Ex Vivo Activation of CD56⁺ Immune Cells That Eradicate Neuroblastoma

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Abstract

Despite the use of intensive contemporary multimodal therapy, the overall survival of patients with high-risk neuroblastoma is still less than 50%. Therefore, immunotherapy without cross-resistance and overlapping toxicity has been proposed. In this study, we report the development of a novel strategy to specifically activate and expand human CD56⁺ (NCAM1) natural killer (NK) immune cells from normal donors and patients with neuroblastoma. Enriched CD56⁺ cells from peripheral blood were mixed with CD56⁻ fraction at 1:1 ratio and cultured in the presence of OKT3, interleukin (IL)-2, and -15 for five days and then without OKT3 for 16 more days. The final products contained more than 90% CD56⁺ cells and could kill neuroblastoma cells effectively that were originally highly resistant to nonprocessed NK cells. Mechanistically, cytolysis of neuroblastoma was mediated through natural cytotoxicity receptor (NCR), DNAX accessory molecule-1 (DNAM-1; CD226), perforin, and granzyme B. Successful clinical scale-up in a good manufacturing practices (GMP)-compliant bioreactor yielded effector cells that in a neuroblastoma xenograft model showed tumor growth and extended survival without GVHD. Investigation of CD56⁺ cells from patients with neuroblastoma revealed a similar postactivation phenotype and lytic activity. Our findings establish a novel and clinically expedient strategy to generate allogeneic or autologous CD56⁺ cells that are highly cytotoxic against neuroblastoma with minimal risk of GVHD.

Introduction

Neuroblastoma is an embryonal tumor of the sympathetic nervous system (1). It is the most common extracranial solid tumor in children, the most frequently diagnosed neoplasm during infancy, and accountable for 15% of all pediatric oncology deaths (2). Attributable to molecular differences (3, 4), the clinical behavior and cure rate differ substantially, ranging from more than 90% cure in patients with locoregional tumors requiring little to no cytotoxic therapy, to less than 50% survival despite aggressive therapy in those 18 months or older at diagnosis with metastatic disease, or those with MYCN gene amplification (5, 6).

The standard treatment of high-risk neuroblastoma includes intensive chemotherapy, radiotherapy, surgery, and autologous hematopoietic stem cell transplantation (7). In addition, maintenance treatment with differentiation agent 13-cis-retinoic acid (isotretinoin) further improves patient’s outcome (8). Because the survival of these high-risk patients is still poor despite intensive contemporary treatment, biologic- or immune-based therapy has been widely studied, given its noncross resistance and less overlapping side effects with current therapies (7, 9, 10). For instance, the use of anti-GD2 (disialoganglioside) monoclonal antibodies (mAb), combined with interleukin (IL)-2 and granulocyte macrophage colony-stimulating factor (GM-CSF) given after completion of standard intensive treatment, has been proven to be beneficial in patients with high-risk neuroblastoma (11). These tumor-targeted mAbs initiate antibody-dependent cell-mediated cytotoxicity (ADCC) primarily through granulocytes, macrophages, and natural killer (NK) cells (12, 13).

The clinical usefulness of NK cell therapy against cancers has been studied particularly in hematologic malignancies (14). NK cells are a subset of cytotoxic lymphocytes that contribute to approximately 5% to 10% of circulating lymphocytes in healthy subjects. Their immunophenotype is defined by the surface expression of CD56 and lack of expression of CD3. A subset of T cells expresses both CD3 and CD56 (CD3⁺CD56⁺ NK T cell) and possesses NK-like cytolytic activity against a variety of cancer cells (15). NK cells participate in innate immunity and play an important role in direct early defense and indirect induction of adaptive immunity against infection or transformation (16). The cells can kill target cells without the need for prior sensitization. The functional activity of NK cells is regulated

