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Research Article
Combination Therapy of Gemcitabine and Doxorubicin to Activate Tumor Immune Microenvironment in Chemotherapy

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Abstract

Background and Objective: Cancer is a major public health problem all over the world. Research on the development of tumor treatment strategies and the pathogenesis is of great importance. This study aims to investigate the combination therapeutic effects of doxorubicin (DOX) and gemcitabine (GEM) on tumor immune microenvironment and chemotherapy. **Materials and Methods:** The marrow cells of mice are induced *in vitro* to obtain Myeloid Inhibitory Cells (MDSC). Cell Counting Kit-8 (CCK8) is used to detect the toxicity of drugs on 4T1 cells. A mouse breast cancer model is established and the changes in tumor size, volume and body weight are determined after administration. The number of related cytokines, MDSC, proliferating cells and $CD8^+$ T cells in the tumor tissues of mice are determined. **Results:** The MDSC is successfully induced *in vitro*, DOX and GEM can inhibit the growth of MDSC and the combination of DOX and GEM can significantly increase the toxicity of drugs to 4T1 cells. *In vivo* experiments show that the combination of DOX and GEM can significantly reduce the volume and mass of tumor, lessen MDSC in tumor tissue, inhibit the proliferation of the tumor cells and increase the number of $CD8^+$ T cells. **Conclusion:** The combination of doxorubicin and gemcitabine can effectively inhibit the proliferation of MDSC cells in the tumor microenvironment, improve immunosuppression in tumor microenvironment and restoring cytotoxic T lymphocyte activity in tumor tissue, thereby activating tumor immune microenvironment and enhancing chemotherapy effect.

Key words: Doxorubicin, gemcitabine, combination therapy, tumor immune, microenvironment

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is a major public health problem that poses a serious threat to human health worldwide[1]. Research on the development of tumor treatment strategies and pathogenesis is still a hot topic in the field of medical research. With the rapid development of biology and molecular biology, studies have found that tumor growth and invasion not only depend on the proliferation of tumor cells themselves, but tumor microenvironment (TME), especially immunosuppressive micro-environment, also plays a crucial role in various stages of tumor development[2-4].

Immunosuppressive micro-environment refers to the situation that during the occurrence and development of tumors, tumor cells will secrete a series of immunosuppressive cytokines such as interleukin-10 (IL-10) and Transforming Growth Factor β (TGF-β) to promote immune cells such as dendritic cells in the tumor microenvironment. Isotropic immunosuppressive cells generated in TME such as tumor-associated macrophages (TAMs), regulatory T cells (Tregs) and Myeloid-Derived Suppressor Cells (MDSC) are transformed[5-7]. With the infiltration of immunosuppressive cells in the tumor site, the immunosuppressive cells will secrete a series of immunosuppressive cytokines within the action of tumor cells to evade immune surveillance and induce apoptosis of T cells, thus enabling the proliferation of tumor cells. Due to the suppressive effects against effector lymphocytes and their abundance in TME, the immunosuppressive cells are a major barrier to tumor immunotherapy[8]. Among immunosuppressive cells, MDSC has the function of regulating tumor growth and targeting metastasis and plays a major role in TME. Therefore, anti-tumor therapy acting on MDSC is a promising anti-tumor therapy strategy[9,10].

Due to the complexity, diversity and heterogeneity of tumor pathogenesis, normal single therapy in tumor treatment usually cannot achieve an ideal therapeutic effect. At present, combination therapy is gradually used to replace single therapy to enhance the effect of drug therapy in clinical treatment[11]. Studies have shown that the chemotherapy drug, Gemcitabine (GEM), can selectively induce the apoptosis of MDSC in the spleen and tumor microenvironment in the mouse tumor model and increase the expression of IL-12 and other pro-inflammatory factors[12,13]. Doxorubicin (DOX) is an anthracycline antibiotic anticancer agent, it is one of the most widely used clinical first-line anticancer drugs[14,15]. Recent studies proved that DOX can also promote the apoptosis of MDSC[16,17]. After treatment of DOX, the number of MDSC in the spleen, peripheral blood and tumor of tumor-bearing mice is significantly reduced and the immunosuppressive activity of residual MDSC is also inhibited. And the depletion of MDSC leads to the increase of granzyme B and interferon-γ produced by effector T cells and NK cells, enabling the activation of the tumor immune microenvironment[17].

Based on the effects of GEM and DOX in tumor immune microenvironment and the strategy of double-drug combination, GEM and DOX were combined in this study to preliminarily evaluate the co-regulatory effects on tumor immune microenvironment and their effects on tumor chemotherapy in tumor-bearing mice.

