

UCLA

UCLA Electronic Theses and Dissertations

Title

Mechanism of Natural Killer Cells Mediated Lysis and Differentiation of Ovarian Tumors

Permalink

<https://escholarship.org/uc/item/9nn225cd>

Author

Chovatiya, Nishant Rajeshbhai

Publication Date

2021

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Mechanism of Natural Killer Cells Mediated Lysis and Differentiation of Ovarian Tumors

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in

Oral Biology

by

Nishant Rajeshbhai Chovatiya

2021

© Copyright by

Nishant Rajeshbhai Chovatiya

2021

ABSTRACT OF THE THESIS

Mechanism of Natural Killer Cells Mediated Lysis and Differentiation of Ovarian Tumors

by

Nishant Rajeshbhai Chovatiya

Master of Science in Oral Biology

University of California, Los Angeles, 2021

Professor Anahid Jewett, Chair

Advances in Cancer Immunology has provided a deeper understanding of function of immune system in the progression of cancer and application of it in cancer. Especially, Natural Killer (NK) cells, without necessity of prior sensitization, have an ability to target cancer cells, specifically cancer stem cells but not their differentiated counterpart. It has been shown that NK cells induced Interferon – gamma (IFN- γ) and Tumor necrosis factor – alpha (TNF- α) leads to the expression of major histocompatibility complex class – I (MHC-class I) on cancer cells surface and induces differentiation in cancer stem cells. In this study, we have studied NK cells mediated lysis and differentiation of seven different types of ovarian cancer cell lines. OVCAR8 and CaOV3 showed highest sensitivity to NK cells mediated cytotoxicity. IFN- γ and TNF- α induced differentiation in all ovarian cancer cell lines being studied, with the exception of OVCAR8 and CaOV3, and enhanced cytotoxicity of chemotherapeutic drug Carboplatin against them.

The thesis of Nishant Rajeshbhai Chovatiya is approved.

Ichiro Nishimura

Nicolas A. Cacalano

Anahid Jewett, Committee Chair

University of California, Los Angeles

2021

DEDICATION

I dedicate this thesis primarily to my parents,

Dayaben

and

Rajeshbhai

who have given me limitless educational opportunities, and to my sister and brother-in-law who have supported me throughout this time, in the faith that this research may, in some way, contribute to the betterment of the Ovarian cancer patients.

VITA

2013-2018

College of Dental Sciences and Research Centre, Gujarat University, India

Table of Contents

DEDICATION	iv
VITA	v
ACKNOWLEDGEMENTS	viii
Introduction	1
Ovarian Cancer	1
OVCAR3	2
OVCAR4	3
OVCAR8	3
SKOV3	4
Kuramochi cells	5
OAW28	6
CaOV3	6
Natural Killer Cells	7
Thesis Outline	9
MATERIALS AND METHODS	10
Cell lines, reagents, and antibodies	10
Bacteria sonication	10
Purification of NK cells from the peripheral blood	11
Surface staining and Flow cytometry analysis	11
Cell differentiation assay	12
Carboplatin	13
Cell death assay	13
⁵¹ Cr release cytotoxicity assay	14
Enzyme-Linked Immunosorbent Assays (ELISAs)	15
Statistical analysis	15
RESULTS	16
Chapter 1: Establish the phenotypic and functional differences and similarities between different ovarian cancer cell lines	16
OVCAR8, SKOV3 and CaOV3 demonstrated the highest growth potential in comparison to other ovarian tumor lines	16
OVCAR8, and CaOV3 demonstrated the highest expression of CD44 and the lowest expression of MHC class I when compared to other ovarian tumor lines	16

IFN- γ and TNF- α treatment of the tumors increased MHC class I expression in all the tumor cells except for OVCAR8 and CaOV3 tumor cells.....	17
Chapter 2: To establish the differential targeting ability of NK cells against ovarian tumors in terms of cytotoxicity and secretion of IFN- γ	32
IFN- γ + TNF- α treatment of the tumors increased differentiation of the tumors in some of the tumors and resulted in less susceptibility to NK cell mediated cytotoxicity with the exception of	32
OVCAR8 and CaOV3 tumor cells which remained susceptible to NK cell mediated cytotoxicity in the presence or absence of IFN- γ + TNF- α treatment.....	32
IFN- γ + TNF- α treatment of the tumors increased differentiation of the tumors in some of the tumors and resulted in less secretion of IFN- γ by the NK cells co-cultured with those tumors, with the exception of OVCAR8 and CaOV3 tumor cells which remained similar in the secretion of IFN- γ by the NK cells when tumors were treated with IFN- γ + TNF- α	33
Chapter 3: Establish the differentiation stages and capabilities of ovarian tumor lines and their susceptibility to carboplatin mediated effects.	39
IFN- γ treatment of the tumors increased differentiation of the tumors in some of the tumors and resulted in more susceptibility to carboplatin mediated decrease in cell growth with the exception of OVCAR8 and CaOV3 tumor cells which remained relatively higher in cell growth when treated with IFN- γ and/or TNF- α	39
IFN- γ treatment of the tumors increased differentiation of the tumors in some of the tumors and resulted in more susceptibility to carboplatin mediated increase in cell death with the exception of SKOV3 and CaOV3 tumor cells which remained in general lower in cell death when treated with and without IFN- γ when compared to other ovarian tumor lines.....	40
Conclusion.....	52
References	53

ACKNOWLEDGEMENTS

I extend my heartiest thanks to my mentor, Dr. Anahid Jewett, who gave me the golden opportunity to do this amazing project, and for her contribution in making me understand intricately the topic of cancer immunology that made this project a success. I would also like to thank my Committee members Dr. Ichiro Nishimura and Dr. Nicholas Cacalano for their guidance and support.

Most importantly, I would like to convey my special thanks of gratitude to all of my lab members, especially Po-Chun Chen, Mengwei Ko and Sara Huerta who have helped me a lot in finishing this project. I could not have done this without you!

Last but not least, I would like to thank Dr. Memarzadeh's team for providing us with tumor cells, technical expertise, data on Ovarian tumors, and to be a backbone in this research work.

Introduction

Ovarian Cancer

Ovarian cancer is a type of cancer involving female reproductive organ, ovaries, which are responsible for producing ova, and hormones – estrogen and progesterone. It usually goes undetected before it spreads to abdominal cavity or into the pelvis, and during this late stage it is very hard to manage ovarian cancer [1]. Based on origin, there are three types of Ovarian cancers.

1. Epithelial: It comprises of 90% of the ovarian cancer and begins in the outermost peripheral epithelium lining the ovaries [1]. It is the most heterogeneous among and is the most fatal gynecological cancer [2].
2. Stromal: It comprises of 7% of the ovarian cancer and begins in the hormone producing cells of the ovaries [1].
3. Germ cell tumors: It is rarest among all and begins in the ova producing cells [1].

As advanced ovarian cancer is still incurable now a days, ovarian cancer remains most lethal gynecological cancer in the western world and is top five leading cause of death due to malignancy in the United States of America [2-4]. Mortality rates for ovarian cancer has not been changed since very long even though after introduction of newer treatments or chemotherapeutic drugs [2]. There are more than 100 epithelial ovarian cancer cell lines exists and we studied the interaction of NK cells with seven of them – OVCAR3, OVCAR4, OVCAR8, SKOV3, Kuramochi, OAW28, and CaOV3 – here in this study, and how NK cells induced differentiation in ovarian cancer cells can enhance cytotoxicity of chemotherapeutic drug (Carboplatin).

OVCAR3

OVCAR3 is an ovarian carcinoma model which was established by T.C. Hamilton, et al. in 1982 from malignant ascites of a patient with ovarian adenocarcinoma [5]. It is mainly being used to study drug resistance in ovarian carcinoma. Presence of surface hormone receptors make it a good candidate for the hormonal therapy research [6]. In addition to hormonal receptors, OVCAR3 expresses MUC16 receptor (Mucin 16), CA-125 (cancer antigen 125), Type I glycoprotein on the surface [7, 15]. It has been known that MUC16 is an inhibitor of NK cell cytotoxicity [8]. It has been established that MUC16 interacts directly with NK cells and incubation of health human NK cells with MUC16 results in marked decrease in surface expression of CD16 on NK cells [8, 9]. In addition, MUC16 acts as an anti-adhesive agent and disables NK cells to form immune synapse on target cells and subsequently restricts NK cell activation and lysis of target cells [10, 11, 12]. And because of higher surface expression of MUC16, health human donors derived NK cells are deficient to lyse OVCAR3 [13, 14]. But MUC16^{low} OVCAR3 cells selectively did conjugate with NK cells about 7 folds more, in comparison to MUC16^{high} OVCAR3 cells, and MUC16^{low} OVCAR3 cells shows higher surface expression of NK cell activating molecules DNAM-1 (CD226) and NKG2D ligands [7]. However, to this date, there has been no studies conducted to determine differentiation stage of OVCAR3 cells. Here in this study, we evaluated effect of key NK cells activating molecules (rh-IFN- γ and rh-TNF- α) and effect of it on chemotherapeutic drugs mediated lysis of OVCAR3 cells.

OVCAR4

OVCAR4 is high-grade serous adenomatic ovarian carcinoma cell line which was established from an ovarian cancer patient resistant to Cisplatin and other multiple chemotherapeutic drugs. It is one of the most representative ovarian cancer cell line for multi-drug resistant ovarian cancer [16, 17]. OVCAR4 has migration and invasion ability and is tumorigenic in nude mice [17, 18]. OVCAR4 shows Leu130VAL mutation in TP53 and has higher surface expression of $\alpha v \beta 3$, a cancer marker integrin [17, 19-20]. In addition to that, OVCAR4 shows higher surface expression of MHC-class I [21]. It has also been shown that NK cells show reduced cytotoxicity against OVCAR4 cells [22]. Concurrently, monocytes stimulated NK cells shows relatively higher CD107a expression when co-cultured with OVCAR4 cells [21]. However, to the date, there has been no studies conducted to identify differentiation status of OVCAR4 cells and its relevance on NK cells mediated cytotoxicity. Here in this study, we have studied differentiation of OVCAR4 cells and its effects on chemotherapeutic drugs mediated lysis.

OVCAR8

OVCAR8 is a high grade ovarian serous adenomatic carcinoma obtained from tumor tissue of platin drug resistant patient [23]. It has been shown that chemotherapeutic drug resistant OVCAR8 displays higher surface expression of CD44 receptor on western blot which correlates with poor clinical outcome [24]. It has also been shown that Paclitaxel treatment in OVCAR8 xenograft model resulted in upregulation of CD44 receptor which can lead to poor outcome and tumor recurrence [24]. Moreover, knockdown of CD44 by CD44-dhRNA using lentivirus-mediated gene transfer system resulted in cell growth retardation and inhibition of spheroid

formation in 3D culture of OVCAR8 cells, and also enhanced drug sensitivity [24]. Moreover, it has also been shown that OVCAR8 displays lower surface expression of HLA-ABC (MHC-class I) [28]. It has been previously shown and criteria has been established in our Lab that poorly differentiated cancer cells show higher surface expression of CD44 and lower surface expression of MHC-class I, CD54, and B7H1 (anti-PD1) [25-27] and are more sensitive to NK cells mediated lysis [29]. Thus, OVCAR8 falls more towards poorly differentiated side on differentiation stage scale which makes them more sensitive to NK cells mediated lysis. Here in this study, we experimented to induce differentiation in OVCAR8 cells using human recombinant NK cells cytokines and subsequently also studied its effects on chemotherapeutic drug mediated lysis of cells.

SKOV3

SKOV3 is an ovarian carcinoma cell line obtained from ascites of patient with serous ovarian cystadenomatous carcinoma [30]. It has been shown that SKOV3 lacks surface expression of MUC16 [31-32] and has ectopic expression of MUC16 C-terminal domain which is responsible for lower lysis by chemotherapeutic platinum drugs [33]. SKOV3 has been known to induce large solid tumors loosely adhered to fat in pelvic region, intestines, and omentum in nude mice [34]. It has been shown earlier that NK cells can limit the growth of SKOV3 tumor cells in a dose and time-dependent manner and can limit the migration/invasion of these cells [35], but SKOV3 cells were comparatively less susceptible to NK cell mediated lysis than K562 and PaCA5061 cells [36]. Moreover, it has been also shown that BALB/c-nude mice with SKOV3 tumor receiving NK cell treatment displays significantly higher expression of MHC-class I expression on tumor cells of xenograft, in comparison to tumors from BALB/c-nude mice

without any treatments [35]. Combined IL-12/IL-15/SR1 generated hematopoietic stem and progenitor cell (HPSC)-derived NK cells mediates prolonged survival of mice bearing SKOV3 tumor and induces anti-tumor effect [37]. Targeted human NK cells to chimeric receptor specific for HER-2 receptor (CR-NK cells) gets activated in relation to magnitude of HER-2 expression on SKOV3 tumor cells and has shown to completely irradiate SKOV3 tumor in ARG2 knockout mice [38]. To this date, tumor cells differentiation stage of SKOV3 and cytotoxic response of chemotherapeutic drugs against SKOV3's differentiation scale has been unanswered. Here in this study, as described earlier, we induced differentiation in SKOV3 tumor cells by key NK cells cytokines and have studied response of these cells to chemotherapeutic drug treatment.

Kuramochi cells

Kuramochi is a high grade serous ovarian adenomatic carcinoma cell line obtained from ascites of a patient with ovarian cancer, and it is relatively resistant to platin drugs [4]. During evaluation of NK cells inhibitory ligands, it has been shown that Carboplatin treatment of Kuramochi cells induces upregulation of HLA-ABC (MHC-class I), and subsequently has shown reduced NK cells mediated lysis [39]. In previous study, Kuramochi cells have formed distinct nodules in mice xenograft model in comparison to large expansile tumors of SKOV3. Kuramochi tumors grew slowly and halted growth at around tumor size of 12 mm³ in comparison to SKOV3 tumor size of 200 mm³ [40]. However, to this date, differentiation status of Kuramochi cells is still unanswered. Here in this study, we researched on different stem cell and differentiated cell markers on Kuramochi cells. We also induced differentiation by exposing Kuramochi cells to IFN- γ and TNF- α and studied the amount of IFN- γ release by NK cells co-cultured with Kuramochi cells and its effect on chemotherapeutic drugs mediated killing.

OAW28

OAW28 is an ovarian high grade serous adenocarcinoma obtained from ascites of a 75-year-old patient with ovarian cystadenocarcinoma [2, 41], and it has a cell doubling time of around 60 hours [41]. OAW28 has lesser surface expression of MUC16 (CA125) in comparison to OVCAR3 [7, 15, 42]. When tested against platin chemotherapeutic drugs, OAW28 was relatively more sensitive than SKOV3 [43]. OAW28 cells are significantly sensitive to all-trans retinoid acid (ATRA) lysis and it significantly inhibits plasmin generation, motility, and invasion of OAW28 cells [44]. In terms of cancer immunology, OAW28 seems to be the least explored cell lines. Here in this study, we studied the immunological counterparts of OAW28, specifically with NK cells, and have shown increase susceptibility of OAW28 cells following induced differentiation by NK cells cytokines – IFN- γ and TNF- α .

CaOV3

CaOV3 is a high grade ovarian serous adenomatous carcinoma obtained from ascites of a patient with ovarian adenocarcinoma [2]. It has an epithelial morphology and forms tightly packed colonies in culture. It is sensitive to ATRA therapy and is tumorigenic when injected into mice [45-47]. CaOV3 displays significantly higher mean fluorescence intensity (MFI) for CD44 receptor in flow cytometry with around 80% of CD44 expression is coming from CD44H. CD44 is the key adhesion molecule for binding of CaOV3 cells to mesothelium and anti-CD44 treatment induces reduction in specific binding of cells [48, 49]. It has been previously shown and criteria has been established in our Lab that poorly differentiated cancer cells show higher surface expression of CD44 and lower surface expression of MHC-class I, CD54, and B7H1 (anti-PD1) [25-27] and are more sensitive to NK cells mediated lysis [29]. It has been shown in

vivo that BALB/c-nude mice implanted with CaOV3 tumor cells shows marked reduction in tumor growth following NK cell treatment in comparison to control mice, and it also increases the median survival time [35]. When evaluated against Cisplatin and Doxorubicin, CaOV3 stem cells displays reduced cytotoxicity and down regulation of MHC-class I on their surface but shows increased lysis by NK cells and upregulation of NKG2D ligands [50]. Here in this study, we have studied differentiation stage of CaOV3, its effect on lysis by NK cells and chemotherapy, and have compared amount of IFN- γ expression by NK cells co-cultured with those cells.

Natural Killer Cells

Natural killer (NK) cells, cytotoxic large granular lymphocytes, is a part of innate immune system, represents around 5 to 20 % of the total lymphocytes in humans, and have an anticancer function. NK cells are identified by lack specific CD3 and T cell receptor on their surface and their expression of CD16 and CD56 receptors, unlike other immune cells, and are activated mainly by cytokine induction [51, 52]. NK cells also induces crucial cytokines and activated adaptive immune system [53]. There are specifically two major subsets of NK cells present in peripheral blood. First is CD16+ CD56dim CD69-, which is the predominant subset, comprises 90% of the NK cells in the circulating blood, and mediates cytotoxicity. Second is CD16- CD56bright CD69+, comprises 10% of the NK cells in the circulating blood and are mainly responsible for the secretion of the cytokines [54].

Perforin, , a membrane-disrupting protein, and granzyme B, a serine protease, are mainly responsible for the NK cells mediated cytotoxicity, and they are responsible for necrosis and apoptosis too [55]. Upon target cells recognition by NK cells, the secretory lysosomes polarize,

and Perforin facilitates delivery of the Granzyme B, which induces caspases mediated cell death [56-58]. In addition, NK cells induce cytotoxicity through the target cells death receptors via surface expression of Fas Ligand, Trail, and Tumor necrosis factor – alpha (TNF- α) [59]. By interacting with its ligand CD95L (CD178/TNFSF6), Fas (CD95/APO-1/TNFRSF6), one type of a cell surface protein of the TNF receptors, can induce apoptosis [59]. NK cells also induce antibody dependent cellular cytotoxicity (ADCC) against tumors and can regulate the function of other cells by secreting cytokines and chemokines [60].

It has been shown that NK cells induce differentiation in the cancer stem cells (undifferentiated or poorly differentiated tumors), specifically by secreting cytokines, such as IFN- γ and TNF- α , which slows-down the tumor progression [61]. Active receptors and co-receptors which recognize ligands on the tumor cells surface induces NK cell activation [62, 63]. The risk of tumor progression gets reduced because of the cytotoxicity of various immune cells and specifically by NK cells infiltration in the tumor [63]. Altered sensitivity and function of NK cells is linked to increased cancer risk diminished NK cells has been found in the cancer patients [64-74].

Thesis Outline

Specific aim 1: To establish the phenotypic and functional differences and similarities between different ovarian tumor lines.

Specific aim 2: To establish the differential targeting ability of NK cells against ovarian tumors in terms of cytotoxicity and secretion of IFN- γ .

Specific aim 3: To establish the differentiation stages and capabilities of ovarian tumor lines and their susceptibility to carboplatin mediated effects.

MATERIALS AND METHODS

Cell lines, reagents, and antibodies

Human immune cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, CA). Seven Ovarian cancer cell lines – OVCAR3, OVCAR4, OVCAR8, SKPV3, Kuramochi, OAW28, and CaOV3 – were obtained from Dr. Sanaz Memarzadeh (G.O. Discovery Lab, UCLA). OVCAR3 and OVCAR4 were cultured in RPMI1640 supplemented with 10% FBS (Gemini Bio-Products, CA). OVCAR8, SKOV3, Kuramochi, OAW28 and CaOV3 were cultured in DMEM 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).

Bacteria sonication

AJ4 is a combination of 4 different strains of gram-positive probiotic bacteria (*Streptococcus thermophiles*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and *Lactobacillus paracasei*) which was used to induce activation of the NK cells. AJ4 was weighed and resuspended in RPMI Medium 1640 containing 10% FBS at a concentration of 10mg per 1mL. The bacteria were thoroughly vortexed, then sonicated on ice for 15 seconds, set at a 60% amplitude. Sonicated samples were then incubated for 30 seconds on ice. After every five pulses,

a sample was taken to observe under the microscope until at least 80 percent of cell walls were lysed. It was determined that approximated 20 rounds of sonication/incubation on ice, were conducted to achieve complete sonication. Finally, the sonicated samples (sAJ4) were aliquoted and stored in a -80 degrees Celsius freezer for future use.

Purification of NK cells from the peripheral blood

Written informed consents, approved by UCLA Institutional Review Board (IRB), were obtained from healthy blood donors, and all procedures were approved by the UCLA-IRB. Peripheral blood was separated using Ficoll-Hypaque centrifugation, after which the white, cloudy layer, containing peripheral blood mononuclear cells (PBMC), was harvested, washed, and re-suspended in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% FBS and plated on plastic tissue culture dishes. After 1-2 hours of incubation, non-adherent, human peripheral blood lymphocytes (PBL) were collected. NK cells were negatively selected and isolated from PBLs using the EasySep® Human NK cell enrichment kit purchased from Stem Cell Technologies (Vancouver, BC, Canada). Isolated NK cells were stained with anti-CD16 antibody, to measure NK cell purity using flow cytometric analysis. The isolated NK cell population was greater than 90% purity. Purified NK cells were cultured in RPMI Medium 1640 supplemented with 10% FBS (Gemini Bio-Products, CA), 1% antibiotic antimycotic, 1% sodium pyruvate, and 1% MEM non-essential amino acids (Invitrogen, Life Technologies, CA).

