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NK cells lyse poorly differentiated but not well-differentiated pancreatic cancer cells; role of NK cells in selection and differentiation of pancreatic cancer stem cells

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

By

Phyu Ou Maung

2014

ABSTRACT OF THE THESIS

NK cells lyse poorly differentiated but not well-differentiated pancreatic cancer cells; role of NK cells in selection and differentiation of pancreatic cancer stem cells

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Phyu Ou Maung

Master of Science in Oral Biology

University of California, Los Angeles, 2014

Professor Anahid Jewett, Chair

Pancreatic cancer remains one of the major unresolved health problems. Conventional cancer treatments have little impact on disease course. The response to conventional therapies such as radiotherapy or chemotherapy is poor and has little or no effect on the natural progress of this malignancy. Therefore, new strategies to tackle pancreatic cancers are needed to reduce delayed diagnosis, relative chemotherapy and radiation resistance, and an intrinsic biologic aggressiveness contributing to the abysmal prognosis associated with pancreatic adenocarcinoma. The background knowledge of understanding tumor microenvironment and mechanism between immune cells and tumorigenesis are still unclear. However, immunosuppression and tumor escape from immune recognition are thought to be the two major

factors responsible for the establishment and progression of cancer. A number of factors responsible for the suppression of NK cell cytotoxicity in humans have been identified previously. In this study, we investigated that NK cells limit the number of stem cells and immune inflammatory cells by selecting those with a greater potential for differentiation in the repair process of the tissues and they also support differentiation of the stem cells and subsequent regeneration of the tissues. Therefore, immunotherapy would be the future goal to achieve success in curing pancreatic cancers.

The thesis of Phyu Ou Maung is approved.

Ichiro Nishimura

Nicholas A Cacalano

Anahid Jewett, Committee Chair

University of California, Los Angeles

2014

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Introduction

Pancreatic cancer is one of the major unresolved health problems. Conventional cancer treatments have little impact on disease course. Ninety-five percent of pancreatic cancers are ductal adenocarcinomas and originate from the lining of the pancreatic duct, which is the exocrine part of the pancreas producing digestive juices. Pancreatic adenocarcinoma is a highly lethal disease and more than 80% of patients are usually diagnosed with pancreatic cancer at a locally advanced or metastatic stage because of late clinical presentation of signs and symptoms and limitations in diagnostic methods. Therefore, although a small number of patients undergo curative surgical resection, most patients capitulate to recurrent and metastatic disease. The response to conventional therapies such as radiotherapy or chemotherapy is poor and has little or no effect on the natural progress of tumors. Despite advances in surgical and medical therapy, little effect has been made on the mortality rate of pancreatic cancers [1,2]. It has the worst prognosis of any major malignancy (3% of 5-year survival rate). Pancreatic cancers rank the fourth most common cause of cancer death yearly in the United States and the twelfth in worldwide [3,4]. One of the major hallmarks of pancreatic cancer is its extensive local tumor invasion and early systemic dissemination. Significant advancements have been made over the past two decades in elucidating important molecular pathways that are involved in pancreatic neoplasia [5]. However, these advancements have not resulted in an improvement in the outcomes for this disease. Therefore, new strategies to tackle pancreatic cancers are needed to reduce delayed diagnosis, relative chemotherapy and radiation resistance, and an intrinsic biologic aggressiveness contributing to the abysmal prognosis associated with pancreatic adenocarcinoma.

Gene and protein expression profiles associated with pancreatic cancers have been widely studied in order to understand the molecular characteristics of pancreatic cancer. However, the heterogeneity of cancer cells within a particular tumor has not been accounted in to these types of studies. Emerging evidence has shown that the capacity of a tumor to grow and propagate is dependent on a small subset of cells. This concept was originally based on the observation that when pancreatic cancer cells of many different types were assayed for their proliferative potential in various *in vitro* or *in vivo* assays, only a minority of cells showed extensive proliferation. This observation supported the idea that malignant tumors are composed of a small subset of distinct cancer stem cells, which have great proliferative potential, as well as more differentiated cancer cells, which have very limited proliferative potential [7]. Therefore, this small subset of cancer stem cells follows the cancer stem cell (CSC) theory.

There is a relationship between chronic inflammation and tumorigenesis which has been recognized in many pathologic conditions, including hepatocellular carcinoma in patients with hepatitis B and C infection, colon cancer in patients with inflammatory bowel disease, esophageal cancer in patients with Barrett's metaplasia, and gastric cancer in patients with chronic *H pylori* infection. A similar relationship has been found between chronic pancreatic inflammation and pancreatic cancer. The patients with hereditary pancreatitis have 40% risk to suffer from pancreatic cancer [8].

Inflammation plays a role in tumorigenesis; however, this correlation has not been proven yet [9]. Only a minority of all cancers are caused by germ line mutations, whereas the vast majority (90%) are linked to somatic mutations and environmental factors. Many environmental causes of pancreatic cancers and their risk factors are associated with some forms of chronic inflammation. Up to 20% of cancers are linked to chronic infections, 30% can be attributed to

tobacco smoking and inhaled pollutants (such as silica and asbestos), and 35% can be attributed to dietary factors (20% of cancer burden is linked to obesity and intake of high caloric beverages) [10].

Chronic inflammation increases cancer risks. Various types of immune and inflammatory cells are frequently present within tumors. Immune cells affect malignant cells through production of cytokines, chemokines, growth factors, prostaglandins, and reactive oxygen and nitrogen species. Inflammation impacts every single step of tumorigenesis, from initiation through tumor promotion, all the way to metastatic progression. In developing tumors anti-tumorigenic and protumorigenic immune and inflammatory mechanisms coexist. If the tumor is not rejected, the protumorigenic effect dominates. Signaling pathways that mediate the protumorigenic effects of inflammation are often subject to a feed-forward loop (for example, activation of NFκB in immune cells induces production of cytokines that activate NFκB in cancer cells to induce chemokines that attract more inflammatory cells into the tumor). Certain immune and inflammatory components may be dispensable during one stage of tumorigenesis but absolutely critical in another stage [11].

Immunosuppression and tumor escape from immune recognition are thought to be the two major factors responsible for the establishment and progression of cancer. A number of factors responsible for the suppression of NK cell cytotoxicity in humans have been identified previously [12-17]. However, the significance and the precise mechanism of NK suppression induced during their interaction with either tumor cells or healthy primary cells are not well understood.

Natural Killer Cells (NK cells) are a type of cytotoxic lymphocyte critical to the innate immune system. The role of NK cells is analogous to that of cytotoxic T cells in the vertebrate

adaptive immune response. NK cells belong to the group of innate lymphoid cells and they are defined as large granular lymphocytes (LGL). They differentiate from the common lymphoid progenitors [18]. NK cells are known to differentiate and mature in the bone marrow, lymph node, spleen, tonsils and thymus where they then enter into the circulation [19].

NK cells provide rapid responses to virally infected cells and respond to tumor formation, acting at around 3 days after infection. Typically immune cells detect MHC presented on infected cell surfaces, triggering cytokine release, causing lysis or apoptosis. NK cells are unique, however, as they have the ability to recognize stressed cells in the absence of antibodies and MHC, allowing for a much faster immune reaction. They were named "natural killers" because of the initial notion that they do not require activation in order to kill cells that are missing "self" markers of major histocompatibility complex (MHC) class 1 [20].

In humans, two distinct NK cell subsets have been characterized according to the cell surface density of CD56 and the expression of CD16 (FcγRIIIa): the $CD56^{dim}$ $CD16^{bright}$ NK cell subset that comprise approximately 90% of circulating NK cells, and the $CD56^{bright}$ $CD16^{neg/dim}$ NK cell subset that accounts for approximately 10% [21]. The $CD56^{dim}$ $CD16^{bright}$ NK subset expresses KIR and/or CD94/NKG2A HLA-specific receptors as well as chemokine receptors such as CXCR1, CX3CR1 and ChemR23; the $CD56^{bright}$ $CD16^{neg/dim}$ NK cell subset expresses NKG2A and the chemokine receptor CCR7 but not killer immunoglobulin-like receptors (KIRs) [21, 22, 23, 24]. Based on their chemokine receptor profile, it is conceivable that $CD56^{dim}$ $CD16^{bright}$ cells may be mainly recruited into inflamed peripheral tissues, whereas $CD56^{bright}$ $CD16^{neg/dim}$ cells could be attracted to secondary lymphoid compartments, such as lymph nodes, in response to CCL19 and CCL21 [25]. Indeed, NK cells localized in inflamed peripheral tissues are mostly $CD56^{dim}$ and express CXCR1 and ChemR23 [23] whereas NK cells localized within

normal non-inflamed lymph nodes are homogeneously characterized by the $CD56^{bright}$ $CD16^{neg/dim}$, KIR^{-}, $CD94/NKG2A^{+}$ surface phenotype [21, 22, 26].

$CD56^{bright}$ NK cells, following monokine stimulation, proliferate and produce immunoregulatory cytokines, including IFN-γ, TNF-α and GM-CSF, whereas $CD56^{dim}$ NK cells are more cytolytic and produce significant amounts of chemokines and proinflammatory cytokines when their activating receptors are engaged. In particular, chemokine production by $CD56^{dim}$ NK cells can be induced by low levels of stimulation (e.g., engagement of individual activating receptors) and occurs more rapidly than the release of IFN-γ and TNF-α [27]. On the other hand, a recent study shows that $CD56^{dim}$ NK cells may also release high amounts of IFN-γ very early after activation [28]. Moreover, $CD56^{dim}$ NK cells represent important effectors not only for their ability to kill abnormal cells but also for their ability to induce rapid inflammatory responses involving the recruitment of other defensive cells and promotion of cellular resistance to infection together with initial shaping of adaptive immune responses. Taken together, these data indicate that both $CD56^{bright}$ and $CD56^{dim}$ NK cells may have an important immunoregulatory role.

We hypothesized in our studies that there are two critical functions of NK cells such as;

1. To limit the number of stem cells and immune inflammatory cells by selecting those with a greater potential for differentiation for the repair of the tissue and
2. To support differentiation of the stem cells and subsequent regeneration of the tissue.

It is shown that freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors. Moreover, NK cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [29, 30, 31, 32]. In addition, NK cell cytotoxicity is suppressed after their interaction with stem cells [33, 34, 35]. In contrast the

interaction of NK cells with the resistant tumors does not lead to suppression of NK cell cytotoxicity toxicity when compared to those dissociated from the NK sensitive target cells [36]. Many mechanisms have been proposed for the functional inactivation of tumor associated NK cells including the over-expression of Fas ligand, the loss of mRNA for granzyme B [14] and decreased CD16 and its associated zeta chain [37]. Traditionally, the suppression of NK cell cytotoxic function after their interaction with the sensitive cells was sometimes perceived to be due to the exhaustion of cytotoxic granules from the NK cells, however, our recent data indicates that such suppression is physiological and it is an important step in maturation of NK cells to support differentiation of other cells, and in the resolution of inflammation [38].

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 [36, 39]. 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 [40, 41, 42]. 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 termed coined by our lab that describes the loss of NK cytotoxicity, but a gain in the ability of NK cells to secrete cytokines.

Therefore, advanced understanding in relationships between immune cells and tumor microenvironments will engage to achieve higher survival rate from cancer related death.

Thesis outline

Specific Aim 1: Characterization of pancreatic cancer cells according to the level of NK cells cytotoxicity and surface expression and introduction of split anergy of NK cells.

Specific Aim 2: Role of anergized NK cells in selection and differentiation of pancreatic cancer stem cells

- **Sub-aim 2A**: Whether anergized NK cells lead to differentiation of pancreatic cancer stem cells.
- **Sub-aim 2B**: Whether the induction of resistance in MP-2s by anergized NK cells is blocked by combination of IFN-γ and TNF-α antibodies.
- **Sub-aim 2C**: Whether the induction of resistance of MP-2s is mediated by the combination of recombinant human IFN-γ and TNF-α.
- **Sub-aim 2D**: Whether differentiation of MP-2s by anergized NK cells lead to decrease secretion of cytokines and chemokines.

Specific Aim 3: Targeting differentiated pancreatic tumors not stem-like tumors by chemotherapeutic drugs

- **Sub-aim 3A:** Whether stem-like pancreatic tumors are resistant to chemotherapeutic drugs.
- **Sub-aim 3B:** Whether stem-like pancreatic tumors differentiated by anergized NK cells become susceptible to chemotherapeutic drugs.

Chapter 1

Specific Aim 1: Characterization of pancreatic cancer cells according to the level of NK cells cytotoxicity and surface expression and introduction of split anergy of NK cells

Introduction:

Mounting effective anti-tumor immune responses by cytotoxic effectors is important for the clearance of tumors. However, accumulated evidence suggests that the cytotoxic function of immune effectors is largely suppressed in the tumor microenvironment by a number of distinct effectors and their secreted factors. The previous studies have shown that increased NK cell function was seen when they were cultured with primary oral squamous carcinoma stem cells (OSCSCs) as compared to their more differentiated oral squamous carcinoma cells (OSCCs). In addition, human embryonic stem cells (hESCs), Mesenchymal Stem Cells (hMSCs), dental pulp stem cells (hDPSCs) and induced pluripotent stem cells (hiPSCs) were significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts or parental cells from which they were derived [43].

It has been also reported that inhibition of differentiation or reversion of cells to a less-differentiated phenotype by blocking NFκB or targeted knock down of COX2 augmented NK cell function significantly [43]. Total population of monocytes and those depleted of CD16 (+) subsets were able to substantially prevent NK cell mediated lysis of OSCSCs, MSCs and DPSCs. Of greater concern is those previous findings suggesting that stem cells are significant targets of the NK cell cytotoxicity [43]. 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.

Split anergy is a term coined by our laboratory for the responses observed by NK cells after their interaction with sensitive target cells or after triggering of CD16 receptors by the antibody in combination with IL-2 treatment. Treatment of NK cells with IL-2 and anti-CD16 mAb induced significantly decreasing the NK cell cytotoxicity while increasing the cytokine secretion capabilities of NK cells [43]. The previous studies had shown the profiles which we had seen when MSCs, hDPSCs, OSCSCs, hESCs, hiPSCs were cultured with NK cells treated with IL-2 and anti-CD16 mAb in which significant decrease in cytotoxicity of NK cells was observed in parallel with increased secretion of IFN-γ (split anergy) [43].

Pancreatic cancer is one of the most life threatening cancers all over the world. The motility rate of pancreatic cancer is high and the estimated number of death is not less than 33,000 people per year only in the USA. This number has been quite steady over the past 3–5 years [3]. Early detection and diagnosis are not easy to establish because signs and symptoms of early stage of pancreatic cancer are similar to general illnesses. Therefore, it is already late if the clinicians find out the diagnosis such as late or advance pancreatic cancer with or without metastasis [2]. The potential treatment procedures are exciting the affected area of pancreas and metastasized organs, giving radiation and chemotherapy. The survival rate of pancreatic cancer is determined by the months after surgery and/or combined therapy of chemotherapy and radiation therapy [2]. The prognosis of pancreatic cancer treatment is extremely low and 6 months to 5 years survival rate may vary from 25% to 5% respectively [3].

Specific aim 1 was to find out the characterization of pancreatic cancer cells, to investigate whether NK cells lysed poorly differentiated but not well-differentiated pancreatic cancer cells according to the level of NK cells cytotoxicity against pancreatic cancer cells and

level of surface expression on tumor cells and to demonstrate the induction of split anergy of NK cells in pancreatic cancer.

Materials and Methods

Cell lines, Reagents and Antibodies

Human pancreatic cancer cell lines Panc-1, MIA PaCa-2 (MP-2), BxPC-3, HPAF-II, Capan-1 were generously provided by Dr. Graham Donald (UCLA, Los Angeles, CA, USA) and pancreatic cancer cell line 12 (PL-12) was kindly contributed by Dr. Nicholas Cacalano (UCLA, Los Angeles, CA, USA). Panc-1, MP-2 and BxPC-3 were cultured DMEM (Dulbecco's Modified Eagle Medium) in supplement with 10% Fetal Bovine Serum (FBS) and 2% Penicillin-Streptomycin (Pen-Strep). HPAF-II, Capan-1 and PL-12 were cultured in RMPI 1640 medium supplemented with 10% FBS and 2% Penicillin-Streptomycin (Pen-Strep). The human NK cells enrichment immunomagnetic negative selection kit was purchased from Stem Cell Technologies (Vancouver, Canada). Isotype control IgG2b κ and antibodies against CD16, CD44, CD54, CD274 (B7-H1, PD-L1) and HLA-A, B, C were purchased from Biolegend (San Diego, CA, USA).

Panc-1

Panc-1 was cultured from a 56-year-old male with an adenocarcinoma in the head of the pancreas, which invaded the duodenal wall. Metastases in one peri-pancreatic lymph node were discovered during a pancreaticoduodenectomy. In culture, the cell line was not found to secrete significant carcinoembryonic antigen [44].

MIA Paca-2 (MP-2)

MP-2 was derived from the pancreas adenocarcinoma of a 65-year-old man who presented with abdominal pain for 6 months and a palpable upper abdominal mass. The tumor involved the body and tail of the pancreas and had infiltrated the periaortic area. The tumor did not express measurable amounts of carcinoembryonic antigen and an alkaline phosphatase stain was negative [45].

BxPC-3

BxPC-3 was cultured from a 61-year-old woman's adenocarcinoma of the body of the pancreas. The patient died 6 months later despite radiation and chemotherapy. No evidence of metastasis was found. Tumors grown in nude mice resemble the primary tumor of the patient and produced carcinoembryonic antigen, human pancreas cancer-associated antigen, human pancreas-specific antigen, and traces of mucin [46].

HPAF-II

HPAF-II was obtained from the ascites of a 44-year-old male with pancreas adenocarcinoma and metastases to the liver, diaphragm and lymph nodes [47].

Capan-1

Capan-1 was obtained from a liver metastasis of a 40-year-old male with an adenocarcinoma in the head of the pancreas. Metastases were present in regional lymph nodes. In euthymic mice, Capan-1 derived tumor-produced mucin and were morphologically and

biochemically similar to the origin of tumor. Although not reported in the original publication, a doubling time of 41 hours was subsequently determined for Capan-1 [48].

PL-12 (Pancreatic cancer cell line 12 or PANC 10.05)

PL-12 is a pancreatic adenocarcinoma epithelial cell line derived in 1992 from a primary tumor removed from the head of the pancreas of a male with pancreatic adenocarcinoma. Panc 10.05 cell lines exhibit a K-ras oncogene mutation at codon 12 where a GGT to GAT mutation resulted in substitution of aspartic acid for glycine. The cells have a reported plating efficiency of 40% [49].

Purification of NK 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).

^{51}Chromium release cytotoxicity assay

^{51}Chromium was purchased from Perkin Elmer (Santa Clara, CA). NK cell cytotoxic function in the experimental cultures and the sensitivity of the target cells to NK cell mediated lysis were determined using the ^{51}Chromium release cytotoxicity assay. Different numbers of purified NK cells were incubated with ^{51}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.

Surface staining

$1x10^5$ of Panc-1, MP-2, BxPC-3, HPAF-II, Capan-1 and PL-12 were stained with isotype control IgG2b κ and antibodies against CD16, CD44, CD54, CD274 (B7-H1, PD-L1) and HLA-A, B, C (Biolegend, San Diego, CA) and incubated them at 4-8°C for 30 minutes. The levels of surface expression were determined by flow cytometry analysis.

