Title

Supercharged NK cells lyse both CSCs/poorly differentiated and well differentiated tumors

Author

Jain, Yash

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Supercharged NK cells lyse both CSCs/poorly differentiated and well differentiated tumors

A thesis submitted in partial satisfaction of

the requirements of the degree Master of Science in Oral Biology

by

Yash Jain

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ABSTRACT OF THE THESIS

Supercharged NK cells lyse both CSCs/poorly differentiated and well differentiated tumors

by

Yash Jain

Master of Science in Oral Biology

University of California, Los Angeles, 2023

Professor Anahid Jewett, Chair

Our previous research has shown that natural killer (NK) cells play a vital role as immune effectors in the selection and differentiation of various cancer stem cells and undifferentiated tumors. They achieve this through the direct killing and the release of IFN-γ and TNF-α, which inhibit tumor growth and curtail metastasis. Mechanistically, our studies have revealed that NK cells expanded by Osteoclasts display the ability to lyse CD4+ T cells while sparing CD8+ T cells, suggesting a potential selective effect of OC-activated NK cells on CD8+ T cell expansion. Consistent with these findings, we observed increased IFN-γ secretion, NK cell-mediated cytotoxicity, and higher proportions of CD8+ T cells in various tissue compartments of oral tumor-bearing hu-BLT mice following immunotherapy with OC-expanded NK cells. In this study, we focused on investigating the lytic and differentiation capabilities of supercharged NK (sNK) cells in ovarian cancer cell lines (OVCAR 4 and OVCAR 8), oral cancer cell lines (OSCC and OSCSC), and a pancreatic cancer cell line (MP 2). Our results demonstrated that sNK cells

exhibited greater lysing activity against both poorly differentiated and well differentiated tumors compared to pNK cells treated with IL-2. Furthermore, the supernatants of sNK cells exhibited significantly higher levels of IFN-γ and induced differentiation by an increase in MHC class1 expressions in all the cell lines tested, except for OVCAR 8. Lastly, we observed a downmodulation of NKG2A and CD94 inhibitory receptors on sNK cells, suggesting potential alterations in their functional properties.

The thesis of Yash Jain is approved.

Ichiro Nishimura

Nicholas A. Cacalano

Anahid Jewett, Committee Chair

University of California, Los Angeles

2023

DEDICATION

I dedicate this thesis primarily to my parents,

Abhay Jain

and

Kavita Jain

whose unwavering support throughout this period and endless provision of educational opportunities have shaped my journey. Additionally, I extend my heartfelt dedication to my sister and grandmother, whose encouragement and steady presence have been instrumental in my success.

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I would also like to thank all of my lab members, especially to Po-Chun Chen, and Sara Huerta, who have guided me, assisted me, and were there for me when I encountered difficulties. I could not have done this without you!

Last but not least, I would like to thank Dr. Sung for all his help with collecting blood samples

Introduction

Natural Killer Cells

NK cells are a type of lymphocyte that originate from the bone marrow and have large granules. They can be identified by the presence of CD16 and CD56 on their surface and the lack of CD3 expression. These cells make up around 5 to 15% of all lymphocytes and can be found in peripheral blood mononuclear cells (PBMCs). NK cells also play a vital role in producing important cytokines and activating the adaptive immune system [1]. In the peripheral blood, there are two main subsets of NK cells. The first subset is characterized by the presence of CD16, CD56dim CD69-. This subset is the predominant one, accounting for 90% of circulating NK cells, and is primarily involved in cytotoxicity. The second subset, which makes up 10% of circulating NK cells, is characterized by the absence of CD16, CD56bright CD69+. These cells are primarily responsible for secreting cytokines [2]. The functional capacity of NK cells is determined by the equilibrium between activating and inhibitory signals transmitted through their surface receptors. These receptors include NKG2D, NKp46, NKp44, and CD54, which are activating receptors, as well as KIR2, which is an inhibitory receptor.

The cytotoxicity mediated by NK cells primarily relies on the actions of perforin, a protein that disrupts cell membranes, and granzyme B, a serine protease. These components are not only responsible for NK cell-induced cytotoxicity but also play a role in processes such as necrosis and apoptosis [3]. In addition, NK cells have the ability to trigger antibody-dependent cellular cytotoxicity (ADCC) against tumors. Furthermore, they can modulate the function of other cells through the secretion of cytokines and chemokines [4]. Studies have demonstrated that NK cells have the capacity to induce differentiation in cancer stem cells, particularly in undifferentiated or poorly differentiated tumors. This process is achieved through the secretion of

cytokines like IFN-γ and TNF-α, which contribute to slowing down the progression of the tumor [5].

OVARIAN CANCER

Ovarian cancer is characterized by the development of cancerous cells in the ovaries, the female reproductive organs responsible for producing ova and hormones like estrogen and progesterone. Typically, ovarian cancer remains undetected until it has spread to the abdominal cavity or pelvic area, making it challenging to effectively manage the disease at this advanced stage [6]. Ovarian cancer continues to be the most fatal gynecological cancer in the Western world and ranks among the top five leading causes of death due to malignancy in the United States of America. Despite the introduction of newer treatments and chemotherapeutic drugs, the mortality rates for ovarian cancer have remained largely unchanged for a significant period of time [7-9]. Over 100 epithelial ovarian cancer cell lines have been identified, and in our study, we specifically examined the interaction between NK cells and supercharged NK cells with two of these cell lines, namely OVCAR 4 and OVCAR 8.

OVCAR 4

OVCAR 4 is considered as one of the highly representative ovarian cancer cell lines, particularly for studying multidrug-resistant ovarian cancer. OVCAR4 is a cell line derived from a patient with high-grade serous adenocarcinoma of the ovary. This cell line was established from an ovarian cancer patient who showed resistance to Cisplatin and several other chemotherapeutic drugs. OVCAR4 exhibits migratory and invasive properties and has the capability to form tumors in nude mice [10-12]. Research has demonstrated a decrease in

cytotoxicity of NK cells against OVCAR4 cells [13]. In contrast, when monocytes are stimulated and co-cultured with OVCAR4 cells, they exhibit relatively higher expression of CD107a [14]. OVCAR4 cells also exhibit a Leu130VAL mutation in the TP53 gene and display elevated surface expression of αvβ3, which is an integrin associated with cancer markers [11, 15-16].

OVCAR 8

Studies have demonstrated that the chemotherapeutic drug-resistant OVCAR8 cells exhibit increased surface expression of the CD44 receptor, as observed through Western blot analysis. This higher expression of CD44 is associated with poor clinical outcomes. Furthermore, it has been observed that treatment with Paclitaxel in an OVCAR8 xenograft model leads to the upregulation of the CD44 receptor, which can contribute to unfavorable outcomes and tumor recurrence [17]. OVCAR8 is a cell line derived from high-grade ovarian serous adenocarcinoma, obtained from tumor tissue of a patient who exhibited resistance to platinum-based drugs [18]. In previous studies conducted in our laboratory, it has been demonstrated and criteria have been established indicating that poorly differentiated cancer cells exhibit elevated surface expression of CD44 and reduced surface expression of MHC-class I, CD54, and B7H1 (anti-PD1). These cells also display greater susceptibility to lysis by NK cells. As a result, OVCAR8, being positioned towards the poorly differentiated end on the differentiation stage scale, is more susceptible to NK cell-mediated lysis [19-22].

Oral Squamous Cell Carcinoma

Oral squamous cell carcinoma (OSCC) is a type of head and neck squamous cell carcinoma (HNSCC), which represents the most prevalent form of cancer originating in the head

and neck region. HNSCC, including OSCC, ranks as the sixth most commonly diagnosed cancer globally in 2018 [23]. The risk factors associated with the development of oral squamous cell carcinoma (OSCC) include tobacco and alcohol usage, as well as infection with Human Papilloma Virus (HPV). Current treatment options for OSCC encompass surgical procedures, radiotherapy, chemotherapy, immunotherapy, or a combination of these approaches. Unfortunately, none of these options have yielded satisfactory outcomes for patients. Despite the emergence of various new treatment strategies, many have failed to achieve a significant clinical response [24,25]. The survival rate for oral squamous cell carcinoma (OSCC), which stands at approximately 40-50%, has remained relatively unchanged over the past several decades [26].

OSCSC(Oral Squamous Carcinoma Stem Cells)

Oral squamous carcinoma stem cells (OSCCs) were obtained by isolating cells from freshly resected tongue tumors. Oral squamous cell carcinomas, characterized as less differentiated oral tumors, exhibit a significantly higher susceptibility to NK cell-mediated cytotoxicity. OSCCs demonstrated minimal to no expression of B7H1 and EGF-R, while displaying increased expression of CD133 and CD44bright. No surface expression of MHC-Class II or CD90 was observed. Furthermore, OSCSCs exhibited minimal to no secretion of IL-6, IL-8, and GM-CSF, while demonstrating higher levels of VEGF secretion in comparison to OSCCs. They exhibit high expression of CD44, EpCAM, CD26, and CD338, while displaying low expression of CD166. These OSCSCs, characterized by such oral stem cell markers, are susceptible to NK cell-mediated cytotoxicity and can stimulate the secretion of IFN-γ [27-28]

OSCC (Oral Squamous Carcinoma Cells)

Freshly resected tongue tumors were used to isolate oral squamous cell carcinomas (OSCC). OSCCs exhibited elevated surface expression of B7H1 and EGF-R, moderate expression of CD44, and no surface expression of CD133. Oral squamous cell carcinoma (OSCC) demonstrated a significantly lower rate of proliferation compared to oral squamous carcinoma stem cells (OSCSC). Compared to their counterpart OSCSC, oral squamous cell carcinomas (OSCCs) display greater resistance and induce little to no IFN-γ secretion by NK cells. Elevated levels of NFκB were observed in these cells [27-28]

Pancreatic Ductal Adenocarcinoma

Despite advancements in cancer therapy, the mortality rate of pancreatic ductal adenocarcinoma (PDA) has seen only slight improvement in recent years. Existing treatments often yield poor responses in PDA patients, resulting in a 5-year survival rate of 10.8%, the lowest among all cancer types. Despite numerous research efforts in cancer treatment, effective therapies or cures for several aggressive cancers remain elusive. This underscores the critical need for the development of highly efficient medical treatments for cancer with minimal side effects. Globally, pancreatic ductal adenocarcinoma (PDA) ranks as the 14th most frequently diagnosed cancer and the 7th leading cause of cancer-related deaths [29].

MP2 and PL-12

The MP2 cell line represents a poorly differentiated pancreatic tumor, while the PL-12 cell line represents a highly differentiated pancreatic tumor. In comparison to the more differentiated pancreatic tumor cell lines PL-12, the MP2 cell line, which represents a stem-like/undifferentiated pancreatic tumor, exhibited higher levels of CD44 and lower levels of

CD54, MHC-I, and MICA. Furthermore, MP2 cells proved to be highly susceptible to lysis mediated by NK cells [30]. When the supernatants from split-anergized NK cells were added to MP2 tumor cells, it was observed that the sensitivity to NK cell-mediated lysis decreased. Furthermore, the expression of B7H1 and MHC-I was upregulated, while CD44 expression was downregulated [31].

AJ2, AJ3 and AJ4 Probiotic Bacteria

AJ2 is a combination of eight distinct strains of gram-positive probiotic bacteria, namely Streptococcus thermophiles, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, and Lactobacillus bulgaricus. When exposed to NK cells, AJ2 demonstrates a synergistic effect by promoting the secretion of a balanced mix of pro-inflammatory and anti-inflammatory cytokines, chemokines, and growth factors. This leads to the modulation of the IFN-γ to IL-10 ratio in NK cells, achieving a desired balance similar to that observed when NK cells are activated with IL-2 and anti-CD16 monoclonal antibody in the absence of bacteria. The administration of such treatment has been shown to induce significant differentiation of cancer stem cells [32-33]. AJ3 is a combination of 3 strains of bacteria from Bifidiobacterium genus whereas AJ4 is a combination of 4 strains of bacteria from Streptococcus and Lactobacillus genus.

