

Cell-Intrinsic MHC-I Expression Sustains NK Cell Effector Function and Its Loss Drives Exhaustion in Triple-Negative Breast Cancer

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Abstract. Triple-negative breast cancer (TNBC), characterized by lack of targetable receptors and an immunosuppressive tumor microenvironment (TME), remains one of the most aggressive breast cancer subtypes with poor prognosis despite emerging immunotherapies. While adaptive immunity has been extensively studied, the role of innate immunity, particularly natural killer (NK) cells, is increasingly recognized. Although NK cells conventionally eliminate MHC-I-deficient tumor cells via "missing-self" recognition, their phenotype in TNBC TME is often compromised, with mechanisms remaining unclear. Here, we combined single-cell RNA sequencing analysis of human TNBC samples with functional in vitro assays to investigate tumor-infiltrating NK cells. Our analysis revealed that TNBC epithelial cells exhibit enhanced MHC-I expression compared to normal breast tissue. However, TNBC-infiltrating NK cells showed high expression of both effector molecules (GNLY, NKG7, PRF1) and inhibitory/exhaustion markers (HAVCR2, LAIR1, TGFB1), suggesting a chronically activated but dysfunctional state. To explore MHC-I function, we established an in vitro model knocking down B2M and HLA-A in NK-92 cells using siRNA. Results demonstrated that MHC-I downregulation does not affect NK cell proliferation but negatively regulates effector function, resulting in reduced degranulation (CD107a), cytokine production (IFN- γ , Granzyme B), and target cell killing. These findings reveal a previously unrecognized cell-autonomous role of MHC-I in sustaining NK cell functionality. Our study identifies NK cell-intrinsic MHC-I expression as a critical regulator of anti-tumor immunity and proposes MHC-I upregulation on NK cells as a potential next-generation immunotherapy strategy for immune checkpoint-resistant TNBC.

Keywords: Triple-negative breast cancer (TNBC), Natural killer (NK) cells, MHC class I, B2M, HLA-A, Immune exhaustion, Tumor microenvironment, Immunotherapy, Cell-intrinsic immunity, Single-cell RNA sequencing.

1. Introduction

Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer characterized by its lack of targetable hormone receptors, including estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2), as well as a high propensity for metastasis [1]. The lack of effective receptor targets in TNBC has led to limited chemotherapeutic agents as well as a poor prognosis [2]. This underscores the urgent need for novel therapeutic strategies, particularly those leveraging the immune system.

Immunotherapies, especially immune checkpoint inhibitors (ICIs), have demonstrated a promising efficiency in cancer treatment through reinvigorating adaptive T cell responses [3]. However, its success in TNBC has been modest and heterogeneous, potentially due to over-reliance on adaptive immunity and underlooking critical innate immune populations such as natural killer cells (NKs) [4]. NK cells are potent cytotoxic lymphocytes with a killer cell immunoglobulin-like receptor (KIR) on their surface, functioning as a classical MHC-I inhibitory receptor. Through a process called "missing-self recognition", NK cells will be activated and eliminate malignant cells with the downregulation of major histocompatibility complex class I (MHC-I) on tumor surface [5]. Therefore, in theory, the pervasive MHC-I loss observed in TNBC should render them more vulnerable to NK cell attack.

Paradoxically, however, NK cells within the TNBC tumor microenvironment (TME) often express a dysfunctional phenotype, characterized by impaired cytotoxicity and increased expression of inhibitory markers such as PD-1, TIGIT, and LAG-3 [6].

Despite the clinical relevance, the transcriptional architecture governing NK cell exhaustion in TNBC remains poorly defined. Most single-cell characterizations have focused on T cells, leaving a major gap in understanding the correlation between tumor intrinsic-MHC I deficiency and NK cell metabolism or function. Specifically, it remains unclear whether chronic MHC I deficiency induces NK cell exhaustion that overrides the initial “missing-self” activation in TNBC cells.

In this study, we sought to resolve this paradox by integrating high-resolution single-cell transcriptomic profiling with functional validation. Based on analysis of exhaustion-associated single-cell transcriptomic data of NK cells in TNBC TME, we identified key regulatory nodes associated with diminished effector functions. To mechanistically link tumor phenotype to NK dysfunction, an in vitro siRNA knockdown model was performed targeting MHC-I components, B2M, and HLA-A. Our findings reveal that while acute MHC-I loss triggers NK activity, sustained exposure to MHC-I-deficient TNBC cells drives a distinct transcriptional exhaustion program, influencing NK cell proliferation and cytotoxic capacity. These findings highlight the previously overlooked aspects of innate immune regulation in TNBC. Although current immunotherapy strategies mainly target adaptive T-cell responses, our research turns its attention to NK cells, whose functional status in TNBC remains insufficiently characterized. By describing the relationship between the intrinsic MHC-I dynamics of tumors and NK cell dysfunction, this work addresses a key gap in this field and provides a conceptual framework for incorporating NK orientation strategies into future TNBC immunotherapy.

2. Materials and Methods

2.1. scRNA-seq Data Acquisition and Curation

Publicly available single-cell RNA sequencing (scRNA-seq) data from human breast tissue were obtained from the NCBI Gene Expression Omnibus (GEO) repository (accession number GSE161529). It contains 52 samples, encompassing a total of 421,761 cells across diverse breast tissue conditions. For the analysis, a curated cohort of 18 samples were included, including normal samples from premenopausal (n=3) and postmenopausal (n=3) donors, BRCA1-associated preneoplastic samples (n=4), BRCA1-associated triple-negative tumors (n=4), and sporadic triple-negative breast tumors (n=3). Detailed sample metadata, including patient identifiers, menopause status, and initial/post-filtering cell counts, are provided (Table 1). All downstream analyses were performed using the R (v.4.5.0) and the Seurat package (v.5.0.0) [7].

Table 1. Cohort Description and Quality Control Metrics

Sample ID	Patient	Description	Menopause	Gender	Number of cells	Number of Cells After Filtering
GSM4909253	0092	Normal tissue, total cells	Pre	Female	4966	3194
GSM4909254	0019	Normal tissue, total cells	Pre	Female	7130	5382
GSM4909265	0233	Normal tissue, total cells	Pre	Female	5665	4035
GSM4909270	0342	Normal tissue, epithelial cells	Post	Female	7856	6205
GSM4909272	0021	Normal tissue, total cells	Post	Female	2463	535
GSM4909276	0372	Normal tissue, total cells	Post	Female	4825	3120
GSM4909277	0894	Normal BRCA1+/- pre-neoplastic, total cells	NA	Female	7850	4964
GSM4909278	0033	Normal BRCA1+/- pre-neoplastic, total cells	NA	Female	6827	2665
GSM4909279	0023	Normal BRCA1+/- pre-neoplastic, total cells	NA	Female	5932	2347
GSM4909280	0090	Normal BRCA1+/- pre-neoplastic, total cells	NA	Female	5663	3408
GSM4909282	0135	Triple negative tumor, total cells	NA	Female	15870	13710
GSM4909283	0106	Triple negative tumor, total cells	NA	Female	1065	714
GSM4909284	0114	Triple negative tumor, total cells	NA	Female	2015	874
GSM4909285	4031	Triple negative tumor (BRCA1), total cells	NA	Female	5581	5056
GSM4909286	0131	Triple negative tumor (BRCA1), total cells	NA	Female	6456	4720
GSM4909287	0554	Triple negative tumor (BRCA1), total cells	NA	Female	9593	2881
GSM4909288	0177	Triple negative tumor (BRCA1), total cells	NA	Female	21130	7212

