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The study on cytotoxicity of cytokines produced by the activated human NKT cells on neuroblastoma

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Purpose : α-Galactosylceramide (α-GalCer)-stimulated human Vα24 natural killer T (NKT) cells exert antitumor activity against some leukemia in a CD1d dependent and TCR-mediated manner, but could not kill CD1d - negative neuroblastoma (NB) cells. There are few reports about the direct antitumor effect of highly secreted cytokines by these cells on activation. In this study, using a cell-free supernatant (SPN) collected from plate bound hCD1d/αGalCer tetramers-stimulated NKT cells, we examined whether they could be helpful in the immunotherapeutic treatment of NB.

Methods : Cells were cultured in IMDM. The cytokines produced by NKT cells were measured with Cytometric Bead Array (CBA) analysis. Cell viability was evaluated by calcein-AM fluorescence with digital image microscopy scanning (DIMSCAN). The percentage of specific apoptosis was calculated by flow cytometric detection of apoptosis using annexin V and 7-AAD.

Results : The activated NKT cells secreted high levels of IL-2, INF-γ, TNF-α. The SPN was significantly cytotoxic against four out of eight tested NB cell lines, through mainly apoptosis as evidenced by annexin-V staining and inhibition with the pretreatment of pancaspase blocker. This apoptosis was significantly inhibited when anti-TNF-α and anti-IFN-γ neutralizing mAbs were used separately and it was completely abolished when the two mAbs were combined.

Conclusion : IFN-γ and TNF-α produced by NKT cells could exert synergistically direct antitumor activity through apoptosis on some NB cell lines. **(Korean J Pediatr 2006;49:439-445)**

Key Words : α-Galactosylceramide (α-GalCer), Vα24 NKT cells, INF-γ, TNF-α, Neuroblastoma (NB)

Introduction

Natural killer T (NKT) cells are a recently defined subset of T lymphocytes, being characterized by the usage of a highly skewed invariant Vα24Jα Q rearrangement paired preferentially with a variable Vβ11 chain and co-expression of high levels of CD161 (NKR-P1A)[1, 2]. These cells are specifically activated by a synthetic glycolipid, α-Galactosylceramide (α-GalCer) in a CD1d-dependent and T-cell receptor-mediated manner[3]. The main feature of NKT cells are their ability to produce large amounts of T helper 1 and 2 cytokines that can link innate to adaptive immune systems[4, 5]. They have been reported as participating in various immune responses such as tumor immunity, protection against infections, regulation of autoimmune diseases, initiation of antigen-specific tolerance, induction of transplantation tolerance and maintenance of the gravid state[6-10].

Neuroblastoma (NB) is the most common extracranial solid tumor in childhood, arising from the sympathetic nervous system. In spite of aggressive therapy involving high dose chemotherapy followed by peripheral blood stem cell transplantation and the administration of retinoic acid, the overall survival of stage IV patients is still poor[11]. A new treatment strategy involving immunotherapy needs to be introduced to overcome the poor prognosis. Previous study showed activated NKT cells could recruit and differentiate mononocyte via production of chemokines, which are capable of providing a source of CD1d for NKT activation in tumor microenvironment[12, 13]. Furthermore, our recent pre-

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liminary data showed that some NB cells could localize NKT cells to tumor site via production of MCP-1. These findings led us to favor NKT cells for treatment of NB, but it was demonstrated they could not directly kill NB cells that do not express CD1d by cell-to-cell interaction[4]. However, it was well known NB was sensitive to IFN-γ or/and TNF-α induced cytotoxicity through modulation of TNFR or FAS/FASL[14, 15]. It has been demonstrated that the activating NKT cells secreted a large amount of cytokines including IFN-γ and TNF-α[4, 5]. Therefore, immunotherapy using NKT cells may be an attractive approach to the treatment of poor risk NB.

In the present study, we demonstrated that NKT cells on activation could exert significant cytotoxicity and apoptosis against some NB through production of IFN-γ and TNF-α without a correlation with NMYC amplification and drug resistance of NB. These findings suggest that production of IFN-γ or TNF-α by NKT cells will be an additional mechanism for their cytotoxicity as well as be helpful for the treatment of NB.