Surface staining and Flow cytometry analysis

1×10^5 cells were used for each sample. All samples were stained with 100 μ L of 1% BSA-PBS (Gemini Bio-Products, CA) and pre-determined optimal concentration of desire

fluorochrome (PE, FITC or PEcy5) conjugated antibodies and incubate at 4°C for 30 min. The sample then was washed and resuspend with 1% BSA-PBS. Intracellular staining was performed as described in manufacturer's protocol. The sample was fixed with fixation buffer for an hour in dark at room temperature. Two ml of perm buffer was added to wash the sample and the sample was centrifuged at 1400 rpm for 5 min. Repeated the washing step once and discarded the supernatants. Resuspend the sample in Perm buffer and added fluorochrome conjugated antibody and incubate in dark at room temperature for 30 minutes. Repeat the washing step with Perm buffer once and 1% BSA/PBS. Resuspend the sample with 1% BSA/PBS. Propidium iodide (PI)(100 µg/ml) was used for cell death assay. Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA) and FlowJo v10.4 (BD, Oregon, USA) were used for analysis.

Cell differentiation assay

Recombinant human Interferon gamma (rhIFN- γ) and recombinant human tumor necrosis factor alpha (rhTNF- α) was purchased from PeproTech (Rocky Hill, NJ). Ovarian cancer cells were treated with different concentration of rhIFN- γ (20ng/ml and 50ng/ml) and rhTNF- α (20ng/ml) or combination of rhIFN- γ (20ng/ml) + rhTNF- α (20ng/ml) or rhIFN- γ (50ng/ml) + rhTNF- α (20ng/ml). After an overnight incubation, all samples were trypsinized, cells were counted after staining with trypan blue, and $\sim 1 \times 10^5$ cells were stained with differentiation cell markers – CD44, MHC-class I, CD54 and B7H1 (PD-L1), and standard surface staining procedure was followed as discussed earlier.

Carboplatin

Carboplatin was received from Tocris Bioscience (Bristol, United Kingdom). 50mg Carboplatin powder was dispensed in 15ml conical tube. 13.46ml of sterilized diH₂O was added into the tube with carboplatin. Then it was mixed thoroughly with pipette to mix the water with the powdered carboplatin. Tube was vortexed to make sure that carboplatin has been dissolved completely. This is how 10mM stock solution of carboplatin was made. After that it was aliquoted and stored at -20C for future use.

Cell death assay

Recombinant human Interferon gamma (rhIFN- γ) and recombinant human tumor necrosis factor alpha (rhTNF- α) was purchased from PeproTech (Rocky Hill, NJ). Propidium iodide (PI) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypan Blue was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ovarian cancer cells were treated with different concentration of rhIFN- γ (20ng/ml and 50ng/ml) and rhTNF- α (20ng/ml) or combination of rhIFN- γ (20ng/ml) + rhTNF- α (20ng/ml) or rhIFN- γ (50ng/ml) + rhTNF- α (20ng/ml). After an overnight incubation, all different samples were treated with different concentration of carboplatin (10 μ M/ml and 30 μ M/ml) for overnight. Next day, all samples were trypsinized, cells were counted after staining with trypan blue, and were stained with Propidium iodine (PI) (PE conjugated) for cell death analysis through flow cytometry. Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA) and FlowJo v10.4 (BD, Oregon, USA) were used for analysis.

⁵¹Cr release cytotoxicity assay

Radioactive ⁵¹Cr was purchased from Perkin Elmer (Santa Clara, CA). Standard ⁵¹Cr release cytotoxicity assay was used to determine NK cell cytotoxicity in the experimental cultures and the sensitivity of target cells to NK cell mediated cytotoxicity. Effector cells (5x10⁴ NK cells/well) were aliquoted into 96-well round-bottom microwell plates (Fisher Scientific, Pittsburgh, PA) and titrated at four to six serial dilutions with RPMI 1640. The target cells (1x10⁴ Ovarian tumor cells) were labeled with 50μCi ⁵¹Cr (Perkin Elmer, Santa Clara, CA) and chromated for 1 hour in CO₂ incubator at 37°C. After incubation period is over, target cancer cells were washed twice with media to remove excess unbound ⁵¹Cr. ⁵¹Cr-labeled target cells (1 x 10⁴ per well) were aliquoted into the 96-well round bottom microwell plates containing effector cells at a concentration of 1x10⁴ cells/well at a top effector: target (E:T) ratio of 5:1. Plates were centrifuged and then incubated for a period of 4 hours. After a 4-hour of an incubation period, the supernatants were harvested from each sample into a glass tube and was counted for released radioactivity using the gamma counter (Beckman Instruments, Fullerton, CA). Total (containing ⁵¹Cr-labeled target cells) and spontaneous (supernatants of target cells alone) release values were measured and used to calculate the percentage specific cytotoxicity. The percentage specific cytotoxicity was calculated using the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Total Cpm} - \text{Spontaneous cpm}}$$

Lytic Unit (LU) 30/10⁶ is calculated by using the inverse of the number of effector cells needed to lyse 30% of target cancer cells x 100.

Enzyme-Linked Immunosorbent Assays (ELISAs)

Human ELISA kit for IFN- γ was purchased from Biolegend (San Diego, CA). ELISA was performed to detect the level of IFN- γ produced from cell cultures. The assay was conducted as described in the manufacturer's protocol. Briefly, 96-well EIA/RIA plates were coated with diluted capture antibody corresponding to target cytokine and incubated overnight at 4°C. After 16-18 hours of incubation, the plates were washed 4 times with wash buffer (0.05% Tween20 in 1xPBS) and blocked with assay diluent (1% BSA in 1xPBS). The plates were incubated for 1 hour at room temperature, on a plate shaker at 200rpm; plates were washed 4 times following incubation. Then, 100 μ L of standards and samples collected from each culture were added to the wells and incubated for 2 hours at room temperature, on the plate shaker at 200rpm. After incubation, plates were washed 4 times, loaded with detection antibody, and incubated for 1 hour at room temperature, on the plate shaker at 200rpm. After 1 hour of incubation, the plates were washed 4 times; wells were loaded with Avidin-HRP solution and incubated for 30 minutes at room temperature, on the plate shaker at 200rpm. After washing the plates 5 times with wash buffer; 100 μ L of TMB substrate solution was added to the wells and plates were incubated in the dark until they developed a desired blue color (or up to 30 minutes). Then, 100 μ L of stop solution (2N H₂SO₄) was added per well to stop the reaction. Finally, plates were read in a microplate reader, at 450nm to obtain absorbance values (Biolegend, ELISA manual).

Statistical analysis

An unpaired, two-tailed student t-test was performed for the statistical analysis of two groups. ***(p value <0.001), **(p value 0.001-0.01), *(p value 0.01-0.05).

RESULTS

Chapter 1: Establish the phenotypic and functional differences and similarities between different ovarian cancer cell lines.

OVCAR8, SKOV3 and CaOV3 demonstrated the highest growth potential in comparison to other ovarian tumor lines.

To study and compare the growth rate of all ovarian cancer cell lines, growth of all ovarian cancer cell lines was determined after every other consecutive day. We observed that OVCAR8, SKOV3 and CaOV3 demonstrates the highest growth potential, OVCAR4 demonstrated medium growth rate, and last 3 cell lines, OVCAR3, Kuramochi, and OAW28 demonstrated the least growth potential among all ovarian cancer cell lines studied. **(Fig. 1).**

OVCAR8, and CaOV3 demonstrated the highest expression of CD44 and the lowest expression of MHC class I when compared to other ovarian tumor lines.

It has been previously researched that lower surface expression of MHC-class I and higher surface expression of CD44 is a characteristic of a poorly differentiated tumor [25-27]. Next, to determine the differentiation status of the ovarian cancer cell lines, surface expression of MHC-class I and CD44 on all ovarian cancer cell lines were determined. PE IgG isotype antibody was used as a control. And to see if the cells are modulating and are showing different expression over the period of time, the experiment was repeated on every other consecutive day. Almost all cell lines, showed relatively stable level of CD44 and MHC-class I **[Fig. 2 to 6]**. The

numbers on bottom right-hand are percentages and the numbers on top right-hand corner are the mean fluorescence intensities of each histogram [Fig. 4 to 6].

OVCAR8 and CaOV3 showed the highest level of CD44 expression, while OVCAR4 and SKOV3 showed medium level of expression, and OVCAR3, Kuramochi, and OAW28 showed the least level of CD44 expression [Fig. 2, 4 to 6]. Correlatively, OVCAR8 and CaOV3 showed least expression of MHC-class I, in comparison to other cell lines, who displayed either medium or higher level of MHC-class I expression [Fig. 3, 4 to 6]. Thus, this experiment shows that OVCAR8 and CaOV3 have the most stem cells like characteristics, OVCAR4 and SKOV3 are in middle in the spectrum, and OVCAR3, Kuramochi, and OAW28 have the most differentiated cells like characteristics.

IFN- γ and TNF- α treatment of the tumors increased MHC class I expression in all the tumor cells except for OVCAR8 and CaOV3 tumor cells.

It has been previously demonstrated that treatment of cancer cells with supernatants from NK cells increases the surface expression of MHC-class I on cancer cells and makes them more differentiated. It has been also found that IFN- γ , secreted by the NK cells, is the chief cytokine responsible for the induction of differentiation [75].

To determine the induction of differentiation in the ovarian cancer cell lines, rh-IFN- γ and rh-TNF- α , in different concentration, either alone or in combination, were used to treat cells overnight. Subsequently, next day, surface expression of CD44, CD54, MHC-class I and B7H1 was determined by flow cytometry. PE IgG isotype antibody was used as a control [Fig. 7 to 14]. All cell lines displayed increase in expression of MHC-class I after treatment with IFN- γ and TNF- α , especially after treatment with combination of IFN- γ and TNF- α , except of OVCAR8

and CaOV3 which showed relatively similar level of expression, in comparison to control [**Fig. 7 to 14**]. This displayed that OVCAR8 and CaOV3 are poorly-differentiated cell lines and are having a stem cells characteristics.

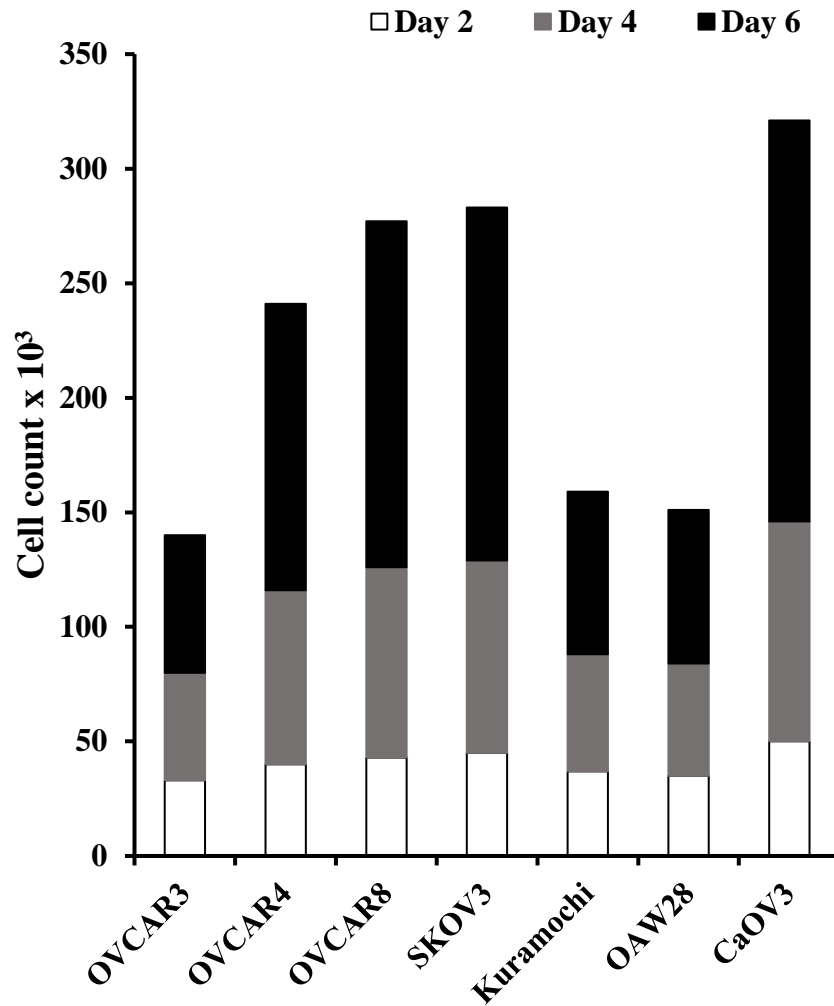


Figure 1: Growth rate of ovarian cancer cell lines.

~30 x 10³ cells of each ovarian cancer cell lines were plated in 3 wells of 12-well plate.

Subsequently, on Day2, Day4, and Day6, one well was trypsinized and living cells were counted after staining with trypan blue. Cell count numbers are shown in x 10³.

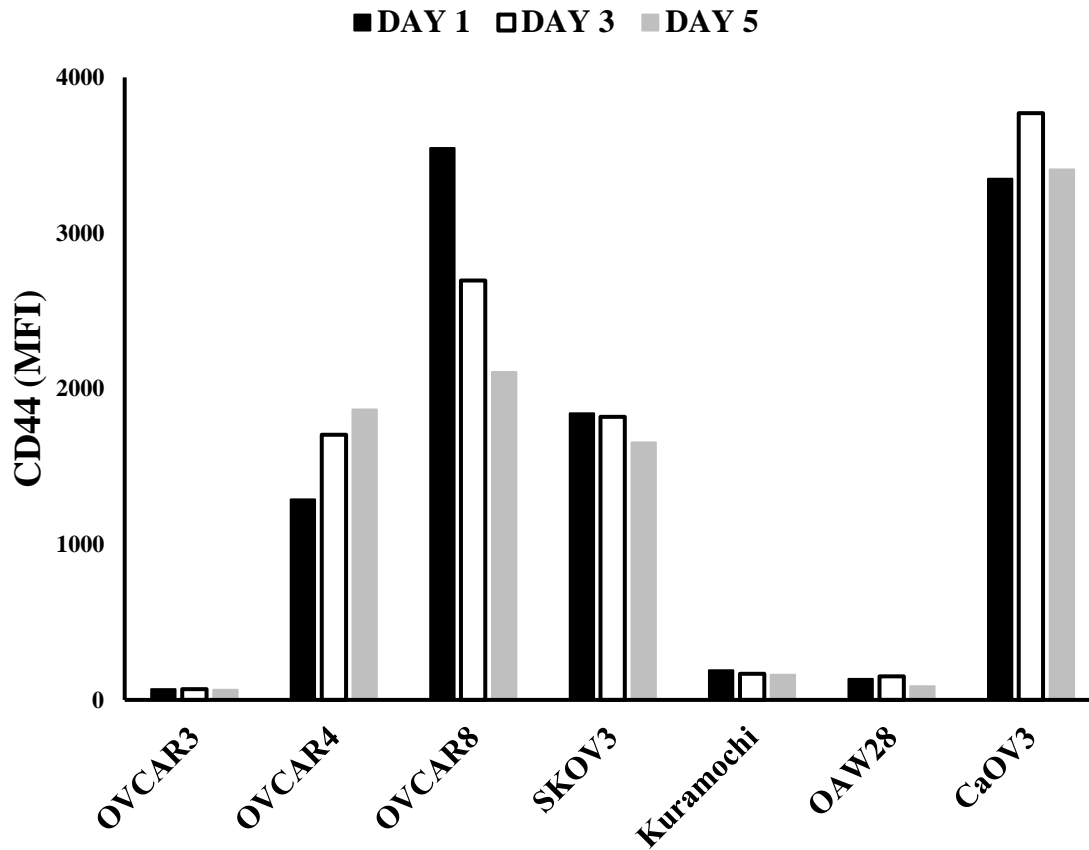


Figure 2: OVCAR8 and CaOV3 displays highest level of CD44 expression.

~50 x 10³ cells of each ovarian cancer cell lines were stained with PE CD44 antibody to determine the surface expression of CD44 on ovarian cancer cells. The experiment was repeated on every other consecutive day (Day1, Day3, and Day5), as detailed in the figure. PE IgG isotype antibody was used as a control. Mean fluorescence intensity (MFI) of CD44 expression was determined, and a bar chart was created, as displayed in the figure.

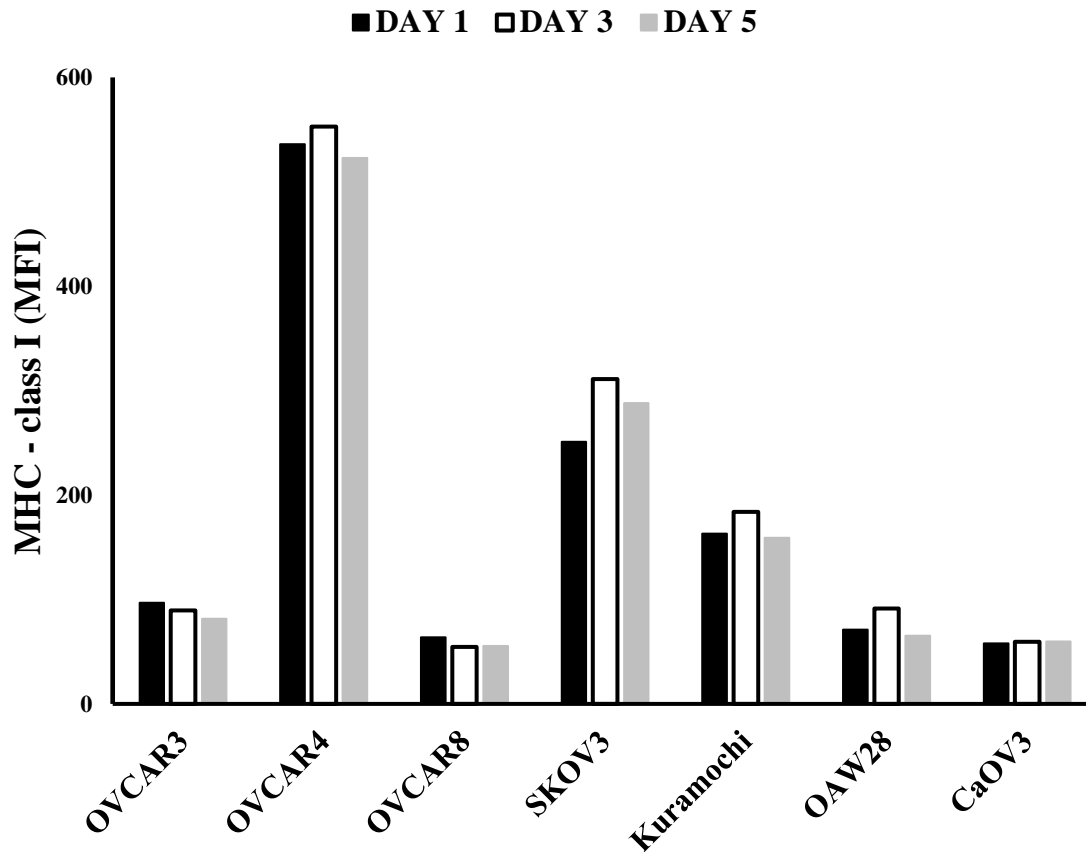
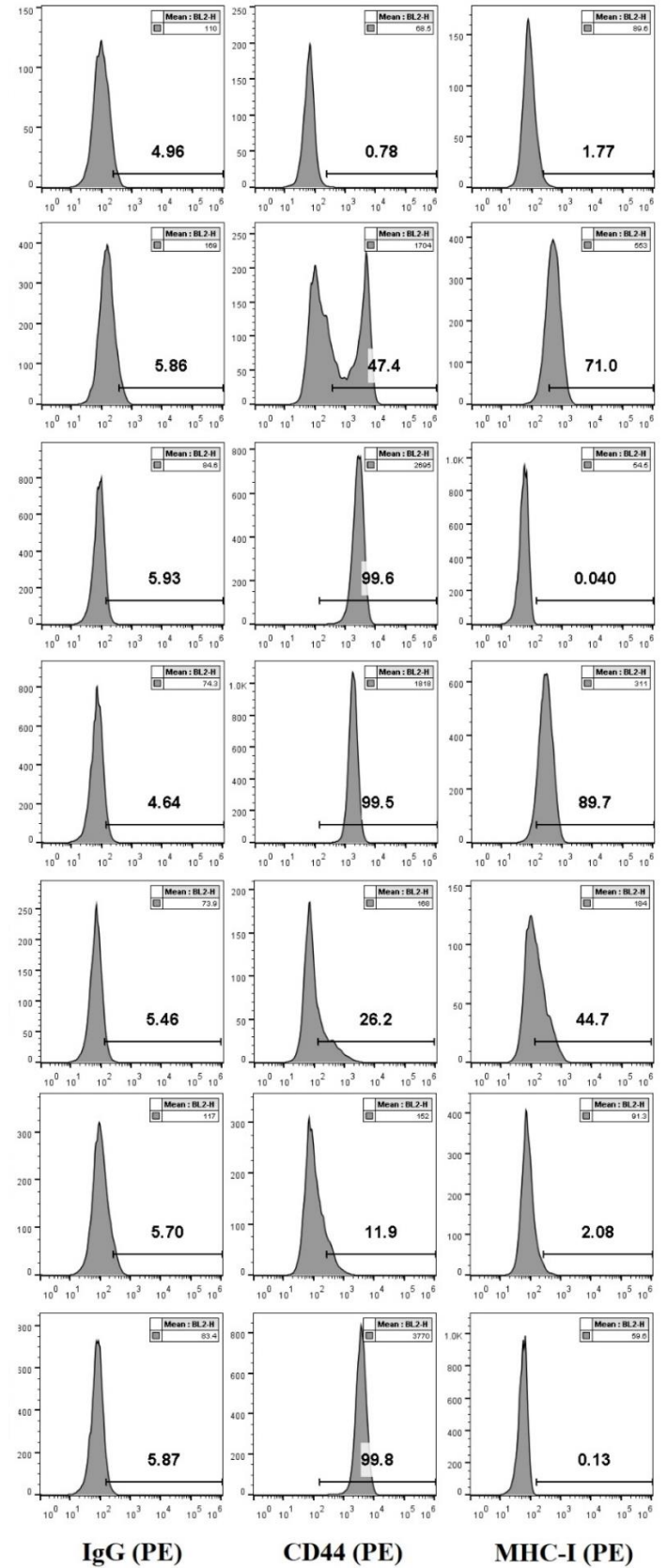
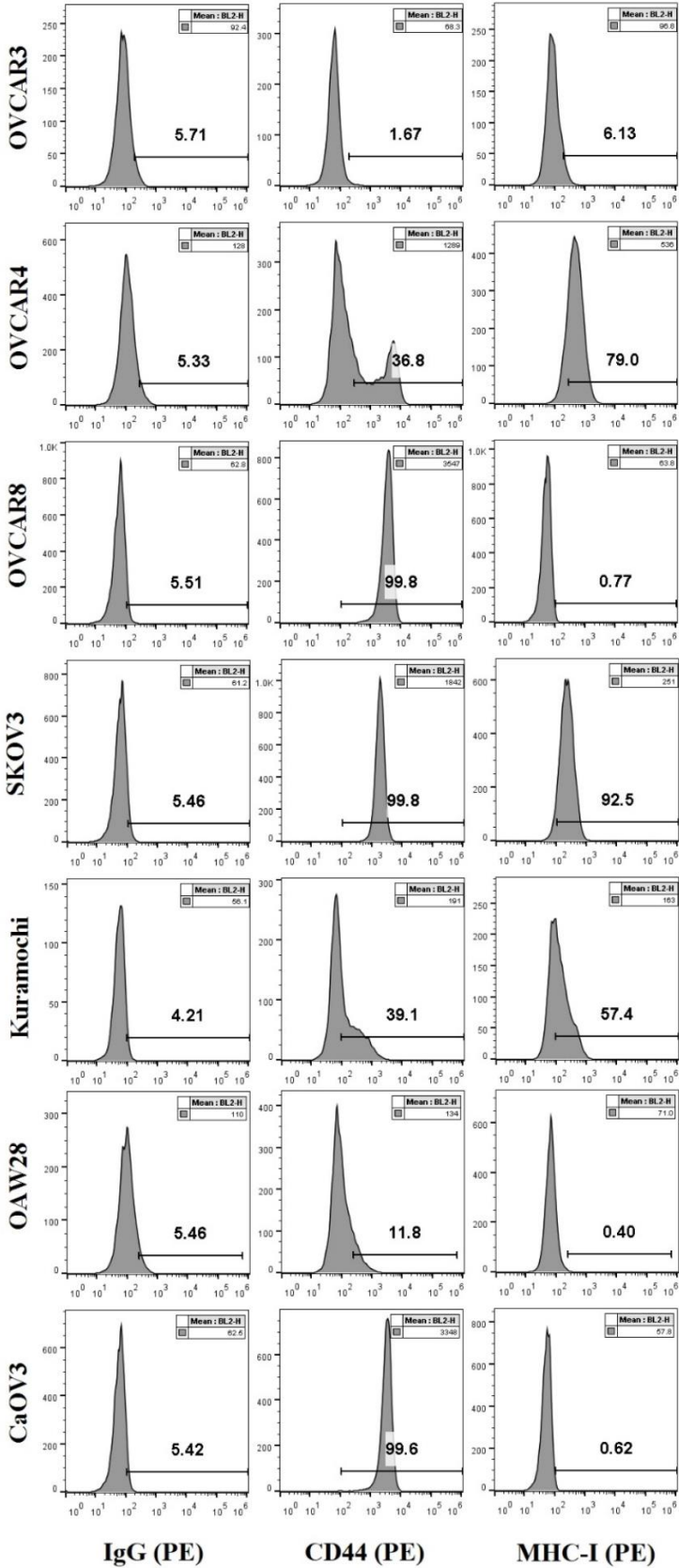


