UCLA
UCLA Electronic Theses and Dissertations

Title
Preferential induction of NK cell mediated Antibody Dependent Cellular Cytotoxicity (ADCC) against differentiated oral and pancreatic tumors expressing MICA/B

Permalink
https://escholarship.org/uc/item/5p85m9v3

Author
Safaie, Tahmineh

Publication Date
2018

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA

Los Angeles

Preferential induction of NK cell mediated Antibody Dependent Cellular Cytotoxicity (ADCC) against differentiated oral and pancreatic tumors expressing MICA/B

A thesis submitted in partial satisfaction of the

requirements of the degree Master of Science in Oral Biology

by

Tahmineh Safaie

2018

© Copyright by

Tahmineh Safaie

2018

ABSTRACT OF THE THESIS

Preferential induction of

NK cell mediated Antibody Dependent Cellular Cytotoxicity (ADCC)

against differentiated oral and pancreatic tumors expressing MICA/B

by

Tahmineh Safaie

Master of Science in Oral Biology

University of California, Los Angeles, 2018

Professor Anahid Jewett, Chair

Natural Killer cells (NK) cells are known to limit growth and expansion of cancer stem cells by direct cytotoxicity, providing key cytokines such as IFN-γ and TNF-α, which drive differentiation of stem-like/poorly differentiated cancer stem cells and through antibody-dependent cell cytotoxicity (ADCC). One of the important surface antigens on transformed cells is Major Histocompatibility Complex class I chain-related protein A and B (MICA/MICB). Induction of NK cell-mediated cytotoxicity resistance and differentiation in the stem cells correlate with the increased expression of CD54, B7H1, and MHCI, and MICA/MICB and antibodies specific to MICA/MICB increased NK cell-mediated ADCC against differentiated oral and pancreatic tumors, while the stem-like/poorly differentiated cancer stem cells were not targeted. This antibody also increased IFN-γ secretion by NK cells when cultured with differentiated oral tumors expressing MICA/MICB. This study also showed that Expanded NK cells target both

undifferentiated and differentiated tumor cells while primary NK cells preferentially target undifferentiated/Stem-like population. In addition, it showed that the combination of IL-2 and antiCD16 treatment induces split anergy in primary NK cells but not in super-charged NK cells and Expanded NK cells were found to mediate lower levels of ADCC than primary NK cells. Knowing the key role of IFN-γ production in differentiation of oral and pancreatic cancer cells, then aimed to determine strategies to increase NK cell-mediated production of IFN-γ and we showed that AJ2 probiotic bacteria and fucoidan extracted from Mekabu could individually increase NK cells' IFN-γ secretion ability. The combination of AJ2 probiotic bacteria and fucoidan extracted from Mekabu resulted in synergic secretion of IFN-γ by NK cells. In conclusion, differentiated and stem-like/ poorly differentiated oral and pancreatic tumors have different expression level of MICA/MICB and can be targeted differentially through ADCC. Primary and Expanded NK cells have very different characteristics and biological functions and all the diverse functions of different subsets of NK cells should be considered in NK cell-immunotherapeutic approaches.

The thesis of Tahmineh Safaie is approved.

Ichiro Nishimura

Nicholas A. Cacalano

Ki-hyuk Shin

Anahid Jewett, Committee Chair

University of California, Los Angeles

2018

DEDICATION

I dedicate this thesis to the women of White Wednesdays campaign in Iran who are fighting bravely for their rights.

TABLE OF CONTENT

Abstract ii
Dedication v
Acknolewdgement vii
Introduction 1
Purpose of the study 13
Methods and Materials 16
Chapter 1: 22
Chapter.2: 33
Chapter.3 40
Discussion 44
Conclusion 49

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Anahid Jewett. I have been extremely lucky to have a mentor who is intelligent, patient, and kind. Thank you for always pushing me and being an incredible role model.

I would like to thank the Committee member for all their time and advise.

I would like to acknowledge my lab mates, especially Meng-wei Ko, Jessica cook and Christin Sutanto who have been my mentors, colleagues and friends. I could not have done this without each one of you!

Thank you Elahe Nadeali. You made my dream come true. Words cannot describe your Kindness.

Finally, heartfelt thanks go out to my beloved husband for his immense sacrifice. Thank you for always supporting my dreams and being my best friend.

Introduction

Natural Killer cells

Natural killer (NK) cells are a subset of lymphocytes with lytic ability, which are known to function within the interface of innate and adaptive immunity. They constitute 10% of peripheral blood mononuclear cells in human blood and are identified by their lack of surface expression of CD3 and positive expressions of CD16 and CD56. NK cells mediate both direct and antibody-dependent cellular cytotoxicity (ADCC) against tumor cells and virally infected cells. They can recognize these cells without prior sensitization [1]. NK cells mediate direct cytotoxicity by releasing pre-formed granules known as perforin and granzyme B, which can induce necrosis and apoptosis. When NK cells recognize their target cells and form the lytic immunological synapse, the secretory lysosome polarizes towards the synapse and move into close proximity with the plasma membrane [2]. Perforin, a membrane-disrupting protein, facilitates delivery of the Granzyme, a serine protease, which cleaves a variety of targets, such as caspases, resulting in cell death [3,4]. NK cells can also mediate cytotoxicity via death receptors on the target cells through surface expression of their ligands such as Fas Ligand, Trail, and TNF-alpha. Fas (CD95/APO-1/TNFRSF6), a cell surface protein that belongs to the tumor necrosis factor receptor family, can mediate apoptosis when bound to its natural ligand, CD95L (CD178/TNFSF6) or stimulated with agonistic antibodies [5]. NK cells can mediate antibody dependent cellular cytotoxicity (ADCC) against tumors and regulate the function of other cells through the secretion of cytokines and chemokines [6].

Two major subsets of NK cells have been identified, one with the surface expression of $CD16^{+++}CD56^{+}$, which is the predominant subset in the circulating blood with high cytotoxicity, whereas the other is $CD16^{-}CD56^{+++}$ subset residing in the mucosa known as the regulatory subset.

Our laboratory has established four different stages of NK cell maturation. Stage one NK cells are $CD16^{+++}$, $CD56^{+}$, $CD69^{-}$, and $CD107a^{-}$ found to select and kill cancer stem-like cells/undifferentiated tumors. Upon IL-2 activation and CD16 receptor triggering, NK cells express $CD16^{+/-}CD56^{++}CD69^{+}CD107a^{+}$ and increase secretion of IFN-γ and TNF-α while exhibiting decreased cytotoxicity. This is the second stage and NK cells in this stage are known as split-anergized NK cells. Without further activation NK cells move towards stage three where they become non-functional and lose their cytotoxicity and cytokine secretion ability. Finally, NK cells may undergo apoptosis giving rise to stage 4 [7] (Fig.1).

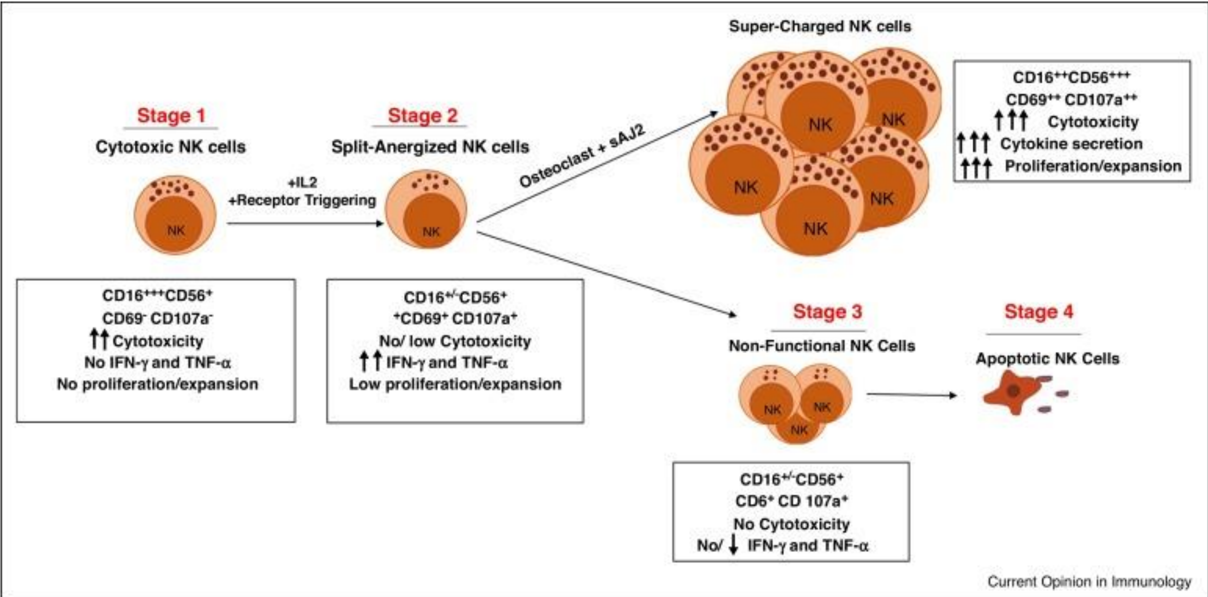


Figure.1 Maturational stages of NK cells.

Antibody-dependent cell-cytotoxicity

Antibody-dependent cellular-cytotoxicity (ADCC), is a mechanism by which immune cells bearing the Fc receptor can kill cells coated with the antibody, upon binding of the Fc receptor to the Fc portion of the antibody. NK cells are one the subset of immune cells that can mediate ADCC through the FcγRIIIA receptor also known as CD16 [6]. The mechanism by which NK cells mediate ADCC is not fully understood. When the effector cell recognizes the target by cross-linking of the Fc receptor and the antibody coating the target cell, the immunoreceptor tyrosine-based activation motifs (ITAMs) gets phosphorylated in the effector cells and leads to the triggering of main downstream signaling pathways in the effector cell to kill the target cell [8]. One of the mechanisms by which NK cells mediate ADCC can be through perforin-granzyme mediated cytotoxicity. [8,9,10]. The role of the FAS ligand in ADCC is unknown but it has been shown that cross-linking of the CD16 receptor on NK cells can upregulate FAS ligand on them which may be indicative of an important role of Fas/Fas-L in ADCC [11].

AJ2 probiotic Bacteria

AJ2 is a combination of eight strains of gram positive probiotic bacteria with the ability to induce synergistic production of IFN-γ when added to IL-2-treated or IL-2 + anti-CD16 monoclonal antibody-treated NK cells (anti-CD16mAb). The combination of strains was used to provide bacterial diversity in addition to synergistic induction of a balanced pro and anti-inflammatory cytokine and growth factor release in NK cells. Moreover, the quantity of each bacteria within the combination of strains was adjusted to yield a closer ratio of IFN-γ to IL-10 to that obtained when NK cells are activated with IL-2 + anti-CD16mAb in the absence of bacteria. The rationale behind such selection was to obtain a ratio similar to that obtained with NK cells activated with

IL-2 + anti-CD16mAb in the absence of bacteria since such treatment provided significant differentiation of the cells [12, 13].

Fucoidan

Fucoidan is a sulfated polysaccharide found on different species of brown algae and brown seaweed such as mozuku, mekabu, limu moui, bladderwrack, and hijiki. Based on the source of extraction, fucoidans may have different chemical compositions. For example, beside the polysaccharide and the sulfate, they might also contain other monosaccharides (e.g. mannose, galactose, glucose, xylose, etc.), uronic acids, acetyl groups, and proteins. For the past decade, fucoidans isolated from different species have been studied due to their varied biological activities, including antitumor and immunomodulatory, and properties [14].

Undaria pinnatifida, also known as mekabu seaweed is another source of fucoidans composed of fucose, galactose, and sulfate. Studies showed that fucoidan can modulate different immune cells such as neutrophils, dendritic cells, and macrophages. It has also been shown that injection of fucoidans inhibited the growth of leukemia in mice through enhancement of NK cells IFN-γ secretion ability [15-17].

Split Anergy

Our laboratory coined the term 'split anergy' to explain the relationship of reduced NK cell cytotoxicity in the presence of augmented secretion of IFN- γ [13]. Split-anergized NK cells promote differentiation of target cells via secreted and membrane-bound factors, increases tumor cell resistance to NK cell-mediated cytotoxicity, as well as inhibits inflammation due to the reduction of cytokine and chemokine production after tumor differentiation [18-23].

Cancer stem cells

Cancer stem cells (CSCs) are the progenies which can create various population of differentiated cells that define the tumor mass. CSCs are like normal stem cells has self-renewal capacity and also can be differentiated but in a dysregulated manner [24]. Bonnet *et al*. were the first to isolate CSCs from acute myeloid leukemia samples [25]. The CSCs from solid tumors were first isolated and identified in breast cancer by Al-Hajj *et al*. They isolated a subpopulation of CD44 cells within the breast cancer tissues having the high tumorigenic capacity [26]. The existence of CSCs is also described in other solid tumors including prostate, melanoma, lung, colon, brain, liver, gastric and pancreatic cancer. [27-33].

Different models have been proposed to explain how CSCs play the role in tumor initiation and heterogenicity, including the CSC or hierarchical model (Fig.2 A) and the stochastic or evolution model (Fig.2 B). Fig.2 C shows a model we proposed. There is a good correlation between our model of tumor development from CSCs and those of the hierarchical and stochastic model proposed previously [34, 35]. In the hierarchical model, CSCs can self-renew and are responsible for the generation of progenies which may or may not have the ability to give rise to tumors [36-38]. In agreement, the well-differentiated tumors in our model have limited capability to proliferate or establish themselves *in vivo*, potentially resembling the progenies within the hierarchical model that lack the ability to establish tumors [39,1, 2]. On the other hand, undifferentiated tumors and potentially moderately differentiated tumors in our model and those of the hierarchical model will be able to establish tumors [2]. There is also a good co-relation with the stochastic model since removal of NK supernatants from the well-differentiated tumors and their *in vitro* culture for over two weeks gradually resulted in the loss of MHC class I and reversion of these tumors to undifferentiated tumors which were capable of establishing tumors *in vivo* [13,40] (Fig. 1). It is of note that when NK-differentiated tumors were implanted in NSG mice no or very slight growth

were seen within the time frames that we studied, which is in contrast to what was observed *in vitro* [2]. Whether immune cells other than NK and T cells or non-immune cells in NSG mice are responsible for exerting continuous pressure on well-differentiated tumors to retain them at the quiescent stage, or we did not allow enough time for the tumors to grow and be detected in NSG mice will require future studies [2, 41, 42]. Moreover, suppression of NK function in patients, in addition to progressive mutations or knock-down of cellular genes in tumors is likely responsible for the decrease in MHC class I and de-differentiation of tumor cells which could potentially be responsible for the reversion of tumors to their less differentiated or CSC stage as seen in the stochastic model [43]. Thus, pressures exerted from the micro-environment, notably through the NK cells and other immune effectors may be able to retain the tumor cells at the less proliferative-quiescent stage, whereas, once those pressures are removed the tumors have the chance to revert to their undifferentiated/CSC stage and gain the ability to invade and metastasize.