Materials and Methods

1. Cell culture

The human NB cell lines were maintained in IMDM (Gibco-BRL products, Gaithersburg, MD, USA) supplemented with 0.7 mM L-glutamine, insulin, and transferrin (5 μg/mL each), selenium (5 ng/mL), and 20% heat-inactivated FBS. Cell lines were cultured at 37℃ in a humidified incubator containing 95% room air plus 5% CO_2 atmosphere. Cells were cultured without antibiotics to facilitate the detection of Mycoplasma, for which all cell lines tested negative. Cells were detached without trypsin from culture plates with the use of a modified Puck's Solution A plus EDTA (Puck's EDTA), which contains 140 mM NaCl, 5 mM KCl, 5.5 mM glucose, 4 mM $NaHCO_3$, 0.8 mM ethylenediamine tetraacetic acid (EDTA), 13 μM Phenol Red, and 9 mM HEPES buffer (pH7.3).

2. NKT cell isolation

Venous blood, collected from healthy adult volunteer, was obtained after informed consent and anti-coagulated with heparin, 100 units/mL. After sedimentation of erythrocytes with dextran, peripheral blood monocytes (PBMC) were obtained from leukocytes by density gradient centrifugation using Hispaque®-1077 (Sigma Diagnostics Inc, St. Louis, MO, USA). NKT cells were isolated from PBMC as described[16] with the addition of FACS® sorting using hCD1d/αGalCer-Tc tetramers (Kirin Brewery Co, Tokyo, Japan). Briefly, PBMC were stained with FITC-conjugated anti-Vα24 mAb C15 (Beckman Coulter-Immunotech, Miami, FL, USA) and incubated with anti-FITC MACS beads according to the manufacturer's protocol. Cells retained in the MACS column (Beckman Coulter-Immunotech, Miami, FL, USA) were selected as Vα24$^+$ cells. Antigen presenting cells (APC) were obtained from Vα24$^+$ fraction after depleting CD3$^+$ cells with anti-CD3 MACS beads (Beckman Coulter-Immunotech, Miami, FL, USA) and APCs (ratio 1 : 10) were co-cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS in the presence of 10 ng/mL αGalCer with 100 U/mL recombinant IL-2 for 3 weeks.

Subsequently, hCD1d/αGalCer tetramer reactive NKT cells were positively sorted using a Coulter® EPICS®Elite flow cytometer (Beckman-Coulter Inc, Miami, FL, USA).

3. Cytometric bead array (CBA) analysis

NKT cells were cultured in plates coated with hCD1d/α-GalCer for 6 hr. Culture medium, 60 μL, was collected and frozen at -80℃ until cytokine analysis. Cytokine concentrations were measured with the CBA kit (BD PharMingen, San Diego, CA, USA), according to the manufacturer's manual, as previously described[17], with modification of data analysis to use GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Briefly, CBA uses a series of beads with discrete fluorescence intensity at 670 nm. Each series of beads is coated with a mAb against a single cytokine (IL-2, IL-4, IL-5, IL-10, IFN-γ, or TNF-α), and the mixture of beads detects six cytokines in one sample. A secondary PE-conjugated mAb stains the beads proportionally to the amount of bound cytokine. A cytokine standard containing a predetermined amounts of all six cytokines was used to prepare 10 serial dilutions, providing a range of concentrations from 20 to 5,000 pg/mL.

After fluorescence intensity calibration and electronic color compensation procedures, standard and test sample were analyzed with a Coulter EPICS Elite flow cytometer.

Fluorescence was excited by the argon laser at 488 nm, and emitted light was measured at 670 and 576 nm. Data was collected using EXPO Analysis software (Beckman-Coulter, Miami, FL, USA). Statistics (median fluorescence intensity values at 576 nm of each bead series) was trans-

ferred to GraphPad Prism. Starting with standard dilutions, the software performed a log transformation of the data, then, fit a curve to the 10 discreet points using a four-parameter logistic model. The created calibration curve for each cytokine was used to estimate the cytokine concentrations in the experimental samples.

