

# Culturing the Human Natural Killer Cell Line NK-92 in Interleukin-2 and Interleukin-15 – Implications for Clinical Trials

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**Abstract.** *Background/Aim: The human natural killer cell line NK-92 is increasingly being used in adoptive cell immunotherapies, either in vitro or in animal models transduced with different chimeric antigen receptor (CAR) constructs. Herein, NK-92 cells were analyzed with respect to their proliferation and cytotoxicity, in the presence of interleukin-2 (IL-2) and interleukin-15 (IL-15). Materials and Methods: A time-resolved fluorometric assay (TDA-labeled K562 target cells) was used for measuring the cytotoxic activity of NK-92 cells treated with IL-2, IL-4, IL-7 and/or IL-15. Their proliferation, in the presence of these common cytokine receptor  $\gamma$  chain ( $\gamma$ c)-dependent cytokines, was measured by traditional tritiated thymidine ( $[^3H]$ -TdR) incorporation. Results: IL-2 and IL-15, but not IL-4 or IL-7, were able to induce a dose-dependent proliferation of NK-92 cells. IL-15 was, depending on the dose and culture time, up to 10 times more potent compared to corresponding concentrations of IL-2, whereas their combination could potentiate the NK-activity almost equally well. No synergistic effects could be noticed with respect to the cytotoxicity and the proliferation of these cells. Conclusion: Data presented here indicate that of the common gamma chain receptor-dependent cytokines tested here, IL-15 alone is able to cultivate and trigger NK-92 cells to such an extent so that they can be used for immune-based cancer therapies. Implications with respect to CAR-transduced NK-92 cells are also discussed.*

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system that were identified in 1975 (1, 2). These cells can lyse virally infected cells as well as cancer

cells without prior sensitization (3). This latter observation has made NK cells very attractive for adoptive cell immunotherapies (4). The most common source of NK cells for this purpose has been mononuclear cells from peripheral blood, collected by apheresis using current good manufacturing processes (cGMP). However, the decreased number of collected NK cells and their limited *ex vivo* therapeutic activity have forced scientists to use additional sources of NK cells, such as the NK-92 cell line (5-7).

Technological advancements have allowed the expansion of NK-92 cells under GMP and several phase I clinical trials have been conducted using these cells with the aim of treating different types of cancers (8, 9). More recently chimeric antigen receptor (CAR)-engineered NK-92 cell lines have also been established for similar purposes (10-12).

NK cells are known for their ability to be kept alive in culture for a long time as well as for their ability to be activated when treated with different types of cytokines (13-15). Many of these cytokines contain the common gamma chain as a receptor component, which is crucial for the signal transduction induced by IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (16). The aim of this study was to find out which cytokines, alone or in combination, can best activate NK-92 cells. The effects of these cytokines on the proliferation of NK-92 cells were also analyzed. The obtained results are discussed with respect to present and future clinical trials conducted using this cell line as a means of adoptive cell immunotherapy.

## Materials and Methods

**Reagents.** Recombinant human IL-2 was purchased from R&D Systems (Minneapolis, MN, USA) and recombinant human IL-4, IL-7 and IL-15 from PeproTech EC (London, UK).

**Cell lines.** The natural killer cell line NK-92 (ImmuneMedicine, Inc. Vancouver, Canada) and the erythroleukemia NK-sensitive target cell line K562, were both 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

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sodium salt (GIBCO) and 10 µg/ml streptomycin sulphate (GIBCO) (17, 18). The culture medium of the NK-92 cell line was always supplemented with 20 U/ml of IL-2, unless otherwise specified.