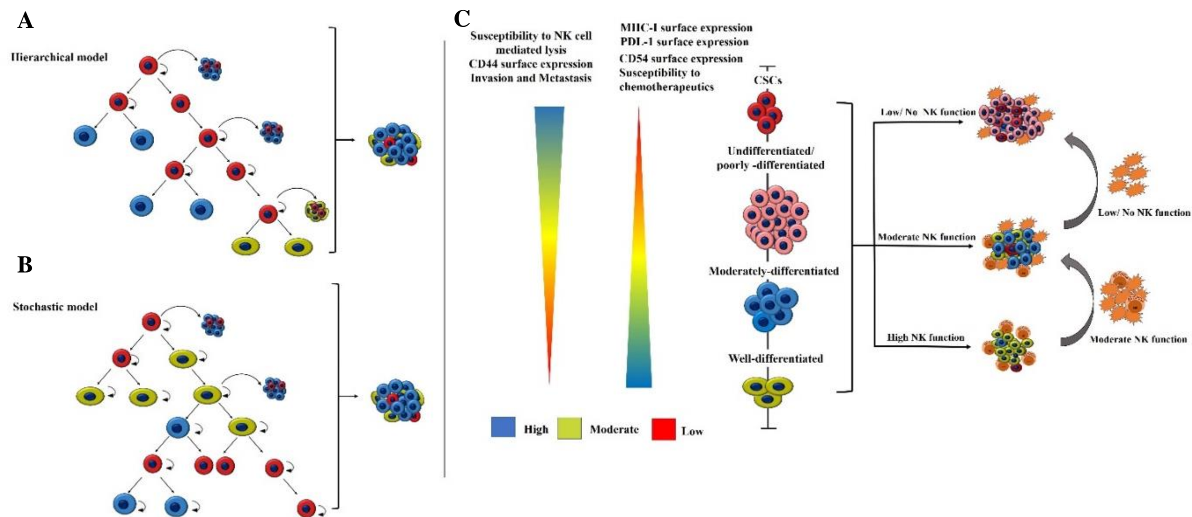


Fig.2 Different models explaining how CSCs contribute to tumor formation

Osteoclasts

Osteoclasts are the large bone cells responsible for bone homeostasis and resorption [44]. Osteoclasts matures via RANKL stimulation and the process is regulated by ICAM-1. Proinflammatory signals can induce expression of ICAM-1 and RANKL on osteoclasts. These signals are mediated by subsets of immune cells. It has been shown that osteoclasts express multiple ligands for both activating and inhibitory NK cell receptors [45].

MICA/MICB

Major Histocompatibility Complex Class I–Related Chains A and B (MICA/MICB) are proteins known to be induced upon stress, damage, viral infection or transformation of cells which act as a 'kill me' signal through the cytotoxic lymphocytes. In contrast to classical MHC class-I molecules, these proteins are not involved in antigen presenting but they are known to be ligands for natural killer group 2D (NKG2D) receptors, a type of receptor on cytotoxic cells. Engagement of NKG2D receptors trigger natural killer (NK) cell-mediated cytotoxicity and provide a costimulatory signal for CD8 T cells and γδ T cells [47]. MICA/B were not thought to be constitutively expressed by healthy normal cells but recently studies have shown that this protein is also expressed on the surface of healthy cells found in the breast, colon, liver, pancreas, stomach, bronchus, bladder and ureter, as well as on smooth muscle cells and/or myofibroblasts within the stomach, small intestine, colon, bladder, cervix, fallopian tube, prostate and ureter [48]. The differential expression of MICA/MICB, based on the differentiation status of the tumor cells, has not be studied. In this study, we will evaluate the expression of MICA/MICB on undifferentiated/stem-like and differentiated oral and pancreatic tumors.

Current state of the art in NK cells immunotherapy

NK cells have very diverse biological functions including significant roles in defense against tumor cells. Based on knowledge of NK cell function and evidence that they become nonfunctional in cancer patients, several approaches have been proposed for the use of NK cells in immunotherapy:

1) Cytokines

Many cytokines such as IL-2, IL-21, IL-12, IL-15, and IFN- γ have been known to activate and boost NK cells function [49]. Cytokines can be used to boost NK cells for immunotherapeutic means, both in vitro and in vivo. Cytokine administration for cancer treatments has been implemented clinically but has never been considered as a success due to severe side-effects and cytotoxicity [50].

2) Antibodies

Antibodies can be applied to NK cell immunotherapy based on different approaches. NK cells can target tumor cells coated with IgG antibodies through the ADCC. There are several monoclonal antibody treatments available specific to different tumor antigens such as the use of anti-CD20 for the treatment of B cell lymphoma or anti-Her2 for the treatment of Her2-overexpressing invasive breast cancer [7]. Antibodies can be also being used to block NK cells' checkpoint inhibitors. Studies have shown that NK cells express checkpoint molecules such as PD-1, CTLA-4, TIM-3 and TIGIT [51]. The role of these checkpoint inhibitor drugs in NK cells have not been well studied.

3) Adoptive transfer of NK cells

Transferring functionally competent NK cells as an immunotherapy approach has been established for many years. NK cells can be harvested from different sources, and their

functional competencies may vary depending on the strategies used to separate, activate or expand them.

Sources of natural killer cells for adoptive transfer:

a) NK cells isolated from Peripheral Blood Mononuclear Cells (PBMCs)

NK cells can be isolated from autologous and allogeneic PBMCs. The strategies to expand and activate NK cells from PBMCs are different. Investigators have tried to Expand NK cells directly from PBMCs without isolating the NK cells population, or by depleting CD3+ cells, or selecting CD16+, or CD16+ and CD56+ cells. Different cells have been used as feeder cells to improve NK cells expansion. Irradiated K562 and OK432 are two of the most popular feeder cells for NK expansions. Different Cytokines and other activators also have been applied to expand NK cells in vitro. IL-2, IL-21, IL-15 and IL-18 are some of the cytokines used for this means [52-54]. Our laboratory has a novel strategy to expand NK cells up to 21,000- to 132,000-fold in 20 days. In this technique NK cells are treated with IL-2 and CD16 antibody and probiotic bacteria and Osteoclasts are used as feeder cells. The Expanded NK cells, called "Super Charged NK cell" have high cytotoxicity and IFN- γ secretion [43].

Autologous NK cells as a source of therapy has not been very effective. We showed that NK cells from cancer patients are less functional both in terms of cytotoxicity and of IFN- γ secretion in vitro [43]. In an in vivo study adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression [55]. In another study autologous NK cells have also demonstrated a limited effect on tumor suppression in malignant glioma [56]. Allogenic NK cells can be a better resource for NK cell therapy since NK cells from healthy donors have better functionality than NK cells in cancer patient [43],

besides the KIR receptor from the donor mismatch the MHC class I of the recipient letting NK cells to skip some of the inhibitory processes [57].

b) Stem cell- derived NK cells

Due to the pluripotency of stem cells, using them as a precursor of NK cells has become one of the interesting sources of NK cells. Generation of NK cells from ESCs and iPSCs generally requires two steps. First, CD34+ hematopoietic precursors must be generated. The CD34+ cells are then sorted and differentiated into NK cells with cytokines and feeder cells (usually murine stromal cells). When NK cells were generated from hESCs, they were mostly CD56+CD45+ NK cells, which also expressed inhibitory and activating receptors typically found on adult NK cells. These NK cells were also able to mediate cytotoxicity against of leukemic cells, K562 (erythroleukemia), and several solid tumors, including breast cancer (MCF7), testicular embryonal carcinoma (NTERA2), prostate cancer (PC3), and glioma (U87) cell lines [58]. A studied showed that when efficacy of Induced pluripotent stem (IPS) cell-derived natural killer cells with NK cells isolated from peripheral blood that had been activated and expanded in long-term culture, and overnight activated Peripheral blood isolated NK cells, were compared, NK cells derived from IPS mediate anti-ovarian cancer killing in NSG mouse at least as well as NK cells isolated from blood [59]. The Kaufman research group established a feeder-free, sorting-free approach to generate NK cells from human ESCs. These authors used a spin-embryoid body (EB) system with BMP4 and VEGF to derive hematopoietic progenitors. After 11 days of culture, the spin-EBs were transferred to the NK cell culture containing the cytokines IL-3, IL-15, IL-7, SCF and Flt3L for 28 days. This feeder-free system can generate NK cells that have no difference from those derived from the murine embryonic liver cell line EL08-1D2 as feeder cell.

Meanwhile, this group established a clinical-scale derivation of NK cells from ESCs and iPSCs without cell sorting and in the absence of feeder cells [60].

c) NK cells isolated from Umbilical Cord blood.

Umbilical cord blood (UCB), has become a known source for NK cells. The NK cells from UCB and peripheral blood (PB) have some differences. UCB NK cells express similar levels of CD56, NKp46, NK30 and NKG2D as PB NK cells but lower levels of CD16 CD2, CD11a, CD18, CD62L), KIRs, DNAM-1, NKG2C, IL-2R, and CD57 and CD8 [61]. UCB has a higher percentage of NK cells but these cells have lower cytotoxicity in comparison to NK cells from PB which could be due to lower levels of Granzyme B and perforin in CB NK cells. Studies have shown that with proper signaling NK cells from UCB can be expanded to create many cells with proper function [62-64].

d) Genetically modified NK cells

The genetic modification can be used to promote the efficacy of NK cells by different means. NK cells can be genetically modified to secrete cytokines in favor of their survival and activation. Engineering Chimeric antigen receptor (CAR) NK cells has currently become the topic of interest. Currently, several tumor antigen-binding domains have been designed as CAR extracellular domains and tested [65-67]. One of the problems associated with genetic modification of NK cells is that these cells are very hard to virally infect and approaches to improve their transfection rate should be introduced.

Although various approaches are being used to use NK cells for cancer therapy, the long-term efficacy of these protocols is not very promising. NK cells have diverse biological functions and to benefit patients the ideal NK cells therapy should focus on boosting all functions of NK cells.

Purpose of the study

Although both direct cytotoxicity and ADCC functions of NK cells have been shown to be crucial for the elimination of tumor cells, it is still unknown whether the same or different subsets of NK cells are able to mediate direct cytotoxicity vs. ADCC. In addition, even though previous reports have shown the increase of MICA/MICB on tumor cells, they did not establish whether expression was limited to undifferentiated or differentiated tumors. In this report we demonstrate that differentiated tumors express MICA/MICB and that primary NK cells are capable of mediating ADCC against differentiated tumors expressing MICA/MICB, whereas super-charged NK cells, even though they express very high levels of direct cytotoxicity, do not mediate appreciable ADCC. In addition, since IFN-γ secretion by the NK cells is crucial for the differentiation of tumors and increased expression of MICA/MICB and increased NK cell-mediated ADCC, we aimed at finding strategies to increase IFN-γ secretion by the NK cells. Collectively, the results establish primary NK cells as mediators of ADCC against MICA/MICB expressing differentiated tumors whereas super-charged NK cells as major effectors in mediating direct cytotoxicity against undifferentiated and differentiated tumors.

Thesis Out line

Specific aim 1: To determine whether NK cells mediate ADCC differentially against well-differentiated and undifferentiated/ stem-like oral and pancreatic tumors.

- Sub-aim 1: S*tudy the differentiation stages of oral and pancreatic tumors based on their surface antigens and their susceptibility to NK cells*
- Sub-aim 2*: To determine whether well-differentiated and undifferentiated/ stem-like oral and pancreatic tumors express distinct levels of MICA/MICB*
- Sub-aim 3*: To study the role of NK cells in mediating ADCC differentially against well-differentiated and undifferentiated/ stem-like oral and pancreatic tumors using antibodies specific to MICA/MICB*
- Sub-aim 4*: To study whether NK cell mediated ADCC through MICA/MICB is responsible for the increased secretion of IFN-γ against well-differentiated and undifferentiated/ stem-like oral tumors*

Specific aim 2: *To delineate the underlying differences between the functions of primary and Expanded NK cells in direct cytotoxicity and ADCC.*

- Sub-aim 1*: To study the primary and Expanded NK cell mediated direct cytotoxicity in undifferentiated and well-differentiated tumor cells*
- Sub-aim 2*: To study whether spilt anergy occurs in both the primary vs Expanded NK cells*
- Sub-aim 3: *Study whether there are differences in NK cell mediated ADCC between primary and Expanded NK cells*

Specific aim 3: We have established that NK cell-mediated IFN-γ production is the key in differentiation of oral and pancreatic cancer cells, therefore we aim at determining strategies to increase NK cell mediated production of IFN-γ

- Sub-aim 1: *Study each of the effect of sAJ2 probiotic bacteria and Mekabue extracted fucoidan on NK cell function.*
- Sub-aim 2: *Study the synergistic effect of AJ2 and Mekabue on IFN-γ secretion by NK cells*

Methods and Materials

Cell Lines, Reagents, and Antibodies

RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, CA, USA) was used for the cultures of human NK cells and monocytes. OSCCs and stem-like OSCSCs were isolated from oral cancer patient tongue tumors at UCLA, and cultured in RPMI 1640 supplemented with 10% FBS (Gemini Bio-Products, CA, USA), 1.4% antibiotic antimycotic, 1% sodium pyruvate, 1.4% non-essential amino acids, 1% L-glutamine, 0.2% gentamicin (Gemini Bio-Products, CA, USA), and 0.15% sodium bicarbonate (Fisher Scientific, PA, USA). Mia-Paca-2 (MP2) was cultured in DMEM with 10% FBS and 1% penicillin and streptomycin (Gemini Bio-Products, CA, USA). Recombinant IL-2 was obtained from NIH-BRB. Recombinant TNF-α and IFN-γ were obtained from BioLegend (San Diego, CA, USA). Anti-MHC class-I was prepared in our laboratory and the 1:100 dilution was found to be the optimal concentration to use. PE conjugated anti-CD54, anti-CD44, anti-B7H1, and anti-MICA/MICB antibodies were obtained from BioLegend (San Diego, CA, USA). Antibody against MICA/MICB was a generous gift from Dr. Jennifer Wu from Feinberg School of Medicine. The human NK and monocyte purification kits were obtained from Stem Cell Technologies (Vancouver, BC, Canada).

Purification of NK cells and T cells from human peripheral blood

Written informed consents, approved by UCLA Institutional Review Board (IRB), were obtained from healthy blood donors, and all procedures were approved by the UCLA-IRB. Peripheral blood was separated using Ficoll-Hypaque centrifugation, after which the white, cloudy layer,

containing peripheral blood mononuclear cells (PBMC), was harvested, washed and resuspended in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% FBS and plated on plastic tissue culture dishes. After 1-2 hours of incubation, non-adherent, human peripheral blood lymphocytes (PBL) were collected. NK cells were negatively selected and isolated from PBLs using the EasySep® Human NK cell enrichment kit and T cells isolation kit, respectively purchased from Stem Cell Technologies (Vancouver, BC, Canada). Isolated NK cells were stained with anti-CD16 and anti-CD3 antibody, respectively, to measure the cell purity using flow cytometric analysis. Purified NK cells were cultured in RPMI Medium 1640 supplemented with 10% FBS (Gemini Bio-Products, CA), 1% antibiotic/antimycotic, 1% sodium pyruvate, and 1% MEM non-essential amino acids (Invitrogen, Life Technologies, CA).

Expansion of NK cells

Human purified and hu-BLT enriched NK cells were activated with rh-IL-2 (1000 U/ml) and anti-CD16mAb (3ug/ml) for 18-20 hours before they were co-cultured with feeder cells and sAJ2. The culture media was refreshed with rh-IL-2 every three days [43].

NK cell supernatants used for stem cell differentiation

As described above, human NK cells were purified from PBMCs of healthy donors. NK cells were treated with a combination of anti-CD16mAb (3 µg/mL) and IL-2 (1,000 U/mL) for 18 hours before supernatants were removed and used for differentiation experiments. The amounts of IFN-γ produced by activated NK cells were assessed with IFN-γ ELISA (BioLegend, CA, USA). OSCSCs were differentiated with a gradual daily addition of increasing amounts of NK cell supernatants. On average, to induce differentiation, a total of 3,500 pg. of IFN-γ containing supernatants were added for 5 days to induce differentiation and resistance of OSCSCs to NK

cell-mediated cytotoxicity and a total of 7000 pg. of IFN-γ containing supernatants were added for 7 days to induce differentiation and resistance of MP2 to NK cell-mediated cytotoxicity. Afterwards, target cells were washed with PBS, detached and used for experiments.

