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Ascorbate in Pharmacological Concentrations Potentiates the Anti-Tumor Activity of NK and CD8$^+$ T Cells and Synergizes with Chemotherapy for Enhanced Anti-Tumor Immune Response against 3D Breast Cancer Spheroid Models

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Abstract: Background: Triple-negative (MDA-MB-231) and hormone-responsive (MCF-7) breast cancers exhibit complex immune-evasive mechanisms within the three-dimensional (3D) tumour microenvironment. While vitamin C (Vit-C) has shown immunomodulatory potential in two-dimensional systems, its efficacy and synergistic potential with chemotherapy in 3D architectures remain poorly defined. Drug resistance and toxicity remain major challenges in breast cancer (BC) therapy. We investigated a novel strategy combining high-dose Vit-C with chemotherapy to enhance anti-tumour immunity in 3D BC models. Methods: MDA-MB-231 and MCF-7 spheroids were established and co-cultured with human natural killer (NK) cells and CD8$^+$ T cells. To isolate functional enhancement from pro-oxidant damage, immune cells were pre-treated with catalase (CAT) and subsequently primed with 1 mM Vit-C for 6 h. These primed effectors were then co-cultured for 24 h (at a 2:1 effector-to-target ratio for single cell types or 1:1:1 for mixed NK+CD8$^+$ T cells) with spheroids that had been pre-conditioned for 24 h with sub-lethal doses of doxorubicin (DOX, 0.5 μM), docetaxel (DOCE, 0.5 μM), Vit-C, or their triple combination. Spheroid viability was assessed via ATP bioluminescence (CellTiter-Glo®), and cytotoxicity was quantified by lactate dehydrogenase (LDH) release. Secreted effector molecules—interferon-gamma (IFN-γ) and granzyme B—were measured in co-culture supernatants by ELISA. Results: Vit-C priming significantly enhanced both NK and CD8$^+$ T cell cytotoxicity against BC spheroids ($p < 0.001$). A potent synergistic effect was observed when Vit-C-primed immune

cells were co-cultured with chemotherapy-conditioned spheroids. This combination drove a massive escalation in IFN-γ and granzyme B production. The triple combination (Vit-C + DOX + DOCE) elicited the most robust response ($p < 0.0001$), yielding IFN-γ concentrations of 18.17 ± 1.476 ng/mL and granzyme B concentrations of 67.20 ± 5.211 ng/mL. This represents a significant 26-fold increase in IFN-γ and a 54-fold increase in granzyme B over untreated controls. Conclusion: This study demonstrates that Vit-C, when combined with sub-lethal chemotherapeutic conditioning, acts as a powerful adjuvant to overcome 3D tumor resistance. These findings provide a strong preclinical foundation for integrating pharmacological Vit-C into current chemo-immunotherapy regimens for BC.

Keywords: breast cancer; 3D tumor spheroid model; vitamin C; immune cells; granzyme B; IFN-γ

1. Introduction

Breast cancer (BC), particularly in its metastatic form, presents significant therapeutic challenges due to its heterogeneous nature and a highly immunosuppressive tumor microenvironment (TME) [1]. While cell-based immunotherapies, such as those leveraging natural killer (NK) cells and cytotoxic $CD8^+$ T cells (also known as cytotoxic T lymphocyte (CTL)), offer immense promise, their effectiveness is often hindered by the hostile environment they encounter within the tumor [1–4]. Compelling research has highlighted the potential of certain agents to reshape the TME and directly augment the function of immune cells. Notably, high-dose ascorbic acid (vitamin C or Vit-C) has emerged as a compound of significant interest, not only for its ability to modulate the microenvironment but also for its documented synergy with immune checkpoint inhibitors [5–7].

High-dose Vit-C can generate reactive oxygen species (ROS), which are toxic to cancer cells but also have the potential to alter the TME and influence immune cell activity [8,9]. Consistent with our previous findings, we demonstrated that in MDA-MB-231 and MCF-7 BC spheroids, high-dose Vit-C induces significant apoptotic cell death by generating a state of oxidative stress. This process is mediated by a surge in intracellular H_2O_2 and the subsequent depletion of the GSH/GSSG ratio. This prior work established that these spheroids, are susceptible to Vit-C-mediated cytotoxicity, which directly informed the design of our current study into combination therapies [10]. However, a significant knowledge gap remains regarding the dual nature of Vit-C; while it serves as a potent pro-oxidant against malignant cells, this same oxidative stress can be detrimental to the effector immune cells themselves, complicating the study of its true immunomodulatory potential.

This challenge is particularly relevant in three-dimensional (3D) tumor spheroids, which are far superior to traditional two-dimensional cultures in mimicking the physiological complexity of solid tumors such as BC [11]. Consequently, understanding whether high-dose Vit-C can effectively stimulate human NK and $CD8^+$ T cells within a physiologically relevant 3D tumor model—while simultaneously mitigating its inherent toxicity to those cells–is critical for developing potential therapeutic strategies.

Moreover, doxorubicin (DOX) and docetaxel (DOCE) are cornerstone anti-cancer agents, classified as an anthracycline and a taxane, respectively [12,13]. DOX functions by disrupting DNA replication and generating cytotoxic free radicals [12], while DOCE targets the microtubule network to inhibit cell division [13]. While both agents are potent cytotoxic chemotherapies, their effects on the immune system are complex; they can both cause immunosuppression and induce immunogenic cell death, which can potentially stimulate a host anti-tumor immune response [12,14].

Despite the established roles of these chemotherapeutic agents and the emerging potential of Vit-C as an immunomodulator, no studies have investigated the combined anti-tumor efficacy of Vit-C, DOX, and DOCE within a physiologically relevant model of 3D BC spheroids co-cultured with human NK and $CD8^+$ T cells. Such a study is essential to determine if a synergistic therapeutic effect can be achieved through this novel triple combination, bridging the gap between conventional chemotherapy and a targeted immunotherapeutic approach.

The novelty of the current study lies in addressing the limitation of effector cell viability in high-oxidative environments through a unique "priming and conditioning" strategy. To date, no studies have investigated the combined anti-tumor efficacy of Vit-C-primed immune cells in conjunction with chemotherapy-conditioned 3D BC spheroids. By pre-treating isolated NK and $CD8^+$ T cells with catalase (CAT), we sought to protect these effectors from Vit-C-induced extracellular ROS, thereby isolating the beneficial immunomodulatory effects of Vit-C from its inherent toxicity. Simultaneously, we conditioned the 3D spheroids with sub-lethal doses of DOX and DOCE to evaluate if such a combination could sensitize the dense tumor architecture to immune infiltration.

Here, our study aimed to investigate the synergistic effects of a combination therapy by priming human NK and $CD8^+$ T cells with high-dose Vit-C (1 mM for 6 h) and then evaluating their anti-tumour activity against drug-treated tumour spheroids (24 h co-culture). We utilized breast cancer spheroids from MDA-MB-231 and MCF-7 cell lines to provide a robust experimental platform. Crucially, the isolated immune cells were first pre-treated with CAT to protect them from the cytotoxic effects of Vit-C-induced ROS, ensuring their viability and functionality. For $CD8^+$ T cells, a 24-h activation with CD3/CD28 Dynabeads™ was performed prior to CAT and Vit-C treatment. These primed immune cells were then added to a co-culture system containing tumour spheroids that had been pre-treated for 24 h with Vit-C, DOX, DOCE, or their triple combination. All co-cultures were performed for 24 h at effector-to-target ratios of 2:1 (for single immune cell types) or 1:1:1 (for mixed NK + $CD8^+$ T cells). This approach allowed us to accurately assess the enhanced ability of the primed immune cells to infiltrate and induce apoptosis in tumor spheroids under the influence of the triple-drug therapy. Our findings will provide a nuanced understanding of the potential of using high-dose Vit-C to prime immune cells for enhanced anti-tumor activity in conjunction with conventional chemotherapies.

2. Materials and Methods

2.1. Cell lines and 2D Monolayer Culture Conditions

MCF-7 and MDA-MB-231 were purchased form the ATCC (American Type Culture Collection, Manassas , VA, USA) and cultured in complete DMEM (Dulbecco's Modified Eagle Medium, Nacalai Tesque, Kyoto, Japan) complemented with 2% L-glutamine (Gibco™ L-Glutamine; Thermo Fisher Scientific, Waltham, MA, USA), 5% fetal bovine serum (FBS, Gibco™ (10270-106) Fetal Bovine Serum; Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin/streptomycin (pen/strep, Gibco™ (10378-016), Thermo Fisher Scientific, Waltham, MA, USA), and 3 g/500 mL Glucose (G7021-100G) D-(+)-Glucose, Sigma, St. Louis, MO, USA). The cells were maintained, passaged until reaching 80% confluency and incubated at 37 °C in a 5% CO_2 incubator.

2.2. Agarose-Coated 96-Well Plate Preparation and 3D Spheroids Formation

To create a concave meniscus bottom for spheroid formation, 1.5% agarose (Promega, Madison, WI, USA) was added to 50 mL of ultrapure water (PURELAB Ultra, ELGA LabWater, High Wycombe, UK) in a 50 mL tube and then autoclaved for 2 min. Subsequently, 65 µL of the warmed solution was carefully dispensed in each well of a 96-(well) flat-bottomed plate (Thermo Fisher Scientific, Waltham, MA, USA). Once solidified, the plate coated with agarose was stored at 4 °C until needed. For spheroid generation, MCF-7 and MDA-MB-231 cells were detached by trypsin (Gibco™ Trypsin-EDTA, Gibco, Grand Island, NY, USA) after reaching confluency, and then counted. Cells were added to reach a density of 5000, 10,000, 15,000, and 20,000 cells/well into the prepared agarose plates. The plates were then centrifuged in a swing bucket rotor at 1000 g for 30 min at room temperature. Finally, the plates were incubated at 37 °C in a 5% CO_2 incubator for 3–5 days to allow spheroid formation.

2.3. Morphological Microscopic Assessment

Spheroid morphology and growth were monitored by acquiring daily images on specified days (3, 6, 9, 12, and 15). Image capture was performed using an inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany) fitted with a 5× objective. Subsequently, the MATLAB-based software Spheroid Sizer (MathWorks, Inc., Natick, MA, USA) was utilized to conduct a quantitative analysis of the spheroid's dimer on day 3, 6, 9, 12, and 15 [15]. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to process the images.

2.4. Drugs Preparation and Treatment

A 200 mM of Vit-C stock solution (L-ascorbate) (A7631-100G, Sigma-Aldrich, Gangnam-Gu, Republic of Korea) was prepared by dissolving 35.224 in 1 mL of ddH_2O, and a working solution was prepared from the stock solution in the DMEM media to have a Vit-C concentrations of 250 uM, 500 uM, and 1 mM, as stated previously [16]. Doxorubicin hydrochloride (DOX; Sigma-Aldrich, Gangnam-Gu, Republic of Korea) and Docetaxel (DOCE; Sigma-Aldrich, Gangnam-Gu, Republic of Korea) were utilized as chemotherapeutic conditioning agents. Primary stock solutions were prepared in dimethyl sulfoxide (DMSO), from which working solutions were freshly prepared in DMEM media to achieve a final concentration of 0.5 µM for both DOX and DOCE The concentration of 0.5 µM was strategically selected based on established preclinical literature to represent a sub-lethal, low-dose conditioning concentration. In conventional 2D monolayer models, reported IC_{50} values for DOX and DOCE in MCF-7 and MDA-MB-231 cells range from 0.005 to 5 µM [17–20]. By utilizing 0.5 µM in our 3D model, we aimed to induce immunogenic stress and modulate the tumor microenvironment architecture without inducing mass independent

apoptosis, thereby facilitating a clear assessment of the synergistic contribution from Vit-C-primed effector cells. DMSO levels were maintained at sub-lethal levels, with the final concentration in any treatment well not exceeding 0.01% (*v*/*v*), a concentration confirmed to have no detectable effect on cell viability. All working concentrations were freshly prepared immediately prior to each experiment, and data were normalized against the vehicle control unless specified otherwise.

2.5. Isolation and Immunophenotypic Characterization of CD8⁺ T Cells and NK Cells

This study was approved by the Human Research Ethics Committee of Universiti Sains Malaysia (JEPeM-USM; study protocol USM/JEPeM/21080576). Following informed consent, peripheral blood was collected from healthy donors into EDTA Vacutainer® tubes (BD Biosciences) and processed within two hours. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. Briefly, blood diluted 1:1 with phosphate-buffered saline (PBS) was layered over Lymphocyte Separation Medium (LSM; Capricorn Scientific) and centrifuged at 800 x g for 20 min. The resulting mononuclear cell layer was collected, washed with PBS, and either used immediately or cryopreserved in freezing medium (20% DMSO in complete RPMI 1640) for long-term storage in liquid nitrogen. $CD8^+$ T cells and NK cells were subsequently isolated from PBMCs by magnetic-activated cell sorting (MACS) using LS columns (Miltenyi Biotec) according to the manufacturer's protocols. All procedures were performed at 4 °C. $CD8^+$ T cells were isolated via positive selection using CD8 MicroBeads (Miltenyi Biotec), where labelled cells were retained on the column and subsequently eluted. Conversely, NK cells were isolated via negative selection using a Human NK Cell Isolation Kit (Miltenyi Biotec), which retains labelled non-NK cells on the column, allowing unlabelled NK cells to be collected in the flow-through.

For immunophenotypic characterization, isolated cells were thawed, counted, and stained with fluorochrome-conjugated antibodies (Miltenyi Biotec) for 10 min at 4 °C in the dark. The panel for $CD8^+$ T cells comprised CD8-APC (clone REA734), CD3-FITC (clone REA613), and CD56-PE (clone REA196). The panel for NK cells comprised CD3-FITC (clone REA613), CD56-PE (clone REA196), and CD45-APC-Vio770 (clone REA747). Following staining, cells were washed and analyzed on a BD FACSCanto™ (BD Biosciences). Data were processed using FlowJo v10.9.0 software, with dead cells and debris excluded based on scatter characteristics and PI fluorescence.

