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Effects of siRNA-mediated TIGIT silencing on NK cell cytotoxicity against triple-negative and luminal breast cancer cells

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ABSTRACT

Objective(s): TIGIT is an inhibitory immune checkpoint receptor expressed on NK and T cells. TIGIT interaction with CD155/CD112 suppresses anti-tumor immunity. We investigated whether siRNA-mediated TIGIT silencing in primary human NK cells enhances cytotoxicity against triple-negative (MDA-MB-231) and luminal (MCF-7) breast cancer cells.

Materials and Methods: NK cells were isolated from three treatment-naïve breast cancer patients and three healthy donors. For cytotoxicity assays, NK cells from the best-expanding donor per group were selected; results are exploratory estimates. Cells were transfected with TIGIT-specific or scrambled siRNA. TIGIT expression was assessed by qRT-PCR, and cytotoxicity was measured using an AlamarBlue assay at an E:T ratio of 5:1.

Results: Baseline TIGIT expression was 1.5-fold higher in patient-derived NK cells (P=0.027). TIGIT silencing increased cytotoxicity against MDA-MB-231 cells by 9 percentage points in healthy donor NK cells (from 41% to 50%) and by 7 percentage points in patient NK cells (from 51% to 58%). Against MCF-7 cells, increases were modest — only 3 to 3.5 percentage points (healthy: from 37.5% to 41%; patient: from 28% to 31%). Cytotoxicity values are descriptive estimates due to single-donor assays. Across all conditions, patient NK cells were more active than healthy NK cells.

Conclusion: TIGIT silencing in primary human NK cells was associated with increased cytotoxicity against MDA-MB-231 cells but not against MCF-7 cells. These preliminary findings support further investigation of TIGIT-targeted NK cell therapy for triple-negative breast cancer.

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Introduction

Breast cancer remains the most frequently diagnosed malignancy among women worldwide. In 2022, approximately 2.3 million new cases and 685,000 deaths were reported globally (1). Despite substantial advances in diagnosis and treatment, breast cancer remains a heterogeneous disease with marked molecular and clinical diversity. Among its subtypes, triple-negative breast cancer (TNBC), defined by the absence of estrogen receptor, progesterone receptor, and HER2 expression, accounts for approximately 15–20% of all breast cancers and is associated with an aggressive clinical course, early metastasis, and poor prognosis (2, 3). MDA-MB-231 is a widely used TNBC cell line with highly invasive characteristics (4). In contrast, MCF-7 is an estrogen receptor-positive luminal breast cancer cell line representing the most common molecular subtype of breast cancer (5,6). While endocrine therapies and CDK4/6 inhibitors have significantly improved outcomes for patients with luminal disease (7), therapeutic options for TNBC remain limited (8), underscoring the need for novel immunotherapeutic approaches. Natural killer (NK) cells are key components of the innate immune system and can recognize and eliminate malignant cells without prior antigen sensitization or major histocompatibility complex restriction. These properties make NK cells attractive candidates for cancer immunotherapy (9, 10). In breast cancer, increased NK cell infiltration has been associated with improved treatment responses and favorable clinical

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outcomes (11). However, prolonged exposure to the immunosuppressive tumor microenvironment can impair NK-cell function, reducing cytotoxicity, diminishing cytokine production, and increasing expression of inhibitory immune checkpoint receptors (12, 13). Among these inhibitory pathways, T-cell immunoreceptor with Ig and ITIM domains (TIGIT) has emerged as a promising therapeutic target. TIGIT is expressed on NK cells, CD8+ T cells, CD4+ T cells, and regulatory T cells (14, 15). Its principal ligands, CD155 and CD112, are frequently overexpressed on tumor cells, including breast cancer cells (16, 17). Upon ligand binding, TIGIT delivers inhibitory signals that suppress NK-cell activation and function, partly through intracellular inhibitory pathways and competition with the activating receptor CD226 for shared ligands (14, 18). Several studies have reported elevated TIGIT expression in breast cancer tissues, where it has been associated with poor clinical outcomes (19–21). In addition, combined TIGIT blockade and MDSC inhibition have been shown to reduce breast cancer bone metastasis in preclinical models, further supporting TIGIT as a potential therapeutic target (22). Most current TIGIT-targeting strategies have relied on monoclonal antibodies, such as tiragolumab. Although early clinical studies demonstrated encouraging activity, subsequent phase III trials failed to achieve their primary endpoints, highlighting the need for alternative approaches and improved patient selection strategies (23). Alternative strategies to enhance NK cell activity, such as bispecific antibodies that redirect NK cells to tumor antigens (e.g., anti-CD16/EGFR), have also been explored (24). However, these approaches may still face limitations related to manufacturing complexity or dependence on specific tumor markers. RNA interference (RNAi) represents one such approach. By degrading target mRNA, small interfering RNA (siRNA) suppresses gene expression at the post-transcriptional level and may provide an alternative means of modulating immune checkpoint signaling (25, 26). Previous studies have reported higher expression of CD155 and CD112 in MDA-MB-231 cells than in MCF-7 cells (27), suggesting that TIGIT signaling may have subtype-dependent functional consequences. In this study, we tested whether siRNA-mediated TIGIT silencing in primary human NK cells could enhance their cytotoxic activity against breast cancer cells and whether the response differed between MDA-MB-231 and MCF-7 breast cancer cell lines.

