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# The Apoptotic Effects of NK-92 Cells Stimulated with an anti-CD226 Antibody on MDA-MB-231 Triple-Negative Breast Cancer Cells

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# Abstract

Research on immunotherapy in breast cancer treatment has recently gained importance. In this context, natural killer (NK) cells have been shown to kill cancer cells without affecting normal cells. Our study used the NK-92 cells that were stimulated with anti-CD226 antibodies (sNK-92) to increase their activity to target MDA-MB-231 triple-negative breast cancer cells. MCF-12A normal breast cells were used as the control in all experiments. The cytotoxic effects of NK-92 and sNK-92 cells on MDA-MB-231 cells were investigated using lactate dehydrogenase tests. The sNK-92 cells were more cytotoxic than NK-92 cells on MDA-MB-231 cells. In contrast, a significant cytotoxic change was not observed in MCF-12A cells cocultured with NK-92 and sNK-92 cells. An increase in granzyme B levels after coculturing with sNK-92 cells was investigated using the granzyme B enzyme-linked immunosorbent assay. The sNK-92 cells secreted more granzyme B than NK-92 cells against MDA-MB-231 cells. This increase was not observed in MCF-12A, indicating that sNK-92 cells specifically target cancer cells. In addition, immunostaining was used to investigate the synthesis level of BAX, CASP3, and CASP9 proteins to determine whether the observed cytotoxic effect was due to apoptosis. These proteins were synthesized more in MDA-MB-231 cells cocultured with sNK-92 than with NK-92 cells. However, no increase in their synthesis was observed in normal breast cells cocultured with NK-92 and sNK-92 cells. In conclusion, NK-92 cells stimulated with anti-CD226 antibodies secrete more granzyme B, resulting in a greater cytotoxic effect by inducing programmed cell death (apoptosis). The fact that the observed effects on breast cancer cells were not observed in normal breast cells indicates that sNK-92 cells specifically target breast cancer cells. These results indicate the potential use of CD226-stimulated NK-92 cells in immunotherapy.


# Introduction

Cancer is a significant health issue characterized by abnormal cell growth and proliferation [1]. Globally, ~ 14 million new cancer cases are reported annually, which is expected to increase in the future [2]. Breast cancer is the most common cancer among women. However, the lack of subtype-specific treatments highlights the urgent need for new therapeutic options. For example, the MDA-MB-231 breast cancer subtype is triple-negative meaning it does not express the estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) making it the most aggressive and challenging to treat using traditional methods. Therefore, more effective and specific treatment strategies are required [3–4]. Consequently, numerous research groups have focused on developing effective therapeutic models for breast cancer. Current traditional treatment methods for breast cancer include surgery, chemotherapy, and radiotherapy. However, hormone therapy, targeted gene therapies, and immunotherapy (biological therapy) have gained recent interest as alternative treatment modalities.

The search for alternative treatments is gaining speed among research groups due to the significant and unavoidable side effects of traditional treatment methods and their prolonged treatment duration. Certain cancer types are unsuitable for chemotherapy, and patients may develop potentially fatal complications, experience a decline in their quality of life, and face a considerable economic burden. These factors highlight the need for new treatment models [5]. Therefore, cancer treatment models are shifting from

traditional methods to cancer-type-specific treatment modalities solely targeting cancer cells without affecting normal cells. Immunotherapy approaches, which aim to directly eliminate cancer cells without harming normal cells, are gaining speed in this direction. These options are also promising for the effective treatment of breast cancer.

Immunotherapy (biological therapy or biotherapy) is a cancer-type-specific treatment modality. It harnesses the body's immune system to combat various diseases, including cancer [6–7]. The potential of tumor-interacting molecules in cancer treatment was first highlighted by Paul Ehrlich's pioneering research in 1900. Immunotherapy aims to stimulate the patient's immune system to kill cancer cells. The immune system can also be enhanced by the body's immune cells, such as macrophages and natural killer (NK), dendritic, T, and B cells, that can be directed toward cancer cells [8].

There is a dynamic balance between cancer cells and the immune system in the body. While the immune system frequently neutralizes cancer cells, it can develop ways to escape them, resulting in uncontrolled cell division and cancer development [9]. Strengthening the immune cells is an important treatment method to increase their effectiveness against cancer cells. Immunotherapy has become a cornerstone of modern oncology, using the body's immune system to fight cancer cells. Cancer immunotherapy aims to activate the immune system against cancer cells by making it more effective and resilient or providing components such as immune system proteins.

Immunotherapy has recently become an important part of treating some cancer types. It is believed that immunotherapy-based treatments will shape future cancer treatment approaches. Therefore, researchers are focusing on approaches that support the immune system and enhance immune cells. Cancer cells express antigens found in normal cells to escape the immune system. However, sometimes the immune system recognizes the cancer cell but fails to mount an adequate response due to the cancer cell's secretion of immunosuppressive factors such as transforming growth factor-beta (TGF-β). Studies have found ways to overcome this by influencing activator receptors that help the immune system recognize and respond to cancer cells or blocking inhibitor receptors with antibodies that have been approved for use in cancer treatment by the US Food and Drug Administration [10–13].

Besides immune cell therapies, studies have also focused on enhancing the immune system to prevent cancer cells from escaping, growing, and spreading. New treatment approaches are being developed to do this [14–16].

NK cells, derived from hematopoietic stem cells in the bone marrow, are a type of lymphocyte that comprises 10% of all lymphocytes in the blood and functions as a specialized defense cell [17]. They play essential roles in the innate and acquired immune systems, activating monocytes and cytotoxic T cells, and also in the maturation of dendritic cells. In addition, they regulate immunoglobulin secretion in B lymphocyte cells. NK cells are crucial to the immune response and do not attack cells expressing major histocompatibility complex-I (MHC-I) molecules at normal levels. Instead, they target cells with decreased or absent MHC-I expression, such as cancer cells or cells infected by viruses.

The most common lysis mechanism of cytotoxic NK cells involves the secretion of lytic granules containing perforin and granzymes. Perforin creates pores in the cell membrane to allow granzymes to enter, and granzymes activate caspases in the apoptotic pathway to kill cancer cells. Additionally, NK cells use Fas ligand (FasL) and tumor necrosis factor (TNF) to affect the external apoptotic pathways of cancer cells and induce cell death. Finally, NK cells impact cancer cells by secreting cytokines interferon (IFN)-γ and TNF-α. These cytokines activate macrophages and dendritic cells, respectively, leading to tumor cell necrosis [18]. Overall, NK cells play a crucial role in activating anti-cancer responses by modulating macrophages, dendritic cells, and T cells by releasing these cytokines [19–20].

NK cells, which comprise about 10% of lymphocytes in the blood, are an essential part of the body's immune system since they can identify and eliminate cancer cells. However, cancer cells can evade NK cells by expressing human leukocyte antigen (HLA)-I molecules, which are also presented by healthy cells.

It is important to activate and increase the persistence of NK cells to prevent tumor cells from escaping this first line of defense. One approach uses activating molecules such as interleukin (IL)-2 to enhance NK cell activity. Another approach uses specific cytokines, inhibitor and activator receptors, and NK cell adoptive transfer to recognize and kill tumor cells more effectively [21]. Different combinations of these methods are being tested to develop the optimal treatment model for each cancer type. NK cell-based cancer immunotherapy is of interest due to its rapid and specific effects against cancer cells without harming healthy cells. NK cells have cytotoxic effects against cancer cells that lead to death via caspase and apoptotic pathways without toxic effects like drugs, making them a promising option for cancer treatment.

