Targeted inhibition of myeloid-derived suppressor cells in the tumor microenvironment by low-dose doxorubicin to improve immune efficacy in murine neuroblastoma

Wei-Li Xu[1], Bao-Jun Shi[1], Suo-Lin Li[1], Feng-Xue Yu[2], Li-Na Guo[2], Meng Li[1], Zhi-Gang Hu[3], Gui-Xin Li[3], Hui Zhou[1]

[1]Department of Pediatric Surgery, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei 050000, China;
[2]Department of Central Laboratory, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei 050000, China;
[3]Department of General Surgery, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei 050000, China.

Abstract

Background: High agglomeration of myeloid-derived suppressor cells (MDSCs) in neuroblastoma (NB) impeded therapeutic effects. This study aimed to investigate the role and mechanism of targeted inhibition of MDSCs by low-dose doxorubicin (DOX) to enhance immune efficacy in NB.

Methods: Bagg albino (BALB/c) mice were used as tumor-bearing mouse models by injecting Neuro-2a cells, and MDSCs were eliminated by DOX or dopamine (DA) administration. Tumor-bearing mice were randomly divided into 2.5 mg/kg DOX, 5.0 mg/kg DOX, 50.0 mg/kg DA, and control groups ($n = 20$). The optimal drug and its concentration for MDSC inhibition were selected according to tumor inhibition. NB antigen-specific cytotoxic T cells (CTLs) were prepared. Tumor-bearing mice were randomly divided into DOX, CTL, anti-ganglioside (GD2), DOX+CTL, DOX+anti-GD2, and control groups. Following low-dose DOX administration, immunotherapy was applied. The levels of human leukocyte antigen (HLA)-I, CD8, interleukin (IL)-2 and interferon (IFN)-γ in peripheral blood, CTLs, T-helper 1 (Thl)/Th2 cytokines, perforin, granzyme and tumor growth were compared among the groups. The Wilcoxon two-sample test and repeated-measures analysis of variance were used to analyze results.

Results: The slowest tumor growth ($F = 6.095$, $P = 0.018$) and strongest MDSC inhibition ($F = 14.632$, $P = 0.001$) were observed in 2.5 mg/kg DOX group. Proliferation of T cells was increased ($F = 448.721$, $P < 0.001$) and then decreased ($F = 2.047$, $P = 0.186$). After low-dose DOX administration, HLA-I ($F = 222.489$), CD8 ($F = 271.686$), Thl/Th2 cytokines, $CD4^+$ and $CD8^+$ lymphocytes, granzyme ($F = 2376.475$) and perforin ($F = 488.531$) in tumor, IL-2 ($F = 62.951$) and IFN-γ ($F = 240.709$) in peripheral blood of each immunotherapy group were all higher compared with the control group (all of P values < 0.05). The most significant increases in the aforementioned indexes and the most notable tumor growth inhibition were observed in DOX+anti-GD2 and DOX+CTL groups.

Conclusions: Low-dose DOX can be used as a potent immunomodulatory agent that selectively impairs MDSC-induced immunosuppression, thereby fostering immune efficacy in NB.

Keywords: Neuroblastoma; Myeloid-derived suppressor cell; Tumor microenvironment; Doxorubicin; Immunotherapy

Introduction

Neuroblastoma (NB) is the most common extracranial malignant tumor in childhood, which is highly malignant and prone to relapse and metastasis. Although many methods including surgery, chemotherapy, and stem cell transplantation have been widely used in the clinic, the survival rate of children with high-risk NB remains very low.[1-3] In recent years, with the progress of tumor biology and immunology, immunotherapy has gradually become a promising new form of tumor treatment. However, to date, there are still some issues, mainly including the tumor immunosuppressive microenvironment, tumor antigen heterogeneity, and short effective time of immune cells *in vivo*, which seriously affect the immune efficacy of NB.[4-7]

Myeloid-derived suppressor cells (MDSCs) are a group of myeloid-derived natural immune cells with inhibitory functions.[8] MDSCs inhibit both natural immunity and T cell adoptive immunity, and can cause tumor immune tolerance, which is the main obstacle that affects immune

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.1097/CM9.0000000000001234

Correspondence to: Prof. Wei-Li Xu, Department of Pediatric Surgery, The Second Hospital of Hebei Medical University, 215 Heping West Road, Shijiazhuang, Hebei 050000, China
E-Mail: drxu9916@163.com

Chinese Medical Journal 2021;134(3)
Received: 13-08-2020 **Edited by:** Ning-Ning Wang

efficacy.[8] In recent years with comprehensive understanding of MDSC differentiation, proliferation, and inhibitory functions, research on tumor immunotherapy that targets MDSCs has advanced significantly. Alizadeh *et al*[9] found that doxorubicin (DOX) selectively clears MDSCs in a breast cancer model, activates effector cells, and has obvious anti-tumor effects. Moreover, the neurotransmitter dopamine (DA) promotes the differentiation and maturation of MDSCs and decreases the inhibitory effect of MDSCs on T cell proliferation.[10] Tumor microenvironment (TME) regulation by targeted inhibition or differentiation of MDSCs improves immune efficacy through various mechanisms, but whether these mechanisms play an effective therapeutic role in NB is unknown.

Therefore, in the present study, bagg albino (BALB/c) mice were used to establish a tumor-bearing model by injection of NB cells, followed by DOX or DA administration to explore the effect and mechanism of targeted inhibition of MDSCs on the killing effect of effector cells *in vivo*. Inhibition of MDSCs is hypothesized to provide a new method to improve immune efficacy in NB, which will be an important theoretical basis to explore new drugs and biological agents.

Methods

Ethical approval

All experiments were conducted in accordance with the principles and procedures outlined in the Guideline of Laboratory Animals of the Institutional Experimental Animal Review Committee (2019-P052). The ethical standards of experiments were in accordance with the guidelines provided by World Medical Association *Declaration of Helsinki* on Ethical Principles for Medical Research Involving Experimental Animals.

Experimental cells and animals

Neuro-2a mouse NB cell line TCM29 was purchased from the Shanghai Cell Bank of the Chinese Academy of Medical Sciences (Shanghai, China) and cultured in glutamine-containing medium with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2. Four-week-old female healthy BALB/c mice, each weighing 8 to 11 g (SCXK Hebei 2008-1-003), were purchased from the Experimental Animal Center of Hebei Province (specific pathogen-free grade; SYXK Hebei 2008-0026) and housed in a specific pathogen-free facility. Their daily routine, diet, and defecation were observed regularly and recorded.

Establishment of the tumor-bearing mouse model and screening of targeted drugs

An NB-bearing mouse model was established by subcutaneous injection of Neuro-2a cells in the logarithmic growth phase at 5×10^7 cells/mL. A total of 250 BALB/c mice were injected with 0.2 mL tumor cell suspension into the lower flank region unilaterally. Successful modeling was assumed when a tumor reached a measurable volume of 100 mm^3 under the inoculated subcutaneous tissue at 5 days after injection. Tumor sizes were determined every 3 days by caliper measurement using the standard formula $1/6\pi$ (length $\times$ width^2). Then, the tumor-bearing mice were randomly divided into the 2.5 mg/kg DOX (Wanle Pharmaceutical, Shenzhen, China) group, 5.0 mg/kg DOX group, 50.0 mg/kg DA (eBioscience, CA, USA) group (hereafter referred to as DOX2.5, DOX5, and DA, respectively), and control group, according to a random number table with 20 mice in each group (the sample size was estimated to meet the design requirements). On days 7 and 12 after inoculation, DOX or DA was injected intravenously in the corresponding groups,[9,10] and the MDSC proportion, T cell cycle, tumor volume, body weight, and tumor weight of tumor-bearing mice in each group were assessed and compared on days 14, 17, and 23 after inoculation. No intervention was applied to the control group. Then the optimal drug and its concentration to inhibit MDSCs were selected according to inhibition of tumor growth.