2.6. Functional Assessment of Vit-C-Treated CD8⁺ T Cells and NK Cells

Following immunophenotypic characterization, the functional capacity of isolated $CD8^+$ T cells and NK cells was evaluated through viability, cytotoxicity, and cytokine production assays under various treatment conditions. All experiments were designed in two complementary phases: (i) assessment of direct Vit-C effects on immune cell function; and (ii) evaluation of how pre-conditioned tumor spheroids modulate immune cell responses.

2.6.1. Cell Viability Following Vit-C Treatment

Vit-C treatment was applied as previously described [5]. Isolated NK cells and $CD8^+$ T cells were seeded at 4×10^4 cells per 100 µL complete RPMI-1640 in round-bottom 96-well plates. To neutralize potential confounding effects of free radicals, cells were pre-incubated with freshly prepared catalase (300 U/mL; Sigma-Aldrich, Cat. No. C1345) for 30 min at 37 °C in a CO_2 incubator. This pre-treatment concentration was strategically selected based on our previous findings demonstrating that 300 U/mL of catalase effectively neutralizes the hydrogen peroxide generated by pharmacological concentrations of ascorbate in 3D models [10].

Following pre-incubation, cells were treated with freshly prepared Vit-C at concentrations of 250 µM, 500 µM, or 1 mM for a duration of 6 h, while control groups received catalase only. This 6-h priming window was designed to allow for intracellular metabolic modulation while minimizing the risk of chronic oxidative stress to the effector cells. Cell viability was subsequently determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Cat. No. G7571) according to the manufacturer's protocol. Luminescence was recorded to ensure that the priming concentrations maintained high effector cell viability prior to their introduction into the 3D spheroid co-culture system.

2.6.1.1. Phase 1: Effect of Vit-C Pre-Treatment on Immune Cell Cytotoxicity

To assess the direct immunomodulatory effects of Vit-C, immune cells were pre-treated with CAT alone or CAT plus 1 mM Vit-C for 6 h, following the established protocol [5]. For $CD8^+$ T cells, an additional activation condition was included using Dynabeads™ Human T-Activator CD3/CD28 (bead-to-cell ratio 1:1; Thermo Fisher Scientific, Cat. No. 11161D) for 24 h prior to Vit-C treatment. Following treatment, cells were washed,

resuspended in fresh medium, and co-cultured with untreated tumor spheroids for 24 h. Cytotoxicity was quantified using the CyQUANT™ LDH Cytotoxicity Assay (Invitrogen, Cat. No. C20301). Supernatants were harvested for IFN-γ and granzyme B quantification by ELISA (Human IFN-γ ELISA Kit, Invitrogen, Cat. No. KHC4021; Human Granzyme B ELISA Kit, Invitrogen, Cat. No. BMS2027-2). For co-cultures with a single immune cell type (NK cells alone or $CD8^+$ T cells alone), the effector-to-target (E:T) ratio was 2:1. For co-cultures containing both NK and $CD8^+$ T cells, the ratio was 1:1:1 (NK: $CD8^+$ T: spheroid). Experimental groups for this phase are detailed in Supplementary Tables S1–S3.

2.6.1.2. Phase 2: Effect of Spheroid Pre-Treatment on Immune Cell Cytokine Production

To evaluate how tumor spheroid pre-conditioning influences subsequent immune responses, MDA-MB-231 and MCF-7 spheroids were treated for 24 h with Vit-C (1 mM), doxorubicin (DOX, 0.5 μM), docetaxel (DOCE, 0.5 μM), or a combination of all three agents. Control spheroids received an equivalent volume of ddH_2O (vehicle control). Following treatment, drug-containing media was removed, and spheroids were washed twice with PBS. Immune cells (NK cells, $CD8^+$ T cells, or a 1:1 mixture) were pre-treated with CAT (300 U/mL, 30 min) followed by 1 mM Vit-C for 6 h as described in Section 2.6.1 (the optimal priming condition from Phase 1). For control conditions, immune cells received catalase only (no Vit-C). After pre-treatment, immune cells were washed and co-cultured with pre-treated spheroids for 24 h. The E:T ratios were as follows: 2:1 for NK cells alone or $CD8^+$ T cells alone; 1:1:1 for mixed NK + $CD8^+$ T cells. Supernatants were harvested for IFN-γ and granzyme B quantification by ELISA (same kits as in 2.6.1.1). Experimental groups are detailed in revised Supplementary Table S4.

2.6.2. LDH Cytotoxicity Assay

Cytotoxicity was measured using the CyQUANT™ LDH Cytotoxicity Assay according to the manufacturer's instructions. Briefly, triplicate samples, spontaneous LDH activity controls (spheroids only), and maximum LDH activity controls (spheroids with 10× lysis buffer) were prepared. Following incubation with reaction mixture for 30 min at room temperature protected from light, stop solution was added. Absorbance was measured at 490 nm and 680 nm using a microplate reader. LDH activity was calculated by subtracting background absorbance (680 nm) from the 490 nm value. Percent cytotoxicity was determined using the formula: % Cytotoxicity = [(Compound-treated LDH activity—Spontaneous LDH activity)/(Maximum LDH activity—Spontaneous LDH activity)] × 100.

2.6.3. IFN-γ and granzyme B Quantification by ELISA

Concentrations of IFN-γ and granzyme B in co-culture supernatants were quantified using commercial ELISA kits following the manufacturers' protocols. Briefly, samples and standards were added to pre-coated microplates and incubated with biotinylated detection conjugates. After washing, streptavidin-horseradish peroxidase (HRP) was added, followed by substrate incubation (TMB for granzyme B). The reaction was terminated with stop solution, and absorbance was measured at 450 nm using a SoftMax Pro 5® microplate reader (Molecular Devices). Cytokine concentrations were determined by interpolation from standard curves.

2.7. Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) from at least three independent experiments, as indicated in the figure legends. Statistical analyses were performed using GraphPad Prism (version 9.0; GraphPad Software, San Diego, CA, USA). Normality was assessed using the D'Agostino-Pearson test. Comparisons between two groups were made using unpaired two-tailed Student's *t*-tests, with Welch's correction applied where variances were significantly different. For comparisons of multiple groups against a single control, one-way analysis of variance (ANOVA) was performed, followed by Dunnett's post-hoc test. Spheroid growth over time was analyzed using two-way repeated measures ANOVA. Statistical significance was set at $p < 0.05$. Exact *p*-values are reported in the figures and corresponding legends.

3. Results

3.1. Human NK Cells and $CD8^+$ T Cells Isolation and Phenotypic Characterization

Flow cytometry confirmed the successful isolation of highly pure and viable populations of both NK cells and $CD8^+$ T cells. Purity for NK cells averaged 88.6% (Figure 1A), while $CD8^+$ T cells demonstrated a high purity of 98.5% (Figure 1C). Viability, assessed by propidium iodide (PI) staining, was also high, at 98.1% for NK cells

(Figure 1B) and 99.9% for $CD8^+$ T cells (Figure 1D), ensuring the presence of intact and healthy cells for subsequent functional assays.

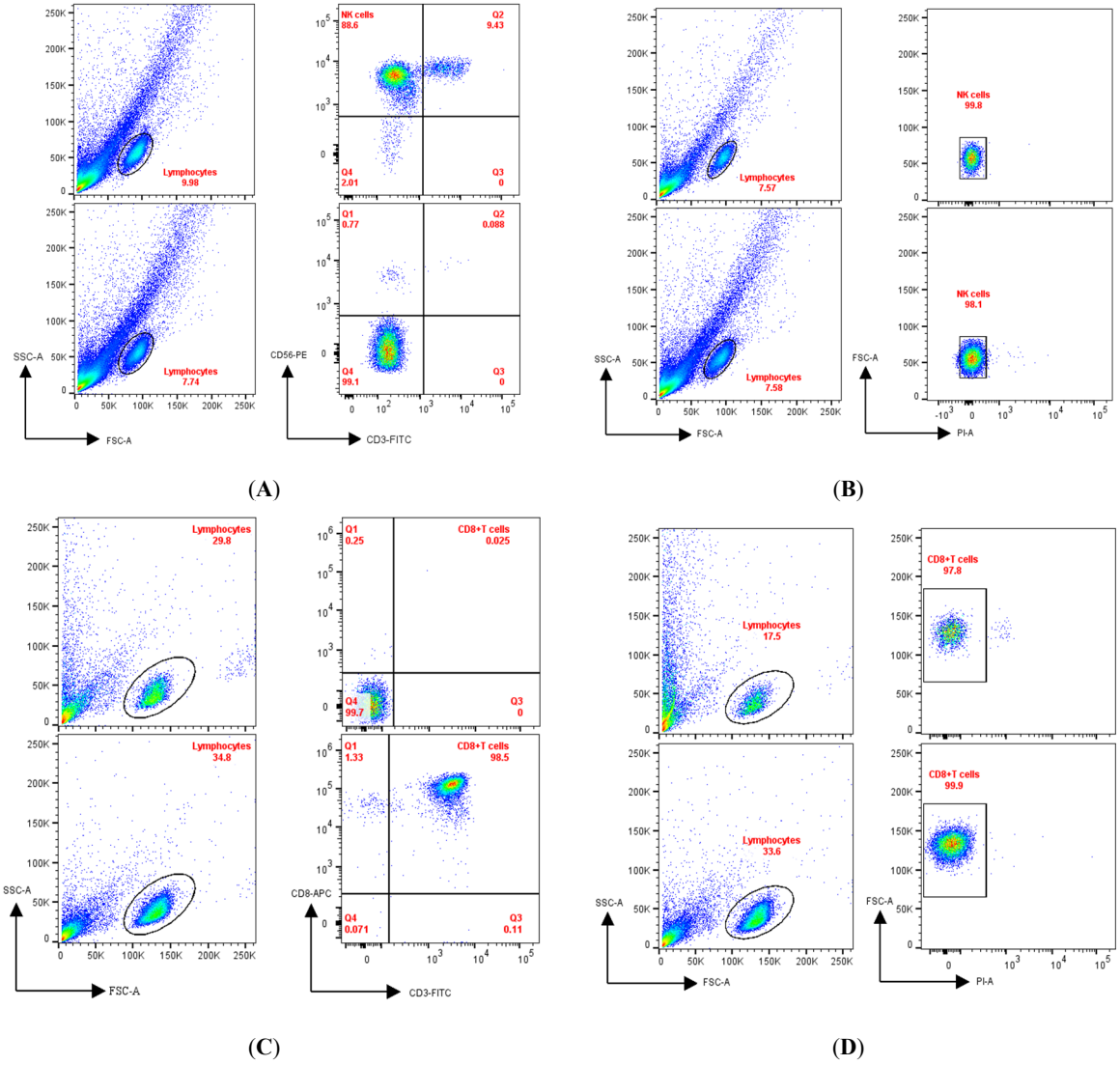


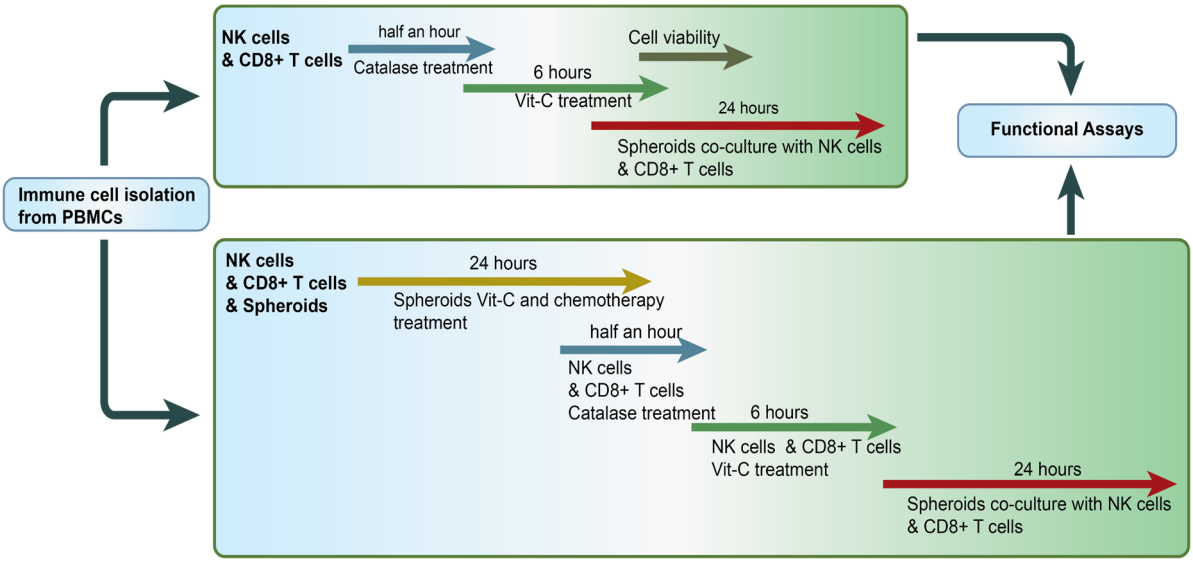
Figure 1. Gating strategy and cell viability analysis for the isolated NK cells and $CD8^+$ T cells. **Panel A**: Within the lymphocyte gate, T cells and NK cells were differentiated. NK cells were identified as the population negative for CD3 (CD3-FITC) and positive for CD56 (CD56-PE). **Panel B**: Viability was assessed specifically within the identified NK cell population by gating using FSC-A and PI-A. **Panel C**: Within the lymphocyte gate, $CD8^+$ T cells were identified. T cells were first gated based on CD3 expression, and $CD8^+$ T cells were then defined as positive for CD8 (CD8-APC). **Panel D**: Viability was assessed specifically within the identified $CD8^+$ T cell population by gating using FSC-A and PI-A. PI, propidium iodide; NK cells, natural killer cells.

