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NATURAL KILLER CELLS EXPANSION FOR ADOPTIVE IMMUNOTHERAPY: COMPARISON OF TWO ISOLATION METHODS, THREE CYTOKINES, IL-2, IL-15, or IL-18 AND IMPACT ON NK CYTOTOXICITY

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ABSTRACT

Natural killer cells are progressively considered as medical tools for cancer immunotherapy. The development of applicable methods to generate large numbers of functional NK cells is a crucial step to maximize the potential of this approach. In this article, NK cells were isolated from PBMCs using 2 different methods. One method with MagniSort Human NK cell Enrichment kit, and the second method isolation with antibodies and complement (cytotoxic method). Purified NK cells were activated *in vitro* by IL-2 and IL-15 for 14 days and we used IL-18 on day 14 for another 48 hours to increase the cytotoxicity. Finally, the HL-60 (Human promyelocytic leukemia *cells)* cell line was used as a target to assess NK *cytotoxic activity*. The purity of NK cells was 86% and 92% by cytotoxic and MACS methods, respectively. NK cells expanded 60-100 fold in day 14. The expanded NK cells were highly cytotoxic (90%) and they were more than 80% viable. Interestingly, the cytotoxic activity of NK cells was decreased in presence of IL-18. In this paper, we present a simple approach that enables the isolation of NK cells without any beads and magnets using mouse complement with anti-CD3 and anti-CD19 and compared it with the routine isolation method, using magnetic cell sorting. This simplified and efficient method for NK cells isolation and activation could be used in future clinical trials.

Keywords: Immunotherapy; NK cells; AML; IL-2; IL-15; IL-18.

INTRODUCTION

Acute myeloid leukemia (AML) is the most prevalent type of acute leukemia in adults. Studies have shown that immunotherapy could be a suitable adjuvant treatment for AML patients. Cell immunotherapy acts primarily through activation of leukemia-specific T cells and natural killer (NK) cells (Showel and Levis, 2009, Smits, et al., 2009). NK cells are necessary components of the innate immune system, which perform their effector functions without requiring pre-stimulation

(Mandal and Viswanathan, 2015). They are determined by the expression of CD56 and the lack of CD3 and T-cell receptor proteins. NK cells represent 5% to 20% of human lymphocytes (Koepsell, et al., 2013, Shook and Campana, 2011). These cells play key roles during early host protection against tumors and viruses by playing two major roles: cytokine production for immune modulation and contact-dependent cytotoxicity. Target cell apoptosis is mainly mediated by granzyme B and perforin pathways and the regulation of immune responses is mediated by the secretion of cytokines. Significant advances have been achieved to establish NK cell adoptive cell therapy by using different isolation and expansion methods. In these methods, expanded and activated NK cells have been obtained using various cytokine treatments including IL-2, IL-15, IL-21 and IL-18, and feeder cells like tumor cell lines and PBMCs, which selectively activate NK cells through cell-cell contact Selvan and Dowling, 2013, Strengell, 2003) Also, different cell culture media have been used to culture NK cells, including X-VIVO, AIM V, stem cell growth medium (SCGM) and Roswell Park Memorial Institute (RPMI), typically supplemented with 5–10% human AB serum to enhance NK function (Dunne, et al., 2001, Pittari, et al., 2015). The potency of these expanded NK cells have been tested in both liquid and solid tumors for possible treatment for a broad variety of cancers (Selvan and Dowling, 2015).

Despite remarkable advances in development of various strategies to optimize the therapeutic value of NK cells, NK cell-based immunotherapy in general has to deal with several objections that until now limits its potency (Granzin, et al., 2015). It is also an expensive therapy and hence, is not available for all the patients suffering from AML in the world.