Assessment of the MDSC proportion and T cell cycle in tumors of each drug screening group

Three mice in each group were sacrificed on days 14, 17, and 23 post-inoculation. The tumor was excised and placed in precooled normal saline to remove blood and then cold erythrocyte lysis buffer was added. The tumor was then fully dissociated by a cell ultrasound pulverizer and centrifuged at 300 $\times$ *g* for 5 minutes. The supernatant was collected and stored at −20°C. The single cell suspension was subjected to erythrocyte lysis and then resuspended in PBS. MDSCs were separated by density gradient centrifugation on a Percoll solution (Cytiva, Amersham, UK). Rat anti-mouse glutathione reductase-1 +-fluorescein isothiocyanate (Gr-1$^+$-FITC) (eBioscience, CA, USA) and rat anti-mouse cluster of differentiation 11b +-phycoerythrin (CD11b$^+$-PE) (eBioscience) antibodies were applied to the MDSCs, according to the manufacturer's instructions. The proportion of Gr-1$^+$CD11b$^+$ MDSCs was determined by flow cytometry (FC500; Beckman, CA, USA). A control group was set up.

CD3$^+$ T cells were purified by negative selection using magnetic activated cell sorting (MACS) and a MagCellect Mouse CD3$^+$ T Cell Isolation Kit (R&D Systems, Shanghai, China), according to the manufacturer's protocol. The samples were collected in PBS and the cell density was adjusted to 1×10^6 cells/mL, followed by centrifugation at 300 $\times$ *g* for 10 minutes. After discarding the supernatant, the cells were resuspended with 100 μL PBS and stained with propidium lodide (PI) in dark for 10 minutes. After adding 500 μL PBS, T cell cycles in the tumor were assessed using a Cell cycle detection kit (Invitrogen, CA, USA) by flow cytometry.

Preparation of NB antigen-specific cytotoxic T lymphocytes (CTLs)

A mononuclear cell suspension derived from the murine spleen was suspended at 2×10^8 cells/mL and incubated with a MagCellect Antibody Cocktail (R&D Systems) to remove undesired cells (non-CD3$^+$ T cells). CD3$^+$ T cells

were purified by negative selection using MACS and a MagCellect Mouse $CD3^+$ T Cell Isolation kit (R&D Systems), according to the manufacturer's protocol. Murine myeloid-derived dendritic cells (DCs) were loaded with tumor antigens and then mixed with a lysate of Neuro-2a cells in the logarithmic growth phase. Tumors loaded with DCs were incubated with $CD3^+$ T cells (DCs: T cells = 1:20) for 3 to 4 days to induce antigen-specific CTLs.

Immunotherapy of NB-bearing mice after low-dose DOX administration

An anti-ganglioside (GD2) antibody is a proven therapeutic agent for GD2-positive NB. Monoclonal antibodies against GD2, such as chimeric mAb ch14.18, have become the benchmark for NB therapies.[11] According to the random number table, NB-bearing mice were randomly divided into six groups including DOX, CTL, anti-GD2, DOX+CTL, DOX+anti-GD2, and control groups with 20 mice in each group (the sample size was estimated to meet the design requirements). On day 7 post-inoculation, low-dose DOX was injected intravenously into the corresponding groups according to the selected dose and no intervention was performed in the control group. On day 9 after inoculation, antigen-specific CTLs were transfused or a mouse anti-GD2 antibody (14.G2a; Abcam, Cambridge, UK) was injected into tumor-bearing mice of the corresponding groups. On days 14, 17, and 23 after inoculation, blood was collected from the eyeball of tumor-bearing mice, tumor tissues and non-tumor tissues (>5 cm away from the tumor edge) were isolated, and the corresponding indexes were measured in subsequent experiments.

Detection of human leukocyte antigen (HLA)-I and CD8 in tumors of each treatment group

Tumor tissues were embedded in paraffin and 4 μm thick tissue sections were prepared, dewaxed, hydrated, and then subjected to antigen retrieval. Staining was carried out using an SP immunohistochemical kit (Santa Cruz Biotechnology, Inc., DE, USA), according to the manufacturer's instructions. The dilutions of a rabbit anti-mouse HLA-I polyclonal antibody and rat anti-mouse CD8 monoclonal antibody were both 1:150. PBS was used as a negative control. Expression of HLA-I and CD8 in each group was compared using an Immunohistochemical Image Optical Density Analysis System (Olympus, Tokyo, Japan) and Image-Pro Plus (Media Cybernetics, Inc., Rockville, USA). According to the gray level of positive immune response images, the appropriate gray segmentation threshold was selected to achieve double threshold segmentation, and the half-gray level target image of the sample was obtained. Then, the intensity and area of positive immunostaining were measured by human-computer interaction mode. Three mice were analyzed in each group, and five visual fields were measured for each mouse. The gray value and area of the measured positive reactant were calculated automatically. The differences in the relative contents of HLA-I and CD8 between the groups were analyzed.

Measurement of interleukin (IL)-2 and interferon (IFN)-γ levels in peripheral blood

Posterior orbital blood (100 μL) was collected from the mice into sterile EP tubes. The blood was centrifuged and the concentrations of IL-2 and IFN-γ in peripheral blood at various time points were measured using ELISA kits (Abcam), according to manufacturer's instructions.

Detection of CTLs, T-helper 1 (Th1)/Th2 cytokines, perforin, and granzyme

Preparation of a homogenate supernatant and lymphocytes from tumor and non-tumor tissues

After tumor-bearing mice were sacrificed, fresh tumor and non-tumor tissues were prepared as a single cell suspension, the supernatant was collected by centrifugation, and the protein concentration was measured by using Bicinchoninic Acid (BCA) Protein Assay Kit (Applygen, Beijing, China). Supernatants were stored at −80°C. After the cells were resuspended, lymphocytes in the tissues were separated using lymphocyte separation solution.

Detection of $CD4^+$ CTLs, and $CD8^+$ CTLs in tumors

The obtained lymphocytes were labeled immediately with fluorescence. A control group was set up. A rat anti-mouse CD3-FITC/CD8-PE antibody (eBioscience) or rat anti-mouse CD3-FITC/CD4-PE antibody (eBioscience) was added to the sample tube, and the corresponding homotype control antibody was added to the control tube. Then, the hemolytic agent was added and the cells were incubated in the dark at room temperature. After washing with PBS and centrifugation at $30 \times g$ for 10 minutes, the supernatant was discarded and the cells were resuspended with PBS and analyzed by the flow cytometer.

Detection of Th1/Th2 cytokines in tumor homogenate supernatants

The supernatant of the tumor homogenate stored at −80°C was thawed at room temperature and then Th1/Th2 cytokines (IL-17A, IFN-γ, tumor necrosis factor (TNF)-α, IL-6, IL-4, and IL-2) were detected using a Cytometric Bead Array (CBA) kit (BD Biosciences, NJ, USA), according to the manufacturer's instructions. Standard control and negative control tubes were set.

Measurement of perforin and granzyme in supernatants of tissue homogenates

The frozen supernatants of tissue homogenates were thawed at room temperature and analyzed using perforin and granzyme ELISA kits (eBioscience). Absorbance at 450 nm was measured using a microplate reader, and perforin and granzyme concentrations were calculated using a standard curve.

Comparison of tumor growth curves

Mice were fed in a sterile purification barrier system with constant temperature (25 ± 2°C) and constant humidity

(45%–50%), and observed for behavior, diet, and defecation. Tumor volumes were calculated according to the formula V=1/6π (length × width^2), and then tumor growth curves were drawn. The body and tumor weights of tumor-bearing mice were also recorded and compared. Deaths of mice were observed, and measurement of the tumor volume was stopped when the death rate of mice in any group reached 50%.