Figure 3: OVCAR8 and CaOV3 displays lowest level of MHC-class I expression.

~50 x 10³ cells of each ovarian cancer cell lines were stained with PE MHC-class I antibody to determine the surface expression of MHC-class I on ovarian cancer cells. The experiment was repeated on every other consecutive day (Day1, Day3, and Day5), as detailed in the figure. PE IgG isotype antibody was used as a control. Mean fluorescence intensity (MFI) of MHC-class I expression was determined, and a bar chart was created, as displayed in the figure.



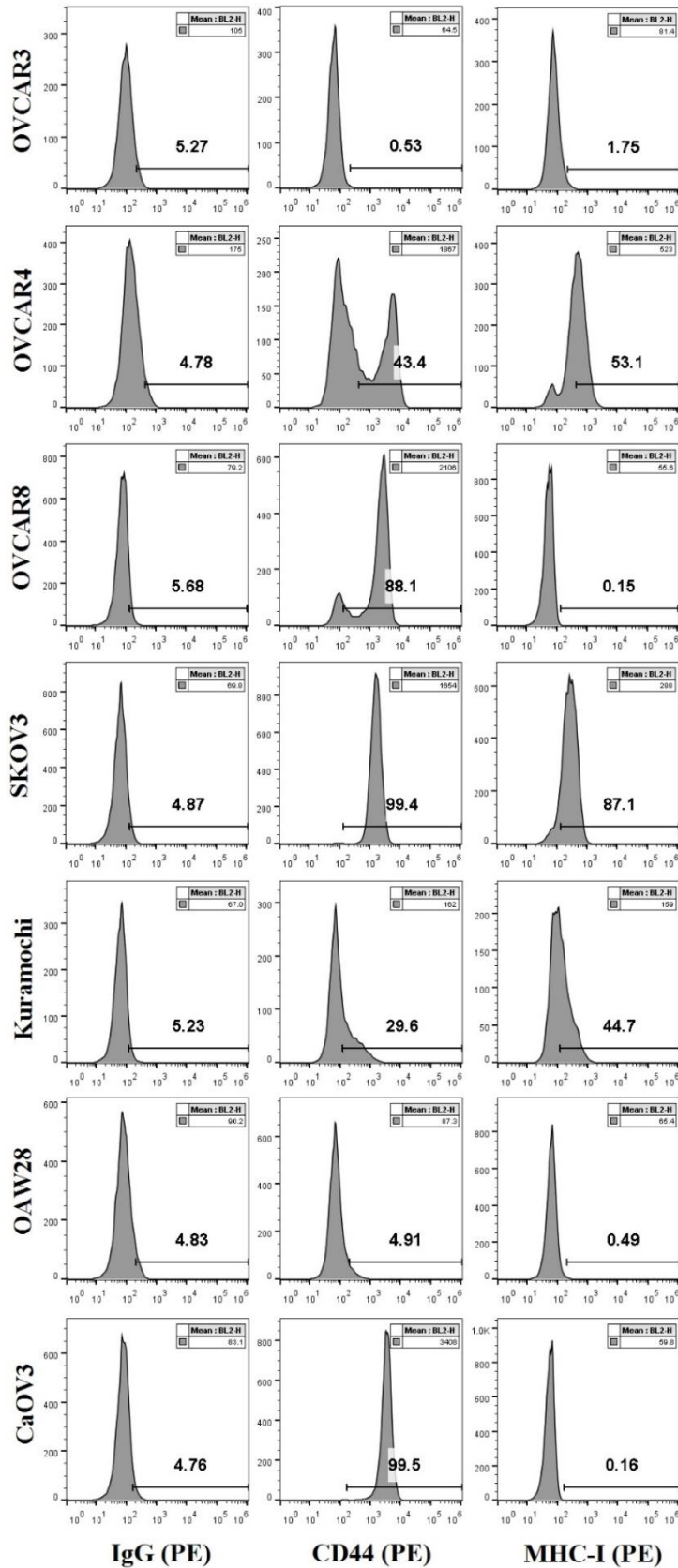


Figure 4. 5 and 6: OVCAR8 and CaOV3 displays highest level of CD44 and lowest level of MHC-class I expression.

~50 x 10³ cells of each ovarian cancer cell lines were stained with PE CD44 and PE MHC-class I antibody. The experiment was repeated on every other consecutive day (Day1, Day3, and Day5), as detailed in the figure. PE IgG isotype antibody was used as a control. The numbers on bottom right-hand are percentages and the numbers on top right-hand corner are the mean fluorescence intensities of each histogram.

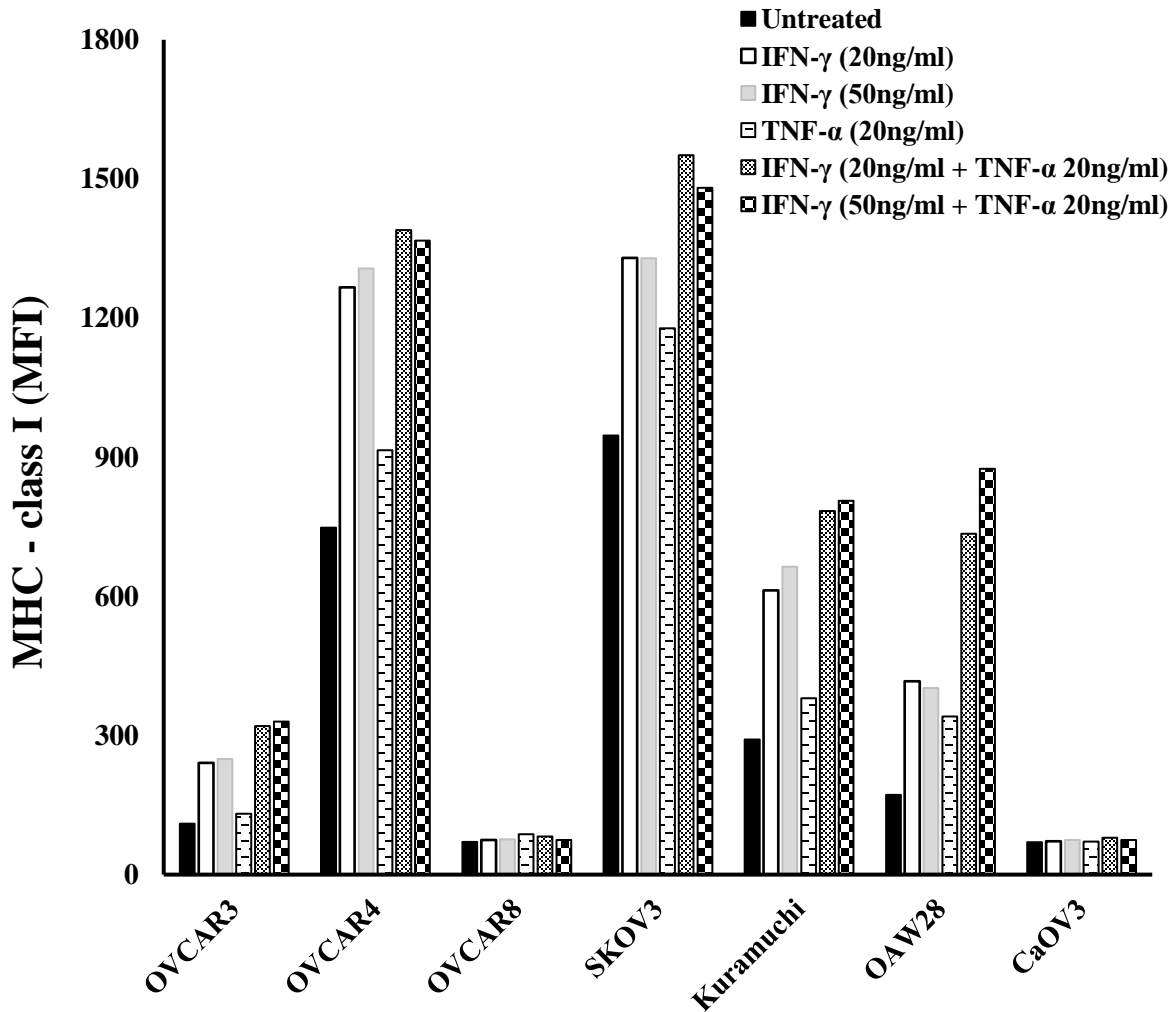


Figure 7: IFN- γ and TNF- α treatment of the tumors increased MHC class I expression in all the tumor cells except for OVCAR8 and CaOV3 tumor cells.

$\sim 2 \times 10^5$ cells of each ovarian cancer cells were plated in 6-well plate and were treated with IFN- γ (20ng and 50ng/ml), TNF- α (20 ng/ml) and a combination of IFN- γ (20ng and 50ng/ml) and TNF- α (20 ng/ml). After an overnight induction, $\sim 50 \times 10^3$ cells were stained with PE MHC-class I antibody to determine surface expression of MHC-class I.

Subsequently, a bar chart of the mean fluorescence intensity (MFI) was created, as displayed in the figure.

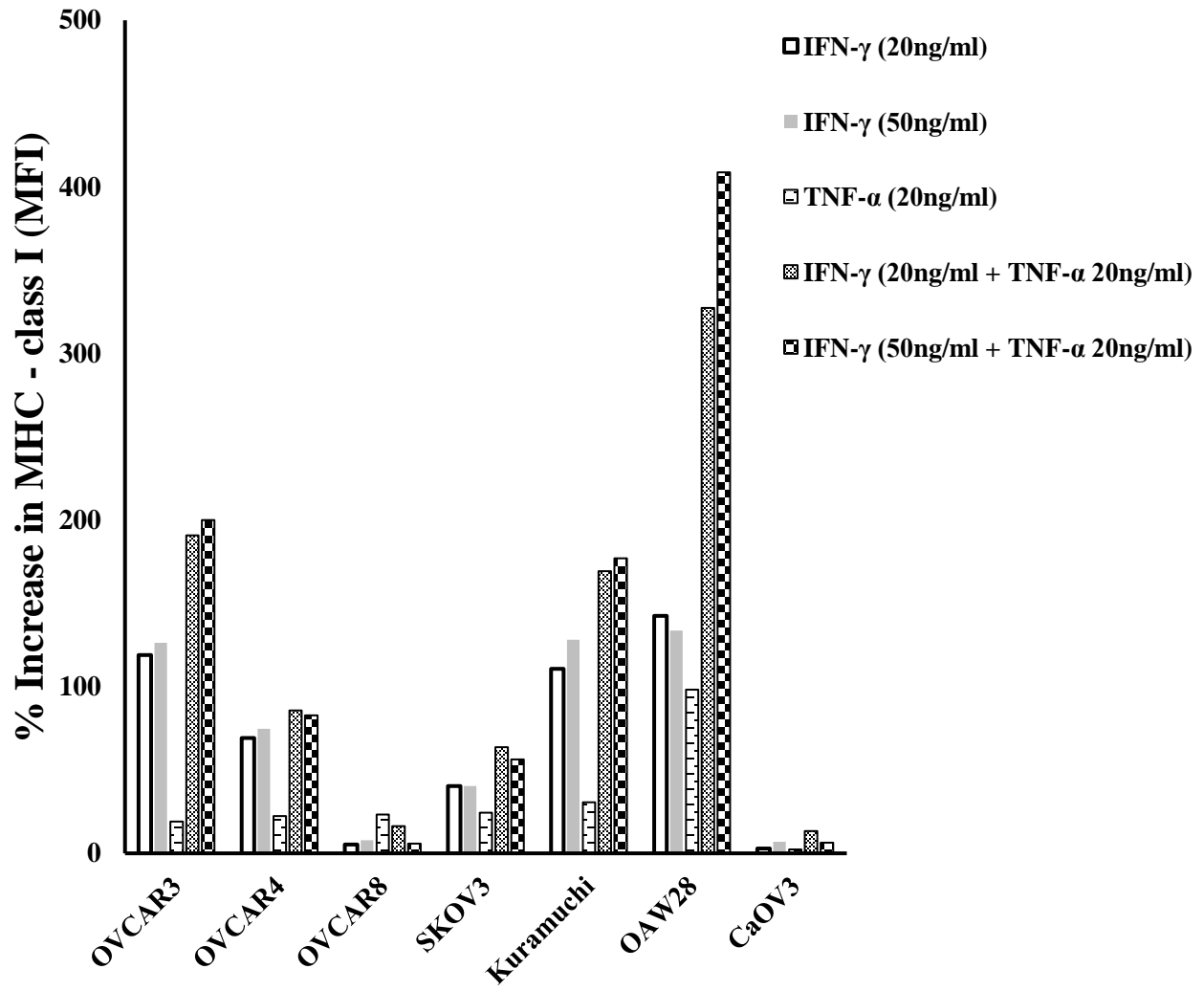
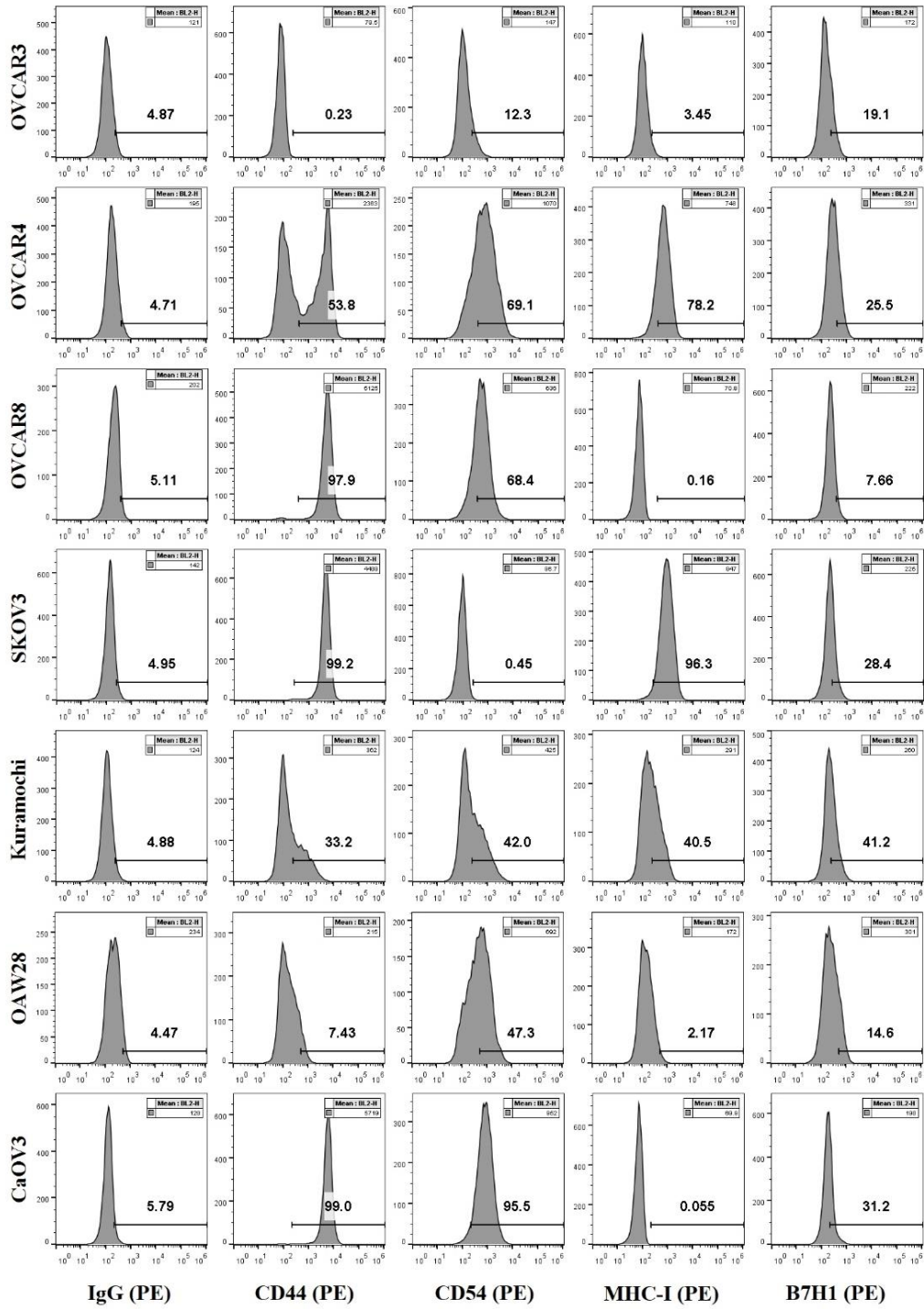