Treating NK cell with Mekabu

The fucoidan extracted from the Mekabu seaweed was purchased from NatureMedic. 12.5 g of the Mekabu extracted fucoidan (Mekabu) was solubilized in 1 mL of PBS·1 and then added to cultures.

Sonicating AJ2

AJ2 was weighed and resuspended in RPMI Medium 1640 containing 10% FBS at a concentration of 10 mg/mL. The bacteria were thoroughly vortexed, then sonicated on ice for 15 seconds, at 6 to 8 amplitudes. Sonicated samples were then incubated for 30 seconds on ice. After every five pulses, a sample was taken to observe under the microscope until at least 80 percent of cell walls were lysed. It was determined that approximated 20 rounds of sonication/incubation on ice, were conducted to achieve complete sonication. Finally, the sonicated samples (sAJ2) were aliquoted and stored in a -80 degrees Celsius freezer.

Generation of osteoclasts

Osteoclasts were generated from PBMC-purified monocytes and cultured in alpha-MEM medium, containing M-CSF (25 ng/mL) and RANK Ligand (RANKL) (25 ng/mL), for 21 days. Medium was refreshed every 3 days with fresh alpha-MEM, containing M-CSF (25 ng/mL) and RANKL (25 ng/mL).

ADCC induction

The target cells ($5 \cdot 10^5$) were labeled with 50 μCi ^{51}Cr (Perkin Elmer, Santa Clara, CA) and chromated for 1 hour. Following incubation, target cells were washed once to remove excess unbound ^{51}Cr. Cells were resuspended in $1 \cdot 10^6$/mL and the treated with the anti-MICA/MICB antibody or Cetaximab (3 μg/mL) and incubated for 30 minutes. Following incubation, target cells were washed again to remove excess unbound antibody and ^{51}Cr. Labeled target cells were cultured with effector cells and the cytotoxicity against target cells were assessed using ^{51}Cr release cytotoxicity assay.

^{51}Cr release cytotoxicity assay

^{51} Cr was purchased from Perkin Elmer (Santa Clara, CA). Standard ^{51}Cr release cytotoxicity assays were used to determine NK cell cytotoxic function in the experimental cultures. The effector cells ($1 \cdot 10^5$ cells/well) were aliquoted into 96-well round-bottom micro-well plates (Fisher Scientific, Pittsburgh, PA) and titrated at 4 to 8 serial dilutions. Target cells ($5 \cdot 10^5$) were labeled with 50μCi ^{51}Cr (Perkin Elmer, Santa Clara, CA) and chromated for 1 hour. Following incubation, target cells were washed twice to remove excess unbound ^{51}Cr. ^{51}Cr-labeled target cells were aliquoted into the 96-well round bottom microwell plates containing effector cells at a concentration of $1 \cdot 10^4$ cells/well at a top effector: target (E: T) ratio of 5:1. Plates were centrifuged and incubated for a period of 4 hours. After a 4-hour incubation period, the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. Total (containing ^{51}Cr labeled target cells) and spontaneous (supernatants of target cells alone) release values were measured and used to calculate the percentage specific cytotoxicity. The percentage specific cytotoxicity was calculated using the following formula:

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

$Lu30/10^6$ is calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells ·100.

Enzyme-Linked Immunosorbent Assays (ELISAs)

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

($2N\ H_2SO_4$) was added per well to stop the reaction. Finally, plates were read in a microplate reader, at 450 nm to obtain absorbance values (BioLegend, ELISA manual).

Surface staining

$1 \cdot 10^5$ cells from each condition were stained in 100 µL of cold 1% BSA-PBS with predetermined optimal concentration of PE conjugated antibodies, as detailed in the experiments, and incubated at 4°C for 30 minutes. Then, cells were washed and resuspended in 1% BSA-PBS. The Epics C (Coulter) flow cytometer was used for cellular surface analysis.

Statistical Analysis

An unpaired or paired two-tailed Student's t-test were performed to compare different groups depending on the experimental design. The p-values were expressed within the figures as follows: ***p-value < 0.001, **p-value: 0.001–0.01, *p-value: 0.01–0.05. The GraphPad Prism software was used to analyze the data.

Chapter 1: **To determine whether NK cells mediate ADCC differentially against well-differentiated and undifferentiated/ stem like oral and pancreatic tumors.**

Results

***NK cells mediate higher cytotoxicity against undifferentiated/stem-like oral and pancreatic tumor cells in comparison to their differentiated compartments*.**

OSCSCs and MP2 displayed higher expression of CD44, and lower expression of MHC-I, MICA and CD54, while the reverse profile was seen in their differentiated counterparts. To differentiate the OSCSCs, and MP2s, the tumor cells were treated with supernatants from split-anergized NK cells as described in the methods and material section. Treatment of OSCSCs and MP2s with split-anergized NK cells' supernatant decreased the CD44 surface expression and increased the MHC-I, MICA and CD54 surface expression. (Fig1. A, B)

As shown previously NK cells mediated much higher cytotoxicity against oral squamous carcinoma stem-like cells (OSCSCs), and undifferentiated/stem-like pancreatic tumor cells (MP2), when compared to the differentiated oral squamous carcinoma cells (OSCCs) and pancreatic tumor cells (PL12). (Fig 1.C, D). The treatment of OSCSCs with split-anergized NK cell supernatants significantly decreased their sensitivity to IL-2-treated NK cell-mediated lysis.

Figure.1

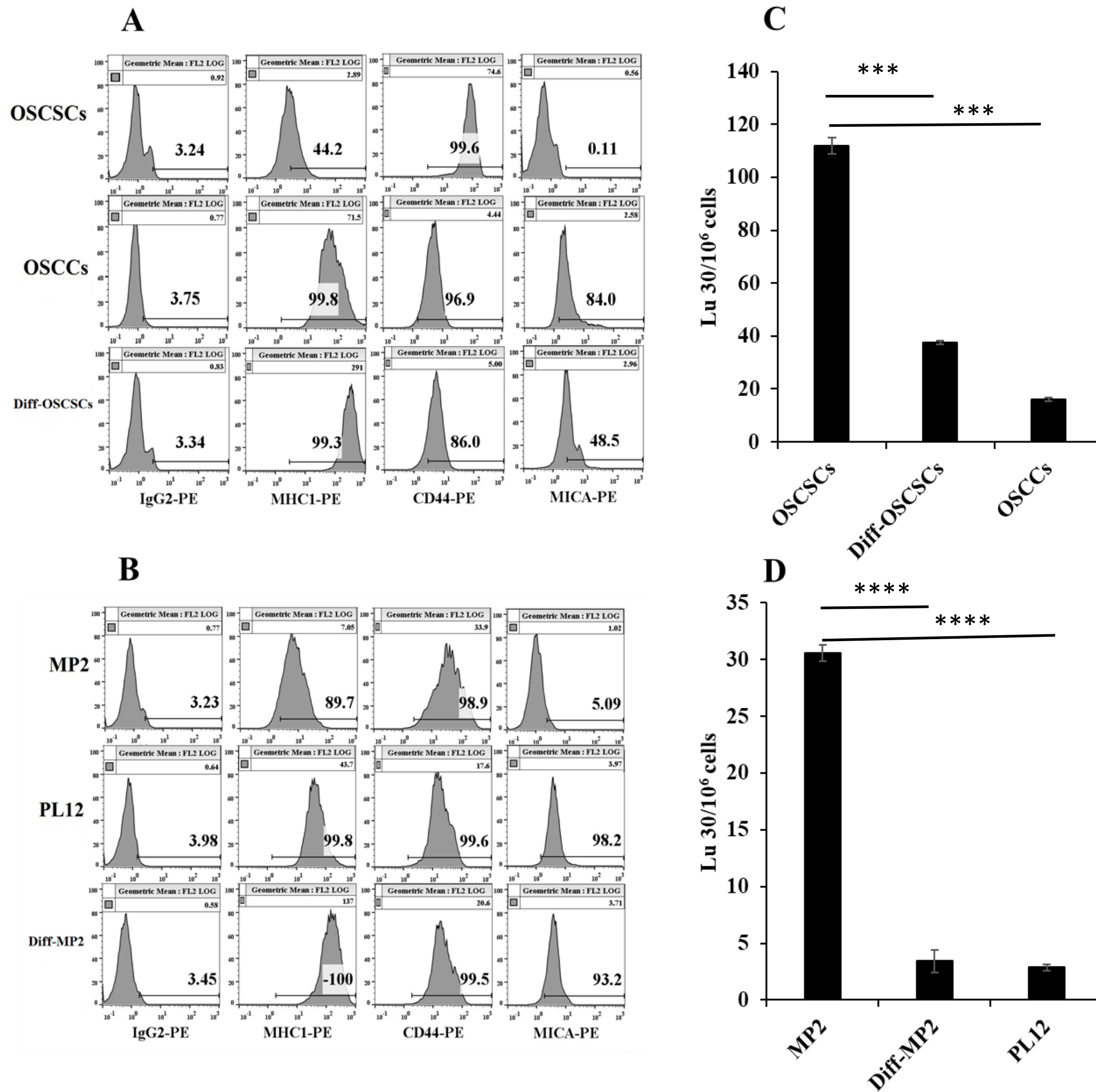


Figure 1. Differentiation stage of oral tumor cells correlates with sensitivity to NK cell-mediated lysis. The OSCSCs and MP2 were differentiated as explained in methods and material. The surface expression of CD44, MHC-I, and MICA on OSCSCs, OSCCs and split-anergized NK cell supernatant-differentiated OSCSCs tumor cells (A), and MP2, PL12, and differentiated MP2(B) was assessed using flow cytometric analysis after staining with respective PE-conjugated antibodies. Isotype control antibodies were used as controls. NK-cell mediated cytotoxicity was determined using a standard 4-hour ^{51}Cr release assay against OSCCs, differentiated OSCCS and OSCSCs (c), and MP2, differentiated MP2, and PL12 (D) tumor cells. Purified NK cells (1Ξ10^6 cells/ml) were treated with IL-2 (1000 U/ml) for 18 hours before they were added to ^{51}Cr labeled tumor cells at various effector to target ratios.

Differentiated pancreatic and oral tumors expressed higher level of MICA/MICB in comparison to their differentiated compartments.

OSCSCs and MP2 displayed lower expression of MICA/MICB, while the reverse profile was seen in their differentiated counterparts, OSCCs and PL12. Treatment of OSCSCs and MP2 with split-anergized NK cell supernatant increased the MICA/MICB surface expression, showing that well-differentiated tumors express higher levels of MICA/MICB in comparison to undifferentiated/stem-like oral and pancreatic tumors (Fig. 2).

Figure. 2

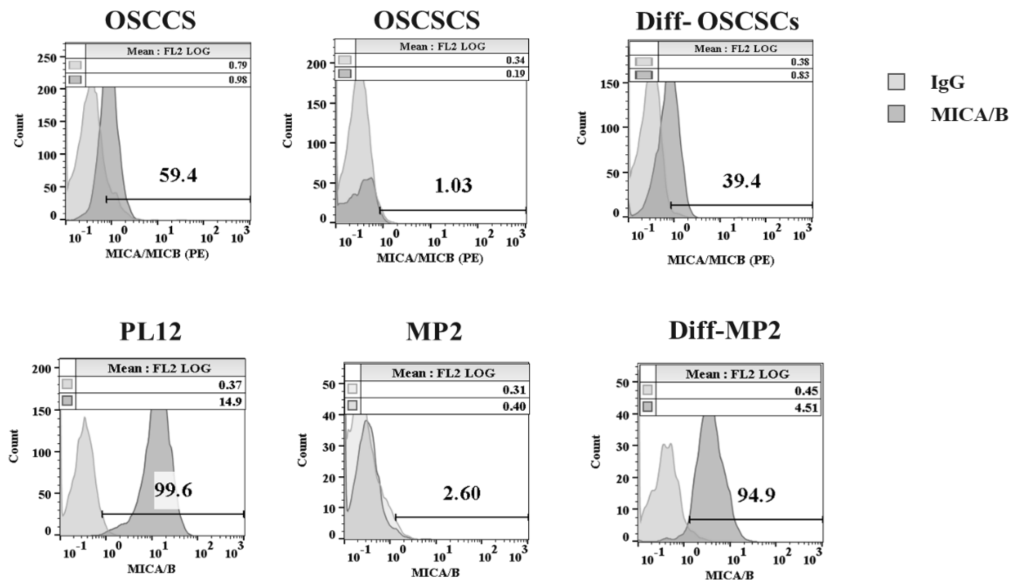


Fig 2. Differentiated oral and pancreatic tumor cells expressed higher MICA/MICB surface expression in comparison to their undifferentiated compartments. The OSCSCs and MP2 were differentiated as explained in methods and material. The surface expression of MICA/MICB on OSCCS, OCSCSs, and split-anergized NK cell supernatant-differentiated MP2, PL12 and split-anergized NK cell supernatant-differentiated OSCSCs (top) PL12, MP2 and NK cell supernatant-differentiated MP2 (bottom) was assessed using flow cytometric analysis after staining with respective PE-conjugated antibodies. Isotype control antibodies were used as controls.

***A**ntibodies specific to MICA/MICB increased NK cell-mediated ADCC against OSCC, while OSCSCs were not targeted*

To study the antibody dependent mediated cytotoxicity (ADCC) in NK cells against differentiated tumor cells expressing high level of MICA/MICB and their undifferentiated Counterparts expressing low levels of MICA/MICB, NK cells were purified from healthy donors. NK cells were treated with IL-2, the combination of IL-2 and anti-CD16mAb, or were left untreated. Their cytotoxicity against the OSCSCs and OSCCs, untreated or treated with the antibody against MICA/MICB, was determined using the ^{51}Cr release assay. Untreated and IL-2 treated NK cells mediated higher cytotoxicity against anti-MICA/B treated OSCCS in comparison to untreated OSCCs (Fig.3 A). The fold increase in cytotoxicity against antiMICA/MICB treated OSCCs was significantly higher in untreated (mean=6.9 fold increase) and IL-2 treated NK (mean=3.1 fold increase) than untreated OSCCs (Fig.3 C, D), while the differences in cytotoxicity of untreated and IL-2 treated NK cells against untreated and anti-MICA/B treated OSCSCS were not significantly different (Fig.3 B, C, D). NK cells treated with combination of IL-2 and anti-CD16mAb did not mediate significant levels of ADCC against OSCSCs and OSCCs. (Fig.3 A, B, C, D).

