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Antibody-dependent cellular cytotoxicity toward neuroblastoma enhanced by activated invariant natural killer T cells

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Key words

Antibody-dependent cellular cytotoxicity, immunotherapy, natural killer cells, natural killer T cells, neuroblastoma

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Funding Information

Japan Society for the Promotion of Science.

Received July 22, 2015; Revised December 15, 2015; Accepted January 3, 2016

Cancer Sci **107** (2016) 233–241

doi: 10.1111/cas.12882

Anti-ganglioside GD2 antibodies mainly work through antibody-dependent cellular cytotoxicity (ADCC) and have demonstrated clinical benefit for children with neuroblastoma. However, high-risk neuroblastoma still has a high recurrence rate. For further improvement in patient outcomes, ways to maximize the cytotoxic effects of anti-GD2 therapies with minimal toxicity are required. Activated invariant natural killer T (iNKT) cells enhance both innate and type I acquired anti-tumor immunity by producing several kinds of cytokines. In this report, we investigated the feasibility of combination therapy using iNKT cells and an anti-GD2 antibody. Although some of the expanded iNKT cells expressed natural killer (NK) cell markers, including FcγR, iNKT cells were not directly associated with ADCC. When co-cultured with activated iNKT cells, granzyme A, granzyme B and interferon gamma (IFNγ) production from NK cells were upregulated, and the cytotoxicity of NK cells treated with anti-GD2 antibodies was increased. Not only cytokines produced by activated iNKT cells, but also NK-NKT cell contact or NK cell-dendritic cell contact contributed to the increase in NK cell cytotoxicity and further IFNγ production by iNKT cells and NK cells. In conclusion, iNKT cell-based immunotherapy could be an appropriate candidate for anti-GD2 antibody therapy for neuroblastoma.

Neuroblastoma (NB), a tumor originating from neural crest cells, is the most common extracranial solid tumor in childhood, and nearly 50% of patients have a high-risk phenotype with a poor long-term survival. More than half of the patients receiving standard therapy, including myeloablative therapy with stem-cell rescue, eventually experience relapse and die from the tumor.(1,2) GD2 is a disialoganglioside expressed on tumors of neuroectodermal origin, such as NB. Because the expression is highly restricted on normal tissues,(3,4) GD2 is a suitable target for immunotherapy. Several clinical trials targeting GD2 have been conducted over the past three decades.(1,3,5,6) These anti-GD2 antibodies mediate tumor cell killing through antibody-dependent cellular cytotoxicity (ADCC), which is mainly induced by FcγR-expressing natural killer (NK) cells or macrophages.(7,8)

Invariant natural killer T (iNKT) cells play an important role in tumor immunity. iNKT cells are activated by a specific glycolipid ligand, α-Galactosylceramide (αGalCer), presented on CD1d molecules. Activated iNKT cells enhance both innate (NK cells) and type I acquired (CTL) immunity.(9) Some reports demonstrate that human iNKT cells activate NK cells through the soluble factors produced by iNKT cells.(10–12) Only one report indicates that ADCC is enhanced by iNKT cells, using anti-MUC1 antibodies;(13) however, the underlying mechanism remains unclear.

In this report, we investigated whether there was any enhancement of ADCC by iNKT cells in humans, and evaluated its mechanism of action using an anti-GD2 antibody and NB tumor cells. We thus explored the feasibility of combination therapy using iNKT cell-based immunotherapy and anti-GD2 antibody therapy in patients with NB.

Material and Methods

Antibodies. Purified mouse anti-human GD2 mAb 14.G2a and purified goat anti-mouse IgG Abs (BD Pharmingen, Franklin Lakes, NJ, USA) were used to detect the GD2 expression by human NB cell lines. The other mAbs used were: FITC-labeled anti-CD3, anti-CD14 (BD Pharmingen), anti-TCR Vα24, PE-labeled anti-TCR Vβ11, (Beckman Coulter, Brea, CA, USA), anti-CD1d, APC-labeled anti-CD3, anti-CD56, PE-Cy7-labeled anti-CD8, anti-CD56, PB-labeled anti-CD4, anti-CD3 (BD Pharmingen), anti-CD16 (BioLegend, San Diego, CA, USA), anti-human TNF-α, IFN-γ, GM-CSF blocking Abs (BioLegend) and anti-human IL-2 blocking Abs (R&D Systems, Minneapolis, MN, USA). The surface phenotypes of PBMC (peripheral blood mononuclear cells) and cultured cells were determined by a FACSCantoII instrument (BD Biosciences, Franklin Lakes, NJ, USA) and were analyzed using the FlowJo software program (Tree Star, Ashland, OR, USA).

Cell lines. The human NB cell lines SK-N-SH,[14] IMR-32[15] and GOTO[16] were obtained from the Japanese Cancer Research Resources Bank. Descriptions of NGP, NMB and NLF cells have been reported previously.[17] LAN-5 cells were kindly provided by Dr R. C. Seegers (Los Angeles, CA, USA).[18] SH-SY5Y cells were kindly provided by Dr A. Nakagawara (Chiba, Japan).[19] Jurkat cells were kindly provided by Dr K. Suzuki.[20] THP-1 was obtained from the ATCC (Manassas, VA, USA).[21] All cell lines were cultured in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FCS.

Cell preparation. PBMC were isolated from healthy donors after obtaining their informed consent, and were separated by density gradient centrifugation using Ficoll-Paque (GE, Fairfield, CT, USA). PBMC were cultured in RPMI 1640 medium containing L-glutamine, penicillin G, streptomycin, 2-mercaptoethanol, HEPES buffer and heat-inactivated 10% FCS for 9–14 days in the presence of 100 U/mL of recombinant human IL-2 (Imunace, Shionogi, Osaka, Japan) and 200 ng/mL of αGalCer (REGiMMUNE, Tokyo, Japan).

