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Establishment of Cytotoxic and Non-cytotoxic NK-92 Cell Clones – Evaluation of Their Function as Target Cells

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Abstract. *Background/Aim: Previous studies have demonstrated that NK cells present in PBMCs might explain why clinical trials conducted with NK-92 as well as CAR modified NK-92 cells have to a large extent failed. Two NK-92 clones with different NK target cell properties have been established and are described here. Materials and Methods: Two NK-92 cell clones, NK-92 clone 1 and clone 2, were established using the limiting dilution technique. A time-resolved fluorometric assay (TDA-labeled NK-92 clone 1, 2 or K562 as target cells) was used for measuring their sensitivities to NK cell-mediated cytolysis and their NKG2D expression was identified with immunoblotting. Results: A striking difference between the NK-92 clones in their cytotoxic capacity against K562 cells was observed. A clear correlation was noticed between these NK-92 clones when used as target cells and their ability to kill K562 cells. A 50:1 effector:target ratio (PBMCs:NK-92 clone 1) gave 6.50±5.44% lysis whereas the corresponding value was 39.9±10.0% with NK-92 clone 2 as target cells. Interestingly, incubating PBMCs in medium for longer times slightly potentiated their NK activity also against the NK-92 clone 1 (E:T ratio 50:1), from 2.5±0.88% lysis (24 h pre-incubation time) to 13.7±9.04% (48 h) and 13.8±6.89% (72 h). Immunoblotting with anti-NKG2D antibodies stained an approximately 34 kDa protein band in lysates prepared from NK-92 clone 1 cells, which corresponds to the NKG2D antigen. A very faint band of the same size was observed in lysates prepared from NK-92 clone 2 cells. Conclusion: The NK-92 clones 1 and 2, established and described here, might turn out to be very useful for finding possible solutions for using NK-92 and CAR NK-92 cells in future treatments of human malignant diseases.*

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

It has been close to 50 years since natural killer (NK) cells were identified by Kiessling *et al*. and Herberman *et al*. (1, 2). These cells belong to the innate immune system and comprise 5% to 15% of the peripheral blood mononuclear cells (PBMCs). Interestingly, they have an ability to kill both some tumor as well as virally infected cells without prior sensitization (3-5). The cell surface proteins involved in the NK cell mediated cytotoxicity process are all germline-encoded receptors and are defined as either killer activation (KARs) or killer inhibitory receptors (5-7). The balance between these inhibition and activation signals determines the final outcome of the target cell (8).

NKG2D is a C-type lectin-like receptor, with its gene located on chromosome 12 (9). It does not form a heterodimeric receptor with CD94 as the other members of the NKG2 family instead NKG2D forms a homodimeric structure that can be found on all NK cells as well as on CD8+ ab and gd T cells (10, 11). NKG2D alone is able to trigger an activation of NK cells and is therefore most likely an important player in the recognition process of target cells (12).

Klingemann and coworkers isolated and established the NK-92 cell line from a 50-year-old patient with a malignant non-Hodgkin's lymphoma in 1992 (13). This cell line, which is dependent on recombinant IL-2, is cell surface marker positive for CD2, CD45, CD56 bright and negative for CD3 and CD34. It also lacks the CD16 receptor and can therefore not mediate antibody-dependent cellular cytotoxicity ADCC (13).

The NK-92 cell line is exceptional, since it lacks almost all inhibitory killing receptors. It has, in addition, high granzyme- and perforin levels, which is more or less a prerequisite for its high cytotoxic activity (14). Altogether, these properties have made the NK-92 cell line attractive in NK-92 or chimeric antigen receptor (CAR)-NK-92 cell therapies. A number of successful preclinical trials have been conducted with these cells, but the results obtained from clinical trials have not been encouraging (15, 16). However, recent data reported by Bergman *et al*. might explain the poor outcome of these clinical trials (17, 18). The NK-92 cells were in these studies in fact demonstrated to be susceptible target cells for PBMCs. Two NK-92 clones are presented

