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Autologous Natural Killer Cell-enrichment for Immune Cell Therapy: Preclinical Setting Phase, Shiraz Experience

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ABSTRACT

Natural killer (NK) cell therapy has proven to be a promising approach for the treatment of malignancies. Osaki method for ex-vivo autologous NK cell expansion has been recently introduced in Japan. To start clinical trial phase I at Shiraz University of Medical Sciences in collaboration with the Japanese group, this preclinical setting study aimed to evaluate the proliferative efficacy of the method, the activation status of expanded autologous NK cells, and the likely unwanted contamination of the final cell product.

Peripheral blood mononuclear cells (PBMCs) were isolated from 5 healthy individuals' peripheral blood and transferred directly to the specified initial culture bag containing anti-CD52 and anti-CD3 and Interleukin (IL)-2. The cells were cultured for 14-17 days in an incubator, during which the cells received condition media, and underwent several passages into bigger culture bags. All the procedures were carried out in a cleanroom and associated facilities. Before and after activation PBMCs were analyzed for their phenotype and cytotoxic activity; using flow cytometry and cytokine release assay.

Our results indicated that NK ($CD3^{-}CD16^{+/-}CD56^{+}$) cells were expanded 510-fold on average (range 200-1100 fold), and the purity of NK cells per whole lymphocytes exceeded 68%. The expanded cells were highly lytic as indicated by in-vitro cytotoxic assay, with a strong expression of Natural killer group 2 member D (NKG2D) and CD16. The prepared final cell products were negative for HCV, HBV, HIV, mycoplasma, and endotoxin.

In the preclinical phase, large numbers of activated and un-contaminated NK cells from healthy individuals' peripheral blood were successfully generated. The method seems to provide ample clean cell product with no contamination and has the potential to be used for NK cell therapy in future clinical trials, suitable to be infused back to the donors in phase I clinical trial.

Keywords: Breast neoplasms; Cell- and tissue-based therapy; Natural killer cells

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INTRODUCTION

Cancer is one of the important causes of death in the Iranian population.[1] A considerable amount of literature has been published demonstrating the immune system's crucial roles in tumor biology.[2,3] Natural killer (NK) cells, which have been defined as CD3-CD16-/+56+ cells, are vital effector cells in the innate immune response against tumor cells.[4] These cells have been well established to be essential in immune surveillance; linking anti-tumor adaptive and innate immune responses.[5] Due to the method of recognition, as well as the natural cytotoxic potential, NK cells not only destroy target tumor cells without prior sensitization but also release crucial cytokines for engaging adoptive immunity into antitumor battle.[6] According to the conventional classification, two subsets of NK cells have been introduced: cytotoxic ($CD56^{dim}$ $CD16^{bright}$) and regulatory ($CD56^{bright}CD16^{dim}$) NK cells.[7] In the more recent publications, however, NK cells are divided, based on the differences in their phenotypes and functions, into three cell subsets including $NK^{cytotoxic}$ ($CD56^{dim}$ $CD11b^{+}$ $CD27^{-}$), $NK^{tolerant}$ ($CD56^{bright}$ $CD27^{-}$ $CD11b^{-}$), and $NK^{regulatory}$ ($CD56^{bright}$ $CD27^{+}$ $CD11b^{+/-}$) cells.[8] Among different NK cell surface molecules, CD16 (FcγRIII) is the most well-known effecter molecule involving in NK-mediated antibody-dependent cellular cytotoxicity (ADCC).[9] Antitumor activity of NK cells made it a promising candidate for cancer immune cell therapy. Although ample data is available verifying the effect of NK cell-based therapy in hematological malignancies[10,11] and solid tumors,[12,13] a major obstacle restricts the clinical use of these natural lymphocytes. Different ex-vivo methods for NK cell expansion have been proposed but obtaining the large numbers of functional NK cells; using ex-vivo culture is very difficult. Several new methods have been developed to overcome this obstacle and produce purified and active NK cells.[14-17] These protocols have some limitations for clinical applications including the low scale of NK cell expansion, low purity, cytotoxic activity, high cost, and complicated protocols.[12,17] A recently published article has illustrated the development of a simple and safe method for ex vivo NK cell expansion. Masuyama and his colleagues introduced a protocol, called the Osaki method, in which they expanded a large number of active NK cells; using anti-CD3, anti-CD52 monoclonal antibodies, and interleukin (IL-2).[18] They injected human pancreatic carcinoma cell line into the peritoneal cavity of NOD rag gamma (NRG) mice with and without expanded NK cells. Better survival was observed in the mice with infused NK cells compared to the control group.[18] Furthermore, injection of expanded NK cell by Osaki method to a patient with metastatic pancreatic cancer showed longer survival in a case report study.[18] This protocol has many advantages for use in adoptive immune cell therapy of patients with cancer and the current study, we expanded cytotoxic NK cells from peripheral blood mononuclear cells of healthy individuals; using the mentioned protocol to check the method, our facilities, and expanded NK cells in our cell processing room in the preclinical phase.

MATERIALS AND METHODS

Subjects

Peripheral blood samples were obtained from five healthy males with informed consent. The study was approved by the ethical committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1396.104).

