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 immune cells (NK cells) from normal donors and neuroblastoma patients. Enriched CD56+ cells from peripheral blood were mixed with CD56− fraction at 1:1 ratio and cultured in the presence of OKT3, IL-2 and IL-15 for 5 days, and then without OKT3 for 16 more days. The final products contained >90% CD56+ cells and could kill neuroblastoma cells effectively that were originally highly resistant to non-processed NK cells. Mechanistically, cytolysis of neuroblastoma was mediated through NCR, DNAM-1 (CD226), perforin, and granzyme B. Successful clinical scale-up in a GMP-compliant bioreactor yielded effector cells that in a neuroblastoma xenograft model slowed tumor growth and extended survival without graft-versus-host disease (GvHD). Investigation of CD56+ cells from neuroblastoma patients revealed a similar post-activation 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 >90% cure in patients with locoregional tumors requiring little to no cytotoxic therapy, to <50% survival despite aggressive therapy in those >18 months of age at diagnosis with metastatic disease, or those with MYCN gene amplification (5, 6).

The standard treatment for high-risk neuroblastoma includes intensive chemotherapy, radiation therapy, 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 non-cross resistance and less overlapping side effects with current therapies (7, 9, 10). For instance, the use of anti-GD2 (disialoganglioside) mAbs, combined with Interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF) given after completion of standard intensive treatment, has been proven to be beneficial in high-risk neuroblastoma patients (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 5-10% 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⁺ NKT 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 by a balance between inhibitory and activating signals generated by the interactions between receptors on NK cell surface and their ligands expressed on target cells (17, 18). Target cell lysis occurs when the signals from activating receptor-ligand interactions dominate over those from inhibitory receptor. The best characterized NK cell receptors are killer cell immunoglobulin-like receptors (KIRs), which recognize major histocompatibility complex (MHC) class I human leukocyte antigen (HLA)-A, -B and -C (19). The other clinically important NK cell receptors include inhibitory receptor NKG2a, and activating receptors natural cytotoxicity receptors (NCRs NKp30, NKp44, NKp46), NKG2D, 2B4, DNAM-1 and NTB-A (20). The mechanisms of NK cell killing include the release of cytotoxic granules containing granzyme and perforin causing target cell apoptosis.
The role of NK and NKT cell therapy in neuroblastoma has not been well established. We and others have shown in preclinical models and preliminary clinical analyses that many immunoregulatory elements may be involved in NK or NKT cell lysis of neuroblastoma cells, including KIR, CD16, TLR9, BDCA4-derived IL6, NCRs and DNAM-1 (21-27). Herein, we studied an expedient CD56+ cell activation strategy that can overcome neuroblastoma resistance with minimal risk of GvHD. Our method was not only able to activate and expand CD56+ NK cells, but also CD56+ NKT cells. We elucidated the receptor-ligand interactions involved in the cell-mediated cytolysis. Large scale activation of CD56+ cells using good manufacturing practices (GMP) was established for allogeneic and autologous settings in preparation for future clinical trials.

Materials and Methods

Cell preparation and isolation

Peripheral blood mononuclear cells (PBMCs) were collected from healthy donors and from neuroblastoma patients who were undergoing autologous stem cell apheresis, with approval from institutional review board (IRB) and written informed consent from donors, patients or legal guardians. Briefly, the specimens were layered over Ficoll-Hypaque gradient (GE Healthcare, Piscataway, NJ) and centrifuged for 20 minutes at 2300 rpm. The light-density fraction was collected, washed, and used immediately. For CD56+ isolation, the PBMCs were labeled with CD56 microbeads (Miltenyi Biotec, Auburn, CA) and separated using autoMACS following the manufacturer’s instruction.