Accordingly, in the present study we decided to test a cost benefit isolation method for NK cells from peripheral blood using mouse complement with anti-CD3 and anti-CD19, and we compared it with the routine isolation method, using magnetic cell sorting. Since the routine method is expensive, we hoped this cytotoxic method could be as useful as the previous one. For expansion of NK cells, we tried different combinations of cytokines, IL-2, IL-15 and IL-18 which have had the best results on NK cells expansion until now. We also tried conditions with and without PBMCs as feeder cells in two different culture media (RPMI, SCGM).

MATERIALS AND METHODS

Cells and cell lines

Primary NK cells were obtained from healthy donors peripheral blood mononuclear cells (PBMCs). The HL-60 cell line was provided by pastor institute, Iran. The cell line was maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The informed consent of all participating subjects was obtained according to the Ethics Committee of the Isfahan University of Medical Sciences.

Natural killer cell enrichment

For NK cell enrichment, peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors buffy coats with Ficoll Paque (Biosera, France) gradient centrifugation and NK cells were obtained by negative purification using MagniSort Human NK cell Enrichment kit (eBioscience, USA) according to manufacturer's instruction. The purity of NK cells (CD3-, CD56+) was assessed by flow cytometry

analysis (FACSCalibur Becton Dickinson, USA).

In the present study, we examined another protocol for NK cell enrichment by using anti CD3, anti CD19 (CMG, Iran) and mouse complement (Inno-train, Germany). PBMCs (10^6 cell) were incubated with anti CD3 (7.5µg), anti CD19 (15 µg) in 0.5 ml RPMI 1640. After 30 minutes of incubation in 37ºC, mouse complement (250 µl) was added. The purity of NK cells was analyzed one hour after incubation in 37ºC, with a flow cytometer (FACSCalibur Becton Dickinson, USA).

Natural killer cell culture and expansion

Enriched NK cells were co-cultured (5×10^5 cells/mL) with 5×10^6 cells/mL gamma irradiated (2500 rad) allogenic PBMCs as feeder cells. Three different culture conditions were as follows (Fig. 1):

I) 10 ng/ml anti CD3 (CMG, Iran), 500 IU/mL human IL-2, 10 ng/mL human IL-15 (eBioscience, USA) and 5% heat inactivated AB serum in CellGro SCGM serum-free medium (CellGenix, Germany).

II) % PHA (Gibco, USA), 500 IU/mL human IL-2 and 10 ng/mL human IL-15 in CellGro SCGM serum-free medium.

In conditions I and II, negative control was included NK cells and feeder cells in SCGM medium without any cytokine treatment.

III) 1% PHA, 200 IU/mL human IL-2 and 10 ng/mL human IL-15 in RPMI 1640 containing 10% FBS and 1% penicillin-streptomycin. NK cells with feeder cells in RPMI without cytokine treatment were used as the negative control.

In all treatments, after 48 hours, the medium was exchanged with fresh medium containing human 500 IU/mL human IL-2 and 10 ng/mL human IL-15 to remove PHA or anti CD3. Every 2 days, half of the tissue

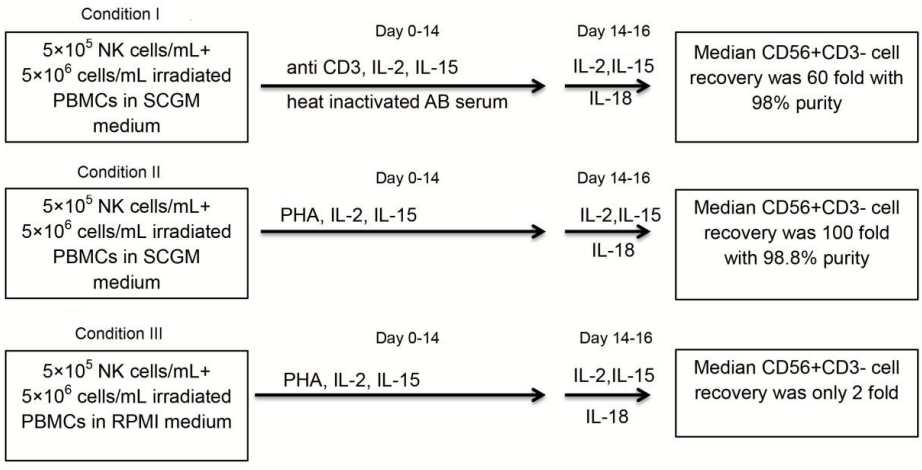

Fig. 1. Schematic diagram of the different culture conditions used for the *ex vivo* generation of CD56+ NK cells from PBMCs. In conditions I–III different combinations of cytokines were tested as described in detail in Materials and Methods.

