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Cytokine-induced killer cells/natural killer cells combined with anti-GD2 monoclonal antibody increase cell death rate in neuroblastoma SK-N-SH cells

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Abstract. Neuroblastoma (NB) is one of the most common extracranial, solid, pediatric malignancies. Despite improvements in conventional therapies, including surgery, chemotherapy and radiation therapy, the prognosis of stage IV NB remains poor, indicating that novel treatment strategies are required. Immunotherapies, such as anti-GD2 monoclonal antibodies, used alone or in combination with cytokines, and peripheral blood mononuclear cells or cord blood mononuclear cells (CBMNCs), have been indicated to cause NB cell death and to prolong patient survival in high-risk NB; however, they remain limited by severe cytotoxicity and side effects. In the present study, it was determined that anti-GD2 monoclonal antibody alone or CBMNC-isolated cytokine-induced killer (CIK)/natural killer (NK) cells alone significantly induced cell death of NB SK-N-SH cells, and the combination of anti-GD2 antibody and CIK/NK cells could significantly increase the cell death rate compared with either treatment alone. In addition, based on a method referred to our previous study, it was identified that a two-cytokine culture system, using interleukin IL-2 and IL-7, effectively stimulated the proliferation of CIK/NK cells. These results serve to suggest a novel treatment strategy for relapsed/refractory NB with high efficiency and few side effects.

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Introduction

Neuroblastoma (NB) is one of the most common extracranial, solid, pediatric malignancies. NB originates from neural crest cells that can be destined for anywhere in the sympathetic nervous system (1), and accounts for 8-10% of pediatric malignancies and 15% of pediatric cancer-associated mortalities worldwide (2). NB is an extremely heterogeneous disease; for low-risk or intermediate-risk NB, the prognosis is favorable after surgical resection alone or with minimal chemotherapy (3). However, >60% of high-risk patients with NB die following recurrence, despite responding well to aggressive chemotherapy at the beginning of treatment (4). This suggests there is a lack of effective salvage regimens for patients with disease-recurrence.

Although the majority of patients with NB achieve complete remission under conventional treatments, including surgery, chemotherapy and radiotherapy, a number of patients still relapse in the late stages of chemotherapy, with acquired multi-drug resistance (MDR) (5). MDR contributes heavily to minimal residual disease (MRD) after chemotherapy, which leads to the recurrence of high-risk NB (6). Despite a large number of innovative studies on MRD, the prognosis of high-risk NB has remained poor over the past decade (7-9). Due to the issues with traditional chemotherapy drugs in NB, including increases in genetic mutations, dysregulated activity of tumor suppressor genes and serious side effects on organs (10-12), the aim of the present study was to identify effective and well-tolerated methods for treating NB.

Ganglioside GD2 is a type of glycosphingolipid molecule that is uniformly expressed on the membrane of neurogenic tumor cells (13). The high expression level of GD2 in NB cells and its restricted distribution in normal tissues indicates that anti-GD2 monoclonal antibodies may be suitable for immunotherapy (14). Four types of anti-GD2 monoclonal antibody have been used in clinical trials, including murine anti-GD2 monoclonal antibody 3F8 (15), chimeric human-murine anti-GD2 monoclonal antibody ch14.18 (16), human anti-GD2 monoclonal antibody hu14.18 and human anti-GD2 monoclonal

antibody hu3F8 (17). Simon *et al* (18) identified in a phase IV clinical trial that patients who receive ch14.18 maintenance treatment after chemotherapy acquire a higher 3-year overall survival compared with those who receive only small doses of maintenance chemotherapy or no maintenance therapy.

Recently, numerous studies have focused on enhancing the treatment effects of anti-GD2 monoclonal antibody (19-21). The mechanisms by which anti-GD2 monoclonal antibody induces apoptosis of NB include complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) (22). However, numerous studies have suggested that the effect of CDC is associated with side effects, such as pain in anti-GD2 monoclonal antibody treatment (23,24). Sorkin *et al* (25) identified that a mutation in anti-GD2 monoclonal antibody can reduce pain so that the tolerance to anti-GD2 monoclonal antibody is increased without a reduction in the killing effect of GD2 antibody. Thus, it may be possible to improve the curative effect of anti-GD2 monoclonal antibody through enhancing ADCC.

The ADCC effect of anti-GD2 monoclonal antibody on NB cells is associated with the Fc receptor (FcR) on killer cells, which combines with the Fc fragment of the anti-GD2 monoclonal antibody, activating ADCC and inducing the apoptosis of NB (26). A number of studies have used anti-GD2 monoclonal antibody combined with granulocyte-macrophage colony stimulating factor (GM-CSF) or interleukin-2 (IL-2), and have demonstrated that combination therapy exerts stronger effects compared with using anti-GD2 monoclonal antibody alone (27-29). This indicates that an increase in the number or activity of killer cells is a key factor in enhancing the efficacy of anti-GD2 monoclonal antibody.

With the development of tumor immunology in recent years, it has been reported that cytokine-induced killer (CIK)/natural killer (NK) cells transfusion, a kind of adoptive cellular immunotherapy, has significant effect in neuroblastosma MRD treatment without obvious side effects (30). It recognizes and kills target tumor cells by binding specific cell surface markers (31). NK cells are one of the most important immune effector cell types in the process of ADCC (32). However, it has been reported (33) that NK function is limited in some patients with neuroblastoma; therefore, the treatment effect of anti-GD2 monoclonal antibody is reduced in these patients (34). CIK cells are *ex vivo*-expanded T lymphocytes (35), a subset of T lymphocytes with a natural killer T-cell phenotype expressing both the CD56 and CD3 markers presents non-histocompatibility complex cytotoxicity against target cells, and exhibits improved anti-tumor activity compared with NK or lymphokine-activated killer cells (36).

In our previous study (37), mononuclear cells (MNCs) from cord blood (CBMNCs) were incubated with IL-2 and IL-7 for 21 days, and the percentage of $CD3^+CD56^+$ CIK cells and $CD3^-CD56^+$ NK cells reached 14.26±1.15 and 29.52±0.89%, respectively, while percentage of total CIK/NK ($CD3^{+/-}CD56^+$) cells reached 43.77±1.93%, which increased significantly compare to 9.31±1.77% before culture. Furthermore, the percentage of $CD3^+CD8^+$ cells was not significantly increased, indicating a risk reduction of having graft versus host disease (GVHD) in patients. It was also identified that FcRγIII (CD16) and lymphocyte function-associated antigen-1 (LFA-1) are highly expressed in these cells. In conclusion, CIK/NK cells can enhance the killing effect of tumor cells by providing the ligands needed. The effect of CIK/NK cells combined with anti-GD2 monoclonal antibody in targeting human NB cells is not yet known, and further studies are required.

