Targeted inhibition of myeloid-derived suppressor cells by doxorubicin to enhance antigen-specific cytotoxic T lymphocytes killing neuroblastoma cells in vitro

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Abstract

Background High agglomeration of myeloid-derived suppressor cell (MDSC) in neuroblastoma (NB) resulted in immune tolerance and impeded therapeutic effects. Doxorubicin (DOX) is currently found the most specific drug to selectively remove MDSC. However, whether these mechanisms can relieve the inhibitory role of MDSC in NB immunotherapy is still remain unclear. Procedure In this study, the inhibitory role of MDSC for NB Ag-specific cytotoxic T lymphocyte (CTL) were investigated in vitro. CTLs, NB cells, MDSCs and DOX were mixed and cultivated in different collocation pattern, the levels of cluster of differentiation 3ζ chain (CD3ζ) and L-selectin in CTL in different groups were detected. Thereafter, the killing rate of neuroblastoma cells, secretion of interleukin-2 and interferon-γ were detected and compared. Results The proliferation and killing effect of CTLs on neuroblastoma cells were all inhibited by MDSC through downregulating CD3ζ ($P = 0.002$; $P = 0.001$) and L-selectin ($P = 0.006$; $P < 0.001$) by reai-time PCR test and by Western-blot analysis respectively. However, this inhibitory role can be effectively reversed by doxorubicin. The significant difference existed in the killing rate between the groups ($P < 0.001$) except between CTL+SK-N-SH group and CTL+SK-N-SH+DOX group ($P > 0.05$). There were significant differences in the levels of IL-2 ($P < 0.001$) and IFN-γ ($P < 0.001$) among the groups. Conclusions This study provided the novel method to enhance immunotherapeutic effects for neuroblastoma by using doxorubicin to targeted inhibition of MDSCs.

Introduction

Neuroblastoma (NB) is the most common pediatric neuroendocrine tumors with high malignancy and early metastasis. The survival rate of children with high-risk NB is still less than 40% although many therapeutic method including surgery, radiotherapy, chemotherapy and stem cell transplantation have been adopted. So, a new therapeutic method is urgently needed to raise curative effect and reduce the recurrence and metastasis for NB [1, 2]. Although immunotherapy in children with solid tumors started lately, many studies have shown that immunotherapy of NB can effectively remove the tumor cells and reduce tumor recurrence and metastasis with higher specificity and less toxicity compared with the traditional therapy [3].

However, in the survival microenvironment of NB, all kinds of complicated immunosuppressive factors impede therapeutic effects and result in immune tolerance, including downregulation of HLA-I antigen and accumulation of various immunosuppressive cells such as myeloid-derived suppressor cell (MDSC), regulatory T cell and tumor associated macrophage, etc [4-8]. Currently, new approaches targeting the tumor microenvironment hold promise for further improvements in survival and long-term quality of life[9]. Furthermore, Jales A found high expression of disialoganglioside in surface of NB resulted in increasing of MDSC agglomeration in tumor microenvironment [10], which triggered tumor immunosuppression and promoted the development and metastasis of tumor. So, in these factors, MDSC may be the main impediment to NB immunotherapy. Some studies described MDSCs caused dissociation between T-cell receptor (TCR) and cluster of differentiation 3ζ (CD3ζ) molecules, disrupting TCR complexes on T cells which result in Ag-specific $CD8^+$T

cell tolerance in cancer [11]. MDSC down-regulate L-selectin levels on naive T cells, decreasing their ability to home to sites where they would be activated [12]. Alizadeh D et al pointed out doxorubicin (DOX) can be used as a potent immunomodulatory agent that selectively impair MDSC-induced immunosuppression in breast cancer[13]. However, whether these mechanisms can explain the inhibitory role of MDSC in NB immunotherapy is still remain unclear.

Therefore, in the present study, we proposed BALB/c mice as the experimental object, successfully prepared NB Ag-specific CTLs, and grouping mixed cultivation of CTL, neuroblastoma cells, MDSC and DOX. Thereafter, the killing rates of CTL to neuroblastoma cells, secretion of Interleukin-2 (IL-2) and interferon-γ (IFN-γ), levels of cluster of differentiation 3ζ chain (CD3ζ) and L-selectin (CD62L) in CTL were detected and compared in different groups respectively in order to explore mechanism of immune tolerance caused by MDSC in NB and provide the new method to enhance the immune therapeutic effects of NB by using doxorubicin to targeted inhibition of myeloid-derived suppressor cells.

