicin exhibit effects on human MDSC comparable to those observed on mouse MDSC, thus supporting the implementation of this drug in clinical chemoimmunotherapeutic approaches.

Discussion

The development of malignant tumors is commonly associated with the occurrence and persistence of an immunosuppressive environment. The expansion of MDSC, a main suppressive cell population (13), has been widely documented in many animal tumor models as well as in patients with different types of cancers and represents a major obstacle for efficient cancer immunotherapy (5, 9, 10). Several strategies have been explored to either eliminate or curtail the immunosuppressive function of MDSC. Depletion of Gr-1⁺ MDSC using anti-Gr-1 monoclonal antibody resulted in restored T cell anti-tumor activity. However, anti-Gr-1 also led to the elimination of mature granulocytes and was associated with severe immunosuppression (13). The promotion of MDSC differentiation using all-trans retinoic acid (ATRA), a natural metabolite of vitamin A, has also been considered. ATRA administration enhanced T lymphocyte function and fostered the efficacy of cancer vaccines (14). In recent years evidence has been provided that chemotherapeutic drugs can not only kill tumor cells, but also enhance anti-tumor immunity through different mechanisms (37). Chemotherapeutic agents can promote the function of antigen presenting cells, NK and T lymphocytes (23, 26, 37) but may also negatively target immunosuppressive cells such as Treg or MDSC (17, 19, 20, 38, 39). For instance, gemcitabine has been reported to deplete MDSC in tumor-bearing mice resulting in enhanced anti-tumor immunity (17–20). Additional reports have demonstrated that 5-Fluorouracil (19), or docetaxel (20) can eliminate, impede the suppressive function or promote the differentiation of MDSC.

Doxorubicin has been a key chemotherapeutic agent used against a variety of human cancers. More recently, this drug has been widely studied for its ability to modulate anti-cancer immunity (23–26, 28). Reports have demonstrated that doxorubicin induces an “immunogenic type” of tumor cell death and promotes anti-tumor immune responses (23). In the current study, we highlight a novel property of doxorubicin: its ability to avert a major mechanism of tumor-induced immunosuppression by eliminating and inactivating tumor-induced MDSC. We established that doxorubicin administration reduced the number of MDSC in the spleen, blood and in the tumor beds of animals bearing established 4T1

mammary tumors. Importantly, doxorubicin selectively eliminated MDSC while enhancing the proliferation status, activation, cytokine production of effector T lymphocytes and/or NK cells. This primary targeting of MDSC while effector T cells (and NK) were spared translated into a substantial increase in effector lymphocytes to MDSC ratios. Compared to the other chemotherapeutic drugs that we evaluated, doxorubicin was endowed with the highest degree of selectivity. Indeed, although associated with a reduction of tumor volume comparable to that observed after doxorubicin treatment, most of these other agents had limited effects on MDSC and some of them, such as cyclophosphamide, even led to a slight increase in MDSC as they targeted T cells. Of therapeutic relevance, the addition of doxorubicin to cyclophosphamide resulted in a significant elimination of MDSC similar to that observed when doxorubicin was administered alone. This peculiar property of doxorubicin further highlights the interest and advantage of using this drug as a potent immunomodulatory agent in chemoimmunotherapeutic approaches.

Further investigation indicated that doxorubicin selectively triggered the apoptotic program of MDSC. No change in the apoptosis rate of T or NK cells was detected post-doxorubicin treatment compared to untreated groups. This selective effect may be partly explained by the preferential targeting of highly proliferative cells by doxorubicin. Indeed, MDSC in untreated tumor-bearing mice expressed high level of Ki67, a marker of cell proliferation (data not shown), while T lymphocyte proliferation was low in untreated tumor-bearing mice. The triggering of apoptosis by doxorubicin through the induction of ROS production has been described in tumor and normal cells such as cardiomyocytes (40). *In vitro*, the ROS scavenger NAC impaired doxorubicin-mediated elimination of MDSC, and MDSC isolated from gp91^{-/-} mice were less sensitive to doxorubicin. The effects of doxorubicin on MDSC were partially impaired in EL4 tumor-bearing gp91^{-/-} mice. These results strongly suggest that, early after its administration, doxorubicin may induce ROS-dependent triggering of the MDSC apoptotic program, resulting in the rapid elimination of most of these cells. Of note, MDSC that were not eliminated and that were found later (5 days) after doxorubicin treatment exhibited an overall impaired suppressive activity which, at this time included a reduction of ROS production.

A previous report has indicated that docetaxel reduced MDSC number by promoting their differentiation into M1 macrophages (20). We did not detect any changes in the expression of M1 or M2 macrophage markers on MDSC following doxorubicin treatment. Additionally, no change in the frequency of macrophages or DC was detected in the treated mice. However, as mentioned above, the suppressive function of the limited number of residual MDSC that were not depleted by doxorubicin was significantly impaired.

