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Role of NK Cells in Selection  
and Differentiation of Lung Tumors.

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

by

Angie Suleny Celis

2014



## **ABSTRACT OF THE THESIS**

### **Role of NK Cells in Selection and Differentiation of Lung Tumors.**

By

Angie Suleny Celis

Master of Science in Oral Biology

University of California, Los Angeles, 2014

Professor Anahid Jewett, Chair

NK cells are large granular lymphocytes known to mediate direct and antibody dependent cellular cytotoxicity (ADCC) against tumors as well as to regulate the function of other cells through the secretion of cytokines and chemokines. In previous experiments our lab has shown that NK cells are significantly more cytotoxic towards stem cells than their differentiated counterparts, as was shown with oral squamous carcinoma stem cells (OSCSCs) versus the differentiated oral squamous carcinoma cells (OSCCs), thus, demonstrating that their stage of differentiation is predictive of sensitivity to NK cell mediated cytotoxicity. The same was seen in human Mesenchymal Stem Cells (hMSCs), human dental pulp stem cells (hDPSCs) and human induced pluripotent stem cells (hiPSCs) in which they were lysed more significantly than their differentiated counterparts. Moreover, interaction of NK cells with stem cells leads to the suppression of NK cell cytotoxicity and in an increase in cytokine secretion termed split energy, however, the interaction of NK cells with resistant tumors does not lead to suppression of NK cytotoxicity or increase in cytokine secretion. NK cells may play an important role in the differentiation of cells.

In this study we examined the role of NK cells in selection and differentiation of lung cancer cells. Our objective was to characterize the lung tumor cell line hA549 and to demonstrate induction of split anergy in NK cells decreases cytotoxicity against hA549, but increases secretion of cytokines, and that energized NK cells support the differentiation of lung cancer stem cells through secreted cytokines. Furthermore, we explored the role of MHC 1 and intracellular signaling pathways in differentiation of lung tumors. In addition, we wanted to demonstrate that undifferentiated or poorly differentiated lung tumors are more resistant to chemotherapy drugs than differentiated lung tumors

hA549 and H292 lung tumor cells, as well as, healthy SCAP cells were used. Human NK cells were purified using negative selection and activated with IL-2 and IL-2 + $\alpha$ CD16mAb. NK cell cytotoxicity was assessed using Cr51 release assay. Lung tumor cells were differentiated using recombinant TNF- $\alpha$ , IFN- $\gamma$ , a combination of TNF- $\alpha$  and IFN- $\gamma$ , or NK IL2 +  $\alpha$ CD16mAb (energized) supernatants. Differentiation was assessed by using surface marker analysis by flow cytometry. Lung tumors were treated with Cisplatin, Paclitaxel, NAC, Cisplatin +NAC, and Paclitaxel + NAC. Cells were stained with Propidium Iodide and cell death was assessed by flow cytometry analysis.

The experimental findings of the project demonstrated that hA549 were sensitive to NK cell mediated cytotoxicity. In addition, based on our accumulated results interaction of NK cells with poorly differentiated tumor cells induced split anergy in NK cells resulting in increased secretion of TNF- $\alpha$  and IFN- $\gamma$  which induced differentiation and resistance of tumor cells. Furthermore, increased expression of MHC class 1 and activation of JNK, AKT, and STAT3 intracellular signaling pathways was correlated with differentiation of hA549 by energized NK supernatants. Thus, NK cells may inhibit or significantly slow the tumor progression 1-by

elimination of cancer stem cells or poorly differentiated tumors and 2- by inducing differentiation of tumors thereby limiting tumor induced inflammation which could further fuel the tumors. In addition, we found that hA549 were more resistant to the chemotherapy drugs, Cisplatin and Paclitaxel, than the more differentiated H292.

The thesis of Angie Suleny Celis is approved.

Ichiro Nishimura

Nicholas A. Cacalano

Anahid Jewett, Committee Chair

University of California, Los Angeles

2014

This thesis is dedicated to:

My mentor, Dr. Anahid Jewett, for giving me the opportunity to be part of an amazing lab whose research has and will continue to contribute to the field of tumor immunology. Thank you for all your support and encouragement, but most of all, your guidance throughout these two years. It is because of you that I have become a better researcher.

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## **Introduction**

### *Innate and Adaptive Immunity*

The mammalian immune system is a complex interacting network that includes actions of both pro-inflammatory and anti-inflammatory mediators to fight pathogens. It is divided into two important parts, innate and adaptive immunity. Innate immunity serves as the primary line of defense against pathogens that can exert rapid effector function. Signaling in innate immunity occurs through a limited repertoire of germline-encoded receptors that recognize patterns of exogenous agents and antigens. Furthermore, the innate immune system depends and is made up of monocytes, macrophages, dendritic cells, mast cells, neutrophils, eosinophils, basophils, and natural killer (NK) cells. In contrast, adaptive immunity is composed of cells with a large repertoire of antigen receptors that are produced by somatic recombination which generates unlimited diversity of recognition response elements (1, 2). Adaptive immunity is characterized by long term persistence of memory cells, such as T cells and B cells, as well as the recognition of specific molecular structures by such cells (2). Upon recognition of foreign structures by T-cells adaptive immune responses are triggered such as direct attack by cytotoxic (CD8) T cells, secretion of cytokines by CD4 T cells, and stimulation of B cells to produce antibodies against antigens (3).

### *NK cell development and biology*

Natural Killer (NK) cells are granular lymphocytes that function at the interference of innate and adaptive immunity (4). Discovered in the early 1970's by accident when investigators were studying specific cytotoxic effects of lymphocytes, it was not until the 1980's that they became generally accepted despite the accumulated evidence (5). NK cells are a subset of

cytotoxic lymphocytes able to recognize and lyse tumor cells and virus infected cells without prior sensitization (6). Traditionally they have been classified as effectors of innate immunity due to the lack of antigen specific cell surface receptors (2). NK cells are known to mediate direct and antibody dependent cellular cytotoxicity (ADCC) against tumors as well as to regulate the function of other cells through the secretion of cytokines and chemokines (7).

NK cells derive from CD34+ hematopoietic stem cells (HSC's) found in the bone marrow. They can be found throughout the body in the spleen, liver, placenta, and peripheral blood (8). Human NK cells are defined phenotypically by the surface expression of CD56 and CD16, and by their lack of CD3 surface expression (9). CD56 is a human neural-cell adhesion molecule, but its function on human NK cells is yet to be understood. Although the function of CD56 is unknown, its expression correlates with the expression of other surface markers that confer important functional properties to NK cells (4). Two subsets of NK cells have been identified based on surface expression of CD56 and CD16. The major subset of NK cells, about 90% of human NK cells, is defined by low expression of CD56 (CD56<sup>dim</sup>) and high expression of CD16 (CD16<sup>bright</sup>). The minor subset makes up approximately 10% of human NK cells and is defined by high expression of CD56 (CD56<sup>bright</sup>) and low or lack of CD16 (CD16<sup>dim/-</sup>) expression (4, 9). The CD56<sup>dim</sup> CD16<sup>bright</sup> cells were found to be the more cytotoxic subset of human NK cells. On the other hand, CD56<sup>bright</sup> CD16<sup>dim/-</sup> NK cells were found to secrete more cytokines such as interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), TNF- $\beta$ , granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-10 (IL-10), and IL13 after being stimulated with pre-inflammatory cytokines (4, 9, 10).

Murine NK cells share many features with human NK cells such as their ability to lyse tumor cells and produce cytokines, but they lack expression of a murine homolog to CD56. This

makes it difficult to compare murine NK cells to human NK cells, thus, limiting studies to in vitro analysis (9, 10). Murine NK cells are divided into four subsets based on CD11B, CD27, CD127, and B220: CD11b<sup>low</sup> CD27<sup>low</sup>, CD11b<sup>low</sup> CD27<sup>high</sup>, CD11b<sup>high</sup> CD27<sup>high</sup>, and CD11b<sup>high</sup> CD27<sup>low</sup>. The CD11b<sup>high</sup> CD27<sup>high</sup> subset produces the largest number of cytokines and has the strongest cytotoxicity, and the CD11b<sup>high</sup> CD27<sup>low</sup> subset has been suggested to resemble the human CD56<sup>dim</sup> CD16<sup>high</sup> NK cell subset(10).

### *Immunosurveillance by NK cells*

NK cells are potent effector cells that function in the surveillance and elimination of infected or transformed cells (11). They circulate in the blood and become activated through cytokines, pathogen derived substances, or through encounters with target cells expressing ligands NK cell receptors recognize (6). Unlike B and T cells whose antigen receptors undergo somatic recombination, NK cell receptors are encoded in the germ line and NK cell function is determined through a balance of signals from activating and inhibitory receptors (6, 11). NK cells have the ability to rapidly destroy targets without prior sensitization through different effector functions including cytolytic granule exocytosis, expression of death receptors, and antibody-dependant cell-mediated cytotoxicity (ADCC) (4, 11).

The granule exocytosis pathway is one of the methods by which NK cells exert cytotoxicity. In NK cells we find granules containing a complex of cytotoxic and pro-apoptotic proteins that together cause death of target cells. Perforin, an important granule protein, is crucial for the function of other granule proteins such as granzymes (6, 12). When an NK cell comes into contact with a target cell it forms an immunological synapse releasing granules containing perforin and granzymes. Perforin creates pores in the plasma membrane of target cells through



which granzymes can diffuse to the cytosol leading to the activation of the caspase cascade and degradation of DNA, thus, inducing apoptosis of target cells (6, 12, 13).

The death-receptor-dependant pathway triggers apoptosis through stimulation of death receptors on the cell membrane. It involves binding of death ligands Factor-related apoptosis ligand (FasL), tumor necrosis factor (TNF), or Tumor necrosis-related apoptosis inducing ligand (TRAIL) on NK cells to their corresponding receptors Fas, TNF receptor, and TRAIL receptor on target cells. Aggregation of receptors causes death domains to colocalize on receptors with adapter molecules such as Fas-associated via death domain (FADD), TNF-receptor 1-associated via death domain (TRADD), and apical pro-caspases leading to activation of caspases and subsequent death of target cells (12-14).

Antibody-dependant cell-mediated cytotoxicity (ADCC) is another function of NK cells. About 90% of human NK cells are CD56<sup>dim</sup>CD16<sup>bright</sup> and are capable of ADCC through expression of CD16. CD16 is a low affinity FcγRIII essential for carrying out ADCC. The Fc portion of CD16 binds to target cells that are Ab coated (opsonized) triggering signals through associated subunits containing immunoreceptor tyrosine-based activation motif (ITAM) which activates ADCC leading to death of target cells (4, 6, 9).

#### *Tumor Surveillance by NK cells*

Tumor cells can be detected by NK cells through a series of receptors able to recognize different ligands. Three major superfamilies of human NK receptors have been studied, killer immunoglobulin (Ig)-like receptor (KIR), the C-type lectin superfamily, and the natural cytotoxicity receptors (NCR) (4). NK cells have the ability to recognize major histocompatibility complex (MHC) class 1 or class 1-like molecules on target cells through KIR and binding of KIR

to its ligand inhibits NK-mediated cytotoxicity (15). The C-type lectin superfamily recognizes non-classical MHC class 1 or class 1-like molecules and binding to its ligand can be inhibitory of activation. Studies have demonstrated that NKR for self MHC class 1 are important for distinguishing autologous normal cells from foreign or transformed cells. However, it has been observed that MHC class 1 is not necessary for protection from NK lysis and inhibition by MHC class 1 is not sufficient to prevent NK cytotoxicity in certain cases. The third superfamily, NCR, are Ig-like activating receptors whose ligands remain poorly defined (4).

### *Cytokines*

NK cells are an important source of immunoregulatory cytokines found to secrete large amounts of TNF- $\alpha$  and IFN- $\gamma$  after target recognition and activation (11). IFN- $\gamma$  is the only member of the type II class of interferons and is produced under pathologic circumstances mainly by NK cells and T-cells (16). IFN- $\gamma$  plays an important role in suppression of pathogens through the containment of the initial infection and promoting an adaptive immune response (12, 17). Production of IFN- $\gamma$  is triggered through interaction of NK cells with target cells and was found to be significantly upregulated by interleukin (IL)-2 and interleukin (IL)-12 (6, 16, 17). IL-2 was found to be important for the development and activation of NK cells (4, 18). TNF-alpha is a type I transmembrane protein with signaling potential as a membrane bound protein or soluble cytokine. It plays an important role in the initiation of inflammatory reactions by the innate immune system and secretion of TNF- $\alpha$  induces activation and migration of macrophages and T-cells to target sites (11, 19).

## *Thesis Outline*

This study has three specific aims in terms of NK cell mediated cytotoxicity against lung tumors, the role of anergized NK cells in differentiation of lung cancer stem cells, and the role of MHC class 1 and intracellular signaling pathways in resistance to NK cell mediated cytotoxicity.

*Specific Aim 1:* Characterize lung tumor cell line hA549 and demonstrate induction of split anergy in NK cells decreases cytotoxicity against hA549 but increases secretion of cytokines.

*Specific Aim 2:* Anergized NK cells support the differentiation of lung cancer stem cells through secreted cytokines.

*Specific Aim 3:* To demonstrate MHC 1 plays an important role on differentiated cells and presence of JNK, AKT, and STAT3 signaling pathways correlates with differentiation of hA549 cells and increased resistance to NK cell mediated cytotoxicity induced by anergized NK cells.

*Specific Aim 4:* Undifferentiated or poorly differentiated lung tumors are more resistant to chemotherapy drugs than differentiated lung tumors

## **Chapter 1:**

*Specific Aim 1:* Characterize lung tumor cell line hA549 and demonstrate induction of split anergy in NK cells decreases cytotoxicity against hA549 but increases secretion of cytokines.

### **Introduction:**

NK cells are known to mediate direct and antibody dependent cellular cytotoxicity (ADCC) against tumors as well as to regulate the function of other cells through the secretion of cytokines and chemokines (7). In previous experiments our lab has shown that NK cells are significantly more cytotoxic towards stem cells than their differentiated counterparts, as was shown with oral squamous carcinoma stem cells (OSCSCs) versus the differentiated oral squamous carcinoma cells (OSCCs), thus, demonstrating that their stage of differentiation is predictive of sensitivity to NK cell mediated cytotoxicity. The same was seen in human Mesenchymal Stem Cells (hMSCs), human Dental Pulp Stem Cells (hDPSCs) and human induced Pluripotent Stem Cells (hiPSCs) in which they were lysed more significantly than their differentiated counterparts (20). Thus, it is an emerging view in our laboratory that the stage of maturation and differentiation of healthy untransformed stem cells as well as transformed tumorigenic cancer stem cells is predictive of their sensitivity to NK cell mediated cytotoxicity (18).

Immunosuppression and tumor escape from immune recognition are thought to be major factors responsible for the establishment and progression of cancer, yet the significance and exact mechanism by which immunosuppression occurs is not understood (18, 20-22). Interaction of NK cells with stem cells leads to the suppression of NK cell cytotoxicity and in an increase in cytokine secretion, however, the interaction of NK cells with resistant tumors does not lead to

suppression of NK cytotoxicity or increase in cytokine secretion (23-27). It has been found that NK's isolated from tumors are not cytotoxic to autologous tumors and NKs obtained from the peripheral blood of cancer patients have significantly reduced cytotoxicity (28, 29). There have been several mechanisms proposed to explain the functional inactivation of tumor-associated NK cells such as over-expression of Fas ligand, the loss of mRNA for granzyme B, and decreased CD16 and its associated zeta chain (23). Phenotypic changes such as the down modulation of CD16 expression has been observed in NK cell cultures with sensitive tumor-target cells, but not resistant tumors, as well as in NK cells from several cancer patients (26, 27). Furthermore, decrease in expression of CD16 was correlated with decrease in NK cytotoxicity suggesting CD16 surface receptor may play an important part in loss of NK cytotoxicity induced by target cells (30-32). In addition, triggering of CD16 on NK cells untreated or IL-2 treated resulted in down modulation of CD16 and subsequent loss of NK cytotoxicity. Split anergy is a term coined by our lab that describes the loss of NK cytotoxicity, but a gain in the ability of NK cells to secrete cytokines.

Lung cancer is the deadliest type of cancer for both men and women, and every year more people die from this type of cancer than breast, colon, and prostate cancer. The leading cause of cancer is smoking, however, about 10-25% of lung cancers occur in never smokers (33). Lung cancer is divided into four major histological types: squamous cell carcinoma, adenocarcinoma, small cell carcinoma, and large cell carcinoma (34).

The following sub aims were done In vitro to characterize the adenocarcinoma cell line hA549 to establish their stage of differentiation and sensitivity to NK cell mediated cytotoxicity, and to demonstrate induction of split anergy in NK cells.

Sub aim 1: hA549 lung tumor cell line is sensitive to NK cell mediated cytotoxicity.

Sub aim 2: IL2+ $\alpha$ CD16mAb treatment induces NK cells to undergo split energy decreasing cytotoxicity but increasing secretion of TNF- $\alpha$  and IFN- $\gamma$ .

## **Materials and Methods**

### Cell lines and Reagents

hA549 cells are human lung alveolar basal epithelial cells derived from human acinar adenocarcinoma with gland formation. The cells were cultured in DMEM (Invitrogen by Life Technologies, CA) supplemented with 3% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA), 1.4% antibiotic-antimycotic, 1% sodium pyruvate, 1.4% non-essential amino acids, 1% L-glutamine (Invitrogen by Life Technologies, CA).

### Antibodies

Antibodies against CD44, CD54, and CD16 were obtained from Biolegend (San Diego, CA).

NK cell purification kits were purchased from Stem Cell Technologies (Vancouver, Canada)

### Purification of Natural Killer Cells

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from blood donors and all procedures were approved by the UCLA-IRB. The Ficoll-Hypaque technique was used to fractionate the blood, and the buffy layer was harvested, washed and re-suspended in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% FBS. Non-adherent human peripheral blood lymphocytes were obtained by sequential incubation

on tissue culture dishes. NK cells were negatively selected and isolated using the EasySep® Human NK cell enrichment kit purchased from Stem Cell Technologies (Vancouver, Canada). The NK cell population was found to have a greater than 90% purity based on flow cytometric analysis of anti-CD16 antibody stained cells.