Materials and methods

Cell lines and animals

SK-N-SH cells, obtained from Shanghai Cell Bank of Chinese Academy of Medical Sciences, were maintained in DMEM (Gibco) with 10% fetal calf serum (Gibco), and cultivated at 37°C in a 5% CO_2humidified incubator. 6 week female BALB/c mice, each weighing 18 to 20 g (SCXK Hebei 2008-1-003), were purchased from the Experimental Animal Center of Hebei Province (SPF grade, SYXK Hebei 2008-0026) and housed in a specific pathogen-free facility. Spirit, diet and defecation of mice were regularly observed and recorded. All experiments were conducted in accordance with the principles and procedures outlined in the Guideline of Institutional Experimental Animal Review Committee.

Separation and purification of MDSC in vitro

BALB/c mice were sacrificed, then bone marrow cells were extracted and made into single cell suspension. After red blood cells having been cracked, cell suspension was administrated by anti-Gr-1 monoclonal Ab (mAb), anti-CD11b mAb, anti-CD80 mAb, anti-F4/80 mAb, anti-CD11c mAb and anti-MHCII mAb. Differentiation of MDSC and proportion of Gr-1^+CD11b^+MDSCs were detected by flow cytometry.

Bone marrow cells were suspended by PBS and MDSC were separated by Percoll Density Gradient Centrifugation. Then, cell suspension was collected and separated by CD11b magnetic bead and purity of Gr-1^+CD11b^+MDSC was detected by flow cytometry.

Preparation of neuroblastoma antigen-specific cytotoxic T lymphocyte (CTL)

Extraction and cultivation of BALB/c mice bone marrow derived **dendritic cell (DC)**

BALB/c mice were sacrificed, then bone marrow cells were extracted and made into single cell suspension. After red blood cells having been cracked, cell suspension was cultivated according to 10^6/ml concentration. Thereafter, rmGM-CSF (20 ng/ml) and rmIL-4 (20 ng/ml) were added. On the sixth day, morphology of DCs was observed by phase contrast microscope and the expressive rate of CD11c, CD86 and MHC-II were detected by flow cytometry.

Tumor antigen loading DC

SK-N-SH cells in logarithmic phase were resuspended and adjusted to 2×10^7/ml concentration. Cells were quickly frozen to -80, then rewarmed in 37 water. After four cycles, cells suspension was centrifuged with 10,000 r/min for 15 minutes and the supernatant was collected and cryopreserved at -80. The lysate equivalent to $2x10^6$ tumor cells was added into per millilite rmedium of DCs and cultivated for 6 days. Four hours later, cells suspension was added rmTNF-α and cultivated to 7th day. Then, the suspension cells were collected and defined tumor antigen loaded DCs. The cell morphology of DCs was observed by phase contrast microscope. Meanwhile, the expression rate of CD11c, CD86 and MHC-II were detected by flow cytometry.

Extraction, identification and cultivation of $CD3^+$T cells

$CD3^+$T cells were extracted from mice spleen lymphocytes suspension by Mouse $CD3^+$Tcell Magnetic Bead. Magnetic-activated cell sorting (MACS) can be repeated for several times and about 3×10^6 T lymphocytes can be extracted from each mouse spleen. The purified lymphocytes were collected and identified purity of $CD3^+$T cells. Concentration of cells was adjusted to 5×10^6/ml and cultivated 2 days in medium containing rmIL-2 (20 ng/ml). Then, cell morphology was observed under the phase contrast microscope and cell vitality was tested by Trypan blue.

Preparation of SK-N-SH Ag-specific CTL and cytokine release test

After $CD3^+$T cells were cultivated for 2 days, DCs loaded tumor antigen were added according to the proportion of DC:T = 1:20. At the same time, rmIL-2 (20 ng/ml) was mixed and cultivated for 3 or 4 days. Thereafter, morphology of T cells was observed under inverted microscope. After T cells had been activated for 72 hours, the supernatants in each group were retained and concentrations of IL-2 and IFN-γ were calculated respectively according to the absorbance value and the standard curve measured by enzyme standard instrument. IL-2 and IFN-γ in the supernatant between the different groups were compared.

