ANTICANCER RESEARCH *40*: 5355-5359 (2020)
doi:10.21873/anticanres.14543

Human NK-92 Cells Function as Target Cells for Human NK Cells – Implications for CAR NK-92 Therapies

HANS BERGMAN, NOORA SISSALA, HENRY HÄGERSTRAND and CHRISTER LINDQVIST

Cell Biology Unit, Faculty of Natural Sciences and Engineering, Åbo Akademi University, Turku, Finland

Abstract. *Background/Aim: Recent studies indicate that chimeric antigen receptor (CAR)-T-cells seem to be superior to CAR modified NK-92 cells. One, at least partial, explanation to this discrepancy has been addressed herein, by having NK-92 cells as target cells in cytotoxicity reactions using peripheral blood mononuclear cells. Materials and Methods: A time-resolved fluorometric assay (TDA-labeled NK-92 or K562 as target cells) was used for measuring the cytotoxic activity of blood mononuclear cells (PBMC). Results: The cytotoxic capacity of the NK-92 cells was initially demonstrated by their ability to efficiently kill K562 cells. Interestingly, having PBMC as effector cells rendered the very same NK-92 cells sensitive to NK-cell mediated cytolysis. A 1:100 target:effector ratio gave 34.1% lysis compared to 72.2% lysis for K562 cells. Incubating PBMC for longer times (24 up to 48 h) potentiated their NK-activity against NK-92 cells even more, reaching a level close to that obtained with K562 cells. Conclusion: This study pinpoints a severe problem that has to be considered in future immune-based cancer therapies with NK-92 as well as CAR-transduced NK-92 cells.*

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system that have the ability to kill cancer as well as virally infected cells without prior sensitization (1). This observation has led to the use of NK cells for therapeutic purposes. Especially hematologic malignancies have turned out to be targets for NK cells collected by apheresis using current good manufacturing processes (cGMP) (2). However, the small number of collected NK cells combined with their limited *ex vivo* therapeutic activity has forced scientists to use NK cell lines (3, 4).

Correspondence to: Christer Lindqvist, Cell Biology, Faculty of Natural Sciences and Engineering, Åbo Akademi University, Tykistökatu 6, FIN-20520, Turku, Finland. Tel: +358 22154053, e-mail: clindqvi@abo.fi

Key Words: Natural killer cells, lysis, NK-92, K562, PBMC.

NK-92 is so far the only FDA-approved cell line to be used in clinical trials and many immunotherapies have in fact already been conducted with this cell line (5). Chimeric antigen receptor modified NK-92 cells (CAR NK-92) (*e.g.* CD19, CD20, CD138 and CS-1 specific) have lately also been generated. Engineered cell lines might become very attractive in future clinical trials; in fact, the first phase I clinical trial has already been conducted with CD33-modified CAR NK-92 cells (4, 6, 7). Altogether, CAR-engineered NK-cells have potential to become the first "off-the-shelf" cellular products for the treatment of cancer. However, data presented here demonstrate a severe drawback for using the NK-92 cell line in these types of clinical trials, since these cells are in fact susceptible target cells for peripheral blood mononuclear cells (PBMC) prepared from normal healthy individuals. This finding and possible ways to circumvent the problem are discussed.

Materials and Methods

Reagents. Recombinant human IL-2 was purchased from PeproTech EC (London, UK).

Cell lines. The natural killer cell line NK-92 (ImmuneMedicine, Inc. Vancouver, Canada), the erythroleukemia NK-sensitive target cell line K562 and the human NK-like cell line YT, were all cultured at 37˚C in RPMI 1640 (GIBCO™ , Invitrogen Corp., Paisley, UK) plus 5% fetal calf serum (FCS; GIBCO™) supplemented with 2 mM L-glutamine, 10 U/ml penicillin G sodium salt and 10 μg/ml streptomycin sulphate (GIBCO™) (8-10). The culture medium of the NK-92 cell line was always supplemented with 20 U/ml of IL-2, but not present in the killing assays. PBMC were isolated from blood donated by healthy volunteers (Finnish Red Cross Blood Service) by density gradient centrifugation on Ficoll-Paque density gradient media (GE Healthcare Life Sciences, CT, USA).