ELISA (enzyme-linked immunosorbent assay)

Human IFN-γ ELISA kit was purchased from Biolegend (San Diego, CA). ELISA was done to detect the amount of IFN-γ produced from different pancreatic cancer cell lines. 96 well plates were coated with 100uL of diluted capture antibody corresponding to target cytokine and

incubated overnight at 4-8°C. In the next day, the plates were washed 4 times with wash buffer (0.05% Tween in 1xPBS). The plates were blocked with assay diluent (1% BSA in 1xPBS) and incubated on a plate shaker shaking at 200 rpm for 1 hour followed by washing 4 times with wash buffer. 100uL of standards and samples were added in duplicate wells and incubated on the plate shaker shaking at 200 rpm for 2 hours at room temperature. After the plates were washed 4 times, 100uL of diluted detection antibody was added to the wells and incubated for 1 hour on the plate shaker at room temperature. Then, the plates were washed with wash buffer and 100uL of diluted Avidi- HRP was added the wells and incubated on the plate shaker shaking at 200 rpm for 30minutes followed by washing the plates for 5 times with wash buffer. Then 100uL of TMB substrate solution was added to the wells. After positive wells turned blue, 100uL of stop solution (2N H_2SO_4) was added and the absorbance was read in a microplate reader capable of measuring absorbance at 450nm within 30 minutes after adding stop solution [Biolegend, ELSA manual].

Results

NK cells were left untreated or treated with anti-CD16 antibody and/or IL-2 for 12-18 hours before they were added to ^{51}Cr labeled Panc-1, MP-2, BxPC-3, HAPF II, Capan-1 and PL-12. As shown in previous studies, using time-lapse microscopic analysis NK cells mediated much higher lysis of stem like OSCSCs when compared to differentiated OSCCs [43]. In accordance to the data obtained with the time-lapse microscopy, both untreated and IL-2 treated NK cells enhanced higher lysis of Panc-1 and MP-2, when compared to BxPC-3, HPAF-II, Capan-1 and PL-12 in ^{51}Cr release assay (Fig-1A). Anti-CD16 mAb treatment inhibited NK cell cytotoxicity against 6 cell lines of pancreatic cancers. The combination of IL-2 and anti-CD16

mAb treated NK cells did not mediate the lysis of Panc-1, MP-2, BxPC-3, HAPF-II, Capan-1 and PL-12 when compared to IL-2 activated NK cells alone (Fig-1A).

Figure-1A

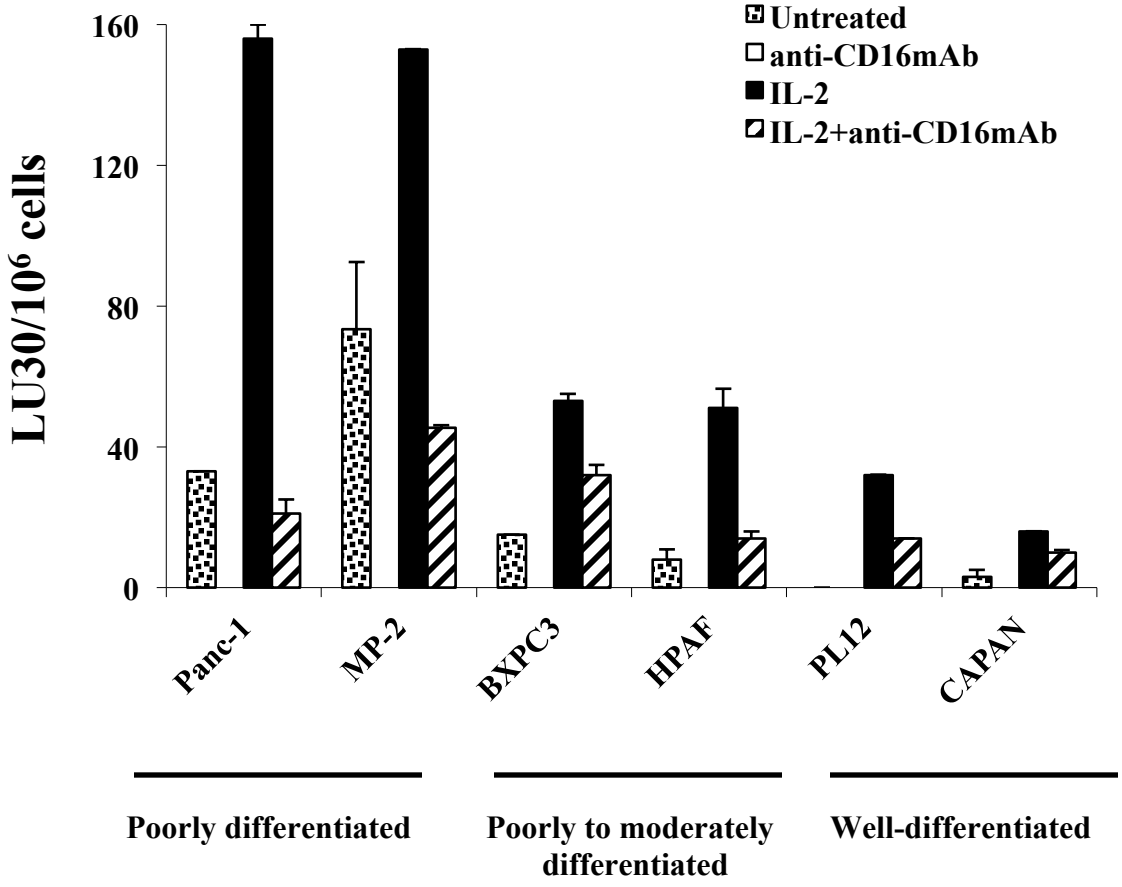

Figure -1A. NK cells lysed poorly differentiated pancreatic cancer cells (Panc-1 and MP2) but not well-differentiated pancreatic cancer cells (Capan-1 and PL-12) while BxPC-3 and HPAF-II were moderately resistant to NK cell mediated cytotoxicity.

NK cells were left untreated or treated with IL-2 (1000 units/ml), anti-CD16mAb (3 mg/ml) or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) for 18 hours overnight before they were used to measure NK cell cytotoxicity against $^{Cr}51$ labeled Panc-1, MP-2, BxPC-3, HPAF-II, Capan-1 and PL-12 cells. After 4 hours of incubation of NK cells with 6 cell lines of pancreatic cancers, the radioactivity released in the supernatants were counted by the gamma counter and the levels of cytotoxicity were determined using LU $30/10^6$. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of Panc-1, MP-2, BxPC-3, HPAF-II, Capan-1 and PL-12.

We had previously characterized stem-like OSCSCs and differentiated OSCCs based on their surface expression, which was also correlated with the level of NK cell cytotoxicity against OSCSCs and OSCCs [43]. We also compared the levels of NK cell resistance to the levels of cell surface receptor modulation on pancreatic cancer cells using CD44, CD54 and MHC-class 1 (Fig- 1B). We found that surface expression of CD44 was higher in Pan-1, MP-2 and BxPC-3 than HPAF-II, Capan-1 and PL-12 (Fig- 1B). However, level of CD54 expression was increased in HPAF-II, Capan-1 and PL-12 when compared to CD54 expression on Panc-1, MP-2 and BxPC-3 (Fig- 1B). Moreover, MHC-class 1 expression was also accelerated in BxPC-3, HPAF-II, Capan-1 and PL-12 where Panc-1 and MP2 showed lower expression of MHC-class 1 (Fig- 1B).

From 6 cell lines of pancreatic cancer, we chose MP-2 representing high NK cell cytotoxic activity and PL-12 as low or resistant to NK cell cytotoxicity. In term of spilt anergy, we demonstrated the concept by culturing $1x10^5$ of MP-2 and PL-12 in 12 well plates on the day before adding NK cells. NK cells were left untreated, treated with anti-CD16 mAb, IL-2 and combination of anti-CD16 mAb and IL-2 on the day before adding to pancreatic cancer cells. 1:1 ratio of NK cells were co-cultured with MP-2 and PL-12 for 16-18 hours in the incubator. After an overnight incubation, the supernatant was removed from the co-cultures and the levels of IFN-γ secretion were determined using specific ELISAs.

The level of IFN-γ secretion was significantly escalated in MP-2 treated with anti-CD16 mAb and IL-2 activated NK cells when compared to IL-2 activated NK cells (Fig- 1C). Moreover, the amount of IFN-γ secretion was noticeably lower in PL-12 than MP-2 not only in anti-CD16 mAb and IL-2 treated NK cells but also in IL-2 treated NK cells alone (Fig- 1C).

Figure-1B

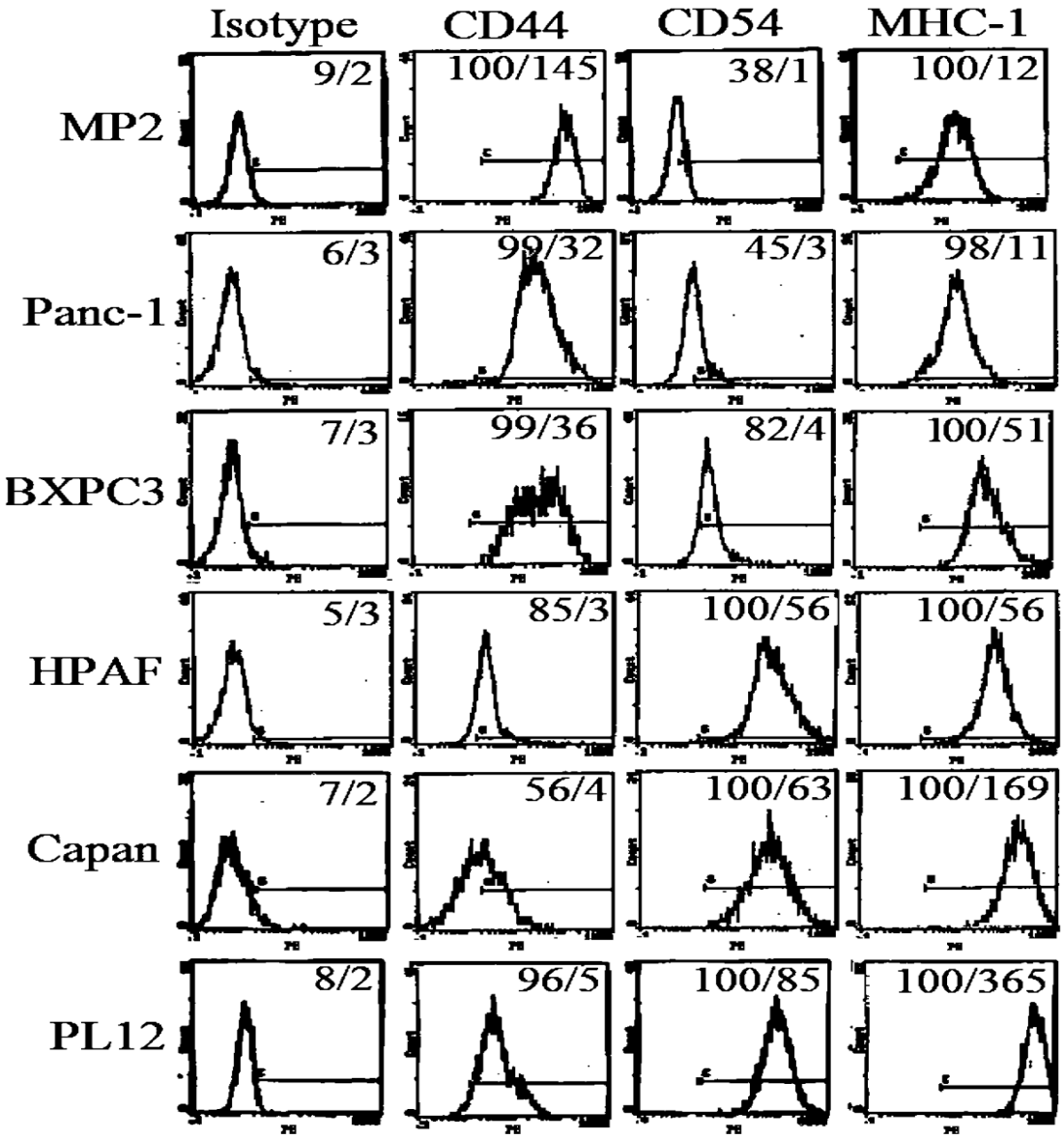

Figure-1B. Detecting the level of CD44, CD54 and MHC-class 1 surface expression on Panc-1, MP2- BxPC-3, HAPF-II, Capan-1 and PL-12

Tumor cells were stained and incubated for 30 minutes with antibodies such as isotype control, CD44, CD54 and primary MHC-class 1 followed by secondary MHC- class 1. Unbound antibodies were washed away by assay diluent. Isotype control samples were used to set gates and analyzed the samples by running in a flow cytometer.

To correlate with NK cell cytoxicity against MP-2 and PL-12 with the level of IFN-γ secretion, IL-2 treated NK cells showed more cytotoxic against MP-2 where IFN-γ secretion was higher in IL-2 and anti-CD16 treated MP-2 not in IL-2 treated NK MP2 (Fig- 1B, 1C). However, we did not see such phenomenon in PL-12. From this result, we investigated that anti-CD16 mAb in combination with IL-2 induced split anergy in NK cells resulting in a great loss of cytotoxicity but significant gain in secretion of IFN-γ against stem-like pancreatic cancers not in well-differentiated pancreatic cancer cells.

Figure-1C

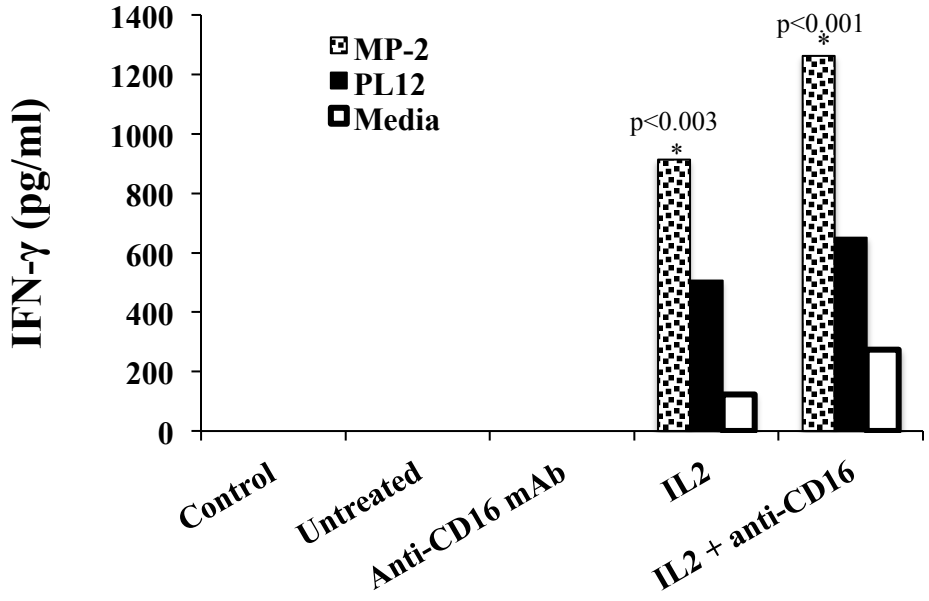

Figure-1C. Level of cytokine secretion in MP-2 and PL-12 co-cultured with NK cells.

NK cells were purified from healthy donors and left untreated or treated with IL-2 (1000 units/ml), anti-CD16 mAb (3 ug/ml) or the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 ug/ml) in the presence or absence of NK cells (1:1 ratio of NK: Tumor cells) for 24 hours. Supernatants were removed from co-cultures and the levels of IFN-γ secretion were determined using specific ELISAs. The "p" values were determined the difference between IFN-γ secretion in IL-2 or IL-2 + anti-CD16 mAb treated NK cells co-cultured with MP-2s and as compared to those co-cultured with Pl-12s. * denotes statistically significant.

Discussion

We had characterized the interaction of pancreatic cancers with NK cells and identified the profile of pancreatic cancer cells, which could distinguish between differentiated NK resistant pancreatic cancers from undifferentiated NK sensitive stem-like pancreatic cancer. As Panc-1 and MP2 were shown high sensitivity to NK cell cytotoxicity, they were represented as poorly differentiated pancreatic cancers. Capan-1 and PL-12 were categorized as well-differentiated pancreatic cancer cells because they were resistant to NK cell cytotoxicity.

The results also indicated that the levels of NK cell cytotoxicity might vary depending on the expression of surface markers on pancreatic cancer cells. According to previous studies, CD44 is the stem cell markers and stem cells are sensitive to cytotoxic activity of NK cells. The loss of MHC class I molecules is a frequent mechanism in experimental and spontaneous tumors to escape recognition and destruction by CTLs [50, 51]. According to the "missing self" hypothesis [52], loss of MHC class 1 molecule should make tumor cells more susceptible to NK immune effector mechanisms, since NK effector cells monitor MHC class I cell surface expression through their specific receptor (KIRs), and eliminate those cells with down-regulated HLA/H-2 class I molecules [53]. When CD44 expression was significantly higher in Panc-1, MP-2 and BxPC-3 than HPAF-II, Capan-1 and PL-12, the surface expressions of CD54 and MHC-class 1 were higher in BxPC-3, HPAF-II, Capan-1 and PL-12. According to surface expression, we categorized Panc-1 and MP2 as poorly differentiated pancreatic cancers because of high expression of CD44 and low expression of MHC1. Capan-1 and PL-12 were classified as well-differentiated pancreatic cancers because they expressed low level of CD44 and high level of CD54 and MHC1.

However, NK cell cytotoxicity against BxPC-3 and HPAF-II were neither as sensitive as Panc-1 and MP-2 nor as resistant as Capan-1 and PL-12. Moreover, surface expression level of CD54 and MHC-class 1 were not as low as Panc-1 and MP-2 or not as high as Capan-1 and PL-12. Therefore, BxPC-3 and HPAF-II were characterized as poorly to moderately differentiated pancreatic cancer cells.

In previous studies and our current studies, it has been shown that NK cells treated with IL-2 and anti-CD16 mAb become anergized and induce split anergy. In figure-1A and 1c, we had confirmed that IL-2 and anti-CD16 mAb activated NK cells were decrease cytotoxic action against stem-like or poorly differentiated pancreatic cancer cells such as Panc-1 and MP-2 while the level of IFN-γ secretion was noticeably high. The loss of cytotoxicity in anergized NK cells is correlated with a significant amount of IFN-γ secretion, which is not seen in untreated NK cells, anti-CD16 mAb treated NK cells or those treated with IL2.

Conclusion

According to the above findings, we characterized pancreatic cancer cells into three profiles; poorly, poorly to moderately and highly or well-differentiated pancreatic cancers. We could also prove that NK cells lysed poorly differentiated but not well-differentiated pancreatic cancer cells according to the level of NK cells cytotoxicity against pancreatic cancer cells and level of surface expression on tumor cells. The induction of split anergy of NK cells was confirmed in poorly differentiated pancreatic cancers showing decrease sensitivity of NK cells while increase level of IFN-γ secretion in IL-2 and anti-CD16 treated NK cell.