Materials and Methods

NK cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Hypaque density-gradient centrifugation (Cytiva, Marlborough, MA, USA). Briefly, whole blood was diluted 1:1 with phosphate-buffered saline (PBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged at 800×g for 20 min at room temperature. NK cells were enriched by negative selection using a magnetic-activated cell sorting (MACS) NK Cell Isolation Kit, human (Miltenyi Biotec, Bergisch Gladbach, Germany; Cat# 130-092-657) according to the manufacturer's instructions. Cell purity was assessed by flow cytometry using fluorochrome-conjugated antibodies against CD56 APC-Vio770 (Miltenyi Biotec, Bergisch Gladbach, Germany) and CD16 BV768 (BD Horizon, BD Biosciences, Franklin Lakes, NJ, USA) on an Attune NxT flow cytometer (Thermo Fisher Scientific, USA). Data were analyzed using FlowJo v10 software (BD Biosciences, Ashland, OR, USA). Purified NK cells were cultured in complete NK MACS Medium, consisting of NK MACS Basal Medium and NK MACS Supplement (Miltenyi Biotec, Cat# 130-114-429) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.3 mg/ml L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), and 0.05 nM β-mercaptoethanol (Merck, Darmstadt, Germany). For expansion, complete NK MACS Medium was supplemented with human IL-2 IS, premium grade (Miltenyi Biotec, Cat# 130-097-746) and human IL-15, premium grade (Miltenyi Biotec, Cat# 130-095-764), and cells were activated with 500 IU/ml IL-2 for 24 hr (PeproTech, USA) at 37 °C in a humidified atmosphere containing 5% CO_2 before transfection. The activating receptor NKp44 has been shown to be up-regulated in certain cancers, and its increased expression correlates with enhanced NK cell cytotoxicity (28).

RNA extraction and cDNA synthesis

Total RNA was extracted from NK cells using TRIzol™ Reagent (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. RNA concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Only RNA samples with A260/A280 ratios between 1.8 and 2.0 were used for subsequent analyses. Extracted RNA was stored at −70 °C until further use. First-strand cDNA was synthesized from 1 µg of total RNA using a cDNA synthesis kit (Thermo Fisher Scientific, Lithuania) according to the manufacturer's instructions. Reverse transcription was performed at 25 °C for 10 min, followed by 50 °C for 40 min and 70 °C for 15 min to terminate the reaction. The synthesized cDNA was stored at −70 °C until quantitative real-time PCR analysis.

Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix (Parstous Corporation, Iran) on an ABI QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA). β-actin was used as the internal reference gene. Primers (listed in Table 1) were synthesized by Bioneer Co. (Daejeon, South Korea). Each 20 µl reaction contained 10 µl SYBR Green Master Mix, 0.5 µM of each primer, and 2 µl cDNA template. Amplification was carried out under

Table 1. Sequences of forward and reverse primers used for quantitative real-time PCR analysis of TIGIT and β-actin gene expression

Name	Type	Strand	Sequence (5′→3′)	Length (nt)
TIGIT	qRT-PCR Primer	Forward	TGGTGGTCATCTGCACAGCAGT	22
TIGIT	qRT-PCR Primer	Reverse	TTTCTCCTGAGGTCACCTTCCAC	23
β-actin	qRT-PCR Primer	Forward	GAGCATCCCCCAAAGTTCAC	20
β-actin	qRT-PCR Primer	Reverse	GGGACTTCCTGTAACAACGC	20

Primers were used with SYBR green master mix on an ABI QuantStudio 5 real-time PCR system
TIGIT: T-cell immunoreceptor with Ig and ITIM domains; qRT-PCR: quantitative reverse transcription PCR

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the following conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec. A melt-curve analysis was performed at the end of each run to verify amplification specificity. All samples were analyzed in duplicate. PCR efficiency was determined using four-point, two-fold serial dilution standard curves, yielding efficiencies of 1.95 for TIGIT and 2.00 for β-actin. Relative gene expression was calculated using the Pfaffl method (29).