Labelling of target cells with benzophenonetetracarboxylic dianhydride (BATDA). K562, YT or NK-92 target cells (1×10^6 in 1 ml RPMI 1640 plus 5% FCS) were first pre-incubated for 15 min at 37˚C before incubation with 20 μM BATDA (PerkinElmer, Inc., Wellesley, MA, USA) for 25 min at 37˚C. The cells were finally washed 4 times in PBS with mild centrifugation (306 × *g*, 2 min) and were adjusted to 5×10^4 cells/ml in RPMI 1640 plus 5% FCS before being mixed with the effector cells.

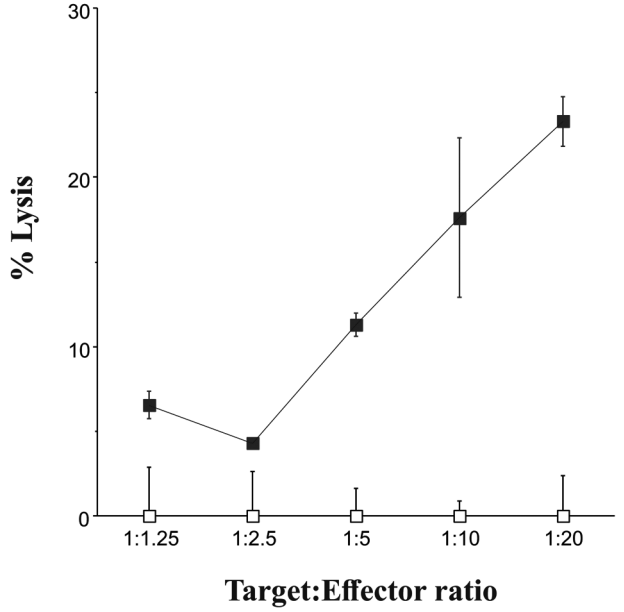

Figure 1. *NK-92 are functional natural killer cells. K562 cells (filled squares) or NK-92 cells (open squares) were used as target cells. Results are expressed as means±SD and are representative of three independent experiments.*

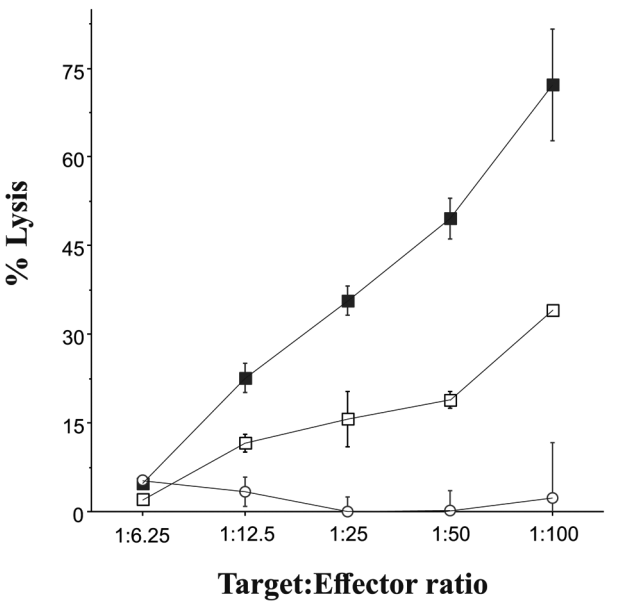

Figure 2. *Blood mononuclear cells as effector cells against K562 (filled squares), NK-92 (open squares) or YT cells (open circles). Results are expressed as means±SD and are representative of three independent experiments.*

