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ORIGINAL RESEARCH

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IL-15 transpresentation by ovarian cancer cells improves CD34$^+$ progenitor-derived NK cell's anti-tumor functionality

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ABSTRACT

Ovarian cancer (OC) is the most lethal gynecological malignancy. As high numbers of Natural Killer (NK) cells in ascites associate with improved survival, the adoptive transfer of allogeneic NK cells is an attractive therapeutic strategy. An approach to further improve NK cell expansion and anti-tumor functionality post-infusion includes IL-15 transpresentation (transIL-15), which involves surface expression of the IL-15 cytokine bound to IL-15Rα. However, others have substantiated that systemic administration of ALT/N-803, a soluble molecule mimicking transIL-15, leads to T cell-mediated rejection of the infused allogeneic NK cell product. In addition, whether transIL-15 induce superior expansion and functionality of our hematopoietic progenitor cell-derived NK cells (HPC-NK) remains understudied. Here, we propose to transfect OC cells with IL-15 and IL-15Rα mRNA and evaluate HPC-NK cell stimulation *in vitro*. Co-transfection of both mRNAs resulted in surface co-expression of both components, thus mimicking the transIL-15. Importantly, co-culture of HPC-NK cells with transIL-15 OC cells resulted in superior proliferation, IFNγ production, cytotoxicity and granzyme B secretion. Furthermore, we observed uptake of IL-15Rα by HPC-NK cells when co-cultured with transIL-15 OC cells, which associates with NK cell long-term proliferation and survival. Superior killing and granzyme B secretion were also observed in transIL-15 OC spheroids. Our results demonstrate that local delivery of IL-15 and IL-15Rα mRNA to OC tumors may be a safer strategy to boost HPC-NK cell therapy of OC through IL-15 transpresentation.

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1. Introduction

Ovarian cancer (OC) is the most common and lethal gynecological malignancy.[1] Approximately 70% of patients show advanced stage disease with <30% 5-year overall survival.[2] This illustrates the urgent need to develop novel therapeutic strategies.

Natural killer (NK) cells show an inherent ability to elicit cytotoxic responses against tumor cells.[3] Interestingly, we previously substantiated that high NK cell frequencies in OC ascites associate with superior survival.[4] Therefore, we have developed a GMP-compliant culture protocol for the generation of billions of NK cells from cord blood-derived hematopoietic progenitor cells (HPC-NK) for OC treatment.[5] HPC-NK cells' safety and efficacy have been investigated for OC (NCT03539406) and is being studied in acute myeloid leukemia (NCT04347616).[6,7] Nonetheless, allogeneic NK cells are detected only up to two weeks after infusion.[8] Such lack of expansion suggests that infusion of billions of HPC-NK cells might not be sufficient to improve OC survival. In addition, NK cells' functionality is reduced shortly after tumor infiltration.[9] Therefore, strategies aimed at improving expansion and anti-tumor functionality of our HPC-NK cells might boost their anti-OC potential.

The expansion and functionality of NK cells can be enhanced with IL-15 stimulation.[10] IL-15 is physiologically expressed as a membrane-bound cytokine together with its alpha receptor component (IL-15Rα).[11] Upon interaction, antigen presenting cells transpresent IL-15 (transIL-15) to T and NK cells. Multiple studies have demonstrated enhanced anti-tumor efficacy when IL-15 cytokine is used in combination with the extracellular fragment of IL-15Rα.[12,13] As a result, multiple studies have addressed the usage of soluble heterodimers of IL-15 and IL-15Rα, also known as IL-15 superagonists, to improve NK cell anti-tumor functionality.[14–16] However, Berrien-Elliott et al. reported that infusion of one of these superagonists, ALT/N-803, can induce CD8$^+$ T cell-mediated rejection of haploidentical infused NK cells.[17] Additionally, we have not observed superior *in vitro* stimulatory effects of ALT/N-803 compared to IL-15 cytokine in our HPC-NK cells.[4] Therefore, local stimulation of NK cells mimicking the membrane-bound IL-15 transpresentation mechanism may be a better strategy to boost their anti-tumor activity and expansion.

In this proof-of-principle study we transfected OC cells with IL-15 and/or IL-15Rα mRNA and evaluated HPC-NK cell stimulation. We confirmed that co-transfection of cells with both mRNAs leads to transpresentation of IL-15 bound to IL-

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15Rα, which ultimately enhances expansion and functionality of HPC-NK cells *in vitro*. We anticipate that local delivery of IL-15/IL-15Rα mRNA to OC cells might be a promising strategy to boost the therapeutic efficacy of HPC-NK cells.

2. Methods and analysis

2.1. Culture of tumor cells

Cell lines were tested mycoplasma-negative with MycoAlertTM Mycoplasma Detection Kit (Lonza). The leukemia cell-line K562 (RRID:CVCL_0004) was cultured in IMDM (Gibco) with 10% heat-inactivated Fetal Bovine Serum (HI-FBS; Integro). SKOV-3 were purchased from ATCC, OVCAR-4 and OVCAR-8 were purchased from the DCTD Tumor Repository, and IGROV-1 and OVCAR-3 were provided by Prof.dr. Boerman, Department of Nuclear Medicine, Radboudumc, Netherlands. IGROV-1, SKOV-3 were cultured in RPMI-1640 (Gibco) with 10% HI-FBS. OVCAR-4 and OVCAR-8 cells were cultured in RPMI-1640 with 10% HI-FBS and 2 mm glutamine (Gibco). OVCAR-3 was cultured in RPMI-1640 with 20% HI-FBS and 1 µg/mL insulin (Merck). Patient-derived ASC009 were obtained from ascites of OC patients after informed consent and kindly provided by Guido Zaman (approved by the Radboudumc Committee for Medical Research Ethics (CMO 2018/4845)),[18] and were cultured in RPMI-1640 with 10% HI-FBS and 2 mm glutamine. All cells were split twice per week and cultured for up to three months at 37°C and 5% CO_2. Adherent cells were washed with PBS and later subjected to trypsin (Life Technologies) for splitting.