MATERIALS AND METHODS

The study was carried out from May, 2021 to December, 2022 in China. Doxorubicin hydrochloride was purchased from a West Asian reagent (Shangdong, China). Gemcitabine hydrochloride was obtained from Sigma Aldrich (Shanghai) Trading Co., LTD. Fluorescein Isothiocyanate (FITC) anti-mouse CD11b antibody was obtained from Biolegeng Beijing Biotechnology Co., Ltd. The RPIM1640 medium was purchased from HyClone (Waltham, MA, USA), PBS phosphate buffer dry powder (0.01M) and BSA-Bovine serum albumin were obtained from Beijing Solaibao Technology Co., Ltd. The Ki67 Polyclonal antibody was purchased from Ebioscience. Female Balb/c mice weighing 18-20 g and aged 8-12 weeks were provided by Chongqing Tengxin Biotechnology Co., Ltd.

All the animal care and experimental protocols were approved and followed the guidelines for the Care and Use of Laboratory Animals of School of Pharmacy and Bioengineering, Chongqing University of Technology.

Methods

Evaluation of the drug effect on MDSC cells *in vitro*

Induction of MDSC *in vitro*: A high dose (10 ng mL^{-1}) of Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) was used to induce mouse bone marrow cells (BMC) to obtain MDSC *in vitro*. The Balb/c mice were anesthetized and sacrificed and soaked in a 75% ethanol solution. The femurs of the mice were separated and the muscle tissue and both ends of the femur were removed. The bone cavity was repeatedly and rapidly rinsed with 1640 culture solution, the washed solution was centrifuged at 2000 rpm for 15 min and the supernatant was removed. The RBC lysis solution and PBS solution were added into the system and the supernatant was removed again by centrifugation. The remained cells were cultured in 1640 tumor condition medium (containing GM-CSF, 10 ng mL^{-1}) for 4 days and the positive rate of MDSCs was detected by flow cytometry.

Inhibitory effect of drugs on MDSC cell growth *in vitro*: After MDSCs were successfully induced *in vitro*, MDSCs were co-cultured with DOX (1 μg mL^{-1}), GEM (2 μg mL^{-1}) and a combination medication of DOX and GEM (DOX+GEM). The effects of each drug on MDSC cells were detected by flow cytometry after 24 hrs.

Toxicity of drugs on tumor cells *in vitro*: To explore the toxic effects of the drugs on tumor cells *in vitro*, the survival rate of murine breast cancer cells (4T1) after being co-cultured with different drugs was detected by Cell Counting Kit-8 (CCK-8). The DOX, GEM and DOX+GEM were completely dissolved by DMSO and then diluted in medium to obtain serial solutions with concentrations of 30, 15, 7.5, 3.75 and 1.875 μg mL^{-1}, respectively. The 4T1 cells with a concentration of 1.5×10^4/mL were inoculated into 96-well plates with 100 μL per well and cultured for 24 hrs in an incubator at 37 with 5% CO_2. As 100 μL of drug solutions were added to each of the 5 groups. After culturing for 24 hrs, cells were cleaned by PBS twice and 10% CCK-8 solution was added into the cells and incubated for 1 hr. The absorption value of each well was measured at 450 nm with a microplate reader and the survival rates of 4T1 cells were calculated.

Evaluation of antitumor efficacy of drugs *in vivo*

Study on tumor inhibitory rate in breast cancer model mice: Twenty female Balb/c mice, weighting 18-20 g, were randomly divided into 4 groups with 5 mice in each group: Model group (Control), DOX treated group (DOX), GEM treated group (GEM), DOX and GEM combined treated group (DOX+GEM). The 4T1 cells, with a concentration of 1×10^7, were resuspended with PBS and placed in the low temperature environment. Then 100 μL 4T1 cells solution was inoculated subcutaneously in the back of mice in each group to establish the breast cancer model. When the subcutaneous tumor volume of mice grew to about 300.0 mm^3, the drugs were administered. The treatment administration referred to the conventional treatment dose, the dose of DOX was 4 and 10 mg kg^{-1} for GEM. The same dose of drugs were injected every four days five times in total. The growth situations of tumor-bearing mice were observed daily and the tumor length (a), width (b) and weight of mice in each group were measured every two days. The tumor volume (v) was calculated according to Eq. 1[18].

$$v = a\times b^2 \quad (1)$$

After the treatment, mice in each group were anesthetized and executed. Tumor tissues were separated, weighed, photographed, fixed with 4% paraformaldehyde and stored in liquid nitrogen for further study. The inhibitory rate of each drug was calculated according to Eq. 2[19].

$$\text{Inhibitory rate (\%)} = \frac{\text{Average tumor weight of the control group-Average tumor weight of the treatment group}}{\text{Average tumor weight of the control group}}\times100 \quad (2)$$

Effects of drugs on expression of inflammatory factors in tumor tissues: In order to explore the effects of different drugs on the microenvironment of mouse tumor tissue, Enzyme-Linked Immunosorbent Assay (ELISA) was used to detect the contents of immune promoting factor TNF-α and IL-12 and the expression of immune suppressive factor IL-10 in the tumor microenvironment.

Effects of drugs on MDSCs cells in mouse tumor tissue: To study the effect of DOX and GEM combined administration on the tumor immune microenvironment of mice, tumor tissues of tumor-bearing mice in each group were isolated after treatment and then dissected. The FITC anti-mouse CD11b antibody was used to label MDSC cells in tumors. The number of MDSC cells in each group was observed by a laser confocal microscope, Nikon Corporation of Japan.