3.2. Vit-C treatment of NK cells and $CD8^+$ T cells

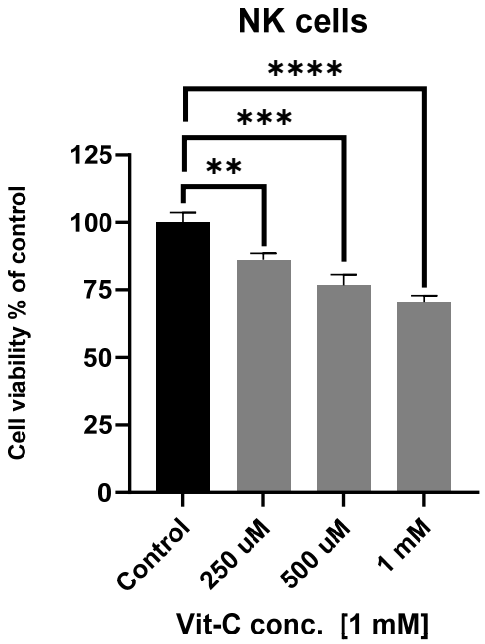
To assess the metabolic viability of immune cells after Vit-C exposure, we used the CellTiter-Glo® assay, which measures cellular ATP levels as a sensitive and quantitative indicator of metabolically active (viable) cells. Unlike propidium iodide (PI) staining–which only identifies cells with irreversibly damaged membranes–ATP measurement captures gradual metabolic decline and is better suited for detecting early, sub-lethal effects of oxidative stress. All data are presented as mean ± SEM of $n = 3$ independent experiments. Treatment of NK cells with Vit-C alone induced a significant, dose-dependent decrease in metabolic activity compared to the untreated control ($p < 0.0001$) (Figure 1B). Dunnett's multiple comparisons test confirmed significant reductions in viability at 250 µM ($p = 0.0070$), 500 µM (mean difference = 1991, $p = 0.0003$), and 1 mM ($p < 0.0001$). Pre-treatment with CAT (300 U/mL) attenuated this decrease ($p = 0.0975$) (Figure 1C), abrogating the significant effect at 250 µM ($p = 0.4297$) and 1 mM ($p = 0.5597$). A significant reduction persisted at 500 µM ($p = 0.0448$), indicating that Vit-

C toxicity to NK cells is predominantly mediated by hydrogen peroxide (H_2O_2), with a minor H_2O_2-independent component at higher intermediate concentrations.

A parallel effect was observed in $CD8^+$ T cells. Vit-C treatment alone significantly decreased viability ($p < 0.0001$) (Figure 1D). Dunnett's multiple comparisons test confirmed significant reductions at 250 µM ($p = 0.0180$), 500 µM ($p < 0.0001$), and 1 mM ($p < 0.0001$). Crucially, pre-treatment with CAT completely abrogated this toxic effect ($p = 0.2169$) (Figure 1E), with viability at all doses returning to levels indistinguishable from the control (250 µM: $p = 0.6062$; 500 µM: $p = 0.9578$; 1 mM: $p = 0.5068$). This demonstrates that the decrease in $CD8^+$ T cell viability is specifically and entirely mediated by extracellular H_2O_2 generated by Vit-C. Based on these findings, the 1 mM concentration of Vit-C+CAT (300 U/mL) was selected for subsequent functional assays. The rationale for this choice was that the concentration eliciting the strongest oxidative stress response (as evidenced by the highest toxicity in Figure 2B,D which was reversible by CAT in Figure 2E,C) would be most likely to potentiate the anti-tumor immune function of NK and $CD8^+$ T cells.



(A)



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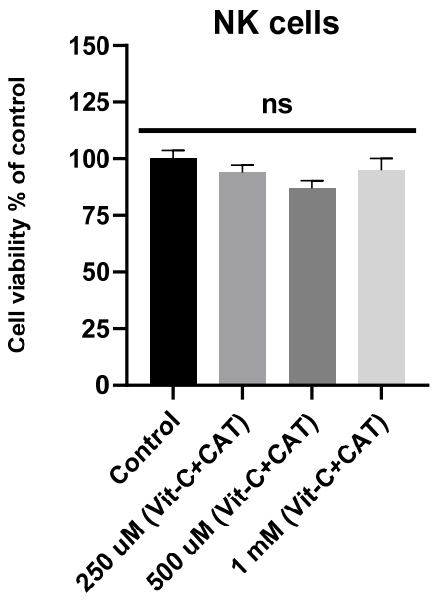


(C)

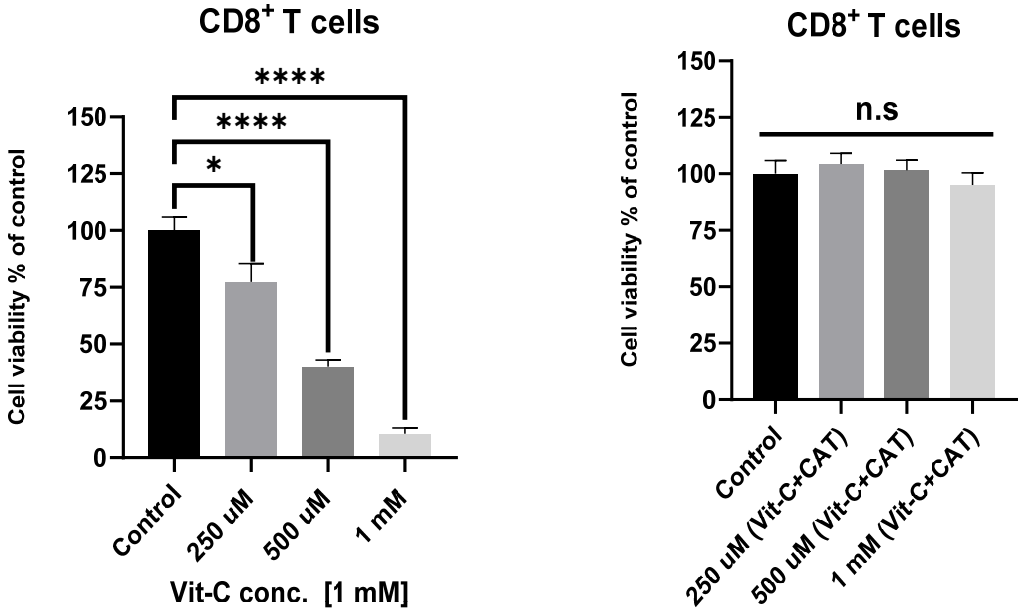


Figure 2. Experimental workflow and the effect of vitamin C-induced oxidative stress on immune cell viability. (**A**) Schematic diagram illustrating the sequential treatment protocol for NK and $CD8^+$ T cells prior to co-culture with breast cancer spheroids. Immune cells were isolated from PBMCs. (**B**,**C**) Viability of NK cells after treatment. (**B**) Cells were treated with increasing doses of Vit-C (250 µM, 500 µM, or 1 mM) for 6 h to assess the direct effect of Vit-C-generated H_2O_2. (**C**) NK cells were pre-treated with CAT (300 U/mL) for 30 min to scavenge H_2O_2 prior to the addition of Vit-C. (**D**,**E**) Viability of $CD8^+$ T cells after treatment under the same conditions as NK cells: (**D**) Vit-C only and (**E**) CAT pre-treatment + Vit-C. Cell viability was assessed using the CellTiter-Glo® Cell Viability Assay. Data are presented as mean ± SEM of $n = 3$ independent experiments. The error bars represent the SEM. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test against the control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, not significant. Abbreviations: Vit-C, vitamin C; NK cells, natural killer celhls; PBMCs, peripheral blood mononuclear cells; SEM, standard error of the mean; H_2O_2, hydrogen peroxide; CAT, catalase.

3.3. The MDA-MB-231 BC Cell Lines Successfully Developed Spheroids with Different Seeding Densities

To establish a robust 3D model, we first optimized the formation of MDA-MB-231 BC spheroids by assessing the impact of initial seeding density (5000, 10,000, 15,000, and 20,000 cells) on spheroid volume over a 15-day period (Figure 3A). A two-way repeated measures ANOVA revealed significant main effects of both seeding density ($p < 0.0001$) and time ($p = 0.0001$), as well as a significant interaction between the two factors ($p = 0.0002$) (Figure 3B), indicating that the increase in volume over time was dependent on the initial cell number.

Post-hoc analysis (Dunnett's multiple comparisons test against Day 3 for each density) detailed this interaction. The lower densities (5000 and 10,000 cells) showed minimal or delayed growth in volume, with significant increases only becoming apparent at later time points (e.g., 5000 cells: Day 9, $p = 0.0235$; 10,000 cells: Day 12, $p = 0.0396$). In contrast, the higher densities (15,000 and 20,000 cells) exhibited robust, continuous volumetric growth from Day 6 onwards (15,000 cells: Day 6, $p < 0.0001$; 20,000 cells: Day 6, $p = 0.0174$) (Figure 3B). Based on these results, a seeding density of 20,000 cells was selected for all subsequent experiments. While the 15,000 cells density produced spheroids with robust growth, the 20,000 cells/well density consistently produced spheroids with a stable diameter from Day 3 to Day 15 (Figure 3A). This reliable growth profile, unlike the variable or non-significant changes seen at lower densities, ensures the establishment of a reproducible model. A well-formed spheroid provides a more physiologically relevant and substantial three-dimensional target, which is essential for accurately assessing the infiltration and cytotoxic activity of immune cells and the efficacy of the drug combination in a co-culture setting. Therefore, this density was chosen as it provides the optimal balance of growth, stability, and integrity necessary for the subsequent therapeutic studies.

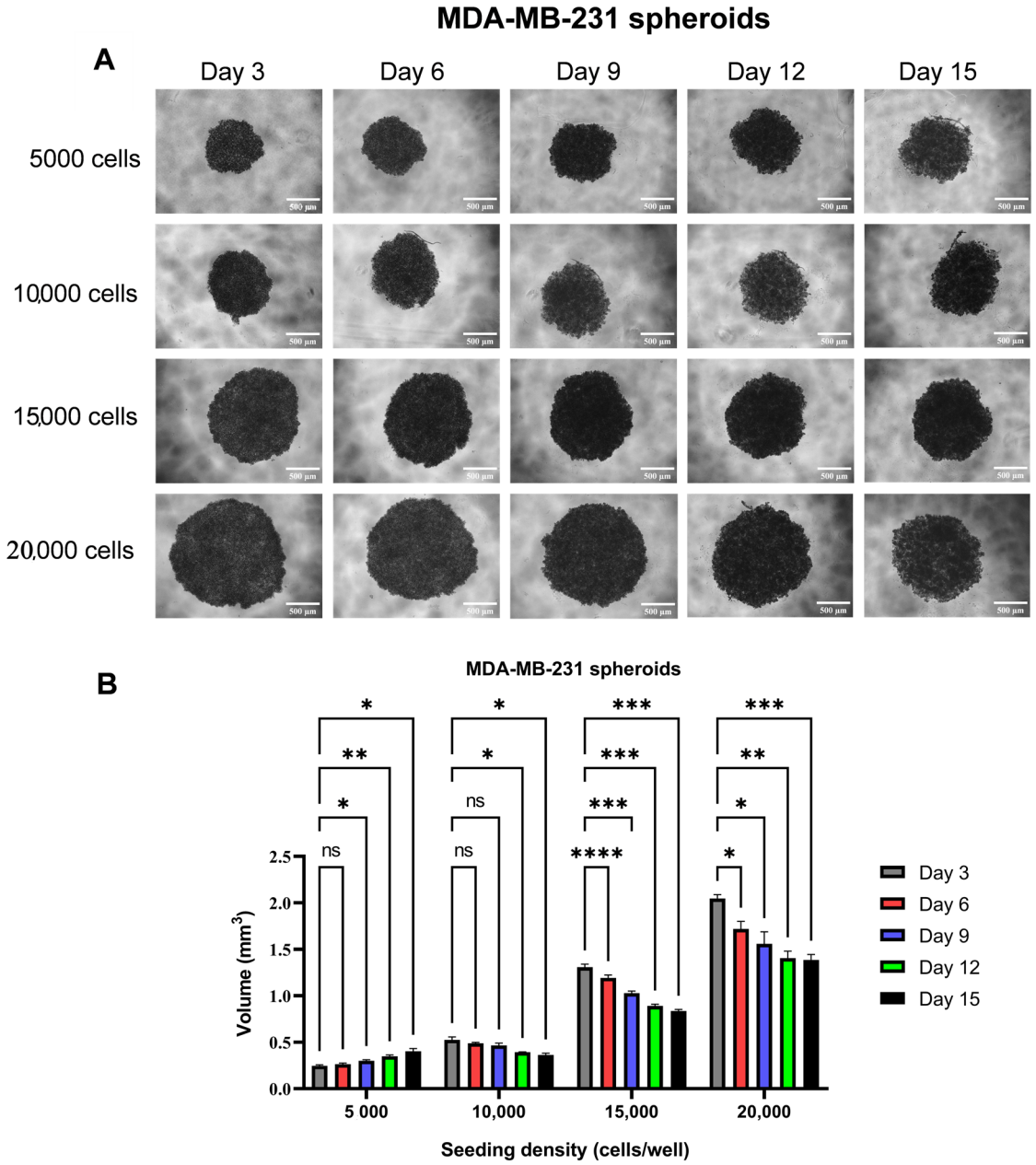


Figure 3. Morphological Evaluation and Growth Kinetics of MDA-MB-231 Spheroids over 15 Days. (**A**) Representative bright-field microscopy images of spheroids formed at different initial seeding densities (5000; 10000; 15000; and 20000 cells) over a 15-day period. Scale bar: 500 µm. (**B**) Quantification of spheroid volume across seeding densities and time. Data are presented as mean ± SEM of $n = 5$ independent spheroids per group. The error bars represent the SEM. Statistical analysis was performed using a two-way repeated measures ANOVA with Geisser-Greenhouse correction, which showed significant main effects of density and time and a significant interaction ($p < 0.0002$ for all). Dunnett's multiple comparisons test was performed against the Day 3 value for each respective seeding density. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, not significant. The 20,000 cells density was selected for subsequent experiments to generate large spheroids capable of developing a necrotic core, mimicking advanced disease. BC, breast cancer; SEM, standard error of the mean.

3.4. The MCF-7 BC Cell Lines Successfully Developed Spheroids with Different Seeding Densities

We next sought to validate our model in the hormone-responsive MCF-7 BC cell line under identical conditions (Figure 4A). Spheroid volume was significantly influenced by both initial seeding density ($p = 0.0004$) and time ($p < 0.0001$). However, unlike the MDA-MB-231 model, the interaction between density and time was not significant ($p = 0.1132$) (Figure 4B), indicating that growth rates were more consistent across the different densities in this cell line.

