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Original Article

Cytoprotective effects of high dose of α-galactosylceramide against activation-induced CD4+ T and CD8+ T cell death as an adjuvant

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Abstract: Objective: To investigate the cytoprotective effects of high dose of α-galactosylceramide (α-GC) on the activation-induced CD4+ T and CD8+ T cell death. Methods: Experimental autoimmune encephalomyelitis (EAE) was induced using adoptive transfer of MOGCD4+ cells treated using α-GC into recipient C57BL/6 mice while the MOGCD4+ cells treated using 0.5% polysorbate were set as vehicle group, based on which to investigate the effects of α-GC on activation induced CD4+ T cell death. Additionally, an EG7 tumor-bearing mice model is established using adoptive transfer of CD8+ T cells, based on which to investigate the effect of α-GC on the apoptosis of CD8+ T cells. Results: A higher induction rate was noticed after adoptive transfer of MOGCD4+ cells treated using α-GC together with the severity of EAE compared with the conventional methods. Longer survival duration was noted in the green fluorescent protein (GFP) labeled MOGT in the α-GC group compared with the vehicle group ($P < 0.05$). Severe inflammatory cell infiltration and myelinoclasis was noted in the white matter of nervous system in the α-GC group. In the EG7 tumor model, more adoptive CD8+ T cells were survived in α-GC group compared with that of vehicle group. The growth of tumor mass was significantly inhibited in α-GC group. Conclusions: high dose of α-GC could be used as an adjuvant for inhibiting activation-induced CD4+ T and CD8+ T cell death. Our study could provide helpful information for the development of adoptive cell therapy with reduced programmed cell death.

Keywords: α-galactosylceramide, experimental autoimmune encephalomyelitis, activation-induced cell death, T cells

Introduction

Adoptive cell immunotherapy (ACI) based on the adoptive transfer of natural or gene-engineered T cells have been widely applied in treating tumor to establish a long-term specific antitumor immunological effect. To date, extensive studies have been performed on the tumor killing effects of cytotoxic T lymphocytes (CTLs) [1-3]. Among this, a new strategy named adoptive regulatory T-cell (Treg) therapy has been extensively studied as it plays pivotal roles in suppressing inflammatory responses and maintaining immune homeostasis [4, 5]. However, as a large number of cells underwent apoptosis after transfusing into the recipients, multiple transfusions were necessary in order to maintain the cell number to exert the tumor killing effects. According to the previous reports, the major obstacles for the adoptive cell therapy are the programmed cell death of T lymphocytes (e.g. CTLs and Treg cells) mediated by Fas/FasL interaction [6, 7]. Therefore, it is necessary to develop an adjuvant which can inhibit the cell apoptosis and enhance the survival duration of the transfused cells in vivo.

β-galactosylceramide (β-GC) has been reported to inhibit cellular apoptosis in human leukemic cell lines and breast cancer cells [8, 9]. In this study, α-GC with similar chemical structure is speculated to show inhibiting effects on apoptosis. To confirm this, we firstly investigated the effects of high doses of α-GC on the apoptosis of cultured CD3+ T cells in vitro, which revealed a protective effect of high doses α-GC on the

apoptosis of CD3+ T cells. Then, an experimental autoimmune encephalomyelitis (EAE) model is used to investigate the apoptosis-inhibiting effects of α-GC on CD4+ T cells as these cells are the effector cells that primed in the periphery and migrate to the central nervous system (CNS) to mediate immune damage to myelin [10]. Besides CD4+ T cells, CD8+ T cells are also considered as a potential target for the therapeutic intervention of EAE [11]. In our study, an EG7 tumor-bearing mice model is established after adoptive transfer of CD8+ T cells, based on which to investigate the effect of α-GC on the apoptosis of CD8+ T cells.

Our study reveals that high doses of α-GC could enhance the severity of EAE through inhibiting the apoptosis of CD4+ T cells. Additionally, a 4.0-fold increase of CD8+ T cells in the total number of donor cells is observed after cell transfer in EG7 tumor model. All these demonstrate that high doses of α-GC could be used as an adjuvant for inhibiting activation-induced CD4+ T and CD8+ T cell death.