4. Cytotoxicity assay

Tumor cell viability was measured by quantifying retained calcein-AM fluorescence with Digital Image Microscopy Scanning (DIMSCAN) as previously described with some modification[18]. 2000-4000 tumor cells were seeded into 96-well plates in 100 μL of complete medium per well. After 24 h incubation for attachment, the desired concentration of the supernatant or cytokines was added in 50 μL/well. The cells were incubated at 37℃ in a humidified atmosphere with 5% CO_2 for the indicated day. Then, after Calcein-AM (Molecular Probes Inc, Eugene, OR) was added to the target cells, 5 μg/mL, the cells were incubated at 37℃ for 30 min and analyzed by DIMSCAN. Data was expressed as % target cell viability (% viability=fluorescence intensity in the experimental well/mean fluorescence intensity in the 6 control wells)×100. The mean % viability±standard deviation for each condition was calculated from 6 replicated experimental wells.

5. Flow cytometric detection of apoptosis using annexin V and 7-AAD

Annexin V-phycoerythrin and the nonvital dye 7-AAD (PharMingen, San Diego, CA, USA) double staining were performed as previously described[19], with minimum modification. After samples were washed twice with cold PBS, 1×10^6 cells/mL, and were resuspended in a binding buffer (10 mM/L 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, pH 7.4, 140 mM/L NaCl, 2.5 mM/L $CaCl_2$). 1×10^5 cells were transferred to a 5 mL culture tube and 5 μL of FITC-Annexin V and 7-AAD was added. The mixture was incubated for 15 minutes in the dark at room temperature. The samples were analyzed by a Coulter® EPICS® Elite flow cytometer. PE and 7-ADD stained cells were excited with 488 nm argon ion-laser. PE emission was detected with a 580±21 nm band pass filter, and 7-ADD-stained cells were detected with a 670 nm long pass filter. The percentage of specific apoptosis was calculated as follows : 100×[experimental apoptosis (%)−spontaneous apoptosis (%)/100%-spontaneuous apoptosis (%)]. Mean and s.d of triplicate are shown, similar results were obtained in three independent experiments. We determined 7-AAD negative/A-V negative cells were living, 7-ADD negative/A-V positive cells were in early apoptosis stage and 7-ADD positive/A-V positive cells were either in late apoptosis stage or already dead.

Results

1. Cytokine production of hCD1d/αGalCer tetramers-stimulated NKT cells

To measure the concentration of cytokines secreted by the activated NKT cells, cell-free supernatants (SPN) acquired from NKT cells stimulated by hCD1d/αGalCer tetramers for 6 hr were analyzed with the CBA assay. The NKT cells produced significant levels of IL-2 (16.2 ng/mL), IL-4 (38.1 ng/mL), TNF-α (331.1 ng/mL), INF-γ (182.3 ng/mL); little IL-5 (0.8 ng/mL) and even less IL-10 (0.2 ng/mL) were secreted (Table 1).

2. The NKT SPN induced direct cytotoxicity against NB cells

Because NKT cells could not kill NB that did not express CD1d by direct cell-to-cell interaction, we have focused on the cell-free SPN acquired after stimulating human NKT cells with a plate bound tetramers for 6 hrs. At first, to investigate whether NB was susceptible to the cytotoxicity of the NKT SPN, we tested the viability of 8 randomly selected NB cell lines using DIMSCAN after incubating with variable diluted concentration of NKT SPN for 6 days. The CHLA-42, CHLA-15, CHLA-171 and CHLA-90 cells were significantly sensitive to the SPN in a concentration-dependent manner whereas SMS-KANR, LA-

Table 1. Cytokine Concentrations of the Supernatant Produced by GalCer-Stimulated NKT Cells

Cytokines	Concentrations (pg/mL)
IL-2	16,227
IL-4	38,126
IL-5	829
IL-10	238
TNF-α	331,053
IFN-γ	182,255

Supernatant (SPN) was acquired after stimulating purified human natural killer T (NKT) cells with plate bound hCD1d/GalCer tetramers for 6 h
The concentration of cytokines was measured in the supernatants using Cytometric bead analysis (CBA) analysis and was proportional (in the range 20-5,000 pg/mL) to the fluorescence intensity at 576 nm (x-axis).