Chapter 2

Specific Aim 2: Role of anergized NK cells in selection and differentiation of pancreatic cancer stem cells

- **Sub-aim 2A**: Whether anergized NK cells lead to differentiation of pancreatic cancer stem cells
- **Sub-aim 2B**: Whether the induction of resistance in MP-2s by anergized NK cells is blocked by combination of IFN-γ and TNF-α antibodies
- **Sub-aim 2C**: Whether the induction of resistance of MP-2s is mediated by the combination of recombinant human IFN-γ and TNF-α
- **Sub-aim 2D**: Whether differentiation of MP-2s by anergized NK cells lead to decrease secretion of cytokines and chemokines

Introduction

Our previous studies indicated that the sensitivity to NK cell lysis is a predictive tool to study the stage of maturation and differentiation of the healthy untransformed stem cells, as well as transformed tumorigenic cancer stem cells. In this regard we have shown that stem like oral tumors OSCSCs are significantly more susceptible to NK cell mediated cytotoxicity when compared to their differentiated counterpart OSCCs which is significantly more resistant [43]. In addition, hESCs and hiPSCs, as well as a number of other healthy normal stem cells such as hMSCs and hDPSCs, were found to be significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts [43]. Moreover, we have investigated that stem like pancreatic tumors such as Panc-1 and MP-2 were significantly more susceptible to NK cell

mediated cytotoxicity; whereas, their well-differentiated counterparts such as Capan-1 and PL-12 were resistant to NK cell cytotoxicity. Based on these results, we suggested that NK cells may play a significant role in differentiation of the cells by providing critical signals via secreted cytokines as well as direct cell-cell contact. In addition, we have shown previously that CD14+HLADR- monocytes can condition NK cells to lose cytotoxicity and secrete inflammatory cytokines [38, 54-57]. The signals received from the stem cells or monocytes alter the phenotype of NK cells and cause NK cells to lose cytotoxicity and change into cytokine producing cells. Therefore, induction of split anergy in NK cells could be an important conditioning step responsible for the repair of tissues during pathological processes irrespective of the type of pathology. Since the generation and maintenance of cancer stem cells is higher, the majority of the NK cells may play as a conditioner to support differentiation and repair of the tissues [38]. Therefore, the phenotype of NK cells in tumor microenvironment as well as in the peripheral blood may resemble to that of the anergic NK cells which decreased NK cell cytotoxicity, acquisition of $CD16^{-/dim}$ $CD56^{dim/+}CD69^{+}$ phenotype and augmented the ability to secrete inflammatory cytokines [58]. These alterations in NK cell effector function are thought to ultimately aid in driving differentiation of a minor population of surviving, healthy, as well as transformed stem cells [38].

In this study, we studied whether anergized NK cells would contribute to differentiation and subsequent resistance of stem cells to NK cell mediated cytotoxicity through cell-cell contact and secreted cytokines such as TNF-α and IFN-γ. Therefore, we chose 2 pancreatic cancer cells; MP-2 representing as poorly differentiated or stem-like pancreatic cancer cells and Capan-1 as well-differentiated pancreatic cancer cells according to characterization and profiles, which were stated in Chapter 1. Moreover, we studied whether anergized NK cells became resistant or

enhanced to NK cell lysis when stem-like pancreatic cancer cells MP-2 were treated with anergized NK supernatants in combination with antibodies against TNF-α and IFN-γ. And if MP-2 differentiated with anergized NK supernatants in the absence or presence of antibodies against TNF-α and IFN-γ, we measured whether the cytokine secretion was shut down or not when compared to untreated MP-2. Then we studied to confirm whether recombinant TNF-α and IFN-γ involved as an important role in differentiation of stem-like pancreatic cancer cells to be more differentiated.

Materials and Methods

Cell lines, Reagents and Antibodies

Dr. Graham Donald (UCLA, Los Angeles, CA, USA) generously gifted human pancreatic cancer cell lines MIA PaCa-2 (MP-2) and Capan-1. MP-2 was cultured using DMEM (Dulbecco's Modified Eagle Medium) in supplement with 10% Fetal Bovine Serum (FBS) and 2% Penicillin-Streptomycin (Pen-Strep). Capan-1 was cultured in RMPI 1640 medium supplemented with 10% FBS and 2% Penicillin-Streptomycin (Pen-Strep). The human NK cells enrichment immunomagnetic negative selection kit was purchased from Stem Cell Technologies (Vancouver, Canada). Isotype control IgG2b κ and antibodies against CD16, CD44, CD54, CD274 (B7-H1, PD-L1) and HLA-A, B, C were purchased from Biolegend (San Diego, CA, USA). FBS and Pen-strep were purchased from Gemini Bio-Products (West Sacramento, CA, USA). DMEM, RPMI and DPBS (Dulbecco's Phosphate Buffered Saline) were purchased from Life Technologies (Los Angeles, CA, USA). Recombinant IL-2 was obtained from NIH- BRB. Recombinant TNF-α and IFN-γ were obtained from Biolegend (San Diego, CA). Antibodies to

TNF-α and IFN-γ were prepared in our laboratory and 1:100 dilution was found to be the optimal concentration to use. Propidium iodide is purchased from Sigma Aldrich (Buffalo, NY).

MIA Paca-2 (MP-2)

MP-2 was derived from the pancreas adenocarcinoma of a 65-year-old man who presented with abdominal pain for 6 months and a palpable upper abdominal mass. The tumor involved the body and tail of the pancreas and had infiltrated the periaortic area. The tumor did not express measurable amounts of carcinoembryonic antigen and an alkaline phosphatase stain was negative [45].

Capan-1

Capan-1 was obtained from a liver metastasis of a 40-year-old male with an adenocarcinoma in the head of the pancreas. Metastases were present in regional lymph nodes. In euthymic mice, Capan-1 derived tumor-produced mucin and were morphologically and biochemically similar to the origin of tumor. Although not reported in the original publication, a doubling time of 41 hours was subsequently determined for Capan-1 [48].

Purification of NK 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). NK cells were left untreated and treated with IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) and kept in the incubator at 37^{o}C for 16-18 hours.

Stem cells differentiation with NK supernatant

Human NK cells were purified from healthy donor's PBMCs as described above. NK cells were left untreated, treated a combination or IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18- 24 hours before the supernatants were removed and used in differentiation experiments. The amounts of IFN-γ produced by activated NK cells were assessed with IFN-γ ELISA (Biolegend, CA). Differentiation of MP-2 was conducted with gradual daily addition of increasing amounts of NK cell supernatant. MP-2 required on average a total concentration of 5000 to 6000 pg of IFN-γ containing supernatants obtained from IL-2+anti-CD16mAb treated NK cells with or without combination of IFN-γ and TNF-α antibodies during a 5 day treatment to induce differentiation. rh IFN-γ (200U/mL) and rh TNF-α (20ng/mL) were treated to tumor cells for 18 hours to induce differentiation by means of recombinant human cytokines. Afterwards, target cells were washed with 1xDPBS, detached and used for experiments.

^{51}Chromium release cytotoxicity assay

^{51}Chromium was purchased from Perkin Elmer (Santa Clara, CA). NK cell cytotoxic function in the experimental cultures and the sensitivity of the target cells to NK cell mediated lysis were determined using the ^{51}Chromium release cytotoxicity assay. Different numbers of purified NK cells were incubated with ^{51}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.

Surface staining

$1x10^5$ of Panc-1, MP-2, BxPC-3, HPAF-II, Capan-1 and PL-12 were stained with isotype control IgG2b κ and antibodies against CD44, CD54, CD274 (B7-H1, PD-L1) and HLA-A, B, C (Biolegend, San Diego, CA) and incubated them at 4-8°C for 30 minutes. The levels of surface expression were determined by flow cytometry analysis.

ELISA (enzyme-linked immunosorbent assay)

Human IFN-γ and IL-8 ELISA kit were purchased from Biolegend (San Diego, CA). ELISA was done to detect the amount of IFN-γ and IL-8 produced from different pancreatic cancer cell lines. 96 well plates were coated with 100uL of diluted capture antibody

corresponding to target cytokine and incubated overnight at 4-8°C. In the next day, the plates were washed 4 times with wash buffer (0.05% Tween in 1xPBS). The plates were blocked with assay diluent (1% BSA in 1xPBS) and incubated on a plate shaker shaking at 200 rpm for 1 hour followed by washing 4 times with wash buffer. 100uL of standards and samples were added in duplicate wells and incubated on the plate shaker shaking at 200 rpm for 2 hours at room temperature. After the plates were washed 4 times, 100uL of diluted detection antibody was added to the wells and incubated for 1 hour on the plate shaker at room temperature. Then, the plates were washed with wash buffer and 100uL of diluted Avidi- HRP was added the wells and incubated on the plate shaker shaking at 200 rpm for 30minutes followed by washing the plates for 5 times with wash buffer. Then 100uL of TMB substrate solution was added to the wells. After positive wells turned blue, 100uL of stop solution ($2NH_2SO_4$) was added and the absorbance was read in a microplate reader capable of measuring absorbance at 450nm within 30 minutes after adding stop solution [Biolegend, ELSA manual].

Results

Sub-aim 2A: Whether anergized NK cells lead to differentiation of pancreatic cancer stem cells

Supernatants from the combination of IL-2 and anti-CD16 mAb treated NK cells induced resistance of MP-2 to NK cell mediated cytotoxicity and increased differentiation antigens on the surface of MP-2.

NK cells were left untreated and treated with IL-2 and anti-CD16 mAB and kept in the incubator for 16-18 hours. Then we induced differentiation of MP-2 by using untreated NK

Figure 2A

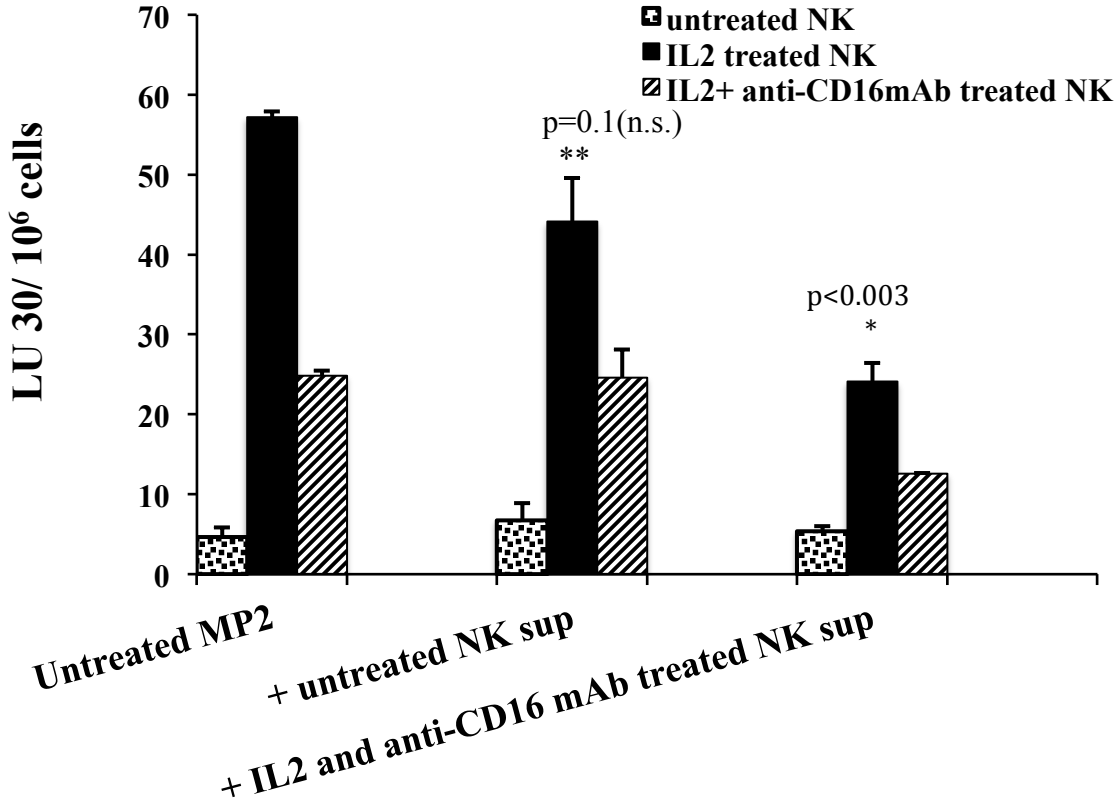

Figure 2A. Anergized NK supernatants induced differentiation of MP-2 increasing resistance to NK cell mediated cytotoxicity.

MP-2 cells were left untreated, treated with untreated NK supernatants and treated with IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) treated NK supernatants. ELISA was run to measure the IFN-γ concentration of supernatants and MP-2 cells were treated with gradual daily addition of increasing the amount of NK supernatants for 5 days. Freahly isolated NK cells were left untreated or treated with IL-2 (1000 units/ml), or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) for 18 hours overnight before they were used to measure NK cell cytotoxicity against $^{Cr}51$ labeled MP-2s. After 4 hours of incubation of NK cells with MP-2 cells, the radioactivity released in the supernatants were counted by the gamma counter and the levels of cytotoxicity were determined using LU $30/10^6$. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of MP-2 cells x100. The "p" values were determined between untreated MP-2s and those of treated with untreated NK supernatants or IL-2 and anti-CD16 mAb treated NK supernatants. * is statistically significant and ** is not significant (n.s.).

supernatants, and IL-2 and anti CD16 mAb treated NK supernatants. The method of differentiation was explained in stem cell differentiation with NK supernatants. MP-2 treated with IL-2 and anti-CD16 mAb treated NK supernatant induced resistance of NK cell mediated cytotoxicity more significantly by IL-2 treated NK cells than by combination of IL-2 and anti-CD16 mAb NK cells (Fig- 2A). Untreated MP-2 and MP-2 treated NK supernatants showed higher levels of cytotoxicity against IL-2 treated NK cells (Fig- 2A). Therefore, supernatants from IL-2 and anti-CD16 mAb treated NK cells enhanced the differentiation of MP-2 and became resistant to NK cell mediated cytotoxicity.

Moreover, we found that there was a correlation between the level of resistance to NK cell cytotoxicity and the degree of differentiation according to the surface receptors. MP-2 treated with IL-2 and anti-CD16 mAb treated NK supernatant expressed higher level of MHC class 1 and B7H1 while there were lower levels of MHC class 1 and B7H1 expression on untreated MP-2 or MP-2 treated with untreated NK supernatants (Fig- 2B). In our previous studies, we have described that more differentiated cancer cells express higher level of CD54, MHC class 1 and B7H1 while stem-like cancer cells express higher level of CD44. We found that levels of CD44 expression were not significantly down-regulated in 3 conditions of MP-2 or CD54 were not also noticeably up-regulated (Fig- 2B). Therefore, we verified that increased level of MHC class 1 and B7H1 expression were related to induction of resistant to NK cell mediated toxicity in MP-2 differentiated with IL-2 and anti-CD16 mAb treated NK supernatants. Unlike stem-like oral cancer cells (OSCSCs) and stem-like lung cancer cells (hA549s), we found that differentiated pancreatic cancer cells induced by anergized NK supernatants showed less significant changes in CD44 and CD54 expression.

Figure 2B

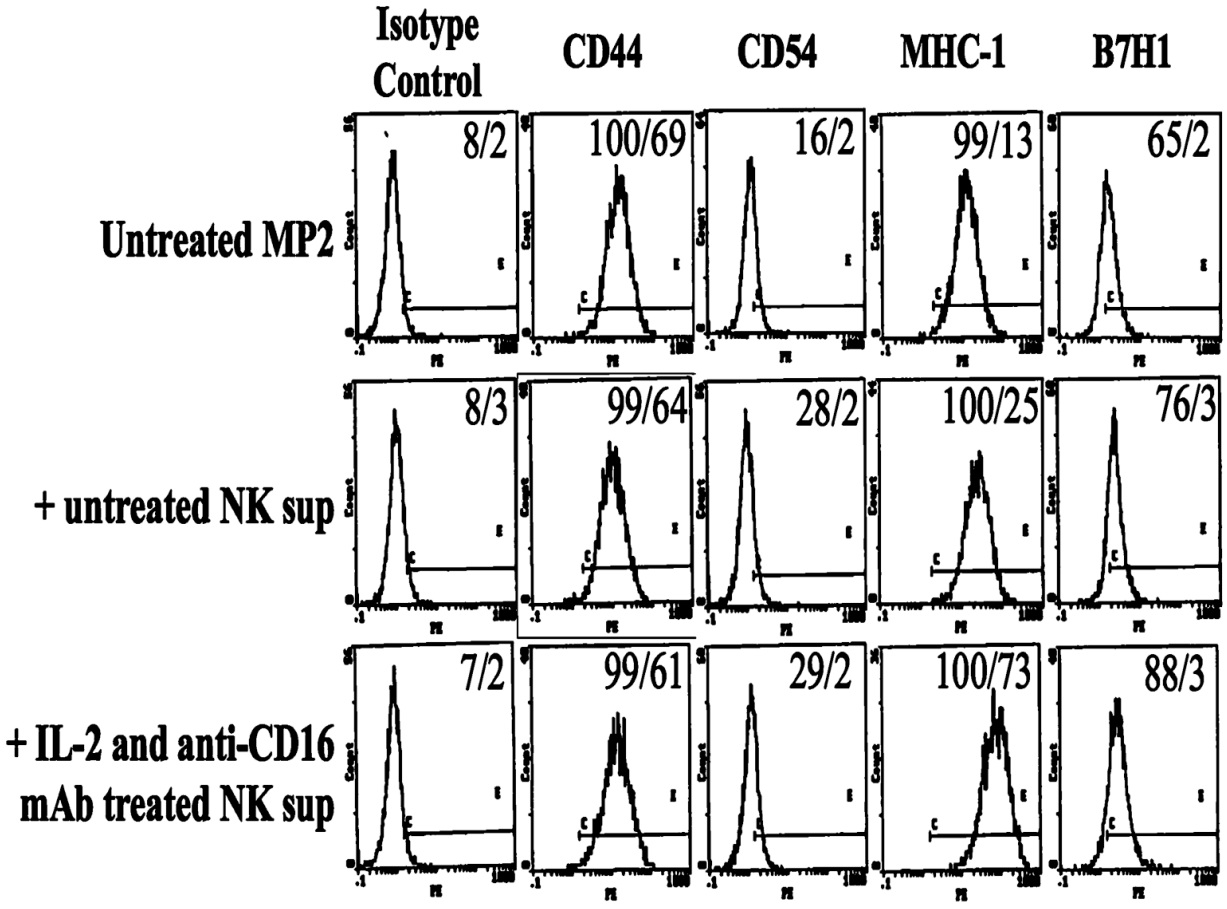

Figure 2B. Differentiation of MP-2 by IL-2 and anti-CD16 treated NK supernatants increased surface expression of MHC1 and B7H1 but no significant changes on CD44 and CD54.

NK cells were purified from healthy donors and left untreated or the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 mg/ml) for 24 hours. Thereafter, the same amounts of supernatants from different treatments of NK cells were removed and added to MP-2S for 5 days. MP-2s were then washed, and the expression of CD44, CD54, MHC Class 1 and B7H1 were assessed after staining with the PE conjugated antibodies using flow cytometry.

Aim 2 Sub-aim 2B: Whether the induction of resistance in MP-2s by anergized NK cells is blocked by combination of IFN-γ and TNF-α antibodies

To examine the mechanisms by which MP-2s become resistant by anergized NK cells, we determined NK cell mediated cytotoxicity when MP-2s were treated with supernatants of NK cells treated with anti-CD16mAb and IL-2 in the presence and absence of combination of IFN-γ and TNF-α antibodies. Therefore, MP-2s were left untreated, treated with untreated NK supernatants and treated with IL-2 and anti-CD16 mAb treated NK supernatant with or without antibodies against IFN-γ and TNF-α for 5 days. Then, MP-2s were then washed with 1xDPBS, detached from the plate and used for $^{Cr}51$ assay and surface staining according to materials and methods.

Induction of resistance in MP-2s to NK cell mediated cytotoxicity by anergized NK cells is reduced by the combination of IFN-γ and TNF-α.

The addition of combination of IFN-γ and TNF-α antibodies in MP-2 treated IL-2 and anti-CD16 mAb NK supernatants significantly overturned the resistance of NK cell mediated cytotoxicity against IL-2 treated NK cells (Fig- 2C). The sensitivities of NK cell mediated cytotoxicity against IL-2 treated NK cells were similar in untreated MP-2 and MP-2 treated with supernatants from IL-2 and anti-CD16 activated NK cells with combination of anti-IFN-γ and anti-TNF-α (Fig- 2C). Therefore, the combination of anti-IFN-γ and anti-TNF-α had strong inhibitory effect on the induction of resistance of MP-2 by IL-2 and anti-CD16 mAb treated NK supernatants and blocked from MP-2 to be differentiated.

