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Diminished cytolytic activity of γδ T cells with reduced DNAM-1 expression in neuroblastoma patients

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ABSTRACT

Neuroblastoma is one of the children's malignant tumors with poor prognosis, as well as high recurrence and metastasis rates after surgical removal and chemotherapy. γδ T-cell based immunotherapy receives increasing attention thanks to the strong cytolytic activity to tumor cells. Our previous data revealed a significant increase in circulating γδ T-cell frequency in NB patients. In the present study, we found that beside a reduction of IFN-γ in serum of NB patients, DNAM-1 expression decreased in both circulating and PAM-expanded NB γδ T cells. Upon PAM stimulation, NB γδ T cells showed a reduced level of cell proliferation. In addition, the cytolytic activity of NB γδ T cells to NB cell lines was proved to be attenuated in a co-culture system. The fact that DNAM-1 neutralizing antibody abolished the tumor cell killing accentuates the indispensable role of DNAM-1 molecule in γδ T-cell cytolytic function.

1. Introduction

Neuroblastoma (NB) is an embryonal tumor of the sympathetic nervous system, arising during fetal or early post-natal life from sympathetic cells originated from the neural crest. It is the most common solid extracranial malignancy of childhood. NB represents about 8% of all malignancies diagnosed in patients younger than 15 years of age but accounts for 15% of pediatric cancer deaths [1]. Moreover, NB tumor progresses rapidly and is prone to early distant metastasis [2]. Despite significant progress in treatment of NB, this disease is usually associated with poor outcome, resulting in a long-term survival rate $< 50\%$ [3]. High recurrence, metastasis and mortality rates discourage the efficacy of the NB treatment relying merely on traditional surgical removal and chemotherapy regimen [4]. While GD2 monoclonal antibody [3], adoptive T-cell transfer are implemented experimentally and clinically [5,6], immunotherapy based on γδ T-cell emerges as one of the new approaches for NB tumor therapy in recent years.

γδ T-cell is a unique and conserved population of lymphocytes that have been the subject with growing interest owing to their essential role mediating various types of immune response and immunopathogenesis [7]. Human γδ T cells account for about 5–10% of $CD3^+$ T cells in the peripheral blood, but constitute a major T-cell subset in other anatomic locations, such as the intestinal lining and mucosal membranes. In contrast to αβ T cells, γδ T cells has restricted TCR repertoire. In human Vγ9Vδ2 T cells account for 50–90% of total γδ T cells [8]. γδ T cells show multiple effects in immune system such as antigen presentation to αβ T cells, defense against infectious pathogens and interactions with B cells or dendritic cells. Especially, γδ T cells exert their function mainly by producing various cytokines and chemokines and mediating cytotoxicity to target cells.

It was demonstrated that γδ T cells could induce effective cytotoxic responses against multiple types of tumors *in vitro*, including renal cell

Abbreviations: NB, neuroblastoma; DNAM-1, DNAX accessory molecule-1; PAM, pamidronate; PVR, poliovirus receptor; AV, Annexin V; CFSE, carboxyfluorescein succinimidyl ester; CDRs, complementarity determining regions; MHC, major histocompatibility complex; MICA/B, MHC class I-related molecule A/B; ULBP, UL16-binding proteins; NKR, NK activating receptor; TIGIT, T cell immunoreceptor with Ig and ITIM domains

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Table 1
Patient information.

Group	Gender	Age	INRG[a] Risk
Healthy controls $n = 35$	Boy (57%) Girl (43%)	2.8 ± 0.5	/
NB patients $n = 25$	Boy (48%) Girl (52%)	3.3 ± 0.3	Low/Intermediate risk (52%) High risk (48%)

[a] INRG, The International Neuroblastoma Risk Group.

carcinoma [9], multiple myeloma [10] and ovarian epithelial carcinoma [11]. Furthermore, both adoptive transfer of *in vitro* expanded γδ T cells or stimulation of γδ T cells *in vivo* were explored clinically for tumors treatment [12–15]. In a trial of adoptive transfer of *in vitro* expanded γδ T cells to non-small cell lung cancer patients, results showed that the peripheral γδ T cell numbers gradually increased with the number of infusion times [16]. In addition, there was a statistically significant correlation between clinical outcome and peripheral Vγ9Vδ2 T cell numbers in advanced breast cancer patients treated with phosphoantigen and IL-2 administration [17]. Similarly, the cytolytic activity of γδ T cells against NB tumor is under active investigation in experimental and clinical settings. NB cell line, as a target, was effectively lysed by *in vitro* expanded γδ T cells in a co-culture system [5,18]. In addition, a phase I study revealed that zoledronate, an γδ T expander, being administrated with IL-2 to NB patients resulted in a good tolerance in NB patients accompanied with an increase number and survival rate of γδ T cells [19].

DNAX accessory molecule-1 (DNAM-1), also known as CD226, is one of NK activating receptors (NKR) expressed by $CD8^+$ T cells, NK cells, γδ T cells and some myeloid cells. DNAM-1 recognizes Nectin-2 (CD112) and poliovirus receptor (PVR, CD155) expressed on a broad range of tumor cells [20]. Binding of DNAM-1 molecule on NK cells to PVR on neuroblastoma cells led to effective tumor killing [21]. It has been well documented that, similar to NK cells, cytotoxic activity of γδ T cells can be rapidly triggered by binding of DNAM-1 to its ligands Nectin-2 or PVR on target tumor cells [22,23].

Our previous study has shown that in NB patients there was an augmentation in circulating γδ T cells [24]. In the present study, we aimed to answer the question whether γδ T cells in NB patients have any functional impairment. Though Luminex cytokine quantification, expression of multiple cytokines mediating tumor elimination, such as IFN-γ, were found to decrease in NB serum. Furthermore, both freshly isolated circulating γδ T cells and pamidronate (PAM)-expanded γδ T cells exhibited a diminished expressions of DNAM-1 in NB patients. In a co-culture system, NB γδ T cells exhibited a reduced killing ability against NB cell lines. To be noted, the specific lysis of γδ T cells to NB cells was virtually abolished by DNAM-1 neutralizing antibody. Results from our study suggest that the reduction of DNAM-1 expression in NB patient is likely responsible for poor cytotoxicity of γδ T cells to tumor.

2. Materials and methods

2.1. Subjects and sample collection

A total of 25 patients (12 boys, 13 girls; mean age 3.3 ± 0.3 years) with NB and 35 healthy children (20 boys, 15 girls; mean age 2.8 ± 0.5 years) under regular physical examination were enrolled between January 2017 and July 2018 from Beijing Children's Hospital (Table 1). The study was approved by the Medical Ethics Committee of Beijing Children's Hospital, Capital Medical University. Written consents were provided by all participants and their parents or legal guardians. Peripheral blood samples from healthy children and NB patients were collected in BD Vacutainer™ plastic blood collection tubes with EDTA K2 as anticoagulant at the onset of the disease. Serum samples were collected in tubes without anticoagulant by a centrifugation at 600*g* for 5 min and the aqueous phase were taken.

