Title
Strategies to Target Pancreatic Cancer Stem Cells using Natural Killer Cells and Chemotherapeutic Drugs

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Strategies to Target Pancreatic Cancer Stem Cells using Natural Killer Cells and Chemotherapeutic Drugs

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

In Oral Biology

by

Caitlin Jean Lanzon

2015
ABSTRACT OF THE THESIS

Strategies to Target Pancreatic Cancer Stem Cells using Natural Killer Cells and Chemotherapeutic Drugs

By
Caitlin Jean Lanzon
Master of Science in Oral Biology
University of California, Los Angeles, 2015
Professor Anahid Jewett, Chair

OBJECTIVES: To demonstrate that similar to differentiated oral tumors, differentiated pancreatic tumors are more resistant whereas their undifferentiated counterparts, or cancer stem cells, are more sensitive to Natural Killer (NK) cell mediated lysis. Furthermore, to reveal that anergized NK cells induce tumor differentiation and to examine the mechanism by which these differentiated cells resist NK cell mediated cytotoxicity. Lastly, to demonstrate that NAC protects tumor cells from lysis by Cisplatin chemotherapeutic treatments, while conversely, when used in conjunction with Paclitaxel, NAC causes a synergistic effect significantly increasing tumor cell lysis versus Paclitaxel treatments alone.

METHODS: Differentiated and undifferentiated, or cancer stem cells, for pancreatic cancer was used to determine their resistance and sensitivity to NK cell mediated
cytotoxicity and secretion of cytokines. The function of NK cells was assessed using $^{51}$Cr release assay, and the secretion of cytokines by cytokine arrays. Flow cytometry was used for surface analysis. Also, tumor cells were cultured with NK supernatants prior to undergoing cytotoxic $^{51}$Cr release assays and flow cytometry surface analysis. In addition, tumor cells were treated with Cisplatin, Paclitaxel, and NAC, and cell death was determined using propidium iodide (PI) staining or ethanol permeabilization, followed by flow cytometry analysis.

RESULTS: Similar to differentiated oral tumors, differentiated tumors of the pancreas are resistant to NK cell mediated cytotoxicity whereas their undifferentiated counterparts or cancer stem cells are significantly more sensitive. Differentiated tumors exhibited lower CD44 and CD54 and higher B7H1 and MICA when compared to undifferentiated or cancer stem cells. Differentiated tumors triggered no or very low levels of IFN-γ secretion by the NK cells. Anergized NK cells release factors that cause tumor cell resistance to NK cell mediated lysis and an increase in B7H1 surface expression on the target cells. Use of anti-B7H1 treatments on these differentiated tumor cells does not restore their sensitivity to NK cell lysis. Furthermore, NAC protects target tumor cells against Cisplatin-mediated killing, yet enhances cell death when used together with Paclitaxel.

CONCLUSIONS: Sensitivity to NK cell mediated lysis is dependent on the stage of differentiation and it is irrespective of the type of cancer. Therefore, NK cells are primary cells to eliminate cancer stem cells. Known inactivation of NK cell cytotoxic function in many cancers including oral tumors may be a major underlying mechanism for the
survival and expansion of cancer stem cells. Therefore, patients with cancer may benefit
from repeated allogeneic NK cell transplantation for specific elimination of cancer stem
cells. There is inactivation of NK cell cytotoxicity function in many cancers, and here we
show that anergized NK cytokines gives tumor cells resistance to NK cell mediated lysis
and up regulates surface expression of B7H1 on the tumor cells. This marker may be
partially responsible for conferring the resistance to NK cell mediated lysis. Knowledge
of tumor cell responses to NK cytokines will aid in determining more effective treatments
for cancer patients. In addition, the known function of NAC to protect cells is not seen
when used with Paclitaxel to lyse tumor cells, therefore patients with cancer may benefit
from this synergistic drug combination. NAC may be a factor that could enhance the
effects of Paclitaxel chemotherapeutic treatments.
The thesis of Caitlin Jean Lanzon is approved.

Nicholas Cacalano

Ichiro Nishimura

Anahid Jewett, Committee Chair

University of California, Los Angeles

2015
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Introduction

The immune system

The immune system is comprised of specific organs, tissues, cells, and molecules, and is defined as the body’s defense against infectious organisms such as bacteria, viruses, fungi, parasites, and other harmful substances. This complex network works to protect the body against diseases through the elimination of the body’s own cells that have become abnormal due to infection with foreign agents or through diseases such as cancer. The immune system can be separated into two major divisions: innate and adaptive immunity. The innate immune system builds the first line of defense against invading pathogens. It provides anatomical barriers and humoral and cellular responses. This immune response is considered unspecific and it does not confer long-lasting immunity to the host (1). In contrast, the adaptive immune system consists of cells and processes that function systemically to eliminate pathogenic challenges and is able to build a long-lasting memory (1).

Natural Killer Cells and Monocytes

These studies concentrate on two specific cells of the innate immune system, natural killer cells (NK cells) and monocytes. NK cells are relatively large cytotoxic cells with perforin- and granzyme-rich granules in their cytoplasm (2-3). NK cells have the ability to lyse virally infected as well as tumor cells by releasing granzymes and perforin when they bind target cells and subsequently release their cytotoxic granules onto the surface of
the bound cells (4-5). This then leads to penetration of the cell membrane and induction of programmed cell death (1). NK cells also play a role in T- and B-cell-mediated immune responses through the secretion of cytokines such as IFN-gamma (4). Furthermore, NK cells constitutively express the heterodimeric IL-2 receptor on its surface, which makes them susceptible to IL-2 stimulation. This gives NK cells the ability to have elevated cytotoxicity levels against target cells (10-12).

Monocytes are phagocytic cells that circulate in blood and migrate into tissue at the site of inflammation or infection. Here they differentiate into either macrophages or dendritic cells depending on the signals they receive (1). The pro-inflammatory CD16+ subset of monocytes produce pro-inflammatory cytokines such as IL-1, TNFα and IL-6 (21-22).

Cancer

Cancer is one of the most common causes of death in the world and has therefore been, and is still the focus of much research. This disease is characterized by uncontrolled cell growth of the body’s own cells (27). Previously it was believed that a tumor consists of a mass of proliferating, genetically altered, homogenous cells. Data has provided that tumors consist of a heterogeneous population of cells of cancer stem cells (CSCs) and their differentiated counterparts (18-33). The CSCs are a small subset of cells within a tumor that can be defined as cells with self-renewing properties and the capacity to initiate a tumor (34). Emerging evidence has suggested that the capability of tumor growth and propagation is dependent on the CSCs. Though cancer stem cells make up
less than 5 percent of a tumor, they may underlie the cancer and be resistant to conventional chemotherapy and radiation treatments.

**Pancreatic Cancer**

Pancreatic cancer is the 4th deadliest cancer in the United States. Most pancreatic cancers are not diagnosed until after the cancer has spread to other organs and is no longer curable. As a result, the extremely high death rate for pancreatic cancer in this country is approximately equal to the incidence rate (6). Researchers have detected cancer stem cells in tumors from patients with pancreatic cancer. These cells demonstrated the stem cell properties of self-renewal and the ability to produce differentiated progeny. Experiments suggest that these cancer stem cells may help explain the aggressive growth and spread of pancreatic tumors seen in patients.

