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Presence of the Transmembrane Protein Neuropilin in Cytokine-induced Killer Cells

EUGENIA V. DÍEZ GARCÍA DE OLALLA[1,2], FRANCESCA GAROFANO[1], HANS WEIHER[2], MICHAEL MUDERS[3], SARAH FÖRSTER[3] and INGO G.H. SCHMIDT-WOLF[1]

[1]*Department of Integrated Oncology, CIO Bonn, University of Bonn, Bonn, Germany;*
[2]*Department of Immunology and Cell Biology, Bonn-Rhein-Sieg University of Applied Sciences, Rheinbach, Germany;*
[3]*Rudolf-Becker-Laboratory, Institute of Pathology, University of Bonn, Bonn, Germany*

Abstract. *Background/Aim: Cytokine-induced killer (CIK) cells are a heterogenous population of immune cells showing promising applications in immunotherapeutic cancer treatment. Neuropilin (NRP) proteins have been proven to play an important role in cancer development and prognosis. In this study, CIK cells were tested for expression of NRPs, transmembrane proteins playing a role in the proliferation and survival of cancer cells. Materials and Methods: CIK cells were analyzed at different time points via flow cytometry and quantitative real-time polymerase chain reaction for neuropilin expression. Results: Phenotyping results showed CIK cells having developed properly, and low levels of NRP2 were detected. On the other hand, no NRP1 expression was found. Two cancer cell lines were tested by flow cytometry: A549 cells expressed NRP1 and NRP2; U251-MG cells expressed high amounts of NRP2. CIK cell showed low levels of NRP2 expression on day 14. Conclusion: The presence of NRP2, but not NRP1, was shown for CIK cells. Recognizing NRP2 in CIK cells might help to improve CIK cell cytotoxicity.*

Neuropilins (NRPs) are non-tyrosine kinase glycoproteins expressed on the surface of cells of all vertebrate animals. There are two main molecules, NRP1 and NRP2 (located on different genes), which act as co-receptors for certain molecules. NRPs bind vascular endothelial growth factors (VEGFs), class III semaphorins, transforming growth factor beta, other growth factors and many more molecules (1). Depending on what they bind, different pathways/signals are either induced or up-regulated. Regarding their effects on tumor cells, interactions of class III semaphorins and NRPs/plexins on cancer cells have been reported to induce inhibition of proliferation, migration, angiogenesis and immune responses (2). On the other hand, when VEGF molecules interact with NRPs, cell survival, proliferation, migration, angiogenesis and metastasis are induced (1).

Previous studies have focused on the role of NRPs on cancer cells, finding a pattern that highlights a positive correlation between the expression of NRPs in tumor cells and the malignancy of the same (1-3). Lal Goel *et al*. found that NRP-2 is associated with high-grade prostate cancer (4), it was also found to be a prognostic marker for survival in prostate cancer (5). Another study showed how the silencing of NRP1 in non-small cell lung carcinomas reduced proliferation and increased radiosensitivity of the tumor cells both *in vitro and in vivo* (6). Therefore, NRPs are becoming a protein of increasing importance in regard to oncology research.

Cytokine-induced killer cells (CIK) are expanded *ex-vivo* from peripheral blood mononuclear cells (PBMCs) of a patient's blood. They were firstly characterized by Schmidt-Wolf *et al*. in 1991 (7) and have been the focus of various prospective oncology therapies since (8). They have certain characteristic features which make them great candidates for adoptive immunotherapy: They are able to lyse tumor cells independently of the major histocompatibility complex, their proliferation rate is also higher than conventional lymphokine-activated killer cells (9), and they are readily available, quick and easy to generate, as well as being relatively cheap.

CIK cells are a heterogenous population composed of natural killer (NK) cells, T-cells and NK T-cells. The phenotype of CIK cells is determined based on the surface markers they express. NK cells express neural cell adhesion molecule (NCAM1 or CD56) but not CD3, T-cells express CD3 but not NCAM1, and NK T-cells, the most cytotoxic sub-group, express both of these molecules. Once isolated

Correspondence to: Ingo G.H. Schmidt-Wolf, Department of Integrated Oncology, University Hospital Bonn, Venusberg-Campus 1, 53127 Bonn, Germany. Tel: +49 22828717050; Fax: +49 2282879080059, e-mail: ingo.schmidt-wolf@ukbonn.de

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CIK cells are mature, they can be analyzed to determine their phenotype (10). When characterizing CIK cells, two T-cell markers are also looked for: CD4 and CD8. CD4 is a marker of helper T-cells, which recruit cytotoxic cells which are $CD8^+$ (11). Therefore, $CD4^+$ and $CD8^+$ populations of CIK cells are expected.