Cell Expansion

Cell processing, expansion, and evaluation were performed by cellular good manufacturing practice (cGMP) facilities in the cell processing cleanroom at Ghadir hospital affiliated to Shiraz University of Medical Sciences, Shiraz, Iran. Adopted from Masuyama et al[18] here after the Osaki method, with some in-home modifications including changes in the total culture days and the days of transferring the cell from the initial bag to the culture flask and then to the expansion bag, was used for NK cells expansion and activation. Briefly, 30-40 mL of heparinized peripheral blood was obtained from each healthy individual. After separating plasma, peripheral blood mononuclear cells (PBMCs) were isolated from blood; using Ficoll-Paque Premium (GE Healthcare, USA) gradient centrifugation. Pre-expansion analysis, killing assay and pathogen-free evaluation tests of the separated cells (described later in this section) were performed on a part of the sample. For the rest of the sample to be expanded, PBMCs were first suspended in natural killer growth medium (NKGM) medium (Cellex, Japan), 10% heat-inactivated autologous plasma, and, depending on the cell number, 25000-50000 U IL-2 (Novartis, Switzerland). The cells were then transferred to the initiation culture bag (Cellex, Japan), coated with anti-CD3 and anti-CD52 antibodies for 2-3 days, followed by cultivating in fresh NKGM and IL-2, in a 225-culture flask (Corning, USA) for a

further 2-3 days. Cellular colonies were then transferred to one or two expansion culture bags (Cellex, Japan) for 14 to 17 days during which the cells were fed, every 2-3 days, by fresh medium plus IL-2 (90000U). Three days before sample collection, 10 ml of culture medium was tested for sterility standards. Finally, the cells were collected between days 14 to 17 based on cell expansion quality. Post-expansion analysis and a killing assay of the separated cells were performed on the expanded samples.

As for the pre-expansion and post-expansion analysis, the following evaluations were performed on the samples.

Pre-and Post-expansion Phenotyping of NK Cells, NK Cell Subsets, T Lymphocytes, B Lymphocytes, and Regulatory T Cells

Before and after expansion, PBMCs or expanded NK cells were stained on the surface by applying antigen-presenting cells (APC)-CD3 (clone:UCHT1), fluorescein isothiocyanate (FITC)-CD16 (clone:B73.1), photoelectric (PE)-CD56 (clone: 5.1H11), FITC-CD4 (clone:SK3), peridinin-chlorophyll-protein complex (Percp)-CD8 (clone:SK1), PE-CD19 (clone: HIB19), PE-CD25 (clone: M-A251), PerCP Cy5.5-NKG2D (clone:1D11), PerCP Cy5.5-CD96 (clone: NK92.39), PerCP Cy5.5-CXCR3 (clone: G025H7), APC-CD11b (clone: ICRF44), PerCP Cy5.5-CD27 (clone: M-T271) monoclonal antibodies (all from BioLegend, USA) and incubated 30 min at room temperature in dark. In a case where intracellular staining was needed (for FoxP3, perforin, and granzyme B molecules), the stained cells were fixed by incubation with 1X Buffer A of FoxP3 Buffer Set (BD Biosciences, USA) or 1% paraformaldehyde (Sigma, Germany) for 15 min at 4 ºC, permeabilized by 1X Buffer C of FoxP3 Buffer Set (BD Biosciences, USA) or 1X perm wash solution (BD Biosciences), followed by intracellular staining with appropriate antibodies (Alexa488-FoxP3 (clone: 259D/C7), PerCP-Cy5.5-perforin (clone: M-T271) (both from BioLegend, USA)) and Alexa flour 647-granzyme B (clone: GB11) (BD Biosciences). The stained samples were then washed and acquired; using a four-color flow cytometer (FACSCalibur, BD Biosciences) at least for 100,000 events. The data were subsequently analyzed by FlowJo software package (version 7.6.2, Ashland, San Diego CA, USA).

Calcein Release Killing (Cytotoxic) Assay

K562 cells (chronic myelogenous leukemia (CML) cell line) (Pasteur Institute, Iran) were selected as the target cells for NK cell cytotoxic activity assay. They were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 medium containing 10% fetal bovine serum (FBS) (Biosera, UK) in a 37ºC, 5% CO2 incubator. The cells were labeled with Calcein AM (BD-USA) and co-cultured with PBMCs as the effector cells before and after expansion in a U bottom 96-well microtiter plate with a different effector (E): target (T) cells ratios in triplicate. Additional wells were used for the assessment of Calcein spontaneous release (only target cells in medium), maximum Calcein release (target cells in 10% Triton X) (Sigma, Germany), and background (medium only). After incubation at 37°C and 5% CO2 for 4 h and centrifugation, the supernatant was harvested and fluorescent intensity was measured at wavelengths 485nm/535nm for 0.1s; using a microplate spectrofluorimeter. The cytotoxic activity was calculated by the following formula:

$$\%\ \text{Cytotoxic activity} = \frac{\text{Experimentalrelease (counts)} - \text{Spontaneousrelease (counts)}}{\text{Maximumrelease (counts)} - \text{Spontaneousrelease (counts)}} \times 100$$

$$\%\ \text{Spontaneous release} = \frac{\text{Spontaneousrelease (counts)} - \text{background (counts)}}{\text{Maximumrelease (counts)} - \text{background (counts)}} \times 100$$