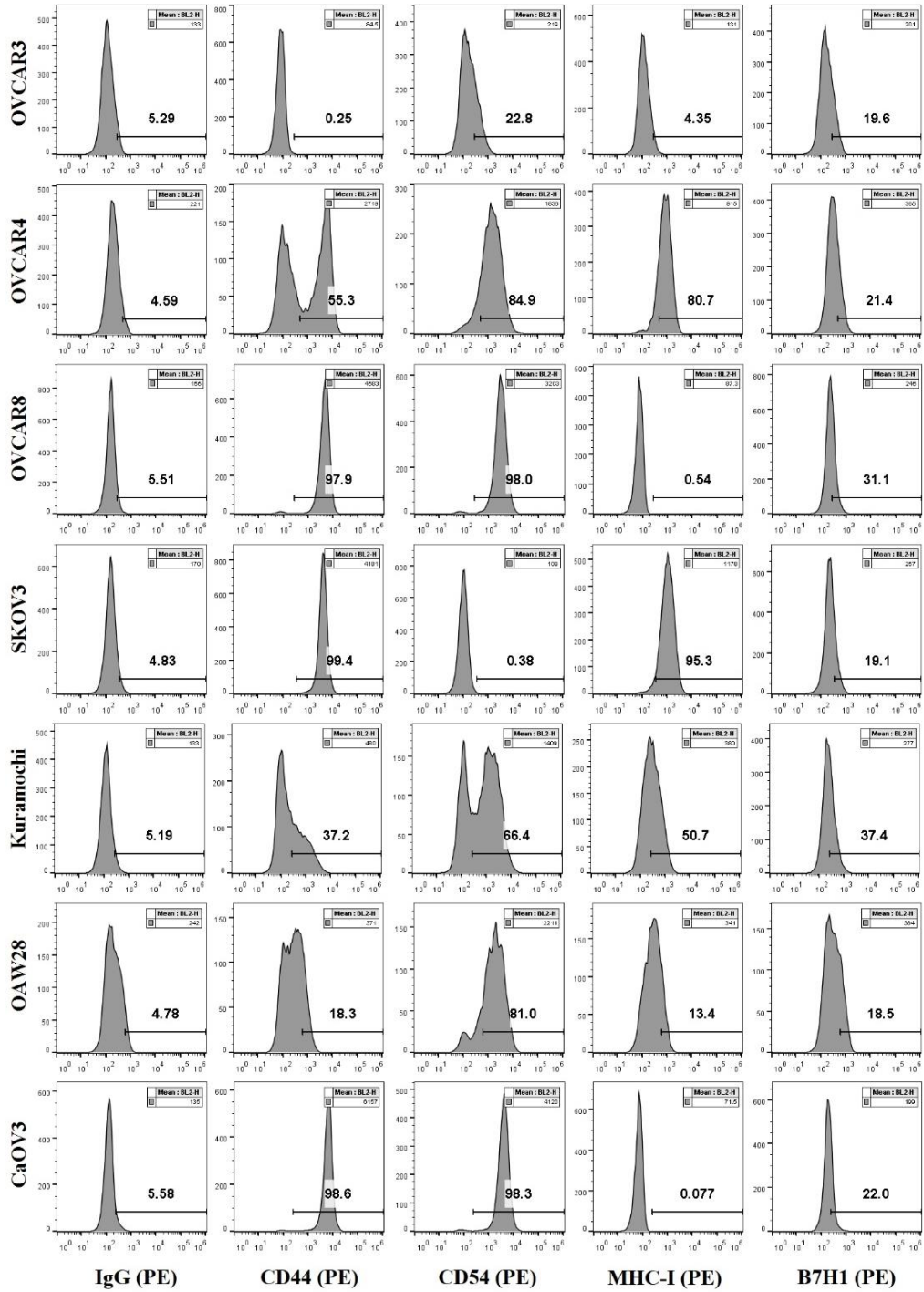
Figure 8: OVCAR8 and CaOV3 displays lowest % increase in MFI of MHC-class I expression.

% increase in the MHC-class I MFI was calculated and a bar chart of which was made, as displayed in the figure.

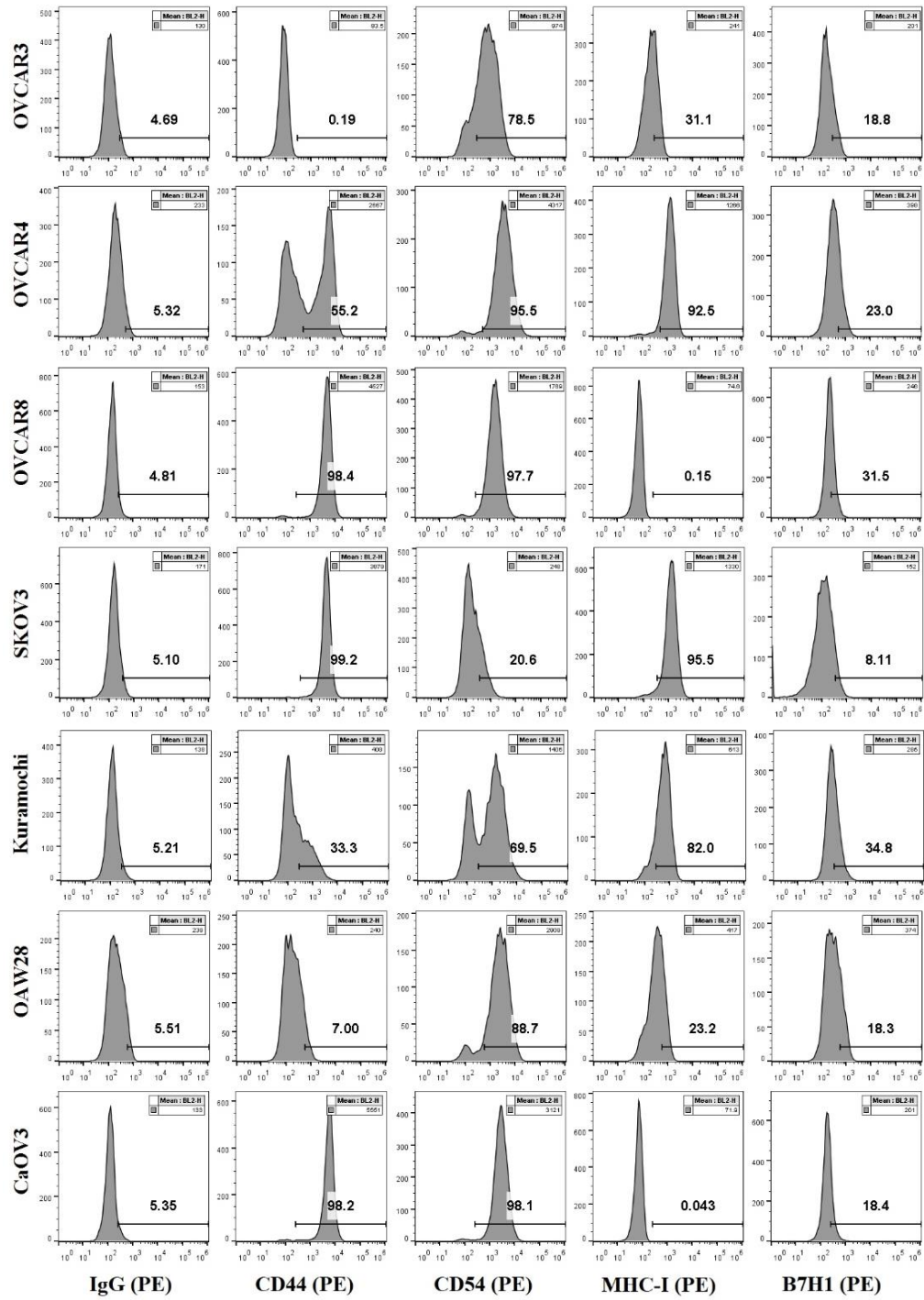
Control



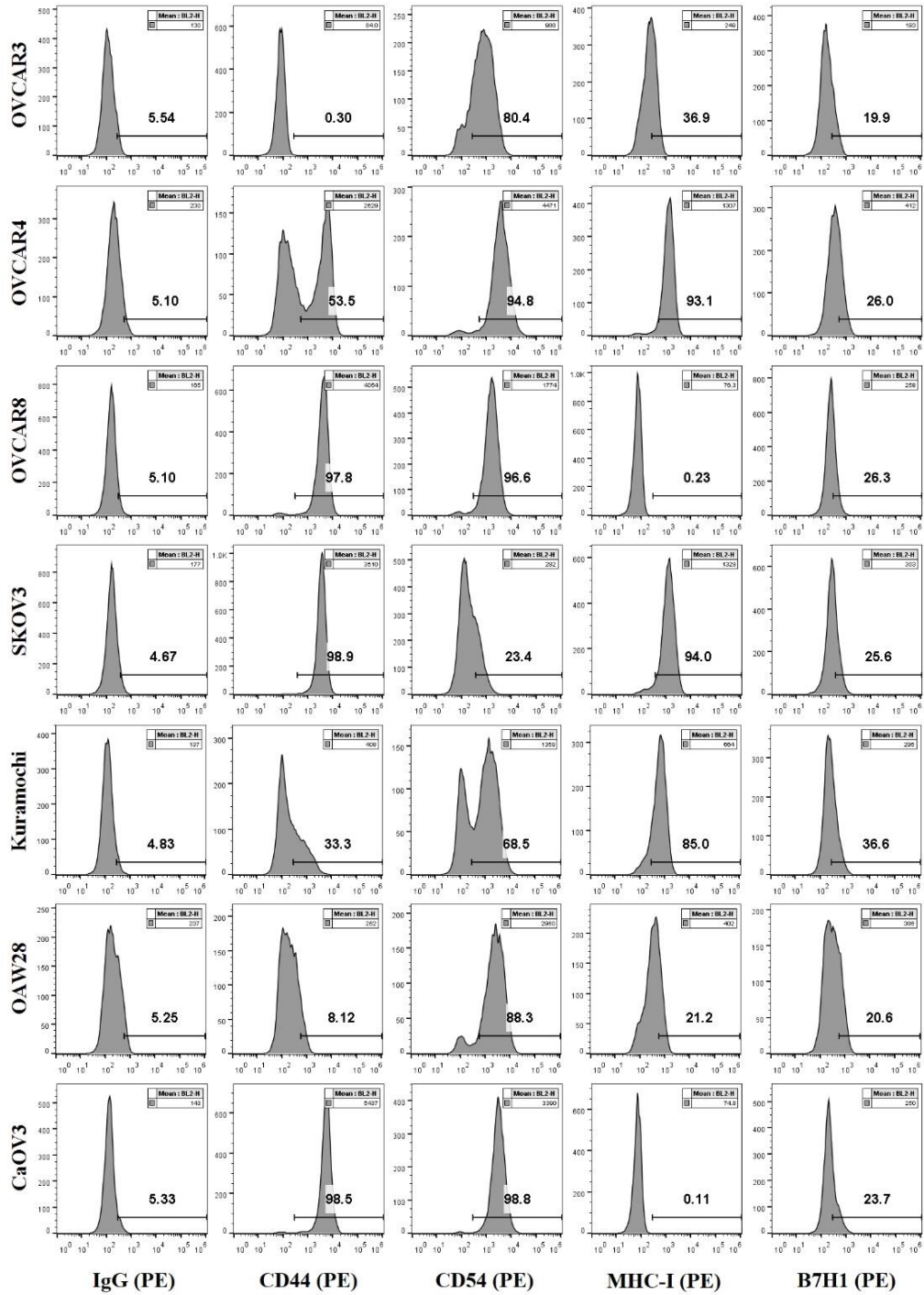
TNF- α 20ng/ml



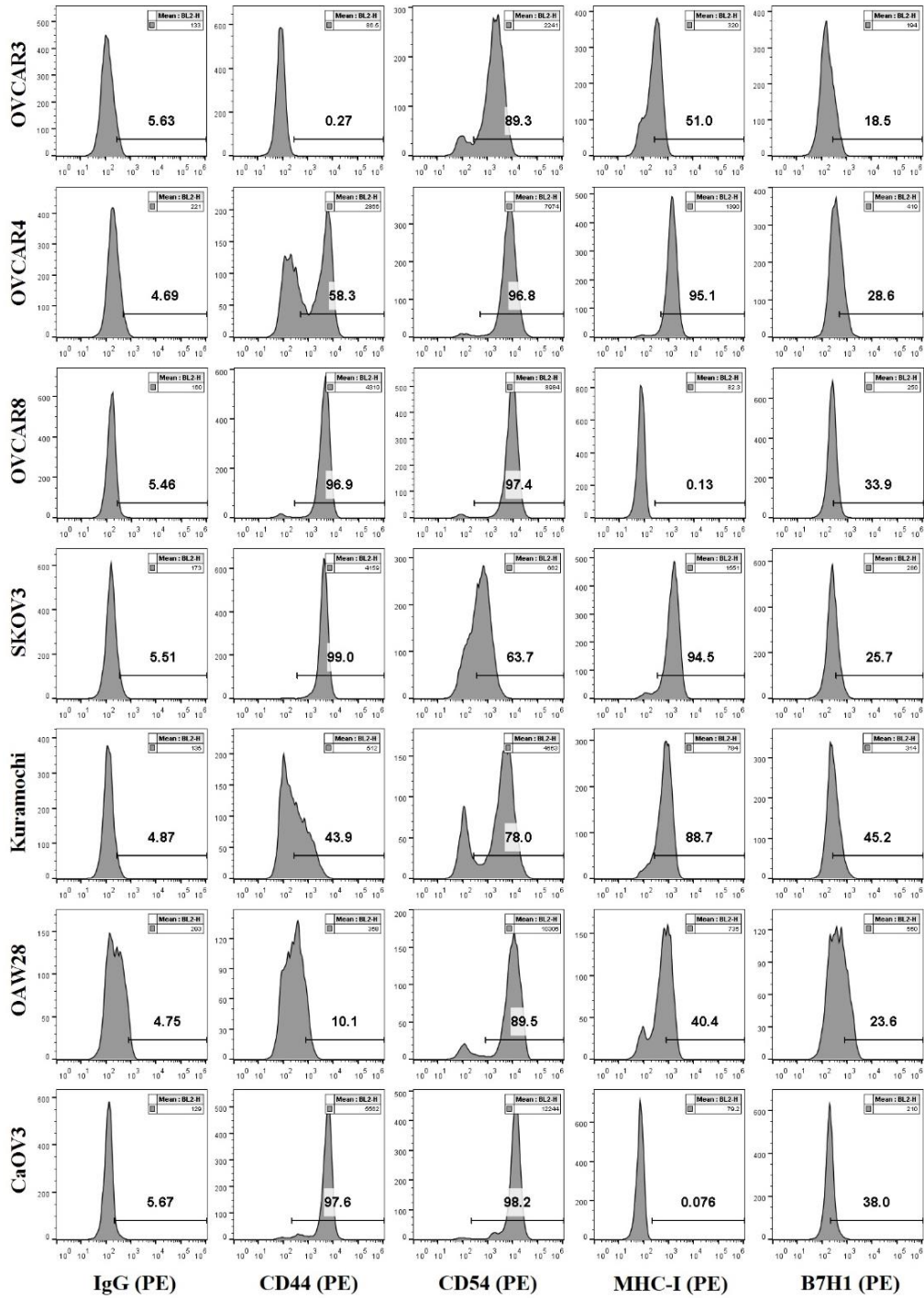
IFN- γ 20ng/ml



IFN- γ 50ng/ml



IFN- γ 20ng/ml + TNF- α 20ng/ml



IFN- γ 50ng/ml + TNF- α 20ng/ml

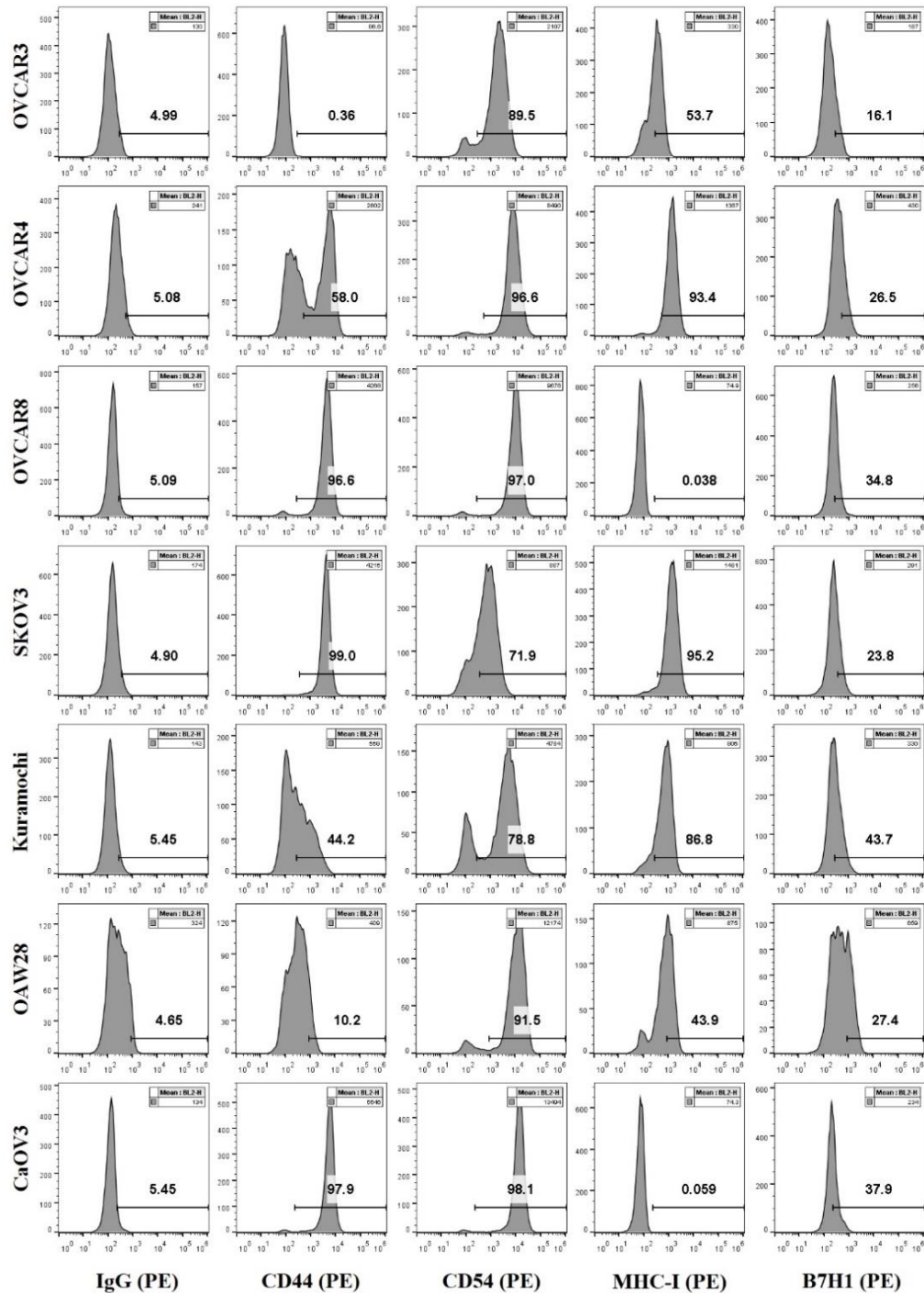


Figure 9 to 14: CD44, CD54, MHC-class I and B7H1 expression on Ovarian cancer cells after IFN- γ and TNF- α treatment.

After IFN- γ and TNF- α treatment, $\sim 50 \times 10^3$ cells of each cancer cell lines were stained with PE CD44, PE CD54, PE MHC-class I and PE B7H1. Isotype IgG antibody was used as a control. The numbers on bottom right-hand are percentages and the numbers on top right-hand corner are the mean fluorescence intensities of each histogram.

Chapter 2: To establish the differential targeting ability of NK cells against ovarian tumors in terms of cytotoxicity and secretion of IFN- γ .

IFN- γ + TNF- α treatment of the tumors increased differentiation of the tumors in some of the tumors and resulted in less susceptibility to NK cell mediated cytotoxicity with the exception of OVCAR8 and CaOV3 tumor cells which remained susceptible to NK cell mediated cytotoxicity in the presence or absence of IFN- γ + TNF- α treatment.

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 [29]. To determine the cytotoxicity of NK cells against ovarian cancer cell lines, cytotoxicity of IL-2 treated NK cells was determined against IFN- γ + TNF- α treated and untreated ovarian cancer cells. Almost all ovarian cancer cell lines, except of OVCAR8 and CaOV3, showed reduced NK cells mediated cytotoxicity after treatment with IFN- γ + TNF- α , in comparison to untreated control ovarian cancer cells (**Fig. 15**). Subsequently, % decrease in the NK cells mediated cytotoxicity was determined, and collectively, OVCAR8 and CaOV3 displayed least decrease in % after treatment of ovarian cancer cell lines with IFN- γ + TNF- α , in comparison to untreated control ovarian cancer cell lines (**Fig. 16**).

IFN- γ + TNF- α treatment of the tumors increased differentiation of the tumors in some of the tumors and resulted in less secretion of IFN- γ by the NK cells co-cultured with those tumors, with the exception of OVCAR8 and CaOV3 tumor cells which remained similar in the secretion of IFN- γ by the NK cells when tumors were treated with IFN- γ + TNF- α

IFN- γ induction is the hallmark of NK cell activation. To determine the activation of NK cells by ovarian cancer cells, ovarian cancer cells, untreated and IFN- γ + TNF- α treated, were cocultured with NK cells, IL-2 treated and IL-2 + sAJ4 treated (**Fig. 17**). Subsequently, induction of the IFN- γ in the coculture was determined. Most increase in IFN- γ induction was found after treatment of NK cells with IL-2 + sAJ4, in comparison to IL-2 treated or untreated NK cells (**Fig. 17**). IFN- γ + TNF- α treatment of ovarian cancer cells induced differentiation in all cancer cell lines, except for OVCAR8 and CaOV3. Thus, IFN- γ secretion was reduced by NK cells cocultured with ovarian cancer cells treated with IFN- γ + TNF- α , except of OVCAR8 and CaOV3 which displayed similar level of secretion of IFN- γ by NK cells even after IFN- γ + TNF- α , in comparison to untreated cancer cells (**Fig. 17**). % decrease in the IFN- γ secretion from the coculture was determined, and it was found that NK cells treated with IL-2 + sAJ4 displayed least % decrease in IFN- γ secretion, in comparison to IL-2 treated NK cells (**Fig. 18**). OVCAR8 and CaOV3 displayed no or minimal level of % decrease in IFN- γ induction by NK cells (**Fig. 18**).