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CD56+ cell activation

Immediately after CD56+ cell isolation, the CD56+ fraction was mixed with the CD56− fraction at a 1:1 ratio. This ratio was chosen on the basis of preliminary experiments showing optimal cytotoxicity and expansion (Supplementary Fig. S1A and S1B). The cells were then seeded at the density of $1 \times 10^6$ cells/mL and activated in a culture cocktail consisting of stem cell growth medium (SCGM; CellGenix GmbH), 500 U/mL IL-2 [aldesleukin (Proleukin); Novartis Pharmaceuticals], 10 ng/mL IL-15 (CellGenix GmbH), 10 ng/mL OKT3 (Biolegend), and 5% human AB serum (Lonzagroup). Under this culture condition, a large amount of IFN-γ was found in the supernatant in addition to IL-6 and -17 (Supplementary Fig. S1C). After 5 days of culture, the cells were washed twice with PBS and reseeded at $1 \times 10^6$ cells/mL with identical culture medium but without OKT3. The cells were replenished with freshly prepared medium every 3 days for 16 more days (total 21 days). In some experiments, PBMCs were not purified for CD56+ cells and were seeded unfraccionated at the density of $1 \times 10^6$ cells/mL (PBMC group). The cells were then activated using the same culture cocktails and maintained using the same procedure as described earlier in the MIX 1:1 group.

Flow cytometry

To determine cell population changes at different time points during the course of CD56+ cell activation, the percentages of T (CD3+CD56−), NK (CD3−CD56+), NK (CD3+CD56+), and CD56+ cells (NK plus NKT) were analyzed using flow cytometry. The following clones of antibody were used for phenotypic analysis: anti-CD158ah (11P86, E6B6.B), anti-CD158bH (CH-L, GLI83), anti-NK11 (DX9), anti-DNAM-1 (DX11), anti-CD11a (HI111), anti-NTB4 (292811), anti-CD24, anti-CD3 (SK7, UCHT1), anti-granzyme B (GB10), anti-NKG2a (Z199), anti-NKp30 (Z25), anti-NKp44 (Z231), anti-NKp46 (BABB81), anti-NKG2D (ID11), anti-CD56 (MY31, N901), and anti-CD14 (MphiP9). Flow cytometry analyses were conducted with LSRII (BD Biosciences) and FlowJo 8.8.6 software (Tree Star).

Neuroblastoma cell lines

The neuroblastoma cell lines were established from patients with different metastatic sites, stages, and N-Myc amplification status (Supplementary Table S1). The CHLA90, CHLA-255 (from Dr. C. Patrick Reynolds of Children’s Hospital of Los Angeles, Los Angeles, CA) and SK-N-SH [American Type Culture Collection (ATCC)] were cultured in Dulbecco’s Modified Eagle’s Medium (Life Technologies, Invitrogen). The NB-1691, NB-1643, NB-EB (from Dr. Peter Houghton, Center for Childhood Cancer, Nationwide Children’s Hospital, Columbus, OH), SK-N-AS (ATCC), and positive control K562 (ATCC) cell lines were cultured in RPMI medium (Cellgro, Mediatech, Inc.). All media were supplemented with 10% FBS (Atlanta Biologicals, Inc.) and penicillin/streptomycin (Life Technologies).

Cytotoxicity assay

To determine the functional changes at different time points during the course of CD56+ cell activation, cytotoxic activity was measured by using the DELFIA BATDA reagent (PerkinElmer Life and Analytical Sciences) following the manufacturer’s instruction. BATDA-labeled neuroblastoma cell lines were used as target cells at an effector:target ratio of 5:1 for 2 hours at 37°C. The fluorescence signals were measured using a Wallac Victor 2 Counter Plate Reader (PerkinElmer Life and Analytical Sciences). Neutralizing antibodies (10 μg/mL each) against Nkp30, Nkp44, Nkp46, and DNAM-1 (all from Biolegend) were added in some experiments.

CD107a degranulation

Effector cells were cocultured with neuroblastoma target cells at 5:1 ratio in the presence of anti-CD107a-PE or -APC antibodies. After 1 hour of coculture, GolgiStop (BD
Biosciences) was added and the cells were incubated for 4 more hours. The cells were then harvested, stained, and analyzed for CD107a degranulation gated on CD45⁺CD56⁻ cells (28).