siRNA-mediated TIGIT knockdown

The TIGIT-specific siRNA duplex was designed in-house following design principles consistent with those employed by the BLOCK-iT™ RNAi Designer (Thermo Fisher Scientific) and siDESIGN Center (Dharmacon) algorithms, to minimize potential off-target effects and avoid stable secondary structure at the target site, while maintaining appropriate strand asymmetry and a favorable thermodynamic energy profile across the duplex. To further reduce the likelihood of off-target silencing, candidate sequences exhibiting low seed-complement frequency were prioritized, and complementarity to 3′ untranslated regions of unrelated transcripts was avoided. The finalized TIGIT-specific siRNA duplex, together with a non-targeting scrambled control siRNA, was chemically synthesized by Bioneer Co. (Daejeon, South Korea). Before transfection, 1×10^5 NK cells were seeded per well in antibiotic-free medium. For transfection, 10 nM siRNA was complexed with Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, Thermo Fisher Scientific, USA) in Opti-MEM™ Reduced Serum Medium (Gibco, Thermo Fisher Scientific, USA) according to the manufacturer's instructions and incubated for 15 min at room temperature. The siRNA-lipid complexes were then added to the cells. After 6 hr of incubation, the transfection medium was replaced with fresh RPMI-1640 supplemented with 100 IU/ml recombinant human IL-2. TIGIT knockdown efficiency was assessed 24 hr post-transfection by qRT-PCR. In addition to the TIGIT siRNA and scrambled siRNA groups, a mock-transfected control group receiving transfection reagent alone was included.

Breast cancer cell culture and co-culture

MCF-7 and MDA-MB-231 breast cancer cell lines were obtained from the Iranian Biological Resource Center (IBRC, Tehran, Iran). MCF-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), whereas MDA-MB-231 cells were cultured in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Both media were supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin–streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Cells were used during the logarithmic growth phase between passages 3 and 8. Target cells were seeded 18 hr before co-culture experiments. For co-culture, both target cell lines were detached, washed, counted, and seeded in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) (without antibiotics) in 96-well plates. NK cells were then added in the same medium after 18 h. Therefore, all cytotoxicity assays were performed under identical culture conditions. Twelve co-culture groups were established across both target cell lines, as summarized in Table 2. For cytotoxicity assays, NK cells transfected with TIGIT-specific siRNA (siTIGIT) or scrambled control siRNA (siCtrl) were added to target cells at an effector-to-target (E:T) ratio of 5:1, a ratio selected based on prior studies demonstrating reliable discrimination of NK cell cytotoxic activity against breast cancer cell lines at this range (9,10). Due to the limited expansion capacity of primary NK cells, cytotoxicity assays were performed using NK cells from one donor in each study group that demonstrated the highest post-transfection cell recovery and viability. Gene-expression analyses by qRT-PCR were performed using samples from all three donors in each group.

Cytotoxicity assay (Alamar blue)

NK cell killing was measured using AlamarBlue™ (Thermo Fisher Scientific) following Nociari *et al.* (30). After 24 hr of co-culture, 10 µl of AlamarBlue was added to each well (10% v/v). Fluorescence was read after 3–4 hr (excitation 530–560 nm; emission 590 nm). Cytotoxicity was calculated as:

$$\%\ Cytotoxicity = \left[1 - \frac{(F_{co-culture} - F_{NK\ alone})}{F_{tumor\ alone}}\right] \times 100$$

Wells with tumor cells alone (0% killing) and NK cells alone (background) were included on each plate. All killing assays were done in technical triplicate.

Statistical analysis

qRT-PCR data obtained from three independent donors

Table 2. Co-culture experimental design for NK cell-mediated cytotoxicity assays following TIGIT silencing

Group	NK cell source	Transfection*	Target cell line	E: T ratio	Donors
1	Healthy Donor	siCtrl	MDA-MB-231	5:1	1
2	Healthy Donor	siTIGIT	MDA-MB-231	5:1	1
3	Healthy Donor	Mock	MDA-MB-231	5:1	1
4	Patient	siCtrl	MDA-MB-231	5:1	1
5	Patient	siTIGIT	MDA-MB-231	5:1	1
6	Patient	Mock	MDA-MB-231	5:1	1
7	Healthy Donor	siCtrl	MCF-7	5:1	1
8	Healthy Donor	siTIGIT	MCF-7	5:1	1
9	Healthy Donor	Mock	MCF-7	5:1	1
10	Patient	siCtrl	MCF-7	5:1	1
11	Patient	siTIGIT	MCF-7	5:1	1
12	Patient	Mock	MCF-7	5:1	1

Mock: transfection reagent only, no siRNA. siCtrl: scrambled non-targeting siRNA
siTIGIT: TIGIT-specific siRNA. For qRT-PCR analyses, all three donors per group were included. E:T, effector-to-target

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per group are presented as mean±standard deviation (SD). Differences in baseline TIGIT expression and post-transfection gene expression between groups were analyzed using two-tailed unpaired Student's t-tests. A *P*-value<0.05 was considered statistically significant. Cytotoxicity data are presented as the mean±SD of technical triplicates. Because cytotoxicity assays were performed using NK cells from a single donor in each study group, these analyses were considered exploratory and intended to provide descriptive estimates of effect size rather than population-level inference. All statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, USA).