2.2. Transfection of OC cells and spheroids

OC cells were cultured in 96-well flat-bottom plates in IMDM with 10% HI-FBS. 24 h after seeding, OC cell monolayers at 70% confluency were transfected. For spheroid formation, SKOV-3 were plated in 96-well plates precoated with 1% agarose (Invitrogen) as previously described,[19] and transfected 72 h after seeding. The transfection agents Lipofectamine MessengerMax (Invitrogen), SAINT-mRNA (Synvolux Products), and TransIT-mRNA (Mirus) were used where indicated according to the manufacturer's indications to encapsulate 100 ng of de-immunized mRNA (Ribopro) encoding GFP (Ribopro),[20] IL-15 (NM_000585.5) or IL-15Rα (NM_002189.4). 50ng of IL-15 and IL-15Rα mRNAs were used (1:1) to obtain transIL-15 targets. GFP images on transfected SKOV-3 spheroids were taken with Zeiss Axio Observer 7 and analyzed with ImageJ. To quantify transfection, OC monolayers were trypsinized as previously indicated, and OC spheroids were disaggregated with TrypLE Express Enzyme (ThermoFisher Scientific) for an hour prior to staining.

2.3. HPC-NK generation and culture

Stem cells were isolated from umbilical cord bloods (following declaration of Helsinki and approved by the Radboud university medical center Committee for Medical Research Ethics CMO2014/226) with CD34 beads (Miltenyi Biotec). $CD34^+$ HPCs were expanded and differentiated into HPC-NK cells as described previously.[5] HPC-NK cells (>95% $CD56^+$) were then directly used for functional assays or cryopreserved. When needed, HPC-NK cells were thawed and cultured for 5–9 days in NK MACS Basal medium (Miltenyi Biotec), 10% pooled human serum (HS, Sanquin), 50 ng/mL IL-15 (Immunotools) and 0.2 ng/mL IL-12 (Immunotools). To adequately evaluate HPC-NK cells' potency, their activation was pre-attenuated overnight with NK MACS with 10%HS and 0.3 ng/mL IL-15. HPC-NK cells were washed prior to functional assays to eliminate any culture cytokines.

2.4. Functional assays

2.4.1. Proliferation assay

Fresh HPC-NK cells were labeled with 10 µM Cell Proliferation Dye eFluor450 (eBioscience) for 10 minutes at 37°C. Unbound dye was quenched with IMDM containing 10% HI-FBS (1:1 v/v) for 5 minutes at 4°C. 33000 eFluor450-labeled HPC-NK cells were then plated with/without targets at 1:1 effector-to-target (E:T) ratio. Proliferation was quantified with flow cytometry three days after co-culture as fold reduction of eFluor450 median fluorescence intensity (MFI) as follows: (1/efluor450 MFI of HPC-NK cells cultured with target cells)/(1/efluor450 MFI of unstimulated HPC-NK cells alone)

2.4.2. Degranulation and potency

Overnight pre-rested 50,000 HPC-NK cells were cultured with/without targets (1.5:1 E:T ratio), or with 25 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) and 1 µg/mL ionomycin (Sigma). Anti-CD107a antibody was added to the co-culture (Supplementary Table S1) to stain for degranulated HPC-NK cells. After 1 h, BD GolgiPlug containing BrefeldinA (BD Biosciences) was added to ensure cytosolic cytokine accumulation, and cells were incubated for 3 additional hours. After 4 h incubation, cells were permeabilized and stained for intracellular cytokines as indicated below.

2.4.3. Killing assay

To distinguish effector from target cells, HPC-NK cells were labeled with Cell Proliferation Dye eFluor450 as previously specified or 20×10^6 cells/mL targets were pre-labeled with 1 µM CFSE (Invitrogen) in PBS for 10 minutes at 37°C. Unbound CFSE was quenched with HI-FBS (1:1 v/v) for 2 minutes at room temperature. Before seeding, HPC-NK and targets were resuspended in IMDM with 10% HI-FBS. After adhesion of 0.3×10^5 OC cells, HPC-NK cells were plated to reach the indicated E:T ratios or numbers. 16-20 h after co-culture, supernatants were frozen for ELISA, and target cells were trypsinized, stained with 7-AAD (Sigma) and counted by flow cytometry. Killing of $CFSE^+$ or $eFluor450^-$ targets was calculated as: (Number of viable targets co-cultured with HPC-NK) /(Number of viable targets alone)x100.

2.5. Flow cytometry analysis

Viability dyes used include Sytox Blue (ThermoFisher Scientific), 7-AAD (Sigma) and Fixable Viability Dye eFluor780 (ThermoFisher Scientific). Cells were resuspended in Fc-blocking buffer containing human immunoglobulins (nanogam, Sanquin Bloodbank) 1:1000 diluted in FACS buffer (PBS containing 0.5% BSA (Sigma)) before staining (Supplementary Table S1). All antibodies were pre-titrated with PBMCs or transfected SKOV-3 cells, when indicated, and diluted in FACS. For intracellular cytokine measurement, cells were pre-permeabilized (Intracellular fixation & Permeabilization Buffer Set, eBioscience), and later stained with antibodies (Supplementary Table S1) in permeabilization buffer (eBioscience). All cells were resuspended in FACS, measured on Gallios, with a maximum detector intensity of 10^3, and analyzed with Kaluza (2.2, Beckman Coulter).

2.6. ELISAs

MaxiSorp ELISA plates (ThermoFisher Scientific) were used. For IFNγ, plates were pre-coated with anti-IFNγ antibody (Invitrogen) overnight, and later blocked for 1 h at room temperature with 1% gelatin in PBS. IFNγ recombinant protein (eBiosciences) was used as standard and detected with anti-IFNγ biotinylated antibody (Invitrogen). Additionally, Granzyme B kit (Mabtech), human IL-15 DuoSet ELISA (R&D systems), ELISA MAX Deluxe Set Human IL-15 (BioLegend) and human IL-15/IL-15R alpha Complex DuoSet ELISA (R&D systems) kits were used following manufacturer's instructions. Unless specified otherwise, the R&D human IL-15 DuoSet ELISA kit was used to measure the secreted IL-15. Streptavidin-HRP (Sanquin), TMB 2-component peroxidase substrate kit (SeraCare) and H_3PO_4 were used to read cytokine concentrations at 450 nm with FlexStation 3 (Molecular devices).

