## Title

Bacteria Induced Split Anergy in NK Cells Drive Maturation, Differentiation and Resistance of Healthy and Transformed Stem Cells



## Author

Bui, Vickie Tam Doan

## Publication Date

2014

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

# Bacteria Induced Split Anergy in NK Cells Drive Maturation, Differentiation and Resistance of Healthy and Transformed Stem Cells

A thesis submitted in partial satisfaction

of the requirements of the degree of Master of Science

in Oral Biology

by

Vickie Tamdoan Bui

2014

## ABSTRACT OF THE THESIS

**Bacteria Induced Split Anergy in NK Cells Drive Maturation, Differentiation and Resistance of Healthy and Transformed Stem Cells**

By

Vickie Tamdoan Bui

Master of Science in Oral Biology

University of California, Los Angeles, 2014

Professor Anahid Jewett, Chair

Natural Killer (NK) cells are part of the innate immune system, they respond to virally infected cells and tumor formation. The NK cells are known to have a crucial role in mediating lysis against a variety of tumor cells through cell-mediated cytotoxicity or antibody-dependent cellular cytotoxicity (ADCC). Accumulated evidence from our lab has shown that anergized NK cells can induce resistance in transformed and healthy stem cells through secreted cytokines and cell-to-cell interaction. We previously showed that NK cells can significantly catalyze cytotoxicity against primary Oral Squamous Carcinoma Stem Cells (OSCSCs), human embryonic stem cells (hESCs), human mesenchymal stem cells (hMSCs), human dental pulp

stem cells (hDPSCs), and induced human pluripotent stem cells (hiPSCs)[1, 2] whereas their differentiated cells lines were more resistant. In addition, our experimental data demonstrated that stem cells become significantly resistant to NK mediated cytotoxicity when they were co-cultured in the presence of monocytes, probiotic bacteria and anti-IL10 monoclonal antibody. Thus, it is an emerging view in our laboratory that the stage of maturation and differentiation of healthy untransformed, as well as transformed tumorigenic, stem cells is predictive of their sensitivity to NK cells' mediated cytotoxicity[3]. We believe that NK cells have the ability to reduce inflammation and increase regeneration of new tissues by two ways: they lyse a small populations of stem cells that are incapable of differentiating and they support the best stem cells to maturation and differentiation after gaining cytokine producing function and up-regulate differentiation markers CD54, B7H1, and MHC-1 and down-regulate stem cell marker, CD44.

We previously found that CD16 receptor caused split anergy on the NK cells in the presence of IL-2. Split anergy was coined by our lab to explain the phenomenon where NK cells lose cytotoxicity but gain the ability to secrete cytokines to support maturation of various types of healthy and transformed stem cell. In this paper, our objective is to study probiotic bacteria because we found that they exacerbate the induction of split anergy on the NK cells through significant secretion of anti-inflammatory and pro-inflammatory cytokines, chemokines, and growth factors compared to CD16 receptor triggered the NK cells alone. We also found that the combination of probiotic bacteria (sAJ2) plus monocytes and anti-IL10 monoclonal antibodies were the ultimate induction of split anergy on NK cells compared to probiotic bacteria, monocytes, or the antibody alone triggering as together these monocytes, bacteria and antibodies have the great capacity to anergize NK cells to speed up the processes of maturation and differentiation of healthy and transformed stem cells and reduce inflammation much more.

Transformed stem cells such as Oral Squamous Cancer Stem Cells (OSCSC), Pancreatic Carcinoma MIA PaCa-2, Human Lung Adenocarcinoma Epithelial Cell Line (A549), human Glioblastoma cell line (X02D) and Human Chronic Myelogenous Leukemia Cell line (K562) as well as non-transformed stem cell, Stem Cell of Apical Papilla (SCAP) were used in a standard $^{51}$Chromium release assay to determine their sensitivity or resistance against NK cell-mediated cytolysis. The secretion of key cytokines by the NK cells induced by probiotic bacteria, such as Interferon-Gamma (IFN-γ), IL-10, and other cytokines, chemokines, and growth factors were determined using Enzyme-Linked Immunosorbent Assays (ELISAs). The death of tumor cells caused by different conditions of NK cell-treated probiotic bacteria were assessed through flow analysis using Propidium Iodide Stain. Transformed and non-transformed stem cells were differentiated using supernatants obtained from untreated NK cells, IL-2 and anti-CD16mAb treated NK cells, and a combination of IL-2 plus anti-CD16mAb and probiotic bacteria, treated NK cells including activators such as monocytes and anti-IL10 monoclonal antibody. We observed a universal step-wise differentiation level of stem cells when they were treated with supernatant of pre-conditioned NK cells that contained various level of IFN-γ production. The level of differentiation of the stem cells was confirmed through surface analysis using surface receptor markers CD54, CD44, B7H1, and MHC -1.

Our experimental findings showed that probiotic bacteria caused significant induction of split anergy on the NK cells via significant secretion of many cytokines and did not induce NK cells mediated cytotoxicity compared to IL-2 plus anti-CD16mAb treated NK cells. Monocytes and anti-IL10 also played a role in split anergy and caused resistance and differentiation of stem cells.

**The thesis of Vickie Tamdoan Bui is approved**

Ichiro Nishimura

Nicholas Cacalano

Anahid Jewett, Committee Chair

University of California, Los Angeles

2014

ACKNOWLEDGEMENTS

I would like to acknowledge my supervisors, colleagues and friends, and my family for your help, support and guidance in completing my Master thesis.

I would like to thank my parents Suu Bui and Huong Phan for their endless love and support and my sister Mai Bui for your encouragement and inspiration.

I would like to express my sincere gratitude to my Principal Investigator, Dr. Anahid Jewett, for your patience, motivation, immense knowledge and guidance through my research as well as your kindness and endless support you have shown me during the past three years it has taken me to complete this thesis.

I also would like to thank the rest of my thesis committee: Dr. Ichiro Nishimura and Dr. Nicholas Cacalano as well as Dr. Anahid Jewett for their insightful comments and review of this paper.

Moreover, I would like to thank my Oral Biology Division Administrator, Megan Scott, for your advice, updates and reminders.

I would like to thank my Program Director, Dr. Shen Hu, for his support and advice at the beginning of my Oral Biology program.

I would like to thank my fellow lab mates in the Weintraub Center for Reconstructive Biotechnology, as well as Helen Tseng (PhD Candidate) and Angie Celis (MS) for your collaboration and discussions.

I would like to thank Martin Hutchinson and Michael Gordon for editing this paper.

I would like to thank David Marino, Terresa Kim, Mohammad Sarwary, Diego Pallavicini, Paul Yang, Pooya Soltanzadeh, Cejo Lonappan and Dae Han Lee for your generous donation of immune cells.

TABLE OF CONTENTS

## LIST OF FIGURES

LIST OF TABLES

## INTRODUCTION

Natural killer (NK) cells comprise 10% to 15% of human peripheral blood lymphocytes (PBL) play an important role in the innate immune system. They are known as first-line defense that mediate spontaneous cytotoxicity against different types of tumor cells and virally infected cells and neoplasia [4] through antibody-dependent cytotoxicity, (ADCC) via the Fcγ receptor CD16, or through direct cell-mediated cytotoxicity [4]. Substantial evidence was shown that low activity of NK cells in the body circulation lead to viral infections, papilla viral infections and cervical carcinoma [6]. Therefore, NK cells are an important component in our immune system to protect us from life-threatening infection [5]. NK cells recognized tumor cells with low class I major histocompatiblity complex (MHC) and mediated cytotoxicity. Two ways in which NK cells can kill target cells are: cytotoxicity and cytokines production. Cytotoxicity in the NK cells occur when they bind to the target cells, become activated and secrete perforin granules and granzymes proteases or secrete death ligand FasL and TRAIL, low L-selectin and intercellular adhesion molecule-1 (ICAM-1). When they bind to the target cells, NK cells also produce large amount of cytokines, such as IFN-γ, TNF-α, granulocyte-macrophages colony-stimulating factor and other cytokines that regulate the immune response[6].

NK cells are heterogeneous cell populations that can be subdivided into several subsets according to their functional and phenotypic differences. The NK cells are defined by their FCγRIII (CD16) subpopulation; the $CD16^{bright}$ population is the most mature, whereas the $CD16^{dim}$ and $CD16^{-}$ populations are immature. Lebow and Bonavida examined the purified cell-sorted NK subsets based on their ability to bind and kill target cells and these subsets consisted of nonbinder free cells, nonkiller binders, and killer binders[7]. About 90% of human NK cells

are $CD56^{dim}/CD16^{+}$ and about 10% of human NK cells are $CD56^{bright}CD16^{dim}$ or $CD56^{bright}CD16^{-}$[8]. $CD56^{dim}$ NK cells are more cytotoxic [9], have high level expression of CD16, and exhibit greater level of ADCC compared to the CD56 $^{bright}$ subset[10]. The CD56 $^{bright}$ human NK cells are able to produce cytokines such as IFN-γ, TNF-β, IL-10, IL-13, and GM-CSF. They lack expression of killer cell immunoglobulin-like receptors (KIRs). They have no granules; therefore, they were not able to show natural-cell-mediated cytotoxicity or antibody-dependent cell-mediated cytotoxicity (ADCC). Both $CD56^{bright}$ and $CD56^{dim}$ NK cells express the activatory NKG2D receptor, which recognizes the MHC-class-1-related molecules MICA and MICB[11]. NK cells have IL-2Rβγ receptors that have an affinity for IL-2 cytokines.

NK cells have the ability to mediate cytotoxicity against healthy and cancer stem cells. Cancer stem cells originate from the normal stem cells that have been subjected to accumulation of multiple mutations for them to become carcinogenic. They have the capacity to self-renewal, differentiate, activate antiapoptotic pathways, and metastasize[12]. In this study, we have shown that as transformed and healthy stem cells go through the processes of maturation and differentiation, they up-regulate or down-regulate expression markers on the cell surfaces that are identifiable to the NK cells to activate or inhibit their cytotoxic function.

*Surface Receptor Markers*

OSCSCs expressed CD44 $^{bright}$ and CD133 oral tumor stem cells markers [1]. CD44s were cell adhesion molecules that contain the HA recognition site that attached to ligands of the extracellular matrix. These glycoproteins have been shown to be involved in cell-to-cell or cell-matrix interactions, cell motility, migration, differentiation, and cell signaling and gene

transcription [13]. CD44 was identified in a small subpopulation of animal breast cancer which contained $CD44^{+}CD24^{-/low}Lineage^{-}$ tumorigenic cells [14]. CD44 expression was also found to be up-regulated in Head and Neck Stem Cell Carcinoma and only the positive subset of $CD44^{+}$ and not $CD44^{-}$ had the capacity to initiate tumor growth [15]. CD44 has been implicated in signal transduction events and triggering of cytotoxic functions of cytotoxic T lymphocyte (CTL) [16-18] and NK cells [17, 19, 20].

CD274 (B7-H1 or PD-L1) is a member of the B7 family that is expressed on dendritic cells, activated immune cells [21]. B7-H1 is also selectively expressed by various cellular components in the tumor microenvironment, where it inhibits tumor-specific T- cell immunity by inducting T cell apoptosis [21]. It is an immune inhibitory molecule that inhibits attack from the innate and adaptive immune responses[22]. B7H1 was identified by searching for homologs for B7-1 and B7-2, the two ligands for CTLA-4 and CD28 [23]. B7H1 is highly expressed within the placenta in allogeneic pregnancies [24]. In addition, Tseng et al showed that oral cancer stem cell expressed low expression of B7H1 compared to their differentiated counterpart [1].

CD54, or ICAM-1, is a ligand for the leukocyte integrin complex CD11a/CD18 (LFA-1) and is expressed at different levels on a variety of cells, including Antigen-presenting cells (APCs), T cells and B cells[25]. Adhesion molecules play an important role in tumor growth, invasion, and metastasis. Cell adhesion molecules (CAMs) are critically involved in NK cells-mediated cytotoxicity. NK cells utilize CAMs to establish initial attachment to target cells [26-28]. LFA-1/ICAM-1 constitutes a dominant adhesion pathway for cytotoxic lymphocytes [26, 29]. CD54 expression on SR-91 was significantly increased by treating TNF-α or IFN-γ cytokines [30].

*Cytokines*

NK cells secrete key cytokines that are important for differentiation of healthy and transformed stem cells. TNF-α is a transmembrane protein with signaling potential as a membrane bound protein or soluble cytokine. TNF-α is known to induce DNA fragmentation in some cells either directly or through induction of other apoptotic pathways such as the Fas-Fas ligand pathway [31]. It plays an important role in the initiation of inflammatory reactions by the innate immune system and involved in maintenance and homeostasis of the immune system, inflammation, host defense and may be a target for cancer therapy [32]. IFN-γ is a member of type II class of interferons that produced under pathologic circumstances mainly by NK, Natural Killer T (NKT) cells and both CD4 and CD8 cytotoxic T lymphocytes [33, 34]. Two cytokines that affect production of IFN-γ on the NK cells are IL-2 and IL-12 and both act to synergistically produce a significant amount of IFN-γ [35]. The cytolytic function of the NK cells is controlled by a number of activating and inhibitory receptors, as well as adhesion molecules [36, 37]. IFN-γ and TNF-α have been shown to be essential in viral and tumor clearance [38, 39]. Wang, R. P et al. showed that the production of IFN-γ and TNF-α by the NK cells are functionally linked to their cytolytic activities and that when these two cytokines worked together synergistically induce cytolysis in insensitive target cells [40].

*Bacteria*

LPS lipopolysaccharide activates macrophages to produce a high level of IFN-γ on the NK cells [8]. Probiotic bacteria were identified in the early 20$^{th}$ century by Elie Metchnikoff, who discovered that some strains of bacteria in the human gut of the micro flora were beneficial to various human processes; and therefore, these good bacteria were named probiotics. They are commonly used in food and supplements to enhance innate immunity such as NK cells activity and to maintain health beneficial microbial balance in the digestive tract [41]. Most probiotics belong to a group of lactic-acid producing bacteria (lactobacilli, streptococci, and bifidobacteria). Probiotic bacteria are one of the most promising areas of research that have found to bring many benefits in the area of cancer prevention, especially colorectal cancer [42], but the consequences of increasing inflammation can have a wide range of effect on other cancer. It could be directly relevant to colon, but indirectly relevant to all other tumors because the facts that NK cells can cause split anergy and decrease inflammation so that the environment is not conducive for other tumors to grow. The probiotics also have an effect on the production of antibodies mainly immunoglobulin A in the intestinal lumen which is a very important antibody for defending against infection in the digestive tract. Probiotics also stimulate the activity of macrophages [43]. Experimental and some clinical evidence suggest that lactic acid bacteria might have beneficial effect on the toxicity of anticancer therapy [44]. During the invasion of pathogen in the intestinal epithelial cells, probiotics have the ability to induce production of pro-inflammatory and anti-inflammatory mediators, which then stimulate the differentiation of immature dendritic cells (DCs) in regulatory dendritic cells (DCreg), regulatory T cells and increase the activity of NK cells, resulting in a local intestinal defense by cytolysis cells [45].

Many mechanisms responsible for the establishment and progression of cancer have been proposed such as immunosuppression, tumor escape from immune recognition, or resistance to the Fas- or perforin-mediated lysis [46, 47]. Effort has gone to reverse such immunosuppression in cancer patients are immerging, particularly the NK cells, and thus requires further investigation.

This study has **three specific aims** and several sub-aims in term of probiotic bacteria induction of split anergy on the NK cells by triggering significant production of pro- and anti-inflammatory cytokines, lack the capability to mediate NK cells cytotoxicity against target cells, and increase resistance of healthy and transformed stem cells.

***Specific Aim 1*:**

Investigate the effect of probiotic bacteria on NK cells. Their role in the induction of split anergy by increasing production of cytokines in the absence of an increase in the NK cell cytotoxic function

***Specific Aim 2:***

Investigate the significance of probiotic bacteria in maturation and resistance of Oral Squamous Cancer Stem Cells (OSCSCs), Pancreas Carcinoma (MIA PaCa-2) and Stem Cells from the Apical Papilla (SCAPs) against the NK cell cytotoxicity through secretion of cytokines and cell-to-cell contact and up-regulation of CD54, B7H1, MHC-1 and down-regulation of CD44

***Specific Aim 3:***

Investigate the function of IL-10 in balanced regulation of cellular differentiation and resistance of tumors

## CHAPTER 1:

***Specific Aim 1*:** Investigate the effect of probiotic bacteria on NK cells. Their role in the induction of split anergy by increasing production of cytokines in the absence of an increase in the NK cell cytotoxic function

## INTRODUCTION:

Immunosuppression and tumor escape from immune recognition are thought to be a major factors responsible for the establishment and progression of cancer. However, neither underlying physiological significance nor the exact mechanisms by which immunosuppression occurs are well understood. It is shown that freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors and NK cells obtained from the peripheral blood of patients with cancer have significant reduced cytotoxic activity [48-50]. There are several mechanisms proposed to explain the functional inactivation of tumor-associated NK cells such as over-expression of Fas ligand, loss of mRNA for granzyme B [51], decreased CD16 and its associated zeta chain[52]. We have previously shown that the triggering of CD16 on untreated or IL-2 treated NK cells was found to result in down-modulation of CD16 receptors and in a great loss of cytotoxicity on the NK cells. In addition, a subset of NK cells was programmed to undergo apoptosis [53-55]. Other studies have also shown that IL-2 activated NK cells undergone cell deaths following cross-linking of the CD16 receptor [56, 57]. Thus, the term "split anergy" was coined by our lab to describe the loss in NK cytotoxicity, but gain in the ability to secrete cytokines, for an example, IFN-γ [1, 58, 59]. NK cells treated IL-2 and anti-CD16mAb are labeled as anergized NK cells as have been shown to secrete high levels of IFN-γ [60, 61]. IFN-

γ and TNF-α have been shown to be essential in viral and tumor clearance [38, 39]. In addition, three subpopulations of NK cells have been identified by their degrees of loss of cytotoxicity: Free, Binder and Killer NK cells [58, 62]. The Free cells exhibited the most cytotoxicity, whereas both Binder and Killer subsets exhibited significant loss of cytotoxicity and secreted significant levels of cytokines and exhibited $CD16^{-}CD56^{dim/-}CD69^{+}$ phenotype[58, 62] which had similar phenotype as the NK cells treated IL-2 and anti-CD16mAb. IFN-γ and TNF-α secretion were found predominately by the binder and killer subsets compared to the free subset which secreted the least IFN-γ and TNF-α[63]. According to Jewett, A. et al, there were three functions of the NK cells when they interacted with sensitive tumor-target cells which were treated with anti-CD16mAb in the presence of IL-2 to induce split anergy: 1-loss of cytotoxicity, 2-gain in the ability to secrete cytokines and 3-death in a small subset of NK cells [3].

Probiotic bacteria have been increasingly studied for their benefit to our immune system. According to Ustunol, Z., adults who consumed lactic acid bacteria, *Lactobacillus*, had shown to increase Interferon and NK lymphocytes activity while children who consumed bifidobacteria had shown to increase total IgA, IgG, IgM anti-rotavirus and lower incidence and severity of diarrhea[43]. Two types of probiotic bacteria that are currently found in yogurt and diet supplements are *Bifidobacterium* and *Lactobacillus* which involve in strengthening the intestinal barrier, modulation of the immune response and fighting of pathogens[64]. In addition, it has been shown that probiotics have some beneficial effects on anticancer treatment for patients who are undergoing radiation and chemotherapy; however, the effects are strain and dose dependent[65].

In the US, 8,000 deaths every year (2%-4%) are due to oral squamous cell carcinomas (OSCC). Oral cancer is ranked in the eight positions in the world and squamous cell carcinomas

represent 90% of all oral malignancies [66]. According to Brinkman, B.M.N et al, the prognosis of patients with oral squamous cell carcinoma (OSCC) remains poor, with a 5-year survival rate of 40%-50% and have not changed significantly for several decades [67]. It was found that OSCC is associated with smokers and alcohol drinkers and in those who have poor oral hygiene[68]. According to Lo et al, the 5-year survival rate for Stage I, II, III, and IV is 75%, 65.6%, 49%, and 30% respectively [69]. Tumor expression of cyclooxygenase-2 (COX-2) was over expressed in OSCC[70] which was associated with higher radio resistance; tumor cells treated in vitro with a COX-2 inhibitor showed better response to radiotherapy[71]. In addition, OSCC is also associated with over expression of Epidermal growth factor receptor (EGFR).

Lung cancer has become one of the world's leading causes of preventable deaths. It accounts for about 28% of all cancer deaths in 2012[72] which is caused by tobacco smoke containing high level of volatile NK-nitrosamines[73]. The survival rate for patients with lung cancer is poor, only 15% of patients survive for 5 years after diagnosis [74]. Detection of lung cancers at stage I can increase the 5-year survival rate to 80%. Lung cancer is usually not detected until it has progressed to an advanced stage because the early-stage disease does not typically cause symptoms. Almost 99% of the tumors in lungs are malignant[75].

Few studies have been conducted on probiotic bacteria and NK cells. Our lab assessed 8 strains of gram positive probiotic bacteria (*Streptococcus thermophilus, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis, Lactobacillus acidophilus, LactobacilluspPlantarum, Lactobacillus casei, and Lactobacillus bulgaricus)* to study their potential role with the NK cells.

The following **eight sub-aims** were demonstrated by *in vitro* assays to show the effect of NK cells-mediated cytotoxicity and secretion of cytokines when induced by probiotic bacteria by measuring IFN-γ and IL-10 production of human NK cells upon bacterial stimulation and NK cells-mediated lysis against cancer stem cells.

***Sub Aim 1*:** Probiotic bacteria induced substantially high levels of IFN-γ by purified untreated NK cells, IL-2 treated NK, and IL-2 plus anti-CD16 mAb treated NK cells

***Sub Aim 2***: Selective secretion of IL-10 by the NK cells triggered by the probiotic bacteria but less when NK cells are activated by IL-2 or IL-2 and anti-CD16 mAb

***Sub Aim 3***: Probiotic bacteria do not change NK cell-mediated cytotoxicity against A549, K562, and OSCSCs

***Sub Aim 4***: Probiotic bacteria were unable to increase NK cell death

***Sub Aim 5:*** Bacteria dosage regulate the level of cytokines secretion and cytotoxicity of NK cells

***Sub Aim 6*:** Sonicated bacteria induced less secretion of IFN-γ and IL-10 on the NK cells in comparison to live bacteria, but no change in cytotoxicity and cells death of the NK cells.

***Sub Aim 7:*** Up-regulation of CD69 expression was observed on the NK cells treated with sonicated probiotic bacteria

***Sub Aim 8:*** More cytokines with substantially higher levels of increase were seen by the treatment of NK cells with probiotic bacteria when compared to those treated with IL-2 or IL-2 and anti-CD16mAb; based on cytokine multiplex analysis.

## MATERIALS AND METHODS

### *A. Cell Culture and Reagents*

**Oral Squamous Cancer Stem Cells (OSCSCs)**

Oral Squamous Cancer Stem Cells (OSCSCs) were isolated from patients at the Department of Head and Neck Surgery at UCLA school of Medicine by Dr. Christian Head. The cells were cultured in RPMI Medium 1640 with L-Glumamine (Cat# 11875-093) supplemented 10% Fetal Bovine Serum (FBS) (Cat# 900-108, Gemini Bio-Products, CA), 1.4% Antibiotic-Antimycotic (100X) (Cat# 15240-062), 1% Sodium Pyruvate (100nM) (Cat# 11360-070), 1.4% MEM Non-Essential Amino Acids (Cat# 11140-050), 0.2% Gentamicin Sulfate (Cat# 400-108, Gemini Bio-Products, CA) and 0.15% Sodium Bicarbonate (Cat# S233-500, Fisher Scientific, CA). The remainder products were purchased from Gibco by Life Technologies (Carlsbad, CA).

**Human lung carcinoma (A549)**

Human A549 cells are human alveolar basal epithelial cells derived from human acinar adenocarcinoma with gland formation. The cells were cultured in Dulbecco modified Eagle Medium (DMEM) containing 4.5g/L D-Glucose and L-Glutamine (Cat# 11965-092) and supplemented with 3% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA), 1.4% MEM Non-Essential Amino Acids (Cat# 11140-050), 1% Sodium Pyruvate (100nM) (Cat# 11360-070), and 1% L-Glutamine, and 1% Penicillin-Streptomycin (Cat# 15140-122). The reminder products were purchased from Gibco by Life Technologies (Carlsbad, CA).

**Human Erythroleukemia Cell Line (K562)**

K562 cell line derived from a patient with chronic myeloid leukemia (CML). The cells were non-adherence and cultured in RPMI Medium 1640 with L-Glumamine (Cat# 11875-093) supplemented 10% Fetal Bovine Serum (FBS) (Cat# 900-108, Gemini Bio-Products, CA), 1% Sodium Pyruvate (100nM) (Cat# 11360-070), 1.4% MEM Non-Essential Amino Acids (Cat# 11140-050),and 1% L-glutamine. The remainder products were purchased from Gibco by Life Technologies (Carlsbad, CA).

*B.* *Antibodies*

a. Recombinant IL-2 was obtained from the NIH repository.
b. PE anti-human CD69 Antibody (Cat#310905) and PE anti-human CD16 Antibody (Cat#302007) and were purchased from Biolegend (San Diego, CA).
c. The Human NK cell Enrichment Kit (Cat# 19055) and Human Monocyte Enrichment Kit without CD16 Depletion (Cat# 19058) was purchased from Stem Cell Technologies (Vancouver, Canada).
d. Human IL-10 ELISA MAX Standard Sets (Cat# 430603) and Human IFN-γ ELISA MAX Standard Sets (Cat# 430103) were purchased from BioLegend (San Diego, CA).
e. The Bio-Plex Pro$^{TM}$ human cytokine 27plex immunoassay (Cat#M50-0KCAF0Y) was purchased from Bio-Rad (Hercules, CA).
f. Propidium Iodide Powder (Cat# P4170) were purchased from Sigma-Aldrich (St. Louis,MO)
g. AJ-1 and AJ-2 were mixtures of eight probiotic bacteria strains formulated by Dr. Anahid Jewett. Each tablet contained 6.28 billion freeze-dried bacteria (*Streptococcus*

*thermophilus, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillusp Plantarum, Lactobacillus casei, and Lactobacillus bulgaricus*).

*C. Purification of Natural Killer Cells and Peripheral Blood Mononuclear Cells*

Human peripheral blood was obtained from healthy donors as per guidelines of the UCLA Human Subject Protection Committee. Written informed consents approved by UCLA-IRB were obtained from all healthy blood donors. The blood was collected through syringed contained heparin and was centrifuged on Ficoll-Hypaque PM400 (Cat#17-0300-50; GE Healthcare, Piscataway, NJ). The buffy layer called Peripheral Blood Mononuclear Cells (PBMC) was harvested by density gradient centrifugation, washed, and resuspended in RPMI Medium 1640 supplemented with 10% FBS. Non-adherent human peripheral blood lymphocytes (PBL) were obtained after 1-2 hours incubation at 37˚C on a plastic 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 greater than 90% purity based on flow cytometric analysis of anti-CD16 antibody stained NK cells.

Purified NK cells were cultured in RPMI Medium 1640 supplemented with 10% FBS (Gemini Bio-Products, CA), 1% Sodium Pyruvate (100nM) (Cat# 11360-070), 1% MEM Non-Essential Amino Acids (Cat# 11140-050), and 1% Antibiotic-Antimycotic(100X) (Cat# 15240-062) (Gibco by Life Technologies, CA).

*D. NK Cell $^{51}$Chromium Release Cytotoxicity Assay*

A standard Chromium Release assay was used to assess the cytolytic function of Natural Killer Cells against the sensitivity of the target cells. The effector cells ($1x10^5$cells/well) were aliquoted into 96-well round bottom microwell plates (Thermo Scientific™ Nunc™, Hudson, NH) and were titrated from four to six serial dilutions. The target cells ($5x10^5$) were labeled with 50μCi Sodium $^{51}$Cr (Perkin Elmer, Shelton, CT) chromate for 1 hour, washed with medium specific for the target cells to remove excess $^{51}$Cr that was not labeled, counted the cells using Hematocytometer, and then washed again to remove excess unbound $^{51}$Cr. The $^{51}$Cr-labeled target cells were aliquoted into 96-well round-bottom micro well plates with the effectors cells at a concentration of $1x10^4$ cells/well to obtain an E:T (effector: target) ratio. The total release contained cell pellets and supernatant were collected and measured while the experimental and spontaneous release samples were centrifuged and incubated for 4 hours at 37˚C in 5%$CO_2$. Afterwards, each supernatant was harvest from each sample and the chromium release from lysed target cells was measured using a gamma counter. The percentage of specific chromium release of each well was calculated using the following formula.

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

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

*E. Enzyme-Linked Immunosorbent Assays (ELISAs) and Multiplex cytokine Assay*

Enzyme-Linked Immunosorbent Assays (ELISAs) were performed on the supernatants harvested after co-cultured experiments to measure the concentration level of cytokines,

chemokines, and growth factors secreted by the NK Cells. Single ELISAs were performed based on the manufactured protocol. Briefly, the 96-well EIA/RIA plates were coated with 100uL of Capture Antibody (1:200) corresponding to the target cytokine and incubated overnight at 4˚C. After 16 hours incubation, the plate was washed 4 times with Wash Buffer (PBS +0.05% Tween-20) and blocked with 200uL Assay Diluent (1%BSA in PBS) for 1 hour. Thereafter, the plates were washed 4 times and 100uL of supernatant collected from experiments and standard dilution were added. After 2 hours, the plates were washed 4 times. Then, 100ul of Detection Antibody (1:200) were added and incubated for 1 hour. Afterward, the plates were washed 4 times and 100uL of Avidin-HRP solution (1:1000) were added and incubated for 30 minutes. Finally, the plates were washed 5 times and 100ul of TMB Substrate Solution were added and incubated in the dark until the wells developed a desired blue color before 100ul of Stop Solution were added to stop the reaction. The plates were read at 450nm to obtain absorbance value. To analyze and obtain the cytokine concentration, a standard curve was generated through 7 serial dilutions of recombinant cytokines.

*F. Surface Staining*

The cells were detached from the tissue culture plates and washed with cold PBS containing 1%BSA. Pre-determined concentration of specific monoclonal antibodies were added to $5x10^4$ cells in 50uL and incubated at 4˚C for 30 minutes. The cells were then washed and re-suspended in PBS containing 1%BSA. The EPICS C (Coulter) flow cytometry was used for analysis and cell sorting.

*G. Propidium Iodide stain (PI)*

The cells were detached from the tissue culture plates and washed with cold PBS containing 1%BSA. About 100ng/mL of PI stain were added to $5x10^4$ cells in 50uL and re-suspend with PBS. The EPICS C (Coulter) flow cytometry was used for analysis and cell sorting. Cells undergoing apoptosis will be detected by measurement of the red fluorescence, indicating the uptake of Propidium Iodide of the gated conjugate population.

H. *Bacteria Sonication*

AJ2 were combination of eight different strains of probiotic bacteria formulated by Dr. Jewett. The whole bacteria was weighted and resuspended in RPMI Medium 1640 Complete containing 10% FBS at a concentration of 10mg per 1mL. The bacteria were vortexed thoroughly and then sonicated for 15 seconds while placing on ice and the Amplitude were set from 6 to 8. After that, the samples were rested for 30 seconds on ice. After every 5 pulses, a small sample was taken to observe under the microscope to obtain at least 80 percentages of bacteria cell walls lyses. Afterward, the sonicated samples were then aliquoted and stored in minus 20 to 80 degrees for long term studies.

## RESULTS:

### Sub Aim 1: Probiotic bacteria induced substantially high levels of IFN-γ by purified untreated NK cells, IL-2 treated NK, and IL-2 + anti-CD16 mAb treated NK cells.

As shown in Figure 1 (most representative data), NK cells without the treatment of probiotic bacteria were unable to induce any IFN-γ secretion; however, when treated with different strains of bacteria moderately induced IFN-γ secretion. Without the treatment of probiotic bacteria, NK treated IL-2 or NK treated IL-2 and anti-CD16 mAb induced some levels of IFN-γ, with IL-2 and anti-CD16mAb treated NK cells having the highest stimulation ability for IFN-γ secretion. However, when NK treated IL-2 and anti-CD16mAb were treated with different bacterial strains, the level of IFN-γ were significantly induced. Three probiotic bacteria strains: *Streptococcus thermophilus, Bifidobacterium longum,* and *Bifidobacterium breve,* and a mixture of eight probiotic strains, AJ2, were frequently observed to produce significantly the highest amount of IFN-γ compared to other five probiotic strains (*Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus Plantarum, Lactobacillus casei, Lactobacillus bulgaricus).*

### Sub Aim 2: Selective secretion of IL-10 by the NK cells triggered by the probiotic bacteria but less when NK cells are activated by IL-2 or IL-2 and anti-CD16 mAb.

As shown in Figure 2, untreated NK cells or those treated with IL-2 or IL-2+anti-CD16 mAb were unable to induce IL-10 secretion. In contrast, probiotic bacteria induced significant amount of IL-10 by the NK cells and the levels of IL-10 secretion decreased when NK cells were treated with IL-2 or IL-2 and anti-CD16mAb triggered by bacteria. *B. Longum, L. Breve*, AJ1

and AJ2 were observed to have significant induction of IL-10 on untreated NK cells while the other six bacteria were moderately induced.

**Sub Aim 3: Probiotic bacteria do not change NK cell-mediated cytotoxicity against A549, K562, and OSCSCs.**

Highly purified human NK cells were left untreated and treated with IL-2 and IL-2+anti-CD16mAb and were cultured with or without three strains of bacteria (*Streptococcus thermophilus, Bifidobacterium longum,* and *Bifidobacterium breve*) and probiotic bacteria mixture, AJ2, which was previously shown to induce the highest level of IFN-γ in NK cells. The co-cultures were incubated for 12-18 hours before they were added to $^{51}$Cr labeled A549 (Figure 3), K562 (Figure 4), and OSCSCs (Figure 5). NK treated probiotic bacteria mediated the same level of cytotoxicity against all three stem-like cell lines compared untreated NK cells mediated cytotoxicity. Untreated NK cells mediated low level of cytotoxicity. Activation of NK cells with IL-2 with or without probiotic bacteria has the highest increased in the level of cytotoxicity against A549 (Figure 3), K562 (Figure 4), and OSCSCs (Figure 5). The presence of probiotic bacteria did not change the level of cytotoxicity. The addition of anti-CD16mAb to NK cells treatead IL-2 inhibited NK cells mediated cytotoxicity against $^{51}$Cr labeled A549 (Figure 3), K562 (Figure 4), and OSCSCs (Figure 5) respectively. The same level of inhibition was also observed when probiotic bacteria were added to NK cells treated IL-2 and anti-CD16mAb, indicating that there was a lack of increase in cytotoxicity when probiotic bacteria were added.

**Sub Aim 4: Probiotic bacteria was unable to increase NK cell death**

As shown in Figure 6, untreated NK or NK treated IL-2 caused less than 1% of cell death in the NK cells. When the NK cells were treated with different probiotic bacteria strains, the percentage of NK cell deaths was induced less than 1%. Similarly, when the NK cells were treated with IL-2 and with different probiotic bacteria strains, the percentage of NK cell deaths induced by probiotic bacteria was also less than 1%. Treatments of NK cells with IL-2 and anti-CD16mAb induced 3% cell deaths in the NK cells. The presence of different strains of probiotic bacteria in NK cells treated with IL-2 and anti-CD16mAb induced less than 1% cell deaths. Therefore, we concluded that the addition of probiotic bacteria to the NK cell treatments did not induce significant cell deaths in the NK cells.

**Sub Aim 5: Bacteria dosage regulate the level of cytokines secretion and cytotoxicity of NK cells**

The highest secretion of IFN-γ (Figure 7) and IL-10 (Figure 8) were seen when AJ1 or AJ2 were treated with NK cells at 5:1 ratio (bacteria: NKs), followed by 1:1 ratio and 0.1:1 ratio.

**Sub Aim 6: Sonicated bacteria induced less secretion of IFN-γ and IL-10 on the NK cells in comparison to live bacteria, but no change in cytotoxicity and cells death of the NK cells**

As shown in Figure 9, NK cells treated with sonicated AJ2 (sAJ2) or live AJ2 mediated similar level of cytotoxicity against OSCSCs compared to untreated NK cells alone. Furthermore, the highest level of cytotoxity against OSCSCs was seen when NK cells were activated with IL-2 with or without sAJ2 or live AJ2. The inhibition of lysis by the NK cells mediated cytotoxicity was observed when the NK cells were treated with IL-2 and anti-CD16mAb with or without live or sonicated AJ2. Although there was no substantial differences

in the level of NK cells mediated cytotoxicity between live and sonicated probiotic bacteria (Figure 9), the level of IFN-γ (Figure 10A) and IL-10 (Figure 10B) secretion triggered by sAJ2 on the NK cells were slightly lower than that of live AJ2. As shown in Figure 11, NK treated IL-2 and anti-CD16mAb induced 14% of cell deaths in the NK cells. The same treatment with sAJ2 or with live AJ2 was showed to have the same level of cell deaths as the control, indicating that neither sAJ2 nor AJ2 induced NK cell deaths.

**Sub Aim 7: Up-regulation of CD69 expression was observed on the NK cells treated with sonicated probiotic bacteria**

As shown in Figure 12, untreated NK cells expressed 4% of CD69, while IL-2 treated NK cells expressed 10% of CD69 and significantly higher MFI (Mean Fluorescence Intensity) compared to untreated NK. NK cells treated IL-2 and anti-CD16mAb expressed 25% of CD69. When sAJ2 was treated with NK, NK treated IL-2, and IL-2 plus anti-CD16mAb, both percentage and mean fluorescence intensity of CD69 increase significantly compared to the NK cells treatments without sAJ2 (Figure 12).

**Sub Aim 8: More cytokines with substantially higher levels of increase were seen by the treatment of NK cells with probiotic bacteria when compared to those treated with IL-2 or IL-2 and anti-CD16mAb; based on cytokine multiplex analysis.**

We also ran multiplex analysis on 27 cytokines, chemokines, and growth factors to compare the level of secretion of untreated NK cells, IL-2 untreated, or IL-2 and anti-CD16mAb treated with or without eight single strains of probiotic bacteria. The chemokines from Table 1 and 2 are MIP-1beta, RANTES, Eotaxin, IP-10, MCP-1, IL-8, and MIP-1alpha; the cytokines from Table 1 and 2 are IL-6, IFN-γ, IL-1ra, IL-5, GM-CSF, TNF-α, IL-2, IL-1β, IL-13, IL-4, IL10, IL-15, IL-7, IL-12p70, IL-17, and IL-9; and the growth factors are Basic FGF, VEGF,

PDGF-BB, and G-CSF. As shown in Table 1 and Table 2, the cytokines that were significantly secreted by the NK cells triggered by probiotic bacteria are IL-6, IFN-γ, TNF-α, IL-1ra, IL-1β, IL-10, and IL-12p70. In addition, probiotic bacteria also triggered NK cells to secrete significant level of IL-8 and G-CSF (Table 2). The cytokines that had no change or low production when treated with probiotic bacteria are IL-5, IL-7, IL-17, and IL-1ra, IP-10, eotaxin, MIP1alpha, MIP1beta, and growth factors Basic FGF, VEGF, and PDGF-BB (Table 2).

**Fig. 1**

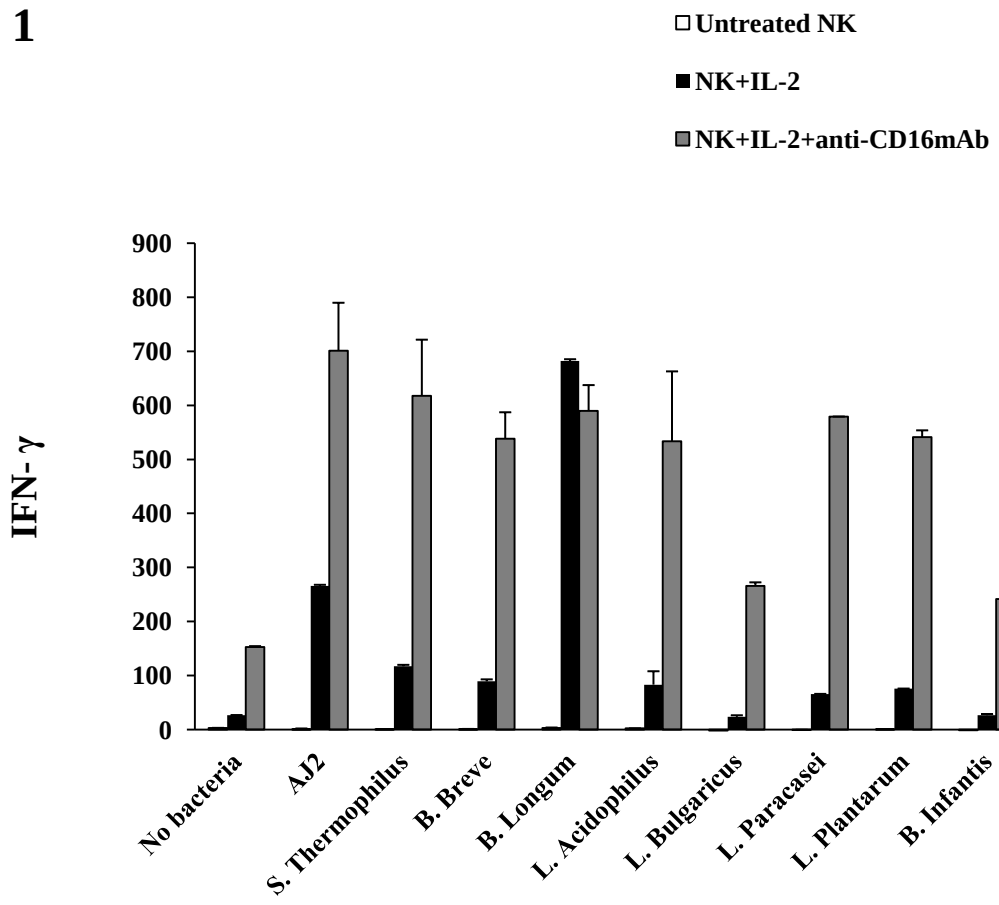



**Figure 1: Probiotic bacteria induced substantially higher levels of IFN-γ by purified untreated NK cells, IL-2 and IL-2 plus anti-CD16 mAb treated NK cells.**

Untreated, IL-2 (1000U/mL) treated, and IL-2 (1000U/mL) + anti-CD16mAb (2.5µg/mL) treated NK cells were incubated with and without various live bacteria at 5:1 (bacteria: NKs) for 12-18 hours before the supernatants were collected and the level of IFN-γ secretion were determined using Human IFN-γ ELISA MAX Standard Sets. The y-coordinate represents concentration in pg/mL.

**Fig. 2**

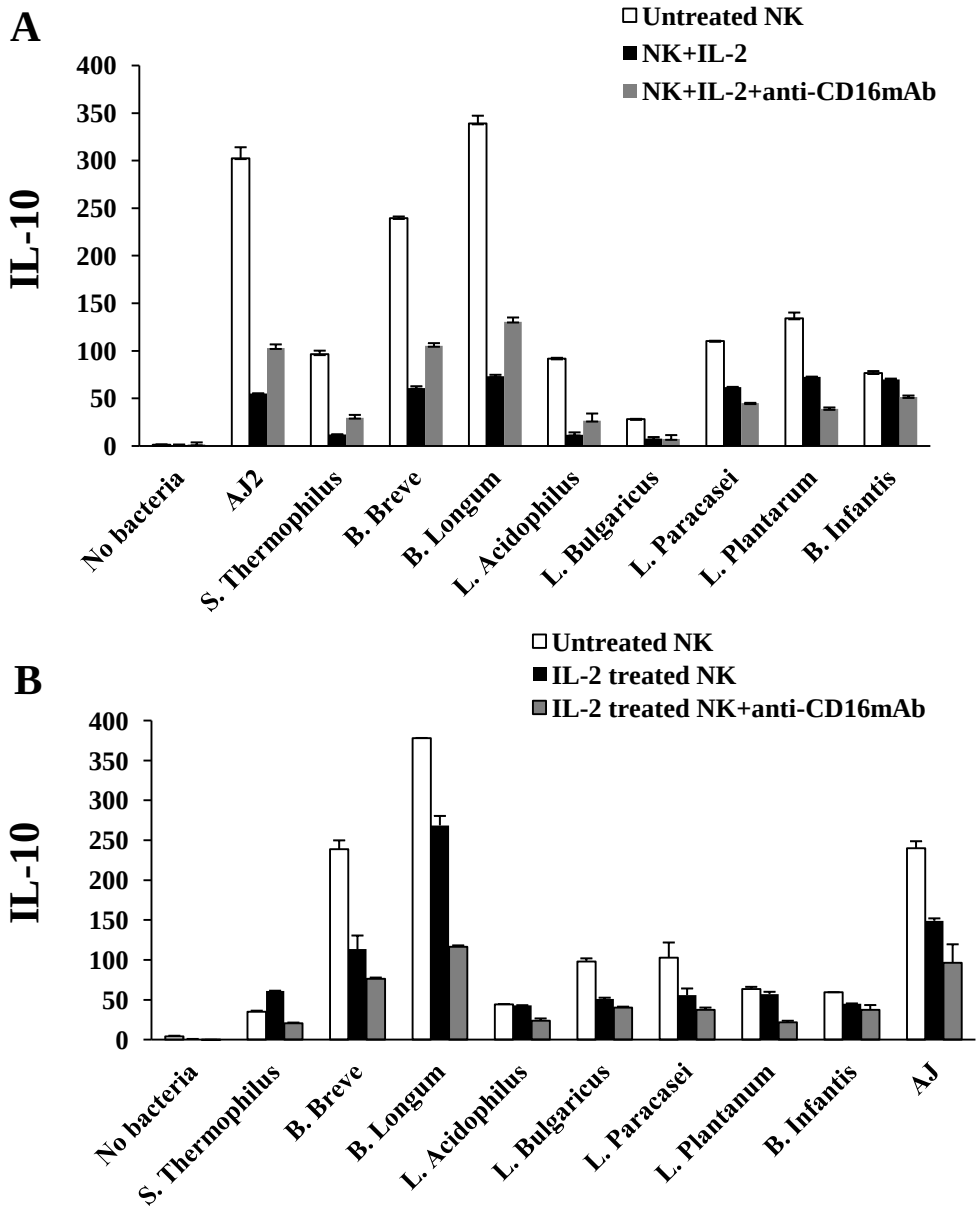



**Figure 2: Selective secretion of IL-10 by the NK cells triggered by the probiotic bacteria and less when NK cells are activated by IL-2 or IL-2 and anti-CD16mAb.**

Untreated, IL-2 (1000U/mL) treated, and IL-2 (1000U/mL) +anti-CD16mAb (2.5µg/mL) treated NK cells were incubated with and without various live bacteria at 3:1 (A) and 5:1 (B) of bacteria: NKs for 12h-18h before the supernatants were collected and the level of IL-10 secretion were determined using Human IL-10 ELISA MAX Standard Sets. The y-coordinate represents concentration in pg/mL.

**Fig. 3**

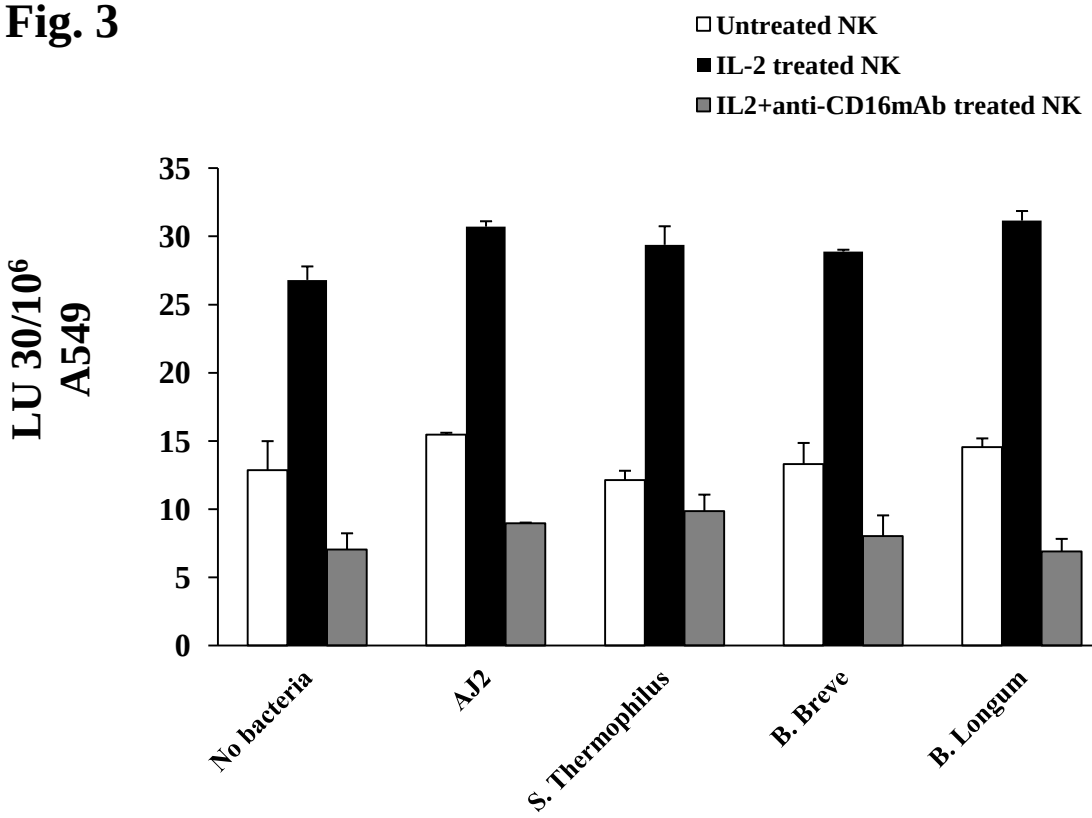



**Figure 3: Probiotic bacteria do not change NK cell-mediated cytotoxicity against A549.**

NK cells were left untreated, treated with IL-2 (1000U/mL) or treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5µg/mL). The three NK cells conditions were incubated with or without different live bacteria at concentration of 3:1 (bacteria: NKs) for 12-18 hours before they were added to $^{51}$Cr labeled A549 cells. NK cell cytotoxicities were determined using a standard 4 hour $^{51}$Cr release assay. The NK cells with A549 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 A549.

**Fig. 4**

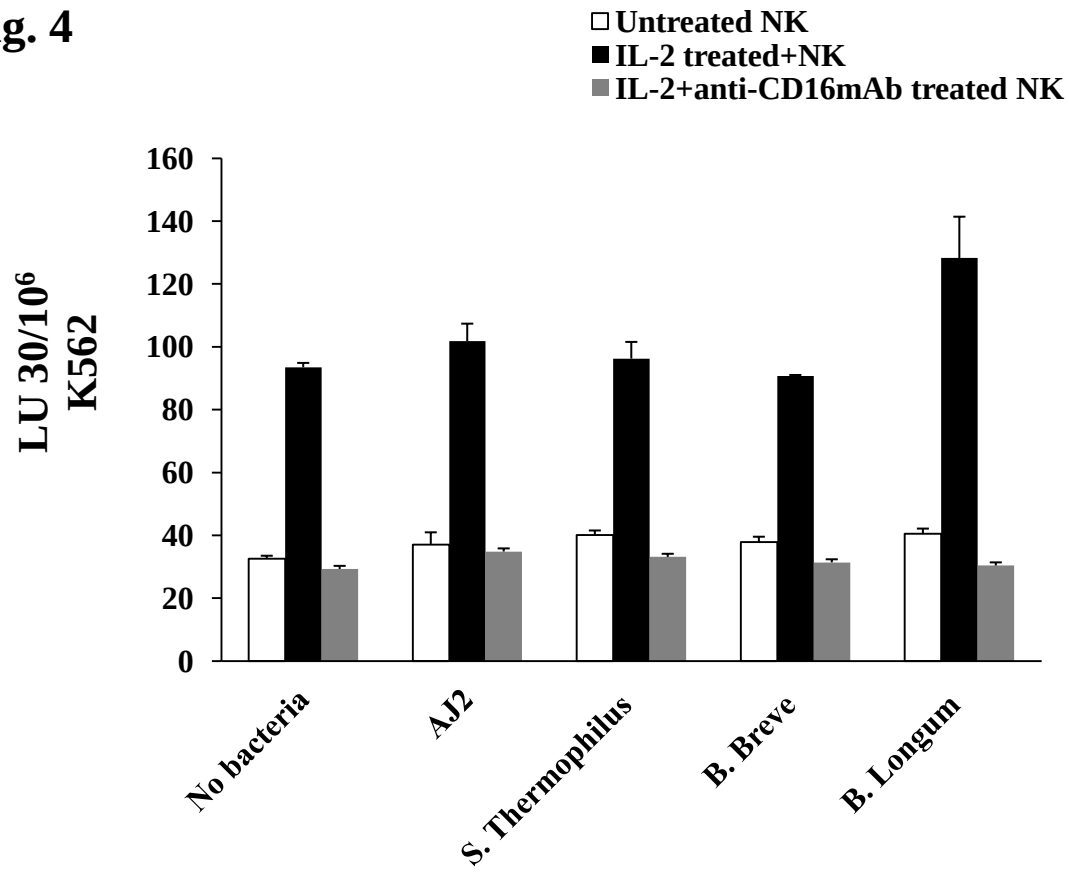



**Figure 4: Probiotic bacteria do not change NK cell-mediated cytotoxicity against K562.**

The NK cells were treated as described in Figure 3. The treatments were incubated for 12-18 hours before they were added to $^{51}Cr$ labeled K562 cells. NK cell cytotoxicities were determined using a standard 4 hour $^{51}Cr$ release assay. The NK cells with K562 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 K562.