Results

NK cell isolation generates a predominantly CD56⁺CD16⁺ population

Flow-cytometric analysis was performed to evaluate the purity of MACS-isolated NK cells. Sequential gating identified single cells (64.25% of total events), followed by lymphocytes (37.28% of single cells) and CD56⁺ cells (73.99% of lymphocytes). Within the CD56⁺ population, 91.20% of cells co-expressed CD16, indicating successful enrichment of NK cells (Figure 1).

Baseline TIGIT expression is higher in patient NK cells

Baseline TIGIT expression levels were determined by averaging the ΔCt values obtained from Mock and siCtrl samples within each donor group. Patient NK cells had 1.5-fold more TIGIT mRNA than healthy donor cells (*P* =0.027; Figures 2A and 2B). The $\log_2$ fold change (Mock Patient vs Mock Health) was approximately +0.6, consistent with the 1.5-fold increase in relative expression. This observation is consistent with the hypothesis that chronic exposure to the tumor microenvironment drives NK cells toward an exhausted, hypo-functional phenotype.

siTIGIT knocks down TIGIT without changing mock or scrambled controls

Mock and siCtrl had similar TIGIT levels in both donor groups (patient: ΔCt 4.73 vs 4.38; healthy: ΔCt 5.56 vs 4.09). These comparable expression levels confirm that neither the transfection procedure nor the scrambled siRNA altered baseline TIGIT expression. TIGIT expression was substantially reduced following siTIGIT transfection at 24 hr post-transfection: 96.5% in patient NK cells (Log_2FC= −4.82, *P*<0.001) and 90.7% in healthy NK cells (Log_2FC= −3.43, *P*<0.001) (Figures 3A and 4A). The corresponding relative fold changes are shown in Figures 3B and 4B. siCtrl/Mock showed no significant difference in either group (ns).

TIGIT knockdown improves killing of MDA-MB-231 but not MCF-7

Mock and siCtrl NK cells showed comparable cytotoxicity against both target lines (MDA-MB-231: healthy Mock 41% vs siCtrl 41%; patient Mock 51% vs siCtrl 51%; MCF-7: healthy Mock 35% vs siCtrl 37.5%; patient Mock 31%

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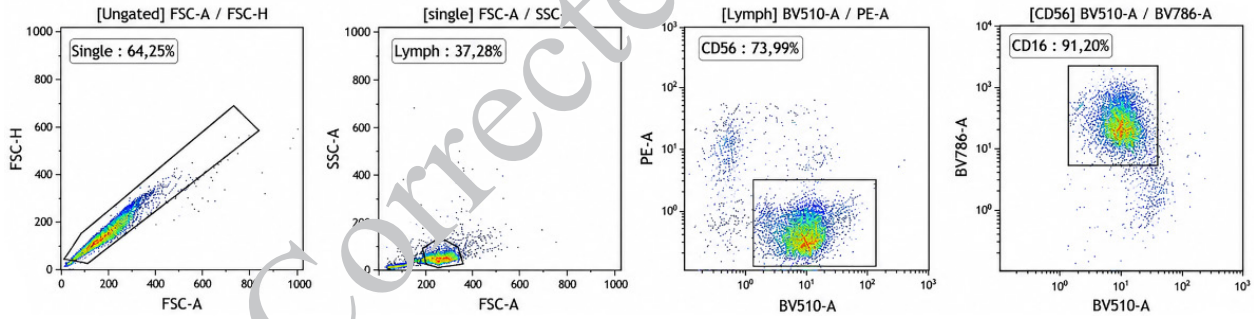


Figure 1. Flow cytometric identification and purity assessment of CD56⁺CD16⁺ natural killer (NK) cells
After gating on single cells (64.25%) and lymphocytes (37.28%), 73.99% of lymphocytes expressed CD56. Among CD56⁺ cells, 91.20% expressed CD16. The final purity of CD56⁺CD16⁺ cells was 91.20%.
NK: Natural killer