Materials and methods

Mice

Male C57BL/6 mice aged 6-12 weeks were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences. GFP-Tg mice of the same C57BL/6 background were kindly provided by Dr XF Zhou (Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences). Mice were maintained under pathogen-free conditions. The animal protocols in this study were approved by the institutional review board of the Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences.

Reagents

α-GC was purchased from Alexis Biochemicals (Miami, FL, US) and was reconstituted in phosphate-buffered saline (PBS) containing 0.5% polysorbate-20 (Sigma-Altrich, St. Louis, MO, US). Collagenase I, collagenase IV, hyaluronidase and DNase were purchased from Sigma-Aldrich (St. Louis, MO, US). Annexin V FITC Apoptosis Detection kit and active form of caspase-3 monoclonal antibody apoptosis detection kit were purchased from BD biosciences (San Jose CA, US). Anti-Mouse CD3 APC, FAS and FASL antibodies for Flow Cytometry were purchased from eBioSciences (San Diego, CA, US). Mouse CD4+ T-cell isolation kit and Mouse CD8+ T-cell isolation kit were purchased from Miltenyi Biotec (Bergish-Gladbach, Germany). OVA-expressing EL4 (EG7) tumor cells were purchased from the American Type Culture Collection.

Flow cytometry

CD3+ T cells were isolated from C57BL/6 mice according to the previous description [12]. Then the cells were incubated on anti-CD3/CD28 (BD Bioscience/Southern Biotech) antibody-coated plates for 72 h. Subsequently, the cells were pulsed with either α-GC (400 ng/ml solved using 0.5% polysorbate, experimental group) or vehicle (0.5% polysorbate, control group) for 12 h prior to cell harvest. For detection of apoptosis, cells were resuspended in phosphate buffered solution (PBS) containing 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, US) for cell surface staining. After preincubation with anti-mouse CD16/32 monoclonal antibody (mAb) for 10 min, the cells were stained at 4°C for 10 min with Annexin V-FITC and 5 μl propidium iodide (PI). To determine the active caspase, the cells were washed, blocked with anti-mouse CD16/32 mAb and stained with APC-anti-mouse CD3. After three washes, the cells were fixed and permeabilized using Cytofix/Cytoperm Plus fixation/permeabilization kit with GolgiPlug in accordance with the manufacturer's instructions. The cells were then stained with PE-anti-mouse Active Caspase. The stained cells were analyzed on a FACS Calibur (BD biosciences, San Jose CA, US) using CellQuest software (BD biosciences, San Jose CA, US) to assess the expression of FAS and FASL.

Induction of EAE by adoptive transfer

The encephalitogenic myelin oligodendrocyte glycoprotein peptide (MOG35-55) with a purity of 95% (BioAsia Biotechnology Co., Ltd, Shanghai, China) was used to evoke MNC separated from C57BL/6 mice after foot-pad immunization for 72 h. Then, the cells were pulsed with α-GC dissolved in 0.5% polysorbate (α-GC group) or 0.5% polysorbate (vehicle group) for 12 h prior to harvest. The resulting cells were intraperitoneally injected into naïve C57BL/6 recipients (1×10^7 cells per mouse) to induce