Degranulation, Lytic Proteins, and Interferon-gamma (IFN-γ) Production Assays

PBMCs (before and after expansion) were incubated with and without K562 cells at 37ºC and 5% CO2 in U bottom 96-well microtiter plate. Following 4 h co-culture, cell mixtures were stained with monoclonal antibodies against APC-CD3, PE-CD56, FITC-CD16, PerCP Cy5.5-CD107a (all from BioLegend, USA). The cells were then fixed, permeabilized, and then stained with PerCP Cy5.5-perforin (BioLegend, USA), Alexa flour 647-granzyme B (BD, USA), and PerCP Cy5.5-IFN-γ (BioLegend, USA). The stained samples were then washed and acquired on a four-color flow cytometer (FACSCalibur, BD Biosciences) for at least 100,000 events. The data were subsequently analyzed by FlowJo software package (version 7.6.2, Ashland, San Diego CA, USA). CD3-CD56+ cells were considered as NK cells and distinguished from K562 cells.

Evaluating the Cells for Culture Contaminations

For HIV, HBV, HCV, and mycoplasma

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contamination tests, enzyme-linked immunosorbent assay (ELISA) kits (all from Cell Biolabs Company, Belgium) were used. Anti-HCV, HBV, HIV, and mycoplasma antigens monoclonal antibodies were coated onto microtiter plates. Briefly, the samples were added to the wells. After 2 h incubation, FITC-conjugated mouse anti-virus Ag antibodies were added and they bind to the antigen captured by the first antibody. Following incubation and wash steps, an HRP-conjugated mouse anti-FITC antibody was added. After washing, a substrate solution reactive with HRP was added to the wells. A colored product was formed in proportion to the amount of viruses' antigen present in the sample. The reaction is terminated by the addition of acid and absorbance is measured at 450 nm.

Statistical Analysis

Nonparametric Mann–Whitney U test was used to determine the differences between frequencies of the cell subsets before and after the expansion process. Statistical analysis was performed; using the SPSS software package (version 11.5; SPSS Inc., Chicago, IL, USA).

RESULTS

Five healthy males participated in the current study. The mean age of them was 32.4±3.1. For gating NK cells, a lymphocyte gate was applied on CD3/Side Scatter (SSC) plot to separate CD3- and CD3+ populations. CD3- gate was then applied on CD16/CD56 plot and NK cells were considered as CD3-CD16+CD56+/- cells which were applied on NKG2D+, CD96+, CXCR3+, perforin, granzyme B/SSC plots, and the percentage of the positive population of NK cells for the mentioned markers were reported in the lymphocyte gate in Table 1 and Figure 1-2.

Table1. The mean percentage of different immune cells by flow cytometry analysis before and after natural killer (NK) cells activation and enrichment in healthy individuals by applying the Osaki method

Immune cell subsets	Before Enrichment Mean%±SD	After Enrichment Mean%±SD
Total NK cells per lymphocyte population		
NK cells ($CD3^{-}CD16^{+/-}CD56^{+}$ lymphocytes)	8.25 ± 3.21	43.24 ± 18.34
NK cell subsets per NK cell population based on the conventional classification i.e. based on the expression of CD3, CD16, and CD56 (7)		
Regulatory NK cell subset (CD3-$CD16^{dim}$ $CD56^{hi}$)	25.321 ± 13.08	7.39 ± 2.34
Cytotoxic NK cell subset (CD3-$CD16^{hi}$ $CD56^{dim}$)	85.76 ± 8.17	39.32 ± 34.50
NK cell subsets per NK cell population based on the new classification, i.e. based on the expression of CD3, CD56, CD27, and CD11b (8)		
Regulatory NK cell subset (CD3- CD56+ CD27+ CD11b+/-)	7.76 ± 4.89	2.94 ± 4.01
Tolerant NK cell subset (CD3- CD56+ CD27- CD11b-)	7.35 ± 2.47	3.91 ± 2.06
Cytotoxcic NK cell subset (CD3- CD56+ CD27- CD11b+)	84.86 ± 7.44	92.96 ± 4.47
NK cells expressing effector/adhesion molecule per lymphocyte population		
CXCR3+ NK cells	2.22 ± 0.01	40.53 ± 1.56
CD96+ NK cells	2.34 ± 2.61	18.53 ± 16
NKG2D+NK cells	7.26 ± 2.45	37.88 ± 1.47
Perforin+ NK cells	7.75 ± 0.24	40.45 ± 0.81
Granzyme B+ NK cells	7.09 ± 1.32	40.62 ± 1.04
Other lymphocyte subsets per lymphocyte population		
B (CD19+) lymphocytes	7.46 ± 5.62	0.14 ± 0.1
Total T cells ($CD3^{+}$ lymphocytes)	69.54 ± 8.75	53.26 ± 19.40
Helper T (CD4+ CD3+) lymphocytes	36.21 ± 0.9	4.07 ± 1.88
Cytotoxic T (CD8+ CD3+) lymphocytes	28.58 ± 0.7	34.57 ± 3.6
NKT ($CD3^{+}CD56^{+}$) lymphocytes	4.36 ± 0.78	25.38 ± 11.02
Regulatory T (Treg) (CD4+CD25+FOXP3+) lymphocytes	0.65 ± 0.41	0.17 ± 0.13
NKG2D+ CD3+ cells	24.33 ± 3.56	49.5 ± 1.87
Perforin+ T (CD3) lymphocytes	10.03 ± 1.21	48.23 ± 0.93
Granzyme B+ of CD8+ cells	3.91 ± 0.34	28.76 ±0.65