**Fig. 5**

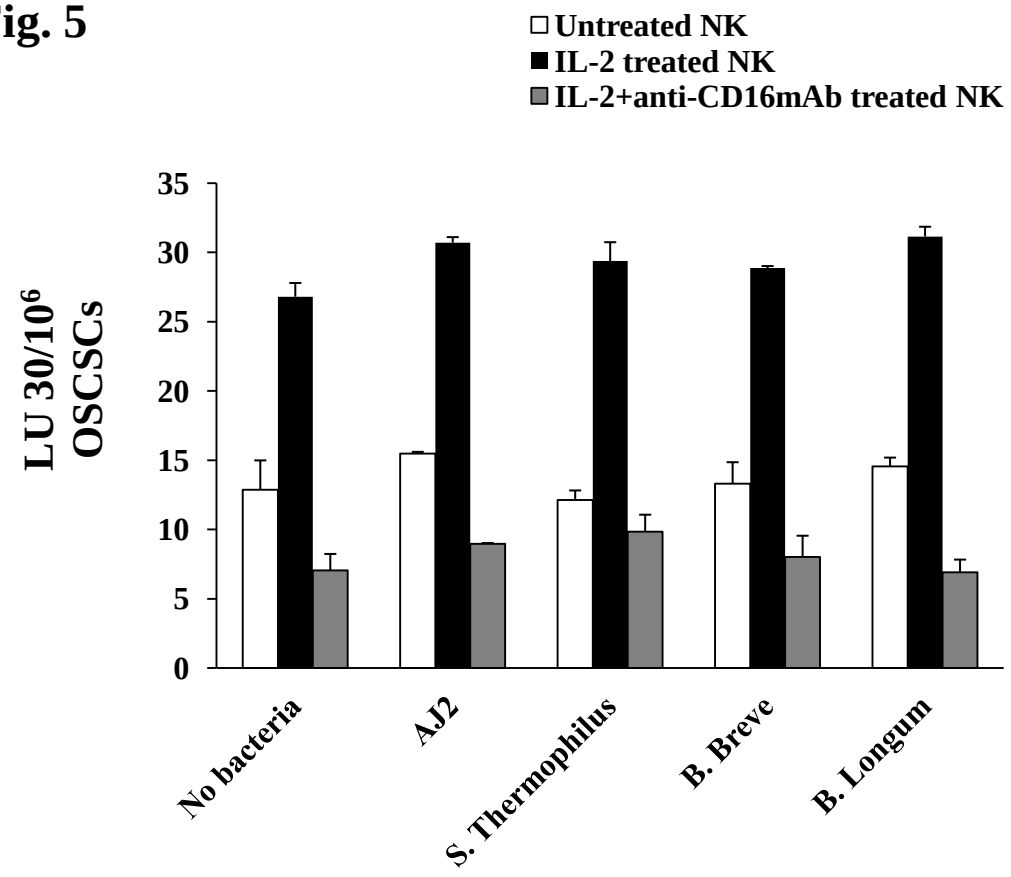



**Figure 5: Probiotic bacteria do not change NK cell-mediated cytotoxicity against OSCSCs.**

The NK cells were treated as described in Figure 3. The treatments were incubated for 12-18 hours before they were added to $^{51}Cr$ labeled OSCSC cells. NK cell cytotoxicities were determined using a standard 4 hour $^{51}Cr$ release assay. The NK cells with OSCSCs 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 OSCSCs.

# Fig. 6

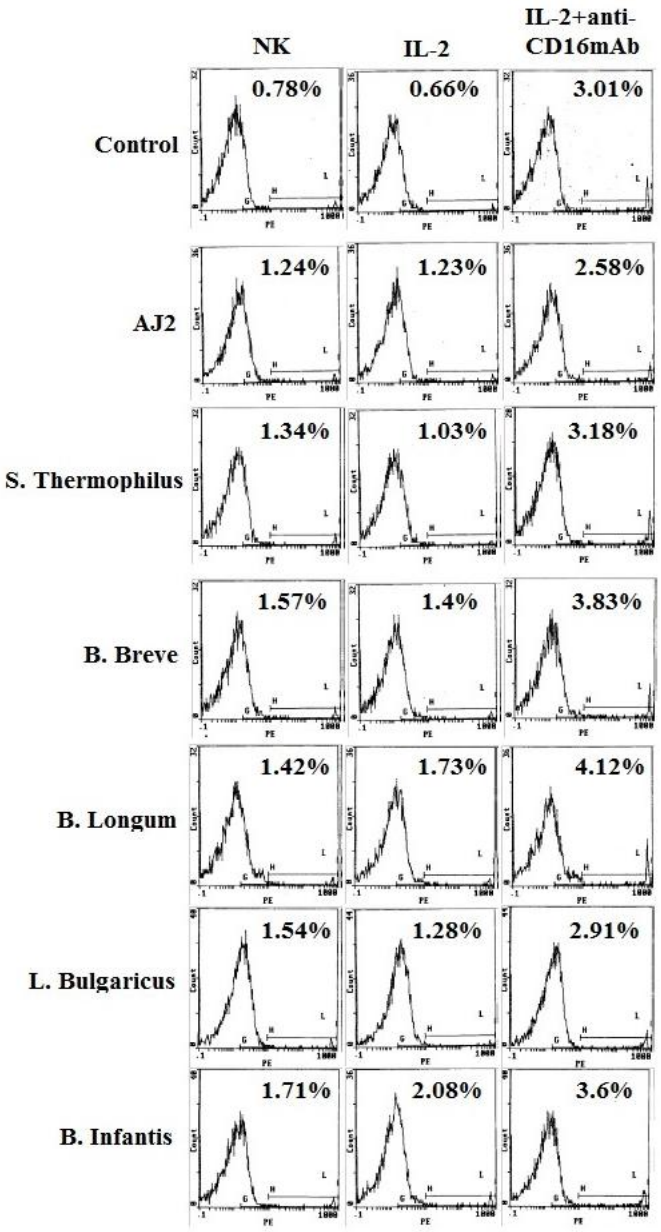



**Figure 6: Probiotic bacteria did not induce significant NK cell deaths.**

Untreated, IL-2 (1000U/mL) treated, or IL-2 (1000U/mL) and anti-CD16mAb (2.5µg/mL) treated NK cells were incubated with and without various live probiotic bacteria strains at 5:1 ratio (bacteria: NKs) for 12-18 hours. After an overnight incubation at 37˚C, $5x10^4$ cells in 50µl was taken from each treatment and treated with Propidium Iodide (100ng/mL) for 5 minutes. Afterward, the cells were resuspended with 400µL PBS and analyzed using flow cytometry. The peak on the left represents live cells, whereas the peak on the right represents dead cells that absorbed red fluorescence from the PI stain.

# Fig. 7

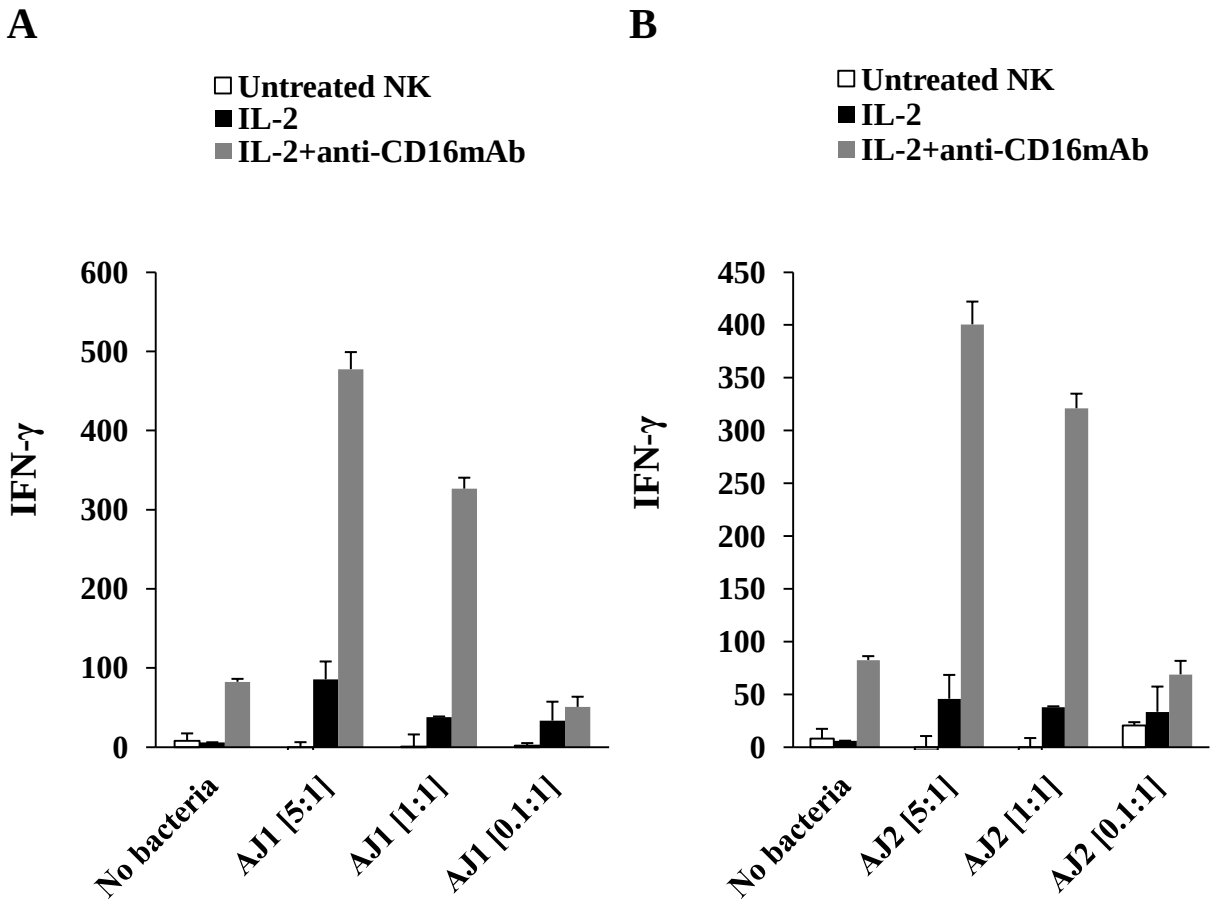



**Figure 7: Dose dependent increase in IFN gamma secretion by probiotic bacteria.**

Untreated, IL-2 (1000U/mL) treated, or IL-2 (1000U/mL) and anti-CD16mAb (2.5µg/mL) treated NK cells were incubated with and without various live probiotic bacteria strains at various ratio of bacteria to NKs for 12-18 hours before the supernatants were collected and the level of IFN-γ secretion were determined using Human IFN-γ ELISA MAX Standard Sets. The y-coordinate represents concentration in pg/mL.

# Fig. 8

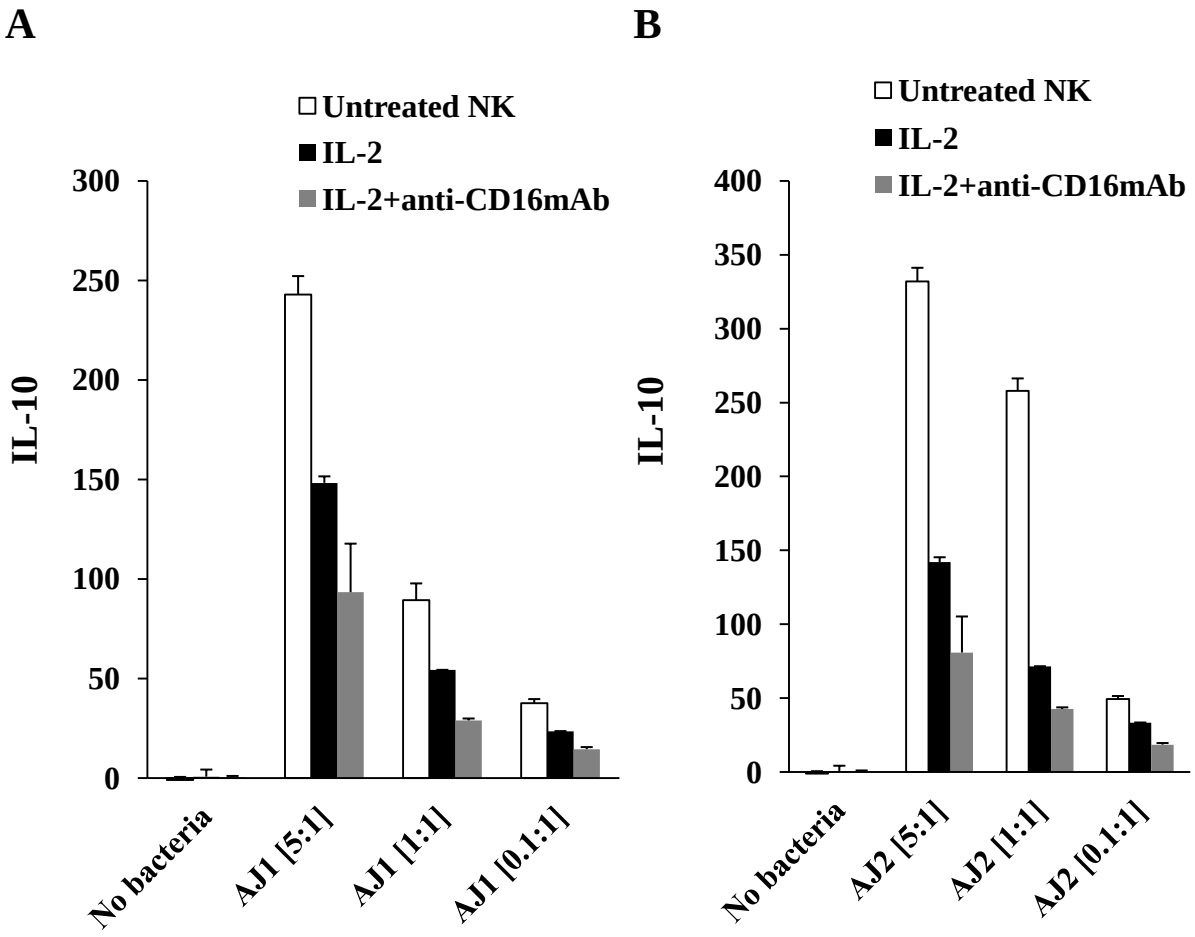



**Figure 8: Dose dependent increase in IL-10 secretion by probiotic bacteria.**

The NK cells treatments were described in Figure 7. The levels of IL-10 secretion were determined using Human IL-10 ELISA MAX Standard Sets. The y-coordinate represents concentration in pg/mL.

# Fig. 9

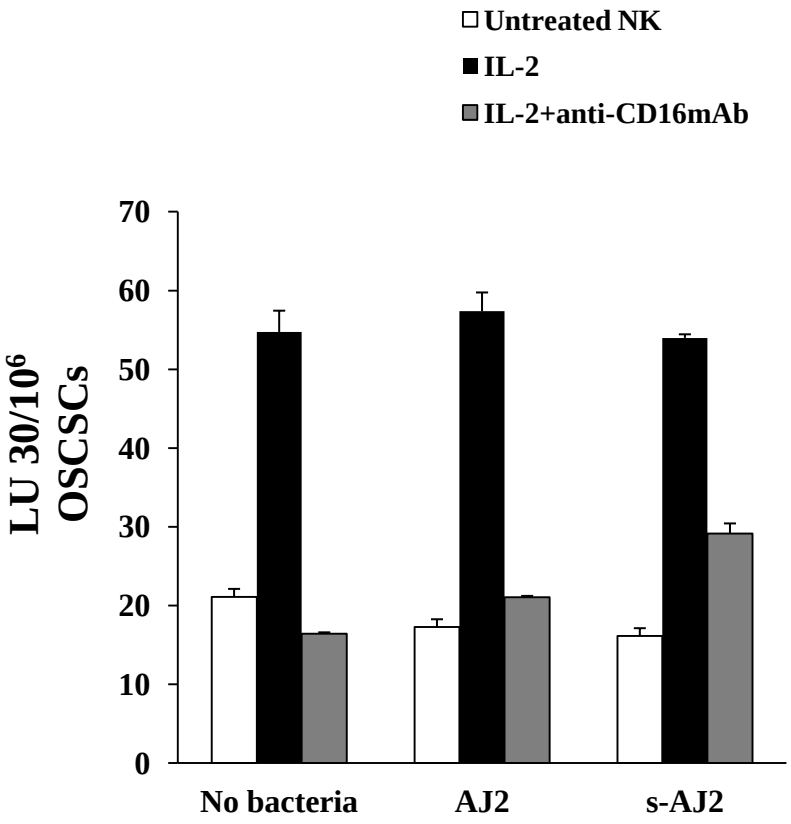



**Figure 9: No differences in NK cell-mediated cytotoxicity against OSCSCs between probiotic bacteria mixtures of live AJ2 and sAJ2.**

NK cells were left untreated, treated with IL-2 (1000U/mL) or treated with IL-2(1000U/mL) and anti-CD16mAb (2.5μg/mL) in the absence and presence of live or sonicated AJ2 (3:1; bacteria:NKs) for 12-18 hours before they were added to $^{51}Cr$ labeled OSCSC cells. NK cell cytotoxicities were determined using a standard 4 hour $^{51}Cr$ release assay. The NK cells with OSCSCs 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 OSCSCs.

# Fig. 10

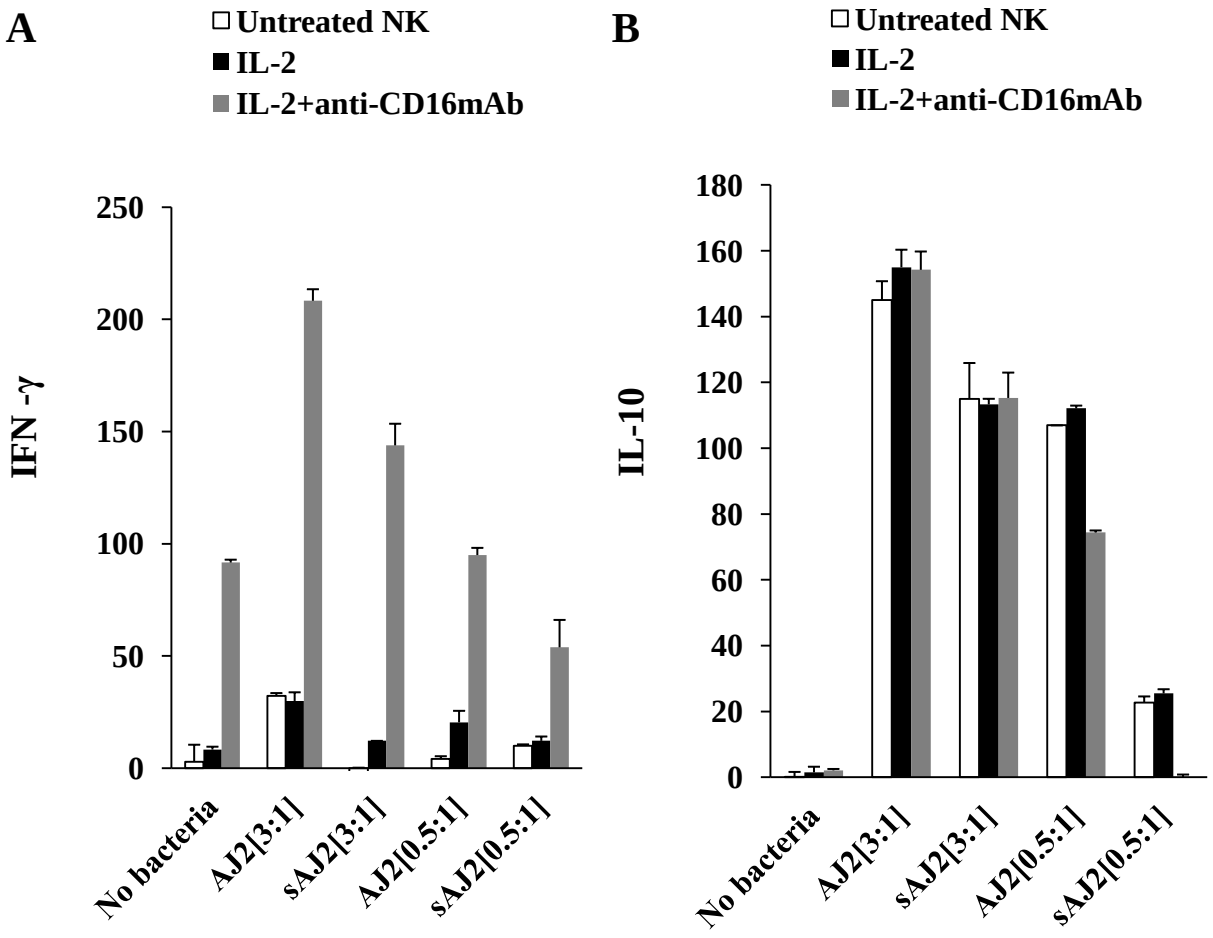



**Figure 10: Sonicated probiotic bacteria induced slightly lower levels of IFN-γ and IL-10 when compared to live probiotic bacteria.**

NK cells were left untreated, treated with IL-2 (1000U/mL) or treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5μg/mL) in the absence and presence of live or sonicated AJ2 (3:1; bacteria:NKs) for 12-18 hours before the supernatants were collected and the level of (A) IFN IFN-γ and (B) IL-10 secretion were determined using Human IFN-γ and Human IL-10 ELISA MAX Standard Sets respectively. The y-coordinate represents concentration in pg/mL.

# Fig. 11

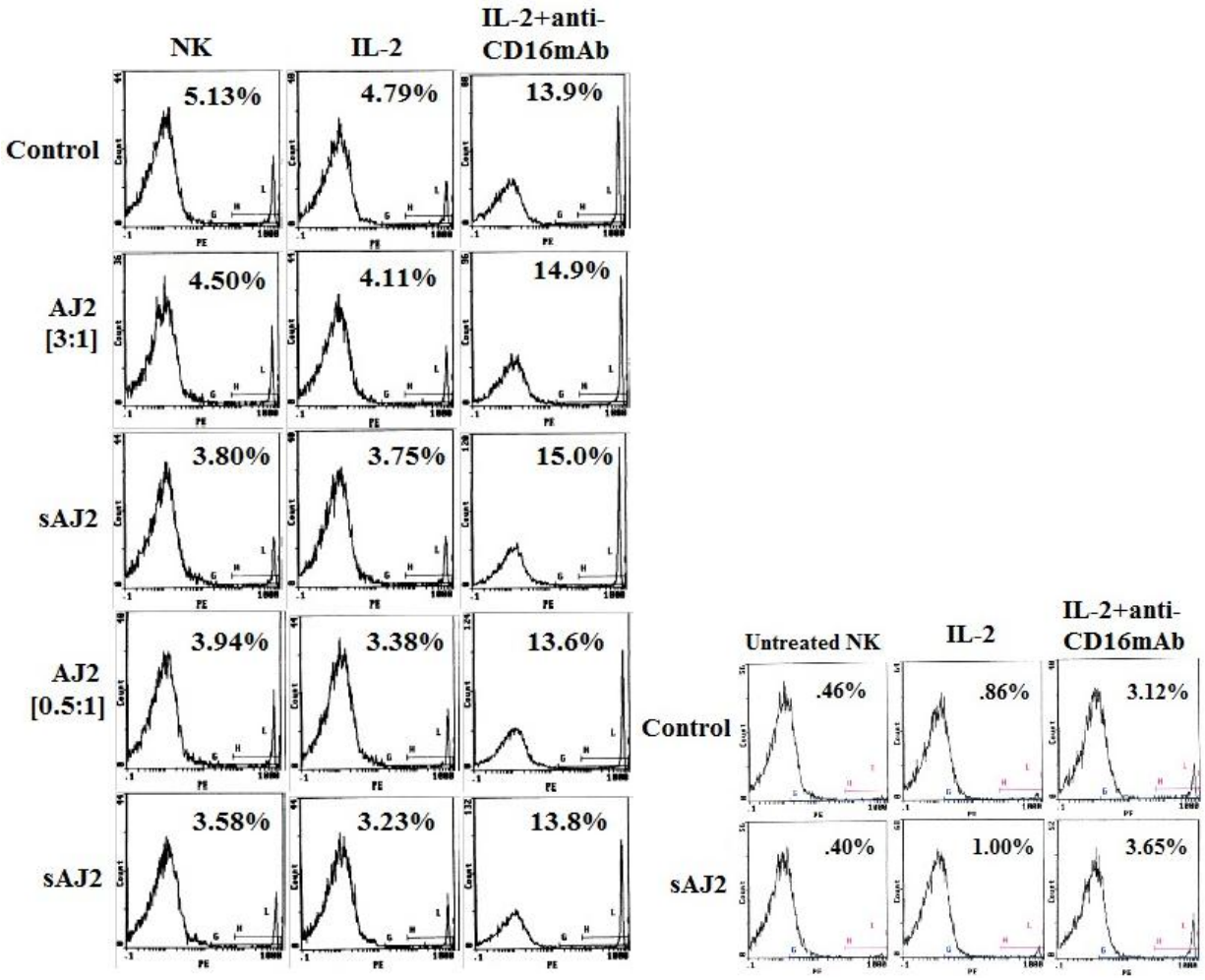



**Figure 11: sAJ2 were unable to increase NK cell death beyond that induced by IL-2 and anti-CD16mAb treated NK cells.**

NK cells were left untreated, treated with IL-2 (1000U/mL) or treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5µg/mL) in the absence and presence of live or sonicated AJ2 (3:1; bacteria:NKs) for 12-18 hours. After an overnight incubation at 37˚C, $5x10^4$ cells in 50µl was taken from each treatment and treated with Propidium Iodide (100ng/mL) for 5 minutes. Afterward, the cells were resuspended with 300µL PBS and analyzed using flow cytometry. The peak on the left represents live cells, whereas the peak on the right represents dead cells that absorbed red fluorescence from the PI stain.

# Fig. 12

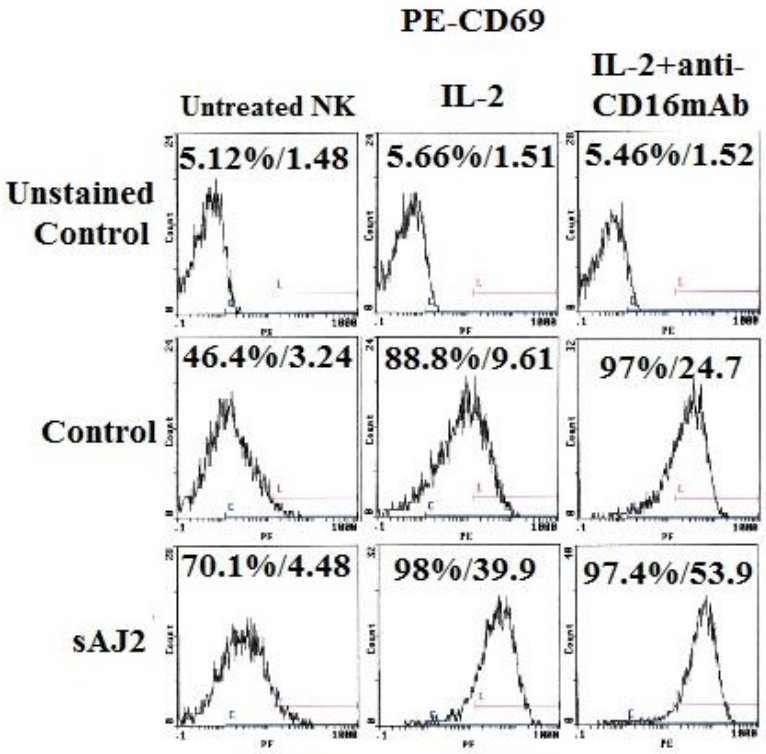



**Figure 12: Sonicated bacteria upregulate CD69 expression on untreated, IL-2 treated and IL-2 and anti-CD16mAb treated NK cells.**

NK cells were left untreated, treated with IL-2 (1000U/mL), or treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5ug/mL) and with or without sAJ2 at concentration of 3:1 (sAJ2: NKs) for 12h – 18h. Afterward, $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against CD69 and the level of surface expression were determined by flow cytometric analysis.

**Table 1**

| | Amounts induced by bacteria when compared to control | Amounts induced by IL-2 or IL-2+anti-CD16 mAb compared to control |
|---|---|---|
| **High** | MIP-1α, IL-1β, IFN-γ, IL-6, MCP-1, IL-1Ra, TNF-α, IL-8, G-CSF, IL-10, IL-12p70 | IFN-γ, TNF-α, IL-1Ra, IP10 |
| **Intermediate** | MIP-1β, VEGF, Eotaxin, IL-4, IL-13 | MIP-1α, IL-4, IL-8, IL-13 |
| **Low** | GM-CSF, Basic FGF, IL-5, IL-9, IL-17 | MIP-1β, IL-1β, GM-CSF, C-CSF, Eotaxin, Basic FGF, IL-5, IL-6, IL7, IL-9, IL-17 |
| **Decrease or no change** | Rantes, PDGF-BB, IL-7, IP-10, IL-15 | Rantes, VEGF, PDGF-BB, IL-10, IL-12p70, IL-15 |

**Table 1: More cytokines with substantially higher levels of increase were seen by the treatment of NK cells with probiotic bacteria when compared to those treated with IL-2 or IL-2+anti-CD16mAb; based on cytokine multiplex analysis.**

Untreated, IL-2 (1000U/mL) treated, or IL-2 (1000U/mL) and anti-CD16mAb (2.5µg/mL) treated NK cells were incubated with and without various live probiotic bacteria at 5:1 ratio (bacteria: NKs) for 12-18 hours before the supernatants were collected and the level of various cytokines secretion were determined using Bio-Plex Pro Human Cytokine 27-plex Assay Kit.

**Table 2**

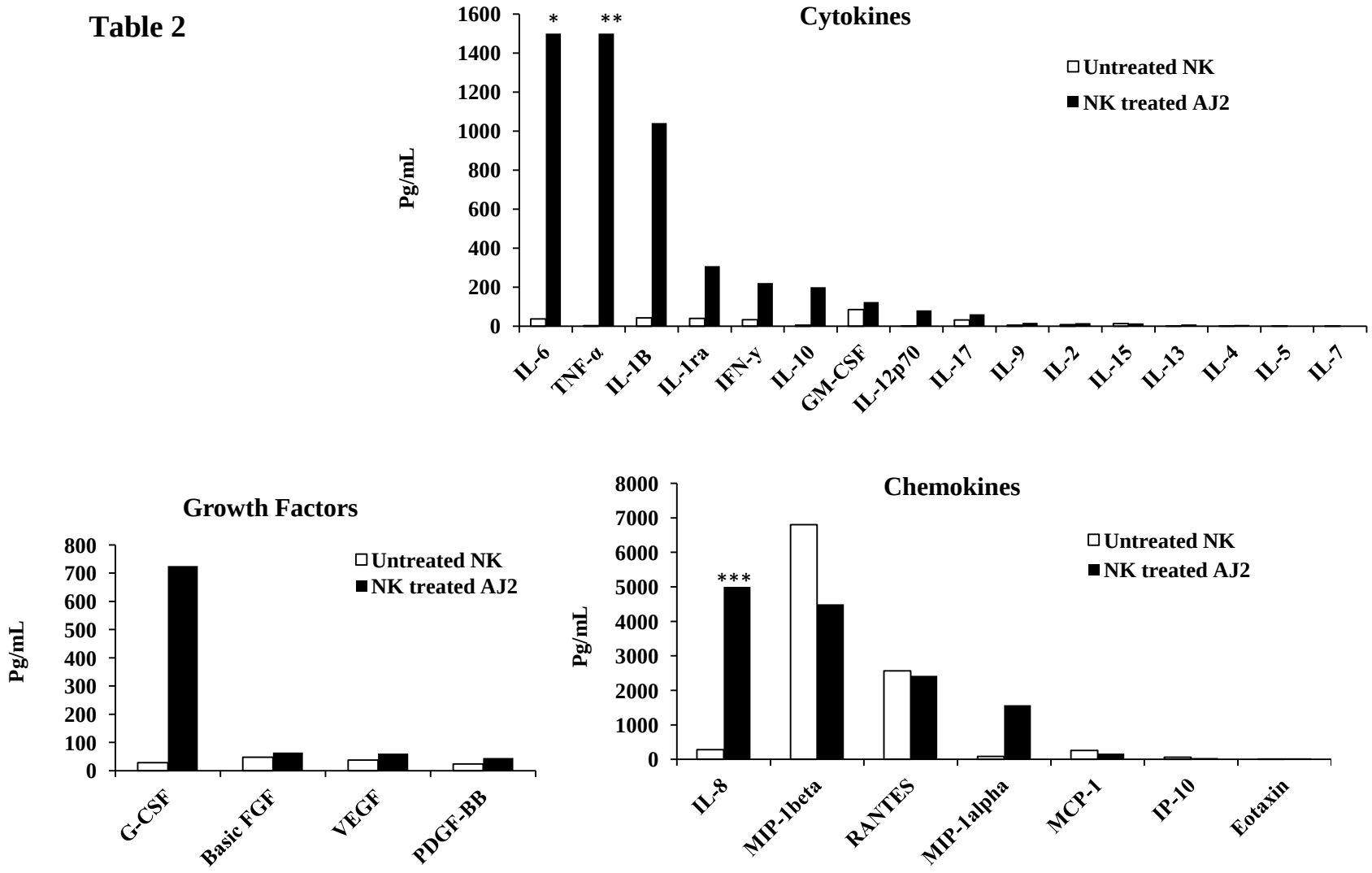



**Table 2: Significant amount of IL-6, IFN-y, IL-1ra, TNF-α, IL-1B, IL-10, IL-12p70, G-CSF, and IL-8 were seen in NK cells triggered by various bifidobacillus and lactobacillus bacteria.**

NK cells were treated as described in Table 1. *20291.67,** 9634.24,*** 12106.84

## DISCUSSION:

Previous studies have shown that purified NK cell CD16+ can be stimulated to secrete cytokines and they can secrete IFN-γ after the NK cells co-cultured with IL-2 in as early as 12 hours treatments. The free NK cell subset is the least mature subpopulation can be stimulated by IL-2 to mature into binder and killers [76]. The binder and killer subsets, however, responded and secreted IFN-γ with different quantitative patterns depending on the stimulus. Therefore, the ability to secrete cytokines by NK subsets correlates with the functional maturation of NK cells[55].

We have previously shown that K562, OSCSCs, hESCs, hMSCs, hDPSCs, and hiPSCs caused a loss in NK cell-mediated cytotoxicity and induced cell deaths in a small subset of NK cells [1, 53, 54]. It was demonstrated that cytolytic $CD56^{dim}CD16^{+}$NK cells released an abundance of IFN-γ at an early stage of their activation [77-79]. After the NK cells bound to target cells, the NK cells undergone phenotypic and functional changes and expressed $CD16^{-}$ $CD56^{dim/-}CD69^{+}$ phenotype [53, 54]. In oral and ovarian cancer patients, the CD16 receptor on NK cells was significantly down-modulated and the cytotoxic function was also decreased [80, 81]. Treatment of NK cells with IL-2 and anti-CD16mAb was shown to induce split anergy by significant decrease of NK cell-mediated cytotoxicity and by significant increase of cytokine secretion capabilities of the NK cells. This study confirmed that the NK cells produced large amount of pro-inflammatory cytokine, IFN-γ when they interact with CD16 receptor in the presence of IL-2. Furthermore, the addition of probiotic bacteria to NK treated IL-2 and anti-CD16mAb significantly accelerated the IFN-γ production capability of the NK cells. IL-10 is an anti-inflammatory cytokine which is also significantly secreted by the NK cells when co-cultured with probiotic bacteria. We observed a seesaw affect between the production of IL-10 and IFN-γ:

untreated NK cells produced the least amount of IFN- γ while they produced the highest amount of IL-10 when compared to NK treated IL-2 or NK treated IL2 and anti-CD16mAb (Figure 1 and Figure 2). In addition, probiotic bacteria induced a lot more IL-10 in the untreated NK and less IL-10 in the NK treated IL-2 or IL-2 and anti-CD16mAb. Because NK cells treated IL-2 or IL-2 and anti-CD16mAb produced more pro-inflamatory cytokines, they mimicked the inflammation condition in the tumor microenvironment as the level of IL-10 is low in these conditions. Because the untreated NK cells produced more IL-10, they kept the cells in the anti-inflammatory environment and did not allow IFN- γ to produce. In another study, it was shown that bifidobacteria were well documented to promote the production of IL-10, which potentially played an important role in the homeostasis of the immune system [77, 82]. Other studies have reported that *Lactobacillus casei* Shirota, L. *acidophilus* ATCC 4356 and *Bifidobacterium breve* ATCC 15700NK enhanced the activity of NK cells and up-regulated CD69, the activation markers on NK cells in human PBMC [2]. CD69 is widely used as an early activation marker for T cells [46].

There are three distinct functional outcomes that were observed in the NK cells when they either interacted with sensitive tumor-target cells or with anti-CD16mAb in the presence of IL-2 treatment to induce split anergy: 1-loss of cytotoxicity, 2-gain in the ability to secrete cytokines and 3-death in a small subset of NK cells[1, 61, 83]. The findings of this study showed that probiotic bacteria did not induce NK cells mediate cytotoxicity against OSCSCs, K562, and A549, nor did they induce significant cell deaths in the NK cells. Furthermore, they triggered the NK cells to produce a lot more pro- and anti-inflammatory cytokines, chemokines, and growth factors compared to the NK cells treated with IL-2 and anti-CD16mAb alone. Therefore, probiotic bacteria is observed to be a potent inducer of split anergy on the NK cells

which is an important physiological factor require to support maturation of stem cells, regeneration of new tissue and reduce inflammation in the tissue microenvironment.

## CONCLUSION:

The results from this study indicated that specific strains of probiotic bacteria are major inducers of split anergy on the NK cells compared to anti-CD16mAb in the NK cells treated IL-2 and anti-CD16mAb (conventional idea of split anergy). Probiotic bacteria were incapable of inducing NK cells-mediated cytotoxicity against tumor cell lines: OSCSCs, K562, and A549 but significantly induced secretion of more anti-inflammatory cytokines (IL-10 and IL-6) and pro-inflammatory (TNF-α and IFN-γ) when they were added to NK cells treated IL-2 and anti-CD16mAb. The activated NK cells that were induced by probiotic bacteria also induced secretions of key cytokines such as IL-1ra, IL-1B, IL-12p70, G-CSF, IL-8 and much more based on Multiplex ELISA analysis. The highest induction of NK cell deaths was caused by the NK cells treated with IL-2 and anti-CD16mAb and the presence of probiotic bacteria treated anergized NK cells (IL-2+anti-CD16mAb) did not induce significant NK cell deaths.

## CHAPTER 2:

***Specific Aim 2:*** Investigate the significance of probiotic bacteria in maturation and resistance of Oral Squamous Cancer Stem Cells (OSCSCs), Pancreas Carcinoma (MIA PaCa-2) and Stem Cells from the Apical Papilla (SCAPs) against the NK cell cytotoxicity through secretion of cytokines and cell-to-cell contact and up-regulation of CD54, B7H1, MHC-1 and down-regulation of CD44

## INTRODUCTION:

It is observed that the NK cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [48-50]. In addition, the NK cell cytotoxicity is suppressed after they interact with the stem cells [84, 85]. We found that the suppression of the NK cells cytotoxicity was not due to the exhaustion of cytotoxic granules but due to the phenotypic changes of the NK cells from the ability to mediate cytotoxicity to the ability to secrete significant amount of cytokines. It has been proposed that the NK cells may play a significant role in differentiation of the cells by providing critical cytokines and cell-cell contacts. Thus, according to Tseng et al, to drive differentiation, the NK cells will have to first receive signals from undifferentiated stem cells or those which have disturbed or defective capabilities to differentiate in order to lose cytotoxicity and to gain a cytokine producing phenotype. These alterations in NK cells effector functions will ultimately aid in driving differentiation of a sub-population of surviving healthy as well as transformed cells[1].

From our previous studies, we have shown that the stage of maturation and differentiation of transformed tumorigenic stem cells as well as healthy untransformed stem cells are predictive of their sensitivity to NK cell lysis [1]. We previously found that stem-like Oral Squamous Stem Cancer Cells (OSCSCs) are more susceptible to NK cells mediated cytotoxicity compared to their differentiated counterparts, Oral Squamous Cancer Carcinoma (OSCC) [1]. In addition, we found that healthy untransformed stem cells hESCs, hiPSCs, hMSCs, and hDPSCs were also more susceptible to NK cell-mediated cytotoxicity compared to their differentiated counterparts [1]. Cancer stem cells, OSCSCs expressed high level of CD133 and CD44, and low level of MHC-1 and B7H1 compared to their differentiated counterparts [1]. Stem cells also exhibit a low level of CD54; however, as they become more differentiated through treatments of anergized NK (IL-2+anti-CD16mAb) supernatants or through cell surface receptor interactions they upregulate CD54 and MHC-1 (manuscript in prep).

Jewett et al. showed that there is a correlation between the ability to secrete TNF-α and IFN-γ by NK cells and their functional maturation[55]. IFN-γ induced by T cells increased B7H1 inhibitory co-stimulatory receptors on MSCs and resulted in the suppression of T cells [86]. In addition, immunosuppressive function of MSCs is elicited by the combination of IFN-γ, TNF-α, IL-1α and IL-1β cytokines[87]. Furthermore, IFN-γ and TNF-α are known to upregulate ICAM-1 expression in tumor cell lines[88] and the productions of IFN-γ and TNF-α by the NK cells are functionally linked to their cytolytic activities[40]. Jewett et al. showed that the decrease in NK cells lysis was paralleled with the significant induction of IFN-γ and TNF-α[60]. Similarly, we also saw the same profile when NK cells were treated with IL-2 and anti-CD16mAb (split anergy) [76]. Since the increase of IFN-γ and TNF-α were shown to suppress NK cell-mediated

cytotoxicity, these cytokines are important mechanism in differentiation stem cells while their antibodies inhibit the differentiation of the cells (manuscript in prep).

Few researchers have studied the mechanism in which NK cells triggered by probiotic bacteria to cause maturation and differentiation of cancer stem cells. Because probiotic bacteria induced significant amounts of IFN-γ and TNF-α compared to NK cells triggered by CD16 receptor, the following **eleven sub aims** were demonstrated by *in vitro* assays to show that probiotic bacteria significantly induce maturation and differentiation of healthy untransformed stem cells as well as transformed stem cells through secreted cytokines and cell-cell contact resulting in resistance of NK cells-mediated lysis and unregulated the expression of CD54, B7H1, and MHC-1 while down-regulated the expression of CD44.

***Sub Aim 1:*** Supernatants from NK cells treated with IL-2 in combination with anti-CD16mAb and sAJ2 were able to induce the highest resistance of OSCSCs against NK cell-mediated cytotoxicity, which correlated with the highest modulation of CD54, B7H1, and MHC-1 and CD44.

***Sub Aim 2:*** Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of OSCSCs to NK cell mediated cytotoxicity induced by the treatment of OSCSCs with IL-2 and anti-CD16mAb treated NK cell supernatants.

***Sub Aim 3:*** Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of OSCSCs to NK cell mediated cytotoxicity induced by the treatment of OSCSCs with IL-2 in combination of anti-CD16mAb and sAJ2 treated NK cell supernatants.

***Sub Aim 4:*** Co-cultures of OSCSCs with paraformaldehyde fixed NK cells treated IL-2+anti-CD16mAb or IL-2 in combination with anti-CD16mAb and sAJ2 induced resistance of OSCSCs against NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of OSCSCs to NK cell mediated cytotoxicity.

***Sub Aim 5:*** Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of OSCSCs to NK cell mediated cytotoxicity induced by the treatment of OSCSCs with IL-2+anti-CD16mAb+sAJ2 treated paraformaldehyde fixed NK cells.

***Sub Aim 6:*** Supernatants from NK cells treated IL-2 and anti-CD16mAb induced resistance of MIA PaCa-2 against NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of MIA PaCa-2 to NK cell mediated cytotoxicity.

***Sub Aim 7:*** Supernatants from NK cells treated IL-2 in combination with anti-CD16mAb and sAJ2 induced resistance of MIA PaCa-2 against NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of MIA PaCa-2 to NK cell mediated cytotoxicity.

***Sub Aim 8:*** Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of MIA PaCa-2 to NK cell mediated cytotoxicity induced by the treatment of MIA PaCa-2 with IL-2+anti-CD16mAb+sAJ2 treated paraformaldehyde fixed NK cells.

***Sub Aim 9:*** Supernatants from NK cells treated with IL-2 in combination with anti-CD16mAb and sAJ2 were able to induce the highest resistance of SCAPs against NK cell-mediated cytotoxicity.

***Sub Aim 10:*** Combination of recombinant TNF-α and recombinant IFN-γ induced resistance of SCAPs to NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of SCAPs to NK cell mediated cytotoxicity.

***Sub Aim 11:*** Supernatants from NK cells treated IL-2 in combination with anti-CD16mAb and sAJ2 induced resistance of SCAPs against NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of SCAPs to NK cell mediated cytotoxicity.

## MATERIALS AND METHODS

A. *Cell Culture and Reagents*

### Oral Squamous Cancer Stem Cells (OSCSCs)

Oral Squamous Cancer Stem Cells (OSCSCs) were isolated from patients at the Department of Head and Neck Surgery at UCLA school of Medicine by Dr. Christian Head. The cells were cultured in RPMI Medium 1640 with L-Glumamine (Cat# 11875-093) supplemented 10% Fetal Bovine Serum (FBS) (Cat# 900-108, Gemini Bio-Products, CA), 1.4% Antibiotic-Antimycotic (100X) (Cat# 15240-062), 1% Sodium Pyruvate (100nM) (Cat# 11360-070), 1.4% MEM Non-Essential Amino Acids (Cat# 11140-050), 0.2% Gentamicin Sulfate (Cat# 400-108, Gemini Bio-Products, CA) and 0.15% Sodium Bicarbonate (Cat# S233-500, Fisher Scientific, CA). The remainder products were purchased from Gibco by Life Technologies (Carlsbad, CA).

### Pancreas Carcinoma cell (MIA PaCa-2)

Pancreas Carcinoma cell (MIA PaCa-2) were a gift from Dr. Graham Donald at the MacDonald Medical Research Laboratory at the University of California, Los Angeles. The cells were cultured in Dubecco Modified Eagle Medium (DMEM) containing 4.5g/L D-Glucose, L-Glutamine, and 110mg/L Sodium Pyruvate (Cat#119955-065) and supplemented with 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA), 1% Penicilin-Streptomycin (Cat# 15140-122). The remainder products were purchased from Gibco by Life Technologies (Carlsbad, CA).

**Stem Cells Apical Papilla (SCAP)**

Stem Cells Apical Papilla (SCAP), identified as an important population of Mesenchymal Stem Cells (MSCs) were a gift from Dr. Avina Paranjpe from the Department of Endodontics at University of Washington, School of Dentistry. The cells were cultured in Dulbecco modified Eagle Medium (DMEM) containing 4.5g/L D-Glucose and L-Glutamine (Cat# 11965-092) and supplemented with 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA), 1% Penicillin-Streptomycin (Cat# 15140-122), 1.4% MEM Non-Essential Amino Acids (Cat# 11140-050), 1% Sodium Pyruvate (100nM) (Cat# 11360-070). The remainder products were purchased from Gibco by Life Technologies (Carlsbad, CA).

*B. Antibodies*

a. Recombinant IL-2 was obtained from the NIH repository.

b. PE anti-human CD16 Antibody (Cat#302007) and PE anti-human CD107a (LAMP-1) Antibody (Cat#328607), PE anti-human CD54 Antibody (Cat# 353106), PE anti-human CD44 Antibody (Cat#33807), PE anti-human CD274 (B7-H, PD-L1) Antibody (Cat#329706), PE Mouse IgG2a, k Isotype Control Antibody (Cat# 400212), PE anti-human HLA-A, B, C Antibody (Cat#311406) were purchased from Biolegend (San Diego, CA).

a. The Human NK cell Enrichment Kit (Cat# 19055) was purchased from Stem Cell Technologies (Vancouver, Canada).

b. Propidium Iodide Powder (Cat# P4170) were purchased from Sigma-Aldrich (St. Louis,MO)

c. AJ2 was mixtures of eight probiotic bacteria strains formulated by Dr. Anahid Jewett. Each tablet contained 6.28 billion freeze-dried bacteria (*Streptococcus thermophilus, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillusp Plantarum, Lactobacillus casei, and Lactobacillus bulgaricus*).

*C. Purification of Natural Killer Cells and Peripheral Blood Mononuclear Cells*

Human peripheral blood was obtained from healthy donors as per guidelines of the UCLA Human Subject Protection Committee. Written informed consents approved by UCLA-IRB were obtained from all healthy blood donors. The blood was collected through syringed contained heparin and was centrifuged on Ficoll-Hypaque PM400 (Cat#17-0300-50; GE Healthcare, Piscataway, NJ). The buffy layer called Peripheral Blood Mononuclear Cells (PBMC) was harvested by density gradient centrifugation, washed, and resuspended in RPMI Medium 1640 supplemented with 10% FBS. Non-adherent human peripheral blood lymphocytes (PBL) were obtained after 1-2 hours incubation at 37˚C on a plastic 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 greater than 90% purity based on flow cytometric analysis of anti-CD16 antibody stained NK cells.

Purified NK cells were cultured in RPMI Medium 1640 supplemented with 10% FBS (Gemini Bio-Products, CA), 1% Sodium Pyruvate (100nM) (Cat# 11360-070), 1% MEM Non-Essential Amino Acids (Cat# 11140-050), and 1% Antibiotic-Antimycotic(100X) (Cat# 15240-062) (Gibco by Life Technologies, CA).

*D. NK Cell $^{51}$Chromium Release Cytotoxicity Assay*

A standard Chromium Release assay was used to assess the cytolytic function of Natural Killer Cells against the sensitivity of the target cells. The effector cells ($1x10^5$cells/well) were aliquoted into 96-well round bottom microwell plates (Thermo Scientific™ Nunc™, Hudson, NH) and were titrated from four to six serial dilutions. The target cells ($5x10^5$) were labeled with 50μCi Sodium $^{51}$Cr (Perkin Elmer, Shelton, CT) chromate for 1 hour, washed with medium specific for the target cells to remove excess $^{51}$Cr that was not labeled, counted the cells using Hematocytometer, and then washed again to remove excess unbound $^{51}$Cr. The $^{51}$Cr-labeled target cells were aliquoted into 96-well round-bottom micro well plates with the effectors cells at a concentration of $1x10^4$ cells/well to obtain an E:T (effector: target) ratio. The total release contained cell pellets and supernatant were collected and measured while the experimental and spontaneous release samples were centrifuged and incubated for 4 hours at 37˚C in 5%$CO_2$. Afterwards, each supernatant was harvest from each sample and the chromium release from lysed target cells was measured using a gamma counter. The percentage of specific chromium release of each well was calculated using the following formula.

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

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.

*E. Enzyme-Linked Immunosorbent Assays (ELISAs)*

Enzyme-Linked Immunosorbent Assays (ELISAs) were performed on the supernatants harvested after co-cultured experiments to measure the concentration level of cytokines,

chemokines, and growth factors secreted by the NK Cells. Single ELISAs were performed based on the manufactured protocol. Briefly, the 96-well EIA/RIA plates were coated with 100uL of Capture Antibody (1:200) corresponding to the target cytokine and incubated overnight at 4˚C. After 16 hours incubation, the plate was washed 4 times with Wash Buffer (PBS +0.05% Tween-20) and blocked with 200uL Assay Diluent (1%BSA in PBS) for 1 hour. Thereafter, the plates were washed 4 times and 100uL of supernatant collected from experiments and standard dilution were added. After 2 hours, the plates were washed 4 times. Then, 100ul of Detection Antibody (1:200) were added and incubated for 1 hour. Afterward, the plates were washed 4 times and 100uL of Avidin-HRP solution (1:1000) were added and incubated for 30 minutes. Finally, the plates were washed 5 times and 100ul of TMB Substrate Solution were added and incubated in the dark until the wells developed a desired blue color before 100ul of Stop Solution were added to stop the reaction. The plates were read at 450nm to obtain absorbance value. To analyze and obtain the cytokine concentration, a standard curve was generated through 7 serial dilutions of recombinant cytokines.

*F. Surface Staining*

The cells were detached from the tissue culture plates and washed with cold PBS containing 1%BSA. Pre-determined concentration of specific monoclonal antibodies were added to $5x10^4$ cells in 50uL and incubated at 4˚C for 30 minutes. The cells were then washed and re-suspended in PBS containing 1%BSA. The EPICS C (Coulter) flow cytometry was used for analysis and cell sorting.

*G. Propidium Iodide stain (PI)*

The cells were detached from the tissue culture plates and washed with cold PBS containing 1%BSA. About 100ng/mL of PI stain were added to $5x10^4$ cells in 50uL and re-suspend with PBS. The EPICS C (Coulter) flow cytometry was used for analysis and cell sorting. Cells undergoing apoptosis will be detected by measurement of the red fluorescence, indicating the uptake of Propidium Iodide of the gated conjugate population.

H. *Bacteria Sonication*

AJ2 were combination of eight different strains of probiotic bacteria formulated by Dr. Jewett. The whole bacteria was weighted and resuspended in RPMI Medium 1640 Complete containing 10% FBS at a concentration of 10mg per 1mL. The bacteria were vortexed thoroughly and then sonicated for 15 seconds while placing on ice and the Amplitude were set from 6 to 8. After that, the samples were rested for 30 seconds on ice. After every 5 pulses, a small sample was taken to observe under the microscope to obtain at least 80 percentages of bacteria cell walls lyses. Afterward, the sonicated samples were then aliquoted and stored in minus 20 to 80 degrees for long term studies.

*I. Natural Killer Cells Fixation*

After purification of the Natural Killer Cells, the cells were left untreated, treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5µg/mL), and combination of IL-2 (1000U/mL) plus anti-CD16mAb (2.5µg/mL) and sAJ2 at ratio of 3:1 (bacteria:NKs) and resuspended with RPMI Medium Complete at 1 million NK cells per Milliliter. After 12-18 hours of incubation in 37˚C,

the cells were fixed with 2% paraformaldehyde in 1X DPBS without Calcium Chloride and Magnesium Chloride (Gibco by Life Technologies, CA) for 12- 15 minutes at room temperature. Afterwards, the cells were washed twice with sterile DPBS (1X) and re-suspended in RPMI Medium 1640 Complete at 1 million NK cells per Milliliter. Then, the cells were used in differentiation experiments of OSCSCs and MIA PaCa-2.

*J. Differentiation of OSCSCs, MIA PaCa-2, and SCAPs with anergized NK cells Supernatants or Fixed anergized NK cells*

After purification of the Natural Killer Cells, the cells were left untreated, treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5μg/mL), and treated with combination of IL-2 (1000U/mL) plus anti-CD16mAb (2.5μg/mL) and sAJ2 at a ratio of 3:1 (bacteria: NKs) and resuspended with RPMI Medium Complete at 1 million NK cells per Milliliter. After 12-18 hours of incubation in 37˚C, the supernatants or the cell pellet from each NK treatment were harvested by centrifugation and then treated (supernatants or fixed NK cells) into culture plates of OSCSCs, SCAPs, or MIA PaCa-2 each day for 3-5 days. The amount of supernatants or fixed cells added to the culture plates each day is determined by several factors: growth rate of the cells, size of the cells, number of non-adherence and adherence cells. Afterward, the OSCSCs, SCAPs, or MIA PaCa-2 were detached from the tissue culture plates by using 0.25% Trypsin-EDTA (1X) (Gibco by Life Technologies, CA) and the sensitivity of the treated cells was evaluated using $^{51}$Chromium Release Assay.