Statistical analysis

All data were analyzed using IBM SPSS Statistics 26.0 software (IBM, NY, USA). The Wilcoxon two-sample test and repeated-measures analysis of variance were used to analyze the results. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of tumor growth between the screening groups and selection of the optimal drug

There were significant differences in the tumor volumes ($F = 6.095$, $P = 0.018$), tumor weight ($F = 224.591$, $P < 0.001$), and body weight ($F = 8.409$, $P = 0.001$) among the various groups. Pairwise comparison showed significant differences in the tumor volume ($P < 0.05$), tumor weight ($P < 0.05$), and body weight ($P < 0.05$) were found between the DOX2.5 group and other groups with the slowest tumor growth, lowest tumor weight, and highest body weight [Figure 1B–1E]. However, no significant differences existed in the tumor volume ($P > 0.05$) or body weight ($P > 0.05$) between DA,

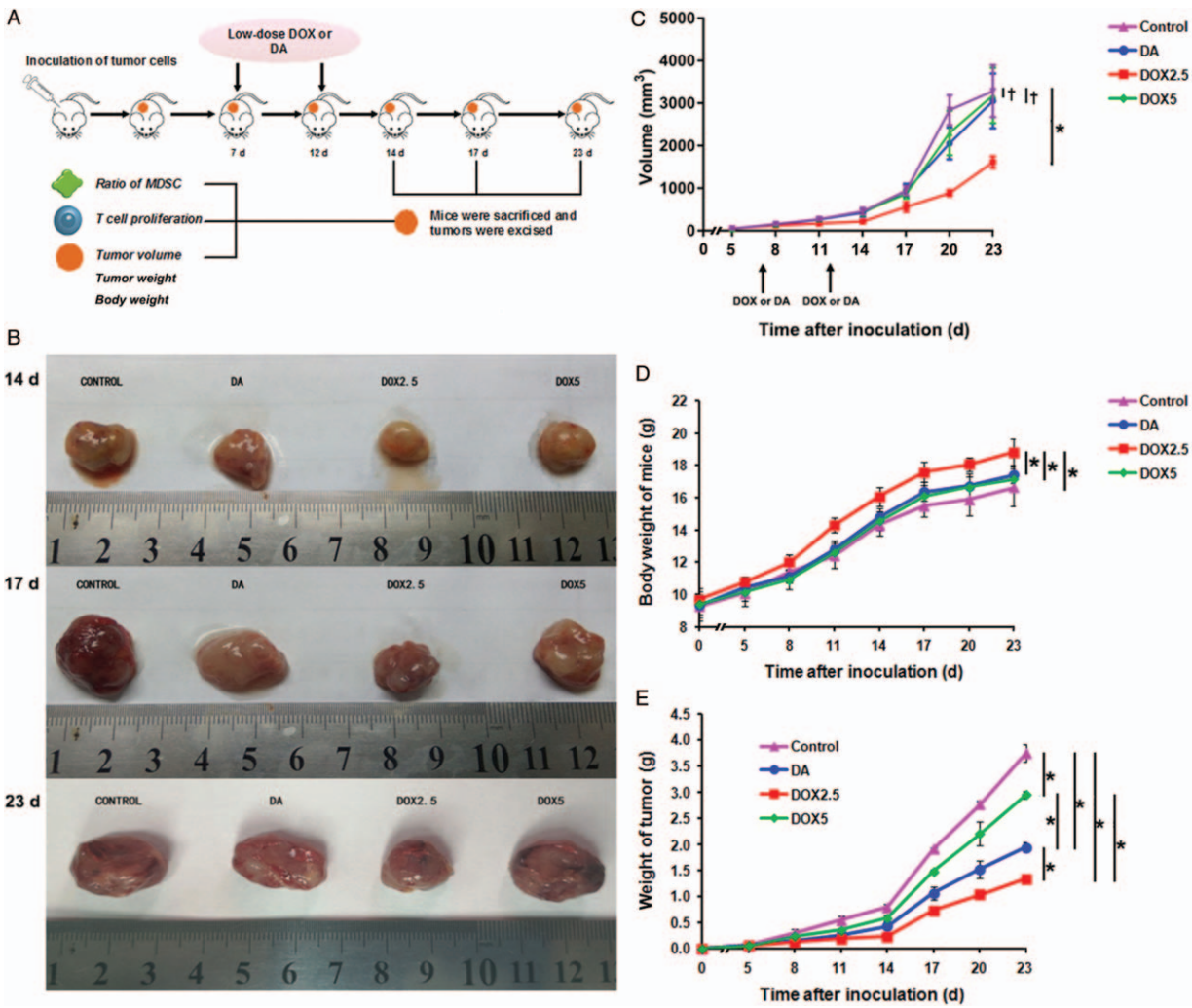

Figure 1: Comparison of tumor growth between the drug screening groups and selection of the optimal drug. (A) A diagram for the experiment of screening the targeted drugs. The tumor-bearing mice were randomly divided into DOX2.5 group, DOX5 group, DA50 group and control group, with 20 cases in each group. The time to inject drugs, detect different indexes and sacrifice NB-bearing mice were clearly introduced. (B) The tumor specimens of each group are shown and compared. (C) On the 7th and 12th days after inoculation, DOX or DA were injected intravenously to the corresponding groups. The difference in tumor volume between the different groups was gradually seen from the 14th day after inoculation. The significant difference occurred between DOX2.5 group and the other groups, with the slowest tumor growth. However, no significant difference existed between the DA group, DOX5 group and control group. (D) There was a significant difference in the body weight between the different groups. The significant difference occurred in the body weight between DOX2.5 group and the other groups, with the highest body weight. (E) A significant difference in the tumor weight between the different groups. The significant difference occurred in the tumor weight between DOX2.5 group and the other groups, with the lowest tumor weight. Significant differences were also shown between the DA, DOX5 and control groups. A repeated-measures analysis of variance was used to analyze the results. $^*P < 0.05$. $^\dagger P > 0.05$ *vs.* control group. DA: Dopamine; DOX: Doxorubicin; MDSC: Myeloid-derived suppressor cell.

DOX5, and control groups, although significant differences were found in the tumor weight ($P < 0.05$) [Figure 1C–1E]. The optimal targeted drug (DOX) to inhibit MDSCs and its concentration (2.5 mg/kg) were selected according to the tumor growth inhibition.

Comparison of the MDSC proportion and T cell cycle in tumors between the drug screening groups

After drug administration, the MDSC proportion in tumors was changed and the T cell cycle also varied. The proportion of Gr-1$^+$CD11b$^+$ MDSCs was notably