**Cancer and the Immune System**

The formation and progression of cancer are thought to be effects of immunosuppression and loss of immunological recognition. Natural Killer cells are a type of cytotoxic lymphocyte that constitute a major component of the innate immune system and should play a major role in the rejection of tumors. Yet, it has been shown that the majority of NK cells have actually lost their cytotoxic function in cancer patients (7). Furthermore, it has been shown that there is depressed NK cell proliferation and function in early stages of HNSCC (38-39) and that oral cancer patients show higher levels of CD16+ cells in their peripheral blood mononuclear cells (PBMCs) compared to healthy controls. These
facts may contribute to the observed decrease in NK cell cytotoxicity in oral cancer patients. Additionally, our group has shown that oral cancer stem cells are significantly more susceptible to NK cell mediated lysis, and their differentiated counterparts are significantly more resistant (7). Also, our group has coined the concept “split anergy” to describe that upon NK cell contact with oral cancer stem cells there is a shift in NK cell effector function from cytotoxic to cytokine secreting. This change allows NK cells to provide critical cytokines that play a significant role in the differentiation of these transformed cells. NK cells therefore may actually contribute to the progression of oral cancer by driving the differentiation of tumor cells and allowing the expansion of the sub-population of oral cancer stem cells (7). In addition, monocytes have been shown to be major inducers of resistance in tumor cells and to cause cancer progression in tumor-bearing hosts (23-25). Consistent with these observations, our group could show recently that this protection mechanism is partially achieved by the ability of monocytes to cause up regulation of NFkB in tumors by their secretion of TNFα (7). Overall, the inability of the body’s immune system to effectively resist tumor formation is a complex question that is beginning to be understood, and the roles that NK cells and monocytes play in this cancer development and progression are becoming more well understood. We are uncovering that these cells are at the forefront of tumor escape from the immune system’s defense, and thus fully understanding the many mechanisms of these cells interactions with various types of cancers is so important in order to more well understand how the body’s own immune system and other therapies could actually be used more effectively to treat cancer.
**Cancer Therapy**

Although there have been great advances in research and the number of tumor therapies available has increased enormously in the last years, cancer therapy is still a challenging task for researchers and clinicians. Patients with distinct types of pancreatic cancer have long-term survival rates that still remain very low, and any increasing knowledge of how tumors work in general, with the immune system, or against cancer therapies will only benefit the advancement in tumor treatment.

**Cisplatin, Paclitaxel, and NAC**

Cisplatin is a chemotherapy drug that reacts in vivo. It binds to and causes cross-linking of DNA, ultimately triggering programmed cell death. Cisplatin is administered intravenously as short-term infusion for treatment of solid malignancies. It is used to treat various types of cancers, including sarcomas and some carcinomas. Cisplatin combination chemotherapy is the cornerstone of treatment of many cancers. Initial responsiveness to Cisplatin is high, but the majority of cancer patients will eventually relapse with Cisplatin-resistant disease. Many mechanisms of Cisplatin resistance have been proposed including changes in cellular uptake and efflux of the drug, increased detoxification of the drug, inhibition of apoptosis, and increased DNA repair. The drug Paclitaxel may be useful in the treatment of Cisplatin-resistant cancer; the mechanism for this activity is unknown.
Paclitaxel is a mitotic inhibitor that is used in cancer chemotherapy. Paclitaxel-treated cells have defects in mitotic spindle assembly, chromosome segregation, and cell division. Currently it is used to treat lung, breast, ovarian, and head and neck cancers, and advanced forms of Kaposi’s sarcoma.

N-acetyl cysteine (NAC) is a pharmaceutical drug and nutritional supplement used primarily as a mucolytic agent and in the management of acetaminophen overdose. Thiols such as NAC are increasingly used in clinical trials of platinum chemotherapy as chemoprotectant agents. NAC can prevent Cisplatin-induced cytotoxicities.

**Aim of Study**

This studies initial aim is to demonstrate that similar to differentiated oral tumors, differentiated Pancreatic tumor cells are more resistant whereas their undifferentiated counterparts, or cancer stem cells, are more sensitive to NK cell mediated lysis. We focus on pancreatic cancer because of its aggressive growth and expansion rates, as well as its resistance to eradication. I continue to examine other forms of cancer including lung, breast, and prostate to further confirm that the observed NK/tumor interaction is a universal phenomena characteristic of all cancers regardless of origin. Furthermore, to reveal that anergized NK cells (those that have lost their cytotoxic function and gained the ability to secrete cytokines) may support tumor differentiation, and to examine the mechanism by which these differentiated cells resist NK cell mediated cytotoxicity. Lastly, this study aims to demonstrate the effects of NAC when used with
chemotherapeutic drugs to treat cancer. We would like to show the duel function of NAC, as it protects tumor cells from lysis by Cisplatin chemotherapeutic treatments, yet conversely, when used in conjunction with Paclitaxel, NAC causes a synergistic effect significantly increasing tumor cell lysis versus Paclitaxel treatments alone.
Materials and Methods

Cell lines and cultivation

Please see Table 1 for cultivation media specificities for each cell line used throughout experimentation.

<table>
<thead>
<tr>
<th>Cultivation Media</th>
<th>Media Composition</th>
<th>Cell Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM complete</td>
<td>DMEM + 10% FBS, 1% Sodium Pyruvate, 1% antibiotic/antimycotic, 1% non-essential amino acids (NEAA) and 1% L-Glutamine</td>
<td>MCF7, PC3, MiaPaca, &amp; Panc-1</td>
</tr>
<tr>
<td>RPMI complete</td>
<td>RPMI 1640 + 10% FBS, 1% Sodium Pyruvate, 1% NEAA and 1% antibiotic/antimycotic</td>
<td>NK cells, monocytes, A549, BxPC3, HPAF4 &amp; Capan-2</td>
</tr>
<tr>
<td>RPMI for OSC’s</td>
<td>RPMI 1640 + 0.75g Sodium Bicarbonate, 10% FBS, 1% antibiotic/ antimycotic, 1.4% Sodium Pyruvate, 1.4% NEAA, 1% L-Glutamine and 0.2% Gentamicin</td>
<td>OSCCs &amp; OSCSCs</td>
</tr>
</tbody>
</table>

Determination of cell counts using a hemocytometer

An adequate volume of cells was diluted with PBS and 10µl of this dilution was pipetted into a hemocytometer. The total cell count was then calculated based on the following formula:

\[(\text{cell count of four big squares} / 4) \times \text{dilution factor} \times \text{total volume (mL)} \times 10^3 = \text{total cell count}\]

Purification of human NK cells and monocytes

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from the blood donors and all the procedures were approved by the UCLA-IRB. To isolate the PBMCs out of blood from a human donor, blood was diluted in a 1:1 ratio using PBS in a culture flask. This blood/PBS mixture was then carefully layered onto pre-aliquoted ficoll in 50mL falcon tubes. After centrifuging for 25 minutes at 2000rpm (brakes set at 3) most of the plasma layer was removed and all PBMCs, the clumpy white layer collecting between the ficoll and the plasma/PBS mixture, were collected in new
50mL falcon tubes. After diluting the PBMCs with PBS at a ratio of 1:1 they were centrifuged for 10 minutes at 2000rpm (brakes set at 9). The supernatant was removed and after breaking the pellet all cells were collected in two 50ml falcon tubes diluted with PBS to a total volume of 100mL. A small aliquot of 50µl was taken and diluted with 200µl of PBS to make a cell count. In the meantime, cells were centrifuged one more time for 7 minutes at 2000rpm. After removing the supernatant and breaking the pellet cell suspension was brought up to 50ml with RPMI complete and ~5x10⁶ cells were plated per 10cm culture plate and incubated for 1 hour to separate adherent from non-adherent cells. After checking that monocytes did adhere supernatants of culture plates were collected in falcon tubes. To get all non-adherent cells (peripheral blood lymphocytes = PBLs) off the plate they were carefully washed three times with PBS. About 7mL of RPMI complete was added per culture plate and these were incubated again to save them for monocyte purification later on.