2.7. Statistical analysis

Statistical analysis was performed using Graphpad Prism (10.1.2). Two-sided Student *t* tests, one-way and two-way ANOVAs with Bonferroni correction were used where indicated. Significance was defined as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

3. Results

3.1. OC cells are effectively transfected with IL-15 and IL-15Ra-encoding mRNA and express membrane-bound IL-15 for transpresentation

To identify the delivery platform yielding the optimal mRNA expression, we first studied multiple transfection reagents (Lipofectamine MessengerMax, SAINT-mRNA or TransIT) in different histopathological OC cell lines (SKOV-3, OVCAR-3/4/8 and IGROV-1). Overall, lipofectamine resulted in high viability and expression (>50%) of IL-15Rα (Supplementary Figure S1a-c) up to 6 days post-transfection (Supplementary Figure S1d and e) and thus was the selected platform for subsequent experiments.

Since lipofectamine-based transfection of SKOV-3 cells compromised viability at 72 h compared to the non-transfected control (NTC) (Supplementary Figure S1f), we studied whether removal of lipofectamine-mRNA lipoplexes 2 h after transfection could preserve viability, as suggested by the manufacturer. Viability at 144 h post-transfection was ≥90% in all transfection conditions when the lipofectamine-mRNA mix was washed away after 2 h (Figure 1a). Notably, receptor expression was generally higher in IL-15Rα than transIL-15, but was overall significantly lower in the washed condition (Figure 1b,c). Most importantly, transIL-15-transfected SKOV-3 showed surface co-expression of IL-15 and IL-15Rα (Figure 1d), suggesting that OC cells could potentially transpresent membrane-bound IL-15. Overall, these results demonstrate that OC cells can be effectively transfected with mRNA *in vitro* to co-express IL-15 and IL-15Rα, and thereby might be able to transpresent IL-15.

Notably, whilst IL-15 was undetectable in the supernatant of NTC and IL-15Rα SKOV-3 cells, its secretion was more pronounced in transIL-15 condition compared to IL-15-transfected cells (Figure 1e). The long signal peptide IL-15 isoform is secreted to a limited degree, being mainly stored intracellularly in the endoplasmic reticulum, and its transport to the plasma membrane is facilitated by IL-15Rα.[11,21] Once on the cell surface, membrane metalloproteases recognize and cleave the extracellular portion of IL-15Rα, thus releasing soluble IL-15/IL-15Rα heterodimeric complexes (hetIL-15)[22] (Figure 1f). By comparing two different IL-15 ELISA assays, we observed that the R&D kit likely recognizes both soluble IL-15 and hetIL-15 (Supplementary Figure S2a). We confirmed this hypothesis by specifically detecting the hetIL-15 in the supernatants of transIL-15, but not of IL-15 SKOV-3 cells (Supplementary Figure S2b). In addition, we observed reduced hetIL-15 secretion when the broad-spectrum matrix metalloprotease inhibitor GM6001 was used (Supplementary Figure S2b). Altogether, these results confirm that both IL-15 and transIL-15 conditions secrete IL-15, and that most of the IL-15 secreted by transIL-15 is present as a heterodimer with cleaved IL-15Rα.

3.2. HPC-NK cells show superior anti-tumor functionality when exposed to transIL-15 SKOV-3 cells

To reduce negative effects on target cell viability and adequately study the anti-tumor functionality of HPC-NK cells at physiologically relevant IL-15 concentrations *in vitro*,[23] we transfected targets with lipofectamine:mRNA lipoplexes that were washed 2 h after incubation (Figure 1e). Surface expression of IL-2Rβ- and IL-2Rγc was validated in our HPC-NK cells, being IL-2Rγc expressed in all HPC-NK cells and at a higher MFI than IL-2Rβ (Figure 2a and Supplementary Figure S3a). Expression of both receptor components suggests that HPC-NK cells could become activated upon (trans)IL-15 stimulation. To address this question, HPC-NK cells were co-