2.2. Data Preprocessing and Quality Control

The raw gene expression matrices were loaded and merged into a unified Seurat object. Quality control filtering was applied to remove low quality cells, excluding cells expressing fewer than 1,000 or more than 6,000 detected genes, as well as cells with mitochondrial gene expression exceeding 20% of total transcripts. Gene expression data were also normalized using Seurat's default normalization method. Highly variable genes were identified using the variance-stabilizing transformation (VST) method, with the top 3,000 variable features selected for subsequent analyses. Data were scaled with ScaleData using the variable features. Principal component analysis (PCA) was performed on the scaled data, and the first 20 principal components were used for graph-based clustering and for uniform manifold approximation and projection (UMAP) visualization (RunUMAP). A preliminary clustering resolution of 0.2 was applied to explore broad cell populations.

2.3. Cell Type Annotation

To distinguish epithelial from immune cells, module scores were calculated for epithelial markers (EPCAM, KRT8, KRT18, KRT19, KRT5, KRT14) and for the pan-immune marker PTPRC (CD45). Cells with an epithelial score above the 80th percentile and a CD45 score below the 80th percentile were classified as epithelial. Within the breast cancer epithelial cells, a subset with high proliferation (cycling score based on MKI67, TOP2A, STMN1, CENPF, CDK1, AURKB, PCNA, BIRC5, TYMS, UBE2C) or high epithelial-mesenchymal transition (EMT) signature (VIM, FN1, SNAI1, SNAI2, ZEB1, ZEB2, ITGA6, ITGB4, LGALS3, ITGA3, ITGA2) was further designated as “tumor-like epithelial” cells. The cycling and EMT scores were computed using AddModuleScore with thresholds at the 80th percentile of the respective scores among breast cancer epithelial cells.

2.4. NK cell identification and Subclustering of breast cancer NK cells

Natural killer (NK) cells were identified based on the expression of canonical markers: cells positive for NKG7, GNLY, or PRF1, and negative for T-cell markers TRAC and CD3D. Breast cancer NK cells were re-analyzed separately. Marker genes for each resulting subcluster were identified using the Wilcoxon rank-sum test (FindAllMarkers) with parameters: only positive markers, minimum fraction of cells expressing the gene in either population of 0.25, and log-fold-change threshold of 0.25. The top 10 markers per cluster (by average log₂ fold change) were visualized in a dot plot.

2.5. NK cell functional scoring

Exhaustion and cytotoxicity module scores were computed for all NK cells (from both cancer and normal groups) using AddModuleScore. The exhaustion signature included PDCD1, TIGIT, LAG3, HAVCR2, CTLA4, and TOX. The cytotoxicity signature included NKG7, GNLY, PRF1, GZMB, GZMH, IFNG, XCL1, and XCL2. For a more comprehensive assessment, linear regression analysis was performed and visualized, comprising additional inhibitory/checkpoint genes such as ENTPD1, TNFRSF9, KLRG1, KLRC1, LAIR1, BTLA, CD96, and others. A core cytotoxic panel (IFNG, GZMB, GZMH, PRF1, GNLY, NKG7, XCL1, XCL2, LAMP1) was used to compute an alternative cytotoxicity score. Differences between groups (breast cancer vs. normal, and TN vs. normal premenopausal) were visualized with violin plots and dot plots.

2.6. Comparative Analyses

A subset analysis was performed comparing triple-negative (TN) tumor samples with normal premenopausal samples. NK exhaustion and cytotoxicity scores were compared between groups. MHC class I expression in epithelial cells was evaluated in normal epithelial cells and tumor-like epithelial cells. Per-sample mean expression values were calculated for downstream correlation analyses. An expanded comparison was conducted between all breast cancer samples (including BRCA1-associated and sporadic TN tumors) and normal samples (premenopausal and

postmenopausal). NK functional scores and epithelial MHC class I expression were evaluated using the same preprocessing and gating strategy.

2.7. MHC class I expression in epithelial cells

Expression of MHC class I genes (B2M and HLA-A) was examined in normal epithelial cells and tumor-like epithelial cells from breast cancer samples. Average expression levels were summarized per sample and visualized using violin plots.

2.8. Correlation between epithelial MHC I and NK cell states

For breast cancer samples, the per-sample mean expression of B2M in tumor-like epithelial cells was correlated with the per-sample mean exhaustion and cytotoxicity scores of cancer-infiltrating NK cells. Pearson correlation analysis was performed, and results were visualized using scatter plots with linear regression lines.

2.9. Cell Culture and Reagent

The human natural killer cell line (NK-92) derived from malignant non-Hodgkin's lymphoma and the chronic myelogenous leukemia cell line K-562 were obtained from Procell Life Science & Technology (NK-92: CL-0530; K-562:CL-0130). NK-92 cells were cultured in NK-92 Cell Specialized Medium (Procell, CM-0530), and K-562 cells were maintained in K-562 Cell Specialized Medium (Procell, CM-0130). All cells were cultured at 37 °C in a humidified incubator with 5% CO₂. Lipofectamine™ 3000 Transfection Reagent (Invitrogen, L3000015) was used for all transfection experiments.

2.10. B2M and HLA-A Knockdown in NK-92 Cells

NK-92 cells were transfected with small interfering RNAs (siRNAs) targeting human B2M or HLA-A. For each gene, three individual siRNA duplexes were initially screened for knockdown efficiency via qPCR and western blot 48 hours post-transfection. The most efficient siRNA for each target was selected for subsequent experiments. A non-targeting control siRNA (siNT) was used as a negative control. 50nM of siRNAs were transfected using Lipofectamine™ 3000 Transfection Reagent according to the manufacturer's instructions. The target sequences of all siRNAs used in this study are provided in Table 2.