To examine the effects of CIK/NK cells combined with anti-GD2 monoclonal antibody in targeting human NB cells, flow cytometry was performed to detect the expression of GD2 and intercellular adhesion molecule-1 (ICAM-1) on the NB cell line SK-N-SH, and CD16 expression on CIK/NK cells. The aim of this study was to investigate the effect of anti-GD2 monoclonal antibody and CIK/NK cells on SK-N-SH cells. The results provide experimental evidence supporting the use of combined treatment with CIK/NK cells and anti-GD2 monoclonal antibody for MDR-NB, in order to improve the prognosis and overall survival of patients.

Materials and methods

Sample collection and cell line culture. Human cord blood samples were obtained from full-term deliveries between January and March 2016 in the Department of Obstetrics, Sun-Yat Sen Memorial Hospital. This study was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital at Sun Yat-sen University (Guangzhou, China). Patients provided informed written consent. A total of 10 patients aged between 24 and 30 years were recruited. Exclusion criteria included premature delivery and high-risk pregnancies. A total of 30 ml human cord blood was collected from each patent and processed immediately or stored in 4˚C for <4 h. MNCs, a group of cells with a one lobed nucleus containing two groups of cells, lymphocytes and monocytes, were isolated using a Ficoll-Paque gradient method (38). A total of 35 ml blood were added slowly into a 50 ml centrifuge tube containing 15 ml Ficoll-Paque Premium (Sigma-Aldrich; Merck KGaA), then centrifuge at 400 x g at room temperature for 20 min. The middle layer cells were collected and washed by phosphate buffer saline (HyClone; GE Healthcare Life Sciences) in triplicate, and cultured in X-VIVO medium (HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), 1% penicillin-streptomycin (Sigma-Aldrich; Merck KGaA), IL-2 (80 ng/ml; Abcam) and IL-7 (40 ng/ml) at 37˚C with 5% CO_2. Half of the medium was replaced and cytokines were re-applied every 3 days. The human NB SK-N-SH cell line was purchased from the Cell Bank of Sun-Yat Sen Medical School, and cultured in DMEM (HyClone; GE Healthcare Life Sciences) supplemented with 12% fetal bovine serum (HyClone; GE Healthcare Life Sciences) and 1% penicillin-streptomycin (Sigma-Aldrich; Merck KGaA) at 37˚C with 5% CO_2.

Characterization of GD2, CD54 and CD16 expression and flow cytometry analysis. Cells were harvested for experiments after 21 days in culture. After cell harvesting, the proportion of $CD3^+CD56^+$ (CIK) cells, $CD3^-CD56^+$ (NK) cells and $CD3^{+/-}CD56^+$ (CIK/NK) cells was detected by flow cytometry. The proportion of CIK cells are a group of immune effector cells characterized by the cell surface marker $CD3^-CD56^+$ and the proportion of NK cells are a type of lymphocytes, which are distinguished by the cell surface marker CD3-CD56+ (39).

Both CIK and NK cells play critical role in immune anti-tumor effect (40). The ratio of CD3+CD56+ CIK, CD3-CD56+ NK or total CD3+/-CD56+ CIK/NK cells accounts for of total mononuclear cells, which was calculated by the following formula: Ratio of CIK cells (%)=number of cells ($CD3^+CD56^+$)/number of cells (all MNCs); ratio of NK cells (%)=number of cells ($CD3^-CD56^+$)/number of cells (all MNCs); ratio of total CIK/NK cells (%)=number of cells ($CD3^{+/-}CD56^+$)/number of cells (all MNCs). The cell surface expression of GD2 and CD54 on SK-N-SH cells and CD16 expression on CIK/NK cells was assessed by staining with 5 μl anti-GD2-phyroerythrin (PE; cat. no. 562100) or anti-CD54-PE-Cy5 (cat. no. 555512) together with anti-45-ECD (cat. no. A07784), or anti-CD16-fluorescein isothiocyanate (cat. no. 555406) antibodies for 20 min at 4˚C. Anti-CD45-ECD were purchased from Beckman Coulter, Inc., and all other antibodies were purchased from BD Biosciences. Cells were then washed by phosphate buffer saline (HyClone; GE Healthcare Life Sciences) and analyzed using a BD FACSVerse (BD Biosciences). Data were analyzed using FlowJo software (FlowJo 7.6.1).

Determination of saturated concentration of anti-GD2 on SK-N-SH cells. To determine the effects of different anti-GD2 monoclonal antibody doses on SK-N-SH cells, the cell death rate of NB cells that had been treated with anti-GD2 monoclonal antibody was assessed by flow cytometry. As a preliminary experiment, the optimum treatment concentration of anti-GD2 antibody was tested. Cells ($1x10^5$) were seeded in a 24-well plate with C-DMEM medium and cultured for 24 h to allow the cells to adhere to the dish, then 0.5, 1, 2, 5, 10, 20 and 50 μg/ml of anti-GD2 antibody was added to each well. IgG1 was added as a control group. After culture for 1 h, cells were harvested using 0.25% trypsin and washed with phosphate-buffered saline (HyClone; GE Healthcare Life Sciences) in triplicate, then cells were stained with GD2-PE/Cy7 antibody for 20 min in the dark and washed. The expression of GD2 on the cell surface was detected using flow cytometry.

Flow cytometry for cell viability assay. For the determination of optimal effector/target (E/T) ratios of CIK/NK cells over SK-N-SH cells, SK-N-SH cells were treated with 0.25% trypsin to detach them from dishes and washed with PBS in triplicate. Cells were labeled with 3 μM 3,3'-dioctadecyloxacarbocyanine perchlorate (DIO; Beyotime Institute of Biotechnology) at 37˚C for 30 min, then $1x10^5$ cells were seeded in a 24-well plate. Different E/T ratios of CIK/NK cells were added and mixed with each well of SK-N-SH cells. The numbers of cells added were $1x10^5$ (E/T=1:1), $2x10^5$ (E/T=2:1), $5x10^5$ (E/T=5:1) and $1x10^6$ (E/T=10:1) (24). The mixed cells were co-cultured in 37˚C and 5% CO_2 for 4 h. The mixed cells were then harvested and washed with PBS in triplicate, and stained with 5 μl propidium iodide (PI; Sigma-Aldrich; Merck KGaA) for 10 min at 4˚C. A flow cytometry assay was performed to detect the percentage of late apoptotic and dead SK-N-SH cells (labeled as DIO^+).