culture medium was replaced with fresh medium including IL-2 and IL-15. After 14 days of co-culture, 100 ng/ml IL-18 (MBL, Japan), 10 ng/ml IL-15 and 500 IU/ml IL-2 were added to the medium for another 48 hours. All cultures were maintained in a humidified incubator with 5% CO2 at 37°C.

IV) The results of expanded NK cells with feeder cells, were compared with the results of expanded NK cells without feeder cells in both RPMI and SCGM medium. Cytokines were added to the cultures every 2 days. Negative control included NK cells in SCGM and RPMI medium without any cytokine treatment. All experiments were done 3 times, independently.

Assay of CD107a degranulation

Freshly isolated NK cellsin day 0 and stimulated NK cells in day 14 and 16, before and after treatment with IL-18, were co-cultured with human HL-60 cell line in a 96-well cell culture plate with an effector/target (E:T) ratio of 10:1. The final volume was 100 µl per well. Then, the cells were stained with FITC-conjugated anti-CD107a mAb (eBioscience, USA) and incubated for 5 h in incubator with 5% CO2 at 37°C. Monensin (eBioscience, USA), with concentration of 2 µmol/L, was added to each well at the last 4 hours of the culture to avoid releasing of the CD107a from NK cell surface. After 5 hours incubation, the cells were stained with PE-conjugated anti-CD3 mAb and PE/CY5-conjugated anti-CD56 mAb. CD107a expression on CD3-CD56+ NK cells was determined with flow cytometry method (FACSCalibur Becton Dickinson, USA).

Natural killer cytotoxicity assay

Stimulated NK cells were co-cultured with human HL-60 cell line in a 24-well cell culture plate with an effector/target (E: T) ratio of 10:1 for 4-6 hours at 37°C. Then, cultured cells were harvested and prepared for staining. After co-incubation of target cells together with effector cells, total cell population was harvested. Staining was performed by FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA). Annexin V- FITC and PI staining was performed with 5µL Annexin V- FITC and 5µL PI and incubated for 15 min at room temperature in the dark. Finally, NK cells viability was analyzed with flow cytometry method (FACSCalibur Becton Dickinson, USA).

The percentage of cytotoxicity was calculated using O¨ner O¨ zdemir et al. method (Özdemir, et al., 2003). According to their formula, we used the percentage of AnnexinV-negative/ PI-negative cells (viable cell population) for the target-cell gate in target-alone (control) and co-culture analysis, to correct for spontaneous apoptosis with the following formula:

Percent Cytotoxicity (PC) = ([Control-viable cell percent] - [Co-incubation-viable cell percent]) ÷ [Control-viable cell percent]

Statistical Analysis

Mann-Whitney U test was used for comparison of the purity of NK cells isolated by different methods. Kruskal-Wallis test was utilized for comparison of CD107a expression and cytotoxicity among different group. P value less than 0.05 was considered as the statistical significance. All experiments were done 3 times and in order to summarize the data, the results have been aggregated and their mean and standard deviation (SD) values have been shown in the graphs.

RESULTS

High Purity of NK Cells Obtained Using of Anti CD3, Anti CD19, and Mouse Complement

We used and compared 2 methods to isolate NK cells from peripheral blood. One was MagniSort Human NK cell Enrichment kit method and the other was one simplified method using antibodies and complement.

Using the first method which was the immune-magnet base method, more than 95% purity of NK cells was confirmed via flow cytometric analysis.