Table 2. siRNA sequences for B2M and HLA-A knockdown

siRNA	Sequence
siHLA-1	Sense Strand: 5'GGAAGAGCUCAGAUAGAAATT 3' Antisense Strand: 5'UUUCUAUCUGAGCUCUUCCTT 3'
siHLA-2	Sense Strand: 5'GCUACUACAACCAGAGCGATT 3' Antisense Strand: 5'UCGCUCUGGUUGUAGUAGCTT 3'
siHLA-3	Sense Strand: 5'GGGAUGGAACCUUCCAGAATT 3' Antisense Strand: 5'UUCUGGAAGGUUCCAUCCTT 3'
siB2M-1	Sense Strand: 5'GCAGAGAAUGGAAAGUCAATT 3' Antisense Strand: 5'UUGACUUCCAUCUCUGCTT 3'
siB2M-2	Sense Strand: 5'CACAGCCCAAGAUAGUUAATT 3' Antisense Strand: 5'UUAACUAUCUUGGGCUGUGTT 3'
siB2M-3	Sense Strand: 5'CGACAUUGAAGUUGACUUATT 3' Antisense Strand: 5'UAAGUCAACUCAAUGUCGTT 3'
siRNA negative control	Sense Strand: 5'UUCUCCGAACGUGUCACGUTT 3' Antisense Strand: 5'ACGUGACACGUUCGGAGAATT 3'
siRNA Human GAPDH	Sense Strand: 5'GUAUGACAACAGCCUCAAGTT 3' Antisense Strand: 5'CUUGAGGCUGUUGUCAUACTT 3'

2.11. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from NK-92 cells using Trizol™ reagent (Invitrogen, 10296028) according to the manufacturer's instructions. RNA concentration and purity were determined using a

NanoDrop spectrophotometer (NanoDrop Lite, Thermo Scientific). Purity was accepted when the A260/A280 ratio was between 1.8 and 2.0.

Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the SuperScript™ III Reverse Transcriptase Kit (Invitrogen, 11752050) with random hexamers, following the manufacturer's protocol. Quantitative real-time PCR was performed on a StepOne™ Real-Time PCR System (Applied Biosystems) using PowerUP™ SYBE™ Green Master Mix according to the thermocycling conditions in Table 3. Relative gene expression was calculated using the formula: $RQ=2^{-(\Delta\Delta Ct)}$ method, normalized to GAPDH. All reactions were performed in technical triplicate.

Table 3. Thermocycling conditions for qPT-PCR

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Degeneration	95°C	10 sec	40
Annealing	58°C	20 sec	40
Extention	72°C	20 sec	40
Melting Curve	95°C	15 sec	1
	60°C	60 sec	
	95°C	15 sec	

2.12. Western Blot Analysis

NK-92 cells were harvested, washed once with ice-cold PBS, and lysed in RIPA buffer (Beyotime, P0013b) supplemented with protease inhibitor cocktail (Roche, 04693116001) for 30 minutes on ice with intermittent vortexing. Lysates were centrifuged at 12,000 rpm for 15 minutes at 4°C, and the supernatant was collected. Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Scientific, 23225) according to the manufacturer's instructions. For electrophoresis, equal amounts of protein (20-30 µg) were mixed with 5×SDS loading buffer and denatured at 95°C for 10 min before immediately cooled on ice. Proteins were separated on SDS-PAGE gels under constant voltage (80 V through stacking gel migration; 120V through the resolving gel) using an electrophoresis apparatus (BayGene, BG-subMIDI).

Proteins were then transferred onto 0.22 µm PVDF membranes (Millipore, ISEQ00010). PVDF membranes were activated in methanol for 2 to 3 min, rinsed briefly in the transfer buffer, and assembled into a wet transfer system at a constant voltage of 65 V for 2 h at 4°C.

After transfer, membranes were blocked in 5% non-fat milk in TBST (Tris-buffered saline containing 0.1% Tween-20) at room temperature for 1 hour. Membranes were then incubated overnight at 4°C with the following primary antibodies diluted in blocking buffer (see Table 4):

Table 4. Antibody type, item number and usage method

Target Protein	Antibody	Catalog No.	Dilution
HLA class I	Mouse monoclonal	Proteintech, 66013-1-Ig	1:2000
Beta-2-Microglobulin	Rabbit polyclonal	Proteintech, 13511-1-AP	1:1000
Beta-actin	Rabbit polyclonal	Proteintech, 20536-1-AP	1:5000

After primary antibody incubation, membranes were washed three times with 1× TBST for 10 min each at room temperature. Membranes were then incubated with HRP-secondary antibodies for 1 hour at room temperature:

1. HRP-conjugated goat anti-rabbit IgG (Servicebio, C030212; 1:5000)
2. HRP-conjugated goat anti-mouse IgG (Servicebio, C030205; 1:5000)

Membranes were washed three times with 1×TBST to remove unbound secondary antibodies. The protein bands were visualized using enhanced chemiluminescence (ECL) substrate (Millipore, WBKLS0500) and imaged with the ChemiDoc MP imaging system (Bio-Rad, 170-8280). Densitometric analysis was performed using software (NIH), with target protein expression normalized to beta-actin.

2.13. Cell Proliferation Assay

NK-92 cell proliferation was assessed using the CellTrace™ CFSE cell proliferation kit (Beyotime, C1031). At 48 hours post-transfection, NK-92 cells were harvested, labeled with CFSE according to the manufacturer's instructions, and reseeded. CFSE fluorescence intensity was analyzed by flow cytometry at 0, 24, 48, 72, and 96 hours. Proliferation was quantified by analyzing the dilution of CFSE fluorescence (decrease in Mean Fluorescence Intensity [MFI]) relative to the 0-hr time point. All experiments were performed in biological replicates.

2.14. NK-92 and K-562 Co-culture Assay

CFSE-labeled K-562 cells (Beyotime, C1031) were co-cultured with NK-92 cells at an effector-to-target (E:T) ratio of 5:1 (NK-92:K-562). For apoptosis analysis, co-cultures were incubated for 12 hours under various experimental groups prior to flow cytometric assessment. For the degranulation and cytokine production assay, co-cultures were incubated for 4 hours in the presence of anti-CD107a antibody. ABD GolgiPlug™ Protein Transport Inhibitor, Brefeldin A (BD Biosciences, 555029), was added for the final hour of incubation to inhibit cytokine secretion. Cells were then harvested for surface and intracellular staining.

2.15. Flow Cytometry Analysis

Single cell suspensions were collected, washed with phosphate-buffered saline (PBS) twice, and stained with the indicated fluorochrome-conjugated surface markers at 4°C for 30 minutes. For intracellular staining (IFN- γ , Granzyme B), cells were fixed and permeabilized following the apoptosis kit protocol or using a standard fixation/permeabilization buffer. The following antibodies were used: FITC anti-human CD56 (Elabscience, E-AB-F1270C; 1:200 dilution), PE anti-human CD107a (Elabscience, E-AB-F1149D; 1:100), PE anti-human IFN- γ (Elabscience, E-AB-F1196D; 1:100), and PE anti-human Granzyme B (BioLegend, 372207; 1:100). NK cell activation and cytotoxic function was assessed based on CD56⁺ population and measuring the percentage of CD107a⁺, IFN- γ ⁺, or Granzyme B⁺ cells.

Apoptosis of K-562 cells was assessed using an aAnnexinV-FITC/PI apoptosis detection kit (Sunbio, FXP018) following the manufacturer's instructions. Data was acquired on a BeamCyte-1026 flow cytometer (Becton Dickinson) and analyzed using FlowJo V10 software.