MDSC inhibiting proliferation of neuroblastoma antigen-specific CTL

The activated CTLs were stained by 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (final concentration of CFSE was 5 μmol/L[14]) and divided into two groups. In one group, CTLs were mixed with $Gr\text{-}1^+CD11b^+$MDSC (4:1) separated by MACS and stimulated proliferation by CD3/CD28 antibody. Meanwhile, in another group, all of the things was same except absence of MDSC. A parallel control group was set up for each group with the same method as the experimental group. After being cultivated for 4 days, the mixed cell system were observed by fluorescence microscopy and detected cell proliferation by flow cytometry.

Detection of CD3ζ and CD62L in CTL by real-time PCR and Western-blot

CTL, CTL+MDSC(1:4) and CTL+MDSC(1:4)+DOX(2μmol/L) were incubated respectively. The contents of CD3ζ and CD62L in CTL of the different groups were assessed and compared by real-time PCR. Total RNA in samples was isolated using the ultra-pure RNA extraction kit according to the manufacturer's guidelines (Cwbio, Co. Ltd.). For reverse transcription, 1 μg of RNA was used to synthesize single-strand cDNA (HiFi-MMLV cDNA first strand synthesis kit; Cwbio, Co. Ltd.) according to the manufacturer's guidelines. Real-time PCR was performed using a fluorescence quantitative PCR amplifier (LightCycler 480 II, Roche Inc., Germany), as described elsewhere. Triplicate reactions were set up for each gene in a 96-well plate. Reaction information is summarized in Table 1. Amplification was performed with the following program: initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 10 s, 58°C for 30 s and 72°C for 30 s. The experimental data were expressed as relative expression using the $2^{-\Delta\Delta^{\hat{}}\tau}$ equation as described previously [15].

Proteins were extracted with RIPA lysis buffer (Invitrogen) and quantitatively detected with the BCA Protein Assay kit (Invitrogen). The samples were separated by 12% SDS-PAGE, transferred to a PVDF membrane, and blocked by 5% BSA. The primary antibodies against each protein were added and incubated at 4°C overnight. Afterward, the second antibodies were added and incubated for 4 h at 20°C. After washing the membrane, chemiluminescence was detected on X-ray film by FluorChem®HD2 (Alpha Innotech, Santa Clara, CA, USA), with GAPDH as the internal reference.

MDSC regulating the killing effect of neuroblastoma antigen-specific CTL in vitro

CTL, CTL+MDSC (1:4), and CTL+MDSC(1:4)+DOX(2μmol/L) were mixed with SK-N-SH cells and incubated respectively (CTL:SK-N-SH = 20:1). At the same time, blank control group, target cells group and effector cells group were set up. CCK-8 was added into the cell system (20 μl/hole) after incubation and absorbance value (A value) was detected in 450 nm wavelength by enzyme standard instrument. The cytotoxic activities of effector cells were evaluated by the killing rate. The killing rate of effector cells was calculated as follow, kill rate (%) = [A value of target cells group - (A value of experimental group - A value

of effector cells group)]/A value of target cells group × 100%. The killing rates of CTLs to SK-N-SH cells between the groups were compared. The secretion levels of IL-2 and IFN-γ in the supernatant between the groups were detected and compared by enzyme-linked immunosorbent assay (ELISA).

Statistical analysis

All data were analyzed using the SAS software (ver. 8 for Windows; SAS Institute Inc., NC, USA). The Wilcoxon two-sample test and a repeated-measures analysis of variance were used to analyze the results. P ¡0.05 was deemed to indicate statistical significance.

Results

MDSC separation, extraction, identification and neuroblastoma Ag-specific CTL preparation, cytokine release test