Effects of drugs on proliferation of tumor cells: To explore the effects of the combination of DOX and GEM on the proliferation of tumor cells in mouse tumor tissues, TRITC was used to label Ki67 cells and the tissues were observed under a laser confocal microscope. The tumor tissues were isolated and sliced, repaired with 0.01 M sodium citrate (pH 6.0) antigen repair solution, then drilled with 0.5% triton, washed with phosphate buffer saline (PBS), sealed with bovine serum albumin (BSA) for 1 hr, incubated with Ki67 antibody overnight at 4 , washed with PBS, incubated with secondary antibody at room temperature for 1 hr at the dark, washed with PBS, incubated with DAPI at room temperature for 0.5 hr, washed with PBS, sealed by glycerin and observed by a confocal laser microscope.

Effect of drugs on the number of $CD8^+$ T cells in tumor tissue: To evaluate whether DOX+GEM combined administration could regulate the tumor immunosuppressive microenvironment by reducing the number of MDSC cells in the tumor and enhancing anti-tumor immunity, the infiltration of $CD8^+$ T cells in the tumor tissues of mice was detected by immunofluorescence staining and observed under laser confocal microscope.

Parameters

Statistical analysis: Statistical analysis in this study was performed using Prism 8.2.0 (GraphPad Software, SanDiego, CA). Analysis of Variance (ANOVA) with two-tailed Student's t-tests was used for experiments with independent continuous variables and more than two groups. All Data were expressed as Mean±Standard Deviation (SD) and significance was assessed when $p<0.05$.

RESULTS

Evaluation of the drug effect on MDSC cells *in vitro*

Induction of MDSC *in vitro*: The MDSC was induced from BMC by GM-CSF, the positive rate of MDSCs was shown in Fig. 1. Compared with the control group, the positive rate of MDSC *in vitro* was 79.9%, indicating that the induction of MDSCs was successfully achieved *in vitro*.

Inhibitory effect of drugs on MDSC cell growth *in vitro*: The effects of drugs on MDSC cells were detected by flow cytometry, results were shown in Fig. 2. The positive rate of MDSC was 28.7 % after being co-cultured with DOX, 25.5% after being co-cultured with GEM and 26.1% after being co-cultured with DOX and GEM simultaneously. The results showed obvious inhibitory effects on MDSC cells by DOX, GEM and DOX+GEM, however, there was no statistical difference in the intensity of action among the three groups.

Toxicity of drugs on tumor cells *in vitro*: The survival rates of 4T1 cells were shown in Fig. 3. Results showed that with the increase of drug concentration, the toxicity of DOX, GEM and DOX+GEM on 4T1 cells increased successively, but compared with single-used drugs, the combination of DOX and GEM exerted better inhibition effects on 4T1 cells.

Evaluation of antitumor efficacy of drugs *in vivo*

Study on tumor inhibitory rate in breast cancer model mice: After treatment with different drugs, the changes in tumor volume in each group were shown in Fig. 4a and e, the weights of tumor tissue were shown in Fig. 4b, the inhibitory rates of drugs in each group were shown in Fig. 4c and the changes in body weight of mice in each group were shown in Fig. 4d. It was found that compared with the control group, DOX, GEM and DOX+GEM administration could reduce the volume and weight of mouse tumors, showing a superior tumor inhibition rate. Compared with the DOX or GEM group, the volume and weight of tumor tissue in the DOX+GEM group increased much less, indicating the combination of two drugs could exert a more significant tumor inhibition effect ($p<0.01$).

The body weight changes of mice in each group were observed and there was no significant difference in the weight of mice in each group, demonstrating that DOX+GEM has no effect on the weight of mice while playing an anti-tumor role.

Effects of drugs on expression of inflammatory factors in tumor tissues: The effects of drugs on the microenvironment of mouse tumor tissue were shown in Fig. 5 study found that DOX, GEM and DOX+GEM all reduced the TNF-α levels in tumor tissues. However, compared with single drug administration, DOX+GEM combined drug intervention could more significantly increase the expression of pro-inflammatory factor IL-12 and could more effectively reduce the expression of immunosuppressive factor IL-12 that would promote tumor proliferation in tumor immune microenvironment ($p<0.001$).