Super-charged NK cells (Osteoclast-expanded NK cells)

Super-charged NK (sNK) cells, which are NK cells that have undergone expansion by osteoclasts, exhibit enhanced rates of proliferation, cytotoxicity, and IFN-γ secretion compared to other myeloid cells such as monocytes and dendritic cells [34]. While cord blood and

iPSC-derived NK cells have the ability to expand in large numbers while maintaining the NK cell phenotype, they are unable to effectively target and lyse oral squamous carcinoma stem-like cells (OSCSCs) or poorly differentiated tumors. Additionally, these cells are not able to produce an adequate amount of IFN-γ compared to primary NK (pNK) cells derived from peripheral blood or super-charged NK (sNK) cells [35].

CD94 and NKG2 receptors

NK cells possess receptors that recognize and monitor the expression of MHC class I molecules. These receptors consist of C-type lectin-like receptors such as CD94/NKG2 and murine Ly49, as well as immunoglobulin-like receptors like human KIR [36]. Among these, the CD94/NKG2 receptors are shared between primates and rodents. CD94/NKG2 receptors are present on most NK cells and a specific group of CD8+ T cells. There are five distinct molecular variants of NKG2 (NKG2A, B, C, E, and H) that have been identified to form heterodimers with the constant CD94 subunit through disulfide linkages [37-38]. NKG2A and NKG2B are derived from a single gene through alternative splicing. These two variants possess two immunoreceptor tyrosine-based inhibitory motifs in their cytoplasmic domains and function as inhibitory receptors when complexed with CD94 [39].

Thesis outline

Specific aim 1: To investigate the production of Interferon-gamma (IFN-γ) levels in PBMC and primary NK and supercharged NK cells following treatment with different activators AJ2, AJ3 and AJ4

- Sub-aim 1: To compare the levels of IFN after treatment with sAJ2, sAJ3, and sAJ4 in both PBMCs and primary NK cells.
- Sub-aim 2: To compare the levels of IFN-gamma in sNK cells and primary NK cells treated with IL-2 and IL-2 with aCD16
- Sub-aim 3: To assess the ability of sNK supernatant to induce differentiation in poorly differentiated tumor cells.

Specific aim 2: To investigate and compare the lysing capacity of supercharged NK (sNK) cells and primary NK cells against poorly differentiated and well differentiated tumor cells using eSight technology

- Sub-aim 1: To assess and compare the cytotoxicity of sNK cells against primary NK cells in both sNK supernatant-treated and untreated ovarian tumor cells.
- Sub-aim 2: To assess and compare the cytotoxicity of sNK cells against primary NK cells in both sNK supernatant-treated and untreated oral tumor cells.
- Sub-aim 3: To assess and compare the cytotoxicity of sNK cells against primary NK cells in both sNK supernatant-treated and untreated pancreatic tumor cells.

Specific aim 3: To establish and compare the expression levels of CD94 and NKG2A receptors on supercharged NK (sNK) cells and primary NK cells.

MATERIALS AND METHODS

Cell lines, reagents, and antibodies

NK cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, CA, USA). OSCSCs were isolated from tongue tumors of oral cancer patients at UCLA and cultured in RPMI 1640 medium supplemented with 10% FBS (Gemini Bio-Products, CA, USA) as well as 1.4% antibiotic antimycotic, 1% sodium pyruvate, 1.4% non-essential amino acids, 1% L-glutamine, 0.2% gentamicin (Gemini Bio-Products, CA, USA), and 0.15% sodium bicarbonate (Fisher Scientific, PA, USA). Mia-Paca-2 (MP2) and PL-12 cells were cultured in DMEM medium with 10% FBS and 1% penicillin and streptomycin (Gemini Bio-Products, CA, USA). OVCAR4 and OVCAR 8 cells were cultured in RPMI1640 supplemented with 10% FBS (Gemini Bio-Products, CA).

Recombinant IL-2 was obtained from NIH-BRB. Antibodies which were used for flow cytometry – isotype control, CD44, MHC-class I, CD54, B7H1 (PD-L1), CD45 (human), CD3, CD4, CD16, CD56, CD8, CD14, and CD19 – were purchased from Biolegend (San Diego, CA). Human NK purification kits were obtained from Stem Cell Technologies (Vancouver, BC, Canada).

Purification of NK Cells from Human Peripheral Blood

Written informed consent was obtained from healthy blood donors, and all procedures were approved by the UCLA Institutional Review Board (IRB). Peripheral blood was processed using Ficoll Hypaque centrifugation to separate the peripheral blood mononuclear cells (PBMCs) from the cloudy white buffy coat. The PBMCs were then washed and resuspended in RPMI 1640 (Invitrogen by Life Technologies, CA) medium supplemented with 10% fetal bovine serum

(FBS). The EasySep® Human NK Cell Enrichment Kit from Stem Cell Technologies (Vancouver, BC, Canada) was utilized for the negative selection and isolation of NK cells from the PBMCs. Flow cytometry analysis was performed using anti-CD45, anti-CD16, anti-CD56, and anti-CD3 antibodies to determine the purity of the isolated NK cells. The purified NK cells were cultured in RPMI 1640 medium supplemented with 10% FBS (Gemini BioProducts, CA), 1% antibiotics/antifungals, 1% sodium pyruvate, and 1% non-essential amino acid MEM (Invitrogen, Life Technologies, CA).

Sonicating AJ2

AJ2 was measured and suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS)(Gemini Bio-Products), reaching a final concentration of 10^6 cells/ml. After every five pulses, a sample was taken and examined under a microscope until at least 80% of the cell wall was dissolved. The sample was then sonicated for 15 seconds while kept on ice, followed by a 30-second incubation on ice. This sonication process was repeated 20 times to ensure complete sonication. Finally, the sonicated samples were divided into smaller portions and stored in a freezer at -80°C.

Generation of Osteoclasts and Expansion of NK Cells

The methodology employed in this study has been extensively detailed in a previous publication. In summary, human NK cells were purified and activated using rhIL-2 (1000 U/ml) and anti-CD16 monoclonal antibody (3 ug/ml) for a duration of 18-20 hours. Subsequently, the activated NK cells were co-cultured with osteoclasts and sAJ2 in a specific ratio of 2:1:4 (NK

cells:osteoclasts:sAJ2). The culture medium was replenished with rhIL-2 every three days to maintain optimal conditions [34].

^{51}Cr release cytotoxicity assay

^{51}Cr was obtained from Perkin Elmer (Santa Clara, California) for use in the standard ^{51}Cr release cytotoxicity assay, which was employed to assess the cytotoxic function of NK cells in the experimental cultures. Effector cells were aliquoted at a concentration of $1x10^5$ cells/well in 96-well round-bottom microplates (Fisher Scientific, Pittsburgh, PA) and titrated with serial dilutions ranging from 4 to 8. Target cells ($1*10^6$) are labeled with 100 μCi of ^{51}Cr (Perkin Elmer, Santa Clara, CA) and stained for an hour. After incubation, the target cells were washed twice to remove excess unbound ^{51}Cr. The^{51}Cr-labeled target cells were aliquoted into 96-well round-bottom microtiter plates containing effector cells at a concentration of $1*10^4$ cells/well, and the effector cell: target (E:T) ratio was 5:1. The plate is centrifuged and placed in an incubator for 4 hours. After a 4-hour incubation period, the supernatant of each sample was collected and the released radioactivity was measured using a gamma counter. The total release (containing ^{51}Cr-labeled target cells) and spontaneous release (containing target cell supernatant only) values were recorded to calculate the percentage of specific cytotoxicity. The following formula calculates the percentage of particular Cytotoxicity:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}}$$

Lu30/106 was calculated using the inverse of the number of effector cells needed to lyse 30% of target cells times 100.

Enzyme-Linked Immunosorbent Assays (ELISA)

The IFN-γ ELISA kit was obtained from BioLegend (San Diego, CA) for the measurement of IFN-γ levels in the cell culture. The ELISA procedure followed the manufacturer's protocol. In summary, a 96-well EIA/RIA plate was coated with a diluted capture antibody specific to the target cytokine and incubated overnight at 4°C. After washing the plate three times with Wash 18 Buffer (0.05% Tween in 1x PBS), it was blocked with Assay Diluent (1% BSA in 1x PBS). The plate was then incubated on a plate shaker at 500 rpm for 1 hour at room temperature and washed again with the wash buffer three times. Next, 100 µl of standards and samples obtained from each culture were added to the wells and incubated on a plate shaker at 500 rpm for 2 hours at room temperature. After incubation, the plates were washed three times with the wash buffer, and the detection antibody was added and incubated on a plate shaker at 500 rpm for 1 hour at room temperature. Following three rounds of washing with wash buffer, Avidin-HRP solution was added to the wells and incubated on a plate shaker at 500 rpm for 30 minutes at room temperature. After three rounds of washing with wash buffer, 100 µL of TMB Substrate Solution was added to the wells, and the plates were incubated in the dark for up to 30 minutes or until the desired blue color developed. The reaction was stopped by adding 50 µl of Stop Solution (2N H2SO4) to each well, and the absorbance values were obtained by reading the plates in a microplate reader at 450 nm (BioLegend, ELISA manual).

Single cell RNA sequencing

We utilized a 10X Chromium machine for the performance of single-cell RNA sequencing. The 10X Chromium Single cell 3’ Reagent kit v3 was used to prepare single-cell cDNA libraries, and the sequencing was conducted on an Illumina Novaseq 6000 instrument.

The sequencing depth aimed for approximately 30 thousand reads per cell. The obtained raw data were demultiplexed and aligned to a customized reference genome (GRCh38). UMI (Unique Molecular Identifier) counts were quantified using the 10X Genomics CellRanger software (v3.0.0) with default settings. For single-cell clustering and cell-cycle scoring, we employed the Seurat package (v3.0). Single-cell regulatory network inference and clustering (SCENIC) analysis were performed using the SCENIC R package (1.2.0) with the hg38 database (https://resources.aertslab.org/cistarget/).

Surface Staining and Cell Death Assays

A total of $30*10^3$ cells from each experimental condition were stained in 100 µL of cold 1% BSA-PBS solution with an optimized concentration of PE-conjugated antibodies, as specified in the experiments. The cells were incubated at 4°C for 30 minutes, followed by washing and resuspension in 1% BSA-PBS. For the cell death assay, $30*10^3$ cells in 100 µl of cold 1% BSA-PBS were stained with 8 mg/ml propidium iodide and adjusted to a final volume of 200 µl with BSA-PBS. Data analysis was performed using the Attune NxT flow cytometer (Waltham, MA) and Flowjo software (Ashland, OR).

Supercharged NK supernatant collection

The methodology employed in this study has been extensively detailed in a previous publication. In summary, human NK cells were purified and activated using rhIL-2 (1000 U/ml) and anti-CD16 monoclonal antibody (3 ug/ml) for a duration of 18-20 hours. Subsequently, the activated NK cells were co-cultured with osteoclasts and sAJ2 in a specific ratio of 2:1:4 (NK cells:osteoclasts:sAJ2). The culture medium was replenished with rhIL-2 every three days to

maintain optimal conditions [34]. On days 15-17 of the co-culture, the cells along with the culture media were harvested and subjected to centrifugation at 1500 RPM for 10 minutes. Care was taken to collect the supernatant without disturbing the cells.

Differentiating cells with Superchrged NK supernatant

$1*10^6$ cells each of OVCAR 4, OVCAR 8, OSCC, OSCSC and MP2 were plated in culture dishes. OVCAR 4 and OVCAR 8 were cultured in RPMI1640 supplemented with 10% FBS (Gemini Bio-Products, CA). OSCC and OSCSC cultured in RPMI 1640 medium supplemented with 10% FBS (Gemini Bio-Products, CA, USA) as well as 1.4% antibiotic antimycotic, 1% sodium pyruvate, 1.4% non-essential amino acids, 1% L-glutamine, 0.2% gentamicin (Gemini Bio-Products, CA, USA), and 0.15% sodium bicarbonate (Fisher Scientific, PA, USA). Mia-Paca-2 (MP2) and PL-12 cells were cultured in DMEM medium with 10% FBS and 1% penicillin and streptomycin (Gemini Bio-Products, CA, USA). After 18-24 hours, half of the culture media was taken out from all the culture dishes and same amount of supercharged NK supernatant was added to these culture dishes. 18-24 hours later, cells were trypsinized and harvested.