## RESULTS

**Sub Aim 1: Supernatants from NK cells treated with IL-2 in combination with anti-CD16mAb and sAJ2 were able to induce the highest resistance of OSCSCs against NK cell-mediated cytotoxicity, which correlated with the highest modulation of CD54, B7H1, and MHC-1 and CD44.**

NK cells were left untreated, treated with sAJ2, treated with IL-2 and anti-CD16mAb, and treated with the combination of IL-2+anti-CD16mAb plus sAJ2 for 12-18 hours before the supernatants were collected and added to OSCSCs each day for 4 days to see a step-wise level of differentiation of OSCSCs. OSCSCs treated with supernatant from untreated NK cells or supernatant from sAJ2 treated NK cells exhibited similar levels of sensitivity as untreated OSCSCs (Figure 13). In addition, the percentage (%) and mean fluorescence intensity (MFI) of differentiation markers CD54, B7H1, MHC-1, and CD44 were also comparable to untreated OSCSCs (Figure 14). OSCSCs treated with supernatants from NK cells treated IL-2 and anti-CD16mAb caused significant resistance against NK cells mediated cytotoxicity (Figure 13). Significant increase in differentiation markers CD54 and MHC- 1, moderate increase in B7H1, and moderate decrease in CD44 were observed in supernatants from NK treated IL-2 and anti-CD16mAb (Figure 14). Addition of sAJ2 to NK treated IL-2 and anti-CD16mAb supernatant caused the highest level of resistance against the NK cells-mediated cytotoxicity above that induced by NK treated IL-2 and anti-CD16mAb (Figure 13). The highest resistance in NK cells-mediated cytotoxicity correlated with the highest surface expressions of CD54, MHC-1, B7H1 and the lowest surface expressions of CD44 in OSCSCs treated NK cells treated with combination of IL-2+ anti-CD16mAb and sAJ2 supernatant (Figure 14).

As shown in Figure 15, the surface expression of CD107a (liposomal-associated membrane protein-1) was low in unstimulated NK cells or NK treated sAJ2. As NK cells were

activated with IL-2 or IL-2 and anti-CD16mAb, the expression of CD107a slightly unregulated. The level of CD107a surface expression in NK cells treated IL-2+anti-CD16mAb and sAJ2 also slightly up regulated (Figure 15).

As shown in Figure 16, the highest level of IFN-γ secretion was seen in the presence of sAJ2 in NK cells treated IL-2 and anti-CD16mAb, followed by NK cells treated with IL-2 and anti-CD16mAb. The lowest level of IFN-γ secretion was seen in untreated NK cells, which correlated with the resistance of OSCSCs to the NK cell-mediated cytolysis (Figure16).

**Sub Aim 2: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of OSCSCs to NK cell mediated cytotoxicity induced by the treatment of OSCSCs with IL-2 and anti-CD16mAb treated NK cell supernatants.**

As shown in Figure 17, supernatant obtained from NK cells treated IL-2 and anti-CD16mAb were able to induce maturation and differentiation of OSCSCs and resisted against NK cells mediated lysis. In addition, the differential marker of CD54 and B7H1 were up-regulated significantly compared to untreated OSCSCs while the stem cells marker CD44 was down-regulated (Figure 18). The expression level of MHC-1 was also up-regulated significantly (Figure 18) which allowed the target cells to escape from being lysed by the NK cells (Figure 17). Treatment of anti-IFN-γ antibody alone to OSCSCs treated NK+IL-2+anti-CD16mAb supernatant was unable to block differentiation and cause resistance against NK cells-mediated cytotoxicity (Figure 17). The expression levels of CD54 and MHC Class 1 were lower and CD44 and B7H1 were higher with the presence of anti-IFN-γ antibody treatment than IL-2 and anti-CD16Ab treated NK cells supernatant (Figure 18). In other words, adding anti-IFN-γ antibody alone was not able to block differentiation of OSCSCs or retain the same surface expression levels as untreated OSCSCs. Similarly, treatment of anti-TNF-α antibody alone to

OSCSCs treated anergized NK supernatant also was unable to block differentiation and cause resistance against NK cells mediated cytotoxicity (Figure 17). The expression levels of CD54 and MHC Class 1 were lower and CD44 and B7H1 were higher with the presence of anti-TNF-α antibody treatment than IL-2 and anti-CD16Ab treated NK cells supernatant (Figure 18). In other words, treatment of anti-TNF-α antibody alone was not able to block differentiation of OSCSCs or retain the same surface expression levels as untreated OSCSCs. However, the presence of both anti-IFN-γ and anti-TNF-α antibodies in OSCSCs treated anergized NK supernatants synergistically blocked differentiation of OSCSCs and significantly susceptible to NK cells mediated cytotoxicity similar to untreated OSCSCs (Figure 17). The presence of both anti-IFN-γ and anti-TNF-α antibodies in OSCSCs treated anergized NK cells supernatants also blocked modulation of CD54, CD44, B7H1 and MHC-1 having the same surface expression as stem-like untreated OSCSCs (Figure 18).

The cell counts obtained from untreated OSCSCs or OSCSCs treated with anergized NK supernatant in the presence of both anti-IFN-γ and anti-TNF-α antibodies after 4 days of treatments observed to have the highest number of attached cells, while OSCSCs treated with anergized NK supernatant alone observed to have the lowest number of attached cells, followed by OSCSCs treated with anergized NK supernatant in the presence of anti-IFN-γ antibody alone and anti-TNF-α antibody alone (Figure 19).

**Sub Aim 3: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of OSCSCs to NK cell mediated cytotoxicity induced by the treatment of OSCSCs with IL-2 in combination of anti-CD16mAb and sAJ2 treated NK cell supernatants.**

We have previously shown that the supernatants from NK treated IL-2 and anti-CD16mAb caused differentiation and resistance in OSCSCs against NK cells-mediated cytolysis. Furthermore, anti-IFN-γ alone or anti-TNF-α alone did not revert differentiation induced by IL-2 treated anti-CD16mAb but the combination of both antibodies had the capability to block differentiation.

The same pattern was also observed in the presence of probiotic bacteria. Untreated OSCSCs or OSCSCs treated sAJ2 did not induce differentiation of OSCSCs and were sensitive to NK cells-mediated cytotoxicity (Figure 20). In addition, their differentiation markers CD54 and B7H1 were comparably low as well as MHC-1 and their stem cell marker CD44 was high (Figure 21). Supernatant obtained from anergized NK cells plus sAJ2 were able to induce maturation, differentiation and resistance of OSCSCs against NK cells-mediated lysis (Figure 20). The differentiation markers CD54, B7H1, MHC-1 on the differentiated OSCSCs were significantly high while the stem cell marker CD44 was significantly low (Figure 21). Treatment of anti-IFN-γ antibody alone to OSCSCs treated anergized NK cells (IL-2+anti-CD16mAb) plus sAJ2 supernatant was not able to block differentiation of OSCSCs (Figure 20) or retain the same surface expression levels as untreated OSCSCs (Figure 21). Similarly, treatment of anti-TNF-α antibody alone to OSCSCs treated anergized NK cells plus sAJ2 supernatant was not able to block differentiation of OSCSCs (Figure 20) or retain the same surface expression levels as untreated OSCSCs (Figure 21), but still significantly induced differentiation of OSCSCs and caused resistance to NK cells-mediated cytotoxicity. However, combination of anti-IFN-γ and anti-TNF-α antibodies added to OSCSCs treated supernatants from NK treated IL-2+anti-

CD16mAb and sAJ2 were able to block differentiation of OSCSCs and resulted in susceptibility to NK cell lysis similar to untreated OSCSCs (Figure 20). Surface expressions CD54, MHC-1, B7H1, and CD44 were closed to untreated OSCSCs (Figure 21).

The presence of sAJ2 in NK treated IL-2 and anti-CD16mAb induced less than 10% cell deaths in OSCSCs (Figure 22).

Figure 23 showed the amount of IFN-γ produced from the NK cells supernatants that were used to treat OSCSCs. The highest level of IFN-γ was seen in NK cells treated IL-2+anti-CD16mAb and sAJ2, which correlated with the highest differentiation of OSCSCs and significantly resistance to NK cell-mediated cytolysis (Figure 23).

**Sub Aim 4: Co-cultures of OSCSCs with paraformaldehyde fixed NK cells treated IL-2+anti-CD16mAb or IL-2 in combination with anti-CD16mAb and sAJ2 induced resistance of OSCSCs against NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of OSCSCs to NK cell mediated cytotoxicity.**

Similar to differentiation of OSCSCs via secretion of cytokines, we also showed that differentiation of OSCSCs can be accomplished through cell-to-cell interaction. As shown in Figure 24, untreated fixed NK cells with 2% paraformaldehyde or untreated OSCSCs did not induce differentiation of OSCSCs and were sensitive to NK cell-mediated cytotoxicity. OSCSC treated fixed NK cells or untreated OSCSCs had similar CD54, MHC-1, B7H1 and CD44 (Figure 25). The presence of probiotic bacteria sAJ2 treated fixed anergized NK cells induced the highest differentiation in OSCSCs compared to OSCSCs treated fixed anergized NK cells without sAJ2; however, fixed anergized NK cells also induce significant differentiation in OSCSCs compared to untreated OSCSCs (Figure 24). The surface expression of CD54 and MHC-1 were up-regulated significantly in sAJ2 treated fixed anergized NK cells while

expression level of CD44 were down-regulated (Figure 25). The CD54 and MHC-1 surface expressions of OSCSCs treated fixed anergized NK cells were also up-regulated but lower compared to sAJ2 treated fixed anergized NK cells and CD44 were comparable to untreated OSCSCs (Figure 25).

Combination of anti-IFN-γ and anti-TNF-α antibodies added to OSCSCs treated fixed anergized NK cells or sAJ2 treated fixed anergized NK cells synergistically blocked differentiation in OSCSCs resulted in susceptibility to NK cells mediated cytotoxicity similar to untreated OSCSCs (Figure 24). In addition, their surface expressions were very similar to untreated OSCSCs (Figure 25).

Figure 26 showed that OSCSCs treated fixed anergized NK cells or sAJ2 treated fixed anergized NK cells induced less than 5% cell death in OSCSCs.

Figure 27 showed that the cell counts obtained from different treatments of OSCSCs with fixed NK cells after 4 days of treatments caused moderate detachment of cells; however, the number of attached cells was higher than the number of detached cells. We also observed fewer numbers of attached cells in the differentiated conditions.

**Sub Aim 5: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of OSCSCs to NK cell mediated cytotoxicity induced by the treatment of OSCSCs with IL-2+anti-CD16mAb+sAJ2 treated paraformaldehyde fixed NK cells.**

OSCSCs treated with fixed anergized NK cells (IL2+anti-CD16mAb) induced significant levels of resistance against NK cell-mediated lysis, but more resistance was observed in the presence of sAJ2 (Figure 28). In addition, there were moderate increases in surface expression CD54 and B7H1, and significant increase in MHC Class 1 observed in supernatants from

NK+IL2+anti-CD16mAb; however, the highest modulation was observed in the presence of sAJ2. There was no change in CD44 surface expression in all treatments (Figure 29). This could be explained that there was a small window of CD44 expression level from the untreated OSCSCs initially.

Treatment of anti-IFN-γ antibody alone to OSCSCs treated fixed anergized NK cells induced by sAJ2 caused moderate resistance in NK cells mediated cytotoxicity (Figure 28). The expression level of CD54, B7H1, and MHC-1 were slightly higher than untreated OSCSCs condition but lower than OSCSCs treated fixed anergized NK cells plus sAJ2 (Figure 29). Similarly, treatment of anti-TNF-α antibody alone to OSCSCs treated fixed anergized NK cells plus sAJ2 significantly induced differentiation in OSCSCs and caused further resistance to NK cell-mediated cytotoxicity (Figure 28). The expression levels of CD54, B7H1, and MHC-1 were significantly higher than untreated OSCSCs condition and expression level CD44 was lower (Figure 29). The combination of anti-IFN-γ and anti-TNF-α antibodies added to OSCSCs before treated with fixed anergized NK cells induced by sAJ2 were able to block differentiation of OSCSCs and caused increased sensitivity to NK cell-mediated lysis (Figure 28). The surface expression of CD54, MHC-1 and CD44 were comparable to untreated OSCSCs (Figure 29).

Figure 30 showed that OSCSCs treated fixed anergized NK cells induced by sAJ2 or in the presence of anti-IFN-γ antibody and/or anti-TNF-α antibody induced less than 5% cell death in OSCSCs.

**Sub Aim 6: Supernatants from NK cells treated IL-2 and anti-CD16mAb induced resistance of MIA PaCa-2 against NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of MIA PaCa-2 to NK cell mediated cytotoxicity.**

We also looked at a different transformed tumorigenic cell line to see whether NK cells can cause differentiation in a variety of cell types of just in OSCSCs. We found that supernatants from the NK cells treatments also differentiated MIA PaCa-2, pancrease carcinoma cell line. Supernatants obtained from anergized NK cells induced differentiation and resistance against NK cell-mediated lysis and addition of anti-IFN-γ antibody alone or anti-TNF-α antibody alone to MIA PaCa-2 treated anergized NK supernatant was not able to revert differentiation to untreated condition (Figure 31). Combination of both anti-IFN-γ and anti-TNF-α antibodies to MIA PaCa-2 treated anergized NK supernatant completely blocked differentiation of MIA PaCa-2 and allowed NK cells to mediate lysis at the same level as untreated MIA PaCa-2 (Figure 31).

Differentiation of MIA PaCa-2 correlated with an increased in surface expression of CD54, B7H1, MHC-1 and decreased in surface expression of CD44 compared to the undifferentiated stem-like MIA PaCa-2 (Figure 32). The addition of anti-IFNγ antibody alone or anti-TNF α antibody alone to MIA PaCa-2 treated NK+IL-2+anti-CD16mAb supernatant were not able to revert CD54, B7H1, and MHC-1 and CD44 from the differentiated condition to untreated condition (Figure 32). However, combination of both anti-IFN-γ anti-TNF α antibodies to MIA PaCa-2 treated with anergized NK supernatant were able to block differentiation and showed comparable expression level of CD54, B7H1, MHC-1 and CD44 as untreated MIA PaCa-2 cell line (Figure 32).

The cell counts obtained from the differentiation of MIA PaCa-2 showed that there were high numbers of attached cells compared to detached cells. The lowest numbers of attached cells were observed in the conditions that show differentiation (Figure 33).

**Sub Aim 7: Supernatants from NK cells treated IL-2 in combination with anti-CD16mAb and sAJ2 induced resistance of MIA PaCa-2 against NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of MIA PaCa-2 to NK cell mediated cytotoxicity.**

Supernatants obtained from anergized NK (IL-2+anti-CD16mAb) and sAJ2 induced significant level of resistance against NK cell-mediated lysis more than NK+IL-2+anti-CD16mAb supernatants and addition of anti-IFN-γ antibody alone or anti-TNF-α antibody alone were not able to block differentiation (Figure 34). Combination of both anti-IFN-γ and anti-TNF-α antibodies to MIA PaCa-2 treated anergized NK and sAJ2 blocked differentiation and resistance against NK cells mediated lysis (Figure 34).

To confirm the level of MIA PaCa-2 differentiation induced by NK cell supernatant, the same population of tumor cells used for chromium release assay was used for surface analysis of CD54, B7H1, MHC Class 1 and CD44 (Figure 35). Addition of anti-IFN-γ antibody alone or anti-TNF-α antibody alone to MIA PaCa-2 treated anergized NK and sAJ2 showed moderate level of induction of expression levels of CD54, B7H1, and MHC Class 1, with anti-TNF-α antibody induced higher induction of MHC-1 and B7H1 than anti-IFN-γ antibody. Combination of anti-IFN-γ and anti-TNF-α antibodies together synergistically reverted surface expression of CD54, B7H1, MHC Class 1 and CD44 in MIA PaCa-2 treated anergized NK induced by sAJ2 to untreated MIA PaCa-2 (Figure 35).

Supernatants obtained from MIA PaCa-2 treatment induced less than 5% cell death from attached cells and less than 15% cell death from detached cells. Probiotic bacteria did not induce cell deaths in MIA PaCa-2 differentiation (Figure36). Moderate numbers of detached cells and high number of attached cells were observed in differentiation of MIA PaCa-2 in all conditions (Figure 37).

The supernatants used to treat MIA PaCa-2 for four days showed that NK cells treated with IL-2 and anti-CD16mAb plus sAJ2 secreted the highest amount of IFN gamma which correlated with highest differentiation (Figure 38).

**Sub Aim 8: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of MIA PaCa-2 to NK cell mediated cytotoxicity induced by the treatment of MIA PaCa-2 with IL-2+anti-CD16mAb+sAJ2 treated paraformaldehyde fixed NK cells.**

We have demonstrated that probiotic bacteria induced differentiation through cell-cell interaction in OSCSCs. We observed the same pattern of differentiation and resistance when we differentiated MIA PaCa-2 with the paraformaldehyde fixed NK cells (Figure 39). The highest level of resistance of MIA PaCa-2 and significant modulation of CD54, MHC-1, B7H1, and CD44 were observed when MIA PaCa-2 was treated with fixed NK cells treated IL-2+anti-CD16mAb+sAJ2 (Figure 39 and Figure 40).

The addition of either anti-IFN-γ antibody alone or anti-TNF-α antibody alone were unable to revert differentiation and resistance against NK cell-mediated lysis (Figure 39) as well as their surface receptor markers (Figure 40). Combination of anti-IFN-γ and anti-TNF-α antibodies together blocked differentiation of MIA PaCa-2 and allowed NK cells to lyse significantly at the same level untreated MIA PaCa-2 (Figure 39 and Figure 40). We also

observed a small window in CD44 surface expression in the untreated MIA PaCa-2 which did not allow us to see the significant changes in CD44 modulation (Figure 40).

Treatments of MIA PaCa-2 with fixed anergized NK cells and sAJ2 induced less than 5% cell death in MIA PaCa-2 (Figure 41). Probiotic bacteria did not induce cell deaths in MIA PaCa-2 differentiation. Moderate numbers of detached cells and high number of attached cells were observed in differentiation of MIA PaCa-2 in all conditions (Figure 42).

**Sub Aim 9: Supernatants from NK cells treated with IL-2 in combination with anti-CD16mAb and sAJ2 were able to induce the highest resistance of SCAPs against NK cell-mediated cytotoxicity.**

Previously we have shown that bacteria induced differentiation in transformed tumorigenic stem cells OSCSCs and MIA PaCa-2 and caused the highest resistance against NK-mediated cytotoxicity. In this study, we demonstrated that probiotic bacteria also induced differentiation in untransformed healthy stem cells. SCAPs cells treated with supernatants from untreated NK cells had similar sensitivity levels as untreated SCAPs (Figure 43). SCAPs treated with supernatants from NK treated IL-2+anti-CD16mAb cells induced significant levels of resistance against NK cell-mediated cytotoxicity (Figure 43). The highest level of resistance was observed when SCAPs were treated with supernatants from NK treated with combination of IL-2 plus anti-CD16mAb and probiotic bacteria, sAJ2 (Figure 43).

**Sub Aim 10: Combination of recombinant TNF-α and recombinant IFN-γ induced resistance of SCAPs to NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of SCAPs to NK cell mediated cytotoxicity.**

We have shown that anti-IFN-γ alone or anti-TNF-α alone were unable to block differentiation of OSCSCs and MIA PaCa-2 and that they require both antibodies to synergistically block differentiation. We want to confirm that IFN-γ and TNF-α were able to induce differentiation of SCAP cells. As shown in Figure 44, SCAP treated recombinant TNF-α induced differentiation and caused resistance against NK cell-mediated cytotoxicity. In addition, treatment of SCAP cells with recombinant TNF-α increased surface expression of CD54, B7H1, and MHC-1 (Figure 45). Treatment of SCAP with recombinant IFN-γ further increased resistance to NK cell-mediated cytotoxicity compared to recombinant TNF-α (Figure 44). Recombinant TNF-α induced differentiation was correlated with increase in CD54, MHC-1, and B7H1 (Figure 45). The combination of both rTNF-α and rIFN-γ synergistically increased resistance against NK cell-mediated cytotxicity (Figure 44) and increased expression of CD54, MHC-1, and B7H1 (Figure 45). Addition of antibodies against rTNF-α and rIFN-γ blocked differentiation making SCAP more sensitive to NK cell-mediated cytotoxicity (Figure 44). Blocking of rTNF-α and rIFN-γ decreased the expression of CD54, MHC-1 and B7H1 similar to the level in untreated SCAPs (Figure 44).

Addition of anti-IFN-γ antibody alone to SCAPs treated anergized NK sup induced moderate differentiation and exhibited moderate resistance to NK cell-mediated cytotoxicity. Furthermore, addition of anti-TNF-α antibody alone to SCAPs treated anergized NK sup induced significant differentiation and exhibited significant resistance to NK cell-mediated cytotoxicity (Figure 46). Only when combination of anti-IFN-γ and anti-TNF-α antibodies together can synergistically block differentiation of SCAPs when treated with anergized NK sup and allowed

NK cells to mediate the highest cytotoxicity against SCAPs treated supernatant from anergized NK cells.

Figure 47 showed that SCAPs treated anergized NK sup in the presence or absence of anti-IFN-γ antibody and/or anti-TNF-α antibody induced less than 10% cell death of attached cells in SCAPs and less than 25% cell death of detached cells.

Figure 48 showed that differentiation of SCAPs using supernatant obtained from anergized NK cells produced very minimal number of detached cells and high number of attached cells. Treatments with NK+IL-2+anti-CD16mAb plus anti-IFN-γ alone or anti-TNF-α antibody alone were observed to have lower number of attached cells.

**Sub Aim 11: Supernatants from NK cells treated IL-2 in combination with anti-CD16mAb and sAJ2 induced resistance of SCAPs against NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of SCAPs to NK cell mediated cytotoxicity.**

As shown in Figure 49, untreated SCAPs or SCAPs treated with untreated NK sup did not induce differentiation and were sensitive to NK cell-mediated cytotoxicity. SCAPs treated with supernatants obtained from anergized NK cells and sAJ2 induced higher level of resistance against NK cell-mediated cytotoxicity. Addition of anti-IFN-γ antibody alone to SCAPs treated with supernatants obtained from anergized NK cells and sAJ2 induced moderate differentiation and caused moderate resistance to NK cell-mediated cytotoxicity. Likewise, addition of anti-TNF-α antibody alone added to SCAPs treated with supernatants obtained from anergized NK cells and sAJ2 induced significant differentiation and significant resistance to NK cell-meditated lysis. However, only when combination of anti-IFN-γ and anti-TNF-α antibodies together when added to SCAPs treated with supernatants obtained from anergized NK cells and sAJ2were able

to block differentiation and allowed NK cells to mediate highest cytotoxicity at the same level as untreated SCAPs.

The expression levels of CD54 and MHC-1 increased in SCAPs treated with anergized NK supernatant and even more significantly increased in SCAPs treated with anergized NK induced by sAJ2 (Figure 50) . Addition of anti-IFN-γ antibody or anti-TNF-α antibody to SCAPs treated anergized NK induced by sAJ2 did not revert CD54 or MHC-1 to undifferentiated condition. Combination of anti-IFN-γ and anti-TNF-α antibodies added to SCAPs treated anergized NK induced by sAJ2 expressed similar expression level of CD54 and MHC Class 1 as undifferentiated SCAPs (Figure 50).

Figure 51 showed that there were minimal numbers of detached cells and high number of attached cells observed in differentiation of SCAPs with various conditions of supernatants. In addition, it was also observed that the most differentiated SCAPs exhibited the less number of attached cells in the differentiation experiments (Figure 51).

**Fig. 13**

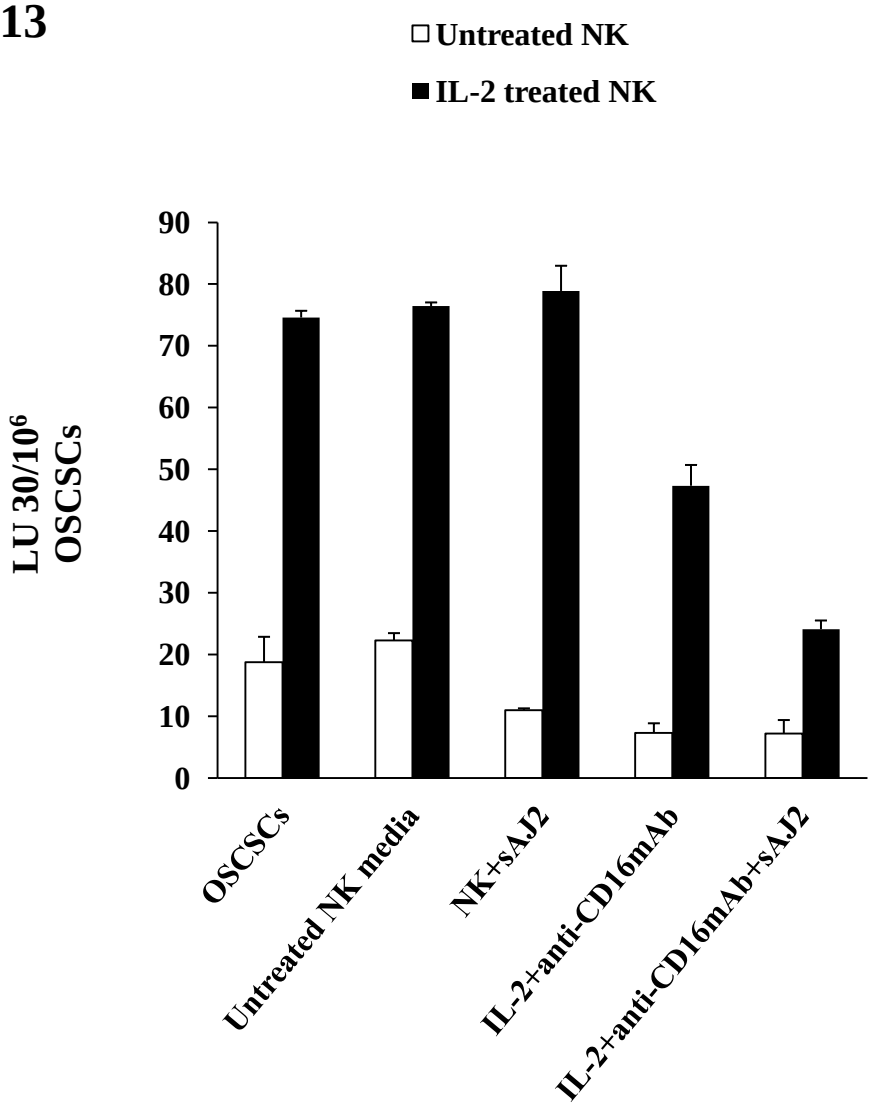



**Figure 13: Supernatants from NK cells treated with IL-2 in combination with anti-CD16mAb and sAJ2 were able to induce the highest resistance of OSCSCs against NK cell-mediated cytotoxicity.**

NK cells were left untreated, treated with sAJ2 (3:1 bacteria: NKs), or treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5ug/mL) with or without the presence of sAJ2 for 12h – 18h. Afterward, the supernatants from each NK sample were harvested and then treated OSCSCs for 4 days. Thereafter, the OSCSCs were detached from the tissue culture plates and their sensitivity to NK cell-mediated lysis was evaluated using 51-Chromium release assay. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of OSCSCs.

# Fig. 14

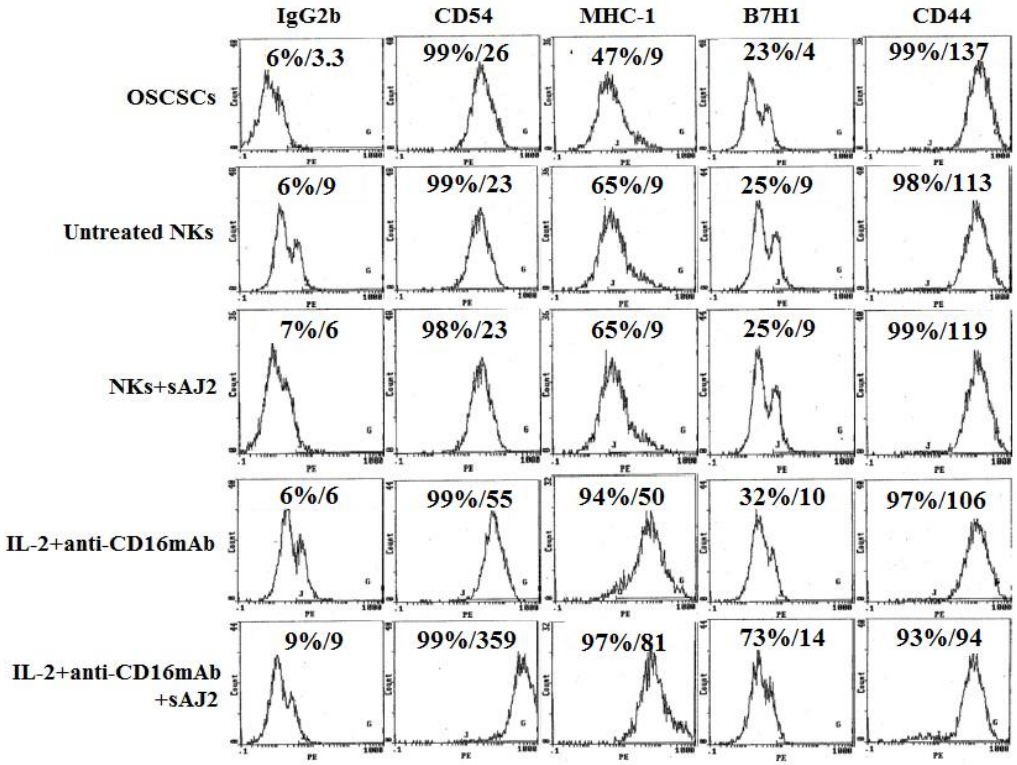



**Figure 14: Increased resistance of OSCSCs to NK cell-mediated cytotoxicity is correlated with the modulation of surface expression of CD54, MHC Class 1, B7H1 and CD44.**

NK cells were treated as described in Figure 13. Afterward, the supernatants from each NK sample were harvested and then treated OSCSCs for 4 days. Thereafter, the OSCSCs were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against isotype control, CD54, MHC-1, B7H1, and CD44 and the levels of surface expression were determined by flow cytometric analysis.

# Fig. 15

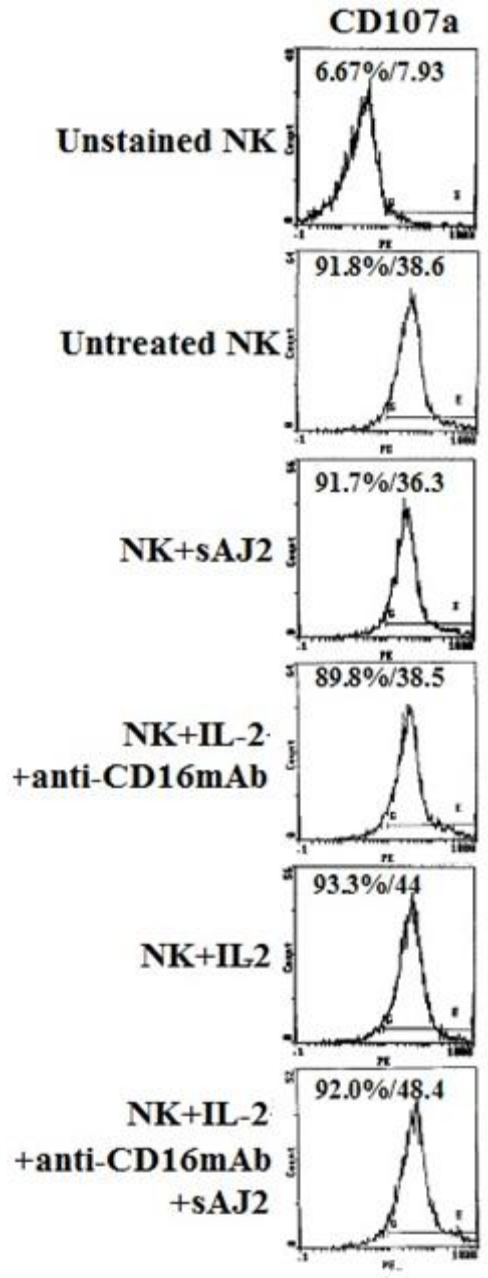



**Figure 15: Slight increases in the expression of CD107a by sAJ2.**

NK cells were treated as described in Figure 13. Afterward, $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against CD107a and the levels of surface expression were determined by flow cytometric analysis.

# Fig. 16

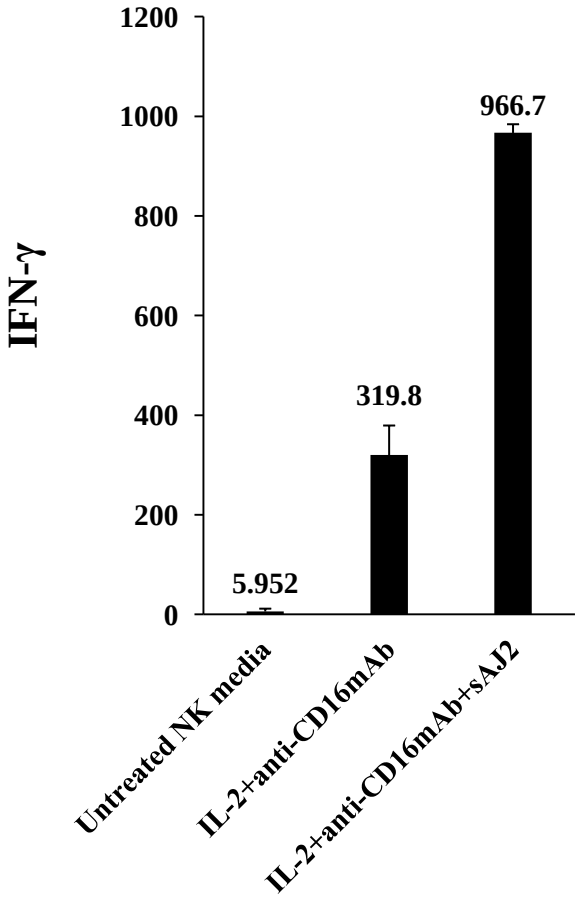
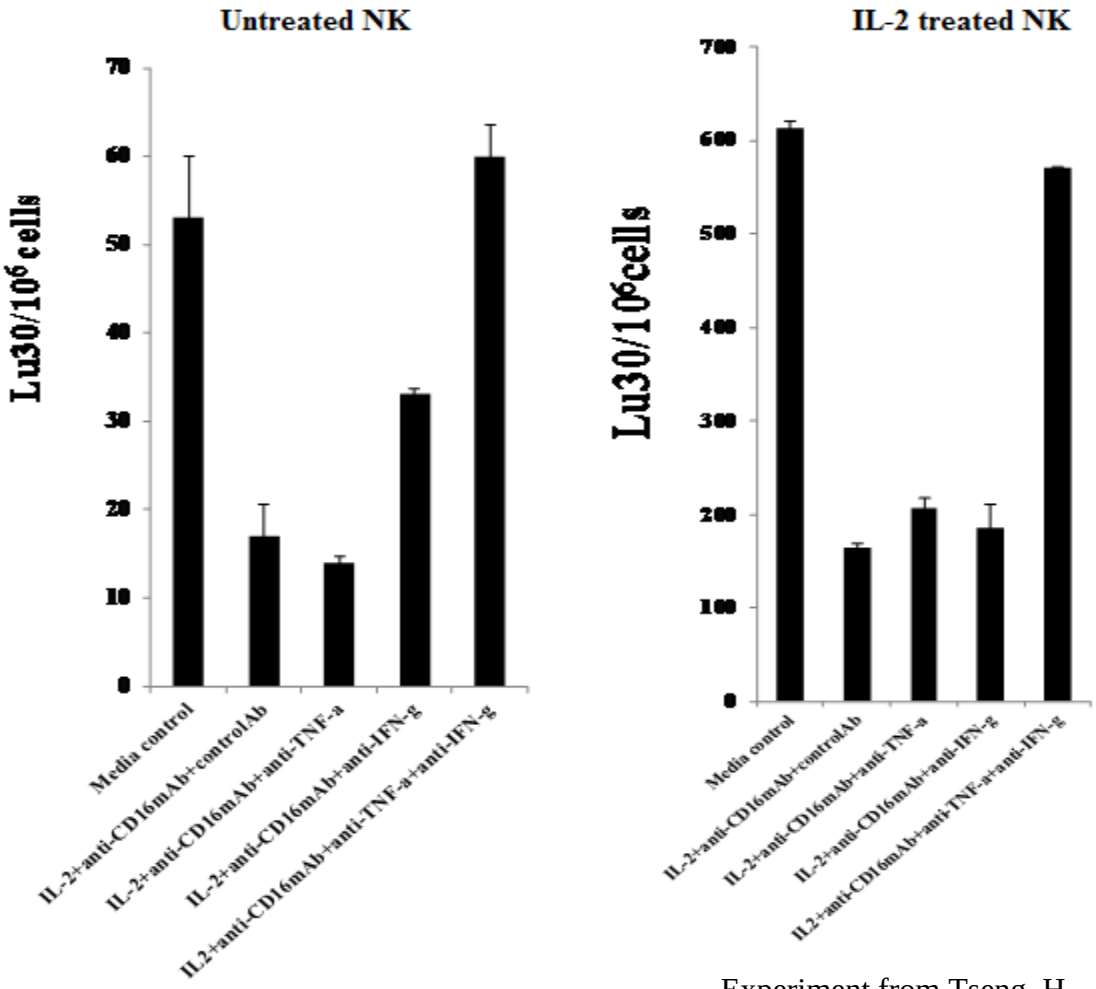



**Figure 16: Supernatant harvested from NK treated IL-2 in combination with CD16mAb plus sAJ2 produced significant amount of IFN-γ which correlated with the resistance of OSCSCs.**

Untreated, or IL-2 (1000U/mL) and anti-CD16mAb (2.5µg/mL) treated NK cells with or without sAJ2 at ratio 3:1 (bacteria : NKs) for 12-18 hours before the supernatants were collected and the level of IFN-γ secretion were determined using Human IFN-γ ELISA MAX Standard Sets. The y-coordinate represents concentration in pg/mL.

# Fig. 17

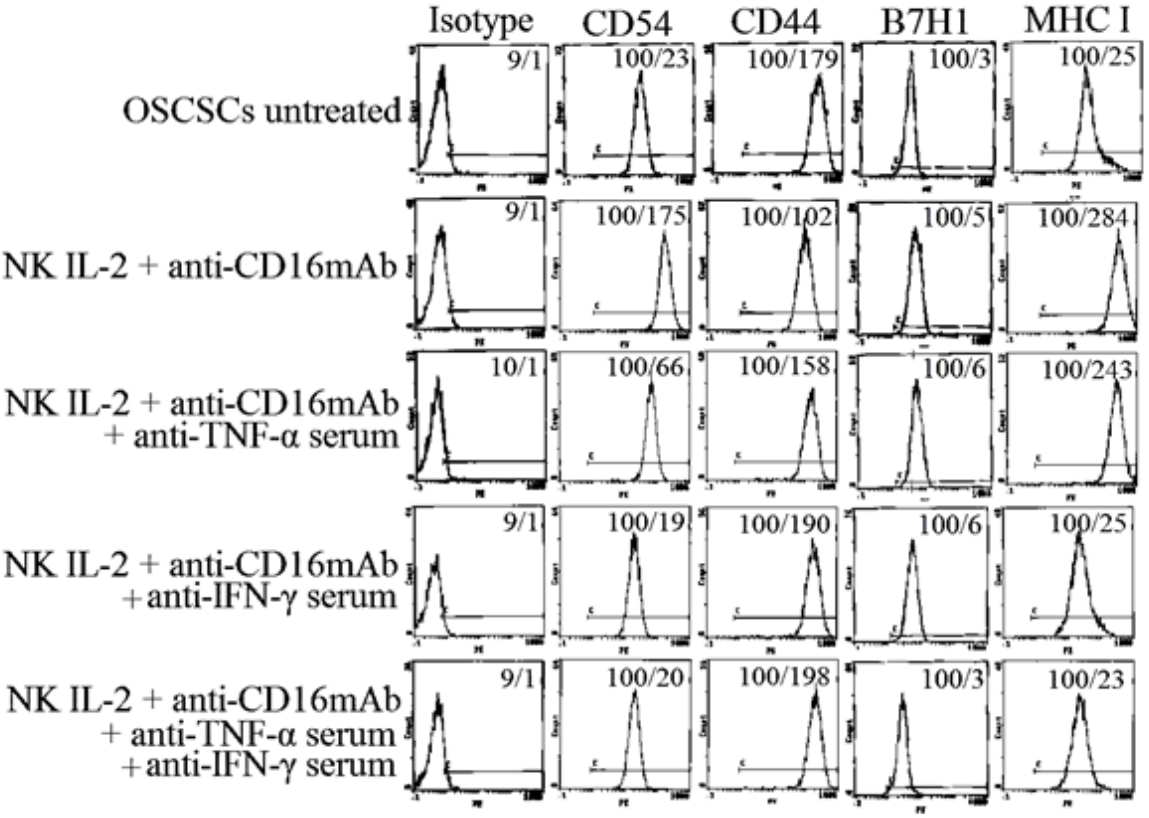



Experiment from Tseng, H.

**Figure 17: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of OSCSCs to NK cell mediated cytotoxicity.**

NK cells were treated with IL-2 (1000 units/ml) and anti-CD16 mAb (2.5 ug/ml) or the combination of IL-2 (1000 units/ml) and anti-CD16 mAb (3 ug/ml) for 24 hours. Afterward, the supernatants were harvested and added to OSCSCs cultures for a period of 5 days. The anti-IFN-γ (1:100) and anti-TNF-α (1:100) antibodies were added to OSCSCSs before the start of treatments. Thereafter, the OSCSCs were detached from the tissue culture plates and their sensitivity to NK cell-mediated lysis was evaluated using 51-Chromium release assay. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of OSCSCs.

# Fig. 18




Experiment from Tseng, H.

**Figure 18: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited modulation of surface receptor expressions on OSCSCs when treated with supernatant from IL-2 and anti-CD16 treated NK cells.**

NK cells were treated as described in Figure 17. Afterward, the supernatants were harvested and added to OSCSCs cultures for a period of 5 days. The anti-IFN-γ (1:100) and anti-TNF-α (1:100) antibodies were added to OSCSCSs before the start of treatments. OSCSCs were then washed, detached from the plates, and the expression levels of CD54, CD44 and MHC class I were assessed with flow cytometry. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentage and the mean channel fluorescence intensity.

# Fig. 19

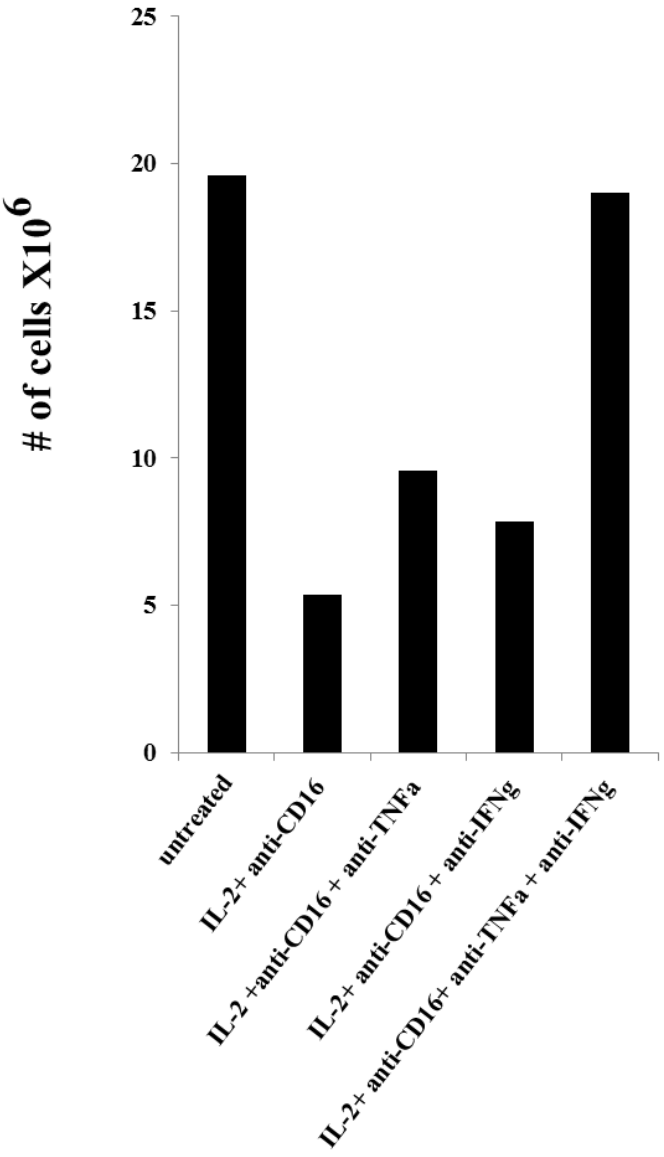



**Figure 19: Supernatant harvested from NK treated with IL-2+CD16mAb inhibited tumor growth of OSCSCs.**

NK cells were treated as described in Figure 17. Afterward, the supernatants were harvested and added to OSCSCs cultures for a period of 5 days. The anti-IFN-γ (1:100) and anti-TNF-α (1:100) antibodies were added to OSCSCSs before the start of treatments. Thereafter, the OSCSCs were detached from the tissue culture plates and were counted using Hemocytometer.

**Fig. 20**

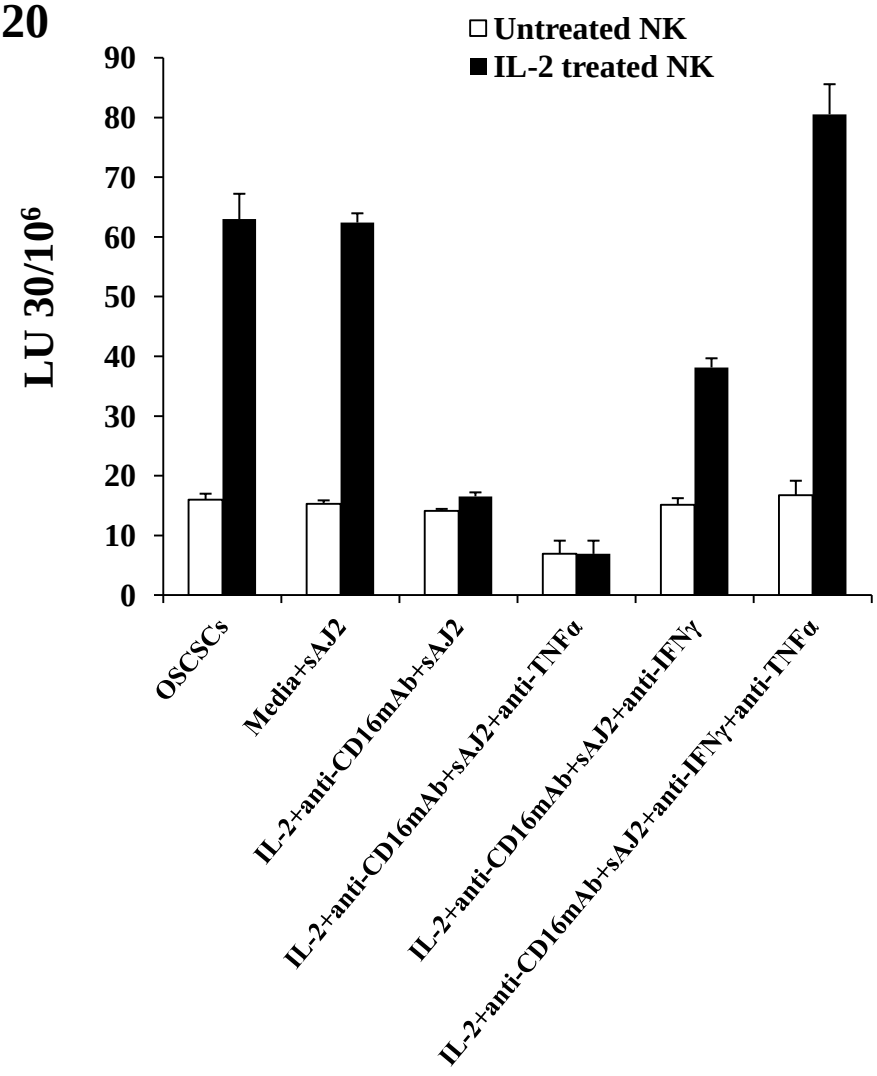



**Figure 20: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of OSCSCs to NK cell mediated cytotoxicity.**

Sonicated AJ2 (3:1 bacteria: NKs), and NK cells treated IL-2 (1000U/mL) and anti-CD16mAb (2.5ug/mL) with or without sAJ2 were incubated for 12h – 18h. Afterward, the supernatants from each NK sample were harvested and then treated OSCSCs for 4 days. The anti-IFN-γ (1:100) and anti-TNF-α (1:100) antibodies were added to OSCSCSs before the start of treatments. Thereafter, the OSCSCs were detached from the tissue culture plates and their sensitivity to NK cell-mediated lysis was evaluated using 51-Chromium release assay. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of OSCSCs.

**Fig. 21**

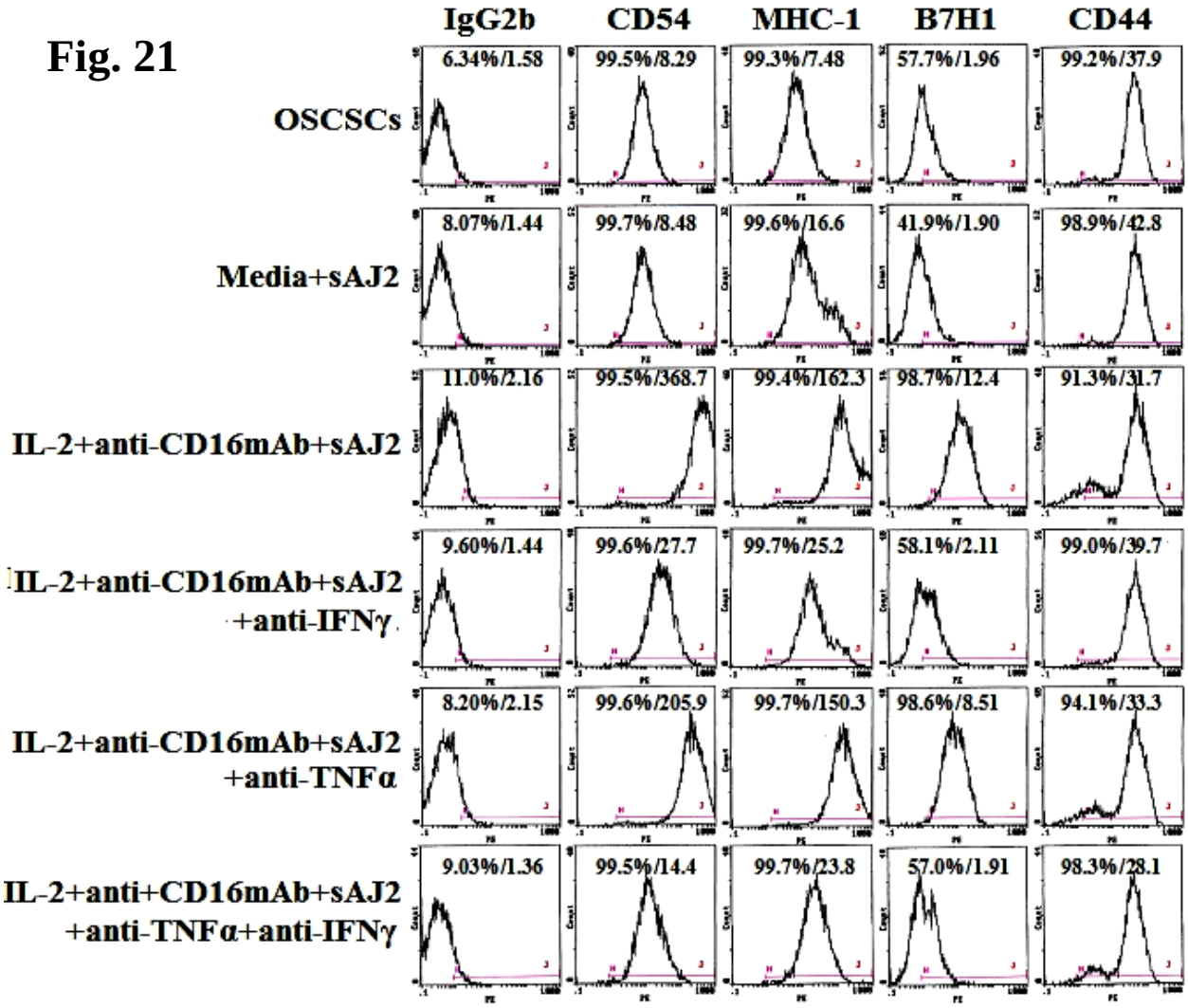



**Figure 21: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited modulation of surface receptor expressions on OSCSCs when treated with supernatants from IL-2+anti-CD16+sAJ2 treated NK cells.**

NK cells were treated as described in Figure 20. Afterward, the supernatants from each NK sample were harvested and then treated OSCSCs for 4 days. The anti-IFN-γ (1:100) and anti-TNF-α (1:100) antibodies were added to OSCSCSs before the start of treatments. Thereafter, the OSCSCs were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against isotype control, CD54, MHC-1, B7H1, and CD44 and the level of surface expression were determined by flow cytometric analysis.

# Fig. 22

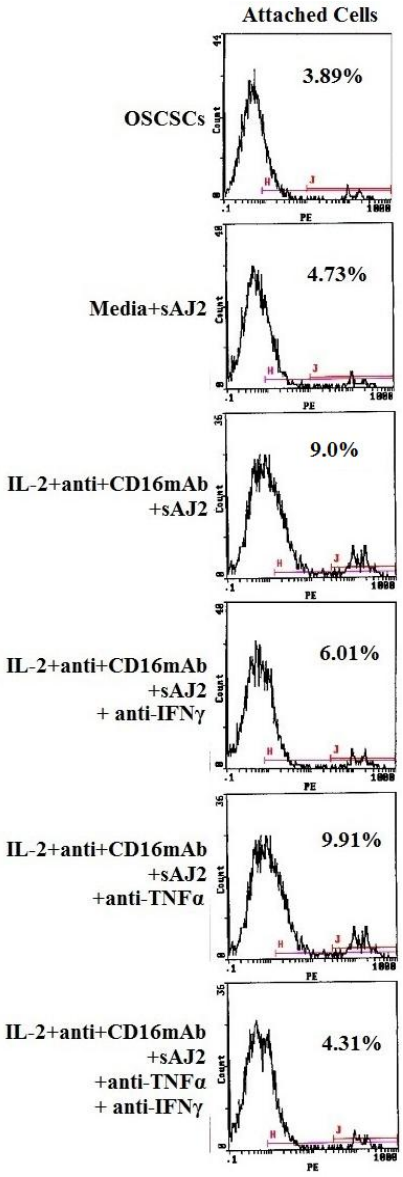



**Figure 22: Supernatant from IL-2+anti-CD16mAb +sAJ2 treated NK cells induced moderate levels of cell deaths in OSCSCs.**

NK cells were treated as described in Figure 20. Afterward, the supernatants from each NK sample were harvested and then treated OSCSCs for 4 days. The anti-IFN-γ (1:100) and anti-TNF-α (1:100) antibodies were added to OSCSCSs before the start of treatments. Thereafter, the OSCSCs were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained with PI (100ng/mL) in 400µl PBS and the level of cell death were determined by flow cytometric analysis.