These cells are generated by isolation of PBMCs from a patient's blood and by cytokine addition, as described in the literature (7, 12). Briefly, PBMCs are isolated, monocytes and granulocytes are eliminated and finally CIK expansion is induced by the addition of interleukin-2 (IL2), IL1β, anti-CD3 and interferon-γ.

Since NRPs have been proven to play a role in up-regulating signals involved with key pathways in cancer cells, it was considered to be of interest to determine whether NRPs are present in CIK cells. Therefore, the aim of this study was to experimentally determine the presence or absence of NRP1 and NRP2 in CIK cells through flow cytometric analysis and quantitative real-time polymerase chain reaction (RT-qPCR).

Materials and Methods

Cell lines, cell cultures and reagents. CIK cells were generated from buffy coats of healthy donors as explained in the literature (7) using the following reagents: Phosphate buffered saline (PBS) (PAN Biotech, Aidenbach, Germany), ethylenediamine tetra-acetic acid (ITW Reagents, Darmstadt, Germany), pancoll (PAN Biotech), distilled water (Gibco/Thermo Fisher Scientific, Waltham, MA, USA), erythrocyte lysis buffer (BioLegend, San Diego, CA, USA), recombinant human interleukin-2 (ImmunoTools, Friesoythe, Germany), recombinant human interferon gamma (ImmunoTools), recombinant human interleukin-1-beta (ImmunoTools) and mouse anti-human CD3 monoclonal antibody (eBioscience - Thermo Fisher Scientific). The buffy coat was stored for a maximum of 24 hours at 4˚C until CIK cell isolation. The CIK cells were incubated at 37˚C, with 5% CO_2 and 95% humidity and kept in a T-175 flask at a density of $1\text{-}2\times10^6$ cells/ml with RPMI 1640 (PAN Biotech) medium supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, ON, Canada) and 1% penicillin/streptomycin (Gibco/Thermo Fisher Scientific) as well as 1 M HEPES (PAN Biotech). The medium was changed every 3-4 days and from day 14, the cells were considered mature CIK cells. The tumor cell line A549 (DSMZ, Braunschweig, Germany), a lung cancer cell line, was cultured in a T-75 flask with 10 ml of RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. The media was changed every 3-4 days and when the cells reached an observable confluence of around 90%, the cell line was seeded at a density of $0.1\text{-}0.5\times10^6$ cells/ml. The cells were incubated at 37˚C, with 5% CO_2 and 95% humidity. The glioblastoma cell line U251-MG (Sigma-Aldrich) was acquired as a cell pellet ready to be thawed and used for flow cytometry antibody-staining. These cells were routinely checked for mycoplasma infection.

Flow cytometry and phenotyping of CIK cells. The cells were prepared for phenotyping at day 0, 1 and 14 onward, by creating a single-cell suspension in PBS at a density of 2×10^7 cells/ml. A staining buffer was also prepared: 0.1 g of bovine serum albumin (Sigma-Aldrich) in 10 ml of PBS. In each tube, 50 μl of the staining buffer were added, as well as 50 μl of the cell suspension. Additionally, making sure to work under dim lighting, monoclonal antibodies were added at the recommended concentrations: for fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD3, phycoerythrin-conjugated mouse anti-human neural cell adhesion molecule (NCAM1), allophycocyanin-conjugated mouse anti-human CD4 and Brilliant Violet 421™ mouse anti-human CD8a, 1 μl was added per tube (all BioLegend); for Pacific Blue™-conjugated mouse anti-human CD20 (BioLegend), FITC-conjugated mouse anti-human NRP1 (CD304) (BioLegend) and AF488-conjugated mouse anti-human NRP2 (R&D systems, Minneapolis, MN, USA), 5 μl were added per tube. Moreover, the tubes were kept on ice in the dark for 20 min. Afterwards, the cells were washed twice with 2 ml of PBS by centrifuging at 405×*g* for 5 min. The pellet was resuspended in 500 μl of PBS. The samples were then analyzed with BD FACS CANTO II and FACS DIVA program (BD Biosciences, San Jose, CA, USA) as explained in the literature (13). The software used to analyze and process the data was FlowJo 10.4.0 (FlowJo-BD Biosciences).