Purified NK cells were cultured in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% FBS (Gemini Bio-Products, CA), 1% antibiotic antimycotic, 1% sodium pyruvate, and 1% nonessential amino acids (Invitrogen by Life Technologies, CA).

### Surface Staining

Staining was done by labeling cells with PE conjugated antibodies against isotype control, CD44, CD54, B7H1, MHC 1 and the levels of surface expression were determined by flow cytometry analysis.

### <sup>51</sup>Chromium release cytotoxicity assay

NK cell cytotoxic function in the experimental cultures and the sensitivity of the target cells to NK cell mediated lysis were determined using the <sup>51</sup>Chromium release cytotoxicity assay. Different numbers of purified NK cells were incubated with <sup>51</sup>Chromium labeled target cells. After a 4-hour incubation period, each supernatant was harvested from each sample and counted for released radioactivity using a gamma-counter. The percentage specific cytotoxicity was calculated as followed

$$\text{Percent cytotoxicity} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Total cpm} - \text{Spontaneous cpm}}$$

Lytic unit  $30/10^6$  was calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells X 100.

### ELISA

ELISA kit was purchased from Biolegend (San Diego, CA). ELISA was done to detect IFN- $\gamma$  and TNF- $\alpha$ . 96 well plates were coated with detection antibody corresponding to target cytokine and incubated overnight at 4-8°. After overnight incubation the plate was washed 4 times with 0.05% Tween in 1xPBS. The plate was blocked for 1hour with 1% BSA in 1xPBS followed by washing 4 times with 0.05% Tween in 1xPBS. 100uL of standard and sample were added in duplicate wells and incubated with shaking for 2 hours. Plates were washed 4 times and 100uL of detection antibody was added for 1 hour with shaking. After washing, 100uL of Avidi-HRP was added for 30 minutes followed by another wash and addition of 100uL of TMB substrate solution. After positive wells turned blue, 100 uL of stop solution was added and the absorbance was read at 450nm.



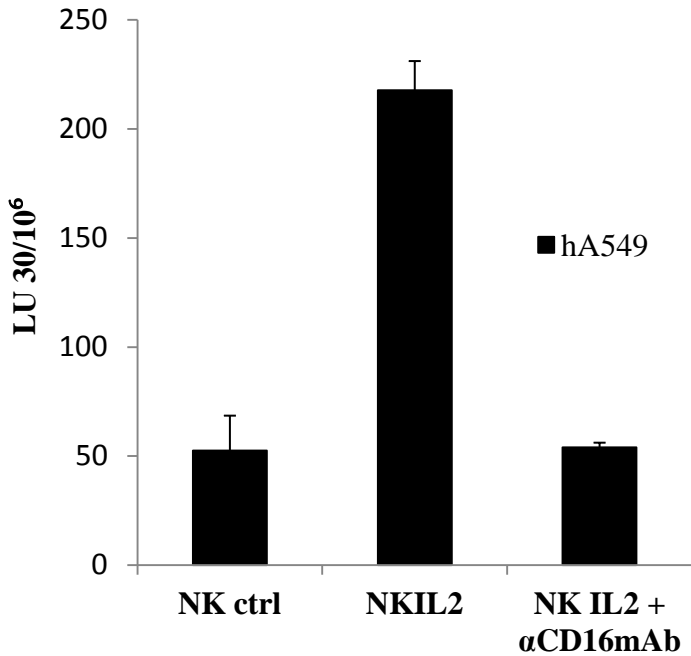
## Results

1. hA549 lung tumor cell line is sensitive to NK cell mediated cytotoxicity.
2. IL2+ $\alpha$ CD16mAb treatment induces NK cells to undergo split energy decreasing cytotoxicity but increasing secretion of TNF- $\alpha$  and IFN- $\gamma$ .

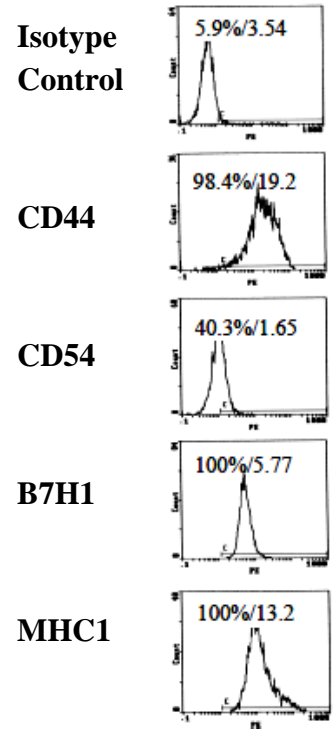
The lung adenocarcinoma hA549 cells were screened to determine sensitivity to NK cell mediated cytotoxicity. Control NK cells which have not been activated are not very cytotoxic against hA549. hA549 cells co-cultured with IL2 activated NK cells for four hours were found to be significantly lysed by NK cells (Figure 1). hA549 were found to express CD44, B7H1, MHC1 and very low CD54 on their surface (Figure 2).

The following experiments were done to demonstrate induction of split energy in NK cells. NK cells that were not activated have low cytotoxicity, very low secretion of TNF- $\alpha$ , and almost no secretion of IFN- $\gamma$  (Figure 3 and 4). Activation of NK cells by IL2 significantly increases their cytotoxic function, however, secretion of TNF- $\alpha$  and IFN- $\gamma$  is low (Figure 3 and 4). Treatment of NK cells with IL-2 + $\alpha$ CD16mAb induced split energy, abolishing their ability to lyse hA549 (Figure 3). Furthermore, decrease in cytotoxic function of IL-2 + $\alpha$ CD16mAb treated NK cells was correlated with a significant increase in secretion of TNF- $\alpha$  and IFN- $\gamma$  compared to untreated and IL2 treated NK cells (Figure 4).

1



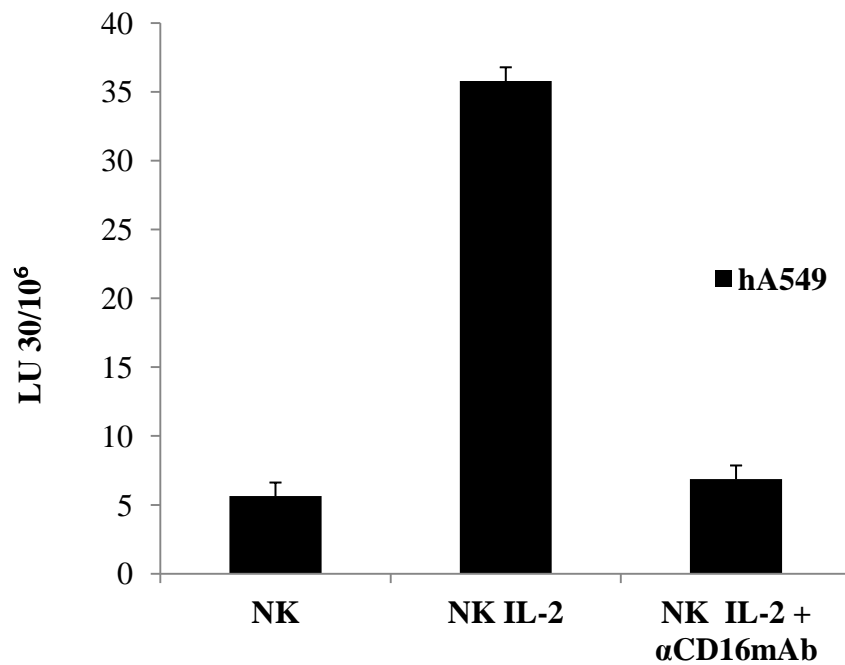
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**Figure 1 and 2.** hA549 were sensitive to NK cell mediated cytotoxicity and were found to express CD44, B7H1, and MHC1 but very low CD54.

NK cells were untreated and IL2 (1000U/mL) treated overnight before they were used to measure NK cell cytotoxicity against Cr51 labeled hA549 cells. After 4 hours of incubation of NK cells with hA549 the radioactivity released in the supernatants were counted by the gamma counter and the levels of cytotoxicity were determined using LU 30/10<sup>6</sup>. LU 30/10<sup>6</sup> denotes the number of NK cell effectors needed to lyse 30% of hA549. hA549 were stained using PE conjugated antibodies against isotype control, CD44, CD54, B7H1 and MHC 1 and the levels of surface expression were determined by flow cytometry analysis.

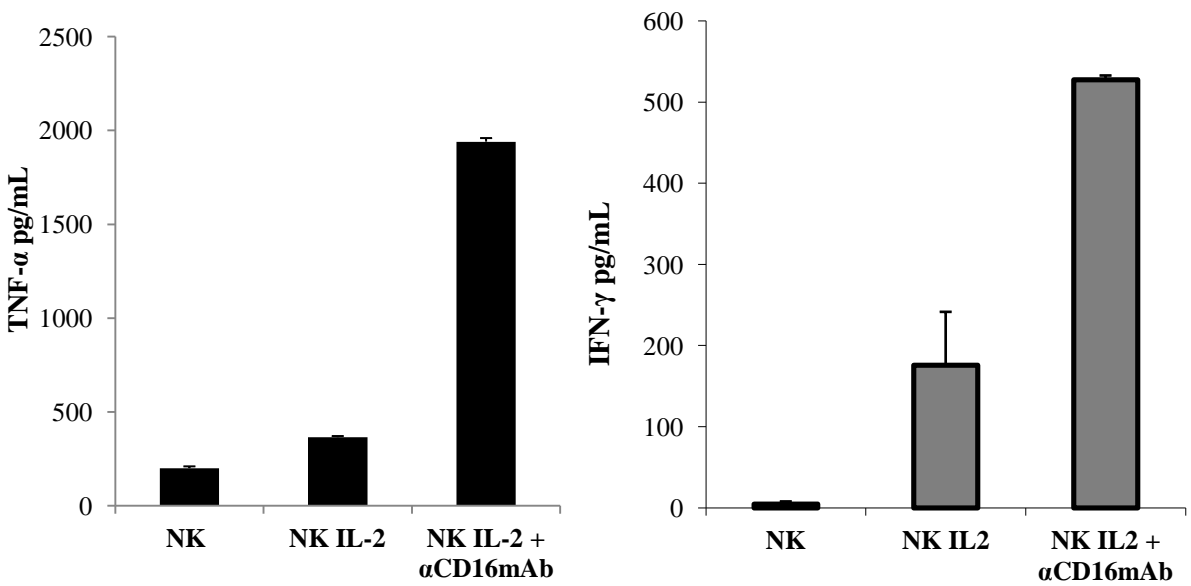
3



**Figure 3:** NK cells treated with IL-2 + $\alpha$ CD16mAb lost cytotoxic function.

NK cells were untreated, IL2 (1000U/mL), and IL2 (1000U/mL) +  $\alpha$ CD16mAb (2.5 $\mu$ g/mL) treated overnight before they were used to measure NK cell cytotoxicity against Cr51 labeled hA549 cells. After 4 hours of incubation of NK cells with hA549 radioactivity released in the supernatants were counted by gamma counter and the levels of cytotoxicity were determined using LU 30/10<sup>6</sup>. LU 30/10<sup>6</sup> denotes the number of NK cell effectors needed to lyse 30% of hA549.

4



**Figure 4:** Treatment of NK cells with IL-2 +αCD16mAb increases secretion of TNF-α and IFN-γ.

NK cells were untreated, IL2 (1000U/mL) treated, and IL2 (1000U/mL) +αCD16mAb (2.5µg/mL) treated overnight. Afterwards, the supernatants were harvested and tested for TNF-α and IFN-γ presence by ELISA.

## **Discussion**

We have characterized the interaction lung cancer cell line hA549 with NK cells and identified key characteristics which could distinguish them between differentiated NK resistant tumors and undifferentiated NK sensitive tumors. hA549 were found to be significantly lysed by NK cells when co-cultured together. Furthermore, increased expression of CD44, B7H1, MHC 1, and very low CD54 in hA549 was found to correlate with sensitivity to NK cell mediated cytotoxicity. CD44 is a stem cell marker found to be significantly higher on poorly differentiated cells. In previous studies it was found OSCSC's were more significantly lysed by NK cells compared to their differentiated counter parts OSCCs. In addition, high expression of CD44 in OSCSCs was correlated with sensitivity to NK cell mediated cytotoxicity. Similar results were seen in hESCs, hiPSCs, hDPSCs, and hMSCs in which they were lysed more significantly than their differentiated counterparts (20). These results indicate a significant correlation between the stage of differentiation of tumors and the level of NK cell mediated lysis. Stem cells and terminally differentiated cells are the two extremes of cell differentiation; however, there are cells that can fall in between. As cells become differentiated they decrease expression of CD44, but increase expression of B7H1, CD54, and MHC (20). Our hA549 cells have expression of CD44, but it is not as high as what was seen with OSCSCs. Furthermore, hA549 express B7H1, which is very low on OSCSCs (20), and MHC1, yet they are very sensitive to NK cell mediated cytotoxicity. Based on this data, we conclude that hA549 cells lie between the two extremes of cell differentiation closer to poorly differentiated cells.

In previous studies and our current study, it has been shown that treatment of NK cells with IL-2 and anti-CD16mAb induces split anergy by decreasing cytotoxicity and increasing cytokine secretion. Our results confirm when NK cells become anergized through treatment of

IL2 + anti-CD16 they lose cytotoxicity against hA549 cells compared to those treated with IL2. The loss of cytotoxicity in energized NK cells is correlated with a significant amount of IFN- $\gamma$  and TNF- $\alpha$  secretion, which is not seen in the control NK cells and those treated with IL2.

## **Conclusion**

In this part of our study we demonstrated hA549 lung tumors are very sensitive to NK cell mediated cytotoxicity and express CD44, B7H1, MHC1, but very low CD54. Based on their phenotype and NK cell mediated cytotoxicity we conclude that hA549 cells are closer to being more like poorly differentiated cells. In addition we found that induction of split anergy through IL2+ $\alpha$ CD16mAb treatment causes NK cells to lose cytotoxicity against hA549, but significantly increase secretion of TNF- $\alpha$  and IFN- $\gamma$ .

## **Chapter 2:**

*Specific Aim 2:* Anergized NK cells support the differentiation of lung cancer stem cells through secreted cytokines.

### **Introduction**

It has been found that NK's isolated from tumors are not cytotoxic to autologous tumors and NKs obtained from the peripheral blood of cancer patients have significantly reduced cytotoxicity (28, 29). Moreover, interaction of NK cells with stem cells leads to the suppression of NK cell cytotoxicity and in an increase in cytokine secretion, however, the interaction of NK cells with resistant tumors does not lead to suppression of NK cytotoxicity or increase in cytokine secretion (23-27). NK cells are conditioned to become anergized through signals from sensitive tumors or healthy untransformed stem cells (25-31). When NK cells become anergized they lose cytotoxicity, but gain the ability to secrete significant amounts of cytokines. Anergized NK cells have been shown to secrete high levels of IFN- $\gamma$  (7, 21). Treatment of IFN- $\gamma$  has been found to protect primary ovarian tumors from lysis by cytotoxic T lymphocytes CTLs, but this has been attributed to alterations on the CTLs (35).

In this study and previous studies by our lab we have demonstrated that treatment of IL2+ $\alpha$ CD16mAb induces split energy in NK cell causing them to lose cytotoxicity but increasing cytokine secretion of TNF- $\alpha$  and IFN- $\gamma$ . We hypothesize that these alterations in effector function of NK cells assist in driving differentiation of lung tumors, thereby, making them resistant to NK cell mediated cytotoxicity. In cancer the generation and maintenance of cancer stem cells is higher, therefore, the majority of NK cells may be conditioned to become anergized

in order to support differentiation and repair of the tissues reducing tumor induced inflammation (23).

The following sub aims were demonstrated by In Vitro assays to show that anergized NK cells support the differentiation of lung tumors through secretion of cytokines.

Sub Aim 1: NK IL2+ $\alpha$ CD16mAb supernatants support the differentiation of lung tumors increasing resistance to NK cell mediated cytotoxicity.

Sub Aim 2: Addition of antibodies against both TNF- $\alpha$  and IFN- $\gamma$  blocked differentiation induced by anergized NK supernatants.

Sub Aim 3: TNF- $\alpha$  and IFN- $\gamma$  secreted by anergized NK cells induce differentiation of lung tumors.

Sub Aim 4: Anergized NK cells support the differentiation of healthy stem cells SCAP increasing resistance to NK cell mediated cytotoxicity.

Sub Aim 5: IFN- $\gamma$  production by NK cells is increased in co-cultures with Monocytes and bacteria.

## **Material and Methods**

### Cell lines and Reagents

hA549 cells are human lung alveolar basal epithelial cells derived from human acinar adenocarcinoma with gland formation. The cells were cultured in DMEM (Invitrogen by Life Technologies, CA) supplemented with 3% Fetal Bovine Serum (FBS) (Gemini Bio-Products,



CA), 1.4% antibiotic-antimycotic, 1% sodium pyruvate, 1.4% non-essential amino acids, 1% L-glutamine (Invitrogen by Life Technologies, CA). Stem cells of the apical papilla (SCAP) is a type of Mesenchymal stem cell (MSC). The cells were cultured in DMEM (Invitrogen by Life Technologies, CA) supplemented with 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA), 1.4% antibiotic-antimycotic, 1% sodium pyruvate, 1.4% non-essential amino acids, 1% L-glutamine (Invitrogen by Life Technologies, CA).

### Antibodies

Antibodies against CD44, CD54, MHC class 1, B7H1 and CD16 were obtained from Biolegend (San Diego, CA).

NK cell purification kits were purchased from Stem Cell Technologies (Vancouver, Canada)

### Bacteria

AJ2 is a bacteria cocktail made by Dr. Anahid Jewett.

