**Autologous NK-cell-enrichment: preclinical setting phase, Shiraz experience**

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**Abstract:**

**Background**

NK cell therapy has proven to be a promising approach for treatment of hematological malignancies and solid tumors. Masuyama et al. have recently introduced a new method for ex-vivo autologous NK cell expansion (Osaki method); resulting in the production of ample active NK cells for a promising cell therapy regimen. In order to start clinical trial phase I at Shiraz University of Medical Sciences in collaboration with Masuyama clinic and St. Luck's International University Hospital, 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.

**Methods**

PBMCs were isolated from 30 ml of 5 healthy individuals' peripheral blood transferring directly to the specified initial culture bag containing antibodies for CD3, CD52 as well as IL-2 cytokine. The cells were cultured for 14-17 days in incubators; during which the cell received condition media, and underwent several passages into bigger culture bags. All the procedure was carried out in the clean room and associated facilities.

**Results**

Our results indicated that NK cells were expanded 510-fold in 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 strong expression of NKG2D and CD16. The prepared final cell products were negative for HCV, HBV, HIV, Mycoplasma and endotoxin.

**Conclusion**

In the preclinical setting phase, large numbers of activated and un-contaminated NK cells from 30 ml of healthy individuals’ peripheral blood were successfully generated. The method seems to provide ample clean cell product with no contamination; suitable to be infused back to the patients in phase I clinical trial.

**Keywords:** NK cells; Cell therapy; Immune cell therapy; Breast cancer
**Background**

Cancer is one of the important causes of death in Iranian population [1]. A considerable amount of literature has been published on the demonstration of the immune system crucial roles in tumor biology [2, 3]. NK cells, which have been defined as CD3- CD16/56+ cells, are vital effector cells in innate immune response against tumor cells [4]. These cells have been well established to be essential in the immune surveillance; linking anti-tumor adoptive and innate immune responses [5]. Thanks to the way 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$^{\text{dim}}$ CD16$^{\text{bright}}$) and regulatory (CD56$^{\text{bright}}$ CD16$^{\text{dim}}$) NK cells [7]. In the more recent publications, NK cells are, however, divided, based on the differences in their phenotypes and functions, into three cell subsets including NK$_{\text{cytotoxic}}$ (CD56$^{\text{dim}}$ CD11b$^+$ CD27$^-$), NK$_{\text{tolerant}}$ (CD56$^{\text{bright}}$ CD27$^+$ CD11b$^-$), and NK$_{\text{regulatory}}$ (CD56$^{\text{bright}}$ CD27$^+$ CD11b$^{+/+}$) cells [8]. Among different NK cell surface molecules, CD16 (FcγRIII) is the most well-known effector 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 an ample data is available verifying the effect of NK cell based therapy in hematological malignancies [10, 11] and solid tumors [12, 13], an important obstacle, however, restricts the clinical use of these natural lymphocytes. Different ex-vivo methods for NK cells expansion has 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 low scale of NK cell expansion, low purity,
cytotoxic activity, high cost and complicated protocols [12, 17] A recent published article has illustrated development of a simple and safe method for the ex vivo NK cells expansion. Masuyama JI and his colleagues expanded a large number of active NK cells using anti-CD3, anti-CD52 monoclonal antibodies and IL-2 [18]. This protocol has many advantages to use for adoptive immune cell therapy of patients with cancer and in the current study, we expanded NK cells from peripheral blood mononuclear cells of healthy individuals using the mentioned protocol to check method, our facilities and expanded NK cells in our cell processing room in the preclinical setting phase.

**Methods**

**Subjects**

To assess the success of the procedure as well as the quality of our cellular products in the preclinical setting phase, peripheral blood samples were obtained from five healthy males.

**Cell expansion**

Cell processing, expansion and evaluation were performed by cellular Good Manufacturing Practice (cGMP) facilities in cell processing clean room at Ghadir hospital affiliated to Shiraz University of Medical Sciences, Shiraz, Iran. Adopted from Masuyama et al [18], here after Osaki method, with some in-home modifications 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 NKGM medium (Cellex, Japan), heat-inactivated autologous plasma and 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, following by cultivating, in fresh NKGM medium and IL-2, in 225-culture flask (Corning, USA) for more 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. Three days before sample collection, 10 ml of culture medium was tested against sterility standards (described later in this section). Finally, the cells were collected between day 14 to 17 based on the cell expansion quality. Post-expansion analysis, and killing assay of the separated cells (described later in this section) were performed on the expanded samples.