The iNKT cells were then isolated using a FITC-labeled anti-Vα24 Ab and anti-FITC microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) by MACS sorting. The selected iNKT cells were cultured with IL-2 for 1 week after separation.

To generate monocyte-derived dendritic cells (moDC), the monocytes were purified using CD14 microbeads (Miltenyi Biotec) and cultured in six-well plates (3×10^5 cells/mL) in complete medium supplemented with 1000 U/mL of GM-CSF (GeneTech, Beijing, China) and 500 U/mL of IL-4 (PeproTech, Rocky Hill, NJ, USA) for 5–7 days. Antigen-presenting cells (APC) were also prepared from PBMC cultured with GM-CSF and IL-2 as previously described with some modification;[22] that is, NK cells were depleted by MACS sorting from PBMC before culturing to avoid carryover of activated NK cells to the cytotoxicity assay. For iNKT cell activation, 200 ng/mL of αGalCer was added the day before mixing together with iNKT cells. The iNKT cells and moDC were mixed together at a ratio of 5:1.

Natural killer cells were separated using an NK cell isolation kit (Miltenyi Biotec). The ratio of NK to NKT cells was also 5:1, unless otherwise stated.

Cytotoxicity assay. For the ^{51}Cr release assay, tumor cells were incubated with 100 µCi sodium chromate (PerkinElmer, Waltham, MA, USA) for 1 h. NK cells with purified anti-GD2 Abs (14.G2a; BD Pharmingen) or isotype controls (BD Pharmingen) were seeded into ^{51}Cr-labeled target cells (1×10^4) at several effector:target (E:T) ratios in triplicate wells. After a 4-h incubation, the radiation dose in the supernatant was measured. The percentage of specific lysis was defined as follows: Cytotoxicity (%) = (cpm experimental − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release) × 100.

The LDH detection assay was performed using an LDH Cytotoxicity Detection Kit (Roche, Mannheim, Germany), according to the manufacturer's recommendations. In brief, NK cells were seeded into target cells (1×10^4) at several E:T ratios in triplicate wells. After a 4-h incubation period, the reaction mixture was added to each well and then was incubated for another 10 min. Stop solution was then added, and the absorbance of the wells was measured by an ELISA reader at 490 nm. The percentage of specific lysis was defined as follows: Cytotoxicity (%) = (((effector-target cell mix − effector cell control) − low control)/(high control − low control)) × 100.

Quantitative RT-PCR. $CD3^-CD56^+$ NK cells were sorted using a FACSAria instrument (BD Biosciences). Total RNA was extracted from NK cells using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) and reverse-transcribed using Superscript II RT and oligo (dT12-18) primers (Invitrogen Life Technologies, Carlsbad, CA, USA).

Quantitative RT-PCR was accomplished with a 7500 Fast Real-Time PCR System using TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA). The probes and primers ordered from Applied Biosystems Custom TaqMan Gene Expression Assays were as follows: *granzyme B* (*GrB*, Hs01554355_m1), *granzyme A* (*GrA*, Hs00989184_m1), *Perforin 1* (Hs00169473_m1) and *interferon gamma* (*IFNγ*, Hs0098291_m1). The relative change in the gene expression was calculated using the ΔCt method using *GAPDH* as a housekeeping gene.

Cytokine measurement. To determine the amount of cytokine secretion, a Bio-Plex assay was performed according to the manufacturer's recommendations using the Bio-Plex 3D Suspension Array System and Bio-Plex Human Cytokine 17-plex Assay (Bio-Rad, Hercules, CA, USA). The cytokines that can be detected using this assay are: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1 (MCAF), MIP-1β and TNF-α. The data were analyzed using the Bio-Plex Manager version 6.1 software program.

Transwell system. Transwell plates with two chambers per well separated by a 400-nm pore membrane (Corning) were used for the transwell assays.

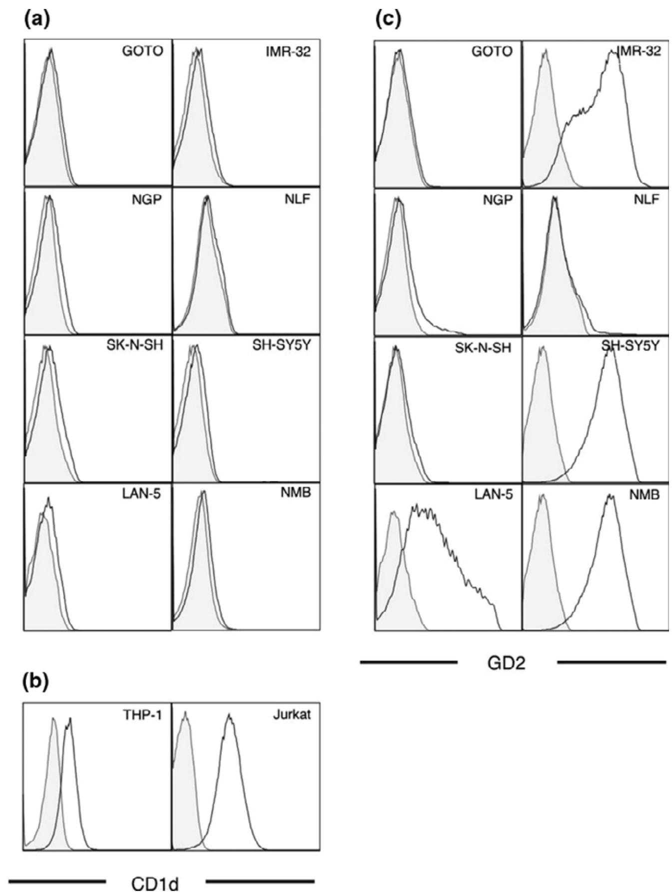

Fig. 1. The expression of CD1d and GD2 on neuroblastoma (NB) cell lines. (a) The surface CD1d expression levels of various NB cell lines are shown. (b) The CD1d expression levels of cell lines from hematological malignancies are shown as positive controls. (c) The GD2 expression levels of various NB cell lines are shown. Shaded histograms represent isotype controls.