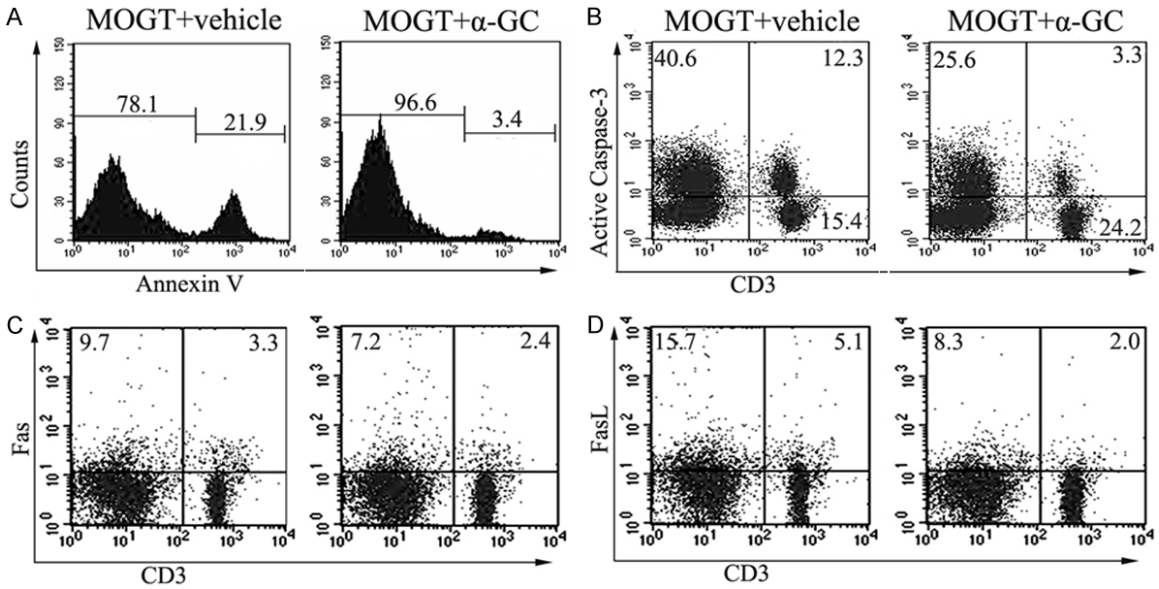

Figure 1. High doses of α-GC inhibits activated-induced apoptosis and the expression of FAS and FASL. FACS staining (A) indicated significant decrease was noted in the apoptosis of CD4+ T cells in the MOGT+α-GC group treated using 400 ng/ml α-GC compared with that of vehicle group treated using 0.5% polysorbate ($P < 0.05$). Remarkable decrease was noted in the activity of caspase-3 in MOGT+α-GC group and vehicle group (B), especially MOGT+α-GC group. FACS staining indicated down-regulation of FAS (C) and FASL (D) in CD4+ T cells of the MOGT+α-GC group compared with that of vehicle group ($P < 0.05$).

Table 1. Effects of α-GC treatment on EAE caused by adoptive transfer

Treatment	Incidence	Onset of disease (d)	Mean maximum score
α-GC/MOGT	12/13	8.2±1.2	2.9±0.5
Vehicle/MOGT	2/13*	11.0±1.0*	0.4±1.0*
α-GC/CD4MOGT	5/10#	12.0±1.0*	1.05±1.25*
Vehicle/CD4MOGT	0/10*	--	--

#$P < 0.05$, compared with other groups; *$P < 0.01$, compared with other groups.

experimental autoimmune encephalomyelitis (EAE). In addition, the resulting cells were also negatively selected using Mouse CD4+ T-cell isolation kit and intraperitoneally injected into naïve C57BL/6 recipients (5×10^6 cells per mouse) to induce EAE. The mice were examined daily for disease symptoms. The incidence rate and incubation period were record. The disease severity was assessed using the EAE scoring scale as previously described [13]. Furthermore, in order to investigate survival information of MOGT cells in recipient mice with α-GC treatment, MOGT cells from GFP+ C57BL/6 mice were transferred to wild C57 mice. On day 4, 9, 13 and 37 after adoptive transfer of donor cells, the recipient tissues of spleen, lymph nodes (LNs) and central nervous system (CNS) were obtained to separate MNC respectively. Fluorescence activated cell sorting (FACS) was used to assessing the proportion of GFP+ MNC.

Histology

Spinal cords were obtained from the sacrificed mice on day 14 after adoptive transfer of CD4+ MOGT cells. Hematoxylin and eosin (HE) staining, fast blue staining and immunohistochemical staining were performed respectively. In brief, the spinal cords were immediately fixed using 4% paraformaldehyde after separation from the sacrificed mice. Paraffin-embedded sections (5~10 μm) were stained with hematoxylin/eosin or Fast blue and then examined by light microscopy.