For the killing effect experiments, cells were treated with IgG only, CIK/NK cells only, anti-GD2 only or CIK/NK combined with anti-GD2. SK-N-SH cells ($1x10^5$) in IgG control group, CIK/NK treated group and CIK/NK + anti-GD2 treated group were labeled with 3 μM DIO for 30 min at 37˚C, while cells in the anti-GD2 treated group were not stained with DIO. SK-N-SH cells were mono-cultured or co-cultured with CIK/NK cells (optimal-fold relative cell number) in the absence or presence of saturated concentration of anti-GD2 monoclonal antibody for 4 h at 37˚C. Cells were washed with PBS in triplicate and apoptotic cells, defined as late apoptotic and necrotic cells, were labeled with 5 μl PI for 10 min at 4˚C before performance of flow cytometry assay. Cell viability was determined using flow cytometry on a BD FACSVerse (BD Biosciences). Data were analyzed by FlowJo software. Each of the experiments were conducted individually, and each of them has a specific control group, therefore the graphs were produced differently according to their own control group.

Cell apoptotic rate in IgG treated group, CIK/NK treated group and CIK/NK+anti-GD2 treated group were calculated using the following equation: Apoptotic rate (%)=DIO^+PI^+ (%)/DIO^+ (%) x100, where DIO^+PI^+ cells were the dead SK-N-SH cells that were previously labeled with DIO and then labeled with PI, and DIO^+ cells were all the SK-N-SH cells, including living and late apoptotic/dead cells.

Statistical analysis. Data were processed using SPSS 20.0 software (IBM Corp.). A Student's t-test or one-way ANOVA with Tukey's post-hoc test was used to compare groups. $P<0.05$ was considered to indicate a statistically significant difference. Data are presented as the mean ± standard deviation. All experiments were conducted in triplicate and repeated three times.

Results

GD2 and CD54 (ICAM-1) are highly expressed in human neuroblastoma SK-N-SH cells. To characterize the molecular basis of the effect of anti-GD2 monoclonal antibody or CIK/NK cells on human NB SK-N-SH cells, the cell surface expression of GD2 and CD54 (ICAM-1) was assessed using flow cytometry analyses. The results showed that GD2 and CD54 (ICAM-1) were highly expressed in 93.37±1.2% (Fig. 1A and C) and 91.72±2.15% (Fig. 1B and C) on human neuroblastoma SK-N-SH cells.

Anti-GD2 monoclonal antibody induces cell death at a saturated concentration. The results indicate that following treatment with low dose anti-GD2 antibody, (<5 μg/ml), the detection of GD2 on the cell surface decreased from >95% (in the control group) to ~75% (5 μg/ml treatment group; $P<0.001$; Fig. 2A). This indicates that anti-GD2 antibody effectively combines with GD2 on the cell surface even at concentrations of <5 μg/ml. However, at doses >5 μg/ml, there was no statistically significant difference in GD2 detection between groups (Fig. 2B), suggesting that increasing the anti-GD2 antibody concentration had no effect on SK-N-SH cells. Therefore, the concentration of 5 μg/ml was selected for subsequent experiments. After 4 h of incubation with 5 μg/ml anti-GD2 antibody 3F8 at 37˚C and 5% CO_2, cells were observed to be larger and rounded in shape compared with cells prior to treatment (Fig. 2C). The results indicate that anti-GD2 antibody treatment led to 8.43±1.06% cell death compared with 0.92±0.05% in the control group ($P<0.001$; Fig. 2D). This demonstrates that treatment with anti-GD2 antibody significantly increased apoptosis compared with the control group.