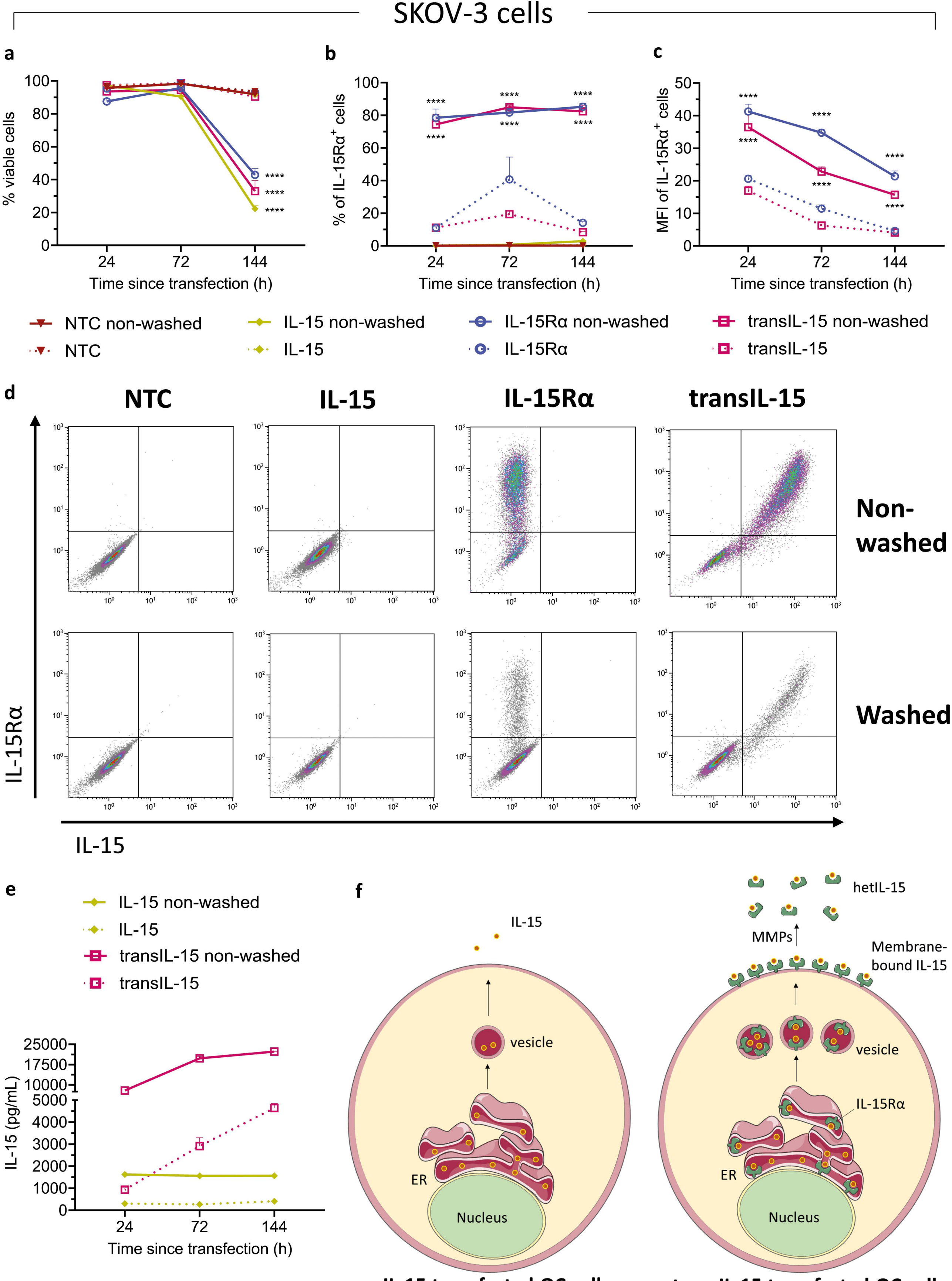

Figure 1. IL-15 and IL-15Rα mRNA are effectively transfected in SKOV-3. Comparison of (a) viability, (b) IL-15Rα$^+$ percentage and (c) IL-15Rα$^+$ MFI of transfected cells measured at the indicated time points. (d) Representative dot plots showing co-expression of IL-15 and IL-15Rα 24 h after transfection with non-washed (upper row) and washed (bottom row) mRNAs. Data derives from 3 independent replicates. (e) IL-15 secretion data from 2–6 independent replicates from one experiment. For all graphs, lipofectamine nanoparticles were either washed 2 h after transfection (dotted lines) or not (continuous lines). (f) Model depicting secretion mechanism of IL-15 by IL-15-transfected OC cells (left) or transIL-15 OC cells (right). All data represents mean values ± SEM. Statistics derive from two-way ANOVA with Bonferroni correction comparing washed and non-washed samples within the same transfection condition and same time point (*$p < 0.05$, ****$p < 0.0001$).