Adoptive transfer of donor CD8+ T cells

Donor GFP+ CD8+ T cells and CD8-depleted splenocytes were isolated from GFP-Tg mice and C57BL/6 mice by magnetic activated cell sorting (MACS; Miltenyi Biotec), respectively. They were cultured with an equal number in the presence of 1 mg/mL OVA peptide (SIINFEKL) for 1 day. The cells were pulsed with either

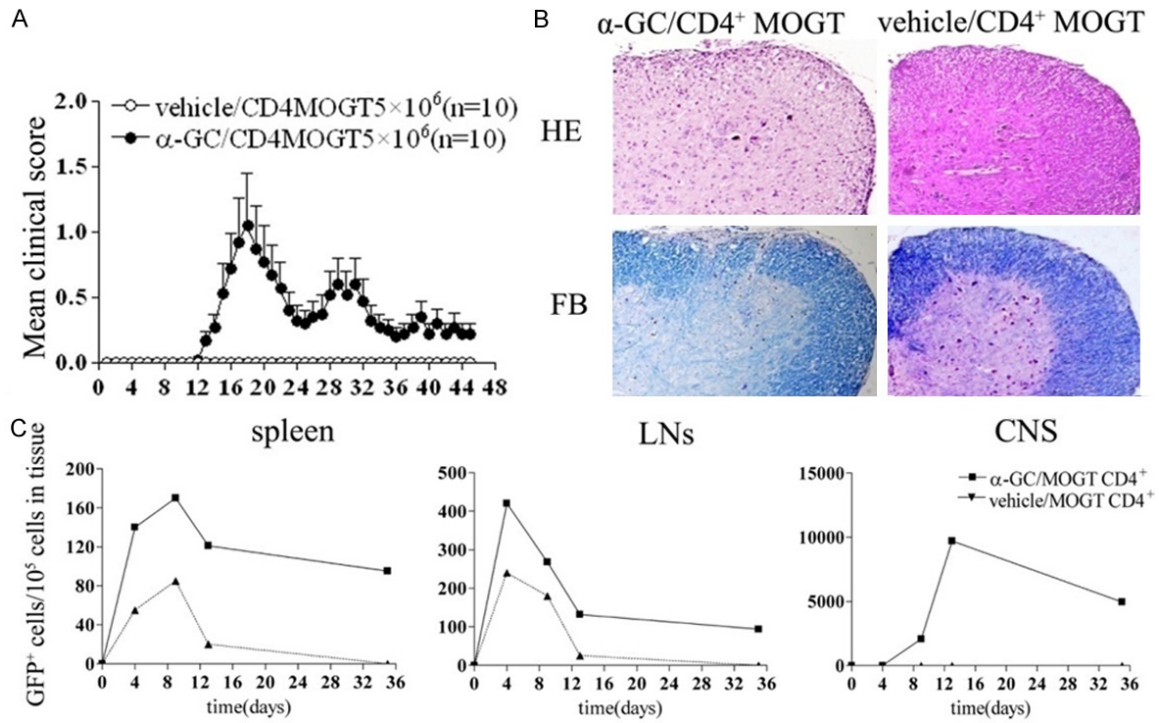

Figure 2. Effects of α-GC on the apoptosis of CD4+ T cells. A: Compared with control group, enhanced EAE was noticed by enhancing the CD4+ T cell responses after administration of α-GC. B: HE staining and Fast blue (FB) staining of spinal cord after adoptive transfer of α-GC. C: The number of GFP labeled MNCs derived from spleen, lymph nodes (LNs) and central nervous system (CNS)

α-GC (400 ng/ml, experimental group) or vehicle (control group) during the last 12 h of culture prior to harvest, and then GFP+ CD8+ T cells were purified by MACS (Miltenyi Biotec) and intraperitoneally transferred into EG7 tumor-bearing C57BL/6 mice (2×10^6 per mouse) until tumor diameters reached an average of 6 to 8 mm. The survival time of tumor-bearing mice was recorded every 3 days and the tumor volume was detected every 2 days. Mice that developed tumors larger than 2,000 mm^3 were sacrificed for ethical reasons. On day 3, 6, 9 and 12 after adoptive transfer of donor cells, the recipient tissues of spleen, LNs and CNS were obtained to separate MNC respectively. Fluorescence activated cell sorting was used to assessing the proportion of GFP+ MNC for each group.