# Fig. 23

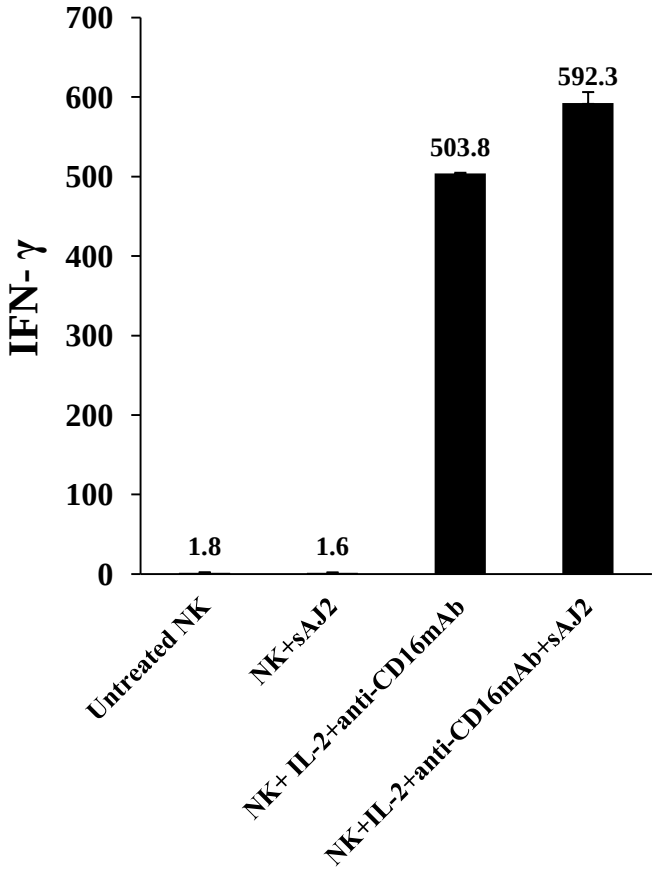



**Figure 23: Supernatant harvested from NK treated IL-2 in combination with CD16mAb and sAJ2 produced significant amount of IFN-γ which correlated with the resistance of OSCSCs.**

Untreated, sAJ2 (3:1 bacteria: NKs) treated, IL-2 (1000U/mL) and anti-CD16mAb (2.5µg/mL) treated NK cells were incubated with and without sAJ2 for 12-18 hours before the supernatants were collected and the level of IFN-γ secretion were determined using Human IFN-γ ELISA MAX Standard Sets. The y-coordinate represents concentration in pg/mL.

# Fig. 24

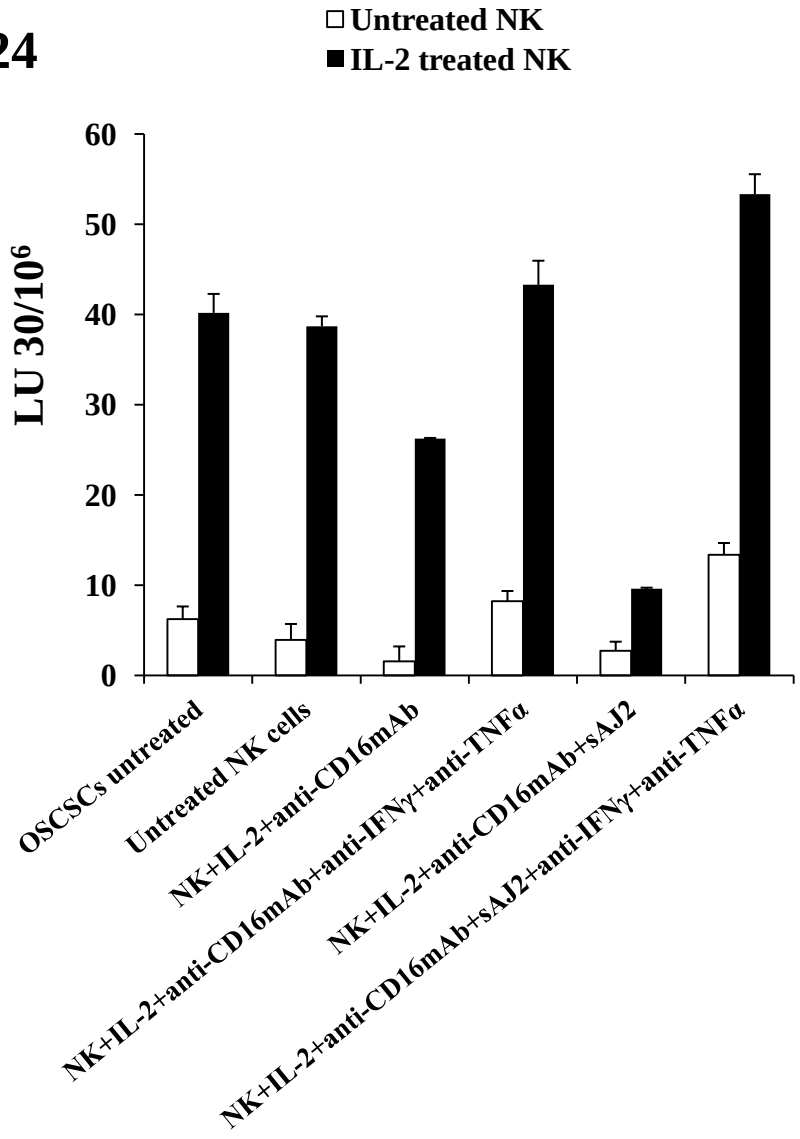



**Figure 24: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of OSCSCs to NK cell mediated cytotoxicity induced by the treatment of OSCSCs with IL-2+anti-CD16mAb or with IL-2+anti-CD16mAb+sAJ2 treated paraformaldehyde fixed NK cells.**

NK cells were left untreated, IL-2 (1000U/mL) and anti-CD16mAb (2.5ug/mL) treated, or IL-2 and anti-CD16mAb with sAJ2 at 3:1 ratios (bacteria: NKs) for 12h – 18h. Afterward, the cells were fixed with 2% formaldehyde and then treated OSCSCs for 4-5 days. Addition of anti-IFN-γmAb (1:100) and anti-TNF-α mAb (1:100) were added to OSCSCSs before the start of treatments. Thereafter, the OSCSCs were detached from the tissue culture plates and their sensitivity to IL-2 treated NK cell-mediated lysis was evaluated using 51-Chromium release assay. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of OSCSCs.

**Fig. 25**

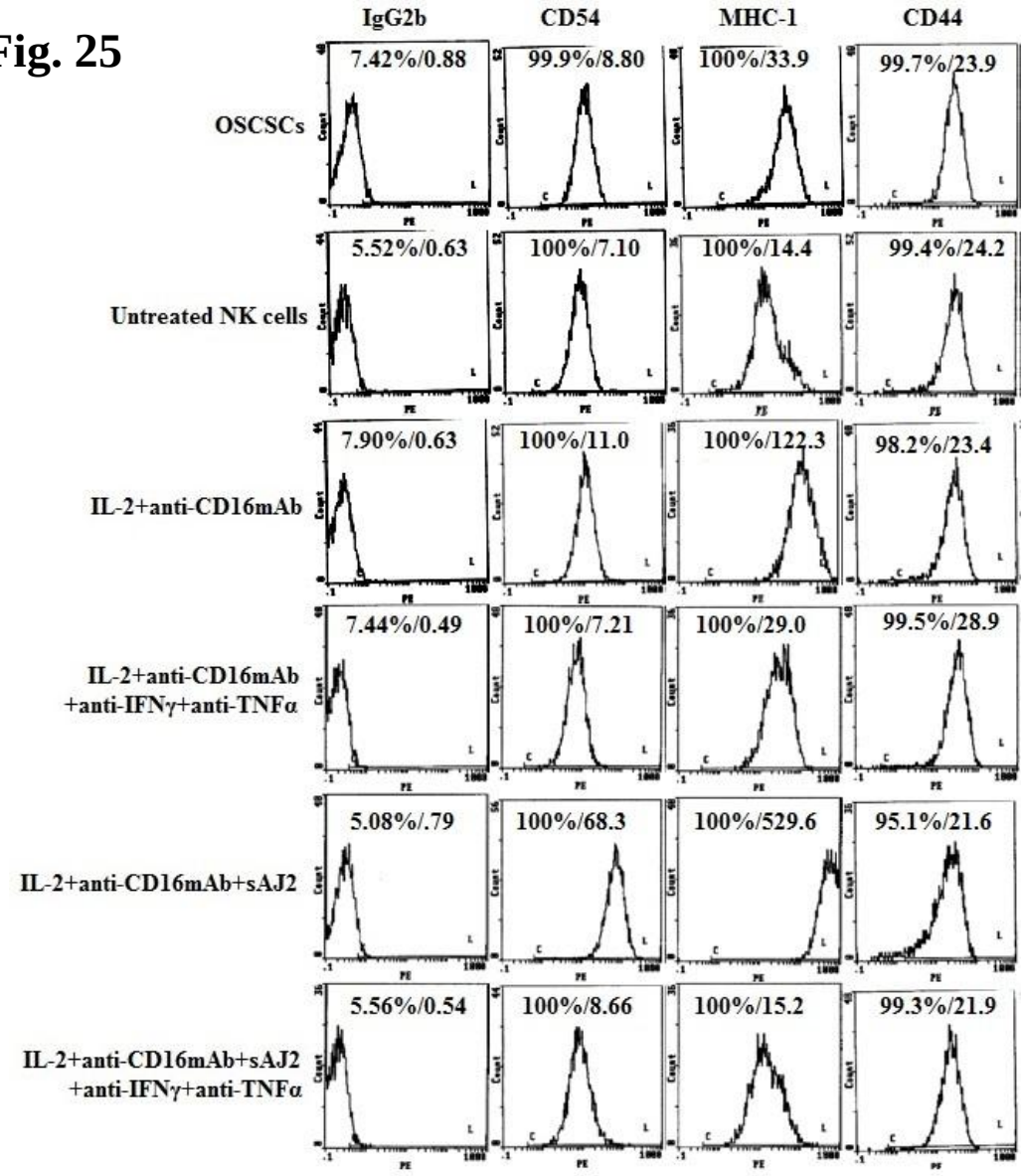



**Figure 25: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited modulation of surface receptor expression on OSCSCs when treated with supernatants from IL-2+anti-CD16mAb and IL-2+anti-CD16mAb+sAJ2 treated NK cells.**

OSCSCs were treated with NK cells supernatants as described in Figure 24. Thereafter, the OSCSCs were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against isotype control, CD54, MHC-1, B7H1, and CD44 and the level of surface expression were determined by flow cytometric analysis.

**Fig. 26**

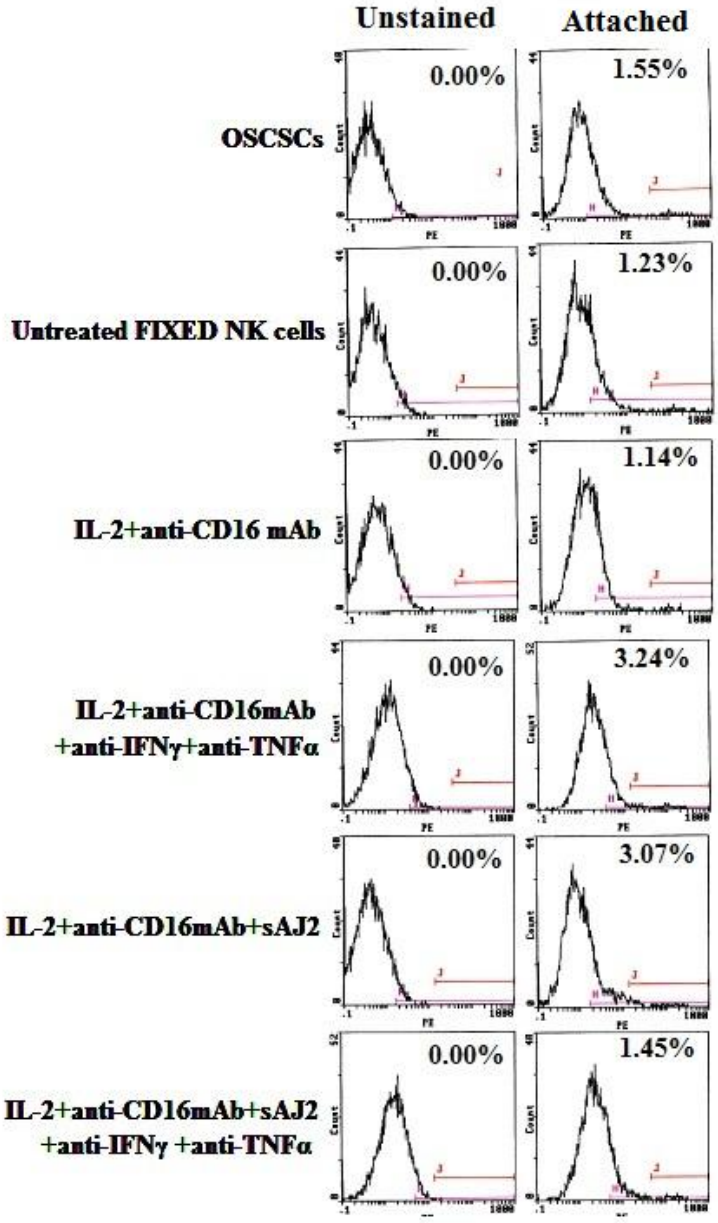



**Figure 26: Paraformadehyde fixed NK cells treated IL-2+anti-CD16mAb or treated IL-2+anti-CD16mAb +sAJ2 did not induce significant cell deaths in OSCSCs.**

OSCSCs were treated with NK cells supernatants as described in Figure 24. Thereafter, the OSCSCs were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using Propidium Iodide (100ng/mL) in 400µl PBS and the levels of cell deaths were determined by flow cytometric analysis.

**Fig. 27**

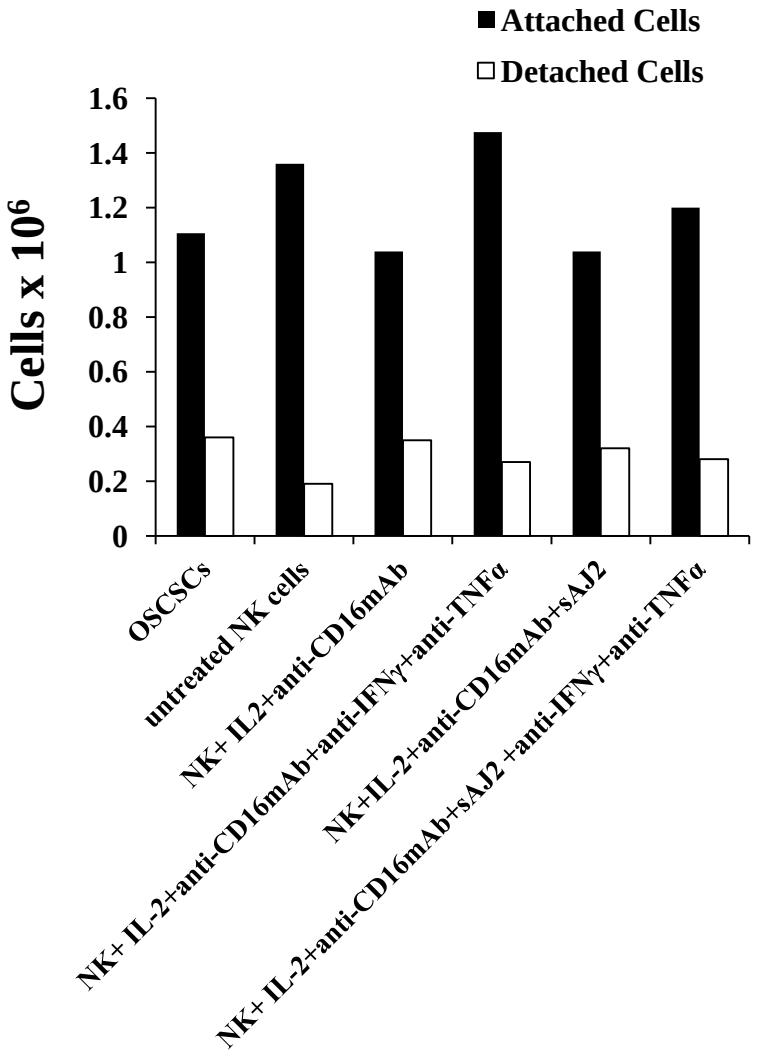



**Figure 27: Paraformaldehyde fixed NK treated with IL-2+CD16mAb or with IL-2 +CD16mAb +sAJ2 inhibited tumor growth of OSCSCs.**

OSCSCs were treated with NK cells supernatants as described in Figure 24. Thereafter, the OSCSCs were detached from the tissue culture plates and were counted using Hemocytometer.

**Fig. 28**

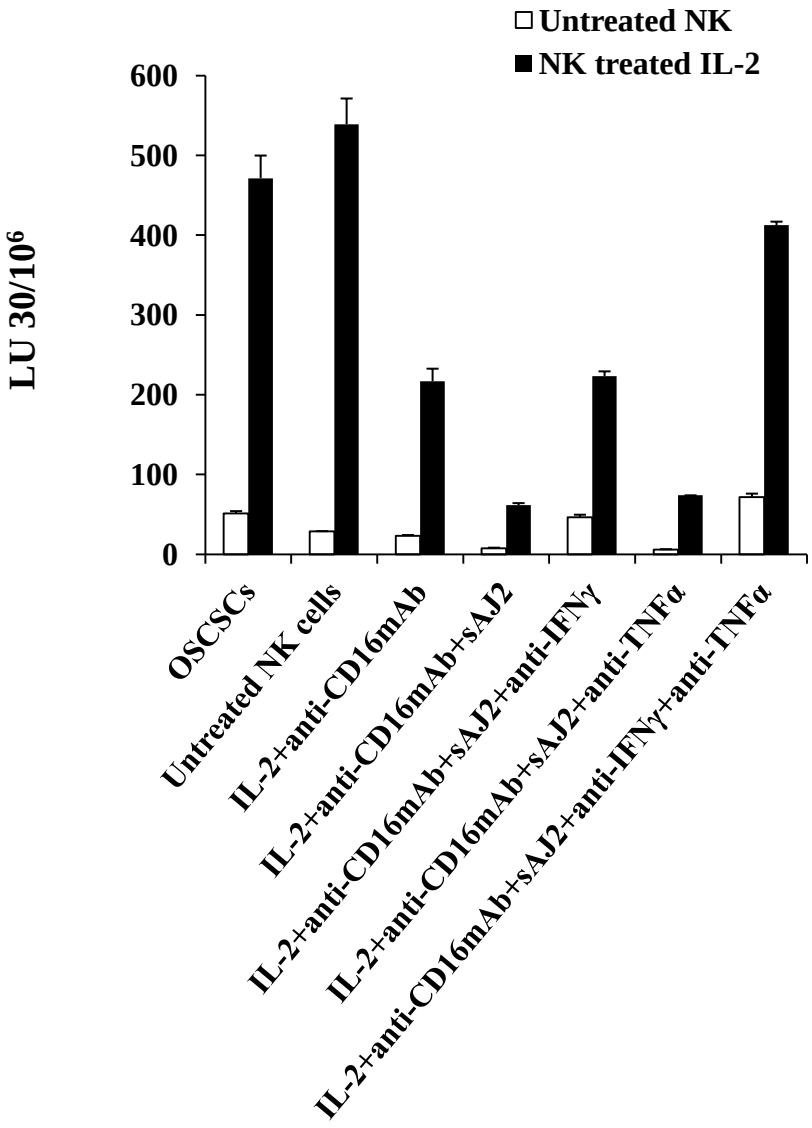



**Figure 28: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of OSCSCs to NK cell mediated cytotoxicity induced by the treatment of OSCSCs with IL-2+anti-CD16mAb+sAJ2 treated paraformaldehyde fixed NK cells.**

NK cells were left untreated, and treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5ug/mL) with or without sAJ2 (3:1; sAJ2: NKs) for 12h – 18h. Afterwards, the cells were fixed with 2% formaldehyde and treated OSCSCs for 4 days. Addition of anti-IFN-γmAb (1:100) alone, anti-TNF-α mAb(1:100) alone, or both anti-IFN-γ mAb and anti-TNF-α mAb were added to OSCSCSs on the same day before the start of treatments. Thereafter, the OSCSCs were detached from the tissue culture plates and their sensitivity to NK cell-mediated lysis was evaluated using 51-Chromium release assay. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of OSCSCs.

# Fig. 29

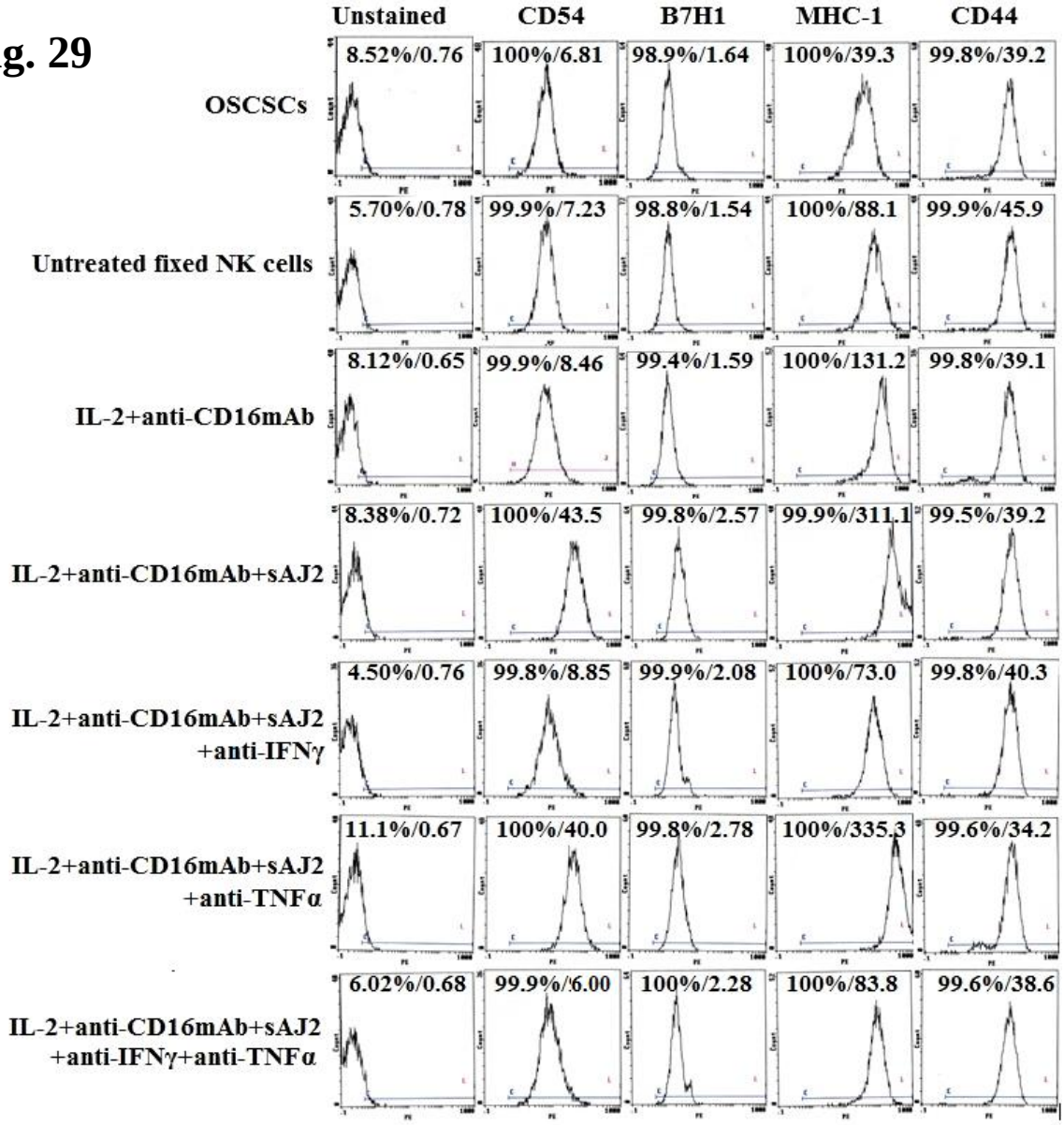



**Figure 29: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited modulation of OSCSCs to NK cell mediated cytotoxicity induced by the treatment of OSCSCs with IL-2+anti-CD16mAb+sAJ2 treated paraformaldehyde fixed NK cells.**

OSCSCs were treated with NK cells supernatants as described in Figure 28. Thereafter, the OSCSCs were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against CD54, MHC-1, B7H1, and CD44 and the level of surface expression were determined by flow cytometric analysis.

# Fig. 30

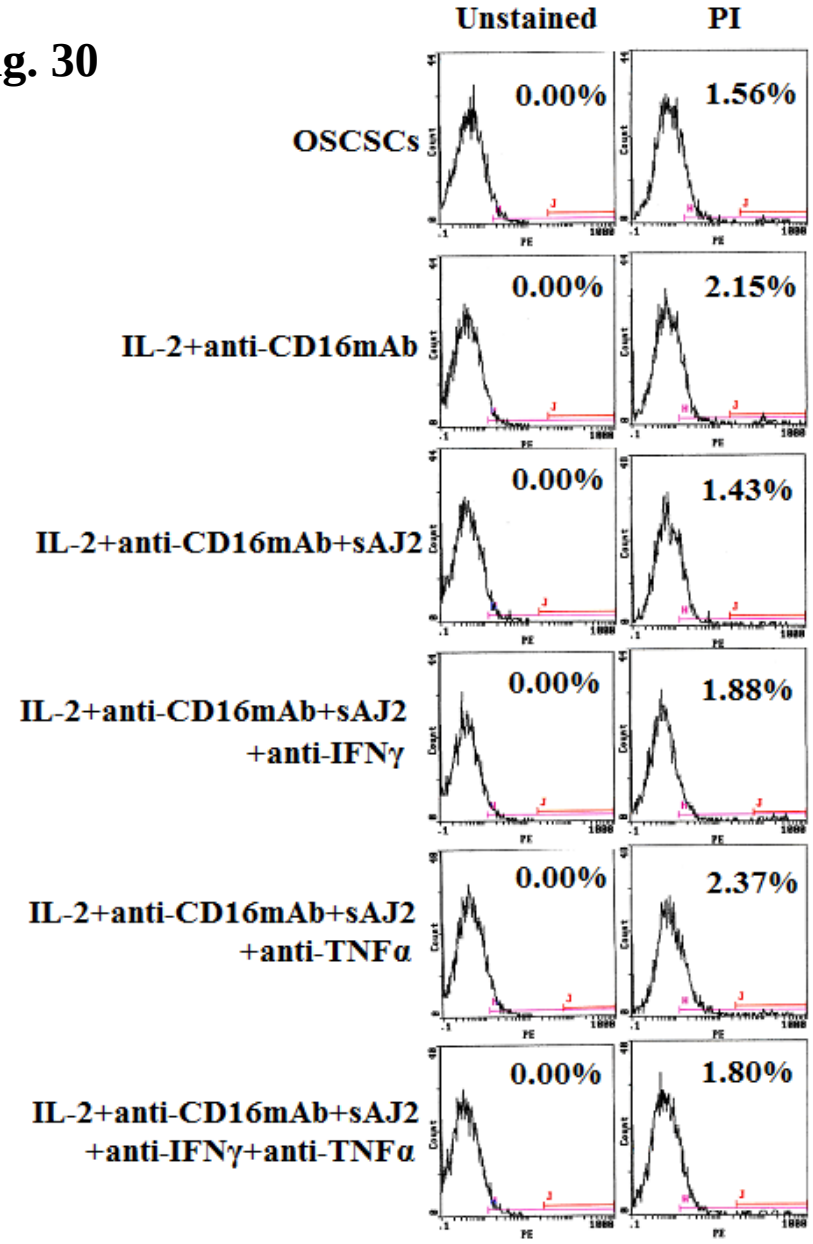



**Figure 30: Paraformadehyde fixed NK cells treated IL-2 with anti-CD16mAb and sAJ2 did not induce significant cell deaths.**

OSCSCs were treated with NK cells supernatants as described in Figure 28. Thereafter, the OSCSCs were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using Propidium Iodide (100ng/mL) in 400µl PBS and the level of cell death were determined by flow cytometric analysis.

**Fig. 31**

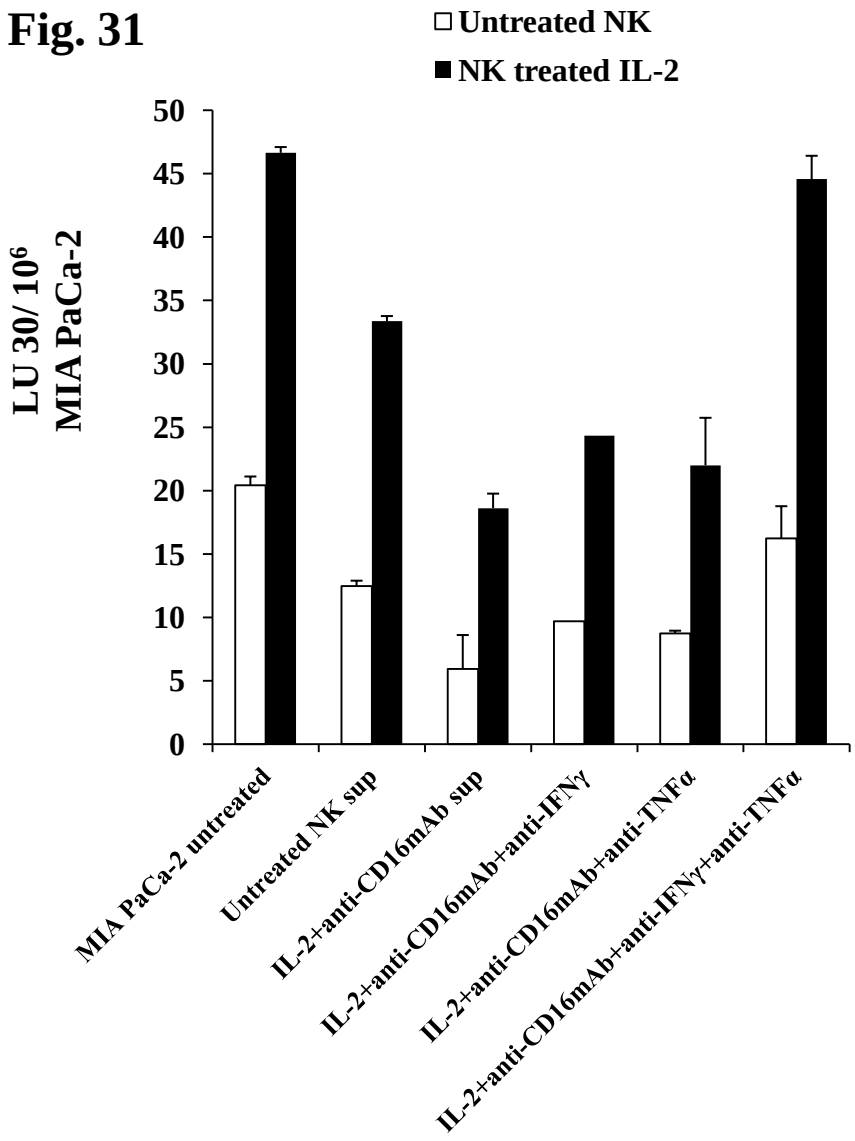



**Figure 31: Supernatant from NK cells treated IL-2 and anti-CD16mAb induced resistance of MIA PaCa-2 against NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of MIA PaCa-2.**

NK cells were left untreated or treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5ug/mL) for 12h – 18h. Afterwards, the supernatants from each NK sample were harvested and then treated MIA PaCa-2 for 4 days. Addition of anti-IFN-γmAb (1:100) alone, anti-TNF-α mAb (1:100) alone, or both anti-IFN-γ mAb and anti-TNF-α mAb were added to MIA PaCa-2 on the same day before the start of treatments. Thereafter, the MIA PaCa-2s were detached from the tissue culture plates and their sensitivity to NK cell-mediated lysis was evaluated using 51-Chromium release assay. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of MIA PaCa-2.

**Fig. 32**

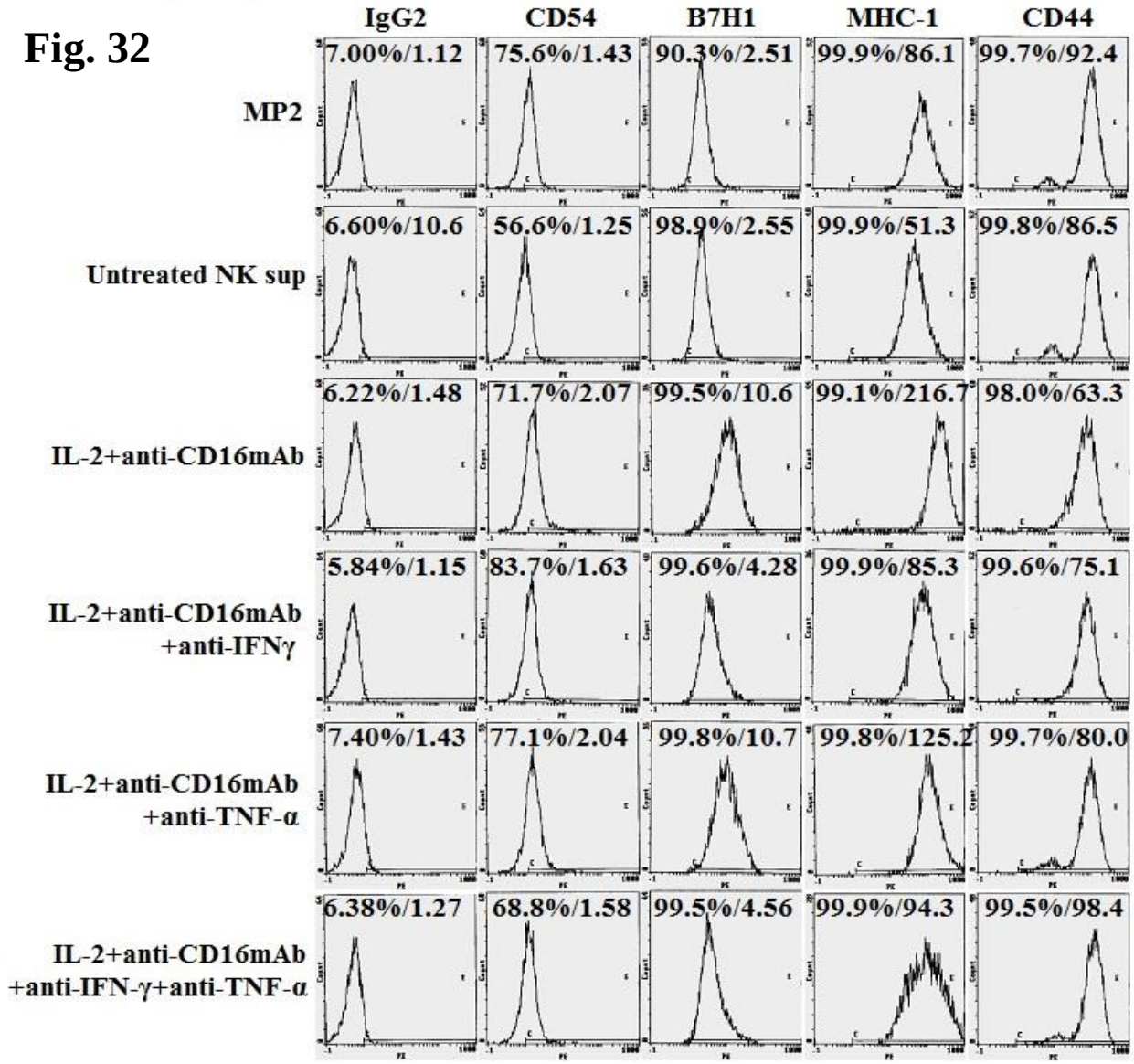



**Figure 32: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited modulation of surface receptor expressions on MIA PaCa-2 when treated with supernatant from IL-2+anti-CD16mAb treated NK cells.**

MIA PaCa-2 was treated with NK cells supernatants as described in Figure 31. Thereafter, the cells were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against isotype control, CD54, B7H1, MHC-1, and CD44 and the level of surface expression were determined by flow cytometric analysis.

**Fig.33**

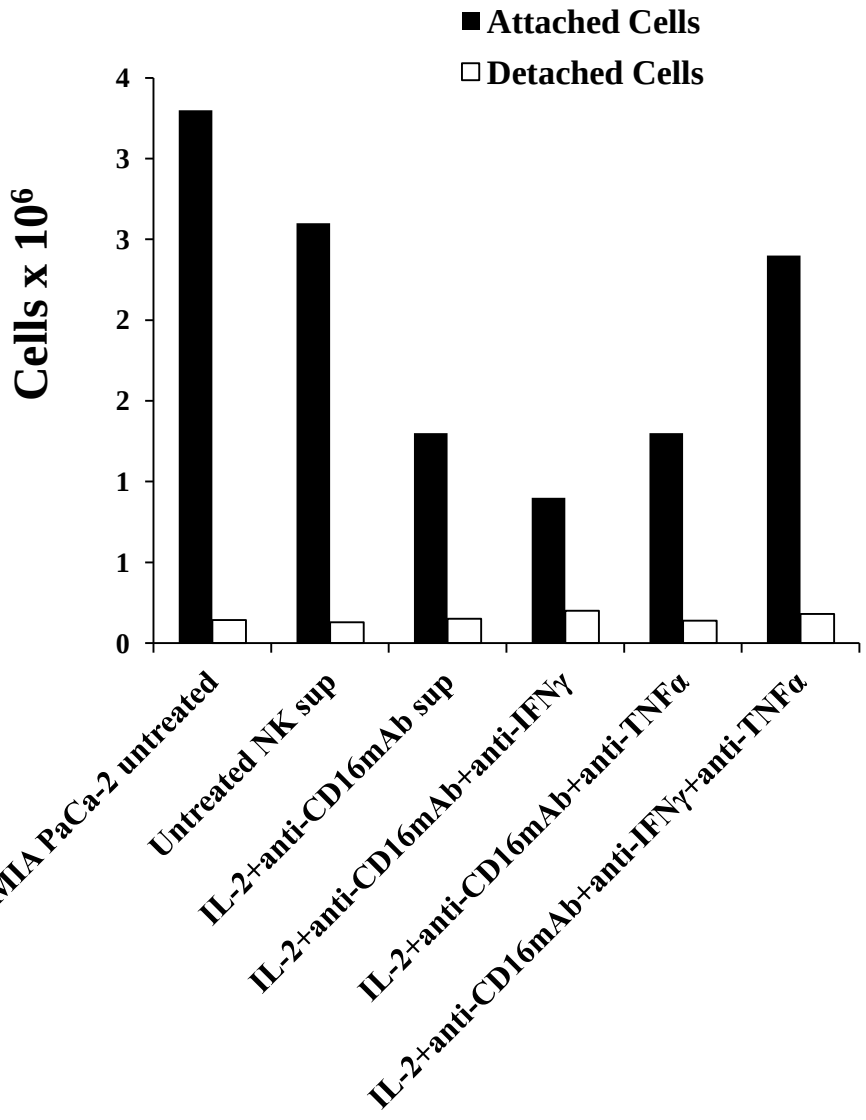



**Figure 33: Supernatant harvested from NK treated IL-2 and CD16mAb inhibited tumor growth of MIA PaCa-2.**

MIA PaCa-2 was treated with NK cells supernatants as described in Figure 31**.** Thereafter, the MIA PaCa-2 were detached from the tissue culture plates and were counted using a Hemocytometer.

**Fig. 34**

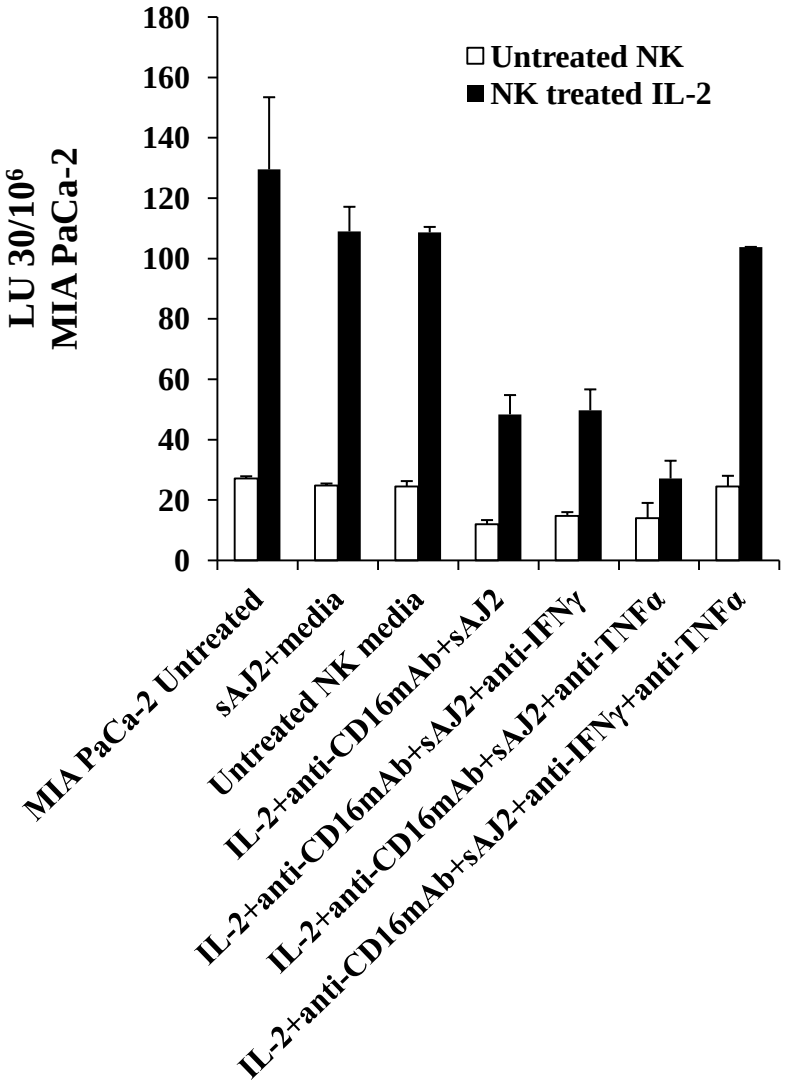



**Figure 34: Supernatant from NK cells treated IL-2 in combination with anti-CD16mAb and sAJ2 were able to induce the highest resistance of MIA PaCa-2 against NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of MIA PaCa-2 to NK cell mediated cytotoxicity.**

MIA PaCa-2 treated with supernatants from sAJ2 alone (3:1 bacteria: NKs), untreated NK cells, or NK treated IL-2 (1000U/mL), anti-CD16mAb (2.5ug/mL) and sAJ2 for 4 days. Addition of anti-IFN-γmAb (1:100) alone, anti-TNF-α mAb (1:100) alone, or both anti-IFN-γ mAb and anti-TNF-α mAb were added to MIA PaCa-2 on the same day before the start of treatments. Thereafter, the MIA PaCa-2s were detached from the tissue culture plates and their sensitivity to NK cell-mediated lysis was evaluated using 51-Chromium release assay. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of MIA PaCa-2.

**Fig.35**

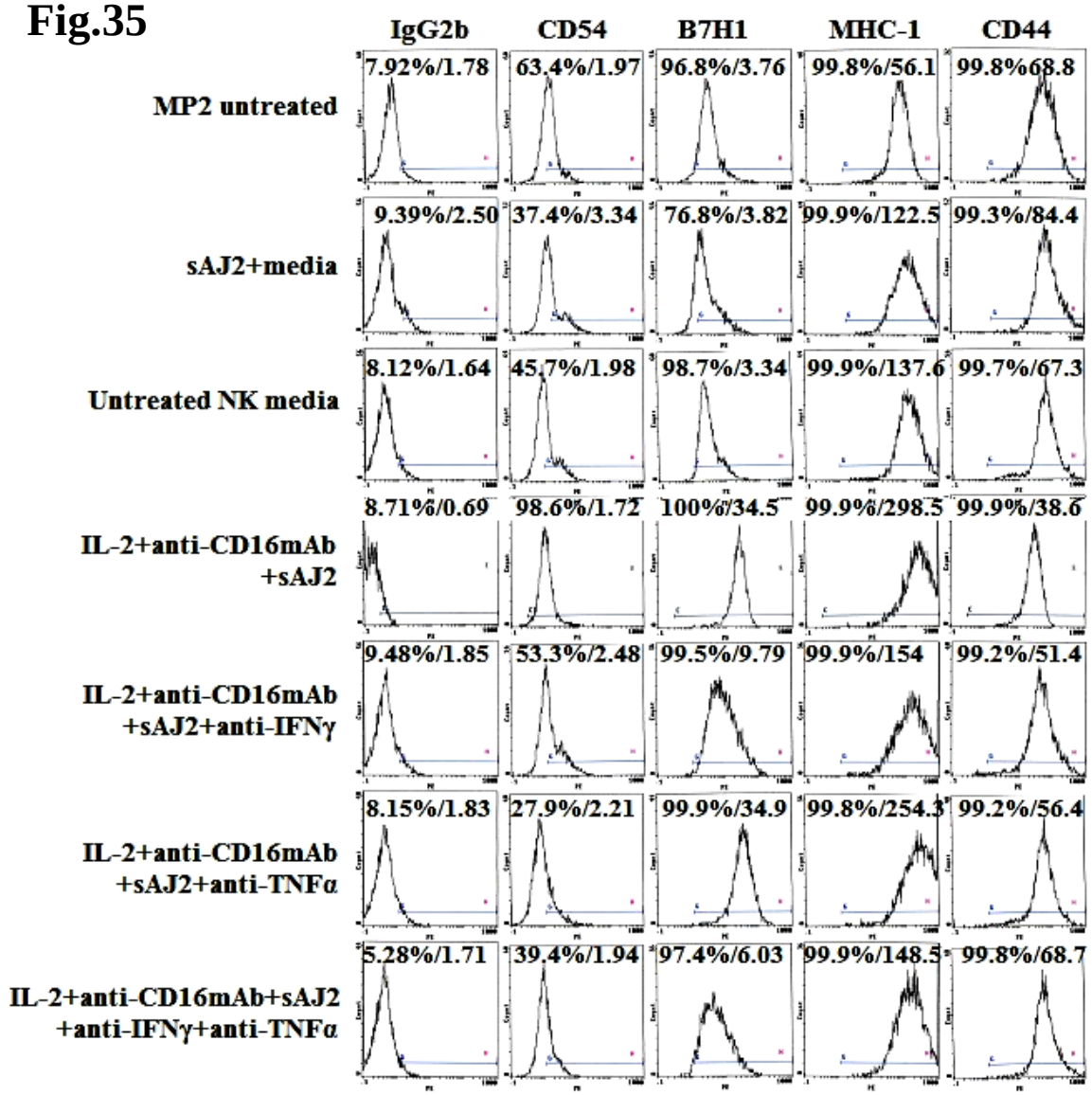



**Figure 35: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited modulation of surface receptor expressions on MIA PaCa-2 when treated with supernatant from IL-2+anti-CD16mAb+sAJ2 treated NK cells.**

MIA PaCa-2 was treated with NK cells supernatants as described in Figure 34. Thereafter, the cells were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against isotype control, CD54, B7H1, MHC-1, and CD44 and the level of surface expression were determined by flow cytometric analysis.

# Fig.36

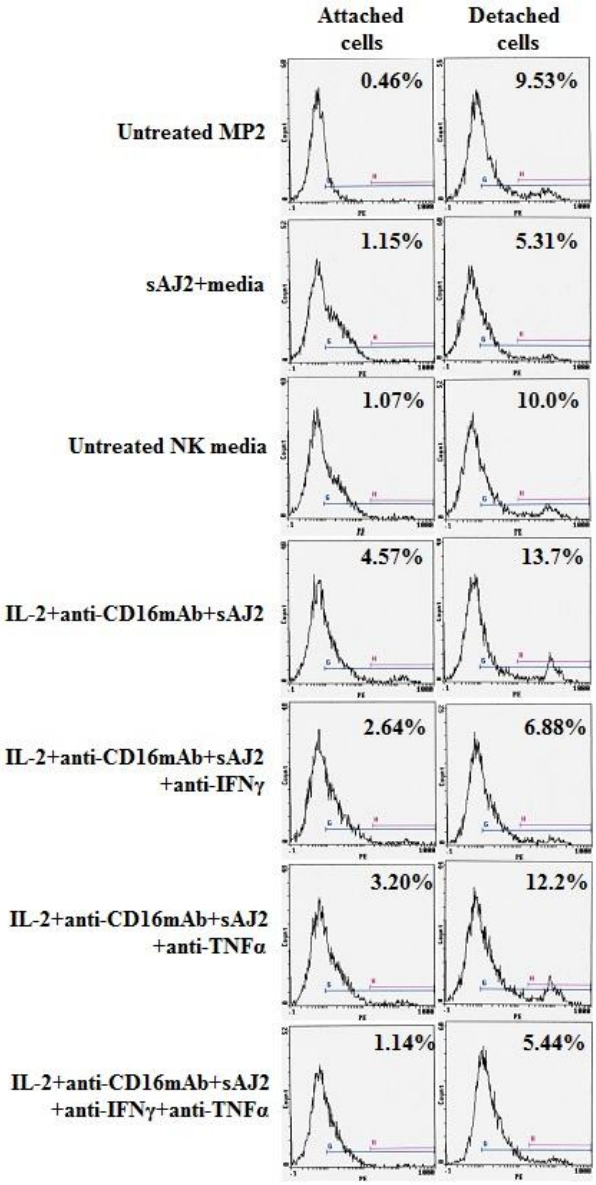



**Figure 36: Supernatant from IL-2+anti-CD16mAb +sAJ2 treated NK cells did not induce significant cell deaths in MIA PaCa-2.**

MIA PaCa-2 was treated with NK cells supernatants as described in Figure 34. Thereafter, adherence cells and the detached cells from the media were counted using Hematocytomer and $5x10^4$ cells from each treatment were stained using Propidium Iodide (100ng/mL) in 400µl PBS. The level of cell deaths was determined by flow cytometric analysis.

**Fig. 37**

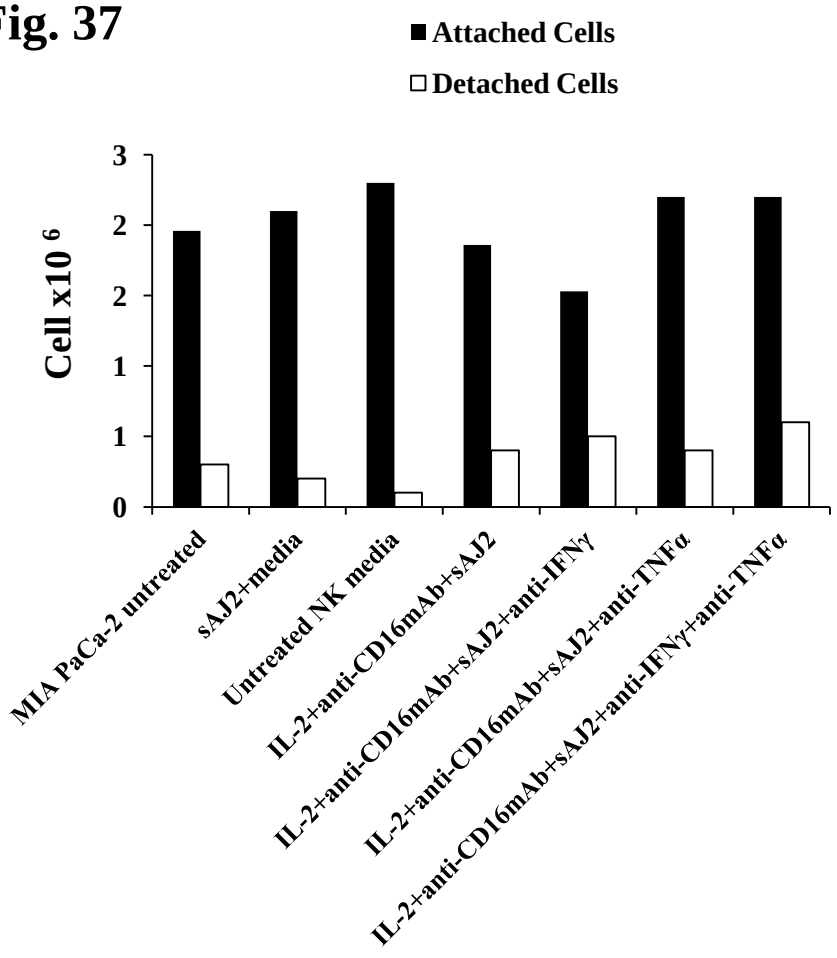



**Figure 37: Supernatant harvested from NK treated IL-2+CD16mAb+sAJ2 inhibited tumor growth of MIA PaCa-2.**

MIA PaCa-2 was treated with NK cells supernatants as described in Figure 34. Thereafter, the adherence and cells from the media of MIA PaCa-2 were counted using Hemocytometer.

# Fig.38

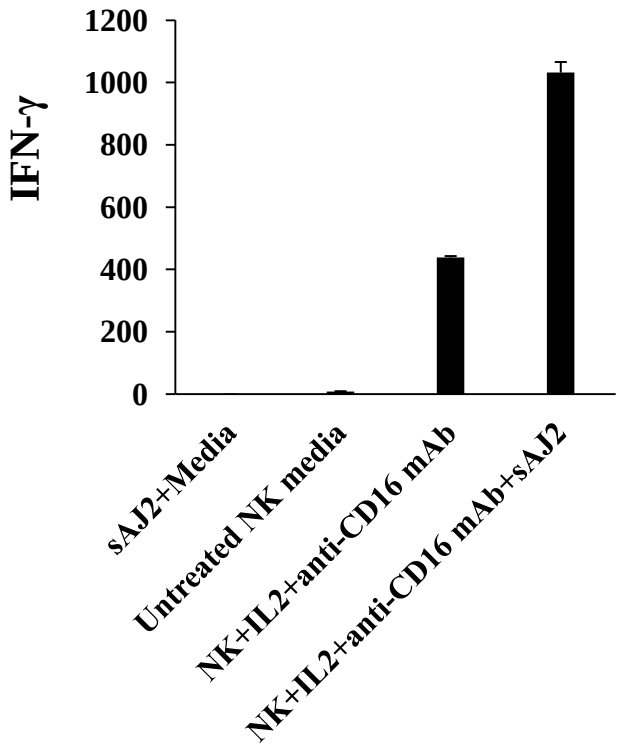



**Figure 38: Supernatant harvested from NK treated IL-2 with CD16mAb plus sAJ2 produced significant amount of IFN-γ which correlated with the resistance of MIA PaCa-2.**

Untreated, sAJ2 (3:1 bacteria: NKs) treated, IL-2 (1000U/mL) and anti-CD16mAb (2.5µg/mL) treated, or IL-2 and anti-CD16mAb plus sAJ2 treated NK cells were co-cultured for 12-18 hours before the supernatants were collected and the levels of IFN-γ secretion were determined using Human IFN-γ ELISA MAX Standard Sets. The y-coordinate represents concentration in pg/mL. Thereafter, the supernatants were used to treat MIA PaCa-2.