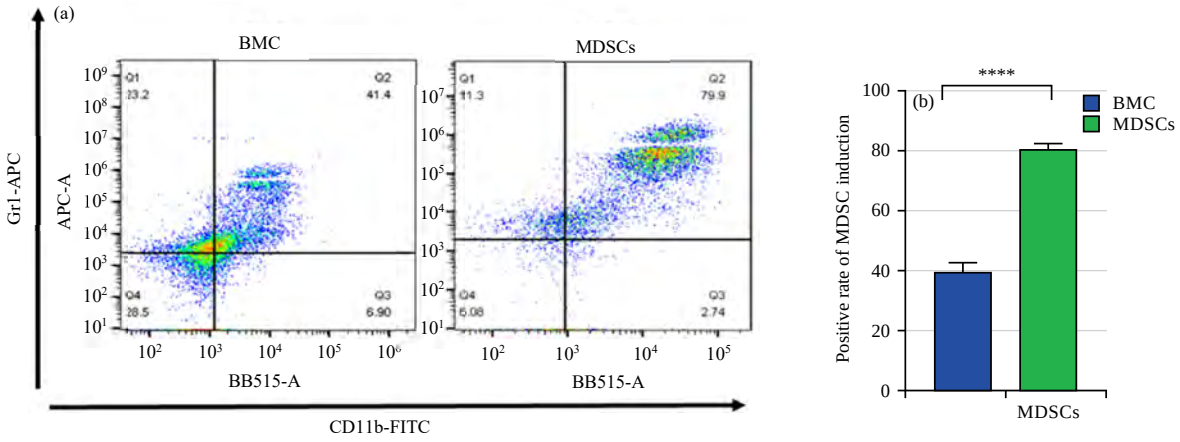

Fig. 1(a-b): (a) Positive rate of MDSC induction by flow cytometry and (b) Quantitative statistics, BMC vs MDSC
****$p<0.0001$ and $n = 3$

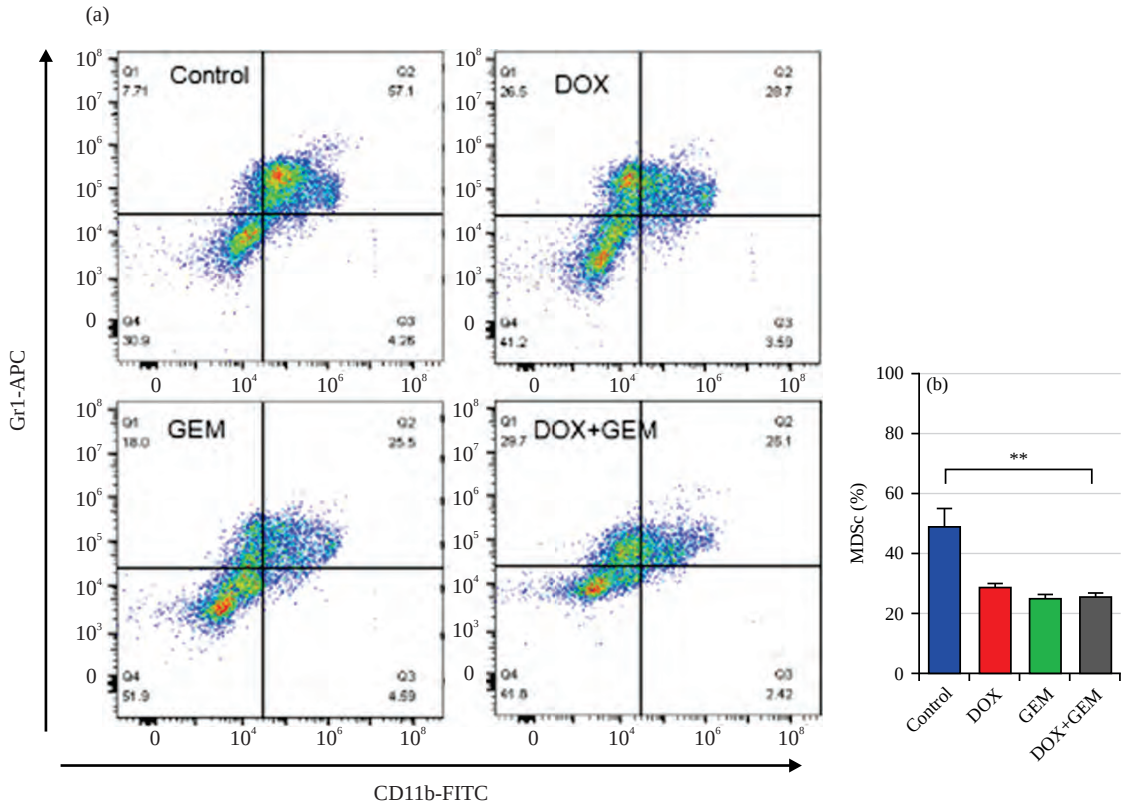

Fig. 2(a-b): (a) Effects of different drugs on MDSC were detected by flow cytometry and (b) Quantitative statistics, Control vs DOX+GEM