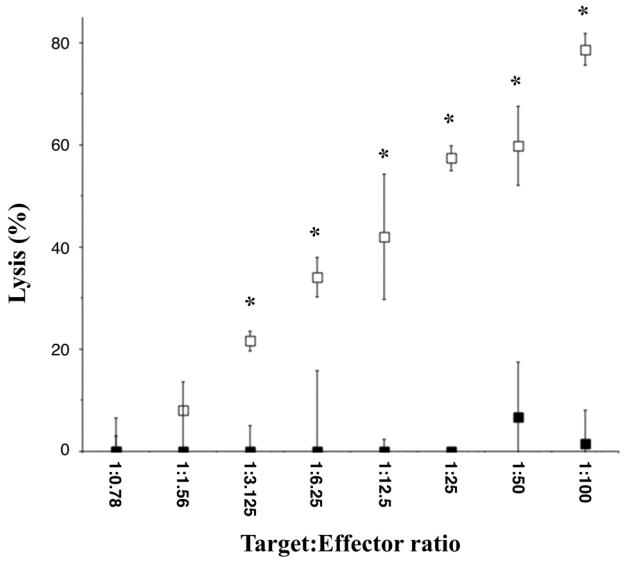

Figure 1. *Cytotoxic activity of NK-92 clone 1 and clone 2 cells against K562 cells. The graph shows the percentage of K562 cell lysis using different target:effector ratios for NK-92 clone 1 (open squares) and NK-92 clone 2 (filled squares). Results are expressed as means±SD and are representative of three independent experiments. *p<0.05 compared to NK-92 clone 2.*

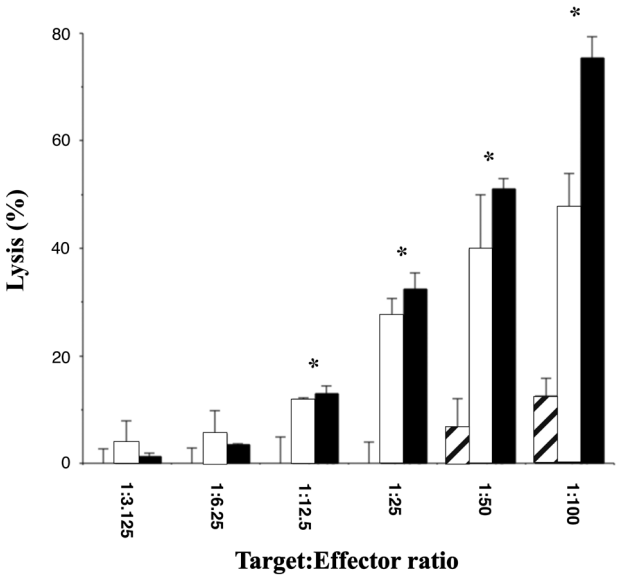

Figure 2. *PBMCs as effector cells against NK-92 clone 1 or clone 2 cells. The graph shows the percentage of NK-92 cell lysis using different target:effector ratios for NK-92 clone 1 (bars with stripes), NK-92 clone 2 (open bars) or K562 (filled bars) cells. Results are expressed as means±SD and are representative of three independent experiments. *p<0.05 compared to NK-92 clone 1.*

here, partially characterized and the outcome of these studies discussed with respect to possible improvements of NK-92 or CAR-NK-92 cell based immunotherapies.

Materials and Methods

Cells and cell lines. The human natural killer cell line NK-92 (ImmuneMedicine, Inc. Vancouver, Canada) and the human erythroleukemia NK-sensitive target cell line K562 were 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™) in a 95% humidified chamber with 5% CO_2 (13, 19). The culture medium of the NK-92 cell line was always supplemented with 20 U/ml of rIL-2 (PeproTech EC, London, UK), 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, Fairfield, CT, USA).

Labelling of target cells with benzophenonetetracarboxylic dianhydride (BATDA). K562 or NK-92 clone 1 or NK-92 clone 2 target cells (1×10^6 in 1ml 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 RPMI 1640 plus 5% FCS with mild centrifugation (306 × *g*, 2 min) and adjusted to 5×10^4 cells/ml before being mixed with the effector cells.

Cytotoxicity assay. The cytotoxicity assay was, with minor modifications performed as described earlier (20). In brief, serial dilutions of PBMCs in 100 μl/well (in triplicates) were added to v-bottomed 96-well microtiter plates (Sarstedt Inc, Nümbrecht, Germany). Target to effector ratios ranged from 1:100 to 1:0.78 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 20μl of supernatant from each well were picked and added to 100 μl europium solution (Eu) (PerkinElmer, Inc.) contained within black flat-bottomed 96-well Costar plates 3916 (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:

$$\text{Specific release} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100\%$$

Immunoblotting. Immunoblot analyses were performed essentially as described by Towbin *et al.* (21). In brief, cell protein solutions diluted in 2× Laemmli sample buffer (Sigma-Aldrich, Saint Louis, MO, USA) were separated on 8-16% Mini-PROTEAN TGX