By percoll density gradient centrifugation, the rate of Gr-1$^+$MDSC, CD11b$^+$MDSC, CD11c$^+$MDSC, CD80$^+$MDSC, F4/80$^+$MDSC and MHC-II$^+$MDSC were 70.4%, 3.5%, 4.8%, 1.2%, 0.3%, 2.1% respectively (Fig. 1A), and the rate of Gr-1$^+$CD11b$^+$MDSC was 22.6% (Fig. 1B). Furthermore, MDSC suspension was sorted by CD11b magnetic bead and purification of Gr-1$^+$CD11b$^+$MDSC was shown 84.6% by flow cytometry (Fig. 1C). By flow cytometry, the expressive rates of CD11c, CD86 and MHC-II on dendritic cells (DCs) wituout antigen-loaded were 10.9%, 3.8% and 27.9% respectively which suggested their weaker antigen presenting ability (Fig. 1D). Most of DCs can be seen adherent growth with different size, star or spindle shape and stretching tubers. At the 7th day, DCs were activated by tumor antigen. DCs in half adherent state increased obviously with radial spike and bigger shape. The expressive rates of CD11c, CD86 and MHC-II were 74.8%, 50.3% and 49.8% respectively which indicated mature DCs with efficient presenting antigen ability (fig. 1E). CD3$^+$T cells, extracted from spleen lymphocytes by MACS, reached 87.3% by flow cytometry. The living cells rate was 96.38% by Trypan blue test. After 3 to 4 days' cultivation with antigen-loaded DCs, antigen-specific CD3$^+$T cells were prepared and gathered into many small colonies. The levels of interleukin-2 (IL-2) (1.092 +/- 0.010 ng/l) and interferon-γ (0.855 +/- 0.038 ng/l) in the supernatant of antigen-loaded CD3$^+$T cells were significantly higher than IL-2 (0.962 +/- 0.007 ng/l) and IFN-γ (0.765 +/- 0.010 ng/l) in the supernatant of CD3$^+$T cells without antigen-loaded (P <0.05).

MDSC inhibiting proliferation of CTL

Under fluorescence microscopy, the number of CTLs was same in the two groups before cultivation (Fig. 2A). However, after having been cultivated for 4 days, cells proliferated obviously but staining intensity weakened in CTL group. On the contrary, in CTL+MDSC group, cell number scarcely increased and staining intensity still remain strong (Fige. 2B).

Analysis of flow cytometry showed consistent results. In CTL+MDSC group, CTLs with strong fluorescence (87.6%) were much more than CTLs with weak fluorescence. However, strong fluorescence CTLs (44.1%) were less than weak fluorescence CTLs in CTL group (Fig. 2C).

Downregulating levels of CD3ζ and CD62L in CTL by MDSC

Respectively, the levels of CD3ζ and CD62L in CTL were detected and compared by reai-time PCR (Fig. 3A) and western-blot analysis (Fig. 3B). In reai-time PCR test, significant difference of CD3ζ ($F = 20.315$, $P = 0.002 < 0.05$) and CD62L ($F = 13.858$,$P = 0.006 < 0.05$) occurred between CTL group, CTL+MDSC group and CTL+MDSC+DOX group. Western-blot analysis showed the same results. Significant difference of CD3ζ ($F = 28.241$, $P = 0.001 < 0.05$) and CD62L ($F = 41.142$, $P< 0.001$) were also seen among the three groups.

Doxorubicin inhibits MDSC then enhances the killing effect of CTL in vitro

Under inverted microscope, the killing process of CTLs to SK-N-SH cells was clearly shown in each group respectively. From day 1 to day 10, we found that in SK-N-SH+CTL+MDSC groups, SK-N-SH cells

still scattered in view with relative regular outline. However, in CTL+SK-N-SH group and CTL+SK-N-SH+MDSC+DOX groups, nearly all of SK-N-SH cells appeared apoptosis or necrosis, and CTLs also decreased significantly. During the whole period, in SK-N-SH group, tumor cells proliferated continuously and kept active state (Fig. 4A). So these results proved that doxorubicin inhibits MDSC then enhances the killing effect of CTL on SK-N-SH cells. The significant difference existed in the killing rate between the groups ($F = 22.386$, $P < 0.001$) except between CTL+SK-N-SH group and CTL+SK-N-SH+DOX group ($P = 0.100 > 0.05$) (Fig. 4B). IL-2 and IFN-γ in the supernatant were detected by ELISA. By repeated measurement analysis of variance, the results showed that there were significant differences in the secretion levels of IL-2 ($F = 192.013$, $P = 0.000 < 0.001$) and IFN-γ ($F = 519.274$, $P = 0.000 < 0.001$) among CTL+SK-N-SH group, CTL+MDSC+SK-N-SH group and CTL+MDSC+SK-N-SH+DOX group (Figs. 4C and 4D).

Discussion

At present, the most effective method of immunotherapy for NB is adoptive transfer chimeric GD2 antigen receptors CTL [6, 16]. However, some phase III clinical trials found that some children with high-risk NB underwent adoptive cell transfer immunotherapy of chimeric GD2 antigen receptors CTL still appeared recurrence and metastasis although the survival rates have improved. This suggested this kind of passive immunotherapy need improve its curative effect by adjusting the immunosuppressive microenvironment [4, 16].