NK cells can specifically target cancer cells without harming healthy cells, setting them apart from chemotherapy drugs [8]. They elicit immune responses against transformed cells either directly via cytotoxicity or by secreting cytokines [22]. Among the various NK cell subsets in peripheral blood, NK-92 cells have the highest cytotoxic potential and are considered suitable for clinical development [23]. Studies are underway to enhance the cytotoxicity of NK cells against cancer cells by targeting the activating receptors on NK cells with molecular antibodies [24]. Creating an appropriate microenvironment for NK cells is also being investigated to increase the effectiveness of NK cell-based therapies. As a result of these studies, the availability of NK-92 cells, which can recognize and kill cancer cells in the immune system, has become a focus for developing effective approaches for cancer treatment.

The activation of NK-92 cells depends on the balance between their inhibitory and activating receptors. These cells function in the direction the balance tilts, which makes supporting activating receptors an effective option to activate NK-92 cells and increase their cytotoxicity against cancer cells. Enhancing this balance with new activators can lead to a more effective defense against cancer cells. One activating receptor that plays a vital role in NK-92 cells is CD226 (formerly DNAM-1; clone: NewE1). CD226 is

involved in forming immune signals and triggers the activation of NK-92 cells when on the cell surface [25–26].

This study presents a solution-oriented preliminary work for treating MDA-MB-231 triple-negative breast cancer. Triggering NK cells using an agonistic antibody targeting the activator receptor CD226 is a specific and exciting approach that increases the anti-cancer efficacy of NK-92 cells, targeting cancer cells specifically. Our team anticipates that targeting the CD226 activator receptor with an antibody will increase NK-92 cells' activity and anti-cancer effect against breast cancer cells. Previous studies have shown that the anti-CD226 clone NewE1 is an activator [26]. However, the effect of anti-CD226 (clone NewE1)-stimulated NK-92 cells on MDA-MB-231 breast cancer cells has not been investigated. This study activates NK-92 cells with anti-CD226 clone NewE1 and explores their anti-cancer effect on MDA-MB-231 breast cancer cells as an antibody therapy, a unique approach. The results obtained from this study are expected to shed light on future in vivo and phase studies on using anti-CD226 clone NewE1in treating MDA-MB-231 breast cancer cases. This study aims to increase the cytotoxic efficacy of NK-92 cells against MDA-MB-231 breast cancer cells by activating CD226 receptors with anti-CD226 clone NewE1 without harming normal cells.

# Materials and Methods

### Cell culture

All cells were maintained in a humidified incubator with 5% $CO_2$ at 37°C. NK-92 cells were cultured in TexMACS medium containing 20% fetal bovine serum (FBS), 500 U/mL IL-2, 50 μM β-mercaptoethanol, 1% L-glutamine, and 1% penicillin/streptomycin. The MDA-MB-231 breast cancer cell line was cultured in DMEM High Glucose medium containing 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. The MCF-12A normal breast cell line was cultured in DMEM-F12 medium containing 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 1% epidermal growth factor, and 3% insulin.

### Activation of NK-92 cells with anti-CD226 antibody clone NewE1

While some antibody clones against the CD226 surface receptor on NK-92 cells have shown agonistic effects (e.g., anti-CD226 clone NewE1), others have shown antagonistic effects [27]. Therefore, an agonistic antibody (anti-CD226 clone NewE1) targeting the CD226 receptor was commercially obtained. NK-92 cells were stimulated with anti-CD226 clone NewE1 to increase their activation (sNK-92 cells). At this stage, the NK-92 cells were cultured, and 1 μg/mL of anti-CD226 agonistic antibody was added after ensuring the culture conditions. The cells were activated under incubation conditions for approximately 20–24 hours [28]. Then, the sNK-92 cells were centrifuged, the medium was removed, and they were prepared for the coculture stages.

### The coculture of active NK-92 cells with MDA-MB-231 and MCF-12A cells

MDA-MB-231 and MCF-12A cells were cocultured with sNK-92 cells. For two different experiments (lactase dehydrogenase [LDH] and apoptosis), $1\times10^4$ and $4\times10^4$ sNK-92 cells were transferred to 96- and 24-well plates previously prepared for MDA-MB-231 and MCF-12A cells, respectively. The coculture experiments used a common culture medium for both cells (used 50% of each cell line's medium). The cells were incubated for four hours at 37°C with 5% $CO_2$ [16].

## Cell viability assay (LDH)

An LDH assay was used to assess the effectiveness of sNK-92 cells against breast cancer cells at the specified concentration. Cancer and normal cells were added to each well with a cell count of $5\times10^3$ and cultured in two different plates. After 24 hours of incubation, the culture medium was removed, and the cells were washed with phosphate-buffered saline (PBS) to prepare them for the coculture experiments. MDA-MB-231 and MCF-12A cells were cocultured with sNK-92 cells at 1:1, 1:5, and 1:10 (target: effector) ratios for four hours at 37°C in a 5% $CO_2$ environment. Experiments were conducted according to the LDH assay protocol. The optical densities (ODs) were measured at wavelengths of 490 nm and 690 nm. Spectrophotometric data were subjected to statistical analyses after being obtained as groups and repeats.

## Immunostaining

Coverslips placed in 24-well plates were coated with poly-L-lysine. MDA-MB-231 or MCF-12A cells were seeded at 40,000 cells per well. After 24 hours, the medium was withdrawn, and cells were washed with PBS and cocultured with NK-92 and sNK-92 cells at a 1:10 ratio (target: effector) for six hours. Then, after the removal of NK-92 and sNK-92 cells, cells were fixed with 3.5% paraformaldehyde. Cells in different wells were incubated with anti-BCL2-associated X apoptosis regulator (BAX; Abcam: ab32503; rabbit), anti-caspase 3 (CASP3; Abcam: ab13847; rabbit), or anti-caspase 9 (CASP9; Abcam: ab202068; rabbit) primary antibodies for 24 hours at 4°C. After removing the unattached primary antibodies with PBS, cells were treated with fluorescein isothiocyanate (FITC)-labeled anti-rabbit secondary antibody (Invitrogen: 656111; rabbit) for two hours at 37°C. Then, after washing twice with PBS, cells were incubated with 7-aminoactinomycin D DNA dye for 30 minutes at 37°C. After washing with PBS, the coverslips were covered with covering media on a slide and imaged under a fluorescent microscope.

## Granzyme B ELISA assay

Based on the results obtained from the LDH cytotoxic experiments, NK-92 and sNK-92 groups with a 1:10 (target: effector) ratio showed the greatest cytotoxicity. Therefore, these groups were selected for granzyme concentration analysis using an enzyme-linked immunosorbent assay. Here, $20\times10^3$ MDA-MB-231 cells were cultured in a 24-well plate, which was incubated at 37°C in a 5% $CO_2$ incubator until they were attached to the plate. The supernatant was aspirated, and the cells were cocultured with NK-92 and sNK-92 cells at a 1:10 (Target: Effector) ratio for four hours. The supernatant was collected and centrifuged at 200 g. The liquid phase was removed and transferred to a tube. The obtained cell pellet

was lysed via the freeze-thaw method and then added to the liquid phase. The Human Granzyme B ELISA Kit protocol was followed. The specified standards were prepared, and the samples were measured spectrophotometrically at 450 nm.

### Statistical analysis

Optical density (OD) values from the LDH cytotoxicity and Granzyme-B ELISA analyses were compared using a one-way analysis of variance in the GraphPad Prism 9 software. Six replicates were performed for each experiment of breast cancer cells cocultured with NK-92 or sNK-92 cells. All experimental groups were compared. The Shapiro–Wilk and Kolmogorov–Smirnov normality tests were used in these experiments. Data were tested to have a normal distribution and were compared using Tukey's significance test and one-way analysis of variance. All results with $P < 0.05$ were considered statistically significant.