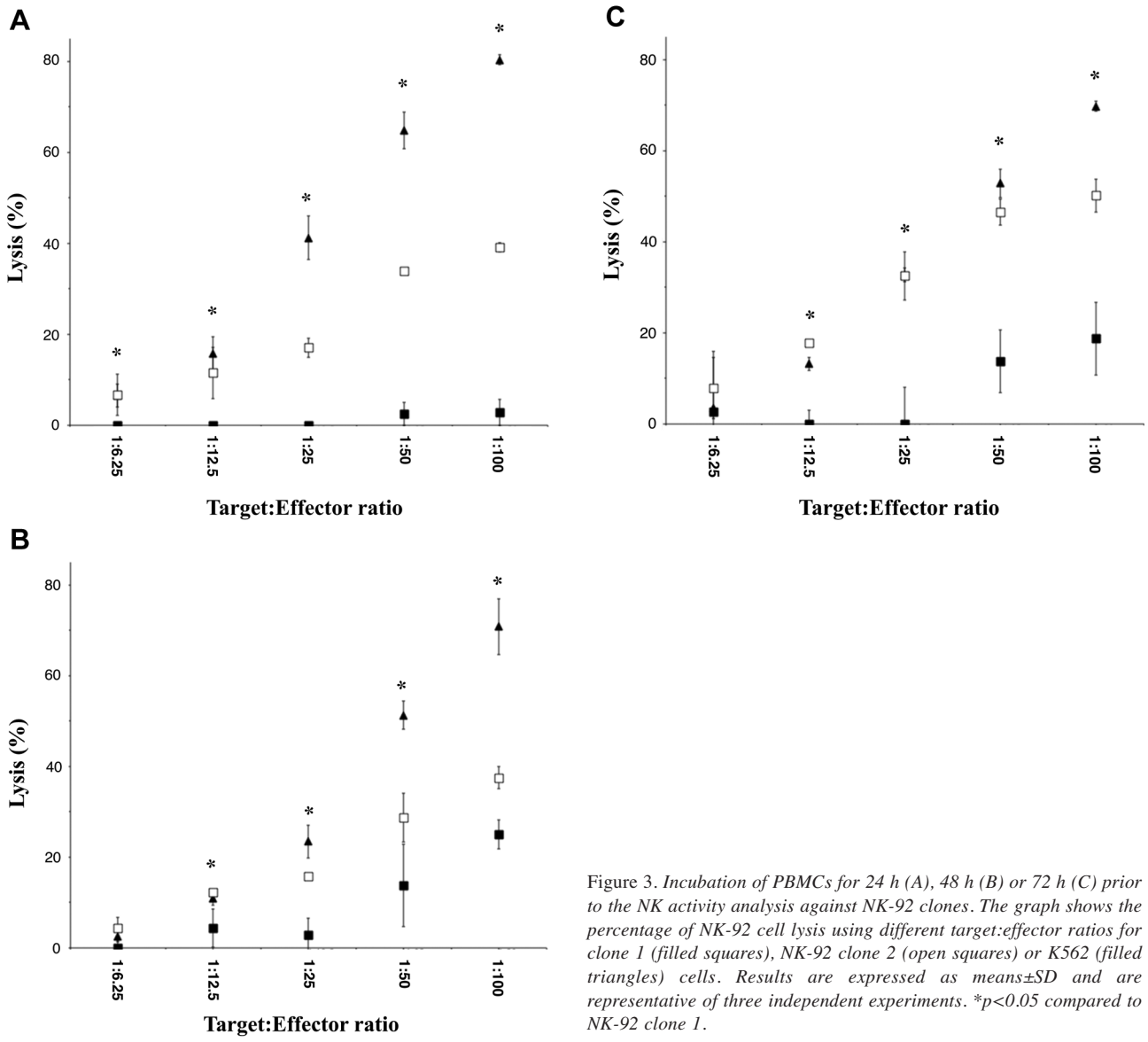

Figure 3. *Incubation of PBMCs for 24 h (A), 48 h (B) or 72 h (C) prior to the NK activity analysis against NK-92 clones. The graph shows the percentage of NK-92 cell lysis using different target:effector ratios for clone 1 (filled squares), NK-92 clone 2 (open squares) or K562 (filled triangles) cells. Results are expressed as means±SD and are representative of three independent experiments. *p<0.05 compared to NK-92 clone 1.*