Preparation of tumor infiltrating lymphocyte

The tumor infiltrating lymphocyte (TIL) was prepared according to the Rosenberg's method [14]. In brief, the tumor tissues of C57BL/6 mice were resected and rinsed by PBS. The tissues were morced and digested with collagenase I, collagenase IV, hyaluronidase and DNase (Sigma-Aldrich, USA). Monoplast suspension was obtained using sterilized net and gradient centrifugation was performed with lydroxypropylmethyl cellulose to isolate TIL.

Statistical analysis

Student's t-test was used to analyze for differences between groups. One-way ANOVA was initially performed to determine whether an overall statistically significant change existed before using the two-tailed paired or unpaired Student's t-test. Differences between tumor survival rates were analyzed using log-rank tests. $P < 0.05$ was considered statistically significant.

Results

Apoptotic rate and active caspase in CD3+ T cells

Annexin V-FITC was used as vital dye and AnnexinV-positive cells were considered as cells underwent apoptosis. Annexin V-FITC

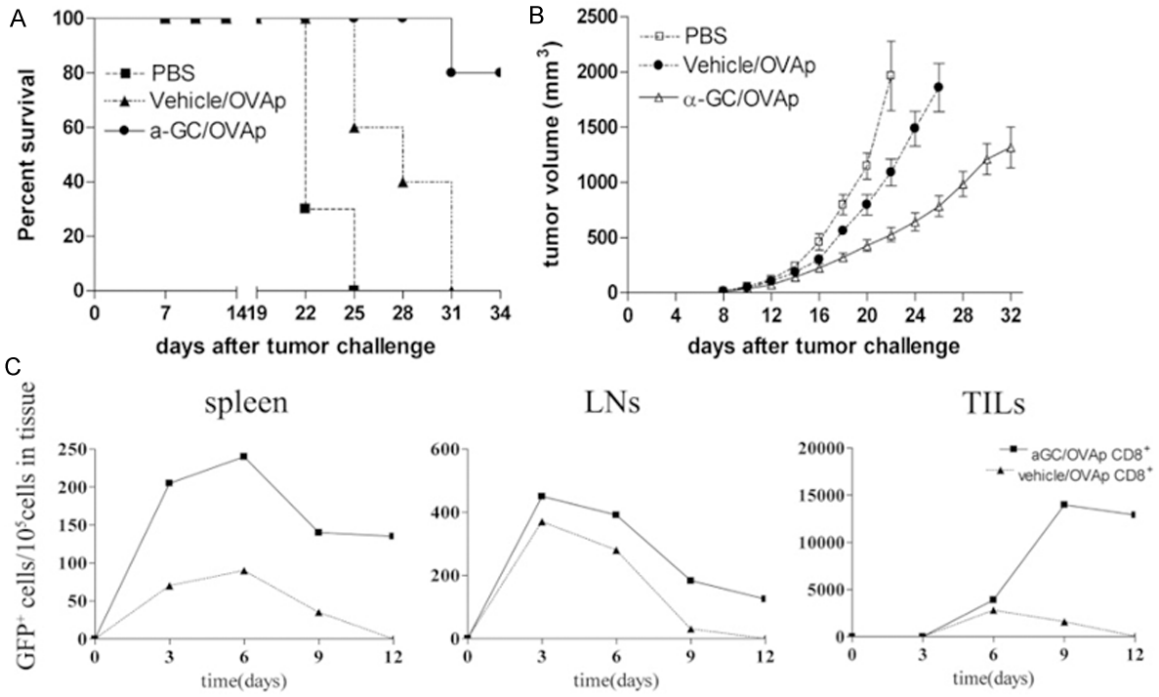

Figure 3. The effect of α-GC on apoptosis of CD8+ T cells. A: The survival rate of tumor bearing mice in different groups. B: The tumor volume of tumor bearing mice in different groups. C: The number of GFP+ cells in tissues of spleen, lymph nodes (LNs) and central nervous system (CNS) separated from sacrificed tumor bearing mice of different groups.

