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Original Article

Enhanced cytotoxicity and underlying mechanism of ex vivo expanded natural killer cells against breast cancer cells

Gege Chen[1*], Rong Wei[1*], Lu Gao[1*], Jun Hou[1], Huiqun Wu[1], Dandan Yu[1], Weibing Zu[2], Yiwen Zhang[1], Xi Sun[1], Gaomei Chang[1], Bojie Dai[3], Yi Tao[1], Jumei Shi[1]

[1]Department of Hematology, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai, P. R. China; [2]Department of General Surgery, Haimen People's Hospital, Jiangsu, P. R. China; [3]School of Life Science and Technology, Tongji University, Shanghai, P. R. China. [*]Equal contributors.

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Abstract: Despite significant advances in treatment, breast cancer, especially the metastatic breast cancer, usually can't be cured by current therapies and has a poor prognosis. To develop more effective therapy for patients with breast cancer, we examined the potential of ex vivo expanded natural killer (NK) cells to exert anti-tumor cytotoxicity. We used K562-mb15-41BBL cells to expand NK cells from peripheral blood mononuclear cells (PBMCs) of healthy donors (HD). We then investigated the expression of NK cell receptors and NK cell cytotoxicity against breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-468 and patient breast cancer cells (n=4), with and without the addition of neutralizing antibodies. As a result, NK cells were sharply expanded to a mean of 254.4-fold (range: 93.4-436.7, n=8). Cell expansion led to NK cell receptor activation, with remarkable up-regulation of activating molecules NKG2D, NKp30, NKp44, CD26, CD69, CD70 and DNAM-1, and resulted in enhanced cytotoxicity against three breast cancer cell lines and primary breast cancer cells (n=4), while sparing patients' normal PBMCs. Blocking studies confirmed that allogeneic NK cell cytotoxicity is established through these multiple activating receptor-ligand interactions. Taken together, the present study demonstrates that large numbers of NK cells with potent cytotoxicity against breast cancer cells can be generated from PBMCs of HD, providing a necessary preclinical evidence for clinical evaluation of these ex vivo expanded NK cells in patients with breast cancer.

Keywords: Natural killer cells, expansion, ex vivo, K562-mb15-41BBL cells, breast cancer

Introduction

Breast cancer is the most common malignancy and the leading cause of cancer-related death in women, with more than 1.6 million new cancer cases diagnosed and 52,180 deaths worldwide in 2012 alone [1]. Despite significant advances in early detection and treatment, a considerable amount of breast cancers metastasize to distant parts of the body. These metastatic breast cancers usually can't be cured by current therapies and have a poor prognosis [2]. In addition, the triple-negative breast cancer, accounting for approximately 15-20% of all breast cancers, also has a poor prognosis owing in large part to the absence of effective targeted therapies [3]. Therefore, there is a high demand for new therapeutic strategies to improve the prognosis of these breast cancer patients.

Natural killer (NK) cells, capable of killing tumor cells and virus-infected cells in the absence of specific immunization, are a subset of peripheral blood lymphocytes and defined by CD56 or CD16 expression and absence of CD3 [4]. Previous studies have indicated that NK cells can exert potent anti-tumor effects on a variety of malignancies including breast cancer in vitro and in animal models [5-12]. Infusion of NK cells obtained from killer immunoglobulin-like receptor ligand-mismatched donors can effectively prevent relapse after allogeneic hematopoietic stem cell transplantation [13-15]. Moreover, allogeneic NK cells are also associated with complete remission in patients with

poor prognosis acute myeloid leukemia [16, 17]. In addition, contrary to T cells, clinical observations and results of animal models show that NK-cell infusion is well-tolerated and does not cause graft-versus-host disease (GVHD) [15, 16]. This feature, combined with their broad-spectrum anticancer activity, suggests that NK cell-based immunotherapy is a promising approach for cancer treatment.

NK cells are equipped with a variety of receptors that can either stimulate NK cell reactivity (activating receptors) or suppress NK cell reactivity (inhibitory receptors) [18, 19]. The NK cell cytotoxicity against target cells is regulated by the balance of inhibitory and stimulatory signals generated by the interaction between these NK cell receptors and target cell ligands [20, 21]. Once the activating signals override the inhibitory signals, NK cells lyse the target cells. Moreover, NK cell cytotoxicity is also affected by the ratio between the number of NK cells and target cells. Thus, to achieve maximum anti-tumor effect, a sufficient number of pre-activated NK cells are needed. Irradiated K562 cells transfected with 41BBL and membrane-bound interleukin (IL)-15 (K562-mb15-41BBL) are previously reported to specifically activate human NK cells and drive these NK cells into the cell cycle, leading to large numbers of highly activated NK cells [22, 23].

In the present study, we used this system to expand NK cells from peripheral blood mononuclear cells (PBMCs) of healthy donors (HD) and investigated whether these expanded NK cells (exp-NK cells) exert potent cytotoxicity against breast cancer cell lines and patient primary breast cancer cells in vitro. Furthermore, we also determined which of the molecular mechanisms might be involved in the interaction between exp-NK cells and patient breast cancer cells.

Materials and methods

Cells and reagents

The breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MB-468 were purchased from the American Type Culture Collection (Manassas, VA, USA). MCF7, MDA-MB-231 and MDA-MB-468 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA, USA). For all cell lines, the media were supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, Carlsbad, CA, USA), and 5 μg/ml plasmocin (InvivoGen, San Diego, CA). The K562-mb15-41BBL cell line was available in our laboratory. Fresh breast tumors were received after surgery from 4 newly diagnostic breast cancer patients who gave informed consent according to procedures approved by the Shanghai Tenth People's Hospital Institutional Review Board. Blood samples were obtained following acquisition of the study participants' written informed consent. The tumors were sterilely minced in DMEM medium to yield less than 2 × 2-mm pieces and cultured in DMEM containing 20% FBS and 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2. Attached cells were propagated after 2-3 weeks. Cells were used as targets in cytotoxicity assays. All fluorescently-labeled antibodies to CD3, CD14, CD19, CD33, CD56, NKG2D, NKp30, NKp44, NKp46, CD26, CD69, CD70, DNAM-1, 2B4, NTB-A, KIR2DL1 (CD158a), KIR2DL2/L3 (CD-158b), KIR3DL1 (NKB1), KIR2DL3 (NKAT2), CD94, TRAIL and Fas-ligand (L) were purchased from BD Biosciences (San Jose, CA, USA). Propidium iodide (PI) and annexin V were also purchased from BD Biosciences (San Jose, CA, USA). Dynabeads CD3 and CD56 were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Concanamycin A (CMA) was purchased from Sigma (St. Louis, MO, USA).