Post-hoc analysis (Dunnett's test against Day 3 for each density) confirmed that all densities eventually produced significant volumetric growth by later time points (e.g., 5000 cells: Day 6, $p = 0.0319$; 20,000 cells: Day 9, $p = 0.0009$) (Figure 4B). To maintain experimental consistency and ensure the formation of large spheroids capable of developing a complex 3D architecture for subsequent treatment, the 20,000 cells density was also selected for the MCF-7 model (Figure 4B). This approach allows for a direct comparative analysis of Vit-C and chemotherapy effects across BC subtypes with differing pathophysiologies.

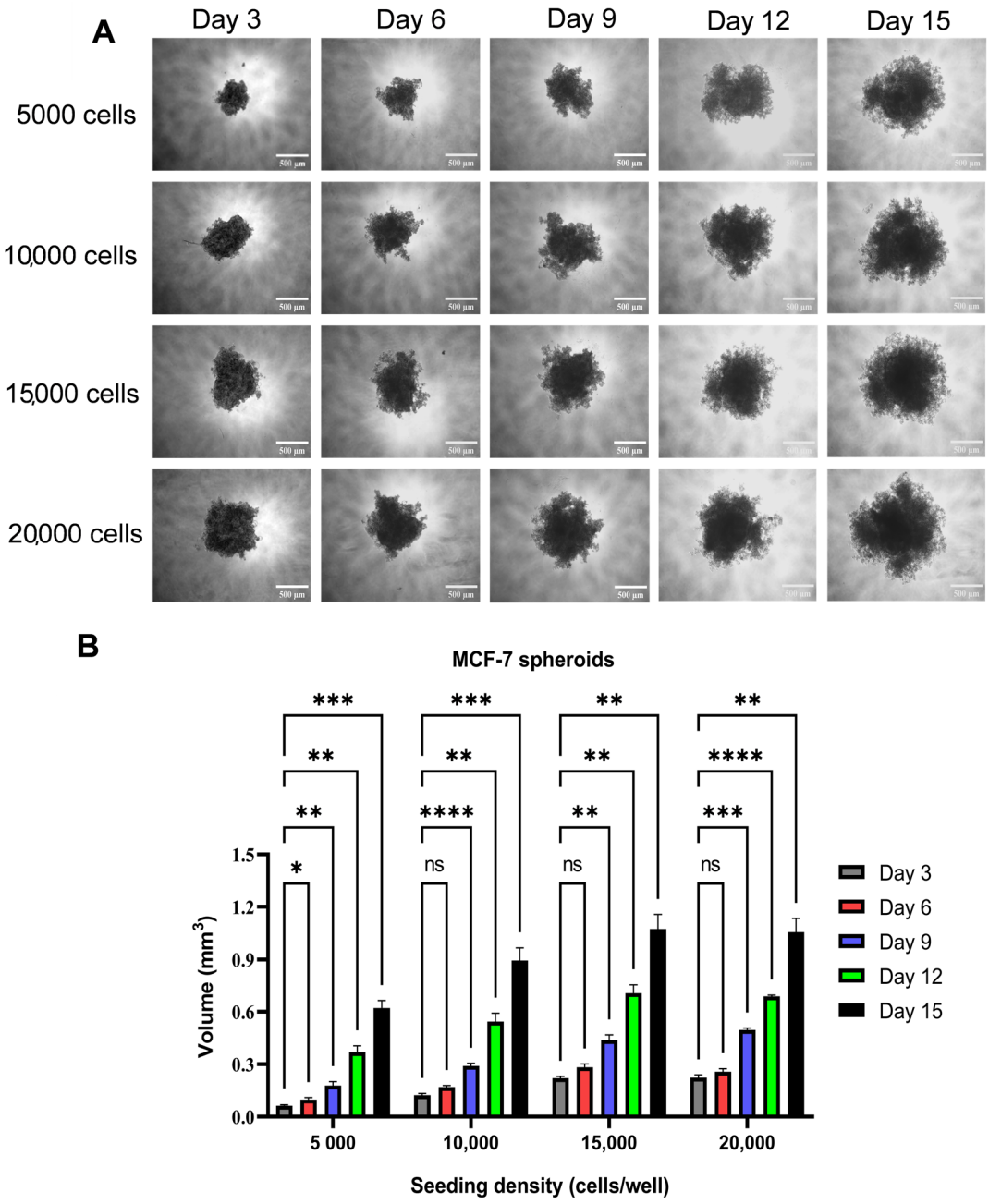


Figure 4. Morphological Evaluation and Growth Kinetics of MCF-7 Spheroids over 15 Days. (**A**) Representative bright-field microscopy images of spheroids formed at different initial seeding densities (5000; 10000; 15000; and 20000 cells) over a 15-day period. Scale bar: 500 µm. (**B**) Quantification of spheroid volume across seeding densities and time. Data are presented as mean ± SEM of $n = 5$ independent spheroids per group. The error bars represent the SEM. Statistical analysis was performed using a two-way repeated measures ANOVA with Geisser-Greenhouse correction, which showed significant main effects of density ($p = 0.0004$) and time ($p < 0.0001$) but no significant interaction ($p = 0.1132$). Dunnett's multiple comparisons test was performed against the Day 3 value for each respective seeding density. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, not significant. The 20,000 cells density was selected for subsequent experiments to maintain consistency with the MDA-MB-231 model. Vit-C, vitamin C; BC, breast cancer; SEM, standard error of the mean.

3.5. Vit-C Enhanced the Function of both NK Cells and CD8⁺ T Cells When Co-Cultured with MDA-MB-231 and MCF-7 Tumor Spheroids

To explore the direct influence of Vit-C on the cytotoxic capabilities of NK cells, following a 24-h co-culture, NK cells pre-treated with Vit-C showed an enhancement in cytotoxicity against tumor spheroids compared to untreated controls, as measured by the CyQUANT™ LDH Cytotoxicity Assay at a 2:1 ratio. Vit-C pre-treatment significantly enhanced the cytotoxic function of NK cells against both BC models. Against triple-negative MDA-MB-231 spheroids, cytotoxicity was augmented by 4.0-fold, increasing from 8.82% to 35.01% (unpaired *t*-test; $p = 0.0045$) (Figure 5A). Similarly, against hormone-responsive MCF-7 spheroids, Vit-C pre-treatment significantly augmented cytotoxicity by 1.7-fold, from 30.32% to 50.59% (unpaired *t*-test; $p = 0.0164$) (Figure 5B). These results demonstrate that pre-treatment with 1 mM Vit-C consistently and significantly enhances the intrinsic cytotoxic capacity of NK cells across BC subtypes.

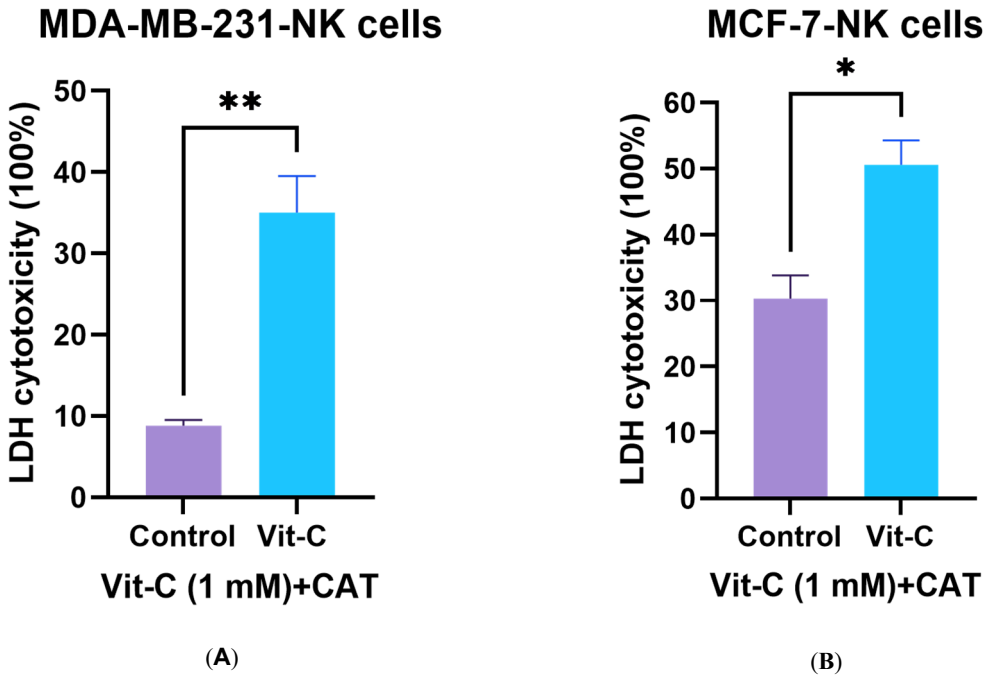


Figure 5. Pre-treatment with 1 mM Vit-C enhances the cytotoxicity of NK cells against BC spheroids. NK cells were pre-incubated with 300 U/mL catalase for 30 min and subsequently primed with 1 mM Vit-C for 6 h before being co-cultured with (**A**) MDA-MB-231 or (**B**) MCF-7 spheroids at a 2:1 E:T ratio for 24 h. NK cell-mediated cytotoxicity was quantified by measuring LDH release using the CyQUANT™ LDH Cytotoxicity Assay. Data are presented as mean ± SEM of $n = 3$ independent measurements. Statistical significance was determined by an unpaired *t*-test comparing the Vit-C primed group to the untreated control. * $p < 0.05$, ** $p < 0.01$; ns, not significant. Vit-C, vitamin C; NK, natural killer; BC, breast cancer; LDH, lactate dehydrogenase; SEM, standard error of the mean; ns, not significant; E:T, effector-to-target.

To investigate the potential synergy between Vit-C and T cell receptor co-stimulation, CD8⁺ T cells were pre-treated with Vit-C (1 mM), CD3/CD28 Dynabeads, or their combination prior to a 24-h co-culture with BC spheroids. Cytotoxicity was assessed using the CyQUANT™ LDH Cytotoxicity Assay at a 2:1 E:T ratio. A one-way ANOVA revealed a significant effect of treatment on CD8⁺ T cell cytotoxicity against both MDA-MB-231 ($p < 0.0001$) (Figure 6A) and MCF-7 ($p < 0.0001$) spheroids (Figure 6B). Post-hoc analysis (Dunnett's test) confirmed that Dynabeads co-stimulation alone did not significantly increase cytotoxicity beyond basal levels against either spheroid type (MDA-MB-231, $p = 0.6872$; MCF-7, $p = 0.9247$).

The post-hoc analysis revealed that the combination of Vit-C and dynabeads generated a robust synergistic response, resulting in a profound 4.10-fold increase in cytotoxicity against MDA-MB-231 spheroids ($p < 0.0001$) (Figure 6A) and a 3.29-fold increase against MCF-7 spheroids ($p < 0.0001$) compared to the untreated control (Figure 6B). This synergy highlights the role of Vit-C as a potent potentiator of CD3/CD28-driven CD8⁺ T cell activation against BC.

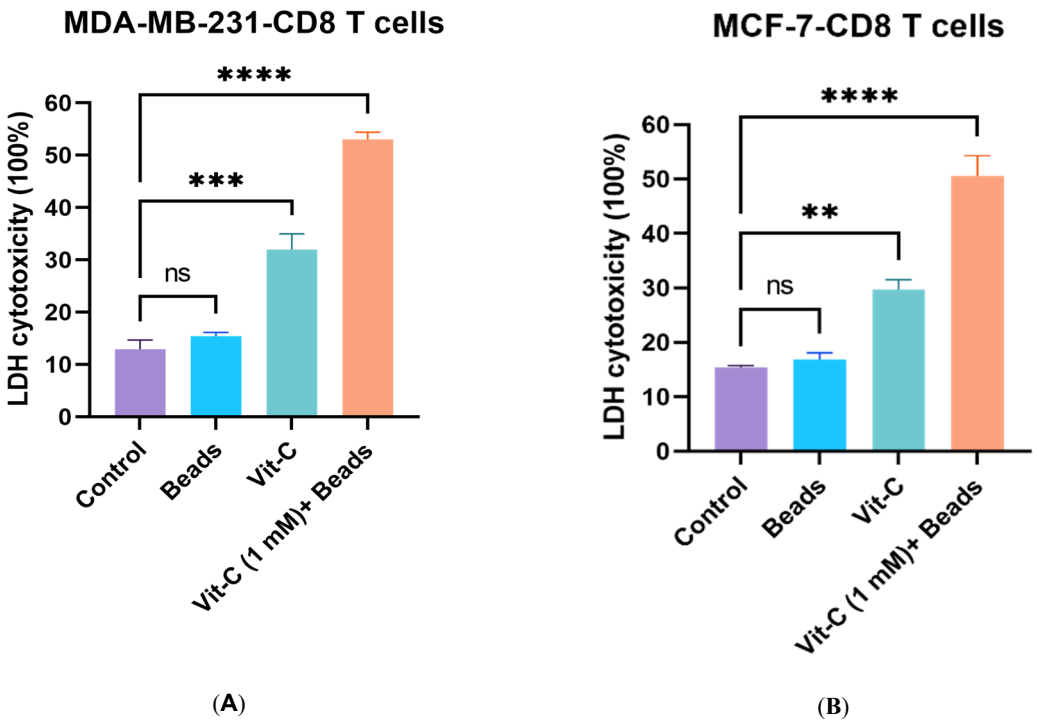


Figure 6. Vit-C synergizes with CD3/CD28 co-stimulation to enhance $CD8^+$ T cell cytotoxicity. $CD8^+$ T cells were activated using CD3/CD28 Dynabeads for 24 h, followed by a 6-h priming period with 1 mM Vit-C in the presence of 300 U/mL CAT. Following the priming period, cells were washed and co-cultured for 24 h with (**A**) MDA-MB-231 or (**B**) MCF-7 spheroids at a 2:1 E:T ratio. Cytotoxicity was quantified by measuring LDH release using the CyQUANT™ LDH Cytotoxicity Assay and is expressed as a percentage of LDH release relative to the control. Data are presented as mean ± SEM of n = *3* independent experiments. The error bars represent the SEM. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test against the control group. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, not significant. Vit-C, vitamin C; SEM, standard error of the mean; LDH, lactate dehydrogenase; E:T, effector-to-target.