Before starting the NK cell purification, cells were counted, centrifuged for 7 minutes at 2000rpm and the cell pellet was transferred to a 15mL polystyrene tube. Cells were then resuspended in the appropriate amount of ice-cold Robosep buffer to obtain 50x10⁶ cells per ml. For isolation of NK cells 13µl of primary antibody of the Human NK enrichment kit (Stem Cell Technologies) were used per mL of cell suspension, mixed and incubated for 10min at room temperature. After incubation time was over EasySep® D Magnetic Particles were vortexed for 30 seconds and twice as much of the volume of the 1st antibody was used and incubated for 5 minutes at room temperature again. When having less than 1x10⁸ cells they were brought up to 5mL with Robosep buffer and when having
more than $1 \times 10^8$ cells brought up to 8mL, placed inside the magnet tube for 2.5 minutes and then solution was poured out into a fresh tube. Tube was washed once again with either 5ml or 8mL of Robosep and placed again into magnet for 2.5 minutes where after liquid was poured out again in a fresh tube. The two different washes were counted and after centrifugation at 1500rpm for 7 minutes brought up to $10 \times 10^6$ cells per mL with RPMI complete.

To isolate and purify monocytes cells were carefully removed from the plate with a cell scraper and transferred to a 50ml falcon tube. To get all cells off the plates they were washed three times with PBS. After counting a small aliquot, cells were centrifuged for 7 minutes at 2000rpm and the cell pellet was transferred to a 5mL polystyrene tube. Cell count was brought down to $50 \times 10^6$ cells per mL with ice cold Robosep buffer and then 25µl of primary antibody of the *Human monocyte enrichment kit w/o CD16 depletion* (Stem Cell Technologies) per mL were used and incubated for 10 minutes at 4°C. Before adding the same amount of magnetic particles to the cells, particles were vortexed for 30 seconds and after addition then cells were incubated for another 5 minutes at 4°C. Cell solution was then brought up to 2.5mL with Robosep buffer, tube was further placed in the magnet for 2.5 minutes and liquid was poured out into a fresh tube. The procedure was repeated once again to get a second wash. After counting monocytes they were centrifuged at 1500rpm for 7 minutes and then brought up to $1 \times 10^6$ cells per mL with RPMI complete.
Stimulation of freshly isolated NK cells

NK control cells were left untreated and were brought up to a concentration of 1x10^6 cells per mL with RPMI complete for further experiments. To activate NK cells, freshly isolated NK cells at a concentration of 10x10^6 cells per mL were incubated with 5µl of rhIL-2 per 1x10^6 cells for 10 minutes. After incubation, cells were brought up to 1x10^6 cells per mL with RPMI complete. For inactivation of NK cells 5µl anti-CD16 per 1x10^6 cells were used for an incubation time of 10 minutes after which they were brought up to 1x10^6 cells per mL. When cells were first inactivated and then rescued again with IL-2 procedure was performed as described for activation and inactivation.

Co-culture of immune cells with oral tumor cells

Oral tumor cells were plated about 24 hours before NK cells were added to let them adhere to the plate. To achieve this, cells were seeded at a density between 1x10^5 and 3x10^5 in a 12well culture plate. The following day, NK cells in different conditions isolated from human blood were added at a ratio of 0.5:1, 0.75:1, or 1:1 to the attached tumor cells. Incubation was continued for 24 hours, where after supernatants were taken. To get rid of any floating cells, supernatants were centrifuged for 6 minutes at 6000rpm and pellet was discarded.

Enzyme linked immunosorbent assay (ELISA)

The IFN-gamma ELISA is a 3-day protocol, which uses alkaline phosphatase instead of horseradish peroxidase as the converting enzyme resulting in higher sensitivity. Wells of
a 96well flat bottom ELISA plate were coated with 50µl of capture antibody (CA)
(4µg/ml) and incubated overnight at room temperature. The following day, wells were
washed twice with washing buffer and then blocked with blocking buffer for a minimum
of 1 hour. Thereafter, plates were washed twice again and 50µl of supernatants of co-
cultures with NK cells (in duplicates) as well as the standard in twelve different dilutions
(starting with 1µg/ml) were added to the wells and incubated overnight at room
temperature. Then, wells were washed four times and 50µl of detection antibody (DA)
was added (0.5µg/ml) containing 20%FBS. After the 1-hour incubation was over, wells
were washed four times again and a 1:1500 dilution of the goat anti-rabbit alkaline
phosphatase antibody (0.7µg/ml) was added supplemented with 20%FBS. Incubation was
performed for one hour in the dark. Then plates were washed and 50µl PNPP substrate
dissolved in alkaline phosphate buffer was added, incubated in the dark, and measured at
several time points at 405nm wavelength. To analyze and obtain the cytokine
concentrations, standard curves were generated by either two or three fold dilution of
recombinant cytokines provided by the manufacturer.

Surface Staining

To stain cells for distinct surface markers e.g. CD133, CD44, CD54, B7H1, MICA, cells
of interest were harvested and washed twice with ice cold PBS containing 1% BSA.
Antibodies targeted against the surface marker of interest were then added to 5x10⁴ cells
in 50µl of ice-cold PBS-BSA using concentrations as indicated in the manufacturer’s
protocol and were incubated for 30min on ice. After incubation time was over, cells were

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washed twice with ice cold PBS containing 1% BSA and brought up in a 1:50 dilution of a PE-conjugated goat anti-mouse secondary antibody if required. After an incubation period of 30 minutes on ice, cells were washed twice again, where after they were measured for PE fluorescence using a flow cytometer.

**Propidium Iodide (PI) Staining**

Samples in 5mL snap cap tubes were centrifuged at 1500rpm for 5 minutes, the liquid was aspirated, the pellet was broken, and then 4ul PI (at 1mg/mL) in 400ul PBS was added to each sample. Samples were then mixed and analyzed using flow cytometry, measuring PE fluorescence.

**Permeablization**

Samples in 5mL snap cap tubes were centrifuged at 1500rpm for 5 minutes, the liquid was aspirated, the pellet was broken and washed with PBS. The samples were then spun down in centrifuge again at 1500rpm for 5 minutes, liquid aspirated, pellet broken, and then 200ul of ice cold 70% denatured ethanol was added to each sample. The samples were incubated on ice in the dark for 30 minutes. After incubation, the cells were washed with ice cold PBS, spun down in centrifuge at 1500rpm for 5 minutes, liquid aspirated, pellet broken. The samples were then washed with ice cold PBS again, same process repeated. After the pellet was broken, 140ul of PI at 100ug/mL was added to each sample. The samples were then incubated on ice in the dark for 1 hour. When the
samples were ready to be analyzed by flow cytometry, and additional 300µl of cold PBS was added to each sample to have sufficient volume as well as to dilute the cells.