### Large-scale activation

Apheresed peripheral blood cells from normal donors were purchased (Key Biologics, Inc.) and purified for CD56⁺ cells on the CliniMACS instrument (Miltenyi Biotec; ref. 23). CD56⁺ cells were then expanded ex vivo in a starting 1:1 ratio of CD56⁺: CD56⁻ cells at a density of 5 × 10⁶ cells/mL in 2 T-300 flasks (Midwest Scientific). The cells were initially cultured in a base medium [SCG1 (Cellgenix), 5% human AB serum (Lonza), 10 ng/mL IL-15 (Cellgenix), 500 U/mL IL-2 (Novartis Pharmaceuticals)], with 10 ng/mL of OKT3 (Centocor Ortho Biotech, L.P.). After 5 days in culture, the cells were harvested from the flasks and washed twice with PBS (Lonza) by centrifugation at 600 × g for 15 minutes. The cells were resuspended in 200 mL of base medium without OKT3 and placed in a WAVE 2L Cellbag prepared per manufacture recommendations (GE Healthcare). The WAVE Bioreactor was maintained at 37°C, 5% CO₂ and rocked at 5 rpm. The culture was monitored for cell density and fed with additional base medium every 2 to 3 days to maintain a cell density of 1 to 2 × 10⁸ cells/mL. When the volume of the culture was greater than 1,000 mL, it was transferred to a WAVE 20L Cellbag, maintained at 37°C, 5% CO₂ and rocking at 9 rpm. Cell density was monitored to maintain the target cell density by adding more medium if needed. If no additional volume was required, cytokines only were added to culture every 2 to 3 days to maintain concentrations equivalent to base medium. On day 21 from the start of the expansion the cells were harvested from the WAVE Cellbag.

### In vivo experiments

Nonobese diabetic/severe combined immunodeficient (NOD/SCID) IL-2⁻/⁻ (NOG) mice of 8- to 12-weeks old were used as a preclinical animal model to determine the efficacy and toxicity of the activated products obtained from large-scale activation. Neuroblastoma cell line NB-EB and SK-N-SH were transduced with lentiviral vector containing MSCV-IRES-YFP (Vector Lab, St. Jude). The YFP⁺ cells were flow-sorted and cloned. Mice were γ-irradiated at a dose of 200 cGy 1 day before 1 × 10⁶ neuroblastoma cells were intraperitoneally injected. On the following day, 1 × 10⁶ MIX 1:1 effector cells obtained from day 0 or 21 large-scale activation were intraperitoneally injected. Human IL-2 (100 U/mL; Novartis Pharmaceuticals) was intraperitoneally injected starting a day after injection of effector cells and continued every 3 days for a total of 3 weeks. To determine the life span of fresh or activated effector cells, mice were sacrificed at regular intervals and their peritoneal fluids, blood, and spleen were collected, immunophenotyped to determine the presence of human cell population. Disease progression of mice was regularly monitored by bioluminescence imaging, and the survival data were recorded. The mice were sacrificed when they displayed signs of significant neuroblastoma progression including paralysis, severely distended abdomen, or more than 20% weight loss.

The experiments were terminated in surviving mice at day 60 after the injection of neuroblastoma cells. All the sick, dead, or sacrificed mice underwent autopsy to examine for the presence of neuroblastoma.

### Statistical analysis

Statistical significance between 2 groups was calculated using Student paired t test. In cases of having more than 2 groups, one-way ANOVA was used. For the survival analysis, Kalper–Meiers curves were constructed and compared by log-rank test. The nominal significance level was set at 0.05.

### Results

#### Overcoming NK cell resistance in neuroblastoma cell lines

We investigated a new CD56⁺ cell activation and expansion strategy to minimize the volume of culture medium and to maximize the cytotoxic activity against neuroblastoma (Fig. 1A). After CD56⁻ cell isolation from PBMCs, the CD56⁺ fraction was mixed with the CD56⁻ fraction in a 1:1 ratio and cocultured for 21 days. The susceptibility of 7 different neuroblastoma cell lines was tested. All 7 cell lines were originally resistant to fresh CD56⁺ cell lysis. Significant increase in cytotoxic activity of activated CD56⁺ cells was observed in all neuroblastoma cell lines (Fig. 1B). The changes in cytotoxic activity of activated CD56⁺ cells were time-dependent. During the 21 days of activation, the longer the culture time, the higher the cytotoxic activity of CD56⁺ cells against neuroblastoma cell lines (Fig. 1C). As for NB-EB, there was an increase in the median-specific lysis from 24.5% on day 14 to 67.6% on day 21. Similar results were also observed in CHLA-90 (from 15.4% to 65.4%) and in SK-N-SH neuroblastoma cells (from 12.8% to 39.4%).