Statistical analysis. The data are expressed as the means ± SD. Statistical analyses were performed using Student's *t*-test.

Results

Expression of CD1d and GD2 on neuroblastoma cell lines. As the activation of iNKT cells is CD1d restricted, the CD1d expression of several NB cell lines was first examined by flow cytometry. As previously described,[23] CD1d was expressed on hematopoietic cells, such as Jurkat and THP-1 cells (Fig. 1b), but it was not expressed on the surface of NB cell lines (Fig. 1a).

The GD2 expression levels of NB cell lines were also examined. GD2 was highly expressed on NMB and SH-SY5Y NB cell lines, whereas GOTO, NGP, NLF and SK-N-SH slightly expressed GD2. LAN-5 and IMR-32 showed a heterogeneous expression of GD2 (Fig. 1c).

Invariant natural killer T cells are not directly associated with antibody-dependent cellular cytotoxicity. To clarify whether iNKT cells can recognize anti-GD2 Ab and are directly associated with ADCC, the FcγR of the expanded iNKT cells were analyzed by flow cytometry. Some of the *in vitro*-expanded iNKT cells, mainly CD56^{+} cells, expressed FcγR. The expression level of FcγR on iNKT cells was lower than that of freshly-isolated NK cells that expressed a high intensity of FcγR (Fig. 2a). Because ADCC caused by NK and iNKT cells might be influenced by the GD2 expression level of NB cell lines, an *in vitro* cytotoxicity assay using NK cells against NB cell lines with various GD2 expression levels was performed. NK cells were cultured for 4 h at various E:T ratios with NB cell lines in the presence of anti-GD2 Abs (14.G2a). ADCC mediated by NK cells toward NMB (high GD2 expression, Fig. 1c) was highest and that toward NLF (low GD2 expression) was lowest. The cytotoxicity toward IMR-32, which had a heterogeneous expression of GD2, was not as high as that against NMB (Fig. 2b). iNKT cell-mediated cytotoxicity toward NMB was not increased by the addition of anti-GD2 Ab (Fig. 2c, right), whereas NK cell-mediated cytotoxicity was dramatically increased by the addition of anti-GD2 Ab

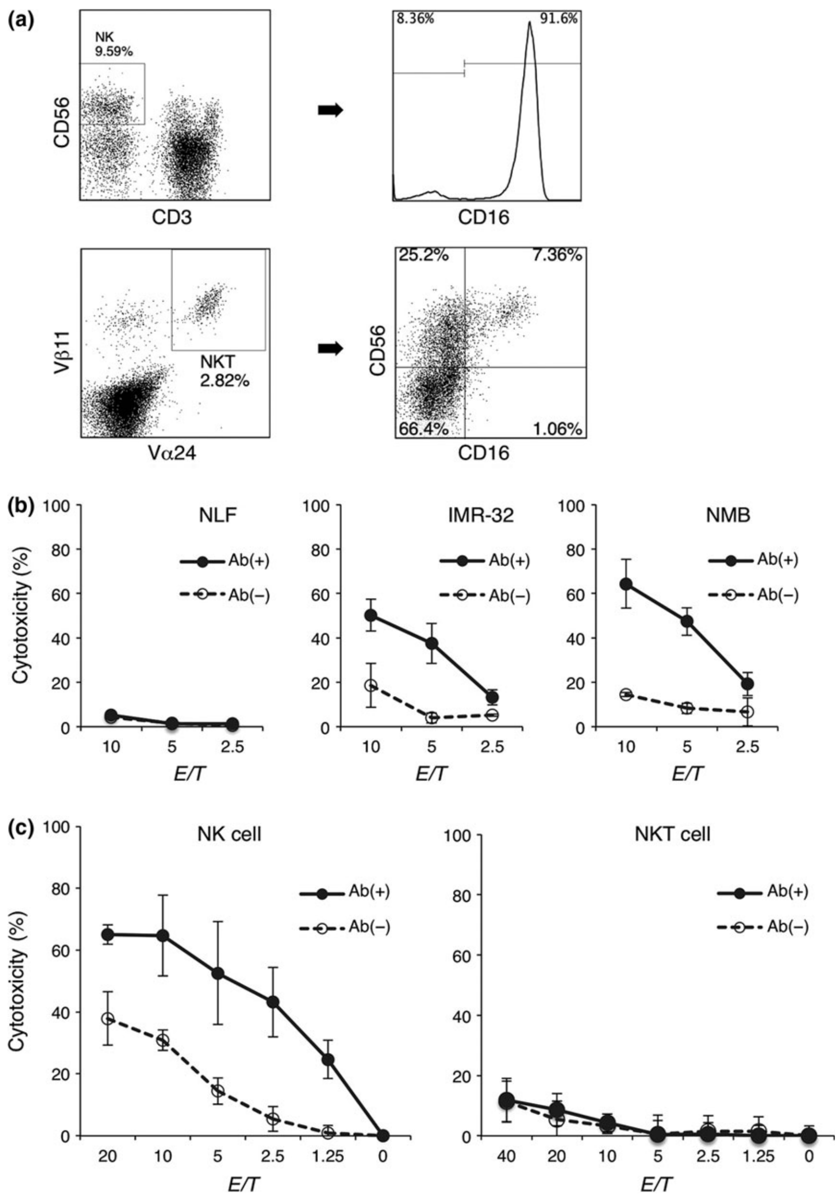

Fig. 2. Natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC) is related to the expression level of the tumor antigen, whereas invariant natural killer T (iNKT) cells themselves do not mediate ADCC. (a) The surface FcγR (CD16) expression of freshly isolated NK cells and expanded iNKT cells is shown. The data are from one representative experiment of a total of five experiments. (b) NK cells were cultured for 4 h at various E:T ratios with NB cell lines with various intensities of the GD2 expression in the presence of anti-GD2 antibodies or isotype controls. (c) NK cells and iNKT cells were cultured for 4 h at various E:T ratios with NMB NB cells.