### Purification of Natural Killer Cells

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from blood donors and all procedures were approved by the UCLA-IRB. The Ficoll-Hypaque technique was used to fractionate the blood, and the buffy layer was harvested, washed and re-suspended in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% FBS. Non-adherent human peripheral blood lymphocytes were obtained by sequential incubation on tissue culture dishes. NK cells were negatively selected and isolated using the EasySep® Human NK cell enrichment kit purchased from Stem Cell Technologies (Vancouver, Canada).

The NK cell population was found to have a greater than 90% purity based on flow cytometric analysis of anti-CD16 antibody stained cells.

The adherent subpopulation of PBMCs was detached from tissue culture plate after a one-hour incubation. The total population of monocytes (CD16+) was purified using an EasySep® purification kit purchased from Stem Cell Technologies (Vancouver, Canada). Monocyte purity greater than 95% was obtained, and confirmed with flow cytometric analysis of CD14 and CD16 stained monocytes.

Purified NK cells and Monocytes were cultured in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% FBS (Gemini Bio-Products, CA), 1% antibiotic antimycotic, 1% sodium pyruvate, and 1% nonessential amino acids (Invitrogen by Life Technologies, CA).

### Surface Staining

Staining was done by labeling cells with PE conjugated antibodies against isotype control, CD44, CD54, MHC class 1, and B7H1 and the levels of surface expression were determined by flow cytometry analysis. Cell death was analyzed by staining cells with propidium iodide (PI).

### <sup>51</sup>Chromium release cytotoxicity assay

NK cell cytotoxic function in the experimental cultures and the sensitivity of the target cells to NK cell mediated lysis were determined using the <sup>51</sup>Chromium release cytotoxicity assay. Different numbers of purified NK cells were incubated with <sup>51</sup>Chromium labeled target

cells. After a 4-hour incubation period, each supernatant was harvested from each sample and counted for released radioactivity using a gamma-counter. The percentage specific cytotoxicity was calculated as followed

$$\text{Percent cytotoxicity} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Total cpm} - \text{Spontaneous cpm}}$$

Lytic unit  $30/10^6$  was calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells X 100.

## Results

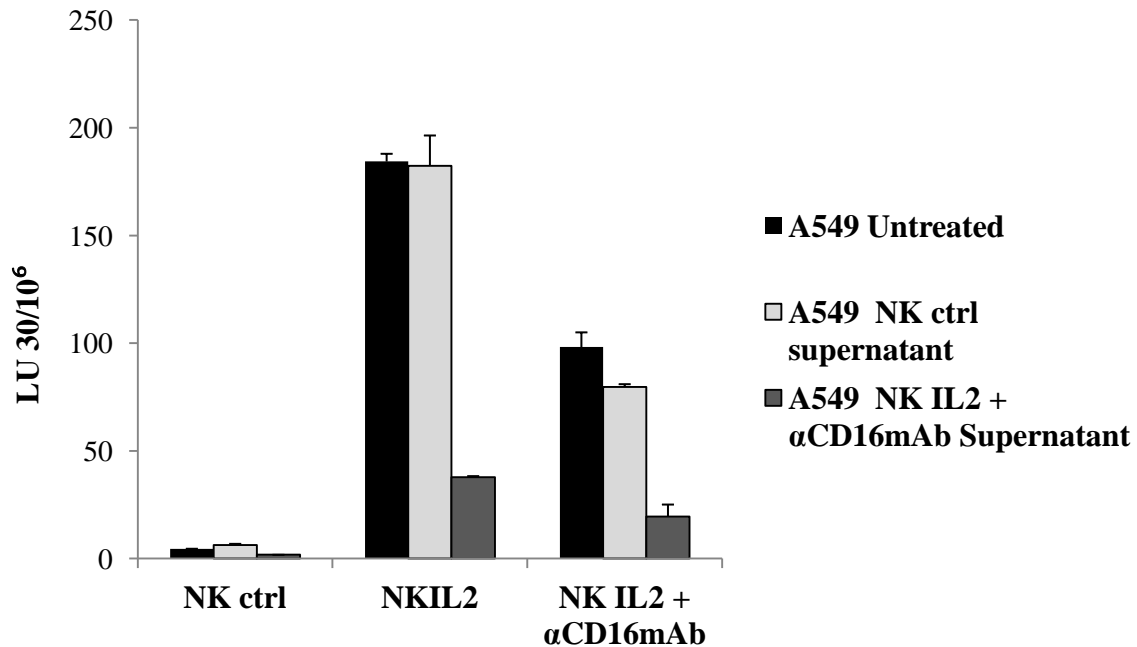
1. NK IL2+ $\alpha$ CD16mAb supernatants support the differentiation of lung tumors increasing resistance to NK cell mediated cytotoxicity.
2. Addition of antibodies against both TNF- $\alpha$  and IFN- $\gamma$  blocked differentiation induced by energized NK supernatants.

NK cells were untreated or energized with treatment of IL2+ $\alpha$ CD16mAb overnight and the supernatants were collected. hA549 cells treated with supernatants from untreated NK cells had similar sensitivity levels as untreated hA549 (Figure 5). In addition, expression of CD44, CD54, B7H1, and MHC1 did not change compared to untreated hA549 (Figure 6). Treatment of hA549 with NK IL2 +  $\alpha$ CD16mAb supernatant induced differentiation of hA549 increasing resistance to NK cell mediated cytotoxicity (Figure 5). NK IL2 +  $\alpha$ CD16mAb supernatant increased expression of MHC1 and B7H1 but only a slight change in CD54 was seen and no change in CD44 expression (Figure 6).

Because it was seen that treatment of NK cells with IL2 +  $\alpha$ CD16 significantly increased secretion of TNF- $\alpha$  and IFN- $\gamma$  (Figure 4), we tested what would happen if we added antibodies against these cytokines. Addition of an antibody against TNF- $\alpha$  alone had a small effect in blocking differentiation and only slightly increased resistance to NK cell mediated cytotoxicity (Figure 7). Expression of MHC1 and B7H1 decreased compared to NK IL-2 +  $\alpha$ CD16mAb treated cells, but expression levels remain high when compared to untreated hA549 (Figure 8). Addition of an antibody against IFN- $\gamma$  alone further increased resistance to NK cell mediated cytotoxicity but not to the extent seen when antibodies against both TNF- $\alpha$  and IFN- $\gamma$  were added to NK IL2 +  $\alpha$ CD16mAb supernatant treated cells (Figure 7). In addition, there was a

decrease in expression of MHC1 and B7H1 when compared to NK IL2 +  $\alpha$ CD16mAb supernatant treated cells as well as NK IL2 +  $\alpha$ CD16mAb supernatant+  $\alpha$ TNF- $\alpha$ mAb treated cells (Figure 8). Addition of both  $\alpha$ TNF- $\alpha$ mAb+  $\alpha$ IFN- $\gamma$ mAb to hA549 treated with NK IL2 +  $\alpha$ CD16mAb supernatant blocked differentiation of hA549 (Figure 7). Blocking caused hA549 to become more sensitive to NK cell mediated cytotoxicity similar to untreated hA549 (Figure 7). Addition of both  $\alpha$ TNF- $\alpha$ mAb+ $\alpha$ IFN- $\gamma$ mAb decreased expression of CD44, MHC1, and B7H1 compared to hA549 cells treated with NK IL2 +  $\alpha$ CD16mAb supernatant (Figure 8). Treatment with NK IL2 +  $\alpha$ CD16 induced less than 10% cell death in hA549 cells (Figure 9).

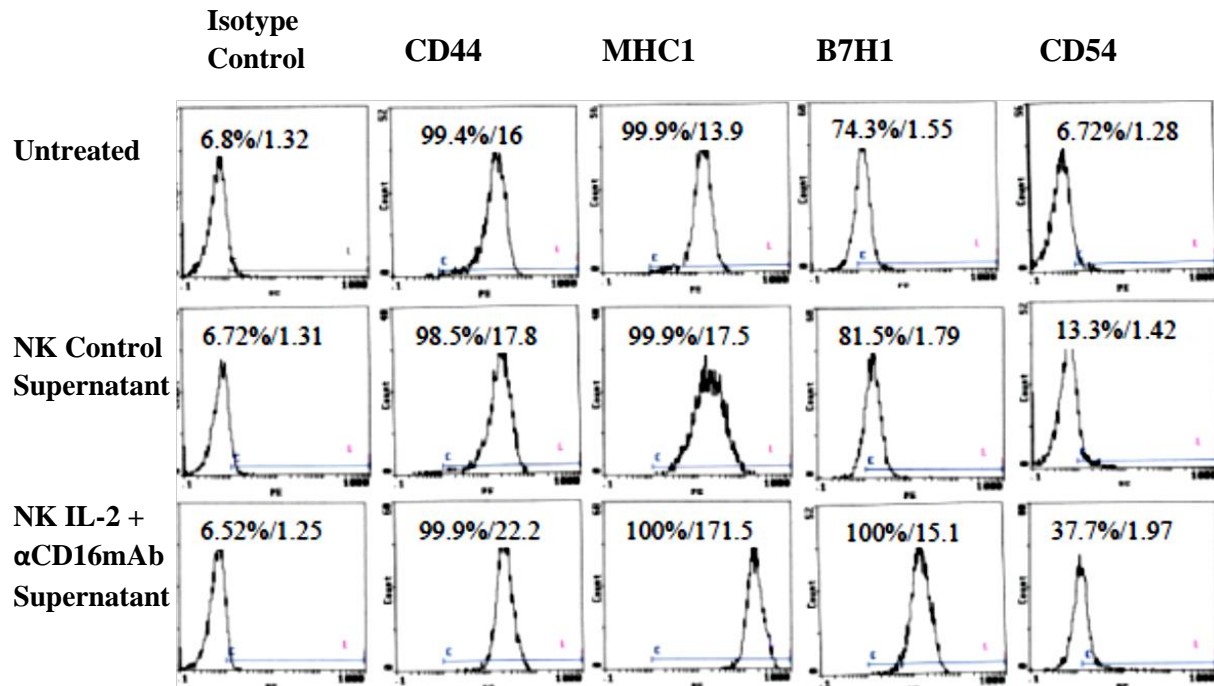
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**Figure 5.** Anergized NK supernatants induced differentiation of hA549 increasing resistance to NK cell mediated cytotoxicity.

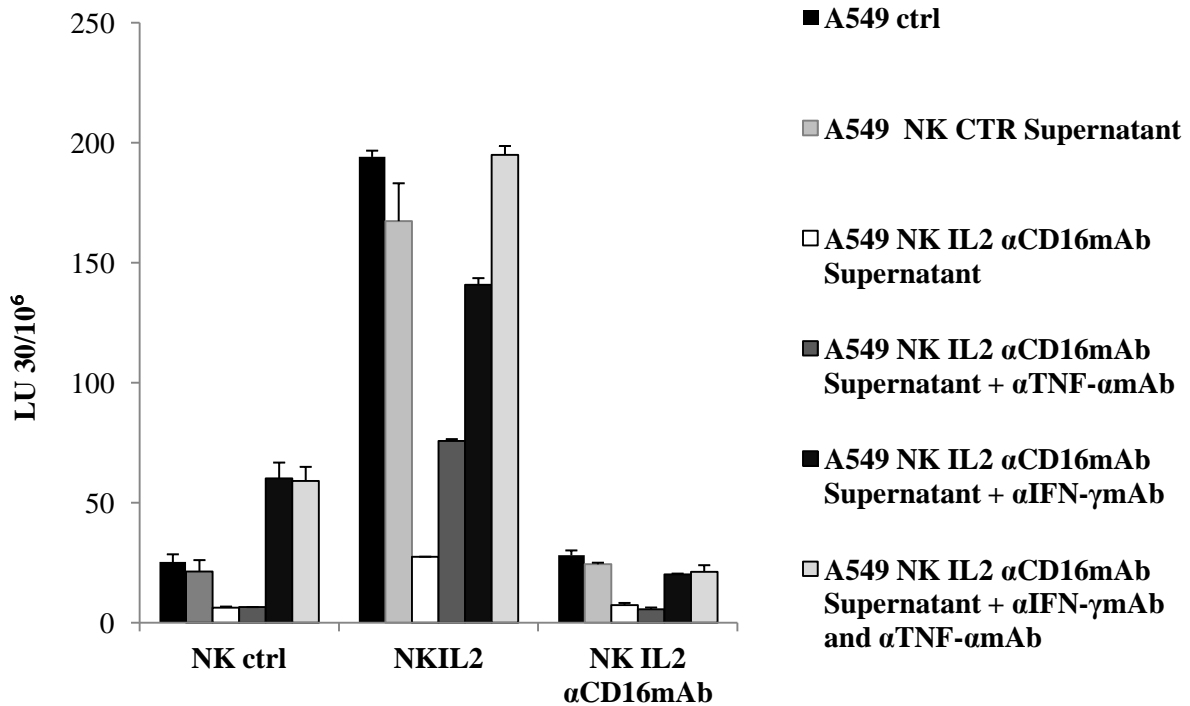
hA549 cells were untreated, treated with supernatants from NK control, or supernatants from NK IL2(1000U/mL) + αCD16mAb(2.5μg/mL) for six days. NK cells were untreated, IL2 (1000U/mL), and IL2(1000U/mL) + αCD16mAb(2.5μg/mL) treated overnight before they were used to measure NK cell cytotoxicity against Cr51 labeled hA549 cells. After 4 hours of incubation of NK cells with hA549 radioactivity released in the supernatants were counted by the gamma counter and the levels of cytotoxicity were determined using LU 30/10<sup>6</sup>. LU 30/10<sup>6</sup> denotes the number of NK cell effectors needed to lyse 30% of hA549.

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**Figure 6.** Differentiation of hA549 by energized NK supernatants increased surface expression of MHC1, B7H1 and CD54, but no change in CD44.

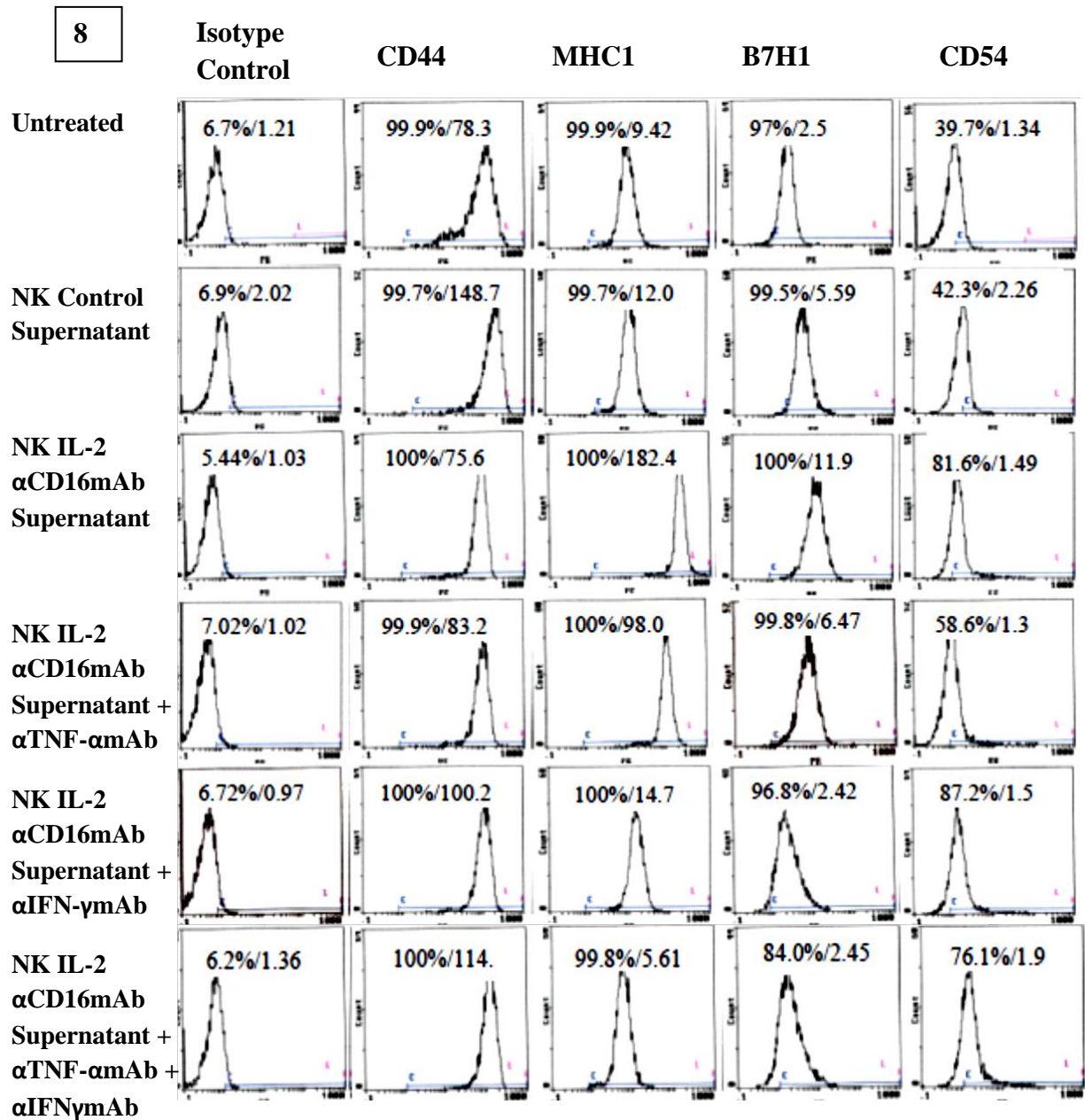
hA549 cells were untreated, treated with supernatants from NK control, or supernatants from NK IL2(1000U/mL) +  $\alpha$ CD16mAb(2.5 $\mu$ g/mL) for six days. hA549 were stained using PE conjugated antibodies against isotype control, CD44, MHC1, B7H1, and CD54 and the levels of surface expression were determined by flow cytometry analysis.