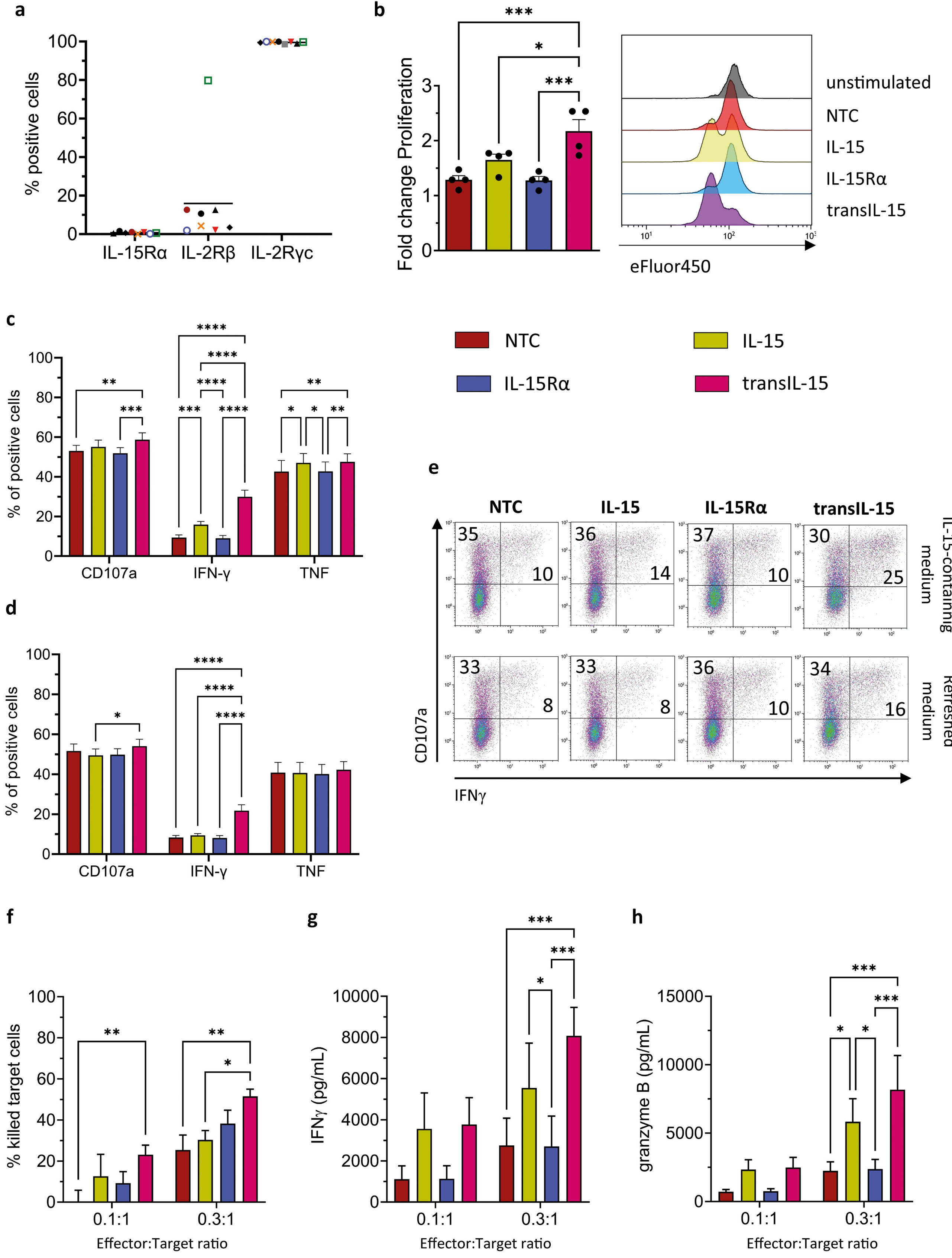

Figure 2. HPC-NK cells show superior functionality upon co-culture with transIL-15-transfected SKOV-3 cells. (a) Percentage of the indicated markers on 8 independent HPC-NK cell donors. (b) Fold change proliferation of HPC-NK cells 3 days after incubation with transfected SKOV-3 cells compared to unstimulated HPC-NK cells alone, and representative histograms showing loss of eFluor450. Data comes from 4 independent experiments, 4 independent fresh donors. (c) Percentage of $CD107a^+$, $IFN\gamma^+$ and TNF^+ in viable $CD56^+$ HPC-NK cultured for 4 h with targets whose media either contains secreted IL-15 or (d) was refreshed just prior to HPC-NK addition. Data comes from 3 independent experiments, 6 independent donors. (e) Representative flow cytometry dot plots of CD107a (y-axis) and IFNγ (x-axis) in HPC-NK cells cultured with transfected SKOV-3 cells whose media was removed (refreshed medium), or not (IL-15-containing medium), prior to plating of the effectors. Numbers represent percentage of positive cells per quadrant. (f) Percentage of target killing and (g) IFNγ and (h) granzyme B secreted by HPC-NK cells after 16-20 h co-culture. Results derive from 2 independent experiments, 4 independent HPC-NK donors. Bars represent mean values ± SEM. Whenever applicable, one or two-way ANOVA within each potency readout or effector-to-target ratio were used for statistical analysis with Bonferroni correction (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

cultured with targets 24 h after transfection to evaluate their functionality.

HPC-NK cells proliferated upon stimulation with (untransfected) K562 positive control cells (Supplementary Figure S3b). Likewise, HPC-NK cell expansion was also observed upon coculture with SKOV-3 target cells (Figure 2b). Interestingly, coculture with transIL-15 SKOV-3 cells induced significantly superior proliferative capacity (Figure 2b). Additionally, HPC-NK cells showed high potency after 4 h of incubation with PMA/ionomycin or K562 (Supplementary figure S3c). In transIL-15 SKOV-3 cells, HPC-NK cells showed a slight improved degranulation and TNF production than NTC and IL-15Rα-transfected cells, but not compared to IL-15 SKOV-3 targets (Figure 2c). Interestingly, 1.9-fold more HPC-NK cells produced IFNγ when co-cultured with transIL-15 SKOV-3 cells compared to IL-15 SKOV-3 cells, and > 3-fold when compared to NTC and IL-15Rα SKOV-3 (Figure 2c). Additionally, there was a trend of slightly higher IFNγ MFI in transIL-15 condition, although this was not deemed as statistically significant (Supplementary Figure S3d). To investigate whether the increase in IFNγ-producing HPC-NK cells was caused by membrane-bound IL-15 or released IL-15, we next washed out all cytokines by refreshing SKOV-3 cell media immediately prior to HPC-NK cell plating. Strikingly, IFNγ production induced by IL-15-transfected SKOV-3 decreased and was comparable to NTC and IL-15Rα conditions (Figure 2d,e). Washed transIL-15 SKOV-3 also resulted in a 37% lower IFNγ-producing HPC-NK cells than non-washed transIL-15 condition, but remained ≥ 2.3-fold significantly higher than NTC, IL-15 and IL-15Rα conditions with refreshed media. Again, no statistically significant differences in MFI values were observed (Supplementary Figure S3e). This suggests that both hetIL-15 and membrane-bound IL-15 collectively induce superior HPC-NK cell functionality in transIL-15 condition. Furthermore, HPC-NK cells demonstrated superior killing when cocultured with transIL-15 cells at low effector-to-target ratios (Figure 2f). These results correlated with superior secretion of IFNγ and granzyme B (Figure 2g,h). Interestingly, we observed that HPC-NK cells took up IL-15Rα from IL-15Rα$^+$ SKOV-3 cells upon cell-to-cell interactions, and this was boosted when OC cells were co-transfected with IL-15 mRNA (Supplementary Figure S4). Overall, HPC-NK cells show superior proliferation, potency and cytotoxic responses when co-cultured with transIL-15-transfected SKOV-3 cells, which is likely induced by both membrane-bound IL-15 and secreted hetIL-15.

3.3. *Transfection of other OC cell types with IL-15 and IL-15Ra mRNA also results in superior HPC-NK cell functionality*

To study whether the superior functionality of HPC-NK cells is translatable to other OC histology subtypes, we transfected high-grade serous OVCAR-4 and low-grade primary ascites-derived ASC009 cells. OVCAR-4 cells were viable at 24 h but sensitive to transfection at 72 h and 144 h (Figure 3a). IL-15Rα surface expression was detected at all three time points, but MFI decreased with time (Figure 3b). Again, transIL-15 showed higher IL-15 secretion over time compared to IL-15 transfection (Figure 3c). In addition, transIL-15 OVCAR-4 cells triggered higher proliferation of HPC-NK cells (Figure 3d). However, percentage of IFNγ-producing HPC-NK cells was comparable between IL-15 and transIL-15 conditions in OVCAR-4 cells (Figure 3e and Supplementary Figure S5a). Similarly, patient-derived ASC009 cells showed comparable transfection profiles, but without differential viability up until 6 days (Figure 3f-h). Again, transIL-15 ASC009 cells induced superior HPC-NK cell proliferation (Figure 3i), and ≥ 2.1-fold IFNγ-producing HPC-NK cells was observed in transIL-15 ASC009 cells (Figure 3j and Supplementary Figure S5b). Overall, these results demonstrate that regardless their histology, transfection of OC cells with IL-15- and IL-15Rα-encoding mRNA improves the therapeutic effect of HPC-NK cells.