**Fig.39**

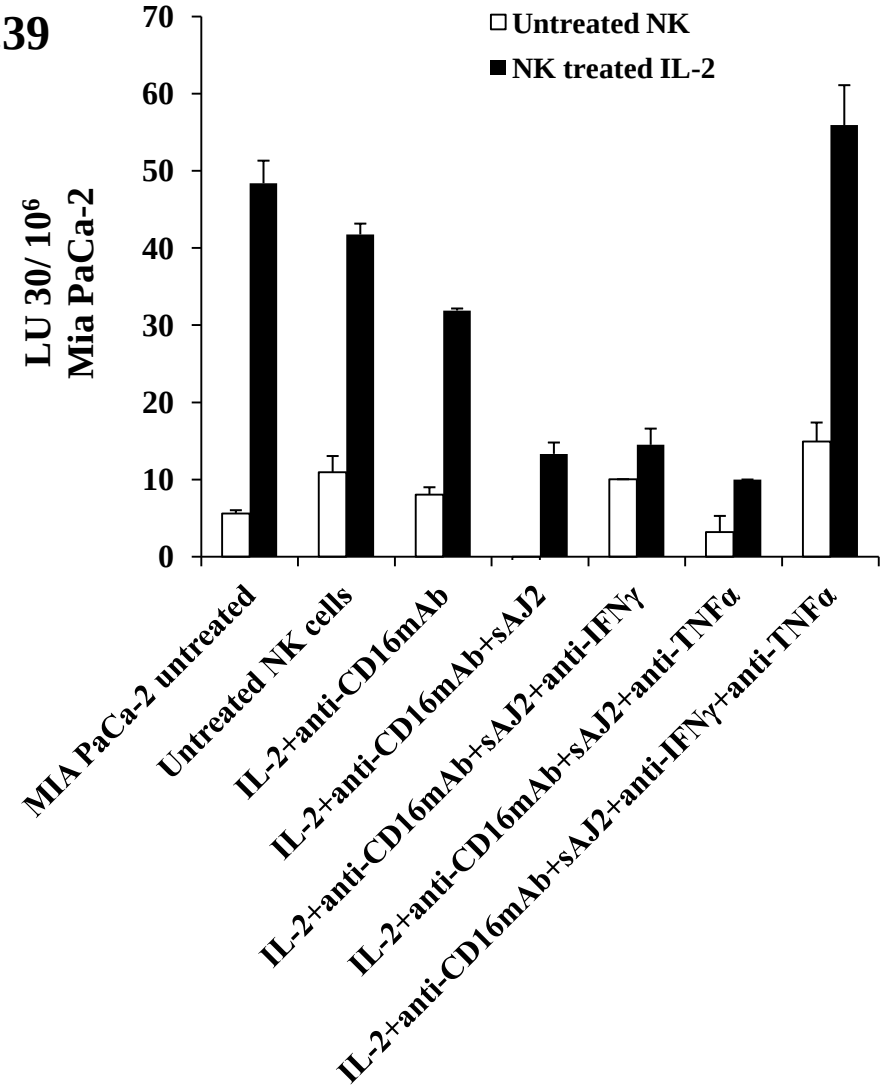



**Figure 39: Paraformaldehyde fixed NK cells treated IL-2 with anti-CD16mAb and sAJ2 induced the highest resistance of MIA PaCa-2 against NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of MIA PaCa-2 to NK cell mediated cytotoxicity.**

NK cells were left untreated or treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5ug/mL) with or without sAJ2 (3:1 bacteria: NKs) for 12h – 18h. Afterwards, the cells were fixed with 2% formaldehyde and used to treat MIA PaCa-2 for 4 days. Addition of anti-IFN-γmAb (1:100) alone, anti-TNF-α mAb (1:100) alone, or both anti-IFN-γ mAb and anti-TNF-α mAb were added to MIA PaCA-2 treated supernatants from NK treated IL-2 and anti-CD16mAb andsJA2. Thereafter, the MIA PaCa-2 was detached from the tissue culture plates and their sensitivity to NK cell-mediated lysis was evaluated using 51-Chromium release assay. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of Mia Capa-2.

# Fig.40

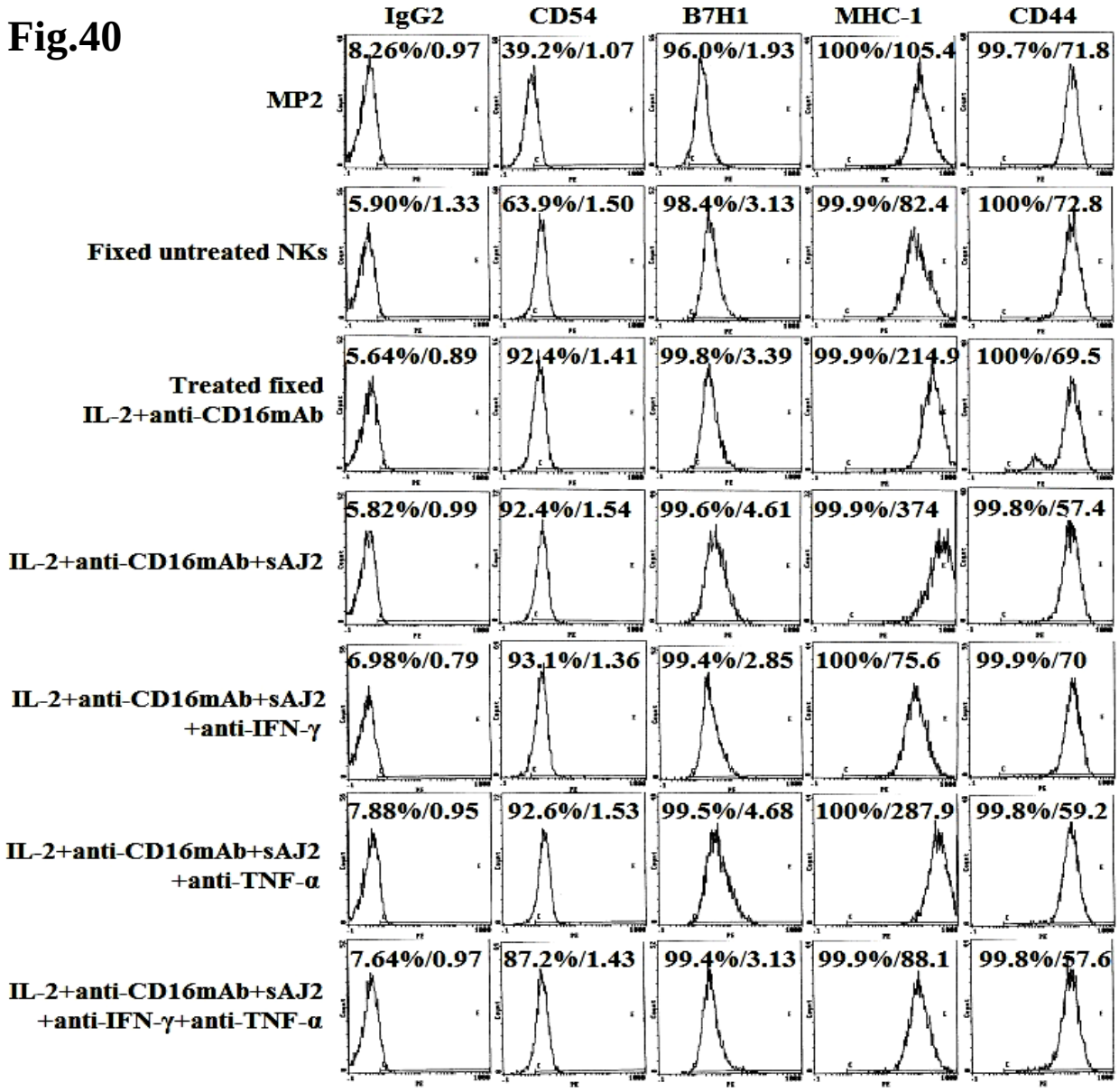



**Figure 40: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited modulation of surface receptor expressions on MIA PaCa-2 when treated with paraformaldehyde fixed IL-2+anti-CD16mAb+sAJ2 treated NK cells.**

MIA PaCa-2 was treated with paraformaldehyde fixed NK cells as described in Figure 39. Thereafter, the MIA PaCa-2 were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against isotype control, CD54, MHC-1, B7H1, and CD44 and the level of surface expression were determined by flow cytometric analysis.

# Fig. 41

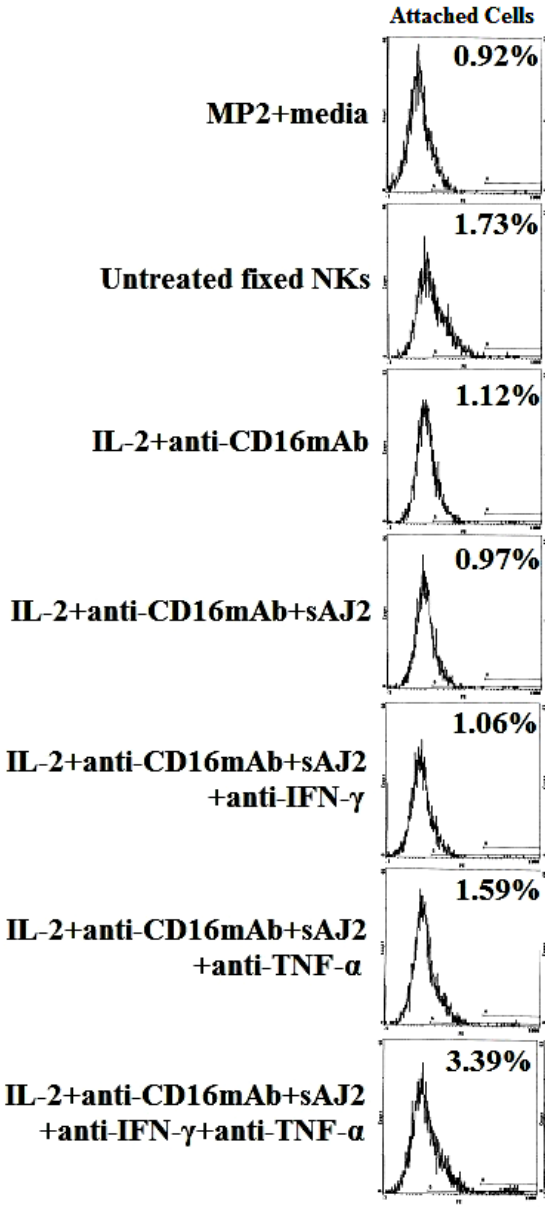



**Figure 41: Paraformaldehyde fixed NK cells treated IL-2 +anti-CD16mAb +sAJ2 did not induce significant cell death in MIA PaCa-2.**

MIA PaCa-2 was treated with paraformaldehyde fixed NK cells as described in Figure 39. Thereafter, the treat MIA PaCa-2 were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using Propidium Iodide (100ng/mL) in 400µl PBS and the level of cell death were determined by flow cytometric analysis.

**Fig. 42**

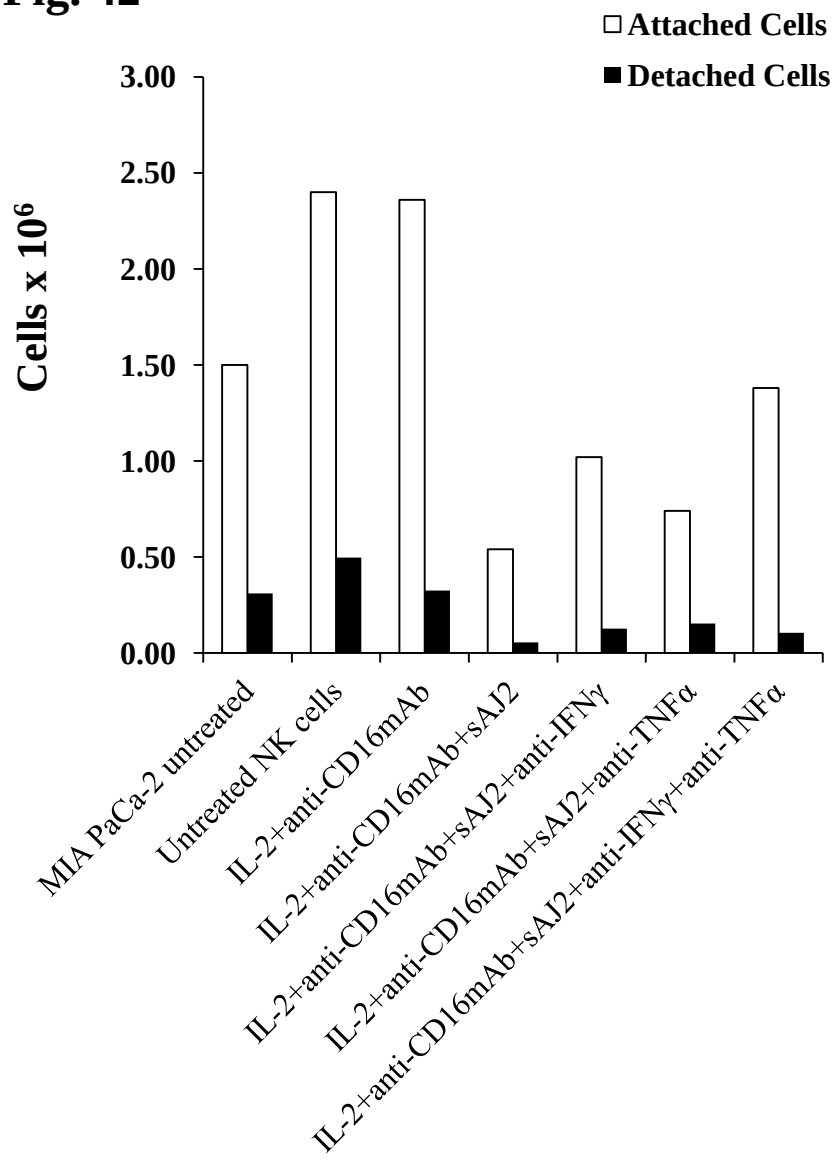



**Figure 42: Paraformaldehyde fixed NK treated IL-2 with CD16mAb and sAJ2 inhibited tumor growth in MIA PaCa-2.**

MIA PaCa-2 was treated with paraformaldehyde fixed NK cells as described in Figure 39. Thereafter, the non-adherence cells from the media of MIA PaCa-2 were collected and the adherence cells were detached from the tissue culture plates and were counted using Hemocytometer.

# Fig.43

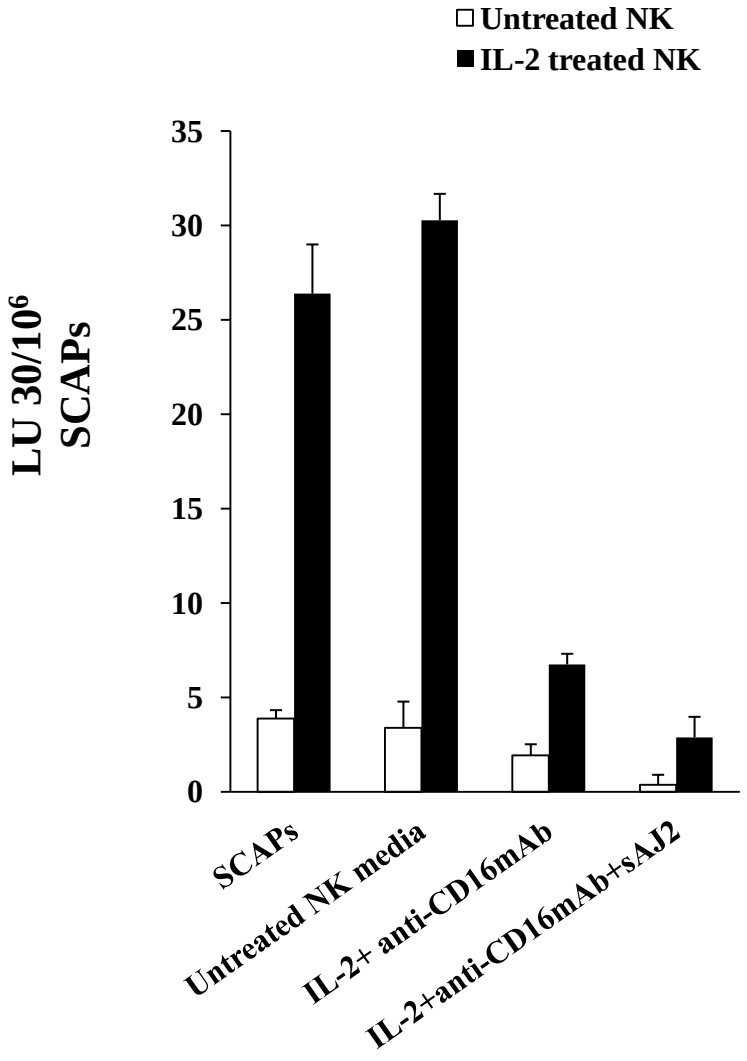
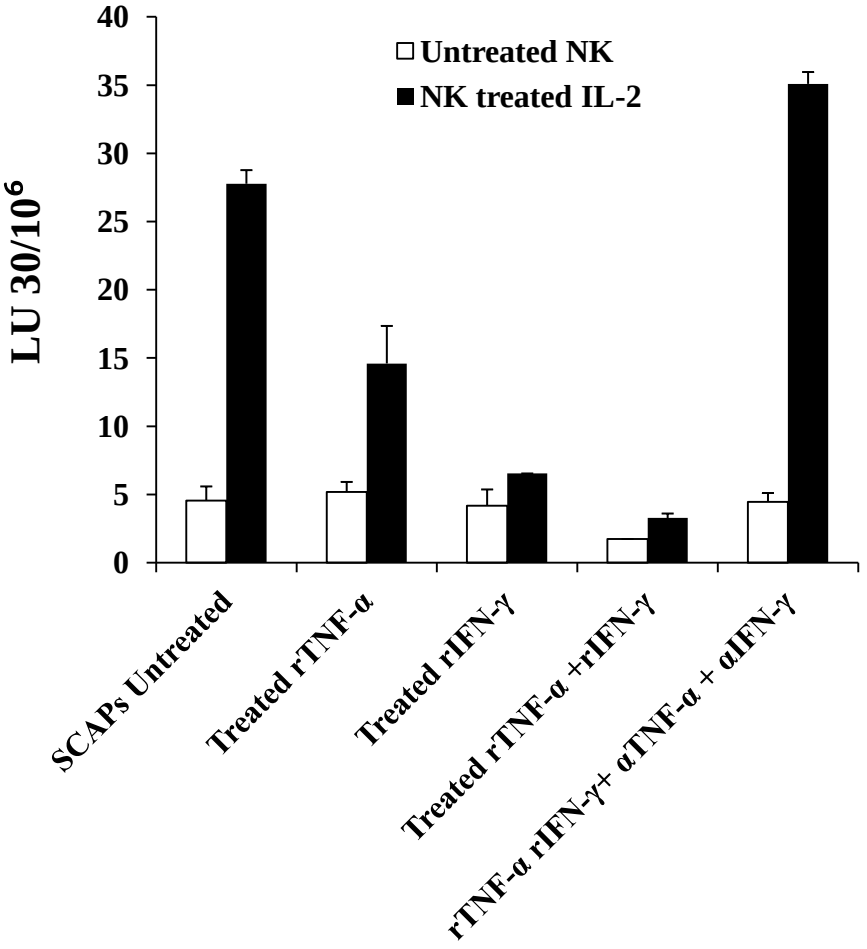



**Figure 43: Supernatant from NK cells treated IL-2 in combination with anti-CD16mAb and sAJ2 were able to induce the highest resistance of SCAPs against NK cell-mediated cytotoxicity.**

NK cells were left untreated or treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5μg/mL) with or without sAJ2 (3:1 sAJ2: NKs) for 12h – 18h. Afterwards, the supernatants from each NK sample were harvested and treated SCAPs passage 5 for 4 days. Thereafter, the SCAPs were detached from the tissue culture plates and their sensitivity to NK cell-mediated lysis was evaluated using 51-Chromium release assay. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of SCAPs.

**Fig. 44**

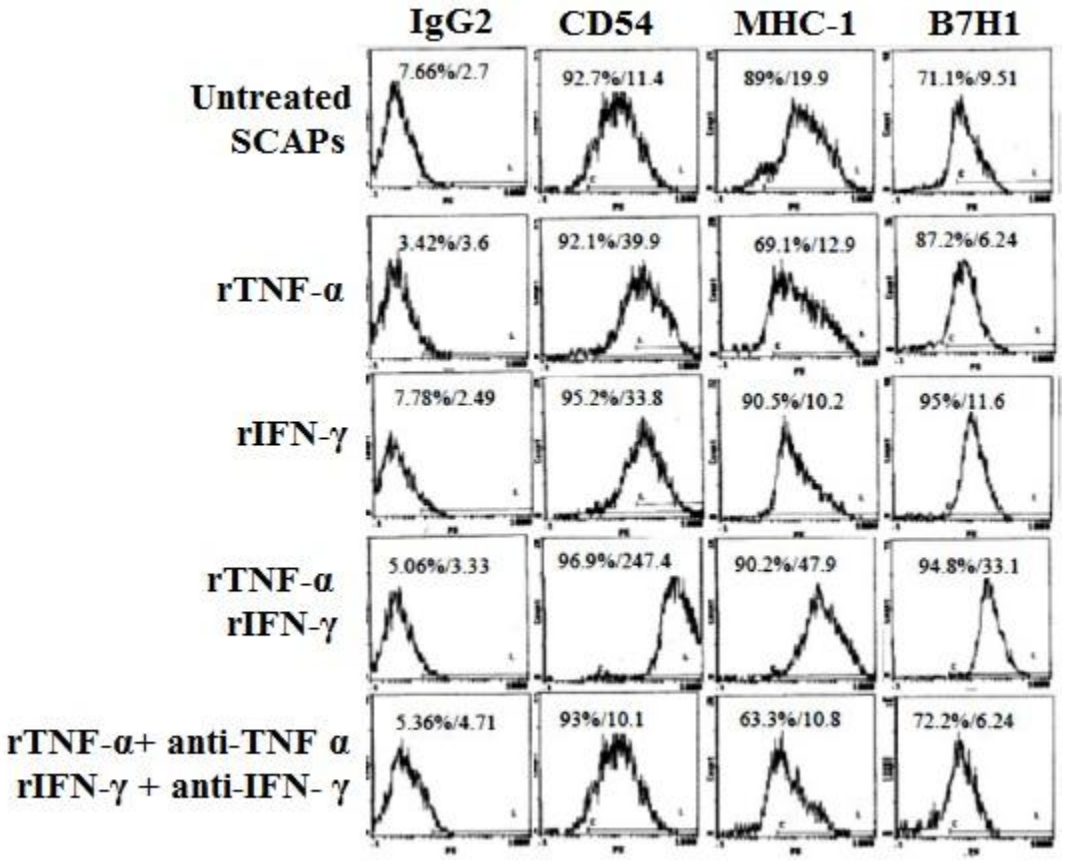



Experiment from Celis, A.

**Figure 44: Combination of recombinant TNF-α and recombinant IFN-γ synergistically induced resistance of SCAPs against NK cell-mediated cytotoxicity. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited resistance of SCAPs against NK cell mediated cytotoxicity.**

SCAP cells were treated with 20ng/mL recombinant TNF-α, 200U/mL recombinant IFN-γ and combination of both, or 20ng/mL rTNF-α+ 200U/mL rIFN-γ+ anti-TNF α(1:100)+anti-IFN- γ (1:100) for 24 hours. NK cells were untreated and treated with IL-2 (1000U/mL) overnight before they were used to measure NK cell cytotoxicity against $^{51}$Cr labeled SCAP cells. After 4 hours of incubation of NK cells with SCAP radioactivity released in the supernatant were read 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 SCAPs.

# Fig. 45




Experiment from Celis, A.

**Figure 45: Combination of recombinant TNF-α and recombinant IFN-γ synergistically induced modulation of surface receptor expressions on SCAPs. Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited modulation of surface receptor expressions on SCAPs.**

SCAP cells were treated with 20ng/mL recombinant TNF-α, 200U/mL recombinant IFN-γ and combination of both, or 20ng/mL rTNF-α+ 200U/mL rIFN-γ+ anti-TNF α(1:100)+anti-IFN- γ (1:100) for 24 hours. Thereafter, the SCAPs were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against isotype control, CD54, MHC-1, B7H1 and CD44 and the level of surface expression were determined by flow cytometric analysis.

**Fig. 46**

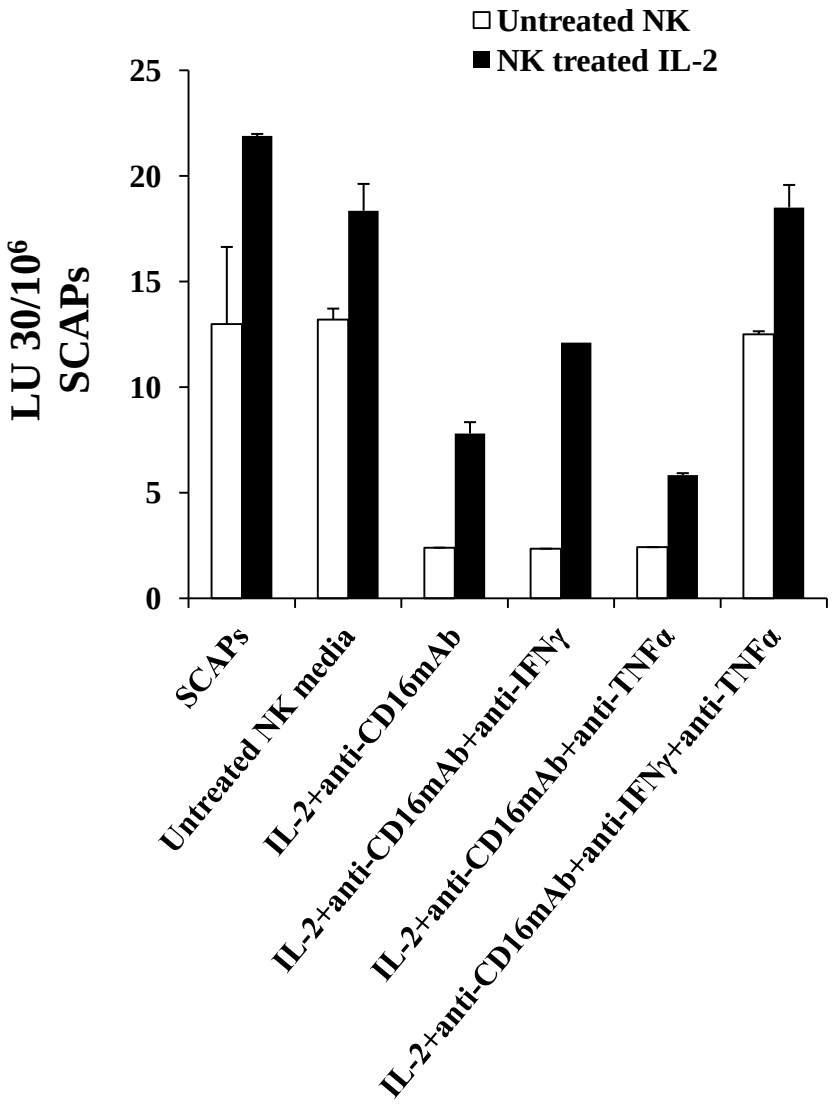



**Figure 46: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited modulation of surface receptor expressions on SCAPs when treated with supernatant from IL-2 and anti-CD16 treated NK cells.**

NK cells were left untreated or treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5ug/mL) for 12h – 18h. Afterward, the supernatants from each NK sample were harvested and then treated SCAPs passage 5 for 5 days. Addition of anti-IFN-γmAb (1:100) alone, anti-TNF-α mAb(1:100) alone, and both anti-IFN-γ mAb+anti-TNF-α mAb were added to SCAPs on the same day before the start of treatments. Thereafter, the SCAPs were detached from the tissue culture plates and their sensitivity to NK cell-mediated lysis was evaluated using 51-Chromium release assay. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of SCAPs.

# Fig. 47

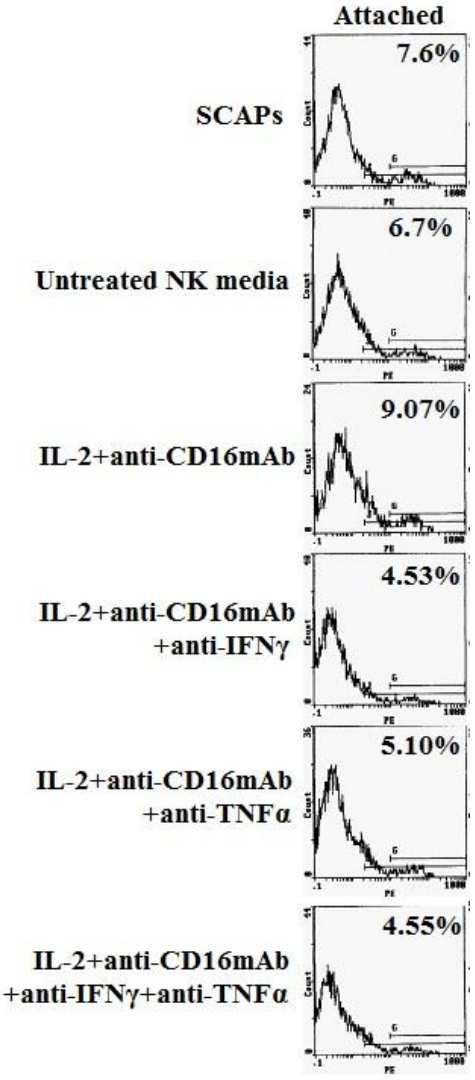



**Figure 47: Supernatants from NK cells treated IL-2 and anti-CD16mAb did not induce significant cell death on SCAPs.**

SCAP passage 5 was treated with supernatants from various NK cells treatments as described in Figure 46. Thereafter, adherence cells were detached from the tissue culture plates and the detached cells from the media treated SCAPs were counted using Hematocytomer and $5x10^4$ cells from each treatment were stained using Propidium Iodide (100ng/mL) in 400µl PBS. The level of cell death was determined by flow cytometric analysis.

**Fig. 48**

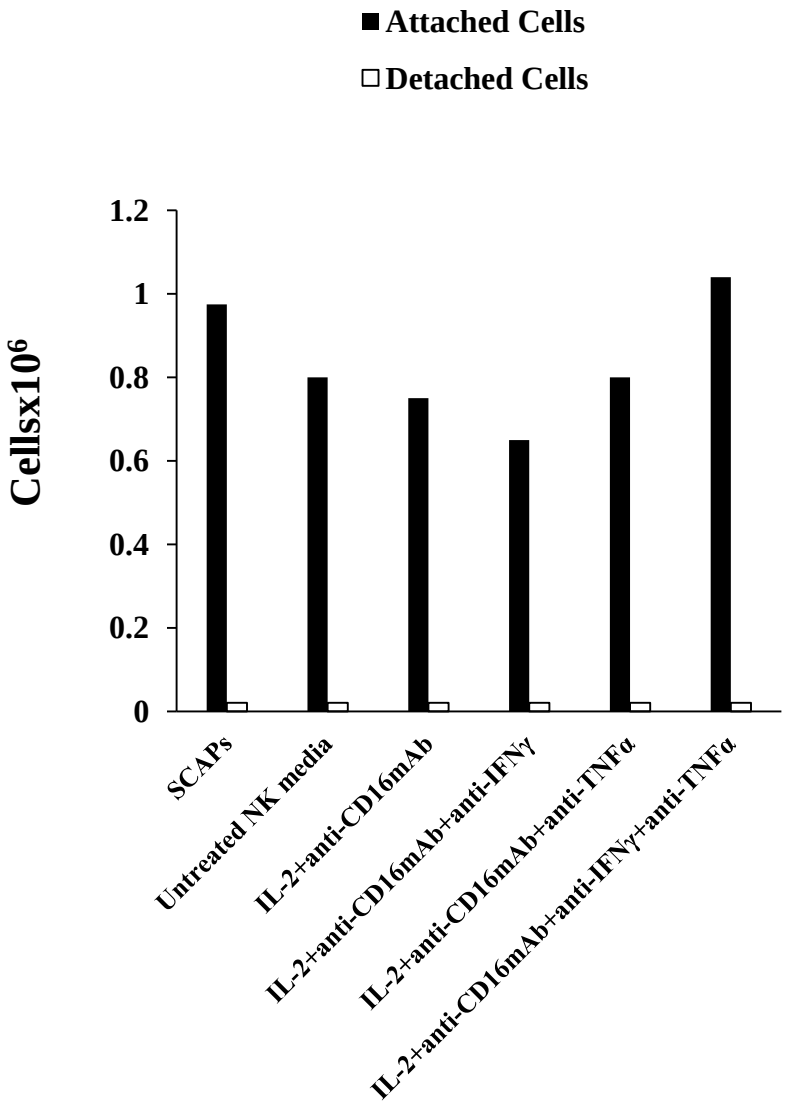



**Figure 48: Supernatants from NK cells treated IL-2 and anti-CD16mAb inhibited tumor growth of SCAPs.**

SCAP passage 5 was treated with supernatants from various NK cells treatments as described in Figure 46. Thereafter, the SCAPs were detached from the tissue culture plates and were counted using a Hemocytometer.

**Fig. 49**

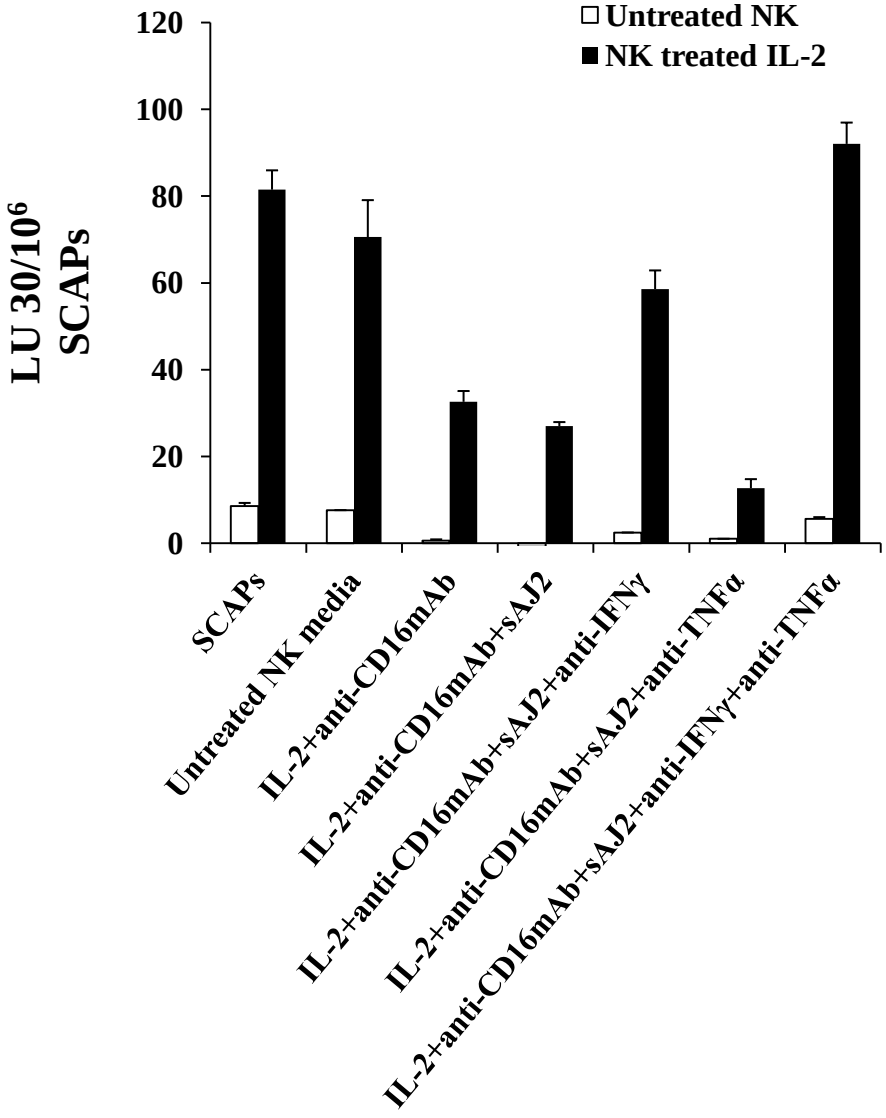



**Figure 49: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited modulation of surface receptor expressions on SCAPs when treated with supernatants from IL-2+anti-CD16+sAJ2 treated NK cells.**

NK cells were left untreated or treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5ug/mL) with or without sAJ2 at concentration 3:1 (sAJ2: NKs) for 12h – 18h. Afterward, the supernatants from each NK sample were harvested and then treated SCAPs passage 4 for 5 days. Addition of anti-IFN-γmAb (1:100) alone, anti-TNF-α mAb (1:100) alone, or both anti-IFN-γ mAb+anti-TNF-α mAb were added to SCAPs on the same day before the start of treatments. Thereafter, the SCAPs were detached from the tissue culture plates and their sensitivity to IL-2 treated NK cell-mediated lysis was evaluated using 51-Chromium release assay. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of SCAPs.

**Fig. 50**

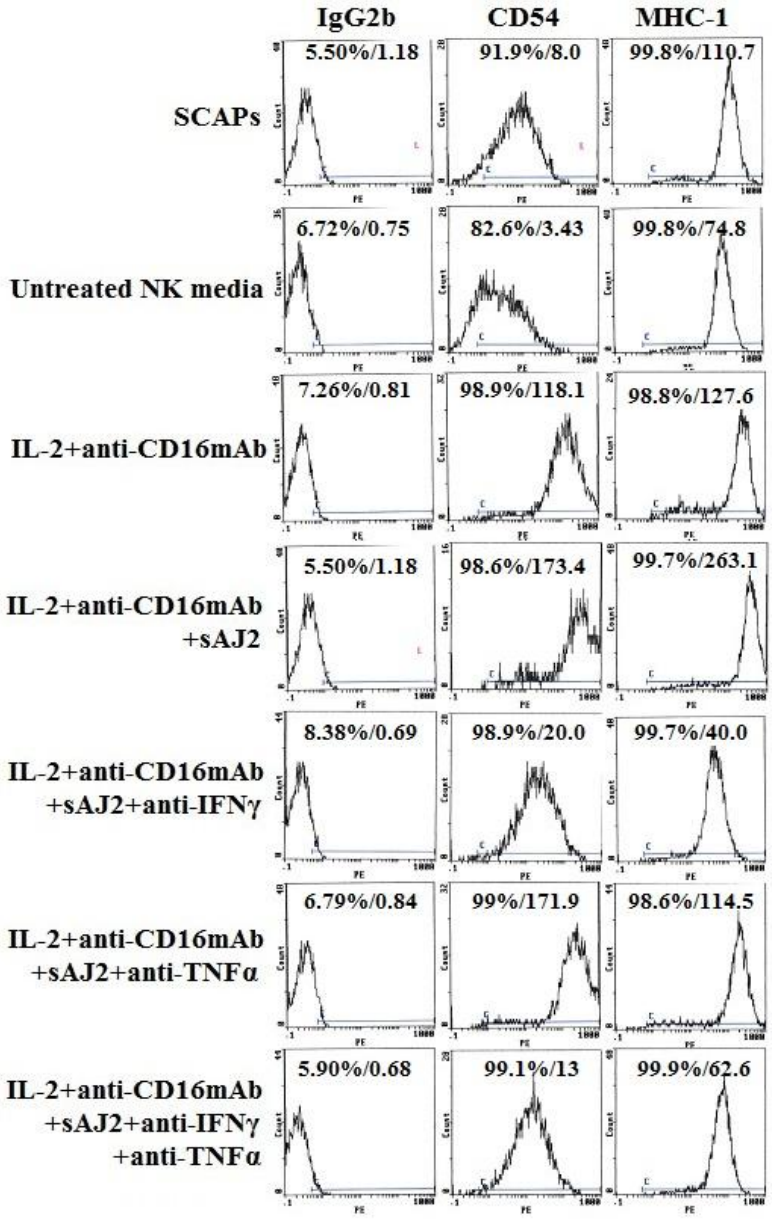



**Figure 50: Combination of anti-IFN-γ and anti-TNF-α antibodies inhibited modulation of surface receptor expressions on SCAPs when treated with supernatants from IL-2+anti-CD16mAb+sAJ2 treated NK cells.**

SCAP passage 4 was treated with supernatants from various NK cells treatments as described in Figure 49. Thereafter, the SCAPs were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against isotype control, CD54 and MHC-1, and the level of surface expression were determined by flow cytometric analysis.

**Fig. 51**

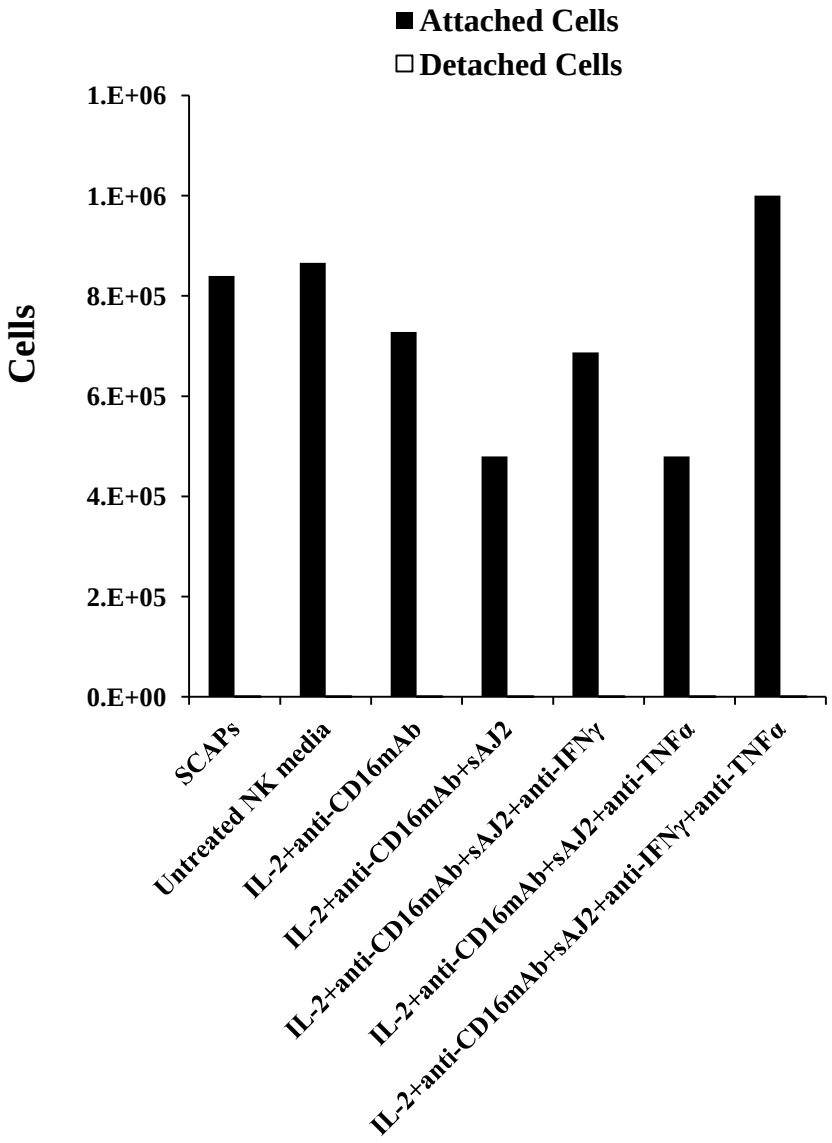



**Figure 51: Supernatants from NK cells treated IL-2 with anti-CD16mAb and sAJ2 inhibited tumor growth of SCAPs.**

SCAP passage 4 was treated with supernatants from various NK cells treatments as described in Figure 49. Thereafter, the SCAPs were detached from the tissue culture plates and were counted using a Hemocytometer.

**DISCUSSION:**

A tumor environment exerts an immunosuppressive effect that could result in the elimination of NK cells[53]. In cancer patients, majority of NK cells have lost cytotoxicity, they may eventually contribute rather than halt the progression of cancer by allowing the growth and expansion of the pool of cancer stem cells. We believe that conditioning of NK cells through key surface receptors and cytokines, differential targeting of stem cells, and differentiated cells by the NK cells to drive differentiation and resistance of undifferentiated tumor and healthy stem cells might provide anti-inflammatory signals resulting in the resolution of inflammation and regeneration of the tissues[60].

Productions of NK IFN gamma and TNF alpha are functionally linked to their cytolytic activities[40]. Our experimental results demonstrated that the degree of differentiation of stem cells by the NK cells correlated with the amount of IFN-γ being produced by the NK cells. The supernatants from the NK cells treated IL-2 plus anti-CD16mAb contained high amount of IFN IFN-γ compared to untreated NK cells; as a result, they were able to induce significant resistance as seen in all three cell lines: OSCSCs, MIA PaCa-2 and SCAPs. IFN-γ was shown to upregulate MHC-1 and downregulate NKG2D ligand expressions in certain tumor cells, thereby rendering them less susceptible to NK lysis[89]. We studied the mechanism that caused differentiation and saw significant up-regulation of surface markers MHC-1, CD54, and B7H1 and down-regulation of CD44, a stem cell marker. Furthermore, addition of probiotic bacteria enhanced IFN-γ production capability on the NK cells; therefore, they caused the NK cells to differentiate stem cells much faster, above that induced by IL-2 plus anti-CD16mAb. Probiotic bacteria also induced significantly high modulation of surface expressions of MHC-1, CD54, B7H1, and CD44 on OSCSCs, MIA PaCa-2, and SCAPs.

The supernatants or paraformaldehyde fixed cells from NK treated IL-2 and anti-CD16mAb with or without probiotic bacteria induced resistance of OSCSCs, MIA PaCa-2 and SCAPs against the NK cells-mediated cytotoxicity. However, bacteria caused significantly more resistance of the stem cells. As stem cells became more resistance, they also exhibited high modulation of surface expressions of CD54, MHC-1, B7H1, and CD44 on OSCSCs, MIA PaCa-2 and SCAPs. IFN-γ and TNF-α are known to upregulate ICAM-1 expression in tumor cell lines[88]. They are pro-inflammatory cytokines that are highly induced in the inflammatory environment. Since NK cells produced high level of IFN-γ and TNF-α and cause resistance of OSCSCs, MIA PaCa-2, and SCAPs, we want to understand if the antibodies against these cytokines will inhibit resistance of OSCSCs, MIA PaCa-2, and SCAPs. The addition of anti-IFN-γ antibody alone or anti- TNF-α antibody alone did not inhibit resistance of OSCSCs, MIA PaCa-2 and SCAPs. However, anti- IFN-γ was observed to play a major role in inhibiting resistance of OSCSCs, MIA PaCa-2 and SCAPs against NK cells mediated cytotoxicity while anti- TNF-α antibody was observed to play a minor role in inhibiting resistance of OSCSCs, MIA PaCa-2 and SCAPs against NK cells mediated cytotoxicity. Nevertheless, combination of both antibodies worked synergistically to inhibit resistance and modulation of surface expression on OSCSCs, MIA PaCa-2 and SCAPs. Jewett et al stated that the failure of cytokines alone to induce the target cell lysis illustrated a synergistic nature between the cytokine production and lytic function of NK cells [61].

Differentiation of OSCSCs and MIA PaCa-2 produced a moderate number of detached cells while differentiation of SCAPs produced low number of detached cells. To be conditioned to drive differentiation, NK cells may have to first receive signals either from healthy stem cells or those which have disturbed or defective capabilities to differentiate[61]. Supernatants or

paraformaldehyde fixed NK cells treated IL-2 and anti-CD16mAb with or without sAJ2 did not induce significant cell deaths in OSCSCs, MIA PaCa-2, or SCAPs. It was observed that as the stem cell became more differentiated, their growth rates slow down.

## CONCLUSION:

In this study, we have demonstrated that anergized NK cells do not contribute to the significant lysis of stem cells, but instead support in the differentiation and resistance against NK cells mediated lysis. We demonstrated that probiotic bacteria induced significant split anergy in NK cells by their lack of increased in cytotoxicity but triggered significant amount of pro-inflammatory and anti-inflammatory cytokines such as IFN-γ and TNFα, thus, mediated differentiation of both transformed stem cells, OSCSCs and MIA PaCa-2, and healthy stem cells, SCAPs and resulted in greater resistance to NK cells mediated cytotoxicity. Supernatants harvested from NK cells treated IL-2 and anti-CD16mAb induced differentiation of OSCSCs, MIA PaCa-2, and SCAPs. Furthermore, the presence of probiotic bacteria was able to accelerate the rate of differentiation of OSCSCs, MIA PaCa-2, and SCAPs when sAJ2 were added to IL-2 and anti-CD16mAb treated NK cells. These levels of differentiation were accessed by using $^{51}$Chromium release assay as target cells became more differentiated they became less susceptible to NK mediated cytotoxicity. We also showed that the mechanism of differentiation of OSCSCs, MIA PaCa-2, and SCAPs by up-regulation of surface expressions of CD54, MHC-1, and B7H1 and down-regulation of surface expression of CD44. We have shown that the two key cytokines, TNF-α and IFN-γ were responsible for differentiation and maturation of OSCSCs, MIA PaCa-2, and SCAPs and that both antibodies much treated together to synergistically inhibited resistance of the stem cells and not when induced by each anti-TNF-α or anti-IFN-γ antibody alone. Paraformaldehyde fixed NK cells treated IL-2+anti-CD16mAb+sAJ2 also caused maturation, differentiation, and resistance of OSCSCs and MIA PaCa-2. Thus, the function of probiotic bacteria was to induce significant split anergy by significantly support the

maturation process of the stem cells and caused resistance of the stem cells against NK cells-mediated cytotoxicity.

## CHAPTER 3:

***Specific Aim 3:*** Investigate the function of IL-10 in balanced regulation of cellular differentiation and resistance of tumors

## INTRODUCTION:

Interleukin (IL)-10 is a cytokine produced by T-helper (Th)-2 cells, natural killer (NK) cells, B-cells and macrophages, which promotes anti-inflammatory responses. IL-10 suppressed the production of pro-inflammatory cytokines such as IL-12 and IL-18 from macrophages, and thus counteracted the production of pro-inflammatory cytokines and lower inflammatory response[90]. IL-10 originated mainly from Kupffer cells in the liver, had a powerful immunosuppressive effect through its actions on macrophages [91, 92]. Scott, M.J., et al showed that NK cells of mice treated with anti-IL10 neutralizing antibody caused an increase in IFN-γ production on the NK cells[93]. IL-10 inhibited the production of IFN-γ in NK cells stimulated by IL-2[94] and it also inhibited the maturation of dendritic cells from monocytes precursors[95]. In addition, studies have shown that IL-10 had an inhibitory activity on the synthesis of IFN-γ by human and mouse NK cells [96-98]. IL-10 had the ability to decrease production of both TNF-α and IL-12 by macrophages which were important for production of IFN-γ by NK cells [98-100].

Monocytes similar to anti-CD16mAb induced split anergy in NK cell resulted in the protection of human Mesenchymal Stem Cells (hMSCs) and human dental pulp stem cells (hDPSCs) from NK cell-mediated lyses while substantially increased the secretion of IFN-γ [1, 59]. Monocytes also protected OSCSCs against NK cell-mediated lyses while substantially

increased secretion of IFN-γ by the NK cells (manuscript in prep). We hypothesized that blocking IL-10 on NK cells triggered by probiotic bacteria (sAJ2) and monocytes significantly up-regulated the production of IFN-γ on NK cells, exacerbated the maturation and differentiation process of OSCSC and X02D cell lines, and instigated resistance against NK cell-mediated cytotoxicity.

NK-92 was a highly cytotoxic NK cell line established from a patient with malignant lymphoma[101]. It required the presence of IL-2 for continuous growth and similar to LAK cells with respect to surface receptor expression and functional abilities[101]. NK-92 lacked CD16 and does not display antibody dependent cell-mediated cytotoxic (ADCC) activity. Our lab has shown that NK-92 secreted a high level of IL-10 compared to IFN-γ and mediated a low level of cytotoxicity against stem cells.

In previous studies conducted by our laboratory, we have demonstrated that the stage of differentiation of the cells is predictive of their sensitivity to NK cell lysis[1]. Thus, the more differentiated the cell line, the more resistant the cell line is to NK cell-mediated lysis.

In this final section of the study, there are **seven** sub aims to demonstrate by *in vitro* assays to assess the role of IL-10, monocytes and probiotic bacteria on NK cells and the modulation of IFN-γ and differentiation of OSCSC and X02D.

***Sub Aim 1*** Addition of anti-IL10mAb to different treated NK cells did not affect NK cells mediated cytotoxicity against OSCSCs.

***Sub Aim 2:*** Monocytes inhibited NK cells cytotoxic function induced significant amount of cytokines and IL-10.

***Sub Aim 3:*** Supernatants from the co-culture of NK cells with monocytes treated with IL-2+ anti-CD16mAb in the presence of anti-IL10mAb induced resistance and modulation of surface expression of CD54 and MHC-1 on OSCSCs.

***Sub Aim 4*****:** IL-10 inhibits IFN-g secretion in the co-cultures of NK cells treated IL-2 and anti-CD16mAb with monocytes and probiotic bacteria.

***Sub Aim 5:*** Supernatants from the co-culture of NK cells treated IL-2 and anti-CD16mAb or untreated NK cells with monocytes and probiotic bacteria in the presence of anti-IL10mAb induced resistance and modulation of surface expressions of CD54, B7H1, MHC-1, and CD44 on OSCSCs.

***Sub Aim 6:*** Supernatants obtained from the co-culture of NK cells with monocytes treated with sAJ2 in the presence of anti-IL10mAb significantly increased CD54 surface receptors of XO2D.

***Sub Aim 7:*** Loss of NK-92 cell-mediated cytotoxicity against OSCSCs and gain in the secretion of cytokine. Addition of anti-IL10mAb to NK-92 suppressed the production of IL-10 but did not induce production of IFN-γ.

## MATERIALS AND METHODS

### *A. Cell Culture and Reagents*

**Primary Human Oral Squamous Cancer Stem Cells (OSCSCs)**

Oral Squamous Cancer Stem Cells (OSCSCs) were isolated from patients at the Department of Head and Neck Surgery at UCLA school of Medicine by Dr. Christian Head. The cells were cultured in RPMI Medium 1640 with L-Glumamine (Cat# 11875-093) supplemented 10% Fetal Bovine Serum (FBS) (Cat# 900-108, Gemini Bio-Products, CA), 1.4% Antibiotic-Antimycotic (100X) (Cat# 15240-062), 1% Sodium Pyruvate (100nM) (Cat# 11360-070), 1.4% MEM Non-Essential Amino Acids (Cat# 11140-050), 0.2% Gentamicin Sulfate (Cat# 400-108, Gemini Bio-Products, CA) and 0.15% Sodium Bicarbonate (Cat# S233-500, Fisher Scientific, CA). The remainder products were purchased from Gibco by Life Technologies (Carlsbad, CA).

**Primary Human Glioblastoma (X02D)**

The primary human glioblastoma were obtained from Dr. Noriyuki Kasahara and Dr. Akihito Inagki from the Department of Digestive Diseases and Gastroenterology, David Geffen School of Medicine at UCLA. The cells were cultured in Dulbecco modified Eagle Medium (DMEM) containing 4.5g/L D-Glucose and L-Glutamine (Cat# 11965-092) and supplemented with 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA), 1% Antibiotic-Antimycotic (100X) (Cat# 15240-062), 1% MEM Non-Essential Amino Acids (Cat# 11140-050), 1% Sodium

Pyruvate (100nM) (Cat# 11360-070). The remainder products were purchased from Gibco by Life Technologies (Carlsbad, CA).