CD56+ cell activation
Immediately after CD56\(^+\) cell isolation, the CD56 positive fraction was mixed with the CD56 negative fraction at a 1:1 ratio. This ratio was chosen based on preliminary experiments showing optimal cytotoxicity and expansion (Supplementary Figure S1a and b). The cells were then seeded at the density of 1×10\(^6\) cells/mL and activated in a culture cocktail consisting of SCGM (CellGenix GmbH, Freiburg, Germany), 500 U/mL IL-2 (Aldesleukin (Proleukin), Novartis Pharmaceuticals, Emeryville, CA), 10 ng/mL IL-15 (CellGenix GmbH), 10 ng/mL OKT3 (Biolegend, San Diego, CA), and 5% human AB serum (Lonza Group, Walkersville, MD). Under this culture condition, a large amount of IFN-\(\gamma\) was found in the supernatant in addition to IL-6 and IL-17 (Supplementary Figure S1c). After 5 days of culture, the cells were washed twice with PBS and reseeded at 1×10\(^6\) cells/mL with identical culture medium but without OKT3. The cells were replenished with freshly prepared medium every three days for 16 more days (total 21 days). In some experiments, PBMCs were not purified for CD56\(^+\) cells and were seeded unfractionated at the density of 1×10\(^6\) cells/mL (PBMC group). The cells were then activated using the same culture cocktails and maintained using the same procedure as described above in the MIX 1:1 group.

**Flow cytometry**

In order to determine cell population changes at different time points during the course of CD56\(^+\) cell activation, the percentages of T (CD3\(^+\)CD56\(^-\)), NKT (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 (11PB6, EB6.B), anti-CD158b (CH-L, GL183), anti-NKB1 (DX9), anti-DNAM-1 (DX11), anti-
CD11a (HI111), anti-NTBA (292811), anti-CD244, anti-CD3 (SK7, UCHT1), antigranzyme B (GB10), anti-NKG2a (Z199), anti-NKp30 (Z25), anti-NKp44 (Z231), anti-NKp46 (BAB281), anti-NKG2D (1D11), anti-CD56 (MY31, N901) and anti-CD14 (MphiP9). Flow cytometry analyses were performed with LSRII (BD Biosciences, San Jose, CA) and FlowJo 8.8.6 software (Tree Star, OR).

**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 CHLA-90, CHLA-255 (from Dr C. Patrick Reynolds of Children's Hospital of Los Angeles) and SK-N-SH (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagles medium (Life Technologies, Invitrogen, Grand Island, NY). The NB-1691, NB-1643, NB-EB (from Dr Peter Houghton, Columbus, OH), SK-N-AS (ATCC) and positive control K562 (ATCC) cell lines were cultured in RPMI medium (Cellgro, Mediatech, Inc., Manassas, VA). All media were supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Inc., Lawrenceville, GA) 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, MA) following the manufacturer’s instruction. BATDA-labeled neuroblasoma cell lines were used as target cells at an effector-to-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 co-cultured with neuroblastoma target cells at 5:1 ratio in the presence of anti-CD107a-FITC or -APC antibodies. After 1 hour of co-culture, GolgiStop™ (BD Bioscience) 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<sup>+</sup>CD56<sup>+</sup> cells (28).

**Large scale activation**

Apheresed peripheral blood cells from normal donors were purchased (Key Biologics, Inc, Memphis, TN) and purified for CD56<sup>+</sup> cells on the CliniMACS instrument (Miltenyi Biotec, Auburn, CA) (23). CD56<sup>+</sup> cells were then expanded ex vivo in a starting 1:1 ratio of CD56<sup>+</sup>:CD56<sup>−</sup> cells at a density of 5 x 10<sup>5</sup> cells/mL in two T-300 flasks (Midwest Scientific, St. Louis, MO). The cells were initially cultured in a base medium [SCGM (Cellgenix), 5% human AB serum (Lonza), 10 ng/mL IL-15 (Cellgenix), 500 units IL-2/mL (Novartis)], with 10 ng/mL of OKT3 (Centocor Ortho Biotech, L.P., Horsham, PA). After 5 days in culture, the cells were harvested from the flasks and washed twice with phosphate-buffered saline (PBS) (Lonza) by centrifugation at 600 x g for 15 min. The cells were re-suspended in 200 mL of base medium without OKT3 and placed in a WAVE 2L Cellbag prepared per manufacture recommendations (GE Healthcare,
Piscataway, NJ). 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 2-3 days to maintain a cell density of 1-2 x 10^6 cells/mL. When the volume of the culture was greater than 1000 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 2-3 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**