RNA isolation and cDNA synthesis. For RNA isolation, CIK cells from the same batch were used on day 0 and day 14, RNeasy mini kit and RNA-free DNase I kit (both from Qiagen, Venlo, the Netherlands) were used and the company's protocol was followed. In regard to the cDNA synthesis, the protocol from the manufacturer (Thermo Fisher Scientific) of the following reagents was followed: 5X reaction buffer, RiboLock RNase inhibitor (200 U/μl), random hexamer primer, dNTP mix, ReverseAid H Minus Reverse Transcriptase (200 U/μl) and nuclease-free water (Qiagen). A thermocycler (AnalytikJena, Jena, Germany) was used for incubation at 25˚C for 10 min, then at 42˚C for 60 min and lastly at 70˚C for 10 min. The cDNA was stored at −20˚C until further use.

Quantitative reverse transcriptase–polymerase chain reaction (RT-qPCR). Following the manufacturer's protocol from Thermo Fisher Scientific for RT-qPCR with SYBR green, the following reagents and instruments were used: a water-bath (Memmert, Schwabach, Germany), vortex machine (Velp Scientifica, Usmate Italy) nuclease-free water, maxima SYBR green (Thermo Fisher Scientific), forward primer, and reverse primer, a 96-well plate, a centrifuge (AG Schorle, BMZ, Universitätsklinikum Bonn, Germany), and an RT-qPCR instrument (4351106, Applied Biosystems -Thermo Fisher Scientific). Three genes were tested: *NCAM1*, *NRP2* and peptidylprolyl isomerase A (*PPIA*; as a positive control). The primers used were the following: *NRP2*: forward: 5'ACCAGAACTGCG AGTGGATT3', reverse: 5'CGATGTTCCCACAGTGTTTG3'; *NCAM1*: forward: 5'GACCAGGTGGAGCCATACTC3', reverse: 5'TTACGGCGTACG TTGTTTCG3'; *PPIA*: forward: 5'GCTGGACCCAACACAAA TGG3', reverse: 5'GGCCTCCACAAT ATTCATGCCT3'. Triplicates were prepared for each sample, two cell types were tested (CIK and U251-MG) and the CIK cells were tested at two time points: day 0 and day 14. A negative control, with nuclease-free water instead of cells, was also used, resulting in a total of 36 samples. The RT-qPCR was run as follows: one cycle of 50˚C for 2 min; one cycle of 95˚C for 10 min; 40 cycles of 95˚C for 10 min, 63˚C for 15 s and 60˚C for 1 min; one cycle of 95˚C for 15 s, 60˚C for 1 min and 95˚C for 30 s; one cycle of 60˚C for 15 s. After the run and after obtaining the results, the expression of the genes in CIK cells relative to those in U251-MG cells, as well as relative to day 0 CIK cells, was calculated