# Results

### Activating NK-92 cells via stimulation with anti-CD226 clone NewE1

NK-92 cells were stimulated with the anti-CD226 antibody clone NewE1, which were termed sNK-92 cells. NK-92 cells were stimulated via incubation with 1 µg/mL of the anti-CD226 antibody for 24 hours at 37°C in 5% $CO_2$ [28]. A FITC-labeled secondary antibody was used to confirm that the administered antibody bound to the CD226 receptor on the NK-92 cells. As shown in (Fig. 1B), the anti-CD226 antibody (green signal) appears localized on the membrane of NK-92 cells.

### Increased cytotoxicity of sNK-92 cells compared to NK-92 against MDA-MB-231 cells

The differential interference contrast images showed that MDA-MB-231 cells' proliferation was decreased by coculturing with NK-92 and sNK-92 cells compared to non-cocultured cells (control group). However, MDA-MB-231 cells' proliferation appeared lower when cocultured with sNK-92 cells than when cocultured with NK-92 cells. In addition, NK-92 and sNK-92 cells are believed not to affect MCF-12A cell proliferation. An LDH assay experiment was conducted to confirm these results (Fig. 1A).

The LDH assay was used to detect cell cytotoxicity after four hours of coculturing NK-92 or sNK-92 cells with MCF-12A normal breast cells or MDA-MB-231 breast cancer cells at a different target: effector ratios. The OD values obtained from the LDH assay were analyzed with the GraphPad Prism 9 software. The results are shown in (Fig. 2). The LDH assay results indicate no significant differences in cytotoxicity rates for MCF-12A (normal breast) cells cocultured with sNK-92 or NK-92 cells at the different target: effector ratios (1:1, 1:5, and 1:10; Fig. 2A). However, cytotoxicity rates were significantly higher for MDA-MB-231 (breast cancer) cells cocultured with sNK-92 cells than with NK-92 cells at the different target: effector ratios (1:1, 1:5, and 1:10; Fig. 2B).

### Increased granzyme B release from sNK-92 and NK-92 cells after coculturing with MDA-MB-231 cells

A standard curve was created using the standard OD values obtained from the Granzyme B ELISA. The samples' OD data were calculated using the standard curve data. The obtained values were statistically analyzed, and the differences between the groups were evaluated for significance. The Granzyme B ELISA results indicate that sNK-92 cells release significantly more granzyme B than NK-92 cells against MDA-MB-231 cells. In contrast, granzyme B release levels from NK-92 and sNK-92 cells did not differ significantly against MCF-12A cells. Therefore, sNK-92 cells release more granzyme B against cancer cells than normal breast cells (Fig. 3).

### Increased apoptotic proteins in cancer cells cocultured with sNK-92 cells

The cytotoxicity experiment results indicate that the effects of NK-92 and sNK-92 cells on cancer cell lines were the highest at a 1:10 ratio. Therefore, only a 1:10 ratio was used in the immunostaining experiments.

BAX, CASP3, and CASP9 protein levels, which play a role in apoptotic pathways, were higher in MDA-MB-231 cells cocultured with sNK-92 cells than with NK-92 cells. In addition, these proteins were synthesized in the cytoplasm. In contrast, their synthesis did not differ significantly between MCF-12A normal breast cells cocultured with sNK-92 or NK-92 cells (Fig. 4, Fig. 5, Fig. 6).

## Discussion

The mechanism by which NK cells recognize and destroy cancer cells is important in immunosurveillance. Therefore, the interaction between NK and cancer cells is an effective approach in cancer immunotherapy. This study demonstrated the cytotoxic and apoptotic effects of anti-CD226 antibody-stimulated NK cells against triple-negative breast cancer MDA-MB-231 cells. The LDH cytotoxicity test showed that sNK-92 cells were significantly more effective than NK-92 cells against MDA-MB-231 cancer cells at increasing ratios of 1:1, 1:5, and 1:10. Additionally, there was no significant effect observed on normal breast cells (MCF-12A) at these ratios. The synthesis of proteins involved in intrinsic and extrinsic apoptotic pathways was examined. CASP3 is present in both intrinsic and extrinsic pathways, while BAX and CASP9 are present only in the intrinsic pathway. The synthesis of these apoptotic pathway proteins was higher in cocultures of NK-92 and sNK-92 cells with MDA-MB-231 cancer cells than normal breast MCF-12A cells. Furthermore, the sNK-92 group was found to synthesize these proteins more than the NK-92 group, leading to the apoptosis of cancer cells via both intrinsic and extrinsic pathways. However, no significant increase in the synthesis of these apoptotic pathway proteins was detected in normal breast cells cocultured with sNK-92 and NK-92 cells. These results show that NK-92 cells stimulated with anti-CD226 have a cytotoxic effect on triple-negative breast cancer cells via apoptotic pathways.

Activating NK cells with agonistic antibodies and increasing their cytotoxicity against target cells is an essential step in cancer immunotherapy [29–30]. Stimulating activator receptors of NK cells and increasing their activity against target cells is a new mechanism of action in immunotherapy to enhance their anti-cancer properties. However, no previous studies have examined the cytotoxic effect of stimulating CD226 activator receptors on breast cancer cells with agonistic antibodies.

The binding of anti-CD226 antibodies to NK-92 cells after 20 hours of incubation was assessed by immunostaining (Fig. 1B). Validation of the antibody-receptor interaction forms the basis of the experiments performed in this study. Rajagopalan et al. (2006) showed via immunostaining that an anti-killer cell immunoglobulin-like receptor, two Ig domains, and long cytoplasmic tail 4 (KIR2DL4) antibody bound to its corresponding activating receptor on NK cells, increasing their IFN-γ production. They analyzed the amount of IFN-γ in the supernatant using an ELISA, noting that this antibody increased IFN-γ secretion [31]. This study stimulated NK-92 cells with an anti-CD226 antibody, successfully enhancing their cytotoxicity against breast cancer cells. The antibody-receptor binding was validated by immunostaining, similar to Rajagopalan et al. (2006).

This study determined that sNK-92 cells showed significantly higher cytotoxicity for breast cancer cells than normal breast cells (Fig. 2). It showed no cytotoxic effect on normal breast cells at all ratios when comparing NK-92 and sNK-92 cells. These results demonstrate no cytotoxic effect of our NK-92 stimulation process on normal breast cells. These results showed a significant increase in cytotoxicity in the cancer group compared to the control group. Additionally, it was determined that sNK-92 cells had a significantly higher cytotoxic effect on breast cancer cells (MDA-MB-231) at three different ratios (1:1, 1:5, 1:10) compared to NK-92 cells. The highest cytotoxic effect observed in the cancer cell experiments belonged to the sNK-92 cells at a ratio of 1:10. Therefore, it was determined that using a high ratio of sNK-92 cells led to an increased cytotoxic effect on cancer cells. To date, no published study has examined the cytotoxic effects of NK-92 cells stimulated with agonistic antibodies to CD226 receptors against breast cancer cells. The cytotoxic effects of NK cells stimulated with other agents and receptors found in the literature have been investigated by our research group and others.

Our previous study showed that simulating NK-92 cells with anti-KIR2DL4 had cytotoxic and apoptotic effects on $HER2^{+}/HER2^{-}$ breast cancer cells [32]. Similar to this study, we showed that NK-92 cells stimulated with a CD226 activator receptor antibody had greater cytotoxic and apoptotic effects on breast cancer cells than unstimulated NK-92 cells.

Jardine et al. (2012) attempted to promote the effect of NK cells against acute lymphoblastic leukemia by inducing the killer cell lectin-like receptor K1 (KLRK1/NKG2D) on NK cells with a proteasome inhibitor (bortezomib), a histone deacetylase inhibitor (valproic acid), and a thiazolidinedione (troglitazone). They found that all three drugs increased NK cell degranulation via the NKG2D-dependent mechanism and enhanced NK-mediated anti-leukemic effects in vivo. Bortezomib was reported to be the most effective of the three agents [33]. While we used a different receptor to stimulate NK cells, our results are similar to that study.