3.4. *Transfection of 3D OC spheroids with IL-15 and IL-15Ra mRNA improves cytotoxic activity of HPC-NK cells*

OC cells in monolayers are easily transfected with lipofectamine-mRNA lipoplexes, but this poorly recapitulates the complexity of OC solid tumors *in vivo* where likely only the outermost cells are exposed to the transfection reagent. As previously reported, transfection of 3D OC spheroids primarily induces protein expression in outer cells (Figure 4a).[20] Transfection of SKOV-3 spheroids with non-washed lipofectamine carrying IL-15- and IL-15Rα-encoding mRNA did not have an impact on cell viability after 24 h (Figure 4b) and resulted in an average receptor expression of 11.6% in IL-15Rα condition and 9.8% in transIL-15 (Figure 4c). Likewise, there was a trend of reduced MFI receptor expression in transIL-15 (Figure 4d). More importantly, IL-15 secretion screening showed average concentrations of 67 pg/mL for IL-15 alone and 546 pg/mL in transIL-15 (Figure 4e), thus matching more physiologically relevant IL-15 concentrations previously observed in the washed transfection of SKOV-3 monolayers (Figure 1e). Not surprisingly, superior HPC-NK cell killing was observed in transIL-15-transfected 3D SKOV-3 cells (Figure 4f) and associated with higher granzyme B secretion (Figure 4g). These results demonstrate that HPC-NK cells become more activated and efficiently kill a transIL-15-trasfected 3D OC models, and thus delivery of IL-15 and IL-15Rα mRNA to OC tumors may be an attractive strategy to locally boost HPC-NK cell anti-tumor functionality through IL-15 transpresentation.

4. Discussion

OC shows a 5-year overall survival of less than 30% in stage III/IV patients.[2] This could be improved with NK cell-based therapies.[4] To this end, our laboratory has developed a GMP-compliant protocol for the generation of billions of HPC-NK cells as off-the-shelf products.[5] However, allogeneic NK cells show poor persistence *in vivo*, and NK cells' functionality is reduced shortly after tumor infiltration.[8,9] While IL-15 stimulation of NK cells is an attractive approach to improve NK expansion and functionality, systemic IL-15 poses toxicity risks and might promote rapid rejection of the allogeneic NK cell product.[4,17,24–26] Here, we aimed to evaluate *in vitro* the