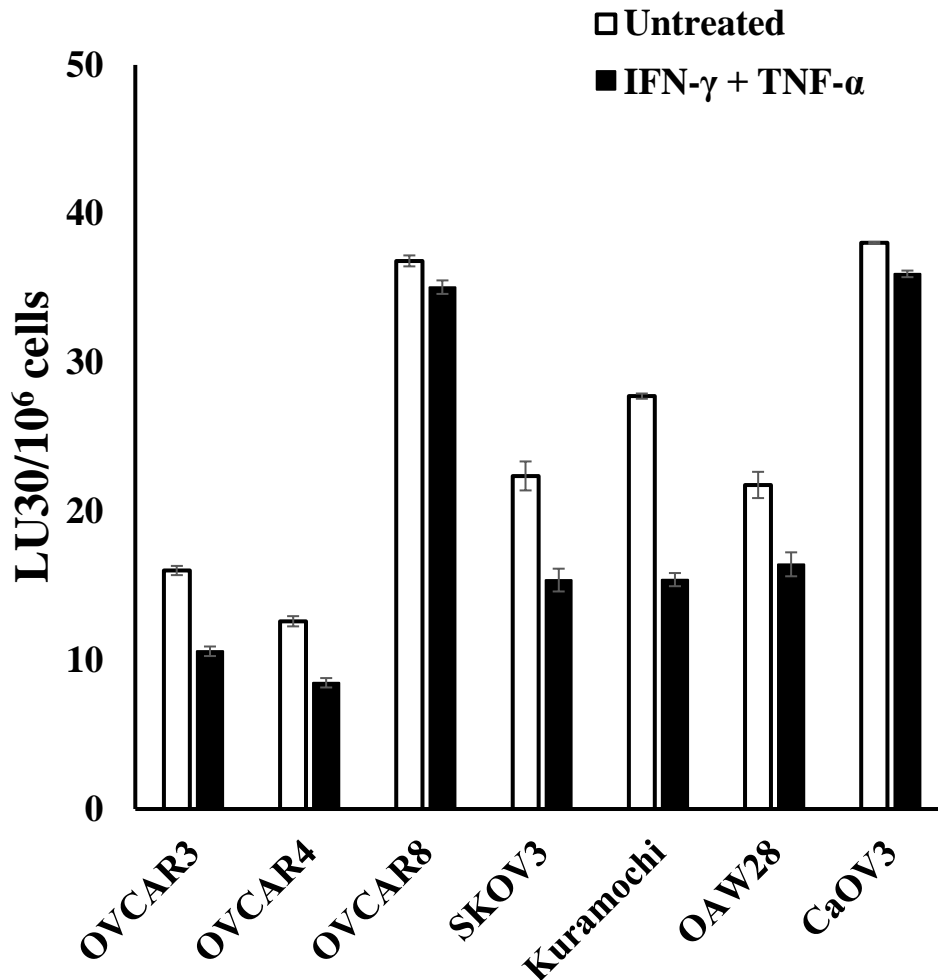


Figure 15: IFN- γ + TNF- α treatment of the tumors resulted in less susceptibility to NK cell mediated cytotoxicity with the exception of OVCAR8 and CaOV3 tumor cells which remained susceptible to NK cell mediated cytotoxicity in the presence or absence of IFN- γ + TNF- α treatment.

L-2 treated NK cells were then used as effector cells against ⁵¹Cr labeled IFN- γ + TNF- α treated ovarian tumor cells. Cytotoxicity was determined using a standard 4-hour ⁵¹Cr release assay and the lytic units 30/10⁶ cells were determined using inverse number of NK cells required to lyse 30% of tumor cells x 100.

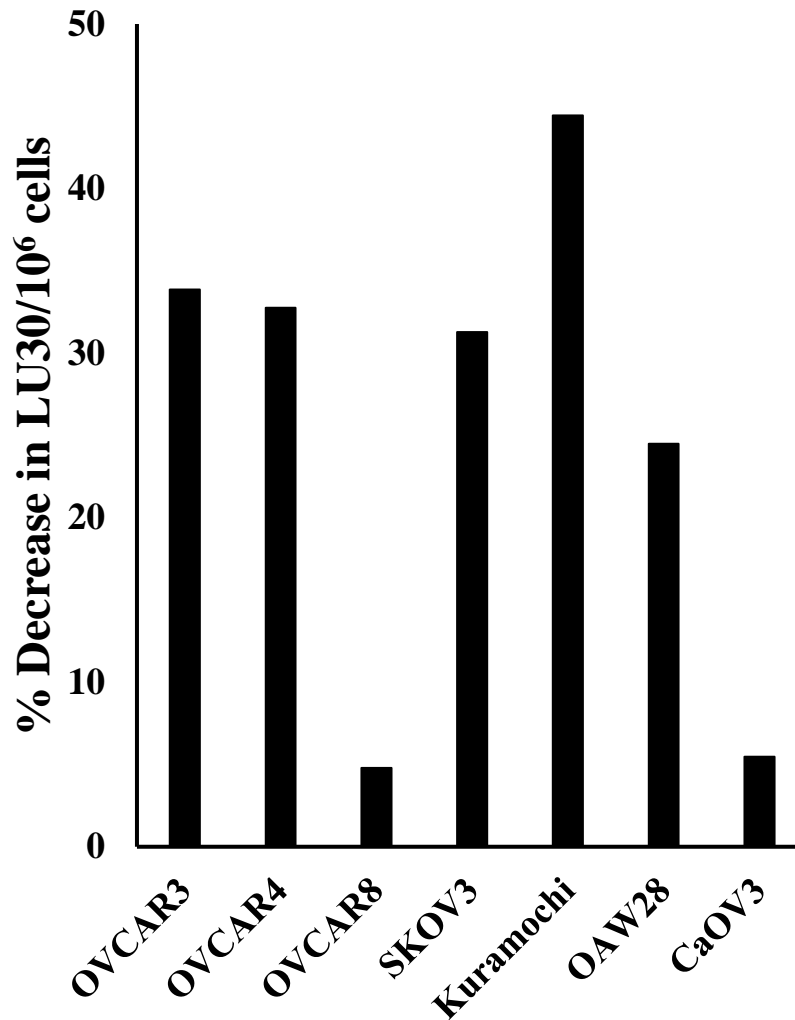
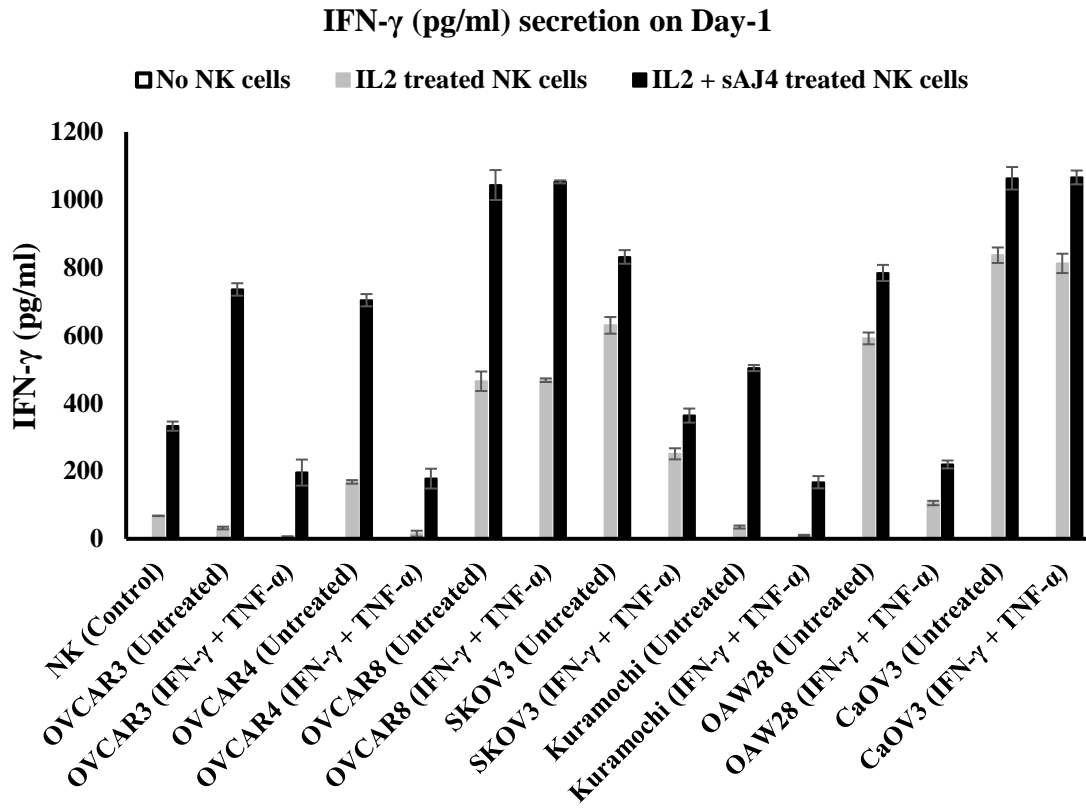
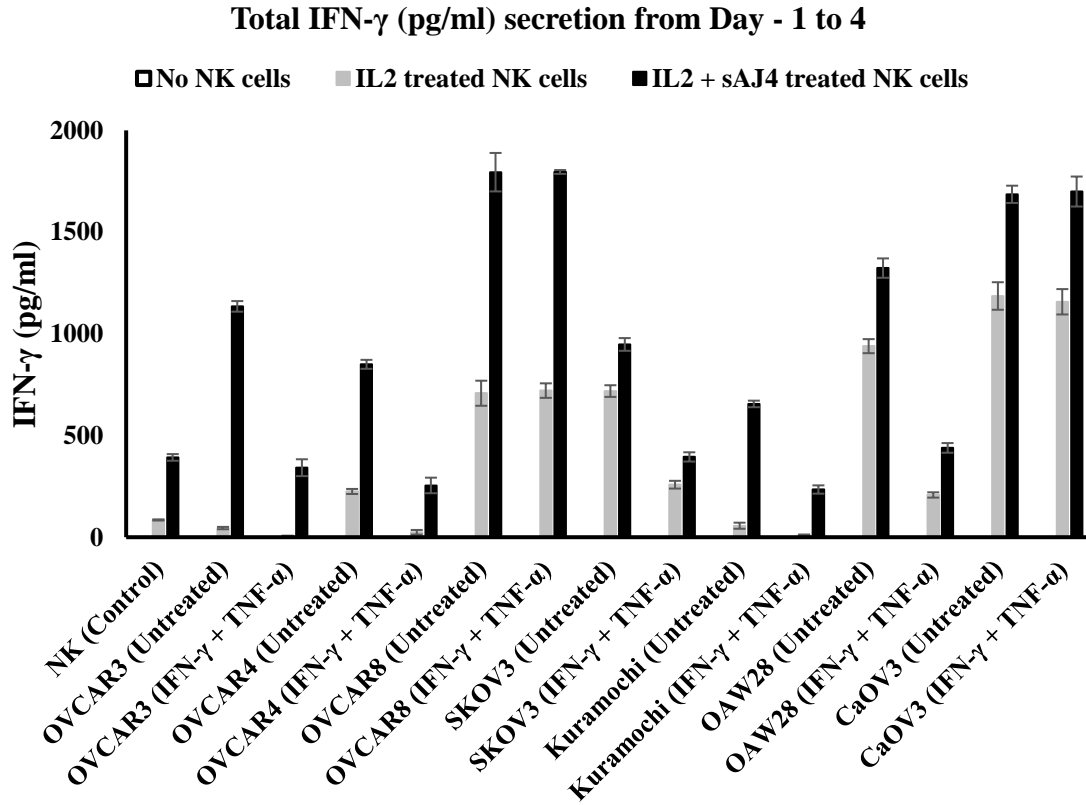


Figure 16: OVCAR8 and CaOV3 shows least decrease in % cytotoxicity of NK cells even after treatment with IFN- γ + TNF- α

A bar chart of % decrease in lytic units 30/10⁶ cells after IFN- γ + TNF- α was created, as displayed in the figure.



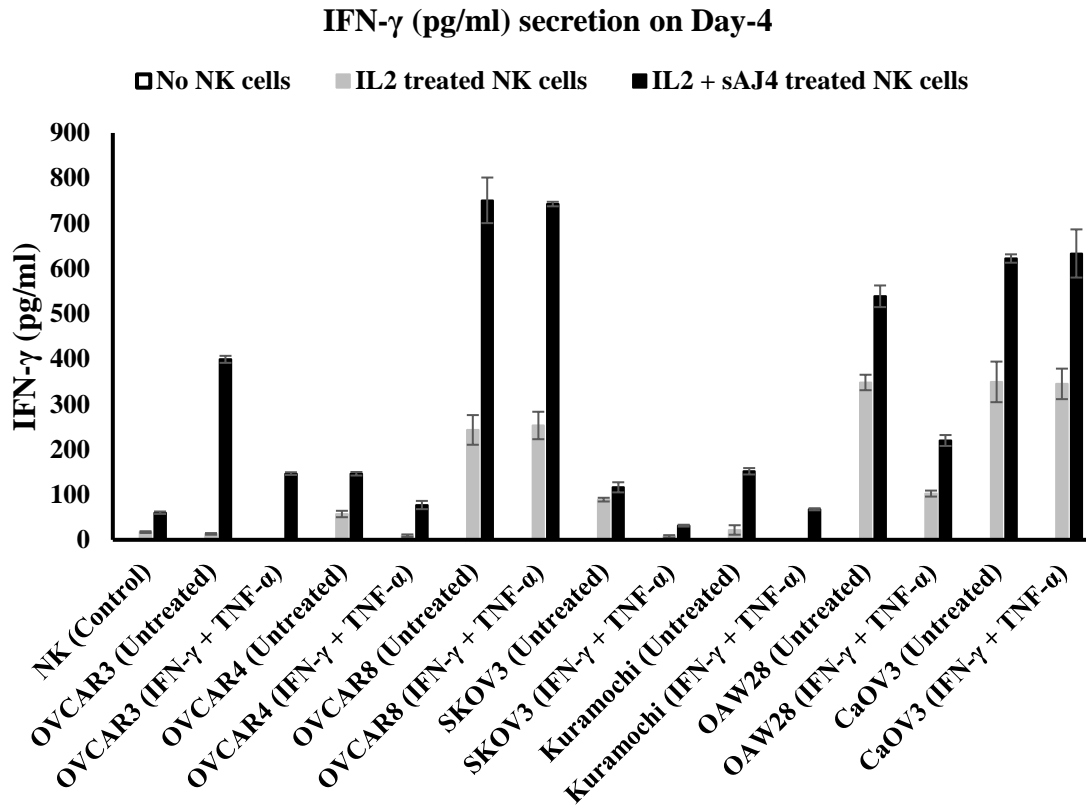


Figure 17: IFN- γ + TNF- α treatment of the tumors resulted in less secretion of IFN- γ by the NK cells co-cultured with those tumors, except of OVCAR8 and CaOV3 tumor cells which remained similar in the secretion of IFN- γ by the NK cells when tumors were treated with IFN- γ + TNF- α .

Untreated and IFN- γ + TNF- α treated ovarian tumor cells were cocultured with IL-2 treated and IL2 + sAJ4 treated NK cells. The supernatants were removed from the co-cultures on Day1 and Day4, and the levels of IFN- γ secretion (in pg/ml) by NK cells were determined using specific ELISA.

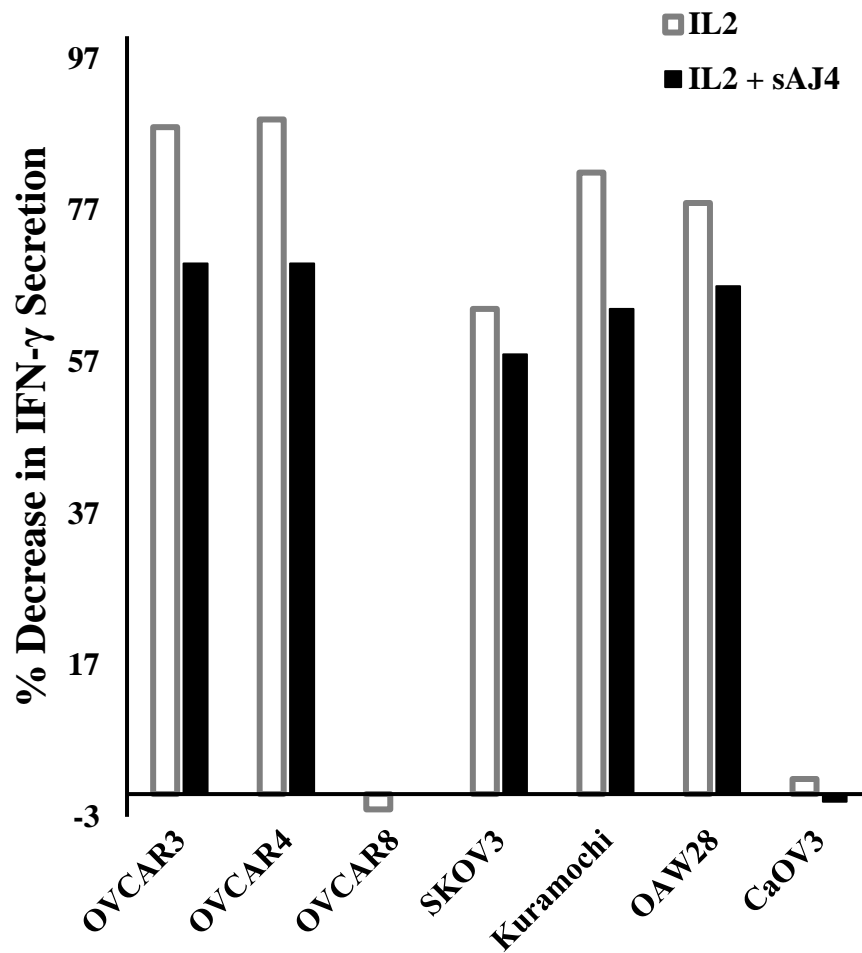


Figure 18: OVCAR8 and CaOV3 shows least % decrease in IFN- γ secretion by NK cells even after treatment with IFN- γ + TNF- α

A bar chart of % decrease IFN- γ secretion by NK cells cocultured with IFN- γ + TNF- α treated ovarian tumor cells was created, as displayed in the figure.

Chapter 3: Establish the differentiation stages and capabilities of ovarian tumor lines and their susceptibility to carboplatin mediated effects.

IFN- γ treatment of the tumors increased differentiation of the tumors in some of the tumors and resulted in more susceptibility to carboplatin mediated decrease in cell growth with the exception of OVCAR8 and CaOV3 tumor cells which remained relatively higher in cell growth when treated with IFN- γ and/or TNF- α .

It has been previously displayed that differentiated cancer cells are more responsive to chemotherapeutic drug mediated cell death, in comparison to poorly differentiated stem like cells. And it has been also shown that NK cells mediated differentiation leads to the increase in targeting of poorly differentiated stem like cancer cells by chemotherapeutic drugs [76]. IFN- γ , secreted by the NK cells, is the chief cytokine responsible for the induction of differentiation [75].

To determine the targeting of ovarian cancer cells by chemotherapeutic drugs, rh-IFN- γ and/or rh-TNF- α treated and untreated ovarian cancer cells were treated with different concentration carboplatin overnight. Subsequently, next day, viable cells were counted after staining with trypan blue (**Fig. 19**). IFN- γ and/or TNF- α treatment of ovarian cancer cells resulted in induction of differentiation in almost all cancer cell lines, with the exception of OVCAR8 and CaOV3, and resulted in increased susceptibility of cancer cells to carboplatin mediated cell death and displayed reduction in cell count with the most cell death after combination treatment of IFN- γ + TNF- α (**Fig. 19**). On the other hand, OVCAR and CaOV3, even after treatment with IFN- γ and/or TNF- α , showed similar level of cell count, in comparison to control, after treatment with carboplatin (**Fig. 19**). % decrease in cell count, after carboplatin

treatment, was determined and it was found that OVCAR8 and CaOV3 displayed minimal level of decrease in % cell count even after treatment of them with IFN- γ + TNF- α , while other ovarian cancer cell lines displayed decrease in % cell count with OVCAR3 being the most (**Fig. 20**).

IFN- γ treatment of the tumors increased differentiation of the tumors in some of the tumors and resulted in more susceptibility to carboplatin mediated increase in cell death with the exception of SKOV3 and CaOV3 tumor cells which remained in general lower in cell death when treated with and without IFN- γ when compared to other ovarian tumor lines.