In tumor immune microenvironment, accumulation of a variety of immunosuppressive cells including MDSC formed the main immunosuppressive factors and resulted in immune tolerance and disability of the immune system [3]. MDSC is a group of innate immune cells originated from myeloid which play a negative immune regulative role in tumor progression [17, 18]. Some studies suggested MDSC inhibit the body's natural immune by inhibiting DC, accelerating polarization of macrophages to M2, decreasing interleukin-12 and reducing function of NK cells. on the other hand, MDSC inhibit T cells adoptive immunotherapy through high expression of arginase-1, inducible nitric oxide synthase and reactive oxygen species [4, 19-21]. Therefore, MDSC induced tumor immune tolerance and became the main impediment to immunotherapy.

The role of MDSC in neuroblastoma still remain unclear although the mechanism of MDSC in other tumors has been explicated too much. In the present study, neuroblastoma Ag-specific CTLs were stained by CFSE. The cytoplasm with fluorescent protein were evenly distributed to the next generation of cells and the fluorescence intensity reduced half when cells proliferated continuously. So, the more generations of cell division, the weaker of cell fluorescence intensity. Therefore, in this study, the fluorescence intensity of CTL became weaker and the number of CTL increased if CTL cultivated without MDSC. Contrarily, cells proliferation decreased and the fluorescence intensity unchanged if CTL cultivated with MDSC. The result fully showed MDSC can inhibit proliferation of neuroblastoma Ag-specific CTL obviously.

In further study, the expressions of CD3ζ and CD62L in Ag-specific CTLs decreased significantly when CTL cultivated with MDSC, but the two proteins raised again when DOX administration. As a chain of CD3 molecules, CD3ζ play a key role in signal transmission inside and outside the cell [22]. The increased expressive activity of ζ chain will promote TCR identified immune signal transmission to intracellular which lead to activation of T cells and produce amounts of cytokines, such as IFN-γ, IL2, etc [23]. Moreover, CD62L is an important molecule involving the extravasation of lymphocytes from the blood and lymphatics, and their homing to lymph nodes and tumors[24-26]. Therefore, the results indicated DOX could targeted relieve the inhibitory role of MDSC on CD3ζ and CD62L in Ag-specific CTL which can promote activation and migration of Ag-specific CTL and effectively kill neuroblastoma cells.

In recent years, some researches have pointed out CTL produce cytotoxicity to target cells by two separate ways, namely Perforin way and PCD way mediated by Fas antigen molecules [27-29]. Of them, Perforin way was a major way and played a significant role in antiviral, intracellular bacteria, tumor and immune pathology, etc[29-31]. During the course of Perforin way, CTL killed tumor meanwhile the cytokines IL-2 and IFN-γ were released. The more obvious of the killing effect, the higher concentrations of the cytokine, and vice versa. In the present study, the killing rate of NB cells and the levels of IL-2 and IFN-γ in supernatant

were all decreased significantly when CTLs cultivated with MDSC. However, this inhibitory role of MDSC can be effectively reversed by DOX administration. Some researches pointed out doxorubicin can selectively eliminates MDSCs and promots the activity of immune effector cells and improves the therapeutic profile of adoptively transferred helper T lymphocytes[13, 32, 33]. So, in this study, we demonstrated the underlying mechanism of targeting inhibition of myeloid-derived suppressor cells by doxorubicin to enhance antigen-specific cytotoxic T lymphocytes killing neuroblastoma cells in vitro.

Conflicts of interest

All authors have declared no financial conflicts regarding this work.

Acknowledgments

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Figure Legends

Figure1 Extraction, identification, purification of myeloid-derived suppressor cell (MDSC) and cultivation and tumor antigen loading of dendritic cell (DC) A, Cells were extracted from bone marrow of BALB/c mice and stained by monoclonal antibodies. By flow cytometry, the expressive rate of Gr-1^+MDSC, $CD11b^+$MDSC, $CD11c^+$MDSC, $CD80^+$MDSC, $F4/80^+$MDSC and MHC-II^+MDSC were 70.4%, 3.5%, 4.8%, 1.2%, 0.3%, 2.1% respectively. **B,** The expressive rate of Gr-1^+CD11b^+MDSC was 22.6%.**C,** After Magnetic-activated cell sorting (MACS) by CD11b magnetic bead, purification of Gr-1^+CD11b^+MDSC reached 84.6%.**D,** Most of dendritic cells wituout antigen-loaded can be seen adherent growth with different size, star or spindle shape and stretching tubers, but part of the cells seemed half adherent state with rough surface. The expressive rates of CD11c, CD86 and MHC-II on DCs were 10.9%, 3.8% and 27.9% respectively by flow cytometry.**E,** At the 7th day, DCs were stimulated and activated by tumor antigen. DCs in half adherent state increased obviously with radial spike and bigger shape. The expressive rates of CD11c, CD86 and MHC-II were 74.8%, 50.3% and 49.8% respectively by flow cytometry.