**p<0.05 and n = 3

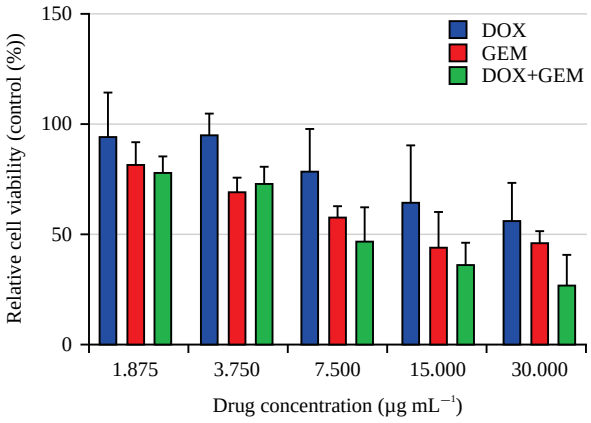

Fig. 3: Detection of drug toxicity on 4T1 cells by CCK-8 method

Effects of drugs on MDSCs cells in mouse tumor tissue: The effects of drugs on MDSCs cells in mouse tumor tissue were shown in Fig. 6. Results showed that a large number of MDSC cells were immersed in the tumor tissue of mice in the control group and the average fluorescence intensity was about 127 by semi-quantitative fluorescence statistics. While compared with the control group, the fluorescence intensity and semi-quantitative fluorescence statistics results of MDSC in other groups were significantly reduced. The reduction was most significant in the DOX+GEM group ($p<0.001$). These results showed that DOX combined with GEM could effectively reduce the MDSC cell invasion in a tumor and then played a role in regulating the tumor microenvironment.

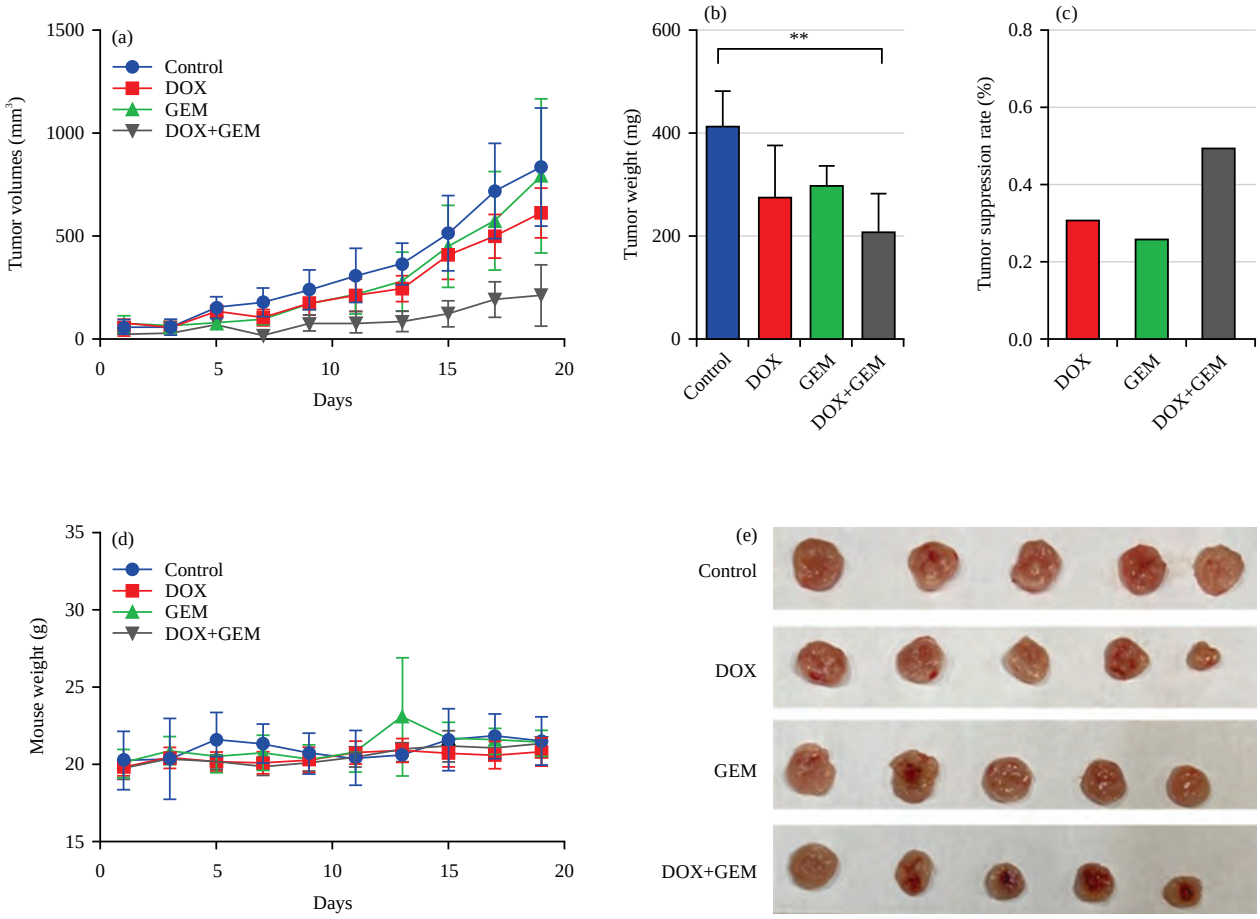

Fig. 4(a-e): Evaluation of anti-tumor efficacy of drugs *in vivo*, (a) Tumor volume changes in each group, (b) Tumor weight of each group, (c) Tumor inhibitory rate in different administration groups, (d) Changes in body weight of mice in each group and (e) Images of tumor *in vitro* of mice in each group