To determine the cell death, rh-IFN- γ and/or rh-TNF- α treated and untreated ovarian cancer cells were treated with different concentration carboplatin overnight. Subsequently, next day, cells were stained with primidone iodine (PI) (**Fig. 21, 23 to 29**). IFN- γ and/or TNF- α treatment of ovarian cancer cells resulted in induction of differentiation in almost all cancer cell lines, with the exception of OVCAR8 and CaOV3, and resulted in increased susceptibility of cancer cells to carboplatin mediated cell death with the most cell death being after combination treatment of IFN- γ + TNF- α (**Fig. 21, 23 to 29**). On the other hand, OVCAR and CaOV3, even after treatment with IFN- γ and/or TNF- α , showed lower level of cell death, in comparison to control, after treatment with carboplatin (**Fig. 21, 23 to 29**). Fold increase in cell death, after carboplatin treatment, was determined and it was found that OVCAR8 and CaOV3 displayed minimal level of increase in cell death even after treatment of them with IFN- γ + TNF- α , while other ovarian cancer cell lines displayed marked level of increase in cell death with OVCAR3 being the most (**Fig. 22**).

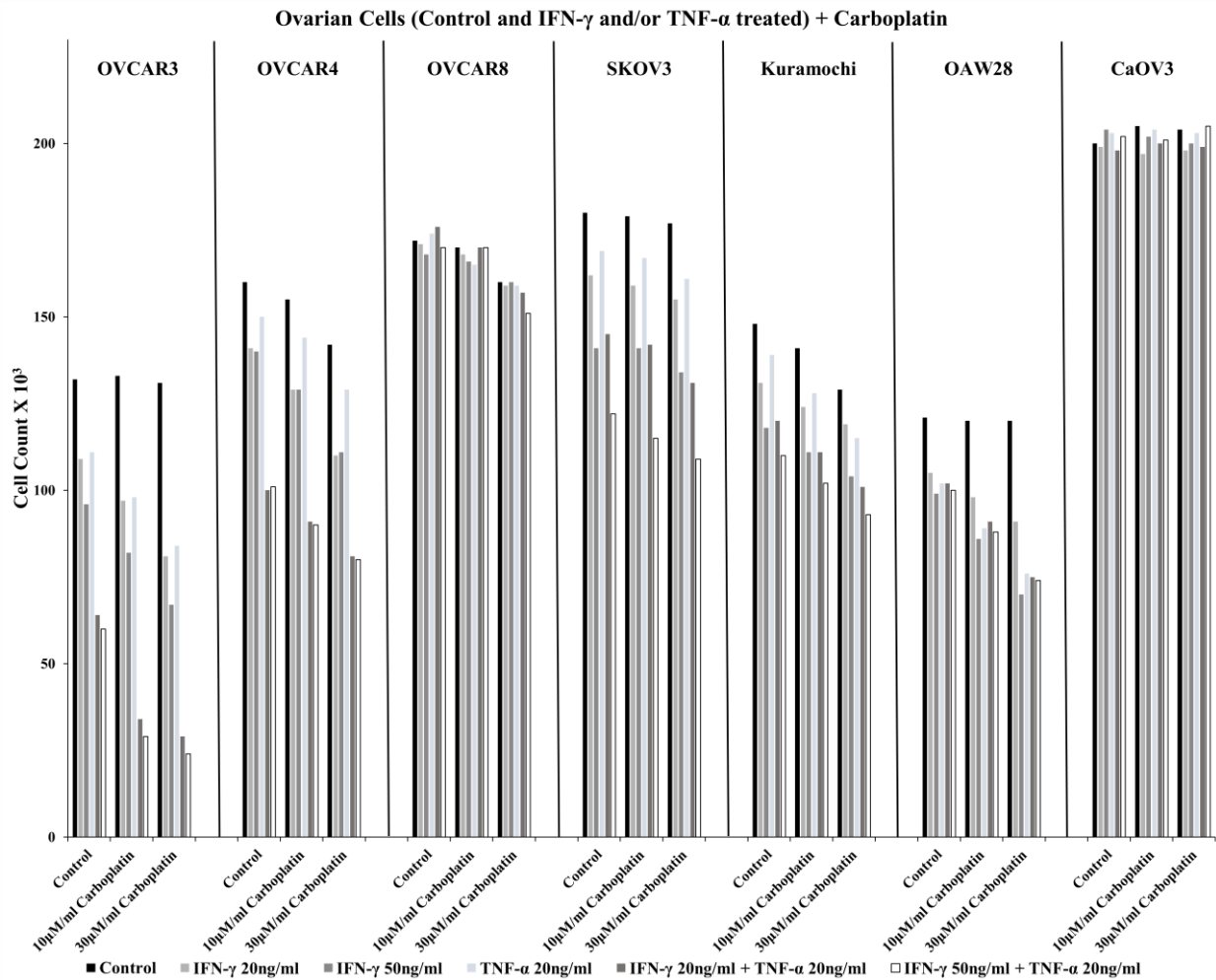


Figure 19: IFN- γ treatment of the ovarian tumors resulted in increased susceptibility of tumor cells to carboplatin mediated decrease in cell growth, except of OVCAR8 and CaOV3 cells.

Ovarian tumor cells were treated with IFN- γ (20ng and 50ng/ml), TNF- α (20 ng/ml) and a combination of IFN- γ (20ng and 50ng/ml) and TNF- α (20 ng/ml). After an overnight induction, they were treated with Carboplatin (Control, 10 μ M/ml, and 30 μ M/ml) overnight. Subsequently, next day, viable cells were counted after staining with trypan blue. Cell count in 10³ is displayed in the figure.

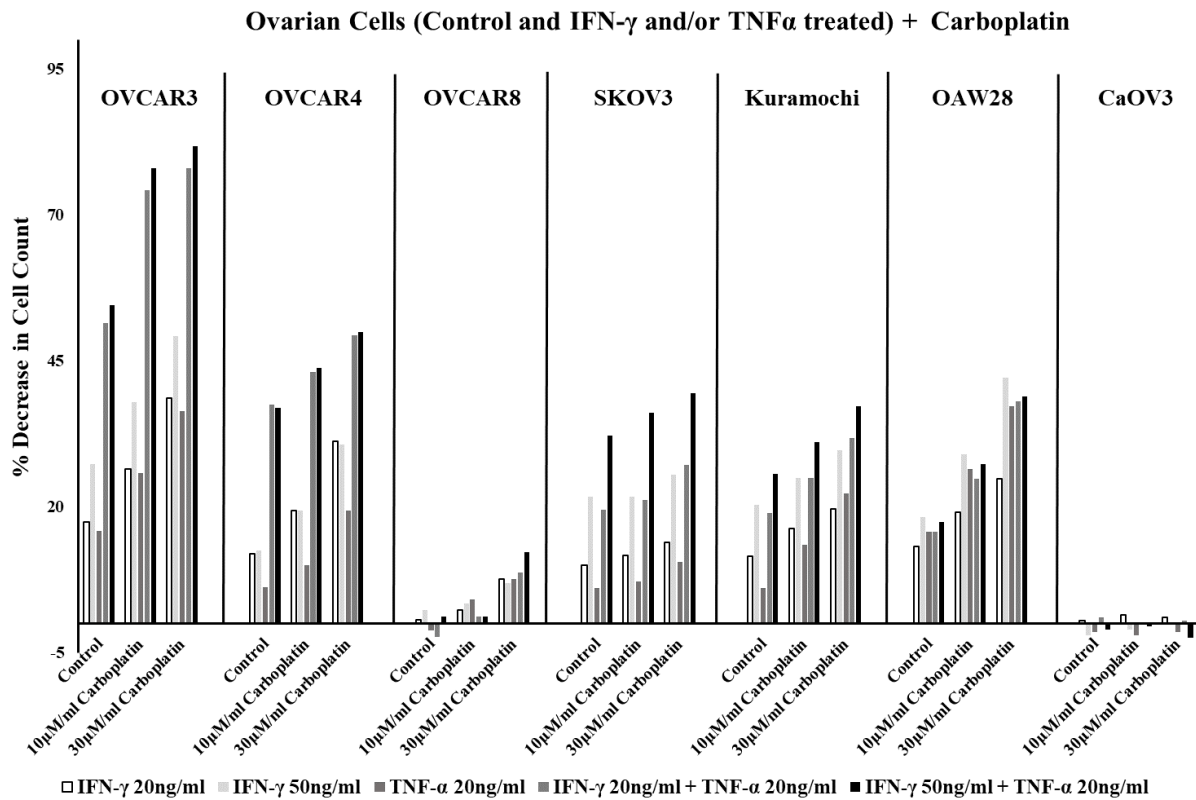


Figure 20: OVCAR8 and CaOV3 displays minimal level of decrease in % cell count even after treatment with IFN- γ + TNF- α .

% decrease in cell count of the IFN- γ and/or TNF- α treated ovarian tumor cells after carboplatin treatment was calculated and a bar chart of which was made, as displayed in the figure.

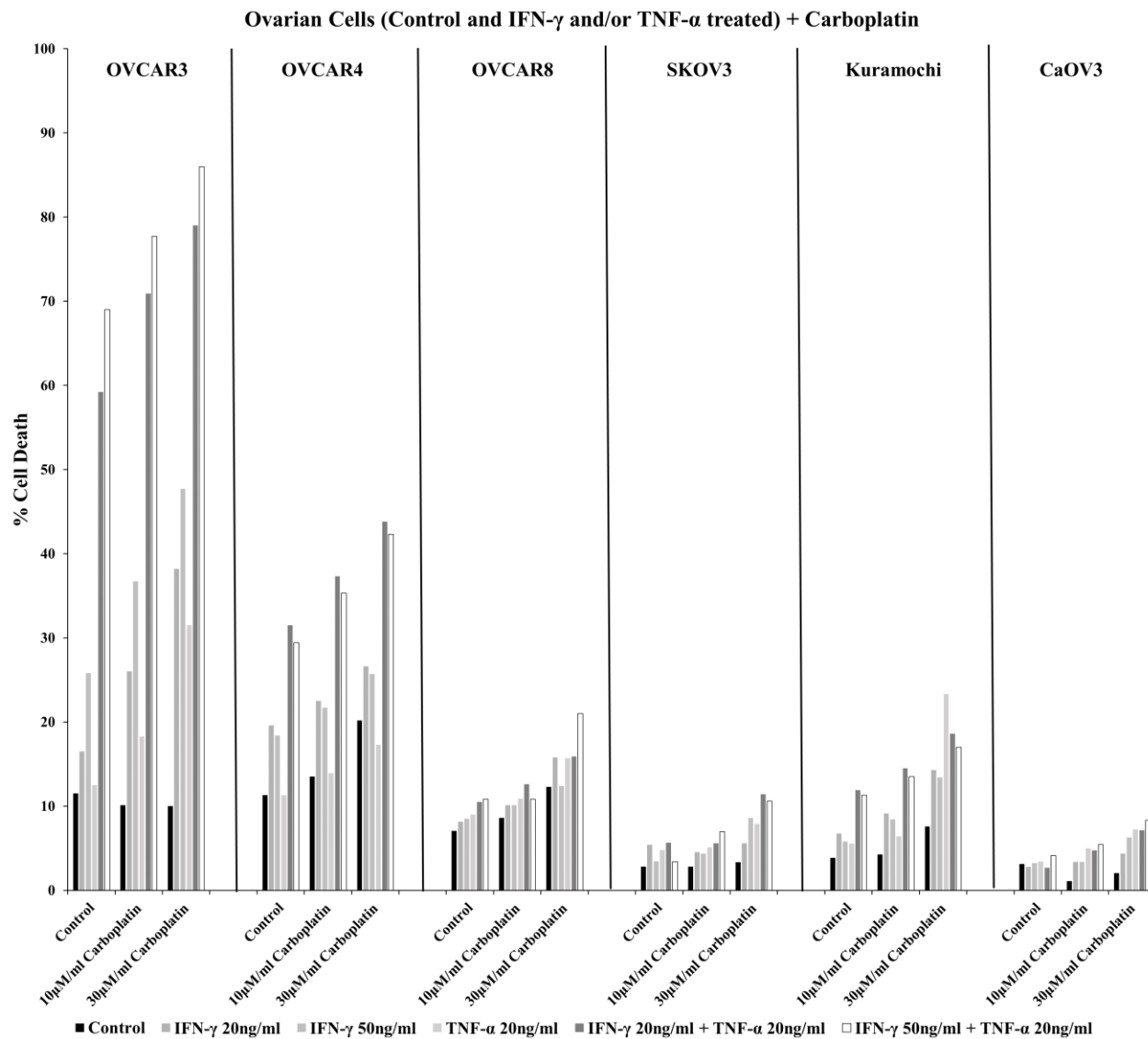


Figure 21: IFN- γ treatment of the ovarian tumors resulted in increased susceptibility of tumor cells to carboplatin mediated cell death, except of OVCAR8 and CaOV3 cells.

Ovarian tumor cells were treated with IFN- γ (20ng and 50ng/ml), TNF- α (20 ng/ml) and a combination of IFN- γ (20ng and 50ng/ml) and TNF- α (20 ng/ml). After an overnight induction, they were treated with Carboplatin (Control, 10µM/ml, and 30µM/ml) overnight. Subsequently, next day, cells were stained with primidone iodine (PI) to determine the cell death. A bar chart of % cell death was made as shown in figure.

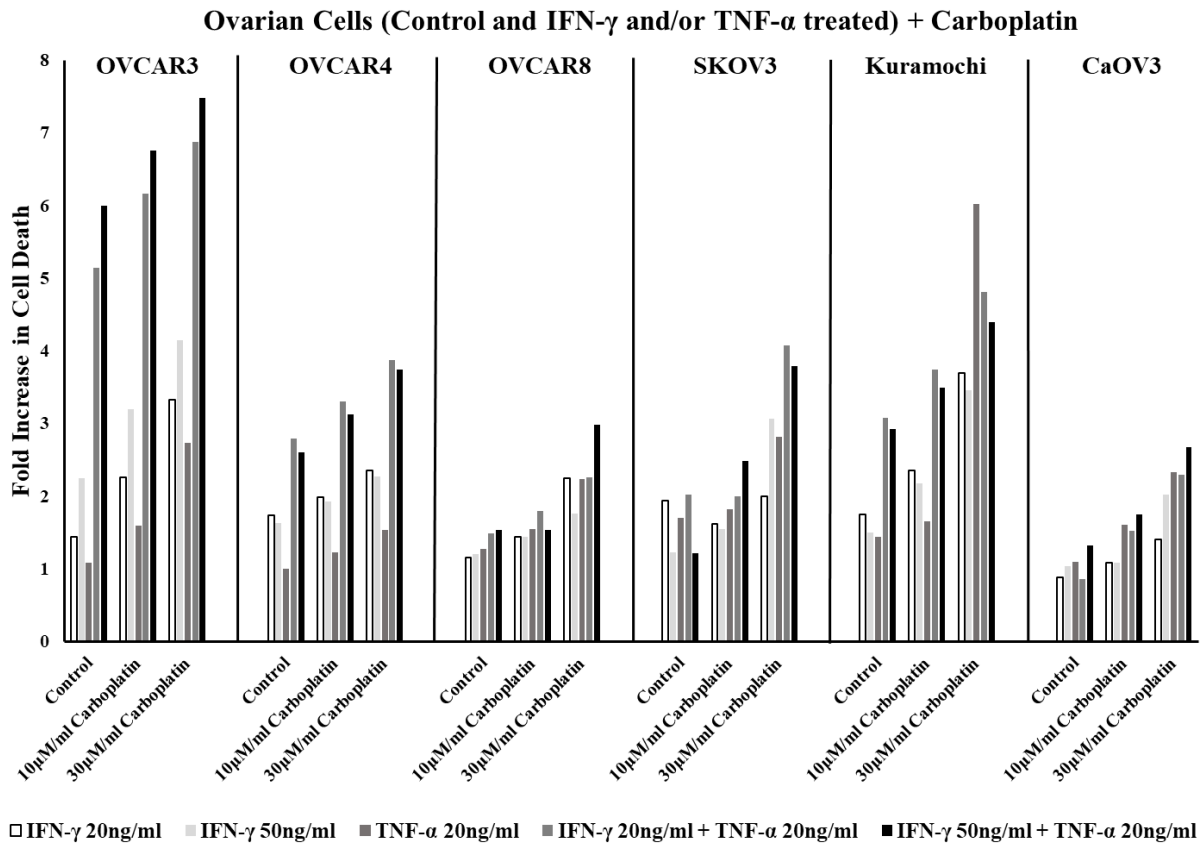
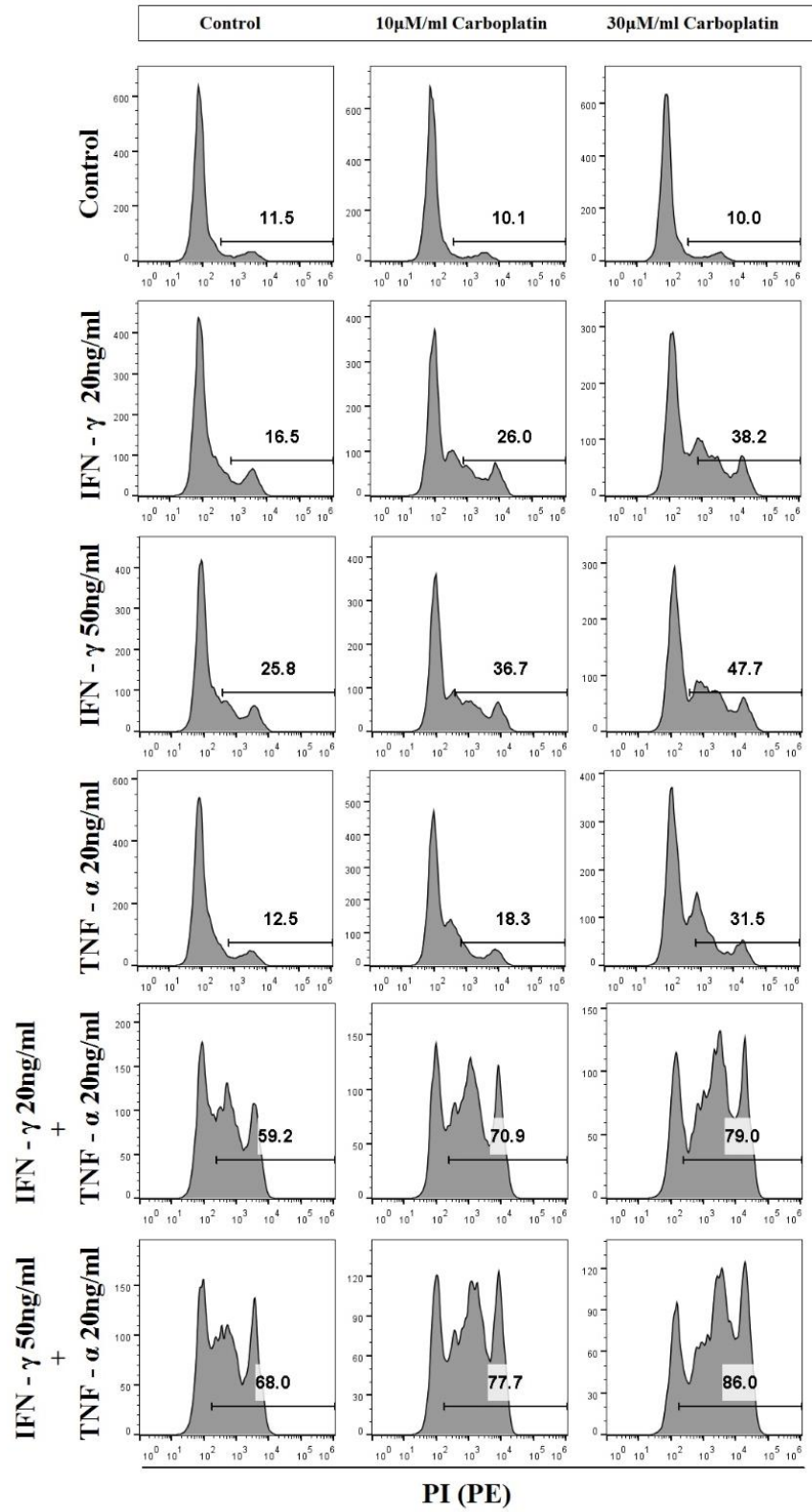


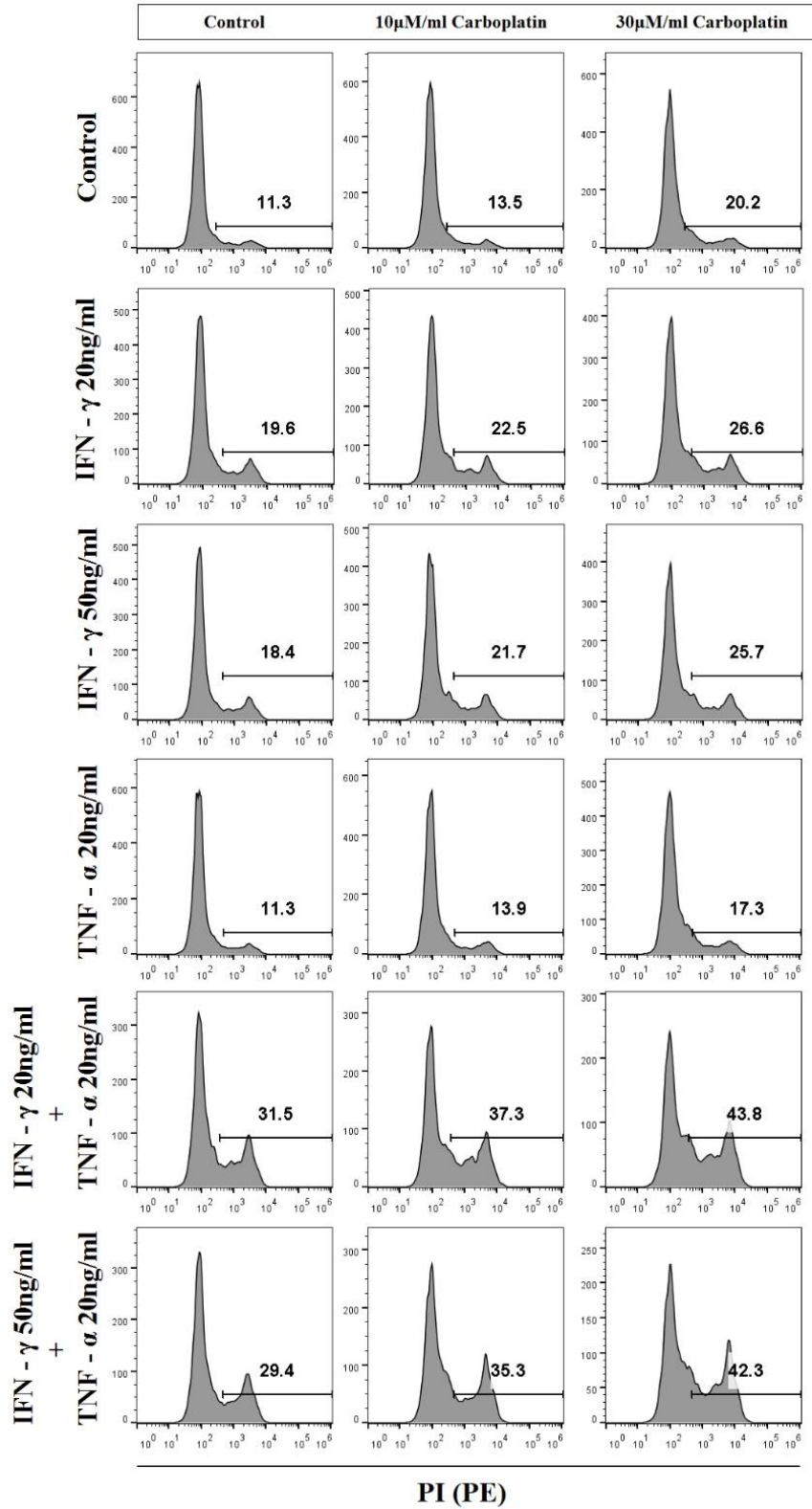
Figure 22: OVCAR8 and CaOV3 displays minimal level of fold increase cell death even after treatment with IFN- γ + TNF- α .