3.6. Vit-C Augment the Production of IFN-γ by NK Cells and $CD8^+$ T Cells in the Co-Culture with MDA-MB-231 and MCF-7 Tumor Spheroids

Following pre-treatment with Vit-C, the co-culture of immune cells with tumor spheroids led to a significant increase in granzyme B production, as quantified by ELISA. When co-cultured with MDA-MB-231 spheroids, Vit-C pre-treated NK cells significantly elevated IFN-γ secretion to 6.684 ± 0.713 ng/mL, a 6.9-fold increase compared to the untreated control (0.966 ± 0.058 ng/mL) (unpaired *t*-test; $p = 0.0153$) (Figure 7A). Vit-C pre-treated $CD8^+$ T cells also significantly increased IFN-γ production to 9.379 ± 1.409 ng/mL versus 1.036 ± 0.349 ng/mL in controls, a 9.1-fold increase (unpaired *t*-test; $p = 0.0041$) (Figure 7B). The most pronounced effect was observed with the combination of pre-treated NK and $CD8^+$ T cells, which synergistically induced IFN-γ secretion to 9.797 ± 1.390 ng/mL, a 9.5-fold increase that was significantly greater than the control (1.029 ± 0.210 ng/mL) (unpaired *t*-test; $p = 0.0032$) (Figure 7C).

For MCF-7 spheroids, Vit-C pre-treated NK cells significantly elevated IFN-γ secretion to 5.129 ± 1.099 ng/mL, a 6.5-fold increase compared to the control (0.786 ± 0.349 ng/mL) (unpaired *t*-test; $p = 0.0292$) (Figure 7D). Pre-treated $CD8^+$ T cells significantly enhanced IFN-γ secretion by 6.9-fold to 6.280 ± 0.719 ng/mL versus control (0.904 ± 0.359 ng/mL) (unpaired *t*-test; $p = 0.0049$) (Figure 7E). The combination of both pre-treated immune cells also significantly enhanced IFN-γ secretion, yielding a 16.6-fold increase to 10.89 ± 1.131 ng/mL compared to the control (0.655 ± 0.105 ng/mL) (unpaired *t*-test; $p = 0.0028$) (Figure 7F). These findings collectively indicate that Vit-C pre-treatment robustly enhances the ability of both NK cells and $CD8^+$ T cells to produce the key anti-tumor cytokine IFN-γ in the TME.

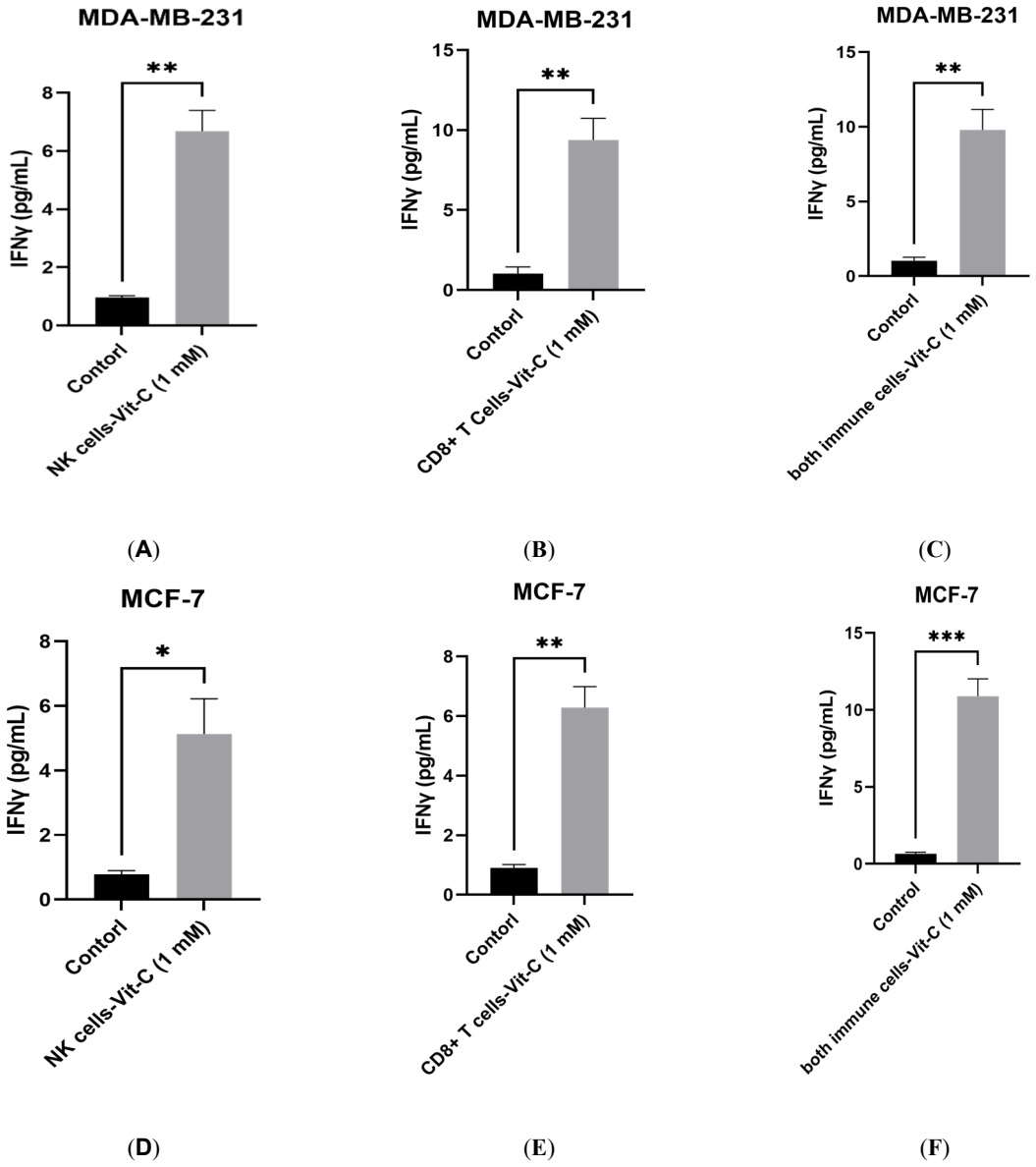


Figure 7. Vit-C enhances IFN-γ secretion by immune cells in a BC spheroid co-culture model. Immune cells were pre-incubated with 300 U/mL CAT and primed with 1 mM Vit-C for 6 h before a 24-h co-culture with tumor spheroids at an E:T ratio of 2:1 for NK cells alone or $CD8^+$ T cells alone, and 1:1:1 for combined NK+$CD8^+$ T cells. $CD8^+$ T cells were activated with CD3/CD28 Dynabeads™ for 24 h prior to the CAT and Vit-C treatment. Following the priming period, cells were washed and introduced to the co-culture system. IFN-γ levels in the supernatant were measured by ELISA. (A) IFN-γ production by NK cells co-cultured with MDA-MB-231 spheroids. (B) IFN-γ production by $CD8^+$ T cells co-cultured with MDA-MB-231 spheroids. (C) IFN-γ production in a combined NK and $CD8^+$ T cell co-culture with MDA-MB-231 spheroids. (D) IFN-γ production by NK cells co-cultured with MCF-7 spheroids. (E) IFN-γ production by $CD8^+$ T cells co-cultured with MCF-7 spheroids. (F) IFN-γ production in a combined NK and $CD8^+$ T cell co-culture with MCF-7 spheroids. Note: The control group in all panels refers to immune cells pre-treated with catalase but not exposed to Vit-C, co-cultured with untreated spheroids. Data are presented as mean ± SEM of $n = 3$ independent experiments. Statistical significance was determined by an unpaired *t*-test comparing the Vit-C group to the untreated control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant. Vit-C, vitamin C; IFN-γ, interferon-gamma; NK, natural killer; SEM, standard error of the mean; ELISA, enzyme-linked immunosorbent assay; E:T, effector-to-target.

3.7. Vit-C augment the Production of Granzyme B by NK Cells and $CD8^+$ T Cells in the Co-Culture with MDA-MB-231 and MCF-7 Tumor Spheroids

Following pre-treatment, the co-culture of immune cells with tumor spheroids led to a significant increase in granzyme B production, as quantified by ELISA. In co-cultures with MDA-MB-231 spheroids, Vit-C pre-treatment significantly enhanced granzyme B secretion across all immune cell populations (Figure 8A–C). Pre-

treated NK cells exhibited a 10.9-fold increase in secretion, reaching 15.32 ± 2.870 ng/mL compared to the control (1.409 ± 1.013 ng/mL) (unpaired *t*-test; $p = 0.0173$) (Figure 8A). Pre-treated $CD8^+$ T cells also showed a significant 11.8-fold increase, with levels at 19.72 ± 4.985 ng/mL versus control (1.672 ± 0.758 ng/mL) (unpaired *t*-test; $p = 0.0355$) (Figure 8B). The most potent effect was observed with the combination of pre-treated NK and $CD8^+$ T cells, which synergistically induced a 64.2-fold increase in granzyme B to 30.96 ± 2.867 ng/mL, a highly significant change from the control (0.482 ± 0.323 ng/mL) (unpaired *t*-test; $p = 0.0009$) (Figure 8C).

This robust enhancement of cytotoxic function was also evident in co-cultures with MCF-7 spheroids. Vit-C pre-treated NK cells significantly increased granzyme B secretion by 10.9-fold to 16.82 ± 2.019 ng/mL (unpaired *t*-test; $p = 0.0016$) (Figure 8D). Pre-treated $CD8^+$ T cells induced a 18.3-fold increase to 26.66 ± 4.104 ng/mL (unpaired *t*-test; $p = 0.0085$) (Figure 8E). The combination of both pre-treated immune cells also triggered a significant 28.1-fold increase, elevating granzyme B to 29.62 ± 5.024 ng/mL compared to the control (1.056 ± 0.159 ng/mL) (unpaired *t*-test; $p = 0.0296$) (Figure 8F). These findings demonstrate that Vit-C pre-treatment is a powerful strategy to boost the cytotoxic potential of both NK and $CD8^+$ T cells, as measured by granzyme B secretion, against BC spheroids.

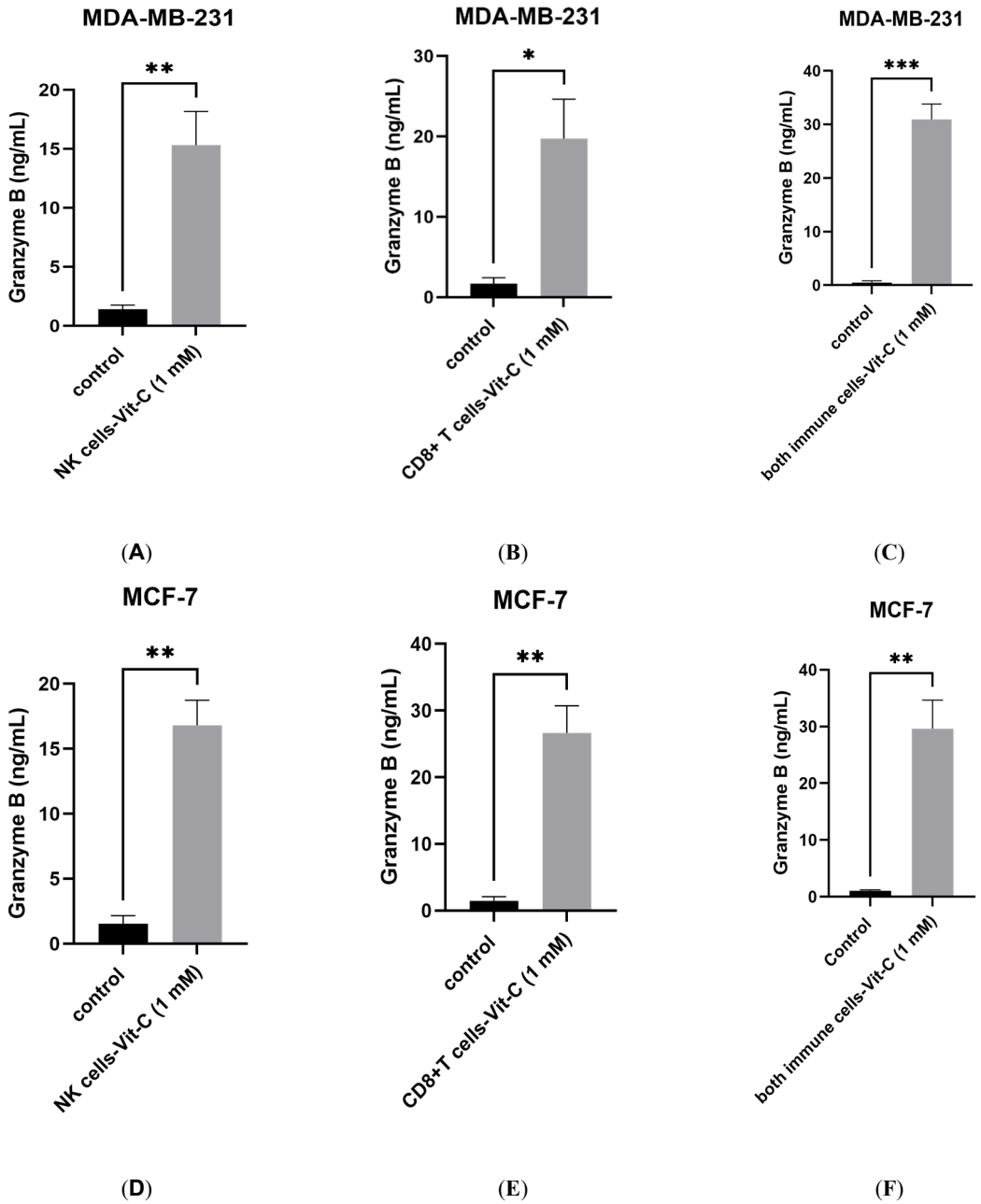


Figure 8. Vit-C pre-treatment enhances Granzyme B secretion in immune cells co-cultured with breast cancer spheroids. Immune cells were pre-incubated with 300 U/mL CAT and primed with 1 mM Vit-C for 6 h before a 24-h co-culture with tumor spheroids at an E:T ratio of 2:1 for NK cells alone or $CD8^+$ T cells alone, and 1:1:1 for combined NK+$CD8^+$ T cells. $CD8^+$ T cells were activated with CD3/CD28 Dynabeads™ for 24 h prior to the CAT

and Vit-C treatment. Following the priming period, cells were washed and introduced to the co-culture system. Granzyme B levels in the supernatant were measured by ELISA. (A) Granzyme B production by NK cells co-cultured with MDA-MB-231 spheroids. (B) Granzyme B production by $CD8^+$ T cells co-cultured with MDA-MB-231 spheroids. (C) Granzyme B production in a combined NK and $CD8^+$ T cell co-culture with MDA-MB-231 spheroids. (D) Granzyme B production by NK cells co-cultured with MCF-7 spheroids. (E) Granzyme B production by $CD8^+$ T cells co-cultured with MCF-7 spheroids. (F) Granzyme B production in a combined NK and $CD8^+$ T cell co-culture with MCF-7 spheroids. Note: The control group in all panels refers to immune cells pre-treated with CAT but not exposed to Vit-C, co-cultured with untreated spheroids. Data are presented as mean ± SEM of $n = 3$ independent experiments. Statistical significance was determined by an unpaired *t*-test comparing the Vit-C group to the untreated control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant. Vit-C, vitamin C; NK, natural killer; SEM, standard error of the mean; ELISA, enzyme-linked immunosorbent assay; E:T, effector-to-target.