**p<0.01, Control vs DOX+GEM and n = 5

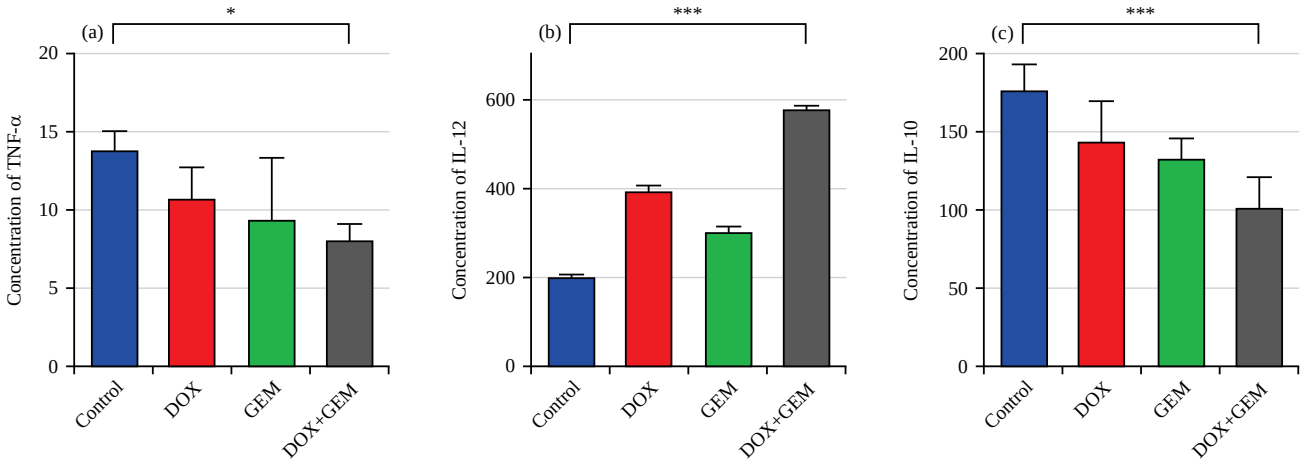

Fig. 5(a-c): Effects of different administration on the expression of (a) TNF-α, (b) IL-12 and (c) IL-10 in tumor tissues

Control vs DOX+GEM, *p<0.05 and ***p<0.001

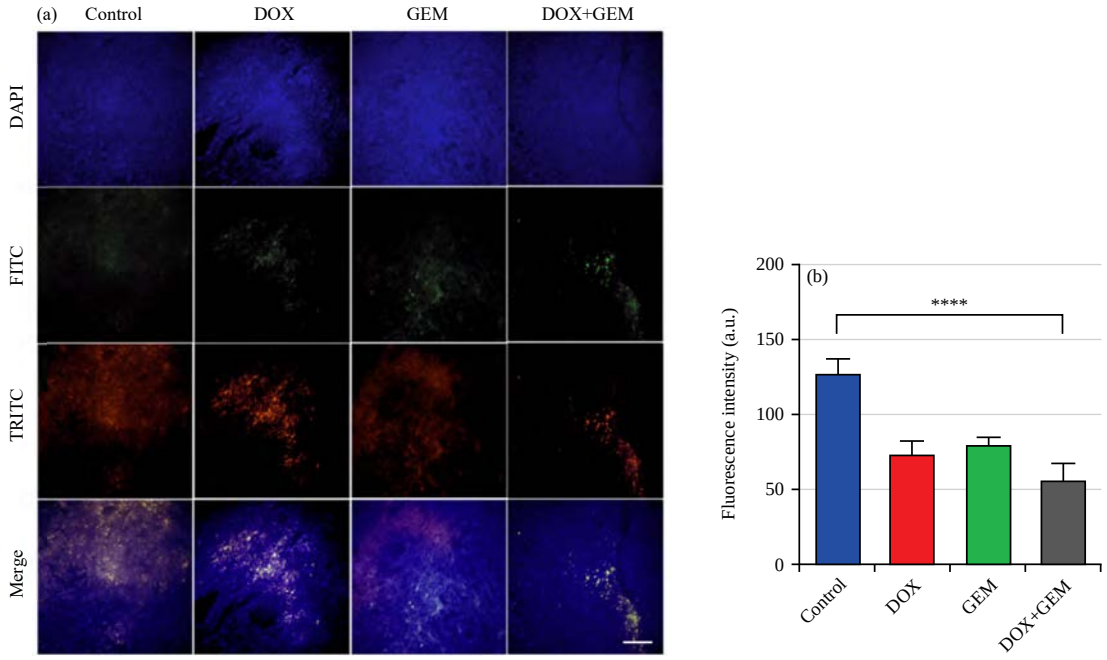

Fig. 6(a-b): (a) Immunofluorescence staining of MDSCs cells in mice tumor tissues (DAPI: Nucleus, FITC: Gr-1, TRITC: CD11b, scale: 100 μm) and (b) Quantitative statistics
*p<0.05 and ***p<0.001