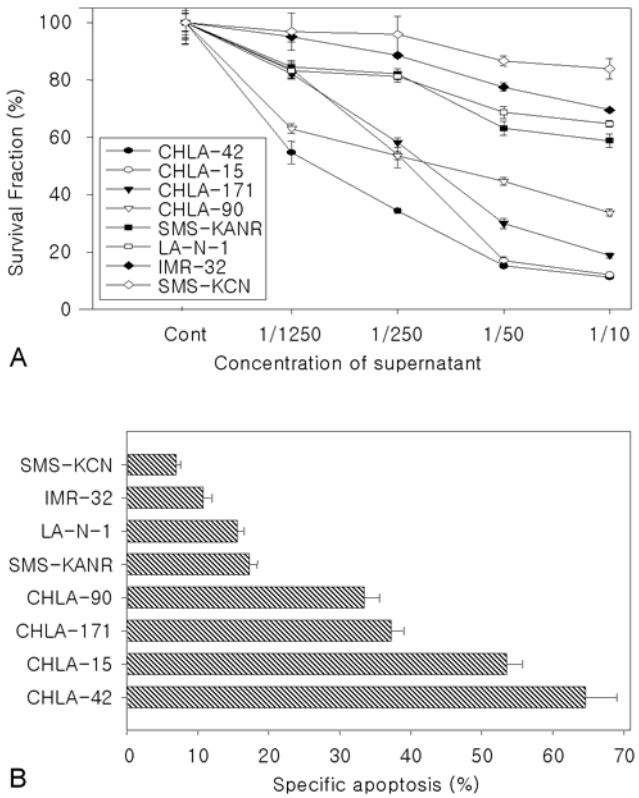

Fig. 1. NKT supernatant induced significant cytotoxicity through apoptosis against some neuroblastoma cells. **(A)** The cells were treated with different concentrations of NKT SPN for 6 d. And then, cells were loaded with calcein-AM (5 μg/mL) and retained calcein fluorescence was measured by DIMSCAN. Each point represents the mean±SD of 6 replicate wells. Results were presented as a percentage of viable cells relative to that in target cells alone. **(B)** Cells were treated with 1/50 SPN for 3 d and were resuspended in annexin V binding buffer. 1×10^5 cells were added 5 μL of FITC-Annexin V and 7-AAD, and was incubated for 15 minutes in the dark at room temperature. The samples were analyzed by a Coulter® EPICS®Elite flow cytometer (Beckman-Coulter Inc, Miami, Fl). Mean and S.D of triplicate are shown, similar results were obtained in three independent experiments.

N-1, IMR-32, SMS-KCN were resistant. Even on 1/50 diluted concentration of SPN, the viability of CHLA-42 and CHLA-15 cells severely decreased to about 10% of control cells and highly drug resistant cell lines, CHLA-171 and CHLA-90 also survived below 50% (Fig. 1A). In order to determine the cytotoxic mechanism of NKT SPN, we next measured apoptotic changes using annexin V-PE and 7-ADD staining after incubating NB cells with 1/50 diluted SPN for 72 hours. This treatment triggered various degrees of specific apoptosis on all tested cell lines that were correlated with the changes in viability (Fig. 1B). In addition, this increased apoptosis in the CHLA15 and CHLA-90 cells were completely blocked after the pretreatment of the

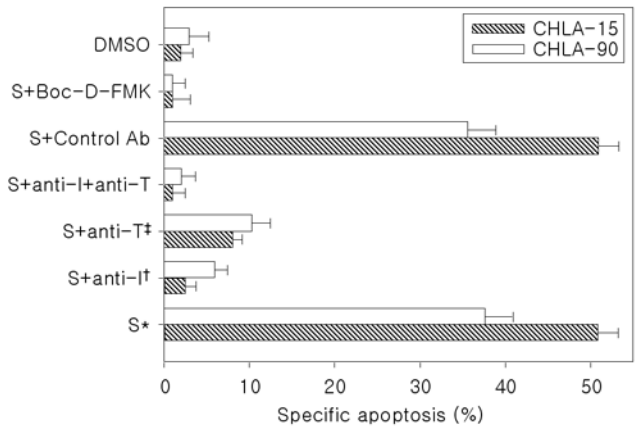

Fig. 2. Anti-IFN- and anti-TNF-α blocking antibody completely inhibited the SPN-induced apoptosis on CHLA-15, 90 cell lines. The supernatant (SPN) were pretreated with 50 μL/mL of anti-IFN-γ, TNF-α and control mAbs for 2 h at 4℃, and then added to cells. The samples were analyzed by flow cytometry using of FITC-Annexin V and 7-AAD. Mean and S.D of triplicate is shown, similar results were obtained in three independent experiments. *Supernatant, †Anti-Interferon-γ mAb, ‡Anti-TNF-α mAb.

broad-spectrum caspase inhibitor BOC-D-FMK, which inhibits most of these proteases (Fig. 2). This data suggest that the NKT SPN could exert direct anti-tumor activity through caspase cascades-dependent apoptosis against some NB.