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

**Cytotoxicity assay.** The cytotoxicity assay was, with minor modifications performed as described earlier (19). In brief, serial dilutions of NK-92 cells 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 10:1 to 1.25:1 following the addition of 100 µl ( $5 \times 10^3$ ) of target cells to each well. Following a short centrifugation ( $34 \times g$ , 1 min), the co-cultures were incubated for 2 h at 37°C in a 95% humidified chamber with 6% CO<sub>2</sub>. They were then centrifuged for 5 min ( $688 \times g$ ), and 20 µl of supernatant from each well were 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 0.5% 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)  $\times 100\%$

**NK-92 proliferation assay.** NK-92 cells were grown in 200 µl cultures ( $2.5 \times 10^5$ /ml, if not otherwise stated) for indicated periods of time. <sup>3</sup>H-thymidine incorporation was determined following a 4-h pulse with 2 µCi/well [methyl-<sup>3</sup>H] Thymidine (Amersham Biosciences, Buckinghamshire, UK, 25 Ci/mmol) at the end of the culture. The plates were subsequently stored overnight at -70°C, and then were thawed and collected using a semiautomatic cell harvester (Skatron, Lier, Norway). Recovered radioactivity was counted using a liquid scintillation counter (Wallac 1410 Liquid scintillation counter; Wallac Oy, Turku, Finland).

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

## Results

**NK-92 cells proliferate in the presence of IL-2 and IL-15, but not of IL-4 and IL-7.** Figure 1 shows the NK-92 cell proliferation in the presence of the common gamma chain receptor-dependent cytokines IL-2, IL-4, IL-7 and IL-15 at

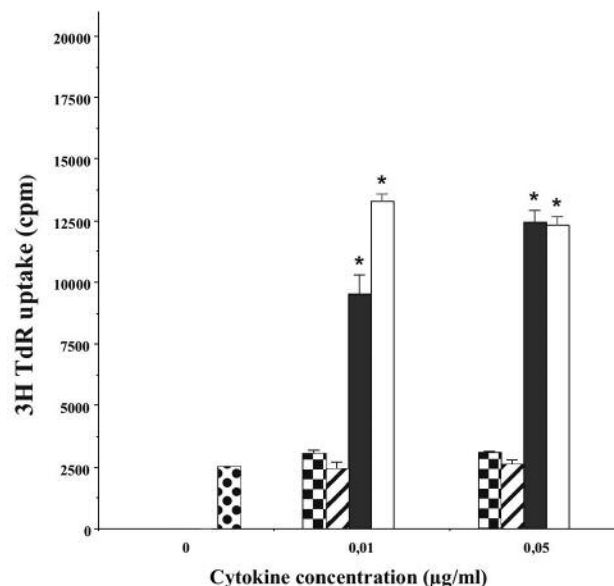


Figure 1. Proliferation of NK-92 cells in the presence of IL-2, IL-4, IL-7 and IL-15. NK-92 cells were cultured in the presence of indicated concentrations of IL-2 (filled), IL-4 (squares), IL-7 (stripes) or IL-15 (open) and in medium alone (black circles;  $2542 \pm 149$  cpm). [<sup>3</sup>H] thymidine uptake was measured after 48 h of culture. Results are expressed as means  $\pm$  SD and are representative of three independent experiments. \**p* < 0.05 as compared to medium alone.

concentrations of 0.01 µg/ml and 0.05 µg/ml. Both IL-4 and IL-7 did not alter proliferation of NK-92 cells, compared to the control (untreated) group. However, cell proliferation was significantly induced by the cytokines IL-2 (0.01 µg/ml:  $9496 \pm 802$ , 0.05 µg/ml:  $12457 \pm 453$  cpm; *p* < 0.05) and IL-15 (0.01 µg/ml:  $13287 \pm 284$ , 0.05 µg/ml:  $12326 \pm 315$  cpm; *p* < 0.05) compared to the control ( $2689 \pm 299$  cpm).

Comparison between the IL-2- and the IL-15-dependent proliferation at 48-96 h indicated that IL-15 is much more potent at all the time points analyzed. In fact, only 0.0005 µg/ml of IL-15 was enough to induce maximal cell proliferation at 48 h, whereas the concentration of IL-2 with the same effect was 0.01 µg/ml (Figure 2A). At 72 h of treatment, maximal cell proliferation was reached by IL-15 at the highest concentration (0.01 µg/ml), whereas IL-2 at the same concentration induced less than 50% of maximal proliferation (Figure 2B). Prolonged proliferation was observed at 96 h in the presence of as little as 0.05 µg/ml of IL-15, while no significant increase in NK-92 cell proliferation was observed at this time with 0.01 µg/ml of IL-2 in the medium (Figure 2C).