Cytotoxicity assay. The cytotoxicity assay was, with minor modifications, performed as described earlier (11). In brief, serial dilutions of NK-92 cells or PBMC in 100 μl/well (in triplicates) were added to v-bottomed 96-well microtiter plates (Sarstedt Inc, Nümbrecht, Germany). Effector to target ratios ranged from 100:1 to 1.25:1 following the addition of 100 μl (5×10^3) of target cells to each well. Following a short centrifugation (34 × *g*, 1 min), the co-cultures were incubated for 2 h at 37˚C in a 95% humidified chamber with 5% CO_2. They were then centrifuged for 5 min (688 × *g*), and 20 μl of supernatant from each well was picked and added to 100 μl europium solution (Eu) (PerkinElmer, Inc.) contained within flat-bottomed 96-well Costar RIA/EIA plates (Corning Inc., Corning, NY, USA). The spontaneous Eu release was determined by incubating the target cells in the culture medium alone instead of having effector cells added, and the maximum Eu release was determined by incubating the target cells in the same medium with 0.05% Triton X-100. The plates containing Eu and the co-culture supernatants were finally shaken for 15 min and the fluorescence of the EuTDA chelates formed was measured using a 1420 Victor multi-label counter (PerkinElmer Inc.). Percentages of specific releases were calculated using the following formula:

Specific Release=(Experimental release-spontaneous release)/(Maximum release-spontaneous release) ×100%

Statistical analysis. Comparisons between treatments were performed using independent samples *t*-test (SPSS 23.0 software; SPSS Inc., Chicago, IL, USA). All *p*-values less than 0.05 were considered statistically significant.

Results

NK-92 cells can lyse K562 cells. The cytolytic capacity of the NK-92 cells used were first analyzed in a regular killing assay by having K562 cells as target cells. Figure 1 shows that a 1:5 ratio of K562:NK-92 cells already gave an NK activity in the range of 10%, that increased to 23.3% with a 1:20 target:effector ratio. When NK-92 cells were used as control targets cells, no killing could be observed at all.

PBMC can kill NK-92 cells. By changing the effector cells from NK-92 to PBMC, but still having K562 cells as target cells, an NK-activity can be noticed from around 5% lysis [PBMC:K562 (6.25:1) cell ratio] up to 72.2% for a 100:1 E:T ratio (Figure 2). Interestingly, NK-92 cells could also be lysed by PBMC. As can be seen in Figure 2, an E:T ratio of 12.5:1 resulted in a 11.6% release, whereas a 100:1 E:T ratio gave 34.1% lysis. Another human NK-like cell line, namely YT, was not sensitive to lysis mediated by PBMC.

Pre-culturing PBMC for 24 to 48 h in medium increased their NK activity against NK-92 cells. Figure 3A shows the NK-activity against NK-92 and K562 cells after culturing PBMC in medium for 24 h. This 24-h pre-culture of PBMCs increased their NK activity against NK-92-cells from 34.1%, without pre-incubation, to 50.2% lysis (1:100 E:T ratio). A similar proportional increase in NK activity was also observed for the