(Fig. 2c, left). When iNKT cells are activated by APC, it is known that iNKT cells produce a substantial amount of IFNγ. Therefore, iNKT cells were cultured with NB cells in the presence of anti-GD2 Abs and the IFNγ production was measured. There was no increase of IFNγ production by iNKT cells with NB cells and antibodies (data not shown).

Natural killer cell activation by invariant natural killer T cells. It has been reported that the cytokines produced by activated iNKT cells can activate and induce the proliferation of NK cells and enhance tumor immunity;[10,11,24] however, precisely which function of NK cells is enhanced remains unclear. To examine whether the expression of Fas ligand (FasL) or cytotoxic granules by NK cells was enhanced by activated iNKT cells, freshly isolated NK cells were incubated together with or without expanded iNKT cells and moDC without exogenous cytokines. The Fas expression of NB cell lines was examined before this experiment (Fig. 3a).

After 2 days of incubation, the expression of CD69 and FasL on $CD3^{-}CD56^{+}$ NK cells was determined by flow cytometry. Compared with NK cells alone, NK cells incubated with iNKT cells and vehicle-pulsed moDC were partially activated. NK cells incubated with iNKT cells and αGalCer-pulsed moDC were activated more efficiently (Fig. 3b). However, there was no effect on the FasL expression of NK cells by bystander iNKT cells (Fig. 3c).

To examine the expression levels of cytotoxic granules, cells cultured for 24 h as described above were collected, and $CD3^{-}CD56^{+}$ NK cells were purified by flow cytometry. mRNA was extracted from NK cells and quantitative RT-PCR was performed. When NK cells were cultured with iNKT cells and moDC, the expression levels of *GrA*, *GrB* and *Perforin* were significantly increased. These increases in *GrA* and *GrB* were augmented when iNKT cells were activated with αGalCer (Fig. 4a–c). *IFNγ* was also significantly increased by bystander-activated iNKT cells (Fig. 4d).

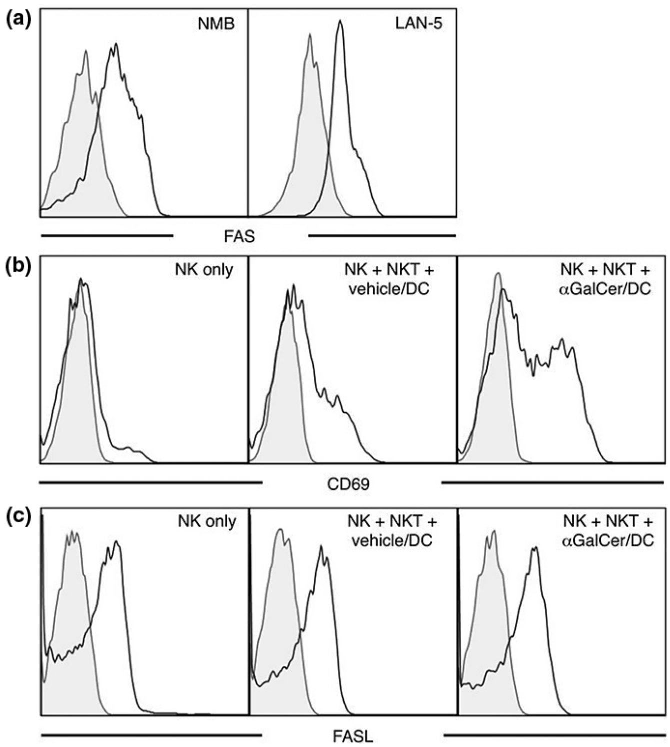

Fig. 3. Activated invariant natural killer T (iNKT) cells have no effect on the natural killer (NK) cell expression of FasL. (a) The Fas expression levels of NMB and LAN-5 NB cells are shown. (b,c) NK cells were cultured with iNKT cells and αGalCer-pulsed or vehicle-pulsed moDC. The surface expression of CD69 (b) and FasL (c) is shown. Representative data from one of three experiments are shown. Shaded histograms represent isotype controls.

Enhancement of natural killer cell-mediated antibody-dependent cellular cytotoxicity by activated invariant natural killer T cells. To measure the actual NK cell cytotoxicity toward NB cells, freshly isolated NK cells were incubated together with or without expanded iNKT cells and moDC without exogenous cytokines for 2 days, and $CD3^{-}CD56^{+}$ NK cells were isolated by cell sorting. Purified NK cells from each condition were mixed together with NMB NB cells (1×10^4 cells/well) in the presence of an anti-GD2 Ab or isotype control, incubated for 4 h, and the LDH release was measured.

Antibody-dependent cellular cytotoxicity of NK cells that were precultured with only iNKT cells (Fig. 5b) or iNKT cells and vehicle-pulsed moDC (Fig. 5c) was slightly increased compared to that of NK cells alone (Fig. 5a). NK cells, precultured with iNKT cells and αGalCer-pulsed moDC, showed higher ADCC compared with those under the other three conditions. Under this condition, the cytotoxicity of the cells without treatment with the anti-GD2 Ab was also increased (Fig. 5d). NK cell cytotoxicity was also increased toward other GD2-positive NB cell lines when NK cells were precultured with iNKT cells and αGalCer-pulsed moDC (Fig. 5e,f).

We previously reported clinical studies using αGalCer-pulsed IL-2/GM-CSF cultured PBMC as APC.[22] ADCC enhancement by iNKT cells stimulated by these APC was also tested. The ADCC of NK cells, precultured with iNKT cells and αGalCer-pulsed IL-2/GM-CSF cultured PBMC, was not as high as the condition using moDC, but much higher than that of NK cells alone (Fig. 5g).