Since doxorubicin administration induced MDSC depletion and was associated with restored T lymphocyte activity, we reasoned that it may create a favorable environment for efficient immunotherapy. Infusion of *in vitro* generated Th1 cells has been reported to promote anti-tumor immunity (35). Th17 lymphocytes have recently emerged as a new effector CD4⁺ T helper cell subset (41) exhibiting effector functions distinct from Th1 and Th2 lymphocytes (42). Th17 have been identified as major contributors to the pathogenesis of multiple autoimmune conditions in animals and humans (42). However, the role of Th17 cells in cancer remains controversial. While some studies have documented the anti-tumoral efficacy of these cells (34, 43–45) others have reported on their immunosuppressive properties (46). These conflicting results related to the pro- versus anti- tumoral properties of Th17 may be explained by the high degree of plasticity of these cells (47–49). Supporting the anti-tumoral role of *in vitro* polarized Th17, Muranski *et al.* demonstrated that adoptive Th17 cell therapy has the potential to eliminate established tumors. The anti-tumoral efficacy of Th17 lymphocytes depended on their ability to produce both IFN γ and IL-17 (43, 44). Consistent with these studies, our own results indicated that Th17 generated from OT-II

mice exhibit anti-tumoral effects against B16-OVA melanoma (unpublished data). In the current study, we demonstrated that the therapeutic efficacy of adoptively transferred Th1 or Th17 lymphocytes was significantly enhanced by doxorubicin administration, resulting in impaired development of the highly metastatic 4T1 mammary carcinoma.

Doxorubicin is widely used in chemotherapeutic regimen primarily for its conventional direct tumoricidal activity. Here we highlight a new application for this drug as a selective MDSC targeting agent, which can be used to overcome a major mechanism of tumor immune evasion. These results advocate for the implementation of doxorubicin in combination strategies to enhance the efficacy of immunotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Doxorubicin eliminates tumor-induced MDSC

A, Schematic of the experimental design followed to evaluate the effects of doxorubicin on MDSC in the 4T1 breast cancer model. Mice were injected orthotopically (mammary fat pad) with 4T1 tumor cells (1×10^6). Doxorubicin (2.5 and 5 mg/kg) was administered intravenously on day 7 and 12 post-tumor injection. Spleen and blood samples were harvested and evaluated on days 14, 17 and 23. B, Proportion of MDSC (CD11b⁺Gr-1⁺) in the spleen of 4T1 tumor-bearing mice post-doxorubicin treatment (right panel) and representative flow cytometry analysis 17 days post-tumor injection (left panel). C, Absolute number of MDSC in tumor-bearing mice treated or not with doxorubicin. D, Proportion of MDSC in the blood of tumor-bearing mice after doxorubicin treatment. E, Confocal

microscopy analysis of CD11b⁺Gr-1⁺ cells in the spleens from untreated or doxorubicin-treated mice 17 days after tumor injection (5 days after the last doxorubicin treatment). CD11b (green), Gr-1 (red) and sytox orange nuclear staining (Nuc, blue). Scale bar 20 μ m. **P* 0.05; ***P* 0.01; ****P* 0.001. n=4 mice per group. Data represent one of 3 experiments performed and analyzed independently.

Figure 2. Doxorubicin increases the frequency, proliferation and cytotoxic activity of effector T lymphocytes and NK

A similar experimental design as described in figure 1 was followed. A, Frequency of CD4⁺ T cells in the spleen (left panel) and blood (right panel) of tumor-bearing mice after doxorubicin treatment. B, Proportion of CD8⁺ T cells in the spleen (left panel) and blood (right panel) of tumor-bearing mice after doxorubicin treatment. C, NK cell frequency in the spleen (left panel) and blood (right panel) of doxorubicin-treated mice. D, Analysis of Ki67 expression after gating on CD4⁺, CD8⁺ T lymphocytes or NK (DX5⁺) cells as indicated (left panel) and related mean fluorescent intensity (MFI) (right panel) (day 17). E, Percent of CD8⁺ T and NK cells expressing granzyme B or perforin in the spleen of doxorubicin-

treated mice 17 days post tumor cell injection. F, Percent of CD3⁺, CD4⁺ and CD8⁺ T lymphocytes expressing IFN γ in the spleen of tumor-bearing mice treated or not with doxorubicin (day 17). **P* 0.05; ***P* 0.01; ****P* 0.001. n=4 mice per group. Data represent one of 3 experiments performed and analyzed independently.