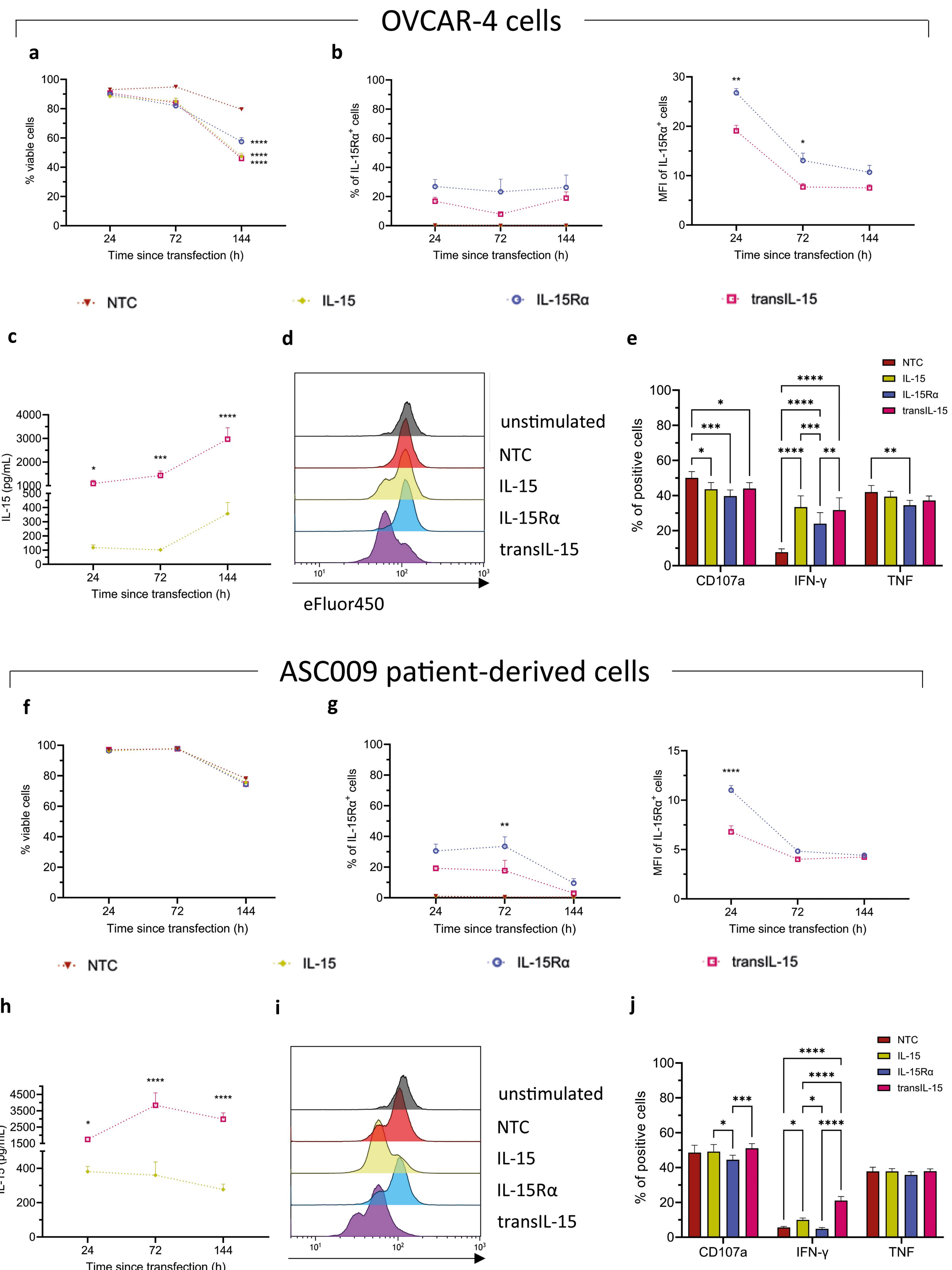

Figure 3. High grade serous OVCAR-4 cells and primary-derived OC cells are effectively transfected and activate HPC-NK cell proliferation and potency. (a) Viability, (b) percentage of IL-15Rα+ cells and MFI within IL-15Rα+ cells and (c) IL-15 secretion of transfected and washed OVCAR-4 cells at the indicated time points. (d) Representative flow cytometry histograms showing loss of eFluor450 after three days in one fresh HPC-NK cell donor. (e) Percentage of CD107a+, IFNγ+ and TNF+ in viable CD56+ HPC-NK cells after 4 h co-culture with OVCAR-4 cells. Data derives from 4 independent donors, one experiment. (f) Viability, (g) percentage of IL-15Rα+ cells and MFI within IL-15Rα+ cells, and (h) IL-15 secretion of transfected and washed ASC009 cells at the indicated time points. (i) Representative flow cytometry histograms showing loss of eFluor450 after three days in one fresh HPC-NK cell donor. (j) Percentage of CD107a+, IFNγ+ and TNF+ in viable CD56+ HPC-NK cells after 4 h co-culture with ASC009 cells. All viability and transfection data comes from 3 independent replicates, one experiment. All potency data derives from 4 independent HPC-NK donors, one experiment. Data represent mean values ± SEM. Two-way ANOVA with Bonferroni correction comparing all conditions within the same time point or potency readout was used (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

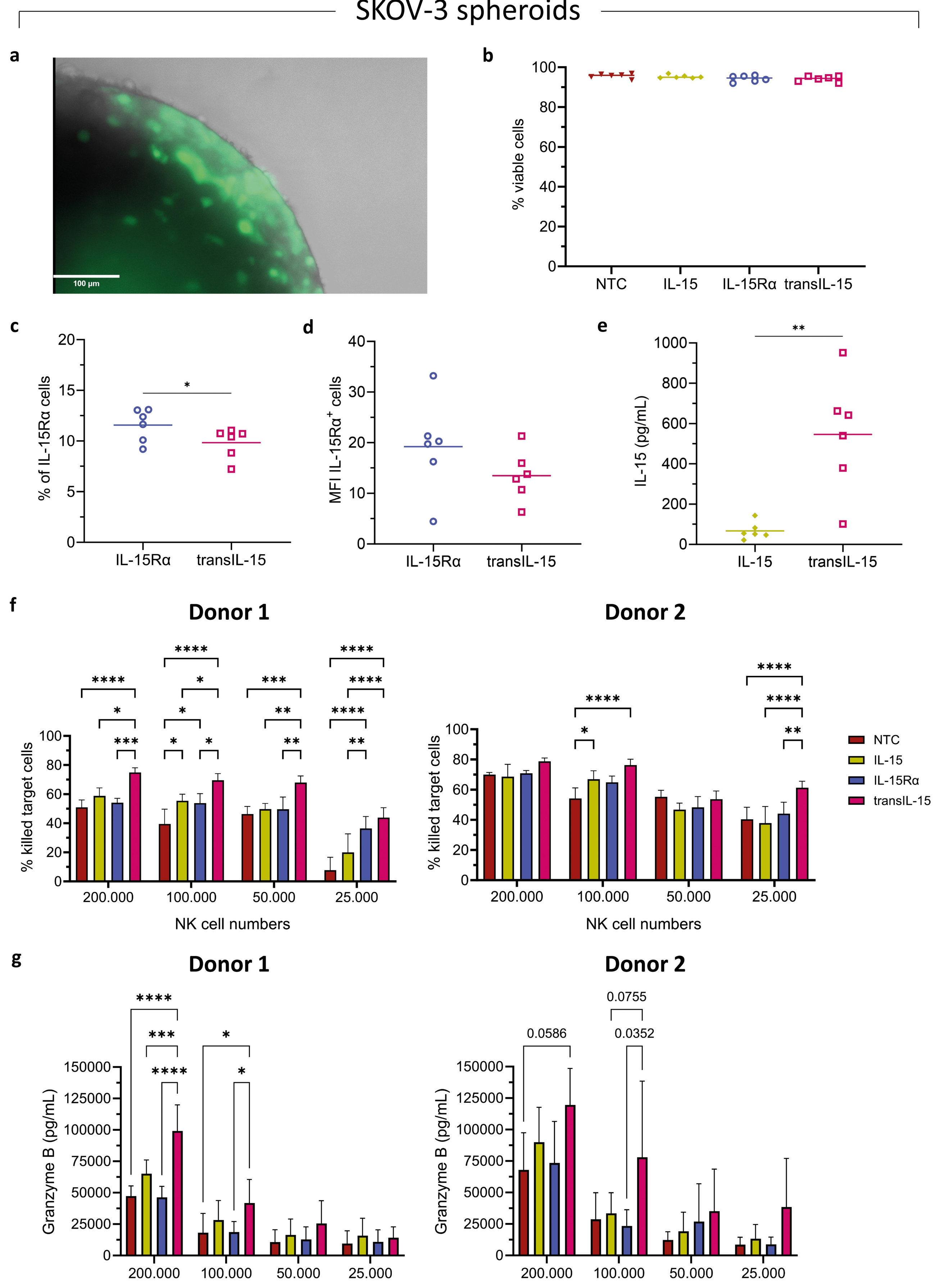

Figure 4. SKOV-3 OC spheroids are effectively transfected with transIL-15, and results in superior HPC-NK-mediated cytotoxicity. (a) Representative image of gfp-transfected, non-washed SKOV-3 spheroids 24 h after transfection (scale bar represents 100 µm). (b) Viability, (c) percentage of IL-15Rα+, (d) MFI of IL-15Rα+ and (e) IL-15 secretion of SKOV-3

feasibility of transfecting OC cells with IL-15 and IL-15Rα mRNA to locally boost HPC-NK cell function and proliferation.