NOD-SCID IL-2γc−/− (NOG) mice of 8-12 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 one day before 1×10^6 neuroblastoma cells were intraperitoneally injected. On the following day, 1×10^6 MIX 1:1 effector cells obtained from Day 0 or Day 21 large-scale activation were intraperitoneally injected. Human IL-2 (100 U/mL, Novartis Pharmaceuticals, Emeryville) was intraperitoneally injected starting a day after injection of effector cells and continued every three days for a total of three weeks. In order 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 two groups was calculated using Students’ paired t-test. In cases of having more than two groups, one-way ANOVA was used. For the survival analysis, Kalper-Meier 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 (Figure 1a). After CD56$^+$ cell isolation from PBMCs, the CD56$^+$ fraction was mixed with the CD56$^-$ fraction in a 1:1 ratio and co-cultured for 21 days. The susceptibility of seven different neuroblastoma cell lines was tested. All seven 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 (Figure 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 (Figure 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 five, NKT cells in four, and equal proportion of NK cells and NKT cells in two (Figure 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 (Figure 2b). Extensive characterization of the phenotypes of the activated CD56+ cells showed that the surface expressions of activating receptors and adhesion molecules (NKp30, NKp44, NKG2D, DNAM-1 and CD11a) were significantly increased on Day 21 activated CD56+ cells compared with Day 0 fresh CD56+ cells (Figure 2c). In contrast, there was no statistical difference in inhibitory receptor expression (KIR2DL1, KIR2DL2/3, KIR3DL1 and NKG2a) between Day 0 and Day 21 CD56+ cells.

**Essential role of natural cytotoxicity receptors (NCRs) and DNAX accessory molecule-1 (DNAM-1) in activated cell-mediated killing of neuroblastoma**
We sought to determine the role of each upregulated receptor (including NKp30, NKp44, NKp46 and DNAM-1) in overcoming neuroblastoma resistance. We performed antibody-blocking experiments using Day 21 activated CD56+ cells as effectors (Figure 3a). Among the seven 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 performed. After co-incubation of activated CD56+ cells (effectors) and neuroblastoma cell lines (targets) at an effector:target ratio of 5:1, degranulation was observed in all experiments (Figure 3b). The levels of CD107a positivity correlated well with the relative levels of cytotoxicity measured by BATDA assay (Figure 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) (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 fold and 111.6 fold
respectively (Figure 4a). Median purity of CD56\(^+\) cells in MIX 1:1 products was 91.5%, compared to 34.3% in PBMC products (Figure 4b). The phenotypes of CD56\(^+\) cells in two cultures are similar, except PBMC products had significant increased in inhibitory KIR2DL1, NKp46 and NTB-A, but not NKp30 and NKG2D as in MIX 1:1 cultures (Supplementary Figure S1d). When used as effectors against 3 representative neuroblastoma cell lines (NB-EB, CHLA-90 and SK-N-SH), activated CD56\(^+\) cells from MIX 1:1 products had superior cytotoxic activity compared to those by the PBMC method (Figure 4c). By defining a < 25% specific lysis as ineffective killing, 6 out of 15 products (40%) by the PBMC method were ineffective against NB-EB. In contrast, only 1 out 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 to 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 good manufacturing practice (GMP)-compliant components. Three separate experiments were performed 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 (Figure 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\(^+\) cells when compared to fresh CD56\(^+\) cells (Figure 5b).

**In vivo anti-tumor and graft-versus-host (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 graft-versus-host disease (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 Figure 6a. Comparing the last time-point on Day 24 and 1\(^{st}\) time-point on Day 4, there was no significant increase in bioluminescence signals in the mice receiving activated products against NB-EB (Figure 6b). Progressive disease, however, was observed in both the control untreated group and fresh cell group. Treatment with activated products, but not fresh products, significantly prolonged the survival of the mice compared to the control group (Figure 6c). Similarly for SK-N-SH, the activated products significantly prolonged the survival of the mice (Figure 6d). In contrast, the mice receiving fresh products died rapidly with clinical signs of severe GvHD, which were confirmed by histopathology (Figure 6e). In skin, there was dense dermal lymphocytic infiltrate with focal vacuolar changes in basal cells, focal separation of dermoeipidermal 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 histopathological 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 of neuroblastoma.