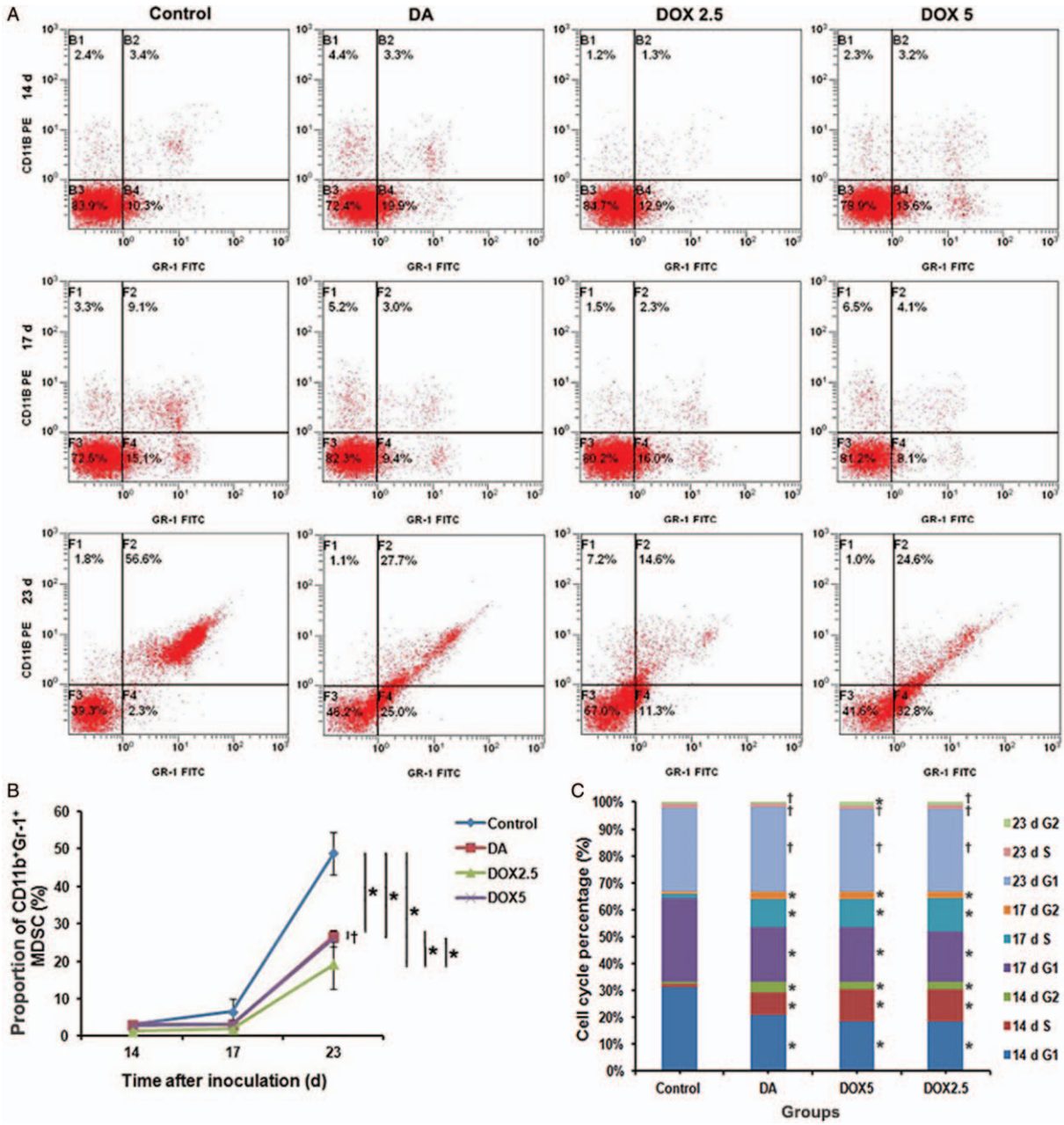

Figure 2: Comparison of the MDSC proportion and T cell cycle in tumors between the drug screening groups. (A) In the flow cytometry, the proportion of Gr-1$^+$CD11b$^+$ MDSCs in the DOX2.5 group, DOX5 group, DA group and control group were shown at the different time points after inoculation, respectively. The proportion of Gr-1$^+$CD11b$^+$ MDSCs notably decreased in tumor tissue after DOX or DA administration, particularly in the DOX2.5 group. (B) By statistical analysis, significant differences were seen between the different groups. The increase in Gr-1$^+$CD11b$^+$ MDSCs was inhibited in the DOX2.5 group, DOX5 group and DA group, particularly in the DOX2.5 group. No difference existed in the proportion of Gr-1$^+$CD11b$^+$ MDSCs between the DOX5 group and DA group. $^*P < 0.05$. $^\dagger P > 0.05$.(C) In tumor, the cell proportions of G1 phase in DOX2.5 group, DA50 group and DOX5 group were all lower than those in the control group on the 14th day after inoculation. The cell proportions of S/G2 phases showed the contrary variation. The proportions of G1/S/G2 cells remained the same tendency in the experimental groups on the 17th day after inoculation. The results showed an obvious proliferation of CD3$^+$ T cells from 14 d to 17 d after inoculation. However, the cell proportions of G1 phase in the experimental groups all increased again on the 23rd day after inoculation and the cell proportions of S/G2 phases decreased contrarily which suggested CD3$^+$ T cells proliferation was inhibited again. The Wilcoxon two-sample test and a repeated-measures analysis of variance were used to analyze the results. $^*P < 0.05$ *vs.* control group $^\dagger P > 0.05$ *vs.* control group. Twenty cases were enrolled in each group. DA: Dopamine; DOX: Doxorubicin; MDSC: Myeloid-derived suppressor cell.

decreased in tumor tissue after DOX and DA administrations, especially in the DOX2.5 group. Statistical analysis revealed significant differences between the various groups ($F = 14.632$, $P = 0.001$; Figure 2A and 2B). The increase in Gr-1$^+$CD11b$^+$ MDSCs was inhibited in DOX2.5, DOX5, and DA groups, particularly in the DOX2.5 group. No difference was observed in the proportion of Gr-1$^+$CD11b$^+$ MDSCs between DOX5 and DA groups ($P > 0.05$).

In tumors, the proportions of cells in G1 phase of DOX2.5, DA, and DOX5 groups were all lower than those in the control group on day 14 after inoculation ($F = 448.721$, $P < 0.001$). The proportions of cells in S/G2 phases showed the opposite trend ($F = 680.833$, $P < 0.001$ / $F = 118.468$, $P < 0.001$). The proportions of G1/S/G2 cells remained the same in the groups on day 17 after inoculation ($P < 0.05$). However, the proportions of cells in G1 phase in the groups were all increased again on day 23 after inoculation ($F = 2.047$, $P = 0.186$), while the proportions of cells in S phase was decreased ($F = 3.844$, $P = 0.057$) [Figure 2C].

Comparison of HLA-I and CD8 in tumors between treatment groups

The expression levels of HLA-I and CD8 on the surface of tumor cells determine whether it can be recognized and killed by effector cells. HLA-I and CD8 were both expressed in the cell membrane. After low-dose DOX administration, the expressions of HLA-I ($F = 222.489$, $P < 0.001$) and CD8 ($F = 271.686$, $P < 0.001$) were significantly higher in each immunotherapy group compared with the control group, particularly in DOX+anti-GD2 and DOX+CTL groups [Figure 3A and 3C]. The expression of HLA-I and CD8 in the immunotherapy groups was first increased at 14 and 17 days after inoculation and then decreased slightly at 23 days after inoculation. However, in the control group, the expression of HLA-I and CD8 was lower and there was no significant change between their expression levels at the various time points. The expression levels of HLA-I and CD8 in the DOX+anti-GD2 group were all higher than those in the anti-GD2 group and the same between DOX+CTL and CTL groups [Figure 3B and 3D].

Comparison of IL-2 and IFN-γ levels in peripheral blood between the treatment groups

IL-2 and IFN-γ are released into peripheral blood during tumor immunotherapy. After low-dose DOX administration, a significant difference was observed in the levels of IL-2 ($F = 62.951$, $P < 0.001$) and IFN-γ ($F = 240.709$, $P < 0.001$) in the peripheral blood of each group compared with those of the control group. IL-2 and IFN-γ levels in the peripheral blood of DOX+anti-GD2, DOX+CTL, and anti-GD2 groups were increased more notably (Figure 4A and 4B). The levels of IL-2 and IFN-γ in peripheral blood of the DOX+anti-GD2 group were all higher than those in