E-sight

xCELLigence RTCA eSight(Agilient, USA) was purchased and cell behaviour and cell function was studied using real time biosensor impedance-based and image-based measurements. The impedance-based xCELLigence technology utilizes proprietary microplates (E-Plates) embedded with gold biosensors at the bottom of each well, which serve to non-invasively quantify cell behavior. Over the course of an experiment, the biosensors monitor cell metrics such as

proliferation, adhesion strength, changes in morphology, migration, and differentiation. On day 1, 50 µl of the respective media was added to each well, and the machine was run once to measure the background. Subsequently, $5*10^3$ target cells (tumor cells) were added per well, and the machine was run overnight. During this time, impedance measurements were taken at 15-minute intervals, and images were captured at 60-minute intervals. After 18-24 hours, effector cells were added to each well in a serial dilution of 2 for each target cell type. The serial dilution began with an Effector:Target (E:T) ratio of 5:1, with $25*10^3$ effector cells, the next dilution being with an E:T ratio of 2.5:1, with $12.5*10^3$ effector cells, followed by 1.25:1 E:T ratio with $6.25*10^3$ cells and finally an E:T ratio of 0.625:1 with $3.125*10^3$ effector cells were added. Impedance readings were recorded at 15-minute intervals, and images were captured at 60-minute intervals for a period of 48-72 hours.

Statistical analysis

A two-tailed, unpaired Student t-test was conducted for statistical analysis. To compare different groups, one-way ANOVA with a Bonferroni post-test was employed. Cytotoxicity and cytokine analysis were performed using duplicate or triplicate samples for assessment. The levels of statistical significance are represented by the following symbols: ***(p-value < 0.001), **(p-value 0.001-0.01), *(p-value 0.01-0.05).

Results and Discussion

Chapter 1: To investigate the production of Interferon-gamma (IFN-γ) levels in PBMC and NK cells following treatment with AJ4 and to assess the ability of sNK supernatant to induce differentiation in poorly differentiated tumor cells.

Increased IFN-γ by sAJ4 treated PBMCs in comparison to sAJ3 and sAJ2 treated PBMCs

We observed increased secretion of IFN-γ in PBMCs when treated with IL-2 and sonicated AJ4 (sAJ4) in comparison to sAJ3 and sAJ2 treated PBMCs **(Fig. 1)** [41]

Increased IFN-γ by sAJ4 treated NK cells in comparison to sAJ3 and sAJ2 treated NK cells

After sorting NK cells from PBMCs, we subjected them to the same treatments as described earlier. Similar to PBMCs, the NK cells exhibited a substantial increase in IFN-γ levels when treated with IL-2 and sAJ4 compared to IL-2+sAJ3 or IL-2+sAJ2 **(Fig. 2)** [41]

Increased IL-10 secretions by sAJ3 treated PBMC and NK cells in comparison to sAJ4 and sAJ2 treated PBMC and NK cells.

We observed increased secretion of IL-10 in PBMCs and NK cells when treated with IL-2+sAJ3 in comparison to IL-2+sAJ2 or IL-2+sAJ4 **(Fig. 3)** [41]

sNK supernatant exhibited the highest levels of IFN-γ release compared to pNK cells treated with IL-2 or IL-2 in combination with aCD16.

The supernatant was obtained from pNK cells after overnight treatment with IL-2 or IL-2 in combination with aCD16, and from sNK cells after 15-16 days of culture. The sNK supernatant exhibited significantly higher levels of IFN-γ compared to pNK cells treated with IL-2 or IL-2 with aCD16 **(Fig.4)**

Treatment of tumors using supernatant of super charged NK cells increased MHC class I in all tumor cells except OVCAR 8 tumors

Previous studies have shown that the treatment of cancer cells with supernatants derived from NK cells leads to an upregulation of MHC-class I expression on the cancer cells, promoting their differentiation. Furthermore, it has been observed that the key cytokine responsible for inducing this differentiation is IFN-γ, which is secreted by the NK cells [40].

To assess the induction of differentiation in ovarian cancer, oral cancer, and pancreatic cancer cell lines, the cells from each cell line OVCAR4, OVCAR8, OSCC, OSCSC and MP2 were treated overnight with supernatant from supercharged NK cells. The following day, flow cytometry analysis was performed to evaluate the surface expression of CD44, CD54, MHC-class I, and B7H1 (PDL-1). A PE IgG isotype antibody was used as a control. The results showed that all cell lines, except OVCAR8, exhibited an increase in MHC-class I expression following treatment with sNK supernatant. OVCAR8 cells, on the other hand, displayed a relatively similar level of expression compared to the control. This finding suggests that OVCAR8 cells are poorly differentiated and possess stem cell characteristics. In addition to the upregulation of MHC class I, we observed a significant increase in CD54 receptors in OVCAR4, OSCC and OSCSC tumor cells (**Fig. 5-8**)

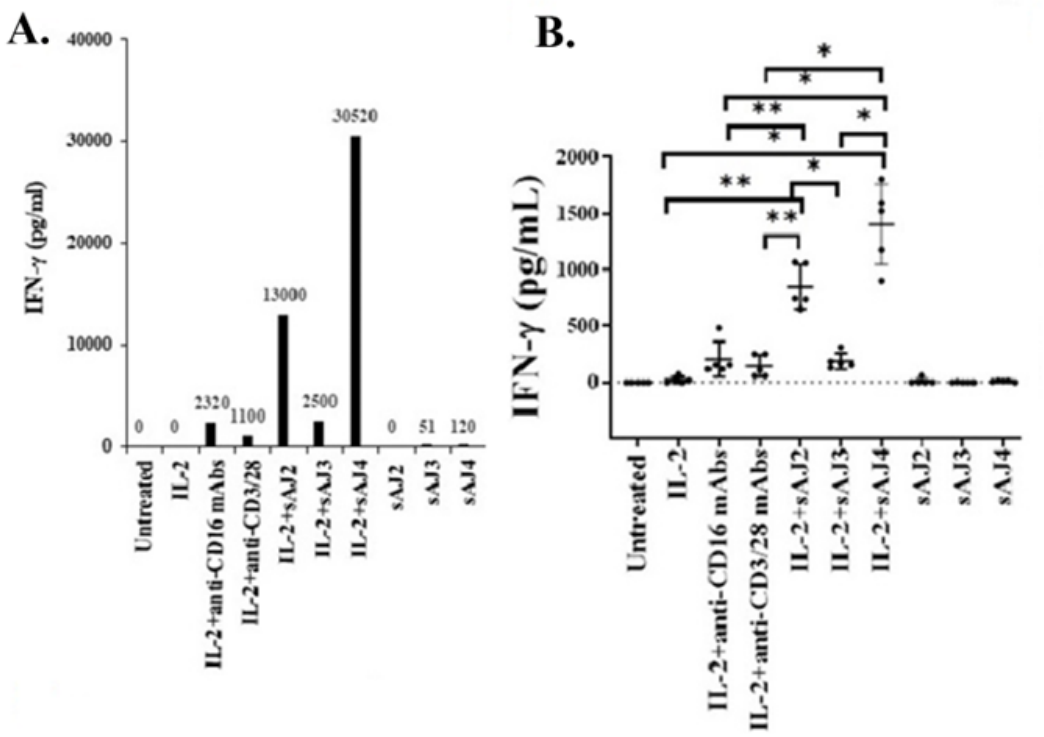


Figure 1. Increased expression of IFN-γ in PBMCs treated with IL-2 in combination with sAJ4

PBMCs were subjected to different treatments, including no treatment, IL-2 alone (1000 U/ml), IL-2 in combination with anti-CD16 mAbs (3 µg/ml), IL-2 in combination with anti-CD3/28 antibody (25 µl/ml), IL-2 in combination with sAJ2 (PBMC:sAJ2, 1:20), IL-2 in combination with sAJ3 (PBMC:sAJ3, 1:20), IL-2 in combination with sAJ4 (PBMC:sAJ4, 1:20), sAJ2 alone (PBMC:sAJ2, 1:20), sAJ3 alone (PBMC:sAJ3, 1:20), or sAJ4 alone (PBMC:sAJ4, 1:20). After an 18-hour incubation period, the supernatants were collected from the PBMCs, and the secretion of IFN-γ was measured using a single ELISA. One of the representative experiments is shown **(A)**, and cumulative samples are shown (n=5) **(B)**. **(p value 0.001-0.01), *(p value 0.01-0.05)

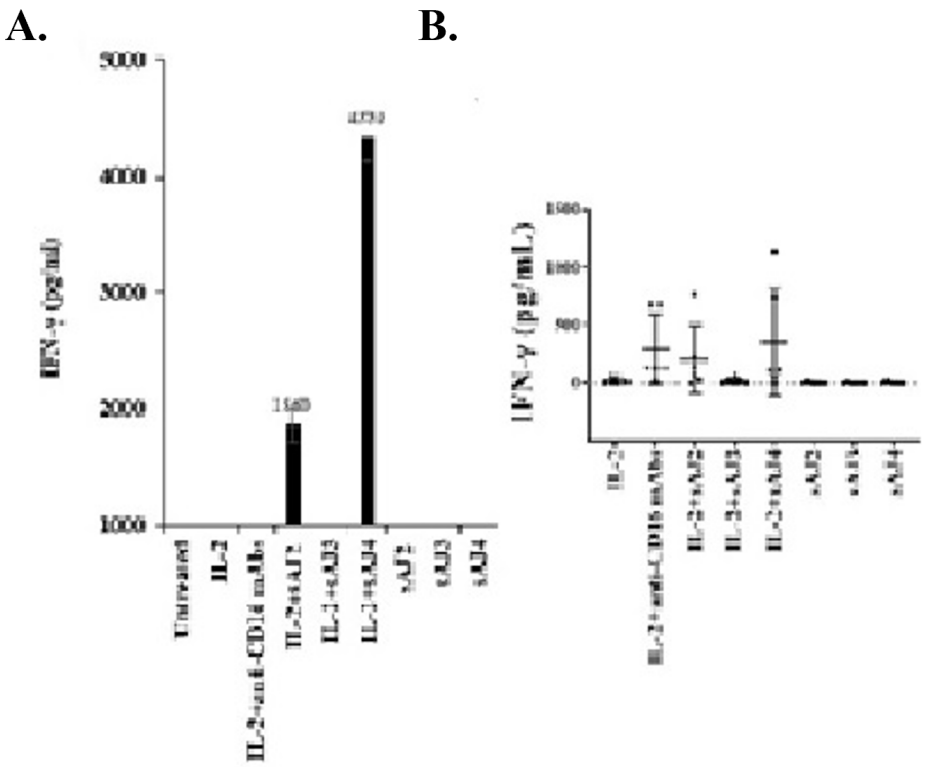


Figure 2: Increased IFN-γ secretions by sAJ4 treated NK cells in comparison to sAJ3 and sAJ2 treated NK cells

NK cells were subjected to various treatments, including being untreated or treated with IL-2 (1000 U/ml) alone, a combination of IL-2 (1000 U/ml) and anti-CD16 mAbs (3 µg/ml), a combination of IL-2 (1000 U/ml) and sAJ2 (NK:sAJ2, 1:20), a combination of IL-2 (1000 U/ml) and sAJ3 (NK:sAJ3, 1:20), a combination of IL-2 (1000 U/ml) and sAJ4 (NK:sAJ4, 1:20), sAJ2 alone (NK:sAJ2, 1:20), sAJ3 alone (NK:sAJ3, 1:20), or sAJ4 alone (NK:sAJ4, 1:20). These treatments were applied for a duration of 18 hours. After the incubation period, the supernatants were collected from the NK cells, and IFN-γ secretion was assessed using a single ELISA. The results of one representative experiment **(A)** as well as the combined data from multiple samples (n=5) are presented **(B)**. ***(p value <0.001), **(p value 0.001-0.01), *(p value 0.01-0.05)