51Cr Release Cytotoxicity Assay

To quantify cytotoxicity levels 1.5-2x10^6 target cells were incubated for 1 hour with 20-40µl of ^51^Cr in a lead container at 37°C. Meanwhile, different titrations of the effector cells were performed. This was done by freshly isolated NK cells (100,000 cells) from human blood were added to the first row of a 96well round bottom culture plate provided with 100µl of RPMI complete per well. Then, wells were resuspended thoroughly and 100µl were transferred from the first into the second row provided with 100µl RPMI complete leading to 50,000 cells in the first row. This was further continued until the last row of the titration. In addition, wells for spontaneous and total release were included containing just 100µl of media at this point. After the chromation time of target cells was over, cells were washed twice with PBS and, after counting, resuspended in media at a final concentration of 1x10^5 cells/ml. Then, 100µl of cell solution (10,000 cells) was added to the wells provided with different titrations of NK cells leading to a starting effector to target ratio (E:T) of 5:1 and were also added to the wells containing just media for the spontaneous and total releases. To allow cell interaction and concentration at the bottom of wells plates were centrifuged for 5 minutes at 1000rpm and then incubated for 4 hours at 37°C. After incubation time was over 100µl of the supernatants were taken and transferred to glass tubes to be measured with a gamma counter. For spontaneous release samples 100µl of the supernatants were taken, whereas for total release wells
were resuspended and then 100µl were transferred to glass tubes. Cytotoxicity was calculated based on the following formula:

\[
\% \text{ Cytotoxicity} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}}
\]

[cpm = count per minute]

LU 30/10^6 is calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells x 100. LU 50/10^6 is calculated by using the inverse of the number of effector cells needed to lyse 50% of target cells x 100.

NK Supernatant recovery and treatment

NK cells were activated using rhIL-2 and/or anti-CD16 mAB as stated previously. Untreated NK cells were left untreated. The cells (at 1x10^6 cells/mL media) were then incubated at 37 degrees and 5% CO2 for 24 hours. After incubation, each NK sample was pelleted by centrifugation at 1500rpm for 4 minutes, and the supernatants were harvested and used immediately or frozen down for later use. For differentiation experiments, tumor target cells were plated in 10 mL of media in 10 cm dishes at varying concentrations, depending on the growth rate of the cell lines. They were allowed to attach for 24 hours, and then 24-hour activated/deactivated/anergized NK supernatants were introduced to the tumors. The cells were cultured for 5-7 days, and then 51Cr
cytotoxicity assays were performed to assess the tumor cells sensitivities to IL-2 activated NK cells mediated lysis. Antibody staining and flow cytometry were also then used to assess any supernatant induced changes in surface expression of B7H1.

**Chemotherapeutic treatments**

Tumor cells were plated at 2x10^5 cells/well in 1mL of media in a 12 well dish. After 24 hours, allowing the cells to attach, the media of each well was changed and appropriate concentration of drugs was added. For Paclitaxel, doses of 10, 20, 30, 40 or 80 µg/mL were used throughout experiments. Treatments with Cisplatin were of 10, 20, 40 or 80 µg/mL. All NAC treatments were 20mM. After 24 hours of incubation, the supernatant from each well was placed in a labeled 5mL snap cap tube. The cells of each well were then trypsinized and added to their supernatant in the respective tubes. The cells and supernatant were spun down at 1500rpm for 5 minutes, the liquid was aspirated, the pellet was broken, and then 4ul PI (at 1mg/mL) in 400ul PBS was added to each sample. The samples were then analyzed for cell death using flow cytometry PE fluorescence.

**Statistical Analysis**

Data were presented as an average ± standard deviation for the statistical analysis. Student t-test was used to show significance.
Results

**Effect of IL-2 activated NK cells on pancreatic, lung, breast and prostate tumors**

IL-2 activated NK cells have significant lysis on tumor cells of the pancreas (BxPC3, Panc-1, Capan-2, HPAF-4), lung (A549), breast (MCF7), and prostate (PC3) (please see Figure 1). Chromium-51 cytotoxicity assays were performed on varying cancer cell lines, and cell death was measured with a gamma-counter. These values were converted into lytic units for more accurate analysis.
Figure 1: 51Cr release assay of untreated and IL-2 activated NK cells on BxPC3 (A), Panc-1 (B), Capan-2 (C), HPAF-4 (D), A549 (E), MCF7 (F), and PC3 (G) tumor cells. Values are represented as average lytic units (LU) ± standard deviation. Differences between untreated NK, and IL-2 treated NK, induced cell death for each of the seven cell lines presented A-G were significant at a p value <0.05. One of a minimum of 2 representative experiments is shown in this figure.
BxPC3 is less differentiated, and a more stem-like pancreatic tumor line based on surface analysis, and more sensitive to NK cell mediated cytotoxicity as reported previously for oral tumors (7) whereas HPAF is more of a differentiated pancreatic cell type and much more resistant to NK cell mediated cytotoxicity (please see Figure 2). The two cell types were selected from 5 pancreatic cell lines based on the highest and the lowest sensitivity to NK cell mediated lysis.

Figure 2: 51Cr release assay of untreated and IL-2 activated NK cells on BxPC3, MiaPaCa, Panc-1, Capan-2, and HPAF-4 tumor cells (A). Values are represented as average lytic units (LU) ± standard deviation. Increase in activated (IL-2) NK susceptibility of BxPC3 compared to HPAF is significant with a p value <0.05. One of a minimum of 2 representative experiments is shown in this figure. Antibody surface staining and flow cytometry results for percent fluorescence of Isotype control mABs, CD54, CD44, MICA, and B7H1 expression for BxPC3 and HPAF tumor cells (B).
The cytotoxic function of purified NK cells were assessed against lung tumors (A549), Breast tumors (MCF7) and Prostate tumors (PC3). A549 was found to be more sensitive to both untreated and IL-2 treated NK cell mediated cytotoxicity than MCF7 or PC3 (please see Figure 3).

![Graph showing 51Cr release assay](image)

**Figure 3**: 51Cr release assay of untreated and IL-2 activated NK cells on A549, MCF7, and PC3 tumor cells (A). Values are represented as average lytic units (LU) ± standard deviation. Differences between A549 and MCF7 or PC3 susceptibility to NKIL2 cell lysis are significant with p-values <0.05. One of a minimum of 2 representative experiments is shown in this figure. Antibody surface staining and flow cytometry results for percent fluorescence of Isotype control mAB and CD44 expression for A549, MCF7, and PC3 tumor cells (B).
**Effect of anergized NK cells on pancreatic and lung tumors**