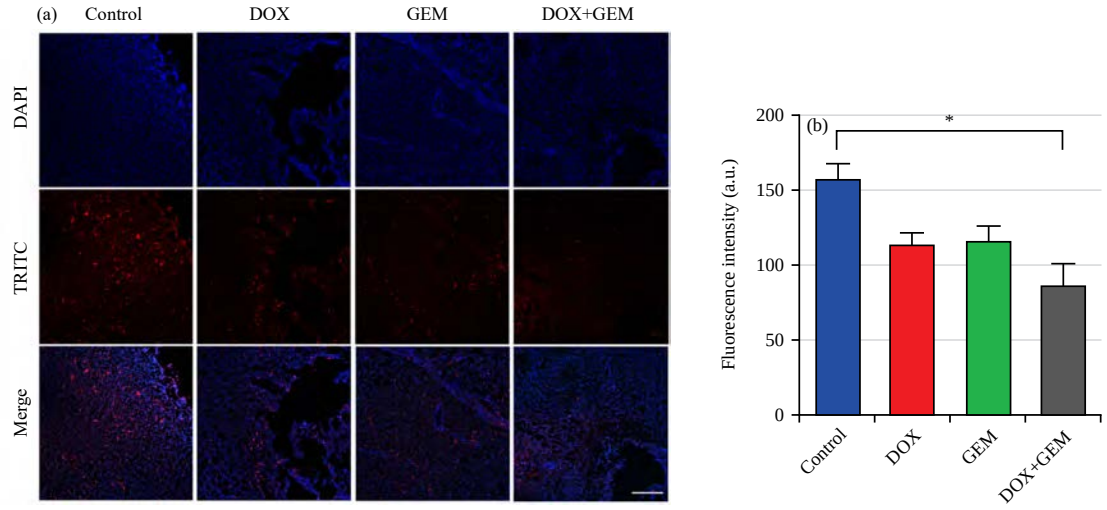

Fig. 7(a-b): (a) KI67 immunofluorescence staining in mouse tumor tissue (DAPI: Nucleus, TRITC: Ki67) and (b) Quantitative statistics
*p<0.05

Effects of drugs on proliferation of tumor cells: The effects of the combination of DOX and GEM on the proliferation of tumor cells were shown in Fig. 7, the study found that DOX+GEM combined administration could significantly reduce cell proliferation in tumor tissues, thus the combined administration could play an excellent role in inhibiting tumor growth.

Effect of drugs on the number of $CD8^+$ T cells in tumor tissue: The results of drugs on the number of $CD8^+$ T cells in tumor tissue were shown in Fig. 8. According to the staining results, little $CD8^+$ T cells were showing red fluorescence in the tumor tissue of the control group and the semi-quantitative fluorescence results showed that the average fluorescence intensity was 63.5, which was significantly lower than that of

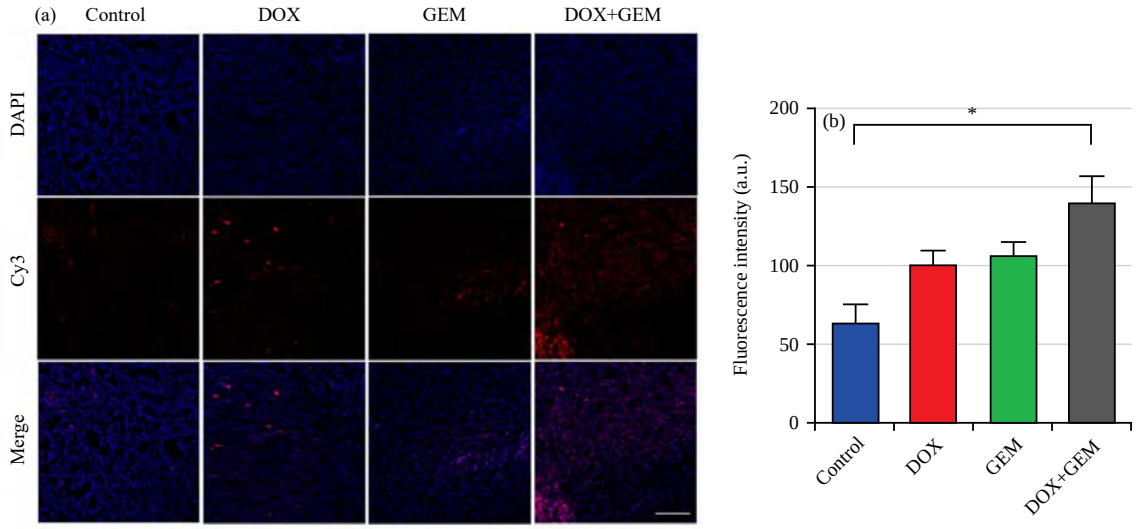

Fig. 8(a-b): Immunofluorescence staining of $CD8^+$ T cells in mouse tumor tissue (DAPI: Nucleus, Cy3: $CD8^+$) and (b) Quantitative statistics
Control vs DOX+GEM and *p < 0.001

other drug-treated groups. Obvious red fluorescence was observed in the mice tumor tissues in DOX, GEM and DOX+GEM groups and the red fluorescence intensity of $CD8^+$ T cells increased most obviously in DOX+GEM group. These results indicated that DOX+GEM combined administration could promote the immune regulation of drugs and thus enhance their antitumor effects.