**Figure 7.** Addition of  $\alpha$ TNF- $\alpha$ mAb+  $\alpha$ IFN- $\gamma$ mAb blocked differentiation of hA549 induced by energized NK supernatants.

hA549 cells were untreated, treated with supernatants from NK control, supernatants from NK IL2(1000U/mL) +  $\alpha$ CD16mAb(2.5 $\mu$ g/mL), and supernatants NK IL2(1000U/mL) +  $\alpha$ CD16mAb(2.5 $\mu$ g/mL) plus the addition of  $\alpha$ TNF- $\alpha$ mAb (1:100),  $\alpha$ IFN- $\gamma$ mAb(1:100), and  $\alpha$ TNF- $\alpha$ mAb(1:100) +  $\alpha$ IFN- $\gamma$ mAb(1:100) for six days. NK cells were untreated, IL2 (1000U/mL), and IL2 (1000U/mL) +  $\alpha$ CD16mAb (2.5 $\mu$ g/mL) treated overnight before they were used to measure NK cell cytotoxicity against Cr51 labeled hA549 cells. After 4 hours of incubation of NK cells with hA549 radioactivity released in the supernatants were counted by the gamma counter and the levels of cytotoxicity were determined using LU 30/10<sup>6</sup>. LU 30/10<sup>6</sup> denotes the number of NK cell effectors needed to lyse 30% of hA549.





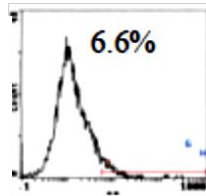
**Figure 8.** Addition of  $\alpha$ TNF- $\alpha$ Ab +  $\alpha$ IFN- $\gamma$ Ab to hA549 treated with energized NK supernatants was correlated with a decrease in MHC, B7H1 and CD54, but no change in CD44.

hA549 cells were untreated, treated with supernatants from NK control, supernatants from NK IL2(1000U/mL) +  $\alpha$ CD16mAb(2.5 $\mu$ g/mL), and supernatants NK IL2(1000U/mL) +  $\alpha$ CD16mAb(2.5 $\mu$ g/mL) plus the addition of  $\alpha$ TNF- $\alpha$ Ab (1:100),  $\alpha$ IFN- $\gamma$ Ab(1:100), and  $\alpha$ TNF- $\alpha$ Ab(1:100) +  $\alpha$ IFN- $\gamma$ Ab (1:100) for six days. hA549 were stained using PE conjugated antibodies against isotype control, CD44, MHC1, B7H1, and CD54 and the levels of surface expression were determined by flow.

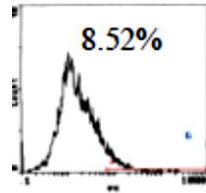
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PI

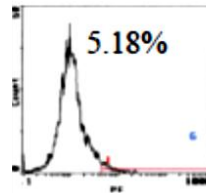
Untreated



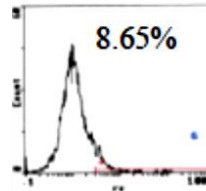
NK Control Supernatant



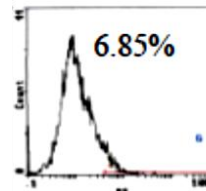
NK IL-2  $\alpha$ CD16mAb  
Supernatant



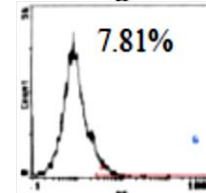
NK IL-2  $\alpha$ CD16mAb  
Supernatant +  $\alpha$ TNF- $\alpha$ mAb



NK IL-2  $\alpha$ CD16mAb  
Supernatant +  $\alpha$ IFN- $\gamma$ mAb



NK IL-2  $\alpha$ CD16mAb  
Supernatant +  $\alpha$ TNF- $\alpha$ mAb +  
 $\alpha$ IFN $\gamma$ mAb

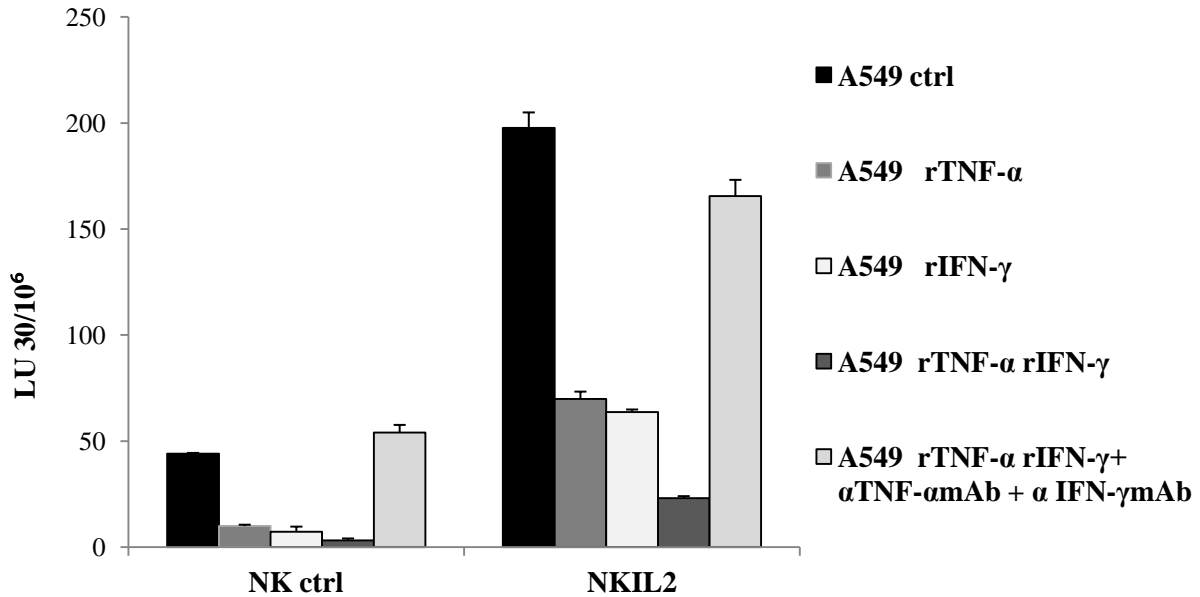


**Figure 9.** Addition of NK IL2 +  $\alpha$ CD16mAb supernatants did not induce cell death in hA549.

hA549 cells were untreated, treated with supernatants from NK control, supernatants from NK IL2(1000U/mL) +  $\alpha$ CD16mAb(2.5 $\mu$ g/mL), and supernatants NK IL2(1000U/mL) +  $\alpha$ CD16mAb(2.5 $\mu$ g/mL) plus the addition of  $\alpha$ TNF- $\alpha$ mAb (1:100),  $\alpha$ IFN- $\gamma$ mAb(1:100), and  $\alpha$ TNF- $\alpha$ mAb(1:100) +  $\alpha$ IFN- $\gamma$ mAb (1:100) for six days. hA549 were stained using PI and the levels of cell death were determined by flow cytometry.

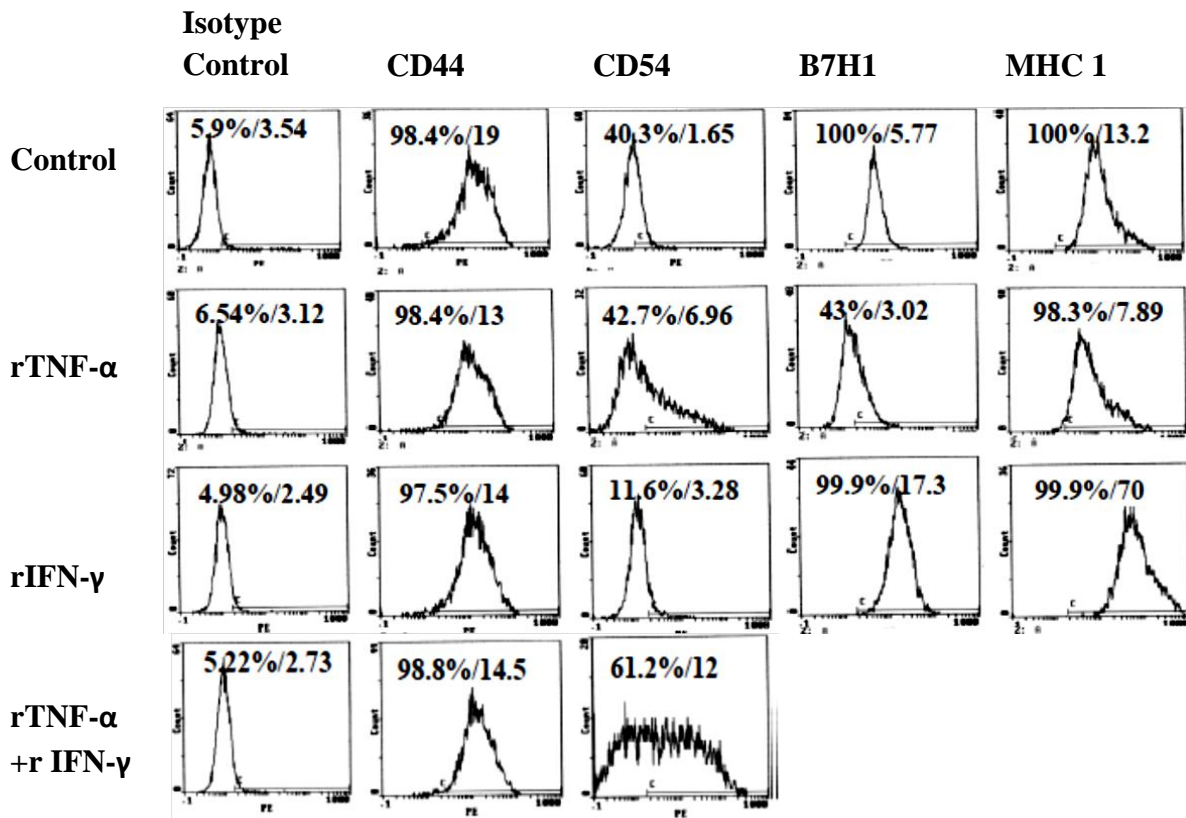
3. TNF- $\alpha$  and IFN- $\gamma$  secreted by anergized NK cells induce differentiation of lung tumors.

We found that addition of antibodies against both TNF- $\alpha$  and IFN- $\gamma$  blocked differentiation induced by NK IL2 + $\alpha$ CD16mAb supernatants, suggesting that TNF- $\alpha$  and IFN- $\gamma$  play an important role in inducing differentiation (Figure 7). To further confirm TNF- $\alpha$  and IFN- $\gamma$  are implicated in inducing differentiation of lung cancer cells we treated hA549 with recombinant TNF- $\alpha$  and IFN- $\gamma$ . hA549 cells treated with rTNF- $\alpha$  were pushed to differentiate becoming resistant to NK cell mediated cytotoxicity (Figure 10). Treatment of hA549 with rIFN- $\gamma$  slightly increased their resistance compared to rTNF- $\alpha$  treatment (Figure 10). The combination of both rTNF- $\alpha$  and rIFN- $\gamma$  worked synergistically to further increase resistance to NK cell mediated cytotoxicity when compared to rTNF- $\alpha$  alone or rIFN- $\gamma$  alone (Figure 10). Similar results were seen with treatment of anergized NK supernatants (Figure 5). Addition of antibodies against TNF- $\alpha$  and IFN- $\gamma$  to hA549 cells treated with both rTNF- $\alpha$  and rIFN- $\gamma$  blocked differentiation causing them to become sensitive to NK cell mediated cytotoxicity similar to the control (Figure 10). The same was seen when we added antibodies against both TNF- $\alpha$  and IFN- $\gamma$  to hA549 treated with anergized NK supernatants (Figure 7). hA549 cells treated with rTNF- $\alpha$  increased expression of CD54, but no change was seen in CD44, MHC 1, and B7H1 surface expression (Figure 11). rIFN- $\gamma$  significantly increased expression of MHC 1 and B7H1 but had no effect on the expression of CD44 and CD54. The combination of both rTNF- $\alpha$  and rIFN- $\gamma$  further increased the expression of CD54 while no change was seen in CD44 expression (Figure 11).



**Figure 10:** rTNF- $\alpha$  and rIFN- $\gamma$  induced differentiation of hA549 increasing resistance to NK cell mediated cytotoxicity and addition of antibodies against TNF- $\alpha$  and IFN- $\gamma$  blocked differentiation.

hA549 cells were treated with 20ng/mL rTNF- $\alpha$ , 200U/mL rIFN- $\gamma$ , a combination of both, or 20ng/mL rTNF- $\alpha$  + 200U/mL rIFN- $\gamma$  + antibodies against rTNF- $\alpha$  and rIFN- $\gamma$  for 24 hours. NK cells were untreated and IL2 (1000U/mL) treated overnight before they were used to measure NK cell cytotoxicity against Cr51 labeled hA549 cells. After 4 hours of incubation of NK cells with hA549 radioactivity released in the supernatants were counted by the gamma counter and the levels of cytotoxicity were determined using LU 30/10<sup>6</sup>. LU 30/10<sup>6</sup> denotes the number of NK cell effectors needed to lyse 30% of hA549.



**Figure 11:** Differentiation of hA549 induced by rTNF- $\alpha$  and rIFN- $\gamma$  was correlated with CD54, B7H1, and MHC 1 and no change in CD44.

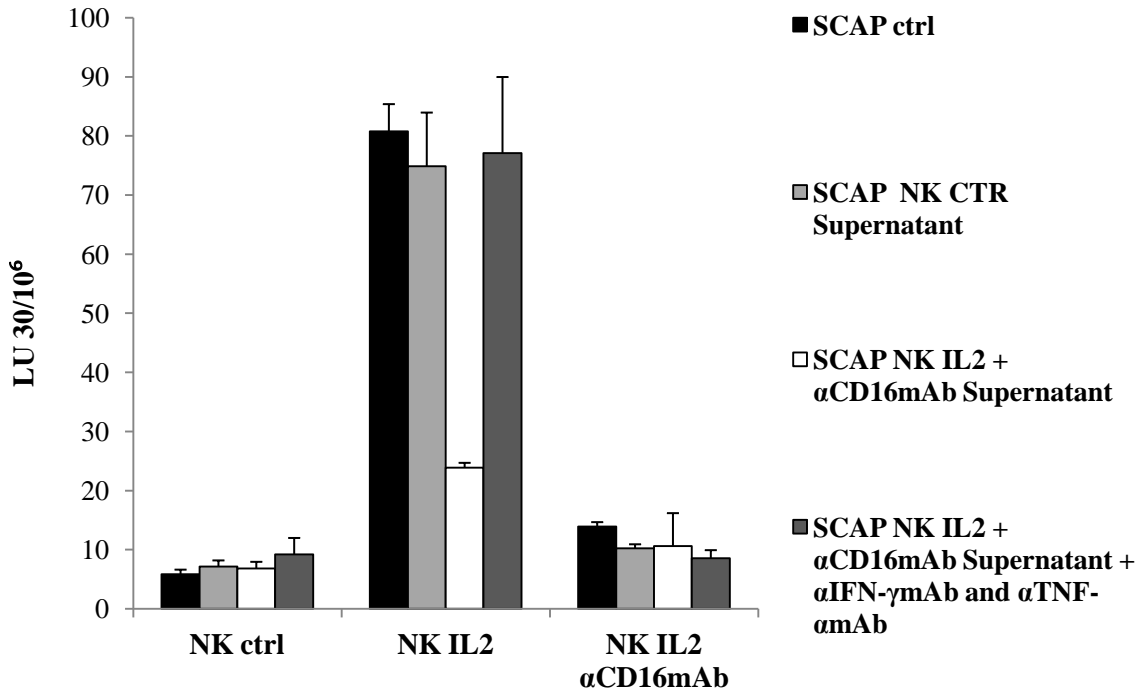
A549 cells were treated with 20ng/mL rTNF- $\alpha$  , 200U/mL rIFN- $\gamma$ , a combination of both, or 20ng/mL rTNF- $\alpha$  + 200U/mL rIFN- $\gamma$  + antibodies against TNF- $\alpha$  and IFN- $\gamma$  for 24hours. hA549 were stained using PE conjugated antibodies against isotype control , CD44, MHC1, B7H1 and CD54 and the levels of surface expression were determined by flow cytometry analysis.

4. Anergized NK cells support the differentiation of healthy stem cells SCAP increasing resistance to NK cell mediated cytotoxicity.

Treatment of SCAP cells with NK control supernatants did not induce differentiation (Figure 12). Levels of MHC1, B7H1, and CD54 remained similar to untreated SCAP cells (Figure 13). Treatment of SCAP with NK IL2 +  $\alpha$ CD16mAb supernatants induced differentiation significantly increasing resistance to NK cell mediated cytotoxicity (Figure 12). Furthermore, addition of NK IL2 +  $\alpha$ CD16mAb supernatants increased surface expression of MHC1, B7H1, and CD54 (Figure13). Addition of antibodies against TNF- $\alpha$  and IFN- $\gamma$  to SCAP cells treated with NK IL2 +  $\alpha$ CD16mAB supernatants blocked differentiation making them more sensitive to NK cell mediated cytotoxicity (Figure 12). In addition, there was a decrease in expression of MHC1, B7H1, and CD54 similar to the levels of untreated SCAP cells (Figure 13). Addition of anergized supernatants did not induce significant cell death in SCAP cells (Figure 14).