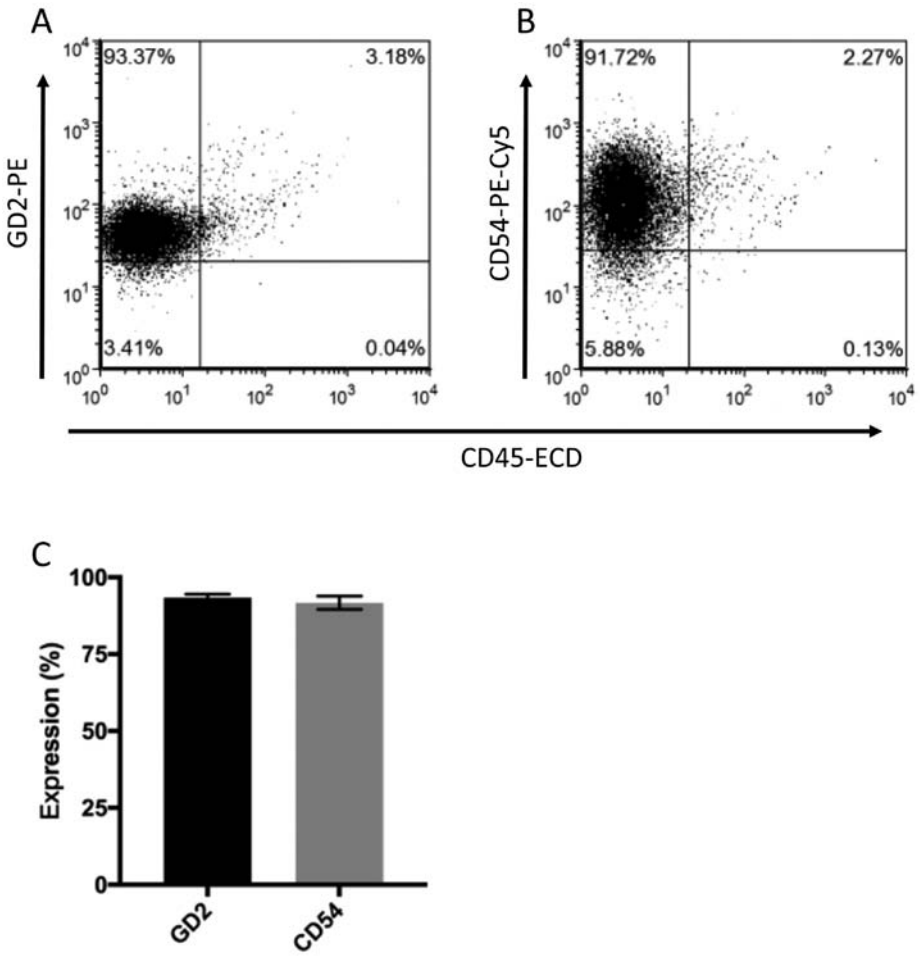

Figure 1. Expression of ganglioside GD2 and CD54 (ICAM-1) on SK-N-SH cells. Flow cytometry analyses were performed to characterize the molecular basis of the effect of anti-GD2 monoclonal antibody or CIK/NK cells on human NB SK-N-SH cells. (A) y-axis represents GD2-PE and x-axis represents CD45-ECD. GD2 was highly expressed (>95%) in SK-N-SH cells. (B) y-axis represents CD54-PE-Cy5 and x-axis represents CD45-ECD. CD54 was expressed in the majority of the SK-N-SH cells detected by flow cytometry. (C) GD2 and CD54 (ICAM-1) were highly expressed on human neuroblastoma SK-N-SH cells. ICAM-1, intercellular adhesion molecule 1; CIK, cytokine-induced killer; NK, natural killer; NB, neuroblastoma.

IL-2 and IL-7-cultured CIK/NK cells partially express CD16. CBMNCs were isolated using a density gradient centrifugation method and cultured in complete X-VIVO medium supplemented with IL-2 and IL-7. The proportion of $CD3^+CD56^+$ CIK cells and $CD3^-CD56^+$ NK cells was evaluated prior to culture, and identified to be 2.20±0.92 and 9.85±1.28%, respectively. In total, $CD3^{+/-}CD56^+$ CIK/NK cells accounted for 12.05±2.09%. After 21 days of culture, the proportion of $CD3^+CD56^+$ CIK cells increased to 43.60±1.98% and $CD3^-CD56^+$ NK cells increased to 10.80±1.73%. In total, $CD3^{+/-}CD56^+$ CIK/NK cells accounted for 54.40±1.56% (Fig. 3A and B). These percentages were increased by 19.8-, 1.1- and 4.5-fold compared with before culture, respectively. As shown in Fig. 3C, CBMNCs were single cells when freshly isolated, and cell colonies gradually formed during 21 days of culture. Expression of CD16 on CIK/NK cells was detected after culture, and the results indicated that CD16 was expressed on the cell surface of 19.01±1.27% of CIK/NK cells (Fig. 3D).

CIK/NK cells lead to cell death at various E/T ratios. To determine whether CIK/NK cells could induce cell death in human NB SK-N-SH cell line and to assess the optimal E/T ratio, a specific killing experiment was performed and a flow cytometry assay was used to analyze apoptotic cells. The results showed that after 4 h of culture, CIK/NK cells had gathered around and adhered to targeted NB SK-N-SH cells. The targeted cells had become larger than normal and some of the cell membranes had burst after adherence by CIK/NK cells (Fig. 4A). According to the aforementioned equation, when E/T ratio was 1:1, the proportion of apoptotic cells was 5.94±0.25%. When E/T ratio was increased to 2:1, 5:1 and 10:1, the proportion of apoptotic cells was 8.33±1.46, 8.56±0.71 and 9.00±0.40%, respectively (Fig. 4B and C). One-way ANOVA indicated that when E/T ratio increased from 1:1 to 2:1, the apoptotic rate increased significantly. However, when the E/T ratio was increased further, there were no significant differences between groups (Fig. 4C). This suggested that an E/T ratio of 2:1 was optimal for cell death, and therefore it was selected for subsequent experiments.

Anti-GD2 monoclonal antibody combined with CIK/NK cells significantly increases apoptotic rate. To further investigate whether combining CIK/NK cells with anti-GD2 monoclonal antibody could enhance the induction of cell death, a combination killing experiment was performed. After incubation with an E/T ratio 2:1 of CIK/NK cells and 5 μg/ml anti-GD2