Fold increase in cell death of the IFN- γ and/or TNF- α treated ovarian tumor cells after carboplatin treatment was calculated and a bar chart of which was made, as displayed in the figure.

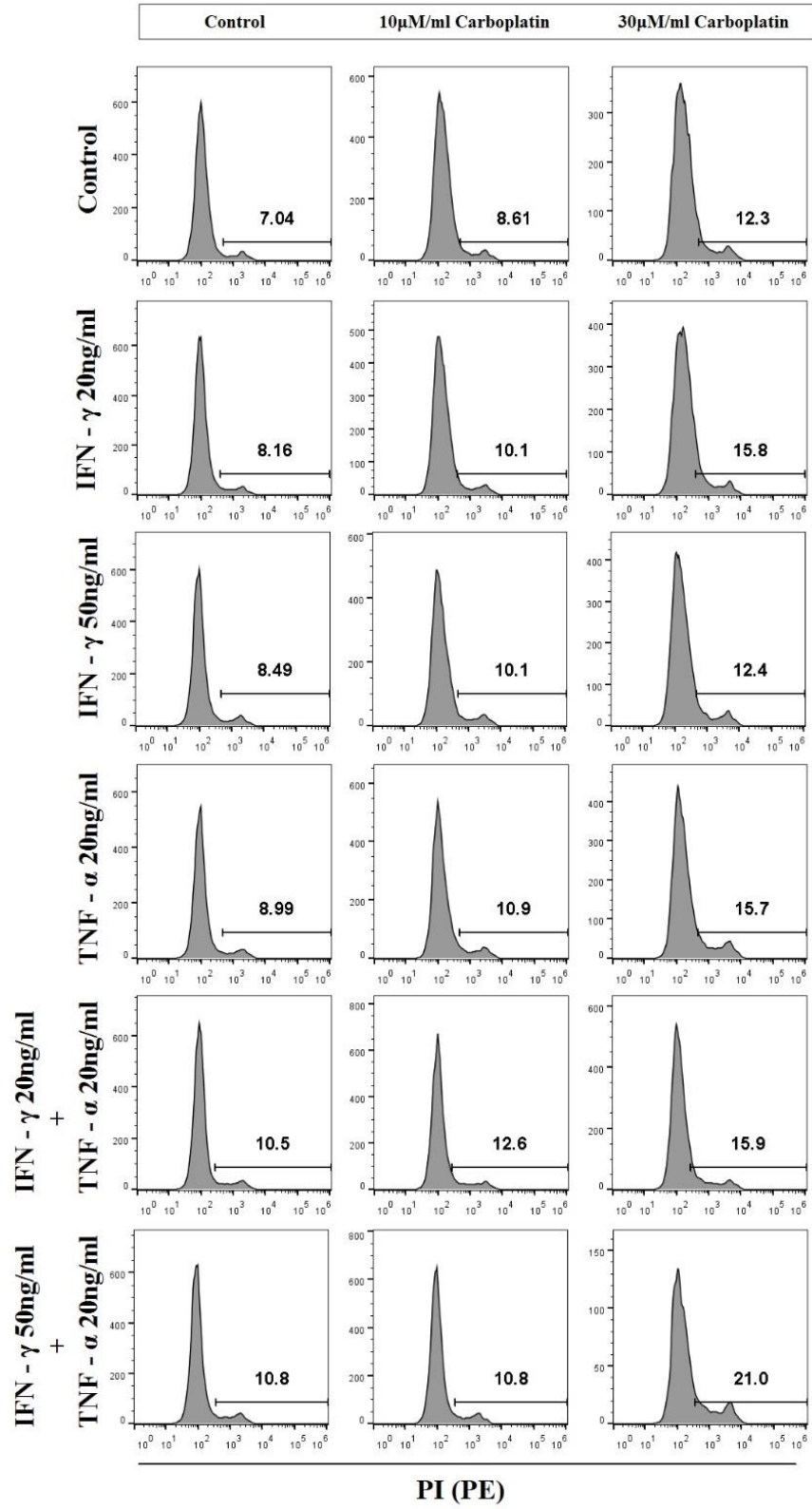
OVCAR3



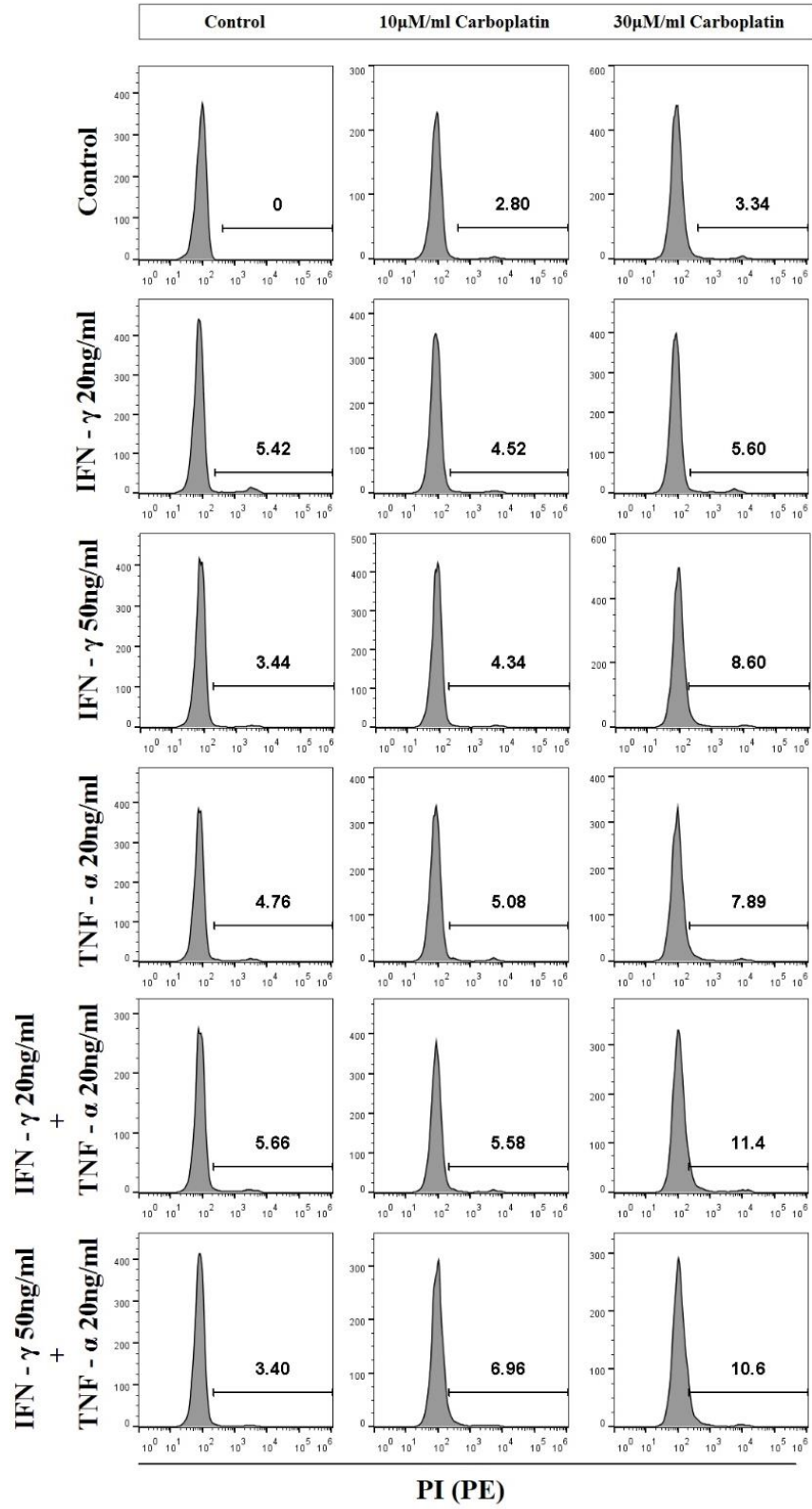
OVCAR4



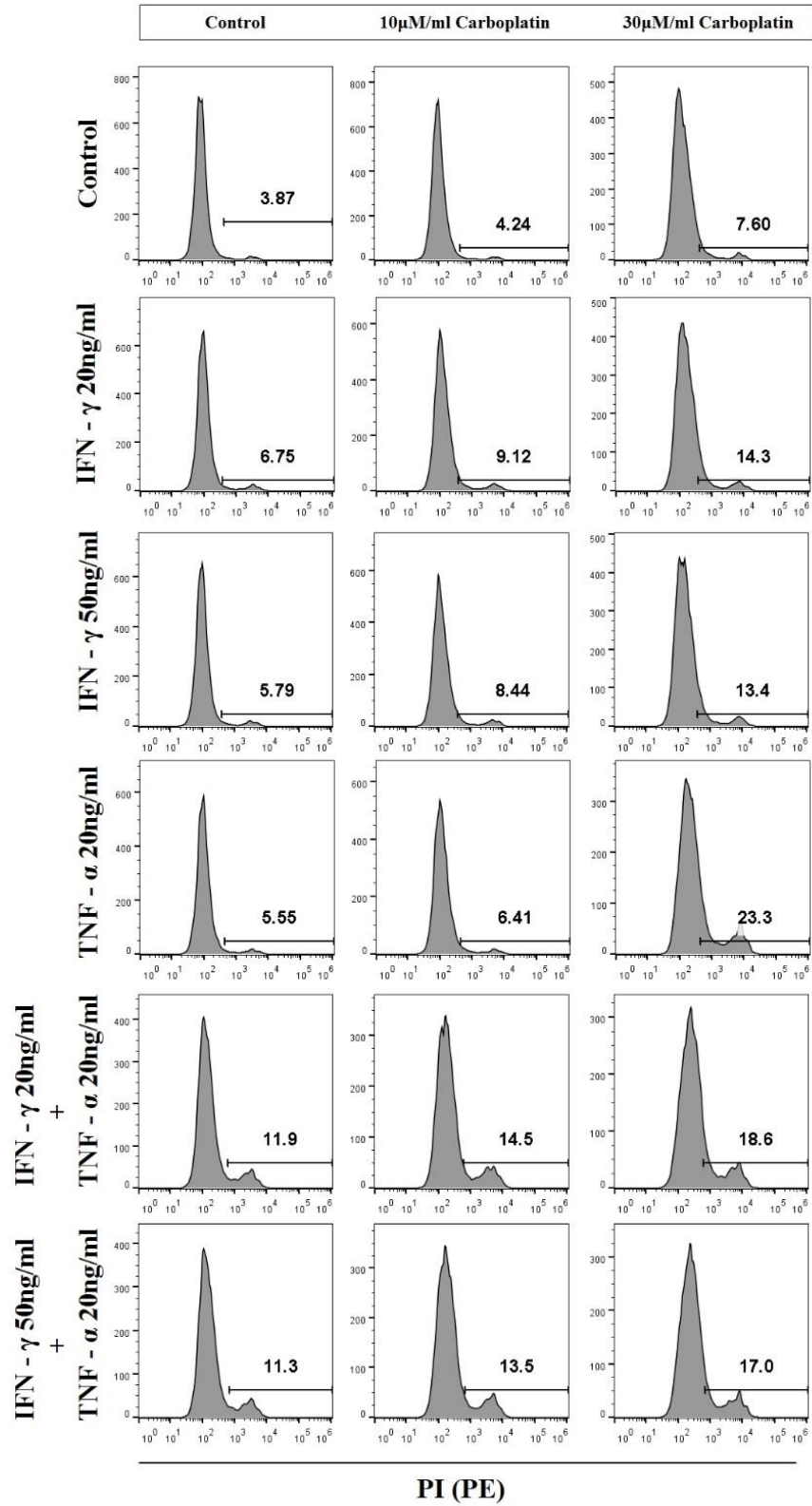
OVCAR8



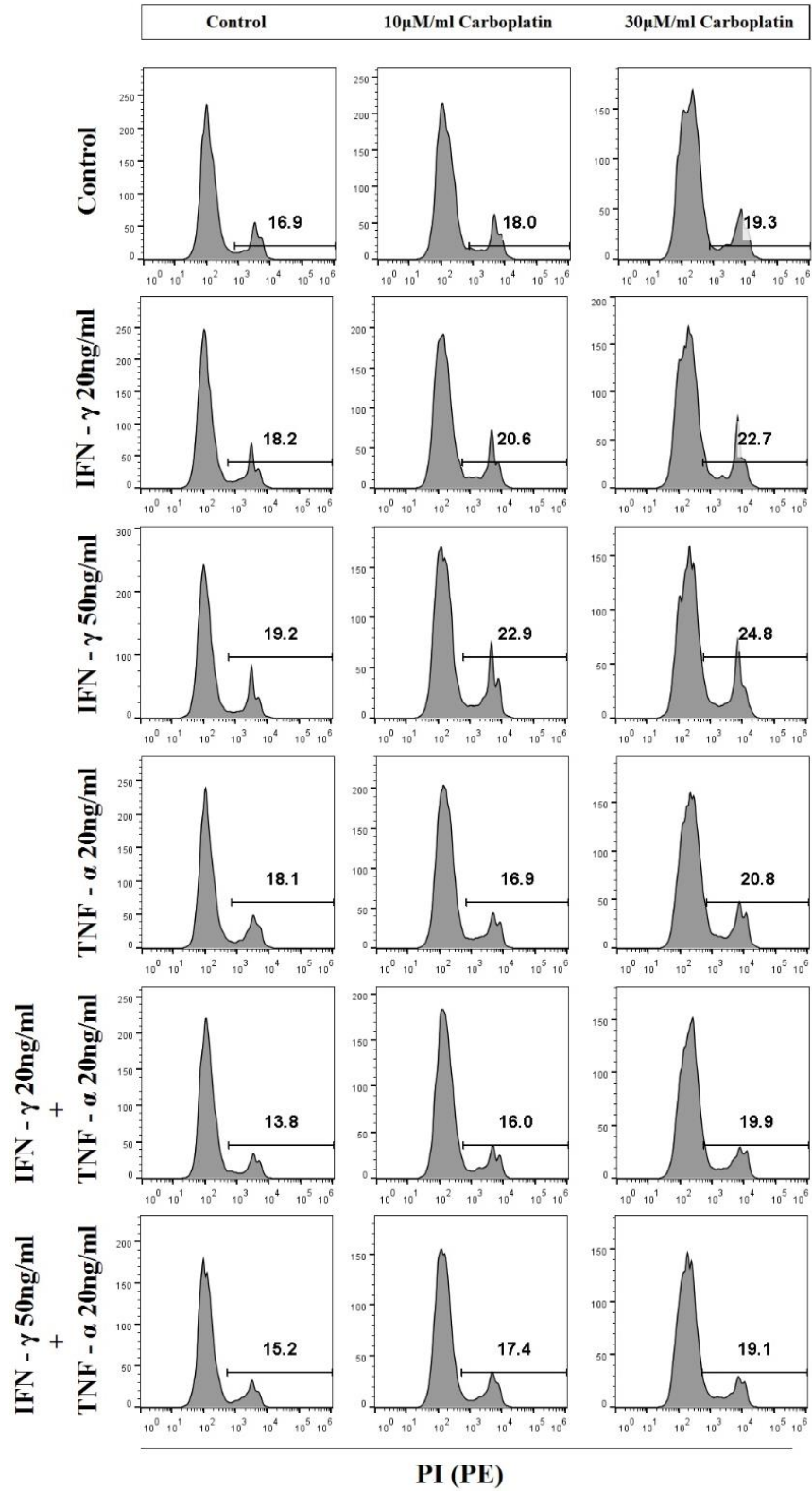
SKOV3



Kuramochi



OAW28



CaOV3

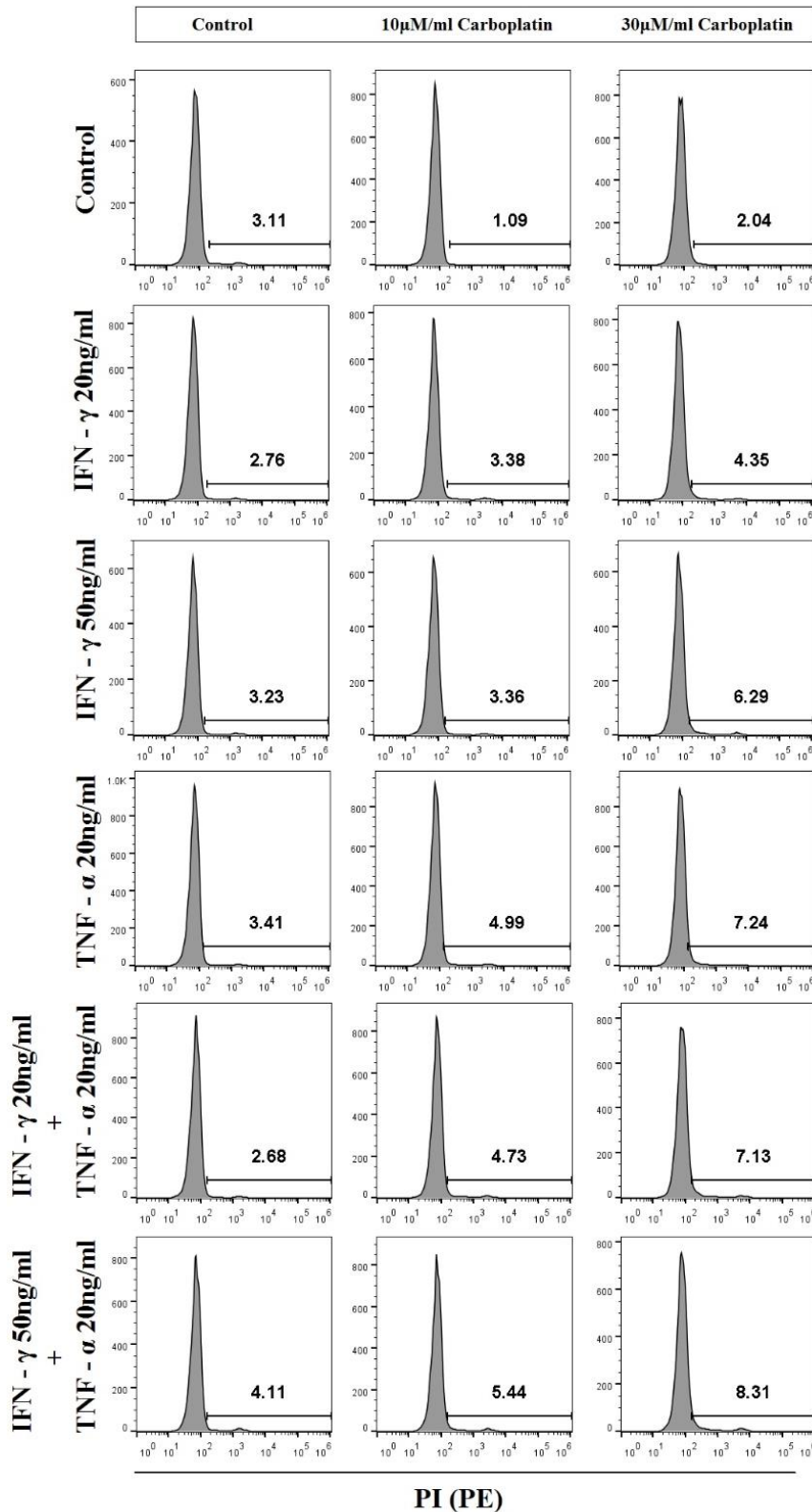


Figure 23 to 29: OVCAR8 and CaOV3 displays lowest and OVCAR3 displays highest susceptibility to carboplatin.