To confirm that TNF- $\alpha$  and IFN- $\gamma$  in anergized NK supernatants were responsible for inducing differentiation of SCAP cells we treated SCAP cells with recombinant TNF- $\alpha$  and IFN- $\gamma$ . rTNF- $\alpha$  treatment of SCAP cells induced differentiation increasing resistance to NK cell mediated cytotoxicity (Figure 15). In addition, treatment of SCAP cells with rTNF- $\alpha$  increased surface expression of CD54 (Figure 16). Treatment of SCAP with rIFN- $\gamma$  further increased resistance to NK cell mediated cytotoxicity compared to rTNF- $\alpha$  treatment (Figure 15). rIFN- $\gamma$  induced differentiation was correlated with increase in CD54, MHC1, and B7H1 (Figure 16). The combination of both rTNF- $\alpha$  and rIFN- $\gamma$  worked synergistically to further increase resistance to NK cell mediated cytotoxicity when compared to rTNF- $\alpha$  alone or rIFN- $\gamma$  alone (Figure 15). Treatment of rTNF- $\alpha$  + rIFN- $\gamma$  further increased expression of CD54, MHC1, and B7H1

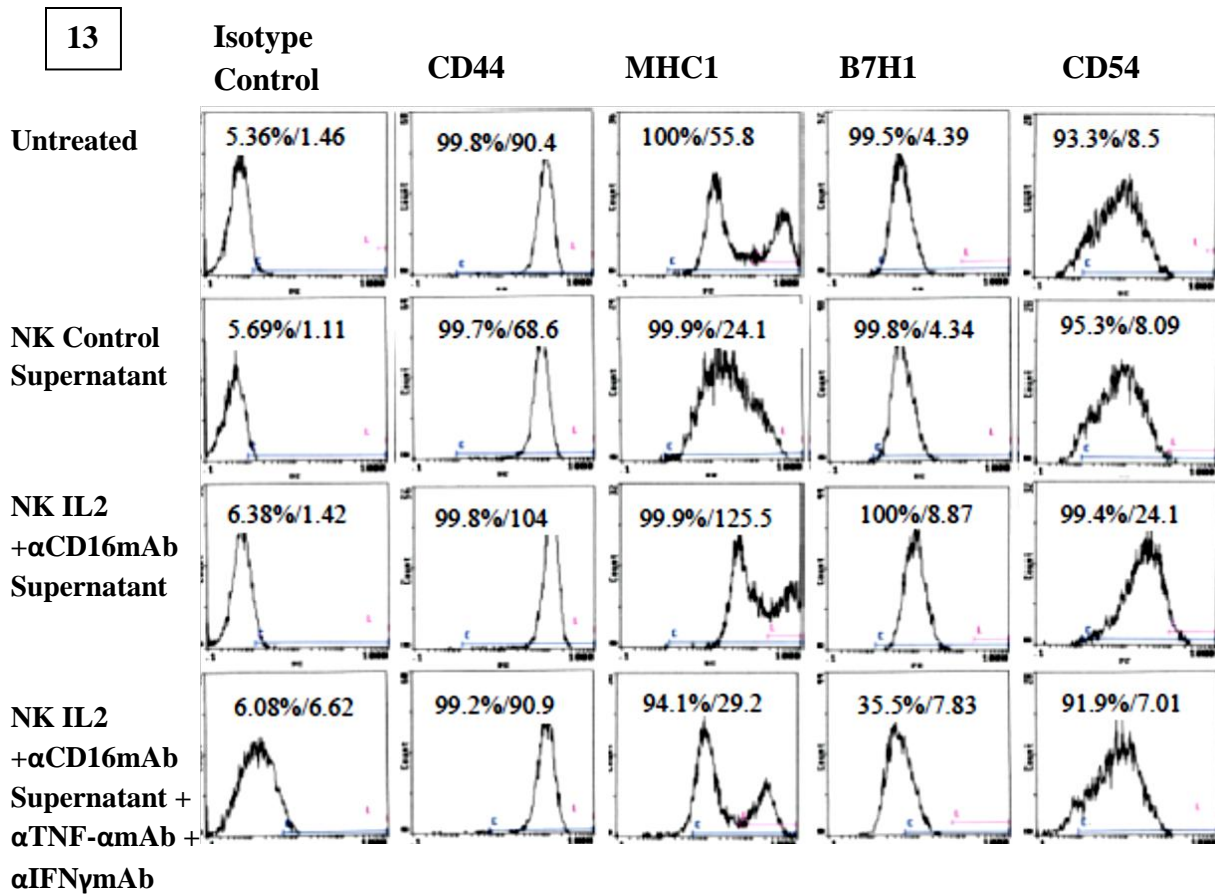
compared to rTNF- $\alpha$  and rIFN- $\gamma$  alone (Figure 16). Addition of antibodies against TNF- $\alpha$  and IFN- $\gamma$  blocked differentiation making SCAP more sensitive to NK cell mediated cytotoxicity (Figure 15). Blocking of TNF- $\alpha$  and IFN- $\gamma$  decreased the expression of CD54, MHC1, and B7H1 similar to levels in untreated cells (Figure 16).



**Figure 12.** NK IL2+ $\alpha$ CD16 supernatants induced differentiation of SCAP cells increasing resistance to NK cell mediated cytotoxicity and addition of  $\alpha$ TNF- $\alpha$ Ab +  $\alpha$ IFN- $\gamma$ Ab blocked differentiation.

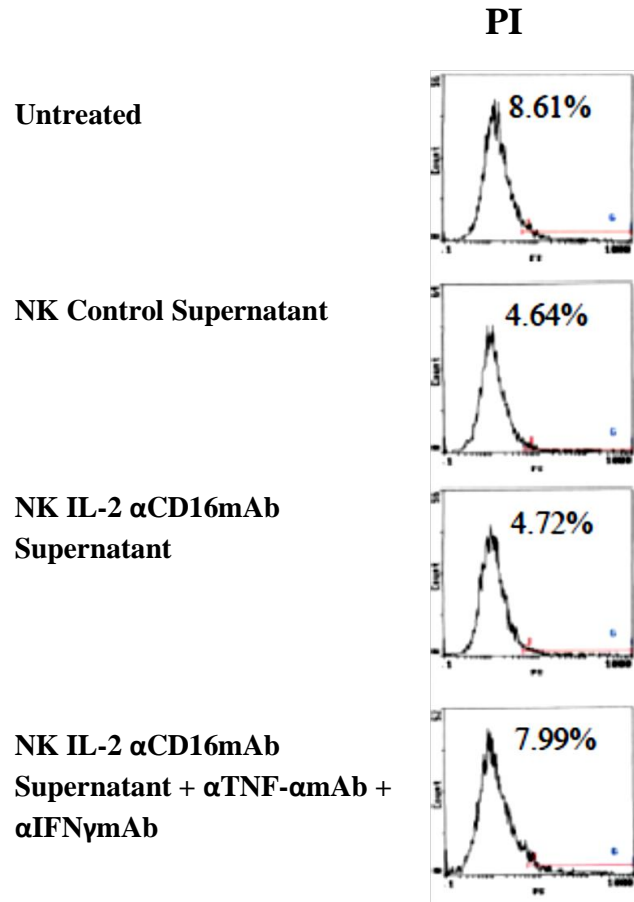
SCAP cells were untreated, treated with supernatants from NK control, supernatants from NK IL2 (1000U/mL) +  $\alpha$ CD16mAb (2.5 $\mu$ g/mL), and supernatants NK IL2 (1000U/mL) +  $\alpha$ CD16mAb (2.5 $\mu$ g/mL) plus the addition of  $\alpha$ TNF- $\alpha$ Ab (1:100) +  $\alpha$ IFN- $\gamma$ Ab (1:100) for six days. NK cells were untreated, IL2 (1000U/mL), and IL2 (1000U/mL) +  $\alpha$ CD16mAb (2.5 $\mu$ g/mL) treated overnight before they were used to measure NK cell cytotoxicity against Cr51 labeled SCAP cells. After 4 hours of incubation of NK cells with SCAP radioactivity released in the supernatants were counted by the gamma counter and the levels of cytotoxicity were determined using LU 30/10<sup>6</sup>. LU 30/10<sup>6</sup> denotes the number of NK cell effectors needed to lyse 30% of SCAP.





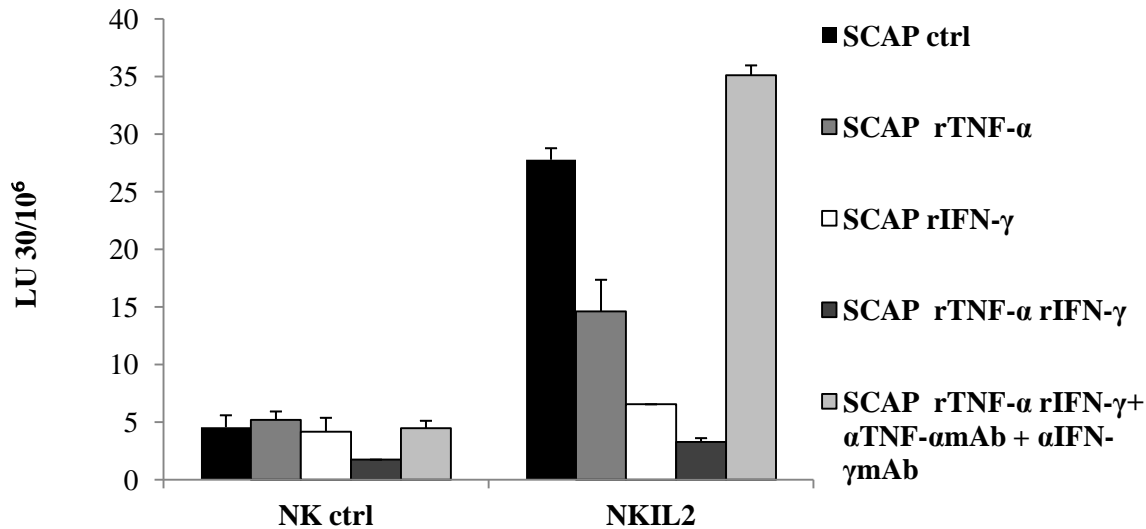
**Figure 13.** Differentiation of SCAP by anergized NK supernatants increased surface expression of MHC1, B7H1, and CD54 and no change in CD44.

SCAP cells were untreated, treated with supernatants from NK control, supernatants from NK IL2(1000U/mL) +  $\alpha$ CD16mAb(2.5 $\mu$ g/mL), and supernatants from NK IL2(1000U/mL) +  $\alpha$ CD16mAb(2.5 $\mu$ g/mL) plus the addition of antibodies against TNF- $\alpha$  and IFN- $\gamma$  for six days. SCAP were stained using PE conjugated antibodies against isotype control, CD44, MHC1, B7H1, and CD54 and the levels of surface expression were determined by flow cytometry analysis.



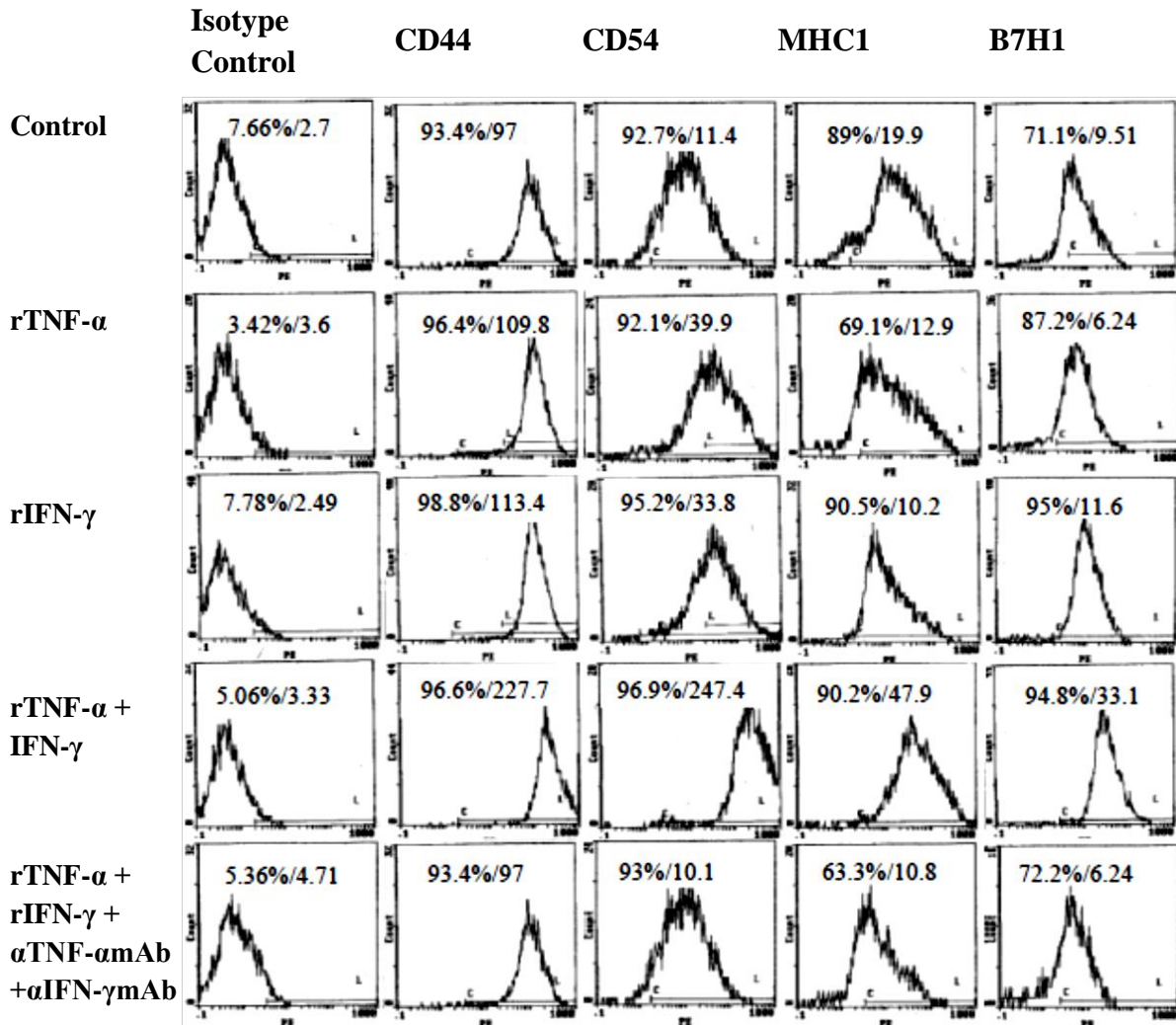
**Figure 14.** Differentiation of SCAP by anergized NK supernatants did not induce cell death.

SCAP cells were untreated, treated with supernatants from NK control, supernatants from NK IL2(1000U/mL) + αCD16mAb(2.5μg/mL), and supernatants from NK IL2(1000U/mL) + αCD16mAb(2.5μg/mL) plus the addition of antibodies against TNF-α and IFN-γ for six days. SCAP were stained using PI and the levels of cell death were determined by flow cytometry analysis.



**Figure 15.** rTNF- $\alpha$  and rIFN- $\gamma$  induced differentiation of SCAP increasing resistance to NK cell mediated cytotoxicity and addition of  $\alpha$ TNF- $\alpha$ mAb+ $\alpha$ IFN- $\gamma$ mAb blocked differentiation.

SCAP cells were treated with 20ng/mL rTNF- $\alpha$  , 200U/mL rIFN- $\gamma$ , a combination of both, or 20ng/mL rTNF- $\alpha$  + 200U/mL rIFN- $\gamma$  + antibodies against TNF- $\alpha$  and IFN- $\gamma$  for 24 hours. NK cells were untreated and IL2 (1000U/mL) treated overnight before they were used to measure NK cell cytotoxicity against Cr51 labeled SCAP cells. After 4 hours of incubation of NK cells with SCAP radioactivity released in the supernatants were counted by the gamma counter and the levels of cytotoxicity were determined using LU 30/10<sup>6</sup>. LU 30/10<sup>6</sup> denotes the number of NK cell effectors needed to lyse 30% of SCAP.



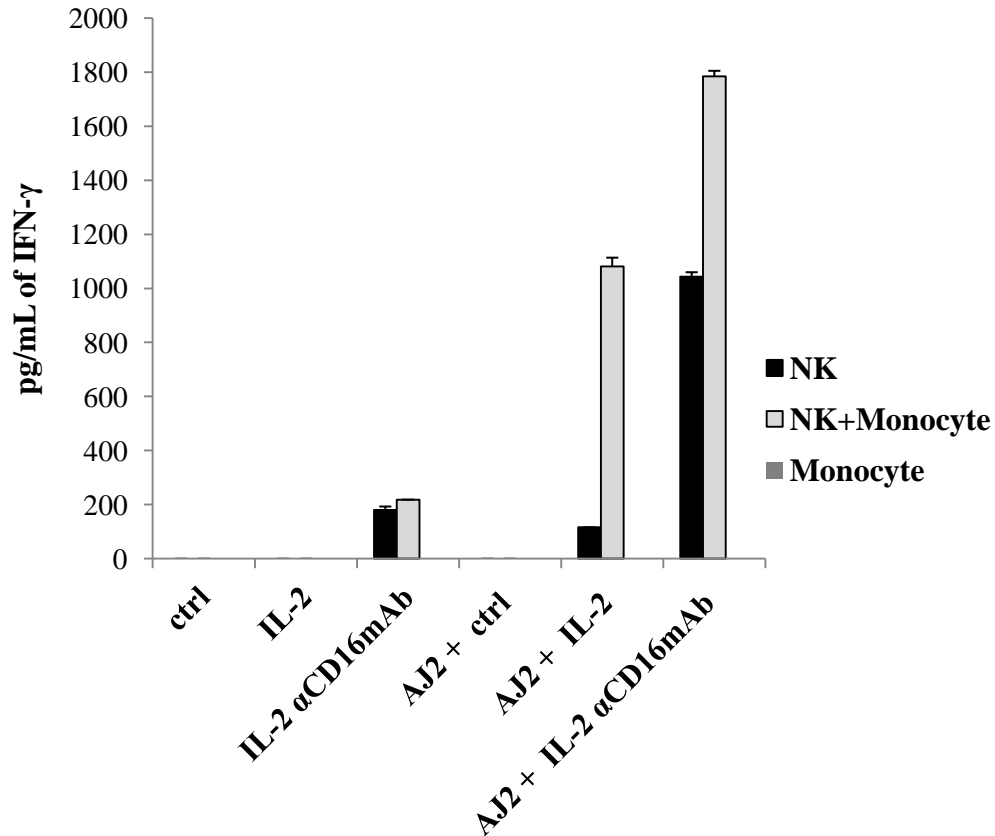
**Figure 16:** Differentiation of SCAP by rTNF- $\alpha$  and rIFN- $\gamma$  was correlated with CD54, MHC1, and B7H1 expression.

