Interleukin-21 Increases Direct Cytotoxicity and IFN-γ Production of Ex Vivo Expanded NK Cells towards Breast Cancer Cells

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Abstract. Background/Aim: Interleukin-21(IL-21) stimulates cytotoxicity and interferon-γ (IFN-γ) production in natural killer (NK) cells. However, little has been reported on the stimulatory effect of IL-21 on ex vivo expanded NK cells. In this study, we examined the cytotoxicity and IFN-γ production of ex vivo expanded, IL-21-stimulated NK cells against trastuzumab-coated breast cancer cells. Materials and Methods: To expand NK cells, peripheral blood mononuclear cells (PBMCs) were isolated and co-cultured with irradiated K562-mb15-41BBL cells in the presence of IL-2 and IL-15 for 3 weeks. After a 4-day stimulation with IL-21, NK cell cytotoxicity and IFN-γ production were measured. Results: NK cells were expanded up to median of 911-fold and represented approximately 94.93% of expanded cells after 21 days. Cytotoxicity of the expanded NK cells against the MCF-7, SKBR3, and T47D cell lines was significantly increased following 4-day stimulation with IL-21. However, antibody-dependent cellular cytotoxicity mediated by trastuzumab was significantly increased only in the SKBR3 cell line, which highly expresses the HER2/neu antigen. IL-21 pre-treatment also increased IFN-γ production in the expanded NK cells in response to the trastuzumab-coated breast cancer cells. Conclusion: IL-21 significantly enhances the cytolytic activity and IFN-γ production of ex vivo expanded NK cells in response to trastuzumab-coated breast cancer cells.

Breast cancer remains the most common malignancy among women worldwide. Mortality rates have fallen over the past two decades because of the introduction of breast cancer screening programs and the development of novel therapies. One of the effective novel therapies is trastuzumab (Herceptin™), a recombinant humanized monoclonal antibody (mAb) to the extracellular domain of the HER2/neu protein. This antigen is overexpressed in about 20-30% of breast cancer cells (1).

It was reported that the action mechanisms of trastuzumab include inhibition of HER2 shedding, inhibition of the phosphatidylinositol 3-kinase (PI3K)-AKT pathway, attenuation of cell signalling, inhibition of tumor angiogenesis, and antibody-dependent cellular cytotoxicity (ADCC). Trastuzumab is very effective in mediating ADCC against HER2-overexpressing tumor targets due to the ability of immune cells, with receptors for the constant region of immunoglobulin G to, recognize antibody-coated tumor cells (2, 3).

It is now well known that natural killer (NK) cells are powerful effector cells that can be directed to eliminate tumor cells through tumor-targeted mAbs including trastuzumab and cetuximab (4). NK cell-mediated ADCC appears to be one of the important mechanisms for these antitumor effects (5). However, ADCC activity mediated by trastuzumab was down-
regulated in patients with gastric cancer mainly because of NK-cell dysfunction (6), cetuximab-mediated ADCC by peripheral blood mononuclear cells (PBMCs) from esophageal cancer patients was impaired (7), and NK activity of PBMCs was also significantly lower in patients with breast cancer than the one observed in healthy individuals (8).

Thus, immunomodulatory cytokines such as interleukin 2 (IL-2), IL-12, or IL-21 have been studied to enhance ADCC of NK cells (9-11). It was reported that IL-21 was able to efficiently restore impaired ADCC by the immune cells of esophageal squamous cell carcinomas patients via the up-regulation of CD247 molecules (9) and enhanced the NK cell cytokine response to trastuzumab-coated HER2-overexpressing tumor cells (11). It was also reported that IL-21, in synergy with IL-15, enhanced the killing capacity of mouse NK cells to the mouse lymphoma cell lines RMA and RMA-S, a mutant RMA cell line lacking MHC class I (12). However, the effect of these cytokines has been evaluated on isolated or purified NK cells and not on expanded NK cells.

Based on our increased understanding of NK cell biology and the introduction of mAb therapy, a combination of mAb therapy with NK cell-based immunotherapy was suggested as a promising approach (13). Stein et al. have suggested that adoptive immunotherapy with allogeneic NK cells and trastuzumab may be an effective combination against breast cancer targets (14). For successful adoptive immunotherapy of cancer in patients with HER2/neu-positive breast cancer, an ex-vivo expansion protocol to produce large numbers of cytotoxic NK cells is required.