DISCUSSION

In this study, we successfully induced MDSCs and found that DOX, GEM and the combination of two drugs can significantly inhibit MDSCs. Immunofluorescence staining of mice tumor tissues showed that DOX+GEM also reduced the number of MDSCs in tumor tissues and showed a better enhancing immunosuppressive effect. The TME denotes non-neoplastic cells, extracellular matrix, immune cells and tumor vasculatures, it has been extensively implicated in tumorigenesis and plays an important role in the various stages of tumor progression[20-22]. In the early stage of tumor formation, tumor cells will secrete a series of immunosuppressive cytokines to enhance the activation and enrichment of immunosuppressive cells in the tumor microenvironment and promote the generation of peripheral blood vessels to accelerate their metastasis. Studies have shown that immunosuppressive microenvironments play an important role in tumor invasion, metastasis and recurrence[23,24]. Therefore, specific regulation of immunosuppression of TME in cancer therapy will greatly improve the effectiveness of cancer therapy. The MDSCs are a major class of immunosuppressive cells in the tumor microenvironment, which play an important role in the process of tumor growth[25]. The MDSCs are derived from bone marrow progenitor cells and aggregate to tumor tissues under tumor induction, inhibit T cell function and induce tumor metastasis.

In the toxicity experiments on 4T1 cells, it was found that DOX+GEM combined administration significantly enhanced the inhibitory effect on 4T1 compared with a single administration, indicating that DOX+GEM combined administration has a better synergistic anti-tumor effect *in vitro*. The 4T1 tumor model in mouse was established and it was found that DOX+GEM combined therapy significantly reduced the tumor volume and weight in mice compared with the drug alone. Cytotoxic $CD8^+$ T cells play a key role in tumor-specific adaptive immune responses and can play an anti-tumor role by attacking tumor cells that express tumor-associated antigenic peptides on the surface containing major histocompatibility complex[26,27]. The existence of immunosuppressive factors in the tumor microenvironment will make $CD8^+$ T cells lose the ability to kill tumor cells, leading to the occurrence of tumor immune escape[28]. Therefore, reversing the depletion of $CD8^+$ T cells at the tumor site and restoring their antitumor activity is one of the main strategies for tumor immunotherapy[29]. In this study, $CD8^+$ T cells in mice tumor tissue were detected and it was found that DOX+GEM combined administration could increase the number of toxic T lymphocytes and $CD8^+$ T cells, thus improving the immunosuppression of tumors.

The TNF-α and IL-12 are important cytokines for tumor killing in tumor immune reaction and IL-10 plays an important role in the immunosuppressive tumor microenvironment, promoting tumor proliferation and angiogenesis[30-32]. Then the expression levels of various cytokines in tumor tissues were detected in the study, which showed that although DOX+GEM combined medication could reduce the expression of TNF-α in tumor, the combined administration would significantly reduce the expression level of immunosuppressive factor IL-10 in tumor tissues and increase the expression of immune promoting factor IL-12. The results showed that the immunosuppressive factors in the tumor microenvironment were significantly inhibited. After KI67 was used to label proliferating cells in tumor tissues, immunofluorescence detection showed that DOX+GEM combined treatment could also significantly inhibit the proliferation of tumor cells. All the above *in vitro* and *in vivo* experiments indicated that DOX+GEM combined administration could inhibit the proliferation of tumor cells and enhance the antitumor effect of drugs.

The study proved the role of tumor microenvironment in tumor pathogenesis, suggesting that tumor immune microenvironment can be used as a target for tumor drug administration regulation. Meanwhile, the combination of drug therapy can achieve better clinical effect than single drug therapy. However the scope of drugs the combination strategy can be applied to has yet to be confirmed.

CONCLUSION

In short, the study proved that DOX+GEM combination administration can effectively reduce the MDSC cells, improve the immunosuppression effect in the tumor microenvironment and restore the cytotoxic T lymphocytes in the tumor tissue, thus activating the tumor immune microenvironment and enhancing the chemotherapy effect of drugs. This study can provide theoretical basis for the combined application of doxorubicin and gemcitabine in clinic and provide a novel target for cancer drug delivery.

SIGNIFICANCE STATEMENT

The combination therapy of gemcitabine and doxorubicin can improve the immunosuppression effect in the tumor microenvironment and restore the cytotoxic T lymphocytes in the tumor tissue, thus activating the tumor immune microenvironment and enhancing the chemotherapy effect of drugs. This study can provide theoretical basis for the combined application of doxorubicin and gemcitabine in clinic and also suggests that the regulation of tumor microenvironment can be used as the target of antitumor drugs.

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