**No synergistic proliferation effect was induced by IL-2 and IL-15.** Figure 3A shows a dose-dependent IL-2-induced proliferation of NK-92 cells at 48 h. Treatment of cells with

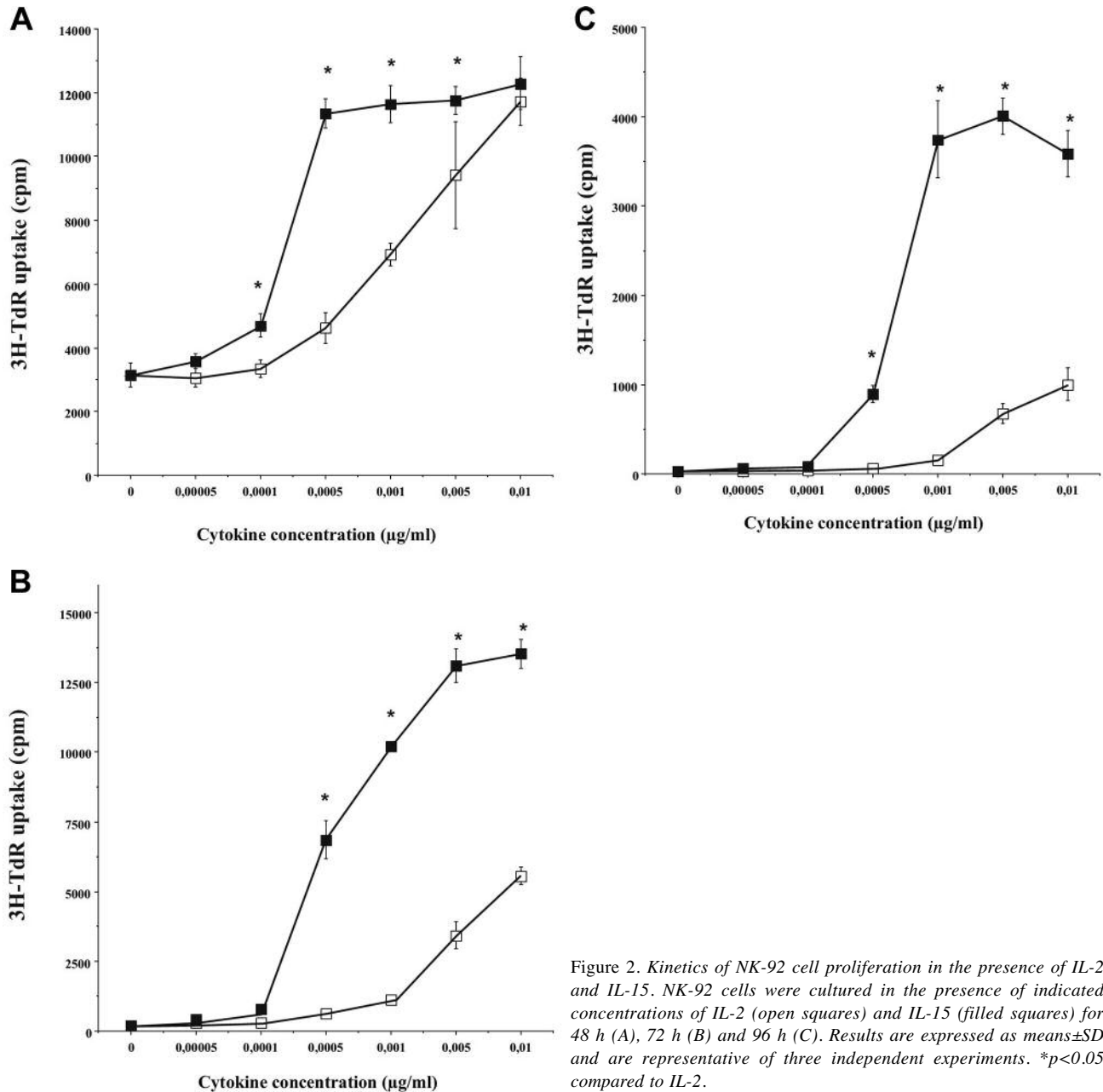


Figure 2. Kinetics of NK-92 cell proliferation in the presence of IL-2 and IL-15. NK-92 cells were cultured in the presence of indicated concentrations of IL-2 (open squares) and IL-15 (filled squares) for 48 h (A), 72 h (B) and 96 h (C). Results are expressed as means $\pm$ SD and are representative of three independent experiments. \* $p$ <0.05 compared to IL-2.