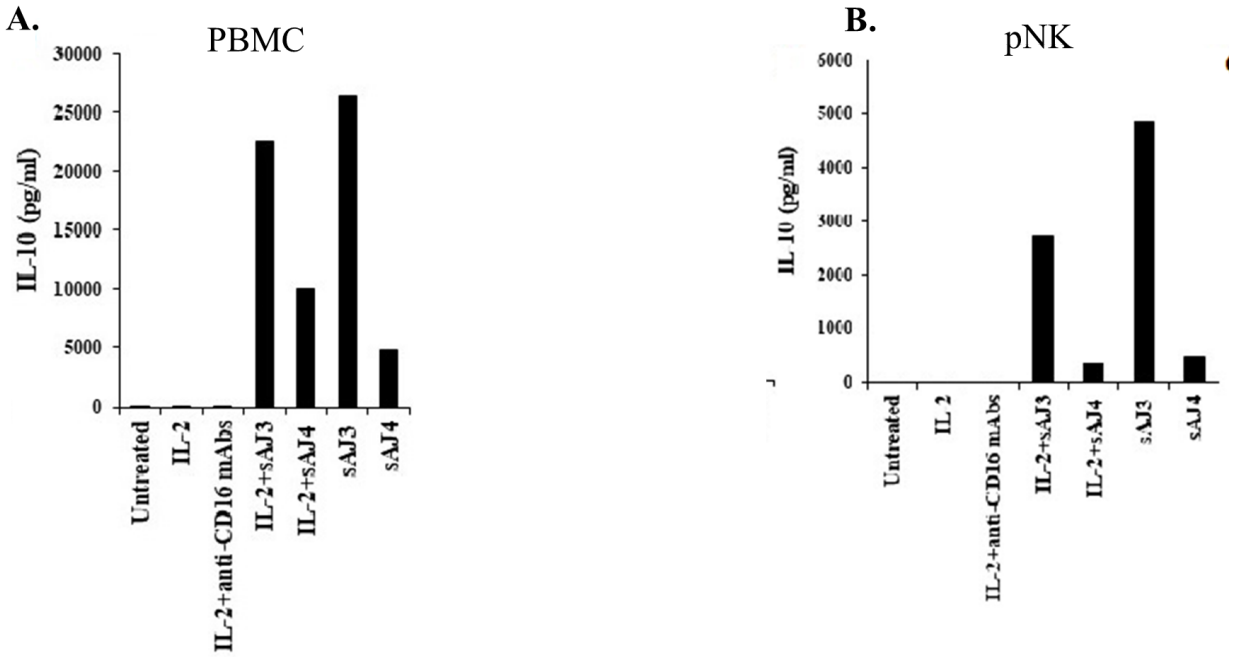


Figure 3: Increased IL-10 secretions by sAJ3 treated NK cells in comparison to sAJ4 and sAJ2 treated PBMC and NK cells.

PBMCs and pNKs were subjected to different treatments for 18 hours, including: being left untreated, treated with IL-2 (1000 U/ml), treated with a combination of IL-2 (1000 U/ml) and anti-CD16 monoclonal antibodies (3 µg/ml), treated with sAJ3 (PBMC:sAJ3, 1:20), treated with sAJ4 (PBMC:sAJ4, 1:20), treated with a combination of IL-2 (1000 U/ml) and sAJ3 (PBMC:sAJ3, 1:20), or treated with a combination of IL-2 (1000 U/ml) and sAJ4 (PBMC:sAJ4, 1:20). Afterward, the supernatants were collected from the PBMCs(**A)** and pNK **(B)** to measure the secretion of IL-10 using a multiplex assay.

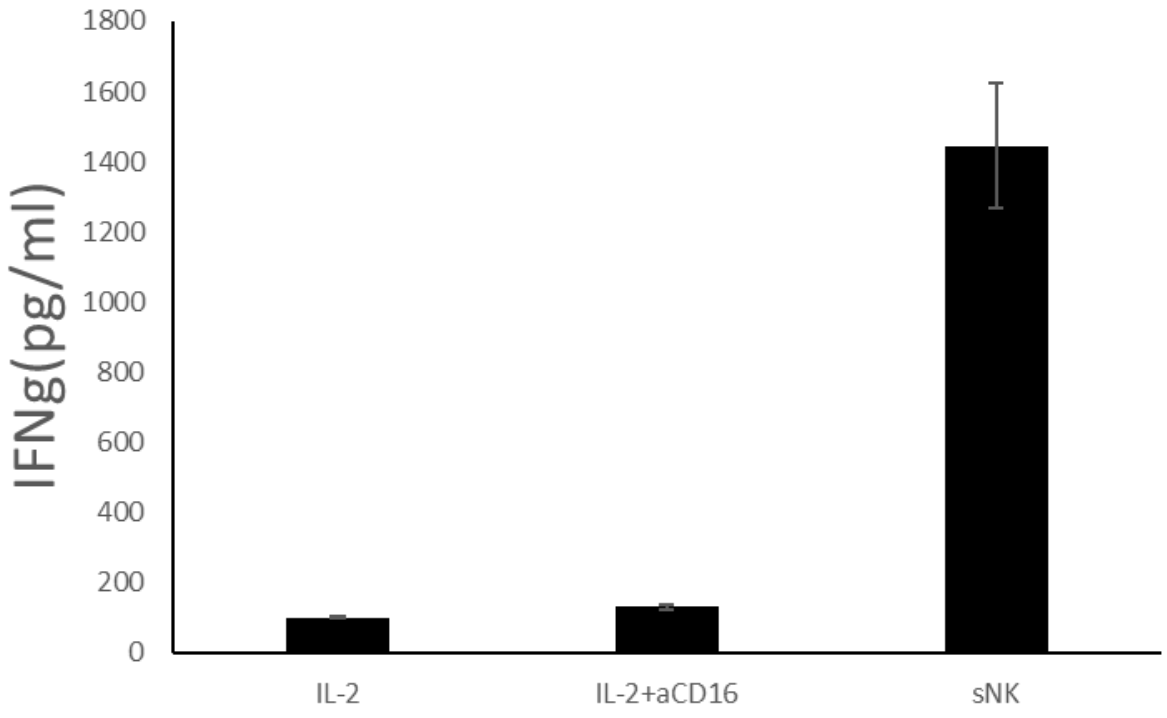


Figure 4: Increased IFN-γ secretion from sNK supernatant in comparison to pNK treated with IL-2 or in combination with IL-2 and aCD16 supernatant.

NK cells were subjected to various treatments, including being untreated or treated with IL-2 (1000 U/ml) alone, a combination of IL-2 (1000 U/ml) and anti-CD16 mAbs (3 µg/ml). These treatments were applied for a duration of 18 hours. After the incubation period, the supernatants were collected from the NK cells, and IFN-γ secretion was assessed using ELISA. sNK cells were cultured following the methodology outlined in the materials and methods section. On day 15 of culture, the supernatant was collected, and the secretion of IFN-γ was assessed using ELISA.

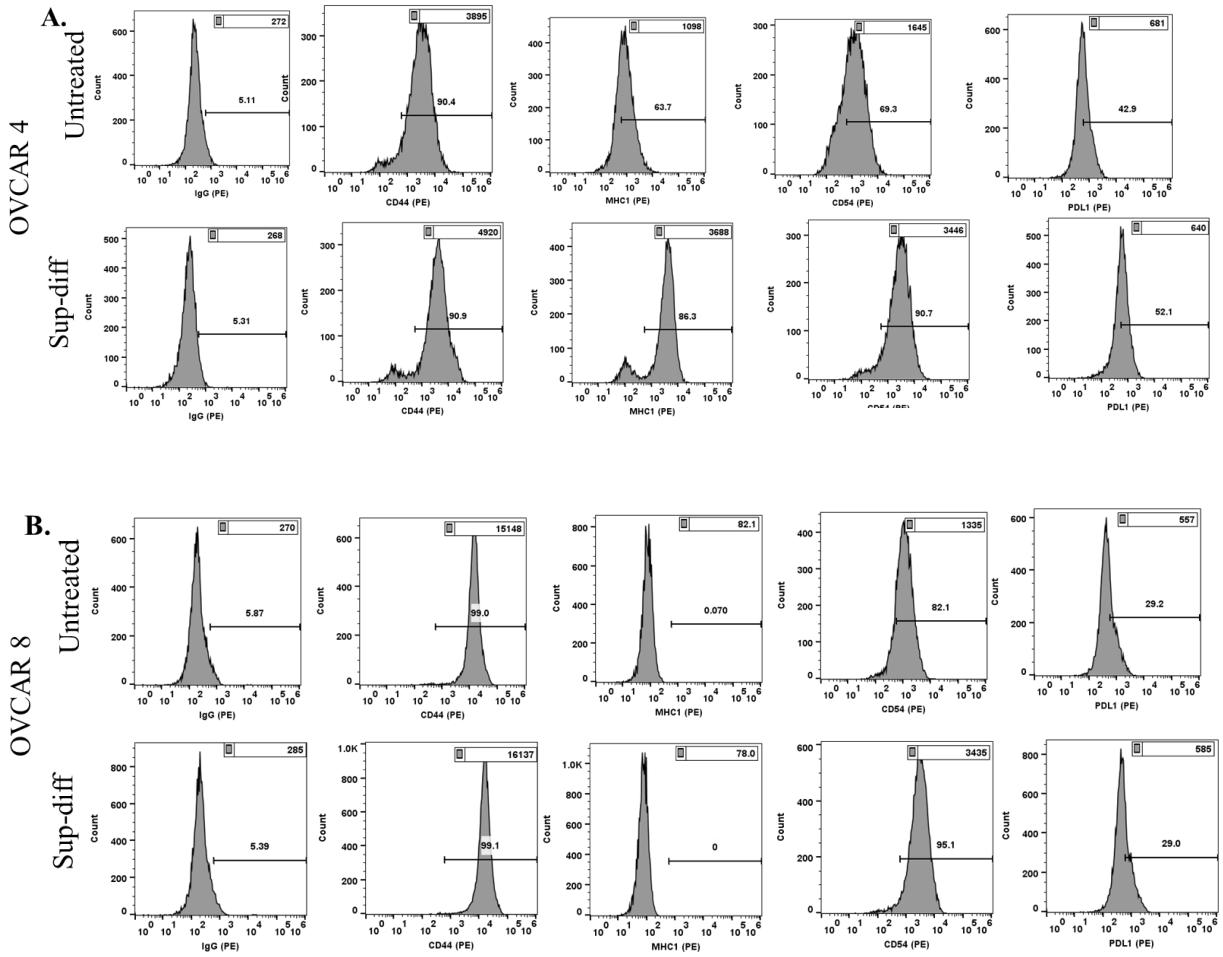


Figure 5: OVCAR 4 shows marked increase in MHC class I expression following supernatant treatment, whereas no increase/ lowest MHC class I is seen in OVCAR 8.

40 x 10^3 cells of each ovarian cancer cell line, OVCAR 4 (**A**) and OVCAR 8 (**B**) with and without sNK supernatant treatment were stained with PE CD44 and PE MHC-class I antibody. A PE IgG isotype antibody served as a control. The percentages indicated in the bottom right-hand corner represent relative values, while the mean fluorescence intensities of each histogram are shown in the top right-hand corner.