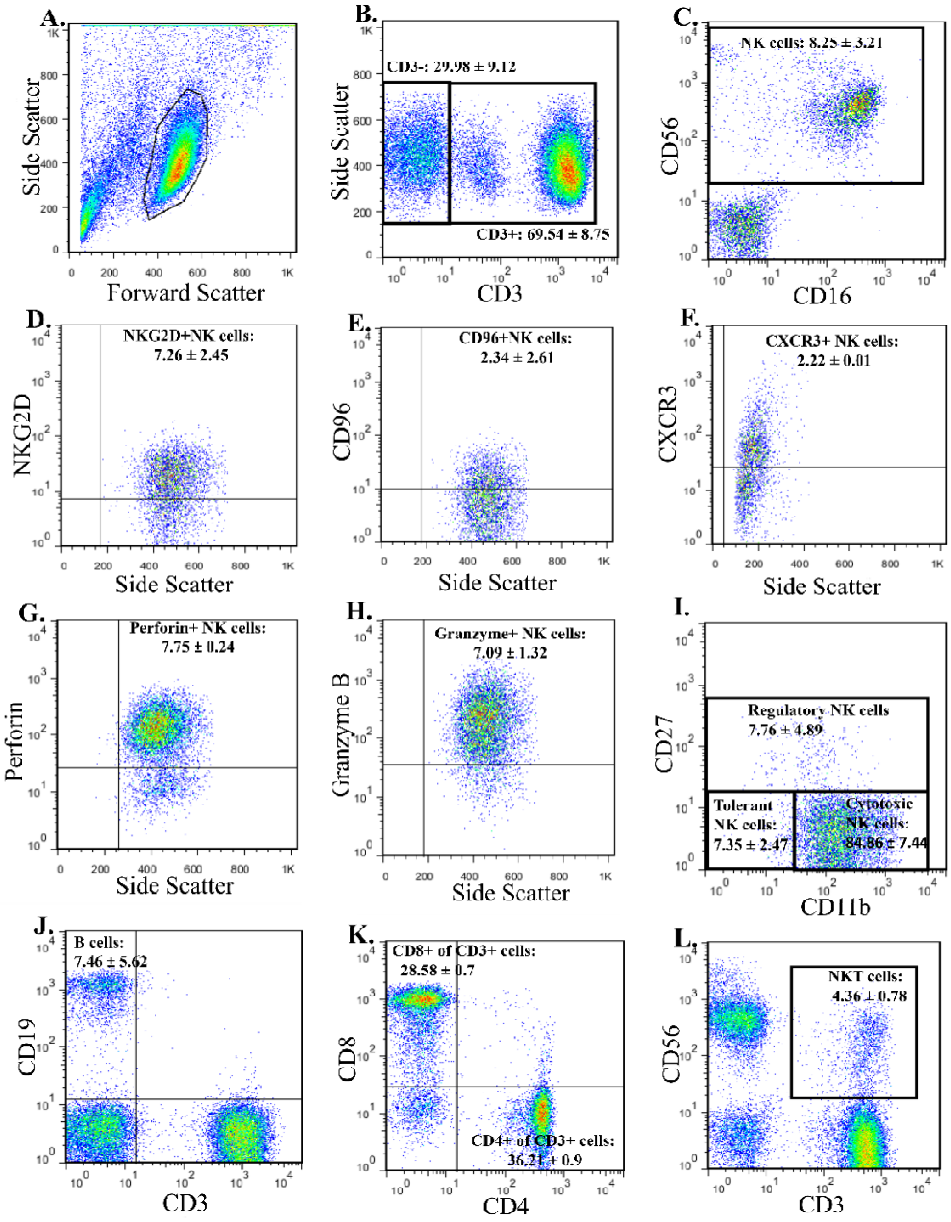

Figure 1.Flow cytometry analysis of the different immune cells in healthy individuals before applying the Osaki method. A. Forward and Side Scatter dot plot with lymphocytes gate. B. Lymphocyte gate was applied on CD3/side scatter (SSC) plot to separate CD3- and CD3+ populations. C. CD3- gate was applied on CD16/CD56 plot and natural killer (NK)cells have been considered as $CD3^{-}CD16^{+}CD56^{+/-}$ cells which were applied on plots in the figures D-H. The frequencies of NKG2D+, CD96+, CXCR3+, perforin+, and granzyme B+ NK cells were then calculated and reported in the lymphocyte gate. I. New classification of NK cells subsets according to CD11b and CD27, $NK^{cytotoxic}$ ($CD56^{dim}$ CD11b+ CD27-), $NK^{tolerant}$ ($CD56^{bright}$ CD27-CD11b-), and $NK^{regulatory}$ ($CD56^{bright}$ CD27+ CD11b+/-) cells was analyzed in CD3-CD56+ cells gate because of 4 color limitation in FACSCalibur flow cytometer. J. B cells in lymphocyte gate. K. CD4+ and CD8+ cells in CD3+ gate. L. NKT cells in the lymphocyte gate.