2.16. Statistical Analysis

All statistical analyses were performed using R (v4.5.0), Seurat (v5.0.0), and GraphPad Prism (v10.0). For in silico single-cell analyses, comparisons between normal and triple-negative (TN) breast cancer samples were conducted using the Wilcoxon rank-sum test as implemented in Seurat. Differential expression analyses were performed to compare B2M and HLA-A expression in epithelial cells, as well as exhaustion- and inhibition-associated genes in NK cells. P-values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) correction. Adjusted p-values (FDR) < 0.05 were considered statistically significant. For in vitro experiments, data are presented as mean $\pm$ standard deviation (SD) from three independent biological replicates. For experiments involving more than two groups (e.g., CD107a, IFN- γ , and Granzyme B flow cytometry analyses, as well as apoptosis assays in NK-92/K-562 co-culture systems), one-way analysis of variance (ANOVA) was applied for comparison, followed by Dunnett's multiple comparisons test to compare each treatment group against the control samples.

3. Results

3.1. Single-cell landscape of breast tissue and immune compartment profiling

To characterize the cellular composition of breast tissue across normal and malignant conditions, we analyzed single-cell RNA sequencing data from 18 curated samples derived from normal breast

tissue, BRCA1-associated preneoplastic tissue, BRCA1 associated TNBC and sporadic TNBC. After quality control filtering, cells were projected into a low-dimensional space using UMAP, revealing distinct clusters corresponding to major cellular compartments, including epithelial, immune, and stromal populations (Figure 1A). We next isolated immune cells from all samples and performed re-embedding analysis. The immune subset UMAP revealed clearly segregated immune populations across the combined cohort, demonstrating transcriptionally distinct immune cell states shared across normal and disease conditions, including T cells, NK cells, and myeloid cells (Figure 1B)

To further evaluate immune composition across clinical groups, immune subsets were visualized separately for each of the five patient categories. While immune populations were present in all conditions, their relative distribution and clustering density varied across groups (Figure 1C). Notably, TN tumor samples exhibited expanded immune representation compared to normal tissues, suggesting an altered immune composition associated with malignant progression. To quantitatively evaluate these differences, we compared immune cell composition across groups. Notably, NK cells accounted for more than 80% of immune cells in both normal premenopausal and postmenopausal tissues. In contrast, TN tumor, BRCA1-associated TNBC, and BRCA1-associated preneoplastic samples exhibited reduced NK cell representation, with NK proportions ranging from approximately 60–70% (Fig. 1D). This reduction was accompanied by a relative expansion of T cell and macrophage populations, indicating a profound remodeling of the immune microenvironment during TNBC progression and a shift in immune composition associated with malignant and high-risk states. Together, these data establish a comprehensive single-cell landscape of breast tissue and provide a framework for subsequent immune-focused analyses.

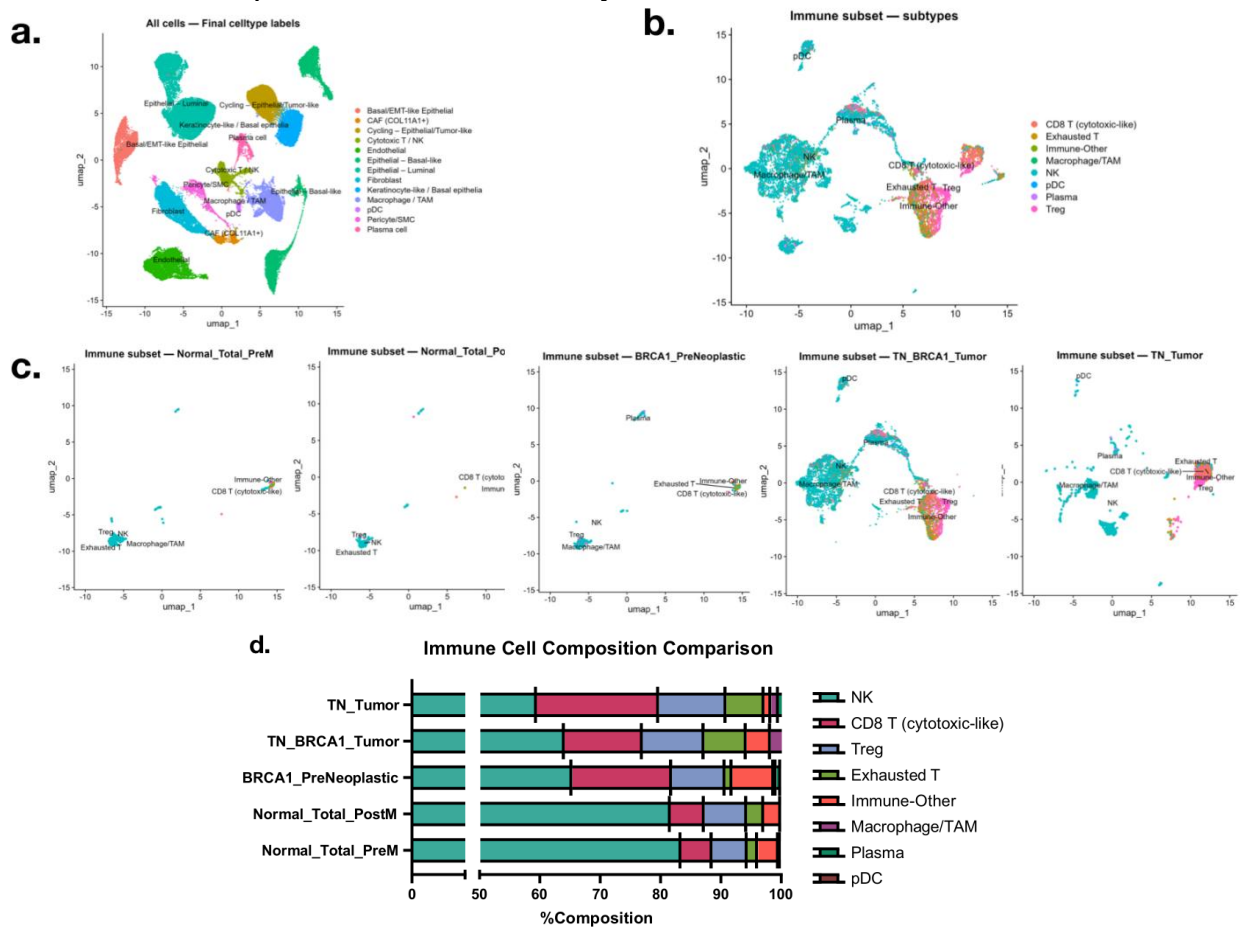


Figure 1. Single-cell landscape of human breast tissue and immune compartment profiling.

(A) UMAP visualization of 42,136 single cells from 18 curated breast samples derived from normal premenopausal, normal postmenopausal, BRCA1-associated preneoplastic, BRCA1-associated TNBC, and sporadic TNBC. Cells are color-coded by major lineage (epithelial, immune, stromal) following graph-based clustering analysis. (B) UMAP embedding of the immune subset

(n=10,284 cells) extracted from all samples and re-analyzed separately. Immune cells were identified based on canonical immune markers and subjected to dimensionality reduction and clustering to resolve transcriptionally distinct immune populations. (C) Immune subset UMAPs displayed separately by clinical group, including normal premenopausal, normal postmenopausal, BRCA1-associated preneoplastic, BRCA1-associated TNBC, and sporadic TNBC samples. Cells are shown using the same embedding space to facilitate cross-group comparison of immune distribution patterns. (D) Quantitative comparison of immune cell composition across clinical groups. Stacked bar plots show the relative proportions of major immune cell subsets—including NK cells, CD8+ T cells, regulatory T (Treg) cells, exhausted T cells, macrophage/tumor-associated macrophage (TAM), plasma cells, plasmacytoid dendritic cells (pDC), and other immune populations. Statistical comparisons of NK cell frequencies between groups were performed using a Wilcoxon rank-sum test (* $P < 0.05$, ** $P < 0.01$).