Figure 2C

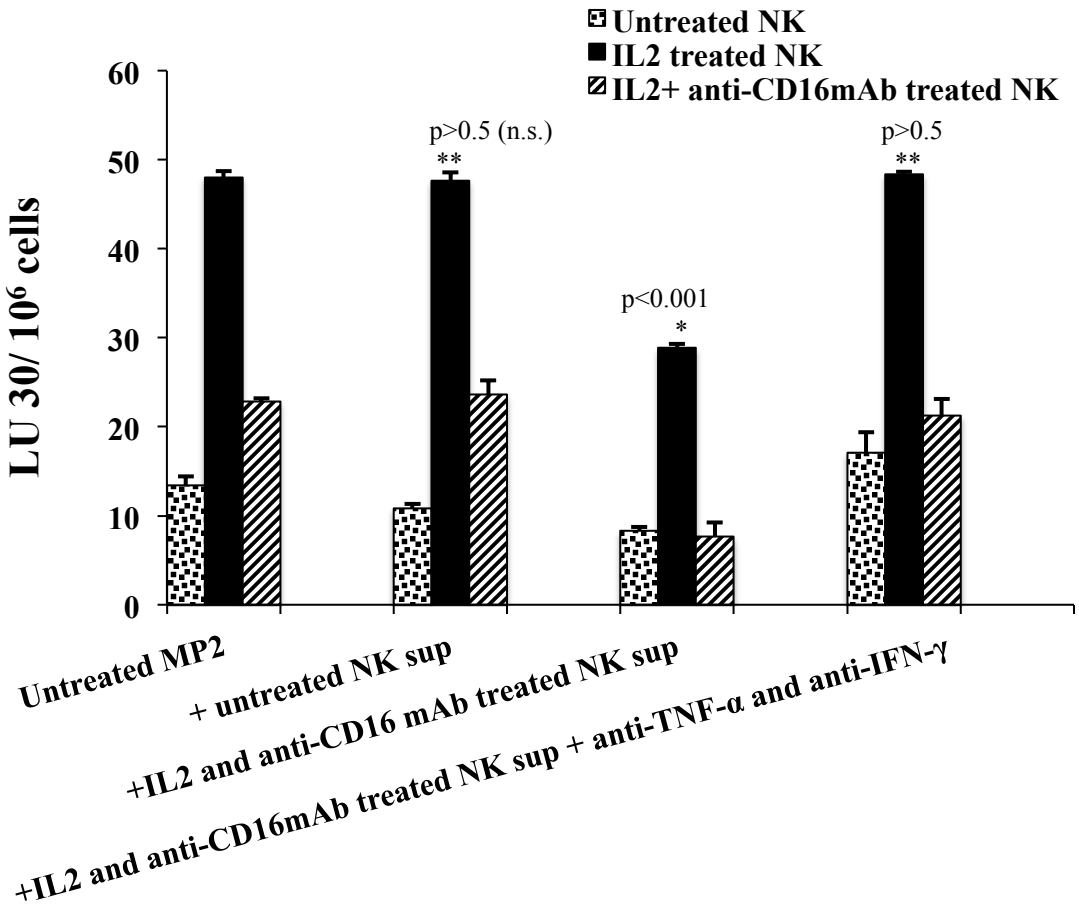

Figure 2C. Combination of IFN-γ and TNF-α antibodies blocked differentiation of MP-2 induced by anergized NK cells and they became sensitive to NK cell mediated cytotoxicity.

MP-2 cells were left untreated, treated with untreated NK supernatants and treated with IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) treated NK supernatants with or without combination of IFN-γ and TNF-α antibodies for 5 days. Freahly isolated NK cells were left untreated or treated with IL-2 (1000 units/ml), or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3 ug/ml) for 18 hours overnight before they were used to measure NK cell cytotoxicity against $^{Cr}51$ labeled MP-2s. After 4 hours of incubation of NK cells with MP-2s, the radioactivity released in the supernatants were counted by the gamma counter and the levels of cytotoxicity were determined using LU $30/10^6$. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of MP-2 cells x100. The "p" values were determined between untreated MP-2s and those of treated with untreated NK supernatants or IL-2 and anti-CD16 mAb treated NK supernatants in the presence and absence of anti-TNF-α and anti-FN-γ. * is statistically significant and ** is not significant (n.s.).

Figure 2D

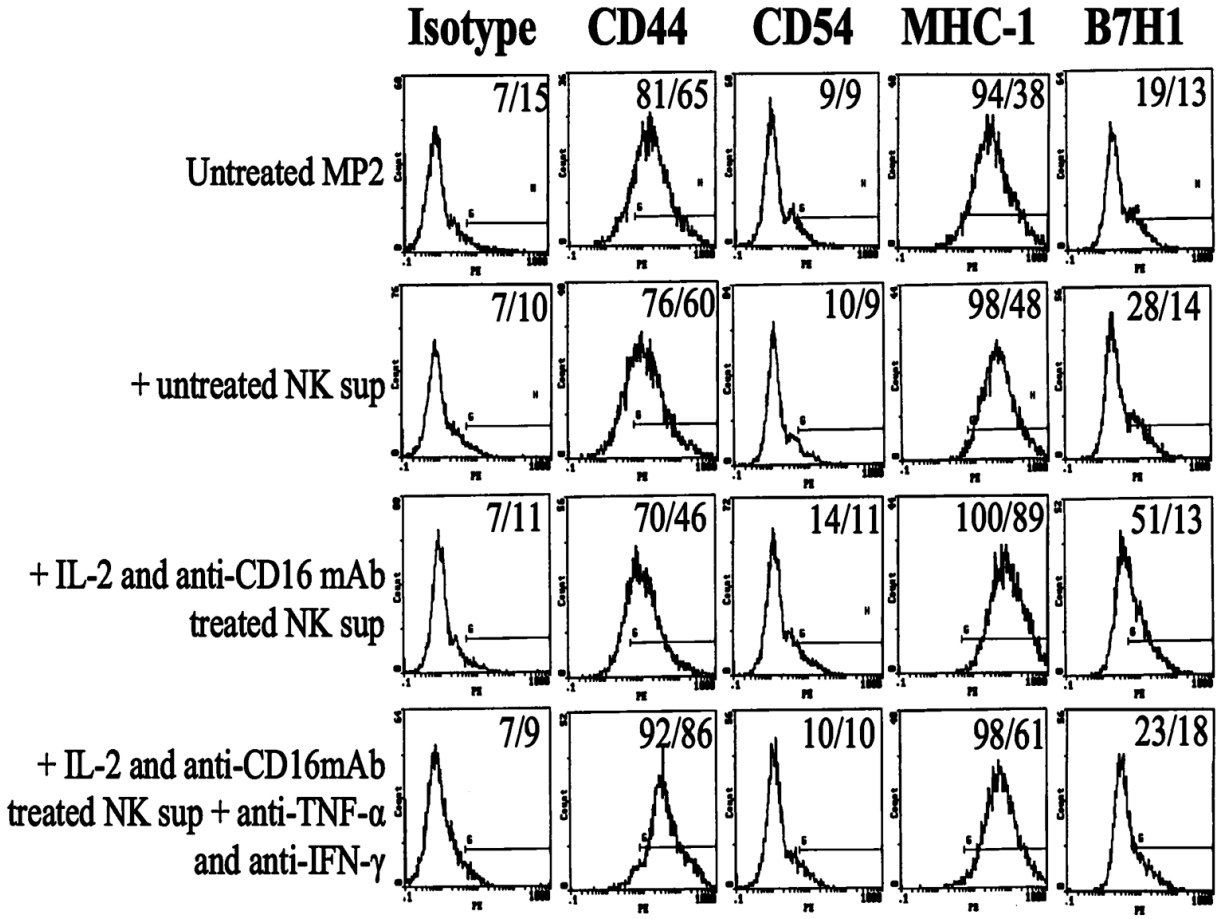

Figure 2D. Addition of combination of IFN-γ and TNF-α antibodies blocked differentiation of MP-2 induced by anergized NK supernatants and expression of CD44, CD54, MHC class 1 and B7H1 remained same profile as untreated MP-2.

NK cells were purified from healthy donors and left untreated or the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 mg/ml) for 24 hours. Thereafter, the same amounts of supernatants from different treatments of NK cells were removed and added to MP-2S for 5 days. MP-2s were then washed, and the expression of CD44, CD54, MHC Class 1 and B7H1 were assessed after staining with the PE conjugated antibodies using flow cytometry.

In figure 2D, resistance of MP-2s induced by supernatants from IL2 and anti-CD16 mAb treated NK cells correlated with increased expression of B7H1 and MHC class I as shown above and the addition of a combination of anti-TNF-α and anti-IFN-γ antibodies prevented the up-regulation of these receptors. CD44 was slightly down-regulated in MP-2 treated with IL2 and anti-CD16 mAb treated NK supernatants when there was increased expression of CD44 in the addition of a combination of anti-TNF-α and anti-IFN-γ antibodies. Moreover, CD54 expression was also not significantly upregulated in differentiated MP-2s with IL2 and anti-CD16 mAb treated NK supernatants with or without antibodies. Therefore, MHC class 1 and B7H1 expressed more significantly in anergized NK cells induced differentiated MP-2s while CD44 and CD54 remained unchanged or insignificantly changed.

Aim 2 Sub-aim 2C: Whether the induction of resistance of MP-2s is mediated by the combination of recombinant human IFN-γ and TNF-α.

To confirm the mechanisms by which MP-2s become resistant to NK cells mediated cytotoxicity by combination of recombinant human IFN-γ and TNF-α and not each cytokine alone, MP-2s were left untreated, treated with rh IFN-γ (200U/mL) alone, treated with rh TNF-α (20ng/mL) alone and combination of rh IFN-γ (200U/mL) and rh TNF-α (20ng/mL) for 18 hours. Then, freshly isolated NK cells were left untreated for 18 hours overnight before they were used to measure NK cell cytotoxicity against $^{Cr}51$ labeled MP-2s. Then, MP-2s were then washed with 1xDPBS, detached from the plate and used for $^{Cr}51$ assay and surface staining according to materials and methods.

Figure 2E

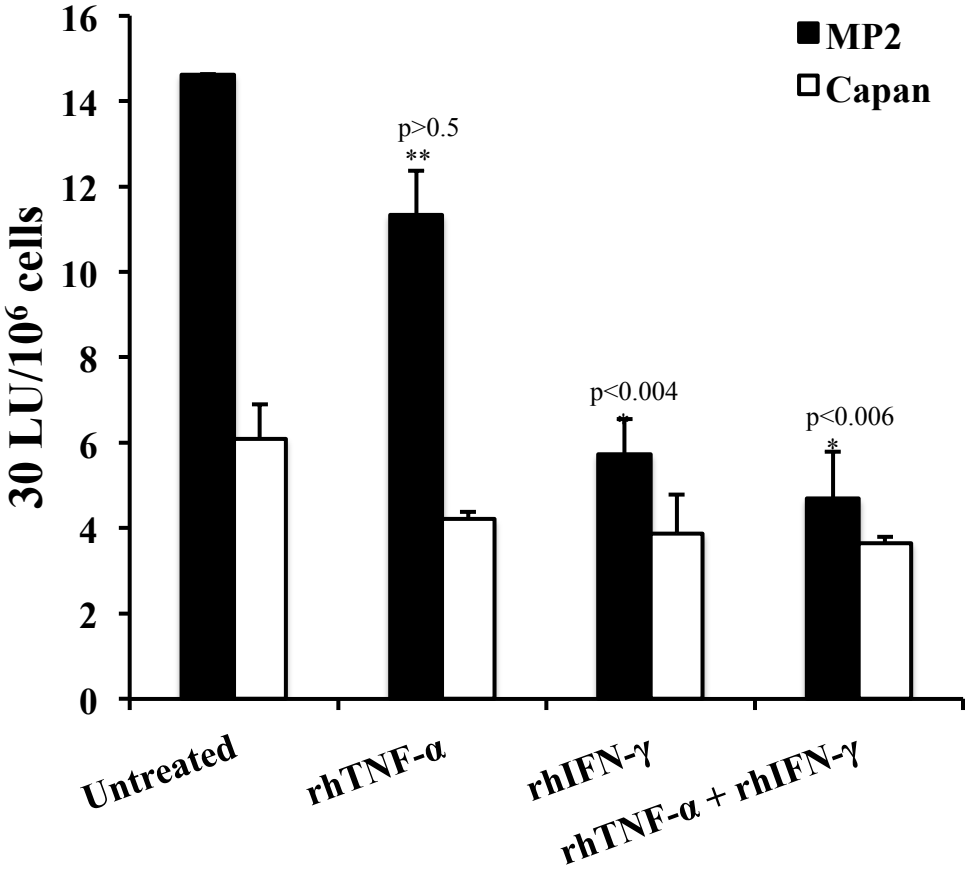

Figure 2E. Addition of combination of recombinant human IFN-γ and TNF-α enhanced significantly differentiation of MP-2 and Capan-1, and increased the resistance to NK cell mediated cytotoxicity against untreated NK cells when compared to addition of each cytokine alone.

MP-2s and Capan-1 were left untreated, treated with rh IFN-γ (200U/mL) alone, treated with rh TNF-α (20ng/mL) alone and combination of rh IFN-γ (200U/mL) and rh TNF-α (20ng/mL) for 18 hours. Freahly isolated NK cells were left untreated for 18 hours overnight before they were used to measure NK cell cytotoxicity against $^{Cr}51$ labeled MP-2s. After 4 hours of incubation of NK cells with MP-2s and Capan-1s, the radioactivity released in the supernatants were counted by the gamma counter and the levels of cytotoxicity were determined using LU $30/10^6$. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of MP-2 and Capan-1 x100. The "p" values were determined between untreated MP-2s and those of treated with rhTNF-α, rhIFN-γ and combined cytokines. * is statistically significant and ** is not significant (n.s.).

Induction of resistance to NK cell mediated cytotoxicity in MP-2 treated with combination of recombinant human IFN-γ and TNF-α and not each cytokine alone.

There was a more significant increase in resistance of NK cell mediated cytotoxicity in MP-2 treated with combination of rh IFN-γ and rh TNF-α than treatment with each cytokine alone (Fig- 2E). There was more resistant to NK cell mediated cytotoxicity in MP-2 treated with rh IFN-γ than that with rh TNF-α (Fig- 2E). Therefore, IFN-γ caused higher degree of differentiation than TNF-α. From this result, we confirmed that combination of IFN-γ and TNF-α had synergy and played an important role in differentiation of stem like pancreatic cancers to become more differentiated, moreover; inducing more differentiation in well-differentiated pancreatic cancer cells Capan-1 causing reduction of sensitivity of NK cell mediated cytotoxicity.

Correlation between the level of resistance to NK cell cytotoxicity and the degree of differentiation according to the surface receptors

MP-2 treated with combination of rh IFN-γ and rh TNF-α showed the highest expressions in CD54, MHC class 1 and B7H1 when compared to MP-2 treated with each cytokine alone (Fig- 2F). However, expression of CD54, MHC class 1 and B7H1 were slightly higher in MP-2 treated with rh IFN-γ alone than treatment with rh TNF-α alone when compared to untreated MP-2 (Fig- 2F). And CD44 expression was significantly down-regulated in MP-2 treated with combination of rh IFN-γ and rh TNF-α when compared to untreated MP-2 (Fig- 2F). In addition, the level of CD54, MHC class 1 and B7H1 expression on tumor cell surfaces showed the degree of differentiation. Therefore, the higher the level of surface expression is, the higher the degree of differentiation is.

Figure 2F

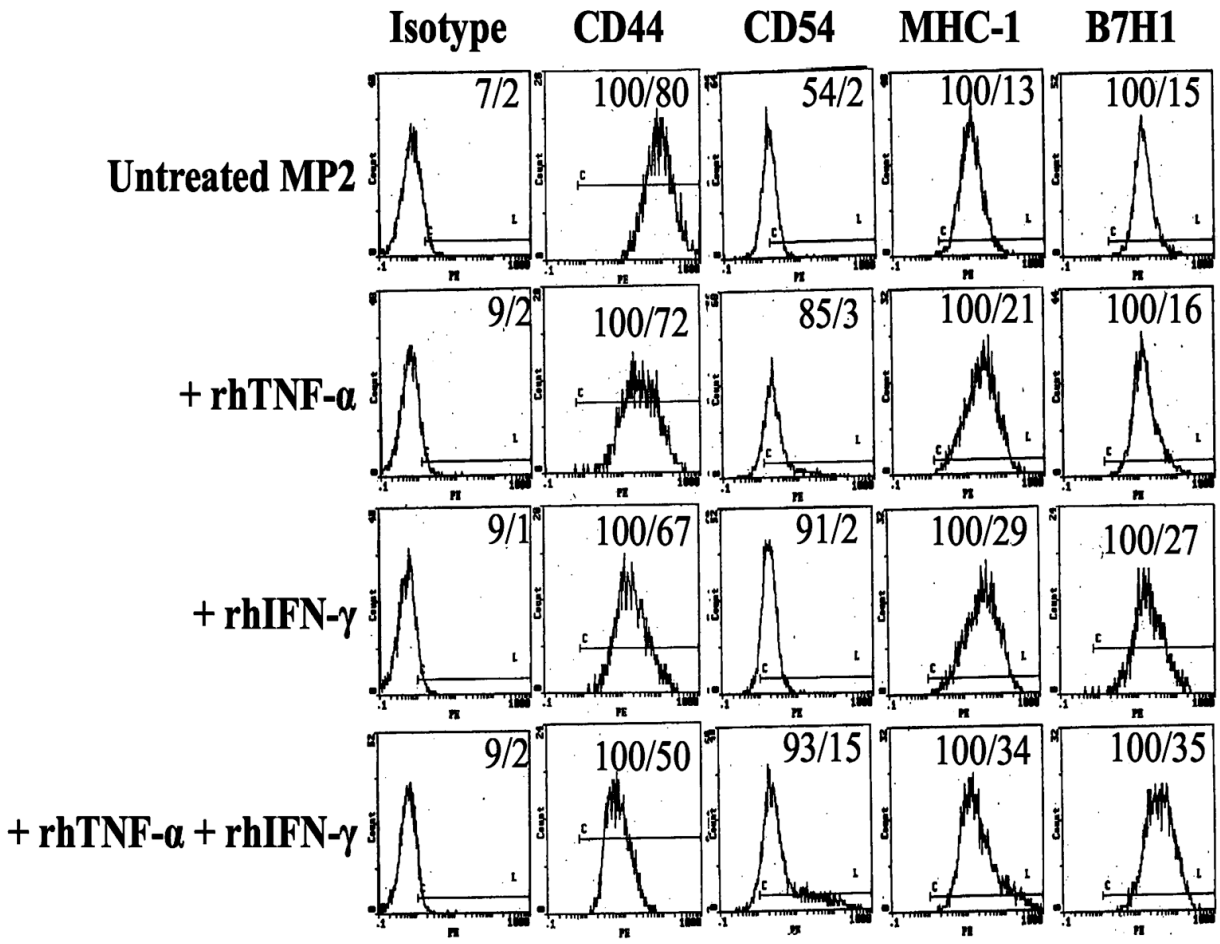

Figure 2F. Addition of combination of recombinant human IFN-γ and TNF-α induced differentiation of MP-2 and expression of CD54, MHC class 1 and B7H1 were upregulated and CD44 showed a decrease expression on surface of MP-2.

MP-2s were left untreated, treated with rh IFN-γ (200U/mL) alone, treated with rh TNF-α (20ng/mL) alone and combination of rh IFN-γ (200U/mL) and rh TNF-α (20ng/mL) for 18 hours. MP-2s were then washed, and the expression of CD44, CD54, MHC Class 1 and B7H1 were assessed after staining with the PE conjugated antibodies using flow cytometry.