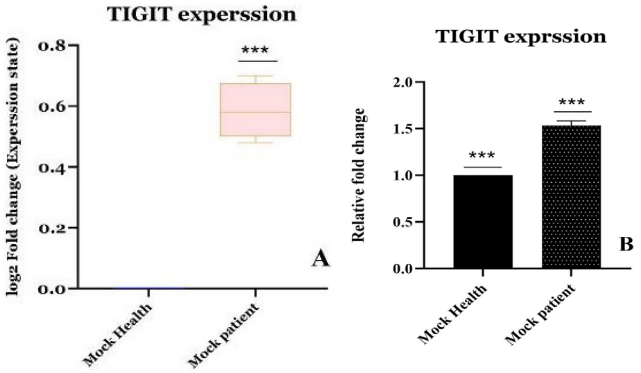


Figure 2. Comparison of baseline TIGIT expression in NK cells derived from healthy donors and patients
(A) Log_2 fold change of TIGIT expression comparing Mock Patient vs Mock Health. (B) Relative TIGIT expression normalized to Mock Health. Bars represent the mean±SD of 3 independent donors per group. **P*=0.027.
TIGIT: T-cell immunoreceptor with Ig and ITIM domains; NK: Natural killer

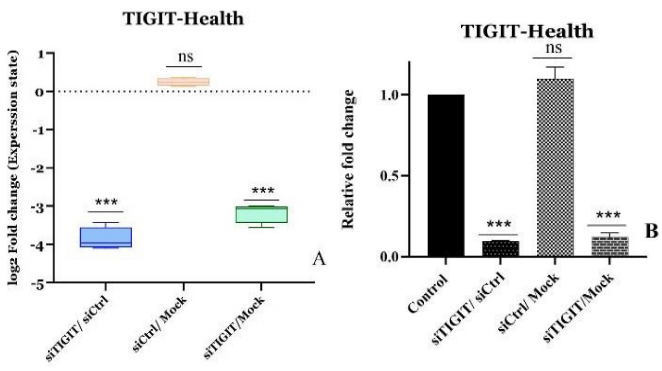


Figure 3. Evaluation of TIGIT knockdown efficiency in NK cells derived from healthy donors
(A) Log_2 fold change of TIGIT expression for the three indicated comparisons (siTIGIT/siCtrl, siCtrl/Mock, siTIGIT/Mock). (B) Relative TIGIT expression normalized to siCtrl. Data represent the mean±SD of 3 independent donors. ****P*<0.001; ns, not significant
TIGIT: T-cell immunoreceptor with Ig and ITIM domains; NK: Natural killer

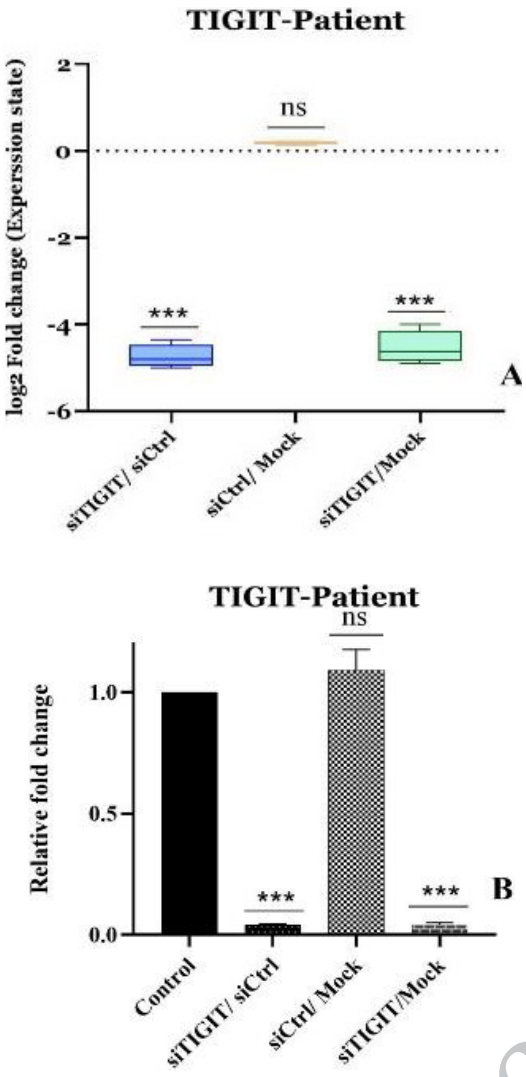


Figure 4. Evaluation of TIGIT knockdown efficiency in NK cells derived from patients

(A) Log_2 fold change of TIGIT expression for the three indicated comparisons (siTIGIT/siCtrl, siCtrl/Mock, siTIGIT/Mock). (B) Relative TIGIT expression normalized to siCtrl. Data represent the mean±SD of independent donors. ***P<0.001; ns, not significant.