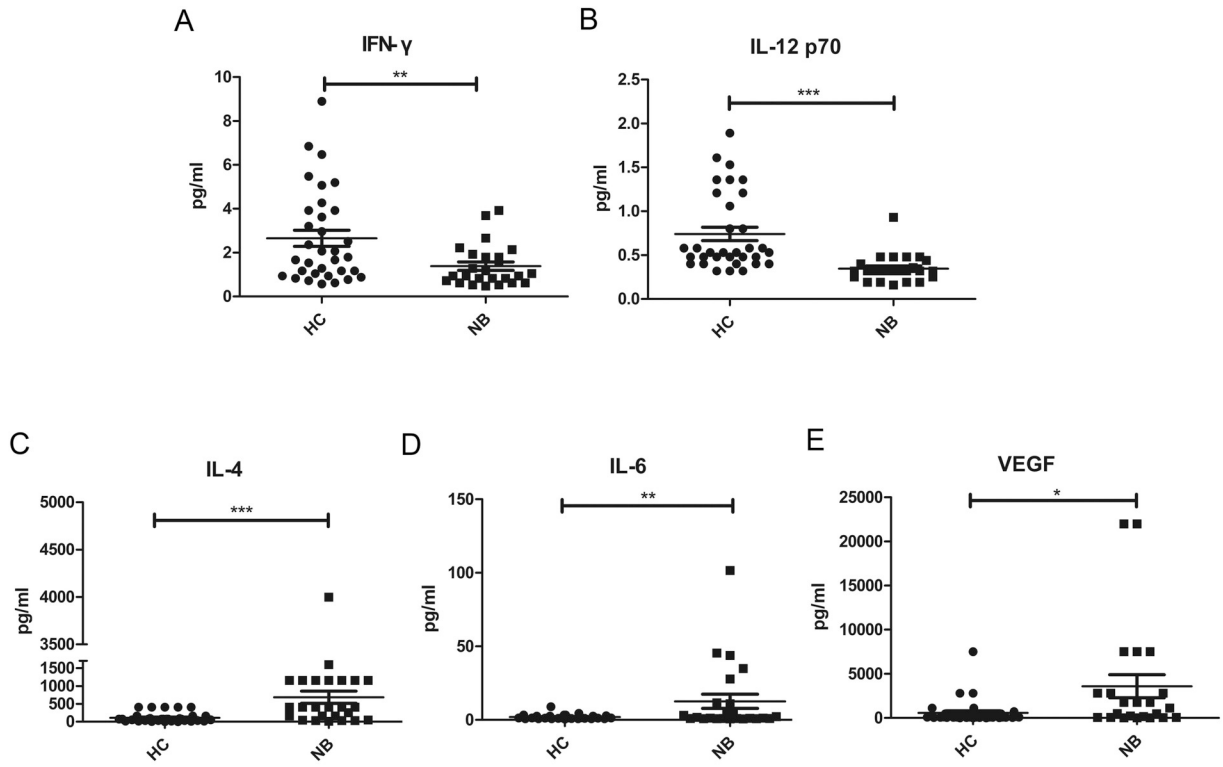

Fig. 1. The cytokine expression in serum of NB patients and healthy controls. Serum samples from NB patients and healthy controls were collected and the concentrations of multiple cytokines were detected using a Luminex200 platform.

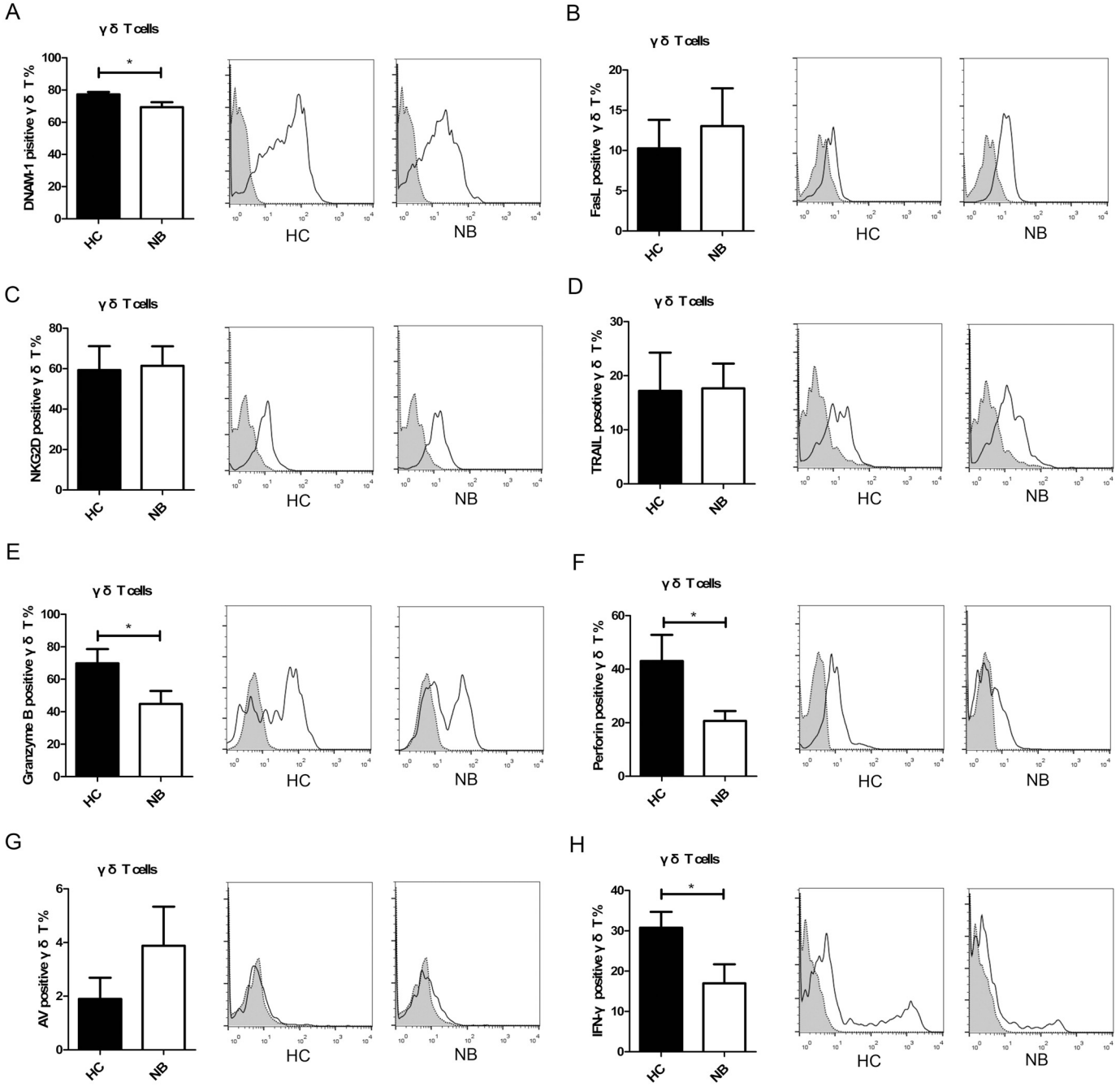

Fig. 2. The cytolytic molecule expression of circulating γδ T cells. PBMC were isolated from peripheral blood of NB patients and healthy controls and were analyzed by BD LSRFortessa flow cytometer. (A) The expression of DNAM-1 in γδ T cells. (B) The expression of FasL in γδ T cells. (C) The expression of NKG2D in γδ T cells. (D) The expression of TRAIL in γδ T cells. (E) The expression of gramzyme B in γδ T cells. (F) The expression of perforin in γδ T cells. (G) The apoptosis of γδ T cells. (H) γδ T cells in PBMC were stimulated with PMA and ionomycin and then the expression of IFN-γ was detected.