Using the second method, the purity of NK cells was 86%. The percentage of T cells was 5% in this approach and comparison between these 2 methods were not significant (*p=0.18*).

Natural Killer Cell Expansion with PHA was Greater than Using Anti CD3 as a Cell Stimulator

The aim of this study was to develop an efficient cytokine-based ex vivo culture system for the expansion of NK cells. To identify a suitable medium and stimulator for expansion and differentiation of NK cells, we tested 2 different media using 3 conditions in a two-step in vitro scheme (Fig. 1).

I: 10 ng/ml anti CD3 as PBMC stimulator in SCGM serum-free medium

When NK cells were co-cultured with irradiated PBMC, median CD56+CD3- cell (representing NK cells) recovery after 14 days was 35-fold, and was 60-fold on day 16. Median percentage of NK cells was 97.35% on day 14 and 98% on day 16; while the CD3+ T cells were 1% (data are not shown).

II: 1% PHA as PBMC stimulator in SCGM serum-free medium

After 14 days of co-culture, median NK cell was 50-fold and, was 100-fold on day 16. Median percentage of NK cells was 98.8% on days 14 and 16. Median percentage of CD3+ T cells was 0.6%.NK cells in negative controls were all dead in day 7 (data are not shown).

III: 1% PHA as PBMC stimulator in RPMI 1640

Using RPMI induced no significant expansion of NK cells. The median expansion on day 14 was 1.7-fold and, was 2-fold after 16 days of culture. NK cells with feeder cells without any cytokines (negative control) were died in day 5 (data are not shown).

IV: NK cell culture without using PBMCs as feeder cells

Using irradiated PBMC cells as feeder cells led to production of significantly higher level of expansion in NK cells than culturing NK cells alone. Treatment with IL-2 alone induced no expansion of CD56+CD3- cells and addition of IL-15 and IL-2 to cultures did not improve NK cell expansion (1.3-fold). We also tested the stimulatory effects of IL-18 and no significant improvements were seen in treatment with this cytokine in combination with IL-15. Pure NK cells as negative controls were all dead in day 5. Due to the lower NK cells proliferation in the third and fourth conditions, we did not perform cytotoxicity assays for these 2 conditions.

PHA and anti CD3 both clearly promoted the expression of CD107a on NK cells

The cytotoxicity assay was performed against HL-60 cell line. Both stimulators

clearly promoted the expression of CD107a on NK cells comparing to negative controls (Fig. 2).

Anti-CD3: The expression of CD107a on NK cells was significantly higher on day 14, before adding IL-18, and on day 16, after adding IL-18, compared to negative control (*p=0.02 for day 0 and 16, p=0.049 for day 0 and 14*) (Fig. 2A).

PHA: The expression of CD107a on NK cells was significantly increased on day 14 before adding IL-18 in comparison to negative control (*p=0.02*). Although the expression of CD107a was increased on day 16, when the IL-18 was added, there were no significant differences between day 16 and negative control (*p=0.53*) (Figs. 2A and 4A).

Natural killer cells cytotoxicity induced by anti-CD3 was more efficient than by PHA

Using anti-CD3*, the mean viability* of the *NK cells* after co-culture with target cells was 92.7% and 96% on day 14 and 16, respectively. NK cell cytotoxicity was 92.8% and 86.4% on day 14 and 16, respectively. *No significant differences* were *observed* in the cytotoxicity of NK cells on day 14 and 16. Only 0.68 % and 1.3% of target cells were alive on the day 14 and 16, respectively (Fig. 3a and b).

Using PHA, NK cell cytotoxicity was 97.9% and 18% on day 14 and 16, respectively. The cytotoxicity of NK cells treated with PHA were significantly higher on day 14 compared to day 16 (*p=0.02*). The viability of CD3-CD56+ NK cells was 95.97% and 90.65% on day 14 and 16, respectively (Fig. 3c and d).