3.8. Direct Treatment of MDA-MB-231 and MCF-7 Tumor Spheroids with High-Dose Vit-C and Low-Dose Chemotherapies Augmented IFN-γ Production by Vit-C Pre-Treated Immune Cells

To evaluate the potential synergy between Vit-C and chemotherapy, immune cells (NK cells, $CD8^+$ T cells, or their combination) were first pre-treated with CAT (300 U/mL, 30 min) followed by 1 mM Vit-C for 6 h (the optimal priming condition from Phase 1). MDA-MB-231 or MCF-7 spheroids were pre-treated for 24 h with Vit-C (1 mM), DOX (0.5 μM), DOCE (0.5 μM), or their combination, then washed and co-cultured with the pre-treated immune cells for 24 h. IFN-γ levels in the supernatant were quantified by ELISA. IFN-γ levels in the supernatant were quantified by ELISA. A one-way ANOVA revealed a significant effect of spheroid treatment on IFN-γ secretion in co-cultures with pre-treated NK cells ($p = 0.0002$) (Figure 9A). Post-hoc analysis (Dunnett's test) confirmed that pre-treating spheroids with DOCE resulted in a 4.74-fold increase to 6.335 ± 0.878 ng/mL ($p = 0.0261$), Vit-C induced a 7.08-fold increase to 9.463 ± 0.878 ng/mL ($p = 0.0011$), and the triple combination yielded a 9.77-fold increase to 13.06 ± 0.878 ng/mL ($p < 0.0001$) compared to the untreated control (1.336 ± 0.878 ng/mL). This synergistic effect was more pronounced in co-cultures with pre-treated $CD8^+$ T cells ($p < 0.0001$) (Figure 9B). Post-hoc analysis showed that all treatments significantly enhanced IFN-γ secretion versus control (1.164 ± 1.357 ng/mL): DOX induced an 8.12-fold increase to 9.451 ± 1.357 ng/mL ($p = 0.0051$), DOCE a 6.87-fold increase to 8.000 ± 1.357 ng/mL ($p = 0.0167$), Vit-C a 10.85-fold increase to 12.63 ± 1.357 ng/mL ($p = 0.0005$), and the triple combination a 15.14-fold increase to 17.63 ± 1.357 ng/mL ($p < 0.0001$).

The most robust effect was observed when both pre-treated NK cells and $CD8^+$ T cells were combined ($p < 0.0001$) (Figure 9C). Dunnett's test confirmed that all spheroid treatments significantly boosted IFN-γ levels relative to the control (0.8927 ± 1.262 ng/mL): DOX induced a 10.73-fold increase to 9.581 ± 1.262 ng/mL ($p = 0.0022$), DOCE a 9.59-fold increase to 8.560 ± 1.262 ng/mL ($p = 0.0053$), Vit-C a 14.06-fold increase to 12.55 ± 1.262 ng/mL ($p = 0.0003$), and the triple combination a 19.75-fold increase to 17.63 ± 1.262 ng/mL ($p < 0.0001$). The post-hoc analysis revealed that the combination of all three treatments generated a robust synergistic response, resulting in the most profound increase in IFN-γ secretion. This synergy highlights the role of Vit-C as a potent potentiator of chemotherapy-induced immune activation against BC.

A parallel synergistic effect was observed in co-cultures with MCF-7 spheroids. With pre-treated NK cells (ANOVA; $p = 0.0002$), all treatments significantly increased IFN-γ versus control (0.8770 ± 1.409 ng/mL): DOX (5.77-fold increase to 5.064 ± 1.409 ng/mL, $p = 0.0439$), DOCE (8.05-fold increase to 7.063 ± 1.409 ng/mL, $p = 0.0046$), Vit-C (8.70-fold increase to 7.632 ± 1.409 ng/mL, $p = 0.0025$), and the triple combination (14.01-fold increase to 12.29 ± 1.409 ng/mL, $p < 0.0001$) (Figure 9D). With pre-treated $CD8^+$ T cells ($F(4,10) = 17.04$, $p = 0.0002$), all treatments were also significant versus control (1.352 ± 1.595 ng/mL): DOX (6.52-fold increase to 8.811 ± 1.595 ng/mL, $p = 0.0030$), DOCE (6.62-fold increase to 8.948 ± 1.595 ng/mL, $p = 0.0026$), Vit-C (7.92-fold increase to 10.71 ± 1.595 ng/mL, $p = 0.0006$), and the triple combination (10.39-fold increase to 14.05 ± 1.595 ng/mL, $p < 0.0001$) (Figure 9E). The strongest synergy was again seen with both immune cells combined ($p < 0.0001$). All treatments significantly increased IFN-γ secretion from the control (0.6967 ± 1.476 ng/mL): DOX (15.72-fold increase to 10.95 ± 1.476 ng/mL, $p = 0.0002$), DOCE (13.92-fold increase to 9.698 ± 1.476 ng/mL, $p = 0.0004$), Vit-C (18.64-fold increase to 12.99 ± 1.476 ng/mL, $p < 0.0001$), and the triple combination (26.08-fold increase to 18.17 ± 1.476 ng/mL, $p < 0.0001$) (Figure 9F). This synergy highlights the role of Vit-C as a potent potentiator of chemotherapy-induced immune activation against BC.

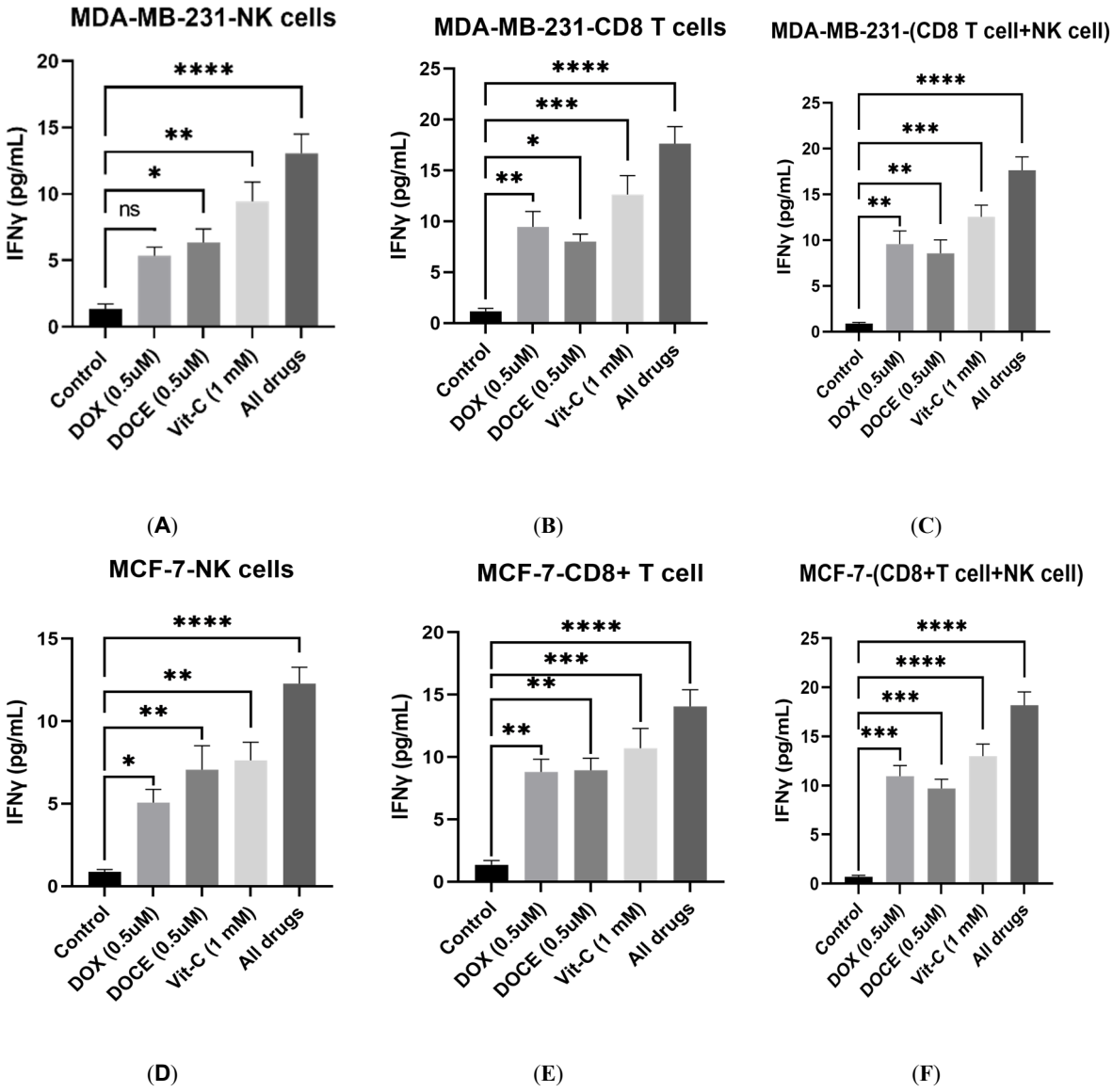
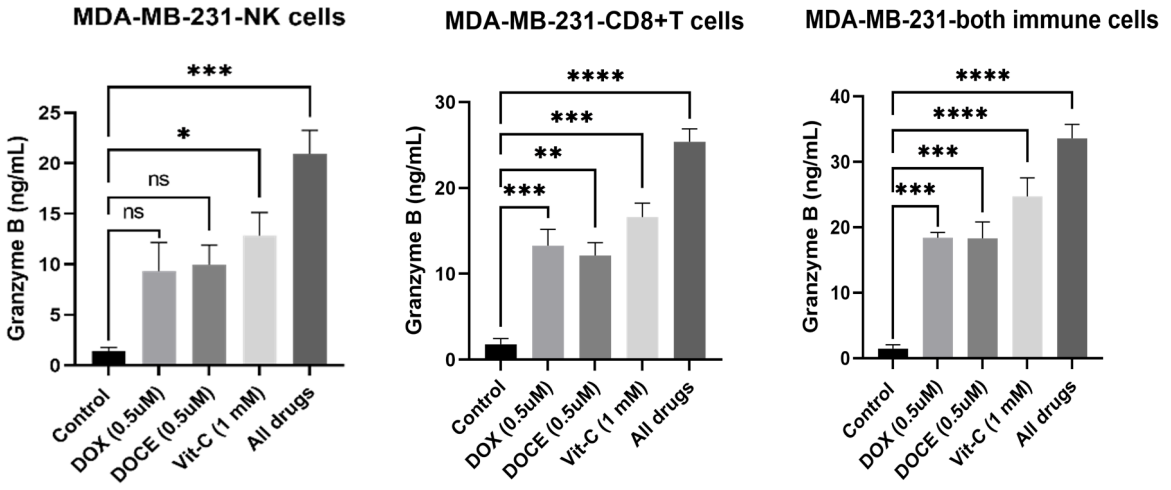


Figure 9. Vit-C synergizes with chemotherapy to enhance IFN-γ secretion in a spheroid co-culture model. Spheroids were pre-treated for 24 h with Vit-C (1 mM), DOX (0.5 μM), and DOCE (0.5 μM), alone or in combination. Immune cells were pre-incubated with CAT (300 U/mL) and primed with 1 mM Vit-C for 6 h. $CD8^+$ T cells were activated with CD3/CD28 Dynabeads™ for 24 h prior to CAT and Vit-C treatment. A 24-h co-culture was then performed, and IFN-γ levels in the supernatant were measured by ELISA. (A) IFN-γ production in co-culture with MDA-MB-231 spheroids and NK cells at an effector-to-target (E:T) ratio of 2:1. (B) IFN-γ production in co-culture with MDA-MB-231 spheroids and $CD8^+$ T cells at an E:T ratio of 2:1. (C) IFN-γ production in co-culture with MDA-MB-231 spheroids and a combined population of NK cells and $CD8^+$ T cells at a 1:1:1 ratio. (D) IFN-γ production in co-culture with MCF-7 spheroids and NK cells at an E:T ratio of 2:1. (E) IFN-γ production in co-culture with MCF-7 spheroids and $CD8^+$ T cells at an E:T ratio of 2:1. (F) IFN-γ production in co-culture with MCF-7 spheroids and a combined population of NK cells and $CD8^+$ T cells at a 1:1:1 ratio. Note: The control group for immune cells refers to cells pre-treated with CAT but not exposed to Vit-C, while for BC cells, spheroids were supplied with ddH_2O. For panels (C) and (F), the 1:1:1 ratio indicates equal numbers of NK cells, $CD8^+$ T cells, and spheroid cells. Data are presented as mean ± SEM from three independent experiments (n=3). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test versus the untreated control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Vit-C, vitamin C; DOX, doxorubicin; DOCE, docetaxel; SEM, standard error of the mean; NK, natural killer; CAT, catalase; ddH_2O, double-distilled water.