As the pre-expansion and post-expansion analysis following evaluations were performed on the samples:

a) Evaluating the percentages of NK cell in total, NK cell subsets, T lymphocytes, B lymphocytes and regulatory T cells by targeting CD3, CD16, CD56, CD11b, CD27, CD4, CD8, CD19, and CD25 molecules using flow cytometry technique
b) Calculating the numbers of NK cells per mL
c) Evaluating the surface expression of NK cell activatory and inhibitory receptors; NKG2D and NKG2A, respectively, NK cell homing receptor CXCR3 and NK cell adhesion molecule CD96 by applying following antibodies and using flow cytometry technique
d) Evaluating the intracellular expression of NK cell death-inducing proteins, granzym and perforin by applying following antibodies and using flow cytometry technique
e) Performing NK cell cytotoxic assay by applying Calcein release assay
f) Performing Pathogen-free evaluation tests for HIV, HBV, HCV and mycoplasma by applying ELISA kits (all from Autobio, China)

**Pre- and post-expansion phenotyping of NK cells, NK cell subsets, T lymphocytes, B lymphocytes and regulatory T cells**

Before and after expansion of NK cells, PBMCs (or expanded NK cells) were stained, on the surface, by applying APC-CD3, FITC-CD16, PE-CD56, FITC-CD4, Percp-CD8, PE-CD19, PE-CD25, PerCP cy5.5-NKG2D, PerCP cy5.5-CD96, PerCP cy5.5-CXCR3, APC-CD11b, PerCP cy5.5-CD27 monoclonal antibodies (all from Biolegend, USA) and incubation 30 minutes at room temperature in dark. In case where intracellular staining was needed (for FoxP3, Perforin and Granzym B molecules), the stained cell were then fixed by incubation with 1X Buffer A of FoxP3 Buffer Set (BD, USA) or 1% paraformaldehyde (Sigma, Germany) for 15 minutes at 4°C, permiabilized by 1X Buffer C of FoxP3 Buffer Set (BD, USA) or 1X permwash solution (BD, USA), following by intracellular staining with appropriate mAbs (Alexa488-FoxP3, PerCP cy5.5-perforin (all from Biolegend, USA) and Alexa flour 647-Granzym B (BD, USA). The stained samples were then acquired using a four-color flowcytometer (BD-USA) at least for 100,000 events. The data were subsequently analyzed by FlowJo software package (version 7.6.2, USA).

**Assessment of Natural Killer cell cytotoxic activity**

- **Calcein release killing assay**

K562 cells (chronic myelogenous leukemia (CML) cell line) were selected as the target (T) cells for NK cell cytotoxic activity assay. The cells were labeled with Calcein AM (BD-USA) and co-cultured with PBMCs as the effector (E) cells before and after expansion in a U bottom 96-well microtiter plate with different effector:target 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) and background (medium only). After incubation at 37°C in 5% CO2 for 4 h and centrifugation, supernatant was harvested and measured using a microplate spectrofluorimeter. The cytotoxic activity were calculated by following formula:

\[
\% \text{ Specific release} = \frac{\text{Experimental release (counts)} - \text{Spontaneous release (counts)}}{\text{Maximum release (counts)} - \text{Spontaneous release (counts)}} \times 100
\]

\[
\% \text{ Spontaneous release} = \frac{\text{Spontaneous release (counts)} - \text{background (counts)}}{\text{Maximum release (counts)} - \text{background (counts)}} \times 100
\]

- **Degranulation, lytic proteins and IFN-γ production assays**

PBMCs (before and after expansion) were incubated with and without K562 cells at 37°C, 5% CO2 in U bottom 96-well microtiter plate. Following a 4-hour 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 and permeabilized. PerCP Cy5.5-Perforin (Biolegend, USA), Alexa flour 647-Granzym B (BD, USA) and PerCP Cy5.5-IFN-γ (Biolegend, USA) production were determined using appropriate antibodies in the effector cells.

