Supporting Information

Palacios *et al.*

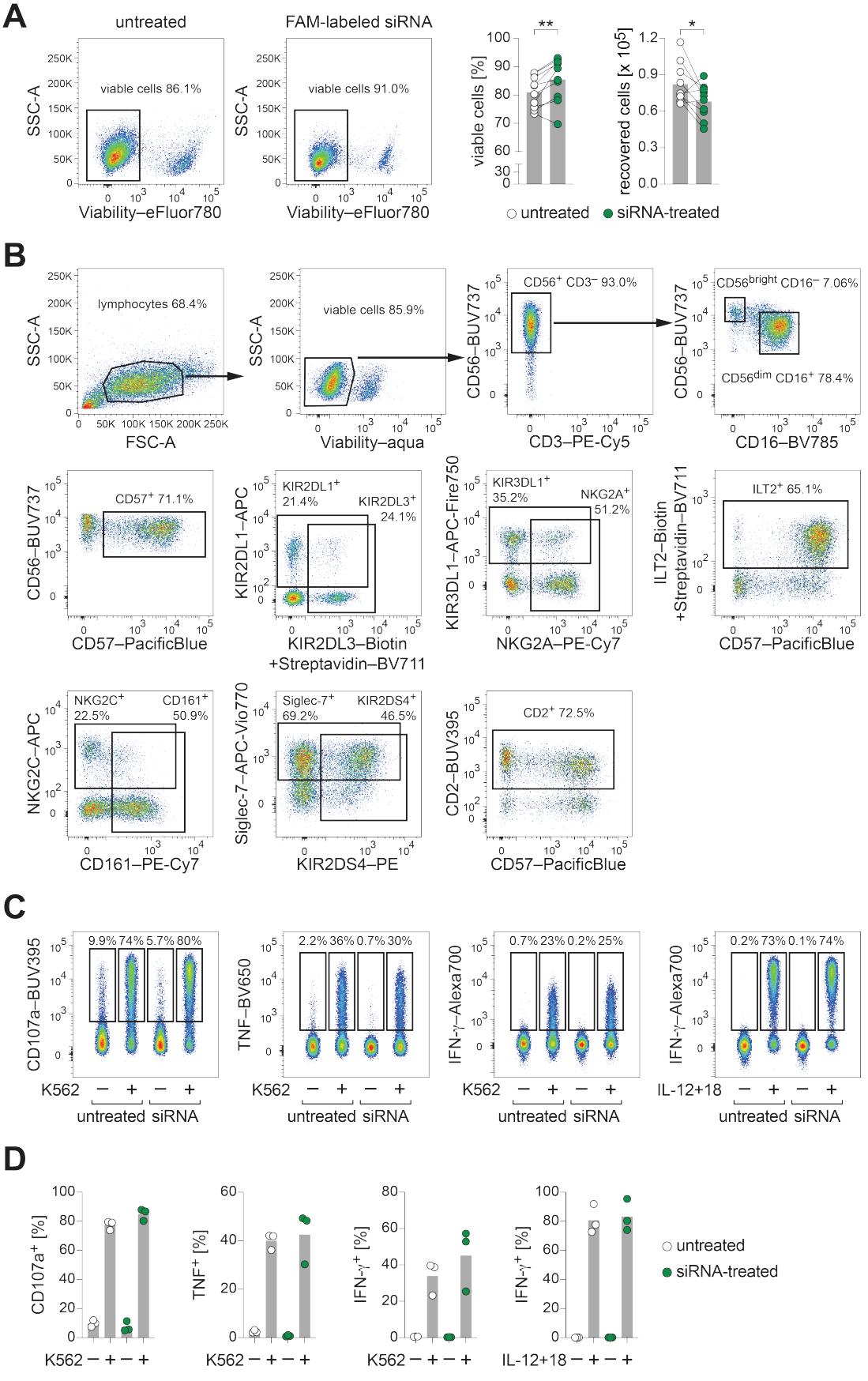

Figure S1. Unaltered viability and function after siRNA uptake.