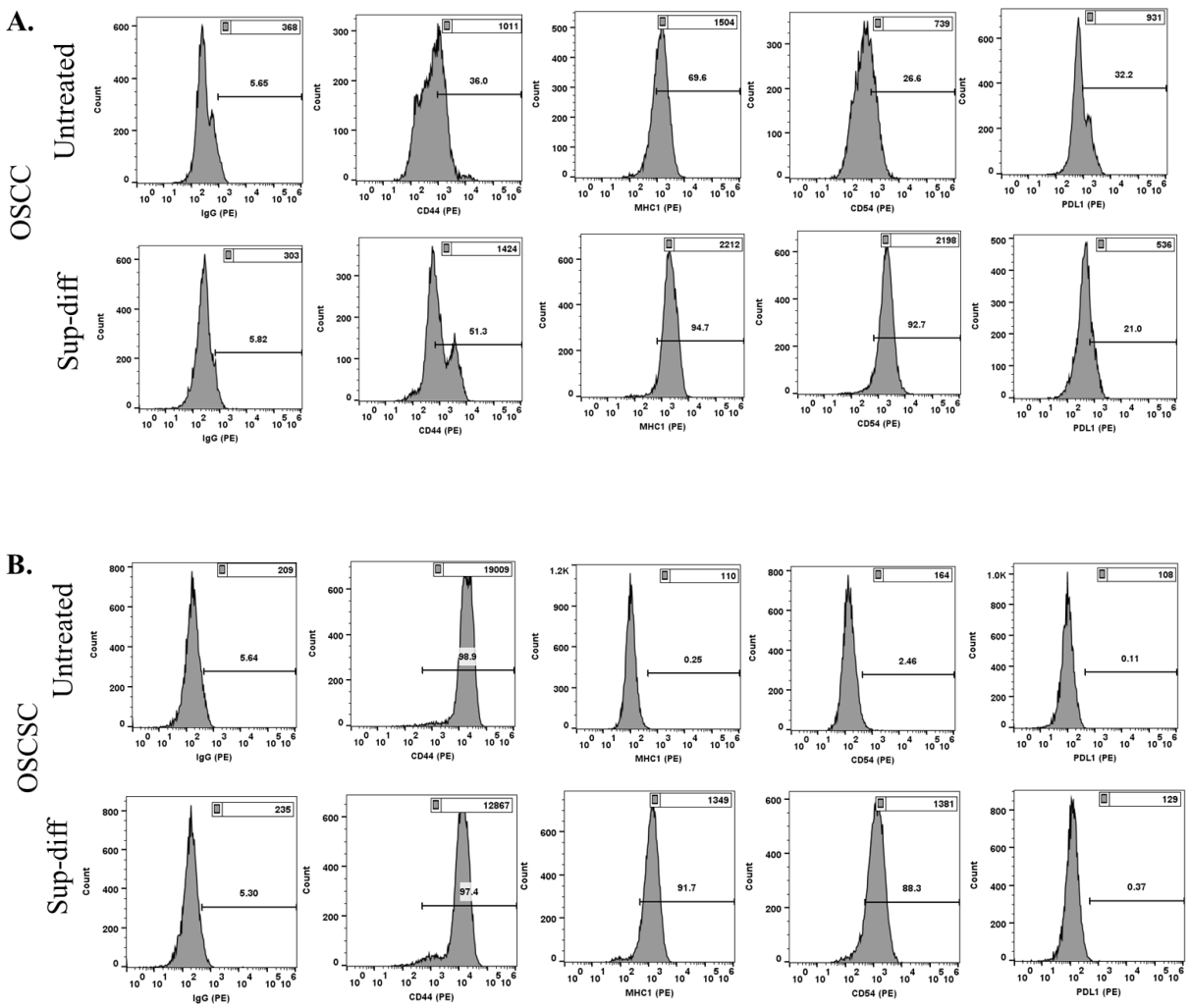


Figure 6: Both OSCC and OSCSC show marked increase in MHC class I expression and CD 54 receptors following sNK supernatant treatment

40 x 10^3 cells of each oral cancer cell line, OSCC (**A**) and OSCSC (**B**) with and without sNK supernatant treatment were stained with PE CD44 and PE MHC-class I antibody. A PE IgG isotype antibody served as a control..The percentages indicated in the bottom right-hand corner represent relative values, while the mean fluorescence intensities of each histogram are shown in the top right-hand corner.

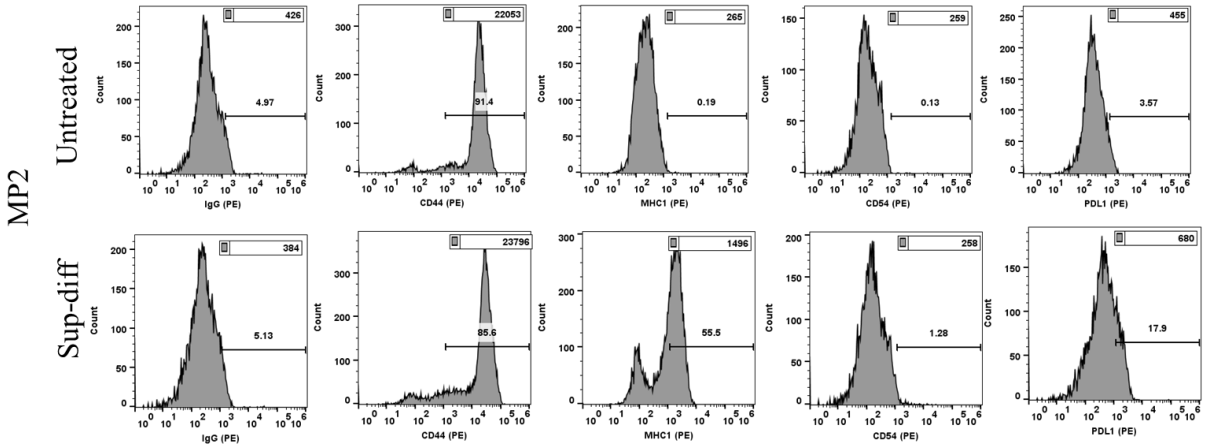
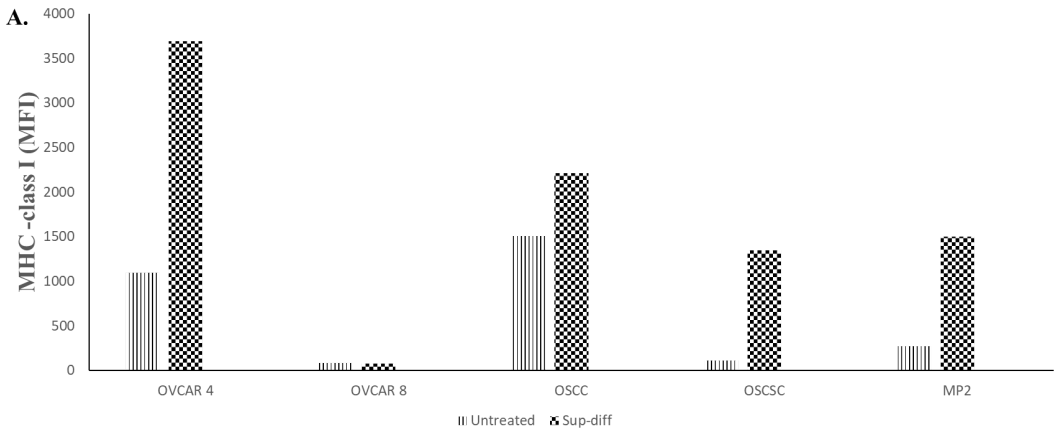


Figure 7: MP2 cells show marked increase in MHC class I expression following sNK supernatant treatment

40 x 10^3 cells of pancreatic cancer cell line, MP2, with and without sNK supernatant treatment were stained with PE CD44 and PE MHC-class I antibody. A PE IgG isotype antibody served as a control..The percentages indicated in the bottom right-hand corner represent relative values, while the mean fluorescence intensities of each histogram are shown in the top right-hand corner.



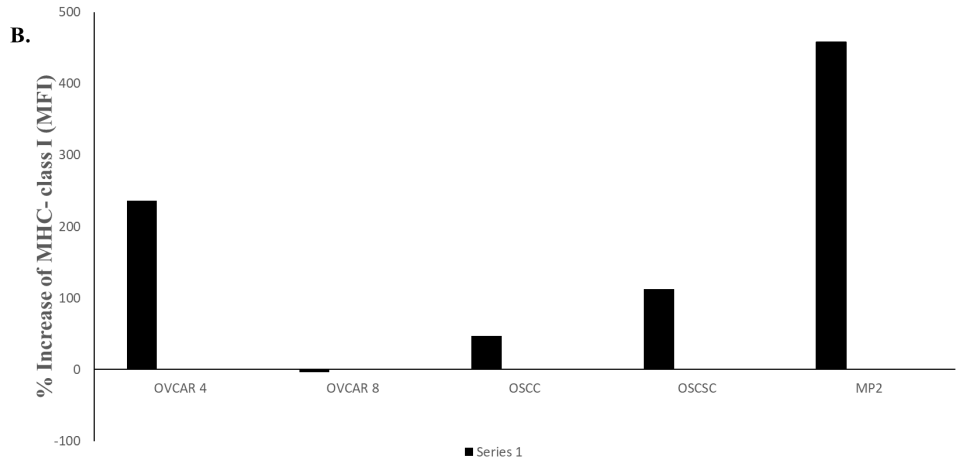


Figure 8: sNK supernatant treatment of the tumors increase MHC class I in all tumor cell lines except OVCAR 8

A total of $1*10^6$ cells from ovarian cancer, oral cancer, and pancreatic cancer cell lines were plated in culture dishes. The cells were then treated with a mixture of 50% sNK supernatant and 50% respective growth media. After an overnight incubation, 40 x 10^3 cells were selected and stained with PE MHC-class I antibody to assess the surface expression of MHC-class I molecules. The mean fluorescence intensity (MFI) values were recorded for each cell line and used to create a bar chart, as shown in Figure **A**. Additionally, the percent increase in MHC-class I MFI was calculated and presented in another bar chart, displayed in Figure **B**.

Chapter 2: To investigate and compare the lysing capacity of supercharged NK (sNK) cells and regular NK cells against poorly differentiated and differentiated tumor cells using E-sight technology

OVCAR 8 cells exhibit a significantly higher growth rate in comparison to OVCAR 4 cells

The growth of OVCAR 8 and OVCAR 4 cells was assessed by plating $1*10^6$ cells per culture dish. Cells were counted 48 hours later using microscopy. It was observed that OVCAR 8 cells exhibited a higher growth rate compared to OVCAR 4 cells.**(Fig.9)**

OSCSC cells exhibit a significantly higher growth rate in comparison to OSCC cells

The growth of OSCSC and OSCC cells was assessed by plating $1*10^6$ cells per culture dish. Cells were counted 48 hours later using microscopy. It was observed that OSCSC cells exhibited a higher growth rate compared to OSCC cells.**(Fig.10)**

Supercharged NK cells showed superior cytotoxic activity in comparison to NK cells in both untreated and sNK supernatant treated well differentiated and poorly differentiated tumor cells

To investigate this, we used five different cell lines- OVCAR 4, OVCAR 8, OSCC, OSCSC and MP2. We also treated all the cell lines with supernatant of supercharged NK cells as described in materials and methods section and compared the cytotoxic activity of sNK cells to NK cells treated with IL-2 (1000 U/ml) or with a combination of IL-2 (1000 U/ml) and anti-CD16 mAbs (3 μg/ml). It has been previously demonstrated that stem-like and poorly differentiated cancer cells are more prone to NK cell mediated cytotoxicity than well

differentiated cancer cells(22). We found that the supernatant caused an increase in MHC class I in all cell lines except OVCAR 8 and made these cells less susceptible to pNK cells. To determine the cytotoxicity of sNK cells against ovarian cancer, oral cancer and pancreatic cell lines, cytotoxicity of IL-2 treated pNK and sNK cells was determined against sNK supernatant treated and untreated ovarian cancer, oral cancer and pancreatic cancer cell lines. All the cell lines showed increased cell death with sNK cells as compared to pNK cells treated with IL-2 (1000 U/ml) or with a combination of IL-2 (1000 U/ml) and anti-CD16 mAbs (3 μg/ml).

In the untreated OVCAR 4 cell line, sNK cells exhibited the highest cytotoxicity compared to pNK cells treated with IL-2. These results align with the findings obtained from the conventional 4-hour ^{51}Cr release assay. The percentage of cytolysis, indicating cell killing, was highest for sNK cells, followed by pNK cells treated with IL-2. The same trend was observed in the OVCAR 4 cell line treated with sNK supernatant, where sNK cells showed the highest cytotoxicity, followed by pNK cells treated with IL-2 **(Figure. 11&12)**

For the OVCAR 8 cell line, the highest cytotoxicity was observed in sNK cells compared to pNK cells treated with IL-2. These findings are consistent with the results obtained from the conventional 4-hour ^{51}Cr release assay. The percentage of cytolysis, indicating cell killing, was highest for sNK cells, followed by pNK cells treated with IL-2. The same trend was observed in the OVCAR 8 cell line treated with sNK supernatant, where sNK cells exhibited the highest cytotoxicity, followed by pNK cells treated with IL-2 **(Figure. 13&14)**

It was also observed that OVCAR 8 cells were targeted more effectively by pNK cells treated with IL-2 compared to OVCAR 4 cells, as OVCAR 8 cells are poorly differentiated and hence more susceptible to pNK cells. However, the maximum cytotoxicity was observed with sNK cells in both cell lines.

In the untreated OSCC cell line, sNK cells displayed the highest cytotoxicity compared to pNK cells treated with IL-2. These results are consistent with the findings obtained from the conventional 4-hour ^{51}Cr release assay. The percentage of cytolysis, indicating cell killing, was highest for sNK cells, followed by pNK cells treated with IL-2 **(Figure. 15)**

For the OSCSC cell line, the highest cytotoxicity was observed in sNK cells compared to pNK cells treated with IL-2. These results align with the findings obtained from the conventional 4-hour ^{51}Cr release assay. The percentage of cytolysis, indicating cell killing, was highest for sNK cells, followed by pNK cells treated with IL-2. The same trend was observed in the OSCSC cell line treated with sNK supernatant, where sNK cells exhibited the highest cytotoxicity, followed by pNK cells treated with IL-2 **(Figure. 16&17)**

It was also found that OSCSC cells were targeted more effectively by pNK cells treated with IL-2 compared to OSCC cells, as OSCSC cells are poorly differentiated and therefore more susceptible to pNK cells. However, the maximum cytotoxicity was seen with sNK cells in both cell lines.