IL-15 (0.005 µg/ml) combined with IL-2 (0.001-0.01 µg/ml) did not alter the proliferation rate to any extent compared to the IL-15 alone (19744 $\pm$ 1185 cpm). Similar results were observed at 72 h (Figure 3B).

*Both IL-2 and IL-15 can potentiate the NK activity equally efficiently.* The clear difference noticed between IL-2- and IL-15-mediated proliferation (Figures 2A-C), suggested a similar trend with their potential to trigger the cytotoxic activity of NK-92 cells. However, when NK-92 cells were

pretreated for 48 h in the presence of either IL-2 or IL-15 at 0.01 µg/ml and 0.1 µg/ml, the NK lytic activity was in the range of 85-90% (K562:NK-92 [1:5] cell ratio). Other target-to-effector ratios did not alter the NK activity to any great extent, regardless of whether IL-2 or IL-15 was present in the culture medium (Table I).

*No synergistic potentiation of the NK activity with IL-2 and IL-15.* Although no synergistic proliferation effects were observed with IL-2 and IL-15, their combined effects on the

Table I. Potentiation of the NK-92 cell activity with interleukin-2 (IL-2) and IL-15.

Cytokine	Concentration ( $\mu\text{g/ml}$ )	Target to effector cell ratio			
		1:1.25	1:2.5	1:5	1:10
Medium	0	37.6 $\pm$ 0.89	56.7 $\pm$ 4.61	67.8 $\pm$ 3.26	89.2 $\pm$ 10.4
IL-2	0.01	71.7 $\pm$ 6.24	88.5 $\pm$ 3.46	91.5 $\pm$ 7.01	100 $\pm$ 14.7
IL-2	0.1	81.3 $\pm$ 0.98	93.8 $\pm$ 3.58	95.8 $\pm$ 5.84	100 $\pm$ 11.5
IL-15	0.01	67.3 $\pm$ 2.20	83.5 $\pm$ 2.53	84.1 $\pm$ 5.20	95.7 $\pm$ 3.04
IL-15	0.1	71.0 $\pm$ 6.00	84.3 $\pm$ 4.46	85.7 $\pm$ 6.49	97.8 $\pm$ 2.60

<sup>a</sup>NK-92 cells ( $1.25 \times 10^5$  cells/ml) were first pretreated, with the indicated concentrations of IL-2 or IL-15, or with medium (control) for 48 h before the cytotoxicity assay with K562 as target cells. The results (% lysis) are expressed as means $\pm$ SD and are representative of three independent experiments.