2.2. PBMC isolation

Freshly isolated EDTA anticoagulated blood was diluted with PBS solution and layered carefully on Ficoll-Hypaque density gradients. After centrifuged at 1000*g* for 20 min at room temperature (RT), interphase cell layer was carefully transferred into a 15-ml tube. The 15-ml tube was then filled with 10 ml PBS, and centrifuged at 600*g* for 5 min. The cell pellet was re-suspended and viability of isolated PBMC was determined by trypan blue exclusion staining.

2.3. Cell lines

Neuroblastoma cell lines SH-SY5Y (CRL-2266), SK-N-BE2 (CRL-2271) and IMR-32 (CCL-127) purchased from American Type Culture Collection (Manassas, VA, USA) were used in this study. SH-SY5Y and SK-N-BE2, immortalized cell lines derived from neuroblastoma metastatic bone marrow, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (fetal bovine serum, Gibco, Invitrogen, Carlsbad, CA, USA). IMR-32, an immortalized cell line generated from neuroblastoma metastatic abdominal mass, was cultured in α-MEM (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS. All cells were cultured

Table 2
The expression of cytolytic molecules and cytokines in γδ T cells.

	Circulating γδ T cells (%)		*P* value	PAM-expanded γδ T cells (%)		P value
	HC	NB		HC	NB	
DNAM-1	77.28 ± 1.47	69.40 ± 3.09	0.019	84.81 ± 2.27	70.59 ± 4.90	0.009
FasL	10.25 ± 3.56	13.04 ± 4.70	0.639	21.08 ± 4.10	11.24 ± 3.92	0.114
NKG2D	59.27 ± 11.93	61.40 ± 9.71	0.891	63.21 ± 4.43	59.43 ± 3.90	0.528
TRAIL	17.20 ± 7.08	17.67 ± 4.58	0.957	74.48 ± 7.38	66.42 ± 7.96	0.468
Granzyme B	69.82 ± 8.78	44.81 ± 8.01	0.044	91.82 ± 2.80	81.08 ± 4.36	0.039
Perforin	43.02 ± 9.79	20.68 ± 3.69	0.047	75.24 ± 10.40	69.90 ± 12.17	0.743
IFN-γ	30.77 ± 3.96	16.98 ± 4.73	0.032	78.71 ± 4.14	55.27 ± 7.52	0.006
Annexin V	1.89 ± 0.79	3.88 ± 1.46	0.225	/	/	/

at 37 °C humidified cell incubator with 5% of CO_2.

2.4. Measurement of serum cytokines

The Human Cytokine/Chemokine Panel MILLIPLEX® MAP kits (Cat. No. HCYTOMAG-60K-13: G-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12(p70), IL-15, IL-17, VEGF, FGF; and Cat. No. HCYP2MAG-62K-04: CXCL12, IL-23, IL-33, TGF-β1) (Merck Millipore, Darmstadt, Germany) were used to measure the serum concentration of multiple cytokines by a Luminex200 platform (Merck Millipore, Darmstadt, Germany). In brief, the plate was incubated with 200 µl wash buffer for 10 min (shaking at RT, 600–800 rpm). The washing buffer was removed subsequently by vacuum filtration and 25 µl of assay buffer (1 × PBS containing 1% bovine serum albumin, 0.05% Tween-20, 0.5 M NaCl, 0.05% sodium azide) was added to the 96-well plate. Next, 25 µl of each standard with a range 0.18 pg/ml to 32,500 pg/ml (depending on the type of cytokine) together with the controls were added to according wells. Following that, 25 µl of assay buffer was added to background wells and 25 µl of sample was added to sample wells. The plate was sealed and incubated at 4 °C (shaking) overnight. The next day, the plate was washed twice with 200 µl wash buffer before 1 h incubation at room temperature in the presence of 25 µl detection antibodies (shaking). Next, each well were incubated with 25 µl Streptavidin-Phycoerythin for 30 min (shaking at RT). After two wash steps, 150 µl sheath fluid was added and the data were collected by the Luminex200 platform. All samples were measured in duplicate.

2.5. Flow cytometry

Freshly isolated PBMC were re-suspended in PBS, containing 5% FBS. For surface marker labeling of γδ T cells, 1×10^6 cells/tube were incubated with different combinations of the following antibodies: TCRγδ-PE, CD3-APC, Annexin V (AV)-APC, CD226 (DNAM-1)-BV785, CD314 (NKG2D)-BV605, CD178 (Fas-L)-BV421, TRAIL-APC. For cytokine and intracellular marker labeling of γδ T cells, the following antibodies were used: IFN-γ-BV650, granzyme B-PE/Cy7, perforin-BV510 (Supplementary Table 1). For surface marker labeling of NB cells, the following antibodies were used: MICA/B-FITC, Nectin-2-PE, PVR-APC, ULBP-1-PerCP, ULBP-2/5/6-PE, ULBP-3-APC (Supplementary Table 1). Isotype-matched controls were included for each labeling. All antibodies were from BioLegend (San Diego, CA, USA) and optimal titer of each antibody was determined before use. Surface marker labeling was performed by antibody incubation for 30 min at 4 °C in dark and washed with PBS. For cytokine detection, γδ T cells were treated with 50 ng/ml PMA, 1 ng/ml ionomycin and GolgiStop protein transport inhibitor (BD Biosciences, San Jose, CA, USA) for 5 h. For cytokine and intracellular marker labeling, cells were permeabilized using Fixation/Permeabilization Solution Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol after surface marker labeling. Finally, cells were re-suspended in 500 µl PBS subjected to events acquisition by BD LSRFortessa flow cytometer (BD Biosciences, San Jose, CA, USA). Analysis of cytolytic molecule, cytokine expression as well as cell apoptosis, were performed by gating TCRγδ$^+$ cells using a Flowjo software (Flowjo LLC, Ashland, Oregon).

2.6. γδ T cell culture

PBMC were cultured in 10% FBS RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) with 10 µg/ml of PAM (Sigma-Aldrich, St Louis, USA) at 37 °C humidified cell incubator with 5% CO_2. Recombinant human IL-2 (R&D System, Minneapolis, MN, USA) was added to a final concentration of 25 ng/ml every 3 days and fresh culture medium was replenished. After 14-day culture, the purity of γδ T cells was determined by flow cytometry with PE-labeled anti-TCRγδ antibody (BioLegend, San Diego, USA). γδ T cell expansion was considered as successful when the TCRγδ$^+$ population represents at least 90% of total cultured cells.

2.7. Carboxyfluorescein succinimidyl ester (CFSE) proliferation assay

After 7-day culture, γδ T cells were re-suspended in 2 ml RPMI 1640 medium and incubated with 2 µM CFSE (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 10 min. The reaction was terminated by cold medium with 10% FBS and placed on ice for 5 min. The stained γδ T cells were washed and re-suspended with RPMI 1640 medium plus 10% FBS. γδ T cells were then seeded (1×10^6/ml) in a 96-well plate in the presence of 25 ng/ml IL-2 and cultured for another 4 days. Cells were analyzed by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). CFSE fluorescence dilution events were calculated by Flowjo software.