Ex-vivo NK cell expansion

NK cells were expanded from PBMCs isolated from human peripheral blood samples from HD by density gradient centrifugation. In brief, PBMCs were incubated with irradiated (100 Gy) K562-mbIL15-41BBL cells at a ratio of 1.5:1 in RPMI-1640 medium with 10% fetal bovine serum and 200 IU/ml human IL-2 (R&D Systems, Minneapolis, MN, USA). Medium was exchanged every 2 days with fresh medium and IL-2. The cells then were re-stimulated with K562 transfectants on day 7 and collected on day 14 for assays. At day 0 and day 14 of co-cultures, the number of NK cells ($CD3^-CD56^+$), NKT cells ($CD3^+CD56^+$), T cells ($CD3^+$), B cells ($CD19^+$), monocytes ($CD33^+CD14^+$), myeloid cells ($CD33^+CD14^-$) and total cells were detected by flow cytometry. The number of viable NK cells was determined by staining with PI and

Table 1. The composition of cell population before expansion and after expansion

Donor	$CD3^-CD56^+$ NK cells (%)		$CD3^+CD56^+$ NKT cells (%)		$CD3^+$ T cells (%)		$CD19^+$ B cells (%)		$CD33^+CD14^+$ monocytes (%)		$CD33^+CD14^-$ myeloid cells (%)	
	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14
1	7.4	71.2	3.6	2.1	59.6	17.3	22.8	9.6	10	1	0.6	1.3
2	6.9	89.3	4.6	3.8	67.5	4.3	13.8	2.9	14.3	0.6	0.3	0.8
3	5.2	76.4	1.8	1.9	71.8	14.6	8.6	6.3	10.5	2.6	3.3	1.7
4	24.8	83.4	2.5	5.2	44.2	8.4	14.3	3.5	11.3	1.5	9.5	0.8
5	4.1	74.5	5.4	3.6	71.7	12.7	11.8	10.5	11.2	0.9	3.1	1.1
6	5.2	96.4	7.3	1.7	72.3	3	16.4	0.3	19.8	0.8	1	0.9
7	14.8	94.0	3.7	2.9	64.5	2.1	9.5	1.8	8.3	1.2	4.2	0.5
8	12.3	97.0	0.4	0.9	48.1	1.1	25.2	0.3	12.6	0.5	2.9	0.4
Mean	10.1	85.3	3.7	2.8	62.5	7.9	15.3	4.4	12.3	1.1	3.1	0.9
SD	7.0	10.4	2.2	1.4	11.0	6.2	6.0	4.0	3.5	0.7	2.9	0.4
Range	4.1-24.8	71.2-97.0	0.4-7.3	0.9-5.2	44.2-72.3	1.1-17.3	8.6-25.2	0.3-10.5	8.3-19.8	0.5-2.6	0.3-9.5	0.4-1.7

NK, natural killer; NKT, natural killer T; SD, standard deviation.

annexin V. Fold expansion was calculated by dividing the number of viable subset cells at day 14 by the number of the cells at the beginning of the culture.

Phenotypic analysis by flow cytometry

At day 0 and day 14 of co-cultures, cells were resuspended at a density of 1×10^6/ml, stained with fluorescent antibody CD3PerCp, CD56FITC and PE coupled antibodies against NK cell activating or inhibitory receptors or indicated antibodies at room temperature away from light for 30 minutes (min), washed with phosphate buffer solution (PBS) for three times, and then detected by flow cytometry to determine the cell surface expression. In the analysis, 10,000 cells were scored.

Intracellular staining

Exp-NK cells were stained for intracellular perforin (PE-conjugated antibody from BD Pharmingen, San Diego, CA, USA) and granzyme B (PE-conjugated antibody from BD Pharmingen, San Diego, CA, USA) after fixation (4% paraformaldehyde) and permeabilization (0.1% saponin, 1% BSA, 0.05% Tween-20 in PBS). They were evaluated on flow cytometric analysis. Data were presented in dot plot.

^{51}Cr release cytotoxicity assay

Before and after 14 days of cell expansion, the $CD3^-CD56^+$ NK cells were separated from PBMCs by magnetic cell sorting system and the cell purity was determined to be more than 95%. If purity of exp-NK cells were > 95%, the NK cells were directly used in cytotoxicity assays. Cytotoxicity mediated by NK cells against MCF-7, MDA-MB-231, MDA-MB-468, patient breast cancer cells (n=4) and patients' normal PBMCs was studied using a standard 4-hour (h) ^{51}Cr release assay. We used 15:1, 7.5:1 and 1:1 of effector-to-target (E:T) ratios for all assays, except when differently indicated. Target cells were first labeled with 100 μCi ^{51}Cr sodium chromate for 1.5 h and then washed extensively. Then effector cells were co-cultured with target cells in 96-well v-bottom plate. After 4 h incubation at 37°C with 5% CO_2, the supernatants were collected for the gamma counter. Specific lysis percentage was calculated as (test release-spontaneous release)/(maximal release-spontaneous release) × 100. All experiments were performed in triplicate wells. For NK cell receptor blocking experiments, we first incubated NK cells with human IgG (1 μg/10^5 cells) (Invitrogen, Carlsbad, CA) on ice for 20 min in order to prevent antibody-dependent cell-mediated cytotoxicity and then incubated at room temperature with various monoclonal antibodies (mAbs) before addition of target cells. Un-conjugated purified antibodies to: isotype, NKG2D, NKp30, NKp44, NKp46, CD26, CD69, CD70, DNAM-1, 2B4 and NTB-1 were used alone or in combination in the blocking experiment. All mAbs were present at the final concentration of 10 μg/ml.

Statistical analysis

The data were analyzed using the Student's t-test to determine their statistical significance.