#### Expansion of both NK and NKT cells with upregulation of expression of activation receptors but not inhibitory receptors

Among the 11 products after 21 days of activation, the predominant population in the final product was NK cells in 5, NKT cells in 4, and equal proportion of NK cells and NKT cells in 2 (Fig. 2A). Both NK and NKT cells were cytolytic, but the activity of NK cells was comparatively higher than that of NKT cells against neuroblastoma cell lines (Fig. 2B). Extensive characterization of the phenotypes of the activated CD56⁺ cells showed that the surface expressions of activating receptors and adhesion molecules (NKp30, NKp44, NKp46, and DNAM-1) in neuroblastoma cells were increased, but the activity of NK cells and NKT cells was not statistically different.

#### Essential role of NCRs and DNAM-1 in activated cell-mediated killing of neuroblastoma

We sought to determine the role of each upregulated receptor (including NKp30, NKp44, and DNAM-1) in overcoming neuroblastoma resistance. We conducted...
antibody-blocking experiments using day 21 activated CD56^+ cells as effectors (Fig. 3A). Among the 7 neuroblastoma cell lines, heterogeneous activation pathways were used to overcome resistance. In general, NCRs and DNAM-1 were involved against most neuroblastoma cell lines, except for CHLA-90 cells that were killed using a DNAM-1–independent pathway. The inhibitory effect was more prominent when multiple receptors were blocked, especially on CHLA-90, CHLA-255, and NB-1691 when the cell lysis was completely abolished after the 3 NCRs and DNAM-1 were blocked.

Degranulation of activated CD56^+ cells triggered by neuroblastoma

To investigate whether the cytotoxicity against neuroblastoma cells involved the granzyme B and perforin pathways, CD107a degranulation assay was conducted. After coincubation of activated CD56^+ cells (effectors) and neuroblastoma cell lines (targets) at an effector:target ratio of 5:1, degranulation was observed in all experiments (Fig. 3B). The levels of CD107a-positivity correlated well with the relative levels of cytotoxicity measured by BATDA assay (Fig. 1B).

MIX 1:1 cultures were superior to PBMC cultures in convenience and in cytotoxicity

We termed our small volume culture method as MIX 1:1, in contrast to the large volume culture method using unfractionated PBMC as starting cells (PBMC method; ref. 29). We found that CD56^- cells from MIX 1:1 products expanded as efficiently as those by the PBMC method. Median CD56^- cell expansion on day 21 was 88.9- and 111.6-fold, respectively (Fig. 4A). Median purity of CD56^- cells in MIX 1:1 products was 91.5%, compared with 34.3% in PBMC products (Fig. 4B). The phenotypes of CD56^- cells in 2 cultures are similar, except PBMC products had significant increase in inhibitory KIR2DL1, NKp46, and NTB-A but not NKp30 and NKG2D as in MIX 1:1 cultures (Supplementary Fig. S1D). When used as effectors against 3 representative neuroblastoma lines, MIX 1:1 cultures had higher cytotoxicity compared with PBMC cultures. Median specific lysis of MIX 1:1 products was 88.9- and 111.6-fold, respectively (Fig. 4B). Median purity of CD56^- cells in MIX 1:1 products was 91.5%, compared with 34.3% in PBMC products (Fig. 4B). The phenotypes of CD56^- cells in 2 cultures are similar, except PBMC products had significant increase in inhibitory KIR2DL1, NKp46, and NTB-A but not NKp30 and NKG2D as in MIX 1:1 cultures (Supplementary Fig. S1D). When used as effectors against 3 representative neuroblastoma lines, MIX 1:1 cultures had higher cytotoxicity compared with PBMC cultures.
neuroblastoma cell lines (NB-EB, CHLA-90, and SK-N-SH), activated CD56\(^+\) cells from MIX 1:1 products had superior cytotoxic activity compared with those by the PBMC method (Fig. 4C). By defining a less than 25% specific lysis as ineffective killing, 6 of 15 products (40%) by the PBMC method were ineffective against NB-EB. In contrast, only...
1 of 15 MIX 1:1 products (6.7%) was unable to lyse NB-EB, with similar results in CHLA-90 and SK-N-SH neuroblastoma cells \( (P = 0.0045) \). Thus, our small volume MIX 1:1 method was comparable with the PBMC method in expansion efficiency but superior to the PBMC method in CD56 purity, functional efficacy, and convenience of preparation.