3.2. TNBC Epithelial Cells Display Elevated Expression of MHC Class I Machinery

To evaluate antigen presentation signatures in tumor epithelial cells, the expression of MHC class I genes (B2M and HLA-A) was compared between clinical groups. Using module scores, we classified malignant epithelial cells based on high proliferative and EMT signatures. A comparative analysis of MHC class I gene expression revealed a counterintuitive finding. Contrary to the classical model of immune evasion by MHC-I downregulation, epithelial cells from both sporadic and BRCA1-associated TNBC samples displayed significantly higher expression of both B2M and HLA-A compared to that of normal breast tissue (Figure 2A). To further refine this comparison, we specifically analyzed triple-negative (TN) tumors against normal premenopausal samples. Consistently, TN tumor epithelial cells exhibited elevated expression of B2M and HLA-A relative to normal premenopausal epithelial cells (Figure 2B). This result suggests that in TNBC, the tumor epithelium does not simply lose MHC-I to evade T cells but may instead engage in a more complex interaction with the immune system, potentially involving chronic stimulation of NK cells.

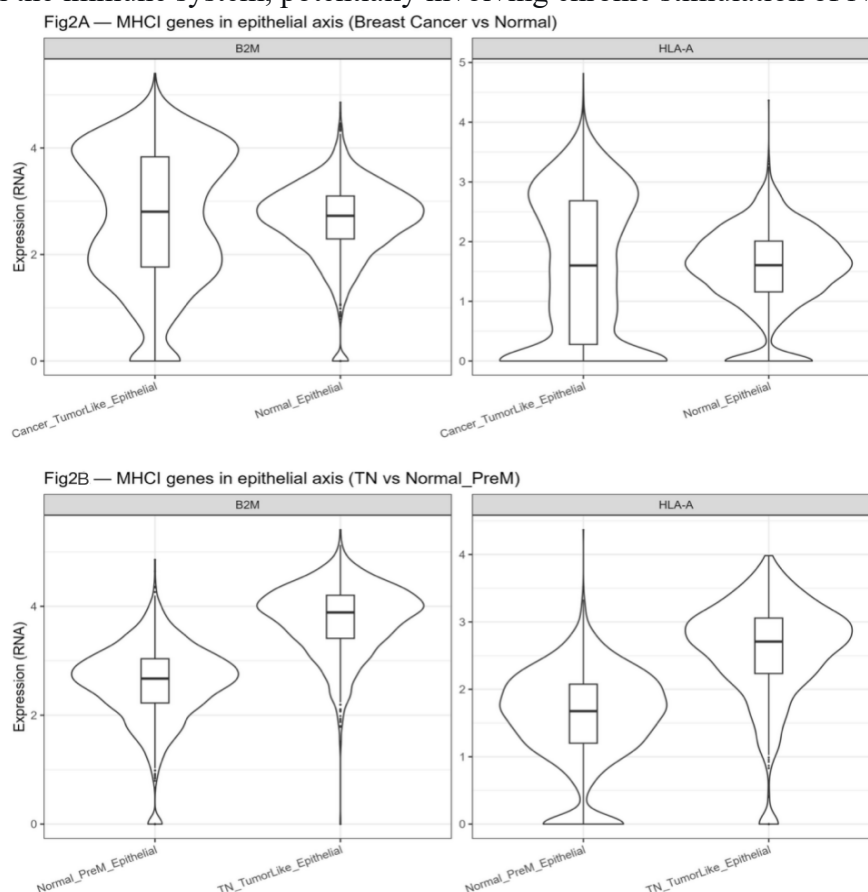


Figure 2. Increased Expression of MHC Class I Genes in TNBC Epithelial Cells.

(A) Violin plots showing the expression of B2M (left) and HLA-A (right) in epithelial cells from normal breast tissue (premenopausal and postmenopausal combined, $n = 6$ samples) versus all breast cancer samples (sporadic TNBC and BRCA1-associated TNBC combined, $n = 7$ samples). Each dot represents a single cell. Horizontal lines within violins indicate the median and quartiles. Statistical significance was assessed using the Wilcoxon rank-sum test with Benjamini-Hochberg correction ($FDR < 0.0001$ for both genes). (B) Violin plots comparing B2M (left) and HLA-A (right) expression in epithelial cells from normal premenopausal tissue ($n = 3$ samples) versus triple-negative (TN) tumor samples (sporadic and BRCA1-associated combined, $n = 7$ samples). Both MHC-I components are significantly elevated in TNBC epithelium relative to normal tissue. **** $FDR < 0.0001$, Wilcoxon rank-sum test.

3.3. NK Cells in the TNBC Microenvironment Exhibit a Distinct Transcriptional State Characterized by High Effector and High Inhibitory Signatures

To further investigate the functional landscape of NK cells across clinical groups, we isolated NK cells and examined the expression of canonical cytotoxic, activation, and inhibitory markers. UMAP visualization of NK cells colored by clinical group revealed broad distribution of NK populations across all conditions, including normal premenopausal, normal postmenopausal, BRCA1-associated preneoplastic, BRCA1-associated TNBC, and sporadic TNBC samples (Figure 3A). Overlaying key NK-associated genes onto the UMAP demonstrated heterogeneous expression patterns of cytotoxic effectors (NKG7, GNLY, PRF1, GZMH, GZMB, IFNG), proliferation marker MKI67, activating receptor FCGR3A, inhibitory/exhaustion-related marker HAVCR2, and additional NK lineage markers (KLRC1, KLRD1). Notably, FCGR3A and HAVCR2 exhibited increased expression frequency and higher average expression levels in TNBC-associated NK cells compared with NK cells from normal tissues, suggesting enrichment of receptor and inhibitory signatures within the tumor microenvironment (Figure 3A).

To quantitatively evaluate NK functional programs across clinical groups, module scores were calculated for cytotoxicity, receptor signaling, inhibitory, and proliferation signatures (Figure 3B). Cytotoxicity scores were uniformly low across all groups, including normal, preneoplastic, and TNBC samples. No marked differences in cytotoxic scores were observed between tumor and normal conditions. In contrast, receptor-associated scores were significantly increased in both sporadic TN tumors and BRCA1-associated TNBC samples compared with normal tissues. NK proliferation scores were modestly elevated in TN tumor and BRCA1-associated TNBC groups relative to other conditions.

To further characterize functional differences between normal and tumor-associated NK cells, we examined the expression of effector/inhibition-associated genes using dot plot visualization (Figure 3B, C). Compared with NK cells from normal tissues, breast cancer-associated NK cells exhibited higher expression frequency and increased average expression levels of key cytotoxic and degranulation-related markers, including GNLY, NKG7, LAMP1, TYROBP, and FCGR3A.