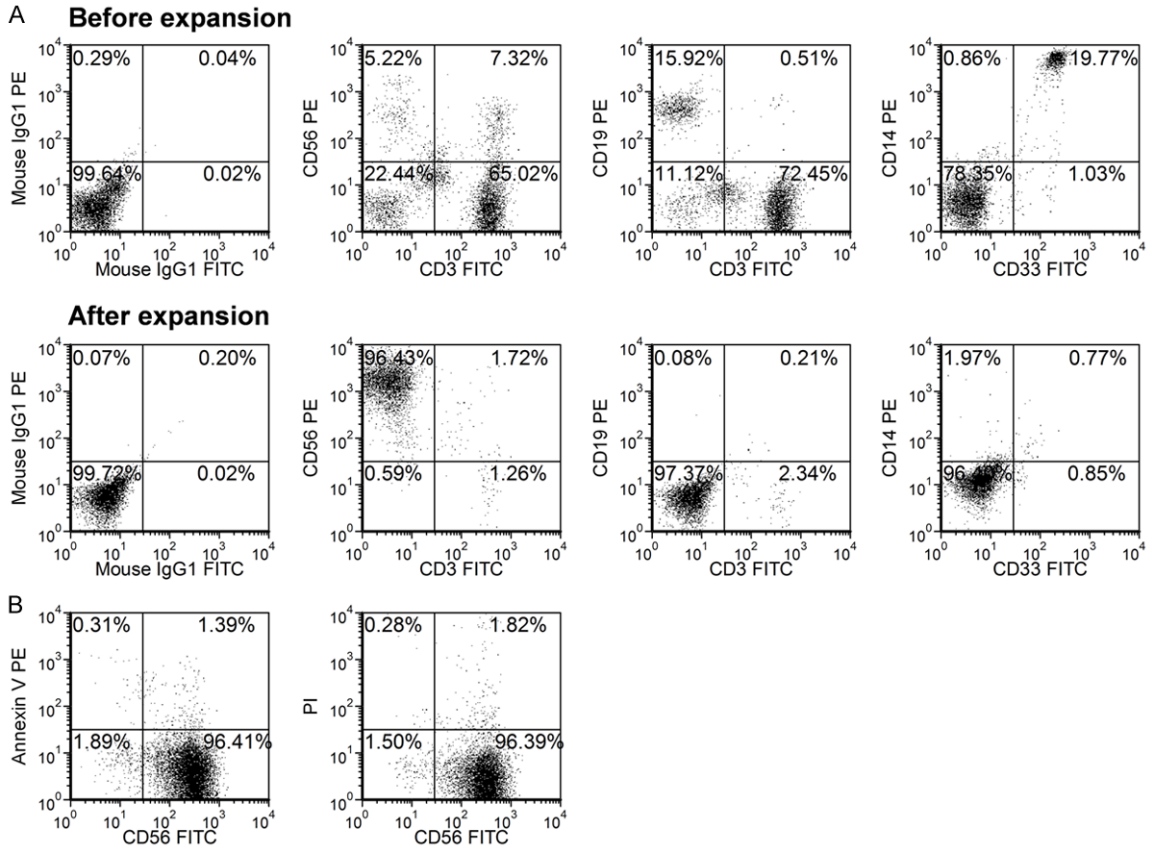


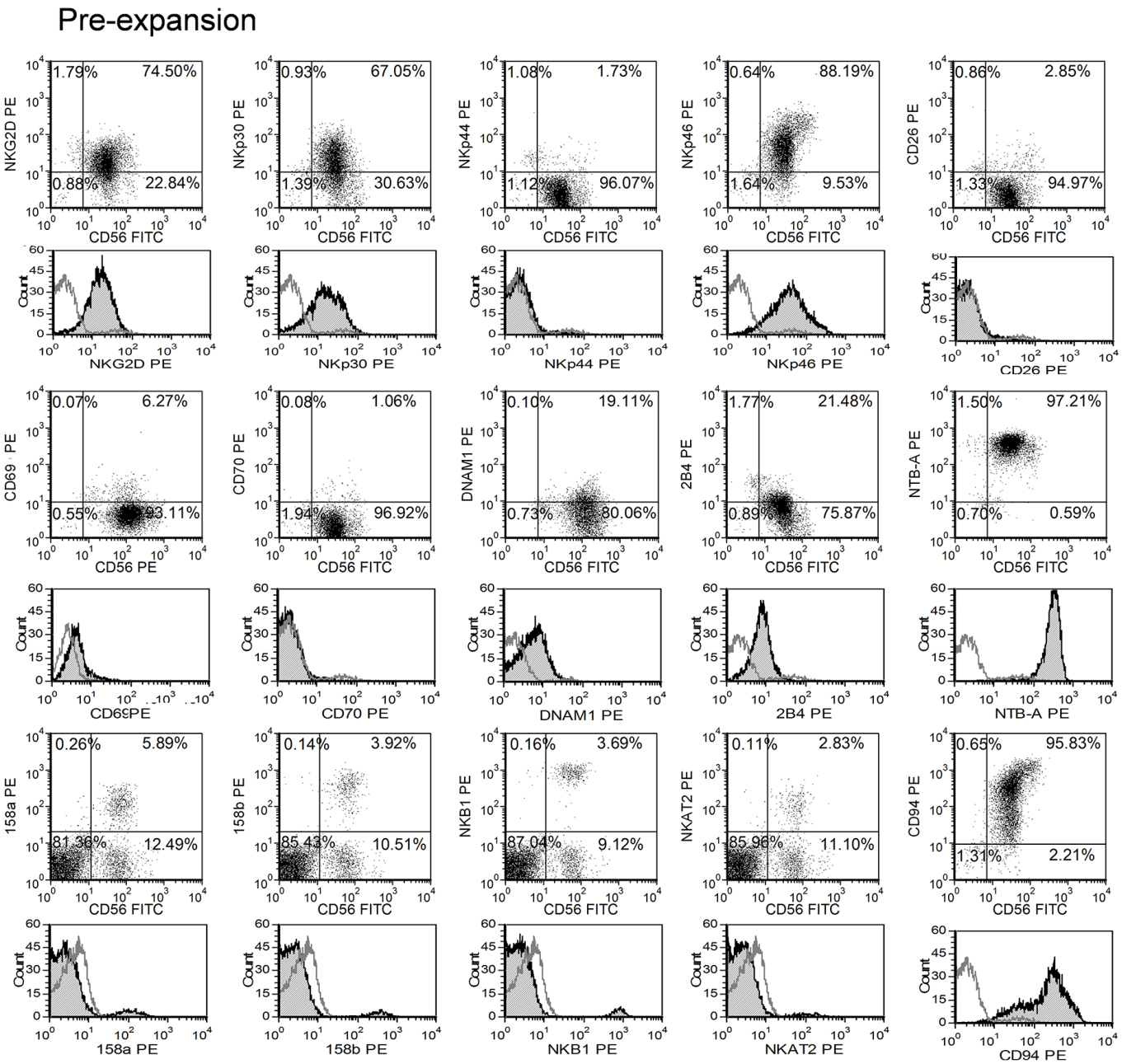
Figure 1. Immunophenotypic features of the cell populations before expansion and after expansion by co-culturing PBMCs with K562-mb15-41BBL cells in the presence of 200 IU/ml of IL-2 using flow cytometry analysis. A. Distribution of cell populations ($CD3^-CD56^+$ NK cells, $CD3^+CD56^+$ NKT cells, $CD3^+$ T cells, $CD19^+$ B cells, $CD33^+CD14^+$ monocytes, $CD33^+CD14^-$ myeloid cells) before expansion and after expansion. B. Viability of expanded NK cells ($CD56^+$, Annexin V^- PI^-) at day 14. The data shown are representative of one of eight healthy donors. NK, natural kill; NKT natural killer T; PI, propidium iodide.

The values were given as mean ± standard deviation (SD). $P < 0.05$ was considered to be significant.

Results

NK cells, but not T cells, are significantly expanded by co-culturing PBMCs with K562-mb15-41BBL cells

To achieve sufficient human NK cells, we developed an approach for expansion of NK cells in vitro by co-culturing PBMCs with K562 transfectants to specifically induce NK cell proliferation. PBMCs from 8 HD were co-cultured with irradiated K562 cell transfectants or normal K562 at a ratio of 1.5:1 for 14 days in the presence of 200 IU/ml of IL-2 or IL-2 alone. The composition of cell populations before expansion and after expansion is depicted in **Table 1** and **Figure 1A**. After two weeks of co-incubation, the cultures were enriched for NK cells (mean: 85.3%, range: 71.2-97.0%) and contained few T cells (mean: 7.9%, range: 1.1-17.3%), compared to PBMCs before stimulation (mean of NK cells: 10.1% and mean of T cells: 62.5%, respectively) (**Table 1**). The exp-NK cells were > 95% viable (Annexin V^- PI^-) at day 14 (**Figure 1B**). In absolute numbers, there was as an average of 254.4-fold (range: 93.4-436.7) expansion of NK cells in the K562-41BBL-IL15 cultures, compared to only an approximate 2-fold expansion of NK cells when cultured with normal K562 or IL2 alone (data not shown). No significant proliferation of T cells was observed (4-fold expansion) at day 14. At the end of incubation, the cell populations only contained a mean of 2.8% NKT cells (range: 0.9-5.2%), 4.4% B cells (range: 0.3-10.5%), 1.1% monocytes (range: 0.5-2.6%), and 0.9% myeloid cells