**GMP-compliant large-scale activation**

To investigate whether our MIX 1:1 activation method could potentially be used clinically, clinical-scale activation experiments of CD56\(^+\) cells were conducted using GMP-compliant components. Three separate experiments were carried out and the culture conditions of the large-scale experiments resembled...
that of the small-scale experiments. Similar increase in surface expression of activated receptors and adhesion molecules (NKp30, NKp44, DNAM-1, NKG2D, and CD11a) were observed on day 21 CD56+ cells (Fig. 5A). Median purity of CD56+ cells on day 21 was 93.4%. Cytotoxic assay against the 2 neuroblastoma cell lines (NB-EB and SK-N-SH) that would be used in the mouse model showed superior lytic activity of activated CD56+ cell lines when compared with fresh CD56+ cells (Fig. 5B).

In vivo antitumor and GVH activity of clinical-scale activated products

A mouse model was used to assess the therapeutic efficacy and GVH potential of the clinical-scale GMP-activated products. We hypothesized that the day 21 products would be more active against neuroblastoma in vivo and would cause less GVHD because of less CD56− T cells in the effector inoculum. NOG mice were xenografted intraperitoneally with neuroblastoma cell lines (NB-EB-YFP and SK-N-SH-YFP) followed by intraperitoneal injection of day 0 fresh or day 21 activated MIX 1:1 products from large-scale activation on the following day. We monitored the progression of tumor growth by bioluminescence as shown in Fig. 6A. Comparing the last time point on day 24 and first time point on day 4, there was no significant increase in bioluminescence signals in the mice receiving activated products against NB-EB (Fig. 6B). Progressive disease, however, was observed in both the control-un-treated group and fresh cell group. Treatment with activated products, but not fresh products, significantly prolonged the survival of the mice compared with the control group (Fig. 6C). Similarly for SK-N-SH, the activated products significantly prolonged the survival of the mice (Fig. 6D). In contrast, the mice receiving fresh products died rapidly with clinical signs of severe GVHD, which were confirmed by histopathology (Fig. 6E). In skin, there was dense dermal lymphocytic infiltrate with focal vacuolar changes in basal cells, focal separation of dermoepidermal junction, and lymphocytic exocytosis. In liver, portal lymphoplasmacytic infiltrate was found with endothelialitis. There was also infiltration of mononuclear inflammatory cells in the bile duct. In the small and large intestine, increased lymphoplasmacytic cells were found in the lamina propria associated with focal cryptitis and abscess. No clinical or histopathologic evidence of GVHD was noted in any of the mice receiving activated products. These findings underscored the relative safety and efficacy of activated products over fresh products in allogeneic immunotherapy for neuroblastoma.

The use of activated product from neuroblastoma patients

Besides allogeneic NK cell therapy, we postulated that aliquots from granulocyte colony-stimulating factor (G-CSF)–mobilized autologous peripheral blood stem cell (PBSC) apheresis products might be potential starting materials for CD56+ cell activation, as autologous stem cell transplantation is widely considered as a standard treatment for patients with high-risk neuroblastoma. The CD56+ cell activation using PBMC method was compared with the MIX 1:1 method. CD56+ cells from MIX 1:1 method had similar expansion (median of 80.27-fold increase) as compared with cells from healthy donors (Fig. 7A). However, CD56− cells could not be expanded at all using the PBMC method, because of the outgrowth by CD56− cells. CD11a, NKp30, NKp44, and DNAM-1 were significantly increased in MIX 1:1 day 21 CD56− cells comparing with day 0 CD56− cells, whereas all inhibitory receptors including KIRs and NKG2a remained unchanged (Fig. 7B). In addition, cytotoxic activity of day 21 activated CD56− cells against neuroblastoma cell lines was
superior to that of day 0 fresh CD56+ cells (Fig. 7C), though lower than those obtained from healthy donor (Fig. 1B), possibly in part because of higher expression of NKG2a and lower expression of NKG2D and 2B4 (Fig. 7D).