**Results**

Five healthy males participated in the current study. The mean percentage of their age was 32.4 ± 3.1. Table 1 and figures 1 and 2 illustrate the mean percentage, per lymphocytes, of different immune cells before and after NK cells enrichment.
| The Immune Cell Subset | Before enrichment | After enrichment |
|------------------------|-------------------|------------------|
|                        | Mean% ± SD        | Minimum %        | Maximum %       | Mean% ± SD | Minimum % | Maximum % |
| Total NK cells         |                   |                  |                 |            |           |           |
| NK cells (CD3-CD16+CD56+ lymphocytes) | 8.25 ± 3.21 | 3.37 | 12.40 | 43.24 ± 18.34 | 19.49 | 68.36 |
| NK cell subsets based on the conventional classification method [7] | | | | |
| Regulatory NK cell subset (CD3-CD16dimCD56hi) | 25.321 ± 13.08 | 6.36 | 35.40 | 7.39 ± 2.34 | 5.09 | 10.20 |
| Cytotoxic NK cell subset (CD3-CD16hiCD56dim) | 85.76 ± 8.17 | 74.40 | 92.90 | 39.32 ± 34.50 | 0 | 91.70 |
| CD3-CD16hi CD56hi NK cells | 24.52 ± 9.72 | 15 | 41 | 83.38 ± 15.75 | 55.30 | 92.60 |
| NK cell subsets based on the recent classification method [8] | | | | |
| Regulatory NK cell subset (CD3-CD56+CD27+CD11b+/−) among NK cell population | 7.76 ± 4.89 | 3.89 | 15.70 | 2.94 ± 4.01 | 0.70 | 10.10 |
| Tolerant NK cell subset (CD3-CD56+CD27-CD11b−) among NK cell population | 7.35 ± 2.47 | 5.54 | 11.60 | 3.91 ± 2.06 | 1.40 | 6.91 |
| Cytotoxic NK cell subset (CD3-CD56+CD27-CD11b+) among NK cell population | 84.86 ± 7.44 | 72.40 | 89.90 | 92.96 ± 4.47 | 85.70 | 97.20 |
| NK cells expressing effector/adhesion molecules | | | | |
| CXCR3+ NK cells | 27.30 ± 0.01 | 27.30 | 27 | 93.75 ± 8.55 | 87.70 | 99.8 |
| CD96+ NK cells | 2.34 ± 2.61 | 0.48 | 6.85 | 18.53 ± 16 | 6.08 | 46.51 |
|                      | Mean   | Min | Max | Mean   | Min | Max |
|----------------------|--------|-----|-----|--------|-----|-----|
| NKG2D+ NK cells      | 7.26 ± 2.45 | 3.14 | 9.74 | 37.88 ± 1.47 | 17.15 | 56.26 |
| Perforin+ NK cells   | 94.26 ± 3.24 | 91.90 | 99.80 | 93.56 ± 4.44 | 88.80 | 97.60 |
| Granzyme+ NK cells   | 86.00 ± 4.24 | 83 | 89 | 93.95 ± 5.72 | 89.90 | 98 |
| Other lymphocytes    |        |     |     |        |     |     |
| Total non-T lymphocytes (CD3⁻) | 29.98 ± 9.12 | 22 | 43 | 46.02 ± 18.83 | 22.10 | 68.70 |
| B (CD19⁺) lymphocytes | 7.46 ± 5.62 | 2.70 | 16.40 | 0.14 ± 0.1 | 0.01 | 0.30 |
| Total T cells (CD3⁺) lymphocytes | 69.54 ± 8.75 | 56.50 | 77.20 | 53.26 ± 19.40 | 31.20 | 77.70 |
| Helper T (CD4⁺ CD3⁺) lymphocytes | 52.08 ± 11.01 | 35.60 | 64.20 | 7.66 ± 9.74 | 1.49 | 24.90 |
| Cytotoxic T (CD8⁺ CD3⁺) lymphocytes | 41.10 ± 8.64 | 31.70 | 53.90 | 64.92 ± 18.55 | 44.40 | 82.10 |
| NKT (CD3⁺CD56⁺) lymphocytes | 4.36 ± 0.78 | 3.67 | 5.57 | 25.38 ± 11.02 | 16 | 42 |
| Regulatory T (Treg) (CD4⁺CD25⁺FOXP3⁺) lymphocytes | 0.65 ± 0.41 | 0.21 | 1.49 | 0.17 ± 0.13 | 0.03 | 0.59 |
| NKG2D+ CD3⁺ cells    | 24.33 ± 3.56 | 18.48 | 28.10 | 49.5 ± 1.87 | 28.49 | 72.26 |
| Perforin+ T (CD3) lymphocytes | 14.43 ± 13.93 | 5 | 38.80 | 90.56 ± 4.82 | 86.30 | 95.80 |
| Granzyme+ of CD8⁺ cells | 13.70 ± 0.34 | 13.72 | 13.4 | 83.20 ±0.65 | 83.40 | 82.80 |