Figure.3

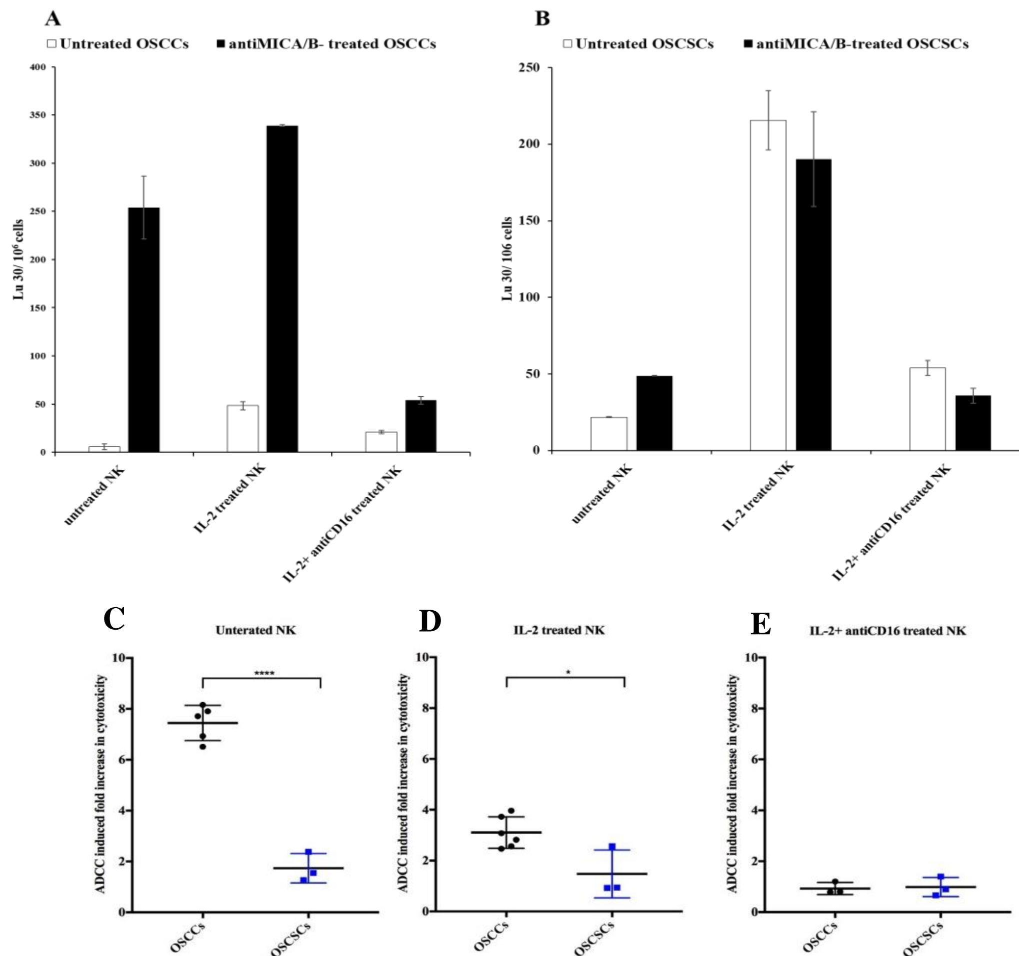


Figure.3 Antibodies specific to MICA/MICB increased NK cell-mediated ADCC against OSCCs, while OSCSCs cells were not targeted significantly through ADCC. Freshly purified NK cells from healthy donors were left untreated, treated with IL-2 (1,000 U/mL) or combination of IL-2 and anti-CD16 mAb (3 µg/ml), for 18 hours. OSCCS, and OSCSCs were labeled with ^{51}Cr, and then left untreated or treated with anti-MICA/MICB antibody (5 µg/ml) for 30 minutes. The unbounded antibodies were washed and the cytotoxicity against the OSCCs (A) and OSCSCs (B) untreated or treated with the antibody against MICA/MICB was determined using the standard 4-hour ^{51}Cr release assay (Fig A & B are Representative of one study)**.** The ADCC induced fold increase in cytotoxicity of untreated (C), IL-2 treated (D) and the IL-2 + anti-CD16 mAb (E) treated NK cells were measured.

Antibodies specific to MICA/MICB increased NK cell-mediated ADCC against PL12, while MP2 cells were not targeted.

To further confirm that the same observation can be seen in pancreatic tumors, the same experiment was conducted with MP2 and PL12. The cytotoxicity against anti-MICA/MICB treated PL12 was 55-fold higher in untreated and 4.3-fold higher in IL-2 treated NK than untreated PL12. (Fig.4 C, D) while the differences in cytotoxicity against untreated and anti-MICA/B treated MP2 were not significant.

Figure.4

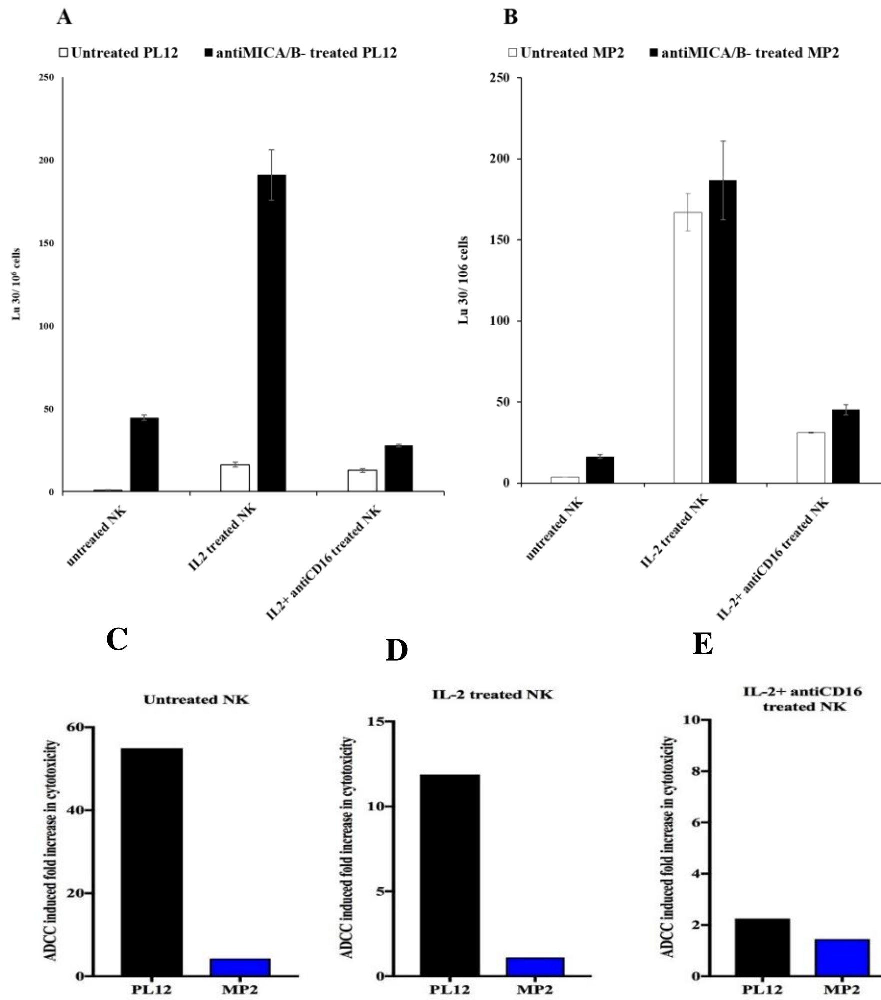


Figure.4 Antibodies specific to MICA/MICB increased NK cell-mediated ADCC against PL12, while MP2 cells were not targeted. Freshly purified NK cells from healthy donors were left untreated, treated with IL-2 (1000 U/mL) or combination of IL-2 and anti-CD16 mAb (3 µg/mL), for 18 hours. PL12, and MP2 were labeled with ^{51}Cr, and then left untreated or treated with anti-MICA/MICB antibody (5 µg/ml) for 30 minutes. The unbounded antibodies were washed and the cytotoxicity against the PL12 (A) and MP2 (B) untreated or treated with the antibody against MICA/MICB was determined using the standard 4-hours ^{51}Cr release assay. The ADCC induced fold increase in cytotoxicity for untreated (C), IL-2 treated (D), and the IL-2 +anti-CD16 mAb treated (E) NK cells were measured.

Differentiation of OSCSCs with split-anergized NK cells supernatant results in their susceptibility to NK cell- mediated ADCC through anti-MICA/MICB antibody.

We previously showed that treatment of OSCSCs and MP2 with split-anergized NK cells' supernatant increases the surface expression of differentiation markers including MICA/MICB. To determine if differentiating oral and pancreatic tumors with spit-anergized NK cell supernatant make them susceptible to NK cell mediated ADCC, OSCSCs and MP2 were differentiated as described in Methods and Material and the cytotoxicity of untreated and IL-2 treated NK cells agains untreated and MICA/MICB treated undifferentiated, split-anergized NK cells supernatant-differentiated, and undifferentiated/stem-like oral and pancreatic tumors were measured. As we have shown before, the untreated and IL-2 treated NK cells mediated ADCC against OSCCSs and PL12 (Fig.5 A, D). The undifferentiated OSCSCs and PL12 were highly susceptible to IL-2 treated NK cell-mediated cytotoxicity, while the cytotoxicity against their supernatant-differentiated counterparts showed a 4-fold decrease in OSCSCs and a 67-fold decrease in MP2. IL-2 treated NK cells showed higher level of cytotoxicity against anti-MICA/MICB treated differentiated OSCSCs and MP2 in comparison to untreated tumors, while the ADCC-mediated lysis did not happen against MP2 or OSCSCs (Fig.4 B, C, E, F), showing that differentiation of OSCSCs and MP2 with split-anergized NK cell supernatant make them susceptible to NK cell mediated ADCC through anti-MICA/MICB antibody.

Figure.5

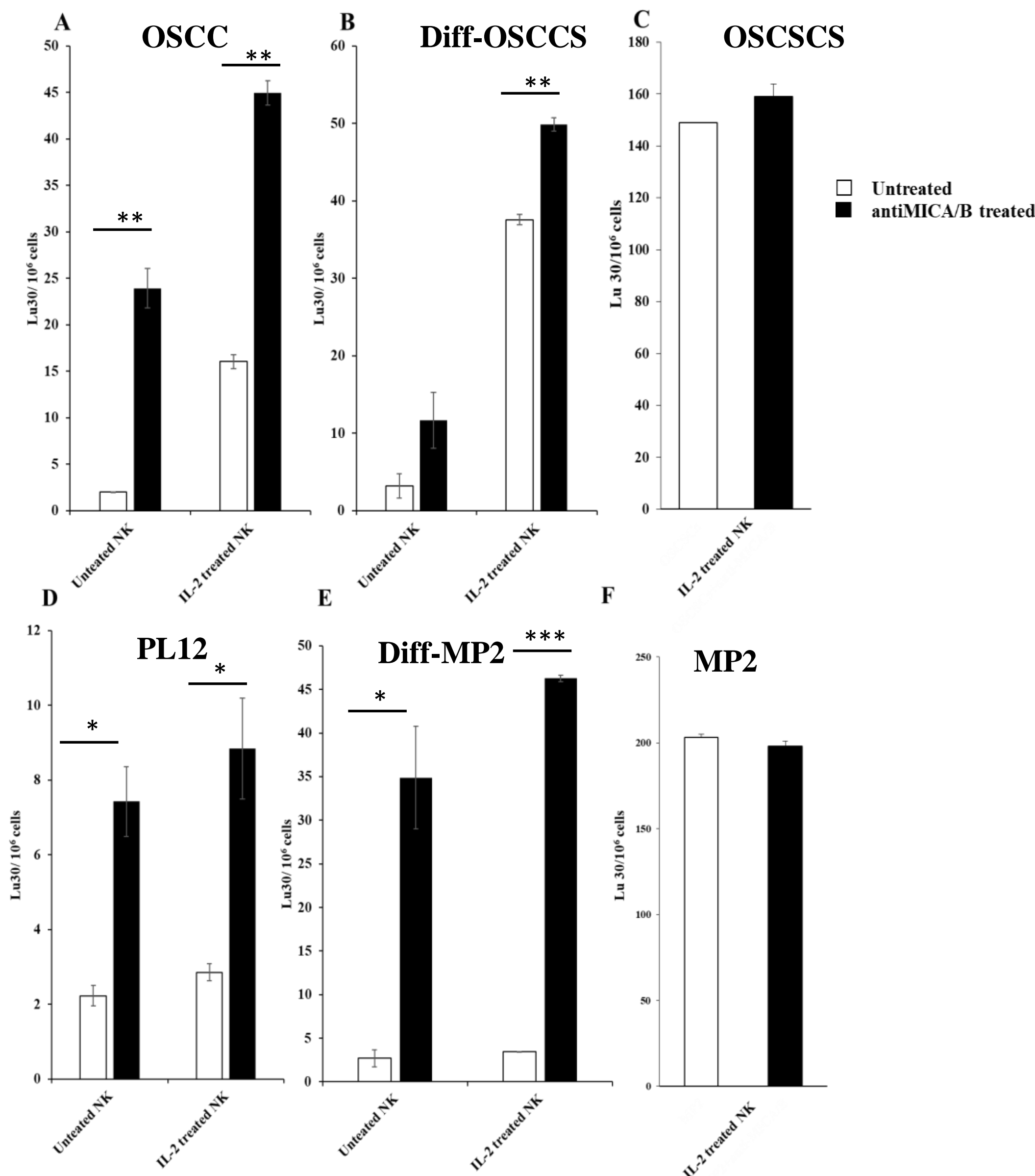


Figure.5 Differentiation of OSCSCs with split-anergized NK cells supernatants results in their susceptibility to NK cell- mediated ADCC through anti MICA/MICB antibody. OSCSCs and MP2 tumor cells were differentiated as described in methods and material section. Freshly purified NK cells from healthy donors were left untreated or treated with IL-2(1,000 U/ml) for 18 hours. OSCCS, differentiated OSCSCs, and OSCSCs, were labeled with ^{51}Cr, and then left untreated or treated with antiMICA/MICB antibody (5 µg / ml) for 30 minutes. The unbounded antibodies were washed and the cytotoxicity of untreated NK cells and/or IL-2 treated against untreated or antiMICA/MICB treated the OSCCs (A), differentiated OSCSCs (B), OSCSCs (C), PL12(D), differentiated MP2 (E), and MP2 (F) were determined using the standard 4-hours ^{51}Chromium release assay.

Antibodies specific to MICA/MICB increased IFN- γ secretion by NK cells when cultured with differentiated Oral tumors expressing MICA/MICB

To determine whether anti-MICA/MICB antibodies can increase the secretion of IFN-γ, NK cells were cultured with untreated or antiMICA/MCB treated OSCCs and OSCSCs. Untreated NK cells did not induce IFN-γ secretion with or without being co-cultured with tumor cells. As we have shown previously, a combination of IL-2 and anti-CD16 mAb induced the highest levels of IFN-γ secretion by NK cells. When IL-2 treated NK cells were co-cultured with OSCCs and OSCSCs, they secreted higher levels of IFN-γ in comparison to the control groups (NK cells alone with no tumor), and OSCSCs caused more secretion of IFN-γ than OSCCs. When IL-2 treated NK cells were cultured with anti-MICA/MICB treated OSCCs they secreted more IFN-γ than NK cells cultured with untreated OSCCs. Secreted levels of IFN-γ in NK cells co-cultured with untreated OSCSCs and anti-MICA/MICB treated OSCCs were not significantly different. The same trend was observed in three different separate experiments (Fig.8 A- C). The amount of IFN-γ secreted by NK cells treated with the combination of IL-2 and anti-CD16mAb, was not significantly different in untreated and anti-MICA/MICB antibody treated OSCCs and OSCSCS.