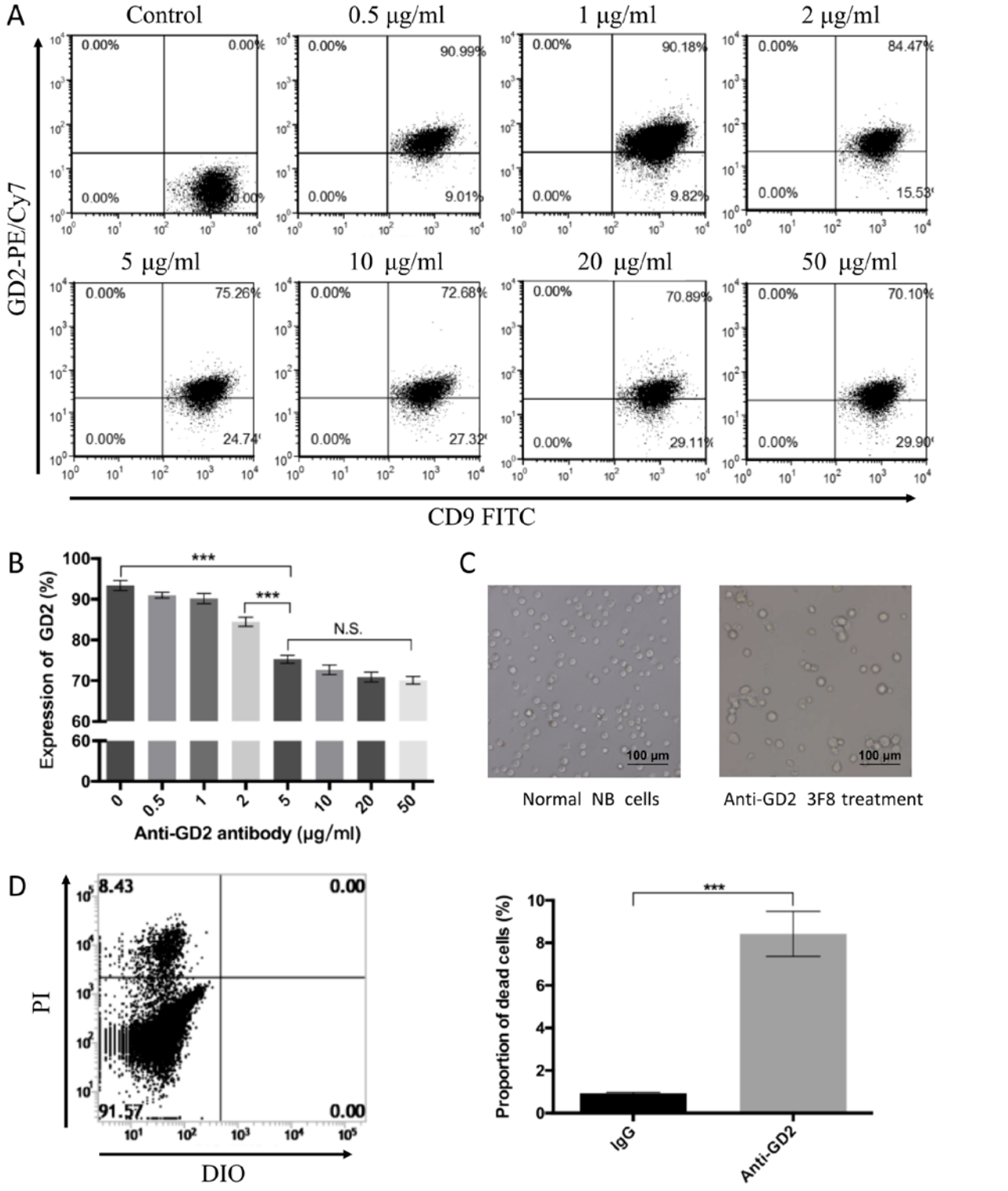

Figure 2. Anti-GD2 monoclonal antibody induces cell death at a saturated concentration. (A) Anti-GD2 antibody at 0.5, 1, 2, 5, 10, 20 or 50 μg/ml was added to each well after culture for 1 h. After treatment with low dose anti-GD2 antibody, (<5 μg/ml), the expression of GD2 on the cell surface decreased from >95% (control group) to ~75% (5 μg/ml treatment group). (B) There was no statistically significant difference in GD2 expression between 5 μg/ml and >5 μg/ml treatment groups. (C) SK-N-SH cells marked by DIO were observed to be larger and more rounded when 5 μg/ml anti-GD2 antibody 3F8 was added. (D) SK-N-SH cells were stained with PI and analyzed by flow cytometry. Anti-GD2 antibody led to 8.43±1.06% cell death compared with 0.92±0.05% in the control group. ***P<0.001. Scale bar, 100 μm. PE, phyroerythrin; N.S., not significant; DIO, 3,3'-dioctadecyloxacarbocyanine perchlorate; PI, propidium iodide; NB, neuroblastoma; FITC, fluorescein isothiocyanate.

antibody 3F8 at 37˚C and 5% CO_2 for 4 h, SK-N-SH cells were observed to be surrounded and adhered by CIK/NK cells (Fig. 5A). All cells were subsequently harvested and analyzed by flow cytometry. The results showed that the combined treatment induced an apoptotic rate of 16.92±0.38%, which was significantly higher than the IgG control group and the CIK/NK cell or anti-GD2 antibody mono-treatment groups (P<0.001; Fig. 5B and C).