Moreover, Capan -1 treated with combination of rh IFN-γ and rh TNF-α exhibited the incredible higher expressions of CD54, MHC class 1 and B7H1 when compared to Capan-1 treated with each cytokine alone (Fig- 2G). And expressions of CD54, MHC class 1 and B7H1 were significantly higher in Capan-1 treated with rh IFN-γ alone than treatment with rh TNF-α alone when compared to untreated Capan-1 (Fig- 2G). And there was a significant down-regulation of CD44 expression in Capan-1treated with combination of rh IFN-γ and rh TNF-α when compared to untreated Capan-1 (Fig- 2G). Therefore, we have confirmed there was strong correlation between the level of resistance to NK cell cytotoxicity and the degree of differentiation according to the surface receptors (Fig- 2E, 2F, 2G). Moreover, we have proved that there is synergistic action of IFN-γ and TNF-α on differentiated cancer cells not only by means of level of surface expression but also by resistance to NK cell mediated cytotoxicity.

Aim 2 Sub-aim 2D: Whether differentiation of MP-2s by anergized NK cells lead to decrease secretion of cytokines and chemokines

We had investigated that there was higher IFN-γ secretion in poorly differentiated pancreatic cancer cells MP-2 co-cultured with IL-2 and anti-CD16 activated NK cells than with IL-2 activated NK cells alone in Chapter 1 (Fig- 1C). We had also studied that poorly differentiated MP-2 became more differentiated by introducing them with IL-2 and anti-CD16 treated anergized NK cells. Therefore, we explored that there were changes in levels of cytokine secretions in untreated MP-2 and MP-2 differentiated by anergized NK cells in the presence or absence of combination IFN-γ and TNF-α antibodies. We also evaluated two cytokine secretions in this specific aim 2 sub-aim 2D, which were IFN-γ and IL-8 by analyzing with ELISA.

Figure 2G

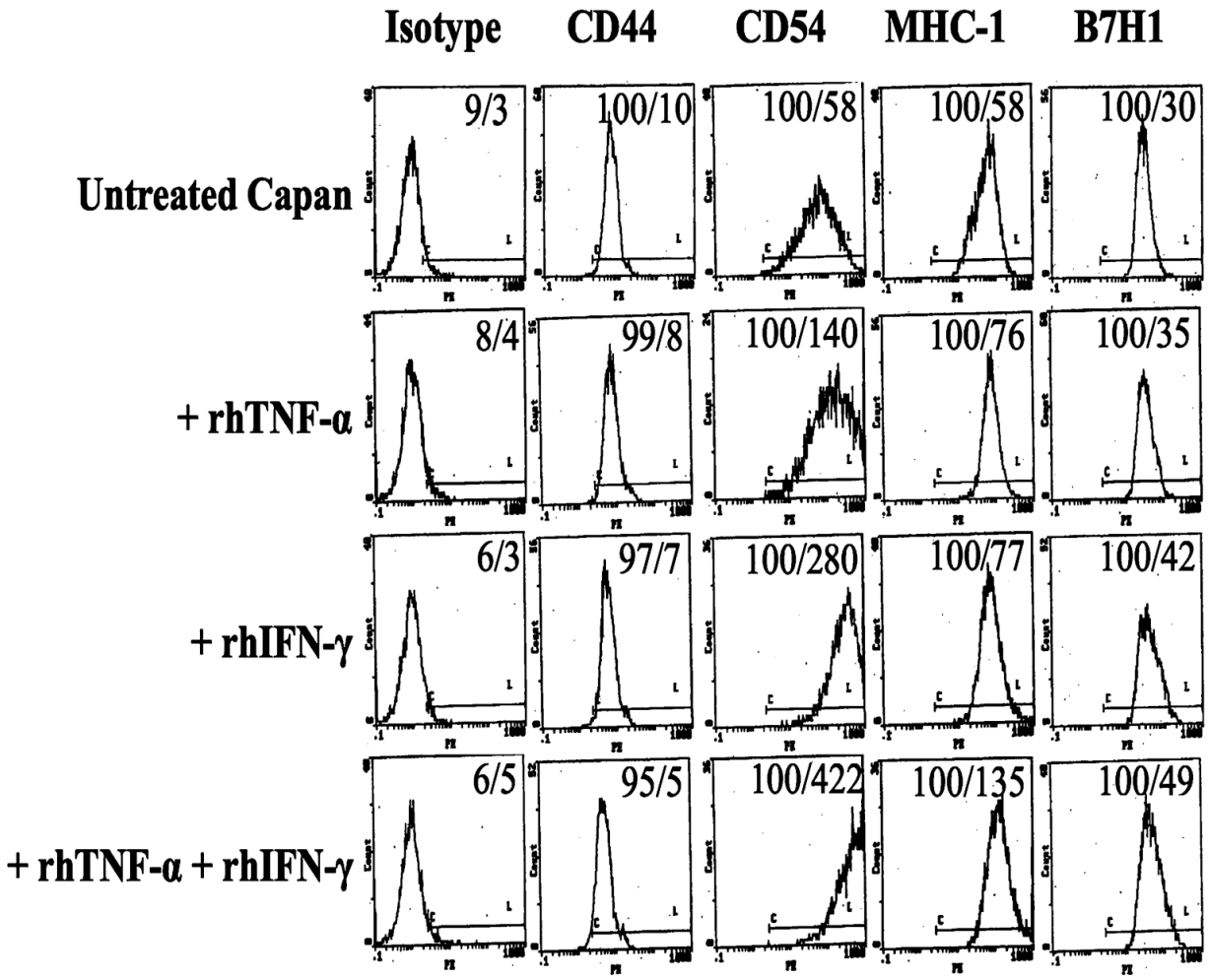

Figure 2G. Addition of combination of recombinant human IFN-γ and TNF-α induced differentiation of Capan-1 and expression of CD54, MHC class 1 and B7H1 were upregulated and CD44 showed a decrease expression on surface of Capan-1.

Capan-1s were left untreated, treated with rh IFN-γ (200U/mL) alone, treated with rh TNF-α (20ng/mL) alone and combination of rh IFN-γ (200U/mL) and rh TNF-α (20ng/mL) for 18 hours. MP-2s were then washed, and the expression of CD44, CD54, MHC Class 1 and B7H1 were assessed after staining with the PE conjugated antibodies using flow cytometry.

Stem cell differentiation by using anergized NK cells was described in material and methods section of Chapter -2. In this sub-aim 2D, we differentiated MP-2 by anergized IL-2 and anti-CD16 treated NK cells with or without combination IFN-γ and TNF-α antibodies and each antibody alone for 5 days. Freshly isolated NK cells were treated with untreated or treated with IL-2 (1000 units/ml) or the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 ug/ml) in the presence or absence of NK cells (1:1 ratio of NK: Tumor cells) for 24 hours. On the same day of NK cells isolation, MP-2s were washed with 1x DPBS and detached from the plates. Then each condition of MP-2s was re-plated to $1x10^5$ cells and kept them overnight in the incubator. $1x10^5$ of NK cells were added and co-cultured with MP-2s for 24 hours. After overnight incubations, supernatants were removed from co-cultures and the levels of IFN-γ and IL-8 secretion were determined using specific ELISAs.

There was a more significant reduction in secretion of IFN-γ and IL-8 in MP-2 differentiated by supernatants of IL-2 and anti-CD16 mAb treated NK cells co-cultured with IL-2 and anti-CD16 activated NK cells than co-culturing with IL-2 activated NK cells when compared to untreated MP-2 or MP-2 treated with untreated NK supernatant (Fig- 2H, I). Interestingly, there was remarkable re-acceleration of IFN-γ and IL-8 secretion in MP-2 differentiated by supernatants of IL-2 and anti-CD16 mAb treated NK cells in the presence of combination IFN-γ and TNF-α antibodies or anti-IFN-γ alone while slightly increased IFN-γ and IL-8 secretion in the presence of TNF-α antibodies alone (Fig- 2H,I).

Therefore, IL-2 and anti-CD16 treated anergized NK cells induced poorly differentiated MP-2 to become more differentiated and IL-2 and anti-CD16 activated NK cells decreased the secretion of cytokines after co-culturing with differentiated MP-2s. Therefore, anergized NK cells and freshly isolated NK cells are important immune cells to fight against inflammation.

Figure 2H

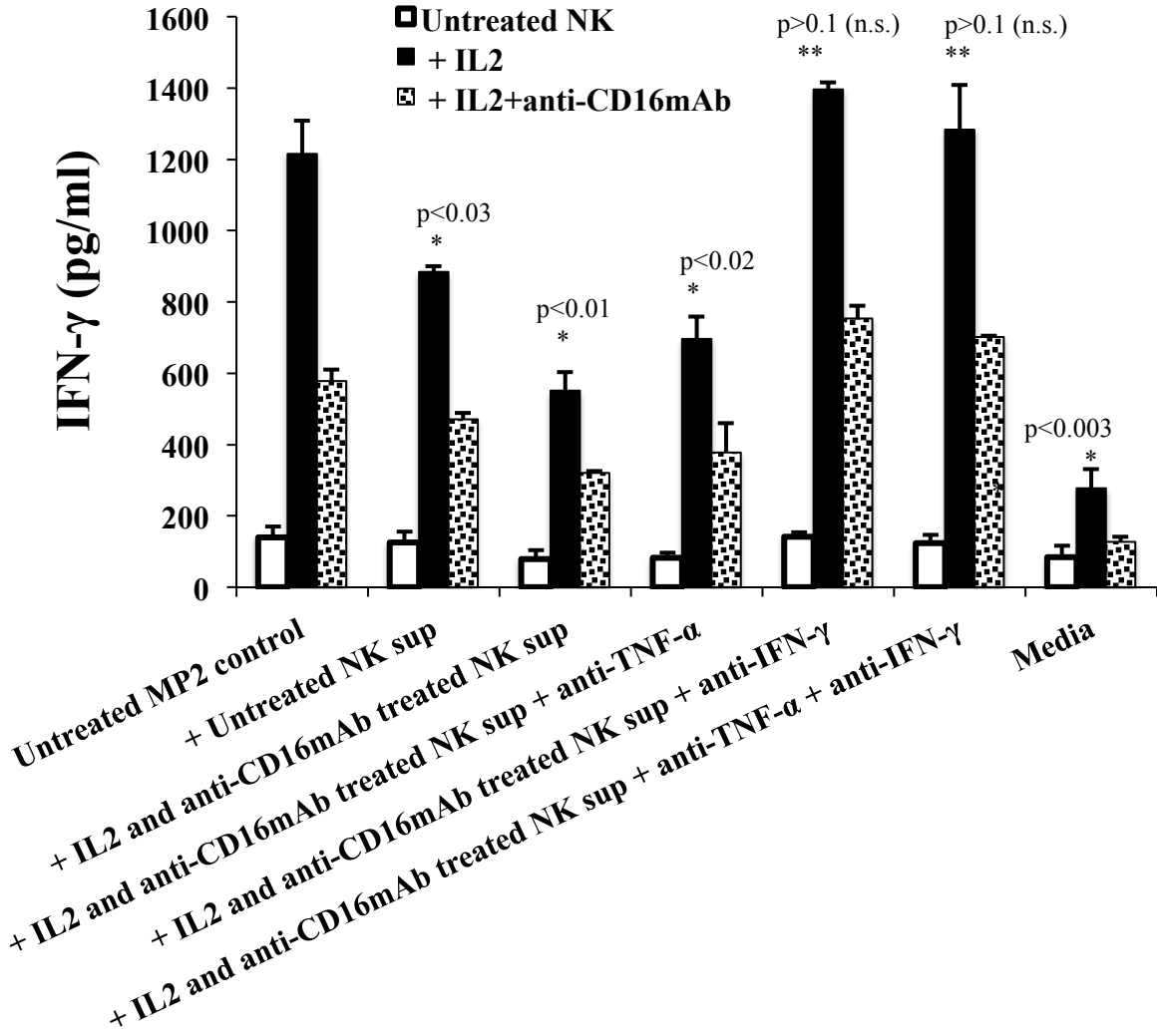

Figure 2H. Significant reduction of IFN-γ secretion was measured in MP-2 treated with anergized NK cells but IFN-γ secretion was reversed in MP-2 treated with anergized NK cells in the presence of anti- IFN-γ alone or combination of IFN-γ and TNF-α antibodies.

MP-2s were left untreated and treated with anergized NK cells with or without combination of IFN-γ and TNF-α antibodies and each antibody alone for 5 days. Freshly isolated NK cells were left untreated or treated with IL-2 (1000 units/ml) or the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 ug/ml) in the presence or absence of NK cells (1:1 ratio of NK: Tumor cells) for 24 hours. After overnight co-culturing of NK cells with MP-2s, supernatants were removed from co-cultures and the levels of IFN-γ secretion were determined using specific ELISAs. The "p" values were determined between untreated MP-2s and those of treated with untreated NK supernatants or IL-2 and anti-CD16 mAb treated NK supernatants in the presence and absence of anti-TNF-α and anti-FN-γ. * is statistically significant and ** is not significant (n.s.).

Figure 2I

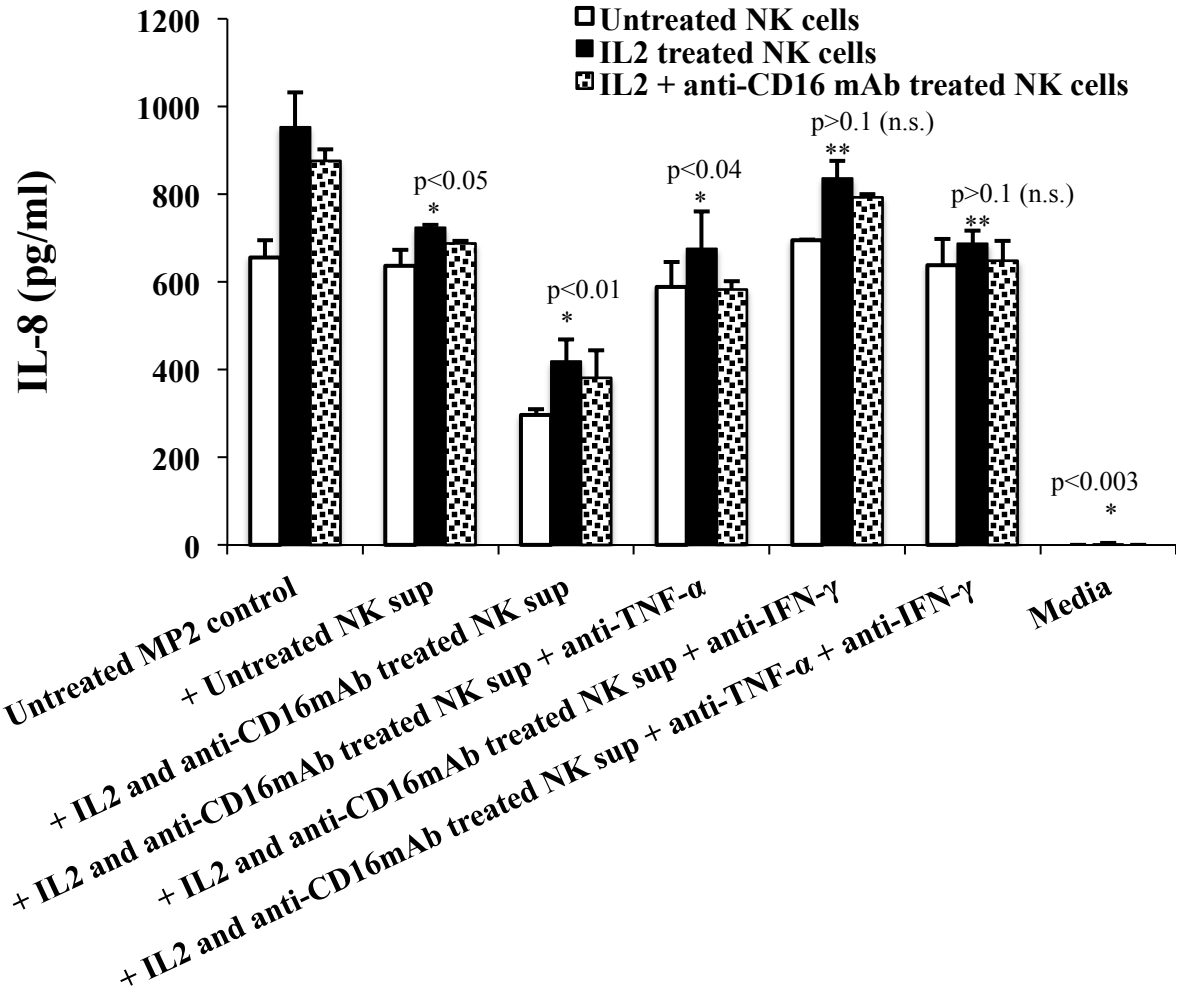

Figure 2I. Significant reduction of IFN-γ secretion was measured in MP-2 treated with anergized NK cells.

MP-2s were left untreated and treated with anergized NK cells with or without combination IFN-γ and TNF-α antibodies and each antibody alone for 5 days. Freshly isolated NK cells were left untreated or treated with IL-2 (1000 units/ml) or the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 ug/ml) in the presence or absence of NK cells (1:1 ratio of NK: Tumor cells) for 24 hours. After overnight co-culturing of NK cells with MP-2s, supernatants were removed from co-cultures and the levels of IFN-γ secretion were determined using specific ELISAs. The "p" values were determined between untreated MP-2s and those of treated with untreated NK supernatants or IL-2 and anti-CD16 mAb treated NK supernatants in the presence and absence of anti-TNF-α and anti-FN-γ. * is statistically significant and ** is not significant (n.s.).

Discussion

In chapter 2, we provided the evidence that conditioned or anergized NK cells have the ability to induce resistance and differentiation of transformed cancer stem cells. The increase in resistance to NK cell mediated cytotoxicity and differentiation in MP-2s was induced by the supernatants of NK cells, which were treated with the combination of IL-2+anti-CD16mAb. Although supernatants from NK cells treated with IL-2 alone had some effect on differentiation and resistance of stem cells, the magnitude of differentiation was much less when compared to the treatment of MP-2s with the supernatants of NK cells treated with the combination of IL-2+anti-CD16mAb. Combination of anti-TNF-α and anti-IFN-γ was necessary for the complete reversal of resistance of stem cells to NK cell mediated cytotoxicity and restoration of growth and expansion of stem cells (Fig- 2A, 2B, 2C, 2D).

Differentiation of MP-2 with the supernatants from the IL-2+anti-CD16mAb treated NK cells inhibited greatly the secretion of cytokines and chemokines induced by the untreated, IL-2 treated and IL-2+anti-CD16 treated NK cells. This observation is of great significance since it indicates that cellular differentiation is an important step in blocking and prevention of inflammation. Indeed, the levels of cytokines and chemokines secreted in the co-cultures of NK cells with IL-2+anti-CD16mAb treated NK supernatant differentiated MP-2 was similar if not identical to the amount secreted by the NK cells in the absence of MP-2 (Fig- 2H, 2I). Similar to the modulation of CD54 and MHC class I, the addition of anti-IFN-γ in the absence of anti-TNF-α to stem cells treated with the supernatants from the NK cells stimulated with IL-2+anti-CD16mAb restored the secretion of cytokines and chemokine in the co-cultures of NK cells with the stem cells.

Induction of split anergy in NK cells is important in conditioning step responsible for the differentiation of cells during pathological processes. In tumors, since the generation and maintenance of cancer stem cells is chronically high, the majority of NK cells including those of the circulating NKs, may be conditioned to support differentiation of the cells and as such the phenotype of NK cells in tumor microenvironment as well as in the peripheral blood may resemble that of the anergic NK cells, i.e., decreased NK cell cytotoxicity, expression of CD16-/dimCD56-/dimCD69+ phenotype and augmented ability to secrete inflammatory cytokines. Therefore, the results from above experiments suggested that there are two very important functions for the NK cells. One potential function of NK cells is to limit the number of stem cells, and second to support differentiation of the stem cells. By eliminating a subset of stem cells or after their interaction with other immune inflammatory cells or effectors of connective tissue, NK cells could then be in a position to support differentiation of remaining stem cells since they will be conditioned to lose cytotoxicity, induce cytokine and growth factor secretion and gain the $CD16^{-/dim}$ $CD56^{dim}$ $CD69^{+}$ phenotype.