(**A**) Purified NK cells were either left untreated in culture medium containing 1 ng/mL IL-15 or treated with 1 µM siRNA in Accell siRNA delivery medium supplemented with 1 ng/mL IL-15 for 96 h and analyzed by flow cytometry. Far left: Representative viability dye staining of untreated NK cells. Middle left: representative viability dye staining of siRNA-treated NK cells. Middle right: frequency of viable NK cells. Far right: absolute count of recovered viable NK cells (input before treatment: $1x10^5$ NK cells).

(**B**) Identification of NK cell sub-populations using flow cytometry. Top row: gating strategy of $CD56^{bright}$ $CD16^-$ and $CD56^{dim}$ $CD16^+$ NK cell populations starting with single events. Middle and bottom row: sub-gating of $CD56^{dim}$ $CD16^+$ NK cells (as gated in top row) according to the expression of the differentiation marker CD57 and selected inhibitory and activating NK cell receptors. For KIR co-expression pattern analysis, Boolean gating of KIR2DL1, KIR2DL3, and KIR3DL1 was employed.

(**C-D**) NK cells were either left untreated in culture medium containing 1 ng/mL IL-15 or treated with 1 µM siRNA in Accell siRNA delivery medium supplemented with 1 ng/mL IL-15 for 96 h, followed by co-culture with K562 cells (at E:T=1) or exposure to 10 ng/mL IL-12 and 10 ng/mL IL-18. (C) Representative functional responses as measured by flow cytometry. Far left: CD107a surface mobilization. Middle left: TNF expression. Middle right and far right: IFN-γ. (D) Summary of functional responses in the indicated conditions.

Data are displayed as mean and individual datapoints (A, D) and representative of n=6-8 donors in 3 independent experiments (B) and n=3 donors in 1 experiment (C) or pooled from n=10 donors in 3 independent experiments (A) and n=3 donors in 1 experiment (D). Symbols represent individual donors (A, D).

Statistical significance was tested using Wilcoxon test (A). $*P < 0.05$, $**P < 0.01$.