In this study, NK cell expansion was accomplished using genetically modified K562 cells expressing 4-1BB ligand and membrane-bound IL-15 (K562-mbIL15-41BBL) developed by the Campana group (15) with slight modification. We modified to express 4-1BB ligand and membrane-bound IL-15 (K562-mbIL15-41BBL, kindly provided by Dario Campana) was used as feeder cells for the activation and expansion of NK cells. All cells were maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin, and 4 mM L-glutamine in a 24-well tissue culture plate. The medium was exchanged every other day with fresh medium including 10 IU/ml of human IL-2. After one week, the concentration of IL-2 was increased to 100 IU/ml and an additional 5 ng/ml of IL-15 was added to the medium, which was exchanged every other day for an additional two weeks. Cell expansion was expressed as the ‘expansion fold’, which was calculated by dividing the output absolute number of NK cells after every seven days of culture by the respective number on day 0.

To evaluate the effect of IL-21 on expanded NK cells, 5 ng/ml of IL-21 was added every other day for the final four days of NK cell expansion. The purity of expanded NK cells was examined by flow cytometry using FITC-conjugated anti-human CD3 Ab and PE-CY5-conjugated anti-human CD56 Ab.

Flow cytometry. To evaluate cell surface expression of MHC class I and HER2/neu, breast cancer cells were harvested by trypsinization and were washed once with ice-cold 1xPBS (1% BSA). Tumor cells (1x 10⁵) were then stained with fluorescence-tagged target mAbs, trastuzumab, or fluorescence-tagged isotype control Ab for 15 min on ice. Trastuzumab-stained cells were washed and incubated with 1x PBS (1% BSA) containing FITC-conjugated anti-human IgG Ab for 15 min on ice. After washing all samples with 1x PBS (1% BSA), stained cells were then fixed in 2% paraformaldehyde and were analyzed on a FACSArria instrument (BD Biosciences, San Jose, CA, USA).

Cytotoxicity assay. EZ-Cytox Cell Viability Assay kit (InBio, Seoul, Korea), which is based on the cleavage of the tetrazolium salt to water-soluble formazan by the succinate-tetrazolium reductase system, present in the mitochondrial respiratory chain of viable cells, was used to measure the cytotoxicity of expanded NK cells. MCF-7 (4x10⁴/well), SKBR3 (8x10⁴/well), and T47D (8x10⁴/well) cells were cultured in a 96-well flat-bottom plate (Corning Incorporated, Corning, NY, USA) in triplicate and cultured at 37°C overnight. The next day, target tumor cells were washed and expanded NK cells cultured in medium supplemented with IL-2 and IL-15 were harvested and resuspended with medium containing no cytokines. Tumor cells were then mixed with expanded NK cells at

**Materials and Methods**

**Cells and culture.** Human breast cancer cell lines MCF-7, SKBR3, and T47D were obtained from the American Type Culture Collection (Manassas, VA, USA). The leukemia cell line K562 modified to express 4-1BB ligand and membrane-bound IL-15 (K562-mbIL15-41BBL, kindly provided by Dario Campana) was used as feeder cells for the activation and expansion of NK cells. All cells were maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified incubator with 5% CO₂.

**Cytokines and antibodies.** Recombinant human IL-2, IL-15, and IL-21 (PeproTech, Rocky Hill, NJ, USA) were resuspended in 1x phosphate buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA). IL-2 and IL-15 were used to expand NK cells and IL-21 (5 ng/ml) was used to stimulate NK cells for 96 h prior to assays for direct cytotoxicity and ADCC of expanded NK cells. Fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 Ab, phycoerythrin (PE)-CY5-conjugated anti-human CD56, PE-conjugated anti-human MHC Class I Ab, PE-conjugated anti-mouse IgG1 Ab, FITC-conjugated anti-human HER2 Ab, FITC-conjugated mouse IgG1 Ab, and FITC-conjugated anti-human IgG1 Ab were used for flow cytometry (BD Biosciences, San Diego, CA, USA). Trastuzumab (Genentech, CA, USA) which is a humanized anti-HER2/neu mAb was used to stain the target tumor cells and to evaluate ADCC effect of expanded NK cells against breast cancer cells.