polyacrylamide gels (BioRad, Hercules, CA, USA) at 200 V for 40 min, according to Laemmli (22), and electrophoretically transferred to nitrocellulose membranes (Immobilon-P, Millipore, Bedford, MA, USA) in transfer buffer [25 mM tris, 192 mM glycine, 20% (v/v) methanol] using a BioRad Transblot cell (BioRad, 1 h at 100 V). The nitrocellulose sheets were then transferred to a membrane blocking solution (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) (16 h, +4˚C). After three washes PBS with 0.05% Tween 20 the filters were immersed into a rabbit anti-human NKG2D antibody solution (Invitrogen, 1:1,000 dilution in PBS + 0.5% BSA). After 24 h incubation (+4˚C) followed by three washes [PBS + Tween 20 (0.05%)], the goat anti-rabbit-IgG (H+L)-HRP labelled antiserum (Invitrogen, 1:4,000 dilution in PBS + 0.5% BSA) was added and incubated for 55 min at 4˚C. The blots were thereafter developed using the SuperSignal™ West Atto Ultimate Sensitivity Substrate (ThermoFisher Scientific) and viewed using the Invitrogen iBright FL1000 Imaging System (ThermoFisher Scientific).

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

A striking difference between NK-92 clone 1 and 2 cells with respect to killing of K562 cells. The NK-92 cells were subjected to successive rounds of limiting dilution in order to establish NK-92 clones with different characteristics. All

cloning steps were performed in the presence of 20 U/ml rIL-2. NK-92 clone 1 and 2 were initially established by analyzing their ability to lyse K562 cells after each round of limiting dilution. Figure 1 shows the cytotoxic activity of two established NK-92 clones (NK-92 clone 1 and clone 2) after a total of eight limiting dilution steps. The cytotoxic activity of the NK-92 clone 1 against K562 cells was 78.7±3.05%, whereas the corresponding lysis value was 1.5±6.58% for the NK-92 clone 2 (E:T ratio 100:1). Even at a 1.56:1 E:T ratio, was the cytotoxic activity of the NK-92 clone 1 as high as 8.01±0.74% (Figure 1).

NK-92 clone 2 is sensitive to PBMCs mediated NK activity. Next both NK-92 clones were analyzed as target cells in a PBMCs mediated NK cytotoxicity assay. As shown in Figure 2, NK-92 clone 2 was close to as sensitive as K562 cells at a 25:1 T:E ratio (27.5±3.17% *vs*. 32.4±3.07% lysis for K562 cells), whereas cells from NK-92 clone 1 were not lysed at all. As expected, these % lysis values increased with higher E:T ratios and also NK-92 clone 1 cells were lysed (12.5±3.35%) at a 100:1 E:T ratio (Figure 2).

Pre-culturing PBMCs for 24 to 72 h in medium increases their NK activity against both NK-92 clones. Figure 3A shows the NK-activity against NK-92 clone 1 and clone 2 cells after culturing PBMCs in medium for 24 h. The % lysis values obtained after a 24-h pre-culture are to a large extent similar to those obtained without any pre-culturing (Figure 2). However, a 48-h pre-culture of PBMCs increased their NK activity against NK-92 clone 1 cells up to 25.0±3.23%, whereas the per cent lysis value was 37.5±2.43%, when NK-92 clone 2 cells were used as target cells (E:T ratio 100:1, Figure 3B). Interestingly, a 72-h pre-culturing increased the NK activity against NK-92 clone 2 cells up to 50.1±3.64% (Figure 3C). A slight decrease in cytotoxicity by time, if anything, was observed when K562 cells were used as target cells (Figure 3A, B and C). Similar proportional decreases/increases in the NK activity were observed for the other E:T ratios, regardless of target cell used.

NKG2D expression on NK-92 clone 1 cells. Immunoblotting with a polyclonal anti-NKG2D antisera stained an approx. 34 kDa protein band in cell lysates prepared from the NK-92 clone 1 (Figure 4, lane 2). A very faint protein band with a corresponding molecular weight was noticed in cell lysates prepared from the NK-92 clone 2 (Figure 4, lane 3).