Figure 3. Doxorubicin selectively induces MDSC apoptosis

A similar experimental design as described in figure 1 was followed. Spleens were collected 5 days after the last doxorubicin administration. A, Spleen samples were labeled for MDSC (CD11b⁺Gr-1⁺) and Annexin V and PI. Representative flow cytometry analysis (left panel, gated on C11b⁺Gr1⁺ cells), and proportion of apoptotic and secondary necrotic MDSC (right panel). B, Detection of caspase-3 cleavage in MDSC isolated from mice treated or not with doxorubicin 17 days after tumor injection. C, Spleen samples were labeled with anti-CD4 or anti-CD8 and Annexin V and PI. Gated CD4⁺ or CD8⁺ T lymphocytes were then analyzed for their Annexin V and PI status. Percent of apoptotic and secondary necrotic CD4⁺ (upper panels) and CD8⁺ (lower panels) T lymphocytes in doxorubicin-treated or untreated mice. Similar experimental design as in A. D, Effects of doxorubicin used at the indicated concentrations on MDSC isolated from 4T1 tumor-bearing mouse spleens (or on the MDSC-depleted cell population) determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays. Cells were cultured in quadruplicate for 30 hr with or without NAC (5 mM). % cell survival=(OD₅₆₀[treated cells] at the indicated doxorubicin concentration)/OD₅₆₀[untreated cells] × 100). E, Analysis of ROS production by MDSC isolated from tumor-bearing mice and treated *in vitro* with the indicated concentrations of doxorubicin and for the indicated period of time. Cells were incubated with Dichlorodihydrofluorescein diacetate (DCFDA) and analyzed by flow cytometry. The mean fluorescent intensity representing ROS levels in MDSC is shown. F, Same as in D, but with MDSC isolated from EL4 tumor-bearing wild-type of gp91^{-/-} mice. **P* 0.05; ***P* 0.01; ****P* 0.001. n=4 mice per group. Data represent one of 3 experiments performed and analyzed independently.

Figure 4. Doxorubicin impairs MDSC immunosuppressive function

A–B, MDSC isolated from untreated or doxorubicin-treated mice were incubated for 4 days with cell trace violet-labeled naïve T cells (MDSC: T cell ratio=1:2). A, Effects of MDSC from the indicated groups of mice on the proliferation of CD4⁺ (left panel) or CD8⁺ (right panel) T lymphocytes assessed by flow cytometry. B, Effects of MDSC on CD25 expression by gated CD4⁺ (upper panels) or CD8⁺ (lower panels) T lymphocytes. C, MDSC isolated from tumor-bearing mice were treated or not *in vitro* with doxorubicin (100 ng/ml, 24 hr) and their ability to impair the proliferation of cell trace violet-labeled naïve CD4⁺ T cells induced by anti-CD3 and anti-CD28-coated activation beads was evaluated by flow

cytometry. * P 0.05; ** P 0.01; *** P 0.001. n=4 mice per group. Data represent one of 2 experiments performed and analyzed independently.

Figure 5. Doxorubicin decreases ROS production and arginase-1 and IDO expression by MDSC
 A similar experimental design as described in figure 1 was followed. Spleens were harvested five days after the last doxorubicin treatment (day 17). A, Analysis of ROS production by MDSC in tumor-free (Tumor-free) or in tumor-bearing (Tumor Bearing) mice treated with the indicated concentration of doxorubicin. Cells were incubated with Dichlorodihydrofluorescein diacetate (DCFDA). Representative flow cytometry analysis of gated CD11b⁺Gr1⁺ cells positive for DCFDA (upper panels). Percent of MDSC positive for DCFDA (left bottom panel). Mean fluorescent intensity representing ROS level in MDSC from the indicated groups (right bottom panel). B, C, Western blot analysis depicting expression of arginase-1 (B) or IDO (C) in MDSC isolated from doxorubicin treated or untreated mice. **P* 0.05; ***P* 0.01; ****P* 0.001. n=4 mice per group. Data represent one of 3 experiments performed and analyzed independently.

Figure 6. The combination of doxorubicin and Th1 or Th17 impairs 4T1 tumor development
 Mice were injected orthotopically (mammary fat pad) with 4T1 tumor cells (1×10^6). Doxorubicin (5 mg/kg) was injected intravenously on day 7 and 12 post-tumor cell injection. Th1 or Th17 lymphocytes were administered on day 9 and 14 post-tumor cell injection, intravenously (1×10^6) and intratumorally (2×10^6). Tumor volume and number of metastatic nodules were evaluated on day 19 post-tumor injection. A, Schematic of the experimental design. B, Number of metastatic nodules (left panel) and tumor volume (right panel). C, Representative flow cytometry analysis of MDSC frequency in mice administered with the indicated therapies. D, Proportion of MDSC, CD4⁺ and CD8⁺ T cells in mice receiving the indicated therapies; n=8 mice per group. Data are representative of 3 independent experiments. **P* 0.05; ***P* 0.01; ****P* 0.001.

Figure 7. Doxorubicin selectively kill MDSC isolated from cancer patients

CD33⁺ cells were isolated from cancer patient PBMCs by magnetic cell sorting. A, Phenotypic analysis of the isolated cells. Representative results of n=10 patients. B, Ability of the CD33⁺ purified cells to impair the proliferation of cell trace violet-labeled T lymphocytes induced with anti-CD3 and anti-CD28-conjugated microbeads (at the indicated MDSC to T cell ratios). PI, proliferation index. C-D, Purified CD33⁺ MDSC or CD33⁻ cells were exposed to the indicated concentrations of doxorubicin for 24 hrs and stained with Annexin V and PI. (% dead cells = % of PI⁺ + % AnnexinV⁺PI⁻ cells). A total of n=10 patients were analyzed. E, Representative dot plots obtained with CD33⁺ MDSC or CD33⁻ cells isolated from a cancer patient. (*P 0.05; **P 0.01; ***P 0.001).