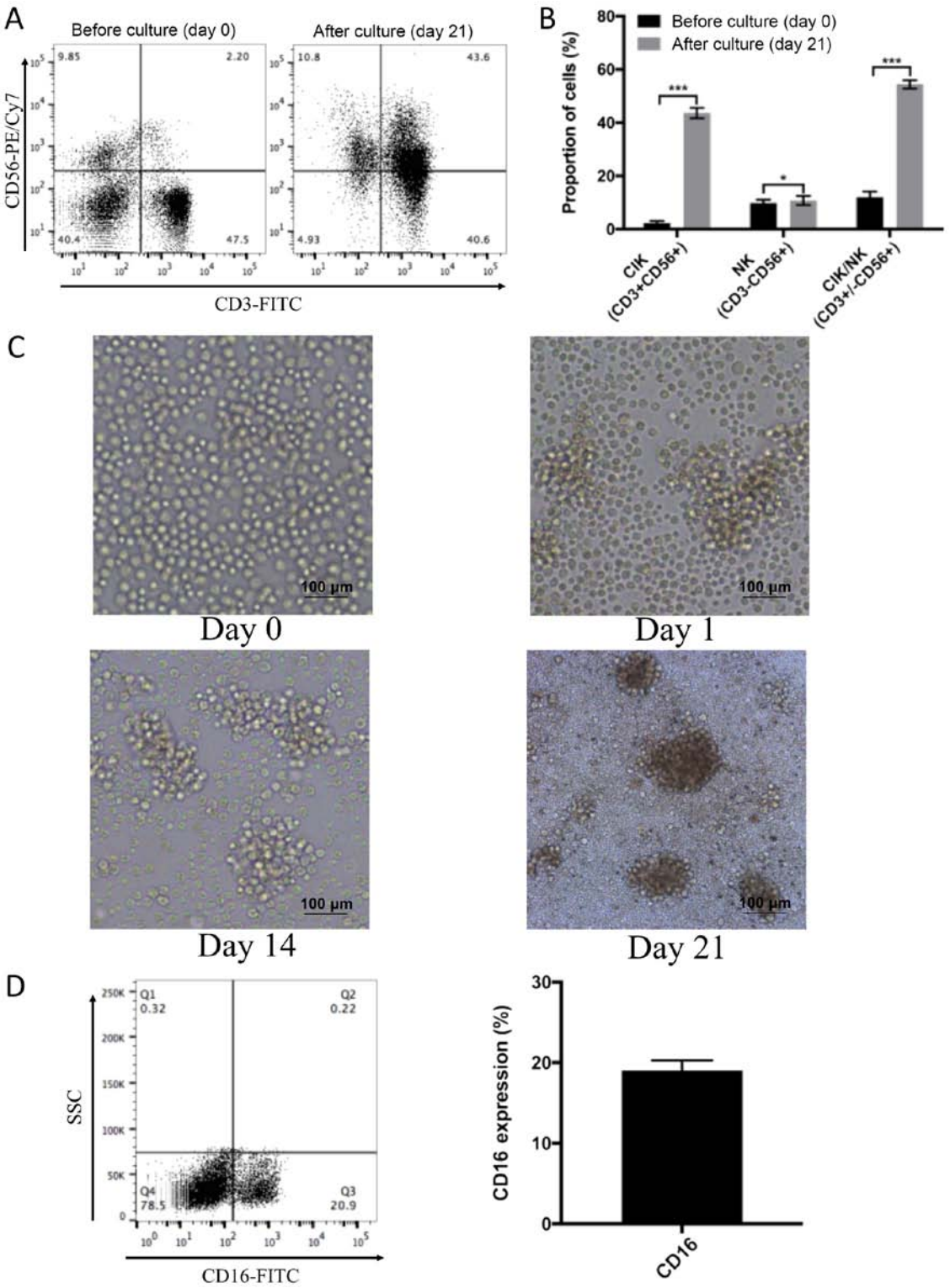

Figure 3. IL-2 and IL-7-cultured CIK/NK cells partially express CD16. (A) The proportion of $CD3^+CD56^+$ CIK cells and CD3-CD56+ NK cells was evaluated prior to culture, and (B) found to be 2.20±0.92 and 9.85±1.28%, respectively, while mixed $CD3^{+/-}CD56^+$ CIK/NK cells was 12.05±2.09%. After 21 days of culture, the proportion of $CD3^+CD56^+$ CIK cells increased to 43.60±1.98, $CD3^-CD56^+$ NK cells to 10.80±1.73 and mixed $CD3^{+/-}CD56^+$ CIK/NK cells to 54.40±1.56%. (C) CBMNCs were all single cells when freshly isolated, and cell colonies gradually formed during 21 days of culture. (D) y-axis showed SSC view, x-axis showed the CD16 was partially expressed on CIK/NK cell surfaces, at a rate of 19.01±1.27%. Scale bar, 100 μm. $^*P<0.05$ and $^{***}P<0.001$ IL, interleukin; PE, phyroerythrin; CIK, cytokine-induced killer; NK, natural killer; CBMNCs, mononuclear cells from cord blood; SSC, side scatter.

Discussion

NB is one of the most common pediatric extracranial solid tumors, and its occurrence and development has marked heterogeneity (41). NB can either differentiate from mature nerve cells or low-stage ganglion tumors spontaneously, or metastasize early with highly malignant characteristics (8). Among high-risk stage IV patients with NB, >60%

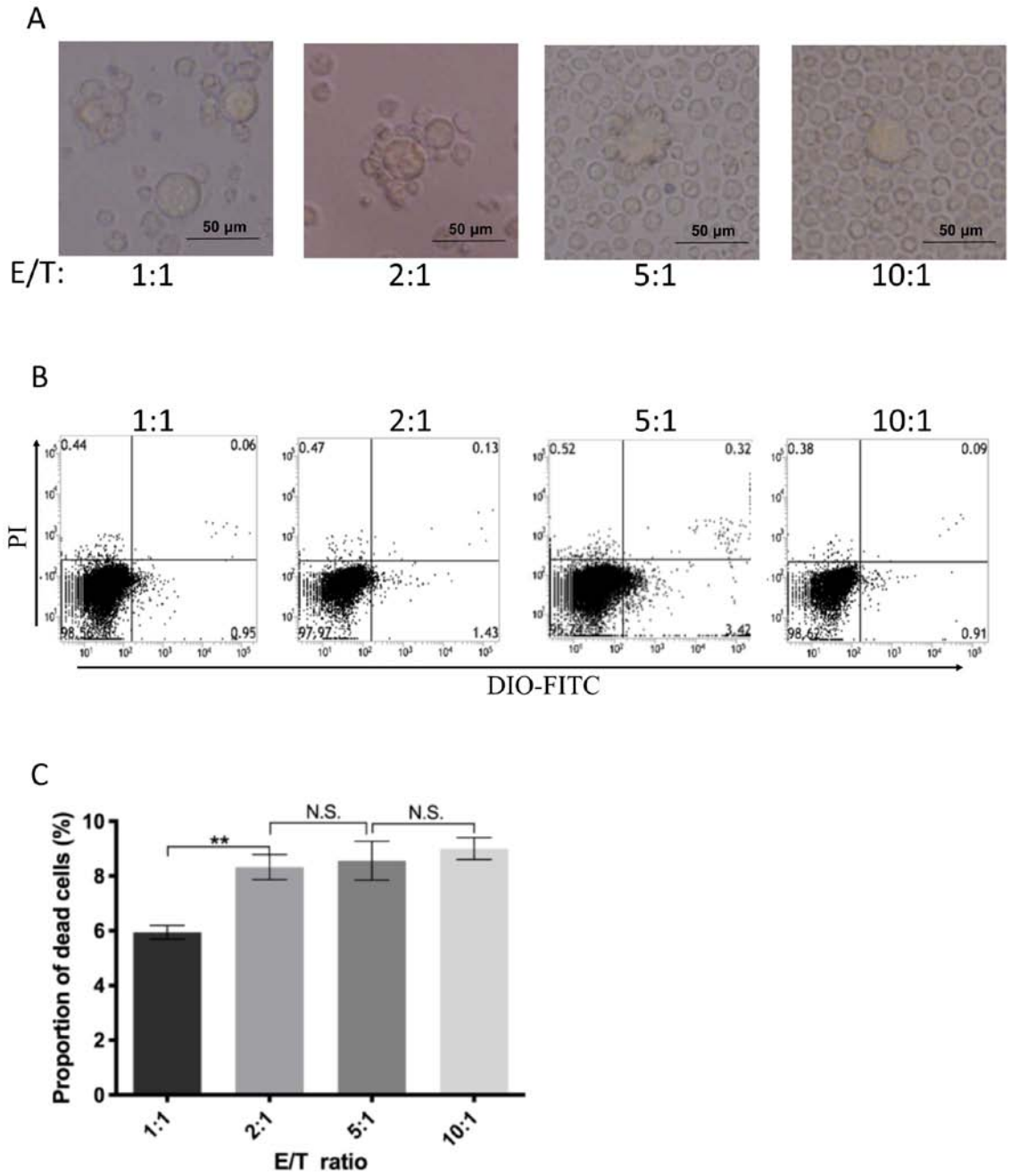

Figure 4. CIK/NK cells lead to cell death at various E/T ratios. (A) Various E/T ratios of CIK/NK cells were added and mixed with each well of SK-N-SH cells and images were obtained after 4 h of co-culture. CIK/NK cells surrounded and adhered to targeted neuroblastoma SK-N-SH cells. Targeted cells became larger than normal. A number of the cell membranes burst after adherence by CIK/NK cells. (B) When the E/T ratio was 1:1, the apoptotic rate was 5.94±0.25%, and when the E/T ratio increased to 2:1, 5:1 and 10:1, the apoptotic cell percentages were 8.33±1.46, 8.56±0.71 and 9.00±0.40%, respectively. (C) When the E/T ratio increased from 1:1 to 2:1, the apoptotic rate significantly increased. However, when the E/T ratio was increased further, there were no significant differences between groups. Scale bar, 50 μm. **P<0.01. CIK, cytokine-induced killer; NK, natural killer; E/T, effector/target; PI, propidium iodide; DIO, 3,3'-dioctadecyloxacarbocyanine perchlorate; N.S., not significant.