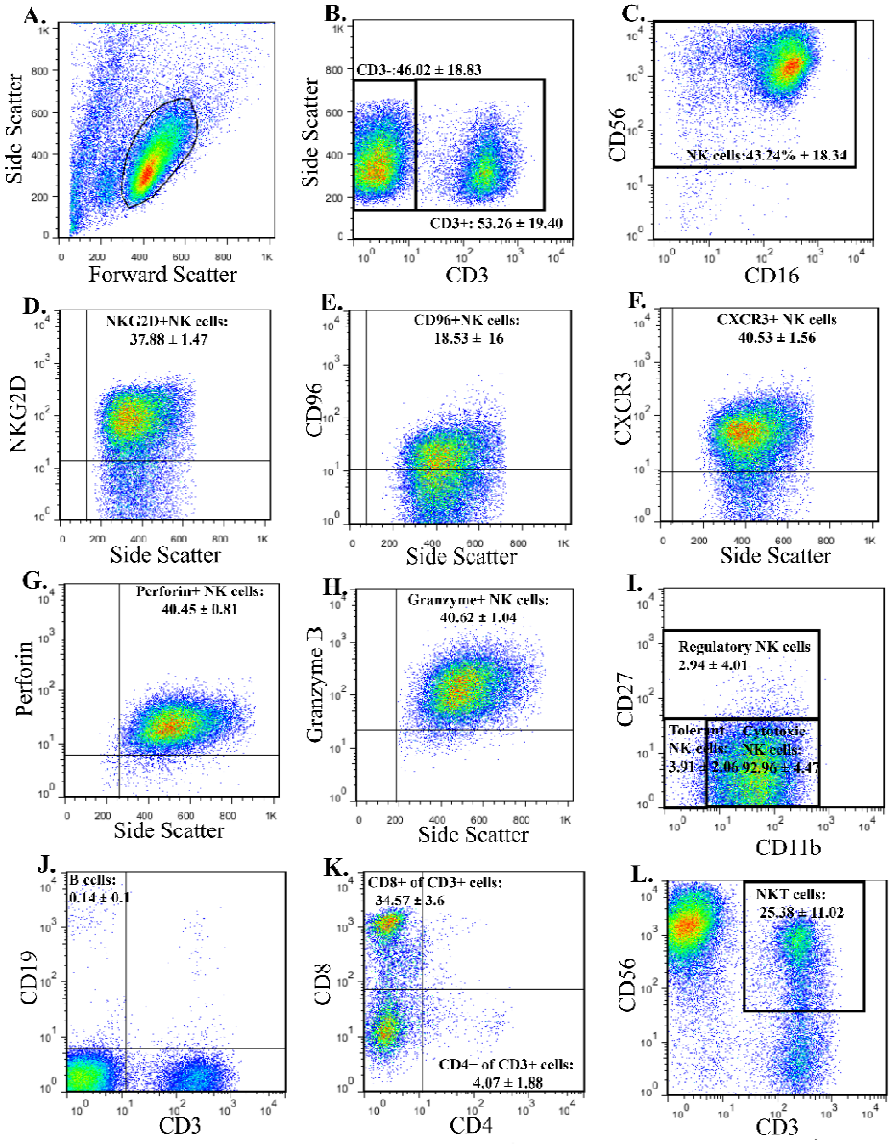

Figure 2.Flow cytometry analysis of the different immune cells in healthy individuals after applying the Osaki method for NK cells enrichment. A. Forward and Side Scatter dot plot with lymphocytes gate. B. Lymphocyte gate was applied on CD3/side scatter (SSC) plot to separate CD3- and CD3+ populations. C. CD3- gate was applied on CD16/CD56 plot and natural killer (NK) cells have been considered as $CD3^{-}CD16^{+}CD56^{+/-}$ cells which were applied on plots in the figures. D-H. The frequencies of NKG2D+, CD96+, CXCR3+, perforin+, and granzyme B+ NK cells were then calculated and reported in the lymphocyte gate. I. New classification of NK cells subsets according to CD11b and CD27, $NK^{cytotoxic}$ ($CD56^{dim}$ CD11b+ CD27-), $NK^{tolerant}$ ($CD56^{bright}$ CD27-CD11b-), and $NK^{regulatory}$ ($CD56^{bright}$ CD27+ CD11b+/-) cells was analyzed in CD3-CD56+ cells gate because of 4 color limitation in FACSCalibur flow cytometer. J. B cells in lymphocyte gate. K. CD4+ and CD8+ cells in CD3+ gate. L. NKT cells in the lymphocyte gate.

Regulatory NK cell subset (CD3-$CD16^{dim}$ $CD56^{hi}$) and cytotoxic subsets (CD3-$CD16^{hi}$ $CD56^{dim}$) of NK cells according to conventional classification were analyzed and reported in CD3-CD16+CD56+/- cells gate. New classification of NK cells subsets according to CD11b and CD27, $NK^{cytotoxic}$ ($CD56^{dim}$ CD11b+

CD27-), $NK^{tolerant}$ ($CD56^{bright}$ CD27-CD11b-), and $NK^{regulatory}$ ($CD56^{bright}$ CD27+ CD11b+/-) cells, was analyzed in CD3-CD56+ cells gate because of 4 color limitation in FACSCalibur flow cytometer. CD4+, CD8+ cells were reported in CD3+ gate.