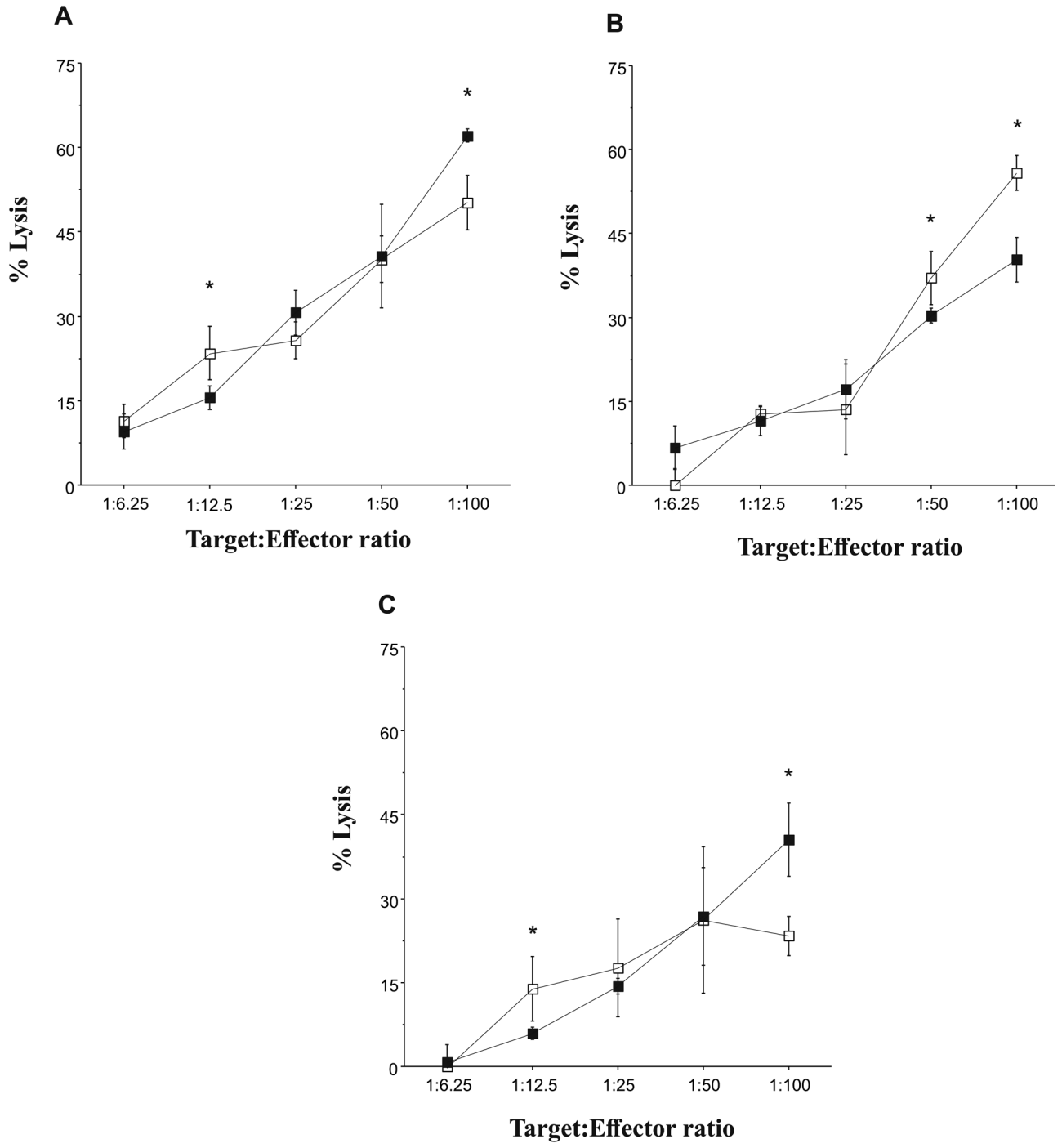

Figure 3. *Incubation of blood mononuclear cells for 24 h (A), 48 h (B) or 72 h (C) prior to the NK activity analysis against K562 (filled squares) or NK-92 (open squares) cells. Results are expressed as means±SD and are representative of three independent experiments. *p<0.05 as compared with K562 cells.*

other PBMC:NK-92 ratios. However, the K562 sensitivity was more or less the same for most of the E:T ratios regardless of whether the PBMC had been pre-incubated for 24 h or not. A 48-h pre-incubation reduced the NK-activity against K562 cells compared to the 24 h pre-culture, whereas the percent release values were close to similar for NK-92 cells (Figure 3B). Pre-culturing the PBMC for 72 h reduced the NK activity for both target cell lines, as shown in Figure 3C.

Discussion

The expansion and maintenance of autologous as well as allogeneic NK cells *ex vivo* in quantities needed for adoptive immunotherapies is rather demanding (12). Different types of cytokines, especially Interleukin-2, are normally added to these culture vessels in order to improve the outcome. However, regulatory T-cells (T_{reg}) that can suppress the NK-activity are also expanded and possibly activated in these *ex vivo* cultures (13). Altogether, these circumstances have made human cell lines with NK properties very attractive for adoptive immunotherapies and the CAR cell engineering technology has accentuated this development even further (4, 14, 15).