Pre-expansion
1.79%
74.50%
0.88%
22.84%
NKG2D PE
CD56 FITC
Count
0.93%
67.05%
1.39%
30.63%
NKp30 PE
1.08%
1.73%
1.12%
96.07%
NKp44 PE
0.64%
88.19%
1.64%
9.53%
NKp46 PE
0.86%
2.85%
1.33%
94.97%
CD26 PE
0.07%
6.27%
0.55%
93.11%
CD69 PE
0.08%
1.06%
1.94%
96.92%
CD70 PE
0.10%
19.11%
0.73%
80.06%
DNAM1 PE
1.77%
21.48%
0.89%
75.87%
2B4 PE
1.50%
97.21%
0.70%
0.59%
NTB-A PE
0.26%
5.89%
81.36%
12.49%
158a PE
0.14%
3.92%
85.43%
10.51%
158b PE
0.16%
3.69%
87.04%
9.12%
NKB1 PE
0.11%
2.83%
85.96%
11.10%
NKAT2 PE
0.65%
95.83%
1.31%
2.21%
CD94 PE

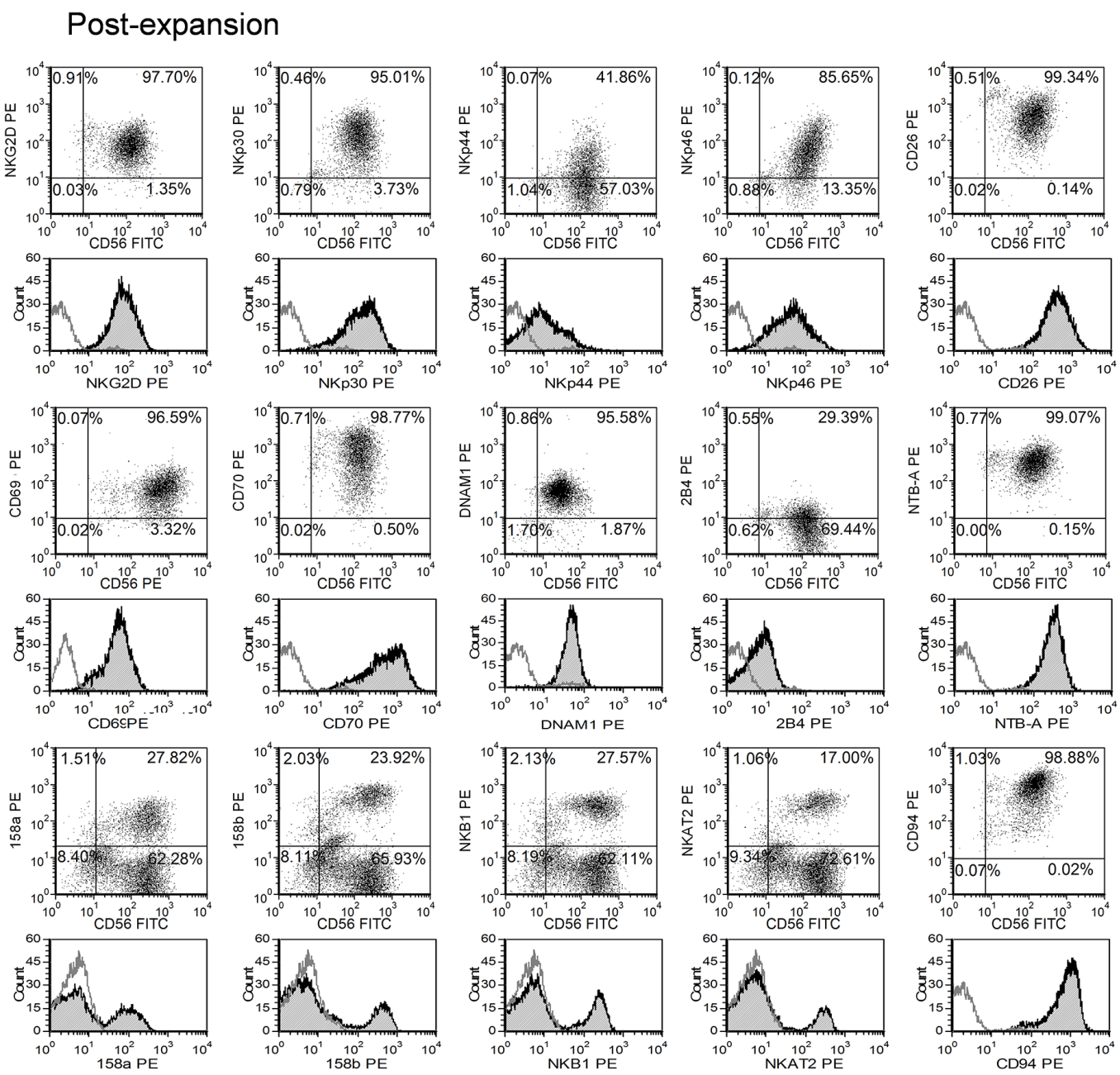

Post-expansion
NKG2D PE
CD56 FITC
0.91%
97.70%
0.03%
1.35%
Count
NKG2D PE
NKp30 PE
0.46%
95.01%
0.79%
3.73%
NKp30 PE
NKp44 PE
0.07%
41.86%
1.04%
57.03%
NKp44 PE
NKp46 PE
0.12%
85.65%
0.88%
13.35%
NKp46 PE
CD26 PE
0.51%
99.34%
0.02%
0.14%
CD26 PE
CD69 PE
0.07%
96.59%
0.02%
3.32%
CD69PE
CD70 PE
0.71%
98.77%
0.02%
0.50%
CD70 PE
DNAM1 PE
0.86%
95.58%
1.70%
1.87%
DNAM1 PE
2B4 PE
0.55%
29.39%
0.62%
69.44%
2B4 PE
NTB-A PE
0.77%
99.07%
0.00%
0.15%
NTB-A PE
158a PE
1.51%
27.82%
8.40%
62.28%
158a PE
158b PE
2.03%
23.92%
8.11%
65.93%
158b PE
NKB1 PE
2.13%
27.57%
8.19%
62.11%
NKB1 PE
NKAT2 PE
1.06%
17.00%
9.34%
72.61%
NKAT2 PE
CD94 PE
1.03%
98.88%
0.07%
0.02%
CD94 PE

Figure 2. Expanded NK cells have an activated phenotype. Flow cytometry confirms the increased cell surface density of NKG2D, NKp30, NKp44, CD26, CD69, CD70 and DNAM-1. Dot plots and histogram overlays are gated on $CD3^{-}CD56^{+}$ lymphocytes. Gray lines are isotype controls and black lines with shade are the indicated marker. One representative donor of 8 healthy donors is shown. NK, natural kill.