Figure.6

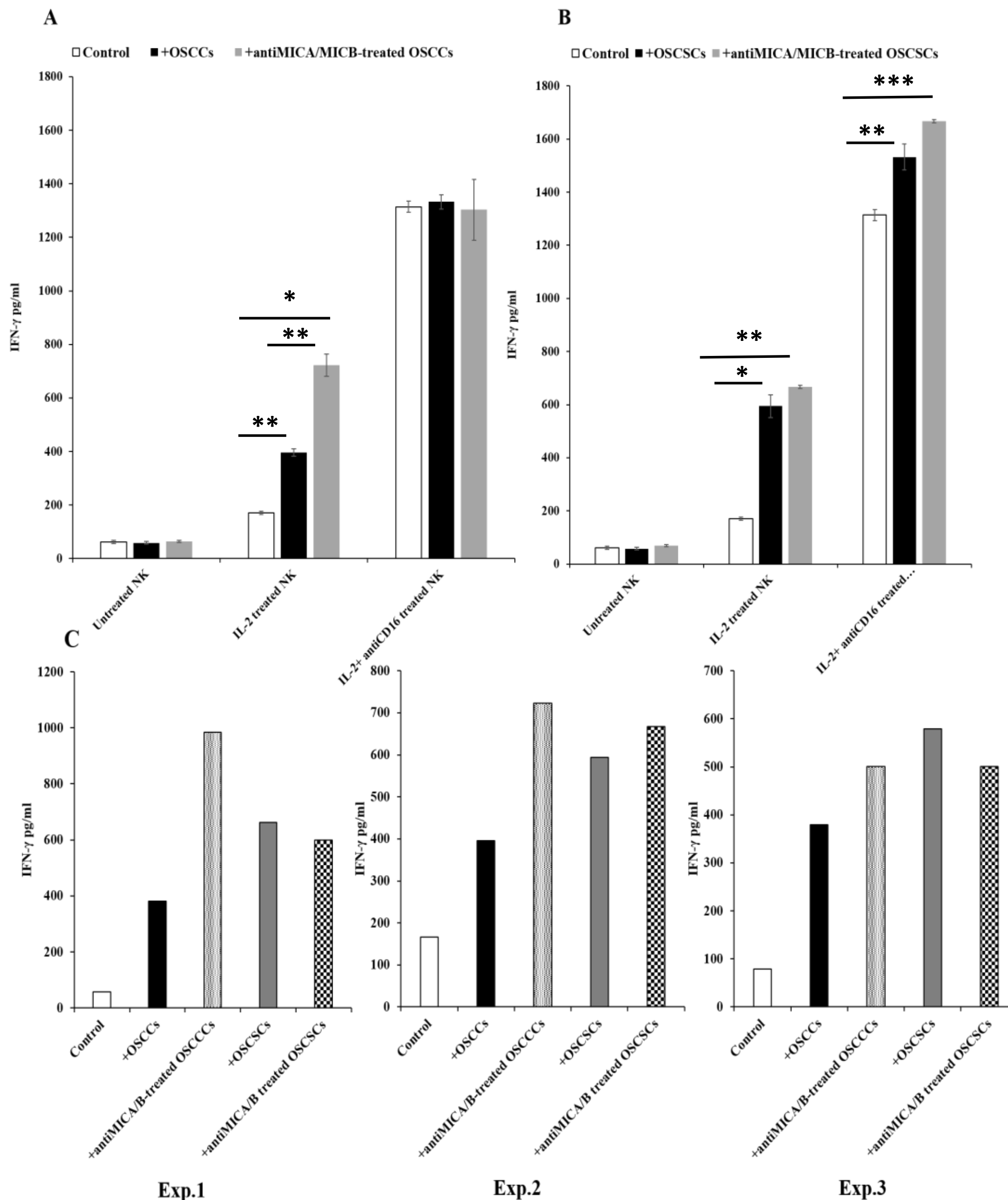


Fig.6 Antibodies specific to MICA/MICB increased IFN-γ secretion by NK cells when cultured with differentiated Oral tumors expressing MICA/MICB. OSCCs and OSCSCs were cultured in absence or presence of anti-MICA/MICB Ab (5 µg/mL) overnight. The unbounded antibodies were washed. Freshly purified NK cells from healthy donors were left untreated, treated with IL-2 (1,000 U/mL) or combination of IL-2 and anti-CD16 mAb (3 µg/mL), for 18 hours and they were co-cultured with OSCCs (A), and OSCSCs (B). After 24 hours the supernatant was collected from the cultures and IFN-g was measured with ELISA. Figure A and B are representative of one experiment. Figure C shows the IFN-g level of IL-2 treated NK co-cultured with untreated or anti-MICA/MICB antibody treated OSCCs and OSCSCs in three different experiments.

Chapter.2: Primary NK cells and Supercharged NK cells function differently in mediating cytotoxicity

Results

Expanded NK cells target both undifferentiated and differentiated tumor cells while primary NK cells preferentially target undifferentiated/Stem-like population.

Our laboratory has developed a novel strategy to expand NK cells called" Super Charged" NK cell, having both high cytotoxicity and cytokine secretion abilities. When comparing the function and surface expression of primary and supercharged NK cells, they show different characteristics (43). We showed that supercharged NK cells have high cytotoxicity against undifferentiated tumor cells, but their function against differentiated tumors was not studied. To compare the cytotoxicity of primary NK and Expanded NK against differentiated and undifferentiated tumor cells, NK cells were Expanded for 15 days and their cytotoxicity against OSCCs, OSCSCs, PL12 and MP2 were measured. Undifferentiated tumor cells were more susceptible to primary NK cell -mediated lysis, but alternatively with the differentiated counterparts, Expanded NK cells from the same donor were able to significantly target both differentiated and undifferentiated tumor cells. The cytotoxicity of Expanded NK cells was 3 to 15-fold higher against OSCCs and 4.7-fold higher against PL12 in comparison to primary NK cells.

Figure. 7

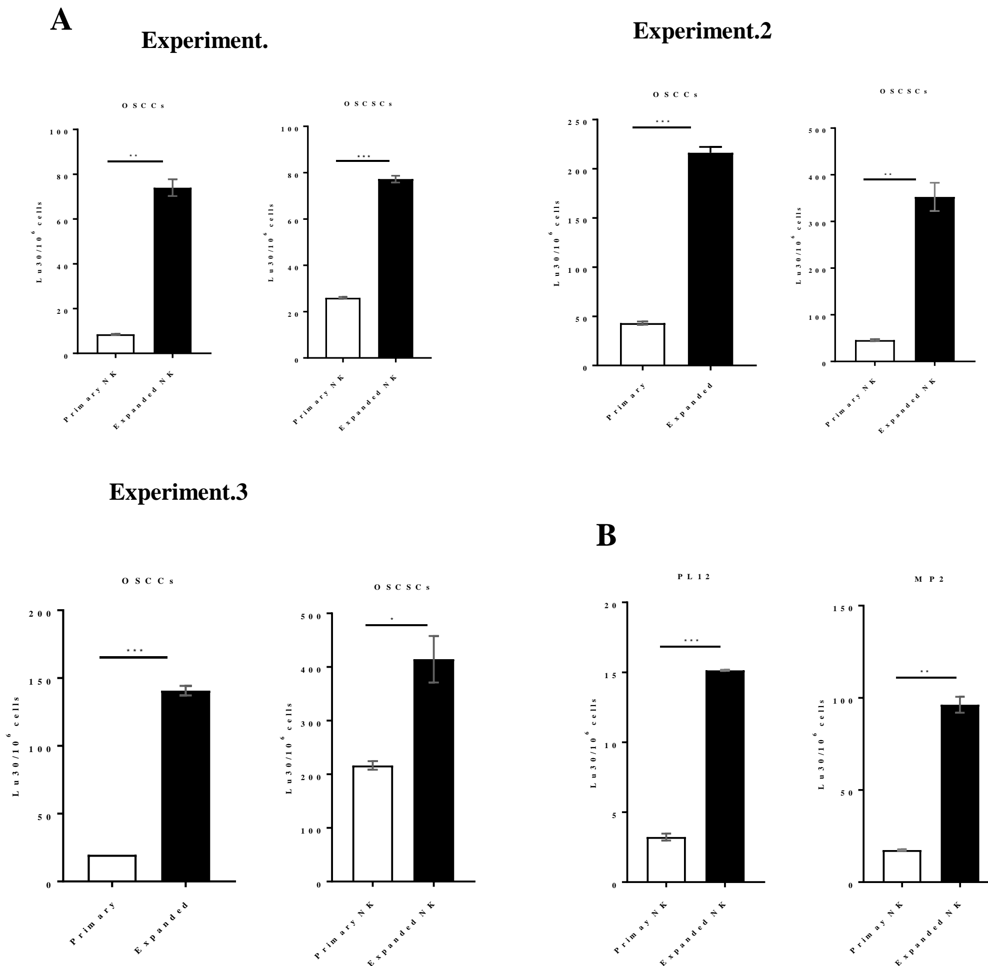


Fig.7 Expanded NK cells target both undifferentiated and differentiated tumor cells while primary NK cells preferentially target undifferentiated/ stem-like population. NK cells were purified from healthy donors' blood and expanded as described in methods and material section. After day 15 of expansion, primary NK cells were purified from the same donor (1×10^{6}cells/mL) and treated with IL-2 (1,000 U/mL) and expanded NK cells reactivated with IL-2 (1,000 U/mL) for 18 hours. OSCCSs and OSCSCs were labeled with ^{51}Cr, and the cytotoxicity of primary and expanded NK cells against OSCCs (left) and OSCSCs (right) were determined in three different experiments, using the standard 4-hours ^{51}Cr release assay (A). PL12 and MP2 were labeled with ^{51}Cr, the cytotoxicity of primary NK cells, and cytotoxicity of expanded NK against MP2and PL12 was determined (Fig B).

Combination of IL-2 and anti-CD16 treatment induces split anergy in primary NK cells but not in super-charged NK cells

Our laboratory has defined a stage of NK cells maturation called "split anergy," that indicates reduced NK cell cytotoxicity in the presence of significant secretion of cytokines. We previously showed that treatment of NK cells with IL-2 and anti-CD16 mAb can induce split anergy in primary NK cells. To determine whether a combination of IL-2 and anti-CD16 mAb treatment will decrease the cytotoxicity in Expanded NK, freshly purified NK cells from healthy donors were Expanded. After being in culture for 15 days, NK cells were purified from the same donors and primary and Expanded NK cells were treated with IL-2 and the combination of IL-2 and anti-CD16 mAb for 18 hours. Then their cytotoxicity against OSCSCs was measured using a standard 4-hour ^{51}Cr release assay. As we have shown previously, the cytotoxicity of IL-2 and anti-CD16 treated primary NK cells against OSCSCs decreased 2.4 to 4.9-fold while the cytotoxicity of IL-2 and anti-CD16 treated Expanded NK cells was almost the same as IL-2 treated Expanded NK (Fig.8).

Figure. 8

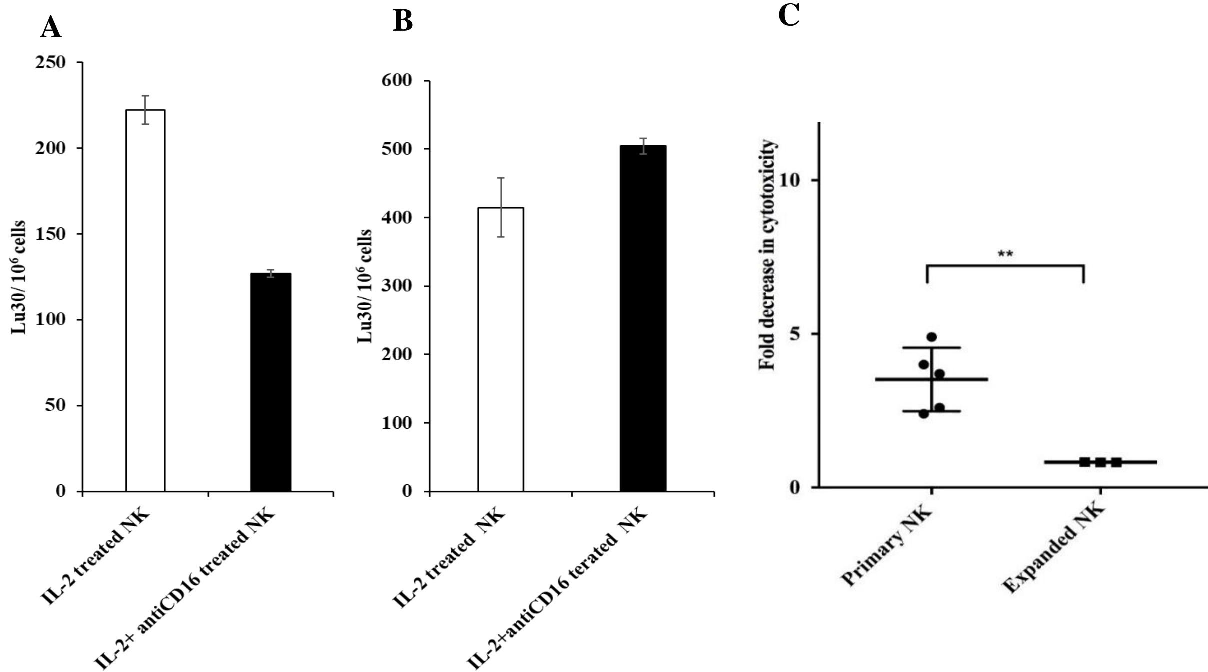
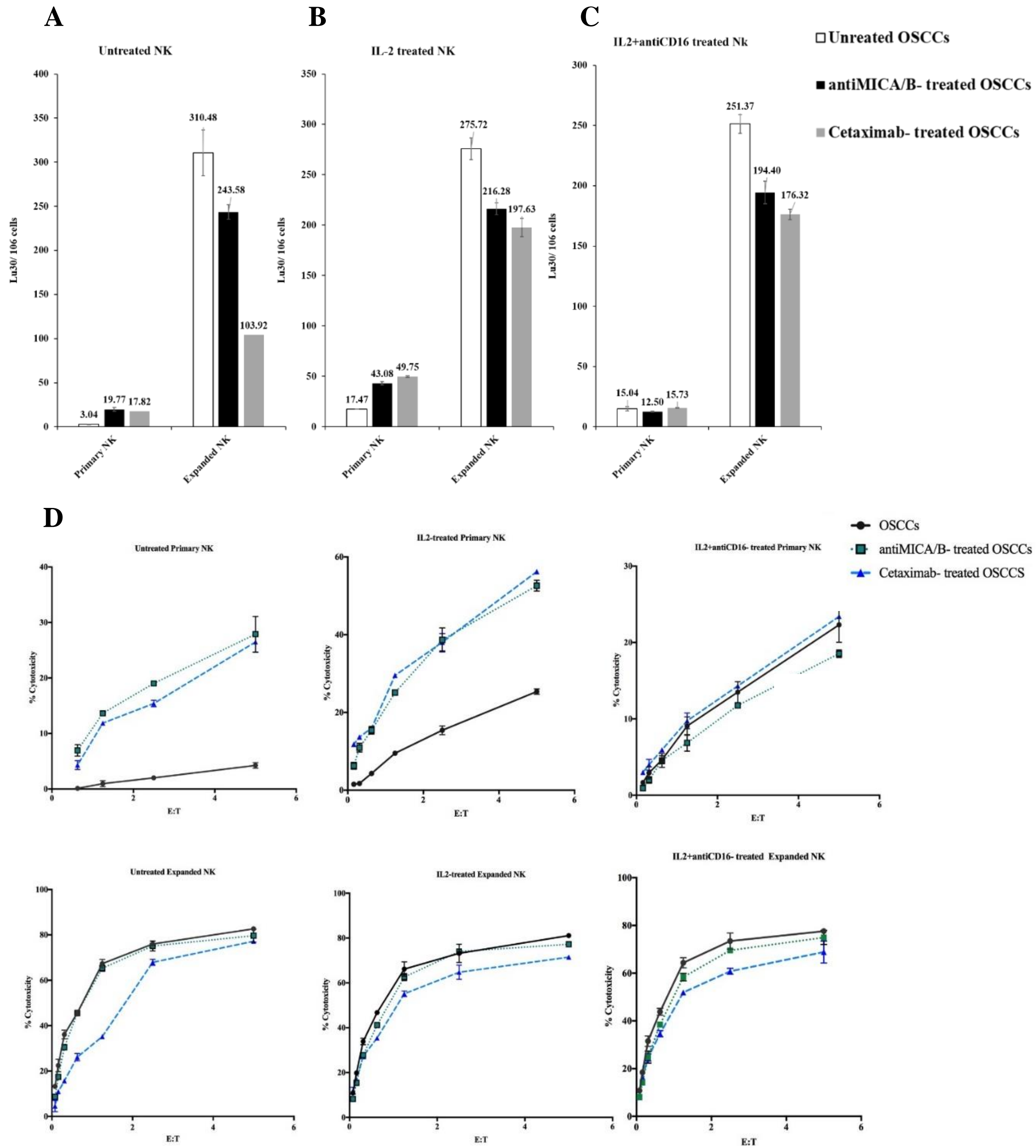


Figure.8 **Combination of IL-2 and antiCD16 treatment induces split anergy in primary NK cells but not in super-charged NK cells.** NK cells were purified from healthy donors' blood and expanded as explained in methods and material section. After day 15 of expansion primary NK cells were purified from the same donor (1×10^6cells/mL) and they were treated with IL-2 (1,000 U/mL), or the combination of IL-2 (1,000 U/mL) and anti-CD16mAb (3 µg/ml) for 18 hours. Expanded NK cells (1×10^6cells/mL) were reactivated with IL-2 (1,000 U/mL), and the combination of IL-2 (1,000 U/mL) and anti-CD16 mAb (3 µg/ml) for 18 hours. OSCSCs were labeled with ^{51}Cr and the cytotoxicity of IL-2- treated and combination of IL-2 and anti-CD16mAb of primary (A) and expanded NK cells (B) against the OSCSCs was determined using the standard 4-hours Chromium-51 release assay. Figure A and B is representative of one separate experiment. The fold decrease in cytotoxicity caused as the result of anti-CD16 mAb treatment was calculated (C).