Lastly, in the untreated MP 2 cell line, sNK cells demonstrated the highest cytotoxicity compared to pNK cells treated with IL-2. These results are consistent with the findings obtained from the conventional 4-hour ^{51}Cr release assay. The percentage of cytolysis, indicating cell killing, was highest for sNK cells, followed by pNK cells treated with IL-2 **(Figure. 18)**

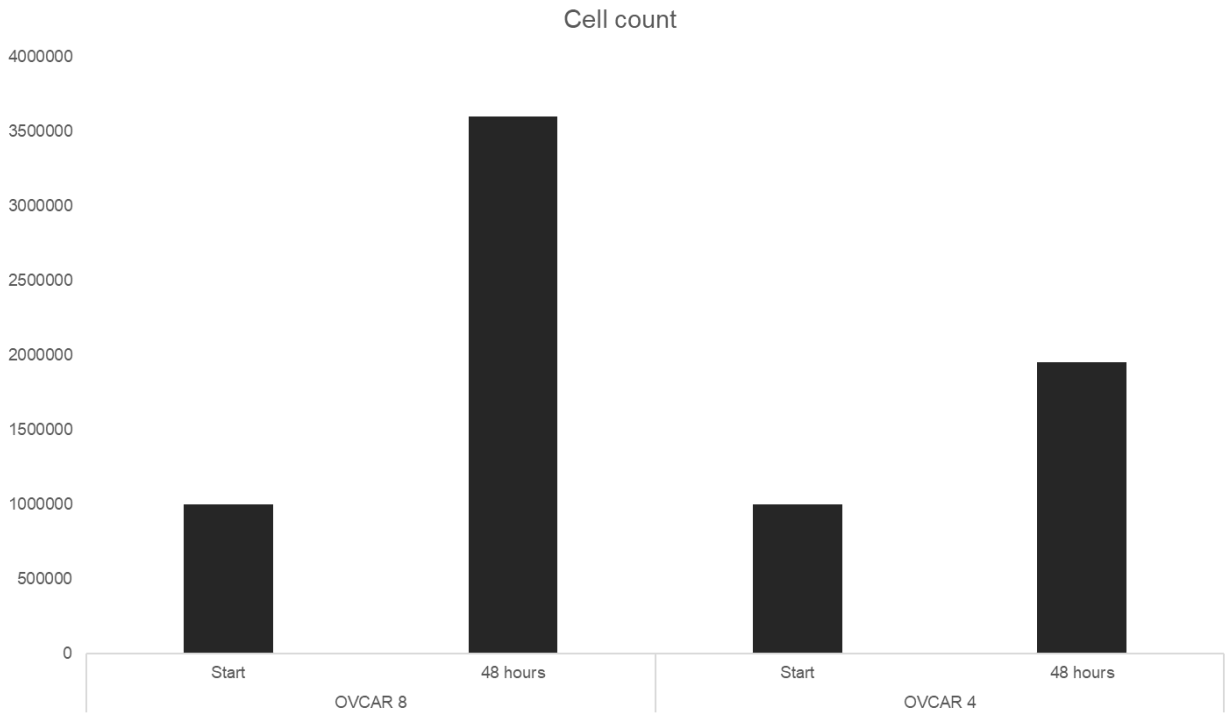


Figure 9: OVCAR 8 cells demonstrate a markedly superior growth rate compared to OVCAR 4 cells:

A total of $1*10^6$ cells from each cell line were seeded in a culture dish. After 48 hours, the cells were trypsinized and counted using microscopy.

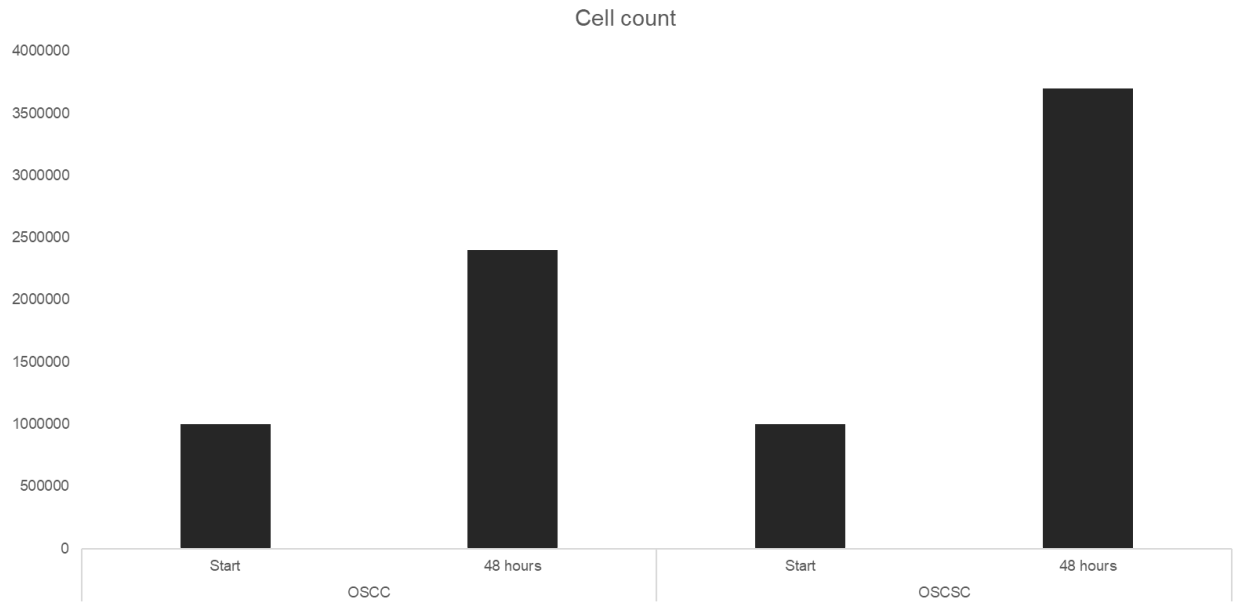


Figure 10: OSCSC cells demonstrate a markedly superior growth rate compared to OSCC cells:

A total of $1*10^6$ cells from each cell line were seeded in a culture dish. After 48 hours, the cells were trypsinized and counted using microscopy.

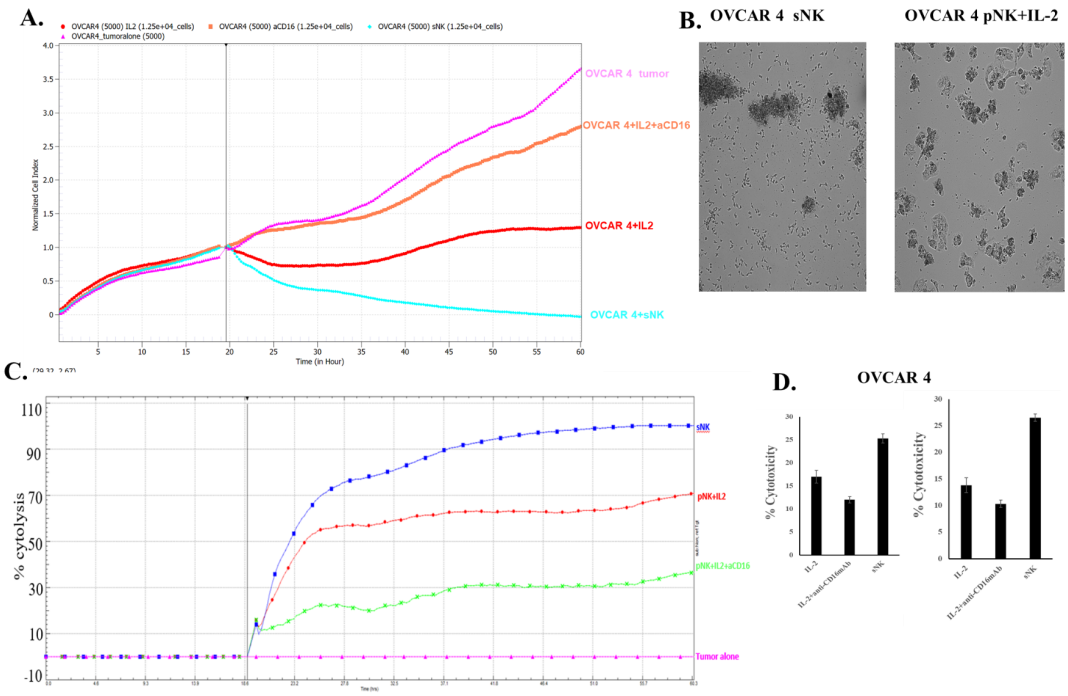


Figure 11: Cytotoxicity of sNK cells is higher compared to pNK with IL-2 in OVCAR 4 cells

In the e-sight microplate, $5*10^3$ cells of the OVCAR 4 cell line were plated. The untreated cells served as the control group. After allowing the cells to grow overnight, effector cells were added to each well following 18 hours of incubation. The cell index corresponding to an E:T ratio of 2.5:1 is illustrated in Figure**(A)**. Real-time images were captured at 48 hours of total incubation time in e-sight Figure **(B)**. The graph in Figure**(C)** represents the percentage of cytolysis. The cytotoxicity of sNK cells, pNK cells treated with IL-2, or a combination of IL-2 and aCD16 was assessed using a standard 4-hour 51Cr release assay Figure **(D)**. .

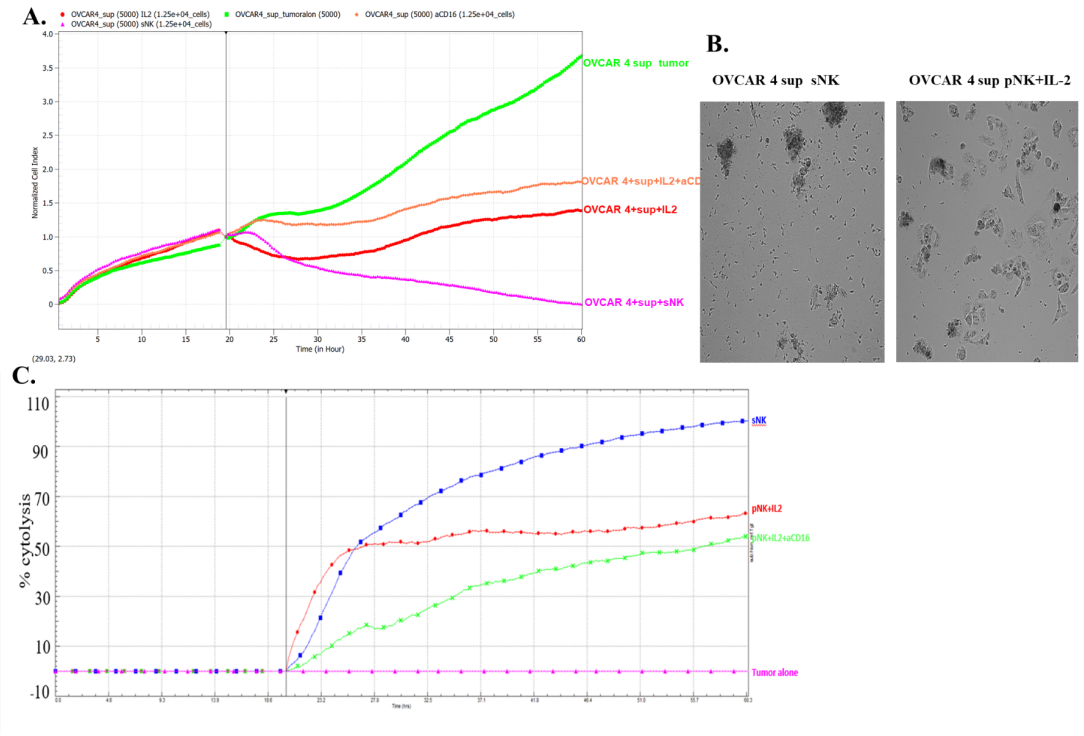


Figure 12: Cytotoxicity of sNK cells is higher compared to pNK with IL-2 in sNK supernatant treated OVCAR 4 cells

In the e-sight microplate, $5*10^3$ cells of the sNK supernatant treated OVCAR 4 cell line were plated. After allowing the cells to grow overnight, effector cells were added to each well following 18 hours of incubation. The cell index corresponding to an E:T ratio of 2.5:1 is illustrated in Figure**(A)**. Real-time images were captured at 48 hours of total incubation time in e-sight Figure **(B)**. The graph in Figure**(C)** represents the percentage of cytolysis.