2.8. Cytotoxicity assays

γδ T cell-mediated cytotoxicity against NB cell lines was assessed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. NB cell lines SH-SY5Y, SK-N-BE2 and IMR-32 were used as target cells. Briefly, before co-culture with target tumor cell line, γδ T cells were prepared in a round-bottom 96-well culture plate through *in vitro* PAM-stimulation in the presence of IL-2 starting from NB patients' and healthy children's PBMC. Wells seeded only with γδ T cells (4×10^5 or 8×10^5/well) served as the spontaneous LDH release control for effector cells and wells seeded only with NB cells (2×10^4/well) served as the spontaneous LDH release control for target cells; the experimental wells were seeded with γδ T cells and NB cells at a ratio of 20:1 or 40:1. In the blocking assays, 10 µg/ml DNAM-1 neutralizing antibody (abcam, Cambridge, MA, USA) was added in the culture, and mouse isotype IgG was added in juxtaposed wells as a control to validate the specificity of the blocking. Cells were centrifuged at 250 × *g* for 4 min at 20 °C after incubation at 37 °C for 7 h. The lysis solution was added to the target cell control wells 45 min prior to supernatant harvest for determination of maximal LDH release. Then, a total of 50 µl supernatant from each well was transferred to a flat-bottom 96-well plate pre-loaded with 50 µl/well reconstituted substrate mix. Following incubation at room temperature in dark for 30 min, 50 µl stop solution

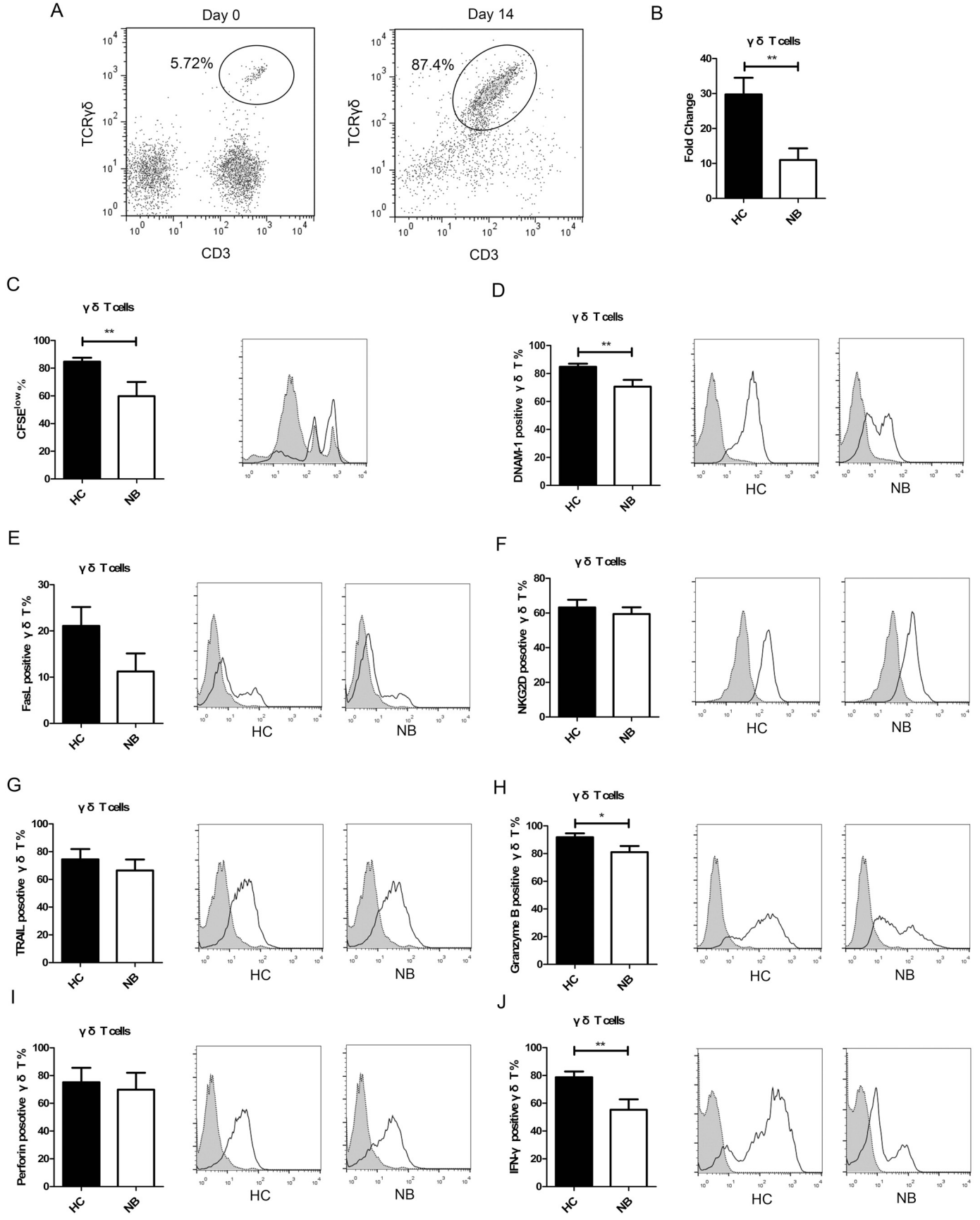

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Fig. 3. The proliferation capacity and cytolytic molecule expression of PAM-expanded γδ T cells. (A) γδ T cells were stimulated with PAM and expanded *in vitro* for 14 days. (B) The cell numbers of PBMC and PAM-expanded γδ T cells were counted and fold change were calculated. (C) The CFSE proliferation assays were performed during *in vitro* culture (from day-7 to day-11) and cells were analyzed by BD FACSCalibur flow cytometer (D) The expression of DNAM-1 in PAM-expanded γδ T cells. (E) The expression of FasL in PAM-expanded γδ T cells. (F) The expression of NKG2D in PAM-expanded γδ T cells. (G) The expression of TRAIL in PAM-expanded γδ T cells. (H) The expression of granzyme B in PAM-expanded γδ T cells. (I) The expression of perforin in PAM-expanded γδ T cells. (J) PAM-expanded γδ T cells were stimulated with PMA and ionomycin and then the expression of IFN-γ was detected.

was added and absorbance at 490 nm was read by TriStar^2 LB 942 Multimode Reader (Berthold, Germany). The cytotoxicity of effector γδ T cells to target tumor cells was calculated as follows: [(Experimental-effector spontaneous-target spontaneous)/(target maximal-target spontaneous)] × 100.

2.9. Statistical analysis

The data were expressed as the mean ± SEM. Statistical analyses were performed using two-tailed Student *t*-tests (unpaired) in Prism 5.0 (GraphPad Software, San Diego, CA, USA). Significant differences between groups are represented by $*p < .05$, $**p < .01$, and $***p < .001$.

3. Results

3.1. NB patients are characterized with a change in serum concentration of multiple cytokines

Multiple cytokines in the serum of NB patients and healthy controls were first examined by a Luminex200 platform. Our results showed that IFN-γ, an important cytokine directing immune-mediated tumor destruction [25], was down-regulated in NB patient (Fig. 1A). Furthermore, while *Th1* cytokine IL-12 expressed at a reduced level (Fig. 1B), *Th2* cytokines IL-4 and IL-6 were greatly up-regulated (Fig. 1C and D) in NB patients compared with those in controls. To be noted, we also detected a remarkable increase in VEGF concentration in NB patients (Fig. 1E). VEGF has been reported as the most important key regulator of angiogenesis in various cancer types [26]. In addition, VEGF also inhibits T-cell development and may contribute to tumor-induced immune suppression [27,28]. Taking together, the bias of increased *Th2/Th1* ratio and increased serum VEGF expression proposed a propensity to pro-tumor microenvironment in NB patients, which has been reported in other tumor types [29].