Primary NK cells mediate Higher levels of ADCC than expanded NK cells

Previously, we have shown that CD16 gets down-modulated on Expanded NK cells [43]. To study the ability of Expanded NK cells in mediating ADCC, the cytotoxicity of primary NK cells and Expanded NK cells on day 15, from the same donor, were measured against untreated, anti-MICA/MICB antibody, and Cetaximab treated OSCCs. While untreated and IL-2 treated primary NK cells mediated higher level of cytotoxicity against anti-MICA/MICB and Cetaximab treated OSCCs than untreated tumors, Expanded NK cells and IL-2-reactivated NK Expanded NK cells did not mediate ADCC against anti-MICA/MICB or Cetaximab treated OSCCs. (Fig. 9 A, B, D and E). When the experiment was repeated with PL12 tumor cells, the same results were observed (Fig.9 D). Both primary and Expanded NK cells treated with the combination of IL-2 and anti-CD16 mAb were not able to mediate ADCC against anti-MICA/MICB antibody treated OSCCs and PL12 (Fig.9 C, F).

Figure.9



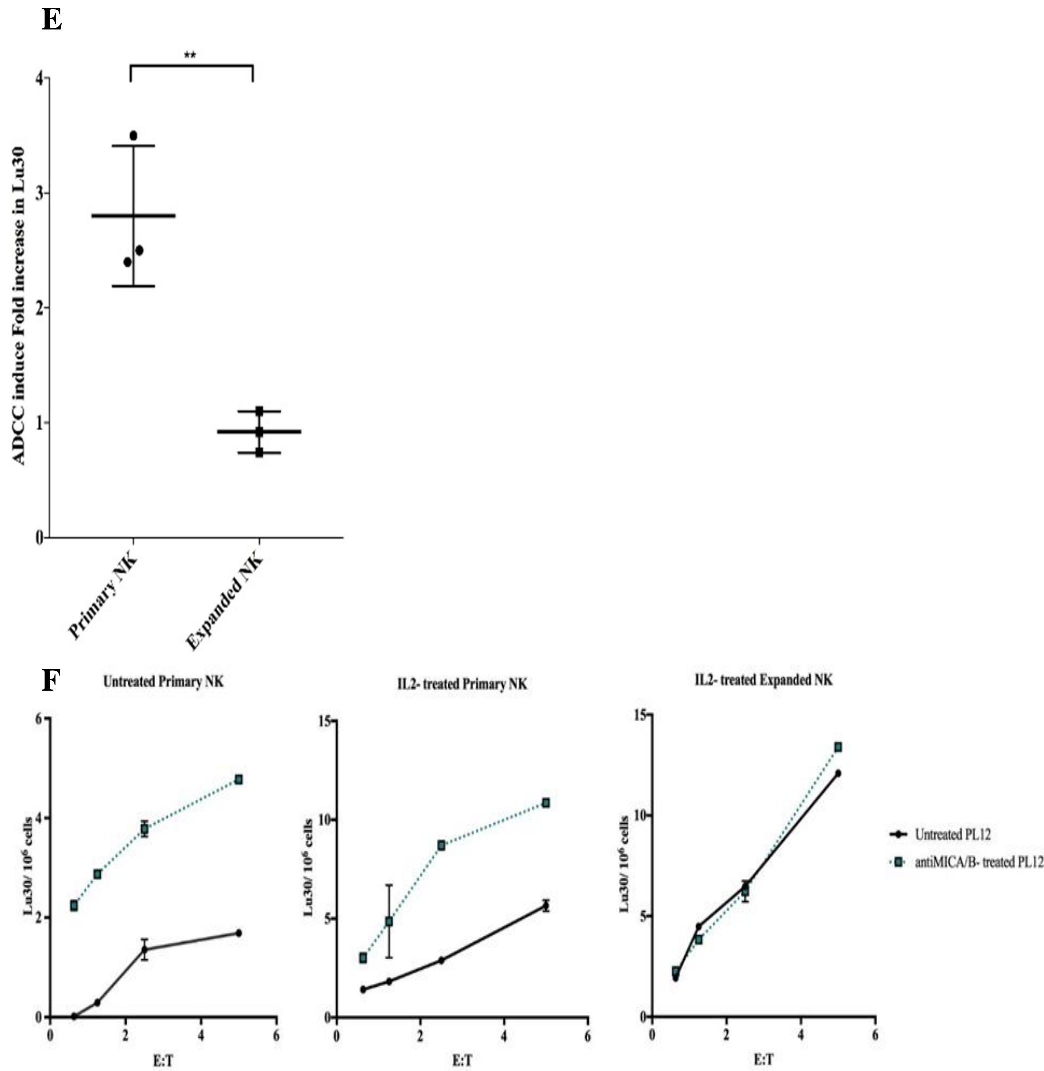


Figure. 9 Primary NK cells mediate higher level of ADCC than expanded NK cells. NK cells were purified from healthy donors' blood and expanded as explained in methods and material section. After day 15 of expansion primary NK cells were purified from the same donor (1×10^6 cells/mL) and they left untreated, treated with IL-2 (1,000 U/mL), and the combination of IL-2 (1,000 U/mL) and anti-CD16mAb (3 µg/mL) for 18 hours. Expanded NK cells (1×10^6 cells/mL) left untreated or reactivated with IL-2 (1,000 U/mL), and the combination of IL-2 (1,000 U/mL) and anti-CD16mAb (3 µg/mL) for 18 hours. OSCCS were labeled with Cr^{51}, and then left untreated or treated with anti-MICA/MICB antibody, or Cetaximab (5 µg/mL) for 30 minutes. The unbounded antibodies were washed and the cytotoxicity of Untreated (A). IL-2- treated (B), and combination of IL-2 and anti-CD16mAb (C) of primary and expanded NK cells against the OSCCs untreated or treated with the antibody against MICA/MICB antibody or Cetuximab was determined using the standard 4-hours Chromium-51 release assay. (Fig. A-C are reprehensive of one study). Figure D Shows the percentage of cytotoxicity for the same experiment. ADCC induced fold increase in IL-2 treated primary and expanded NK cells were calculated, (E)Cytotoxicity of untreated, IL-2- treated (1,000 U/mL) primary NK, and day15 IL-2-reactivated expanded NK (1,000 U/ml) form the same donor against the PL12 untreated or treated with the antibody against MICA/B (5 µg/mL) was determined using the standard 4-hours Chromium-51 release assay. (E)

Chapter.3: **We have established that NK cell-mediated IFN-γ production is the key in differentiation of oral and pancreatic cancer cells, therefore we aim at determining strategies to increase NK cell mediated production of IFN-γ.**

Results

Treatment of NK cells with each AJ2 probiotic bacteria and Mekabue extracted fucoidan Extract increased NK cells IFN-γ secretion ability but does not elevate NK cell mediated cytotoxicity.

Previously we have established that IFN-γ secreted by NK cells have a significant role in differentiation of tumor cells. To look for the strategy to increase NK cell-mediated production of IFN-γ, the effect of fucoidan and AJ2 probiotic bacteria on function of NK cells was studied. To study the effect of fucoidan on NK cells function, purified NK cells from healthy donors were treated over 24 hours with IL2 and different concentrations of D-fucoidan extracted from *Undaria pinnatifida* known as Mekabu. Treatment of NK cells with Mekabu for 24 hours significantly increased their ability to secrete IFN-γ but decreased their cytotoxicity, pushing the NK cells to a stage known as split-anergy (Fig.10 A, B).

To study the effect of AJ2 probiotic bacteria on the function of NK cells, purified NK cells were treated with IL-2 for 18 hours and they were treated with the sAJ2 probiotic bacteria for 24 hours. Activated NK cells with sAJ2 induced higher level of IFN-γ, while their ability to mediated cytotoxicity did not change. (Fig.10 C, D).

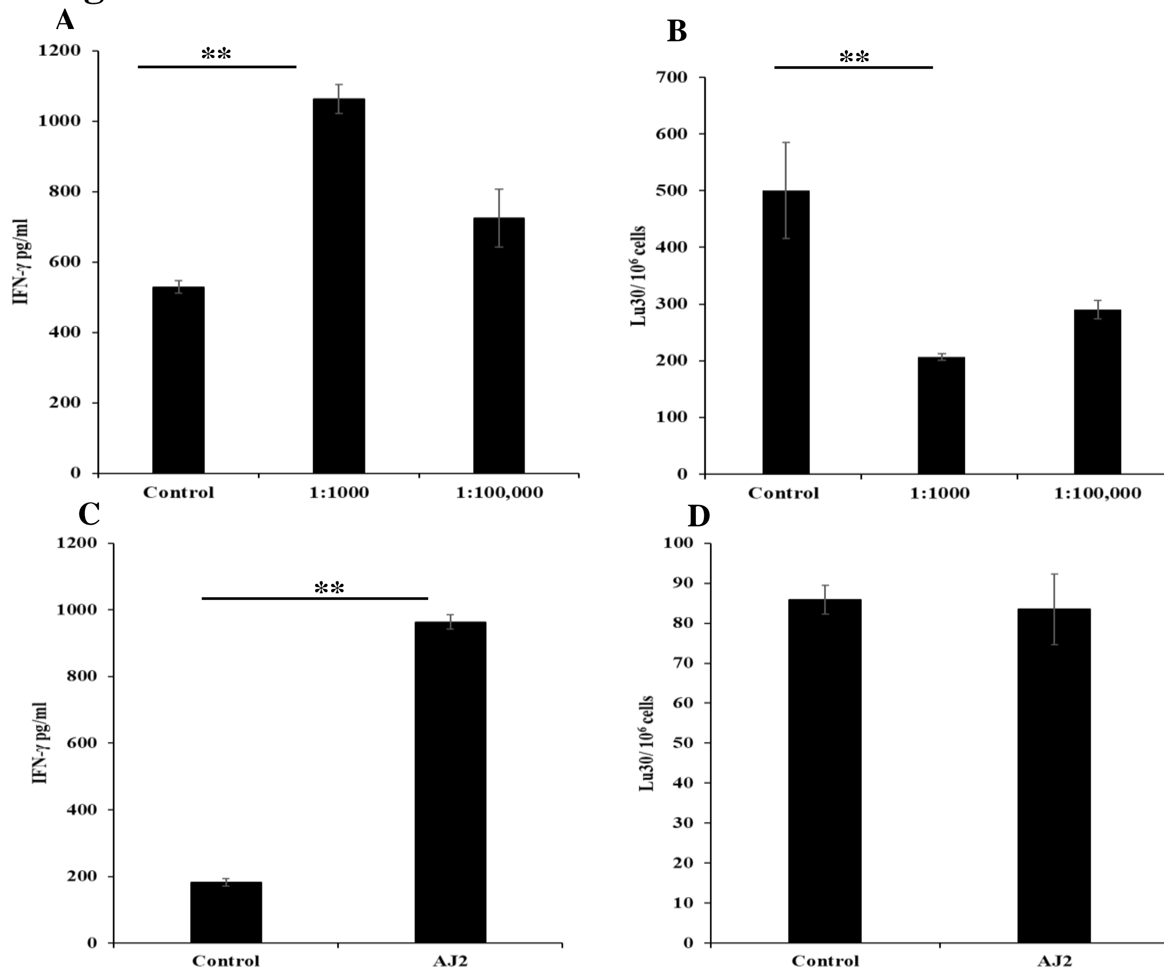


Figure 10. Mekabue extracted Fucoidan at a specific concentration induced split-anergy in NK cells, and A2 probiotic bacteria increased IFN-γ secretion in NK while their ability to mediate cytotoxicity remains unchanged. Mekabue were solubilized in PBS (12.5 mg/ml), Purified NK cells (1x106 cells/ml) were treated with IL-2 (1000 U/ml) for 18 hours and the NK cells were cultured in absence or presence of Mekabue in 1:1000, and 1: 100,000 titrations. The supernatant was harvested from the culture after 24 hours and the IFN-γ was measured by ELISA (Fig.2 A). Cells were resuspended in fresh media (1x10^6 cells/ml). NK cells were then used as effector cells against ^{51}Cr labeled OSCSCs. NK cell mediated cytotoxicity was determined using a standard 4-hour ^{51}Cr release assay and the lytic units determined as described in methods and materials (Fig.2 B).

AJ2 probiotic bacteria and Mekabu can synergistically induce IFN-γ secretion in IL-2- treated NK cells.

To study the synergistic effect of AJ2 and Mekabu on IFN-γ induction by NK cells, purified NK cells from healthy donors were treated with IL-2 for 18 hours and then they were left untreated, treated with AJ2, Mekabu, or both. The cultures' supernatants were collected after 24 hours and IFN-γ levels were measured. NK cells treated with AJ2 or Mekabu induced significantly higher levels of IFN-γ in comparison to the control group (Fig.11 A, B). The treatment combination of AJ2 and Mekabu significantly induced higher secretion of IFN-γ compared to the control and the NK cells treated with only AJ2 or Mekabu groups. (Fig.11 A, B).