staining analysis showed significant decrease was noted in the apoptosis rate of activated CD3+ T cells in the α-GC group compared with that of vehicle group (3.4% vs. 21.9%, $P < 0.05$, **Figure 1A**). Meanwhile, obvious inhibition for production of active caspase3 was noticed in α-GC group compared with that of vehicle group (3.3% vs. 12.3%, $P < 0.05$, **Figure 1B**). Further, FAS and FASL expression in CD3+ T cells were significantly decreased in experimental group compared with these of control group as revealed by FACS analysis (**Figure 1C** and **1D**).

Pathopoiesia of adoptive transfer EAE enhanced after α-GC interference

The effects of α-GC on EAE development was investigated after the EAE model was induced by adoptive transfer. **Table 1** summarized the incidence rate, onset of disease period and severity score of the EAE in the α-GC/MOGT group, Vehicle /MOGT group, α-GC/CD4 MOGT group, and vehicle/CD4MOGT group, respectively. The results indicated that statistical differences were noted in the incidence of EAE ($P < 0.05$), onset of disease ($P < 0.01$), and mean score ($P < 0.01$) of α-GC/CD4 MOGT group compared with the other groups (**Figure 2A**).

HE staining indicated that after inducing of EAE by CD4+ MOGT adoptive transfer, mononuclear cellular (MNC) infiltrations were significantly increased surrounding small vessels of the spinal cord in α-GC treatment group compared with that of the control group. Immunohistochemical staining revealed that the MNCs involved in the cellular infiltration were CD4+ T cells, and few CD8+ T cells were identified. Compared with the vehicle group, CD4+ T cellular infiltrations showed obvious increase in α-GC/CD4MOGT. Compared with the vehicle group, significant impairment was noticed in the myelin sheath profile of spinal cord in α-GC treatment group as revealed by fast blue staining. Additionally, severe demyelination and increased vacuole-like absences were noted in the peripheral blue parts (**Figure 2B**).

To further analyze the survival of α-GC treated MOGT in the recipient mice, GFP mice and wild type C57 mice were selected as donor mice and recipient mice, respectively. After adoptive