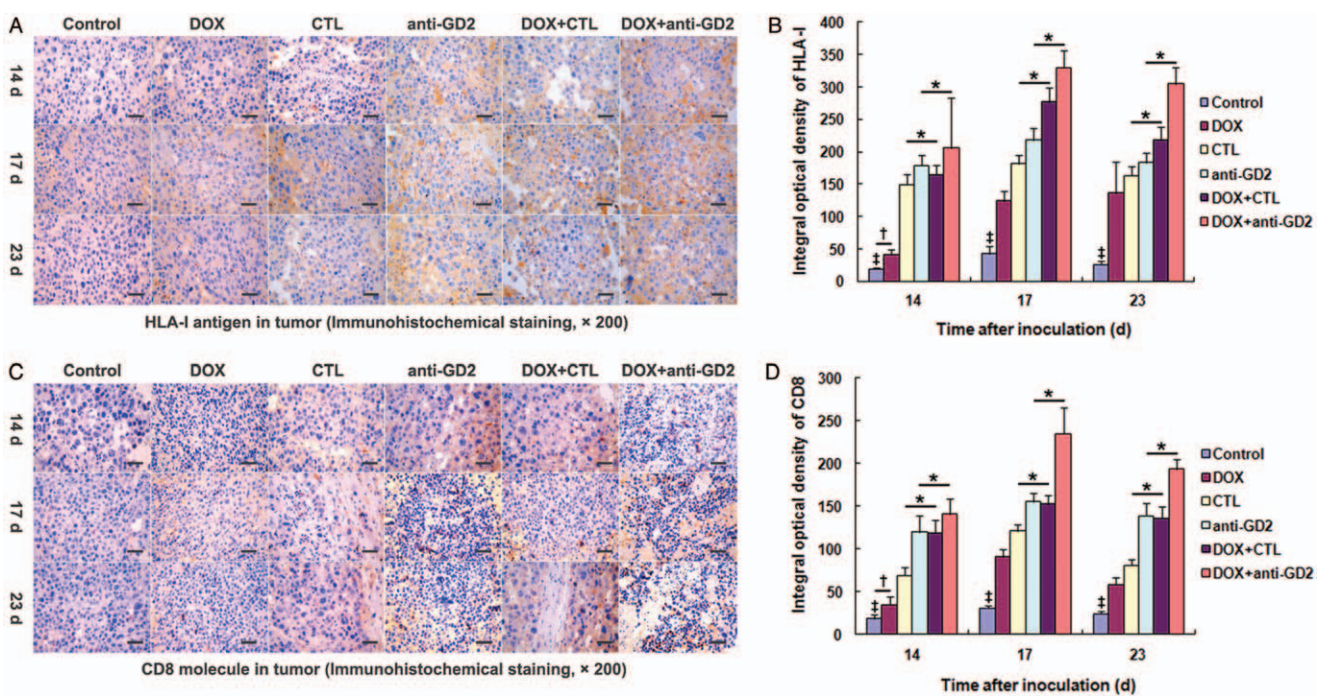

Figure 3: Comparison of HLA-I and CD8 in tumors between the treatment groups. (A and C) HLA-I and CD8 were both expressed brown-yellow in the cell membrane. After low-dose DOX administration, the expressions levels of HLA-I and CD8 in each immunotherapy group were all significantly higher compared with the control group, especially in DOX+anti-GD2 group and DOX+CTL group. (B and D) The expressions of HLA-I and CD8 in the immunotherapy groups increased firstly at the 14th and 17th days after inoculation, and then decreased slightly at the 23rd day after inoculation. However, in the control group, the expressions of HLA-I and CD8 was lower and no significant change was observed at the different time points. The expressions of HLA-I and CD8 in the DOX+anti-GD2 group were all higher compared with the anti-GD2 group, and the same as between the DOX+CTL group and CTL group. On the 14th day after inoculation, no difference existed between the DOX group and control group; but the differences were shown at the 17th and 23rd day after inoculation. The Wilcoxon two-sample test was used to analyze the results. $^*P < 0.05$; $^\dagger P > 0.05$ *vs.* control group; $^\ddagger P < 0.05$ *vs.* all the other groups except DOX group. Twenty cases were enrolled in each group. *Scale bar* = 100 μmol/L. DA: Dopamine; DOX: Doxorubicin; HLA: Human leukocyte antigen; IHS: Immunohistochemical staining; CTL: cytotoxic T lymphocyte.

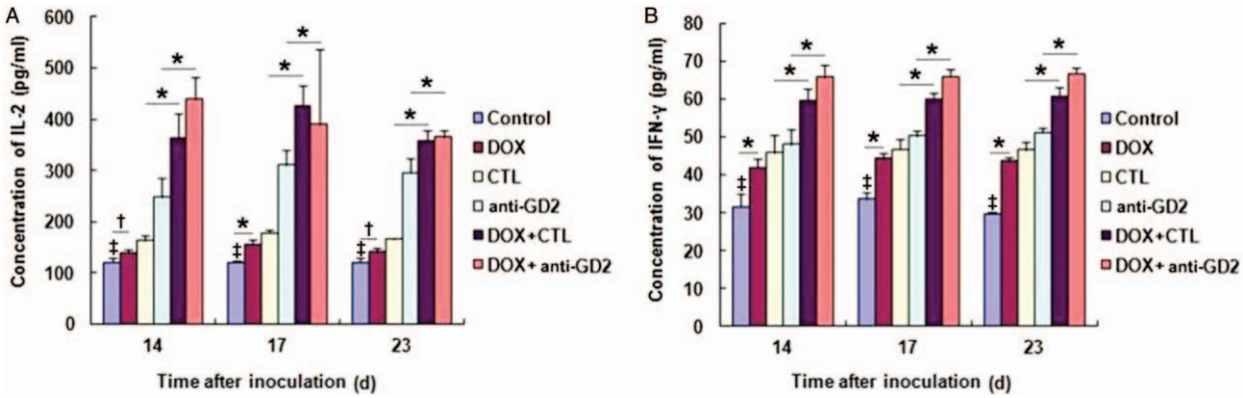

Figure 4: Comparison of IL-2 and IFN-γ levels in peripheral blood between the treatment groups. After low-dose DOX administration, significant differences were observed in IL-2 (A) and IFN-γ (B) levels in the peripheral blood of each group. Specifically, the levels of IL-2 and IFN-γ in the DOX+anti-GD2 group, DOX+CTL group and anti-GD2 group increased more than those in the control group. The levels of IL-2 and IFN-γ in DOX+anti-GD2 group were all higher compared with the anti-GD2 group, the same as between the DOX+CTL group and CTL group. On the 14th and 23rd days after inoculation, no difference existed in the level of IL-2 between the DOX group and control group; but the differences were shown in the level of IFN-γ on the 14th, 17th, and 23rd days after inoculation. The Wilcoxon two-sample test was used to analyze the results. $^{*}P < 0.05$; $^{\dagger}P > 0.05$ *vs.* control group; $^{\ddagger}P < 0.05$ *vs.* DOX+anti-GD2 group, DOX+CTL group and anti-GD2 group. Twenty cases were enrolled in each group. CTL: Cytotoxic T lymphocyte; DOX: doxorubicin; GD2: Ganglioside; IL: Interleukin; IFN: Interferon.