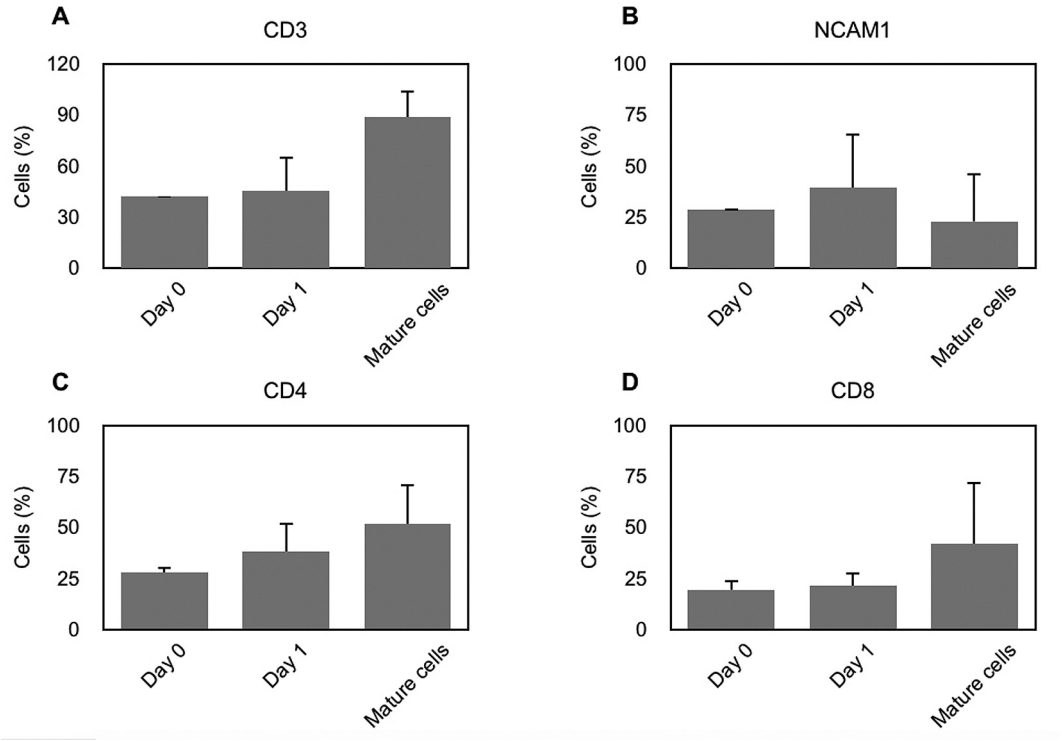

Figure 1. *Phenotyping of cytokine-induced killer (CIK) cells (single markers) by flow cytometry. The percentage of CIK cells expressing CD3 (A), neural cell adhesion molecule (NCAM1) (B), CD4 (C) and CD8 (D) at different time points of cell development. Data are presented as the mean±SD. The sample size on day 0 was two, day 1 three and for mature cells four.*

using the Ct values obtained and the formulae: $\Delta Ct = Ct_{target\ gene} - Ct_{housekeeping\ gene}$, $\Delta\Delta Cq = \Delta Ct_{treated} - \Delta Ct_{control}$, ratio = $2^{(-\Delta\Delta Cq)}$; he SD was also calculated as shown in the protocol (14).

Statistical analysis. Descriptive data and graphics were prepared using Numbers (Apple, Cupertino, CA, USA). Sample means were calculated and reported with the standard deviation. Results are also presented as fold expression of the control, where one-fold represents the same level of expression as the control.

Results

Phenotyping of CIK cells through flow cytometry. The generated CIK cell population was characterized based on the number of cells expressing specific cell surface markers: CD3, NCAM1, CD4 and CD8. In Figure 1A, it can be observed that the percentage of cells expressing CD3 increased from day 0 to day 14. Figure 1B shows that the percentage of $NCAM1^+$ cells increased from day 0 to day 1, after which it decreased by day 14. Regarding Figure 1C, $CD4^+$ cells increased from day 0 to day 14. Lastly in Figure 1D, the proportion of $CD8^+$ cells also increased from day 0 to day 14. Figure 2 shows that the percentage of cells in the lymphocyte population increased with time up to day 14, whereas the populations of monocytes and granulocytes decreased as the CIK cell population matured. It can also be seen that the population of $CD3^+NCAM1^+$ cells decreased on day 1, after which it increased. Regarding the populations of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells, both increased with time.

Determination of NRP expression by flow cytometry. The levels of NRP1 and NRP2 expression were measured by flow cytometry using labelled antibodies. Two cell lines were taken as positive controls, U251-MG for NRP2 and A549 for NRP1 and NRP2. Different CIK cell batches were isolated and tested at different time points. In Figure 3, the results obtained for the expression of NRP1 and NRP2 in CIK cells and A549 cells can be seen. The graph shows the mean expression of all of the tested batches normalized against the antibody controls of each measurement which had identical isotypes to the tested antibodies. A549 cells had a level of NRP2 expression similar to that of CIK cells on both day 1 and day 14. On the other hand, NRP1 expression in CIK cells at all time points averaged the same or lower than the isotype control.