**Table1.** The mean, minimum and maximum percentage of different immune cells in lymphocytes gate by flowcytometry analysis before and after NK cells activation and enrichment in healthy individuals by applying Osaki method.

Our results indicated that total NK (CD3⁻CD56⁺/CD16⁺) cells were expanded 510-fold in average, (range 200-1100 fold), and the purity of NK cells per whole lymphocytes exceeded 68% after enrichment. (43.24% ± 18.34 Vs. 8.25 ± 3.21).
The percentage of CD3+ cells population decreased after NK cell enrichment (69.54 ± 8.75 Vs. 53.26 ± 19.40) in contrast to CD3- cells population (29.98 ± 9.12 Vs. 46.02 ± 18.83).

After the cell expansion process, the majority of CD3+ expanded cells were demonstrated to be CD3+CD8+ cells, approximately 64.92%, while the frequency of CD3+CD4+ cells as well as Treg cells reduced from 52.08% to 7.66% and 0.65 ± 0.41 to 0.17 ± 0.13 in the final cell product, respectively.

After cell expansion and activation, the frequency of NK cells expressed the activating NKG2D receptor, CD96 and CXCR3 were higher than the cells before expansion (37.88%, 18.53% and 93.75%, respectively).

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 less than 4% of the expanded NK cells. The dominated population of NK cells were CD16hi CD56dim NK cells (85.76 ± 8.17%) and CD16hi of CD56hi NK cells (83.38 ±15.75) before and after enrichment process, respectively.

Perforin and Granzyme B expressing NK cells were illustrated not to be dramatically different before and after enrichment (94.26% and 86% vs. 93.56% and 93.95%). However aforementioned lytic proteins in CD3+ cells showed an intensely rise after cell processing (14.43 % and 13.70% vs. 90.56 % and 83.20%).
Figure 1. Flowcytometry analysis of the different immune cells before Osaki method of NK cells enrichment in healthy individuals

A. Forward and Side Scatter dot plot and lymphocytes gate

B. CD3- and C3+ cell population in lymphocyte gate

C. NK cells: 8.25 ± 3.21

D. NKG2D+ NK cells: 7.26 ± 2.45

E. CD96+ NK cells: 2.34 ± 2.61

F. CXCR3+ NK cells: 27.30 ± 0.01

G. Perforin+ NK cells: 94.26 ± 3.24

H. Granzyme B+ NK cells: 86.00 ± 4.24

I. Regulatory, Tolerant and cytotoxic subsets of NK cells

J. B cells: 7.46 ± 5.62

K. CD8+ of CD3+ cells: 41.10 ± 8.64

L. NKT cells: 4.36 ± 0.78
Figure 2. Flow cytometry analysis of the different immune cells after Osaki method of NK cells enrichment in healthy individuals

A. Forward and Side Scatter dot plot and lymphocytes gate
B. CD3- and C3+ cell population in lymphocyte gate
C. NK cells in CD3- cell population
D. NKG2D+ NK cells
E. CD96+ NK cells
F. CXCR3+ NK cells
G. Perforin+ NK cells
H. Granzyme B+ NK cells
I. Regulatory, Tolerant and cytotoxic subsets of NK cells
J. B cells
K. CD4+ of CD3+ cells
L. NKT cells
Calcein release killing assay

The expanded cells were highly lytic as indicated by in-vitro Calcein release cytotoxic assay. The cytotoxic activity ratio of the expanded cells to the cells before processing was revealed to be 3.19 folds, Figure 3.