SCAP cells were treated with 20ng/mL rTNF- $\alpha$  , 200U/mL rIFN $\gamma$ , a combination of both, or 20ng/mL rTNF- $\alpha$  + 200U/mL rIFN $\gamma$  + antibodies against TNF- $\alpha$  and IFN $\gamma$  for 24 hours. SCAP were stained using PE conjugated antibodies against isotype control, CD44, MHC1, B7H1, and CD54 and the levels of surface expression were determined by flow cytometry analysis.

5. IFN- $\gamma$  production by NK cells is increased in co-culture with Monocytes and bacteria.

As seen in previous experiments in the present study, control NK cells do not secrete IFN- $\gamma$  and those treated with IL-2 secrete extremely low or no IFN- $\gamma$  (Figure 4 and 17). Treatment of NK cells with IL2+ $\alpha$ CD16mAb increases secretion of IFN- $\gamma$  (Figure 4 and 17). When NK cells are cultured with the bacteria AJ2 we see no increase of IFN- $\gamma$  in the control NK cells, but we do see an increase in IL2 activated NK cells compared to those activated with IL2 without bacteria. Addition of AJ2 to IL2+ $\alpha$ CD16mAb significantly increases secretion of IFN- $\gamma$  compared to IL2+ $\alpha$ CD16mAb treated NK cells without the presence of bacteria. IL2+ $\alpha$ CD16mAb NK cells cultured with Monocytes increased secretion of IFN- $\gamma$ , however, addition of Monocytes to control and IL2 NK cells did not induce production of IFN- $\gamma$ . When we culture NK cells with Monocytes in the presence of AJ2 we see a significant increase in IFN- $\gamma$  in IL-2 and IL2+ $\alpha$ CD16mAb activated NK cells. Production of IFN- $\gamma$  is much higher than IL2+ $\alpha$ CD16mAb NK cells and IL2 or IL2+ $\alpha$ CD16mAb activated NK cells cultured with AJ2 only. Monocytes do not produce IFN- $\gamma$ , thus, IFN- $\gamma$  detected is due increased secretion of IFN- $\gamma$  by NK cells (Figure 17).

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**Figure 17:** NK cells cultured with bacteria or Monocytes increased secretion of IFN- $\gamma$ .

NK cells were untreated, IL2 (1000U/mL) treated, and IL2 (1000U/mL) + $\alpha$ CD16mAb (2.5 $\mu$ g/mL) treated overnight. NK cells were cultured with Monocytes (2 NK: 1 Monocyte), bacteria (1 NK: 1AJ2), or bacteria + Monocytes overnight. Afterwards, the supernatants were harvested and tested for IFN- $\gamma$  presence by ELISA.

## Discussion

To examine the functional role of energized NK cells on lung tumors, hA549 were treated with supernatants from energized NK cells. Control supernatants, shown to secrete very low TNF- $\alpha$  and IFN- $\gamma$ , did not induce differentiation of hA549 cells. Treatment of energized NK cell supernatants pushed hA549 cells to differentiate leading to an increase in resistance to NK cell mediated cytotoxicity. Differentiation of hA549 was correlated with a significant increase of MHC 1, moderate increase of B7H1, and slight increase of CD54, but no change in CD44 surface expression. These results demonstrated that energized NK cells are able to support the differentiation of lung tumors. In differentiation experiments with oral tumors our lab has found addition of cytokines and energized NK supernatants increased induction of cell death in a fraction of tumors (manuscript in progress). Thus, we analyzed the cell death in hA549 treated with NK IL-2+ $\alpha$ CD16mAb and found that treatment did not induce cell death. This data suggested that low cytotoxicity of NK cells against hA549 differentiated with NK IL2+ $\alpha$ CD16mAb was due to resistance and not induction of cell death.

Because IL2+ $\alpha$ CD16mAb treated, or energized, NK cells were shown to secrete increased amounts of TNF- $\alpha$  and IFN- $\gamma$ , we added antibodies against both of these cytokines to see the effect it had on cells treated with NK IL2+CD16mAb supernatants. Addition of an antibody against TNF- $\alpha$  blocked differentiation very little compared to addition of an antibody against IFN- $\gamma$ . IFN- $\gamma$  seems have more weight in inducing differentiation compared to TNF- $\alpha$ . Only when we added the combination of both antibodies against TNF- $\alpha$  and IFN- $\gamma$  do we see complete blocking of differentiation which brought us to believe that it is the synergistic function of TNF- $\alpha$  and IFN- $\gamma$  secreted by energized NK cells that induces differentiation of lung tumors. To further demonstrate the role of TNF- $\alpha$  and IFN- $\gamma$ , hA549 were treated with recombinant

TNF- $\alpha$  and IFN- $\gamma$ . Each cytokine alone was able to induce minimal differentiation, but it was the addition both cytokines that drove differentiation of hA549. Addition of antibodies against both TNF- $\alpha$  and IFN- $\gamma$  blocked differentiation causing hA549 to become sensitive to NK cell mediated cytotoxicity similar to untreated cells. Together these results confirmed that it is the synergistic effect of TNF- $\alpha$  and IFN- $\gamma$  secreted by energized NK cells that push lung tumors to differentiate thereby becoming resistant to NK cell mediated cytotoxicity.

Similar results were seen when SCAP cells were treated with energized NK supernatants and recombinant TNF- $\alpha$  + IFN- $\gamma$  in which they were pushed to differentiate becoming more resistant to NK cell mediated cytotoxicity. In addition, there was an increase in surface expression of CD54, MHC class 1, and B7H1, but no change in CD44. There was also no induction of cell death. This data demonstrates that energized NK cells do not discriminate between healthy cells and transformed cells. The cytokines secreted are capable of differentiating transformed as well as healthy untransformed stem cells.

AJ2 was shown to significantly increase secretion of IFN- $\gamma$  by IL2 and IL2+ $\alpha$ CD16mAb activated NK cells. IFN- $\gamma$  secretion was significantly increased to very high levels with the addition of Monocytes in the presence of AJ2. Monocytes are part of the myeloid-derived suppressor cells (MDSC), a heterogeneous population that consist of myeloid progenitor cells and immature myeloid cells. These cells have been found to suppress adaptive immune responses, as well as, regulate innate immune responses (47). MDSCs have been shown to inhibit T cell proliferation and activation, suppress NK cell cytotoxicity, and suppress maturation of DCs, which together contribute to the negative regulation of immune responses and the promotion of immune escape of tumors and pathogens (48). The results presented in this study demonstrate Monocytes may not be the bad effectors they were thought to be because their



interaction with NK cells leads to an increase in IFN- $\gamma$  production, thus, contributing to differentiation of cells. Together these results suggest that interaction of NK cells with bacteria or Monocytes is another method through which NK cells can become conditioned to support differentiation of cells.

Induction of split anergy could be an important conditioning step for the repair of tissues during pathological processes irrespective of the type of pathology. In cancer the generation and maintenance of cancer stem cells is higher, therefore, the majority of NK cells may be conditioned to become anergized in order to support differentiation and repair of the tissues reducing tumor induced inflammation (23). The data presented in this part of the study and other data from our laboratory suggest that the same mechanism is likely responsible for shaping the survival and maturation of healthy stem cells. The degree of anergy may be directly proportional to amount of cancer stem cells, stage of differentiation, and metastatic potential of tumors (21).

## **Conclusion**

In this part of the study we demonstrate that anergized NK cells are capable of supporting differentiation of lung tumors through the secretion of TNF- $\alpha$  and IFN- $\gamma$ , thus, contributing to resistance against NK cell mediated cytotoxicity. Supernatants from IL-2 +  $\alpha$ CD16mAb treated NK cells induced differentiation of hA549 increasing expression of MHC class 1, B7H1, and CD54 and resistance to NK cell mediated cytotoxicity. Furthermore, supernatants from IL-2 +  $\alpha$ CD16mAb treated NK cells also induced differentiation of the healthy stem cells, SCAP, leading to increase in surface expression of CD54, MHC class1, and B7H1, as well as an increase in resistance to NK cell mediated cytotoxicity. In addition, we demonstrated that

bacteria and Monocytes induce NK cells to secrete high levels of IFN- $\gamma$  which allows them to support differentiation of cells. Together these results suggest one of the roles of NK cells is to support differentiation and promote tissue regeneration thereby limiting inflammation.

### **Chapter 3:**

*Specific Aim 3:* To demonstrate MHC 1 plays an important role on differentiated cells and presence of JNK, AKT, and STAT3 signaling pathways correlates with differentiation of hA549 cells and increased resistance to NK cell mediated cytotoxicity induced by anergized NK cells.

#### **Introduction**

NK cells recognize tumor cells based on “missing self” and “induced self” signals on target cells (36). NK cells primarily target cells that have down regulated expression of Major Histocompatibility Complex 1 (MHC class 1), which is expressed in almost every healthy cell in the body (“missing self”). MHC 1 is a ligand for killer cell immunoglobulin-like receptor (KIR). This inhibitory receptor prevents killing of normal cells and monitors the production of IFN- $\gamma$ , TNF- $\alpha$ , and granulocyte macrophage-colony stimulating factor (GM-CSF) by NK cells. However it has been observed that MHC 1 expression is not always necessary for protection from NK cell lysis and inhibition by MHC 1 is not always sufficient to prevent NK cell lysis (4). In the present study we have seen a significant increase of MHC 1 when cells are differentiated by anergized NK supernatants. Increase in MHC1 has been correlated with resistance to NK cell mediated cytotoxicity, thus, we hypothesize MHC1 has an important role in the differentiation of hA549.

Changes in the environment cause cells to respond via a diverse array of intracellular signaling pathways that relay, amplify, and integrate signals from external stimuli resulting in a genomic and physiological response. In the mammalian system such responses include cellular proliferation, differentiation, development, the inflammatory response and apoptosis (37). The c-

Jun NH<sub>2</sub>-terminal kinase (JNK) pathway represents one subgroup of Mitogen-activated proteins (MAP) kinases that is activated by cytokines and exposure to environmental stress. JNK becomes activated through phosphorylation and one of its major targets is the transcription factor Activator protein-1 (AP-1) (37-39). JNK has been found to have a role in cell death, cancer, diabetes and metabolizing, and survival signaling under specific circumstances. AKT, also known as Protein kinase B, is a serine/threonin specific protein kinase first identified as a retroviral oncogene. The AKT pathway can regulate cell growth, apoptosis, and tumor-related diseases, thus, it plays an essential role in physiological as well as pathological signaling mechanisms, but one of its most important functions is that AKT is a survival factor for cells (40). The signal transducer and activator of transcription 3 (STAT3) is part of a family of seven proteins which relay signals from activated cytokine and growth factor receptors in the plasma membrane to the nucleus. The STAT3 signaling pathway has been found to regulate cell differentiation, proliferation, apoptosis, angiogenesis, metastasis, and immune responses (41).

In this part of the project we wanted to study the role of MHC 1 in differentiation, as well as the expressions of JNK, AKT, and STAT3 intracellular signaling pathways in hA549 differentiated by anergized NK supernatants.

Sub aim 1: Addition of anti-MHC 1 inhibits function of activated NK cells in co-culture with hA549, but increases sensitivity of differentiated hA549 in co-culture with activated NK cells.

Sub aim 2: Presence of JNK, AKT, and STAT3 correlates with differentiation of hA549 induced by anergized NK supernatants and resistance to NK cell mediated cytotoxicity.

## **Material and Methods**

### Cell lines and Reagents

A549 cells are human lung alveolar basal epithelial cells derived from human acinar adenocarcinoma with gland formation. The cells were cultured in DMEM (Invitrogen by Life Technologies, CA) supplemented with 3% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA), 1.4% antibiotic-antimycotic, 1% sodium pyruvate, 1.4% non-essential amino acids, 1% L-glutamine (Invitrogen by Life Technologies, CA).

### Antibodies

NK cell purification kits were purchased from Stem Cell Technologies (Vancouver, Canada)

### Purification of Natural Killer Cells

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from blood donors and all procedures were approved by the UCLA-IRB. The Ficoll-Hypaque technique was used to fractionate the blood, and the buffy layer was harvested, washed and re-suspended in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% FBS. Non-adherent human peripheral blood lymphocytes were obtained by sequential incubation on tissue culture dishes. NK cells were negatively selected and isolated using the EasySep® Human NK cell enrichment kit purchased from Stem Cell Technologies (Vancouver, Canada).

The NK cell population was found to have a greater than 90% purity based on flow cytometric analysis of anti-CD16 antibody stained cells.

Purified NK cells were cultured in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% FBS (Gemini Bio-Products, CA), 1% antibiotic antimycotic, 1% sodium pyruvate, and 1% nonessential amino acids (Invitrogen by Life Technologies, CA).

#### <sup>51</sup>Chromium release cytotoxicity assay

NK cell cytotoxic function in the experimental cultures and the sensitivity of the target cells to NK cell mediated lysis were determined using the <sup>51</sup>Chromium release cytotoxicity assay. Different numbers of purified NK cells were incubated with <sup>51</sup>Chromium labeled target cells. After a 4-hour incubation period, each supernatant was harvested from each sample and counted for released radioactivity using a gamma-counter. The percentage specific cytotoxicity was calculated as followed

$$\text{Percent cytotoxicity} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Total cpm} - \text{Spontaneous cpm}}$$

Lytic unit  $30/10^6$  was calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells X 100.

#### Luminex

The following was done using the MILLIPLEX map protocol. After treatments, cells were washed with ice cold Buffered Saline (PBS or TBS) and drain. Added ice-cold 1X MILLIPLEX<sup>®</sup>

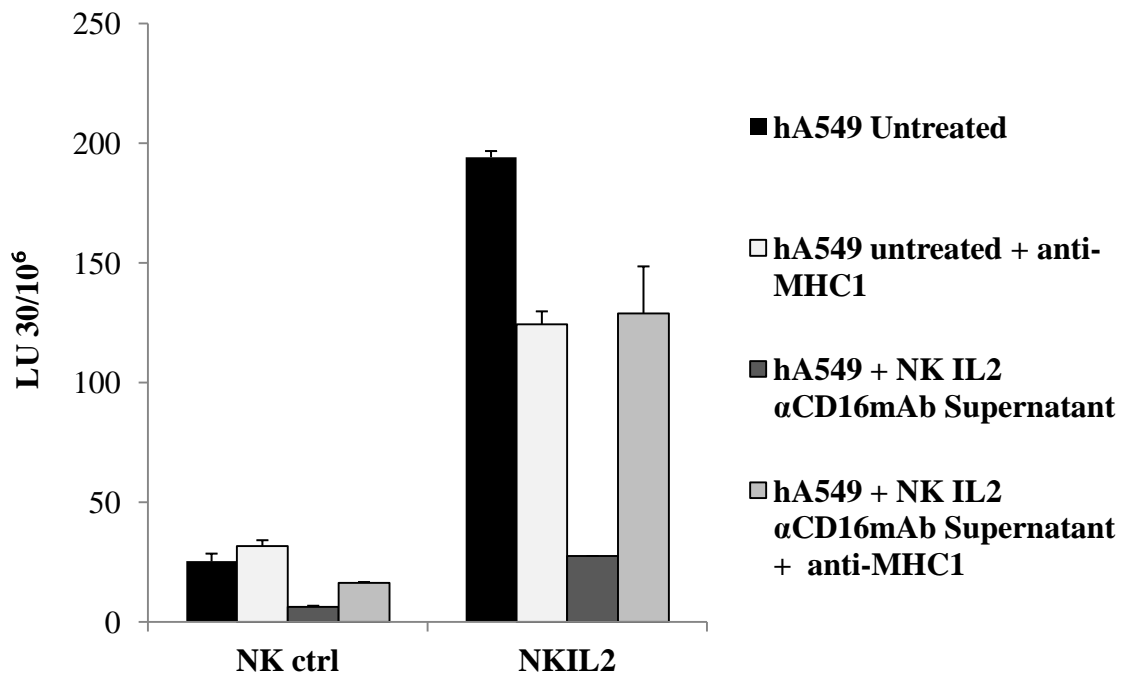
MAP Lysis Buffer with freshly added protease inhibitors to cells (0.6 mL per 150 mm dish, 0.3 mL per 100 mm dish, or 0.1 mL per well of 24-well plate). Then adherent cells were scraped off the dish with a cell scraper and the cell suspension was transferred into a centrifuge tube and gently rocked for 10-15 minutes at 4°C. The particulate matter was removed by centrifuging at highest speed for 5 minutes and the liquid containing the protein lysate was collected. The lysate was diluted to obtain 25µg of total protein per well. 50µL of assay buffer was added and shaken for 10 minutes at room temperature. 25µL of 1x beads was added to wells followed by 25uL of control and sample lysates and incubated overnight at 4°C with shaking. After overnight incubation we washed twice with assay buffer and added 25µL of detection antibody. After 1 hour incubation, detection antibody was removed and 25µL of streptavidin-PE (SAPE) was added. After 15 minute incubation 25uL of amplification buffer was added and incubated for another 15 minutes. After incubation streptavidin-PE/amplification buffer was removed and beads were resuspended in 150µL of assay buffer. The results were read using an appropriate Luminex instrument.

**Results:**

1. Addition of Anti-MHC 1 inhibits function of activated NK cells in co-culture with hA549, but increases sensitivity of NK IL2+ $\alpha$ CD16 supernatant differentiated hA549 in co-culture with activated NK cells.

MHC 1 is one of the surface receptors that is significantly increased when hA549 cells are differentiated by energized NK supernatants (Figure 6 and 8), as well as in SCAP cells differentiated with energized NK supernatants (Figure 13). To determine the role of MHC 1 in differentiation we added an antibody against MHC 1. Addition of anti-MHC 1( $\alpha$ PA2.6) to co-culture of poorly differentiated h549 and activated NK cells decreased the cytotoxicity of NK cells. hA549 became more resistant to NK cell mediated cytotoxicity when compared to the control (Figure 18). Addition of NK IL2 +  $\alpha$ CD16 supernatants, as seen previously, differentiated hA549 cells making them more resistant to NK cell mediated cytotoxicity. However, addition of anti-MHC 1 to co-culture of differentiated hA549 and activated NK cells significantly increased the sensitivity of differentiated hA549 to NK cell mediated cytotoxicity compared to those treated with energized NK supernatants and no anti-MHC 1 (Figure 18).