**Isolation of human peripheral blood mononuclear cells and culture of NK cells.** Written informed consent was obtained from healthy donors and PBMCs were isolated by density-gradient centrifugation with Ficoll-Hypaque (d=1.077 g/ml, Lymphoprep™; Axis-Shield, Oslo, Norway) and then washed in PBS. The generation of ex vivo activated and expanded NK cells was conducted as previously described (15, 16) with slight modification. Briefly, freshly prepared PBMCs were co-cultured with 100 Gy gamma-ray irradiated K562-mbIL15-41BBL cells in RPMI1640 media with 10% heat-inactivated FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 4 mM L-glutamine in a 24-well tissue culture plate. The medium was exchanged every other day with fresh medium including 10 IU/ml of human IL-2. After one week, the concentration of IL-2 was increased to 100 IU/ml and an additional 5 ng/ml of IL-15 was added to the medium, which was exchanged every other day for an additional two weeks. Cell expansion was expressed as the ‘expansion fold’, which was calculated by dividing the output absolute number of NK cells after every seven days of culture by the respective number on day 0.

To evaluate the effect of IL-21 on expanded NK cells, 5 ng/ml of IL-21 was added every other day for the final four days of NK cell expansion. Thus, immunomodulatory cytokines such as interleukin 2 (IL-2), IL-12, or IL-21 have been studied to enhance ADCC of NK cells (9-11). It was reported that IL-21 was able to efficiently restore impaired ADCC by the immune cells of esophageal squamous cell carcinomas patients via the up-regulation of CD247 molecules (9) and enhanced the NK cell cytokine response to trastuzumab-coated HER2-overexpressing tumor cells (11). It was also reported that IL-21, in synergy with IL-15, enhanced the killing capacity of mouse NK cells to the mouse lymphoma cell lines RMA and RMA-S, a mutant RMA cell line lacking MHC class I (12). However, the effect of these cytokines has been evaluated on isolated or purified NK cells and not on expanded NK cells.

Based on our increased understanding of NK cell biology and the introduction of mAb therapy, a combination of mAb therapy with NK cell-based immunotherapy was suggested as a promising approach (13). Stein et al. have suggested that adoptive immunotherapy with allogeneic NK cells and trastuzumab may be an effective combination against breast cancer targets (14). For successful adoptive immunotherapy of cancer in patients with HER2/neu-positive breast cancer, an ex-vivo expansion protocol to produce large numbers of cytotoxic NK cells is required.

In this study, NK cell expansion was accomplished using genetically modified K562 cells expressing 4-1BB ligand and membrane-bound IL-15 (K562-mbIL15-41BBL) developed by the Campana group (15) with slight modification. We evaluated whether IL-21 enhances the cytolytic activity and ADCC of the expanded NK cells against trastuzumab-coated HER2/neu-expressing breast cancer cells. Furthermore, the effect of IL-21 stimulation on the production of interferon (IFN)-γ, by the expanded NK cells, was evaluated.
For the ADCC assay, breast cancer cells were treated with trastuzumab (100 μg/ml) diluted in culture media at 37˚C for 30 min and unbound trastuzumab was removed by washing with medium prior to admixture with NK cells. Plates were then centrifuged at 1500 rpm for 3 min and incubated at 37˚C for 3 h. After adding 20 μl WST-1 solution (ItsBio) to the well, plates were incubated for 1 h and placed on ice for 10 min to stop the reaction. Absorption at 450 nm was measured using the Infinite M200 PRO (Salzburg Umgebung, Salzburg, Austria) device to determine the amount of formazan produced by live cells. All experiments were performed in triplicate from a single donor.

The percentage of cytotoxicity was calculated by the following equation:

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100\% - 100 \times \left( \frac{A_{450} \text{ of NK cell-treated target cells} - A_{450} \text{ of NK cells}}{A_{450} \text{ of target cells} - A_{450} \text{ of target cells with no WST-1 (background of target cells)}} \right)
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Enzyme-linked immunosorbant assay (ELISA). IFN-γ production in response to trastuzumab-coated breast cancer cells was examined by ELISA from expanded NK cells pretreated with or without IL-21. Briefly, MCF-7 (4x10^5/well), SKBR3 (8x10^5/well), and T47D (8x10^4/well) cells were placed in a 96-well flat-bottom microplate in triplicate and cultured at 37˚C overnight. The next day, tumor cells were washed and treated with 100 μg/ml trastuzumab for 30 min at 37˚C. After washing off unbound trastuzumab with medium, expanded NK cells were added to target tumor cells at a 2:1 E:T ratio. After 24-h co-culture, cell-free culture supernatants were harvested and analyzed for IFN-γ production using commercially available ELISA kits (BD Biosciences, San Diego, CA, USA). All procedures were performed according to the manufacturer’s instructions and the concentration of IFN-γ was determined via the use of standard curve regression analysis.