Since the NK-92 cell line is a realistic alternative for the clinic, either as such or as a CAR engineered variant, we initially confirmed its NK activity with erythroleukemia K562 cells (Figure 1). Interestingly and to our great surprise, when these NK-92 cells were used as target cells, they could easily be killed by PBMCs isolated from normal healthy donors (Figure 2). The percent lysis values observed for K562 cells were more than two times higher compared to NK-92 cells at 25:1 to 100:1 effector:target ratios. The results in Figure 2 demonstrate that NK-92 are in fact susceptible to NK cell mediated lysis, which might well explain the rather weak outcome of the "first-in-man clinical trial" conducted by Tang *et al*. with CD33-CAR NK-92 cells, as well as the findings that CAR-T-cells in general seem to be superior to CAR NK-92 cells (7, 16). The human NK-like leukemia cell line YT, another potential cell line for adoptive immunotherapies, was not sensitive at all (Figure 2), an observation that now makes the YT cell line more attractive for adoptive immunotherapies compared to NK-92 cells.

By pre-culturing the PBMCs in normal medium for 24 h up to 72 h, compared to no pre-culturing conditions (Figure 2), generated cells that could lyse NK-92 cells even better (Figure 3A and B) and with an almost similar killing pattern as with K562 cells (Figure 3A-C).

Whether the increased NK activity against NK-92 cells by time is due to down-regulation of certain regulatory T cells or NK-cells with some kind of regulatory properties, mediated by soluble factors or already known cytokines or direct cell-to-cell contacts, remains to be seen. There are several issues that have to be clarified in order to further develop effective NK-92 cell-based immunotherapies.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

HB performed the majority of the experiments and contributed to the writing of the manuscript. NS performed part of the experiments and contributed to the writing of the manuscript. All Authors discussed the results and contributed to the final manuscript.

References

1 Trinchieri G: Biology of natural killer cells. Adv Immunol *47*: 187-376, 1989. PMID: 2683611. DOI: 10.1016/s0065-2776(08)60664-1

2 Fang F, Xiao W and Tian Z: NK cell-based immunotherapy for cancer. Semin Immunol *31*: 37-54, 2017. PMID: 28838796. DOI: 10.1016/j.smim.2017.07.00

3 Galluzzi L, Vacchelli E, Bravo-San Pedro JM, Buqué, A, Senovilla L, Baracco EE, Bloy N, Castoldi F, Abastado JP, Agostinis P, Apte RN, Aranda F, Ayyoub M, Beckhove P, Blay JY, Bracci L, Caignard A, Castelli C, Cavallo F, Celis E, Cerundolo V, Clayton A, Colombo MP, Coussens L, Dhodapkar MV, Eggermont AM, Fearon DT, Fridman WH, Fučíková J, Gabrilovich DI, Galon J, Garg A, Ghiringhelli F, Giaccone G, Gilboa E, Gnjatic S, Hoos A, Hosmalin A, Jäger D, Kalinski P, Kärre K, Kepp O, Kiessling R, Kirkwood JM, Klein E, Knuth A, Lewis CE, Liblau R, Lotze MT, Lugli E, Mach JP, Mattei F, Mavilio D, Melero I, Melief CJ, Mittendorf EA, Moretta L, Odunsi A, Okada H, Palucka AK, Peter ME, Pienta KJ, Porgador A, Prendergast GC, Rabinovich GA, Restifo NP, Rizvi N, Sautès-Fridman C, Schreiber H, Seliger B, Shiku H, Silva-Santos B, Smyth MJ, Speiser DE, Spisek R, Srivastava PK, Talmadge JE, Tartour E, Van Der Burg SH, Van Den Eynde BJ, Vile R, Wagner H, Weber JS, Whiteside TL, Wolchok JD, Zitvogel L, Zou W and Kroemer G: Classification of current anticancer immunotherapies. Oncotarget *5*: 12472-12508, 2014. PMID: 25537519. DOI: 10.18632/oncotarget.2998