It has been reported that the soluble factors produced by iNKT cells, such as IFNγ and IL-2, can enhance the NK cell function,[10] but it has not been clarified as to whether cell–cell contact between NK cells and iNKT cells or NK cells and moDC also contributes to further NK cell activation. To analyze the role of cell–cell contact, purified NK and iNKT cells with moDC were co-cultured in a Transwell system. NK cells were purified after 48 h, and a cytotoxicity assay was performed using NMB NB cells. As a result, NK cell cytotoxicity was enhanced by soluble factors that were produced from the iNKT cell–moDC interaction (Fig. 6a–c) as expected; however, this was further enhanced by culturing them together with iNKT cells and moDC (Fig. 6d,e).

Using the Transwell system, the cytokine production was also measured by the Bio-Plex System. Using Transwell 12-well plates, NK cells (1.0×10^6 cells/well), iNKT cells (2.0×10^5 cells/well) and moDC (4.0×10^4 cells/well) were seeded with a medium volume of 500 μL in the upper well and 1.5 mL in the lower well. Supernatants were collected from the lower wells after a 2-day incubation period. Cytokines that are known to induce NK cell activation, such as IFNγ, IL-2 and TNFα, were mainly produced by iNKT cell–moDC contact and enhanced by αGalCer presentation (Fig. 6f). The same result could be observed for GM-CSF, Th2 type cytokines and CCL4 (MIP-1β, data not shown). Interestingly, cell–cell contact between NK cells and iNKT cells or moDC dramatically increased the IFNγ production (Fig. 6f).

To assess the responsible cytokines for ADCC enhancement, neutralization of IFNγ, IL-2, TNFα and GM-CSF was

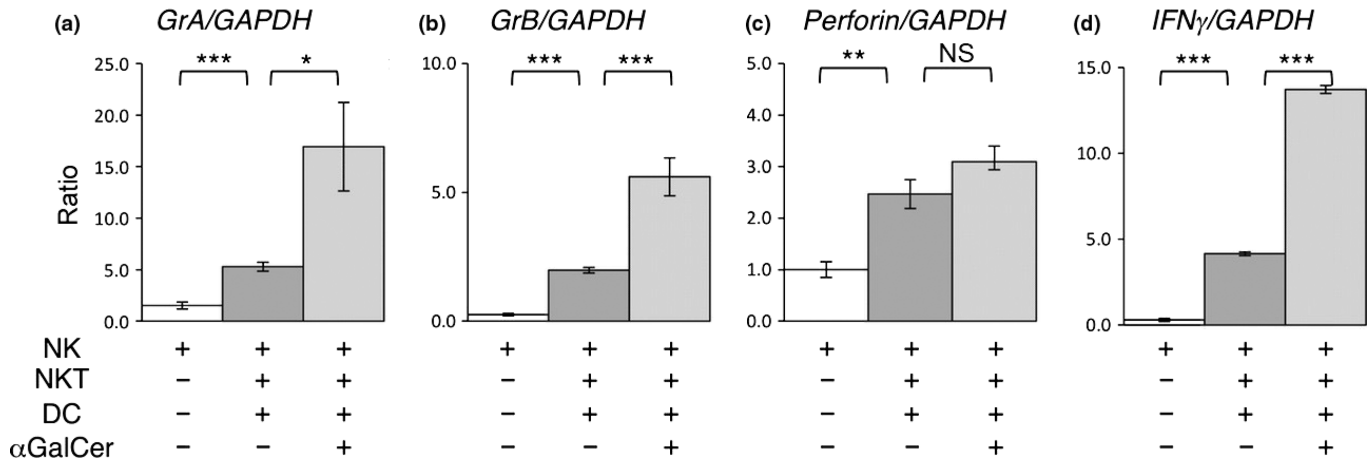

Fig. 4. The production of cytotoxic granules and interferon gamma (IFNγ) by natural killer (NK) cells were enhanced by invariant natural killer T (iNKT) cell activation. NK cells (1×10^6) were cultured for 24 h with iNKT cells (5×10^5) and αGalCer-pulsed or vehicle-pulsed moDC (1×10^5). RNA was isolated from NK cells, and quantitative RT-PCR was performed to evaluate the mRNA expression levels of *GrA* (a), *GrB* (b), *Perforin* (c) and *IFN*γ (d) relative to that of *GAPDH*. Representative data from one of three independent experiments are shown. The statistical analysis was performed using Student's *t*-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. NS, not significant.