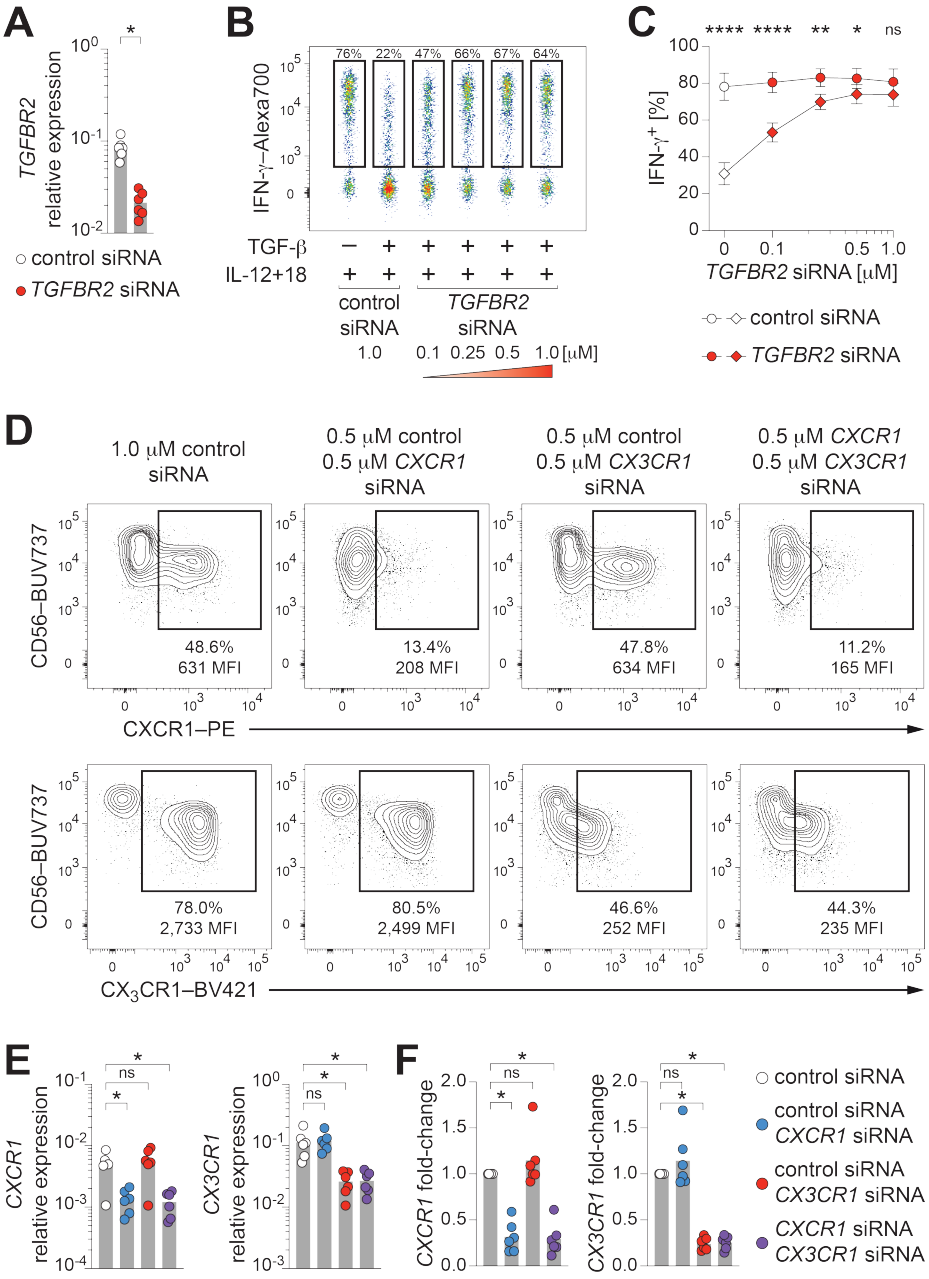

Figure S2. Dose-dependent and multiplexed knock-downs.

(**A**) NK cells were treated with control siRNA or *TGFBR2* siRNA for 96 h and *TGFBR2* mRNA expression was assessed relative to *GAPDH* using RT-qPCR.

(**B-C**) NK cells were treated with 1.0 μM control siRNA or varying concentrations of *TGFBR2* siRNA, followed by exposure to 20 ng/mL TGF-β and stimulation with 10 ng/mL IL-12 and 10 ng/mL IL18. (B) Representative IFN-γ expression as determined by flow cytometry after treatment of NK cells with siRNA at the indicated concentration

followed by cytokine exposure. (C) Summary of IFN-γ expression by NK cells treated with 1.0 μM control siRNA (white symbols) or varying concentrations of *TGFBR2* siRNA (red symbols), followed by exposure to TGF-β (diamonds) or not (circles) and subsequent stimulation with IL-12 and IL-18.

(**D-F**) NK cells were treated with control siRNA or combinations of siRNA targeting *CXCR1* or *CX3CR1*. (D) Representative chemokine receptor protein expression as determined by flow cytometry. (E) Chemokine receptor mRNA expression relative to *GAPDH* as assessed by RT-qPCR. Left: *CXCR1*. Right. *CX3CR1*. (F) Chemokine receptor mRNA expression as fold-change to control siRNA. Left: *CXCR1*. Right. *CX3CR1*.

Data are displayed as mean and individual datapoints (A, E, and F) or mean ± SEM (C) and representative of n=4 donors in 1 experiment (B) and n=8 donors in 3 independent experiments (D) or pooled from n=6 donors in 2 independent experiments (A), n=4 donors in 1 experiment (C), and n=6 donors in 2 independent experiments (E, F). Symbols represent individual donors (A, E) or mean (C).

Statistical significance was tested using Wilcoxon test (A), two-way repeated-measures ANOVA (C), or Friedman test with Dunn's test (E, F). **P* < 0.05, ***P* < 0.01, *****P* < 0.0001.