3.2. NB circulating γδ T cells expressed reduced levels of cytolytic molecules

To understand the function of γδ T cells in NB, the expression of cell activating markers (DNAM-1, FasL, NKG2D and TRAIL) and intracellular cytolytic granular molecules (granzyme B and perforin) on freshly isolated γδ T cells were examined. As shown in Fig. 2, the γδ T cells, regardless of healthy control or NB patient group, had a general expression pattern: a high level of DNAM-1, low levels of FasL and TRAIL and medium levels of NKG2D, granzyme B and perforin. However, as far as the expression levels of DNAM-1, granzyme B and perforin are concerned, γδ T cells from NB patients exhibited significant reduced levels compared to those in controls (Fig. 2A, E and F, Table 2). The expression of FasL, NKG2D and TRAIL, however, showed no difference between patients and controls (Fig. 2B, C and D, Table 2). The reduced expression of cytolytic molecules DNAM-1, granzyme B and perforin in NB patients suggested a compromised γδ T-cell cytotoxicity to NB tumor. Poor cytotoxicity of NB γδ T cells was unlikely a result from a decreased survival, since there is no evidence of apoptosis rate change in patients' γδ T cells based on our Annexin V^+ assay by flow cytometry (Fig. 2G). Worth to mention, the intracellular expression of IFN-γ in γδ T cells showed a remarkable decrease in NB patients (Fig. 2H, Table 2), which was consistent with what has been found in NB serum.

3.3. PAM-expanded NB γδ T cells are featured with a reduced proliferation capacity and diminished expression of cytolytic molecules

It is well documented that phosphoantigens could activate γδ T cells *via* TCR complementarity determining region (CDR) recognition [30]. To understand if there is any difference in γδ T-cell proliferation capacity and cytolytic activity between NB patients and healthy controls, we stimulated isolated PBMC in *petri dish* with PAM and performed an *in vitro* γδ T-cell expansion. In Fig. 3A, the proportion of γδ T cells was increased to about 90% in day 14. During the *in vitro* culture, absolute cell number counts and CFSE cell proliferation assays revealed that γδ T cells from healthy controls generated more progeny through cell division than γδ T cells from NB patients (Fig. 3B and C). Similar to the cells at rest, PAM-expanded γδ T cells from healthy controls expressed higher levels of DNAM-1 and granzyme B compared with γδ T cells from patients (Fig. 3D and H, Table 2). Much to our surprise, the expression level of perforin, which had being seen to decrease in freshly isolated resting γδ T cells from NB patients, showed no difference from that of controls upon PAM stimulation; neither did the expression levels of FasL, NKG2D and TRAIL which agreed with that had been found in freshly isolated resting γδ T cells (Fig. 3E, F, G and I, Table 2). The PAM-stimulated γδ T cells from healthy controls, as expected, expressed IFN-γ at a much higher level in comparison to that in NB patient γδ T cells (Fig. 3J, Table 2).

3.4. γδ T cells from NB patients exhibit significant poor cytolytic activity

Production of IFN-γ and expression of cytolytic granules are major assembly for γδ T-cell cytotoxic function against malignant cells *in vivo* [7]. We have demonstrated a decrease in expression levels of multiple activating molecules in NB γδ T cells and verified that the proliferation capacity of NB γδ T cells were attenuated upon PAM-stimulation. To understand if these changes in NB γδ T cells adversely affected their tumoricidal ability, we co-cultured *in vitro* expanded γδ T cells with NB cell lines SH-SY5Y, SK-N-BE2 and IMR-32. Indeed, as shown in Fig. 4A, B and C, γδ T cells form NB patients exhibited a diminished cytolytic activity towards all three types of NB cell lines.

γδ T cells kill activated, infected, stressed, and transformed cells *via* various strategies including but not limited to engagement of death-inducing molecules (DNAM-1, NKG2D and FasL) and release of cytotoxic effector molecules (granzyme B and perforin) [31,32]. The death-inducing molecules generally bind to corresponding ligands or receptors to induce tumor cell death. For instance, DNAM-1 recognizes its ligands Nectin-2 and PVR [20]; and NKG2D recognizes MHC class I-related molecule A/B (MICA/B) and UL16-binding proteins (ULBP) [33]. We were interested in knowing the expression pattern of these ligands in the NB cells used in the present study. Results in Fig. 4D, E and F revealed that all three types of NB cell lines expressed extremely low level of Fas, MICA/B and ULBP-1/2/3/5/6. However, NB cell line SH-SY5Y and SK-N-BE2 expressed high levels of DNAM-1 ligands Nectin-2 and PVR, and cell IMR-32 expressed high level of PVR only, which is consistent with previous reports [34]. The expression pattern implied that γδ T cells probably recognizes NB cells through DNAM-1 instead of NKG2D or FasL. To testify whether DNAM-1 is the key player directing the cytolytic activity against NB cells, we performed cytotoxicity assays in the presence or absence of DNAM-1 neutralizing antibody and found that the specific lysis of NB cells by γδ T cells was abolished in anti-DNAM-1 treated group (Fig. 4G). Our results indicated