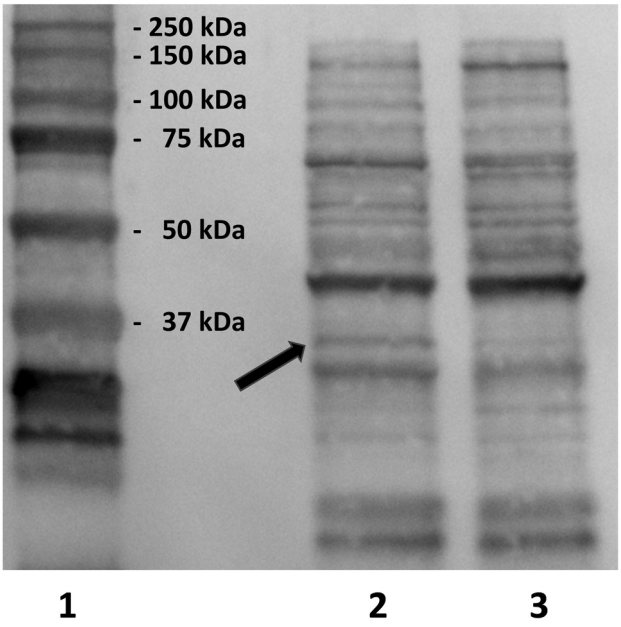

Figure 4. *Immunoblotting of lysates prepared from NK-92 clone 1 cells (lane 2) or NK-92 clone 2 cells (lane 3) with a rabbit anti-NKG2D antiserum. The NKG2D protein is indicated with an arrow. Molecular size markers are in lane 1.*

Discussion

Great enthusiasm was generated especially among immunologists in the mid 70s after the discovery of NK cells. A cellular "tool" had been found that could identify and eliminate cells undergoing neoplastic transformation. Later it was demonstrated that these cells could also eliminate some virally infected cells, as well as enhance antibody and T cell responses (23). A number of NK based clinical trials have been conducted since then and lately also with NK cell lines such as NK-92 or CAR-NK-92 cells (15, 16, 24). However, the NK-92 based preclinical studies that generated great hope among oncologists, have turned into disappointment, when the results from the clinical trials were presented (15, 16). Findings by Bergman *et al*. have provided a likely explanation to these clinical failures, namely that the NK-92 cells are themselves susceptible target cells for PBMCs prepared from normal healthy individuals (17).

In an effort to better characterize the mechanisms and thereby in the long run perhaps find some hints on how to overcome the poor outcomes of the NK-92/CAR-NK-92 based clinical trials cloning attempts were conducted with the NK-92 cells. As demonstrated in Figure 1, two NK-92 clones were established with drastic differences (57.4±2.48% for NK-92 clone 1 *vs*. 0.0±0.49% for NK-92 clone 2 at E:T ratio 25:1) in their abilities to lyse K562 cells. Interestingly, when the very same cell clones were analyzed for their abilities to function as NK target cells, NK-92 clone 2 cells turned out to be almost as sensitive as K562 cells (27.5±3.17% for NK-92 clone 2 cells compared to 32.4±3.07% for K562 cells at E:T ratio 25:1). The corresponding E:T ratio with NK-92 clone 1 cells resulted in no lysis at all (0.0±3.91%, Figure 2). However, it is important

to mention that the preincubation of PBMCs for 24-72 hours in medium alone, generated an increased NK activity against both NK-92 clones. NK-92 clone 1 cells were lysed close to similar extents with PBMCs pre-incubated for 48 hours as for 72 hours (Figure 3A, B and C). A similar observation has been previously reported with NK-92 cells (17).

A classic property of NK cells is that no prior sensitization or proliferation of specific clones is needed in order for them to execute their lethal hits. A shortage in MHC class I expression functions as a triggering signal for the killing process to start. Important molecules in this effector:target recognition process are the inhibitory and activation receptors (5-8). Since NK-92 cells lack inhibitory receptors, we focused our interests on the activation receptors (14). Of the killer activation receptors analyzed (p30, p46 and NKG2D) only NKG2D expression levels were found to differ between the two clones. As can be seen in Figure 4 (lane 3), NK-92 clone 2 cells express very low amounts of NKG2D receptors compared to NK-92 clone 1 (lane 2). This latter observation, combined with their low ability to kill K562 cells and high sensitivity to be killed by PBMCs partly supports findings reported by Liu *et al*. (25). They suggest NKG2D to be a "master switch" in determining the activation status of NK cells, in addition to the expression of NKG2D ligands (NKG2DLs) in target cells (25, 26). Furthermore, several g chain-related cytokines have also been reported to modulate the NKG2D expression, which fit well with the results obtained from earlier PBMC:NK-92 cytotoxic models (18, 28).

The NK-92 clones 1 and 2 that are described here, might be valuable tools for further characterization of the mechanisms involved and thereby hopefully in obtaining knowledge on how to circumvent the problems that the clinical trials using NK-92 and CAR-NK-92 cells have encountered.

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 article. EH and CL performed part of the experiments and contributed to the writing of the article. All Authors discussed the results and contributed to the final article.

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