Concurrently, these cells displayed elevated expression of multiple inhibitory and regulatory genes, including HAVCR2, TGFB1, TNFRSF14, LAIR1, and IL10RA. This transcriptional pattern—high effector potential coupled with high inhibitory receptor expression—is a hallmark of chronically activated, yet functionally restrained, immune cell state, resembling T cell exhaustion. This suggests that NK cells in TNBC are not simply inactive but are highly activated counterbalanced by potent suppressive signals from the tumor microenvironment.

Importantly, these exhausted NK cells function in an MHC-I-rich tumor microenvironment, with inhibitory receptors (e.g., KIRs, NKG2A) actively engaging with tumor cell HLA molecules. This led us to hypothesize that the MHC-I expression level on the NK cell itself—a largely unexplored variable—might modulate its susceptibility to exhaustion and its overall effector function.

(a) UMAP visualization of NK cells (n = 6,821 cells) colored by clinical group, including normal premenopausal, normal postmenopausal, BRCA1-associated preneoplastic, BRCA1-associated TNBC, and sporadic TNBC samples. Feature plots display the expression of key NK-associated genes projected into the UMAP, including cytotoxic effectors (NKG7, GNLY, PRF1, IFNG, GZMH, GZMB), proliferation marker (MKI67), receptor-associated marker (FCGR3A), inhibitory/exhaustion-related marker (HAVCR2), and lineage markers (KLRC1, KLRD1). (B) Violin plots showing module scores for NK cytotoxicity, receptor signaling, inhibitory, and proliferation programs across the five clinical groups. Cytotoxicity scores are uniformly low across conditions. Receptor/activation and proliferation scores are elevated in sporadic TN tumors and BRCA1-associated TNBC samples relative to normal tissues, whereas inhibitory scores showed no marked differences between groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Wilcoxon rank-sum test. (C) Dot plot showing the expression of selected gene sets in NK cells from normal breast tissue (n = 6 samples) versus breast cancer tissue (sporadic and BRCA1-associated TNBC, n = 7 samples). Top: Effector and degranulation-associated genes (GNLY, NKG7, LAMP1, TYROBP, FCGR3A). Bottom: Exhaustion and immune checkpoint-related genes (HAVCR2, TGFB1, TNFRSF14, LAIR1, IL10RA, ENTPD1). Dot size represents the percentage of NK cells expressing the indicated gene, and color intensity reflects average expression level. Tumor-associated NK cells display increased frequency and intensity of both effector genes and multiple inhibitory receptors compared to normal NK cells, consistent with a “stressed” yet functionally restrained phenotype.

3.4. Tumor-intrinsic MHC-I deficiency directly drives NK cell dysfunction In Vitro

Our single-cell data raised a critical mechanics question: Does chronic exposure to MHC-I-deficient tumor cells actively drive the NK cell exhaustion program observed in vivo? To address this, we established an in vitro co-culture system using TNBC cells in which MHC-I expression was stably reduced. We screened three independent siRNAs targeting the essential MHC-I components, beta-2-microglobulin (B2M) (si-B2M-1, si-B2M-2, si-B2M-3) and HLA-A (si-HLA-A-1, si-HLA-A-2, si-HLA-A-3) using qRT-PCR and western blot analysis 48 h post-transfection.

Quantitative PCR analysis demonstrated that all three B2M-targeting siRNAs reduced B2M mRNA expression compared to the non-targeting control (siNC), with si-B2M-1 achieving the most potent and significant suppression (*** $P < 0.001$) (Figure 5A). Similarly, among the HLA-A-targeting siRNAs, si-HLA-A-3 exhibited the strongest reduction in HLA-A transcript level (**** $P < 0.0001$), while si-HLA-A-1 and si-HLA-A-2 showed comparatively moderate but significant knockdown efficiency (Figure 4A).

Consistent with the transcriptional findings, western blot analysis confirmed marked decreases in B2M and HLA-A protein levels following transfection with the most effective siRNAs. Cells transfected with si-HLA-A-3 showed substantially reduced HLA-A protein levels (~40 kDa) compared to control and siNC-transfected cells, while si-B2M-1 transfection resulted in pronounced loss of B2M protein (~12 kDa) (Figure 4B). Densitometric quantification of three independent experiments confirmed that both si-B2M-1 and si-HLA-A-3 significantly reduced their respective protein levels compared to the siNC control (**** $P < 0.0001$) (Figure 4C).

These data demonstrate efficient and specific knockdown of MHC-I components at both the transcriptional and translational levels in NK-92 cells. Based on these validation results, si-B2M-1 and si-HLA-A-3 were selected for all subsequent functional studies examining the cell-intrinsic role of MHC-I in NK cell effector function.

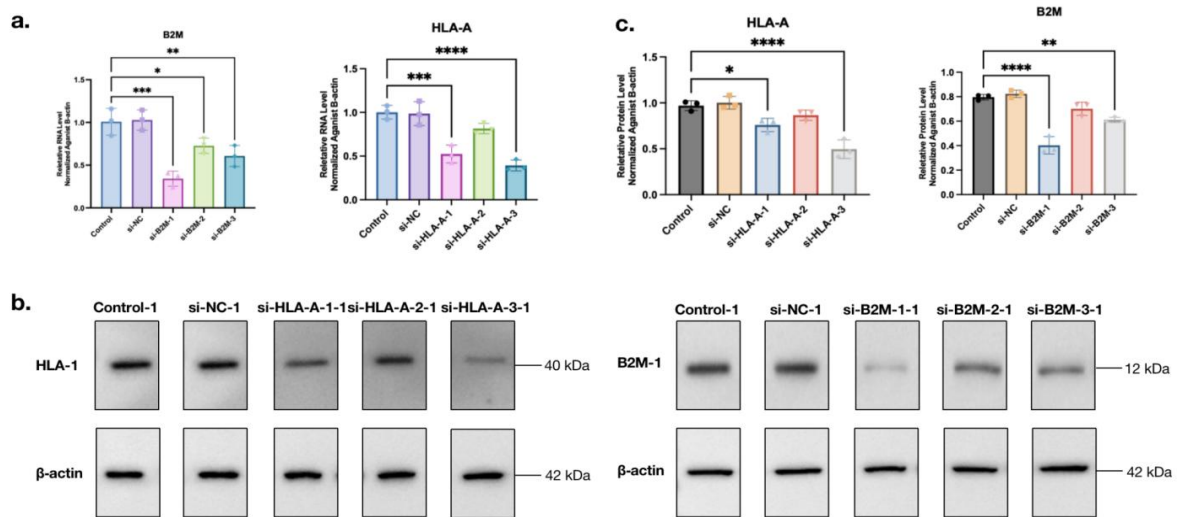


Figure 4. Efficient siRNA-Mediated Knockdown of MHC-I Components in NK-92 Cells.