Methods

Cells and cell lines

Buffy coats from healthy donors were obtained from the Department of Clinical Immunology and Transfusion Medicine, Karolinska Institute as approved by the Ethical Review Board Stockholm (DNR 2020-05289). All participants provided informed consent. PBMC were isolated from buffy coats with standard density gradient centrifugation and either used directly or cryopreserved in FBS (Gibco/ThermoFisher) containing 10% (V/V) DMSO (Sigma) and stored in the vapor phase of liquid nitrogen. NK cells were purified from PBMC by magnetic separation using human NK cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions.

K562 cells were maintained in culture medium (RPMI-1640 supplemented with 2 mM glutamine, 10% (V/V) FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin; all Gibco/ThermoFisher).

siRNA

Accell siRNA with proprietary chemical modifications were obtained from Dharmacon/Horizon Discovery. The following siRNA reagents (catalogue number) were used in this study: Accell Green Non-targeting Control siRNA (D-001950-01-20), Accell Non-targeting Control siRNA (individual siRNA #1, D-001910-01-20), Accell Human *B2M* siRNA (individual siRNA #13, A-004366-13-0020), Accell Non-targeting Control siRNA (SMARTpool, D-001910-10-20), Accell Human *TGFBR2* siRNA (SMARTpool, E-003930-00-0020), Accell Human *CXCR1* siRNA (SMARTpool, E-005646-00-0020), and Accell Human *CX3CR1* siRNA (SMARTpool, E-005471-00-0020).

If not indicated otherwise, siRNA treatment was performed in Accell siRNA delivery medium (B-005000-100).

Reverse transcription quantitative PCR (RT-qPCR)

After 96 h of siRNA treatment, $1x10^5$ NK cells were subjected to RNA isolation using RNeasy Micro Plus Micro Kit (Qiagen) according to the manufacturer's instructions. Total RNA was reverse-transcribed with the QuantiTect Reverse Transcription Kit (Qiagen) and qPCR was performed in triplicates with QuantiTect SYBR Green PCR Kit (Qiagen) and QuantiTect Primer Assays (Qiagen) in a QuantStudio 5 instrument (ThermoFisher). Data were analyzed using ThermoFisher Connect (ThermoFisher) and are presented relative to *GAPDH* expression or as fold-change between treatment conditions as indicated. The following primer assays (catalogue number) were used in this study: Hs_GAPDH_1_SG (QT00079247), Hs_B2M_1_SG (QT00088935), Hs_TGFBR2_1_SG (QT00014350), Hs_CXCR1_1_SG (QT00212919), and Hs_CX3CR1_1_SG (QT00203434; all Qiagen).

Flow cytometry

Flow cytometric analyses were performed following established guidelines [1]. In brief, cell suspensions were incubated with combinations of fluorochrome-conjugated antibodies at optimized concentrations in PBS for 20 min at room temperature (RT). Viable cells were identified with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit or eBioscience Fixable Viability Dye eFluor 780 (both ThermoFisher). Streptavidin-BV711 (BD Biosciences) was used as secondary reagent in combination with biotinylated antibodies. Chemokine receptor staining was performed for 20 min at 37°C prior to staining of other surface antigens. Samples were fixed before acquisition with BD

Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions, stored in PBS containing 2% (V/V) FBS and 2 mM EDTA. Samples were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo v10.7.1 (BD Biosciences). NK cells were identified as single viable $CD3^-$ $CD56^+$ lymphocytes (Supporting Information Fig. S1B for gating strategy) and further stratified into subsets as indicated.