Our results indicated that total NK ($CD3^-$ $CD56^+/CD16^{-/+}$) cells were expanded 510-fold on average, (range 200-1100 fold), and the purity of NK cells per whole lymphocytes exceeded 68% after enrichment concerning the percentage before that. (43.24% Vs. 8.25, p=0.008).

After the cell expansion process, the majority of CD3+ expanded cells were demonstrated to be CD3+CD8+ cells, approximately 64% of CD3+ cells (and 34.57% in the lymphocyte gate). However, this increase was not statistically significant (p=0.1). The frequency of CD3+CD4+ cells significantly reduced from 36.21% to 4.07% (p=0.016). Furthermore, Treg cells showed a decreasing trend from 0.65 to 0.17 (p=0.06) in the final cell product.

After the cell expansion, the frequency of NK cells that expressed the activating NKG2D receptor (37.88% Vs 7.26, p=0.008), CD96 (18.53% Vs. 2.34, p=0.016), and CXCR3 (40.53% Vs 2.22, p=0.002) were significantly higher than the cells before expansion in the lymphocyte gate.

According to the conventional classification, the dominated population of NK cells was $CD16^{hi}$ $CD56^{dim}$ (85.76 %) before the enrichment process and then decreased to 39.32% after the process. However, NK cells consist of 83.3% $CD16^{hi}$ $CD56^{hi}$ NK cells after the enrichment process compared to 24.52% in non-processed cells. The cytotoxic (CD11b+ CD27-) and regulatory (CD11b-/+ CD27+) subsets of NK cells comprised about 92.96% and 2.94% of the expanded NK cells, respectively. The tolerant subset of NK cells (CD11b- CD27-) consisted of less than 4% of the expanded NK cells. All NK cell subsets differences were statistically significant (p=0.009) before and after cell expansion.

Although perforin and granzyme B expressing NK cells were illustrated as not being dramatically different before and after enrichment (94.26% and 86% vs.93.56% and 93.95%, p<0.05), the percentage of NK cells with expression of the aforementioned lytic proteins was higher in the lymphocyte gate due to NK cell increase in the final cell product (7.74% and 40.45% vs.7.09% and 40.65%, p=0.03). Nevertheless, perforin and granzyme B expressing CD3 cells showed a significant rise after cell processing in the CD3+ cells population (14.43 % vs. 90.56 % and 13.70% vs. 83.20%, p=0.02) as well as in lymphocyte gate (10.03 vs. 48.23 and 3.91 vs. 28.76, p=0.03).

Calcein Release Killing Assay

The expanded cells were highly lytic as indicated by in-vitro Calcein release cytotoxic assay. In the different ratios of the effector (E): target (T) cells (3, 10, and 30), the cytotoxic activities of the cells were demonstrated to be 15.25, 18.63, and 29.9 before the process and 50.49, 64.5, and 82.19 after the process of expansion, respectively and they increased significantly by increasing the ratio from 3 to 10 (p=0.022 for all conditions). The average cytotoxic activity of the activated cells to not-activated cells before processing in all ratios was revealed to be 3.19 fold, Figure 3.

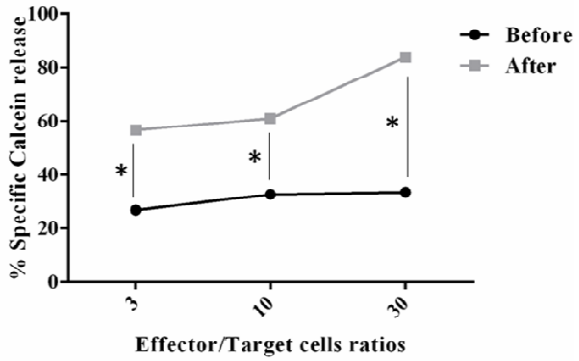

Figure 3. Calcein release killing assay before and after the Osaki method of natural killer (NK) cells enrichment. Effector peripheral blood mononuclear cells (PBMCs)/ Target cells (K562 cells) ratios were 3, 10 and 30,*p<0.05

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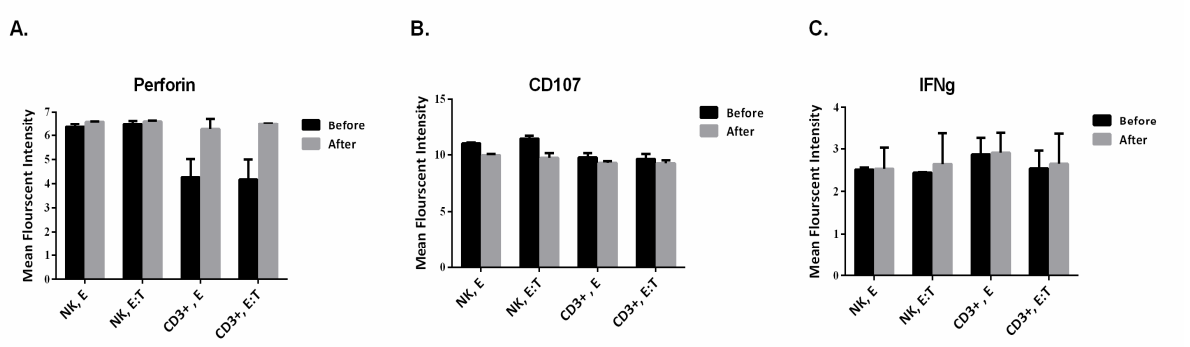