![Calcein release killing assay](image)

**Figure 3.** Calcein release killing assay before and after Osaki method of NK cells enrichment. Effector (PBMCs)/Target cells (K562 cells) ratios were 30, 10 and 3

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 to be higher in E and E/T cells after expansion compared to the ones before the process. However, CD107a staining MFI was decreased following cell expansion.
Contamination test out

The prepared final cell products were shown to be negative for HCV, HBV, HIV, Mycoplasma and endotoxin.

Discussion

Convincing evidences 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 in 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 Interleukin (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 KIR mismatch particularly in the patients with hematological malignancies, and administration of NK cell lines particularly NK-92 which observed to be a safe cell therapy approach. Furthermore, NK cells have recently attracted attentions 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 indicated 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 sufficient number of the target cells. Our results indicated that following applying this method, total NK cells (CD3-CD56+/CD16+-) were expanded 510-fold in average (ranging 200-1100 fold). The mean percentage of NK cells per lymphocytes was 8.25 ± 3.21 before expansion, which extended to 43.24 ± 18.34 after expansion (Maximum 68%).

Wang et al. has 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 in 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; and they finally acquired a 280-fold NK cells proliferation [16]. Arai et al have utilized recombinant IL-2 for allogeneic NK-92 cells expansion and illustrated a 250 fold of 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 total non-T lymphocytes, B cells, total T cells, helper T lymphocytes, cytotoxic T cells, NK T 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 foremost cytotoxic immune cell subsets (NK and cytotoxic T cells) have been observed to be increased after cell processing by Osaki method.

Beside 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), CD56hi CD16dim regulatory NK cells were observed to be decreased after expansion (25.321 ± 13.08 Vs. 7.39 ± 2.34). Unexpectedly the CD16hi CD56dim cell subset which are known conventionally as cytotoxic subset was also observed to be decreased after expansion (85.76 ± 8.17 Vs. 39.32 ± 34.50). However and interestingly a third subset with the bright expression of both markers (CD16hi CD56hi) comprising 83% percent of the total expanded NK cells observed to be significantly increased following cell expansion and enrichment (24.52 ± 9.72 Vs. 83.38 ±15.75 before and % after expansion). Evaluation of NK cell subsets were 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 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), are cytotoxic NK cells. Consistently, the cytotoxic assay of the expanded cell by Calcein released method demonstrated that the cytotoxic activity of the final cell product has raised 3-fold compared to the cells before expansion. IFN-γ were 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 following by flowcytometry analysis.

The ability of the expanded NK lymphocytes to infiltrate and to persist in the tumor microenvironment has been considered as the forth important characteristic of the cells in an expansion process. As an essential chemokine-chemokine receptor for NK cells homing, CXCL10-CXCR3 axis has been illustrated to be related in higher infiltration of NK cells into tumor microenvironment as well as tumor draining lymph nodes [26]. CD96 is an important adhesion molecule involved in NK cell-target cell adhesion by ligation with CD155 [27]. 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 ± 0.01 Vs. 93.75 ± 8.55, and 2.34 ± 2.61 Vs. 18.53 ± 16 respectively). These observations suggest that Osaki method is able to increase the ability of the expanded cells to be recruited to tumor environment and to be efficiently engaged with the target cells; a suggestion which 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 take into account in any cell therapy based approach. NKG2D, as an NK cells activating receptor, demonstrated to be involved in tumor growth suppression in several research [28]. In the present study, after NK cells enrichment, not only the production of IFN-γ and the cytolytic activity of the expanded cells against K562 cell line were increased (as previously described), but also the percentages of the NK cells positive for NKG2D, one of the main NK-activating receptor was also significantly increased (37.88 ± 1.47 Vs. 7.26 ± 2.45). Evaluating the expression of lytic proteins including Perforin and Granzyme B in enriched NK cells were illustrated not to be dramatically changed after enrichment. In fact in total NK cell population before and after expansion, the percentages of NK cells positive for theses lytic proteins was around 90%. 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 (14.43 ± 13.93 Vs. 90.56 ± 4.82 and 13.70 ± 0.34 Vs. 83.20 ±0.65). These observations collectively suggest the expanded cells are well armed, are able to be effectively activated and to effectively kill target cell by means of their effector lytic molecules.