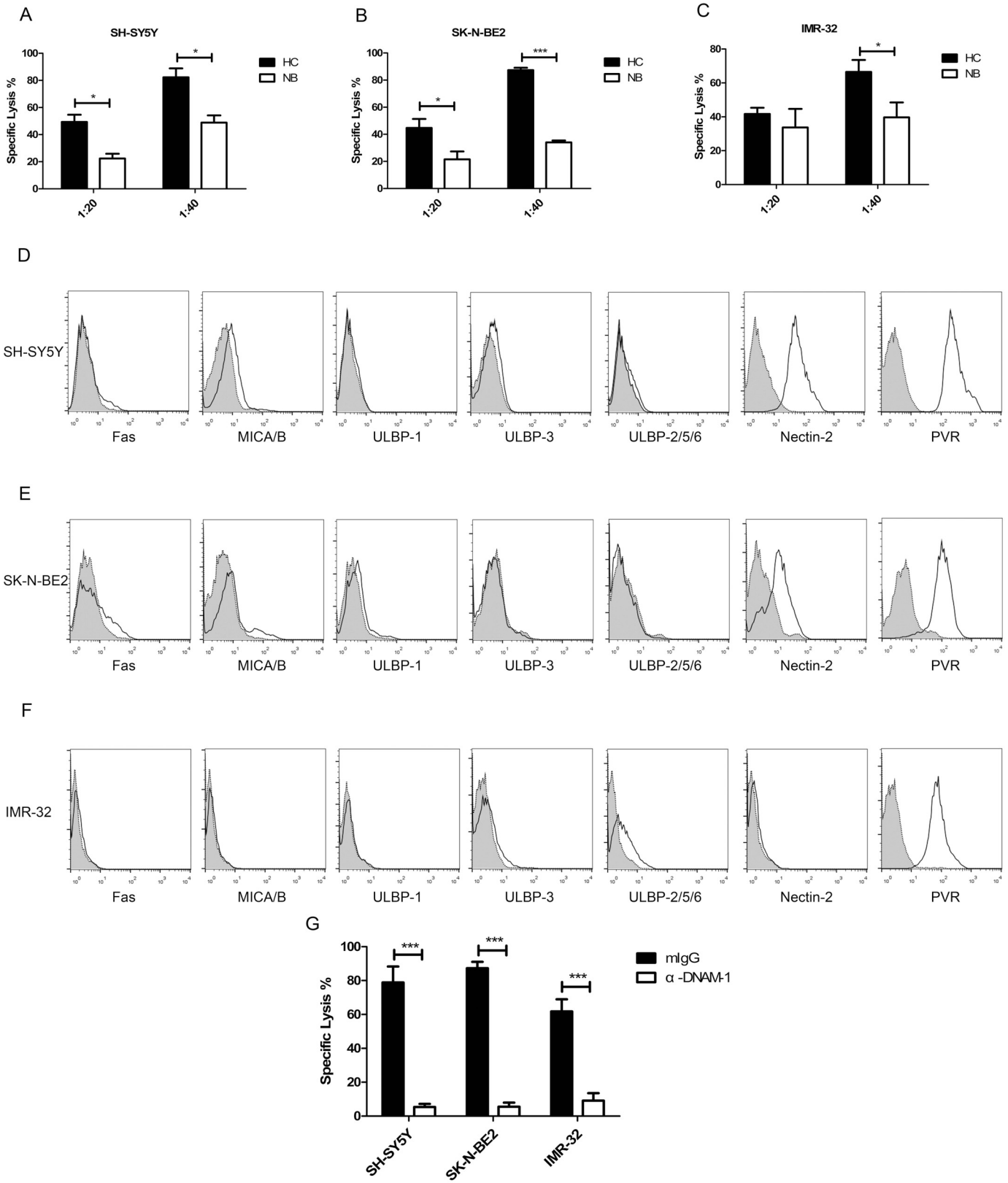

Fig. 4. The cytolytic activity of PAM-expanded γδ T cells. (A-C) The cytotoxic assays were performed and PAM-expanded γδ T cells were cultured with NB cell lines SH-SY5Y (A), SK-N-BE2 (B) and IMR-32 (C) at the ratio of 1:20 or 1:40. (D–F) The expression of Fas, MICA/B, ULBP-1/2/3/5/6, Nectin-2 and PVR were detected in SH-SY5Y (D), SK-N-BE2 (E) and IMR-32 (F). (G) The cytotoxic assays were performed using neutralizing antibody against DNAM-1 and mouse IgG.

that DNAM-1 played an indispensable role in directing cytolytic function of γδ T cells against NB cells, and suggested that reduction of DNAM-1, granzyme B and IFN-γ expression in NB patients is likely one of major culprits leading to the insufficient ability of γδ T cells for the tumor elimination.

4. Discussion

γδ T cells, implementing anti-tumor surveillance and bridging the gap between innate and adaptive immunity, are considered to be a potent candidate for tumor immunotherapies [35]. In neuroblastoma, γδ T cells exert important cytotoxic effects against tumor cells [5,18]. IFN-γ, one of the major *Th1* cytokines for T-cell activation and cytolytic function, was expressed at a much lower level in NB serum. The fact of *Th1* cytokine decrease and a concurrent increase in serum concentrations of IL-4 and IL-6 in patients suggested a *Th2* bias environment, which is believed to promote tumor development [36]. In cellular level, we found that main cytolytic factors including granzyme B, perforin as well as IFN-γ were down-regulated in freshly isolated patient γδ T cells indicating that γδ T cells from patients were installed with a subdued cytolytic function. This has been further supported by the fact that upon PAM-stimulation, *in vitro* expanded activating NB γδ T cells remained a significant lower expression level of these cytolytic factors compared to that in controls. Perforin, as an exception, had a trend of decreased expression in NB γδ T cells yet not reach to a statistical difference. This could be attributable to the gross up-regulation of perforin expression upon activation by PAM for both NB γδ T cells and control cells.

In contrast to αβ T cells, γδ T cells do not usually express CD4 or CD8 lineage markers, and they do not require conventional major histocompatibility complex (MHC) for antigen presentation [37]. Furthermore, Vγ9Vδ2 T cells can be expanded *in vitro* with a large number following the stimulation with phosphoantigens in the presence of IL-2. These characteristics make γδ T-cell an attractive candidate for adoptive cell transfer therapy. Administration of zoledronate-activated Vγ9Vδ2 T cells was evidenced as a safe and promising immunotherapy approach for treatment of patients with multiple myeloma in a clinical study [38]. It has been documented that infusion and *in vivo* expansion of haploidentical γδ T cells led to a complete remission in three out of four malignant leukemia patients [15]. γδ T cells were also proven to induce specific lysis of NB cells *in vitro* and treatment with zoledronate and IL-2 to NB patients was safe and feasible [19,39,40]. Adoptive cell transfer therapy of *in vitro* expanded γδ T cells and induce expansion of γδ T cells *in vivo* were promising approaches for NB treatment, particularly in the condition of that GD2 antibody-based immunotherapy [41] or manipulation of other immune cells [6,42] become unavailable or ineffective. In the present study, we showed that even there was an augment of γδ T cells in PBMC of NB patients, these cells were poor responders to PAM stimulation with less capacity for proliferation as well as a diminished cytolytic activity presented by reduced IFN-γ and granzyme B expression. This impaired function of NB γδ T cells not only results in an incompetent immune function to fend off the tumor but also makes it not possible to use tumor infiltrated γδ T cells from patients for autologous adoptive cell transfer therapy. It appeared paradoxical that γδ T cells in NB patient showed a higher percentage *in vivo* while these cells presented with a subdued proliferation *in vitro* without a change in cell survival, as revealed in our cell apoptosis assay and microarray-based mRNA quantification (no significant difference in the expression of major apoptosis related genes, such as Bcl-2 and Bim, between healthy controls and NB patients). Other mechanisms might involve in maintaining the homeostasis of these immuno-incompetent otherwise intact γδ T cells in NB patients.

DNAM-1 was initially identified as a molecule promoting cytotoxicity and cytokine secretion by immune cells. Evidence showed that induced DNAM-1 expression on activated γδ T cells promotes various types of tumor cell lysis, including hepatocellular carcinoma cells [22], primary multiple myeloma cells [43] and leukemia cells [44]. Meantime, it has been demonstrated that DNAM-1 ligands expression on cancer cells were up-regulated in the process of cell lysis during a chemotherapy regimen [45]. Our data revealed that the DNAM-1 expression maintained at a consistently lower level in NB γδ T cells no matter if they were freshly isolated or being *in vitro* expanded by PAM stimulation. This at least partially explained the diminished cytolytic activity of NB γδ T cells to NB cell lines in a co-culture setting. Abolishing the tumor cell lysis by γδ T cells in the presence of DNAM-1 neutralizing antibody in our co-culture system immediately validated the irreplaceable role of DNAM-1 in mediating tumor killing. It is envisaged that manipulating DNAM-1 expression in NB patient γδ T cells could become a valuable avenue to boost the effectiveness of γδ T-cell based immunotherapy in the future.