**Natural Killer Cells (NK-92)**

NK-92 is derived from peripheral blood mononuclear cells. The cells were cultured in Alpha Minimum Essential medium without ribonucleosides and deoxyribonucleosides but with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate supplemented with 0.2 mM inositol; 0.1 mM 2-mercaptoethanol; 0.02 mM folic acid; 100-200 U/ml recombinant IL-2; 10% horse serum and 10% Fetal Bovine Serum (FBS) (Cat# 900-108, Gemini Bio-Products, CA), 1% MEM Non-Essential Amino Acids (Cat# 11140-050), 1% Sodium Pyruvate (100nM) (Cat# 11360-070) and 1% Penicillin-Streptomycin (Cat# 15140-122).

*B.* *Antibodies*

a. Recombinant IL-2 was obtained from the NIH repository.
b. PE anti-human CD16 Antibody (Cat#302007), PE anti-human CD54 Antibody (Cat# 353106), PE anti-human CD44 Antibody (Cat#33807), PE anti-human CD274 (B7-H1, PD-L1) Antibody (Cat#329706), PE Mouse IgG2a, k Isotype Control Antibody (Cat# 400212), PE anti-human HLA-A, B, C Antibody (Cat#311406) were purchased from Biolegend (San Diego, CA)
c. The Human NK cell Enrichment Kit (Cat# 19055) was purchased from Stem Cell Technologies (Vancouver, Canada).
d. LEAF ™ Purified anti-human IL-10 Antibody (Clone: JES#-19F1) was purchased from BioLegend (San Diego, CA)

e. The Human Monocyte Enrichment Kit without CD16 Depletion (Cat# 19058) was purchased from Stem Cell Technologies (Vancouver, Canada).

f. Propidium Iodide Powder (Cat# P4170) were purchased from Sigma-Aldrich (St. Louis, MO)

g. AJ2 was mixtures of eight probiotic bacteria strains formulated by Dr. Anahid Jewett. Each tablet contained 6.28 billion freeze-dried bacteria (*Streptococcus thermophilus, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillusp Plantarum, Lactobacillus casei, and Lactobacillus bulgaricus*).

*C. <u>Purification of Natural Killer Cells and Peripheral Blood Mononuclear Cells</u>*

Human peripheral blood was obtained from healthy donors as per guidelines of the UCLA Human Subject Protection Committee. Written informed consents approved by UCLA-IRB were obtained from all healthy blood donors. The blood was collected through syringed contained heparin and was centrifuged on Ficoll-Hypaque PM400 (Cat#17-0300-50; GE Healthcare, Piscataway, NJ). The buffy layer called Peripheral Blood Mononuclear Cells (PBMC) was harvested by density gradient centrifugation, washed, and resuspended in RPMI Medium 1640 supplemented with 10% FBS. Non-adherent human peripheral blood lymphocytes (PBL) were obtained after 1-2 hours incubation at 37˚C on a plastic 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 greater than 90% purity based on flow cytometric analysis of anti-CD16 antibody stained NK cells.

The adherence subpopulation of PMBCs was detached from the tissue culture plate after 1-2 hours incubation in 37˚C. The human monocytes were negatively selected and isolated using EasySep® Human Monocyte Enrichment kit without CD16 Depletion purchased from Stem Cell Technologies (Vancouver, Canada). The population was found to have greater than 90% purity based on flow cytometric analysis of anti-CD14 monoclonal antibody stained monocytes.

Purified NK cells were cultured in RPMI Medium 1640 supplemented with 10% FBS (Gemini Bio-Products, CA), 1% Sodium Pyruvate (100nM) (Cat# 11360-070), 1% MEM Non-Essential Amino Acids (Cat# 11140-050), and 1% Antibiotic-Antimycotic(100X) (Cat# 15240-062) (Gibco by Life Technologies, CA).

*D. NK Cell $^{51}$Chromium Release Cytotoxicity Assay*

A standard Chromium Release assay was used to assess the cytolytic function of Natural Killer Cells against the sensitivity of the target cells. The effector cells ($1x10^5$cells/well) were aliquoted into 96-well round bottom microwell plates (Thermo Scientific$^{TM}$ Nunc$^{TM}$, Hudson, NH) and were titrated from four to six serial dilutions. The target cells ($5x10^5$) were labeled with 50μCi Sodium $^{51}$Cr (Perkin Elmer, Shelton, CT) chromate for 1 hour, washed with medium specific for the target cells to remove access $^{51}$Cr that were not labeled, counted the cells using Hematocytometer, and then washed again to remove access un-bound $^{51}$Cr. The $^{51}$Cr-labeled target cells were aliquoted into 96-well round-bottom micro well plates with the effectors cells at a concentration of $1x10^4$ cells/well to obtain an E:T (effector:target) ratio. The total release contained cell pellets and supernatant were collected and measured while the experimental and spontaneous release samples were centrifuged and incubated for 4 hours at 37˚C in 5%$CO_2$.

Afterwards, each supernatant was harvest from each sample and the chromium release from lysed target cells was measured using a gamma counter. The percentage of specific chromium release of each well was calculated using the following formula.

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

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.

*E. Enzyme-Linked Immunosorbent Assays (ELISAs)*

Enzyme-Linked Immunosorbent Assays (ELISAs) were performed on the supernatants harvested after co-cultured experiments to measure the concentration level of cytokines, chemokines, and growth factors secreted by the NK Cells. Single ELISAs were performed based on the manufactured protocol. Briefly, the 96-well EIA/RIA plates were coated with 100uL of Capture Antibody (1:200) corresponding to the target cytokine and incubated overnight at 4˚C. After 16 hours incubation, the plate was washed 4 times with Wash Buffer (PBS +0.05% Tween-20) and blocked with 200uL Assay Diluent (1%BSA in PBS) for 1 hour. Thereafter, the plates were washed 4 times and 100uL of supernatant collected from experiments and standard dilution were added. After 2 hours, the plates were washed 4 times. Then, 100ul of Detection Antibody (1:200) were added and incubated for 1 hour. Afterward, the plates were washed 4 times and 100uL of Avidin-HRP solution (1:1000) were added and incubated for 30 minutes. Finally, the plates were washed 5 times and 100ul of TMB Substrate Solution were added and incubated in the dark until the wells developed a desired blue color before 100ul of Stop Solution were added to stop the reaction. The plates were read at 450nm to obtain absorbance value. To analyze and

obtain the cytokine concentration, a standard curve was generated through 7 serial dilutions of recombinant cytokines.

*F.* *Surface Staining*

The cells were detached from the tissue culture plates and washed with cold PBS containing 1%BSA. Pre-determined concentration of specific monoclonal antibodies were added to $5x10^4$ cells in 50uL and incubated at 4˚C for 30 minutes. The cells were then washed and re-suspended in PBS containing 1%BSA. The EPICS C (Coulter) flow cytometry was used for analysis and cell sorting.

*G.* *Propidium Iodide stain (PI)*

The cells were detached from the tissue culture plates and washed with cold PBS containing 1%BSA. About 100ng/mL of PI stain were added to $5x10^4$ cells in 50uL and re-suspend with PBS. The EPICS C (Coulter) flow cytometry was used for analysis and cell sorting. Cells undergoing apoptosis will be detected by measurement of the red fluorescence, indicating the uptake of Propidium Iodide of the gated conjugate population.

H. *Bacteria Sonication*

AJ2 were combination of eight different strains of probiotic bacteria formulated by Dr. Jewett. The whole bacteria was weighted and resuspended in RPMI Medium 1640 Complete containing 10% FBS at a concentration of 10mg per 1mL. The bacteria were vortexed

thoroughly and then sonicated for 15 seconds while placing on ice and the Amplitude were set from 6 to 8. After that, the samples were rested for 30 seconds on ice. After every 5 pulses, a small sample was taken to observe under the microscope to obtain at least 80 percentages of bacteria cell walls lyses. Afterward, the sonicated samples were then aliquoted and stored in minus 20 to 80 degrees for long term studies.

*I. Differentiation of OSCSCs and XO2D with anergized Natural Killer Cells Supernatant*

After purification of the Natural Killer Cells, the cells were treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5µg/mL) and in combination with sAJ2 at a ratio of 2:1 (sAJ2: NKs) and/or anti-IL10mAb (10µg/mL) and/or Monocytes (1 Monocytes: 2 NKs). The cells were then resuspended with RPMI Medium Complete at 1 million NK cells per Milliliter. After 12-18 hours of incubation in 37˚C, the supernatants from each NK treatment were harvested by centrifugation and then treated 6-well culture plates of OSCSCs or XO2D each day for 2-5 days. The amount of supernatants added to the culture plates each day is determined by several factors: growth rate of the cells, size of the cells, number of non-adherence and adherence cells. Afterward, the OSCSCs or XO2D were detached from the tissue culture plates by using 0.25% Trypsin-EDTA (1X) (Gibco by Life Technologies, CA) and the sensitivity of the treated cells was evaluated using $^{51}$Chromium Release Assay.

## RESULTS:

### Sub Aim 1: Addition of anti-IL10mAb to different treated NK cells did not affect NK cells mediated cytotoxicity against OSCSCs.

As shown in Figure 52 and 53, untreated NK cells mediated low cytotoxicity and the level remained unchanged in the presence of probiotic bacteria or probiotic bacteria and anti-IL10mAb. IL-2 activated NK cells mediated high level of cytotoxicity and similarly the level remained unchanged in the presence of probiotic bacteria or probiotic bacteria plus anti-IL10mAb. IL-2 activated NK cells treated with anti-CD16mAb (anergized NK) induced split anergy by suppressing NK cytotoxic function; the level remained unchanged in the presence of probiotic bacteria or probiotic bacteria and anti-IL10mAb. Similarly we did not see an increase in the level of IFN-γ when anti-IL10mAb was added into untreated NK, NK treated IL-2 or NK treated IL-2 and anti-CD16mAb (Figure 54A). However, anti-IL10mAb blocked the production of IL-10 on NK cells triggered by probiotic bacteria (Figure 54B).

### Sub Aim 2: Monocytes inhibited NK cells cytotoxic function induced significant amount of cytokines and IL-10.

The addition of anti-IL10mAb to untreated monocytes, treated with IL-2, or treated with IL-2 and anti-CD16mAb did not induce secretion of IFN-γ (Figure 55) similar to NK treated cells. Probiotics bacteria also did not induce secretion of IFN-γ on monocytes (Figure 55). Monocytes alone did not induce NK cells to secrete IFN-γ; however monocytes induced anergized NK cells to secrete significant amounts of IFN-γ after 106 hours (Figure 56 A-B). In Figure 57C, untreated monocytes secreted a large amount of IL-10 compared to untreated NK

cells and when monocytes treated IL-2 and anti-CD16mAb the level of IL-10 increased. Monocytes co-cultured with anergized NK cells synergistically induced IL-10 production (Figure 56C). When we looked at cytotoxic function of untreated monocytes, or monocytes treated IL-2, or monocytes treated IL-2 and anti-CD16mAb, we saw that they did not mediate cytotoxicity against OSCSCs or SCAP (Figure 57). However, monocytes co-cultured with NK cells caused a loss in NK cell-mediated cytotoxicity against OSCSCs and SCAPs (Figure 57).

**Sub Aim 3: Supernatants from the co-culture of NK cells with monocytes treated with IL-2+ anti-CD16mAb in the presence of anti-IL10mAb induced resistance and modulation of surface expression of CD54 and MHC-1 on OSCSCs.**

We have previously demonstrated that differentiation of healthy as well as transformed stem cells corrected with resistance from NK cells lyses and up-regulation of MHC-1, CD54, and B7H1 and down regulation of CD44. As shown in Figure 58, monocytes and monocytes+anti-IL10mAb did not induce differentiation of OSCSCs. The surface expressions of CD54, CD44, and B7H1 were similar to untreated OSCSCs with slightly higher but not a significant increase in MHC-1 surface expression. In addition, NK treated monocytes supernatant with or without anti-IL10mAb did not induce differentiation of OSCSCs (Figure 58) and CD44, B7H1, and CD54 were similar to untreated OSCSCs as well, with slight increase in MHC-1 but not as significant (Figure 58). When monocytes are activated with IL-2 and anti-CD16mAb with or without anti-IL10mAb and then treated OSCSCs, we observed that the CD54 and MHC-1 surface expressions were similar to untreated OSCSCs (Figure 59). Supernatants from NK treated IL-2 and anti-CD16mAb with or without anti-IL10mAb induced the same level of differentiation of OSCSCs with an increase of CD54 and MHC-1 (Figure 59) and resistance to NK-mediated cytotoxicity (Figure 60). When monocytes are co-cultured with anergized NK cells with or without anti-

IL10mAb and treated OSCSCs, the surface expression of CD54 and MHC-1 increased significantly without anti-IL10mAb and significantly resisted NK-mediated lysis (Figure 60) and with anti-IL10mAb increased CD54 and MHC-1 substantially (Figure 59) and resisted NK-mediated lysis to a significant level (Figure 60).

Treatment of OSCSCs with anergized NK and monocytes supernatant induced 26% cell deaths in attached OSCSCs and 67% cell deaths in detached OSCSCs compared to 2% cell deaths in attached and 27% cell deaths in detached OSCSCs of untreated OSCSCs (Figure 61). The addition of anti-IL10mAb did not significantly induce cell deaths in OSCSCs.

There was low number of detached cells and high number of attached cells in differentiation of OSCSCs (Figure 62).

IFN-γ ELISA showed that untreated monocytes or monocytes treated anti-IL10mAb or monocytes treated IL-2 and anti-CD16mAb with or without anti-IL10mAb did not induce secretion at day 1 to day 8 (Figure 63). NK treated IL-2 and anti-CD16mAb with or without anti-IL10mAb secreted similar level of 860pg/mL at day 1 and decreased drastically at day 4 (Figure 63). Monocytes co-cultured with NK treated IL-2 and anti-CD16mAb with or without anti-IL10mAb had the highest production of IFN-γ between 1200pg/mL to 1300pg/mL. The level remained high at day 4, decreased slightly at day 6, and significantly dropped at Day 8 (Figure 63). Figure 63 showed that the conditions that caused resistance of stem cells secreted the most IFN-γ.

**Sub Aim 4: IL-10 inhibits IFN-g secretion in the co-cultures of NK cells treated IL-2 and anti-CD16mAb with monocytes and probiotic bacteria.**

Figure 64 showed that monocytes treated IL-2 or treated IL-2 and anti-CD16mAb did not mediate cytotoxicity against OSCSCs. The level of monocytes mediated cytotoxicity did not change in the presence of sAJ2. Untreated NK cells moderately mediated cytotoxicity against OSCSCs and when treated with IL-2 increased the level of cytotoxicity significantly. Addition of anti-CD16mAb to NK cell treated IL-2 inhibited lysis against OSCSCs. When monocytes were added to NK cells, the level of cytotoxicity against OSCSCs decreased. Addition of sAJ2 to monocytes and NK cells was observed to have the lowest cytotoxicity level (Figure 64). In contrast, the treatments of NK cells with monocytes secreted high amount of IFN-γ compared to NK cells without treatment of monocytes (Figure 65 A). More induction of IFN-γ was observed when probiotic bacteria sAJ2 were added into the co-cultured of monocytes and NK cells treated IL-2 and anti-CD16mAb (Figure 65A). The addition of anti-IL10mAb to the co-culture of monocytes and NK cells treated IL-2 with antiCD16mAb and sAJ2 increased production of IFN-γ (Figure 65B). Monocytes or NK treated monocytes did not produce much IL-10; however the addition of sAJ2 to monocytes or NK treated monocytes secreted significant amount of IL-10 (Figure 66).

**Sub Aim 5: Supernatants from the co-culture of NK cells treated IL-2 and anti-CD16mAb or untreated NK cells with monocytes and probiotic bacteria in the presence of anti-IL10mAb induced resistance and modulation of surface expressions of CD54, B7H1, MHC-1, and CD44 on OSCSCs.**

NK cells were pretreated with different spilt anergy inducers as described in Figure 63. The experiment was co-cultured for 8 days and the supernatants were collected at 1 day, 4 days, 6 days, and 8 days to observe the ability of NK cells to secrete IFN-γ. As shown in Figure 67,

NK treated IL-2 and anti-CD16mAb produced 800pg/mL of IFN-γ and the addition of anti-IL10mAb to NK treated IL-2 and anti-CD16mAb also induced similar level of IFN-γ in NK cells (Figure 67). When the NK cells were treated with monocytes and sAJ2, a high induction of IFN-γ was observed and the addition of anti-IL10mAb to monocytes treated NK cells exacerbated IFN-γ secretion. The level of IFN-γ decreased after 4 days in NK treated IL-2 and anti-CD16mAb and NK treated IL-2 and anti-CD16mAb and anti-IL10mAb; however, the level of IFN-γ secretion remained high in NK cells treated monocytes with or without anti-IL10mAb (Figure 67). After 6 days, level of IFN-γ decreased in NK cells treated IL-2 + CD16mAb + sAJ2, but not in NK cells treated IL-2 + CD16mAb + sAJ2 + anti-IL10mAb while any treatment of NK with monocytes remained high on 6 days. On 8 days, the levels of IFN-γ decreased in all conditions (Figure 67).

The level of IFN-γ secretion correlated with the level of differentiation of OSCSCs. OSCSCs cells treated with supernatants from NK cells sAJ2 and anti-IL10mAb had similar sensitivity levels as untreated OSCSCs (Figure 68). Supernatant from NK treated IL-2+anti-CD16mAb+sAJ2 induced significant resistance against NK mediated cytotoxicity and addition of anti-IL10mAb caused further resistance (Figure 68). Supernatant from NK treated monocytes and sAJ2 also induced significant resistance against NK mediated cytotoxicity and significantly more when anti-IL10mAb was added (Figure 68). Supernatant from NK treated IL-2+anti-CD16mAb+sAJ2+monocytes and addition of anti-IL10mAb had the highest resistance against NK cells mediated cytotoxicity (Figure 68).

The expression levels of CD54, B7H1, MHC Class 1 and CD44 of OSCSCs treated supernatant from NK treated sAJ2 and anti-IL10mAb was similar to untreated OSCSCs (Figure 69). There were moderate increases in surface expression of CD54, B7H1, and MHC Class 1 and

a moderate decrease in CD44 observed in OSCSCs treated with supernatants from NK treated IL-2+anti-CD16mAb plus sAJ2. The expression levels of CD54, B7H1 and MHC-1 increased further with the addition of anti-IL10mAb (Figure 69). The OSCSCs treated supernatant from NK+sAJ2+monocytes had a moderate increases in CD54, B7H1, and MHC Class 1 and a moderate decrease in CD44 compared to untreated OSCSCs, but the level is slightly lower than OSCSCs treated with supernatants from NK treated IL-2+anti-CD16mAb plus sAJ2. However, OSCSCs treated supernatant from NKs+sAJ2+Monocytes+anti-IL10mAb had significant increases in CD54, B7H1, and MHC Class 1 and the CD54 and B7H1 expressions were higher compared to OSCSCs treated with supernatants from NK treated IL-2+anti-CD16mAb plus sAJ2 and anti-IL10mAb (Figure 69). But the highest surface expressions of CD54, MHC Class 1, and B7H1 were observed in OSCSCs treated with supernatants obtained from combination of IL-2, anti-CD16mAb, sAJ2, monocytes and anti-IL10mAb treated NK cells, while the surface expression CD44 had the greatest decrease compared to all treatments (Figure 69).

Two conditions that were shown to induce the most cell deaths in OSCSCs were supernatant from anergized NK cells treated sAJ2 and monocytes with or without anti-IL10mAb. The level of cell deaths in attached cells were 11% and 19% observed in the most differentiated conditions while the less differentiated conditions induced less than 3% cell deaths in attached cells (Figure 70). Anergized NK cells treated sAJ2 and monocytes with or without anti-IL10mAb induced 60% to 70% cell deaths in detached cells compared to untreated OSCSCs (Figure 70).

Figure 71 showed that OSCSCs treated with supernatant from anergized NK treated sAJ2 and monocytes without or with anti-IL10mAb had the lowest number of attached cells and highest number of detached cells.

**Sub Aim 6: Supernatants obtained from the co-culture of NK cells with monocytes treated with sAJ2 in the presence of anti-IL10mAb significantly increased CD54 surface receptors of XO2D.**

We have demonstrated that supernatants from anergized NK cells treated monocytes, anti-IL10mAb induced differentiation in OSCSCs. We want to look at a differentiated cells type, X02D to see if conditioned NK cells also can differentiated X02D. As shown in Figure 72A, X02D treated supernatant from monocytes treated IL-2 and anti-CD16mAb with or without anti-IL10mAb were unable to differentiate and had similar surface expressions B7H1, CD54, MHC-1 and CD44 as untreated X02D. Supernatants from anergized NK and anergized NK treated anti-IL10mAb induced differentiation and significantly up-regulated CD54, MHC-1 while B7H1 was moderately up-regulated and CD44 was moderately down regulated (Figure 72A). Supernatants from monocytes treated anergized NK cells with and without anti-IL10mAb significantly induced differentiation and significantly up-regulated B7H1, CD54, MHC-1, and down regulated CD44. Supernatant from anergized NK cells induced moderate differentiation and anergized NK cells triggered sAJ2 significantly induced differentiation of X02D (Figure 72B) as determined by increased expression levels of B7H1, CD54, MHC-1, decreased expression levels of CD44. Supernatants from NK treated monocytes and sAJ2 also induced differentiation with or without anti-IL10mAb. Increased surface expression levels B7H1and CD54 and decreased surface expression level CD44 were observed in the presence of anti-IL10mAb treatment while the surface expression MHC-1 decreased slightly. The highest increase in B7H1, CD54, MHC-1 and highest decreased in CD44 was seen in supernatant from anergized NK cells treated monocytes and sAJ2 (Figure 72B).

Differentiation of X02D with supernatants from anergized NK cells treated monocytes, sAJ2, and anti-IL10mAb induced less than 5% cell deaths in attached cell and between 30%-

40% cell deaths in detached cells compared to 1% cell deaths in attached cells and 35%-45% cell deaths in detached cells from untreated X02D (Figure 73). In other words, supernatants from anergized NK cells treated monocytes and IL-10mAb and sAJ2 did not induce cell deaths in attached and detached cells of X02D (Figure 73). Differentiation of X02D produced few number of detached cells and a high number of attached cells. In addition, the most differentiated condition was shown to have the least number of attached in differentiation of X02D (Figure 74).

**Sub Aim 7: Loss of NK-92 cell-mediated cytotoxicity against OSCSCs and gain in the secretion of cytokine. Addition of anti-IL10mAb to NK-92 suppressed the production of IL-10 but did not induce production of IFN-γ.**

NK cells and NK-92 cells were left untreated, treated with IL-2, and treated with IL-2 and anti-CD16mAb with and without sAJ2. NK-92 mediated lower cytotoxicity against OSCSCs compared to primary NK cells and the level of cytotoxicity did not change when sAJ2 or anti-IL10R mAb or anti-IL10mAb was added (Figure 75).

Untreated NK-92 secreted a moderate amount of IL-10 compared to no secretion of IL-10 seen in untreated primary NK cells. NK-92 treated with IL-2 and IL-2 plus anti-CD16mAb secreted a high level of IL-10 compared to NK cells treated IL-2 plus sAJ2 or NK cells treated IL-2 and anti-CD16mAb plus sAJ2. Addition of anti-IL10mAb and anti-IL10RmAb blocked the production of IL-10 in NK-92 (Figure 76).

NK-92 cells secreted very low amounts of IFN-γ compared to primary NK cells and blocking IL-10 and IL-IL10R in NK-92 did not increase the secretion of IFN-γ (Figure 77).

**Fig. 52**

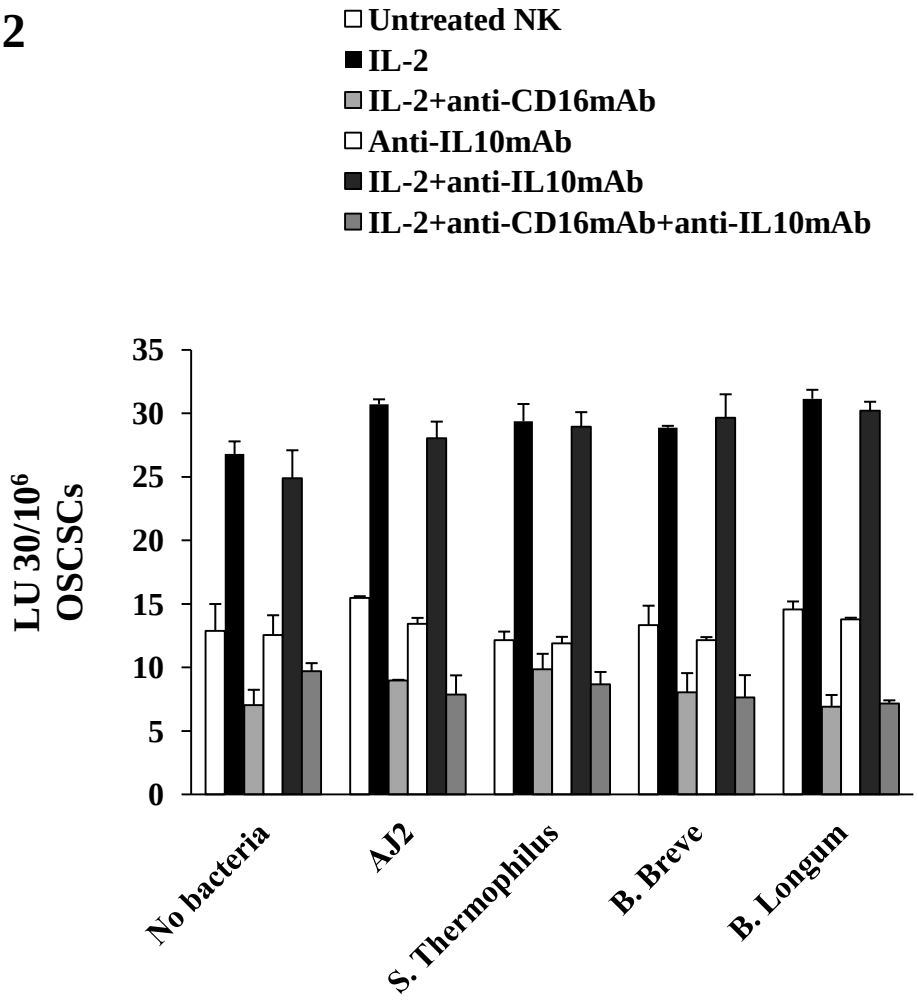



**Figure 52: Addition of anti-IL10mAb to different treated NK cells did not affect NK cells mediated cytotoxicity against OSCSCs.**

NK cells were left untreated, or treated with IL-2 (1000U/mL) or IL-2 (1000U/mL) + anti-CD16mAb (2.5µg/mL) and/or AJ2 (3:1 bacteria: NKs) in the presence and absence of anti-IL10mAb (10µg/mL) for 12-18 hours before they were added to $^{51}$Cr labeled OSCSCs. NK cell cytotoxicities were determined using a standard 4 hour $^{51}$Cr release assay. The NK cells with OSCSCs 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 OSCSCs.

# Fig. 53

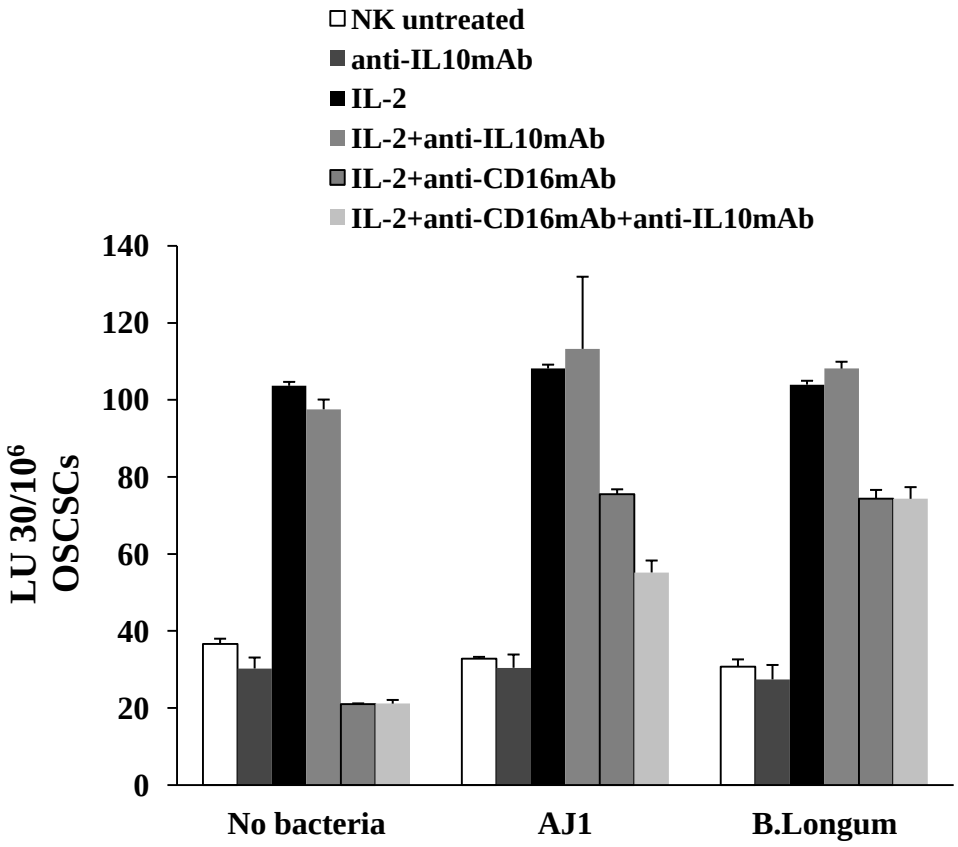



**Figure 53: Addition of anti-IL10mAb and AJ1 to NK cells did not affect NK cells mediated cytotoxicity against OSCSCs.**

NK cells were left untreated, or treated with IL-2 (1000U/mL) or IL-2 (1000U/mL) + anti-CD16mAb (2.5µg/mL) and/or AJ1 (3:1 bacteria: NKs) in the presence and absence of anti-IL10mAb (10µg/mL) for 12-18 hours before they were added to $^{51}$Cr labeled OSCSCs. NK cell cytotoxicities were determined using a standard 4 hour $^{51}$Cr release assay. The NK cells with OSCSCs 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 OSCSCs.

# Fig. 54

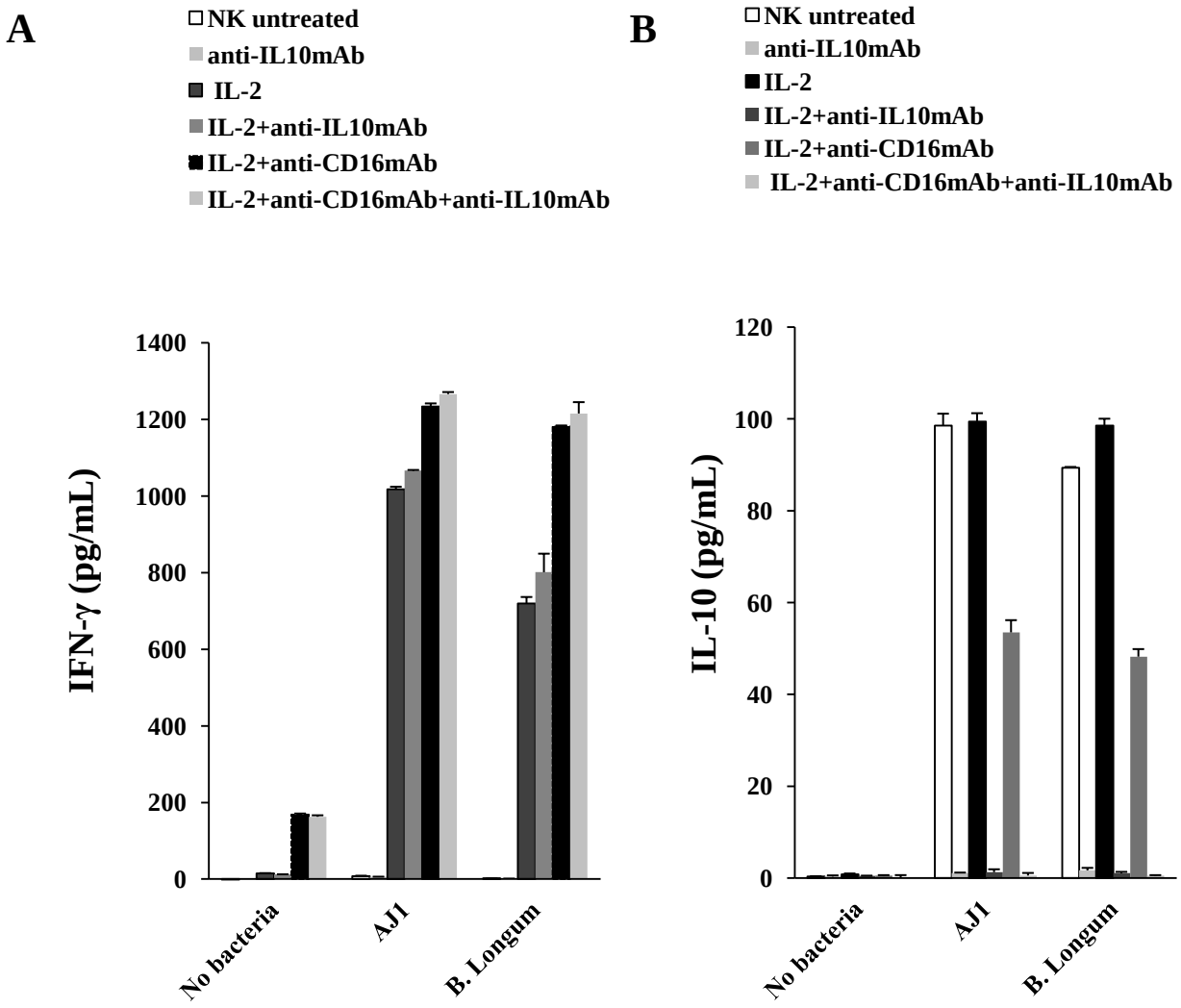



**Figure 54: Addition of anti-IL10mAb to NK cells inhibited secretion of IL10, but did not induce IFN-γ on NK cells in the presence to probiotic bacteria.**

NK cells were left untreated, or treated with IL-2 (1000U/mL) or IL-2 (1000U/mL) + anti-CD16mAb (2.5µg/mL) and/or AJ1 (3:1 bacteria: NKs) in the presence and absence of anti-IL10mAb (10µg/mL) for 12-18 hours before the supernatants were collected and the level of IFN-γ (A) and IL-10(B) secretion were determined using Human IFN-γ or IL-10 ELISA MAX Standard Sets.

**Fig. 55**

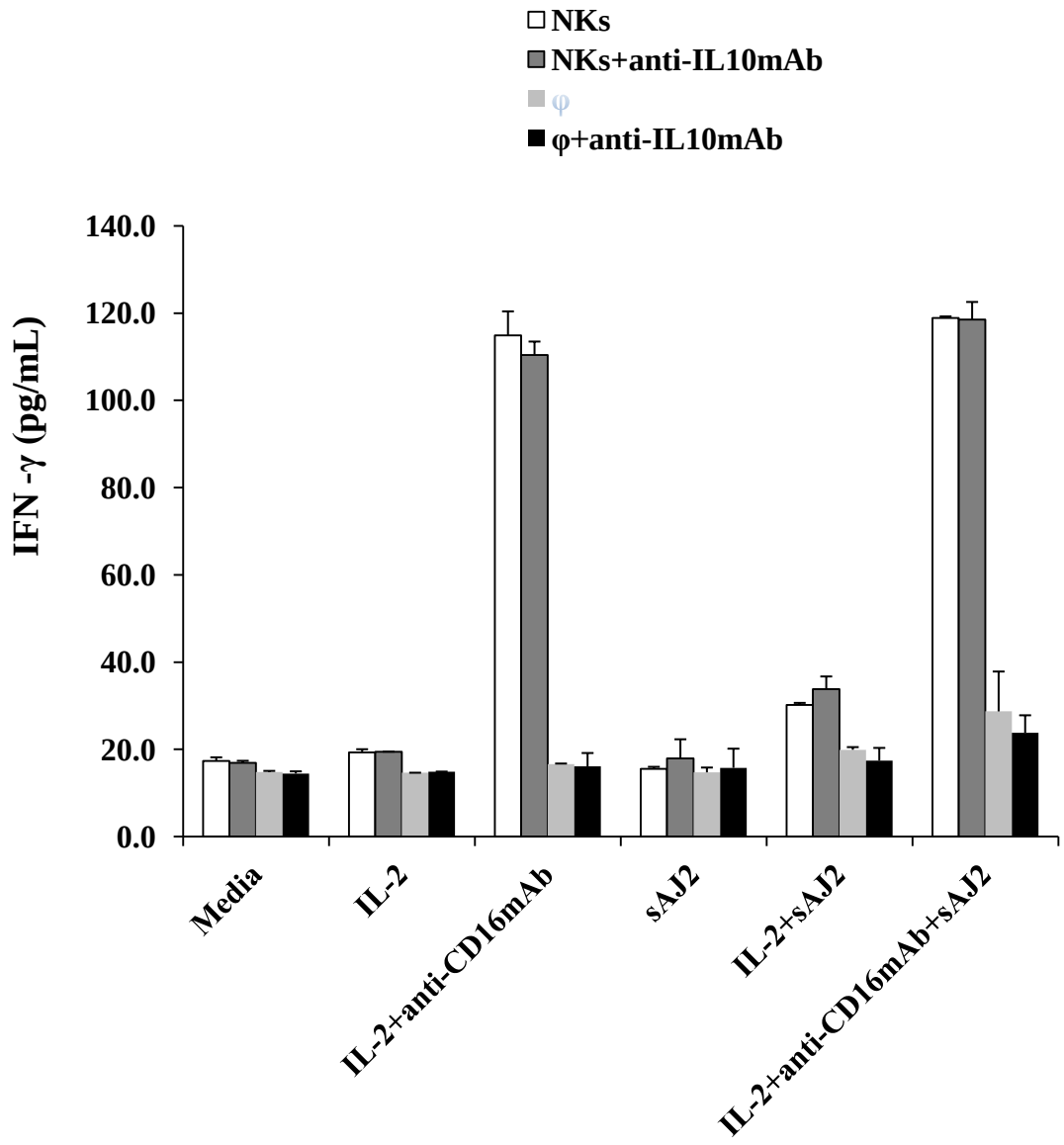
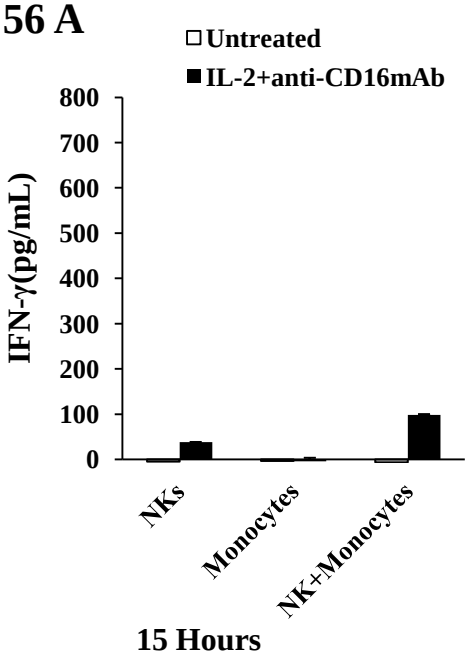


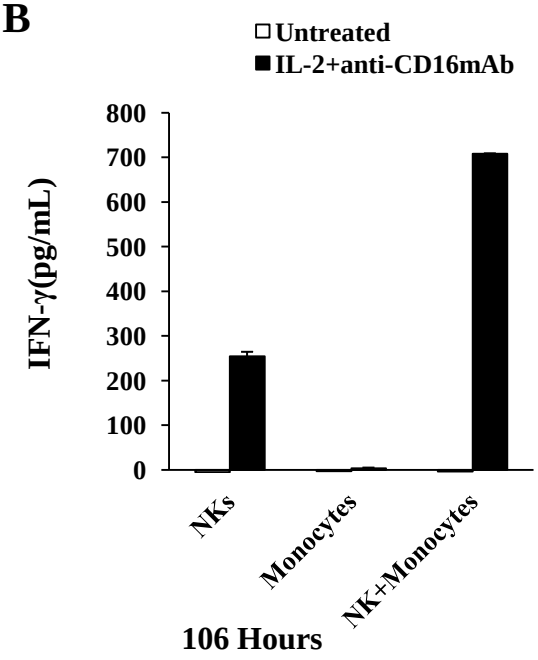

**Figure 55: Addition of anti-IL10mAb to monocytes triggered by AJ2 did not induce IFN-γ.**

NK and monocytes cells were left untreated, or treated with IL-2 (1000U/mL) or IL-2 (1000U/mL) + anti-CD16mAb (2.5µg/mL) and/or sAJ2 (2:1 bacteria: NKs) in the presence and absence of anti-IL10mAb (10µg/mL) for 12-18 hours before the supernatants were collected and the level of IFN-γ secretion were determined using Human IFN-γ ELISA MAX Standard Sets

**Fig. 56 A**




**B**




**C**

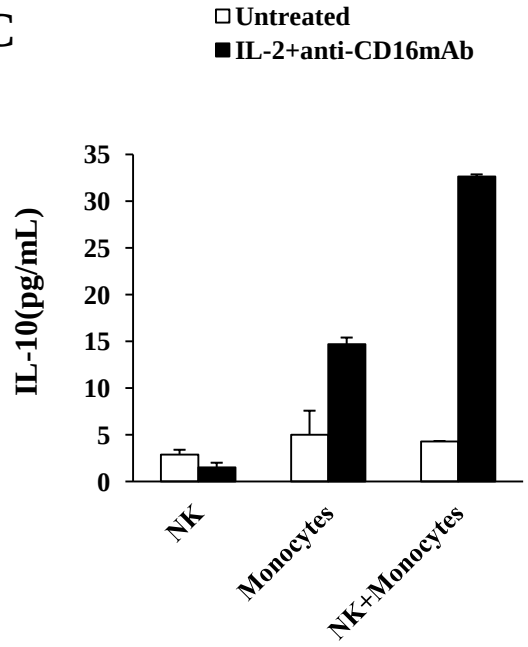



**Figure 56: Co-cultured of monocytes and NK cells treated IL-2 and anti-CD16mAb induced secretion of IFN-γ and IL-10.**

NK and Monocytes cells were left untreated and treated with IL-2 (1000U/mL) and anti-CD16mAb (2.5µg/mL) and in combination of NK and Monocytes (3NKs:1Monocytes) for 12-18 hours before the supernatants were collected and the level of IFN-γ (A-B) and IL-10 (C) secretion were determined using Human IFN-γ and IL-10 ELISA MAX Standard Sets

# Fig. 57

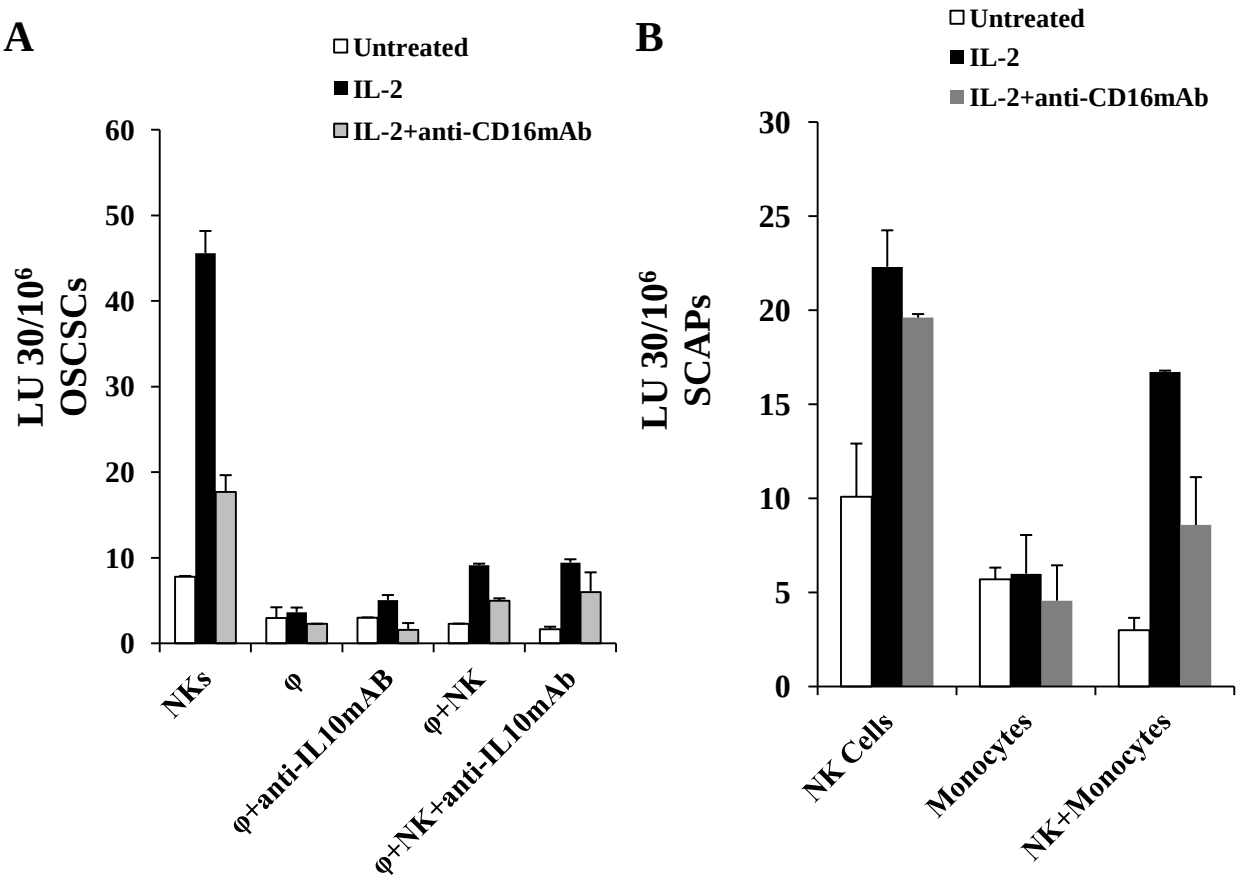



**Figure 57: Monocytes inhibited NK cells mediated cytotoxcity against OSCSCs and SCAPs.**

NK cells were left untreated, treated with IL-2 (1000U/mL), IL-2+anti-CD16mAb (2.5µg/mL) and combination of Monocytes and NKs (1:3 Monocytes: NKs) and/or anti-IL10mAb (10µg/mL) for 12-18 hours before they were added to $^{51}Cr$ labeled OSCSC cells. NK cell cytotoxicities were determined using a standard 4 hour $^{51}Cr$ release assay. The NK cells with OSCSCs (A) /SCAPs (B) 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 OSCSCs and SCAPs. The symbol φ represents monocytes.

# Fig. 58

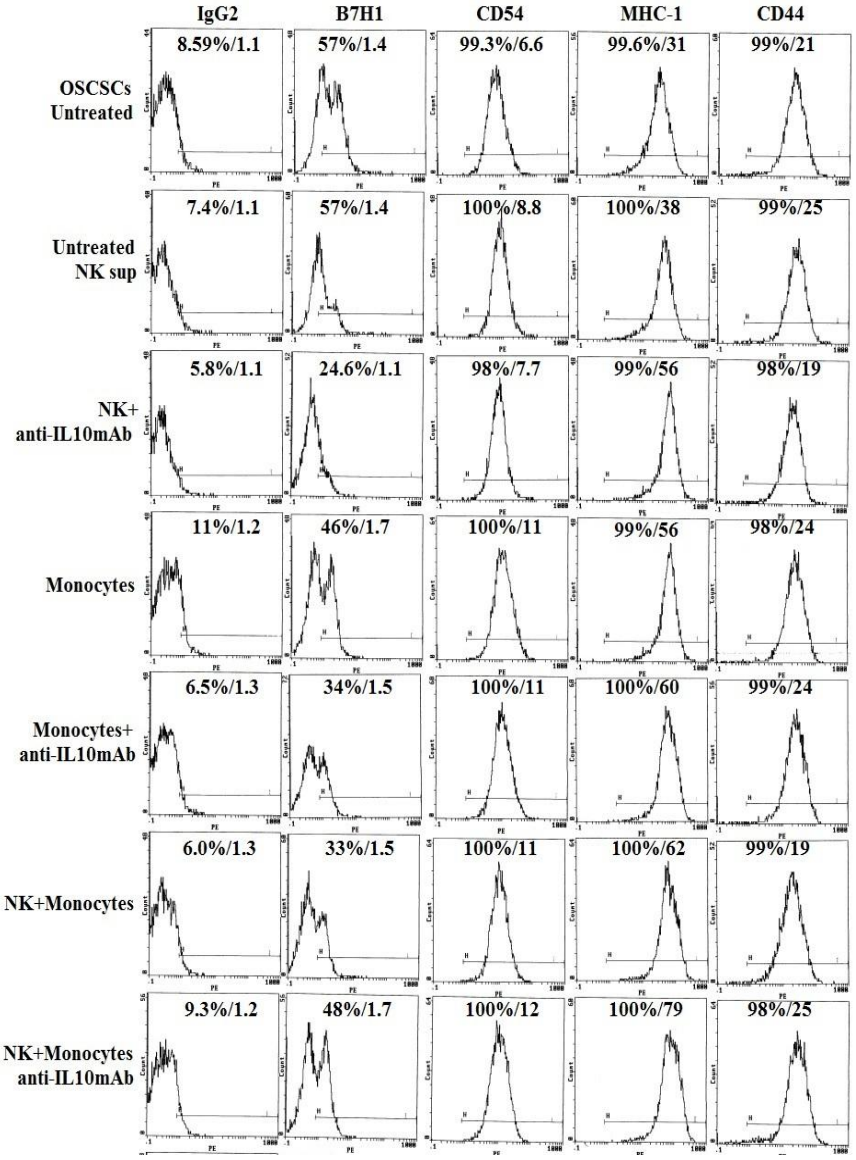



**Figure 58: Supernatants from untreated monocytes or monocytes cultured with untreated NK cells in the presence of anti-IL-10 antibody induced slight modulation of MHC-1 while no changes were seen for CD44, B7H1, and CD54 when compared to untreated OSCSCs.**

NK cells were treated with anti-IL10mAb (10μg/mL), monocytes (1:2 φ: NKs), or monocytes and sAJ2 (2:1 bacteria: NKs) or combination of monocytes, anti-IL10mAb and sAJ2 for 12-18 hours. The supernatant from each sample was collected and treated OSCSCs for 4 days. Afterwards, the OSCSCs were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against isotype control, CD54, MHC-1, B7H1, and CD44 and the level of surface expression were determined by flow cytometric analysis.

# Fig. 59

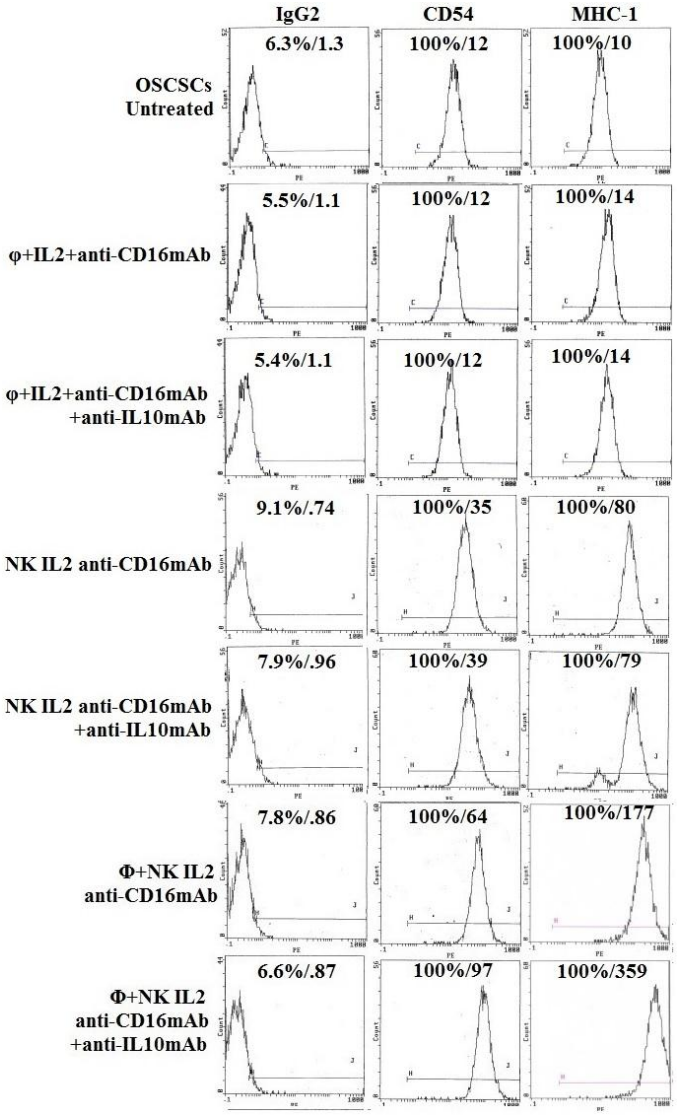



**Figure 59: Supernatants obtained from the co-culture of NK cells with monocytes treated with IL-2+ anti-CD16mAb in the presence of anti-IL10mAb significantly increased CD54 and MHC-1 surface receptors.**

NK cells were left untreated, treated with IL-2 (1000U/mL), IL-2+anti-CD16mAb (2.5µg/mL) and in combination of monocytes and NKs (1:3 Monocytes: NKs) and/or anti-IL10mAb (10µg/mL) for 12-18 hours. Afterwards, the supernatants from each NK sample were harvested and then treated OSCSCs for 3 days. Thereafter, the OSCSCs were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against isotype control, CD54, MHC-1 and the level of surface expression were determined by flow cytometric analysis.