NRP2 mRNA detection through RT-qPCR. One batch of CIK cells was analyzed on days 0 and 14 through RT-

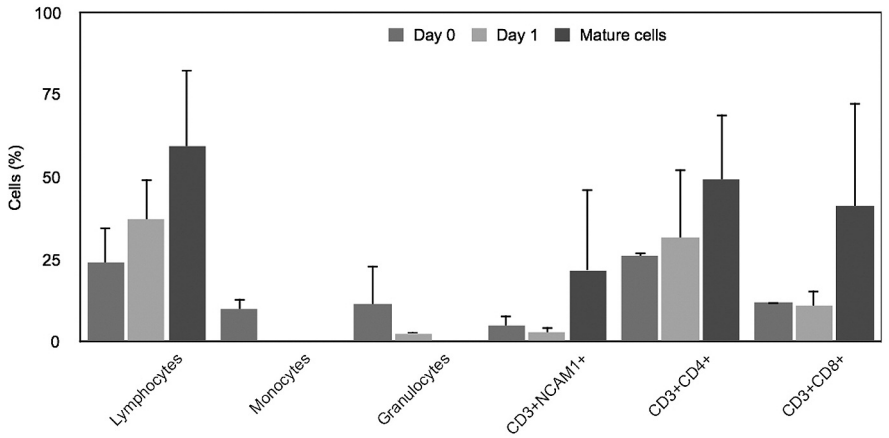

Figure 2. *Phenotyping of cytokine-induced killer (CIK) cells (double-positive markers) and peripheral blood mononuclear cell (PBMC) populations using CD3, CD4, CD8, and neural cell adhesion molecule (NCAM1) expression. CIK cells were analyzed on day 0, 1 and day 14 (mature cells). The different PBMC populations (lymphocytes, monocytes and granulocytes) were gated in the obtained fluorescence-activated cell sorting dot plot (FSC-A vs. SSC-A) and the percentage of cells belonging to those populations are than seen as a percentage. The percentage of double-positive populations, $CD3^+NCAM1^+$, $CD3^+CD4^+$ and $CD3^+CD8$, are also shown. Data are presented as the mean±SD. The sample size on days 0 and 1 was three, and for mature cells was four.*

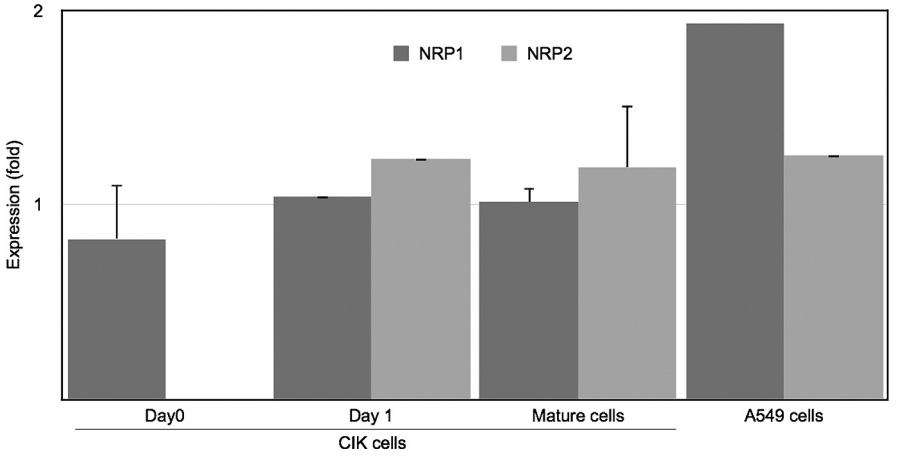

Figure 3. *Expression of neuropilin-1 (NRP1) and neuropilin-2 (NRP2) in A549 and cytokine-induced killer cells (CIK cells), normalized against the control antibody with identical isotype using flow cytometry. Fold expression is shown, where 1 equals the same level of expression as the control antibody with identical isotype. Data are presented as the mean±SD. The sample size for NRP1 in CIK cells on day 0 was two, on day 1 was one and for mature cells was two; for NRP2 no data were collected on day 0, on day 1 the sample size was one and for mature cells it was five. The sample size in A549 cells for NRP1 was two and for NRP2 was three.*