The inability of patient NK cells to contain cancer stem cells due to the proliferating cancer stem cells and conversion of NK cells to cytokine secreting cells may likely be one mechanism by which cancer progresses and metastasizes. Poorly differentiated tumors have unfavorable prognosis since the microenvironment of these tumors are likely to condition a great majority if not all of the NK cells to support the differentiation of newly generated cancer stem cells, and as such these patients are likely to have more NK cells with no or low cytotoxic function. Therefore, there should be two distinct strategies to eliminate tumors, one targets stem cells and the other targets differentiated cells [54, 55, 38, 56, 59]. Since cancer stem cells were found to be more resistant to chemotherapeutic drugs but sensitive to NK cell mediated killing

while differentiated oral tumors were more resistant to NK cell mediated killing but relatively more sensitive to chemotherapeutic drugs, combination therapy should be considered for the elimination of both undifferentiated and differentiated tumors. In addition, since a great majority of patient NK cells have modified their phenotype to support differentiation of the cells, i.e., have lost cytotoxic function, they may not be effective in eliminating the cancer stem cells. Therefore, these patients may benefit from repeated allogeneic NK cell transplantation for elimination of cancer stem cells. In this regard depletion of NK anergizing effectors such as monocytes in the tumor microenvironment which condition NK cells to lose cytotoxicity, via radiation or chemotherapeutic drugs before allogeneic NK cell transplantation should provide a theoretic strategy for targeting of cancer stem cells by the NK cells. However, this strategy may also halt or decrease the ability of NK cells to drive optimal differentiation of the tumors and tilt the balance towards a more inflammatory tumor microenvironment that may run the risk of fueling the growth and expansion of more cancer stem cells.

Conclusion

It is possible that the successful cancer therapy may lie between a balance in the two above-mentioned approaches depending on the type of the tumor and the status of patients' immune system. The most dangerous and devastating outcome of the cancer is its ability to deplete NK cells and other immune inflammatory cells. In this case, not only cancer stem cells will be surviving but they will also remain poorly differentiated, which may establish a vicious cycle of tumor growth and loss of immune effectors in the tumor microenvironment and in the periphery. Therefore, NK cell immunotherapy in pancreatic cancer patients should be highly beneficial.

Chapter 3

Specific Aim 3: Targeting differentiated pancreatic tumors not stem-like tumors by chemotherapeutic drugs

- **Sub-aim 3A:** Whether stem-like pancreatic tumors are resistant to chemotherapeutic drugs
- **Sub-aim 3B:** Whether stem-like pancreatic tumors differentiated by anergized NK cells become susceptible to chemotherapeutic drugs

Introduction

Pancreatic ductal adenocarcinoma (PDA) remains a uniformly lethal disease with a catastrophic 5-year survival rate of less than 5% [60]. Despite intensive preclinical and clinical research efforts to tackle this disastrous disease, the oncologic management of PDA patients has hardly changed over the last several decades and remains one of the major challenges in clinical oncology. The poor responsiveness to standard single and combination chemotherapies is reflected in a median survival of 6–11 months in advanced disease, and emphasizes the desperate need for novel therapies [61, 62]. The high mortality rate is a result of multiple factors including late diagnosis, early systemic spread and a poor response to chemotherapy and/or radiotherapy [63]. A striking histological feature of PDA is the extremely dense and highly abundant tumor stroma consisting of activated cancer-associated fibroblasts (CAFs), infiltrating immune cells, and perturbed vascular cells that form a reactive, inflammatory, immunosuppressive, and highly dynamic tumor microenvironment around neoplastic ductal cells. More than in any other solid malignancy, the micro-environmental network of soluble cytokines, growth factors, proteases,

and additional extracellular matrix (ECM) components has increasingly been connected to support cancer cell proliferation, differentiation, invasion, early metastasis, and therapeutic resistance in PDA [64]. Therefore, we need to understand more about the molecular mechanisms of pancreatic cancer pathogenesis and to develop effective treatments for pancreatic cancer.

Paclitaxel was discovered in a US National Cancer Institute program at the Research Triangle Institute in 1967 and named it as Taxol. Later, it was discovered in endophytic fungi from the bark of *Taxus brevifolia*, which synthesize Paclitaxel [65]. Paclitaxel is a taxoid antineoplastic agent indicated as first-line and subsequent therapy for the treatment of advanced carcinoma of the ovary, and other various cancers including breast cancer. Paclitaxel is also a microtubule-targeted agent widely used in cancer therapy. Its primary cellular effect is to cause abnormal stabilization of the dynamic microtubule polymerization, leading to the failure of mitosis.

In addition, Paclitaxel also alters other cellular functions such as intracellular signaling, organelle transportation and locomotion. Paclitaxel induces abnormal arrays or "bundles" of microtubules throughout the cell cycle [66]. Recent studies showed that paclitaxel is able to induce early reactive oxygen species (ROS) production in cancer cells and hydrogen peroxide (H_2O_2). They are found to be involved in paclitaxel-induced cancer cell death *in vitro* and *in vivo* [67]. In our studies, we would like to propose Paclitaxel enhanced the cell death in poorly or well-differentiated pancreatic cancer cell lines (MP-2 or Capan-1 and PL-12, respectively). Moreover, if we induced poorly differentiated pancreatic cancer cells (MP-2) to be more differentiated by using anergized NK supernatants, paclitaxel induced cell death would be higher or would remain as low as untreated poorly differentiated pancreatic cancer cells.

Cisplatin or cis-diamminedichloroplatinum (CDDP) is an antineoplastic in the class of

alkylating agents and is used to treat various forms of cancer. Alkylating agents are so named because of their ability to add alkyl groups to many electronegative groups under conditions present in cells [68]. They stop tumor growth by cross-linking guanine bases in DNA double-helix strands - directly attacking DNA. This makes the strands unable to uncoil and separate. As this is necessary in DNA replication, the cells can no longer divide [69]. In addition, these drugs add methyl or other alkyl groups onto molecules where they do not belong which in turn inhibits their correct utilization by base pairing and causes a miscoding of DNA [70].

Alkylating agents are cell cycle nonspecific and have three different mechanisms all of which achieve the same end result as disruption of DNA function and cell death.

1. CDDP induces DNA strand crosslinks and DNA damage recognition and exerts its cytotoxic properties by reacting with DNA, which eventually culminates in irreversible apoptosis [71].
2. CDDP primarily interacts with the N7-sites of purine residues in DNA to form DNA-DNA inter-strand and intra-strand crosslinks. These crosslinks block DNA replication and transcription followed by DNA damage recognition by over 20 proteins including human mutS homolog 2 (hMSH2) of the mismatch repair (MMR) complex [72].
3. The putative role of these DNA damage recognition proteins and the induction of mispairing of the nucleotides lead to mutations and transmit DNA damage signals to downstream signaling cascades involving p53, MAPK, and p73, which ultimately induce apoptosis [73].

CDDP is used to treat many types of solid malignancies, however, it is widely prescribed for testicular, ovarian, bladder, lung, stomach and pancreatic cancers regardless of its high toxicity or primary and secondary resistance of cancer cells to CDDP [74, 75]. Moreover, the

mechanisms of CDDP resistance have been suggested to involve in reduced intracellular CDDP accumulation [76], increased inactivation of CDDP by thiol-containing molecules [77], increased DNA damage repair, and inhibition of transmitted DNA damage recognition to apoptotic pathways [78].

Therefore, CDDP is not recommended to use as monotherapy to prevent drug resistance and CDDP is now used with Gemcitabine and some other anti-cancer drugs to enhance the survival rate of pancreatic cancer after chemo-radiotherapy [79]. In this study, we proposed whether CDDP enhanced the cell death in poorly or well-differentiated pancreatic cancer cell lines (MP-2 or Capan-1 and PL-12, respectively). Moreover, if we induced poorly differentiated pancreatic cancer cells (MP-2) to be more differentiated by using anergized NK supernatants, we studied whether CDDP induced cell death would be higher or would remain as low as untreated poorly differentiated pancreatic cancer cells.

Some studies have shown that CDDP induced apoptosis caused nephrotoxicity, neurotoxicity, myelotoxicity and ototoxicity. However, N-acetyl cysteine (NAC) has protective against CDDP induced toxicities by blocking the caspase-signaling pathway [80]. NAC is a cysteine analog with free radical scavenging activity that is gaining use as a chemoprotective agent [81]. NAC is also a membrane permeable aminothiol compound with diverse functions. NAC is shown to be the precursor of glutathiones (GSH) with a significant anti- oxidant effect. Although previously published reports on the function of NAC have largely been concentrated on its anti-oxidant effect, recent reports have underscored the significance of this compound in inhibition of proliferation and induction of differentiation [82]. NAC has also been used as an anti-inflammatory compound [83]. Therefore, NAC is shown to have a number of different functions depending on the nature of the cells and their stage of maturation. However, the

primary functions of NAC are inhibition of cell death by HEMA induced apoptotic cell death and restoration of the function of DPSCs and oral epithelial cells. NAC inhibits HEMA mediated toxicity through induction of differentiation in DPSCs since the genes for dentin sialoprotein, osteopontin, osteocalcin, and Alkaline Phosphatase which are induced during differentiation are also induced by NAC [84]. In our studies, we would like to find out whether the combined treatment of NAC and CDDP or NAC and Paclitaxel prevented CDDP or Paclitaxel induced apoptosis in poorly differentiated pancreatic cancer cells (MP-2) and in well-differentiated pancreatic cancer cells (PL-12 and Capan-1). Moreover, we would like to study which combined drug treatment (NAC and CDDP or NAC and Paclitaxel) accelerates tumor cell apoptosis in poorly differentiated pancreatic cancer cells (MP-2) and in differentiated MP-2 induced by anergized NK supernatants.

Material and Methods

Cell lines, Reagents and Antibodies

Human pancreatic cancer cell lines MIA PaCa-2 (MP-2) and Capan-1 were generously provided by Dr. Graham Donald (UCLA, Los Angeles, CA, USA) and pancreatic cancer cell line 12 (PL-12) was kindly contributed by Dr. Nicholas Cacalano (UCLA, Los Angeles, CA, USA). MP-2 was cultured DMEM (Dulbecco's Modified Eagle Medium) in supplement with 10% Fetal Bovine Serum (FBS) and 2% Penicillin-Streptomycin (Pen-Strep). Capan-1 and PL-12 were cultured in RMPI 1640 medium supplemented with 10% FBS and 2% Penicillin-Streptomycin (Pen-Strep). OSCCs and stem-like OSCSCs were isolated from oral cancer patient tongue tumors at UCLA, and cultured in RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS), 1.4% antibiotic antimycotic, 1% sodium pyruvate, 1.4% non-essential amino

acids, 1% L-glutamine, 0.2% gentamicin (Gemini Bio-Products, CA) and 0.15% sodium bicarbonate (Fisher Scientific, PA). The human NK cells enrichment immunomagnetic negative selection kit was purchased from Stem Cell Technologies (Vancouver, Canada). Recombinant IL-2 was obtained from NIH- BRB. Isotype control IgG2b κ and antibodies against CD16 were purchased from Biolegend (San Diego, CA, USA). Propidium iodide and N-acetyl cysteine (NAC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Cisplatin (CDDP) and Paclitaxel were purchased from Bristol-Myers Squibb (New York, NY, USA). FBS and Pen-strep were purchased from Gemini Bio-Products (West Sacramento, CA, USA). DMEM, RPMI and DPBS (Dulbecco's Phosphate Buffered Saline) were purchased from Life Technologies (Los Angeles, CA, USA).

MIA Paca-2 (MP-2)

MP-2 was derived from the pancreas adenocarcinoma of a 65-year-old man who presented with abdominal pain for 6 months and a palpable upper abdominal mass. The tumor involved the body and tail of the pancreas and had infiltrated the periaortic area. The tumor did not express measurable amounts of carcinoembryonic antigen and an alkaline phosphatase stain was negative [45].

Capan-1

Capan-1 was obtained from a liver metastasis of a 40-year-old male with an adenocarcinoma in the head of the pancreas. Metastases were present in regional lymph nodes. In euthymic mice, Capan-1 derived tumor-produced mucin and were morphologically and biochemically similar to the origin of tumor. Although not reported in the original publication, a doubling time of 41 hours was subsequently determined for Capan-1 [48].

PL-12 (Pancreatic cancer cell line 12 or PANC 10.05)

PL-12 is a pancreatic adenocarcinoma epithelial cell line derived in 1992 from a primary tumor removed from the head-of-the-pancreas of a male with pancreatic adenocarcinoma. Panc 10.05 cell lines exhibit a K-ras oncogene mutation at codon 12 where a GGT to GAT mutation resulted in substitution of aspartic acid for glycine. The cells have a reported plating efficiency of 40% [49].

Purification of NK 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). NK cells were left untreated and treated with IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) and kept in the incubator at 37^{o}C for 16-18 hours.

Stem cells differentiation with NK supernatant

Human NK cells were purified from healthy donor's PBMCs as described above. NK cells were left untreated, treated a combination or IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 18- 24 hours before the supernatants were removed and used in differentiation experiments. The amounts of IFN-γ produced by activated NK cells were assessed with IFN-γ ELISA (Biolegend, CA). Differentiation of MP-2 was conducted with gradual daily addition of increasing amounts of NK cell supernatant. MP-2 required on average a total concentration of 5500 pg of IFN-γ containing supernatants obtained from IL-2+anti-CD16mAb treated NK cells with our without combination of IFN-γ and TNF-α antibodies during a 5 day treatment to induce differentiation. Afterwards, target cells were washed with 1xDPBS, detached and used for experiments.

Determination of Apoptosis

Propidium iodide (PI) solution was be used in evaluation of apoptosis, cell viability and cell cycle analysis by flow cytometry. 1mg of PI powder was diluted with 1 ml of DPBS and used 5ul of PI solution for each condition of target cells. After 15 minutes of incubation on ice, PI stained cells were analyzed by flow cytometry. Flow cytometric analysis was performed using EPICs-ELITE flow cytometer (Coulter, Miami, FL).

Results

Aim 3 sub-aim 3A: Whether stem-like pancreatic tumors are resistant to chemotherapeutic drugs

NAC induced apoptosis to MP-2, Capan-1, PL-12, OSCC and OSCSC

Poorly differentiated pancreatic cancer cells MP-2, well-differentiated pancreatic cancer cells Capan-1 and PL-12, OSCCs and stem-like OSCSCs were treated with or without 20nM of NAC for 24 hours. The cells were detached from the plates and 5ul of 1mg/ml of PI solution were stained and analyzed in flow cytometer. NAC induced apoptosis in NAC treated MP-2, Capan-1, PL-12, OSCC and OSCSC when there was less cell death in cells without NAC treatment (Fig-3A). Moreover, NAC accelerated more cell death in well-differentiated pancreatic cancer cells Capan-1 and PL-12, and well-differentiated oral cancer cells OSCC rather than poorly differentiated pancreatic cancer cells MP-2 nor oral cancer stem cells OSCSC (Fig-3A). Therefore, NAC induced apoptosis significantly in well-differentiated pancreatic cancer and OSCSCs rather than poorly differentiated pancreatic cancer or OSCCs.

NAC inhibited CDDP mediated apoptosis significantly in well-differentiated pancreatic cancer cells PL-12 and Capan-1

MP-2, PL-12 and Capan-1 were left untreated, treated with CDDP and treated with NAC with or without CDDP for 24 hours. 100ug/ml of CDDP was treated to specific conditions of cells and 20nM concentration of NAC was used which was determined as the optimal dose for protection of CDDP mediated cell death. The cells were detached from the plates and 5ul of 1mg/ml of PI solution were stained and analyzed in flow cytometer.

Figure 3A

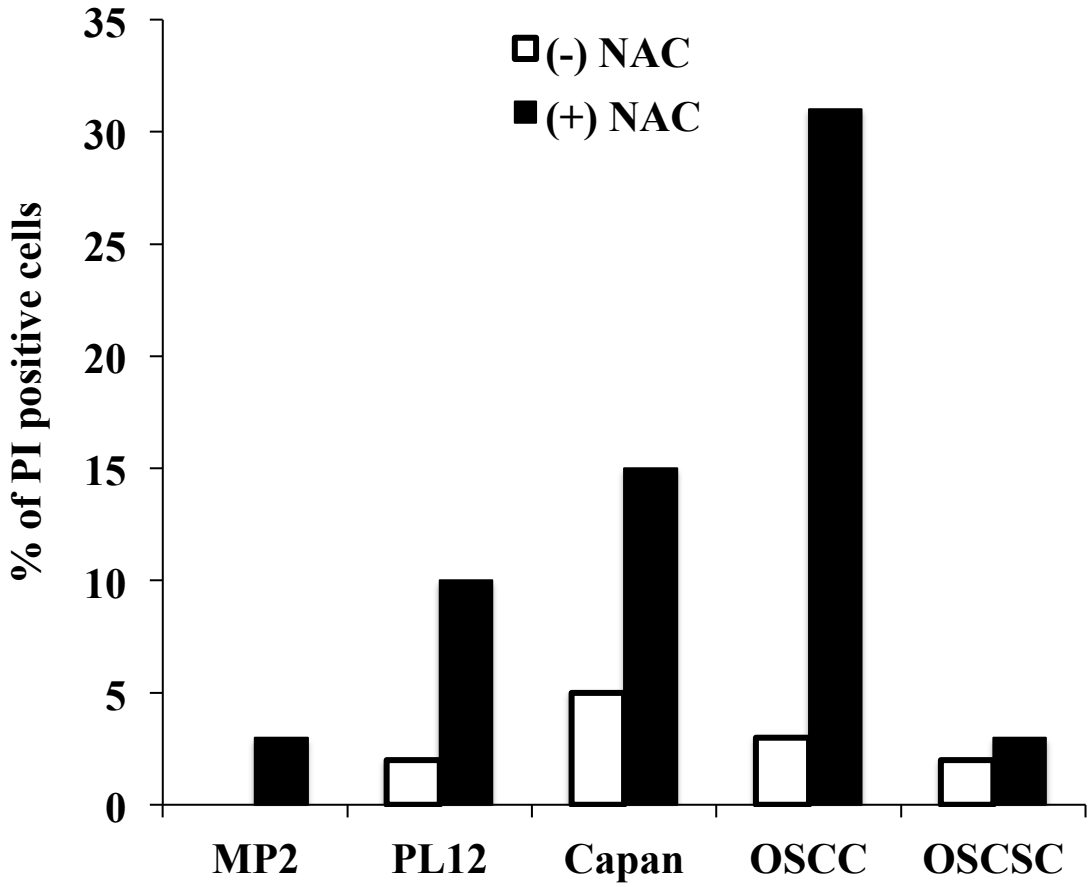

Figure 3A. NAC induced higher level of apoptosis in well differentiated PL-12, Capan-1 and OSCC treated with NAC compared to NAC treated poorly differentiated MP-2 and OSCSC.

MP-2, PL-12, Capan-1, OSCC and OSCSC were cultured for 2 days to have 90% confluence in 10 cm square plates. Then they were detached by using trypsin, spinned down, removed the supernatants, broke the pallets and re-suspended to 1 million per ml. And each cell line was plated into $1x10^5$ cells in 24 well plates and let them adhere to the wells for 24 hours. On the next day, each cell line was treated with or without 20nM of NAC and kept in the incubator for 24 hours. The cells were detached from the plate, determined the apoptosis by staining them with Propidium iodide and analyzed with flow cytometer.

CDDP induced apoptosis was significantly increased in untreated PL-12 and Capan-1 when compared to untreated MP-2 (Fig- 3B). Moreover, there was a remarkable acceleration of CDDP induced apoptosis in untreated OSCC when compared to untreated OSCSC whose level of CDDP induced apoptosis was noticeably lower than untreated OSCC (Fig-3C).