Untreated NK cells, anti-CD16 mAb treated NK cells (inactivated), IL-2 treated NK cells (activated), and IL2 in combination of anti-CD16mAb treated NK cells (anergized) were used to assess cytotoxicity levels with the different treatments. Chromium-51 cytotoxicity assays were performed on two cancer cell lines, and cell death was measured with a gamma-counter. These values were converted into lytic units for better analysis. IL-2 activated NK cells have significant lysis on tumor cells of the pancreas and lung. Anergized NK cells have significantly less cytotoxic effects than IL-2 activated NK cells (please see figures 4A and 5A). Also, untreated NK cells, anti-CD16 mAb treated NK cells, IL-2 treated NK cells (activated), and IL2 in combination of anti-CD16mAb treated NK cells (anergized) were co-cultured with pancreatic, lung, breast, and prostate tumor cells for 24 hours at an Effector:Target ratio of 0.5:1. After NK/tumor interaction, IFN-gamma secretion by the NK cells was assessed using ELISAs. Significant increases in cytokine secretion are observed in the co-cultures with anergized NK cells (please see figures 4B and 5B).
Figure 4: 51Cr release assay of untreated, anti-CD16 mAB treated, IL-2 treated, and IL-2 + anti-CD16mAB treated NK cells on BxPC3 pancreatic tumor cells (A). Values are represented as average lytic units (LU) ± standard deviation. IFN-gamma secretion by NK cells after co-culture with BxPC3 tumor cells for 24 hours at an effector:target (E:T) ratio of 0.5:1. Significant increase in cytotoxicity with IL-2 treated NKS versus untreated NKS, and significant increase in resistance between IL2+antiCD16 mAB treated NKS versus IL-2 treated NKS is found with p values <0.05. ELISA was performed, and cytokine concentrations were calculated using the generation of standard curve by two-fold dilution of recombinant IFN-gamma provided by the manufacturer. Values are represented as average concentrations (pg/mL) ± standard deviation. Significant increase in INF-g with IL-2+antiCD16 mAB treated NKS versus IL2 activated NKS are seen with p value <0.05. One of a minimum of 2 representative experiments is shown in this figure.
Figure 5: 51Cr release assay of untreated, anti-CD16 mAB treated, IL-2 treated, and IL-2 + anti-CD16mAB treated NK cells on A549 lung tumor cells (A). Values are represented as average lytic units (LU) ± standard deviation. IFN-gamma secretion by NK cells after co-culture with A549 tumor cells for 24 hours at an effector:target (E:T) ratio of 0.5:1. Significant increase in cytotoxicity with IL-2 treated NKs versus untreated NKs, and significant increase in resistance between IL2+antiCD16 mAB treated NKs versus IL-2 treated NKs is found with p values <0.05. ELISA was performed, and cytokine concentrations were calculated using the generation of standard curve by two-fold dilution of recombinant IFN-gamma provided by the manufacturer. Values are represented as average concentrations (pg/mL) ± standard deviation. Significant increase in INF-g with IL-2+antiCD16 mAB treated NKs versus IL2 activated NKs are seen with p value <0.05. One of a minimum of 2 representative experiments is shown in this figure.
A549 lung tumors were cultured with supernatants removed from untreated NK, anti-CD16 mAb treated NK cells, IL-2 treated NK cells, and IL2 in combination of anti-CD16mAb treated NK cells. NK treatments were carried out for 24 hours before the supernatants were removed and used to treat A549 lung tumors for 5 days. As shown in the figure, treatment of A549 with supernatants from the anergized NK cells (IL-2+anti-CD16mAb) for 5 days caused the most resistance to NK cell mediated lysis when exposed to IL-2 treated NK cells (please see figure 6A). Please note the killing of A549 without the NK supernatants are not shown in this figure. Anti-CD16mAb or IL-2 treated NK cell supernatants also caused resistance in A549 cells when compared to A549 treated with supernatants removed from untreated NK cells. In addition, anergized NK supernatants cause the highest increase in B7H1 surface expression (please see figure 6B). Therefore, these results indicated that anergized NK cells are important for the differentiation and resistance of lung tumors.
Figure 6: 51Cr release assay of IL-2 treated NK cells on A549 tumor cells after culture with varying NK supernatants for 5-7 days. Values are represented as average lytic units (LU) ± standard deviation. Decrease in NKIL2 + antiCD16 supernatant treated tumor susceptibility compared to NK supernatant treatment alone is significant with p value <0.05. One of a minimum of 2 representative experiments is shown in this figure. Antibody surface staining and flow cytometry results for percent fluorescence of B7H1 expression for A549 tumor cells after culture with varying NK supernatants for 5-7 days (B).
Similarly, BxPC3 pancreatic tumors were cultured with supernatants removed from untreated NK, anti-CD16 mAb treated NK cells, IL-2 treated NK cells, and IL2 in combination of anti-CD16mAb treated NK cells, yet this time in the presence and absence of monocytes. NK treatments were carried out for 24 hours before the supernatants were removed and used to treat BxPC3 pancreatic tumors for 5 days. As shown in the figure, treatment of pancreatic cells with supernatants from the anergized NK cells (IL-2+anti-CD16mAb) for 5 days caused the most resistance to NK cell mediated lysis when exposed to IL-2 treated NK cells; monocytes induced even further resistance to NK cell mediated lysis (please see figure 7A). Please note the killing of BxPC3 without the NK supernatants are not shown in this figure. Anti-CD16mAb or IL-2 treated NK cell supernatants also caused resistance in BxPC3 cells when compared to BxPC3 treated with supernatants removed from untreated NK cells. In addition, anergized NK supernatants cause the highest increase in B7H1 surface expression; monocytes induced even further increase in B7H1 surface expression (please see figure 7B). Therefore, these results indicated that anergized NK cells are important for the differentiation and resistance of pancreatic tumors, and monocytes protect these pancreatic tumors from NK cell mediated lysis and confer even further differentiation of these cells.
Figure 7: 51Cr release assay of IL-2 treated NK cells on BxPC3 tumor cells after culture with varying NK supernatants, with or without monocyte supernatants, for 5-7 days. Values are represented as average lytic units (LU) ± standard deviation. Decreases in susceptibility to NK cell mediated lysis between tumors with monocytes present compared to those without are significant with p values <0.05. One of a minimum of 2 representative experiments is shown in this figure. Antibody surface staining and flow cytometry results for percent fluorescence of B7H1 expression for BxPC3 tumor cells after culture with varying NK supernatants, with or without monocyte supernatants, for 5-7 days (B).
Similarly, A549 tumors were cultured with supernatants removed from untreated NK, anti-CD16 mAb treated NK cells, IL-2 treated NK cells, and IL2 in combination of anti-CD16mAb treated NK cells. NK treatments were carried out for 24 hours before the supernatants were removed and used to treat A549 tumors for 5 days. As shown in the figure, treatment of lung cells with supernatants from the anergized NK cells (IL-2+anti-CD16mAb) for 5 days caused the most resistance to NK cell mediated lysis when exposed to IL-2 treated NK cells (please see figure 8). Please note the killing of A549 without the NK supernatants are not shown in this figure. In addition, these supernatant treated cells were treated with anti-B7H1 mAB prior to 51Cr release assays, to assess whether blocking surface B7H1 restores the tumor cells sensitivity to IL-2 activated NK cell mediated lysis. No significant decrease in tumor resistance was observed with these antibody treatments. Therefore, these results indicated that anergized NK cells are important for the differentiation and resistance of lung tumors, and blocking surface expression of B7H1 does not restore the sensitivity of these tumor cells to NK cell mediated lysis.
Figure 8: 51Cr release assay of IL-2 treated NK cells on A549 tumor cells, with or without anti-B7H1 mAB treatments, after culture with varying NK supernatants for 5-7 days. Values are represented as average lytic units (LU) ± standard deviation.

No significant differences could be obtained with and without anti-B7H1 treatments for NK-IL2 or NK-IL2 + antiCD16 mAB Supernatant treatment of A549 lung tumors (P=0.5994 and P=0.5036 respectively).
Effect of the combination of NAC and Paclitaxel on oral, pancreatic, lung, prostate and breast tumors

Dose dependent effect of Cisplatin and Paclitaxel on two oral tumors. Stem-like oral tumors, oral squamous carcinoma stem cells (OSCSCs), and differentiated oral tumors, oral squamous carcinoma cells (OSCCs).

Cisplatin has increased effect on differentiated OSCCs whereas it does not kill OSCSCs. Paclitaxel affects both cell types similarly (please see figure 9). Propidium iodide (PI) staining and flow cytometry were used to assess cell death.
Figure 9: PI staining and flow cytometry analysis of cell death after treatment of OSCCs and OSCSCs with varying concentrations (10, 20, 30, 40, or 80 ug/mL) of Cisplatin (cis) or Paclitaxel (pax).
Dose dependent increase in Cisplatin mediated killing of pancreatic cells (BXPC3).

NAC inhibits Cisplatin-mediated killing of pancreatic cells (BXPC3) (please see figure 10). Propidium iodide (PI) staining and flow cytometry were used to assess cell death.