NK cell cytotoxicity in negative control was 43% and NK cell viability was 89.7% (Fig. 3e). Our results after stimulation of feeder cells with anti-CD3 mAb showed that the differences between negative control, day 14, and day 16 were not significant (*p=0.06*). Although, treating with PHA, the difference between the cytotoxicity on days 14 and 16 was statistically significant (*p=0.02*), it was not statistically significant between control and 14 or 16 (*p=0.53*) (Fig. 3B and 4B).

DISCUSSION

Natural killer cells are considered as potential tools for cancer immunotherapy. Based on the success achieved in AML, a number of clinical trials are being carried out to specify the efficacy of NK cell infusion for cancer treatment (Lim, et al., 2015). NK cells from healthy donors are prepared by *in vitro* expansion and activation and are then injected to cancer patients (Sutlu and Alici 2009). In order to utilize therapeutic use of NK cells, a sufficient number of highly enriched NK cells must be obtained. In previous studies, various methods of *ex vivo* NK cell expansion have been developed. PBMCs collected by leukapheresis are often used as a general source of NK cells (Lim, et al., 2013), however accessing to leukapheresis is not always possible. In this study, peripheral blood-derived NK cells was obtained from healthy donors which is currently the main source for NK cells for clinical use (Cany, et al. 2015). Anti-CD3, anti-CD19 and mouse complement were used in lymphocytotoxicity assay and our results showed that this method can be used as a cost benefit alternative for the routine method (Hakemi, 2012).

In contrast to T cells that can even be expanded clonally with the help of cytokines and lectins, human NK cells cannot proliferate without feeder cells (Luhm, et al., 2012). Accordingly in our study, NK cells did not expand at all without feeder cells and

they were all dead on the day 5-7.In previous studies, different attempts were tried to stimulate NK cell proliferation with irradiated feeder cells such as PBMCs, EBVLCLs or engineered leukemic cell lines (Lim, et al. 2013). The present work included irradiated allogeneic PBMCs supplied with anti CD3 or PHA at the beginning of the expansion. Stimulation of T cells withanti-CD3 among irradiated feeder cells is utilized by several clinical protocols for NK cell cultivation and can induce NK cells proliferation, probably by activation of T cells (Alici, et al., 2008, Barkholt, et al., 2009, Carlens, et al., 2001, Guven, et al., 2003, Sutlu, et al., 2010, Granzin, et al. 2017).

In addition, studies have shown allogeneic PBMCs may be even more efficient than autologous PBMCs as feeder. For example, in one study, 300-fold NK expansion achieved with irradiated allogeneic PBMC feeder cells while by using of irradiated autologous PBMC feeder cells from the patients only 169-fold expansion was obtained (Luhm, et al., 2002, Kim, et al. 2013). Interestingly, infusion of NK cells with alloreactive T cells did not cause graft-