Figure 4. A. Lytic protein (perforin) B. Degranulation marker (CD107a) C. Interferon-gamma (IFN-γ) production assayed by flow cytometry in Effector (E) and effector: target (E:T) cells before and after the Osaki method of natural killer (NK) cells enrichment. The data were statistically insignificant in all conditions, $p>0.05$

Degranulation, Lytic Proteins, and IFN-γ Production Assays

The mean fluorescent intensity (MFI) of perforin and IFN-γ staining in the NK and CD3+ cells were shown not to be considerably higher in E and E/T cells after expansion compared to the ones before the process of expansion. However, CD107a staining MFI was decreased following cell expansion (Figure 4).

Contamination Test Out

The prepared final cell products were shown to be negative before and after the expansion process for HCV, HBV, HIV, mycoplasma, and endotoxin.

DISCUSSION

Convincing evidence revealed that NK cells, as a significant armament of the innate immune system, play undisputable roles in immune surveillance against different types of cancers.[19] Accordingly, NK cells have been at the center of many therapeutic approaches by scientists from all over the world.[20-22] There are five main immunotherapy approaches focusing on NK lymphocytes: systemic administration of recombinant cytokines involved in NK cell activation including IL-2, IL-15, and IL-12, systemic administration of recombinant monoclonal antibodies with the potency to trigger NK cell-mediated ADCC; autologous adoptive NK cells transfer, allogeneic adoptive NK cells transfer after selected Killer cell immunoglobulin-like receptors (KIR) mismatch, particularly in the patients with hematological malignancies, and administration of NK cell lines particularly NK-92, which is observed to be a safe cell therapy approach. Furthermore, NK cells have recently attracted attention for chimeric antigen receptor (CAR) genetic engineering.[23] Diverse methods for expansion and activation of NK cells have been introduced in the last decade. The majority of these methods, however, have been shown to suffer from serious confines in terms of clinical applications including low scale cell expansion, low purity, low cytotoxic activity, high cost, and the complexity of the protocol.[12,17]

In the present study, and as a prerequisite to start autologous NK cell-enriched therapy phase I clinical trial in Shiraz, Iran, the NK cell enrichment in whole PBMCs of five healthy individuals has been performed; using a method adopted from Masuyoma et al[18] with some modifications. One of the most significant issues in immune cell processing protocols is to end with a sufficient number of effector cells. Our results indicated that following the application of this method, total NK cells (CD3-CD56+/CD16+/-) were expanded 510-fold on average (ranging 200-1100 fold). The mean percentage of NK cells per lymphocyte was 8.25 before expansion, which was extended to 43.24 after expansion (maximum 68%).

Wang et al have compared four different protocols of NK cell expansion; using different cocktails of cytokines including IL-2, IL-15, IL-7, IL-18 as well as OKT3. Although PBMCs of advanced solid cancer patients (but not the healthy normal individuals) have been used for the expansion process in the mentioned study, their results demonstrated a 43-fold NK cell expansion on average (ranging 40-46-fold).[24] Fujisaki et al developed an NK cell expansion protocol by applying IL-2, IL-15, and 4-1BB ligand stimulation on the PBMCs from healthy donors; they finally acquired

280-fold NK cell proliferation.[16] Arai et al have utilized recombinant IL-2 for allogeneic NK-92 cell expansion and illustrated a 250 fold cell expansion.[25] Therefore, the average expansion of the Osaki method seems worthy in comparison with the other protocols' expansion rate.