3. The IFN-γ and TNF-α in the NKT SPN cooperatively mediated cytotoxicity

To study whether the apoptosis by the SPN was induced by IFN-γ and TNF-α that existed in high quantities in the SPN, we performed blocking experiments with 50 μg/mL of anti-IFN-γ or/and anti-TNF-α antibody on the CHLA-15 and CHLA-90 cells. Pretreatment of the anti-IFN-γ or anti-TNF-α antibody alone inhibited about 70-80% or 80-90% of specific apoptosis by SPN, respectively. And the combination of both blocking antibody completely abolished SPN-induced apoptosis (Fig. 2). This finding indicates that IFN-γ and TNF-α produced by the activated NKT cells cooperatively induced the direct anti-tumor activity against some NB cells.

Discussion

Previous studies have shown human NKT cells, activated by CD1d- and ligand dependent manners, could rapidly produce large amount of various cytokines including IFN-γ and TNF-α[4, 5]. Here the purified NKT cells were iso-

lated by flow cytometric cell sorting with newly developed CD1d/αGalCer tetramers, and stimulated with the tetramer for obtaining SPN. With this SPN and cytokines blocking mAb, we demonstrated IFN-γ and TNF-α produced by activated NKT cells could synergistically exert direct anti-tumor activity through mainly apoptosis.

Although there was a paradoxical study that NKT cells suppressed cytotoxic T lymphocyte (CTL)-mediated tumor immunosurveillance in mice, they have been considered to play an essential role in the development of early innate anti-tumor immunity and linkage to adaptive tumor immunity[6, 20-22]. It was demonstrated that human NKT cells could directly kill target tumor cells through release of cytoplasmic perforin/granzyme even though it was strictly limited to only CD1d+ some leukemia (AML M4, M5, juvenile myelomonocytic leukemia)[23, 24]. Using CD1d+ dendritic cells as a source of CD1d, it was reported αGalCer-stimulated NKT cells also could induce apoptosis in their respective receptor-expressing cells through expression of FASL or TRAIL[23, 25]. In addition, they were reported to induce an anti-proliferative effect against melanoma via release of IFN-γ[26]. Our observations suggest that IFN-γ and TNF-α produced by activated NKT cells could be an additional direct mechanism through which they can kill malignant tumor cells.

As well as above direct cytotoxic mechanism the activated NKT cells have been demonstrated to induce potent NK cell-mediated cytotoxicity by producing IFN-γ or IL-2 in mice and human studies[4, 27], and to recruit and differentiate monocyte through release of MIP-1a, MIP-1b, GM-CSF and IL-4, which support CD1d for activation of NKT cells and facilitate the development of CTL[12, 13]. As a matter of fact, because most human tumor cells did not express antigen-presenting molecules, CD1d, NKT cells cannot directly kill them by cell-to cell interaction. However, monocyte lineage cells, including monocytes, monocyte derived dendritic cells and macrophages, were reported to be constitutive and low expression of CD1d but to provide extremely potent antigen presenting capacity for NKT cells[28, 29]. Therefore, using DC cells as a source of CD1d in tumor bed, we may design therapeutic modalities. Additionally, our unpublished data showed some NB could produced MCP-1 localizing NKT cells to tumor site. These findings suggest NKT cells may perform central crucial roles in immunotherapy using immune cells, which affect tumor environments, and control from innate to adaptive immunity.