**Figure 18.** Anti-MHC1 causes activated NK cells to lose cytotoxicity against A549, but increases sensitivity of differentiated hA549 to NK cell mediated cytotoxicity.

hA549 cells were untreated and treated with supernatants from NK IL2(1000U/mL) +  $\alpha$ CD16mAb(2.5 $\mu$ g/mL) for six days. NK cells were untreated and IL2 (1000U/mL) treated overnight before they were used to measure NK cell cytotoxicity against Cr51 labeled hA549 cells. Anti-MHC1 ( $\alpha$ PA2.6mAb) was added in 1:100 ratio to a portion of Cr51 labeled untreated A549 and NK IL2 (1000U/mL) +  $\alpha$ CD16mAb (2.5 $\mu$ g/mL) supernatant treated hA549 before 4 hour incubation with NK cells. After 4 hours of incubation of NK cells with hA549 radioactivity released in the supernatants were counted by the gamma counter and the levels of cytotoxicity were determined using LU 30/10<sup>6</sup>. LU 30/10<sup>6</sup> denotes the number of NK cell effectors needed to lyse 30% of hA549.

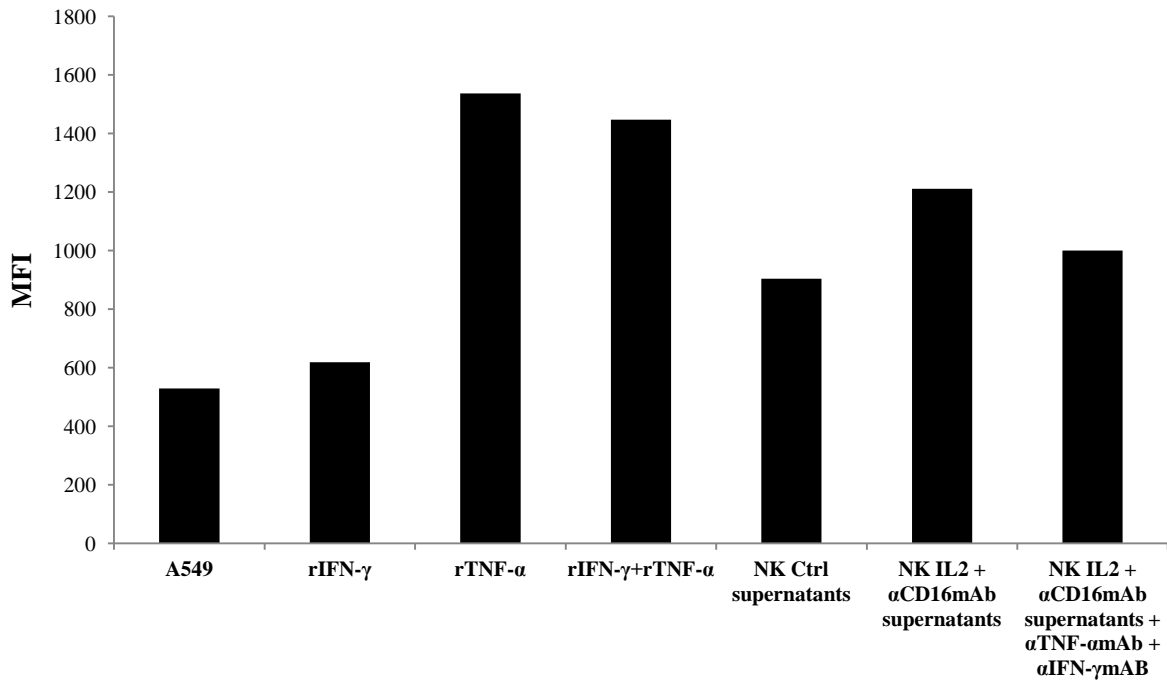
2. Presence of JNK, AKT, and STAT3 correlates with differentiation of hA549 induced by anergized NK supernatants and resistance to NK cell mediated cytotoxicity.

In the following early preliminary results we examined presence of various transcriptional pathways that might be involved in the differentiation of hA549 by anergized NK supernatants. When we examine the JNK pathway we find that rIFN- $\gamma$  did not induce pJNK presence. rTNF- $\alpha$ , on the other hand, increases presence of pJNK and similar results are seen with rTNF- $\alpha$ + rIFN- $\gamma$ . This could be due to rTNF- $\alpha$  and not rIFN- $\gamma$ , since rIFN- $\gamma$  was not seen to induce pJNK. Treatment of hA549 with control supernatants also increased pJNK, but the levels of pJNK were not as high as rTNF- $\alpha$  or NKIL2+ $\alpha$ CD16mAb supernatants. hA549 treated with NK IL2+ $\alpha$ CD16mAb supernatants further increased pJNK and addition of antibodies against rTNF- $\alpha$  and rIFN- $\gamma$  decreased levels of pJNK (Figure 19).

Treatment of hA549 with rIFN- $\gamma$  increased pAKT compared to the control. Similar levels of pAKT were seen with treatment of rTNF- $\alpha$ . rIFN- $\gamma$ +rTNF $\alpha$  only slightly increased pAKT compared to rTNF- $\alpha$  and rIFN- $\gamma$ . Control supernatant treatment was also capable of increasing presence of pAKT in hA549 and the levels were similar to those induced by rTNF- $\alpha$  and rIFN- $\gamma$ . NK IL2+ $\alpha$ CD16mAb supernatant showed the highest increase of pAKT in hA549 compared to the other treatments and addition of antibodies against rTNF- $\alpha$  and rIFN- $\gamma$  decreased the levels of pAKT (Figure 20).

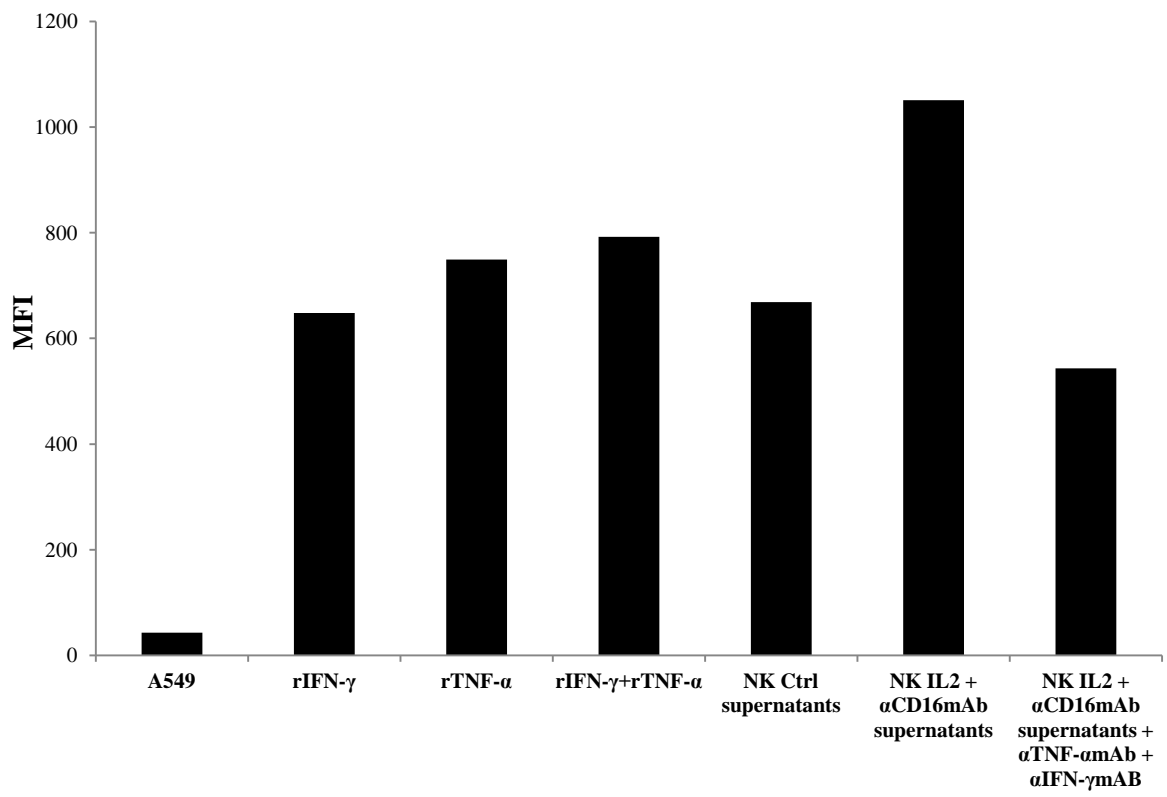
STAT3 on the other hand does not increase in hA549 treated with rTNF- $\alpha$ , rIFN $\gamma$ , rTNF- $\alpha$ +IFN- $\gamma$ . Control supernatants were able to increase STAT3 only slightly compared to rTNF- $\alpha$ , rIFN- $\gamma$ , and rTNF- $\alpha$ +IFN- $\gamma$ . However, we see the highest increase of STAT3 in hA549 when

they are differentiated with NK IL2+ $\alpha$ CD16 supernatants. Interestingly, addition of antibodies against TNF- $\alpha$  and IFN- $\gamma$  was able to decrease the levels of STAT3 (Figure 21).



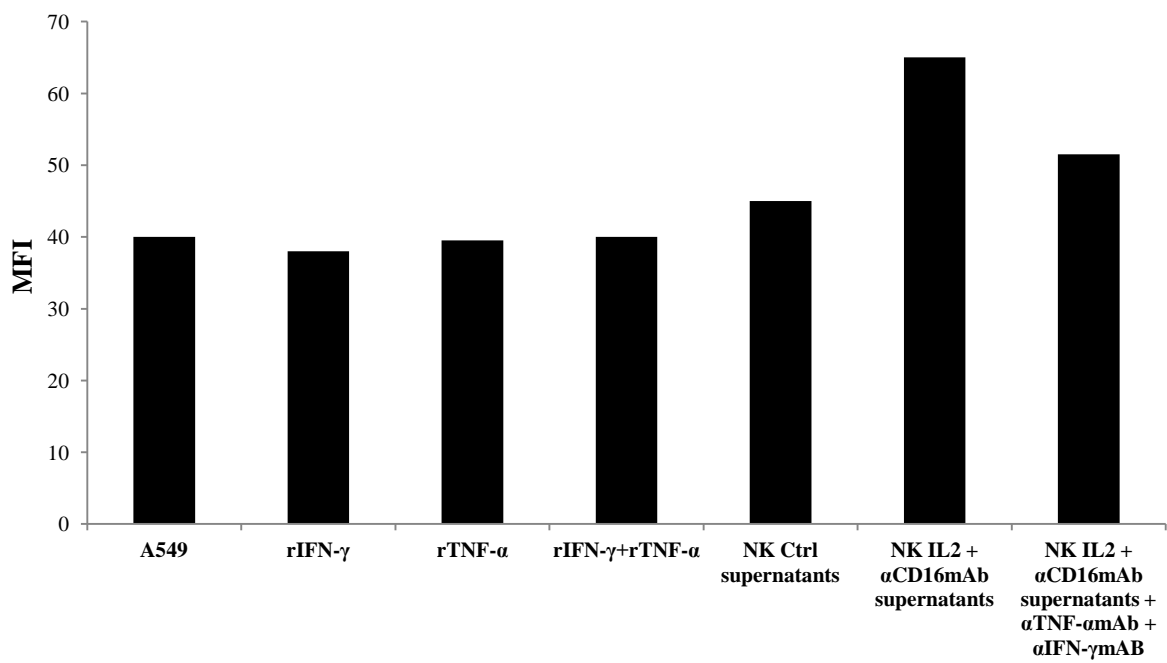
**Figure 19:** Differentiation of hA549 induced by rTNF- $\alpha$ , rIFN- $\gamma$  + rTNF- $\alpha$ , and NK IL2 +  $\alpha$ CD16 supernatants increases presence of pJNK.

On the day of treatment media was removed from hA549 cells and fresh media was added. hA549 were incubated for 1 hour at 37°C. Following 1 hour incubation cells were treated with 20ng/mL rTNF- $\alpha$ , 200U/mL rIFN $\gamma$ , a combination of both, 500uL of NK control supernatants, 500uL of NK IL2 +  $\alpha$ CD16mAb supernatants, or 500uL of NK IL2 +  $\alpha$ CD16mAb supernatants plus the addition of antibodies against TNF- $\alpha$  and IFN- $\gamma$  for 1 hour. Protein was extracted using 200uL of protein lysis buffer and 100uL of protein inhibitor. Presence of pJNK was determined using Luminex.



**Figure 20:** Differentiation of hA549 induced by NK IL2 +  $\alpha$ CD16mAb increased presence of pAKT compared to differentiation induced by rIFN $\gamma$ , rTNF- $\alpha$ , and rIFN $\gamma$ + rTNF- $\alpha$ .

On the day of treatment media was removed from hA549 cells and fresh media was added. hA549 were incubated for 1 hour at 37°C. Following 1 hour incubation cells were treated with 20ng/mL rTNF- $\alpha$  , 200U/mL rIFN- $\gamma$ , a combination of both, 500uL of NK control supernatants, 500uL of NK IL2 +  $\alpha$ CD16mAb supernatants, or 500uL of NK IL2 +  $\alpha$ CD16mAb supernatants plus the addition of antibodies against TNF- $\alpha$  and IFN- $\gamma$  for 1 hour. Protein was extracted using 200uL of protein lysis buffer and 100uL of protein inhibitor. Presence of pAKT was determined using Luminex.



**Figure 21:** Differentiation of hA549 induced by NK IL2 +  $\alpha$ CD16mAb increased presence of STAT3.

On the day of treatment media was removed from hA549 cells and fresh media was added. hA549 were incubated for 1 hour at 37°C. Following 1 hour incubation cells were treated with 20ng/mL rTNF- $\alpha$  , 200U/mL rIFN- $\gamma$ , a combination of both, 500uL of NK control supernatants, 500uL of NK IL2 +  $\alpha$ CD16mAb supernatants, or 500uL of NK IL2 +  $\alpha$ CD16mAb supernatants plus the addition of antibodies against TNF- $\alpha$  and IFN- $\gamma$  for 1 hour. Protein was extracted using 200uL of protein lysis buffer and 100uL of protein inhibitor. Presence of STAT3 was determined using Luminex.

## Discussion

In our previous experiments in which we induce differentiation of hA549 with energized NK supernatants we have shown a significant increase of MHC 1 surface expression. In addition, this increase in MHC 1 surface expression has been correlated with resistance of hA549 to NK cell mediated cytotoxicity. We have shown similar results with the healthy stem cells SCAP, suggesting that this phenomenon is not exclusive to lung tumors. Because MHC class 1 is one of the receptors whose expression was significantly increased as cells become differentiated, we wanted to explore its importance in resistance of tumors to NK cell mediated cytotoxicity. Thus, an antibody against MHC 1 was added to co-cultures of activated NK cells plus hA549, as well as activated NK cells plus differentiated hA549. Addition of an antibody against MHC 1 to co-cultures of undifferentiated hA549 and activated NK cells increased resistance to NK cell mediated cytotoxicity. hA549 are poorly differentiated cells expressing low MHC 1, thus, it may be that adding anti-MHC 1 is not targeting these cells. It has been shown that addition of anti-MHC to activated T cells suppresses their function (42). It's possible when NK cells become activated addition of anti-MHC 1 can target MHC1 on NK cells and block their function, which would explain the small decrease in cytotoxicity. On the other hand, when we added anti-MHC 1 to co-culture of differentiated hA549 and activated NK cells we saw an exacerbation of lysis by NK cells compared to differentiated A549 not treated with anti-MHC 1. It is not clear whether this is due to ADCC or covering of inhibitory ligands on NK cells. Our results suggest that MHC1 is important for the function of NK cells but only when NK cells interact with differentiated cells not stem cells.

Changes in the environment cause cells to respond via a diverse array of intracellular signaling pathways that relay, amplify, and integrate signals from external stimuli resulting in a

genomic and physiological response (37). In this part of the study we examined possible involvement of various intracellular signaling pathways. These are very early preliminary studies but they provide important insight into the possible regulation of differentiation of hA549 induced by energized NK cells. We demonstrate as hA549 cells become differentiated they increase important transcription factors and signaling molecules. It was found that pJNK and pAKT were expressed more when hA549 were differentiated with cytokines as well energized NK supernatants. Control supernatants were also seen to increase pJNK and pAKT. In the present study we have found that control supernatants secrete very small amounts of TNF- $\alpha$ , thus, it is a possibility that the presence of TNF- $\alpha$  is responsible for the increase in pJNK and pAKT seen in hA549 cells treated with control supernatants. The JNK pathway has been found to regulate cell death and in some cases cell survival (36-38). AKT has been shown to be an important survival factor for cells and increase of AKT correlates with increase survival (39), which could possibly explain why there was very little induction of cell death in hA549 differentiated with energized NK supernatants.

In addition, treatment of hA549 cells with rIFN- $\gamma$ , rTNF- $\alpha$ , and rIFN- $\gamma$ +rTNF- $\alpha$  did not increase STAT3. Control supernatants was able to increase STAT3 but very little compared to the other treatments. This could be triggered by the presence of other cytokines which we have not screened for. The highest STAT3 expression was found in hA549 cells differentiated with NK IL2+ $\alpha$ CD16mAb suggesting increased expression is specific to supernatants from energized NK cells. Interestingly, addition of antibodies against TNF- $\alpha$  and IFN- $\gamma$  to hA549 cells treated with energized NK supernatants decreased STAT3, which suggest TNF- $\alpha$  and IFN- $\gamma$  are involved in increasing STAT3. It's possible that TNF- $\alpha$  and IFN- $\gamma$  are working synergistically with IL6, found to be secreted by energized NK cells, or other cytokines, which have not been



screened for. This would explain why recombinant TNF- $\alpha$  and IFN- $\gamma$  did not show STAT3 increase. In previous studies glioblastoma multiforme tumors shown to constitutively express STAT3 were found to be resistant to NK cell mediated cytotoxicity (Cacalano and Jewett, unpublished data). Based on this data and the presence of STAT3 on our differentiated hA549 cells, we conclude that the resistant cells are in fact the differentiated cells, and this might be an important mechanism through which resistance could be induced. Presence of such pathways, suggest they may play an important role in regulating differentiation and survival of hA549 induced by energized NK supernatants, but more studies need to be done to truly understand the implications of these pathways in differentiation induced by energized NK cells.