Statistics. Statistical analyses on cytotoxicity and IFN-γ production of expanded NK cells were carried out to evaluate the statistical significance of the differences between groups using Wilcoxon test, and a p-value of ≤0.05 considered significant.

Results

Ex vivo expansion of human NK cells. Human NK cells were expanded from normal donor PBMCs using K562-mb15-41BBL cells with a slight modification to improve the rate of proliferation. We added 5 ng/ml of IL-15 to the medium after seven days of culture and IL-15 was replenished every two days (for details, see the Materials and Methods). In the presence of 5 ng/ml of IL-15 as well as 100 IU/ml of IL-2, NK cells vigorously proliferated over a period of three weeks (Figure 1). Median NK cell recovery was 11-fold (range, 4- to 41-fold) after 7 days and was increased to 204-fold (64- to 643-fold) after 14 days and 911-fold (258- to 3226-fold) after 21 days.

Expression of MHC class I molecules and Her2/neu on breast cancer cell lines. We first examined the surface expression of MHC class I molecules (which act as ligands of NK cell inhibitory receptors) on breast cancer cells by flow cytometry prior to evaluating the cytotoxicity of expanded NK cells. The T47D cell line expressed high levels of MHC class I molecules (mean fluorescence intensity, 88.22), while MCF-7 (MFI=7.11) and SKBR3 (MFI=2.61) expressed low basal level of these molecules on their cell surface (Figure 2A). We next investigated the expression of HER2/neu (the target antigen of trastuzumab) on breast cancer cells by flow cytometry prior to their use in an ADCC assay. Among the three cell lines examined, only SKBR3 cells (MFI, 92.65) expressed high levels of the HER2/neu antigen. T47D (MFI=9.23) and MCF-7 (MFI=5.18) cells showed lower expression levels of HER2/neu (Figure 2B). Similar results were observed in a binding assay using trastuzumab (17) (Figure 2C). Trastuzumab bound differentially to breast cancer cells according to their documented expression levels of HER2/neu.
Cytotoxicity of expanded NK cells in response to trastuzumab-coated breast cancer cells. The direct cytotoxic effects of expanded NK cells against breast cancer cell lines including MCF-7, SKBR3, and T47D were next examined (Figure 3). With regard to the cytotoxicity of expanded NK cells in response to trastuzumab-coated breast cancer cells, significant enhancement was observed in MCF-7, SKBR3, and T47D cells ($p<0.05$ at an E:T ratio of 2:1 and 4:1; Figure 3).

IL-21 enhances direct cytotoxicity of expanded NK cells against MCF-7, SKBR3, and T47D and ADCC of expanded NK cells against SKBR3. We examined the effect of IL-21 on the direct cytotoxicity and ADCC of expanded NK cells against breast cancer cells which differentially express HER2/neu as targets. After 4-h co-culture, expanded NK cells pretreated with 5 ng/ml of IL-21 for 4 days significantly enhanced cytotoxicity compared with IL21-untreated NK cells ($p<0.05$ at an E:T ratio of 1:1 and 2:1; Figure 4).

IL-21 also enhances ADCC of expanded NK cells against SKBR3 cells. However, trastuzumab-mediated ADCC activity was not significantly increased by expanded NK cells pretreated with IL-21 against MCF-7 and T47D.