TIGIT (T cell immunoreceptor with Ig and ITIM domains) expressed on lymphocytes exert the immunoinhibitory effect by competing with DNAM-1 for the same ligands PVR and Nectin-2 on target cells [46] with stronger affinity [47]. Our data indicated that TIGIT expressed in PAM-expanded NB γδ T cells with a higher level than that in healthy controls (data not shown). It is boldly to suggest that the tilted expression of immunoinhibitory receptor TIGIT and immunoactivating receptor DNAM-1 in a pro-tumor microenvironment could be the culprit for insufficient γδ T cell tumoricidal ability in NB patients. That being said, evidence from deep analysis of tumor infiltrated γδ T cells is required to consolidate these findings.

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Competing interests

The authors have declared that no competing interest exists.

References

[1] A.M. Davidoff, I. Fernandez-Pineda, V.M. Santana, S.J. Shochat, The role of neoadjuvant chemotherapy in children with malignant solid tumors, Semin. Pediatr. Surg. 21 (2012) 88–99.
[2] Y.B. Luo, X.C. Cui, L. Yang, D. Zhang, J.X. Wang, Advances in the surgical treatment of neuroblastoma, Chin. Med. J. 131 (2018) 2332–2337.
[3] S. Gholamin, H. Mirzaei, S.M. Razavi, S.M. Hassanian, L. Saadatpour, A. Masoudifar, S. ShahidSales, A. Avan, GD2-targeted immunotherapy and potential value of circulating microRNAs in neuroblastoma, J. Cell. Physiol. 233 (2018) 866–879.
[4] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2017, CA Cancer J. Clin. 67 (2017) 7–30.
[5] K.E. Schilbach, A. Geiselhart, J.T. Wessels, D. Niethammer, R. Handgretinger, Human gammadelta T lymphocytes exert natural and IL-2-induced cytotoxicity to neuroblastoma cells, J. Immunother. 23 (2000) 536–548.
[6] S.B. Whittle, V. Smith, E. Doherty, S. Zhao, S. McCarty, P.E. Zage, Overview and recent advances in the treatment of neuroblastoma, Expert. Rev. Anticancer. Ther. 17 (2017) 369–386.
[7] P. Vantourout, A. Hayday, Six-of-the-best: unique contributions of γδ T cells to immunology, Nat. Rev. Immunol. 13 (2013) 88–100.
[8] A.C. Hayday, [gamma][delta] cells: a right time and a right place for a conserved third way of protection, Annu. Rev. Immunol. 18 (2000) 975–1026.
[9] E. Viey, G. Fromont, B. Escudier, Y. Morel, S. Da Rocha, S. Chouaib, A. Caignard, Phosphostim-activated gamma delta T cells kill autologous metastatic renal cell carcinoma, J. Immunol. 174 (2005) 1338–1347.
[10] M. Burjanadzé, M. Condomines, T. Reme, P. Quittet, P. Latry, C. Lugagne, F. Romagne, Y. Morel, J.F. Rossi, B. Klein, Z.Y. Lu, *In vitro* expansion of gamma delta T cells with anti-myeloma cell activity by Phosphostim and IL-2 in patients with multiple myeloma, Br. J. Haematol. 139 (2007) 206–216.
[11] H. Zhao, X. Xi, L. Cui, W. He, CDR3δ -grafted γ9δ2T cells mediate effective antitumor reactivity, Cell. Mol. Immunol. 9 (2012) 147–154.
[12] J. Nakajima, T. Murakawa, T. Fukami, S. Goto, T. Kaneko, Y. Yoshida, S. Takamoto, K. Kakimi, A phase I study of adoptive immunotherapy for recurrent non-small-cell lung cancer patients with autologous γδ T cells, Eur. J. Cardiothorac. Surg. 37 (2010) 1191–1197.
[13] A. Cordova, F. Toia, C. La Mendola, V. Orlando, S. Meraviglia, G. Rinaldi, M. Todaro, G. Cicero, L. Zichichi, P.L. Donni, N. Caccamo, G. Stassi, F. Dieli, et al.,