# Fig. 60

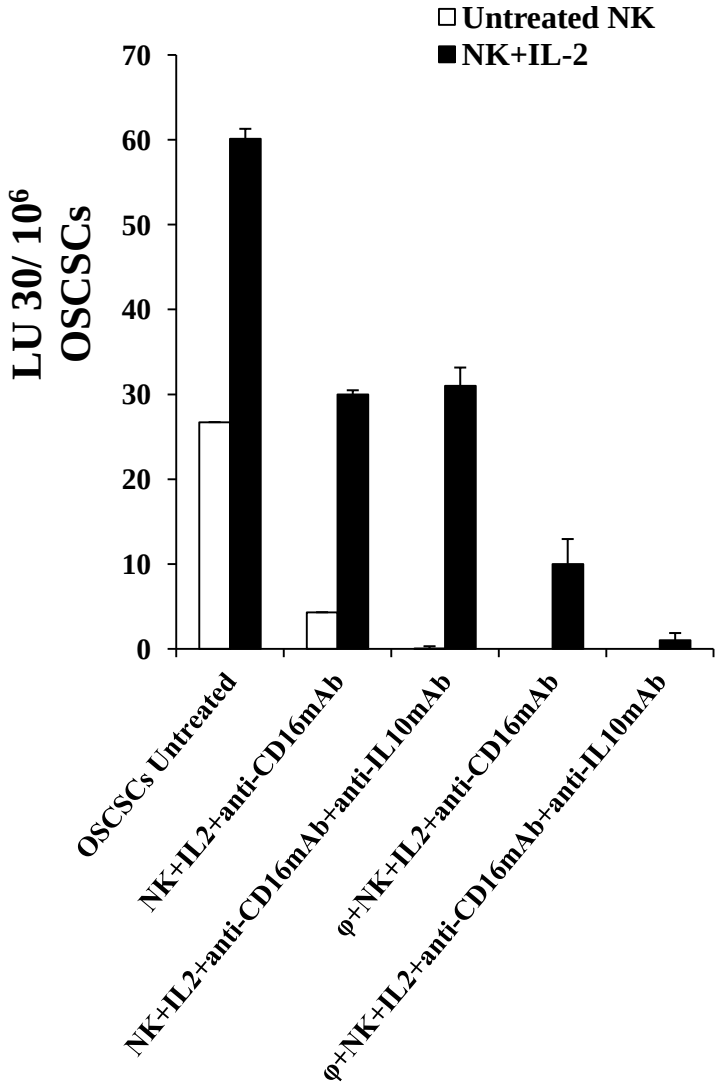



**Figure 60: Supernatants obtained from the co-culture of NK cells with monocytes treated with IL-2+ anti-CD16mAb in the presence of anti-IL10mAb caused the highest resistance of OSCSCs against NK cell-mediated cytotoxicity.**

NK cells were left untreated, treated with IL-2 (1000U/mL), IL-2+anti-CD16mAb (2.5μg/mL) and in combination of monocytes and NKs (1:3 Monocytes: NKs) and/or anti-IL10mAb (10μg/mL) for 12-18 hours. Afterwards, the supernatants from each NK sample were harvested and then treated OSCSCs for 3 days. Thereafter, the OSCSCs were detached from the tissue culture plates and their sensitivity to NK cell-mediated lysis was evaluated using 51-Chromium release assay. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of OSCSCs.

# Fig. 61

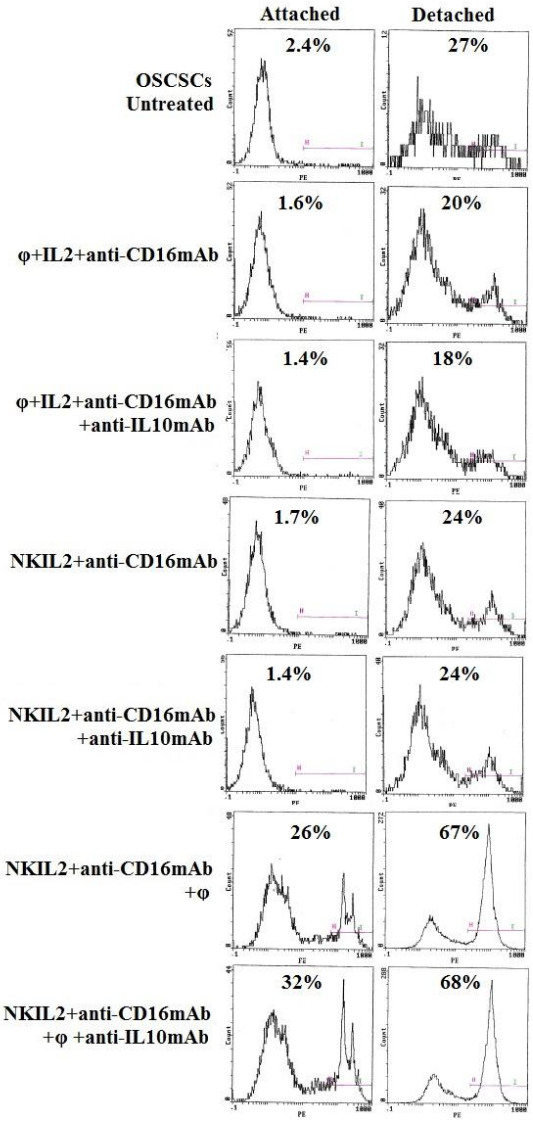



**Figure 61: Supernatants obtained from the co-culture of NK cells with monocytes treated with IL-2+ anti-CD16mAb induced cell deaths in OSCSCs.**

NK cells were left untreated, treated with IL-2 (1000U/mL), treated with IL-2+anti-CD16mAb (2.5µg/mL) and a combination of monocytes and NKs (1:3 Monocytes: NKs) and/or anti-IL10mAb (10µg/mL) for 12-18 hours. Afterwards, the supernatants from each NK sample were harvested and then treated OSCSCs for 3 days. Thereafter, the treat OSCSCs were detached from the tissue culture plates and 5x104 cells from each treatment were stained using Propidium Iodide (100ng/mL) in 400µl PBS and the level of cell death were determined by flow cytometric analysis.

# Fig. 62

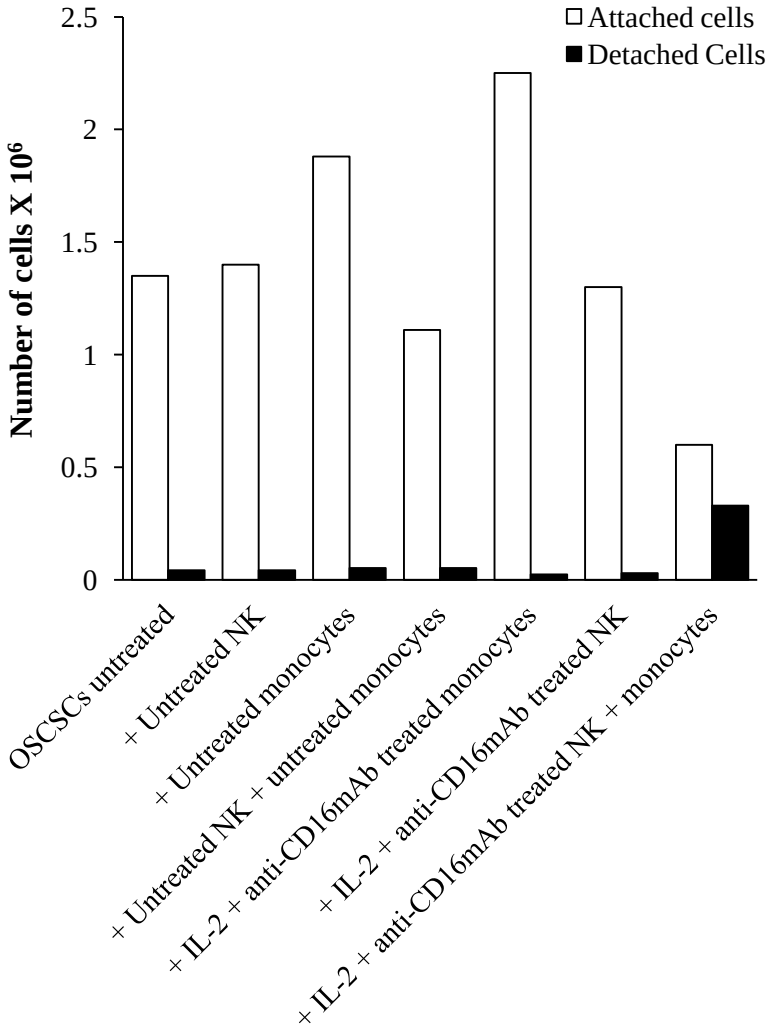



**Figure 62: Supernatant from the co-cultured of monocytes and NK cells treated IL-2 and anti-CD16mAb inhibited cell growth of OSCSCs.**

NK cells were left untreated, treated with IL-2 (1000U/mL), IL-2+anti-CD16mAb (2.5µg/mL) and in combination of monocytes and NKs (1:3 Monocytes: NKs) and/or anti-IL10mAb (10µg/mL) for 12-18 hours. Afterwards, the supernatants from each NK sample were harvested and then treated OSCSCs for 3-4 days. Thereafter, the non-adherence cells from the media were collected and the adherence cells were detached from the tissue culture plates and were counted using Hemocytometer. Total number of cells was calculated as total cells counted in $4mm^2$ divided by cells per $mm^2$ multiplied by Volume $x10^4$.

# Fig. 63

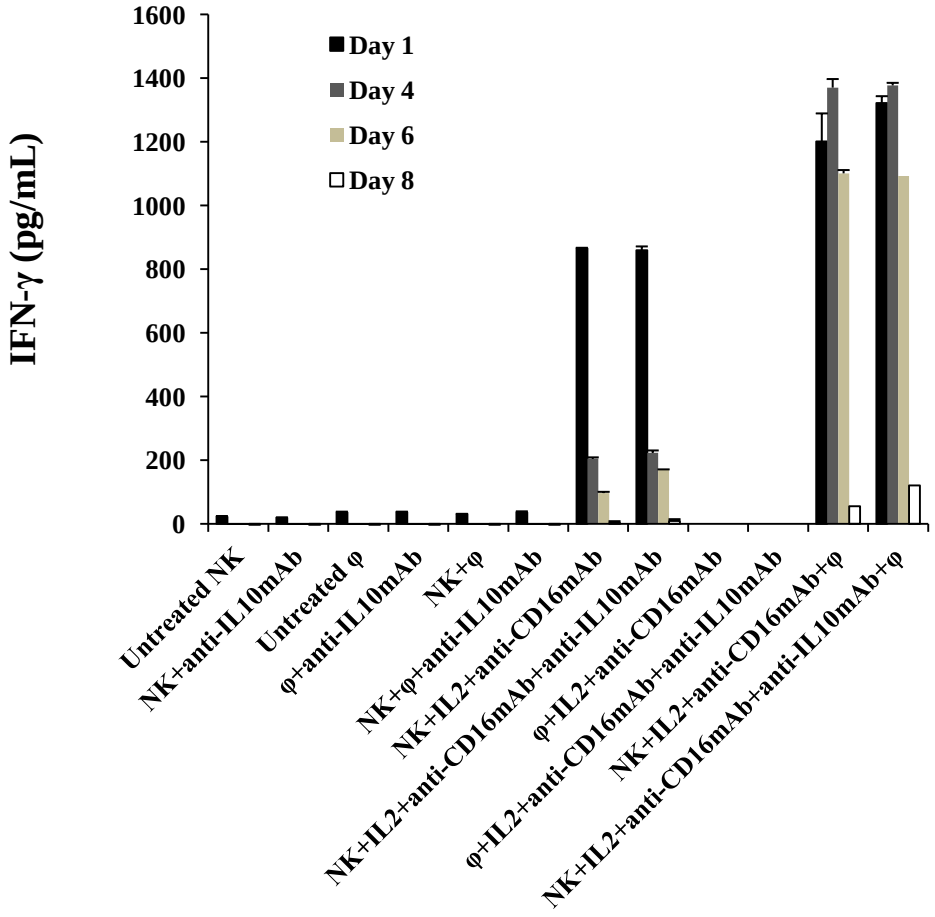



**Figure 63: Monocytes triggered secretion of IFNγ on NK cells until 8 days.**

NK cells were left untreated, treated with IL-2 (1000U/mL), IL-2+anti-CD16mAb (2.5µg/mL) and in combination of monocytes and NKs (1:3 Monocytes: NKs) and/or anti-IL10mAb (10µg/mL) for 12-18 hours before the supernatants were collected and the level of IFN-γ secretion were determined using Human IFN-γ ELISA MAX Standard Sets

**Fig. 64**

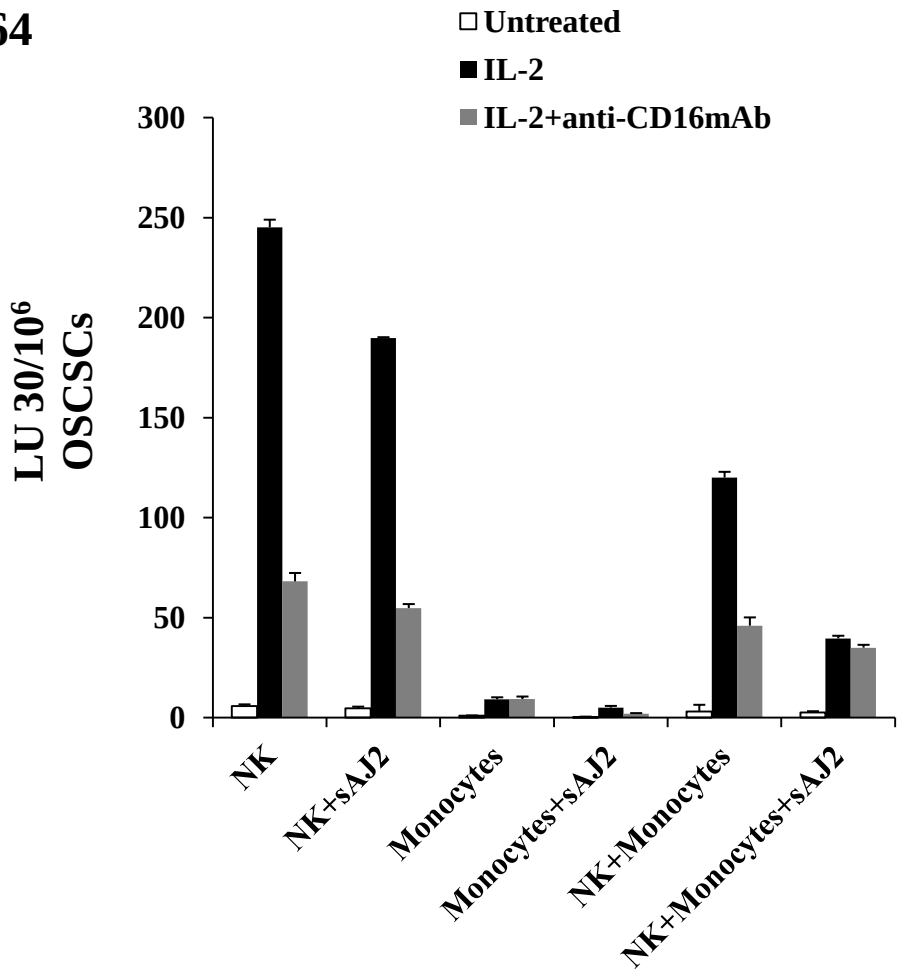



**Figure 64: Co-cultured monocytes and NK cells treated IL-2 with anti-CD16mAb and sAJ2 inhibited NK cells mediated cytotoxicity against OSCSCs.**

NK cells were left untreated and treated with IL-2 (1000U/mL) and IL-2+anti-CD16mAb (2.5µg/mL) and in combination of sAJ2 (3:1 sJA2: NKs) and/ or monocytes (1:1 Monocytes: NKs) for 12-18 hours before they were added to $^{51}Cr$ labeled OSCSC cells. NK cell cytotoxicities were determined using a standard 4 hour $^{51}Cr$ release assay. The NK cells with OSCSCs 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 OSCSCs.

# Fig. 65

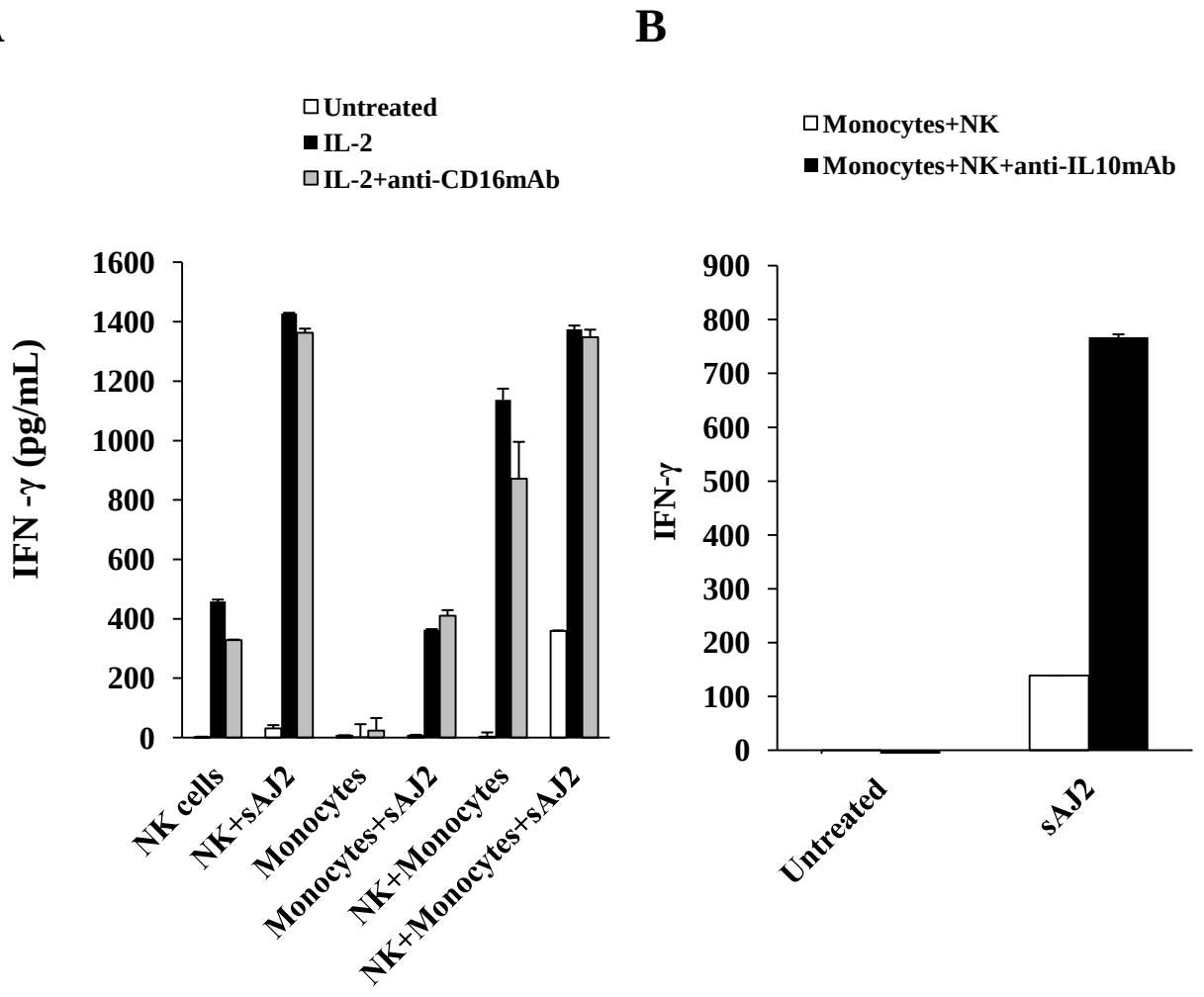



**Figure 65: IL-10 inhibits IFN-γ secretion in the co-cultures of NK cells treated IL-2 and anti-CD16mAb with monocytes and probiotic bacteria**

NK cells were left untreated and treated with IL-2 (1000U/mL) and IL-2+anti-CD16mAb (2.5µg/mL) and in combination of sAJ2 (3:1 sJA2: NKs) and/ or monocytes (1:1 Monocytes: NKs) for 12-18 hours before the supernatants were collected and the level of IFN gamma secretion were determined using Human IFN-γ ELISA MAX Standard Sets.

# Fig. 66

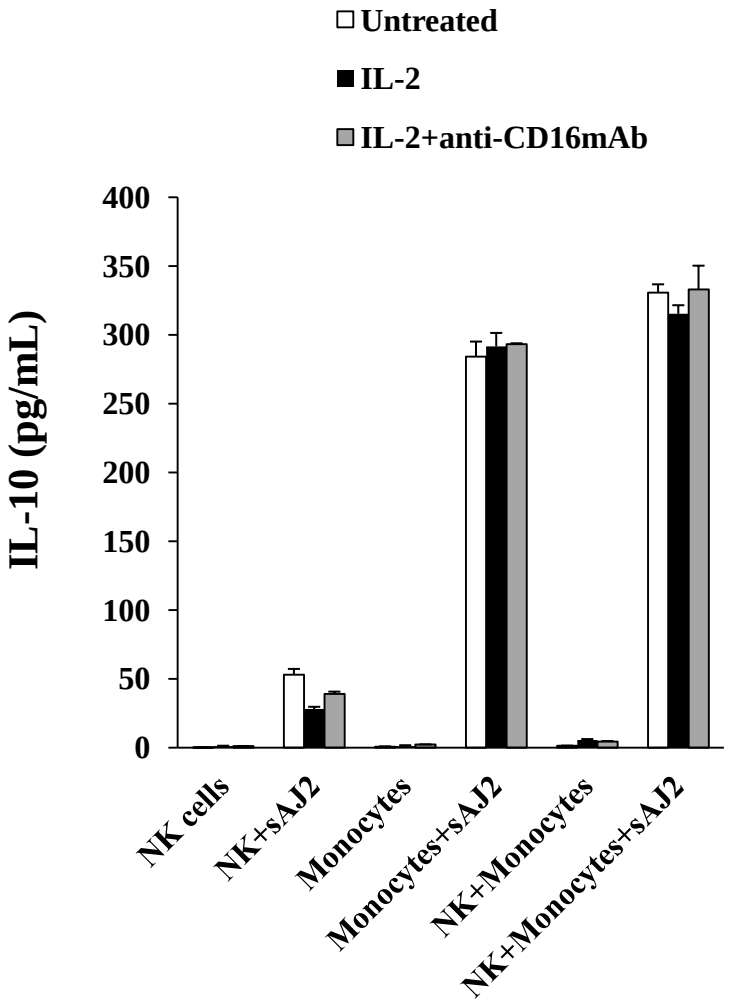



**Figure 66: The co-culture of monocytes and NK cells treated IL-2 and anti-CD16mAb secreted high level of IL-10 in the presence of probiotic bacteria.**

NK cells were left untreated and treated with IL-2 (1000U/mL) and IL-2+anti-CD16mAb (2.5µg/mL) and in combination of sAJ2 (3:1 sJA2: NKs) and/ or monocytes (1:1 monocytes: NKs) for 12-18 hours before the supernatants were collected and the level of IL-10 secretion were determined using Human IL-10 ELISA MAX Standard Sets.

# Fig. 67

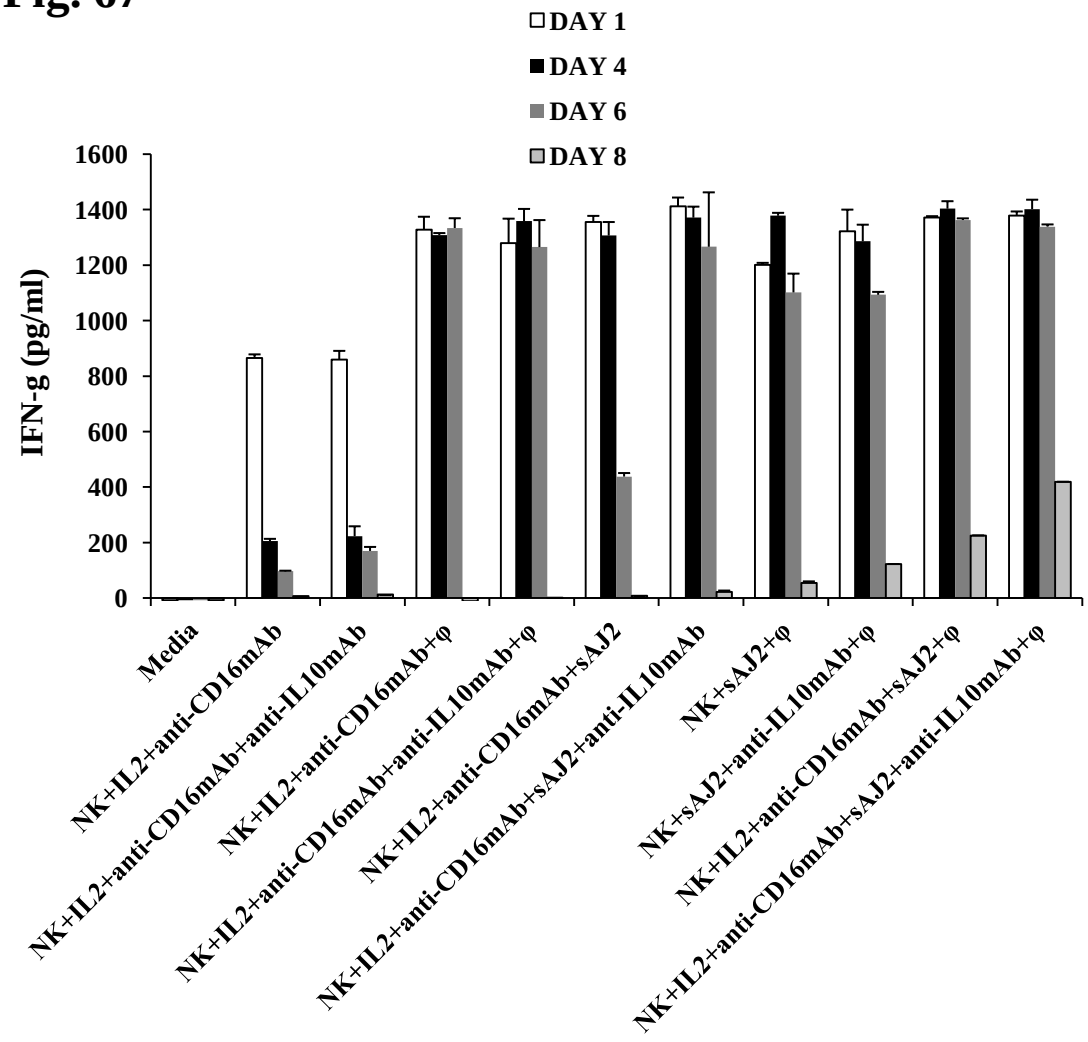



**Figure 67: Combination of probiotic bacteria, monocytes and anti-IL10mAb treated anergized NK cells continuously secreted IFN-γ until 8 days.**

NK cells treated sAJ2 (2:1 bacteria: NKs), IL-2 (1000U/mL) +anti-CD16mAb (2.5μg/mL) and in combination of monocytes (1:3 φ: NKs) and/or anti-IL10mAb (10μg/mL) and/or sAJ2 for 12-18 hours, 4 days, 6 days, and 8 days. The supernatants were collected each time and the levels of IFN-γ secretion were determined using Human IFN-γ ELISA MAX Standard Sets. The symbol φ represents monocyte.

**Fig. 68**

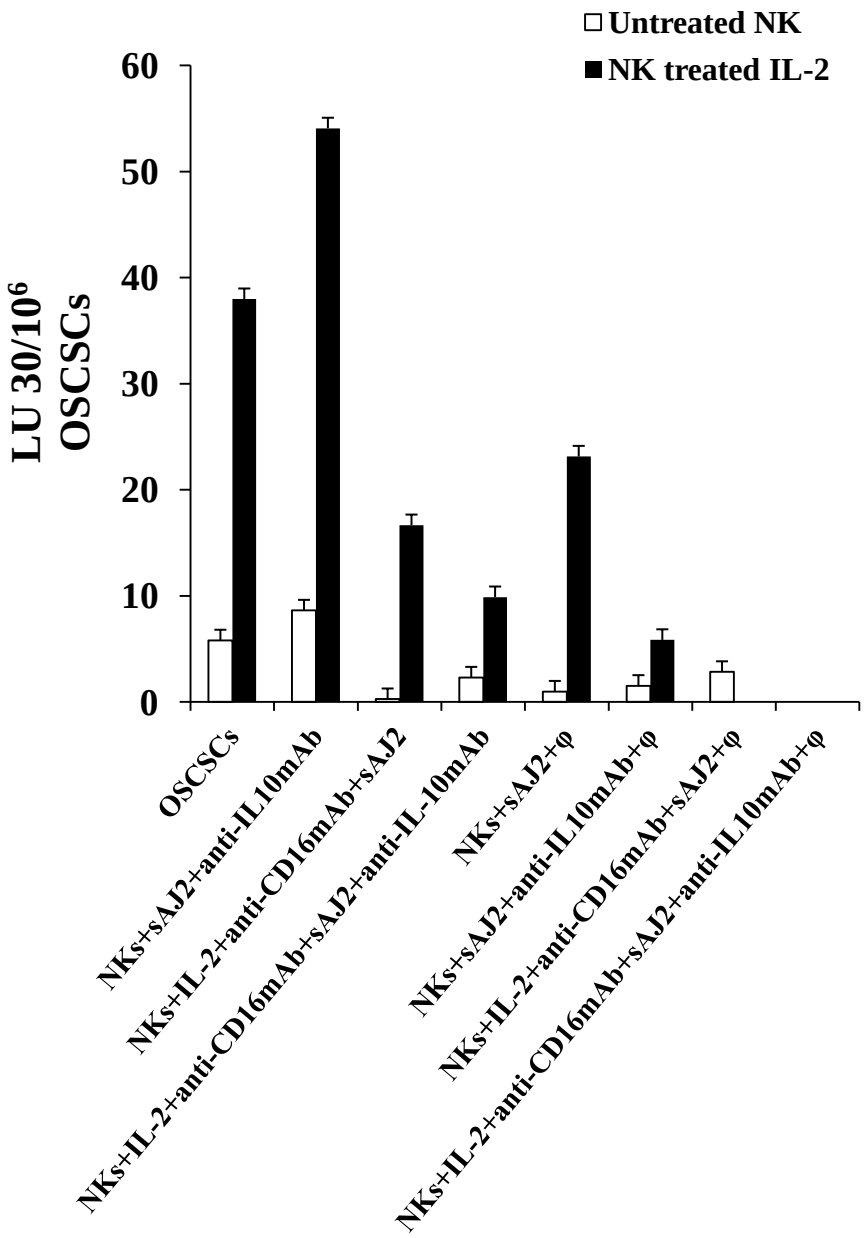



**Figure 68: Supernatant from the co-cultured of monocytes and NK cells treated IL-2 with anti-CD16mAb and sAJ2 in the presence of anti-IL10mAb induced the highest resistance of OSCSCs against NK cell-mediated cytotoxicity.**

As described above in the figure, NK cells were treated with IL-2 (1000U/mL), anti-CD16mAb (2.5ug/mL), Monocytes (1:3 φ: NKs), anti-IL10mAb (10μg/mL), and sAJ2 (2:1 bacteria: NKs) for 12h – 18h. Afterwards, the supernatants were collected and treated OSCSCs for 2 days. Thereafter, the OSCSCs were detached from the tissue culture plates and their sensitivity to NK cell-mediated lysis was evaluated using 51-Chromium release assay. LU $30/10^6$ denotes the number of NK cell effectors needed to lyse 30% of OSCSCs. The symbol φ represents monocyte.

# Fig. 69

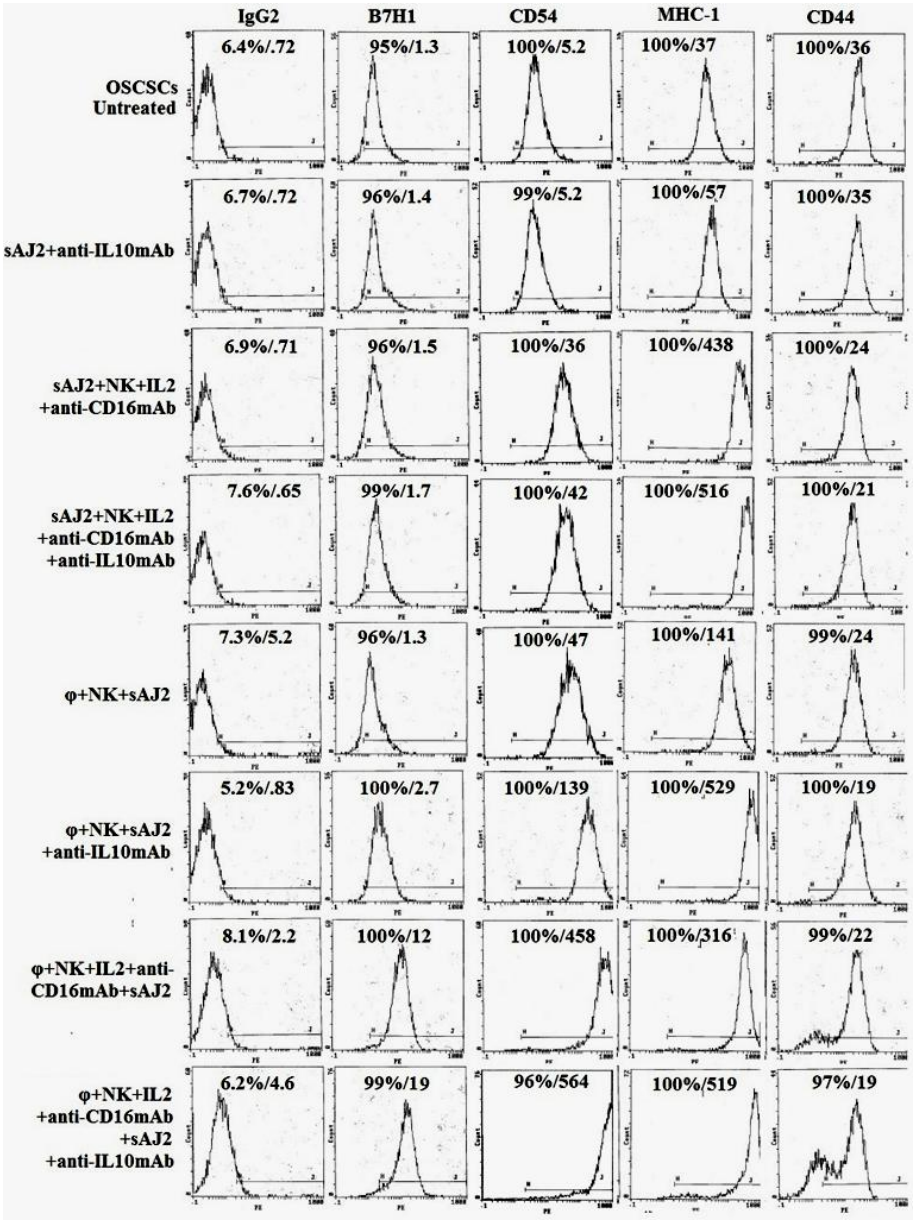



**Figure 69: Supernatant from the co-cultured of monocytes and NK cells treated IL-2 with anti-CD16mAb and sAJ2 in the presence of anti-IL10mAb induced the highest modulation of surface expressions of CD54, MHC-1, B7H1, and CD44.**

NK cells were pre-treated for 12-18 hours described in Figure 68. Afterward, the supernatants were collected and treated OSCSCs for 2 days. Thereafter, the OSCSCs were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against isotype control, CD54, MHC-1, B7H1, and CD44 and the level of surface expression were determined by flow cytometric analysis. The symbol φ represents monocyte.

# Fig. 70

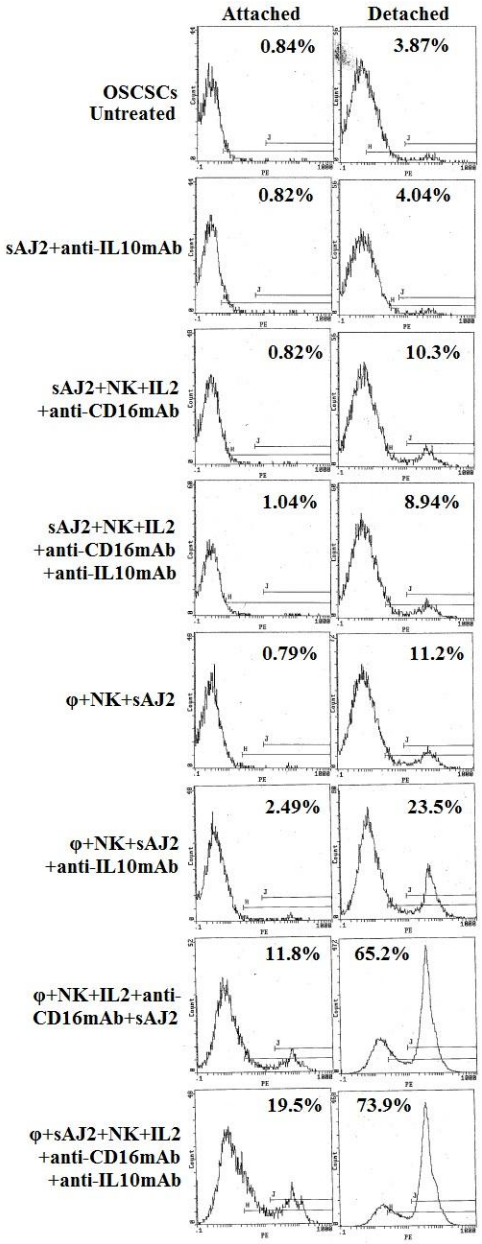



**Figure 70: Supernatant from the co-cultured of monocytes and NK cells treated IL-2 with anti-CD16mAb and sAJ2 in the presence of anti-IL10mAb induced cell deaths in OSCSCs.**

NK cells were pre-treated for 12-18 hours described in Figure 68. Afterward, the supernatants from each NK sample were harvested and then treated OSCSCs for 2 days. Thereafter, adherence cells and the detached cells from the media were counted using Hematocytomer and $5x10^4$ cells from each treatment were stained using Propidium Iodide (100ng/mL) in 400µl PBS. The level of cell death was determined by flow cytometric analysis.

# Fig. 71

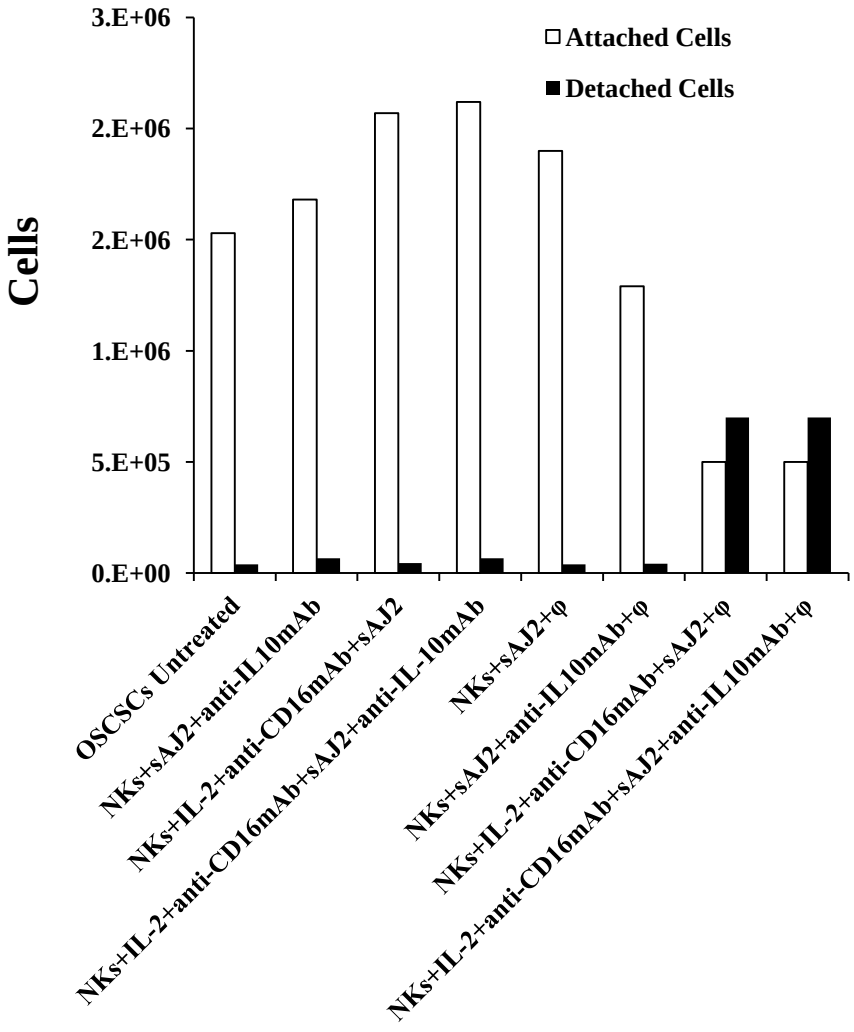
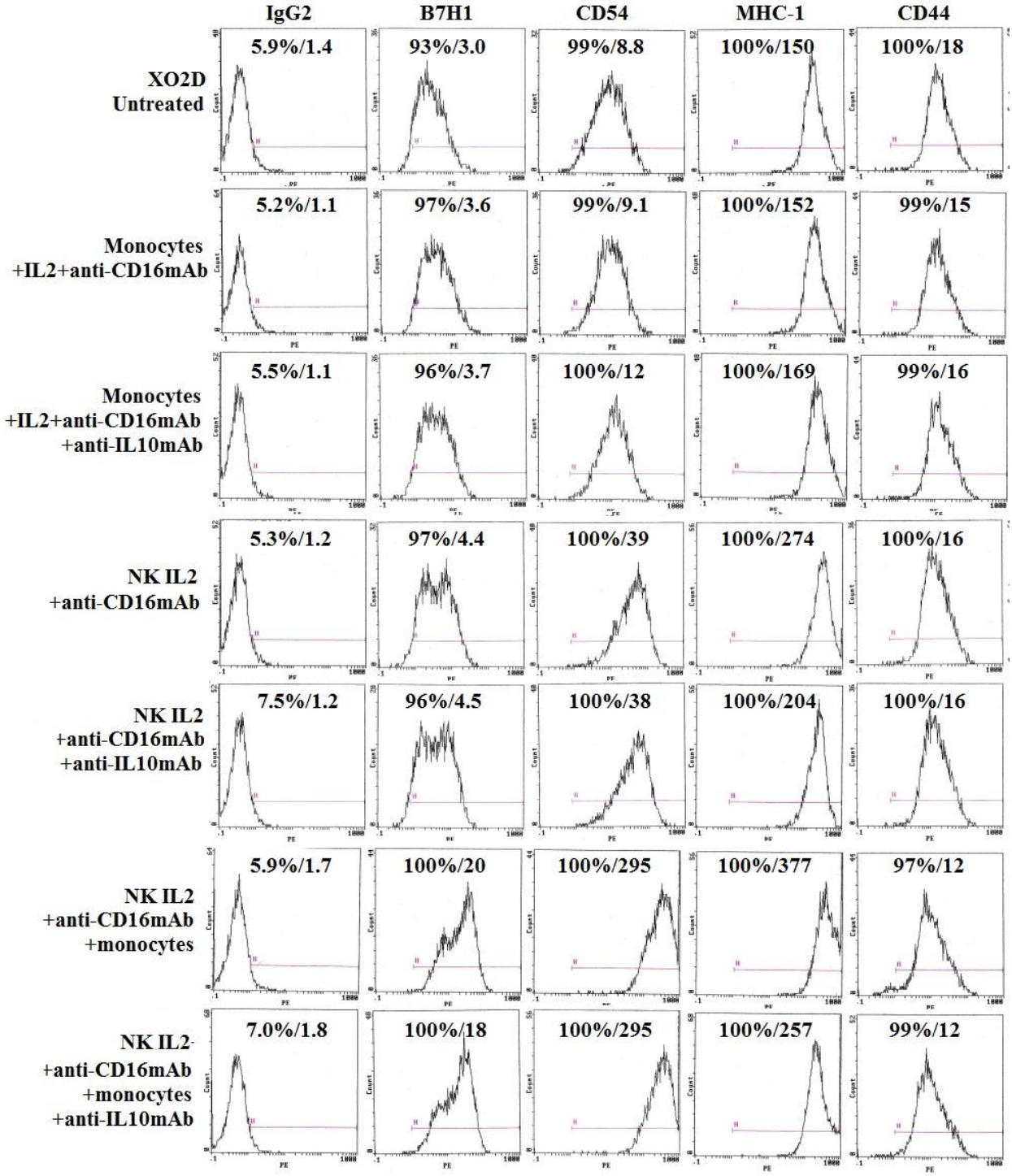



**Figure 71: Supernatant from the co-cultured of monocytes and NK cells treated IL-2 with anti-CD16mAb and sAJ2 in the presence of anti-IL10mAb inhibited cell growth and caused high number of detached cells in OSCSCs.**

NK cells were pre-treated for12-18 hours described in Figure 68. Afterwards, the supernatants from each NK sample were harvested and then treated OSCSCs for 3 days. Thereafter, the non-adherence cells from the media were collected and the adherence cells were detached from the tissue culture plates and were counted using Hemocytometer. Total number of cells was calculated as total cells counted in $4mm^2$ divided by cells per $mm^2$ multiplied by Volume $x10^4$.

# Fig. 72 A


IgG2
B7H1
CD54
MHC-1
CD44
XO2D
Untreated
5.9%/1.4
93%/3.0
99%/8.8
100%/150
100%/18
Monocytes
+IL2+anti-CD16mAb
5.2%/1.1
97%/3.6
99%/9.1
100%/152
99%/15
Monocytes
+IL2+anti-CD16mAb
+anti-IL10mAb
5.5%/1.1
96%/3.7
100%/12
100%/169
99%/16
NK IL2
+anti-CD16mAb
5.3%/1.2
97%/4.4
100%/39
100%/274
100%/16
NK IL2
+anti-CD16mAb
+anti-IL10mAb
7.5%/1.2
96%/4.5
100%/38
100%/204
100%/16
NK IL2
+anti-CD16mAb
+monocytes
5.9%/1.7
100%/20
100%/295
100%/377
97%/12
NK IL2-
+anti-CD16mAb
+monocytes
+anti-IL10mAb
7.0%/1.8
100%/18
100%/295
100%/257
99%/12

# Fig. 72B

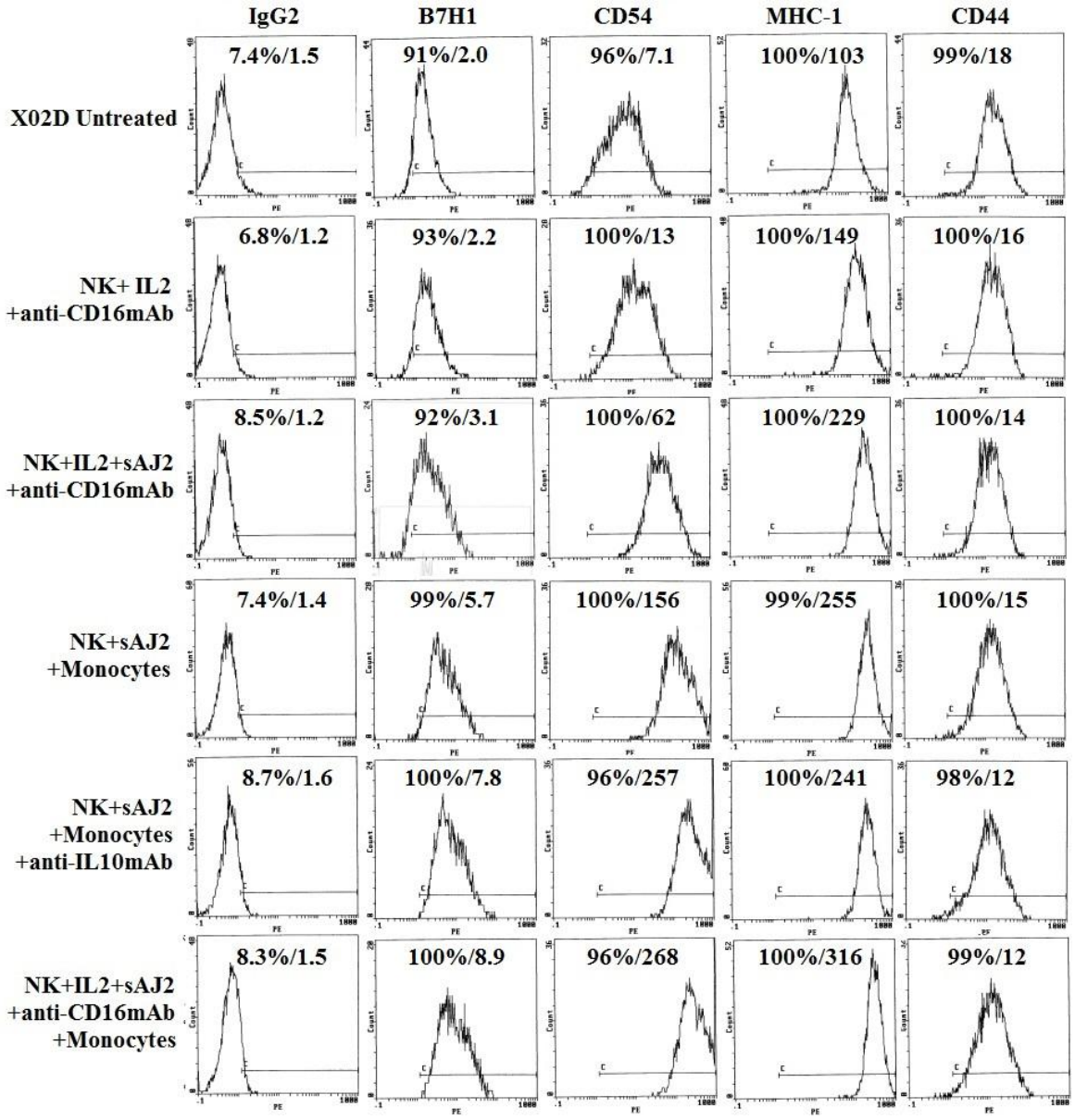



**Figure 72: The presence of anti-IL10mAb in the supernatants from the co-cultured of monocytes and NK cells treated IL-2+anti-CD16mAb+sAJ2 or monocytes and untreated NK cells+sAJ2 induced modulation of XO2D.**

NK cells were treated with IL-2 (1000U/mL), anti-CD16mAb (2.5ug/mL), monocytes (1φ: 3NKs), sAJ2 (2sAJ2: 1NKs), for 12h – 18h before the supernatants from each NK sample were harvested and then treated XO2D for 5 days. Thereafter, the XO2D were detached from the tissue culture plates and $5x10^4$ cells from each treatment were stained using PE conjugated antibodies against isotype control, CD54, MHC-1, B7H1, and CD44 and the level of surface expression were determined by flow cytometric analysis. The symbol φ represents monocyte.

# Fig. 73

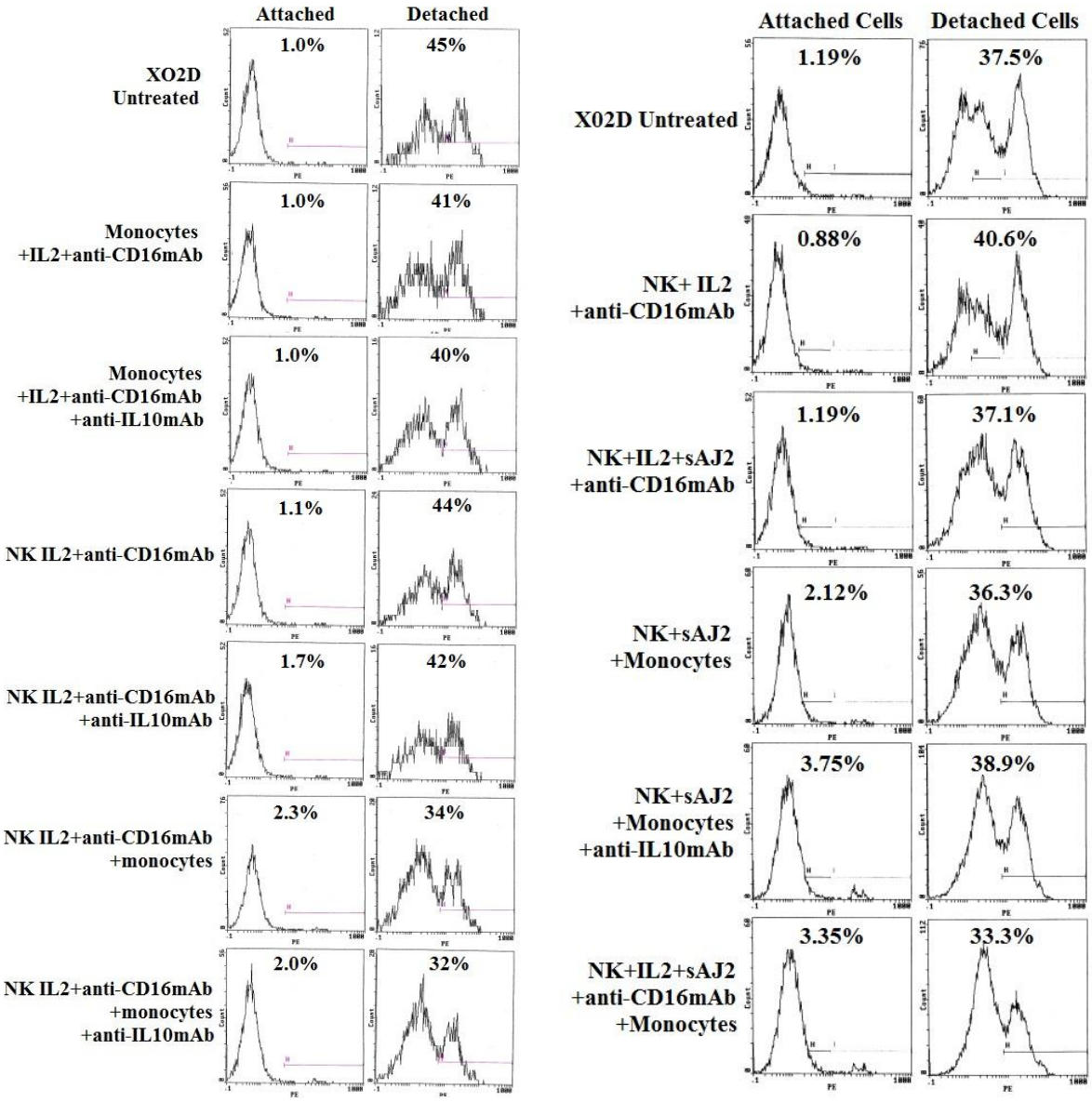



**Figure 73: Supernatant from the co-cultured of monocytes and NK cells treated IL-2 with anti-CD16mAb induced moderate cell deaths in X02D.**

NK cells were pre-treated for12-18 hours described in Figure 72. Afterwards, the supernatants from each NK sample were harvested and then treated XO2D for 5 days. Thereafter, adherence cells and the detached cells from the media were counted using Hematocytomer and $5x10^4$ cells from each treatment were stained using Propidium Iodide (100ng/mL) in 400µl PBS. The level of cell death was determined by flow cytometric analysis.