We confirmed that NAC induced apoptosis was also tremendously escalated in NAC treated PL-12, Capan-1 and OSCC than NAC treated MP-2 and OSCSC (Fig- 3B, 3C). However, NAC interestingly inhibited CDDP induced cell death in NAC treated MP-2, PL-12, Capan-1, OSCC and OSCSC (Fig- 3B, 3C). From figure 3B and 3C, we could determine that NAC provided the inhibitory action against CDDP.

NAC triggered Paclitaxel induced cell death not only in pancreatic cancer cells but also in oral cancer cells.

MP-2, PL-12 and Capan-1 were left untreated, treated with Paclitaxel and treated with NAC with or without Paclitaxel for 24 hours. 5 different concentrations of Paclitaxel were treated to specific conditions of cells. We calculated the dosage of Paclitaxel according to C_{max} (maximum plasma concentration) and MP-2, PL-12, Capan-1, OSCC and OSCSC were treated with 10nM, 200nM, 600nM and 1000nM concentration of Paclitaxel. Then, 20nM concentration of NAC was used which was determined as the optimal dose for protection of Paclitaxel mediated cell death after 24 hours. The cells were detached from the plates and 5ul of 1mg/ml of PI solution were stained and analyzed in flow cytometer.

Figure 3B

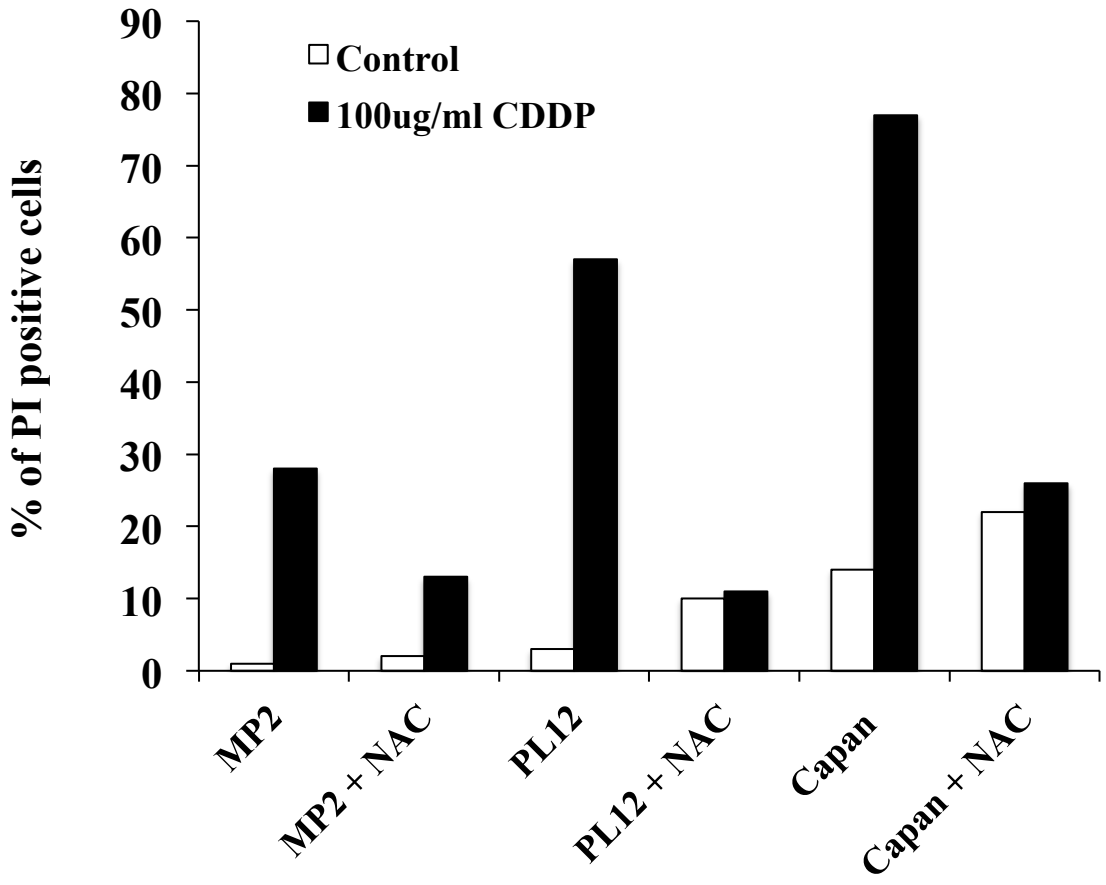

Figure 3B. NAC inhibited CDDP induced apoptosis in NAC treated MP-2, PL-12 and Capan-1.

MP-2, PL-12 and Capan-1 were cultured for 2 days to have 90% confluence in 10 cm square plates. Then they were detached by using trypsin, spinned down, removed the supernatant, broke the pallets and re-suspended the cells to 1 million per ml. And each cell line was plated into $1x10^5$ cells in 24 well plates and let them adhere to the wells for 24 hours. On the next day, each cell line was treated with 100ug/ml of CDDP in the presence or absence of 20nM of NAC and kept in the incubator for 24 hours. The cells were detached from the plate, determined the apoptosis by staining them with propidium iodide and analyzed with flow cytometer.

Figure 3C

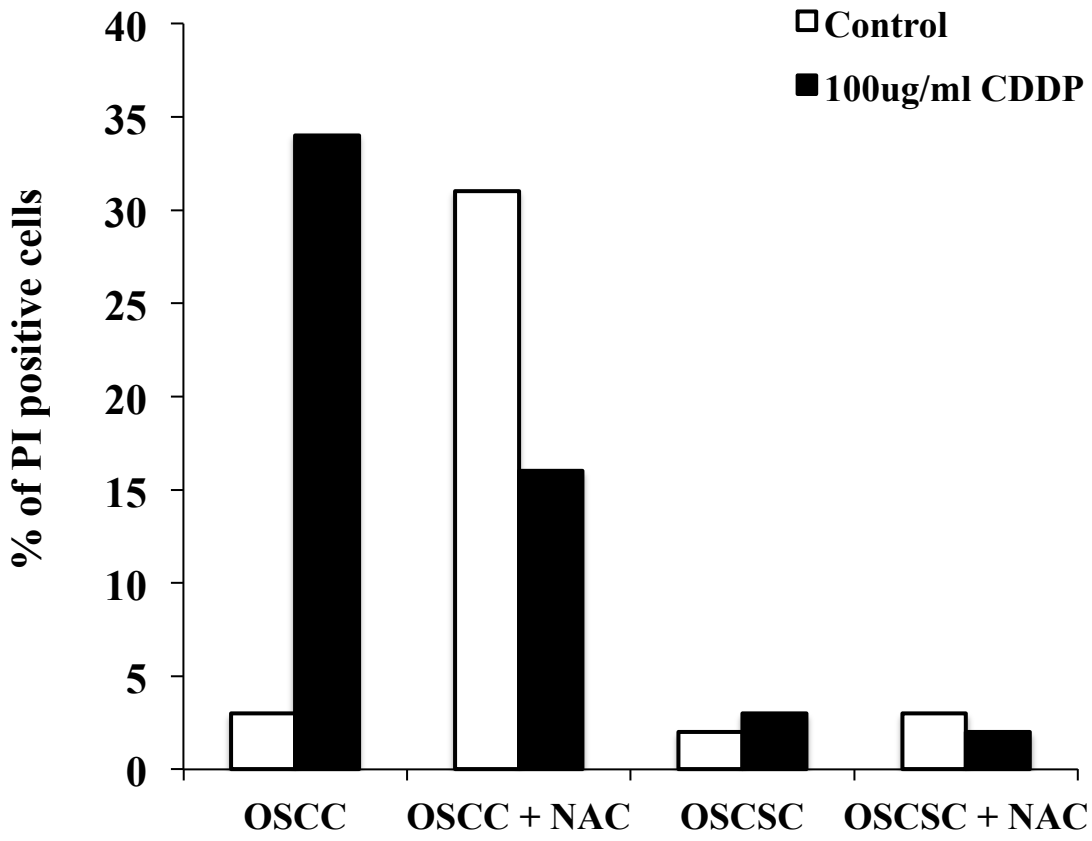

Figure 3C. NAC significantly inhibited CDDP induced apoptosis in NAC treated OSCC.

OSCC and OSCSC were cultured for 2 days to achieve 90% confluence in 10 cm square plates. Then they were detached by using trypsin, spinned down, removed the supernatant, broke the pallets and re-suspended the cells to 1 million per ml. And each cell line was plated into $1x10^5$ cells in 24 well plates and let them adhere to the wells for 24 hours. On the next day, each cell line was treated with 100ug/ml of CDDP in the presence or absence of 20nM of NAC and kept in the incubator for 24 hours. The cells were detached from the plate, determined the apoptosis by staining them with propidium iodide and analyzed with flow cytometer.

In figure 3D, there was no significant induction of NAC mediated apoptosis in Paclitaxel and NAC treated MP-2 when compared to Paclitaxel treated MP-2 without NAC. MP-2 treated with NAC and 1000nM didn't even accelerate the apoptosis of MP-2. However, Capan-1 and PL-12 treated with NAC and Paclitaxel showed there was synergistic action between NAC and Paclitaxel on Capan-1 and PL-12 because there was detectable acceleration of NAC induced cell death in Capan-1 and PL-12 treated with NAC and different doses of Paclitaxel when compared to Paclitaxel treated Capan-1 and PL-12 without NAC. We added the same dosages of Paclitaxel and same concentration of NAC to MP-2, PL-12 and Capan-1. However, there were different responses on each cell line. NAC triggered Paclitaxel-induced apoptosis in well differentiated PL-12 and Capan-1 not in poorly differentiated MP-2. Moreover, Paclitaxel mediated more cell death in higher dose in PL-12 and Capan-1 (Fig- 3D)

There was also same phenomenon occurred in OSCC and OSCSC (Fig- 3E). OSCC treated with NAC and Paclitaxel responded more cell death than OSCC treated with Paclitaxel alone. OSCSC also responded cell death but not as much as OSCC. Therefore, NAC induced Paclitaxel mediated apoptosis more in OSCC than in OSCSC. Moreover, NAC had the synergistic action on OSCC and OSCSC treated with Paclitaxel, which enhanced more cell death in combined treatment rather than treating Paclitaxel or NAC alone to OSCC or OSCSC. They were also dose-dependent. The higher the dose of Paclitaxel, the more cell death was induced by NAC (Fig- 3E).

As of specific aim 3 sub-aim 3 A, we had proved that there was resistance in lysis stem-like pancreatic cancer cells treated with chemotherapeutic drugs such as Cisplatin (CDDP) and Paclitaxel with or without NAC (N-acetyl cysteine). Moreover, we found that NAC treated

pancreatic cancer showed more cell death in well-differentiated cancers than poorly differentiated one. NAC inhibited CDDP mediated cell death in pancreatic cancer cells and oral cancer cells while NAC accelerated Paclitaxel mediated cell death in those cancer cells. On the other hand, NAC exhibited more significant cell death in well differentiated PL-12, Capan-1 and OSCC rather than poorly differentiated MP-2 and OSCSC. Therefore, combination of Paclitaxel and NAC had synergistic action on pancreatic as well as oral cancer cells but more significant in well differentiated oral and pancreatic cancer cells such as OSCC, PL-12 and Capan-1.

Aim 3 sub-aim 3B: Whether stem-like pancreatic tumors differentiated by anergized NK cells become susceptible to chemotherapeutic drugs.

NAC induced apoptosis to differentiated MP-2.

We induced differentiation of MP-2 by using untreated NK supernatants, and IL-2 and anti CD16 mAb treated NK supernatants with or without antibodies combination of IFN-γ and TNF-α according to technique stated in materials and methods section. Undifferentiated and differentiated MP-2s were treated with or without NAC. In figure 3F, MP-2 differentiated with IL-2 and anti CD16 mAb treated NK supernatants showed more cell death in treatment without NAC when compared to untreated or undifferentiated MP-2, MP-2 differentiated with untreated NK supernatants and MP-2 differentiated with IL-2 and anti-CD16 mAb treated NK supernatants with combined IFN-γ and TNF-α antibodies (Fig- 3F).

Figure 3D

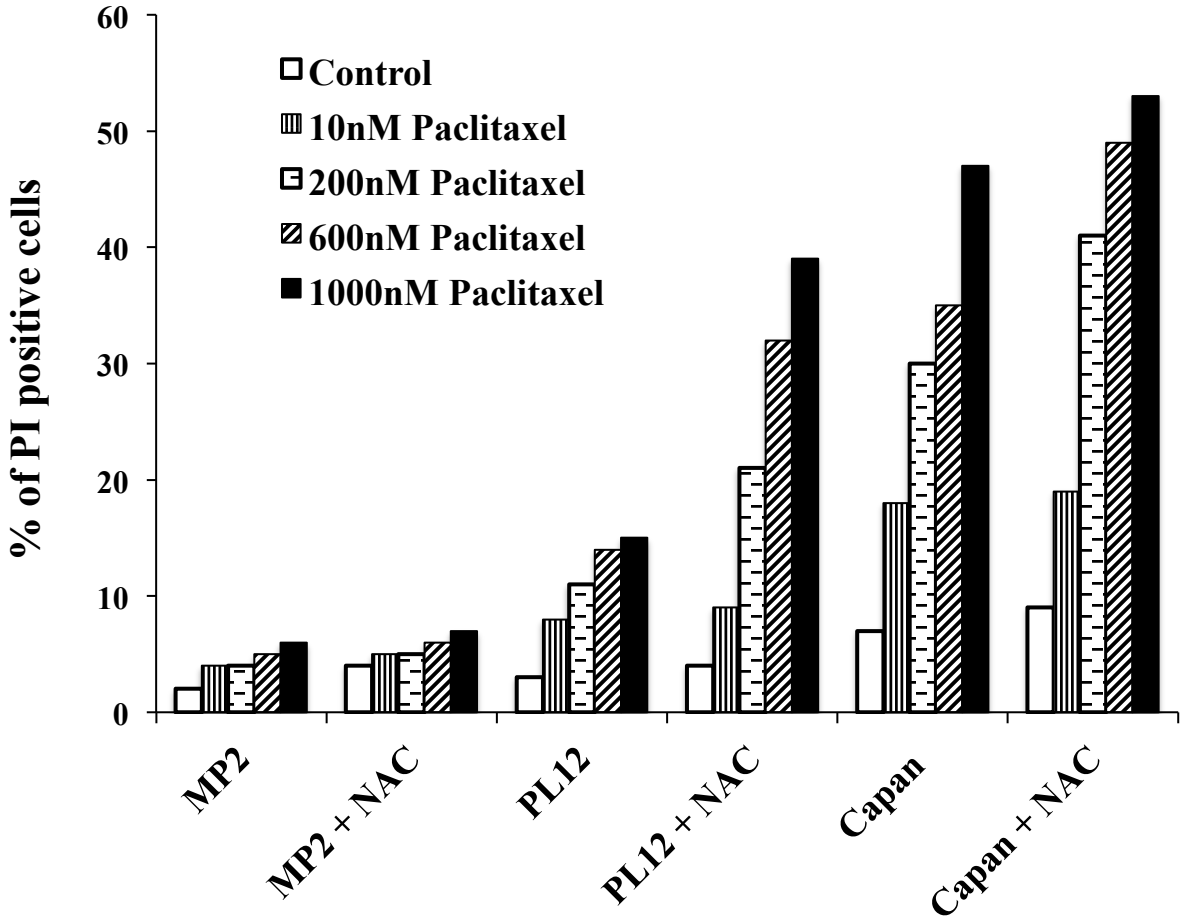

Figure 3D. NAC induced Paclitaxel mediated apoptosis in PL-12 and Capan-1.

MP-2, PL-12 and Capan-1 were cultured for 2 days to have 90% confluence in 10 cm square plates. Then they were detached by using trypsin, spinned down, removed the supernatant, broke the pallets and re-suspended the cells to 1 million per ml. And each cell line was plated into $1x10^5$ cells in 24 well plates and let them adhere to the wells for 24 hours. On the next day, each cell line was treated with 10nM, 200nM, 600nM and 1000nM of Paclitaxel in the presence or absence of 20nM of NAC and kept in the incubator for 24 hours. The cells were detached from the plate, determined the apoptosis by staining them with propidium iodide and analyzed with flow cytometer.

Figure 3E

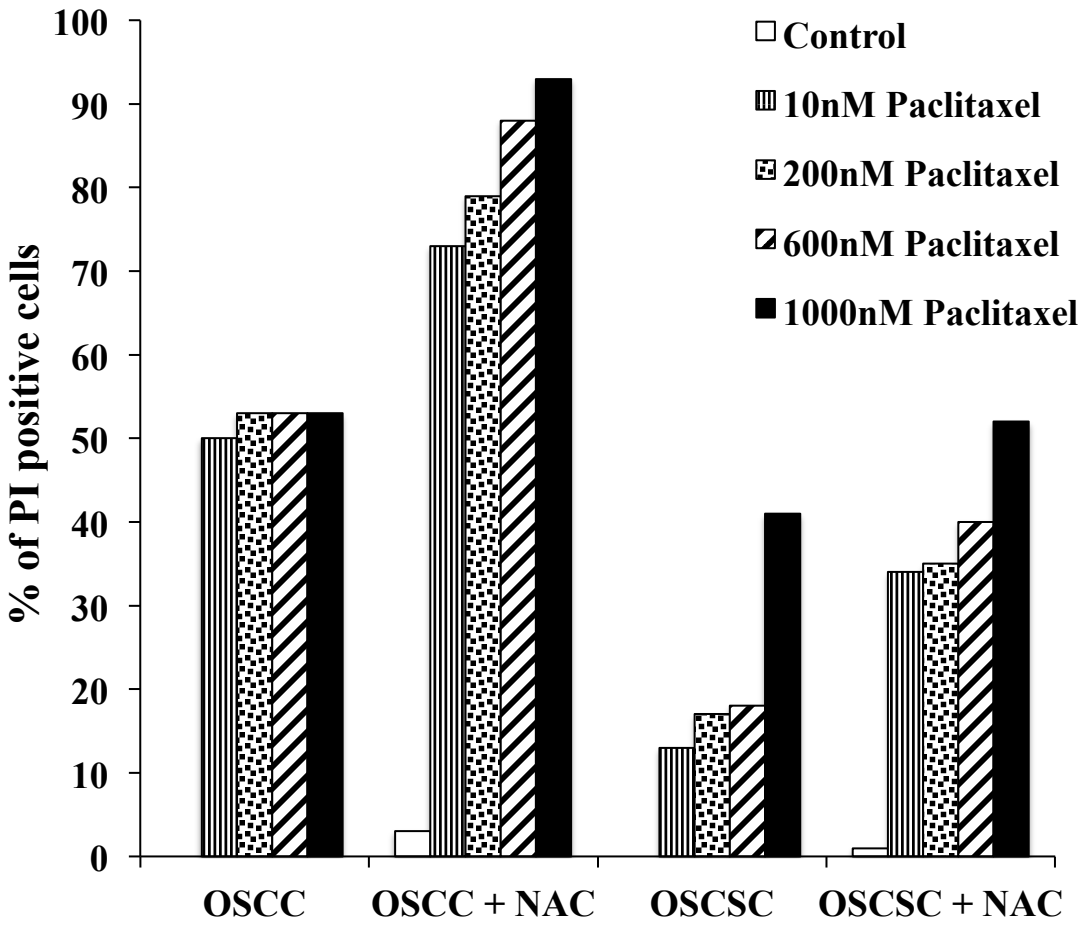

Figure 3E. NAC noticeably induced Paclitaxel mediated apoptosis in NAC treated OSCC.