Wang et al. (2012) increased the activity of NK cells obtained from umbilical cord blood using cytokines (IL-12 and IL-15). Their cytotoxicity against the K562 cell line was determined by WST-1 analysis, showing that induced cells were more cytotoxic than non-induced cells [34]. These findings were similar to the results obtained from our cytotoxicity experiments. Karlıtepe et al. (2021) also investigated the cytotoxic effect of NK cells obtained from untreated umbilical cord blood against breast cancer using the WST-1

assay. Unlike our study, they used total NK cells from umbilical cord blood without any induction process. Freshly harvested NK cells from cord blood showed significant cytotoxicity when cocultured with breast cancer cell lines compared to the control group [35].

Von Strandmann et al. (2006) designed a novel recombinant bi-specific protein (ULBP2-BB4) to activate NK cells via NKG2D. The binding of this protein to the NKG2D receptor strongly activated NK cells and increased their IFN-γ secretion. This activation induces NK cells and elicits cytotoxic effects against multiple myeloma cells in vitro. In an in vivo study, NK cells activated by ULBP2-BB2 inhibited tumor growth [36]. Similar to our study, they increased activation by stimulating a receptor on NK cells. However, our study confirmed the cytotoxic effect of sNK-92 cells using granzyme experiments (Fig. 3). Our study showed that sNK-92 cells secrete higher amounts of granzymes than NK-92 cells.

Turaj et al. (2018) evaluated the anti-tumor effect of NK cells against lymphoma by stimulating the TNF receptor superfamily member 4 (TNFRSF4/OX40/CD134) activation receptor with an agonistic anti-CD134 antibody. This study reported that the OX40 receptor, a TNF receptor family member, had anti-tumor effects in NK cells in various malignancies. NK cells stimulated with agonistic antibodies showed increased IFN-γ production and cell cytotoxicity [37]. While this study used different agonistic antibodies and receptors, the cytotoxic results obtained were similar to ours.

Pahl et al. (2018) showed that the AFM13 agonistic antibody increased the cytotoxic effect of NK cells. NK cells induced with the AFM13 agonistic antibody for a certain period were investigated for their cytotoxic effect against lymphoma cell lines, obtaining significant results. The induced NK cells affected lymphoma cell lines by increasing their cytotoxicity and IFN-γ release [28]. Similar to our study, this study evaluated the effect of agonistic antibody-stimulated NK cells against breast cancer cell lines and found that their cytotoxic effect was increased.

Published studies have shown that agonist antibodies targeting TNF receptor superfamily member 9 (TNFRSF9/CD137), OX40, TNF receptor superfamily member 18 (TNFRSF18/GITR), CD27, CD28, and inducible T cell costimulatory (ICOS) glycoproteins, which are all activating receptors on the NK cells' transmembrane, transmit activating signals to lymphocytes by binding to these receptors. These responses, which strengthen the cellular immune response, have been shown to have anti-tumor effects in mouse models [38]. Similarly, in the studies included in this review, activation was increased by stimulating immune cells externally with agonists [39–41].

Experiments, phase, and clinical studies on combined treatment approaches are ongoing to achieve the most effective treatment model with chemotherapy and clinically used antibodies. This study focused on increasing the activity of NK-92 cells by stimulating their CD226 receptor. Its results showed a significant increase in the cytotoxic properties of all treated groups compared to the control group.

Our immunostaining experiment showed that the synthesis of apoptotic proteins such as BAX, CASP3, and CASP9 in MDA-MB-231 cancer cells was significantly higher in cocultures with sNK-92 cells than NK-92 cells. This finding indicates that the greater cytotoxic effect of sNK-92 cells compared to NK-92 cells

occurs through apoptotic pathways. Additionally, when MCF-12A normal breast cells were cocultured with sNK-92 and NK-92 cells, there was no increase in the synthesis of these apoptotic proteins. This result indicates that the apoptotic pathways of MCF-12A cells were not activated by sNK-92 and NK-92 cells.

This study evaluated the levels of proteins involved in the extrinsic and intrinsic apoptotic pathways. It examined the levels of BAX and CASP9 in the intrinsic pathway and CASP3 in both the extrinsic and intrinsic pathways. Their synthesis was higher in cancer cells than in normal breast cells cocultured with NK-92 and sNK-92. Additionally, their synthesis was higher in the sNK-92 group than in the NK-92 group, leading to apoptosis in cancer cells via both intrinsic and extrinsic pathways. In contrast, their synthesis did not increase significantly in normal breast cells cocultured with sNK-92 or NK-92 cells. Based on these findings, it was concluded that CD226-stimulated NK-92 cells had a lethal effect on MDA-MB-231 triple-negative breast cancer cells via apoptotic pathways.

Zhang et al. (2021) showed that the methionine enkephalin (MENK) pentapeptide activated NK cells against lung cancer in mice, increasing Granzyme B and IFN-γ secretion. Granzyme B and IFN-γ target the apoptotic pathways of tumor cells. This study analyzed the synthesis of apoptotic proteins (BAX, B-cell leukemia/lymphoma 2 [BCL2], and CASP3) in lung cancer cells cocultured with stimulated NK cells by western blot analysis [42]. They observed that their synthesis was elevated in cells cocultured with the stimulated NK cells. This study demonstrated that stimulated NK cells are highly effective against lung cancer cells. The study by Zhang et al. is similar to ours, activating NK cells and analyzing the synthesis of apoptotic proteins. However, their NK cells were stimulated against lung cancer with a peptide.

Cui et al. (2020) induced the cytotoxic effect of NK-92 cells against colon cancer using IL-2 and IL-15 and examined the synthesis of apoptotic pathway proteins BCL2 and BAX [43]. Their results showed increased BAX protein synthesis compared to the reference protein. This increase was similar to our study results.

# Conclusion

Current research focuses on targeting the receptors on NK-92 cells to increase their cytotoxicity and promote them effectively against target cells. In this study, stimulating the CD226 activator receptor on NK-92 cells against target cells is a unique contribution to the literature. The results provide basic information, will pave the way for further studies, and shed light on in vivo and clinical studies. Preclinical studies on the stimulation and autologous and allogeneic use of NK-92 cells have the potential to be translated into clinical applications. The results of these cytotoxicity experiments were supported by immunostaining analysis. The images showed the increased synthesis of apoptotic pathway proteins in breast cancer cells cocultured with sNK-92 cells compared to NK-92 cells. These results are significant regarding leading cancer cells to death via the apoptotic pathway. This study's results suggest that NK-92 cells stimulated with anti-CD226 antibodies can be evaluated for clinical use in clinical trials and in vivo studies. Further studies are needed to support the therapeutic use of this antibody.

# Declarations

### Authors' contributions

MD and NK designed the experiments, developed the methodology analyzed results, prepared data presentations, and wrote and reviewed the draft manuscript. MD was the project supervisor.


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### Data availability

All datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

### Declarations

**Competing interests**, The authors do not declare any conflict of interest.

**Ethical approval** Not applicable

**Consent to participate** Authors agreed to participate in this research.

**Consent for publication** Authors has approved the last version of the manuscript for its submission.

# References


1. Braakhuis, B.J., Tabor, M.P., Kummer, J.A., Leemans, C.R., Brakenhoff, R.H. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. Cancer research, 2003; 63, 1727-1730.
2. Zhou, J. 2014. Advances and prospects in cancer immunotherapy. New Journal of Science, 2014.
3. Cancer Research Institute. "Immunotherapy for Breast Cancer". https://www.cancerresearch.org/immunotherapy/cancer-types/breast-cancer. Date of access: 05 Temmuz 2019.
4. World Health Organization. "What is Cancer". https://www.who.int/cancer/en/, Son erişim tarihi: 05 Temmuz 2019.
5. Wayteck, L., Breckpot, K., Demeester, J., De Smedt, S. C., & Raemdonck, K. A personalized view on cancer immunotherapy. Cancer Lett, 2014; 352(1), 113-125. doi:10.1016/j.canlet.2013.09.016
6. Herbert, T.B., Cohen, S. Stress and immunity in humans: a meta-analytic review. Psychosomatic Medicine, 1993; 55, 364-379.