Discussion

High-risk neuroblastoma is one of the most aggressive cancers in children (4). Over the past decade, the addition of isotretinoin and immunotherapy with anti-GD2 mAbs in combination with IL-2 and GM-CSF has emerged as promising therapy for high-risk neuroblastoma (8, 11). Despite this advance and those in intensive chemotherapy, surgery, and radiation, the survival of patients remains unsatisfactory (9, 10). Recently, following the success of NK cell therapy in hematologic malignancies (14, 30, 31), investigators have begun studying similar therapy in neuroblastoma (23, 32, 33). We and others have shown that several novel NK cell activation methods using various cytokine combinations such as IL-12 and -18 or using genetic modification may improve the efficacy of NK cells in preclinical models (34–37). Herein, we further developed a simpler method to activate human CD56+ NK and NKT cells efficiently in relatively small volume without the need for genetic modification or artificial supportive cells. Using our new strategy, we generated adequate number of effector cells with potent activity against NK-resistant neuroblastoma with minimal risk of GVHD.

One key advantage of our culture method is relatively small volume. With the lower percentage of CD56+ cells in the PBMC population compared with that in MIX 1:1 (~5% in PBMC and 50% in MIX 1:1), more starting cells are needed using the PBSC method to achieve a similar final CD56+ cell yield. The more starting cells are needed, the more culture medium and handling are required, thus increasing the cost and risk of contamination. Another major advantage is the reduction in risk of GVHD. In contrast to the PBMC method in which two thirds of the cells in the end products were CD56−/CD3+, our end products using the MIX 1:1 method were always more than 90% CD56+ (Fig. 4B), with very few CD3+ CD56− cells detected. Thus, the extra step of CD3+ depletion before cell infusion would not be necessary in the allogeneic setting to avoid GVHD. In fact, when the products were injected into the mice, none of them developed GVHD, which was in sharp contrast to those receiving day 0 fresh products in which many developed severe signs and symptoms of GVHD, which was confirmed by histopathologic findings. This observation suggested that our method not only increased antineuroblastoma activity but also decreased the risk of GVHD.

Several mechanisms may be involved in overcoming NK-resistance, including reduction of inhibitory signaling or augmentation of activating pathways. Recently, NCRs and DNAM-1 have been reported to be involved specifically in NK cell recognition of neuroblastoma (26, 27). Using our culture approach, increased surface expression of activating receptors NCRs, NKG2D, and DNAM-1 was noted on the CD56+ cells. Importantly, there was no significant increase in inhibitory receptor expression. Antibody-blocking

Figure 5. GMP-compliant activation of CD56+ cells resembled those of small-scale activation in terms of phenotype and cytotoxicity. Flow cytometry analysis of day 21 activated cells in expression of KIR (KIR2DL1, 2DL2/3, and 3DL1), NKG2a, NKp30, NKp44, NKp46, NKG2D, DNAM-1, NTB-A, 2B4, and CD11a. Bars represent the median surface expression. B, cytotoxicity of fresh (white) and activated (black) CD56+ cells against NB-EB and SK-N-SH (effector: target ratio of 5:1).
experiments confirmed that NCR and DNAM-1 pathways were essential for activated CD56\(^+\) cell-mediated killing of neuroblastoma. Without blocking NKG2D, a combination of antibodies against the 3 NCRs and DNAM-1 could already abolish lysis of all neuroblastoma lines to less than 5% of those of negative controls. These data suggest that the NKG2D pathway is nonessential in our activated cell killing, in line with our finding that NKG2D ligands were not detected on any of our neuroblastoma cells (data not shown). Nevertheless, as killing was always more potent when multiple activating receptors were triggered, our finding underscores that more than one activating pathway should be optimized for future neuroblastoma therapy.