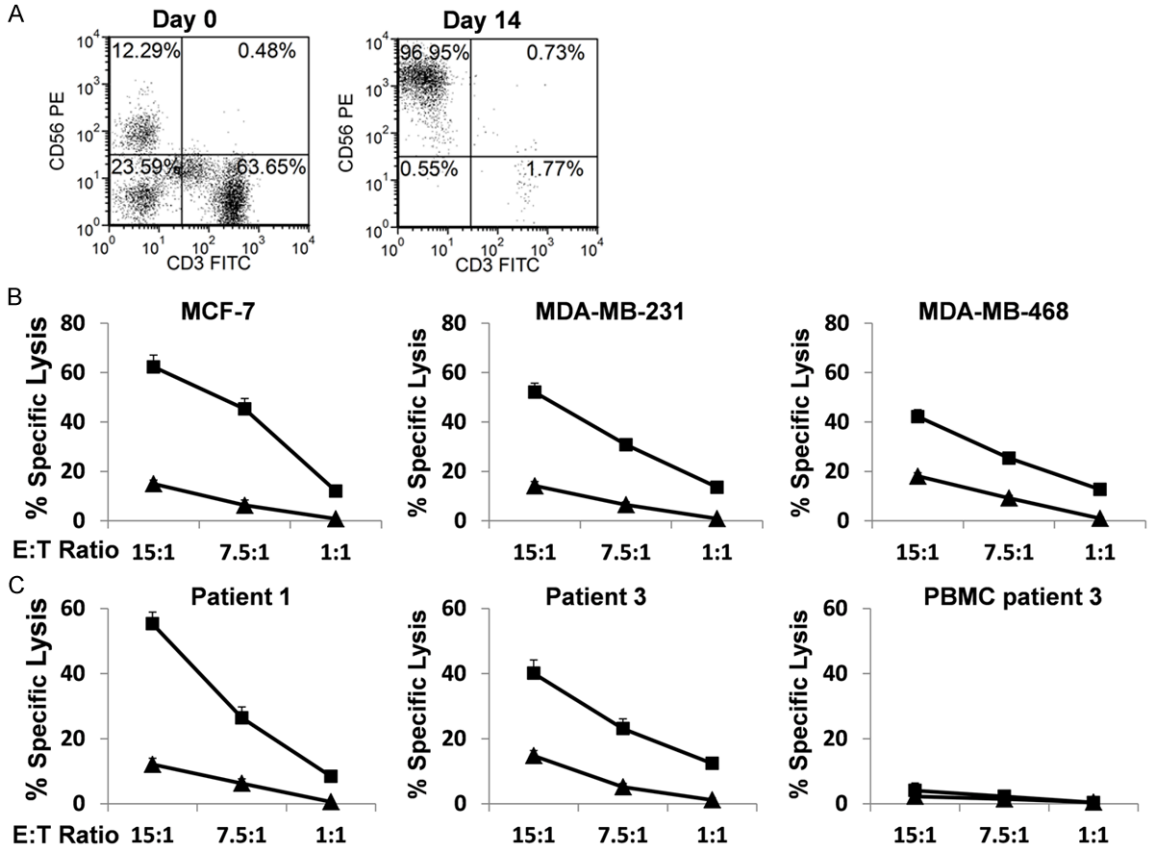


Figure 3. Cytolytic NK cells are efficiently expanded from PBMCs of healthy donor. A. In the presence of K562-IL15-41BBL, expanded cells become significantly enriched ($P < 0.05$) in NK cells ($CD56^{+}CD3^{-}$) after 14 days of culture; B. Expanded NK cells from one donor were evaluated for cytolytic activity using 4 h ^{51}Cr release assays. Ex-vivo expanded NK cells (■), but not freshly purified non-expanded NK cells (▲), efficiently lysed breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MB-468; C. Expanded NK cells (■), but not freshly purified non-expanded NK cells (▲), obviously lysed patient breast cancer cells, but not allogeneic patient's PBMC. The mean ± SD of percentage cytotoxicity is shown from triplicate wells from one representative donor. One representative donor of 8 healthy donors is shown. NK, natural kill; PBMC, peripheral blood mononuclear cell.

(range: 0.4-1.7%). However, cultures with either normal K562 with IL-2 or IL-2 alone completely failed to induce NK cell proliferation and, therefore, are not described later.

Ex vivo expansion of NK cells turns the NK cell receptor balance towards activation

To determine whether the cell surface expression associated with activating, natural cytotoxicity and inhibitory molecules of NK cells expanded from PBMCs was different from non-exp-NK cells, flow cytometry analysis of both non-exp-NK cells and exp-NK cells from 8 HD was performed. The change in cell surface expression of NK cells of one representative donor is shown in both dot plot and histogram overlays (**Figure 2**). There was an increased expression of the C-type lectin-activating receptor NKG2D and natural cytotoxicity receptors (NKp30, NKp44), which are important to activating NK cells [24, 25]. There was also an elevated expression of other activating molecules such as CD26, CD69, CD70 and DNAM-1 on exp-NK cells. However, NKp46, 2B4 and NTB-A, which may be involved in inducing NK

cell cytotoxicity [26, 27], were not significantly changed. In addition, the repertoire and percentage positive of inhibitory KIR receptors (2DL1, 2DL2/L3, 3DL1, 2DL3 and CD94) was comparable on the cell surface of exp-NK cells, when compared with NK cells before expansion. Surface expression profile of these activating and inhibitory receptors of non-exp-NK cells and exp-NK cells from all other seven HD was similar. These data indicate that NK cells post expansion may have a phenotype associated with a greater activation state.

Exp-NK cells are highly and specifically cytotoxic against both breast cancer cell lines and patient breast cancer cells

We observed that there was a shift in favor towards expression of activating molecules on the surface of exp-NK cells. Using 4 h ^{51}Cr release assay, we next tested anti-breast cancer potential of exp-NK cells and non-exp-NK cells from eight HD PBMCs against MCF-7, MDA-MB-231, MDA-MB-468, patient breast cancer cells (n=4) and patient non-cancer cells (patients' PBMCs, n=2). ^{51}Cr release assay of exp-NK cells from 8 donors showed more efficiently killing of MCF-7 (mean: 61%; range: 52-72%), MDA-MB231 (mean: 57%; range: 49-65%), and MDA-MB-468 (mean: 47%; range: 42-56%) at a ratio of 15 NK effectors to 1 target cell, compared with non-exp-NK cells against MCF-7 (mean: 16%; range: 13-19%), MDA-MB231 (mean: 14%; range: 12-18%), and MDA-MB-468 (mean: 15%; range: 10-18%), respectively ($P < 0.05$). Importantly, exp-NK cells were highly active and killed patient primary breast cancer cells very avidly in 4 h ^{51}Cr-release assays. The mean lysis of patient cancer cells (n=4) by exp-NK cells was 48% (range: 40-55%) at a 15:1 E:T ratio, compared to non-exp-NK cells (mean: 15%; range: 12-16%). The NK cell activity differences at 15:1 E:T ratio against primary breast cancer cells between non-exp-NK cells and exp-NK cells were significant ($P < 0.05$). Notably, there was no killing of patient normal PBMCs. **Figure 3** showed cytolytic capacity of exp-NK cells from one of eight HD against three breast cancer cell lines (**Figure 3B**), two patient tumor cells and one patient's PBMCs (**Figure 3C**), compared with the capacity of non-exp NK cells. These data indicate that exp-NK cells achieved much higher specific lysis of patient tumor cells at 15:1 E:T ratios, when compared side-by-side with non-exp-NK cells. They may overcome the inhibitory signaling from patient breast cancer cells and have potential for clinical immunotherapy of patients with breast cancer.