7. Schenk, D. Amyloid-β immunotherapy for Alzheimer's disease: the end of the beginning, Nature Reviews Neuroscience, 2002;3, 824.
8. Aslan, G. Tümör İmmünolojisi Tumour Immunology. Turk Journal Immunology, 2010; 15, 1.
9. Sharma, P., Hu-Lieskovan, S., Wargo, J.A., Ribas, A. Primary, adaptive, and acquired resistance to cancer immunotherapy, Cell, 2017;168, 707-723.
10. Lesokhin, A.M., Callahan, M.K., Postow, M.A., Wolchok, J.D. On being less tolerant: enhanced cancer immunosurveillance enabled by targeting checkpoints and agonists of T cell activation, Science Translational Medicine, 2015;25(7), 280.
11. Stojanovic, A., Fiegler, N., Brunner-Weinzierl, M., Cerwenka, A. CTLA-4 is expressed by activated mouse NK cells and in hibits NK cell IFN-γ production in response to mature dendritic cells, The Journal of Immunology, 2015; 192, 4184-4191.
12. Huang, B.Y., Zhan, Y.P., Zong, W.J., Yu, C.J., Li, J.F., Qu, Y.M., Han, S. The PD-1/B7-H1 pathway modulates the natural killer cells versus mouse glioma stem cells. PloS One, 2015;18(10), 8.
13. Westin, J.R., Chu, F., Zhang, M., Fayad, L.E., Kwak, L.W., Fowler, N., Romaguera, J., Hagemeister, F., Fanale, M., Samaniego, F. Safety and activity of PD1 blockade by pidilizumab in combination with rituximab in patients with relapsed follicular lymphoma: a single group, open-label, phase 2 trial. The lancet oncology 2014; 15, 69-77.
14. Gardner, R., Wu, D., Cherian, S., Fang, M., Hanafı, L.-A., Finney, O., Smithers, H., Jensen, M.C., Riddell, S.R., Maloney, D.G. Acquisition of a CD19-negative myeloid phenotype allows immune escape of MLL-rearranged B-ALL from CD19 CAR-T-cell therapy, Blood, 2016; 127, 2406-2410.
15. Parlar, A., Sayitoglu, E.C., Ozkazanc, D., Georgoudaki, A.M., Pamukcu, C., Aras, M., Josey, B.J., Chrobok, M., Branecki, S., Zahedimaram, P. Engineering antigen-specific NK cell lines against the melanoma-associated antigen tyrosinase via TCR gene transfer, European Journal of Immunology, 2019; 49(8), 1278-1290.
16. Sayitoglu, E.C., Georgoudaki, A-M., Chrobok, M., Ozkazanc, D., Josey, B.J., Arif, M., Kusser, K., Hartman, M., Chinn, T.M., Potens, R., Pamukcu, C., Krueger, R., Zhang, C., Mardinoglu, A., Alici, E., Temple, H.T., Sutlu, T., and Doganay- Duru., A. Boosting natural killer cell-mediated targeting of sarcoma through DNAM-1 and NKG2D, Frountiers Immunology, 2020; 11,40.
17. Sun, J.C., Lanier, L.L. NK cell development, homeostasis and function: parallels with CD8+ T cells, Nature Reviews Immunology, 2011; 11, 645.
18. Liu, C., Yu, S., Kappes, J., Wang, J., Grizzle, W.E., Zinn, K.R., Zhang, H.-G. Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host, Blood, 2007;109, 4336-4342.
19. Scott, P. IFN-gamma modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. J Immunol, 1991; 147(9), 3149-3155.
20. Brinkman, B., Zuijdeest, D., Kaijzel, E.L., Breedveld, F.C., Verweij, C.L. Relevance of the tumor necrosis factor alpha (TNF alpha)-308 promoter polymorphism in TNF alpha gene regulation. Journal of inflammation 1995; 46, 32-41.

21. Ljunggren, H.G., Malmberg, K.J. Prospects for the use of NK cells in immunotherapy of human cancer, Nature Reviews Immunology, 2007; 7, 329.
22. Pegram,H.J.,Andrews,D.M.,Smyth,M.J.,Darcy,P.K.,Kershaw,M.H. Activatingandinhibitory receptors of natural killer cells, Immunology and Cell Biology, 2011; 89, 216-224.
23. Schönfeld, K., Sahm, C., Zhang, C., Naundorf, S., Brendel, C., Odendahl, M., Nowakowska, P., Bönig, H., Köhl, U., Kloess, S. Selective inhibition of tumor growth by clonal NK cells expressing an ErbB2/HER2-specific chimeric antigen receptor, Molecular Therapy, 2015; 23, 330-338.
24. Guillerey, C., Huntington, N.D., Smyth, M.J. Targeting natural killer cells in cancer immunotherapy. Nature Immunology, 2016; 17, 1025-1036.
25. Hou, S., K. Ge, X. Zheng, H. Wei, R. Sun, and Z. Tian. CD226 protein is involved in immune synapse formation and triggers Natural Killer (NK) cell activation via its first extracellular domain. Journal of Biological Chemistry, 2014; 289:6969-6977.
26. Gaud, G., Lesourne, R. & Love, P.E. Regulatory mechanisms in T cell receptor signalling. Nat Rev Immunol 2018; 18, 485–497 https://doi.org/10.1038/s41577-018-0020-8
27. Jin, H., Ko, M., Choi, D., Kim, J. H., Lee, D., Kang, S.-H., Kim, I., Lee, H. J., Choi, E. K., Kim, K., Yoo, C., & Park, Y. CD226hiCD8+ T Cells Are a Prerequisite for Anti-TIGIT Immunotherapy. Cancer Immunology Research, 2020; 8(7), 912-925. https://doi.org/10.1158/2326-6066.CIR-19-087
28. Pahl, J. H. W., Koch, J., Götz, J.-J., Arnold, A., Reusch, U., Gantke, T., Cerwenka, A. CD16A Activation of NK Cells Promotes NK Cell Proliferation and Memory-Like Cytotoxicity against Cancer Cells. Cancer Immunology Research, 2018; 6(5), 517. doi:10.1158/2326-6066.CIR-17-0550
29. Lin W, Voskens CJ, Zhang X, et al. Fc-dependent expression of CD137 on human NK cells: insights into “agonistic” effects of anti-CD137 monoclonal antibodies. Blood, J Am Soc Hematol. 2008;112(3):699–707.
30. Melero, I., Hirschhorn-Cymerman, D., Morales-Kastresana, A., Sanmamed, M. F., & Wolchok, J. D. Agonist antibodies to TNFR molecules that costimulate T and NK cells. Clinical Cancer Research, 2013; 19(5), 1044-1053.
31. Rajagopalan, S., Bryceson, Y. T., Kuppusamy, S. P., Geraghty, D. E., van der Meer, A., Joosten, I., & Long, E. O. Activation of NK cells by an endocytosed receptor for soluble HLA-G. PLoS Biol, 2006; 4(1), e9. doi: 10.1371/journal.pbio.0040009
32. Kilic, N., Dastouri, M., Kandemir, I. et al. The effects of KIR2DL4 stimulated NK-92 cells on the apoptotic pathways of HER2 + /HER-breast cancer cells. Med Oncol 2023; 40, 139 https://doi.org/10.1007/s12032-023-02009-6
33. Jardine, L., Hambleton, S., Bigley, V., Pagan, S., Wang, X.-N., & Collin, M. Sensitizing primary acute lymphoblastic leukemia to natural killer cell recognition by induction of NKG2D ligands. Leukemia & lymphoma,2013; 54(1), 167-173.
34. Wang J, Sun Z-M, Cao L-L, Li Q. Biological characteristics of cord blood natural killer cells induced and amplified with IL-2 and IL-15. Zhongguo Shi Yan Xue Ye Xue Za Zhi. 2012;20(3):731–5.