TIGIT: T-cell immunoreceptor with Ig and ITIM domains; NK: Natural killer

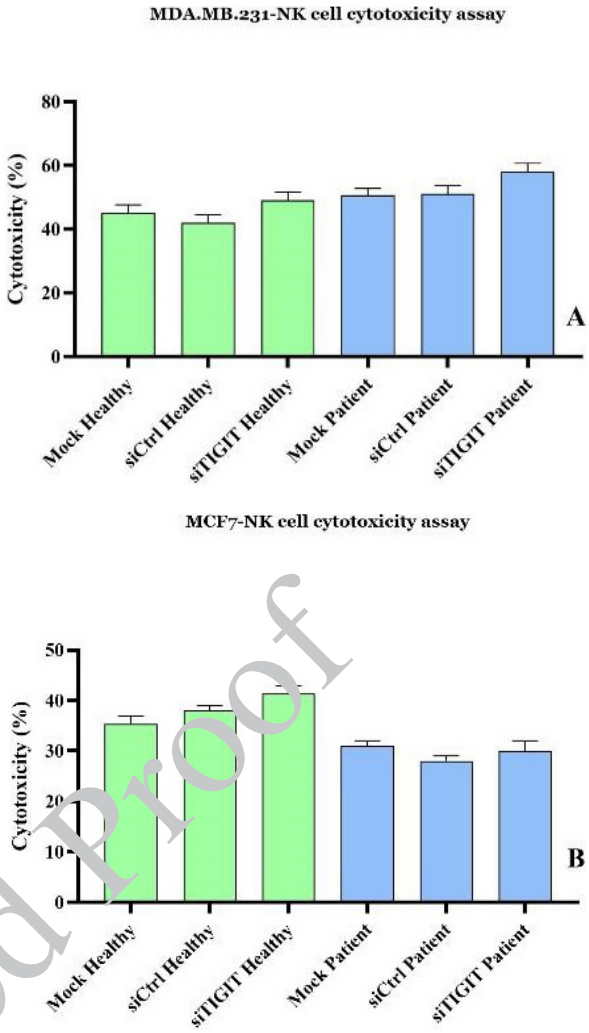


Figure 5. Effects of TIGIT knockdown on NK cell-mediated cytotoxicity

NK cells from healthy donors (H) and breast cancer patients (P) were transfected with siCtrl, siTIGIT, or left untreated (Mock). Cytotoxic activity against (A) MDA-MB-231 and (B) MCF-7 cells was measured after 24 hr co-culture using the AlamarBlue assay. Bars represent the mean±SD of technical triplicates (n=3 technical replicates). (A) Against MDA-MB-231 cells, TIGIT silencing was associated with a 9 percentage-point increase in cytotoxicity in healthy donor NK cells (from 41% to 50%) and a 7 percentage-point increase in patient-derived NK cells (from 51% to 58%). (B) Against MCF-7 cells, only modest increases were observed (healthy: from 37.5% to 41%; patient: from 28% to 31%). All cytotoxicity values represent the mean±SD of technical triplicates from a single donor (n=1 biological replicate) per group and are presented as descriptive estimates only.

TIGIT: T-cell immunoreceptor with Ig and ITIM domains; NK: Natural killer

vs siCtrl 28%), confirming that neither the transfection procedure nor the scrambled siRNA altered baseline NK cell function. The siCtrl group was therefore used as the reference condition for all subsequent comparisons with siTIGIT. Against MDA-MB-231 cells, TIGIT silencing was associated with a 9 percentage-point increase in cytotoxicity in healthy donor NK cells (from 41% to 50%) and a 7 percentage-point increase in patient-derived NK cells (from 51% to 58%). Against MCF-7 cells, only modest increases were observed in both groups (healthy: from 37.5% to 41%; patient: from 28% to 31%). Because these assays were performed using NK cells from a single donor per group, the observed differences are reported as descriptive estimates of effect size. They are not subjected to formal statistical inference. Patient NK cells were more active than healthy NK cells across all conditions, and MDA-MB-231 cells were more susceptible to NK-mediated killing than MCF-7 cells at baseline (Figures 5A and 5B).

Discussion

Three main findings emerge from this study. siRNA-mediated TIGIT knockdown proved technically feasible in primary NK cells from both healthy individuals and treatment-naïve breast cancer patients. TIGIT silencing enhanced cytotoxicity selectively against MDA-MB-231 cells, whereas responses against MCF-7 cells remained largely unchanged. Taken together, these observations point to a subtype-dependent role for TIGIT in restricting NK cell function in breast cancer. Patient-derived NK cells showed 1.5-fold higher baseline TIGIT expression than cells from healthy donors (P=0.027), a finding that aligns with earlier work linking TIGIT up-regulation in breast cancer to poor clinical outcomes (19–21). Sustained exposure to CD155 and CD112 on tumor cells likely drives this elevation by