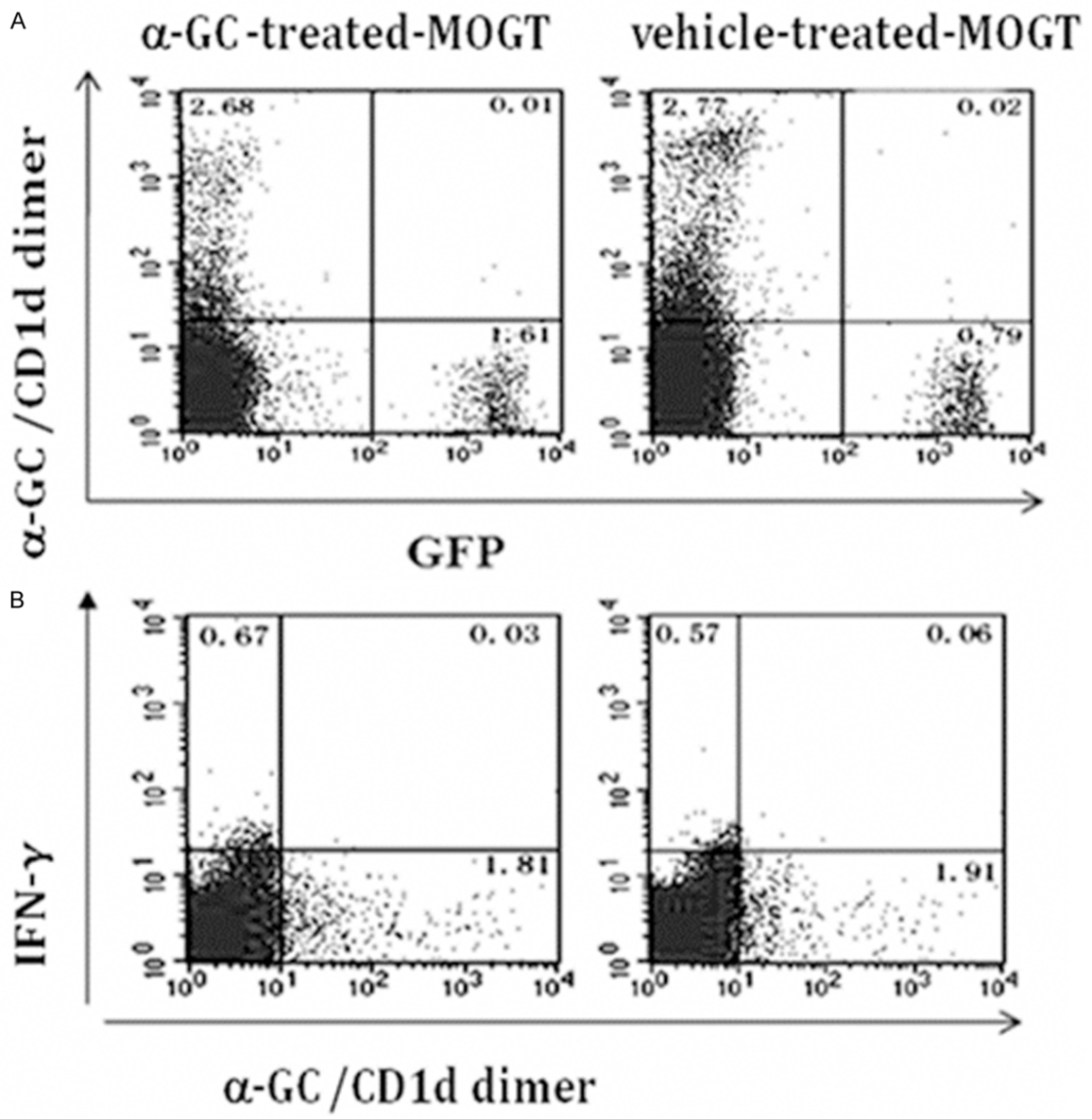

Figure 4. Adoptive transfer of α-GC caused no effects on the number (A) and the activation (B) of NKT cells. After adoptive transfer of high dose of α-GC in recipient mice, the spleens were obtained at 12 h, 2 d, 4 d, and 8 d, respectively. On this basis, the MNCs were obtained. The function and number of NKT was detected using FACS method. The activation of NKT was displayed by the staining of IFN-γ.

transfer, the animals were sacrificed at 4 d, 9 d, 13 d and 37 d to obtain spleen, lymph nodes (LNs), central nervous system (CNS), respectively. FACS analysis demonstrated that more GFP positive cells were identified in α-GC treatment group than the vehicle group at different time points. In particular, the number of GFP positive cells showed significant increase in CNS on day 9 after adoptive transfer, and reached the peak level on day 13. In the vehicle group, no GFP positive cells were identified in spleen and LNs, GFP+ cells were not detected after day 37 in vehicle group, while massive GFP+ cells were still detected in α-GC group (**Figure 2C**).