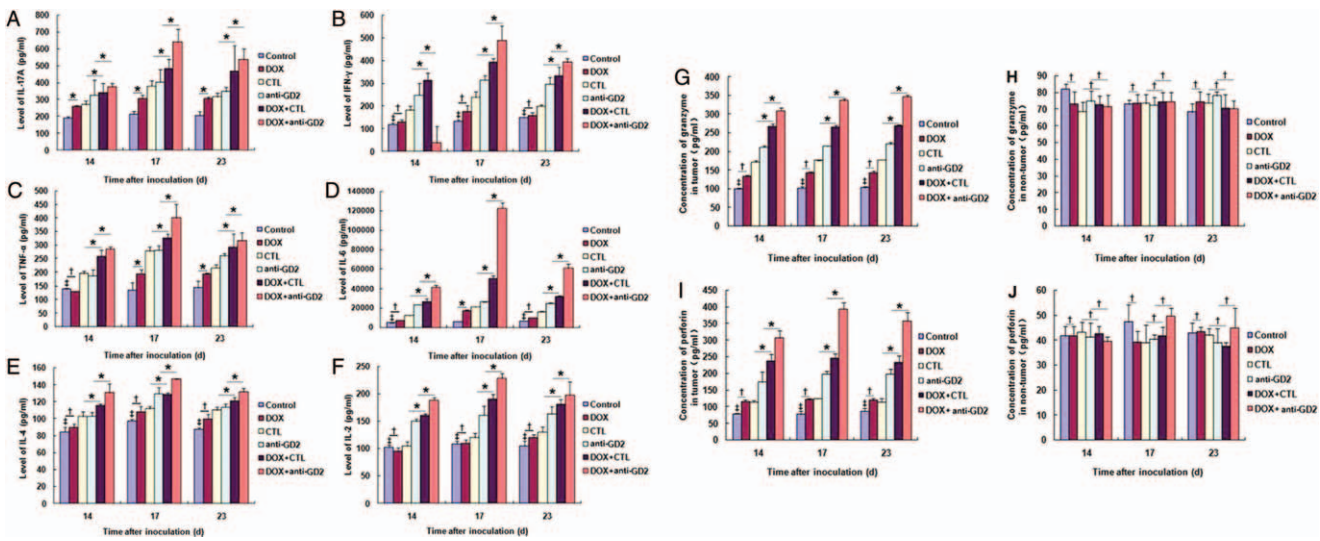

Figure 5: Comparison of Th1/Th2 cytokines, granzyme and perforin in tumors between the treatment groups. The expression levels of IL-17A, IFN-γ, TNF-α, IL-6, IL-4 and IL-2 showed significant differences in tumor tissues between the different groups, with 20 cases in each group. Specifically, the levels in the DOX+anti-GD2 group, DOX+CTL group and anti-GD2 group were increased compared with the control group. The levels of IL-17A, IFN-γ, TNF-α, IL-6, IL-4 and IL-2 in tumors of the DOX+anti-GD2 group were all higher compared with the anti-GD2 group, the same as between DOX+CTL group and CTL group. No difference existed in the levels of IFN-γ, IL-4 and IL-2 between the DOX group and control group; but the differences were shown in the level of IL-17A, TNF-α and IL-6 on the 14 th or 17 th or 23 rd days after inoculation. (A) IL-17A; (B) IFN-γ; (C) TNF-α; (D) IL-6; (E) IL-4; (F) IL-2. Significant differences were shown in the concentrations of granzyme and perforin in tumor tissues between the different groups (G and I). However, there were no significant differences in the concentration of granzyme and perforin in non-tumor tissues between the different groups (H and J). An apparent increase in DOX+anti-GD2 group, DOX+CTL group and anti-GD2 group was observed compared with those in control group. The concentrations of granzyme and perforin in tumors of the DOX+anti-GD2 group were all higher compared with the anti-GD2 group, the same as between DOX+CTL group and CTL group. No difference existed between the DOX group and control group. The Wilcoxon two-sample test was used to analyze the results. $^{*}P < 0.05$. $^{\dagger}P > 0.05$. $^{\ddagger}P < 0.05$ *vs.* all the other groups except DOX group. DOX: Doxorubicin; IL: Interleukin; IFN: Interferon; TNF: tumor necrosis factor; GD2: Ganglioside; CTL: Cytotoxic T lymphocyte.

the anti-GD2 group and the same between DOX+CTL and CTL groups (all of P values < 0.05).

Comparison of CTLs, Th1/Th2 cytokines, perforin, and granzyme in tumors between the treatment groups

CTL infiltration and release of Th1/Th2 cytokines, perforin, and granzyme in tumors reflected the immunotherapy state in each treatment group. The proportions of $CD4^+$ and $CD8^+$ CTLs in tumor tissue were significantly higher compared with those in non-tumor tissue. The proportions of $CD4^+$ and $CD8^+$ CTLs in the tumor tissue of each group were in the order of DOX+anti-GD2, anti-GD2, DOX+CTL, CTL, DOX, and control groups from high to low.

There were significant differences in the expression levels of IL-17A ($F = 10.762$, $P = 0.006$), IFN-γ ($F = 50.272$, $P < 0.001$), TNF-α ($F = 65.847$, $P < 0.001$), IL-6 ($F = 301.880$, $P < 0.001$), IL-4 ($F = 45.182$, $P < 0.001$), and IL-2 ($F = 86.316$, $P < 0.001$) in tumor tissues between the various groups [Figure 5A–5F]. Their levels in DOX

+anti-GD2, DOX+CTL, and anti-GD2 groups showed more increases compared with those in the control group ($P < 0.05$). The levels of IL-17A, IFN-γ, TNF-α, IL-6, IL-4, and IL-2 in tumors of the DOX+anti-GD2 group were all higher than those in the anti-GD2 group and the same between DOX+CTL and CTL groups (all of P values < 0.05).

Significant differences were observed in the concentrations of granzyme ($F = 2376.475$, $P < 0.001$) and perforin ($F = 488.531$, $P < 0.001$) in tumor tissues between the various groups. However, there was no significant difference in the concentrations of granzyme ($F = 0.510$, $P = 0.764$) and perforin ($F = 1.650$, $P = 0.221$) in non-tumor tissues between the various groups. More apparent increases were observed in DOX+anti-GD2, DOX+CTL, and anti-GD2 groups compared with the control group (Figure 5G–5J). The concentrations of granzyme and perforin in tumors of the DOX+anti-GD2 group were all higher compared with those in the anti-GD2 group and the same between DOX+CTL and CTL groups (all of P values < 0.05). No difference existed between DOX and control groups ($P > 0.05$).

Comparison of tumor growth between the treatment groups

The tumor growth exactly reflected the immune efficacy in each treatment group. There was a significant difference in the tumor volume ($F = 45.639$, $P < 0.001$), tumor weight ($F = 697.051$, $P < 0.001$), and body weight ($F = 30.238$, $P < 0.001$) between the groups. Pairwise comparison showed significant differences occurred in the tumor volume among the various groups, except in DOX and CTL groups ($P = 0.066$), and DOX+CTL and DOX+anti-GD2 groups ($P = 0.073$). Significant differences were also found in the tumor and body weights among the various groups, except in anti-GD2 and CTL groups ($P = 0.974$, $P = 0.286$), and DOX+CTL and DOX+anti-GD2 groups ($P = 0.662$, $P = 0.980$). Of note, apparent inhibition of