Figure 10: PI staining and flow cytometry analysis of cell death after treatment of BxPC3 pancreatic tumor cells with varying concentrations (10, 20, 40, or 80 ug/mL) of Cisplatin (cis) with or without treatment of N-acetyl cysteine (NAC) at 20mM/mL.
Dose dependent synergistic induction of cell death by NAC and Paclitaxel in BXPC3 pancreatic cells (please see figure 11A); please note HPAF is more resistant to Paclitaxel and NAC mediated cell death than BXPC3. Propidium iodide (PI) staining and flow cytometry were used to assess cell death. Results were further confirmed by assessing DNA fragmentation using ethanol permeablization (please see figure 11B).

**Figure 11**: PI staining and flow cytometry analysis of cell death after treatment of BxPC3 pancreatic tumor cells with varying concentrations (10, 20, or 30 ug/mL) of Paclitaxel (pax) with or without treatment of N-acetyl cysteine (NAC) at 20mM/mL (A). Ethanol permeablization and measurement of DNA fragmentation of the BXPC3 tumors after treatment with Paclitaxel (B).
Dose dependent synergistic effect of NAC and Paclitaxel on lung (A549), prostate (PC3) and breast (MCF7) tumors (please see figure 12). Propidium iodide (PI) staining and flow cytometry were used to assess cell death.

Figure 12: PI staining and flow cytometry analysis of cell death after treatment of A549, MCF7, and PC3 tumor cells with varying concentrations (10, 20, or 30 ug/mL) of Paclitaxel (pax) with or without treatment of N-acetyl cysteine (NAC) at 20mM/mL.
A549 lung tumors were cultured with supernatants removed from untreated NK, anti-CD16 mAb treated NK cells, IL-2 treated NK cells and IL2 in combination of anti-CD16mAb treated NK cells. NK treatments were carried out for 24 hours before the supernatants were removed and used to treat A549 lung tumors for 5 days. All the NK supernatant treated lung tumors were then washed and treated with different concentrations of Cisplatin as shown in the figure and incubated overnight before they were stained with propidium iodide to determine cell death in each sample. As shown in the figure treatment of A549 with supernatants from the anergized NK cells (IL-2+anti-CD16mAb) for 5 days and then exposed to Cisplatin resulted in the highest induction of cell death by Cisplatin. Untreated NK sup or those treated with anti-CD16mAb or IL-2 treated NK cell supernatants also caused low to moderate increases in Cisplatin mediated death of A549 cells when compared to A549 with media alone without NK cell supernatants (please see figure 13). Therefore, these results indicated that anergized NK cell supernatant can not only differentiate the lung cells and cause resistance against NK cell mediated cytotoxicity but it can also make the lung tumors more sensitive to chemotherapeutic drugs such as Cisplatin mediated cells death.
Figure 13: PI staining and flow cytometry analysis of cell death after treatment of A549 cells treated with NK supernatants for 5-7 days, with varying concentrations (20, 40, or 80 ug/mL) of Cisplatin (cis) (A and B). Supernatants from IL-2+anti-CD16mAb treated NK cells differentiate tumors and result in their susceptibility to Cisplatin mediated cell death.
Discussion

Immunosuppression and tumor escape from immune recognition are thought to be integral factors in the establishment and progression of cancer. Natural Killer cells are a type of cytotoxic lymphocyte that constitute a major component of the innate immune system and should play a major role in the rejection of tumors. Yet, it has been shown that the majority of NK cells have actually lost their cytotoxic function in cancer patients. Many factors responsible for the suppression of NK cell cytotoxicity in humans have been identified. The cytotoxic function of NK cell is greatly suppressed after their interaction with cancer stem cells (7). Hence, uncovering the details of NK cells’ cytotoxicity and their interactions with tumors is very important in order to understand and effectively design immunotherapy tumor treatments. In addition, any advance in knowledge of chemotherapeutic treatments alone, with additives, as well as in conjunction with NK tumor function could only aid in the progression of tumor therapy, using this knowledge to implement combinations of NK therapy and chemotherapies to successfully target the tumor cells of interest. These are the broad based goals that the focuses of these studies aim to help complete.

We have characterized the interaction of two pancreatic cancer cell lines and lung, breast, and prostate cancer cell lines with NK cells, and further identified several important profiles that could distinguish between differentiated NK resistant pancreatic, breast, and prostate tumors from undifferentiated NK sensitive pancreatic and lung tumor stem cells. Our results indicated that this observed interaction between NK cells and
tumor cells could be a universal phenomenon. That is, differentiated tumor cells are more resistant to NK cell mediated cytotoxicity, and cancer stem cells are more sensitive to lysis, for all cancers, irrespective of origin. Also, we have uncovered properties explaining the concept “split anergy”, and how in vitro studies can be formulated to aid in the understanding the in vivo interactions of anergized NK cells and tumor resistance and differentiation. We have uncovered some of the physiological roles that anergized NK cells have with cancer stem cells, and how these cells are inducing resistance to NK lysis in these tumors. In addition, we have revealed that contrary to it’s indicated protective effect when used with Cisplatin, N-acetyl cysteine (NAC) and Paclitaxel treatments synergize to actually increase tumor cell death. NAC may therefore be an important factor that could enhance the effectiveness of some chemotherapeutic treatments.