(a) Relative mRNA expression levels of B2M (left) and HLA-A (right) in NK-92 cells 48 h after transfection with indicated siRNAs (Table 2). A non-targeting siRNA (siNC) was used as the negative control. Gene expression was quantified by qRT-PCR, normalized to GAPDH, and expressed relative to siNC using the $2^{-\Delta\Delta C_t}$ method. Data are presented as mean $\pm$ SD from three independent biological replicates, each analyzed in technical triplicate. *** $P < 0.001$, **** $P < 0.0001$ by one-way ANOVA with Dunnett's post-test compared to siNC. (b) Representative western blot images showing protein expression of HLA-A (top, ~40kDa) and B2M (bottom, ~12kDa) following siRNA-mediated knockdown. B-actin (~42kDa) was used as a loading control. (c) Densitometric quantification of Western blot results from three independent experiments. Protein expression levels were normalized to β -actin and expressed relative to the siNC control group. **** $P < 0.0001$ by unpaired t-test

Following validation of efficient MHC-I silencing, we next investigated the functional consequences of B2M and HLA-A knockdown on NK cell fitness and effector activity. We co-cultured K-562 cells with NK-92 that were either control (siNC-transfected) or deficient in MHC-I (si-B2M-1 or si-HLA-A-3 transfected). To assess proliferative capacity, NK cells were labeled with CFDA-SE and monitored over a 96-hour time course (Figure 5A). All three groups exhibited a similar progressive decrease in fluorescence intensity, reflecting comparable cell division rates. No significant differences in proliferation kinetics were observed between control and MHC-I-silenced conditions.

We next evaluated NK-mediated cytotoxicity by measuring target cell apoptosis (Figure 5B). One-way ANOVA analysis demonstrated a marked reduction in apoptotic target cell rate in both si-B2M-1 and si-HLA-A-3 groups compared with control (**** $P < 0.0001$). These findings indicate significantly impaired NK killing capacity upon loss of tumor MHC-I expression.

To directly evaluate NK effector responses, tumor cells transfected with control, si-B2M-1, or si-HLA-A-3 were co-cultured with NK cells for 4 hours. GolgiStop (2 μ L/mL) was added during the final hour of incubation to enable intracellular cytokine accumulation prior to flow cytometric analysis. NK cells were identified by CD56 expression, and functional readouts were quantified by dual staining for CD56 together with CD107a, GZMB, and IFN- γ . Consistent with impaired cytotoxic function, the frequency of CD56⁺CD107a⁺ NK cells was significantly reduced in both si-B2M-1 and si-HLA-A-3 groups compared with control (one-way ANOVA, **** $P < 0.0001$), indicating decreased degranulation capacity (Figure 5C). Similarly, the proportions of CD56⁺GZMB⁺ and CD56⁺IFN- γ ⁺ NK cells were markedly decreased following MHC-I knockdown (**** $P < 0.0001$ for both comparisons). These results demonstrate a significant reduction in NK cytotoxic granule release and cytokine production upon loss of tumor MHC-I expression.

These results demonstrate a previously unrecognized, cell-intrinsic role for MHC-I in maintaining NK cell effector function. Loss of B2M or HLA-A on NK cells themselves leads to a profound

functional impairment, characterized by reduced degranulation, cytokine production, and cytotoxic capacity. This finding suggests that the MHC-I complex on NK cells is not merely an inhibitory ligand for other immune cells but plays a critical role in sustaining the NK cell function. In the context of TNBC, where the tumor microenvironment is rich in inflammatory and suppressive signals, the downregulation of MHC-I on infiltrating NK cells could actively contribute to their exhausted phenotype and explain the disconnect between their high effector gene expression and poor functional output observed in our single-cell data.

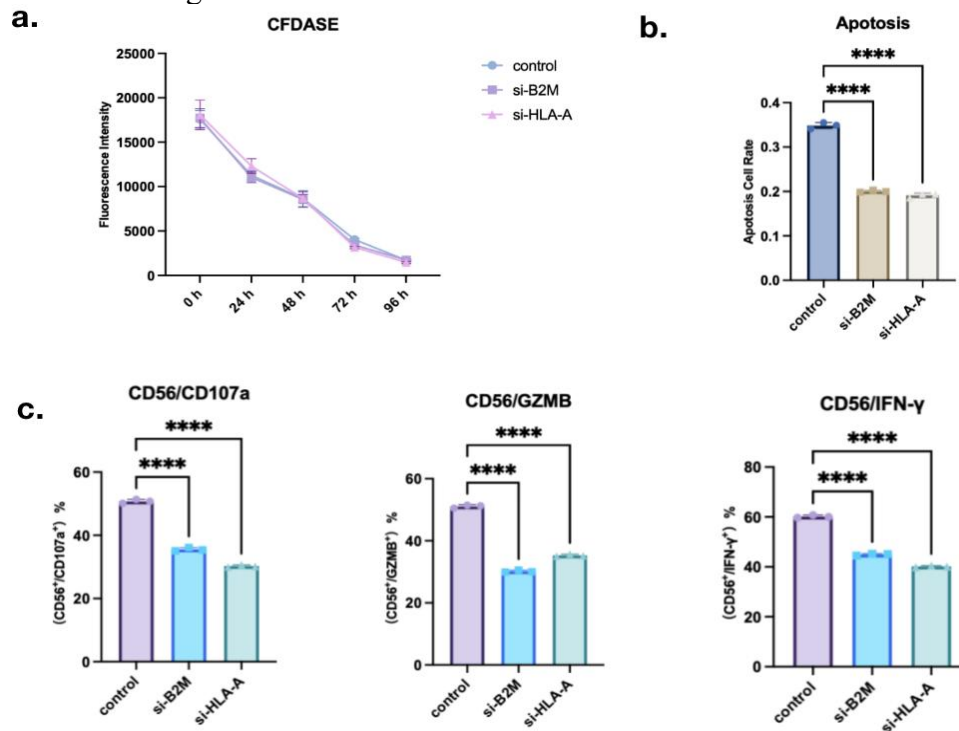


Figure 5. Cell-Intrinsic MHC-I Loss Impairs NK Cell Effector Function and Cytotoxic Capacity.