Tumor inhibiting effects of CD8+ T cells mediated by α-GC

To investigate whether α-GC loading would influence the effector function of the transferred CD8+ T cells, we transferred vehicle/

OVAp or OVAp/αGalCer into EG7 tumor-bearing mice. As shown in **Figure 3**, OVAp/αGalCer significantly inhibited the tumor growth, and 80% of the recipient mice survived until the termination of the study. On the contrary, a mortality of 100% was reported in the PBS group and vehicle group, respectively (**Figure 3A**). For the tumor growth, OVAp/αGalCer remarkably inhibited the growth of tumor mass compared with the other two groups (**Figure 3B**). To investigate the potential role of CD8+ T cells on the tumor inhibition, the number of CD8+ cells was determined in spleen, LNs and TILs, respectively. The results revealed a 4.0-fold increase of CD8+ T cells in the total number of donor cells after transfer. In addition, a longer survival time was noted in the CD8+ T cells after interference of α-GC (**Figure 3C**).

Discussion

Programmed cell death of T lymphocytes has been considered as the main obstacle for the cancer patients underwent adoptive cell therapy. The Fas/FasL interaction has been well acknowledged in modulating the programmed cell death of T cells [15-17]. We aim to develop an adjuvant to inhibit or eliminate the loss of transfused cells mediated by programmed cell death. Our study revealed that α-GC could be used as a vaccine adjuvant through enhancing the activity of CD4+ and CD8+ T cells.

Studies have been carried out to determine the properties of α-GC as a novel vaccine adjuvant that induces potent CD4+ and CD8+ T cells in animal models although the clinical responses were less impressive [18, 19]. Therefore, there are still disputes on the property of α-GC. In this study, we aim to identify whether α-GC could be used be as an adjuvant to enhance the immune function of CD4+ and CD8+ T cells in vivo. In our study, an effective adoptive transfer EAE model with characteristics of less time consuming, high incidence rate and severe pathological features was induced. Conventionally, the induction of EAE is through adoptive transfer with activated T cells specific for myelin Ag. The method is technical demanding which poses great strict to the animal selection and treatment such as dosing regimen. In particular, additional cytokines (e.g. IL-12 and IL-18) were required to enhance the pathogenesis [20, 21]. In our study, we have developed an easy-to-perform EAE model, which only involves the adoptive transfer of activated MOGT through high dose of α-GC treatment. The model was superior to the conventional ones at the terms of incidence rate, onset of disease, and the severity. The in vivo study also demonstrated α-GC could enhance the severity of EAE though inhibiting the apoptosis of CD4+ cells. Our study could provide helpful information for the modification and/or innovation of CD4+ T cells based immunotherapy, such as treating autoimmune disease using in vitro proliferation of Treg cells. Meanwhile, we demonstrated that α-GC could lead to a 4.0-fold increase of CD8+ T cells in the total number of donor cells after transfer in EG7 tumor model. Further, longer survival duration was noted in the recipient mice with adoptive transfer of donor activated cell through high dose of α-GC treatment.

In previous studies, NKT cells have been reported to be associated with the enhancing of CD4+ and CD8+ T cell responses after interference of α-GC [18, 19]. For example, Choi et al showed the iNKT activation was involved in the cell-to-cell interaction to activate CD8+ T cells to absorb α-GC via CD1d independent mechanism in vitro [18]. Also, Hermans et al proposed that the enhanced response of CT4+ and CD8+ T cells with α-GC was directly attributed to the activation of NKT cells [19]. However, our study indicated that high doses of α-GC treatment caused no effects on the activation of NKT. In addition, the number of NKT cells showed no statistical difference after administration of cells treated by α-GC compared with the control group (**Figure 4**). Therefore, we speculated that high doses of α-GC involved in the enhancing of CD4+ and CD8+ T cell responses not through the activation of iNKT cells. In the future studies, we will focus on the targets of α-GC during the enhancing of CD4+ and CD8+ T cell responses in vivo.

In conclusion, in this study, we found that α-GC as an adjuvant have a novel property of anti-apoptosis effects rather than inducing the activation of NKT cells. α-GC can enhance immune response as an adjuvant by protecting the activated CD4+ T cell in the EAE model induced by adoptive transfer of MOGT, and activating CD8+ T cells in EG7 immunotherapy model. Our study could provide a reference for the clinical practices of immunotherapy, especially tumor immunotherapy.

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Disclosure of conflict of interest

None.

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