## **Conclusion**

In this part of the study we found addition of anti-MHC to co-culture of undifferentiated A549 and NK cells caused reduced NK cell cytotoxicity. However, addition of anti-MHC to co-cultures of differentiated hA549 and NK cells caused differentiated hA549 to become sensitive to NK cell mediated cytotoxicity. Furthermore, the preliminary studies of signaling pathways demonstrated the presence of JNK, AKT, and STAT3 in hA549 cells differentiated by energized NK supernatants. Together these results suggest 1- MHC1 is important for NK cell function when they interact with differentiated cells, but not stem cells and 2- the presence of JNK, AKT, and STAT3 intracellular signaling pathways may provide an important insight into the regulatory mechanisms involved in differentiation of hA549 induced by energized NK supernatants.

## **Chapter 4:**

*Specific Aim 4:* Undifferentiated or poorly differentiated lung tumors are more resistant to chemotherapy drugs than differentiated lung tumors.

### **Introduction**

One of the major goals of cancer chemotherapy is to induce tumor cells to undergo apoptosis following exposure to antitumor agents. Cisplatin (cis-diammine-dichloro-platinum) is one of the most potent antitumor agents known targeting a wide variety of tumors. It's had a major impact in cancer medicine, changing the course of therapeutic management of several tumors. Cisplatin primarily targets DNA causing damage which leads to activation of signals that induce apoptosis. However, after treatment tumors have been shown to acquire resistance to Cisplatin which is a major limitation of the drug (43).

Paclitaxel is another chemotherapeutic agent used as a first line therapy for many cancers such as lung, breast, ovarian, and head and neck. It is a natural product obtained from the North American Pacific yew tree and belongs to the taxane class of drugs. Paclitaxel works by inhibiting the dissociation rate of the tubulin subunits from the tubule leading to the formation of microtubule bundles, arresting cells in mitosis. In addition, Paclitaxel has been found to have antiangiogenic activity (44).

N-acetylcysteine (NAC), the acetylated variant of the amino acid L-cysteine, is an antioxidant with many important clinical applications. It has the ability to support the body's antioxidant and nitric oxide systems during stress, infections, and inflammatory conditions. Furthermore, NAC has other applications including prevention of chronic obstructive pulmonary disease exacerbation, prevention of contrast-induced kidney damage during imaging procedures,

attenuation of illness from the influenza virus when started before infection, treatment of pulmonary fibrosis, and treatment of infertility in patients with clomiphene-resistant polycystic ovary syndrome (45, 46).

In previous experiments in our lab we have seen that differentiated cells are more susceptible to chemotherapy drugs, whereas, undifferentiated cells are more resistant. The following sub aims were demonstrated by In Vitro assays to explore the effect of the chemotherapy drugs Cisplatin and Paclitaxel, as well as, the antioxidant NAC on lung tumors.

Sub Aim 1: Poorly differentiated cells are more resistant to Cisplatin and addition of NAC to Cisplatin treated cells rescues them from Cisplatin induced cell death.

Sub Aim 2: Paclitaxel and NAC work synergistically to increase cell death in lung tumors.

## **Material and Methods**

### Cell lines and Reagents

A549 cells are human lung alveolar basal epithelial cells derived from human acinar adenocarcinoma with gland formation. The cells were cultured in DMEM (Invitrogen by Life Technologies, CA) supplemented with 3% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA), 1.4% antibiotic-antimycotic, 1% sodium pyruvate, 1.4% non-essential amino acids, 1% L-glutamine (Invitrogen by Life Technologies, CA). H292 were derived from a lymph node metastasis of a pulmonary mucoepidermoid carcinoma. The cells were cultured in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA), 1.4% antibiotic-antimycotic, 1% sodium pyruvate, 1.4% non-

essential amino acids, 1% L-glutamine (Invitrogen by Life Technologies, CA), 0.2% gentamicin (Gemini Bio-Products, CA) and 0.15% sodium bicarbonate (Fisher Scientific, CA).

### PI Staining

Cell death was analyzed by staining cells with propidium iodide (PI).

### Chemotherapy Drugs and NAC

Cisplatin (1mg/mL) (Ronald Reagan Medical Center Pharmacy, UCLA)

Paclitaxel (6 mg/mL) (Ronald Reagan Medical Center Pharmacy, UCLA)

N-Acetylcysteine (Sigma, CA)

## Results

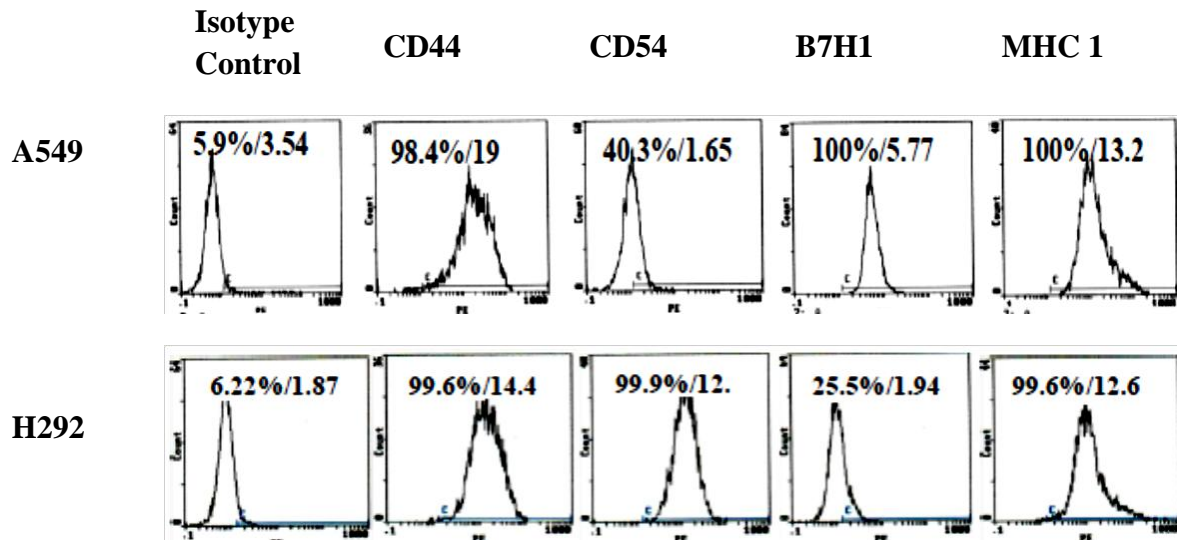
1. Poorly differentiated cells are more resistant to Cisplatin and addition of NAC to Cisplatin treated cells rescues them from Cisplatin induced cell death.
2. Paclitaxel and NAC work synergistically to increase cell death in lung tumors.

Surface staining was done on hA549 and H292 to explore the differences in the cell lines. Expression of CD44 and MHC 1 surface markers were very similar in both cell lines. Expression of CD54 was much higher on H292 than hA549. B7H1 surface expression was higher on hA549 than H292 (Figure 22).

To determine the effect of the Cisplatin and NAC on the lung tumors hA549 and H292 we ran a cell death assay. Control hA549 and H292 do not show any cell death and addition of NAC alone did not increase cell death. When we treat the cells with Cisplatin we see an increase in cell death of hA549, but it is not as high as H292. H292 is much more sensitive to Cisplatin than hA549. Interestingly, addition of NAC to Cisplatin treated cells protects both hA549 and H292 from Cisplatin induced cell death bringing the percentage of cell death close to that of the control (Figure 23).

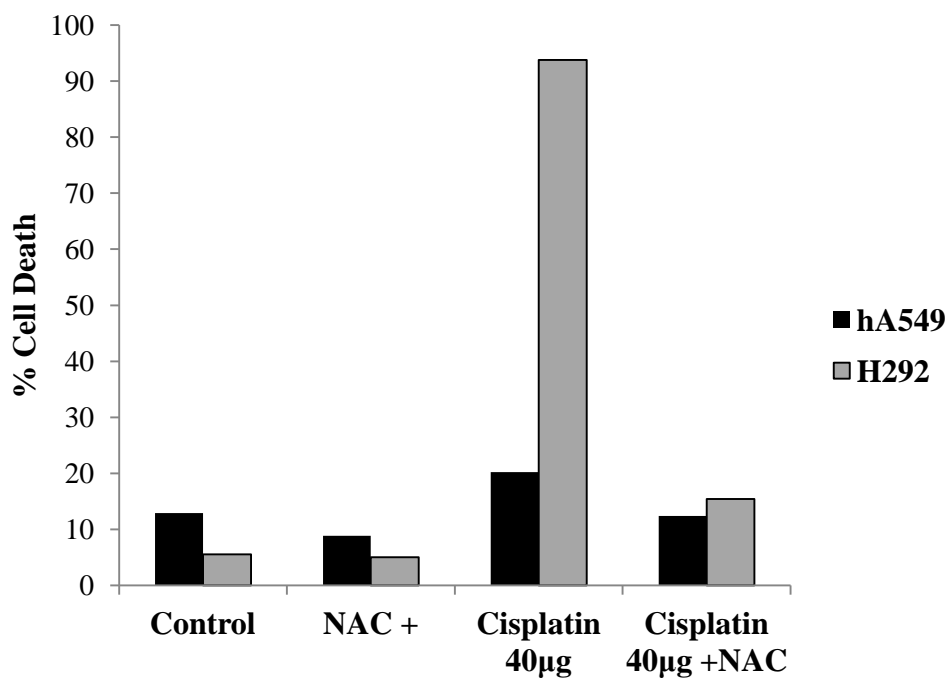
To explore the effect of Paclitaxel and NAC we ran a cell death assay. We found that both hA549 and H292 were sensitive to Paclitaxel. As the concentration was increased so was the percentage of cells undergoing cell death. At the highest concentration 40 $\mu$ g Paclitaxel we see higher cell death for H292 than hA549. When we add NAC to the different concentrations of Paclitaxel we see a synergistic effect between Paclitaxel and NAC increasing the percentage of

cell death compared to Paclitaxel alone. This is seen at every concentration of Paclitaxel and in both hA549 and H292 (Figure 24).



**Figure 22.** Expression of B7H1 is higher on A549 cells and CD54 is higher on H292, but CD44 and MHC1 expression is similar.

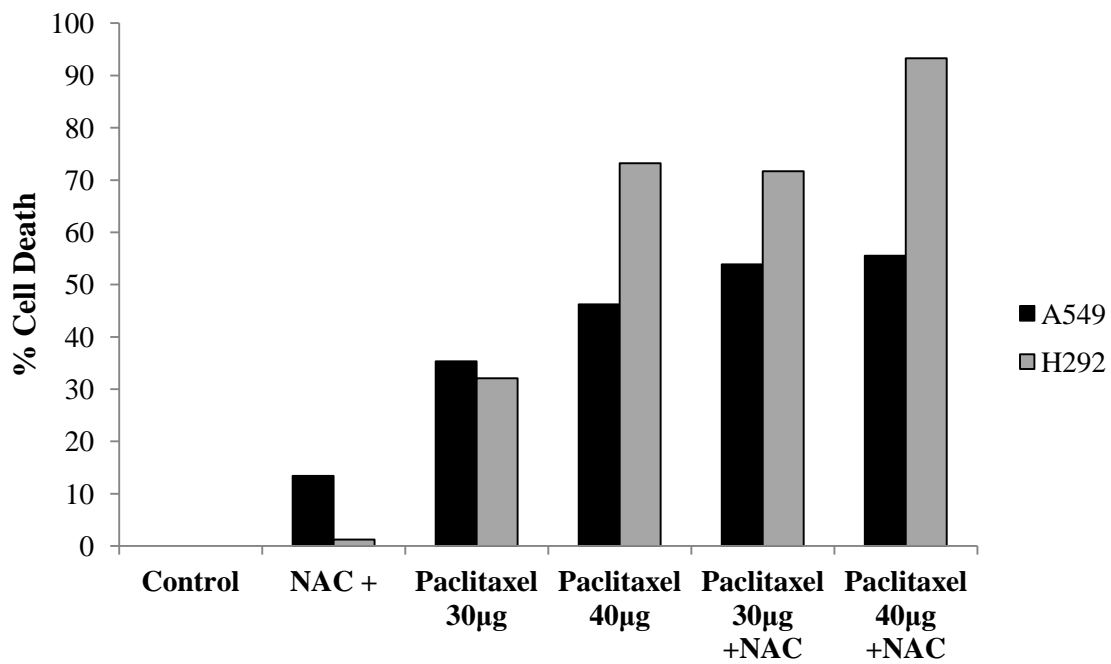
hA549 and H292 were stained using PE conjugated antibodies against isotype control, CD44, CD54, B7H1 and MHC 1 and the levels of surface expression were determined by flow cytometry analysis.



**Figure 23 A and B.** H292 cells are more sensitive to Cisplatin than A549 and NAC rescues cells from Cisplatin induced cell death.

A549 and H292 cells were treated with 40µg Cisplatin, NAC, or 40µg Cisplatin + NAC for 24 hours. Afterwards half of the cells were stained with PI and the levels of cell death were determined by flow cytometry analysis.





**Figure 24.** Paclitaxel and NAC work synergistically to increase cell death in A549 and H292.

A549 and H292 cells were treated with Paclitaxel (30µg and 40µg), NAC, or Paclitaxel (30µg and 40µg) + NAC for 24 hours. Afterwards the cells were stained with PI and the levels of cell death were determined by flow cytometry analysis.

## Discussion

In recent studies by our lab, we have found that undifferentiated or poorly differentiated tumors are resistant to chemotherapy drugs and as cells become more differentiated their sensitivity to these drugs increases. In the present study we examine the effect of the chemotherapy drugs, Cisplatin and Paclitaxel, on lung tumors. We use hA549, an adenocarcinoma, and H292, a mucoepidermoid carcinoma. Although these cells are different, we believe they are at different differentiation stages, thus, we are able to show the effect of the chemotherapy drugs on lung tumors at different levels of differentiation. In the present study we have established that hA549 cells are poorly differentiated based on their sensitivity to NK cell mediated cytotoxicity and surface marker expression. When we treated hA549 with Cisplatin we found that they were resistant to the drug and the same was seen with the 40 $\mu$ g Paclitaxel treatment. H292 are thought to be more differentiated based on their surface expression. In our surface staining we find that CD44 and MHC 1 is very similar in both cell lines, however, CD54 is much higher on H292. CD54 is one of the surface markers that is found to be elevated when cells are more differentiated. In addition, H292 cells were found to be very sensitive to Cisplatin and to Paclitaxel at 40 $\mu$ g. Together these results suggest that H292 is the more differentiated cell line. The results presented in this part of the study agree with what we see in the oral tumors, in which the poorly differentiated tumors are more resistant to these chemotherapy drugs and as they become more differentiated their sensitivity increases.

NAC is an antioxidant with many important functions, thus, we wanted to study its effect in combination with Cisplatin and Paclitaxel. Interestingly, when we treat cells with Cisplatin and NAC we find that they rescue cells from Cisplatin induced cell death. Addition of NAC significantly reduces the amount of cell death in both hA549 and H292. Cisplatin is one of the

most effective drugs against cancer, but its usefulness is limited by its toxicity to normal tissues. These results demonstrate that NAC is capable of reducing Cisplatin toxicity, decreasing the level of cell death. Unlike Cisplatin, addition of NAC to Paclitaxel treated cells did not rescue them from Paclitaxel induced cell death. What is important to note is that addition of NAC to Paclitaxel treated cells increased the amount of cell death in both cell lines, suggesting NAC is working synergistically with Paclitaxel increasing toxicity towards lung tumors. These results are significant in that they demonstrate important functions of NAC which have not been explored before.

## **Conclusion**

In this part of the study we explored the effect of NAC with Cisplatin and Paclitaxel on lung tumors. We demonstrated that H292, the more differentiated cell line, were more sensitive to Cisplatin and Paclitaxel than hA549. In addition, we found that NAC rescued hA549 and H292 from Cisplatin induced cell death, but worked synergistically with Paclitaxel to increase cell death in both cell lines. From these results and other studies in our lab, we believe that poorly or undifferentiated cells are more resistant to chemotherapy drugs and as they become more differentiated their sensitivity increases. Furthermore, we demonstrate important functions of NAC which have not been explored before.

## Final Conclusion

Advances in our understanding of anti-tumor immune responses and cancer biology have revealed complex and dynamic interactions between the immune effectors and target cells. NK cell function has been attributed to the killing of tumors, but accumulated evidence from our lab has shown that NK cell functions go beyond cytotoxicity. We believe NK cells are not only important for the removal of stem cells, but also for their differentiation and regeneration of tissue, as well as, reducing inflammation. In this study we were able to further investigate the roles of NK cells in the selection and differentiation of lung cancer, as well as, the effect of chemotherapy drugs, Paclitaxel and Cisplatin, on lung tumors. Our findings show:

1. Based on their phenotype and NK cell mediated cytotoxicity, hA549 cells were found to be closer to poorly differentiated cells than they were to more differentiated/terminally differentiated cells.
2. Anergized NK cells support the differentiation of lung tumors through secretion of TNF- $\alpha$  and IFN- $\gamma$ , thereby, making them more resistant to NK cell mediated cytotoxicity.
3. MHC 1 plays an important role in differentiation of hA549 and expression of JNK, AKT, and STAT3 signaling pathways correlates with differentiation of hA549 cells and increased resistance to NK cell mediated cytotoxicity induced by anergized NK cells.
4. Undifferentiated or poorly differentiated lung tumors are more resistant to chemotherapy drugs than differentiated lung tumors

Based on our accumulated results we conclude that NK cells may inhibit or significantly slow the tumor progression 1-by elimination of cancer stem cells or poorly differentiated tumors,

and 2- by inducing differentiation of tumors thereby limiting tumor induced inflammation which could further fuel the tumors.

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