The use of activated product from neuroblastoma patients

Besides allogeneic NK cell therapy, we postulated that aliquotes from 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 high-risk neuroblastoma patients. 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 to cells from healthy donors (Figure 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, while all inhibitory receptors including KIRs and NKG2a remained unchanged (Figure 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 (Figure 7c), though lower than those obtained from healthy
donor (Figure 1b), possibly in part because of higher expression of NKG2a and lower expression of NKG2D and 2B4 (Figure 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 in 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 demonstrated that several novel NK cell activation methods using various cytokine combinations such as IL12 and IL18 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 modifications 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 to 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−, our end products using the MIX 1:1 method were always >90% CD56+ (Figure 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 histopathological findings. This observation suggested that our method not only increased anti-neuroblastoma 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 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 non-essential 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.

Since 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 (Figure 5), and their efficacy in controlling neuroblastoma was confirmed in the mouse experiments. Notably, the MIX 1:1 activated cells were not only more effective, but they 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 to normal PBMCs, a lower frequency of CD56+ cells was consistently observed in neuroblastoma patients after GCSF mobilization (5-10% in normal versus 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 were successfully achieved by the MIX 1:1 approach (Figure 7). Given that most high-risk neuroblastoma patients will receive autologous HSCT (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 AML patients
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 $3 \times 10^5$/kg starting CD56$^+$ cells is 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 comparing to neuroblastoma patients, 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.
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Figure legends

Figure 1

Increase in cytotoxicity of CD56+ cells from the MIX 1:1 activation method. (A) Schematic presentation of the MIX 1:1 strategy used for activating CD56+ cells. (B) Cytotoxicity of Day 0 fresh (blue) and Day 21 activated (red) CD56+ cells against NB-1691, NB-1643, NB-EB, SKN-AS, CHLA-90, CHLA-255 and SK-N-SH neuroblastoma cells. (C) Increase in cytotoxicity of CD56+ cells overtime against NB-EB, CHLA-90 and SK-N-SH during the 21 days of cell activation. Effector to target ratio was 5:1. Bars
represent the median specific lysis. The significance levels were as follows: ** $p<0.01$; *** $p<0.001$.

**Figure 2**
Expansion of CD56$^+$ NK and NKT cells with increased expression of activating receptors, but not inhibitory KIRs. (A) Percentages of CD56$^+$CD3$^+$ (NKT) and CD56$^+$CD3$^-$ (NK) cells in CD56$^+$ cells on Day 0 and 21 from MIX 1:1 culture. Data are from 11 independent experiments. (B) Cytotoxicity of Day 21 activated CD56$^+$ NKT and NK cells against neuroblastoma cell-lines. The NKT and NK cells were flow-sorted and used as effector at effector: target ratio 5:1. The cytotoxicity of NK cells were normalized as 100% and compared with that of NKT cells. Data are from representative of three independent experiments. (C) Flow cytometry 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 on CD56$^+$ cells. * $p<0.05$.

**Figure 3**
Activated CD56$^+$ cells could use different pathways to overcome NK-resistance. (A) Blocking experiments used anti-NKp30, NKp44, NKp46 and DNAM-1 antibodies, with the Day 21 activated CD56$^+$ cells as effectors and neuroblastoma cell lines as targets. Effector to target ratio was 5:1. Cell lysis was almost completely abolished after NCRs and DNAM-1 were blocked. Results were normalized to the groups with isotype control. Data represented the mean +/- SEM. The significance levels were as follows: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. (B) Degranulation of activated CD56$^+$ cells triggered by
neuroblastoma cells. CD107a assay was performed with Day 21 activated CD56+ cells from MIX 1:1 culture. Numbers in the gated regions are the percentage of CD107a+ cells. Data are representative of three independent experiments.

Figure 4
Activation of CD56+ cells by MIX 1:1 method was superior to that by larger volume PBMC method. (A) Comparison of fold increase in CD56+ cell numbers between PBMC and MIX 1:1 methods. Bars represent the median of the fold increase after 21 days. (B) Distribution of CD56+ cells and CD56− cells on Day 21 using PBMC and MIX 1:1 method. The results were from seven different independent experiments on PBMC cultures and ten on MIX 1:1 cultures. (C) Cytotoxicity of Day 21 activated CD56+ cells from PBMC and MIX 1:1 cultures against neuroblastoma cell-lines NB-EB, CHLA-90 and SK-N-SH. Bars represent the median percent lysis. The gray areas (< 25%) are the thresholds to define resistance to NK cell-mediated cytotoxicity.