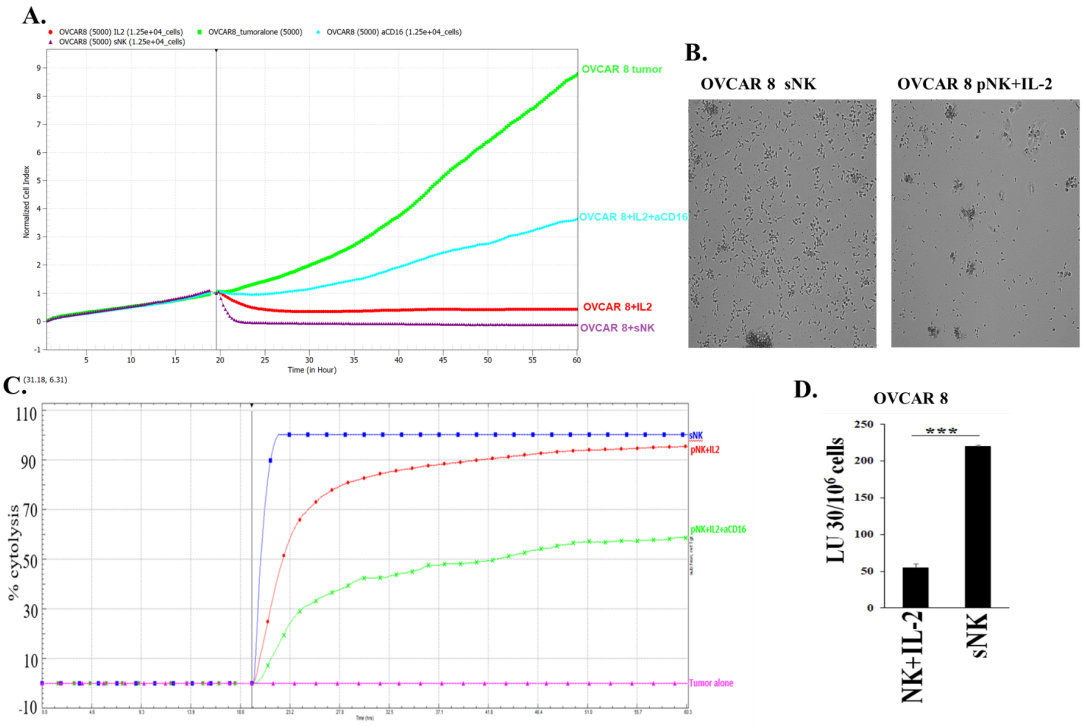


Figure 13: Cytotoxicity of sNK cells is higher compared to pNK with IL-2 in OVCAR 8 cells

In the e-sight microplate, $5*10^3$ cells of the OVCAR 8 cell line were plated. After allowing the cells to grow overnight, effector cells were added to each well following 18 hours of incubation. The cell index corresponding to an E:T ratio of 2.5:1 is illustrated in Figure**(A)**. Real-time images were captured at 48 hours of total incubation time in e-sight Figure **(B)**. The graph in Figure**(C)** represents the percentage of cytolysis. The cytotoxicity of sNK cells, pNK cells treated with IL-2, or a combination of IL-2 and aCD16 was assessed using a standard 4-hour 51Cr release assay Figure **(D)**. ***(p value <0.001)

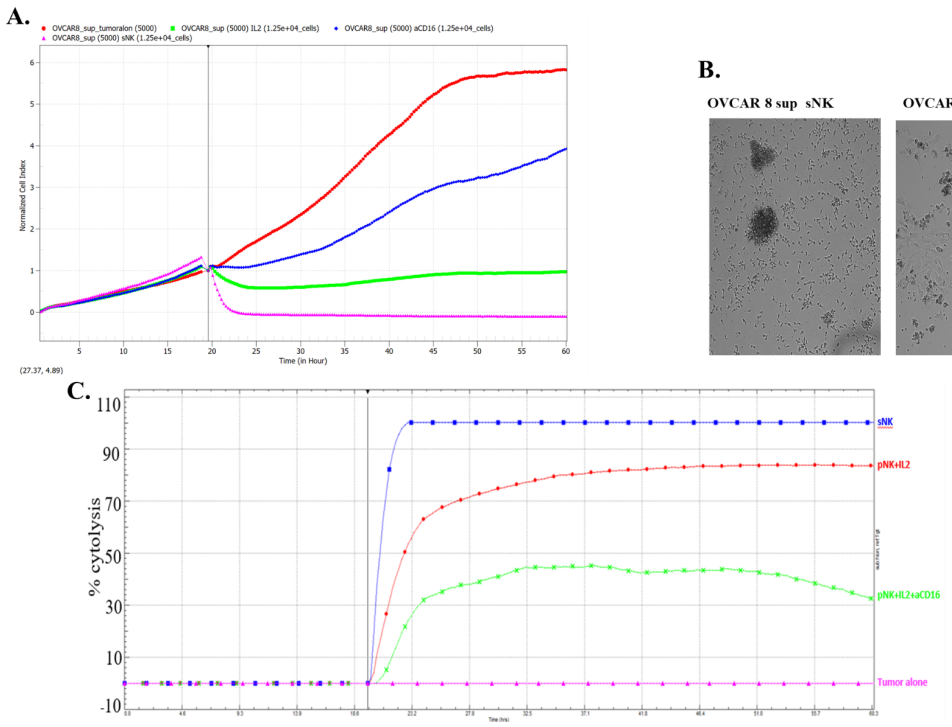


Figure 14: Cytotoxicity of sNK cells is higher compared to pNK with IL-2 in sNK supernatant treated OVCAR 8 cells

In the e-sight microplate, $5*10^3$ cells of the sNK supernatant treated OVCAR 8 cell line were plated. After allowing the cells to grow overnight, effector cells were added to each well following 18 hours of incubation. The cell index corresponding to an E:T ratio of 2.5:1 is illustrated in Figure**(A)**. Real-time images were captured at 48 hours of total incubation time in e-sight Figure **(B)**. The graph in Figure**(C)** represents the percentage of cytolysis.

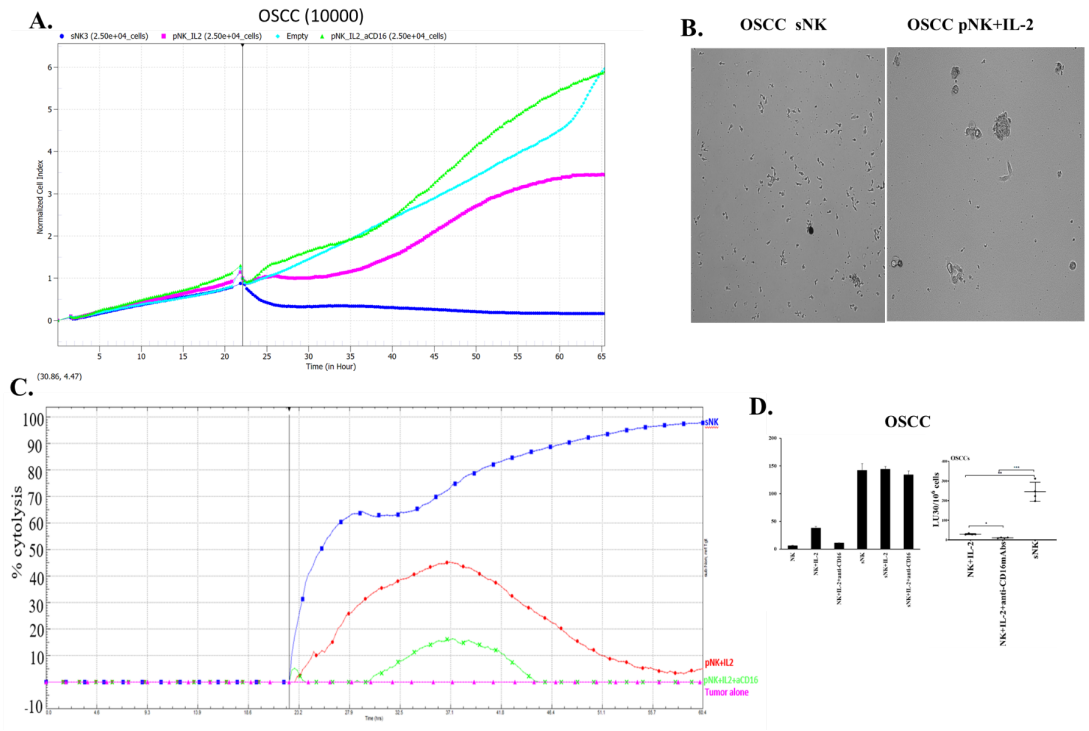


Figure 15: Cytotoxicity of sNK cells is higher compared to pNK with IL-2 in OSCC cells

In the e-sight microplate, $10*10^3$ cells of the OSCC cell line were plated. After allowing the cells to grow overnight, effector cells were added to each well following 18 hours of incubation. The cell index corresponding to an E:T ratio of 2.5:1 is illustrated in Figure**(A)**. Real-time images were captured at 48 hours of total incubation time in e-sight Figure **(B)**. The graph in Figure**(C)** represents the percentage of cytolysis. The cytotoxicity of sNK cells, pNK cells treated with IL-2, or a combination of IL-2 and aCD16 was assessed using a standard 4-hour 51Cr release assay Figure **(D)**. *** (p value < 0.001), ** (p value 0.001-0.01), * (p value 0.01-0.05).

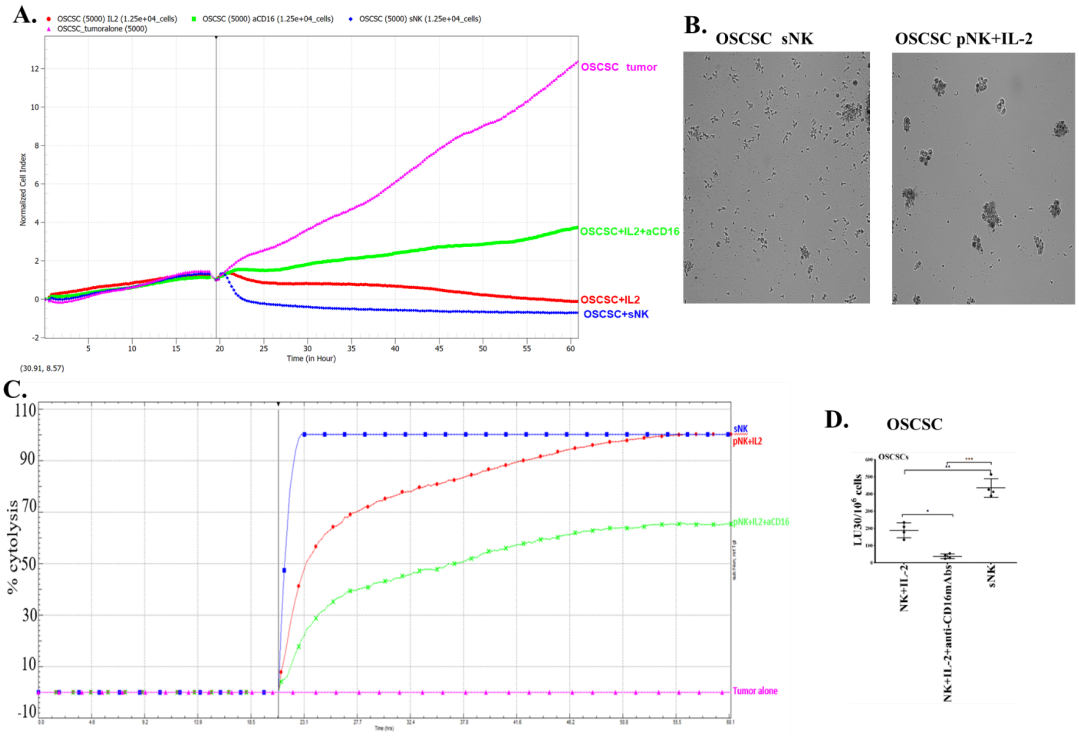


Figure 16: Cytotoxicity of sNK cells is higher compared to pNK with IL-2 in OSCSC cells