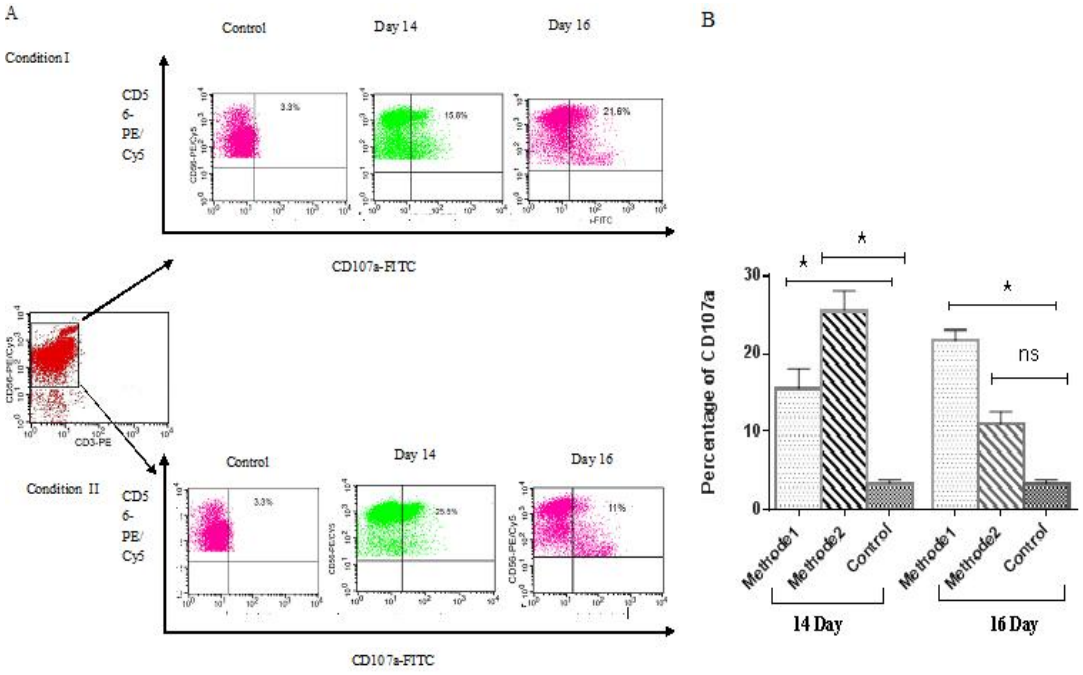

Fig. 2. (A) Representative dot plots depict the expression of CD107a on NK cells. CD3−CD56+ NK cells were gated. Values in quadrants represent the percentages of CD3−CD56+ NK cells that express CD107a marker in conditions I and II. (B) Pooled data showed the percentages of CD107a expression by NK cells after stimulation by two conditions. Multiple comparisons were first made among the three groups using the Kruskal-Wallis H non-parametric test. Then the comparisons between groups were performed using the Mann-Whitney U test. *P<0.05 compared with negative control.

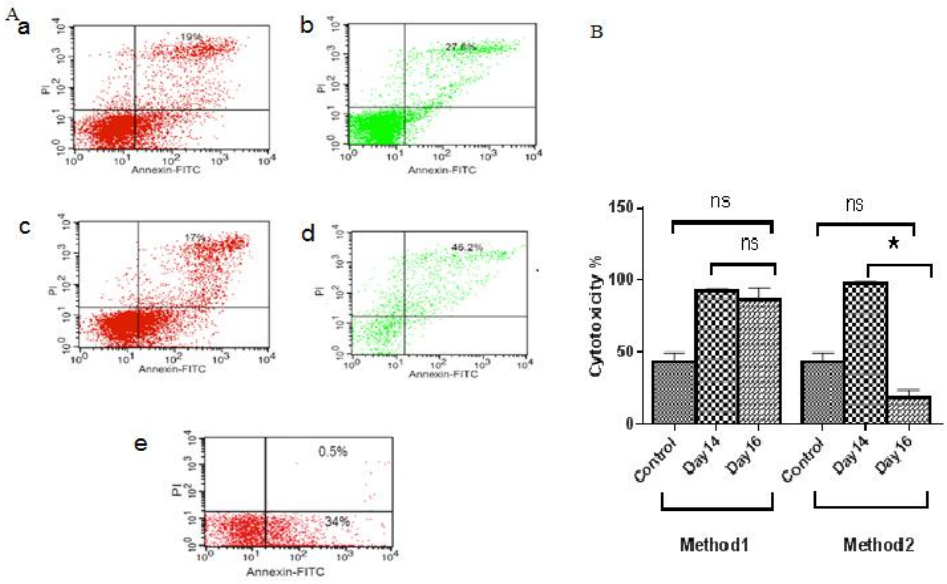

Fig. 3. (A) Representative NK cells cytotoxicity that confirmed with FACS Caliber instrument. The cytotoxicity was evaluated by FITC Annex in V apoptosis detection kit I against HL-60 cells at E:T ratio 10:1. a and b) NK cells cytotoxicity on day 14 and 16 respectively in first condition. C and d) NK cells cytotoxicity on day 14 and 16 respectively using second condition. e) NK cells cytotoxicity on day 0. (B) Pooled data showed the percentages NK cell cytotoxicity by NK cells after stimulation by two conditions. Multiple comparisons were first made among the three groups using the Kruskal-Wallis H non-parametric test. Then the comparisons between groups were performed using the Mann-Whitney U test. *P<0.05 compared with negative control.

versus-host disease (GVHD) side effects in a trial with five cancer patients which suggests using the cultivated cellular product in an allogeneic setting (Barkholt, et al. 2009).