We transfected six different OC cell lines and primary ascites-derived OC cells with different histopathology with IL-15- and IL-15Rα-encoding mRNA, with detectable expression still 6 days post-transfection. In addition, co-culture of transIL-15 OC cells (including SKOV-3, OVCAR-4 and primary ASC009 cells) with IL-2Rβ- and IL-2Rγc-expressing HPC-NK cells resulted in up to 20% superior killing in monolayers and spheroids, and up to 2-fold more IFNγ-producing HPC-NK cells and > 30% higher proliferative capacity in monolayers compared to IL-15. Notably, similar stimulation was previously reported in murine NK cells targeting IL-15Rα-transfected murine colorectal cancer cells.[12] Although the improved in vitro killing capacity that we observed was moderate, heterodimeric IL-15 shows superior stability and half-life in vivo than soluble IL-15 cytokine.[27] In addition, we previously reported more striking differences in spheroid killing capacity when HPC-NK cells were stimulated for long-term with the IL-15 superagonist ALT/N-803.[28] Therefore, the seemingly moderate superior anti-tumor functionality in transIL-15 compared to IL-15 alone will likely be more pronounced in an in vivo setting. Furthermore, we previously found no differences in IFNγ production when HPC-NK cells were stimulated with the soluble ALT/N-803 superagonist vs. soluble IL-15 cytokine alone.[4] Conversely, in this study we found that transIL-15 condition results in superior HPC-NK cell functionality compared to IL-15 transfection condition. We hypothesize that this effect could be (partly) due to the increased secretion of IL-15 in the presence of IL-15Rα, an effect previously reported by Bergamaschi, C. et al.[29] Alternatively, the superior stimulatory effect might be due to expression of both membrane-bound IL-15 and secreted soluble hetIL-15 in the transIL-15 condition, as opposed to ALT/N-803 that only mimics soluble hetIL-15 form. In line with this, we found that superior HPC-NK IFNγ production was mediated by both membrane-bound IL-15 on the surface of OC cells and secretion of hetIL-15. Furthermore, we observed transfer of IL-15Rα from transIL-15 OC cells to HPC-NK cells, a mechanism previously linked to superior survival of IL-15-responding immune cells that could further improve the therapeutic effect of HPC-NK cells.[30] Overall, these results go in concomitance with previous findings where IL-15 coupled with IL-15Rα results in improved expansion and functionality of NK cells.[16,31–33]

Previous (clinical) studies have evaluated the safety and efficacy of cytokine therapy for cancer, including IL-15. Soluble IL-15 alone has been evaluated for the treatment of metastatic melanoma and renal cell carcinoma, but resulted in detectable grade three toxicities and no detectable clinical responses, probably due to the induction of only transient expansion of peripheral blood-derived NK cells up to a month.[34] On the contrary, clinical evaluation of the IL-15 superagonist ALT/N-803 proved to be safe and resulted in an overall response rate of 19% in cancer patients that relapsed after stem cell transplantation.[14] Therefore, use of IL-15 superagonists mimicking transIL-15 stimulation appeared to be more effective than IL-15 cytokine alone for cancer treatment. However, additional evaluation of the ALT/N-803 superagonist in acute myeloid leukemia patients resulted in a limited clinical response of NK cell-treated patients compared to other cytokines, such as IL-2.[17] This was associated with $CD8^+$ T cell-mediated rejection of the allogeneic haploidentical NK cells two weeks after infusion. In addition, another phase I trial revealed no observed clinical response in ALT/N-803-treated patients with solid tumors, suggesting soluble heterodimeric IL-15 might not be enough to stimulate potent anti-tumor responses.[35] In line with this finding, we did not detect superior functionality of our HPC-NK cells with ALT/N-803 compared to IL-15 alone in vitro for OC.[4] In this context, local delivery of IL-15 and IL-15Rα mRNAs to the tumor might be an attractive strategy to boost expansion and functionality of the allogeneic (HPC-)NK cells whilst minimizing the toxicity issues associated with IL-15 cytokine alone and improving clinical efficacy associated with ALT/N-803. The combination of both membrane-bound IL-15 and hetIL-15 at the tumor site might significantly improve clinical efficacy of our HPC-NK cell product.

In conclusion, we here substantiate that transfection of OC cells with IL-15 and IL-15Rα mRNAs results in superior HPC-NK cell functionality and expansion *in vitro*, laying the groundwork for the *in vivo* transfection of OC tumors with transIL-15. For this, the mode of injection (intravenous vs. intraperitoneal) and type of nanoparticle (nature of nanoparticle, and targeting vs. non-targeting) should be addressed. Notably, given the clinical approval of SARS-CoV-2 vaccines and the siRNA drug patisiran, lipid nanoparticles represent the most accessible mRNA delivery platform.[36,37] In addition, use of non-targeting nanoparticles will transfect both tumor and normal stroma cells, but this will likely not activate cytotoxic responses against healthy cells due to lack of activating ligands.[20] Therefore, intraperitoneal infusion of non-targeting lipid nanoparticles would be the most adequate option for mRNA delivery, as it simplifies GMP production, treatment and bypasses liver-mediated clearance of the therapeutic product.[20,38,39] Overall, we believe that the implementation of these transIL-15 nanoparticles could potentially translate into improved survival in NK cell-treated OC patients.

spheroids 24 h after transfection. Data comes from 6 independent spheroids, 4 independent experiments. (f) Percentage of target cell death and (g) granzyme B secretion by HPC-NK cells co-cultured with spheroids for 16-20 h. For each donor, data comes from 2 independent experiments, 4 independent replicates per experiment. All data represent mean values ±SD. When applicable, student t-tests or two-way ANOVA with Bonferroni correction to compare different transfection conditions within the same effector-to-target ratios were used (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

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Data availability statement

Data can be made available by the corresponding author upon reasonable request.

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