4 Suck G, Odendahl M, Nowakowska P, Seidl C, Wels WS, Klingemann HG and Tonn T: NK-92: an 'off-the-shelf therapeutic' for adoptive natural killer cell-based cancer immunotherapy. Cancer Immunol Immunother *65*: 485-492, 2016. PMID: 26559813. DOI: 10.1007/s00262-015-1761-x

5 Klingemann H, Boissel L and Toneguzzo F: Natural killer cells for immunotherapy – Advantages of the NK-92 cell line over blood NK cells. Front Immunol *7*: 91-97, 2016. PMID: 27014270. DOI: 10.3389/fimmu.2016.00091

6 Jiang H, Zhang W, Shang P, Zhang H, Fu W, Ye F, Zeng T, Huang H, Zhang X, Sun W, Man-Yuen Sze D, Yi Q and Hou J: Transfection of chimeric anti- CD138 gene enhances natural killer cell activation and killing of multiple myeloma cells. Mol Oncol *8*: 297-310, 2014. PMID: 24388357. DOI: 10.1016/j.molonc.2013.12.001

7 Tang X, Yang L, Li Z, Nalin, AP, Dai H, Xu T, Yin J, You F, Zhu M, Shen W, Chen G, Zhu X, Wu D and Yu J: First-in-man clinical trial of CAR NK-92 cells: safety test of CD33-CAR NK-92 cells in patients with relapsed and refractory acute myeloid leukemia. Am J Cancer Res *8*: 1083-1089, 2018. PMID: 30323981.

8 Gong J-H, Maki G and Klingemann HG: Characterization of a human cell line (NK-92) with phenotypocal and functional characteristics of activated natural killer cells. Leukemia *8*: 652-658, 1994. PMID: 8152260.

9 Lozzio CB and Lozzio B: Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood *45*: 321-324, 1975. PMID: 163658.

10 Yodoi J, Teshigawara T, Nikaido K, Fukui K, Noma T, Honjo T, Takigawa M, Sasaki M, Minato N and Tsudo M: TCGF (IL 2)-receptor inducing factor(s). I. Regulation of IL 2 receptor on a natural killer-like cell line (YT cells). J Immunol *134*: 1623-1630, 1985. PMID: 2578514.

11 Blomberg K, Hautala R, Lövgren J, Mukkala V-M, Lindqvist C and Åkerman K: Time resolved fluorometric assay for natural killer activity using target cells labelled with a fluorescence enhancing ligand. J Immunol Meth *193*: 199-206, 1996. PMID: 8699033. DOI: 10.1016/0022-1759(96)00063-4

12 Klingemann HG and Martinson J: Ex vivo expansion of natural killer cells for clinical applications. Cytotheraphy *6*: 15-22, 2004. PMID: 14985163. DOI: 10.1080/14653240310004548

13 Smyth MJ, Teng MW, Swann J, Kyparissoudis K, Godfrey DI and Hayakawa Y: CD4+CD25+ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. J Immunol *176*: 1582-1587, 2006. PMID: 16424187. DOI: 10.4049/jimmunol.176.3.1582

14 Harnack U, Johnen H and Pecher G: Natural killer cell line YT exerts cytotoxicity against CD86+ myeloma cells. Anticancer Res *31*: 475-479, 2011. PMID: 21378326.

15 Ingegnere T, Mariotti FR, Pelosi A, Quintarelli C, De Angelis B, Tumino N, Besi F, Cantoni C, Locatelli F, Vacca P and Moretta L: Human CAR NK Cells: A new non-viral method allowing high efficient transfection and strong tumor cell killing. Front Immunol *10*: 957-966, 2019. PMID: 31114587. DOI: 10.3389/fimmu.2019.00957

16 Alcantara M, Tesio M, June CH and Hout R: CAR T-cells for T-cell malignancies: challenges in distinguishing between therapeutic, normal, and neoplastic T-cells. Leukemia *32*: 2307-2315, 2018. PMID: 30315238. DOI: 10.1038/s41375-018-0285-8

Received June 21, 2020
Revised July 9, 2020
Accepted July 10, 2020