Initially we sought to show that NK cells kill tumor cells. NK cells are cytotoxic cells that have the ability to lyse virally infected as well as tumor cells by releasing granzymes and perforin when they bind target cells and subsequently release their cytotoxic granules onto the surface of the bound cells causing programmed cell death. NK cells constitutively express the heterodimeric IL-2 receptor on its surface, which makes them susceptible to IL-2 stimulation. This gives NK cells the ability to have elevated cytotoxicity levels against target cells (10-12). Here we show that when NK cells are activated with IL-2 they significantly lyse tumor cells of varying origin. We proved this effect on several pancreatic cancer cells (BxPC3, HPAF4, Panc-1, MiaPaca, and Capan2) as well as lung (A549), breast (MCF7), and prostate (PC3) tumor cells.
Previously our group has shown that oral cancer stem cells are more susceptible to NK cell mediated cytotoxicity when compared to their respective differentiated counterparts (7). They proved that IL-2 activated NK cells mediate much increased cytotoxicity on primary oral squamous carcinoma stem cells (OSSCCs) versus their differentiated counterparts, oral squamous carcinoma cells (OSCCs). We wanted to expand these findings by putting emphasis and focus on the mechanistic properties relating to NK interaction with pancreatic cancer due to this type of cancer’s extreme resistance to conventional therapies. Pancreatic cancer is very hard to eradicate, thus uncovering how our immune system interacts with these types of tumor cells could aid in increasing tumor therapy effectiveness. Here we were able to advance these properties of oral tumors to pancreatic cancer, proving increased lysis of pancreatic cancer stem cells (BxPC3s), but not their differentiated counterparts (HPAFs) by NK cells. In addition, we characterized A549, MCF7, and PC3 tumor cell lines amongst each other, and found that A549 seems to be a more stem like cell population when compared to MCF7 and PC3, and indeed NK cells mediate higher cytotoxicity on these cells when compared to MCF7 or PC3. Thus, we have essentially universalized the idea that differentiated tumor cells are more resistant to NK cell mediated cytotoxicity, and cancer stem cells are more sensitive to lysis, for all cancers, irrespective of tumor origin. We started out the studies with five pancreatic tumor cell lines: BxPC3, HPAF4 (HPAF), Panc-1, MiaPaca, and Capan2. We ran cytotoxicity assays on these cells in order to determine their relative sensitivity or resistance to NK cell mediated lysis. We used chromium-51 release assays. The gamma counter reading for the measurement of the amount of chromium in each
sample was directly related to the amount of death of the tumor cells. We converted these readings into lytic units, which represent the inverse of the amount of NK cells it takes to kill 30% of the tumor cells, multiplied by 100. These conversions allow all values to be easily analyzed and comparisons to be made between the varying cell lines within a single experiment. These experiments proved BxPC3 to be the most sensitive to NK cell mediated lysis, and HPAF to be the most resistance. From these findings, we performed surface analyses of these cells to confirm the characterization and identification of these two cell lines. We used monoclonal antibody staining for isotype control, CD54, CD44, MICA, B7H1, and CD133. HPAFs were found to have higher surface expression of B7H1 and MICA and moderate expression of CD44 and CD54, whereas BxPC3 expressed no or very low expression of B7H1 and MICA and increased expression of CD44 and CD54. No surface expression of CD133 (data not shown) could be seen on either tumor type. Furthermore, BxPC3s were smaller in size and proliferated at a much higher rate when compared to HPAF cells (data not shown). We then chose these two cells lines to represent a pancreatic cancer stem cell population, BxPC3, and its differentiated counterpart, HPAF, based on their phenotypic characteristics and sensitivity to NK cell mediated cytotoxicity. Hence, we used these two pancreatic tumor cell lines to further study NK cell function. Similar experiments were performed on lung (A549), breast (MCF7), and prostate (PC3) tumors. These studies proved A549 to be the most sensitive cells to NK cell mediated lysis, while MCF7 and PC3 showed similar higher resistances to NK cell cytotoxicity. Upon surface analysis, results confirmed A549 to be the most stem cell population (highest level of CD44 surface expression),
while the other two cell lines represent differentiated tumor cell populations. A549s were also smaller in size and proliferated at a much higher rate when compared to MCF7s or PC3s (data not shown). Collectively these results expanded the previous findings that transformed stem cells of varying origin, pancreas, lung, breast, and prostate, are highly susceptible to NK cell mediated lysis compared to their respective differentiated counterparts. Thus, the stage of maturation and differentiation of tumor cells is predictive of their sensitivity to NK cell lysis, regardless of origin.

With this expansion on NK cell lysis function, our next goal was to confirm the concept of NK cell “split anergy,” and to expand the idea of it’s effects on cancer cells with our cell lines of choice, in order to, once again, expand the concept to encompass tumor cells in general, rather than oral tumor cells specifically. First we had to answer the question of how to induce split anergy in NK cells. In vitro, our group has shown previously that the act of “anergizing” NK cells (activating with IL-2 and treating with anti-CD16 mAB) causes a switch in effector function from cytotoxic to cytokine secreting. A decrease in cytotoxicity of these cells is paralleled with an increase in IFN-gamma secretion. This is significant because in vivo it has been shown that NK cells which have no or low CD16 receptor expression have decreased competence to mediate cytotoxicity, yet have the ability to secrete significant amounts of cytokines (“split anergy”). Since it has also been shown that after NK cells interact with tumor cells in vivo they lose their cytotoxic function and gain in cytokine secretion phenotype and down modulate CD16, it is tempting to speculate that in vivo identified CD16 negative NK cells and in vitro tumor induced CD16 negative NK cells may have similar
developmental pathways since they have similar if not identical functional properties. Thus, we can explore the interaction that NK cells in a cancer patient’s body have with the tumor cells present through co-culture and cytotoxicity assays. Here we were able to trigger CD16 receptors on NK cells using anti-CD16 mAB, giving split anergy of the NK cells, which significantly decreased their cytotoxic function and increased IFN-gamma secretion when incubated with pancreatic and lung. Anergized NK cells have significantly lower cytotoxicity on BxPC3 and A549 tumor cells, and this decrease in lysis is coupled to significant increases in IFN-gamma secretion with tumor cell interaction. Cytotoxicity was measured using chromium-51 release assays, and cytokine secretion was analyzed using enzyme linked immunosorbant assay (ELISA) for the protein IFN-gamma.

Next, in order to uncover some of the physiological roles that anergized NK cells have on stem cells, we chose to culture lung (A549) and pancreatic (BxPC3) tumor stem cells with the supernatants of NK cells of varying treatments (untreated, anti-CD16 mAB deactivated, IL-2 activated, and IL-2 + anti-CD16 mAB anergized), and then compare the sensitivities of these cultured tumor cells to IL-2 activated NK cell mediated lysis. With both A549s and BxPC3s, we found a significant increase in resistance to lysis with the tumor cells that were treated with the supernatants of the anergized NK cells. These supernatants contained all of the cytokines that anergized NK cells secrete, and thus it is these cytokines that induced resistance to these tumor stem cells. We also performed antibody staining and flow cytometry to analyze any changes in surface expression of these supernatant treated tumor cells. We found that when cultured with the anergized
NK cell cytokines, the tumor stem cells had a significant increase in B7H1 expression, a marker for cell differentiation. Thus, we can conclude that anergized NK cells secrete factors that cause differentiation of lung and pancreatic stem cells. Since anergized NK cells have significant increases in IFN-gamma secretion, we can speculate that these NK cells have lost their cytotoxic function, gained in IFN-gamma cytokine secretion, and herein supported the differentiation of lung and pancreatic tumor stem cells.

Furthermore, through this we have actually started to unravel the mechanism of these cytokine secreting NK cells inducing resistance to tumor cells from NK cell mediated lysis. Up regulation of B7H1 on the tumor cells displays that it may be an important factor that confers resistance to NK lysis on NK-induced differentiated tumor cells. B7H1 may therefore be an important factor of differentiation, survival, and function of tumors during their interaction with NK cells in the body. We also used antibodies to block B7H1 expression on these supernatant treated cells to see if we could restore the tumor cells sensitivity to IL-2 activated NK cell lysis, but did not see significant changes in tumor cell cytotoxicity. Further studies using genetic modulation of B7H1 could further reveal its importance in the resistance of these tumor cells. In addition, we added the affects of monocytes in split anergy to this type of experimental protocol to see if the monocytes were able to protect the stem cells even further from NK cell mediated cytotoxicity. Indeed, we found that when NK cells interact with monocytes prior to supernatant harvest, it causes even further decrease in cytotoxicity and increase in B7H1 expression of BxPC3 cells. Overall, we have uncovered that anergized NK cells support the differentiation of pancreatic and lung tumors, and increase their resistance against NK
cell mediated lysis. Based on the results we can speculated that the inability of cancer patient NK cells to kill cancer stem cells is due to flooding of NK cells by proliferating cancer stem cells and the consequent conversion of NK cells to cytokine secreting cells. This may likely be a mechanism by which cancer stem cells remain viable and proliferate.

The second large branch of our study explores factors relating to Cisplatin and Paclitaxel chemotherapeutic treatments. Cisplatin and Paclitaxel are two drugs used in chemotherapy that have different mechanisms for inducing cell lysis. Cisplatin reacts in vivo by binding to and causing cross-linking of DNA, ultimately triggering programmed cell death. In turn, Paclitaxel is a mitotic inhibitor that produces defects in mitotic spindle assembly, chromosome segregation, and cell division in target cells. So basically, in addition to the immunotherapeutic avenues that we have until this point focused on, we also wanted to uncover some of the physiological roles of these chemotherapy drugs, and the roles that N-acetyl cysteine (NAC) may play when used with these drug treatments.