Molecular mechanisms are involved in the interaction between exp-NK cells and patient breast cancer cells

Since activating receptors NKG2D, NKp30, NKp44, CD26, CD69, CD70 and DNAM-1 were significantly up-regulated in exp-NK cells, we performed blocking studies by using neutralizing antibodies, alone or in combination, to NKG2D, NKp30, NKp44, CD26, CD69, CD70 and DNAM-1 to investigate the contribution of these activating receptors to the cytotoxicity of exp-NK cells against patient breast cancer cells. As expected, exp-NK cell cytotoxicity was significantly reduced when activating receptor-ligand interactions were inhibited by these blocking antibodies (**Figure 4**). The stronger inhibition of NK cell killing against patient tumor cells was observed when used anti-NKG2D, anti-NKp30 + NKp44, anti-CD26, anti-CD69, anti-CD70, anti-DNAM-1 or all antibodies, giving mean of inhibition as 42%, 49%, 23%, 28%, 34%, 39% and 96% in each group, respectively, at 15:1 E:T ratio ($P < 0.05$) (**Figure 4A**). However, anti-NKp46, anti-2B4 and anti-NTB-A antibodies, which were not significantly changed in exp-NK cells when compared with non-exp-NK cells, had no discernible effect on the cytotoxicity of exp-NK cells against primary cancer cells ($P > 0.05$) (**Figure 4A**). These results point to cooperative role for NKG2D, NKp30, NKp44, CD26, CD69, CD70 and DNAM-1 in the cytotoxicity of exp-NK cells against patient breast cancer cells. Finally, the primary mechanism of exp-NK cell killing of patient breast cancer cells was perforin-mediated, as the perforin inhibitor CMA greatly reduced killing ($P < 0.05$), while blocking TRAIL and Fas-L did not have a significant impact on killing ($P > 0.05$) (**Figure 4B**). We also found that more than 90% of exp-NK cells have high expression of perforin, granzyme A and granzyme B, whereas no marked expression of TRAIL and Fas-L was detected on exp-NK cells (**Figure 4C**).

Discussion

NK cells are a type of innate immunocytes that can directly induce the death of tumor cells and virus-infected cells in the absence of prior immune sensitization of the host [12, 28].

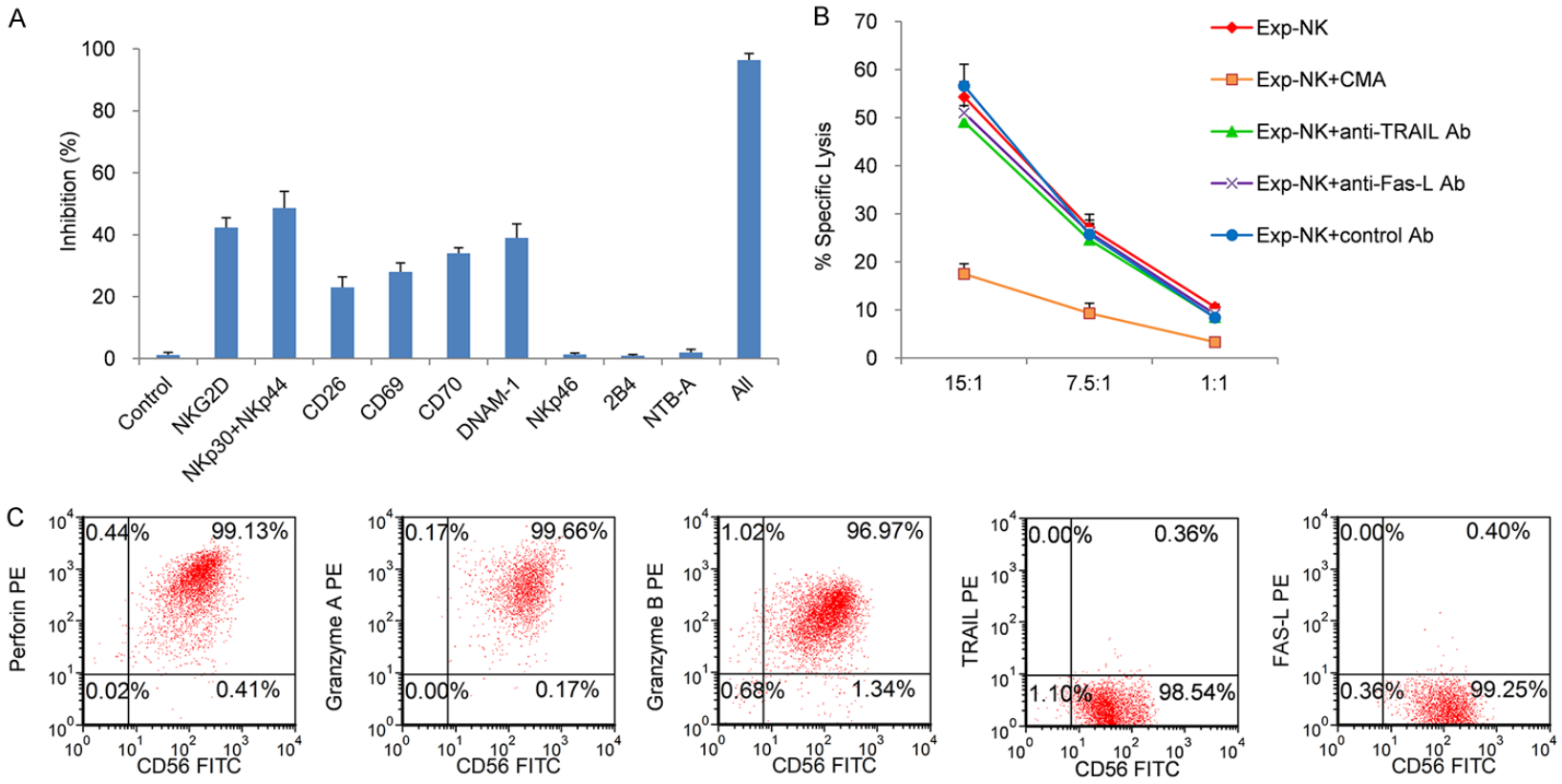


Figure 4. Expanded NK cells (one representative donor, unselected, purity > 95%) kill primary breast cancer cells and this killing is mediated by critical activating receptor-ligand interactions and the perforin pathway. A. Blocking critical NK cell activating receptors on NK cells can inhibit killing of primary tumor cells at 15:1 E:T ratio; B. HD-derived exp-NK cells kill primary breast cancer cells via a perforin pathway. Blocking effectors with anti-TRAIL or anti-FAS-L antibodies did not significantly reduce the level of killing whereas the addition of the perforin inhibitor CMA to the assay reduced killing appreciably. The mean ± SD from triplicate wells is shown; C. Exp-NK cells have high cytoplasmic granular contents of Perforin, Granzyme A and Granzyme B. Flow cytometric analysis confirms the presence of cytoplasmic granules in appreciable amounts in exp-NK cells. Representative FACS plots are shown. The numbers in each quadrant are the percentages based on total $CD3^-CD56^+$ NK cells. NK, natural kill; Exp-NK, expanded NK; Ab, antibody.