35. Karlitepe A, Atakul T, Kilic Eren M. Cytotoxic Effect of cord blood derived natural killer cells on breast cancer cells. Turkish J Oncol/Türk Onkoloji Dergisi 2021;36(2)
36. von Strandmann, E. P., Hansen, H. P., Reiners, K. S., Schnell, R., Borchmann, P., Merkert, S., Purr, I. A novel bispecific protein (ULBP2-BB4) targeting the NKG2D receptor on natural killer (NK) cells and CD138 activates NK cells and has potent antitumor activity against human multiple myeloma in vitro and in vivo. Blood, 2006; 107(5), 1955-1962.
37. Turaj, A. H., Cox, K. L., Penfold, C. A., French, R. R., Mockridge, C. I., Willoughby, J. E., ... & Lim, S. H. Augmentation of CD134 (OX40)-dependent NK anti-tumour activity is dependent on antibody cross-linking. Scientific Reports, 2018; 8(1), 2278.
38. Sanmamed, M. F., Pastor, F., Rodriguez, A., Perez-Gracia, J. L., Rodriguez-Ruiz, M. E., Jure-Kunkel, M., & Melero, I. Agonists of co-stimulation in cancer immunotherapy directed against CD137, OX40, GITR, CD27, CD28, and ICOS. Paper presented at the Seminars in oncology 2015.
39. Song, J., So, T., Cheng, M., Tang, X., & Croft, M. Sustained survivin expression from OX40 costimulatory signals drives T cell clonal expansion. Immunity, 2005; 22(5), 621-631.
40. Ascierto, P. A., Simeone, E., Sznol, M., Fu, Y. X., & Melero, I. Clinical experiences with anti-CD137 and anti-PD1 therapeutic antibodies. In Seminars in oncology 2010; 37, 5, 508-516
41. Kim, B. S., Kim, J. Y., Kim, E. J., Lee, J. G., Joo, D. J., Huh, K. H., ... & Kim, Y. S. Role of thalidomide on the expression of OX40, 4-1BB, and GITR in T cell subsets. In Transplantation proceedings 2016; 48, 4, 1270-1274 Elsevier.
42. Zhang S, Liu N, Ma M, et al. Methionine enkephalin (MENK) suppresses lung cancer by regulating the Bcl-2/Bax/Caspase-3 signaling pathway and enhancing natural killer cell-driven tumor immunity. Int Immunopharmacol 2021; 98:107837 doi: 10.1016/j.intimp.2021.107837.
43. Cui, F., Qu, D., Sun, R., Zhang, M., & Nan, K. NK cell-produced IFN-γ regulates cell growth and apoptosis of colorectal cancer by regulating IL-15. Experimental and therapeutic medicine, 2020; 19(2), 1400- 1406.

# Figures

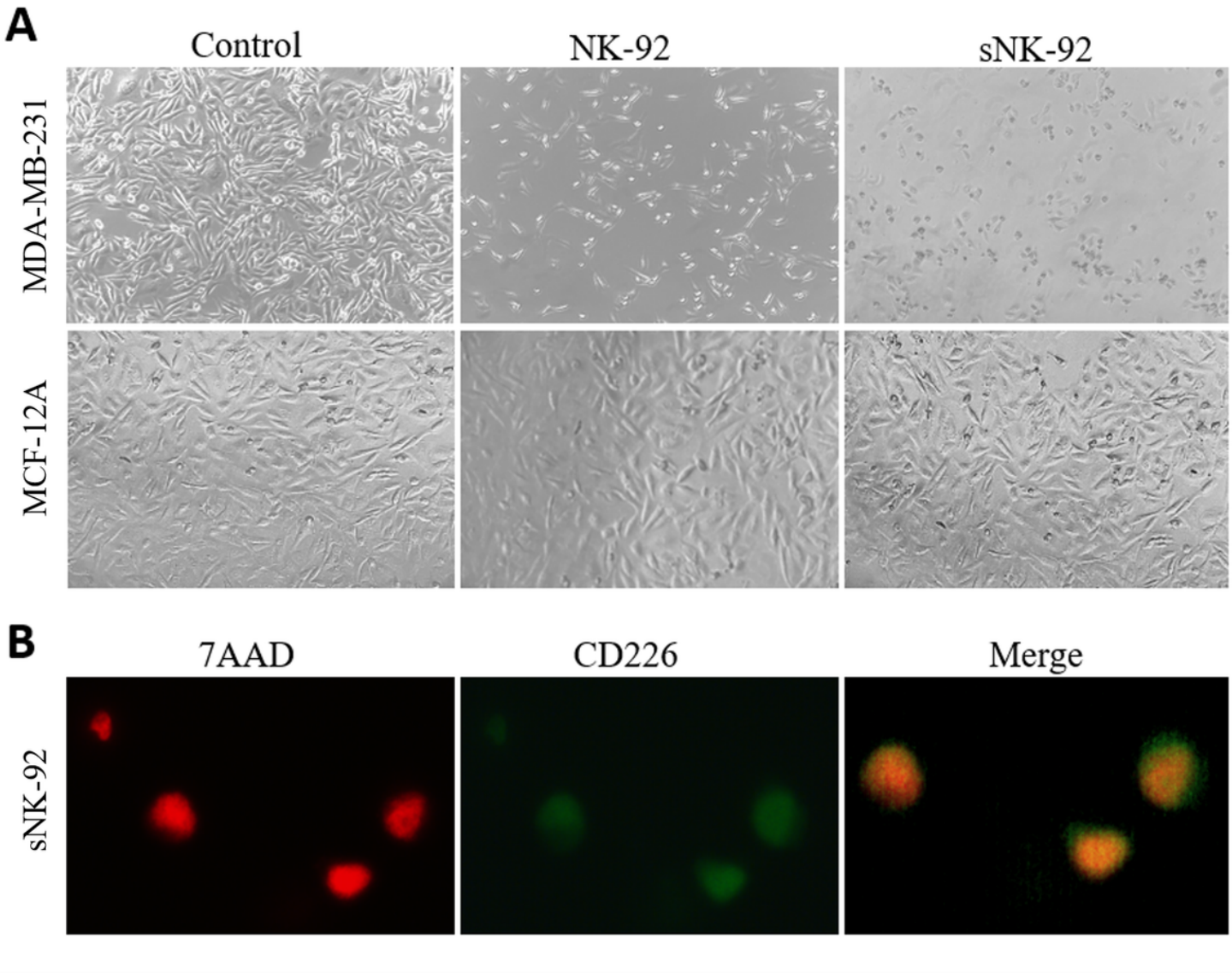
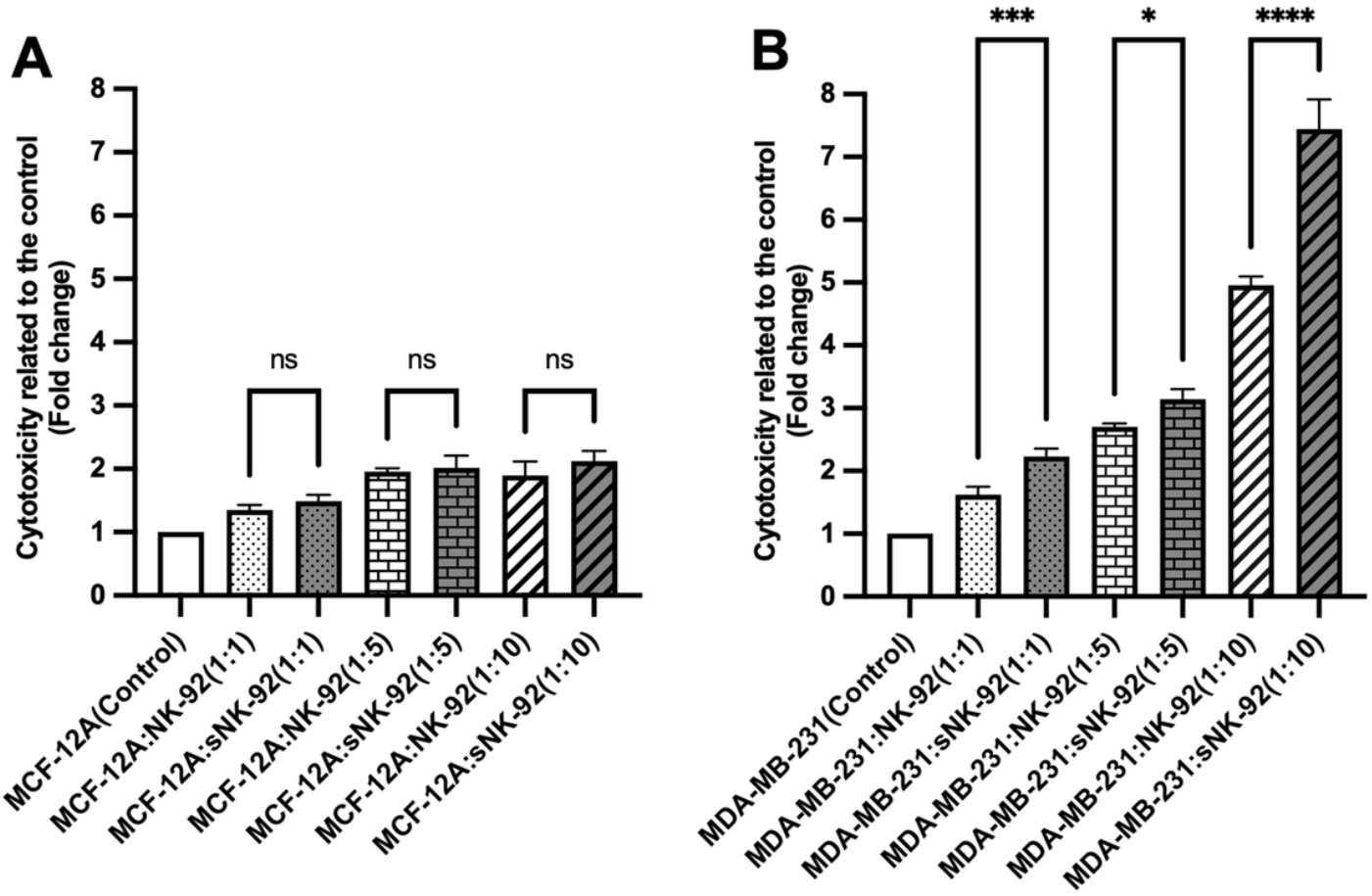