Figure2 Myeloid-derived suppressor cell (MDSC) inhibiting proliferation of neuroblastoma antigen-specific cytotoxic T lymphocyte (CTL) Neuroblastoma antigen-specific CTLs in the two groups were stained by 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE).**A,** Under fluorescence microscopy, the same number of CTLs was seen in CTL group and CTL+MDSC group before cultivation. **B,**After having been cultivated for 4 days, cell proliferated obviously but fluorescent intensity weakened in CTL group. However, in CTL+MDSC group, cell fluorescent intensity remain strong and cell number scarcely increased. **C,** By flow cytometry, the results showed the consistent results with the view in microscopy. After cultivation, In CTL+MDSC group, the rate of CTLs with strong fluorescence was 87.6%. However, only 44.1% of CTLs with strong fluorescence were found in CTL group.

Φιγυρε3 Εξπρεσσιονς οφ ςλυστερ οφ διφφερεντιατιον 3ζ (ˆΔ3ζ) ανδ Λ-σελεςτιν (ˆΔ62Λ) ιν ˆΤΛ ιν διφφερεντ γρουπς The levels of CD3ζ and CD62L in CTL were detected and compared by reai-time PCR and western-blot analysis respectively. **A,** In reai-time PCR test, significant difference of CD3ζ ($F = 20.315$, $P = 0.002 < 0.05$) and CD62L ($F = 13.858$, $P = 0.006 < 0.05$) occurred between CTL group, CTL+MDSC group and CTL+MDSC+DOX group. **B,** Western-blot analysis showed that significant difference of CD3ζ ($F = 28.241$, $P = 0.001 < 0.05$) and CD62L ($F = 41.142$, $P < 0.001$) were seen among the three groups. $^*P$ ¡0.05; $^{**}P$ ¡0.01.

Figure4 Immunosuppressive action of MDSC on neuroblastoma antigen-specific CTL and doxorubicin inhibits MDSC then enhances the killing effect of CTL in vitro Under inverted microscope, the killing process of CTLs to SK-N-SH cells was clearly shown in each group respectively. **A** , As the control group, In SK-N-SH group, tumor cells proliferated continuously and kept active state. At d1, CTLs and SK-N-SH cells were mixed and no obvious difference was seen between the groups. At d2, in CTL+SK-N-SH group and CTL+SK-N-SH+MDSC+DOX group, CTLs and SK-N-SH cells gathered and began to interact. But CTLs and SK-N-SH cells scattered in view in CTL+SK-N-SH+MDSC group. At d4, in CTL+SK-N-SH+MDSC group, CTLs and SK-N-SH cells all proliferated slowly. However, in the other two groups, CTL proliferated but SK-N-SH cells began to deform. At d7, in CTL+SK-N-SH group and CTL+SK-N-SH+MDSC+DOX group, SK-N-SH cells furtherly deformed and lost their cellular shape meanwhile CTLs began to decrease. However, SK-N-SH cells kept regular shape in CTL+SK-N-SH+MDSC group. At d10, in SK-N-SH+CTL+MDSC groups, SK-N-SH cells still scattered in view with relative regular outline. However, in the other two groups, nearly all of SK-N-SH cells appeared apoptosis or necrosis, and CTLs also decreased significantly.**B,** The significant difference existed in the killing rate between the groups ($F = 22.386$, $P < 0.001$) except between CTL+SK-N-SH group and CTL+SK-N-SH+DOX group ($P = 0.100 > 0.05$). **C and D,** Interleukin-2 (IL-2) and interferon-γ (IFN-γ) in the supernatant were detected by ELISA. By repeated measurement analysis of variance, the results showed that there were significant differences in the secretion levels of IL-2 ($F = 192.013$, $P = 0.000 < 0.001$) and IFN-γ ($F = 519.274$, $P = 0.000 < 0.001$) among CTL+SK-N-SH group, CTL+MDSC+SK-N-SH group and CTL+MDSC+SK-N-SH+DOX group. $^*P$ ¡0.05.

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