IL-21 enhances IFN-γ production by expanded NK cells co-cultured with trastuzumab-coated breast cancer cells. We assessed the IFN-γ production of expanded NK cells pretreated without and with IL-21 in response to trastuzumab-coated and uncoated tumor cells. Target tumor cells were treated with or without trastuzumab and then co-cultured at a 2:1 E:T ratio with expanded NK cells that had been pretreated with IL-21 or control solution. After 24-h incubation, cell-free supernatants were harvested and analyzed for IFN-γ content. Breast cancer cells cultured without expanded NK cells did not secrete IFN-γ when treated with trastuzumab, confirming that the IFN-γ was produced from expanded NK cells. Basal levels of IFN-γ secretion by expanded NK cells was observed when they were cultured without target tumor cells, while high levels of IFN-γ secretion were observed when NK cells were co-cultured with target tumor cells (Figure 5). Expanded NK cells alone produced minimal level
of IFN-γ, regardless of pretreatment with IL-21. On the other hand, pretreatment of expanded NK cells with IL-21 markedly enhanced IFN-γ production in response to breast cancer cells and this effect was further enhanced when SKBR3 cells were coated with trastuzumab.

**Discussion**

This study shows that IL-21 significantly enhances the direct cytolytic activity of expanded NK cells against breast cancer cells and promotes production of IFN-γ. IL-21 enhancement of NK cell ADCC was also observed in a HER2/neu overexpressing cell line.

For the successful adoptive immunotherapy of NK cells in patients with cancer, an ex vivo expansion protocol for the generation of large numbers of cytotoxic NK cells is required.

Several protocols for NK cell expansion have been developed that use various cytokines including IL-2 and/or IL-15 with an anti-CD3 antibody (Orthoclone OKT-3) (18) or accessory cells which give additional signals to enhance proliferations of NK cell (14). Allogeneic mononuclear cells, mitogen activated lymphocytes, umbilical cord mesenchymal cells and cancer cell lines such as HFWT (a Wilms tumor-derived cell line), Epstein–Barr virus -transformed B lymphoblastoid cell lines (EBV-LCL), and K562 cells have been used for the NK cell expansion. Recently, it was reported that genetically modified K562 cells expressing 4-1BBL could be used for NK cell expansion, along with either of these molecules; a membrane-bound IL-15, MHC class I related chain A (MICA), CD86 and a membrane-bound IL-21 (19, 20).

In this study, NK cell expansion was accomplished using K562-mbIL15-41BBL developed by the Campana group (15).
with slight modification including adding soluble IL-15 (5 ng/ml) after one week of culture. We found that we could expand NK cells up to about 911-fold, while it was reported that the original protocol expanded NK cells up to 277-fold. The Campana group previously reported that the \textit{ex vivo} expanded NK cells were considerably more cytotoxic than non-stimulated NK cells against AML, EWS, and rhabdomyosarcoma cell lines (21, 22). Voskens \textit{et al.} reported that NK cells expanded by using the K562-mbIL15-41BBL technique also mediated ADCC in an autologous and allogeneic setting by mAb that are currently being used to treat patients with select solid tumors. For example, ADCC against gastric tumor cells could be enhanced in the presence of cetuximab (Erbitux®), a chimeric mAb that reacts with the epidermal growth factor receptor (23).

The direct cytotoxic effect and ADCC of expanded NK cells used in this study were evaluated against breast cancer cell lines coated with trastuzumab. Expanded NK cells were highly cytotoxic against breast cancer cells even at low E:T ratio, compared with previous reports using purified normal NK cells (11, 24). In those reports, large amount of purified NK cells (\textit{e.g.} E:T ratio=50:1) were used to obtain maximal cell killing target. Expanded NK cells used in this study exhibited much higher cytotoxicity against MCF-7 cells compared with NK cells employed by another group (23). Although T47D express higher levels of MHC class I than MCF-7 and SKBR3, T47D was the most sensitive to expanded NK cells among the three breast cancer cell lines. This result corresponds to a previous report, that there was little correlation between pediatric solid tumor expression of MHC class I and their susceptibility to expanded NK cell cytotoxicity (22). These results indicate that \textit{ex vivo} expanded NK cells possess some mechanism to overcome the inhibitory signal mediated by MHC class I, because the expression level of activating receptors and molecules, which are implicated in the natural cytotoxicity, are up-regulated in expanded NK cells (21, 23, 25). Notably, the level of expression of Killer-cell immunoglobulin-like receptors molecules in expanded NK cells is lower than the one of NK cells before culture.