qPCR. As a positive control, the cell line U251-MG was also tested since it is a cell line that was shown to express a high amount of NRP2 (15). Three parameters were measured: Relative expression of *NRP2, NCAM1* and *PPIA*. The relative expression of the genes in CIK cells was compared to those of the positive control cell line U251-MG. The results can be seen in Table I. CIK cell expression was normalized firstly against the expression in U251-MG cells. This showed the expression of *NRP2* increased from day 0 to day 14 but this was not very clear because the U251-MG cell line expressed both genes at a higher level. When expression was normalized against that of day 0 CIK cells, the pattern of expression was clearer. In Figure 4, the expression of NRP2 and NCAM1 in CIK cells on day 14 were normalized against the expression of those same genes on the same CIK cells at day 0. The results showed

Table I. *Quantitative real-time polymerase chain reaction results and calculated relative expression of neuropilin-1 (NRP1) and neural cell adhesion molecule (NCAM1) on cytokine-induced killer cells (CIK). ΔCt, ΔΔCt and ratio values were calculated as per the protocol (14) in the Materials and Methods section. The expression of the genes was normalized against U251-MG cells and against CIK cells on day 0. Peptidylprolyl isomerase A (PPIA) was used as a housekeeping gene. The sample size for all Ct values was three, except for CIK cells on day 0 and 14 testing NCAM1, where one outlier was excluded for each triplicate and therefore the sample sizes for each was two.*

Target	Sample		Mean Ct	Ct SD	ΔCt	ΔCt SD	ΔΔCt	ΔΔCt SD	Ratio	SD	ΔΔCt	ΔΔCt SD	Ratio	SD
NCAM1	U251-MG		21.690	0.190	3.820	0.236	0	0.236	1	0.1777	-	0.236	-	
	CIK cells	Day 0	27.415	0.120	3.805	0.134	–0.015	0.134	1.0105	0.0986	0	0.134	1	0.0976
		Day 14	24.875	0.601	3.535	0.651	–0.285	0.651	1.2184	0.6947	0.270	0.651	1.2058	0.6875
NRP2	U251-MG		19.490	0.060	1.620	0.152	0	0.152	1	0.1114	-	0.152	-	
	CIK cells	Day 0	36.930	0.000	13.320	0.060	11.700	0.060	0.0003	0.0000	0	0.060	1	0.0425
		Day 14	31.570	0.640	10.230	0.687	8.610	0.687	0.0026	0.0016	3.090	0.687	8.5150	5.1945
PPIA	U251-MG		17.870	0.140	-	-	-	-	-		-	-	-	
	CIK cells	Day 0	23.610	0.060	-	-	-	-	-		-	-	-	
		Day 14	21.340	0.250	-	-	-	-	-		-	-	-	

a clearer fold increase in expression of NRP2 from day 0 to day 14.

Discussion

NRPs have been proven to play a role in cancer progression and severity, and therefore it has become a protein of interest in oncology studies. In this report the focus was on the study of this transmembrane protein in a specific immune-system cell-type: CIK cells. These cells have been and are being studied with the purpose of using them for adoptive immune-therapies directed against cancer. Since as far as we are aware the presence of NRPs have never been reported on CIK cells, this study was focused on determining this. The two NRPs were tested: NRP1 and NRP2 and flow cytometry and RT-qPCR were performed. As a positive control, the cell lines A549 and U251-MG were used, since they have been already proven to express NRP1 and/or NRP2 (16, 17).

Firstly, the CIK cells to be tested were characterized by flow cytometry. The results can be seen in Figures 2 and 3. Of all CIK cells, 86.5% of the population are expected to express CD3, 28.5% NCAM1, 45.4% CD4 and 47.7% CD8 (10). The phenotyping results seen in Figure 1 of cell-surface marker expression are in accordance with the literature (10), despite our testing a relatively low number of cell batches. The error bars (representing the standard deviation of the mean) indicate for the broad range of the samples.