We implemented incremental drug treatments on varying tumor cell lines. After the drugs were allowed to react with the cells for about 24 hours, the cells and supernatants were harvested, and propidium iodide staining followed by flow cytometry analyses for PE fluorescence were performed to assess cell death. Our studies revealed that Cisplatin has increased effect on differentiated OSCCs whereas it does not kill OSCSCs, while Paclitaxel affects both cell types similarly. We used these very well established primary oral tumor cells to show that Cisplatin has increased cell death with differentiated oral tumor cells, and low or no lysis on oral tumor stem cells. Paclitaxel did not have
significant differences in lysis between the two cell types. We also found a dichotomy in the effect of NAC when used in conjunction with these two different drug treatments. NAC inhibits Cisplatin mediated killing of pancreatic tumor cells (BxPC3s), but conversely synergizes with Paclitaxel inducing increased cell death in pancreatic (BxPC3), lung (A549), breast (MCF7), and prostate (PC3) tumor cell lines.

Data has shown that thiols such as NAC are increasingly used in clinical trials of Cisplatin chemotherapy as chemoprotectant agents. These studies illustrated that NAC can prevent Cisplatin-induced cytotoxic side effects. We confirmed these results with our BxPC3 cell line. Our data showed that NAC rescued the pancreatic tumor cells from Cisplatin-induce lysis, thus we used these experiments as controls, hence confirming our observed synergy of NAC and Paclitaxel to indeed be significant. We wanted to insure that our synergistic observations were not an observation specific to these cell lines only or effects from NAC regardless of the chemotherapy drug used, and that this observed increase in cell lysis is with Paclitaxel treatments specifically. These experiments prove that NAC possesses multiple functions when used with chemotherapeutic treatments: decreased or increased cytotoxicity, and thus can be used in varying treatment plans to produce different desired outcomes.

With so much effort being focused on immunotherapy and chemotherapy mechanisms and treatments, experimental studies to examine the combination of some of these tumor treatment options seemed like a great next step to this project. We wanted to uncover some of the physiological roles of anergized NK cells in chemotherapeutic treatments. We decided to use anergized NK cell supernatants (as well as the other NK
supernatants of varying treatments to use for comparison) to differentiate A549 lung tumors, and then subsequently perform Cisplatin treatments on these cells. This experimental design thus represents the effects that Cisplatin has on tumor cells in the body that are inherently being affected by the present non-cytotoxic/cytokine secreting anergized NK cells. Treatment of A549 with supernatants from the anergized NK cells for 5 days and then exposed to Cisplatin resulted in the highest induction of cell death by Cisplatin. Untreated NK supernatant or those treated with anti-CD16mAb or IL-2 treated NK cell supernatants also caused low to moderate increases in Cisplatin mediated death of A549 cells when compared to A549 with media alone without NK cell supernatants. These results indicate that anergized NK cell supernatant can not only differentiate the lung cells and cause resistance against NK cell mediated cytotoxicity but it can also make the lung tumors more sensitive to chemotherapeutic drugs such as Cisplatin mediated cell death.

Significance of these experiments can be confirmed through the fact that data has revealed that tumors are not homogeneous. Tumors are heterogeneous populations of cells containing cancer stem cells (CSCs) and their differentiated counterparts. Therefore, one way to target the tumor population as an entirety would be to use two distinct strategies to eliminate tumors, one, which targets stem cells, and the other, which targets differentiated cancer cells. In theory, based on our previous data, this could be achieved in cancer patients by the use of a combination of activated NK cells that should target the stem cells, alongside chemotherapeutic treatments such as Cisplatin that would eliminate differentiated tumor cells. However, since the great majority of patient NK cells
have modified their phenotype to support differentiation of the tumor cells, they may not be effective in eliminating the target cancer stem cells. Therefore, cancer stem cells may accumulate and eventually result in the demise of the patient. These patients may therefore, benefit from the repeated allogeneic NK cell transplantation for elimination of cancer stem cells.

NK immunotherapy and chemotherapy are at the forefront of tumor treatments, thus understanding their individual mechanistic affects, as well as how these systems could work together to produce the most effective tumor therapies is very important. Our data gives great evidence to the therapeutic effects that repeated allogeneic NK transplants could have on tumor patients. We have made much progress in understanding this effector/target cell interaction, and how treatments must be designed in order to produce the desired results. Our discovered synergistic effects of Paclitaxel and NAC could also be a great advancement in treatment design, as could be the combination of NK cells effects and chemotherapy drugs.

There are many avenues in which I would like explore to further my studies. Using shRNA to block the expression of B7H1 would aid in revealing its importance in tumor cell resistance to NK cell mediated lysis. Over expression of this protein could also provide significant data to further our understanding of their observed up regulated surface expression from anergized NK cytokines. In addition, uncovering the mechanistic effects of NAC causing cell death synergy with Paclitaxel would be a focus that I would like to concentrate on.
The ongoing experiments in our laboratory elucidated the mechanisms governing pancreatic tumor cell survival and differentiation, and furthermore, provided potential therapeutic strategies for cancer treatment. In vitro experiments demonstrated that NK cells play a significant role in limiting growth and expansion of pancreatic stem-line tumors. They demonstrated that stem-like/poorly differentiated pancreatic tumors are lysed by the NK cells, whereas their differentiated counterparts are more resistant to NK cell mediated lysis. The data indicated that NK cells are able to limit the growth and expansion of pancreatic tumors by providing IFN-g and TNF-a, the combination of which drives differentiation of stem-like/poorly differentiated pancreatic tumors. The data suggested that tumor differentiation by NK cells limits cell growth and, in addition, inhibits inflammatory cytokine release which could fuel tumor growth. Furthermore, in vivo studies verified that poorly differentiated pancreatic tumors formed large tumors and metastasized to liver and lungs in NSG (immunodeficient) mice, whereas their NK differentiated tumors were unable to form tumors in the pancreas of these same mice. Also, well differentiated pancreatic tumors when implanted in the pancreas formed much smaller tumors, and the mice survived for a long period of time, whereas those implanted with stem-like/poorly differentiated tumors all died a few weeks after implantation and tumor growth (manuscript in prep). Further studies on cancer therapy were also performed. We demonstrated that differentiated tumors responded to and were lysed by chemotherapeutic drugs cisplatin and paclitaxel, whereas stem-like/poorly differentiated tumors were highly resistant. Furthermore, the addition of NAC with paclitaxel induced cell death whereas it inhibited cisplatin mediated cell death demonstrating distinct
mechanisms of drug effects. We also demonstrated that NAC induced an increase in cell death of differentiated oral and pancreatic tumors, but had no/low effect on stem-like/poorly differentiated tumors. Fascinatingly, in vivo NK therapy was also performed. Single injections of expanded potent NK cells were able to prevent tumor growth and also mediated significant differentiation of the tumors and resistance of tumor to NK cell mediated cytotoxicity. Together, these results are in great agreement with the in vitro studies, indicating that differentiation is an important step in inhibition of tumor progression and cessation of inflammation. It is likely that continued supplementation with NK cells may be required for the complete eradication of the tumors since the removal leads to reversion of the tumor growth. Collectively, these results indicate the significance of NK cell function in successful treatment of the patients both with and without chemotherapeutic drugs. Patients with advanced tumors usually present with loss of NK cell numbers and function and therefore are at risk for cancer progression. In addition, since in these patients chemotherapy usually is ineffective in controlling the tumor growth, a combination of chemotherapy and immunotherapy should be effective in controlling the progression of the cancer (Ou Maung et al, manuscript in prep).
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