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maintaining TIGIT signaling through SHP-1 and SHP-2 (14,18); at the same time, TIGIT competes with CD226 for the same ligands, progressively tilting the receptor balance toward inhibition (18). Importantly, our observation that patient NK cells, despite higher inhibitory receptor levels, remained responsive to *ex vivo* IL-2 stimulation suggests that these cells may not be terminally exhausted; rather, they could be primed for functional restoration upon appropriate immunomodulatory intervention. Against this backdrop, the 90.7–96.5% mRNA knockdown achieved here is notable. By eliminating the mRNA template, siTIGIT halts de novo protein production (25,26) — an effect that may carry particular weight in a tumor microenvironment where TIGIT signaling is continuously engaged. The most striking finding was the divergent responses of MDA-MB-231 and MCF-7 cells. MDA-MB-231 cells carry substantially higher CD155 and CD112 than MCF-7 cells (16, 27). This ligand-density-dependent pattern mirrors findings in ovarian cancer, where siRNA-mediated knockdown of CD155, but not CD112, on tumor cells significantly reduced NK cell reactivity, confirming CD155 as the dominant TIGIT/DNAM-1 ligand governing NK cell responsiveness (31). When TIGIT is the dominant brake on NK cell activation — as it appears to be against CD155-high targets — removing it shifts the balance decisively toward killing. For MCF-7 cells, where CD155/CD112 density is lower, TIGIT is unlikely to be the rate-limiting inhibitory signal; other checkpoints presumably govern the interaction instead. This interpretation is consistent with the principle of checkpoint hierarchy (32), which holds that therapeutic benefit depends on targeting whichever receptor actually dominates the inhibitory tone in a given effector–target pair. For NK cells facing MCF-7, KIR–MHC-I interactions and the PD-1/PD-L1 axis are plausible candidates for that dominant role, and both remain intact after TIGIT knockdown (32, 33). Notably, although the two target cell lines were maintained in different media prior to the assay, all co-culture experiments were performed in a single uniform medium (RPMI-1640 supplemented with 10% FBS) after the cells had been detached, washed, and counted. This procedural standardization minimizes the likelihood that the different maintenance media influenced the observed differences in NK cell cytotoxicity. These data also bear on a practical question raised by the failure of tiragolumab in phase III trials: under what conditions does TIGIT blockade actually work (23)? Antibody-based strategies face at least three obstacles — incomplete receptor occupancy, Fc-mediated elimination of TIGIT-expressing effector cells, and unpredictable variation in tumor CD155 levels (23). None of these apply to the siRNA approach. There is no Fc domain, knockdown operates upstream of receptor recycling, and the selectivity we observed between CD155-high and CD155-low targets fits a ligand-density model of checkpoint regulation rather neatly. Measuring CD155 on tumor biopsies could therefore prove useful as a selection criterion for future trials of TIGIT-directed NK cell therapy. The superior cytotoxic activity of patient-derived NK cells relative to healthy-donor cells across all conditions deserves comment. At first glance, this seems paradoxical — chronically tumor-exposed NK cells are generally expected to be functionally impaired. However, several non-mutually exclusive mechanisms may explain this apparent contradiction. First, *ex vivo* IL-2 stimulation at 500 IU/ml may preferentially rescue cytotoxic function in tumor-primed NK cells, a pattern documented in exhaustion-reversal settings where brief cytokine stimulation restores effector capacity more efficiently in previously activated cells than in naive ones (34,35). Second, breast cancer patients may harbor an expanded pool of adaptive NK cells (CD56dimCD57+CD16+) that arise following cytomegalovirus exposure or chronic antigen stimulation; these cells display enhanced antibody-dependent cellular cytotoxicity and are less susceptible to tumor microenvironment-mediated suppression than conventional NK cells (36, 37). Third, cytotoxicity assays used NK cells from a single donor per group, and the observed difference may partly reflect inter-individual variation rather than a systematic patient-versus-donor effect. Resolving this question will require subset-level phenotyping — including CD57, NKG2C, and CD16 — alongside biological replication across multiple donors in future work. For luminal breast cancer, TIGIT silencing alone appears insufficient. This is not entirely surprising. Luminal tumors are inherently less immunogenic, and removing a single checkpoint creates space for compensatory inhibitory pathways to fill (32). Several combination strategies could address this. Simultaneous knockdown of TIGIT and PD-1 or TIM-3 — both elevated on exhausted NK cells in breast tumors (12, 33) — would attack the problem from multiple angles. CDK4/6 inhibitors, which enhance tumor immunogenicity in breast cancer models (38), offer a pharmacological route to making MCF-7-like targets more susceptible. CAR-NK cells co-expressing tumor-targeting receptors such as anti-HER2 alongside TIGIT knockdown represent a more ambitious but mechanistically coherent approach, providing a strong activating signal that may overcome checkpoint compensation (39, 40). The broader feasibility of such strategies is supported by advances in off-the-shelf allogeneic NK cell platforms, which could facilitate scalable clinical translation (41). Genetic disruption of TIGIT in NK cells has also shown promise in preclinical work, most notably a recent study in which CRISPR-Cas9-mediated TIGIT knockout in *ex vivo* expanded human NK cells significantly increased cytotoxicity against multiple cancer cell lines (18, 42). Notably, another study using simultaneous CRISPR-Cas9 TIGIT knockout and EGFR-CAR transgenesis reported a contrasting result in an MDA-MB-231 intraperitoneal xenograft model, where TIGIT knockout did not improve tumor control (43), a discrepancy attributable to differences between CAR-driven and endogenous NK-cell signaling; our *in vitro* findings with non-engineered primary NK cells against MDA-MB-231 cells are therefore not directly comparable to that *in vivo* CAR-NK setting. Moreover, even advanced CAR-NK designs, including those with dual-engine configurations (44), face challenges such as persistence, trafficking, and antigen escape in solid tumors. Regarding methodological limitations, cytotoxicity assays used NK cells from a single donor per group—a practical necessity given the challenge of expanding primary NK cells from multiple donors in parallel. By contrast, qRT-PCR data incorporated all three donors per group and therefore offer a more robust picture of TIGIT expression. Cytotoxicity results are accordingly treated as exploratory throughout. NK cell viability after transfection was not formally measured; however, the near-identical TIGIT expression and killing activity in mock-transfected and scrambled siRNA groups suggest that