eventually die of relapse even after comprehensive treatment including surgery, radiotherapy or chemotherapy (42). The reason for relapse in patients with stage IV NB is the presence of chemotherapy-resistant NB cells, which induces MRD (5). Chemotherapy is the most effective treatment for NB; however, its effectiveness is constrained by the presence of MDR in tumor cells. Furthermore, increasing the dose of chemotherapeutic drugs may lead to severe toxic effects in multiple organs, including the heart, liver, kidney and ear (43). This suggests that it is necessary to identify a more effective and tolerable treatment strategy for MDR NB.

In recent years, immunotargeting treatment of MDR NB has been demonstrated to significantly prolong patient survival (44). Following treatment with anti-GD2 monoclonal antibody combined with IL-2, GM-CSF or cis-retinoic acid, the 5-year overall survival time of relapsed/refractory patients with NB has improved by 20-30% (45,46). This finding suggests that anti-GD2 monoclonal antibody has enormous potential for the treatment of relapsed/refractory NB.

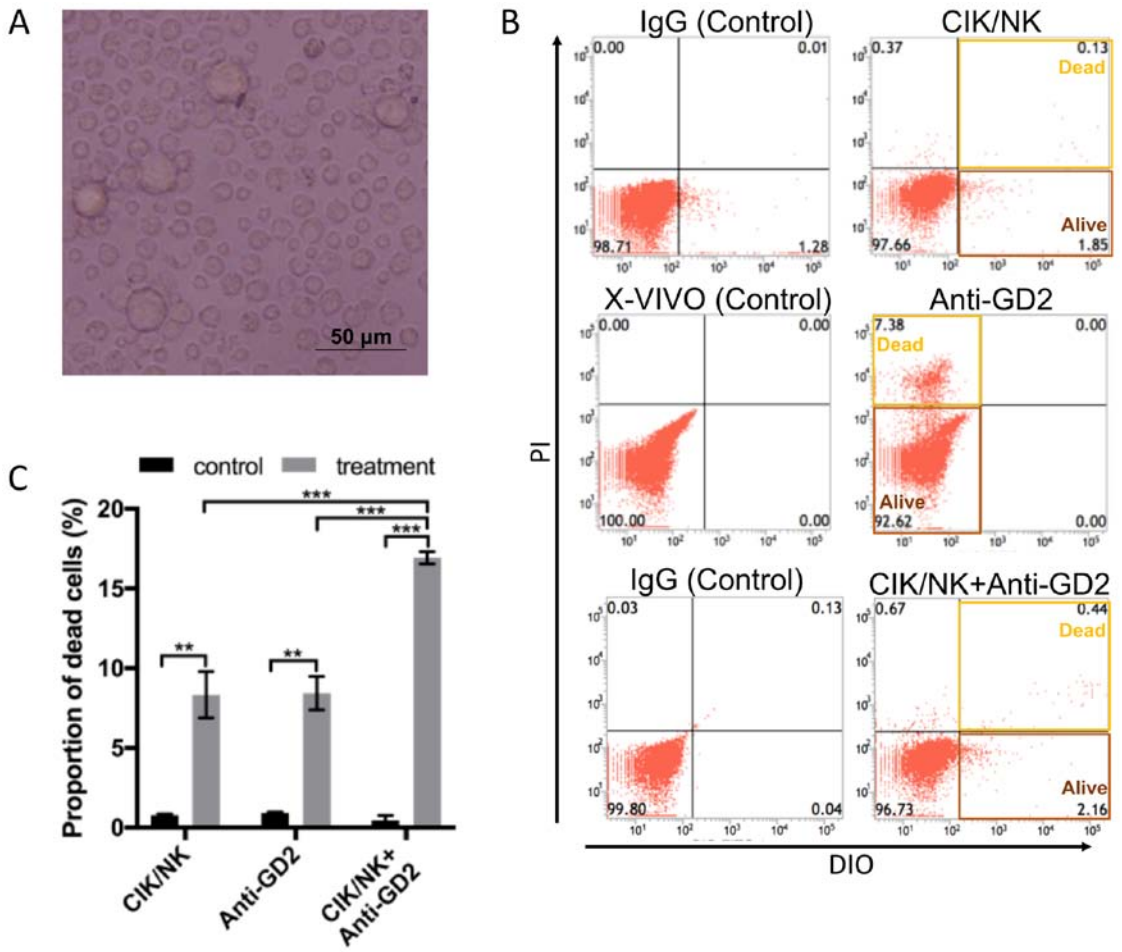

Figure 5. Anti-GD2 monoclonal antibody combined with CIK/NK cells significantly increases apoptotic rate. (A) The SK-N-SH cells that were treated with E/T ratio 2:1 of CIK/NK cells and 5 μg/ml anti-GD2 antibody 3F8 were observed to be surrounded and adhered by CIK/NK cells. (B and C) The combined treatment induced an apoptotic rate of 16.92±0.38%, which was significantly higher compared with the IgG control group and the CIK/NK cell or anti-GD2 antibody mono-treatment groups. Scale bar, 50 μm. **P<0.01 and ***P<0.001. CIK, cytokine-induced killer; NK, natural killer; PI, propidium iodide; DIO, 3,3'-dioctadecyloxacarbocyanine perchlorate.

Anti-GD2 monoclonal antibody has been found to stimulate a CDC killing effect by specifically binding to tumor cell surface GD2 or activating the ADCC effect by binding to Fc receptors on killer cells (17-23). However, the dosage of anti-GD2 antibody is restricted by severe side effects, such as intolerable pain and allergy (47). This suggests that a novel strategy based on anti-GD2 antibody is required.