(a) MHC-I loss does not affect NK cell proliferation. NK-92 cells were transfected with siNC (control), si-B2M-1, or si-HLA-A-3 and labeled with CFDA-SE. CFSE fluorescence intensity was measured by flow cytometry at 0, 24-, 48-, 72-, and 96-hours post-labeling. Proliferation is represented as the progressive dilution of CFSE signal (decrease in Mean Fluorescence Intensity [MFI]) over time relative to the 0-hour time point. All three groups exhibited comparable proliferation kinetics, indicating that MHC-I expression is not required for basal NK cell proliferative capacity. Data are representative of three independent experiments. (B) MHC-I-deficient NK cells exhibit impaired cytotoxic activity against target cells. Control (siNC) or MHC-I-deficient (si-B2M-1, si-HLA-A-3) NK-92 cells were co-cultured with CFSE-labeled K-562 target cells at an effector-to-target (E:T) ratio of 5:1 for 12 hours. Target cell apoptosis was assessed by Annexin V/PI staining followed by flow cytometry. The percentage of apoptotic target cells (Annexin V⁺ and/or PI⁺) was significantly reduced in co-cultures containing MHC-I-deficient NK cells compared to control NK cells. Data are presented as mean $\pm$ SD from three independent experiments. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparisons test. ****P < 0.0001. (C) MHC-I-deficient NK cells display reduced degranulation and cytokine production. Control or MHC-I-deficient NK-92 cells were co-cultured with K-562 target cells (E:T ratio 5:1). Cells were harvested, stained for CD107a, CD56 and intracellular Granzyme B (GZMB) and IFN- γ , and analyzed by flow cytometry. Quantitative summaries show the frequency of CD56⁺ NK cells positive for CD107a (degranulation marker), Granzyme B (cytotoxic granule protein), and IFN- γ (effector cytokine). MHC-I-deficient NK cells (si-B2M-1 and si-HLA-A-3) exhibited significantly reduced frequencies of all three functional markers compared to control NK cells. Data are presented as mean $\pm$ SD from three independent experiments. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparisons test. ****P < 0.0001.

4. Discussion

Triple-negative breast cancer (TNBC) is a clinically aggressive and biologically heterogeneous subtype of breast cancer characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and HER2 expression as well as having a highly immunologically complex tumor microenvironment, all of which cause it to be malignant [8]. While immune checkpoint have shown promise in various cancer types, its efficiency in TNBC has been compromised, likely due to over-reliance on T cell response and an underappreciated role of innate immune population such as NK cells [4]. Classically, reduced MHC-I expression is expected to trigger NK cell activation through the “missing-self” mechanism to efficiently kill MHC class I-deficient tumor cells [9]. However, the TNBC tumor microenvironment is far more complex with dysfunctional, exhausted NK phenotype [10]. The mechanisms driving this dysfunction have remained poorly defined, representing a critical gap in our understanding of TNBC immuno-biology.

In this study, we used large-scale single-cell transcriptomic profiling with functional validation experiments to investigate how tumor-intrinsic MHC-I dynamics shape NK cell states in TNBC. Our data challenges several existing assumptions against MHC-I in NK cell effector functions. First, we show a coordinated pattern of epithelial MHC-I upregulation in TNBC compared with normal tissue. Second, we demonstrate that NK cells infiltrating this MHC-I high TME shows a distinct transcriptional characterized by high level of effector molecules (*GNLY*, *NKG7*, *PRF1*) and inhibitory/exhaustion markers (*HAVCR2*, *LAIR1*, *TGFB1*), indicating a chronically activated yet dysfunctional phenotype. Third, and most importantly, our functional studies indicate that the loss of MHC-I on NK cells, via siRNA-targeting *B2M* or *HLA-A*, negatively regulates their cytokine production, and cytotoxicity independent of cell proliferation. This suggests a critical cell-autonomous role for the MHC-I complex in maintaining NK cell fitness and effector function.

Even though our data that TNBC epithelial cells express high MHC-I (Figure 2) seems counterintuitive against the canonical model of tumor immune evasion through MHC-I downregulation, this observation aligns with emerging literature suggesting that the relationship between MHC-I expression and immune evasion is context-dependent. In highly inflamed tumors, such as many TNBCs, persistent interferon- γ signaling upregulates MHC-I expression, which enhances CD8⁺ T cells-mediated killing while inhibiting NK cells activation, potentially contributing to chronic NK cell stimulation and eventual exhaustion [11]. This is further supported by our single-cell data which shows elevated effector and inhibitory gene expression (Figures 3 and 4) in TNBC-infiltrating NK cells—a transcriptional signature strikingly similar to that of exhausted T cells in chronic infection and cancer [12].

Our data first report a cell-autonomous role for the MHC-I complex in sustaining NK cell cytotoxicity as we show that MHC-I expression on NK cells is required for effector function. Distinct from the conventional view that MHC-I on NK cells primarily serve as inhibitory receptors for interaction with other NK cells or T cells, our data indicates that the loss of B2M or HLA-A directly impairs the NK cell’s ability for IFN- γ and Granzyme B production (Figure 6). Our findings offer a new perspective for understanding the NK cell dysfunction in TNBC. In inflammatory TME, NK cells are in a state of chronic activation—the “stressed” phenotype observed in our scRNA-seq data marked by upregulating both effector and inhibitory. The situation is further exacerbated by the MHC-I downregulation on NK cells, potentially induced by chronic stimulation, suppressive cytokines (e.g., TGF- β), or metabolic stress within the TME. This loss of MHC-I further impairs the NK cell’s cytotoxicity, rendering it dysfunctional despite being transcriptional active. The high MHC-I expression on TNBC tumor cells is, therefore, further enhanced the suppressive TME, providing continuous inhibitory signals to KIRs on already-compromised NK cells.

Our findings have suggested novel immunotherapies that are underappreciated in the past. Current immunotherapies aimed at reactivating NK cells—such as anti-NKG2A antibodies (monalizumab) or KIR-blocking antibodies (lirilumab). These would be less effective if the NK cells lack MHC-I and are therefore functionally compromised at a basal level [13]. Our current results suggest that restoring MHC-I expression on NK cells is vital for NK cell-directed immunotherapies, perhaps through

cytokine support (e.g., IL-15) or metabolic modulation. MHC-I expression level on NK cells could also serve as a predictive biomarker for response to NK-mediated therapies.

Despite the promising results, our study presents several limitations that need to be addressed in future work. First, our single-cell analyses were performed on a public dataset with a limited sample size. It is therefore necessary to validate the results in larger, independent cohorts across various TNBC subtypes. Second, even though our study employs a well-established model of NK-92 cell line for NK cell biology, it may not fully recapitulate the complexity of primary human NK cells. As a result, future studies could validate the findings with primary NK cells isolated from healthy donors and from TNBC patients. Third, our in vitro model of acute MHC-I knockdown with siRNA does not fully capture the chronic loss of MHC-I occurring in vivo. Hence, using an in vivo mouse model with conditional MHC-I deletion in NK cells would provide a more physiologically relevant assessment. Finally, even though the K-562 target cell line is useful for studying NK cell activation, it does not represent the TNBC TME and future studies employing MHC-I-high and MHC-I-low TNBC cell lines would be more helpful to model the full spectrum of tumor-immune interactions.

Overall, this study reveals a critical and previously underappreciated role for cell-intrinsic MHC-I expression in sustaining NK cell effector function. Our findings challenge the conventional view that MHC-I merely serve as an inhibitory ligand for NK cell activation and suggest its new role in NK effector function. In a highly inflammatory and suppressive TME, such as TNBC, MHC-I deficiency might be a key driver for NK cell exhaustion, offering a new perspective for next-generation immunotherapies as restoration of NK cell-intrinsic MHC-I could enhance the efficacy of NK cell-directed treatments. Future work should focus on unravelling the molecular mechanisms behind inhibition of NK cell function through MHC-I downregulation, identifying the signals within the TME that drive its downregulation, and exploring whether MHC-I expression on patient NK cells predicts clinical outcomes to immunotherapy.

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