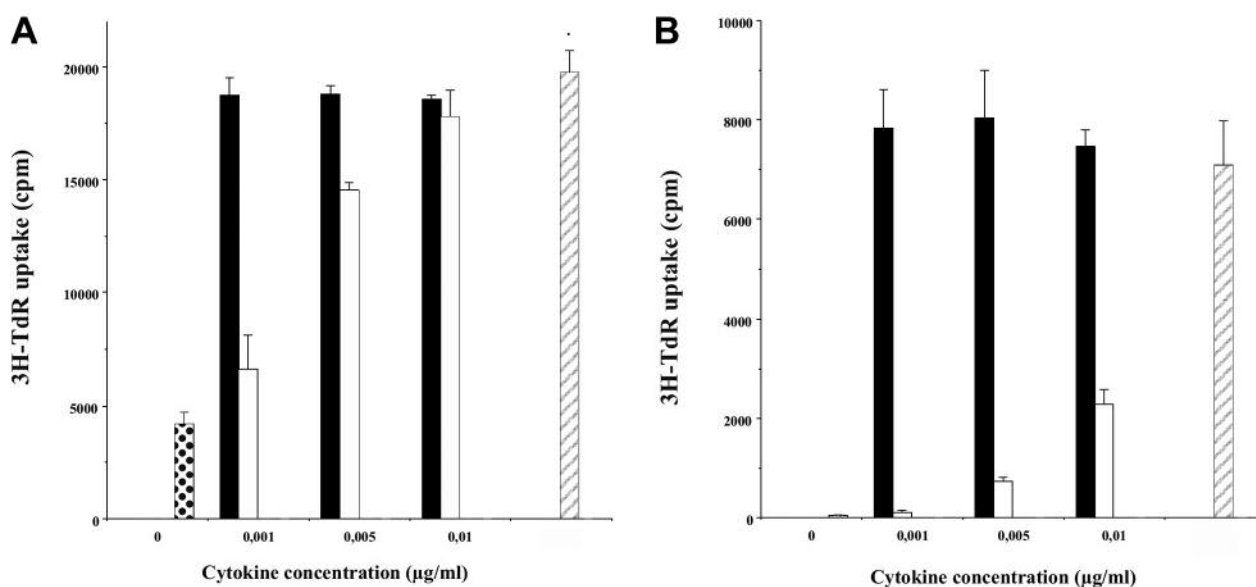


Figure 3. No synergistic effect of IL-2 and IL-15 on the NK-92 cell proliferation. NK-92 cells ( $2 \times 10^5/\text{ml}$ ) were cultured for 48 h (A) or 72 h (B) in the presence of indicated concentrations of IL-2 alone (open) or together with 0.005  $\mu\text{g/ml}$  of IL-15 (filled). Culture for 48 h in 0.005  $\mu\text{g/ml}$  of IL-15; 19744 $\pm$ 1185 (stripes), medium only; 4210 $\pm$ 489 (black circles) and 72 h culture in only 0.005  $\mu\text{g/ml}$  of IL-15; 6988 $\pm$ 1158 (stripes), medium only; 51 $\pm$ 13 (black circles). Results are expressed as means $\pm$ SD and are representative of three independent experiments.

NK activity could be different. To test this, NK-92 cells were cultured for 48 h in the presence of IL-2 or IL-15 alone, or combined at a concentration of 0.1  $\mu\text{g/ml}$ . Pretreating the cells with IL-2 or IL-15 dramatically triggered the NK activity compared to effector cells in medium alone. We observed an almost 40% increase in the lytic activity of NK-92 cells in the presence of these cytokines, independently of the target-to-effector cell ratio analyzed. However, combination of the two cytokines did not improve or alter the NK activity any further (Figure 4).

## Discussion

Since the early eighties, NK cells are known to be activated by “soluble factors”, which were later defined as cytokines (20). A great number of cytokines has since then been analyzed with respect to their effect on the activity, proliferation, production and secretion of cytotoxic granules of the NK cells (13-15, 21). Since NK cells have the ability to kill tumor cells without prior sensitization and restriction of human leukocyte antigens (HLA), they have been