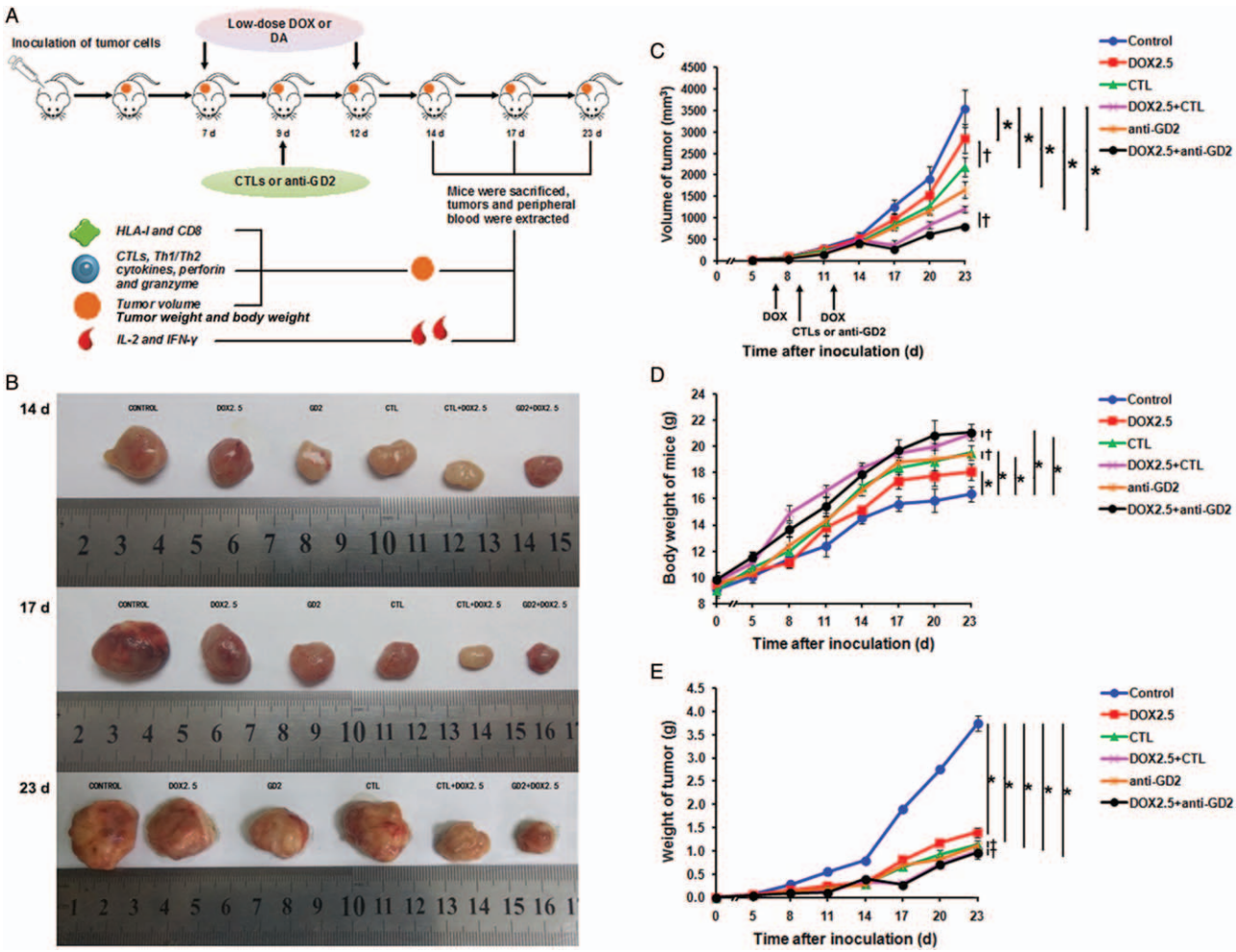

Figure 6: Comparison of tumor growth between the treatment groups. (A) A diagram for immunotherapy of NB-bearing mice. The tumor-bearing mice were randomly divided into DOX group, CTL group, anti-GD2 group, DOX+CTL group, DOX+anti-GD2 group and control group, with 20 cases in each group. The time to inject drugs, detect different indexes and sacrifice NB-bearing mice were clearly introduced. (B) The tumor specimens of each group are shown and compared. (C) The significant differences occurred among all the groups other than the DOX2.5 group and CTL group, DOX2.5+CTL group and DOX2.5+anti-GD2 group. In particular, an apparent inhibition of tumor growth was observed in the DOX2.5+anti-GD2 group and DOX2.5+CTL group. The tumor volume at 17th day was slightly lower than that at 14th day, which may be related to acceleration of T cells proliferation and killing effect in tumor with low-dose of DOX from 14th day to 17th day. However, from 17th day to 23rd day, T cell proliferation is inhibited again, so tumor volume increase again. (D) The significant differences were shown in the body weight among the different groups, except the anti-GD2 group and CTL group, DOX+CTL group and DOX+anti-GD2 group. (E) The significant differences existed in the tumor weight among the different groups, except the anti-GD2 group and CTL group, DOX+CTL group and DOX+anti-GD2 group. A repeated-measures analysis of variance was used to analyze the results. $^{*}P < 0.05$. $^{\dagger}P > 0.05$. CTL: cytotoxic T lymphocyte; DOX: Doxorubicin; GD2: Ganglioside.

tumor growth existed in DOX+anti-GD2 and DOX+CTL groups [Figure 6A–6E].

Discussion

NB is the most common neuroendocrine malignant tumor in childhood, which is susceptible to relapse and metastasis.[12,13] NB immunotherapy effectively removes tumor cells and decreases the recurrence rate because of its higher specificity and lower toxicity compared with the traditional treatment.[14] At present, the most effective method of NB immunotherapy is to inject an anti-GD2 antibody or gene-engineered vaccine, or adoptively transfuse chimeric antigen receptor T (CAR-T) cells.[14] However, in phase III clinical trials, although the survival rate of children with NB has improved by alternate use of chimeric mAb ch14.18 (γ1, κ) specific for GD2 combined with GM-CSF/IL-2 or CAR-T cells, some children with high-risk NB still experience relapse and metastasis. These results suggest that passive immunotherapy may need to adjust the immunosuppressive microenvironment to improve the efficacy of immunotherapy.[4,11,15]

MDSCs are a group of myeloid-derived natural immune cells with inhibitory functions, which play a negative immunomodulatory role in tumor progression.[8] They inhibit the functions of DCs by increasing the level of IL-10, which accelerates the polarization of macrophages to the M2 type, decreases the production of IL-12, and decreases the functions of NK cells to inhibit innate immunity *in vivo*. T cell adoptive immunity is inhibited by high expression of arginase-1, inducible nitric oxide synthetase, reactive oxygen species, and Treg amplification induced by TGF-β and IL-10.[8] Thus, MDSCs play a major role in the tumor microenvironment (TME) by promoting tumor immune escape and reducing immune efficacy.[13,16] In recent years, research on MDSCs as a regulatory target has gradually emerged to improve tumor immune efficacy.[17] Parihar *et al* showed that NK cells with chimeric activation of NKG2D receptor eliminate MDSCs, and rescue and improve the killing activity of CAR-T cells in solid tumors.[18] In animal experiments, it was also found that MDSC reduction enhances the anti-tumor effect of leukocyte infusion in NB-bearing mice.[19]

In the screening of targeted drugs, there was no significant difference in the tumor growth and weights among DOX5, DA, and control groups. However, the tumor volume and weight of the DOX2.5 group showed the slowest increase and the most significant difference. Therefore, as a type of chemotherapeutic drug, the low-dose DOX administration unexpectedly had a greater role in tumor inhibition compared with high-dose DOX administration, which suggested that other mechanisms may be involved in immunotherapy. Moreover, in tumors, the proportion of MDSCs was decreased notably and proliferation of T cells was first increased then decreased after low-dose DOX administration. DOX at a high concentration may cause toxicity and side effects in the immune system of mice. However, a low concentration of DOX not only clears MDSCs, but also causes no damage to the immune system of mice and promotes T cell proliferation steadily. Therefore, DOX cooperates with the immune killing effect of the immune system on tumors and achieves a better therapeutic effect. The inhibitory effect on tumors may be dependent on the concentration of DOX and its clearance efficiency of MDSCs. The specific mechanism needs to be verified by further experiments. Yuan *et al* found that nano-DOX is a cell inhibitor with good host tolerance, which avoids chemotherapy resistance in the tumor microenvironment of triple-negative breast cancer.[20] Moreover, to date, DOX is the most specific drug to selectively clear MDSCs.[9] Thus, low-dose DOX might play an important role in removing MDSCs from the TME of NB to accelerate tumor inhibition. Although some studies have shown that high-dose DOX inhibits tumor growth more than low-dose DOX in some cancers, it may be because the excessive dose of DOX only acts as a cytotoxic drug rather than an immunomodulator or different tumors have different responses and tolerance to DOX.

To verify whether low-dose DOX improved the immune efficacy of NB, tumor-bearing mice were injected with an anti-GD2 antibody and/or adoptively transfused with antigen-specific CTLs after low-dose DOX administration. The results showed that the therapeutic effect was improved significantly and tumor growth was apparently inhibited. The survival state of mice was restored and their body weight was increased by inhibition of tumor growth.

A recent study found that the level of NF-κB in NB cells is low.[21] NF-κB is a transcription factor that controls the expression of MHC molecules. The low level of MHC-I makes NB cells difficult to recognize by antigen-presenting cells and T cells. With the development of tumors, tumor-infiltrating lymphocytes are replaced by immature immune cells, whose frequency can predict the prognosis.[22-24] Thus, in the present study with the elimination of MDSCs by low-dose DOX administration, HLA-I and CD8 in tumors were increased and tumor-infiltrating lymphocytes were also increased, which made NB cells easier to recognize and be killed. Simultaneously, the levels of Th1/Th2 cytokines (IL-17A, IFN-γ, TNF-α, IL-6, IL-4, and IL-2) were improved, the release of perforin and granzyme was increased, and IL-2 and IFN-γ in peripheral blood were also increased. However, it is of interest that most of these immune factors were notably improved in groups with low-dose DOX administration, including DOX+anti-GD2 and DOX+CTL groups, but this effect did not occur in the groups of low-dose DOX administration alone. This suggested that low-dose DOX did not act as a cytotoxic drug, but as an immunomodulator. When low-dose DOX was combined with the anti-GD2 antibody or CTLs, the tumor-killing effect was enhanced and more cytokines were released.

The present results indicate that DOX can be used as not only 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. MDSC elimination by low-dose DOX administration causes effector cells to inhibit tumor immune tolerance and the killing effect of transfusing CTLs or anti-GD2 on tumor cells was enhanced significantly, more Th1/Th2 cytokines were

secreted, and more granzyme and perforin were released, leading to obviously inhibited growth of tumors.

In conclusion, the future of cancer patient care is the combination of immunotherapy with therapies that improve effector functions and synergize with therapies that target protector functions. The present study highlights a new application for DOX as a selective MDSC-targeting agent that can be used to overcome a major mechanism of tumor immune evasion. Thus, our results advocate the implementation of DOX in combination strategies to enhance the efficacy of immunotherapy. Tesi *et al* suggested that the best combination therapy will be immunotherapy combined with a therapy that targets MDSCs, a major player in the TME.[8] Overall, low-dose DOX provides a novel approach for anti-MDSC combination therapy for NB.