OSCC and OSCSC were cultured for 2 days to have 90% confluence in 10 cm square plates. Then they were detached by using trypsin, spinned down, removed the supernatant, broke the pallets and re-suspended the cells to 1 million per ml. And each cell line was plated into $1x10^5$ cells in 24 well plates and let them adhere to the wells for 24 hours. On the next day, each cell line was treated with 10nM, 200nM, 600nM and 1000nM of Paclitaxel in the presence or absence of 20nM of NAC and kept in the incubator for 24 hours. The cells were detached from the plate, determined the apoptosis by staining them with propidium iodide and analyzed with flow cytometer.

Moreover, there was a tremendous rise of NAC-induced apoptosis in MP-2 differentiated with IL-2 and anti CD16 mAb treated NK supernatants. Untreated MP-2, MP-2 treated with untreated NK supernatant and MP-2 differentiated with IL-2 and anti CD16 mAb treated NK supernatants with combined IFN-γ and TNF-α antibodies also showed NAC-induced apoptosis, however, the level of NAC-induced apoptosis was not as highly significant as MP-2 treated with IL-2 and anti CD16 mAb treated NK supernatants. Looking at it the other way, antibodies against IFN-γ and TNF-α blocked the differentiation of MP-2 with IL-2 and anti CD16 mAb treated NK supernatants and became resistant to NAC-induced apoptosis. Therefore, NAC induced more cell death in differentiated MP-2 treated with IL-2 and anti CD16 mAb treated NK supernatants (Fig- 3F)

NAC inhibited CDDP induced apoptosis in undifferentiated and differentiated MP-2s

We induced differentiation of MP-2 by using untreated NK supernatants, and IL-2 and anti-CD16 mAb treated NK supernatants with or without antibodies combination of IFN-γ and TNF-α according to technique stated in materials and methods section. Undifferentiated and differentiated MP-2s were treated 100ug/ml of CDDP with or without NAC. Figure 3G had shown that NAC induced more cell death in NAC treated undifferentiated MP-2, MP-2 treated with untreated NK supernatants and MP-2 treated with IL-2 and anti-CD16 mAb treated NK supernatants with antibodies combination of IFN-γ and TNF-α, however, NAC induced more cell death in MP-2 treated with IL-2 and anti-CD16 mAb treated NK supernatants without antibodies combination of IFN-γ and TNF-α.

In addition, CDDP induced cell death in all conditions of MP-2s, more significantly in MP-2 treated with IL-2 and anti-CD16 mAb treated NK supernatants without antibodies against

Figure 3F

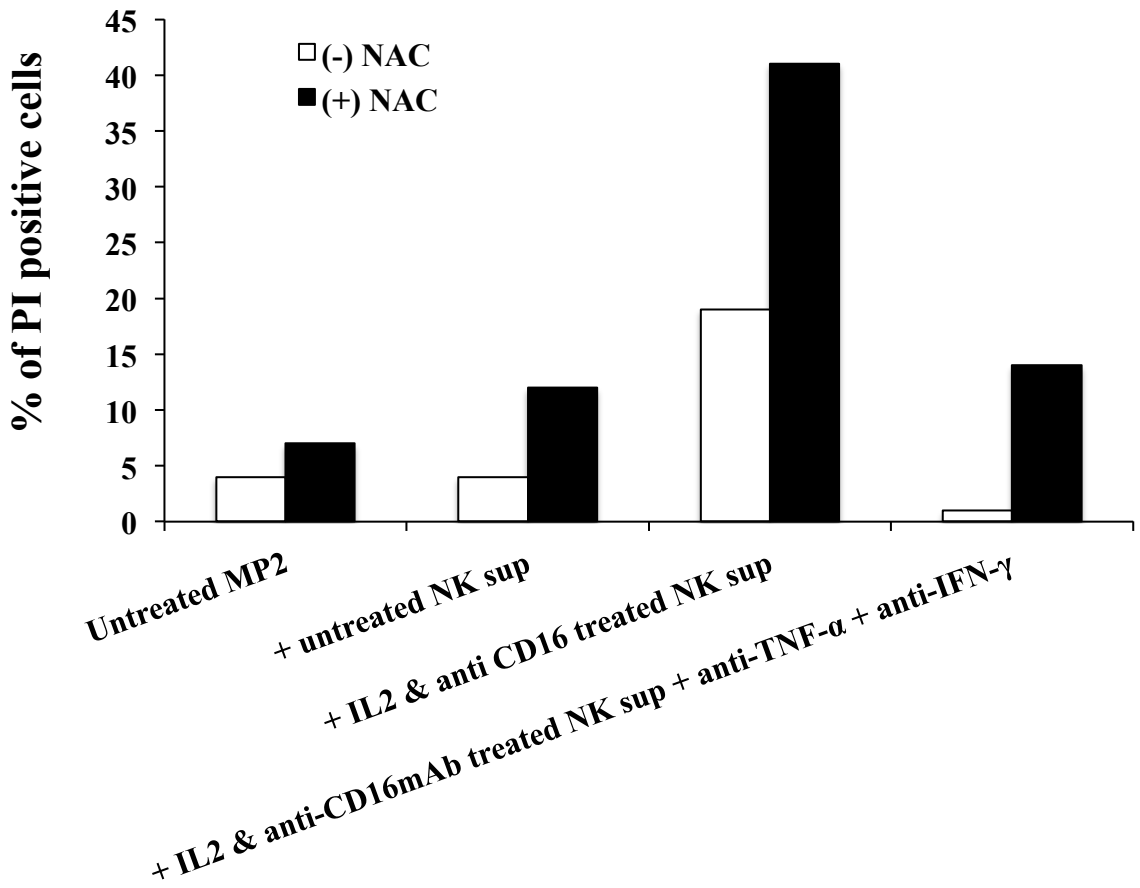

Figure 3F. NAC induced more cell death in differentiated MP-2.

NK cells purified from healthy donors were left untreated or treated with a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 24 hours. The amounts of IFN-γ produced by activated NK cells were assessed with IFN-γ ELISA. On the next day, MP-2s were left untreated, treated with untreated NK supernatants and treated with IL-2 and anti-CD16 mAb treated NK supernatants with or without combination of antibodies against IFN-γ and TNF-α for 5 days to induce differentiation. On 6^{th} day, each condition of MP-2 was detached, re-plated to $1x10^5$ into 24 well plates and let them adhere to the plate for 24 hours. On the next day, NAC were treated and kept in the incubator for 24 hours. The cells were detached from the plate, determined the apoptosis by staining them with propidium iodide and analyzed with flow cytometer.

Figure 3G

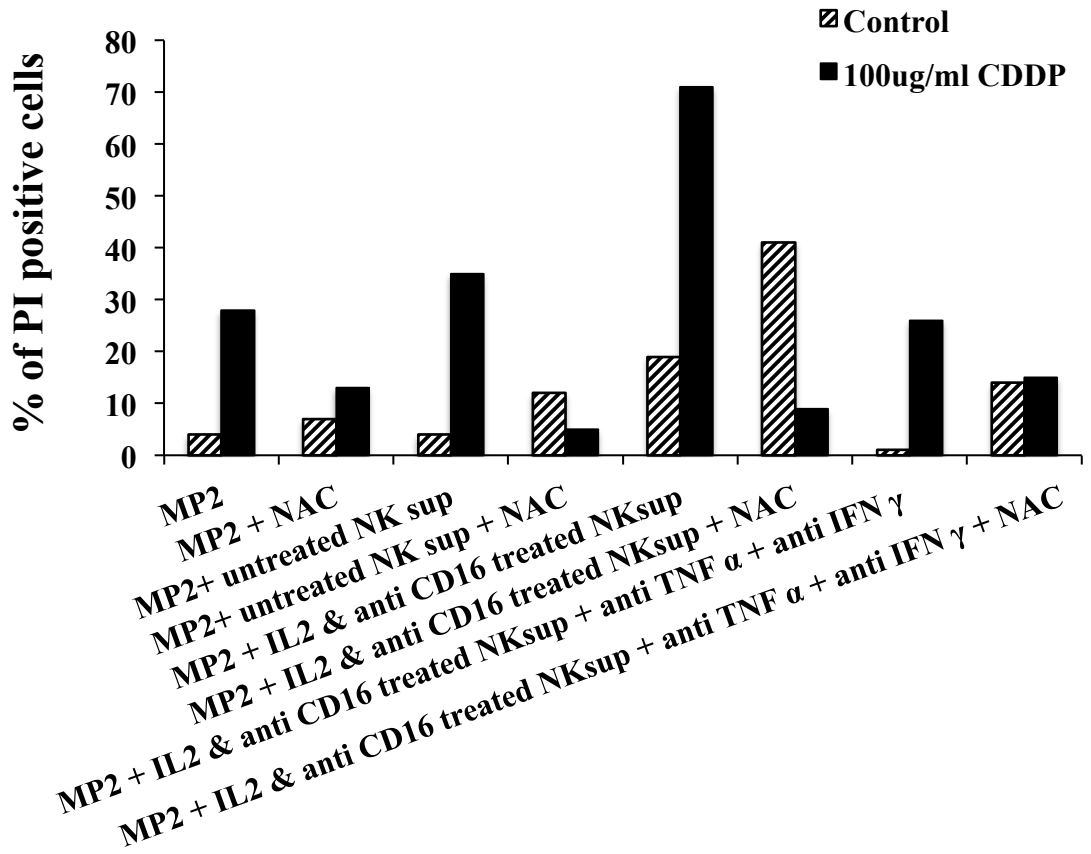

Figure 3G. NAC inhibited CDDP induced cell death in untreated and differentiated MP-2s.

NK cells purified from healthy donors were left untreated or treated with a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 24 hours. The amounts of IFN-γ produced by activated NK cells were assessed with IFN-γ ELISA. On the next day, MP-2s were left untreated, treated with untreated NK supernatants and treated with IL-2 and anti-CD16 mAb treated NK supernatants with or without combination of antibodies against IFN-γ and TNF-α for 5 days to induce differentiation. On 6^{th} day, each condition of MP-2 was detached, re-plated to $1x10^5$ into 24 well plates and let them adhere to the plate for 24 hours. On the next day, 100ug/ml of CDDP was added to each condition of MP-2 with or without NAC and kept in the incubator for 24 hours. The cells were detached from the plate, determined the apoptosis by staining them with propidium iodide and analyzed with flow cytometer.

IFN-γ and TNF-α combination (Fig- 3G). Therefore, we confirmed that CDDP induced more cell death in more differentiated MP-2s rather than untreated or less differentiated MP-2s. Interestingly, each condition of MP-2 treated with NAC showed inhibition of CDDP induced cell death more significantly in MP-2 treated with untreated NK supernatant and treated with IL-2 and anti-CD16 mAb treated NK supernatants (Fig- 3G).

NAC triggered Paclitaxel induced cell death not only in pancreatic cancer cells but also in oral cancer cells.

MP-2s were left untreated and treated with IL-2 and anti-CD16 mAb treated NK supernatants with or without antibodies against IFN-γ and TNF-α combination for 5 days. Then, untreated and differentiated MP-2s with or without antibodies were treated with 10nM, 200nM and 600nM Paclitaxel with or without NAC. We found that NAC enhanced Paclitaxel mediated apoptosis in untreated MP-2 and differentiated MP-2 with IL-2 and anti-CD16 mAb treated NK supernatants with or without antibodies against combination of IFN-γ and TNF-α, more significantly in differentiated MP-2 without antibodies. We found that there was no synergy between Paclitaxel and NAC on differentiated MP-2 and it could be additive effect (Fig- 3H). Moreover, Paclitaxel induced more cell death in differentiated MP-2 without antibodies against combination of IFN-γ and TNF-α when compared to untreated MP or differentiated MP-2 with antibodies against IFN-γ and TNF-α (Fig- 3H). Therefore, we had proved that there was a increase level of tumor cell lysis induced by Paclitaxel with NAC in differentiated MP-2 treated with IL-2 and anti-CD16 mAb treated NK supernatants without antibodies against combination of IFN-γ and TNF-α (Fig- 3H).

Figure 3H

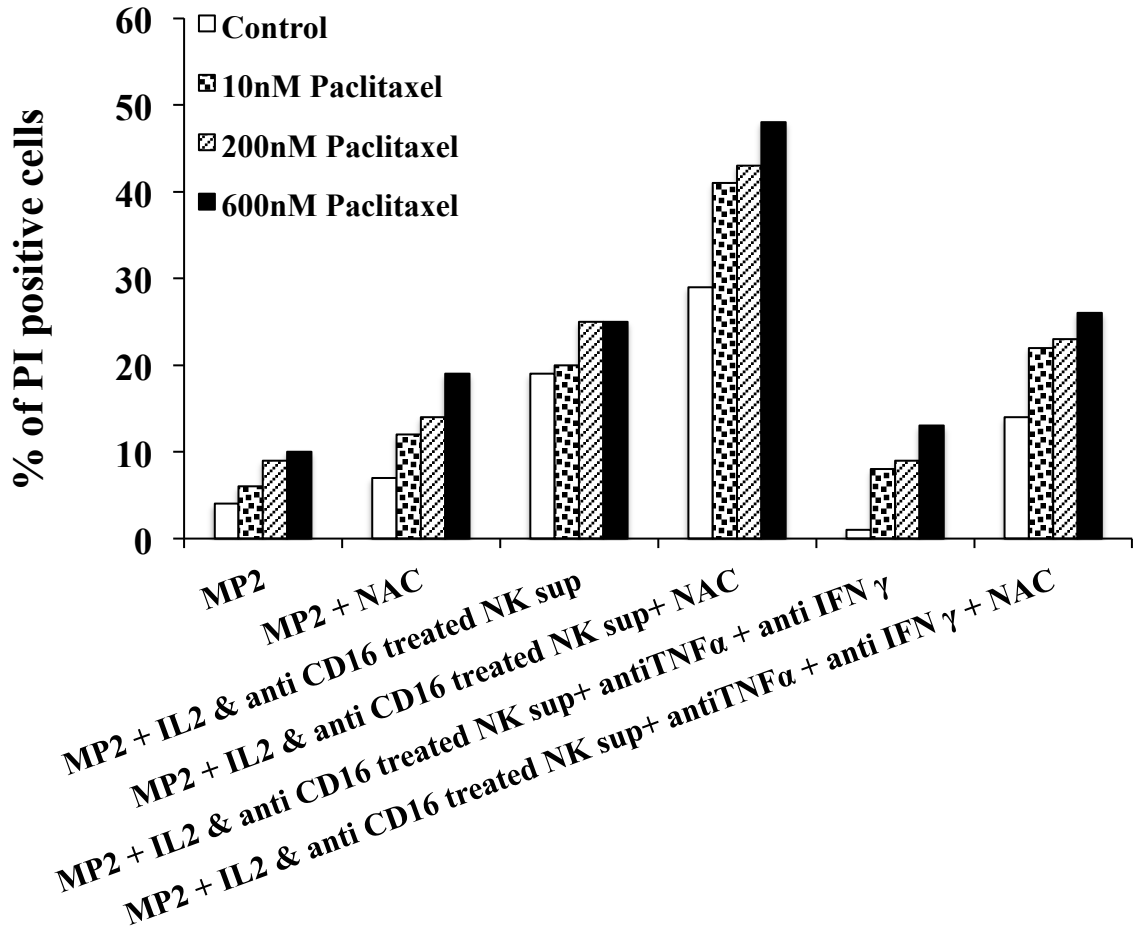

Figure 3H. NAC increased Paclitaxel induced cell death in untreated and differentiated MP-2s.

NK cells purified from healthy donors were left untreated or treated with a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 24 hours. The amounts of IFN-γ produced by activated NK cells were assessed with IFN-γ ELISA. On the next day, MP-2s were left untreated and treated with IL-2 and anti-CD16 mAb treated NK supernatants with or without combination of antibodies against IFN-γ and TNF-α for 5 days to induce differentiation. On 6^{th} day, each condition of MP-2 was detached, re-plated to $1x10^5$ into 24 well plates and let them adhere to the plate for 24 hours. On the next day, 10nM, 200nM and 600nM of Paclitaxel were added to each condition of MP-2 with or without NAC and kept in the incubator for 24 hours. The cells were detached from the plate, determined the apoptosis by staining them with propidium iodide and analyzed with flow cytometer.

Discussion

We had proved that poorly differentiated pancreatic cancer cells or oral cancer stem cells were resistant to single drug or multi-drugs of anti-cancer treatment while well-differentiated pancreatic cancers and oral squamous cell carcinomas were very sensitive to both single or multi-drug chemotherapy. In addition, NAC caused increae apoptosis of more diffrentiated cancer cells rather than poorly differentiated cancer cells. Moreover, we demonstrated that NAC enhacned Paclitaxel induced apoptosis more significantly in well-differentiated cancer cells such as PL-12, Capan-1 and OSCC while less significant in poorly-differentiated cancer cells such as MP-2 and OSCSC. Theforefore, we stated that NAC had synergistic effect on Pacltiaxel treated well-differentiated tumor cells. Reversely, we found that NAC inhibited CDDP induced cell death not only in well-differentiated but also in poorly differentiated cancer cells. However, NAC decreased significantly in CDDP mediated cell death in well-differentiated cancer cells such as PL-12, Capan-1 and OSCC.

As of what we had shown in chapter 1, MP-2 were poorly differentiated pancreatic cancer cells. We had already proved in chapter 2 that poorly differentiated MP-2 became more differentiated and behaved like well differentiated PL-12 or Capan-1. Therefore, in this chapter 3, we induced differentiation of MP-2 by using untreated anergized NK supernatants, IL-2 and anti-CD16 mAb treated anergized NK supernatants in the presence or absence of antibodies against combination of IFN-γ and TNF-α. We had also studied how MP-2 behaved if we differentiated them with or without antibodies against combination of IFN-γ and TNF-α. We found that antibodies against combination of IFN-γ and TNF-α blocked MP-2 to be more differentiated by anergized NK supernatants and became resistant to CDDP or Paclitaxel

treatment. Therefore, we believed that if we could increase the cytokine secretions in tumor microenvironment, the combined chemotherapy would be more effective than single therapy. Upon what we had shown in result section of Chapter 3, differentiated MP-2s were become more sensitive to CDDP or Paclitaxel induced apoptosis than untreated MP-2. And even in differentiated MP-2s, CDDP or Paclitaxel promoted more cell death in MP-2 treated with IL-2 and anti-CD16 mAb treated NK supernatants when compared to less cell death in MP-2 treated with untreated NK supernatants.

Therefore, we believed that if we could differentiate poorly differentiated cancer cells to become more differentiated by using IL-2 and anti-CD16 treated anergized NK supernatants, chemotherapy therapy would achieve greater success and higher survival from cancer related death. In addition, immune-chemotherapy would be advanced cancer treatment era.

Conclusion

According to the above findings, we had studied that there was resistance in tumor cell lysis in stem-like pancreatic cancer cells and increased apoptosis in well-differentiated cancer cells. Moreover, we had demonstrated that if we differentiated the poorly differentiated pancreatic cancers to be more differentiated, they became sensitive to chemotherapeutic drugs not only in single drug therapy but also in multi-drugs therapy.

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. Effectors of the immune system are known to shape tumor cells (immunoediting) and select for cancers with reduced immunogenicity and enhanced capacity to actively induce immunosuppression. However, the same effector mechanisms are likely responsible for the selection of healthy stem cells with enhanced capacity to induce immunosuppression for the ultimate goal of the regeneration of damaged or disturbed tissues. Therefore, we studied to characterize the pancreatic cancers using NK cells and tumor makers in terms of NK cell cytotoxicity and surface expressions. As we have studied, NK cells lyse stem cells or poorly differentiated cells. We have investigated that we differentiated the poorly differentiated pancreatic cancers to more differentiated by anergized NK cells and analyzed the sensitivity of NK cell cytotoxicity against target cells. Moreover, stem cells are resistant to chemotherapy while well-differentiated cells are sensitive. We have also examined that differentiated stem cells became sensitive to the treatment of anti-cancer drugs and our vitro chemo-experiment was successful. Therefore, combination of immunotherapy and chemotherapy would be the superior era for prevention and curing cancer.

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