3.9. Direct Treatment of MDA-MB-231 and MCF-7 Tumor Spheroids with High-Dose Vit-C and Low-Dose Chemotherapies Augmented Granzyme B Production by Vit-C Pre-Treated Immune Cells

To investigate the synergy between Vit-C and chemotherapy, breast cancer spheroids were pre-treated with Vit-C (1 mM), DOX (0.5 μM), DOCE (0.5 μM), or their combination prior to a 24-h co-culture with pre-activated immune cells. Granzyme B levels in the supernatant were quantified by ELISA. In co-cultures with MDA-MB-231 spheroids, the effect of spheroid treatment on granzyme B secretion varied by immune cell type. In co-cultures with pre-treated NK cells, one-way ANOVA revealed a significant overall effect ($p = 0.0011$). Post-hoc analysis (Dunnett's test) showed that pre-treating spheroids with Vit-C (1 mM) resulted in a significant 9.14-fold increase to 12.87 ± 2.990 ng/mL ($p = 0.0108$) and the triple combination yielded a significant 14.88-fold increase to 20.95 ± 2.990 ng/mL ($p = 0.0003$) compared to the untreated control (1.408 ± 2.990 ng/mL). Pre-treatment with DOX (9.339 ± 2.990 ng/mL; p = 0.0737) or DOCE (9.944 ± 2.990 ng/mL; $p = 0.0530$) did not significantly increase granzyme B levels above control (Figure 10A). In co-cultures with pre-treated $CD8^+$ T cells ($p < 0.0001$), all treatments significantly enhanced granzyme B secretion versus control (1.776 ± 2.116 ng/mL): DOX induced a 7.48-fold increase to 13.28 ± 2.116 ng/mL ($p = 0.0010$), DOCE a 6.83-fold increase to 12.13 ± 2.116 ng/mL ($p = 0.0021$), Vit-C an 9.36-fold increase to 16.62 ± 2.116 ng/mL ($p = 0.0001$), and the triple combination a 14.29-fold increase to 25.39 ± 2.116 ng/mL ($p < 0.0001$) (Figure 10B). The most robust effect was observed when both pre-treated immune cells were combined ($p < 0.0001$). All spheroid treatments significantly boosted granzyme B levels relative to the control (1.447 ± 2.820 ng/mL): DOX induced a 12.74-fold increase to 18.43 ± 2.820 ng/mL ($p = 0.0005$), DOCE a 12.65-fold increase to 18.30 ± 2.820 ng/mL ($p = 0.0005$), Vit-C a 17.09-fold increase to 24.73 ± 2.820 ng/mL ($p < 0.0001$), and the triple combination a 23.21-fold increase to 33.59 ± 2.820 ng/mL ($p < 0.0001$) (Figure 10C).

A more pronounced synergistic effect was consistently observed in co-cultures with MCF-7 spheroids. With pre-treated NK cells (ANOVA, $F(4,10) = 19.01$, $p = 0.0001$), all treatments significantly increased granzyme B versus control (1.792 ± 3.390 ng/mL): DOX induced an 8.96-fold increase to 17.85 ± 3.390 ng/mL (p = 0.0027), DOCE a 7.03-fold increase to 14.39 ± 3.390 ng/mL ($p = 0.0130$), Vit-C a 12.62-fold increase to 24.41 ± 3.390 ng/mL ($p = 0.0002$), and the triple combination a 15.19-fold increase to 29.02 ± 3.390 ng/mL ($p < 0.0001$) (Figure 10D). With pre-treated $CD8^+$ T cells ($p < 0.0001$), all treatments were also highly significant versus control (2.004 ± 4.072 ng/mL): DOX induced an 11.93-fold increase to 25.90 ± 4.072 ng/mL ($p = 0.0006$), DOCE a 16.24-fold increase to 34.55 ± 4.072 ng/mL ($p < 0.0001$), Vit-C a 20.74-fold increase to 43.56 ± 4.072 ng/mL ($p < 0.0001$), and the triple combination a 30.09-fold increase to 62.30 ± 4.072 ng/mL ($p < 0.0001$) (Figure 10E). The strongest synergy was again seen with both immune cells combined ($p < 0.0001$). All treatments significantly increased granzyme B secretion from the control (1.213 ± 5.211 ng/mL): DOX induced a 17.12-fold increase to 21.98 ± 5.211 ng/mL ($p = 0.0085$), DOCE a 13.07-fold increase to 17.07 ± 5.211 ng/mL ($p = 0.0390$), Vit-C a 29.14-fold increase to 35.34 ± 5.211 ng/mL ($p = 0.0002$), and the triple combination a 54.39-fold increase to 67.20 ± 5.211 ng/mL ($p < 0.0001$) (Figure 10F). This robust, consistent synergy across both BC models, resulting in up to a 54-fold increase in cytotoxic granzyme B secretion, underscores the role of Vit-C as a potent potentiator of chemotherapy-induced immune activation.



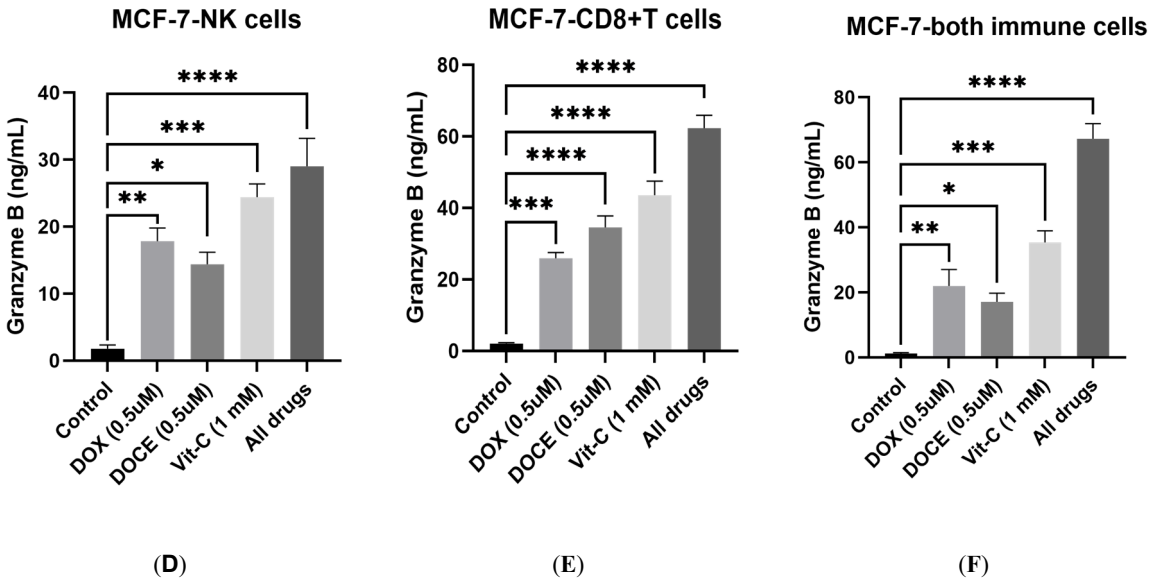


Figure 10. Vit-C synergizes with chemotherapy to enhance Granzyme B secretion in a spheroid co-culture model. Spheroids were pre-treated for 24 h with Vit-C (1 mM), DOX (0.5 μM), and DOCE (0.5 μM), alone or in combination. Immune cells were pre-incubated with CAT (300 U/mL) and primed with 1 mM Vit-C for 6 h. $CD8^+$ T cells were activated with CD3/CD28 Dynabeads™ for 24 h prior to CAT and Vit-C treatment. A 24-h co-culture was then performed, and Granzyme B levels in the supernatant were measured by ELISA. (A) Granzyme B production in co-culture with MDA-MB-231 spheroids and NK cells at an effector-to-target (E:T) ratio of 2:1. (B) Granzyme B production in co-culture with MDA-MB-231 spheroids and $CD8^+$ T cells at an E:T ratio of 2:1. (C) Granzyme B production in co-culture with MDA-MB-231 spheroids and a combined population of NK cells and $CD8^+$ T cells at a 1:1:1 ratio. (D) Granzyme B production in co-culture with MCF-7 spheroids and NK cells at an E:T ratio of 2:1. (E) Granzyme B production in co-culture with MCF-7 spheroids and $CD8^+$ T cells at an E:T ratio of 2:1. (F) Granzyme B production in co-culture with MCF-7 spheroids and a combined population of NK cells and $CD8^+$ T cells at a 1:1:1 ratio. Note: The control group for immune cells refers to cells pre-treated with CAT but not exposed to Vit-C, while for BC cells, spheroids were supplied with ddH_2O. For panels (C) and (F), the 1:1:1 ratio indicates equal numbers of NK cells, $CD8^+$ T cells, and spheroid cells. Data are presented as mean ± SEM from three independent experiments (n=3). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test versus the untreated control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Vit-C, vitamin C; DOX, doxorubicin; DOCE, docetaxel; SEM, standard error of the mean; NK, natural killer; CAT, catalase; ddH_2O, double-distilled water.

4. Discussion

This study successfully established the necessary in vitro foundations—highly pure populations of human natural killer (NK) cells and $CD8^+$ T cells and robust three-dimensional spheroid models of triple-negative and hormone-responsive breast cancer—to demonstrate that pharmacological ascorbate (vitamin C, Vit-C) acts as a powerful modulator of innate and adaptive immune cell function. Our key findings show that Vit-C pre-treatment alone markedly augments the secretion of the critical effector molecules interferon-gamma (IFN-γ) and granzyme B. Furthermore, we identified a profound synergistic effect when Vit-C was combined with sub-lethal conditioning doses of the chemotherapeutic agents, doxorubicin (DOX) and docetaxel (DOCE). This triple combination therapy, targeting the tumor spheroids, elicited the most potent immune activation, resulting in dramatic, synergistic increases in cytotoxic output—with granzyme B levels increasing by over 50-fold in the most responsive co-culture conditions. The consistency of these results across both BC models, underscores the broad potential of Vit-C as an adjuvant to enhance chemo-immunotherapy.

The initial generation of spheroids, established at various cell densities, allowed us to define the ideal growth kinetics and identify a reproducible model. Our data on spheroid growth, particularly the consistent and stable diameter achieved with a seeding density of 20,000 cells/well on Day 15, provided the foundation for subsequent experiments. This optimized model served as the second-generation platform for all our functional assays, including the co-culture experiments with immune cells and various treatments. By rigorously characterizing our spheroid model before use, we ensured that our downstream results, from cytotoxicity assays to cytokine

measurements, were conducted on a robust and physiologically relevant system, effectively bridging the gap between simplified 2D monolayers and complex *in vivo* architectures.

Moreover, BC is a complex disease characterized by a highly heterogeneous TME, which significantly impairs the immune response by inhibiting effector immune cells [4]. This inhibition creates an environment that facilitates tumor growth and complicates treatment strategies. Specifically, BC cells directly suppress the effective immune response of both NK cells and $CD8^+$ T cells through various mechanisms [2]. These cells directly target cancer cells through the secretion of perforin and granzyme B, while also producing IFNγ to enhance their own activation and that of other immune effectors like c-DC1 and M1 macrophages [1]. However, cancer cells evade the anti-tumor immune response by expressing various inhibitory receptors, including PD-L1 and CTLA4, and by secreting inhibitory mediators such as IL-10 and TGF-β, which suppress NK cells and $CD8^+$ T cells [1,2]. For a comprehensive overview of immune suppressive mechanisms and their relation to cancer immune phenotypes, we refer readers to Turan et al. [21]. This indicates that the enhancement or restoration of the functionality of NK cells and $CD8^+$ T cells through agents like Vit-C in 3D spheroid model is of paramount importance, as these structures provide a more accurate representation of the physical and chemical barriers found within the TME.

A critical distinction emerging from our data is the dual role of Vit-C. While its initial cytotoxic effect on immune cell viability was unequivocally mediated by extracellular H_2O_2 (and completely abrogated by CAT), the subsequent potent functional enhancement—marked increases in IFN-γ and granzyme B secretion during co-culture—likely occurs through a separate, ROS-independent mechanism. This suggests that while oxidative stress may initially threaten cell survival, the functional boost in anti-tumor performance is likely driven by metabolic or epigenetic pathways that are isolated when cells are protected by CAT. This finding aligns with and clarifies prior work, suggesting that the functional benefits of Vit-C can be harnessed independently of its cytotoxic properties [5].

The existing literature on Vit-C's immunomodulatory effects presents a nuanced picture. Previous *in vivo* studies have demonstrated enhanced immune function with supplementation [6,7,17,18], but the direct cellular mechanisms often remained unclear. In vitro studies directly applying Vit-C to immune cells have shown promise [5,19], yet the specific context of a 3D TME and the delineation of cytotoxic versus functional effects were not fully explored. Our study bridges this gap by systematically demonstrating that direct Vit-C priming, when combined with CAT protection, potently enhances immune cell function within a sophisticated 3D co-culture model. This is particularly significant as we observed that high-dose Vit-C (1 mM) enhances the secretion of IFN-γ and granzyme B, and when combined with sub-lethal (0.5 μM) DOX and DOCE conditioning, markedly increases the sensitivity of tumor spheroids to immune-mediated killing.

Several studies found that Vit-C mediate the proliferation and enhance the cytotoxic function and tumor infiltration of NK cells and $CD8^+$ T cells in multiple in vitro and *in vivo* models [5,7,22,23]. A study involving mice deficient in L-gulono-γ-lactone oxidase (Gulo), the enzyme necessary for the final step in *de novo* Vit-C synthesis, revealed that $Gulo^{-/-}$ mice with Vit-C depletion exhibited decreased survival time, which was linked to impaired NK cell function resulting from Vit-C deficiency [22]. High-dose Vit-C supplementation significantly restored NK cell function in these mice. Vit-C stimulated the expression of CD69 and NKG2D, resulting in increased secretion of perforin, granzyme B, and IFN-γ (Figure 11) [22]. While studies by Magrì et al. and others observed comparable findings *in vivo* [6,7,23], the direct administration of Vit-C to effector cells in a controlled 3D environment remained under-documented. Our research demonstrates that direct administration of Vit-C to NK and $CD8^+$ T cells effectively enhances their cytotoxic response, a finding that is consistent with prior 2D studies [5,24] but now validated within a more complex tissue-like architecture. Notably, the inclusion of CAT in our protocol addressed a critical oversight in many previous reports by mitigating the pro-oxidant damage to immune cells, thereby allowing the non-ROS-dependent functional enhancements to manifest.