With regard to the expression level of HER2/neu and binding of trastuzumab on tumor cells, expression of HER2/neu on SKBR3 was high, whereas the ones of Her2/neu on T47D and MCF-7 were very low in flow cytometric analysis. The expression results of Her2/neu are in line with the fact that the breast cancer cell lines SKBR3, T47D and MCF-7 have high, low, and no HER2 gene amplification, respectively (26, 27).

With regard to trastuzumab-mediated ADCC of expanded NK cells used in this study, significant enhancement was observed in response to high HER2/neu-expressing SKBR3 cells coated with trastuzumab and was also observed in response to low HER2/neu-expressing T47D and MCF-7. These results are consistent with the finding that HER-2-non-amplified breast cancer cells including T47D and MCF-7, with low but detectable levels of HER-2 protein, can bind trastuzumab and be lysed by expanded NK cells (28). Our results correspond to the previous study in which expanded NK cells were capable of lysing autologous and allogeneic EGFR expressing lung cancer cells coated with cetuximab (23).

Based on a previous report about NK cell activation by IL-21 (9), we explored IL-21 would be also suitable for enhancing the effect of direct cytotoxicity and ADCC of \textit{ex vivo}-expanded NK cells against trastuzumab-coated and uncoated breast cancer cell lines. Since expanded NK cells are already activated by IL-2 and IL-15 in the medium during the expansion, we sought to determine whether IL-21 could exert a synergistic effect, what concentration of the IL-21 is suitable and when the optimal timing for the addition of IL-21 during the culture is. Based on preliminary study, adding IL-21 (5 mg/ml) every other day for four days after the two-week expansion point was the optimal method.
Cytotoxicity of expanded NK cells pretreated with IL-21 against MCF-7, SKBR3, and T47D was significantly enhanced as compared with IL21-untreated NK cells. This result accords with the one of previous reports using purified NK cells stimulated with IL-21 (11, 24). With regard to trastuzumab-mediated ADCC activity, it was not significantly increased by expanded NK cells treated with IL-21 against trastuzumab-coated MCF-7 and T47D as compared with the enhancement of their direct cytotoxicity. This result is different from the previous report that IL-21 significantly enhanced trastuzumab-mediated ADCC activity against high HER2-expressing TE4 and low HER2-expressing KYSE50 cells (9). These results indicated that the ADCC effect of IL-21 on expanded NK cells was less than the one observed on unexpanded NK cells. It can be explained through this result; expanded NK cells have higher direct cytotoxicity than unexpanded NK cells, so that the high cytotoxicity of expanded NK cells may mask the moderate degree of ADCC effect.

The ability of expanded NK cells to produce IFN-γ in response to breast cancer cells was also evaluated. IFN-γ is an important cytokine which exerts antiproliferative and pro-apoptotic effects on tumor cells (29-31) and IFN-γ has also been shown to be an important component of the IL-21-mediated antitumor response (32). In the current study, IL-21 treatment of expanded NK cells resulted in high levels of IFN-γ production in response to MCF-7, T47D and SKBR3 cells, regardless of trastuzumab coating. Moreover, expanded NK cells pre-treated with IL-21 produced the highest level of IFN-γ in response to trastuzumab-coated MCF-7 and SKBR3. This result is consistent with the findings of Roda et al. who have reported that co-stimulation of NK cells with IL-21 and trastuzumab-coated breast cancer cells caused higher levels of IFN-γ production (11, 24).

One limitation of this study was that we did not show how expanded NK cells pre-stimulated with IL-21 enhanced their direct cytotoxicity, ADCC, and production of IFN-γ against trastuzumab-coated human breast cancer cells. Further studies are needed to understand these mechanisms.

In this report, we demonstrated that expanded NK cells, pre-stimulated with IL-21, exhibit enhanced direct cytotoxicity and an abundant production of IFN-γ against trastuzumab-coated human breast cancer cells, while having only an effect on ADCC against a HER2/neu high-expressing cell line. The minimal ADCC of expanded NK cells pre-stimulated with IL-21 against HER2/neu low-expressing cell line might be explained by the fact that high direct cytotoxicity of expanded NK cells may overshadow the ADCC effect. These results suggest that ex vivo expanded NK cells pre-treated with IL-21 would be considerably useful as a cellular source for NK cell adoptive transfer for the treatment of patients with breast cancer receiving trastuzumab, for HER2/neu-overexpressing breast cancer.

**Conflict of Interest**

The Authors have no competing interests.

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