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-to-target ratio of 5:1).
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 untreated 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 +/- 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 200X magnification. The lines on pictures were scale bars in μm. 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.

Figure 7

Efficient expansion of CD56⁺ cell with MIX 1:1 method using neuroblastoma patient cells. (A) Fold increase of the CD56⁺ cells after 21 days of CD56⁺ activation. (B) Mean fluorescence intensity of NK cell receptors were compared and summarized. (C) Cytotoxicity of fresh (blue) and Day 21 MIX 1:1 activated (red) CD56⁺ cells against
seven neuroblastoma cells. (D) Comparison of NKG2a, NKG2D, and 2B4 expression levels on CD56+ cells of activated products from normal donors and neuroblastoma patients. Bars represent medians. The significance levels were as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. 
**Figure 1**

A

PBMCs ↓

CD56+ isolation

CD56 negative  CD56 positive

(1:1 ratio of neg : pos fraction)

CD56+ cell activation

B

Fresh CD56+ cells

Activated CD56+ cells

% Specific lysis

NB-1691  NB-1643  NB-EB  SK-N-AS  CHLA-90  CHLA-255  SK-N-SH

C

NB-EB

CHLA-90

SK-N-SH

% Specific lysis

Time (Day)

0  14  21

0  20  40  60  80  100

** * ***

*** ***

*** ***
Figure 2

A

B

C

NK
NKT

Percentage of cells

Time (Day)

Specific lysis

NB-1691 NB-1643 NB-EB CHLA-90 CHLA-255

KIR2DL1

KIR2DL2/3

KIR3DL1

NKG2a

NKp30

NKp44

NKp46

NKG2D

DNAM-1

NTB-A

2B4

CD11a
Figure 3

A

![Figure A showing relative specific lysis for different cell lines and conditions.](image)

**Relative % Specific Lysis (E/T ratio 5:1)**

B

![Figure B showing CD56+ cell alone, NB-EB alone, and combinations.](image)

**CD56**

- **CD56** cell alone
- **CD56** and NB-EB
- **CD56** and CHLA-90
- **CD56** and SK-N-SH
Figure 4

A

Fold Increase

PBMCs MIX 1:1

Day 21 MIX 1:1

PBMCs

CD56+

CD56-

CD56

Day 21

B

C

% Specific lysis

PBMCs MIX3

PBMCs MIX3

PBMCs MIX3

NB-EB CHLA-90 SK-N-SH
Figure 5

A

- KIR2DL1
- KIR2DL2/3
- KIR3DL1
- NKG2a
- NKp30
- NKp44
- NKp46
- NKG2D
- DNAM-1
- NTB-A
- 2B4
- CD11a

B

% Specific lysis

Fresh CD56+  Activated CD56+

NB-EB  SK-N-SH
Figure 6

A

Day 14

NB-EB

NB-EB + fresh product

NB-EB + activated product

B

Log_{10} [bioluminescence]

Time (Day)

Control

Fresh MIX 1:1

Activated MIX 1:1

C

Percent survival

Time (Day)

Control

Fresh MIX 1:1

Activated MIX 1:1

D

Percent survival

Time (Day)

Control

Fresh MIX 1:1

Activated MIX 1:1

E

skin

liver

Small intestine

large intestine
Figure 7

A

Fold increase

250
200
150
100
50
0
PBMCs MIX 1:1

B

CD11a

NKp30

NKp44

DNAM-1

Mean Fluorescence Intensity of CD11a

Mean Fluorescence Intensity of NKp30

Mean Fluorescence Intensity of NKp44

Mean Fluorescence Intensity of DNAM-1

Fresh CD68* Activated CD68*

KIR2DL1

KIR2DL2/3

KIR3DL1

NKG2a

Mean Fluorescence Intensity of KIR2DL1

Mean Fluorescence Intensity of KIR2DL2/3

Mean Fluorescence Intensity of KIR3DL1

Mean Fluorescence Intensity of NKG2a

Fresh CD68* Activated CD68*

C

Fresh NK

Activated NK

% Specific Lysis

D

Mean Fluorescence Intensity of NKG2a

Mean Fluorescence Intensity of NK2D0

Mean Fluorescence Intensity of 2B4

normal donor NB patient

normal donor NB patient

normal donor NB patient
Ex vivo activation of CD56+ immune cells that eradicate neuroblastoma

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