In the e-sight microplate, $5*10^3$ cells of the OSCSC cell line were plated. After allowing the cells to grow overnight, effector cells were added to each well following 18 hours of incubation. The cell index corresponding to an E:T ratio of 2.5:1 is illustrated in Figure**(A)**. Real-time images were captured at 48 hours of total incubation time in e-sight Figure **(B)**. The graph in Figure**(C)** represents the percentage of cytolysis. The cytotoxicity of sNK cells, pNK cells treated with IL-2, or a combination of IL-2 and aCD16 was assessed using a standard 4-hour 51Cr release assay Figure **(D)**. *** (p value < 0.001), ** (p value 0.001-0.01), * (p value 0.01-0.05)

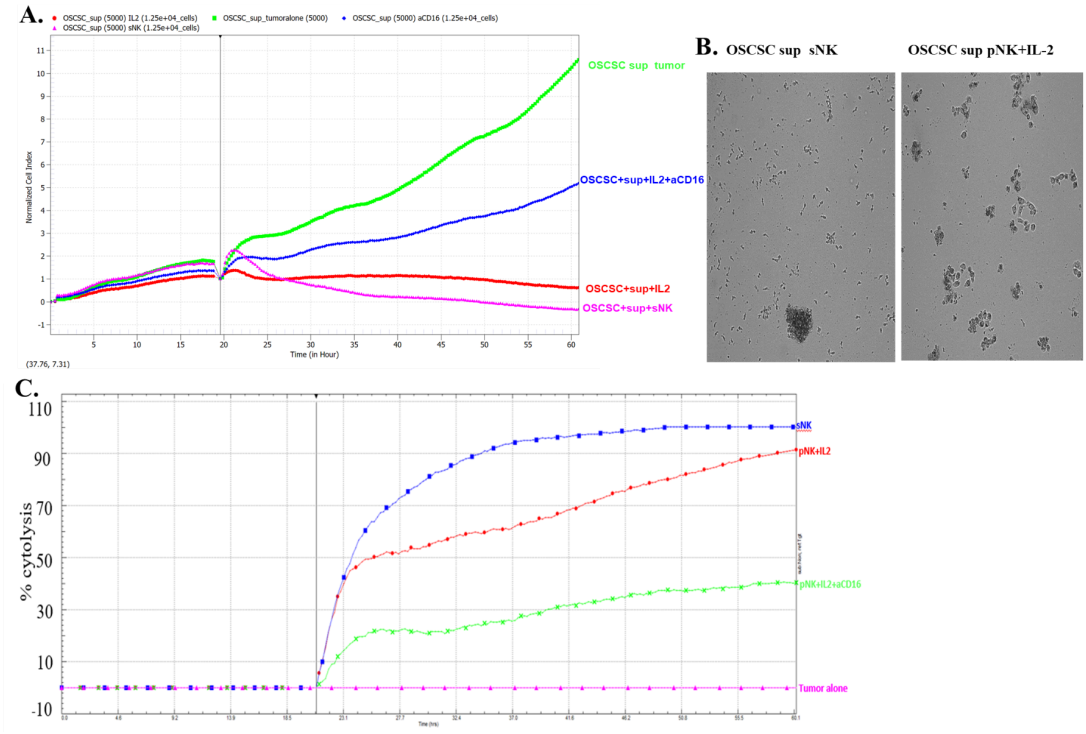


Figure 17: Cytotoxicity of sNK cells is higher compared to pNK with IL-2 in sNK supernatant treated OSCSC cells

In the e-sight microplate, $5*10^3$ cells of the sNK supernatant treated OSCSC cell line were plated. After allowing the cells to grow overnight, effector cells were added to each well following 18 hours of incubation. The cell index corresponding to an E:T ratio of 2.5:1 is illustrated in Figure**(A)**. Real-time images were captured at 48 hours of total incubation time in e-sight Figure **(B)**. The graph in Figure**(C)** represents the percentage of cytolysis.

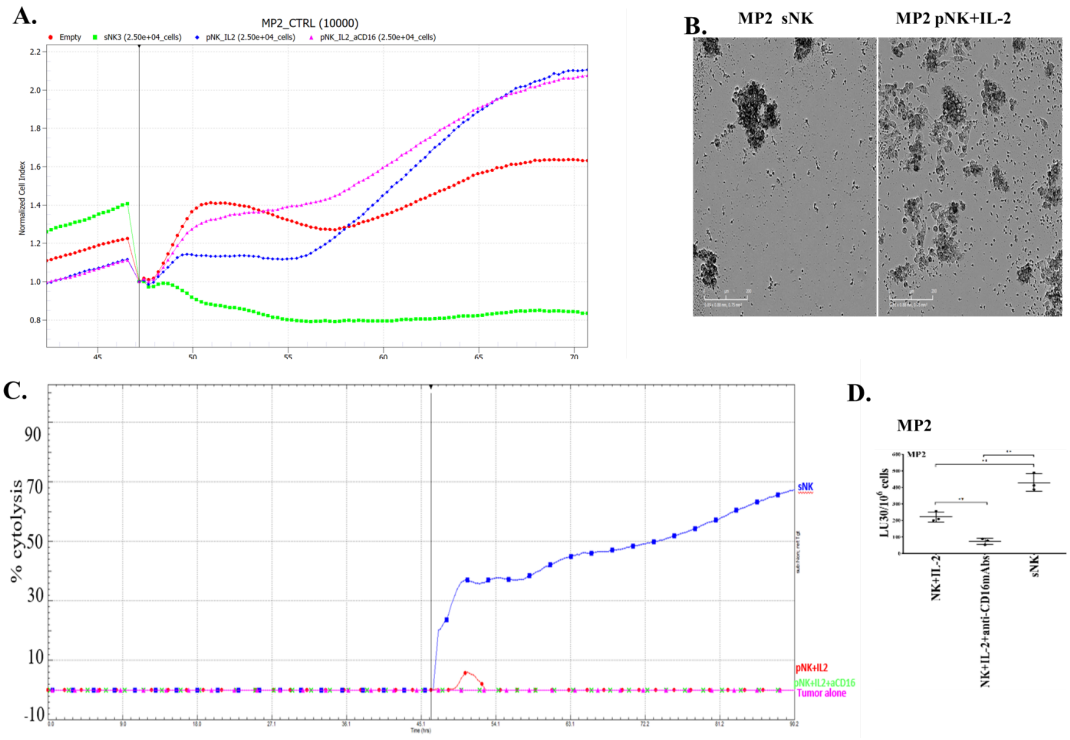


Figure 18: Cytotoxicity of sNK cells is higher compared to pNK with IL-2 in MP 2 cells

In the e-sight microplate, $5*10^3$ cells of the MP 2 cell line were plated. After allowing the cells to grow overnight, effector cells were added to each well following 18 hours of incubation. The cell index corresponding to an E:T ratio of 2.5:1 is illustrated in Figure**(A)**. Real-time images were captured at 48 hours of total incubation time in e-sight Figure **(B)**. The graph in Figure**(C)** represents the percentage of cytolysis. The cytotoxicity of sNK cells, pNK cells treated with IL-2, or a combination of IL-2 and aCD16 was assessed using a standard 4-hour 51Cr release assay Figure **(D)**. *** (p value < 0.001), ** (p value 0.001-0.01), * (p value 0.01-0.05)

Chapter 3: To establish and compare the expression levels of CD94 and NKG2A receptors on supercharged NK (sNK) cells and primary NK cells.

CD94 and NKG2A receptors are downregulated on supercharged NK cells

Single-cell RNA sequencing revealed differential expression patterns of CD94 and NKG2A receptors among supercharged NK (sNK) cells and IL-2 treated or untreated primary NK (pNK) cells. CD94 receptor levels were found to be lowest in sNK cells compared to IL-2 treated and untreated pNK cells. In contrast, NKG2A receptor levels were lowest in IL-2 treated pNK cells. Untreated pNK cells exhibited the highest expression levels of both CD94 and NKG2A receptors among the tested cell populations, surpassing sNK cells and IL-2 treated pNK cells **(Fig.19)** (manuscript in preparation)

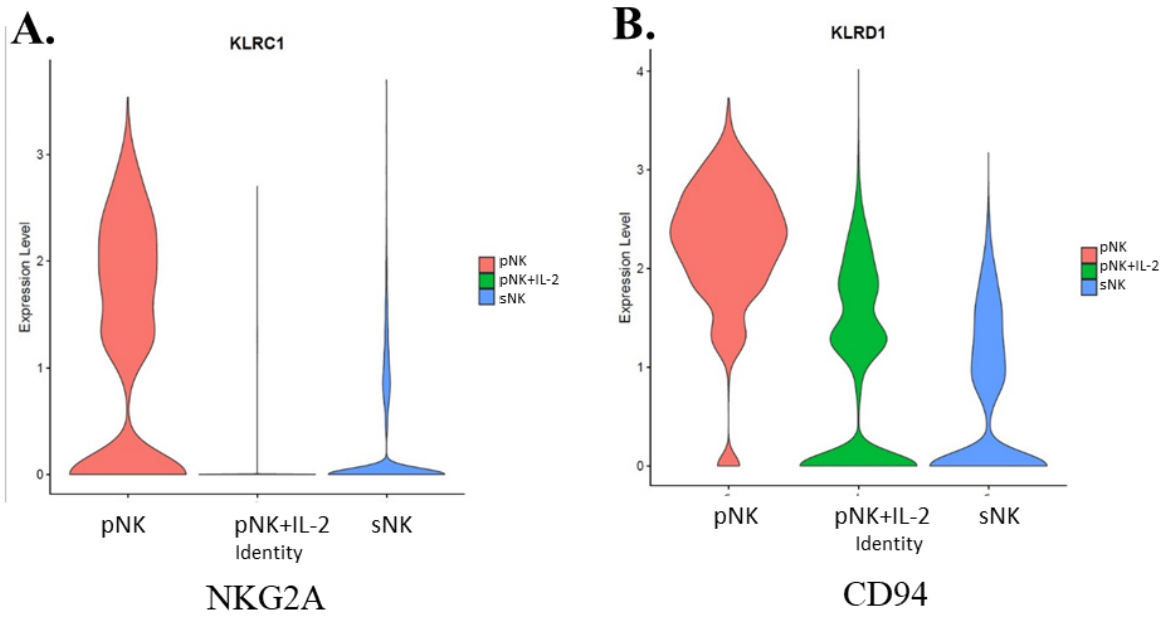


Figure 19: sNK cells show reduced expression of NKG2A and CD94 receptors

Single-cell RNA sequencing was performed on sNK, pNK and pNK treated with IL-2 and violin graphs were made for NKG2A receptors (A) and CD94 receptors (B).

Conclusion

In summary, our findings indicate that pNK cells treated with IL-2+sAJ4 exhibited the highest levels of IFN-γ, while IL-2+sAJ3 treatment resulted in the lowest levels, and IL-2+sAJ2 treatment showed moderately higher levels. Treatment of OVCAR 4, OVCAR 8, OSCC, OSCSC, and MP 2 cell lines with supernatant from supercharged NK (sNK) cells induced differentiation and upregulated MHC class-I receptors in all tumor lines, except for OVCAR 8. Moreover, Supercharged NK cells showed superior cytotoxic activity in comparison to primary NK cells in both untreated and sNK supernatant treated well differentiated and poorly differentiated tumor cells. Furthermore, we found that sNK cells expressed lower levels of inhibitory receptors NKG2A and CD94.

From this study, we have gained insights into the ability of sNK cells to lyse both stem-cell-like and well differentiated tumors. These cells also exhibit higher cytotoxicity compared to pNK cells. The supernatant of sNK cells induced differentiation, potentially slowing tumor growth. We also observed downmodulation of CD94 and NKG2A receptors on sNK cells. In future studies, it would be intriguing to examine the response of supernatant-treated and untreated tumors to sNK cells in animal models. Additionally, further investigation is warranted to understand the mechanisms underlying the higher cytotoxicity and the ability of sNK cells to lyse both stem-cell-like and well differentiated tumors.

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