Because the ultimate goal of our cell activation strategy was to translate to clinical therapy, we examined the feasibility of clinical-scale activation, the efficacy and toxicity of in vivo administration, and the possibility of using patients’ cells as starting cells. Our clinical-scale activated CD56\(^+\) cells revealed similar phenotypes and functions as those derived from the small-scale approach (Fig. 5), and their efficacy in controlling neuroblastoma was confirmed in the mouse experiments. Notably, the MIX 1:1 activated cells not only were more effective, but also reduced the risk of GVHD when compared with PBMC. This finding has significant implications in allogeneic CD56\(^+\) cell therapy, either alone or in combination with anti-GD2 ADCC, as any immunosuppressive prophylaxis or treatment of GVHD will hinder the efficacy of tumor control. Compared with normal PBMCs, a lower frequency of CD56\(^+\) cells was consistently observed in patients with neuroblastoma after G-CSF mobilization (3%–10% in normal vs. 0.28% on average from 5 patient samples). With such a low beginning amount of CD56\(^+\) cells, we could not expand CD56\(^+\) cells using the PBMC method. However, expansion and activation of CD56\(^+\) cells as starting cells. Our clinical-scale activated CD56\(^+\) cells revealed similar phenotypes and functions as those derived from the small-scale approach (Fig. 5), and their efficacy in controlling neuroblastoma was confirmed in the mouse experiments. Notably, the MIX 1:1 activated cells not only were more effective, but also reduced the risk of GVHD when compared with PBMC. This finding has significant implications in allogeneic CD56\(^+\) cell therapy, either alone or in combination with anti-GD2 ADCC, as any immunosuppressive prophylaxis or treatment of GVHD will hinder the efficacy of tumor control. Compared with normal PBMCs, a lower frequency of CD56\(^+\) cells was consistently observed in patients with neuroblastoma after G-CSF mobilization (3%–10% in normal vs. 0.28% on average from 5 patient samples). With such a low beginning amount of CD56\(^+\) cells, we could not expand CD56\(^+\) cells using the PBMC method. However, expansion and activation of CD56\(^+\)

Figure 6. The mice treated with activated CD56\(^+\) cells had significant reduction of tumor growth and longer survival. A, representative bioluminescence pictures of NB-EB-ununtreated control mice and treated mice on day 14. B, progression of NB-EB as detected by bioluminescence in untreated control (closed circle) and mice receiving MIX 1:1 of fresh products (close triangle) or day 21 activated products (open circle). Data were plotted as mean \pm SEM. C, Kaplan–Meier survival curve of NB-EB control mice (closed circle) and those treated with fresh (close triangle) or day 21 activated (open circle) products. D, Kaplan–Meier survival curve of SK-N-SH control mice (closed circle) and those treated with fresh (close triangle) or day 21 activated (open circle) products. E, representative histopathologic evidences of GVHD were noted in the skin, liver, small intestine, and large intestine of the SK-N-SH mice treated with fresh products. The pictures were taken with >200 magnification. The lines on pictures were scale bars in micrometer. The numbers of mice included in this experiment were as follows: NB-EB group (control, \(n = 27\); fresh product, \(n = 17\); and activated product, \(n = 20\)); SK-N-SH group (control, \(n = 32\); fresh product, \(n = 10\); and activated product, \(n = 25\)). The significance levels were as follows: *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\).
cells were successfully achieved by the MIX 1:1 approach (Fig. 7). Given that most patients with high-risk neuroblastoma will receive autologous hematopoietic stem cell transplantation (HSCT; ref. 38), a small aliquot from an autologous stem cell collection may be used conveniently as an alternative starting cells for CD56\(^+\) activation. Our previous study in patients with acute myelogenous leukemia (AML) infused a median of 3 \(\times\) 10\(^7\) CD56\(^+\) cells/kg (31). Assuming a 100-fold expansion of CD56\(^+\) cells with our culture system, only 3 \(\times\) 10\(^5\) starting CD56\(^+\) cells are needed (equivalent to 21.4% of CD34\(^+\) fraction of PBSC products, based on an average of 0.28% CD56\(^+\) and 5 \(\times\) 10\(^8\) white blood cells/kg in the PBSC collections). The activated CD56\(^+\) cells could then be given before autologous HSCT as part of the conditioning regimen, or after HSCT in conjunction with standard anti-GD2 and cytokine therapy. Although our data revealed higher cytotoxic activity of CD56\(^+\) cells from allogeneic healthy donors than patients with neuroblastoma, autologous activated CD56\(^+\) cells from patients were still highly lytic, and therefore may be used as an alternative or supplement to allogeneic activated CD56\(^+\) cells.

In conclusion, our activation method yields abundant allogeneic or autologous CD56\(^+\) cells that may overcome inherent NK-resistance in neuroblastoma through the NCR, DNAM-1, and perforin pathways. This method reduces the technical difficulty, cost, and GVHD risk.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

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