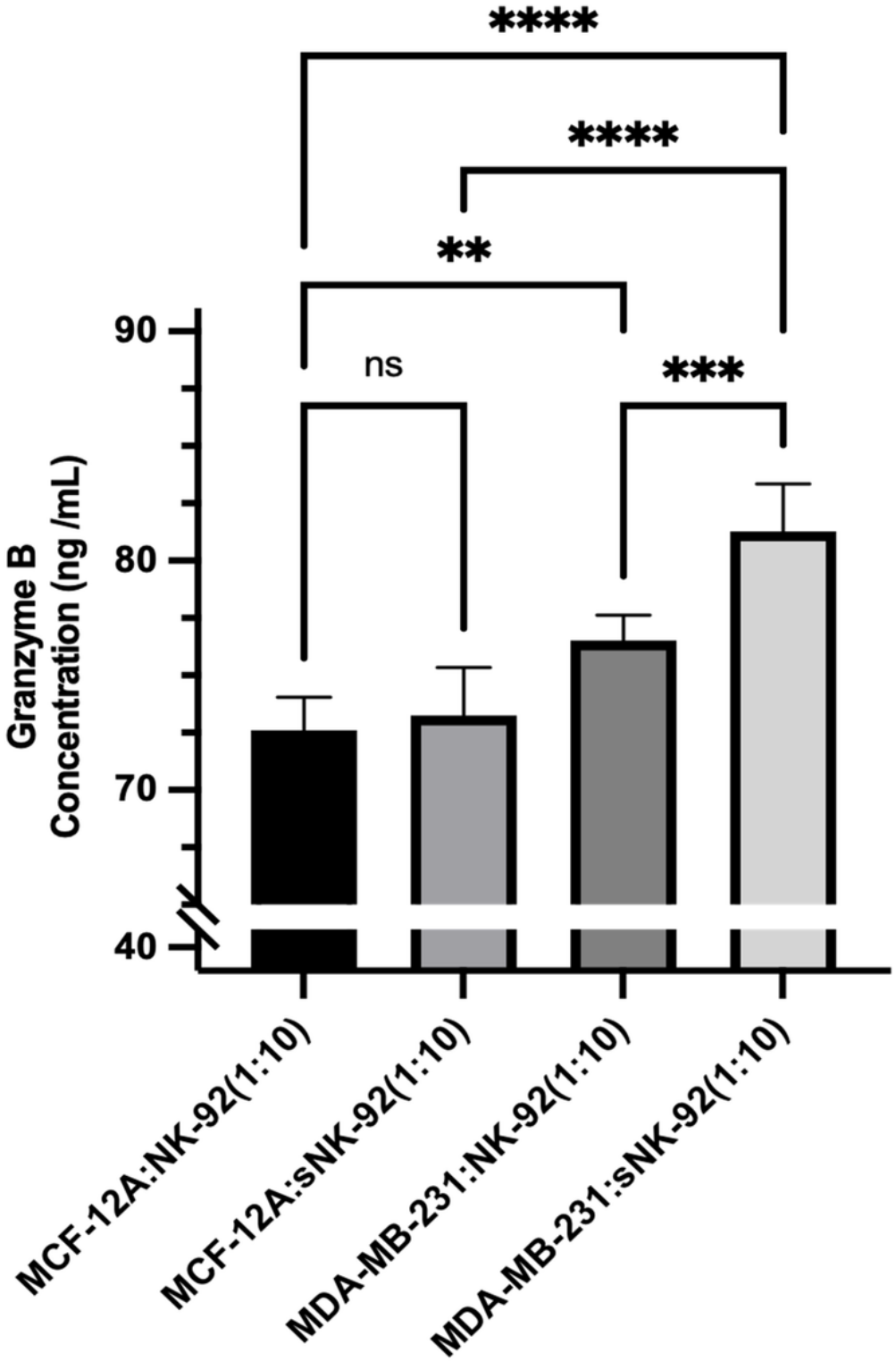

**Figure 1**

(a) DIC images (10X) of MDA-MB-231 and MCF-12A cells alone and cocultured with NK-92 and sNK-92. (b) Binding of anti-CD226 and CD226 receptors. 7-AAD was used as a DNA stain (red signal) and FITC was used as a secondary antibody. The green signal showed that the antibody is localized on the NK-92 cell membrane (100Xfluorescent microscope).