# Fig. 74

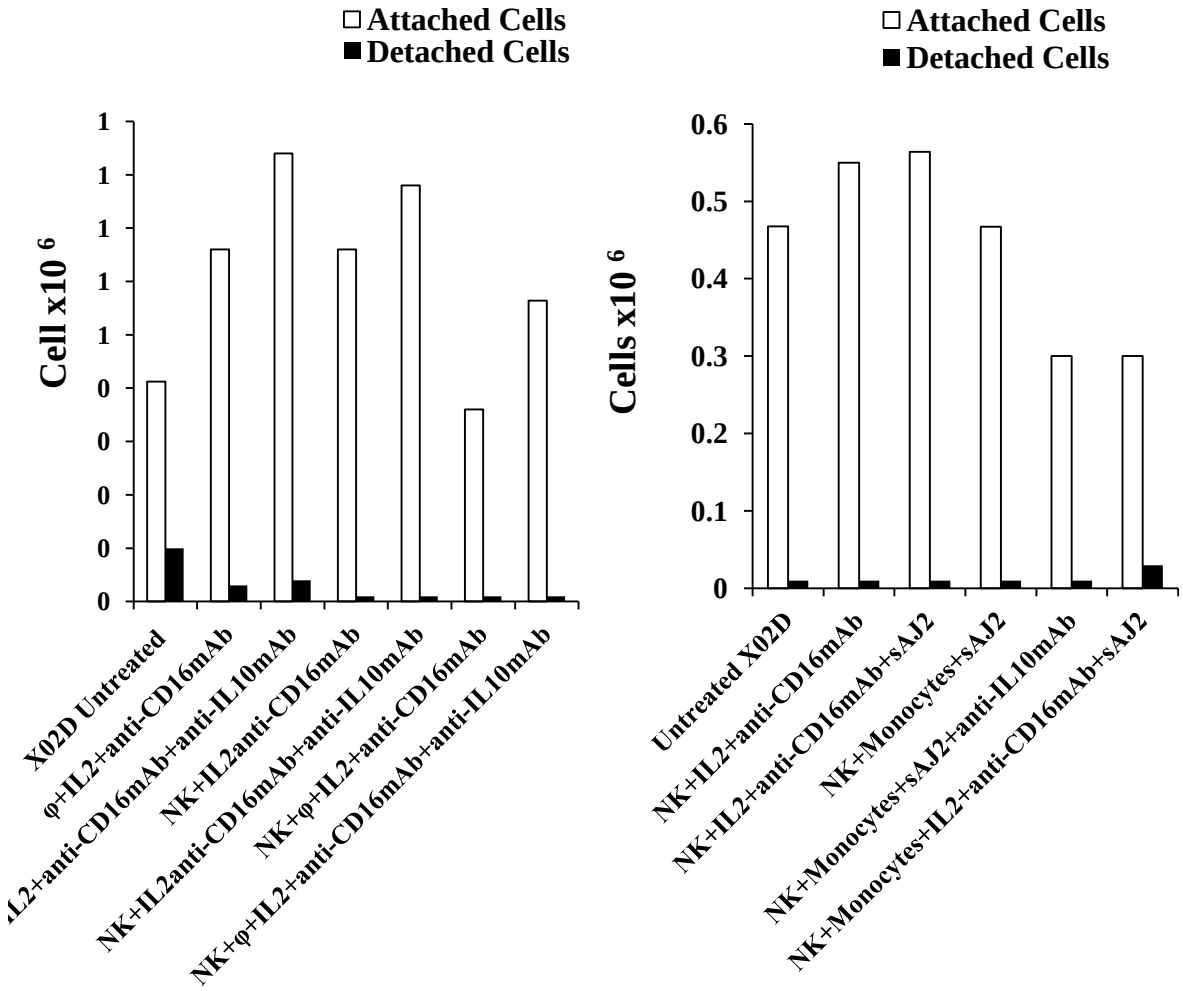



**Figure 74: Supernatant from the co-cultured of monocytes and NK cells treated IL-2 with anti-CD16mAb and sAJ2 in the presence of anti-IL10mAb inhibited cell growth of X02D.**

NK cells were pre-treated for12-18 hours described in Figure 72. Afterwards, the supernatants from each NK sample were harvested and then treated XO2D for 5 days. Thereafter, the non-adherence cells from the media were collected and the adherence cells were detached from the tissue culture plates and were counted using Hemocytometer. Total number of cells was calculated as total cells counted in $4mm^2$ divided by cells per $mm^2$ multiplied by Volume $x10^4$.

**Fig. 75**

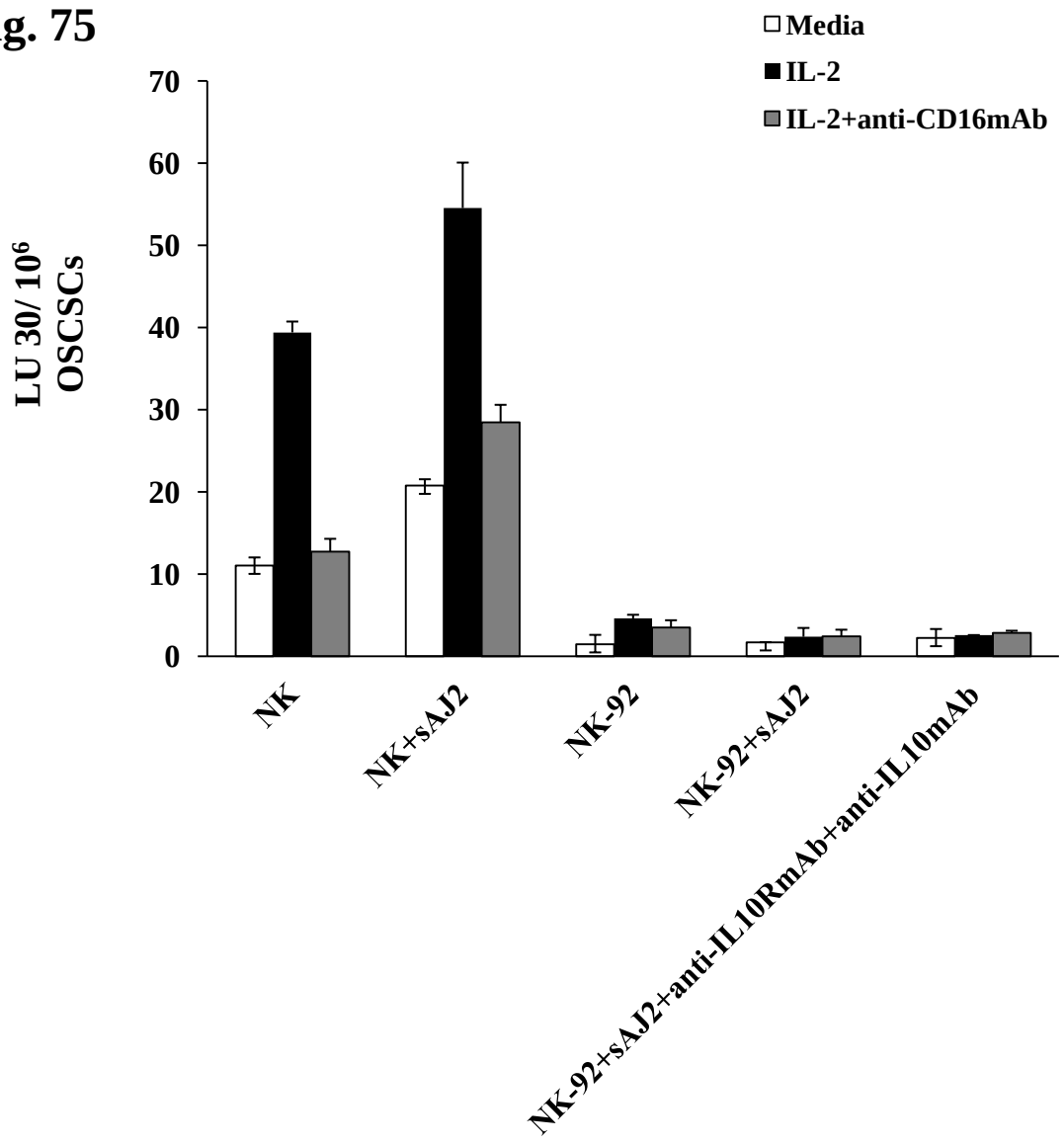



**Figure 75: Addition of anti-IL10mAb to NK-92 cells treated probiotic bacteria did not affect NK cells mediated cytotoxicity against OSCSCs.**

NK cells were left untreated, or treated with IL-2 (1000U/mL) or IL-2 (1000U/mL) + anti-CD16mAb (2.5µg/mL) plus AJ1 (3:1 bacteria: NKs) and anti-IL10 mAb (10mg/mL) for 12-18 hours before they were added to $^{51}$Cr labeled OSCSCs. NK cell cytotoxicities were determined using a standard 4 hour $^{51}$Cr release assay. The NK cells with OSCSCs 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 OSCSCs.

**Fig. 76**

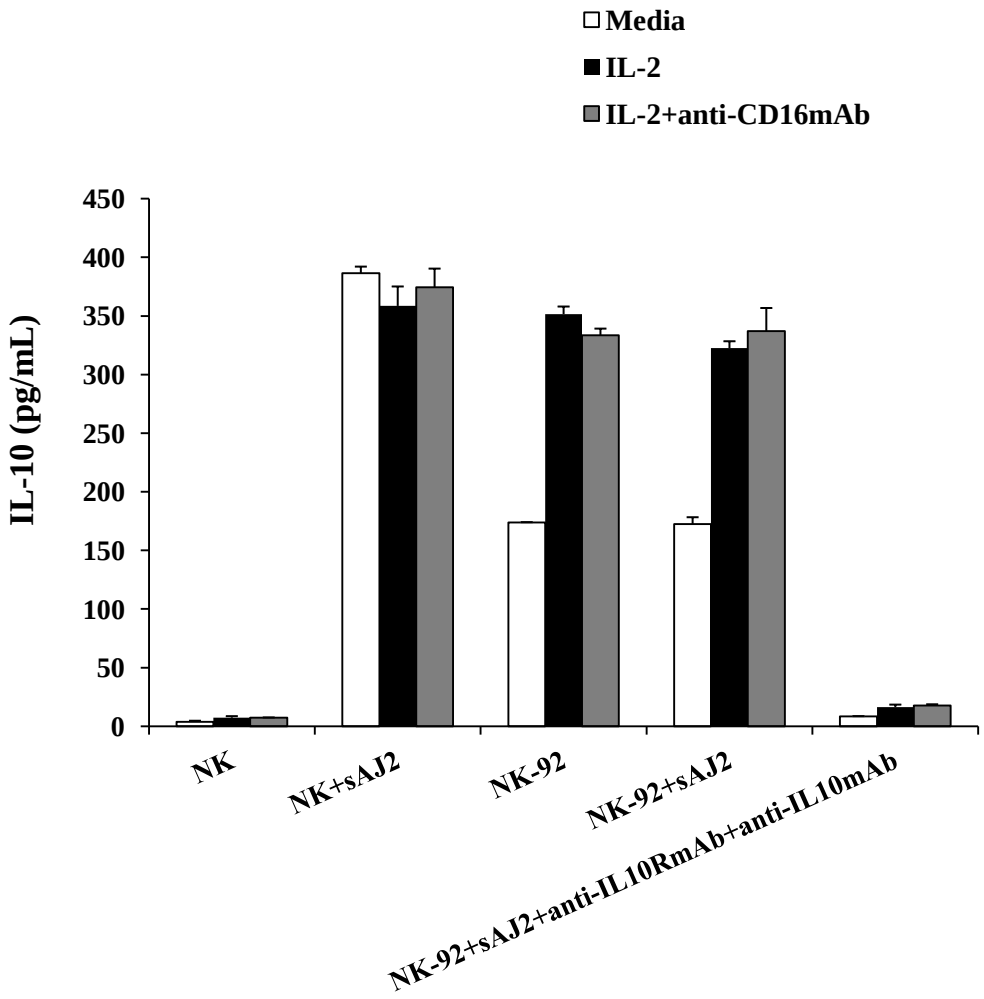



**Figure 76: NK-92 induced high level of IL-10; addition of IL10mAb and IL-10 receptor mAb inhibited secretion of IL-10 on NK-92.**

Untreated, IL-2 (1000U/mL) treated, and IL-2 (1000U/mL) +anti-CD16mAb (2.5µg/mL) treated NK and NK-92 cells were co-cultured with and without sAJ2 at 2:1 (bacteria: NKs) and with anti-IL10mAb (10mg/mL) and anti-IL10RmAb (5mg/mL) for 12h-18h before the supernatants were collected and the level of IL-10 secretion were determined using Human IL-10 ELISA MAX Standard Sets.

**Fig. 77**

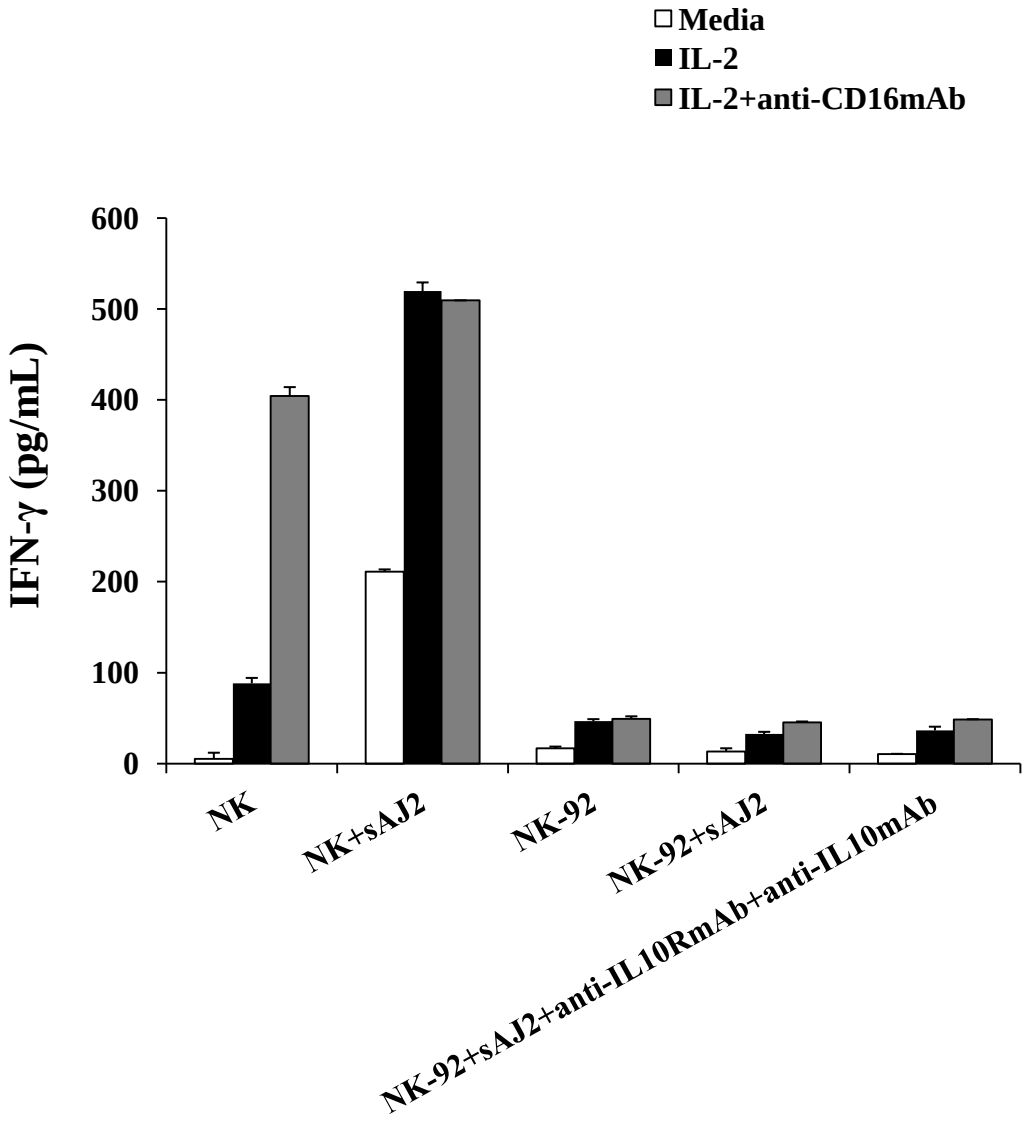



**Figure 77: Addition of anti-IL10 and anti-IL10R on NK-92 did not affect IFN-γ production.**

Untreated, IL-2 (1000U/mL) treated, and IL-2 (1000U/mL) +anti-CD16mAb (2.5µg/mL) treated NK and NK-92 cells were co-cultured with and without sAJ2 at 2:1 (bacteria: NKs) and with anti-IL10mAb (10mg/mL) and anti-IL10RmAb (5mg/mL) for 12h-18h before the supernatants were collected and the level of IFN-γ secretion were determined using Human IFN-γ ELISA MAX Standard Sets.

**DISCUSSION:**

In the tumor microenvironment, the NK cells interact with the monocytes and other immune cells and lyse a small population of stem cells before they become anergized to secrete cytokines to support maturation and differentiation of the stem cells. The additions of anti-IL10mAb to the NK cells have no significant changes in the level of cytotoxicity on the NK cells against OSCSCs nor does the antibody induce IFN-γ on the NK cells. The addition of anti-IL10mAb blocked the production of IL-10 on the NK cells. Furthermore, purified monocytes were observed to produce very low level of IFN-γ and when treated with anti-IL10mAb did not induce secretion of IFN-γ. Monocytes co-cultured with untreated NK cells produced low level of IFN-γ; however, when NK cells were treated with IL-2 and anti-CD16mAb and co-cultured with monocytes significantly induced IFN-γ. The level of IFN-γ plateau when probiotic bacteria or anti-IL10mAb were treated with monocytes co-cultured with NK treated IL2 and anti-CD16mAb. The important role of anti-IL10mAb was not observed in ELISA due to plateau effect; however, in a long term differentiation experiment using OSCSCs and X02D cell lines, anti-IL10mAb was able to significantly induce maturation, differentiation and resistance of OSCSCs and X02D when co-cultured with activated monocytes and NK cells or/and sAJ2. Monocytes are potent inducers of IFN-γ on NK cells, very similar to probiotic bacteria. Other studies have shown that monocytes, but not monocyte-depleted PBMNC, secreted IL-12, TNF alpha, and IL-10 in response to *L. casei* strain *Sirota*[2]. Monocytes inhibited the function of NKs mediated cytotoxicity. Monocytes have the ability to block NK cells from lysing OSCSCs or SCAPs. The supernatants from NK treated monocytes and anti-IL10mAb minimally unregulated CD54, MHC-1, B7H1 and CD44 in OSCSCs and X02D; however, the presence of probiotic bacteria with NK treated monocytes and anti-IL10mAb significantly up-regulated

CD54, MHC-1 and B7H1 and down-regulated CD44 in OSCSCs and X02D. Monocytes with NK treated IL-2 and anti-CD16mAb without bacteria still able to differentiate stem cells, but the level of differentiation increased in the presence of probiotic bacteria.

Because IL-10 has an inhibitory activity on the synthesis of IFN-γ by human NK cells [96-98], it blocks differentiation of stem cells by inhibiting the secretion of IFN-γ on NK cells. Supernatants from NK cells treated IL-2+anti-CD16mAb+sAJ2+monocytes+anti-IL10mAb induced the highest level of IFN-γ and caused differentiation of stem cells in just 2 days. Furthermore, the supernatants from the highest level of IFN-γ caused strong selection of stem cells and therefore a great number of stem cells undergo cell deaths as observed in the medium. According to Sellon R K et al, mouse deficient of IL-10 caused more inflammation [102]. IL-10 played a significant role in regulating tumor progression and differentiation of stem cells. Based on our accumulated data, IL-10 is important in keeping the differentiation of stem cells in a well balanced state. IL-10 decreases the differentiation level of stem cells; it balances the level of differentiation so that the cells will not be pushed to undergo cell deaths and remained differentiated to perform their functions (Figure 60, Figure 61, Figure 68, and Figure 70). The less IL-10 the more resistance of the stem cells to NK cells mediated cytotoxicity; which correlated with surface expressions.

Monocytes triggered secretion of IFN-γ on NK treated IL-2+anti-CD16mAb until 8 days. Bacteria induced secretions of IFN-γ on NK treated IL-2+anti-CD16mAb until 6 days. It is not clear whether monocytes are more potent inducers of IFN-γ on the NK cells over bacteria because the concentrations were not optimized. The NK cells treated IL-2 and anti-CD16mAb with monocytes and bacteria without anti-IL10mAb caused induction of IFN-γ while the presence of anti-IL10mAb expanded the lifespan of secretion of the NK cells (Figure 67).

NK-92 cells also caused split anergy by losing their cytotoxic function and gaining the ability to secrete cytokine, IL-10; however, their role in supporting differentiation of stem cells is still not clear.

## CONCLUSION:

This study has demonstrated that monocytes as well as probiotic bacteria, AJ2, are a powerful inducers of IFN-γ secretion when combined with the NK cells and they significantly drive differentiation and resistance of OSCSCs and X02D. IL-10 cytokine has an inhibitory effect on the secretion of IFN-γ of the NK cell. NK synergized with monocytes to produce IL-10; therefore, we see differences when we added anti-IL10mAb. NK cells treated with IL-2, anti-CD16mAb, monocytes, anti-IL10mAb and probiotic bacteria (AJ2) synergistically pushed stem cells to the next highest level of differentiation and maturation, which significantly shielded OSCSC and X02D from the NK-mediated lysis. The supernatant from NK cells treated IL-2 and anti-CD16mAb with sAJ2, monocytes, and anti-IL10 mAb contained the highest concentration of IFN-γ and supported the differentiation of stem cells much faster, resulted in the highest resistance to NK cell-mediated cytotoxicity. The highest level of differentiation was confirmed through a significant decrease in CD44 expression and significant increase in CD54, B7H1, and MHC-1 expressions. Co-cultured of monocytes with NK treated IL-2 and anti-CD16mAb induced cell deaths in OSCSCs and X02D, but not induced by probiotic bacteria or anti-IL10mAb.

**FINAL CONCLUSION:**

Our lab focused immensely on the mechanism by which NK cells can become anergized in order to support tissue regeneration of the epithelial cells and the maturation and differentiation of transformed and non-transformed stem cells. Based on the accumulated data from this study, we have shown that the stages of maturation and differentiation of the tumor cells are predictive of their sensitivity to NK cells cytotoxicity. We have shown that probiotic bacteria have the ability to anergize NK cells at a significant level by secreting significant amounts of pro-inflammatory and anti-inflammatory cytokines and by mediating maturation and differentiation of OSCSCs, MIA PaCa-2, SCAPs and X02D at a faster rate. At this point of the study, we have demonstrated that the greatest level of anergy in NK cells was when NK cells interacted with combination of anti-CD16mAb, IL-2, monocytes, anti-IL10mAb, and probiotic bacteria. Furthermore, we have shown that increasing CD54, MHC Class 1 and B7H1 and decreasing CD44 surface expression of stem cells were the mechanism in which NK cells differentiated stem cells. Anti-IL10mAb pushed stem cells to differentiate much faster while IL-10 decreased the level of differentiation.

In tumors since the generation and maintenance of cancer stem cells is higher, the majority (or all) of the NK cells may be conditioned or "anergized" to support differentiation, which may be one mechanism by which cancer progress and metastasize. Because anergized NK cells have the ability to drive differentiation, they may have the ability to stop the progress of inflammation since differentiated cells are not targeted by the NK cells. Therefore, although

immunosuppression in the tumor microenvironment is not helpful for the patient, it is indeed an important function that may not only stimulate differentiation, but it may also halt inflammation.

We believe that there are a few possible strategies by the NK cells to eliminate tumors, to target the stem cells and differentiated cells. Since cancer stem cells were found to be sensitive to NK cells-mediated lysis but resistance to chemotherapeutic drugs while differentiated tumors were more sensitive to chemotherapeutic drugs but resistance to NK cells-mediated lysis, combination immunotherapy of cancers should be considered for the elimination of both undifferentiated and differentiated tumors. In addition, cancer patients may be benefit from repeated transplantation of allogeneic NK cells, ingestion of probiotic bacteria as an adjunctive therapy to anticancer treatment, and blocking the effect of IL-10 to cancer patients may be able to break the tumor-specific immune tolerance.

The continuing investigation of how stem cells interact with the immune system may open up a new scientific field-the immunology of stem cells. Our future study is to study probiotic bacteria interaction with NK cells through Toll-Like Receptors 2, 4 and 9. We want to study the cytokine receptors that are responsible for differentiation of stem cells such as IFN gamma Receptor, TNF alpha Receptor, IL-10 Receptor. In addition, we also want to examine the role of anergized NK cells on differentiation of stem cells induced by probiotic bacteria *in vivo.*

# Fig. 78

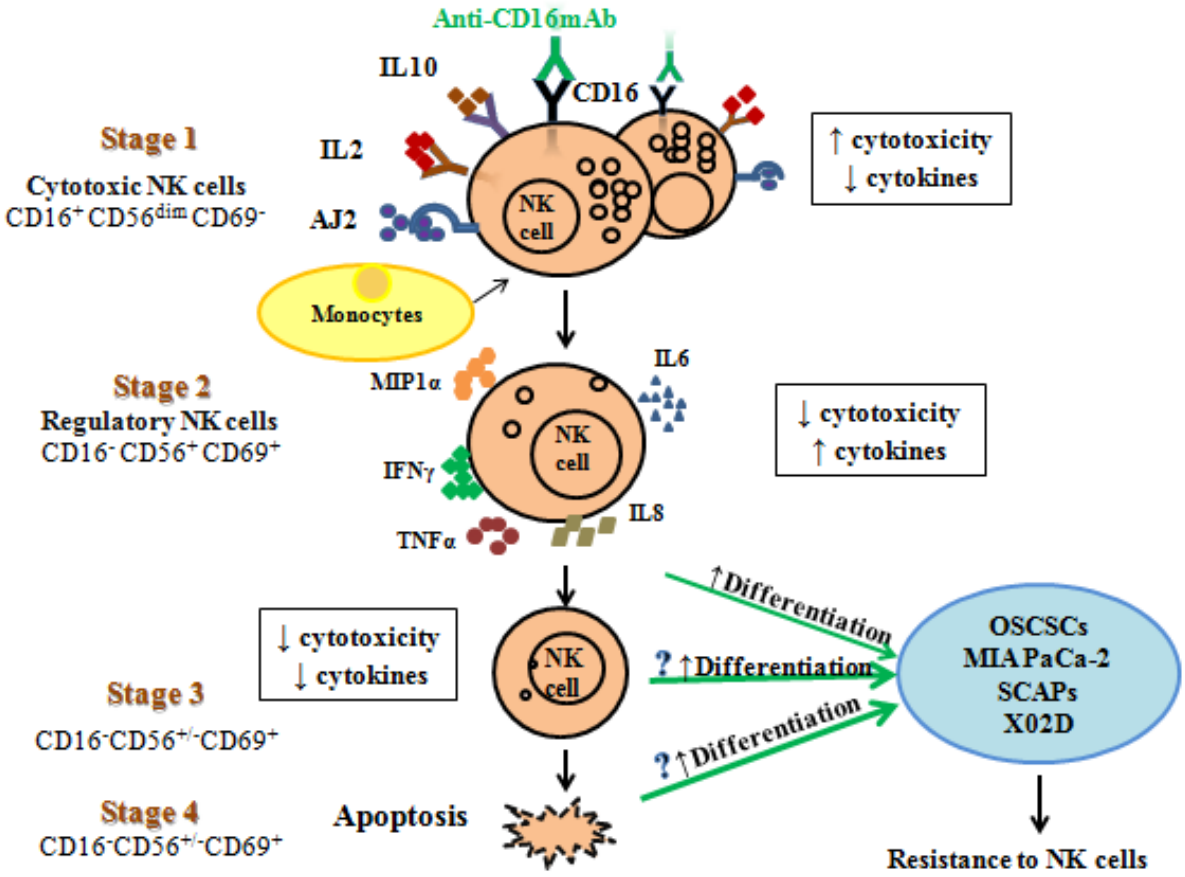



**Figure 78: A schematic representation of hypothetical model of stages of differentiation of NK cells to support maturation of OSCSCs, MIA PaCa-2, X02D and SCAPs**

There are four hypothetical stages of NK cells. In **Stage 1**, the NK cells are highly cytotoxic and they secrete low level of cytokines, this stage is known as "Cytotoxic NK cells". In **Stage 2**, by interacting with IL-2, anti-CD16mAb, monocytes, anti-10mAb, and probiotic bacteria, the NK cells are conditioned to lose cytotoxicity and gain the ability to secrete cytokines to support tissue healing, maturation and differentiation of transformed and non-transformed stem cells; this stage is known as "Regulatory NK cells". In **Stage 3**, the NK cells lose both cytotoxicity and cytokines secretion ability; their ability to support differentiation is still not clear. In **Stage 4**, the NK cells undergo apoptosis and their ability to support differentiation is still not clear.

# Fig. 79

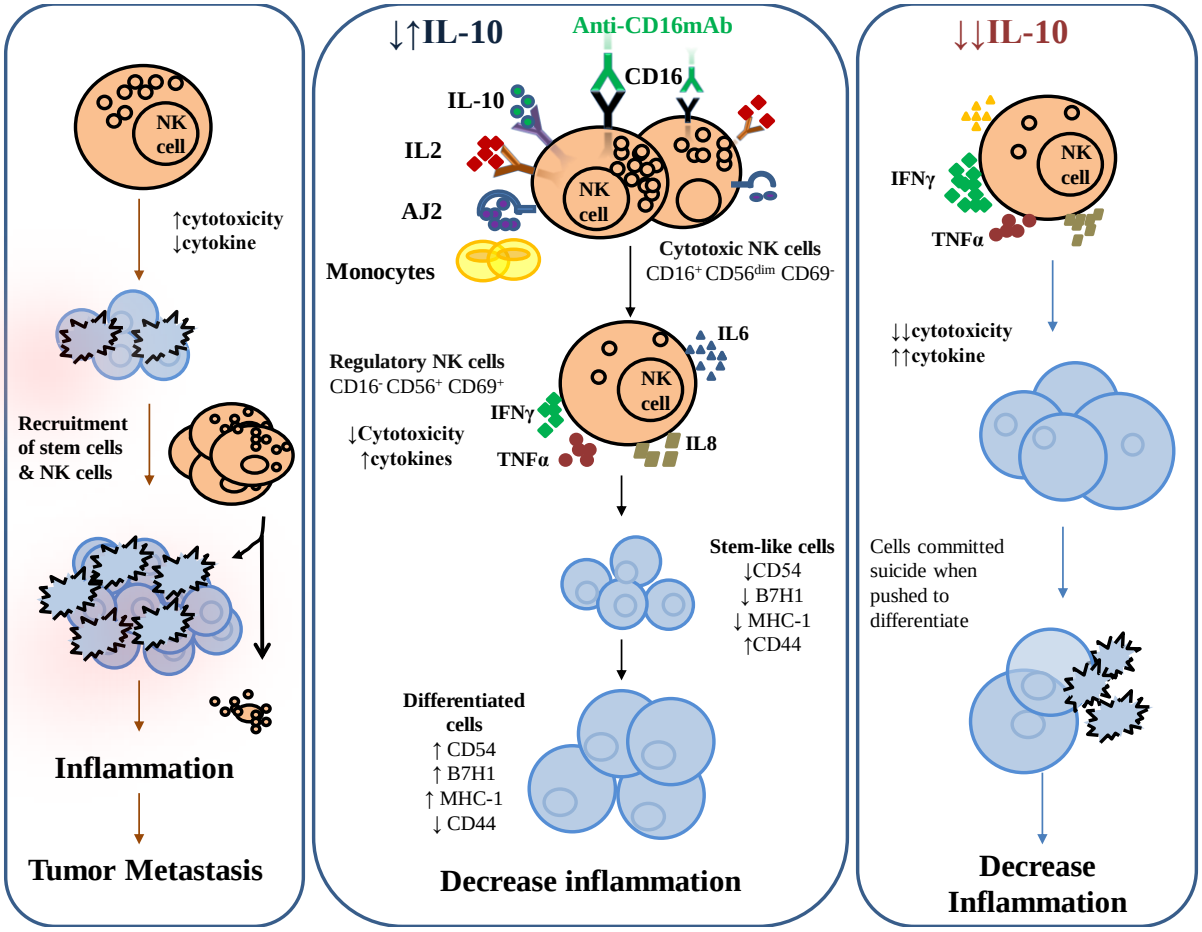



**Figure 79: Schematic representation of IL-10 regulating differentiation of stem cells**

*Left*: NK cells are cytotoxic and are not triggered to secrete cytokines IL-10 or IFN-γ. They kill stem cells and conditioned to support differentiation of the stem cells. However, tumor progresses much faster than NK cells can keep up to mediate cytotoxicity. The end stage of cancer happens when NK cells are depleted, tumor proliferate, exacerbate inflammation and tumor metastasis occurs.
*Middle*: A balanced of pro-and anti-inflammatory cytokines supported differentiation of stem cells in a balance state and less inflammation occurs when there was no cell death.
*Right*: Blocking IL-10 pushed NK cells to differentiate stem cells much faster and caused differentiated stem cells to commit suicide. The lost of differentiated cells required more regulatory NK cells to differentiate new cells. The death of differentiated cells does not cause inflammation.

## REFERENCES


1. Tseng, H., et al., *Increased Lysis of Stem Cells but Not Their Differentiated Cells by Natural Killer Cells; De-Differentiation or Reprogramming Activates NK Cells.* Plos One, 2010. **5**(7).
2. Shida, K., et al., *Essential roles of monocytes in stimulating human peripheral blood mononuclear cells with Lactobacillus casei to produce cytokines and augment natural killer cell activity.* Clinical and Vaccine Immunology, 2006. **13**(9): p. 997-1003.
3. Jewett, A. and H.C. Tseng, *Tumor Induced Inactivation of Natural Killer Cell Cytotoxic Function; Implication in Growth, Expansion and Differentiation of Cancer Stem Cells.* Journal of Cancer, 2011. **2**: p. 443-457.
4. Trinchieri, G., *BIOLOGY OF NATURAL-KILLER CELLS.* Advances in Immunology, 1989. **47**: p. 187-376.
5. Ballas, Z.K., et al., *A PATIENT WITH SIMULTANEOUS ABSENCE OF CLASSICAL NATURAL-KILLER-CELLS (CD3-, CD16+, AND NKH1+) AND EXPANSION OF CD3+, CD4-, CD8-, NKH1+ SUBSET.* Journal of Allergy and Clinical Immunology, 1990. **85**(2): p. 453-459.
6. Farag, S.S., et al., *Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect.* Blood, 2002. **100**(6): p. 1935-1947.
7. Lebow, L.T. and B. Bonavida, *PURIFICATION AND CHARACTERIZATION OF CYTOLYTIC AND NONCYTOLYTIC HUMAN NATURAL-KILLER-CELL SUBSETS.* Proceedings of the National Academy of Sciences of the United States of America, 1990. **87**(16): p. 6063-6067.
8. Cooper, M.A., et al., *Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset.* Blood, 2001. **97**(10): p. 3146-3151.
9. Lanier, L.L., et al., *THE RELATIONSHIP OF CD16 (LEU-11) AND LEU-19 (NKH-1) ANTIGEN EXPRESSION ON HUMAN PERIPHERAL-BLOOD NK CELLS AND CYTOTOXIC LYMPHOCYTES-T.* Journal of Immunology, 1986. **136**(12): p. 4480-4486.
10. Nagler, A., et al., *COMPARATIVE STUDIES OF HUMAN FCRIII-POSITIVE AND NEGATIVE NATURAL-KILLER CELLS.* Journal of Immunology, 1989. **143**(10): p. 3183-3191.
11. Bauer, S., et al., *Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA.* Science, 1999. **285**(5428): p. 727-729.
12. Wicha, M.S., S.L. Liu, and G. Dontu, *Cancer stem cells: An old idea - A paradigm shift.* Cancer Research, 2006. **66**(4): p. 1883-1890.
13. Goodison, S., V. Urquidi, and D. Tarin, *CD44 cell adhesion molecules.* Journal of Clinical Pathology-Molecular Pathology, 1999. **52**(4): p. 189-196.
14. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(7): p. 3983-3988.
15. Krump, M. and J. Ehrmann, *Differences in CD44s expression in HNSCC tumours of different areas within the oral cavity.* Biomedical Papers-Olomouc, 2013. **157**(4): p. 280-283.
16. Galandrini, R., et al., *ANTIBODIES TO CD44 TRIGGER EFFECTOR FUNCTIONS OF HUMAN T-CELL CLONES.* Journal of Immunology, 1993. **150**(10): p. 4225-4235.

17. Galandrini, R., et al., *HYALURONATE IS COSTIMULATORY FOR HUMAN T-CELL EFFECTOR FUNCTIONS AND BINDS TO CD44 ON ACTIVATED T-CELLS.* Journal of Immunology, 1994. **153**(1): p. 21-31.
18. Bourguignon, L.Y.W., et al., *HYALURONIC ACID-INDUCED LYMPHOCYTE SIGNAL-TRANSDUCTION AND HA RECEPTOR (GP85/CD44)-CYTOSKELETON INTERACTION.* Journal of Immunology, 1993. **151**(12): p. 6634-6644.
19. Sconocchia, G., J.A. Titus, and D.M. Segal, *CD44 IS A CYTOTOXIC TRIGGERING MOLECULE IN HUMAN PERIPHERAL-BLOOD NK CELLS.* Journal of Immunology, 1994. **153**(12): p. 5473-5481.
20. Tan, P.H.S., et al., *ENHANCEMENT OF NATURAL-KILLER ACTIVITY BY AN ANTIBODY TO CD44.* Journal of Immunology, 1993. **150**(3): p. 812-820.
21. Francisco, L.M., P.T. Sage, and A.H. Sharpe, *The PD-1 pathway in tolerance and autoimmunity.* Immunological Reviews, 2010. **236**: p. 219-242.
22. Chao, M.P., I.L. Weissman, and R. Majeti, *The CD47-SIRP alpha pathway in cancer immune evasion and potential therapeutic implications.* Current Opinion in Immunology, 2012. **24**(2): p. 225-232.
23. Zhang, X.W., et al., *Structural and functional analysis of the costimulatory receptor programmed death-1.* Immunity, 2004. **20**(3): p. 337-347.
24. Guleria, I., et al., *A critical role for the programmed death ligand 1 in fetomaternal tolerance.* Journal of Experimental Medicine, 2005. **202**(2): p. 231-237.
25. Vanseventer, G.A., et al., *THE LFA-1 LIGAND ICAM-1 PROVIDES AN IMPORTANT COSTIMULATORY SIGNAL FOR T-CELL RECEPTOR-MEDIATED ACTIVATION OF RESTING T-CELLS.* Journal of Immunology, 1990. **144**(12): p. 4579-4586.
26. Storkus, W.J. and J.R. Dawson, *TARGET STRUCTURES INVOLVED IN NATURAL KILLING (NK) - CHARACTERISTICS, DISTRIBUTION, AND CANDIDATE MOLECULES.* Critical Reviews in Immunology, 1991. **10**(5): p. 393-416.
27. Patarroyo, M., et al., *LEUKOCYTE-CELL ADHESION - A MOLECULAR PROCESS FUNDAMENTAL IN LEUKOCYTE PHYSIOLOGY.* Immunological Reviews, 1990. **114**: p. 67-108.
28. Ritz, J., et al., *CHARACTERIZATION OF FUNCTIONAL SURFACE-STRUCTURES ON HUMAN NATURAL-KILLER CELLS.* Advances in Immunology, 1988. **42**: p. 181-211.
29. Schmidt, R.E., et al., *FUNCTIONAL-CHARACTERIZATION OF LFA-1 ANTIGENS IN THE INTERACTION OF HUMAN NK CLONES AND TARGET-CELLS.* Journal of Immunology, 1985. **135**(2): p. 1020-1025.
30. Maki, G., et al., *Induction of sensitivity to NK-mediated cytotoxicity by TNF-alpha treatment: possible role of ICAM-3 and CD44.* Leukemia, 1998. **12**(10): p. 1565-1572.
31. Maciejewski, J., et al., *FAS ANTIGEN EXPRESSION ON CD34(+) HUMAN MARROW-CELLS IS INDUCED BY INTERFERON-GAMMA AND TUMOR-NECROSIS-FACTOR-ALPHA AND POTENTIATES CYTOKINE-MEDIATED HEMATOPOIETIC SUPPRESSION IN-VITRO.* Blood, 1995. **85**(11): p. 3183-3190.
32. Balkwill, F., *TNF-alpha in promotion and progression of cancer.* Cancer and Metastasis Reviews, 2006. **25**(3): p. 409-416.
33. Billiau, A., et al., *Immunomodulatory properties of interferon-gamma - An update*, in *Molecular Mechanisms of Fever*, M.J. Kluger, T. Bartfai, and C.A. Dinarello, Editors. 1998. p. 22-32.

34. Robertson, M.J. and J. Ritz, *BIOLOGY AND CLINICAL RELEVANCE OF HUMAN NATURAL-KILLER-CELLS.* Blood, 1990. **76**(12): p. 2421-2438.
35. Arase, H., N. Arase, and T. Saito, *Interferon gamma production by natural killer (NK) cells and NK1.1(+) T cells upon NKR-P1 cross-linking.* Journal of Experimental Medicine, 1996. **183**(5): p. 2391-2396.
36. Long, E.O., *Negative signaling by inhibitory receptors: the NK cell paradigm.* Immunological Reviews, 2008. **224**: p. 70-84.
37. Moretta, L., et al., *Human NK cells and their receptors.* Microbes and Infection, 2002. **4**(15): p. 1539-1544.
38. Ikeda, H., L.J. Old, and R.D. Schreiber, *The roles of IFN gamma in protection against tumor development and cancer immunoediting.* Cytokine & Growth Factor Reviews, 2002. **13**(2): p. 95-109.
39. Balkwill, F., *Tumour necrosis factor and cancer.* Nature Reviews Cancer, 2009. **9**(5): p. 361-371.
40. Wang, R.P., et al., *Natural killer cell-produced IFN-gamma and TNF-alpha induce target cell cytolysis through up-regulation of ICAM-1*. Journal of Leukocyte Biology, 2012. **91**(2): p. 299-309.
41. Fuller, R., *PROBIOTICS IN MAN AND ANIMALS.* Journal of Applied Bacteriology, 1989. **66**(5): p. 365-378.
42. Fotiadis, C.I., et al., *Role of probiotics, prebiotics and synbiotics in chemoprevention for colorectal cancer.* World Journal of Gastroenterology, 2008. **14**(42): p. 6453-6457.
43. Ustunol, Z. and J.J. Pestka, *Probiotics in health: Their immunomodulatory potential against allergic disorders.* Journal of Animal Science, 2004. **82**: p. 274-274.
44. Fuccio, L., et al., *Effects of Probiotics for the Prevention and Treatment of Radiation-induced Diarrhea.* Journal of Clinical Gastroenterology, 2009. **43**(6): p. 506-513.
45. Shida, K. and M. Nanno, *Probiotics and immunology: separating the wheat from the chaff.* Trends in Immunology, 2008. **29**(11): p. 565-573.
46. Sancho, D., M. Gomez, and F. Sanchez-Madrid, *CD69 is an immunoregulatory molecule induced following activation.* Trends in Immunology, 2005. **26**(3): p. 136-140.
47. Orleans-Lindsay, J.K., et al., *Acute myeloid leukaemia cells secrete a soluble factor that inhibits T and NK cell proliferation but not cytolytic function - implications for the adoptive immunotherapy of leukaemia.* Clinical and Experimental Immunology, 2001. **126**(3): p. 403-411.
48. Tsuboi, I., et al., *NONSTEROIDAL ANTIINFLAMMATORY DRUGS DIFFERENTIALLY REGULATE CYTOKINE PRODUCTION IN HUMAN-LYMPHOCYTES - UP-REGULATION OF TNF, IFN-GAMMA AND IL-2, IN CONTRAST TO DOWN-REGULATION OF IL-6 PRODUCTION*. Cytokine, 1995. **7**(4): p. 372-379.
49. Miescher, S., et al., *PREFERENTIAL CLONOGENIC DEFICIT OF CD8-POSITIVE LYMPHOCYTES-T INFILTRATING HUMAN SOLID TUMORS.* Cancer Research, 1988. **48**(24): p. 6992-6998.
50. Han, X., et al., *Tumor lymphocytes in patients with advanced ovarian cancer: Changes during in vitro culture and implications for immunotherapy*. Gynecologic Oncology, 1997. **65**(3): p. 391-398.
51. Mulder, W.M.C., et al., *T cell receptor-zeta and granzyme B expression in mononuclear cell infiltrates in normal colon mucosa and colon carcinoma.* Gut, 1997. **40**(1): p. 113-119.

52. Nakagomi, H., et al., *DECREASED EXPRESSION OF THE SIGNAL-TRANSDUCING ZETA-CHAINS IN TUMOR-INFILTRATING T-CELLS AND NK CELLS OF PATIENTS WITH COLORECTAL-CARCINOMA.* Cancer Research, 1993. **53**(23): p. 5610-5612.
53. Jewett, A. and B. Bonavida, *Target-induced inactivation and cell death by apoptosis in a subset of human NK cells.* Journal of Immunology, 1996. **156**(3): p. 907-915.
54. JEWETT, A. and B. BONAVIDA, *TARGET-INDUCED ANERGY OF NATURAL-KILLER CYTOTOXIC FUNCTION IS RESTRICTED TO THE NK-TARGET CONJUGATE SUBSET.* Cellular Immunology, 1995. **160**(1): p. 91-97.
55. Jewett, A., et al., *Differential secretion of TNF-alpha and IFN-gamma by human peripheral blood-derived NK subsets and association with functional maturation.* Journal of Clinical Immunology, 1996. **16**(1): p. 46-54.
56. Ortaldo, J.R., A.T. Mason, and J.J. Oshea, *RECEPTOR-INDUCED DEATH IN HUMAN NATURAL-KILLER-CELLS - INVOLVEMENT OF CD16.* Journal of Experimental Medicine, 1995. **181**(1): p. 339-344.
57. Azzoni, L., et al., *LIGAND-BINDING TO FC-GAMMA-R INDUCES C-MYC-DEPENDENT APOPTOSIS IN IL-2-STIMULATED NK CELLS.* Journal of Immunology, 1995. **154**(2): p. 491-499.
58. Jewett, A., M. Cavalcanti, and B. Bonavida, *Pivotal role of endogenous TNF-alpha in the induction of functional inactivation and apoptosis in NK cells.* Journal of Immunology, 1997. **159**(10): p. 4815-4822.
59. Jewett, A., et al., *Strategies to Rescue Mesenchymal Stem Cells (MSCs) and Dental Pulp Stem Cells (DPSCs) from NK Cell Mediated Cytotoxicity.* Plos One, 2010. **5**(3).
60. Jewett, A., Y.G. Man, and H.C. Tseng, *Dual Functions of Natural Killer Cells in Selection and Differentiation of Stem Cells; Role in Regulation of Inflammation and Regeneration of Tissues.* Journal of Cancer, 2013. **4**(1): p. 12-24.
61. Jewett, A., et al., *Natural Killer Cells Preferentially Target Cancer Stem Cells; Role of Monocytes in Protection Against NK Cell Mediated Lysis of Cancer Stem Cells.* Current Drug Delivery, 2012. **9**(1): p. 5-16.
62. Bonavida, B., L.T. Lebow, and A. Jewett, *NATURAL-KILLER-CELL SUBSETS - MATURATION, DIFFERENTIATION AND REGULATION.* Natural Immunity, 1993. **12**(4-5): p. 194-208.
63. Jewett, A., et al., *Differential secretion of TNF-alpha and IFN-gamma by human peripheral blood-derived NK subsets and association with functional maturation.* Journal of Clinical Immunology, 1996. **16**(1): p. 46-54.
64. Marco, M.L., S. Pavan, and M. Kleerebezem, *Towards understanding molecular modes of probiotic action.* Current Opinion in Biotechnology, 2006. **17**(2): p. 204-210.
65. Mego, M., et al., *Probiotic bacteria in cancer patients undergoing chemotherapy and radiation therapy.* Complementary Therapies in Medicine, 2013. **21**(6): p. 712-723.
66. Massano, J., et al., *Oral squamous cell carcinoma: Review of prognostic and predictive factors.* Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics, 2006. **102**(1): p. 67-76.
67. Brinkman, B.M.N. and D.T. Wong, *Disease mechanism and biomarkers of oral squamous cell carcinoma.* Current Opinion in Oncology, 2006. **18**(3): p. 228-233.
68. Leite, I.C.G. and S. Koifman, *Survival analysis in a sample of oral cancer patients at a reference hospital in Rio de Janeiro, Brazil.* Oral Oncology, 1998. **34**(5): p. 347-352.

69. Lo, W.L., et al., *Outcomes of oral squamous cell carcinoma in Taiwan after surgical therapy: Factors affecting survival.* Journal of Oral and Maxillofacial Surgery, 2003. **61**(7): p. 751-758.
70. Itoh, S., et al., *Immunohistochemical study on overexpression of cyclooxygenase-2 in squamous cell carcinoma of the oral cavity: its importance as a prognostic predictor.* Oral Oncology, 2003. **39**(8): p. 829-835.
71. Terakado, N., et al., *Overexpression of cyclooxygenase-2 is associated with radioresistance in oral squamous cell carcinoma.* Oral Oncology, 2004. **40**(4): p. 383-389.
72. *Cancer Facts and Figure 2012.* 2012.
73. Takano, H.-C.Z.a.Y., *NNK-Induced Lung Tumors: A Review of Animal Model.* J. Oncol., 2011.
74. Bhatnagar, S., D.P. Katare, and S.K. Jain, *Serum-based protein biomarkers for detection of lung cancer.* Central European Journal of Biology, 2014. **9**(4): p. 341-358.
75. Vollmer, E., et al., *Tumors in the lung - morphologic features and the challenge of integrating biomarker signatures into diagnostics.* Romanian Journal of Morphology and Embryology, 2010. **51**(4): p. 607-614.
76. Jewett, A. and B. Bonavida, *PIVOTAL ROLE OF ENDOGENOUS TNF-ALPHA IN THE IL-2-DRIVEN ACTIVATION AND PROLIFERATION OF THE FUNCTIONALLY IMMATURE NK FREE SUBSET.* Cellular Immunology, 1993. **151**(2): p. 257-269.
77. Helwig, U., et al., *Lactobacilli, bifidobacteria and E-coli nissle induce pro- an anti-inflammatory cytokines in peripheral blood mononuclear cells.* World Journal of Gastroenterology, 2006. **12**(37): p. 5978-5986.
78. Juelke, K., et al., *CD62L expression identifies a unique subset of polyfunctional CD56(dim) NK cells.* Blood, 2010. **116**(8): p. 1299-1307.
79. De Maria, A., et al., *Revisiting human natural killer cell subset function revealed cytolytic CD56(dim)CD16(+) NK cells as rapid producers of abundant IFN-gamma on activation.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(2): p. 728-732.
80. Kuss, I., et al., *Clinical significance of decreased zeta chain expression in peripheral blood lymphocytes of patients with head and neck cancer.* Clinical Cancer Research, 1999. **5**(2): p. 329-334.
81. Lai, P., et al., *Alterations in expression and function of signal transducing proteins in tumor-associated T and natural killer cells in ovarian carcinoma.* Biochemical Society Transactions, 1997. **25**(2): p. S218-S218.
82. Lammers, K.M., et al., *Immunomodulatory effects of probiotic bacteria DNA: IL-1 and IL-10 response in human peripheral blood mononuclear cells.* Fems Immunology and Medical Microbiology, 2003. **38**(2): p. 165-172.
83. Jewett, A., C. Head, and N. Cacalano, *Emerging mechanisms of immunosuppression in oral cancers.* Journal of Dental Research, 2006. **85**(12): p. 1061-1073.
84. Aggarwal, S. and M.F. Pittenger, *Human mesenchymal stem cells modulate allogeneic immune cell responses.* Blood, 2005. **105**(4): p. 1815-1822.
85. Selmani, Z., et al., *Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4(+)CD25(high)FOXP3(+) regulatory T cells.* Stem Cells, 2008. **26**(1): p. 212-222.

86. Sheng, H.M., et al., *A critical role of IFN gamma in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1.* Cell Research, 2008. **18**(8): p. 846-857.
87. Ren, G., et al., *Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide.* Cell Stem Cell, 2008. **2**(2): p. 141-150.
88. Most, J., et al., *REGULATION OF THE EXPRESSION OF ICAM-1 ON HUMAN MONOCYTES AND MONOCYTIC TUMOR-CELL LINES.* Journal of Immunology, 1992. **148**(6): p. 1635-1642.
89. Bui, J.D., et al., *IFN-Dependent down-regulation of the NKG2D ligand H60 on tumors.* Journal of Immunology, 2006. **176**(2): p. 905-913.
90. Scott, M.J., et al., *Genetic background influences natural killer cell activation during bacterial peritonitis in mice, and is interleukin 12 and interleukin 18 independent.* Cytokine, 2004. **28**(3): p. 124-136.
91. Seki, S., et al., *Role of liver NK cells and peritoneal macrophages in gamma interferon and interleukin-10 production in experimental bacterial peritonitis in mice.* Infection and Immunity, 1998. **66**(11): p. 5286-5294.
92. Emmanuilidis, K., et al., *Critical role of Kupffer cell-derived IL-10 for host defense in septic peritonitis.* Journal of Immunology, 2001. **167**(7): p. 3919-3927.
93. Scott, M.J., et al., *Interleukin-10 suppresses natural killer cell but not natural killer T cell activation during bacterial infection.* Cytokine, 2006. **33**(2): p. 79-86.
94. Peppa, D., et al., *Blockade of Immunosuppressive Cytokines Restores NK Cell Antiviral Function in Chronic Hepatitis B Virus Infection.* Plos Pathogens, 2010. **6**(12).
95. Buelens, C., et al., *Interleukin-10 prevents the generation of dendritic cells from human peripheral blood mononuclear cells cultured with interleukin-4 and granulocyte/macrophage-colony-stimulating factor.* European Journal of Immunology, 1997. **27**(3): p. 756-762.
96. Hsu, D.H., K. Moore, and H. Spits., *Differential effects of interleukin-4 and -10 on interleukin-2 induced interferon-y synthesis.* International Immunology, 1992. **4**: p. 563-569.
97. Sher, A., I. P. Oswald, S. Hieny, and R. T. Gazzinelli, *Tosoplasma gondii induces a T-independent IFN-y response in natural killer cells that requires both adherent accessory cells and tumor necrosis factor alpha.* . Jounral of Immunology 1993. **150**: p. 3982-3989.
98. Tripp, C.S., S. F. Wolf, and E.R. Unanue, *Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiological antagonist.* Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**: p. 3725-3729.
99. Gazzinelli, R.T., et al., *INTERLEUKIN-12 IS REQUIRED FOR THE T-LYMPHOCYTE-INDEPENDENT INDUCTION OF INTERFERON-GAMMA BY AN INTRACELLULAR PARASITE AND INDUCES RESISTANCE IN T-CELL-DEFICIENT HOSTS.* Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**(13): p. 6115-6119.
100. Hsieh, C.S., et al., *DEVELOPMENT OF TH1 CD4+ T-CELLS THROUGH IL-12 PRODUCED BY LISTERIA-INDUCED MACROPHAGES.* Science, 1993. **260**(5107): p. 547-549.

101. Gong, J.H., G. Maki, and H.G. Klingemann, *CHARACTERIZATION OF A HUMAN CELL-LINE (NK-92) WITH PHENOTYPICAL AND FUNCTIONAL-CHARACTERISTICS OF ACTIVATED NATURAL-KILLER-CELLS.* Leukemia, 1994. **8**(4): p. 652-658.

102. Sellon RK, Tonkonogy S, Schultz M, Dieleman LA, Grenther W, Balish E, Rennick DM, Sartor RB. *Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice*. Infect Immun. 1998. **66**: p. 5224–31.