Characterization of human gammadelta T lymphocytes infiltrating primary malignant melanomas, PLoS One 7 (2012) e49878.
[14] M. Wilhelm, M. Smetak, K. Schaefer-Eckart, B. Kimmel, J. Birkmann, H. Einsele, V. Kunzmann, Successful adoptive transfer and *in vivo* expansion of haploidentical γδ T cells, J. Transl. Med. (2014) 2–5.
[15] C. Zou, P. Zhao, Z. Xiao, X. Han, F. Fu, L. Fu, γδ T cells in cancer immunotherapy, Oncotarget. 8 (2017) 8900–8909.
[16] M. Sakamoto, J. Nakajima, T. Murakawa, T. Fukami, Y. Yoshida, T. Murayama, S. Takamoto, H. Matsushita, K. Kakimi, Adoptive immunotherapy for advanced non-small cell lung cancer using zoledronate-expanded γδ T cells: a phase I clinical study, J. Immunother. 34 (2011) 202–211.
[17] S. Meraviglia, M. Eberl, D. Vermijlen, M. Todaro, S. Buccheri, G. Cicero, C. La Mendola, G. Guggino, M. D'Asaro, V. Orlando, F. Scarpa, A. Roberts, N. Caccamo, G. Stassi, F. Dieli, A.C. Hayday, *In vivo* manipulation of Vgamma9Vdelta2 T cells with zoledronate and low-dose interleukin-2 for immunotherapy of advanced breast cancer patients, Clin. Exp. Immunol. 161 (2010) 290–297.
[18] J. Chargui, V. Combaret, V. Scaglione, I. Iacono, V. Péri, D. Valteau-Couanet, M. Dubrel, E. Angevin, A. Puisieux, F. Romagne, C. Bergeron, Bromohydrin pyrophosphate-stimulated Vgamma9delta2 T cells expanded ex vivo from patients with poor-prognosis neuroblastoma lyse autologous primary tumor cells, J. Immunother. 33 (2010) 591–598.
[19] J.G. Pressey, J. Adams, L. Harkins, D. Kelly, Z. You, L.S. Lamb Jr., *In vivo* expansion and activation of γδ T cells as immunotherapy for refractory neuroblastoma: a phase 1 study, Medicine (Baltimore) 95 (2016) e4909.
[20] C. Bottino, R. Castriconi, D. Pende, P. Rivera, M. Nanni, B. Carnemolla, C. Cantoni, J. Grassi, S. Marcenaro, N. Reymond, M. Vitale, L. Moretta, M. Lopez, A. Moretta, Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule, J. Exp. Med. 198 (2003) 557–567.
[21] R. Castriconi, A. Dondero, M.V. Corrias, E. Lanino, D. Pende, L. Moretta, C. Bottino, A. Moretta, Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: critical role of DNAX accessory molecule-1-poliovirus receptor interaction, Cancer Res. 64 (2004) 9180–9184.
[22] O. Toutirais, F. Cabillic, G. Le Friec, S. Salot, P. Loyer, M. Le Gallo, M. Desille, C.T. de La Pintière, P. Daniel, F. Bouet, V. Catros, DNAX accessory molecule-1 (CD226) promotes human hepatocellular carcinoma cell lysis by Vgamma9Vdelta2 T cells, Eur. J. Immunol. 39 (2009) 1361–1368.
[23] B. Silva-Santos, K. Serre, H. Norell, γδ T cells in cancer, Nat. Rev. Immunol. 15 (2015) 683–691.
[24] W. Mou, W. Han, X. Ma, X. Wang, H. Qin, W. Zhao, X. Ren, X. Chen, W. Yang, H. Cheng, X. Wang, H. Zhang, X. Ni, H. Wang, J. Gui, γδTFH cells promote B cell maturation and antibody production in neuroblastoma, BMC Immunol. 18 (2017) 36.
[25] Q. Gao, F. Li, S. Wang, Z. Shen, S. Cheng, Y. Ping, G. Qin, X. Chen, L. Yang, L. Cao, S. Liu, B. Zhang, L. Wang, Y. Sun, Y. Zhang, A cycle involving HMGB1, IFN-γ and dendritic cells plays a putative role in anti-tumor immunity, Cell. Immunol. (2018) (pii: S0008-8749(18)30382-4).
[26] Y. Hori, K. Ito, S. Hamamichi, Y. Ozawa, J. Matsui, I.O. Umeda, H. Fujii, Functional characterization of VEGF- and FGF-induced tumor blood vessel models in human cancer xenografts, Anticancer Res. 37 (2017) 6629–6638.
[27] J.E. Ohm, D.I. Gabrilovich, G.D. Sempowski, E. Kisseleva, K.S. Parman, S. Nadaf, D.P. Carbone, VEGF inhibits T-cell development and may contribute to tumor-induced immune suppression, Blood. 101 (2003) 4878–4886.
[28] T. Voron, O. Colussi, E. Marcheteau, S. Pernot, M. Nizard, A.L. Pointet, S. Latreche, S. Bergaya, N. Benhamouda, C. Tanchot, C. Stockmann, P. Combe, A. Berger, F. Zinzindohoue, H. Yagita, E. Tartour, J. Taieb, M. Terme, VEGF-A modulates expression of inhibitory checkpoints on CD8+ T cells in tumors, J. Exp. Med. 212 (2015) 139–148.
[29] D.E. Johnson, R.A. O'Keefe, J.R. Grandis, Targeting the IL-6/JAK/STAT3 signalling axis in cancer, Nat. Rev. Clin. Oncol. 15 (2018) 234–248.
[30] R.S. Patil, S.A. Bhat, A.A. Dar, S.V. Chiplunkar, The Jekyll and Hyde story of IL17-producing γδT cells, Front. Immunol. 6 (2015) 37.
[31] G. Qin, H. Mao, J. Zheng, S.F. Sia, Y. Liu, P.L. Chan, K.T. Lam, J.S. Peiris, Y.L. Lau, W. Tu, Phosphoantigen-expanded human gammadelta T cells display potent cytotoxicity against monocyte-derived macrophages infected with human and avian influenza viruses, J. Infect. Dis. 200 (2009) 858–865.
[32] S. Dhar, S.V. Chiplunkar, Lysis of aminobisphosphonate-sensitized MCF-7 breast tumor cells by Vγ9Vδ2 T cells, Cancer Immun. 10 (2010) 10.
[33] C. Niu, H. Jin, Li M1, S. Zhu, L. Zhou, F. Jin, Y. Zhou, D. Xu, J. Xu, L. Zhao, S. Hao, W. Li, J. Cui, Low-dose bortezomib increases the expression of NKG2D and DNAM-1 ligands and enhances induced NK and γδ T cell-mediated lysis in multiple myeloma, Oncotarget 8 (2017) 5954–5964.
[34] A. Dondero, F. Pastorino, M. Della Chiesa, M.V. Corrias, F. Morandi, V. Pistoia, D. Olive, F. Bellora, F. Locatelli, A. Castellano, L. Moretta, A. Moretta, C. Bottino, R. Castriconi, PD-L1 expression in metastatic neuroblastoma as an additional mechanism for limiting immune surveillance, Oncoimmunology 5 (2015) e1064578.
[35] M. Bonneville, R.L. O'Brien, W.K. Born, Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity, Nat. Rev. Immunol. 10 (2010) 467–478.
[36] N. Takahashi, M. Sugaya, H. Suga, T. Oka, M. Kawaguchi, T. Miyagaki, H. Fujita, S. Sato, Thymic stromal chemokine TSLP acts through Th2 cytokine production to induce cutaneous T-cell lymphoma, Cancer Res. 76 (2016) 6241–6252.
[37] E.J. Adams, W.L. Havran, Introduction to cellular immunology special issue on γδ T cells, Cell. Immunol. 296 (2015) 1–2.
[38] Y. Abe, M. Muto, M. Nieda, Y. Nakagawa, A. Nicol, T. Kaneko, S. Goto, K. Yokokawa, K. Suzuki, Clinical and immunological evaluation of zoledronate-activated Vgamma9gammadelta T-cell-based immunotherapy for patients with multiple myeloma, Exp. Hematol. 37 (2009) 956–968.
[39] E. Di Carlo, P. Bocca, L. Emionite, M. Cilli, G. Cipollone, F. Morandi, L. Raffaghello, V. Pistoia, I. Prigione, Mechanisms of the antitumor activity of human Vγ9Vδ2 T cells in combination with zoledronic acid in a preclinical model of neuroblastoma, Mol. Ther. 21 (2013) 1034–1043.
[40] J.P. Fisher, M. Yan, J. Heuijerjans, L. Carter, A. Abolhassani, J. Frosch, R. Wallace, B. Flutter, A. Capsomidis, M. Hubank, N. Klein, R. Callard, K. Gustafsson, J. Anderson, Neuroblastoma killing properties of Vδ2 and Vδ2-negative γδT cells following expansion by artificial antigen-presenting cells, Clin. Cancer Res. 20 (2014) 5720–5732.
[41] N. Berois, E. Osinaga, Glycobiology of neuroblastoma: impact on tumor behavior, prognosis, and therapeutic strategies, Front. Oncol. 4 (2014) 114.
[42] R. Handgretinger, P. Schlegel, Emerging role of immunotherapy for childhood cancers, Chin. Clin. Oncol. 7 (2018) 14.
[43] A. Knight, S. Mackinnon, M.W. Lowdell, Human Vdelta1 gamma-delta T cells exert potent specific cytotoxicity against primary multiple myeloma cells, Cytotherapy. 14 (2012) 1110–1118.
[44] V. Pistoia, N. Tumino, P. Vacca, I. Veneziani, A. Moretta, F. Locatelli, L. Moretta, Human γδ T-cells: from surface receptors to the therapy of high-risk leukemias, Front. Immunol. 9 (2018) 984.
[45] A. Soriani, C. Fionda, B. Ricci, M.L. Iannitto, M. Cippitelli, A. Santoni, Chemotherapy-elicited up-regulation of NKG2D and DNAM-1 ligands as a therapeutic target in multiple myeloma, Oncoimmunology. 2 (2013) e26663.
[46] B. Zhang, W. Zhao, H. Li, Y. Chen, H. Tian, L. Li, L. Zhang, C. Gao, J. Zheng, Immunoreceptor TIGIT inhibits the cytotoxicity of human cytokine-induced killer cells by interacting with CD155, Cancer Immunol. Immunother. 65 (2016) 305–314.
[47] K.E. Pauken, E.J. Wherry, TIGIT and CD226: tipping the balance between costimulatory and coinhibitory molecules to augment the cancer immunotherapy toolkit, Cancer Cell 26 (2014) 785–787.