Figure.11

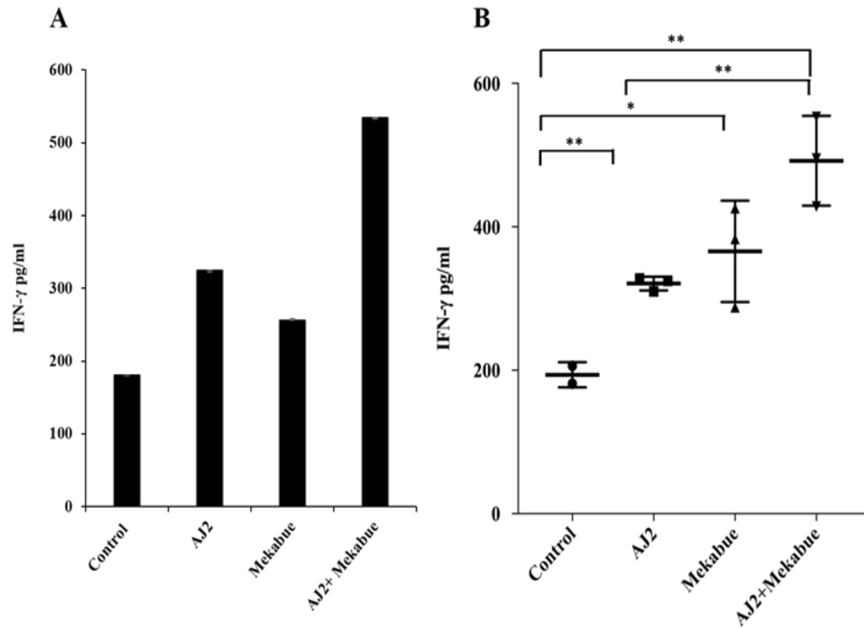


Figure.3 AJ2 probiotic bacteria and Mekabue can synergistically induce IFN- γ secretion in IL2- treated NK cells. Purified NK cells (1x10^6 cells/ml) were treated with IL-2 (1000 U/ml) for 18 hours and the NK cells were cultured in absence or presence of Mekabue in 1:1000,000 titration, sAJ2 at 1:0.5 ratio (NK: sAJ2), or combination of sAJ2 and Mekabue. After 24 hours the supernatant was collected and IFN- γ was measured by ELISA. Fig.3 A is reprehensive of one experiment), Fig.3 Shows the scatter plot for three experiments.

Discussion

We have previously shown that NK cells target poorly differentiated cells or stem cells with lower expression of key differentiation antigens [68-70]. In our studies, we characterized the link between the stage of maturation and differentiation of tumors and their sensitivity to NK cell-mediated lysis. In this regard, we have shown that stem-like/poorly differentiated oral and pancreatic tumor cells were significantly more susceptible to NK cell-mediated cytotoxicity, whereas, their differentiated counterparts were significantly more resistant (Fig.1).

Furthermore, we demonstrated that differentiated oral, pancreatic tumor cells, and cancer stem cells/poorly differentiated tumor cells differentiated in vitro with supernatants from split-anergized NK cells became resistant to NK cell-mediated cytotoxicity. Unlike the CSCs/poorly differentiated tumor cells, both patient-derived differentiated tumor cells and split-anergized NK supernatant-differentiated tumor cells exhibited upregulated CD54, B7H1, and MHC class I surface expression and demonstrated decreased CD44 expression (Fig.1)

When it comes to NK cells' immunity, MICA/MICB antigens play an important role since it is a well-known ligand for NKG2D and it gets expressed upon stress, damage, viral infection or transformation of cells which act as a 'kill me' signal through the cytotoxic lymphocytes. We showed that MICA/MICB does not get upregulated on all tumor cells but is correlated with the differentiation stages of the cells. We showed that differentiated oral, pancreatic tumor cells, and cancer stem cells/poorly differentiated tumor cells differentiated in vitro with supernatants from split-anergized NK cells express a higher level of MICA/MICB in comparison to their stem-like/undifferentiated counterparts (Fig.2). We previously showed that primary NK cells preferentially target stem-like/undifferentiated cells [1, 68]. This finding does not correlate with the fact that well-differentiated cells express a higher level of MICA/MICB which is a ligand for

an activator receptor on NK cells. This fact can explain the unknown roles of MICA/MICB ligands and its receptors. Since there was a difference in the pattern of MICA/MICB expression in stem-like/undifferentiated and well-differentiated oral and pancreatic tumor, we then studied whether NK cells mediate ADCC differentially against these tumors based on the antibody specific to MICA/MICB. We showed that antibodies specific to MICA/MICB increased NK cell-mediated ADCC against well-differentiated and cancer stem cells/poorly differentiated tumor cells differentiated in vitro with supernatants from split-anergized NK cells. Alternatively, stem-like/undifferentiated tumor cells were not targeted by untreated and IL-2 treated primary NK cells through ADCC which correlated with the expression level of MICA/MICB. When NK cells were treated with anti-CD16 mAb, NK cells were not able to mediate ADCC since the CD16 receptor was masked by the antibody (Fig.3-5). Furthermore, we showed that antibody specific to MICA/MICB increased IFN-γ secretion by NK cells when cultured with differentiated oral tumors expressing MICA/MICB, but not stem-like/undifferentiated oral tumor cells. The effect of ADCC on IFN-γ secretion by NK cells has not yet been well studied. A study showed that when NK cells were cultured with target cells and the antibody specific to the antigen they present, cytokines such as TNFα, IL-6, IFN-γ, or chemokines such as IL-8 and MCP-1 were found to be significantly enhanced [71].

Next, we delineated the underlying differences between the functions of primary and Expanded NK cells in direct cytotoxicity and ADCC. We have established a novel strategy to expand NK cells by IL-2, antiCD16 mAb, and sAJ2 probiotic bacteria activation and Osteoclasts as feeder cells. This strategy leads to NK cells called "Super Charged "NK cell, having high cytotoxicity and high levels of IFN-γ secretion [43]. When we compared the cytotoxicity of NK cells against stem-like/undifferentiated oral and pancreatic tumors with their well-differentiated counterparts

we showed that Expanded NK cells target both undifferentiated and differentiated tumor cells while primary NK cells preferentially target undifferentiated/stem-like populations. The cytotoxicity of Expanded NK cells was 3 to 15-fold higher against OSCCs and 4.7-fold higher against PL12 in comparison to primary NK cells (Fig.7). This shows the extend well-differentiated tumor cells can become sensitive to Expanded NK cell-mediated cytotoxicity can be different based on the biology of these cells and maybe their resistance to primary NK cells mediated cytotoxicity. In the study done by *Lee et al*, they evaluated the antitumor cytotoxicity of NK cells expanded by anti-CD16 and autologous irradiated PBMCs, against an MHC class I-negative cell line (K562) and MHC class I-positive cell line (MCF-7, and SW480). They saw that MCF-7 and SW480 cells, which both expressed MHC-I and MICA, were sensitive to NK cell-mediated lysis of Expanded NK cells. They did not measure the cytotoxicity of primary NK cells against these cell lines to compare with Expanded NK cells, but MHC-I negative cells were moderately sensitive to NK cell-mediated lysis in comparison to K562 cells which were highly sensitive to NK cells [72]. The mechanism by which Expanded NK cells can target cells expressing MHC-I is unknown. Expanded NK cells express a higher level of NKG2D than primary NK cells. We showed that MICA/MICB, one of the NKG2D ligands, expresses highly on well-differentiated cells which also express a high level of MHC-I. Therefore, it could be possible that since Expanded NK cells express a higher level of NKG2D, the NKG2D-MICA/MICB mediated lysis became the dominant mechanism of cytotoxicity in Expanded NK cells, leading to the targeting of both undifferentiated and differentiated tumor cells.

One of the novel findings of our laboratory is defining the stage of maturation in NK cells called split anergy which can be initiated by targeting different receptors on NK cells including the CD16 receptor. We showed that when NK cells were treated with the combination of IL-2 and

antiCD16 mAb, their cytotoxicity decrease but their ability to secrete IFN- γ significantly increase in comparison to IL-2 treated NK cells [13]. Furthermore, we investigated that whether spilt anergy occurs in both the primary and Expanded NK cells. The results showed that treating Expanded NK cells with the combination of IL-2 and antiCD16 did not result in a decrease in their cytotoxicity (Fig. 8). One of the receptors that get downmodulated on the surface of Expanded NK cells is CD16 and this could explain why antiCD16 treatment in Expanded NK cells cannot push them towards the split anergy stage. Since the level of CD16 is different on primary and Expanded NK cells, next we compare the ADCC in primary and Expanded NK cells. When the level of ADCC was measured in untreated and IL-2 treated NK cells against well-differentiated Oral tumors treated with antiMICA/MICB antibody and also Cetaximab (antibody against EGFR receptor), despite primary NK cells, Expanded NK cells were not able to mediate ADCC and even some levels of inhibition was seen in antibody treated tumor cells in comparison to the untreated cells (Fig.9). The reason why Expanded NK cells mediate lower level of ADCC could be due to the downmodulation of CD16 receptor on their surface. Although Expanded NK cells express some levels of CD16, but it could be assumed that a distinct level of CD16 is required for NK cells to be able to mediate ADCC s it is known that during HIV-1 infection, NK cells are known to express low levels of CD16 and exhibit reduced ADC [73].

In our previous finding, we showed that induction of split anergy in NK cell effector function is thought to ultimately aid in driving differentiation of healthy, as well as transformed stem-like cells [68, 69]. Differentiation of tumor cells is one of the important role of NK cells since the differentiated tumor micro environment become less invasive and more susceptible to chemotherapeutics. Our group has previously shown that cytokines that are secreted by NK cells, primarily IFN-γ and TNF-α, are responsible for the differentiation of cancer stem cells

(CSCs) and result in the increase in differentiation antigens such as MHC class I, CD54, B7H1, and MICA and decrease in CD44 [13]. This study also demonstrates that exposing oral and pancreatic cancer stem cells to IFN-γ secreted by NK cells activated with IL-2 and CD16 triggered, resulted in upregulation of differentiation the same differentiation markers a respectively the differentiated tumor cells, lose their cytotoxicity against NK cells (Fig. 1). This study and our previous extensive work on AJ2 probiotic bacteria illustrate the profound capability probiotic bacteria has on NK cells to induce a significant increase in cytokine secretion, known as split anergy. AJ2 is a combination of 8 strains of probiotic bacteria for their ability to induce significant secretion of IFN-γ when added to IL-2 or IL-2+anti-CD16mAb treated NK cells [11]. The ratio of bacteria added to create sAJ2 was adjusted to yield a ratio of IFN-γ to IL-10 for when cells are activated with IL-2 or IL-2+anti-CD16mAb without bacteria. This ratio was established to obtain a similar ratio when NK cells are activated with IL-2+anti-CD16mAb without bacteria since this NK treatment provided increased differentiation of stem cells. IL-10, an anti-inflammatory cytokine, was taken into consideration to balance the significant amount of IFN-γ secreted by cells during the process of differentiation. This combination of bacterial strains was selected due to its optimal induction of pro- and anti-inflammatory cytokine and growth factors by the NK cells.

Fucoidan is a sulfated polysaccharide, can be extracted from different species of brown algae and brown seaweed. This compound has been known to have immunomodulatory effects on immune cells. Although studies have shown that Fucoidan can decrease tumor size, have antitumor activities, and lead to higher survival in tumor-induced mouse [15-17], the exact role of Fucoidan on NK cells function has not been well studied. In this study, we showed that Mekabu extracted Fucoidan increased NK cells IFN- γ secretion ability but decreased NK cell-

mediated cytotoxicity which is a profile of split anergized NK cells. Seeking strategies to push NK cells to secrete the highest level of IFN-γ, we looked at synergistic effect of Mekabu and AJ2 probiotic bacteria on NK cell-mediated IFN-γ secretion and we showed that NK cells treated with AJ2 or Mekabu induce significantly higher level of IFN- γ in comparison to untreated, sAJ2, or Mekabu treated IL-2 treated NK cells (Fig.11). NK cells express different families of Toll-like receptors (TLRs) [74]. The gram-positive bacteria in the probiotic bacteria can trigger NK cells TLRs via their cell wall components. A study showed that cytokine induction by both *B. breve* and the *lactobacilli* is strongly dependent on TLR9 since blocking of TLR9 resulted in decreased production of IL-10 and IFN-γ in PBMCs [75] Fucoidan from seaweeds is independent ligands for TLR-2 and TLR-4. The reason that NK cells produce a higher level of IFN-γ in presence of both AJ2 probiotic bacteria and Mekabu could be since these compound target different family of TLRs on NK cells.

Conclusion

In conclusion, differentiation stage of pancreatic cancer cells correlated directly with the resistance to NK cell-mediated cytotoxicity and expression of key surface antigens. Differentiation by NK cells is very important for the effective targeting of cancer stem cells/ undifferentiated tumor cells. As IFN-γ plays a critical rule in differentiation, looking for treatment strategies to push NK cells to produce higher levels of IFN-γ is a critical step in eliminating tumors. Combination of probiotic AJ2 bacteria with fucoidan extracted from Mekabu seaweed can cause higher secretion of IFN-γ by NK cells.

Oral and pancreatic tumor cells have specific pattern of MICA/MICB antigen expression as differentiated tumor cells express higher levels of MICA/MICB than stem-like/ undifferentiated tumor cells. Since well differentiated cells express higher levels of MICA/MICB NK cells

mediate higher levels of ADCC through antibody specific to MICA/MICB against these cells than their undifferentiated compartments. Furthermore, Primary and Expanded NK cells have very different characteristic and biological functions and all diverse functions of different subsets NK cells should be considered in NK cell- immunotherapeutic approaches.

References

1. Jewett A, Man Y-G, Tseng H-C. Dual Functions of Natural Killer Cells in Selection and Differentiation of Stem Cells; Role in Regulation of Inflammation and Regeneration of Tissues. *Journal of Cancer*. 2013;4(1):12-24.
2. Topham NJ, Hewitt EW. Natural killer cell cytotoxicity: how do they pull the trigger? *Immunology*. 2009;128(1):7-15.
3. Jordan S., & Zuhair, K. Natural killer cells in human health and disease. *Clinical Immunology*. 2006; 118 (1):1-10.
4. Yoon, S. R., Kim, T., & Choi, I. Understanding of molecular mechanisms in natural killer cell therapy. *Experimental & Molecular Medicine.* 2015; *47*(2).
5. Zamai L, Ahmad M, Bennett IM, Azzoni L, Alnemri ES, Perussia B. Natural Killer (NK) Cell–mediated Cytotoxicity: Differential Use of TRAIL and Fas Ligand by Immature and Mature Primary Human NK Cells . *The Journal of Experimental Medicine*. 1998;188(12):2375-2380.
6. Wang W, Erbe AK, Hank JA, Morris ZS, Sondel PM. NK Cell-Mediated Antibody-Dependent Cellular Cytotoxicity in Cancer Immunotherapy. *Frontiers in Immunology*. 2015; 6:368.
7. Kaur, K., Nanut, M. P., Ko, M., Safaie, T., Kos, J., & Jewett, A. Natural killer cells target and differentiate cancer stem-like cells/undifferentiated tumors: Strategies to optimize their growth and expansion for effective cancer immunotherapy. *Current Opinion in Immunology.* 2018.51: 170-180.
8. Wang W, Erbe AK, Hank JA, Morris ZS, Sondel PM. NK Cell-Mediated Antibody-Dependent Cellular Cytotoxicity in Cancer Immunotherapy. *Frontiers in Immunology*. 2015; 6:368.