Moreover, $CD8^+$ T cells, integral to the adaptive immune system, necessitate activation by antigen-presenting cells (APCs) that display tumor antigens through MHC-I. This MHC-I engages with the TCR-CD3 complex on $CD8^+$ T cells and co-stimulatory receptors on APCs, including CD28, which subsequently interacts with CD80 and CD86 on $CD8^+$ T cells [25]. Subsequently, $CD8^+$ T cells in the present research, after treatment with dynabeads (CD3/CD28) followed by Vit-C, exhibited markedly improved functionality, resulting in elevated IFN-γ secretion and increased cytotoxicity, as evidenced by high granzyme B production during co-culture with MDA-MB-231 and MCF-7 spheroids. The findings correspond with an earlier study that demonstrated comparable results for granzyme B production, though not for IFN-γ secretion [5]. This indicates that $CD8^+$ T cells need support from APC cells and Vit-C to exhibit increased cytotoxicity. Furthermore, Vit-C may also act as a metabolic adjuvant that synergizes with TCR-mediated signals to drive peak effector performance.

Numerous studies have examined the direct addition of Vit-C to cancer cells, followed by co-culture with Vit-C-pretreated NK cells and $CD8^+$ T cells, to assess whether Vit-C enhances the cytotoxic activity of these

immune cells against cancer cells. Vit-C was found to induce TET-2 expression, leading to a global 5hmC content increase [5,26]. Specifically, the application of Vit-C in lymphoma cells markedly enhanced the fraction of hydroxymethylation (5hmC), indicative of demethylation, which corresponded with greater sensitivity to cytotoxic $CD8^+$ T cell-mediated killing, as demonstrated by increased granzyme B production [5]. This increase in 5hmC content suggests a potential role for Vit-C in regulating DNA demethylation processes, which could have implications for gene expression and epigenetic modifications. The results were validated through both in vitro and *in vivo* studies, demonstrating that Vit-C markedly induces epigenetic changes enhancing TET-2 activity, thereby promoting the production of IFN-γ through the JAK2/STAT1 signaling pathway, which subsequently facilitates increased $CD8^+$ T cell tumor infiltration [23]. In vitro, Vit-C was shown to negatively regulate PD-L1 expression through ROS-mediated inhibition of pSTAT3 (Figure 11). The reduction in PD-L1 expression correlated with heightened infiltration and cytotoxic activity of $CD8^+$ T cells within the TME *in vivo* [27].

In this research, direct addition of Vit-C alone to MDA-MB-231 and MCF-7 in their 3D structures increased their sensitivity to the Vit-C pre-treated NK cells and $CD8^+$ T cells-mediated cytotoxic killing of $CD8^+$ T cells, as evidenced by the elevation of granzyme B levels as well as enhanced IFN-γ production. However, the addition of combined Vit-C, DOX, and DOCE sensitized MDA-MB-231 and MCF-7 spheroids even more significantly, especially in the groups co-cultured with both Vit-C pre-treated NK cells and $CD8^+$ T cells. The amount of the produced IFN-γ and granzyme B was augmented significantly compared to Vit-C sensitization alone. This suggests that Vit-C, DOX, and DOCE acted synergistically to sensitize MDA-MB-231 and MCF-7 spheroids; however, the exact mechanisms were not discussed in this research. Further investigations are needed to elucidate the precise pathways through which these agents interact and enhance immune responses ; while the exact molecular pathways were not the primary focus of this research, our data providing a strong foundation for several likely mechanisms.

A possible mechanism by which Vit-C sensitizes MDA-MB-231 and MCF-7 spheroids to NK cell and $CD8^+$ T cell cytotoxic killing is to induce epigenetic changes by restoring the function of TET-2 as well as down-regulating PD-L1 expression. DOX down-regulates PD-L1 expression [28], whereas DOCE up-regulates it [29]. This suggests that the inclusion of Vit-C and DOX may collaboratively counteract any DOCE-induced PD-L1 elevation, effectively maintaining a more favorable "immune-open" window. Additionally, DOCE induces cell cycle arrest, which, in combination, may sensitize MDA-MB-231 and MCF-7 spheroids to NK cell- and $CD8^+$ T cell-mediated killing. This modulation of PD-L1 expression could enhance the recognition and elimination of cancer cells by immune cells, thereby improving the efficacy of immunotherapeutic strategies. Additionally, the restoration of TET-2 function may lead to further alterations in gene expression patterns that favor an anti-tumor immune response, while DOX, and DOCE can disrupt DNA replication, and induce cellular arrest thus together with Vit-C they sensitize cancer cell for NK cell and $CD8^+$ T cell cytotoxic killing. Moreover, Vit-C, DOX, and DOCE were observed to up-regulate Fas, TRAIL, and TRAILR, thereby enhancing the vulnerability of MDA-MB-231 and MCF-7 spheroids to killing by NK cells and $CD8^+$ T cells [26,30–33]. In fact, Vit-C and DOCE can epigenetically enhance the expression of TRAIL. Vit-C-mediated TET activation led to TRAIL overexpression [26], whereas DOCE-mediated downregulation of enhancer of zeste homolog 2 (EZH2) also resulted in TRAIL overexpression [34]. Together, these convergent pathways increase the susceptibility of the 3D spheroid architecture to the cytotoxic machinery of primed immune cells.

Furthermore, aside from the mechanisms previously discussed regarding the modulation of NK cell and $CD8^+$ T cell activity by Vit-C, it is noteworthy that Vit-C possesses the capability to inhibit HIF-1α in BC [35]. Hypoxia undeniably represents a fundamental characteristic of solid tumors and is thoroughly documented in 3D tumor spheroids across various cancers, including breast cancer [36]. Hypoxia triggers the activation of a crucial transcription factor, HIF-1α, which subsequently initiates numerous signaling and metabolic pathways that facilitate tumor growth by promoting metastasis and suppressing the anti-tumor immune response [36]. HIF-1α is recognized for its role in inducing resistance to DOX and DOCE in BC [37,38]. Consequently, the inhibition of HIF-1α by Vit-C may reverse this inherent chemotherapy resistance, making the hypoxic core of MDA-MB-231 and MCF-7 spheroids significantly more vulnerable to the cytotoxic effects of infiltrating NK and $CD8^+$ T cells. Collectively, these mechanisms increase the effectiveness of immunotherapies for BC, indicating that the combination of Vit-C with sub-lethal chemotherapeutic conditioning represents a promising strategy to address drug resistance and enhance patient outcomes (Figure 11). Additional research is required to comprehensively understand the mechanisms involved and to refine treatment protocols.

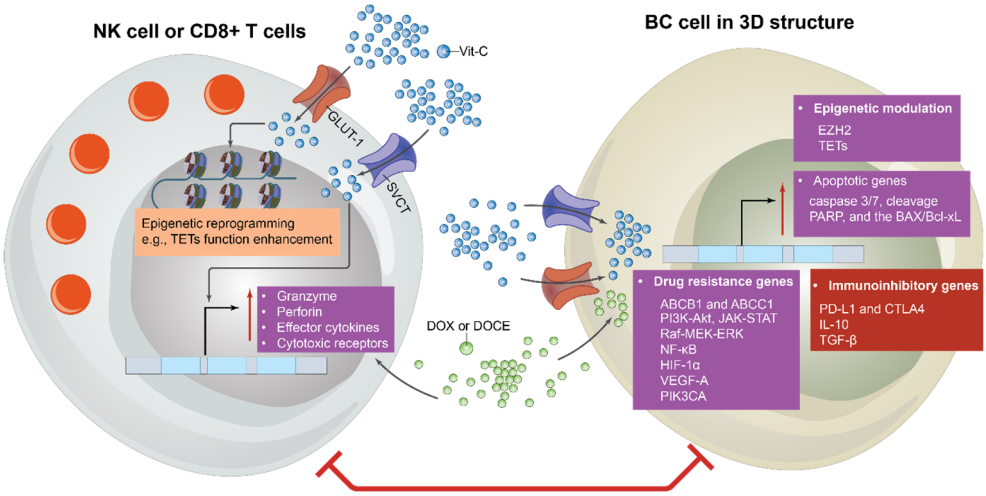


Figure 11. The interaction between Vit-C-treated immune cells and 3D BC spheroids. Proposed mechanisms of the addition of Vit-C to NK cells and $CD8^+$ T cells alone or to DOCE, and DOX can augment the antitumor immune response of each cell leading to enhanced suppressive activity.

5. Limitations of the Study

Despite the robust findings presented herein, it is important to acknowledge several limitations inherent to this study. Firstly, while our 3D spheroid model offers a more physiologically relevant system than traditional 2D cultures, it remains a simplification of the complex human TME. The absence of key stromal and immune cells, such as cancer-associated fibroblasts, endothelial cells, and immunosuppressive populations like regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs), means we cannot assess how these potent inhibitory factors might modulate the beneficial effects of Vit-C priming and combination therapy observed in our system.

Furthermore, while we conclusively established that the initial cytotoxic effect of Vit-C is mediated by extracellular H_2O_2, the precise intracellular mechanisms within NK cells and $CD8^+$ T cells responsible for the enhanced functional capacity—such as specific metabolic reprogramming, activation of key signaling pathways (e.g., NF-κB, NFAT), or epigenetic modifications—were not fully elucidated. A deeper mechanistic insight would significantly strengthen our understanding of how oxidative stress translates to improved anti-tumor function.

Finally, the therapeutic strategy of pre-treating both immune cells and spheroids, though highly effective in vitro, presents a significant translational challenge. The clinical feasibility of such a sequential approach is complex. Future work must focus on translating these findings into more applicable *in vivo* models to investigate the pharmacokinetics of achieving effective local concentrations and to explore the efficacy of concurrent, rather than sequential, administration schedules.

6. Conclusions

This study establishes a robust and physiologically relevant 3D spheroid model of BC and demonstrates a novel strategy to enhance tumor immunity by combining high-dose Vit-C with conventional chemotherapy. Our findings reveal that the cytotoxic effect of high-dose Vit-C on immune cells is mediated by oxidative stress, this pro-oxidant damage can be entirely mitigated by CAT. This approach facilitates the safe priming of NK cells and $CD8^+$ T cells to augment their cytotoxic capabilities and enhance their secretion of key effector molecules, including IFN-γ and granzyme B.

The co-culture experiments further highlight the synergistic potential of this strategy, particularly when targeting the complex architecture of a 3D tumor mass. We demonstrate that the combined treatment of tumor spheroids with Vit-C and sub-lethal conditioning doses of DOX and DOCE significantly enhances immune cell-mediated killing. This triple combination consistently resulted in the highest levels of cytotoxicity and cytotoxic molecule secretion across both triple-negative (MDA-MB-231) and hormone-responsive (MCF-7) models, effectively lowering the threshold for immune-mediated apoptosis.

Moreover, while our study used established BC cell lines, the extension to patient-derived spheroids or ex vivo tumor fragments would increase clinical translatability. A recent study by Ramasubramanian et al. [39], xdemonstrated the feasibility of using live tumor fragments from core needle biopsies to assess immunotherapy responses, addressing tumor heterogeneity. Similar approaches could be applied to test Vit-C and chemotherapy combinations in a personalized manner. Additional research is required to comprehensively understand the precise molecular pathways involved and to refine treatment protocols for potential clinical translation.

In conclusion, this research provides compelling evidence that high-dose Vit-C can be used as an effective priming agent to boost innate and adaptive anti-tumor immunity. By bridging the gap between 2D cell culture and complex tumor environments, our results provide a strong preclinical rationale for further *in vivo* investigation of a novel combinatorial immunotherapy that integrates high-dose Vit-C with conventional chemotherapeutic agents for the treatment of BC.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/260420095247605 0/TI-26030126-SM-FC-done.pdf. Table S1: Experimental groups for $CD8^+$ T cell and spheroid co-culture. Table S2: Experimental groups for NK cell and spheroid co-culture. Table S3: Experimental Groupings with Untreated MDA-MB-231 and MCF-7 Spheroids. Table S4: Experimental Groupings for cytokine and Granzyme B production with MDA-MB-231 and MCF-7 Spheroids.

Author Contributions

A.M., M.H., R.H., K.H., N.F.M., A.H.M., M.A.A.-H., and R.M.: Conceptualization; A.M.; M.H., M.T. and A.H.M.: Experimental procedures and data analyses. A.M., M.T., and M.H: Writing—Original draft preparation; R.M., K.H., A.H.M., N.F.M. and R.H.: Writing—Review and Editing; K.H., N.F.M., R.H., R.M., and M.A.A.-H.: Supervision and Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was approved by the Human Research Ethics Committee of Universiti Sains Malaysia (JEPeM-USM) under the study protocol code USM/JEPeM/21080576. All human donors provided informed consent to participate in the study.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest

The authors declare no conflicts of interest.

Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

Abbreviations

5hmC	Hydroxymethylation
APC	Antigen-Presenting Cell
BC	Breast Cancer
CAT	Catalase
CD	Cluster of Differentiation (e.g., CD3, CD8, CD28)
CTLA	Cytotoxic T-Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
DOCE	Docetaxel

DOX	Doxorubicin
ELISA	Enzyme-Linked Immunosorbent Assay
E:T Ratio	Effector-to-Target Ratio
EZH2	Enhancer of Zeste Homolog 2
Fas	FS-7-associated surface antigen (CD95)
Gulo	L-gulono-γ-lactone oxidase
H_2O_2	Hydrogen Peroxide
HIF-1α	Hypoxia-Inducible Factor 1-alpha
IFN-γ	Interferon Gamma
IL-10	Interleukin 10
LDH	Lactate Dehydrogenase
MHC-I	Major Histocompatibility Complex Class I
NK cell	Natural Killer cell
PD-L1	Programmed Death-Ligand 1
PI	Propidium Iodide
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
TCR	T-cell Receptor
TET2	Ten-Eleven Translocation 2
TGF-β	Transforming Growth Factor Beta
TIME	Tumor Immune Microenvironment
TME	Tumor Microenvironment
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRAILR	TRAIL Receptor
Vit-C	Vitamin C

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