CIK cells are mononuclear cells that can be isolated from peripheral blood or cord blood. The cells are cultured with various cytokines and are $CD3^+CD56^+$ (48). Due to the expression of CD3 and CD56 on the cell surface, CIK cells not only demonstrate anti-tumor activity, but also have non-major histocompatibility complex-restricted killing activity (49). The killing effect of CIK cells on tumor cells is mainly via expression of LFA-1, which specifically binds to ICAM-1 (CD54) on the surface of tumor cells, resulting in the secretion of benzyloxycarbonyl-L-lysine thiobenzyl ester esterase (granzyme A) that can penetrate the cell membrane and lead to tumor cell death (50). However, previous experiments have demonstrated that the efficacy of CIK/NK cells alone for the treatment of patients with high-risk stage IV NB is limited (51). This is due to the large amount of complement fragments produced by anti-GD2 antibodies during the killing of NB tumor cells, which can cause severe pain (52). Since anti-GD2 is already in late phase of clinical trials (25), numerous evidence has reported that it is effective for the treatment of neuroblastoma, despite of the tolerable side effects, and it shows no harm to normal cells. CIK/NK cell transfusion is a type of adoptive cellular immunotherapy (35), which is commonly used in clinical treatment. It recognizes and kills target tumor cells by binding to specific cell surface markers based on the immune system (17). To the best of our knowledge, there is no evidence showing that CIK/NK cells identify and kill normal cells. Therefore, maximizing the anti-GD2 antibody-mediated ADCC effect is a feasible research direction for improving anti-GD2 antibody treatment for relapsed/refractory NB.

In the present study, the cytokine combination of IL-2 and IL-7 was used for culturing CBMNCs to obtain CIK/NK cells, based on our previous study (37). After 21 days, the proportion of $CD3^+CD56^+$CIK and $CD3^-CD56^+$ NK cells was 43.60±1.98 and 10.80±1.73%, respectively, and the proportion of CIK/NK mixed cells was 54.40±1.56%, which indicated increases of 19.8-, 1.1- and 4.5-fold, respectively. CD16 expression on the surface of CIK/NK cells was 19.01±1.27% after culture, which was similar to the results reported by Bonanno *et al* (53). Compared with the 'classical' CIK amplification system (54)

(IL-2, IL-1a, IFN-γ and anti-CD3 monoclonal antibodies), not only were NK cells effectively expanded, but also the proportion of $CD8^+$ cells had not been significantly increased, which could lead to a decreased rate of GVHD (55). This suggests that the two-cytokine culture system provides effective targeting of anti-GD2 antibodies to NB cells and involves a simple preparation process with fewer side effects.

The findings of the present study showed that when various E/T ratios of CIK/NK cells were implemented, the higher the E/T ratio, the more CIK/NK cells adhered around NB tumor cells. After 4 h of treatment, some NB cells exhibited cell membrane rupture and died, indicating that CIK/NK cells had anti-tumor activity against NB cells *in vitro*. The killing rate gradually increased as E/T ratio was increased, from 5.94±0.26% at the 1:1 ratio to 9.00±0.40% at the 10:1 ratio, suggesting that an increase in effector cells could enhance the anti-tumor effect to a certain extent. It was also found that at E/T >2:1, the cell apoptotic rate curve began to stabilize, suggesting that when CIK/NK cells reached a certain threshold, they could not further increase the apoptotic rate of NB tumor cells. Therefore, clinical application of CIK/NK cell immunotherapy for NB tumors should use an infusion of an appropriate number of cells, in order to achieve maximum efficacy, while minimizing side effects, including GVHD caused by infusion of immune cells.

For the anti-GD2 antibody experiment, it was identified that GD2 was highly expressed on SK-N-SH cells, which was also highly expressed on other malignancies, including bladder cancer (56), breast cancer (57) and some other solid tumors in children (58). The effects of different concentrations of anti-GD2 antibody on NB cells was examined. The experimental results showed that when the antibody concentration reached 5 μg/ml, there were no significant changes in the expression rate of anti-GD2 antibody on the cell surface with increases in concentration. This finding was similar to the results of Esser *et al* (59). Anti-GD2 antibody mediates the ADCC effect of immune cells on tumor cells *in vivo*; the Fc segment of anti-GD2 antibody binds to the FcR on the immune cell surface and stimulates the immune cell to release perforin and granzyme that can cleave the NB cell membrane (20). In addition, the anti-GD2 antibody itself can also form antigen-antibody complexes by binding to GD2 antigen on the surface of NB cells, activating the classical complement pathway (CDC pathway) for cell death (60). This study's results showed that the apoptotic rate under anti-GD2 monoclonal antibody alone was 8.43±1.06% at 5 μg/ml. However, activation of the classical complement pathway requires the binding of two or more antibodies to the Fc segment (61), therefore anti-GD2 antibody killing of target cells through the CDC pathway is not a highly effective therapeutic method, which is consistent with our *in vitro* results.

However, CIK/NK cells combined with anti-GD2 antibody only increased the cell death rate to 16.92±0.38%, which was <20% in the present study. This may be explained by the low expression of FcRγIII (CD16) on the surface of CIK/NK cells, which was only 19.01±1.27%. FcRγIII (CD16) activates the ADCC pathway by combining with Fc region of the anti-GD2 antibody (62). However, this study's bi-factor (IL-2 and IL-7) culture system has no effect on increasing the expression of CD16 on these cells, therefore no comparison is necessary for the expression of CD16 before and after culture. The expression of CD16 on CIK/NK cells was tested after culture, in order to determine the possible proportion of CIK/NK cells involved in anti-GD2-mediated ADCC effect, which may provide an explanation to the low killing effect of combined therapy. Seidel *et al* (63) showed long treatment time may be beneficial for increasing killing efficacy. Furthermore, in this experiment we defined late apoptosis and cell death induced by CIK/NK cells only, anti-GD2 only or CIK/NK cells combined with anti-GD2 as effective killing, which does not include early apoptosis, therefore PI single staining was performed instead of Annexin-V/PI double staining, which may give another explanation to the low killing effect.

In conclusion, this study provided supporting evidence for treating relapsed/refractory NB with CIK/NK cells combined with anti-GD2 antibodies. Immunotherapy combined with monoclonal antibodies is a potential novel strategy for treating relapsed and refractory NB; however, further studies are required in order to validate this method.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CZ and XX performed the experiments and wrote the manuscript. KH designed the experiments and collected the samples. XP analyzed the experimental data. YL, LL and WW performed data analysis and revised the manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital at Sun Yat-sen University (Guangzhou, China). This study was performed in compliance with the Helsinki Declaration. All patients gave written informed consent to participate in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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