Acknowledgments

The authors thank Hui-Zhen Geng and Jie Zhang for their technical assistance, and the development facility for their help with sorting dendritic cells and myeloid-derived suppressor cells. The authors also thank Professor Zhen-Yun Mou (Department of Statistics Research Office, Hebei Medical University) for his help with statistical analysis and Mitchell Arico from Edanz Group (https://en-author-services.edanzgroup.com/ac) for editing a draft of this manuscript.

Funding

This study was supported by the grant from the National Natural Science Foundation of China (No. 81472503).

Conflicts of interest

None.

References

1. Yang L, Li BS, Chen J. Research progress of neuroblastoma immunotherapy with target of ganglioside GD2 [in Chinese]. Chin J Pediatr 2016;54:476–478. doi: 10.3760/cma.j.issn.0578-1310.2016.06.023.
2. Armideo E, Callahan C, Madonia L. Immunotherapy for high-risk neuroblastoma: management of side effects and complications. J Adv Pract Oncol 2017;8:44–55. doi: 10.6004/jadpro.2017.8.1.4.
3. Luo YB, Cui XC, Yang L, Zhang D, Wang JX. Advances in the surgical treatment of neuroblastoma. Chin Med J 2018;131:2332–2337. doi: 10.4103/0366-6999.241803.
4. Kholodenko IV, Kalinovsky DV, Doronin II, Deyev SM, Kholodenko RV. Neuroblastoma origin and therapeutic targets for immunotherapy. J Immunol Res 2018;2018:7394268. doi: 10.1155/2018/7394268.
5. Zhong X, Zhang Y, Wang L, Zhang H, Liu H, Liu Y. Cellular components in tumor microenvironment of neuroblastoma and the prognostic value. Peer J 2019;7:e8017. doi: 10.7717/peerj.8017.
6. Ye MJ, Liu GB, Dong KR. Advances of CAR-T cell immunotherapeutic targeting for malignant tumors of children [in Chinese]. Chin J Pediatr Surg 2019;40:370–373. doi: 10.3760/cma.j.issn.0253-3006.2019.04.017.
7. Delloye-Bourgeois C, Bertin L, Thoinet K, Jarrosson L, Kindbeiter K, Buffet T, *et al*. Microenvironment-driven shift of cohesion/detachment balance within tumors induces a switchtoward metastasis in neuroblastoma. Cancer Cell 2017;32:427–443. e8. doi: 10.1016/j.ccell.2017.09.006.
8. Tesi RJ. MDSC; the most important cell you have never heard of. Trends Pharmacol Sci 2019;40:4–7. doi: 10.1016/j.tips.2018.10.008.
9. Alizadeh D, Trad M, Hanke NT, Larmonier CB, Janikashvili N, Bonnotte B, *et al*. Doxorubicin eliminates myeloid-derived suppressor cells and enhances the efficacy of adoptive T-cell transfer in breast cancer. Cancer Res 2014;74:104–118. doi: 10.1158/0008-5472.CAN-13-1545.
10. Wu J, Tang N, Gong ZZ, Zhou JF, Cai W. Study on the effect of neurotransmitter dopamine on the function of MDSC and tumor growth [in Chinese]. J Southeast Univ (Medical Edition) 2013;32:723–727. doi:10.3969/j.issn.1671-6264.2013.06.013.
11. Ladenstein R, Pötschger U, Valteau-Couanet D, Luksch R, Castel V, Yaniv I, *et al*. Interleukin 2 with anti-GD2 antibody ch14.18/CHO (dinutuximab beta) in patients with high-risk neuroblastoma (HR-NBL1/SIOPEN): a multicentre, randomised, phase 3 trial. Lancet Oncol 2018;19:1617–1629. doi: 10.1016/S1470-2045(18)30578-3.
12. Almstedt E, Elgendy R, Hekmati N, Rosén E, Wärn C, Olsen TK, *et al*. Integrative discovery of treatments for high-risk neuroblastoma. Nat Commun 2020;11:71. doi: 10.1038/s41467-019-13817-8.
13. Zhao Q, Jin M, Zhang DW, Zhao W, Wang XS, Yue ZX, *et al*. Serum interleukin-6 level and the rs1800795 polymorphism in its gene associated with neuroblastoma risk in Chinese children. Chin Med J 2018;131:1075–1078. doi: 10.4103/0366-6999.230719.
14. Morandi F, Frassoni F, Ponzoni M, Brignole C. Novel immunotherapeutic approaches for neuroblastoma and malignant melanoma. J Immunol Res 2018;2018:8097398. doi: 10.1155/2018/8097398.
15. Zhao J, Huang J. Breast cancer immunology and immunotherapy: targeting the programmed cell death protein-1/programmed cell death protein ligand-1. Chin Med J 2020;133:853–862. doi: 10.1097/CM9.0000000000000710.
16. Vanichapol T, Chutipongtanate S, Anurathapan U, Hongeng S. Immune escape mechanisms and future prospects for immunotherapy in neuroblastoma. Biomed Res Int 2018;2018:1812535. doi: 10.1155/2018/1812535.
17. Eissler N, Sveinbjörnsson B, Kock A, Johnsen JI, Kogner P. Immune suppression by myeloid-derived suppressor cells, MDSCs, in MYCN-driven neuroblastoma provides a potential target for cancer immunotherapy. JITC 2014;2 (Suppl 3):203. doi: 10.1186/2051-1426-2-S3-P203.
18. Parihar R, Rivas C, Huynh M, Omer B, Lapteva N, Metelitsa LS, *et al*. NK cells expressing a chimeric activating receptor eliminate MDSCs and rescue impaired CAR-T cell activity against solid tumors. Cancer Immunol Res 2019;7:363–375. doi: 10.1158/2326-6066.CIR-18-0572.
19. Dierckx de Casterlé I, Fevery S, Rutgeerts O, Poosti F, Struyf S, Lenaerts C, *et al*. Reduction of myeloid-derived suppressor cells reinforces the anti-solid tumor effect of recipient leukocyte infusion in murine neuroblastoma-bearing allogeneic bone marrow chimeras. Cancer Immunol Immunother 2018;67:589–603. doi: 10.1007/s00262-017-2114-8.
20. Yuan SJ, Xu YH, Wang C, An HC, Xu HZ, Li K, *et al*. Doxorubicin-polyglycerol-nanodiamond conjugate is a cytostatic agent that evades chemoresistance and reverses cancer-induced immunosuppression in triple-negative breast cancer. J Nanobiotechnology 2019;17:110. doi: 10.1186/s12951-019-0541-8.
21. Jabbari P, Hanaei S, Rezaei N. State of the art in immunotherapy of neuroblastoma. Immunotherapy 2019;11:831–850. doi: 10.2217/imt-2019-0018.
22. Spel L, Schiepers A, Boes M. NFκB and MHC-1 interplay in neuroblastoma and immunotherapy. Trends Cancer 2018;4:715–717. doi: 10.1016/j.trecan.2018.09.006.
23. Faè DA, Martorelli D, Mastorci K, Muraro E, Col JD, Franchin G, *et al*. Broadening specificity and enhancing cytotoxicity of adoptive T cells for nasopharyngeal carcinoma immunotherapy. Cancer Immunol Res 2016;4:431–440. doi: 10.1158/2326-6066.CIR-15-0108.
24. Bashiri Dezfouli A, Salar-Amoli J, Pourfathollah AA, Yazdi M, Nikougoftar-Zarif M, Khosravi M, *et al*. Doxorubicin-induced senescence through NF-kB affected by the age of mouse mesenchymal stem cells. J Cell Physiol 2020;235:2336–2349. doi: 10.1002/jcp.29140.

How to cite this article: Xu WL, Shi BJ, Li SL, Yu FX, Guo LN, Li M, Hu ZG, Li GX, Zhou H. Targeted inhibition of myeloid-derived suppressor cells in the tumor microenvironment by low-dose doxorubicin to improve immune efficacy in murine neuroblastoma. Chin Med J 2021;134:334–343. doi: 10.1097/CM9.0000000000001234