Ovarian tumor cells were treated with IFN-γ (20ng and 50ng/ml), TNF-α (20 ng/ml) and a combination of IFN-γ (20ng and 50ng/ml) and TNF-α (20 ng/ml). After an overnight induction, they were treated with Carboplatin (Control, 10µM/ml, and 30µM/ml) overnight. Subsequently, next day, cells were stained with primidone iodine (PI) to determine the cell death.

Conclusion

In conclusion, OVCAR8, SKOV3, and CaOV3 demonstrates the highest growth potential in comparison to other ovarian tumor lines. OVCAR8 and CaOV3 have the highest expression of CD44 and the lowest expression of MHC-class I when compared to other ovarian tumor lines, and treatment of ovarian tumors with IFN- γ and TNF- α increases the MHC-class I expression in all the ovarian tumor cells with the exception of OVCAR8 and CaOV3 tumor cells. In addition, IFN- γ and TNF- α treatment of the ovarian tumor cells increased the differentiation of the ovarian and lessened the susceptibility to NK cell mediated cytotoxicity and resulted in reduced secretion of IFN- γ by the NK cells co-cultured with those tumors, except of OVCAR8 and CaOV3 which remained susceptible to NK cell mediated cytotoxicity in the presence or in the absence of IFN- γ and TNF- α treatment. Because of the IFN- γ and TNF- α treatment induced differentiation in the ovarian tumors, except of OVCAR8 and CaOV3, ovarian tumors become more susceptible to carboplatin mediated decrease in cell growth and increase in cell death.

From this study, we now understand role of NK cells in ovarian cancer. In future, it would be interesting to study this NK cells and Ovarian tumors interactions in the animal models. Specifically, OVCAR8 and CaOV3 which are sensitive to NK cells mediated cytotoxicity but not to chemotherapeutic drugs, adoptive NK cells immunotherapy can be a breakthrough treatment. And in case of other ovarian tumors, adoptive NK cells mediated cytotoxicity can induce differentiation and enhance the cytotoxicity of chemotherapeutic drugs.

References

1. Mayo Foundation for Medical Education and Research. (2019, July 25). *Ovarian cancer*. Mayo Clinic. <https://www.mayoclinic.org/diseases-conditions/ovarian-cancer/symptoms-causes/syc-20375941>.
2. Beaufort, C. M., Helmijr, J. C., Piskorz, A. M., Hoogstraat, M., Ruigrok-Ritsstier, K., Besselink, N., ... & Helleman, J. (2014). Ovarian cancer cell line panel (OCCP): clinical importance of in vitro morphological subtypes. *PloS one*, 9(9), e103988.
3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin*. 2015;65:5–29
4. Haley, J., Tomar, S., Pulliam, N., Xiong, S., Perkins, S. M., Karpf, A. R., ... & Mitra, A. K. (2016). Functional characterization of a panel of high-grade serous ovarian cancer cell lines as representative experimental models of the disease. *Oncotarget*, 7(22), 32810.
5. Hamilton, T. C., Young, R. C., McKoy, W. M., Grotzinger, K. R., Green, J. A., Chu, E. W., ... & Ozols, R. F. (1983). Characterization of a human ovarian carcinoma cell line (NIH: OVCAR-3) with androgen and estrogen receptors. *Cancer research*, 43(11), 5379-5389.
6. *NIH:OVCAR-3 [OVCAR3] (ATCC® HTB-161™)*. NIH:OVCAR-3 [OVCAR3] ATCC ® HTB-161™ Homo sapiens ovary aden. (n.d.). <https://www.atcc.org/products/all/HTB-161.aspx>.
7. Gubbels, J. A., Felder, M., Horibata, S., Belisle, J. A., Kapur, A., Holden, H., ... & Patankar, M. S. (2010). MUC16 provides immune protection by inhibiting synapse formation between NK and ovarian tumor cells. *Molecular cancer*, 9(1), 1-14.

8. Patankar, M. S., Jing, Y., Morrison, J. C., Belisle, J. A., Lattanzio, F. A., Deng, Y., ... & Clark, G. F. (2005). Potent suppression of natural killer cell response mediated by the ovarian tumor marker CA125. *Gynecologic oncology*, *99*(3), 704-713.
9. Belisle, J. A., Gubbels, J. A., Raphael, C. A., Migneault, M., Rancourt, C., Connor, J. P., & Patankar, M. S. (2007). Peritoneal natural killer cells from epithelial ovarian cancer patients show an altered phenotype and bind to the tumour marker MUC16 (CA125). *Immunology*, *122*(3), 418-429.
10. Davis, D. M., Chiu, I., Fassett, M., Cohen, G. B., Mandelboim, O., & Strominger, J. L. (1999). The human natural killer cell immune synapse. *Proceedings of the National Academy of Sciences*, *96*(26), 15062-15067.
11. Orange, J. S., Harris, K. E., Andzelm, M. M., Valter, M. M., Geha, R. S., & Strominger, J. L. (2003). The mature activating natural killer cell immunologic synapse is formed in distinct stages. *Proceedings of the National Academy of Sciences*, *100*(24), 14151-14156.
12. Lanier, L. L. (2003). Natural killer cell receptor signaling. *Current opinion in immunology*, *15*(3), 308-314.
13. Fuchshuber, P. R., & Lotzova, E. (1992). Differential oncolytic effect of NK-enriched subsets in long-term interleukin-2 cultures. *Lymphokine and cytokine research*, *11*(5), 271-276.
14. Fuchshuber, P. R., Lotzova, E., & Pollock, R. E. (1991). Antitumor activity, growth, and phenotype of long-term IL-2 cultures of human NK and T lymphocytes. *Lymphokine and cytokine research*, *10*(1-2), 51-59.
15. Gubbels, J. A., Belisle, J., Onda, M., Rancourt, C., Migneault, M., Ho, M., ... & Patankar, M. S. (2006). Mesothelin-MUC16 binding is a high affinity, N-glycan dependent

- interaction that facilitates peritoneal metastasis of ovarian tumors. *Molecular cancer*, 5(1), 1-15.
16. Johnson, S. W., Laub, P. B., Beesley, J. S., Ozols, R. F., & Hamilton, T. C. (1997). Increased platinum-DNA damage tolerance is associated with cisplatin resistance and cross-resistance to various chemotherapeutic agents in unrelated human ovarian cancer cell lines. *Cancer research*, 57(5), 850-856.
17. *OVCAR-4 Human Ovarian Cancer Cell Line*. (2016). Endmillipore.
https://www.emdmillipore.com/US/en/product/OVCAR-4-Human-Ovarian-Cancer-Cell-Line,MM_NF-SCC258#overview
18. Mitra, A. K., Davis, D. A., Tomar, S., Roy, L., Gurler, H., Xie, J., ... & Burdette, J. E. (2015). In vivo tumor growth of high-grade serous ovarian cancer cell lines. *Gynecologic oncology*, 138(2), 372-377.
19. Ikediobi, O. N., Davies, H., Bignell, G., Edkins, S., Stevens, C., O'Meara, S., ... & Wooster, R. (2006). Mutation analysis of 24 known cancer genes in the NCI-60 cell line set. *Molecular cancer therapeutics*, 5(11), 2606-2612.
20. Shaw, S. K., Schreiber, C. L., Roland, F. M., Battles, P. M., Brennan, S. P., Padanilam, S. J., & Smith, B. D. (2018). High expression of integrin $\alpha\beta3$ enables uptake of targeted fluorescent probes into ovarian cancer cells and tumors. *Bioorganic & medicinal chemistry*, 26(8), 2085-2091.
21. Gottschalk, N., Lang, S., Kimmig, R., Singh, M., & Brandau, S. (2012). Monocytes and the 38kDa-antigen of mycobacterium tuberculosis modulate natural killer cell activity and their cytotoxicity directed against ovarian cancer cell lines. *BMC cancer*, 12(1), 1-11.

22. Agilent Technologies, Inc., Lamarche, B. L., Velez, J. V., & Zhao, L. Z. (2021, February). *Label-Free Assay for NK Cell-Mediated Cytolysis*.
<https://www.agilent.com/cs/library/applications/application-nk-cell-mediated-cytolysis-xCELLigence-5994-1694en-agilent.pdf>
23. Haley J, Tomar S, Pulliam N, Xiong S, Perkins SM, Karpf AR, Mitra S, Nephew KP, Mitra AK. Functional characterization of a panel of high-grade serous ovarian cancer cell lines as representative experimental models of the disease. *Oncotarget*. 2016 May 31;7(22):32810-20. doi: 10.18632/oncotarget.9053.
24. Gao, Y., Foster, R., Yang, X., Feng, Y., Shen, J. K., Mankin, H. J., ... & Duan, Z. (2015). Up-regulation of CD44 in the development of metastasis, recurrence and drug resistance of ovarian cancer. *Oncotarget*, 6(11), 9313.
25. Cabrera C.M., Nieto A., Cortes J.L., Montes R.M., Catalina P., Cobo F., Barroso-Del-Jesus A., Concha A. The low rate of HLA class I molecules on the human embryonic stem cell line HS293 is associated with the APM components' expression level. *Cell Biol. Int.* 2007;31:1072–1078
26. Suarez-Alvarez B., Rodriguez R.M., Calvanese V., Blanco-Gelaz M.A., Suhr S.T., Ortega F., Otero J., Cibelli J.B., Moore H., Fraga M.F., et al. Epigenetic Mechanisms Regulate MHC and Antigen Processing Molecules in Human Embryonic and Induced Pluripotent Stem Cells. *PLoS ONE*. 2010;5:e10192
27. Kaur, K., Kozłowska, A. K., Topchyan, P., Ko, M. W., Ohanian, N., Chiang, J., ... & Jewett, A. (2020). Probiotic-treated super-charged NK cells efficiently clear poorly differentiated pancreatic tumors in Hu-BLT mice. *Cancers*, 12(1), 63.

28. Natoli, M., Bonito, N., Robinson, J. D., Ghaem-Maghami, S., & Mao, Y. (2020). Human ovarian cancer intrinsic mechanisms regulate lymphocyte activation in response to immune checkpoint blockade. *Cancer Immunology, Immunotherapy*, 1-11.
29. Tseng, H. C., Inagaki, A., Bui, V. T., Cacalano, N., Kasahara, N., Man, Y. G., & Jewett, A. (2015). Differential targeting of stem cells and differentiated glioblastomas by NK cells. *Journal of Cancer*, 6(9), 866.
30. Fogh, J., Fogh, J. M., & Orfeo, T. (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *Journal of the National Cancer Institute*, 59(1), 221-226.
31. Shaw, T. J., Senterman, M. K., Dawson, K., Crane, C. A., & Vanderhyden, B. C. (2004). Characterization of intraperitoneal, orthotopic, and metastatic xenograft models of human ovarian cancer. *Molecular therapy*, 10(6), 1032-1042.
32. Suh, K. S., Park, S. W., Castro, A., Patel, H., Blake, P., Liang, M., & Goy, A. (2010). Ovarian cancer biomarkers for molecular biosensors and translational medicine. *Expert review of molecular diagnostics*, 10(8), 1069-1083.
33. Fogh, J. (1986). Human tumor lines for cancer research. *Cancer investigation*, 4(2), 157-184.
34. Shaw, T. J., Senterman, M. K., Dawson, K., Crane, C. A., & Vanderhyden, B. C. (2004). Characterization of intraperitoneal, orthotopic, and metastatic xenograft models of human ovarian cancer. *Molecular therapy*, 10(6), 1032-1042.
35. Sun, Y., Yao, Z., Zhao, Z., Xiao, H., Xia, M., Zhu, X., ... & Sun, C. (2018). Natural killer cells inhibit metastasis of ovarian carcinoma cells and show therapeutic effects in a

- murine model of ovarian cancer. *Experimental and therapeutic medicine*, 16(2), 1071-1078.
36. Bröker, K., Sinelnikov, E., Gustavus, D., Schumacher, U., Pörtner, R., Hoffmeister, H., ... & Dammermann, W. (2019). Mass production of highly active NK cells for cancer immunotherapy in a GMP conform perfusion bioreactor. *Frontiers in bioengineering and biotechnology*, 7, 194.
37. Hoogstad-van Evert, J. S., Bekkers, R., Ottevanger, N., Jansen, J. H., Massuger, L., & Dolstra, H. (2020). Harnessing natural killer cells for the treatment of ovarian cancer. *Gynecologic oncology*, 157(3), 810-816.
38. Kruschinski, A., Moosmann, A., Poschke, I., Norell, H., Chmielewski, M., Seliger, B., ... & Charo, J. (2008). Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. *Proceedings of the National Academy of Sciences*, 105(45), 17481-17486.
39. Gonzalez, V. D., Huang, Y. W., Chen, S. Y., Delgado-Gonzalez, A., Donoso, K., ... & Fantl, W. (2020). High-Grade Serous Ovarian Tumor Cells Modulate NK Cell Function to Create an Immune-Tolerant Microenvironment. *bioRxiv*.
40. Elias, K. M., Emori, M. M., Papp, E., MacDuffie, E., Konecny, G. E., Velculescu, V. E., ... & Drapkin, R. (2015). Beyond genomics: critical evaluation of cell line utility for ovarian cancer research. *Gynecologic oncology*, 139(1), 97-103.
41. Wilson, A. P., Dent, M., Pejovic, T., Hubbard, L., & Radford, H. (1996). Characterisation of seven human ovarian tumour cell lines. *British journal of cancer*, 74(5), 722-727.
42. Sanches, R., Kuiper, M., Penault-Llorca, F., Aunoble, B., D'Incan, C., & Bignon, Y. J. (2000). Antitumoral effect of interleukin-12-secreting fibroblasts in a mouse model of

ovarian cancer: implications for the use of ovarian cancer biopsy-derived fibroblasts as a vehicle for regional gene therapy. *Cancer gene therapy*, 7(5), 707-720.

43. Hills, C. A., Kelland, L. R., Abel, G., Siracky, J., Wilson, A. P., & Harrap, K. R. (1989). Biological properties of ten human ovarian carcinoma cell lines: calibration in vitro against four platinum complexes. *British journal of cancer*, 59(4), 527-534.
44. Lokman, N. A., Ho, R., Gunasegaran, K., Bonner, W. M., Oehler, M. K., & Ricciardelli, C. (2019). Anti-tumour effects of all-trans retinoid acid on serous ovarian cancer. *Journal of Experimental & Clinical Cancer Research*, 38(1), 1-12.
45. Buick, R. N., Pullano, R., & Trent, J. M. (1985). Comparative properties of five human ovarian adenocarcinoma cell lines. *Cancer research*, 45(8), 3668-3676.
46. Yaginuma, Y., & Westphal, H. (1992). Abnormal structure and expression of the p53 gene in human ovarian carcinoma cell lines. *Cancer research*, 52(15), 4196-4199.
47. Shayesteh, L., Lu, Y., Kuo, W. L., Baldocchi, R., Godfrey, T., Collins, C., ... & Gray, J. W. (1999). PIK3CA is implicated as an oncogene in ovarian cancer. *Nature genetics*, 21(1), 99-102.
48. Cannistra, S. A., DeFranzo, B., Niloff, J., & Ottensmeir, C. (1995). Functional heterogeneity of CD44 molecules in ovarian cancer cell lines. *Clinical cancer research*, 1(3), 333-342.
49. Cannistra, S. A., Kansas, G. S., Niloff, J., DeFranzo, B., Kim, Y., & Ottensmeier, C. (1993). Binding of ovarian cancer cells to peritoneal mesothelium in vitro is partly mediated by CD44H. *Cancer research*, 53(16), 3830-3838.

50. Koh, J., Lee, S. B., Park, H., Lee, H. J., Cho, N. H., & Kim, J. (2012). Susceptibility of CD24+ ovarian cancer cells to anti-cancer drugs and natural killer cells. *Biochemical and biophysical research communications*, 427(2), 373-378.
51. Perera Molligoda Arachchige, A. S. (2021). Human NK cells: From development to effector functions. *Innate Immunity*, 27(3), 212-229.
52. Poli, A., Michel, T., Thérésine, M., Andrès, E., Hentges, F., & Zimmer, J. (2009). CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology*, 126(4), 458-465.
53. Vivier, E., Tomasello, E., Baratin, M., Walzer, T., & Ugolini, S. (2008). Functions of natural killer cells. *Nature immunology*, 9(5), 503-510.
54. Hashemi, E., & Malarkannan, S. (2020). Tissue-resident NK cells: development, maturation, and clinical relevance. *Cancers*, 12(6), 1553.
55. Jewett A, Man Y-G, Tseng H-C. Dual Functions of Natural Killer Cells in Selection and Differentiation of Stem Cells; Role in Regulation of Inflammation and Regeneration of Tissues. *Journal of Cancer*. 2013;4(1):12-24.
56. Topham NJ, Hewitt EW. Natural killer cell cytotoxicity: how do they pull the trigger? *Immunology*. 2009;128(1):7-15.
57. Jordan S., & Zuhair, K. Natural killer cells in human health and disease. *Clinical Immunology*. 2006; 118 (1):1-10.
58. Yoon, S. R., Kim, T., & Choi, I. Understanding of molecular mechanisms in natural killer cell therapy. *Experimental & Molecular Medicine*. 2015; 47(2).
59. Zamai L, Ahmad M, Bennett IM, Azzoni L, Alnemri ES, Perussia B. Natural Killer (NK) Cell mediated Cytotoxicity: Differential Use of TRAIL and Fas Ligand by Immature and

Mature Primary Human NK Cells . *The Journal of Experimental Medicine*.
1998;188(12):2375-2380.

60. Wang, W., Erbe, A. K., Hank, J. A., Morris, Z. S., & Sondel, P. M. (2015). NK cell-mediated antibody-dependent cellular cytotoxicity in cancer immunotherapy. *Frontiers in immunology*, 6, 368.
61. Lanier, L.L., NK cell recognition. *Annu Rev Immunol*, 2005. 23: p. 225-74.
62. Poli, Aurélie et al. CD56bright Natural Killer (NK) Cells: An Important NK Cell Subset. *Immunology*, 2009. 126(4): p. 458–465.
63. Bui, V.T., et al., Augmented IFN- γ and TNF- α Induced by Probiotic Bacteria in NK Cells Mediate Differentiation of Stem-Like Tumors Leading to Inhibition of Tumor Growth and Reduction in Inflammatory Cytokine Release; Regulation by IL-10. *Front Immunol*, 2015. 6: p. 576.
64. Hersey, P., et al., Low natural-killer-cell activity in familial melanoma patients and their relatives. *Br J Cancer*, 1979. 40(1): p. 113-22.
65. Kuss I, Saito T, Johnson JT, Whiteside TL. Clinical significance of decreased zeta chain expression in peripheral blood lymphocytes of patients with head and neck cancer. *Clin Cancer Res*, 1999 5(2): p. 329–34.
66. Larsen, S.K., Y. Gao, and P.H. Basse, NK cells in the tumor microenvironment. *Crit Rev Oncog*, 2014. 19(1-2): p. 91-105.
67. Lee, June-Chul, Yong-Oon Ahn, Dong-Wan Kim, and Dae Seog Heo. Elevated TGF- β 1 Secretion and Downmodulation of NKG2D Underlies Impaired NK Cytotoxicity in Cancer Patients. *Journal of Immunotherapy*, 2004. 172: 7335–7340.

68. Imai, K., et al., Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet*, 2000. 356(9244): p. 1795-
69. Jewett A, Teruel A, Romero M, Head C, Cacalano N. Rapid and potent induction of cell death and loss of NK cell cytotoxicity against oral tumors by F(ab')₂ fragment of anti-CD16 antibody. *Cancer Immunol Immunother*, 2008. 57(7): p.1053–66.
70. Pietra, G., et al., Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. *Cancer Res*, 2012. 72(6): p. 1407-15.
71. Hershey, P. et al. Relationship of cell-mediated cytotoxicity against melanoma cells to prognosis in melanoma patients. *British Journal of Cancer* 37.4 (1978): 505–513. Print.
72. Lai P, Rabinowich H, Crowley-Nowick PA, Bell MC, Mantovani G, Whiteside TL. Alterations in expression and function of signal-transducing proteins in tumor-associated T and natural killer cells in patients with ovarian carcinoma. *Clin Cancer Res* (1996) 2(1): p.161–73.
73. Burke, S., et al., New views on natural killer cell-based immunotherapy for melanoma treatment. *Trends Immunol*, 2010. 31(9): p. 339-45.
74. Brittenden, Julie, S. D. Heys, J. Ross, and O. Eremin. Natural killer cells and cancer. *Cancer* 77.7, 1996. 77 (7): p. 1226-1243.
75. Kozłowska, A. K., Tseng, H. C., Kaur, K., Topchyan, P., Inagaki, A., Bui, V. T., ... & Jewett, A. (2016). Resistance to cytotoxicity and sustained release of interleukin-6 and interleukin-8 in the presence of decreased interferon- γ after differentiation of

glioblastoma by human natural killer cells. *Cancer Immunology, Immunotherapy*, 65(9), 1085-1097.

76. Kozłowska, A. K., Topchyan, P., Kaur, K., Tseng, H. C., Teruel, A., Hiraga, T., & Jewett, A. (2017). Differentiation by NK cells is a prerequisite for effective targeting of cancer stem cells/poorly differentiated tumors by chemopreventive and chemotherapeutic drugs. *Journal of Cancer*, 8(4), 537.