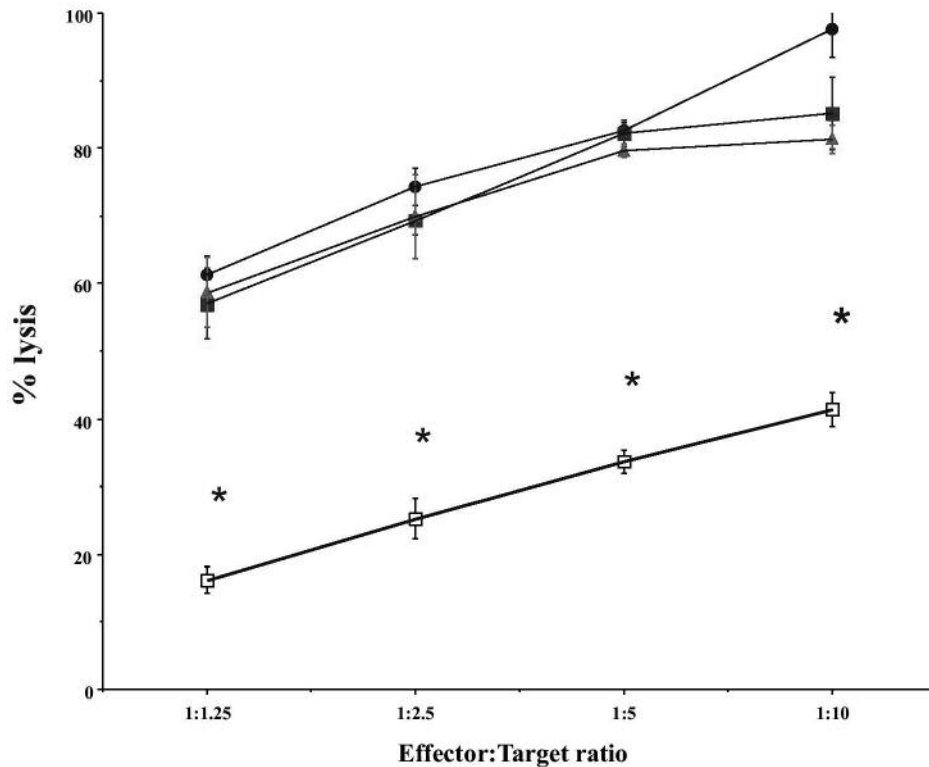


Figure 4. No synergistic effect of IL-2 and IL-15 on the NK-92 activity. NK-92 cells ( $1.25 \times 10^5/\text{ml}$ ) were cultured with  $0.1 \mu\text{g}/\text{ml}$  of IL-2 (filled circles),  $0.1 \mu\text{g}/\text{ml}$  of IL-15 (filled squares),  $0.1 \mu\text{g}/\text{ml}$  of IL-2+ $0.1 \mu\text{g}/\text{ml}$  of IL-15 (filled triangles) or in medium alone (open squares) for 48 h and were subsequently analyzed for their NK activity against K562 cells. Results are expressed as means $\pm$ SD and are representative of three independent experiments. \* $p < 0.05$  compared to medium alone.

successfully used, albeit to a limited extent, in anticancer immunotherapy (6, 22). The next step in adoptive cellular immunotherapy is to use more robust NK cell lines, such as NK-92. However, an even more encouraging future awaits with the development and use of CAR-engineered NK cell lines (11). More specifically, it has been demonstrated that CAR-expressing NK-92 cells exert enhanced anti-tumor effects and selective cytotoxicity against tumor cells. Moreover, CAR expression is more stable and homogeneous in NK-92 cells, which might further improve the outcome of an *in vivo* treatment (23, 24).

In order to successfully perform an *ex vivo* expansion of NK cells and in particular of NK-92 cells, several parameters have to be evaluated and monitored. It is evident that IL-2 and especially IL-15 are crucial for these cells' expansion, while NK-92 cells cannot be maintained in IL-4 and IL-7 containing media, despite the presence of the common gamma chain receptor in all these cytokines, an observation also partly confirmed by Gong *et al.* and Törnroos *et al.* (17, 25). The important role of IL-2 and IL-15 with respect to the survival of NK cells in general, has been previously well-described by Carson *et al.*, although

information regarding IL-15-induced proliferation of NK-92 cells is limited (21, 26, 27). Our data here clearly demonstrate that IL-15 is much more potent at any given concentration compared to IL-2. It is, therefore, of importance that this great difference between the two cytokines is taken into consideration in the future clinical adoptive cellular immunotherapy trials, especially if using NK-92 cells. Trials reported so far have only used IL-2 for the *ex vivo* expansion of these cells (8, 9).

Interestingly, both IL-2 and IL-15 were able to potentiate the activity of NK-92 cells independently of cytokine concentrations and effector-to-target ratios (Table I). Similar results have also been reported for NK-cells isolated from human peripheral blood (28). However, our data on proliferation and activation efficiency of NK-92 cells show no synergistic effect when combining these two cytokines. These results can benefit the scientific community as well as patients in both technical and financial terms, as the *ex vivo* expansion of NK-92 cells can be done in IL-15-containing media alone. Additional studies need to be set in order to engineer CAR NK-92 cells transfected with the *IL-15* gene that can be safely used in clinical trials.

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