Our results showed that RPMI does not have enough supplements for NK cells proliferation which confirm the earlier report (Dunne, et al., 2001). We showed that SCGM was a very suitable medium to support NK cell proliferation, however, in our study the NK cell proliferation was less than of some other studies (Heiskala, et al., 1987). One reason for this finding *may* be the low number of NK cells and irradiated PBMCs at the beginning of the culture that we used. The high number of NK cells can be provided by leukapheresis, and for handling more amount of NK cells, culture bags should be used while our cultures were performed in T25 flasks.

In addition, our result showed that between the 2 days of 14 until 16, more than two fold NK cells expansion was obtained. Therefore, the longer culture period until 21-day probably stimulate a greater NK cell expansion. The expansion of NK cells in presence of PHA at the beginning was more compared to using anti CD3. It is likely that the more proliferation of NK cells in presence of PHA is due in part by a direct action of this activator on NK cells and

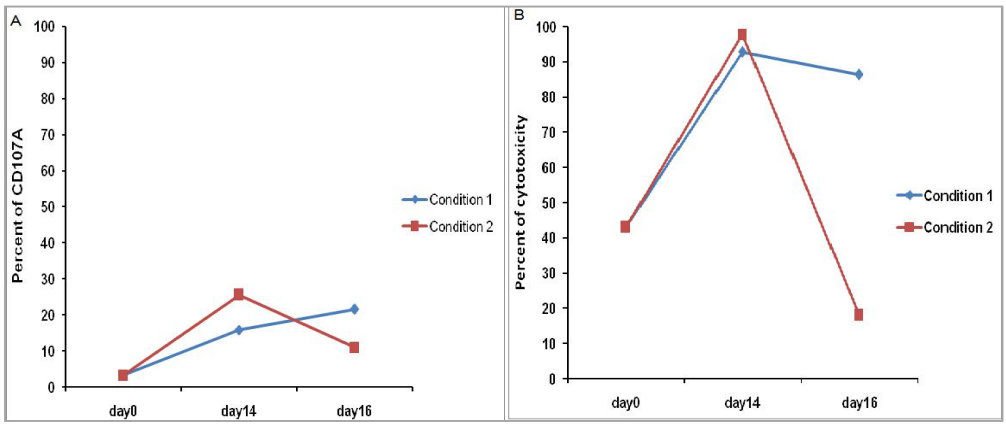

Fig. 4. (A) Comparison of CD107a expression between using condition I and II in 3 days (0, 14, 16). Values in quadrants represent the percentages of CD3−CD56+ NK cells that express CD107a marker in conditions I and II and negative control. (B) Comparison of NK cell cytotoxicity between using condition I and II in 3 days (0, 14, 16). Values in quadrants represent the percentages of CD3−CD56+ NK cells cytotoxicity in conditions I and II and negative control.

in part by the products released from activated PBMCs, which can more stimulate NK cells.

After 2 weeks of proliferation we tried to determine the NK cell cytotoxicity after treatment with IL-18. The expression of CD107a against HL-60 cells on NK cells cultured with anti CD3 was clearly increased after adding IL-18. However, in the condition of stimulating of PBMCs using PHA, the CD107a expression on NK cells was significantly decreased after adding IL-18. Our results from anti-CD3 treated condition suggested a role of IL-18 in augmenting the activation of NK cells, which is consistent with the previously published studies (Lusty, et al. 2017, Qi et al. 2014). Studies have shown that IL-18 can potently co-stimulate activated NK cells by ligation of Fc receptors and, synergistically, with IL-2 increases CD107a degranulation of human NK cells in vitro (Qi, et al., 2014, Srivastava, et al., 2013).