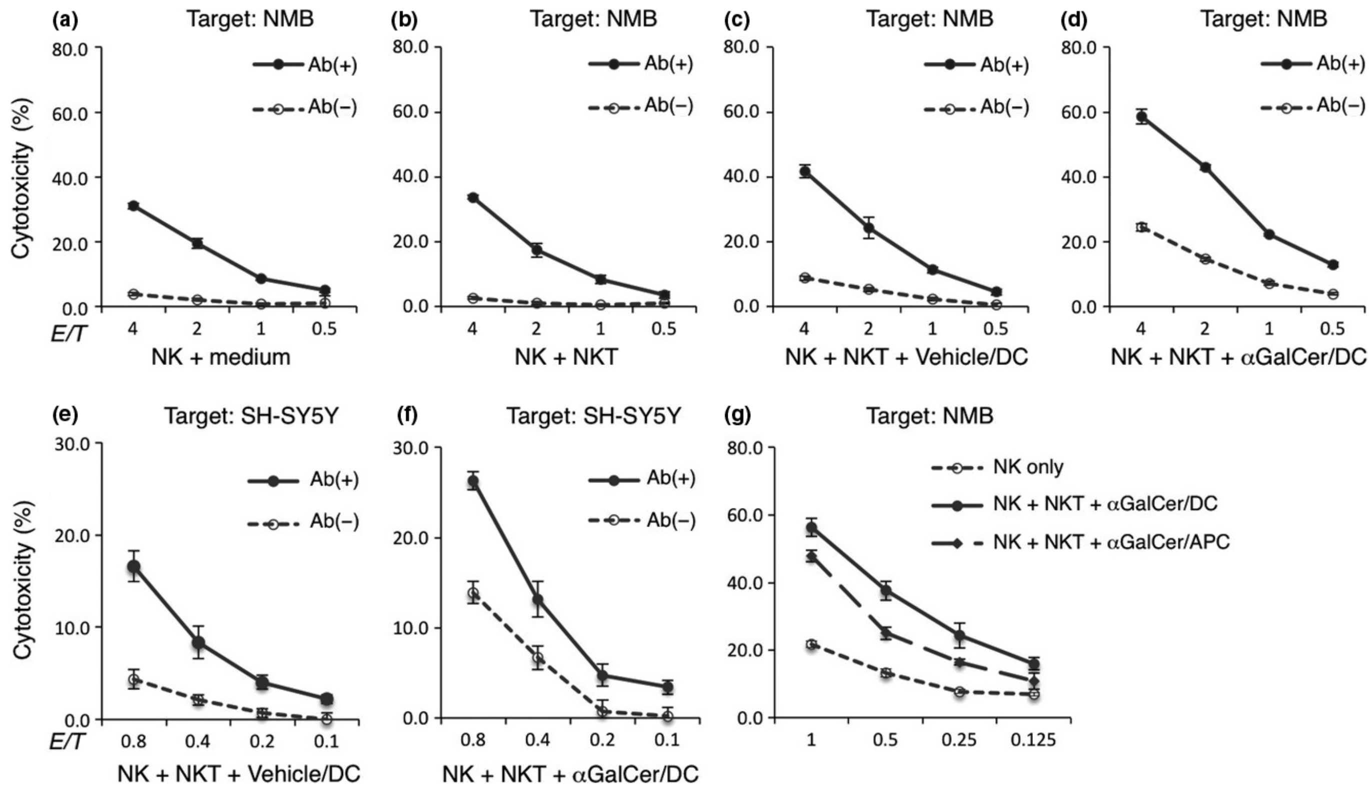

Fig. 5. Antibody-dependent cellular cytotoxicity (ADCC) of natural killer (NK) cells was enhanced by activated invariant natural killer T (iNKT) cells. NK cells were cultured with iNKT cells and αGalCer-pulsed or vehicle-pulsed moDC without exogenous cytokines for 2 days. $CD3^-CD56^+$ NK cells were then purified by MACS and mixed together with NMB NB cells with an anti-GD2 antibody or isotype control. The cytotoxicity was measured by the LDH release. (a) NK cells only. (b) NK cells cultured with iNKT cells without moDC. (c) NK cells cultured with iNKT cells with vehicle-pulsed moDC. (d) NK cells cultured with iNKT cells with αGalCer-pulsed moDC. The cytotoxicity toward SH-SY5Y NB cells was also measured. (e) NK cells cultured with iNKT cells with vehicle-pulsed moDC. (f) NK cells cultured with iNKT cells with αGalCer-pulsed moDC. In another experiment, NK cells were cultured with iNKT cells and αGalCer-pulsed IL-2/GM-CSF cultured PBMC. (g) The cytotoxicity of purified NK cells with anti-GD2 antibody toward NMB NB cells was measured and compared with the condition above. All measurements were performed in triplicate. Representative data from one of three independent experiments are shown.

performed. Using Transwell plates, NK cells were seeded in the lower well, iNKT cells and moDC were seeded in the upper well, and neutralizing mAbs or isotype controls were also added. NK cells were collected after 48 h, and cytotoxicity assays were performed using NMB NB cells with anti-GD2 Abs. None of the neutralizing mAbs abrogated the cytotoxicity of NK cells activated by iNKT cells and αGalCer-pulsed moDC (Fig. 7a–d).