On day 0, a lower proportion of cells expressed CD3, NCAM1, CD4 and CD8 than did mature CIK cells, which is consistent with the literature (18). Regarding the results for day 1, the percentage of $CD3^+$, $CD4^+$ and $CD8^+$ cells was higher in comparison to day 0, but lower than in mature CIK cells. The only discrepancy in Figure 1 can be seen for the percentage of CIK cells expressing NCAM1 on day 1, which

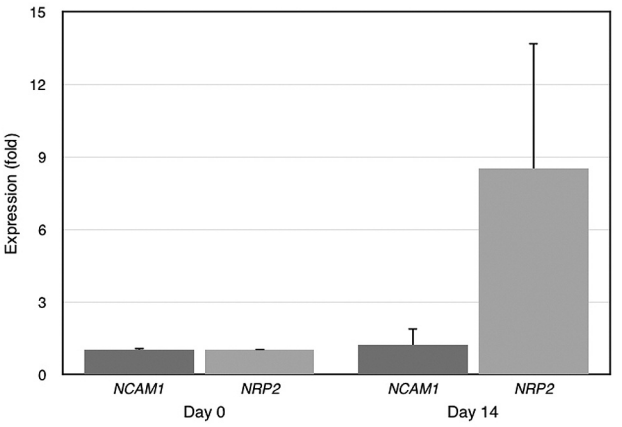

Figure 4. *Graph showing the relative expression of neuropilin-2 (NRP2) and neural cell adhesion molecule (NCAM1) on day 0 and day 14 in cytokine-induced killer cells (CIK). Expression in CIK and U251-MG cells was analyzed through quantitative real-time polymerase chain reaction using SYBR green as the reporting dye. The expression levels of CIK cells on day 14 were normalized against the expression of CIK cells on day 0. Data are presented as the mean±SD fold expression, where 1 represents the same expression as that at day 0.*

was higher on day 1 and day 0 than on day 14. Zhao *et al*. found similar results, where the percentage of NCAM1-expressing cells on day 0 was 26.8% but that in mature CIK cells was only 24% (18). This is a very small difference in percentage but similar results can also be seen in Figure 1 of this study, where on day 0, 28.9% of cells expressed NCAM1 while on day 14, 23% did. This leads to the conclusion that the natural expression pattern of NCAM1 in CIK cells is such that as cells mature, the $NCAM1^+$ population decreases.

In this study, the population of lymphocytes increased as expected after day 0 (Figure 2), since the CIK cells are immune-system cells of the lymphocyte population. Monocytes were present on day 0, but not on days 1 and 14. This is due to monocytes being adherent cells, which by the protocol, are to be excluded on day 0 immediately before interferon-γ was added. Regarding granulocytes, the population disappeared after day 1 since the cytokine addition on that day makes the cells differentiate into lymphocytes. Figure 2, therefore, shows how the CIK cell batches used for the further experiments developed properly and according to literature (7).

Figure 2 also shows how the proportion of $CD3^+NCAM1^+$ cells decreased slightly from day 0 to day 1 and increased again on day 14. These findings are consistent with the literature (19), where the percentage of $CD3^+NCAM1^+$ cells was below 3% on day 0, decreased slightly on day 1 and then increased to around 35% on day 14. The results for $CD3^+CD4^+$ cells are slightly different from those of the literature (19), since on day 14, the population comprised around 50%, whereas in the literature the population decreased to about 20% on day 14. Lastly, the finding for the $CD3^+CD8^+$ population was similar to results in the literature for days 0 and 1 but on day 14, the obtained result was about 40%, whereas Meng *et al*. shows values of around 80%. This would mean that the cells used in this study were not as cytotoxic, since that characteristic is attributed to $CD8^+$ cells (20). Still, there are publications in which the population of $CD3^+CD4^+$ cells was reportedly higher than that of $CD3^+CD8^+$ cells (21). Therefore, even though the CIK cells tested would not be as cytotoxic (20), they still fall within the parameters of previously reported CIK cells (21).

Another important point to consider when analyzing the results is that the phenotype of each CIK cell batch is patient-dependent (22) and, therefore, discrepancies in phenotyping between patients is expected. This can be seen when comparing the results in Figure 1B to the results in Figure 4. In Figure 1B, the average proportion of $NCAM1^+$ cells can be seen to decrease from day 0 to day 14. However, the standard deviation was large on day 14, showing that the proportion of $NCAM1^+$ cells in some batches increased as they matured. In Figure 4, on the other hand, an increase in NCAM1 expression from day 0 to day 14 can be seen.