NB is the most common extracranial solid tumor among children derived from the neural crest. Although recently considerable progress has been made in the treatment of stage IV NB, such as myeloablative chemoradiotherapy with autologus bone marrow transplantation and 13-cis retinoic acid, the overall survival is still poor. Therefore a new treatment strategy including immunotherapy could support another approach to improve patients with this poor prognosis. In fact this has been attempted in a wide range of clinical and research fields including neutrophil with antibody dependent cellular cytotoxicity (ADCC), NK cell and CTL, especially in minimal residual disease setting after intensive anticancer therapy[30, 31]. However, the immunotherapeutic approach using NKT cells has its limitations because αGalCer-stimulated NKT cells can not kill the $CD1d^{-}$ NB cells. Here, alternatively, we found that INF-γ and TNF-α produced by NKT cells might induce significant anti-tumor cytotoxicity through apoptosis against some NB cells. Previous studies showed both cytokines synergistically exert differentiation and growth inhibition against NB cells through regulation of TNF R[14]. And it was reported IFN-γ could induce apoptosis against NB cells through cooperation with expression of MycN or up-regulation of CD95/CD95L[32, 33]. In our study, the main cytotoxic mechanism was apoptosis via common caspase pathway as evidenced by annexin-V staining and inhibition with the pretreatment of pancaspase and caspase-8 blocker, but we cannot exclude growth inhibition in some sensitive cell lines that did not measure specific apoptosis. This study provided the first observation of IFN-γ and TNF-α synergistically inducing apoptosis against neuroblastoma; previous studies have demonstrated only synergistic effects of growth inhibibition. Also, although previous study showed that using tetracycline-controlled expression of MycN with SH-EP NB cell lines[33], MycN cooperate with IFN-γ for triggering apoptosis, we could not find significant relationship between cytokines-mediated cytotoxicity and levels of MycN expression (data not shown). Interestingly, the most drug-resistant CHLA-90 and CHLA-51 cells, which were established from a tumor relapse in bone marrow after myeloablative chemoradiotherapy supported by autologus bone marrow transplantation, were strikingly suppressed by the SPN. This finding suggested that a therapeutic strategy using NKT cells might be used to treat some NB patients, especially in minimal residual disease and terminally relapsing patients.

Our results indicate that NKT cells activated by CD1d/αGalCer tetramers could induce anti-tumor activity against some NB through apoptosis by producing IFN-γ and TNF-α. Production of cytokine produced by activated NKT cells may be an important possible mechanism of the anti-tumor effect of NKT cells. This finding suggested the possibility that an immunotherapeutic strategy using NKT cells might be attempted in the treatment of NB patients.

한 글 요 약

활성화된 자연살상 T 세포(NKT)에서 생성된 사이토카인에 의한 신경모세포종의 세포독성에 관한 연구

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목 적 : α-Galactosylceramide (GalCer)로 자극한 자연살상 T 세포(NKT)는 CD1d 및 T 세포 수용체(T cell receptor) 의존적으로 일부 백혈병에서 항암효과를 발현하나, CD1d음성인 신경모세포종에서는 세포독성을 유도할 수 없다. 이들 NKT세포의 활성화 시 분비되는 많은 양의 사이토카인의 직접적인 항암효과에 대해서는 소수의 보고가 있다. 본 연구에서는 hCD1d/α-GalCer tetramer로 NKT세포를 자극하여 얻은 상청액(supernatant)을 이용하여 NKT세포에 의한 신경모세포종의 치료적 접근의 가능성을 알아보았다.

방 법 : 신경모세포종 세포 주를 IMDM 배지에 배양하였고, NKT세포에서 분비되는 사이토카인 양은 cytometric bead array (CBA)분석으로 측정하였다. 세포 생존율은 calcein-AM 형광물질을 이용하여 digital image microscopy scanning (DIMSCAN)으로 측정하였고 특이 세포고사(specific apoptosis)는 annexin V and 7-AAD 염색 후 유식세포분석기를 통하여 산출하였다.

결 과 : 활성화된 NKT세포는 많은 양의 IL-2, IL-4, INF-γ와 TNF-α을 분비하였다. NKT 자극 후 얻어진 상청액은 8개의 신경모세포종 세포 주 중 4개에서 의미있는 세포독성을 나타냈으며, 그 기전은 annexin-V 염색이나 pancaspase 억제제의 전 처치 실험으로 세포고사을 통하여 유도됨을 알 수 있었다. 그리고 이들 세포고사 유도는 anti-TNF-α, anti-IFN-γ 중화항체의 단독투여 시 현저히 감소하였고 동시투여 시에는 완전하게 억제되었다.

결 론 : NKT 세포의 활성화에 의해 분비된 IFN-γ와 TNF-α가 일부 신경모세포종 세포 주에서 협동적 세포 독성을 유도하였다.

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