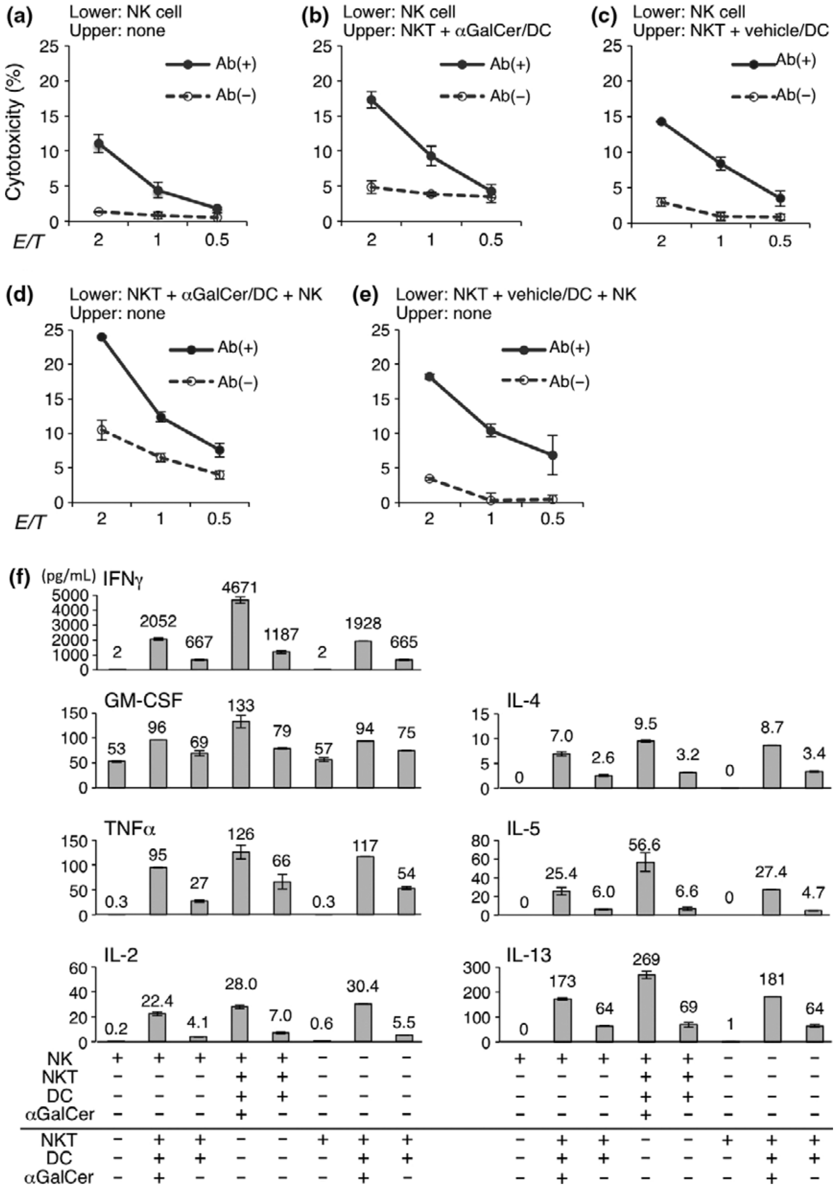

Fig. 6. Not only soluble factors produced by invariant natural killer T (iNKT) cell-moDC contact, but also the cell–cell contact of natural killer (NK) cells, iNKT cells and DC is important for the enhancement of ADCC. NK cell culture was performed by using a Transwell system. Purified NK cells were cultured in the lower wells, and iNKT cells with αGalCer-pulsed or vehicle-pulsed moDC were added in the upper wells (b,c) or lower wells (d,e). Two days later, the cells in the lower wells were collected. $CD3^{-}CD56^{+}$ NK cell purification was performed by MACS, and the purified cells were mixed together with NMB NB cells with an anti-GD2 antibody or isotype control. The cytotoxicity was measured by the LDH release assay. Measurements were performed in triplicate. In another experiment, NK cells, iNKT cells and moDC were cultured in Transwell plates for two days, and the supernatants in the lower wells were collected. A cytokine analysis was performed using the Bio-Plex assay (f). Only 7 representative cytokines out of a total of 17 are shown in the figure.

In Figure 4, we showed the expression level of *IFNγ* of NK cells to increase when activated by iNKT cells. To investigate the importance of IFNγ in ADCC, neutralization of IFNγ from NK cells was also performed. NK cells were precultured with iNKT cells and αGalCer-pulsed moDC for 2 days, and then purified again for the cytotoxicity assays. The cytotoxicity assays were performed under anti-GD2 Abs with or without anti-IFNγ Abs. With anti-IFNγ Abs, ADCC was found to slightly decrease (Fig. 7e).

Discussion

Early clinical studies with murine anti-GD2 mAbs, 3F8 and 14.G2a, showed the safety of the treatment and some limited antitumor effects in patients with refractory or metastatic NB.[3,5,25] The anti-GD2 Ch14.18 chimeric mAb, which was developed from 14.G2a to diminish its immunogenicity, was combined with IL-2 and GM-CSF, which significantly improved the outcome in high-risk NB patients.[1] Combining the monoclonal antibody, 3F8, with GM-CSF and 13-*cis*-retinoic acid also showed promising effects.[6] Cytokines are used to augment ADCC, which is the main mechanism by which anti-GD2 Ab stimulates effector cells, such as NK cells, macrophages and neutrophils. However, several toxic effects, such as capillary leak syndrome and hypersensitivity reactions, were reported,[1] and the effects of the antibodies were limited to controlling minimal residual disease. Therefore, it is considered necessary to seek better methods to maximize the cytotoxic effects on the tumor and to minimize the toxic effects of anti-GD2 therapies. The hu14.18-IL2 fusion protein consists of the humanized 14.18 anti-GD2 mAb linked to IL-2, and it is one of the potential approaches to address these challenges.[26] Other strategies involve β-glucan, which enhances the function of human NK cells,[27] and fenretinide, a synthetic derivative of vitamin A, which enhances the sensitization of NB cells toward anti-GD2 antibodies.[28]

Killer cell immunoglobulin-like receptor (KIR)-KIR-ligand mismatch has been reported to be a predictor of a good outcome, thus suggesting that NK cells play an important role in the effects of anti-GD2 antibodies.[6,29] In this report, we