Recent studies have demonstrated that NK cell based adoptive cancer immunotherapy is a promising approach for the treatment of malignant diseases [29]. Because NK cells represent a small fraction of PBMCs and the NK cells acquired from HD or patients with cancer always show low cytotoxicity against tumor cells [7, 8]. Hence, ex vivo expansion and activation of NK cells is necessary before applying them to adoptive cancer immunotherapy. Several methods for NK cell expansion using accessory cells like K562-mb15-41BBL cells and HFWT cells or stimulants such as IL-2, IL-15 and OKT3 have been reported [23, 30]. Such exp-NK cells showed potent antitumor activity in vitro to a variety of malignancies including acute myeloid leukemia, Ewing's sarcoma, multiple myeloma, gastric, lung, prostate, and breast cancer [7, 8, 31-33]. In the current study, we used K562-mb15-41BBL cells to expand NK cells from PBMCs of HD. The results suggested that NK cells were sharply expanded by this protocol. Moreover, activation receptors that mediate target cell killing were increasingly expressed on the exp-NK cells. This indicated that these exp-NK cells might possess enhanced cytotoxicity. Indeed, further data revealed that these exp-NK cells with increased activation receptors exerted more powerful cytotoxicity against the breast cancer cell lines as well as primary breast cancer cells.

In the context of adoptive cellular immunotherapy, infusions of donor cells containing activated T cells would possibly result in unacceptable GvHD, particularly in recipients of HLA-haploidentical or mismatched transplants. Indeed, there are many protocols for NK cell expansion. Most of these protocols, however, induce large quantities of T lymphocytes [23, 34]. In contrast, the expansion method used in our study did not stimulate T cell proliferation. In the setting of allogeneic NK cell therapy, this could be an important practical advantage as it would help to avoid the risk of GvHD. In addition, the results showed that the exp-NK cells did not present enhanced cytotoxicity against allogeneic PBMC from the breast cancer patients compared with these non-exp-NK cells, an indication that despite the increased expression of activation receptors on the exp-NK cells, the inhibitory signals were dominant between NK cells and normal PBMC and able to control the NK cell cytotoxicity against non-malignant cells. Taking together, our data suggested that the adoptive cellular immunotherapy using NK cells expanded by co-culture with K562-mb15-41BBL cells could be a safe therapeutic regimen for patients with breast cancer.

Previous studies have indicated that multiple NK cell receptors working via different signaling pathways act together to achieve maximum cytolytic activity [8, 33, 35, 36]. In the present study, we postulate that the activating receptors NKG2D, NKp30, NKp44, CD26, CD69, CD70 and DNAM-1 may be involved in NK cell cytotoxicity, since the presence of blocking antibodies against these receptors, alone or in combination, significantly inhibited the lytic activity of NK cells. Although no significant change was observed in NKp46, 2B4 and NTB-A expression on exp-NK cells, we tested the involvement of these activating receptors in allogeneic breast cancer cell cytotoxicity. The results suggested that NKp46, 2B4 and NTB-A have nothing to do with the increased cytolysis of breast cancer cells. The fact that NK cell cytotoxicity was almost completely inhibited by a combination of all anti-NKG2D, NKp30, NKp44, CD26, CD69, CD70 and DNAM-1 antibodies may indicate that these cytotoxic receptors may be involved in the interaction between exp-NK cells and patient breast cancer cells. Perforin/granzyme- and Fas-based cytolytic pathways are two major mechanisms of NK cell-mediated cytotoxicity. In this study, the primary mechanism of NK cell killing of patient breast cancer cells was perforin/granzyme-mediated, as the perforin/granzyme inhibitor CMA greatly inhibited killing, while blockings of TRAIL and Fas-Ligand did not have a significant impact on expanded NK cell lysis of tumor cells.

In conclusion, the ex vivo exp-NK cells pre-activated by K562-mb15-41BBL cells might be a useful cellular source for NK cell adoptive immunotherapy for the patients with breast cancer. The activity of exp-NK cells could be further enhanced by adding a KIR blocking antibody, as has been attempted in other malignancies [37]. An important issue that remains to be addressed is the ability of adoptive NK cells to infiltrate into solid tumor. We postulate that such ability may be enhanced by transfecting NK cells with chimeric receptors specific for cell surface molecules expressed on breast

cancer cells. Clinical studies are needed to confirm this hypothesis, as well as to evaluate the therapeutic potency and safety of infusion of large amounts of ex-vivo expanded allogeneic NK cells in breast cancer patients.

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Disclosure of conflict of interest

None.

Address correspondence to: Yi Tao and Jumei Shi, Department of Hematology, Shanghai Tenth People's Hospital, Tongji University School of Medicine, 301 Middle Yanchang Road, Shanghai 200072, P. R. China. E-mail: taoyi018@139.com (YT); shijumei@tongji.edu.cn (JMS)

References

[1] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015; 136: E359-386.

[2] Nieto Y. The verdict is not in yet. Analysis of the randomized trials of high-dose chemotherapy for breast cancer. Haematologica 2003; 88: 201-211.

[3] Audeh MW. Novel treatment strategies in triple-negative breast cancer: specific role of poly(adenosine diphosphate-ribose) polymerase inhibition. Pharmgenomics Pers Med 2014; 7: 307-316.

[4] Caligiuri MA. Human natural killer cells. Blood 2008; 112: 461-469.

[5] Geller MA, Cooley S, Judson PL, Ghebre R, Carson LF, Argenta PA, Jonson AL, Panoskaltsis-Mortari A, Curtsinger J, McKenna D, Dusenbery K, Bliss R, Downs LS and Miller JS. A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. Cytotherapy 2011; 13: 98-107.

[6] Dewan MZ, Terunuma H, Takada M, Tanaka Y, Abe H, Sata T, Toi M and Yamamoto N. Role of natural killer cells in hormone-independent rapid tumor formation and spontaneous metastasis of breast cancer cells in vivo. Breast Cancer Res Treat 2007; 104: 267-275.

[7] Mimura K, Kamiya T, Shiraishi K, Kua LF, Shabbir A, So J, Yong WP, Suzuki Y, Yoshimoto Y, Nakano T, Fujii H, Campana D and Kono K. Therapeutic potential of highly cytotoxic natural killer cells for gastric cancer. Int J Cancer 2014; 135: 1390-1398.

[8] Garg TK, Szmania SM, Khan JA, Hoering A, Malbrough PA, Moreno-Bost A, Greenway AD, Lingo JD, Li X, Yaccoby S, Suva LJ, Storrie B, Tricot G, Campana D, Shaughnessy JD Jr, Nair BP, Bellamy WT, Epstein J, Barlogie B and van Rhee F. Highly activated and expanded natural killer cells for multiple myeloma immunotherapy. Haematologica 2012; 97: 1348-1356.