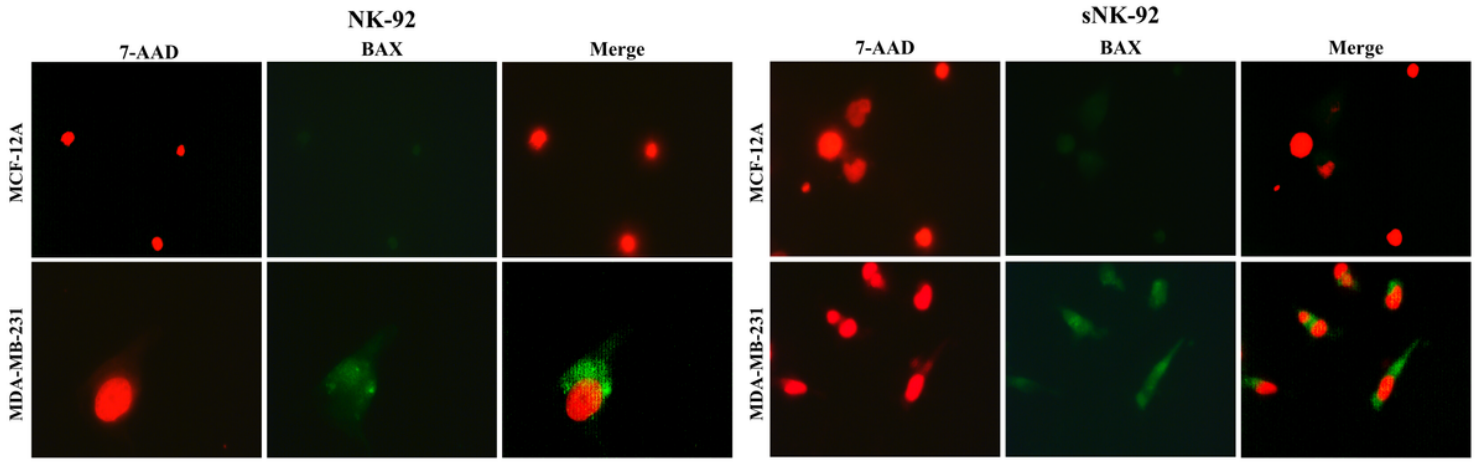

## Figure 2

LDH assay results of MCF-12A and MDA-MB-231 coculture with NK-92 and sNK-92. A. MCF-12A coculture with a different target: effector ratio of NK-92 and sNK-92. B. MDA-MB-231 coculture with a different target: effector ratio of NK-92 and sNK-92. (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ ns: not significant.)



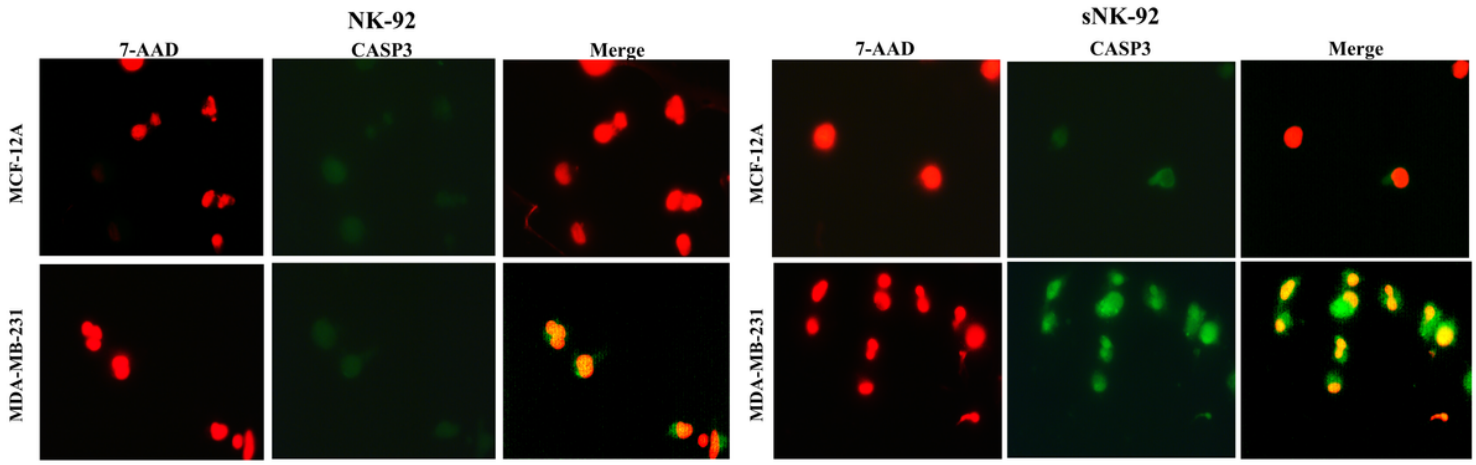

## Figure 3

Granzyme B concentration release from NK-92 and sNK-92 against MCF-12A and MDA-MB-231 (* $p<0.05$)




## Figure 4

Immunofluorescent staining of BAX in MCF-12A and MDA-MB-231 cells after coculturing with NK-92 and sNK-92. Green fluorescent signals indicate BAX protein synthesis and localization in cells. The red signal indicates nuclei stained with DNA dye (7-AAD). (100 X)




## Figure 5

Immunofluorescent staining of CASP3 in MCF-12A and MDA-MB-231 cells after coculturing with NK-92 and sNK-92. Green fluorescent signals indicate CASP3 protein synthesis and localization in cells. The red signal indicates nuclei stained with DNA dye (7-AAD). (100 X)

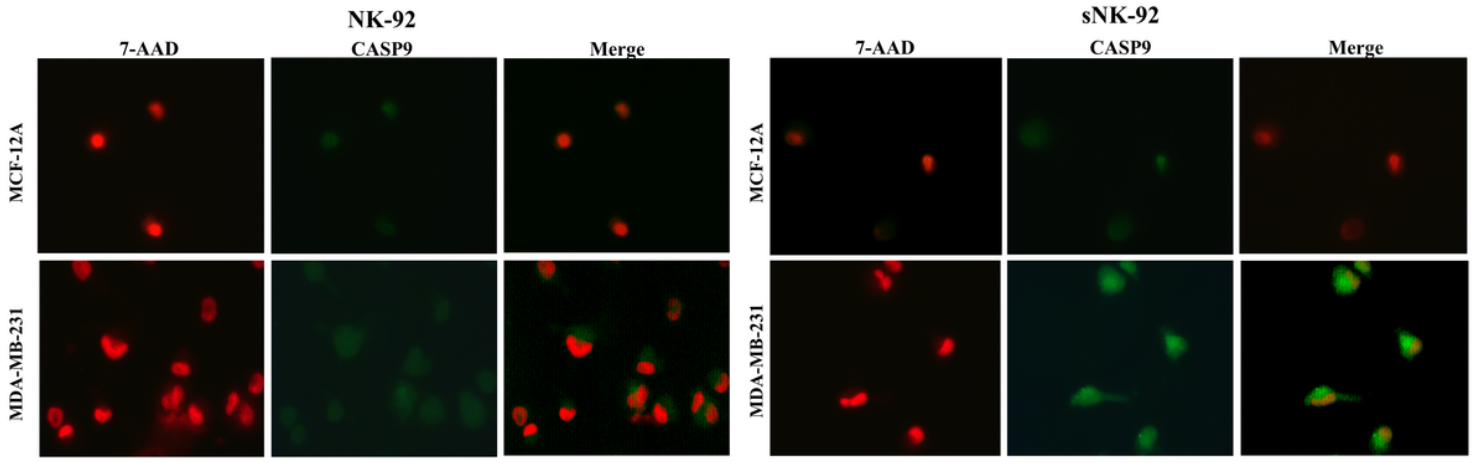



**Figure 6**

Immunofluorescent staining of CASP9 in MCF-12A and MDA-MB-231 cells after coculturing with NK-92 and sNK-92. Green fluorescent signals indicate CASP9 protein synthesis and localization in cells. The red signal indicates nuclei stained with DNA dye (7-AAD). (100 X)

# Supplementary Files

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