The following antibodies (clone, RRID) were used in this study: anti-CD56-BUV737 (NCAM16.2, AB_2860005), anti-IFN-γ-Alexa700 (B27, AB_396977), anti-CX_3CR1-BV421 (2A9-1, AB_2744471), anti-CD2-BUV395 (RPA-2.10, AB_2744356), anti-CD107a-BUV395 (H3A4, AB_2739073; all BD), anti-CXCR1-PE (8F1/CXCR1, AB_439815), anti-TNF-BV650 (Mab11, AB_2562741), anti-KIR3DL1-APC-Fire750 (DX9, AB_2687394), anti-CD16-BV785 (3G8, AB_2563803), anti-CD161-PE-Cy7 (HP-3G10, AB_11126745), anti-β_2m-APC (2M2, AB_10641281), mouse IgG1 isotype control-APC (MOPC-21, AB_2888687), anti-HLA class I-FITC (W6/32, AB_314873), mouse IgG1 isotype control-FITC (MOPC-21, AB_326429; all BioLegend), anti-CD3-PE-Cy5 (UCHT1, AB_2827419), anti-NKG2A-PE-Cy7 (Z199, AB_2687887; both Beckman Coulter), anti-KIR2DL1-APC (REA284, AB_2752101), anti-KIR2DL3-Biotin (REA147, AB_2655344), anti-KIR2DS4-PE (JJC11.6, AB_871619), anti-NKG2C-APC (REA205, AB_2727980), anti-ILT2-Biotin (REA998, AB_2727627), and anti-Siglec-7-APC-Vio770 (REA214, AB_2657541; all Miltenyi Biotec).

Absolute counts of viable cells

To determine the absolute counts of viable NK cells that were recovered after treatment, 20 µL CountBright Absolute Counting Beads (ThermoFisher) were added directly to cultured cells prior to processing and subsequently prepared for flow

cytometric analysis as outlined above. After acquisition, the absolute counts of single viable $CD56^+$ $CD3^-$ lymphocytes were calculated relative to the recorded numbers of counting beads according to the manufacturer's instructions.

NK cell functional assays

For functional assays, $1x10^5$ siRNA-treated NK cells were either left untreated or co-cultured with $1x10^5$ K562 target cells in 200 µL culture medium in V-bottom 96-well plates for 6 h at 37 °C. To detect degranulation, anti-CD107a antibodies were added at the start of the co-culture and GolgiStop (containing monensin) as well as GolgiPlug (containing Brefeldin A, both BD Biosciences) were added after 1 h. After 6 h of co-culture, the cells were stained for surface markers and fixed and permeabilized (BD Cytofix/Cytoperm, BD Biosciences) prior to intracellular staining of IFN-γ and TNF in BD Perm/Wash Buffer (BD Biosciences) for 30 min at 4 °C.

Alternatively, siRNA-treated NK cells were stimulated by addition of 10 ng/mL IL-12 (PeproTech) and 10 ng/mL IL-18 (R&D Systems) for 24 h with GolgiStop and GolgiPlug (both BD Biosciences) present during the last 5 h. Cytokine-stimulated NK cells were surface stained and fixed, followed by intracellular staining of IFN-γ as above.

TGF-β suppression assay

To assess suppression of NK cell cytokine production by TGF-β, purified NK cells were either treated with control siRNA or *TGFBR2* siRNA at the indicated concentrations for 72 h. Next, NK cells were exposed to 5 ng/mL IL-15 alone or 5 ng/mL IL-15 combined with 20 ng/mL TGF-β1 (Miltenyi Biotec). After 24 h of exposure, NK cells were stimulated with IL-12 and IL-18 as above.

Statistical analysis

Statistical parameters (sample size of individual donors as biological replicates, number of performed independent experiments, statistical tests, and significance) are reported in the figure legends and/or displayed in the figures. Two groups of paired samples were analyzed with two-tailed Wilcoxon signed-rank test and three or more paired groups were analyzed with Friedman test and Dunn's post-test to correct for multiple comparisons. Paired samples with two variables (i.e., different concentrations of different treatments) were analyzed with repeated-measures two-way ANOVA with Bonferroni correction. All statistical analyses were performed in Prism 9 (GraphPad Software) with a confidence level of 0.95 and $P > 0.05$ was considered not significant (ns).