Besides NK cells, the mean percentages of the other immune cell subsets including non-T lymphocytes, B cells, total T cells, helper T lymphocytes, cytotoxic T cells, NKT lymphocytes, as well as regulatory T (Treg) cells were observed to be 29.98 vs. 46.02, 7.46 vs.0.14, 69.54 vs. 53.26, 52.08 vs. 7.66, 41.10 vs. 64.92, 4.36 vs. 25.38 and 0.65 vs. 0.17 respectively, before and after expansion in our study. Accordingly, two leading cytotoxic immune cell subsets (NK and cytotoxic T cells) have been observed to be increased after cell processing by the Osaki method. In phase I clinical trial in Vietnam, autologous NK cells and cytotoxic T lymphocytes were expanded; using anti-CD16 and anti-CD3 antibodies and then were injected to 10 patients with lung, liver, and colon cancer. After 20–21 days of culture, the average number of cytotoxic T lymphocytes and NK cells increased 488.5- and 542.5-fold, respectively. Survival and quality of life were increased in most patients.[26] Besides the number and the purity, another important entity in NK cell-enrichment is to yield cytotoxic subset. Considering the conventional classification (based on the expression of CD16 and CD56), $CD56^{hi}$ $CD16^{dim}$ regulatory NK cells were observed to be decreased after expansion (25.321 vs. 7.39). Unexpectedly the $CD16^{hi}$ $CD56^{dim}$ cell subset which is known conventionally as the cytotoxic subset was also observed to be decreased after expansion (85.76 vs. 39.32). However, and interestingly, a third subset with the bright expression of both markers ($CD16^{hi}$ $CD56^{hi}$) comprised 83% of the total expanded NK cells was observed to be significantly increased following cell expansion and enrichment (24.52 vs. 83.38 before and after expansion). Evaluation of NK cell subsets was also assessed based on the new calcification method applying the expression level of CD56, CD11b, and CD27.[8] Accordingly, the percentages of both regulatory (CD3- CD56+ CD27+ CD11b+/-) and tolerant (CD3- CD56+ CD27- CD11b-) NK subsets were decreased after expansion, and the dominated population of the final NK cells after the expansion was observed to be cytotoxic NK cells subset (CD3-CD56+CD11b+CD27-). Consequently, it can be suggested that what we observed based on the older classical classification, i.e. the dominant expanded cells with both CD16 and CD56 over-expression (CD16 hi CD56 hi), might be cytotoxic NK cells. Considering the increase in the $CD27^{-}CD11b^{-}$ NK cells population in melanoma[27] and their association with tumor progression in hepatocellular carcinoma,[28] having dominant cytotoxic NK cells subset population in the final cell product of Osaki method seems to be promising for application in the treatment of patients with cancer.

Subsequently, the cytotoxic assay of the expanded cell by Calcein released method demonstrated that the cytotoxic activity of the final cell product has increased 3-fold compared to the cells before expansion. IFN-γ was also observed to be higher in the cells after NK cell enrichment as illustrated by co-cultivation of K562 target cell line with the expanded cells followed by flow cytometry analysis.

The ability of the expanded NK lymphocytes to infiltrate and to persist in the tumor microenvironment has been considered as the fourth important characteristic of the cells in an expansion process. As an essential chemokine-chemokine receptor for NK cells homing, the CXCL10-CXCR3 axis is related to higher infiltration of NK cells into tumor microenvironment as well as tumor-draining lymph nodes.[29] CD96 is an important adhesion molecule involved in NK cell-target cell adhesion by ligation with CD155.[30] In the present study, the percentages of NK cells expressing CXCR3 and adhesion molecule and CD96 were revealed to be significantly increased in expanded cell product (27.30 vs. 93.75, and 2.34 vs. 18.53 respectively). These observations suggest that the Osaki method can increase the ability of the expanded cells to be recruited to the tumor environment and to be efficiently engaged with the target cells, a suggestion that needs more functional assays to be fully elucidated.

As the last but not the least entity, the ability of the expanded NK cells to be activated and to kill target cells efficiently should be taken into account in any cell therapy-based approach. NKG2D, as an NK cells activating receptor, has been demonstrated to be involved in tumor growth suppression in several research studies.[31] In the present study, after NK cells enrichment, not only were the production of IFN-γ and the cytolytic activity of the expanded cells against the K562 cell line increased (as previously described) but also the percentage of the NK cells positive for

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NKG2D was also significantly increased (37.88 vs. 7.26). Evaluating the expression of lytic proteins including perforin and granzyme B in enriched NK cells was illustrated as not being dramatically changed after enrichment. In fact, in the total NK cell population before and after expansion, the percentage of NK cells positive for these lytic proteins was around 90%. However, the percentage of NK cells with the expression of the aforementioned lytic proteins rose in the lymphocyte gate (7% to 40%) due to NK cell frequency increase in the final cell product. Interestingly, and as a co-finding, the cell expansion method in the present study was observed to increase the percentages of CD8+ T lymphocytes expressing Granzyme, as well as the percentages of T lymphocytes (most likely CD8+) expressing perforin (10.03 vs. 48.23 and 3.91 vs. 28.76). These observations collectively suggest the expanded cells are well-armed, able to be effectively activated, and kill target cells; using their effector lytic molecules.

In our study, the NK cell enrichment process was performed in a cleanroom. Assessing the final cell product by standard clinical tests revealed negative HCV, HBV, HIV, mycoplasma, and endotoxin contamination before and after the procedure. Consequently, and as a prerequisite to start autologous NK cell-enriched therapy phase I clinical trial in Shiraz, this method seems to provide ample clean cell product with no contamination and is safe to be infused back to the patients in phase I clinical trial.

As a limitation in the current study, the cells were from healthy donors in which NK cells are not expected to be spontaneously suppressed. The challenge is whether we can get the same results by the Osaki method in the clinical trial study in which PBMCs will be obtained from the end-stage patients with breast cancer.

As a preclinical setting phase of NK cell enrichment for implication in immunotherapy, the Osaki method adopted from Masuyama et al,[18] has been demonstrated to expand a considerable number of un-contaminated NK cells. Not only NK cells but also cytotoxic T cells seem to be increased after cell processing by this method. The dominant expanded NK cell subset is suggested to be cytotoxic NK cells. The data have illustrated that the final cell product processed by this method is suitable for infusion back to the donors in phase I clinical trial for refractory breast cancer.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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