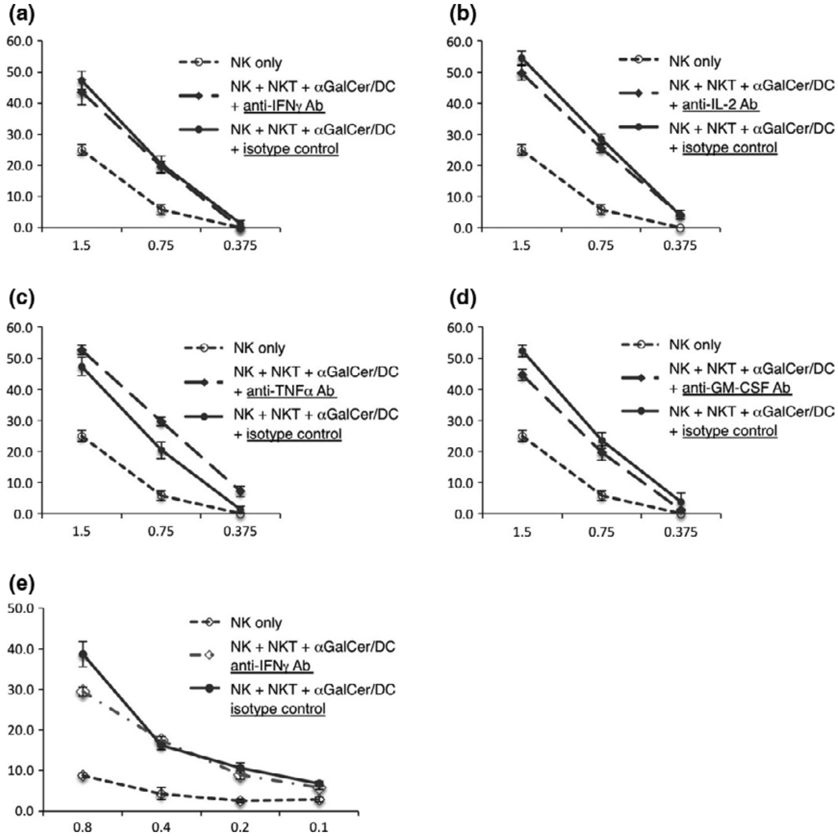

Fig. 7. Blocking antibodies against IFNγ, IL-2, TNFα and GM-CSF did not abrogate the ADCC of activated NK cells. Using Transwell plates, NK cells were seeded in the lower wells, and invariant natural killer T (iNKT) cells and moDC were seeded in the upper wells. Neutralizing mAbs against IFNγ (a), IL-2 (b), TNFα (c) and GM-CSF (d) or isotype controls were also added. Two days later, NK cells were collected from the lower wells and cytotoxicity assays were performed using NMB NB cells with anti-GD2 Abs. (e) NK cells were precultured with iNKT cells and αGalCer-pulsed moDC for two days, and $CD3^{-}CD56^{+}$ NK cells were then purified by MACS and mixed together with NMB NB cells. The cytotoxicity assays were performed under anti-GD2 Abs with or without anti-IFNγ Abs.

revealed that iNKT cells can effectively enhance the cytotoxicity of NK cells and can enhance ADCC toward GD2-expressing tumors, and therefore, may be good candidates for combination therapy with anti-GD2 antibodies.

Activated human iNKT cells show a strong anti-tumor effect against various malignant tumors[30,31] and produce high amounts of cytokines, such as IFNγ and IL-4, and activate other anti-tumor effector cells.[32,33] In murine models, endogenous iNKT cells showed anti-tumor responses following systemic treatment with αGalCer or αGalCer-pulsed DC as a result of NK cell trans-activation by iNKT cells.[33,34] Patients with some malignant diseases show a decreased number or functionally-impaired iNKT cells in human PBMC.[35] It was also reported that NB patients whose tumors have iNKT cell infiltration demonstrated a trend toward better survival, corresponding with MYCN non-amplification.[36] Therefore, the expansion and/or activation of iNKT cells in patients with malignant diseases would be considered to be meaningful therapy.

The CD1d expression has been reported in several types of lymphoma and leukemia, suggesting that these malignancies could be targeted for direct iNKT cell cytotoxicity.[23] For CD1d-negative tumors, such as NB and other solid tumors, indirect iNKT cell cytotoxicity, including activation of other anti-tumor effector cells, could be expected using *ex vivo*-activated iNKT cells and/or αGalCer-pulsed APC. A series of clinical studies of αGalCer-presenting APC for non-small cell lung cancer and head and neck squamous cell carcinoma have been conducted without inducing severe adverse events.[35,37–41] In these reports, endogenous iNKT cells, stimulated by αGalCer-pulsed APC, expanded and produced IFNγ preferentially, and, in turn, activated NK cells to produce IFNγ. These responses might have resulted in the suppression of tumor growth and prolonged survival.[42]

Considering the combination of iNKT cells and tumor antigen-specific antibodies, the direct effects of iNKT cells on tumor cells with antibodies cannot be ignored. As we herein have reported, some of the expanded iNKT cells expressed FcγR, suggesting the possibility of antibody recognition by iNKT cells. However, neither the augmentation of iNKT cell cytotoxicity nor the IFNγ production induced by adding antibodies was observed (Fig. 2c). Regarding the role of iNKT cells in modulating the NK cell function, the cytotoxicity of NK cells co-cultured with activated iNKT cells remained increased under both conditions with and without αGalCer-pulsed moDC (Figs 5,6). Previous reports suggested the importance of cytokines that could activate NK cells produced by iNKT cells, such as IFNγ, IL-2 and TNFα;[10,11] however, in our experiments, blocking these cytokines did not diminish NK cell cytotoxicity. Blocking only one cytokine might, therefore, not be sufficient, or there may be other cytokine candidates for NK cell activation that are produced by iNKT cells, such as IL-15 or IL-21.[43]

Interferon gamma is crucial for anti-tumor immunity because it is involved in the activation of cytotoxic cells and the suppression of tumor growth.[44] According to the results of the Transwell assay, IFNγ was secreted by both iNKT and NK cells, particularly when NK cells, iNKT cells and moDC were co-cultured in the same well. Furthermore, under this condition, our data showed increased GM-CSF secretion by iNKT cells. GM-CSF is a cytokine that can enhance the function or proliferation of macrophages and granulocytes, taking part in ADCC, and has been used with anti-GD2 antibodies in clinical trials.[1,6] These findings suggest that activated iNKT cells may contribute to ADCC not only by NK cell activation, but also by enhancing macrophage or granulocyte-induced tumor killing.

In conclusion, activated iNKT cells enhanced NK cell-induced ADCC, mainly via the upregulation of GrA and GrB. The IFNγ secretion by NK cells and iNKT cells was synergistically increased, suggesting that this led to further anti-tumor effects. Both iNKT cell-based therapy and anti-GD2 antibody therapy are being investigated in ongoing clinical trials. In addition, there are several mAb therapies targeting other cancers. Clinically, iNKT cells or anti-tumor mAb therapy alone have not resulted in sufficient anti-tumor effects; therefore, combination therapy may be more efficacious. Although further confirmation of our results is needed, especially using *in vivo* models, the feasibility of combination immunotherapy using an anti-GD2 antibody and iNKT cells in patients with NB has been suggested.

Acknowledgments

This work was supported by a Grant-in-Aid for Young Scientists (B) from Japan Society for the Promotion of Science (No. 25861664).

Disclosure Statement

The authors have no conflict of interest to declare.

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