Kurimoto et al. have shown induction of IL-18 receptor (IL-18R) expression on T cells was moderately in presence of PHA. They suggest that optimum induction of IL-18R expression on T cells, needs the presence of stimulatory cytokines together with a stimulatory signal through the T cell receptor/CD3 complex (Kunikata, et al., 1998). This is consistent with our findings as stimulation of NK cells in presence of allogeneic PBMCs, cytokines and anti-CD3 in our study probably inducedIL-18R expression strongly on T cells while PHA did not. Interestingly, Sivalingam *et al.* in a study on PBMC from rheumatoid arteritis (RA) patients and healthy subjects have found that PHA treatment decreased IL-18 gene and protein expression after 72h in both groups (Sivalingam, et al., 2007).

In the other study, Dayton et al. have found in the presence of low levels of IL-2, IL-18 induced NK cell apoptosis and inhibited NK cell expansion while in the high concentrations, IL-2 overcame these effects

of IL-18, and high concentrations of IL-2 elevated the stimulatory effect of IL-18 on NK cells (Huang, et al., 2010).

Therefore, decrease of NK cytotoxicity after adding IL-18 in the presence of PHA in our experiments may be related to suppressor effect of PHA or low levels of IL-2. Additionally, Duarte et al. (2002) have demonstrated a functional disadvantage for PHA-based protocols for transduction of human T-lymphocytes. Their results showed that PHA can lead to an impairment of the immune competence of T cells *in vitro*, and they identified expansion protocols based on CD3/CD28 stimulation maintain the ability of the target cells to respond *in vitro* to either allogeneic stimulation or viral antigens. So, the possibility of decreased ex vivo cytotoxicity of NK cells in the presence of PHA implicates its uselessness as aNK cell activator. However, more detailed studies are needed to assess the effect of PHA on NK cytotoxicity (Huang, et al., 2010).

CD107a expression correlates with both NK cell-mediated lysis of target cells and cytokine secretion and addition of CD107a expression of NK cells after IL-2 stimulation is parallel to the increase of cytotoxicity (Aktas, et al., 2009, Alter, et al. 2004). In the present study, the combination of IL-2 and IL-15 significantly increased the expression of CD107a on NK cells comparing to negative control. Interestingly, the results using combination of IL-2, IL-15 and IL-18 were not significantly different from using IL-2 and IL-15 cocktail. In consistent with our results, Riley *et al.* have indicated a synergy between IL-15, IL-2, and to a lesser extent IL-18 in NK cell activation (Nielsen, et al., 2016). They also have shown that IL-18 and IL-2 in a positive feedback loop induce CD25 and IL-18Rα expression on NK cells and IL-18 synergizes with FcγRIII (CD16) signaling to augment antibody-dependent cellular cytotoxicity (ADCC).

Cumulatively, the evidence suggests that the diverse conditions such as mitogenes, cytokines, culture media, and allogeneic lymphocytes have different effects on *ex vivo* expansion of cytotoxic NK cells.

CONCLUSION

In the present study, we established a simplified and efficient method for NK cells isolation from healthy volunteers. We showed that, NK cells were effectively expanded with irradiated allogeneic PBMCs in the presence of OKT3and IL-2, IL-15 in SCGM medium resulting in a highly pure population of CD3-CD56+NK cells within 14 days which is shorter than the most presented conditions with 21 days of culture. The NK cells were highly cytotoxic against HL-60 cell line at E:T ratio of 10:1. Using IL-18 did not induce further expansion or cytotoxic activity in NK cells treated with anti-CD3 and even it severely reduced the PHA treated NK cells cytotoxicity. Thus, using only IL-2 and IL-15 plusanti CD3 in SCGM medium for 14 days is enough for producing a large number of activated NK cells.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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