Step-by-step protocol: siRNA treatment of NK cells

1. Materials

- 5X siRNA Buffer (Horizon Discovery, Catalog no.:B-002000-UB-100)
- RNase-free Water (Horizon Discovery, Catalog no.:B-003000-WB-100)
- NK Cell Isolation Kit, human (Miltenyi Biotec, Catalog no.: 130-092-657)
- Accell siRNA Delivery Medium (Horizon Discovery, Catalog no: B-005000-100)
- Penicillin-Streptomycin (ThermoFisher, Catalog no.: 15140122)
- Accell siRNA as desired, either as SMARTpool or individual sequences (see **Note 1**)
- Accell Non-targeting Control siRNA (SMARTpool or individual sequence, matched to siRNA-of-interest) to determine baseline cellular response

2. Methods

2.1. Protocol for reconstitution of siRNA

1. Dilute 5X siRNA Buffer with RNase-free water to 1X siRNA buffer
2. Briefly centrifuge the vial containing siRNA at 14,000 *g*
3. Add the appropriate volume of 1X siRNA Buffer to the lyophilized siRNA to obtain the desired stock concentration (see **Note 2**)
4. Mix by pipetting up-and-down five times without introducing air bubbles into the solution
5. Incubate at room temperature for 30 min while shaking at 50 rpm or gently flicking the vial repeatedly

6. Verify reconstitution and determine concentration of reconstituted siRNA using a spectrophotometer (see **Note 3**)
7. Aliquot reconstituted siRNA to minimize freeze-thaw cycles and store at -20 °C

2.2. Protocol for siRNA treatment of NK cells

1. Obtain PBMC by density gradient centrifugation of whole blood or buffy coats; alternatively, thaw previously cryopreserved PBMC as described elsewhere
2. Purify NK cells with method of choice, e.g., by using human NK cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions
3. Determine viable cell count by trypan blue exclusion using a hemocytometer or an automated counting device and transfer desired number of viable NK cells into a 15 or 50 mL tube
4. Centrifuge the sample at 300 *g* for 10 min and aspirate the supernatant completely
5. Wash sample by adding 1 mL pre-warmed PBS per $1x10^6$ cells, centrifuge at 300 *g* for 10 min, and aspirate the supernatant completely
6. Resuspend cells in pre-warmed Accell siRNA delivery medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 ng/mL IL-15 to a final cell density of $1x10^6$ cells/mL (see **Note 4**)
7. Dispense 100 µL of cell suspension (containing $1x10^5$ cells) into the wells of a U-bottom 96-well plate (see **Note 5**)
8. Add desired amount of Accell siRNA directly into the cell suspension and mix well

9. Incubate at 37 °C, 5% CO_2, and 95% humidity for 48 to 96 h, depending on the intended downstream assay(s) (see **Note 6**)

Notes

Note 1: For most targets, Accell siRNA reagents are available in either individual format (one siRNA sequence) or SMARTpool format (four siRNA sequences targeting different sections of the same gene-of-interest). We recommend using the appropriate Non-targeting Control siRNA (either individual sequence or SMARTpool).

Note 2: For ease of dilution, 100 µM is recommended as stock concentration.

Note 3: Reconstitution can be quality-controlled by standard UV spectrophotometry. Stock solutions of 100 µM siRNA contain approx. 1,350 ng/µL RNA, but RNA concentrations may vary depending on the exact sequence and are strongly influenced by potential fluorescent labels of the siRNA in question.

Note 4: We supplement the medium with 1 ng/mL IL-15 once and do not re-add the cytokine or replace the medium with fresh medium containing fresh cytokine. Results may vary and should be optimized, considering NK cell survival and activation for the intended application.

Note 5: $1x10^5$ purified NK cells in 100 µL medium per well of a U-bottom 96-well plate is our preferred format. The protocol can be up-scaled to other formats, but when using F-bottom plates, it is recommended to adjust volumes and cell numbers to account for possible evaporation and reduced cell-cell contact.

Note 6: The exact timing required for knock-down should be determined separately for each gene-of-interest. Generally, we observed efficient knock-down at 48-96 h for transcript levels and at 72-96 h for protein levels.

References

1 **Cossarizza, A.et al.,** *Eur J Immunol* 2017. **47**: 1584-1797.