[9] Verhoeven DH, de Hooge AS, Mooiman EC, Santos SJ, ten Dam MM, Gelderblom H, Melief CJ, Hogendoorn PC, Egeler RM, van Tol MJ, Schilham MW and Lankester AC. NK cells recognize and lyse Ewing sarcoma cells through NKG2D and DNAM-1 receptor dependent pathways. Mol Immunol 2008; 45: 3917-3925.

[10] Honorati MC, Neri S, Cattini L and Facchini A. IL-17 enhances the susceptibility of U-2 OS osteosarcoma cells to NK cell lysis. Clin Exp Immunol 2003; 133: 344-349.

[11] Castriconi R, Dondero A, Cilli M, Ognio E, Pezzolo A, De Giovanni B, Gambini C, Pistoia V, Moretta L, Moretta A and Corrias MV. Human NK cell infusions prolong survival of metastatic human neuroblastoma-bearing NOD/scid mice. Cancer Immunol Immunother 2007; 56: 1733-1742.

[12] Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM and Ugolini S. Innate or adaptive immunity? The example of natural killer cells. Science 2011; 331: 44-49.

[13] Ruggeri L, Aversa F, Martelli MF and Velardi A. Allogeneic hematopoietic transplantation and natural killer cell recognition of missing self. Immunol Rev 2006; 214: 202-218.

[14] Giebel S, Locatelli F, Lamparelli T, Velardi A, Davies S, Frumento G, Maccario R, Bonetti F, Wojnar J, Martinetti M, Frassoni F, Giorgiani G, Bacigalupo A and Holowiecki J. Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. Blood 2003; 102: 814-819.

[15] Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F, Martelli MF and Velardi A. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 2002; 295: 2097-2100.

[16] Curti A, Ruggeri L, D'Addio A, Bontadini A, Dan E, Motta MR, Trabanelli S, Giudice V, Urbani E, Martinelli G, Paolini S, Fruet F, Isidori A, Parisi S, Bandini G, Baccarani M, Velardi A and Lemoli RM. Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute

myeloid leukemia patients. Blood 2011; 118: 3273-3279.
[17] Rubnitz JE, Inaba H, Ribeiro RC, Pounds S, Rooney B, Bell T, Pui CH and Leung W. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. J Clin Oncol 2010; 28: 955-959.
[18] Chaix J, Tessmer MS, Hoebe K, Fuseri N, Ryffel B, Dalod M, Alexopoulou L, Beutler B, Brossay L, Vivier E and Walzer T. Cutting edge: priming of NK cells by IL-18. J Immunol 2008; 181: 1627-1631.
[19] Vivier E, Nunes JA and Vely F. Natural killer cell signaling pathways. Science 2004; 306: 1517-1519.
[20] McQueen KL and Parham P. Variable receptors controlling activation and inhibition of NK cells. Curr Opin Immunol 2002; 14: 615-621.
[21] Moretta L and Moretta A. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. EMBO J 2004; 23: 255-259.
[22] Imai C, Iwamoto S and Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. Blood 2005; 106: 376-383.
[23] Fujisaki H, Kakuda H, Shimasaki N, Imai C, Ma J, Lockey T, Eldridge P, Leung WH and Campana D. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. Cancer Res 2009; 69: 4010-4017.
[24] Nieto-Velazquez NG, Torres-Ramos YD, Munoz-Sanchez JL, Espinosa-Godoy L, Gomez-Cortes S, Moreno J and Moreno-Eutimio MA. Altered expression of natural cytotoxicity receptors and NKG2D on peripheral blood NK cell subsets in breast cancer patients. Transl Oncol 2016; 9: 384-391.
[25] Mavoungou E, Bouyou-Akotet MK and Kremsner PG. Effects of prolactin and cortisol on natural killer (NK) cell surface expression and function of human natural cytotoxicity receptors (NKp46, NKp44 and NKp30). Clin Exp Immunol 2005; 139: 287-296.
[26] Messmer B, Eissmann P, Stark S and Watzl C. CD48 stimulation by 2B4 (CD244)-expressing targets activates human NK cells. J Immunol 2006; 176: 4646-4650.
[27] Stark S and Watzl C. 2B4 (CD244), NTB-A and CRACC (CS1) stimulate cytotoxicity but no proliferation in human NK cells. Int Immunol 2006; 18: 241-247.
[28] Marcus A, Gowen BG, Thompson TW, Iannello A, Ardolino M, Deng W, Wang L, Shifrin N and Raulet DH. Recognition of tumors by the innate immune system and natural killer cells. Adv Immunol 2014; 122: 91-128.
[29] Geller MA and Miller JS. Use of allogeneic NK cells for cancer immunotherapy. Immunotherapy 2011; 3: 1445-1459.
[30] Berg M, Lundqvist A, McCoy P Jr, Samsel L, Fan Y, Tawab A and Childs R. Clinical-grade ex vivo-expanded human natural killer cells up-regulate activating receptors and death receptor ligands and have enhanced cytolytic activity against tumor cells. Cytotherapy 2009; 11: 341-355.
[31] Cho D, Shook DR, Shimasaki N, Chang YH, Fujisaki H and Campana D. Cytotoxicity of activated natural killer cells against pediatric solid tumors. Clin Cancer Res 2010; 16: 3901-3909.
[32] Roberti MP, Rocca YS, Amat M, Pampena MB, Loza J, Colo F, Fabiano V, Loza CM, Arriaga JM, Bianchini M, Barrio MM, Bravo AI, Domenichini E, Chacon R, Mordoh J and Levy EM. IL-2- or IL-15-activated NK cells enhance Cetuximab-mediated activity against triple-negative breast cancer in xenografts and in breast cancer patients. Breast Cancer Res Treat 2012; 136: 659-671.
[33] Voskens CJ, Watanabe R, Rollins S, Campana D, Hasumi K and Mann DL. Ex-vivo expanded human NK cells express activating receptors that mediate cytotoxicity of allogeneic and autologous cancer cell lines by direct recognition and antibody directed cellular cytotoxicity. J Exp Clin Cancer Res 2010; 29: 134.
[34] Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, Matory YL, Skibber JM, Shiloni E, Vetto JT, et al. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N Engl J Med 1985; 313: 1485-1492.
[35] Alici E, Sutlu T, Bjorkstrand B, Gilljam M, Stellan B, Nahi H, Quezada HC, Gahrton G, Ljunggren HG and Dilber MS. Autologous antitumor activity by NK cells expanded from myeloma patients using GMP-compliant components. Blood 2008; 111: 3155-3162.
[36] Bryceson YT, March ME, Ljunggren HG and Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. Blood 2006; 107: 159-166.
[37] Romagne F, Andre P, Spee P, Zahn S, Anfossi N, Gauthier L, Capanni M, Ruggeri L, Benson DM Jr, Blaser BW, Della Chiesa M, Moretta A, Vivier E, Caligiuri MA, Velardi A and Wagtmann N. Preclinical characterization of 1-7F9, a novel human anti-KIR receptor therapeutic antibody that augments natural killer-mediated killing of tumor cells. Blood 2009; 114: 2667-2677.