9. Aziz M, Mahmood F, Mata M, et al. Development of IgG Mediated Antibody Dependent Cell-mediated Cytotoxicity (ADCC) in the Serum and Genital Mucosa of HIV Seroconverters. *Journal of AIDS & clinical research*.
10. Richard K, Mann BJ, Stocker L, et al. Novel Catanionic Surfactant Vesicle Vaccines Protect against Francisella tularensis LVS and Confer Significant Partial Protection against F. tularensis Schu S4 Strain. Burns DL, ed. *Clinical and Vaccine Immunology.* 2014;21(2):212-226.
11. Eischen, C M., Schilling, J D.,Lynch, D H., Krammer, P H., Leibson, P. Fc receptor-induced expression of Fas ligand on activated NK cells facilitates cell-mediated cytotoxicity and subsequent autocrine NK cell apoptosis. *The Journal of Immunology*. 1996; 56 (8) 2693-2699.
12. Bui, V. T., Tseng, H.-C., Kozlowska, A., Maung, P. O., Kaur, K., Topchyan, P., & Jewett, A. Augmented IFN-γ and TNF-α Induced by Probiotic Bacteria in NK Cells Mediate Differentiation of Stem-Like Tumors Leading to Inhibition of Tumor Growth and Reduction in Inflammatory Cytokine Release; Regulation by IL-10. *Frontiers in Immunology*. 2015; *6*: 576.
13. Tseng, H.-C., Bui, V., Man, Y.-G., Cacalano, N., & Jewett, A.Induction of Split Anergy Conditions Natural Killer Cells to Promote Differentiation of Stem Cells through Cell–Cell Contact and Secreted Factors. *Frontiers in Immunology*. 2014; *5*: 269.
14. Li, B., Lu, Fei,. Wei, X., Zhao, R. Fucoidan: Structure and Bioactivity. *Molecules. 2008; 13:* 1671-1695.
15. Maruyama, H., Tamauchi, H., Iizuka, M., & Nakano, T. The Role of NK cells in Antitumor Activity of Dietary Fucoidan from Undaria pinnatifida Sporophylls (Mekabu). *Planta Medica. 2006; 72*(15): 1415-1417.

16. Maruyama, H., Tamauchi, H., Iizuka, M., & Nakano, T. The Role of NK cells in Antitumor Activity of Dietary Fucoidan from Undaria pinnatifida Sporophylls (Mekabu). *Planta Medica,72*(15), 1415-1417.

17. Jiao, G., Yu, G., Zhang, J., & Ewart, H. S. Chemical Structures and Bioactivities of Sulfated Polysaccharides from Marine Algae. *Marine Drugs*. 2011; *9*(2):196–223.

18. Jewett A, Bonavida B. Target-induced anergy of natural killer cytotoxic function is restricted to the NK-target conjugate subset. Cell Immunol 1995; 160(1): 91–97. 25.

19. Jewett A, Bonavida B. Target-induced inactivation and cell death by apoptosis in a subset of human NK cells. *J Immunol*. 1996; 156(3):907–15

20. Jewett A, Gan XH, Lebow LT, Bonavida B. Differential secretion of TNF-alpha and IFNgamma by human peripheral blood-derived NK subsets and association with functional maturation. J *Clin Immunol*. 1996; 16(1):46–54

21. Jewett A, Cavalcanti M, Bonavida B. Pivotal role of endogenous TNF-alpha in the induction of functional inactivation and apoptosis in NK cells. *J Immunol*. 1997;159(10):4815–4822

22. Jewett A, Bonavida B. MHC-Class I antigens regulate both the function and the survival of human peripheral blood NK cells: role of endogenously secreted TNF-alpha. *Clin Immunol.* 2000; 96(1):19–28

23. Jewett A, Cacalano NA, Head C, Teruel A. Coengagement of CD16 and CD94 receptors mediates secretion of chemokines and induces apoptotic death of naive natural killer cells. *Clin Cancer Res.* 2006; 12(7):1994–2003.

24. Carnero, A., & Lleonart, M. The hypoxic microenvironment: A determinant of cancer stem cell evolution. *Inside the Cell. 2015; 1*(2): 96-105.

25. Donnet, D., Dick, JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med.* 1977; 3(7):730-7

26. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA.* 2003; 100:3983–3988.

27. Parmiani, G. Melanoma Cancer Stem Cells: Markers and Functions. *Cancers*. 2016; *8*(3): 34.

28. Badeaux, M. A., & Tang, D. G. Prostate Cancer Cell Heterogeneity and Prostate Cancer Stem Cells. *Cancer Stem Cells. 2014;*

29. Hardavella, G., George, R., & Sethi, T. Lung cancer stem cells—characteristics, phenotype. *Translational Lung Cancer Research*. 2016; *5*(3): 272–279.

30. Garza-Treviño, E. N., Said-Fernández, S. L., & Martínez-Rodríguez, H. G. Understanding the colon cancer stem cells and perspectives on treatment. *Cancer Cell International*. 2015; *15*(1): 2.

31. Lathia, J. D., Mack, S. C., Mulkearns-Hubert, E. E., Valentim, C. L. L., & Rich, J. N. (2015). Cancer stem cells in glioblastoma. *Genes & Development*. 2015; *29*(12): 1203–1217.

32. Nio, K., Yamashita, T., & Kaneko, S. The evolving concept of liver cancer stem cells. *Molecular Cancer*. 2017;*16*: 4.

33. Rao, C. V., & Mohammed, A. New insights into pancreatic cancer stem cells. *World Journal of Stem Cells*. 2015; *7*(3): 547–555.

34. Syachandran A, Dhungel B, Steel JC. Epithelial-to-mesenchymal plasticity of cancer stem cells: therapeutic targets in hepatocellular carcinoma. *Journal of hematology & oncology.* 2016, **9**(1):74.

35. Shackleton M: Normal stem cells and cancer stem cells: similar and different. *Seminars in cancer biology.* 2010, 20(2):85-92.

36. Song Y, Wang Y, Tong C, Xi H, Zhao X, Wang Y, Chen L: A unified model of the hierarchical and stochastic theories of gastric cancer. *British journal of cancer* 2017, 116(8):973-989.
37. 4. Baccelli I, Trumpp A: The evolving concept of cancer and metastasis stem cells. *The Journal of Cell Biology* 2012, 198(3):281-293.
38. 5. Plaks V, Kong N, Werb Z: The Cancer Stem Cell Niche: How Essential is the Niche in Regulating Stemness of Tumor Cells? *Cell stem cell* 2015, 16(3):225-238.
39. Kozlowska AK, Topchyan P, Kaur K, Tseng H-C, Teruel A, Hiraga T, Jewett A: Differentiation by NK cells is a prerequisite for effective targeting of cancer stem cells/poorly differentiated tumors by chemopreventive and chemotherapeutic drugs. *Journal of Cancer* 2017, 8(4):537-554.
40. Tseng HC, Cacalano N, Jewett A: Split anergized Natural Killer cells halt inflammation by inducing stem cell differentiation, resistance to NK cell cytotoxicity and prevention of cytokine and chemokine secretion. *Oncotarget* 2015, 6(11):8947-8959.
41. 11. Kozlowska AK, Kaur K, Topchyan P, Jewett A: Adoptive transfer of osteoclast-expanded natural killer cells for immunotherapy targeting cancer stem-like cells in humanized mice. *Cancer immunology, immunotherapy : CII* 2016, 65(7):835-845.
42. 12. Kozlowska AK, Kaur K, Topchyan P, Jewett A: Novel strategies to target cancer stem cells by NK cells; studies in humanized mice. *Frontiers in bioscience (Landmark edition)* 2017, 22:370-384.
43. 13. Kaur K, Cook J, Park SH, Topchyan P, Kozlowska A, Ohanian N, Fang C, Nishimura I, Jewett A: Novel Strategy to Expand Super-Charged NK Cells with Significant Potential to Lyse and Differentiate Cancer Stem Cells: Differences in NK Expansion and Function between Healthy and Cancer Patients. *Frontiers in immunology* 2017, 8:297.

44. Tanaka, Y., Nakayamada, S., & Okada, Y. Osteoblasts and Osteoclasts in Bone Remodeling and Inflammation. *Current Drug Target -Inflammation & Allergy*. 2005; 4(3): 325-328.

45. Feng, S., Madsen, S. H., Viller, N. N., Neutzsky-Wulff, A. V., Geisler, C., Karlsson, L., & Söderström, K. Interleukin-15-activated natural killer cells kill autologous osteoclasts via LFA-1, DNAM-1 and TRAIL, and inhibit osteoclast-mediated bone erosionin vitro. *Immunology*. 2015; 145(3): 367-379.

46. Weiss-Steider, B., Soto-Cruz, I., Martinez-Campos, C. A., & Mendoza-Rincon, J. F. Expression of MICA, MICB and NKG2D in human leukemic myelomonocytic and cervical cancer cells. *Journal of Experimental & Clinical Cancer Research. 2011; 30*(1): 37.

47. Bauer, S. Activation of NK Cells and T Cells by NKG2D, a Receptor for Stress-Inducible MICA. *Science. 1999; 285*(5428): 727-729.

48. Ghadially, H., Brown, L., Lloyd, C., Lewis, L., Lewis, A., Dillon, J., Wilkinson, R. W. MHC class I chain-related protein A and B (MICA and MICB) are predominantly expressed intracellularly in tumour and normal tissue. *British Journal of Cancer*. 2017; *116*(9): 1208–1217.

49. Wu, Y., Tian, Z., & Wei, H. Developmental and Functional Control of Natural Killer Cells by Cytokines. *Frontiers in Immunology*. 2017;*8*: 930.

50. Floros, T., & Tarhini, A. A. Anticancer Cytokines: Biology and Clinical Effects of IFN-α2, IL-2, IL-15, IL-21, and IL-12. *Seminars in Oncology*. 2015; *42*(4): 539–548.

51. Beldi-Ferchiou, A., & Caillat-Zucman, S. Control of NK Cell Activation by Immune Checkpoint Molecules. *International Journal of Molecular Sciences*. 2017; *18*(10): 2129.

52. Lapteva, N., Duret, A. G., Sub, J., Rollins, L. A., Huye, L. L., Fang, J. Rooney, C. M. Large-scale *ex vivo* expansion and characterization of natural killer cells for clinical applications. *Cytotherapy*. 2012; *14*(9): 1131–1143.

53. Deng, X., Terunuma, H., Nieda, M., Xiao, W., & Nicol, A. Synergistic cytotoxicity of ex vivo expanded natural killer cells in combination with monoclonal antibody drugs against cancer cells. *International Immunopharmacology. 2012;14*(4): 593-605. doi:10.1016/j.intimp.2012.09.014

54. Romee, R., Rosario, M., Berrien-Elliott, M. M., Wagner, J. A., Jewell, B. A., Schappe, T.,Fehniger, T. A. Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Science Translational Medicine*. 2016;*8*(357): 357.

55. Parkhurst, M. R., Riley, J. P., Dudley, M. E., & Rosenberg, S. A. Adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*. 2011; *17*(19): 6287–6297.

56. E. Ishikawa, K. Tsuboi, K. Saijo, H. Harada, S. Takano, T. Nose, T. Ohno. Autologous natural killer cell therapy for human recurrent malignant glioma *Anticancer Res*. 2004; 24(3BO:1861-1871.

57. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295:2097–2100.

58. Woll, P. S., Grzywacz, B., Tian, X., Marcus, R. K., Knorr, D. A., Verneris, M. R., & Kaufman, D. S. Human embryonic stem cells differentiate into a homogeneous population of natural killer cells with potent in vivo antitumor activity. *Blood*. 2009; 113(24): 6094–6101.

59. Hermanson, D. L., Bendzick, L., Pribyl, L., McCullar, V., Vogel, R. I., Miller, J. S., Kaufman, D. S. Induced pluripotent stem cell-derived natural killer cells for treatment of ovarian cancer. *Stem Cells (Dayton, Ohio)*. 2016; *34*(1): 93–101.

60. Li, Y., Hermanson, D. L., Moriarity, B. S., & Kaufman, D. S. (2018). Human iPSC-Derived Natural Killer Cells Engineered with Chimeric Antigen Receptors Enhance Anti-tumor Activity. *Cell Stem Cell. 2018 23*(2.

61. Sarvaria, A., Jawdat, D., Madrigal, J. A., & Saudemont, A. Umbilical Cord Blood Natural Killer Cells, Their Characteristics, and Potential Clinical Applications. *Frontiers in Immunology*. 2017; 8:329.

62. Shah, N., Martin-Antonio, B., Yang, H., Ku, S., Lee, D. A., Cooper, L. J. N., Shpall, E. J. Antigen Presenting Cell-Mediated Expansion of Human Umbilical Cord Blood Yields Log-Scale Expansion of Natural Killer Cells with Anti-Myeloma Activity. *PLoS ONE*. 2013; *8*(10): e76781.

63. Ayello, J., Hochberg, J., Flower, A., Chu, Y., Baxi, L. V., Quish, W., Cairo, M. S. Genetically re-engineered K562 cells significantly expand and functionally activate cord blood natural killer cells: Potential for adoptive cellular immunotherapy. *Experimental Hematology*. 2017;46: 38-47.

64. Veluchamy, J. P., Heeren, A. M., Spanholtz, J., van Eendenburg, J. D. H., Heideman, D. A. M., Kenter, G. G., de Gruijl, T. D. High-efficiency lysis of cervical cancer by allogeneic NK cells derived from umbilical cord progenitors is independent of HLA status. *Cancer Immunology, Immunotherapy*. 2017: *66*(1), 51–61.

65. Li, L., Liu, L. N., Feller, S., Allen, C., Shivakumar, R., Fratantoni, J., Peshwa, M.Expression of chimeric antigen receptors in natural killer cells with a regulatory-compliant non-viral method. *Cancer Gene Therapy*. 2010; 17(3): 147.

66. Chu, Y., Hochberg, J., Yahr, A., Ayello, J., Ven, C. V., Barth, M.,Cairo, M. S. Targeting CD20 Aggressive B-cell Non-Hodgkin Lymphoma by Anti-CD20 CAR mRNA-Modified Expanded Natural Killer Cells In Vitro and in NSG Mice. *Cancer Immunology Research*. 2014; 3(4): 333-344.

67. Kruschinski, A., Moosmann, A., Poschke, I., Norell, H., Chmielewski, M., Seliger, B., Charo, J. Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105(45):17481–17486.

68. Tseng, H.-C., Arasteh, A., Paranjpe, A., Teruel, A., Yang, W., Behel, A.,Jewett, A. Increased Lysis of Stem Cells but Not Their Differentiated Cells by Natural Killer Cells; De-Differentiation or Reprogramming Activates NK Cells. *PLoS ONE*. 2208; *5*(7): e11590.

69. Tseng HC, Bui V, Man YG, Cacalano N, Jewett A. Induction of Split Anergy Conditions Natural Killer Cells to Promote Differentiation of Stem Cells through Cell-Cell Contact and Secreted Factors. Frontiers in immunology. 2014; 5:269

70. 19. Jewett A, Arasteh A, Tseng HC, Behel A, Arasteh H, Yang W. et al. Strategies to rescue mesenchymal stem cells (MSCs) and dental pulp stem cells (DPSCs) from NK cell mediated cytotoxicity. PLoS One. 2010;5: e9874

71. Kircheis, R., Halanek, N., Koller, I., Jost, W., Schuster, M., Gorr, G., … Nechansky, A. Correlation of ADCC activity with cytokine release induced by the stably expressed, glyco-

engineered humanized Lewis Y-specific monoclonal antibody MB314. *mAbs*. 2012; 4(4): 532–541.

72. Lee, H., Son, C., Koh, E., Bae, J., Kang, C., Yang, K., & Park, Y. Expansion of cytotoxic natural killer cells using irradiated autologous peripheral blood mononuclear cells and anti-CD16 antibody. *Scientific* Reports. 2017; 7(1).
73. Alter G, Altfeld M. NK cells in HIV-1 infection: Evidence for their role in the control of HIV-1 infection. *Journal of internal medicine*. 2009;265(1):29-42.
74. Guo, Q., & Zhang, C. Critical role of Toll-like receptor signaling in NK cell activation. *Chinese Science Bulletin. 2012;* 57(24): 3192-3202.
75. Plantinga, TS., Maren WWC., Bergenhenegouwen , J., et al. Differential Toll-Like Receptor Recognition and Induction of Cytokine Profile by Bifidobacterium breve and Lactobacillus Strains of Probiotics . *Clinical and Vaccine Immunology:* CVI. 2011;18(4):621-628.