The isolated CIK cells were tested by flow cytometry at different time points for the expression of the transmembrane NRP proteins. The cells lines U251-MG and A549 were also tested and taken as positive controls. The cell line U251-MG showed 36.6-fold expression of NRP2 when compared to the isotype. This is consistent with the literature (15). The A549 cell line also produced the expected results regarding NRP1 expression, concordant with the literature (6). However, NRP2 expression on A549 cells seemed to be very low, which is a discrepancy from the literature (23, 24), where NRP2 showed a clear positive band in western blots.

The expression level of NRP1 in CIK cells on day 0, 1 and 14 was equivalent to that of the isotype control. Regarding NRP2, on day 1 and day 14 expression was low. The standard deviation was large on day 14 for NRP2 since there was one sample in which expression was 1.76-fold. Since the phenotype of CIK cells varies from patient to patient, it could be hypothesized that NRP expression also does. From the results obtained, this cannot be proven since the sample size was too small, but it can be seen that NRP expression is not consistent in all CIK cell batches.

Further experiments would be necessary to determine whether the expression of NRPs in CIK cells is patient-dependent. In fact, Nasarre *et al*. obtained immunofluorescence results showing that not all A549 cells tested expressed similar amounts of NRP2 (24). It should therefore be considered that like A549 cells, NRP2 expression on CIK cells might vary from cell to cell. Another point to note is that flow cytometric analysis may not be the best method to determine the presence of NRP, since there might be intracellular presence of the protein. Therefore, future experiments should attempt to determine the presence of NRPs in CIK cells by other techniques such as western blotting. In fact, Nasarre *et al*. detected the presence of NRP2 in A549 cells through western blotting, and obtained clear positive results (24), as did Dong *et al*. (6), whereas through flow cytometry in our experiments, as seen in Figure 3, NRP2 seemed to be expressed at very low levels.

Lastly, one batch of CIK cells was taken to perform quantitative RT-qPCR, in which the samples were collected on day 0 and 14 after isolation. Since no cells were detected as being positive for NRP1, its RNA was not investigated. There was an 8.5-fold increase of NRP2 expression from day 0 to day 14 in CIK cells as seen in Figure 4 but the expression was still low when compared to that of the cell line U251-MG (Table I). Regarding the expression of NCAM1, the results in Table I and Figure 4 were as expected (10).

In future experiments, it would be of interest to retest the CIK cells using a cell line which expresses less NRP2, for example the A549 cell line. This cell line has already been tested through flow cytometry as seen in Figure 3, and would make a desirable future positive control. This cell line would also be a good choice since as seen in Figure 3, the A549 cells showed a level of NRP2 expression similar to that of CIK cells.

Finding proof of the presence of NRP2 on CIK cells might be advantageous for cancer therapy studies, since the presence of NRP has been proven to increase cancer progression and tumor cell migration, among others (1). Therefore, a possible way forward with this study might be to assess the importance of NRPs on CIK cells in regard to their cytotoxic effect on tumor cells. In other words, to see if there is a difference in the cytotoxicity of CIK cells against normal tumor cells *versus* against the same NRP-silenced cancer cells. Such a difference

could be advantageous for cancer therapy since, as previously described by Prud'homme *et al*. (1), the interaction of VEGF with NRPs on the membrane of cancer cells leads to cell survival, proliferation, migration, angiogenesis and metastasis of tumor cells. Therefore, it is possible that VEGF expressed by CIK cells (19) interacts with NRPs on tumor cells and may counteract the cytotoxic effect desired from immunogenic therapies. By silencing NRP, the susceptibility of the treated tumor cells to CIK cell therapy might be increased.

In conclusion, it can be stated that NRP2 is expressed on CIK cells. On the other hand, there is evidence to support the claim that NRP1 is not present on CIK cells. This might prove important in future studies to improve the cytotoxicity of CIK cells.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

EDGO prepared the cells, analyzed the samples, analyzed the data and wrote the article. FG aided in all practical work and guided the project as well as coordinated the experiments and corrected the article. HW corrected the article and aided in guiding the writing and editing. MM provided the U251-MG cell line as well as resources and guidance for the RT-qPCR experiment. SF aided in the preparation of samples for RT-qPCR and aided in analyzing the results. ISW provided resources, guided the project, supervised the development of the article, corrected and edited the article.

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