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RNAiMAX did not appreciably compromise NK cell viability or function under the conditions used.

A broader caution is also warranted. Immune checkpoint pathways serve dual purposes—suppressing tumor immunity and preventing autoimmunity. Disrupting them, even briefly and locally, carries a risk of off-target immune activation. The results reported here are early-stage and require considerably more work before any clinical translation can be seriously considered.

Several limitations warrant acknowledgment. First, TIGIT knockdown was assessed only at the mRNA level by qRT-PCR, and changes in TIGIT protein expression were not confirmed by flow cytometry or Western blotting. Second, cytotoxicity assays were performed using NK cells from only one donor per group; therefore, these findings should be considered preliminary and require confirmation in studies involving multiple independent donors. Finally, although the AlamarBlue assay provided an estimate of cancer cell viability, we did not use Annexin V/PI or another complementary assay to characterize cell death directly. Future studies should address these limitations by including protein-level validation, multiple independent donors, and complementary assays to more directly confirm NK cell-mediated killing.

Conclusion

TNBC remains one of the harder problems in breast cancer oncology, and new therapeutic options are sorely needed. This study shows that siRNA-mediated silencing of TIGIT in primary human NK cells is achievable and produces a measurable gain in cytotoxicity against MDA-MB-231 cells. The contrasting outcome in MCF-7 cells suggests that TIGIT's functional importance as an inhibitory checkpoint is tied to the target's CD155/CD112 expression profile. This dependency could help guide patient selection in future TIGIT-directed immunotherapy. That said, the findings carry real limitations: small donor numbers, cytotoxicity data from a single donor per group, and the absence of protein-level confirmation of knockdown. Larger studies with rigorous biological replication ($n \geq 10$ per group), western blot or flow cytometric verification of TIGIT protein loss, and *in vivo* validation are needed before the approach can move forward with confidence. Whether combining TIGIT silencing with blockade of PD-1, TIM-3, or other co-inhibitory receptors can extend efficacy to luminal disease is a question worth pursuing.

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Ethical Approval and Study Design

This study employed primary human NK cells to assess the technical feasibility of siRNA-mediated TIGIT silencing and to generate preliminary data for future, larger investigations. Blood was collected from three treatment-naïve breast cancer patients (age range 39–47 years) and three age- and sex-matched healthy donors. All participants provided written informed consent. The ethics committee at Shahrekord University of Medical Sciences, Iran, approved the study (Code: IR.SKUMS.REC.1402.041). The work followed the Declaration of Helsinki.

Authors' Contributions

Z BR conceived the study, conducted the experiments, collected the data, and wrote the original draft. N B designed and optimized the qRT-PCR assay, interpreted the data, and reviewed and edited the manuscript. F G performed siRNA transfection experiments and maintained breast cancer cell lines for co-culture assays. H Y conducted AlamarBlue cytotoxicity assays and contributed to data interpretation. M GS performed statistical analysis, prepared figures, and edited the manuscript. K A supervised the study, designed the study, reviewed the manuscript, and served as corresponding author. A S performed flow cytometry analysis, isolated N K cells, reviewed the manuscript, and served as co-corresponding author. All authors read and approved the final version.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

Two large language models, Claude Sonnet 5 (Anthropic) and DeepSeek-V4 (DeepSeek, Hangzhou, China), were used solely for grammar and language editing. They were not used to generate data, run statistics, interpret results, or draw conclusions. All scientific work is the authors' own. The final manuscript was reviewed and approved by all co-authors before submission.

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