In our study, NK cell enrichment process was performed in the clean room. Assessing the final cell product by standard clinical tests revealed negative HCV, HBV, HIV, Mycoplasma and endotoxin contamination before and after 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; safe to be infused back to the patients in phase I clinical trial.

**Conclusion**

As a preclinical setting phase of NK cell enrichment for implication in immunotherapy, method adopted from Masuyama et al [18]; seems to expand a huge number of un-contaminated NK cells. Not only NK cells, but also cytotoxic T cells seems to be increased after cell processing by this method. The dominant expanded NK cell subset seems to be cytotoxic NK cells and to have significant increased IFN-γ release, overexpressed chemokine receptor for NK cells homing, over expressed cell adhesion molecule involved in NK cell-target cell adhesion and over expressed NKG2D, as an NK cells activating receptor. The expanded cells seems to have enough lytic molecules Granzyme and Perforin, have their cytotoxic activity increased significantly and are able to kill target tumor cells. These observations support the idea that the expanded cells are able to be effectively recruited and to meritoriously target the tumor cells in tumor microenvironment. The expansion method seems not only to expand and to activate NK cells but also to affect the other immune cells all in favor of antitumor immunity. The data have illustrated that the final cell
product processed by this method is suitable to be infused back to the patients in phase I clinical trial for refractory breast cancer.

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 or not we can get the same results when the cells are extracted from end-stage patients (in clinical trial) have the same responses to expansion method.

Abbreviations
NK: Natural killer
PBMC: Peripheral blood mononuclear cells
CD: Cluster of differentiation
CXCR3: C-X-C motif chemokine receptor 3
HCV: Hepatitis C virus
HIV: Human immunodeficiency viruses
HBV: Hepatitis B virus

Declarations
Ethics approval and consent to participate
This project has been approved by the ethic committee at Shiraz University of Medical Sciences, and informed consent was obtained from all subjects before sampling.

Availability of data and materials
Not applicable.

Competing interests
Authors state that Jun-Ichi Masuyama is the head of Cellex Company from which the culture media and bags were obtained. Other authors declare no conflict of interest.

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Authors' contributions
SR performed setting practical parts of experiments, analyzing and interpreting the data and writing the article. YH participated in teaching, analyzing and interpreting the data. JM, AR and RV performed the data analysis, data interpretation and the article revision. NE participated in
teaching, analyzing, interpreting the data and writing the article. All authors read and approved the final manuscript

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References:

1. Mousavi SM, Gouya MM, Ramazani R, Davanlou M, Hajsadeghi N, Seddighi Z. Cancer incidence and mortality in Iran. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2009;20(3):556-63.
2. Nagarajan D, McArdle SEB. Immune Landscape of Breast Cancers. Biomedicines. 2018;6(1).
3. Passarelli A, Mannavola F, Stucci LS, Tucci M, Silvestris F. Immune system and melanoma biology: a balance between immunosurveillance and immune escape. Oncotarget. 2017;8(62):106132-42.
4. Ljunggren HG, Malmberg KJ. Prospects for the use of NK cells in immunotherapy of human cancer. Nature reviews Immunology. 2007;7(5):329-39.
5. Sun JC, Lanier LL. Natural killer cells remember: an evolutionary bridge between innate and adaptive immunity? European journal of immunology. 2009;39(8):2059-64.
6. Martin-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A, Sallusto F. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nature immunology. 2004;5(12):1260-5.
7. Poli A, Michel T, Theresine M, Andres E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. Immunology. 2009;126(4):458-65.
8. Fu B, Tian Z, Wei H. Subsets of human natural killer cells and their regulatory effects. Immunology. 2014;141(4):483-9.
9. Rezvani K, Rouce RH. The Application of Natural Killer Cell Immunotherapy for the Treatment of Cancer. Frontiers in immunology. 2015;6:578.
10. Cheng M, Chen Y, Xiao W, Sun R, Tian Z. NK cell-based immunotherapy for malignant diseases. Cellular & molecular immunology. 2013;10(3):230-52.
11. Rubnitz JE, Inaba H, Ribeiro RC, Pounds S, Rooney B, Bell T, Pui CH, Leung W. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010;28(6):955-9.
12. Sakamoto N, Ishikawa T, Kokura S, Okayama T, Oka K, Ideno M, Sakai F, Kato A, Tanabe M, Enoki T, Mineno J, Naito Y, Itoh Y, Yoshikawa T. Phase I clinical trial of autologous NK cell therapy using novel expansion method in patients with advanced digestive cancer. Journal of translational medicine. 2015;13:277.
13. 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, Miller JS. A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. Cytotherapy. 2011;13(1):98-107.
14. Koehl U, Brehm C, Huenecke S, Zimmermann SY, Kloess S, Bremm M, Ullrich E, Soerensen J, Quaiser A, Erben S, Wunram C, Gardlowski T, Auth E, Tonn T, Seidl C, Meyer-Monard S, Stern M, Passweg J, Klingebiel T, Bader P, Schwabe D, Esser R. Clinical grade purification and expansion of NK cell products for an optimized manufacturing protocol. Frontiers in oncology. 2013;3:118.
15. Spanholtz J, Tordoir M, Eissens D, Preijers F, van der Meer A, Joosten I, Schaap N, de Witte TM, Dolstra H. High log-scale expansion of functional human natural killer cells from umbilical cord blood CD34-positive cells for adoptive cancer immunotherapy. PloS one. 2010;5(2):e9221.
16. Fujisaki H, Kakuda H, Shimasaki N, Imai C, Ma J, Lockey T, Eldridge P, Leung WH, Campana D. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. Cancer research. 2009;69(9):4010-7.
17. Childs RW, Berg M. Bringing natural killer cells to the clinic: ex vivo manipulation. Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program. 2013;2013:234-46.
18. Masuyama JI, Murakami T, Iwamoto S, Fujita S. Ex vivo expansion of natural killer cells from human peripheral blood mononuclear cells co-stimulated with anti-CD3 and anti-CD52 monoclonal antibodies. Cytotherapy. 2015.
19. Sharma P, Kumar P, Sharma R. Natural Killer Cells - Their Role in Tumour Immunosurveillance. Journal of clinical and diagnostic research : JCDR. 2017;11(8):BE01-BE5.
20. Daher M, Rezvani K. Next generation natural killer cells for cancer immunotherapy: the promise of genetic engineering. Current opinion in immunology. 2018;51:146-53.
21. Mehta RS, Randolph B, Daher M, Rezvani K. NK cell therapy for hematologic malignancies. International journal of hematology. 2018;107(3):262-70.
22. Van Audenaerde JRM, Roeyen G, Darcy PK, Kershaw MH, Peeters M, Smits ELJ. Natural killer cells and their therapeutic role in pancreatic cancer: A systematic review. Pharmacology & therapeutics. 2018.
23. Becker PS, Suck G, Nowakowska P, Ullrich E, Seifried E, Bader P, Tonn T, Seidl C. Selection and expansion of natural killer cells for NK cell-based immunotherapy. Cancer immunology, immunotherapy : CII. 2016;65(4):477-84.
24. Wang X, Li L, Yu J, Li H, Qi J, Zhang P, Yu W, Ren X, Cao S. Comparison of four kinds of NK cell in vitro expansion methods. Chinese Journal of Cancer Biotherapy. 2013;20(3):336-41.
25. Arai S, Meagher R, Swearingen M, Myint H, Rich E, Martinson J, Klingemann H. Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: A phase I trial. Cytotherapy. 2008;10(6):625-32.
26. Bernardini G, Antonangeli F, Bonanni V, Santoni A. Dysregulation of Chemokine/Chemokine Receptor Axes and NK Cell Tissue Localization during Diseases. Frontiers in Immunology. 2016;7:402.
27. Fuchs A, Cella M, Giurisato E, Shaw AS, Colonna M. Cutting edge: CD96 (tactile) promotes NK cell-target cell adhesion by interacting with the poliovirus receptor (CD155). Journal of immunology (Baltimore, Md : 1950). 2004;172(7):3994-8.
28. Dhar P, Wu JD. NKG2D and its ligands in cancer. Current opinion in immunology. 2018;51:55-61.